PhD. Thesis

Modelling of microalgae-bacteria consortia for wastewater treatment

Modelado de los consorcios microalgas-bacterias para el tratamiento de aguas residuales

University of Almería

Department of Chemical Engineering

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

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A mi familia,

To my family,

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RESUMEN

La demanda de agua limpia ha estado en continuo crecimiento en el último siglo y se espera que aumente aún más durante las próximas décadas. Este incremento es debido al aumento de la población mundial, asociado con el incremento del consumo individual de agua y el rápido desarrollo de la agricultura intensiva, la industrialización y la urbanización. Además, la descarga de efluentes con altas concentraciones de nutrientes y materia orgánica promueve procesos de eutrofización en las reservas de aguas naturales, ayudados en gran medida por el aumento de las temperaturas oceánicas globales. En Europa, la Directiva del Consejo 91/271/EEC regula el tratamiento de aguas residuales urbanas para proteger el medio ambiente, estableciendo diferentes límites de descarga de efluentes en cuanto a la demanda química de oxígeno (DQO), nitrógeno (N) y fósforo (P). Para garantizar estos estándares, diferentes procesos de tratamiento de aguas residuales tanto físicos como químicos o biológicos se han implementado con éxito en todo el mundo, cada uno con sus ventajas y limitaciones.

En las plantas de tratamiento de aguas residuales, el tratamiento más empleado es el proceso de fangos activados. A pesar de las altas tasas de eliminación de DQO, N y P obtenidas por los procesos de lodos activados, la aireación, los tiempos de mezcla y los reactivos necesarios para llevar a cabo el proceso siguen causando altas demandas de energía y elevados costos operativos. Las pérdidas potenciales de nutrientes y las emisiones de gases de efecto invernadero (CO_2, NO_x, CH_4) también se plantean como las principales desventajas de los tratamientos convencionales. Para superar estos inconvenientes, se ha propuesto como solución sostenible el tratamiento de aguas residuales mediante consorcios de microalgas-bacterias. Esta tecnología aprovecha la luz solar renovable, minimiza la emisión de gases de efecto invernadero a la atmósfera y permite la recuperación de nitrógeno y fósforo a la vez que se generan bioproductos de alto valor añadido a partir de la biomasa generada.

En los consorcios microalgas-bacterias tienen lugar múltiples interacciones entre las microalgas y los distintos grupos de bacterias presentes en las aguas residuales y en el medioambiente. Estas interacciones son dinámicas y están determinadas por las variables ambientales y operacionales en las que se desarrollan. Factores como la composición de las aguas residuales o la disponibilidad de nutrientes, junto con la disponibilidad de luz, son críticos para determinar el crecimiento específico de microalgas y bacterias. Otros parámetros que también juegan un papel relevante son el pH, la temperatura y las concentraciones de oxígeno disuelto. Además, estas interacciones determinan el éxito de los procesos porque permiten la eliminación de los

sustratos presentes en las aguas residuales. Así, conocer las interacciones microbianas en los sistemas microalgas-bacterias es fundamental para controlar y maximizar el rendimiento y la eficacia de estos sistemas.

Por ello, en este trabajo se ha desarrollado un modelo matemático como potente herramienta para evaluar las principales poblaciones microbianas que aparecen en el tratamiento de aguas residuales con microalgas, así como la dinámica de los compuestos que en ellas se encuentran. Para desarrollar dicho modelo matemático, se adaptaron las técnicas tradicionales de respirometría para evaluar los organismos fotoautótrofos, dando lugar a la foto-respirometría, que permite eestimar tanto las actividades bacterias como la de microorganismos productores de oxígeno. Se aplicó la foto-respirometría para determinar la influencia de variables ambientales (luz, temperatura, pH y oxígeno disuelto) y operacionales (disponibilidad de nutrientes) en la actividad de microalgas, bacterias heterótrofas y bacterias nitrificantes. Estos datos fueron esenciales para determinar los parámetros cinéticos correspondientes a cada población, lo que permitió desarrollar un modelo biológico para los consorcios microalgas-bacterias que aparecen en los procesos de tratamiento de aguas residuales. Los parámetros cinéticos, junto con los datos reales del cultivo de microalgas-bacterias permitieron desarrollar, calibrar y validar un modelo matemático, denominado modelo ABACO, a escala de laboratorio utilizando aguas residuales reales. El modelo ABACO fue desarrollado utilizando el software MATLAB y está disponible como una herramienta de descarga que permite evaluar la influencia de cada variable ambiental y operacional en las poblaciones de microalgas y bacterias.

La última fase de desarrollo de la Tesis consistió en la mejora del modelo biológico distinguiendo dos poblaciones diferentes dentro del grupo de los nitrificantes. Además, los parámetros cinéticos para las poblaciones de bacterias se determinaron nuevamente utilizando una nueva metodología de respirometría aplicada al cultivo de microalgas-bacterias producido con digestato proveniente de una digestión anaeróbica en lugar de aguas residuales primarias. Los datos experimentales obtenidos se ajustaron con las ecuaciones correspondientes para obtener los parámetros cinéticos que se pueden utilizar para mejorar el modelo ABACO en trabajos futuros.

ABSTRACT

Clean water demand has continued to grow in the last century, and this trend is expected to continue to grow during the coming decades. The mains reasons include the expected increase of the worlds' population, associated with rising individual water consumption and the rapid development of intensive agriculture, industrialization, and urbanization in developing countries. In addition, the discharge of urban, agricultural, and industrial effluents with high nutrient concentrations and organic matter is the main cause of severe eutrophication, aided by climate change and the increasing temperature of the ocean. In Europe, the Council Directive 91/271/EEC regulates urban wastewater treatment to protect the water reservoirs, by setting different maximum discharge limits for chemical oxygen demand (COD), nitrogen (N) and phosphorus (P). To ensure these standards, physical, chemical, and biological wastewater treatment processes have been successfully implemented worldwide, each strategy with its own strengths and challenges.

In wastewater treatment plants, the most widely applied strategy is the utilization of activated sludge processes. Despite the high COD, N, and P removal capacities obtained by activated sludge processes, high energy demands, and operating costs caused by aeration, mixing, and the reagents required to operate the process remains a challenge. Potential nutrient losses and greenhouse gas emissions are also reported as major disadvantages of conventional processes. To overcome these drawbacks, the treatment of wastewater using microalgae-bacteria consortia has been proposed as a sustainable and innovative solution. This technology exploits sunlight, which is free and unlimited, minimize the release of greenhouse gases (CO_2, NOx, CH_4) into the atmosphere, and allows recovering nitrogen and phosphorous while simultaneously generating valuable bio-products from the produced biomass.

In microalgae-bacteria consortia, multiple interactions take place between microalgae and the most abundant bacterial groups. These interactions are dynamic and depend on both, environmental and operational variables. Factors such as the composition of wastewater or nutrient availability, in addition to light availability are critical in determining the specific growth of microalgae and bacteria. Other important factors affecting the process include as the pH, temperature and dissolved oxygen concentration. These interactions determine the success of the processes because they allow recovering the nutrients present in wastewater while producing valuable biomass. Thus, understanding the microbial interactions in microalgal systems, which is a challenging task, is essential to control and maximize the yield of these systems.

In this work, a mathematical model was developed as a powerful tool to evaluate the main microbial populations that appear in microalgae wastewater treatment as well as the main nutrients present in the media. To develop the mathematical model, a new methodology, termed as photo-respirometry, was developed based on already available techniques. Photo-respirometry was applied to determine the influence of environmental and operational variables on the performance of microalgae, heterotrophic bacteria, and nitrifying bacteria. These data were essential to determine the corresponding kinetic parameters of each population, which allowed developing a biological model for microalgae-bacteria consortia in wastewater treatment processes. The kinetic parameters together with experimental data of microalgae-bacteria cultures allowed developing, calibrating and validating a mathematical model, named ABACO. The ABACO model was developed using the software MATLAB and it is available as an easy and downloadable tool that permits the assessment of the influence of each environmental and operational variable on the microalgal and bacterial populations.

The last development phase of this Thesis consisted of the improvement of the biological model distinguishing two different populations within the nitrifiers group. In addition, the kinetic parameters for bacterial populations were determined again using a novel respirometry methodology applied to microalgal-bacterial cultures produced with anaerobic digestate as the source of nutrients instead of primary wastewater. The experimental data obtained were fitted with the corresponding equations to obtain the kinetic parameters that could be used to improve the ABACO model in future works.

CONTENTS

LIST OF ACRONYMS

ASM: Activated Sludge Models

AS: Activated Sludge Process

AOB: Ammonium-Oxidizing Bacteria

C: Carbon

CO_2: Carbon Dioxide

DO: Dissolved Oxygen

HBRR: Heterotrophic Bacteria Respiration Rate

HET: Heterotrophic Bacteria

IWA: International Water Association

MA: Microalgae

MB: Microalgae-Bacteria

MNPR: Microalgal Net Photosynthetic Rate

MRR: Microalgal Respiration Rate

N: Nitrogen

NBRR: Nitrifying Bacteria Respiration Rate

NIT: Nitrifying Bacteria or Nitrifiers

NH_4^+: Ammonium

NO_3^-: Nitrate

NO_2^-: Nitrite

NOB: Nitrite-Oxidizing Bacteria

O_2: Oxygen

P: Phosphorous

PAOs: Polyphosphate-Accumulating Organisms

PWW: Primary domestic WasteWater (PWW)

sOUR: Specific Oxygen Uptake Rate

TN: Total Nitrogen

TP: Total Phosphorus

WW: Wastewater

HRAP: Wastewater High-rate Algal Ponds

WWT: Wastewater treatment

WWTP: Wastewater Treatment Plant

LIST OF SYMBOLS

$PO_2max,_{ALG}$	Maximun photosinthesys rate
$\mu,_{MAX,ALG}$	Maximun microalgae growth rate
Iav	Average Irradiance
$Ik,_{ALG}$	Constant representing the affinity of algae to light
$n,_{ALG}$	Form parameter
$Tmin,_{ALG}$	Minimal microalgae temperature
$Tmax,_{ALG}$	Maximum microalgae temperature
$Topt,_{ALG}$	Optimum microalgae temperature
$pHmin,_{ALG}$	Minimal microalgae pH
$pHmax,_{ALG}$	Maximum microalgae pH
$pHopt,_{ALG}$	Optimum microalgae pH
$DO_2max,_{ALG}$	Maximum dissolved oxygen
$m,_{ALG}$	Form dissolved oxygen parameter
$RO_2max,_{ALG}$	Maximum microalgae respiration rate
$RO_2min,_{ALG}$	Minimum microalgae respiration rate
$Ik\_res,_{ALG}$	Constant representing the affinity of algae to light
$n\_resp,_{ALG}$	Form respiration parameter
$K_{C,ALG}$	Microalgae half-saturation constant on CO_2
$I_{CO2,ALG}$	Microalgae inhibition constant on CO2
$k_{S,NH4,ALG}$	Microalgae half-saturation constant for N- NH_4^+
$K_{I,NH4,ALG}$	Microalgae inhibition constant for N- NH_4^+
$k_{S,NO3,ALG}$	Microalgae half-saturation constant for N-NO_3^-
$K_{I,NO3,ALG}$	Microalgae inhibition constant for N- NO_3^-
$k_{S,P,ALG}$	Microalgae half-saturation constant for P
$RO_2max,_{HET}$	Maximum heterotrophic respiration rate
$\mu,_{MAX,HET}$	Maximun heterotrophic growth rate
$Tmin,_{HET}$	Minimal heterotrophic bacteria temperature
$Tmax,_{HET}$	Maximum heterotrophic bacteria temperature
$Topt,_{HET}$	Optimum heterotrophic bacteria temperature
$PHmin,_{HET}$	Minimal heterotrophic bacteria pH
$PHmax,_{HET}$	Maximum heterotrophic bacteria pH
$PHopt,_{HET}$	Optimum heterotrophic bacteria pH
$K_{S,DO2,HET}$	Heterotrophic bacteria half-saturation constant for dissolved oxygen
$K_{S,NH4,HET}$	Heterotrophic bacteria half-saturation constant for N- NH_4^+

$K_{S,P,HET}$	Heterotrophic bacteria half-saturation constant for P-PO4
$K_{S,Ss,HET}$	Heterotrophic bacteria half-saturation constant for biodegradable organic matter
$RO_{2max,NIT}$	Maximum nitrifiying respiration rate
$\mu_{,MAX,NIT}$	Maximun nitrifying growth rate
$Tmin_{,NIT}$	Minimal nitrifiying bacteria temperature
$Tmax_{,NIT}$	Maximum nitrifiying bacteria temperature
$Topt_{,NIT}$	Optimum nitrifiying bacteria temperature
$pHmin_{,NIT}$	Minimal nitrifiying bacteria pH
$pHmax_{,NIT}$	Maximum nitrifiying bacteria pH
$PHopt_{,NIT}$	Optimum nitrifiying bacteria pH
$K_{S,DO2,NIT}$	Nitrifiying bacteria half-saturation constant for dissolved oxygen
$K_{I,DO2,NIT}$	Nitrifiying bacteria inhibition constant for dissolved oxygen
$K_{C,NIT}$	Nitrifiying saturation half-constant for CO_2
$K_{S,NH4,NIT}$	Nitrifiying bacteria half-saturation constant for $N\text{-}NH_4^+$
$K_{S,P,NIT}$	Nitrifiying bacteria half-saturation constant for P

1.HYPOTHESIS AND OBJECTIVE

1.1. Hypothesis

The overexploitation of water reservoirs is a serious global concern. The discharge of effluents containing an excessive amount of nutrients such as nitrogen (N) and phosphorus (P) in addition to other contaminants such as heavy metals and other compounds of emerging concern, inevitably leads to eutrophication and pollution of water reservoirs (Cao et al. 2019). The actual situation is critical and has become a central aspect of the 2030 Agenda of the EU and on the Sustainable Development Goals of the United Nations. Both insists on the need to seek solutions that ensure sufficient and safe water supplies for everyone (Pacheco et al. 2020). The high concentration of N and P in wastewater (WW) makes it a potential culture medium for the growth of photo-autotrophic organisms such as microalgae (MA) and cyanobacteria.

Coupling microalgae production and wastewater bioremediation allows processing WW while simultaneously producing value biomass (Craggs et al. 2013). This biological treatment is performed by complex microalgae-bacteria (MB) consortia. MB interactions have been well-known for a long time as they were already described by Ostwald in 1953 (Oswald et al. 1953). These interactions occur in the area surrounding microalgal cells, where metabolites are exchanged between MA and bacteria (Amin et al. 2012) and are species-specific as the microenvironment of each MA is different. Currently, it is accepted that the interactions between MA and bacteria have potential to improve microalgal biomass production (Fuentes et al. 2016) along with nutrient removal/recovery (Acién et al. 2016). This powerful interaction is based on oxygen/carbon dioxide (O_2/CO_2) and nutrients/products exchange. Under illumination, MA perform photosynthesis, consuming CO_2 as a carbon (C) source and producing O_2. This O_2 is essential for the degradation of organic matter present in wastewater by heterotrophic bacteria (HET). Simultaneously, during bacterial oxidation of organic matter, CO_2 is produced and is available for MA to produce photosynthesis (Quijano et al. 2017). Nitrifying bacteria or nitrifiers (NIT) also are present in wastewater and have a symbiotic relationship with MA. Indeed, these microorganisms transform ammonium into nitrate using the O_2 produced by MA (Vargas et al. 2016).

To optimize and maximize these beneficial interactions at an industrial scale, it is essential to develop powerful and easy-to-implement tools allowing to understand and optimize its performance. In this way, in recent decades multiple mathematical models have been developed capable of predicting how these populations perform in microalgal-based wastewater treatment (WWT), as the activated sludge models (ASM) already proposed (Solimeno and García 2017). However, these mathematical models still face

several challenges. On the one hand, these models are overparameterized and contemplate multiple biological processes at the same time, which complicates their application in the regular operation of microalgal WWT systems. On the other hand, most of them are built with parameters obtained from the literature, adapted from ASM or obtained by calibration, so it is necessary to review these values and obtain them through experimental tests. To carry out this last aspect, it is necessary to have simple, low time-consuming and reproducible methodologies and protocols that allow obtaining the biological parameters of MA and bacteria in the laboratory.

Currently, photo-respirometric tests enable estimating different kinetic and stoichiometric parameters of the MB consortia that could be used for building biological models (Rossi et al. 2020b). Photo-respirometry is proposed as an alternative to long batch experiments since it is a cheap technique, easy to use, less time consuming and can be carried out in almost any biological or engineering laboratory (Sforza et al. 2019). In addition, it is also possible to use photo-respirometry later to validate the biological models, which gives rise to continuous feedback between the mathematical models and the respirometric tests.

1.2. Objective

The main objective of this Thesis was to increase the understanding and deepen the knowledge of the MB consortia in WWT through the development of a mathematical model, which allows predicting the dynamics of the main microbial populations (MA, HET and NIT) based on the environmental and operational variables. Furthermore, a second goal for this purpose was to develop a photo-respirometric methodology able to differentiate the activity of MA, HET and NIT in MB cultures, which allowed determining the kinetics parameters of these microbial populations under specific conditions of light, temperature, pH, DO, N and P. Kinetic parameters were essential to develop and calibrate the mathematical model for MB wastewater treatment.

To achieve this main general goal, the following specific objectives were completed (Figure 1):

1. To develop a fast and straightforward methodology to identify the main microbial populations (MA, HET and NIT) existing in MB suspensions. For this purpose, a specific photo-respirometric protocol was developed and validated, which can be used to estimate microbial populations in MB cultures and determine their kinetic parameters (Sánchez-Zurano et al. 2020).

2. To determine the effect of environmental and operational conditions such as light, temperature, pH, DO, nutrients concentration (N, P) on microalgal and bacterial

activities using the photo-respirometric methodology developed. These experimental data allowed obtaining the kinetic parameters of the microbial populations and developed a biological models with multiple factors for each population (MA, HET and NIT) (Sánchez-Zurano et al. 2021; Sánchez-Zurano et al. 2021).

3. To develop, calibrate and validate a mathematical model for MB consortia in wastewater treatment at lab-scale using Matlab Software (ABACO model). This model is suitable to simulate the biomass concentration of each microbial population over time along with the evolution of the main dissolved components in the MB system. Also, an interactive tool was developed to understand and analyze the effect of each variable on the MA and bacterial activities in the MB culture (Sánchez-zurano et al. 2021).

4. To improve the biological features of the MB mathematical model, the NIT group was divided in two main populations, the ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). For this purpose, new respirometric trials were performed to obtain the kinetics parameters for bacterial populations (HET, AOB, and NOB) that allowed increases the knowledge of the MB interactions in wastewater treatment (Sánchez-Zurano et al. 2022).

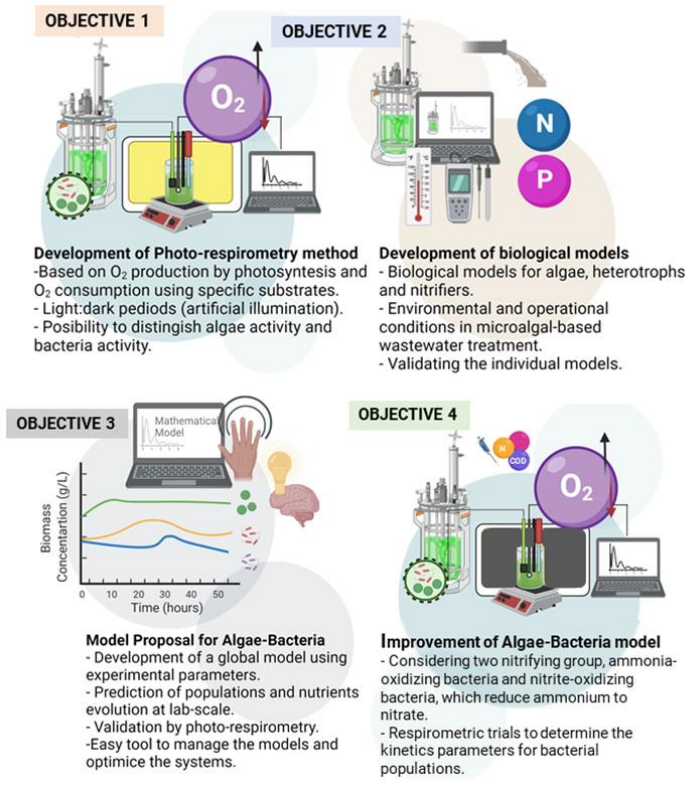


Figure 1.- The main objectives proposed to achieve the objectives planned.

2.INTRODUCTION

2.1. Why does wastewater treatment impact the global water crisis?

In the last 100 years, the demand for clean water never stopped growing and it is expected that it will continue to grow in the coming years. There are several reasons for this increase, primarily (i) the world population growth and rising individual consumption; (ii) the development of intensive agriculture; (iii) the rapid industrialization and urbanization or (iv) the discharge of effluents with values of contamination in nutrients such as P and N higher than safe (Cao et al. 2019; Giri 2021).

Against this background, the current state of WW management is in transition. In Europe, in the last two decades, the implementation of the Urban Waste Water Treatment European Directive (91/271/EEC) involved a significant improvement in river water quality. This directive sets effluent discharge limits for chemical oxygen demand (COD) at 125 mg L^{-1} O_2, for total nitrogen (TN) at 10 or 15 mg L^{-1} for population equivalence (PE) of >100 k or < 100 k, respectively, and for total phosphorus (TP) at 1 or 2 mg L^{-1}. To assure these standards, numerous wastewater treatment processes (physical, chemical and biological) have been implemented, each one with its strengths and limitations (Waqas et al. 2020). Traditionally, in a wastewater treatment plant (WWTP) primary treatment is applied to separate the particulate pollutants such as debris, sand, oils, and particulate wastes in a large settling tank. After that, a biological secondary treatment stage eliminates the colloidal and dissolved wastes, the most well-known being the activated sludge process (AS) (Hreiz et al. 2015). To remove excess nutrients such as C, P, and N from sewage, there are diverse groups of microorganisms, that comprise the activated sludge microbiome and acts as the active biological component that bioremediates the influent wastewaters (Johnston et al. 2019).

Despite this technology providing satisfactory levels nutrients removal, it demands high energy and imposes high operating costs, in addition to wasteful practices of potential nutrients and release of significant sources of greenhouse gases (Mennaa et al. 2019; Capodaglio and Olsson 2019; Mantovani et al. 2020). By 2050, the next generation of WW management technologies will be hugely influenced by the need to reduce greenhouse gas emissions regulations that promote recovering resources and materials (water, energy, nutrients) (Soares 2020; Mannina et al. 2021).

Accordingly, microalgae-bacteria WWT provides a sustainable strategy in the global WWT industry, as this environmentally friendly technology avoid the release of greenhouse gases (CO_2, NO_x, CH_4), allow to recover of N and P, and generates valuable biomass while WW is treated (Craggs et al. 2014; Do et al. 2022). Therefore, microalgae-based WWT provides substantial advantages over traditional WWT systems using AS

(Beuckels et al. 2015). For instance, the costs associated with microalgae-based WWT along with the energy requirements (are lower compared to the conventional treatment processes (0.2 €/m^3, 0.5 kWh/m^3), because the contribution of oxygen by microalgal photosynthesis could reduce the requirement for aeration, which is a costly process in WWTP (Vargas et al. 2016). Furthermore, the nutrient (N, P) removal efficiency by MA is higher than AS systems; MA are capable of capturing and fixing CO_2, and even though microalgal biomass generated using WW is a great sustainable alternative for animal and aquaculture feed (Nagarajan et al. 2020). All these advantages make microalgae-based WWT a promising platform for integral WW valorization (Posadas et al. 2017).

2.2. Microalgae-bacteria interactions for wastewater treatment

Microalgae-based WWT is performed by complex MB consortia, first reported in wastewater high-rate algal ponds (HRAP) (Oswald et al. 1953). However, the associations of MA and cyanobacteria with other aerobic or anaerobic microorganisms have been known for years in diverse natural environments (Subashchandrabose et al. 2011). These interactions take place in the microenvironment immediately surrounding individual algal cells, known as the phycosphere (González-González and De-Bashan 2021). The term 'Phycosphere' was used for the first time in 1972 (Bell and Mitchell, 2016), and it was defined as "a zone that may exist extending outward from an algal cell or colony for an undefined distance, in which the bacterial growth is stimulated by the extracellular products of the algae". The exchange of metabolites and other chemical compounds in this area governs MB relationships, which encompassing different types of interactions such as mutualism, commensalism, antagonism, parasitism and competition. Despite, these interactions taking place in each individual phycosphere, they exert their influence on an ecosystem-scale on fundamental processes like nutrients consumption and its regeneration (Seymour et al. 2017).

As occurs in nature, the use of MA for WWT includes the establishment of MB interactions through the phycosphere, which determine the WWT efficiency (Wirth et al. 2020). The phycosphere is comparable with an oasis for bacteria because it is in this area where gas exchange occurs, by which bacteria supply CO_2 to the MA and the MA excrete O_2, essential for bacterial growth (González-González and De-Bashan 2021). Moreover, this interaction is beneficial for MA because bacteria play a key role in providing phytohormones and macro-and micronutrients to MA, resulting in a notably enhanced growth rate of MA. This has been observed eliminating the phycosphere bacterial communities, thus giving rise to a very slow microalgal growth (Ramanan et al. 2016).

Using molecular sequencing technology it was previously demonstrated that in a typical wastewater treatment plant are present up to 3000 different microbial species. However, most of them are in very low abundance and presumably are not relevant to the treatment processes, with only a few hundred being abundant and important (Nierychlo et al. 2020). This fact has traditionally resulted in the focus on only a few microbial groups in WWT processes, which are currently considered in microalgae-bacteria WWT because they interact with MA, and influence nutrient removal/recovery. In the field of WWT, the N and P sources are classified mainly as nitrogen in form of ammonium (**NH_4^+**), nitrogen in form of nitrate (**NO_3^-**) and phosphorous in form of phosphate (**PO_4^{-3}**), and its concentration along with the **organic matter** concentration, usually measured as Chemical Oxygen Demand (**COD**) in the WWTP, has a strong influence on microbial populations (Higgins et al. 2018).

Indeed, the basis of the biological removal in the wastewater treatment ponds is the symbiotic interactions of **MA** and **HET**. Under light conditions, MA realize the photosynthetic activity, which releases O_2 that is used as an electron acceptor by HET to degrade organic matter. In turn, CO_2 from the bacterial mineralization is used as a C source by microalgal cells, completing the photosynthetic cycle (Muñoz and Guieysse 2006; Subashchandrabose et al. 2011). Apart from HET, in sewage treatment processes the **NIT** is one of the most important bacterial groups. It is responsible for producing large amounts of **$N–NH_4^+$** through the decomposition of organic matter. NIT are autotrophic bacteria that perform nitrification in the presence of DO. Specifically, it's possible to distinguish between **ammonia-oxidizing bacteria (AOB)** which transform the NH_4^+ to nitrite (NO_2^-), and subsequently, **nitrite-oxidizing bacteria (NOB)** that transform NO_2^- to NO_3^- (Prosser 1990).

In MB processes, complex interactions occur between MA, AOB and NOB. On the one side, some studies have proposed that MA could stimulate the nitrification process because they release O_2 via oxygenic photosynthesis, thereby stimulating NH_4^+ oxidation (Risgaard-Petersen et al. 2004). However, previous studies have revealed that nitrifiers' growth was inhibited by a high level of DO (Prosser 1990), which in microalgae cultures may exceed 300 %Sat during the sunlight (Chisti 2016). Moreover, It has been suggested that MA inhibit nitrification by reducing NH_4^+ availability for AOB (Risgaard-Petersen et al. 2004). On the other side, AOB can proliferate faster than NOB in the culture, resulting in a NO_2^- accumulation in the systems which can inhibit photosynthetic electron transport in microalgal cells (González-Camejo et al. 2020). Therefore, AOB and NOB play an important role in MB consortia and its balance is strongly affected by the operational conditions of the MB systems (Sánchez-Zurano et al. 2021).

Also, in WWT bacteria may appear which reduce NO_3^{-2} and NO_2^{-} to nitrogen gas (denitrification process). These bacteria, named **denitrifiers**, are a group of HET that use NO_2^{-} or NO_3^{-} as the electron acceptor in the respiration process and obtain energy from organic substances. Denitrifiers are active under anoxic conditions, which means that the DO concentration should be less than 0.5 mg/L. However, a low DO concentration is not usual in MA cultures because MA produce O_2 under light conditions, which can inhibit the denitrification process (Jia and Yuan 2017) (Figure 2).

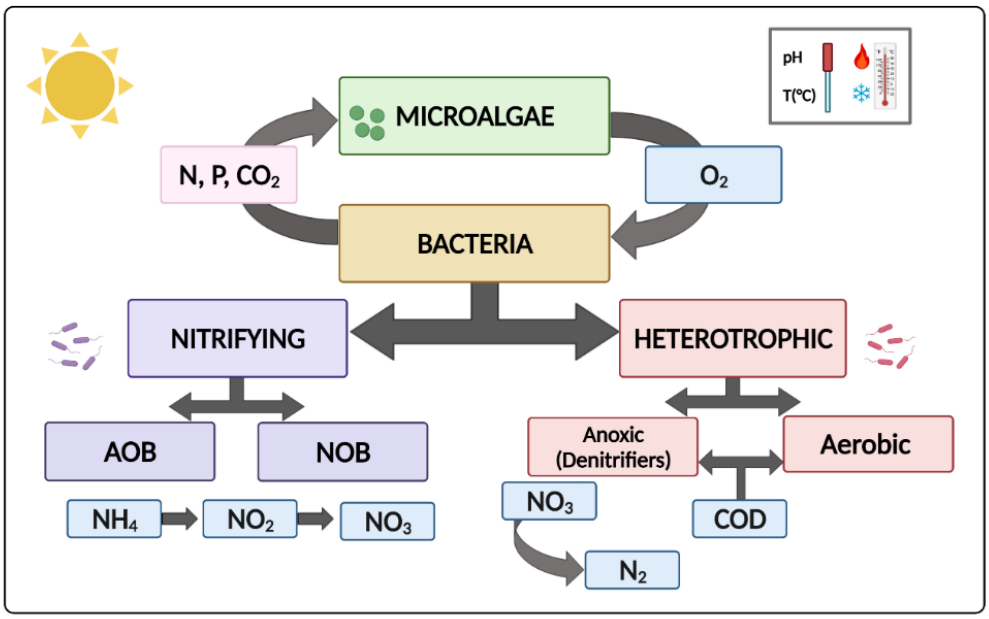


Figure 2.- Major interactions between MA and bacteria in microalgal-based wastewater treatment. Microalgae produce O_2 by photosynthesis while consuming CO_2 and nutrients (principally N and P). At the same time, aerobic heterotrophic bacteria use the O_2 to oxidize the organic matter present in the influent. The O_2 is also used by Nitrifying bacteria to oxidize the NH_4^+ to the most nitrogen form, NO_3^-.

Regarding the **PO_4^{-3}**, conventional activated sludge plants typically have a poor phosphorus removal efficiency and, to improve the biological phosphorus removal, they favor the development of **polyphosphate-accumulating organisms (PAOs)**. These bacteria assimilate phosphate as polyhydroxyalkanoates under anaerobic conditions while releasing the phosphate under the aerobic stage (Fallahi et al. 2021). Conversely, MA can accumulate polyphosphate directly. Under some environmental/operational conditions, MA may perform the luxury P-uptake phenomena, defined as the uptake of P by microalgal cells beyond that required for growth, and storage of **PO_4^{-3}** within the biomass as PolyP (>1% P dry weight). Also, MA can preserve to preserve their poly PolyP granules for several days, whereas bacteria tend to rapidly re-release their stored P (Slocombe et al. 2020). In MB cultures, one advantage is that the phenomenon of

PO_4^{-3} released by PAOs under aerobic conditions will be assimilated by MA, contributing to complete PO_4^{-3} removal (Zhang et al. 2021).

Therefore, achieving correct associations between MA and bacteria is the key to the success of biological microalgal-based WWT. However, they are complex interactions, which require a deep biological and engineering knowledge of the process, and powerful tools that allow their monitoring and optimization (Solimeno and García 2017).

2.3. Biological models for microalgae-bacteria consortia

For decades, researchers have looked for tools that will allow them to gain insights into the complex interactions that occur in biological systems. Currently, one of the best available tools is mathematical modelling. Most of the models are based on experimental observations and allow to save experimental difficulties such as carrying out rapidly on experiments that are not currently experimentally feasible (Brodland 2015). Particularly, mathematical models have proven to be useful tools to assess and optimize the performance of MB consortia in biological WWT systems (Solimeno and García 2019). Most of the biological MB models for WWT are based on the coupling of the traditional biological processes in AS (most of them are bacteria-made), together with the phototrophic processes that occur in microalgae/cyanobacteria cultures.

2.3.1. Activated sludge models: the role of bacteria

Traditional bacterial models for activated sludge are inspired by the mechanistic models developed by the International Water Association (IWA), known as the Activated Sludge Model (ASM) series. ASM models allow the description of the biological phenomena taking place in AS, it is of great importance because provides researchers and practitioners with a standardized set of basic models for biological WWT processes (Damayanti et al. 2009). In 1987, the first model (ASM1, Activated Sludge Model No 1) was developed to obtain a simple model to understand the biological processes that appear in activated sludge systems (Henze et al. 1987). The ASM1 model included the presence of two microbial populations: autotrophs and heterotrophs. The autotrophs are assumed to be NIT. Their growth is associated with the conversion of soluble ammonium nitrogen into soluble nitrate and nitrite nitrogen. The heterotrophic biomass could grow in aerobic conditions and anaerobic conditions (denitrifying heterotrophic biomass), they remove soluble organic carbon and they are responsible for biodegradation of slowly biodegradable particulate matter and biodegradable organic nitrogen (Nelson et al. 2019). Over the following decades, the IWA task group developed new activated sludge models (ASM2, ASM2d, ASM3) that allowed to improve the modelling of the biological

systems introducing more microbial populations and biological processes (Henze, M., Gujer, W., Mino, T., & van Loosdrecht 2000).

Nowadays, ASM models are still considered the most important mathematical models for WWT simulations despite some drawbacks such as the values of coefficients related to bacteria processes (growth and decay) are considered constant for any given type of wastewater or the kinetic parameters were calibrated in a close temperature and pH range (Solimeno and García 2017).

2.3.2. Microalgal biological models

Many experimental studies were conducted to assess the effect of individual parameters such as light, temperature, nutrients (N, P, C), pH, salinity, and DO on microalgal growth or its photosynthetic activity. Based on this information, multiple models have been developed to describe microalgal photosynthesis and growth kinetics (Darvehei et al. 2018). In general, microalgal kinetic models could be classified into three groups considering (Lee et al. 2015): (i) Under light-saturating conditions the growth of microalgal cells depends on the availability of nutrients such as N, P and C, in the culture media, thus these models only consider a single substrate factor (N, P or C); (ii) Under nutrients excess conditions the nutrients are presented in excess in the media, so the light intensity is the limiting factor for the growth; and (iii) Under multifactor limiting conditions all the factors determining the performance of microalgal cells such as the nutrient's availability, the light intensity, and the environmental and operational conditions, influences the overall performance of the system.

Currently, most of the current models for MA recognize the effect of multiple limiting factors. These models considering multiple factors provide a better explanation of the microalgal growth which is based on either threshold or multiplicative theories (Lee et al. 2015). The threshold theory, also called the minimum law, considers that the overall growth rate is affected only by the most limited resource among all resources required by cell growth (Lee and Zhang 2016), while the multiplicative theory assumes that all variables are simultaneously influencing the overall microalgal growth rate. Currently, the first approach is the most common used for microalgal models such as adopted by Costache et al. (Costache et al. 2013) to describe the influence of environmental parameters (light, temperature, pH and DO) on the photosynthesis rate of *Scenedesmus almeriensis*. Moreover, the multiplicative model was used by Franz et al. to determine the growth rate of *Chlamydomonas reinhardtii* as a function of light, temperature, DO and nutrients (A et al. 2012) and by Solimeno et al. (Solimeno et al. 2015) to evaluate the growth of *Scenedesmus sp.* as a function of light intensity and temperature, as well

as the availability of N and other nutrients. In large scale systems, the impossibility of controlling all the culture parameters, multiple variables models are preferred (Fernández et al. 2014; Hoyo et al. 2022).

2.3.3. Microalgae-bacteria biological models

The most preliminary MB model for WWT was developed in 1983 by Buhr and Miller (Buhr and Miller 1983). This model considered the existence of two main populations, MA and HET, the phenomena of nitrification and denitrification, which may be expected to occur in the high-rate wastewater treatment pond was ignored. In this model, the algal growth was limited by CO_2, N, and light availability, while HET were limited by organic substrates, DO and N. From it, different mathematical models were developed and validated using different types of WW and production systems. One of the most cited models was developed by Reichert et al. (Reichert et al. 2001) 2001. In reality, the model, **River water quality model no. 1** (RWQM1), was developed for water quality management, especially in rivers, but it was used as a basic model for MA treatment systems as it considered MA as well as bacteria. This model was considered as a real compact model for microalgae WWT. However, the application of RWQM1 model was scarce and limited because the model structure was complex, the model was often overparameterized and it needed a better knowledge about algae processes (Fu et al. 2020).

Other models were proposed for MB consortia. Sah et al. developed a comprehensive model of WWT in secondary facultative ponds (Sah et al. 2011). The model was based on the ASM2 from the ASM series (Henze, M., Gujer, W., Mino, T., & van Loosdrecht 2000) for describing aerobic and anoxic bacteria processes, and the part related to algae growth was based on the RWQM1. This model along with RWQM1 was considered the most complex model for microalgae-based wastewater treatment, although they did not consider important issues that happen in MB cultures as C limitation on the growth of MA and autotrophic bacteria, and the possible effect of high DO concentration in mixed liquor on microalgal performance.

In 2016, Wágner et al., developed an extension of the ASM2d to introduce the green microalgal growth (ASM-A) using the systematic approach of the ASM framework. To develop the microalgal model, the authors used some parameters from the literature and others obtained experimentally using laboratory-scale tests. The most remarkable aspect was that they considered both photoautotrophic and heterotrophic microalgal growth and nutrient uptake and storage (Droop model). Despite this model predicting some interactions between bacteria and MA, direct interactions between algal and

bacterial growth were not considered, and bacterial processes were assumed negligible during the experiments. For this reason, this model was only considered a possible extension to be integrated into the conventional ASM models.

For the same year, Zambrano et al., developed a simple MB model to simplify the expressions for the process rates and to reduce the number of the model components and parameters that include the majority of biological models. In this model, the authors considered the existence of two main populations: algae and bacteria, that perform the nitrification process. The concept of the model was easy: the algae grow with light, consume substrate containing either C or N and produce DO. The carbon component in the substrate is modelled as dissolved carbon dioxide, and the N is modelled as dissolved ammonium and nitrate, whereas the bacteria grow with dissolved oxygen and ammonium and produce nitrate and dissolved carbon dioxide. The bacteria processes were inspired in ASM models while the algal part was based on Solimeno et al. 2015. Despite its simplicity, the model gave a good starting point for further research in describing the dynamic behavior of the consortia of MB, since on many occasions, the over parameterization masks the real dynamics of the biological system.

One year later (2017), Solimeno et al., completed the microalgal model (BIO_ALGAE) previously developed (Solimeno et al. 2015), including crucial improvements for microalgal processes as well as bacteria processes in WWT systems. The authors considered four main populations: MA, HET, AOB and NOB. The inorganic C limitation for MA and NIT was one of the main features included in the model. This model was improved in BIO_ALGAE 2 (Solimeno et al. 2019), which introduced some new aspects in the biological model as the effect of culture conditions prevailing in microalgal cultures (pH, temperature, DO) on microalgal and bacterial activities. BIO_ALGAE 2 used cardinal equations to represent the inhibitory effects on the growth response of MA and bacteria at inadequate culture conditions, such as low and high pH and temperature respectively. The model was based on the cardinal temperature model (CTMI) (Bernard and Rémond 2012). The cardinal model allows obtaining the minimum, maximum and optimal values of temperature culture conditions, such as pH and temperature, for MA and bacteria.

Currently, one of the most advanced models for treating WW with MA is the ALBA model (Casagli et al. 2021b). The ALBA model was based on mass balances of COD, C, N and P, but also H and O. It described the growth and interactions among MA, HET, AOB, NOB and denitrifiers in pilot-scale raceway reactor. The model was validated along the different seasons over more than one year. Despite this model along with its

predecessors, showed a high short term and long-term prediction capability, they had two main drawbacks:

1. They were hyper parameterized, which make their application in the operation of regular wastewater treatment systems complicated.
2. Most of the parameters were extracted from the literature or obtained from calibration, so they needed experimental validation.

Therefore, this last aspect open a fundamental discussion when developing biological models, what tools or techniques are available for their development and validation? Which of them are feasible in research laboratories and are reproducible?

2.4. Photo-respirometry for microalgae-bacteria modelling

During the last years, different methods to study microalgal–bacterial community interactions have been proposed. Proposed technologies include microscopy, cell sorting, omics, and genetic engineering, all of them showed different limitations, advantages and disadvantages (Mu et al. 2021). However, these technologies were sometimes not available because are expensive and large time-consuming.

Therefore, developing cheap and fast methods for the assessment of microorganisms' activity in microalgae-based wastewater systems is essential. In this sense, respirometric techniques have an interesting role in monitoring and optimization of biological processes. Respirometry has been traditionally applied to AS to evaluate microbial load at different treatment stages (Spanjers and Vanrolleghem 1995). Moreover, these techniques were expanded to phototrophic cultures to study MA and cyanobacteria photosynthetic activity (Dubinsky et al. 1987; Decostere et al. 2013; Sforza et al. 2019). In recent years, respirometric techniques have been adapted to be applied in mixed cultures whit microorganisms that are oxygen-consuming and oxygen-producing. This technique was called **Photo-respirometry** (Ariza 2018).

The photo-respirometry allows evaluating the contribution of the main microbial populations that appear in the MA consortia (heterotrophic biomass, NIT and MA) depending on the production and consumption of oxygen through specific protocols based on the use of substrates and selective inhibitors (Rossi et al. 2020c). During the last years, many different protocols were developed for conducting respirometric tests on microalgal-bacterial cultures, given the lack of official guidelines. Briefly, they are all based on alternating periods of light and darkness at different times. During periods of light, the Oxygen Production Rate (OPR) due to the photosynthetic process can be measured, while in periods of darkness, the Oxygen Consumption Rate (OCR) of the

endogenous cultures can be measured. Following these, microbial activity measurements based on oxygen consumption can be properly detected by adding substrates/inhibitors that allow to selectively activate/inactivate specific microalgal or bacterial metabolisms (Rossi et al. 2018; Petrini et al. 2020). In the case of heterotrophic biomass, organic substrates such as sodium acetate are used, while for NIT, it would be possible to distinguish between AOB, which are mainly detected using ammonium chloride, and NOB, whose activity is detected with sodium nitrite (Rossi et al. 2020b).

Moreover, in recent years, respirometry has been applied in microalgae-bacteria modelling because traditional batch experiments based on measures of biomass (or chlorophyll) to obtain a kinetic characterization of the biological processes are expensive and time-consuming. Respirometry is considered as an alternative since it is a low-cost, fast, non-destructive, and non-invasive approach to determine kinetic data (Sforza et al. 2019; Flores-Salgado et al. 2021). From a practical point of view, respirometric protocols based on light/darkness, using specific substrates and inhibitors, allow evaluating the activity of each microbiological population under different environmental and operational conditions. These experimental data are a powerful tool to develop and calibrate microalgal-based WWT models quickly and at a low cost. A simple approximation of this methodology was developed in 2016 (Decostere et al. 2016). In this work, the authors calibrated and validated microalgal growth as a function of inorganic C, N and P concentrations to predict nutrient removal by microalgal biomass in WW using combined respirometric–titrimetric data. A similar approach was later used by performing photo-respirometric tests to calibrate the most sensitive parameters (maximum growth rate of bacteria and algae, yield coefficients on N, affinity constant for CO_2) in algae-bacteria models (Zambrano et al. 2016).

This methodology, together with other simple methodologies such as the traditional plate count and other more rigorous ones, such as flow cytometry, epi-fluorescence microscopy, metabarcoding or metatranscriptomic, give rise to an in-depth knowledge of microalgae-bacteria systems, and they open a field of possibilities to develop and validate biological models experimentally, which can be continually fed back with new data generated. Therefore, this thesis is focused on developing a simple and robust biological model that describes MB interactions in WW. To build this model, photo-respirometry was used to determine the characteristic parameters of MA and bacteria under different environmental and operational conditions. Moreover, photo-respirometry was used as a tool to validate the proposed biological models and to establish feedback between the mathematical modelling and the photo-respirometric technique.

3.MATERIALS AND METHODS

3.1. Microorganisms and culture conditions

The experiments performed in the University of Almeria (Spain) were performed using the microalga *Scenedesmus almeriensis* (CCAP 276/24, Culture Collection of Algae and Protozoa of the Centre for Hydrology and Ecology, Ambleside, UK). *S.almeriensis* is characterized by a high growth rate both in fertilizer- and WW-based media, with an optimal growth temperature of 35°C, and capable of withstanding up to 48°C. This strain is also tolerant to high irradiances (Sánchez et al. 2008). The inoculum of this strain was grown photoautotrophically in a spherical flask (1.0 L capacity) and renewed weekly with fresh modified Arnon medium (Allen and Arnon 1955) (Table 1). The microalgal culture was continuously aerated with air containing 1 % CO_2 to keep the pH constant at 8.0. The spherical flasks were maintained at 24 ± 1 °C; the temperature was controlled by regulating the air temperature inside the chamber. The culture was artificially illuminated on a 12:12 h light:dark cycle using four Philips PL-32W/840/4p white-light lamps, providing an irradiance of 750 $\mu mol_{photons}/m^2 \cdot s$ on the surface of the reactor. During the experimental trials, *S. almeriensis* was produced using different cultivation systems and using both, the modified Arnon medium used as the control and different types of WW namely. Primary domestic WasteWater (PWW), pig manure WW and agricultural waste leachates. For the experiments carried out in Politecnico di Milano (Italy), a natural bloom of microalgae grown under outdoor environmental conditions was used. The predominant microalgal genus observed by microscopy were *Chlorella* and *Scenedesmus*.

Table 1. Average composition of the modified Arnon medium. Concentrations expressed as $mg \cdot L^{-1}$. COD: chemical oxygen demand, TC: total carbon; TN: total nitrogen; TP: total phosphorous.

Parameters	Arnon media
pH	7.5 ± 0.2
COD	16.0 ± 1.2
Sulphate	6.3 ± 0.8
Nitrogen-Nitrate	140.0 ± 4.5
Chloride	78.9 ± 2.1
Sodium	276.1 ± 7.9
Potassium	325.1 ± 6.3
Calcium	364.9 ± 5.5
Magnesium	12.2 ± 0.6
Phosphorus-Phosphate	39.3 ± 3.1
Nitrogen-Ammonium	0.0 ± 0.1
Iron	5.0 ± 0.3
Copper	0.02 ± 0.0
Manganese	0.5 ± 0.02
Zinc	0.06 ± 0.01
Boron	0.4 ± 0.03
TC	52.4 ± 4.9
TN	140.0 ± 4.5
TP	39.3 ± 3.1

Values from the Arnon media correspond to the mean mean ± SD (n=3).

3.2. Lab-scale photobioreactors

Laboratory-scale experiments were performed using four cylindrical photobioreactors made of polymethylmethacrylate (0.08 m in diameter, 0.2 m in height and 1 L capacity) (Figure 3). The reactors were inoculated with 20% of *S. almeriensis* cultures at a concentration of 0.8 g/L and filled up to 0.8L with culture medium. The photobioreactors were operated indoors but simulating outdoor sunlight conditions. To simulate the outdoor solar cycle, the reactors were artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5). The maximum irradiance (PAR) inside the reactors without cells was 1000 $\mu molm^{-2}\ s^{-1}$, measured using an SQS-100 spherical quantum sensor (Walz GmbH, Effeltrich, Germany) at midday. Firstly, the photobioreactors were operated in batch mode for 7 days to obtain the maximum biomass concentration, that was stable for three days. Then, they were operated in semi-continuous mode by removing 20% of the culture every day and replacing it with a fresh culture medium. The concentration reached during the

continuous period was 0.6 ± 0.2 g/L. The dissolved oxygen in the culture was controlled below 200% Sat by on-demand injection of air to avoid negative effects because of excessive dissolved oxygen accumulation. Also, the pH was controlled at 8.0 using pure on-demand CO_2 injections. The culture temperature was kept at 25 ± 0.2 °C by controlling the temperature of the culture chamber in which the photobioreactors were located.

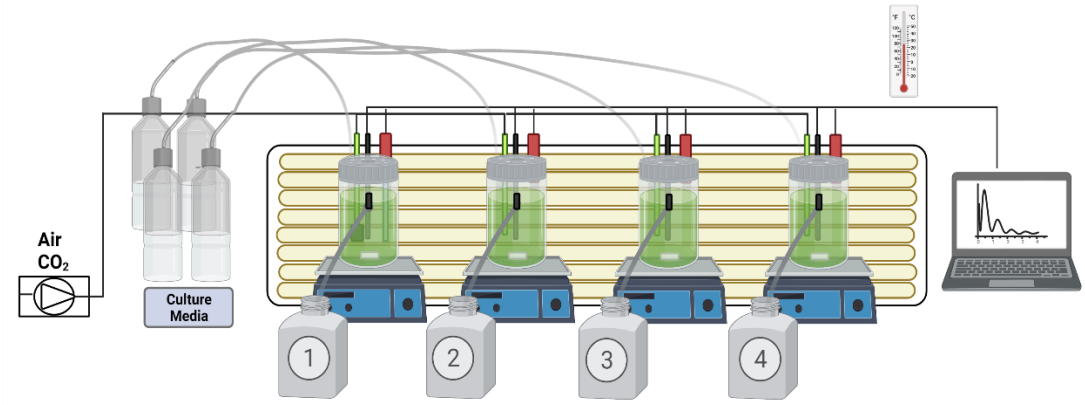


Figure 3.- Scheme of lab-scale hand-made photobioreactors utilized during the experiments.

3.3. Monitoring of the biological systems

The biomass concentration (Cb) of the microalgal cultures was measured daily by dry weight. Aliquots containing 100 mL of the culture were filtered through Macherey-Nagel MN 85/90 glass fibre filters. For that, the filters were placed on a büchner funnel made of porcelain in contact with a vacuum flask connected to a vacuum pump. After that, the filters were dried in an oven at 80ºC for 24 h.

The status of the cultures being produced under different environmental and operational conditions was evaluated by measuring the maximum quantum yield of the photosystem PSII. The photosynthetic activity can be determined in a non-intrusive way under different conditions through the fluorescence of chlorophyll associated with photosystem II. For these measurements, an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic) was used. For this purpose, the microalgal culture was previously incubated for 15 minutes under dark conditions to ensure that all the reaction centres were closed, then the optimal quantum yield (Fv / Fm) was determined.

3.4. Microalgal Demo Plant

MB samples for respirometric trials were obtained from the microalgal production plant is located at IFAPA La Cañada, next to the University of Almería in Spain. The facilities include (i) lab-scale photobioreactors as bubble columns (0.250 L) and stirrer-tank reactors (1L), (ii) closed photo-reactors equipped with bubble columns (100 L) and

closed photobioreactors (tubular photobioreactors of 3 m^3), (iii) an open reactor module under a greenhouse equipped with raceway reactors (3 raceway of 80 m^2) and (2 thin-layer reactors of 60 m^2 and 120 m^2), with a total area of 400 m^2, (iv) an open-air reactor module equipped with raceway reactors (2 raceways of 80 m^2, 3 raceways of 10 m^2) and thin-layer (1 thin-layer reactor of 30 m^2, and 2 thin-layer reactors of 10 m^2), with a total area of 800 m^2, and (v) an industrial reactor module equipped with a raceway reactor of large scale (700 m^2). All these reactors are monitored and controlled online, being operated automatically according to industry standards. For the respirometric experiments these systems were maintained with different culture media (freshwater and WW media). More details in Section 4.1. “A novel photo-respirometry method to characterize consortia in microalgae-related wastewater treatment processes”.

3.5. Analytical methods

Some standard official methods were performed to analyze the chemical composition of wastewaters used and the microalgae-bacteria cultures. The nitrate was measured by measuring optical density at 220 nm and 275 nm (Nitrate Standard for IC: 74246) using a Thermo ScientificTM GENESYS 10S UV-Vis spectrophotometer. The ammonium was quantified according to the Nessler method (Ammonium standard for IC: 59755). The phosphate was measured by visible spectrophotometry through the phospho-vanado-molybdate complex (Phosphate Standard for IC: 38364). The Chemical Oxygen Demand (COD) was determined by spectrophotometric measurement using Hach-Lange kits (LCI-400).

3.6. Modelling approach

To achieve the main objective of the Thesis, developing and validating a biological model for microalgae-bacteria wastewater treatment, a modelling approach was defined. The biological model is a series of equations representing the different biological processes in which microalgal and bacterial populations participate. To achieve this purpose, it was essential to define the different steps to be followed such as experimental tests, individual models’ definition and individual model validations. Next, a global model was proposed, then performing the calibration of the global model and its validation with new experimental data.

Firstly, photo-respirometric experiments were done to obtain reliable data for the mathematical model. The experimental data were filtered to reduce the dispersion and ensure adequate data quality. The experimental data allowed to assess the influence of each environmental and operational variable on MA, HET and NIT, which were fitted to

the corresponding equations using Statgraphics Centurion XVI software package and Microsoft Excel. After this step, the kinetics parameters for the microbial populations considering the variable study were obtained. Then, the proposed models along with the corresponding parameters were used to develop individual models to describe the growth of MA, HET and NIT in WWT conditions. Consecutively, the individual models allowed to propose a global mathematical model with multiple processes, which include both particulate and dissolved components. Afterwards, the model parameters (previously obtained by the individual models) were adapted by the calibration process using part of the data from the experiments. Matlab Software was used to carry out the model calibration process using genetic algorithms through the Genetic Algorithm Optimization Toolbox (GAOT), based on (Houck et al.)

Finally, the model validation with experimental data was performed using Matlab Software. Then, the modelling process ends when the model validation succeeds with adequate goodness of fitting to the real data.

3.7. Statistical analyses

Data analysis was carried out using the Statgraphics Centurion XVI software package, in which non-linear regression was used to fit experimental data to the proposed models and to determine the characteristic parameter values. Also, this package allowed to obtain the statistical analyses of the experimental data. Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was $p < 0.05$.

4. RESULTS AND DISCUSSION

To develop a mathematical model of MB interactions in WWT, a photo-respirometric protocol was first developed to differentiate the three main microbial populations that appear in these systems (MA, HET, and NIT). The protocol was used to study the effect of environmental and operational variables such as light, temperature, pH, DO, and N and P concentrations on their activity. These generated data were fitted to individual mathematical models, widely used in the WWT literature. With the coupling of the proposed models and their corresponding parameters, the ABACO biological model was proposed, which was calibrated and validated on a laboratory scale. This model was integrated into an interactive tool that allowed simulating the effect of each parameter that influenced the biological system in real-time. Finally, a few improvements to the model were proposed, starting with the incorporation of two different population withing the NIT group (AOB and NOB), and the use of novel improved respirometry techniques to obtain new model parameters to reduce uncertainty and the utilization of parameters obtained from the literature.

4.1. A novel photo-respirometry method to characterize consortia in microalgae-related wastewater treatment processes.

4.1.1. Photo-respirometer

To develop a photo-respirometric protocol that allows distinguishing between the activity of MA, HET, and NIT, the required equipment was first designed and developed. The developed device allowed users to measure any variation in DO in MB samples under operational and environmental controlled conditions (irradiance, temperature, pH, DO, nutrients concentration, etc.). The photo-respirometer consisted of an 80 mL jacketed transparent cylindrical glass flask, connected to a temperature-controlled water reservoir for the control of device's temperature. The reactor was magnetically stirred at 250 rpm and artificially illuminated using two power-controlled LED lamps (Secom Iluminacion 4125015085DR, Spain) placed to the right and left of the glass chamber (Figure 4). The average irradiance inside of the culture (I_{av}) can be automatically regulated and controlled to achieve the desired value once the sample was added. The average irradiance was measured using a QSL-1000 sensor (Walz, Germany). Moreover, the unit was equipped with a gases diffuser that can supply a low flow rate of the desired gas (air, O_2, N_2 and CO_2) to modify the culture's dissolved oxygen or pH. The photo-respirometer is also equipped with sensors for temperature (PT-100), pH (Crison 5343, Barcelona, Spain) and dissolved oxygen (Crison 5002, Barcelona, Spain) located inside the flask (Figure 4). The entire system was computer-controlled using DaqFactory software.

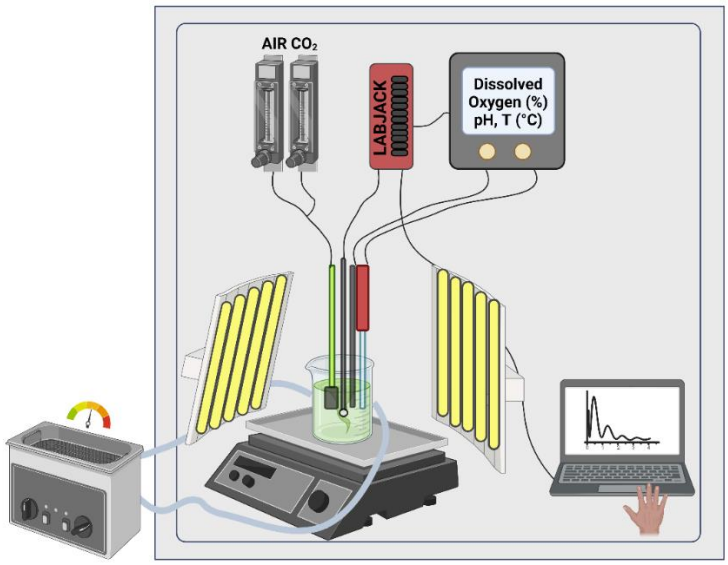


Figure 4.- The layout of the respirometer.

4.1.2. Photo-respirometric protocol

A novel protocol was developed to determine the microalgal, heterotrophic and nitrifying activity in MB suspensions. The photo-respirometric method allowed distinguishing between the photosynthetic activity of MA under light conditions and the respiratory activity of bacteria when using specific substrates in the dark. That is, it allowed distinguishing the contribution of each microbial population in terms of production and consumption of DO. The first step to distinguish between the microbial population was to subject the MB culture to nutrient starvation. In this respect, the culture was continuously illuminated at 200 $\mu molm^{-2}\cdot s^{-1}$ and aerated at 0.2 $v\cdot v^{-1}\cdot min^{-1}$ for 24 hours to remove the organic matter and the NH_4^+ present in the medium. This point was essential to ensure that the culture was without organic matter and NH_4^+ to have a bacteria response at adding the specific substrates.

Once the culture overpassed the starvation period, cycles of light and darkness were applied, along with the addition of substrates and inhibitors according to the protocol described below. Each light and dark period took four minutes, on which the variation of DO concentration over time was measured and registered by the respirometer. The first minute of exposure was considered adaptation time and it was discarded. The variations of DO concentration under four different conditions allowed calculating the respective metabolic rates. In the section below, each metabolisms determination (MA, HET and NIT) are described in detail (Figure 5), including the expected biological reactions related to the DO concentration under the condition exposed:

A. **Microalgal Net Photosynthetic Rate (MNPR).** To determine microalgal activity, a sample from the starvation culture was placed in the glass flask inside the photo-respirometer. Firstly, the air was provided by the diffuser to start the measurements at 100 %Sat. The next step was to expose the MB culture to four light-dark cycles of four minutes each to measure and register the variation in DO concentration. Between the dark and light periods, the air was provided to recover the 100 %Sat. Under light periods, MA released O_2 that was generated by photosynthetic activity while the oxygen was consumed during the dark periods by the culture (endogenous respiration). The Microalgal Oxygen Production Rate (MOPR) was calculated as the slope of the DO concentration (generation) during the light period whereas the Endogenous Oxygen Consumption Rate (EOCR) was determined as the slope of the DO concentration (consumption) during the dark period. Finally, The MNPR was calculated as the difference between the MOPR minus the EOCR, divided by the dry weight of total biomass (Cb) (Equation 1). And the Microalgal Respiration Rate (MRR) was established as the EOCR.

$$\mathrm{MNPR} = \frac{\mathrm{MOPR} - \mathrm{EOCR}}{Cb} \qquad \text{Equation 1}$$

B. Heterotrophic Bacteria Respiration Rate (HBRR). To determine the HBRR, a new sample from the starvation culture was placed in the glass flask inside of the photo-respirometer equipment. Before making measurements, the air was provided by the diffuser until reaching 100 %Sat. Then, a specific organic substrate such as sodium acetate was used to detect the HBRR. For this purpose, 0.8 mL of sodium acetate (30 $g \cdot L^{-1}$) was added before starting the respirometric measurements. Acetate has been described as a substrate for use in wastewater respirometry tests (Spanjers and Vanrolleghem 1995). The next step consists of applying four light-dark cycles of four minutes each to MB culture and registering the variation in DO concentration under dark periods. Between the dark and light periods, the air was provided to recover the 100 %Sat. Under dark periods, the O_2 was consumed by endogenous respiration and heterotrophic respiration, it was namely Heterotrophic Oxygen Consumption (HOC). The HBRR is calculated as the slope of HOC minus the EOCR, divided by the dry weight of total biomass (Cb) (Equation 2).

$$\mathrm{HBRR} = \frac{\mathrm{HOC} - \mathrm{EOCR}}{Cb}$$ Equation 2

C. **Total Ammonium Respiration Rate (TARR).** To determine the TARR, a new sample from the starvation's culture was placed in the glass flask inside of the photo-respirometer equipment. Before making measurements, the air was provided by the diffuser until reaching 100 %Sat. Then, nitrogen substrate as ammonium chloride was used to detect the Microalgae Ammonium Respiration Rate (MARR) and Nitrifying Bacteria Respiration Rate (NBRR). For this purpose, 0.8 mL of ammonium chloride (3 $g \cdot L^{-1}$) was added before starting the respirometric measurements. The next step consists of applying four light-dark cycles of four minutes each to MB culture and registering the variation in DO concentration under dark periods. Between the dark and light periods, the air was provided to recover the 100 %Sat. Under dark periods, the O_2 was consumed by endogenous respiration, microalgal ammonium respiration and nitrifying bacteria respiration, it is namely Ammonium Oxygen Consumption (AOC). The TARR is calculated as the slope of AOC minus the EOCR, divided by the dry weight of total biomass (Cb) (Equation 3).

$$\mathrm{TARR} = \frac{\mathrm{AOC} - \mathrm{EOCR}}{Cb}$$ Equation 3

D. **Microalgal Ammonium Respiration Rate (MARR).** To determine the MARR, a new sample from the starvation's culture was placed in the glass flask inside of the photo-respirometer equipment. Before making measurements, the air was provided by the diffuser until reaching 100 %Sat. Then, N-Allylthiourea (ATU) (1 $g \cdot L^{-1}$) and ammonium chloride (3 $g \cdot L^{-1}$) was used to detect the MARR. For this purpose, 0.6 mL of ATU and 0.8 mL of ammonium chloride were added before starting the respirometric measurements. The next step consists of applying four light-dark cycles of four minutes each to MB culture and registering the variation in DO concentration under dark periods. Between the dark and light periods, the air was provided to recover 100 %Sat. Under dark periods, the O_2 was consumed by endogenous respiration and microalgae ammonium respiration, it is namely Microalgae Ammonium Oxygen Consumption (MAOC). The MARR is calculated as the slope of MAOC minus the (EOCR), divided by the dry weight of total biomass (Cb) (Equation 4).

$$\mathrm{MARR} = \frac{\mathrm{MAOC} - \mathrm{EOCR}}{Cb} \qquad \text{Equation 4}$$

E. **Nitrifying Bacteria Respiration Rate (NBRR).** Therefore, the NBRR was calculated as the difference between the TARR rate and the MARR (Equation 5).

$$\mathrm{NBRR} = \mathrm{TARR} - \mathrm{MARR} \qquad \text{Equation 5}$$

Respirometric measurements were performed between 80 and 130 %Sat. The oxygen mass transfer ($K_L a$) was minimum in this operating conditions. The $K_L a$ was determined in order to correct the influence of oxygen desorption on the MA and bacteria measurements. The method used consisted in measuring the DO concentration versus time profiles in the same chemical-physical conditions set during the experiments. The final value obtained was 1.08 h^{-1}. This value was used to correct the different metabolic responses (Sforza et al. 2019). Once the protocol was established, it was intended to optimize the intensity of light to determine the photosynthetic activity, as well as the biomass concentration at which to carry out the experiments to measure both the photosynthetic activity and the bacteria activities.

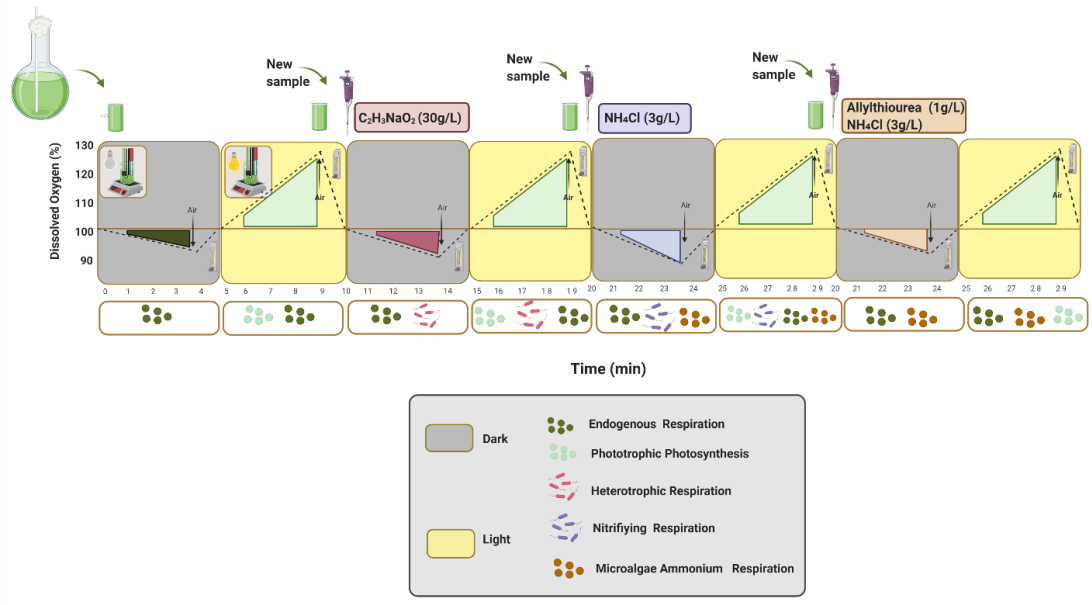


Figure 5.- Schematic photo-respirometric protocol to estimate the Microalgal Net Photosynthesis rate (MNPR), the Heterotrophic Bacteria Respiration Rate (HBRR) and Nitrifying Bacteria Respiration Rate (NBRR). Firstly, the MNPR is determined by alternating dark and light periods without any substrate. Then, HBRR is estimated using a new sample from the microalgae-bacteria culture and adding sodium acetate as a specific organic substrate. Also, for determining NBRR it is necessary to carry out a respirometric test using ammonium chloride as a substrate and another test with a new sample adding ammonium chloride and ATU. The figure shows the variation in dissolved oxygen with time in each dark and light phase before and after the addition of substrates that activate bacterial populations.

4.1.3. Light and biomass concentration conditions for photo-respirometry

Concerning the average irradiance, results showed that at low irradiance values (50 µmol photons/m^2·s) the microalgal photosynthesis rate was 16±4.3 $mgO_2 \cdot g^{-1} \cdot h^{-1}$ increasing with light availability up to 102.2 ± 3.5 $mgO_2 \cdot g^{-1} \cdot h^{-1}$at 500 $\mu molm^{-2} \cdot s^{-1}$, and then remaining constant up to values of 2000 $\mu molm^{-2} \cdot s^{-1}$ (Figure 6A). Regarding the heterotrophic and nitrifying activity, they did not show significant differences regardless of the irradiance values imposed. The microalgal activity was maximal at values of 500 $\mu molm^{-2} \cdot {}^{-1}$ but to avoid saturation during photosynthesis, an irradiance of 200 $\mu molm^{-2} \cdot s^{-1}$ was selected for the photo-respirometric measurements. Moreover, under real conditions, the cultures are mainly photo-limited, the average irradiance being from 100 to 300 $\mu molm^{-2} \cdot s^{-1}$ (Molina Grima et al. 1999).

Regarding biomass concentration, there was a significant relationship between the production/uptake of oxygen and the relative biomass concentration in the cultures ($p < 0.05$). For this reason, the optimal biomass concentration for respirometric measurements were determined. This variable greatly impacted the method's precision and sensitivity. Consequently, experiments were performed to determine the MNPR, HBRR and NRR at different biomass concentrations in MB cultures. The results showed that at 0.1 $g \cdot L^{-1}$, the MNPR was 30.41 ± 1.1 $mgO_2 \cdot g^{-1} \cdot h^{-1}$ while the HBRR and NBRR by the HET and NIT was much lower, 0.5 ± 1.2 and 0 ± 1.1 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, respectively, not being possible to obtain an adequate measure of heterotrophic and nitrifying activity at biomass concentrations of 0.1 $g \cdot L^{-1}$ (Figure 6B). A similar trend was observed at 0.2 $g \cdot L^{-1}$. At this concentration, the MNPR, HBRR and NBRR were 36.8 ± 0.9, 1.2 ± 1.1, 1.1 ± 1.25 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, respectively. The photo-respirometric trial at 0.4 $g \cdot L^{-1}$ allowed obtaining 35.8 ± 0.9 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, and the HBRR and NBRR were 1.9 ± 1.1 and 2.6 ± 1.2 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, respectively. These values were close the MNPR, HBRR and NBRR at 0.5 $g \cdot L^{-1}$ (37.1 ± 1.3, 1.9 ± 1.1, 2.4 ± 0.9 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, respectively). At 0.8 $g \cdot L^{-1}$, the MNPR was 32.9 ± 1.3 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, while the HBRR and NBRR were 1.4 ± 1.3, 2.2 ± 1.2 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, respectively. Therefore, a concentration of 0.4-0.5 $g \cdot L^{-1}$ allowed to obtain comparable values of MNPR, HBRR and NBRR. Moreover, the bacterial respiration rates were not within the limits of error. Thus, a concentration above 0.5 $g \cdot L^{-1}$ was selected, avoiding working at high biomass concentrations, in which two problems could appear. On the one hand, shaded areas could appear that would affect the MNPR values, and on the other hand, not all MB cultures reach a concentration greater than 0.5 $g \cdot L^{-1}$, since

they depend on factors such as the production system, the culture used and the variables of operation (Acién et al. 2016).

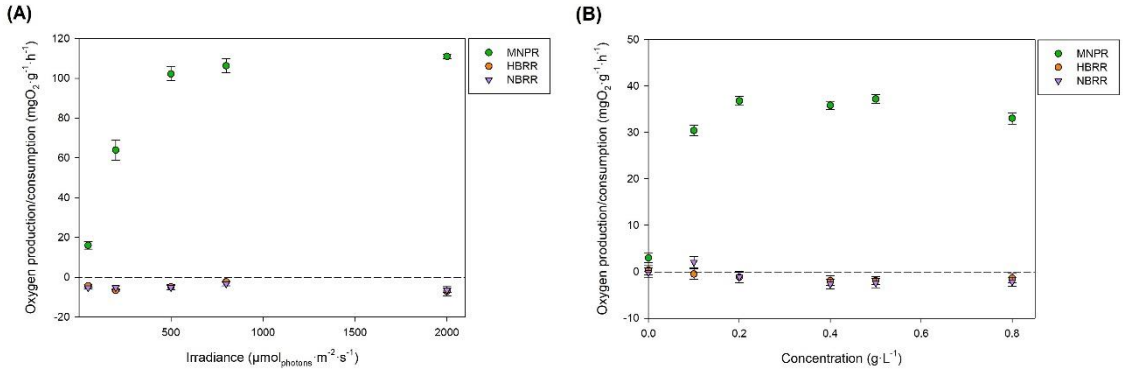


Figure 6.- Influence of irradiance (A) and biomass concentration (B) on microalgal, heterotrophic and nitrifying activity in microalgae-bacteria cultures. Error bars indicate the standard deviations obtained from four measurements. Abbreviations: MNPR, microalgal net photosyntetic rate; HBRR, heterotrophic bacteria respiration rate; NBRR: nitrifying bacteria respiration rate. Values correspond to the mean ± SD (n = 3).

4.1.4. Photo-respirometric evaluation of the microbial activity in different water types

Once the methodology was defined, it was used to assess the microbial activity in different production systems feed with diverse culture media: Freshwater (Arnon Media and Chemical Fertilizers), PWW, Pig Manure WW and Agricultural Waste Leachate (Figure 7). Results showed that the MNPR was higher than HBRR and NBRR by heterotrophic and nitrifying bacteria in all cases ($p<0.05$) Results showed that the MNPR was strongly affected by the type of culture media utilized. It was maximal in systems fed with freshwater containing modified Arnon and fertilizers (114.3 ± 1.7 $mgO_2 \cdot g^{-1} \cdot h^{-1}$). It decreased when different wastewaters were used ($p<0.05$).

On the other side, the HBRR was maximum using leachate of vegetal compost as culture medium (8.8 ± 1.3 $mgO_2 \cdot g^{-1} \cdot h^{-1}$) while the HBRR using animal manure as culture media was 7.6 ± 0.4 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, corresponding to the highest HBRR values measured during the photo-respirometric trials. These results agreed with the high chemical oxygen demand (COD) values recorded in these types of wastewaters (Acién et al. 2016),

The NBRR values were from 0.2 ± 0.5 to $mgO_2 \cdot g^{-1} \cdot h^{-1}$ $mgO_2/g_{biomass} \cdot h$ in MB cultures fed with freshwater and leachate of vegetal compost, respectively.

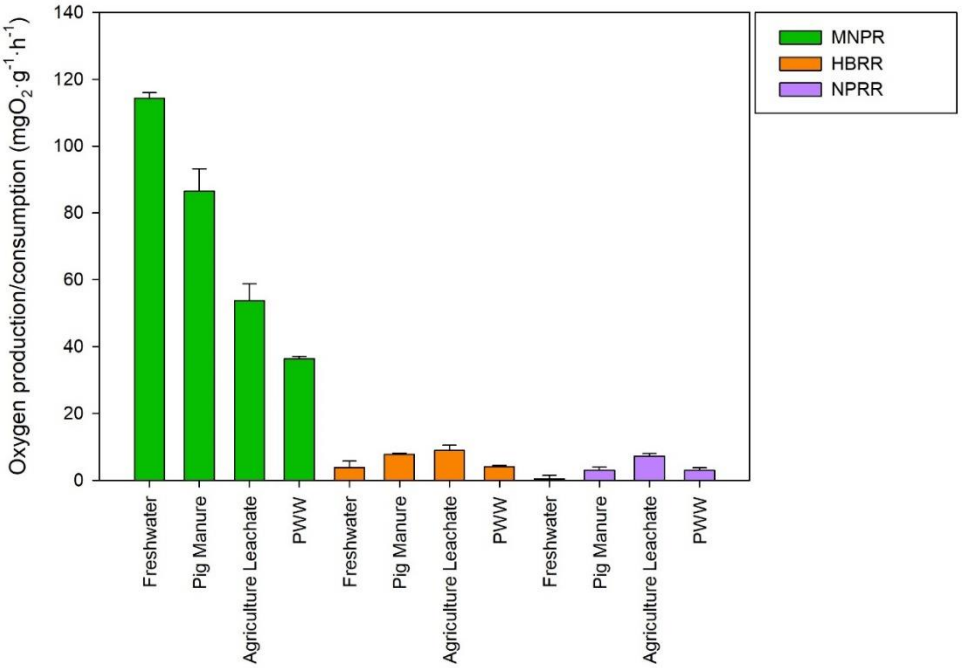


Figure 7.- Microalgal, heterotrophic and nitrifying activity (NBRR, HBRR and NBRR) in the microalgal production systems performed with freshwater and chemical fertilizers, pig manure wastewater, agricultural waste leachate and PWW. Abbreviations: MNPR, microalgal net photosyntetic rate; HBRR, heterotrophic bacteria respiration rate; NBRR: nitrifying bacteria respiration rate. Values correspond to the mean ± SD (n = 4).

Therefore, the experimental data confirms that, (i) photo-respirometry is an adequate methodology to evaluate MB suspensions and estimate the different microbial populations that appear in MB systems, (ii) in terms of oxygen production/uptake, MA are the main microorganisms contributing to the MB consortia, HET maintain a relatively stable contribution whatever the operational conditions, whereas the NIT contribution largely depends on the nitrogen load and the microalgal performance. However, comparing these novel data with literature data were not possible, because most of the paper were focus on phototrophic cultures without bacteria contributions or the studies determined the bacterial activities in AS systems, where MA were not considered.

4.2. Modelling of photosynthesis and respiration rate for microalgae–bacteria consortia.

Photo-respirometry allowed to determine the microalgal activity, heterotrophic activity and nitrifying activity in MB cultures by measuring the oxygen production/consumption under specific conditions. These measurements were rapid and easily obtainable (Tang et al. 2014; Petrini et al. 2020). Thus, this methodology led to estimate microbial activity under different environmental and operational conditions such as light, temperature, pH and DO in MB cultures using PWW as culture media. Also, the experimental data could be fit to suitable mathematical equations (Figure 8).

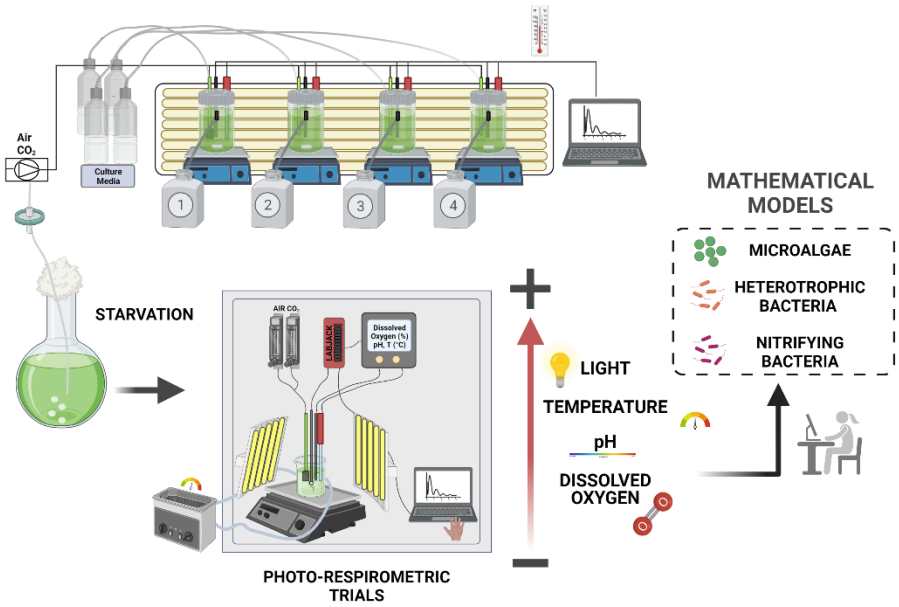


Figure 8.- Scheme of photo-respirometric trials performed to model microalgal, heterotrophic and nitrifying activity as a function of light, temperature, pH and DO concentration to which the cells were exposed.

4.2.1. Effect of the light on microalgae-bacteria consortia

Regarding irradiance, the MNPR was zero at zero irradiance and increased with irradiance to a maximum of 106 ± 5.1 $mgO_2 \cdot g^{-1} \cdot h^{-1}$ at an irradiance of 650 $\mu molm^{-2} \cdot s^{-1}$, then remained constant at higher irradiances. Photo-inhibition was not observed at high irradiance values. However, in previous studies, in which *Scenedemus almeriensis* was produced with freshwater, the MNPR increased with light availability up to values of 400 $\mu molm^{-2} \cdot s^{-1}$, remaining constant up to values of 1.000 $\mu molm^{-2} \cdot s^{-1}$, and finally decreased at higher irradiances (Costache et al. 2013). The data were fitted to the Molina model (Grima et al. 1994b) (Equation 6), in which the MNPR was a function of specific maximum photosynthetic rate ($PO_{2,max,ALG}$), average irradiance (I_{av}), irradiance constant that

represented the affinity of algae to light ($I_{k,ALG}$) and a form parameter ($n_{,ALG}$) (Figure 9A). The HBRR and the NBRR were not significantly affected by the irradiance.

$$MNPR=\frac{PO_{2,max,ALG}\cdot I_{av}^{n,ALG}}{I_{k,ALG}^{n}+I_{av}^{n,ALG}} \quad \text{Equation 6}$$

On the other hand, the MRR was evaluated during the dark periods after the illuminated periods. The respiration rate was 3.4± 0.3 $mgO_2 \cdot g^{-1} \cdot h^{-1}$ at zero irradiance, increasing with irradiance up to 16.4± 2.8 $mgO_2 \cdot g^{-1} \cdot h^{-1}$ at an irradiance of 1000 $\mu molm^{-2} \cdot s^{-1}$, then remaining constant up to 2000 $\mu molm^{-2} \cdot s^{-1}$ (

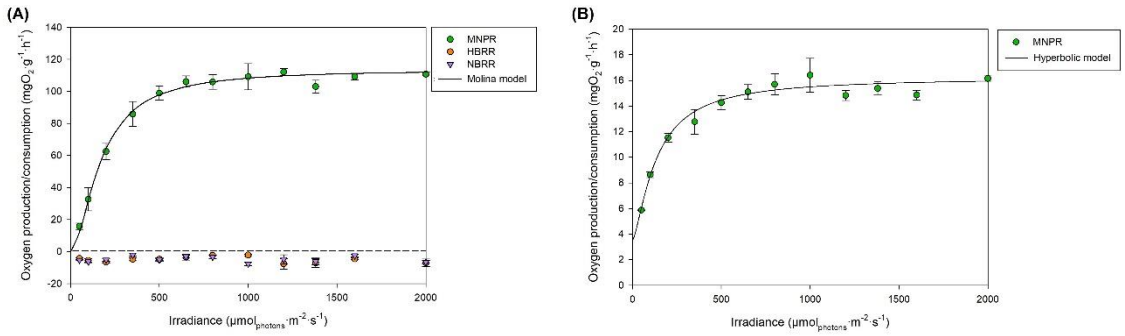


Figure 9B). Experimental data were fitted to the hyperbolic model with no inhibition (Equation 7) and the characteristic parameters values were determined ($RO_{2,min,ALG}$= 3.4 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, $RO_{2,max,ALG}$= 12.7 $mgO_2 \cdot g^{-1} \cdot h^{-1}$, $n_{,r,ALG}$=1.4, $Ik_{,res,ALG}$= $\mu molm^{-2} \cdot s^{-1}$).

$$MRR = RO_{2,min,ALG} + \frac{RO_{2,max,ALG}\cdot {I_{av}}^{n,res,ALG}}{{I_{k,res,ALG}}^{n,r,ALG}+{I_{av}}^{n,res,ALG}} \quad \text{Equation 7}$$

This trend was like observed in previews work when phytoplankton were exposed to high and low irradiances. (Grobbelaar and Soeder, 1985).

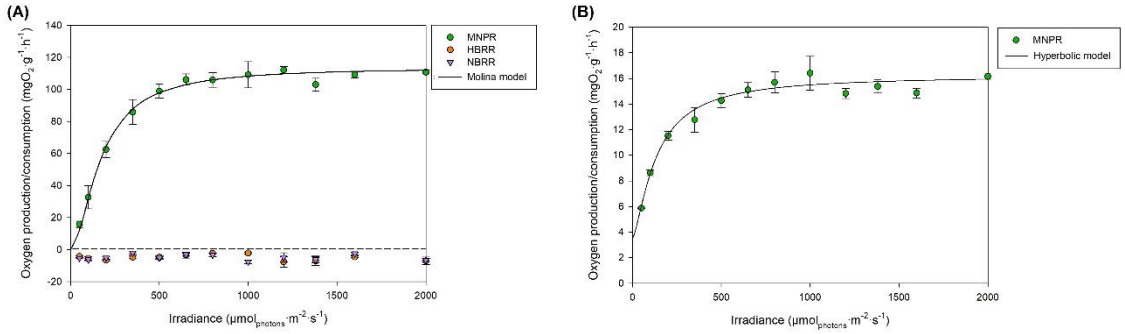


Figure 9.- Influence of average irradiance on the microalgal, nitrifying and heterotrophic activity at 24 °C (A). Influence of average irradiance on the photo-respiration rate of the microalgal culture at 24 °C (B). Lines correspond to fit the proposed models Abbreviations: MNPR, microalgal net photosyntetic rate; HBRR, heterotrophic bacteria respiration rate; NBRR, nitrifying bacteria respiration rate. Values correspond to the mean ± SD (n = 3).

4.2.2. Effect of temperature on the behavior of microalgae-bacteria consortia

Temperature is a crucial culture condition that determines the microbial structure of the community and the performance of WWT processes. Photo-respirometric trials allowed to determine the influence of temperature on MA, HET and NIT, and obtained the MNPR, HBRR and NBRR at different temperatures. The experimental values were normalized based on the maximum value obtained in the trials for MA ($MNPR_{MAX}$) HET ($HBRR_{MAX}$) and NIT ($NBRR_{MAX}$). The normalized values of MNPR, HBRR and NBRR were fitted to the cardinal model developed for bacteria (Rosso et al. 1993) and further validated for microalgae (Bernard and Rémond 2012) (Equation 8, Equation 9 and Equation 10). The cardinal model is a simple equation that considers maximum, minimum, and optimal temperature values. Currently, cardinal equations are well accepted in the MB models because they are helpful to represent experimental data and make the models easy to understand (Rossi et al. 2020a).

$$\frac{MNPR}{MNPR_{MAX}} = \frac{(T - Tmax_{ALG})(T - Tmin_{ALG})^2}{(Topt_{ALG} - Tmin_{ALG})\left((Topt_{ALG} - Tmin_{ALG})(T - Topt_{ALG})\right) - \left((Topt_{ALG} - Tmax_{ALG})(Topt_{ALG} + Tmin_{ALG} - 2T)\right)} \quad \text{Equation 8}$$

$$\frac{HBRR}{HBRR_{MAX}} = \frac{(T - Tmax_{HET})(T - Tmin_{HET})^2}{(Topt_{HET} - Tmin_{HET})\left((Topt_{HET} - Tmin_{HET})(T - Topt_{HET})\right) - \left((Topt_{HET} - Tmax_{HET})(Topt_{HET} + Tmin_{HET} - 2T)\right)} \quad \text{Equation 9}$$

$$\frac{NBRR}{NBRR_{MAX}} = \frac{(T - Tmax_{NIT})(T - Tmin_{NIT})^2}{(Topt_{NIT} - Tmin_{NIT})\left((Topt_{NIT} - Tmin_{NIT})(T - Topt_{NIT})\right) - \left((Topt_{NIT} - Tmax_{NIT})(Topt_{NIT} + Tmin_{NIT} - 2T)\right)} \quad \text{Equation 10}$$

Where: T_{MIN} is the minimum cardinal temperature below which the MNPR, HBRR and NBRR were zero (°C), T_{OPT} is the optimal temperature for which the activity was

maximum (°C), T_{MAX} is the maximum cardinal temperature above which the activity was zero (°C).

Concerning the microalgal activity, the MNPR was maximal at a temperature of 30 °C, lower than the optimum temperature of *Scenedesmus almeriensis* previously reported (Costache et al. 2013). The microalgal activity decreased at lower and higher temperatures, with zero activity at temperatures below 5°C and above 49 °C. Other authors showed that *Scenedemus* did not grow at 42 °C (Westerhoff et al. 2010). However, photo-respirometric tests were performed at short periods of exposure, so that the photosynthetic response of the MB culture could be conditioned by varying the exposure time, even so exposing the microalgal culture at moderate temperatures (Karemore et al. 2020).

The bacterial respiration rates were also significantly affected by the temperature ($p<0.05$). The HBRR showed their optimum temperature at 36 °C, progressively decreasing as the temperature increased. From 44 °C, it was reduced by 50% and at 47 °C, it no longer showed activity. For the nitrifying bacteria, temperature effects were more complex. The main reason is that the nitrification process in wastewater is the most temperature-sensitive step among the microbial activities because nitrifiers could decrease by 50 % with each temperature decrease of 10 °C (Ge et al. 2015). Results showed the optimal temperature was 34 °C and a wide range of activity from 0 to 49 °C, appreciating a strong decrease in the activity below 34 °C (Figure 10).

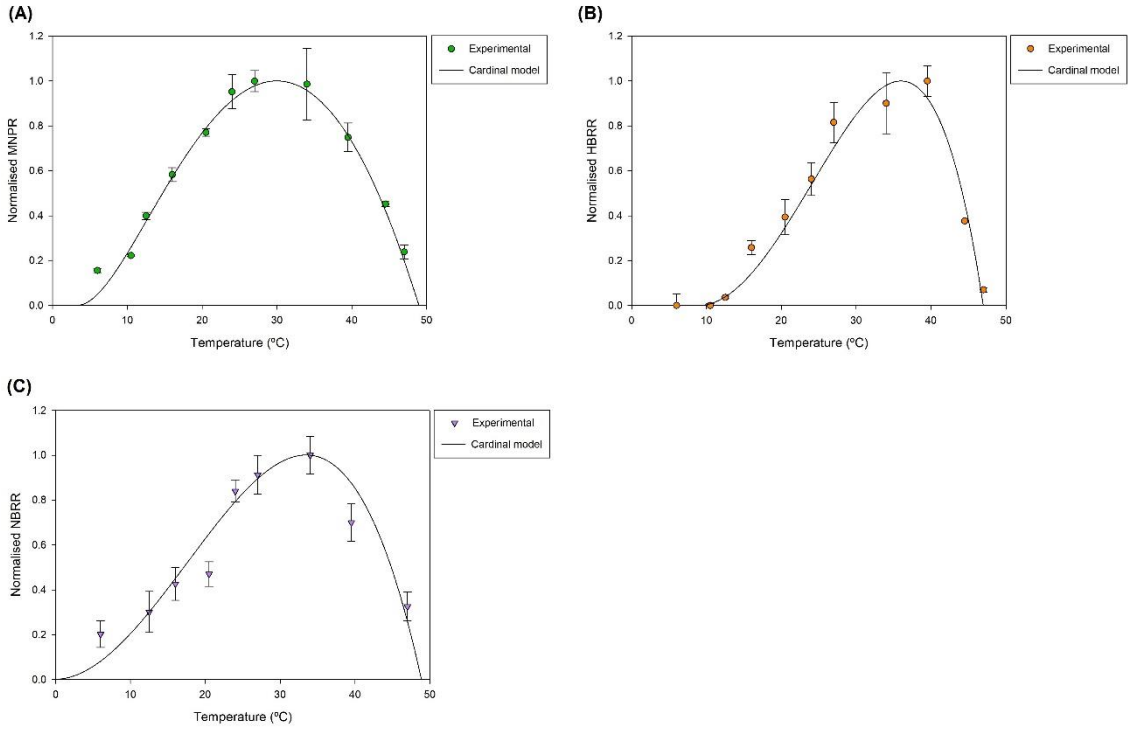


Figure 10.- Influence of the temperature on the microalgal (A), heterotrophic (B) and nitrifying (C) activity. Lines correspond to fit the proposed models Abbreviations: MNPR, microalgal net photosyntetic rate; HBRR, heterotrophic bacteria respiration rate; NBRR, nitrifying bacteria respiration rate. Values correspond to the mean ± SD (n = 3).

4.2.3. Effect of the pH on the behaviour of microalgae-bacteria consortia

The pH is one of the parameters that conditions the activity of the MB consortium in the treatment of wastewater. Each group of microorganisms has a pH range at which its metabolism works optimally. In the case of MB consortia, photosynthetic activity leads to an increase in the pH of the medium, which can influence bacterial activity. However, in most large-scale MB systems, the pH is controlled by injecting CO_2 on demand at a fixed set point, which is normally between 7 and 8 (Casagli et al. 2021a). Therefore, the influence of pH on MNPR, HBRR and NBRR was evaluated. The experimental data obtained were normalized based on the maximum value obtained for MA ($MNPR_{MAX}$) HET ($HBRR_{MAX}$) and NIT ($NBRR_{MAX}$), and were adjusted to the corresponding mathematical equations (Equation 11, Equation 12 and Equation 13). To evaluate the dependence on the pH value, the cardinal pH model was fitted to experimental data:

$$\frac{MNPR}{MNPR_{MAX}} = \frac{(pH - pHmax_{ALG})(pH - pHmin_{ALG})^2}{(pHopt_{ALG} - pHmin_{ALG})\left((pHopt_{ALG} - pHmin_{ALG})(pH - pHopt_{ALG})\right) - \left((pHopt_{ALG} - pHmax_{ALG})(pHopt_{ALG} + pHmin_{ALG} - 2pH)\right)} \quad \text{Equation 11}$$

$$\frac{HBRR}{HBRR_{MAX}} = \frac{(pH - pHmax_{HET})(pH - pHmin_{HET})^2}{(pHopt_{HET} - pHmin_{HET})\left((pHopt_{HET} - pHmin_{HET})(pH - pHopt_{HET})\right) - \left((pHopt_{HET} - pHmax_{HET})(pHopt_{HET} + pHmin_{HET} - 2pH)\right)} \quad \text{Equation 12}$$

$$\frac{NBRR}{NBRR_{MAX}} = \frac{(pH - pHmax_{NIT})(pH - pHmin_{NIT})^2}{(pHopt_{NIT} - pHmin_{NIT})\left(((pHopt_{NIT} - pHmin_{NIT})(pH - pHopt_{NIT})) - ((pHopt_{NIT} - pHmax_{NIT})(pHopt_{NIT} + pHmin_{NIT} - 2pH))\right)}$$ Equation 13

In general, where: pH_{MIN} is the minimum cardinal pH value below which the MNPR, HBRR or NBRR are zero (-), pH_{OPT} is the optimal pH value for which the the MNPR, HBRR or NBRR are maximum (-), pH_{MAX} is the maximum cardinal pH value above which the the MNPR, HBRR or NBRR are zero (-).

Concerning the influence of the pH on microalgal activity, the MNPR was maximal at pH 8. At pH values lower than 7, the microalgal activity reduced slowly as it happened at pH values higher than 9. For example, at a pH value of 7 the microalgal activity was 10% lower than the optimal value measured at a pH value of 8, while at a pH 6, MNPR was 25% lower that the optimal value. Results were consistent with previous works that reported an optimal growth rate of *Scenedesmus sp*. at pH ranges from 7 to 9 (Difusa et al. 2015).

The maximal HBRR was measured at pH 9, and it decreased strongly at lower and high pH values. Nitrifiers showed an optimal pH at 8.5, with a higher tolerance to high pH values. For example, at a pH value of 10, the activity of heterotrophic bacteria and nitrifiers was 15% and 5% lower that the optimal value, respectively (Figure 11).

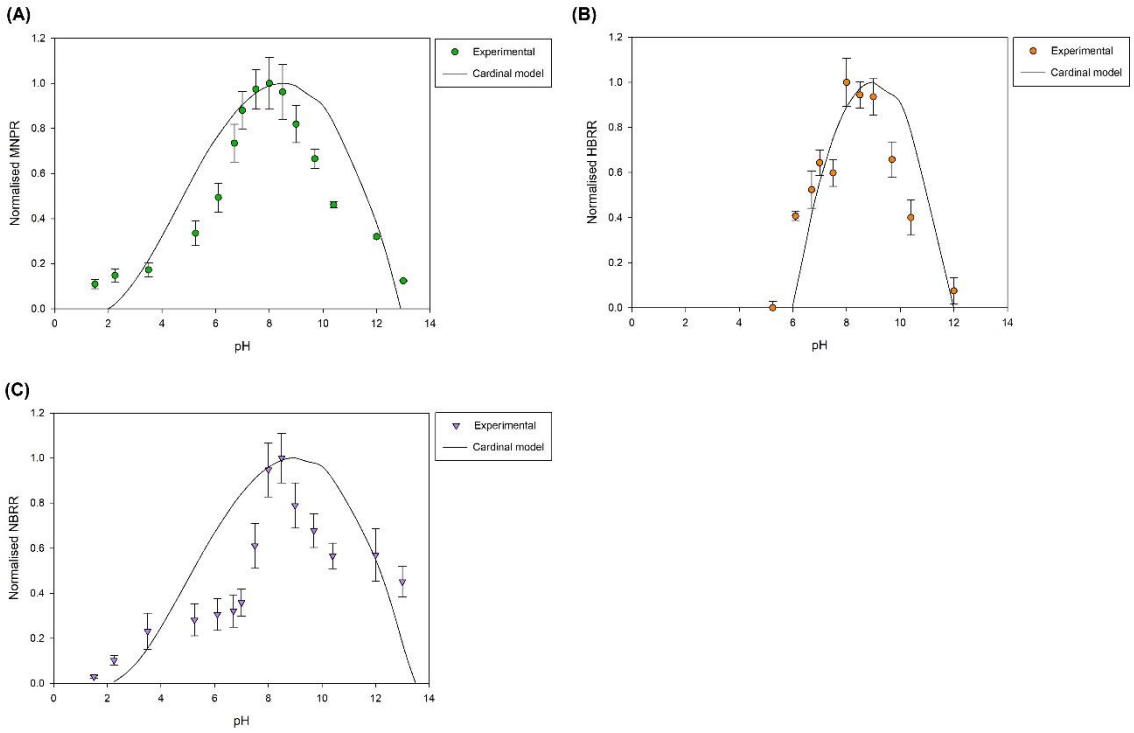


Figure 11.- Influence of the pH on the microalgal (A), heterotrophic (B) and nitrifying (C) activity. Lines correspond to fit the proposed models. Abbreviations: MNPR, microalgal net photosyntetic

rate; HBRR, heterotrophic bacteria respiration rate; NBRR, nitrifying bacteria respiration rate. Values correspond to the mean ± SD (n = 3).

4.2.4. Influence of the dissolved oxygen on the behavior of microalgae-bacteria consortia

Oxygen build-up in the culture is a major problem in MB suspensions as oxygen is a by-product of photosynthesis and its concentration can reach over four times of air saturation in the culture, which could constrain the photosynthetic activity (Darvehei et al. 2018). Therefore, the influence of DO on MA, HET and NIT were assessed through photo-respirometric experiments. The experimental data obtained were normalized based on the maximum value obtained for MA ($MNPR_{MAX}$) HET ($HBRR_{MAX}$) and NIT ($NBRR_{MAX}$) and were adjusted to the mathematical equations (Equation 14, Equation 15, and Equation 16).

$$\frac{MNPR}{MNPR_{MAX}} = 1 - \left(\frac{DO_2}{DO_{2,\,MAX,\,ALG}}\right) \qquad \text{Equation 14}$$

Where: DO_2 is the DO concentration ($mg \cdot L^{-1}$) and DO_2 is the maximum DO value above which the MNPR is zero ($mg \cdot L^{-1}$).

$$\frac{HBRR}{HBRR_{MAX}} = \frac{DO_2}{DO_2 + K_{S,\,DO,\,HET}} \qquad \text{Equation 15}$$

Where: DO_2 represents the concentration of DO ($mg \cdot L^{-1}$) and $K_{S,DO,HET}$ is the heterotrophic half-saturation constant for DO ($mg \cdot L^{-1}$).

$$\frac{NBRR}{NBRR_{MAX}} = \frac{DO_2}{(DO_{2,} + K_{S,DO2,NIT})\left(1 + \frac{DO_2}{K_{I,DO2,NIT}}\right)} \qquad \text{Equation 16}$$

Where: DO_2 represents the concentration of DO ($mg \cdot L^{-1}$), $K_{S,DO2,NIT}$ is the nitrifying half-saturation constant for DO ($mg \cdot L^{-1}$) and $K_{I,DO2,NIT}$ is the nitrifying inhibition constant for DO ($mg \cdot L^{-1}$).

The experimental data fitted to the mathematical equations are shown in

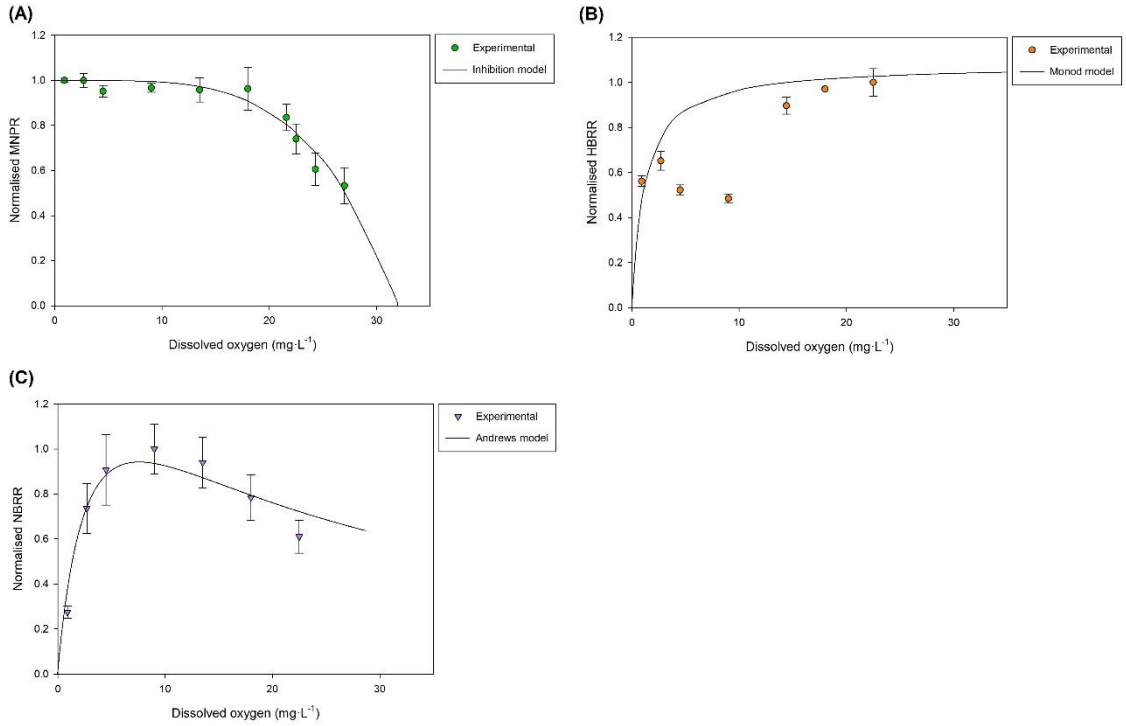


Figure 12. Concerning the microalgal activity, the MNPR was maximal from 0 mg·$L^{-1}$to 9 mg·L^{-1}, and it decreased up to zero at 32 mg·L^{-1}. This effect was first discovered by Warburg in 1920, who observed that photosynthesis of *Chlorella* was dropped significantly when culture was exposed to pure oxygen. Furthermore, in the last years, many researchers have reported a decrease in biomass productivity when a high concentration of DO was present in microalgal cultures because of extremely high concentration of DO could involve a photooxidative death (Darvehei et al. 2018). For instance, *Scenedesmus sp.* cultures showed an inhibited growth at DO concentrations higher than 25 mg·L^{-1}(Barceló-Villalobos et al. 2019). Heterotrophic bacteria supported a wide range of dissolved oxygen concentrations and can be active even at low DO concentrations (<0.9 mg·L^{-1}). In turn, nitrifiers showed maximal activity within a narrow range of dissolved oxygen concentration (5-10 mg·L^{-1}) because an inhibitory effect happened at concentrations above 10 mg·L^{-1} (Figure 12).

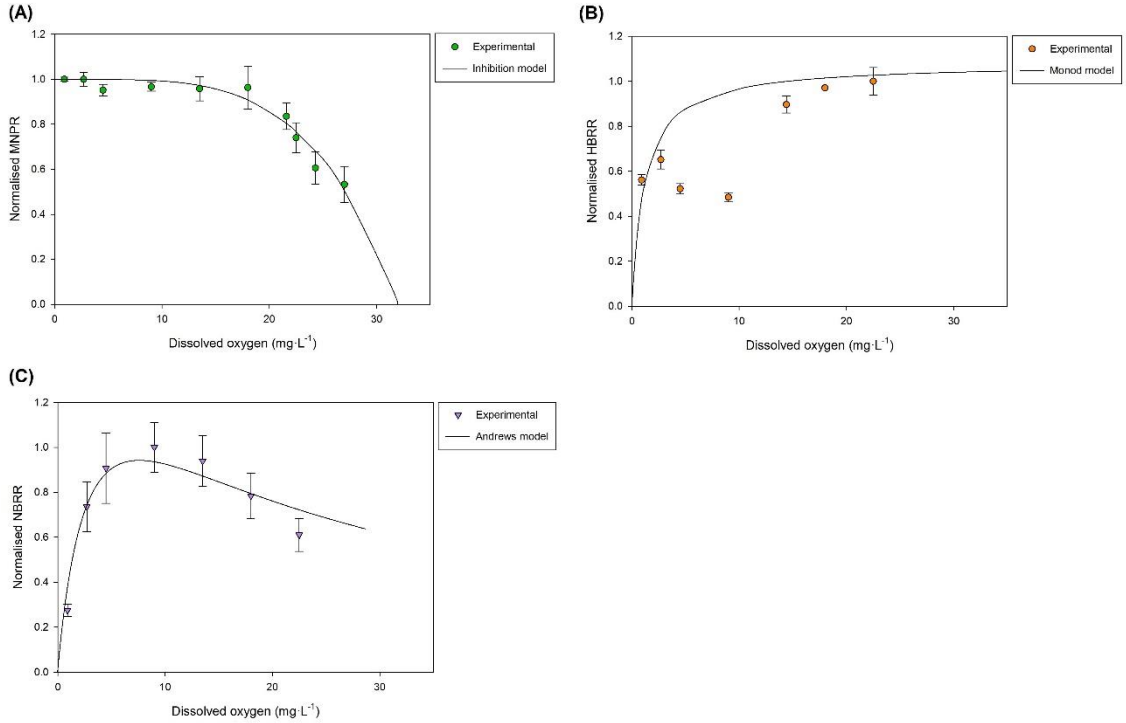


Figure 12.- Influence of the dissolved oxygen on the microalgal (A), heterotrophic (B) and nitrifying (C) activity. Lines correspond to fit the proposed models. Abbreviations: MNPR, microalgal net photosyntetic rate; HBRR, heterotrophic bacteria respiration rate; NBRR, nitrifying bacteria respiration rate. Values correspond to the mean ± SD (n = 3).

4.2.5. Mathematical models and validation

The adjustment of the experimental data obtained for MA, HET and NIT to the corresponding mathematical equations, allowed obtaining the kinetic parameters of these populations as a function of light, temperature, pH and DO (Table 2) With these equations and their corresponding parameters, a model (based on oxygen production/consumption) capable of predicting the activity of each microbial was proposed.

Table 2.- Values for the proposed model's parameter characteristics.

Microalgal Net Photosynthetic Rate			**Heterotrophic Respiration Rate**			**Nitrifying Respiration Rate**		
Parameter	**Value**	**Units**	**Parameter**	**Value**	**Units**	**Parameter**	**Value**	**Units**
$PO_2max,_{ALG}$	113	$mgO_2 \cdot g^{-1} \cdot h^{-1}$	$RO_2max,_{HET}$	5.4	$mgO_2 \cdot g^{-1} \cdot h^{-1}$	$RO_2max,_{NIT}$	4.4	$mgO_2 \cdot g^{-1} \cdot h^{-1}$
$Ik,_{ALG}$	168	µmolm-2·s-1	$Tmin,_{HET}$	9	°C	$Tmin,_{NIT}$	0	°C
$N,_{ALG}$	1.7		$Tmax,_{HET}$	47	°C	$Tmax,_{NIT}$	49	°C
$Tmin,_{ALG}$	3.4	°C	$Topt,_{HET}$	36	°C	$Topt,_{NIT}$	33.6	°C
$Tmax,_{ALG}$	49	°C	$pHmin,_{HET}$	6		$pHmin,_{NIT}$	2	
$Topt,_{ALG}$	30	°C	$pHmax,_{HET}$	12		$pHmax,_{NIT}$	13.4	
$pHmin,_{ALG}$	1.8		$pHopt,_{HET}$	9		$pHopt,_{NIT}$	9	

$pHmax_{,ALG}$	12.9		$K_{S,DO2,HET}$	1.98	mgO_2/L	$K_{S,DO2,NIT}$	1.08	mgO_2/L
$pHopt_{,ALG}$	8.5					$K_{I,DO2,NIT}$	104.9	mgN/L
$DO_2max_{,ALG}$	32	$mg \cdot L^{-1}$						
$m_{,ALG}$	4.15							
$RO_2max_{,ALG}$	12.7	$mgO2/g_{biomass} \cdot h$						
$RO_2min_{,ALG}$	3.4	$mgO2/g_{biomass} \cdot h$						
$Ik\_res_{,ALG}$	134	$\mu E/m^2 \cdot s$						
$n\_Res_{,ALG}$	1.4							

For MA, it was considered that light is the determining factor, and the one that establishes the maximum value of MNPR that can be obtained according to the irradiance in the culture. However, this value can be modified by other parameters such as temperature, pH and DO just like the BIOALGAE model (Solimeno et al. 2017). These parameters have a normalized and multiplicative effect on the model developed for MA ($PO2_{ALG}$). Its value in the model fluctuates between 0 and 1 depending on how the value of temperature, pH and DO affects the normalized MNPR. Therefore, the activity of microalgae is determined by light, temperature, pH and DO (Equation 17). The proposed model for MA also included the effect of endogenous respiration of the culture. Working under optimal conditions for all parameters (temperature, pH and DO), allowed obtaining the maximal microalgal activity as a function of light.

Regarding the HET and NIT, their activity did not depend on the light, so a constant rate of respiration in darkness was obtained. This value was affected by the other parameters (temperature, pH and DO). In the heterotrophic model ($RO2_{Het}$) (Equation) and nitrifying model ($RO2_{Nit}$) (Equation) model, the respiration rate varied as a function of the multiplicative effect of temperature, pH, and DO concentration.

$$\mathrm{PO2_{ALG}} = \mathrm{PO2(I)} \cdot \overline{\mathrm{PO2(T)}} \cdot \overline{\mathrm{PO2(pH)}} \cdot \overline{\mathrm{PO2(DO2)}} - \mathrm{RO2(I)} \quad \text{Equation 17}$$

$$\mathrm{RO2_{Het}} = \mathrm{RO2(I)} \cdot \overline{\mathrm{RO2(T)}} \cdot \overline{\mathrm{RO2(pH)}} \cdot \overline{\mathrm{RO2(DO2)}} \quad \text{Equation 18}$$

$$\mathrm{RO2_{Nit}} = \mathrm{RO2(I)} \cdot \overline{\mathrm{RO2(T)}} \cdot \overline{\mathrm{RO2(pH)}} \cdot \overline{\mathrm{RO2(DO2)}} \quad \text{Equation 19}$$

To validate each one of the proposed models, photo-respirometric trials were performed modifying the environmental and operational parameters of the system (light, temperature, pH and DO concentration). These experimental data were correlated with the simulated data obtained with the mathematical models and kinetic parameters proposed for each population (Figure 13).

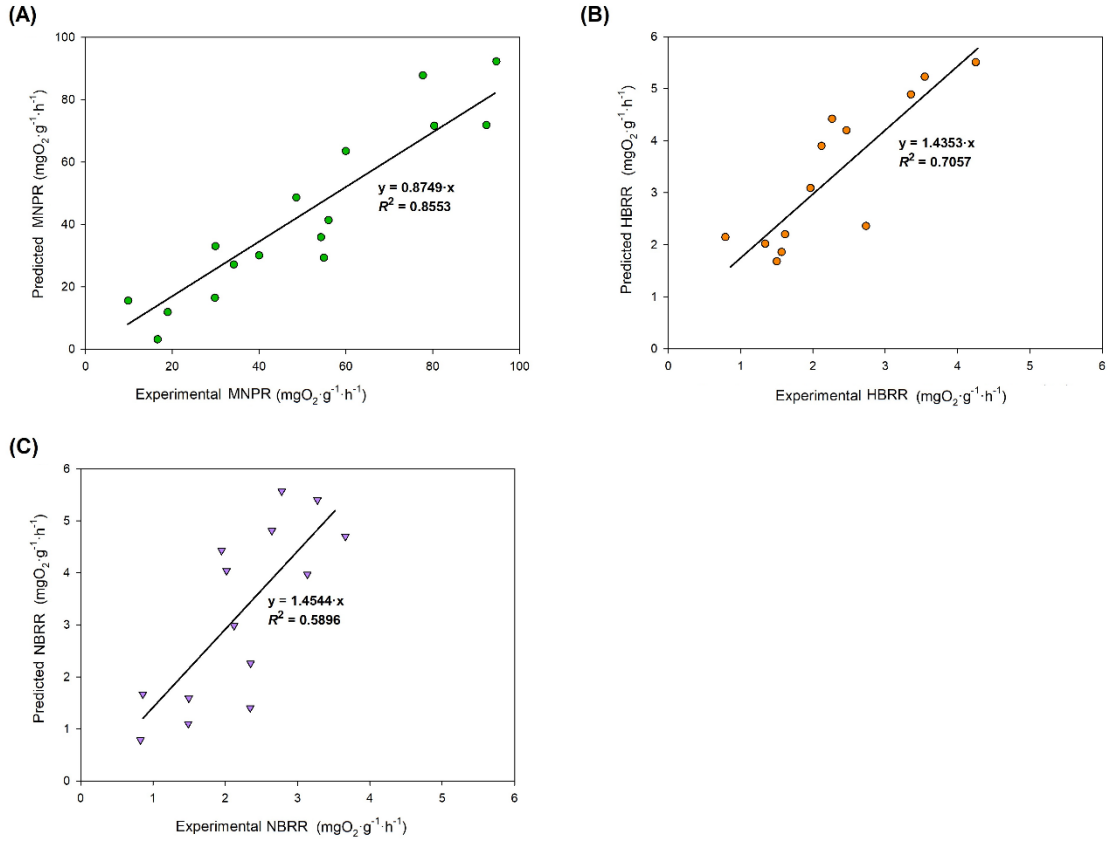


Figure 13.- Correlation between the experimental data and simulations for microalgae (A), heterotrophic bacteria (B) and nitrifiers (C). Abbreviations: MNPR, microalgal net photosyntetic rate; HBRR, heterotrophic bacteria respiration rate; NBRR, nitrifying bacteria respiration rate.

Results demonstrated that photo-respirometric trials allowed the evaluation of the influence of environmental and operational variables (light, temperature, pH and DO) on MA, HET and NIT. Moreover, photo-respirometry allowed modelling their activity to obtain useful simulation tools. The experimental data fitted the simulated data with R^2 values of 0.96; 0.96 and 0.91 for MA, HET and NIT which demonstrated the model's reliability.

4.3. Modelling of photosynthesis, respiration, and nutrient yield coefficients in *Scenedemus almeriensis* culture as a function of nitrogen and phosphorus.

Once the effect of light, temperature, pH and DO concentration on the activity of the MA and bacteria was evaluated and modelled, the influence of other operational variables was further investigated. In this sense, the influence of the concentration of N and P on microalgal activity was studied; specifically, it was investigated as the concentration of N (in the form of NH_4^+ and NO_3^-) and the concentration of phosphorus (in the form of PO_4^{-3}) affects the MNPR and MRR. Experiments were also performed to determine the

coefficient yields, both for N and P, in microalgal cultures. These data along with the photosynthetic and reparation rates allowed to development of simulation tools to assess and optimize microalgal production systems with different culture media (

Figure **14**).

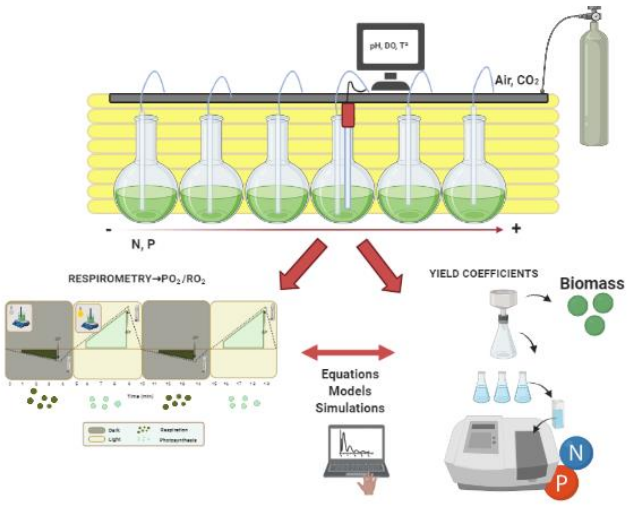


Figure 14.- Evaluation of nitrogen and phosphorous concentration in microalgal cultures.

4.3.1. Influence of the nitrogen and phosphorous concentration on the photosynthetic and respiration rates of microalgae

The use of wastewater for MA production has several advantages; for example, MA can be grown using both organic and inorganic compounds. Inorganic compounds include compounds such as NH_4^+, NO_3^- and PO_4^{-3}, which are already present in the wastewater, avoiding the cost of nutrient supplementation. However, WW has a significantly variable composition, since it depends on many factors such as the time of year, location, industrial waters that discharge into the urban network, or activities of the population (Acién et al. 2016). Therefore, the MNPR and MRR were evaluated and modelled at different concentrations of $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$. The concentrations varied from 0-200, 0-220, 0-30 mg/L for $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$, respectively (Figure 15, Figure 16).

For this purpose, photo-respirometric trials were performed varying the concentration of one substrate (N or P) and maintained the other one constant following the modified Arnon medium (see Material and Methods section).

The experimental data for MNPR and MRR were normalized to the maximal values obtained in the trials,and fitted to the corresponding equations. The dependence on nutrients ($N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$) was expressed as either the Monod function with

nutrient limitation in Equation 20 and Equation 21, or Andrews kinetics, if inhibition at high substrate concentrations occurred, as shown in Equation 22 and Equation 23.

$$\frac{\mathrm{MNPR}}{\mathrm{MNPR_{MAX}}} = \frac{\mathrm{S}}{\mathrm{S}+\mathrm{K_S}} \qquad \text{Equation 20}$$

$$\frac{\mathrm{MRR}}{\mathrm{MRR_{MAX}}} = \frac{\mathrm{S}}{\mathrm{S}+\mathrm{K_S}} \qquad \text{Equation 21}$$

Where: S represents the concentration of the relevant substrate ($N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$) ($mg \cdot L^{-1}$) and K_S is the half-saturation constant for the substrate (mg/L).

$$\frac{\mathrm{MNPR}}{\mathrm{MNPR_{MAX}}} = \frac{\mathrm{S}}{\mathrm{S}+\mathrm{K_S}+\left(\frac{\mathrm{S}}{\mathrm{S}_I}\right)^2} \qquad \text{Equation 22}$$

$$\frac{\mathrm{MRR}}{\mathrm{MRR_{MAX}}} = \frac{\mathrm{S}}{\mathrm{S}+\mathrm{K_S}+\left(\frac{\mathrm{S}}{\mathrm{S}_I}\right)^2} \qquad \text{Equation 23}$$

Where: S represents the concentration of the relevant substrate ($N\text{-}NO_3^-$, $N\text{-}NH_4^+$, $P\text{-}PO_4^{3-}$) ($mg \cdot L^{-1}$), K_S is the half-saturation constant for the substrate (mg/L) and S_I represents the inhibition constant for the substrate ($mg \cdot L^{-1}$).

The results were fitted with the corresponding models and the values of the kinetic parameters are shown in Table 3.

Table 3.- Values for the proposed model's parameter characteristics and confidence intervals.

Nitrate models			Ammonium models			Phosphate models		
Parameter	**Value**	**Units**	**Parameter**	**Value**	**Units**	**Parameter**	**Value**	**Units**
$K_{S,N\text{-}NO3^-}$	2.77±0.28	$mgN\text{-}NO_3^- \cdot L^{-1}$	$K_{S,N\text{-}NH4^+}$	1.54±0.15	$mgN\text{-}NH_4^+ \cdot L^{-1}$	$K_{S,\ P\text{-}PO4}$	0.43±0.06	$mg\ P\text{-}PO_4^{3-} \cdot L^{-1}$
$K_{I,N\text{-}NO3^-}$	386.6±42.5	$mgN\text{-}NO_3^- \cdot L^{-1}$	$K_{I,N\text{-}NH4^+}$	571±49.2	$mgN\text{-}NH_4^+ \cdot L^{-1}$	$K_{R,\ P\text{-}PO4}$	0.35±0.03	$mg\ P\text{-}PO_4^{3-} \cdot L^{-1}$
$K_{R,N\text{-}NO3^-}$	1.02±0.12	$mgN\text{-}NO_3^- \cdot L^{-1}$	$K_{R,N\text{-}NH4^+}$	0.65±0.08	$mgN\text{-}NH_4^+ \cdot L^{-1}$			
$K_{I,R,N\text{-}NO3^-}$	279±25.4	$mgN\text{-}NO_3^- \cdot L^{-1}$	$K_{I,R,N\text{-}NH4^+}$	205±21.3	$mgN\text{-}NH_4^+ \cdot L^{-1}$			

Results demonstrated that N, both in the form of $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$, influenced in the same way on MNPR, with inhibition taking place from 50 $mgN \cdot L^{-1}$up to 200 $mgN \cdot L^{-1}$, whereas no inhibition by P was observed in the range of conditions studied. These results were crucial to understand and assess the importance of the concentration of the nutrient in the performance of MA cultures, which is a decisive factor together with operational factors such as the pH, temperature and DO concentrations. These models

should be considered in microalgae-related systems during their optimization when using inorganic fertilizers or WW as a culture medium. In the former, it is necessary to optimize the culture medium composition according to the systems' performance and nutrient demand. In the latter, the challenge is to determine the optimal conditions for maximizing the recovery of nutrients and the biomass production capacity. The main reason is that the composition of the wastewater cannot be modified cost-effectively.

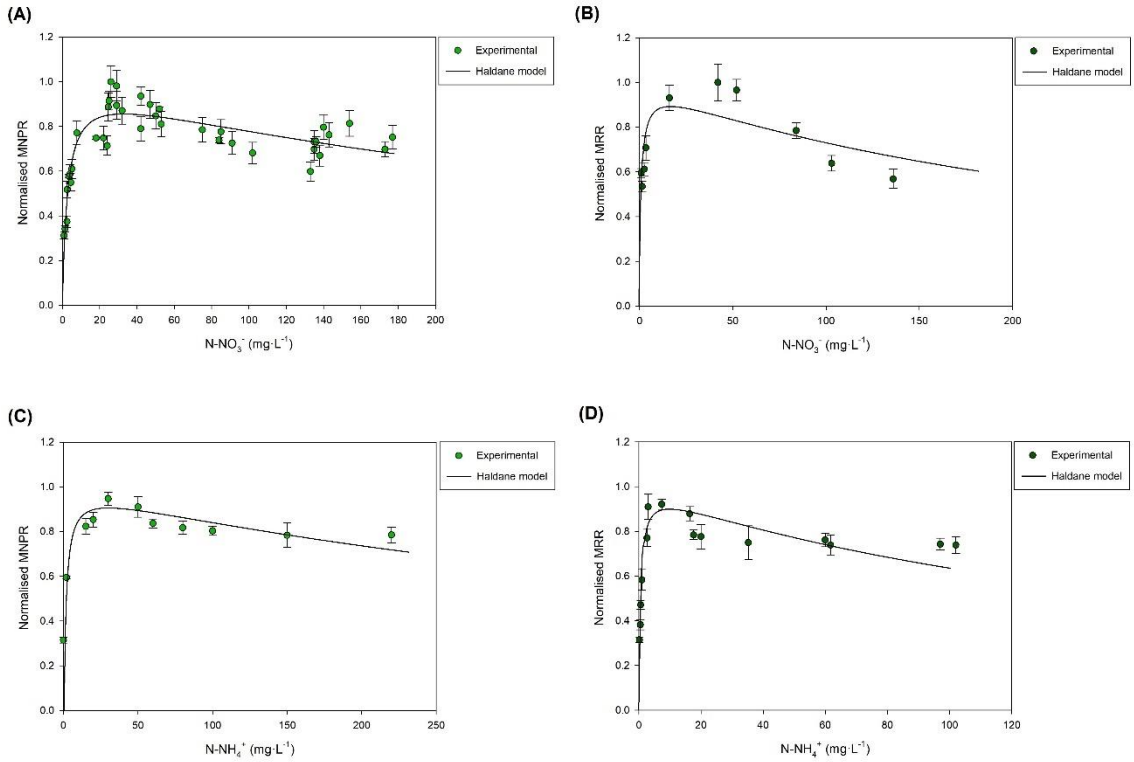


Figure 15.- Influence of $N\text{-}NO_3^-$ on the normalized MNPR of *S. almeriensis* (A) and on the normalized MRR of *S. almeriensis* (B). Normalized MNPR (C) and normalized MRR (D) as a function of $N\text{-}NH_4^+$. Lines correspond to fit the proposed models. Values correspond to the mean ± SD (n = 3).

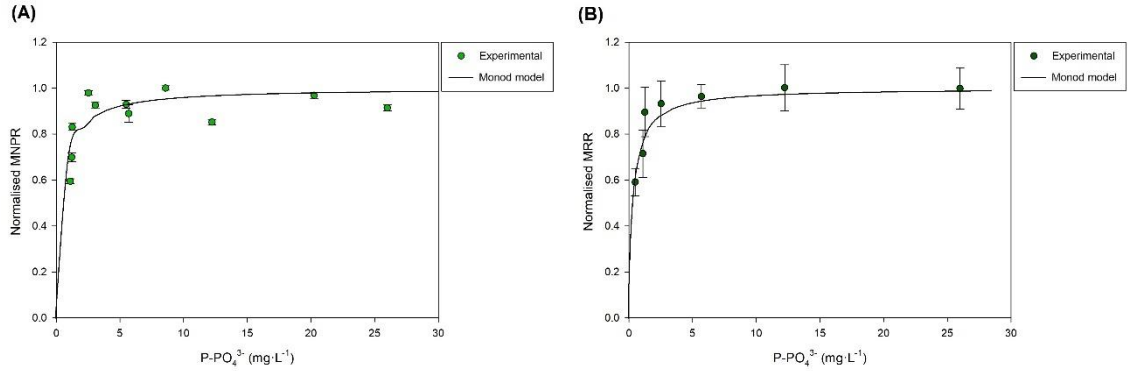


Figure 16.- Influence of $P\text{-}PO_4^{3-}$ on the normalized MNPR of *S. almeriensis* (A) and the normalized MRR of *S. almeriensis* (B). Lines correspond to the fit of the proposed models. Values correspond to the mean ± SD (n = 3).

Results demonstrated that nitrogen, both in the form of $N\text{-}NO_3^-$ and $N\text{-}NH_4^+$, influenced in the same way on microalgal cells, with inhibition taking place from 50 $mgN \cdot L^{-1}$, whereas no inhibition by phosphorous was observed in the range of conditions studied. These results were crucial to understand and assess the importance of the concentration of the nutrient in the performance of microalgae cultures, which is a decisive factor together with operational factors such as the pH, temperature and DO concentrations. These models should be considered in microalgae-related systems during their optimization when using inorganic fertilizers or WW as a culture medium. In the former, it is necessary to optimize the culture medium composition according to the systems' performance and nutrient demand. In the latter, the challenge is to determine the optimal conditions for maximizing the recovery of nutrients and the biomass production capacity. The main reason is that the WW composition cannot be modified cost-effectively.

4.3.2. Coefficient yield of microalgae as a function of nitrogen and phosphorous concentration

Although it is generally assumed that N and P coefficient yields for MA are constant, previews works described that N and P coefficient yields vary as a function of culture conditions (Gómez-Serrano et al. 2015; Morales-Amaral et al. 2015). For this reason, in the present work, experimental runs were performed to determine if nutrient concentrations in the culture media influenced the coefficient yields. Results showed that the N and P coefficient yields increased as N or P concentrations in the culture medium increased, observing a peak at 70 $mgN\text{-}NO_3^- \cdot L^{-1}$ and 18 $mgP\text{-}PO_4^{3-} \cdot L^{-1}$, respectively.

Regarding N, the coefficient yield ranged from 0.02 to 0.09 $gN\text{-}NO_3^- \cdot g^{-1}$. Concerning P, results showed that the P yield coefficient ranged from 0.004 to 0.014 $gP\text{-}PO_4^{3-} \cdot g^{-1}$. The variability in nutrient uptake has been widely described particularly of phosphorus, whose consumption depends on the concentration in the medium. This phenomenon, by which microalgal cells are capable of taking up and storing nutrients in larger amounts than necessary for immediate growth, is termed "luxury uptake", which are initiated by excess P availability (Solovchenko et al. 2019). P is stored within the biomass in the form of polyphosphate as acid-soluble or acid-insoluble polyphosphate.

4.3.3. Performance of microalgal culture as a function of the culture medium composition

Once the effects of N and P concentrations were assessed and modelled, simulations were performed to determine the performance of MA as a function of the composition of the culture medium. These simulations were performed considering different culture

media, from the standard culture medium prepared using fertilizers to different types of WW (PWW, pig manure and centrate) and WW treated according to the regulations. Two possibilities were included in this work: treated WW with the maximum nutrient concentration for safe disposal (10 $mgN \cdot L^{-1}$) and treated WW complying with the new limits (5 $mgN \cdot L^{-1}$) (European Directive 91/271/CEE).

Figure 17A shows the normalized MNPR rate as a function of the N and P concentrations when using different culture media. Regarding N, results show that the normalized MNPR was maximal when WW and WW after treatment were used to produce MA, whereas it was reduced because of N limitation when depurated WW was used ($p<0.05$). In turn, the use of pig manure or centrate as a culture medium involved the decrease of the MNPR because of nutrients inhibition. Concerning the influence of P, no limitation/inhibition was observed, except when the depurated WW was used as the culture medium. Therefore, the study of both nutrients together shows that N concentration usually determines the performance of MA. The simulations showed that the MNPR of *S. almeriensis* decreased sharply when using manure or centrate as the culture medium. In contrast, *S. almeriensis* performed at its maximal capacity when using WW and treated WW as the culture medium. Moreover, a simulation was developed considering both the influence of nutrients on MNPR and MRR and the yield coefficients determined as a function of N and P. This simulation allowed estimating the amount of biomass, which can be produced per litre of culture medium used for microalgal production (Figure 17B).

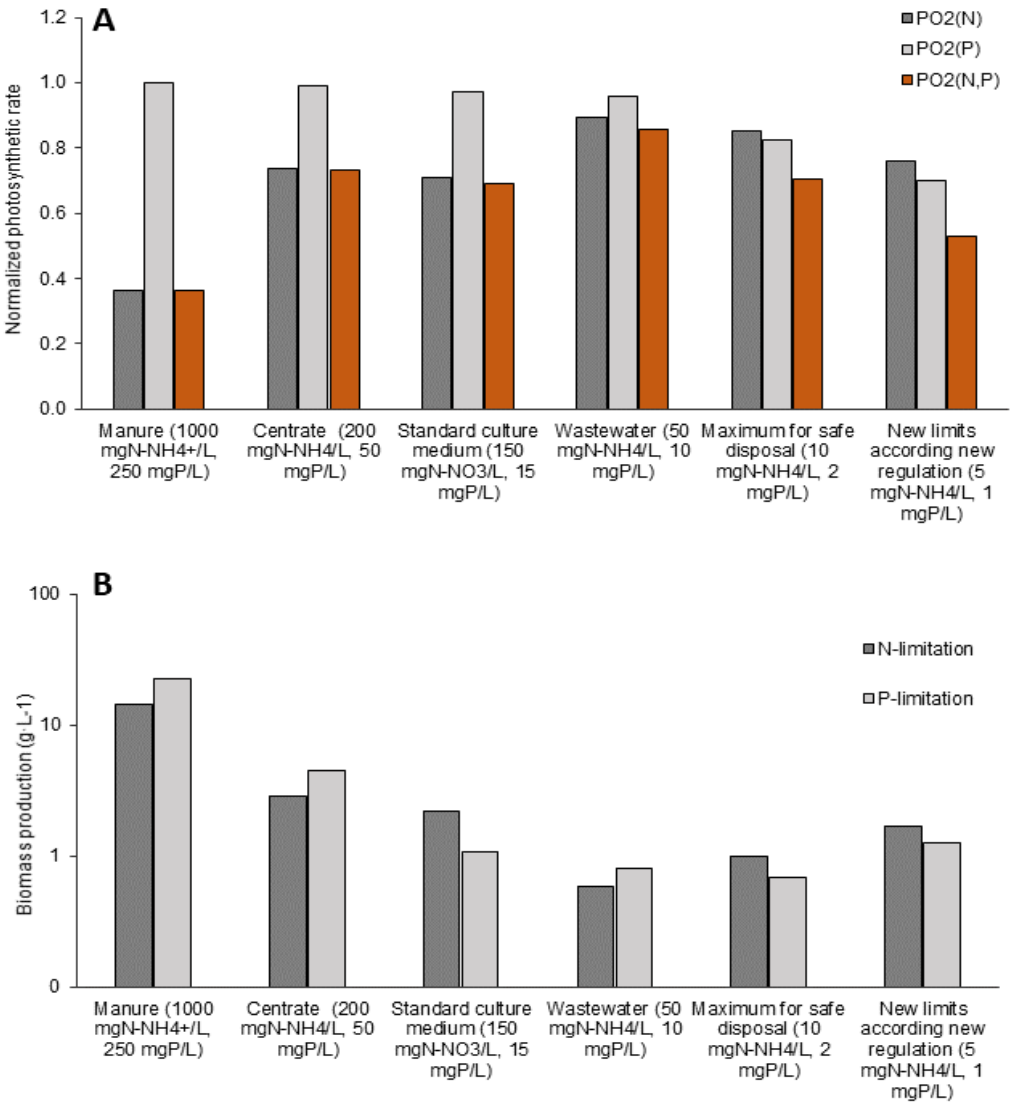


Figure 7.- Simulations of the nitrogen and phosphorous influence in different culture media on the normalized photosynthetic rate (A), and biomass production (B).

Results suggested that the use of pig manure allows producing up to 14.3 g of biomass per litre of manure. This production capacity was limited by the N concentration in the medium, while up to 22.7 g of biomass per litre could be produced considering the P concentration. In the case of centrate and WW, the maximal biomass production capacity was 2.9 and 0.6 g of biomass per litre respectively, considering N as limiting nutrient. However, in the case of treated WW, P was the limiting nutrient for MA production. Considering the P concentration, it was possible to produce 0.7 and 1.3 g of biomass per litre using treated WW with the maximum nutrient concentration for safe disposal and treated WW complying to the new limits, respectively. In addition, the use of PWW allowed to produce 0.6 and 0.8 g of biomass per litre, considering the concentration of N or P, respectively. These values were comparable to those obtained using raceway

reactors in Almeria fed with treated WW, where the biomass productivity also depends on the design of the reactor and the operational conditions (Morillas-España et al. 2021).

These simulations were just theoretical because in an actual microalgal facility the WW with high nutrient concentration, such as pig manure or centrate, most often needs to be diluted before use as the culture medium to avoid inhibition effects caused by an excess of NH_4^+ or other micropollutants, such as heavy metals, and because of their color and turbidity, which could reduce light availability (Acién et al. 2016; García et al. 2017). For that, knowing the exact composition of the WW to be treated is essential for an optimal treatment process and biomass production, not only to avoid inhibition processes but also to determine if additional C, N, or P need to be added when a low nutrient concentration appears in the medium.

4.4. ABACO: A NEW MODEL OF MICROALGAE-BACTERIA CONSORTIA FOR BIOLOGICAL TREATMENT OF WASTEWATERS

Most of the mathematical models found in literature use the kinetic parameters of bacterial activity from the ASM (Henze et al. 2015), while the information of microalgal parameters in WWT systems is scarce. Therefore, in this work, the kinetic coefficients obtained through photo-respirometric experiments for MA, HET and NIT, along with the mathematical models developed based on oxygen production/consumption, were used to develop a biological model of MB for WWT.

The new MB mathematical model, named ABACO was developed, calibrated and validated with experimental data from duplicate lab-scale photobioreactors using pig slurry as culture medium. The implementation of the MB model was performed in Matlab software, and it allowed to simulate the dynamics of different components in the biological system and the relative proportion of microalgae, heterotrophic bacteria and nitrifiers. The calibration was performed using genetic algorithms, which allowed determining their value from the minimization of a given cost function. In addition, the calibration process allowed estimating the percentages of each microbial population in the consortia. Also, these percentages were validated with experimental data and performing photo-respirometric tests.

4.4.1. ABACO model: concept

The biological model for microalgae-based WWT involves the interaction of three main populations. Under illumination, microalgae (X_{ALG}) fix CO_2 and release oxygen O_2 while assimilating nutrients, such as N and P from NH_4, NO_3^- and PO_4^{2+}. The oxygen produced by photosynthesis is used for the degradation of the biodegradable soluble organic matter (BSOM) by heterotrophic bacteria (X_{HET}). BSOM was considered as a fraction of COD contained in the WW. In turn, during bacterial oxidation of BSOM, CO_2 is produced and is available for photosynthesis and nitrification. During nitrification, nitrifying bacteria (X_{NIT}) transform NH_4^+ already contained at the inlet culture medium into NO_3^-, simultaneously consuming the O_2 produced by photosynthesis (Figure).

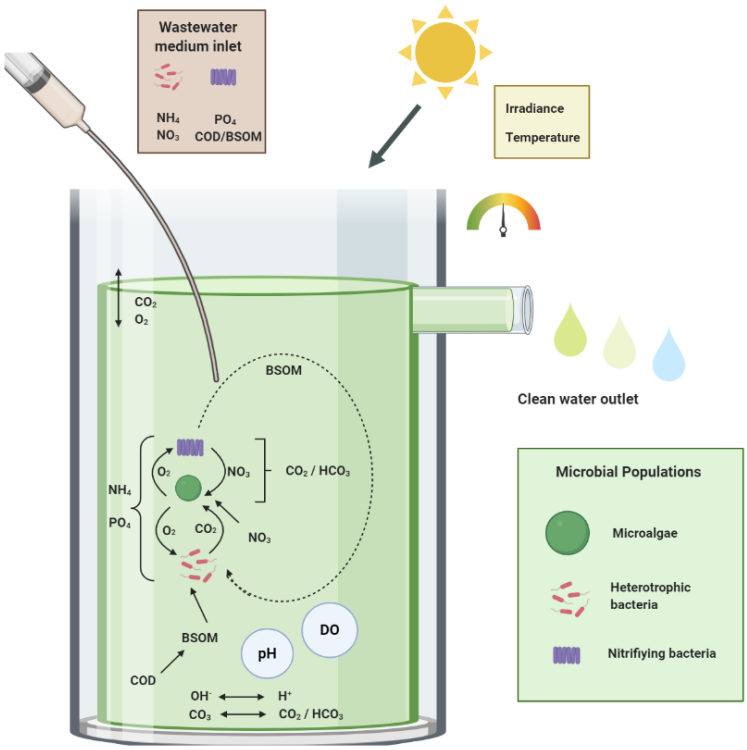


Figure 18.- Scheme of biological processes considered in ABACO model for microalgae-bacteria wastewater treatment.

4.4.2. ABACO model: components

The model considers components (10) – 3 particulate and 7 dissolved – implicated as variables in the biological processes that occur during microalgae-bacteria WWT.

1.1.1.1. Particulate components

- **X_{ALG} [g·m^{-3}]**: Microalgal biomass. Microalgae biomass concentration increases due to autotrophic growth of MA, using light as an energy source and of CO_2 as a carbon source, whereas it reduces by endogenous respiration and decay of microalgae. The global balance to estimate the microalgae biomass concentration in the system is given by Equation 24.

$$V \cdot X_{ALG} \cdot \mu_{ALG} = Q_h \cdot X_{ALG} + V \cdot \frac{dX_{ALG}}{dt} \qquad \text{Equation 24}$$

where $\boldsymbol{V}$ [m^3] is the volume in the reactor, $\boldsymbol{X_{ALG}}$ [g m^{-3}] is the microalgae biomass concentration, $\boldsymbol{\mu_{ALG}}$ [day^{-1}] is the microalgae specific growth rate and $\boldsymbol{Q_h}$ [m^3 s^{-1}] represents the harvesting flow rate. The specific growth rate $\boldsymbol{\mu_{ALG}}$ is mainly a function of light availability inside the reactor, summarized by the average

irradiance inside the culture $\boldsymbol{I_{av}}$ (Grima et al. 1994a), and modified by the influence of different environmental and operational variables such as temperature $\left(\overline{\boldsymbol{\mu_{ALG}}}(\boldsymbol{T})\right)$, pH $\left(\overline{\boldsymbol{\mu_{ALG}}}(\boldsymbol{pH})\right)$, dissolved oxygen concentration $\left(\overline{\boldsymbol{\mu_{ALG}}}(\boldsymbol{DO_2})\right)$ and CO_2 $\left(\overline{\boldsymbol{\mu_{ALG}}}(\boldsymbol{CO_2})\right)$. Moreover the influence of nutrients availability such as ammonium nitrogen $\left(\overline{\boldsymbol{\mu_{ALG}}}([\boldsymbol{N-NH_4}])\right)$, phosphate phosphorus $\left(\overline{\boldsymbol{\mu_{ALG}}}([\boldsymbol{P-PO_4}])\right)$ and nitrate nitrogen concentrations $\left(\overline{\boldsymbol{\mu_{ALG}}}([\boldsymbol{N-NO_3}])\right)$, and the microalgae maintenance $(\boldsymbol{m_{ALG}})$, is considered as showed in Equation .

$$\mu_{ALG} = \left(\mu_{ALG}(I_{av}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO_2) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}(N) \cdot \overline{\mu_{ALG}}([P-PO_4])\right) - m_{ALG} \qquad \text{Equation 25}$$

MA can grow using both NH_4^+ and NO_3^- as a nitrogen source. Therefore, there is a process rate for the growth of microalgae with ammonium and another one for nitrate consumption, thus Equation becomes Equation and Equation for considering this phenomenon. Equation considering the use of NO_3^- is only considered when there is no NH_4^+ in the culture medium.

$$\mu_{ALG} = \left(\mu_{ALG}(I_{av}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO_2) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}([N-NH_4]) \cdot \overline{\mu_{ALG}}([P-PO_4])\right) - m_{ALG} \qquad \text{Equation 26}$$

$$\mu_{ALG} = \left(\mu_{ALG}(I_{av}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO_2) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}([N-NO_3]) \cdot \overline{\mu_{ALG}}([P-PO_4])\right) - m_{ALG} \qquad \text{Equation 27}$$

- **X_{HET} [g·m^{-3}]**: Heterotrophic bacteria. HET grow using organic matter as the source of energy and C. These bacteria are aerobic then consume O_2 produced during the photosynthesis process. The endogenous respiration and decay are responsible for the heterotrophic biomass loss. HET are present in the system, and also they enter daily into the system with the inlet wastewater. They are removed through harvesting and dilution. The global balance to estimate the heterotrophic bacteria concentration is given by Equation .

$$Q_d \cdot X_{HET,in} + V \cdot X_{HET,out} \cdot \mu_{HET} = Q_h \cdot X_{HET,out} + V \cdot \frac{dX_{HET,out}}{dt} \qquad \text{Equation 28}$$

where $\boldsymbol{Q_d}$ [m^3 s^{-1}] is the dilution flow rate, $\boldsymbol{X_{HET,in}}$ [g m^{-3}] is the heterotrophic bacteria inlet concentration, $\boldsymbol{\mu_{HET}}$ [day^{-1}] is the specific growth rate of heterotrophic bacteria and $\boldsymbol{X_{HET,out}}$ [g m^{-3}] is the heterotrophic bacteria concentration in the reactor. As with microalgal processes, heterotrophic processes include both heterotrophic growth and heterotrophic maintenance.

The heterotrophic specific growth rate $\boldsymbol{\mu_{HET}}$ is modelled as the product of maximum growth rate ($\boldsymbol{\mu_{HET,max}}$) and switching functions for environmental parameters such as temperature $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{T})\right)$, pH $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{pH})\right)$ and dissolved oxygen $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{DO_2})\right)$; in addition to biodegradable soluble organic matter $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{BSOM})\right)$, ammonium nitrogen $\left(\overline{\boldsymbol{\mu_{HET}}}([\boldsymbol{N-NH_4}])\right)$ and phosphate phosphorous $\left(\overline{\boldsymbol{\mu_{HET}}}([\boldsymbol{P-PO_4}])\right)$ (Equation). The rate of the heterotrophic maintenance ($\boldsymbol{m_{HET}}$) considers the endogenous respiration of the heterotrophic bacteria and the heterotrophic decay. The specific growth rate for heterotrophic bacteria is expressed by Equation where $\boldsymbol{\mu_{HET,max}}$ [day^{-1}] is the maximum specific growth rate for heterotrophic bacteria, whereas $\boldsymbol{m_{HET}}$ [day^{-1}] represent the endogenous respiration of the heterotrophic bacteria and the heterotrophic decay.

$$\mu_{HET} = \mu_{HET,max} \cdot \left(\overline{\mu_{HET}}(T) \cdot \overline{\mu_{HET}}(pH) \cdot \overline{\mu_{HET}}(DO_2) \cdot \overline{\mu_{HET}}([N-NH_4]) \cdot \overline{\mu_{HET}}([P-PO_4]) \cdot \overline{\mu_{HET}}(BSOM)\right) - m_{HET} \quad \text{Equation 29}$$

- **X_{NIT} [g·m^{-3}]: Nitrifying bacteria**. These bacteria are supplied to the system with the inlet wastewater and are also removed during harvesting. Nitrifiers are aerobic and use CO_2 as a carbon source. The concentration of nitrifying bacteria increases due to growth but is also decreased by endogenous respiration and decay. The global balance to estimate the concentration of nitrifying bacteria is given by Equation .

$$Q_d \cdot X_{NIT,in} + V \cdot X_{NIT,out} \cdot \mu_{NIT} = Q_h \cdot X_{NIT,out} + V \cdot \frac{dX_{NIT,out}}{dt} \quad \text{Equation 30}$$

where $\boldsymbol{X_{NIT,in}}$ [g m^{-3}] is the nitrifying bacteria inlet concentration, $\boldsymbol{\mu_{NIT}}$ [day^{-1}] is the nitrifying bacteria-specific growth rate and $\boldsymbol{X_{NIT,out}}$ [g m^{-3}] is the nitrifying bacteria concentration in the reactor. The processes related to nitrifying bacteria include both autotrophic growth and maintenance. The rate of the autotrophic growth is modelled as the product of maximum growth rate ($\boldsymbol{\mu_{NIT,max}}$) and switching functions for environmental parameters, such as temperature $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{T})\right)$, pH $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{pH})\right)$ and dissolved oxygen $\left(\overline{\boldsymbol{\mu_{HET}}}(\boldsymbol{DO_2})\right)$; in addition to ammonium nitrogen $\left(\overline{\boldsymbol{\mu_{HET}}}([\boldsymbol{N-NH_4}])\right)$ and phosphate phosphorous $\left(\overline{\boldsymbol{\mu_{HET}}}([\boldsymbol{P-PO_4}])\right)$ (Equation). The rate of maintenance ($\boldsymbol{m_{NIT}}$) considers the endogenous respiration of the nitrifying bacteria and nitrifying decay. The Equation represents the nitrifying bacteria-specific growth rate where $\boldsymbol{\mu_{NIT,max}}$ [day^{-1}] is the maximum specific growth rate for nitrifying bacteria and $\boldsymbol{m_{NIT}}$ [day^{-}

[1] is the endogenous respiration of the nitrifying bacteria and the nitrifying maintenance.

$$\mu_{NIT} = \mu_{NIT,max} \cdot \left(\overline{\mu_{\mathrm{NIT}}}(T) \cdot \overline{\mu_{\mathrm{NIT}}}(pH) \cdot \overline{\mu_{NIT}}(DO_2) \cdot \overline{\mu_{NIT}}(CO_2) \cdot \overline{\mu_{NIT}}([N-NH_4]) \cdot \overline{\mu_{NIT}}([P-PO_4])\right) - m_{NIT} \quad \text{Equation 31}$$

1.1.1.2. Dissolved components

Apart from the particulate components, the ABACO model includes 7 dissolved components: dissolved oxygen (**O_2**), dissolved carbon dioxide (**CO_2**), chemical oxygen demand (**COD**), biodegradable organic soluble matter (**BSOM**), ammonium nitrogen (**N-NH_4^+**), nitrate nitrogen (**N-NO_3^-**), and phosphate phosphorous (**P-PO_4^{3-}**). To study the evolution of these components in the system, the corresponding mass balances were carried out. These components enter the system through the culture medium, are removed by harvesting, and produced and/or consumed by particulate components.

Most of the kinetic parameters and yield coefficients described in the ABACO model were determined experimentally, only a few were obtained from the literature. However, using different culture media for MB production involves some model parameter presenting some uncertainty. This situation leads to the need for a biological model that allows adapting its parameters for each situation. Therefore, a calibration method is presented using genetic algorithms that can estimate the characteristic parameters of the model from experimental data measured in the microalgae-bacteria system.

4.4.2. Calibration process

The proposed equations for each model component included a series of characteristic parameters whose exact values were unknown, or the values were known in a defined range. The uncertainty in the value of these parameters led to the need for a calibration process, which was performed using genetic algorithms. The calibration parameters were related to the maximum growth rates for each microbial population and the nutrients generation/consumption coefficients. In addition to the parameters described in the table, it was possible to estimate the percentages of each particulate component in the system through the calibration process.

Figure shows the calibration results obtained in the estimation of the model components. The percentages of each microbial population are estimated in Figure A. Figure B represents the biomass concentration for each organism in the reactor (MA, HET and NIT), in addition to the total biomass concentration, expressed as the sum of the

individual concentrations, and the experimental measurements. Figure C represents the estimated PO_4^{2-} concentration and the experimental data while Figure D shows the estimated NH_4^+ concentration and the experimental values. Figure E showed the estimated NO_3^- concentration and the experimental measurements. Finally, Figure F represented the estimated BSOM, compared with the experimental measurement.

The model was able to predict the dynamics of the components, both particulate and soluble. It is observed how the model is capable of reproducing behaviors of the system, such as the increase in nutrients as NO_3^-, and the reach of the MB culture at a steady state. However, a significant deviation of the experimental points from one day to the next is observed, so a greater number of experimental data could improve the accuracy of the trends predicted by the model.

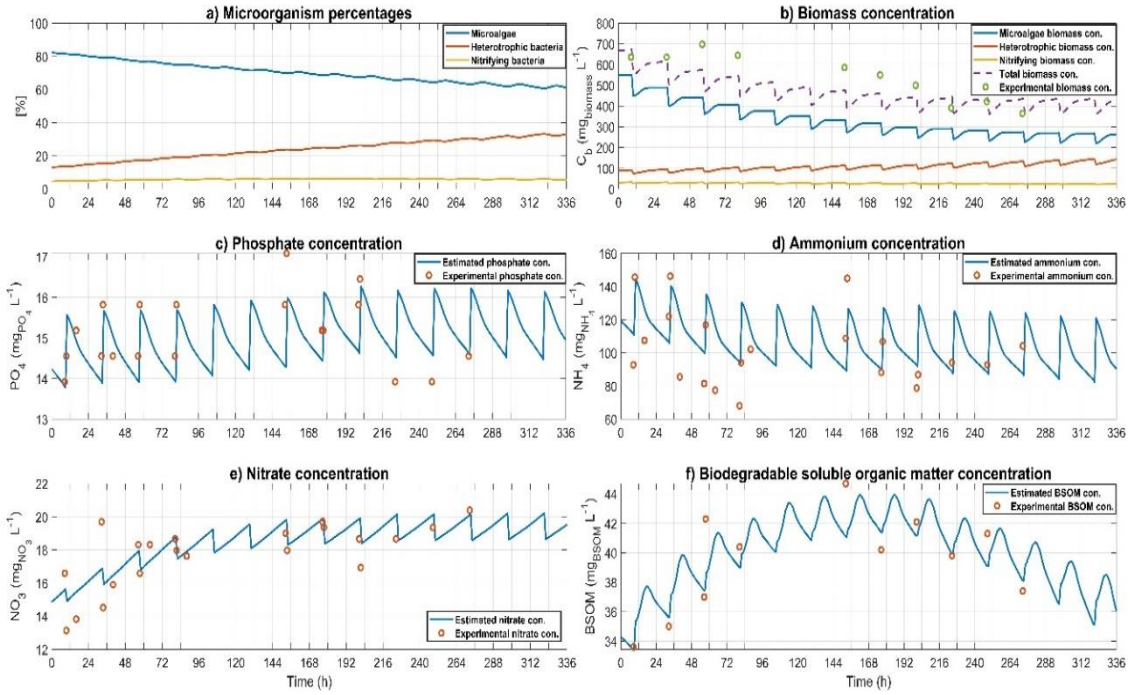


Figure 19.- Results of the calibration process in the ABACO model.

4.4.3. Validation process

After the calibration, the results were validated using a new experimental data set (Figure 17). Results showed the percentage of each microorganism over time (Figure 17A), and the biomass concentrations of each one (Figure 17B). The concentration of microalgae decreased up to achieve the steady-state, while the heterotrophic bacteria slightly grew, and the nitrifying bacteria remained constant. The estimation of the nutrient concentrations (PO_4^{2-}, NH_4^+, NO_3^-) was shown in Figure 17C, Figure 17D and Figure

17E, respectively. Moreover, the BSOM estimation was represented in Figure 17F and showed the same trend that the calibration results.

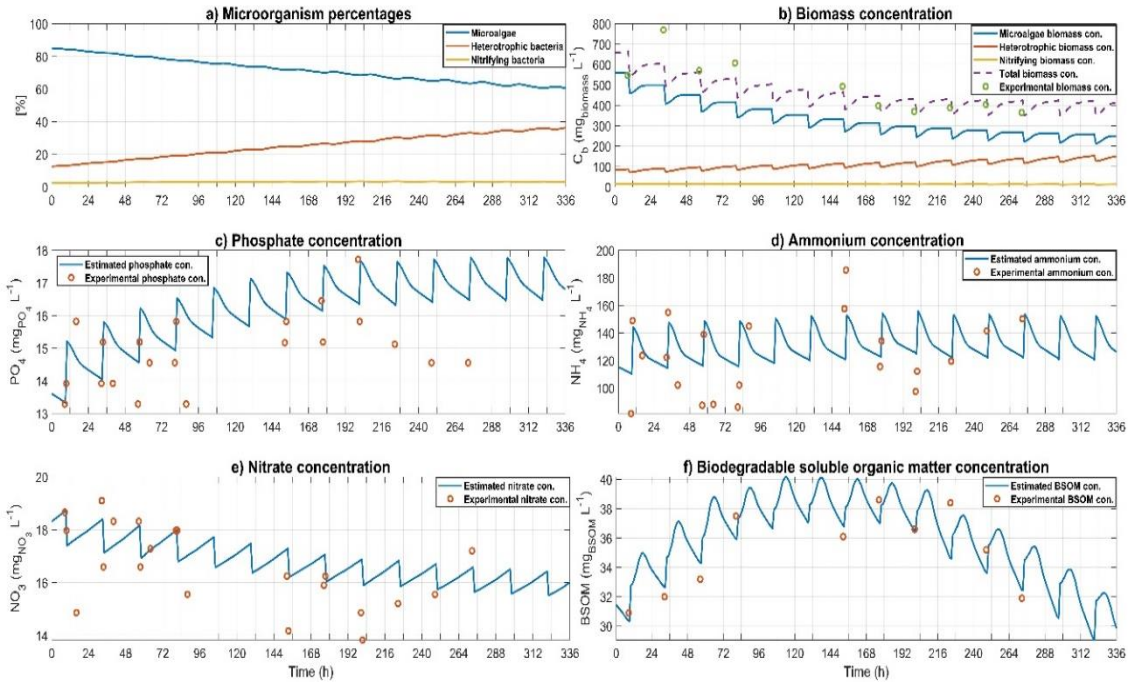


Figure 17.- Results of the validation process in the ABACO model.

4.4.4. Photo-respirometry for model validation

At the end of the experimental trials, some photo-respirometric tests were performed to compare the microbial percentages obtained with the calibration process, validation process and photo-respirometric techniques (Figure 18). The MNPR rate was 15.8 ± 2.3 $mgO_2 \cdot L^{-1} \cdot h^{-1}$, the HBRR was 2.2 ± 0.8 $mgO_2 \cdot L^{-1} \cdot h^{-1}$ and the NBRR was 0.27 ± 0.1 $mgO_2 \cdot L^{-1} \cdot h^{-1}$. In terms of oxygen production/consumption these values corresponded to a concentration of 86.7 % of MA, 11.8% of HET, and 1.5 % of NIT, while the percentages determined in the calibration process were 82.1% of MA, 13.2% of HET and 4.7% of NIT. For the validation processes, the percentages were 85% of MA, 12.6% of HET and 2.4% of NIT.

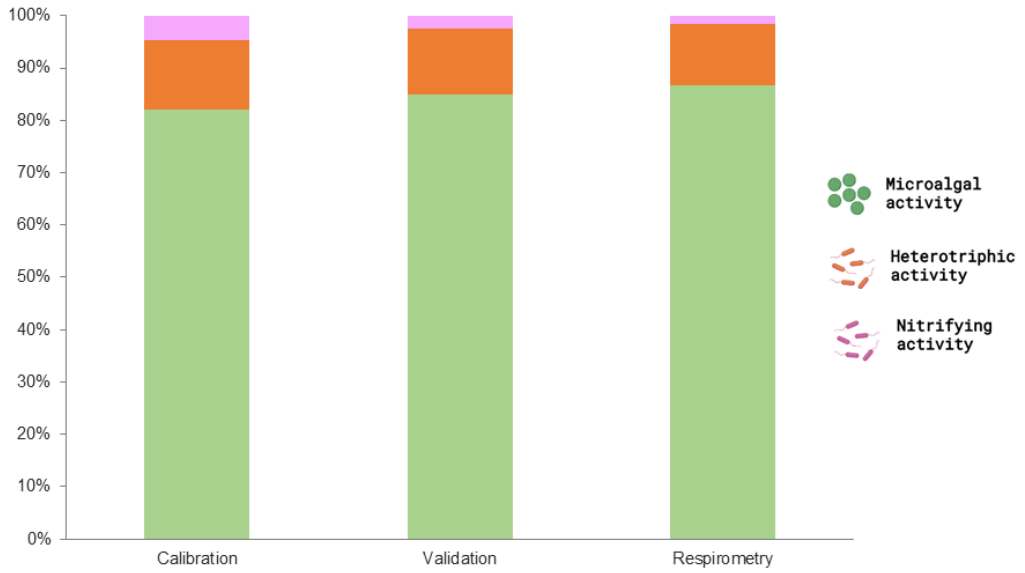


Figure 18.- Microbial percentages obtained during the calibration process, validation process and experimental photo-respirometric trials.

These results showed that after calibrating and validating the ABACO model, it was capable of reproducing results of the dynamics observed in experimental trials. In addition, the results demonstrate that photo-respirometry not only allowed to obtain the kinetic parameters of the microalgae-bacteria consortia for the development of mathematical models but also validating their results.

4.5. AN INTERACTIVE TOOL FOR SIMULATION OF BIOLOGICAL MODELS INTO WASTEWATER TREATMENT WITH MICROALGAE.

Virtual labs and interactive tools have been proposed to allow the simulation of complex mathematical models and control systems quickly and easily. Moreover, these tools allow real-time interaction between the modification of model parameters and the visualization of results (Guzmán et al. 2012). Once the ABACO model was calibrated and validated, the main processes related to MA and bacteria were used to develop an interactive tool to visualize the productivity of the biological system as a function of the main environmental and operational variables (Figure 19). The tool allows the visualization in real-time and instantaneously the productivity of the MB system and the influence of each variable on MA, HET and NIT. For the simulations, experimental environmental and operational data of a raceway reactor were introduced as inputs to the model, while the composition of the culture medium was designed by the user.

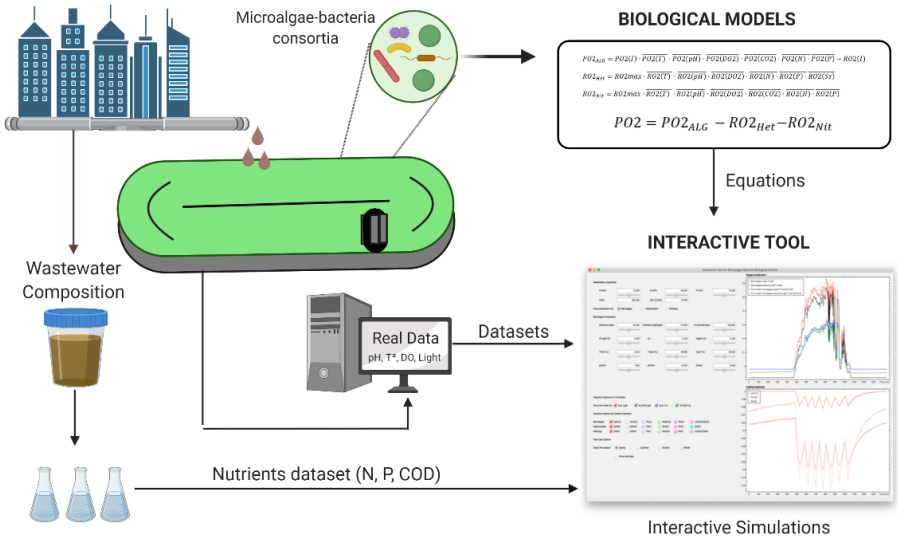


Figure 19.- Interactive tool based on ABACO model for microalgae-bacteria WWT.

4.5.1. Biological models for the interactive tool

The ABACO model was developed considering the performance of MA, HET and NIT as a function of environmental and operational variables such as light, temperature, pH, DO, N, P and COD. The kinetics parameters of ABACO model were obtained based on oxygen production/consumption in microalgae-bacteria WWT considering the parameters that determine the activity of each one. The same concept based on oxygen

production/consumption by MA, HET and NIT under different environmental and operational conditions was used to design the interactive tool, which was capable of showing a Net Oxygen Production Rate (PO_2) over 24 hours.

Four scenarios were proposed in the tool based on: (1) there are only microalgal cells in WWT and they are affected by solar radiation (Equation); (2) three populations coexist in WWT (MA, HET and NIT) affected only by solar radiation (Equation 6); (3) the WWT is performed by microalgal cells and its activity is a function of solar radiation, temperature, pH, DO, N and P (Equation 7); (4) finally, the more complex model involves MA, HET and NIT, affected by solar radiation, temperature, pH, DO, N and P (Equation 8).

$$PO_2 = PO2_{ALG} \cdot [I] \quad \text{Equation 22}$$

$$PO_2 = PO2_{ALG}\ [I] - RO2_{HET} - RO2_{NIT} \quad \text{Equation 6}$$

$$PO_2 = PO2_{ALG}\ ([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[CO2]} \cdot \overline{[N]} \cdot \overline{[P]}) \quad \text{Equation 7}$$

$$PO_2 = PO2_{ALG}\ \left([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[CO2]} \cdot \overline{[N]} \cdot \overline{[P]}\right) - RO2_{HET}([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[N]} \cdot \overline{[P]}) - RO2_{NIT}([I] \cdot \overline{[T]} \cdot \overline{[pH]} \cdot \overline{[DO]} \cdot \overline{[CO2]} \cdot \overline{[N]} \cdot \overline{[P]}) \quad \text{Equation 8}$$

4.5.2. Design of the interactive tool

The developed tool is freely available through http://www.eu-sabana.eu/ at the Data and Software website section and does not require a Sysquake license to be run. Windows and Mac versions are available. Moreover, a short video tutorial can be found to describe the main capabilities of the interactive tool.

The graphic part of the tool was organized to facilitate the understanding of the main biologic components that appear in the biological models together with the operational and environmental parameters that affected them (Figure 20). The users can easily work simulating all the proposed models on the screen at the simultaneously, which is very useful for a deeper understanding of the fundamentals of the process. Moreover, it is possible to visualize the four scenarios throughout the four seasons of the year. All the models can be simulated for 24 h, where experimental data of solar radiation, temperature, pH and DO concentration are used as inputs. The inputs can be modified for each season of the year. However, the user can load their data from the “Load data” option available in the software. Instructions are also given on the tools’ website.

The left-hand side of the screen is the parameter section, which is divided into two parts: the model parameters part, which is located at the upper area of the screen, and the graphical option parameters located at the lower part of the screen. From the former, it is possible to modify the concentrations of nutrients ($mg \cdot L^{-1}$) in the WW: COD, $N\text{-}NO_3^-$, $N\text{-}NH_4^+$, and $P\text{-}PO_4^{-3}$. Moreover, all the model parameters for MA, HET and NIT can be interactively modified in this area. On the other hand, the graphical parameter option is focused on switching on/off the graphical results of the models. Two groups of checkboxes are available to show or to hide the plots for the different simulated models or the cardinal elements at the right-hand side of the screen. Furthermore, an option to select the season of the year is shown. Once the season of the year is selected, the real data (that includes solar radiation, temperature, pH and DO concentration) of a characteristic day for the selected season is used as input to the models as commented above. Together with this option, a checkbox called “Real Data” is also available to show or to hide the used real data in the plots.

The right-hand side of the screen shows the graphical results of the simulated models or the real data used as inputs to the model. When the “Real Data” checkbox is switched on, the real input data is shown in the graphics. The DO and the pH are shown at the top, and the solar radiation and the medium temperature are shown at the bottom. However, when the “Real Data” checkbox is switched off (option by default) the simulation results for the models are presented.

The graphic at the upper part of this area shows the results for the four scenarios described by equations. The plots in the lower graphic show all the components involved in the different models. Notice that these two graphics only show those results that were selected in the graphical parameter option of the tool.

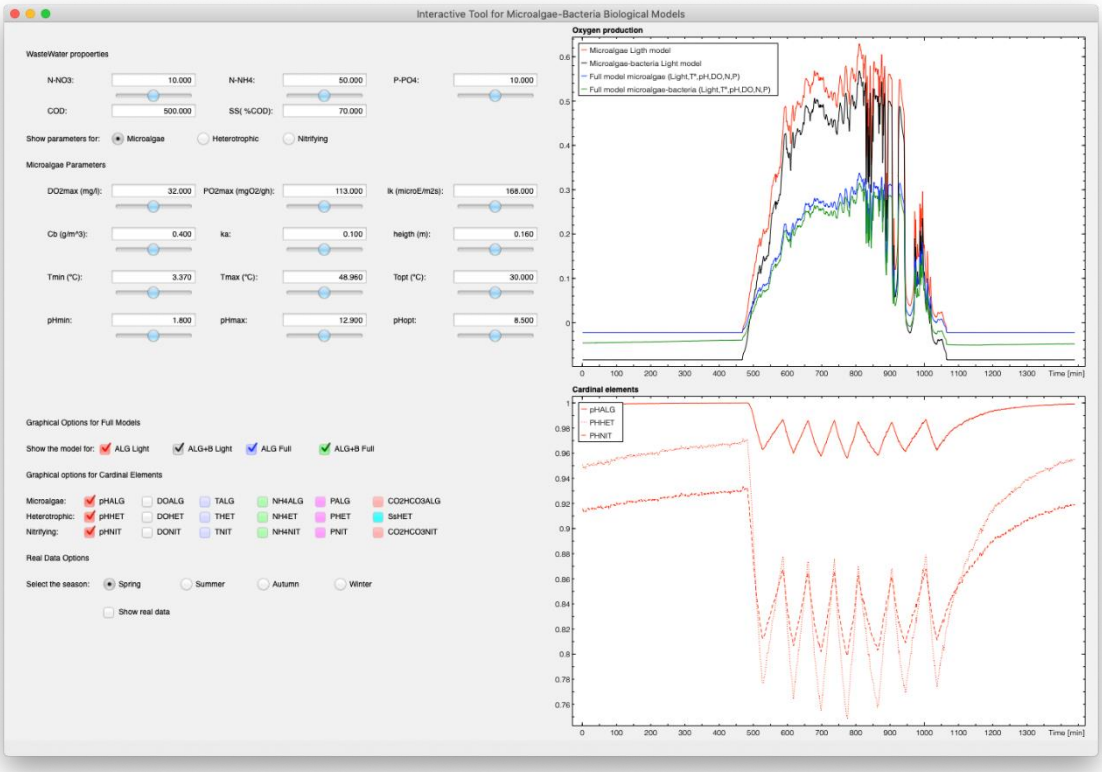


Figure 20.- Main screen of the interactive tool for ABACO model.

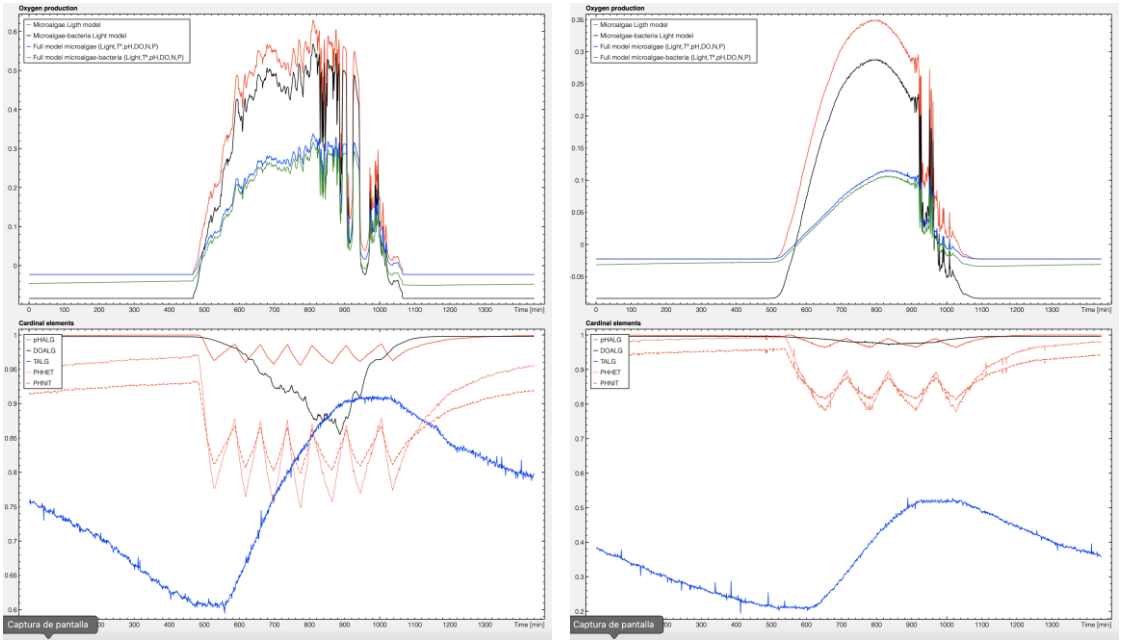
4.5.3. Use of the interactive tool to analyze the influence of environmental variables on the performance of the system

One of the most interesting applications of the tool was its capacity to evaluate the influence of season (environmental conditions) on the MB system considering the characteristic parameters of MA, HET and NIT. A default the kinetic parameters of ABACO model were used. The interactive tool permits the user to calculate the productivity of the system (considering the four possible scenarios) when the cultivation is performed in spring or winter (

Figure **21**). The first part of the screen shows the productivity in terms of net oxygen production rate considering the four scenarios described in the tool. Results demonstrated that productivity was strongly affected by the season of the year ($p<0.05$).

In the last part of the screen, the normalizing influence of each variable of the models was shown. Results showed that the temperature was a crucial parameter in microalgal productivity because other parameters such as pH and DO concentrations could be easily and economically controlled (Costache et al. 2013; Duarte-Santos et al. 2016). However, in microalgal cultures: i) it is costly to control the temperature of the culture; (ii) optimal temperature depends on each MA; (iii) temperature fluctuations depend on geographic localization and season of the year (Ras et al. 2013). These simulations

along with economic strategies such as regulating the weight of the cultures or covering the systems (greenhouse effect), will allow cost-effectively maximizing the biomass productivity (Rodríguez-Miranda et al. 2020).



(A) Spring season results (B) Winter season results

Figure 21.- Interactive tool to analyze the influence of environmental variables on microalgae-bacteria productivity.

The ABACO model included many equations and parameters that were difficult to interpret simply and practically. Therefore, the use of interactive tools is a useful alternative to facilitate the understanding of microalgae-bacteria processes and was a possible solution to predict the productivity of the MB system and consequently, to avoid long experiments, waiting time, and additional costs.

4.6. RESPIROMETRIC ASSESSMENT OF POPULATIONS IN ACTIVATED SLUDGE AND MICROALGAE-BASED WASTEWATER TREATMENT.

After developing and validating the ABACO model, the next step was to improve the biological processes related to bacterial performance. The ABACO model developed included many kinetic parameters determined experimentally by photo-respirometry, especially related to the microalgal activity. However, the model has two main challenges: i) Differentiating between the two populations that complain the nitrifiers group, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB); (ii) obtaining and assessing the bacterial kinetic coefficients by experimental tests. Therefore, a respirometric study on the dependence of bacterial activities on environmental (temperature, pH and DO) and nutrients (COD, N, and P) conditions commonly experienced in outdoor MB reactors is mandatory. The procedure was developed and applied to samples of a conventional activated sludge tank (fed on municipal wastewater), and an MB raceway reactor. The methodology allowed to model the behavior of the main aerobic bacterial populations in the two systems (i.e., HET, AOB, and NOB), and to identify related kinetic parameters (Figure 22)

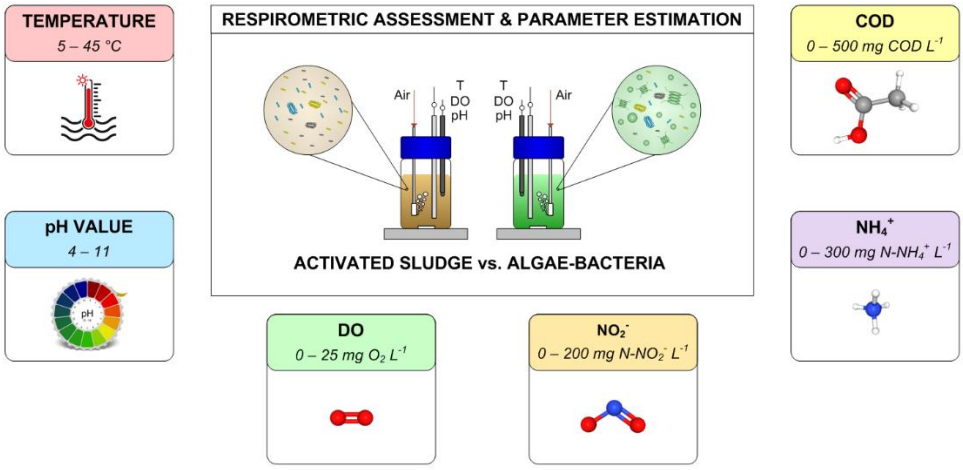


Figure 22.- Respirometry for determining kinetic parameters of bacterial populations in microalgae-bacteria systems and activated sludge processes.

4.6.1. Effect of temperature

The effect of temperature on bacterial populations of AS and MB cultures is shown in Figure 23. The values of the bacterial respiration rate for HET. AOB and NOB were normalized, being SOUR is the Specific Oxygen Uptake Rate, $SOUR_{MAX}$ is the maximum Specific Oxygen Uptake Rate. The trend for all populations followed the typical asymmetric bell curve of bacterial cultures (Rosso et al. 1995), in which the optimal

temperature is closer to the maximum than to the minimum temperature (Equation 9), where T_{MIN} is the minimum cardinal temperature below which the respiration rate is zero [°C], T_{OPT} is the optimal temperature for which the respiration rate is maximum, or equal to one in the case on normalized data [°C], T_{MAX} is the maximum cardinal temperature above which the respiration rate is zero [°C].

$$\frac{SOUR}{SOUR_{MAX}} = \begin{cases} 0, \text{ if } T<T_{MIN} \\ \dfrac{(T-T_{MAX})\cdot(T-T_{MIN})^2}{(T_{OPT}-T_{MIN})\cdot\left((T_{OPT}-T_{MIN})\cdot(T-T_{OPT})-(T_{OPT}-T_{MAX})\cdot(T_{OPT}+T_{MIN}-2\cdot T)\right)}, \text{ if } T_{MIN}<T<T_{MAX} \\ 0, \text{ if } T>T_{MAX} \end{cases} \quad \text{Equation 9}$$

Regarding the estimated optimal temperatures, these ranged from 30 to 36°C, a common range for a wide variety of bacterial strains and species (Rosso et al. 1993). For AS, optimal temperatures were 36.1°C for HB, 30.2°C for AOB and 36.0°C for NOB, while in AB samples, the optimum for growth resulted to be 35.6°C for HB, 34.1°C for AOB and 34.5°C.

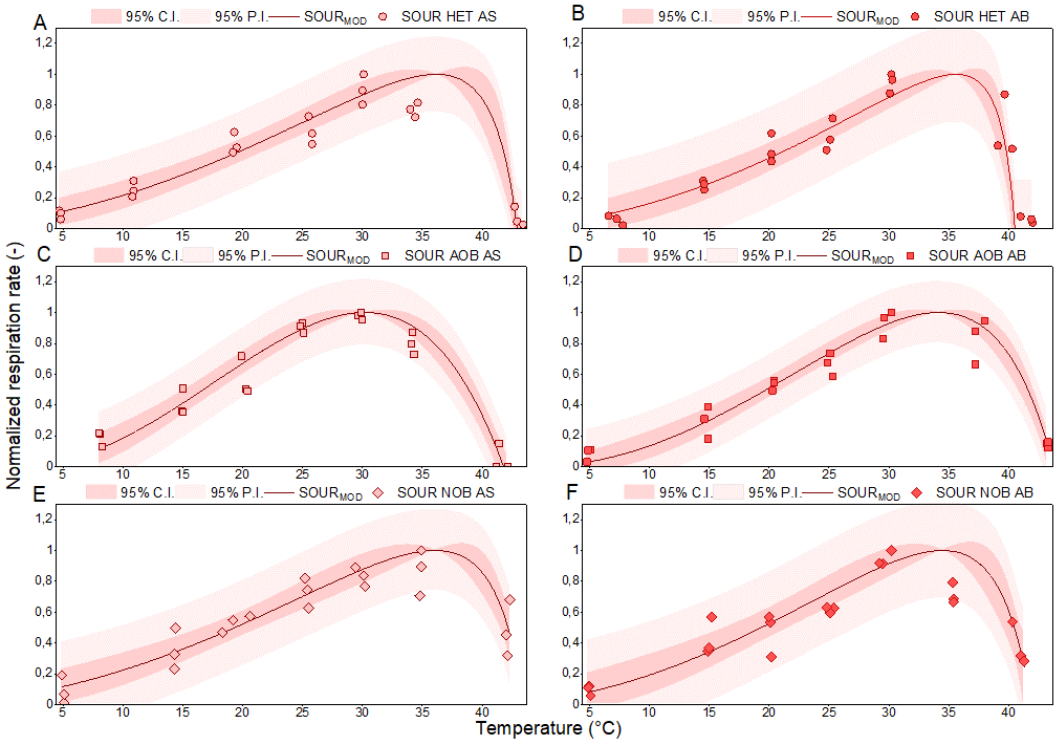


Figure 23.- Effect of temperature on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F). Abbreviations: SOUR, Specific Oxygen Uptake Rate; $SOUR_{MAX}$, the maximum Specific Oxygen Uptake Rate.

4.6.2. Effect of pH

The effect of different pH values on AS and MB is shown in Figure 24. The cardinal model (Equation 10) described well the respirometric dataset of all bacterial populations, where pH_{MIN} is the minimum cardinal pH value below which the respiration rate is zero [-], pH_{OPT} is the optimal pH value for which the respiration rate is maximum, or equal to one in the case on normalized data [-], pH_{MAX} is the maximum cardinal pH value above which the respiration rate is zero [-].

$$\frac{SOUR}{SOUR_{MAX}} = \begin{cases} 0, \text{ if } pH < pH_{MIN} \\ \frac{(pH-pH_{MIN})(pH-pH_{MAX})}{(pH-pH_{MIN})(pH-pH_{MAX})-(pH-pH_{OPT})^2}, \text{ if } pH_{MIN} < pH < pH_{MAX} \\ 0, \text{ if } pH > pH_{MAX} \end{cases} \quad \text{Equation 10}$$

As a general trend, bacteria in both AS and MB systems could resist quite large intervals of pH, still showing some residual activity at pH 4 – 11. Regarding the optimal pH, estimated values varied among the different populations (between 7.5 and 9.5). For AS, optimal pH values were 8.0 for HB, 8.5 for AOB and 7.5 for NOB, while the estimates for MB were shifted towards higher optima (8.8 for HB, 9.5 for AOB and 7.8 for NOB), suggesting that bacteria adapted in the AB system to more alkaliphilic conditions. The higher pH values promoted by photosynthetic CO_2 uptake in microalgae-based wastewater treatment processes can explain these higher pH optima in MB compared to AS.

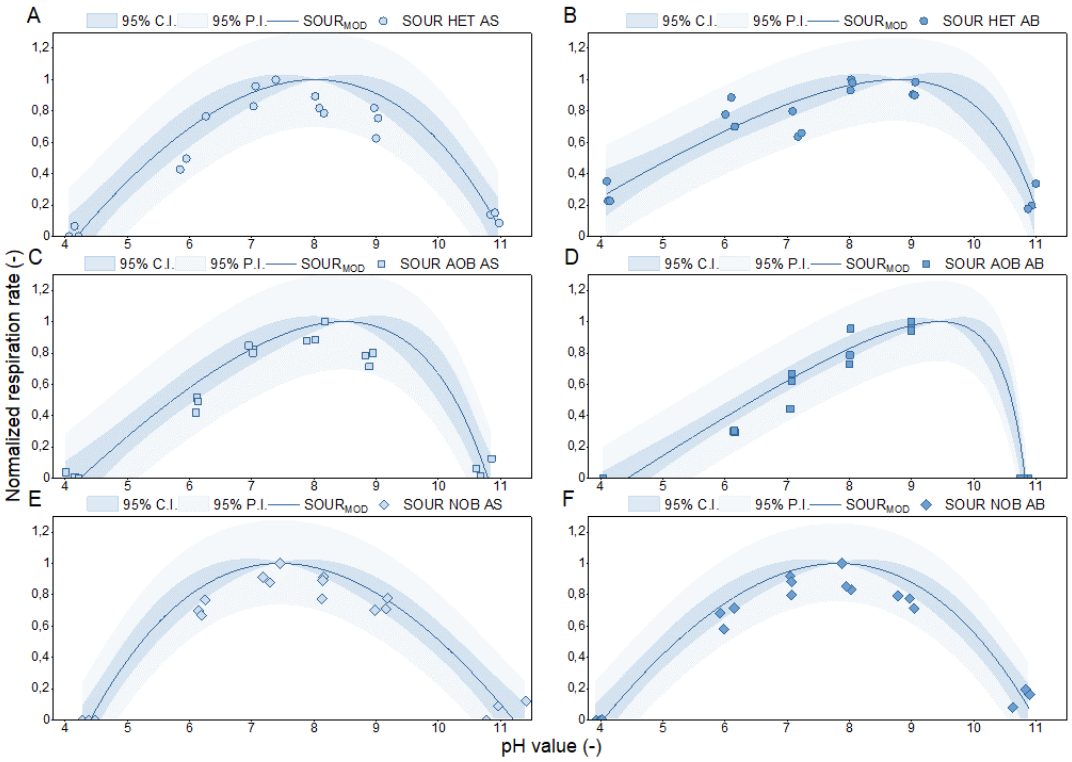


Figure 24.- Effect of pH on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F). Abbreviations: SOUR, Specific Oxygen Uptake Rate; $SOUR_{MAX}$, the maximum Specific Oxygen Uptake Rate.

4.6.3. Effect of dissolved oxygen

The dependence on DO was expressed as either a Monod function with nutrient limitation (Equation) where S represents either the concentration of dissolved oxygen ($mg \cdot L^{-1}$), K_S is the half-saturation constant for dissolved oxygen ($mg \cdot L^{-1}$); or Haldane kinetics, if inhibition at high concentrations occurred (Equation 29), where K_I is the inhibition constant for DO, corresponding to 50% inhibited growth ($mg \cdot L^{-1}$).

$$\frac{SOUR}{SOUR_{MAX}} = \frac{S}{S+K_S}$$ Equation 28

$$\frac{SOUR}{SOUR_{MAX}} = \frac{S}{S+K_S+\frac{S^2}{K_I}}$$ Equation 29

The DO concentration had a relevant effect on bacterial respiration rates. This parameter has an important inhibitory effect on heterotrophic bacteria and AOB sampled from MB culture, while almost no inhibition was observed at high DO concentrations for all activated sludge bacterial populations and NOB in both systems (Figure 25). As an explanation for this fact, possible inhibitory effects of DO concentrations far above air

saturation were previously reported to occur because of the DO diffusion through the membranes and to cause oxidative stress in cells. However, this inhibitory effect only seems to be related to long-term exposure times to dissolved oxygen oversaturation. Therefore, the differences observed between the two treatment systems could be because, in AS, the microorganisms are rarely exposed to high DO concentrations, while in AB cultures the frequent exposition to high DO concentrations (caused by the algal photosynthetic activity), could have led to long-term cell stress (Baez and Shiloach 2014).

For Het, AOB and NOB, estimated half-saturation constants in activated sludge were 0.5, 1.6, and 2.1 mg $DO \cdot L^{-1}$, respectively. In MB cultures, the half-saturation was lower, corresponding to 0.1, 0.9, and 0.9 mg $DO \cdot L^{-1}$, for heterotrophic bacteria, AOB and NOB, respectively. By comparing the dissolved oxygen dependence for the three populations in both systems, it was confirmed, as previously reported for activated sludge samples, that oxygen half-saturation constants for heterotrophic bacteria were generally lower than for nitrifiers. Moreover, several flocs appear in activated sludge while algae-bacteria systems are more dispersed cells. Therefore, the higher diffusion resistance in activated sludge because of the floc formation can explain the higher saturation constants observed in activated sludge, along with the less substrate inhibition. The lower affinity to oxygen for NOB compared to AOB was also described in several studies, and this fact was often adopted as a selective strategy for a partial nitrification in the raceway reactors, allowing to wash out NOB and achieve stable accumulation of NO_2^- (Rongsayamanont et al. 2010).

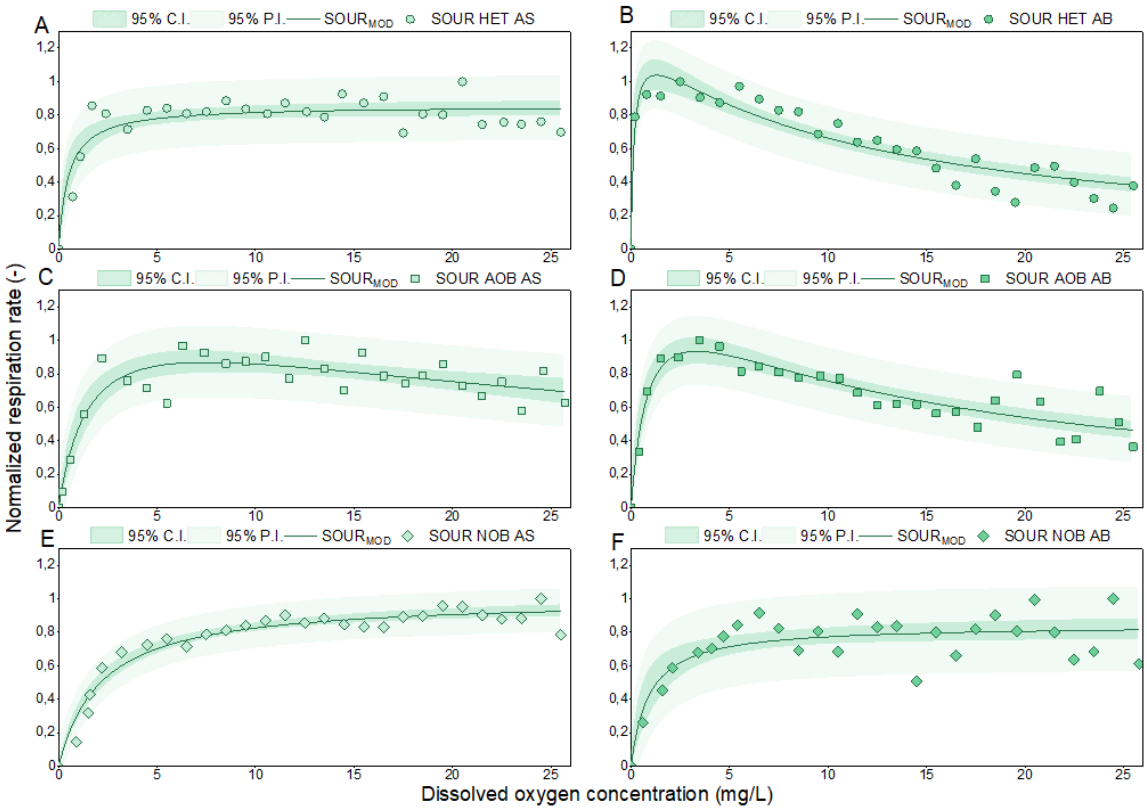


Figure 25.- Effect of dissolved oxygen on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F). Abbreviations: SOUR, Specific Oxygen Uptake Rate; $SOUR_{MAX}$, the maximum Specific Oxygen Uptake Rate

4.6.4. Effect of substrates

The influence of substrates concentration on HET, AOB and NOB was fitted to Monod and Haldane models. The effects of nutrient concentrations on bacterial populations of AS and MB cultures are reported in Figure . For heterotrophic bacteria, the half-saturation constants for COD were quite similar in both activated sludge and algae-bacteria cultures, only showing a slightly lower substrate affinity in AS (3 mg COD·L^{-1}), compared to AB (2 mg COD·L^{-1}). Half-saturation constants found in this study for activated sludge were close to previous works (Ellis et al. 1996), though a wide range of values is available in the literature, reaching up to one order of magnitude more than those found in this work, i.e. up to 20 – 45 mg COD·L^{-1}, depending on process characteristics. Regarding algae-bacteria cultures, as no experimental determination of the half-saturation values is available in the literature for heterotrophic bacteria, mathematical models describing the growth of activated sludge consortia generally assume the value of 20 mg COD·L^{-1} from the ASMs (Solimeno et al. 2019; Sánchez-

zurano et al. 2021), or more similar values to those experimentally found in this study (4 mg COD·L^{-1}), as reported by other sources (Casagli et al. 2021b).

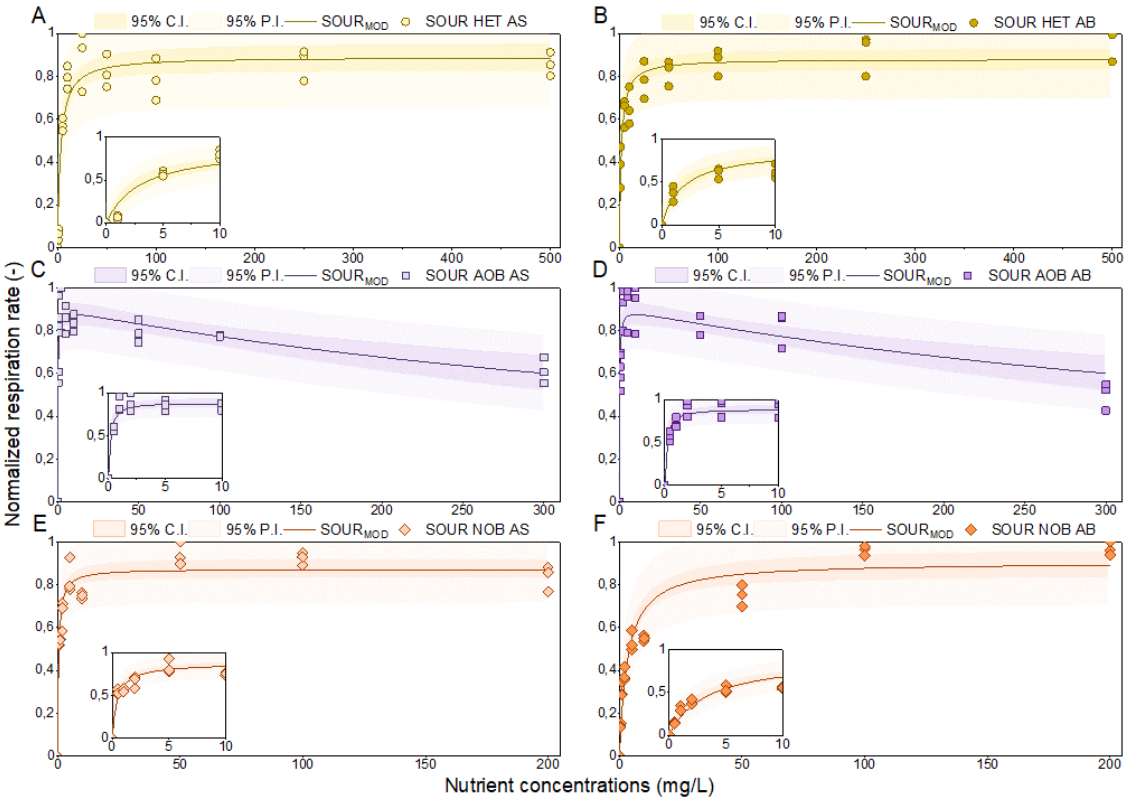


Figure 29.- Effect of substrates on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F). Abbreviations: SOUR, Specific Oxygen Uptake Rate; $SOUR_{MAX}$, the maximum Specific Oxygen Uptake Rate

The activity of the main bacterial populations (HET, AOB and NOB) in AS and MB cultures were sensitive to changes in culture conditions, such as temperature, pH and DO. Specifically, bacterial activities were strongly influenced by the pH and DO values, however, these parameters can be cost-effectively controlled to target optimal values, while temperature control may be more difficult. Along with environmental variables, the concentration of nutrients in biological systems severely affected the microbial activity, with ammoniacal nitrogen originating a strong inhibitory effect. The kinetic modelling and parameter estimation using experimental data are crucial to design and adequately manage biological wastewater treatment systems based on AS and MB consortia, as well as to define control strategies that maximize the treatment efficiency and to guarantee the stability of these processes.

5. CONCLUSIONS

Different methodologies, developed from a biological and engineering point of view, were proposed along the Ph.D. for the study, evaluation, optimization, and control of microalgae-bacteria interactions during wastewater treatment. These interactions are crucial to optimize microalgae-bacteria-based systems.

In this way, the first research work focused on the development of a fast and economical tool that allowed distinguishing between microalgal and bacteria metabolism in microalgae wastewater treatment. The second research work was devoted to using this technique, photo-respirometry, to evaluate the effect of the environmental variables (light, temperature, pH and dissolved oxygen concentration) on microalgal and bacterial activity. The third research work dealt with using the photo-respirometer to evaluate and determine the influence of the culture media composition on microalgal activity. These three works allowed the development of the fourth work, which aimed to create a mathematical model for microalgae-bacteria interactions using kinetic parameters obtained experimentally. Then, the mathematical model was used to develop an interactive tool, the fifth work, which allowed to understand easily the effect of each environmental and operational parameter in microalgae-bacteria wastewater treatment. Finally, the thesis project culminated with research focused on improving some aspects of the biological part of the model; for example, the different bacteria metabolism that appear in the microalgae wastewater treatment, as well as the enhancements to the respirometric techniques to distinguish each microbial population, which was developed based on the experience acquired up through the thesis development, literature review of the state-of-art in this field and international research with experts in this topic.

The main conclusion of the whole work is that mathematical models for microalgae-bacteria interactions, developed using accurate techniques, are essential tools for the industrial implementation of microalgae-bacteria related processes. Their use can help to improve the fundamental performance of microalgae and bacteria populations in such processes and reduce the cost of microalgae-based wastewater treatment operating under optimal conditions as a function of their localization, operation mode, and control strategies.

This overall conclusion is supported by the specific conclusions of each of the sections addressed in this Thesis, which are summarized below. Based on these findings, some ideas for future research works are also provided.

5.1. Anovel photo-respirometry method to characterize consortia in microalgae related wastewater treatment processes.

- Respirometry is a traditional technique applied for the study of bacteria in activated sludge processes that can be adapted to phototrophic organisms such as microalgae.
- Despite being a technique capable for being used in this field of study, it must be optimized before being used as a monitoring tool for microalgae-bacteria systems. It requires specific equipment and the development of protocols to allow differentiating between populations of microalgae, heterotrophic bacteria and nitrifying bacteria in a reliable and reproducible way.
- The use of photo-respirometry allows evaluating how each microbial population contributes in terms of oxygen consumption and production to the microalgae-bacteria consortia. In addition, it is possible to use this strategy to identify how these populations are determined by the type of culture medium used and the production system used.

5.2. Modelling of photosynthesis and respiration rate for microalgae–bacteria consortia.

- Photo-respirometry allows assessing the influence of environmental and operational variables on the microalgal and bacterial activity during microalgae-based wastewater treatment processes.
- Light has a significant effect on the microalgal activity, which can be modified as a function of other parameters including temperature, pH or dissolved oxygen concentration. The bacterial activity, both heterotrophic and nitrifying activity does not depend on light availability, but is dependent on temperature, pH and dissolved oxygen concentration that have a significant effect on their performance.
- The effect of light, temperature, pH and dissolved oxygen concentration on microalgae and bacteria can be modelled using the developed mathematical models for microalgae and heterotrophic and nitrifying bacteria.
- These models were successfully validated with experimental data obtained from microalgae-bacteria cultures.

5.3. Modelling of photosynthesis, respiration, and nutrient yield coefficients in *Scenedemus almeriensis* culture as a function of nitrogen and phosphorus.

- The influence of the concentration of nutrients on the microalgal activity can be evaluated using respirometric protocols in a fast and economic way avoiding the need for performing long traditional experiments.

- The concentration of nitrogen and phosphorous determines the activity of microalgal cells, which can be limited or inhibited under certain conditions. Therefore, the culture media used for microalgae production is a crucial factor. The use of wastewater for microalgae production involves a variable concentration of the main substrates such as ammonium, nitrate or phosphate, which will determine the success of the process.
- The influence of these parameters should be evaluated and determined to obtain kinetic parameters that allow improving the mathematical models for microalgae-bacteria wastewater treatment.
- The use of the simulation tool developed could be used as an accurate, free and rapid strategy to estimate the performance and the productivity of biological systems.

5.4. ABACO: A New Model of Microalgae-Bacteria Consortia for Biological Treatment of Wastewaters

- The kinetic parameters obtained for microalgae, heterotrophic bacteria and nitrifiers allowed developing a mathematical model for simulating microalgae-bacteria wastewater treatment processes. The model included the main populations involved in microalgae-based wastewater treatment together with the most important dissolved components that are consumed or/and produced by the cells.
- The mathematical model was successfully calibrated using Genetic Algorithms, which led to obtaining the maximal growth rate for each population together with the yield coefficient for each substrate.
- The model was calibrated and validated using two different data sets obtained from two lab-scale photobioreactors.
- Photo-respirometry allowed validating the final microbial composition of the microalgae-bacteria cultures and these values were compared with the values estimated by the mathematical model.
- The microalgae-bacteria model designed is a powerful tool to develop control strategies for maximizing the biomass productivity of the system and the capacity of the process to remove/recover nutrients.

5.5. An interactive tool for simulation of biological models into wastewater treatment with microalgae.

- Interactive tools enable us to understand and simplify mathematical models with many compounds, variables, and parameters, such as the ABACO model. In addition, the tool also enables the user to observe the influence of each variable on the performance of each population. The tool was validated using experimental data from microalgae-bacteria cultures produced following industrial practices.
- The tool allows visualizing the variables that are responsible for decreasing the productivity of the system and therefore to improve the control and performance of the system. These variables depend on the localization and the operational conditions of the systems. The latter can be easily modified by the user.
- The tool shows that the light determines the maximal productivity of the microalgal system; which is modified as a function of the rest variables. Both, the temperature and the culture medium composition have a strong influence on the production of the system. The pH and the dissolved oxygen concentration have a lower influence as both parameters can be easily controlled.

  The interactive tool allows performing experiments online and avoid carrying out long time consuming and expensive experiments. Also, it allows taking control strategies as a function of the environmental and operational data to maximize microalgal production.

5.6. Respirometric assessment of bacterial kinetics in algae-bacteria and activated sludge processes

- Respirometry allow distinguishing between AOB and NOB within the nitrifying bacteria in a fast and reliable way.
- The characteristic parameters for heterotrophic bacteria, AOB and NOB are different between AB and AS, especially for the influence of the dissolved oxygen concentration. Also, these values are different to the previous one, obtained using primary wastewater instead of anaerobic digestate.
- The influence of the substrate concentration on the heterotrophic, AOB and NOB activity was strong, especially at low and high concentrations.
- This trial allowed improving the ABACO model introducing two new populations and more accurate values for the kinetic parameters as a function of the origin or localization of the microalgae-bacteria cultures.
- New control and optimization strategies can be developed using the kinetic parameters obtained in this research project.

6. RECOMMENDATIONS FOR FUTURE RESEARCH

This Thesis provides a large number of experimental data and modelling approaches for the microalgae-bacteria interactions that occur during microalgae-based wastewater treatment. Although it may serve as a reference in this research field in microalgae technology, there is still room for investigating and developing new models and operating strategies. The future challenges and gaps to be filled in this field of research are summarized below.

There are well-known models in the literature that can be successfully applied to evaluate the microalgae-bacteria interactions that take place during wastewater treatment. Most of these approaches assume the kinetic parameters for microbial populations found in the literature and they are over-parameterized, which limits their practical application and difficult the studying of the effect of a single variable. In addition, most of them were validated using artificial wastewater, not considering the variability in the composition of wastewater, which is a reality. Consequently, the development of robust models that can be adapted to any wastewater is required. To develop these models, it is essential to establish an adequate methodology that allows obtaining the specific kinetic parameters rapidly and accurately. Photo-respirometry should be improved to make fine measurements because most of the microbial populations appear at low concentrations and they are difficult to distinguish and quantify in terms of oxygen consumption. Moreover, the photo-respirometry technique could be complemented with other techniques that allow improving our knowledge about microalgae-bacteria interactions such as fluorescence microscopy, flow cytometry, metabarcoding or meta transcriptomic. Within the genetic approach, some works have been developed during the PhD thesis focused on determining how environmental and operational variables along with photo-bioreactor designs determine the microbial composition and structure. These studies enabled me to identify the AOB and NOB, which are strongly influenced by operational parameters, and can be correlated with respirometry results. In the end, all this information will be the key to the success of the improvement of the mathematical models for microalgae-bacteria.

7. CONTRIBUTIONS TO SCIENTIFIC JOURNALS

This PhD thesis is presented as a compendium of publications according to modality A of the normative of the University of Almería (article 24). This normative establishes that any PhD thesis can be presented in the compendium modality if it is supported by at least three scientific contributions. This thesis project is supported by 6 scientific articles in journals ranked in the JCR. The articles have been included in this section according to the normative. The reader must take into consideration that each of the papers contains its specific bibliography, which is not related to the main bibliography section of the present document. In addition, it should be remarked that apart from the journal papers, the research work developed during the PhD project resulted in contributions to the general magazine, international conferences, and national conferences.

Each of the contributions has been referred to in the corresponding section among the ones described above. Besides, the PhD candidate made an international research stay in the Politecnico di Milano (Milano, Italy).

Aside from the outcomes included in this chapter, the PhD candidate has participated in different contributions directly derived from the activity carried out during the thesis project. However, these publications have been included in Section 7 according to the normative of the University of Almería.

7.1. A novel photo-respirometry method to characterize consortia in microalgae related wastewater treatment processes.

Research in this field is supported by the following journal publication:

Title	A novel photo-respirometry method to characterize consortia in microalgae related wastewater treatment processes
Authors	**A. Sánchez-Zurano**, C. Gómez-Serrano, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima
Journal	Algal Research
Year	2019
Volume	47
Pages	101858
DOI	https://doi.org/10.1016/j.algal.2020.101858
IF (JCR 2019)	4.401
Categories	Biotechnology & applied microbiology (32/165)

Contribution of the Ph.D. candidate
The Ph.D. candidate, A. Sánchez-Zurano, is the main contributor and first author of this paper.

Aside from the main contribution, the following contributions were presented at international conferences:

- **A. Sánchez-Zurano**, C. Gómez-Serrano, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima, "A novel photorespirometry method to characterize microalgae-bacteria consortia in wastewater treatment". Event: IWA Conference on Algal Technologies and Stabilization Ponds for Wastewater Treatment and Resource Recovery 2019. Type of presentation: Oral. Place: Valladolid, Spain.
- **A. Sánchez-Zurano**, C. Gómez-Serrano, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima, "Measuring the interactions of photosynthesis and respiration in microalgae-bacteria consortia for wastewater treatment". Event: 9th International Conference on Algal Biomass, Biofuels and Bioproducts (AlgalBBB 2019). Type of presentation: Poster. Place: Boulder, CO, USA.

Besides, it resulted also in the following contributions to national conferences:

- **A. Sánchez-Zurano**, C. Gómez-Serrano, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima, "A novel photorespirometry method to characterize microalgae-bacteria consortia in wastewater treatment". Event: VII Simposio de investigación en ciencias experimentales 2018. Type of presentation: Poster. Place: Almeria, Spain.
- **A. Sánchez-Zurano**, C. Gómez-Serrano, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima, "Activity assessment of microalgae bacteria consortia based on ammonium consumption". Event: II Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias 2019. Type of presentation: Poster. Place: Almeria, Spain.

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A novel photo-respirometry method to characterize consortia in microalgae-related wastewater treatment processes

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ABSTRACT

In this paper, a new photo-respirometry method for determining the rates of the main metabolic processes of microalgae-bacteria consortia in microalgae-based wastewater treatment processes has been developed and tested. The proposed protocol consists on applying dark and light periods to a microalgae-bacteria consortium in the presence of different substrates and measuring the rate of oxygen production. This allows determining the activity of microalgae, heterotrophic bacteria and nitrifying bacteria separately.

The method has been optimized in terms of the operation strategy, including the starvation period required, the biomass concentration and the irradiance during the measurements. Results show that a starvation period of one to three days is necessary depending on the nutrient concentration. The optimal experimental conditions determined were a biomass concentration of 0.5 g/L and an irradiance of 200 μmol photons/m^2·s. Furthermore, microalgae-bacteria samples from seven photobioreactors (indoor and outdoor) with different nutrient sources have been evaluated applying the methodology proposed. Regardless of the wastewater type, the microalgae activity is the main metabolic process, with heterotrophic activity increasing along with the chemical oxygen demand (COD) in the wastewater. Nitrifying activity was only observed when high ammonium concentrations were present. The developed method is a powerful tool to adequately manage and operate wastewater treatment processes using microalgae/bacteria consortia, providing valuable information to model wastewater treatment systems with microalgae and determine kinetic parameters.

1. Introduction

The use of microalgae-bacteria consortia in multiple biotechnology processes, such as in wastewater treatment, has required an understanding of the mechanisms involved in microalgae-bacteria interaction [1]. Knowledge of the microalgae-bacteria consortia which appear in the treatment of wastewaters (from urban, industrial, agricultural and animal-use sources) is essential to maximise the benefits of microalgae wastewater treatment, as previously reported [2–4]. The schematic functioning of this consortia has been previously described. When illuminated, the microalgae consume inorganic carbon, nitrogen and phosphorus, as well as other compounds, to produce biomass while simultaneously releasing oxygen from photosynthesis [5,6]. This activity is beneficial in wastewater treatment processes because the oxygen produced by microalgae can be used by aerobic bacteria to biodegrade pollutants, so they are capable of oxidizing organic matter into inorganic compounds mainly containing nitrogen and phosphorus [7]. Moreover, the carbon dioxide produced by bacterial respiration is consumed by the microalgae, completing a photosynthesis-respiration cycle [8].

In microalgae-based wastewater treatment, it is considered that an equilibrium exists between microalgae and bacteria-related processes. However, this is not always true because, depending on the operational conditions, the prevalence of microalgae or bacteria varies greatly [9]. Accordingly, recent studies have shown that the bacterial contribution to a consortium's performance is lower than that from the microalgae; this is due to the fact that the bacteria's metabolism is faster than the microalgae's so only a low bacterial mass is necessary to degrade organic compounds into inorganic compounds. Moreover, the amount of oxygen produced in this process by the microalgae population is far higher than that required by the low bacterial mass. Consequently, the relationship between microalgae and bacteria in a consortium is determined by the wastewater composition and its feed rate. For this reason, it is essential to understand and model these phenomena so as to adequately design and operate microalgae-based systems for wastewater treatment [10].

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Similarly, inspired by classic respirometric techniques in the activated sludge process, some authors started to apply respirometry to study microalgae activity and to determine microalgae kinetic parameters [11–13]. Despite respirometry has been considered for years an adequate approach to rapidly determine microalgae activity, the methods applied were often ambiguous on what exact test devices are needed or test conditions. In this way, [14] developed a standard procedure to determine algal activities through specific oxygen production rate (SOPR) in the light and specific oxygen uptake rate (SOUR) in the dark, which could allow for determination of microalgae growth kinetic. Other authors have applied similar respirometry techniques in microalgae cultures but shortening the duration of the dark-light cycles to a few minutes that are sufficient to obtain enough dissolved oxygen measurements in order to estimate the influence of different environmental factors [13,15,16]. However, respirometry methods have been generally applied to pure microalgae cultures, while in wastewater processes is indispensable to consider the existence of microalgae-bacteria consortia. On this issue, some authors have started to develop respirometry methods for studying microalgae-bacteria consortia by evaluating both microalgae and bacterial activity, specifically nitrifying activity in microalgae wastewater treatment [17].

This work aims to develop a complete photo-respirometry method to quantify the microalgae-bacteria consortia found in wastewater treatment processes, distinguishing between microalgae, heterotrophic and nitrifying activity using the oxygen production/consumption rates. The method's operational conditions have been optimized to define a standardized protocol for characterizing this type of consortia. Furthermore, the developed method has been used to compare the composition of microalgae-bacteria consortia prevailing in different wastewater treatment processes and in pure microalgae cultures, thus showing the large variability of these types of consortia. The methodology described here is a valuable tool for optimizing any microalgae-based process, especially those related to wastewater treatment, which are expected to expand greatly in the near future.

2. Materials and methods

2.1. Photosynthesis and respiration rate measurements

A photo-respirometer device was designed and built in-house. This equipment allows to determine any variation in dissolved oxygen concentration in microalgae culture samples under controlled conditions. It comprises an 80 mL jacketed transparent cylindrical glass flask (connected to a temperature-controlled water reservoir for the device's temperature control), which is magnetically stirred and artificially illuminated using two power-controlled LED lamps (Secom Iluminacion 4125015085DR, Spain) placed to the right and left of the glass chamber (Fig. 1A). The light provided by the lamps can be automatically regulated to obtain the desired irradiance inside the centre of the chamber once the sample is added. The device is also equipped with a diffuser through which gases (air, O_2, N_2 and CO_2) can be supplied at a low flow rate to modify the culture's dissolved oxygen or pH. To achieve this, the device is also equipped with sensors for irradiance (QSL-1000, Walz, Germany), temperature (PT-100), pH (Crison 5343, Barcelona, Spain) and dissolved oxygen (Crison 5002, Barcelona, Spain) located inside the flask.

An adequate protocol was developed to determine the microalgae cultures' photosynthesis and respiration rates. The developed methodology allows to distinguish between the metabolisms of the three main populations: the microalgae, the heterotrophic bacteria and the nitrifying bacteria. Firstly, samples of the microalgae cultures were taken and subjected to nutrient starvation (continuous light of 200 $\mu Em^{-2}s^{-1}$ and an aeration rate of 0.2 $v{\cdot}v^{-1}{\cdot}min^{-1}$) to remove the organic matter and the ammonium present in the medium. Subsequently, the samples were placed inside the jacketed flask and the variation in dissolved oxygen over time was measured under different conditions. The temperature was controlled at 24–25 °C in the culture. To determine the microalgae's net photosynthesis rate and the respiration rates of the heterotrophic and nitrifying bacteria, each sample was subjected to four light–dark periods of 4 min, during which the variation in dissolved oxygen over time was measured and registered. These values allow calculating the respective metabolic rates. The first minute of exposure was disregarded as it was considered to be adaptation time.

The variation in dissolved oxygen was measured in the 90–130%Sat range (i.e., when the level of dissolved oxygen was between 90% and 130% with respect 100% that corresponds to air saturation), in which the oxygen mass transfer was determined. The entire system was computer-controlled using DaqFactory software. In the following section, each part of the process is described in detail, including the expected biological reactions affecting the dissolved oxygen concentration:

- Microalgae net photosynthesis rate (MNPR). A culture sample was placed inside the photo-respirometer and then exposed to four light–dark cycles of 4 min each to measure and register the variation in dissolved oxygen under every condition. Between the dark and light periods, air was provided in order to recover the 100%Sat of the dissolved oxygen. During the light periods, oxygen generation is expected as a result of the active photosynthesis in microalgae whereas during the dark periods, the oxygen is consumed by the endogenous respiration rate. Endogenous respiration is defined as the culture's oxygen consumption rate when subjected to starvation, which is indicative of the active biomass concentration [18]. The microalgae net photosynthesis rate was calculated as the difference between the slope of the oxygen production during the light period minus the slope of the oxygen consumption during the dark period.
- Heterotrophic bacteria respiration rate (HBRR). Another culture sample was used for this measurement. Now, 0.8 mL of sodium acetate (30 g/L) were added as an organic matter source. Acetate has been described as a substrate for use in wastewater respirometry tests [18]. The sample was exposed to four light–dark cycles of 4 min each. Between each light and dark period, air was provided in order to recover the 100%Sat of the dissolved oxygen. The oxygen consumption in the dark phase allows to determine the oxygen consumed by the heterotrophic biomass. The respiration rate of the heterotrophic bacteria was calculated as the slope of the oxygen consumption with sodium acetate minus the slope of the oxygen consumption during the dark period in the endogenous culture.
- Nitrifying bacteria respiration rate (NBRR). Another sample of culture was used for this measurement. For this procedure, 0.8 mL of ammonium chloride (3 g/L) was added as the ammonium source. Among the different ammonium sources that have been used to evaluate nitrifying activity in activated sludge processes and microalgae-bacteria consortia, ammonium chloride has been the most extensively utilized [17,18]. The sample was exposed to four light–dark cycles of 4 min each. In the middle of each light and dark period, air was provided in order to recover the 100%Sat of dissolved oxygen. The oxygen consumption in the dark phase allows to determine the oxygen consumed by nitrifying biomass. The nitrifying bacteria's respiration rate was calculated as the slope of the oxygen consumption with ammonium chloride minus the slope of the oxygen consumption during the dark period in the endogenous culture.

A simplified scheme of the proposed methodology is shown in Fig. 1B. During the dark phases (D1-D4), the dissolved oxygen is consumed by microalgal-bacterial endogenous respiration. During the light phase (L1-L4), the microalgae perform photosynthesis and the dissolved oxygen production increases while, simultaneously, it is consumed by the respiration processes. The microalgae net photosynthesis rate (MNPR) is calculated as the difference between the oxygen production

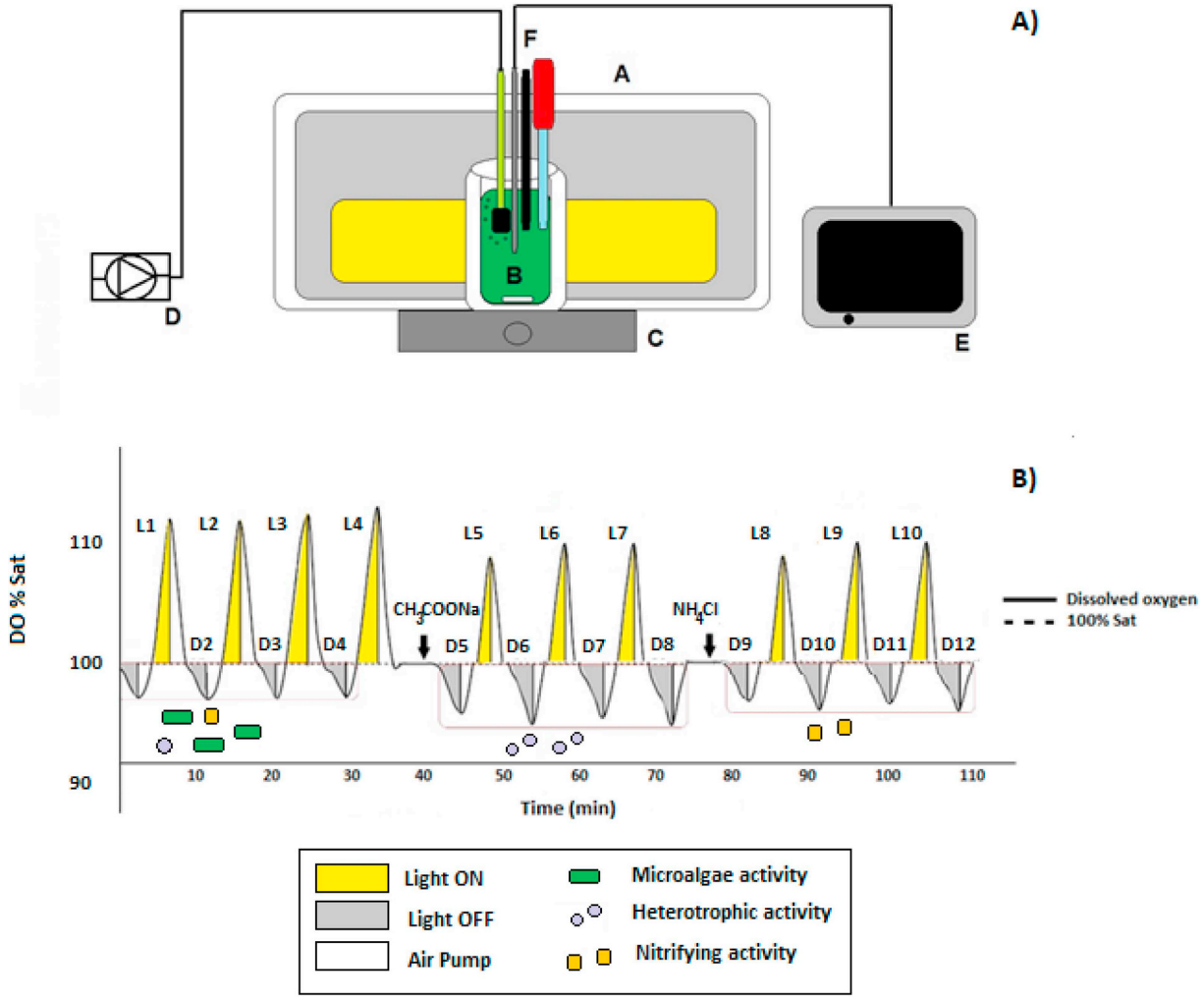

Fig. 1. Layout of the respirometer (A: lights, B: 250 mL glass flask, C: magnetic mixer, D: air pump, E: multi-meter and data logger, F: DO probe) (A). Expected result of a respirometric test to estimate the microalgae net photosynthesis rate (MNPR), the heterotrophic bacteria respiration rate (HBRR) and nitrifying bacteria respiration rate (NBRR). Dark-light periods are reported, showing the variation in dissolved oxygen with time in each of the phases before and after the addition of substrates which activate bacterial populations (B).

rate (OPR) during the light period minus the oxygen consumption rate (OCR) during the dark period, divided by the dry weight of total biomass (Cb) (Eq. 1).

$$MNPR = \frac{OPR - OCR}{Cb} \quad (1)$$

After adding sodium acetate, the same measurements are performed to determine the heterotrophic metabolism, always starting with a new sample. During the dark phases (D5-D8), the dissolved oxygen is consumed by heterotrophic biomass. Thus, the heterotrophic bacteria respiration rate (HBRR) was calculated as the difference between the heterotrophic oxygen consumption (HOCR) rate, after providing acetate, and the oxygen consumption rate (OCR) without adding sodium acetate, divided by the dry weight of the total biomass (Eq. 2).

$$HBRR = \frac{HOCR - OCR}{Cb} \quad (2)$$

Similarly, the nitrifying bacteria respiration rate (NBRR) was calculated by adding ammonia chloride as a substrate in a new sample of the culture. NBRR is determined as the difference between the nitrifying oxygen consumption (NOCR) rate after providing ammonium chloride (D9-D12) and the oxygen consumption rate (OCR) without adding ammonium chloride, divided by the dry weight of the total biomass (Eq. 3).

$$NBRR = \frac{NOCR - OCR}{Cb} \quad (3)$$

Four measurement replicates of each biological activity were done.

2.2. Oxygen mass transfer determination

In order to correct the influence of desorption on the metabolic activity measurements, the oxygen mass transfer coefficient ($\boldsymbol{K_L a}$) in absence of aeration was determined experimentally. The method used consisted in measuring the dissolved oxygen concentration versus time profiles in the same chemical-physical conditions set during the experiments. For this, a cell-free sample was placed in the measurement device and the concentration of oxygen was increased to 130%.sat by bubbling with the pure O_2 gas. After this, the bubbling was stopped and the variation in oxygen concentration ($\boldsymbol{C_{O2}}$) with time was monitored for around 4 h. The $\boldsymbol{K_L a}$ in the system quantifies the proportionality between the oxygen exchange between the liquid and gas phases and the driving force expressed as ($\boldsymbol{C_{O2}}^* - \boldsymbol{C_{O2}}$) leading to the following elementary mass balance:

$$\frac{dCO2}{dt} = K_L a\,(C_{02}^* - C_{O2}) \quad (4)$$

Where dC_{O2}/dt is the oxygen accumulation expressed as the derivate of C_{O2} (mg/L) over time, K_La is the global oxygen mass transfer

Table 1
Composition of the waters used as influent in the cultivation systems.

Parameters	Cultivation systems			
	Arnon medium	Primary domestic wastewater	Pig manure wastewater	Agricultural waste leachates
pH	7.9 ± 0.2	7.6 ± 0.1	7.7 ± 0.0	–
Conductivity, mS/cm^{-1}	2.3 ± 0.1	1.9 ± 0.1	17 ± 0.2	–
Turbidity, FTU	0.0 ± 0.0	17 ± 0.4	7.3*10^3 ± 3.1	1.9*10^3 ± 0.9
SST, g/L	0.6 ± 0.0	0.6 ± 0.1	12 ± 0.1	8.2 ± 2.4
N-NH_4, mg/L	0.0 ± 0.0	59 ± 0.8	2.9*10^3 ± 1.2	3.9*10^3 ± 3.1
N-NO_3, mg/L	1.4*10^2 ± 2.2	2.9 ± 0.1	7.4*10^2 ± 1.2	1.9*10^2 ± 2.4
P-PO_4, mg/L	30.9 ± 0.7	11.1 ± 0.3	1.3*10^2 ± 1.5	8.2*10^2 ± 1.8
COD, mg/L	–	5*10^2 ± 3.2	2.2*10^4 ± 5.6	3.3*10^4 ± 4.9

Data shown are the mean ± SD (n = 3).

coefficient (h^{-1}), and C_{O2}^* is the oxygen saturation concentration in the liquid.

Eq. 4 can be rearranged as follow:

$$K_L a \int_0^t dt = \int_{C_{O2o}}^{C_{O2}} \frac{dC_{O2}}{C_{02}^* - C_{O2}} \quad (5)$$

Leading to

$$ln\left(\frac{C_{02}^* - C_{O2o}}{C_{02}^* - C_{O2}}\right) = t \cdot K_L a \quad (6)$$

This means that K_La can be obtained from data of $C_{O2,t}$ vs. as the slope of the plot of $ln\left(\frac{C_{02}^* - C_{O2o}}{C_{02}^* - C_{O2}}\right)$ against time. The final value obtained was 1.08 h^{-1}. This value was used to correct the different metabolic responses as described by [13].

2.3. Respiration rate measurements by inhibiting nitrifying activity

The methodology was enhanced in order to distinguish between the ammonium consumption by nitrifying bacteria and by microalgae inhibiting the nitrifying activity of the ammonia-oxidizing bacteria. For this, allylthiourea solution (ATU) (1 g/L) was used as inhibitor, which was dosed in order to achieve ATU concentration of 10 mg/L. Then, the following measurements described in detail were done.

- Firstly, a culture sample was placed inside the photo-respirometer and exposed to four light–dark cycles of 4 min each to measure and register the variation in dissolved oxygen under light-dark periods. During the dark periods, the oxygen is consumed by the endogenous respiration rate. The oxygen consumption rate (OCR) during the dark period was calculated as the slope of dissolved oxygen consumption during the dark period.
- Next, a fresh sample was placed in the equipment in order to determine the total ammonium respiration rate (TARR) by the culture. For this, 0.8 mL of ammonium chloride solution (3 g/L) was added as nutrient source. The sample was then exposed to four light–dark cycles of 4 min each. The ammonium oxygen consumption rate (AOCR) in the dark phase was calculated as the slope of dissolved oxygen consumption with ammonium chloride minus the dissolved oxygen consumption during the dark period in the endogenous culture.
- Then, in order to determine the ammonium respiration due to the microalgae activity, the microalgae ammonium respiration rate (MARR) was calculated using another sample. In this measurement, ATU and ammonium chloride were added in order to inhibit ammonia oxidizing bacteria and measure the microalgae ammonium respiration. The sample was exposed to four light–dark cycles of 4 min each. The microalgae ammonium oxygen consumption rate (MAOCR) in the dark phase was calculated as the slope of dissolved oxygen consumption with ATU and ammonium chloride minus the dissolved oxygen consumption during the dark period in the endogenous culture.

The proposed protocol was used to determine the total ammonium respiration rate (TARR):

$$TARR = \frac{AOCR - OCR}{Cb} \quad (7)$$

The microalgae ammonium respiration rate (MARR):

$$MARR = \frac{MAOCR - OCR}{Cb} \quad (8)$$

Thus, the nitrifying ammonium respiration rate (NARR) was calculated as the difference between the total ammonium respiration rate (TARR) rate and the microalgae ammonium respiration rate (MARR).

$$NARR = TARR - MARR \quad (9)$$

2.4. Microorganisms and culture conditions

2.4.1. Samples from laboratory culture

The strain *Scenedesmus almeriensis* was used as the control microorganism. Stock cultures were maintained photo-autotrophically in spherical flasks (1.0 L capacity) using Arnon medium [19]. The culture was continuously bubbled with an air–1% CO_2 mixture to control the pH at 8.0. The culture was artificially illuminated in a 12:12 h L/D cycle using four Philips PL-32W/840/4p white-light lamps, providing an irradiance of 750 μE/m^2 s. For the experiments, this inoculum was transferred to laboratory-scale photobioreactors and industrial-scale outdoor photobioreactors. Details on the reactors and culture medium used in each experiment are given below. The average composition of the wastewaters used is reported in Table 1.

2.4.2. Samples from spherical flasks fed with Arnon medium with acetate

Experiments were performed in 4 spherical flasks (1.0 L capacity) filled up to 650 mL with Arnon medium complemented with different acetate concentrations and 20% of *Scenedesmus almeriensis* inoculum. The Arnon medium, except the organic substrates, was sterilised in an autoclave at 128 °C for 21 min. The organic nutrient (acetate) was separately sterilised by filtration through 0.2 μm pore membranes. The reactors were operated in batch mode for 96 h. The nutrient concentrations used were 0.005, 0.01 and 0.05 M, and a photoautotrophic control. Each reactor was aerated at a rate of 0.2 v/v/min, with CO_2 injected on demand (pH = 8). The reactors were artificially illuminated continuously using eight 28 W fluorescent tubes (Philips Daylight T5). The temperature was kept at 25 °C.

2.4.3. Samples from bubble columns fed with crop residue leachate

Experiments were performed in 12 bubble column-type reactors with spherical bases (3 cm in diameter, 45 cm in height and with a 300 mL capacity) filled up to 250 mL with leachate from crop residues diluted in water (10% crop residues, 90% water) and 20% of

Scenedesmus almeriensis inoculum. Each reactor was aerated at a rate of 0.2 v/v/min, with CO_2 injected on demand at pH = 8. Eight 28 W fluorescent tubes (Philips Daylight T5) were used to artificially illuminated the reactors, in order to simulate daylight cycle. The cultures' temperature was kept at 25 °C. The reactors were operated in batch mode for 6 days, after which they were operated in a semi-continuous mode (20%) (i.e., every morning a 20% of the volume of each reactor volume was harvested during approximately 3 h, while an equal volume of medium was introduced during the same time interval).

2.4.4. Samples from stirred-tank reactors fed with sewage

Experiments were performed in four 1 L stirred-tank reactors (9 cm in diameter, 30 cm in height and with a 1.5 L capacity) operated in the laboratory but simulating outdoor conditions. These reactors were filled with 1 L of sewage taken directly after primary treatment from the wastewater treatment plant in Roquetas de Mar (Almería) and 20% of *Scenedesmus almeriensis* inoculum. To prevent the adverse effect of excessive dissolved oxygen accumulation, the dissolved oxygen was controlled and kept below 200%Sat by supplying air on demand; CO_2 was also injected on demand to control the pH at 8. The reactors were artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5) on a simulated daylight cycle. The cultures' temperature was kept at 25 °C. The reactors were operated in batch mode for 6 days, after which they were operated in continuous mode. For this, 20% of culture volume was harvested every day and replaced with fresh culture medium.

2.4.5. Samples from an outdoor raceway reactor fed with sewage

A 32 m^2 (4.4 m^3) open raceway reactor operated at a 0.12 m water depth was used. The reactor is equipped with a 1 m^3 sump where pH is controlled at 8. In the raceway reactor, the culture is circulated at 0.2 m s^{-1} using a rotating paddlewheel actuated by an electric motor [20]. A SCADA system monitors and controls the reactor's overall operation, including environmental parameters such as solar radiation and ambient temperature, and culture parameters such as pH, temperature and dissolved oxygen. The experiments were performed in semi-continuous mode, by initially filling the reactor with wastewater inoculated with 10% total volume of *Scenedesmus almeriensis* culture, which was operated in batch mode for one week, after which it was operated in semi-continuous mode at a daily dilution rate of 20%.

2.4.6. Samples from an outdoor thin-layer cascade reactor fed with diluted manure

An open 32 m^2 (1.4 m^3) thin-layer cascade reactor operated at a 0.02 m water depth was used. The reactor is equipped with a 0.8 m^3 sump where pH is controlled at 8 by the on-demand injection of pure CO_2 at 5 l min^{-1}, or air supplied at 50 l min^{-1} to remove oxygen [20]. A SCADA system monitors and controls the reactor's overall operation, including environmental parameters such as solar radiation and ambient temperature, and culture parameters. The experiments were performed in semi-continuous mode by initially filling the reactor with pig manure diluted in water (10% pig manure, 90% water) and inoculated with a 10% total volume of *Scenedesmus almeriensis* culture from a 3.0 m^3 tubular photobioreactor, which was operated in batch mode for one week, after which it was operated in semi-continuous mode at a daily dilution rate of 30%.

2.4.7. Samples from an outdoor tubular photobioreactor fed with fertilizers

A 3.0 m^3 capacity industrial tubular photobioreactor (T-PBR) was used for the *S. almeriensis* culture. The facility consists of ten tubular fence-type photobioreactors built as previously described [21]. The reactors were bubbled at a constant airflow rate of 200 l·min^{-1} while the pH is controlled (pH = 8) by the on-demand injection of pure CO_2 at 3 l min^{-1}. The culture temperature was controlled by passing cooling water. The reactors were operated in continuous mode by harvesting 20% of the culture volume every day, which was replaced by fresh medium.

2.5. Biomass concentration and analytical methods

The biomass concentration (Cb) was measured by dry weight. The biomass concentration includes both microalgae, bacteria and inert suspended particles. It was used 100 mL aliquots of the culture filtered through Macherey-Nagel glass fiber MN 85/90. Then, the filters were dried in an oven at 80 °C for 24 h. Standard official methods approved by the Spanish Ministry of Agriculture [22] were used to check that the samples used were in starvation. Furthermore, the same methods were applied to analyse the composition of the wastewater samples and the water from the reactors. The phosphorus was measured by visible spectrophotometry through the phospho-vanado-molybdate complex. Nitrates were quantified at between 220 and 275 nm using a spectrophotometer. Ammonium was measured using the Nessler reactive method. The Total Chemical Oxygen Demand (COD) was determined by spectrophotometric measurement using Hach-Lange kits (LCl-400).

2.6. Software and statistical analysis

The data acquisition and control software DaqFactory (Azeotech, USA) was used to gather the photosynthesis and respiration rate data. All the experiments were performed at least by triplicate to allow calculating the mean values and standard deviation that are shown. Statistical analysis of data was carried out with the software Microsoft Excel.

3. Results and discussion

3.1. Development of the proposed methodology

To carry out the photo-respirometry methodology it was necessary to subject previously the culture samples to starvation to eliminate ammonium and organic matter. Then it is possible to add specific substrates that allow distinguishing between the respiration rates of the two types of bacteria studied. Heterotrophic biomass uses substrate consisting of carbonaceous material; therefore, it was checked for the absence of organic matter in the samples after starvation. Nitrifying bacteria, on the other hand, are autotrophic bacteria which use dissolved carbon dioxide to oxidise ammonia to nitrite and nitrite to nitrate. Thus, starvation is also applied in order to eliminate the initial ammonium in the culture. Then the addition of ammonium chloride is used to distinguish the nitrifying activity [23] of the sample. It was experimentally determined that one day of starvation is sufficient for primary domestic wastewater, two days for animal manure wastewater while three days are needed for lixiviated compost wastewater. After the starvation period, different respirometric measurements were performed using microalgae-bacteria cultures obtained from wastewater to check the viability of the methodology. A preliminary example of one functional test is shown in Table 2. It summarizes the overall values determined and the standard deviation of the measurements. It is possible to observe that during the light phase, the dissolved oxygen level increased rapidly from 100%Sat to 110%Sat.; the OPR was 16.7

Table 2
Results obtained in preliminary tests showing the method precision.

Parameters	Rates
OPR, mgO_2/L·h	16.7 ± 1.1
OCR, mg O_2/L·h	−1.9 ± 0.2
MNPR, mgO_2/gbiomass·h	37.9 ± 1.5
HBRR, mg O_2/gbiomass·h	−11.5 ± 1.3
NBRR, mg O_2/gbiomass·h	−7.2 ± 0.6

Values correspond to the mean ± SD (n = 4).

mgO_2/L·h. During the dark phase, the dissolved oxygen level dropped to 98%Sat.; the OCR was 1.9 mgO_2/L·h. From these values, it was calculated that the microalgae net photosynthesis rate (MNPR) was 37.9 mgO_2/gbiomass·h, a value normalized to the biomass dry weight. Another sample of culture was used to determine the heterotrophic bacteria respiration rate (HBRR), which was calculated by adding sodium acetate to the sample after starvation. In this case, the dissolved oxygen level decreased to 93.9%Sat., resulting in a HBRR of 11.5 mgO_2/gbiomass·h. Lastly, the nitrifying activity was determined by adding ammonium chloride as the nitrogen source. In these experiments the dissolved oxygen concentration during the dark phase dropped to 96.9%Sat., corresponding to an NBRR of 7.2 mgO_2/gbiomass·h. The results confirm the precision of the measurements, with the standard deviation being less than 10% of the values obtained.

The results reported here show that a respirometry method based on oxygen production/consumption is a useful and rapid technique. This permitted to study the contribution of each population in the microalgae-bacteria consortium by distinguishing the oxygen production rate (OPR) from microalgae photosynthetic activity, the oxygen consumption rate (OCR) from endogenous respiration, the microalgae net photosynthesis rate (MNPR), the heterotrophic bacteria respiration rate (HBRR), and the nitrifying bacteria respiration rate (NBRR). The results from these preliminary tests, and its variability, were comparable to previous studies focusing on the activity of microalgal-bacterial wastewater consortia using respirometric tests. The oxygen consumption rate (OCR) results from endogenous respiration (3.8 mgO2/gbiomass·h) were quite similar to the results described by [17] of 4.3 and 4.1 mg O2/gTSS.h, and were within the range indicated by [24] (0.9–5.1 mg O2/gTSS).

3.2. Determination of optimum light availability and biomass concentration

One of the main factors influencing microalgae behaviour is light availability, this is the irradiance to which the cells are exposed in the culture. This is determined by the external irradiance and the biomass concentration as well as the culture geometry and size (diameter in our case). To determine the optimal irradiance to carry out the measurements, experiments were performed using samples from laboratory stirred-tank reactors fed with sewage. These samples were selected because they are the most relevant in terms of the further application of the methodology proposed. The experiments were carried out at a fixed biomass concentration of 0.5 g/L, the irradiance inside the sample, measured with the interior sensor, was modified by changing the external irradiance. The results show that, at low irradiance values (50 μmol photons/m^2·s), the microalgae photosynthesis rate is also low, increasing with light availability up to values of 500 μmol photons/m^2·s, and then remaining constant up to values of 2000 μmol photons/m^2·s (Fig. 2). Regarding the heterotrophic and nitrifying bacteria, they did not show significant differences regardless of the irradiance values imposed. Microalgae activity, on the other hand, was maximal at values of 500 μmol photons/m^2·s but to avoid saturation during photosynthesis, an irradiance of 200 μmol photons/m^2·s was selected for the measurements. As with the previews studies, the results have verified that under real conditions, the cultures are mainly photo-limited, the average irradiance being from 100 to 300 μmol photons/m^2·s [15,25,26]. Regarding the biomass concentration, there is a direct relationship between the production/uptake of oxygen and the relative biomass concentration in the cultures. For this reason, determining the optimal biomass concentration at which the measurements should be performed was essential. This variable greatly impacts the method's precision and sensitivity. Consequently, experiments were also performed using microalgae cultures from laboratory stirred-tank reactors fed with sewage as the most representative sample type, with distilled water as the control. Measurements were carried out at different biomass concentrations up to 0.8 g/L, determining the three main metabolisms: (i) photosynthesis by microalgae, (ii) respiration by heterotrophic bacteria and (iii) respiration by nitrifying bacteria (Fig. 3). The results show that even for low biomass concentration values, as low as 0.1 g/L, the photosynthesis rate was high enough to provide a statistically significant response, much larger than the measurements' standard deviation. The oxygen production rate's standard deviation, based on the photosynthesis rate, was similar for every biomass concentration tested, so values from 0.1 to 0.8 g/L can be used for the standard method. However, the oxygen consumption rate from the respiration measured was much lower than from photosynthesis; hardly measurable at biomass concentrations of 0.1 g/L. Only at a biomass concentrations of 0.2 g/L there was a measurable oxygen consumption rate for bacteria but too close to the measurement error, so we decided to choose a biomass concentration of 0.5 g/L and not higher to prevent severe light attenuation effects and because most samples are obtained with a concentration close to 0.5 g/L.

3.3. Determination of mixotrophic activity

In the literature, results about mixotrophic metabolism in microalgae-bacteria culture are ambiguous. Mixotrophic activity in microalgae is relevant because it could significantly affect the oxygen production and consumption rate, especially when carbonaceous substrates such as sodium acetate are used. Accordingly, some experiments were designed to evaluate the potential mixotrophic metabolism of microalgae in order to ensure that sodium acetate is consumed by heterotrophic bacteria when it is used as a substrate in respirometric tests.

On the one hand, samples from an outdoor raceway reactor fed with sewage were taken and the heterotrophic activity was evaluated following the proposed respirometric methodology. To ensure that this activity was due to the heterotrophic biomass and not to the microalgae activity of the wastewater culture, samples of the same culture were filtered through 0,9 μm fiber filters to separate the biomass from the liquid phase.

Then, the heterotrophic activity of the filtrate was evaluated by respirometric tests. The results showed that heterotrophic activity in microalgae wastewater samples was 2.3 mgO_2/L·h while 2.1 mgO_2/L·h was the heterotrophic activity in the liquid phase using acetate as a substrate (Fig. 4). This experiment was essential to ensure that the mixotrophic behaviour of the microalgae can be neglected because the heterotrophic activity was the same with or without microalgae, being this activity caused by the heterotrophic biomass from the wastewater. The small difference between the two measurements could be due to the heterotrophic biomass that is retained in the filter along with the microalgae.

Once the mixotrophic activity in wastewater cultures has been discarded, the possible mixotrophic growth of *Scenedesmus almeriensis* cultures using sodium acetate as an organic substrate has been studied. In this way, the methodology described in section “2.4.2 Samples from spherical flasks fed with Arnon medium with acetate” was applied.

The results show that the growth of *Scenedesmus almeriensis* is the same under photoautotrophic conditions and using 0.005 M, 0.01 M and 0.05 M sodium acetate (data not shown). In this sense, it can be ruled out that *Scenedesmus almeriensis* uses sodium acetate for its growth. These data demonstrate that the main microalgae presents in the cultures does not use sodium acetate in the short term. This results allowed discarding the influence of sodium acetate consumption during respirometric tests, although the possibility that they were capable of using acetate for mixotrophic growth in the long term cannot be ruled out, but in any case it requires a long adaptation time or it is only consumed under conditions of strong light limitation [27].

3.4. Evaluation of the metabolisms prevailing in different cultures

Once the methodology was defined, it could be used to evaluate the metabolism in different samples from different culture media and reactors (both in the laboratory and outdoors) (Table 1). The results show

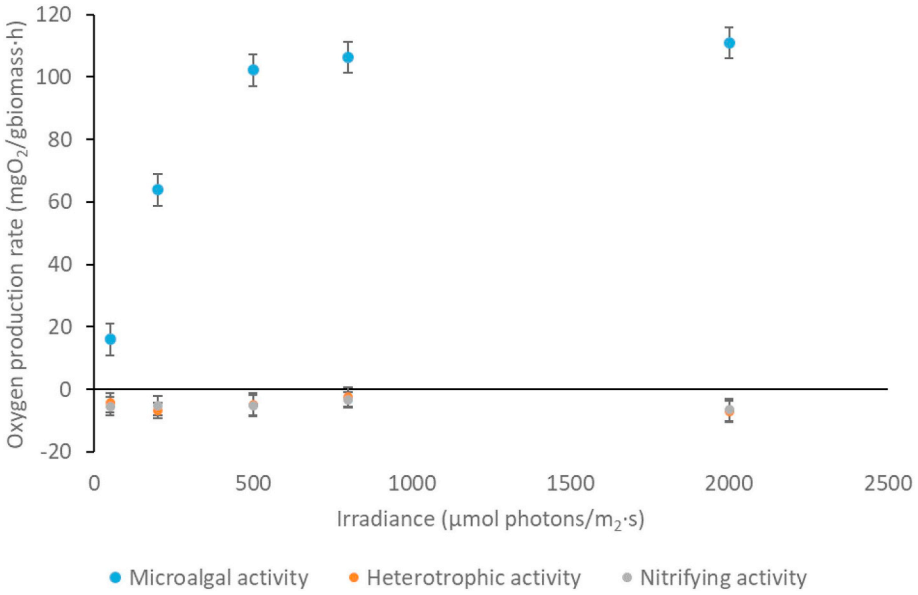

Fig. 2. Influence of irradiance on the different net metabolisms considered: microalgal activity, heterotrophic activity and nitrifying activity. Values correspond to the mean ± SD (n = 3).

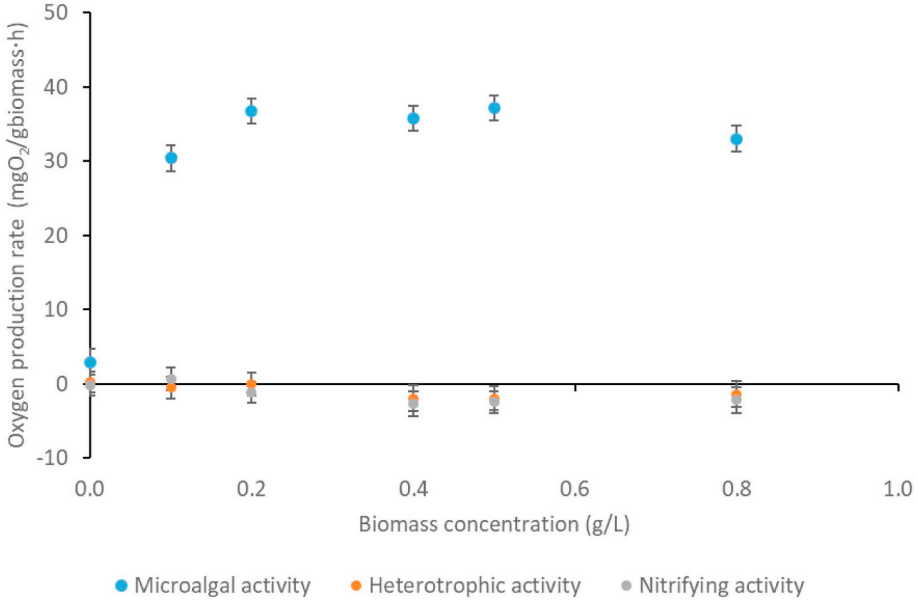

Fig. 3. Influence of biomass concentration on the different metabolisms considered: microalgal activity, heterotrophic activity and nitrifying activity. Values correspond to the mean ± SD (n = 3).

that the oxygen production rate from photosynthesis was higher than the heterotrophic and nitrifying activity in all cases, with maximal values being obtained from pure microalgae cultures (Fig. 5); especially in *S. almeriensis* culture from spherical flasks (129 mgO_2/gbiomass·h). The microalgae net photosynthetic activity from *S. almeriensis* in bubble columns was comparable to the activity in spherical flasks (118.9 mgO_2/gbiomass·h). The photosynthetic activity of *S. almeriensis* in the tubular photobioreactor was likewise very similar to that in culture produced using animal manure in a thin-layer reactor (94.3 and 92.5 mgO_2/gbiomass·h, respectively). These results suggest that using pig manure as the microalgae substrate is an excellent alternative method for treating animal manure and producing microalgae biomass. Although the most common way of reusing pig manure is to spread it on farmland, some authors have described how it can be used to produce microalgae biomass [28,29]. Not only have we been able to demonstrate that pig manure serves as a good microalgae substrate, but the data also show the high microalgae activity achieved from using agricultural leachate wastes as the substrate, with a microalgae activity of 56.1 mgO_2/gbiomass·h. In the outdoor raceway reactor using primary domestic wastewater, microalgae activity was lower (37.8 mgO_2/gbiomass.h), similar to that achieved using the laboratory-scale photobioreactor (34.3 mgO_2/gbiomass·h) As previous studies reported, this was possible because the thin-layer reactor was more photosynthetically efficient at producing *Scenedesmus* sp. than the raceway reactor and the closed tubular photobioreactor [20,30].

Heterotrophic activity in the activated sludge treatment process has been studied and described for decades because it is responsible for oxidizing the organic material and is capable of forming flocs, which also facilitate effluent clearing [31]. Accordingly, it is necessary to determine the heterotrophic population which appears in microalgae-bacteria consortia wastewater treatment. Our results show that heterotrophic activity was very similar in vegetal compost leachate culture

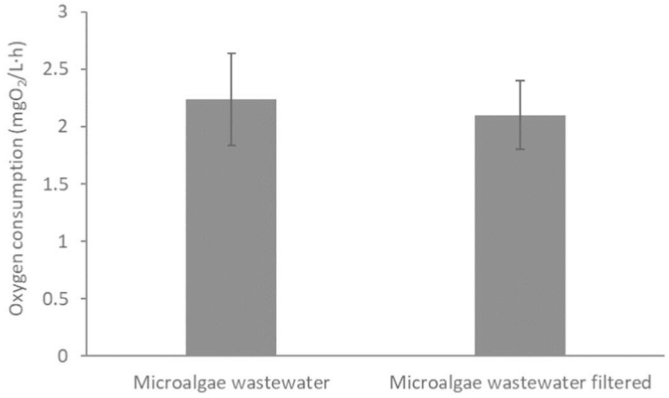

Fig. 4. Influence of microalgae presence in the oxygen consumption by heterotrophic bacteria when sodium acetate was used as a substrate.
Values correspond to the mean ± SD (n = 3).

(8.9 mg O_2/gbiomass.h) and animal manure culture (7.6 mg O_2/gbiomass.h), corresponding to the highest heterotrophic activity values measured. These results were in agreement with the chemical oxygen demand (COD) values recorded in animal manure and compost leachate, corresponding to 20.200 mg/L and 33.200 mg/L, respectively. The results from the two systems using primary domestic wastewater were comparable, showing that heterotrophic activity was present at a similar level, though slightly lower in the raceway reactor (2.6 mg O_2/gbiomass.h) than in the laboratory reactors (4 mg O_2/gbiomass.h). The respiration rate using *S. almeriensis* culture in the pilot column system (3.8 mgO_2/gbiomass.h) and in the tubular cultures (3.7 mgO_2/gbiomass.h) was similar to that obtained from wastewater, indicating that organic matter removal from wastewater treatment using microalgae was quite efficient. The heterotrophic activity measured in the laboratory cultures (2.3 mgO_2/gbiomass) was the expected response because preview studies have described that most microalgae culture collections exist in a non-axenic state because other organisms, such as bacteria and microfungi, are present in the culture due to co-insolation [32].

Regarding the experimental measurement of the nitrifying activity, after the starvation period, it was necessary to check that the nitrogen remaining in the cultures in the form of ammonium was below 2 $mg{\cdot}L^{-1}$. The results showed that maximal nitrifying activity was obtained using leachate from crop residues as the culture media (8.4 mgO_2/gbiomass·h). These results were supported by previous studies on agricultural waste composting, which described the presence of ammonia-oxidizing archaea and bacteria; these transform NH_3 to NO^{-3} during nitrification. [33]. The nitrifying activity measured in *S. almeriensis* laboratory cultures was 5.6 mgO_2/gbiomass·h, higher than the nitrifying activity measured when different types of wastewater were used. The nitrifying activity was also measured for *S. almeriensis* in tubular reactors (3.2 mgO_2/gbiomass.h). A similar value was obtained when animal manure was used as the substrate (3 mgO2/gbiomass·h). Nitrification in samples obtained from microalgae cultures grown in animal manure have been reported by some authors; this is because nitrogen in the form of ammonia nitrogen is present at very high concentrations in animal manures such as pig waste [34,35], with ammonium comprising up to 70% of the nitrogen present in liquid manure [36]. The animal manure used in this study contained up to 3.1 g NH_4/L and it was diluted to 10% for use in the microalgae cultures. The results for the two systems using primary domestic wastewater were comparable although they show that nitrifying activity was present at a slightly higher level in the laboratory reactors (2.9 mg O_2 /gbiomass.h) than in the external raceway (0.6 mg O_2/gbiomass.h), with both primary domestic wastewaters containing a low ammonium concentration (70 mg/L) diluted by 20–10%, respectively.

3.5. Further improvements of the methodology

After standardizing the proposed respirometric method and protocol, the method needed to be further improved in certain aspects such as finding specific nitrifying inhibitors to help discriminating microalgae activity and activity from nitrifying bacteria when ammonium chloride is used as a substrate. In this regard, tests have been carried out using allylthiourea (ATU) solution, known to cause the inhibition of ammonia-oxidizing bacteria (AOB) in the respirometric tests, in order to definitively discriminate between ammonium consumption by nitrifying activity and by microalgae activity. In this way, the methodology described in Section 2.3, "Respiration rate measurements inhibiting nitrifying activity", was applied using samples from an outdoor

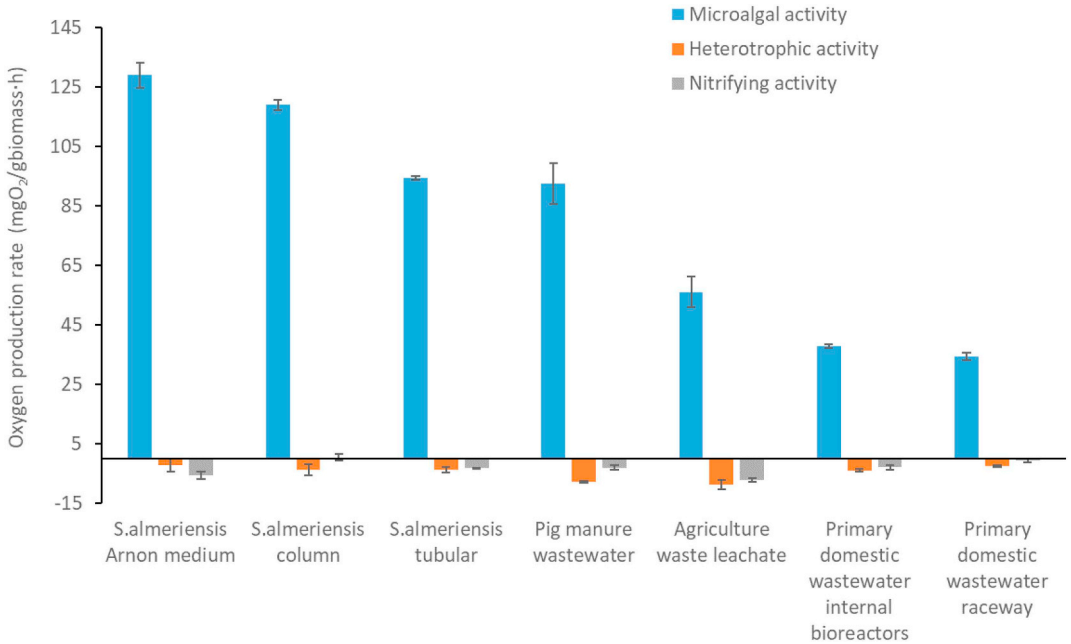

Fig. 5. Distribution of microalgae activity, nitrifying bacteria activity and heterotrophic bacteria activity in pure *S. almeriensis* cultures and in the different wastewaters used. Values correspond to the mean ± SD (n = 4).

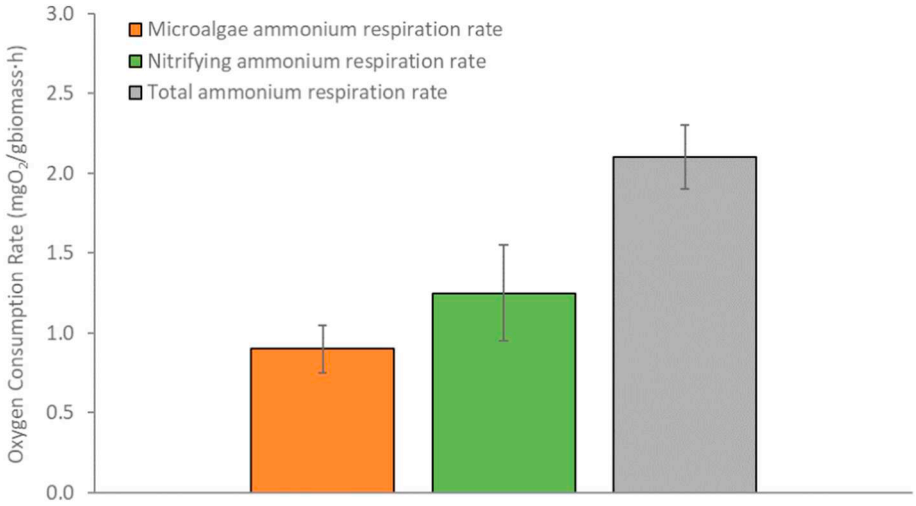

Fig. 6. Determination of total ammonium respiration rate, microalgae ammonium respiration rates and nitrifying ammonium respiration rate in sewage using the respirometric protocol with ATU as inhibitor solution. Values correspond to the mean ± SD (n = 3).

raceway reactor fed with sewage. These tests showed a total ammonium respiration rate when using ammonium chloride as a substrate of 2.1 mgO_2/gbiomass·h, being the respiration of the nitrifying ammonium respiration rate of 1.2 mgO_2/gbiomass·h and the microalgae ammonium respiration rate of 0.9 mgO_2/gbiomass·h (when ATU is applied) (Fig. 6). This methodology is consistent with the one proposed by [17], and helps to optimize and improve respirometric techniques to study the main microbiological metabolisms in wastewater treatment. These results suggested that it is necessary to carry out some tests with ATU before applying the methodology in order to ensure what ammonium percentage is consumed by nitrifying bacteria.

4. Conclusions

The photo-respirometry method developed allows quantifying the contribution of the three main microorganism types that appear in wastewater treatment: microalgae, heterotrophic bacteria and nitrifying bacteria in term of oxygen production and oxygen consumption. The correct application of the proposed methodology was due to the standardization of the photo-respirometry method, including the starvation period required, the established protocol, the substrates and inhibitors applied the biomass concentration and irradiance during the measurements, etc. The method has been applied to microalgae/bacteria consortia established in different wastewater treatment systems (different reactors, water types and operating conditions). The data confirm that in respirometric term, microalgae are the main microorganisms contributing to the microalgae-bacteria consortia, heterotrophic bacteria maintain a relatively stable activity whatever the operational conditions whereas the nitrifying bacteria activity largely depends on the nitrogen load and the microalgae activity. This method is a powerful tool for improving the performance of microalgae-based wastewater treatment processes, for obtaining kinetic parameters of microalgae and bacteria and, even so, for studying the complex relationship between microalgae and nitrifying bacteria.

CRediT authorship contribution statement

A. Sánchez-Zurano: Conceptualization, Methodology, Software, Formal analysis, Investigation, Writing - original draft. **C. Gómez-Serrano:** Validation, Resources, Supervision. **F.G. Acién-Fernández:** Supervision, Writing - review & editing, Funding acquisition. **J.M. Fernández-Sevilla:** Project administration, Funding acquisition, Writing - review & editing. **E. Molina-Grima:** Writing - review & editing, Funding acquisition.

Declaration of competing interest

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

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Statement of informed consent, human/animal rights

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

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7.2. Modelling of photosynthesis and respiration rate for microalgae–bacteria consortia.

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Aside from the main contribution, the following contributions were presented at international conferences:

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Besides, it resulted also in the following contribution to a national conference:

- **A. Sánchez-Zurano**, C. Gómez-Serrano, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima, “Modelización de la tasa de fotosíntesis y respiración de los consorcios microalgas-bacterias en aguas residuales”. Event: I Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias 2018. Type of presentation: Poster. Place: Almeria, Spain.

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ARTICLE

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Modeling of photosynthesis and respiration rate for microalgae–bacteria consortia

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Abstract

In this article, the influence of culture conditions (irradiance, temperature, pH, and dissolved oxygen) on the photosynthesis and the respiration rates of microalgae–bacteria consortia in wastewater treatment was analyzed. Specifically, some short photo-respirometric experiments, simulating outdoor raceway reactors, were performed to evaluate the response of microalgae, heterotrophic bacteria, and nitrifying bacteria to variations in environmental parameters. Results demonstrate that irradiance is the most dominant variable to determine microalgae photosynthesis rates. However, reduction in microalgae activity was not observed at higher irradiance, ruling out the existence of photoinhibition phenomena. Related to heterotrophic and nitrifying bacteria, their activities were strongly affected by the influence of temperature and pH. Moreover, the effect of dissolved oxygen concentrations on microalgae, and bacteria activities was studied, displaying a reduced photosynthetic rate at dissolved oxygen concentrations above 20 mg/L. Data have been used to develop an integrated model for each population (microalgae, heterotrophic bacteria, and nitrifying bacteria) based on considering the simultaneous influence of irradiance, temperature, pH, and dissolved oxygen. The models fit the experimental results in the range of culture conditions tested, and they were validated using data obtained by the simultaneous modifications of the variables. These individual models serve as a basis for developing a global biologic microalgae–bacteria model for wastewater treatment to improve the optimal design and management of microalgae-based processes, especially outdoors, where the cultures are subject to variable daily culture conditions.

KEYWORDS
bacteria, heterotrophic, microalgae-wastewater, nitrification, photosynthesis, respiration

1 | INTRODUCTION

Currently, the demand for clean water has become a worldwide requirement and impose an investment in water management about of €150 billion per year. As a result, conventional wastewater treatment processes offer a treatment composed of several stages based on physical, chemical and biological methods which provide satisfactory levels of nutrient removal (carbon, nitrogen, phosphorous, etc.; Cabanelas et al., 2013). However, these common systems require a high complexity and electrical power consumption. As a solution to beat the disadvantages associated to the commonly used wastewater treatment methods, the search for environmentally friendly

alternatives has brought the interest in biological treatment using microalgae (Park & Craggs, 2010). Not only further reducing microalgae wastewater treatment the cost of the process but also it avoids the nutrients loss such as nitrates, ammonia and phosphates, which can be used for algae growth (Li et al., 2019). Microalgae wastewater treatment is performed by complex microalgae–bacteria consortia which vary as a function of the environmental and operational conditions (Acién et al., 2016). Despite the relevant role of microalgae and bacteria improving wastewater treatment efficiency was put into evident in late 1950s (Oswald et al., 1953), compared with conventional technologies, little is known about the internal functioning of microalgae–bacteria wastewater processes [6]. From a macro point of view, several authors have described this synergistic performance of microalgae–bacteria in wastewater. In the presence of light, microalgae perform photosynthesis in which reduce carbon dioxide (CO_2) and produce oxygen. The released oxygen is used by aerobic bacteria (heterotrophic bacteria) for the degradation of organic compounds present in wastewater. Concurrently, bacteria produce CO_2 through aerobic respiration, which is essential for photosynthesis (Muñoz et al., 2009; Petrini et al., 2018; Quijano et al., 2017; Zambrano et al., 2016). In addition to heterotrophic bacteria, the nitrifying bacteria, which perform the nitrification process, establish different interactions with microalgae. On the one side, it has been proposed that microalgae may stimulate nitrification process by increasing the dissolved oxygen by oxygenic photosynthesis and thereby stimulating NH4 oxidation. However, other authors have suggested that microalgae could suppressed nitrification activity by reducing N-NH4 availability (Risgaard-Petersen et al., 2004).

Therefore, within these systems occur multiple physical, chemical and biological processes which should be evaluated in detail. Within this context, it is mandatory to get insight of the complex interaction between microalgae and bacteria, mathematical models being a powerful tool to predict the performance and to optimize the design of the microalgae–bacteria wastewater processes (Solimeno & García, 2017). In this direction, for convectional wastewater technologies, different bacteria models have been developed and promoted by the International Water Association (IWA; former International Association on Water Pollution Research and Control; Henze et al., 1999; Henze et al., 2015). On the other side, several microalgae mathematical models to understand microalgae growth have been validated. First, multiple models which only taking account one factor to predict microalgae growth were developed (Eilers & Peeters, 1988; Grima et al., 1994). And gradually numerous microalgae models which consider more than one factor have been described (Costache et al., 2013; Ippoliti et al., 2016). The most recent models have introduced different environmental and substrate factors which determine microalgae activity. However, very little research has focused on developing dynamic models to understand the interactions between microalgae and bacteria in wastewater treatment. Despite the first simple microalgae-bacteria model developed in 1983 (Buhr & Miller, 1983), it is not until recently that interest has focused on developing microalgae–bacteria models which combine the overall biochemical processes involved in these systems and the simultaneous effects of environmental parameters on biomass growth, highlighting BIO_ALGAE model (Solimeno et al., 2017).

Traditionally, respirometric tecniques have been proposed as a rapid tool for bacteria characterization in activated sludge processes (Ellis et al., 1996). Currently, this technology has been extended to microalgae–bacteria cultures (Flores-Salgado et al., 2021; Rossi et al., 2018). In microalgae–bacteria cultures, the use of respirometry allows one to determine the phototrophic activity by measuring the oxygen production rate (OPR) under light conditions and the oxygen uptake rate in the dark. The methodology allows to assess heterotrophic and nitrifying activity in microalgal–bacterial consortia too (Petrini et al., 2020; Sánchez-Zurano et al., 2020). These measurements, which are based on oxygen production/consumption, are rapid and easily obtainable (Tang et al., 2014).

Given the need for progress in this area, in this paper, the influence of major environmental parameters (irradiance, temperature, pH, and dissolved oxygen) was evaluated by determining the net microalgae photosynthesis rate and the respiration rates of both heterotrophic and nitrifying bacteria in wastewater processes using respirometric tecniques. The results were used to develop individual models that allow the simulation of the net microalgae photosynthesis rate and respiration rates of the two bacteria populations (heterotrophic and nitrifying bacteria) under different culture conditions. The models were validated by experimental data compiled under different culture conditions tested. According to these results, the developed models are useful tools to optimize the design and operation control of photobioreactors developing an overall model which integrate microalgae and bacteria activities.

2 | MATERIALS AND METHODS

2.1 | Microorganisms and culture conditions

The *Scenedesmus almeriensis* strain was used as the control microorganism. Stock cultures were maintained photo-autotrophically in spherical flasks (1.0 L capacity) using Arnon medium (Allen & Arnon, 1955). The culture was continuously bubbled with an air–1% CO_2 mixture to control the pH at 8.0. The culture temperature was set at 22°C, controlled by regulating the air temperature in the chamber. The culture was artificially illuminated in a 12:12 h L/D cycle using four Philips PL-32W/840/4p white-light lamps, providing an irradiance of 750 $\mu E/m^2$ s on the spherical 1.0 L flask surface.

For the experiments, this inoculum was transferred to laboratory-scale photobioreactors. Details of the reactor and culture medium used in each one are given below. The average composition of the wastewaters used is reported in Table 1.

2.2 | Laboratory photobioreactors

Experiments were performed in four stirred-tank reactors made with polymethylmethacrylate (0.08 m in diameter, 0.2 m in height, and

TABLE 1 Composition of the waters used as effluent in the cultivation system

Parameters	Primary domestic wastewater	Arnon medium
pH	7.6 ± 0.2	7.8 ± 0.2
Conductivity, mS/cm^{-1}	1.8 ± 0.3	2.3 ± 0.2
Turbidity, FTU	16.9 ± 0.5	0.0 ± 0.0
SST, g/L	0.4 ± 0.1	0.6 ± 0.0
COD, mg/L	511.0 ± 5.3	16.0 ± 1.2
Sulfate, mg/L	98.1 ± 6.4	6.3 ± 0.8
Nitrogen-Nitrate, mg/L	23.2 ± 1.7	139.9 ± 3.1
Chloride, mg/L	411.6 ± 23.5	78.9 ± 2.1
Sodium, mg/L	222.5 ± 12.1	276.1 ± 7.9
Potassium, mg/L	8.6 ± 1.6	325.1 ± 6.3
Calcium, mg/L	30.1 ± 0.2	364.9 ± 5.5
Magnesium, mg/L	54.1 ± 14.1	12.2 ± 0.6
Phosphorus-phosphate, mg/L	15.8 ± 0.9	41.1 ± 4.3
Nitrogen-ammonium, mg/L	137.6 ± 6.2	0.0 ± 0.2
Iron, mg/L	0.19 ± 0.01	5.0 ± 0.3
Copper, mg/L	0.09 ± 0.07	0.02 ± 0.0
Manganese, mg/L	0.03 ± 0.01	0.5 ± 0.02
Zinc, mg/L	0.10 ± 0.08	0.06 ± 0.01
Boron, mg/L	0.35 ± 0.09	0.4 ± 0.03

Note: Values correspond to the mean ± *SD*.
Abbreviation: COD, chemical oxygen demand.

with a 1 L capacity) operated in the laboratory but simulating outdoor raceway reactors. These reactors were filled with sewage taken directly after primary treatment from the wastewater treatment plant in Roquetas de Mar (Almería) and 20% of *S. almeriensis* inoculum. First, they were operated in batch mode for 6 days to achieve a high biomass concentration, next it being operated in continuous mode, by replacing 20% of the culture volume daily with fresh wastewater. Once the reactors volume was renewed twice and the reactors concentration remained stable, it was considered that the reactors have reached steady state. To prevent the adverse effect of excessive dissolved oxygen accumulation, the dissolved oxygen was controlled below 200% saturation by supplying air on demand; CO_2 was also injected on demand to control the pH at 8. Concerning illumination, the reactors were artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5) on a simulated solar cycle. The maximum irradiance (PAR) inside the reactors in the absence of cells was 1000 μE m^{-2} s^{-1}, measured using an SQS-100 spherical quantum sensor (Walz GmbH). The culture temperature was kept at 25°C by controlling the temperature of the ambient air of the culture chamber in which the reactors were located.

2.3 | Measurement of photosynthesis and respiration rates

A photo-respirometer was used to obtain the microalgae net photosynthesis rate and the bacteria respiration rates for the biologic model. The protocol and methodology applied allow to distinguish between the metabolisms of the three main populations which appear in microalgae–bacteria wastewater treatment: the microalgae, the heterotrophic bacteria, and the nitrifying bacteria. The equipment operation is based on determining any variation in dissolved oxygen concentration in microalgae–bacteria culture samples under controlled conditions. An adequate protocol was applied to determine the microalgae net photosynthesis rate, the heterotrophic respiration rate, and the nitrifying respiration rate. First, samples of the microalgae–bacteria cultures should be taken and subjected to nutrient starvation (continuous light of 200 μEm^{-2} s^{-1} and an aeration rate of 0.2 v·v^{-1}·$^{min-1}$) during 24 h to remove the organic matter and the ammonium present in the medium. Subsequently, the methodology consists on placing a sample of the culture inside the photo-respirometer and subjecting to four light–dark periods of 4 min each one during which the variation in dissolved oxygen under different conditions is measured. In the following section, the determination of each microbial metabolism is described, including the expected biological reactions affecting the dissolved oxygen concentration:

- For evaluating the microalgae net photosynthesis rate of each microalgae–bacteria culture, a sample of the culture is exposed to four light–dark cycles of 4 min each to measure and register the variation in dissolved oxygen. Air is provided through the diffuser to recover the 100% saturation between the dark and light cycles. The first minute of exposure (light and dark phases) was disregarded as it was adaptation time. During the light phases, the photosynthetic microalgae generated dissolved oxygen while this dissolved oxygen is consumed by the endogenous respiration during the dark periods. Therefore, the endogenous respiration activity or the oxygen consumption rate (OCR) was obtained through linear regression analysis of DO data (negative trend) in the dark, while the OPR was obtained through linear regression analysis of DO data (positive trend) in the light. Thus, the microalgae net photosynthesis rate was calculated as the difference between the slope of the OPR during the light period minus the slope of the OCR during the dark period.
- Subsequently, another sample of the culture was used to determine the heterotrophic respiration rate. For this purpose, 0.8 ml of sodium acetate (30 g/L) was added to the sample and it was exposed to four light–dark cycles of 4 min each one. The respiration rate of the heterotrophic bacteria was calculated as the slope of the oxygen consumption with sodium acetate minus the slope of the oxygen consumption during the dark period in the endogenous culture.

- By following the same method, another sample was used to measure the nitrifying respiration rate of the culture. However, the nitrifying activity was determined using 0.8 ml of ammonium chloride (3 g/L) instead of sodium acetate. The respiration rate of the nitrifying bacteria was calculated as the slope of the oxygen consumption with ammonium chloride minus the slope of the oxygen consumption during the dark period in the endogenous culture.

Finally, to correct the influence of oxygen desorption on the photo-respirometric measurements, the oxygen mass transfer coefficient (K_La) was calculated and included. This coefficient was measured in the system according to (Equation 1).

$$\frac{dC_{O_2}}{dt} = K_La\left(C^*_{O_2} - C_{O_2}\right) \quad (1)$$

where dC_{O_2}/dt is the oxygen accumulation expressed as the derivate of C_{O_2}(mg/L) over time, K_La is the global oxygen mass transfer coefficient (h^{-1}), and $C^*_{O_2}$ is the oxygen saturation concentration in the liquid. The final value obtained was 1.08 h^{-1}. A further detailed description of the equipment, the experimental protocol and the metabolic rates calculations are described in (Sánchez-Zurano et al., 2020).

The protocol described below was applied to determine the microalgae net photosynthesis rate and bacteria respiration rates exposed to different irradiancies inside the glass chamber. Furthermore, experiments were performed also modifying the temperature by heating/cooling the samples. According to the pH, it was adjusted in the samples from the laboratory photobioreactor by adding HCl or NaOH. Finally, experiments were performed modifying the dissolved oxygen by bubbling pure oxygen or pure nitrogen into the sample. For each measurement, a new sample of culture in steady state, coming from the laboratory photobioreactors described in Section 2.2, was used, to avoid accumulation of effects.

2.4 | Biomass concentration and analytical methods

The microalgae biomass concentration was measured by dry weight. It was used 100 ml aliquots of the culture filtered through a pre-dried 1 μm filter (Macherey-Nagel GmbH & Co. KG). Then, the filters were dried in an oven at 80°C for 24 h. Standard official methods were used to analyse the composition of the wastewater samples and the water from the reactors. The phosphate was measured by visible spectrophotometry through the phospho–vanado–molybdate complex (Phosphate Standard for IC: 38364). The nitrate was quantified by measuring optical density at 220 nm and 275 nm (Nitrate Standard for IC: 74246). The ammonium was measured according to the Nessler method (Ammonium standard for IC: 59755). The chemical oxygen demand (COD) was determined by spectrophotometric measurement using Hach–Lange Kits (LCI-400).

2.5 | Software and statistical analysis

The DaqFactory program (Azeotech) was used to gather the photosynthesis and respiration rate data. Data analysis was carried out using the Statgraphics Centurion XVI software package, in which nonlinear regression was used to fit experimental data to the proposed models, and to determine the characteristic parameter values. These models were used to obtain simulations in Microsoft Excel. All the experiments were performed at least by triplicate to allow calculating the mean values and standard deviation that are shown.

3 | RESULTS AND DISCUSSIONS

During the last decades, different types of mathematical models have been presented to describe the growth of microalgae and bacteria influenced by environmental variables (Béchet et al., 2013; Buhr and Miller, 1983; Sah et al., 2011; Zambrano et al., 2016). As with all microalgae pure culture (Costache et al., 2013; Ippoliti et al., 2016), under unlimited nutrient conditions, the most important factors for microalgae–bacteria wastewater processes are irradiance, temperature, pH, and dissolved oxygen (Solimeno et al., 2017). To optimize the productivity of the microalgae–bacteria systems, the influence of these factors should be analyzed in the laboratory simulating outdoor environmental changes to develop models that can be integrated in outdoors photobioreactors. Therefore, to model the activity of both microalgae and bacteria cells to environmental conditions, samples from the lab-scale cultures were collected and used to determine the photosynthesis and respirations rates of the three main populations.

Regarding irradiance, the net photosynthesis rate was zero at zero irradiance and increased with irradiance to a maximum of 106 $mgO_2/g_{biomass}$·h at an irradiance of 650 μE/m^2·s, then remained constant at higher irradiance. Photo-inhibition was not observed at high irradiance values, keeping the photosynthetic activity as was reported using the green microalga *Chlorella vulgaris* by (Yun & Park, 2003). The lack of photo-inhibition in cells activity is in breach of previous studies with pure *Scenedemus almeriensis* culture in which the photosynthesis rate increased with light availability up to values of 400 μE/m^2·s, remaining constant up to values of 1.000 μE/m^2·s, and finally decreased at higher irradiances (Costache et al., 2013). Despite several light intensity models developed to describe microalgae photosynthesis and growth kinetics (Aiba, 1982; Eilers & Peeters, 1988), experimental data have been fitted to the Molina model (Equation 2; Grima et al., 1994), in which the net photosynthesis rate is a function of specific maximum photosynthetic rate (PO_2, max), average irradiance (I_{av}), constant representing the affinity of algae to light (I_k), and a form parameter (n). By fitting experimental data to this equation, the characteristic parameter values were determined (PO_2, max = 113 $mgO_2/g_{biomass}$·h, n = 1.68, I_k = 168 μE/m^2·s), verifying that the model reproduces the behavior of the measurements performed. Heterotrophic and nitrifying bacteria respiration remained constant along the irradiance values applied, that any light intensity model was used. As a result, heterotrophic bacteria

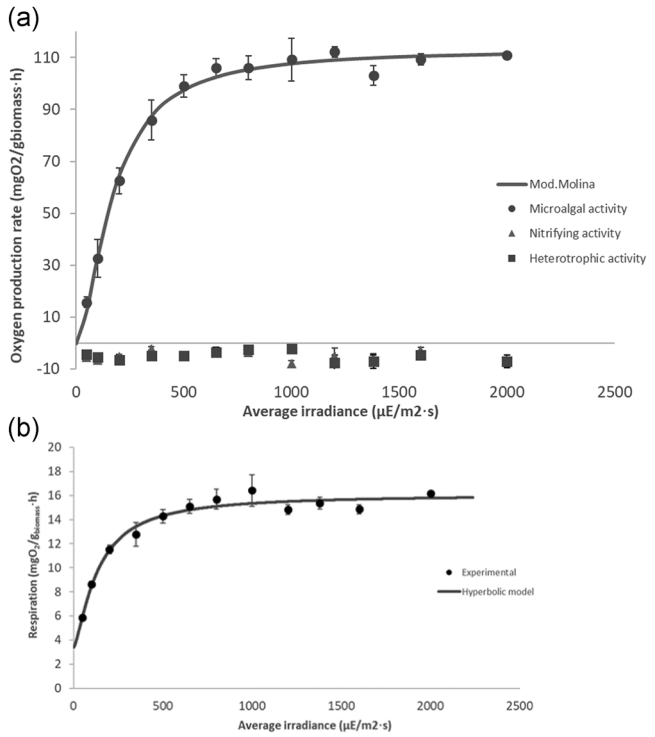

FIGURE 1 Influence of average irradiance in the culture on the oxygen production rate of microalgae, nitrifying bacteria and heterotrophic bacteria at 24°C (a). Influence of average irradiance on the respiration rate of microalgae at 24°C (b). Lines correspond to fit the proposed models (Equations 2 and 3). Values correspond to the mean ± *SD* ($n = 3$)

respiration rate was 5.8 $mgO_2/g_{biomass}$·h and nitrifying bacteria respiration rate was 4.4 $mgO_2/g_{biomass}$·h (Figure 1a).

$$PO_2 = \frac{PO_{2,max} \cdot I_{av}{}^n}{I_k{}^n + I_{av}{}^n} \quad (2)$$

Regarding microalgae respiration, it may be influenced by several factors such as temperature, oxygen tension, exogenous substrates, and so forth. The illumination level before the measurement also influences the microalgae respiration rates that are found to be higher when the microalgae algae exposed to high irradiance while lower respiration rates were measured after weaker illumination conditions (Grobbelaar & Soeder, 1985). This tendency related with the light exposition was observed in this study too. The respiration rate was 3.4 $mgO_2/g_{biomass}$·h at zero irradiance, increasing with irradiance up to 16.4 $mgO_2/g_{biomass}$·h at an irradiance of 1000 µE/m^2·s, then remaining constant up to 2000 µE/m^2·s (Figure 1b). Experimental data have been fitted to the hyperbolic model with no inhibition (Equation 3) and the characteristic parameter values were determined (RO_2 min = 3.4 $mgO_2/g_{biomass}$·h, RO_2, max = 12.7 $mgO_2/g_{biomass}$·h, $n\_r = 1.4$, $I_{k\_res} = 134$ µE/m^2·s).

$$RO_2 = RO_2\text{min} + \frac{RO_{2,max} \cdot I_{av}{}^{n\_r}}{I_{k\_res}{}^{n\_r} + I_{av}{}^{n\_r}} \quad (3)$$

As in previous studies (Acién Fernández et al., 1999; Fernández et al., 1998), results have verified that under real conditions, the cultures are mainly photo-limited, the average irradiance being from 100 to 300 µE/m^2·s. The net photosynthesis rate was saturated at 500 µE/m^2·s, taking this value, 200 µE/m^2·s was selected as a constant light intensity to determine the influence of the other main environmental parameters, which could modulate the response of the net photosynthesis rate and the bacteria respiration rate to irradiance (such as temperature, pH and dissolved oxygen). These experiments will allow to determine the normalized net photosynthesis rate and bacteria respiration rates as a function of these culture conditions.

Consequently, experiments were performed by modifying the temperature of the cultures to calculate microalgae net photosynthesis rate and bacteria respiration rates over a wide range of temperatures. Temperature has been pointed as one of the main environmental conditions which determining the structure of the microbial community and the process performance (such as the nitrification activity) in wastewater treatment (Chen et al., 2017). Concerning the microalgae activity, the net photosynthesis rate was maximal at a temperature of 30°C, lower than the optimum temperature of the *S. almeriensis* reported by (Costache et al., 2013). However, other authors showed the maximum specific growth rate of *Scenedesmus* sp. at 25°C and a wide activity range at temperatures from 10°C to 30°C (Xin et al., 2011). In this way, the microalgae activity decreased at high temperatures, with zero activity at extreme temperatures above 49°C. According to these data, previews studies reported that *Scenedemus* did not grow at 42°C (Westerhoff et al., 2010). Note that these experiments have been carried out at short periods of exposure, so that the photosynthetic response of the culture could be conditioned by varying the exposure time, even at moderate temperatures such as 35°C (Karemore et al., 2020).

Bacterial growth is quite dependent to the temperature. For them, as the temperature rises, enzyme reactions in the cell proceed at more fast rates, with rates approximately doubling with every 10°C increase. However, above a certain temperature, growth slows and, if the temperature continues to increase, bacteria could die (Rajeshwari et al., 2000; Spellman, 1999). This effect has been observed in the experiments, in which the oxygen consumption rate of the heterotrophic bacteria increases at high values of temperature, showed their optimum activity at 36°C and the higher rate of respiration was at 39.5°C. For nitrifying bacteria, temperature effects are more complex, because influence in nitrifiers viability and its activity rates (Hülsen et al., 2016). Traditionally, nitrification process in wastewater has been considered the most temperature-sensitive step among the microbial activities, due to the fact that nitrifying activity could decrease by 50% with each temperature decrease of 10°C (Wang & Li, 2015). Results showed the optimal temperature was 30°C and a wide range of activity from 0°C to 49°C, appreciating a strong decrease in activity below 30°C. Just like heterotrophic bacteria, nitrifying bacteria are especially sensitive to high temperature, the activity of them being zero at extreme temperatures (50–60°C; Henze et al., 2001; Figure 2).

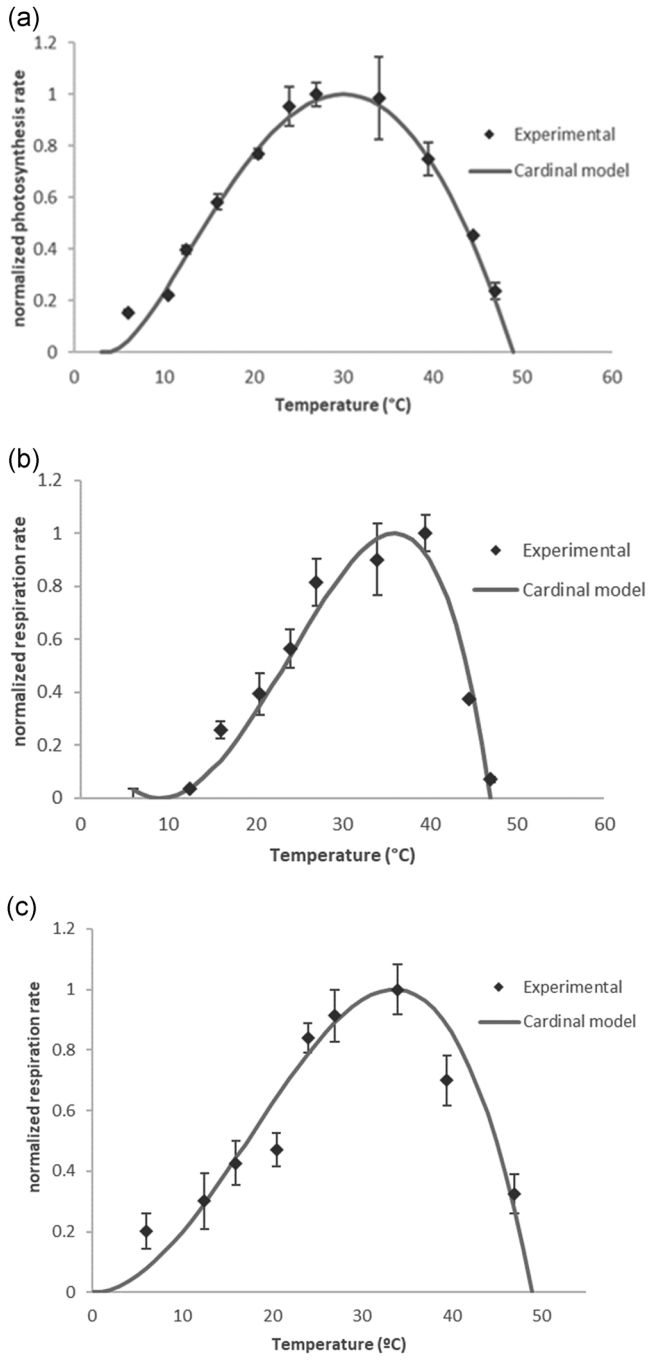

FIGURE 2 Influence of temperature on the normalized photosynthesis rate of microalgae (a), normalized heterotrophic bacteria respiration rate (b), and nitrifying bacteria respiration rate (c). Lines correspond to fit the proposed models (Equation 4). Values correspond to the mean ± *SD* ($n = 3$)

The influence of temperature in the normalized net photosynthesis rate and the normalized respiration rate for heterotrophic and nitrifying bacteria, was fitted to the cardinal model developed for bacteria (Rosso et al., 1993) and validated for microalgae (Bernard & Rémond, 2012; Equation 4). The cardinal model is a simple equation which considers a maximum, a minimum and an optimal value, the values of the variable (for instance, temperature) only existing on the range between maximum and minimum tolerable values (Ippoliti et al., 2016). Currently, cardinal equations are well accepted in the microalgae–bacteria models because they are helpful to represent experimental data and make the models straightforward to understand (Rossi et al., 2020).

$$RO_2(T) = \frac{(T - Tmax)(T - Tmin)^2}{(Topt - Tmin)(((Topt - Tmin)(T - Topt)) - ((Topt - Tmax)(Topt + Tmin - 2T)))} \quad (4)$$

Concerning to the influence of pH on microalgae activity, the normalized net photosynthesis rate was considered fairly high at pH from 7.5 to 8.5 with an optimum pH above 8.5. At pH values lower than 7.0, the photosynthesis rate reduced slowly just like pH values higher than 9.0. Photosynthesis took place even at high pH values, but at pH 13, the photosynthesis rate was zero. It is important to note that experiments were performed by modifying the pH through the addition of sodium hydroxide or hydrochloric acid. However, because the system was not bubbled, no decarbonation took place although concentrations of different carbonate–bicarbonate–carbonic acid species modified as a function of pH (Costache et al., 2013). The results agree well with the reported literature (Difusa et al., 2015; Gardner et al., 2011) where a pH trend ranging from 7.0 to 9.0 provided favorable condition for high growth rate of *Scenedesmus*.

Bacteria, just as happens with the microalgae, do not tolerate pH values below 4 or above 9,5. Despite most wastewater treatments work at a pH near to neutral, there are situations that involve excursions to high or low pH values. Certain bacterial strains are still capable of working at these pH values (acidophiles, neutrophiles, and alkalinophiles) in wastewatater (Gerardi, 2006). The maximal heterotrophic bacteria respiration rate was at pH 8 and, it keeps up to pH 9, but their tolerance to low pH values were lower than microalgae. Under pH 6, heterotrophic bacteria did not show activity. Despite there is a wide range in the reported pH optima (pH 6.5–8.6) for nitrifying bacteria in the activated sludge process and there is general agreement that as the pH shifts to the acid range, the rate of nitrification decline (Cheremisinoff, 1997). The results showed an optimal pH higher, above 9.7, with tolerance to high values of pH (Figure 3). The observed influence of pH on net photosynthesis rate and bacteria respiration rate exhibit similar behavior to that previously observed for temperature. Thus, cardinal model allowed to model the response of the net photosynthesis rate and bacteria respiration rate to pH (Equation 5).

$$RO_2(pH) = \frac{(pH - pHmax)(pH - pHmin)^2}{(pHopt - min)(((pHopt - pHmin)(pH - pHopt)) - ((pHopt - pHmax)(pHopt + pHmin - 2pH)))} \quad (5)$$

The effect of dissolved oxygen on microalgae and bacteria activity was also studied (Figure 4). According to the microalgae activity, at low dissolved oxygen concentrations and at saturation concentration (9.0 mg/L), the net photosynthesis rate was maximal, decreasing until zero activity at 32 mg/L. Previous studies reported

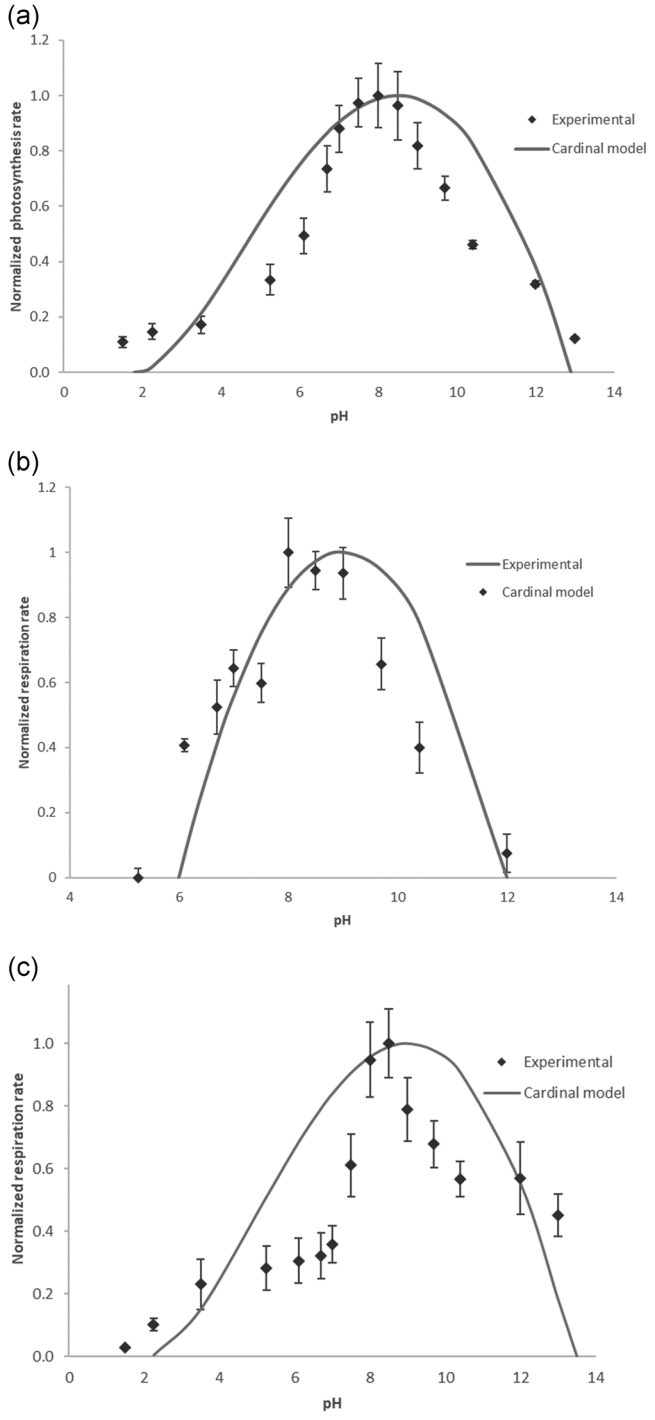

FIGURE 3 Influence of pH on the normalized photosynthesis rate of microalgae (a), normalized heterotrophic bacteria respiration rate (b), and normalized nitrifying bacteria respiration rate (c). Lines correspond to fit the proposed models (Equation 5). Values correspond to the mean ± *SD* (*n* = 3)

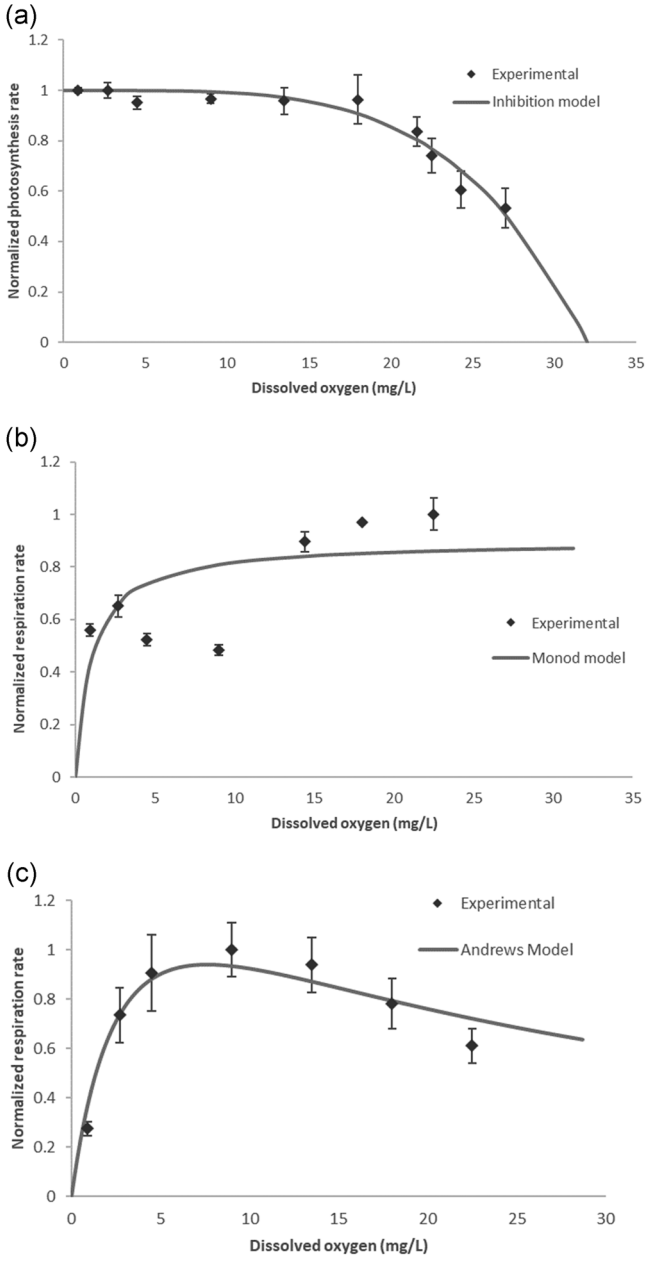

FIGURE 4 Influence of dissolved oxygen on the normalized photosynthesis rate of microalgae (a), normalized heterotrophic bacteria respiration rate (b), and normalized nitrifying bacteria respiration rate (c). Lines correspond to fit the proposed models (Equations 6–8). Values correspond to the mean ± *SD* (*n* = 3)

that oxygen levels above air saturation could inhibit photosynthesis in some microalgae. Also oxygen accumulation could be the cause of photo-oxidation in microalgae culture (Molina Grima et al., 1999). These data were comparable with previews results using *Scenedesmus* cultures, in which the maximal dissolved oxygen concentration tolerable by the culture was 25 mg/L (225%Sat.; Barceló-Villalobos et al., 2019). However, other microalgae species have showed less tolerance under extreme dissolved oxygen concentrations. For instance, photosynthesis activity of some polar sea ice microalgae was restricted at dissolved oxygen concentration above 20 mg/L (McMinn et al., 2005), even so strains such as *Isochrysis galbana* reduce the photosynthesis rate to zero at 20 mg/L (Ippoliti et al., 2016). To model the microalgae's response to dissolved oxygen concentration, an equation considering the inhibition by

product was used, previously reported (Costache et al., 2013; Ippoliti et al., 2016; Equation 6).

$$RO_2(DO_2) = 1 - \left(\frac{DO_2}{DO_{2,max}}\right)^m \quad (6)$$

It is mandatory to determine bacteria activity under different dissolved oxygen concentration because of the competitive interaction of heterotrophs and nitrifies for dissolved oxygen is well known for years (Furumai & Rittmann, 1992). Heterotrophic bacteria support a wide range of dissolved oxygen values, being able to live at very low values (<0.9 mg/L) and their activity increases with increasing dissolved oxygen, fitting experimental data to Monod equation (Equation 7).

$$RO_2(DO_2) = \frac{DO_2}{DO_2 + Ks} \quad (7)$$

Concerning the effect of dissolved oxygen on nitrification in wastewater treatment, one of the earliest works to quantify nitrification in wastewater treatment processes was that performed by A. L. Downing and Associates at the Water Pollution Lab at Stevenage (Downing & Scragg, 1958). They reported that the respiration rate in activated sludge plants fall off when the DO concentration fell below 0.3 mg/L, while several years after this initial work, other authors reported that nitrifying growth is inhibited even so at dissolved oxygen levels below 2.0 mg/L (Abbassi et al., 2000). Also it has been described that the rate of nitrification in wastewater treatment increases as the DO concentration is increased to 7 or 8 mg/L (Stenstrom & Poduska, 1980). According to high levels of dissolved oxygen for nitrification, respirometric experiments have been shown that high oxygen concentrations are initially inhibitory but acclimatization occurs after several days at high oxygen levels according to previous authors (Charley et al., 1980). The findings of this study showed an optimal range of dissolved oxygen between 5 and 13 mg/L, decreasing slowly at high values of dissolved oxygen. Furthermore, nitrifying bacteria were capable to tolerate low levels of oxygen, showing activity at 0.9 mg/L. From the nitrifying bacteria respiration rate variation with the dissolved oxygen concentration, an Andrews equation (Andrews et al., 1968), similar to that used by heterotrophic bacteria but taking into account the inhibition by product has been applied (Equation 8).

$$RO_2(DO_2) = \frac{DO_2}{(DO_2 + Ks)(1 + \frac{DO_2}{Ki})} \quad (8)$$

According to these results, the characteristic parameter values were determined (Table 2) and the microalgae net photosynthesis and bacteria respiration rates could be modeled by combining these equations to obtain a general equation representing the overall behavior based on the observed patterns. Thus, Equations (9–11) allow to model the microalgae net photosynthesis rate, heterotrophic bacteria respiration rate, and nitrifying bacteria respiration rate as a function of the culture conditions (irradiance, temperature, pH, and dissolved oxygen) to which the cells are exposed.

$$PO2_{ALG} = PO2(I)\cdot\overline{PO2}(T)\cdot\overline{PO2}(pH)\cdot PO2(\overline{DO2}) - RO2(I) \quad (9)$$

$$RO2_{Het} = RO2(I)\cdot\overline{PO2}(T)\cdot\overline{PO2}(pH)\cdot PO2(\overline{DO2}) \quad (10)$$

$$RO2_{Nit} = RO2(I)\cdot\overline{PO2}(T)\cdot\overline{PO2}(pH)\cdot PO2(\overline{DO2}) \quad (11)$$

TABLA 2 Values for the proposed model's parameter characteristics

Microalgae net photosynthesis rate			Heterotrophic respiration rate			Nitrifying respiration rate		
Parameter	Value	Units	Parameter	Value	Units	Parameter	Value	Units
PO_2max	113	$mgO_2/g_{biomass}\cdot h$	RO_2max	5.4	$mgO_2/g_{biomass}\cdot h$	RO_2max	4.4	$mgO_2/g_{biomass}\cdot h$
Ik	168	$\mu E/m_2\cdot s$	Tmin	9	°C	Tmin	0	°C
n	1.7		Tmax	47	°C	Tmax	49	°C
Tmin	3.4	°C	Topt	36	°C	Topt	33.6	°C
Tmax	49	°C	pHmin	6		pHmin	2	
Topt	30	°C	pHmax	12		pHmax	13.4	
pHmin	1.8		pHopt	9		pHopt	9	
pHmax	12.9		$K_{S,DO2}$	1.98	mgO_2/L	$K_{S,DO2}$	1.08	mgO_2/L
pHopt	8.5					$K_{I,DO2}$	104.9	mgN/L
DO_2max	32	mgO_2/L						
m	4.15							
RO_2max	12.7	$mgO_2/g_{biomass}\cdot h$						
RO_2min	3.4	$mgO_2/g_{biomass}\cdot h$						
Ik_res	134	$\mu E/m^2\cdot s$						
n_r	1.4							

To validate the proposed models, some experiments were performed modifying the culture conditions studied (irradiance, temperature, pH, and DO2). With these experimental data and the simulated data from the developed models and the characteristic parameter values, it was possible to determine a correlation between them. Figure 5 showed that the microalgae model fitted the experimental data of the net photosynthesis rate with a correlation coefficient of 0.87. The models were validated for heterotrophic and nitrifying bacteria activity too. Using the experimental data changing the values of the environmental parameters and the simulated data from the model, was possible correlating them. The bacteria models fitted the experimental data of the heterotrophic and nitrifying respiration rate with a correlation coefficient of 0.73 and 0.64, respectively. The results obtained demonstrated that the models fitted experimental values determined indoors and allow the identification of the characteristic parameter values for the microalgae-bacteria consortia in wastewater.

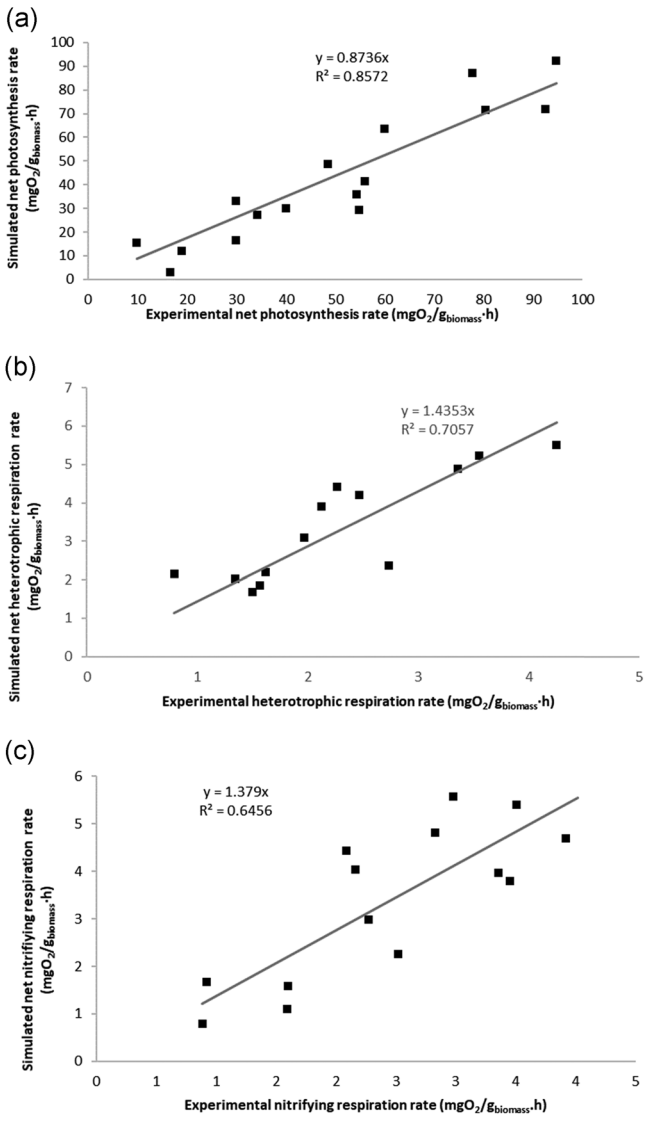

FIGURE 5 Correlation between the experimental and simulated values of (a) the net photosynthesis rate, (b) the heterotrophic bacteria respiration rate, and (c) the nitrifying bacteria respiration rate

4 | CONCLUSIONS

Environmental parameters (Irradiance, temperature, pH, and dissolved oxygen) are significant variables determining the performance of microalgae–bacteria consortia in wastewater treatment. In this study, it has been developed and validated for first time, a microalgae–bacteria model based on the photosynthesis and respiration rates for microalgae wastewater processes. Next steps are aimed at validating the proposed models in outdoors conditions using industrial-scale raceway photobioreactors. This implementation will allow to design and management microalgae wastewater processes improving the productivity and reducing the cost of the systems.

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CONFLICTS OF INTEREST

There are no potential financial or other interests that could be perceived to influence the outcomes of the research. No conflicts, informed consent, human or animal rights applicable. All authors confirmed the manuscript authorship and agreed to submit it for peer review.

AUTHOR CONTRIBUTIONS

Ana Sánchez Zurano conducted methodology, investigation, formal analysis, and writing-original draft. Cintia Gómez Serrano provided conceptualization, data curation, and resources. Francisco Gabriel Acién Fernández assisted in supervision, writing-reviewing, and funding acquisition. José María Fernández Sevilla did the formal analysis, software, and supervision. Emilio Molina Grima did writing- reviewing and editing, project administration, and funding acquisition.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Modelling of photosynthesis, respiration, and nutrient yield coefficients in *Scenedemus almeriensis* culture as a function of nitrogen and phosphorus

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Abstract
Photo-respirometric tecniques are applied for evaluating photosynthetic activity in phototrophic organisms. These methods allow to evaluate photosynthetic response under different conditions. In this work, the influence of nutrient availability (nitrate, ammonium, and phosphate) on the photosynthesis and respiration of *Scenedesmus almeriensis* was studied using short photo-respirometric measurements. Both photosynthesis and respiration increasing until saturation value and consecutively diminishing, presenting inhibition by high concentrations. Regarding the influence of phosphorus concentration in microalgae cells, a similar hyperbolic trend was observed but no inhibition was observed at high concentration. Based on these experimental data, the respiration, and the photosynthesis rate of *S. almeriensis* were modelled using Haldane equation for nitrate and ammonium data, and Monod equation for phosphate data. In addition, experiments were performed to determine the yield coefficients for both nitrogen and phosphorus in *S. almeriensis* cultures. The data showed that the nitrogen and phosphorous coefficient yields are not constant, being modified as a function of nutrients concentration, presenting the luxury uptake phenomena. Finally, the proposed models were incorporated into a simulation tool to evaluate the photosynthetic activity and the nutrient yield coefficients of *S. almeriensis* when different culture media and wastewaters are used as a nitrogen and phosphorous source for its growth.

Key points
- *Microalgal photosynthesis/respiration vary as a function of nutrients availability.*
- *Photosynthesis inhibition appears at high N-NO_3^- and N-NH_4^+ concentrations.*
- *Nutrient yield coefficients are influenced by luxury uptake phenomenon.*

Keywords Microalgae · Photosynthesis · Respiration · Nitrogen · Phosphorus · Modelling

Introduction

Over the past centuries, CO_2 concentration in the atmosphere has greatly increased, mainly because of human activities and it leads to known climate change events. Climate change comes along with global consequences on an environmental, social, and economic scale. As a solution to beat these consequences, there is growing interest in developing alternatives for CO_2 capture, including photosynthetic microorganisms (Aghaalipour et al. 2020). Microalgae cultivation has proposed as a highly promising biological method of CO_2 because the generated biomass can be used widely (Rodas-Zuluaga et al. 2021). Microalgae biomass have become an eco-friendly alternative in emerging industrial sectors such as aquaculture and animal feed, human nutrition, cosmetics, biofertilizers, and biofuels (Chisti 2008; Acién et al. 2017). However, their large-scale application is still limited by the specific requirements for biomass growth. Microalgae production involves both the maintenance of adequate culture conditions (light, pH, temperature, and dissolved oxygen), related to the reactor design and operating conditions, and the optimal supply of nutrients (carbon, nitrogen, phosphorus, etc.), which affects the production cost (Posten 2009; Acién et al. 2012). An inadequate nutrient supply

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can greatly reduce the performance of microalgae cells. In general, nutrients usually present in excess, meaning that most processes operate under nutrient-saturation conditions. Nutrients are generally provided as fertilizers to minimize cost; nevertheless, this still represents a relevant contribution to the overall final cost, which ranges from 5 to 20% depending on the production technology (Acién et al. 2012). To reduce the nutrient contribution to the final biomass production cost, utilizing wastewater for microalgae cultivation has been proposed. The advantage of using wastewater as culture medium is that the microalgae can be grown using both organic and inorganic compounds, such as phosphates, ammonium, and nitrates, which are already present in the wastewater, avoiding the cost of nutrients supplementation. At the same time, the wastewaters are treated and can be reused for multiple purposes (Rawat et al. 2011; Acién et al. 2016). Moreover, these sewage treatment systems based on microalgae can be optimized by an adequate CO_2 supplementation, which allow to obtain high biomass productivity and nutrients removal (Molino et al. 2019).

Several works have revealed the robustness of microalgae-based wastewater systems in terms of biomass productivity and the high contaminant removal rates, the focus being on developing mathematical models capable of simulating and optimizing microalgae wastewater treatment. Although the first microalgae models were based on single factors, such as light intensity (Molina-Grima et al. 1994), nitrogen (Smit 2002), or phosphorus (Sommer 1991), the current models have introduced multiple factors affecting microalgae performance such as irradiance, temperature, pH, and dissolved oxygen (Costache et al. 2013; Ippoliti et al. 2016). However, the use of wastewater for microalgae production involves not only microalgae performance but also different bacterial populations appear in these systems, which increases the complexity of the mechanistic models (Solimeno et al. 2015). Various types of mathematical models have been developed for understanding the interaction between the microalgae and the bacteria. Since Buhr and Miller (1983) developed the first mathematical model to describe microalgae and bacteria growth in wastewater, multiple microalgae-bacteria models for wastewater treatment have been proposed and validated (Reichert and Vanrolleghem 2001; Sah et al. 2020; Solimeno et al. 2019, 2017; Wágner et al. 2016; Zambrano et al. 2016).

Many microalgae-bacteria models have been validated in terms of the influence of nutrient availability on microalgae/bacteria consortia performance, and considerable knowledge has been accrued regarding the behavior of both heterotrophic and nitrifying bacteria as a function of nutrient concentration. Nonetheless, the performance of microalgae cells has hardly been studied. For instance, the mechanistic models (ASM1, ASM2, ASM2D, and ASM3) of the Activated Sludge Model (ASM) series, promoted by the International Water Association, already consider the influence of organic carbon sources, ammonium, nitrate, and phosphorus on bacterial performance—the variation in growth rates based on the concentration of the respective nutrients fitting the Monod model, with constant coefficient yields being determined for each microorganism type and nutrient type (Gernaey et al. 2004; M Henze et al. 2015). Of the scarce information available regarding microalgae performance, BIOALGAE is one of the most nutrient-complete models (Solimeno et al. 2017). Most papers in the literature provide information on experiments carried out under excess nutrient conditions, focusing on maximizing the microalgal cell performance. Conversely, other papers looking at nutrient limitation conditions focus on the kinetics of secondary metabolite accumulation. However, little information is available regarding the influence of nutrient concentration on microalgal cell performance (Fernandes et al. 2016; Mc Gee et al. 2020).

For the bacteria characterization of activated sludge, respirometric techniques have been applied as a rapid tool to ascertain kinetic growth parameters (Ellis et al. 1996). Over recent years, this respirometry, which has traditionally been applied to bacteria in wastewater, has been extended to phototrophic cultures. In algal cultures, the use of respirometry allows one to determine the phototrophic activity by measuring the oxygen production rate (*OPR*) under light conditions and the oxygen uptake rate (OUR) in the dark. These measurements, which are based on oxygen production/consumption, are rapid and easily obtainable (Tang et al. 2014; Sánchez-Zurano et al. 2020). In fact, respirometric methods have been evaluated and applied to photosynthetic cultures for biokinetic parameter determination (Decostere et al. 2013). This methodology allows one to determine the effect of culture parameters on microalgae activity and to measure kinetic parameters such as the nutrients' half-saturation constants, thus avoiding batch experiments, which are very time consuming (> 10 days); in addition, the results might be affected by biomass debris formation (Robertson et al. 1998; Sforza et al. 2019).

In this work, a photo-respirometric method is proposed as a simple, innovative, and rapid method to measure kinetic parameters in microalgae cultures. Respirometry was applied to measure the nutrient saturation coefficients of *Scenedesmus almeriensis*, relating to the main nutrients present in the wastewater (nitrate, ammonium, and phosphate). The respirometric experiments allow to determine the kinetic parameters of the net photosynthesis rate and the net respiration rate under autotrophic conditions. Experiments were also performed to determine the coefficient yields, both for nitrogen and phosphorus, in *S. almeriensis* cultures. This study allowed an in-depth analysis of the importance of adequate nutrient supplementation in the microalgae cultivation. All the obtained parameters allow to increase the

understanding of the effect of nutrients on microalgae-based processes and to improve the current mechanistic models for microalgae-bacteria systems.

Materials and methods

Microalgal species and culture conditions

The microalga *S. almeriensis* CCAP 276/24 was obtained from the culture collection of the Department of Chemical Engineering of the University of Almería. The inoculum of this strain was grown photoautotrophically in a Erlenmeyer spherical flask (1.0 l capacity) and inoculated weekly with fresh modified Arnon medium (Allen and Arnon 1955) (Table 1). The culture was continuously supplied with an air–1%CO_2 mixture to control the pH at 8.0. The Erlenmeyer spherical flask was maintained at 24 °C, controlled by regulating the air temperature in the chamber. The culture was artificially illuminated on a 12:12 h L/D cycle using four Philips PL-32 W/840/4p white-light lamps, providing an irradiance of 750 µE/m^2 s on the spherical 1.0 L flask surface.

Experimental set-up

To evaluate the oxygen production/consumption rates of *S. almeriensis* as a function of nutrient availability, experiments were performed in Erlenmeyer spherical flasks (1.0 L capacity) filled to 650 mL with Arnon medium, modified according to the specific assay, and 20% of *S. almeriensis* inoculum. To study the effect of the concentration of each main nutrient (nitrogen and phosphorous), the other one was maintained in the same concentration that established Arnon medium. Moreover, the rest of the minor and major nutrients were kept as defined the protocol. Three sets of experiments were performed: (i) at different nitrate concentrations from 0 to 200 mgN·L^{-1}, maintaining of phosphate at the concentration that indicate Arnon medium, (ii) without nitrate but using ammonium as a nitrogen source, at different concentrations from 0 to 200 mgN·L^{-1}, and (iii) at different phosphate concentrations from 0 to 30 mgP·L^{-1}, maintaining nitrogen in form of nitrate as a nitrogen source (at the concentration that indicate Arnon medium). The modified Arnon mediums were sterilized in an autoclave at 120 °C for 20 min. The Erlenmeyer spherical flasks were operated in batch mode to take samples for the respirometric tests and nutrient yield coefficient determination. Each reactor was aerated at a rate of 0.2 v/v/min with CO_2 injected on demand (pH = 8). The reactors were continuously illuminated artificially using eight 28 W fluorescent tubes (Philips Daylight T5), providing an irradiance of 1350 µE/m^2 s on the spherical 1.0 L flask surface.

Table 1 Average composition of the modified Arnon medium. Concentrations expressed as mg·L^{-1}

Parameters	Arnon
pH	7.5 ± 0.2
COD	16.0 ± 1.2
Sulphate	6.3 ± 0.8
Nitrogen-nitrate	140.0 ± 4.5
Chloride	78.9 ± 2.1
Sodium	276.1 ± 7.9
Potassium	325.1 ± 6.3
Calcium	364.9 ± 5.5
Magnesium	12.2 ± 0.6
Phosphorus-phosphate	39.3 ± 3.1
Nitrogen-ammonium	0.0 ± 0.1
Iron	5.0 ± 0.3
Copper	0.02 ± 0.0
Manganese	0.5 ± 0.02
Zinc	0.06 ± 0.01
Boron	0.4 ± 0.03
TC	52.4 ± 4.9
TN	140.0 ± 4.5
TP	39.3 ± 3.1

Values correspond to the mean ± SD

Respirometric measurements

To determine the oxygen production rate and oxygen consumption rate of *S. almeriensis*, a photo-respirometer was used. This device allows one to measure the variation in the dissolved oxygen concentration in microalgae samples under different conditions. The oxygen measurements were performed in a jacketed 60 mL glass flask which was mixed by a magnetic stirrer. The glass flask was artificially illuminated using two controlled LED lamps situated to the right and left of the flask. The desired irradiance inside the flask could be automatically controlled. The dissolved oxygen concentration in the microalgae samples was continuously measured by a sensor (Crison 5002, Barcelona, Spain) located inside the glass flask. There were also sensors for temperature, pH, and irradiance placed within the flask. As the temperature was controlled at 24 °C, the temperature effect was disregarded in the growth kinetic parameters. The reliability of this method was highlighted by (Sánchez-Zurano et al. 2020), since the authors proposed a standardization of the photo-respirometry method, defining a protocol to follow, the biomass concentration and irradiance used during the measurements, and the oxygen mass transfer coefficient (K_La) used to correct the influence of oxygen desorption on the photo-respirometric measurements (Sánchez-Zurano et al. 2020).

The influence of the oxygen desorption on the respirometric measurements was corrected using the oxygen mass transfer coefficient ($\boldsymbol{K_L a}$). This value was determined in absence of aeration experimentally. The method used consisted in measuring the dissolved oxygen concentration versus time profiles in the same chemical-physical conditions applied during the respirometric tests. For this, a cell-free sample was placed in the measurement device and the concentration of oxygen was increased to 130%Sat by bubbling with the pure O_2 gas. After this, the bubbling was stopped and the variation in oxygen concentration ($\boldsymbol{C_{O2}}$) with time was monitored for around 4 h. The ($\boldsymbol{K_L a}$) in the system quantifies the proportionality between the oxygen exchange between the liquid and gas phases and the driving force expressed as ($C^*_{02} - C_{O2}$) leading to the following elementary mass balance:

$$\frac{d\mathrm{CO2}}{dt} = K_L a\left(C^*_{02} - C_{O2}\right) \tag{1}$$

The determination of the $\boldsymbol{K_L a}$ is described in details by Sánchez-Zurano et al. 2020. The $\boldsymbol{K_L a}$ value obtained was 1.08 h^{-1}.

The protocol proposed relies on the measurement of oxygen produced or consumed by microalgal biomass under different nitrogen and phosphorous concentrations. The procedure proposed is based on the oxygen production/consumption under cycles of light and dark as a function of a single variable at a time, while keeping the other variable constant. These produced/consumed oxygen measurements allow us to determine the net photosynthesis rate and the net respiration rate, respectively. The methodology consists of inoculating the Erlenmeyer spherical flaks at different stages with different concentrations of the studied variable and waiting 30 min for acclimatization. After that time, samples of each microalgae culture were taken to measure the oxygen production during the light phases and the oxygen consumption during the dark phases (Fig. 1). Each culture sample was placed inside the photo-respirometer and then exposed to light–dark cycles of 4 min each to measure and record the variation in dissolved oxygen under each condition (Fig. 1). The first minute of exposure was disregarded as it was considered an adaptation time. Between the dark and light periods, air was provided to recover the 100%Sat of the dissolved oxygen. During light periods, oxygen generation is expected as a result of the active photosynthesis carried out by the microalgae whereas during the dark periods, oxygen is consumed by the endogenous respiration rate. The microalgae's oxygen production rate (*OPR*) was calculated from the slope of the dissolved oxygen concentration over the last 3 min of the light phases $\left(\frac{d[O2]_L}{dt}\right)$, dividing by the biomass concentration (*Cb*) (Eq. 2).

$$OPR = \frac{1}{Cb}\left(\frac{d[O2]_L}{dt}\right) \tag{2}$$

Similarly, the oxygen consumption rate (*OCR*) was calculated from the slope of the dissolved oxygen concentration over the last 3 min of the dark phases $\left(\frac{d[O2]_D}{dt}\right)$, dividing by the biomass concentration (*Cb*) (Eq. 3).

$$OCR = \frac{1}{Cb}\left(\frac{d[O2]_D}{dt}\right) \tag{3}$$

Finally, the net photosynthesis rate (*NPR*) was calculated as the difference between the oxygen production rate and the oxygen consumption rate (Eq. 4). In addition, the microalgae respiration rate (*MRR*) was defined as the oxygen consumption rate (Eq. 5).

$$NPR = OPR - OCR \tag{4}$$

$$MRR = OCR \tag{5}$$

The maximal photosynthetic and respiratory activities, measured under an increasing nutrient concentration, were

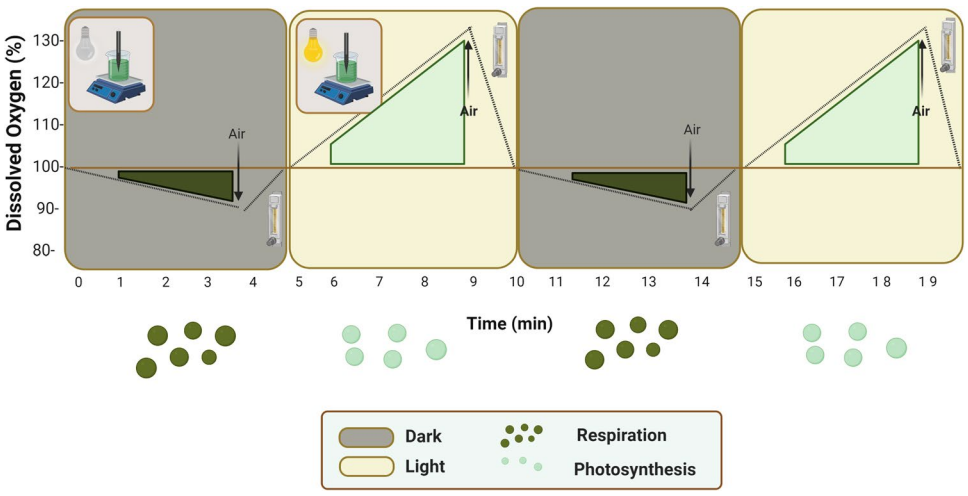

Fig. 1 Typical result of a respirometric test. Dark and light phases are reported together with the addition of air to recover 100% dissolved oxygen

used to normalize the experimental data obtained from 0 to 1. Each *OPR* and *OCR* value was estimated as the average of at least four measurements (i.e., four dark–light cycles of 4:4 min each).

Estimation of the nutrient yield coefficients

The coefficient yield for the macronutrients (nitrogen and phosphorus) was determined as the variation of the substrate to biomass concentration ratio; that is to say, the coefficient yield was defined as the amount of substrate consumed over the amount of microalgae produced. Determining these coefficients is mandatory for optimizing the mathematical models which simulate the biomass growth and the nutrient removal in microalgal processes. The nitrogen/biomass yield and phosphorous/biomass yield were expressed in g N/g dry biomass and g P/g dry biomass, respectively.

For this purpose, samples from each spherical glass flask containing the different concentrations of nitrogen and phosphorus were taken over 24 h to determine the biomass concentration by the dry weight and to measure the nutrients in the sample's supernatant.

Biomass concentration and analytical methods

The biomass concentration (Cb) was measured by dry weight. Aliquots containing 100 mL of the culture were filtered through the Macherey–Nagel MN 85/90 glass fiber filters. Then, the filters were dried in an oven at 80 °C for 24 h. Standard official methods were used to analyze the composition of the wastewater samples and the water from the reactors. The phosphate was measured by visible spectrophotometry through the phospho-vanado-molybdate complex (Phosphate Standard for IC: 38,364). The nitrate was quantified by measuring optical density at 220 nm and 275 nm (Nitrate Standard for IC: 74,246). The ammonium was measured according to the Nessler method (Ammonium standard for IC: 59,755).

Software and statistical analysis

The DaqFactory data acquisition and control software (Azeotech, USA) were used to gather the photosynthesis and respiration rate data. All the measurements were performed in triplicate (at least) to allow us to calculate the mean values and standard deviations shown. Data analysis was carried out using the Statgraphics Centurion XVI software package, in which non-linear regression was used to fit experimental data to the proposed models, and to determine the characteristic parameter values. These models were used to obtain simulations in Microsoft Excel.

Results

Influence of the nutrient concentration on the photosynthesis and respiration rates

To study the influence of nitrate on *S. almeriensis* performance, concentrations ranging from 0 to 200 mgN·L^{-1} were assayed, which correspond to a nitrate range from 0 to 900 mgNitrate·L^{-1}. Experiments performed in which the nitrogen in form of nitrate concentration in the culture medium was modified have shown that both the net photosynthesis rate and the net respiration rate increase hyperbolically with the nitrogen concentration, achieving a maximum value in the 20–40 mgN·L^{-1} range; above this value, both the net photosynthesis rate and the net respiration rate decrease (Fig. 2). According to these figures, inhibition by nitrate does take place, even at moderate concentrations of 200 mgN-NO_3^-·L^{-1} (approximately 40 mgN·L^{-1}); this has not been widely reported. Data processing was subsequently carried out to calculate the normalized maximum net photosynthesis and respiration rates, being 130 and 25 mgO2·$g_{biomass}^{-1}$·h^{-1} for the specific maximum photosynthetic rate ($PO_{2,max}$) and the specific maximum respiration rate ($RO_{2,max}$), respectively. Experimental data have been fitted to a model which considers inhibition by substrate, such as the Haldane equation (Eq. 6) (Armstrong 1930), in which the net photosynthesis (PO_2) rate is a function of the nitrogen concentration (N-NO_3^-), the nitrogen half-saturation constant ($K_{S,N\text{-}NO3^-}$), and the inhibition parameter constant (K_I). By fitting experimental data to this equation, the characteristic parameter values were determined ($K_{S,N\text{-}NO3^-} = 2.77$ mgN-NO_3^-·L^{-1} and $K_{I,N\text{-}NO3^-} = 279$ mgN-NO_3^-·L^{-1}), verifying that the model reproduces the behavior of the measurements performed.

$$\overline{PO_2([N-NO_3^-])} = \frac{[N-NO_3^-]}{[N-NO_3^-] + K_{S,N-NO3-} + \frac{[N-NO_3^-]^2}{K_{I,N-NO3-}}} \quad (6)$$

Concerning the respiration rate, which was determined by oxygen measurements in the dark, the data also show a pattern of inhibition by substrate, The respiration rate is zero at a null nitrogen in form of nitrate concentration but increases with the concentration to reach a maximum at 20 mgN·L^{-1} (approximately 90 mgNitrate·L^{-1}); it then decreases at higher nitrogen concentrations. The data have also been fitted to the Haldane equation (Eq. 7). The characteristic parameter values obtained were as follows: $K_{R,\ N\text{-}NO3^-} = 1.02$ mgN-NO_3^-·L^{-1} and $K_{I,R,N\text{-}NO3^-} = 279$ m g N-NO_3^-·L^{-1}. The results show that the selected microalgae only need low nitrogen concentrations to perform the photosynthesis and respiration properly.

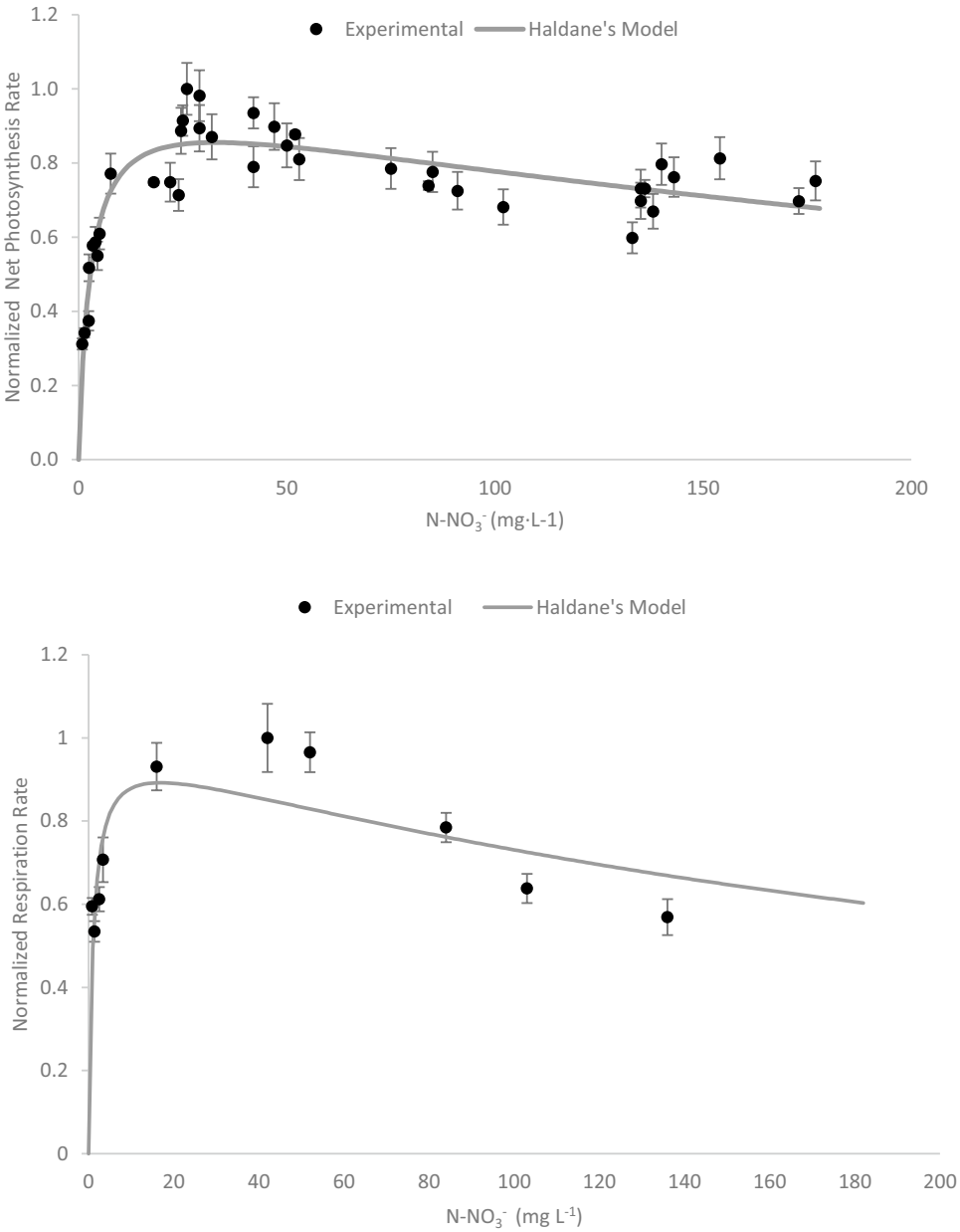

Fig. 2 Influence of nitrogen in form of nitrate on the normalized photosynthesis rate of *S. almeriensis* (A) and on the normalized respiration rate of *S. almeriensis* (B). Lines correspond to the fit of the proposed models (Eq. 6, Eq. 7)

$$\overline{RO_2(\left[N-NO_3^-\right])} = \frac{\left[N-NO_3^-\right]}{\left[N-NO_3^-\right] + K_{R,N-NO3-} + \frac{\left[N-NO_3^-\right]^2}{K_{I,R,N-NO3-}}} \quad (7)$$

To determine the behavior of *S. almeriensis* with respect to N-NH_4^+, experiments were performed at concentrations ranging from 0 to 250 mgN·L^{-1}, which corresponds to ammonium range of 0 to 320 mg N-NH_4^+ L^{-1} (Fig. 3). The results showed a similar trend as previously found with nitrate both the net photosynthesis rate and the net respiration rate increased along with the N-NH_4^+ concentration until a value of 10–20 mg N-NH_4^+ L^{-1} was reached; above this value, both the net photosynthesis rate and the net respiration rate decreased. As before, a model considering the existence of inhibition by substrate has been used to fit the experimental results. These experimental data were modelled using the Haldane equation (Eq. 8, Eq. 9), in which the characteristic parameter values for the net photosynthesis rate (PO_2) were determined ($K_{S,N\text{-}NH4}{}^+ = 1.54$ mgN-NH_4^+·L^{-1} and $K_{I,N\text{-}NH4} = 571$ mgN-NH_4^+), verifying that the model reproduces the behavior indicated by the measurements. For the respiration rate (RO_2), the kinetic parameters for the ammonium concentrations were calculated ($K_{R,\,N\text{-}NH4}{}^+ = 0.65$ mgN-NH_4^+·L^{-1} and $K_{I,R,N\text{-}NH4} = 205$ mgN-NH_4^+·L^{-1}).

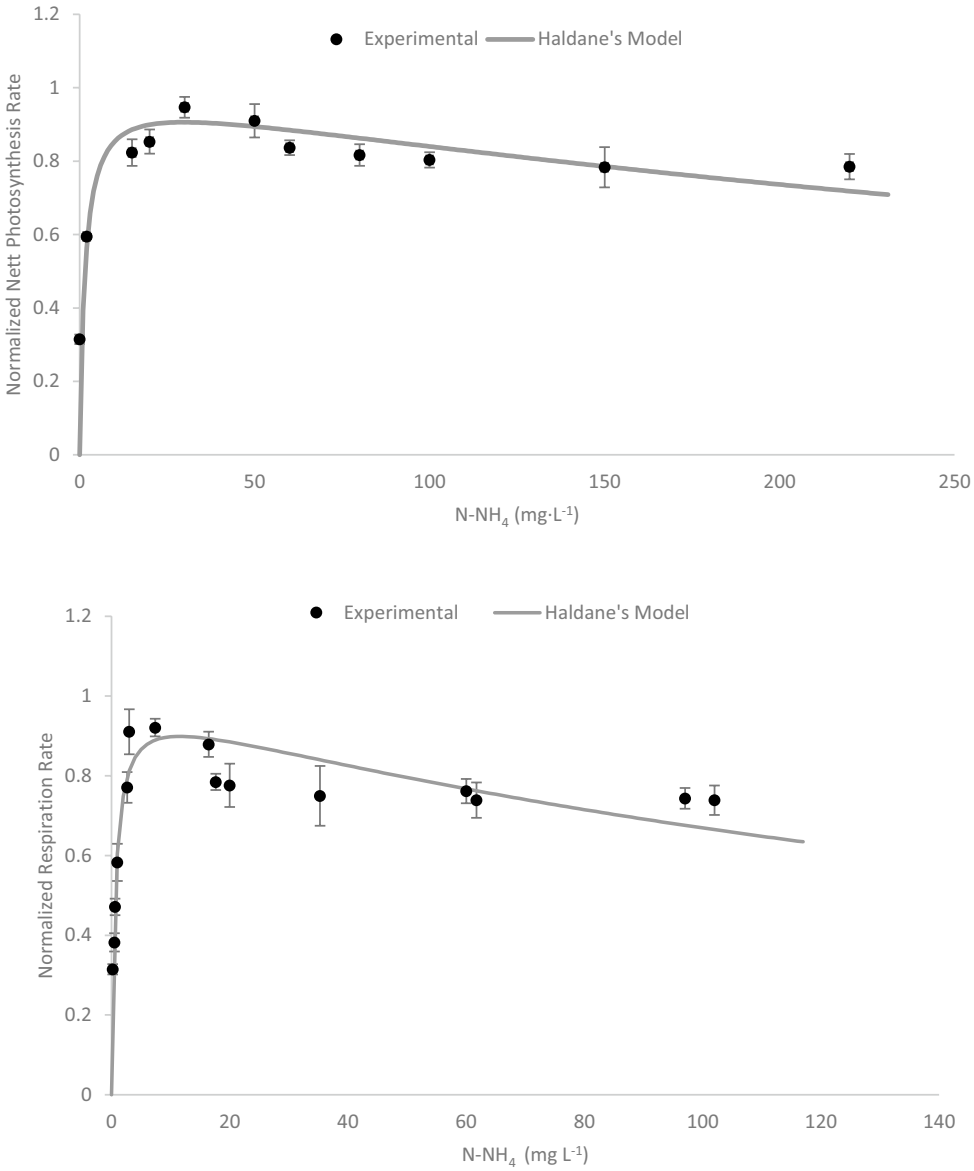

Fig. 3 Influence of nitrogen in form of ammonium on the normalized photosynthesis rate of *S. almeriensis* (A) and on the normalized respiration rate of *S. almeriensis* (B). Lines correspond to the fit of the proposed models (Eq. 8, Eq. 9)

$$\overline{PO_2(\left[N-NH_4^+\right])} = \frac{\left[N-NH_4^+\right]}{\left[N-NH_4^+\right] + K_{S,N-NH4+} + \frac{\left[N-NH_4^+\right]^2}{K_{I,N-NH4+}}} \quad (8)$$

$$\overline{RO_2(\left[N-NH_4^+\right])} = \frac{\left[N-NH_4^+\right]}{\left[N-NH_4^+\right] + K_{R,N-NH4+} + \frac{\left[N-NH_4^+\right]^2}{K_{I,R,N-NH4+}}} \quad (9)$$

Concerning to the phosphorous, in this work, the experiments were performed up to a concentration of 120 mg PO_4^{3-}·L^{-1}, which corresponds to 40 mg P-PO_4^{3-}·L^{-1}. The results showed that the net photosynthesis and respiration rates hyperbolically increased with the phosphorous concentration in the concentration range assayed, with no inhibition being observed at higher concentrations (Fig. 4). To fit the experimental data, the Monod model has been used (Eq. 10), in which the characteristic parameter values for the net photosynthesis rate and the net respiration rate were determined ($K_{S,P\text{-}PO4}$ = 0.43 mg P-PO_4^{3-}·L^{-1} and $K_{R,\ P\text{-}PO4}$ = 0.35 mg P-PO_4^{3-}·L^{-1}).

$$\overline{PO_2(\left[P-PO_4^{3-}\right])} = \frac{\left[P-PO_4^{3-}\right]}{\left[P-PO_4^{3-}\right] + K_{S,P-PO4}} \quad (10)$$

$$\overline{RO_2(\left[P-PO_4^{3-}\right])} = \frac{\left[P-PO_4^{3-}\right]}{\left[P-PO_4^{3-}\right] + K_{R,P-PO4}} \quad (11)$$

In summary, the values obtained for all the characteristic parameters are shown in Table 2.

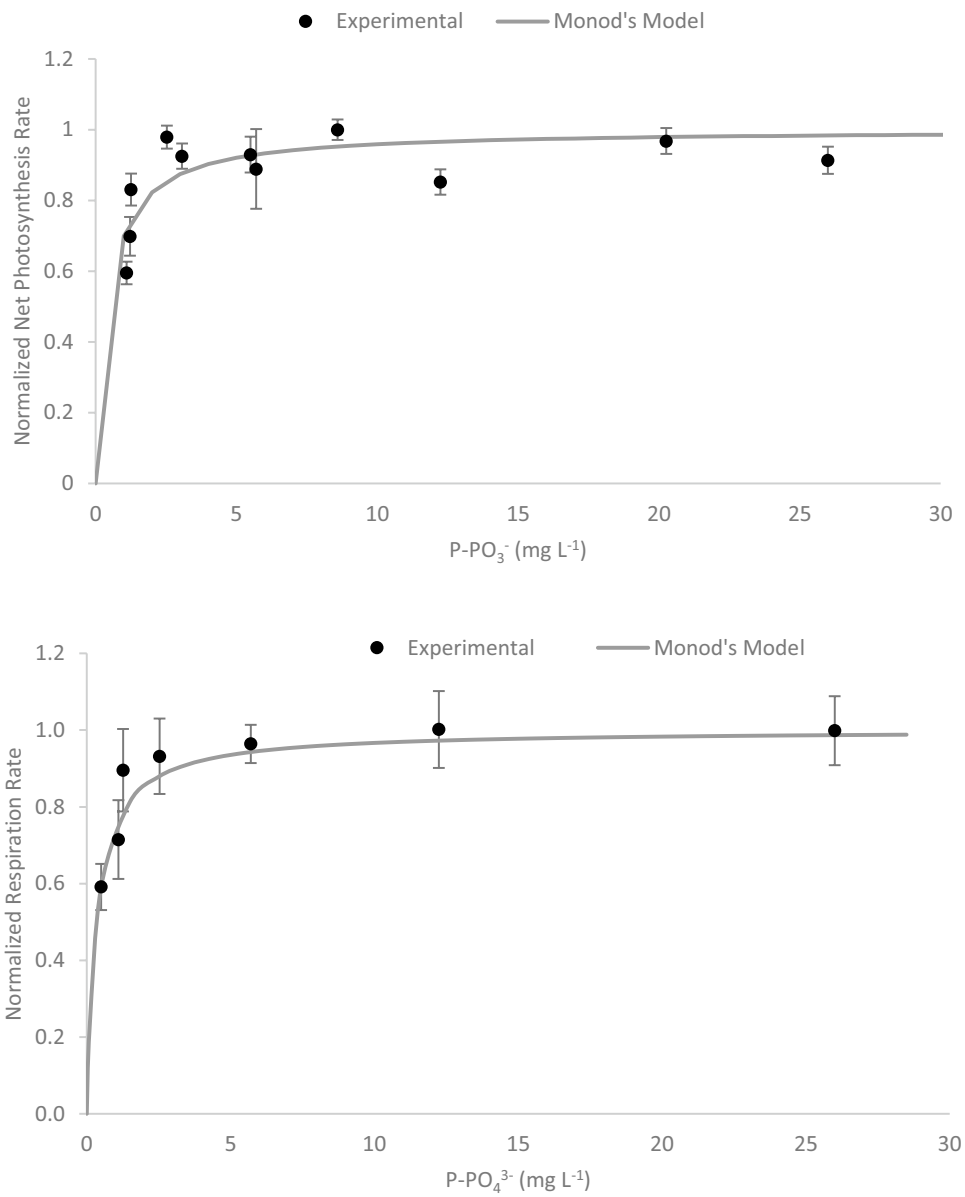

Fig. 4 Influence of phosphorus on the normalized photosynthesis rate of *S. almeriensis* (A) and on the normalized respiration rate of *S. almeriensis* (B). Lines correspond to the fit of the proposed models (Eq. 10)

Table 2 Values for the proposed model's parameter characteristics and confidence intervals

Nitrate models			Ammonium models			Phosphate models		
Parameter	Value	Units	Parameter	Value	Units	Parameter	Value	Units
$K_{S,N\text{-}NO3}^-$	2.77 ± 0.28	mgN-NO_3^-·L^{-1}	$K_{S,N\text{-}NH4}^+$	1.54 ± 0.15	mgN-NH_4^+·L^{-1}	$K_{S,\ P\text{-}PO4}$	0.43 ± 0.06	mg P-PO_4^{3-}·L^{-1}
$K_{I,N\text{-}NO3}^-$	386.6 ± 42.5	mgN-NO_3^-·L^{-1}	$K_{I,N\text{-}NH4}^+$	571 ± 49.2	mgN-NH_4^+·L^{-1}	$K_{R,\ P\text{-}PO4}$	0.35 ± 0.03	mg P-PO_4^{3-}·L^{-1}
$K_{R,N\text{-}NO3}^-$	1.02 ± 0.12	mgN-NO_3^-·L^{-1}	$K_{R,N\text{-}NH4}^+$	0.65 ± 0.08	mgN-NH_4^+·L^{-1}			
$K_{I,R,N\text{-}NO3}^-$	279 ± 25.4	mgN-NO_3^-·L^{-1}	$K_{I,R,N\text{-}NH4}^+$	205 ± 21.3	mgN-NH_4^+·L^{-1}			

Influence of nutrient concentration on the yield coefficients

Once the influence of the nutrient concentrations on the photosynthesis and respiration rates of *S. almeriensis* cells had been determined, experiments were also performed to determine the yield coefficients. Experiments were performed under the same conditions as before, in the same concentration ranges, to determine if nutrient concentrations influence the coefficient yield values.

The data show that the nitrogen and phosphorous coefficient yields are not constant, being modified as a function of the nutrient's concentration (Fig. 5). The results show that the nitrogen and phosphorous coefficient yields increase as nitrogen or phosphorus increase in the culture medium, observing a peak at 70 mgN-NO_3^-·L^{-1} and 18 mgP-PO_4^{3-}·L^{-1}, respectively. Modelling this phenomenon is complex, since if the trend of the experimental data is considered, one might think that a certain inhibition appears in the yield coefficients. However, it would not be an inhibition, but the data show a variability in the value of the yield coefficients due to its relationship with the concentration of nitrogen and phosphorus in the medium. To model this phenomenon, the sum of two equations has been applied—the hyperbolic equation and the cardinal equation. The former, which is typically used for microbial growth kinetics, has been used to explain the increase in the nitrogen and phosphorous coefficient yields as the nitrogen or phosphorous

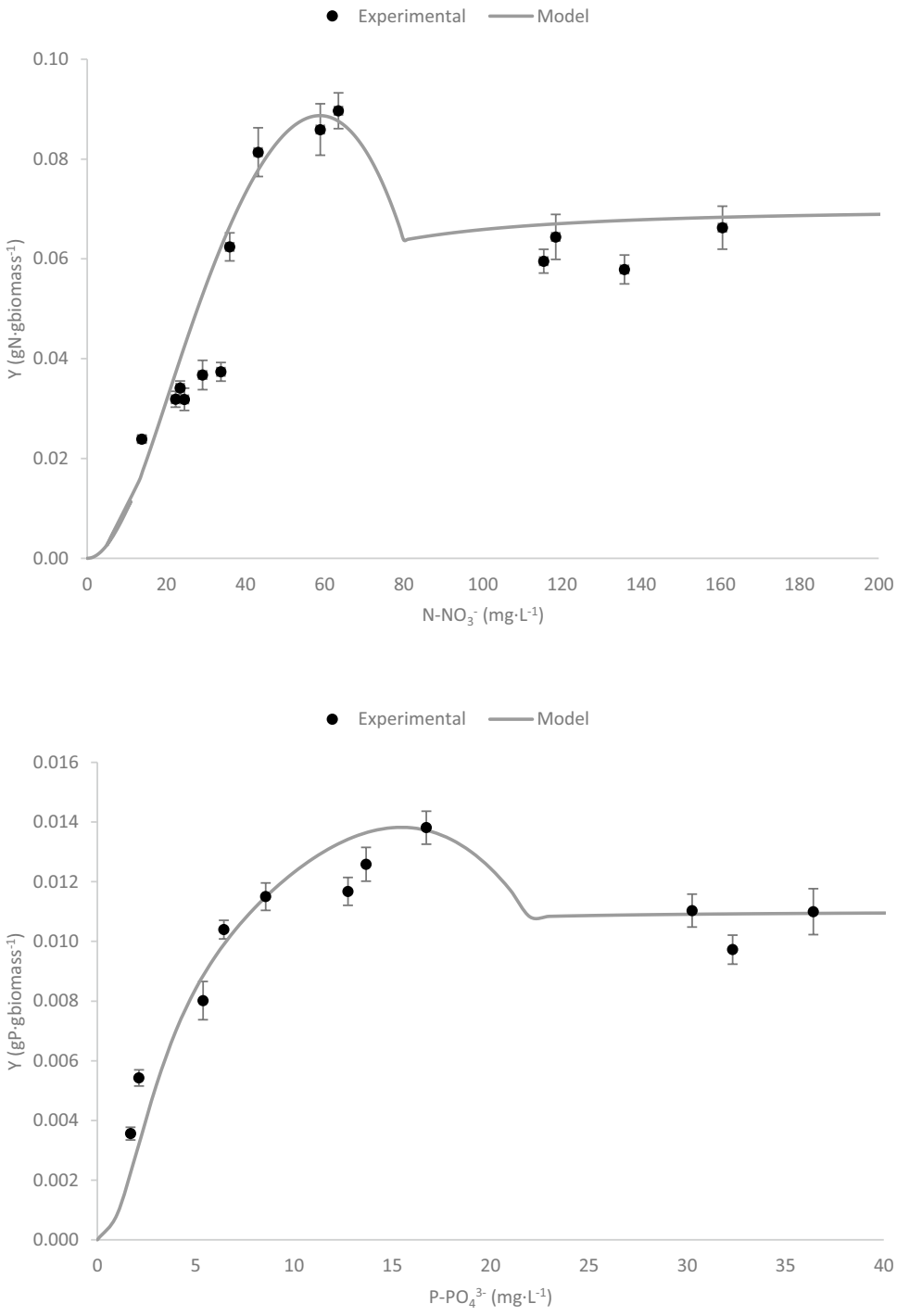

Fig. 5 Nutrient yield coefficients of *S. almeriensis*: nitrogen yield coefficient (A); phosphorous yield coefficient (B). Lines correspond to the fit of the proposed models (Eq. (11), Eq. (12))

concentrations increase in the medium. In addition, to describe the peaks observed both in the nitrogen and phosphorous coefficient yields, the cardinal equation has been applied within the minimum and maximum ranges established. The cardinal model allows one to define the maximal, minimal, and optimal conditions for whichever variable, fitting its influence into the biological system performance as a Gaussian function (Bernard and Rémond 2012). Using the cardinal equation allows one to obtain the "optimal nutrient concentration value" in which the nitrogen and phosphorous yield coefficients are higher. Regarding nitrogen, the coefficient values obtained ranged from 0.02 to 0.09 $gN\text{-}NO_3^-\cdot g_{biomass}^{-1}$. Concerning the phosphorus, the results showed that the phosphorous yield coefficient ranged from 0.004 to 0.014 $gP\text{-}PO_4^{3-}\cdot g_{biomass}^{-1}$ at the phosphorous concentrations tested. Subsequently, the nitrogen and phosphorous yield coefficients were fitted to the sum of the hyperbolic and cardinal models (Eq. 12, Eq. 13) from which the characteristic parameter values for the nitrogen yield coefficient ($Y_{gN/gbiomass,\,max}=0.07$ $gN\text{-}NO_3^-\cdot g_{biomass}^{-1}$, $K_{S,YN}=25$ $mgN\text{-}NO_3^-\cdot L^{-1}$, $m=2$, $N_{max}=80$ $mgN\text{-}NO_3^-\cdot L^{-1}$, $N_{min}=10$ $mgN\text{-}NO_3^-\cdot L^{-1}$, $N_{opt}=55$ $mgN\text{-}NO_3^-\cdot L^{-1}$) and phosphorous yield coefficient ($Y_{gP/gbiomass,\,max}=0.011$ $gP\text{-}PO_4^{3-}\cdot g_{biomass}^{-1}$, $K_{S,YP}=3.2$ $mgP\text{-}PO_4^{3-}\cdot L^{-1}$, $m=2.14$, $P_{max}=22$ $mgP\text{-}PO_4^{3-}\cdot L^{-1}$, $P_{min}=2$ $mgP\text{-}PO_4^{3-}\cdot L^{-1}$, $P_{opt}=15$ $mgP\text{-}PO_4^{3-}\cdot L^{-1}$) were determined (Table 3).

$$Y_{N/biomass}=\left[\frac{Y_{N/biomass,max}\cdot[N]^m}{[N]^m+K_{S,YN}{}^m}\right]+\left[\frac{(N-Nmax)(N-Nmin)2}{(Nopt-Nmin)(((Nopt-Nmin)(N-Nopt))-((Nopt-Nmax)(Nopt+Nmin-2N)))}\right] \quad (12)$$

$$Y_{P/biomass}=\left[\frac{Y_{P/biomass,max}\cdot[P]^m}{[P]^m+K_{S,YP}{}^m}\right]+\left[\frac{(P-Pmax)(P-Pmin)2}{(Popt-Pmin)(((Popt-Pmin)(P-Popt))-((Popt-Pmax)(Popt+Pmin-2P)))}\right] \quad (13)$$

Performance of S. almeriensis cells as a function of the culture medium

Once the effects of nitrogen and phosphorus were evaluated and modelled, both for the photosynthesis rate and for the respiration rate, simulations were performed to determine the performance of *S. almeriensis* cells as a function of the culture medium used to produce them. These simulations were performed mainly considering the culture media, from the standard culture medium prepared using fertilizers to the different wastewater types, even including wastewater that had been depurated in accordance with the regulations. Wastewater that has already been treated should contain a low nutrient concentration (5–10 mg-$N\cdot L^{-1}$ and 1–2 mg-$N\cdot L^{-1}$). In this work, we considered two possibilities: treated wastewater with the maximum nutrient concentration for safe disposal (10 mg-$N\cdot L^{-1}$) and treated wastewater complying to the new limits (5 mg-$N\cdot L^{-1}$) (European Directive 91/271/CEE).

Figure 6A shows the normalized photosynthesis rate as a function of the nitrogen and phosphorous concentration when using different culture media. Concerning nitrogen, the results shows that the normalized photosynthesis rate was maximal when using wastewater and wastewater after treatment, whereas it reduced because of nitrogen limitation when totally depurated wastewater was used. Conversely, when using manure or centrate as the culture medium, the photosynthesis rate decreased as a result of inhibition; this included fertilizers with high nitrogen concentrations. Regarding phosphorus, a different trend was observed. No inhibition was observed as a result of excess phosphorus, regardless of the culture medium used.

Table 3 Values for nitrogen and phosphorous yield and confidence intervals

Nitrogen yield model			Phosphorous yield model		
Parameter	Value	Units	Parameter	Value	Units
$Y_{gN/gbiomass,\,max}$	0.07 ± 0.008	$g\ N\text{-}NO_3^-\cdot g_{biomass}^{-1}$	$Y_{gP/gbiomass,\,max}$	0.011 ± 0.001	$gP\text{-}PO_4^{3-}\cdot g_{biomass}^{-1}$
$K_{S,\,YN}$	25 ± 2.7	$mg\ N\text{-}NO_3^-\cdot L^{-1}$	$K_{S,\,YP}$	3.2 ± 0.34	$mg\ P\text{-}PO_4^{3-}\cdot\cdot L^{-1}$
m	2 ± 0.2	-	m	2.14 ± 0.22	-
N_{max}	80 ± 7.2	$mg\ N\text{-}NO_3^-\cdot L^{-1}$	P_{max}	22 ± 2.3	$mg\ P\text{-}PO_4^{3-}\cdot L^{-1}$
N_{min}	10 ± 0.9	$mg\ N\text{-}NO_3^-\cdot L^{-1}$	P_{min}	2 ± 0.3	$mg\ P\text{-}PO_4^{3-}\cdot\cdot L^{-1}$
N_{opt}	55 ± 4.9	$mg\ N\text{-}NO_3^-\cdot L^{-1}$	P_{opt}	15 ± 1.7	$mg\ P\text{-}PO_4^{3-}\cdot\cdot L^{-1}$

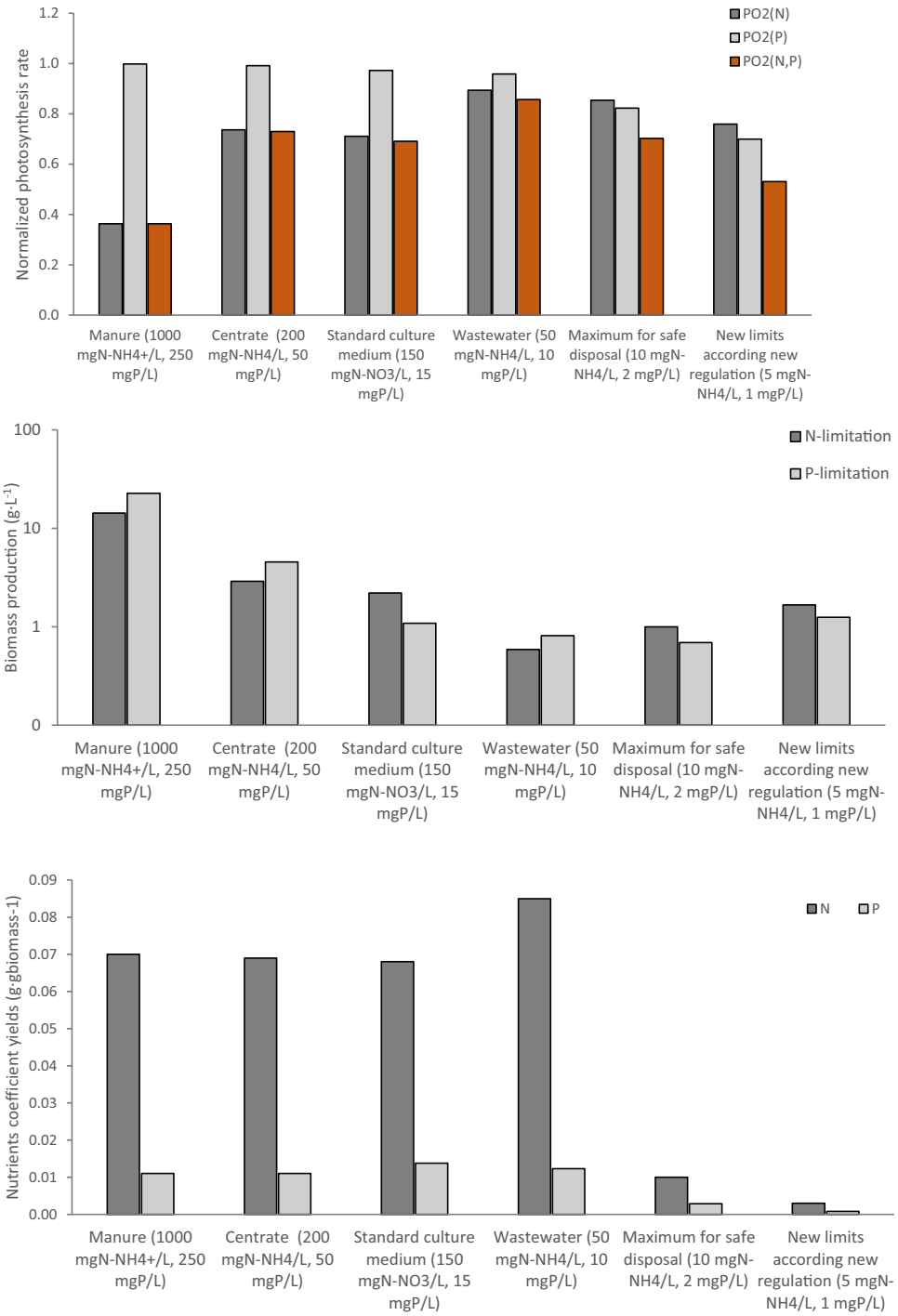

Fig. 6 Simulations of the nitrogen and phosphorous effect in different culture media on the normalized photosynthesis rate (A), the nutrient yield coefficient (B), and biomass production (C)

A limitation in the photosynthesis rate only took place when totally depurated wastewater was used as the culture medium. Because the performance of the photosynthetic process is a function of both nitrogen and phosphorous availability, the performed simulations showed the photosynthesis rate of *S. almeriensis* decreased sharply when using manure or centrate as the culture medium. In contrast, *S. almeriensis* performed at its maximal capacity when using wastewater and treated wastewater as the culture medium.

The same scenarios were used to simulate the nutrient yield coefficients as a function of the nitrogen and phosphorus contained in the culture media (Fig. 6B). The results show that *S. almeriensis* consumed from 0.003 to 0.085 $gN \cdot g_{biomass}{}^{-1}$, with maximal values being obtained when using wastewater and standard culture media, whereas both

were reduced when excess or limiting concentrations of nitrogen were provided. The same behavior was observed for the phosphorous yield coefficients, which varied from 0.001 to 0.014 $gP \cdot g_{biomass}^{-1}$, with maximal values also being obtained when using wastewater and standard culture medium.

Due to the diverse nutrient availability in the different culture media and the above-described variation in the yield coefficients as a function of nutrient availability, to calculate how much biomass can be produced per liter of culture medium for the different culture media is an interesting parameter (Fig. 6C). This analysis can be performed considering either N or P as the limiting nutrient, thus allowing us to identify which is the limiting factor when using the different culture media. The data shows that when using manure, up to 14.3 g of biomass can be produced per liter of manure, this production capacity being limited by the nitrogen concentration in the effluent, with the phosphorous content producing up to 22.7 g of biomass per liter. This biomass production capacity per liter of effluent was less for the other culture media. In the case of centrate, the maximal biomass production capacity was 2.9 g of biomass per liter, with nitrogen as the limiting nutrient. When using wastewater, the maximal biomass production capacity was 0.6 g of biomass per liter, again with the nitrogen concentration as the limiting factor. Also, phosphorous is the limiting nutrients when treated wastewater is used as a culture medium, so it is theoretically possible to produce 0.7 and 1.3 g of biomass per liter using treated wastewater with the maximum nutrient concentration for safe disposal and treated wastewater complying to the new limits, respectively.

Discussion

Nitrate is the most convectional source of nitrogen used in microalgae cultures. In large-scale production systems, it is supplied in excess to avoid nutrient limitation (above 1000 mgNitrate·L^{-1}, which corresponds to 225 mgN·L^{-1}) (Acién et al. 2012). In the case of wastewaters, nitrogen mainly comes in the form of ammonium, with only minor concentrations of nitrate are detected when nitrification takes place, and always below 220 mgNitrate·L^{-1} (approximately 50 mgN·L^{-1}). To study the influence of nitrate concentration on *Scenedesmus almeriensis* performance, concentrations ranging from 0 to 200 mgN·L^{-1} were assayed, which correspond to a nitrate range from 0 to 900 mgNitrate·L^{-1}. By fitting experimental data to the Haldane equation, the nitrogen half-saturation constant ($K_{S,N\text{-}NO3^-}$ = 2.77 mgN-NO_3^-·L^{-1}) and the inhibition parameter constant ($K_{I,\ N\text{-}NO3^-}$ = 279 mgN-NO_3^-·L^{-1}) were determined. The nitrogen half saturation constant described for different *Scenedesmus* strains varies widely. An early kinetic model of *Scenedesmus dimosphus* growth and nutrient uptake proposed a nitrogen half-saturation constant of 0.018 mgN·L^{-1} using nitrate as the nitrogen source (Kunikane and Kaneko 1984), which is considerably less than that proposed in this work ($K_{S,N\text{-}NO3^-}$ = 2.77 mg·N-NO_3^-·L^{-1}). In addition, recent research indicates the same variability with respect to the nutrient kinetic parameters. For instance, the nitrogen half-saturation constant obtained when *Scenedesmus* sp. is cultivated at different nitrate concentrations was 11.8 mgN·L^{-1}. Furthermore, the authors did not observe microalgae growth inhibition as high nitrate concentration. However, it is important to note that no more than 25 mgN·L^{-1} was tested (Xin et al. 2010). Another previous work in which the nitrogen half-saturation constant was determined in an airlift-raceway reactor, using both *Scenedesmus* sp. and *Nannochloropsis salina*, showed a nitrogen half-saturation constant of 0.2 mgN·L^{-1} (Ketheesan and Nirmalakhandan 2013). Therefore, comparing the saturation coefficients collected in the bibliography together with the parameters determined in this study is especially difficult, since in each case a specific methodology (respirometric or through traditional tests), different nutrients and study times are applied. Concerning the respiration rate, the characteristic parameter values obtained were $K_{R,\ N\text{-}NO3^-}$ = 1.02 mgN-NO_3^-·L^{-1} and $K_{I,R,N\text{-}NO3^-}$ = 279 mg N-NO_3^-·L^{-1}. The results show that the selected microalgae only need low nitrogen concentrations to perform the photosynthesis and respiration properly.

Regarding the influence of N-NH_4^+, this is the most frequent nitrogen source in wastewater, with concentrations ranging from 0 to 130 mg N-NH_4^+·L^{-1}. It has been widely reported that N-NH_4^+ reduces the performance of microalgae cultures, especially at concentrations above 100 mgN·L^{-1} (approximately 130 mg N-NH_4^+·L^{-1}) (Cabanelas et al. 2013). The results showed both the net photosynthesis rate and the net respiration rate increased along with the N-NH_4^+ concentration until a value of 10–20 mg N-NH_4^+·L^{-1} was reached; above this value, both the net photosynthesis rate and the net respiration rate decreased. These experimental data were fitted using the Haldane equation, in which the characteristic parameter values for the net photosynthesis rate (PO_2) were determined ($K_{S,N\text{-}NH4^+}$ = 1.54 mgN-NH_4^+ L^{-1} and $K_{I,\ N\text{-}NH4^+}$ = 571 mgN-NH_4^+). Moreover, the kinetic parameters for the respiration rate (RO_2) were $K_{R,\ N\text{-}NH4^+}$ = 0.65 mgN-NH_4^+·L^{-1} and $K_{I,R,\ N\text{-}NH4^+}$ = 205 mgN-NH_4^+·L^{-1}. Despite the scarcity of nutrient half saturation constants obtained by respirometric tests, these results are comparable with a previous work in which the ammonia half-saturation constant for the Chlorophyta microalgae *Chlorella protothecoides* was determined ($K_{S,NH4^+}$ = 14.23 mgN-NH_4^+·L^{-1} (Sforza et al. 2019). The ammonia saturation coefficient described for *Chlorella protothecoides*, which was obtained using a similar respirometric protocol, was higher than for the same parameter in *S. almeriensis*.

Furthermore, the respirometric experiments with *Chlorella protothecoides* did not show ammonia inhibition. However, the tests were performed in the 0–40 mgN-$NH_4^+ \cdot L^{-1}$ range, which is significantly lower than the range tested here with *S. almeriensis*. The tests described in this work reached fairly high ammonia concentrations, which might explain the photosynthetic inhibitory effect. Rossi et al. (2020) used photo-respirometric tests to determine the $EC_{50,NH3}$, which represents the free ammonia concentration causing a 50% inhibition of photosynthetic activity in a microalgae monoculture. They evaluated two *Scenedesmus* strains, *S.quadricuada* and *S.obliquus*, which showed an $EC_{50,NH3}$ of 77.7 and 52.6 mg$NH_3 \cdot L^{-1}$, respectively (Rossi et al. 2020). At these concentrations, *S. almeriensis* showed a reduction in net photosynthesis of 20% and 10%, respectively, lower than that described for the other strains. However, the exposure time for *S. quadricuada* and *S. obliquus* was longer than that for *S. almeriensis*, which might have affected the results.

Apart from respirometric experiments, previous works have evaluated the influence of ammonia concentration on microalgae growth. These experiments founded that specific microalgae growth rate values showed no obvious differences to those in which the ammonia concentration was below 15–20 mgN-$NH_4^+ \cdot L^{-1}$. However, when the free ammonia increased above 30–40 mgN-$NH_4^+ \cdot L^{-1}$, the specific growth rate decreased. Compared to the optimal growth rate, the specific growth rate decreased by more than 50% and 80% when the free ammonia concentration increased to 30–40 mgN-$NH_4^+ \cdot L^{-1}$ and 50–60 mgN-$NH_4^+ \cdot L^{-1}$, respectively (Tan et al. 2016). These results showed an inhibitory effect at lower concentrations than those proposed in this work. Thus, it is essential to point out that the inhibitory effects seen in the short respirometric test could be aggravated if the test were longer.

As the data reported here show, *S. almeriensis* microalgae photosynthesize properly whether ammonium (or ammonia; note that they are in chemical equilibrium) or nitrate is used as the nitrogen source. The lab-scale experiments developed in this work have been performed using pure *S. almeriensis* cultures in which nitrate and ammonium have been tested separately. However, when microalgae are used to treat wastewater, both nitrate and ammonium appear as contaminants. To improve the microalgae wastewater treatment models, they should take into account that ammonium is generally preferred when both ammonium and nitrate are present (Mengesha et al. 1999; Solimeno et al. 2015).

Regarding phosphorus, this appears in the natural environment and wastewater in many forms such as orthophosphate (containing one phosphate unit), polyphosphate, pyrophosphate, metaphosphate, and their organic complexes. However, the main form from which microalgae acquire phosphorus is inorganic phosphate P-PO_4^{3-} (orthophosphate) (Procházková et al. 2014; Khanzada 2020). Thus, most of the culture media reported for microalgae production contain phosphate in phosphorous form. The phosphorous concentration in regular microalgae culture media is much lower than the nitrogen concentration (up to ten times lower) whereas in some culture media, such as Arnon, it is even higher. In wastewater, the usual phosphorous concentration is much lower than the nitrogen concentration, with values ranging from 0 to 20 mg P-$PO_4^{3-} \cdot L^{-1}$ (Acién et al. 2016). Wastewaters coming from the mineral fertilizer industry can also contain high phosphorous concentrations, from 13 to 60 mg P-$PO_4^{3-} \cdot L^{-1}$ (Moreno Osorio et al. 2019). In this work, the experiments were performed at 120 mg $PO_4^{3-} \cdot L^{-1}$, which corresponds to 40 mg P-$PO_4^{3-} \cdot L^{-1}$. The characteristic parameter values for the net photosynthesis rate and the net respiration rate related to the phosphorous concentration were $K_{S,P\text{-}PO4} = 0.43$ mg P-$PO_4^{3-} \cdot L^{-1}$ and $K_{R,\ P\text{-}PO4} = 0.35$ mg P-$PO_4^{3-} \cdot L^{-1}$.

The phosphorous half-saturation constant obtained in the respirometric tests closely corresponds to the value obtained for of *Scenedesmus* sp. grown in batch mode in culture media modified with different phosphorous concentrations ($K_{S,\ P\text{-}PO4} = 0.28$ mg P-$PO_4^{3-} \cdot L^{-1}$) (Xin et al. 2010). However, these values are higher than those reported for *Scenedesmus obliquus*, which was studied in a mineral medium at different phosphorous and temperature values. The phosphorous half-saturation constant described ranged from 0.2 to 1.33 μM, which corresponds to 0.006 to 0.04 mg P-$PO_4^{3-} \cdot L^{-1}$ (Martínez et al. 1999). In addition, these authors reported growth inhibition at high phosphorous concentrations, which was not observed in this study. Despite most of the references revealing low phosphorous half-saturation coefficient values, a similar photo-respirometric work with *Chlorella protothecoides* showed a phosphorous half-saturation coefficient of 1.8 mg P-$PO_4^{3-} \cdot L^{-1}$. In short experiments, which take a few minutes, the observed effect of phosphorus on increased microalgae photosynthesis is due to phosphorous incorporation into the microalgal biomass, which could be used for metabolism (Sforza et al. 2019).

In respect of the yield coefficients; that is to say, how much of the nutrients are consumed from the culture medium per mass unit of already-produced biomass. Experiments were performed under the same concentration ranges as before, to determine if nutrient concentrations influence the coefficient yield values, as has previously been reported (Gómez-Serrano et al. 2015; Morales-Amaral et al. 2015). The data show that the nitrogen and phosphorous coefficient yields are not constant, being modified as a function of the nutrient's concentration. The results show that the nitrogen and phosphorous coefficient yields increase as nitrogen or phosphorus increase in the culture medium. This variability in phosphorus uptake has already been previously described in a mixed microalgal consortium dominated by *Scenedesmus* at increasing phosphate concentrations. In

practice, when phosphate aqueous concentration increased from 5 to 15 mgP-PO_4^{3-}·L^{-1}, the microalgal acid soluble polyphosphate content increased up to three times (Powell et al. 2009). This phenomenon, by which microalgae cells are capable of taking up and storing more nutrients in larger amounts than necessary for immediate growth, is termed "luxury uptake" (Solovchenko et al. 2019). Apart from nutrients concentration, environmental variables such as temperature or light intensity may influence on luxury uptake of phosphorus by microalgae too (Powell et al. 2008).

Modelling this phenomenon is complex, since if the trend of the experimental data is considered, one might think that a certain inhibition appears in the yield coefficients. However, it would not be an inhibition, but the data show a variability in the value of the yield coefficients due to its relationship with the concentration of nitrogen and phosphorus in the medium. Regarding nitrogen, the coefficient values obtained ranged from 0.02 to 0.09 gN-NO_3^-·$g_{biomass}^{-1}$, which were in the same range as applied by Reichert et al. (2001) their mathematical models, with 0.065 gN·$g_{COD\text{-}ALG}^{-1}$ (Reichert and Vanrolleghem, 2001). Concerning the phosphorus, there are fewer references available in the literature related with phosphorus consumption by microalgae. The results showed that the phosphorous yield coefficient ranged from 0.004 to 0.014 gP-PO_4^{3-}·$g_{biomass}^{-1}$ at the phosphorous concentrations tested. Within this range, most of the previously described values in wastewater treatment appear (Reichert and Vanrolleghem 2001; Solimeno et al. 2017).

As previously explained for the kinetic parameters regarding the influence of nitrogen and phosphorous availability on the photosynthesis rate, the information found in the literature on the nitrogen and phosphorous coefficient yields is also highly variable. This may be due to the wide variety of microalgae strains and culture conditions tested. On the other hand, both the specific strain requirements and the methodology applied are complex and diverse.

Because the performance of the photosynthetic process is a function of both nitrogen and phosphorous availability, the performed simulations showed the photosynthesis rate of *S. almeriensis* decreased sharply when using manure or centrate as the culture medium. When using this strain to treat these effluents, great attention must be given to the effluent dosage in the reactor. In contrast, *S. almeriensis* performed at its maximal capacity when using wastewater and treated wastewater as the culture medium, making this strains' application highly recommendable for wastewater treatment processes. The variation in the photosynthesis rate of *S. almeriensis* at different nutrients concentrations must be taken into account due to its influence on the oxygen production rate and related dissolved oxygen concentration, which determine the required mass transfer capacity and the overall design of the reactor.

Furthermore, an analysis was performed to determine how much biomass can be produced per liter of culture medium using the yield coefficients determined previously and the culture media proposed. The data showed that when using manure, up to 14.3 g of biomass can be produced per liter of manure considering the nitrogen concentration in the effluent and up to 22.7 g of biomass can be produced with the phosphorous content. Related to the other culture media such as centrate, it is possible to achieve 2.9 g of biomass per liter with nitrogen as the limiting nutrient. The use of wastewater with high contents in nitrogen as an ammonium form (manure or centrate), making it necessary to dilute this effluent prior to use as the culture medium inside the reactor to prevent to avoid inhibition caused by an excess of ammonium or others micropollutants, such as heavy metals, and because the color they have prevent light penetration (Acién et al. 2016; García et al. 2017). For that, knowing the exact composition of the wastewater to be treated is mandatory for an optimal treatment process and biomass production, not only to avoid inhibition processes but also to determine if additional carbon, nitrogen, or phosphorus need to be added when a low nutrient concentration appear. When using standard culture medium was phosphorus the limiting factor, but this could easily be corrected for by modifying its input into the culture medium, whereas modifying the effluent composition is a far more difficult matter. Also, phosphorous is the limiting nutrients when treated wastewater is used as a culture medium, being possible to produce 0.7 and 1.3 g of biomass per liter using treated wastewater with the maximum nutrient concentration for safe disposal and treated wastewater complying to the new limits, respectively.

In summary, results demonstrated that the photosynthesis rate and the respiration rate of *Scenedesmus almeriensis* vary as a function of nutrient availability (N-NO_3^-, N-NH_4^+, and P-PO_4^{3-}). Regarding nitrogen, both in the form of N-NO_3^- and N-NH_4^+, a similar trend was observed with inhibition taking place at high concentrations, whereas no inhibition by phosphorous was observed. Regarding the nutrient yield coefficients, data show that the luxury uptake phenomenon appears at increasing nutrient concentrations, while above a limit, the nutrient yield coefficients remain constant. Both the photosynthesis/respiration rates and the nutrient yield coefficients have been modelled as a function of nutrient availability in the medium. To the best of our knowledge, this is the first time that such models have been proposed, including the luxury uptake phenomenon in microalgae cultures. These results highlight the importance of the concentration of nutrients in the microalgae culture, which is a decisive factor together with operational factors such as the pH of the culture or the temperature. With the aim of working in the most optimal conditions possible since it is crucial to achieve the maximum performance in microalgae cultures. These models must be considered

in microalgae-related systems in order to optimize them, whether using inorganic fertilizers or wastewater. In the former, it is necessary to optimize the culture medium composition according to the system performance and nutrient demand. In the latter, the challenge is to determine the optimal conditions for maximizing the nutrient removal and biomass production capacity because the wastewater composition cannot be modified.

Author contribution Ana Sánchez Zurano: methodology, research, formal analysis, and writing—original draft. Cintia Gómez Serrano: conceptualization, data curation, and resources. Francisco Gabriel Acién Fernández: supervision, writing—reviewing, and funding acquisition. José María Fernández Sevilla: formal analysis, software, and supervision. Emilio Molina Grima: writing—reviewing and editing, project administration, and funding acquisition.

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Data availability The data that support the findings of this study are available from the corresponding author on request.

Declarations

Competing interests The authors declare no competing interests.

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7.4. ABACO: A New Model of Microalgae-Bacteria Consortia for Biological Treatment of Wastewaters.

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Article

ABACO: A New Model of Microalgae-Bacteria Consortia for Biological Treatment of Wastewaters

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Abstract: Microalgae-bacteria consortia have been proposed as alternatives to conventional biological processes to treat different types of wastewaters, including animal slurry. In this work, a microalgae-bacteria consortia (ABACO) model for wastewater treatment is proposed, it being calibrated and validated using pig slurry. The model includes the most relevant features of microalgae, such as light dependence, endogenous respiration, and growth and nutrient consumption as a function of nutrient availability (especially inorganic carbon), in addition to the already reported features of heterotrophic and nitrifying bacteria. The interrelation between the different populations is also included in the model, in addition to the simultaneous release and consumption of the most relevant compounds, such as oxygen and carbon dioxide. The implementation of the model has been performed in MATLAB software; the calibration of model parameters was carried out using genetic algorithms. The ABACO model allows one to simulate the dynamics of different components in the system, and the relative proportions of microalgae, heterotrophic bacteria, and nitrifying bacteria. The percentage of each microbial population obtained with the model was confirmed by respirometric techniques. The proposed model is a powerful tool for the development of microalgae-related wastewater treatment processes, both to maximize the production of microalgal biomass and to optimize the wastewater treatment capacity.

Keywords: microalgae; bacteria; modelling; wastewater treatment; nutrients; photobioreactor

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1. Introduction

One of the most critical environmental challenges of the 21st century envisaged by humanity is the expansion of the population, which will result in increased urban wastewater production [1] and large amounts of animal slurry caused by the rise in meat production [2,3]. The world's growing population, along with (i) a rapid industrialization, (ii) intensive agriculture, (iii) the effluent discharged below an environmentally safe level, and (iv) the lack of technologies to reclaim used water could lead to a scarcity of clean water in many countries [4]. The current conventional wastewater treatment methods have become quickly outdated because they need a lot of land, intensive energy input, and a lot of money [5]. As an alternative strategy to beat these disadvantages, microalgae-based wastewater treatment is gaining an increased importance in the context of European bioeconomy, because of its potential to treat wastewater, recover nutrients of wastewater, and produce a large variety of valuable compounds with applications in agriculture, aquaculture, and food production, among others [6–8]. The use of microalgae for wastewater treatment involves the emergence of complex microalgae–bacteria consortia which vary as functions of environmental and operational conditions [9].

Microalgae are photosynthetic microorganisms that grow using inorganic carbon (CO_2) as a carbon source, and light as an energy source. During this growth, microalgae release oxygen which can use by heterotrophic bacteria to oxidate the organic matter present in influent wastewater. At the same time, heterotrophic bacteria supply CO_2 for photosynthetic activity, completing the cycle. Besides, the oxygen produced by microalgae can be used by nitrifying bacteria to oxidize the ammonium to nitrate (nitrification process), consuming CO_2 as a carbon source too [10–12]. Since microalgae–bacteria consortia in wastewater treatment was described in 1953 by [13], multiple microalgae–bacteria models have been described and validated [14–17]. These mathematical models offer great appeal to studying microalgae–bacteria interactions because they can provide useful tools for design and control purposes, in addition to model simulators, which can all lead to an increase the process efficiency [18].

In most of the proposed mathematical models, the part related to the activity of the bacteria is widely obtained and validated through the Activated sludge models (ASM) [19]. However, information on microalgae parameters in wastewater treatment systems is scarce. Therefore, in this work, a new microalgae–bacteria mathematical model named ABACO is proposed; the characteristic parameters of microalgae in it were obtained experimentally in previous works [20,21]. Thus, the main purpose of this study was to develop, calibrate, and validate the whole microalgae and bacteria model with experimental data from duplicate laboratory-scale photobioreactors using pig slurry as a nutrient source. The implementation of the microalgae–bacteria model has been performed in MATLAB software, and it allows one to simulate the dynamics of different components in the system and the relative proportions of microalgae and bacteria. Moreover, the model has a series of parameters whose exact values are unknown, being within a range. The calibration of these parameters has been carried out using genetic algorithms, which allow determining their values from minimizations of given cost functions. This calibration procedure provides a simple and fast adjustment method for the characterization of the model parameters, even allowing recalibration with different scenarios in a very easy way, such as for different strains and culture mediums. Moreover, notice that thanks to the proposed calibration process, it is possible to estimate the percentage of each species in the reactor, which is also a relevant contribution of the methodology proposed in this work.

2. Materials and Methods

2.1. Microorganisms and Culture Conditions

The microalgal strain used to inoculate the photobioreactors was *Scenedesmus almeriensis*. The stock culture of *Scenedesmus almeriensis* was maintained photo-autotrophically in spherical flasks (1 L capacity) using the Arnon medium [22]. The microalgal culture was continuously bubbled with CO_2-enriched air (1%), which allowed us to control the pH at 8.0. The air temperature in the chamber was controlled in order to obtain a desire temperature (22 °C). The culture temperature was set at 25 °C, controlled by regulating the air temperature in the chamber. The culture was artificially illuminated in a 12:12 h L/D cycle using four Philips PL-32W/840/4p white-light lamps, providing an irradiance of 750 $\mu E/m^2$ s on the spherical 1.0 L flask surface. Two laboratory-scale photobioreactors were inoculated using the culture stock. The average composition of the Arnon medium used is reported in Table 1.

2.2. Laboratory Photobioreactors

Two hand-made photobioreactors made with polymethylmethacrylate (0.08 m in diameter, 0.2 m in height and with a 1 L capacity) were used to perform the experiments (Figure 1). The reactors were inoculated with 20% of *Scenedesmus almeriensis* and diluted pig slurry (20%). The photobioreactors were operated in the laboratory but simulating outdoor conditions prevailing in outdoor raceway reactors. Firstly, the photobioreactors were operated in batch mode for 5 days to obtain a high biomass concentration. Afterwards, they were operated in continuous mode by removing 20% of the culture every day and replacing

it with fresh piggery wastewater. The dissolved oxygen in the culture was controlled below 200 % Sat to avoid negative effects because of excessive dissolved oxygen accumulation. For that, air was supplied on demand. Additionally, the pH was controlled at 8.0 using CO_2 injections. To simulate the outdoor solar cycle, the reactors were artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5). The maximum irradiance (PAR) inside the reactors without cells was 1000 μEm^{-2} s^{-1}, measured using an SQS-100 spherical quantum sensor (Walz GmbH, Effeltrich, Germany). The culture temperature was kept at 25 °C by controlling the temperature of the culture chamber in which the photobioreactors were located. The average composition of the piggery wastewater used is reported in Table 1.

Table 1. Average compositions of the culture medium and piggery wastewater used as the influent in the bioreactors. Concentrations expressed as mg × L^{-1}.

Parameters	Piggery Wastewater	Arnon Medium
pH	8.1 ± 0.3	7.5 ± 0.2
COD	2181.7 ± 100.9	16.0 ± 1.2
Nitrogen-Nitrate	56.5 ± 2.7	140.0 ± 4.5
Chloride	2060.2 ± 23.5	78.9 ± 2.1
Potassium	1800 ± 1.6	325.1 ± 6.3
Calcium	350.1 ± 0.2	364.9 ± 5.5
Magnesium	108.2 ± 14.1	12.2 ± 0.6
Phosphorus-Phosphate	119.2 ± 5.1	39.3 ± 3.1
Nitrogen-Ammonium	1495.6 ± 17.7	0.0 ± 0.1
Iron	4.8 ± 0.01	5.0 ± 0.3
Copper	1.1 ± 0.1	0.02 ± 0.00
Manganese	2.6 ± 0.0	0.5 ± 0.02
Zinc	20.1 ± 0.2	0.06 ± 0.01
Boron	5.3 ± 0.1	0.4 ± 0.0

Figure 1. Laboratory photobioreactors used for performing the experiments.

2.3. Biomass Concentration and Analytical Methods

The biomass concentration (Cb) was measured by dry weight. For that, aliquots (100 mL) of each photobioreactors the culture were filtered through the Macherey–Nagel MN 85/90 glass fiber filters. Then, the filters were dried in an oven at 80 °C for 24 h. Standard official methods were used to analyze the composition of the piggery wastewater and the supernatants from microalgae–bacteria cultures. The phosphate was measured by visible spectrophotometry through the phospho-vanado-molybdate complex (phosphate standard for IC: 38364). The nitrate was quantified by measuring optical density at 220 nm and 275 nm (nitrate Standard for IC: 74246). The ammonium was measured according to the Nessler method (ammonium standard for IC: 59755). The chemical oxygen demand (COD) was determined by spectrophotometric measurement using Hach–Lange kits (LCl-400).

2.4. Model Calibration and Validation

MATLAB Software was used to carry out the model calibration process using genetic algorithms through the Genetic Algorithm Optimization Toolbox (GAOT), based on [23]. Additionally, the model validation with experimental data was performed using MATLAB Software.

2.5. Respirometry: Measurements of the Photosynthesis and Respiration Rates

In order to validate experimentally the percentage of each microbial population proposed in the biological model, respirometric measurements were performed when at steady state. The percentages of microalgae and bacteria in the culture were estimated as functions of the microalgae net photosynthesis rate, the heterotrophic respiration rate, and the nitrifying respiration rate, respectively. These measurements were performed with handmade photo-respirometer equipment. This equipment is described in detail in [11]. The method allows one to determine the photosynthesis and respiration rates through the variations in dissolved oxygen concentrations in microalgae–bacteria cultures, as described in detail in [11].

For evaluating the microalgae net photosynthesis rate of each microalgae–bacteria culture, a sample of the culture was exposed to four light–dark cycles of four minutes each to measure and register the variation in dissolved oxygen. During the light phases, the photosynthetic microalgae generated dissolved oxygen, and this dissolved oxygen was consumed by the endogenous respiration during the dark periods. Thus, the microalgae net photosynthesis rate was calculated as the difference between the slope of the oxygen production during the light period and the slope of the oxygen consumption during the dark period. Subsequently, another sample of the culture was used to determine the heterotrophic respiration rate. For this purpose, 0.8 mL of sodium acetate (30 g/L) was added to the sample and it was exposed to four light–dark cycles of 4 min each. The respiration rate of the heterotrophic bacteria was calculated as the slope of the oxygen consumption with sodium acetate minus the slope of the oxygen consumption during the dark period in the endogenous culture. By following the same method, another sample was used to measure the nitrifying respiration rate of the culture. However, the nitrifying activity was determined using 0.8 mL of ammonium chloride (3 g/L) instead of sodium acetate. The respiration rate of the nitrifying bacteria was calculated as the slope of the oxygen consumption with ammonium chloride minus the slope of the oxygen consumption during the dark period in the endogenous culture [11].

Finally, in order to correct the influence of oxygen desorption on the photo-respirometric measurements, the oxygen mass transfer coefficient (K_La) was calculated. This coefficient was measured in the system according to Equation (1).

$$\frac{dX_{O_2}}{dt} = K_La\left(X^*_{O_2} - X_{O_2}\right) \quad (1)$$

where $\frac{dX_{O_2}}{dt}$ is the oxygen accumulation expressed as the derivate of X_{O_2} (mg/L) concentration over time, K_La is the global oxygen mass transfer coefficient (h^{-1}), and $X^*_{O_2}$ is the oxygen saturation concentration in the liquid. Further detailed descriptions of the equipment, the standard protocol, and the metabolic rate calculations are in [11].

3. Results

This section, divided into four parts, presents the results obtained for the joint model of microalgae biomass production combined with pig slurry treatment. The first part provides a description of the mass balances of the model related to the process. The second part shows the mathematical background relative to the growth rate of the species involved. The third part shows the calibration process and the results. Finally, in the fourth part, the validation results obtained for the model are presented.

3.1. Model Concept

In microalgae-based wastewater treatment, different types of microbial consortia appear as a function of environmental and operational conditions. Figure 2 shows the biological process taking place in the reactor when using wastewater (i.e., diluted pig slurry) as the culture medium. Under illumination, microalgae (X_{ALG}) fix carbon dioxide (CO_2) and release oxygen (O_2) while assimilating nutrients, such as ammonium (NH_4), nitrate (NO_3), and phosphate (PO_4). The O_2 produced by the photosynthesis is essential for the degradation of the biodegradable soluble organic matter (BSOM) by heterotrophic bacteria (X_{HET}), BSOM being a fraction of total organic matter (COD) contained in wastewater. In turn, during bacterial oxidation of soluble organic matter, CO_2 is produced, it being available for photosynthesis and the nitrification process. During nitrification, nitrifying bacteria (X_{NIT}) transform NH_4 already contained at the inlet culture medium into NO_3, while also consuming O_2 produced through photosynthesis.

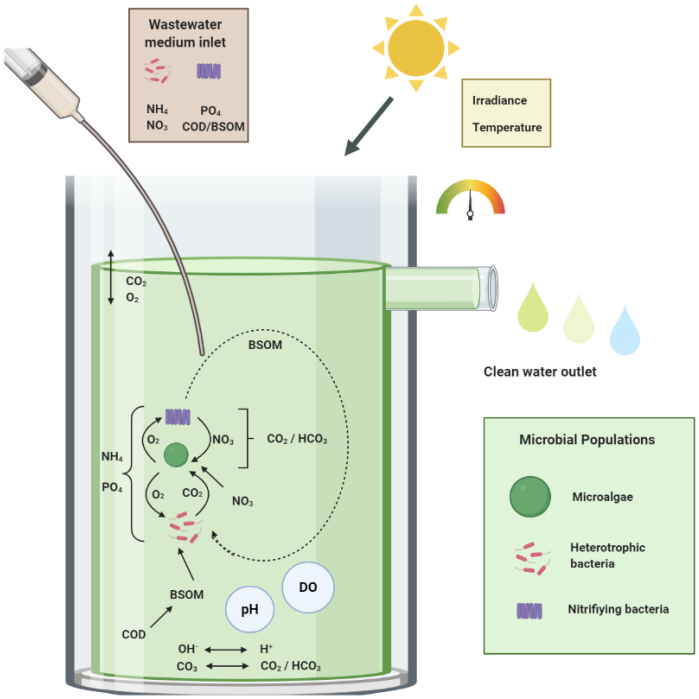

Figure 2. Biological process for microalgae biomass production coupled with wastewater treatment.

The developed model includes the mass balances of major compounds involved into the biological process, in addition to the growth rate of the different species involved (microalgae and bacteria) as a function of culture conditions and nutrients availability. Starting from known initial conditions and variables already measured in the reactor it is possible to simulate the evolution of the system over time, thus the variation of both compounds and microorganisms. Figure 3 shows the most relevant inputs and outputs of the model, and the initial conditions required. The inputs for the model are the variables commonly measured in photobioreactors such as irradiance, dissolved oxygen, pH and temperature. The model outputs are the concentrations of major microorganisms already considered such as microalgae, heterotrophic bacteria and nitrifying bacteria; in addition to the concentration of major components and nutrients involved into the biological process such as oxygen, carbon dioxide, total inorganic carbon, ammonium, nitrate, phosphate, and BSOM. For the right estimation of the evolution of the system it is necessary to establish values for the initial conditions, which correspond to the initial concentrations of the nutrients and the total biomass, the initial percentages of species in the photobioreactor and the calibration parameters.

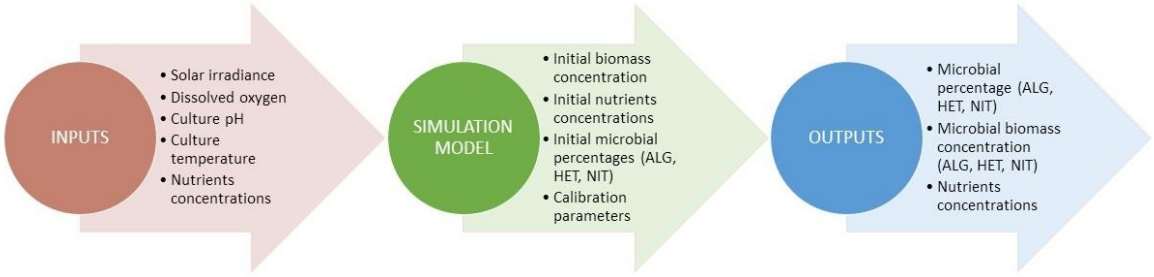

Figure 3. Model input-output diagram.

3.2. ABACO Model

The biological model has been applied to the treatment of diluted pig slurry as a relevant type of wastewater. The model has been developed considering the main microalgal and bacterial processes that simultaneously occur in the microalgae-based wastewater treatment. An initial dynamic model considering the influence of main environmental variables (irradiance, temperature, pH and dissolved oxygen) on microalgae and bacteria growth was developed by [20]. The model equations were inspired in the BIOALGAE model [17], and it was already validated, the model allowing one to simulate the effect of environmental conditions on the photosynthesis and respiration rate of microalgae–bacteria consortia. Distinctions were performed among activity of microalgae, heterotrophic and nitrifying bacteria [11]. The BIOALGAE model has been improved in this work by considering the influence of nutrients concentration (CO_2, N-NH_4^+, N–NO_3^-, P–PO_4^{2-} and BSOM) in the microalgae and bacteria growth and coefficient yields, resulting in the new ABACO model. The parameters of the model related with the microalgae activity were determined experimentally [21], while the bacterial parameters were obtained from the Activated Sludge Models (ASM) [19,24].

3.2.1. Microalgae Biomass

The microalgal cells are present in the photobioreactors, it not being feed to the system with the influent wastewater. Part of the microalgae biomass is removed every day with the effluent as a function of imposed dilution rate (inverse of hydraulic retention time). Microalgae biomass concentration increases due to autotrophic growth of microalgae, using light as energy source and of CO_2 as carbon source, whereas it reduces by endogenous respiration and decay of microalgae. These last phenomena represent the autoxidation of microalgae, where they metabolize their own cellular material. The global balance to estimate the microalgae biomass concentration is given by Equation (2).

$$V \cdot X_{ALG} \cdot \mu_{ALG} = Q_h \cdot X_{ALG} + V \cdot \frac{dX_{ALG}}{dt} \quad (2)$$

where V [m^3] is the volume in the reactor, X_{ALG} [g m^{-3}] is the microalgae biomass concentration, μ_{ALG} [day^{-1}] is the microalgae specific growth rate and Q_h [m^3 s^{-1}] represents the harvesting flow rate.

The specific growth rate μ_{ALG} is mainly a function of light availability inside the reactor, summarized by the average irradiance inside the culture I_{av} [25], and modified by the influence of different variables such as temperature ($\overline{\mu_{ALG}}(T)$), pH ($\overline{\mu_{ALG}}(pH)$), dissolved oxygen ($\overline{\mu_{ALG}}(DO_2)$) and CO_2 ($\overline{\mu_{ALG}}(CO_2)$). In addition the influence of nutrients availability such as ammonium nitrogen ($\overline{\mu_{ALG}}([N-NH_4])$), phosphate phosphorus ($\overline{\mu_{ALG}}([P-PO_4])$) and nitrate nitrogen ($\overline{\mu_{ALG}}([N-NO_3])$), and the microalgae maintenance (m_{ALG}), is considered as shown in Equation (3).

$$\mu_{ALG} = (\mu_{ALG}(I_{av}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO_2) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}(N) \cdot \overline{\mu_{ALG}}([P-PO_4])) - m_{ALG} \quad (3)$$

Microalgae can growth using both ammonium and nitrate as a nitrogen source. Then, there is a process rate for the growth of microalgae using ammonium and another one when using nitrate, thus Equation (3) becomes as Equations (4) and (5) for considering this phenomenon. Notice that Equation (5) considering the consumption of nitrate is only used when there is not ammonium in the system.

$$\mu_{ALG} = (\mu_{ALG}(I_{av}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO_2) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}([N-NH_4]) \cdot \overline{\mu_{ALG}}([P-PO_4])) - m_{ALG} \quad (4)$$

$$\mu_{ALG} = (\mu_{ALG}(I_{av}) \cdot \overline{\mu_{ALG}}(T) \cdot \overline{\mu_{ALG}}(pH) \cdot \overline{\mu_{ALG}}(DO_2) \cdot \overline{\mu_{ALG}}(CO_2) \cdot \overline{\mu_{ALG}}([N-NO_3]) \cdot \overline{\mu_{ALG}}([P-PO_4])) - m_{ALG} \quad (5)$$

As observed from Equation (3), during the microalgae growth, it is assumed that two main process occur: the microalgal growth and the microalgal maintenance. The microalgae growth rate is modeled as the product of a maximum growth rate ($\mu_{ALG,max}$) as expressed in Equation (6), algae biomass concentration (X_{ALG}) as shown in Equation (7), and switching functions for environmental parameters (irradiance, temperature, pH and dissolved oxygen), carbon dioxide, nitrogen and phosphorous (Equation (9)–(15)). The rate of the microalgae maintenance (m_{ALG}) considers the endogenous respiration of the microalgae and the microalgae decay (Equation (8)).

Taking the model described by Molina et al. in [25], the light limitation growth model can be expressed as follows:

$$\mu_{ALG}(I_{av}) = \frac{\mu_{ALG,max} \cdot {I_{av}}^n}{{I_k}^n + {I_{av}}^n} \quad (6)$$

where $\mu_{ALG,max}$ [day^{-1}] is the maximum microalgae growth rate, I_{av} [$\mu E\ m^{-2}\ s^{-1}$] is the average irradiance inside de culture it summarizing the light availability inside the reactor, I_k [$\mu E\ m^{-2}\ s^{-1}$] is the irradiance constant (equivalent to irradiance required to achieve half of the maximal growth rate) and n is a form parameter. The average irradiance is expressed as follows:

$$I_{av} = \frac{I_0}{K_a \cdot X_{ALG} \cdot h}\left(1 - e^{-K_a \cdot X_{ALG} \cdot h}\right) \quad (7)$$

where I_0 [$\mu E\ m^{-2}\ s^{-1}$] is the irradiance on the reactor surface, K_a [$m^2\ g^{-1}$] is the biomass extinction coefficient and h [m] is the culture depth in the reactor.

The endogenous respiration term can be expressed as follows:

$$m_{ALG} = m_{min,alg} + \frac{m_{max,alg} \cdot {I_{av}}^{n_{resp}}}{{I_{k,resp}}^{n_{resp}} + {I_{av}}^{n_{resp}}} \quad (8)$$

where $m_{min,alg}$ and $m_{max,alg}$ [day^{-1}] represent the minimum and maximum respiration rates, $I_{k,resp}$ [$\mu E\ m^{-2}\ s^{-1}$] is the irradiance required to stop photosynthesis and start respiration process, and n_{resp} is the form parameter for respiration.

The influence of temperature, pH, dissolved oxygen and nutrients concentration into the microalgae growth rate are included as normalized values, then it varying between 0 and 1. Therefore, when the culture conditions are optimal these terms are equal to 1 and the specific growth rate is only a function of light availability, achieving the maximal value at irradiances upper than saturation irradiance. However, if culture conditions are not optimal the respective normalized values are lower than 1, directly reducing the microalgae growth rate whatever the irradiance. The temperature index $\overline{\mu_{ALG}}(T)$, expressed by Bernard et al. in [26], represents the influence of temperature on microalgae growth. The temperature index can be expressed as follows:

$$\overline{\mu_{ALG}}(T) = \frac{(T - T_{max,\ ALG})(T - T_{min,\ ALG})^2}{(T_{opt,\ ALG} - T_{min,\ ALG})(((T_{opt,\ ALG} - T_{min,\ ALG})(T - T_{opt,\ ALG})) - ((T_{opt,\ ALG} - T_{max,\ ALG})(T_{opt,\ ALG} + T_{min,\ ALG} - 2 \cdot T)))} \quad (9)$$

where T [°C] is the culture temperature, whereas T_{max} [°C], T_{min} [°C] and T_{opt} [°C] are the respective maximal, minimal and optimal temperature for the microalgae strain. As for the

temperature term, the pH term $\overline{\mu_{ALG}}(pH)$ represents the influence of pH on microalgae growth. It can be expressed by a cardinal formula as follows:

$$\overline{\mu_{ALG}}(pH) = \frac{(pH-pH_{max,\ ALG})(pH-pH_{min,\ ALG})^2}{(pH_{opt,\ ALG}-pH_{min,\ ALG})(((pH_{opt,\ ALG}-pH_{min,\ ALG})(pH-pH_{opt,\ ALG}))-((pH_{opt,\ ALG}-pH_{max,\ ALG})(pH_{opt,\ ALG}+pH_{min,\ ALG}-2\cdot pH)))} \quad (10)$$

where pH is the culture pH, whereas pH_{max}, pH_{min} and pH_{opt} the respective maximal, minimal and optimal pH for the microalgae strain.

The dissolved oxygen term $\overline{\mu_{ALG}}(DO_2)$ depends on a maximum value, determined by the strain, which represents the dissolved oxygen concentration that can be accumulated in the culture without being detrimental to microalgae growth. It can be expressed as the following equation:

$$\overline{\mu_{ALG}}(DO_2) = 1 - \left(\frac{DO_2}{DO_{2,max}}\right)^m \quad (11)$$

where DO_2 [%] is the culture dissolved oxygen, $DO_{2,max}$ [%] is the maximum amount of dissolved oxygen for the microalgae strain, m is a form parameter.

The concentration of nutrients in the culture medium (wastewater) can be also a limiting factor for microalgae growth. The influence of carbon dioxide $\overline{\mu_{ALG}}(CO_2)$ is described as follows:

$$\overline{\mu_{ALG}}(CO_2) = \frac{X_{CO_2} + X_{HCO_3}}{K_{S,C,ALG} + X_{CO_2} + X_{HCO_3} + \frac{X_{CO_2}{}^{n_{C,ALG}}}{K_{I,C,ALG}}} \quad (12)$$

where X_{CO_2} [g m^{-3}] is the carbon dioxide concentration, X_{HCO_3} [g m^{-3}] is the bicarbonate concentration, $K_{S,C,ALG}$ [g m^{-3}] is the microalgae half-saturation constant for carbon, $K_{I,C,ALG}$ [g m^{-3}] is the microalgae inhibition constant for carbon, and $n_{C,ALG}$ is the microalgae form parameter for carbon. The influence of ammonium nitrogen $\overline{\mu_{ALG}}([N-NH_4])$ is represented by the following equation:

$$\overline{\mu_{ALG}}([N-NH_4]) = \frac{X_{NH_4}}{X_{NH_4} + K_{S,NH_4,ALG} + \frac{X_{NH_4}{}^{n_{NH_4,ALG}}}{K_{I,NH_4,ALG}}} \quad (13)$$

where X_{NH_4} [g m^{-3}] is the ammonium nitrogen concentration, $K_{S,NH_4,ALG}$ [g m^{-3}] is the microalgae half-saturation constant for ammonium, $K_{I,NH_4,ALG}$ [g m^{-3}] is the microalgae inhibition constant for ammonium, and $n_{NH_4,ALG}$ is the microalgae form parameter for ammonium. The influence of nitrate nitrogen $\overline{\mu_{ALG}}([N-NO_3])$.is represented by the following equation:

$$\overline{\mu_{ALG}}([N-NO_3]) = \frac{X_{NO_3}}{X_{NO_3} + K_{S,NO_3,ALG} + \frac{X_{NO_3}{}^{n_{NO_3,ALG}}}{K_{I,NO_3,ALG}}} \quad (14)$$

where X_{NO_3} [g m^{-3}] is the nitrate nitrogen concentration, $K_{S,NO_3,ALG}$ [g m^{-3}] is the microalgae half-saturation constant for nitrate, $K_{I,NH_4,ALG}$ [g m^{-3}] is the microalgae inhibition constant for nitrate, and $n_{NO_3,ALG}$ is the microalgae form parameter for nitrate. The influence of phosphate phosphorus $\overline{\mu_{ALG}}([P-PO_4])$ is represented by the following equation:

$$\overline{\mu_{ALG}}([P-PO_4]) = \frac{X_{PO_4}}{X_{PO_4} + K_{S,PO_4,ALG}} \quad (15)$$

where X_{PO_4} [g m^{-3}] is the phosphate phosphorus concentration and $K_{S,PO_4,ALG}$ [g m^{-3}] is the microalgae half-saturation constant for phosphate.

3.2.2. Heterotrophic Bacteria

Heterotrophic bacteria are already present in the influent wastewater, then they are supplied to the system with the inlet wastewater also it being removed with the harvested flow rate as a function of imposed dilution rate. Heterotrophic bacteria grow using the organic matter as source of energy and carbon. These bacteria are aerobic then consuming O_2 produced during the photosynthesis process. The endogenous respiration and the decay are responsible for the heterotrophic biomass lost. The global balance to estimate the heterotrophic bacteria concentration is given by Equation (16).

$$Q_d \cdot X_{HET,in} + V \cdot X_{HET,out} \cdot \mu_{HET} = Q_h \cdot X_{HET,out} + V \cdot \frac{dX_{HET,out}}{dt} \quad (16)$$

where Q_d [$m^3\ s^{-1}$] is the dilution flow rate, $X_{HET,in}$ [$g\ m^{-3}$] is the heterotrophic bacteria inlet concentration, μ_{HET} [day^{-1}] is the specific growth rate of heterotrophic bacteria and $X_{HET,out}$ [$g\ m^{-3}$] is the heterotrophic bacteria concentration in the reactor.

As with microalgal processes, heterotrophic processes include both the heterotrophic growth and the heterotrophic maintenance. The heterotrophic specific growth rate μ_{HET} is modeled as the product of maximum growth rate ($\mu_{HET,max}$) and switching functions for environmental parameters such as temperature ($\overline{\mu_{HET}}(T)$), pH ($\overline{\mu_{HET}}(pH)$) and dissolved oxygen ($\overline{\mu_{HET}}(DO_2)$); in addition to biodegradable soluble organic matter ($\overline{\mu_{HET}}(BSOM)$), ammonium nitrogen ($\overline{\mu_{HET}}([N-NH_4])$) and phosphate phosphorous ($\overline{\mu_{HET}}([P-PO_4])$) (Equation (17)). The rate of the heterotrophic maintenance (m_{HET}) considers the endogenous respiration of the heterotrophic bacteria and the heterotrophic decay. The specific growth rate for heterotrophic bacteria is expressed from the following equation:

$$\mu_{HET} = \mu_{HET,max} \cdot (\overline{\mu_{HET}}(T) \cdot \overline{\mu_{HET}}(pH) \cdot \overline{\mu_{HET}}(DO_2) \cdot \overline{\mu_{HET}}([N-NH_4]) \cdot \overline{\mu_{HET}}([P-PO_4]) \cdot \overline{\mu_{HET}}(BSOM)) - m_{HET} \quad (17)$$

where $\mu_{HET,max}$ [day^{-1}] is the maximum specific growth rate for heterotrophic bacteria, whereas m_{HET} [day^{-1}] represent the endogenous respiration of the heterotrophic bacteria and the heterotrophic decay.

The temperature and pH terms ($\overline{\mu_{HET}}(T)$ and $\overline{\mu_{HET}}(pH)$) are also based on the cardinal model, so they are identical to those previously expressed for microalgae. These terms depend on the maximum ($T_{max,\ HET}$ and $pH_{max,\ HET}$), minimum ($T_{min,\ HET}$ and $pH_{min,\ HET}$) and optimal ($T_{opt,\ HET}$ and $pH_{opt,\ HET}$) values of temperature and pH for heterotrophic bacteria, such as expressed in Equations (18) and (19), respectively.

$$\overline{\mu_{HET}}(T) = \frac{(T-T_{max,\ HET})(T-T_{min,\ HET})^2}{(T_{opt,\ HET}-T_{min,\ HET})(((T_{opt,\ HET}-T_{min,\ HET})(T-T_{opt,\ HET}))-((T_{opt,\ HET}-T_{max,\ HET})(T_{opt,\ HET}+T_{min,\ HET}-2\cdot T)))} \quad (18)$$

$$\overline{\mu_{HET}}(pH) = \frac{(pH-pH_{max,\ HET})(pH-pH_{min,\ HET})^2}{(pH_{opt,\ HET}-pH_{min,\ HET})(((pH_{opt,\ HET}-pH_{min,\ HET})(pH-pH_{opt,\ HET}))-((pH_{opt,\ HET}-pH_{max,\ HET})(pH_{opt,\ HET}+pH_{min,\ HET}-2\cdot pH)))} \quad (19)$$

The influence of dissolved oxygen $\overline{\mu_{HET}}(DO_2)$ is expressed as follows:

$$\overline{\mu_{HET}}(DO_2) = \frac{DO_2}{DO_2 + K_{S,DO_2,\ HET}} \quad (20)$$

where $K_{S,DO_2,\ HET}$ [$g\ m^{-3}$] is the heterotrophic bacteria half-saturation constant for dissolved oxygen. The influence of ammonium nitrogen $\overline{\mu_{HET}}([N-NH_4])$ is represented by the following equation:

$$\overline{\mu_{HET}}([N-NH_4]) = \frac{X_{NH_4}}{X_{NH_4} + K_{S,NH_4,HET}} \quad (21)$$

where $K_{S,NH_4,HET}$ [g m^{-3}] is the heterotrophic bacteria half-saturation constant for ammonium. The influence of phosphate phosphorus $\overline{\mu_{HET}}([P-PO_4])$ is represented by the following equation:

$$\overline{\mu_{HET}}([P-PO_4]) = \frac{X_{PO_4}}{X_{PO_4} + K_{S,PO_4,HET}} \quad (22)$$

where $K_{S,PO_4,HET}$ [g m^{-3}] is the heterotrophic bacteria half-saturation constant for phosphate. The influence of biodegradable soluble organic matter $\overline{\mu_{HET}}(BSOM)$ is represented by the following equation:

$$\overline{\mu_{HET}}(BSOM) = \frac{X_{BSOM}}{X_{BSOM} + K_{S,BSOM,HET}} \quad (23)$$

where X_{BSOM} [g m^{-3}] is the concentration of biodegradable soluble organic matter (BSOM) in the reactor and $K_{S,BSOM,HET}$ [g m^{-3}] is the heterotrophic bacteria half-saturation constant for BSOM.

3.2.3. Nitrifying Bacteria

Nitrifiying bacteria can be also supplied to the reactor with the wastewater supplied to the reactor, it being also removed during harvesting. Nitrifying bacteria perform the nitrification process, thus oxidizing ammonium to nitrate. These microorganisms are aerobic, then requiring oxygen, also using CO_2 as a carbon source. The concentration of nitrifying bacteria increases due to growth but also decrease by endogenous respiration and decay. The global balance to estimate the concentration of nitrifying bacteria is given by Equation (24).

$$Q_d \cdot X_{NIT,in} + V \cdot X_{NIT,out} \cdot \mu_{NIT} = Q_h \cdot X_{NIT,out} + V \cdot \frac{dX_{NIT,out}}{dt} \quad (24)$$

where $X_{NIT,in}$ [g m^{-3}] is the nitrifying bacteria inlet concentration, μ_{NIT} [day^{-1}] is the nitrifying bacteria specific growth rate and $X_{NIT,out}$ [g m^{-3}] is the nitrifying bacteria concentration in the reactor.

The processes related with nitrifying bacteria include both autotrophic growth and maintenance. The rate of the autotrophic growth is modeled as the product of maximum growth rate ($\mu_{NIT,max}$) and switching functions for environmental parameters, such as temperature ($\overline{\mu_{HET}}(T)$), pH ($\overline{\mu_{HET}}(pH)$) and dissolved oxygen ($\overline{\mu_{HET}}(DO_2)$); in addition to ammonium nitrogen ($\overline{\mu_{HET}}([N-NH_4])$) and phosphate phosphorous ($\overline{\mu_{HET}}([P-PO_4])$) (Equation (25)). The rate of maintenance (m_{NIT}) considers the endogenous respiration of the nitrifying bacteria and nitrifying decay. The following equation represents the nitrifying bacteria specific growth rate:

$$\mu_{NIT} = \mu_{NIT,max} \cdot (\overline{\mu_{NIT}}(T) \cdot \overline{\mu_{NIT}}(pH) \cdot \overline{\mu_{NIT}}(DO_2) \cdot \overline{\mu_{NIT}}(CO_2) \cdot \overline{\mu_{NIT}}([N-NH_4]) \cdot \overline{\mu_{NIT}}([P-PO_4])) - m_{NIT} \quad (25)$$

where $\mu_{NIT,max}$ [day^{-1}] is the maximum specific growth rate for nitrifying bacteria and m_{NIT} [day^{-1}] is the endogenous respiration of the nitrifying bacteria and the nitrifying maintenance.

As for the heterotrophic bacteria, the temperature and pH terms ($\overline{\mu_{NIT}}(T)$ and $\overline{\mu_{NIT}}(pH)$) are expressed the same form. These terms depend on the maximum ($T_{max,\ NIT}$ and $pH_{max,\ NIT}$), minimum ($T_{min,\ NIT}$ and $pH_{min,\ NIT}$) and optimal ($T_{opt,\ NIT}$ and $pH_{opt,\ NIT}$) values of temperature and pH for nitrifying bacteria, such as expressed in Equations (26) and (27), respectively.

$$\overline{\mu_{NIT}}(T) = \frac{(T-T_{max,\ NIT})(T-T_{min,\ NIT})^2}{(T_{opt,\ NIT}-T_{min,\ NIT})(((T_{opt,\ NIT}-T_{min,\ NIT})(T-T_{opt,\ NIT}))-((T_{opt,\ NIT}-T_{max,\ NIT})(T_{opt,\ NIT}+T_{min,\ NIT}-2\cdot T)))} \quad (26)$$

$$\overline{\mu_{NIT}}(pH) = \frac{(pH-pH_{max,\ NIT})(pH-pH_{min,\ NIT})^2}{(pH_{opt,\ NIT}-pH_{min,\ NIT})(((pH_{opt,\ NIT}-pH_{min,\ NIT})(pH-pH_{opt,\ NIT}))-((pH_{opt,\ NIT}-pH_{max,\ NIT})(pH_{opt,\ NIT}+pH_{min,\ NIT}-2\cdot pH)))} \quad (27)$$

The influence of dissolved oxygen $\overline{\mu_{NIT}}(DO_2)$ is represented by the following equation:

$$\overline{\mu_{NIT}}(DO_2) = \frac{DO_2}{(DO_2 + K_{S,DO_2,\,NIT}) \cdot \left(1 + \frac{DO_2}{K_{I,DO_2,\,NIT}}\right)} \quad (28)$$

where $K_{S,DO_2,\,NIT}$ [g m^{-3}] is the nitrifying bacteria half-saturation constant for dissolved oxygen and $K_{I,DO_2,\,NIT}$ [g m^{-3}] is the nitrifying bacteria inhibition constant for dissolved oxygen.

The influence of carbon dioxide $\overline{\mu_{NIT}}(CO_2)$ is described as follows:

$$\overline{\mu_{NIT}}(CO_2) = \frac{X_{CO_2} + X_{HCO_3}}{K_{S,C,NIT} + X_{CO_2} + X_{HCO_3}} \quad (29)$$

where $K_{S,C,NIT}$ [g m^{-3}] is the nitrifying bacteria half-saturation constant for carbon.

The influence of ammonium nitrogen $\overline{\mu_{NIT}}([N - NH_4])$ is represented by the following equation:

$$\overline{\mu_{NIT}}([N - NH_4]) = \frac{X_{NH_4}}{X_{NH_4} + K_{S,NH_4,NIT}} \quad (30)$$

where $K_{S,NH_4,NIT}$ [g m^{-3}] is the nitrifying bacteria half-saturation constant for ammonium.

The influence of phosphate phosphorus $\overline{\mu_{NIT}}([P - PO_4])$ is represented by the following equation:

$$\overline{\mu_{NIT}}([P - PO_4]) = \frac{X_{PO_4}}{X_{PO_4} + K_{S,PO_4,NIT}} \quad (31)$$

where $K_{S,PO_4,NIT}$ [g m^{-3}] is the nitrifying bacteria half-saturation constant for phosphate.

3.2.4. Dissolved Oxygen

During the photosynthesis, microalgae release O_2 and its consumed by aerobic bacteria respiration and microalgae respiration. The dissolved oxygen is measured and represents a model input.

3.2.5. Dissolved Carbon Dioxide

Carbon dioxide is generated during the aerobic respiration (bacteria and microalgae), and is consumed by nitrifying bacteria as carbon source and by microalgae for the photosynthetic process. The concentration of CO_2 is determined by the total inorganic carbon concentration and the presence of bicarbonate buffer. Thus, it is assumed that CO_2 is always in chemical equilibrium with bicarbonate (HCO_3) and carbonate (CO_3). The following equilibrium constant between carbon dioxide, carbonate and bicarbonate is defined:

$$K_1 = \frac{[X_{HCO_3}][H^+]}{[X_{CO_2}]} = 10^{-6.381} \quad (32)$$

$$K_2 = \frac{[X_{CO_3}][H^+]}{[X_{HCO_3}]} = 10^{-10.377} \quad (33)$$

where X_{HCO_3} is the bicarbonate concentration, X_{CO_2} is the carbon dioxide concentration, X_{CO_3} is the carbonate concentration, and H^+ is the concentration of hydrogen ions, which can be obtained from the pH by means of the following equation:

$$H^+ = 10^{-pH} \quad (34)$$

Assuming a total inorganic carbon concentration X_{C_T} of 0.1 [g L^{-1}], the concentration of bicarbonate and carbon dioxide can be obtained from the following equations:

$$X_{HCO_3} = \frac{(H^+ \cdot X_{C_T})}{(K_2 + H^+ + H^{+2})} \tag{35}$$

$$X_{CO_2} = \frac{(X_{HCO_3} \cdot H^+)}{K_1} \tag{36}$$

3.2.6. Chemical Oxygen Demand

Chemical oxygen demand (COD) of the inlet wastewater is mainly related with the organic matter already present on it. The COD includes the total organic matter, both the biodegradable and the no biodegradable organic matter. Additionally, it is produced during the microbial decay, and the biodegradable fraction is consumed by heterotrophic bacteria.

3.2.7. Biodegradable Organic Soluble Matter.

The biodegradable organic matter dissolved is the fraction of the organic matter which is available for biodegradation by heterotrophic bacteria X_{HET}. It is introduced in the influent wastewater and is produced by microbial decay. X_{BSOM} is removed by heterotrophic consumption and during the dilution process, such as expressed in the following equations:

$$\begin{gathered} Q_d \cdot X_{BSOM,in} + V \cdot \\ \cdot \left(X_{ALG} \cdot \mu_{alg} \cdot Y_{gen}\left[\frac{BSOM}{alg}\right] + X_{het,out} \cdot \mu_{het} \cdot Y_{gen}\left[\frac{BSOM}{het}\right] + X_{nit,out} \cdot \mu_{nit} \cdot Y_{gen}\left[\frac{BSOM}{nit}\right] \right) = \\ = Q_h \cdot X_{BSOM,out} + V \cdot \\ \cdot \left(X_{het,out} \cdot \mu_{het} \cdot Y_{con}\left[\frac{PO_4}{het}\right] \right) + V \cdot \frac{dX_{BSOM,out}}{dt} \end{gathered} \tag{37}$$

where $X_{BSOM,in}$ [g m^{-3}] is the inlet BSOM concentration, $X_{BSOM,out}$ [g m^{-3}] the BSOM concentration in the reactor, $Y_{gen}\left[\frac{BSOM}{alg}\right]$ [-] represents the BSOM generation rate from microalgae, $Y_{gen}\left[\frac{BSOM}{het}\right]$ [-] is the BSOM generation rate from heterotrophic bacteria, $Y_{gen}\left[\frac{BSOM}{nit}\right]$ [-] is the BSOM generation rate from nitrifying bacteria, and $Y_{con}\left[\frac{PO_4}{het}\right]$ [-] shows the BSOM consumption rate from heterotrophic bacteria.

3.2.8. Ammonium Nitrogen

Different forms of nitrogen can be found in wastewater. Ammonium nitrogen is introduced in the system though the influent wastewater, it being consumed by microalgae, heterotrophic bacteria, and nitrifying bacteria. Besides, ammonium nitrogen is generated by microbial decay. The ammonium nitrogen concentration is modelled by the following equation:

$$\begin{gathered} Q_d \cdot X_{NH_4,in} = Q_h \cdot X_{NH_4,out} + V \cdot \\ \cdot \left(X_{ALG} \cdot \mu_{alg} \cdot Y_{con}\left[\frac{NH_4}{alg}\right] + X_{het,out} \cdot \mu_{het} \cdot Y_{con}\left[\frac{NH_4}{het}\right] + X_{nit,out} \cdot \mu_{nit} \cdot Y_{con}\left[\frac{NH_4}{nit}\right] \right) + V \cdot \frac{dX_{NH_4,out}}{dt} \end{gathered} \tag{38}$$

where $X_{NH_4,in}$ [g m^{-3}] is the ammonium nitrogen inlet concentration, $X_{NH_4,out}$ [g m^{-3}] represents the outlet ammonium nitrogen concentration, $Y_{con}\left[\frac{NH_4}{alg}\right]$ [-] shows the ammonium consumption rate from microalgae, $Y_{con}\left[\frac{NH_4}{het}\right]$ [-] is the ammonium consumption rate from heterotrophic bacteria and $Y_{con}\left[\frac{NH_4}{nit}\right]$ [-] shows the ammonium consumption rate from nitrifying bacteria.

3.2.9. Nitrate Nitrogen

Nitrogen in form of nitrate enters in the system through the influent wastewater and it is produced during nitrification by nitrifying bacteria. It is consumed by microalgae

cells when ammonium is not presented or have been consumed. The nitrate nitrogen concentration is modelled by the following equation:

$$Q_d \cdot X_{NO_3,in} + V \cdot X_{NO_3,out} \cdot \mu_{nit} \cdot Y_{gen}\left[\frac{NO_3}{nit}\right] = Q_h \cdot X_{NO_3,out} + V \cdot \cdot \left(X_{ALG} \cdot \mu_{alg} \cdot Y_{con}\left[\frac{NO_3}{alg}\right]\right) + V \cdot \frac{dX_{NO_3,out}}{dt} \quad (39)$$

where $X_{NO_3,in}$ [g m^{-3}] is the inlet nitrate nitrogen concentration, $X_{NO_3,out}$ [g m^{-3}] represents the outlet nitrate nitrogen concentration, $Y_{gen}\left[\frac{NO_3}{nit}\right]$ [-] is the nitrate generation rate from nitrifying bacteria and $Y_{con}\left[\frac{NO_3}{alg}\right]$ [-] shows the nitrate consumption rate from microalgae.

3.2.10. Phosphate Phosphorous

Phosphorous is contained into the wastewater both as organic and inorganic. Organic phosphorous is transformed into inorganic during degradation of biodegradable organic matter then the phosphate phosphorous concentration corresponding to total phosphorous available. Phosphate phosphorous is introduced in the system with influent wastewater, it being produced during decay of all microbial populations. It is consumed during the growth of microalgae, heterotrophic bacteria and nitrifying bacteria. The phosphate phosphorus concentration is modelled by the following equation:

$$Q_d \cdot X_{PO_4,in} = Q_h \cdot X_{PO_4,out} + V \cdot \cdot \left(X_{ALG} \cdot \mu_{alg} \cdot Y_{con}\left[\frac{PO_4}{alg}\right] + X_{het,out} \cdot \mu_{het} \cdot Y_{con}\left[\frac{PO_4}{het}\right] + X_{nit,out} \cdot \mu_{nit} \cdot Y_{con}\left[\frac{PO_4}{nit}\right]\right) + V \cdot \frac{dX_{PO_4,out}}{dt} \quad (40)$$

where $X_{PO_4,in}$ [g m^{-3}] is the inlet phosphate phosphorus concentration, $X_{PO_4,out}$ [g m^{-3}] is the outlet phosphate phosphorus concentration, $Y_{con}\left[\frac{PO_4}{alg}\right]$ [-] represents the phosphate consumption rate from microalgae, $Y_{con}\left[\frac{PO_4}{het}\right]$ [-] is the phosphate consumption rate from heterotrophic bacteria and $Y_{con}\left[\frac{PO_4}{nit}\right]$ [-] is the phosphate consumption rate from nitrifying bacteria.

Although growth rate models for the different microorganisms are well defined, the consumption and generation parameters of nutrients associated with each species present some uncertainty. The production of microalgae using wastewater as culture medium presents diverse variability in the model parameters. Depending on the type of wastewater and its components, the generation and consumption parameters associated with microalgae and bacteria may vary. This fact raises the need for a model that allows adapting its parameters for each situation. Therefore, a calibration method is presented using genetic algorithms that is capable of estimating the characteristic parameters of the model from experimental data measured in the reactor.

3.3. Experimental Datasets

Experimental data for model calibration and validation were collected from two laboratory-scale photobioreactors, which were fed with pig slurry diluted at 20%. The concentrations of biomass and the major nutrients (N–NH_4^+, N–NO_3^-, P–PO_4^{2-}, COD) both at the inlet wastewater and inside the reactor were measured. The descriptions of the reactors and the probes used to collect the data (temperature, pH, DO, and light), along with the methods used to measure biomass and nutrients, are shown in Section 2.

3.4. Calibration Process

The already shown equations of the model include of a series of characteristic parameters whose exact values are unknown, or the values are known in a defined range. The uncertainty in the values of these parameters imposes the need for a calibration process, which has been carried out through genetic algorithms. Calibration using genetic algorithms results in a useful and reliable method for the estimation of uncertain parameters, since it allows optimizing a cost function that measures the deviation of the output of the model from that of the real system by modifying the parameter values between the

established limits. The ranges of the estimated parameters have been obtained from the cited literature, and from experience in the design of the installation.

The calibration process using genetic algorithms was implemented in MATLAB using the Genetic Algorithm Optimization Toolbox (GAOT), based on [1], with an initial population of 50 phenotypes (solutions) and a termination condition of 50 generations. This method starts with an initial set of calibration parameters and runs the model to obtain the error. The cost function is computed as the sum of the individual root mean square error (RMSE) functions for the simulated organism and nutrients (total biomass, ammonium, nitrate, phosphate, and BSOM) and the real measured values, expressed as the following equation:

$$J = \left(\sqrt{\sum_{i=1}^{N} \frac{\left(Cb_{total_{est}}(i) - Cb_{total_{real}}(i)\right)^2}{N}}\right) + \left(\sqrt{\sum_{i=1}^{N} \frac{\left(X_{NH_{4,est}}(i) - X_{NH_{4,real}}(i)\right)^2}{N}}\right) + \left(\sqrt{\sum_{i=1}^{N} \frac{\left(X_{NO_{3,est}}(i) - X_{NO_{3,real}}(i)\right)^2}{N}}\right)$$

$$+ \left(\sqrt{\sum_{i=1}^{N} \frac{\left(X_{PO_{4,est}}(i) - X_{PO_{4,real}}(i)\right)^2}{N}}\right) + \left(\sqrt{\sum_{i=1}^{N} \frac{\left(X_{BSOM_{est}}(i) - X_{BSOM_{real}}(i)\right)^2}{N}}\right)$$

where $Cb_{total_{est}}$ $\left[g\ m^{-3}\right]$ is the estimated total biomass concentration (microalgae + heterotrophic bacteria + nitrifying bacteria); $Cb_{total_{real}}$ $\left[g\ m^{-3}\right]$ is the experimental total biomass concentration measured. The rests of the parameters also describe the differences between the estimated concentrations and the experimentally measured ones for all elements. N represents the size of the data vector.

The calibration parameters are related to the maximum growth rates for the microorganisms, and the coefficients of generation and nutrient consumption. Table 2 lists the descriptions of all the calibration parameters, and the values obtained as a result of the calibration process.

In addition to the parameters described in the table, through this calibration process, it is possible to estimate the percentage of each species in the reactor. The experimental measurement of the concentration of the species of bacteria is something complex to carry out and highlights the need for a simple way of being able to estimate the percentage of each species within the reactor. Therefore, for both the calibration and validation data, the genetic algorithm method was used to determine the initial percentage of each species. In this way, the calibration process acts as a tool to estimate the percentages of microalgae and bacteria involved in the reactor from the measurements of total biomass and nutrients in it.

Data used during the calibration process correspond to the experimental measurements from during for 14 consecutive days. The imposed culture conditions were equivalent to that found in a raceway reactor, with light and dark cycles representing day and night. In addition, pH and dissolved oxygen were controlled by injecting CO_2 and air. Figure 4 represents the experimental data measured, which correspond to measurements of irradiance, pH, dissolved oxygen, and temperature, in addition to measurements of total biomass dry weight (microalgae, heterotrophic bacteria, and nitrifying bacteria) and measurements of nutrients (ammonium, nitrate, phosphate, and BSOM).

Figure 5 represents the calibration results obtained in the estimation of the model variables. This figure is made up of six independent graphs that represent different variables estimated in the model. Figure 5a represents the percentage of each species of microorganisms within the reactor. Figure 5b represents the biomass concentration for each organism in the reactor (microalgae, heterotrophic bacteria, and nitrifying bacteria), in addition to the total biomass concentration, expressed as the sum of the individual concentrations, and the experimental measurements. Figure 5c represents the estimated phosphate concentration and the experimental data. Figure 5d shows the estimated ammonium concentration and the experimental values. Figure 5e represents the estimated nitrate concentration and the experimental measurements. Finally, Figure 5f represents the estimated biodegradable soluble organic matter concentration, compared with the experimental measurement.

As a result of the calibration, initial percentages of 82.1% for microalgae, 13.2% for heterotrophic bacteria, and 4.7% for nitrifying bacteria have been established. Looking at Figure 5a,b, it is observed how the concentration of microalgae decreases until reaching a steady state. On the other hand, the concentration of heterotrophic bacteria grows slightly, consuming ammonium and organic matter, while the concentration of nitrifying bacteria remains constant. The sum of the concentration of each species represents the total biomass concentration (dashed line), which properly fit to the experimental data.

Table 2. Calibration parameters for the ABACO model.

Symbol	Parameter	Value	Unit
$\mu_{alg,max}$	Microalgae maximum growth rate	1.591	day^{-1}
$\mu_{het,max}$	Heterotrophic bacteria maximum growth rate	1.235	day^{-1}
$\mu_{nit,max}$	Nitrifying bacteria maximum growth rate	0.730	day^{-1}
$m_{min,alg}$	Microalgae endogenous respiration minimum rate	0.01	day^{-1}
$m_{max,alg}$	Microalgae endogenous respiration maximum rate	0.276	day^{-1}
$Y_{con}\left[\frac{NH_4}{alg}\right]$	Ammonium consumption rate from microalgae	0.369	$g_{NH_4}\ g_{alg}^{-1}$
$Y_{con}\left[\frac{NO_3}{alg}\right]$	Nitrate consumption rate from microalgae	0.214	$g_{NO_3}\ g_{alg}^{-1}$
$Y_{con}\left[\frac{PO_4}{alg}\right]$	Phosphate consumption rate from microalgae	0.008	$g_{PO_4}\ g_{alg}^{-1}$
$Y_{gen}\left[\frac{BSOM}{alg}\right]$	BSOM generation rate from microalgae	0.148	$g_{BSOM}\ g_{alg}^{-1}$
$Y_{con}\left[\frac{NH_4}{het}\right]$	Ammonium consumption rate from heterotrophic bacteria	0.299	$g_{NH_4}\ g_{het}^{-1}$
$Y_{con}\left[\frac{PO_4}{het}\right]$	Phosphate consumption rate from heterotrophic bacteria	0.017	$g_{PO_4}\ g_{het}^{-1}$
$Y_{gen}\left[\frac{BSOM}{het}\right]$	BSOM generation rate from heterotrophic bacteria	0.153	$g_{BSOM}\ g_{het}^{-1}$
$Y_{con}\left[\frac{BSOM}{het}\right]$	BSOM consumption rate from heterotrophic bacteria	0.478	$g_{BSOM}\ g_{het}^{-1}$
$Y_{con}\left[\frac{NH_4}{nit}\right]$	Ammonium consumption rate from nitrifying bacteria	3.224	$g_{NH_4}\ g_{nit}^{-1}$
$Y_{gen}\left[\frac{NO_3}{nit}\right]$	Nitrate generation rate from nitrifying bacteria	0.355	$g_{NO_3}\ g_{nit}^{-1}$
$Y_{con}\left[\frac{PO_4}{nit}\right]$	Phosphate consumption rate from nitrifying bacteria	0.182	$g_{PO_4}\ g_{nit}^{-1}$
$Y_{gen}\left[\frac{BSOM}{nit}\right]$	BSOM generation rate from nitrifying bacteria	0.149	$g_{BSOM}\ g_{nit}^{-1}$

Although the experimental concentrations of nutrients (phosphate, ammonium, nitrate, and BSOM) are very scattered, a trend is observed for each. The estimated values for the elements presented in Figure 5c–f fit correctly within the experimental data. The total RMSE value obtained through the cost function during calibration was 25.93, which is an acceptable value, since, with the exception of ammonia, the range of variation of the variables analyzed is small. An error of 0.076 was obtained for total biomass concentration, an error of 0.9 for phosphate, an error of 20.76 for ammonium, an error of 1.27 for nitrate, and an error of 2.94 for BSOM.

3.5. Validation

The validation data used to verify the values of the characteristic parameters obtained during the calibration process were obtained in a separate vessel reactor, operated in parallel with the one used for calibration. These data collect the experimental measurements from 14 days, represented in Figure 6.

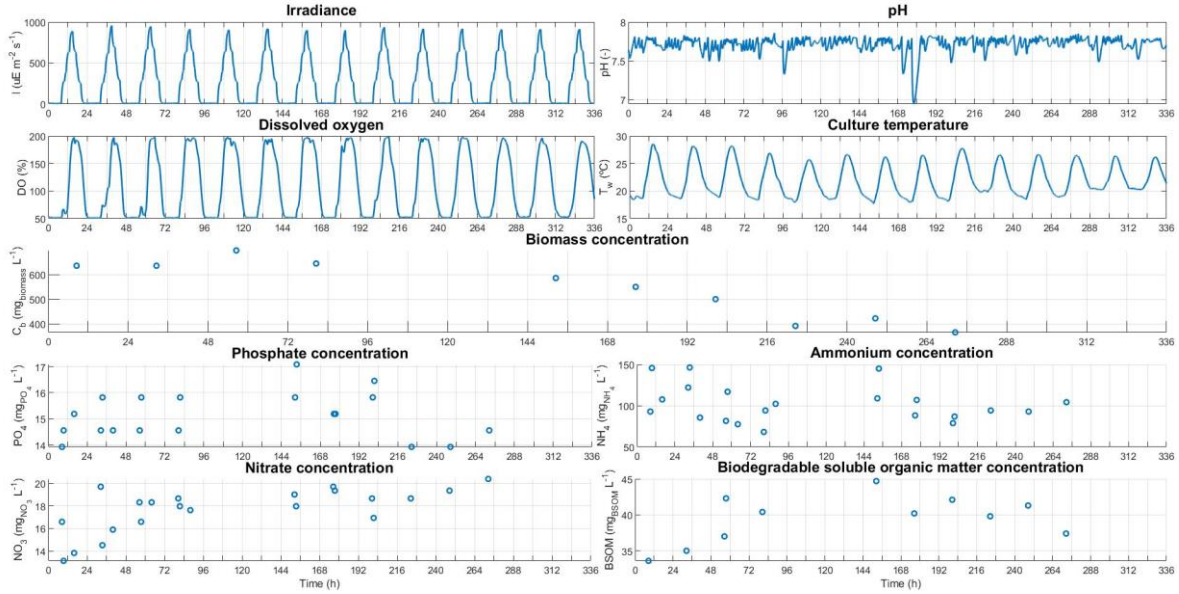

Figure 4. Input variables for calibration.

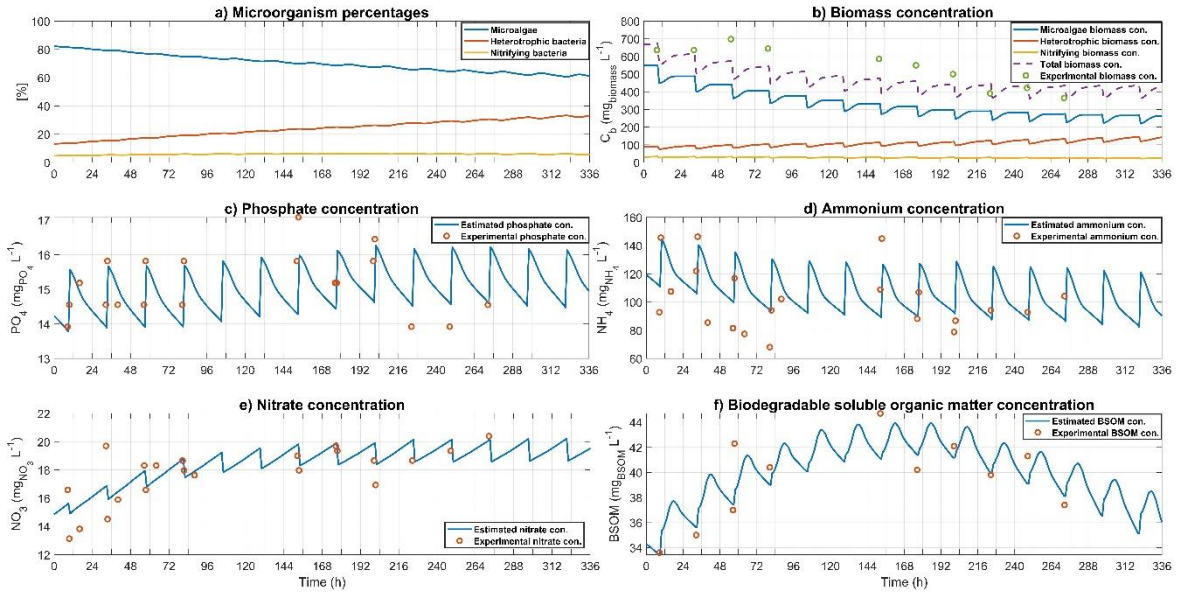

Figure 5. Calibration results for the biomass production model with wastewater medium. (**a**) Microalgae and bacteria percentages inside the reactor; (**b**) Microalgae and bacteria biomass concentration; (**c**) Phosphate concentration inside the reactor; (**d**) Ammonium concentration inside the reactor; (**e**) Nitrate concentration inside the reactor; (**f**) Biodegradable soluble organic matter concentration inside the reactor.

For the validation process, calibration using genetic algorithms has been used to determine the initial percentages of microorganisms in the reactor. In this way, it is possible to estimate the starting points for the concentration of microalgae and bacteria. In this case, the initial percentages obtained were 85% for microalgae, 12.6% for heterotrophic bacteria, and 2.4% for nitrifying bacteria, very similar to the percentages obtained during the calibration test. After this initial point, the concentrations of all the elements in the reactor were estimated and compared with the points measured experimentally, represented in Figure 7.

Figure 7a,b shows trends in biomass concentrations similar to those obtained during calibration. The concentration of microalgae decreases till achieving steady state, the

heterotrophic bacteria slightly grow, and the nitrifying bacteria remain constant. The total concentration correctly resembles the trend shown by the experimental measurements.

The estimation of the phosphate concentration (Figure 7c) shows an increasing trend, slightly away from the center of the measurement points. However, the estimation is within the range of the experimental values. The concentration of ammonium (Figure 7d) maintains a good trend within the established range, as does the estimated nitrate concentration (Figure 7e). Finally, the BSOM estimation (Figure 7f) shows a trend similar to the calibration results, within the experimental points. In this case, the total RMSE error was 30.25, slightly higher for calibration.

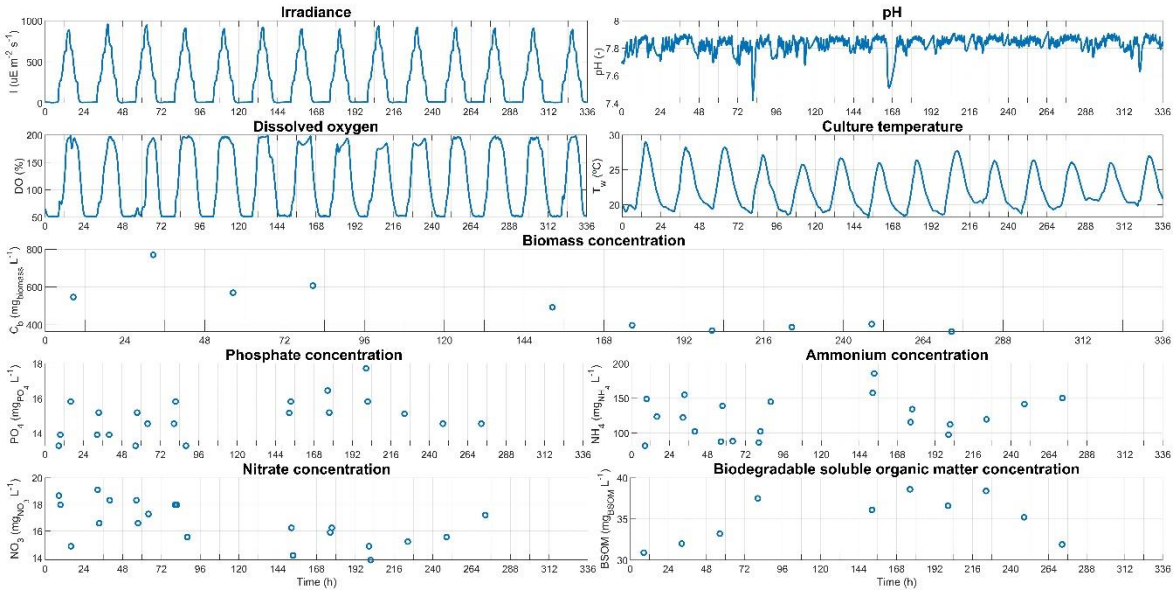

Figure 6. Input variables for validation.

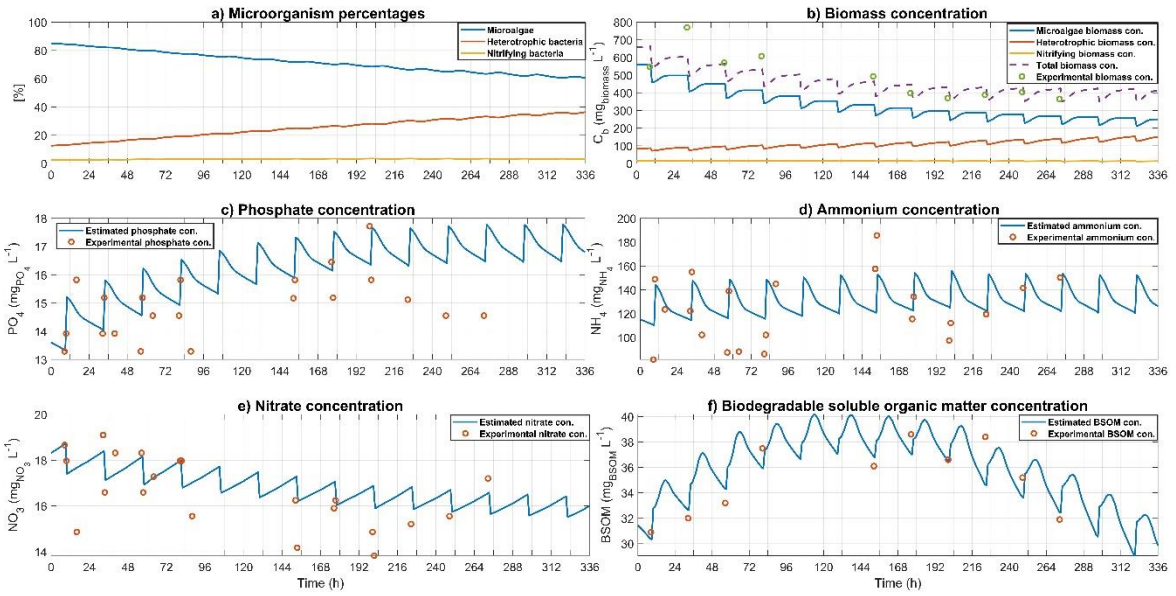

Figure 7. Validation results for the biomass production model with wastewater medium. (**a**) Microalgae and bacteria percentages inside the reactor; (**b**) Microalgae and bacteria biomass concentration; (**c**) Phosphate concentration inside the reactor; (**d**) Ammonium concentration inside the reactor; (**e**) Nitrate concentration inside the reactor; (**f**) Biodegradable soluble organic matter concentration inside the reactor.

Regarding errors, for the total biomass concentration, an error of 0.077 was obtained, a value almost identical to the result obtained in calibration. The error obtained for phosphate was 1.33, higher than the result obtained in the calibration. The error obtained for ammonia was 25.89, also higher than the result obtained in calibration. The error for nitrate was 1.7, slightly higher than the calibration result. Finally, the error obtained for the BSOM was 1.25, lower than the result obtained in calibration.

These results, at a preliminary level, show a good trend in the estimation of the elements of the model. Certain discrepancies in the results, as in the case of phosphate, may have been due to approximations and considerations in the input parameters of the model, such as the concentrations of the nutrients in the dilution medium, which change over time and have been considered constant.

More details about the parameters used in ABACO model can be found in Appendix A.

3.6. Respirometric Measurements

The respirometric measurements allowed us to determine the microalgal photosynthesis rate, the heterotrophic respiration rate, and the nitrifying respiration rate in the cultures. The microalgal photosynthesis rate was 15.8 ± 2.3 mgO_2 L^{-1} h^{-1}, the heterotrophic respiration rate was 2.2 ± 0.8 mgO_2 L^{-1} h^{-1}, and the nitrifying respiration rate was 0.27 ± 0.1 mgO_2 L^{-1} h^{-1}. These values correspond to 86.7% microalgae, 11.8% heterotrophic bacteria, and 1.5 % nitrifying bacteria. These values closely approximate those determined by calibration (82.1% for microalgae, 13.2% for heterotrophic bacteria, and 4.7% for nitrifying bacteria) and validation (85% for microalgae, 12.6% for heterotrophic bacteria, and 2.4% for nitrifying bacteria) processes (Figure 8).

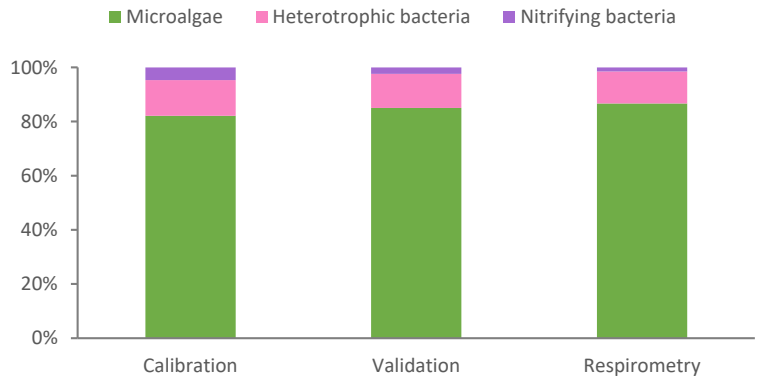

Figure 8. The microbial percentages obtained during the calibration process, the validation process, and the experimental respirometric measurements.

3.7. Discussion

The combination of microalgae biomass production processes and wastewater (diluted pig slurry) treatment is a cost-effective goal that poses several challenges. On the one hand, the already used wastewater contains high amounts of nutrients that allow microalgae and bacteria growth [27]. On the other hand, the lower energy demand for the microalgae–bacteria wastewater treatment, along with the ability of microalgae for CO_2 fixation, significantly increases the environmental sustainability of this eco-friendly technology [28]. Apart from multiple biological models proposed for microalgae–bacteria wastewater treatment using different types of effluents, scarce information is available about the use of animal manure as a nutrient source [29]. In this work, an integral microalgae and bacteria model named ABACO was developed, calibrated, and validated with experimental data from duplicate laboratory-scale photobioreactors using pig slurry as a nutrient source. The implementation of the model allowed us to simulate the dynamics of different components in the system and the relative proportions of microalgae and bacteria. The values of several model parameters were calibrated using genetic algorithms. Addi-

tionally, the percentage of each microbial population present in the microalgae–bacteria culture was estimated.

The results obtained from the comparison between the estimated values with respect to the measured experimental data have been satisfactory. The concentrations of the elements were adjusted within the range formed by the measurement points, despite being scattered data. The percentages of microalgae and bacteria within the reactor over time showed values close to those obtained in the literature [17,29]. Additionally, these percentages were estimated by a respirometric method, in which the microalgae, heterotrophic bacteria, and nitrifying bacteria showed estimated values that were close to those determined by calibration and validation processes. When analyzing the errors obtained for both cases, it becomes clear that faithfully estimating the evolution in the concentration of the different elements in the reactor is a complex process. The experimental values are very scattered, and that hinders their continuous evolution estimation. Even so, the results obtained are within the ranges of variation of the measurements taken.

The complexity in measuring individual concentrations of each species highlights the need for a reliable estimation method. Due to the calibration using genetic algorithms, it is possible to estimate the percentage of each microorganism in the reactor. From experimental data, the model allows one to determine the initial percentage of each element and estimate its evolution over time. In this way, the model can act as a simulator to predict the behavior of organisms based on the concentrations of nutrients present in the reactor medium. This model and the calibration parameters obtained will serve as the basis for the development of simulation models where the production of microalgae biomass is combined with wastewater treatment.

4. Conclusions

The microalgae–bacteria model proposed has demonstrated itself to be a useful tool for understanding the microalgal–bacterial interaction in wastewater treatment. The calibration carried out by means of genetic algorithms opens the door to a simple method of adjusting the various parameters that make up the model, so that it can be recalibrated from experimental measurements of different medium and culture scenarios, since the concentrations of nutrients vary from one type of medium to another. Therefore, the model could be applied to different strains, both microalgae and bacteria, by recalibrating the parameters based on a set of experimental data. The next step is focusing on the validation of the biological model in large-scale photobioreactors in order to find the optimal conditions for wastewater treatment, nutrient recovery, and biomass production, thereby enabling the sustainability of the process.

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Appendix A

Table A1. Variables for the proposed ABACO model.

Variables of the Biologic Models	
Heterotrophic Bacteria	
$\mu_{het,max}$	Heterotrophic bacteria maximum growth rate
T_{min}	Minimal heterotrophic bacteria temperature
T_{max}	Maximum heterotrophic bacteria temperature
T_{opt}	Optimum heterotrophic bacteria temperature
pH_{min}	Minimal heterotrophic bacteria pH
pH_{max}	Maximum heterotrophic bacteria pH
pH_{opt}	Optimum heterotrophic bacteria pH
$K_{S,DO_2,\ HET}$	Heterotrophic bacteria half-saturation constant for dissolved oxygen
$K_{S,NH_4,HET}$	Heterotrophic bacteria half-saturation constant for N-NH_4
$K_{S,PO_4,HET}$	Heterotrophic bacteria half-saturation constant for P-PO_4
$K_{S,BSOM,HET}$	Heterotrophic bacteria half-saturation constant for biodegradable soluble organic matter
$Y_{con}\left[\frac{NH_4}{het}\right]$	Ammonium consumption rate from heterotrophic bacteria
$Y_{con}\left[\frac{PO_4}{het}\right]$	Phosphate consumption rate from heterotrophic bacteria
$Y_{gen}\left[\frac{BSOM}{het}\right]$	BSOM generation rate from heterotrophic bacteria
$Y_{con}\left[\frac{BSOM}{het}\right]$	BSOM consumption rate from heterotrophic bacteria
Nitrifiying Bacteria	
$\mu_{nit,max}$	Nitrifiying bacteria maximum growth rate
T_{min}	Minimal nitrifiying bacteria temperature
T_{max}	Maximum nitrifiying bacteria temperature
T_{opt}	Optimum nitrifiying bacteria temperature
pH_{min}	Minimal nitrifiying bacteria pH
pH_{max}	Maximum nitrifiying bacteria pH
pH_{opt}	Optimum nitrifiying bacteria pH
$K_{S,DO_2,\ NIT}$	Nitrifiying bacteria half-saturation constant for dissolved oxygen
$K_{I,DO_2,\ NIT}$	Nitrifiying bacteria inhibition constant for dissolved oxygen
$K_{S,C,NIT}$	Nitrifiying saturation half-constant for CO_2
$K_{S,NH_4,NIT}$	Nitrifiying bacteria half-saturation constant for N-NH_4
$K_{S,PO_4,NIT}$	Nitrifiying bacteria half-saturation constant for P-PO_4
$Y_{con}\left[\frac{NH_4}{nit}\right]$	Ammonium consumption rate from nitrifying bacteria
$Y_{gen}\left[\frac{NO_3}{nit}\right]$	Nitrate generation rate from nitrifying bacteria
$Y_{con}\left[\frac{PO_4}{nit}\right]$	Phosphate consumption rate from nitrifying bacteria
$Y_{gen}\left[\frac{BSOM}{nit}\right]$	BSOM generation rate from nitrifying bacteria

Table A2. Values for the proposed ABACO model's characteristic parameters.

Microalgae Net Photosynthesis Rate			
Parameter	**Value**	**Units**	**Source**
$\mu_{alg,max}$	1.591	day^{-1}	Calibrated
I_k	168	$\mu E \cdot m^{-2} \cdot s^{-1}$	Sánchez-Zurano et al., 2020
n	1.700	-	Sánchez-Zurano et al., 2020
T_{min}	3.400	°C	Sánchez-Zurano et al., 2020
T_{max}	49	°C	Sánchez-Zurano et al., 2020
T_{opt}	30	°C	Sánchez-Zurano et al., 2020
pH_{min}	1.800	-	Sánchez-Zurano et al., 2020
pH_{max}	12.900	-	Sánchez-Zurano et al., 2020
pH_{opt}	8.500	-	Sánchez-Zurano et al., 2020
$DO_{2,max}$	32	$mg_{O_2} \cdot L^{-1}$	Sánchez-Zurano et al., 2020
m	4.150	-	Sánchez-Zurano et al., 2020
$m_{max,alg}$	0.010	day^{-1}	Calibrated
$m_{min,alg}$	0.276	day^{-1}	Calibrated
$I_{k,resp}$	134	$\mu E \cdot m^{-2} \cdot s^{-1}$	Sánchez-Zurano et al., 2020
n_{resp}	1.400	-	Sánchez-Zurano et al., 2020
$K_{S,C,ALG}$	$4 \cdot 10^{-3}$	$mg_C \cdot L^{-1}$	BIO_ALGAE
$K_{I,C,ALG}$	120	$mg_C \cdot L^{-1}$	BIO_ALGAE
$K_{S,NH_4,ALG}$	1.540	$mg_N \cdot L^{-1}$	Sánchez-Zurano et al., 2020. Under rev.
$K_{I,NH_4,ALG}$	571	$mg_N \cdot L^{-1}$	Sánchez-Zurano et al., 2020. Under rev.
$K_{S,NO_3,ALG}$	2.770	$mg_N \cdot L^{-1}$	Sánchez-Zurano et al., 2020. Under rev.
$K_{I,NO_3,ALG}$	386.600	$mg_N \cdot L^{-1}$	Sánchez-Zurano et al., 2020. Under rev.
$K_{S,PO_4,ALG}$	0.430	$mg_P \cdot L^{-1}$	Sánchez-Zurano et al., 2020. Under rev.
$Y_{con}\left[\frac{NH_4}{alg}\right]$	0.369	$g_{NH_4} \cdot g_{alg}^{-1}$	Calibrated
$Y_{con}\left[\frac{NO_3}{alg}\right]$	0.214	$g_{NO_3} \cdot g_{alg}^{-1}$	Calibrated
$Y_{con}\left[\frac{PO_4}{alg}\right]$	0.008	$g_{PO_4} \cdot g_{alg}^{-1}$	Calibrated
$Y_{gen}\left[\frac{BSOM}{alg}\right]$	0.148	$g_{BSOM} \cdot g_{alg}^{-1}$	Calibrated
Heterotrophic Respiration Rate			
Parameter	**Value**	**Units**	**Source**
$\mu_{het,max}$	1.235	day^{-1}	Calibrated
T_{min}	9	°C	Sánchez-Zurano et al., 2020
T_{max}	47	°C	Sánchez-Zurano et al., 2020
T_{opt}	36	°C	Sánchez-Zurano et al., 2020
pH_{min}	6	-	Sánchez-Zurano et al., 2020
pH_{max}	12	-	Sánchez-Zurano et al., 2020
pH_{opt}	9	-	Sánchez-Zurano et al., 2020
$K_{S,DO_2,\ HET}$	1.980	$mg_{O_2} \cdot L^{-1}$	Sánchez-Zurano et al., 2020
$K_{S,NH_4,HET}$	0.500	$mg_N \cdot L^{-1}$	ASM
$K_{S,PO_4,HET}$	0.010	$mg_P \cdot L^{-1}$	ASM
$K_{S,BSOM,HET}$	20	$mg_{BSOM} \cdot L^{-1}$	ASM
$Y_{con}\left[\frac{NH_4}{het}\right]$	0.299	$g_{NH_4} \cdot g_{het}^{-1}$	Calibrated
$Y_{con}\left[\frac{PO_4}{het}\right]$	0.017	$g_{PO_4} \cdot g_{het}^{-1}$	Calibrated
$Y_{gen}\left[\frac{BSOM}{het}\right]$	0.153	$g_{BSOM} \cdot g_{het}^{-1}$	Calibrated
$Y_{con}\left[\frac{BSOM}{het}\right]$	0.478	$g_{BSOM} \cdot g_{het}^{-1}$	Calibrated

Table A2. *Cont.*

Nitrifying Respiration Rate			
Parameter	**Value**	**Units**	**Source**
$\mu_{nit,max}$	0.730	day^{-1}	Calibrated
T_{min}	0	°C	Sánchez-Zurano et al., 2020
T_{max}	49	°C	Sánchez-Zurano et al., 2020
T_{opt}	33.600	°C	Sánchez-Zurano et al., 2020
pH_{min}	2	-	Sánchez-Zurano et al., 2020
pH_{max}	13.400	-	Sánchez-Zurano et al., 2020
pH_{opt}	9	-	Sánchez-Zurano et al., 2020
$K_{S,DO_2,\ NIT}$	1.080	$mg_{O_2} \cdot L^{-1}$	ASM
$K_{I,DO_2,\ NIT}$	104.900	$mg_{O_2} \cdot L^{-1}$	ASM
$K_{S,C,NIT}$	0.500	$mg_C \cdot L^{-1}$	ASM
$K_{S,NH_4,NIT}$	1	$mg_N \cdot L^{-1}$	ASM
$K_{S,PO_4,NIT}$	0.010	$mg_P \cdot L^{-1}$	ASM
$Y_{con}\left[\frac{NH_4}{nit}\right]$	3.224	$g_{NH_4} \cdot g_{nit}^{-1}$	Calibrated
$Y_{gen}\left[\frac{NO_3}{nit}\right]$	0.355	$g_{NO_3} \cdot g_{nit}^{-1}$	Calibrated
$Y_{con}\left[\frac{PO_4}{nit}\right]$	0.182	$g_{PO_4} \cdot g_{nit}^{-1}$	Calibrated
$Y_{gen}\left[\frac{BSOM}{nit}\right]$	0.149	$g_{BSOM} \cdot g_{nit}^{-1}$	Calibrated

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7.5. An Interactive Tool for Simulation of Biological Models Into the Wastewater Treatment With Microalgae.

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An Interactive Tool for Simulation of Biological Models Into the Wastewater Treatment With Microalgae

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This paper presents a novel simulation tool to understand and analyze biological models for wastewater treatment processes using microalgae. The models for this type of processes are very complex to be analyzed because of the very different phenomena, variables and parameters involved. The model already included in the tool has been validated at controlled conditions simulating outdoor ones, it being useful to simulate real outdoor cultures. The major contribution of the proposed tool is that these models can be easily and interactively simulated and compared. The tool allows simulating biological models only considering microalgae or including the microalgae-bacteria consortium. Moreover, the simulations can be done only using the solar radiation contribution or by adding the environmental and bacteria effects as cardinal terms. Furthermore, the effects of the wastewater properties or different microalgae strains can be evaluated. The interactive simulations can be performed for selected days as representative of the different year seasons that are already preloaded in the tool. However, the user can also load data from other locations to simulate the models under particular conditions.

Keywords: microalage, modelling, bacteria, wastewater, interactive learning environments

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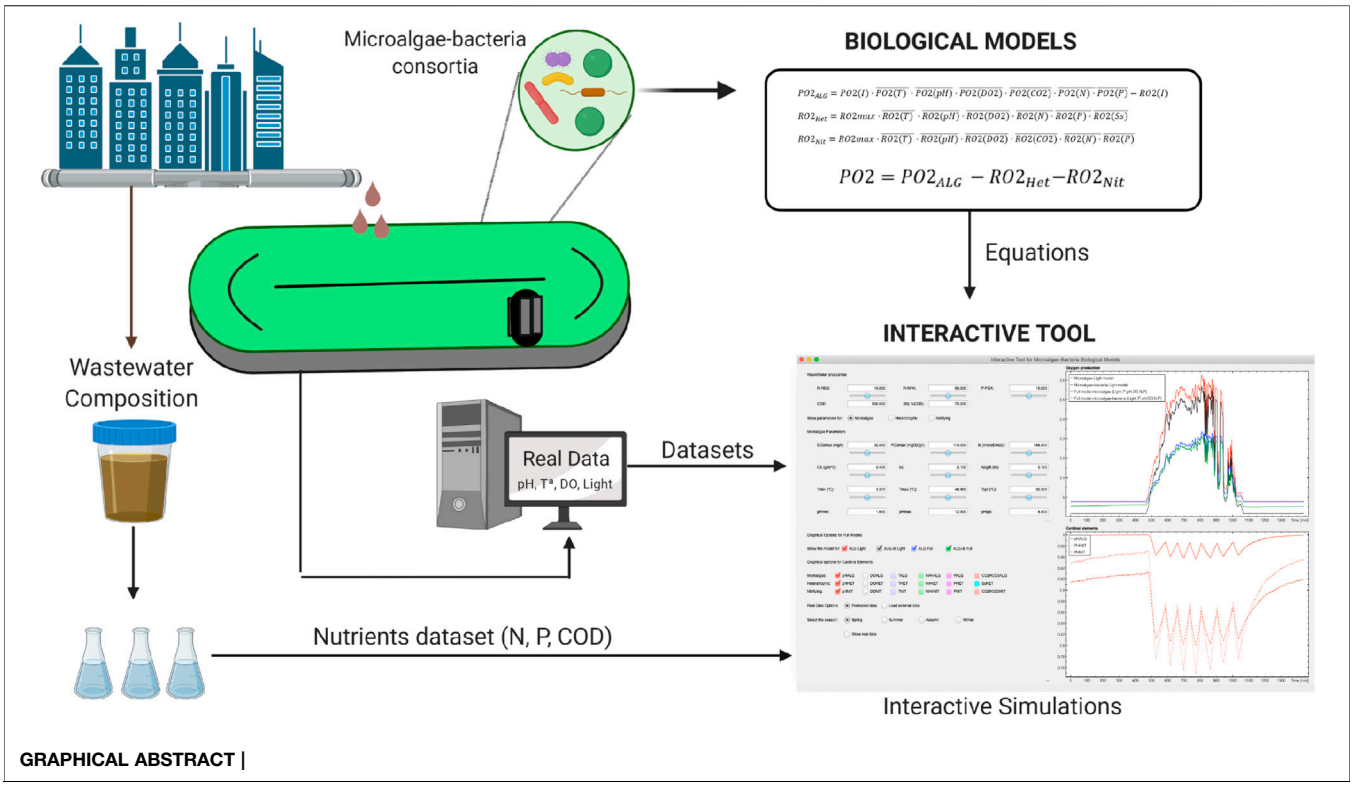
HIGHLIGHTS:

Understanding biological models using interactive tools.
Analysis of weather, nutrients and strain type effects on oxygen production.
Studying microalgae-bacteria consortia models with interactivity.

INTRODUCTION

Water has become a scarce and limited resource due to its growing consumption in developed industrial countries, contamination of water sources and the lack of efficient technologies for retrieving more usable water (Li et al., 2019). The large volume of wastewater generated should be treated before being discharged into natural watercourses or reused because untreated wastewater discharge pollutes the water bodies and spreads water-related diseases (Singh, 2021). Although the conventional wastewater treatments have shown adequate nutrients removal levels, they imply a high economic cost and a resource waste (Acién et al., 2016). Therefore, in order to solve this situation, some eco-friendly alternatives have appeared for wastewater treatment, which allow obtaining a treated effluent of good quality, an efficient nutrient recovery, and production of energy and/or bioproducts at low cost (Puyol et al., 2017; Patel et al., 2021). From these alternatives, a microalgae-based wastewater treatment process is one of the most promising



GRAPHICAL ABSTRACT |

technologies for the advanced treatment and nutrient recovery of wastewater, and it has attracted more and more attention in recent years. The reason for this increased interest is that the use of microalgae has a dual benefit: economic wastewater treatment and microalgae biomass production, that can be subsequently converted into added-value products such as biofertilizers or animal feed (Craggs et al., 2013; .Guzmán et al., 2020; Acién et al., 2017; Li et al., 2019; Suganya et al., 2016).

Microalgae-based wastewater treatment is performed by complex microalgae-bacteria consortia which varies as a function of the environmental and operational conditions, especially the composition of the wastewater being processed (Acién et al., 2016). Although these interactions have been studied for many years, it is still challenging to understand what are the main processes that occur in microalgae-bacteria systems because most of them take place simultaneously and are strongly interdependent (García et al., 2006). Addressing this challenge, mathematical models have been proposed as a useful tool to understand and optimize biological systems (Bernstein and Carlson, 2012; Klanchui et al., 2012) such as microalgae-bacteria processes (Solimeno and García, 2019; Casagli et al., 2021). During the last decades, different types of mathematical models have been developed for understanding this interaction between microalgae and bacteria for wastewater treatment systems. Since Buhr and Miller (1983) developed the first mathematical model to describe microalgae and bacteria growth in wastewater, more complex models have progressively emerged (Buhr and Miller, 1983; Reichert and Vanrolleghem, 2001; Zambrano et al., 2016; Solimeno et al., 2017). However, due to the complexity of these models and the high number of parameters (more than 50 parameters), it is very difficult to understand and analyze the effect of all of them in a simple way. As a result, many simulations have to be carried out in order to study the effect of all the components involved in the process. This difficulty is evident at both the research level, when a deep analysis of the system is required, and at the understanding level, when the objective is to learn the concepts related to this type of systems.

In recent years, interactive tools and virtual laboratories have been presented as tools that allow simulating highly complex models and control systems quickly and easily. Especially, the interactive tools provide a high potential allowing a real-time interaction between the modification of parameters and the visualization of results (Guzmán et al., 2012). In essence, interactive tools show a graphical interface with dynamic and clickable components, which can be changed in order to visualize the system response immediately, which naturally lends itself to interactivity (Sánchez et al., 2005). These tools have been used successfully in the field of Control Engineering leading to very interesting results (Dormido et al., 2003; Guzmán et al., 2006). Moreover, specific virtual labs have been developed to simulate microalgae growth in photobioreactors, which helps to learn how a microalgae-based system work and to understand how the essential variables are involved in the algae growth (Dormido et al., 2014). However, to the best of the authors' knowledge, no interactive tool or virtual laboratory microalgae-bacteria processes has been developed to date.

Therefore, this work aims to develop an interactive tool based on microalgae mathematical models to visualize the productivity of the system as a function of the main environmental and operational variables that determine microalgal performance. The effect of each parameter is possible to visualize in real time and instantaneously. Specifically, the proposed interactive tool includes four possible models: the microalgae model only with the light effect, the microalgae-bacteria model only with the light effect, and these same two extended models including the effect of other parameters, such as pH, temperature, dissolved oxygen, nitrogen and phosphorous. Furthermore, it is possible to modify the rest of the biological parameters associated with the selected strain as well as the properties of wastewater to be treated. All these analyses can be carried out taking climate and reactor data (solar radiation, pH, dissolved oxygen, and culture temperature) as inputs to the models for different seasons of the year. All the proposed models included in the tool have been validated using experimental data, thus demonstrating its reliability. It is important to remark that the main contribution of this work is the proposed Interactive Tool and the combined implementation of the biological models. The software tool allows to immediately observe the effect of more than 50 parameters interactively, which is not possible in a classical static simulation.

This work is organized as follows. First, the biological models used in the tool are summarized. Next, the models are validated using experimental data obtained at controlled conditions. Then, a detailed description of the interactive tool developed is presented. Afterwards, several illustrative examples are provided to show how the interactive tool can be used under various types of possible scenarios.

MATERIAL AND METHODS

Biological Model

Microalgae wastewater treatment is performed by complex microalgae-bacteria consortia which vary as a function of the environmental and operational conditions (Acién et al., 2016). Under illumination, **microalgae** perform photosynthesis that turns carbon dioxide (CO_2) and water into organic molecules. In this process, microalgae reduce CO_2 and split water to release oxygen (O_2). This oxygen is essential for the degradation of organic compounds present in wastewater by aerobic bacteria (**heterotrophic bacteria**). In turn, during bacterial oxidation of organic matter, carbon dioxide is produced and is available for photosynthesis, thereby completing the cycle (Zambrano et al., 2016; Quijano et al., 2017). Apart from heterotrophic bacteria, other bacteria populations appear in wastewater and establish interactions with microalgae, emphasizing the **nitrifying bacteria**, which perform the nitrification process. During nitrification, nitrifying bacteria transforms ammonium, input from sewage into nitrate, in the presence and with the consumption of oxygen produced through photosynthesis (Vargas et al., 2016) (see **Figure 1**).

According to the simple scheme proposed, an equilibrium appears in wastewater treatment between the microalgae and the bacteria through the gas exchanges such as oxygen production/consumption. On the one hand, microalgae produce oxygen by photosynthesis and consume part of them for endogenous respiration. Moreover, heterotrophic and nitrifying bacteria consume the released oxygen by respiration. In fact, this makes possible to develop mathematical models based on net oxygen production by microalgae-bacteria consortia in wastewater treatment systems. As a result, and through oxygen production, it is possible to obtain models to predict the biomass productivity in these complex systems.

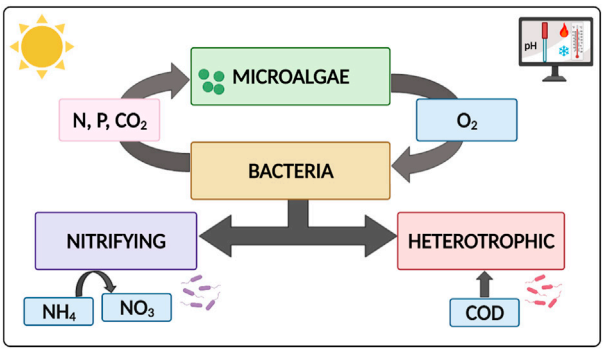


FIGURE 1 | Overall scheme of microalgae-bacteria interaction in wastewater treatment systems.

Mathematical Model Background

Sánchez-Zurano et al. (2021a) developed a dynamic model considering several important environmental parameters (light intensity, temperature, pH, and dissolved oxygen) on microalgae and bacteria growth (Sánchez Zurano et al., 2021b). The model equations were built using experimental data related to the influence of the environmental parameters on the photosynthesis rate and the respiration rate of microalgae-bacteria consortium, distinguishing between microalgae activity, heterotrophic activity, and nitrifying activity and by considering the methodology proposed by (Sánchez-Zurano et al., 2020a). After that, the model was validated using experimental data from a laboratory culture of *Scenedesmus* sp. growing with wastewater. In reality, three models, one for each microbial population, were developed in order to assemble all of them in one global model. The models were based on measuring the oxygen production rate (PO_2) for microalgae and oxygen respiration rate for bacteria (RO_2), both under different conditions of light intensity, temperature, pH and dissolved oxygen. The light is considered the decisive parameter which allows obtaining the maximal productivity, and the rest of the parameters (temperature, pH and dissolved oxygen) provide a normalized effect in the models (0–1) and modify the productivity obtained by the light influence. Besides, it must be considered that microalgae-bacteria systems are used to treat wastewaters with different composition, including those from industrial, agricultural and municipal sources. For that, along with environmental conditions, the chemical characteristics of these wastewaters

may severely influence microalgae productivity (Kube et al., 2018). Therefore, it is mandatory to include the influence of the main nutrients present in wastewater into the microalgae-bacteria mathematical models.

Apart from light, microalgae require nitrogen (N) and phosphorus (P) for their autotrophic growth, in which they fix inorganic carbon (CO_2 and HCO_3). Concerning nitrogen, ammonium (NH_4^+) is the main nitrogen form in wastewater, while nitrate (NO_3^-) usually appears in a low concentration. Most microalgae can utilize nitrogen in different forms. However, ammonium was known to be preferred by many microalgae because it requires less energy for assimilating (Kim et al., 2016). For that, in the biologic models, the effect of nitrogen on microalgae activity is calculated as a function of the nitrogen in form of ammonium (N-NH_4^+) present in the culture. In place, only if there is not ammonium in the medium, microalgae consume nitrate and its activity depends on nitrogen in form of nitrate (N-NO_3^-) concentration. Concerning bacteria activities, the effect of nutrients is considered too. For heterotrophic respiration, the concentration of both N-NH_4^+ and P-PO_4^{3-} is included in the model along with the concentration of biodegradable organic matter (Ss), which is calculated as a certain percentage of the total organic matter concentration (COD). During bacterial organic matter oxidation, CO_2 is produced, and it is available for photosynthesis and nitrification processes. Moreover, the normalized effect of the N-NH_4^+ and P-PO_4^{3-} concentration is taken into account to determine the nitrifying respiration. Therefore, the equations proposed by Sánchez-Zurano et al. (2020c) can be improved by adding the terms related to nitrogen and phosphorous availability (Sánchez-Zurano et al., 2020b), and the effect of the biodegradable organic matter and the inorganic carbon as follows:

$$PO2_{ALG} = PO2(I)_{ALG} \cdot \overline{PO2(T)_{ALG}} \cdot \overline{PO2(pH)_{ALG}} \cdot \overline{PO2(DO2)_{ALG}} \cdot \overline{PO2(CO2)_{ALG}} \cdot \overline{PO2(N)_{ALG}} \cdot \overline{PO2(P)_{ALG}} - RO2(I)_{ALG} \quad (1)$$

$$RO2_{HET} = RO2(I)_{HET} \cdot \overline{RO2(T)_{HET}} \cdot \overline{RO2(pH)_{HET}} \cdot \overline{RO2(DO2)_{HET}} \cdot \overline{RO2(N)_{HET}} \cdot \overline{RO2(P)_{HET}} \cdot \overline{RO2(Ss)_{HET}} \quad (2)$$

$$RO2_{NIT} = RO2(I)_{NIT} \cdot \overline{RO2(T)_{NIT}} \cdot \overline{RO2(pH)_{NIT}} \cdot \overline{RO2(DO2)_{NIT}} \cdot \overline{RO2(CO2)_{NIT}} \cdot \overline{RO2(N)_{NIT}} \cdot \overline{RO2(P)_{NIT}} \quad (3)$$

Previous models allowed to assemble a global model, ABACO model, to calculate the oxygen production by a microalgae-bacteria consortium in wastewater treatment (**Eq. 4**):

$$PO2 = PO2_{ALG} - RO2_{HET} - RO2_{NIT} \quad (4)$$

Finally, ABACO model was calibrated and validated in the laboratory photobioreactors using pig slurry as a nutrient source, demonstrating the validity of the developed model (Sánchez-Zurano et al., 2021b).

Biological Models for the Interactive Tool

As described earlier, processes that occur in microalgae systems are difficult to understand because most of them take place simultaneously and they are strongly interdependent. For that, it is very challenging to understand a microbiological system where a wide variety of metabolic processes coexist and are affected by multiple variables such as solar radiation, temperature, pH, etc. For these reasons, the implementation of an interactive tool that allows the visualization of all these variables and checking their effect on the microalgae-bacteria system is especially useful. The interactive tool proposed in this work includes four different oxygen production models for the microalgae-wastewater treatment problem. A more comprehensive version of the models described in the "Mathematical model background" section is implemented because the effect of the principal nutrients in wastewater treatment (nitrogen and phosphorous) has been also included. The four scenarios proposed in the tool are based on: 1) there are only microalgal cells in wastewater treatment and they are affected by solar radiation; 2) three populations coexist in wastewater treatment (microalga, heterotrophic bacteria and nitrifying bacteria) affected only by solar radiation; 3) the wastewater treatment is performed by microalgae cells and its activity is a function of solar radiation, temperature, pH, dissolved oxygen, nitrogen and phosphorous; 4) finally, the more complex model involves both microalgae and bacteria (heterotrophic and nitrifying bacteria) affected by solar radiation, temperature, pH, dissolved oxygen, nitrogen and phosphorous.

The next sections briefly describe these four different models. Notice that the simulation and comparison of these four different models will allow to obtain a better understanding of the microalgae-bacteria consortium and the effect of all the environmental and operation variables and parameters. More details about the models, the equations and the parameters can be found in **Supplementary Table 1**.

Light Microalgae Model

The simplest models for describing microalgae growth in different cultivation systems are based on the effect of solar radiation. Despite several light intensity models have been developed to describe microalgae photosynthesis and growth kinetics (Aiba, 1982; Eilers and Peeters, 1988), one of the most accepted models nowadays is the Molina model (Grima et al., 1994) in which the oxygen production is a function of specific maximum photosynthetic rate ($PO_{2,max}$), average irradiance (Iav), photosynthetic parameter constant (Ik), and a form parameter (n). Using this simple model, it is possible to determine what is the oxygen production by microalgae in a wastewater system according to the following equation:

$$PO_2 = PO2_{ALG} \cdot [I] \quad (5)$$

when only solar radiation is considered. This simplification of the model described by Sánchez-Zurano et al. (2021a) allows to offer an idea of the maximal productivity obtaining in a wastewater bioreactor in which microalgae are the only microbial population

FIGURE 2 | Raceway reactor. The real input data for simulating the seasons of the year were collected from the sensor located in it.

and the rest of the operational and environmental parameters do not affect the process.

Light Microalgae-Bacteria Model

Despite the usefulness of the previous model, the actual integrated model considering simultaneous growth of microalgae and bacteria in wastewater treatment should be considered. For this reason, it is recommendable to add the effect of bacteria populations to the model presented in **Eq. 6**. Thus, if the presence of microalgae and bacteria consortia is considered in wastewater treatment, and only the influence by solar radiation is considered, the new equation is described as follows:

$$PO_2 = PO2_{ALG}\ [I] - RO2_{HET} - RO2_{NIT} \tag{6}$$

Microalgae Full Model

On the other hand, another comprehensive model for microalgae activity can be obtained by extending the model in **Eq. 7** to influence both the environmental parameters (light intensity, temperature, pH and dissolved oxygen) and operational conditions such as culture media composition (nitrogen and phosphorous) and inorganic carbon availability. The inorganic carbon has been calculated according to the equilibrium with carbonate and bicarbonate species by considering a total inorganic carbon (TIC) vale of 100 $g\,L^{-1}$ and using the real pH values included in the tool for each specific day. Therefore, a new model only for microalgae cells which oxygen production is determined by solar radiation and the effect of other normalized factors can be obtained:

$$PO_2 = PO2_{ALG}\left([I]\cdot\overline{[T]}\cdot\overline{[pH]}\cdot\overline{[DO]}\cdot\overline{[CO2]}\cdot\overline{[N]}\cdot\overline{[P]}\right) \tag{7}$$

Microalgae-Bacteria Full Model

Finally, the most complex model for microalgae-bacteria systems can be obtained by including microalgae activity, heterotrophic activity and nitrifying activity. All these microbial populations are affected by the environmental and operational parameters, being solar radiation the decisive factor for determining oxygen production by the consortia. The full model including all the possible parameters and populations is the most realistic model described in this work and the final model used for calculating the real oxygen production of the system (**Eq. 8**):

$$\begin{aligned} PO_2 = {} & PO2_{ALG}\left([I]\cdot\overline{[T]}\cdot\overline{[pH]}\cdot\overline{[DO]}\cdot\overline{[CO2]}\cdot\overline{[N]}\cdot\overline{[P]}\right) \\ & - RO2_{HET}\left([I]\cdot\overline{[T]}\cdot\overline{[pH]}\cdot\overline{[DO]}\cdot\overline{[N]}\cdot\overline{[P]}\right) \\ & - RO2_{NIT}\left([I]\cdot\overline{[T]}\cdot\overline{[pH]}\cdot\overline{[DO]}\cdot\overline{[CO2]}\cdot\overline{[N]}\cdot\overline{[P]}\right) \end{aligned} \tag{8}$$

Raceway Reactor: Real Dataset

The real data included in the interactive tool for each season (spring, summer, autumn and winter) were collected from a raceway reactor located at the IFAPA Research Centre (Almería, Spain) (**Figure 2**). The raceway reactor consists of a polypropylene algal pond of two 50 m length channels (0.46 m high × 1 m wide) connected by 180°bends at each end, with a 0.59 m^3 sump (0.65 m long × 0.90 m wide × 1 m deep) located 1 m along one of the channels (Barceló-Villalobos et al., 2018). Guide vanes made of polypropylene were placed in the bends of the photobioreactors. In the raceway reactor, the microalgae-bacteria culture is circulated using a rotating paddlewheel actuated by an electric motor. pH is controlled to 8.0 by on-demand injection of pure CO_2 in the sump. Also, air is supplied to remove excess dissolved oxygen on demand. Environmental parameters such as pH, temperature and dissolved oxygen in the culture were measured using appropriate probes (5083 T and 5,120, Crison, Barcelona, Spain), connected to an MM44 control-transmitter unit (Crison Instruments, Spain), and data acquisition software (Labview, National Instruments) providing complete monitoring and control of the installation. Also, the solar radiation was collected using an adequate sensor (Pyranometer Kipp & Zonen CM 6B). The reactor is

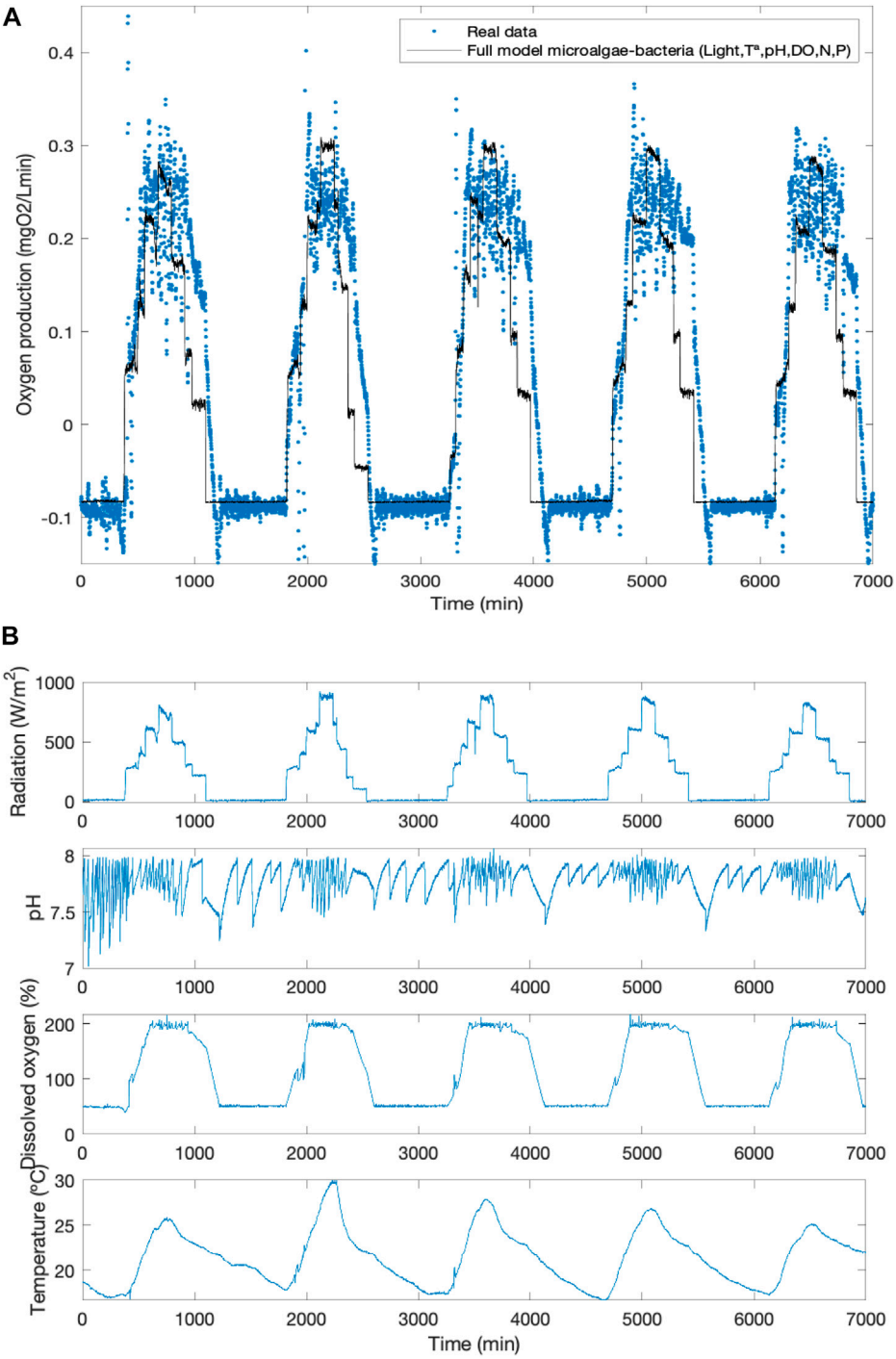


FIGURE 3 | Experimental validation of the full microalgae-bacteria model proposed **(A)**, Real input data from the laboratory stirred-tank reactor used for the validation **(B)**.

operated in semi-continuous mode throughout the year collecting daily and replaced with fresh wastewater.

By default, representative data from raceway reactors located in Almería (Spain) are preloaded in the tool. However, the user can load his/her own data for a particular analysis. Notice that the dissolved oxygen, pH and temperature balances are not included in the tool, since the objective is the comparison of biological models under the same reactor and weather conditions.

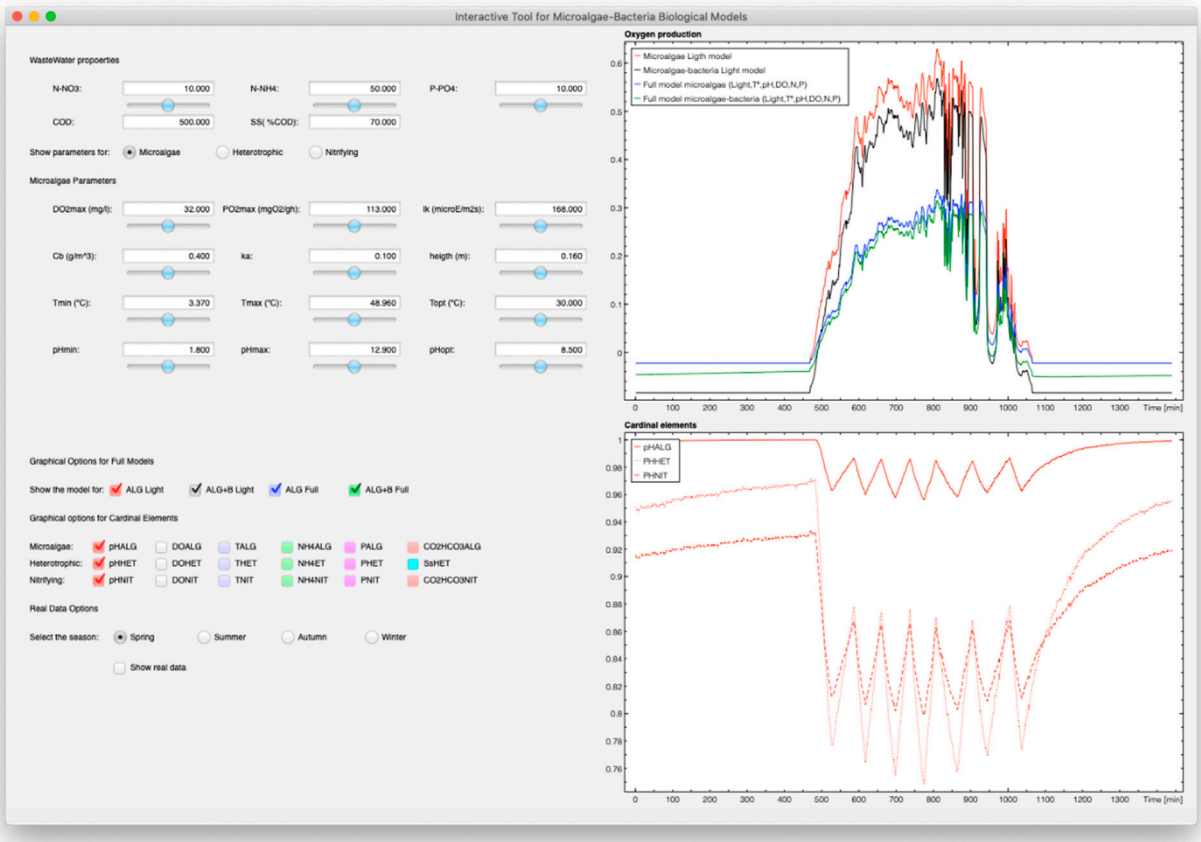


FIGURE 4 | Main screen of the interactive tool.

Laboratory Stirred-Tank Reactor: Biologic Model Validation

To validate the model implemented in the proposed interactive tool, experiments were performed in a stirred tank reactor (0.08 m in diameter, 0.2 m in height and with a 1 L capacity) operated at laboratory but simulating outdoor conditions. The reactor was filled with 20% of *Scenedesmus* sp. inoculum and primary domestic wastewater. The reactor was artificially illuminated using eight 28 W fluorescent tubes (Philips Daylight T5) on a simulated daylight cycle. The culture conditions inside the reactor, such as pH (Crison 5,002, Barcelona, Spain), temperature and dissolved oxygen (Crison 5,002, Barcelona, Spain) were monitored. To prevent the adverse effect of excessive dissolved oxygen accumulation, the dissolved oxygen was controlled and kept below 200%Sat by supplying air on demand. CO_2 was likewise injected on demand to control the pH at 8. The oxygen mass transfer coefficient (K_La) in the stirred tank reactor was 0.9 h^{-1}. The reactor was operated in batch mode for 6 days, after which it was operated in continuous mode to reach the steady state. For this, 20% of the culture volume was harvested every day and replaced with fresh culture medium. The data from the steady state was used for the validation process, in which the composition of the wastewater was: 146 mg L^{-1} $N\text{-}NH_4$, 3.8 mg L^{-1} $N\text{-}NO_3$, 15.2 mg L^{-1} $P\text{-}PO_4$ and 391 mg L^{-1} COD.

Programing Software to Implement the Interactive Tool

Sysquake (Anon), a Matlab-like language with fast execution and excellent facilities for interactive graphics, was used to code the interactive tool for these microalgae-bacteria models. Sysquake allows that the tool is delivered as a stand-alone executable that is readily accessible for free for both professionals and students (Díaz and Dormido, 2015).

RESULTS AND DISCUSSION

Full Biologic Model Validation

An experimental validation has been performed to compare the experimental oxygen production by a microalga-bacteria culture

and the oxygen production predicted by the full microalgae-bacteria model under particular operational conditions. In practice, the real data obtained from a laboratory stirred-tank reactor, fed with primary domestic wastewater, were compared with the simulated oxygen production by the full microalgae-bacteria model given by **Eq. 8**, which considers the effect of the whole environmental and operational conditions (light, temperature, pH, dissolved oxygen, nitrogen and phosphorous). For the purpose of this comparative being as precise as possible, the real data from the sensors located in the stirred-tank reactor (radiation, pH, dissolved oxygen and temperature) were introduced in the biologic model to simulate the oxygen production by the microalgae-bacteria consortia. Furthermore, the wastewater properties introduced in the biologic model and used for the simulation were the same nutrient measurements described in "3.1. Laboratory stirred-tank reactor: Tool validation". Specifically, a five days dataset from the laboratory reactor under different environmental conditions was used. For the validation, the oxygen production was determined as a function of dissolved oxygen in the reactor measured and taking into account the oxygen mass transfer in the reactor. Real data are shown in **Figure 3A** by dots, while the full model values are represented by a solid line. From this figure, it can be observed that the model highly fits the experimental oxygen produced by the real system for the given days, which confirms the validation of the oxygen production model included in the interactive tool. The maximal oxygen production measured and simulated was 0.3 mgO_2/Lmin throughout the test. In **Figure 3B**, data of radiation, pH, dissolved oxygen and temperature during the 5 days dataset measured in the laboratory stirred-tank reactor are shown, which were the inputs used for the proposed biological model to simulate the data presented in **Figure 3A**. As observed, these results suggested that the biologic model is capable to reproduce the behavior of the microalgae-bacteria consortia despite the wide environmental variability in parameters as temperature.

Interactive Tool Description

Once the theoretical concepts of the microalgae-bacteria models have been summarized, the functionality of the developed tool is described in this section. The tool is freely available through http://www.eu-sabana.eu/ at the Data and Software website section and does not require a Sysquake license to be run. Windows and Mac versions are available for free. On the other hand, a short video tutorial can be found to describe the main capabilities of the tool. **Figure 4** shows the main screen of the tool.

The graphic part of the tool has been organized in order to facilitate to the user an understanding of the main biologic components which appear in the models along with the operational and environmental parameters that affected them. Moreover, different microalgae strains and bacteria properties can also be analyzed. The users can easily work simulating all of the proposed models on the screen at the same time, which is very useful for a deeper understanding. Moreover, it is possible to visualize the models throughout the four seasons of the year. All the models are always simulated for 24 h, where real data for pH, dissolved oxygen, solar radiation, and medium temperature are used as input to the models. These real data are modified according to the selected season of the year. Data from the raceway reactor described in *Interactive Tool Description* is preloaded in the tool by default. However, the user can load his/her own data from the "Load data" available in the tool. Instructions are given at the tool website. In the following, the main features and options of the tool are described.

The left-hand side of the screen is the parameter section, which is divided into two parts: the model parameters part that is located at the upper area of the screen, and the graphical option parameters located at the lower part of the screen. From the model parameters part, it is possible to modify the different wastewater properties such as organic matter concentration (COD), readily biodegradable soluble organic matter (Ss), the nitrogen in form of nitrate $N\text{-}NO_3^-$, nitrogen in form of ammonium $N\text{-}NH_4^+$, and phosphorous $P\text{-}PO_4^{-3}$ concentrations. Moreover, all the model parameters for microalgae, heterotrophic bacteria and nitrifying bacteria (see **Supplementary Tables 2, 3**) can be interactively modified in this area. Each parameter value in this area can be changed by using a text field or a slider element. The last one allows easily observing the effect of the corresponding parameter in an interactive manner. On the other hand, the graphical parameter option is focused on switching on or off the graphical results of the models. Two groups of checkboxes are available to show or to hide the plots for the different simulated models or the cardinal elements at the right-hand side of the screen. Furthermore, an option to select the season of the year is shown. Once the season of the year is selected, the real data (that includes pH, dissolved oxygen, solar radiation and medium temperature) of a characteristic day for the selected season is used as input to the models as commented above. Together with this option, a checkbox called "Real Data" is also available to show or to hide the used real data in the plots.

The right-hand side of the screen is devoted to showing the graphical results of the simulated models or the real data used as inputs to the model. When the "Real Data" checkbox is switched on, the real input data is shown in the graphics. The dissolved oxygen and the pH are shown at the top, and the solar radiation and the medium temperature are shown at the bottom. However, when the "Real Data" checkbox is switched off (option by default) the simulation results for the models are presented. As mentioned previously, all the models are simulated for a whole day, where the time scale is shown in minutes. The graphic at the upper part of this area shows the results for the four biological models described by **equations 5–8**. The plots in the lower graphic show all the cardinal elements involved in the different models. Notice that these two graphics will only show those results that are selected in the graphical parameter option of the tool.

Illustrative Examples

As described above, the multiple options available in the tool allow simulating a large number of possible scenarios with

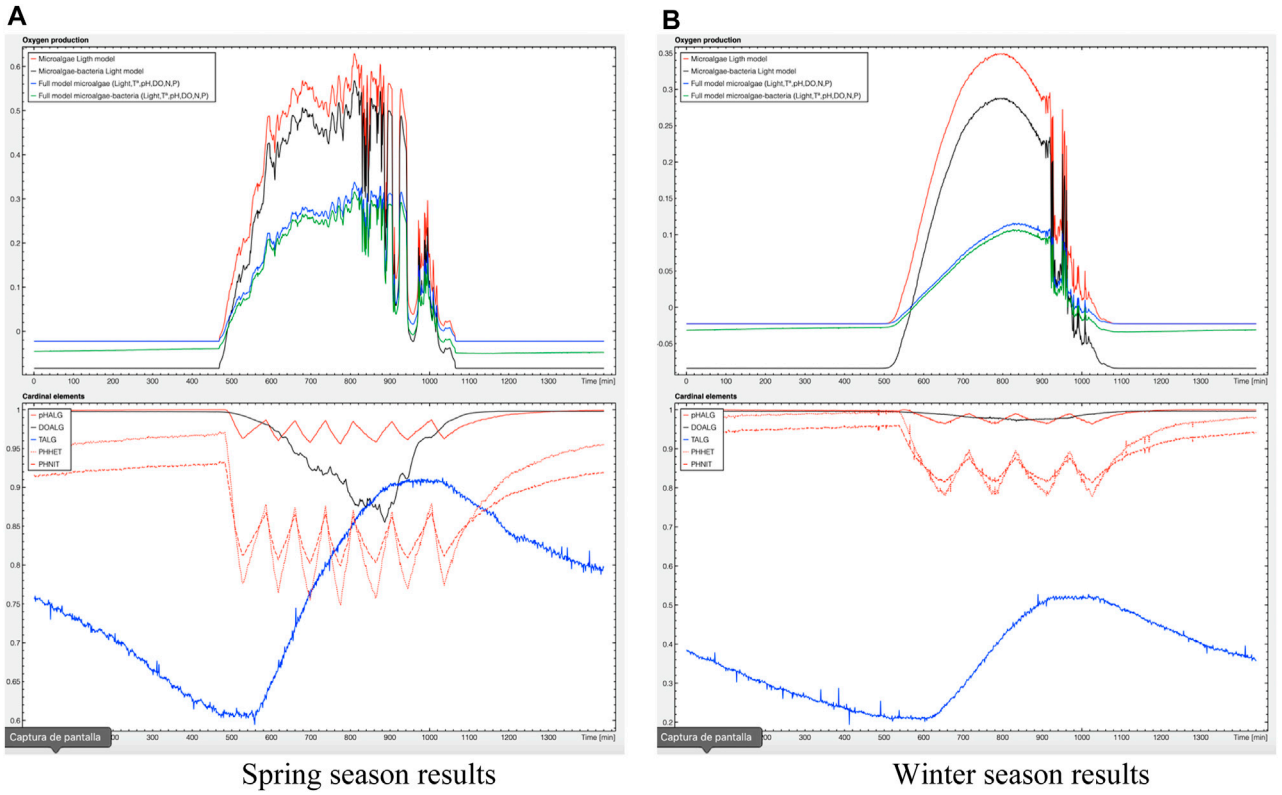


FIGURE 5 | Biological models results for different seasonal data to demonstrate the environmental variables effect on the productivity of the microalgae systems.

scientific-practical interest. Some of the main ideas that can be analyzed with the tool are listed below:

- To simulate the oxygen production by a microalgae-based system considering the presence or absence of bacteria populations.
- To simulate different microalgae strains with different properties and to observe their behavior under different weather conditions and under the presence/absence of bacteria.
- To evaluate the effect of different biomass concentrations or medium height in the reactor.
- To analyze the differences between the "theoretical oxygen production" (determined by solar radiation parameter) and the more realistic oxygen production, including the environmental and operational variables.
- To take into account and modifying the biological parameters which determining the microalgae and bacteria activities.
- To analyze and to visualize the effect of each factor involved in the biological models. For instance, the effect of each cardinal term.
- To evaluate the biological models in all seasons of the year (summer, spring, autumn and winter).
- To visual the real data used for the simulation tool and to relate it with the effect of the different elements.

In the following, a set of illustrative examples are exposed to show the usefulness of the tool. Notice that the interactive capabilities of the tool are difficult to be shown in a written document. So, we encourage the reader to download it and to evaluate the examples by himself/herself.

Importance of Environmental Factors on Microalgae Productivity

The impact of environmental variables on microalgae growth in outdoor cultivation has been widely reported [33]. Compared to laboratory cultivation, in which most of the environmental conditions are controlled, the diurnal and seasonal fluctuations in irradiance and temperature in outdoor cultures involve potential complications on microalgae activity. This effect has been evaluated in **Figure 5** by using the tool, where the four possible biological models presented in this work have been simulated using data from spring and winter seasons. **Figure 6** shows the used real input data of pH, dissolved oxygen, medium temperature and solar radiation for these two seasons. The upper plots in **Figures 5A,B** show the oxygen production by the four biological models for a full day. In this simulation, it can be fully appreciated an oxygen production slight downturn in the models with bacteria compared to biological models which considering only microalgae activity. However, it is remarkable the strong decrease in the oxygen production when environmental and operational factors are considering in microalgae models and microalgae-bacteria

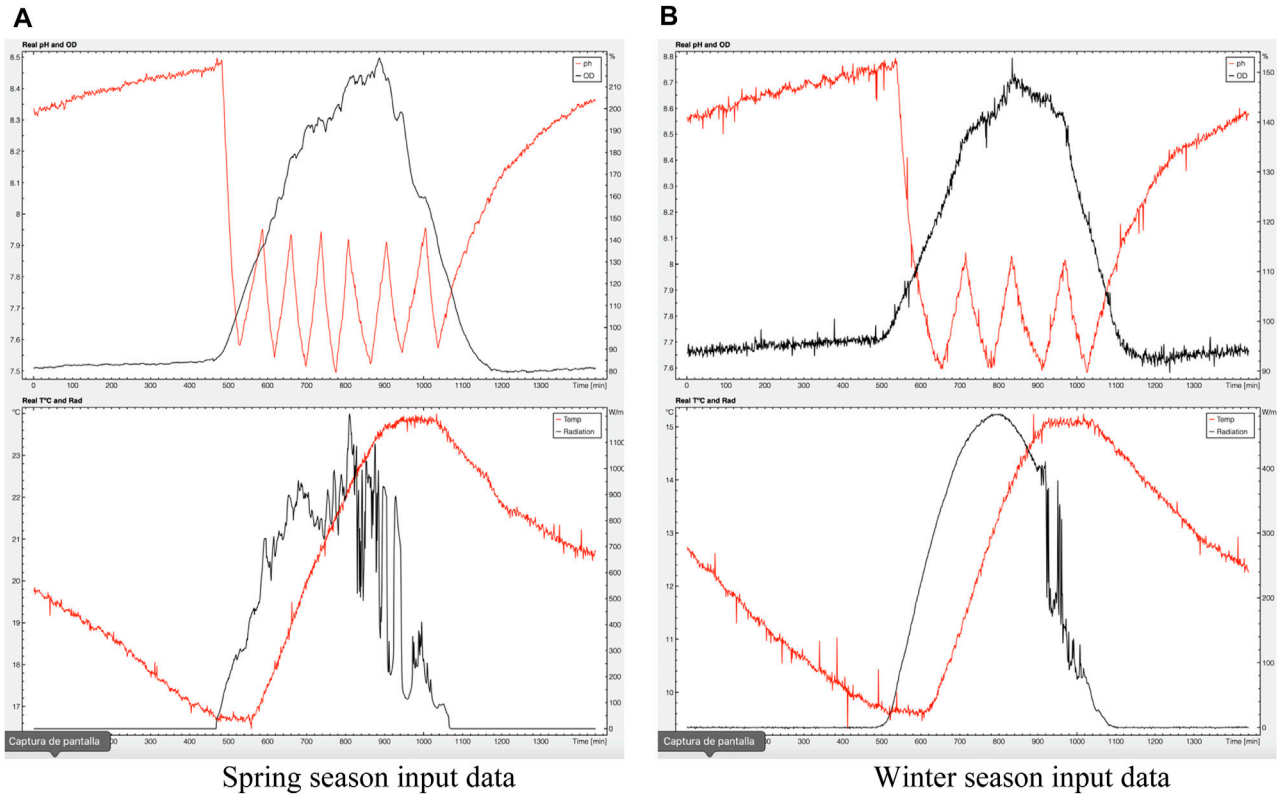


FIGURE 6 | Real input data for the examples showed in **Figure 2** to demonstrate the environmental variables effect on the productivity of the microalgae systems.

models. In the bottom plots of **Figures 5A,B**, the normalized effect of the environmental parameters (pH, dissolved oxygen and temperature) on the microalgae cells is shown.

For microalgae growth, pH is one of the most important factors because it deeply influences most of the enzymatic reactions. Despite a majority of microalgae strains have the optimal pH in the neutral to slightly alkaline range, this is characteristic of each one and should be considered in the mathematical models. Moreover, it should be noted that during the photosynthetic activity, an increase in pH is produced and it should be controlled to avoid inhibitory effects. Currently, a possible strategy to mitigate this negative effect on microalgae activity is to control the pH with injection of CO_2 on demand (Duarte-Santos et al., 2016). As may be seen in the figure, a good pH control make it possible that microalgae can be cultivated in optimal conditions and their production is not affected by abrupt pH changes due to photosynthetic activity. A similar behavior is observed in the dissolved oxygen because microalgae release a large quantity of oxygen during the light hours by photosynthesis. Part of this oxygen is removing by the oxygen desorption process while the rest remains in the culture reducing the photosynthesis rate and favoring the photorespiration of the culture (Costache et al., 2013; Rubio et al., 1999). To solve this problem, most microalgae systems have an air injection system to remove the oxygen excess. Therefore, if pH and dissolved oxygen are well controlled, they will not have a drastic negative effect on productivity.

Finally, the temperature is one of the most crucial factors in the open microalgae-bacteria systems because can modify the microalgae-bacteria growth. To evaluate the temperature effect on microalgae system, three questions should be considered: i) it is not feasible to control the temperature of the culture; ii) optimal temperature depends on each microalgal species (mesophilic, thermophilic and psychrophilic strains); iii) temperature fluctuations depend on geographic localization and season of the year (Ras et al., 2013). In the figure, it is observed that during the spring season, the temperature in the light hours rises up to 24–25°C, which are considering favorable values for the microalgae strain evaluated (*Scenedesmus almeriensis*). However, when it is evaluating the oxygen production in winter (**Figure 3B**), the situation is markedly different due to the low temperatures (10–15°C). No different effects with respect to the rest of the variables (dissolved oxygen and pH) between both seasons were observed. As multiple authors have described, the temperature has remarkable effects on microalgae systems which implicated that below or above optimal temperatures, the activity drastically decreases (Costache et al., 2013; Ras et al., 2013). Therefore, biological models coupled with models of the temperature evolution in the reactors could be especially useful for researchers in microalgae fields to predict the weather impact on the microalgae cultures. These predictions/simulations, along with economic strategies such as regulated the weight of the cultures or covered the systems (greenhouse effect), will allow maximizing the biomass productivity.

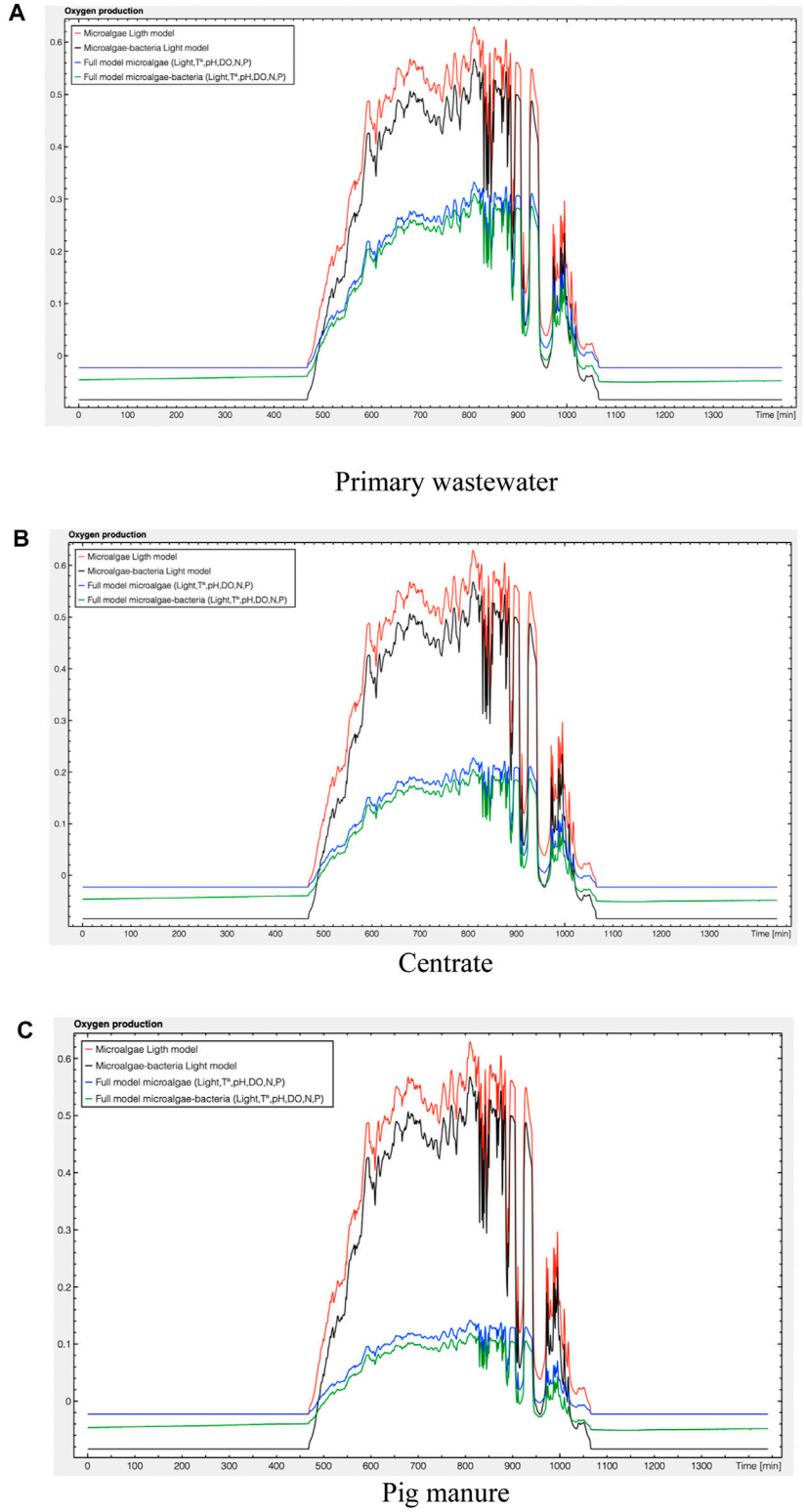


FIGURE 7 | Application of the biological models to predict the productivity of the microalgal based system using different types of wastewater: **(A)** wastewater from primary treatment; **(B)** centrate from anaerobic digestion; **(C)** Pig manure.

TABLE 1 | Composition of the wastewaters used for the simulations.

Parameter (mg·L^{-1})	Primary treatment	Centrate from anaerobic digestion	Pig manure
COD	500	300	22,000
N-NO_3	2.4	5.3	740
N-NH_4	62.6	506.5	2,900
P-PO_4	11.3	12	130

Influence of Wastewater Composition on Microalgae-Bacteria Systems

Microalgae-based technologies have been used for the treatment of human sewage, industrial wastes, and other wastes such as piggery effluent or effluent from food processing factories (Abdel-Raouf et al., 2012). However, most of the microalgae systems used wastewaters from the wastewater treatment plant, in which different types of wastewater are found, noting wastewaters after primary treatment when the solids and fats are removed or the centrate from anaerobic digestion, which contains a high contaminant concentration (Acién et al., 2016). The composition of these wastewaters varies significantly for some reasons such as the location tested and the variations in water consumption in households or the predominant activities in the surrounding area (agriculture, industry, farms, etc.) (Henze et al., 2015). All these wastewaters contain the main nutrients required for microalgae and bacteria growth: organic matter (COD), nitrogen (N) and phosphorus (P). However, the concentrations of them vary between the wastewater used and have the potential to affect the productivities of microalgae-bacteria cultures significantly. To evaluate the influence of nitrogen, phosphorous and organic matter concentrations on microalgae-bacteria systems, the use of three types of wastewater for microalgae production has been simulated with the tool: primary domestic wastewater, centrate from anaerobic digestion, and pig manure during the summer season (**Figure 7**). The composition of these wastewaters is shown in **Table 1** (Acién et al., 2016). Wastewater from primary treatment contains an adequate level of nutrients for microalgae growth, being ammonium the most frequent nitrogen source with concentrations ranging from 0 to 100 mg L^{-1}, while the concentration of nitrate is significantly less. In **Figure 7A**, it possible to appreciate the oxygen production expected when primary domestic wastewater is used. With these wastewater properties and using the spring real data, the maximal oxygen production obtained with the full microalgae-bacteria model would be 0.2 mgO_2/L min. Given these nutrients values, one may note that both heterotrophic and nitrifying bacteria are in optimal growth conditions ranged the normalized effect of nitrogen, phosphorous and organic matter between 0.9 and 1. For microalgae activity, the nitrogen concentration is remarkable because its normalized effect is 0.7, which corresponds to 30% less than the possible maximal value. However, the use of centrate (**Figure 7B**) and pig manure (**Figure 7C**) for microalgae production, involve a strong reduction in oxygen production, being 0.12 and 0.06 mgO_2/L min respectively. These data are caused by the high values of ammonium in the medium (506.5 mg L^{-1} N-NH_4^+ for centrate and 2,900 mg L^{-1} N-NH_4^+ for pig manure). It has been largely reported that ammonium reduces the performance of microalgae cultures, especially at concentrations upper than 100 mg L^{-1} (Cabanelas et al., 2013). Values above this limit are commonly found in centrate and animal manure, making it necessary to dilute this effluent prior to use as the culture medium inside the reactor (García et al., 2017). For that, knowing the exact composition of the wastewater to be treated is mandatory for an optimal treatment process and biomass production, especially to determine if additional carbon, nitrogen, or phosphorus need to be added when a low nutrient concentration appears. By contrast, the use of wastewaters that contain high nutrients values (such as centrate or animal manure), require a prior dilution to avoid inhibition caused by an excess of ammonium or others micropollutants, such as heavy metals. Moreover, this type of wastewaters should be diluted before use, because of their color could prevent light penetration (Acién et al., 2016). Therefore, this interactive tool allows us to simulate different scenarios, without the need to carry out long experiments, to obtain an approximation of the productivity of the wastewater treatment process based on microalgae-bacteria consortia.

Production of Specific Microalgae in Wastewater for Industrial Purposes

Currently, the production of some specific microalgal strains has attracted considerable interest worldwide due to their applications in biofuels, animal food ingredients or agriculture. However, a pure production of microalgae may remain costly for animal feed or agricultural purposes. Despite the wastewater cultivation has made microalgae biotechnology sustainable and economically viable, several other questions related to microalgae production should be considered. As previously mentioned, if pH and dissolved oxygen are controlled and an adequate composition is achieved in the influent wastewater, the two determining factors for production would be light and temperature. In Almería, the light conditions are adequate to maintain production throughout the year (San Pedro et al., 2015). However, the temperature is still a challenge. In this example, we propose the production of two strains of microalgae with different industrial/ commercial interests in Almería (using the actual real data registered) and the minimum, maximum and optimal temperatures reported in (Bernard and Rémond, 2012). The proposed strains are Dunaliella tertiolecta (Tamin = 5°C; T^amax = 38.9°C; T^aopt = 32.6°C) and Nannochloropsis oceanica (T^amin = -0.2°C; T^amax = 33.3°C; T^aopt = 26.7°C). The first one, Dunaliella tertiolecta has been proposed as a potential candidate for biofuels production because of its high oil content and rapid growth rates (Tang et al., 2011). In **Figure 8**, the oxygen production by Dunaliella tertiolecta is shown in spring (**Figure 8A**), summer (**Figure 8B**), autumn (**Figure 8C**) and winter (**Figure 8D**) along with the cardinal effect of temperature using its temperature specific parameters. The rest of parameters

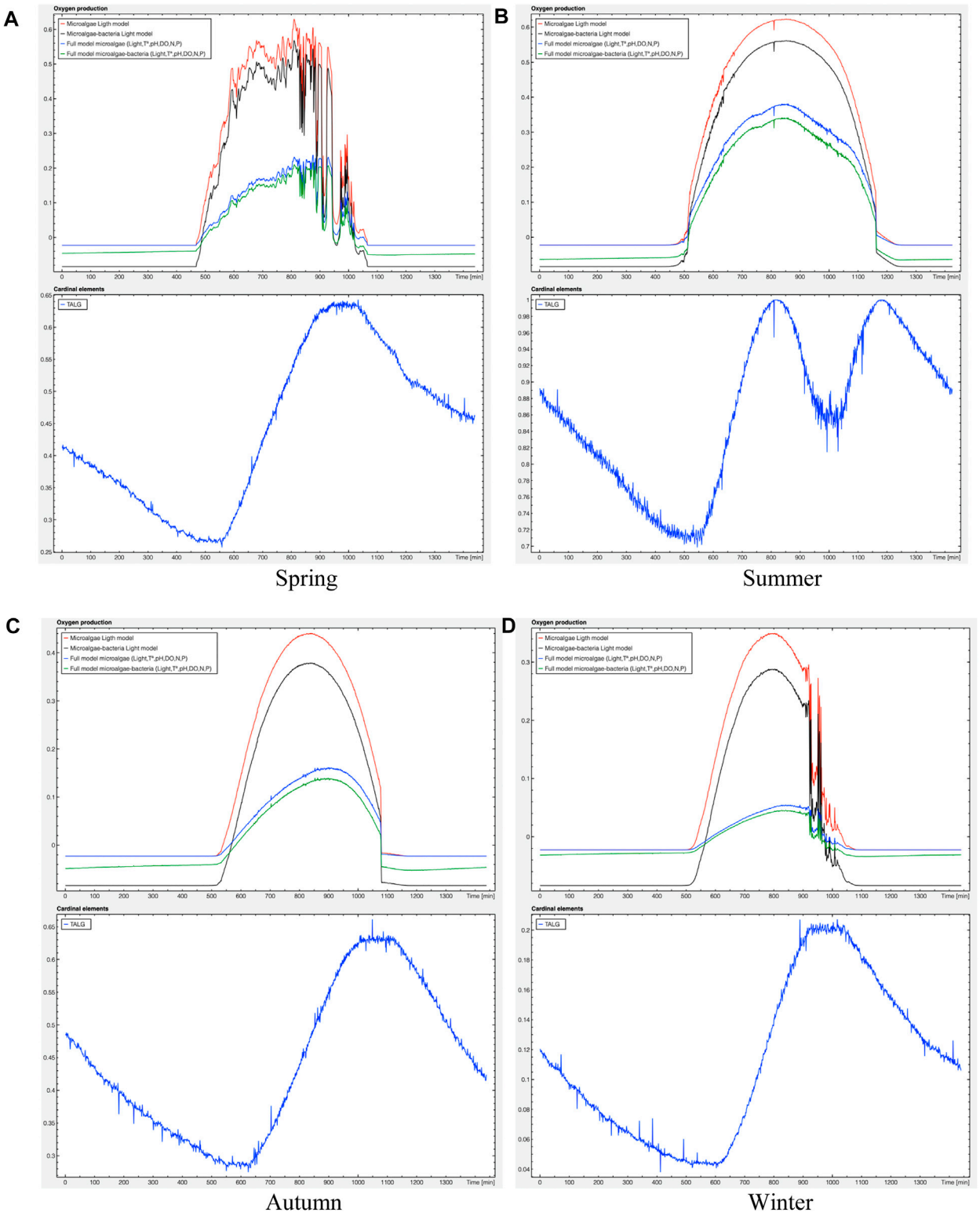


FIGURE 8 | Oxygen production by Dunaliella tertiolecta during the seasons of the year using the proposed biological models, along with the temperature effect using its temperature specific parameters: spring (Panel **8A**) summer (Panel **8B**), autunm (Panel **8C**), winter (Panel **8D**).

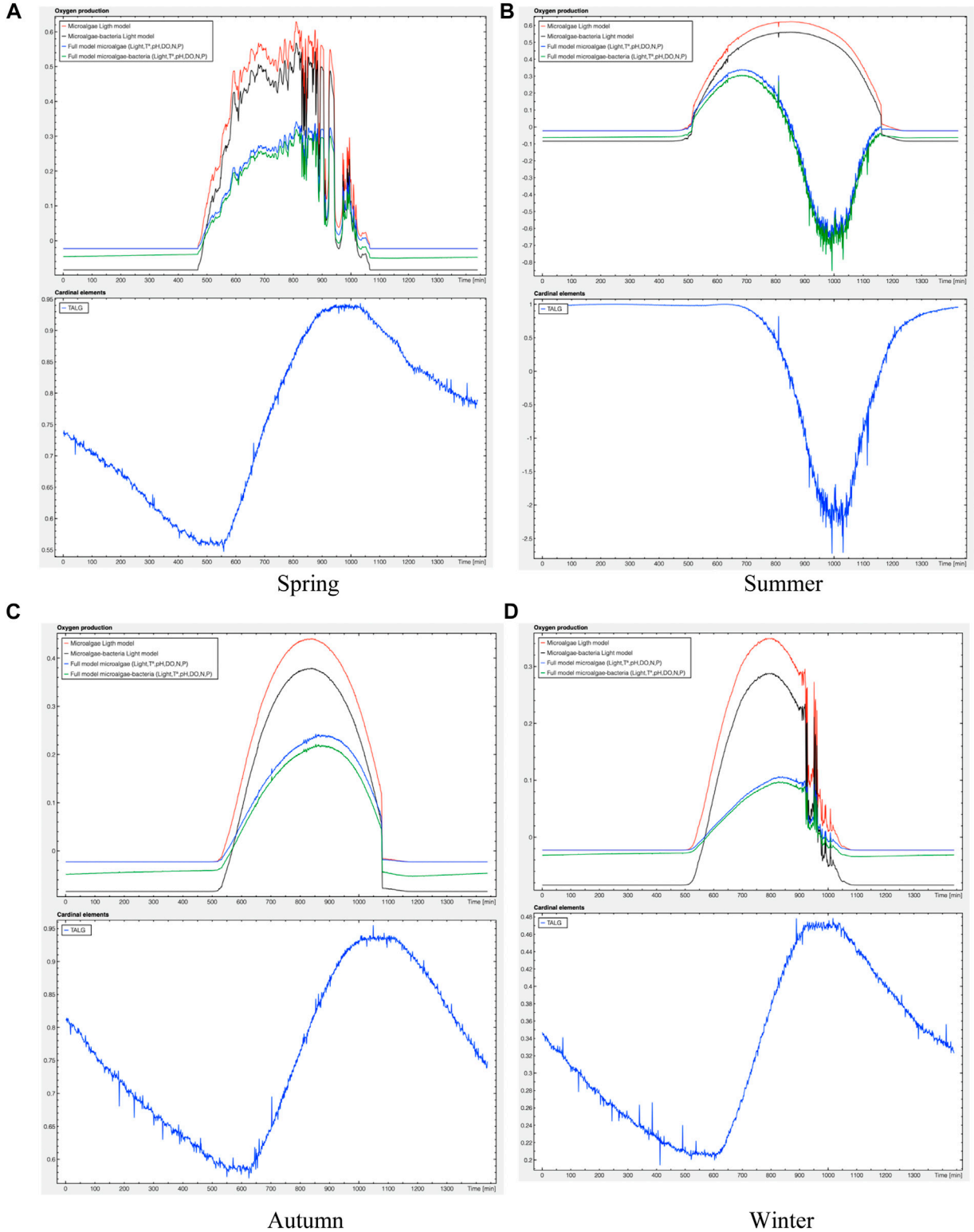


FIGURE 9 | Oxygen production by Nannochloropsis oceanica during the seasons of the year using the proposed biological models, along with the temperature effect using its temperature specific parameters: spring (Panel **9A**) summer (Panel **9B**), autunm (Panel **9C**), winter (Panel **9D**).

are the predetermined by the model and the wastewater used is from primary treatment. The model shows the strong influence of temperature on oxygen production by Dunaliella tertiolecta. Due to its optimal temperature is above 30°C, the major productivity is observed in summer by reaching an oxygen production around 0.22 mgO2/Lmin. The oxygen production decreases both in spring and autumn owing to the falling temperature, which a temperature effect on microalgae cells of 0.6–0.7 at noon. Moreover, the microalgae activity has reduced sharply in winter, being the oxygen production 0.03 mgO2/Lmin. This is presumably because of the low temperatures registered in the winter season with a cardinal effect ranged from 0.05 to 0.2. These results reveal that this strain should be produced in summer periods and even so in spring or autumn seasons (accepting the decrease in productivity), but it is not recommendable in winter. Another microalga with special interest because of its significant capacity to accumulate lipids and various bioactive compounds is Nannochloropsis, widely used for biodiesel fuel and as an aquaculture feed (Li et al., 2020). In **Figure 9**, the production of Nannochloropsis oceanica has been evaluated in Almería during spring (**Figure 9A**), summer (**Figure 9B**), autumn (**Figure 9C**) and winter (**Figure 9D**). Both the oxygen production and the temperature cardinal effects are shown. In these figures, it is possible to observe that the best period to cultivate Nannochloropsis oceanica is spring with an oxygen production around 0.2 mgO2/Lmin. This productivity decreases in autumn (0.15 mgO2/Lmin), while it is not possible to produce this strain in summer because of the high temperatures such as it can be seen in **Figure 9B**, with negative values in the temperature cardinal element. Besides, the production in winter is possible but obtaining a low productivity (0.06 mgO2/Lmin). The results from this section reveal the importance of learning about the selected strains before their large-scale production because of the biological aspects of each microalgal strain are crucial to maximize the economic efficiency of the process. In addition, it should be borne in mind that the production of strains has been evaluated using the environmental conditions of Almería, which must be adapted to other locations, since some microalgae cultures in Almería in summer are unfeasible due to high temperatures but may be perfectly valid in the same season but in another location.

CONCLUSION

Microalgae-bacteria biological models have been proposed and experimentally validated. These models include many equations and parameters and are difficult to interpret in a simple and practical way. However, the use of interactive tools is presented as a promising alternative not only for understanding microalgae-bacteria processes, but also it is a possible solution to predict the productivity of microalgae-bacteria system and consequently, to avoid long experiments, waiting time, and additional costs. Furthermore, the tool allows to obtain fast simulations and make interactive comparisons that very useful for a deep understanding. Notice that some examples have been included in this paper to show the capabilities of the proposed tool, but many other scenarios can be easily simulated with a high scientific interest.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AS-Z: investigation, data curation and original draft preparation/writing. JG: Conceptualization, software and reviewing. FA: Conceptualization, supervision, and funding acquisition. JF-S: Editing and funding acquisition.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fenvs.2021.721324/full#supplementary-material

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7.6. Respirometric assessment of bacterial kinetics in algae-bacteria and activated sludge processes.

Research in this field is supported by the following journal publication:

Title	Respirometric Assessment Of Bacterial Kinetics In Algae-Bacteria And Activated Sludge Processes
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- A. Sánchez-Zurano, S. Rossi, F.G. Acién-Fernández, J.M. Fernández-Sevilla, E. Molina-Grima, E. Ficara, “Evaluación respirométrica de la cinética bacteriana en procesos microalgas-bacterias y lodos activados”. Event: META 2022. Place: Sevilla, Spain.

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Respirometric assessment of bacterial kinetics in algae-bacteria and activated sludge processes

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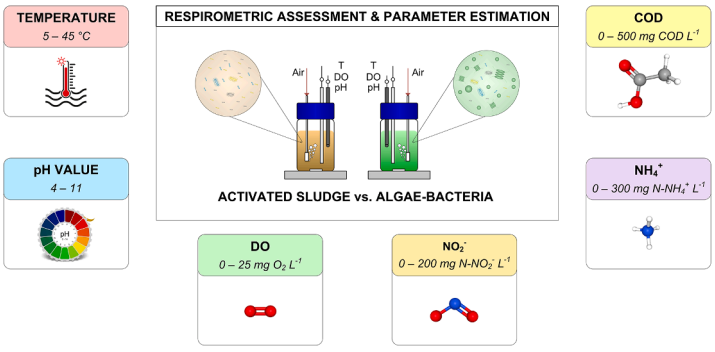
HIGHLIGHTS

- Respirometric procedure to estimate bacterial activities in algae-bacteria systems.
- Comparison of bacterial kinetic parameters in algae-bacteria and activated sludge.
- Strong influence of pH, temperature, oxygen, and substrates on oxygen uptake rates.
- Cardinal models described the effects of different temperature and pH on bacteria.
- Monod/Andrews models described the effects of oxygen and substrate availability.

GRAPHICAL ABSTRACT



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ABSTRACT

Algae-bacteria (AB) consortia can be exploited for effective wastewater treatment, based on photosynthetic oxygenation to reduce energy requirements for aeration. While algal kinetics have been extensively evaluated, bacterial kinetics in AB systems are still based on parameters taken from the activated sludge models, lacking an experimental validation for AB consortia. A respirometric procedure was therefore proposed, to estimate bacterial kinetics in both activated sludge and AB, under different conditions of temperature, pH, dissolved oxygen, and substrate availability. Bacterial activities were differently influenced by operational/environmental conditions, suggesting that the adoption of typical activated sludge parameters could be inadequate for AB modelling. Indeed, respirometric results show that bacteria in AB consortia were adapted to a wider range of conditions, compared to activated sludge, confirming that a dedicated calibration of bacterial kinetics is essential for effectively modelling AB systems, and respirometry was proven to be a powerful and reliable tool to this purpose.

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1. Introduction

Biological secondary treatment is traditionally employed in wastewater treatment plants (WWTPs) to remove the dissolved nutrients (i.e., carbon (C), nitrogen (N) and phosphorous (P) compounds), the most widely applied system being the activated sludge (AS) process (Hreiz et al., 2015; Orhon, 2015). In this bioremediation process, diverse groups of microorganisms are responsible for wastewater treatment, the main actors being heterotrophic bacteria (HB), and autotrophic nitrifying bacteria, i.e., ammonia-oxidizing Bacteria (AOB), and nitrite-oxidizing bacteria (NOB). Despite the high Chemical Oxygen Demand (COD), N and P removal efficiencies obtained by AS processes, high energy demands, and operating costs are caused by aeration, mixing, and reagents required to properly operate the process (Crini and Lichtfouse, 2019). Nutrient losses and greenhouse gas emissions are also reported as major disadvantages of conventional bioremediation systems (Campos et al., 2016; Capodaglio and Olsson, 2019). To overcome these drawbacks, algae-bacteria (AB) consortia has been proposed as a sustainable alternative, as this biotechnology exploits renewable sunlight, consumes atmospheric CO_2, and allows for N and P removal and recovery, while generating valuable bio-products from the algal biomass (Chan et al., 2022; Mantovani et al., 2020; Mennaa et al., 2019; Nguyen et al., 2019).

AB consortia are generally cultivated for wastewater treatment in large-scale outdoor raceway ponds (RWPs), that are exposed to continuous variations in the environmental conditions (mainly: temperature, irradiance, and evaporation rates), driving the algal and bacterial growth kinetics to follow both daily and seasonal patterns. As a result, other key parameters such as pH and dissolved oxygen (DO) noticeably vary during the day, further influencing the water chemistry and growth kinetics (Casagli et al., 2021a; Robles et al., 2020). Moreover, the availability of nutrients in AB wastewater systems is in low amounts, or in large excess, introduce other limitation or inhibition factors for both the algal and bacterial growth (Aparicio et al., 2022; Rossi et al., 2020a; Rossi et al., 2020b; Rossi et al., 2020c). This makes microalgae-bacteria modelling especially challenging, and imposes the calibration of many kinetic parameters, for which few experimental guidelines have been released (Shoener et al., 2019). Within this context, respirometry have been widely applied as a rapid tool for the assessment of kinetic parameters of AS, aimed at the calibration of Activated Sludge Models (ASM) (Henze et al., 2015; Mainardis et al., 2021). On the other hand, photo-respirometry can be successfully applied to assess the activity of AB consortia treating municipal and industrial wastewaters (Flores-Salgado et al., 2021; Rossi et al., 2018; Sánchez-Zurano et al., 2020). Respirometric data have been profitably used to calibrate mathematical models describing AB systems for wastewater treatment (Casagli et al., 2021b; Sánchez-zurano et al., 2021). However, only a few studies proposed specific protocols to assess bacterial activities in AB systems (Flores-Salgado et al., 2021; Sánchez-Zurano et al., 2020), these experiments being generally conducted on the algal biomass alone, which has more inter-species variability than bacteria (Rossi et al., 2020b). In AB modelling, it is generally assumed that the bacterial populations can be modelled by using parameters that are conventionally adopted in ASMs. However, adaptation phenomena in the AB unit can be expected, driven by continuous environmental perturbations and nutrient/substrates competition with algal species (Fallahi et al., 2021; González-Camejo et al., 2020a; González-Camejo et al., 2020b; Ramanan et al., 2016).

In this work, a comprehensive respirometric study of the dependence of bacterial activities on environmental conditions (temperature, pH and DO) and substrates concentrations (COD, N-NH_4^+, and N-NO_2^-) was carried out. The procedure was developed and applied to samples collected from a full scale conventional activated sludge tank (fed on municipal wastewater), and from a pilot-scale algae-bacteria raceway pond (fed on the liquid fraction of anaerobic digestate), both in operation at the same WWTP. The respirometric study allowed to model the behaviour of aerobic bacteria (namely, HB, AOB, and NOB) in the two systems, and to identify the most relevant kinetic parameters describing the effect of the above-mentioned conditions. Parameter identification using experimental data made it possible to compare these effects on each bacterial population, aiming at providing a suitable methodology and an extensive dataset to calibrate existing AB growth models, thus improving their efficiency, and making them effective tools for the improvement of the AB-based process performances. Indeed, by a model-based optimization of crucial parameters, such as temperature, DO, pH and nutrient loads, biomass production and wastewater treatment efficiency can be enhanced.

Table 1
Comparison of the main wastewater characteristics and reactor conditions in the two treatment systems. Results are reported as average ± standard deviation. n. a.: not available.

Parameter	Unit	Activated Sludge (AS)		Algae-Bacteria (AB)	
		Influent	Reactor	Influent	Reactor
N-NH_4^+	mg N·L^{-1}	29.4 ± 10.9	1.0 ± 0.9	219 ± 51	34.5 ± 21.1
N-NO_2^-	mg N·L^{-1}	–	n.a.	0.31 ± 0.08	75.5 ± 50.9
N-NO_3^-	mg N·L^{-1}	–	10.1 ± 3.1	0.32 ± 0.75	58.3 ± 57.2
Total N	mg N·L^{-1}	32.1 ± 10.3	6.4 ± 3.6	220 ± 52	168 ± 71
P-PO_4^{3-}	mg P·L^{-1}	n.a.	0.74 ± 0.30	9.2 ± 2.4	6.3 ± 2.5
Total P	mg P·L^{-1}	4.6 ± 2.2	0.8 ± 0.4	13.1 ± 1.1	12.4 ± 1.5
COD_S	mg COD·L^{-1}	n.a.	n.a.	164 ± 60	205 ± 154
COD_{TOT}	mg COD·L^{-1}	308 ± 128	17.9 ± 8.7	301 ± 40	669 ± 103
$BOD_{TOT,5}$·COD_{TOT}^{-1}	–	0.57 ± 0.09	n.a.	0.30 ± 0.08	0.09 ± 0.01
TSS	g TSS·L^{-1}	0.15 ± 0.08	7.3 ± 1.3	0.21 ± 0.09	0.47 ± 0.24
OD_{680}	–	n.a.	n.a.	0.10 ± 0.06	0.42 ± 0.21
HRT	d	n.a.	0.7 ± 0.1	n.a.	15.0 ± 0.0
Temperature	°C	n.a.	18.3 ± 4.6	n.a.	20.2 ± 6.0
Temperature range	°C	n.a.	13.0–21.9	n.a.	4.7–32.4
pH	–	7.5 ± 0.3	6.9 ± 0.1	8.6 ± 0.2	7.1 ± 0.3
pH range	–	n.a.	6.7–7.1	n.a.	5.9–7.9
DO	mg DO·L^{-1}	n.a.	0.27 ± 0.43	n.a.	8.1 ± 3.1
DO range	mg DO·L^{-1}	n.a.	0.0–3.4	n.a.	0.3–20.8

2. Materials and methods

2.1. Wastewater characteristics, treatment systems and climate

The biomass used for respirometric tests was sampled from two wastewater treatment systems, both located in the WWTP of Bresso-Niguarda (Milan, Italy): (i) a full-scale AS tank receiving pre-treated (screening, sand/grit removal, primary settling) municipal wastewater, and (ii) a pilot-scale AB RWP, treating the liquid fraction of centrifuged digestate originated from the anaerobic digestion of excess activated sludge.

The AS tank (6100 m^3) was operated continuously with an average HRT of 17 h. The tank was located outdoor, and it was subject to weather conditions and low temperatures during winter. However, the large volume and the fact that it was built underground, made it possible to maintain relatively high temperatures throughout the year (13–22 °C). The influent wastewater had average total COD, NH_4^+, and total P concentrations of 307 mg COD·L^{-1}, 22.9 mg N-NH_4^+·L^{-1}, and 4.6 mg

Table 2
Experimental design describing the environmental conditions and nutrient concentrations maintained during respirometric tests.

Parameter	Unit	Target Populations	Range tested
Temperature	°C	HB, AOB, NOB	5–45
pH value	–	HB, AOB, NOB	4–11
Dissolved oxygen	mg DO·L^{-1} (%DO_{SAT})	HB, AOB, NOB	0–25 (0–275%)
COD	mg COD·L^{-1}	HB	0–500
NH_4^+	mg N·L^{-1}	AOB	0–300
NO_2^-	mg N·L^{-1}	NOB	0–200

P·L^{-1}, respectively. Under typical operational conditions, the average pH and DO values in the AS tank were 6.9 pH units and 0.3 mg DO·L^{-1}.

The AB cultivation unit was a 0.87 m^3 RWP, which was operated in continuous mode to maintain an HRT of 6 d. The reactor had a 0.15 m liquid height and a total surface of 5.8 m^2. The RWP was located into a greenhouse, to mitigate the cold winter conditions of Northern Lombardy. More details about the AB unit are available in a previous work (Mantovani et al., 2020). The AB culture was periodically examined for identifying algal species and other microorganisms. During the experimentation, the dominant algae species were *Scenedesmus sp.* and *Chlorella sp.* ($1.6{\cdot}10^6 \pm 1.9{\cdot}10^6$ and $1.1{\cdot}10^6 \pm 1.3{\cdot}10^6$ cells·mL^{-1}, respectively, on average). The temperature range and the maximum solar radiation to which the algal culture was exposed in the RWP were 5–32 °C and 1010 W·m^{-2}, respectively. The pH was maintained within 6–8 by temporized bubbling of pure CO_2 coming from the full-scale biogas upgrading unit. The DO was measured online, reaching minimum and maximum values of 0.3–20.8 mg DO·L^{-1}, respectively. The influent digestate had average concentrations of 220 mg N-NH_4^+·L^{-1}, 177 mg COD·L^{-1}, and 9 mg P-PO_4^{3-}·L^{-1}, respectively. Nitrite was almost absent in both the WW and influent digestate (<0.3 mg N-$NO2^-L^{-1}$), however partial nitrification occurred in the RWP, with NO_2^- accumulation peaks up to 144 mg N-NO_2^-·L^{-1}. The main characteristics of the influent wastewater and treatment systems are reported in Table 1.

2.2. Respirometric device

The respirometer used to evaluate respiration rates was composed of two 500 mL glass bottles filled to 300 mL with biomass suspensions. The device was equipped with probes for temperature, DO and pH. Also, the device was equipped by a control unit collecting and communicating the data every 3 s. Tests were conducted in a thermostatic chamber to maintain the desired temperatures. The pH was controlled at the desired levels by automatic titration of concentrated HCl or NaOH solutions (0.1–0.5 M), while the DO concentration was controlled by on-demand aeration. A more detailed description of the respirometric equipment is available elsewhere (Rossi et al., 2020a,b, 2021).

2.3. Respirometric procedures and experimental design

The experimental procedure was inspired by typical respirometric protocols available for the AS process (Vanrolleghem et al., 1999) and for AB consortia (Rossi et al., 2020a,b; Sánchez-Zurano et al., 2020). For each parameter tested (i.e., temperature, pH, DO, and substrate concentrations), a different respirometric protocol was applied for each bacterial population (AOB, NOB, HB).

First, AB samples were concentrated 10 times by centrifugation at 10,000 g (Filtermaxx VWO, USA) to remove residual culture nutrients and to adjust the biomass concentration, then the samples were immediately resuspended in fresh Bold's Basal Medium (BBM) to avoid stress on the algal populations, which could have resulted in the release of organic matter (González-Camejo et al., 2020b). The composition of the BBM used to resuspend the biomass was previously described (Rossi et al., 2021). AS samples were left under dark conditions for 24 h, bubbling unfiltered ambient air to reach substrate depletion and endogenous conditions.

After pre-treatments, the environmental conditions were modified according to the experimental design (see Table 2), and the tests started. Respirometric protocols were constituted by a series of three re-aeration cycles. The environmental parameter under investigation was later varied, and the re-aeration cycles were further repeated for all parameters' combination. When a certain parameter was varied, all other parameters were kept at reference levels (T = 20 °C, pH = 7.5, DO = 5–6 mg DO·L^{-1}), and the substrate concentrations were maintained to non-limiting concentrations. The control was reactor A (with substrate availability), and the limited reactor was reactor B (with no substrates or with inhibitors). For tests performed on AOB, NH_4^+ (100 mg N·L^{-1}) was added in both respirometric vessels, while reactor B was supplemented with 10 mg·L^{-1} of ATU to stop ammonia oxidation (Rossi et al., 2018). For NOB and HB, reactor A was maintained under endogenous and substrate-limited conditions, while the reactor B was supplemented with the relevant substrate (NO_2^- and COD at concentrations of 25 mg N·L^{-1} and 100 mg COD·L^{-1}, respectively (Sánchez-Zurano et al., 2020). In substrates tests, each substrate was added through successive spikes, aimed at increasing the substrate availability, up to the desired concentrations (see also Section 2.4). The tests were divided into four series, each targeting temperature, pH, dissolved oxygen, and substrate concentrations. The values of the parameters to be tested were chosen to cover their range in outdoor AS and AB systems (see Table 1), as summarised in Table 2.

2.4. Numerical methods

A detailed description of numerical methods to calculate the OURs is reported in previous studies (Rossi et al., 2020a,b, 2021; Sánchez-Zurano et al., 2020). Briefly, a DO mass balance was applied to the respirometric bottles (Eq. (1)), allowing to define relevant rates affecting the dynamic evolution of the DO during each phase, i.e.: the oxygen uptake rate (OUR) and the oxygen transfer rate (OTR).

$$\frac{d\,(DO)}{d\,(t)} = OUR_i + OTR, \quad (i = 1, \ldots, 3) \tag{1}$$

where: OUR_i is the oxygen uptake rate for the considered phase *i* [mg DO·L^{-1}·h^{-1}], and OTR is the oxygen transfer rate for the given respirometer characteristics [mg DO·L^{-1}·h^{-1}].

To describe the oxygen mass transfer, the following equation was used (Eq. (2)):

$$OTR = \theta^{(T-T_{REF})}{\cdot}k_L a_{20}{\cdot}(DO_{SAT}\text{-}DO) \tag{2}$$

where: $\theta = 1.024$ [−] is the temperature correction coefficient according to previous guidelines (ASCE, 1993), T_{REF} = 20 °C is the reference temperature, k_La_{20} = 1.06 [h^{-1}] is the volumetric gas–liquid mass transfer coefficient, estimated through dedicated reaeration tests in clean water at 20 °C (see also supplementary material), DO_{SAT} [mg DO·L^{-1}] is the oxygen saturation at the considered temperature, calculated from the appropriate Henry constant (Rossi et al., 2021).

Given that the DO dynamics were recorded online, and the OTR was calculated by knowing the volumetric mass transfer coefficient k_La, the OUR could be estimated for each phase. Specific oxygen uptake rates ($SOUR_i$, [mg DO·g TSS^{-1}·h^{-1}]) were calculated for each phase, by dividing the obtained OUR_i values by the TSS concentration expressed in [g TSS·L^{-1}] (Eq. (3)).

$$SOUR_i = \frac{OUR_i}{TSS}, \quad (i = 1, \ldots, 3) \tag{3}$$

The bacterial activity of each population ($SOUR_X$ in Eq. (4), where X = HB, AOB, or NOB) was determined by difference among the activity recorded in the control reactor A and the limited reactor B.

$$SOUR_{X,i} = SOUR_{A,i} - SOUR_{B,i} \ (X = HB,\ AOB,\ NOB;\ i = 1, \ldots, 3) \tag{4}$$

Table 3
Results of parameter identification for heterotrophic bacteria (HB), ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB) in activated sludge (AS) and algae-bacteria (AB) consortia. Results are expressed as value (standard error). n.a.: not applicable.

Variable	Fitting Function	Model parameter	Unit	HB		AOB		NOB	
				AS	AB	AS	AB	AS	AB
T	CTMI (Eq. (6))	T_{MIN}	°C	−7.7 (4.3)	−4.3 (4.2)	1.3 (2.5)	0.48 (2.88)	−7.8 (5.4)	−4.3 (5.2)
		T_{OPT}	°C	36.1 (1.1)	35.59 (0.87)	30.19 (0.84)	34.13 (0.80)	36.0 (1.3)	34.4 (1.3)
		T_{MAX}	°C	42.90 (0.14)	40.52 (0.17)	41.78 (0.31)	43.75 (0.26)	43.43 (0.73)	41.85 (0.50)
pH	CPM (Eq. (7))	pH_{MIN}	–	4.19 (0.17)	2.93 (0.50)	4.24 (0.21)	4.43 (0.22)	4.40 (0.10)	4.04 (0.13)
		pH_{OPT}	–	8.02 (0.28)	8.77 (0.35)	8.48 (0.26)	9.45 (0.18)	7.46 (0.23)	7.83 (0.23)
		pH_{MAX}	–	11.06 (0.14)	11.13 (0.11)	10.80 (0.08)	10.82 (0.02)	11.21 (0.17)	11.00 (0.13)
DO	Monod (Eq. (8)), Andrews (Eq. (9))	k_{DO}	mg DO·L^{-1}	1.25 (0.25)	n.a.	n.a.	n.a.	2.12 (0.16)	2.28 (0.41)
		α	L·d·mg DO^{-1}	n.a.	8.75 (3.06)	0.59 (0.15)	1.45 (0.31)	n.a.	n.a.
		DO_{OPT}	mg DO·L^{-1}	n.a.	1.29 (0.21)	6.64 (0.62)	3.27 (0.29)	n.a.	n.a.
COD	Monod (Eq. (8))	k_{COD}	mg COD·L^{-1}	4.39 (0.83)	3.46 (0.61)	n.a.	n.a.	n.a.	n.a.
NH_4^+	Andrews (Eq. (9))	α	L·d·mg N^{-1}	n.a.	n.a.	3.08 (0.60)	2.38 (0.32)	n.a.	n.a.
		NH_{4OPT}	mg N·L^{-1}	n.a.	n.a.	10.55 (3.08)	10. 86 (0.98)	n.a.	n.a.
NO_2^-	Monod (Eq. (8))	k_{NO2}	mg N·L^{-1}	n.a.	n.a.	n.a.	n.a.	0.76 (0.12)	4.58 (0.59)

To compare test results among different conditions, OUR data were finally normalized by the maximum experimental value recorded, i.e., $SOUR_{MAX}$ ($SOUR_{NORM,X,i}$, Eq. (5)).

$$SOUR_{NORM,X,i} = \frac{SOUR_{X,i}}{SOUR_{MAX}} \quad (X = HB, AOB, NOB;\ i = 1, \ldots, 3) \tag{5}$$

To model the respiration dependence from each tested condition, commonly applied models were used (Casagli et al., 2021b; Sánchez-zurano et al., 2021; Solimeno et al., 2019).

The temperature dependence was modelled using the cardinal temperature model with inflection (CTMI), shown in Eq. (6) (Rosso et al., 1995):

$$\frac{SOUR}{SOUR_{MAX}} = \begin{cases} 0, \text{ if } T < T_{MIN} \\ \dfrac{(T\text{-}T_{MAX})\cdot(T\text{-}T_{MIN})^2}{(T_{OPT}\text{-}T_{MIN})\cdot((T_{OPT}\text{-}T_{MIN})\cdot(T\text{-}T_{OPT})\text{-}(T_{OPT}\text{-}T_{MAX})\cdot(T_{OPT}+T_{MIN}\text{-}2\cdot T))}, \text{ if } T_{MIN} < T < T_{MAX} \\ 0, \text{ if } T > T_{MAX} \end{cases} \tag{6}$$

Where: T_{MIN} is the minimum cardinal temperature below which the respiration rate is zero [°C], T_{OPT} is the optimal temperature for which the respiration rate is maximum [°C], T_{MAX} is the maximum cardinal temperature above which the respiration rate is zero [°C].

To evaluate the dependence on the pH value, the cardinal pH model (CPM) in Eq. (7) (Rosso et al., 1995) was fitted to experimental data:

$$\frac{SOUR}{SOUR_{MAX}} = \begin{cases} 0, \text{ if } pH < pH_{MIN} \\ \dfrac{(pH\text{-}pH_{MIN})\cdot(pH\text{-}pH_{MAX})}{(pH\text{-}pH_{MIN})\cdot(pH\text{-}pH_{MAX})\text{-}(pH\text{-}pH_{OPT})^2}, \text{ if } pH_{MIN} < pH < pH_{MAX} \\ 0, \text{ if } pH > pH_{MAX} \end{cases} \tag{7}$$

where: pH_{MIN} is the minimum cardinal pH value below which the respiration rate is zero [-], pH_{OPT} is the optimal pH value for which the respiration rate is maximum [-], pH_{MAX} is the maximum cardinal pH value above which the respiration rate is zero [-].

The dependence on DO and nutrients (NH_4^+, NO_2^-, COD) was expressed as either the Monod function with nutrient limitation in Eq. (8) (Monod, 1942), or Andrews kinetics, if inhibition at high substrate concentrations occurred, as shown in Eq. (9) (Turon et al., 2015).

$$\frac{SOUR}{SOUR_{MAX}} = \frac{S}{S + K_S} \tag{8}$$

where: S represents either the concentration of DO or the relevant substrate (NH_4^+ for AOB, NO_2^- for NOB, and COD for HB) [mg·L^{-1}], K_S is the half-saturation constant for DO or for the substrate [mg·L^{-1}];

$$\frac{SOUR}{SOUR_{MAX}} = \frac{S}{S + \frac{SOUR_{MAX}}{\alpha}\cdot\left(\frac{S}{S_{OPT}}\text{-}1\right)^2} \tag{9}$$

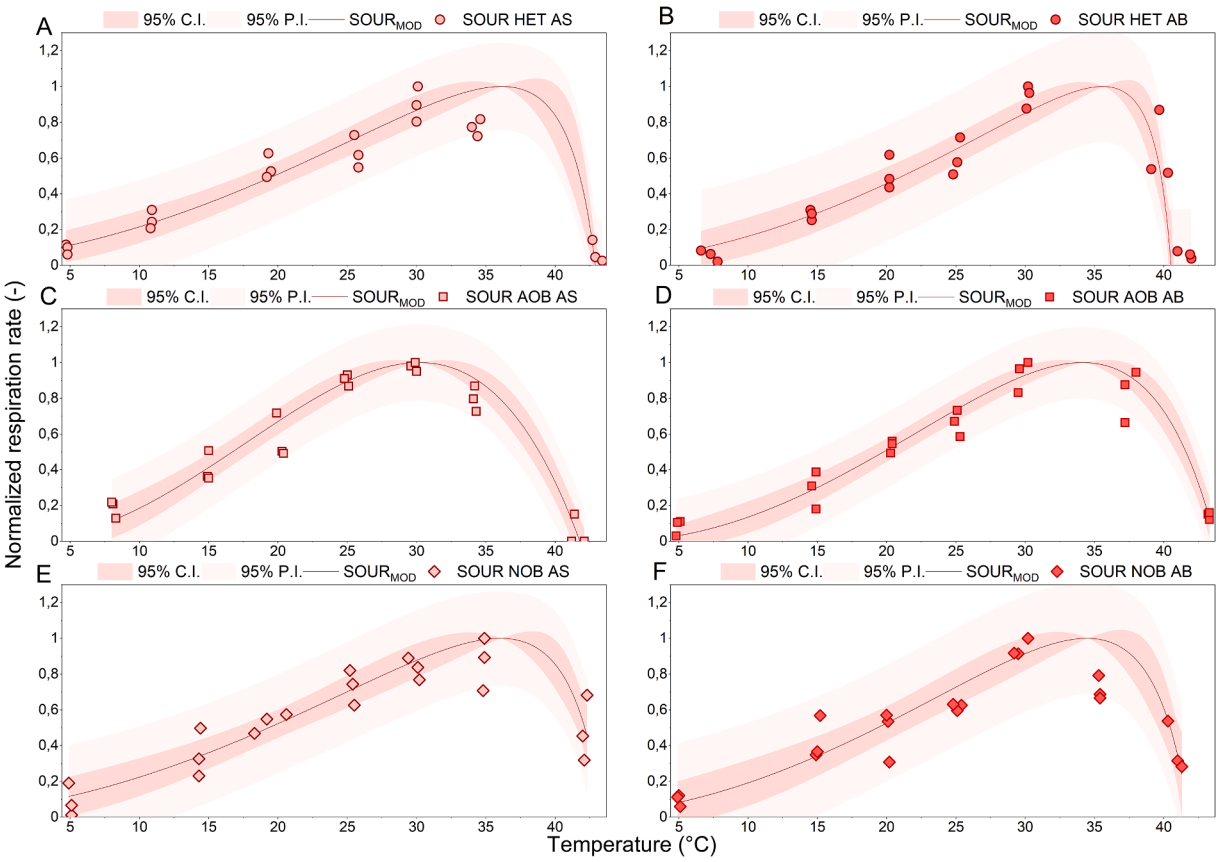

Fig. 1. Effect of temperature on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

where: α [L·d·mg^{-1}] is the initial slope coefficient; S_{OPT} [mg·L^{-1}] is the optimum concentration of DO or of the considered substrate, for which the respiration rate is maximum.

2.5. Statistical methods and software

Raw data were exported in the software Excel 365 (Microsoft) and organized as input tables for subsequent elaborations. Data were then imported in MATLAB R2021b (The Mathworks) and the SOUR values were estimated from raw data using the *Optimization Toolbox* (function: *lsqcurvefit*). Further elaborations, and data plotting, was performed using OriginPro 2020b (OriginLab Corporation), including SOUR data normalization, nonlinear curve fitting, and model statistics. The reduced chi-squared, residual sum of squares, and the adjusted r-squared were used to express the goodness of fit for selected models. Confidence and prediction intervals were calculated at the significance level of 95% (α = 0.05).

2.6. Analytical methods and reagents used

The TSS concentrations were measured according to Standard Methods (APHA, 2017). Triplicate measurements of the OD_{680} were assessed using a 1-cm path plastic cuvette and read using a spectrophotometer (Hach Company, Model: DR 3900). For AB, the linear regression among the TSS and OD_{680} was used to rapidly estimate the TSS concentration based on faster OD_{680} measurement. All chemicals used to prepare synthetic media (i.e., the synthetic BBM and the concentrated NH_4, NO_2, COD and ATU solutions) were reagent-grade from Sigma-Aldrich.

3. Results and discussion

The results of the fitting procedures for the AS and AB samples (i.e., the estimated model parameters describing the dependence on temperature, pH, DO and substrates) are given in Table 3, respectively. Detailed residual analyses and model statistics, including the reduced Chi-square, residual sum of squares, and adjusted R-square, are reported in see supplementary materials. All models were able to represent the experimental dataset (adjusted R-square values: 0.54–0.94), confirming both the adequacy of the equations used in algae-bacteria models, and the versatility of respirometric tests to determine bacterial kinetics rapidly and reliably.

Results can be discussed by considering that AS and AB were sampled from systems subjected to different conditions, which could have potentially strong impacts on the response of bacterial communities and their kinetic parameters. As the AS and AB conditions significantly varied, bacterial response was expected to reflect certain differences among the two samples. Regarding the distinct conditions experienced by bacteria, the main differences mainly arose from the presence of microalgae, the reactor geometry/scale, and the wastewater characteristics, influencing the environmental conditions of the suspension and the composition and activities of the microbial community. In particular, the scale and geometry of reactors mainly had an impact on the thermal properties and thermal inertia of the biomass suspension. Indeed, the AS had a volume of 6100 m^3, which is several orders of magnitude higher than the scale of the RWP (0.87 m^3). In addition, the AS tank of the Bresso-Niguarda plant was built underground, so that thermal excursions were further buffered, and the AS culture had a more stable temperature throughout the year. On the other hand, the AB pond had higher thermal dispersions, since the pilot plant floor was also in

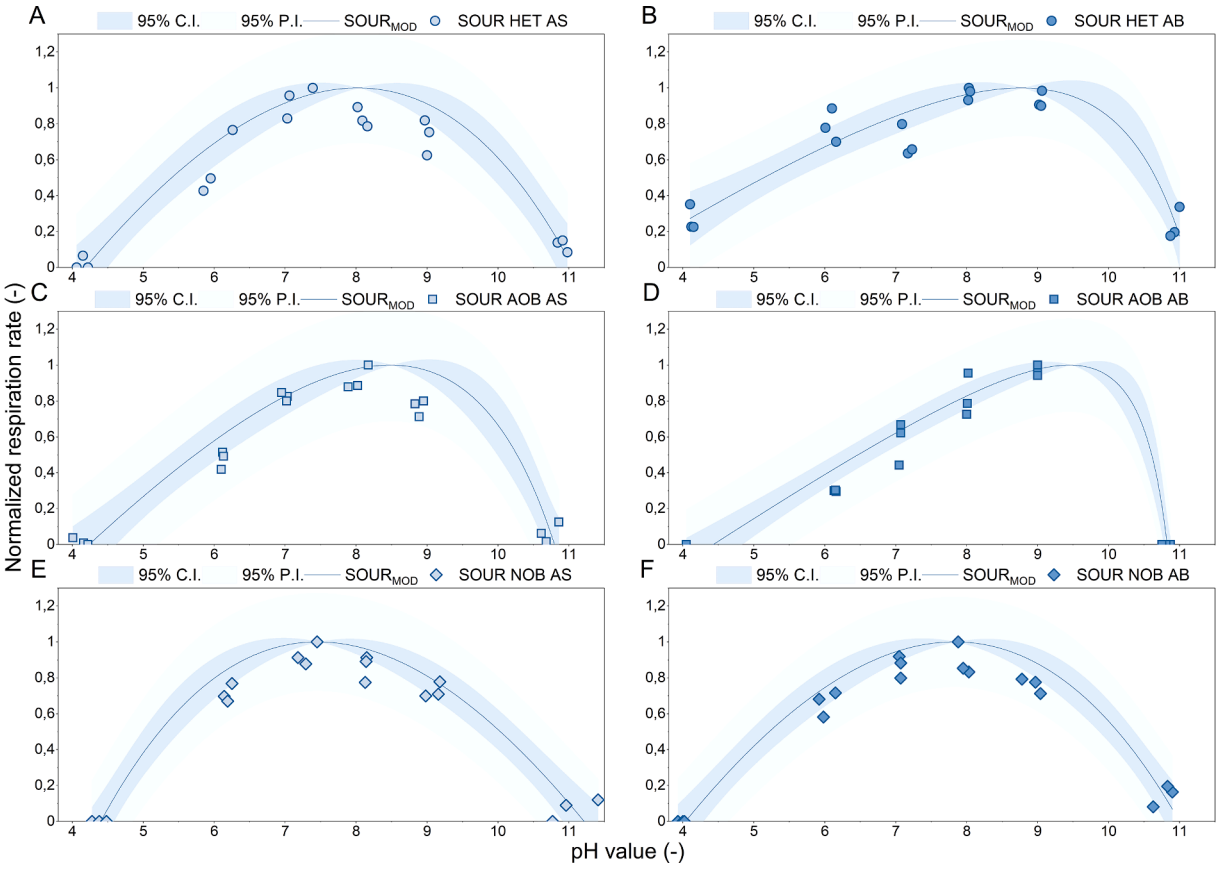

Fig. 2. Effect of pH on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

exchange with the surrounding air, being placed on a metal structure at approximately 0.8 m above the ground. The geometry of the reactor was further responsible for emphasizing thermal variations, as RWPs are specifically designed to maximize the surface/volume ratio, and to minimize the liquid height, thus maximizing the light penetration for photosynthesis. Indeed, typical liquid heights for RWPs are in the range of 0.1–0.3 m, compared to AS systems in which the tank height can typically reach 3–6 m, depending on the type of installed aeration devices. In addition, the pH and DO were subject to a marked increase in the algal-bacterial suspension, following the photosynthetic activity. Another major difference among the two systems is that the biomass concentration was kept at very high levels in AS (up to 3.7 g TSS·L^{-1}, through settled sludge recirculation). On the contrary, lower TSS concentrations were reached in AB (up to a maximum value of 0.9 g TSS·L^{-1}), as it is common in these systems, to guarantee a sufficient light penetration.

Regarding nutrient sources, as reported in Table 1, the AS received primary effluent, rich in degradable organic matter, and with moderate concentrations of N and P. On the contrary, the AB was fed on nutrient-rich digestate with low concentrations of biodegradable organic matter (Akhiar et al., 2017). This possibly led to the predominance, in the AB system, of autotrophic microalgae and nitrifying bacteria over HB, as testified by the high NH_4^+ removal rates (greater than 84%) and the high NO_X effluent concentrations (up to 245 mg N-NO_2^-+NO_3^-·L^{-1}), coupled with a negligible soluble COD removal efficiency. It should be finally noticed that the bacterial biomass concentrations in AB systems are generally much lower than the algal concentrations, typically showing a ratio of bacterial to algal TSS lower than 1:10 (Casagli et al., 2021a). On the contrary, the biomass in the AS tank is practically constituted only by bacterial biomass, and the concentration of HB in AS is expected to be much higher than AOB and NOB, due to the larger availability of degradable organics and the high growth rates of HB. In the following sections, the results obtained for each parameter are reported and discussed.

3.1. *Effect of temperature*

The effect of temperature on bacterial populations of AS and AB is reported in Fig. 1. As shown, the trend for all populations followed the typical asymmetric curve of algal and bacterial cultures (Rosso et al., 1995), in which the optimal temperature is closer to the maximum than to the minimum temperature. The values of the parameters estimated for the CTMI are reported in Table 3.

Regarding the estimated optimal temperatures, these ranged from 30 to 36 °C, which is common for a wide variety of bacterial strains and species (Rosso et al., 1995). For AS, optimal temperatures were 36.1 °C for HB, 30.2 °C for AOB and 36.0 °C for NOB, while in AB samples, the optimum for growth resulted to be 35.6 °C for HB, 34.1 °C for AOB and 34.5 °C for NOB. Since, to the best knowledge of the authors, no studies are available in which the cardinal temperatures were experimentally determined for AS, it is not possible to directly compare these results with other literature experiences. However, previous studies reported that the optimal temperature for nitrifiers is approximately 30 °C, even though values close to the maximum activity could be observed in the entire range from 15 °C to 35 °C (Shammas, 1986), with a strong decrease in the activity below 15 °C, and almost no activity could be detected at 5 °C. In this work, the optimal temperature for AOB was found to be lower in AS than in AB, while the optimum for NOB was similar in both systems. In most cases, the dependence of AS on temperature is modelled by using Arrhenius-type models, which is only suitable to represent the activity below the optimal temperature, therefore no maximum values are found in the literature.

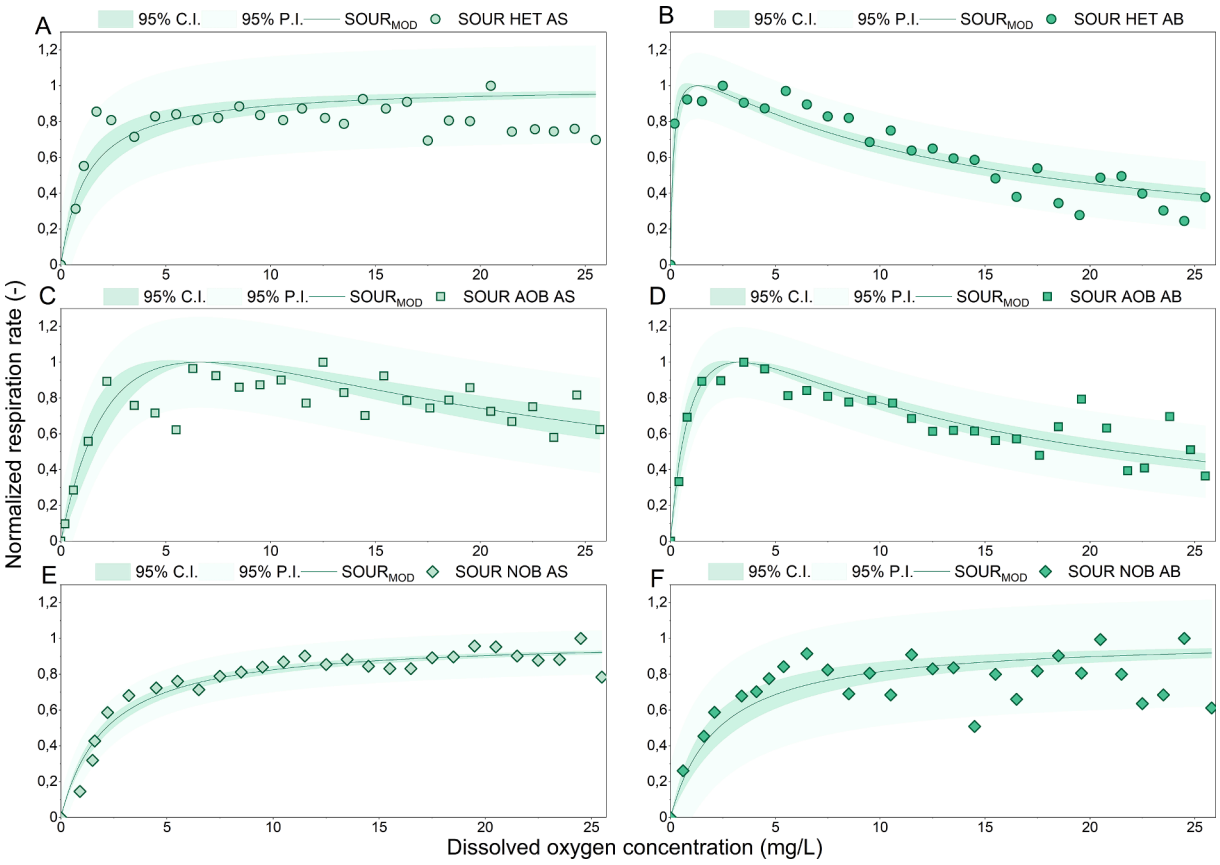

Fig. 3. Effect of dissolved oxygen on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

Regarding bacterial populations in AB, the temperature dependence was recently modelled based on the CTMI (Sánchez Zurano et al., 2021), obtaining very similar results for HB (T_{OPT} = 36 °C) and for nitrifying bacteria, even if these were assessed as a single bacterial group (T_{OPT} = 33.6 °C). These results were also used as nominal values for the ABACO model (Sánchez-zurano et al., 2021). A further confirmation on the reliability of respirometric estimates comes from the calibrated cardinal temperature models available in recently published AB models. In both the ALBA (Casagli et al., 2021b) and BIO_ALGAE (Solimeno et al., 2019), calibrated values for bacteria are close to the reported estimations.

As previously reported in other studies, bacteria can survive in a wide range of temperatures (Alisawi, 2020; Rosso et al., 1995). This was also confirmed by the experimental data, for which the thermal niche was of 40–50 °C. The minimum tolerable temperature among all bacterial populations spanned from −7.7 °C to 1.3 °C, although with large standard errors, suggesting that the experimental design should be improved to better target this parameter. These results were higher than those reported in a similar AB system (Sánchez Zurano et al., 2021), though in this case the climate conditions at which the AB was operated were much warmer than those described here, possibly suggesting an adaptative behaviour. All the bacterial populations could resist up to more than 40 °C, and maximum tolerable temperatures ranged from 40.5 °C to 43.8 °C, coherently with previous findings (Sánchez Zurano et al., 2021). Maximum temperatures were more precisely identified (i.e., with lower standard errors) compared to minimum temperatures (see Table 3 and supplementary material for more details). The results reported in this section are a great example of how respirometric methods could allow gathering relevant information related to wastewater treatment processes. In particular, the use of the CTMI allows to predict the effect of temperature on bacteria, over a wide range of operational conditions. However, despite the availability of this useful model, the majority of literature works on activated sludge bacteria make use of Arrhenius-type equations, which are only suitable to describe the effect of temperature below the optimum, providing no information about bacterial decay at high temperatures. Furthermore, the existence of slight, yet potentially relevant differences among the kinetic models for bacterial communities in activated sludge and AB were quantified in this work.

3.2. *Effect of pH values*

The effect of different pH values on AS and AB is shown in Fig. 2. The CPM described well the respirometric dataset of all bacterial populations, with satisfactory fits and acceptable adjusted r-square values (0.84–0.90).

The findings indicated that bacteria in both AS and AB systems could resist to wide intervals of pH, still showing some residual activity at pH 4 and 11. Regarding the optimal pH, estimated values varied among the different populations (between 7.5 and 9.5). For AS, optimal pH values were 8.0 for HB, 8.5 for AOB and 7.5 for NOB, while the estimates for AB were shifted towards higher optima (8.8 for HB, 9.5 for AOB and 7.8 for NOB), suggesting that bacteria adapted in the AB system to more alkaliphilic conditions. These higher pH optima in AB compared to AS can be indeed explained by the higher pH values promoted by photosynthetic CO_2 uptake in algae-based wastewater treatment processes (according to the results reported in Table 1, the pH in the RWP was on average 7.1, with peaks up to 7.9). Previous studies have confirmed that in AS bacterial consortia, a pH ranging from 6.8 to 8.5 resulted in a high microbial activity and rate of biodegradation (Zhou et al., 2019). For HB, high consumption rates could be reached in a wide range of pH values (from 3

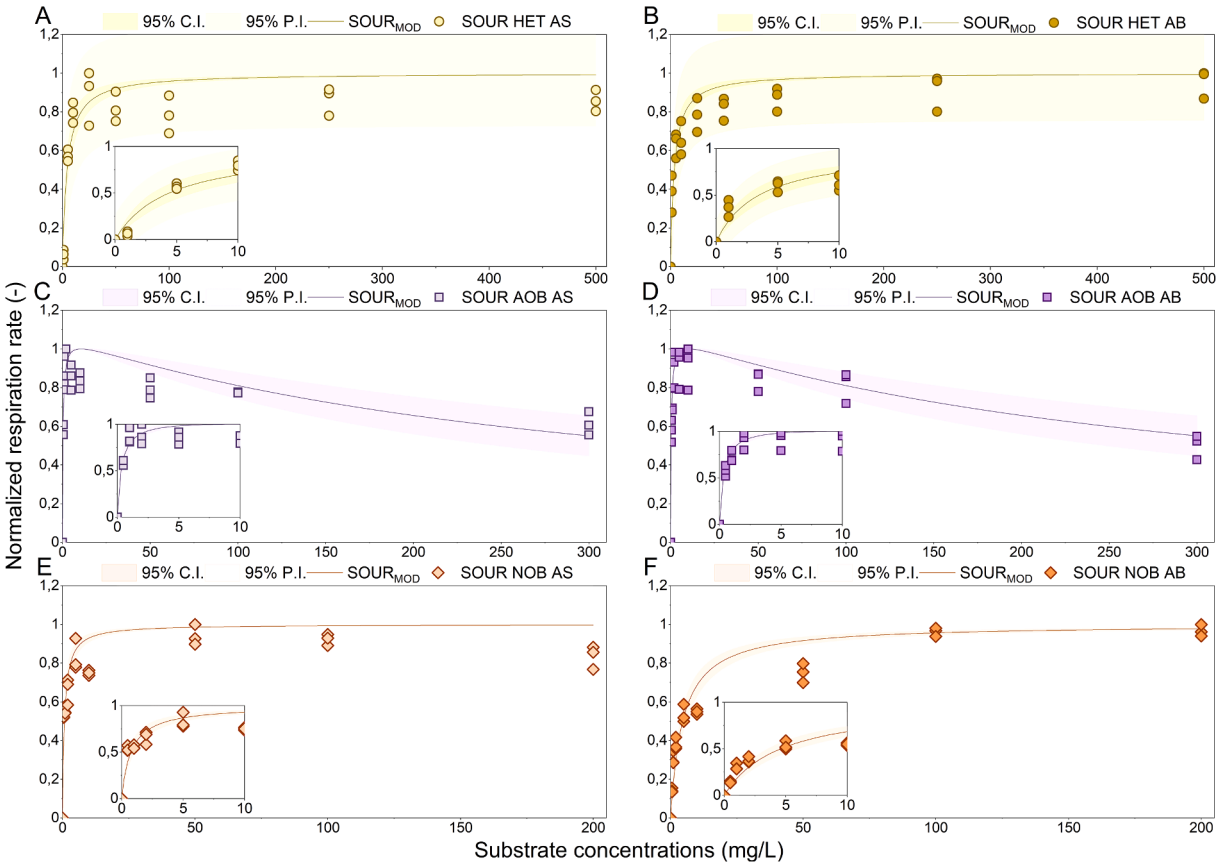

Fig. 4. Effect of substrates on bacterial populations in activated sludge (AS) and algae-bacteria (AB) samples: heterotrophic bacteria in AS (A), heterotrophic bacteria in AB (B), ammonia-oxidizing bacteria in AS (C), ammonia-oxidizing bacteria in AB (D), nitrite-oxidizing bacteria in AS (E), nitrite-oxidizing bacteria in AB (F).

to 9), with a very sharp drop at higher pH. Also, the pH dependence curve for nitrifiers is quite flat-topped, showing similarly high activities at pH values from 7 to 9.5 and with very little activity, or complete inactivation, only below pH 6 and above pH 10 (Shammas, 1986).

Concerning AB consortia, only a few studies have recently reported the use of the CPM to describe the effect of pH on bacterial populations. Results from Sánchez Zurano et al. (2021) and the ALBA model (Casagli et al., 2021b) were fairly consistent with the parameter estimates given in Table 3. Similarly, calibrated values adopted in the BIO_ALGAE model (Solimeno et al., 2019) are very close to the estimates proposed in this study. In these studies, describing the pH dependence for bacterial populations in AB, the pH_{MIN}, pH_{OPT}, and pH_{MAX} assumed values in the following ranges: 2.0–6.0, 7.0–9.0 and 11.0–13.4, respectively, further confirming the ability of bacteria to grow in a wide range of pH conditions.

3.3. Effect of dissolved oxygen

As depicted in Fig. 3, DO have a relevant effect on bacterial respiration rates. DO had an important inhibitory effect on HB and AOB sampled from AB, while almost no inhibition was observed at high DO concentrations, for all AS bacterial populations and for NOB in both systems. As an explanation for this fact, possible inhibitory effects of DO concentrations far above air saturation were previously reported to occur because of the DO diffusion through the membranes and to cause oxidative stress in cells (Baez and Shiloach, 2014). However, this inhibitory effect only seems to be related to long-term exposure times to DO oversaturation. Therefore, the differences observed between the two treatment systems could be because in AS, the microorganisms are rarely exposed to high DO concentrations, while in AB cultures the frequent exposition to high DO concentrations (caused by the algal photosynthetic activity), could have led to long-term cell stress (Baez and Shiloach, 2014). Estimated half-saturation constants for DO in AS samples were 1.2 and 2.1 mg $DO{\cdot}L^{-1}$, respectively, for HB and NOB, and the optimal DO for AOB was 6.6 mg $DO{\cdot}L^{-1}$. In AB samples, a similar half-saturation coefficient was found for NOB (2.3 mg $DO{\cdot}L^{-1}$), and the optimal DO concentrations for HB and AOB were 1.3 and 3.3 mg $DO{\cdot}L^{-1}$, respectively (Table 3). By comparing the DO dependence for the three populations in both AS and AB, it was confirmed, as previously reported for AS samples (Daebel et al., 2007), that the affinity for oxygen in HB was generally higher than for nitrifiers. Moreover, the AS had floccular nature, while AB grew as suspended cells. The presence of bacterial aggregates can cause a higher resistance to diffusion in AS, that could explain the lower affinity for DO observed in AS. The lower oxygen affinity for NOB compared to AOB was also described in several studies, and this was often adopted as a selective strategy in partial nitritation reactors, allowing to wash out NOB and to achieve stable accumulation of NO_2^- (Blackburne et al., 2008).

3.4. Effect of substrate limitation / inhibition

The effects of nutrient concentrations on bacterial populations of AS and AB are reported in Fig. 4. For HB, the half-saturation constants for COD were quite similar in both AS and AB (Fig. 4A and B, respectively), only showing a slightly lower substrate affinity in AS (4.4 mg $COD{\cdot}L^{-1}$), compared to AB (3.5 mg $COD{\cdot}L^{-1}$). Half-saturation constants found in this study for AS were very close to previous studies (Orhon, 2015), though a wide range of values is available in the literature, reaching up to one order of magnitude more than those found in this work, i.e. up to 20–45 mg $COD{\cdot}L^{-1}$, depending on process characteristics (Esquivel-Rios et al., 2014). Regarding AB, as no experimental determination of the half-saturation values is available in the literature for HB, mathematical

models describing the growth of AB consortia generally assume a value of 20 mg COD·L^{-1} from the ASMs (Sánchez-zurano et al., 2021; Solimeno et al., 2019), or more similar values to those experimentally found in this study (4 mg COD·L^{-1}), as reported by Casagli et al. (2021b).

Regarding the effect of nutrients for nitrifying bacteria, the results for AOB are given in Fig. 4C (for AS) and D (for AB), while the results for NOB are reported in Fig. 4E (for AS) and F (for AB). When looking at the parameter estimates for AOB, inhibitory effects of NH_4^+ occurred in both AS and AB, as further emphasized by the low optimal ammonia concentrations ($NH_{4,OPT}$ = 10.5 mg N·L^{-1} and 10.8 mg N·L^{-1} for AS and AB, respectively). This was coherent with the findings reported in previous studies for AS bacteria (Kim et al., 2006) and might be due to the generation of small amounts of free ammonia (FA), even if the pH was kept at 7.5 to minimize this effect. Indeed, FA is a strong growth/activity inhibitor for several types of microorganisms (Rossi et al., 2020b). However, no evidence is directly available for bacterial populations in AB consortia, and the dependence of bacterial growth on ammoniacal nitrogen is generally evaluated in AB models based on a Monod-type function, i.e., not considering substrate inhibition. The findings reported in the present study suggest that a deeper investigation should be conducted, to define whether the inhibitory effect is to be attributed to FA or the ammonium ions. These results were coherent with the experimental determinations of the half-saturation constants for ammoniacal nitrogen in AS: the ASMs and other studies reported values ranging from 0.4 to 5.2 mg N·L^{-1} (Henze et al., 2015; Iacopozzi et al., 2007; Leyva-Díaz et al., 2020), though a large variability was again found for this parameter, reaching in some cases values up to 9–40 mg N·L^{-1} (Terada et al., 2013). Very similar results were also obtained for AOB in AB consortia Due to the unavailability of experimental studies on bacterial activities, the only possible comparisons can be made with AB models, in which the assumed half-saturation constant for ammoniacal nitrogen range from 0.5 mg N·L^{-1} (Casagli et al., 2021b; Reichert et al., 2001; Solimeno et al., 2019), up to 1 mg N·L^{-1} (Sánchez-zurano et al., 2021).

Regarding NOB, a similar effect was observed for the AS and AB samples, since in both ecosystems a Monod-type curve could fit well the experimental values. However, results suggested that NOB populations in AS had a higher substrate affinity (K_{NO2} = 0.8 mg N·L^{-1}) compared to AB (K_{NO2} = 4.6 mg N·L^{-1}). Such values are highly consistent with recent respirometric studies reporting kinetic parameters of NOB for AS samples (Iacopozzi et al., 2007; Jiménez et al., 2012), along with recent AB models considering two-step nitrification (Casagli et al., 2021b; Solimeno et al., 2019). It should be finally noticed that NO_2^- concentrations in the AB cultivation system reached up to 144 mg N·L^{-1}. On the other hand, no relevant NO_2^- accumulation was ever recorded in the AS tank, due to the high nitrite-oxidizing activity, that always resulted in complete nitrification, regardless of the operational conditions. However, no explanation regarding the phenomenon of incomplete nitrification in AB reactors could be provided, based on the results of this study. Therefore, it is suggested that further experimental work is conducted, to evaluate other possible factors which could result in limited NOB growth, such as the half-saturation constants for NOB on other nutrients than nitrite (e.g., for inorganic carbon, or phosphorus). Results of kinetic parameter identification for nutrients are reported in Table 3.

4. Conclusions

The tested environmental and operational conditions were demonstrated to strongly impact each bacterial population (HB, AOB and NOB). Furthermore, bacteria in activated sludge and AB were differentially sensitive to the tested conditions, especially with respect to pH, dissolved oxygen, and nitrite concentration. This finding demonstrates that kinetic models developed and calibrated on the activated sludge process cannot be directly extended to AB processes. Respirometric techniques proved to be an essential tool to identify and calibrate proper kinetic models for the AB process, to be eventually applied as an optimization tool to improve the efficiency and stability of AB-based wastewater treatment.

CRediT authorship contribution statement

A. Sánchez-Zurano: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **S. Rossi:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft, Writing – review & editing, Visualization, Supervision. **J.M. Fernández-Sevilla:** Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **G. Acién-Fernández:** Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **E. Molina-Grima:** Validation, Formal analysis, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **E. Ficara:** Conceptualization, Methodology, Validation, Formal analysis, Resources, Data curation, Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.127116.

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8. OTHER CONTRIBUTIONS

Aside from this work, several collaborations have been performed resulting in publications derived directly from the thesis. In addition, the PhD candidate has participated in several research projects and contributed as a reviewer on several occasions. Apart from the research activities, the PhD candidate has also been involved in teaching activities and has collaborated in one group of innovation and good teaching practices, resulting in a publication at a national conference. All this information is summarized in this chapter.

7.1. Scientific papers

During the development of the thesis, several collaborations with partners from the University of Almería and other national and international institutions, such as Gruppo Ricicla in the Università degli Studi di Milano were performed. This research work resulted in 16 publications in JCR journals. The topic of each of the publications was diverse, from microalgae for wastewater treatment up to microalgae for food innovation.

- Barceló-Villalobos M., Serrano C.G., **Sánchez-Zurano, A**., García L.A., Maldonado S.E., Peña J., Fernández F.G., Variations of culture parameters in a pilot-scale thin-layer reactor and their influence on the performance of Scenedesmus almeriensis culture. Bioresource Technology Reports, 6, 190-197, 2019.

- **Sánchez-Zurano, A**., Garrido-Cárdenas, J.A., Gómez, C., Morales Amaral M., Acién-Fernández, F.G., Fernández Sevilla, J.M., Molina, E. Year-long assessment of a pilot-scale thin-layer reactor for microalgae wastewater treatment. Variation in the microalgae-bacteria consortium and the impact of environmental conditions. Algal Research, 50, 101983, 2020.

- **Sánchez-Zurano, A**., Morillas-España, A., González-López C.V., Lafarga, T., Optimisation of protein recovery from arthrospira platensis by ultrasound-assisted isoelectric solubilisation/precipitation. Processes, 8, 12, 1-13, 2020.

- **Sánchez-Zurano, A**., Ciardi, M., Lafarga, T., Fernández Sevilla, J.M., Bermejo, R., Molina, E Role of microalgae in the recovery of nutrients from pig manure. Processes, 9, 2, 1-11, 2020.

- Lafarga, T., Rodríguez-Bermúdez, R., Morillas-España, A., Villaro, S., García-Vaquero, M., Morán, L., **Sánchez-Zurano, A**., González-López C.V., Acién-Fernández, F.G. Consumer knowledge and attitudes towards microalgae as food: The case of Spain. Algal Research, 54, 102174, 2021.

- Lafarga, T., **Sánchez-Zurano, A**., Morillas-España, A., Acién-Fernández, F.G. Extremophile microalgae as feedstock for high-value carotenoids: A review. International Journal of Food Science and Technology, 56, 10, 4934 – 4941, 2021.

- Hernández-López I., Benavente Valdés J.R., Castellari M., Aguiló-Aguayo I., Morillas-España A., **Sánchez-Zurano, A.,** Acién-Fernández F.G., Lafarga T. Utilisation of the marine microalgae Nannochloropsis sp. and Tetraselmis sp. as innovative ingredients in the formulation of wheat tortillas, 58, 102361, 2021.

- Lafarga, T., **Sánchez-Zurano, A**., Villaró, S., Morillas-españa, A., Acién, G. Industrial production of Spirulina as a protein source for bioactive peptide generation. Trends in Food Science & Technology, 116, 176-185, 2021.

- Villaró S., Ciardi M., Morillas-España A., **Sánchez-Zurano, A**., Acién-fernández G. Lafarga T. Microalgae derived astaxanthin: Research and consumer trends and industrial use as food, Foods, 10, 10, 2303, 2021.

- **Sánchez-Zurano, A**., Lafarga, T., Morales, M., Gómez-Serrano, C., Acién, G., Molina, E. Wastewater treatment using Scenedesmus almeriensis: effect of operational conditions on the composition of the microalgae-bacteria consortia. Journal of Applied Phycology, 33, 3885–3897, 2021.

- Morillas-España A., **Sánchez-Zurano, A**., Lafarga, T., Acién-Fernández, F.G., Rodríguez-Miranda, E., Gómez Serrano, C., González-López, C.V. Year-long evaluation of microalgae production in wastewater using pilot-scale raceway photobioreactors: Assessment of biomass productivity and nutrient recovery capacity. Algal Research, 60, 102500, 2021.

- Morillas-España A., **Sánchez-Zurano, A**., Lafarga, T., Morales, M., Gómez Serrano, C., Acién-Fernández, F.G., González-López, C.V. Improvement of wastewater treatment capacity using the microalga Scenedesmus sp. and membrane bioreactors. Algal research, 60, 102516, 2021.

- Morillas-España, A., **Sánchez-Zurano, A**., Gómez, C., Ciardi, M., Acién-Fernández, F.G., Clagnan, E., Adani., Lafarga, T. Potential of the cyanobacteria Anabaena sp. and Dolichospermum sp. for being produced using wastewater or pig slurry: Validation using pilot-scale raceway reactors. Algal Research, 60, 102517, 2021.

- **Sánchez-Zurano, A**., Morillas-España, A., Gómez-Serrano, C., Ciardi, M., Acién, G., Lafarga, T. (2021) Annual assessment of the wastewater treatment capacity of the microalga Scenedesmus almeriensis and optimisation of operational conditions. Scientific Reports, 11, 21651, 2021.

- Morillas-España A., Lafarga, T., **Sánchez-Zurano, A**., Acién-Fernández, F.G., Rodríguez-Miranda, E., Gómez Serrano, C., González-López, C.V. Microalgae based wastewater treatment coupled to the production of high value agricultural products: Current needs and challenges. Chemosphere, 291, 2022.

- Moran, L., Bou, Gemma., Aldai., N., Ciardi, M., Morillas-España, A., **Sanchez-Zurano**, **A.,** Barrón, L., Lafarga, T. Characterisation of the volatile profile of microalgae and cyanobacteria using solid-phase microextraction followed by gas chromatography coupled to mass spectrometry. Scientific Reports, 12, 2022.

7.2. International conferences

Similarly, the different collaborations mentioned above and other partners resulted in 5 contributions to international conferences also covering topics related to the thesis as microalgae-bacteria interactions, microalgae-based wastewater treatment or other microalgal-based processes:

- Aparicio, S., Sánchez-Zurano, A., Borras-Falomir, L., Robles, A., Acién Fernández, F.G., Seco, A. "Effect of ammonium concentration on microalgae photosynthesis". Event: IWA Young Water Professionals Spain Conference 2019. Type of presentation: Poster. Place: Madrid, Spain.

- Sánchez-Zurano, A., Garrido-Cárdenas, J.A., Gómez, C., Morales Amaral M., Acién-Fernández, F.G., Fernández Sevilla, J.M., Molina, E. "Annual performance of a microalgae-bacteria consortia based wastewater treatment process using a pilot scale thin-layer reactor". Event: ALGAEUROPA 2020. Type of presentation: Poster. Place: Online.

- Morillas-España A., **Sánchez-Zurano, A**., Lafarga, T., Morales, M., Gómez Serrano, C., Pinar, M., Acién-Fernández, F.G., Fernadez-Sevilla, J.M. "Evaluation of Operational Conditions on the Performance of Microalgae-Based Wastewater Treatment. Variation in the Bioremediation Capacity of Primary Urban Wastewater and Microalgae-Bacteria Consortia". Event: 29th European Biomass Conference and Exhibition 2021. Type of presentation: Poster. Place: Online.

- **Sánchez-Zurano, A**., Garrido-Cárdenas, J.A., Gómez, C., Morales Amaral M., Acién-Fernández, F.G., Fernández Sevilla, J.M., Molina, E. "Yearly assessment of a pilot scale thin-layer reactor for microalgae wastewater treatment. Variation of the microalgae-bacteria consortium and impact of the environmental conditions". Event: 7th Congress of the International Society for Applied Phycology 2021. Type of presentation: Poster. Place: Online.

- **Sánchez-Zurano, A**., Lafarga, T., Morales, M., Gómez-Serrano, C., Acién, G., Molina, E. "Microalgae-based wastewater treatment: Understanding the effect of operational conditions on biomass productivity, nutrients removal, and composition of the microalgae-bacteria consortium". Event: 10th International Conference on Algal Biomass, Biofuels and Bioproducts 2021. Type of presentation: Poster. Place: Online.

7.3. National conferences

In addition, the PhD candidate participate in some national conferences:

- Jiménez-Veuthey, M., Morillas-España A., **Sánchez-Zurano, A**., Flores, M., Acién-Fernández, F.G. "Biorremediación de efluentes porcinos en biorrecator de capa fina empleando Nannochloropsis gaditana". Event: 6º Simposio Argentino de Procesos Biotecnológicos 2021. Type of presentation: Oral. Place: Online.

- Ciardi, M., **Sánchez-Zurano, A**., Fernandez-Sevilla, J.M., Acién-Fernández, F.G. "Recuperación de agua para el tratamiento de purines con microalgas". Event: III Congreso de Jóvenes Investigadores del Mar 2021. Type of presentation: Poster. Place: Motril, España.

- Jiménez-Veuthey, M., Morillas-España A., Sánchez-Zurano, A., Navarro, E., Acién-Fernández, F.G. “Production of nannochloropsis gaditana in outdoor thin-layer reactor using pig slurry as sole nutrients source”. IX Simposio de investigación en ciencias experimentales 2019. Type of presentation: Poster. Place: Almeria, Spain.

- Villaro, S., Sánchez-Zurano, A., Morillas-España A., Acién-Fernández, F.G., González-López, C.V., Lafarga, T. “Optimisation of the protein recovery from a. platensis by ultrasound-assisted isoelectric solubilisation-precipitation”. IX Simposio de investigación en ciencias experimentales 2019. Type of presentation: Poster. Place: Almeria, Spain.

7.4. Collaboration in research projects

- Research project: Sustainable algae biorefinery for agriculture and aquaculture (SABANA) Funding agency: H2020-EU.3.2.5 Code: 727874 Contribution: Researcher Start-End date: 01/12/2016 – 31/10/2021 Budget: 10.646.000€

  Research project: Developing early-warning systems for improved microalgae production (PRODIGIO) Funding agency: H2020-EU.3.3.2 Code: 101007006 Contribution: Researcher Start-End date: 01/01/2021 – 30/12/2023 Budget: 2.452.941€

- Research contract: Eco-friendly and sustainable new family of biopesticides based on microalgae via circular economy (ALGAENAUTS) Contractor: Biorizon Biotech SL (Almería, Spain) Contribution: Researcher Start-End date: 01/01/2022 – 30/06/2023 Budget: 55.000€

- COST Action: Applications for zoosporic parasites in aquatic systems (ParAqua) Funding agency: European Cooperation in Science and Technology (COST) Code: CA20125 Contribution: Researcher Start-End date: 02/11/2021 – 01/11/2025 Budget: X€

7.5. Communication and dissemination

- Title: ¿Qué beneficios tiene la spirulina, el alimento de los astronautas? Publisher: The Conversation. Date: 25/02/2021 Reads: 97.000 (21/02/2022). Available: https://theconversation.com/que-beneficios-tiene-la-espirulina-el-alimento-de-los-astronautas-155097.

- Title: Depuradoras de microalgas: ahorran energía, absorben CO2 y producen fertilizantes sostenibles. Publisher: The Conversation. Date: 04/07/2021 Reads: 4000 (21/02/2022). Available: https://theconversation.com/depuradoras-de-microalgas-ahorran-energia-absorben-co-sub-2-sub-y-producen-fertilizantes-sostenibles-162808.

- Title: Progress and Challenges in Microalgal Large-Scale Production. Publisher: PRODIGIO Project. Data: 2021 Available: https://prodigio-project.eu/progress-and-challenges-in-microalgal-large-scale-production/

- Title: Microalgas y bacterias para la Europa post-covid. Publisher: iAgua Magazine. Date: 15/03/2022.

7.6. Teaching activities

As a complement to the research work, the Ph.D. candidate has participated in teaching activities, namely X hours. The subjects taught include Chemistry and Bioreactors. Moreover, the Ph.D. candidate has also collaborated in an Innovation and Good Teaching Practices group, devoted to the improvement of teaching activity and the quality of learning of students at the University of Almería. One of the results of this project was presented as a contribution to the following national conference:

- Casa, J.L., Pinna-Hernández, G., Fernández-Sevilla, J.M., Esteban, B., Sánchez-Zurano, A., García-Sánchez, J., Sánchez, J.A. “Process dynamics through the Easy Java Simulations tool (EJS)”. Jornadas de Innovación Docente y Experiencias Profesionales 2020. Type of presentation: Poster. Place: Almeria, Spain.

In addition, a contribution to the following international journal has been performed:

- **Sanchez-Zurano**, **A.,** Fernández-Sevilla, J.M., Esteban, B., Pinna-Hernández, G., Casas, J.L. Virtual Labs for the study of enzymatic stirred tank bioreactors. Computer Applications in Engineering Education, 1-12, 2022.

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