PhD Thesis

MICROALGAE IN AQUACULTURE WASTEWATER: A NEW FORECASTING METHOD OF PRODUCTION IN A MARINE SYSTEM

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THESIS DISSERTATION OF THE PHD TITLE

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Abstract

In the field of aquaculture, the main microalgae application is animal nutrition, in which they can be used as an unprocessed component, or as dried material for feed preparations. Moreover, microalgae can assimilate the main nutrients dissolved in aquaculture wastewater reducing the environmental impact of aquaculture and at the same time producing valuable biomass.

Because of the variability of wastewater, it is not easy to predict the microalgae production, nevertheless, the mathematical model could offer the possibility to study microalgae growth in different conditions.

Mathematical models are used to forecast algal productivity and nutrient removal efficiency in synthetic media and in urban wastewater, but they were never been implemented and calibrated for aquaculture wastewater.

The main outcome of the present Ph.D. thesis was to calibrate and validate the integrated mechanistic model BIO_ALGAE with experimental data obtained from the cultivation of marine microalgae in aquaculture wastewater. This model includes crucial physical and biokinetic processes to simulate microalgae growth in wastewater, and in a different type of

photobioreactors. BIO_ALGAE was used to understanding the slight diurnal variations, which could have not been detected with experimental samples.
Preliminary respirometric tests were carried out on the microalgal-bacterial suspension. These respirometric outputs were compared with process rates affecting dissolved oxygen dynamics computed by the mathematical model.

In the experimental part of this thesis project, the productivity and capacity in the bioremediation of three marine microalgae species, *Tetraselmis suecica*, *Dunaliella tertiolecta* and *Isochrysis galbana* was investigated and compared. Wastewater generated during the production of grey mullet (*Mugil cephalus*) and sea bream (*Sparus aurata*) was used as culture medium.
The experiments were conducted in batch and in semi-continuous conditions using column photobioreactors with differents volumes.

It is known that under different stress conditions, the microalgae produce bioactive compounds, therefore, aquaculture wastewater was used as substitute synthetic cultivation medium to test the production of lipids, proteins, and carbohydrates in the microalgal biomass. Moreover, these species were cultivated in unsterilized culture media, and this reduces energy

consumption, costs, and efforts.

This study confirms the potential to employ *Tetraselmis suecica* in an Integrated Multi-Trophic Aquaculture system for biomass production and bioremediation of wastewater and identifies *Dunaliella tertiolecta* as another valid candidate species.

T. suecica was therefore selected for the validation of BIO_ALGAE model.

For the first time, BIO_ALGAE model was applied in aquaculture system and highlights a good agreement between experimental data and simulations.

This model has proved to be an efficient tool to understand microalgae production in aquaculture wastewater treatment and to simulate the dynamics of different conditions in closed photobioreactors. Indeed, BIO_ALGAE describes the factors that influence microalgae growth and this is a useful approach to predict microalgal biomass with the optimization of the operating conditions.

Resumen

En el campo de la acuicultura, la principal aplicación de las microalgas es la nutrición animal, en la que se pueden usar como un componente no procesado o como material seco para la preparacion de pienso. Además, las microalgas pueden tener el potencial de asimilar los principales nutrientes disueltos en las aguas residuales de la acuicultura y, por lo tanto, pueden ayudar en el tratamiento y al mismo tiempo producir biomasa de alto valor comercial.

Debido a la variabilidad de estas aguas residuales, no es fácil predecir la producción de microalgas, pero los modelos matemáticos podrían ofrecer la posibilidad de estudiar el crecimiento de las microalgas en diferentes condiciones.

Los modelos matemáticos, se han utilizado para simular la productividad de algas y la eficiencia de eliminación de nutrientes en medios sintéticos y en aguas residuales urbanas, mientras que, en lo que respecta a las aguas residuales de acuicultura, todavía no se ha implementado ni calibrado un modelo matemático.

El principal resultado de la presente tesis doctoral fue calibrar y validar el modelo mecanístico integrado, BIO_ALGAE, con datos experimentales obtenidos del cultivo de microalgas

marinas en aguas residuales de acuicultura. Este modelo incluye procesos físicos y bioquinéticos cruciales para simular el crecimiento de microalgas en aguas residuales y diferentes fotobiorreactores. BIO_ALGAE se utilizó para comprender las variaciones diurnas, que no se pudieron detectar con muestras experimentales.

Se llevaron a cabo pruebas respirométricas preliminares en la suspensión microalgas-bacterias. Los datos de salida de la respirométria se compararon con las tasas de proceso que afectan la dinámica del oxígeno disuelto obtenidas por el modelo matemático.

En la parte experimental de este proyecto de tesis, se investigó y comparó la productividad y la capacidad en la biorremediación de tres especies de microalgas marinas, *Tetraselmis suecica*, *Dunaliella tertiolecta* y *Isochrysis galbana*. Como medio de cultivo se utilizaron las aguas residuales generadas durante la producción de salmonete (*Mugil cephalus*) y dorada (*Sparus aurata*). Los experimentos se realizaron en condiciones "batch" y semi-continuas utilizando fotobiorreactores de columna con diferentes volúmenes.

Se sabe que bajo diferentes condiciones de estrés, las microalgas producen compuestos bioactivos, por lo tanto, las aguas residuales de la acuicultura se utilizaron como medio de cultivo

sintético sustituto para probar la producción de lípidos, proteínas y carbohidratos en la biomasa de microalgas. Además, estas especies se cultivaron en medios de cultivo no esterilizados, y esto reduce el consumo de energía, los costos y los esfuerzos de producción.

Este estudio confirma la posibilidad de emplear *Tetraselmis suecica* y *Dunaliella tertiolecta* en un sistema integrado de acuicultura multitrófica para la producción de biomasa y biorremediación de aguas residuales, sin embargo, *Tetraselmis suecica* demostró mayor eficiencia de remoción de nutrientes y mayor crecimiento. Por lo tanto, se seleccionó *T. suecica* para la validación del modelo BIO_ALGAE.

Por primera vez, el modelo BIO_ALGAE se aplicó en el sistema de acuicultura y destaca un buen acuerdo entre los datos experimentales y las simulaciones.

Este modelo ha demostrado ser una herramienta eficiente para comprender la producción de microalgas en el tratamiento de aguas residuales de acuicultura y para simular la dinámica de diferentes condiciones en fotobiorreactores cerrados. De hecho, BIO_ALGAE describe los factores que influyen en el crecimiento de las microalgas y este es un enfoque útil para predecir la biomasa de microalgas con la optimización de las condiciones operativas.

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Acronyms and Abbreviations

ASM	Activated Sludge Model
AGRIS	Agency for Agricultural Research in Sardinia
ANOVA	Analysis of Variance
ASM1	Activated Sludge Model No.1
ASM2	Activated Sludge Model No.2
ASM2d	Activated Sludge Model No.2d
ASM3	Activated Sludge Model No.3
AW	Aquaculture Wastewater
BF	Biological Filter
BOD	Biochemical Oxygen Demand
c	chiller
CCAP	Culture Collection for Algae and Protozoa
CFP	Common Fisheries Policy
COD	Chemical Oxygen Demand
CWM1	Constructed Wetland Model No.1
DIN	Dissolved Inorganic Nitrogen
DIP	Dissolved Inorganic Phosphorous
DO	Dissolved Oxygen
DW	Dry Weight
EB	Extraction Buffer
EU	European Union
HRAPs	High Rate Algal Pond
HRT	Hydraulic Retention Time

HSD	Honestly significant difference test
IMTA	Integrated Multi-Trophic Aquaculture
ISPRA	Italian Institute for Environmental Protection and Research
IWA	International Water Association
MF	Mechanical Filter
MSFD	Marine Strategy Framework Directive
OPR_{MA}	Oxygen Production Rate by microalgae
OPR_{NIT}	Oxygen Production Rate by nitrifiers
OPR_R	Oxygen Production Rate by microalgae and heterotrophic bacteria
OPR_S	Oxygen Production Rate
OUR	Oxygen Uptake Rate
PBR	Photobioreactor
PLC	Programmable Logic Controller
PS	Protein Skimmer
RAS	Recirculation Aquaculture System
RMSE	Root Mean Square Error
RWQM1	River Water Quality Model No. 1
SE	Standard Error
SS	Suspended Solids
TP	Total Phosphorus
TSS	Total Suspended Solids
VSS	Volatile Suspended Solid
WFD	Water Framework Directive

WSPs	Waste Stabilization Ponds

List of symbols

f_{ALG}	Production of inert particulate organic matter in endogenous respiration of microalgae
$f_{T,FS}$	Thermic photosynthetic factor
$f_{T,MB}$	Thermal factor for bacteria
f_{PR}	Photorespiration factor
f_{XI}	Production of inert particulate organic matter in endogenous respiration of heterotrophic bacteria
i	Number of components
$i_{C,ALG}$	Fraction of carbon in microalgae
$I_{CO2,ALG}$	Inhibition constant of microalgae on dissolved carbon
$i_{H,ALG}$	Fraction of hydrogen in microalgae
$i_{N,ALG}$	Fraction of nitrogen in microalgae
$i_{O,ALG}$	Fraction of oxygen in microalgae
j	Number of processes
k	Number of components
$K_{a,NH3}$	Mass transfer coefficient for ammonia
$K_{a,O2}$	Mass transfer coefficient for oxygen
$K_{a,CO2}$	Mass transfer coefficient for dioxide carbon
$K_{C,ALG}$	Saturation constant of microalgae on dissolved carbon
$K_{C,AOB}/K_{C,NOB}$	Saturation constant of nitrifying bacteria for bicarbonate

$k_{death,ALG}$	Decay constant of microalgae
$k_{death,H}$	Decay constant of heterotrophic bacteria
$k_{death,AOB}$/ $k_{death,NOB}$	Decay constant of nitrifying bacteria
$k_{eq,c}$	Dissociation constant
$k_{eq,1}$	Dissociation constant of $CO_2 \leftrightarrow HCO_3^-$
$K_{eq,1}$	Chemical equilibrium of $CO_2 \leftrightarrow HCO_3^-$
$k_{eq,2}$	Dissociation constant of $HCO_3^- \leftrightarrow CO_3^{2-}$
$K_{eq,2}$	Chemical equilibrium of $HCO_3^- \leftrightarrow CO_3^{2-}$
$k_{eq,3}$	Dissociation constant of $NH_4^+ \leftrightarrow NH_3$
$K_{eq,3}$	Chemical equilibrium of $NH_4^+ \leftrightarrow NH_3$
$k_{eq,w}$	Dissociation constant of $H^+ \leftrightarrow OH^-$
$K_{eq,w}$	Chemical equilibrium of $H^+ \leftrightarrow OH^-$
K_{HYD}	Hydrolysis rate constant
$K_{I,NH4}$	Ammonia inhibition constant of nitrite oxidizing bacteria
$K_{N,ALG}$	Saturation constant of microalgae on nitrogen species
K_N	Saturation constant of heterotrophic bacteria on nitrogen species
$K_{NH4,AOB}$	Saturation constant of ammonium oxidizing bacteria on ammonium
$K_{NO3,H,ANOX}$	Saturation constant of heterotrophic bacteria for nitrate
$K_{NO2,H,ANOX}$	Saturation constant of heterotrophic bacteria for nitrite
$K_{NO2,NOB}$	Saturation constant of nitrite oxidizing bacteria for nitrite

$K_{O2,AOB}$/ $K_{O2,NOB}$	Saturation constant of nitrifying bacteria for dissolved oxygen
$K_{O2,H}$	Saturation constant of heterotrophic bacteria for dissolved oxygen
K_{PR}	Inhibition constant of photorespiration
$k_{resp,ALG}$	Endogenous respiration constant
$k_{resp,AOB}$/$k_{resp,NOB}$	Endogenous respiration rate of nitrifying bacteria
$k_{resp,H}$	Endogenous respiration rate of heterotrophic bacteria
$K_{S,H}$	Saturation constant of heterotrophic bacteria for readily biodegradable soluble organic matter
K_S	Half-saturation coefficient for substrates
S	Limiting substrate concentration
S_c	Concentration of species in equilibrium
S_{CO2}	Carbon dioxide
S_{CO3}	Carbonate
S_g	Gas concentration in water
S_g^{WAT}	Gas saturation concentration in water
S_H	Hydrogen ions
S_{HCO3}	Bicarbonate
S_{NH3}	Ammonia nitrogen
S_{NH4}	Ammonium nitrogen
S_{NO2}	Nitrite nitrogen
S_{NO3}	Nitrate nitrogen
S_{O2}	Dissolved oxygen

$S_{O2^{SAT}}$	Dissolved oxygen air saturation
S_{OH}	Hydroxide ions
S_{PO4}	Phosphate phosphorus
S_S	Readily biodegradable soluble organic matter
T_{OPT}	Optimum temperature
u	Culture velocity
$v_{i,j}$	Stoichiometric coefficients
x	Vector of components xi
x_*	Random vector
x_i	Parameters of sensitivity analysis
X_{AOB}	Ammonium oxidizing bacteria
X_C	Sum of particulate components
X_H	Heterotrophic bacteria
X_I	Inert particulate organic matter
X_{NOB}	Nitrite oxidizing bacteria
X_S	Slowly biodegradable particulate organic matter
x_1	Open or resting state
x_2	Closed or activated state
x_3	Inhibited state
y	Model output
Y_{ALG}	Yield of microalgae
Y_{AOB}	Yield of ammonium oxidizing bacteria
Y_H	Yield of heterotrophic bacteria

Y_{NOB}	Yield of nitrite oxidizing bacteria
$Y_{H,NO3}$	Yield of heterotrophic bacteria on nitrate
$Y_{H,NO2}$	Yield of heterotrophic bacteria on nitrite
Y_{HYD}	Hydrolysis saturation constant

Greek symbols

α	Activation rate
β	Inhibition rate
γ	Production rate
δ	Recovery rate

1

Introduction

1.1 Scenario: the problem of the aquaculture wastewater

Over the past 20 years the significant growth in fisheries and aquaculture production has enhanced world's capacity to consume diversified and nutritious food (FAO, 2016). Aquaculture production of fish and shellfish was the main contributor to the fastest growing food production sector, in response to the high demand for marine products all over the world (Tacon et al., 2011). Recent estimates indicate that in 2016 about 59.6 million people around the world were engaged in the primary sector of capture fisheries and aquaculture (FAO, 2018). Almost all fish produced from aquaculture is destined for human consumption, but by-products may be used also for non-food purposes. Based on FAO's analysis, production of aquatic animals from aquaculture in 2016 amounted to 80.0 million tonnes, whereas 90.9 million tonnes for capture fisheries production (FAO, 2018).

Aquaculture activities introduce into the environment wastewater, which is characterized by high quantities of non-consumed nutrients (Munday et al., 1992; Pillay, 1992), and if discharged without any treatment could cause water pollution by eutrophication. For this reason it is necessary to develop new aquaculture methods, compatible with current legislation and sustainable both economically and ecologically. Scientific

research is working to expand the range of livestock species, to improve the quality of products and to reduce the environmental impact that this type of production activity can generate.

New technologies and strategies are being studied with the aim of removing contaminants as well as chemical compounds from aquaculture wastewater. To this end, there is a growing trend to use microalgae with a double benefit, on one side for nutrient remediation and on the other side for biomass generation. In this context, microalgae represent an opportunity to produce important by-products, as well as energy or animal feed. The idea to use intensively microalgae for wastewater treatment was originally developed in the 1950s in California (Oswald and Gotaas, 1957; Oswald, 1963) and numerous researchers have contributed developing techniques to exploit the microalgae's fast growth and nutrient removal capacity. This has to be seen as a low-cost process and it is considered as one of the most efficient and environmental friendly alternatives, compared to conventional techniques. Even though in the last decade have been developed new techniques for the production of microalgae in aquaculture wastewater, the number of scientific papers is still rather limited (Borges et al., 2005; Guo et al., 2013; Michels et al., 2014; Sirakov et al., 2014; Khatoon et al., 2016; Guldhe et al., 2017).

Microalgae culture systems are generally classified according to their engineering and hydraulic characteristics in: (1) open systems (including ponds, deep channels, shallow circulating units, etc.); (2) closed systems, commonly named photobioreactors (PBR) (Chaumont, 1993).

Despite the several benefits in the use of microalgae, its commercial-scale production is still developing due to the high production costs. As regarding microalgae culture systems in aquaculture context, it is necessary a deep and realistic knowledge of the inner functioning to predict performance and optimize reactor design and costs.

Mathematical models for microalgae have been proven to be useful tools for design, analysis, operation and control of wastewater treatment systems and biomass production. Nowadays, models have become essential for testing operational scenarios in wastewater systems aiming to improve the removal efficiency at the lowest operational cost.

Several mathematical and numerical models for predicting microalgae biomass growth in photobioreactors and ponds have been published (Kroon et al., 1989; Sukenik and Livne, 1991; Behrenfeld and Falkowski, 1997; Bernard, 2011; Packer et al., 2011; Bernard and Rémond, 2012; James et al., 2013; Béchet et al., 2013).

It was recently developed a mathematical model to simulate the growth interaction of microalgae and bacteria in wastewater systems. This model called BIO_ALGAE was built by coupling the own models of the authors (Solimeno et al., 2015) with the modified ASM3 (Activated Sludge Model No.3) (Iacopozzi et al., 2007). This model permits to infer the relative proportion of microalgae and bacteria in mixed culture systems, and to make predictions on biomass production and nutrients uptake. Mathematical models for microalgae in aquaculture systems are only at a very initial research stage and are not common because of the variability of the process. The characteristics of wastewater in an aquaculture system are very variable and depend on a great number of factors such as the breed species and the season, making the forecast more difficult. Therefore, it is important to experiment and develop new tools for coupling mathematical models with microalgae production and bioremediation in aquaculture wastewaters.

2

Objectives and thesis outline

2.1 Objectives

The general scope of this Ph.D. project was to enhance the cultivation of marine microalgae in aquaculture wastewater (AW) coupling with new forecasting systems.

In this research, we calibrated and validated the innovative integrated mathematical mechanistic model BIO_ALGAE in aquaculture systems, with the aim to simulate the growth, and nutrients uptake for marine microalgae in aquaculture wastewater. We tested the adaptability of marine microalgae species in wastewater from two different aquaculture systems. The first was a pilot hatchery system for the reproduction and rearing of grey mullet (*Mugil cephalus*), and the second one is an intensive land-based aquaculture production system of sea bream (*Sparus aurata*). We aimed to obtain biochemical products by microalgae, promoting at the same time responsible and sustainable aquaculture with the use of wastewater.

This research has been divided into two main parts: the first with the microalgae cultivation and data collection and the second one with development and validation of the mathematical model (BIO_ALGAE) (Figure 2.1).

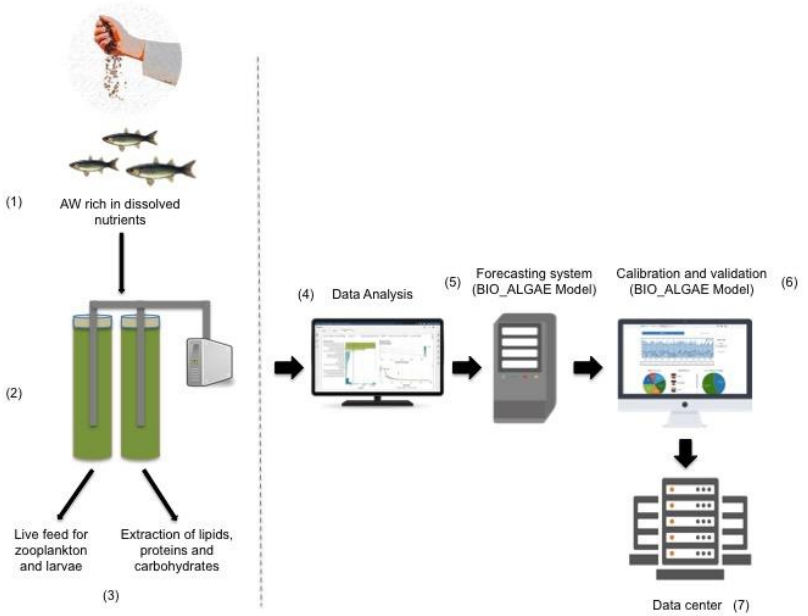

Figure 2.1: General scheme: Marine fish aquaculture in land based systems, and wastewater production rich in dissolved nutrients (1) is used in PBR as growth medium to test different microalgae species (2).

The microalgal biomass is used as live feed in aquaculture systems and for the extraction of lipids, proteins and carbohydrates (3).

The data collected in this first phase is analyzed statistically (4) and used for the calibration and validation for BIO_ALGAE model (5), (6). The simulations produced will be used to optimize biomass production and nutrients removal efficiency in other studies (7).

The specific objectives of this research are:

1. to evaluate and compare the capability of different marine microalgae for the removal of dissolved inorganic nutrients (nitrogen and phosphorous) and

biomass yield in aquaculture wastewater in batch mode;

2. to implement and calibrate a mechanistic microalgae model BIO_ALGAE for aquaculture wastewater in order to simulate the uptake of nutrients (N, P) and the biomass production of two microalgae species in batch conditions;
3. perform a validation to mechanistic microalgae model BIO_ALGAE for marine microalgae production and nutrients uptake in continuous mode, and at the same time to evaluate the possibility of including these forecasting methods within aquaculture systems;
4. to determine the biochemical composition of microalgae cultivated in aquaculture wastewater.

The project aims at broadening the knowledge about the role of microalgae in aquaculture systems through an innovative approach based on the development of new technology to forecast biomass production.

2.2 Thesis outline

Beside the introduction and the state of the art (Chapters 1 and 3), this Ph.D. thesis contains three experiments, developed to reach the specific objectives. These experiments are organized in chronological order of the works performed.

Chapter 4: *Bioremediation of aquaculture wastewater from Mugil cephalus (Linnaeus, 1758) with different microalgae species.*

This chapter meets the first objective of the thesis.

Is evaluated and compare the biomass yield and the capability of three different marine microalgae species, for the removal of dissolved inorganic nutrients (nitrogen and phosphorous) from aquaculture wastewater. It's used untreated grey mullet (*Mugil cephalus*) wastewater as culture medium. Nutrient uptake and biomass production are evaluated in batch conditions using two completely mixed bubble column photobioreactors of 6 L.

Chapter 5: *Production of microalgae in aquaculture wastewater and calibration of the mechanistic microalgae model BIO_ALGAE.*

This experiment is related to the second objective.

Two out of three species used in the previous chapter are selected for the cultivation in grey mullet aquaculture wastewater using a column photobioreactors of 120 L in a batch condition. The total lipid content is analyzed at the end of the experiment. The data collected are used for the first time, to implement and calibrate the microalgae-bacteria mechanistic model BIO_ALGAE for aquaculture wastewater in order to simulate the uptake of nutrients (N, P) and the biomass production of these marine microalgae.

Chapter 6: *Validation of the BIO_ALGAE Model in aquaculture systems for the semi-continuous production of Tetraselmis suecica.*

This chapter meets objectives 3 and 4.

Is used only the microalga that in the previous studies has better adapted in the aquaculture wastewater. This species (*Tetraselmis suecica*) is cultivated for biomass production in a semi-continuous system with wastewater from an intensive aquaculture system. The aim is to validate the mechanistic model BIO_ALGAE in order to simulate the production and nutrients uptake of this marine microalga in a semi-continuous system and with two different HRTs. The biochemical composition of biomass is analyzed and respirometric tests are performed to assess the oxygen uptake rates and oxygen production rates.

In Chapter 7 the main conclusions of this research are presented.

3

State of the art

3.1 Aquaculture systems

Traditional aquaculture has existed for over a thousand years, however its value in the food production sector has been recently recognized and this has led to strong investments in this field (Holmer et al., 2007; Turchini et al., 2010). Since the late 1980s, aquaculture was responsible for the growth in fish supply for human consumption (FAO, 2016) and in the last decades the production of aquatic animals (captured and farmed combined) has increased from approximately 26% in 2000 to 45% in 2015 (FAO, 2017).

Traditional intensive aquaculture is based on the production of a single commercial species, fed with formulated feeds, which uses natural resources and causes environmental changes because of wastewater, which is characterized by high quantities of non-consumed nutrients (Munday et al., 1992; Pillay, 1992). Aquaculture it is often subject to limitations, such as the availability of suitable sites and the different uses of marine areas (Cataudella and Spagnolo, 2011). In addition, aquaculture represents one of the major contributors to the increasing levels of organic waste and toxic compounds in the marine environment (Vezzulli et al., 2008; Gondwe et al., 2012). There are different aquaculture technologies and their environmental impact can be highly variable in marine systems. Typical

systems and processes are: (1) open systems, where water is constantly replenished from the sea or (2) closed systems with a continuous clean, aeration and recirculation through the system (VanGorder, 1990).

Several countries around the world are experimenting new models of integrated aquaculture to reduce the environment pollution, which involves the production of many species with a high commercial value and different trophic levels (Figure 3.1). Through these systems it is possible to breed a great diversity of species, by adapting them to the geographical context and responding to market demands.

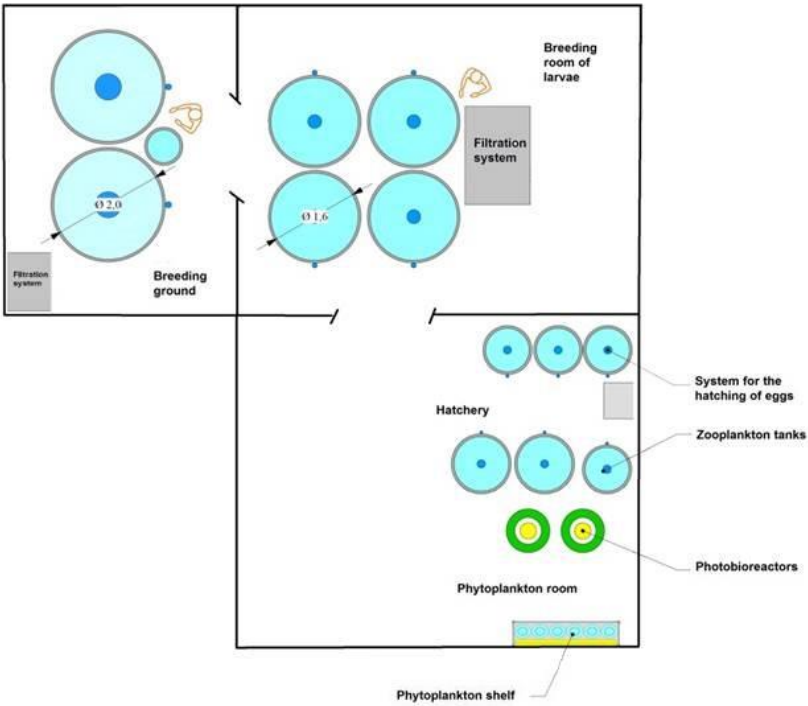

Figure 3.1: Experimental laboratories of Integrated aquaculture in International Marine Centre (Oristano, Sardinia - Italy). The system is composed by an incubation system, and breeding of larvae and juveniles of species of commercial interest. The breeding room has an independent recirculation system with biological and mechanical filters, UV lamp and refrigerators. There are two tanks of 2.5 m^3 for the reproduction, and 4 tanks of 2 m^3 for the larvae. Laboratories are also organized for breeding of benthic animals such as the sea urchin.

Recirculating aquaculture (RAS) is a method of farming fish or other aquatic organisms by reusing the water in the production. These aquaculture systems can operate in outdoor or indoor.

This technology is based on the use of mechanical and biological filters, and the system can be used for any species grown in aquaculture such as fish, shrimps, clams, etc. This system is used mainly when water availability is restricted enabling, to recycle the 90-99% of the water (Badiola et al., 2012). The system reduces water usage and improves waste management and nutrient recycling. European countries such as United Kingdom, Ireland, Italy and Norway have promoted RAS as one of the possible solutions and opportunities to further develop aquaculture (Eurostat, 2010; Eurostat, 2011; Badiola et al., 2012). Moreover, RAS makes intensive fish production compatible with environmental sustainability, because effluent is treated before final discharge. The waste treatment may include devices for sludge thickening, sludge digestion and for inorganic phosphate and nitrogen removal (Jaap van Rijn, 2013). At the same time the purified water is subsequently recirculated in the system (Bovendeur et al., 1987; Eding and van Weerd, 1999).

Among the various integrated systems, the "IMTA" Multi-Trophic Aquaculture is the most renowned (Lorkowski et al., 2012). The term “multi-trophic” refers to the several aquatic species of different trophic or nutritional levels being reared in the same system (Neori et al., 2007; Chopin et al., 2006). IMTA has as primary objectives the reduction of pollution and the

increase of productivity, making aquaculture more sustainable all over the world (Butterworth et al., 2010). These systems include both land-based and offshore mariculture systems (Shpigel et al., 2013). Each species is bred in a separate module, fishes are fed with artificial food, the suspended solids (feces and feed) are consumed by filter feeders and detritivores that can be used directly for human consumption (Barrington et al., 2009). At the same time, the final products of the metabolism of these organisms, like dissolved nutrients (nitrogen and phosphorus), are assimilated by primary producers (micro and/or macroalgae).

The first integrated land-based cultures of marine fish and shellfish and phytoplankton were described by Hughes-Games (1977) and Gordin et al. (1981). Manzi et al., (1988) and Wang (1990) described intensive pond polyculture systems, composed by shrimps, phytoplankton, and bivalves that supported good survival and high yields. Miller (1989) described commercial land-based polyculture of abalone and sea urchins. Krom et al. (1989) and Erez et al. (1990) studied a semi-intensive "green-water" system (seabream and grey mullet pond) that supported dense populations of diatoms, excellent for feeding oysters.

Nowadays, technologies are well established and typically include two or three species, but there are many combinations of organisms: shellfish-shrimp, fish-shrimp and seaweed-shrimp,

fish-sea urchin, fish-polychaetes (Figure 3.2) (Troell et al., 2003; Hambrey and Tanyaros, 2003).

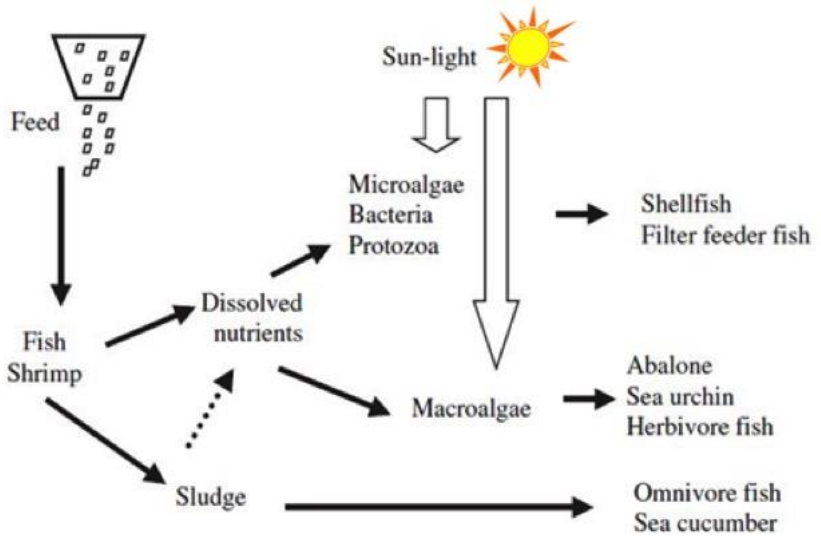

Figure 3.2: A diagram of optional nutrient pathways to crops and waste in the IMTA System (Neori et al., 2017).

These systems are modular and adaptable for several culture combinations that have an important commercial value. For example, a land-based integrated seabream–shellfish–seaweed farm of 1 ha can produce 25 tons of fish, 50 tons of bivalves and 30 tons fresh weight of seaweeds annually. A different system can produce in 1 ha 55 tons of seabream, with 385 of fresh weight of seaweed (Neori et al., 2004). In the IMTA system there must be an equilibrium in the biological and chemical

processes, in particular between nutrient production by the main organism, nutrient uptake capacity by algae, and then for the consumption of these by algivores (Shpigel et al., 2013).

In these systems algae play a double role (both ecological and productive), thus through their photosynthetic activity they introduce new energy as organic carbon and can also be used in other industrial sectors as biomass (if they are not used in the same system). In general, the IMTA systems used macroalgae as biofilters (FAO, 2009). Nowadays, the use of microalgae as biofilter is not common, but it has been demonstrated that they can effectively treat aquaculture wastewater (Cai et al., 2013; Milhazes-Cunha et al., 2017; Andreotti et al., 2017).

3.2 Microalgae in aquaculture

Microalgae are autotrophic photosynthetic organisms that usually constitute the first step of the aquatic food chain. Microalgae are a key food source in aquatic environments, ensuring the flow of matter and energy necessary for the maintenance of heterotrophic organisms.

Microalgal biotechnology only began to develop in the middle of the last century. Nowadays, there are numerous commercial applications of microalgae, especially in the areas of pharmaceuticals, agriculture, pollution control, cosmetics and

energy (Pulz and Gross, 2004; Spolaore et al., 2006; Rosenberg et al., 2008). Microalgae can be used to enhance the nutritional value of animal feed and play a crucial role in aquaculture because their biomass contains products with a commercial importance such as proteins, lipids, carbohydrates, and pigments (Figure 3.3) (Torzillo and Vonshak, 2004; Hu, 2014). Under normal culture conditions, the typical content in the microalgal biomass is 25%–50% of total protein, 5%–40% carbohydrate, and 10%–30% lipid (Brown et al., 1997). It was observed that, in the late-logarithmic growth phase, microalgae contain typically 30-40 % protein, 10-20 % lipids and 5-15 % carbohydrates (Brown et al., 1997, Renaud et al., 1999).

Figure 3.3: diagram of the main uses of microalgal biomass. Microalgae are photosynthetic organisms whose growth is appropriately favored by nutritive salts, light and carbon dioxide. Microalgae are suitable to produce lipids and other biomolecule that can be use for biofuels production, for human and animal consumption and in pharmaceuticals and cosmetics areas.

Concerning aquaculture application, microalgae are widely used as feed for rearing larvae and juveniles of many species of great economic value: mollusks, crustaceans, gastropods and fish (freshwater and marine). They have also a key role in growing different kinds of zooplankton (rotifers, cladocerans, copepods), which are used as live food in crustacean and finfish farming.

Under different culture conditions, microalgae have the capacity to modify their internal composition and therefore their nutritional value (Enright et al., 1986a; Brown et al., 1997). The chemical composition of microalgae depends on some factors. For example the lipid contents depends on the species, cell density, age of the culture, harvesting phase and growth conditions (temperature, lighting, nutrients, etc.) (Impiccini et al., 1996; Mayer et al., 1997).

When microalgae are cultivated under nutrient starvation, the chlorophyll production and biomass productivity decreas due to the allocation of fixed carbon, which is not used for growth (Monfet et al., 2017). The typical lipid content in algal cell can be increased limiting the nitrogen (Benemann and Oswald, 1994). Indeed, limiting the nitrogen the algal growth decrease and lipid content increase up to 40% (Park et al., 2011). In stationary phase, when nitrogen is limiting, the proximate composition of microalgae can change significantly. For example, carbohydrate levels can double at the expense of protein (Harrison et al., 1990; Brown et al., 1993b). In the same way, phosphorus can be the limiting nutrient to promote lipid production (Monfet et al., 2017).

Several hundred species of microalgae have been studied as feed in aquaculture, but only a few species currently have a real application (Priyadarshani et al., 2012).

Microalgae must have different characteristics to be useful in aquaculture:

- size for ingestion (1 to 15 μm for filter feeders and 10 to 100 μm for grazers) (Jeffrey et al., 1992; Kawamura et al., 1998);
- readily digestive for animals;
- fast growth rates;
- being stable in culture to fluctuations in temperature, light and nutrients;
- have a good nutrient composition, including absence of toxins that might be transferred up the food chain (Brown and Robert, 2002).

For their growth microalgae need vital elements such as light, water, carbon dioxide and nutrients such as nitrogen (N), phosphorus (P) and potassium (K), also silica (Si) and iron (Fe), and other trace elements.

When the N:P ratio deviates from the optimal value, algae might accumulate nutrients without biomass production (Monfet et al., 2017). In algal biomass the N:P ratio can vary from 4:1 to 40:1 depending on the species and nutrient availability (Craggs et al., 2011). Usually, the inorganic forms of nitrogen and phosphorus are directly accessible to microalgae. In particular, nitrogen

compounds are bioavailable for microalgae in various forms, but the predominant are NO_3^-, NH_4^+ and urea (Price et al., 1985; Kristiansen and Lund, 1989). When these sources are simultaneously presented, ammonia is preferred, followed by nitrate and urea (Harrison et al., 1985; Levasseur et al., 1990; 1993). When $NH_4^+$and NO_3^- are absent, algae may use nitrite (Chen et al., 2012), but at the same time it has been demonstrated that high nitrite concentrations are toxic and inhibiting the growth (Chen et al., 2011).

The uptake of nutrients depends on several factors such as environmental conditions, species, nutrient ratios and growth rates. It is also necessary to achieve a right balance between these different elements and in the presence of favourable environmental conditions, microalgae usually double their biomass in 24 h (3.5 h in the exponential growth phase), with a very short harvesting cycle (1-10 days) (Singh et al., 2010; Pfromm et al., 2011; Thurmond et al., 2011).

3.3 Microalgae in aquaculture wastewaters

A great number of studies have demonstrated the capacity of microalgae to remove nitrogen and phosphorus from wastewaters or seen in another way, the capacity of wastewaters to sustain algal growth (Monfet et al., 2017). The use of wastewater for microalgae cultivation as a substitute for the synthetic medium has the potential to reduce production costs (Cai et al., 2013).

Thanks to their ability to uptake the nutrients quickly, several authors have evaluated the possibility to employ microalgae for treating wastewater from fish or shrimp production plants (Riaño et al., 2011; Michels et al., 2014; Nasir et al., 2015). The integration of wastewater remediation and production of microalgae in aquaculture systems is an alternative method to optimize the use of the resources. In fact, it offers combined advantages of treating the wastewater and simultaneously producing algal biomass, which can further be exploited for producing valuable products (Lam and Lee, 2012; Christenson and Sims, 2011). In this way, the waste is considered a resource for algae growth, which in turn restores water quality reducing the environmental impact of fish culture. Algae offer economic returns because of direct sales of biomass (Chopin et al., 1999), in addition, the feeding costs for herbivores are reduced as well

as the pumping costs through recirculation and the wastewater treatment (Bolton et al., 2009; Nobre et al., 2010).

The assimilation of nitrogen and phosphorus in wastewater is coupled and biomass production is maximized with an optimal N:P ratio (Liu et al., 2011). At an industrial-scale, cultivation of microalgae in aquaculture wastewater in modern reactors and with controlled parameters has not yet been done. Several studies at a lab-scale and pilot-scale experiments demonstrated that microalgae had a high nutrient removal rates (in terms of NH_4^+, NO_3^-, NO_2^- and PO_4^+) ranging between 75% and 100% (Borges et al., 2005; Freire et al., 2013; Michels et al., 2014;).

Lefebvre et al. (2004) changed the classical concept of IMTA (fish-bivalve-macroalgae) drawing attention to microalgae, through the system of a fish-phytoplankton-bivalve design based on the potential of microalgae to uptake nutrients. In this system, the cultivation of marine microalgae (e.g. *Isochrysis*, *Tetraselmis* or *Phaeodactylum*) had a great potential for bivalve feeding as well as for wastewater treatment (Milhazes-Cunha et al., 2017). However, further studies on the growth of microalgae in aquaculture wastewater must be carried out. Indeed, the characteristics of these effluents can be species-specific and all microalgae should be analyzed (Milhazes-Cunha et al., 2017). For example, *Isochrysis galbana* have a much lower productivity than *Tetraselmis suecica* when cultivated in the

same aquaculture wastewater (Borges et al., 2005). On the contrary, instead, Freire et al., (2013) have successfully cultivated the *Isochrysis* genus in fish farm effluents, with a production of 0.08 g L^{-1} d^{-1}.

Nowadays, very few studies have analyzed the microalgal biomass composition produced in aquaculture wastewater. In a recent experiment it was observed that *Chlorella sorokiniana* had a biomass productivity of approximately 500 mg L^{-1} d^{-1} in aquaculture wastewater of a freshwater system. This biomass presented a lipid productivity of approximately 150 mg L^{-1} d^{-1}, carbohydrate productivity of 170 mg L^{-1} d^{-1} and protein productivity of 140 mg L^{-1} d^{-1} (Guldhe et al., 2017).

Khatoon et al., (2016) compared the growth, productivity, and approximate composition of *Chaetoceros calcitrans*, *Nannochloris maculate*, and *Tetraselmis chuii* cultivated in wastewater collected from aquaculture shrimps and Conway medium. This study highlighted that lipid productivity in *N. maculate* was higher when cultured in wastewater than in the Conway medium, while there were no differences for *C. calcitrans* and *T. chuii* when cultivated in the two media. *N. maculate* and *T. chuii* in aquaculture wastewater showed a higher lipid and protein content compared to *C. calcitrans*. On the contrary, the carbohydrate content has not been influenced by the two culture media (Table 3.1).

In aquaculture wastewater, nitrogen and phosphorous content are lower than in synthetic culture medium, which could cause a stress condition for microalgae. It was demonstrated that under this stress conditions, microalgae usually tend to accumulate more lipids and carbohydrates (Singh et al., 2015; Sarat Chandra et al., 2016; Ansari et al., 2017). Most of the studies that used wastewater as a culture medium for microalgae were focused on lipid accumulation, while few works were published on the carbohydrate and protein content (Ansari et al., 2017).

Table 3.1: The content of lipids, carbohydrates, and proteins in microalgae cultivated in different aquaculture wastewater (AW).

Microalgae	Medium	Lipids %	Carbohydrates %	Proteins %	Reference
Scenedesmus sp.	AW	31.6	-	-	HF Ma et al. (2012)
Chlorella sp.	AW (Axenic condition)	12.5	-	-	Halfhide et al. (2014)
Chlorella sp.	AW (Non-axenic)	50.4	-	-	Halfhide et al. (2014)
C. calcitrans	AW	~15	~16	~20	Khatoon et al. (2016)
N. maculate	AW	~17	~19	~28	Khatoon et al. (2016)
T. chuii	AW	~12	~17	~34	Khatoon et al. (2016)
S. obliquus	AW	30.85	35.05	19.52	Ansari et al. (2017)
C. sorokiniana	AW	31.85	35.43	28.81	Ansari et al. (2017)
A. falcatus	AW	35.9	33.88	30.59	Ansari et al. (2017)
T. chuii	AW+Conway medium	28	22	45	Khatoon et al. (2018)
T. chuii	AW	17.6	12.1	33.7	Khatoon et al. (2018)

3.4 Aquaculture wastewater

Typical aquaculture wastewater (AW) includes feces and nutrients from excretion by aquatic animals, as well as uneaten feeds, chemicals substances and antibiotics. A significant discharge of these wastewaters into lakes, rivers, estuaries or seas it may cause adverse environmental impacts. Water quality is a critical factor in aquaculture systems. Optimal water quality varies by species and must be monitored to ensure growth and survival.

In aquaculture industry, water quality parameters that are commonly monitored include: temperature, dissolved oxygen, pH, alkalinity, hardness, ammonia, and nitrites.

The compounds present in wastewater can be found in the form of dissolved substances or suspended solids. The concentrations are commonly low if compared with municipal wastewater. In aquaculture effluents the values are generally about 14 mg/L for suspended solids, 100 – 150 mg/L for COD, 1.4 and 0.13 mg/L for total nitrogen and total phosphorous respectively (Cripps and Kelly, 1996).

Dissolved contaminants include ammonia, nitrate, phosphate and organic matter. Suspended solids are usually uneaten food

and fish feces that can make natural waters more turbid and eventually form organic deposits on the bottom of water bodies. It is therefore very important to quantify the fish waste productions to monitor risks in the marine environment.

3.4.1 Dissolved contaminants in aquaculture systems

The pollution load in aquaculture wastewater depends on factors such as the species produced, life stage, size, rearing system, diet and environmental parameters (Foy and Rosell, 1991a; Lazzari and Baldisserotto, 2008).

Most dissolved compounds are feed-derived waste, antibiotics, and hormones, as mentioned before (Tacon et al., 1995).

Concentrations of dissolved nitrogenous and phosphorous compounds in effluents depend on the quantity of un-metabolized food and on the digestibility of the raw material (Kaushik, 1998).

The most abundant dissolved compounds are the nitrogenous wastes excreted by fish because of aminoacid degradation in the body (Mugg et al., 2007). Teleost fish digest the proteins in their feed and excrete ammonia through their gills and in their feces as end-products of protein catabolism whereas phosphate and urea are excreted by the kidney (Lemarié et al., 1998). The amount of ammonia excreted by the fish is related to the feeding rate and the protein level in feed (Hargreaves and Tucker, 2004).

In general, ammonia represents 75-90% of the total nitrogenous loss (Handy and Poxton, 1993). In aquaculture facilities, ammonia is monitored, because it is highly toxic to fish, dangerous short-term levels start at about 0.6 mg/L (Ogbonna and Chinomso, 2010).

Total ammonia nitrogen is composed of toxic (un-ionized) ammonia (NH_3) and nontoxic (ionized) ammonium (NH^+_4). When ammonia gas (NH_3) dissolves in the water, it reacts with the water to give ammonium ions NH_4^+ while some remains unionized as dissolved NH_3 (Figure 3.4).

In culture systems, the equilibrium between NH_3 and NH_4^+ is affected by temperature and pH (pK_a= 9.25). As the pH increases, the amount of toxic NH_3 increases, which can be harmful to fish. Fish have different tolerances of ammonia, trout reduce their growth rate over a level of 0.0125 mg/L, whereas catfish have damage to gills over 0.12 mg/L of NH_3 (Mugg et al., 2007).

The loss or the transformation of ammonia takes place mostly with two processes: the uptake of ammonia by algae and the nitrification process by bacteria. The assimilation by microalgae is important to reduce the amount of ammonia dissolved in the water.

In nitrification ammonia is aerobically converted to nitrate by nitrifying bacteria. This process transforms NH_4^+ to NO_3^- and is often coupled to denitrification in which activity of denitrifiers reduce NO_3^- to nitrogen gas (N_2), that is subsequently lost to the atmosphere (Knowles, 1982) (Figure 3.4).

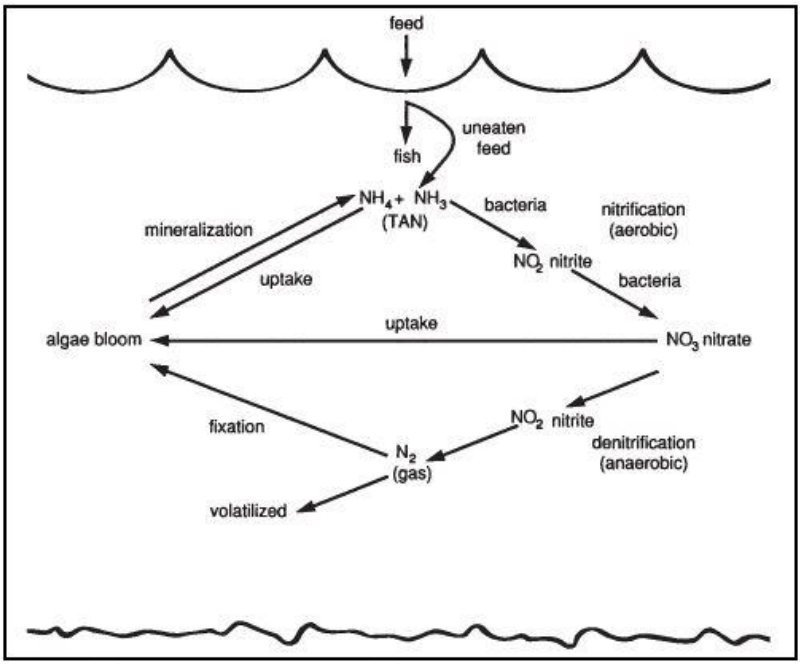

Figure 3.4: Nitrogen cycle in a fish pond (Durborow, 1997).

In aquaculture wastewater, nitrates have a range between 2 to 110 mg/L (Lowrey et al., 2014; Gao et al., 2016). Conversion of ammonia to nitrate does not remove the total dissolved nitrogen from the aquaculture system, it simply makes the form of nitrogen less toxic to the cultured organisms. Temperature, ammonia concentration and dissolved oxygen are the main factors that affect nitrification rate (Hargreaves and Tucker, 2004).

The nitrification process can be promoted on surfaces of biological filters in recirculating or water reuse systems. During the oxidation of ammonium to nitrate it is obtained nitrite (NO_2^-) as intermediate product. In oxygenated aquaculture waters, the nitrite concentration is typically less than 0.005 mg/L but can reach concentration of 50 mg/L (Avnimelech et al., 1986; Kamstra et al., 1996). This may result in a damage to aquatic organisms or in a mass fish mortality (Svobodova and Kolarova, 2004; Svobodova et al., 2005a). Studies on fish and crustaceans have showed that nitrite induced a large variety of physiological disturbances (Jensen, 1995; 2003). The nitrite is toxicant for fish and interfering in numerous physiological functions including ion regulatory, respiratory, cardiovascular, endocrine and excretory processes (Kroupova et al., 2005). Nitrite toxicity to fish varies considerably and depends on several external and internal factors. The most important are water quality (e.g. pH,

temperature, cation, anion and oxygen concentration), length of exposure, fish species, fish size and age, and individual fish susceptibility (Kroupova et al., 2005).

The ammonia (53–68%) and urea (6–10%) are the most important forms of nitrogenous waste in juvenile rainbow trout, but have a considerable excretion effect on nitrogen as amino acids (4–10%) and as protein (3–11%) (Kajimura et al., 2004). Outputs of creatine and creatinine contributed only as a small fraction to total nitrogen excretion (<1.4%), whereas trimethylamine, trimethylamine oxide, uric acid, and nitrite + nitrate, were not excreted in detectable quantities.

The quantities of total phosphorus (TP) in aquaculture wastewater fluctuate between 2 and 50 mg/L (Lowrey et al., 2014; Gao et al., 2016). Most of the phosphorus in aquaculture effluent originates from animal feed. When this phosphorus is ingested by fish either becomes incorporated into the fish body or is excreted into the environment. The content, solubility, and availability of phosphorus in formulated fish diets may vary with the types of ingredients used. Phosphorus in fish meal is in the form of tricalcium phosphate, which remains almost inaccessible to many cultivated species. The other chemical forms presented in the diet have significant problems in the digestibility as bone, phytin-P or organic P (Azevedo et al., 1998). These products could be present at levels potentially high

enough to cause environmental damage (Sugiura et al., 2000a).

The best method to manage the phosphates in the effluent of aquaculture consists in controlling and limiting the quantities in the feed (Lall, 1991). In most of fish and crustacean feeds the requirements of ranged from 0.3 to 0.8% of the dry weight of the diet (Penaflorida, 1998). Phosphorus uptake depends also on the growth rate (Jahan et al., 2002). In fish, a non-fecal phosphorus excretion occurs even if the intake of phosphorous is zero (Rodehutscord et al., 2000).

Concentrations of the main nutrients in aquaculture systems, found in different studies are reported in the Table 3.2.

Table 3.2: Values of the main nutrients found in aquaculture wastewater systems in different studies

N-NO2 (mg/L)	N-NO3 (mg/L)	P-PO4 (mg/L)	NH_4-N (mg/L)	COD (mg/L)	Microalgae	Aquaculture	Reference
0.1	1.1	0.3	-	-	*Skeletonema costatum*	*Psetta maxima*	Hussenot et al., (1998)
0.1	0.2	0.4	-	-	*S. costatum*	*Dicentrarchus labrax*	Hussenot et al., (1998)
-	-	-	13.7	-	*Oocystis* sp.	*Onkhorynchus mykiss*	Riaño et al., (2011)
-	-	-	17.3	-	*Oocystis* sp.	*O. mykiss*	Riaño et al., (2011)
0.2	1.7	0.2	0.5	-	*Platymonas sub cordiformis*	-	Guo et al., (2013)
0.1	12.2	6.8	5.6	-	*Chlorella* sp.	*Lates calcarifer*	Lananan et al., (2014)
0.1	40.7	-	0.5	115	*Tetraselmis*		Michels et al., (2014)
-	17.6	16.9	-	238	*Chlorella sp. Scenedesmus*	Tilapia	Halfhide et al., (2014)
-	-	2.6	-	-	*Chlorella* sp	*Clarias gariepinus*	Nasir et al., (2015)
0.1	2		-	-	*Chlorella vulgaris, Scenedesmus obliquus*	*Penaeus vannamei Boone*	Gao et al., (2016)
3.8	3.5	7.2	6.1	-	*Chaetoceros calcitrans, Nannochloris maculate, Tetraselmis chuii*	Shrimp	Khatoon et al., (2016)

-	-	6	-	-	*C. vulgaris*	-	Blanco-Carvajal et al., (2017)
	40.7	8.8	5.3	96	*C. sorokiniana*	Nile tilapia	Guldhe et al., (2017)
5.5	40.7	8.8	5.3	96	*C. sorokiniana, S. obliquus, Ankistrodesms falcatus*	Nile tilapia	Ansari et al., (2017)
4.1	-	5.6	5.3	-	*T. chuii*	-	Khatoon et al., (2018)

About carbon dioxide in fish-ponds, it is mainly produced by the respiration of fish, microalgae and other animals. Decomposition of organic matter is also a source of carbon dioxide in aquaculture facilities. During the day, oxygen is supplied by photosynthesis of algae and other aquatic plants, whereas during the night, photosynthesis ceases, and the algae, sediment, and fish consume oxygen (Hargreaves and Brunson, 1996). The daily pattern of carbon dioxide concentration is generally opposite to that of dissolved oxygen (Figure 3.5).

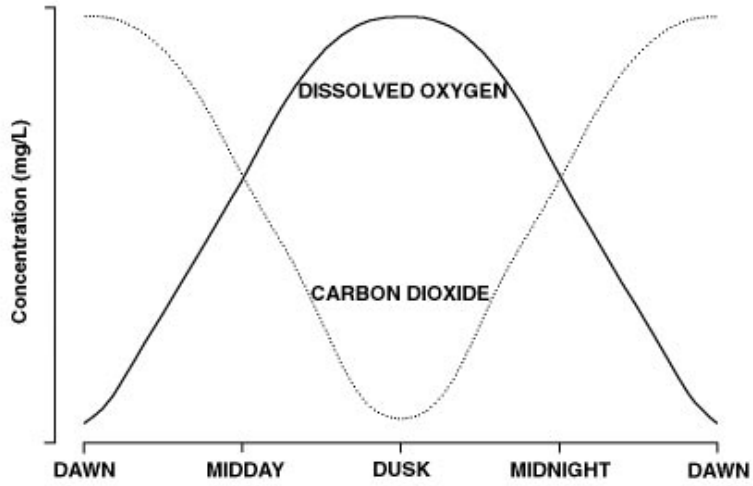

Figure 3.5: The daily cycle of oxygen and carbon dioxide in a fish pond (Hargreaves and Brunson, 1996)

During the day algae assimilate the CO_2 from the water. Therefore, the carbon dioxide concentration is lowest in the late

afternoon (can be almost 0 mg/L), while the dissolved oxygen is highest. The CO_2 concentration grow during the night with the respiration of the organisms in the pond, reaching a peak at dawn (usually around 10 to 15 mg/L) (Hargreaves and Brunson, 1996).

Daily fluctuation of CO_2 may cause animal toxicity. In response to a difference concentration of CO_2 between the blood and water, fish can release CO_2 through the gills. If the concentrations of carbon dioxide environment are high, fish will have difficulty to reduce concentrations internal carbon dioxide (Hargreaves and Brunson, 1996). This toxicity is attributed to the Bohr effect when the decrease in the ability of the fish's hemoglobin to transport oxygen because of the elevated level of CO_2. This indicates that high CO_2 levels compromised fish respiration.

3.4.2 Particulate contaminants in aquaculture systems

Suspended solids (SS) are usually defined as the particles greater than 2 µm in size in the water column (Mugg et al., 2007). Solids concentrations in aquaculture effluents usually range from 5 to 50 mg/L (Cripps and Bergheim, 2000). These concentrations depend by the rate of change of water, the hydrology of the tanks and the stocking density (Turcios and Papenbrock, 2014). Furthermore, the quality of feed, the feed rate, and the feeding method can influence the production and composition of SS.

The amount of waste also depends on the season, in fact, with the high temperature the feeding rates tend to increase.

In a properly managed aquaculture, approximately 30% of the feed used will become particulate contaminants (Miller and Semmens, 2002) (Figure 3.6).

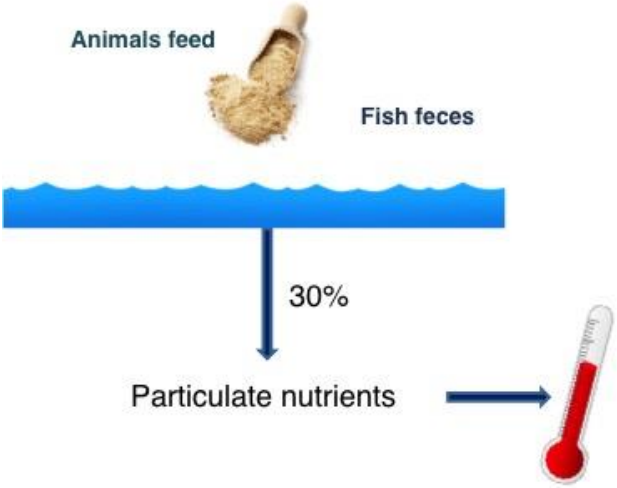

Figure 3.6: In aquaculture system approximately the 30% of the feed used becomes particulate contaminants. This causes an increase in temperature, and the amount of waste is higher in the summer when the feed rate is higher.

The quantity of the unconsumed feed can be reduced with a correct feeding regime that provides the right amount of food when the fish require it (Cripps and Bergheim, 2000). The quality of the ingredients in the feed play also an important role due to the effect on feed and feces stability in the water. As a result, it gives a reduction in the quantity of faecal solids produced. To maintain waste integrity, with subsequent slower fragmentation rates, a primary treatment, or solid waste removal, should be done as soon as possible (Cripps and Bergheim, 2000).

Feed and fecal wastes also contribute to increase the levels of BOD, total nitrogen and total phosphorus (Alabaster, 1982). The suspended solids are composed in general by a 7–32% of total nitrogen and by a 30–84% of total phosphorus (Cripps and Bergheim, 2000).

The intensification of the practice of aquaculture, in response to market pressures, has led companies to increase the stocking density of fish resulting in the production of large volumes of suspended solids. Concentrations are usually low in the effluents of facilities in open systems. In recirculating systems, effluents

tend to have higher concentrations, but all fish farms have concentrations significantly lower than those found in treatment systems for domestic wastewater.

Through hydrolysis, suspended solids can be transformed to provide substrates (dissolved nutrients) (Castine et al., 2013). It is very important to know the composition of a waste to maintain SS at acceptable levels for discharging or recycling (Turcios and Papenbrock, 2014). An appropriate treatment technology and waste management technique can be employed to reduce the production of suspended solids, and to facilitate their removal (Cripps and Bergheim, 2000).

3.4.3 Regulation of environmental impacts in Europe

One of the purposes of the EU Common Fisheries Policy (CFP) is to take measures to mitigate the impact of aquaculture on the environment. As already mentioned, aquaculture activities may apply pressure and produce a huge impact upon aquatic ecosystems. For example, wastewater quality is determined by the concentration and amount of nutrients in the discharge water and the flow rate of the effluent. Aquaculture systems require high-quality waters, therefore, management measures which introduce and maintain best practices for the protection of the environment are essential to the industry functioning.

Aquaculture involves different application areas, many of which are already regulated. The EU's water policy has been regulated throughout two instruments: the Water Framework Directive (WFD), covering inland and coastal waters, and the Marine Strategy Framework Directive (MSFD), covering marine waters. The WFD was published and entered into force in December 2000. The fundamental aim of the WFD is to maintain, improve and protect the ecological status of the aquatic environment. This extends from rivers, lakes, and ground-waters through to transitional (including estuaries) and coastal waters. Includes five classes for the ecological status classification: high, good, moderate, poor and bad. Article 4 of this directive, requires that

the Member States prevent deterioration of the ecological of surface waters, to take all the necessary measures to progressively reduce pollution and phase out the emissions, discharges, and losses of priority hazardous substances.

The MSFD (2008/56/EC) of the European Parliament and of the Council (17 June 2008) establishes a framework within the Member States with the aim to achieve good environmental status in marine waters by 2020. It concerns aspects of the environmental status of the coastal waters, which are not already addressed by the WFD or other Community legislation.

The evaluation criteria associated with the two Directives MSFD and WFD differ due to the geographical scale. The "good environmental status" in MSFD is not exactly equivalent to "good ecological status" in WFD.

In both Directives, the chemical quality, the effects of nutrient enrichment, and aspects of ecological quality and hydro-morphological quality are closely related.

The WFD and the MSFD do not contain specific obligations for aquaculture (COM 2016, 178 final). The aquaculture industry must observe the requirements of the national legislation that implements those Directives in each Member State.

In fact, the Annex II, section 1.4 of the WFD requires each Member State to manage and maintain information on the type

and measure of anthropogenic pressures on surface waters. For MSFD instead, aquaculture is potentially relevant as regards the reduction in contaminants and litter in the marine environment, with improved water quality and reduction of contamination of the fish produced.

3.4.4 Management of aquaculture wastewater in Italy

Italy has an important role in European aquaculture, contributing to 12.6% of EU aquaculture production volume (FAO, 2016). Italian aquaculture produces according to high environmental standards. ISPRA (Italian Institute for Environmental Protection and Research) annually presents an estimate of potential environmental pressures related to aquaculture activities by geographical area and uses data collected from aquaculture farms in Italy pursuant to the Regulation (EC) No. 762/2008 of the European Parliament and of the Council.

In Italy, the regulatory competence of aquaculture activities is delegated to each region, which can delegate other local authorities to manage it.

Italian legislation defines the environmental regulation through the legislative decree 3 April 2006, n. 152. Article 101 of the Decree regulates the discharges according to the quality objectives of the water bodies and must, in any case, respect the limit values present in Attachment 5 to the third part of the

decree. Wastewater from aquaculture and fish farming facilities is related as domestic wastewater. These waters are characterized by a density of breeding equal to or less than 1 kg per square meter of water or in which a flow of water equal to or less than 50 L per minute second is used.

The main purposes of this decree concern:

· Prevention and reduction of water pollution;

· Reduction of pollutants for already polluted water;

· Improvement of the general state of the water;

· Protection of water intended for particular uses in order to maintain natural self-purifying water capacity and consequently protecting plant and animal communities.

Companies must adopt the best techniques available to eliminate or reduce hazardous substances present in the drains. The individual industries must therefore evaluate and characterize their waste.

3.5 Microalgae mechanistic models

Modern microalgal biotechnologies require new tools to forecast bioremediation and biomass production. Microalgae growth depends on many factors such as light, temperature, nutrients availability (e.g. nitrogen and phosphorus) as well as on certain inhibitory conditions (e.g. excess of oxygen), which have multiple interactions between one another. This complexity has encouraged the development of different mathematical models in the last years (e.g. Bernard et al., 2009; Solimeno et al., 2015; Solimeno et al., 2017).

Mathematical models help to study and discern the simultaneous effect of the different factors affecting algal growth, and allow forecasting algal production. Monod and Droop equations are usually adopted to describe nutrient limitation of microalgae (Sommer, 1991). The Droop quota model was published in 1968 and was the first model that related the process rates and the content of microalgae cell (Sommer, 1991; Richmond, 2004). This model originated as an empirical description of the relationship between the cell quota (an amount of a resource within a cell, hence 'cell quota') and the organism's steady-state growth rate. This was in contrast with the previous Monod model (1950), which was based on the concept of the growth

controlling substrate. This substrate is called "limiting substrate" to indicate that the growth rate was correlated to the concentration of a particular metabolite. In this formulation, the growth rate (μ) was related to the culture concentration of the limiting nutrient (S). The Monod model has the same formulation of the Michaelis - Menten model of enzyme kinetics. This model was adopted to describe nutrient use by marine microalgae and nutrient competition between algae (Dugdale, 1967; O'Brien, 1974; Petersen, 1975).

The general specific growth rate for Monod formulation is:

$$\mu\ [T^{-1}] = \mu_{max} * S/K_S + S \qquad (1.1)$$

where μ_{max} $[T^{-1}]$ is the maximum specific growth rate, S $[M\ L^{-3}]$ is the dissolved nutrient concentration, and Ks $[M\ L^{-3}]$ is the half-saturation constant of the Monod mode.

In the last two decades, complex microalgae-bacteria mechanistic models have been developed to treat wastewater and at the same time producing valuable biomass (Solimeno et al., 2017).

Due to the multiple factors involved in wastewater treatment, and for the interaction between organisms, these models are inherently complex. The River Water Quality Model No. 1 (RWQM1) is an indicative model for water quality management, especially in rivers, and it was used as basic model for

microalgae treatment systems because it considers microalgae as well as bacteria. RWQM1 is based on mass balance of chemical elements expressed as Biochemical Oxygen Demand (BOD). The RWQM1 also includes chemical equilibrium of nitrogen, carbon and phosphorus species and considers 26 processes and 24 components (9 particulate and 15 soluble). RWQM1 kinetic expressions are grounded on switching functions of nutrient availability, light, and temperature (Monod, Lambert and Beer's Law, and Arrhenius equations, respectively).

Sah et al. (2011) mechanistic model simulate the wastewater treatment in facultative ponds. This model was constructed coupling the ASM2 model (Activated Sludge Model No.2) (Henze et al., 1995) and CWM1 (Constructed Wetland Model No.1; Langergraber et al., 2009) for describing bacteria processes and RWQM1 for simulating microalgae growth. This model considers 19 processes and 18 components (9 particulate and 9 soluble) and processes rates are based on Monod type rate equations, while light attenuation and temperature are based on Lambert Beer's Law and Arrhenius type equation, respectively.

Zambrano et al. (2016) developed a mechanistic model to describe the growth of microalgae and bacteria consortia in a photobioreactor. The model was inspired by the ASM1 for bacteria processes and by BIO_ALGAE model for microalgae

growth. This model considers 6 processes and 6 components (2 particulate and 4 soluble).

The ASM-A model (Wágner et al., 2016) describes microalgae growth in waste stabilization ponds (WSPs), in High Rate Algal Pond (HRAPs) and closed photobioreactors fed with wastewater. This model was developed as an extension to the Actived Sludge Model No. 2d (ASM-2d) (Henze et al., 1999). The ASM-A model only shows the biochemical processes related to microalgae, where N, P limitations are described according to Droop formulation while the consumption of inorganic C is formulated using Monod kinetics.

3.5.1 BIO_ALGAE

BIO_ALGAE model was mainly built by coupling the model RWQM1 (Reichert et al., 2001) with the modified ASM3 (Iacopozzi et al., 2007), and was implemented in COMSOL MultiphysicsTM platform. This model is applicable for photobioreactors, WSPs and HRAPs.

BIO_ALGAE model was used to simulate microalgae and bacteria population dynamics. It is based on Monod type functions for carbon, nitrogen and phosphorus limitations. The other relevant features are the reaction's temperature dependence, light attenuation, and photorespiration. It also included pH dynamics and the effect of an excess of oxygen.

Temperature dependence for microalgae and bacteria was described using Arrhenius type equation, while the dynamic model by Eilers and Peters was used to describe the effect of light intensity on photosynthesis in microalgae (Eilers and Peters, 1988).

The model considers the 19 components (6 particulate and 13 dissolved) included in the common nomenclature of the International Water Association (IWA) model. Particulate and dissolved components implicated as variables in the physical, chemical and biokinetic processes are described in Solimeno et al. (2017), as reported in Table 3.3.

Table 3.3: particulate and dissolved components implicated as variables in the physical, chemical and biokinetic processes

Component	Description
S_{NO3}	Nitrate nitrogen
S_{NO2}	Nitrite nitrogen
S_{NH3}	Ammonia nitrogen
S_{NH4}	Ammonium nitrogen
S_{PO4}	Phosphate phosphorus
S_{CO2}	Carbon dioxide
S_{CO3}	Carbonate
S_{HCO3}	Bicarbonate
S_H	Hydrogen ions
S_{OH}	Hydroxide ions
S_S	Readily biodegradable soluble organic matter
S_{O2}	Dissolved oxygen
S_I	Soluble inert organics
X_H	Heterotrophic bacteria
X_I	Inert particulate organic matter
X_S	Slowly biodegradable particulate organic matter
X_{AOB}	Ammonium oxidizing bacteria
X_{NOB}	Nitrite oxidizing bacteria
X_{ALG}	Microalgae biomass

BIO_ALGAE is the only model that implements the inhibitory effect of high concentrations of carbon dioxide, and this is very

important in closed photobioreactors with CO_2 injection in which partial pressures above 0.6 atm can acidify the culture medium (Silva and Pirt, 1984).

As previously mentioned, pH dynamics are included in BIO_ALGAE. In microalgae-bacteria systems pH greatly changes following daily and seasonal rhythms. The fluctuations depend on photosynthetic activity, which impacts bicarbonate buffer system producing pH changes (Sutherland et al., 2014; Solimeno et al., 2015). The influence of pH on photosynthesis rate and bacteria growth can be easily implemented as the Arrhenius equation proposed in the model of Costache et al. (2013) for microalgae growth. Recently, a cardinal pH sub-model was included to BIO_ALGAE to represent the inhibitory effects on the growth response of microalgae and bacteria at elevated pH (Sutherland et al., 2014). This model contains three values of pH ($pH_{i,max}$, $pH_{i,min}$ and $pH_{i,opt}$). $pH_{i,min}$ and $pH_{i,max}$ represent the lower and higher limit that each microorganism can support, while $pH_{i,opt}$ is the optimal pH, and "i" is the ith species of microorganism (Solimeno et al., 2019, submitted)

As already discussed, the microalgae temperature dependence is described with a normal distribution by BIO ALGAE model. The thermic photosynthetic factor is highest at the optimal temperature (T_{opt} = 25 °C) and declines as temperature deviate from the optimum towards either higher or lower limits. Just like

pH, a modification was recently done for temperature (Solimeno et al., 2019 submitted). It was implemented a cardinal temperature sub-model that replacing the normal distribution, described in Solimeno et al. (2015).

Another new important feature added to BIO_ALGAE model was the implementation of CO_2 injection for both carbon supply and pH control (Solimeno et al., 2019, submitted).

As regard the nutrients, carbon, nitrogen, and phosphorus are in chemical equilibrium, which is affected by pH. Phosphorus is largely available in wastewater (Larsdotter, 2006), and generally is not considered in wastewater models, however, BIO_ALGAE model includes phosphorus limitations. The model considers only phosphate as phosphorus species, so phosphorous equilibrium is neglected.

Oxygen exchange with the atmosphere is very limited in closed photobioreactors (Weissmand and Goebel, 1987; Costache et al., 2013), and for this reason, BIO_ALGAE considers the excess of dissolved oxygen in the culture medium.

This model implements the attenuation of the light intensity using Lambert-Beer's Law, where the intensity depends on the presence of particulate components inside the reactors, and also by the depth of the system.

BIO_ALGAE considers endogenous respiration and decay as two different processes. The endogenous respiration produces

CO_2 and inert organic matter (X_I), while the decay transforms alive biomass into slowly biodegradable particulate organic matter (X_S) and inert (X_I) organic matter (Van Loosdrecht and Henze, 1999). X_S originating from decay process is assumed to be 80% of the total loss of microalgae biomass (Solimeno et al., 2017).

The growth rate of microalgae has a great influence on the simulation response, and for BIO_ALGAE it was calibrated as μ_{ALG}= 1.5 d^{-1}.

3.5.2 Model description

Figure 3.7, shows a general representation of the conceptual model BIO_ALGAE and describes the microalgal-bacterial interactions.

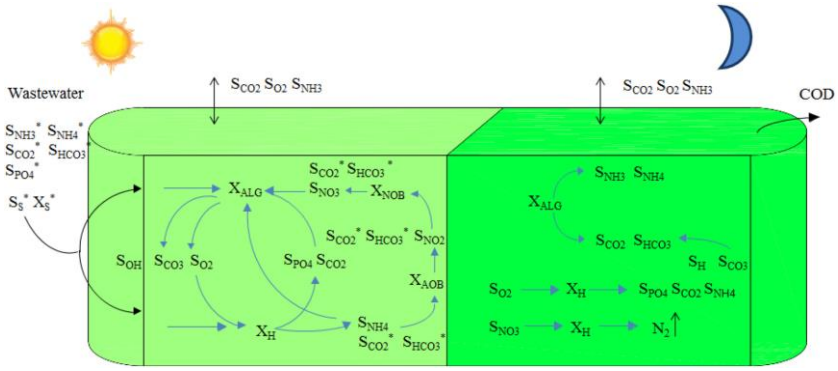

Figure 3.7: Schematic representation of the conceptual integrated model. Show the main algal-bacterial interactions in a high rate algal pond, during the day (left) and

night (right). Are marked with (*) all components that enter in the ponds with the influent, while the processes are indicated by arrows (Solimeno et al., 2017).

Light activates the photosynthetic processes in microalgae (X_{ALG}), that grow and fix inorganic carbon (S_{CO2} and S_{HCO3}). At the same time, it consumes substrates like S_{NH4}, S_{NO3}, and S_{PO4} in the wastewater and supply oxygen (S_{O2}) required by heterotrophic bacteria (X_H) to oxidize organic matter (SS, X_S). CO_2 is produced in the bacterial organic matter oxidation. During nitrification two types of bacteria participate: (X_{AOB}) ammonium oxidizing bacteria that convert ammonia and ammonium to nitrite (S_{NO2}), and (X_{NOB}) nitrite oxidizing bacteria that convert nitrite to nitrate (S_{NO3}) (Diehl, 2007).

Microalgal activity causes an increase of hydroxide ion concentrations (S_{OH}) and therefore of pH. This result in a displacement of the bicarbonate-carbonate equilibrium, with the formation of carbonate (S_{CO3}), phosphorus precipitation and ammonia volatilization (Serodes et al., 1991; Nurdogan and Oswald, 1995).

In darkness there is a net CO_2 release operated by heterotrophic bacteria (X_H) and microalgae (X_{ALG}) because of the oxidation of organic matter and endogenous respiration. This involves an increase of hydrogen ions with a consequent decrease in pH and a transformation of carbonate into bicarbonate (S_{HCO3}).

In the presence of light, this bicarbonate can be used as a substrate.

Denitrification occurs when the oxygen level is low and nitrate is the only source of oxygen. This reduction of the oxygen level in the water is caused by microalgae respiration and bacterial growth. During denitrification, the denitrifying bacteria reduce nitrate (S_{NO3}) into nitrogen gas under anoxic conditions. These microalgae and bacteria processes are influenced by temperature which also affects chemical equilibria, pH and gas solubility (Bouterfas et al., 2002).

3.6 Appendix

Model process

Table A3.1 shows the processes included in the complete model (bacteria and microalgae) and the equations describing their rates.

Table A3.1: Mathematical description of the processes of the model (processes rates).

Processes	**Process rate [M L^{-3} T^{-1}]**
Microalgae (X_{ALG}) processes	
1a. Growth of X_{ALG} on S_{NH4}	$\rho_{1a} = \mu_{ALG} \cdot f_{T,FS}(T) \cdot \eta_{PS}(I, S_{O2}) \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,ALG} + S_{CO2} + S_{HCO3} + \frac{{S_{CO2}}^2}{I_{CO2,ALG}}} \cdot \frac{S_{NH3} + S_{NH4}}{K_{N,ALG} + S_{NH3} + S_{NH4}} \cdot \frac{S_{PO4}}{K_{P,ALG} + S_{PO4}} \cdot X_{ALG}$
1b. Growth of X_{ALG} on S_{NO3}	$\rho_{1b} = \mu_{ALG} \cdot f_{T,FS}(T) \cdot \eta_{PS}(I, S_{O2}) \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,ALG} + S_{CO2} + S_{HCO3} + \frac{{S_{CO2}}^2}{I_{CO2,ALG}}} \cdot \frac{S_{NO3}}{K_{N,ALG} + S_{NO3}} \cdot \frac{K_{N,ALG}}{K_{N,ALG} + S_{NH3} + S_{NH4}} \cdot \frac{S_{PO4}}{K_{P,ALG} + S_{PO4}} \cdot X_{ALG}$
2. Endogenous respiration of X_{ALG}	$\rho_2 = k_{resp,ALG} \cdot f_{T,FS}(T) \cdot \frac{S_{O2}}{K_{O2,ALG} + S_{O2}} \cdot X_{ALG}$
3. Decay of X_{ALG}	$\rho_3 = k_{death,ALG} \cdot f_{T,FS}(T) \cdot X_{ALG}$
Heterotrophic bacteria (X_H) (aerobic and denitrifying activity)	
4a. Aerobic growth of X_H on S_{NH4}	$\rho_{4a} = \mu_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{S_{O2}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NH4} + S_{NH3}}{K_{N,H} + S_{NH4} + S_{NH3}} \cdot X_H$
4b. Aerobic growth of X_H on S_{NO3}	$\rho_{4b} = \mu_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{S_{O2}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO3}}{K_{N,H} + S_{NO3}} \cdot X_H$

5. Anoxic growth of X_H on S_{NO2} (denitrification on S_{NO2})	$\rho_5 = \mu_H \cdot \eta_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{K_{O2,H}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO2}}{K_{NO2,H,anox} + S_{NO2}} \cdot X_H$
6. Anoxic growth of X_H on S_{NO3} (denitrification on S_{NO3})	$\rho_6 = \mu_H \cdot \eta_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{K_{O2,H}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO3}}{K_{NO3,H,anox} + S_{NO3}} \cdot X_H$
7. Aerobic endogenous respiration of X_H	$\rho_7 = k_{resp,H} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,H} + S_{O2}} \cdot X_H$
8. Anoxic endogenous respiration of X_H	$\rho_8 = k_{resp,H} \cdot \eta_H \cdot f_{T,MB}(T) \cdot \frac{K_{O2,H}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO3} + S_{NO2}}{K_{NO3,H,anox} + S_{NO2} + S_{NO3}} \cdot X_H$
9. Decay of X_H	$\rho_9 = k_{death,H} \cdot f_{T,MB}(T) \cdot X_H$
Autotrophic bacteria (nitrifying activity)	
10. Growth of ammonia oxidizing bacteria (X_{AOB})	$\rho_{10} = \mu_{AOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,AOB} + S_{O2}} \cdot \frac{S_{NH3} + S_{NH4}}{K_{NH4,AOB} + S_{NH4} + S_{NH3}} \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,AOB} + S_{CO2} + S_{HCO3}} \cdot X_{AOB}$
11. Growth of nitrite oxidizing bacteria (X_{NOB})	$\rho_{11} = \mu_{NOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,NOB} + S_{O2}} \cdot \frac{K_{I,NH4}}{K_{I,NH4} + S_{NH4} + S_{NH3}} \cdot \frac{S_{NO2}}{K_{NO2,NOB} + S_{NO2}} \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,NOB} + S_{CO2} + S_{HCO3}} \cdot X_{NOB}$
12. Endogenous respiration of X_{AOB}	$\rho_{12} = k_{resp,AOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,AOB} + S_{O2}} \cdot X_{AOB}$
13. Endogenous respiration of X_{NOB}	$\rho_{13} = k_{resp,NOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,NOB} + S_{O2}} \cdot X_{NOB}$
14a. Decay of X_{AOB}	$\rho_{14a} = k_{death,AOB} \cdot f_{T,MB}(T) \cdot X_{AOB}$

14b. Decay of X_{NOB}	$\rho_{14b} = k_{death,NOB} \cdot f_{T,MB}(T) \cdot X_{NOB}$
Hydrolysis, Chemical equilibrium and Transfer of gases	
15. Hydrolysis	$\rho_{15} = k_{HYD} \cdot \frac{X_S/X_H}{Y_{HYD} + (X_S/X_H)} \cdot X_H$
16.Chemical equilibrium $CO_2 \leftrightarrow HCO_3^-$	$\rho_{16} = k_{eq,1} \cdot (S_{CO2} - S_H S_{HCO3}/K_{eq,1})$
17. Chemical equilibrium $HCO_3^- \leftrightarrow CO_3^{2-}$	$\rho_{17} = k_{eq,2} \cdot (S_{HCO3} - S_H S_{CO3}/K_{eq,2})$
18. Chemical equilibrium $NH_4^+ \leftrightarrow NH_3$	$\rho_{18} = k_{eq,3} \cdot (S_{NH4} - S_H S_{NH3}/K_{eq,3})$
19. Chemical equilibrium $H^+ \leftrightarrow OH^-$	$\rho_{19} = k_{eq,w} \cdot (1 - S_H S_{OH}/K_{eq,w})$
20. Oxygen transfer to the atmosphere	$\rho_{20} = k_{a,O2} \cdot (S_{O2}^{WAT} - S_{O2})$
21. Carbon dioxide transfer to the atmosphere	$\rho_{21} = k_{a,CO2} \cdot (S_{CO2}^{WAT} - S_{CO2})$
22. Ammonia transfer to the atmosphere	$\rho_{22} = k_{a,NH3} \cdot (-S_{NH3})$

Algal processes

- Microalgae growth (processes 1a and 1b) is expressed as the product of their maximum specific growth rate (μ_{ALG}) [T^{-1}], by their concentration at a specific point in time (X_{ALG}) and by corrective factors (Monod functions) that limit or inhibit their growth. Microalgae grow in the presence of carbon dioxide (S_{CO2}) and bicarbonate (S_{HCO3}). Silva and Pirt (1984) showed that high concentration of carbon dioxide can inhibit the microalgae growth.

When ammonium (or ammonia) and nitrate are both present in the substrate, ammonium is generally preferred (Stewart, 1974; Syrett, 1981).

η_{PS} [-] is the photosynthetic factor that considers the effects of light intensity (I) [M T^{-3}] and excess of oxygen (S_{O2}) [M L^{-3}] on photosynthesis (see Solimeno et al., 2015). $f_{T,FS}$ [-] is the thermic photosynthetic factor that takes into account the effects of temperature on microalgae growth and also on endogenous respiration and inactivation processes (processes 1a, 1b, 2 and 3 respectively). The thermic photosynthetic factor is represented in the model following the work of Dauta et al. (1990).

- Endogenous respiration (process 2). This process is expressed as the product between the maximum rate of endogenous

respiration ($k_{resp,ALG}$) [T^{-1}], the thermic photosynthetic factor, the Monod function and the concentration of microalgae (X_{ALG}).

- Decay of microalgae (process 3). The process is expressed as the product of the maximum rate of inactivation ($k_{death,ALG}$) [T^{-1}] by the concentration of microalgae and by thermic photosynthetic factor (the same as for growth) (Reichert et al., 2001).

Heterotrophic bacteria (X_H) (aerobic and denitrifying activity)

- Aerobic and anoxic growth of heterotrophic bacteria (X_H) (Processes 4a, 4b, 5 and 6). The growth of these bacteria was modeled with Monod kinetics. Anoxic processes include an additional reduction factor (μ_H). In aerobic conditions, heterotrophic bacteria assimilate the readily biodegradable substrate (S_S) (from influent or produced during the hydrolysis of biodegradable particulate organic matter (X_S)) and grow consuming both ammonium and ammonia (S_{NH4}, S_{NH3}) and nitrate (S_{NO3}).

Processes 5 and 6 show the denitrification with S_{NO2} and S_{NO3} as substrates for heterotrophic bacteria (Iacopozzi et al., 2007). $f_{T,MB}$ is an Arrhenius type thermal factor for modeled the temperature dependence of bacterial processes (Langergraber et al., 2009; Reichert et al., 2001; Sah et al., 2011).

- Aerobic and anoxic endogenous respiration of heterotrophic bacteria (X_H) (Processes 7 and 8). In these processes, the Monod

function introduces oxygen and nitrogen as limiting factors. They are modeled as the product between the maximum rate of endogenous respiration ($k_{resp,H}$), the thermal factor, and the concentration of heterotrophic bacteria. CO_2 is produced during respiration and transforms alive biomass into inert organic matter (X_I).

- Decay of heterotrophic bacteria (X_H) (Process 9). This process transforms living biomass into slowly biodegradable (X_S) and inert (X_I) organic matter (Van Loosdrecht and Henze, 1999). Is calculated as the product of the maximum rate of decay ($k_{decay,H}$) by the concentration of bacteria and the thermal factor.

Autotrophic bacteria (nitrifying activity)

- Growth of autotrophic bacteria (X_{AOB} and X_{NOB}) (Processes 10 and 11). These bacteria operate for the biological conversion of ammonium to nitrate nitrogen (nitrification) using molecular oxygen as an electron acceptor.

- Endogenous respiration of autotrophic bacteria (X_{AOB} and X_{NOB}) (Processes 12 and 13). These processes are modeled as the product between the maximum rate of endogenous respiration, the concentration of bacteria, the thermal factor and the Monod function.

- Decay of autotrophic bacteria (X_{AOB} and X_{NOB}) (Process 14). This process is modeled in the same way as the decay of

heterotrophic bacteria using different decay rates, $k_{death,AOB}$ and $k_{death,NOB}$, respectively for X_{AOB} and X_{NOB}.

Hydrolysis, Chemical equilibrium, and Transfer of gases

- Hydrolysis (Process 15). This process transforms slowly biodegradable particulate organic matter (X_S) into readily biodegradable soluble organic matter (S_S) catalyzed by heterotrophic bacteria.

- Chemical equilibrium reactions, (processes 16, 17, 18 and 19). These processes affect carbon, nitrogen and the balance of hydrogen and hydroxide ions. The rates of these chemical reactions are obtained with the following general equation of Batstone et al., (2002).

- Transfer of gases to the atmosphere (processes 20, 21 and 22). These transfers between water and atmosphere are given by the general equation of Batstone et al., (2002)

4

Bioremediation of aquaculture wastewater from *Mugil cephalus* (Linnaeus, 1758) with different microalgae species

This chapter is based on the article:

- V. Andreotti, A. Chindris, G. Brundu, D. Vallainc, M. Francavilla, J. García, Bioremediation of aquaculture wastewater from *Mugil cephalus* (Linnaeus, 1758) with different microalgae species. Chem. Ecol. 33 (8) (2017) 750–761.

4.1 Introduction

Aquaculture is one of the fastest-growing food producing sectors in the world, providing almost about 50% of all fish for human consumption; within 2030, this share is projected to rise to 62% (FAO, 2014). On the other hand, aquaculture represents one of the major contributors to the increasing levels of dissolved and particulate nutrients in the aquatic ecosystems (Lamprianidou et al., 2015). A high nutrient loading into the aquatic environment, in particular nitrogen and phosphorus may cause eutrophication, oxygen depletion and siltation (Burford et al., 2003).

With the aim to reduce the impacts of traditional aquaculture, several countries around the world are developing Integrated Multi-Trophic Aquaculture (IMTA) systems, which reuses the wastewaters for the growth of micro and macroalgae. Indeed, aquaculture wastewater provides nutrients (ammonia, nitrite, nitrate, dissolved organic nitrogen and phosphate) (Converti et al., 2006; Soletto et al., 2005; Abe et al., 2002), which can be used for the production of microalgae. The uptake of dissolved nutrients by microalgae is considered as the main way to remove nitrogen in aquaculture wastewaters (Attasat et al., 2013; Sirakov et al., 2013).

Previous studies showed that it is possible to remove nutrients from wastewater (fishes and shrimp production plants) employing microalgae and macroalgae as key elements in biological treatments (Gao et al., 2016; Michels et al., 2014; Sirakov and Velichkova, 2014; Bartoli et al., 2005; Borges et al., 2005; Lefebvre et al., 2004; Hussenot et al., 1998; Lefebvre et al., 1996; Hammouda et al., 1995; Shpigel et al., 1993). This phycoremediation is an eco-friendly method that offers the advantage to be a low-cost way to nutrient removal (Mulbry et al., 2008). In addition, biomass produced through bioremediation could have multi-purpose uses including fuels, fertilizers, fine chemicals production and feed in aquaculture (Mulbry et al., 2006; Vilchez et al., 1997).

One of the most common microalgae species employed in aquaculture bioremediation wastewater is *Tetraselmis* spp. (Michels et al., 2014; Sirakov and Velichkova, 2014; Borges et al., 2005). A recent study showed for the first time that it is possible to use *Tetraselmis suecica* for nutrient assimilation of fishfarm wastewater throughout its cultivation in controlled photobioreactors (Michels et al., 2014).

The aim of this study is to evaluate and compare the capability of *T. suecica*, *Isochrysis galbana* and *Dunaliella tertiolecta*, widely used in aquaculture as a feed for rotifers (Mason, 1963), echinoderms (Brundu et al., 2016a, 2016b; Paredes et al., 2015;

De La Uz et al., 2013; Azad et al., 2011; Miller and Emlet 1999; Zamora and Stotz 1994), filter feeders (Nevejan et al., 2003; Carboni et al., 2016) and fin fishes (Fabregas et al., 1986), for the removal of dissolved inorganic nutrients (nitrogen and phosphorous) from aquaculture wastewater. We evaluated the biomass yield of these species in controlled bubble column annular photobioreactors, by using untreated mullet wastewater as culture medium. Contrarily to previous studies that sterilized the wastewater before its use for bioremediation to eliminate zooplankton, bacteria and suspended solids (Michels et al., 2014), we avoided the use of expensive pretreatment procedures as filtration and sterilization, aiming to reduce the costs of seawater treatment and simulate more real operation conditions of a wastewater treatment system.

4.2 Materials and methods

4.2.1 Aquaculture wastewater

Aquaculture wastewater was provided by an experimental fish hatchery located in the International Marine Centre - IMC Foundation (Oristano, Sardinia, Italy). Juveniles of grey mullet *Mugil cephalus* (Linnaeus, 1758) were obtained in the laboratory and reared in a recirculating aquaculture system (RAS) consisting of four tanks of 2000 L volume. In this system, the tanks were linked in a single biological (trickling filter) and cartridge mechanical filter (10 μm) and supplied with UV lamp (UVPE5, 80 W) and protein skimmer (Panaque) (Figure 4.1). Temperature of seawater was maintained at 23 ± 2°C (mean ± SE), pH 7.5 ± 0.1 and salinity 37.0 ± 1.0 ppt.

Fishes were stocked at an average density of 0.5 g body weight/L. Tanks were monitored daily for checking mortality; the uneaten food and faeces were siphoned out twice a week for maintaining good water quality. A 30% water exchange was weekly performed, and a part of this 30% was employed as wastewater in our experiment.

Wastewater was taken at the inlet of the tank, after UV lamp.

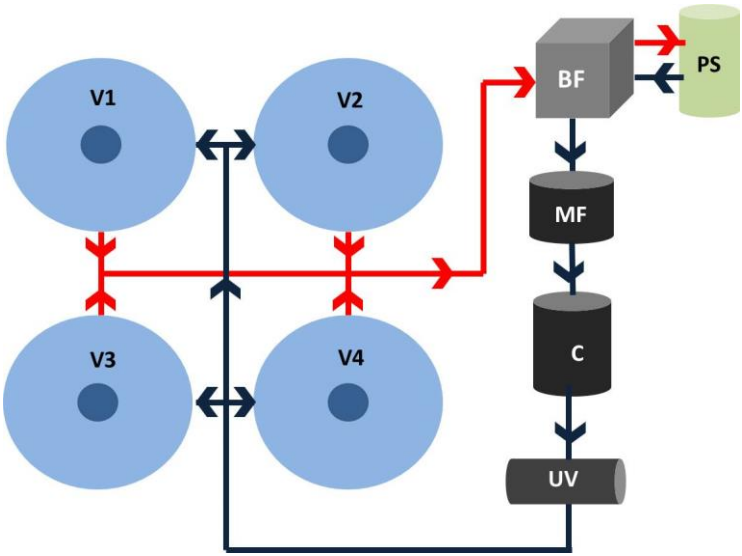

Figure 4.1: Recirculating aquaculture system (RAS) for rearing of juvenile grey mullets *Mugil cephalus*, consisting of four circular fiberglass tanks with 2000 L volume (V1, V2, V3 and V4). The system was equipped with biological (BF) and mechanical filter (MF), protein skimmer (PS), chiller (C) and UV lamp (UV). Red arrow = seawater outlet; black arrow = seawater intake.

4.2.2 Microalgae culture

The microalgae species were provided by the Agency for Agricultural Research in Sardinia (AGRIS) and sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland). Pre-culture inocula were permanently kept in Erlenmeyer flasks in Pyrex glass with a total capacity of 2 L, closed with cotton and covered with gauze and alluminium foil (Figure 4.2). Natural seawater was autoclaved at 121°C for 30 min and enriched with Guillard F/2 medium (Guillard 1975;

Guillard and Ryther 1962). Cultures were exposed to a constant illumination (155 μ mol/s/m^2) provided by four fluorescent lamps (OSRAM type Natura). Continuous aeration 3 L/min was supplied by a peristaltic pump (ECOH Air Pump) and the temperature was maintained at 23°C by air conditioning.

Figure 4.2: Pre-culture inocula in Erlenmeyer flasks in Pyrex glass enriched with Guillard F/2 medium (International Marine Centre - IMC Foundation)

4.2.3 Experimental design

Nutrient uptake and biomass production of *T. suecica*, *I. galbana* and *D. tertiolecta* were evaluated during seven days in batch conditions using two completely mixed bubble column photobioreactors of 6 L. Five runs were done for a total of three replicates per treatment; therefore, the wastewater was not the same for all the runs.

Lighting system was composed by four neon daylight lamps (four fluorescent lamps type cool daylight, OSRAM Lumilux

FQ 24W/865), with a light intensity of 100 μ mol/s/m^2. This system was monitored with a Programmable Logic Controller (PLC) that it is a device that performs discrete or continuous control logic in process plant or factory environments. These controllers are hardware and software engineered microcomputers, used to provide industrial control operations (Netto et al., 2013). Reactors were equipped with temperature and aeration regulation control system; temperature was maintained at 23°C, aeration was ensured by a blower at a flow rate of 3 L/min. On the contrary, pH was not controlled and resulted at 7.7 ± 0.2. Phytoplankton laboratory-culture methods and photobiorectors operation were adopted according to Saiu et al. (2016) (Figure 4.3).

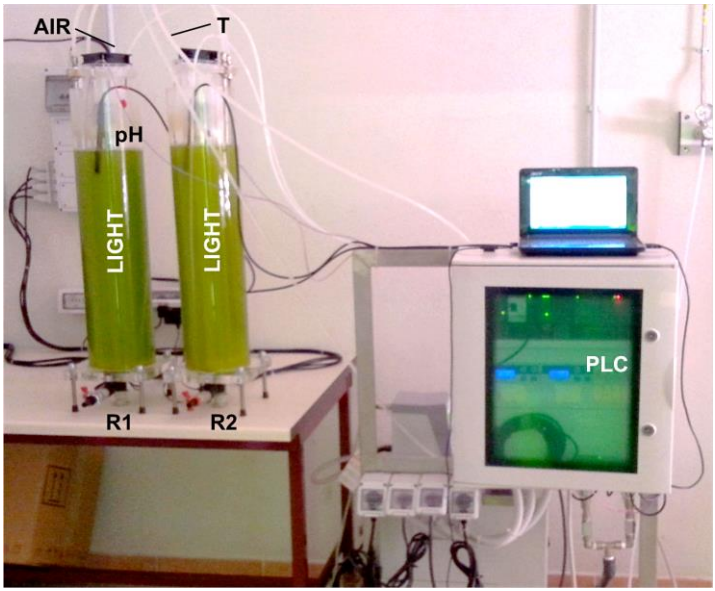

Figure 4.3: Bubble column annular photobioreactors of 6 L volume (R1 and R2) used for the growth of phytoplankton, supplied with LIGHT, Programmable Logic Controller (PLC), gentle aeration (AIR), probes for temperature (T) and pH (pH).

Microalgae growth was measured as dry weight (DW) biomass (Clasceri et al., 1999). DW was measured once a day in 40 mL of water sample previously filtered through 0.45-μm Whatman Grade GF/C Glass Microfiber filters.

After filtration, filters were washed with 20 mL of deionized water to remove salts and dried in an oven at 105°C until constant weight, following Saiu et al. (2016). The supernatant liquid fraction obtained after filtration was used for nitrate, nitrite, ammonia and phosphorous analysis. In order to monitor the microalgae nutrient uptake, nutrients were daily analysed by an automatic chemical analyser μCHEM based on Loop Flow Analysis (Systea, Italy). Microalgae removal efficiencies of dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorous (DIP) were calculated according to the method used by Michels et al. (2014), as follows:

N removal efficiency (%) = ((DIN influent − DIN effluent)/ DIN influent) x 100,

P removal efficiency (%) = ((DIP influent − DIP effluent)/ DIP influent) x 100.

DIN values were calculated as the sum of nitrite (NO_2^-), nitrate

(NO_3^-) and ammonia (NH_4^+), while DIP corresponded to the total dissolved phosphate (PO_4^{3-}) in mg/L.

4.2.4 Statistical analysis

Data were analysed by Statistica 6.1 StatSoft, Inc. (2004). Differences in the removal efficiencies among microalgae species were analysed using analysis of variance (ANOVA, $\alpha = 0.05$). Shapiro Wilk' s W test was used to verify the normality of the data distribution and Levene' s test was used to verify the homogeneity of variances. Biomass was analysed using repeated-measures ANOVA, with species as independent factor and days as repeated factor. Tukey' s honestly significant difference (HSD) test was used to evaluate all pair-wise treatment comparisons ($\alpha < 0.05$).

4.3 Results

The nutrient concentration of the wastewater was regularly measured before each experiment (Table 4.1). It was possible to observe that the composition of wastewater was very similar in each experiment, being nitrate the N species with the highest concentration.

Table 4.1. Dissolved nutrients in the *Mugil cephalus* wastewater. Values are expressed as mean ± SE (n= 3)

	Tetraselmis suecica	***Dunaliella tertiolecta***	***Isochrysis galbana***
NO_3^- -N (mg/L)	4.1 ± 0.4	4.2 ± 0.1	4.2 ± 0.4
NO_2^- -N (mg/L)	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1
NH_4^+ -N (mg/L)	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
PO_4^{3-} -P (mg/L)	0.3 ± 0.1	0.6 ± 0.1	0.6 ± 0.1

4.3.1 Nutrients removal efficiency

At the end of the experiment, a clearly higher DIN removal efficiency ($p < 0.001$) resulted for *T. suecica* (94.40 ± 0.97%, mean ± SE) and *D. tertiolecta* (95.44 ± 0.29%) in comparison with *I. galbana* (66.02 ± 1.52%). There were no statistical differences between the three species in the removal efficiency of DIP at the end of the experiments (Table 4.2). However, differences were found in terms of DIP removal rate (mg P/L/d), which was mainly related to the different DIP concentration in influent wastewater.

Table 4.2. Influent and effluent DIN and DIP values (mg/L) and removal efficiency (%) of *Tetraselmis suecica*, *Dunaliella tertiolecta* and *Isochrysis galbana*. Values are expressed as mean ± SE (n= 3). Superscripts indicate significant differences among species ($p < 0.001$).

	Tetraselmis suecica	***Dunaliella tertiolecta***	***Isochrysis galbana***
DIN Influent (mg/L)	4.5 ± 0.5	4.6 ± 0.1	4.6 ± 0.5
DIN Effluent (mg/L)	0.3 ± 0.1	0.2 ± 0.1	1.6 ± 0.1
DIN %	94.4 ± 1.0 [a]	95.4 ± 0.3 [a]	66.0 ± 1.5 [b]
DIP Influent (mg/L)	0.3 ± 0.01	0.6 ± 0.1	0.6 ± 0.1
DIP Effluent (mg/L)	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1
DIP %	96.0 ± 2.5	91.2 ± 2.3	91.9 ± 4.0

T. suecica and *D. tertiolecta* showed a similar pattern of nutrient uptake (Figure 4.4 A, C). Both species removed more than 90% of DIN and DIP after 2 and 1 days, respectively. On the contrary, *I. galbana* showed a slower nutrient uptake, lower than 35% and 80% removal for DIN and DIP, respectively, after 2 days (Figure 4.4 (B)). The nutrient uptake of DIN showed significant differences between *I. galbana* and the other two phytoplankton species ($p < 0.001$).

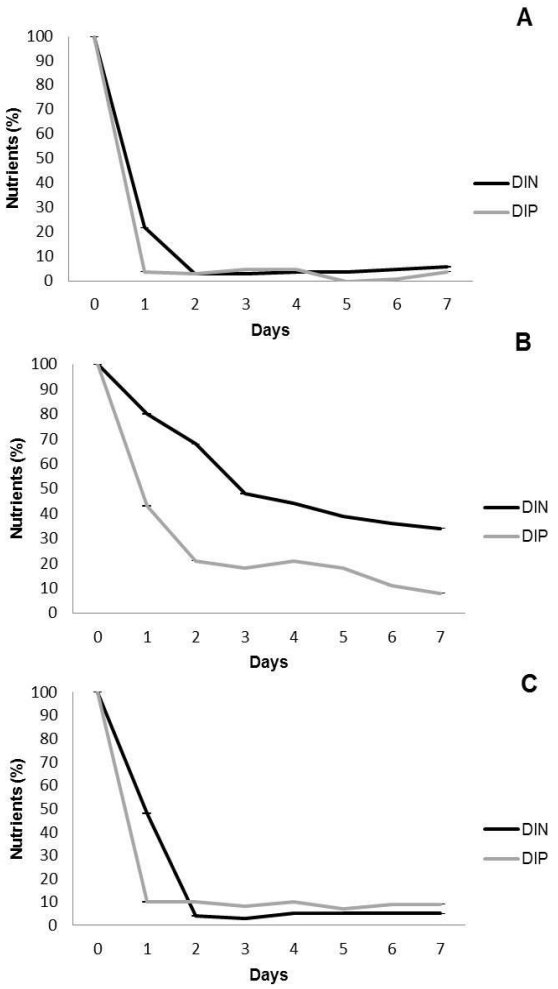

Figure 4.4: Nutrient uptake (%) of Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorous (DIP) for *Tetraselmis suecica* (A), *Isochrysis galbana* (B) and *Dunaliella tertiolecta* (C), during 7 days. Values are expressed as mean ± SE (n = 3).

4.3.2 Biomass yield

Ciliate protozoan *Paramecium* spp. was observed in all cultures through the duration of the experiment, but we did not evaluate the abundance of this species. This was mainly due to lack of the wastewater pretreatment procedures (i.e. filtration and sterilization). We found a significant difference in biomass yield among the three species (repeated-measures ANOVA, $p < 0.001$). *T. suecica* resulted in a higher DW (570 ± 15 mg/L, mean ± SE) than *I. galbana* (117 ± 11 mg/L) from 3 days up to the end of the experiment, 603 ± 34 mg/L for *T. suecica* and 161 ± 24 mg/L for *I. galbana*. We found an intermediate behaviour of *D. tertiolecta* in terms of biomass concentration that reached the value of 380 ± 37 mg/L at the end of the experiment (Figure 4.5). According to our test batch results, the volumetric productivity achieved by each of three species was 86.14 ± 5 mg/L/d for *T. suecica*, 54.26 ± 5 mg/L/d for *D. tertiolecta* and 23 ± 4 mg/L/d for *I. galbana*, respectively.

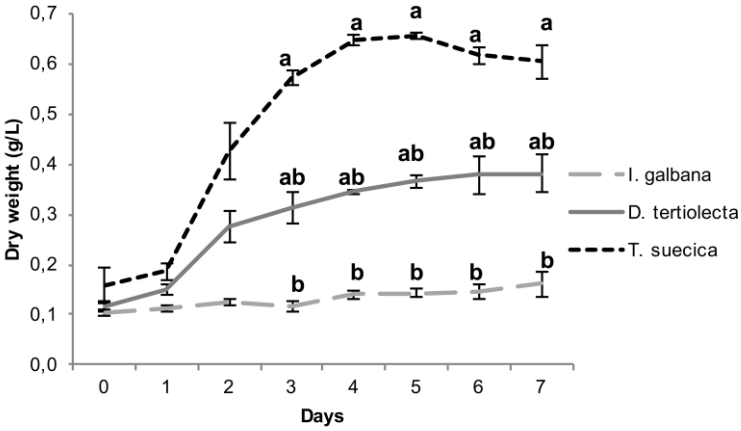

Figure 4.5: Microalgal growth curves as DW (g/L) of *Tetraselmis suecica*, *Isochrysis galbana* and *Dunaliella tertiolecta*, during 7 days. Values are expressed as mean ± SE (n = 3). Superscripts indicate significant differences among species ($p < 0.001$).

4.4 Discussion

In this study, we tested the ability of three microalgae species to remove dissolved nutrients in the wastewater of a hatchery pilot rearing system of *M. cephalus*. We found two out of three species, *T. suecica* and *D. tertiolecta*, able to remove more than 90% of the DIN and DIP after two days of treatment. Differently, the phytoplankton species *I. galbana* employed 7 days to remove 92% of DIP, while DIN was not completely removed at the end of the experiment (66%).

This is the first time that *D. tertiolecta* was used as aquaculture wastewater species, whereas previous studies obtained efficient results by using *T. suecica*. Michels et al. (2014), showed that with a biomass concentration of 0.5 g/L, *T. suecica* resulted in a removal efficiency of 49.4% for N and 99.0% for P, after 15 days and using a continuously operated tubular photobioreactor. These authors obtained a higher N removal efficiency (95.7 ± 1.0%) after addition of extra orthophosphate to compensate the insufficient amount of DIP in the wastewater. On the other hand, contrary, culturing *T. suecica* under batch condition, the maximum P removal was obtained of only 52– 63% at 8 days, even after nutrient (+N) ratio correction (Borges et al., 2005).

The growth of microalgae is influenced by the culture medium composition and variables such as temperature, light intensity

and pH (Molina et al., 1991). Moreover, it was previously observed that other factors are determinant for the growth of phytoplankton, as the N:P ratio. Once microalgae reach the stationary phase the biomass concentration increases with the N:P ratio up to different levelling-off values, which depends upon temperature, with concentration remaining nearly constant for values beyond this point (Molina et al., 1991). At 25°C, the N:P levelling-off value registered for *Tetraselmis* spp. (Michels et al., 2014) is lower than values registered in the wastewater used for this study, 18 for *D. tertiolecta*, 16.3 for *I. galbana* and 32 for *T. suecica*.

Our results show that the concentration of nutrients decreases after 2 days, but the biomass yield of *T. suecica* and *D. tertiolecta* increases beyond 2 days. This indicates that the growth of phytoplankton depends on the stored intra-cellular pool of nutrient, rather than only on the extracellular nutrients into the culture medium, as previously reported by Lemesle et al. (2008).

In this study, the highest biomass concentration (DW) was obtained with *T. suecica*, 603 ± 34 mg/L, while 380 ± 37 and 161 ± 24 mg/L were recorded for *D. tertiolecta* and *I. galbana*, respectively, at the end of the experiment. We hypothesize that these differences were related to the different species-specific cell size. Indeed, *T. suecica* has the largest median cell volume

(300 μm^3), followed by *D. tertiolecta* (170 μm^3) and *I. galbana* (40 – 50 μm^3) (FAO, 2004).

The results showed that *I. galbana* was not suitable for the nutrient removal of *M. cephalus* aquaculture wastewater due to its low biomass yield and removal efficiency of DIN and DIP. We hypothesize that the ciliate *Paramecium* spp. influenced negatively the growth of *I. galbana* due to the habits of this organism to feed on other live microorganisms (Wichterman 1986). *Paramecium* spp. was observed also in the cultures of *T. suecica* and *D. tertiolecta*, but the presence of this protozoan did not seem to affect the growth of these phytoplankton species. *I. galbana* is smaller than the other two species, therefore, it could be easily preyed by the zooplankton. Moreover, it has been previously reported a large spectrum of antimicrobial activity and antibiotic substances of the genus *Tetraselmis* spp. (Austin et al., 1992; Austin and Day, 1990) and *Dunaliella* spp. (Chang et al., 1993), which could limit the negative effects of *Paramecium* spp. on the growth of cultures. When aquaculture wastewater is used as a nutrient source for algae, sterilization may be necessary to minimize the negative effects of bacteria and other organisms on the algae growth (Cai et al., 2013; Stein, 1979).

However, sterilization process increases the capital cost of the algae cultivation system, representing a negative point for an

efficient phytoplankton bioremediation system at a large scale. Indeed, microalgae production, must be a low-cost system, easily installable and maintainable (Cai et al., 2013). Avoiding pretreatment and sterilization of wastewater, as in our experiment, could positively contribute in a reduction of management costs, as energy, technology and manual labour. Moreover, it was demonstrated that microalgae cultures with protozoans such as *Paramecium* spp. represent suitable diets for fish fries (FAO, 1980).

During last decade, research efforts have been focused towards the development of more efficient, higher surface-to-volume ratio photobioreactors for microalgae cultivation (Tredici, 2004; Rodolfi et al., 2008). This is the first study that compared the ability of these three microalgae species in nutrient removal of aquaculture wastewater by using controlled bubble column annular photobioreactors. Interestingly, our results show a higher volumetric productivity for three tested species compared to literature data. Gao et al. (2016) recently tested the cultivation in batch conditions of *Chlorella vulgaris* and *Scenedesmus obliquus* shrimp *Penaeus vannamei Boone* in wastewater. The better performance in terms of biomass production was recorded for *C. vulgaris* (7.3 mg/L/d) in comparison with *S. obliquus* (6.2 mg/L/d). *C. vulgaris* was exploited again in a membrane photobioreactor for continuous biomass production, resulting in

a biomass yield of 42.6 mg/L/d. Ansari et al. (2017) used the aquaculture wastewater as a nutrient substrate for the cultivation of *Scenedesmus obliquus*, *Chlorella sorokiniana* and *Ankistrodesmus falcatus* in 1 L conical flask. This study reported a biomass concentration of 1.25 g/L for *S. oliquus*, 1.51 g/L for *C. sorokiniana* and 2.25 g/L for *A. falcatus*. A nitrates removal efficiency of 77.70% (*S. obliquus*), 75.76% (*C. sorokiniana*) and 80.85% (*A. falcatus*) was obtained.

Sirakov and Velichkova (2014) tested the removal efficiency of *Nannochloropsis oculata* and *Tetraselmis chuii* in wastewater originated from semi-closed RAS and by using a 500 mL Erlenmeyer flask. *T. chuii* decreased the concentration of phosphorus to 79%, while *N. oculata* decreased it to 52.3%.

4.5 Conclusion

This study confirms the potential of *T. suecica* in the assimilation of nutrients dissolved in aquaculture wastewater and in the production of biomass. *D. tertiolecta* also resulted suitable for bioremediation, removing more than 90% of DIN and DIP. Differently from *I. galbana*, *T. suecica* and *D. tertiolecta* were able to grow well in no sterilized culture media contaminated with bacteria and zooplankton (*Paramecium* spp.), reflecting in the potential to reduce manual labour and energy

costs for pretreatment of culture medium in a phytoplankton bioremediation system. *T. suecica* and *D. tertiolecta* are valid candidates for the use in IMTA systems. They can be cultivated for bioremediation of finfish or shrimp wastewater and biomass produced can be re-used as live-feed for hatchery-grown of herbivorous and filter feeders (Alsull and Omar, 2012; Michels et al., 2014). Previous studies analysed the production of lipid, proteins and carbohydrates in *T. suecica*, *I. galbana* and *D. tertiolecta* using synthetic culture media (Pusceddu and Fabiano, 1996; Chen et al., 2011), but further researches are required in order to assess the biochemical composition of these species cultivated in aquaculture wastewater and to evaluate their effects as live-feed.

5

Production of microalgae in aquaculture wastewater and calibration of the mechanistic microalgae model BIO_ALGAE

This chapter is based on the article:

- V. Andreotti, A. Solimeno, A. Chindris, F. Marazzi and J. García, Growth of *Tetraselmis suecica* and *Dunaliella tertiolecta* in aquaculture wastewater: numerical simulation with the BIO_ALGAE model. Water Air Soil Pollut (2019) 230: 60. https://doi.org/10.1007/s11270-019-4122-0

5.1 Introduction

In the last five decades, microalgae biotechnology has been constantly developing (Muller-Feuga et al., 2007). Microalgae have the capacity to remove the macronutrients dissolved in wastewater, in particular, nitrogen and phosphorus, and at the same time, to produce biomass that can be used as such or as a source of valuable compounds (Christenson and Sims, 2011; Lam and Lee, 2012).

Some studies have been recently carried out to explore the use of microalgae for the treatment of aquaculture wastewater and the production of biomass (Michels et al., 2014; Velichkova et al., 2014; Lananan et al., 2014; Gao et al., 2016; Ansari et al., 2017; Andreotti et al., 2017). Aquaculture wastewater is composed mainly by nitrogenous components (ammonia, nitrite, nitrate), phosphorus, and organic carbon (Nasir et al., 2015; Wuang et al., 2016). Its composition is related to the nature and quantity of feed, the species being reared, and the type of system in operation. In aquaculture, microalgae are used also as a feed additive in the commercial rearing or as live food for many aquatic animals in freshwater and in marine systems (Mata et al., 2010; Guedes and Malcata, 2012). Microalgae are therefore

the source of fatty acids, proteins, essential amino acids, and pigments, and for this reason, they have an important nutritional role for marine animals (Berge and Barnathan, 2005). The composition of microalgal cells depends on the conditions of the culture (Guiheneuf et al., 2008; Pal et al., 2011; Alsull and Omar, 2012), namely on the culture age, on the light characteristics and intensity, on nutrient source and availability, and on the cell density (Alsull and Omar, 2012).

The yield of commercially valuable products from microalgae could be improved by inducing environmental stress conditions (Ansari et al., 2017). It was demonstrated that lipid accumulation in microalgae cells increases under nutrient-deficient conditions (Xin et al., 2010) and can reach 85% of the dry weight (Chisti 2007a, b; Rodolfi et al., 2009). Mata et al., (2010) reported that for marine microalgae, the total lipid content per dry mass values is species-specific and can vary from 22.7 to 29.7% in *Nannochloropsis oculata*, from 7 to 40% in *Isochrysis galbana* and from 8.5 to 23% in *Tetraselmis suecica*. Other studies showed that the macromolecular content is related to the growth phase of the culture (Brown et al., 1997; Renaud et al., 1999).

Due to their nutritional value, two unicellular green marine microalgae *Dunaliella* and *Tetraselmis* have been used in aquaculture as feed for live preys of fish larvae, for penaeid shrimp larvae, and *Tetraselmis* also for bivalve mollusk larvae (Muller-Feuga et al., 2007).

Dunaliella tertiolecta is simple to cultivate, highly salt tolerant (Chen et al., 2011), and it has been reported to have a lipid concentration of 36–42% (Tsukahara and Sawayama, 2005). In addition to this, it was demonstrated that *Dunaliella* spp. can increase their lipid accumulation when nitrogen starvation occurs (Guevara et al., 2005; Chen et al., 2011). Chen et al., (2011) identified the nutritional requirements for *D. tertiolecta* growth and neutral lipid production in a synthetic medium and showed that this organism was able to use either ammonium or nitrate as a nitrogen source. As to phosphorus, starvation seems to have little effect on growth and lipid accumulation, apparently due to intracellular phosphate storage (Chen et al., 2011).

Tetraselmis spp. can accumulate lipids (approximately 20–30% on dry weight basis) and tolerate a wide range of environmental conditions (Chini Zittelli et al., 2006; Rodolfi et al., 2009).

In aquaculture wastewater, *T. suecica* and *D. tertiolecta*

showed a similar pattern of nutrient assimilation, being able to remove more than 90% inorganic nitrogen and inorganic phosphorus after 2 days and 1 day, respectively (Andreotti et al., 2017).

Due to the influence of many parameters, such as nutrient availability, light, oxygen, and temperature, it is not easy to predict the growth of microalgae, but mathematical models offer the possibility to study microalgae growth in different bioreactors (Bitog et al., 2011). Several models have been developed to predict algal productivity and nutrient removal efficiency in synthetic media and in urban wastewater (Mairet et al., 2011; Reichert et al., 2001; Bernard et al., 2016; Solimeno et al., 2015, 2016). About aquaculture wastewater, fewer experiences are reported (Lamprianidou et al., 2015; Kiridi and Ogunlela, 2016), and a mathematical model has not yet been implemented and calibrated.

This work is a base to create with the help of the mathematical model a platform that will be used in the aquaculture systems to design and operate an efficient and sustainable microalgae cultivation. The integral mechanistic model BIO_ALGAE calibrated and validated in closed and open reactors provides new analysis into the functioning of microalgae culture, and is useful to understand simultaneous

effects of factors affecting microalgae growth (Solimeno et al., 2016).

In detail, the aim of this study was to implement and calibrate the microalgae-bacteria mechanistic model BIO_ALGAE for aquaculture wastewater in order to simulate the uptake of nutrients (N, P) and the biomass production of *T. suecica* and *D. tertiolecta*. The total lipid content was also investigated at the end of the experiment.

5.2 Materials and methods

5.2.1 Microalgae and wastewater

Microalgae were obtained from the Agency for Agricultural Research in Sardinia (AGRIS, Italy) and sourced from the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland, UK).

Inocula were grown in fully controlled photobioreactors (6 L volume each), with natural seawater (NSW) enriched with Guillard F/2 medium (Guillard et al., 1962, 1975). The culture procedures and the photobioreactors operation were carried out according to Saiu et al. (2016).

The aquaculture wastewater (AW) was obtained from a grey mullet fish hatchery located in the International Marine Centre - IMC Foundation (Oristano, Sardinia, Italy), where fish were reared in a recirculating aquaculture system (RAS) consisting of 4 tanks of 2000 L volume each (Andreotti et al., 2017) (Figure 5.1). The tanks were monitored daily, the seawater temperature was maintained at 20.3 ± 1.9 °C, salinity was 36.6 ± 1.0 g/L, DO (dissolved oxygen) 8.1 ± 1.2 mg/L and pH 7.5 ± 0.1. Weekly, 30% of the water in the tanks was discharged and replaced by clean seawater, and a part of the outflowing 30% (AW) was used

as culture medium for microalgae experiments. Average concentrations of nitrate nitrogen (mg/L), nitrite nitrogen (mg/L), ammonium nitrogen (mg/L) and orthophosphate (mg/L) were 3.32 ± 0.17, 0.11 ± 0.02, 0.28 ± 0.05 and 0.63 ± 0.01, respectively.

Figure 5.1: Grey mullet fish hatchery located in the International Marine Centre - IMC Foundation (Oristano, Sardinia, Italy)

5.2.2. Analyses

NO_3^--N, NO_2^--N, NH_4^+-N and PO_4^{3-}P concentrations were measured by an automatic chemical analyzer µCHEM based

on Loop Flow Analysis (Systea, Italy), and for the quality control it was used the Matrix Spiking method (NMKL, 2012). Microalgal concentration was measured as mg TSS/L, according to the method used by Saiu et al. (2016) for seawater culture samples. Algal growth was assessed by following the TSS data collected during the exponential growth phase. The microalgal growth rate was estimated by daily measurement of biomass concentration as reflected in dry weight. The specific value (μ in day^{-1}) was calculated as the slope of the line fitting the TSS mg/L data plotted in a log [TSS(t)/TSS(0)] versus time graph.

Lipids were extracted from the biomass collected at the end of each experiment. 100 mg of microalgae, previously lyophilized at -80 °C, were suspended in 10 mL of chloroform-methanol 2:1 according to Folch et al. (1957). The solution was vortex mixed for 30 s, sonicated for other 30 s and then centrifuged at 3.000 rpm for 5 min. The liquid fraction was filtered using GF/C filter paper in a funnel and the remaining solids were re-extracted with 5 mL of chloroform–methanol 2:1 (Ryckebosch et al., 2012). The solvent was removed by evaporation and after that the lipid content was determined gravimetrically. The percent lipid content was calculated with reference to the weight of dry

biomass (Ryckebosch et al., 2012).

The lipid productivity in mg/L/d was calculated according to Singh et al., (2015):

Lipid productivity (mg/L/d) = Biomass productivity (mg/L/d) * (Lipid content % /100).

5.2.3 Culture systems and photobioreactors

To start the experiments, aliquots of microalgae suspensions were collected from the 6 L photobioreactors in the exponential growth phase when the microalgal concentration was approximately 0.13 g TSS/L.

Two completely mixed bubble column photobioreactors of 120 L were used in batch condition for 7 days (Figure 5.2). Four consecutive replicates for each species were done.

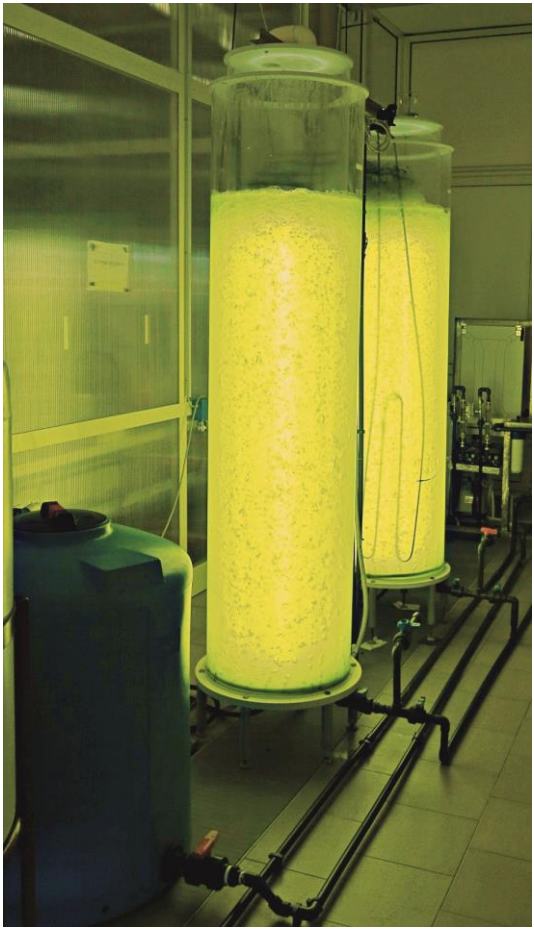

Figure 5.2: Column photobioreactors of 120 L with a temperature and aeration regulation control system. The system was monitored with a Programmable Logic Controller (PLC).

As the experiments were carried out in different periods, the nutrient concentrations of AW used for the two species were not the same, as shown in Table 5.1.

Table 5.1: Nutrient concentrations in the AW used for the two microalgal species (mg/L). Values are expressed as mean ± SE (n=4).

	T. suecica	*D. tertiolecta*
NO_2^- -N	0.073±0.001	0.156±0.009
NO_3^- -N	3.755±0.016	2.878±0.038
NH_4^+-N	0.144±0.001	0.408±0.031
PO_4^{3}-P	0.657±0.002	0.613±0.018

Light was provided by fluorescent lamps (Cool Daylight - 58W/865 Lumilux) for 24/24. Photosynthetically active radiation (PAR) was 150 (μmol/s/m^2) in the external part of photobioreactor. The cultures were maintained at constant temperature (23°C). Dissolved oxygen (DO) concentration was 8.0 ± 2 mg/L and pH was 8.0 ± 2. PH and temperature were measured every 10 minutes. The airflow was constant at 2 m^3/h.

A sample of each culture was collected daily to analyze the microalgae growth and the nutrient concentrations in the culture medium.

5.2.4 Statistical analysis

Data analysis was performed using R Studio (Version 1.0.153—© 2009–2017 R Studio, Inc.). Differences in the removal efficiencies and biomass as mg TSS/L among microalgae species were analyzed using all 4 replicates (R1 to R4). Normality and homogeneity of data were examined using Shapiro Wilk's W test. The statistical significance of the differences between experimental data of the two algal species was determined for all the measured parameters by the Kruskal–Wallis test ($\alpha = 0.05$). Model data of nitrogen, phosphorous, and biomass production were compared to experimental data by the root mean square error (RMSE).

All data are expressed as mean ± standard error (SE).

5.2.5 BIO_ALGAE model

BIO_ALGAE model has been described in Solimeno et al. (2017) and was used to simulate mixed cultures of microalgae and bacteria. This model was implemented in COMSOL Multiphysics™ v5.3 software and was basically constructed through the RWQM1 (Reichert et al., 2011), with modifications of ASM3 (Iacopozzi et al., 2007).

The kinetic expressions of BIO_ALGAE are based on Monod type functions for carbon, nitrogen, and phosphorus limitation. C was included as limiting factor because in

some cases, namely when intense photosynthesis raises pH to very high values, CO_2 can be no more available as it turns to carbonate. This model is applicable for waste stabilization ponds, high-rate algal ponds, and photobioreactors.

The model considers the 19 components (6 particulate and 13 dissolved) included in the common nomenclature of the International Water Association (IWA) model (Solimeno et al., 2016). Particulate and dissolved components are implicated as variables in the physical, chemical, and biokinetic processes (Solimeno et al., 2015; Solimeno et al., 2017a, b). The particulate components is composed by heterotrophic bacteria (X_H), nitrifying bacteria (X_{AOB}, X_{NOB}), microalgae (X_{ALG}), organic inerts (X_I), and biodegradable materials (X_S). Instead, dissolved components include the inert organic matter (S_I) and biodegradable organic matter (S_S), nitrogen fractions (S_{NH3}, S_{NH4}, S_{NO2}, S_{NO3}), phosphate (S_{PO4}), oxygen (S_{O2}), and inorganic carbon components such as (S_{CO2}, S_{HCO3}, S_{CO3}) hydroxyl ions (S_{OH}) and hydrogen ions (S_H).

For the calibration, the sum of NO_2^-–N and NO_3 –N was used. The experimental data on biomass were expressed as total suspended solids (TSS), while the simulation provided

both TSS and by X_{ALG} (mg TSS algal biomass/L). TSS is the sum of all particulate components including microalgae and bacteria biomass, and X_{ALG} is the concentration of microalgae (mg TSS algal biomass/L) (Solimeno et al., 2015, 2017a).

To simplify presentation of the simulation results, Tables A5.1 and A5.2 in the appendix present the biokinetic processes and the matrix of stoichiometric parameters. Values of biokinetic, physical, and chemical parameters are shown in Tables A5.3 – A5.4. Mathematical expressions of the stoichiometric coefficients of each process are also shown in Table A5.5.

5.3. Results

5.3.1. Nutrient removal and biomass production

At the beginning of experiments, the concentrations of *T. suecica* and *D. tertiolecta* were 96.9 ± 4.7 mg TSS/L and 88.1 ± 6.7 mg TSS/L, respectively. As shown in Fig. 5.3, the growth of the two microalgae had similar trends, but the statistical analysis demonstrated a significant difference between them for biomass production ($p < 0.05$).

T. suecica showed a better performance in terms of biomass productivity in batch culture (reaching a maximum of 460.0

± 29.8 mg TSS/L at the end of the experiment) than *D. tertiolecta* (329.4 ± 11.0 mg TSS/ L). This is also confirmed by the daily biomass production during the 7 days that was 65.7 ± 4.3 mg/L/day for *T. suecica* and 47.1 ± 1.6 mg/L/day for *D. tertiolecta*. In both cases, the exponential phase lasted 96 h. In that time range, the density reached 433.8 ± 17.4 and 313.8 ± 15.8 mg TSS/L for *T. suecica* and for *D. tertiolecta*, respectively (Fig. 5.3). The biomass production per day in this phase was 83.8 ± 4.4 mg/L/day for *T. suecica* and 56.4 ± 5.1 mg/L/day for *D. tertiolecta*.

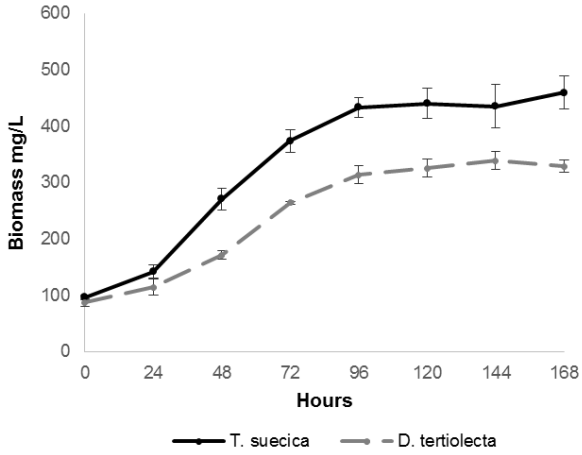

Figure 5.3: Biomass algal concentration measured as mg TSS/L (mean (n=4) ± standard error) for *T. suecica* and *D. tertiolecta* during the experiments.

The specific microalgal growth rate in exponential phase (μ in day^{-1}) was 0.16 day^{-1} for *T. suecica* and 0.15 day^{-1} for *D. tertiolecta*.

Figures 5.4 and 5.5 show the decrease of dissolved inorganic nitrogen (DIN) and phosphorus (DIP) concentrations, during the 7 days of treatment, for the two cultures, respectively. DIN was the sum of NO_2^-–N, NO_3^-–N, and NH_4^+–N in mg/L, while DIP was the total dissolved orthophosphate (PO_4^{3-}–P mg/L).

In 7 days, the total DIN removal efficiency % was 98 ± 0.6% for *T. suecica*, and 97 ± 1.5% for *D. tertiolecta*. During the exponential growth phase, the daily removal rate was 0.88 ± 0.05 mg N/L/day for *T. suecica*, and 0.96 ± 0.01 mg N/L/day for *D. tertiolecta* ($p > 0.05$).

The complete removal occurred after 72 h in the case of *D. tertiolecta* and after 120 h in the case of *T. suecica*.

The total DIP removal efficiency was similar for the two species: 97 ± 1.2% for *T. suecica* and 99 ± 0.7% and *D. tertiolecta* respectively ($p > 0.05$). As also shown in figure 5.5, the DIP was completely removed after 24 h in both cases, with a removal rate in the exponential phase of 0.81 ± 0.05 and 0.93 ± 0.02 mg/L/day for *T. suecica* and *D. tertiolecta*, respectively.

The total lipid content after 7 days was very different in the two species, being 75.8 ± 1.6% in the biomass of *T. suecica*, while only 23.2 ± 2.0% in the biomass of *D. tertiolecta*. The lipid accumulation rate was also lower for *D. tertiolecta* (11.1 mg/L/day) than for *T. suecica* (49.8 mg/L/day).

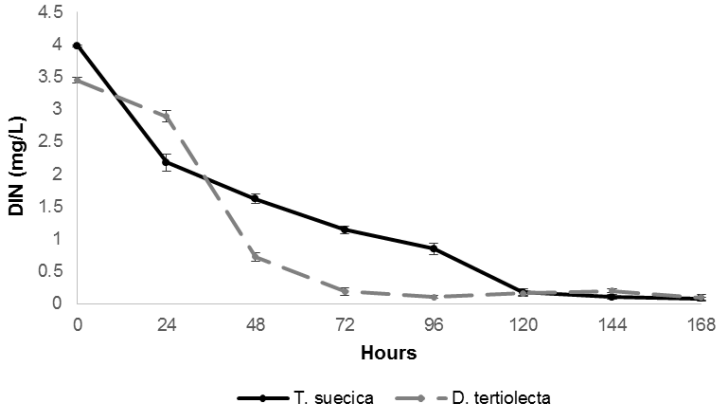

Figure 5.4: Decrease in concentration (mg/L) of Dissolved Inorganic Nitrogen (DIN) for *T. suecica* and *D. tertiolecta*, (n=4).

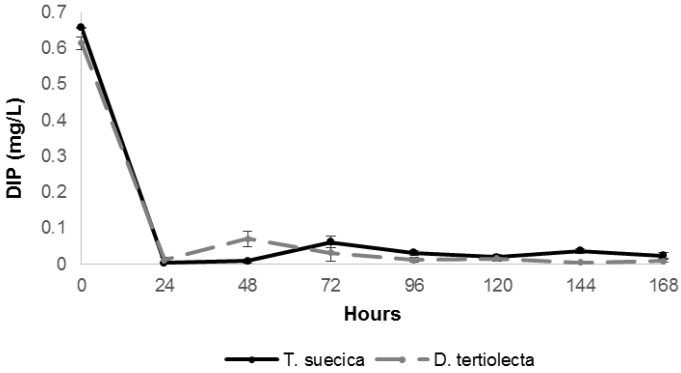

Figure 5.5: Decrease in concentration (mg/L) of Dissolved Inorganic Phosphorous (DIP) for *T. suecica* and *D. tertiolecta* (n=4).

5.3.2. Implementation of BIO_ALGAE model

The model was calibrated using the data for the 7 days of batch experimentation and it was conducted comparing simulated and experimental data curves. For the calibration, only two replicates of experimental data (R1–R2) were used. Unlike the original model (Solimeno et al. 2017a) that considers relevant features such as light attenuation, photorespiration, and temperature dependency, for this experiment, light and temperature were constant and, thus, were not considered growth-limiting factors. The initial values of the parameters of concern are shown in Table 5.2.

Table 5.2: Values of the components of concern at the beginning of the experiment. All components are described in detail in Solimeno et al., (2017a).

Component	Concentration	Units
S_{NO3}	2.98	gN-NO_3 m^{-3}
S_{NO2}	0.14	gN-NO_2 m^{-3}
S_{NH3}	0.41	gN-NH_3 m^{-3}
S_{NH4}	1.6	gN-NH_4 m^{-3}
S_{PO4}	0.65	gP-PO_4 m^{-3}
S_{CO2}	0.145	gC-CO_2 m^{-3}
S_{CO3}	0.866	gC-CO_3 m^{-3}
S_{HCO3}	35.00	gC-HCO_3 m^{-3}
S_H	1.78 10^{-9}	gH m^{-3}
S_{OH}	4.69 10^{-6}	gH-OH m^{-3}
S_S	2	gCOD m^{-3}
S_{O2}	8.74	gO_2 m^{-3}
S_I	8	gCOD m^{-3}
X_H	1	gCOD m^{-3}
X_I	10	gCOD m^{-3}
X_S	1	gCOD m^{-3}
X_{AOB}	0.05	gCOD m^{-3}
X_{NOB}	0.05	gCOD m^{-3}
X_{ALG}	80	gTSS m^{-3}

The kinetic expressions of BIO_ALGAE are based on Monod-type functions. The Monod equations do not consider the variable cell quota (intracellular nutrient

concentration), as the Droop model does (Droop, 1968). This variable is important if the growth depends also (or chiefly) on a stored intracellular pool of nutrient, and not only on the nutrients available in the growth media, as in the Monod equations. In fact, BIO_ALGAE has been developed for microalgae growing in urban wastewaters, where normally the availability of nutrients is high.

Nutrients in AW have much lower concentrations than those in urban wastewater, so they can have a completely different influence on microalgae growth. In fact, in most experimental works, microalgae cultivation in AW included nutrient addition to increase production (Michels et al., 2014; Guldhe et al., 2017). On the contrary, in our work, N and P in AW were depleted in few days, but no nutrient addition was provided, and algal growth did not stop. This suggested that growth was more closely related to the intracellular nutrient concentration than to the external one (Lemesle and Mailleret, 2008) and this, in turn, could depend on the fact that the algal biomass used for the experiment had been previously grown in a nutrient-rich medium. The use of nutrient-rich inoculum for batch experiments could preclude to find the correct relationships between external nutrient concentrations and algal growth.

One of the aims of the work is to calibrate BIO_ALGAE model to use it to predict algal growth in batch experiments as a function of nutrient availability. The theoretical initial concentrations able to sustain the observed
growth were calculated according to external data (Lemesle and Mailleret, 2008). The model has been programmed to have an input of N and P in the system.

Various concentrations were tested to obtain the amount of biomass indicated in the experimental data. The obtained data (19 mg NO_3^-–N/L and 8 mg PO_4^{3-}–P/L) were then used as input for the calibration of the model.

The RMSE values obtained comparing the experimental data with the model simulations are presented in Table 5.3. Values of RMSE are near 0 and this confirms that the model fits experimental data well.

Table 5.3: Values of RMSE for the two microalgae species. These values were obtained comparing model simulations with experimental data. n = 8 for NO_2^--N + NO_3^- -N ($RMSE_N$), PO_4^{3-}-P ($RMSE_P$) and total suspended solid concentrations ($RMSE_{BIOMASS}$).

Specie	**$RMSE_N$**	**$RMSE_P$**	**$RMSE_{BIOMASS}$**
T. suecica	0.41	0.14	0.05
D. tertiolecta	0.55	0.04	0.02

In detail, the comparison between experimental and simulated data shows how for *T. suecica* the two curves X_{ALG} and TSS follow quite well the same pattern of the experimental data (R1–R2) (Fig. 5.6 A). After 50 h, some differences between the two curves can be observed, but these differences are not statistically significant ($RMSE_{BIOMASS}$ 0.05).

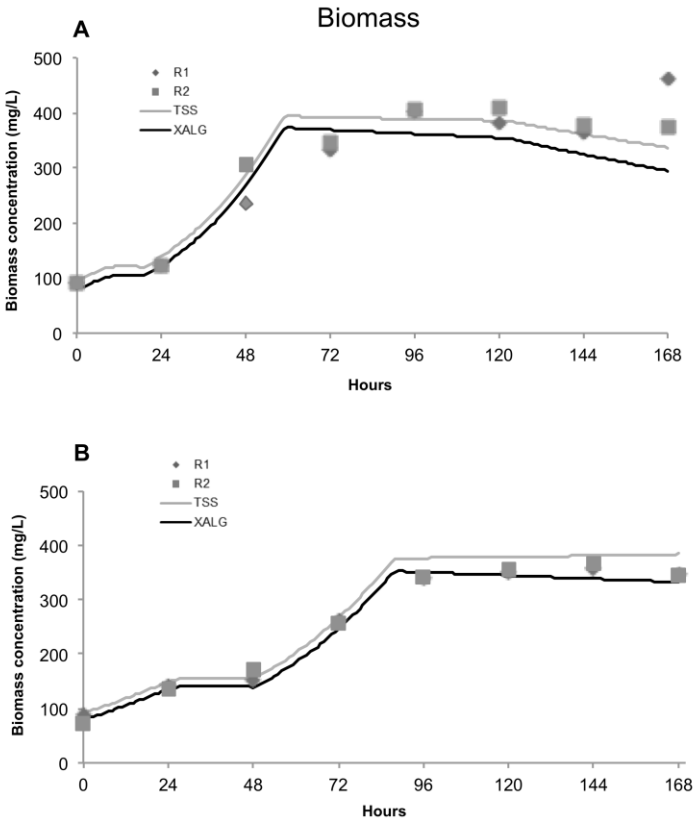

Figure 5.6: Trend of biomass concentration with time in the experimental trial (mg TSS/L, average of the two replicates) and according to BIO_ALGAE simulation (TSS and X_{ALG}) for *T. suecica* (A) and for *D. tertiolecta* (B).

After 72 h, the maximum values (nearly 400 mg/L) were reached and after that a slow decrease occurred, so that a true steady state did not take place. As previously told, at the end of the experiment the simulated and experimental data did not agree anymore. Also for *D. tertiolecta*, the predicted curves were very similar to the experimental ones ($RMSE_{BIOMASS}$ 0.02) (Table 5.3) but their shape was different from those derived from *T. suecica* experiments. In the first 24 h, no lag phase was observed for *D. tertiolecta*, and the biomass density increased, even if slowly (Fig. 5.6 B). Between 24 and 48 h, the data show a sort of steady state while the exponential growth occurred between 48 and 96 h, when TSS and X_{ALG} reached their maxima (just a little lower than for *T. suecica*), to keep nearly constant afterwards (Fig. 5.6 B). As to nutrient removal, the simulations of the sum of NO_3^-–N + NO_2^-–N and the PO_4^{3-}–P represent quite well the experimental data in *T. suecica* (Fig. 5.7). Instead, in *D. tertiolecta*, the simulation curve of NO_3^-–N + NO_2^-–N has a rapid decrease at 24 h, while in the real data the concentrations of these nutrients

begin to drop after 48 h (Fig. 5.8). Simulated phosphorus concentrations fitted well the experimental data for the two microalgae ($RMSE_P$ 0.14 for *T. suecica* and 0.04 for *D. tertiolecta*), although these data showed a non-constant distribution after 24 h.

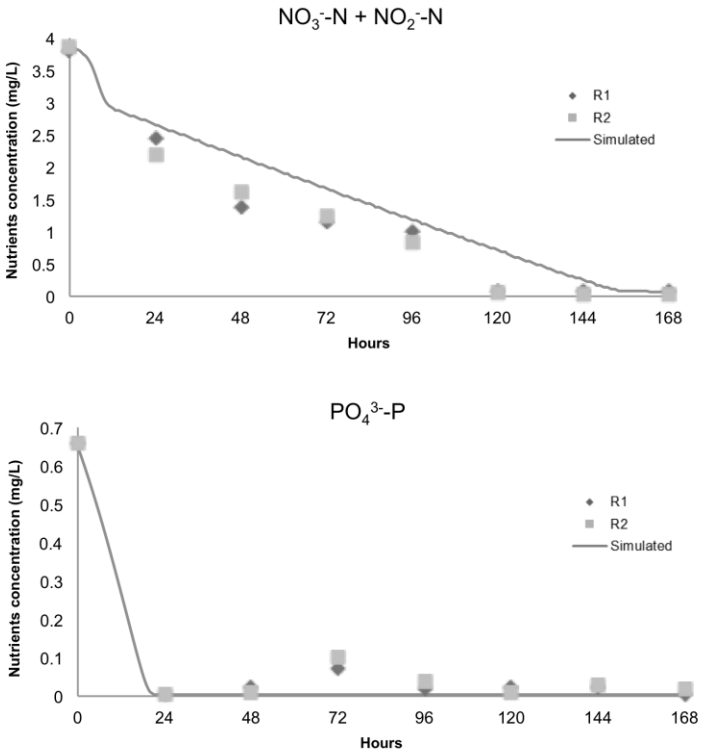

Figure 5.7: Nutrient removal for *T. suecica*, experimental data (R1, R2) and BIO_ALGAE simulation curves in mg/L.

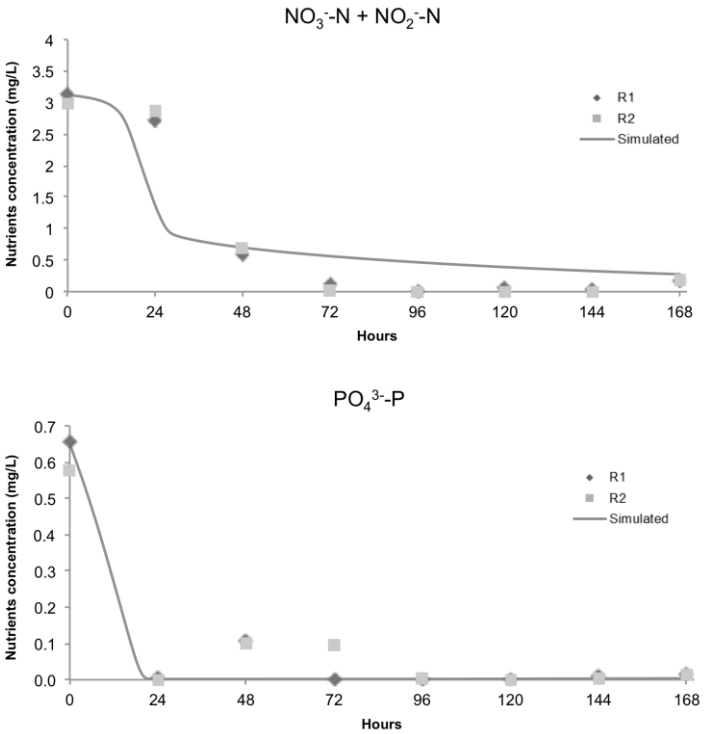

Figure 5.8. Nutrient removal for *D. tertiolecta*, experimental data (R1, R2) and BIO_ALGAE simulation curves in mg/L.

5.4 Discussion

This work has confirmed that aquaculture wastewater is suitable for the cultivation of *T. suecica* and *D. tertiolecta*. In a previous experiment with reactors of 6 L and the same AW, biomass production was of 86.14 ± 5 mg/L/day for *T. suecica*, and 54.26 ± 5 mg/L/day for *D. tertiolecta* (Andreotti et al. 2017), while in the present work, with 120-L reactors, biomass production was lower for *T. suecica* (65.71 ± 4.25 mg/L/day) and similar for *D. tertiolecta* (47.05 ± 1.57 mg/L/day). This variation could depend on the different nutrients' concentration of the wastewater in two experiments. However, the biomass production was not affected by the low nitrogen values in aquaculture. This is also confirmed in a recent study, in which a *D. tertiolecta* cell size increase was observed under nitrogen starvation conditions (Chen et al., 2011). The results obtained by Michels et al., (2014), instead, range between 0.46 and 0.52 g/L/day of biomass production with extra addition of phosphorus in the aquaculture wastewater. Gao et al., (2016) cultivated *Chlorella vulgaris* and *Scenedesmus obliquus* in aquaculture wastewater and obtained lower biomass productions: 7.3 and 6.2 mg/L/day, respectively. Khatoon et al., (2016) made a comparison between

Tetraselmis chuii growth in aquaculture wastewater and *T. chuii* growth in a synthetic medium and observed no significant differences ($p > 0.05$) in terms of biomass production in two different mediums.

The specific microalgal growth rate (μ in day^{-1}) obtained in the exponential phase (0.16 day^{-1} for *T. suecica* and 0.15 day^{-1} for *D. tertiolecta*) was calculated with experimental data and was estimated only on TSS value. Gao et al. (2016) obtained in the first 6 days of the experiment an average specific growth rate of 0.17 day^{-1} for *Chlorella vulgaris* and 0.15 day^{-1} for *Scenedesmus obliquus* cultivated in AW. Another recent study demonstrates that *Tetraselmis chuii* cultured in synthetic medium and in AW showed a similar growth rate of 0.71 day^{-1} and 0.72 day^{-1} respectively (Khatoon et al., 2018). The typical range for growth rate values obtained in literature is 0.4–2 day^{-1} (Reichert et al., 2011). These different values could be determined by the cultivation system or by the amount of nutrients in the wastewaters, which has been demonstrated important factors for the microalgae growth (Xin et al., 2010; Tang et al., 2012). In this study, AW was analyzed for the presence of nitrates, nitrites, ammonia, and phosphates that are essential for microalgae cultivation. For both species, the

removal efficiency exceeded 95% for DIN and DIP, reaching higher levels than in the previous study (Andreotti et al., 2017) and in various literature data. Michels et al. (2014) showed that *T. suecica* has a removal efficiency of 49.4% for N and 99.0% for P in AW, while Lowrey (2011) used *Tetraselmis* sp. in a dairy wastewater obtaining a reduction of 51% of total nitrogen, and of 40% of total phosphorus. Wu et al., (2015) cultivated *D. tertiolecta* in a saline sewage (13 ± 0.2 mg/L of nitrate as nitrogen mg/L and 14.7 ± 0.1 mg/L of orthophosphate) and the removal percentage was 60 ± 5.4% for nitrate and 70 ± 13.5% for orthophosphate after 6 days. The higher results obtained in the present study may be related to the initial concentration of nutrients in the wastewater and microalgae strains used (Zhou et al., 2012). However, further studies on the microalgae growth in AW must be carried out, because of its suitability in effluents that can be species specific and no microalgae should be neglected (Milhazes-Cunha and Otero, 2017). For example, recent studies (Borges et al., 2005; Andreotti et al., 2017) demonstrated that *Isochrysis galbana* has a lower productivity than *T. suecica* when cultivated in the same aquaculture wastewater. On the contrary, Freire et al. (2013) and Zheng et al. (2011)

successfully cultivated the *Isochrysis* genus in fish farm effluents.

Nowadays, very few studies analyzed the microalgal biomass composition produced in aquaculture wastewater.

Ansari et al., (2017) have obtained a total lipid percentage of 30.85% for *Scenedesmus obliquus*, 31.85% for *Chlorella sorokiniana*, and 35.90% for *Ankistrodesmus falcatus* grown in aquaculture wastewater.

Another recent study extracted from *C. sorokiniana* cultivated in aquaculture wastewater the 39.1% of lipids and calculated a daily production of 138.17 mg/L/day (Guldhe et al., 2017). *T. suecica* cultivated in artificial seawater showed a different response to nutrient deprivation, with a lipid content of 22% in the nitrogen starved culture, 27% under nitrogen and phosphorus starvation and 29% in a culture with enough content of nutrients (Bondioli et al., 2012). Furthermore, this species cultivated in f/2 culture medium has a lipid content of 4.85% (Kim et al., 2001). The lipid productivity for *T. suecica* observed in this study was higher compared to the previously reported studies, and this result allows us to confirm that these wastewaters are suitable for the production of lipids in *T. suecica*. *Dunaliella* sp. is also known to respond to nitrogen

starvation by increasing lipid production (Lombardi and Wangersky, 1995; Guevara et al., 2005). The nitrogen and phosphorous content were lower in our wastewaters than in synthetic media. This probably caused a nutrient stress and the consequent reduction of microalgal growth and increase of lipid concentration as already observed in other studies (Guldhe et al., 2017).

For the first time, BIO_ALGAE model was applied in aquaculture system and was able to fit very well for the species studied both in terms of biomass and nutrients uptake, indicating a good agreement between our real data and simulations. In fact, all the parameters previously used in BIO_ALGAE model, including the sensitive parameters, were used as such in this work. The sensitivity analysis was not conducted and all parameters (Table A5.3) have proved suitable for this type of wastewater, making BIO_ALGAE useful in different conditions.

As previously mentioned, BIO_ALGAE model relies on Monod kinetics, which growth depends of the extracellular available nutrient and does not take into account of internal reserves of nutrients (cell quotas) as in Droop model. Our results show that after 24 h, the biomass continues to grow during a few days after nutrient exhaustion. We solved this

problem assuming an external input of nutrients after its total consumption and it was necessary to calculate a new concentration of nitrogen and phosphorous in the culture to simulate the real data. The amount of internal nutrients was calculated by the experimental data, according to Lemesle and Mailleret, (2008). This calculation strategy has allowed us to adapt to experimental data to the model parameters, making it suitable for the objectives of this work. In this circumstance, this model has had a simple and effective application in aquaculture systems.

In intensive aquaculture systems with continuous production of wastewater, nutrients are never limiting; for this reason, BIO_ALGAE seemed a suitable choice, foreseeing an actual future application of it for a continuous production of microalgae in aquaculture. This model describes the factors that influence microalgae growth and this is a useful approach to predict microalgal biomass production optimizing the operational conditions. The Monod model, in a system with a continuous supply of external substrate, guarantees modeling accuracy, which makes it preferable to the Droop's model.

Other important aspects are the starvation conditions, as in our experimentation; in fact, the growth rate of the biomass

can be related to the internal concentration of the limiting element (Bernard, 2011). As an example, the correlation between maximum uptake velocities and cell quota for limiting nutrient may need to be modified if phosphate or iron are limiting factors. This depends by the greater potential for luxury uptake of phosphorus and iron relative to nitrogen (Mc-Carthy, 1980; Morel, 1987).

Chen et al. (2011) showed that *D. tertiolecta* had internal phosphate stores enough for the synthesis of lipids in phosphate-deficient cultures. BIO_ALGAE model was developed for municipal wastewater with a high concentration of nitrogen and phosphorous. In AW the content of N and P is lower and influenced by several factors, including the area used for culture, the bred species, the production level, and the profile of the waterbody (Islam, 2005). The content of these nutrients in the feed has decreased, especially for N (Islam, 2005). Despite this, the simulation curves of the nutrient removal (NO_3^-–N + NO_2^-–N and PO_4^3–P) produced by BIO_ALGAE represent accurately the experimental data for two microalgae. It has already been demonstrated that these microalgae species are able to compete with other microorganism, specifically ciliates (Austin et al., 1992, Chang et al., 1993; Andreotti et

al., 2017). Accordingly, in our work, it has not been performed a sterilization process. In this way, by avoiding pretreatment and sterilization of wastewater, the management costs are reduced, as well as energy and manual labor.

The mathematical models offer a great opportunity to predict microalgae growth permiting to control the parameters and increasing the bioreactor efficiency. The control and the forecast of variables that limit the microalgae productivity, such as light intensity, pH, temperature, nutrient concentration, and the photobioreactors design, will allow to increase biomass production at an industrial system (Oswald, 2001; Rodriguez-Mata et al., 2016).

This study improved the knowledge about the role of microalgae in aquaculture systems through an innovative approach based on the development of new technology to forecast biomass production.

5.5 Conclusion

The present study demonstrated that *T. suecica* and *D. tertiolecta* are suitable for upscale in vertical column photobioreactors with a volume of 120 L. Using aquaculture

wastewater as culture medium, nutrient removal was greater than 95%. Moreover, *T. suecica* has been able to produce more than 75% of total lipid content, whereas *D. tertiolecta* only 23%, and it is possible to confirm that nitrogen stress has disproportionate effects in different ways on growth and lipid content between the species. These microalgae are valid candidates for a second use in aquaculture systems as live feed for hatchery-grown herbivorous and filter feeders (Alsull and Omar, 2012). Despite this, further studies are necessary to analyze the protein and lipid composition of these species.

This research also proved for the first time the applicability of the BIO_ALGAE model to simulate the growth of these microalgae and the assimilation of nutrients in aquaculture wastewater. The model was calibrated by comparing simulated results to experimental data during 7 days of batch experiment. The results of the calibration indicate that the model was able to reproduce with a good degree the assimilation of nutrients. However, further modifications will be necessary as regards the biomass production.

The possibility of applying BIO_ALGAE model to predict use of microalgae for wastewater treatment and the biomass production use for feed in aquaculture is a new aspect that

should be developed with further studies.

The next approach towards better understanding the wastewater aquaculture treatment with microalgae will imply predicting the growth and nutrient uptake using the model in a continuous system.

5.6 Appendix

Table A5.1 Mathematical description of the processes of the model (processes rates).

Processes	**Process rate [M L^{-3} T^{-1}]**
Microalgae (X_{ALG}) processes	
1a. Growth of X_{ALG} on S_{NH4}	$\rho_{1a} = \mu_{ALG} \cdot f_{T,FS}(T) \cdot \eta_{PS}(I, S_{O2}) \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,ALG} + S_{CO2} + S_{HCO3} + \frac{{S_{CO2}}^2}{I_{CO2,ALG}}} \cdot \frac{S_{NH3} + S_{NH4}}{K_{N,ALG} + S_{NH3} + S_{NH4}} \cdot \frac{S_{PO4}}{K_{P,ALG} + S_{PO4}} \cdot X_{ALG}$
1b. Growth of X_{ALG} on S_{NO3}	$\rho_{1b} = \mu_{ALG} \cdot f_{T,FS}(T) \cdot \eta_{PS}(I, S_{O2}) \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,ALG} + S_{CO2} + S_{HCO3} + \frac{{S_{CO2}}^2}{I_{CO2,ALG}}} \cdot \frac{S_{NO3}}{K_{N,ALG} + S_{NO3}} \cdot \frac{K_{N,ALG}}{K_{N,ALG} + S_{NH3} + S_{NH4}} \cdot \frac{S_{PO4}}{K_{P,ALG} + S_{PO4}} \cdot X_{ALG}$
2. Endogenous respiration of X_{ALG}	$\rho_2 = k_{resp,ALG} \cdot f_{T,FS}(T) \cdot \frac{S_{O2}}{K_{O2,ALG} + S_{O2}} \cdot X_{ALG}$
3. Decay of X_{ALG}	$\rho_3 = k_{death,ALG} \cdot f_{T,FS}(T) \cdot X_{ALG}$
Heterotrophic bacteria (X_H) (aerobic and denitrifying activity)	
4a. Aerobic growth of X_H on S_{NH4}	$\rho_{4a} = \mu_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{S_{O2}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NH4} + S_{NH3}}{K_{N,H} + S_{NH4} + S_{NH3}} \cdot X_H$
4b. Aerobic growth of X_H on S_{NO3}	$\rho_{4b} = \mu_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{S_{O2}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO3}}{K_{N,H} + S_{NO3}} \cdot X_H$
5. Anoxic growth of X_H on S_{NO2} (denitrification on S_{NO2})	$\rho_5 = \mu_H \cdot \eta_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{K_{O2,H}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO2}}{K_{NO2,H,anox} + S_{NO2}} \cdot X_H$
6. Anoxic growth of X_H on S_{NO3} (denitrification on S_{NO3})	$\rho_6 = \mu_H \cdot \eta_H \cdot f_{T,MB}(T) \cdot \frac{S_S}{K_{S,H} + S_s} \cdot \frac{K_{O2,H}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO3}}{K_{NO3,H,anox} + S_{NO3}} \cdot X_H$
7. Aerobic endogenous respiration of X_H	$\rho_7 = k_{resp,H} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,H} + S_{O2}} \cdot X_H$
8. Anoxic endogenous respiration of X_H	$\rho_8 = k_{resp,H} \cdot \eta_H \cdot f_{T,MB}(T) \cdot \frac{K_{O2,H}}{K_{O2,H} + S_{O2}} \cdot \frac{S_{NO3} + S_{NO2}}{K_{NO3,H,anox} + S_{NO2} + S_{NO3}} \cdot X_H$
9. Decay of X_H	$\rho_9 = k_{death,H} \cdot f_{T,MB}(T) \cdot X_H$
Autotrophic bacteria (nitrifying activity)	

10. Growth of ammonia oxidizing bacteria (X_{AOB})	$\rho_{10} = \mu_{AOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,AOB} + S_{O2}} \cdot \frac{S_{NH3} + S_{NH4}}{K_{NH4,AOB} + S_{NH4} + S_{NH3}} \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,AOB} + S_{CO2} + S_{HCO3}} \cdot X_{AOB}$
11. Growth of nitrite oxidizing bacteria (X_{NOB})	$\rho_{11} = \mu_{NOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,NOB} + S_{O2}} \cdot \frac{K_{I,NH4}}{K_{I,NH4} + S_{NH4} + S_{NH3}} \cdot \frac{S_{NO2}}{K_{NO2,NOB} + S_{NO2}} \cdot \frac{S_{CO2} + S_{HCO3}}{K_{C,NOB} + S_{CO2} + S_{HCO3}} \cdot X_{NOB}$
12. Endogenous respiration of X_{AOB}	$\rho_{12} = k_{resp,AOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,AOB} + S_{O2}} \cdot X_{AOB}$
13. Endogenous respiration of X_{NOB}	$\rho_{13} = k_{resp,NOB} \cdot f_{T,MB}(T) \cdot \frac{S_{O2}}{K_{O2,NOB} + S_{O2}} \cdot X_{NOB}$
14a. Decay of X_{AOB}	$\rho_{14a} = k_{death,AOB} \cdot f_{T,MB}(T) \cdot X_{AOB}$
14b. Decay of X_{NOB}	$\rho_{14b} = k_{death,NOB} \cdot f_{T,MB}(T) \cdot X_{NOB}$
Hydrolysis, Chemical equilibrium and Transfer of gases	
15. Hydrolysis	$\rho_{15} = k_{HYD} \cdot \frac{X_S/X_H}{Y_{HYD} + (X_S/X_H)} \cdot X_H$
16.Chemical equilibrium $CO_2 \leftrightarrow HCO_3^-$	$\rho_{16} = k_{eq,1} \cdot (S_{CO2} - S_H S_{HCO3}/K_{eq,1})$
17. Chemical equilibrium $HCO_3^- \leftrightarrow CO_3^{2-}$	$\rho_{17} = k_{eq,2} \cdot (S_{HCO3} - S_H S_{CO3}/K_{eq,2})$
18. Chemical equilibrium $NH_4^+ \leftrightarrow NH_3$	$\rho_{18} = k_{eq,3} \cdot (S_{NH4} - S_H S_{NH3}/K_{eq,3})$
19. Chemical equilibrium $H^+ \leftrightarrow OH^-$	$\rho_{19} = k_{eq,w} \cdot (1 - S_H S_{OH}/K_{eq,w})$
20. Oxygen transfer to the atmosphere	$\rho_{20} = k_{a,O2} \cdot (S_{O2}^{WAT} - S_{O2})$
21. Carbon dioxide transfer to the atmosphere	$\rho_{21} = k_{a,CO2} \cdot (S_{CO2}^{WAT} - S_{CO2})$
22. Ammonia transfer to the atmosphere	$\rho_{22} = k_{a,NH3} \cdot (-S_{NH3})$

Table A5.2. Matrix of stoichiometric parameters that relate processes and components through stoichiometric coefficients in Table A5.5.

	S_{NH4}	S_{NH3}	S_{NO3}	S_{NO2}	S_{CO2}	S_{HCO3}	S_{CO3}	S_{PO4}	S_{O2}	S_H	S_{OH}	S_S	S_I	X_{ALG}	X_S	X_I	X_H	X_{AOB}	X_{NOB}
ρ_{1a}	$v_{1,1a}$				$v_{5,1a}$			$v_{8,1a}$	$v_{9,1a}$	$v_{10,1a}$				$v_{14,1a}$					
ρ_{1b}			$v_{3,1b}$		$v_{5,1b}$			$v_{8,1b}$	$v_{9,1b}$	$v_{10,1b}$				$v_{14,1b}$					
ρ_2	$v_{1,2}$				$v_{5,2}$			$v_{8,2}$	$v_{9,2}$	$v_{10,2}$				$v_{14,2}$					
ρ_3	$v_{1,3}$				$v_{5,3}$			$v_{8,3}$	$v_{9,3}$	$v_{10,3}$				$v_{14,3}$	$v_{15,3}$	$v_{16,3}$			
ρ_{4a}	$v_{1,4a}$				$v_{5,4a}$			$v_{8,4a}$	$v_{9,4a}$	$v_{10,4a}$		$v_{12,4a}$					$v_{17,4a}$		
ρ_{4b}			$v_{3,4b}$		$v_{5,4b}$			$v_{8,4b}$	$v_{9,4b}$	$v_{10,4b}$		$v_{12,4b}$					$v_{17,4b}$		
ρ_5				$v_{4,5}$	$v_{5,5}$			$v_{8,5}$		$v_{10,5}$		$v_{12,5}$					$v_{17,5}$		
ρ_6			$v_{3,6}$		$v_{5,6}$			$v_{8,6}$		$v_{10,6}$		$v_{12,6}$					$v_{17,6}$		
ρ_7	$v_{1,7}$				$v_{5,7}$			$v_{8,7}$	$v_{9,7}$	$v_{10,7}$							$v_{17,7}$		
ρ_8	$v_{1,8}$		$v_{3,8}$	$v_{4,8}$	$v_{5,8}$			$v_{8,8}$		$v_{10,8}$							$v_{17,8}$		
ρ_9															$v_{15,9}$	$v_{16,9}$	$v_{17,9}$		
ρ_{10}	$v_{1,10}$			$v_{4,10}$	$v_{5,10}$			$v_{8,10}$	$v_{9,10}$	$v_{10,10}$								$v_{18,10}$	
ρ_{11}			$v_{3,11}$	$v_{4,11}$	$v_{5,11}$			$v_{8,11}$	$v_{9,11}$	$v_{10,11}$									$v_{19,11}$
ρ_{12}	$v_{1,12}$				$v_{5,12}$			$v_{8,12}$	$v_{9,12}$	$v_{10,12}$								$v_{18,12}$	
ρ_{13}	$v_{1,13}$				$v_{5,13}$			$v_{8,13}$	$v_{9,13}$	$v_{10,13}$									$v_{19,13}$
ρ_{14a}															$v_{15,14a}$	$v_{16,14a}$		$v_{18,14a}$	
ρ_{14b}															$v_{15,14b}$	$v_{16,14b}$			$v_{19,14b}$
ρ_{15}	$v_{1,15}$				$v_{5,15}$			$v_{8,15}$		$v_{10,15}$		$v_{12,15}$	$v_{13,15}$		$v_{15,15}$				
ρ_{16}					$v_{5,16}$	$v_{6,16}$				$v_{10,16}$									
ρ_{17}						$v_{6,17}$	$v_{7,17}$			$v_{10,17}$									
ρ_{18}	$v_{1,18}$	$v_{2,18}$								$v_{10,18}$									
ρ_{19}										$v_{10,19}$	$v_{11,19}$								
ρ_{20}									$v_{9,20}$										
ρ_{21}					$v_{5,21}$														
ρ_{22}		$v_{2,22}$																	

Table A5.3 Values of biokinetic, chemical and physic parameters.

Parameters	Description	Value	Unit	Source
Microalgae (X_{ALG})				
μ_{ALG}	Maximum growth rate of X_{ALG}	1.6	d^{-1}	Solimeno et al. 2017
$k_{resp,ALG}$	Endogenous respiration constant of X_{ALG}	0.05	d^{-1}	Reichert et al. 2001
$k_{death,ALG}$	Decay constant of X_{ALG}	0.05	d^{-1}	Reichert et al. 2001
$K_{C,ALG}$	Saturation constant of X_{ALG} for carbon species	0.004	$gC\ m^{-3}$	Novak and Brune, 1985
$I_{CO2,ALG}$	Carbon dioxide inhibition constant of X_{ALG}	120	$gC\ m^{-3}$	Silva and Pirt, 1984
$K_{N,ALG}$	Saturation constant of X_{ALG} for nitrogen species	0.1	$gN\ m^{-3}$	Reichert et al. 2001
$K_{O2,ALG}$	Saturation constant of X_{ALG} for S_{O2}	0.2	$gO_2\ m^{-3}$	Reichert et al. 2001
$K_{P,ALG}$	Saturation constant of X_{ALG} for S_{HPO4}	0.02	$gP\ m^{-3}$	Reichert et al. 2001
Heterotrophic bacteria (X_H)				
μ_H	Maximum growth rate of X_H	1.3	d^{-1}	Solimeno et al. 2017
η_H	Anoxic reduction factor for X_H	0.6	−	Gujer et al. 1999

$k_{resp,H}$	Endogenous respiration rate of X_H	0.3	d^{-1}	Reichert et al. 2001
$K_{O2,H}$	Saturation constant of X_H for S_{O2}	0.2	$gO_2\ m^{-3}$	Reichert et al. 2001
$K_{N,H}$	Saturation constant of X_H for nitrogen species	0.2	$gN\ m^{-3}$	Reichert et al. 2001
$K_{S,H}$	Saturation constant of X_H for S_S	20	$gCOD\ m^{-3}$	Henze et al. 2000
$K_{NO3,H,anox}$	Saturation constant of X_H for S_{NO3}	0.5	$gN\ m^{-3}$	Reichert et al. 2001
$K_{NO2,H,anox}$	Saturation constant of X_H for S_{NO2}	0.2	$gN\ m^{-3}$	Reichert et al. 2001
$k_{death,H}$	Decay constant of X_H	0.3	d^{-1}	Solimeno et al. 2017
Autotrophic bacteria: ammonia oxidizing bacteria (X_{AOB}) and nitrite oxidizing bacteria (X_{NOB})				
μ_{AOB}	Maximum growth rate of X_{AOB}	0.63	d^{-1}	Gujer et al. 1999
μ_{NOB}	Maximum growth rate of X_{AOB}	1.1	d^{-1}	Gujer et al. 1999
$K_{O2,AOB}/K_{O2,NOB}$	Saturation constant of X_{AOB} and X_{NOB} for S_{O2}	0.5	$gO_2\ m^{-3}$	Reichert et al. 2001
$K_{NH4,AOB}$	Saturation constant of X_{AOB} on S_{NH4}	0.5	$gN\ m^{-3}$	Reichert et al. 2001
$K_{I,NH4}$	Ammonia inhibition constant of X_{NOB}	5.0	$gN\ m^{-3}$	Henze et al. 2000
$K_{NO2,NOB}$	Saturation constant of X_{NOB} for S_{NO2}	0.5	$gN\ m^{-3}$	Henze et al. 2000

$K_{C,AOB}/K_{C,NOB}$	Saturation constant of X_{AOB} and X_{NOB} for carbon species	0.5	gC m^{-3}	Henze et al. 2000
$k_{resp,AOB}/k_{resp,NOB}$	Endogenous respiration rate of X_{AOB} and X_{NOB}	0.05	d^{-1}	Reichert et al. 2001
$k_{death,AOB}/$ $k_{death,NOB}$	Decay constant of X_{AOB} and X_{NOB}	0.2	d^{-1}	Henze et al. 2000
Hydrolysis				
k_{HYD}	Hydrolysis rate constant	3.0	d^{-1}	Reichert et al. 2001
Photorespiration factor of microalgae				
K_{PR}	Inhibition constant of photorespiration	0.03	–	Solimeno et al. 2015
τ	Coefficient of excess dissolved oxygen	3.5	–	Fernández et al. 2014
S_{O2}^{SAT}	Saturation concentration of oxygen in the air	9.07	gO_2 m^{-3}	Fernández et al. 2014
Thermal factor of microalgae and bacteria				
T_{OPT}	Optimum temperature for microalgae growth	25	°C	Dauta et al. 1990
s	Normalized parameter	30	–	Dauta et al. 1990
θ	Temperature coefficient for bacteria	1.07		Von Sperling, 2005
Light factor of microalgae				

α	Activation rate	0.001935	$(\mu E\ m^{-2})^{-1}$	Wu and Merchuk, 2001
β	Inhibition rate	5.7E-7	$(\mu E\ m^{-2})^{-1}$	Wu and Merchuk, 2001
γ	Production rate	0.1460	s^{-1}	Wu and Merchuk, 2001
δ	Recovery rate	0.0004769	s^{-1}	Wu and Merchuk, 2001
K_I	Biomass extinction coefficient	0.07	$m^2\ g^{-1}$	Molina-Grima et al. 1994

Parameters	**Equations**
Chemical equilibrium $CO_2 \leftrightarrow HCO_3^-$.	$K_{eq,1} = 10^{17.843-\frac{3404.71}{273.15+T}-0.032786(273.15+T)}$
Chemical equilibrium $HCO_3^- \leftrightarrow CO_3^{2-}$	$K_{eq,2} = 10^{9.494-\frac{2902.39}{273.15+T}-0.02379(273.15+T)}$
Chemical equilibrium $NH_4^+ \leftrightarrow NH_3$	$K_{eq,3} = 10^{2.891-\frac{2727}{(273.15+T)}}$
Chemical equilibrium $H^+ \leftrightarrow OH^-$	$K_{eq,w} = 10^{-\frac{4470.99}{273.15+T}+12.0875-0.01706(273.15+T)}$

Kinetics parameters				
$k_{eq,1}$	Dissociation constant of $CO_2 \leftrightarrow HCO_3^-$.	10000	d^{-1}	Reichert et al. 2001
$k_{eq,2}$	Dissociation constant of $HCO_3^- \leftrightarrow CO_3^{2-}$	1000	d^{-1}	Reichert et al. 2001

$k_{eq,3}$	Dissociation constant of $NH_4^+ \leftrightarrow NH_3$	1000	d^{-1}	Reichert et al. 2001
$k_{eq,w}$	Dissociation constant of $H^+ \leftrightarrow OH^-$	1000	$g\ m^{-1}\ d^{-1}$	Reichert et al., 2001
Transfer of gases to the atmosphere				
$K_{a,O2}$	Mass transfer coefficient for oxygen	0.16	h^{-1}	Solimeno et al. 2017
$K_{a,CO2}$	Mass transfer coefficient for dioxide carbon	0.14	h^{-1}	Solimeno et al. 2017
$K_{a,NH3}$	Mass transfer coefficient for ammonia	0.14	h^{-1}	Solimeno et al. 2017

Table A5.4 Values of fractions of carbon, hydrogen, oxygen and nitrogen in microalgae and bacteria biomass.

Parameters	Description	Value	Unit	Source
Fractions of microalgal biomass (X_{ALG})				
$i_{C,ALG}$	Fraction of carbon in microalgae	0.387	gC $gCOD^{-1}$	Reichert et al. 2001
$i_{H,ALG}$	Fraction of hydrogen in microalgae	0.075	gH $gCOD^{-1}$	Reichert et al. 2001
$i_{O,ALG}$	Fraction of oxygen in microalgae	0.538	gO $gCOD^{-1}$	Reichert et al. 2001
$i_{N,ALG}$	Fraction of nitrogen in microalgae	0.065	gN $gCOD^{-1}$	Reichert et al. 2001
$i_{P,ALG}$	Fraction of phosphorus in microalgae	0.01	gP $gCOD^{-1}$	Reichert et al. 2001
Fractions of bacteria biomass (X_H, X_{AOB}, X_{NOB})				
$i_{C,BM}$	Fraction of carbon in bacteria	0.323	gC $gCOD^{-1}$	Reichert et al. 2001
$i_{H,BM}$	Fraction of hydrogen in bacteria	0.060	gH $gCOD^{-1}$	Reichert et al. 2001
$i_{O,BM}$	Fraction of oxygen in bacteria	0.155	gO $gCOD^{-1}$	Reichert et al. 2001
$i_{N,BM}$	Fraction of nitrogen in bacteria	0.075	gN $gCOD^{-1}$	Reichert et al. 2001
$i_{P,BM}$	Fraction of phosphorus in bacteria	0.018	gP $gCOD^{-1}$	Reichert et al. 2001
Fractions of slowly biodegradable substrates (X_S)				
$i_{C,XS}$	Fraction of carbon in X_S	0.318	gC $gCOD^{-1}$	Reichert et al. 2001
$i_{H,XS}$	Fraction of hydrogen in X_S	0.045	gH $gCOD^{-1}$	Reichert et al. 2001
$i_{O,XS}$	Fraction of oxygen in X_S	0.156	gO $gCOD^{-1}$	Reichert et al. 2001
$i_{N,XS}$	Fraction of nitrogen in X_S	0.034	gN $gCOD^{-1}$	Reichert et al. 2001
$i_{P,XS}$	Fraction of phosphorus in X_S	0.005	gP $gCOD^{-1}$	Reichert et al. 2001

Fractions of inert particulate organics (X_I)				
$i_{C,XI}$	Fraction of carbon in X_I	0.327	gC $gCOD^{-1}$	Reichert et al. 2001
$i_{H,XI}$	Fraction of hydrogen in X_I	0.037	gH $gCOD^{-1}$	Reichert et al. 2001
$i_{O,XI}$	Fraction of oxygen in X_I	0.150	gO $gCOD^{-1}$	Reichert et al. 2001
$i_{N,XI}$	Fraction of nitrogen in X_I	0.016	gN $gCOD^{-1}$	Reichert et al. 2001
$i_{P,XI}$	Fraction of phosphorus in X_I	0.005	gP $gCOD^{-1}$	Reichert et al. 2001
Fractions of readily biodegradable substrates (S_S)				
$i_{C,SS}$	Fraction of carbon in S_S	0.318	gC $gCOD^{-1}$	Reichert et al. 2001
$i_{H,SS}$	Fraction of hydrogen in S_S	0.045	gH $gCOD^{-1}$	Reichert et al. 2001
$i_{O,SS}$	Fraction of oxygen in S_S	0.156	gO $gCOD^{-1}$	Reichert et al. 2001
$i_{N,SS}$	Fraction of nitrogen in S_S	0.034	gN $gCOD^{-1}$	Reichert et al. 2001
$i_{P,SS}$	Fraction of phosphorus in S_S	0.005	gP $gCOD^{-1}$	Reichert et al. 2001
Fractions of soluble inert organics (S_I)				
$i_{C,SI}$	Fraction of carbon in S_I	0.327	gC $gCOD^{-1}$	Reichert et al. 2001
$i_{H,SI}$	Fraction of hydrogen in S_I	0.037	gH $gCOD^{-1}$	Reichert et al. 2001
$i_{O,SI}$	Fraction of oxygen in S_I	0.150	gO $gCOD^{-1}$	Reichert et al. 2001
$i_{N,SI}$	Fraction of nitrogen in S_I	0.016	gN $gCOD^{-1}$	Reichert et al. 2001
$i_{P,SI}$	Fraction of phosphorus in S_I	0.005	gP $gCOD^{-1}$	Reichert et al. 2001
Fractions of inert produced by biomass degradation				
f_{ALG}	Production of X_I in endogenous respiration of microalgae	0.1	gCOD $gCOD^{-1}$	Sah et al. 2011
f_{SI}	Production of S_I in hydrolysis of X_S	0	gCOD $gCOD^{-1}$	Henze et al. 2000

f_{XI}	Production of X_I in endogenous respiration of bacteria	0.1	gCOD gCOD^{-1}	Sah et al. 2011
Yield of biomass				
Y_{ALG}	Yield of X_{ALG}	0.62	gCOD gCOD^{-1}	Reichert et al. 2001
Y_H	Yield of X_H when using S_{O2} as electron acceptor	0.6	gCOD gCOD^{-1}	Reichert et al. 2001
$Y_{H,NO3}$	Yield of X_H when using S_{NO3} as electron acceptor	0.5	gCOD gCOD^{-1}	Reichert et al. 2001
$Y_{H,NO2}$	Yield of X_H when using S_{NO2} as electron acceptor	0.3	gCOD gCOD^{-1}	Reichert et al. 2001
Y_{AOB}	Yield of X_{AOB}	0.13	gCOD gCOD^{-1}	Reichert et al. 2001
Y_{NOB}	Yield of X_{NOB}	0.03	gCOD gCOD^{-1}	Reichert et al. 2001
Y_{HYD}	Hydrolysis saturation constant	1	gCOD gCOD^{-1}	Reichert et al. 2001

Table A5.5 Mathematical expressions of the stoichiometric coefficients of each process.

Stoichiometric coefficients	**Unit**
Growth of X_{ALG} on S_{NH4}	
$v_{1,1a} = -i_{N,ALG}$	gN gCOD^{-1}
$v_{5,1a} = -i_{C,ALG}$	gC gCOD^{-1}
$v_{8,1a} = -i_{P,ALG}$	gP gCOD^{-1}
$v_{9,1a} = (8i_{C,ALG}/3 + 8i_{H,ALG} - i_{O,ALG} - 12i_{N,ALG}/7 + 40i_{P,ALG}/31)/2$	gO_2 gCOD^{-1}
$v_{10,1a} = i_{N,ALG}/14 - 2i_{P,ALG}/31$	gH gCOD^{-1}

$v_{14,1a} = 1$	gCOD $gCOD^{-1}$
Growth of X_{ALG} on S_{NO3}	
$v_{3,1b} = -i_{N,ALG}$	gN $gCOD^{-1}$
$v_{5,1b} = -i_{C,ALG}$	gC $gCOD^{-1}$
$v_{8,1b} = -i_{P,ALG}$	gP $gCOD^{-1}$
$v_{9,1b} = (8i_{C,ALG}/3 + 8i_{H,ALG} - i_{O,ALG} + 20i_{N,ALG}/7 + 40i_{P,ALG}/31)/2$	gO_2 $gCOD^{-1}$
$v_{10,1b} = -i_{N,ALG}/14 - 2i_{P,ALG}/31$	gH $gCOD^{-1}$
$v_{14,1b} = 1$	gCOD $gCOD^{-1}$
Endogenous respiration of X_{ALG}	
$v_{1,2} = i_{N,ALG} - f_{ALG}\, i_{N,XI}$	gN $gCOD^{-1}$
$v_{5,2} = i_{C,ALG} - f_{ALG}\, i_{C,XI}$	gC $gCOD^{-1}$
$v_{8,2} = i_{P,ALG} - f_{ALG}\, i_{P,XI}$	gP $gCOD^{-1}$
$v_{9,2} = ((i_{O,ALG} - f_{ALG}\, i_{O,XI}) - 8(i_{H,ALG} - f_{ALG}\, i_{H,XI}) - 8/3\,(i_{C,ALG} - f_{ALG}\, i_{C,XI}) + 12/7\,(i_{N,ALG} - f_{ALG}\, i_{N,XI}) - 40/31\,(i_{P,ALG} - f_{ALG}\, i_{P,XI}))/2$	gO_2 $gCOD^{-1}$
$v_{10,2} = -1/14\,(i_{N,ALG} - f_{ALG}\, i_{N,XI}) + 2/31\,(i_{P,ALG} - f_{ALG}\, i_{P,XI})$	gH $gCOD^{-1}$
$v_{14,2} = -1$	gCOD $gCOD^{-1}$

Decay of X_{ALG}	
$v_{1,3} = i_{N,ALG} - (1 - f_{ALG})Y_{ALG}\, i_{N,XS} - f_{ALG}Y_{ALG}\, i_{N,ALG}$	gN $gCOD^{-1}$
$v_{5,3} = i_{C,ALG} - (1 - f_{ALG})Y_{ALG}\, i_{C,XS} - f_{ALG}Y_{ALG}\, i_{C,ALG}$	gC $gCOD^{-1}$
$v_{8,3} = i_{P,ALG} - (1 - f_{ALG})Y_{ALG}\, i_{P,XS} - f_{ALG}Y_{ALG}\, i_{P,ALG}$	gP $gCOD^{-1}$
$v_{9,3} = -((i_{O,ALG} - f_{ALG}\, i_{O,XI}) - 8(i_{H,ALG} - f_{ALG}\, i_{H,XI}) - 8/3\,(i_{C,ALG} - f_{ALG}\, i_{C,XI}) + 12/7\,(i_{N,ALG} - f_{ALG}\, i_{N,XI}) - 40/31\,(i_{P,ALG} - f_{ALG}\, i_{P,XI}))/2$	gO_2 $gCOD^{-1}$
$v_{10,3} = -1/14\,(i_{N,ALG}\,(1 - f_{ALG})Y_{ALG}\, i_{N,XS} - f_{ALG}Y_{ALG}\, i_{N,XI}) + 2/31\,(i_{P,ALG}\,(1 - f_{ALG})Y_{ALG}\, i_{P,XS} - f_{ALG}Y_{ALG}\, i_{P,XI})$	gH $gCOD^{-1}$
$v_{14,3} = -1$	gCOD $gCOD^{-1}$
$v_{15,3} = (1 - f_{ALG})$	gCOD $gCOD^{-1}$
$v_{16,3} = f_{ALG}Y_{ALG}$	gCOD $gCOD^{-1}$
Aerobic growth of X_H on S_{NH4}	
$v_{1,4a} = i_{N,SS}/Y_H - i_{N,BM}$	gN $gCOD^{-1}$
$v_{5,4a} = i_{C,SS}/Y_H - i_{C,BM}$	gC $gCOD^{-1}$
$v_{8,4a} = i_{P,SS}/Y_H - i_{P,BM}$	gP $gCOD^{-1}$
$v_{9,4a} = (-(1 - Y_H)/\,Y_H)/2$	gO_2 $gCOD^{-1}$
$v_{10,4a} = -1/14\,(i_{N,SS}/Y_H - i_{N,BM}) + 2/31\,(i_{P,SS}/Y_H - i_{P,BM})$	gH $gCOD^{-1}$
$v_{12,4a} = -1/Y_H$	gCOD $gCOD^{-1}$

$v_{17,4a} = 1$	gCOD $gCOD^{-1}$
Aerobic growth of X_H on S_{NO3}	
$v_{3,4b} = i_{N,SS}/Y_H - i_{N,BM}$	gN $gCOD^{-1}$
$v_{5,4b} = i_{C,SS}/Y_H - i_{C,BM}$	gC $gCOD^{-1}$
$v_{8,4b} = \left(i_{P,SS}/Y_H - i_{P,BM}\right)$	gP $gCOD^{-1}$
$v_{9,4b} = (-(1 - Y_H)/\ Y_H)/2$	gO_2 $gCOD^{-1}$
$v_{10,4b} = -1/14\left(i_{N,SS}/Y_H - i_{N,BM}\right) + 2/31\left(i_{P,SS}/Y_H - i_{P,BM}\right)$	gH $gCOD^{-1}$
$v_{12,4b} = -1/Y_H$	gCOD $gCOD^{-1}$
$v_{17,4b} = 1$	gCOD $gCOD^{-1}$
Anoxic growth of X_H on S_{NO2}	
$v_{4,5} = -(1 - Y_{H,NO2})/(1.71Y_{H,NO2})$	gN $gCOD^{-1}$
$v_{5,5} = \left(i_{C,SS}/Y_{H,NO2} - i_{C,BM}\right)$	gC $gCOD^{-1}$
$v_{8,5} = \left(i_{P,SS}/Y_{H,NO2} - i_{P,BM}\right)$	gP $gCOD^{-1}$
$v_{10,5} = 1/24\left(i_{O,SS}/Y_{H,NO2} - i_{O,BM}\right) - 1/3\left(i_{H,SS}/Y_{H,NO2} - i_{H,BM}\right) - 1/9\left(i_{C,SS}/Y_{H,NO2} - i_{C,BM}\right) - 1/93\left(i_{P,SS}/Y_{H,NO2} - i_{P,BM}\right)$	gH $gCOD^{-1}$
$v_{12,5} = -1/Y_{H,NO2}$	gCOD $gCOD^{-1}$
$v_{17,5} = 1$	gCOD $gCOD^{-1}$

Anoxic growth of X_H on S_{NO3}	
$v_{3,6} = -(1 - Y_{H,NO3})/(1.14Y_{H,NO3})$	gN $gCOD^{-1}$
$v_{4,6} = (1 - Y_{H,NO3})/(1.14Y_{H,NO3})$	gN $gCOD^{-1}$
$v_{5,6} = (i_{C,SS}/Y_{H,,NO3} - i_{C,BM})$	gC $gCOD^{-1}$
$v_{8,6} = (i_{P,SS}/Y_{H,NO3} - i_{P,BM})$	gP $gCOD^{-1}$
$v_{10,6} = 1/14\,(i_{N,SS}/Y_{H,NO3} - i_{N,BM}) + 2/31\,(i_{P,SS}/Y_{H,NO3} - i_{P,BM})$	gH $gCOD^{-1}$
$v_{12,6} = -1/Y_{H,NO3}$	gCOD $gCOD^{-1}$
$v_{17,6} = 1$	gCOD $gCOD^{-1}$
Aerobic endogenous respiration of X_H	
$v_{1,7} = i_{N,BM} - f_{XI}\, i_{N,XI}$	gN $gCOD^{-1}$
$v_{5,7} = i_{C,BM} - f_{X1}\, i_{C,XI}$	gC $gCOD^{-1}$
$v_{8,7} = i_{P,BM} - f_{X1}\, i_{P,XI}$	gP $gCOD^{-1}$
$v_{9,7} = -(1 - f_{X1})/2$	gO_2 gCOD-1
$v_{10,7} = -1/14\,(i_{N,BM} - f_{XI}\, i_{N,XI}) + 2/31\,(i_{P,BM} - f_{XI}\, i_{P,XI})$	gH gCOD-1
$v_{17,7} = -1$	gCOD $gCOD^{-1}$
Anoxic endogenous respiration of X_H	

$v_{1,8} = i_{N,BM} - f_{XI}\, i_{N,XI}$	gN $gCOD^{-1}$
$v_{3,8} = (f_{XI} - 1)/1.14$	gN $gCOD^{-1}$
$v_{4,8} = (1 - f_{XI})/1.14$	gN $gCOD^{-1}$
$v_{5,8} = i_{C,BM} - f_{XI} i_{C,XI}$	gC $gCOD^{-1}$
$v_{8,8} = i_{P,BM} - f_{XI} i_{P,XI}$	gP $gCOD^{-1}$
$v_{10,8} = 1/40\,(i_{O,BM} - f_{XI} i_{O,XI}) - 1/5\,(i_{H,BM} - f_{XI} i_{H,XI}) - 1/15\,(i_{C,BM} - f_{XI} i_{C,XI}) + 1/35\,(i_{N,BM} - f_{XI} i_{N,XI}) - 1/31\,(i_{P,BM} - f_{XI} i_{P,XI})$	gH $gCOD^{-1}$
$v_{17,8} = -1$	gCOD $gCOD^{-1}$
Decay of X_H	
$v_{15,9} = (1 - f_{XI})$	gCOD $gCOD^{-1}$
$v_{16,9} = f_{XI}$	gCOD $gCOD^{-1}$
$v_{17,9} = -1$	gCOD $gCOD^{-1}$
Growth of ammonia oxidizing bacteria (X_{AOB})	
$v_{1,10} = -1/Y_{AOB}$	gN $gCOD^{-1}$
$v_{4,10} = 1/Y_{AOB} - i_{N,BM}$	gN $gCOD^{-1}$
$v_{5,10} = -i_{C,BM}$	gC $gCOD^{-1}$
$v_{8,10} = -i_{P,BM}$	gP $gCOD^{-1}$

$v_{9,10} = (1 - 3.43/Y_{AOB})/2$	gO_2 $gCOD^{-1}$
$v_{10,10} = 2/14Y_{AOB} - 1/14\,(i_{N,BM}) - 2/31\,(i_{P,BM})$	gH $gCOD^{-1}$
$v_{18,10} = 1$	gCOD $gCOD^{-1}$
Growth of nitrite oxidizing bacteria (X_{NOB})	
$v_{3,11} = 1/Y_{NOB} - i_{N,BM}$	gN $gCOD^{-1}$
$v_{4,11} = -1/Y_{NOB}$	gN gCOD-1
$v_{5,11} = -i_{C,BM}$	gC $gCOD^{-1}$
$v_{8,10} = -i_{P,BM}$	gP $gCOD^{-1}$
$v_{9,11} = (1 - 1.14/Y_{NOB})/2$	gO_2 $gCOD^{-1}$
$v_{10,11} = -1/14\,(i_{N,BM}) - 2/31\,(i_{P,BM})$	gH $gCOD^{-1}$
$v_{19,11} = 1$	gCOD $gCOD^{-1}$
Endogenous respiration of X_{AOB}	
$v_{1,12} = i_{N,BM} - f_{XI}\, i_{N,XI}$	gN $gCOD^{-1}$
$v_{5,12} = i_{C,BM} - f_{XI} i_{C,XI}$	gC $gCOD^{-1}$
$v_{8,12} = i_{P,BM} - f_{XI} i_{P,XI}$	gP $gCOD^{-1}$
$v_{9,12} = -(1 - f_{XI})/2$	gO_2 $gCOD^{-1}$

$v_{10,12} = -1/14\left(i_{N,BM} - f_{XI}\, i_{N,XI}\right) + 2/31\left(i_{P,BM} - f_{XI}\, i_{P,XI}\right)$	gH $gCOD^{-1}$
$v_{16,12} = f_{XI}$	gCOD $gCOD^{-1}$
$v_{18,12} = -1$	gCOD $gCOD^{-1}$
Endogenous respiration of X_{NOB}	
$v_{1,13} = i_{N,BM} - f_{XI}\, i_{N,XI}$	gN $gCOD^{-1}$
$v_{5,13} = i_{C,BM} - f_{XI} i_{C,XI}$	gC $gCOD^{-1}$
$v_{8,13} = i_{P,BM} - f_{XI} i_{P,XI}$	gP $gCOD^{-1}$
$v_{9,13} = -(1 - f_{XI})/2$	gO_2 $gCOD^{-1}$
$v_{10,13} = -1/14\left(i_{N,BM} - f_{XI}\, i_{N,XI}\right) + 2/31\left(i_{P,BM} - f_{XI}\, i_{P,XI}\right)$	gH $gCOD^{-1}$
$v_{16,13} = f_{XI}$	gCOD $gCOD^{-1}$
$v_{19,13} = -1$	gCOD $gCOD^{-1}$
Decay of X_{AOB} and X_{NOB}	
$v_{15,14a} = (1 - f_{XI})$	gCOD $gCOD^{-1}$
$v_{16,14a} = f_{XI}$	gCOD $gCOD^{-1}$
$v_{18,14a} = -1$	gCOD $gCOD^{-1}$
$v_{15,14b} = (1 - f_{XI})$	gCOD $gCOD^{-1}$

$v_{16,14b} = f_{XI}$	gCOD $gCOD^{-1}$
$v_{19,14b} = -1$	gCOD $gCOD^{-1}$
Hydrolysis	
$v_{1,15} = -(1 - f_{SI})i_{N,SS} - f_{SI}i_{N,SI} + i_{N,XS}$	gN $gCOD^{-1}$
$v_{5,15} = i_{C,XS} - (1 - f_{SI})Y_{HYD}i_{C,SS} - f_{SI}Y_{HYD}i_{C,SI}$	gC $gCOD^{-1}$
$v_{8,15} = i_{P,XS} - (1 - f_{SI})Y_{HYD}i_{P,SS} - f_{I,XS}Y_{HYD}i_{P,SI}$	gP $gCOD^{-1}$
$v_{10,15} = -1/14\left(i_{N,XS} - (1 - f_{SI})Y_{HYD}i_{N,SS} - f_{SI}Y_{HYD}i_{N,SI}\right) + 2/31\left(i_{P,XS} - (1 - f_{SI})Y_{HYD}i_{P,SS} - f_{SI}Y_{HYD}i_{P,SI}\right)$	gH $gCOD^{-1}$
$v_{12,15} = (1 - f_{SI})Y_{HYD}$	gCOD $gCOD^{-1}$
$v_{13,15} = (f_{SI})Y_{HYD}$	gCOD $gCOD^{-1}$
$v_{15,15} = -1$	gCOD $gCOD^{-1}$
Chemical equilibria $CO_2 \leftrightarrow HCO_3^-$	
$v_{5,16} = -1$	gC gC^{-1}
$v_{6,16} = 1$	gC gC^{-1}
$v_{10,16} = 1/12$	gH gC^{-1}
Chemical equilibria $HCO_3^- \leftrightarrow CO_3^{2-}$	
$v_{6,17} = -1$	gC gC^{-1}

$v_{7,17} = 1$	gC gC^{-1}
$v_{10,17} = 1/12$	gH gC^{-1}
Chemical equilibria $NH_4^+ \leftrightarrow NH_3$	
$v_{1,18} = -1$	gN gN^{-1}
$v_{2,18} = 1$	gN gN^{-1}
$v_{10,18} = 1/14$	gH gN^{-1}
Chemical equilibria $H^+ \leftrightarrow OH^-$	
$v_{10,19} = 1$	gH gH^{-1}
$v_{11,19} = 1$	gH gH^{-1}
Oxygen transfer to the atmosphere	
$v_{9,20} = 1$	–
Carbon dioxide transfer to the atmosphere	
$v_{5,21} = 1$	–
Ammonia transfer to the atmosphere	
$v_{2,22} = 1$	–

6

Validation of the BIO_ALGAE Model in aquaculture systems for the semi-continuous production of *Tetraselmis suecica*

This chapter is based on the article:

- V. Andreotti, A. Solimeno, S. Rossi, F. Marazzi, V. Mezzanotte, E. Ficara and J. García, Validation of the BIO_ALGAE Model in aquaculture system for the semi-continuous production of *Tetraselmis suecica*. (In preparation).

6.1 Introduction

Microalgae play a key role as nutrients in aquatic environments, guaranteeing the flow of matter and energy required for the maintenance of heterotrophic organisms. Nowadays, microalgae have been exploited in numerous commercial applications such as, pharmaceuticals, agriculture, pollution control, cosmetics and energy (Pulz and Gross, 2004; Spolaore et al., 2006; Rosenberg et al., 2008). At commercial scale, the use of microalgal technologies is often limited to the production of valuable products that can ensure the return on investment (Barsanti, 2018). The microalgal products (e.g., carotenoids, poly-unsaturated fatty acids, proteins, carbohydrates etc.) can be used to enhance the nutritional value of food and animal feed (Torzillo, 2004; Hu, 2014). In aquaculture, microalgae are widely employed for the direct consumption by molluscs and penaeid shrimps or indirectly, as food for zooplankton (crustaceans, rotifers, cladocerans, copepods) which in turn is fed to fish or prawn larvae (Muller-Fuega, 2007).

Tetraselmis sp. is a typical marine microalga widely used as live feed in aquaculture industry due to their high nutritional quality (Liao, 1983; Jefrey, 1994). *T. suecica* is employed as food for live preys of fish larvae, for bivalve mollusc larvae

and for penaeid shrimp larvae (Muller-Fuega, 2007). *T. suecica* was one of the first heterotrophic microalgae that appeared on the aquaculture market as food for rotifers (Day et al., 1991). This specie is an optimal source of long-chain Poly-Unsaturated Fatty Acids (PUFAs) and its lipid, carbohydrate and protein composition can be easily changed controlling the cultivation conditions and the nutrients concentrations (Fabregas et al., 1984; Cid et al., 1992; Otero and Fabregas, 1997; D'Souza and Kelly, 2000; Fabregas et al., 2001).

T. suecica has been recently used for the bioremediation of aquaculture wastewaters (Michels et al., 2014; Andreotti et al., 2017, Andreotti et al., 2019), and it was shown that the use of waste streams improves the economics of microalgal biomass production (Dickinson, 2013; Nayak, 2016). Aquaculture Wastewaters (AWs), can be considered as an alternative to synthetic growth media for marine microalgae: in a previous batch experiment (Andreotti et al., 2019), this species showed a daily biomass production of 65.7 ± 4.2 mg L^{-1} d^{-1}, with removal efficiencies of 98.0 ± 0.6% and 96.6 ± 1.2% for dissolved inorganic nitrogen (DIN) and dissolved inorganic phosphorus (DIP), respectively. A lipid content higher than 75% was also obtained, making *T. suecica* a valid candidate for

a reuse in aquaculture as feed in hatcheries of herbivorous and filter feeders (Alsull et al., 2012).

In the field of microalgal biotechnologies, modelling tools have been often adopted to forecast the bioremediation efficiency of the system, to describe microalgae growth and to estimate the biomass production at the given atmospheric conditions (Moreno-Grau et al. 1996; Reichert et al. 2011; Bitog et al. 2011). Most of microalgae-bacteria models (Reichert et al., 2001; Sah et al. 2011) do not combine biochemical processes with physical and environmental factors on biomass growth. The mechanistic BIO_ALGAE model was developed to simulate microalgae and bacteria dynamics in different wastewaters, including physical, chemical and biokinetic processes (Solimeno et al., 2015; 2017a). The application of mathematical models in aquaculture systems is still in an initial stage of the research, and only few studies have been reported so far (Lamprianidou et al., 2015; Kiridi and Ogunlela, 2016). The BIO_ALGAE Model was initially developed for urban wastewaters with high nutrient concentrations. Recently, the model was selected to simulate the biomass production of marine microalgae and its nutrients uptake in AW (Andreotti et al., 2019). The model was used with experimental data obtained growing *T. suecica* with annular photobioreactors in

batch conditions, using AW as N- and P-sources. The results of the modelling study confirmed its effectiveness in describing algal kinetics in these systems (Andreotti et al., 2019). With the help of the mathematical model, we want to create a modern technology to forecast the sustainable production of microalgae in an aquaculture system.

The aim of this study was to simulate the production and nutrients uptake of marine microalga *T. suecica* in lab-scale column photobioreactors (PBRs) using aquaculture wastewater. This system was fed under semi-continuous operation, in order to perform a validation of a mathematical model previously implemented to describe the growth of this microalga specie. The total lipid content, proteins, and carbohydrates were analyzed, and the effects of two different hydraulic retention time (HRT) were investigated to determine the optimal conditions for nutrient removal and biomass production. Respirometric tests were also performed to assess the oxygen uptake rates and oxygen production rates by the microalgae-bacteria consortia developed during the lab-scale cultivation.

6.2 Material and methods

6.2.1 Aquaculture wastewater and microalgae cultivation

Aquaculture wastewater (AW) was collected from the aquaculture rearing tank of a sea bream factory (*Sparus aurata*) located in Sant'Antioco, in the inner part of a lagoon in the south-east of Sardinia (Italy). The tank had 50.000 sea breams, with an average weight of 62.6 g and a biomass weight of 3.1 Kg.

The AW was stored at -18°C for further utilization, without any sterilization treatment.

T. suecica was obtained from the Agency for Agricultural Research in Sardinia (AGRIS) and sourced by the Culture Collection for Algae and Protozoa (CCAP: Oban, Scotland).

Before the experiment, the inoculum was grown in batch condition in Erlenmeyer flasks (2 L volume), under continuous aeration and artificial illumination (12:12 h light:dark photoperiod) with a Photosynthetically Active Radiation (PAR) of 118 ± 2.1 μmol m^{-2} s^{-1}. Natural seawater enriched with Guillard F/2 medium (Guillard et al., 1962; 1975), was used as growth medium.

6.2.2 Photobioreactors and experimental design

T. suecica was grown under laboratory conditions using two column PBRs (reactor A and B), made in Poly-Methyl-Methacrylate, with an operational volume of 3.5 L and a diameter of 10 cm. Two HRTs of 10 and 7 days were analyzed (RUN_1 and RUN_2 respectively), and each RUN had a duration of 3 HRT, then 30 days for RUN_1 and 21 days for RUN_2. Two replicates for each RUN (Reactor A and Reactor B) were performed (Figure 6.1).

The PBRs were fed under semi-continuous operations by automatically switching On/Off of a peristaltic pump (Watson Marlow 323). The pump was switched On for 1 h, in order to feed the influent at 12:00 am and 12:00 pm every day, with an average AW flow rate of 4.2 L^{-1} d^{-1} for RUN_1 and 6 L^{-1} d^{-1} for RUN_2.

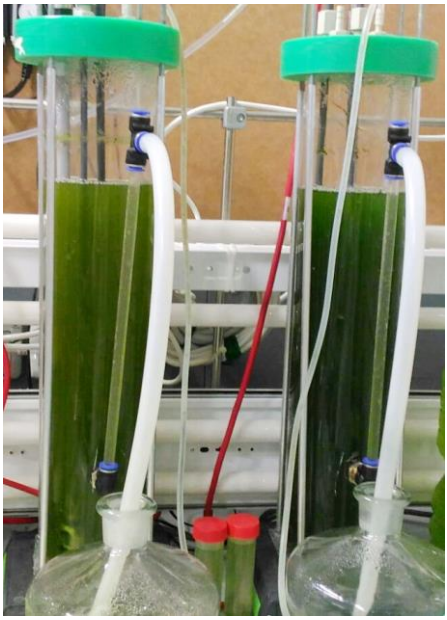

Figure 6.1: two column PBRs used for the experiment. This system is located in Politecnico di Milano, (Department of Civil and Environmental Engineering)

At the beginning of the experiment, $NaNO_3$ and K_2HPO_4 were supplemented to the wastewater to increase the initial concentrations of N and P to 20 mg N/L and 10 mg P/L, respectively.

The pH and the temperature values were measured by a multiparametric probe (Hamilton, Polilyte Plus, PHI Arc 325). The average ambient temperature during the experimental period was 27.5 ± 0.2 °C. pH was maintained at the value of

8.2 ± 0.5 by a pH-controlled injection of pure CO_2 gas. The PBRs were illuminated using 4 fluorescent lamps (OSRAM Fluora, 18W 77) and the Photosynthetically Active Radiation (PAR) was 122 ± 23 and 123 ± 29 μmol m^{-2} s^{-1}, measured at the surface of Reactor A and B, respectively. The light:dark cycle was set to 12:12. Non-sterilized air was pumped into the reactors at the constant rate of 1.8 L/min. The mixing of the culture was guaranteed by a magnetic mixer with a speed of 150 RPM.

Microalgae were inoculated to reach a dry weight concentration of 500 mg TSS L^{-1}, and after the inoculation, microalgae were acclimated for 3 days in batch conditions.

The initial characterization of AW for the two RUNs are reported in Table 6.1.

The wastewater had a soluble COD concentration of 165 mg L^{-1} for RUN_1 and 155 mg L^{-1} for RUN_2.

Table 6.1: Chemical characterization of the AW used to grow *T. suecica* after the addition of nitrate and phosphate

	NO_3^- -N (mg L^{-1})	NO_2^- -N (mg L^{-1})	NH_4+-N (mg L^{-1})	PO_4^{3-} - P (mg L^{-1})	Conductivity (mS cm^{-1})	pH
RUN_1	18.2	0.5	0.4	10.8	51.8	8.2
RUN_2	19.7	0.7	0.4	11.1	53.1	8.3

6.2.3 Analytical measurements

For the monitoring of the PBRs performance, samples were collected every 3 days at the same time of the day and analyzed for their biomass and nutrients concentrations.

Microalgal growth was monitored through dry weight measurements (as total suspended solids, TSS, and volatile suspended solids, VSS) (Saiu et al., 2016; APAT IRSA-CNR 2090). Determination of ammonia (NH_4^+-N), nitrite (NO_2^--N), nitrate (NO_3^--N), and phosphate (PO_4^{3-}-P), was carried out by an automatic chemical analyzer based on Loop Flow Analysis (μCHEM, Systea, Italy). The outlet samples were collected and analyzed every 3 days and at the same time of the day. The removal efficiencies of inorganic nitrogen and phosphorus were calculated by referring to the Dissolved Inorganic Nitrogen (DIN) and Dissolved Inorganic Phosphorus (DIP), where DIN was the sum of nitrite, nitrate and ammonia, while DIP corresponded to the total dissolved phosphate according to Michels et al. (2014).

COD was quantified using spectrophotometric test kits (Hach-Lange LCK 314) on 0.45-μm filtered samples.

6.2.4 BIO_ALGAE Model for aquaculture wastewater

The BIO_ALGAE model is based on Monod kinetics and was built combining the RWQM1 model (Reichert et al., 2001) with the modified ASM3 (Iacopozzi et al., 2007). This model is useful to forecast on biomass production and nutrients uptake by microalgae and it is applicable to waste stabilization ponds, high rate algal ponds, and photobioreactors. A detailed description of the model is presented in previous works (Solimeno et al., 2015, 2017a, 2017b). In brief, the BIO_ALGAE model considers 19 components (6 particulate and 13 dissolved) and integrates various processes that take place in microalgae systems, like biokinetic, chemical and physical processes. It includes photorespiration, pH dynamics, solar radiation, light attenuation and transfer of gases to the atmosphere, but the major innovative feature is the integration of inorganic carbon as a limiting nutrient for microalgae. The model was implemented in COMSOL MultiphysicsTM platform. The calibration of the BIO_ALGAE Model for aquaculture wastewater has been described in Andreotti et al. (2019).

In this work, BIO_ALGAE was implemented and validated using real datasets collected from the PBR operation. The initial values of the components used as input data in the model

are shown in Table 6.2.

The concentration of each particulate component (described in detail in Solimeno et al., 2017a) at the beginning of the experiment was not known, therefore, the initial ratios of X_{ALG}, X_S, X_I, X_H, X_{AOB}, and X_{NOB} concentrations were quantified from initial TSS value (Reactor A), based on previous simulation tests (Solimeno et al., 2017a; Andreotti et al., 2019).

Table 6.2: Values of the components of at the beginning of the experiment. All components are described in Solimeno et al. (2017a).

Component	Concentration RUN_1	Concentration RUN_2	Units
S_{NO3}	17.7	18.8	$gN\text{-}NO_3\ m^{-3}$
S_{NO2}	0.5	0.4	$gN\text{-}NO_2\ m^{-3}$
S_{NH3}	0.41	0.41	$gN\text{-}NH_3\ m^{-3}$
S_{NH4}	0.9	1.9	$gN\text{-}NH_4\ m^{-3}$
S_{PO4}	10.9	11.5	$gP\text{-}PO_4\ m^{-3}$
S_{CO2}	0.145	0.145	$gC\text{-}CO_2\ m^{-3}$
S_{CO3}	0.866	0.866	$gC\text{-}CO_3\ m^{-3}$
S_{HCO3}	35.00	35.00	$gC\text{-}HCO_3\ m^{-3}$
S_H	$1.78\ 10^{-9}$	$1.78\ 10^{-9}$	$gH\ m^{-3}$
S_{OH}	$4.69\ 10^{-6}$	$4.69\ 10^{-6}$	$gH\text{-}OH\ m^{-3}$
S_S	2	2	$gCOD\ m^{-3}$
S_{O2}	7.8	7.5	$gO_2\ m^{-3}$
S_I	8	8	$gCOD\ m^{-3}$
X_H	1	1	$gCOD\ m^{-3}$

X_I	10	10	gCOD m^{-3}
X_S	1	1	gCOD m^{-3}
X_{AOB}	0.05	0.05	gCOD m^{-3}
X_{NOB}	0.05	0.05	gCOD m^{-3}
X_{ALG}	484	400	gTSS m^{-3}

6.2.5 Respirometric characterization

Respirometric tests were conducted on the microalgal-bacterial suspension to define the microalgal and nitrifying activities, expressed as Oxygen Production Rate (OPR) and Oxygen Uptake Rate (OUR), respectively, and the light respiration rate for microalgae. In the protocol, the light regime is repeatedly switched on/off and selective inhibitors are dosed to suppress the activity of nitrifying bacteria. The protocol was previously applied to determine the activity of microalgal/bacterial consortia treating anaerobic effluents from municipal sludge dewatering (Rossi et al., 2018). Experiments were performed in an automated respirometer, in which the DO concentration and the pH were acquired every three seconds and maintained in the desired range by automatic injection of air/N_2 and by adding 0.1 M solutions of HCl and NaOH. During light phases, the light intensity was maintained at 113.1 ± 0.3 µE m^{-2} s^{-1} by means of dimmable fluorescent light bulbs (OSRAM Fluora, 2x18W). The light absorbance at 680 nm of the sample of the

algal suspension was 0.6 ± 0.1 during the respirometric tests. The tests were performed at the ambient temperature of 25.0 ± 1.1 °C. Results of the tests performed at the end of RUN_2 were then compared with the predictions obtained by the BIO_ALGAE model.

6.2.6 Biomass productivity and composition

Biomass volumetric productivity (P) was calculated according to equation 1 (Dalrymple, 2013):

$$P = (Q*C) / V \quad (1)$$

Where: Q is the daily flow rate (L/d), C the algae biomass concentration (g TSS L^{-1}) and V is the volume of the photobioreactor (L).

Lipids, proteins and carbohydrates were extracted from the biomass collected at the end of the two RUNs. For lipids and proteins analyses, microalgae were previously frozen and lyophilized at -80 °C. Lipids were then extracted from the biomass using a chloroform–methanol extraction solution (2:1 v/v), according to Folch et al. (1957). Biomass residues were removed by filtration using GF/C filter paper, and the solvent was subsequently removed by evaporation. The lipid content

was determined gravimetrically (Ryckebosch et al., 2012; Andreotti et al., 2019). The percent lipid content was referred to the biomass dry weight (Ryckebosch et al., 2012) and the lipid productivity was calculated according to Singh et al. (2015).

Proteins extraction was carried out following the method of Unterlander et al. (2017) for soluble proteins in microalgae. For the extraction, the protocol of intracellular APases from plant tissues and suspension cell cultures (Veljanovski et al., 2006; Tran et al., 2010a) was used.

Dry biomass was weighed, frozen in liquid N_2 and ground with sea sand, obtaining a powdered material. The extraction buffer (EB) was added before centrifuging (5 min at 11000 rpm, 4 °C). Protein concentrations were then determined using bovine γ-globulin as standard (Bradford, 1976). Protein productivity was determined according to the equation used by Guldhe et al. (2017).

Total carbohydrates were analyzed using the colorimetric method with phenol and sulphuric acid, according to Dubois et al. 1956. Concentrated phenol (90%) and H_2SO_4 (95%) solutions were added to the sample, then the absorbance at the wavelength of 490 nm was measured spectrophotometrically. A calibration curve was prepared using glucose as a standard

(Prajapati et al., 2013). Carbohydrates productivity was determined according to Guldhe et al. (2017).

6.2.7 Statistical Analysis

Differences in the nutrients removal efficiencies and biomass production between RUN_1 and RUN_2 were analyzed statistically. Normality and homogeneity of data were examined using Shapiro Wilk's W test. Statistical tests were performed using R Studio (Version 1.0.153 – © 2009-2017 R Studio, Inc.). One-way analysis of variance was used to determine whether differences in two HRTs were significant. An α level of 0.05 was considered. All data are expressed as average value ± Standard Error (SE).

The root mean square error (RMSE) was used to compare the model data with experimental data.

6.3 Results and discussion

6.3.1 Nutrient removal efficiencies and biomass productivity

Typically, aquaculture wastewater contains a range of all nutrients needed for microalgae growth and can therefore be used directly for microalgae cultivation (Andreotti et al., 2017;

Ansari et al., 2017). Although N:P ratios in marine fish farms effluents are not far from the Redfield ratio of 16:1 (Lefebvre et al., 2004), the nutrient concentrations are generally low, in order to comply with the discharge limits. Therefore, nitrate and phosphate salts were supplemented to reach the national AW discharge limits (20 mg NO_3-N/L and 10 mg PO_4-P/L), to sustain microalgal productivity and to avoid nutrient limitation. For RUN_1, the average of initial DIN for Reactor A and Reactor B were 18.5 ± 0.6 mg L^{-1}, whereas the initial DIP was 10.9 ± 0.1 mg L^{-1}. In RUN_2 the initial values were 20.4 ± 0.6 mg L^{-1} for DIN and 11.4 ± 0.2 mg L^{-1} for DIP.

Figures 6.2 and 6.3 show the consumption of DIN and DIP in the two RUNs. The effluent nutrient concentrations decreased remarkably during the first week of operations and later they stabilized to lower values. In RUN_1 the average DIN and DIP percentage removal efficiencies were 99.82 ± 0.03 % and 97.18 ± 0.01 %, respectively. Similar efficiencies were reached in RUN_2 where the values of DIN and DIP removal efficiencies were 98.98 ± 0.26 % and 92.25 ± 0.90 %, respectively. Between the two RUNs, no significant differences were highlighted ($p > 0.05$).

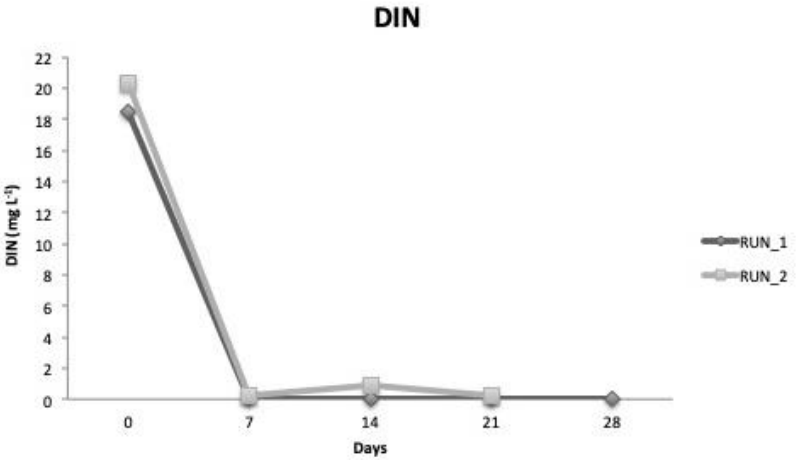

Figure 6.2: Effluent concentration of Dissolved Inorganic Nitrogen (DIN) for RUN_1 and RUN_2

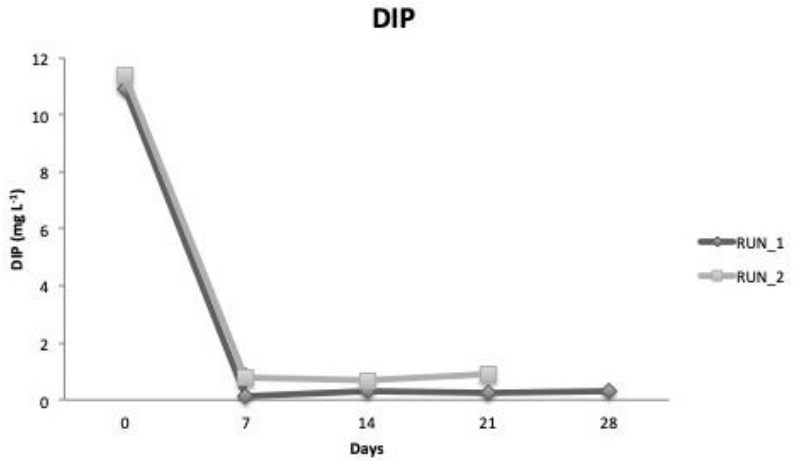

Figure 6.3: Effluent concentration of Dissolved Inorganic Phosphorus (DIP) for RUN_1 and RUN_2

These results are in agreement with our previous findings, where *T. suecica* cultivated in batch removed more than 90% of DIN and DIP in less than 7 days (Andreotti et al., 2017; 2019). Michels et al. (2014) obtained N and P removal efficiencies of 95.7% and a 99.7%, respectively, when extra phosphate was added to the wastewater. The addition of 10 mg P L^{-1} in the culture of *T. suecica*, with an initial biomass concentration of about 1 g L^{-1}, a P removal efficiency of 99.7 ± 0.1% was obtained (Michels et al., 2014). Patel et al. (2012) monitored the growth and P uptake of *T. suecica* in batch conditions, under different P loading rates. In conditions of 10 mg P L^{-1}, *T. suecica* removed the 79.4% of Total Phosphorous in 8 days (Patel et al., 2012). Also, it was demonstrated that P-uptake was species-specific and depended on the treatment process, the biomass productivity and the intracellular storage capacity (Ruiz et al., 2013b). Our results suggest that a high percentage of phosphorous is initially absorbed by the microalgae within a few hours. In fact, when analyzing the nutrient concentration in the morning, before the entrance of the new wastewater, a DIP concentration near zero could be observed. This meaning that the phosphorous added within the previous 12 h was already absorbed. This rapid removal of P

was mostly due to algal metabolic uptake, because the chemical precipitation was discouraged by controlling the pH value (Chinnasamy, 2010a).

T. suecica was proven to be a suitable species for the removal of N and P, both in batch and continuous systems. Thus, the cultivation of *T. suecica* in AW seems to be a feasible means for removing nutrients from these streams, as also suggested by the results of the feasibility and economic assessment of Heo et al. (2015).

Figure 6.4 shows the trial of TSS in the two RUNs. Microalgae exhibited a short exponential growth phase of about one week. In this initial phase, the biomass concentration increased until reaching its maximum value. The presence of this exponential phase shows that the microalga had excellent adaption characteristics to the AW used in this trial.

Biomass concentration in the bioreactor increased from around 500 mg TSS L^{-1}, reaching the maximum concentration of about 900 mg TSS L^{-1} after 6 days in RUN_1. In the RUN_2 the maximum concentration was reached after 9 days at 550 mg TSS L^{-1}.

During RUN_1, the biomass productivity was 66 ± 4 mg L^{-1} d^{-1} and 68 ± 4 mg L^{-1} d^{-1} for Reactor A and Reactor B,

respectively. RUN_2 showed lower biomass productivity, with 50 ± 2 mg L^{-1} d^{-1} for Reactor A and 48 ± 1 mg L^{-1} d^{-1} for Reactor B (Table 6.3).

In both RUN_1 and in RUN_2, no significant differences were observed between Reactor A and Reactor B in terms of TSS (Figure 6.4) and VSS. The productivity of TSS and VSS was significantly higher ($p < 0.05$) in RUN_1 than in RUN_2. The average ratio between VSS and TSS was 0.91 mg L^{-1} for RUN_1 and 0.92 mg L^{-1} for RUN_2.

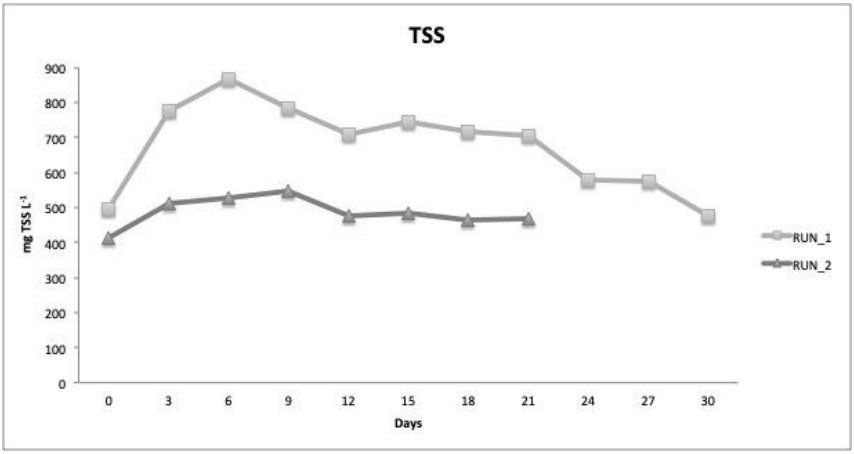

Figure 6.4: Biomass algal concentration measured as mg TSS L^{-1} for *T. suecica* in RUN_1 and RUN_2

These results agree with our previously work in batch conditions (Andreotti et al., 2019), where was observed a biomass production of 65.7 ± 4.2 mg L^{-1} d^{-1} for *T. suecica* in AW. Higher biomass productions were observed for *T. suecica* ranging from 460 to 520 mg L^{-1} d^{-1} when extra P was added in the influent AW (Michels et al., 2014). These differences could be explained by an accurate analysis of biomass and the effluents composition. In fact, it was demonstrated that the maximum biomass concentration is determined by the balance between the nutrient concentration in the wastewater and the elemental composition of the biomass (Wang and Lan, 2011). It is also important to know the nutritional history of microalgae (Voltolina et al., 1998) and to acclimate the biomass to the effluents before their cultivation, in order to increase biomass production (Borges et al., 2005). Moreover, further studies have demonstrated that microalgal production can be designed in such a way that the inorganic nutrients from the wastewater can be used, at constant nitrogen and phosphorous uptake and that waste of valuable nutrients is avoided (Chuntapa et al., 2003; Acién et al., 2012). Therefore, AW could substitute synthetic media, giving the opportunity of significantly reducing operational costs in microalgae cultivation. In addition to this, some authors showed that

microalgae cultivated in these wastewaters could be re-used in aquaculture and a further reduction of costs can be achieved (Malibari et al., 2018). In fact, in aquaculture farming industry, about 70% of the hatchery costs are due to microalgae culture (Borowitzka, 1997).

6.3.2 Simulation results with BIO_ALGAE Model

For the validation of the BIO_ALGAE Model, monitoring data obtained for Reactor A (biomass and nutrient concentrations) were used. Model data were compared to experimental data with the RMSE. Values near zero indicate that the model fits well with the experimental data (Bennett et al., 2013).

Figures 6.5 to 6.8 show the experimental and simulated nutrients concentrations in the PBRs. Simulated curves had a wavelike-trend, indicating a good accuracy of the model to reproduce the growth and nutrient dynamics during daytime and night.

The model was implemented with a notable simplification of bacteria process and they were considered at a low level (X_{AOB} and X_{NOB} of 0.05 gCOD m^{-3}). This value is significantly low in comparison to heterotrophic bacteria (X_H=1 gCOD m^{-3}). These results agreed with previous studies that demonstrated a very low amount of nitrifying's bacteria in comparison to other

bacteria groups (Krasnits et al., 2009; Samsó and García, 2013). Therefore, in this AW the nitrification processes by bacteria could be neglected.

As reported by many authors (Kaplan et al., 1986; Shi et al., 2000; Grobbelaar, 2004; Wilhelm et al., 2006), the preferential N-source for microalgae growth is NH_4^+ because it requires less energy than in the case of NO_3^-. As it is shown in figures 6.5, 6.6 and 6.7, the concentrations of NH_4^+ and NO_2^- were lower than NO_3^- although an high variability in their values occurred. A reduced ammonium availability in AWs was reported by the previous studies where the initial ammonium concentrations ranged from 0.37 mg L^{-1} to 0.48 mg L^{-1} (Michels et al., 2014; Velichkova et al., 2016). These values agree with the initial concentration applied in this study (0.66 mg L^{-1}). In aquaculture systems, ammonium from protein metabolism is one of the main nutrients available in the wastewater but it is quickly converted into nitrite and subsequently to nitrate by aerobic nitrifying biofilters. The reduced NH_4^+-availability has been recognized as responsible of a higher accumulation of alternative nitrogen sources such as nitrite and nitrate.

The simulated ammonium and nitrate concentrations matched the trend of the experimental measurements with a satisfactory

degree of accuracy (Figures 6.5 and 6.6). In fact, as shown in Table 6.3, the root mean square error of the simulation was low in relation to measured values ($RMSE_{NH4}$ = 0.28 mg NH_4^+ -N L^{-1} and $RMSE_{NO3}$ = 0.97 mg NO_3^- -N L^{-1} for RUN_1; $RMSE_{NH4}$= 0.85 NH_4^+ -N and $RMSE_{NO3}$ = 0.66 for RUN_2 mg NO_3^- -N L^{-1}). This is considered a good agreement between experimental data and simulations (Willmott et al., 1985; Bennet et al., 2013).

Higher concentration of NH_4^+ was obtained during the night and at the same time a very low concentration during the day. Also nitrite simulated curves represented well the trend of experimental data ($RMSE_{NO2}$ = 0.04 mg NO_2^- -N L^{-1} for RUN_1 and 0.34 mg NO_2^- -N L^{-1} for RUN_2) despite the biochemical instability and the low concentration of this nutrient, which was always below 0.5 mg NO_2^- -N L^{-1} from the second sampling day until the end of the experiment (Figure 6.7). Indeed, it was shown that nitrite is the most transient form of nitrogen (Taziki et al., 2015).

Table 6.3: Values of RMSE obtained in the model validation for RUN_1 and RUN_2.

RUN_1: n = 5 for nitrate, nitrite, phosphate and ammonium and n = 11 for total suspended solid concentrations. RUN_2: n = 4 for nitrate, nitrite, phosphate and ammonium and n = 8 for total suspended solid concentrations.

	$RMSE_{NO3}$	$RMSE_{NO2}$	$RMSE_{NH4}$	$RMSE_{PO4}$	$RMSE_{TSS}$
RUN_1	0.97	0.04	0.28	0.19	46.25
RUN_2	0.66	0.34	0.85	0.49	41.89

It is possible to conclude that, under ammonium limiting concentrations, nitrates are used as nitrogen source for microalgae growth. Moreover, the low concentration of nitrate in the culture medium could have limited the activity of microalgae. As can be seen (Figure 6.6), microalgae consumed nitrate quickly in the first days, and with the simulated curves, was possible to predict the behavior of microalgae in nitrate assimilation with a daily variation. The model was sensitive enough to show slight diurnal variations, although have not been detected with experimental samples.

Phosphorus represents a limiting nutrient in almost natural aquatic ecosystems (Correll, 1999) whereas it is typically available in wastewater streams (Larsdotter, 2006). For this reason, P is not usually considered in models simulating the growth of microalgae in wastewater. BIO_ALGAE includes P-limitation by means of a Monod function, like the other nutrients (i.e., C and N) (Solimeno et al., 2017). As it showed in figure 6.8, the simulated curves of PO_4-P accurately represented experimental data for RUN_1 ($RMSE_{PO4}$ = 0.19 mg PO_4-P L^{-1}) and RUN_2 ($RMSE_{PO4}$ = 0.49 mg PO_4-P L^{-1}).

The quantities of total phosphorus (TP) in aquaculture wastewater fluctuate between 2 and 50 mg L^{-1} (Lowrey et al., 2014; Gao et al., 2016), which are generated from animal feed. It was demonstrated that P concentrations higher than 6 mg L^{-1} could cause rapid microalgae blooms (Ahmad et al., 2013). The phosphorus in excess is assimilated in microalgae cells, like proteins and polyphosphates granules (Rawat et al., 2011), and these reserves can be used for the growth when the nutrients conditions in the environment are limited. P removal in wastewater depends not only by the microalgal uptake rates but also by environmental conditions such as pH and dissolved oxygen (DO). In these experiments, pH was controlled by CO_2 injection and DO were constant, so it was concluded that the phosphate in the AW was assimilated by microalgae cells.

In our model, TSS was calculated as the sum of all particulate components, including microalgae and bacterial biomass (Solimeno et al., 2015; 2017a). According to figure 6.9, during the first 6 days), the conditions were more favorable and microalgae faced an increase in their concentration (without lag phase) followed by a slight decrease after this period. Microscopic observations during the experimental phase highlighted an irrelevant concentration of bacteria in comparison to microalgae, which is usual in these closed

PBRs. Therefore, the nitrification processes by bacteria was considered negligible.

In figure 6.9, the simulated curve of the microalgal biomass production follows the same trend of the experimental data for both RUNs ($RMSE_{TSS}$ = 46.25 mg TSS L^{-1} for RUN_1 and $RMSE_{TSS}$ = 41.89 mg TSS L^{-1}). As shown by the simulated results, during the day photosynthesis predominates over respiration and the biomass concentration increases. On the contrary, during the night, photosynthesis did not occur and respiration prevailed.

The biomass production results confirmed the findings of a previous work (Andreotti et al., 2019), in which the BIO_ALGAE model described with good accuracy the growth of *T. suecica* in AW. The model, previously calibrated in batch conditions in AW, allow to make predictions of microalgae production in a semi-continuous system with other different environmental factors, such as temperature, CO_2 injection, and nutrients.

However, the results of the simulations indicated that the model was able to accurately reproduce microalgae growth and changes in nutrient concentrations. Otherwise it will require a further verification with other real datasets of aquaculture wastewater.

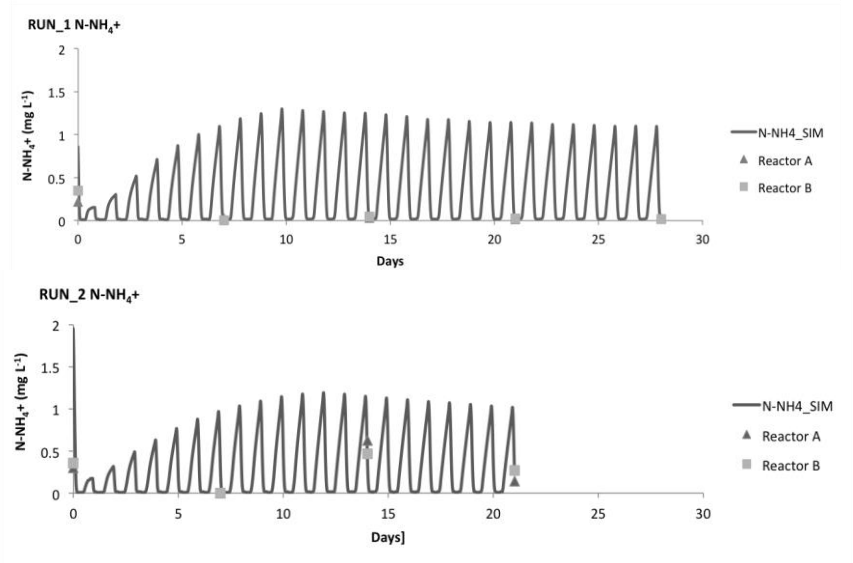

Figure 6.5: Experimental data (triangles and squares) and simulated curve (grey line) of ammonium in RUN_1 and RUN_2

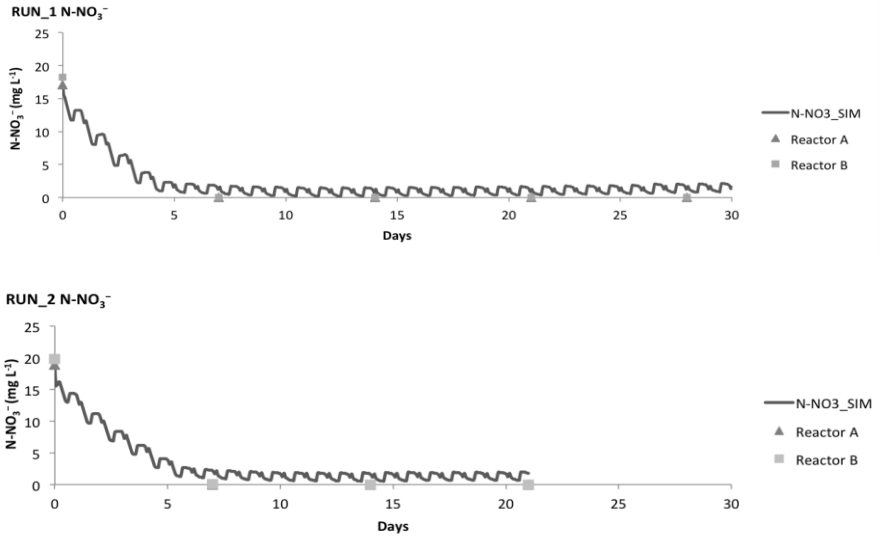

Figure 6.6: Experimental data (triangles and squares) and simulated curve (grey line) of nitrate in RUN_1 and RUN_2

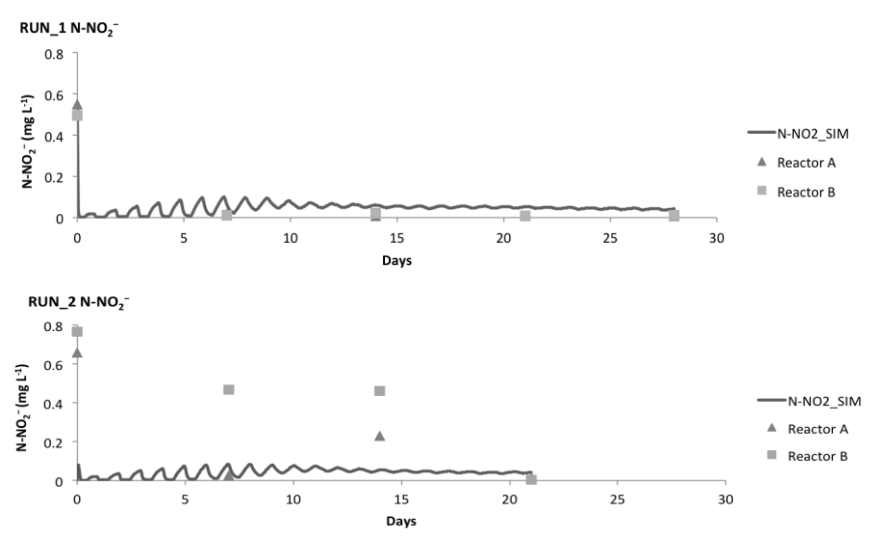

Figure 6.7: Experimental data (triangles and squares) and simulated curve (grey line) of nitrite in RUN_1 and RUN_2

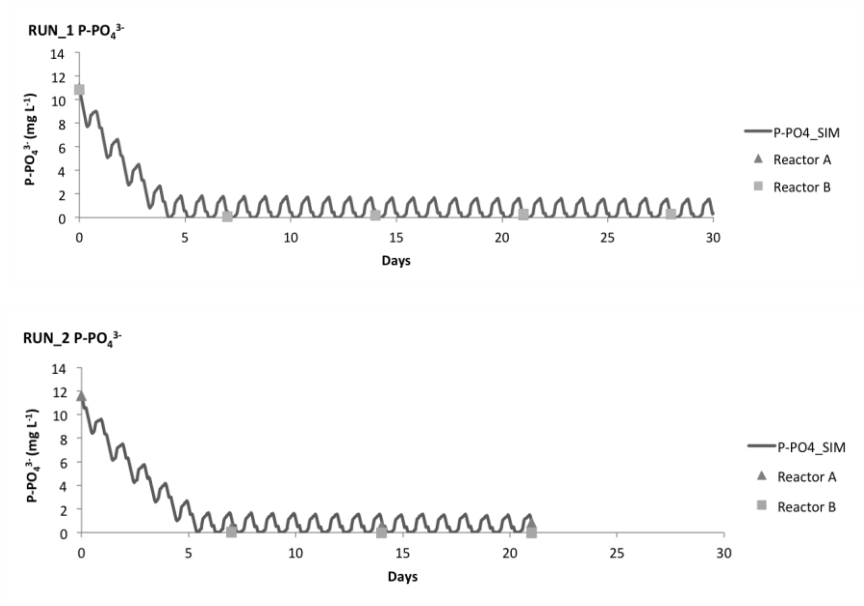

Figure 6.8: Experimental data (triangles and squares) and simulated curve (grey line) of phosphate in RUN_1 and RUN_2

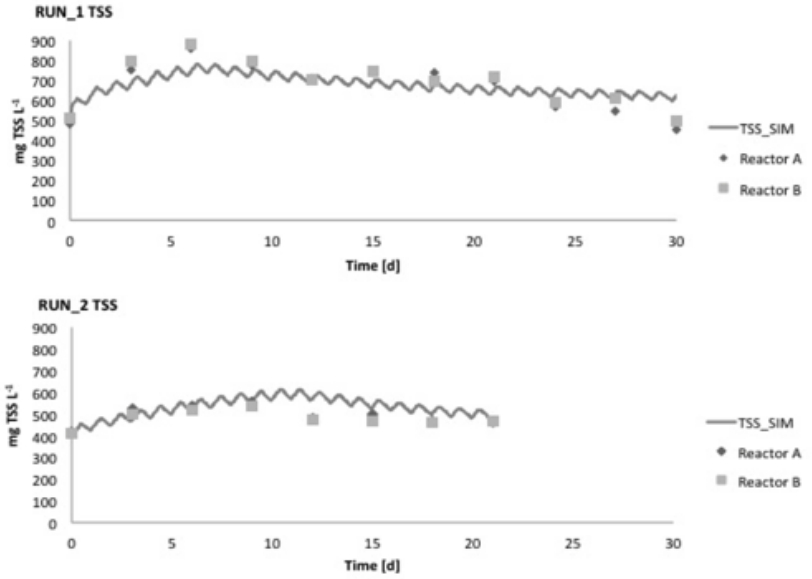

Figure 6.9: Experimental data (diamonds and squares) and simulated curve (grey line) of TSS in RUN_1 and RUN_2

6.3.3 Biochemical composition of biomass

The storage products are depleted for energy supply according to their energy content, from lipids to carbohydrates to proteins (Wilhelm et al., 2006). In this work carbohydrates and lipid contents were higher in RUN_1 than in RUN_2 as shown in the table 6.4. On the other hand, protein content was higher in RUN_2 (72.08 % for reactor A and 91.81 % for reactor B) than RUN_1 (37.27 % for reactor A and % for 50.20 % for reactor B) ($p < 0.05$).

Table 6.4: Biomass productivity (mg L^{-1} d^{-1}) of *T. suecica* and carbohydrats, lipids and protein content (%) and productivity (mg L^{-1} d^{-1}) for RUN_1 and RUN_2.

RUN_1

	Biomass productivity (mg L^{-1} d^{-1})	**Carbohydrate content (%)**	**Carbohydrate Productivity (mg L^{-1} d^{-1})**	**Lipids content (%)**	**Lipids Productivy (mg L^{-1} d^{-1})**	**Protein content (%)**	**Protein Productivity (mg L^{-1} d^{-1})**
Reactor A	66.55±3.95	9.13	6.07	20.01	13.32	37.27	24.81
Reactor B	68.53±3.69	10.62	7.28	25.06	17.17	50.20	34.40

RUN_2

	Biomass productivity (mg L^{-1} d^{-1})	**Carbohydrate Content (%)**	**Carbohydrate Productivity (mg L^{-1} d^{-1})**	**Lipids content (%)**	**Lipids Productivity (mg L^{-1} d^{-1})**	**Protein content (%)**	**Protein Productivity (mg L^{-1} d^{-1})**
Reactor A	49.26±1.60	4.75	2.34	16.54	8.15	72.08	35.51
Reactor B	48.02±1.39	5.42	2.60	17.71	8.51	91.81	44.09

The total lipid content differed from the previous experiment in batch condition where total lipid was 76% of the biomass of *T. suecica* and the lipid production rate was 49.8 mg L^{-1} d^{-1} (Andreotti et al., 2019). The lipid productivity observed in this work in semi-continuous mode was lower if compared to other similar studies. In general, microalgae have an inter- and intraspecific variability in lipid composition affected by culture conditions (Roessler, 1990; Hu et al., 2008).

Bondioli et al. (2012) showed that *T. suecica* cultivated in a semi-continuous mode in artificial seawater had a lipid content of 22% in the nitrogen-starved culture and a 27% under nitrogen and phosphorus starvation. In addition, N and P deprivation caused a production of proteins of the 10% and carbohydrates more than 50%. In this study, a similar lipid production was obtained by using AW, with 20.01 % and 25.06 % for Reactor A and Reactor B in RUN_1, while in RUN_2 16.54 % and 17.71 % for Reactor A and Reactor B respectively (Table 6.4). Kim et al. (2001) observed that *T. suecica* in synthetic medium have a composition of 44.9% of protein, 4.8% of lipid and 24.05% of carbohydrates. Similarly, in our work, the proteins content was higher than lipids and carbohydrate. This could depend on the addition of N and P in the AW in this experiment. The protein content of *T. suecica* is

influenced by the nutrient concentration (Fabregas et al., 1984). Indeed, it was shown that a nitrogen and phosphorus deprivation caused a dramatic decrease of proteins in *T. suecica*, which was compensated by an increase of carbohydrates (Bondioli et al., 2012). At the same time, the production of sugars and lipids are competing processes in the microalgae metabolism. Kim et al., (2016) highlighted that in *Tetraselmis* sp. if the accumulation of lipids increases, the carbohydrate levels, and biomass production decrease. The reason of this pattern is due to the shift cells from the carbon flux towards the synthesis of carbohydrates instead to the accumulation of lipids (Pereira et al., 2018). Malibari et al., (2018) showed that lipid accumulation in *Tetraselmis* sp. was higher when the microalga was cultivated in AW than in synthetic growth medium.

In general, the accumulation of these products is most probably a survival response at non-optimal conditions, which are stressful for the species. The variations in the nutrient composition can take place very quickly and may be useful to develop new cultivation systems, such as the techniques of biphasic culture for the accumulation of lipids (Mendoza et al., 2008).

6.3.4 Respirometric tests

Preliminary respirometric tests were carried out on the algal-bacterial suspension, to specifically assess the presence of nitrifying bacterial activity in the sample, expressed as Oxygen Production Rates (OPR, mg O_2 L^{-1} h^{-1}), according to the procedure adopted by Rossi et al. (2018) for microalgae-bacteria consortia cultivated in anaerobic digestion effluents. Process rates affecting DO dynamics (photosynthesis, respiration and decay of algae; respiration and activity of nitrifying and heterotrophic bacteria) were computed by the mathematical model and compared to respirometric outputs. In the respirometer, nutrients (ammonia and nitrite) were dosed at the beginning of the test, so in the model output the term representing nutrient limitation was removed.

The OPRs detected by the respirometric assay (photosynthetic O_2 production by microalgae, OPR_{MA}, dark O_2 consumption by microalgae and heterotrophic bacteria, OPR_{R*}, O_2 consumption by nitrifiers, OPR_{NIT}) are compared to the corresponding model outputs in Figure 6.10.

In general, the results obtained with the experimental setup showed a low variability, suggesting that respirometric procedures could be successfully applied to AW bioreactors. The production of oxygen by microalgae obtained during

respirometric tests reached the values of 10.7 and 10.2 mg O_2 L^{-1} h^{-1} in the two replicates, respectively. These values are similar with the model outputs and with the results obtained in other studies (Wang et al., 2015; Arbib et al., 2017; Rossi et al., 2018).

Regarding microalgal respiration during the dark phases and the contribution of heterotrophic bacteria (OPR_{R*}), results deriving from the respirometric protocol (2.8 and 3 mg O_2 L^{-1} h^{-1}) were not in agreement with the simulation, where O_2-consuming process resulted underestimated (0.2 mg O_2 L^{-1} h^{-1}). This was probably due to the stress condition connected to the lack of a light-acclimation phase in the test conditions. To support our hyphothesis, Ruiz-Martinez et al. (2016) reported respiration rates in the range of about 0.4-1 mg O_2 L^{-1} h^{-1}, during the first 50 h exposition to darkness. However, different values from literature data may be also attributed to differences in the respirometric setup and protocols, microalgae species and initial nutrient concentrations, having all these parameters a crucial importance in the definition of the microalgal OPR. It is important to stress that our respirometric protocol is not able to discriminate between the oxygen consumption due to microalgae and heterotrophic bacteria respiration, therefore another explanation for the different results obtained might

come from the underestimation of heterotrophic activity by the BIO_ALGAE model.

The experimental OURs obtained for nitrifying bacteria suggest that nitrifying bacteria were present, even if very low nitrification rates were detected during the two replicated experiments (0.7 and 0.9 mg O_2 L^{-1} h^{-1}, respectively). The simulated outputs showed no bacterial activity in any case and a possible explanation for this difference with the simulation results may be found in the initial choice of bacterial concentrations. In this sense, the design and execution of specific respirometric tests could be useful for the optimization and calibration of the initial conditions of the mathematical model.

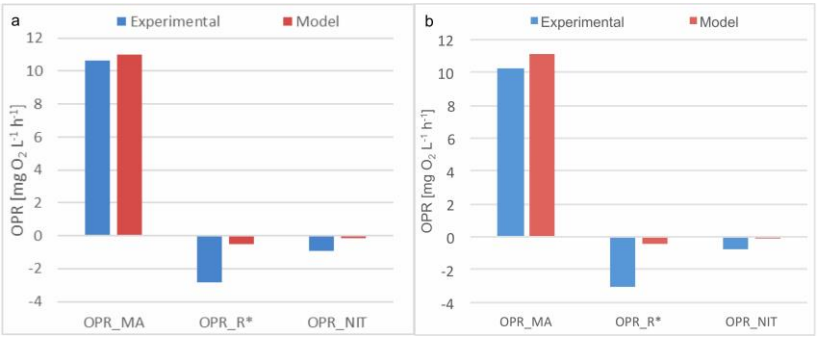

Figure 6.10: Measured and modelled photosynthetic oxygen production by microalgae (OPR_{MA}), dark oxygen consumption of microalgae and heterotrophic

bacteria (OPR_{R^*}), and oxygen production rate by nitrifiers (OPR_{NIT}). a) and b) represent the two replicates.

6.4 Conclusions

The results of this study showed that *T. suecica* was suitable for the growth in tubular photobioreactors in semi-continuous systems with aquaculture wastewater. This microalga has removal efficiency higher than 90% for DIN and DIP and supplementation of N and P enhanced the maximum biomass production in comparison to previous experiments (Andreotti et al., 2019). Moreover, biomass produced using aquaculture wastewater has shown high productivities for lipids, carbohydrate, and proteins, which could be used for applications in animals feed. The cultivation at controlled conditions in closed photobioreactors ensures constant high productivity and high quality of the feedstock.

BIO_ALGAE model was proven to be a useful tool to simulate microalgae production and the uptake of nutrients in aquaculture wastewater and could be applied to predict the performance under different operating conditions, for the design, optimization and control of the entire process. The respirometric tests showed that the protocol used could be

successfully used to estimate the photosynthetically-produced oxygen by microalgae, which is available for bacterial oxidation of ammonia and organic substrates. The presence of a minimal nitrifying activity and of an increased respiration rate, compared to the results obtained by the model, confirmed the respirometric tests, suggesting that a respirometric calibration should be performed to estimate uncertain parameters (kinetic parameters or biomass initial conditions), obtaining more realistic results from the mathematical model.

Aquaculture wastewater can be considered as a cost-effective and available medium for microalgae production. In addition to cost reduction, the use of the wastewater also contributes to the development of a more sustainable aquaculture production. This means reduce the nitrogen and phosphorus, which are the main end-products of fish effluents, and also reduce their negative effect both in the rearing water and in the environment.

7

Conclusions

7.1 General conclusions

In this chapter, a review of the main results and the final conclusions obtained during this research work are described.

Summarizing, this thesis project consists of two main research lines: the cultivation of marine microalgae in AW, and the calibration and validation of the mechanistic model BIO_ALGAE in aquaculture systems.

Microalgae are valuable resources to the environment that offer a solution to both environmental pollution with the wastewater treatment and with the biomass production.

It was demonstrated that aquaculture wastewater is a valid substitute to the synthetic medium for the microalgae growth and has the potential to reduce production costs of biomass and produce at the same time valuable biochemical products (Lam and Lee, 2012; Cai et al., 2013, Michels et al., 2014; Nasir et al., 2015; Andreotti et al., 2017).

A critical point to the advancement of the use of microalgae in aquaculture systems has been a lack of technological tools for the forecast to wastewater quality requirements and sustainable biomass production. Coupling a mathematical model with the microalgae processes in aquaculture could be represents an

implement to overcome the bottlenecks in aquaculture marine systems.

For this purpose, the broader aspect of this research was to enhance and adapt the integral mechanistic model BIO_ALGAE in order to simulate the microalgae growth and inorganic nutrients uptake in aquaculture wastewater.

Three marine microalgae species widely used in aquaculture systems as a live feed, have been cultivated in AW, but only two species were selected for the calibration of the BIO_ALGAE model in batch systems. After that, the model was validated with experimental data obtained in a semi-continuous system and the effect of different HRT were evaluated.

The biomass production as mg TSS algal biomass/L, the uptake of nutrients (N, P), the microalgal compositions in term of lipids, proteins, and carbohydrates were also investigated.

From the specific objectives of this thesis, it was possible to obtain the following conclusions:

- it was compared the efficiency of the cultivation of the microalgae, *T. suecica*, *I. galbana* and *D. tertiolecta* in grey mullet *M. cephalus*

wastewater using two column photobioreactors of 6 L.

This was done with the aim of reducing the impacts of traditional aquaculture on the environment and to promote responsible and sustainable integrated aquaculture systems.

D. tertiolecta and *T. suecica* has removed more than 90% of the dissolved inorganic nitrogen (DIN) and phosphorous (DIP) in the wastewater. This confirms that these species are suitable for use in bioremediation, as previously observed for *T. suecica* by other authors (Borges et al., 2005; Michels et al., 2014; Sirakov and Velichkova, 2014). This microalga obtained the highest biomass production of 86.14 ± 5 mg/L/d, while only 54.26 ± 5 mg/L/d for *D. tertiolecta*. Instead, *I. galbana*, has not proved adapt for growth in these AW probably due to the presence of ciliate *Paramecium* spp.

It was demonstrated that the genus *Tetraselmis* spp. (Austin et al., 1992; Arora et al., 2012) and *Dunaliella* spp. (Chang et al., 1993) have a large spectrum of antimicrobial activity, so it is

possible to use AW without sterilization process, reducing at the same time the production cost of the microalgae in aquaculture systems.

In this first part of this research, it was demonstrated that *T. suecica* and *D. tertiolecta* are valid candidate for the employement in integrated aquaculture systems, and can be cultivated in AW.

- *T. suecica* and *D. tertiolecta* were cultivated in AW using two column photobioreactors of 120 L. Despite the scale-up, these two species have confirmed suitable for the growth in *M. cephalus* wastewater with a biomass production of 65.71 ± 4.25 mg/L/d for *T. suecica* and 47.05 ± 1.57 mg/L/d for *D. tertiolecta*. At the same time it was demonstrated that the total lipid content was higher in *T. suecica* (75.8 ± 1.6%) than in *D. tertiolecta* (23.2 ± 2.0%).

  BIO_ALGAE model was calibrated with experimental data in order to predict algal growth in batch experiments as a function of nutrient availability. In conclusion, the results of

the calibration demonstrated that the model was able to reproduce the assimilation of nutrient and biomass production.

- This study confirmed that *T. suecica* is suitable for the growth in column photobioreactors in semi-continuous systems with aquaculture wastewater. The addition of N and P enhance biomass production in comparison to previous experiments in batch conditions. No significant differences were observed for two RUNs in terms of nutrients uptake. On the contrary, as regard, the productivity of TSS and VSS was higher in RUN_1 than in RUN_2.

  BIO_ALGAE model was validated with experimental data for the two RUNs, and was proven to be a useful tool to simulate nutrients uptake by *T. suecica* and biomass production in aquaculture wastewater for both HRTs. The Model allowed as to show slight diurnal variations, which could have not been detected with experimental samples.

  Results obtained with preliminary respirometric tests could be successfully applied to AW

bioreactors. This protocol could be used to estimate the photosynthetically-produced oxygen by microalgae, which is available for bacterial oxidation of ammonia and organic substrates. This test confirms the results obtained by the model, namely the presence of minimal nitrifying activity and of increased respiration rates. In this way, a respirometric calibration could be usefull to estimate uncertain parameters (kinetic parameters or biomass initial conditions), allowing to obtain even more realistic results from the mathematical model.

- As regards the biochemical composition of the biomass cultivated in aquaculture wastewater, we demonstrated that in batch conditions *T. suecica* had a higher production of lipids than *D. tertiolecta*. Nitrogen and phosphorus starvation could cause an increase of lipids content in the biomass, as highlighted comparing the results obtained in batch and in semi-continuous conditions with two differents wastewater. In fact, the addition of $NaNO_3$ and

K_2HPO_4 to the wastewater, in order to increase the initial concentrations of N and P to 20 mg N/L and 10 mg P/L, led to a decrease in the lipid percentage in the biomass of *T. suecica*. On the contrary, in these conditions, this microalga showed a proteins content higher than lipids and carbohydrate for both RUNs. Moreover, we confirmed what has been shown by other authors, namely that the production of sugars and lipids are competing processes in the microalgae metabolism. In fact, in this species if the accumulation of lipids increases, the carbohydrate levels, and biomass production decrease (Kim et al., 2016).

The cultivation of microalgae in these controlled conditions with closed photobioreactors could lead to constant production of high-quality products.

7.2 Future perspectives

The next straightforward step for the future is to test this microalga in an integrated aquaculture system. With this study, we demonstrated that it is possible to re-use the wastewater of the grey mullet (*M. cephalus*) and sea bream (*S. aurata*) to produce microalgae. Those results created the conditions to continue the experimental work with the possibility to test this biomass as feed in aquaculture systems. Applicability of the process should be verified for different species in an IMTA systems, for example for the larvae of sea urchins, mussels or fish larvae. Another important use could be the extraction of bio-compounds from microalgae cells (lipids, carbohydrates, proteins) as ingredients for feed formulation.

This integrated multi-trophic aquaculture approach is possible only after different microbiology analysis of biomass and tests of mortality for the larvae.

Therefore, it is appropriate to develop a better and economic quality control, which minimizes the chance of contamination and the variation in composition of the microalgal biomass produced.

The laws in force are not clear about the fate of algae grown on wastewaters, so it is important to check the feasibility of the

process.

Nowadays, the costs of energy, investment and production in these closed systems are still relatively high (Molina Grima et al., 2003; Norsker et al., 2011). Therefore, a cost-profit analysis is needed for the integration of these cultivation techniques into the commercial aquaculture system.

As regard the application of BIO_ALGAE model, improvements should be focused on biomass production, in order to improve the forecasts on the microalgae growth.

Further studies should be aimed to validate the prediction of growth and nutrient uptake in large-scale production system in terms of number of replicates and total biomass.

With these systems it will then be possible to apply this mathematical model for the development of a platform/APP that can be used by companies to predict microalgae production and the removal efficiency.

Another important aspect should be the technology transfer: to encourage aquaculture enterprises to use microalgae as a sustainable resource, testing new tools for the prediction and remote control of parameters in the microalgae cultivation.

8

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