UNIVERSIDADE FEDERAL DE ALAGOAS - UFAL
CENTRO DE TECNOLOGIA - CTEC
PROGRAMA DE PÓS-GRADUAÇÃO EM
ENGENHARIA QUIMICA

FRANCINE PIMENTEL DE ANDRADE

USE OF MICROALGA AND FILAMENTOUS FUNGI IN THE TREATMENT OF INDUSTRIAL EFFLUENTS (WHEY AND PETROLEUM PRODUCED WATER)

MACEIÓ
2023

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PhD thesis presented to the Post-graduate Program of Chemical Engineering at the Federal University of Alagoas as a partial requirement for the obtaining title of PhD in Chemical Engineering.

Supervisor: Prof. Dr. Carlos Eduardo de Farias Silva

MACEIÓ

2023

Catalogação na Fonte
Universidade Federal de Alagoas
Biblioteca Central
Divisão de Tratamento Técnico
Bibliotecário: Marcelino de Carvalho Freitas Neto – CRB-4 – 1767

A553u Andrade, Francine Pimentel de.
Use of microalga and filamentous fungi in the treatment of industrial effluents (whey and petroleum produced water) / Francine Pimentel de Andrade. – 2023.
206 f. il. : figs. ; tabs. color.

Orientador: Carlos Eduardo de Farias Silva.
Tese (Doutorado em Engenharia Química). Universidade Federal de Alagoas. Centro de Tecnologia. Programa de pós-Graduação em Engenharia Química. Maceió, 2023.

Inclui bibliografias.

1. Biodegradação ambiental. 2. *Tetradesmus obliquus*. 3. *Cunninghamella echinulata*. 4. Consórcio microbiano. I. Título.

CDU: 579

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Approved in: Maceió, May 9, 2023.

EXAMINATION BOARD

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ABSTRACT

This work aimed to obtain information regarding the use of microalgae and the cocultivation of microalgae with other microorganisms (bacteria, filamentous fungi and yeasts) in the treatment of wastewater, specifically the dairy wastewater, and petroleum produced water. For this, a literature review regarding open system cultivation revealed information about the ability to remove contaminants (mainly Chemical Oxygen Demand (COD), Total Nitrogen (TN) and Total Phosphorus (TP)) present in industrial effluents by microalgae and their consortia with other microbial groups. Microalgae-bacteria consortia are used mainly due to the great capacity to remove organic matter from bacteria and better assimilation of nitrogen and phosphorus by microalgae. On the other hand, lines of research using the consortium of microalgae with yeasts and filamentous fungi have gained attention, first because yeasts can accumulate a high lipid content, as well as microalgae, and can therefore be used, for example, in the production of biodiesel, and in the case of filamentous fungi to increase the capacity of effluent treatment containing molecules complex organic compounds (the metabolic power of fungi is superior to that of microalgae) and helps in the process of harvesting microalgal biomass. But it is recognized that the biotechnological applications for these two groups can be expanded, therefore, research is necessary. The type of bioreactor and mode of operation significantly influence the effluent treatment process using microalgae. Thus, a review of the operating modes showed that batch and fed-batch cultivations present lower risks of contamination, while the continuous and semi-continuous modes have higher productivity rates. Combining the use of effluents, reactors and mode of operation in conjoint with the nutritional and environmental requirements, can reach removal rates for COD, nitrogen and phosphorus greater than 90%. After, a kinetic model was developed to describe simultaneous removal of organic carbon, nitrogen and phosphorus, and microbial growth in wastewater treatment using microalgae, applying the n-order model for contaminant consumption, Monod (a limiting substrate) and Silva and Cerqueira (multiple substrates) for microbial growth. The results demonstrate the ability of these models to predict the treatment behavior of different industrial effluents. Additionally, an experimental procedure was performed, in which the cultivation of the microalgae Tetradesmus obliquus in the treatment of whey in open ponds was evaluated through experiments carried out at different organic loads (0.5-4% v/v) and light intensities (25-200 μmol m^{-2} s^{-1}). It was possible to efficiently remove COD, nitrogen and phosphorus with rates greater than 80% at higher light intensities (25-200 μmol m^{-2} s^{-1}) in all analysed organic loads, with emphasis on the lower ones (0.5 and 1% v/v) because they have a final concentration of contaminants in accordance with the legislation. In addition, a literature review on the biological treatment of oil produced water was carried out, and it was shown that there are few published works when compared to other effluents, mainly due to the characteristic of this wastewater, such as xenobiotic substances, high oil and grease content, and high salinity. In this sense, physical and chemical processes are more applied, although they are more effective when the treated water is used for reinjection in the wells, but due to the high salinity and the presence of toxic compounds, it is suggested the bioremediation of the effluent when the water produced it can be used for other noble purposes such as irrigation. Studies using bacteria, microalgae, filamentous fungi and yeasts were found, the first one being the most prominent, but showing the potential and need for further studies with other microbial groups. In this sense, experiments were conducted to treat produced water in a bubble column reactor using the co-culture of microalgae (*Tetradesmus obliquus*) and filamentous fungi (*Aspergillus niger, Penicillium oxalicum and Cunninghamella echinulata*). The species *C. echinulata* achieved higher TOG removal rates (90-95%), initially with 312-2500 mg L^{-1}, being more efficient than microalgae for this parameter. At different salinity concentrations (5-50 g L^{-1}), and T. obliquus remained alive up to 25 g L^{-1}, while the fungus *C. echinulata* grew at all salinity concentrations and removed TOG at rates between (80-95%). Finally, the co-culture of *T. obliquus-C. echinulata* removed up to 63.4 and 36.58% of nitrogen and phosphorus, with initial concentrations between 50-150 and 30 mg L^{-1}, respectively.

Keywords: Biorremediation, *Tetradesmus obliquus*, *Cunninghamella echinulata*, Microbial consortium

RESUMO

Este trabalho teve como objetivo obter informações sobre o uso de microalgas e o cocultivo de microalgas com outros microrganismos (bactérias, fungos filamentosos e leveduras) no tratamento de águas residuais, especificamente o efluente da indústria de laticínios, soro de leite, e petróleo, denominado água produzida. Para isso, uma revisão da literatura em relação ao cultivo em sistema aberto revelou informações sobre a capacidade de remover contaminantes (principalmente Demanda Química de Oxigênio (DQO), Nitrogênio Total (TN) e Fósforo Total (TP) presentes em efluentes industriais por microalgas e seus consórcios com outras grupos microbianos. Os consórcios microalga-bactéria são utilizados principalmente devido à grande capacidade de remover matéria orgânica das bactérias e melhor assimilação de nitrogênio e fósforo pelas microalgas. Por outro lado, linhas de pesquisa utilizando o consórcio de microalgas com leveduras e os fungos têm ganhado atenção, primeiro porque as leveduras podem acumular um alto teor lipídico, assim como as microalgas, podendo assim ser utilizadas, por exemplo, na produção de biodiesel, e no caso dos fungos filamentosos para aumentar a capacidade de efluentes tratamento com moléculas orgânicas complexas (o poder metabólico dos fungos é superior ao das microalgas) e ajuda no processo de colheita da biomassa microalgal. Mas é reconhecido que as aplicações biotecnológicas para esses dois grupos podem ser ampliadas, portanto, pesquisas são necessárias. O tipo de biorreator e modo de operação influenciam significativamente no processo de tratamento de efluentes usando microalgas. Assim, uma revisão sobre os modos de operação mostrou que cultivos em batelada e batelada alimentada apresentam menores riscos de contaminação, enquanto os modos contínuo e semicontínuo maiores taxas de produtividade. Combinando o uso de efluentes, reatores e modo de operação que atendam as necessidades nutricionais e ambientais para o cultivo de microalgas, taxas de remoção de DQO, nitrogênio e fósforo podem ser superiores a 90%. Foi desenvolvido um modelo cinético para descrever a remoção simultânea de carbono orgânico, nitrogênio e fósforo, e o crescimento microbiano no tratamento de águas residuais utilizando microalgas, aplicando o modelo de ordem *n* para o consumo de contaminantes, Monod (um substrato limitante) e Silva e Cerqueira (múltiplos substratos) para o crescimento microbiano, os resultados demonstraram a capacidade desses modelos em predizer o comportamento do tratamento de diferentes efluentes industriais. Também foi realizado procedimento experimental, no qual o cultivo da microalga *Tetradesmus* sp. no tratamento de soro de leite em lagoas abertas foi avaliado, esta espécie foi capaz de remover a DQO, NT e PT, representando uma alternativa de tratamento sustentável, além da produção de biomassa microalgal, com os experimentos realizados em baixas intensidade de luz (25-50 µmol m^{-2} s^{-1}) capaz de remover mais DQO enquanto intensidades mais altas removeram mais NT e PT (100-200 µmol m^{-2} s^{-1}). Além disso, uma revisão da literatura sobre o tratamento biológico da água produzida mostrou que existem poucos trabalhos quando comparado com outros efluentes, principalmente pela característica desse efluente por possuir diversas substâncias xenobióticas, alto teor de óleos e graxas e alta salinidade. Nesse sentido, os processos físicos e químicos são mais utilizados, embora sejam mais eficazes quando a água tratada é utilizada para reinjeção nos poços, mas devido à alta salinidade e à presença de compostos tóxicos, sugere-se a biorremediação do efluente quando o a água pode ser utilizada para outros fins nobres como a irrigação. Foram encontrados estudos utilizando bactérias, microalgas, fungos filamentosos e leveduras, sendo os dois primeiros os de maior destaque. Foram conduzidos experimentos de tratamento de água produzida em um reator de coluna de bolhas utilizando o co-cultivo de microalgas (*Tetradesmus obliquus*) e fungos filamentosos (*Aspergillus niger*, *Penicillium oxalicum* e *Cunninghamella echinulata*). A espécie *C. echinulata* alcançou maiores taxas de remoção de TOG (90-95%), inicialmente com 312-2500 mg L^{-1}. Em diferentes concentrações de salinidade (5-50 g L^{-1}), a *T. obliquus* manteve-se viva até 25 g L^{-1}, *C. echinulata* cresceu em todas as concentrações de salinidade e removeu TOG com taxas entre (80-95%). O co-cultivo *T. obliquus-C. echinulata* removeu até 63,4 e 36,58% de nitrogênio e fosforo, respectivamente.

Palavras-chaves: Biorremediação, *Tetradesmus obliquus*, *Cunninghamella echinulata*, Consórcio microbiano

FOREWORD

This research project was a collaboration between CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico do Brasil – Brazilian Council of Scientific and Technological Development) and Federal University of Alagoas through the Project number 440070/2019-8 – Formação de Mestres e Doutores em Petróleo, Gás e Biocombustíveis (Training of Masters and Doctors in Oil, Gas and Biofuels).

The research work was completely performed at Technology Center (CTEC) under the supervision of Prof. Carlos Eduardo de Farias Silva.

As a tangible result of the work completed during the Ph. D. school, some publications and chapters of books has been produced, as listed below.

Published and accepted papers in referred journals and books directly linked to the PhD Thesis

1. De Farias Silva CE, De Oliveira Cerqueira RB, De Lima Neto CF, **De Andrade FP,** De Oliveira Carvalho F, Tonholo J. (2020) Developing a kinetic model to describe wastewater treatment by microalgae based on simultaneous carbon, nitrogen and phosphorous removal. Journal Of Environmental Chemical Engineering 8. https://doi.org/10.1016/j.jece.2020.103792
2. **De Andrade FP**, De Farias Silva CE, Medeiros JA, Vieira RC, De Sá Filho MLF. (2022) Consortium between microalgae and other microbiological groups: a promising approach to emphasise the sustainability of open cultivation systems for wastewater treatment. Journal of Water Process Engineering 50. https://doi.org/10.1016/j.jwpe.2022.103211
3. De Andrade FP, Medeiros JA, De Farias Silva CE, De Sá Filho MLF. Chapter 7: Bioreactors and modes of operation for microalgae wastewater treatment. Ambati RR, De Carvalho JC, Soccol SR, Ravishankar GA. (2023) Algae mediated bioremediation: Treatment of Industrial wastewater streams and carbon dioxide capture. Wiley Publishers, USA.
4. De Andrade FP, De Farias Silva CE, Dos Santos J, Ribeiro TRM, Medeiros JA, Do Nascimento MAA, Santos GKS, Dos Santos Carneiro W, Almeida RMRG, De Oliveira AMM, Feijó FM, da Silva Costa MM, De Andrade Lima GS, Ribeiro Junior KAL,

Tonholo J. (2023) Dairy wastewater treatment by *Tetradesmus* sp. 15 in open system: molecular identification and the effect of light intensity and organic load in the process. Energy, Ecology and Environment.

Other published and accepted papers in referred journals and books

1. De Farias Silva CE, Vieira RC, Da Silva ICC, De Oliveira Cerqueira RB, Andrade NP, Claudino Da Silva F, **De Andrade FP**, De Souza Abud AK, Andreola K, Taranto OP. (2021) Combining fruit pulp and rice protein agglomerated with collagen to potencialize it as a functional food: particle characterization, pulp formulation and sensory analysis. Journal of Food Science and Technology 58: 4194-4204. https://doi.org/10.1007/s13197-020-04892-7
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4. Lopes EWR, Carneiro WS, Silva CEF, Vitorino AFRA, Filho LFS, **De Andrade FP**. (2023) A procedure to implement kinetic modelling of wastewater treatment by microalgae considering multiple contaminant removal. Energy, Ecology and Environment.

Papers accepted for publication in Referred Journals

1. Da Silva AHG, **De Andrade FP**, De Farias Silva CE, Medeiros JA, Santos GKS, Do Nascimento MAA, Tonholo J, Almeida RMRG. (2023) Biological Treatment of Petroleum Produced Water Ex-Situ Using Microorganisms: an overview, main developments and challenges. Energy, Ecology and Environment.

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°C - Degree Celsius
μE m^{-2} s^{-1} – MicroEinsteins per square meter per second
μg - Microgram
μm – Micrometer
μmol - Micromol
μmol m^{-2} s^{-1} - Micromol per square meter per second
μmol photons s^{-1} - Micromol photons per second
ABR - Anaerobic baffled reactor
Al- Aluminum
API - American Petroleum Institute
b - Barrel
B - Boron
Ba - Barium
BG-11 – Blue-Green medium
BOD - Biochemical Oxygen Demand
BTEX - Benzene, toluene, ethylbenzene and xylenes
BRT - Biomass retention time
C – Carbon
C/N - Carbon per Nitrogen
Ca- Calcium
$CaCl_2.2H_2O$ - Calcium chloride dihydrate
CAD - combined wastewater of anaerobic digestion
cells mL^{-1} - Cells per mililiter
cm - Centimeter
cm s^{-1} - Centimeter per second
CO_2 - Carbon dioxide
$COCl_2.6H_2O$ - Cobaltous chloride hexahydrate
COD - Chemical oxygen demand
$Co(NO_3)_2.6H_2O$ - Cobalt(II) nitrate hexahydrate
$CuSO_4.5H_2O$ - Copper Sulfate Pentahydrate
d – day
DIN - Dissolved inorganic nitrogen
DIP - Dissolved inorganic phosphorus
DO - Dissolved oxygen
EDTA - Ethylenediaminetetraacetic acid
FAME - Fatty acid methyl ester
Fe - Iron
$FeSO_4.7H_2O$ - Iron Sulfate II Oso heptahydrate
FG - Flue gas
g - Gram
g L^{-1} - Gram per liter
g L^{-1} day^{-1} - Gram per liter per day
GRG - Generalized Reduced Gradient
h – Hour
HCl - Hydrochloric acid
H_2S - Hydrogen sulphide
H_3BO_3 - Boric acid
HRAP - Hight-Rate Algal Ponds
HRP - High-rate ponds
HRT - Hydraulic retention time
ind. mL^{-1} - individuals per milliliter
K - Potassium
K_2HPO_4 - Dipotassium phosphate
KH_2PO_4 - Monopotassium phosphate
KNO_3 - Potassium nitrate
L - Liter
L $cycle^{-1}$ - Liter per cycle
L min^{-1} - Liter per minute
LSC - Luminescent solar concentrator
lx – Lumen per square meter
lux - Lumen per square meter
m – meter
m^3 – cubic meters
MBR - membrane bioreactor
mg – milligram
Mg -Magnesium
mg L^{-1} - Miligram per liter
mg L^{-1} d^{-1} - Miligram per liter per day
mg O_2 L^{-1}
$MgSO_4.7H_2O$ - Magnesium Sulfate Heptahydrate
min - minute
mL - Miligram
mL min^{-1} - Miligram per minute
Mn - Manganese
$MnCl_2.H_2O$ - Manganese(II) chloride monohydrate
$MnCl_2.4H_2O$ - Manganese(II) chloride tetrahydrate
MPBR - Membrane photobioreactor
MPE - Model's predictive error
N – Nitrogen
N/P - Nitrogen per phosphorus
Na - Sodium
NA - Not analysed

NaCl Sodium chloride
Na_2CO_3 - Sodium carbonate
Na_2MG EDTA - Ethylenediaminetetraacetic acid disodium magnesium salt
$Na_2MoO_4.2H_2O$ - Sodium Molybdate Dihydrate
$NaNO_3$ - Nitrate sodium
NH_3-N - Ammonium-Nitrogen
NH_4^+ - Ammonium
NH_4^+-N - Ammonium-Nitrogen
Ni - Nickel
nm - Nanometer
NO_3^- - Nitrate
NO_3^--N - Nitrate -Nitrogen
NORM - Naturally occurring radioactive materials
NPD - Naphthalene, phenanthrene, dibenzothiophene
NSO - Nitrogen, sulphur and oxygen
NTU - Nephelometric Turbidity Unit
O_2 - Oxygen Gas
O&G - oils and greases
OD - Optical density
OPEC- Organization of Petroleum Exporting Countries
P – Phosphorus
PAHs - Polyaromatic hydrocarbons
PBR – Photobioreactore
PDA - Potato Dextrose Agar
PHAs – Polihidroxiácidos
Phos - Phosphates
PO_4^{3-} - Phosphate
PO_4^{3-}-P - Phosphate-Phosphorus
PS - Primary settling
PW - Produced water
Ra - Radium
rpm - Revolutions per minute
s - Second
SCOD - Soluble chemical oxygen demand
SDZ - Sulfadiazine
spores L^{-1} - Spores per liter
TDS - Total dissolved solids
Ti- Titanium
TN - Total Nitrogen
TOC - Total organic carbon
TOG - Total oil and grease content
tonnes/ha/year - Tonnes per hectare per year
TP - Total Phosphorus
TPhos – Total Phosphates
TPH - Total Petroleum Hydrocarbons
TSS - Total soluble solids
U - Uranium
v/v - Volume per volume
vvm - Gas flow per volume of medium
W m^{-2} - Watt per square meter
w/w - weight per weight
Zn – Zinc
$ZnSO_4.7H_2O$ - Zinc sulfate heptahydrate

CONTENTS

Chapter 1: Introduction

Many industries need large volumes of potable water in their production processes, either as an integral part of their products or for cleaning after the process. Thus, high amounts of effluents are generated, in which the direct release of these wastewaters into the environment without treatment or with minimal treatment resulting in serious environmental problems. According to the UNESCO report (2021), about 80% of different wastewater produced in the world is inappropriately discharged in the environment.

Industrial effluents are different in their composition and volume because they are composed, for example, of oils and greases, nutrients, organic compounds and heavy metals. However, to determine an adequate treatment for these wastewaters, it is necessary to evaluate the physical, chemical and microbiological characteristics, such as, for example, COD, BOD, pH, alkalinity, total dissolved and suspended solids, nitrogen and phosphorus, metals, indicator microorganisms, toxicity, among others (SELVASEMBIAN et al., 2022).

Among the food industries, the dairy industry stands out for the volume of effluents generated, in which about 6 to 10 L of wastewater are produced per liter of processed milk, producing around 4 to 11 Mt of dairy effluents per year. These wastewaters can be classified according to their origin and composition, as process waters, cleaning wastewaters and sanitary wastewaters. They contain suspended and dissolved solids, soluble organics, lactose, nutrients, fats, sulfates, chlorides and high concentrations of BOD and COD (AHMAD et al., 2019).

Whey, the main by-product resulting from milk processing, is the aqueous portion released after the coagulation step in the conventional cheese production process (OLIVEIRA, BRAVO and TONIAL, 2012), approximately 9 L of whey are generated per kilo of cheese produced (CAPRIO et al., 2022). When considered residual effluent, if it is released in water bodies without proper treatment, it can cause serious damage to the aquatic environment, such as eutrophication, and when discarded in soil, it can change the physical and chemical characteristics, resulting in a decrease in agricultural productivity, due to its high levels of BOD (89-90,000 mg L^{-1}) and COD (40-48,000 mg L^{-1}), and large amounts of nitrogen and phosphorous (AHMAD et al., 2019).

In the oil industry, during the oil and gas extraction activity, large amounts of effluents are generated, called produced water (PW), which is the water extracted of the reservoir together with the oil and gas. PW can be generated from two processes, one occurs when the water is injected in the well to bring the oil to the surface mixing with it, and the other when the water is mixed with the oil during the extraction process in offshore wells (AL-GHOUTI et al., 2019).

The volume and characteristics of the PW vary with the geographic location and geology of the reservoir, oil composition and water injection history (AMMAR, KHADIM and MOHAMED, 2018). According to AL-KAABI et al. (2021), the PW volume increases with increasing age of the reservoir due to the decrease in oil and gas production, where the PW:extracted oil ratio can change from 3:1 to more than 10:1 (v/v) , and from 3:1 to 4:1 (v/v) for shale reservoirs.

The composition of produced water is complex, the main constituents are oils and greases (O&G), it may contain suspended solids, salinity, and microorganisms and organic acids, polycyclic aromatic hydrocarbons (PAHs), phenols, BTEX (benzene, toluene, ethylbenzene and xylene), high concentrations of BOD and COD, in addition to chemicals such as biocides and corrosion inhibitors added in the well's operational processes (AL-GHOUTI et al., 2019).

Due to its high polluting potential, the release of whey and PW into water bodies and/or soil can cause several damages to ecosystems, causing damage to aquatic life, agriculture and consequently the health of animals, including the human beings. Therefore, efficient treatments of these wastewaters are necessary before reuse or disposal in the environment to avoid severe environmental impacts, and biological oxidation (bioremediation) using microorganisms (Bacteria, yeasts, filamentous fungi and microalgae) are widely used.

Bioremediation has been considered an alternative to solve the problems related to the release of effluents mentioned above, however, there are several gaps in the metabolic abilities of microorganisms in contaminated environments that need to be investigated. This is a biological degradation mechanism that uses microorganisms to reduce/eliminate various organic and inorganic pollutants through biochemical processes of these beings (AKANSHA et al., 2020). In this thesis the focus will be on the use of microalgae and filamentous fungi in the bioremediation of whey and water produced from petroleum.

Microalgae are unicellular photoautotrophic beings that use light and CO_2 as an energy source and are able to reduce inorganic nutrients into biomass that can be used for the production of value-added bioproducts, such as biofuels and food additives (ALI et al., 2021). These microorganisms have been shown to be effective in wastewater treatment due to their good adaptation, high growth rate at low cost, nutrient removal, and productivity of lipid/carbohydrate-rich biomass (BENTAHAR et al., 2019). In turn, filamentous fungi, heterotrophic microorganisms, are an alternative for the remediation of several contaminants, due to their resistance to heavy metals, good adaptability to changes in pH and temperature and presence of metals, high growth rate and production of extracellular enzymes that act in the degradation of polluting compounds (AKHTAR and MANNAN, 2020).

Despite the advantages of using microalgae in the bioremediation of effluents, the cost of harvesting (use of energy and chemical agents) is one of the obstacles of this type of treatment, characteristics such as its small size, low cell density and negatively charged surfaces make the traditional biomass harvesting techniques difficult, such as centrifugation, flocculation, filtration, and others. An effective alternative for this process using microalgal biomass is the co-cultivation of microalgae and filamentous fungi, in which the formation of sedimentable pellets occurs, facilitating the recovery process by simple operations, in addition there is no need to use chemical agents (PEI, REN and LIU, 2021). Furthermore, the metabolic and gas exchanges in this mutualistic relationship can make these microorganisms less dependent on external sources of nutrients, in which the fungus uses the oxygen provided by the microalgae through photosynthesis, and the microalgae have access to nutrients due to the extracellular enzymatic actions of the fungi. that convert macromolecules into soluble nutrients, thus ensuring the consumption of organic matter and consequently the decrease in COD, in addition the fungus returns CO_2 to the medium leaving it available for microalgae (ZHAO et al., 2019; CHU et al., 2021; LENG et al., 2021).

In recent years, several studies have shown efficiency in the treatment of different types of wastewater using the symbiosis between microalgae and filamentous fungi, namely: municipal wastewater (Zhou et al., 2012) swine effluent (Wrede et al., 2014; Guo et al., 2013), arsenic-contaminated wastewater (LI, ZHANG and YANG, 2019), pharmaceutical groups (BODIN et al., 2016), gold-containing wastewater (Shen and Chirwa, 2020), secondary effluents generated by seafood processing industries (SRINUANPAN et al., 2018), molasses wastewater (YANG, LI and WANG, 2019) and cassava wastewater (PADRI et al., 2022).

In this sense, the use of microalgae or microalgae/filamentous fungi was sought in the treatment of whey and PW to remove the content of oils and greases, COD, nitrogen and phosphorus from these effluents with consequent formation of microbial biomass by testing different configurations. and operations of open pound and bubble column bioreactors. Specifically, the objectives were:

• Carry out reviews on the use of microalgae and/or in association with other microorganisms (bacteria, filamentous fungi and yeasts) in the treatment of different wastewaters (nutritional, environmental and operational conditions);

• Develop a kinetic model to describe the simultaneous consumption of substrates and microbial growth using microalgae for wastewater treatment;

• Evaluate the treatment of whey using the microalgae *Tetradesmus obliquus*, in an open system with different concentrations of contaminants and light intensity;

• Investigate the use of the microalgae Tetradesmus obliquus and co-culture with filamentous fungi in the remediation of oil produced water in a bubble column reactor with different concentrations of total oil hydrocarbons, salinity and nitrogen.

For this purpose, the thesis was organized into six chapters. In **Chapter 1**, a bibliographic review was carried out to collect and discuss the available information on the use of microalgae and other microorganisms in the treatment of effluents. Nutritional, environmental and operational conditions for microalgae cultivation were also discussed. In **Chapter 2**, types of bioreactors and mode of operation for wastewater remediation using microalgae were discussed. The behaviour of microalgal biomass production and substrate removal in different operation modes were shown.

Additionally, the **Chapter 3**, shows the development of the Silva and Cerqueira model to describe the simultaneous removal of multiple substrates in the microalgal growth kinetics. On the other hand, in **Chapter 4**, the batch culture of *Tetradesmus obliquus* in an open bioreactor was evaluated in the remediation of whey, dairy effluent, with different concentrations of contaminants and light intensity.

Finally, in **Chapter 5**, a review on ex-situ biological treatment of produced water, effluent from the oil industry, was carried out to identify the available information and the main bottlenecks to adequately develop this process, which were applied in **Chapter 6**, where the treatment of synthetic produced water using filamentous fungi and in coculture with the microalgae *T. obliquus* was studied. The adaptability of microorganisms to limiting conditions such as salinity and concentration of contaminants was analysed.

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Chapter 2: Consortium between microalgae and other microbiological groups: a promising approach to emphasise the sustainability of open cultivation systems for wastewater treatment

Abstract

The application of microalgae in bioremediation of urban and industrial effluents has shown excellent results, both in terms of their ability to remove pollutants, mainly nitrogen and phosphorus and in the way that this system operates, which does not require large structures and is easy to handle. Microalgae are microscopic beings that have a high photosynthetic efficiency and are able to adapt to the most diverse types of effluents and environmental conditions due to mixotrophic characteristics. The treatment performed by these microorganisms can be done in a variety of systems, whether open, closed or in a hybrid model, so, the choice is directly correlated with the desirable characteristics, in terms of the design and configuration of the system. The open system has several economic and operational advantages and for this reason it has been widely used for effluent treatment. Furthermore, it is important to highlight that there is a mechanism of proto-cooperation between microalgae and contaminating microorganisms of the open system, mainly with bacteria, filamentous fungi and yeasts. In this work, the mechanisms, advantages and bottlenecks of the use of symbiotic systems in the bioremediation of urban and industrial effluents will be discussed, mainly considering the feasibility and stability of open systems.

Keywords: Microalga; Yeast; Bacteria; Fungi; Biological treatment; Bioremediation.

This article was published in Journal of Water Process Engineering (De Andrade FP, DE Farias Silva CE, Medeiros JA, Vieira RC, De Sá Filho MLF. (2022) Consortium between microalgae and other microbiological groups: a promising approach to emphasise the sustainability of open cultivation systems for wastewater treatment. Journal of Water Process Engineering, v. 50. https://doi.org/10.1016/j.jwpe.2022.103211).

2.1 Introduction

The demand for potable water and the issues concerning the contamination of water bodies are two of the greatest global challenges of the century. Several processes have been proposed for treating domestic, industrial and agro-industrial wastewaters such as bioremediation, which uses either a single or a consortium of microorganisms - bacteria, microalgae, fungi and protozoa (aeration tanks, anaerobic ponds, aerobic and anaerobic bioreactors, activated sludge, biological filters and biological nutrient removal).

Microalgae need a sufficient (organic or inorganic) carbon source, other nutrients, and light for photosynthesis. Nonetheless, they can adjust their internal structure to ensure a more efficient use of the available resources in the environment (MATA, MARTINS and CAETANO, 2010; ROSERO-CHASAY et al., 2021). The advantages of exploring these photosynthetic organisms for industrial applications, mainly in the environmental sector, are relevant to ensure a more sustainable future. These microorganisms can promote the carbon cycle and consequently its environmental renewal, which is vital because carbon is the most prevalent component in this biomass (representing approximately 50% of its dry weight) (CHO et al., 2013; ZHENG et al., 2018).

In the last decades, researchers have focused on the use of microalgae and their consortia with other microorganisms (other algae, bacteria, yeast and fungi) in wastewater treatment as an alternative and low-cost process for producing and recovery of microalgal biomass.

The use of polyculture for nutrient removal can be very advantageous, as the combination of microorganisms with different metabolic activities and diverse adaptive capacity can result in higher rates of nutrient absorption and biomass production (GONÇALVES et al., 2017). This occurs because in most cases, the formation of a consortium benefits both species involved, mainly as a result of the gas exchange, in which the microalgae through photosynthesis releases O_2 that helps the growth of other microorganisms (bacteria and fungi, for example), which by respiration can release CO_2 favouring the development of the microalgae, or by the action of extracellular enzymes sometimes released by these microorganisms that act in the breakdown of macromolecules facilitating the absorption of certain nutrients by the microalgae (ROSERO-CHASAY et al., 2021; LENG et al., 2021). However, there are still gaps that need to be further assessed to ensure a full understanding of metabolic interactions between these microorganisms, as well as regarding the best cultivation systems in terms of the most suitable physical and environmental operational conditions to treat the wastewater.

Microalgae cultivation in closed bioreactors is demonstrated to be the most efficient biomass production system, given the possibility of a better control of the operational/nutritional/environmental parameters such as pH, temperature, mixing rate, light intensity, CO_2 and nutrient concentration, among others. In addition, closed bioreactors facilitate aseptic conditions, although installation and maintenance costs are high. A more economical and sustainable alternative include open cultivation systems, which is enable to use natural light, ensuring easier control with lower costs and total aseptic conditions are not required. Nevertheless, the main drawback of open cultivation systems is the contamination. Contamination can present beneficial results when the synergy between microorganisms promotes biomass growth and contaminant removal. However, the negative effect of contamination includes the considerable decrease of biomass production and consequently dead of desired species reducing the efficiency of the process in remove contaminants (MILANO et al., 2016; LI et al., 2019; VEERABADHRAN et al., 2021).

This review provides an overview of current sustainable phycoremediation technologies for wastewater treatment, focused on open cultivation systems for value-added biomass production. In this regard, this work carries out a bibliographic prospection of articles and patents in order to gather the main sources of information to support the understanding of the subject proposed. In this sense, it reports on the nutritional needs of microalgae, as well as the advantages and challenges of the processes using symbiotic relationships between microalgae and other microorganisms. Finally, it presents the challenges and needs for intensifying research on this promising strategy.

2.2 Types of systems for wastewater treatment by microalgae

Microalgae-based wastewater treatment has been the focus of several studies which seek to improve the efficiency of the process and demonstrate its viability in operational and economic terms. Nevertheless, in practice, its application is still limited, thus treatment systems used in this type of bioremediation are usually found at a laboratory or pilot scale.

According to Li et al. (2010), overall, treatment systems can be divided in groups according to their design and configuration. Therefore, they can be presented as traditional open systems, enclosed photobioreactors (PBRs) or hybrid systems. It is important to point out that the choice of reactor used in microalgae-based wastewater treatment depends on an initial data assessment, namely wastewater nutrient profile, parameter adjustment requirements, volumetric concentration, likely microalgae species employed, biomass development capacity, among others. These are important specifications, as they directly influence the reactor design and the type of operation, being crucial to ensure stability (YIN et al., 2020). The main bioreactor configurations are showed in **Figure 2.1**.

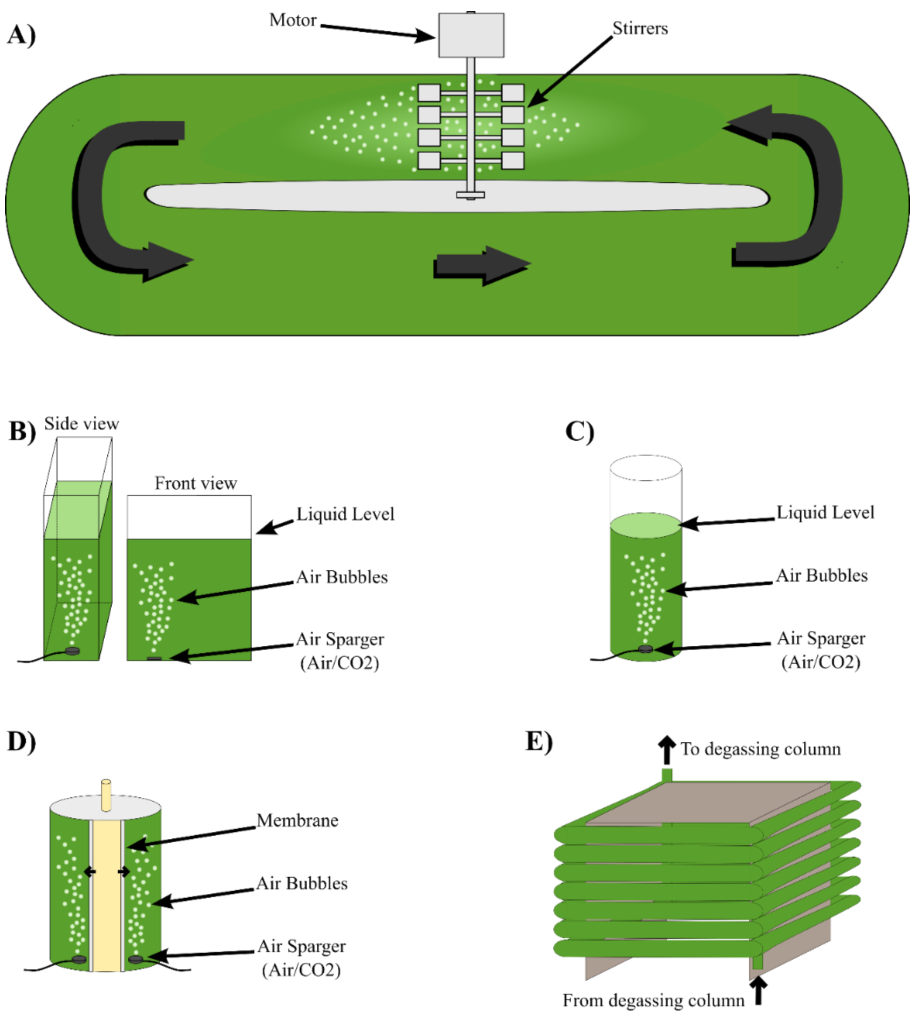

Figure 2.1 - Simplified schematics of different types of systems for microalgae cultivation. A) Open raceway pond; B) Flat panel PBR; C) Bubble column PBR; D) Membrane PBR; E) Bio-coil tubular PBR.

Open microalgae cultivation systems are widely used for industrial purposes, with studies proving the efficiency of this method for wastewater treatment applications. Open ponds, raceway ponds and tanks are common examples of this type of treatment system. For instance, raceway ponds (high-rate algal ponds) are an evolution of the open ponds, usually installed with paddle wheels for mixing to ensure the circulation of microalgae, preventing precipitation and improving the photosynthetic rate. In terms of operating conditions, open systems are more easily operated, with a low energy consumption and lower associated costs. However, issues related to poor lighting distribution and the needed for larger areas in the treatment plants installation, and if aeration is used, high CO_2 is lost to the environment (especially when used in high concentrations mixed with atmospheric air) (LI et al., 2010; ACIÉN et al., 2012; YIN et al., 2020).

It is worth mentioning that most effluents are unsterilised, thus with a natural microbial load. However, the treatment of large volumes of wastewater makes unfeasible the sterilisation of the wastewater if the purpose is contaminant removal, only. On the other hand, if high-value products can be obtained, it can be an option considering the economic return involved. Therefore, open systems can be a more direct option and easily adaptable. In addition, the assessment of the microbial load (either from microalgae or other microbial groups) is essential to maintain a symbiotic relation (LI et al., 2010; ACIÉN et al., 2012; YIN et al., 2020; MOHSENPOUR et al., 2021).

Closed systems, more specifically photobioreactors (PBR), have several designs, with their horizontal tubular design being more commonly used at an industrial scale and for microalgae cultivation. This configuration has many advantages such as greater control of parameters (pH, temperature, CO_2, O_2 and other nutrients), minimising contamination by other microorganisms, as well as ensuring high biomass productivity rates. The use of this type of reactor is limited due to high operating and capital costs, with its application being limited to processes with high-value products. Nevertheless, its use for wastewater treatment is seen as advantageous given the high-quality wastewater product (purity). In addition, the use of microalgae in this reactor type can lead to drawbacks such as the formation of algae biofilm on the reactor surface, thus limiting light penetration into the culture, and possibility of cell structure damage as a result of high shear stresses resulted by the operational characteristic of this type of bioreactor (LI et al., 2010; YIN et al., 2020).

However, some hybrid systems are being developed aimed at reducing the limitations associated to standard configurations. For instance, membrane photobioreactors are capable of promoting more efficient treatment and higher biomass production with much lower operating costs when compared to closed photobioreactors

(PBR). Thus, the growth experienced by the membrane sector can lead to even lower costs, with this reactor type being a promising alternative to traditional models (LI et al., 2010; BILAD et al., 2014; MARBELIA et al., 2014; LUO et al., 2019; YIN et al., 2020).

Microalgae biofilm consists of a symbiotic association between microalgae and other microorganisms, such as bacteria and fungi. This type of biofilm is usually formed on solid surfaces or in the interior of materials. Its application in remediation processes has various advantages, such as a greater production and easiness of microalgal biomass recovery, making the process less costly. Some researchers have analysed the relationship between the cultivation system (constantly submerged, intermittently submerged and perfused) and attachment growth system. Studies have shown that the use of materials such as nylon and stainless steel in microalgae attachment generate 2.8 times greater biomass production than the open pond systems. Nevertheless, it is worth pointing out that the use of this wastewater treatment method depends on the wastewater's nutrient composition and on the surface area available for developing the biofilm (LI et al., 2010; BERNER, HEIMANN and SHEEHAN, 2015; MANTZOROU and VERVERIDIS, 2019). According to Palma et al. (2017), the use of Chlorella-like microalgae biofilm in the treatment of mine tailing waters, containing heavy metals, promoted a considerable removal of pollutants, allowing such system to be used for large-scale remediation.

2.3 Desired wastewater properties: microalgal nutrient requirements

Microalgae are microorganisms that can be found in various water environments, ranging from freshwater to marine environments or even wastewaters. Similar to plants, microalgae are photosynthetic microorganisms capable of growing in growth media with reasonable nutrient availability, namely carbon (C), either in its organic or inorganic form, nitrogen (N) (in its organic or inorganic form, namely ammonia, nitrite and nitrate), phosphorus (P) and other elements that are essential to certain species (micronutrients and trace elements). Its use in bioremediation processes has been increasingly considered given their low costs, easy operation, as robust environments and complex cultivation systems are not required, besides not making use of any fertilizer type, as well the high growth rate and the possibility to use the biomass formed during the treatment process for noble purposes (LI et al., 2010; MANIRAFASHA et al., 2016; TANG et al., 2020).

In short, for the microalgae development, an adequate amount of light, water, nitrogen, carbon, phosphorus and other metal ions should be available in the medium (wastewater). It is important to stress the relevant presence of metal ions, as they favour osmoregulation and osmoadaption, thus contributing to the photosynthetic metabolic pathway. Regarding light availability, studies show that both the intensity and the light/dark relationship (photoperiod) can influence the microalgal development. For instance, assays developed under artificial light point out that continuous lighting (24 h) promotes a much greater reduction in total phosphorus when compared with solar radiation, which provides approximately 12 hours of light and 12 hours of darkness (depending on the location and season of the year). On the other hand, in terms of light intensity, some analyses show that the increase in this parameter can reduce microalgae phosphorus absorption (it is necessary to evaluate the biological characteristics of the strains), though increasing the uptake of other compounds such as COD (chemical oxygen demand) and nitrogen (SCHENK et al., 2008; MORONE et al., 2019).

Another factor of great importance concerns the C/N and N/P ratios, not only in terms of nutrient absorption, but also regarding the requirement of competitivity between the species found in the medium. In urban wastewaters, these ratios are often lower than what is recommended to ensure rapid microalgae growth. Studies which sought to understand the C/N ratio and establish a confidence interval for this parameter to ensure efficient wastewater treatment have demonstrated that C/N ratios between 5 and 10 lead to greater COD reduction in the medium, while higher ratios (close to 20) result in a reduction in microalgae biomass development. This reduction implies a low pollutant removal rate, probably inhibiting microalgae activity due to excess nutrients. In addition, it may lead to metabolic consequences, such as the inability of proportionally absorbing carbon and nitrogen, with the latter being the main structural component together with carbon; or the formation of excess toxic metabolites as acids in the metabolization of organic matter (SCHENK et al., 2008; LI et al., 2010).

Regarding the nitrogen/phosphorus ratio, data found in the literature show that both elements are crucial factors for microalgae development. According to Li et al. (2010), this ratio can vary between 6.8 and 10 to ensure good biomass development and efficient wastewater treatment. Moreover, it is worth pointing out that the possible adjustments to nutrient conditions in the cultivation medium can favour biomass development and optimise wastewater treatment (LI et al., 2010; MORONE et al., 2019).

Leong and Chang (2020) analysed the use of microalgae in the bioremediation of heavy metals usually found in industrial effluents. The study states that this type of microorganism presents various advantages, ranging from its ability to adapt to the medium, resistance to extreme environmental conditions, besides its high photosynthetic efficiency. The research showed that microalgae are capable of absorbing heavy metals such as boron, cobalt, iron and molybdenum, as trace elements for enzymatic process of the cell metabolism. Nonetheless, one of the drawbacks of its use lies on the fact that heavy metals often can be absorbed by other microorganisms that can compete for nutrients with microalgae. In addition, the variability of nutrients found in this type of effluent can hamper microalgae development due to excessive or deprivation of carbon, phosphorus and nitrogen, and the high turbidity caused by the excess of these metals can limit light penetration, hindering the photosynthetic process (LI et al., 2010).

Li et al. (2010), discuss the viability of using microalgae in the treatment of municipal wastewaters, which often present lower nutrient content than synthetic media used in laboratory. As effluents contain metals, wastewaters can also present variability in the nutrient composition, either due to rainfall or due to the variability of their forming sources. Barsanti and Gualtieri (2014) state that previous studies have already proven that microalgae are capable of efficiently absorbing nutrients found in this type of effluent, consequently reaching a good growth rate, promoting wastewater treatment. This is possible as microalgae development is linked with its metabolic functions. Thus, these microorganisms require the absorption of nutrients such as nitrogen, phosphorus, as well as carbon dioxide (CO_2) or some type of organic matter which is used as a carbon source during the synthesis of proteins, nucleic acids, phospholipids and other cellular structural components. According to Li et al. (2010), the ideal municipal wastewater treatment using microalgae depends on the presence of adequate levels of nitrogen (N), phosphorus (P) and chemical oxygen demand (COD), with values of approximately 130, 200 and 2250 mg/L, respectively. De Farias Silva et al. (2019) point out that a COD of up to 5,000 mg/L is possibly treatable depending on the microalgae species, as well as on the availability of other nutrients, cultivation system and possibility of symbiotic relationships with other microbial groups. Nonetheless, values higher than these usually exhibit toxicity to this microbial group or causes high instability of the system (for example, significant changes in the pH needing constant control during the process). Moreover, authors have discussed the benefits of CO_2 addition to the medium, as some results have indicated that the addition of high CO_2 concentrations promoted an increase in microalgae biomass productivity, consequently leading to an upsurge in pollutant removal rates, especially when associated to greater light availability. However, it is important to assess the cost of this process, as well as the advantages of its use, in economic, environmental and operating terms, because artificial concentrated CO_2 injection in the system can increase up to 50% of cultivation costs for microalgae (DE FARIAS SILVA et al., 2016; DE FARIAS SILVA, SFORZA and BERTUCCO, 2017).

2.4 Bibliographic and patent prospection of microalgae and their consortia for wastewater treatment

Seeking new available technologies, a technological prospection of scientific articles and patent databases was carried out aiming to verify the literature availability of the subject. The prospection was based on the review of scientific articles using the databases Science Direct (2021), Capes Journals (2021), Scielo (2021) and Scopus (2021). For patent prospection, the following databases were used: the Brazilian National Institute of Industrial Property (INPI) (2021), World Intellectual Property Organization (WIPO) (2021), and the free version of Patent Inspiration (2021), EspaceNet Patent search (2021) and Derwent World Patents Index (2021).

Table 2.1 presents the results of the analysis of the number of scientific articles published in specialised journals.

Table 2.1 - Number of articles retrieved per database based on the keywords used.

KEYWORDS	DATABASES			
	Capes Journal	**Science Direct**	**Scopus**	**Scielo**
“Microalgae” and “Wastewater Treatment”	8,851	8,619	21,806	14
“Microalgae” and “Wastewater Treatment” and “Consortium”	981	1,575	4,466	1
“Microalgae” and “Wastewater Treatment” and “Consortium” and “Bacteria”	793	1,405	3,525	0
“Microalgae” and “Wastewater Treatment” and “Consortium” and “Yeasts”	254	480	934	0
“Microalgae” and “Wastewater Treatment” and “Consortium” and “Fungi”	293	601	1,096	0
TOTAL	**11,172**	**12,680**	**31,827**	**15**

Table 2.1 shows the great importance of the subject of wastewater treatment using microalgae, which resulted in over 55,000 documents in the databases searched (even

though overlap can occur between the databases). It is also possible to observe the high number of articles related to microalgae and bacteria consortia, with a total of 5,723 documents, with the number of documents related to consortia using fungi and yeast being 3 times lower, with a total of 1,668 and 1,990, respectively. Nonetheless, most works available are mainly related to the application of microalgae in the removal of a specific pollutant, such as in the treatment of industrial wastewaters (MÁRQUEZ et al., 2017; PETRINI, FOLADORI and ANDREOTTOLA, 2018), municipal wastewater (DE GODOS et al., 2011; GUDE, 2018; ESPANA et al., 2021), biorefineries (PESSÔA et al., 2021; MAGALHÃES et al., 2021; PALACIOS, LÓPEZ and BASHAN, 2022), reduction of organic compounds in oil refinery wastewaters (GAUR, NARASIMHULU and PYDISETTY, 2018), wastewater treatment from meat processing and leather manufacturing (HU and MENESES, 2019; PENA et al., 2020), among others. In addition, these studies used microalgae-bacteria consortia for effluent treatment (RODRIGUES et al., 2020; JIANG, LI and PEI, 2021; CAI et al., 2021; SOROOSH, OTTERPOHL and HANELT, 2022), biodiesel production (PADRI et al., 2022), dairy effluent (YANG et al., 2018; BISWAS et al., 2021; TALAPATRA et al., 2021), insecticide removal (MOJIRI et al., 2022), biotreatment of landfill leachate (ZHAO et al., 2014; TIGHIRI and ERKURT, 2019), biogas sludge treatment (LI et al., 2021), nitrification by microalga-bacteria consortia for effluent treatment (FÉRNANDEZ, SALCES and GONZÁLEZ 2011; ARUN RAMASAMY and PAKSHIRAJAN, 2021). In a consortium between yeast and microalgae for lipid production in wastewater (LING et al., 2014); municipal wastewater treatment (WALLS et al., 2019), and in a consortium between microalgae and fungi in piggery effluents (WREDE et al., 2014); remediation of municipal wastewater (ZHOU et al., 2012); biogas treatment (ZHAO et al., 2019; ZHANG et al., 2021); treatment of pharmaceutical groups (BONDIN et al., 2016); treatment of wastewater contaminated with arsenic (LI, ZHANG and YANG, 2019); revision on the consortium for effluent treatment (CHU et al., 2021; LENG et al., 2021), co-cultivation for biomass harvesting (ZHANG et al., 2012; ZHOU et al., 2013; MACKAY et al., 2015; ZAMALLOA et al., 2017; PEI, REN and LIU, 2021).

The change in the number of documents related to the use of consortia in several treatments published over the years (1980 and 2021) was verified in the Scopus database (2021), highlighting the increased of this research topic. This analysis showed a significant increase on research regarding the topic over the last years, having been observed that from 2009 that there was an increasing interest on the use of microalgae associated with bacteria, mainly; in the treatment of industrial effluents, as shown in **Figure 2.2**.

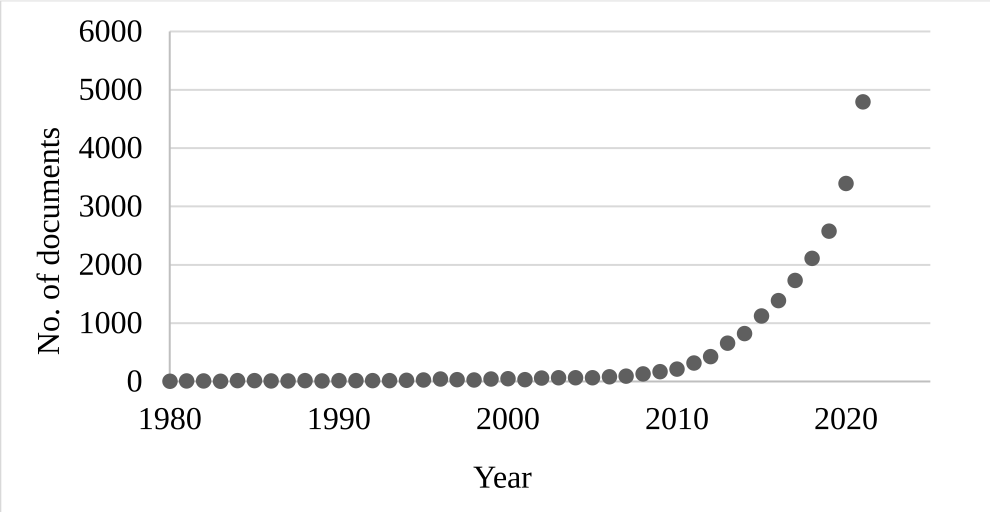

Figure 2.2 - Number of documents published over the years in the Scopus database, using the keywords "Microalgae" and "Wastewater Treatment".

Similarly, the period for collecting information regarding the patents available in the databases was between 1997 and 2022. Some patents are protected by the secrecy period, though they were included in the present research as showed in **Table 2.2**.

Table 2.2 - Number of documents retrieved in the different patent databases using different keywords.

KEYWORDS	DATABASES				
	INPI	**WIPO**	**Derwent**	**EspaceNet**	**Patent Inspiration**
"Microalgae" and "Wastewater Treatment"	5	753	96	3,315	114
"Microalgae" and "Wastewater Treatment" and "Consortium"	0	62	1	75	40
"Microalgae" and "Wastewater Treatment" and "Consortium" and "Bacteria"	1	57	1	66	37
"Microalgae" and "Wastewater Treatment" and "Consortium" and "Yeasts"	0	28	0	11	21
"Microalgae" and "Wastewater Treatment" and "Consortium" and "Fungi"	0	30	0	27	20
TOTAL	**6**	**930**	**98**	**3,494**	**232**

Table 2.2 shows the great importance of effluent treatment using microalgae, resulting in approximately 4,760 documents in the databases searched. Nevertheless, the patents related to consortia is low, with 162 documents on microalgae-bacteria consortia, 60 and 77 on microalgae-yeast and microalgae-fungi consortium, respectively. It is also important to highlight that only 10 documents directly report on effluent treatment, such as the cultivation of microalgae in wastewaters (YANN, 2014; JUNYUAN et al., 2017), wastewater treatment using microalgae consortia (YEAN, 2016; YEAN, 2018), effluent treatment for biofuel production (CHINNASAMY et al., 2010), microalgae cultivation in a wastewater dominated by carpet mill effluents (XIAOQING et al., 2020), bioremediation in industrial effluents (NAIK, 2018), development of a microalgae-bacteria symbiotic system for pyridine biodegradation (SHEN and CHIRWA, 2020).

2.5 Microalga-bacterium consortium

The presence of toxic organic compounds and some heavy metals in wastewaters can be lethal for microalgae. Studies have shown that treatment processes of these effluents can be potentialized by microalgae-bacteria consortia when compared to individual cultures of these species. According to Saravanan et al. (2021), this symbiotic relationship can take place in 3 different forms: mutualism, commensalism and parasitism. The algae-bacteria interaction mainly occurs through physical contact, substrate exchange, signal transduction or horizontal gene transfer, modifying the physiology and metabolism of both. Therefore, wastewater-native bacteria interact directly or indirectly with the microalgae, leading to an improvement or inhibition of the microorganisms present.

The microalgae consortium (photosynthetic microorganisms) and heterotrophic bacteria is a promising alternative for biological wastewater treatment, in order to avoid the external supply of CO_2 and reduce CO_2 emissions, ensuring a less costly and more ecologically sustainable treatment pathway. The treatment is undertaken through a photosynthetic process, in which microalgae use light and CO_2 and produce oxygen, while bacteria use oxygen to remove nitrogen through nitrification-denitrification and organic matter bio-oxidation, thus producing CO_2, allowing microalgae to carry out mixotrophy, contributing to the removal of organic matter (GONÇALVES, PIRES and SIMÕES, 2017; PETRINI et al., 2020) as showed in **Figure 2.3**.

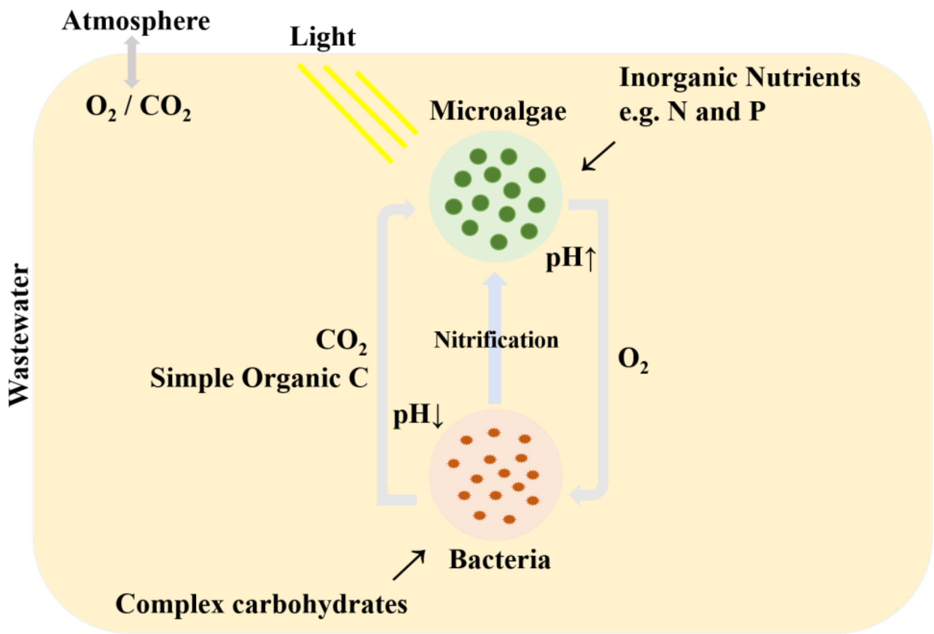

Figure 2.3 - Simplified mechanism of interactions between microalgae and bacteria in wastewater treatment.

In addition to oxygen – carbon dioxide exchange, studies show that these mutual interactions are very complex, and can be either cooperative or competitive. Some advantages of algae-bacteria consortia include synergistic relationships between microorganisms, resistance to adverse conditions, oxygen that is released from algae photosynthesis which reduces costs through aeration for bacteria development, CO_2 assimilation by algae reduces the environmental impact, greater efficiency in the removal of nutrients and COD reduction of autonomous systems of algae and bacteria, among others. On the other hand, some of the substances excreted by bacteria can hamper algae growth, or some algae presenting an antibacterial effect. In addition, the increase in pH and temperature due to the association to algae metabolism can be prejudicial to the bacteria strains (GONÇALVES et al., 2017; RODRIGUES et al., 2020).

This symbiotic relationship was successfully applied in the treatment of various wastewaters. **Table 2.3** presents an overview of the COD, nitrogen and phosphorus removal efficiency by the different microalgae/cyanobacteria and bacteria consortia in different effluent types. With these data in mind, the use of microalgae-bacteria polyculture for effluent bioremediation has proven to be an advantageous alternative, resulting in greater contaminant removal rates, as well as higher nutrient absorption and biomass production. Main microalgal strains used include the genera *Chlorella*, *Scenedesmus* (*Acutodemus/Tetradesmus*) and *Chamydomonas*. For cyanobacteria, *Anabaena*, *Microcystis* and *Oscillatoria* are cited. In terms of bacteria, as the range is larger, the use of natural microbiota from wastewater treatment plants or from the natural environment is more versatile.

It is important to point out that only the removal rate of the pollutants is not sufficient to consider the process efficient because it depends on the initial concentration of them, so; always should be evaluated both parameters, as presented in **Table 2.3**.

Table 2.3 - Performance of contaminants in some effluents by microalgae/cyanobacteria and bacteria consortia.

Microorganisms	Effluent	Cultivation Conditions	Effluent Characteristics			Removal			Reference
			COD (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})	COD (%)	TN (%)	TP (%)	
Chlorella vulgaris and native bacteria	Anaerobic digestion effluent from biogas project	Batch PBRs (800 mL working volume), aring with compressed air containing 2.5% CO_2, pH 7.0 ± 0.2, temperature of 30 ± 1ºC, with light of µmol m^{-2} s^{-1} (white light) 12h a day for 10 days	167*	16.17	7.47	99	100	99	Xie et al. (2018)
Scenedesmus obliquus, *Chorella vulgaris* microalgae and *Anabaena sp.* cyanobacteria and bacteria (*Pantoea agglomerans* and *Raoultella terrígena*)	Olive oil washing wastewater	Pre-treatment with activated charcoal for colour and turbidity removal. Closed tubular PBR (14,5 L) with internal continuous recycling of 0.35 L min^{-1}, light intensity of 450 ± 50 µmol m^{-2} s^{-1}, magnetic stirring of 200 rpm, 5 days	1508.5 ± 44.6	25.2 ± 5.6	10.6 ± 3.6	79.76	98.01	97.17	Márquez et al. (2017)
Chlorella sp., *Chlamydomonas sp. and Scenedesmus sp*. and native bacteria	Municipal wastewater	18% culture for wastewaters per volume, bioreactor capacity of 1 L, light intensity of 120 µmol m^{-2} s^{-1}, 9 days, pH 7.2	530	--	7.2	84.3	--	59.4	Fito and Alemu (2019)
Eukaryotic microalgae, prokaryotic cyanobacteria and bacteria	Municipal wastewater	Bench-scale cylindrical PBR with a working volume 2L, light intensity of 25 ± 5 µmol m^{-2} s^{-1}, light/dark photoperiod of 16:8 h, cycle of 48 h, feeding rate of 0.7 L $cycle^{-1}$, 22.2 ºC, 7 months	262 ± 97	54 ± 18	4.7 ± 1.8	85 ± 8	98 ± 2	50 ± 19	Petrini, Foladori and Andreottola (2018)
Chorella prothothecoides and *Brevundimonas diminuta* bacteria (isolated from activated sludge)	Real effluent	Drechel bottles of 250 mL, microalgae-bacteria proportion of 1:1, CO_2 injection at 1 L h^{-1}, light intensity of 10 µmol m^{-2} s^{-1}, 24 ºC	225	22	3.5	92	80	71	Sforza et al. (2018)

Chlorella sp. BWY-1 and activated sludge bacteria system	Liquid digestion of piggery effluent	Airlift PBR, 0.5 mg L^{-1} DW *Chlorella sp.* Aeration rate of 1.0 cm s^{-1} for 12h at daylight, air filters of 0.22 µm in the system's input and output, temperature of 25 ºC, 5 mg L^{-1} of DO concentration and 6 h HRT	Na	500	40	na	30	50	Jiang et al. (2018)
Coelastrum microporum and activated sludge	Municipal wastewater	Cylindrical plastic buckets (15 L working volume), algae-sludge proportion of 40:1, aeration of 0.2 vvm, light intensity of 120 µmol m^{-2} s^{-1}, light/dark photoperiod 12:12h, temperature of 25 ºC for 10 days	166.9	40.1	7.1	73.3	94.8	98.6	Lee et al. (2019)
Chlorella vulgaris (CPCC 90) and activated sludge	Secondary wastewater of anaerobic MBR of synthetic malting	Microalgae-bacteria membrane photobioreactor (9.64 L working volume), microalgae-sludge proportion of 1:3, O_2 injection of 3.39 ± 0.16 L min^{-1}, light intensity of 8,400 lux, 300 days divided into 4 phases	1106.17 ± 20.05	136.72 ± 8.17	24.63 ± 1.13	90-94	13-20	24-49	Zhang et al. (2021)
Microalgae consortium, mainly of *Picochlorum* sp. And *Stichococcus* sp., bacteria and native fungi community	Synthetic saline wastewater	The pilot plant consists of bed reactor (630 L) and feed-recirculation tank with 500L, light intensity of 240 µmol m^{-2} s^{-1}, light/dark photoperiod of 16:8h, pH 6.7 – 7.5, 6 hours.	600	22	5	99	99	95	Babatsouli et al. (2015)
Microlagas *Chlorella sp.*, *Scenesdesmus sp.*, and *Stigeoclonium sp.*; *Microcystis sp.* and *Oscillatoria sp.* cyanobacteria	10% (v/v) landfill leachate	10 L photobioreactor, batch, 3:1 microalgae:bacteria, light intensity of 76 µmol m^{-2} s^{-1}, stirring of 75 rpm, oxygen dissolved between	9360.8	3805	97.48***	90.1-92.34	99.1-99.4	98,88 - 99,39	Tighiri and Erkurt (2019)

		5-8 mg L^{-1}, pH 6.5-8.5, temperature of 25º, 18 days							
Cholrella pyrenoidosa and native bacteria	Landfill leachate enriched with municipal sewage (30%)	3L tubular photobioreactor, light intensity of (8,000 lux) 148 µmol m^{-2} s^{-1}, aeration rate of 1 L min^{-1}, 20 days	1700 (LL) and 330 (MS)	228.4 (LL) and33.3 (MS)	31.6 (LL) and 4.3 (MS)****	81	70	89	Nair and Nagendra (2018)
Chlorella, diatoms, filamentous cyanobacteria and heterotrophic bacteria	Municipal wastewater	2L cylindrical photobioreactor, light intensity of 30 µmol m^{-2} s^{-1}, light/dark photoperiod of 16:8h, feeding in the beginning of the dark phase, magnetic stirring of 200 rpm, temperature of 22.8 ºC	257 ± 91	54 ± 22	4.8 ± 1.5	86.2	88.33	47,92	Foladori et al. (2018)
Coelastrella sp. UKM4 and native bacteria	Anaerobic efluente of palm oil	2L flasks , 20% (v/v), light intensity of 230 µmol m^{-2} s^{-1}, airing at 0.25 vvm, 30 days	1251-2119	65-151 **	60-142 ***	~ 32	~ 88	~ 12	Udaiyappan et al. (2020)
Chlamydomonas sp. UKM6 and native bacteria						~ 21	~ 85	< 10	
Scenedesmus sp. UKM9 and native bacteria						~ 43	~ 90	~ 60	
Ochromonas microalgae and *Leptolyngbya sp.*cyanobacteria	Synthetic medium A	4L rectangular photobioreactors, average inoculum concentration of 75.1 ± 17.5 mg L^{-1}, pH between 7 and 9	39 ± 4.0	296.87 ± 5.0	12.96 ± 1.9***	ND	0	98.3 ± 0.02	Tsolcha et al. (2018)
	Synthetic medium V		4.48 ± 2.0	5.63 ± 0.2	1.9***	ND	95.7 ± 0.1	92.6 ± 0	
	Poplar sawdust hydrolysate 8%		1500 ± 210.0	36.73 ± 0.2	1.8 ± 0.32***	34.2 ± 4.7	25.2 ± 1	26.4 ± 0.5	

	Grass hydrolysate 25%		2414.5 ± 29.5	119.2 ± 10.8	12.7 ± 0.06***	88.7 ± 3.3	88.2 ± 1.2	97.5 ± 0.3	
	Second cheese whey A		3240 ± 132.0	91.93 ± 5.0	18.5 ± 0.09***	94 ± 1	91.2 ± 0.6	83.8 ± 0.4	
	Second cheese whey B		1578 ± 75.0	24.69 ± 1.7	9.63 ± 0.16***	93.5 ± 6.95	72.6 ± 1.9	83.2 ± 0.2	
Microalgae consortium (*Phormidium* (71%), *Oocystis* (20%) and *Microspora* (9%)) and activated sludge	Potato processing diluted 4x	Glass bottles with 1 L working volume, filtered in filters of 0,40 µm, magnetic stirring with tap water, light intensity of 76 ± 4 µmol m^{-2} s^{-1}, light/dark photoperiod of 12:12h, temperature of 30 °C	na	17.25	1.5	na	60 ± 0.4	na	Posadas et al. (2014)
	Fish processing diluted 2x		na	41	3	na	85 ± 1	Na	
	Animal feed processing diluted 2x		na	98.5	13.5	na	62 ± 2	83 ± 5	
	Coffee manufacturing diluted 100x		na	7.66	0.59	na	80 ± 4	Na	
	Yeast production diluted 10x		na	7.03	0.7	na	50 ± 1	Na	
Desmodesmus sp. microalgae, unidentified coccal microalgal sp., diatoms *Nitzchia* sp. and cyanobacteria *Phormidium* sp. , *Oscillatoria sp.*	Upflow anaerobic sludge blanket digestion (UASB) of a paper manufacturer	5L tubular photobioreactor, light/dark photoperiod of 12:12h, light intensity of 122 µmol photons m^{-2} s^{-1}, stirring of 120 rpm	na	30.8 ± 2. 5	7.5 ± 0.5	na	76.5 ± 5.2	73.4 ± 2.4	Hende et al. (2017)

NA – Not analysed, * SCOD – soluble chemical oxygen demand, ** ammoniacal nitrogen, *** phosphate and **** orthophosphate.

2.6 Microalga and yeast consortium

Microalgae are known by their oxygen production and their efficiency in removing nutrients such as nitrogen and phosphorus from wastewaters. On the other hand, organic matter removal is low in these microorganisms due to their slow growth, requiring greater cultivation times to reach high biomass and lipids concentration (in the context of biodiesel from microbial sources which microalgae is widely linked) even though they can use heterotrophy (mixotrophic beings). Nonetheless, oleaginous yeasts are highly efficient options for organic matter removal and lipids production from wastewaters. In this regard, microalgae-yeast consortia prove to be a promising alternative for effluent treatment through oxygen from microalgae photosynthesis using yeast and carbon gas through yeast respiration for microalgae, improving lipids and biomass production (DIAS et al., 2019). In addition, lipid-rich biomass from the microalgae-yeast consortium can be used for the production of added-value products, such as biodiesel, as aforementioned, and its recovery can be improved due to the flocculation properties of some yeasts, besides the efficiency in holding microalgae which helps on its recovery, decreasing the harvesting costs, as displayed **Figure 2.4**.

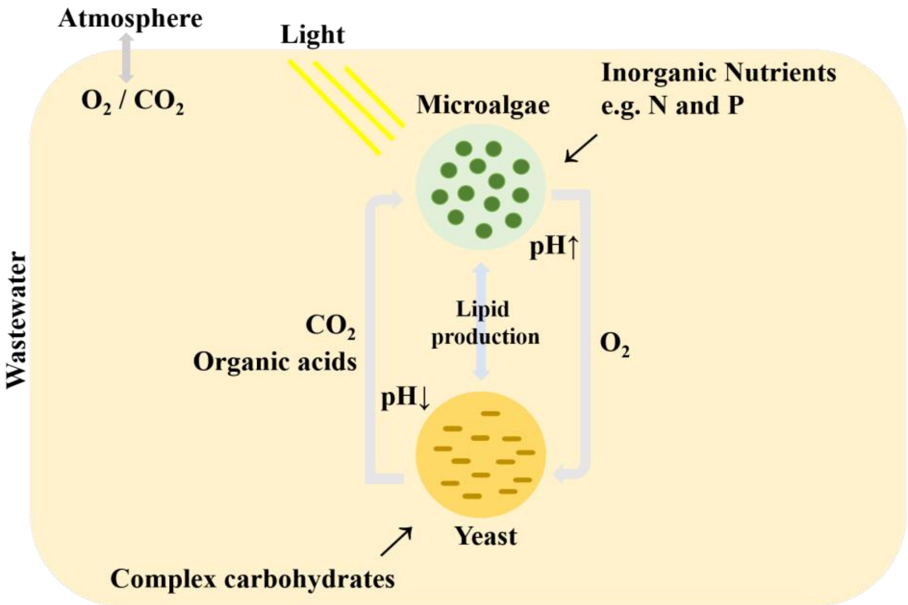

Figure 2.4 - Simplified mechanism of interactions between microalgae and yeast in wastewater treatment.

Yeasts have not been widely explored for effluent treatment probably due to their non-sterile conditions and the needed to manage the fermentation/respiration aspects, because to improve biomass production and consequently the contaminants removal, respiration should be favoured, i.e., oxygen is necessary (aerobic respiration – aeration). Nevertheless, their composition is indicative of a better nutrient removal capacity in wastewaters when compared to microalgae, where 3-5% of its dry cell weight consists of phosphorus and 0.87% in the case of microalgae, with nitrogen concentration being 10% higher when compared to the 6% in microalgae (WALLS et al., 2019), even though these % can change according to the environmental, nutritional and operating parameters, because they have a biochemical plasticity which can lead to much higher values than those previously mentioned (DE FARIAS SILVA et al., 2016). In this regard, *Chlorella vulgaris* presents the following composition: 2-14% nitrogen and 0.5-4% phosphorus, in dry weight (adjusting itself to light, nitrogen and phosphorus availability) (DE FARIAS SILVA and SFORZA, 2016).

Yeasts are considered more efficient oleaginous microorganisms when compared to others (algae, bacteria and mould) due to their capacity of accumulating more lipids. In addition, they present a high growth rate, being easily cultivated at a large scale (KARIM et al., 2021). The lipids rate in microalgae and its composition can be affected by environmental and chemical cultivation conditions, namely by pH, light intensity, temperature, stirring rate, carbon sources and nutrient concentration (phosphorus and nitrogen). These lipids can be classified as neutral, crude and total, with neutral lipids being the most commonly used in biodiesel production through transesterification (SUASTES-RIVAS et al., 2020).

Researchers have suggested three types of microalgae-yeast mutualistic symbiosis mechanisms, such as the exchange of primary metabolites, interchange of cofactors and hormones, as well as the formation of particular physical microenvironments. Nonetheless, such mechanisms are not entirely well known (ASHTIANI et al., 2021), the choice of combination of these microorganisms must take into account cultivation conditions, as microalgae prefer higher pH values, while oleaginous yeasts grow better in acid medium, a buffer can be created in the cultivation if the conditions are managed adequately. Another restriction is related to organic carbon, which should be provided to the system in suitable amounts to ensure high-quality though non-significant lipids production, as excess primary metabolites such as acids and ethanol can hinder microalgae development (PADRI et al., 2021).

Ling et al. (2014) analysed lipids production using the mixed *Chlorella pyrenoidosa* microalgae and *Rhodosporidium toruloides* yeast culture in rice wine distillery effluent with municipal wastewater at a ratio of 1:1 (SCOD – Soluble COD of

17,150 mg L^{-1}, TN (Total Nitrogen - of 720 mg L^{-1}, TP – Total Phosphorous - of 349 mg L^{-1}, pH 3.8). Yeast-microalgae co-cultivation with an initial cell density of $2x10^{7}$:$5x10^{6}$ cells mL^{-1}, at 30 ºC and 2.93 W m^{-2}, light/dark photoperiod of 12:12h, light intensity of 265 µmol m^{-2} s^{-1}, have reached lipids production of 4.60 ± 0.36 g L^{-1}, with SCOD, TN and TP removal of 95.34 ± 0.07, 51.18 ± 2.17 and 89.29 ± 4.91%, respectively, after 5 days of cultivation without pH adjustment (a buffer phenomenon).

Walls et al. (2019) studied nutrient removal in non-sterile municipal wastewaters (enriched with 10 and 20 g L^{-1} glucose to increase COD, initially with of 95 ± 24.7 mg L^{-1}, 15.0 ± 4.9 mg L^{-1} of nitrate, 133.1 ± 13.9 mg L^{-1} of total ammoniacal nitrogen and 67.5 ± 19.5 mg L^{-1} of orthophosphate) through the consortium consisting of *Scenedesmus obliquus* microalgae and wild yeast, with an initial biomass concentration of 0.2 g L^{-1} (inoculum) (94% in microalgae weight and 6% in yeast weight). Nutrient removal by the co-culture was of 93 and 97% for nitrate, 93 and 95% for total ammoniacal nitrogen, 91 and 94% for orthophosphate, in 3 days of cultivation, and total biomass concentrations after cultivation were 1.85 ± 0.26 to 2.74 ± 0.43 g L^{-1} for cultures of 10 and 20 g L^{-1}, respectively. Nonetheless, the culture with the greatest glucose concentration favoured yeast and bacteria growth, with the presence of microalgae being 18% lower in terms of the average number of cells.

Suastes-Rivas et al. (2020) analysed the optimised distribution of fatty acid methyl esters (FAMEs) under the individual and combined effect of micronutrients in the culture media, with the experimental design considering a multi-level (3^3) strategy. The concentrations found were of 0.35, 0.7 and 1.05 g L^{-1} of $NaNO_3$, 0.11, 0.08 and 0.095 g L^{-1} of K_2HPO_4 and 0.0649, 0.0949 and 0.1249 g L^{-1} of $FeSO_4.7H_2O$. The microalgae-yeast co-culture was isolated from a municipal wastewater treatment station, consisting of 68% *Scenedesmus obliquus*, 29% *Scenedesmus sp.* and 3% of some yeast species, among them 43% *Candida pimensis.* Cultivation took place in 1 L PBR, with a photoperiod of 12:12h, light intensity of 400 µmol m^{-2} s^{-1}, 24 ± 1 ºC and aired at a constant flow rate of 0.25 vvm. The highest lipids contents (27.77 and 20.84%) occurred in assays with N/P ratios of 4:1 and 3:1, respectively. High-saturated fatty acids were obtained for high N/P ratios (6:1 and 11:1), while unsaturated fatty acids under a N/P ratio of 9:1. A lower iron concentration was observed to be the main factor to promote higher FAME concentrations desired for the production of high-quality biodiesel. Thus, the effect of iron was more significant than the N/P ratio, despite the latter modifying lipids concentration and the fatty acid composition of the microorganisms.

Ashtiani et al. (2021) analysed the influence of age of microalgae pre-culture (*Chlorella vulgaris)* and yeast (*Rhodotorula glutinis)* on biomass and lipids content in the co-culture. The inoculum seed ages in the co-culture were 7 days for microalgae (logarithmic phase, 0.88 g L^{-1} of biomass) and 5 days for yeasts (stationary phase, 11.12 g L^{-1} of biomass). The assays were carried out in 250 mL flasks, at 26 ºC, 90 rpm, light intensity of 800 µmol m^{-2} s^{-1}, for 11 days, inoculated with ratios of 1:1, 1:2 and 2:1 (v/v) of yeast to microalgae. As anticipated, a higher lipids production was observed at a microalgae/yeast ratio of 2:1 after 5 days, with a lipids content of approximately 5 times higher than in pure cultures. On the following days, there was a decrease in lipids production due to the internal degradation of lipids stored in yeasts due to CO_2 deficiency. In addition, the synergistic impacts of the consortium were also analysed on enzyme expression. For *in vitro* assays, at a microalgae/yeast ratio of 2:1, the co-culture increased approximately 6- and 5-fold the concentration of nervonic acid (C24:1) and behenic acid (C22:0), respectively. In the yeast residual cell-free medium, with a 2:1 ratio, the microalgae resulted in an upsurge of 9- and 6-fold in nervonic acid (C24:1) and behenic acid (C22:0), respectively, when compared to the monocultures.

Based on the aforementioned studies, it can be observed that the microalgae-yeast consortium has proven to be promising in the bioremediation of wastewaters and in biomass production, leading to a significant increase in the molecular compounds of microorganisms, namely high-quality added-value lipids content. Nevertheless, further research is necessary to better understand the mutualistic relationship between these microorganisms, besides the optimisation of processes and considering different yeasts and microalgae strains.

2.7 Microalgae and filamentous fungus consortium

One of the most self-sufficient symbiotic associations are the lichens, stable symbiosis between some filamentous fungi species (mycobionts) and green algae and/or cyanobacteria (photobionts). This mutualistic relationship consumes oxygen and organic carbon, such as sugars and nutrients, provided by algae through photosynthesis. In exchange, algae have protection and access to nutrients through the action of extracellular fungal enzymes which convert microcellular organic matter into soluble and easily absorbable nutrients (WREDE et al., 2014; LUTZU and DUNFORD, 2018; ZHAO et al., 2019; CHU et al., 2021). The simultaneous metabolic exchange between species of these co-cultivation systems can make them less dependent on external nutrient sources (nitrogen and phosphorus), as well as the transformation of external CO_2 and sunlight into bio-sustainable products (LI et al., 2020).

Several studies have shown that microalgae have a high potential in the bioremediation of different wastewater types. In addition, microalgae biomass has a high added value, and can be used as raw material for several products, namely biofuels, biofertilizers, biochemicals, among others. However, the high operating costs of conventional (physical and chemical) biomass recovery methods have promoted the use of bio-flocculation for microalgae harvesting, such as the co-culture between filamentous fungi and microalgae forming sustainable pellets by gravity, being considered a cheaper and more sustainable system (ZHANG and HU, 2012; ZHOU et al., 2013; MACKAY et al., 2015; PEI, REN and LIU, 2021). The se interactions are visualized in **Figure 2.5**.

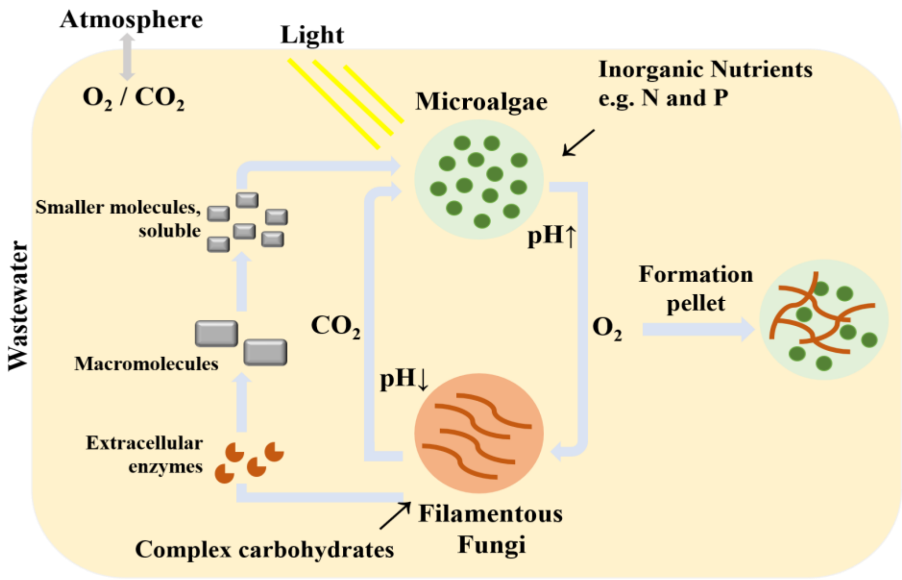

Figure 2.5 - Simplified mechanism of interactions between microalgae and fungi in wastewater treatment.

Pellet formation is intimately related to agitating effects, with a very low stirring rate preventing the aggregation of conidia or spores. Nonetheless, under rapid rates, they tend to break up or form smaller and smoother pellets. The ideal agitation rate is not yet known, though it is thought to be related to the needs of each microorganism in the demand for oxygen. Another factor which interferes in pellet formation is the pH. Acidic environments are favourable to fungi growth. Nevertheless, microalgae grow better in alkaline conditions, thus it is important to select fungi that adapt well to a wide pH range.

Moreover, fungi and microalgae present opposite electrical conditions, and their co-culture tend to be neutral (a buffering phenomenon as occurs with yeast-microalga) (LENG et al., 2021). However, Chu et al. (2021) concluded that the ideal pH for pellet harvesting is specific to each microorganism species, with further analysis being needed to optimise co-cultivation conditions in future research.

In Zhou et al. (2012), the co-culture was formed by *Aspergillus sp.* fungi and *Chlorella vulgaris* microalgae, being used in the remediation of municipal wastewaters (COD = 1660 ± 40.1 mg L^{-1}; TN = 97.2 ± 6.8 mg L^{-1} and TP = 51.2 ± 7.2 mg L^{-1}). The process was carried out under 100 rpm agitation for 24 hours, having reached removal rates of 62.5, 58.9 and 89.8% for COD, TN and PT, respectively.

Wrede et al. (2014) used *Aspergillus fumigatus* species and several microalgae species, having verified a high efficiency of anaerobically digested piggery effluent, reaching better results with co-cultivation than with isolated fungi and microalgae species, with nitrogen and phosphorus removal rates of approximately 90%. Initial NH_4^+-N and PO_4^{-3}-P concentrations in the effluent were of 680.7 mg L^{-1} and 145.7 mg L^{-1}, respectively.

Zhao et al. (2019) used co-pelletization of microalgae (*Chlorella vulgaris)* and fungi (*Ganoderma lucidum*) in the treatment of biogas slurry from an anaerobic digestion reactor in a pig farm wastewater treatment plant (COD = 1061.51 ± 26.23 mg L^{-1}, NT = 182.64 ± 11.68 mg L^{-1}, TP = 17.96 ± 1.93 mg L^{-1} and CO_2 = 36.17 ± 1.97 v/v). The optimised experiment took place under the following conditions: sludge/mixed medium ratio of 3:7, fungus:microalga ratio of 1:10 (1.0×10^6 of *G. lucidum* spores L^{-1}), light intensity 200 μmol m^{-2} s^{-1}, photoperiod of 12:12h, red:blue light 5:5, temperature of 28°C, magnetic stirring of 160 rpm, for 8 days. The COD, TN, TP and CO_2 removal efficiencies were of 92.17 ± 5.28, 89.83 ± 4.36, 90.31 ± 4.69 and 74.26 ± 3.14, respectively.

Under similar conditions, microalgae (*Chlorella vulgaris)* and fungi *(Ganoderma lucidum)* were cultivated in medium supplemented with strigolactone (10^{-9} M of GR24), light intensity of 225 μmol m^{-2} s^{-1}, red:blue light of 5:5, light/dark photoperiod of 12:12h, temperature of 25.2 °C for 7 days for crude biogas and biogas slurry treatment (COD = 1671.28 ± 44.39 mg L^{-1}, TN = 107.54 ± 7.75 mg L^{-1} and TP = 25.12 ± 2.47 mg L^{-1}). Obtaining the maximum COD, TN and TP efficiency of 76.35 ± 6.87, 78.77 ± 7.13 and 79.49 ± 7.43%, respectively (Zhang et al. 2021).

Li, Zhang and Yang (2019) assessed the effects of nitrogen, glucose and phosphorus in the remediation of arsenic-contaminated wastewaters using fungi-algae pellets (*Aspergillus oryzae* and *Chlorella vulgaris)* (26 °C, 125 rpm, 112 μmol m^{-2} s^{-1}, photoperiod of 12:12h, for 7 days). The pellets showed a high potential in the bioremediation of arsenic-contaminated wastewaters due to the high tolerance and capacity of accumulating the contaminant.

Bodin et al. (2016) used the consortium formed by *Chlorella vulgaris* microalgae and *Aspergillus niger* fungi for the treatment of pharmaceutical groups usually found in aquatic environments. The results showed that the use of bio-pellets was effective in the removal of the 7 types of substances analysed (with initial concentration between 8 and 11 mg L^{-1}, each), especially ranitidine, with a removal percentage of 50 ± 19%.

Shen and Chirwa (2020) studied the potential of live and lyophilized fungi-algae pellets (*Aspergillus niger* and *Tetradesmus obliquus)* as biosorbents for remediating wastewaters containing gold (30 mg L^{-1}). The lyophilized pellets showed greater storage and adsorption potential than live pellets, absorbing approximately 97.7% gold from multi-metallic wastewaters in the column reactor.

Srinuanpan et al. (2018) used pellets formed by *Trichoderma reesei* and *Scenedesmus sp*. fungi in the remediation of secondary, non-sterile effluents, generated by the seafood processing industry (pH 7.7, 1,239 mg L^{-1} of COD, TN 144 mg L^{-1} and TP 18.6 mg L^{-1}). This resulted in the removal rate of 74, 44 and 93% for COD, TN and TP, respectively.

Therefore, based on these studies, the microalgae-fungi consortium has shown to be more advantageous in the treatment of wastewaters when compared with the axenic cultures. Nonetheless, there are still several gaps to be filled on this subject, highlighting the need for optimising culture parameters in future researchers in order to reach even more efficient results and even how to apply the fungus-alga biomass produced from a biotechnological point of view.

2.8 Feasibility of the open cultivation system for symbiotic association using microalgae and other microbiological groups

As previously pointed out, microalgae can be cultivated in different system types. These range from litres to billion litres, as well as unsophisticated systems, individual cultures in the case of open tanks under natural light and temperature conditions, with low or zero control, or sophisticated system, with closed photobioreactors, where it is possible to control cultivation parameters (KIM et al., 2017; KUMAR et al., 2018).

One of the main problems faced in open microalgae cultivation systems is contamination by other microorganisms. Biological contamination can reduce product synthesis or inhibit microalgae cell growth. Nonetheless, research in metabolic engineering has invested in solutions to reduce or mitigate these adverse conditions (VEERABADHRAN et al., 2021).

According to Kim et al. (2017), approximately 95% of total microalgae production takes place in open systems, with roughly 20,000 tonnes of microalgae/year, reaching productions of almost 180 tonnes/ha/year, under natural interference of other microorganisms in the cultivation medium. However, biomass production in closed photobioreactors is higher than 1,500 tonnes/ha/year, due to operating conditions optimisation.

Open microalgae cultivation systems can be raceway ponds, tanks or circular ponds, though High-Rate Algal Ponds (HRAP) are more commonly used. HRAPs have a high microalgae biomass production with commercial applications, mainly for biofuel production purposes (ARITA, PEEBLES and BRADLEY, 2015; MILANO et al., 2016). HRAPs are compared to a running track or an oval pond, 15 to 80 cm deep, allowing solar light to enter with the entire bubble column euphotic, facilitating photosynthesis in the entire system. One important variable is the quality of solar light incidence in the system. Thus, it is important to ensure that the pond is well-located to allow adequate light irradiation, which also interferes on the choice of microalgae species to be used in the system. These systems usually present paddle wheels, ensuring the mixing of the liquid and biomass, optimising nutrient distribution, microalgae suspension in the bubble column and gas exchange (CHEAH et al., 2015; MILANO et al., 2016).

One of the main advantages of open pond systems include their large surface area, optimising CO_2 sequestration, besides of the liquid evaporation what can increase the temperature in the bioreactor. These systems are usually applied in large scale microalgal cultivation, due to their low cost and easy operation, maintenance and cleaning after cultivation. However, these open systems are also exposed to climate variation, which can affect light intensity, temperature, water evaporation, resulting in significant volume losses and impacting on culture stability, low mass transfer due to the inefficient mixture, hampering biomass productivity and quality/quantity (GRUBIŠIĆ, ŠANTEK and ŠANTEK, 2019). In addition, the needed of large land areas for implementing the ponds, but these can be set up in unfertile lands, avoiding competition with food production.

Due to limitations in water evaporation at a similar rate to land crops, photoautotrophic biomass production in open systems is limited to only some species, with many being unable to be maintained for long periods of time due to risk of contamination (MILANO et al., 2016). Despite the need for more control in cultivation conditions in open systems, these are successfully used at a commercial scale for *Spirulina platensis, Haematococcus* sp., *Dunaliella salina* microalgae, as well as for genera *Chlorella* sp., *Scenedesmus/Acutodesmus/Tetradesmus* and *Nannochloropsis* sp., among others (GRUBIŠIĆ, ŠANTEK and ŠANTEK, 2019).

Open natural ponds for microalgae cultivation include circular ponds, commonly used in Japan, Taiwan and Indonesia, for cultivating the *Chlorella* species; raceway ponds, used in the cultivation of the *Arthrospira* species in the United States and *Dunaliella salina* in Israel; natural *Dunaliella salina* ponds in Mexico and Australia; inclined systems, used in Bulgaria for genera *Arthrospira* and *Scenedesmus,* and *Chlorella* in Australia. In turn, tanks are usually used for production at a lower scale for genera used in aquaculture, namely *Nannochloroposis oculate* (BOROWITZKA, 2005; ARITA, PEEBLES and BRADLEY, 2015).

Aimed at improving microalgae biomass productivity, researchers have studied and developed CO_2 injection systems, or injection systems using combustion gas (FG – flue gas) applied in open systems (CHEAH et al., 2015), being advantageous for reducing greenhouse gas emissions. For instance, Yadav, Dukey and Sen (2020) assessed the capacity of culture *Chlorella vulgaris* in the sequestration of combustion gases and biomass growth in open air pond systems under different CO_2 concentrations (0.04% - only air, and 10 ± 2%, for batch and semi-continuous cultures). For batch experiments with and without FG, the CO_2 fixation rate and maximum biomass production were of 8.92 ± 0.28 mg L^{-1} d^{-1} and 0.11 ± 0.08 g L^{-1}, 33.79 ± 4.02 mg L^{-1} d^{-1} and 0.3 ± 0.13 g L^{-1}, respectively. In turn, for the semi-continuous mode with FG addition, mass density of 0.428 ± 0.12 g L^{-1} and CO_2 fixation rate of 102.66 ± 5.77 mg L^{-1} d^{-1} was obtained, being higher.

Some studies focus on the use of extremophilic species, growing under extreme temperature, pH and salinity conditions, being successfully produced at a commercial scale, being sustainable and reliable in an open-air system. For instance, the *Dunaliella salin*a species has a high growth rate in saline waters, due to its high intracell glycerol content, contributing towards the protection against osmotic pressure. *Spirulina platensis* and *Spirulina maxima* are capable of surviving and growing well at a high pH, between 9 and 11.5 (YADAV, DUBEY and SEN, 2020; ZHU, JIANG and FA, 2020).

Aimed at optimising microalgae cultivation conditions in open systems, researchers also have focused on attempting to solve or minimise any environmental, physical and even economic cultivation issues. For instance, Baldev et al. (2018), cultivated *Chorella vulgaris* microalgae in an open pond system (35,000 L, 15 rpm, cell density of 2 x 10^6 cells mL^{-1}), with a low-cost optimised medium (urea, superphosphate and potassium at 91.9, 72.9 and 62.7 mg L^{-1}, respectively, and pH 8.03) for biomass production aimed at biodiesel production. This ensured biomass productivity of 31.5 mg L^{-1} d^{-1} and lipids content of 25 ± 5%.

Ketheesan and Nirmalakhandan (2012), cultivated *Scenedesmus* sp. in an airlift-driven raceway reactor (23 L, air rate of 2,400 mL min^{-1}, circulation rate of 10 cm s^{-1} initial inoculum density of 0.15 g L^{-1}, light intensity of 80 μmol m^{-2} s^{-1} for continuous mode and 110 μmol m^{-2} s^{-1} for batch mode). Maximum biomass productivity was of 0.085 g L^{-1} day^{-1} for the batch mode with CO_2 at 1% for 15 days. For the continuous mode, with 1% CO_2 for 18 days, maximum biomass and lipids productivity were of 0.19 and 0.04 g L^{-1} dia^{-1}, respectively.

Raeisossadati, Moheimani and Parlevliet (2019) analysed red and blue luminescent solar concentrators (LSCs) to increase *Arthrospira platensis* biomass and phycocyanin productivity in 21cm deep outdoor raceway ponds, at a mixing rate of 11 cm s^{-1}, light intensity of 34 and 4.5 µmol photons s^{-1} for red and blue LSCs, respectively. Biomass and phycocyanin productivity increased by 26 and 44%, respectively, when using red LSCs. However, blue LSCs did not result in a significant increase in biomass productivity.

2.9 Contamination by protozoa in cultivation systems

Biological contaminants such as zooplankton, bacteria, fungi, protozoa, other algae and viruses, can have either a positive or negative effect on microalgae cell growth. In open cultivation systems, contamination will eventually occur through gas exchange (CO_2 and O_2) between air and liquid culture, through the contact with animals and insects, or even through the water used.

As previously mentioned, open systems have the greatest disadvantage of greater susceptibility to contamination by predator species. These predators can drastically reduce production yields or, in extreme cases, leading to total cultivation loss. Once an important competitor has established residence in the pond, it becomes extremely difficult to eradicate it (MILANO et al., 2016). Therefore, it is necessary to develop ways of eliminating/inhibiting the action of these contaminants without hindering the development of the desired species.

Protozoa are natural bacteria and microalgae predators and have been used as bio-indicators in community assays due to their short generation time and rapid responses to environmental changes. The body-size spectrum of protozoa communities was successfully employed to analyse the defence capacity of *Chlorella* sp. and *Nannochloropsis oceanica* protozoa under protozoa herding. Protozoa communities were exposed to different concentrations of both microalgae - 10^0 (control), 10^4, 10^5, 10^6 and 10^7 cells mL^{-1} for a period of 9 days each. In sum, both species showed a strong defence effect in the body-size spectrum, in which the body-size distinction of protozoa communities showed a significant reduction in microalgae concentrations higher than 10^6 cells mL^{-1}) (WANG et al., 2017). Under the same conditions, Gui et al. (2020) explored functional distinction measures based on biological characteristics to identify the effect of microalgae protection against protozoa. In addition, results showed that functional distinction measures present a downward trend of these microorganisms throughout the concentration gradient of both aforementioned microalgae.

According to Zhu, Jiang and Fa (2020), biological contamination can occur in any cultivation phase. Nonetheless, microalgae are capable of adapting to the environment, tolerating extreme environmental conditions, while biological contaminants require specific conditions to survive. Therefore, environmental control is a way of inhibiting contamination, such as by maintaining culture pH under alkaline conditions (10-11), as well as the rapid increase in light intensity to 30,000 lux, salinity above 15% (NaCl) and temperature increase can all have a positive influence on microalgae in terms of protozoa development.

Zhao et al. (2021) state that extracellular microalgae excretion inhibits protozoa development. In this regard, the authors carried out a controlled laboratory assay to evaluate the inhibitory effect on ciliated *Euplotes vannus* growth under different densities of marine microalgae *Nannochloropsis oceanica* (high, moderate and low density, respectively). Maximum population densities of *N. oceanica* in the plateau phase were of 210, 350 and 300 ind. mL^{-1} for cultivation with high, moderate and low algae density, respectively. Preliminary results confirmed that protozoa growth was inhibited in the medium with high microalgae density, suggesting that extracellular excrements of *N. oceanica* have the potential of controlling/inhibiting protozoa contamination in large scale microalgae cultivation.

2.10 Conclusion and future works

Bioremediation in different wastewaters in open microalgae cultivation systems and their symbiotic relationship with other microorganisms have a great potential for solving environmental issues, besides reducing demand for supplies, such as clean water, energy, CO_2 and nutrient injection for microalgae cultivation. Also noteworthy are the advantages of co-cultivation systems in wastewater when compared to axenic cultures, especially in terms of the degradation of organic compounds, phosphorus and nitrogen absorption and CO_2 sequestration. These benefits are observed due to the synergistic relationship between these consortia, such as metabolic and gas exchanges, as well as the high pH adjustment in the medium. In addition, microalgae contribute to renewable energy production, namely of biodiesel and biogas, as well as valuable co-products, such as fertilizers, animal feed, cosmetics, among others.

As it was possible to see in the entire manuscript, bacteria are generally used with microalgae to treat wastewater, probably, because they are the main agent used in secondary wastewater treatment (for organic matter removal, mainly) and microalgae have efficiency in remove nitrogen and phosphorous. For yeast-microalga consortium, the main application today is regarding the production of lipids for high-value products or biodiesel production, but more research is needed in order to improve this application range. On the other hand, fungus-microalga consortium is used mainly for effluent with recalcitrant pollutants or to improve the microalgal harvesting in the biological process, but as the use of yeast, this type of consortium should be more studied because the biotechnological plasticity of these microorganisms can help in the obtaining of better economic applications helping the sustainability and feasibility of the system.

In the past decades, many studies have been successfully carried out at a laboratory scale. Nevertheless, a lot still needs to be done to understand prevailing gaps of these mutualistic relationship and optimal cultivation conditions in large scale cultivation for industrial purposes, ensuring high efficiency levels, and finding promising alternatives for recovering the biomass produced. Economic and life cycle assessment are need in order to provide information regarding the feasibility of the process in large scale.

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Chapter 3: Bioreactors and Operation Modes for Microalgae Wastewater Treatment

Abstract

Microalgae are used in wastewater treatment for various reasons (e.g., high growth rate, mixotrophic metabolism, high affinity for assimilating nitrogen and phosphorus, and the ability to form a symbiosis with chemo-heterotrophic microorganisms when performing photoautotrophy). To achieve effective microalgae cultivation and wastewater treatment, it is crucial to carefully select the appropriate bioreactor and operation mode, which influence the pollutant removal rate and biomass productivity. In this chapter, the main bioreactors used, and the operation modes carried out with them will be highlighted, showing the performance described in the literature in terms of pollutant removal and biomass production. Removal rates exceeding 90% for chemical oxygen demand, nitrogen, and phosphorus can be achieved if these operational factors (bioreactor and operation mode) are appropriately selected in conjunction with nutritional factors (initial contaminant concentration and inoculum concentration) and environmental requirements (primarily temperature, pH, and light intensity).

Keywords: Biological process; Microalgal system; Operational parameters; Contaminant removal.

This chapter was accepted for publication as: De Andrade FP, Medeiros JA, De Farias Silva CE, De Sá Filho MLF. Chapter 7: Bioreactors and modes of operation for microalgae wastewater treatment. Ambati RR, De Carvalho JC, Soccol SR, Ravishankar GA. (2023) Algae mediated bioremediation: Treatment of Industrial wastewater streams and carbon dioxide capture. Wiley Publishers, USA.

3.1 Introduction

Microalgal biomass is promising for applications in food, cosmetics, pharmaceuticals and other biotechnological processes due to its nutritional properties and natural pigments (AMBATI et al., 2019; GOKARE and AMBATI et al., 2019a; 2019b; 2021). In addition, the cultivation process is a critical factor as it can result in high concentrations of lipids and carbohydrates, which can serve as an alternative source for biofuel production (AMBATI and GOKARE, 2019; DE ANDRADE et al., 2022; MAURYA et al., 2022).

This interest is primarily related to the ease of cultivation of these microorganisms, as their nutritional requirements are simple, usually requiring light (natural or artificial), CO_2, and nutrients (e.g., nitrogen and phosphorus) (YAAKOB et al., 2021). Therefore, they can be cultivated in salt water, fresh water, or wastewater. Aspects related to the cost of the culture medium, energy consumption for aeration, and possible supplementation of the air with CO_2, as well as lighting (if provided artificially), are obstacles to ensuring the economic viability of microalgal cultivation.

In this sense, wastewater can provide a nutrient-rich culture medium at no significant additional cost, and after the microalgae have grown in it, treated water and microbial biomass can be produced as products. Therefore, research has been conducted in order to optimize the growth of microalgae, evaluating factors that may interfere during the process, such as type of reactor, C/N and N/P ratio and operation mode (DE ANDRADE et al., 2022).

The microalgae are grown in photobioreactors (PBRs), which can be categorized as open (e.g., lagoons and tanks) or closed (e.g., tubular or membrane) systems (MADHUBALAJI et al., 2019). These have different configurations. Open systems are in contact with the atmosphere and are therefore not fully axenic, whereas closed systems minimize contact with the environment outside the reactor. One of the main advantages of closed systems is the ability to reduce contaminants. In addition, important parameters (e.g., pH, temperature, light intensity, CO_2 flow rate, and agitation) can be better controlled (SARADA et al., 2012). Therefore, these systems have a higher associated cost compared to open systems, which are simpler but more economical (due to their greater susceptibility to external variations) (LI et al. 2019; DE ANDRADE et al. 2022).

The operation mode can be batched or continuous, as well as their variations (e.g., fed-batch and semi-continuous), which not only change the way the substrate is introduced into the reactor but mainly the tendency of microalgal biomass growth and harvesting. The operation mode used in the process depends on both the destination of this microorganism and the availability of the cultivation structure, as well as the sensitivity of the microorganism to nutritional, environmental, and hydrodynamic regimes (PETER et al.; 2022).

In this chapter, a comprehensive overview of microalgal biomass cultivation methods is presented, focusing on the main types of bioreactors utilized, optimal growth medium conditions, and operation modes of cultivation. Moreover, this chapter discusses the studies carried out on the subject matter, addressing both the advantages and limitations of these methods.

3.2 Bioreactor Types and Operating Conditions

Microalgae cultivation can be performed in both open and closed systems. Open systems (e.g., open ponds, raceways, and tanks) are widely used in microalgae-based wastewater treatment. Advantages of this type of system include ease of construction and operation compared to closed systems, low energy consumption, and the ability to use non-arable land for microalgae cultivation. However, some disadvantages (e.g., poor light distribution, losses due to evaporation, predation, the need for large land areas, lack of control over weather conditions, and poor CO_2 dissolution from the air into the water) limit microalgae growth and result in low biomass productivity (YIN et al., 2020). High-rate ponds (HRP) are a low-energy, low-cost alternative for wastewater treatment. They are shallow and open ponds, 30 to 40 cm deep, with paddle wheels to promote agitation and prevent microalgae sedimentation. In addition, they can significantly reduce land use and water resources (BEHERA et al., 2019).

Tubular photobioreactors (vertical or horizontal), bubble columns, flat panels, and hybrid systems are examples of some bioreactors that can be used as closed systems for microalgal cultivation, of which tubular PBRs are the most commonly used industrially (LI et al., 2019). Unlike open systems, closed systems have better control over essential factors for microalgal growth and cultivation conditions, less water and CO_2 loss, and less risk of contamination by predators, allowing for higher concentrations of microalgal biomass. Some of the major drawbacks of the closed cultivation system are the high costs associated with installation, maintenance, and operation as well as the significant energy consumption (YIN et al., 2020).

3.3 Operation Modes in Microalgae Cultivation

The difference in cultivation in batch, fed-batch, semi-continuous and continuous reactors is mainly due to the way the substrate (culture medium) is introduced into the reactor (PETER et al., 2022).

In general, the batch system consists of a operation where there is no input or output of inputs during the reaction period, and the tank is completely emptied at the end of the process, except for air or chemicals for specific purposes during cultivation, such as pH controllers or antifoaming agents. As for microalgal biomass growth, the duration of the process is related to the time required to reach the maximum cell density (MATHIMANI et al., 2019). It has advantages such as efficient nutrient removal and low risk of contamination but also has disadvantages such as low volumetric biomass production and is limited to one cultivation cycle, which also has lost time (related to cleaning and starting a new batch) (YIN et al., 2020). The inoculum can be taken from a previous fermentation (recirculated), which saves costs.

Fed-batch is the main variation of the batch process, in which the process starts as a batch for biomass accumulation, and as the nutrient concentration decreases or runs out, one or more nutrients are added continuously or intermittently at a pre-defined constant or increasing/decreasing flow rate for biomass growth/maintenance and product formation, been all discharged at the end of the process. Its advantage is the maintenance of a low substrate concentration in the medium (avoiding substrate inhibition or toxicity), making it suitable for processes where the microorganism may be inhibited by high concentrations of contaminants present in the wastewater (GUAJARDO, SCHREBLER and DE MARIA, 2019; WANG et al., 2023).

The continuous system, on the other hand, is characterized by the initial introduction of a portion of the substrate (culture medium) and the inoculum, which are maintained in the reactor for a predetermined period until the culture stabilizes (usually a batch pre-step to promote propagation and adaptability of the microorganism). From this point on, the medium (wastewater) is continuously pumped in and the fermented medium is discharged at the same rate (usually by overflow) to maintain a constant volumetric rate within the reactor. Since the reactor volume is constant because the input volume is equal to the output volume, determining a hydraulic retention time (HRT) (θ) (relation between the volume and volumetric flow rate), and initially, there is a transient period in which the culture shifts from the batch regime to the continuous process until it stabilizes and reaches a steady state (contaminant and biomass concentrations remain constant) (COELHO et al., 2014). Some advantages of this system are that growth rates can be regulated and maintained for long periods and biomass concentration can be controlled by varying the dilution rate ($D = 1/\theta$), but some disadvantages are that long-term growth increases the risk of contamination and the original microbial strain may be lost (YIN et al., 2020). Continuous systems that work well always have higher productivity than batch processes because they have microalgae in high metabolic activity and concentration throughout the cultivation period, in addition to not having frequent downtime (as they operate continuously for months).

The difference between the continuous and the semi-continuous mode is related to the fact that in the semi-continuous mode, the process initially runs as a batch (microorganism/inoculum proliferation and culture adaptability, as mentioned for the continuous mode). However, after the first cycle of the process, part of the reactor volume is removed, and a new substrate is added corresponding to the removed volume in a fast way, thus, the volume remains constant during the cultivation (TAN et al., 2018). High volumetric productivity of biomass, high wastewater treatment capacity, and simple operation (based on a single variable – the percentage of volume to be removed and reintroduced into the system) are some of the advantages of semi-continuous operation. However, its disadvantages include high operating costs, the uncertainty of long-term operational stability, and greater susceptibility to contamination compared to batch operation (YIN et al., 2020). However, the semi-continuous mode has been shown to be the most promising operating mechanism for the growth of these microorganisms, as it can combine a shorter retention time with efficient biomass production (PETER et al., 2022).

The semi-continuous operation mode offers a number of advantages for microalgae cultivation compared to the batch mode, especially for large-scale production (TAN et al., 2018). This is because batch systems require a larger number of reactors to operate simultaneously to achieve relevant biomass production; moreover, this mode of operation has a long cultivation time and a period of inactivity after reactor discharge (lost time) (HE, YANG and HU, 2016). In the semi-continuous mode of operation, discharges occur regularly, facilitating cell separation in shorter periods, and since part of the reaction medium remains in the reactor, the remaining biomass acts as inoculum for continues the cultivation (serving as a highly concentrated and active inoculum). In addition, there is no lag phase of growth as the biomass is acclimated to the medium

(HAN et al., 2013). **Table 3.1** summarizes the main advantages and limitations of each operation mode.

Table 3.1 - Advantages and limitations of each operation modes. A) Batch, B) Fed-batch, C) Semicontinuous when the volumetric replacement time is higher than μ, D) Semicontinuous Semicontinuous when the volumetric replacement time is lower than μ, and E) Continuous. μ - Growth rate, S – substrate/contaminant/nutrient, and X – microbial/microalgal biomass.

Operation mode	Advantages	Bottlenecks
Batch (Discontinuous)	• High efficiency of contaminants/nutrients removal • Low risk of contamination • Easy operation and configuration	• Low biomass productivity • High Frequency of lost time (required to cleaning and start a new batch) • Cost of inoculum propagation
Fed-batch	• Possibility to adjust contaminant/nutriente concentration (minimizing or avoid substrate inhibition) • Hydric resources economy	• It is necessary to know microrganismo kinetics well • The feed must be compatible with the consumption of contaminant or additional batch fermentation time will be required • Less uniformity in product concentration
Semicontinuous	In comparison with a batch: • Long-term operation without the need for re-inoculation • High biomass volumetric productivity • High nutrient removal efficiency • Shorter hydraulic retention time In comparison with the continuous: • One variable to control (volume withdraw/replacement) • Microbial stress is lower	In comparison with a batch: • High operating cost • Possibility of long-term operation instability • High risk of contamination
Continuous	• High metabolic activity of the microorganism • Regulated growth rate • Controlled biomass concentration • Reproducible results	• Higher operating cost • High possibility of long-term operation instability • Higher risk of contamination • The original strain can be lost due contamination or genetic adaptations • Low frequency of lost time

In all modes inoculum can be recirculated in the system to reduce production costs (batch and fed-batch) or improve system productivity (semicontinuous or continuous).

Therefore, it is evident that not only nutritional and environmental factors affect microalgae growth, but also the mode of operation of the system, resulting in different rates of biomass production and pollutant removal (**Figure 3.1**). It is worth noting that, unlike other systems, the fed-batch regime operates in a transient regime due to variations in cell, biomass, and product concentrations as well as the increase in reactor volume over time, while other systems maintain a constant volume even in semi-continuous mode due to very rapid volume removal and insertion (PETER et al., 2022; TAN et al., 2018).

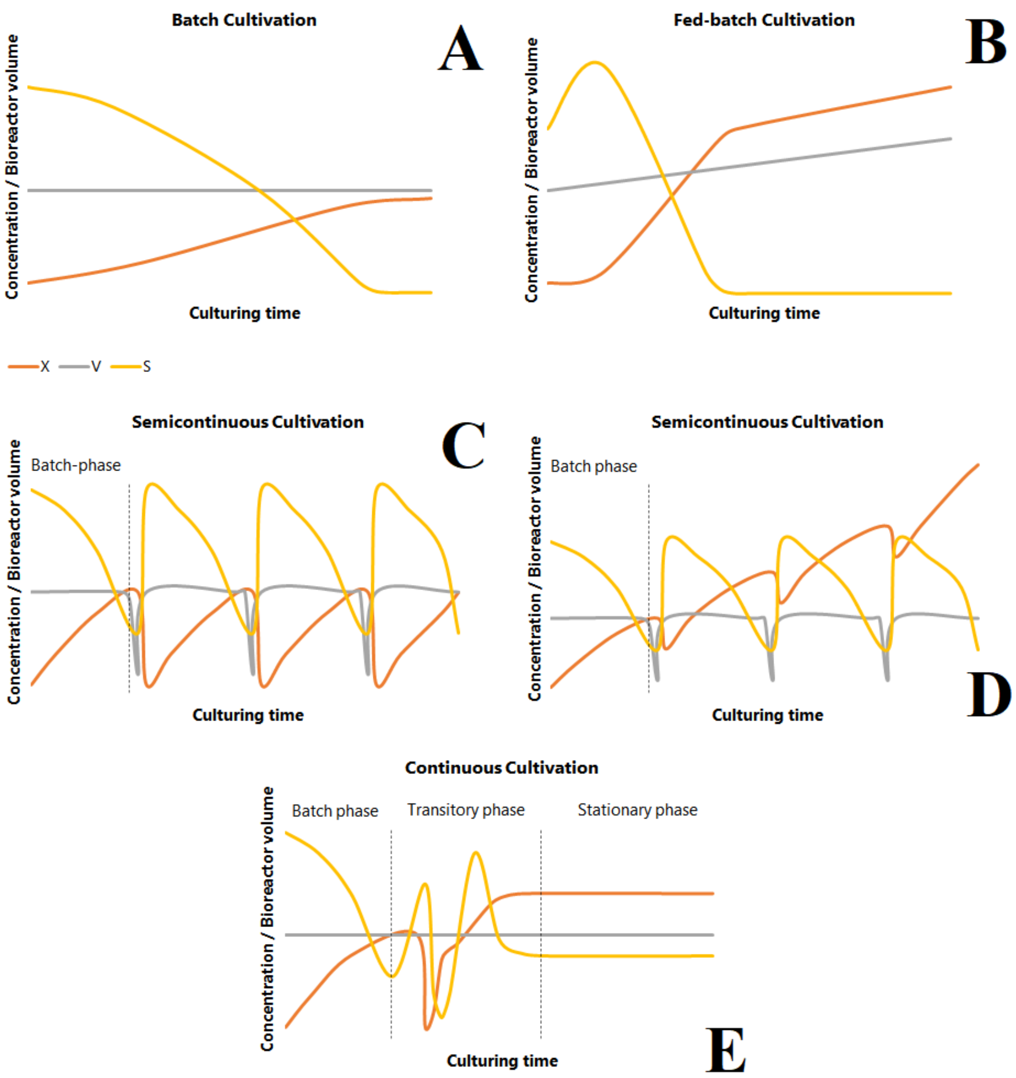

Figure 3.1 - Kinetic and volumetric profiles of the operating modes. X- biomass concentration, S – contaminant/substrate/nutrient concentration, and V – bioreactor volume. A – Batch, B – Fed-batch, C – Semicontinuous with volumetric replacement time lower than the growth rate (t < μ), D - Semicontinuous with volumetric replacement time higher than the growth rate (t > μ), and E – Continuous modes.

The following are examples of some wastewater treatment processes using different bioreactor configurations and operating modes, with comments on their efficiencies in microbial biomass production and pollutant removal (summarized in **Table 3.2**).

Table 3.2 - Wastewater treatment efficiencies in different bioreactor configurations and operation modes.

Species	Type of photobioreactor/ System (open/closed)/ Operation mode	Experimental Conditions	Microalgal Biomass Yield	Contaminant Initial Concentration / Removal Rate (%)	Reference
Chlorella vulgaris	1 L cylindrical glass reactor, 0.1 m diameter Open system Batch	Wastewater from anaerobic digestion. Working volume 0.2 L, turbidity 175 NTU, magnetic stirring, light intensity 5300 lx 12h per day, 24 °C, pH 7.	21.3 ± 1.4 µg mL^{-1} chlorophyll (3.49 × 10^7 microalgae cells)	NH_4^+-N = 350 mg L^{-1} / 98.7%; COD = 28.1 ± 0.5 mg L^{-1} / 77.2%; final pH = 10.4 ± 0.1	Greses et al. (2022)
Tetradesmus sp.	Flat panel Batch	Dairy wastewater, useful volume 40L, artificially aerated at a flow rate of 35 L min-1, solar illumination (12:12h light/dark cycle), 28°C, pH 7, 12 days.	2.38 g L^{-1}; Specific growth rate of 433.55 mg d^{-1}	COD = 3600 mg L^{-1} / 95.5%; Nitrates = 145.93 mg L^{-1} / 65,26%; Phosphates = 175.97 mg L^{-1} / 57.36%	Kiran e Mohan (2022)
Tetradesmus obliquus PF3	Column PBR Batch	Non-sterile wastewater, 600 mL working volume, 60 mL min^{-1} aeration flow, 10% CO_2 added, 6000 lux light intensity, 25 °C, 5 days	1.8 g L^{-1}	COD = 267 mg L^{-1} / 90%; N = 43 mg L^{-1} / 93.2%; P = 4.9 mg L^{-1} / 99%	Ma et al. (2020)

Chlorella vulgaris	Vertical bubble column PBR with 12 tubes Open Fed-batch	Urban wastewater, 12 days	0.1 g L^{-1} d^{-1}	COD = 101 mg L^{-1} / 36%; TN = 151 mg L^{-1} / 84%; TP = 23.8 mg L^{-1} / 95%	Gouveia et al. (2016)
Scenedesmus obliquus		Urban wastewater, 13 days, additional feeding with 50 L of wastewater was performed on day 8	0.4 *g* L^{-1} d^{-1}	COD = 131 mg L^{-1} / 63%; TN = 222.2 mg L^{-1} / 95%; TP = 29.9 mg L^{-1} / 92%	
Consortium C	Vertical bubble column PBR with 12 tubes Open Semi-continuous	Urban wastewater, 103 days, 60 L wastewater feed and 30 L culture recovery on days 37, 47, 57, 62, 72, 83, and 93	0.9 *g* L^{-1} d^{-1}	COD = 147 mg L^{-1} / 64%; TN = 473 mg L^{-1} / 98%; TP = 47.3 mg L^{-1} / 100%	
Chlorella thermophila	*Erlenmeyer flasks* Batch	Cattle wastewater (2.5%) combined with domestic wastewater, aeration rate 0.5 vvm, light intensity 100 µmol m^{-2} s^{-1}, 25 °C	2.17 g L^{-1}	NH_4^+ ≅ 600 mg L^{-1} / >95%; NO_3^- ≅ *85* mg L^{-1} >95%; PO_4^3 ≅ *40* mg L^{-1} / >99%	Jain, Mishra e Mohanty (2022)
	Fed-batch	Cattle wastewater (1.5%) combined with domestic wastewater,	4.52 g L^{-1}	NH_4^+ ≅ 380 mg L^{-1} / >95%; PO_4^3 ≅ 22.5-12.5 mg L^{-1} / >99%	

		aerated (0.5 vvm), light intensity 100 μmol m^{-2} s^{-1}, 25 °C, HRT of 14 days, the reactor was supplemented whenever the concentration of NH_4^+ in the wastewater fell to half of the initial value (< 200 mg L^{-1})			
Consortium consisting mainly of *Chlorella* and *Scenecesmus*	Fed-batch	Textile wastewater, working volume 4.5 L, light intensity 170.21 μmol m^{-2} s^{-1}, aeration rate 0.2 vvm, pH 8.2–9, which operated for 5 cycles of 95 days	2.57 and 1.95 for OD (optical density) of 680 and 750 nm, respectively	COD = 2200 mg L^{-1} / 52%; TN = 380.5 mg L^{-1} / 71%; TP = 94 mg L^{-1} / 98%	Kumar et al. (2018)
Tetraselmis suecica	Semi-continuous	Aquaculture wastewater, PBR fed automatically, 150 rpm agitation, 1.8 L m^{-1} aeration rate, 120 μmol m^{-2} s^{-1} light intensity (12:12 h), 27 °C, pH 8.2, HRT 7 days, re-feeding every 3 days.	0.9 g L^{-1}	N = 20 mg L^{-1}; 99.82%; P = 10.8 mg L^{-1} / 97.18%	Andreotti et al. (2020)

Consortium of filamentous cyanobacteria (*Geitlerinema* sp.) and microalgae (*Scenedesmus* sp. and *Coellastrella* sp.)	Bubble Column PBR Semi-continuous	Low-load domestic wastewater, working volume of 4L, aeration with a flow rate of 1 L min^{-1}, the pressure of 1 PSI, light intensity of 75 μmol m^{-2} s^{-1}, 16-20 °C, pH 8-8.8, the PBR was operated for 4 months with HRT 10 days, withdrawal of 0, 4 L of mixed liquor and refeed of 0.4 L of wastewater, followed by HRT 8 days withdrawal of 0.5 L of mixed liquor and refeed of 0.5 L of wastewater, and finally, HRT 6 days withdrawal of 0.6 L of mixed liquor and refeed of 0.6 L of wastewater.	HRT_{10} = 0.5 g L^{-1}; HRT_8 = 0.3 g L^{-1}; HRT_6 = 0.09 g L^{-1};	HRT_{10}: NH_4^+ -N = 52,5 mg L^{-1} / ≅ 82%; PO_4^{3-} -*P* ≅ 7 mg L^{-1} / 90% TDH_8: NH_4^+ -N ≅ 50 mg L^{-1} / ≅ 90%; PO_4^{3-} -*P* ≅ 6 mg L^{-1} / 85% TDH_6: NH_4^+ -N ≅ 60 mg L^{-1} / ≅ 50%; PO_4^{3-} -*P* ≅ 7 mg L^{-1} / 38%	Sólis-Salinas et al. (2021)
Chlorella vulgaris	Semi-continuous	Chicken compost as substrate (3.3% w/w nitrogen [as N]), 17 cycles of 3 days	0,1 g L^{-1} day^{-1}	Removal rate of NO_3^- between 50-60%	Tan et al. (2018)

Chlorella vulgaris	Cylindrical MPBR Continuous	Secondary wastewater, working volume of 4L, aeration rate of 0.5 L min^{-1}, light intensity of 101.5 to 112.3 µmol m^{-2} s^{-1} (4:1 red/blue light), 25 – 30°C, pH 6.8-7.2 controlled by CO_2 injection, 130 days, 2-day HRT and 21.1-day biomass retention time (BRT).	1.035 e 1.524 g L^{-1}	Dissolved inorganic nitrogen = 15 mg L^{-1} / 74,53-88,27%; Dissolved inorganic phosphorus = 0,8 mg L^{-1} / 82.5-98.75%	Gao et al. (2018)
Consortium consisting of *Chlorella* sp p., *Scenedes mus* spp., an d cyanobacteri a	2 parallel PBR, Plexiglas column Semi-continuous	Textile digital printing wastewater, 3L working volume, 300 rpm agitation, light intensity 100 µmol m^{-2} s^{-1}, light/dark cycle 12:12), pH 7.5 - 8.7, 50 days with 13 days DTH	6 x 10^6 cells mL^{-1}	COD = 764 ± 128 mg L^{-1} / 21.9%; Phosphate = 2 ± 1 mg L^{-1} / 81.5%; NH_4 -N = 200 mg L^{-1} / 9.15%	Marazzi et al. (2023)
Chlorella sp p.			8.6 x 10^6 cells mL^{-1}	COD = 764 mg L^{-1} / 26.9%; Phosphate = 2 mg L^{-1} / 65% NH_4 -N = 200 mg L^{-1} / 11.15%	
Chlorella sp. G-9	MPBR Continuous	Synthetic wastewater, working volume 1 L, air containing 4% CO_2, light intensity 140 µmol m^{-2} s^{-1}, 28	TDH4 = 284.8 mg L^{-1} TDH1 = 367.9 mg L^{-1}	Sulfadiazine = 0.5 mg L^{-1} HRT_4: 57.8-89.7%; HRT_2: 54.7-91.7%; HRT_1: 54.6-93.5%	Gao et al. (2023)

		°C, 63 days with HRT of 4, 2 and 1 days, BRT of 20 days.			
Chlorella vulgaris	1 L, 0.1 m diameter cylindrical glass reactor Open system Continuous	Anaerobic digestion wastewater. Working volume 0.2 L, turbidity 175 NTU, magnetic stirrer, light intensity 5300 lx 12h per day, 24 °C, pH 7, HRT 10 days, for 110 days.	25.2 µg mL $^{-1}$ de chlorophyll	NH_4^+ -N = 350 mg L^{-1} / 100%	Greses et al. (2022)

Greses et al. (2022) used dry anaerobic digestion wastewater as a medium to cultivate *Chlorella vulgaris*. Magnetically stirred batch reactors with a working volume of 0.2 L were used and operated under 12 h illumination conditions, at 24°C and an initial pH of 7. The experiments were varied in turbidity and nitrogen concentration such that the condition with a turbidity of 175 NTU and an initial nitrogen concentration of 350 mg L^{-1} provided a maximum microalgal growth of 21.3 µg mL^{-1} of chlorophyll (3.49×10^7 microalgal cells), resulting in a maximum removal of 98.7% for nitrogen and 77.2% for COD (initially at 28.1 g L^{-1}).

Using a batch mode of operation, Kiran and Mohan (2022) evaluated the potential of *Tetradesmus sp*. for the remediation of dairy wastewater in a flat-panel photobioreactor. The reactor used had a useful volume of 40 L, was artificially aerated (35 L min^{-1}), exposed to solar illumination (12:12h light/dark), and the pH and temperature parameters were kept constant at 7 and 28°C, respectively. After a retention time of 12 days, the authors achieved residual levels of COD (3600 mg L^{-1}), nitrates (145.93 mg L^{-1}), and phosphates (175.97 mg L^{-1}) of 160 mg L^{-1}, 50.69 mg L^{-1}, and 75.04 mg L^{-1}, respectively. In addition, biomass production reached a concentration of 2.38 g L^{-1} with a specific growth rate of 433.55 mg d^{-1}.

Ma et al. (2020) used *Tetradesmus obliquus* PF3 to treat unsterilized wastewater and evaluated the accumulation of carbohydrates in biomass. Column PBRs with a working volume of 600 mL were used with aeration at a flow rate of 60 mL min^{-1} (plus the addition of 10% CO_2), artificial illumination of 6000 lux, and a constant temperature of 25°C. After 5 days of cultivation in batch, the microalgal biomass had a maximum concentration of 1.8 g L^{-1} with a maximum specific growth rate of 1.8 d^{-1}. The COD removal rate (initially at 267 mg L^{-1}) was 90%, whereas the removal rates of the nutrients phosphorus (initially at 4.9 mg L^{-1}) and nitrogen (initially at 43 mg L^{-1}) were 99% and 93.2%, respectively.

Gouveia et al. (2016) used different microalgae species (e.g., *Chlorella vulgaris, Scenedesmus obliquus,* and *Consortium* C) isolated from wastewater and inoculated in an outdoor discontinuous fed-batch vertical bubble column PBR to treat urban wastewater. The *Chlorella vulgaris* experiment was conducted in September (COD = 101 mg L^{-1}, TN = 151 mg L^{-1}, TP = 23.8 mg L^{-1}) and lasted 12 days, with maximum productivity reached on the 5th day (100 mg L^{-1} d^{-1}) – maximum removal rates were 84% for TN on the 11th day, 95% for phosphorus on the 8th day, and 36% for COD on the 4th day. In October (COD = 131 mg L^{-1}, TN = 222.2 mg L^{-1}, TP = 29.9 mg L^{-1}), a 13-day experiment was conducted with *Scenedesmus obliquus*, with the addition of 50 L of wastewater on the 8th day; the maximum productivity of 400 mg L^{-1} d^{-1} was reached on the 9th day, and the removals of TN and phosphorus were 95% and 92%, respectively, on the 13th day; a COD removal rate of 63% was reached on the 5th day. The *Consortium* C experiment was conducted in November (COD = 147 mg L^{-1}, TN = 473 mg L^{-1}, TP = 47.3 mg L^{-1}) and lasted 103 days in semi-continuous feeding mode. Each feeding included about 60 L of primary wastewater and had a recovery of about 30 L of concentrated culture, which occurred on the 37th, 47th, 57th, 62nd, 72nd, 83rd, and 93rd days. The maximum productivity achieved was 900 mg L^{-1} d^{-1} on the 71st day, and the maximum removal rates were 98% for total nitrogen on the 36th day, 100% for phosphorus on the 83rd day, and 64% for COD on the 12th day.

Jain, Mishra and Mohanty (2022) studied the use of cattle wastewater (NH_4^+ = 22358 mg L^{-1}, PO_4^{3-} = 760 mg L^{-1}) as a supplement for the growth of *Chlorella thermophila* using batch and fed-batch operations. The experiments were performed in 250 mL Erlenmeyer flasks, aerated (0.5 vvm), and constantly illuminated (100 μmol m-2 s^{-1}) at 25°C. Eight concentrations of cattle wastewater (1-4.5%) combined with domestic wastewater (NH_4^+: 44.7 mg L^{-1}; PO_4^{3-}: 10.7 mg L^{-1}) were evaluated for discontinuous operation. The media with a concentration of 1.5 to 2.5% achieved a higher biomass production, reaching values of 2.17 g L^{-1} (2.5%) between the 8th and 9th day of incubation. Regarding nutrient removal, up to a concentration of 2.5%, the removal rate of NH_4^+ was over 95%, with the medium with a concentration of 2.5% achieving the lowest residual amount of this pollutant at 591.19 mg L^{-1}. In addition, PO_4^{3-} removal was about 99% for all concentrations of cattle wastewater studied. In the fed-batch mode, the three best conditions from the previous study (1.5-2.5%) were selected, and in this case, an intermittent feeding strategy was used during a retention period of 14 days. Biomass production in this system was 2.5 times higher than the yield obtained in batch mode, and nutrient removal analysis showed that in all cases the concentration of PO_4^{3-} was reduced by about 90% during the first few days. However, for NH_4^+, on the 3rd day of treatment, its concentration was still close to 50% in all cases, and after the second feeding, the medium with the highest concentration of cattle wastewater (2.5%) presented a stationary mode in the removal of this pollutant, highlighting the reduction of the same in concentrations of cattle wastewater of 1.5 and 2%.

Kumar et al. (2018) studied the treatment of textile wastewater (COD = 22 g L^{-1}, NT = 380.5 mg L^{-1}, TP = 94 mg L^{-1}) using a mixed consortium of microalgae (consisting mainly of *Chlorella* and *Scenedesmus*). The system consisted of an PBR with a working volume of 4500 mL, artificially illuminated (170.21 µmol m^{-2} s^{-1}), aerated (0.2 vvm), with pH maintained between 8.2 and 9, and operated in a fed-batch mode for five cycles (95 days), such that the reactor received 2.5 L of fresh textile wastewater at the end of each cycle. The research showed that it was possible to achieve organic removal efficiencies of 98%, 71%, and 52% for TP, NT, and COD, respectively. Biomass growth was measured by optical density (OD), and at the end of the 5th cycle, ODs of 2.57 and 1.95 were obtained for 680 and 750 nm, respectively.

Using the microalgae *Tetraselmis suecica*, Andreotti et al. (2020) studied the remediation of aquaculture wastewater in reactors operated in semi-continuous mode. The photobioreactors had agitation (150 rpm) and aeration (1.8 L min^{-1}), and the conditions of temperature, pH, and light intensity were kept constant at 27.5°C, 8.2 and 120 µmol m^{-2} s^{-1} (12:12h), respectively. Initially, the process was subjected to a batch period of three days to reach steady state; then, two hydraulic retention times (HRTs) were evaluated – 7 and 10 days, each one with 3 cycles of operation. The system with the shorter HRT had a higher biomass production (approximately 900 mg TSS L^{-1}) in 6 days, with a maximum daily productivity of 68 mg L^{-1} day^{-1}, and as a consequence, the removal of nitrogen (initially 20 mg L^{-1}) and phosphorus (initially 10.8 mg L^{-1}) in these reactors was also higher, reaching percentages of 99.82 and 97.18%, respectively.

In the study carried out by Sólis-Salinas et al. (2021), the biomass formed by a consortium of filamentous cyanobacteria (*Geitlerinema* sp.) and microalgae (*Scenedesmus* sp. and *Coellastrella* sp.) was cultivated in low-strength domestic wastewater. In this case, a bubble column photobioreactor with a working volume of 4 L, operated in semi-continuous mode, was used. The medium was agitated by air injection at a flow rate of 1 L min^{-1}, with a pressure of 1 psi and a light intensity of 75 μmol m^{-2} s^{-1}. The system was operated for four months, and hydraulic retention times (HRTs) of 10, 8, and 6 days were tested with similar carbon and nutrient loads. The use of a 10-day HRT showed the best N-NH_4^+ uptake rate (initially at 52.5 mg L^{-1}) consuming 6 mg L^{-1} d^{-1}, up to 90% removal of phosphorus (initially at 5 mg L^{-1}) in the last days of operation, and a maximum biomass content of 0.05 g L^{-1}, which was higher than the other HRTs.

Tan et al. (2018) used *Chlorella vulgaris* in a semi-continuous mode of operation for a growth optimization study using chicken manure as a substrate (3.3% w/w nitrogen [as N]). The study showed that the semi-continuous cultivation (initiated after the microalgae had reached its stationary phase – 12 days) could be operated for 17 cycles of 3 days each, with the removal/Insertion of 30% (v/v) of the culture medium, and produced a biomass production of up to 0.10 g L^{-1} d^{-1}, which was better than the batch system (12 days of cultivation at pH 3 in a 1 L photobioreactor), which reached a maximum production of 0.06 g L^{-1} d^{-1}. In addition, it was observed that nutrient removal was uniform during the cycles (50 to 60% nitrate removal), except for the 18th cycle, where nitrate removal was lower than the others (40%).

Gao et al. (2018) used *Chlorella vulgaris* to evaluate nutrient removal from secondary wastewater (initial concentration of 15 mg L^{-1} dissolved inorganic nitrogen [DIN] and 0.8 mg L^{-1} dissolved inorganic phosphorus [DIP]) and biomass production in a membrane photobioreactor (MPBR) with a working volume of 4 L and different hydraulic retention times (HRTs) in continuous operation. The best results were obtained for an MPBR with a stable operation of 130 days, an HRT of 2 days, and a biomass retention time (BRT) of 21.1 days. This model achieved biomass concentrations ranging from 1.035 to 1.524 g L^{-1} throughout the cultivation period and residual nutrient concentrations ranging from 1.76 to 3.82 mg L^{-1} for DIN and 0.01 to 0.14 mg L^{-1} for DIP.

In the study by Marazzi et al. (2023), textile digital printing wastewater (COD = 764 mg L^{-1}; phosphate = 2 mg L^{-1}; ammonia = 200 mg L^{-1}) was used as a substrate for microalgal biomass growth using two different inocula, one of which was a consortium (*Chlorella* spp., *Scenedesmus* spp., and cyanobacteria) (A) and the other only the microalga *Chlorella* spp. (B). For this purpose, a system consisting of four parallel photobioreactors (two with inoculum A and the others with inoculum B) with 3 L working volume, agitation (300 rpm), constant illumination (about 100 μmol m^{-2} s^{-1}; light/dark cycle of 12:12) and pH between 7.5 and 8.7 was used. Initially, the reactors were operated in batch mode for 15 days to allow the microalgae to acclimate to the medium, after which the pumps were turned on to operate the system in continuous mode with a hydraulic retention time (HRT) of 13 days for 50 days. During the discontinuous operation, the reactors receiving inoculum A (2.8×10^4 cells mL^{-1}) increased the biomass concentration by about three times, while those receiving inoculum B (8.2×10^3 cells mL^{-1}) increased it by four times. In terms of pollutant removal, system A achieved a minimum concentration of $N\text{-}NH_4^+$ of 156 mg L^{-1}, while system B achieved 170 mg L^{-1}. In the continuous tests, a high cell density was maintained throughout the process, with the average cell count for reactors A and B being 6×10^6 cells mL^{-1} and 8.6×10^6 cells mL^{-1}, respectively. At the end of the operation (from day 37), reactor A had a lower cell density compared to B. Regarding COD removal, an average residual COD of 596.7 mg L^{-1} and 558.7 mg L^{-1} was obtained for systems A and B, respectively. For nutrients, the residual concentrations of ammonium were 181.7 mg L^{-1} and 177.7 mg L^{-1} for systems A and B, respectively, and for phosphate, the concentrations ranged from 0.37 mg L^{-1} to 0.7 mg L^{-1}.

Gao et al. (2023) investigated the removal of sulfadiazine (SDZ) from synthetic wastewater using the microalga *Chlorella* sp. G-9 as a remediation technique. The batch experiment was performed in Erlenmeyer flasks (200 mL working volume) with different SDZ concentrations (0 to 100 mg L^{-1}), agitation (100 rpm), and illumination (161.1 μmol m^{-2} s^{-1}). The next step was carried out continuously in membrane photobioreactors (MPBRs) with a working volume of 1 L, receiving air containing 4% CO_2 and constant illumination (140 μmol m^{-2} s^{-1}) at a temperature of 28°C. The experiment included three MPBRs (membrane photobioreactor) operated with different hydraulic retention times (HRTs) (4, 2, and 1 days) for 63 days, with an average biomass retention time (BRT) of 20 days. The batch system showed that SDZ could inhibit the microalgae such that increasing its concentration (above 0.5 mg L^{-1}) had a negative effect on microalgal growth. Thus, in media without SDZ or with low concentrations (0.5 mg L^{-1}), the biomass concentration after 20 days was 92.9 and 81.6 mg L^{-1}, respectively. SDZ removal was

54%, 40%, 34%, and 19% for initial concentrations of 0.5, 1.0, 10.0, and 100 mg L^{-1}, respectively. During the continuous experiments, the authors found that a concentration of 0.5 mg L^{-1} of SDZ allowed a high biomass yield, and even at higher concentrations (100 mg L^{-1}), the biomass concentration remained at high levels, although there was significant fluctuation. Furthermore, the HRTs influenced the final biomass concentration such that shorter hydraulic retention times resulted in higher concentrations.

3.4 Conclusions and Future Prospects

Different operating modes result in different biomass productivities, which directly affect the percentage of pollutants removed from the wastewater. The choice of an open or closed system depends on the use of biomass after cultivation. Typically, open systems are more commonly used because the wastewater is contaminated with a microbial load and the main objective is to have treated water. If the biomass is to be used for a finer application, such as food, pharmaceutical or cosmetic, it is necessary to pre-treat the wastewater to ensure the required quality of the final product, which favors closed photobioreactors. The batch process, although simpler by putting everything in at the beginning and removing it at the end, generally offers lower productivity compared to other modes of operation, so the batch process is carried out to verify the technical viability of the process and then try another mode of operation that presents stability. Hydrodynamic stress is the main factor, in addition to nutritional and environmental factors, which is purely operational, which will dictate the best bioreactor and operating mode for a specific microalgal species. It is necessary to extend the study of wastewater treatment with bioreactors of different typologies and operating modes to seek greater optimizations of the wastewater treatment process, improving the economic viability of the process.

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Chapter 4: Developing a kinetic model to describe wastewater treatment by microalgae based on simultaneous carbon, nitrogen and phosphorous removal

Abstract

This work developed a kinetic model that could be used to describe wastewater treatment by microalgae using multiple and simultaneous contaminant removals, linking them to biomass production. Based on literature, kinetic models commonly used in wastewater treatment by microalgae were tested in published data and demonstrated limitation in kinetic prediction. In this sense, a sequential study to develop a kinetic model that could be applied and validated in similar random bioprocesses was made. For the contaminant removal, the *n*-th order kinetic model was the most efficient. On the other hand, for biomass production, the Monod (one limiting substrate) and Silva and the Cerqueira (multiple limiting substrates) models were considered efficient to describe wastewater treatment using COD (Chemical Oxygen Demand)/TOC (Total Organic Carbon), nitrogen and phosphorus content. The search for kinetic constants to describe biomass production was considered suitable, with a satisfactory predictive error of the calibrated model.

Keywords: n-th Order; Kinetics; Contaminant removal; Biological treatment; Monod model.

This article was published in Journal of Environmental Chemical Engineering (De Farias Silva CE, Cerqueira RBO, Lima Neto CF, De Andrade FP, Carvalho FO, Tonholo J. (2020) Developing a kinetic model to describe wastewater treatment by microalgae based on simultaneous carbon, nitrogen and phosphorous removal. Journal of Environmental Chemical Engineering, v. 8. https://doi.org/10.1016/j.jece.2020.103792).

4.1 Introduction

Microalgae are promising alternatives for biomass production and contaminant removal, due to their faster growth rates when compared to superior plants, ensuring greater productivity of biomass per unit area. Moreover, there is a wide variety of microalgae which can be found in lakes or oceans, with a unique biochemical plasticity (microalgal metabolism reacts to the different biochemical fractions to be produced, such as proteins, carbohydrates and/or lipids (KLINTHONG et al., 2015; GAO et al., 2016; SILVA et al., 2017a; DAS et al., 2019a,b).

One of the greatest costs involve microalgal cultivation, with some studies citing that these cultivation costs represent on average 80% of production costs. Therefore, the search for low-cost substrates is highly necessary, in order to enable the application of microalgae at a larger scale. Another factor that has an increasing effect on costs is often associated to the need of aeration with concentrated carbon gas, supplementing the media (vitamins and phosphate and nitrogen salts, for instance), as well as energy consumption (artificial lighting, stirring and/or pumping) (MITRA et al., 2012; PEREZ-GARCIA and BASHAN, 2015; WANG et al., 2016; SALATI et al., 2017).

Nevertheless, the use of urban and industrial effluents can eliminate the costs associated with the addition of nutrients in the culture media. Therefore, the combination of wastewater treatment, microalgae and biofuel production is a promising alternative for the recovery of nutrients and to increase the value of produced biomasses. In addition, the increasing consumption of drinking water, as well as the greater need for water treatment to be used in human activities are considered among the greatest priorities of the century (CABANELAS et al., 2013; CUELLAR-BERMUDEZ et al., 2017).

There are great environmental advantages in exploring photosynthetic organisms to be applied for industrial, food, biofuel, cosmetic, fertilizer purposes, among others, as they contribute to a more sustainable solution to the future, as long as they promote the carbon cycle, renewing carbon sources (CHO et al., 2013).

A patent search highlights the increased interest on this subject, with 149 applications using keywords *microalgae* + *wastewater* + *treatment* since year 2000. As an illustrative example, Hui and col. (2019) developed a process using microalgae coupled to ion transfer membrane in wastewater treatment for nitrogen and phosphorus removal technology with the biological electrochemical technology of an organic binder, and a bloom of nitrogen and phosphorus removal of sewage, claiming it is simple to operate, easy to control and a highly-efficient wastewater treatment (2019CN-0657353). In turn, Jianfeng and col. (2019) proposed an economical, environment-friendly method and apparatus for treating aquaculture wastewater to effectively remove antibiotics and organic carbon, nitrogen and phosphorus nutrients based on *Chlorella* species (2019CN-0649361).

One of the greatest drawbacks of bioprocesses at a larger scale, involving microalgal cultivation, lies on the difficulty of being able to simulate and control the optimal operating conditions of bioreactors (SILVA et al., 2019a; SILVA an BERTUCCO, 2019). In order to better estimate and optimise the productivity of microalgae under different conditions, it is necessary to rely on process modelling, which can provide useful information regarding the performance of microalgal cultivation systems. Therefore, it is crucial to develop a process model, as well as a growth kinetic model for describing microalgae cultivation (LEE et al., 2015).

Several papers have focused on urban/industrial/agroindustrial wastewater treatment, providing valuable experimental data on this subject, though without the application of a growth kinetic model (HODAIFA et al., 2013; CAPORGNO et al., 2015; CHEAH et al., 2016; GONZÁLEZ-FERNÁNDEZ et al., 2016; MASSA et al., 2017). Some examples of the models developed regarding contaminant removal, include a first-order kinetic model for contaminant removal and/or the Monod Model for a limiting substrate (WANG et al., 2014; SFORZA et al., 2014; TERCERO et al., 2014), a Verhuls logistic kinetic model for microbial growth and an associated model for substrate growth (RUIZ et al., 2013a,b; MANNA et al., 2015), and multiplicative factors for temperature, nitrogen and phosphorous considering a natural environment (lake) (HAARIO et al., 2009). Lee et al. (2015) points out that most kinetic models developed tend to be descriptive models, in an attempt to simplify the difficulties of measuring several variables to be able to develop satisfactory explanatory models, with the Monod model being increasingly used.

However, the most of these kinetic models are used in specific studies and make its application limited. It is also worth pointing out that little is found in the literature regarding kinetic models that can be used in this mixotrophic process, for the removal of multiple contaminants (mainly organic carbon, nitrogen and phosphorus), and not only for biomass production, but also for optimising the treatment and reuse of water from the process (LEE et al., 2015). With this in mind, the study of the behaviour of a process involving wastewater treatment using microalgae through a kinetic model for contaminant removal (substrate consumption) and biomass production is of great importance.

Accordingly, this article was aimed at developing a kinetic model to describe substrate consumption (mainly organic carbon, nitrogen and phosphorus, simultaneously) and microbial growth using microalgae for wastewater treatment, applied to data available in published literature, considering the physical meaning of the kinetic constants and the adjustment coefficient (model predictive error).

4.2 Materials and Methods

As highlighted in the Introduction section, the methodology adopted in this work initially consisted of a study, based on published literature, regarding kinetic models that could be used to describe the phases of the process - substrate consumption (contaminants) and biomass production during wastewater treatment by microalgae. By identifying the kinetic models and their respective constants, a research was carried out to determine whether the models found, and their parameters, could be applied in similar bioprocess conditions, using new data values (validation of the model previously studied).

4.2.1 Cultivation parameter and experimental data of the initial modelling

The data used in the present work were obtained in the literature from articles that did not perform any modelling of their experimental data. The initial tests were carried out using data from Shen et al. (2015), given the coordinated variations in contaminant concentrations, mainly of total organic carbon (TOC) and total nitrogen content (TN).

In that study, different concentrations of organic carbon were used, which were obtained from the addition of glucose in pre-determined concentrations for obtaining a simulated municipal effluent, combined with: 180.3 mg L^{-1} KNO_3 (25 mg L^{-1} NO_3–N), 13 mg L^{-1} KH_2PO_4 (3 mg L^{-1} PO_4^{3-}–P), 37.5 mg L^{-1} $MgSO_4.7H_2O$, 18 mg L^{-1} $CaCl_2.2H_2O$, 3 mg L^{-1} citric acid, 5 mg L^{-1} $FeSO_4.7H_2O$, 0.5 mg L^{-1} EDTA, 10 mg L^{-1} Na_2CO_3 and 1.0 mL L^{-1} of A5 + Co solution. The A5 + Co solution contained 2.86 g L^{-1} of H_3BO_3, 1.81 g L^{-1} $MnCl_2.H_2O$, 222 mg L^{-1} $ZnSO_4.7H_2O$, 79 mg L^{-1} $CuSO_4.5H_2O$, 390 mg L^{-1} $Na_2MoO_4.2H_2O$ and 49 mg L^{-1} $Co(NO_3)_2.6H_2O$.

Lastly, the concentrations of total organic carbon tested were as follows: 20, 40, 60, 80 and 120 mg L^{-1}. The initial nitrogen and phosphorus concentrations remained constant, being equal to 25 and 3 mg L^{-1}, respectively; similar to the concentrations of the original effluent considered. The media conditions included pH 7.0-8.0, light intensity of 40 µmol photons m^{-2} s^{-1} and temperature of 25 ºC in a photobioreactor (PBR) aerated with an airflow of 300 mL min^{-1}. In this paper, no phosphorus concentration was not used, as the consumption profiles were similar in all experiments, i.e. no significant variation was observed, being, thus, disregarded in the analysis of the kinetic model.

4.2.2 Definition of the kinetic models for contaminant removal and cell growth

4.2.2.1 Substrate consumption (Nutrient/Contaminant)

A study was carried out with the main contaminants, which included total nitrogen and total organic carbon or COD (chemical oxygen demand). Wang et al. (2014) used a first-order kinetic reaction to study a contaminant removal model, with the Monod Equation being applied to describe biomass production. For a better analysis to be carried out, a comparison between first-order (Equation 4.1), second-order (Equation 4.2) and *n*-th order (Equation 4.3) kinetic models was performed, in order to analyse the respective adjustments, as well as the similarities and differences in the representation of the experimental data and how these models could influence the calculation of the concentration of biomass produced during the process for obtaining the best fit.

$$S_i = S_0 e^{-kt} \qquad \text{(1st order)} \qquad (4.1)$$

$$S_i = \frac{S_0}{(1+(S_0 kt))} \qquad \text{(2nd order)} \qquad (4.2)$$

$$S_i = ({S_0}^{1-n} + (n-1)kt)^{\frac{1}{1-n}} \qquad (n\text{-th order}) \qquad (4.3)$$

where S_i is the nutrient/contaminant concentration at time *i*, S_0 is the initial nutrient/contaminant concentration (t = 0), both expressed in mg L^{-1}; *k* is the reaction constant, in day^{-1} (first-order), L mg^{-1} day^{-1} (second-order) and (L $mg^{-1})^{n-1}$ day^{-1}, *t* is the time (days) and *n* is substrate consumption kinetic order.

3.2.2.2 Model for Cell Growth

In order to represent the production of biomass over time, the classic Monod reaction for microbial kinetics (Equation 4.4) and the modified Monod reaction were used, with the latter considered a *n*-th order Monod modified model with some modifications and called the Silva and Cerqueira model in this paper (Equation 4.5). Some models take into account light intensity and kinetic models considering multiple factors, such as light and substrate, for example (LEE et al., 2015). However, due to nitrogen and organic carbon limitations in wastewater treatment by microalgae, light is provided in high intensity and, generally, constant on the cultivation/treatment system (RUIZ et al., 2013a,b; TERCERO et al., 2014; WANG et al., 2014; MENNAA et al., 2015; MASSA et al., 2017), even though it can be an important variable on the process (LEE et al. 2015). Nevertheless, a lack of data (experiments performed in different light intensities with a growth curve coupled to contaminants removal kinetics) to validate this factor is observed, thus, it is not possible to include this variable in the present study, with light intensity being considered constant in all cases (based on experimental results).

$$\mu = \mu_{max} \frac{Si}{K_{s,i}+Si} \qquad \text{(Monod)} \qquad (4.4)$$

$$\mu = \mu_{max} \frac{Si^m}{{K_{s,app}}^m+Si^m} \qquad \text{(Silva and Cerqueira Model)} \qquad (4.5)$$

where: μ represents the specific growth rate (day^{-1}); *Si* the substrate concentration; μ_{max} the maximum growth rate of the microorganism (day^{-1}); $K_{S,i}$ is the Monod half-saturation constant; *m* the Silva and Cerqueira model order and $K_{S,app,i}$ the apparent half-saturation constant (mg L^{-1}), correlated with the Monod half-saturation constant, where $K_{S,app,i} = K_{S,i}{}^{p}$, with *p* representing a fitting constant.

The kinetic constants for the components of each model were estimated using Solver® ME, through the Generalized Reduced Gradient (GRG) method and validated by the Method of Least Squares. The model's predictive error (MPE) between the theoretical curve and the experimental points was calculated using Equation 4.6 to validate the models tested in the present work.

$$MPE\ (\%) = \frac{100}{n} . \sum_{i=1}^{n} \left| \frac{x_{exp}-x_{calc}}{x_{calc}} \right| \qquad (4.6)$$

where: x_{exp} represents the experimental data, x_{calc} represents the calculated data and *n* the number of experimental points.

4.2.3 Investigation of wastewater treatment modelling

As previously pointed out, the additional modelling performed using other data found in the literature was carried out aimed at investigating the possibility of generalising the kinetic models implemented in the previous phase.

Firstly, data from Cho et al. (2013) was used. This study used total nitrogen and total phosphorus due to their high variations, being important for the implementation of the kinetic models. In the given study, *Chlorella sp.* was cultivated in a 1 L PBR reactor, under continuous light intensity of 200 µmol m^{-2} s^{-1} and constant aeration of 1% CO_2, at an airflow of 0.4 L min^{-1}. The initial pH and temperature were set to 7 ± 0.1 and 30 ± 2 ºC, respectively. The authors studied the capacity of the microalga in treating different types of effluents, with combined wastewater of anaerobic digestion (CAD) and the effluent of anaerobic digestion from a primary settling tank (PS).

In addition, the modelling for the system studied by González-Fernández et al. (2016) was also studied. In that work, the authors used a combination of microalgae (*Chlorella vulgaris*, *Scenedesmus obliquus* and *Chlamydomonas reinhardtii*) grown in an urban wastewater from a Wastewater Treatment Plant in Valladolid (Spain). The physicochemical characterisation of the effluent presented a COD of 259.5 mg L^{-1}, with nitrogen in the form of ammonia at 80 mg L^{-1} and 14.5 mg L^{-1} phosphate. The microalgae were cultivated in a photobioreactor with a 1L photobioreactor with a water jacket (at a corresponding temperature of 23 ºC), under 550 lux light and a photoperiod of 14hrs.

Therefore, based on the data presented, the present work sought the models that best fit the data of the process in study. Thus, a study was carried out on the models that best described the consumption of nutrients/contaminants (COD, total nitrogen, total phosphorus and organic carbon), which are most commonly present in the characterisation of the various types of effluents and studies published in the literature (SILVA et al., 2019b), as well as in the production of biomass resulting from the process.

4.3 Results and Discussion

The present study was aimed at analysing contaminant removal and biomass production using various kinetic models developed in other articles using microalgae. The removal of each substrate at a time was first observed in each case, as well as the production of microalgae. With this in mind, reference articles were used for the collection of data and for verifying the substrate consumption kinetics, as well as biomass production. Different kinetic orders were tested for substrate consumption, aimed at obtaining a better modelling of the process. Subsequently, a modified Monod kinetic model (Silva and Cerqueira Model) was tested, considering multiple substrates and including a validation step of the model.

4.3.1 Contaminant removal and biomass production using first, second and n-th order kinetics

4.3.1.1 First and second-order kinetics

The first and second-order models were not satisfactorily adjusted to the experimental data, for TOC and TN (Total Nitrogen), with the model's predictive error (MPE) ranging between 10 and 58%, thus, considered high.

It is important to point out the following inconsistencies of the models considered in this step: 1) The models for substrate consumption exhibited a better fit with the second-order model for TOC and the first-order kinetic model for TN; 2) Nevertheless, biomass production was modelled in the same way for both models, and they were not considered a suitable model to predict the entire growth curve, since only one part of the exponential growth phase was efficiently simulated.

Nonetheless, positive aspects could also be observed, following the Monod kinetics: 1) The values of μ_{max} (1.15-1.72 day^{-1}) are found in the literature (Lee et al. 2015; Silva et al. 2016; Silva et al. 2017b); 2) The value of Ks was constant and approximately similar in all cases, both in terms of TOC and TN. $Ks_{,TOC}$ exhibited a value of 23.12 ± 2.91 mg of TOC L^{-1} for both kinetic models, and $Ks_{,TN}$ was equal to 12.33 ± 1.55 mg of TN L^{-1} for a first-order consumption kinetics and equal to 12.77 ± 1.59 mg of TN L^{-1} for a second-order consumption kinetics. Tercero et al. (2014) found a half-saturation constant of total nitrogen equal to $K_{S,TN}$ = 23.4 mg L^{-1}, close to what was found in the present work.

Following the analysis of the model's predictive error obtained for the first and second-order kinetic models, it was considered suitable to evaluate the behaviour of the process for a *n*-th order kinetic model, in an attempt to reach a more efficient modelling of substrate consumption (thus, reducing the error between the experimental and the calculated data), and consequently enabling the application of the Monod kinetics for the entire growth curve.

4.3.1.2 *n*-th order substrate kinetics

As previously mentioned, in an attempt to find a single model that could describe the process of biomass production, while also minimising the error associated with the substrate consumption kinetics, a *n*-th order kinetic modelling was performed.

The modelling was repeated for the following concentrations: 20-120 mg L^{-1} of TOC (Total Organic Carbon) and TN (Total Nitrogen), aiming at finding the most suitable kinetic order, as well as the respective kinetic constant for substrate consumption, having subsequently calculated the error associated to each concentration used. At first, it was verified whether the same kinetic order would be applicable to all concentrations, or if each concentration would, otherwise, follow a different order. This analysis is illustrated in **Figure 4.1**. It is observed that with both concentrations, TOC substrate consumption was efficiently modelled.

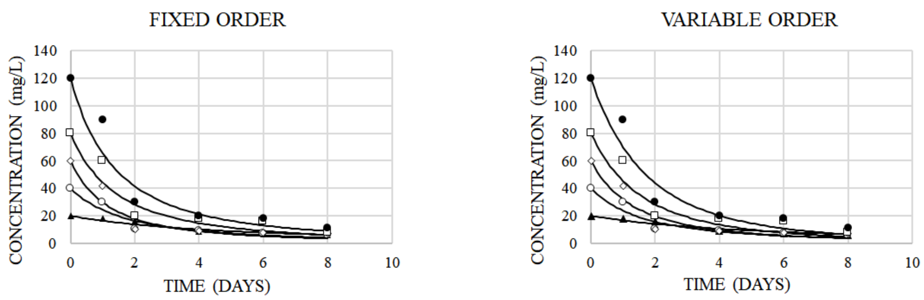

Figure 4.1 - Comparison of substrate consumptions for fixed and variable kinetic orders. Initial TOC concentrations are respectively, (●), (□), (◊), (○) and (▲) for 120, 80, 60, 40 and 20 mg L^{-1}. Standard deviation of the experimental points was lower than 5%.

In turn, for both cases, a comparison between the kinetic constants and the kinetic order, in terms of a fixed and variable *n*-th order, can be observed in **Figure 4.2**.

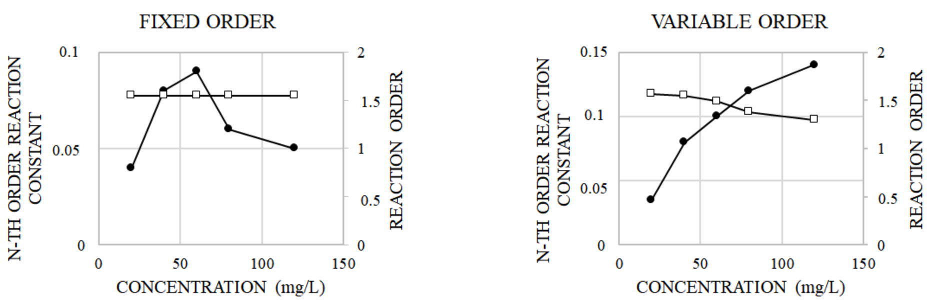

Figure 4.2 - Comparison between the kinetic constants and the kinetic order for TOC consumption. (●) represents the reaction constant and (□) the reaction order. Standard deviation of the experimental points was lower than 5%.

According to **Figure 3.1**, the difference between both cases did not show any significant variation. When considering a variable kinetic order, the constants were inversely proportional, while, for a fixed order, the kinetic constants increased up to a certain concentration and then decreased (with a possible substrate inhibition being observed, which is common with microalgae and cyanobacteria (LEE et al., 2015; SILVA et al., 2016)). This behaviour can be associated to the availability of substrate to the microalgae. Thus, a fixed order was adopted to describe TOC consumption, given that the variation between the values modelled (**Figure 3.1**) was not significant, also enabling a better application of the Silva and Cerqueira model (proposed in the present article). Therefore, it can be observed that only the values of μ_{max} and Ks suffered variations. The results obtained for a kinetic order of 1.55 (reason why the models of first (1) and second (2) order did not fit well the experimental data i.e., because the reaction order more suitable was between them), as well as the reaction constants and respective MPE for TOC consumption are presented in **Table 4.1**.

Table 4.1 - Kinetic order and kinetic constants for TOC and TN consumption.

TOC	TOC Concentration (mg L^{-1})				
	20	40	60	80	120
Kinetic order (*n*)	1.55				
k (L $mg^{-1})^{(n-1)}(day^{-1})$	0.04	0.075	0.09	0.06	0.05
MPE (%)	9.70	16.76	17.65	17.57	21.84
TN	20	40	60	80	120
Kinetic order (*n*)	1.55				
k $(Lmg^{-1})^{(n-1)}(day^{-1})$	0.07	0.07	0.11	0.08	0.10
MPE (%)	32.09	38.92	23.53	24.19	25.55

When comparing the errors obtained for the first and second-order reactions with the errors found for the *n*-th order reaction, it was found that the latter errors were lower than the former (sometimes, more than 50% lower). Therefore, it can be implied that the use of a *n*-th order consumption kinetics is more efficient for the factor studied (TOC). Regarding TN, a fixed kinetic order equal to that found for TOC consumption was used, given that the use of a same kinetic order to describe substrate consumption can help to simplify the models. **Table 3.1** also presents the respective constants and MPE calculated for each TN concentration.

However, the use of a kinetic order equal to 1.55 (the same used for TOC) to describe TN consumption was not efficient in all cases. In some cases, the error was slightly higher when compared to the first and second-order consumption kinetics. This behaviour can either be due to the error associated to the experimental data obtained, or that a fixed kinetic order is not applicable in this case, with the substrate consumption order being different than the kinetic order to describe biomass production. This case will be more thoroughly discussed in the following section, with the use of multiple substrates in the model.

The kinetic data found showed to be physically plausible, as well as similar to the results found by Wang et al. (2014), who obtained values of k between 0.05-0.16 day^{-1} for the removal of total nitrogen during wastewater treatment, though using a first-order kinetic model. In turn, Tercero et al. (2014) and Mennaa et al. (2015), despite analysing microbial growth kinetics in terms of contaminant variation, did not determine the order of substrate consumption kinetics.

4.3.2 Monod Kinetics considering one substrate

After determining the parameters for substrate consumption, the parameters to describe biomass production were also established, considering an n-th order substrate consumption and each substrate separately. In this case, the Monod equation was also used (Equation 3.4), with the following concentrations being used in the biomass production modelling: 20-120 mg L^{-1}, analysing TOC and TN separately. As the Monod constants had already been determined in the previous model, the same values were used as much as possible, being only slightly changed, depending on the case, in order to optimise the proposed modelling. **Table 4.2** presents the results obtained, as well as the mean squared errors for each case.

When using a n-th order to describe substrate consumption and the Monod model for biomass production, a small error was observed. Therefore, it can be concluded that when only using a limiting substrate, the Monod model can be applied, thus, corroborating the findings by Ruiz et al. (2013a), Wang et al. (2014), Tercero et al. (2014), and demonstrated by Lee et al. (2015) as being the most commonly used model for modelling microalgae growth, in its simple or modified form.

Consequently, the *n-th* order kinetics was extremely satisfactory to describe substrate consumption. **Figure 4.3** illustrates substrate consumption and biomass production and the respective simulations.

Table 4.2 - Monod constants and *n*-th order kinetic constants for TOC consumption.

TOC	Concentration (mg L^{-1})				
	20	40	60	80	120
Kinetic order (*n*)	1.55				
k $(Lmg^{-1})^{(n-1)}(day^{-1})$	0.04	0.075	0.09	0.06	0.05
μ_{max} (day $^{-1}$)	1.37	1.38	1.38	1.06	0.97
$K_{S,COT}$ (mg of TOC L^{-1})	27.88	22.33	21.55	19.78	20.87
	21.55 ± 2.16 (average and deviation from the five growth curves considered)				
MPE (%)	20.05	14.95	14.30	18.51	21.34

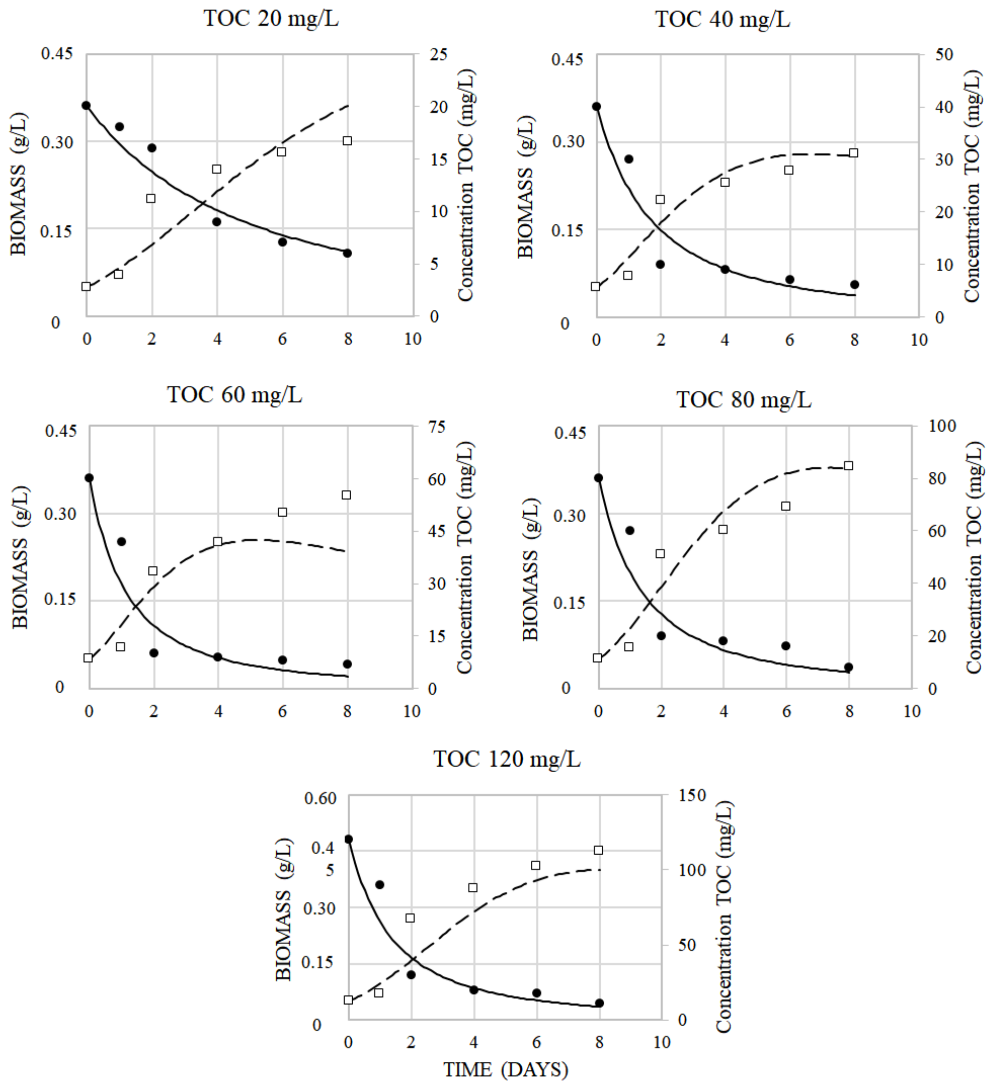

Figure 4.3 - Biomass production and TOC substrate consumption for each concentration and kinetic order. (●) represents the experimental values for substrate consumption and (□) for the biomass produced. Lines represent the simulation of the models using (---) for biomass production and (▬) for substrate consumption. Standard deviation of the experimental points was lower than 5%.

Subsequently, the same procedures were carried out considering TN as substrate. The results for TN consumption, as well as the MPE for biomass production at each concentration are presented in **Table 4.3**. The results for TN were similar to the ones observed for TOC, with only the Monod model being used. **Figure 4.4** represents TN consumption and biomass production. By observing the results found, it can be noted that both μ_{max} and $K_{S,NT}$ are close to the figures found by Haario et al. (2009), Hodaifa et al. (2012), Ruiz et al. (2013a), Tercero et al. (2014) and Mennaa et al. (2015), who found values for this constant ranging between 0.1-1.25 day^{-1}.

Table 4.3 - Monod constants, *n*-th order kinetic constants for TN consumption.

TN	Concentration (mg L^{-1})				
	20	40	60	80	120
Kinetic order (*n*)	1.55				
k (Lmg^{-1})$^{(n-1)}$(day^{-1})	0.07	0.07	0.085	0.08	0.10
μ_{max} (day $^{-1}$)	1.13	1.17	1.33	1.30	1.33
$K_{S,TN}$	14.51	15.18	14.12	14.12	10.08
(mg of TN L^{-1})	14.12 ± 1.41				
	(average and deviation from the five growth curves considered)				
MPE (%)	12.52	15.90	13.47	13.88	17.31

When the Monod model was applied, only one limiting substrate was used. However, among the nutrients studied, the carbon source (in terms of TOC or COD), nitrogen and phosphorus are considered equally important for simulating the process, with the data being also more easily found in the articles published (2019). Although temperature and light intensity are also important (2015), they were not considered in the present work, given that most of the experimental data available, and the number of points required for modelling, consider constant light intensity and temperature.

Therefore, aimed at seeking a model that considered multiple contaminants, a modified Monod Model was developed and tested (Silva and Cerqueira Model), having simultaneously considered the influence of more than one limiting substrate, though still using the data available from Shen et al. (2015) for TOC and TN.

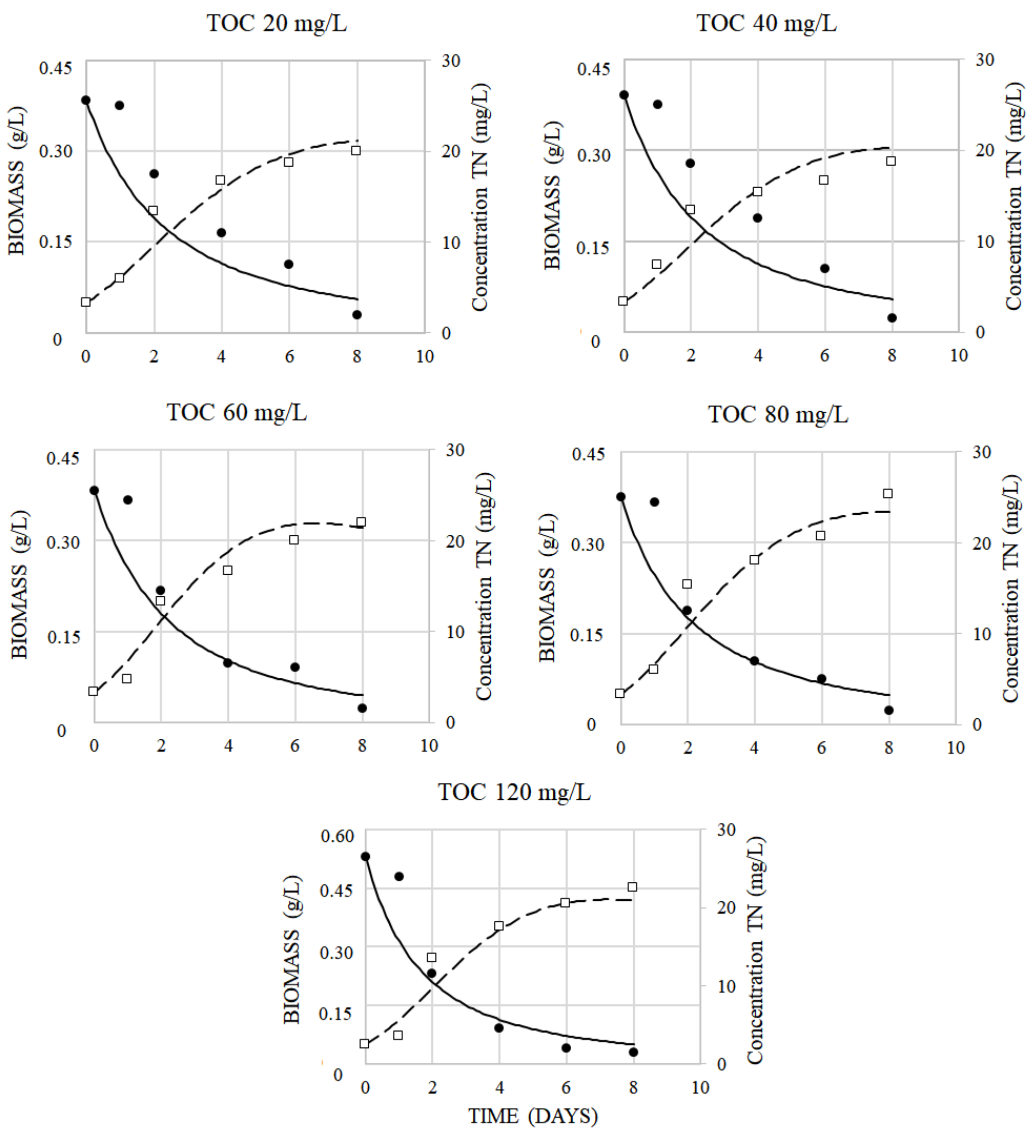

Figure 4.4 - Biomass production and TN substrate consumption for each concentration and kinetic order. (●) represents the experimental values for substrate consumption and (□) for the biomass produced. Lines represent the simulation of the models using (---) for biomass production and (▬) for substrate consumption. Standard deviation of the experimental points was lower than 5%.

4.3.3 Silva and Cerqueira Model considering multiple substrates

In theory, models considering multiple factors are the most reliable forms of modelling the effect of substrates on microalgae growth, especially when considering limiting substrates simultaneously, as is the case of wastewater treatment (LEE et al., 2015). This relationship is usually based on the Monod equation, presented in Equation 4.7.

$$\mu = \mu_{max} \frac{S_1}{(K_{S,1}+S_1)} \frac{S_2}{(K_{S,2}+S_2)} \cdots \frac{S_n}{(K_{S,n}+S_n)} \tag{4.7}$$

where: μ_{max} is the specific growth rate in day^{-1}, S_i substrate concentration in mg/L and $K_{S,i}$ the half-saturation constant for each substrate in mg L^{-1}.

However, modelling was not consistent, having represented biomass production as a function of substrates (TOC and TN) poorly. In order to verify if the kinetic order of the Monod equation was appropriate, a *m*-th order was added to the equation, as represented in Equation 4.8.

$$\mu = \mu_{max} S^m / ({K_{S,app}}^m + S^m) \tag{4.8}$$

where: μ_{max} is the specific growth rate in day^{-1}, S substrate concentration in mg/L, $K_{S,app}$ is the apparent half-saturation constant for the substrate (mg L^{-1}).

Nevertheless, the sensitivity to the value of $K_{S,app}$ on the system proved to be high, indeed, having the effect of an apparent *Ks*. Thus, an adjustment factor was used in Equation 4.9.

$$K_{S,app} = {K_S}^p \tag{4.9}$$

where: $K_{S,app}$ is the apparent half-saturation constant for the substrate (S), *Ks* is the half-saturation constant, p is the curve-fitting constant for K_S, with the same constant being used for all substrates.

Therefore, a modified model (Silva and Cerqueira Model), considering TOC or COD, TN and TP (total phosphorus) can be represented by Equation 4.10.

$$\mu = \mu_{max} \frac{{S_{COD}}^m}{(({K_{S,COD}}^p)^m + {S_{COD}}^m)} \cdot \frac{{S_{TN}}^m}{(({K_{S,TN}}^p)^m + {S_{TN}}^m)} \cdot \frac{{S_{TP}}^m}{(({K_{S,TP}}^p)^m + {S_{TP}}^m)} \tag{4.10}$$

The results of the constants obtained for the *n*-th order model were used for TOC and TN, as they were considered satisfactory to simulate the consumption of the contaminant. Thus, the consumption of both substrates was considered to represent biomass production. The values obtained for the constants in this model, as well as the model's predictive error (MPE) calculated for each concentration, are presented in **Table 4.4**. These values show the success of the model proposed, as only a single equation is used to describe the substrate consumption and one equation to describe biomass production using multiple substrates with a MPE around 20-30%, which is graphically suitable to model the experimental data, i.e. a MPE value of 20-30% showed to be sufficient for the study performed. **Figure 4.5** illustrates the adjustments for the curve representing the consumption of the substrates and biomass growth.

Table 4.4 - Kinetic constants using Silva and Cerqueira Model.

TOC Concentration (mg L^{-1})	20	40	60	80	120
Silva and Cerqueira Model kinetic order (m)	0.75	0.70	0.60	0.55	0.72
			0.60 ± 0.06 (average and deviation from the five growth curves considered)		
Fitting constant for K_S (p)	0.62	0.70	0.60	0.51	0.46
			0.58 ± 0.07 (average and deviation from the five growth curves considered)		
μ_{max} (day $^{-1}$)	1.10	1.38	1.38	1.03	0.97
$\boldsymbol{K_{S,TOC}}$ (mg of TOC $L^{-1})^{m.p}$	27.88	22.33	21.55	20.87	20.87
$\boldsymbol{K_{S,NT}}$ (mg of TN $L^{-1})^{m.p}$	14.51	15.18	14.12	14.12	10.08
MPE (%) to TOC	9.70	19.66	28.19	33.60	21.19
			22.47 ± 5.62 (Average and deviation of the MPE for the five substrate consumption curves)		
MPE (%) to TN	27.11	25.11	13.89	24.19	25.55
			23.17 ± 3.09 (Average and deviation of the MPE for the five substrate consumption curves)		
MPE (%) to Growth curve	22.63	16.57	13.66	22.95	27.25
			20.61 ± 3.66 (Average and deviation of the MPE for the five growth curves)		

For substrate consumption, model constants used the values presented in Tables 41-4.3.

The study carried out by Shen et al. (2015) supported the verification of the models to be used. Accordingly, it was concluded that the best models for describing substrate consumption and biomass production were the Monod Model (for one substrate) and the Silva and Cerqueira Model (multiple substrates) when a *n-th* order kinetics is used for substrate consumption. However, for a better demonstration of the results obtained with the application of the type of system considered, other studies were used for validation.

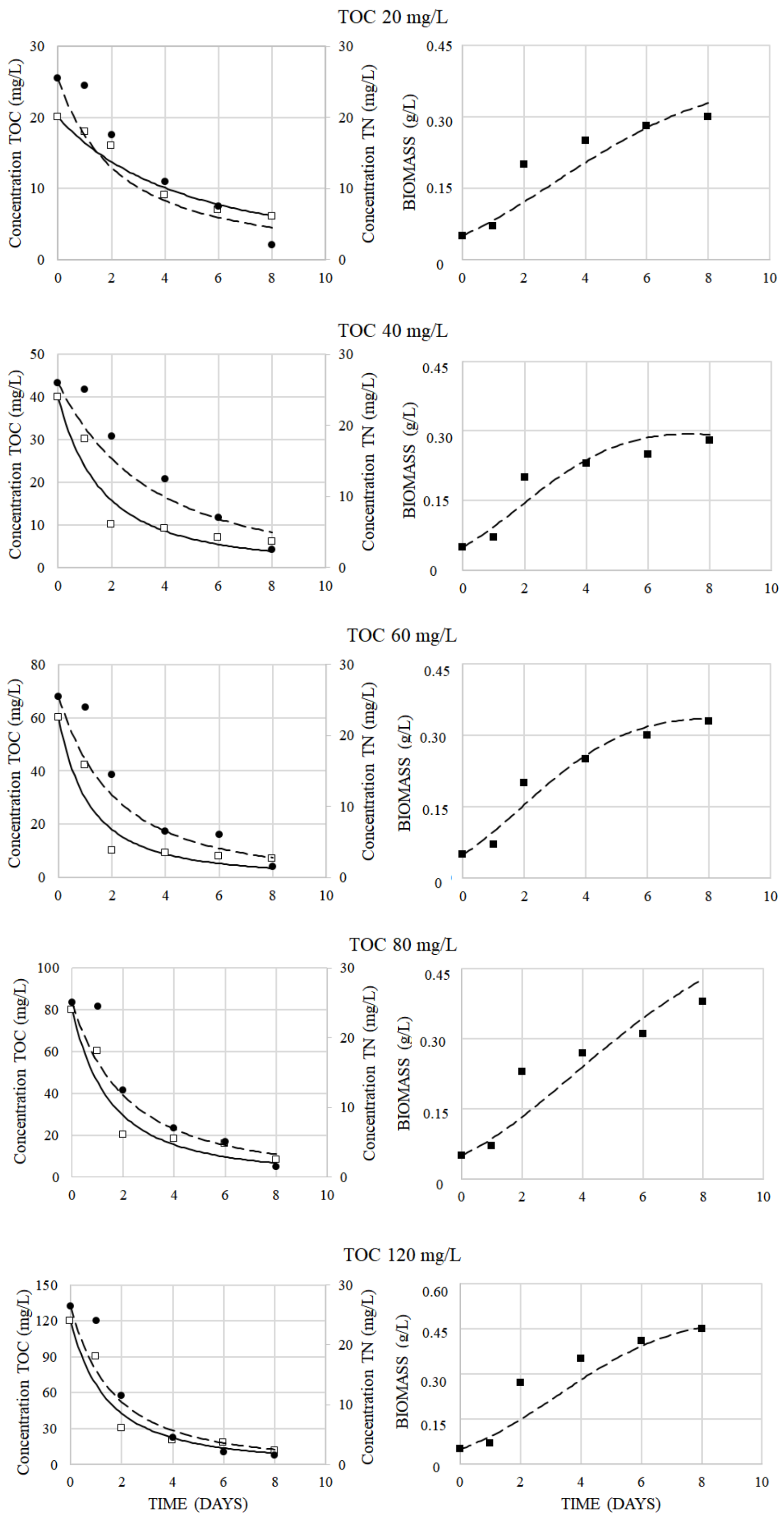

Figure 4.5 - Biomass production, and TOC and TN substrate consumption for each concentration. On the left, (●) represents the experimental values for TN and (□) for TOC for the substrate consumption curves. Lines represent the simulation of the models using (---) for TN and (▬) for TOC consumption. On the right, (■) represents the produced biomass and the line represents the simulation of the model for biomass production (---). Standard deviation of the experimental points was lower than 5%.

4.3.4 Model validation using other experimental data

4.3.4.1 Data from the study carried out with anaerobic digestion wastewater treated by microalgae

In the study carried out by Cho et al. (2013), the modelling was only performed for *n*-th order substrate consumption kinetics, with the Monod model being applied when considering the contaminants (TN – Total Nitrogen and TP – Total Phosphorous) separately, and the Silva and Cerqueira model when simultaneously using both contaminants.

Table 4.5 - Kinetic constants for TN and TP, substrate consumption and cell growth.

Effluent	CAD	PS
Substrate Consumption – TN		
Kinetic order (n)	1.19	1.69
k (L mg^{-1})$^{(n-1)}$(day^{-1})	0.24	0.099
MPE (%)	31.28	27.12
Cell Growth (Monod) – TN		
$\mu_{\boldsymbol{max}}$ (day $^{-1}$)	1.83	2.99
$\boldsymbol{K_{S,NT}}$ (mg of TN L^{-1})	21.81	21.21
MPE (%)	23.32	17.11
Substrate Consumption – TP		
Kinetic order (n)	1.77	1.70
k (L mg^{-1})$^{(n-1)}$(day^{-1})	0.16	0.170
MPE (%)	23.23	25.19
Cell Growth (Monod) – TP		
$\mu_{\boldsymbol{max}}$ (day $^{-1}$)	5.59	3.81
$\boldsymbol{K_{S,PT}}$ (mg of TN L^{-1})	8.93	8.93
MPE (%)	22.62	19.59
Cell Growth (Silva and Cerqueira) – TN and PT		
Silva and Cerqueira Model kinetic order (m)	1.57	0.60
Fitting constant for K_S (p)	0.23	0.53
$\mu_{\boldsymbol{max}}$ (day $^{-1}$)	1.83	2.99
MPE (%) to TN	31.38	17.26
MPE (%) to TP	23.23	25.19
MPE (%) to Growth curve	21.24	7.42

Firstly, substrate consumption was analysed using only TN and then only TP (**Table 4.5**). The procedure adopted was similar to the one used for obtaining the constants necessary for the Monod model, with CAD and PS being subsequently implemented as the initial guesses for the Silva and Cerqueira model in both cases. The results of the simulations are presented in **Figure 4.6**.

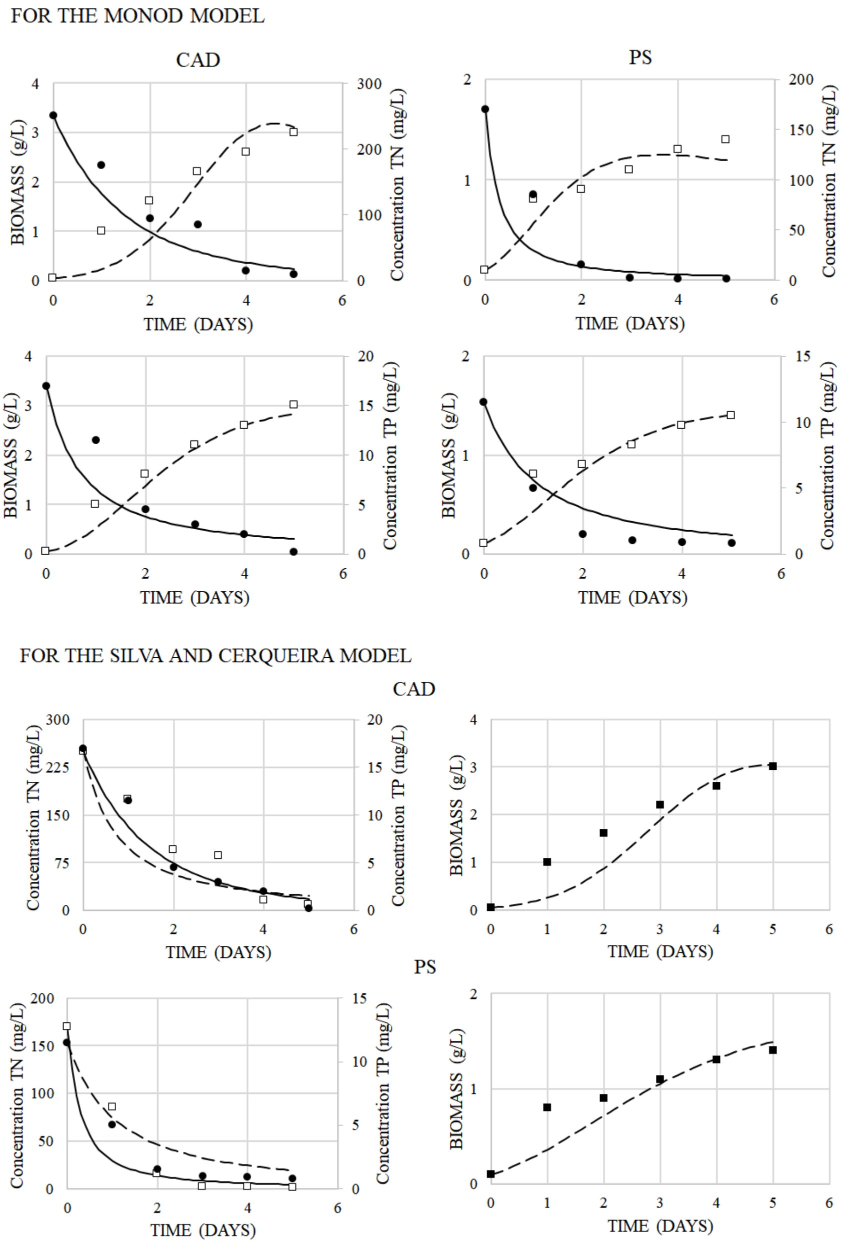

Figure 4.6 - Biomass production and substrate consumption. For the Monod model, (●) represents the experimental values for the substrate and (□) for biomass produced. Lines represent the simulation of the models using (---) for biomass and (▬) for substrate consumption. For the Silva and Cerqueira model, (●) represents the experimental values for TN and (□) for TP for substrate consumption curves. Lines represent the simulation of the models used in the curve for TN (---) and for TP consumption (▬). (■) for biomass produced and the line represents the simulation of the models for biomass production (---). Standard deviation of the experimental points was lower than 5%.

4.3.4.2 Data from the study carried out with real urban wastewater treated by microalgae

In the study carried out by González-Fernández et al. (2015), the concentrations of COD, TN and TP are described in such a way that it enables to repeat the same procedure previously described for the data from Shen et al. (2015) and Cho et al. (2013). The results of the kinetic parameters are presented in **Table 4.6**, with the simulations being illustrated in **Figure 4.7**.

Table 4.6 - Kinetic constants for COD, TN and TP, substrate consumption and cell growth.

Parameters			
Substrate Consumption	COD (mg L^{-1})	AMMONIA (mg L^{-1})	PHOSPHATE (mg L^{-1})
Kinetic order (n)	1.34	1.68	1.52
k (L $mg^{-1})^{(n-1)}(day^{-1})$	0.05	0.040	0.140
MPE (%)	23.51	25.34	18.48
Microalgae growth (Monod)			
μ_{max} (day $^{-1}$)	0.18	0.39	0.89
$\boldsymbol{K_S}$ (mg of Substrate L^{-1})	27.00	21.31	18.03
MPE (%)	6.59	12.69	20.67
Microalgae growth (Silva and Cerqueira)			
Silva and Cerqueira Model kinetic order (m)			0.30
Fitting constant for K_S (p)			0.52
μ_{max} (day $^{-1}$)			0.60
MPE (%) to substrate consumption	23.51	25.34	18.47
MPE (%) to growth curve			11.63

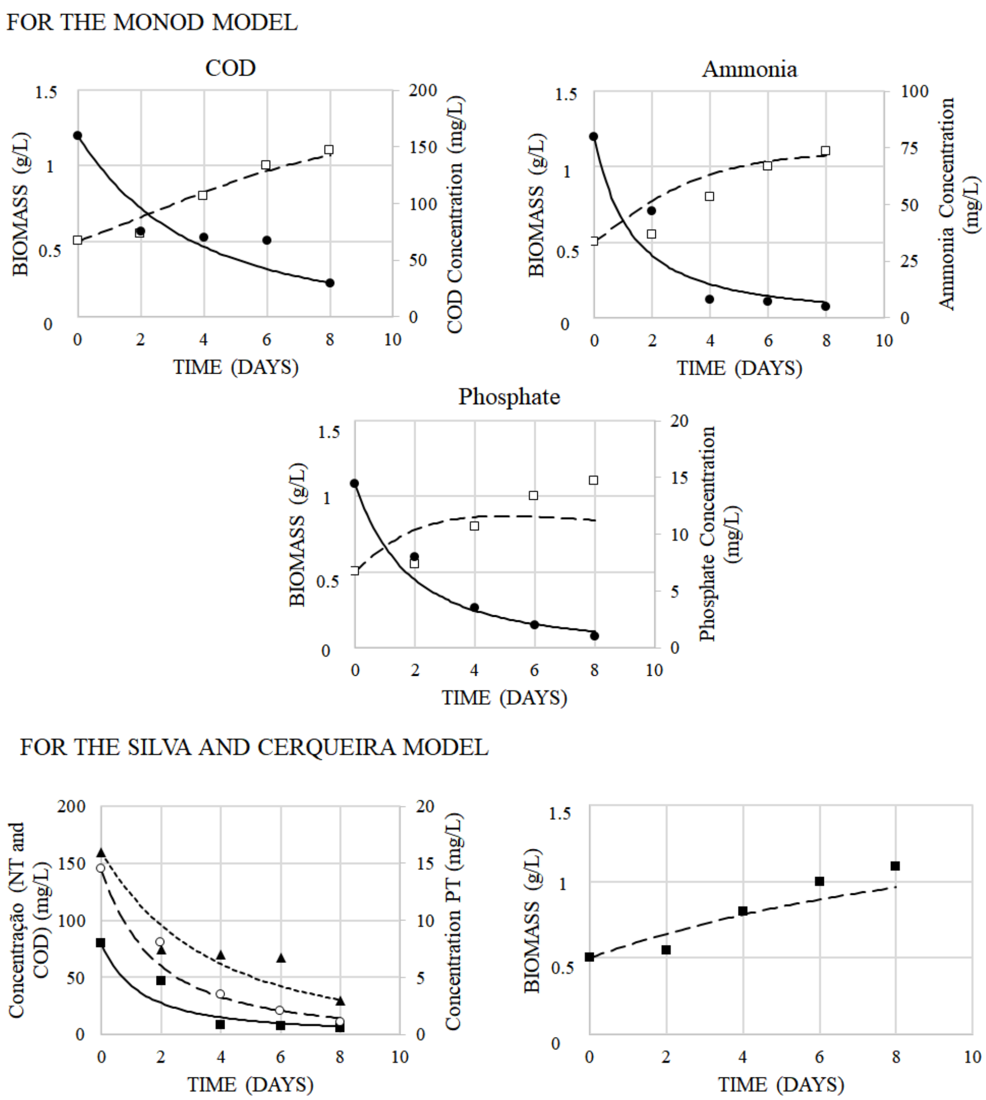

Figure 4.7 - Biomass production and consumption of each substrate. For the Monod model, (●) represents the experimental values for the substrate and (□) for biomass produced. Lines represent the simulation of the models using (---) for biomass and (▬) for substrate consumption. For the Silva and Cerqueira model, (▲) represents the experimental values for COD, (■) for TN and (○) for TP in the substrate consumption curves. Lines represent the simulation of the models used for COD (...), TN (---) and TP consumption (▬). (■) for biomass produced and the dashed line represents the simulation of the models for biomass production (---). Standard deviation of the experimental points was lower than 5%.

4.3.4.3 Discussion on the kinetic constants

The comparison between the kinetic parameters found include k, μ_{max} and K_S, which can be found in the literature. On the other hand, the comparison of the parameters m and p is difficult, as they are specific to the model developed in this article, which is an empirical model.

Regarding the parameter k, Wang et al. (2014) obtained values ranging between 0.05-0.16 day^{-1} when using first-order kinetics; a range close to those values found with the modelling carried out in the present article.

In terms of μ_{max}, values between 0.1-3.5 day^{-1} are found in the literature (Lee et al. 2015; Silva et al. 2016; Silva et al. 2017b), with several other works on effluents obtaining the same range, such as in the works carried out by Haario et al. (2009), Hodaifa et al. (2012), Ruiz et al. (2013a), Terceiro et al. (2014) and Mennaa et al. (2015), Massa et al. (2017). The results of the modelling carried out fit this profile, except for TP when using the Monod model in the work carried out by Cho et al. (2013). The modelling performed in the present work, using the Silva and Cerqueira model, also falls within this range, corroborating the additional advantage of using multiple substrates rather than one single substrate coupled to Monod kinetics.

As for K_S, the results often found in the literature included the same contaminants used in the present work (TOC, COD, TN and TP), with TN and TP being more easily compared. It is important to point out that the articles considered involved wastewater treatment, with the addition of high CO_2 concentrations, light intensity, as well as the concentration of other nutrients having great interference on the data obtained and, consequently, on the modelling and calculation of the parameters. Lee et al. (2015), in their compilation of kinetic data from microalgae cultivation, state values of K_S for TN and TP that vary from µg to g L^{-1}, in terms of the order of magnitude. However, these contaminants are usually represented in mg L^{-1} in effluents.

Wang et al. (2014) found values of $K_{S,TP}$ between 3.01-4.20 mg of TP L^{-1} (*Chlorella* and *Microctinium*). In turn, Tercero et al. (2014) and Sforza et al. (2014) found values of $K_{S,TN}$ and $K_{S,TP}$ of 23.4 and 28.2 mg N and P L^{-1}, respectively, when considering urban wastewater. Haario et al. (2009) found $K_{S,TN}$ of 7.9 mg L^{-1}. All these results are close to those found in the modelling performed in the present article. Regarding these parameters (TN and TP), microalgae exhibit extremely high TN and TP assimilation and metabolic capacities, with rapid microalgae growth and high productivity, even in values of 500 mg TN L^{-1} (nitrogen) and 200 mg of TP L^{-1} (phosphorus), for instance (Silva et al. 2016). As for COD, values of 5,000 mg L^{-1} or less can cause an inhibitory effect depending on the species, requiring greater consideration (SILVA et al., 2019b).

4.4 Conclusion

From the results obtained, it is possible to observe that the first and second-order kinetic models were not able to satisfactorily represent the consumption of substrate. On the other hand, the application of a *n*-th order kinetic model led to a significant decrease of the mean squared error for the contaminants chemical oxygen demand (COD), total organic carbon (TOC), total nitrogen and total phosphorus. When coupled with the microalgae growth model, it was observed that, when considering only one substrate and using *n*-th order kinetics to simulate substrate consumption, the Monod model could be applied. Nevertheless, when coupling multiple factors, the Silva and Cerqueira model was satisfactorily adjusted to the cases applied. Therefore, it can be argued that the search for kinetic constants to describe biomass production was successful. Finally, this work reinforces the importance of the study of simplified kinetic models for different processes, especially for modelling wastewater treatment with microalgae, which is a process that is not often described in the literature.

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Chapter 5: Dairy wastewater treatment by *Tetradesmus obliquus* in open system: the effect of light intensity and organic load in the process

Abstract

In this study, the potential of the microalgae *Tetradesmus obliquus* to be used in bioremediation of effluents was evaluated in the treatment of dairy wastewater through mixotrophic cultivation in open system. Experimental conditions were tested in different whey concentrations (0.5, 1, 2 and 4% v/v) and light intensities (25, 50, 100 and 200 µmol m^{-2} s^{-1}) for 14 days. The whey was characterized with high contents of Chemical Oxygen Demand (COD) (52,886 mg O_2 L^{-1}), Total Nitrogen (TN) (1,563 mg L^{-1}) and Total Phosphorus (TP) (663.5 mg L^{-1}). It was found that the presence of exogenous microorganisms did not inhibit microalgae growth and they alone did not treat efficiently the wastewater (control). Dry cell weight (microbial sludge) reached values between 200-600 mg L^{-1}. Increasing whey concentration was positive for COD removal capacity in terms of the amount removed, reaching up to 80% of removal rate, even though be better to work up to 1% of diluted whey. Higher TN (83-94%) and TP (almost 100%) removal rates were obtained when higher light intensities (100 and 200 µmol m^{-2} s^{-1}) and lower concentrations (0.5 and 1% of whey) were applied. Nitrogen and phosphorus content in biomass varied between 4-11% and 0.5-1.4% (dry cell weight), respectively.

Keywords: Bioremediation, biological process, wastewater treatment, dairy industry.

This article was published in Energy, Ecology and Environment (De Andrade FP, De Farias Silva CE, Dos Santos J, Ribeiro TRM, Medeiros JA, Do Nascimento MAA, Santos GKS, Dos Santos Carneiro W, Almeida RMRG, De Oliveira AMM, Feijó FM, da Silva Costa MM, De Andrade Lima GS, Ribeiro Junior KAL, Tonholo J. (2023) Dairy wastewater treatment by *Tetradesmus* sp. in open system: molecular identification and the effect of light intensity and organic load in the process. Energy, Ecology and Environment, v. 8, p. 356-369. https://doi.org/10.1007/s40974-023-00278-5).

5.1 Introduction

Microalgae offer advantages and disadvantages when applied to wastewater treatment, depending on the culture conditions (nutritional, environmental and operating conditions). In mixotrophic cultivation, microalgae can grow either autotrophically using CO_2 as carbon source and light as an energy source, and heterotrophically metabolizing organic compounds as a carbon source and in the absence of light, combining the advantages of both cultures by increasing biomass production, improving contaminant removal capacity and decreasing cultivation costs (BEHERA et al., 2019).

Many industries produce large volumes of effluents in their production process, which, when disposed without proper treatment, can lead to the pollution of water bodies promoting serious damage to the environment, and the dairy industry is one of them. Therefore, there is a growing interest in the use of biological processes for the treatment of industrial and agro-industrial wastewater, such as bioremediation using microorganisms.

Dairy effluents is the wastewater originated from the processing of milk and its derived products, such as cheese, butter, yoghurt, powdered milk and other milk by-products. Dairy wastewater is difficult to treat by conventional methods due to its high COD (chemical oxygen demand), BOD (biochemical oxygen demand) and high concentration of other nutrients/contaminants (such as nitrogen and phosphorus) (KAUR, 2021).

Whey is the aqueous part of milk and represents about 85 to 95% of milk volume generally resulted from cheese production. The composition varies according to its origin and the processing technique employed. It is rich in inorganic nutrients, such as ammonia, and phosphates (Phos), and organic nutrients (mainly lactose). Approximately 50% of the whey produced in the world is discharged into the sewage system without proper treatment (BENTAHAR et al., 2019).

Researchers have successfully used microalgae species as a biological method for dairy wastewater treatment and simultaneous biomass production. To mention, Chandra et al. (2021) remediated dairy wastewater using a culture of polymicroalgae *(Chlorella minutissima, Scenedesmus abundans, Nostoc muscorum* and *Spirulina* sp.), Salati et al. (2017) cultured *Chorella vulgaris* using cheese whey under mixotrophic and continuous culture conditions and Talaprata et al. (2021) studied the application of *Tetraselmis indica* in dairy wastewater. Specifically, a *Tretadesmus sp.* was applied to treat dairy wastewater

(COD = 3600 mg L^{-1}; NO_3^- = 158,69 ± 2,40 mg L^{-1}; PO_4^{3-} = 175,97 ± 1,81 mg L^{-1}) in a flat-panel photobioreactor using a daily photoperiod of 12/12 hours (light/dark) for 12 days. The study shows that the microalga decreased contaminants concentration of COD, nitrate and phosphate to 160 mg L^{-1}, 50,69 mg L^{-1} e 75,04 mg L^{-1}, respectively (KIRAN and MOHAN, 2022). However, a specific work evaluating the organic load and effect of light intensity in open system was not found in literature.

Microalgae cultivation in open systems is simpler and more economical compared to closed systems. However, the lack of control over climatic conditions and contamination by predators are the main disadvantages decreasing the productivity regarding closed systems (BEHERA et al., 2018). Some biological contaminants such as zooplankton, bacteria, other algae and viruses, can significantly restrict microalgae growth (WANG et al., 2013). For wastewater treatment, open systems are suitable from a technical and economic point of view, as there is no feasibility in sterilizing the effluents on industrial scale to promote the treatment in axenic system (SHASHIREKHA et al., 2016; MOHSENPOUR et al., 2021).

On the other hand, cooperative interactions can be established by different types of microorganisms forming consortia, e.g., microalgal-bacterial consortia. The consortium consisting of algae and bacteria can be applied for wastewater treatment to avoid the external supply of oxygen and carbon dioxide gas, allowing the assimilation of nutrients in the biomass and reducing CO_2 emissions to the atmosphere, especially when an open system is considered.

Many studies cite microalgal growth as a limiting factor for obtaining a high efficiency in the removal of nutrients/pollutants, mainly nitrogen and phosphorus, when cultivated together with contaminating microorganisms from the natural environment. However, algae growth can also be stimulated. Thus, in open systems, a good control of the microbial population during the removal of organic matter is necessary managing nutritional/environmental/operating parameters (DE FARIAS SILVA et al., 2019).

This work aimed to apply a species of *Tetradesmus* in the treatment of whey in open system (open pond), evaluating the biomass production and the removal rate of COD, TN and TP in different organic loads and light intensities.

5.2 Methodology

5.2.3 Dairy wastewater

The whey, a by-product of curd cheese production, was collected at the Federal Institute of Alagoas - Campus Satuba (9º34'29.08 "S 35°49'15.25 "O) (Satuba, Alagoas, Brazil). The effluent went through a process of separation of solid particles by centrifugation (3500 rpm and 15 min) (ACB Labor Dry-Bloch Centrifuge), filtration on qualitative filter paper, and then frozen (-12 to -18 ºC) aiming effluent conservation.

5.2.4 Experimental set-up

The bioreactors were built in transparent acrylic vases (PoliControl®) with the following dimensions:18.7 cm x 12 cm x 12 cm. The useful volume in each assay was of 1.5 L, varying whey concentrations (0.5, 1, 2 and 4% v/v) sterilized at 121 ºC, 1 atm for 15 min in autoclave. Inoculum concentration was of approximately 50 mg L^{-1} of dry cell weight. The assays were magnetically shaken, and pH was controlled daily and adjusted between 8 and 8.5 during 15 days at room temperature (30-35 ºC).

The assays consisted in monitoring the influence of light intensity (measured with a Delta Ohm 2302.0 radiometer) applying 25, 50, 100 and 200 µmol m^{-2} s^{-1}, provided frontally on one of the bioreactor’s side surfaces, verifying microalgae growth and the removal of contaminants under surface aeration (totalizing 16 assays performed in duplicate). Control assays were performed at the same organic loads, light intensities and experimental conditions to verify the effective contribution of the microalgal presence in the treatment since the system was open but without microalgal inoculation.

5.2.5 Analytical Methods

After the treatment, the samples went through centrifugation at 3,000 rpm for 20 min. Then, mainly for COD determination, the samples were respectively passed through 0.20 µm filters **(Chromastore®**) to eliminate possible solids. The solids separated in the centrifugation step were used for cell dry weight analysis in an oven at 55 ºC until constant weight. The chemical oxygen demand was analyzed by the dichromate digestion method and read in a UV-vis spectrophotometer (Shimadzu, UVmini 1240) at 600 nm (AOAC, 2002). For the total nitrogen analysis, the procedure was based on Kjeldahl method, consisting of a process consisting of 3 steps, digestion of the sample (digester block TECNAL, model TE007 MP), distillation (nitrogen distiller TECNAL, model TE-0363) and titration with standardized HCl (IAL, 2005). Total phosphorus (TP) was estimated by the ascorbic acid method and read in UV-vis spectrophotometer (Shimadzu, UVmini 1240) at 706 nm (AOAC, 2002). The pH was determined electronically with a previously calibrated equipment (TECNAL) to pH 4.0 and 7.0 standards.

5.2.6 Calculations of % N and P removed in biomass

For the calculations of the percentage of nitrogen and phosphorus in the biomass (microbial sludge), it was assumed that these cellular components were recovered from the wastewater by microalgal biomass, using equations 5.1 and 5.2, respectively:

$$\frac{N}{X}(\%) = \frac{(TN_{final} - TN_{initial})}{(DW_{final} - DW_{initial})} * 100 \qquad (5.1)$$

where: N/X = percentage of nitrogen in biomass absorbed during treatment (%). TN = total nitrogen (mg L^{-1}). DW = dry cell weight (mg L^{-1}).

$$\frac{P}{X}(\%) = \frac{(TP_{final} - TP_{initial})}{(DW_{final} - DW_{initial})} * 100 \qquad (5.2)$$

where: P/X = the percentage of phosphorus in biomass absorbed during treatment (%). TP = total phosphorus (mg L^{-1}). DW = dry cell weight (mg L^{-1}).

5.3 Results and Discussion

5.3.2 Characterization of whey

Whey is a by-product characterized by having high levels of organic matter, as well as nutrients essential for microalgal metabolism, such as nitrogen and phosphorus. **Table 5.1** presents the data obtained from the characterization of the whey collected to be used as remediated effluent in this research in comparison with the data found in literature. High COD, TN and TP concentrations were observed. However, the differences observed in the values are probably due to various agronomic factors, namely source of milk, types of processing, among other factors.

Table 5.1 - Physicochemical characteristics of whey.

Type of Whey	COD (mg L^{-1})	TN (mg L^{-1})	TP (mg L^{-1})	pH	Reference
Curd cheese whey	52,886 ± 269.25	1,563± 35.0	663.50 ± 12	6.33 ± 0.5	This work
Raw cheese whey	68,600 ± 3300	1,120 ± 10	500 ± 1.8	4.9 ± 0.27	Saddoud et al. (2007)
Cheese whey	147,000 ± 7,000	805 ± 48	400 ± 20	5.26	Salati et al. (2017)
Second whey cheese	1,100 ± 97 to 7,295 ± 80.9	32.9 ± 0.3 to 102.5 ± 5	1.2 ± 0.29 to 44.8 ± 0.34 (TPhos)	7 to 7.5	Tsolcha et al. (2016)
Whey	17,806.36	383	396.87	5-6	Patel et al. (2020)

COD - chemical oxygen demand. TN - total nitrogen. TP - total phosphorus. TPhos – total phosphate.

5.3.3 pH

pH is an important parameter in wastewater biological treatment systems because microbial growth is pH dependent. Considering microalgae, their development is associate to alkaline pH, favouring pollutant removal and is correlated to their metabolic requirements (DE ANDRADE et al., 2022).

For this reason, the pH was measured daily in all assays and adjusted to the initial range of 8-8.5. For the more diluted assays, an increase in pH (9-11) was observed, due to the excretion of alkaline metabolites by the microalgae from the biodegradation of organic matter and the uptake of CO_2 (TSOLCHA et al., 2016). However, the more concentrated assays showed a decrease in pH values, between 4 and 5, due to probable metabolization of organic compounds resulting in the excretion of organic acids during the metabolization of organic matter (DOGARIS et al., 2020). Liang et al. (2013) studied the effect of pH on an algae-bacteria combined system of *Chorella vulgaris* and *Bacillus licheniformis* on nitrogen and phosphorus removal in synthetic medium (initial NH_4^+ concentration and TP of 20 mg N L^{-1} and 4 mg L^{-1}, respectively). The pH of the combined system significantly reduced from neutral to acidic, from 7 to 3.5, in the removal process accompanied by a decrease in Chlorophyll *a* content. With pH adjusted to neutral, higher removal efficiencies of NH_4^+ (86%) and TP (93%) were obtained along with higher Chlorophyll *a* content and recovery of algal cells compared to those without pH regulation.

5.3.4 Biomass produced

For microalgae, mixotrophic microorganisms, light supply strongly influences cell growth. **Figure 5.1** shows that the growth of *Tetradesmus* sp. microalgae increased gradually under all culture conditions, either by increasing whey concentration (using myxotrophy) or by higher light intensity. Thus, resulting in a higher biomass production and showing a positive effect of organic matter concentration and light intensity. The maximum dry cell weight values were between 200-600 mg L^{-1}, after 14 days of cultivation, except for assay 1 (0.5% and 25 μmol photons m^{-2} s^{-1}) that reached a maximum dry cell weight of 66 mg L^{-1}. This possibly occurred due to the combination of low whey concentration and light intensity. According to Choi et al. (2016), microalgal growth is proportional to contaminant removal, mainly organic carbon, nitrogen and phosphorus as well as light intensity.

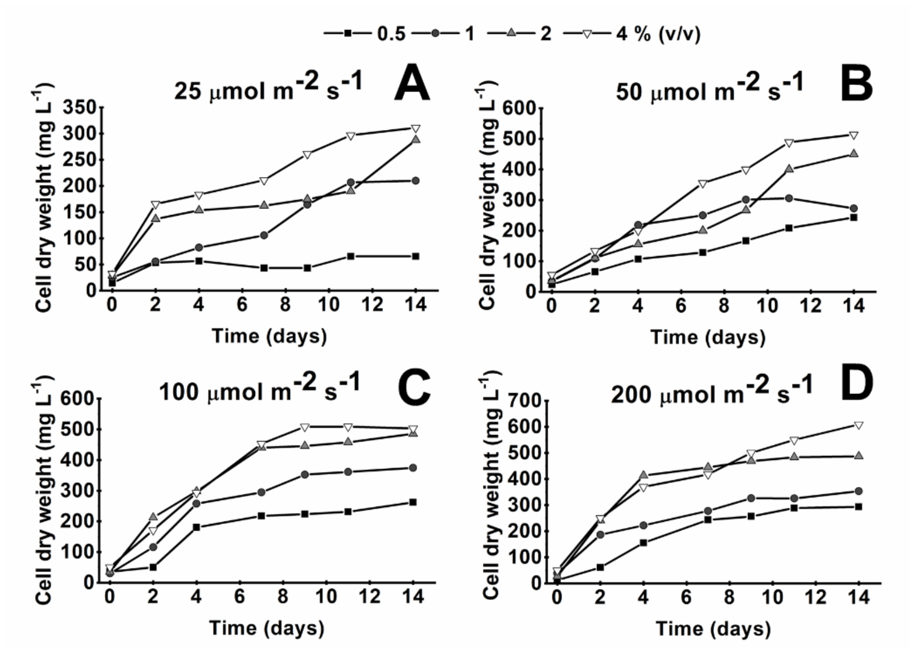

Figure 5.1 - Biomass growth curves for *Tetradesmus obliquus* under different whey concentrations (% v/v) and light intensities: (A) 25, (B) 50, (C) 100 and (D) 200 μmol m^{-2} s^{-1}.

In open systems, the interaction of microalgae and, mainly, bacteria can result in a series of positive and/or negative mechanisms, such as resource exchange or competition. In this work, it was found that the presence of exogenous bacteria or other microorganisms (since the medium was sterilized before the assays) did not inhibit microalgal growth. The same was observed by Sforza et al. (2018), who compared the growth of a microalgae-bacteria consortium in real effluent (*Chlorella protothecoides*, and bacteria obtained from activated sludge) with the microalgal monoculture in a real and sterile effluent. Makut et al. (2019) observed a significant improvement in the growth of associated microalgae (consortium consisting of two microalgae, *Chlorella sorokiniana* and *Chlorella sp.* and two bacteria, *Klebsiella pneumoniae* and *Acinetobacter calcoaceticus*) when compared to the isolated microalga.

5.3.5 COD Removal

Higher COD removal rates reached more than 80% (**Figure 5.2**). In addition, increasing the organic load improved whey treatment, except for the experiment applying 4% and 25 μmol photons m^{-2} s^{-1}**,** probably due to the presence of organic compounds that can be absorbed by the myxotrophic microalgae, as well as excess nitrogen and phosphorus that support this higher metabolization of organic load. Autotrophic microalgae are able to remove inorganic nutrient and CO_2. However, they are not efficient in removing a lot of organic matter from wastewaters. Thus, growing them mixotrophically aid to remove organic nutrients (PATEL et al., 2020). Furthermore, with light intensity increasing, higher COD removal rates were visualized, which could be associated with a higher efficiency from the combination of heterotrophic and autotrophic routes (photosynthesis). Patel et al. (2020) studied the use of mixotrophic cultivation of *Chlorella Protothecoides* microalgae to perform remediation of chemically-pretreated and diluted whey (COD = 1,693.70 mg L^{-1}), under constant lighting conditions of 150 μmol m^{-2} s^{-1}, 120 rpm, for 9 days, achieving a reduction of 92.6%.

European legislation foresees a maximum effluent discharge concentration of 125 mg L^{-1} for COD and a minimum removal rate of 75% (EEC, 1991, DE FARIAS SILVA et al., 2020). All experiments carried out at light intensities of 100-200 μmol m^{-2} s^{-1} and dilution rates of 0.5 and 1% reached this treatment standard, being recommended form an operational point of view to treat te wastewater in one single step. On the other hand, for whey diluted 2 and 4%, even though the COD residual be higher than 125 mg L^{-1}, high COD content was removed reaching around 80% of removal rate, and a process based in two sequential steps can be designed, mainly if a continuous process is applied.

It is noticed that the microalga was efficient in the removal of COD in the above conditions and constitutes an alternative for the secondary treatment of effluents. These results agree with the study conducted by Shen et al. (2017), who reported high COD removal efficiency (97%) in the treatment of synthetic effluent (COD = 1159.2 ± 12.6 mg L^{-1}) by microalgal-bacterial consortium (*Chlorella vulgaris* - *Pseudomonas putida*), under batch culture conditions, with light intensity of 200 μmol photons m^{-2} s^{-1}, at 26 ± 2 °C for 48h. Similarly, a COD removal percentage of 90-94% was reported by Zhang et al. (2021), who applied *Chlorella vulgaris* microalgae (CPCC 90) and activated sludge to treat secondary effluent from a synthetic anaerobic malting MBR (COD = 1106.17 ± 20.05 mg L^{-1}) in a membrane photobioreactor, with microalgae and sludge ratio of 1:3, O_2 injection of 3.39 ± 0.16 L min^{-1}, light intensity of 8400 lux, for 300 days divided into 4 phases.

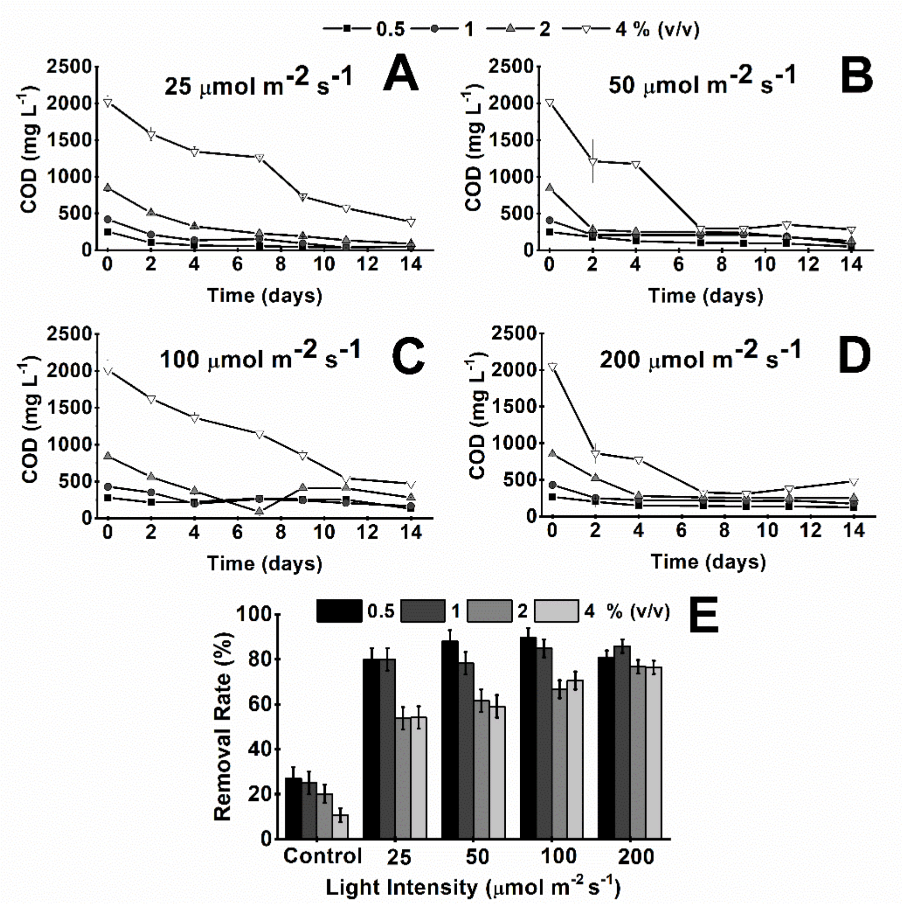

Figure 5.2 - *Tetradesmus obliquus* efficiency in COD removal under different whey concentrations (% v/v) and light intensities: (A) 25, (B) 50, (C) 100 and (B) 200 μmol m^{-2} s^{-1}. (E) COD removal rate (%).

5.3.6 Total Nitrogen Removal

The best TN removal rates were obtained in the assays with higher light intensities (50, 100 and 200 μmol m^{-2} s^{-1}), within a range of 83-94% (**Figure 5.3**), with a substantial drop in concentrations for levels lower than 10-15 mg L^{-1} in accordance with European legislation that stabilish a final TN concentrations must be under the permissible limit of 15 mg L^{-1} and a minimum removal rate of 70-80% (EEC, 1991). Microalgae are microorganisms with a high capacity for nitrogen accumulation and metabolization.

Nitrogen accounts for 4 to 14% of their dry cell weight, being an essential constituent for all protein structures and functions within cells (DE FARIAS SILVA and SFORZA, 2016).

Microalgae can be able to metabolize the nitrogen present in whey. For example, Riaño et al. (2016) applied *Chlorella sorokiniana* microalgae in the treatment of a whey previously treated anaerobically (735 mg L^{-1} of ammonia). The assay was conducted in an open photobioreactor, mechanically stirred, and continuously illuminated by four fluorescent lamps at 54 µE m^{-2} s^{-1}, reaching 92% of removal rate after 5 days. In addition, Patel et al. (2020) achieved 98.4% of TN removal in the treatment of a chemically pretreated and diluted (25:75) whey (383 mg L^{-1} of TN) using mixotrophic cultivation of *Chlorella protothecoides* microalgae under constant light intensity (150 µmol m^{-2} s^{-1}) and stirring (120 rpm) for 9 days.

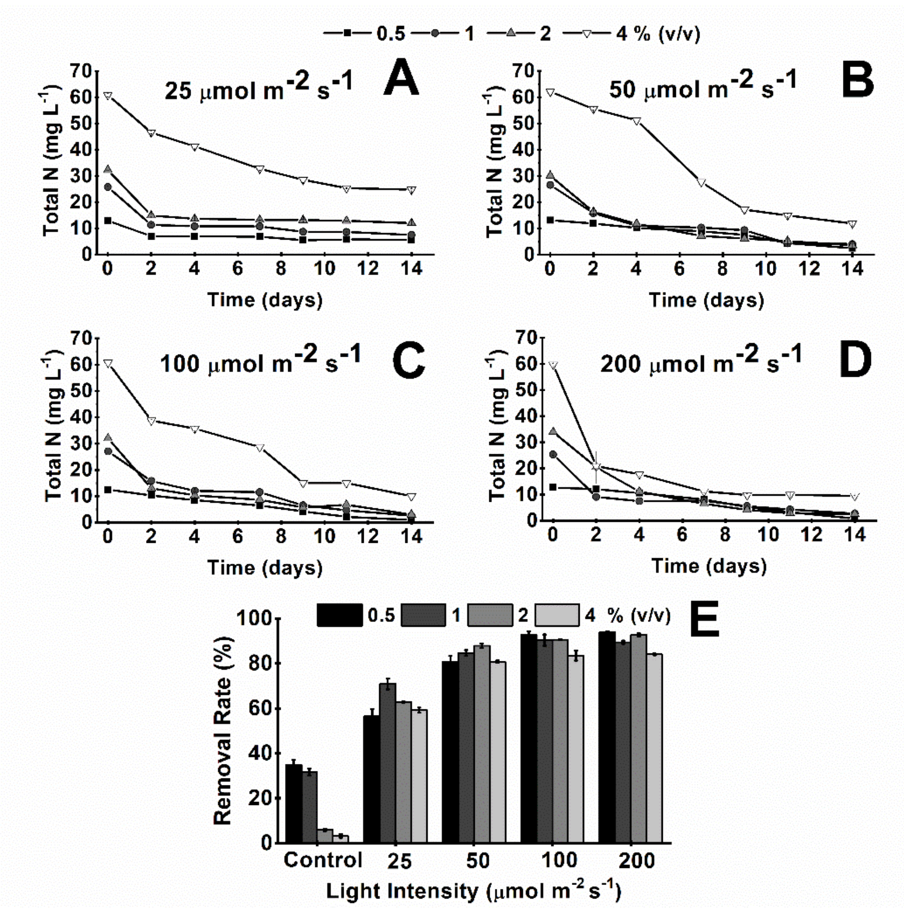

Figure 5.3 - Performance of *Tetradesmus obliquus* in Total Nitrogen (TN) removal under different whey concentrations (%) and light intensities: (A) 25, (B) 50, (C) 100 and (B) 200 µmol m^{-2} s^{-1}. (E) TN removal rate (%).

5.3.7 Total Phosphorus Removal

Higher phosphorus removal rates were obtained in the assays with higher light intensities (100 and 200 µmol m^{-2} s^{-1}) and lower whey concentrations (0.5 and 1% v/v), reaching removal rates of almost 100% (**Figure 5.4**), with a substantial drop in concentrations in the first 10 days of cultivation and within the regulated discharge rates parameters by European legislation (between 1-2 mg L^{-1} of TP and a minimum removal rate of 80%) (ECC, 1991). The experiments with 2 and 4% of whey, phosphorus concentration was in excess and for this reason was not efficiently removed even though 80% of removal rate be reached.

Phosphorus has an important role in cellular metabolism, forming various structures and components essential for microalgal growth, nucleic acid production and for the production of value-added products such as polyunsaturated fatty acids and astaxanthin (PATEL et al., 2020). According to Tighiri and Erkut (2019), P removal rate can occur by biomass assimilation including the possibility of intracellular polyphosphate formation.

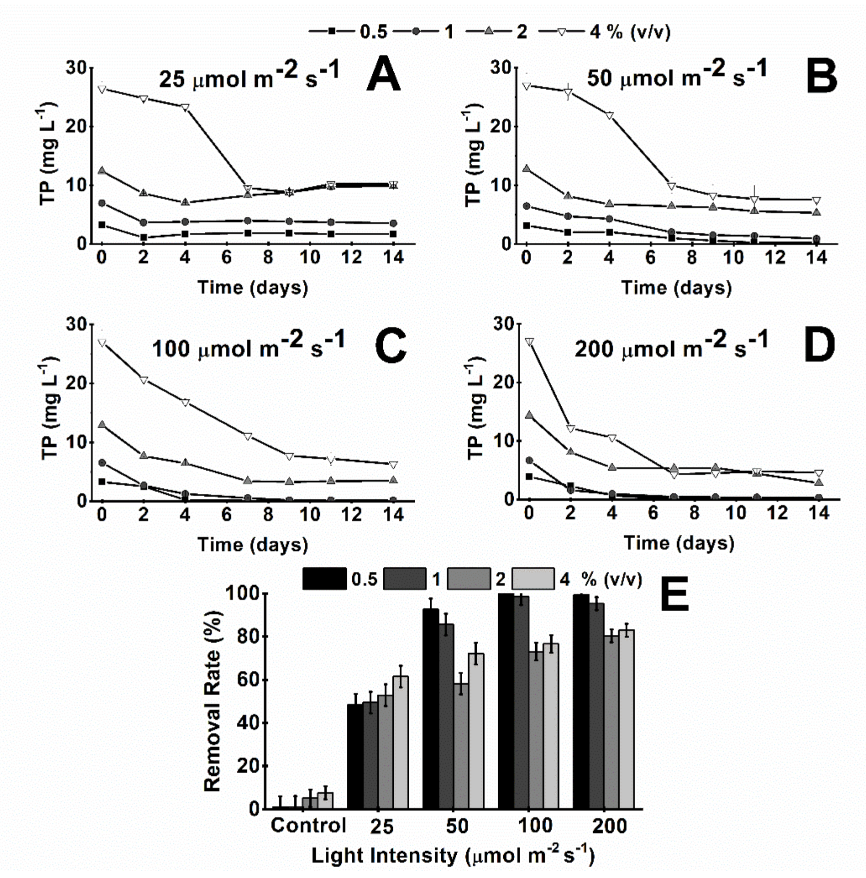

Figure 5.4 - Performance of *Tetradesmus obliquus* in Total Phosphorus (TP) removal under different whey concentrations (%) and light intensities: (A) 25, (B) 50, (C) 100 and (B) 200 µmol m^{-2} s^{-1}. (E) TP removal rate (%).

Similar to the results of total nitrogen removal, some works emphasize the high capacity of phosphorus removal from effluents by microalgae. For example, the mixotrophic cultivation of *Chlorella protothecoides* in the remediation of whey (150 μmol m^{-2} s^{-1}, 120 rpm, 25:75 dilution rate, 9 days) achieved 79.9% of TP removal rate, initially with 397 mg L^{-1} of TP (PATEL et al., 2020). Sforza et al. (2018) exploited the specific interactions between *Chorella prothothecoides* microalgae and *Brevundimonas diminuta* bacteria (isolated from activated sludge) to improve nutrient removal from real effluent (TP = 3.5 mg L^{-1}), achieving 71% of TP removal rate. Tsolcha et al. (2018) obtained up to 84% of phosphate removal rate (TPhos) from second whey with 9.63-18.5 mg L^{-1} initial phosphate concentration (TPhos), respectively.

5.3.8 Percentages of N and P in biomass

The percentage uptake of N and P observed in microalgal biomass were between 4-11% for N/X and 0.5-1.4% for P/X. Hence, the N/P ratio found in the treatment was around 8:1.

De Farias Silva and Sforza (2016) found that for *Chlorella vulgaris* grown under different environmental conditions (light intensity and cultivation time were mainly checked), as well as nutritional conditions (amounts of N and P available), the % of N and P in biomass varied between 4-14% for N/X and 0.5-3.5% for P/X.

This ratio N/P of 8:1 was equal or close to ratios found in the literature. For example, Xin et al. (2010) studied *Scenedemus* sp. microalga and the N/P ratio for nutrient removal and concluded that under N/P ratio between 5:1-8:1, nitrogen and phosphorus could be efficiently removed. On the other hand, Shashirekha et al. (2016) found a N/P ratio of 1.75.

In microalgal cultivation, N/P ratio is important not only nutrient absorption, but also as competitiveness requirement of the microorganisms found in the medium (DE ANDRADE et al., 2022). According to Li et al. (2019), microalgal growth rate influences directly in nitrogen and phosphorous removal, thus, to obtain an efficient wastewater treatment, N/P ratio should be between 6.8-10. Higher ratios can cause phosphorus limitation and lower ratios, nitrogen limitation. Stumm's empirical nutriente relation to microalga eis cited as $C_{106}H_{263}O_{110}N_{16}P$ (N/P ratio = 16:1) (XIN et al., 2010).

Additionally, Vrede et al. (2002) found that the atomic C:N:P ratio of exponentially grown bacterioplankton varied 32:6.4:1. For carbon-limited grown cells, it was 34:9.2:1, for nitrogen-limited 42:7:1 and for phosphorus-limited 172:16:1. The general average can be represented by the ratio 45:7.4:1.

5.4 Conclusion

In the present study, microalgal *Tetradesmus obliquus* carried out the treatment of whey under different organic loads and light intensities. Results showed that high light intensities increased contaminants removal efficiency by *Tetradesmus obliquus* LCE-01, being limited by the organic load. It is worth to mention that there were no additional costs with forced aeration. Finally, results were in accordance with the removal rates defined by European legislation for the treatment of diluted whey, in concentrations of 0.5 and 1% v/v considering the three analyzed parameters (COD – 125 mg L^{-1}, TN – 15 mg L^{-1}, and TP – 1-2 mg L^{-1}), even though applying a dilution rate of 2 and 4% high amount of these contaminants were removed, but an additional step to decrease them is necessary. Results show that integrate the use of dairy wastewater for microalgae cultures is beneficial to minimize freshwater use, reduce the cost of nutrient addition, reach high contaminant removal rate and produce microbial biomass which has biotechnological potential.

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Chapter 6: Biological Treatment of Petroleum Produced Water Ex-Situ using Microorganisms: an overview, main developments and challenges

Abstract

Large volumes of a potentially polluting effluent are generated during oil extraction, denominated production water or produced water (PW). PW is characterised by high concentrations of contaminants, such as COD, nitrogen and phosphorus, heavy metals, hydrocarbons and others. This review aims to analyse ex-situ biological treatment methods using microorganisms for PW. There are several consolidated physical and chemical treatments of PW. However, they present high operation costs and may raise the final value of the product. Thus, the biological treatment of PW performed ex-situ by microorganisms has been the goal of research in recent decades, in order to develop an efficient and less costly process when compared to conventional treatments, resulting in microbial biomass and clean water. Ex-situ biological treatment by microorganisms are carried out in acclimated bioreactors, with environmental (salinity, pH, temperature and light intensity (for microalgae)), nutritional (macro and micronutrients, and contaminants concentration to avoid nutrient limitation or substrate inhibition, mainly caused by hydrocarbons) and operating adaptations (type of bioreactor, class of microorganisms, treatment time and mode of operation (batch or continuous)) to maximize the treatment performance, which are promising reaching high removal rates of oil and greases (TOG), nitrogen, phosphorus, metals and other contaminants. Bacteria are the most applied microorganism even though microalgae, yeast and filamentous fungi be tested in the last decade. Advantages and limitations of each class of microorganisms is presented in this review, and more research and technological development is expected in future for this research topic.

Keywords: bacteria, microalgae, fungus, yeast, petroleum industry, biological challenges

This article was published in Energy, Ecology and Environment (Da Silva AHG, De Andrade FP, De Farias Silva CE, Medeiros JA, Santos GKS, Do Nascimento MAA, Tonholo J, Almeida RMRG. (2023) Biological Treatment of Petroleum Produced Water Ex-Situ Using Microorganisms: an overview, main developments and challenges. Energy, Ecology and Environment, v. 8, p. 289-303. https://doi.org/10.1007/s40974-023-00284-7).

6.1 Introduction

The extraction of oil and gas, one of the most important energy sources in the world, produces a large amount of wastewater, called production water (PW), with highly polluting characteristics. This effluent contains wide-ranging concentrations of naturally occurring impurities, such as hydrocarbons, inorganic salts, metals, phenols and radioactive materials (AMMAR, KHADIM and MOHAMED, 2018; COSTA et al., 2022). Chemical, physical, and biological treatments methods can be Applied to remediate PW, but the choice is dependent on the effluent composition. Commonly, monosystems are not enough to attend the legislation limits of PW discharge and reuse (GHAFOORI et al., 2022).

To date, the existing treatments of oil production water are efficient for reusing water to be used in the re-injection in oil and gas reservoirs. In this case, PW re-injection into depleted oil reservoirs and saline aquifers is the most widely used disposal method but has become obsolete mainly due to the reduction of injection sites, high operating costs, and more restrictive release regulations (EZENNÚBIA and VILCÁEZ, 2023). On the other hand, when water is treated, it can be reused for irrigation. However, if contaminants are present, they can accumulate in crops and soil, contaminating water bodies (SEDLACKO et al., 2020).

The limited capacity to dispose of PW in large volumes has encouraged the search for new, cheaper, and more sustainable treatment technologies. In general, conventional physical and chemical treatments have a high process cost, high consumption of energy and chemicals (AL-KAABI et al., 2021), resulting in an increase in the value of oil. Chemical remediation methods, such as flocculation and coagulation, are not effective in removing diluted components, in addition to concentrating toxic metals in the sludge generated in the process (GHAFOORI et al., 2022). Consolidated physical technologies, such as membrane filtration, encounter the high concentration of petroleum hydrocarbons as a barrier, which can lead to rapid incrustation of membrane filters, negatively affecting the removal of salinity and toxic metals. Biological treatment processes, such as activated sludge, can efficiently remove petroleum hydrocarbons and other contaminants, with high salinity being the limiting factor for the microbial community. The dilution of PW can be an alternative to reduce the inhibitory effect of salinity, however it would increase the operational value of the process, but the water can be also recirculated in the process after the treatment (EZENNÚBIA and VILCÁEZ, 2023).

In recent years, several literature review papers have pointed to the use of different effective processes to treat produced water, citing a comprehensive study by Ghafoori et al. (2022) about recent advances and challenges in the most commonly treatment technologies and hybrid systems applied, pointing out the advantages and disadvantages of each method, and future perspectives. Samuel et al. (2022) critically analyzed the properties of Oilfield Produced Waste (OPW) and different treatment technologies, including physical-chemical, biological and physical processes, with a main focus on membrane technologies. Abujayyab (2022) performed a metadata review and analysis evaluating the effects of different operational conditions on the biological removal of COD, such as temperature, salinity, oxygen availability, type of microorganism, technology, and scale of treatment. However, comments on biological treatments ex-situ using different types of microorganisms, in particular bacteria, microalgae, and fungi, are not widespread.

This review reports the main characteristics of PW, such as volume and composition, demonstrating its high polluting power and the need for efficient treatment methods for the destination of this effluent. It provides an overview of the current types of PW treatment, citing some advantages and disadvantages of the available technologies, with emphasis on the biological treatment ex-situ and its ability to remove contaminants present in PW. Finally, it presents the need for investment in new research to optimise the promising use of microorganisms in PW remediation.

6.2 The Petroleum Industry

As society evolved, a worldwide dependence on energy sources has emerged, namely on fossil sources, such as oil and natural gas. According to the U.S. Energy Information Administration, global oil production in 2022 reached about 100 million barrels per day (b d^{-1}), with 34.19% of that amount produced by countries which are part of the Organization of Petroleum Exporting Countries (OPEC). In the same year, North America was the continent with the highest oil production (27.85 million b d^{-1}), especially due to the high contribution of the United States, which reached 20.25 million b d^{-1}, becoming the country with the highest oil production. In second place in this ranking is Russia, with a production of 10.94 million b d^{-1} and, among the OPEC countries, Saudi Arabia stands out with a production of 10.43 million b d^{-1}. In 2022, Brazil's production was of 3.76 million b d^{-1}, which places it in 7^{th} place among the countries with the highest oil production (EIA, 2023).

However, as in other industrial sectors, the extraction of oil and gas generates high volumes of by-products with high polluting power, such as produced water (PW), making it a major problem for the energy sector.

6.3 Production and characteristics of produced water

Produced water, also known as formation water or connate water, is the waste generated in larger quantities during the extraction of oil and gas. When it is obtained, it is confined during the formation of the subsoil and is brought to the surface along with the products of interest (AL-KAABI et al., 2021). According to the origin of PW, it can be named as oilfield, natural gas and coal bed methane (IGUNNU and CHEN, 2012; AL-KAABI et al., 2021).

In 2020, the daily global production of oil was of 80 million barrels, while the daily generation of produced water was of approximately 320 million barrels (Dudek et al. 2020). In this sense, it is generally mentioned that 3-4 barrels of PW for one barrel of oil (AL-KAABI et al., 2021; ABUJAYYAB, HAMOUDA and HASSAN, 2022).

PW/hydrocarbon ratios are evolving globally, as conventional oil and gas reservoirs are ageing and consequently producing more waste (PW), forming a low proportion of oil (AL-GHOUTI et al., 2019; ABUJAYYAB, HAMOUDA AND HASSAN, 2022). In this regard, it is estimated that by 2025 the PW/hydrocarbon ratio will reach 12:1 (v/v) (JIMÉNEZ et al., 2018; AL-KAABI et al., 2021). It is important to note that there is no standard volume of production of this effluent, being generated in different quantities in each country (COSTA et al., 2022).

According to Huang et al. (2021), about 14.5 billion m^3 of produced water is generated annually. Of this amount, only 60% have an appropriate final destination, and the remainder is irregularly discarded. From an environmental point of view, there is concern about this effluent, especially due to its composition of organic compounds such as benzene, toluene, polycyclic aromatic hydrocarbons, phenol and organic acids, as well as inorganic compounds such as arsenic, cadmium, copper, nickel, lead and radionuclides, besides other chemicals that are potential pollutants of the aquatic ecosystem (GONDIM et al., 2017). Given this growing perspective with respect to the generation of PW, associated with the need of its appropriate discharge, the market for managing and reusing this effluent is expected to expand (SALEM and THIEMANN, 2022).

Produced water has a complex and variable composition and usually consists of a combination of formation water (confined by a layer of rock and soil) and injected water. When hydrocarbon and gas are removed, the pressure in the reservoirs is restricted, being necessary to inject water on the water layer of the reservoir to maintain hydraulic pressure and boost the extraction of oil, thus generating a liquid waste known as injected water (MUNIRASU, HAIJA and BANAT, 2016; Jiménez et al., 2018). In addition, PW may also contain hydrocarbons (SEDLACKO et al., 2020) and chemicals from the production and treatment procedures (JIMÉNEZ et al., 2018; AL-GHOUTI et al., 2019).

In the characterisation of this effluent, a combination of organic and inorganic compounds is found in varying concentrations. Its physical and chemical properties are influenced by conditions such as the geological location of the field, its own geological formation, the life of its reservoirs and the type of hydrocarbon produced (AL-KAABI et al. 2021; AL-GHOUTI et al., 2019). The main compounds of produced water can be observed in **Figure 6.1**.

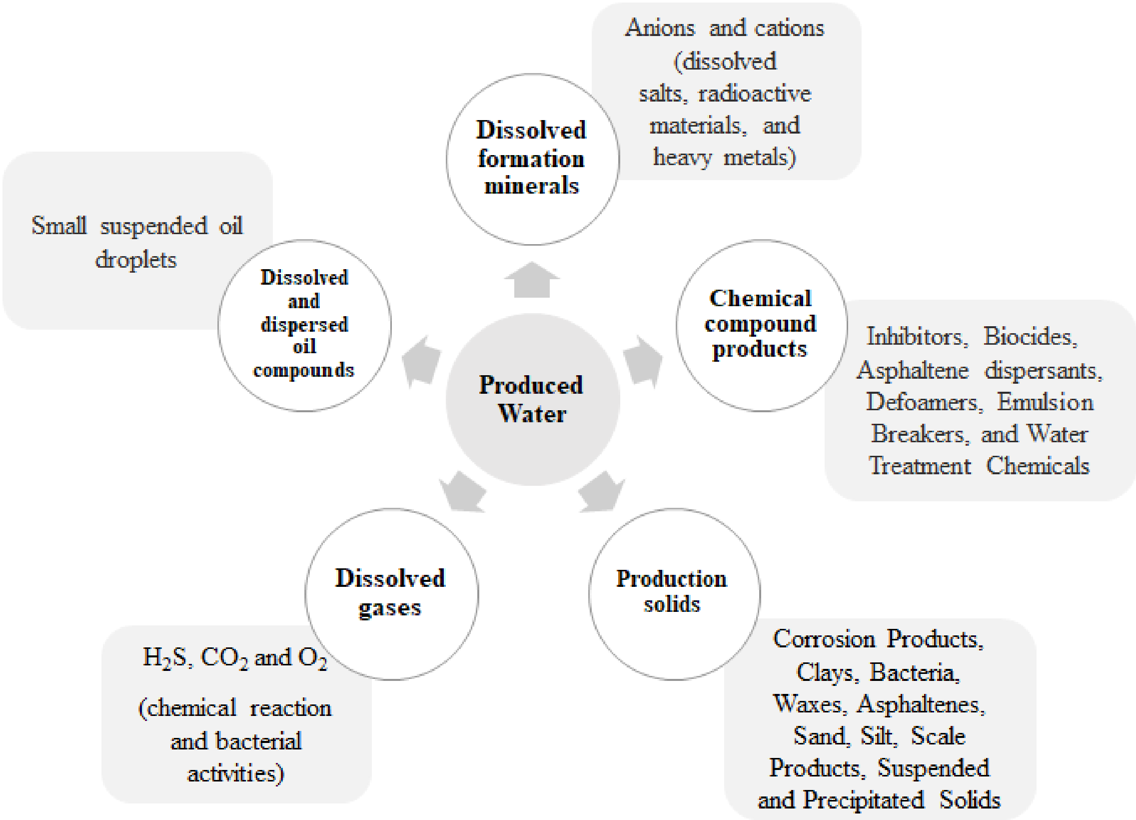

Figure 6.1 - Main compounds of produced water. Source: Adapted from Costa et al. (2022).

Since oil is a mixture of hydrocarbons, including benzene, toluene, ethylbenzene and xylenes (BTEX), naphthalene, phenanthrene, dibenzothiophene (NPD), polyaromatic hydrocarbons (PAHs) and phenols, water cannot dissolve all these hydrocarbons, so most of them are dispersed in water (DUDEK et al., 2020). The volumes of dissolved and suspended oil present in produced water (before treatment) are related to the following conditions: oil composition, pH, salinity, TDS (total dissolved solids), temperature, oil/water ratio, type and amount of oilfield chemicals and various stability compounds (waxes, asphaltenes, and fine solids) (FAKHRU'L-RAZI et al., 2009; JIMÉNEZ et al., 2018).

The inorganic molecules dissolved in PW contain anions, cations, heavy metals, and naturally occurring radioactive materials (NORM). Produced water consists of a wide range of cations and anions that have similar concentration for different metals (COSTA et al., 2022). The common cations present in produced water are Na, Ca, K, Mg, Fe, Al, B, Ba, Cu, Li, Zn, Ti, Mn and the main anions are chloride, sulphate, sulphite, bicarbonate, nitrate, nitrite and others (MUNIRASU, HAIJA and BANAT 2016). It is important to note that the existence of certain cations and anions can cause inorganic fouling of production equipment or in the well (DUDEK et al., 2020)

In addition, traces of various heavy metals are found in PW, such as cadmium, chromium, copper, lead, mercury, nickel, silver and zinc, in various concentrations that are influenced by the age of the reserves and the geology of the formation, but it can reach values between 102 to 105 times of that found in seawater (FAKHRU'L-RAZI et al., 2009; JIMÉNEZ et al., 2018). The most abundant radioactive materials (NORM) in produced water are 226 Ra and 228 Ra and, depending on the geology, some wells may also have 238 U and 235 U (MUNIRASU, HAIJA and BANAT, 2016).

Moreover, in oil and gas exploration, the chemical agents used vary according to the various production systems and are usually inserted into the hydrocarbon or gas well to minimise operational problems such as fouling and corrosion, acting in the inhibition of hydration and as biocides and assisting in water treatment from their action as flocculants and antifoams, emulsion breakers, reverse emulsion breakers and coagulants, which are used in the recovery and pumping of hydrocarbons (AL-GHOUTI et al., 2019). Thus, these chemical agents act by improving the process of oil, gas and water extraction and consequently avoiding the corrosion of pipelines and formation of methane hydrate in the gas production system, despite being a different source of contamination of the effluent.

Production solids originate from a variety of organic and inorganic materials that are together with produced water. Floating or drifting materials can also be found in water, such as mud, silt, sand, algae and plankton (JIMÉNEZ et al. 2018; AL-GHOUTI et al., 2019). Dissolved gases are generated spontaneously by the action of bacteria or by chemical reactions in water (IGUNNU and CHEN, 2012), and the most common are volatile hydrocarbons, carbon dioxide, oxygen and hydrogen sulphide (CO_2, O_2 and H_2S) (DUDEK et al., 2020; COSTA et al., 2022).

6.4 Produced water treatment methods

Produced water is seen as a major issue in the oil industry. Besides being generated in large volumes, it still has a high concentration of organic and inorganic compounds with high polluting power. The treatment of this effluent is seen as an alternative for its reuse in agricultural, industrial areas and even in the oil extraction process itself. According to the literature, the main objective of the remediation process is to reduce the content of oil and grease, soluble organics, suspended solids, dissolved gases, natural radioactive materials and salts (DOLAN, CATH and HOGUE, 2018; DUDEK et al., 2020).

In this sense, different treatment techniques are studied to adapt this effluent to the discharge standards required in each region. TOG (total oil and grease content) is one of the main parameters measured to quantify the pollution load of this effluent. Although each country or region has its own legislation (**Table 6.1**), in general, the average monthly TOG for regular discharge into marine environments should be between 30 and 40 mg L^{-1}. Other pollution parameters are also evaluated, such as chemical oxygen demand (COD). According to the monthly effluent discharge limits in China, COD levels cannot exceed 100 mg L^{-1}. Some countries are also attempting to establish more stringent measures for the disposal of this effluent in order to minimise environmental damage (NEFF, 2002; EPA, 2002; TELLEZ, NIRMALAKHANDAN and GARDEA-TORRESDEY, 2002).

Besides these parameters, the levels of compounds such as salts, hydrocarbons, organic acids and phenols are also quantified and used to validate the remediation process of this effluent (JIMÉNEZ et al., 2018). It is important to note that the concentration of each pollutant in treated produced water can vary according to its destination, which can either be for disposal or reuse, such as in irrigation as cited by Suhane, Dewan and Mohaimin (2021).

Table 6.1 - Regulated limits of oil and grease standards in produced water for disposal.

REGIONS	DAILY LIMIT (mg L^{-1})	MONTHLY LIMIT (mg L^{-1})	REFERENCES
Brazil (*offshore*)	42	29	Brasil (2007)
Brazil (discharge near the coast)	20	-	Brasil (2011)
China	-	10	Tellez, Nirmalakhandan and Gardea-Torresdey (2002)
United States	42	29	EPA (2002)
Australia (*offshore*)	30	-	NEFF (2002)
North Sea, the Mediterranean Sea, Arabian Gulf and Asia (*offshore*)	-	40	
Canada	60	30	
Nigeria	72	40	
Northeast Atlantic	-	30	

The remediation of this type of fluid can be performed using chemical, physical and biological methods, with the first two methods being the most widely used, especially when the treatment units are built on marine platforms. However, some limitations can be found in these techniques, especially with regard to the high cost associated with the chemicals used and the operation required for physical treatment. Additionally, produced water is characterised by having a varied composition and, due to the complexity of this treatment, an association of different techniques is often required (ESTRADA and BHAMIDIMARRI, 2016; JIMÉNEZ et al., 2018).

In general, the literature cites that the treatment process of produced water is preferably formed by three stages (pre-treatment, main treatment and final or polishing treatment), as shown in **Figure 6.2**.

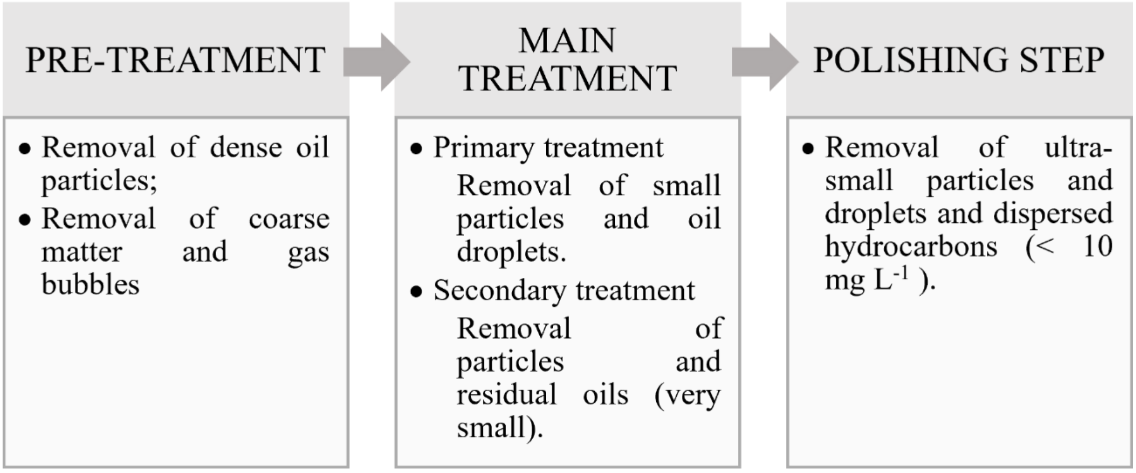

Figure 6.2 - Flowchart of the steps of produced water treatment. Source: Adapted from Al-Ghouti et al. (2019).

Physical, chemical and biological treatment mechanisms have different roles when used in combination in the remediation of produced water and can be used at different stages of the process. For instance, biological methods can be used in the pre-treatment or main stage, when the plant is land-based. On the other hand, physical methods such as centrifugation, filtration and adsorption are applied in the main or polishing treatment steps, while chemical methods such as flotation, oxidation and precipitation are usually used in secondary treatment.

Some studies have evaluated the application of physical and chemical methods, mainly adsorption, membrane filtration and chemical precipitation, in the reduction of the pollutant load found in produced water. However, studies that seek to reduce the cost associated to the process as well as to boost the removal of contaminants are investing in biological processes that mainly use activated sludge, lagoons and biological filters. The starting point of the evaluation of this type of treatment is associated with the presence of native microorganisms in this effluent, which use its polluting load as a source of nutrients for the functioning of their metabolic activities and thus promote its remediation. Besides these microorganisms, commercial microorganisms, specific groups and even acclimated sewage sludge can be used (JIMÉNEZ et al., 2018; AL-GHOUTI et al., 2019).

The process of biological treatment of produced water is also known as biological oxidation. In this case, microorganisms consume the hydrocarbons present in the effluent through biodegradation and bioflocculation mechanisms. The main advantage of this type of treatment is the ability to reduce pollutant levels (metals, COD and contaminants in general) over a wide range of pH, temperature and salinity levels (KATSOYIANNIS and

ZOUBOULIS, 2004, JIMÉNEZ et al., 2018). Guerra, Dahm and Dundorf (2011) also point out that this type of mechanism achieves higher levels of efficiency when the remediated produced water has COD values less than 400 mg L^{-1}, BOD less than 50 mg L^{-1} and oil levels less than 60 mg L^{-1}.

The biological processes usually used for the remediation of produced water can be carried out solely by microorganisms or by an association with other treatment methods. Membrane bioreactors, for instance, combine the use of microorganisms and liquid-solid separation (physical method), while biofilms only make use of different microorganisms. Their application is very convenient for this type of effluent, as they are highly resistant to shock loads and have a great adaptation to the environment even under adverse conditions. Other methods include the use of wetlands and lagoons, activated sludge and anaerobic and bioelectrochemical processes (HASNINE et al., 2017; ABUJAYYAB, HAMOUDA and HASSAN, 2022).

Table 6.2 presents some works that have been carried out in the field of produced water treatment using three types of treatment cited (physical, chemical and biological). Comparing the data presented in the table, mainly in relation to the type of treatment used and TOG and COD removal efficiencies, it is possible to observe that biological treatments are capable of reaching yields of pollutant removal as high as the methods traditionally used (physical and chemical). In addition, the information contained in the literature enables to conclude that bioremediation is a less costly process, making it a promising treatment mechanism for produced water.

Table 6.2 - Removal efficiency of some contaminants present in PW using different types of treatment.

TYPE OF TREATMENT	TREATMENT PRINCIPLE	INITIAL EFFLUENT CHARACTERISTICS (mg L^{-1})	REMOVAL RATE (%)	REFERENCES
Biological	The biosurfactant-producing bacterium *Acinetobacter sp.* Y2 was used to remove chemical oxygen demand (COD) and hydrocarbon fractions.	COD[a] 6646.7	76.7 (COD[a])	Zhou et al (2020)
	Using microalgae *Nannochloropsis oculata* with the objective of removing oils and grease, as	TOG[b] 540 COD[a] 1300	99.6 - 89 (TOG[b]) 54 - 90 (COD[a])	Ammar, Khadim and Mohamed (2018)

	well as chemical oxygen demand (COD).			
	The microalga *Isochrysis galbana* was applied in the treatment for the removal of oil and grease, as well as chemical oxygen demand (COD).	TOG[b] 540 COD[a] 1300	68 - 82 (TOG[b]) 56 - 83 (COD[a])	
	Using *Dunaliella salin* microalgae to treat produced water and obtain biodiesel as a by-product.	NO_3 622 PO_4 300	65 (Nitrogen) 40 (Phosphorus)	Tabeli et al (2016)
	The fungus *Aspergillus niger* was applied to reduce hydrocarbon fractions in produced water.	n-alkanes 608 aromatics 13.88 NSO Comp.[c] 12.68 PAHs[d] 0.833	90.7 (n-alkanes) 95.32 (aromatics) 92.43 (NSO compound[c]) 90.4 (PAHs[d])	Okoro (2008)
	The fungus *Penicillium sp.* was applied to reduce hydrocarbon fractions in produced water.	n-alkanes 608 aromatics 13.88 NSO Comp.[c] 12.68 PAHs[d] 0.833	89.3 (n-alkanes) 93 (aromatics) 87.1 (NSO compound[c]) 99.7 (PAHs[d])	
	The fungus *Aspergillus fumigatus* was used in the reduction of hydrocarbon fractions in produced water.	n-alkanes 608 aromatics 13.88 NSO Compound[c] 12.68 PAHs[d] 0.833	87 (n-alkanes) 88.6 (aromatics) 90.4 (NSO compound[c]) 98 (PAHs[d])	Okoro and Amund (2010)
Physical	It consists of treatment steps using ceramic membranes.	Initial oil concentration 564	99.5 (oil)	Ebrahimi et al (2010)
	Tubular UF module equipped with polyvinylidene fluoride membranes modified by inorganic nano-sized alumina particles used for chemical oxygen demand removal.	COD[a] 637 TOC[e] 214.9	90.14 (COD[a]) 98.04 (TOC[e])	Lia et al (2006)
	PVC hollow fibre alloy membranes, aeration tank, air flotation and sand filter	COD[a] between 380 and 490	80 (COD[a]) 90 (TOG[b])	Qiao et al (2008)

	were combined for the treatment of oily effluent.	TOG[b] between 50 and 200		
	Cellulose acetate hollow fibre membranes for wastewater treatment.	COD[a] 985 TOG[b] 230	97.7 (COD[a]) 98.3 (TOG[b])	He and Jiang (2008)
	Coalescence/filtration which uses porous materials containing impurities.	COD[a] 320 TOG[b] 20	68.1 (COD[a]) 95.8 (TOG[b])	Multon and Viraraghavan (2006)
Chemical	Ozonation combined with H_2O_2 applied in the removal of dissolved organic compounds from produced water.	Organic acids in the range 7 and 760	78 (acetic acid)	Jiménez et al (2019)
	Using semiconductor photocatalysis as a treatment for hydrocarbon removal.	COD[a] between 3500 and 4000	85 (COD[a])	Adams, Campbell and Robertson (2008)
	Combined process that included flocculation, Fenton oxidation and sequencing batch reactor (SBR) for treatment of produced water.	COD[a] of approximately 393.16	76.6 (COD[a])	Yang et al (2014)
	Based on the electrochemical process using double active metal and graphite as anodes and iron as cathode.	COD[a] 5800	90 (COD[a])	Ma and Wang (2006)

[a]Chemical Oxygen Demand, [b]Total Oil and Grease Content, [c]Nitrogen, Sulphur and Oxygen, [d]Polycyclic Aromatic Hydrocarbons, [e]Total Organic Carbon.

6.5 Biological treatment of produced water

As previously pointed out, there are several types of treatment for PW (physical, chemical and biological). However, these are linked to high energy consumption, the use of chemicals and sludge formation. Thus, PW treatments with appropriate disposal, reuse or re-injection standards can cost from 7 to 52% of the total well operating cost (CABRERA et al., 2022). Among the treatment types, biological treatment is considered one of the least costly. Moreover, microorganisms present in PW, such as algae, fungi and bacteria, can be used in the treatment of this effluent (AL-GHOUTI et al., 2019).

Biological treatments of PW can adequately remediate nutrients, organic compounds, heavy metals and other pollutants in different environmental conditions, such as under different levels of salinity, pH and temperature (ABUJAYYAB, HAMOUDA and HASSAN, 2022). Environmental factors are species-dependent, because the growth of each type of microorganism responds to these parameters individually. For example, bacteria generally prefer pH close to neutrality, while fungi prefer acidic pH and microalgae alkaline pH. In addition, microalgae to perform autotrophy/mixotrophy need light intensity input (DE ANDRADE et al., 2022). Regarding salinity and temperature, the differences are even greater within the same class of microorganisms, depending on how halo and thermotolerant they are adapted, generally associated with the region where they were isolated (OJAGH, FALLAH and NASERNEJAD, 2020; EZENNÚBIA and VILCÁEZ, 2023).

Regarding nutritional parameters, it is important to pay attention to the main macro and micronutrients necessary for microbial growth. The main macronutrients are carbon, nitrogen and phosphorus. The excess or lack of some of these nutrients can cause a limitation during the treatment process. Therefore, it is possible that there is a need for supplementation of some nutrient (DE ANDRADE et al., 2022).

In addition to environmental and nutritional factors, operational factors are extremely important, such as the type of bioreactor, mode of operation (batch and continuous), hydraulic retention time, aeration rate and agitation, which are applied differently according to the class. of applied microorganisms, for example activated sludge, fixed-film, anaerobic digestion, and membrane bioreactors technologies were already tested for produced water (CAMARILLO and STRINGFELLOW, 2018; ACHARYA et al., 2020).

Biological treatments can be in-situ when carried out at the effluent/waste generation site itself, stimulating the natural microbiota or inserting a microorganism with remedial potential, or ex-situ, when the effluent is conditioned in an adapted treatment system composed mainly of the bioreactor. A summary of the types of in-situ and ex-situ biological treatments is presented in the **Figure 6.3**. In in-situ processes, bioaugmentation and biostimulation are most applied when the biodegradation of contaminants can be conducted slowly or incompletely. Ex-situ processes are designed to increase treatment efficiency, being faster and more efficient in the contaminant removal (GHAFOORI et al., 2022). Recent advances on ex-situ biological treatment applying microorganisms in bioreactors in PW treatment are discussed in the following subsection.

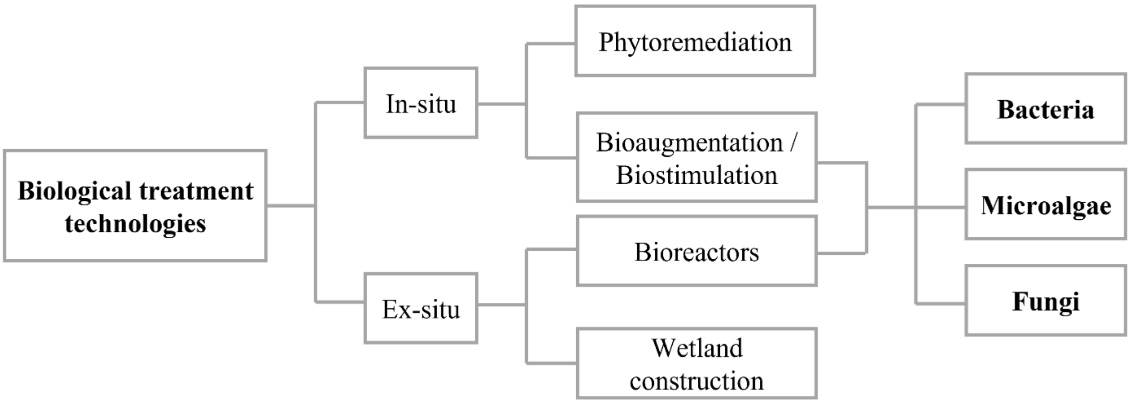

Figure 6.3 - Biological technologies for PW treatment. Source: Adapted from Ghafoori et al. (2022).

The abilities to biologically degrade and remove the contaminants present in the PW vary with the characteristics of the pollutants, the microbial strain used and its nutritional and environmental needs, in addition to the system used (operating variables). Some advantages and disadvantages when using bacteria, microalgae and fungi to bioremediate PW are presented in the **Table 6.3**.

Table 6.3 - Advantages and limitations of PW treatment methods using bacteria, microalgae and fungi.

Microorganism	**Advantages**	**Limitation**
Bacteria (aerobic)	Greater efficiency in the degradation of alkanes and hydrocarbons, compared to anaerobic pathways	Higher production of microbial sludge, compared to anaerobic pathways
Bacteria (anaerobic)	Less sludge production, lower energy expenditure, greater efficiency in the removal of heavy metals, TOG, and solids, in relation to aerobic pathways	Consumes compounds such as alkanes and hydrocarbons more slowly compared to aerobic pathways
Microalgae	High capacity in the absorption and metabolization of metals, Capable to remove COD and TOG, BTEX can be used as a carbon source by some species, good adaptation to different environmental conditions, and higher metabolization of nitrogen and phosphorous compounds respect to the microorganism class	May be inhibited by high concentrations of hydrocarbons and salinity, for example
Filamentous fungi and yeasts	Resistant to hydrocarbons; resistant to heavy metals, good adaptation to different environments, and high ability to remove pollutants such as COD	Most filamentous fungi are not able to fully degrade aromatic hydrocarbons

6.5.1 Bioremediation of PW by bacteria

These treatments can occur via aerobic and anaerobic processes, with aerobic treatment standing out. Nonetheless, anaerobic treatment presents some advantages over aerobic treatment despite its limiting points (HUANG et al., 2021). Some of the advantages of anaerobic treatment over aerobic are liquid energy recovery, lower sludge production, higher loading rates, efficiency in the removal of heavy metals, oil and grease (TOG) and solids. Nevertheless, the degradation of alkanes and hydrocarbons in anaerobic processes occur at slower rates when compared to aerobic processes (SUDMALIS et al., 2018). Some applications are detailed below.

An immobilized culture of *Bacillus* sp. (M-12) was able to significantly decrease the COD of crude oil wastewater from 2600 to 240-260 mg L^{-1} under aerobic conditions in a continuous system (LI et al., 2005). Using an anaerobic baffled reactor (ABR), a microbial community including *Clostridia*, *Methanosarcina*, *Methanothrx* sp. and *Rhodopseudomonas* reduced COD and oil content by 65 and 88%, respectively, of water produced containing heavy oil with poor nutrients (COD:TN:TP, 1200:15:1) and high salt concentration (1.15 –1.46%) (JI et al., 2009).

Ezennúbia and Vilcáez (2023) examined the feasibility of stimulating the activity of indigenous oil-degrading anaerobic microcosms by providing CO_2 and protein-rich matter (yeast extract and isolated soy protein), and adjusting physicochemical conditions (pH, temperature, and potential oxidation-reduction (ORP)) of PW in closed anaerobic tanks using formation water from the Stillwater and Cushing oil fields of Oklahoma, USA. The results show that the stimulation method works at mesophilic (25°C) and thermophilic (50°C) temperatures, isolated soy protein can be used as a substitute for yeast extract, and the degradation of oil hydrocarbons was increased with reduction of ORP, and oil concentrations reduced 40-90% in 7–35 days of treatment.

High salinity concentration in PW (NaCl > 35 g L^{-1}) decrease the treatment efficiency by activated sludge, mainly due to microorganism plasmolysis. Thus, halophilic, and halotolerant bacteria present themselves as a viable alternative for the bioremediation of PW with medium to high salinity concentration (OJAGH, FALLAH and NASERNEJAD, 2020). This is demonstrated by Pendashteh et al. (2011), who used halophilic bacteria to treat hypersaline (TDS = 35 g L^{-1}) synthetic PW using MBR (membrane bioreactor), achieving removal efficiencies of 97.5, 97.3 and 99.0% for COD, TOC and TOG, respectively. In a previous study, Pendashteh et al. (2010) obtained a

COD and O&G removal rate using synthetic PW higher than 90% with a TDS of 35 g L^{-1} and of 74 e 63% when the TDS was 250 g L^{-1}, respectively.

Sharghi, Bonakdarpour and Pakzadeh (2014), evaluated the application of a bacteria consortium moderately halotolerant using a membrane bioreactor to treat synthetic hipersaline PW (100, 150, 200 e 250 g L^{-1} de NaCl), obtaining removal rate of COD and O&G between 81.6–94.6% and 84.8–94.0%, respectively. Similarly, Sharghi and Bonakdarpour (2013) studied the performance of organic pollutants removal in different organic loads (0.3 a 2.6 g L^{-1} d^{-1} de DQO and 50 g L^{-1} of NaCl) reaching removal efficiencies between 83-93% and 95-99% for DQO and O&G, respectively.

Ojagh, Fallah and Nasernejad (2020) carried out a stepwise adaptation strategy to acclimate the gram-positive bacterium *Rhodococcus eryhtropolis* to synthetic PW. The maximum COD removal in synthetic PW (600 mg L^{-1} of COD) was of 97% in the presence of 25 g L^{-1} of NaCl after 2 days. Then, the adapted cells were used in the treatment of real PW with 530 mg L^{-1} of COD and 62 g L^{-1} of salinity, achieving 52% COD removal efficiency after 2 days of treatment.

Tellez, Nirmalakhandan and Gardea-Torresdey (2002) carried out continuous flow activated sludge treatment in PW (COD: 431±25 mg L^{-1}; TOG: 147±35 mg L^{-1}; TPH: 126±30 mg L^{-1}; BTEX: 7.7±2 mg L^{-1}; n-alkanes: 115±30 mg L^{-1}). After 120 days, the activated sludge unit achieved removal rates of 92% for COD, 98% for TOG, 98 % for TPH, 99% for BTEX and 98% for n-alkanes.

Khadam, Agab and Saad (2009) studied the biodegradation of hydrocarbons in PW using native bacterial cultures. The experiments showed that all hydrocarbon contents (nC_4-nC_{34}) were reduced to zero in 36 h in batch and continuous cultures. The BOD and COD contents were reduced to approximately 94% and 97%, respectively.

Huang et al. (2021) performed anaerobic treatment of PW using a microbial consortium from anaerobic digestion from a brewery wastewater treatment plant. The initial COD and TOC levels of PW were 46,580 and 13,590 mg L^{-1}, respectively. The micro composites were prepared in flasks containing 64 mL of PW and inoculum in a 2:1 ratio, diluting COD and TOC concentrations by about 1/3, 31,380 mg L^{-1} of COD and 9,530 mg L^{-1} of TOC and were statically incubated at 30°C in the dark, pH 7.99, for 40 days. After incubation, COD was reduced by 93% and TOC by 89%, with a final pH of 6.18 and cumulative methane yield of 33.9 mmol g^{-1} carbon (65.9% of the maximum theoretical yield).

Zhou et al. (2020) used the indigenous bacterium *Acinetobacter* sp. Y2 isolated from hydraulic fracturing backflow and produced water (HF-FPW). The bioaugmentation treatment occurred in 250 mL conical flasks containing 100 mL of HF-FPW, inoculated with 1 mL of Y2 culture mixture and 100 mg L^{-1} of yeast extract, and pH adjusted to 7-7.5, for 7 days, achieving 76.73% removal of COD, 93.94% of n-alkanes and 77.18% of PHAs, initially at 6646.7 mg L^{-1}, 2635.4 mg L^{-1} and 918.6 μg L^{-1}, respectively.

Based on the studies cited, it is noted that the treatment using bacteria has proved to be promising in the bioremediation of PW, reaching significant values in the removal of contaminants. However, salinity is an important factor for this microbial group and for determining the treatment conditions (aerobic or anaerobic) and the culture to be used in the process. The availability of nitrogen is mentioned as an important factor, generally, supplemented as yeast extract or isolated soy protein, but also ammonia and nitrate salts can be used.

6.5.2 Bioremediation of PW from microalgae

The treatment of wastewater using microalgae has proven to be a sustainable alternative due to their ability to use certain contaminants as a source of nutrients, besides having a high capacity in the absorption and metabolization of metals. For instance, BTEX present in PW can be used as a carbon source for some species of microalgae. However, there may be inhibition in their growth due to increased concentration of BTEX and contact time (AL GHOUTI et al., 2019). Takáčová et al. (2015), added BTEX, at a concentration of 100 µg L^{-1}, as the only carbon source to a culture of *Parachlorella kessleri*, with the aim of evaluating the biodegradation of BTEX. In 48h, 63% of toluene and 40% of benzene and xylenes were degraded, but only 30% of ethylbenzene was degraded after 72h. It was reported that the growth of *P. kessleri* was minimally inhibited after 48 h (13%) due to higher concentrations of metabolic intermediates such as catechol or methyl catechol.

Typically, PWs present toxic compounds that can inhibit the growth of microalgae, as example, metals, volatile organic compounds, and radionuclides, nonetheless, they present concentrations of nitrogen and phosphorus in their composition, which are required nutrients for microalgal metabolism. Nutrient supplementation can represent up to 45% of cultivation costs, and the presence of N and P in wastewaters decrease these costs (HOPKINS et al., 2019). The high salinity in PW is a challenge for microalgae cultivation. Nevertheless, the treatment can be satisfactory using species resistant to hypersalinity, or by diluting the effluent. This type of cultivation/treatment produces biomass, which can be used as raw material for the production of value-added products (ALSARAYREH et al., 2022). Some authors tried to treat PW applying microalgal technology and they are described below.

Hakim et al. 2018 carried out an isolation of microalgae in regions with high oil content and verified their adaptability of cultivation in produced water. After initial studies, the promising species were *Menoraphidium, Chlorella, Neochloris, Scenedesmus, Dictyosphoerium*. However, some of these genera are more often found in freshwater.

Arriada and Abreu (2014) only evaluated the growth viability of the marine microalgae *Nannochlopsis oculata* in different concentrations of produced water. *N. oculata* showed significant growth in diluted produced water with a standard medium (50% of dilution v/v), although obtaining lower biomass yields for the medium with 100% effluent.

Hopkins et al. (2019) showed that a microalgal co-cultivation composed by *Cyanobacterium aponinum* (cyanobacterium), *Parachlorella kessleri* (microalga) and several halotolerant bacteria, mainly; can grow in PW with high salinity range (between 15-60 g L^{-1} of TDS) obtaining dry cell weights between 46-51 mg L^{-1} d^{-1}, decreasing as function of higher salinity.

Ammar, Khadim and Mohamed (2018), evaluated the feasibility of cultivation of marine microalgae *Nannochoropsis oculate* and *Isochrysis galbana* in PW using different effluent loadings (10 to 50%) with BG-11 medium modified with salt water (salinity 35 g L^{-1}), initial COD of 1,300 mg L^{-1} and initial TOG of 540 mg L^{-1}. After 21 days, significant biomass yields were obtained, 1.123, 1.0166, 0.856 and 0.31 g L^{-1} for *Nannochloropsis oculata* and 1.01, 0.899, 0.638 and 0.314 g L^{-1} for *Isochrysis galbana* at 0, 10, 25 and 50% of PW, respectively. Oil and COD removals decreased with increasing PW loads. COD removals were from 54% to 90% for *Nannochloropsis oculata* and 56% to 83% for *Isochrysis galbana. Nannochloropsis oculata* removed 66.5% and 89% of oil and *Isochrysis galbana removed 68% to 82% of oil when grown at 50% and 10% PW loading, respectively.*

Tabeli et al. (2016) showed that the application of PW increased biomass production and lipid content of *Dunaliella salina* by 120% and 65% compared to the control (seawater), respectively. The marine microalgae showed good adaptation to salinity fluctuations (1:1 diluted PW with seawater), achieving removal rates of 66% for

NO_3 and 40% for PO_4, initially with 622 mg L^{-1} and 300 mg L^{-1}, respectively, and heavy metal biosorption rates of 90% for Ni and 80% for Zn, initially with 5 mg L^{-1} and 2 mg L^{-1}, respectively.

Das et al. (2019) used *Chlorella* sp. to treat produced water supplemented with nitrogen and phosphorus. After 15 days, the microalgal biomass yield was 1.72 g L^{1}, achieving 73% TOC and 92% TN removal, despite being a more prominent species in freshwater.

Thus, based on these studies, it is observed that the treatment from different species of microalgae has shown to be efficient in the removal of contaminants present in PWs, such as COD and TOG, and good adaptation to the medium with different salinity concentrations, despite PW having some components that may present toxicity to microalgae. Yet, there are several gaps to be filled on this subject, showing the need for future research to optimise culture parameters to achieve better results, mainly in terms of metabolism and adaptability to adverse composition that is found in produced water.

6.5.3 Bioremediation of PW from filamentous fungi and yeasts

A variety of filamentous fungi and yeasts isolated from hydrocarbon contaminated areas are capable of mineralising petroleum compounds (GARGOURI et al., 2015). Most filamentous fungi are not able to fully degrade aromatic hydrocarbons, rendering them only into products with lower toxicity and more susceptible to decomposition by bacteria (STELIGA, 2012). The significant removal capacity of hydrocarbons by fungi is demonstrated in some studies cited below. However, the number of works available in the literature regarding the use of fungi to remediate PW is low, being more expressive the amount of works in bioremediation by fungi in soil contaminated by oil.

Gargouri et al. (2015) showed that two yeast strains isolated from industrial refinery effluents, *Candida tropicalis* and *Trichosporon asahii*, present a strong ability to biodegrade hydrocarbons and completely metabolise n-alkanes.

On the other hand, Okoro (2008) studied the use of pure cultures of the filamentous fungi *Penicillium* sp. and *Aspergillus niger* in the biodegradation of hydrocarbons in PW with an oil and grease content of 1407 mg L^{-1}, containing various petroleum hydrocarbon fractions, including n-alkanes (608 mg L^{-1}), aromatics (13.88 mg L^{-1}), NSO compounds (12.68 mg L^{-1}) and PAHs (0.833 mg L^{-1}). After 120 days treatment, the results showed that the biodegradation rates were slightly higher using *A. nigger* than *Penicillium* sp, in which the *Penicillium* sp. culture achieved an TOG content removal of

94.86%, reducing to 65.5 mg L^{-1} of n-alkanes, 0.98 mg L^{-1} of aromatics, 1.64 mg L^{-1} of NSO compounds and 0.0021 mg L^{-1} PAHs, and the *A. niger* removed oil and grease content by 95.8%, reducing to 56.5 mg L^{-1} of n-alkanes, 0.65 mg L^{-1} of aromatics, 0.96 mg L^{-1} of NSO compounds and 0.008 mg L^{-1} PAHs. Similarly, Okoro and Amund (2010), using a culture of *Aspergillus fumigatus*, were able to reduce hydrocarbons to 78.5 mg L^{-1} of n-Alkanes, 1.58 mg L^{-1} of aromatics, 1.22 mg L^{-1} of NSO compounds (1.22 mg L^{-1}) and 0.0168 mg L^{-1} of PAHs.

Finally, Filamentous fungi isolated from soils and waters contaminated by petroleum hydrocarbons were used in PW remediation. The species *A. niger*, *A. flavus*, *A. fumigatus* and *Penicillium* sp. were able to remove between 43-47% of BOD, initially with 500 mg L^{-1}, and 99.9% of TPH was removed compared to the initial concentration of 770 mg L^{-1}. The mix of these fungi showed better COD, TSS and TPH removals than the axenic fungi (AI-JAWHARI, MHAIL and ALI, 2015).

6.6 Conclusions and future prospects

This review verified the performance of various types of PW treatment and conditions. It is noted that there are different types of treatment that make the effluent suitable to meet the limits determined by the regulations for disposal or reuse. The choice of a suitable treatment should take into account some factors, such as the characteristics of produced water, geographical location, operational cost, desired by-products and others. Ex-situ biological treatment by microorganisms highlighted the effect of critical conditions such as PW dilution and salinity concentration, and emphasize a system composed by a bioreactor, mainly; designed to perform the treatment faster and with efficiency of contaminants removal.

Besides the biological treatment of PW is promising, most of bioreactors need acclimation periods which is a challenge from a large-scale point of view. Thus, the screening of tolerant microorganisms together with environmental and nutritional adaptations and applying suitable operating conditions (type of bioreactor and system, a good hydraulic retention time and different mode of operation (batch or continuous)) aid to overcome this bottleneck.

There are fewer studies available in the literature in this field when compared to the treatment of other urban and industrial effluents, and bacteria are the main target of study, although the use of microalgae, yeasts and filamentous fungi are also considered.

Further research is needed to better determine the most promising classes of microorganisms and evaluate the use of axenic or mixed cultures (consortia) and optimise operating conditions in the biological treatment of PW. Several works show that halophyte microorganisms are more efficient to treat PW, however, more investigation is necessary to identify and understand their metabolism during the bioreactor operation. But dilution can be an alternative to apply non-halotolerant organisms looking for a treated water recycling strategy inside of the treatment system.

Furthermore, in order to scale up the processes, life cycle assessment and economic analysis are required. Although biological treatment is considered in isolation, it is important to emphasise that it can be a strong ally in the complementation of treatment when physical and chemical processes are also applied.

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Chapter 7: Treatment of Oil-Produced Water Using a Fungi-Microalgae Consortium

Abstract

This study investigated the effect on the treatment of oil-produced water by varying the initial TPH (total petroleum hydrocarbons) concentration of the effluent between 312 and 2500 mg L^{-1}, the salinity between 5-50 g L^{-1}, and at different concentrations of nitrogen (25, 50, and 100 mg L^{-1}) and phosphorus (approximately 30 mg L^{-1}). A synthetic effluent (modified BG-11) with crude oil as a carbon source was used. The microorganisms used were the microalga *Tetradesmus obliquus* LCE-01 and the filamentous fungi *Aspergillus niger*, *Penicillium oxalicum*, and *Cunninghamella echinulata*. The experiments were performed in an aerated bubble column reactor at a rate of 0.5 vvm, using illumination of 100 µmol m^{-2} s^{-1} (for microalgae experiments) and room temperature between 30 and 35 °C. By cultivating all fungi and microalgae separately and in co-culture, it was found that the highest contribution to TPH removal was made by the filamentous fungi, with *C. echinulata* achieving removal rates between 90 and 95%. With respect to salinity, it was observed that *T. obliquus* was able to survive up to concentrations of 25 g L^{-1}, and *C. echinulata* not only grew in all saline concentrations tested but also significantly removed TPH at rates between 80 and 95%. It was observed that the co-cultivation of the fungus with the microalgae removed higher percentages of nitrogen, at 63.4%, 44.4%, and 31.7% of the initial concentrations of 25, 50, and 100 mg L^{-1}, respectively; a similar average of 36.58 ± 4.82% of phosphorus was found for all experiments.

Keywords: Bubble Column Reactor; Environmental Biotechnology; Bioremediation; *Tetradesmus obliquus*; *Cunninghamella echinulata*

This chapter will be submitted to the Journal Energy, Ecology and Environment

7.1 Introduction

The process of extracting oil, whether from underground reserves, onshore or offshore, remains one of the world’s most important sources of energy and chemicals, even though it is a nonrenewable resource (COSTA et al., 2022; ANP, 2022). However, this industry produces an effluent called produced water (PW), which is the largest residue from oil production. Projections indicate that four barrels of produced water are currently produced for every barrel of oil (DUDEK et al., 2020). Its major constituents are dissolved and dispersed oil compounds, dissolved formation minerals, production chemical compounds, production solids (including formation solids, corrosion and scale products, bacteria, waxes, and asphaltenes), and dissolved gases (COSTA et al., 2022). Therefore, there is a lot of concern and action focused on both the environment to minimize the polluting effects of this type of wastewater and on the availability of renewable raw materials in the coming years so that technological progress can continue in balance with nature.

The chemical and physical methods currently used to treat PW have some disadvantages, such as high costs and the generation of toxic waste. There is a need to seek more economical and ecologically sustainable alternatives, such as biological treatment, also known as bioremediation, which uses microorganisms to degrade/detoxify pollutants in wastewater and can use bacteria, fungi, and microalgae (VARJANI, 2016).

Filamentous fungi can degrade hydrocarbons and oil derivatives due to their enzymatic plasticity; in addition, they can remediate liquid effluents and contaminated soils (SINGH and WARD, 2004; BARBIERI and GALLI, 2012). On the other hand, microalgae can consume phosphorus, nitrogen, organic carbon, and components of many effluents (KUMAR et al., 2022). Many researchers report a good relationship between these microorganisms in nature, which is called lichens or symbiotic associations (DAS et al., 2022).

Many filamentous fungi isolated from hydrocarbon-contaminated areas can mineralize petroleum compounds but are more commonly used in *in situ* bioremediation of contaminated soils (GARGOURI et al., 2015) because halotolerant bacteria grow faster and are easier to manage in bioreactors. However, filamentous fungi can efficiently mineralize petroleum hydrocarbons when acclimated and introduced into operating conditions that respect their biological limitations. The species diversity of filamentous fungi used for contaminant removal in the petroleum industry includes *Aspergillus*, *Penicillium*, *Fusarium*, *Verticillium*, and *Phanerochaete* (GHOSH et al., 2023). Some researchers, such as Okoro (2008) and Okoro and Amund (2010), have investigated the use of pure cultures of filamentous fungi *Penicillium sp.*, *Aspergillus niger*, and *Aspergillus fumigatus* in the biodegradation of hydrocarbons (n-alkanes, aromatics, and compounds with NSO). Finally, Al-Hawash et al. (2019) and Al-Jawari et al. (2015) mention the species *Aspergillus sp.* RCF-1 and *A. niger*, *A. flavus*, *A. fumigatus*, and *Penicillium sp*. in the bioremediation of petroleum wastewater.

Regarding microalgae, we can mention the study of Das et al. (2019), in which *Chlorella sp*. was used to treat produced water supplemented with nitrogen and phosphorus, and Arriada and Abreu (2014), who only evaluated the growth viability of the marine microalga *Nannochlopsis oculata* in different concentrations of produced water. Studies using filamentous fungi and microalgae for produced water treatment are limited, and no study of their consortium for this specific wastewater was found, which requires further research in this specific area.

This study investigated the effect on produced water treatment with oil, varying the initial TPH (total petroleum hydrocarbons) concentration of the effluent between 312 and 2500 mg L^{-1}, salinity between 5 and 50 g L^{-1}, and different concentrations of nitrogen (25, 50, and 100 mg L^{-1}) and phosphorus (approximately 30 mg L^{-1}).

7.2 Methodology

7.2.1 Species of filamentous fungi and microalgae

The microalga used was *Tetradesmus sp*. LCE-01. It was maintained on solidified nutrient agar medium (Kasvi®) and in liquid medium for use as an inoculum in BG-11 (blue-green medium) (RIPPKA et al., 1979). The filamentous fungi used were *Penicillium oxalicum* Currie & Thom URM 7170, *Aspergillus niger* Thiegh URM 7282, and *Cunninghamella echinulata* Thaxt URM 7150, obtained from the URM Mycoteca at the Federal University of Pernambuco. The fungal species were maintained on PDA (Potato Dextrose Agar) medium. For the spore suspension, a sterile solution of Triton 0.1% (v/v) was used after an average growth period of 10 days, and the spore concentration was determined from the Neubauer chamber counting procedure.

7.2.2 Efluente sintético utilizado nos experimentos

The synthetic effluent was based on the BG-11 culture medium (RIPPKA et al., 1979), specifically used for microalgae cultivation, while also considering the Bushnell-Haas medium used in hydrocarbon degradation tests by fungal species (ATAKPA et al., 2022; BEMGUENAB and CHIBANI, 2021), in order to meet both nutritional needs of both microbial groups (**Table 7.1**), and crude oil supplied by Petrobras from the Pilar field.

Table 7.1 - Composition of the mineral medium used in the experiments.

Compound	**Modified BG-11 (g/ L)**
Na_2MG EDTA	0,002
Ferric ammonium citrate	0,024
Citric acid.1H_2O	0,024
$CaCl_2.2H_2O$	0,072
$MgSO_4.7H_2O$	0,150
K_2HPO_4	0,170
H_3BO_3	0,003
$MnCl_2.4H_2O$	0,002
$ZnSO_4.7H_2O$	0,0002
$CuSO_4.5H_2O$	0,00008
$COCl_2.6H_2O$	0,0005
$NaMoO_4.2H_2O$	0,0004
Na_2CO_3	0,02
$NaNO_3$	1,5

Regarding the petroleum, provided by Petrobras - Pilar Field - Alagoas - Brazil, the company provided a report specifying that it has a density of 0.7781 g cm-3 and API gravity of 49.4 (PETROBRAS, 2022).

7.2.3 Sistema de tratamento

The bioreactors, cylindrical Drechsel® glass bottles of 500 mL (60 mm outer diameter), were used to simulate the behavior of a bubble column reactor and received 400 mL of useful volume. For experiments with microalgae, the system was exposed to constant artificial light at 100 µmol photons m^{-2} s^{-1} (Delta Ohm 2302.0 radiometer). The system was aerated using a JEBO 660 compressor (2 L min^{-1}) at a rate of 0.5 vvm. pH is one of the most important environmental parameters to ensure the proper functioning of microbial cultivation (ZAINITH et al., 2021). Therefore, it was monitored in all experiments to evaluate its behavior in the growth of the fungus and microalgae separately and in their co-culture, aiming to control it in the best way possible without interfering with the metabolism of both microbial groups.

During inoculation, the concentration of fungal spores used was 10^4 spores mL^{-1}, and about 50 mg L^{-1} for the microalgae concentration. The pH of these media was adjusted according to the needs of the microorganism used so that for media with only microalgae the pH was kept between 8 and 8.5, while for filamentous fungi and microalgae-fungi consortia, the pH was kept between 7 and 7.5. All experiments lasted 7 days and were performed in duplicate.

7.2.4 Preliminary experiments

Initially, synthetic produced water (BG11 culture medium modified with crude oil) with a concentration of 500 mg L^{-1} oil was used in all experiments. In these experiments, the three fungal species and the microalgae were used alone or in co-culture, in addition to a control experiment (without the addition of any microorganisms), since aeration was performed in a non-axenic manner.

7.2.5 Optimization of environmental and nutritional conditions

All experiments were performed under the same experimental conditions as described in **Section 7.2.4**, with the appropriate modifications noted below.

7.2.6 Evaluation of the influence of the concentration of TPH on the treatment

After testing which fungus was most efficient at removing oil and grease, the effect of oil concentration in the treatment was evaluated by applying initial concentrations of TPH ranging from 312 to 2500 mg L^{-1} to the system.

7.2.7 Evaluation of the influence of salinity on the treatment

Next, the effect of salinity on the growth and TPH removal capacity of the fungus and microalgae was evaluated to verify if there was any significant limitation that could compromise the treatment process, as real PW can contain significant salt content;

however, there are exceptions depending on the physical and chemical conditions of the well. The applied salinity varied between 5 and 50 g L^{-1}. The initial concentration of TPH was 2500 mg L^{-1} in all experiments.

7.2.8 Evaluation of nitrate and phosphorus consumption on the treatment process

Finally, the removal of nitrogen and phosphorus in the effluent is verified in order to adapt the synthetic effluent to the nutritional needs of the microorganisms. Values between 25, 50, and 100 mg L^{-1} of nitrogen in the form of nitrate were used and the concentration of total dissolved phosphorus was around 30 mg L-1. The initial TPH used was 2500 mg L^{-1}.

7.2.9 Analyses carried out

For the determination of oils and fats (TPH), the gravimetric method was used by extraction with hexane at a ratio of 5:1 sample:hexane. Hexane was added to the sample and shaken for up to 5 minutes, then transferred to a separating flask. After 10 minutes, the solvent-containing portion was transferred to an oven at 55 ºC in a previously tared crucible for a maximum of 48 hours to verify the complete evaporation of the solvent and to correlate the dry material with a previously calibrated standard curve. The dry weight of the cells was determined gravimetrically by drying the solid fraction obtained by centrifugation at 3500 rpm for 15 minutes of the aqueous suspension after hexane extraction until a constant weight was obtained.

Total phosphorus was determined by the ascorbic acid method (APHA, 2018a). Nitrate was determined by the cadmium reduction method (APHA, 2018b). Nitrite was determined by the ferrous sulfate solution method (APHA, 2018c). Ammoniacal nitrogen was determined by the Kjeldahl method used for agro-industrial effluents, consisting of a 2-step methodology: distillation and titration (IAL, 2005). The pH was determined electronically using a previously calibrated pH meter (TECNAL).

7.3 Results and Discussion

As mentioned above, the company provided the density and API gravity of the oil. The API gravity was established by the American Petroleum Institute (API) for better commercial identification of different types of oil. Therefore, the crude oil collected was characterized as light (PETROBRAS, 2017). Thus, it is possible to verify that the oil was composed mainly of alkanes.

Filamentous fungi have been widely used as bioremediators of petroleum and its derivatives and have also been applied for the treatment of produced water (AI-JAWHARI et al., 2015). In addition, microalgae are present in some studies for the treatment of oil-produced water (GILLARD et al., 2021). However, compared to physical and chemical methods, few studies use biological treatment, although it is a method with lower cost and better environmental sustainability (DELL'ANNO, 2021). Biological treatment still has limitations, mainly due to the recalcitrant nature of petroleum and the presence of other contaminants in produced water as well as the high salinity of the effluent (DAS et al., 2019).

In this sense, it is necessary to carry out more studies to search for microorganisms with higher degradation capacity, as well as wastewater treatment systems that guarantee a better adaptability to them for the removal of contaminants present in the produced water.

First, we sought to select a filamentous fungus of interest among those available (*Aspergillus niger, Penicillium oxalicum, and Cuninghamella echinulata*) and verify its adaptability to the microalgae *Tetradesmus obliquus* for co-cultivation for produced water treatment. Then, the fungal capacity to remove TPH at different initial petroleum concentrations was evaluated. Then, the survival capacity of the microalgae and the filamentous fungus (*Cuninghamella echinulata*) at different salinities was studied, as it is an important characteristic for microbial cultivation in produced water. In the case of co-cultivation with microalgae, the studies were intensified to verify the ability of microalgae to remove additional contaminants in produced water, mainly in the form of nitrogen and phosphorus, since they have a high capacity for their removal in effluents.

7.3.1 Screening of filamentous fungi

In a bubble column reactor, with an initial TPH concentration of 500 mg L^{-1} using modified BG-11, the ability of the above-mentioned filamentous fungi to remove TPH alone and in co-culture with the microalgae was evaluated. For comparative purposes, a control experiment and an experiment with the microalgae alone were performed because the forced aeration (0.5 vvm) was not sterile. In the experiments with the microalgae, a lateral light intensity of 100 µmol m^{-2} s^{-1} was used in the reactors.

As showed in **Figure 7.1**, filamentous fungi were efficient in removing TPH from the effluent between 80 and 90%, with the most promising species being *Aspergillus niger* and *Cunninghamella echinulata*.

According to Al-Hawash (2018) and Dell'Anno (2021), specific genera of *Cladosporium*, *Aspergillus*, *Cunninghamella*, *Penicillium*, and *Fusarium* have been reported in the bioremediation of aliphatic hydrocarbons and the degradation of aromatic hydrocarbons. *Penicillium oxalicum*, despite belonging to a genus commonly cited for oil bioremediation (AI-JAWHARI et al., 2015; MAAMAR et al., 2020), was only able to remove about 55-60% of TPH, which may be due to environmental, nutritional, or operational conditions of the reactor used.

Microalgae have not had a significant impact on the removal of TPHs from wastewater, likely due to the fact that petroleum is composed of hydrocarbons and unicellular microalgae have difficulty metabolizing them directly. However, some studies have demonstrated the potential use of microalgae for TPH removal. For example, Ammar et al. (2018) investigated the feasibility of cultivating the marine microalgae Nannochoropsis oculata and *Isochrysis galbana* in produced water (at different effluent loads: 10 to 50%) using a modified BG-11 medium with salt water (salinity 35 g L^{-1}), with an initial TPH concentration of 540 mg L^{-1}. *Nannochloropsis oculata* removed 66.5 to 89% and *Isochrysis galbana* removed 68 to 82% of TPH when grown at 50% and 10% AP loads, respectively.

However, it is also interesting to mention that despite not efficiently metabolizing TPHs, the microalga was able to grow and produce biomass in equal or greater quantities than the filamentous fungi, probably performing photosynthesis by capturing carbon dioxide from the air injected into the reactor. And it was able to be cultivated together with the filamentous fungi, consequently producing more biomass than the fungi grown alone, thus acting synergistically.

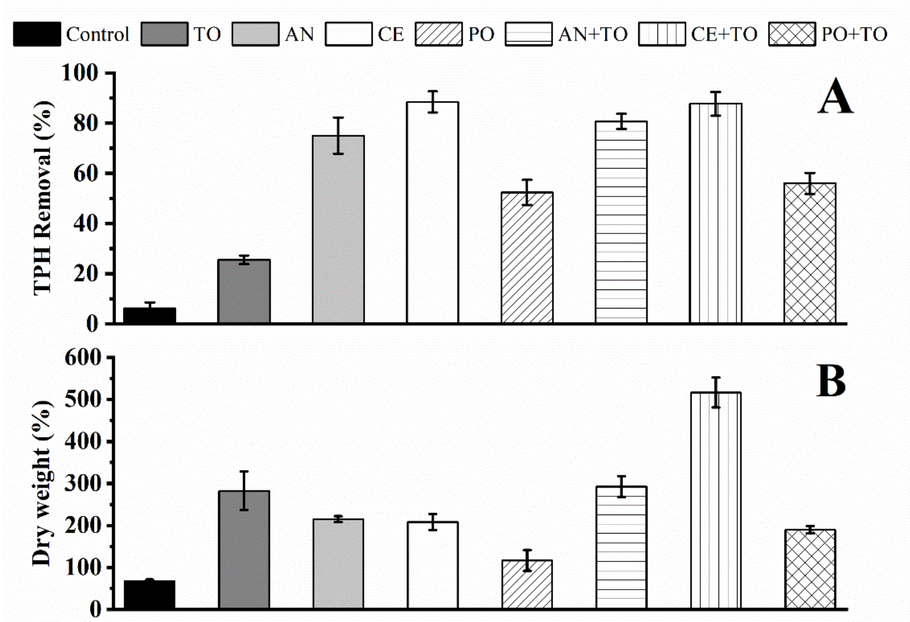

Figure 7.1 - Screening of filamentous fungi in synthetic produced water. A) TPH removal (%) and B) dry weight. TO: *Tetradesmus obliquus*, AN: *Aspergillus niger*, CE: *Cunninghamella echinulate*, PO: *Penicillium oxalicum.*

Some studies show that the synergism between filamentous fungi and microalgae can be applied to wastewater treatment. The study by Zorn et al. (2020) aimed to evaluate the formation of consortium biomass between the filamentous fungus *Mucor circinelloides* and the microalga *Chlorella vulgaris*. A synthetic medium containing glucose (2 g L^{-1}) and essential mineral nutrients for these microorganisms was used. After 180 hours, *C. vulgaris* and *M. circinelloides* cultures reached biomass concentrations of about 0.75 and 1 g L^{-1}, respectively. The biomass of the consortium, composed mainly of fungal mycelia (with microalgal cells contributing in the range of 11.9 ± 1.1%), reached values close to 2 g L^{-1}.

According to the work of Tang et al. (2019), the consortium consisting of the fungus (*Aspergillus sp*. XJ-2) and the microalga (*Chlorella sorokiniana* XJK) performed better than the single system in terms of nutrient removal and biomass formation in simulated Dispersed Red 3B wastewater (COD = 545 mg L^{-1}, TP = 20 mg L^{-1}, and NH_4^+-N = 100 mg L^{-1}). Cultures were performed in flasks containing 100 mL of medium, at 25°C, 160 rpm, pH = 6, for 4 days, in a fungus:microalgae ratio of 1:2. The monocultures of *Chlorella sorokiniana* XJK and *Aspergillus sp*. XJ-2 removed only 55% and 50%, 50% and 50%, and 75% and 70% of COD, TP and $NH4^+$-N, respectively. While the removal rate of COD, TP and NH_4^+-N when the consortium was used reached 93.9%, 83.9% and 87.6%, respectively. The fungus produced a biomass of 0.75 g L^{-1} and the microalgae produced 0.35 g L^{-1} when grown separately. However, the total biomass of the co-cultured microorganisms was 1.42 g L^{-1}.

Another interesting characteristic observed is that the cultivation of filamentous fungi during the 7-day culture did not show a significant pH variation in the bioreactors, starting at 7.5-8.0 and remaining within this range without the need for adjustment. However, for the microalgae, although the initial pH of the medium was in the same range, it continuously increased to a pH of up to 10.5, typical of photosynthetic microorganisms (DE ANDRADE et al., 2022), although no adjustment was necessary since this microalgal species can be easily cultivated within this range.

On the other hand, when the microorganisms were co-cultured, daily pH adjustment to values of 7.3-7.5 was required to accommodate both species, as biological buffering in the synthetic effluent was not efficient when the synthetic effluent was used. In some studies, depending on the characteristics of the effluent, buffering may be present in the fungus-microalgae co-culture without the need for biological control (LIN et al., 2022).

Considering everything that was mentioned, it was decided to continue the work with the fungus *Cunninghamella echinulata*, for the following reasons: higher capacity for TPH removal from synthetic effluent; higher production of microbial biomass with consequent better adaptability of the fungus-microalgae consortium; and lower amount of studies on the fungus as a bioremediator of petroleum and its derivatives.

7.3.2 Evaluation of TPH removal capacity by *Cunninghamella echinulata*

Observing that the filamentous fungus was mainly responsible for the removal of TPH from the effluent and to verify its efficiency even at high petroleum concentrations, experiments were carried out with initial petroleum concentrations ranging from 312 to 2500 mg L^{-1}.

These concentrations of TPH are commonly found in produced water (OKORO and AMUND, 2010; AMMAR et al., 2018). It was observed that the fungus was able to grow well and treat TPH in the synthetic wastewater at all concentrations tested, with a removal rate between 95-98% (**Figure 7.2C**). It was also observed that the production of microbial biomass was proportional to the initial petroleum concentration (**Figure 7.2A**), reaching almost 1 g L^{-1} dry weight when 2500 mg L^{-1} petroleum was used.

On the other hand, microalgae produced about 400-500 mg L^{-1} biomass but removed very little of the TPH present in the effluent (about 30%), and their efficiency decreased with increasing TPH concentration (**Figure 7.2B and 7.2D**). In the literature, it is possible to find that microalgae remove TPH from the effluent, but at low concentrations, as verified by Ammar et al. (2018), who had to dilute the TPH -containing produced water with an initial concentration of 540 mg L^{-1}. They showed that the final biomass concentrations of the microalgae Nannochloropsis oculata and *Isochrysis galbana* decreased with increasing PW percentage (due to toxicity). The biomass concentration decreased from 1.0166 to 0.31 g L^{-1} for *Nannochloropsis oculata* and from 0.899 to 0.314 g L^{-1} for *Isochrysis galbana* when the effluent loading increased from 10% to 50%. *Nannochloropsis oculata* removed 66.5% and 89% of the oil and *Isochrysis galbana* removed 68% to 82% of the oil when cultivated at 50% and 10% effluent load, respectively.

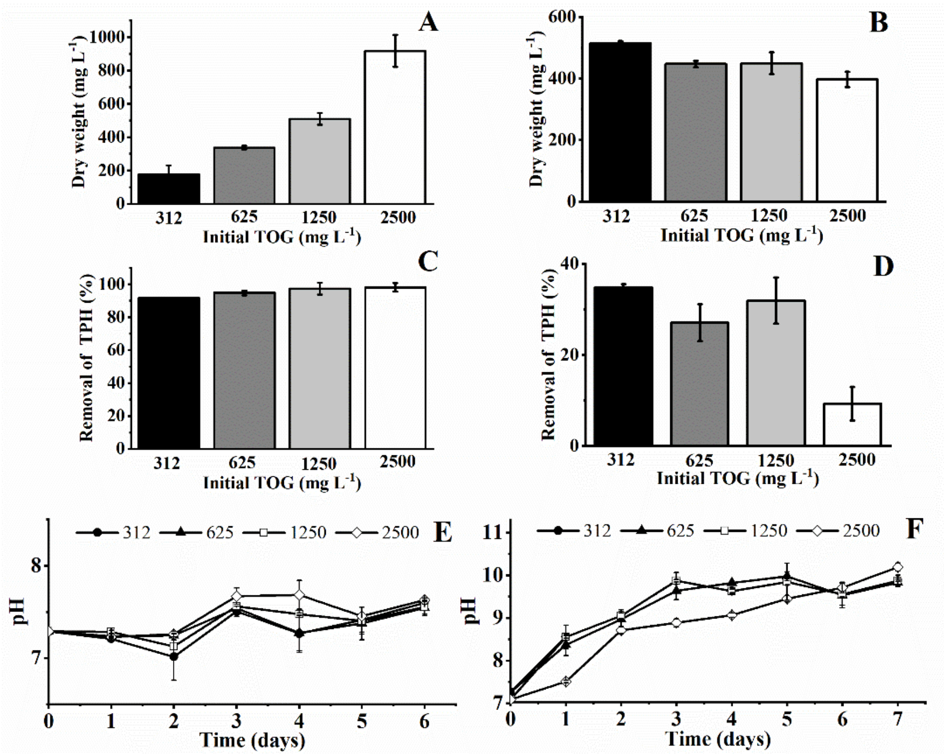

Figure 7.2 - Treatment of synthetic produced water at different oil concentrations A) and B) TPH removal, C) and D) dry weight, and D) and E) pH during cultivation by *Cunninghamella echinulata* (A, C, and E) and *Tetradesmus obliquus* (B, D, and F), respectively.

Finally, as previously verified, there was no significant change in the pH range during fungal cultivation in synthetic produced water that did not require pH control (**Figure 7.2E**). However, with respect to microalgae, the pH continuously increased to reach values around 10 (**Figure 7.2F**). The results obtained agree with Xie et al. (2013), who conducted a study to optimize the co-cultivation of microalgae (*Chlorella vulgaris*) with filamentous fungus (*Cunninghamella echinulata*) to achieve complete removal of individual algal cells from the liquid medium by pelleting. One of the parameters evaluated was the pH in a monoculture and co-culture cultivation system. Through this evaluation, it was found that in the monoculture system, *Chlorella vulgaris* developed better in alkaline pH (close to 8), while the fungus *Cuninghamella echinulata* showed superior growth in an acidic medium (between 5 and 6.3). However, in co-cultivation, the pH tended to decrease to values between 3.5 and 5, which was different from what occurred in our work but showed that pH imbalance can occur in the co-cultivation system.

On the other hand, the study developed by Qiao et al. (2022) confirms that *Cunninghamella* has greater efficiency at pH values between 6 and 7, as their research showed greater efficiency in the co-cultivation between the fungus *Cunninghamaella echinulata* and the yeast *Trichosporon fermentans* at pH values between 6 and 7 in soybean oil wastewater, which is consistent with the conditions adjusted in the present study.

7.3.3 Study of the adaptability of fungus and microalgae to salinity

In this step, the survivability of the fungus *Cuninghamella* and the microalgae *Tetradesmus* in different salinities of the synthetic wastewater was verified, since salinity is an important parameter to evaluate the treatability of the wastewater (SILVA, 2021).

The salinity of PW from offshore exploration has high salt concentrations, around 30 g L^{-1} (ZANDONADE and SANJOMBI, 2015). Similarly, PW from onshore exploration has a salinity higher than seawater (35 g L^{-1}) (OJAGH, FALLAH and NASERNEJAD, 2020), but it should be noted that these values can be much higher. This is a major disadvantage for biological treatment because most terrestrial microorganisms grow better at low salinity. Regarding the adaptability to salinity, microorganisms are classified as non-halophilic (NaCl concentrations less than 0.2 M, about 12 g L^{-1}), but if they tolerate higher salt concentrations, they are called halotolerant. Microorganisms that grow in salt concentrations between 12 and 30 g L^{-1} are slightly halophilic (considered marine organisms). Moderately halophilic organisms adapt better to saline conditions containing 30 and 150 g L^{-1} NaCl. Finally, extreme halophiles have satisfactory growth in salt concentrations higher than 150 g L^{-1} NaCl (ŚLIZEWSKA, 2022).

In this sense, values between 0 (control condition) and 50 g L^{-1} of NaCl added to the synthetic produced water were chosen, verifying both the removal of TPH and the production of microbial biomass and pH during fermentation, with a constant initial concentration of TPH equal to 2500 g L^{-1} (highest concentration) (the same experimental setup as in the preceding steps was used).

Increasing salinity in the effluent resulted in a reduction in fungal pellet production from 1200 to 900 mg L^{-1} and in produced water treatment from 95% to approximately 80% for effluent without and with 50 g L^{-1} NaCl addition, respectively (**Figure 7.3A and C**). Increased salinity hinders the survival of most living organisms due to osmotic and ionic stress (CORRAL, 2019). However, some organisms, such as fungi, develop other adaptation mechanisms (ŚLIZEWSKA, 2022).

With respect to microalgae (especially those living in freshwater), increased salinity affects their development and photosynthesis, causing irreparable damage to the photosynthetic system. In addition, salt stress affects the activity of certain specific enzymes, thus affecting metabolic processes (QIU, 2022; JI et al., 2018). Therefore, a preliminary study varying the salt concentration is necessary, as produced water typically has a high salt concentration.

Regarding the microalgal cultivation at different NaCl concentrations using the same synthetic produced water, it was confirmed that the studied microalgae did not significantly remove TPH from the wastewater, removing only between 3-10% of the initial TPH. On the other hand, it was observed that the microalgae remained alive in reasonable quantities in all experiments between 0 and 25 g L^{-1} NaCl, although the cell growth was negatively influenced by the salt concentration, especially at 50 g L^{-1}, reaching a maximum dry weight of about 300 mg L^{-1} for the condition without added salinity and decreasing to values <50 mg L^{-1} biomass for 50 g L^{-1} NaCl (**Figure 3B and D**).

The behavior obtained in this study is consistent with the literature. For example, Ji et al. (2018) evaluated the physiological and biochemical effects of salt stress (0 to 12 g L^{-1}) on the freshwater microalga Scenedesmus obliquus XJ002. They found that with increasing salinity, there was a decrease in biomass production, chlorophyll and carotenoid content. In addition, salt stress affected the development of the oxygen-evolving complex (OEC) and the reaction center of PSII (photosystem II), reducing electron transport on the donor and acceptor sides of the reaction center and affecting the absorption, transfer, and utilization of light energy.

In addition, Yang et al. (2022) found lower biomass production with increasing salinity (9 to 36 g L-1) in both freshwater microalgae (*Chlorella sorokiniana* GEEL-01 and *Desmodesmus asymmetricus* GEEL-05) evaluated during the experiments. It is noteworthy that the microalgae were completely inhibited at 36 g L^{-1} NaCl, but removed 60 to 80% of total nitrogen (TN) up to 27 g L^{-1}. Both strains completely degraded total phosphorus (TP) after 8 days of cultivation in wastewater with up to 18 g L^{-1} NaCl, while 40 to 80% of TP was removed below 27 g L^{-1}, confirming the results of this study.

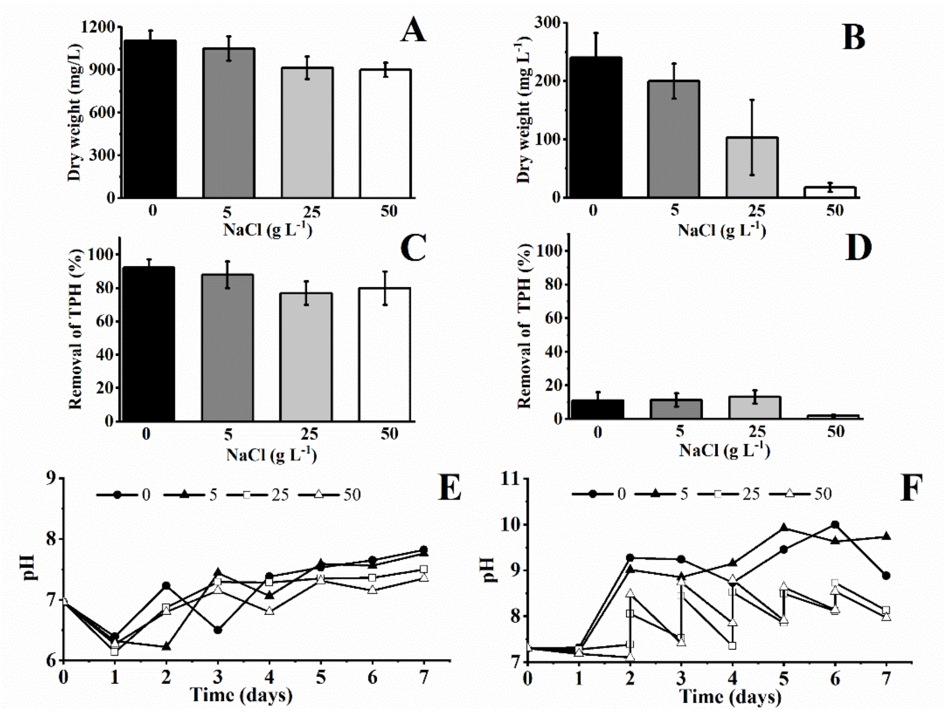

Figure 7.3 - Treatment of synthetically produced water at different NaCl concentrations. A) and B) dry weight, C) and D) TPH removal and E) and F) pH during cultivation of *Cunninghamella echinulata* (A, C and E) and *Tetradesmus obliquus* (B, D and F), respectively.

Despite these limitations due to high salinity, current research confirms the use of microalgae in produced water bioremediation. This can be achieved through dilution or gradual adaptation of the microorganism to the effluent. Hakim et al. (2018) isolated microalgae in regions with high oil content and tested their adaptability to produced water cultivation. After initial studies, promising species were identified, including *Menoraphidium, Chlorella, Neochloris, Scenedesmus, and Dictyosphoerium*. However, some of these genera are more common in freshwater. Hopkins et al. (2019) showed that a microalgal co-culture consisting of *Cyanobacterium aponinum* (cyanobacterium), *Parachlorella kessleri* (microalga), and several halotolerant bacteria can grow in produced water with a high salinity range (between 15 and 60 g L^{-1} TDS – total dissolved solids) and achieve biomass productivity of 46 to 51 mg L^{-1} d^{-1}, which decreases at higher salinities.

Das et al. (2019) used Chlorella sp. to treat produced water supplemented with nitrogen and phosphorus. After 15 days, the microalgal biomass yield was 1.72 g L^{-1}, achieving removals of 73% for COD and 92% for TN, despite being a species more prominent in freshwater. Thus, based on these studies, it is observed that the treatment by different species of microalgae was efficient in removing contaminants present in PWs, such as COD, TOC, and TPH, and has a good adaptation to the environment with different salinity concentrations, although PW has some components that may be toxic to microalgae.

Regarding the pH during cultivation for fungal growth, it can be seen that the behavior was similar in the four experiments, remaining in a constant range between 6.0-7.5 (**Figure 3E**), similar to the experiments of the previous steps.

One event that differed from previous studies of microalgal growth was that at high salt concentrations (25 and 50 g L^{-1} NaCl), the pH during cultivation tended to decrease to values close to 7.0 (**Figure 3F**), requiring daily monitoring, and this may have been one of the reasons for significantly lower microalgal biomass production compared to experiments with 0 and 5 g L^{-1} NaCl, as pH directly affects microalgal cultivation. A possible cause of this phenomenon is the fact that petroleum residues contain free hydrogen, which causes acidification of the medium. This causes damage to equipment and marine transport and makes the aquatic environment inhospitable to microorganisms (ALAZAIZA et al., 2022).

For microalgae, the ideal pH is alkaline, including for Tetradesmus obliquus, preferably between 8 and 9.5 (CASSINI et al., 2017; ROSLI et al., 2020), but as this is directly related to salinity and pH, it needs to be better studied from a cellular and chemical perspective.

7.3.4 Evaluation of nitrogen and phosphorus removal by fungus and fungus-microalgae consortium

After verifying that the microalgae alone did not efficiently remove TPH from the effluent, especially at high concentrations, experiments were conducted to evaluate different concentrations of nitrate (in the form of nitrogen at 25, 50, and 100 mg L^{-1}) and phosphate (in the form of phosphorus around 30 mg L^{-1}) with the fungus alone and in co-culture with the microalgae to verify if there was complementation in the removal of these contaminants when the microalgae were present.

Figure 7.4 shows that the removal was significantly higher when the fungus was grown in co-culture with the microalgae. Microalgae are great removers of nitrogen and phosphorus from wastewater, pollutants responsible for the phenomenon of eutrophication in water bodies (DE ANDRADE et al., 2022). The fungus alone was able to remove about 10 and 15% of the initial nitrogen (nitrate) (**Figure 7.4A),** while when cultivated together with the microalgae, they removed 63.4, 44.4, and 31.7% of the nitrate present in the effluent for initial concentrations of 25, 50, and 100 mg L^{-1} (**Figure 7.4B**).

In terms of effluent phosphorus removal, the profiles for the fungus alone were similar at the three initial nitrate concentrations used, with a removal rate of about 22.28 ± 4.13%. Similarly, the removal rates in the co-culture experiments were close to each other, resulting in 36.58 ± 4.82%. It is important to note that the removal of phosphorus was much higher (almost 15%) when the microalgae were present in co-culture with the fungus than when the fungus was grown alone.

Several studies are cited in the literature that deals with the effluent treatment and demonstrate the capacity of *Tetradesmus* (phylogenetically correlated with *Scenedesmus*, *Desmodesmus*, and *Acutodesmus*) for high nitrogen and phosphorus assimilation. For example, Kim et al. (2015) treated anaerobic digester effluent with low carbon content (COD = 660 mg L^{-1}) and high nutrient content (NH_3-N = 273 mg L^{-1}, TP = 58.75 mg L^{-1}) with the microalgae *Scenedesmus sp*. (initial biomass concentration of 0.09 g L^{-1}). The experiments were carried out in flasks containing 200 mL of autoclaved wastewater under constant agitation of 150 rpm, constant temperature of 27 °C, continuous light of 140 µmol photons m^{-2} s^{-1} and CO_2 supplementation for 23 days. This treatment was quite effective, achieving removal efficiencies of > 99.19% for nitrogen and 98.01% for phosphorus and a final biomass concentration of 8.55 g L^{-1}.

Another example is the work developed by Fontoura et al. (2017), who cultured Scenedesmus sp. in tannery wastewater and evaluated its ability to remove pollutants (NH_3-N = 343 mg L^{-1}, P = 6.6 mg L^{-1}, COD = 4000 mg L^{-1}). The cultures were carried out in 1000 mL flasks with different concentrations of wastewater (20% to 100%), different light intensities (80 to 200 µmol photons m^{-2} s^{-1}) with a 12:12 h day/night cycle, at room temperature (25 °C) and constant aeration by bubbling compressed air (1 L min^{-1}) at the bottom of the flasks for 24 days. The culture showed a maximum biomass concentration (900 mg L^{-1}) and a maximum removal of NH_3-N (85.63%), P (96.78%), and COD (80.33%) in the effluent with a concentration of 88.4% and a light intensity of 182.5 µmol photons m^{-2} s^{-1}. Gil-Izquierdo et al. (2021) used a consortium of microalgae

(*Monoraphidium* sp., *Desmodesmus subspicatus*, *Nannochloris* sp.) to evaluate the pollutant removal capacity of FBRs to minimize the eutrophication process of a pond (NO^{3-} = 274 mg L^{-1}; PO_4^{3-} = 19.1 mg L^{-1}). Cultivation was performed outdoors (light and ambient temperature) and in batch PBRs (capacity of 6 L) connected to a timer for pulsed injection of air and CO_2 every 20 min. The microalgae consortium achieved nitrate and phosphate removal rates of 89.9% and 99.7%, respectively.

Finally, Salazar et al. (2023) evaluated the nutrient removal capacity of Tetradesmus obliquus in wastewater from a commercial hydroponic greenhouse (Experiment 1: NO^{3-}-N = 284.8 ± 0.18 mg L^{-1}, PO_4^{3}-P = 15.3 ± 0.01 mg L^{-1}, pH = 6.9; Experiment 2: NO^{3-}-N = 235 ± 0.29 mg L^{-1}, PO_4^{3}-P = 8. 8 ± 0.04 mg L^{-1}, pH = 7.7), in a 65 L tubular PBR, with an approximate light intensity of 320 µmol photons m^{-2} s^{-1} and a 17:7 h (light:dark) photoperiod, inoculated with an initial dry weight of 0.2 g L^{-1} of T. obliquus, a constant pH of 7.5 with automatic CO_2 injection, for 20 days. In both experiments, 100% removal of PO_4^{3}-P was achieved on day 3. In Experiment 1, the culture was able to remove 97.5% and 100% of NO^{3-}-N on days 13 and 15, respectively, and continued to grow until the end of the experiment, reaching a maximum dry weight of 4.8 ± 0.02 g L^{-1}. In Experiment 2, the culture achieved 100% removal of NO^{3-}-N on day 8 and accumulated a maximum dry weight of 6.2 ± 0.03 g L^{-1} (day 20).

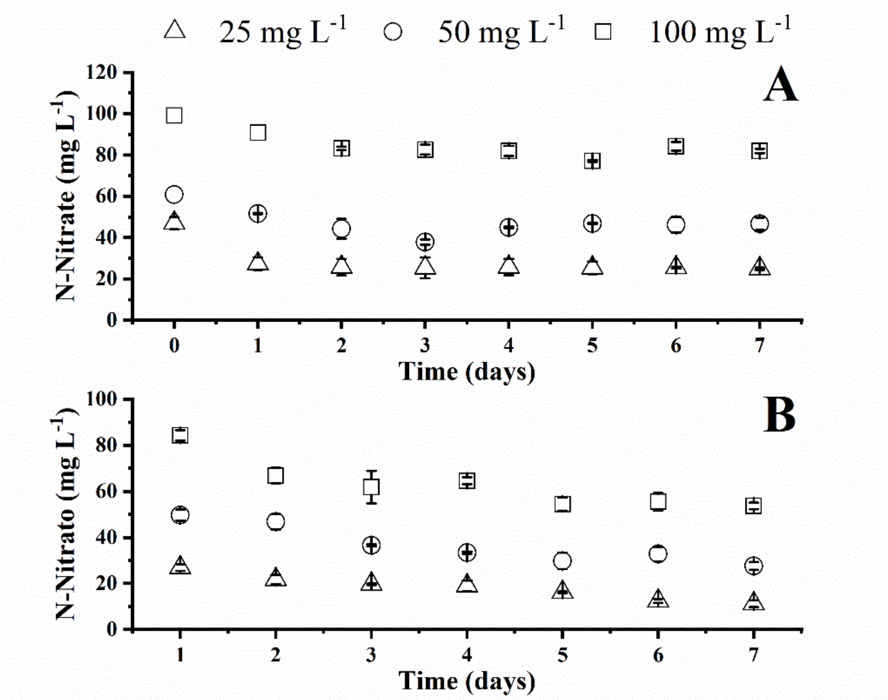

Figure 7.4 - Treatment of synthetic produced water at different nitrogen concentrations by *Cunninghamella echinulata* (A) and its co-culture with *Tetradesmus obliquus* (B).

Some studies show the ability of co-cultivation of filamentous fungi and microalgae for wastewater treatment, called artificial "lichens", discussing the mutual benefits they obtain and the advantages that can be applied to wastewater treatment, achieving higher values than when cultivated separately. For example, Wrede et al. (2014) evaluated the ability of the fungus *Aspergillus fumigatus* in co-culture with the microalgae *Thraustochytrid* (Af/Thr) and *Tetraselmis chuii* (Af/Tc) to absorb the main nutrients (NH^{4+}-N = 680.7 ± 23.1 mg L^{-1} and PO_4^{-3}-P = 145.4 ± 13.7 mg L^{-1}) from pig wastewater diluted with sterile seawater. After 48 h of Af/Thr incubation, NH^{4+}-N concentration was reduced from 164.3 ± 13.2 mg L^{-1} to 22.2 ± 5.8 mg L^{-1} (86% removal) and PO_4^{-3}-P concentration was reduced from 38.7 ± 3.4 mg L^{-1} to 11.8 ± 2.1 mg L^{-1} (69% removal). Af/Tc reduced NH^{4+}-N to 36.9 ± 10.0 mg L^{-1} (77%) and PO_4^{-3}-P to 19.0 ± 5.6 mg L^{-1} (51%). These removal efficiencies were higher than those obtained separately by *Thraustochytrid sp*. (30% and 18%, respectively), *A. fumigatus* (43% and 31%, respectively), and *T. chuii* (32% and 40%, respectively).

Another study conducted by Yang et al. (2019) showed that co-cultivation of *Chlorella vulgaris* and *Aspergillus* sp. in molasses effluent (NT = 407.5 mg L^{-1}, NH_3^-N = 170 mg L^{-1}, FT = 30.4 mg L^{-1}) had better performance in nutrient removal than monocultures. The microalgae and fungi were co-cultured in flasks containing 100 mL of sterilized molasses at an inoculation ratio of 100:1 (microalgal cells and fungal spores – initial density of fungal spores was 1.5 × 10^4 mL^{-1}), shaken at 80 rpm, 35°C, for 5 days. The TN removal efficiencies by microalgae, fungi, and co-culture systems reached 44.39%, 18.20%, and 67.09%, respectively. The introduction of fungi into the microalgae system increased the NH_3-N removal from 79.64% to 94.72%. Finally, the TP removal efficiency of the co-culture system reached 88.39%, while the microalgal and fungal monocultures removed about 30% and 40%, respectively, at the end of the cultivation period.

Song et al. (2022) used the fungus *Penicillium sp*. in mono- and co-culture with the microalga *Chlorella pyrenoidosa* at different inoculum concentrations (*Chlorella pyrenoidosa*: 2 × 10^6, 5 × 10^6, and 8 × 10^6 cells mL^{-1} with dry weights of 0.10, 0.25, and 0.40 g L^{-1}; *Penicillium* sp. 10^3, 10^4, and 10^5 spores mL^{-1}; ratio of microalgae cells to fungal spores: 50:1, 500:1, and 5000:1) to remediate high nutrient levels in soy sauce wastewater (COD = 4440 ± 235.6 mg L^{-1}, NH^{4+}-N = 154.5 ± 2.6 mg L^{-1}, TN = 172.4 ± 10.9 mg L^{-1}, TP = 24.9 ± 2.2 mg L^{-1}). All experiments were performed in 100 mL culture flasks with continuous shaking at 120 rpm at 28 °C for 6 days and, for experiments with microalgae, with the continuous light incidence of 120 μmol photons m^{-2} s^{-1}. In monoculture, the microalgae with an initial dry weight of 0.25 g L^{-1} achieved nutrient removal rates of 564.7 ± 13.1, 20.2 ± 0.8, 22.3 ± 0.4, 3.3 ± 0.01 mg L^{-1} d^{-1} for COD, NH^{4+}-N, TN, and TP, respectively, and reached a maximum biomass yield of 2.2 g L^{-1}. The fungal monoculture, 10^5 spores mL^{-1}, achieved nutrient removal rates of 356.2 ± 9.1, 9.7 ± 0.3, 10 ± 0.3, 0.9 ± 0.02 mg L^{-1} d^{-1} for COD, NH^{4+}-N, TN, and TP, respectively, and a biomass yield of 0.29 ± 0.01 g L^{-1}. Compared to the monocultures, the *C. pyrenoidosa-Penicillium sp*. consortium (5000:1) showed a higher biomass yield (2.8 ± 0.07 g L^{-1}) and nutrient removal rates of 616.7 ± 8.6, 22.8 ± 0.6, 25.0 ± 0.7, and 4.1 ± 0.18 mg L^{-1} d^{-1} for COD, NH^{4+}-N, TN, and TP, respectively.

Finally, Wang et al. (2022) used a combination of the fungus *Aspergillus oryzae* and the microalga *Chlorella pyrenoidosa* to remove nutrients from potato starch wastewater (COD = 12266.82 ± 754.26 mg L^{-1}, TN = 611.30 ± 1.78 mg L^{-1}, and TP = 49.59 ± 1.45 mg L^{-1}). The initial density of fungal spores was fixed at 5, 10, 20, and 30 spores mL^{-1}, and the density of *C. pyrenoidosa* was fixed at an algae/fungus ratio of 4000:1. All cultures were maintained at 25°C on an orbital shaker at 150 rpm with a light intensity of 30 μmol m^{-2} s^{-1}. *C. pyrenoidosa* showed low final removal efficiencies for the main pollutants, only 53.33%, 52.81%, and 42.18% for COD, TN, and TP, respectively. A. oryzae showed better performance in pollutant removal than *C. pyrenoidosa*, with final removal efficiencies of 77.38%, 73.96%, and 82.57% for COD, TN, and TP, respectively. The co-culture of *C. pyrenoidosa* and *A. oryzae* showed significant synergistic effects in the removal of nutrients from potato starch wastewater, as the removal efficiency of COD, TN, and TP reached 92.08%, 83.56%, and 96.58%, respectively.

Regarding the dry cell weight and TPH removal in experiments with different concentrations of nitrogen (in the form of nitrate), it was observed that a higher availability of nitrogen favored cell growth as well as TPH removal, whether with the fungus alone or with its co-culture with the microalgae (**Figure 7.5**).

As previously observed, the pH variation in the experiments showed stability in terms of fungal growth, with initial acidification but stability around 7.5 after 2 to 3 days of cultivation, requiring no pH adjustment (**Figure 7.6A**). On the other hand, in the co-culture with the microalgae, there is an alkalization of the medium to high values around 9.0, requiring daily correction to values close to 7.5 (**Figure 7.6B**). This phenomenon has been observed before, but it is important to emphasize that further studies are needed regarding the influence of this pH variation on the survival of both microbial species, as well as its interference in the removal of contaminants such as TPH, nitrogen and phosphorus.

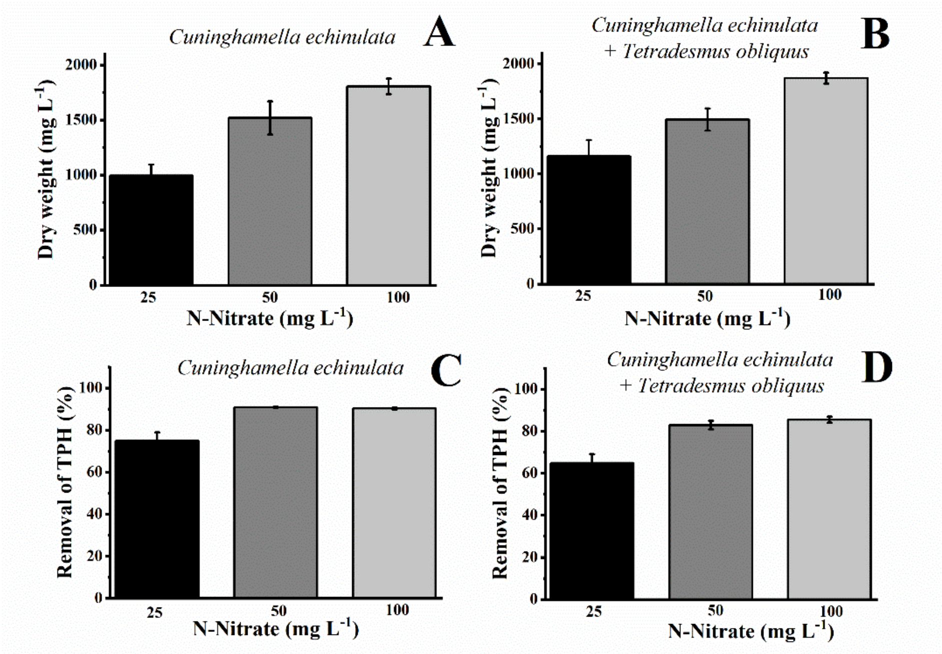

Figure 7.5 - Dry cell weight and TPH removal during the treatment of synthetic produced water at different nitrogen concentrations by *Cunninghamella echinulata* (A and C) and its co-culture with *Tetradesmus obliquus* (B and D), respectively.

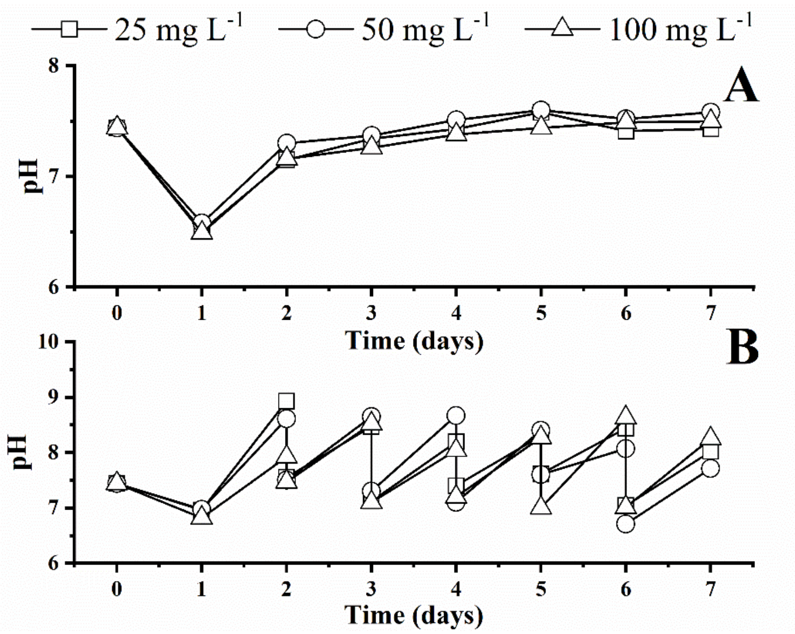

Figure 7.6 - pH variation during the treatment of synthetic produced water at different nitrogen concentrations by *Cuninghamella echinulata* (A) and its co-culture with *Tetradesmus obliquus* (B), respectively.

In the study by Yang et al. (2019), after five days of cultivation in molasses wastewater (pH = 6.12), the pH value of the monospecific culture of fungi (*Aspergillus sp.*) decreased rapidly to around 5, while for microalgae (*Chlorella vulgaris*) the pH value increased to values close to 10. However, the pH in the co-culture remained stable between 6 and 7.5. Song et al. (2022) cultivated the microalga *Chlorella pyrenoidosa* and the fungus *Penicillium sp*. in soy sauce wastewater. After six days, the pH values of the microalgae culture and the microalgae-fungus consortium gradually increased to about 9.5, while for the fungus culture, the pH value remained between 7.5 and 8. These results corroborate the behavior found in the current study.

7.4 Conclusion

The study demonstrated that the fungus *Cunninghamella echinulata* is efficient in removing TPHs from liquid effluent and can maintain this efficiency even at high salt concentrations, a characteristic that may be present in real effluent. Although the microalga *Tetradesmus obliquus* is not efficient in TPH removal, it plays an important role in symbiosis with the fungus, significantly improving the removal of environmentally hazardous contaminants such as nitrogen and phosphorus. One parameter that must always be taken into consideration is the pH since the filamentous fungus tends to keep it at slightly acidic to neutral levels, while the microalgae tend to raise it to very alkaline levels. Therefore, to carry out their co-cultivation, the pH must be controlled at values between 7-7.5. The study demonstrated the applicability of microorganisms in co-culture for the biological treatment of produced water. Moreover, the microorganisms can produce a relevant amount of biomass that can be used in other biotechnological processes.

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Chapter 8: Conclusions

In this thesis, it was possible to perceive that the treatment of effluents using microalgae is a topic of current interest due to the efficiency that can be achieved in the remediation of urban and (agro)industrial effluents verified in the literature. It was also noticed that the types of bioreactors and the consortium with other microbial groups (bacteria, filamentous fungi and yeasts) can enhance the effluent treatment, increasing the removal of chemical oxygen demand (COD), nitrogen and total phosphorus from the effluents. The microbial consortium exhibits additional advantages, due to the existing synergy between microalgae (autotrophic/myxotrophic) and these other heterotrophic microbial groups, complementing their biochemical functions.

In addition, the operation mode of the treatment process constitutes an important operational parameter to achieve greater treatment efficiency. The modes discussed were batch, batch-fed, semi-continuous and continuous, which are adapted to effluents of different types reaching high rates of contaminant removal. While batch and fed-batch systems have the advantages of reducing contamination risks, the semi-continuous and continuous modes have higher productivity due to the lack of lost time (between one process and another) and the needed for inoculum propagation.

By testing kinetic models to better model the treatment of effluents by microalgae, it was found that the *n* order model, with *n* between 1-2 (first and second order) was the most adequate to predict the removal of contaminants (such as COD, nitrogen and phosphorus) of the effluents. For cell growth, the Monod Model was able to be applied, however, it has the limitation that it can only be applied considering a single substrate/contaminant, and in the effluent several contaminants need to be evaluated concomitantly. In this sense, the Silva and Cerqueira Model was developed, and was able to model microbial cell growth during the treatment of effluents considering multiple removal of contaminants.

In the treatment of whey by microalgae in an open system, the positive effect of light intensity on the removal of DOC, nitrogen and phosphorus by the microalgae was also verified. Furthermore, the effluent could be efficiently treated with initial COD around 2000 mg L^{-1}. It was noticed that the pH at high organic loads, COD > 1000 mg L^{-1}, required daily adjustment to remain in the best alkaline range for microalgae activity, showing high sensitivity in the process.

In addition, it was possible to evaluate different technologies for the treatment of oil produced water, effluent generated in high quantities for this important world energy and chemical sector; whether physical, chemical or biological methods. Regarding biological methods, mainly those conducted ex-situ in bioreactors, it was noticed that studies are limited and that there is still much to be studied in terms of adapted species, cultivation systems, types of bioreactors and effluent adaptations. Mostly bacteria are used for the treatment, but even limited, the few works that apply microalgae, filamentous fungi, and yeasts, show great potential for the removal of oil and grease content (TOG) and other contaminants. However, salinity imposes itself as the most impeding parameter for the application of microorganisms to treat this effluent, since it can contain a higher concentration than sea water (> 35 g L^{-1}). In this sense, it is necessary to adjust its salinity or supplement it with other nutrients that are not in this mineral effluent and look for microorganisms adaptable to the conditions of the effluent, mainly the halotolerant ones.

In the experiments treating produced water, using a synthetic effluent, it was possible to perceive the behaviour of the microalgae *Tetradesmus obliquus* and the filamentous fungus *Cunninghamella echinulata* in the removal of TOG and other contaminants from the effluent and in different salinities. It was noticed that the fungus was the main responsible for the removal of TOG from the effluent and adapted to salinities of up to 50 g L^{-1}, while the microalgae remained alive up to 25 g L^{-1}. By intercropping the microalgae with the fungus, greater removals of other contaminants from the effluent, nitrogen and phosphorus, were achieved. The literature cites the microalgae as a great nitrogen and phosphorus remover from the effluent, thus combining it with the action of the filamentous fungus maximizes the treatment potential of the produced water.