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PROGRAMA DE MAESTRÍA Y DOCTORADO EN INGENIERÍA
ENERGÍA – ENERGÍA Y MEDIO AMBIENTE

WASTEWATER MICROALGAL BIOMASS PRODUCTION IN OUTDOOR CONDITIONS: BIOFUEL FEEDSTOCK AND NUTRIENT REMOVAL POTENTIAL

TESIS
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The research contribution of this thesis is presented in “journal format” as two academic papers for possible publication in scientific journals. Keeping on format employed, thesis is structured as follows: Chapter I include a general introduction to the contributions presented in this work; Chapter II present a literature review in this research area and precedent works; Chapter III presents the general and particular research objectives; Chapter IV contents materials and methods; Chapters V and VI include the corresponding manuscript for contributions 1 and 2 respectively and are based on specific objectives. Finally in Chapter VII general conclusions were summarised.

Con el objetivo de transitar de una economía energética linear basada en fuentes fósiles a una economía energética circular de fuentes renovables, diversos trabajos se han enfocado en integrar el concepto de biorrefinería con el aprovechamiento de flujos residuales para la generación de biomasa con fines energéticos. Por lo anterior, el objetivo del presente trabajo fue comparar el rendimiento y las características como materia prima para biocombustibles, de la biomasa microalgal cultivada bajo condiciones semi-ambientales en dos diferentes medios de cultivo; agua residual sanitaria y agua estéril enriquecida con fertilizante foliar. De forma simultánea se evaluó la remoción de nutrientes en el agua residual por efecto del metabolismo microbiano, y así analizar su influencia en las características de calidad del agua residual. Dos experimentos tipo batch se realizaron de forma paralela, se establecieron dos bloques experimentales con tres réplicas cada uno, para ello, seis fotobiorreactores de placa plana fueron alimentados con los medios de cultivo a un volumen total de 20 L y se inocularon con un cultivo mixto de microalgas en concentración del 10% v/v. Se realizó el monitoreo cada dos días durante 14 días de las condiciones semi-ambientales en las que se desarrolló el experimento, así como del crecimiento microalgal, temperatura y pH dentro de los biorreactores. De igual forma se monitoreó la concentración de la demanda química de oxígeno, amoniaco, nitratos, nitritos y fosfatos así como de tres características físico-químicas del agua residual. Al final del experimento se determinó la cantidad de biomasa obtenida, el rendimiento de lípidos y almidón en base seca para cada bloque experimental, así como el porcentaje y la tasa de remoción de nutrientes. No se observó diferencia estadísticamente significativa entre la biomasa obtenida en los dos medios de cultivo ($p<0.05$), el rendimiento final de biomasa cultivada con fertilizante fue de 0.253 g L^{-1}, y de 0.312 g L^{-1} para la cultivada en agua residual. El contenido de lípidos crudos fue significativamente mayor para la biomasa cultivada en agua residual, mientras que el contenido de almidón fue similar para ambos medios. Se observaron altos valores de eliminación para nitrógeno amoniacal, DQO y nitratos con porcentajes de remoción del 81.11%, 63.99% y 50.50% respectivamente. Se concluyó que bajo condiciones semi-ambientales y empleando agua residual como medio de cultivo, es posible obtener buenos resultados para el rendimiento de biomasa microalgal, y al mismo tiempo lograr altas eficiencias de remoción de nutrientes y mejorar la calidad en aguas residuales sanitarias.

As a challenge for the transition from linear-fossil-fuel economy to circular economy base on renewable energy, many studies have been developed and integrated the biorefinery concept with residual flow utilization and valorization, to produce biomass for energetic issues. The aim in the present work was to compare the microalgal biomass production in outdoor conditions for two different culture medium: sanitary wastewater, and freshwater enrichment with foliar fertilizer. Simultaneously, nutrient removal by microbial metabolism was evaluated in both culture medium. Two parallel batch experiments were carried out by triplicate, for this purpose, six flat-plates photobioreactors were filled at a total volume of 20 L, and inoculated with 10% v/v microalgae mixed-culture. Outdoor conditions were monitored every 2 days during 14 days of experimentation, at the same time, microalgal growth, potential hydrogen, and temperature were followed. Concurrently, Chemical Oxygen Demand, ammonia, nitrates, nitrites, and phosphate concentrations during the experiment were determined, as well as three physical characteristics in wastewater. At the end of the experiment, biomass, crude lipid, and starch yields in dry-weight were calculated for each experimental block, as well as the nutrient removal rate and removal percentage. No statistical difference was found in biomass obtained in wastewater and control cultures ($p<0.05$), the biomass yield for the control medium was 0.253 g L^{-1}, and 0.312 g L^{-1} for wastewater medium. Crude lipids content was significantly higher in wastewater biomass while starch content was similar for both culture mediums. High removal nutrient values were observed for ammonia, COD, and nitrates, with removal percentages of 81.11%, 63.99%, and 50.50%, respectively. It was concluded that under outdoor conditions and employing sanitary wastewater as a culture medium, it is possible to achieve similar biomass yield than those obtained in conventional culture, and at the same time, it is possible to attain high nutrient removal values and improve sanitary wastewater quality.

CHAPTHER I
General Introduction

Microalgae and cyanobacteria have been utilized as food in México since antiquity, and currently being extensively investigated as a sustainable solution for the production of renewable energy a high-value products (Zhang et al., 2015). The production of more than one type of biofuel from microalgal biomass or additional co-products, increases the valorization and efficient utilization of biomass, and also, environmental impacts could be mitigated (Venkata Mohan, Rohit, Chiranjeevi, Chandra, & Navaneeth, 2015). For a sustainable transition to bioeconomy, a cascading use of biomass in biorefinery approach is a new trend defined as sustainable processing of biomass into a spectrum of bio-based products and bioenergy, in examples, chemicals, materials, human food, animal feed, fuels, power and/or heat could be obtained (IEA, 2019).

Focusing on microalgae as a biofuel feedstock, another attraction is that microalgae can effectively grow in conditions where require minimal freshwater input, nutrients and land, moreover, wastewater is a potentially sustainable growth medium for microalgal feedstock, due to easy availability and higher organic content (Pittman, Dean, & Osundeko, 2011). Integrating the biorefinery concept with wastewater treatment will provide efficient utilization of biomass, reduces its overall residual waste, and favors sustainable-circular economy, combination of bioenergy production from microalgal biomass with wastewater management is part of a transition from linear-fossil-based to a circular waste-based bioeconomy (Venkata Mohan et al., 2015).

Integrated biorefinery concept with wastewater management consists of the use of different types of wastewater as a growth medium in microalgae cultivation, while wastewater is simultaneously treated. Biomass obtained can be converted into bioenergy products by biochemical conversion as transesterification, anaerobic digestion, and fermentation, or by thermochemical conversion as gasification, pyrolysis, and liquefaction (Venkata Mohan et al., 2015).

On the other hand, energy-efficient cultivation is the major bottleneck for microalgal biomass production on a large scale. For this reason, outdoor cultures are proposed as a solution for energy savings, due to using sunlight as a light source for microalgae cultures could greatly improve the net energy ratio values (Ekendahl et al., 2018). The need to reduce the energy consumption of indoor crops as well as the water footprint and nutrient consumption have promoted studies to

evaluate the simultaneous treatment of wastewater and the outdoor production of microalgae for energy purposes. This study aims to evaluate the influence of a mixed-culture of microalgae in nutrient removal of sanitary wastewater and to evaluate the biomass obtained as a potential raw material for biofuels. The growth of microalgae was compared to sanitary wastewater against a commercial culture medium in outdoor conditions; at the same time, it assesses if the culture can remove nutrients and improve wastewater quality at the end of culture time.

CHAPTHER II
Literature Review

2.1 Microalgae as a third generation biofuel feedstock

In recent years microalgae is a growing trend as an alternative for sustainable and low-cost biofuel feedstock. Third-generation biofuel feedstock uses photosynthetic organisms and involved "microalgae-to-biofuels" technology, it consists basically of processing microalgae biomass for biofuel production. Microalgae are a class of microorganisms that exhibit a large biological diversity and metabolism plasticity, they are regarded as unicellular photoautotrophic organisms and can be prokaryotic and eukaryotic. Microalgae are important members of biota and they can recycle organic and inorganic carbon, water and nutrients (i.e., nitrogen and phosphorous), using sunlight as an energy source through photosynthesis to produce organic matter most commonly named biomass (Lü, Sheahan, & Fu, 2011)(Torres, 2018).

Microalgae produce compounds that can be used in industries such as food, biomaterials or to produce energy. Focus on energy utilization microalgae synthesizes carbohydrates (mainly in starch granules form) and lipids, the two major precursors of sugar and lipid-based fuels. Biofuels that can be obtained from essential biomolecules are biodiesel, bio-oil, bioethanol, charcoal, fuel gas, hydrogen, methane, butanol, acetone and also electricity (Bekirogullari, Fragkopoulos, Pittman, & Theodoropoulos, 2017).

Carbohydrates are the major products derived from photosynthesis and the carbon fixation metabolism (i.e., the Calvin cycle). Some microalgae contain a high amount of carbohydrates in the cell wall (mainly in cellulose and soluble polysaccharides forms) and plastids (mainly in the form of starch) that can be used for the production of sugar-based biofuels like ethanol or butanol (Menegazzo & Fonseca, 2019). Most green species accumulate carbohydrates in the form of starch granules localized in chloroplasts, here, carbon source is assimilated and converted from Glucose-1-P into ADP-Glucose. In an important starch synthesis step, glucose units from ADP-Glucose are then transferred to pre-existing water-soluble polysaccharides, forming an elongating chain of amylopectin and amylose employing starch synthases and branching enzymes (Torres, 2018). Figure 2.1 shows a representation of starch and lipid pathways based on the *Chlamydomonas reinhardtii* metabolism, an ideal model organism for the understanding of various fundamental

mechanisms in microalgae (Lü et al., 2011). Most studied eukaryotic microalgae species are *Chlamydomonas reinhardtii* and *Chlorella vulgaris. C. rheinhardii* can synthesize around 17% carbohydrates on a dry-weight basis and *C. vulgaris* can accumulate around 12-17%. *Scenedesmus obliquus* is another highly studied microalgae, this specie accumulate 10-17% of this biomolecule (Milano *et al*., 2016).

Lipids in microalgae serve as a major building block in cell membranes as structural components (i.e., phospholipids, sulfolipids, and galactolipids), they can be also stored in cytosolic and/or plastidic as energy storage reserves in form of lipid bodies, those bodies are commonly formed during stress conditions, through to the adaptations of their biochemical metabolic pathways and cellular composition in response to external conditions (Jacob-lopes, 2018).

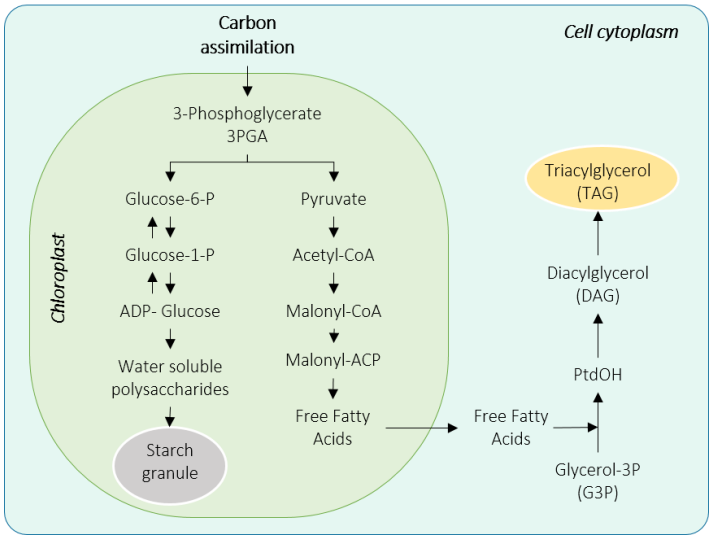


Figure 2.1 | Schematic representation of the starch and lipid pathways in microalgae (based on *C. reinhardthii* metabolism) (Torres, 2018).

Microalgae lipids consist of neutral lipid triacylglycerol (TAG lipids), which include saturated and unsaturated fatty acids, those are better suited for the production of biodiesel via transesterification. The most nature abundant lipid class is glycerolipids and its biosynthesis arises from the action of acetyl-CoA carboxylase (ACCase) and fatty acid synthase (FAS). As a committed step in fatty acid biosynthesis pathway, malonyl-CoA is produced by the action of ACCase, this enzyme is regarded as central in regulating the initiation of fatty acid biosynthesis. Following the synthesis of malonyl-CoA, malonate is transferred to a small polypeptide called acyl carrier protein (ACP) and following the synthesis of malonyl-ACP, the fatty acid synthesis in plastids continues in the chloroplast stroma (Torres, 2018)(Riekhof & Benning, 2009).

As an example, microalgae lipids accumulation capacity can be of 21% for *Chlamydomonas reinhardtii*, *Chlorella vulgaris* can accumulate around 14-22% and *Scenedesmus obliquus* 12-14% (Milano *et al*., 2016).

In respect to microalgae cellular metabolism, they can grow in different ways focusing on the predominant form of nutrition, microalgae may grow based on four types of cell metabolism: autotrophy, heterotrophy, mixotrophy, and photoheterotrophy. Autotrophic organisms obtain energy and electrons by CO_2 reduction through the absorption of solar energy and substrates oxidation (mostly water). Heterotrophic organisms use only organic compounds as carbon energy (i.e. glucose or acetic acid). Mixotrophic growth consists of growth in both metabolism types, autotrophic and heterotrophic, where organic compounds and CO_2 can be assimilate depending on the growing conditions. In this case, mixotrophic microorganisms synthesize compounds characteristic of both types of metabolisms, showing high production rates. Concerning photoheterotrophic metabolism, organisms require light as an energy source and organic compounds as a carbon source (Jacob-lopes, 2018).

2.2 The role of microalgae in wastewater treatment

Wastewater is the liquid waste fraction that has been contaminated after use and is generated in any residence, public institutions and industrial or commercial establishments. Wastewater can be classified according to the generation point as industrial and sanitary/municipal. Sanitary wastewater is generated in residential zones or commercial and public facilities while industrial wastewater is predominantly shaped by industrial processes discharges. Wastewater flow is collected in sewage systems for transporting to a treatment plant or ultimately, might be conducted to receiving water bodies. If untreated wastewater is discharged directly in water bodies, pollutants can cause ecosystem deterioration. Contaminants present in wastewater can be eliminated by physical, chemical and/or biological methods, these are combined and complemented to lead different stages of a treatment system.

There are four basic stages in treatment systems: pre-treatment, primary treatment, secondary treatment, and tertiary treatment. Pre-treatment consists of removing large solids as solid waste, sand that can result in system operational problems, and fats and oils. Roughing, screening and desanding are examples of this stage. In primary treatment, suspended solids and organic matter are eliminated, sieving and sedimentation are physics operations used at this stage. Secondary

treatment is aimed at the elimination of suspended solids and biodegradable organic compounds, this stage includes activated sludge biological process, fixed bed reactors, and pounds-sedimentation systems. After secondary treatment, nutrient control and removal is an important issue for different reasons: the nutrients in excess discharged in receptors body waters can stimulate or accelerate eutrophication and nitrification process, limiting oxygen sources and aquatic plants can proliferate. The most important nutrients in wastewater are nitrogen and phosphorous, the excess amount removal can be performed by chemical, physical and biological methods, these latter methods are mostly for organic matter and nutrients remove in sanitary wastewaters.

In biological wastewater treatment bacteria are the primary organisms present, however fungi, protozoa, rotifers and algae also play an important role. Microalgae species can be part of the microbiological community, they are tolerant of stressful wastewater conditions and can efficiently remove nutrients while they use it for cell growth. It has been appreciated that microalgae can be potentially utilized for low-cost and environmentally friendly wastewater treatment. Traditional biological wastewater processes can remove phosphates and total nitrogen until 2 m L^{-1} and 15-25 mg L^{-1} respectively (Metcalf, 2014). In contrast, better values in treated wastewaters with microalgae have been reported, where values were reduced around 0.5 mg L^{-1} and 5 mg L^{-1} respectively (Lau, Tam, & Wong, 1995). The efficiency at removing nutrients will depend on the species, in example, some unicellular green microalgae widely used in wastewater treatments are *Chlorella* and *Scenedesmus,* both can efficiently remove over 90% of nitrogen and 80% of phosphorous content from the primary treated sewage and very high complete removal (>80%) of ammonia and nitrate from secondary treated wastewater (Lau et al., 1995)(Bekirogullari et al., 2017). Following the above, due to its efficiently nutrient removal potential, microalgae cultures can be proposed as a possible tertiary wastewater treatment stage. The feasibility of using microalgae in wastewater treatment as a supplement for tertiary treatment has been studied by many researchers, also its potential for biomass productivity could be ≈2 g L^{-1} for *Chlorella kesslery* (Olguín, 2012) and lipid accumulation (4-26%) for different species cultivated on municipal wastewater (Venkata Mohan et al., 2015).

México has an opportunity area for wastewater treatment and biomass production from microalgae, due to its high volume of wastewater generation, medium treatment systems coverage, and its environmental conditions. In 2017, 234.9 m^3 s^{-1} of municipal wastewater was

discharged in the country, from this, 91.6% (215.2 $m^3 s^{-1}$) was collected by the municipal sewerage system and only 57.7% was processed in wastewater treatment plants, mostly by biological processes (activated sludge). Besides, treated wastewater directly reused (before discharge) was estimated as 39.8 $m^3 s^{-1}$ (CONAGUA, 2018). These statistics shows that is necessary to propose and adapt new technologies to recover waste products and value them as other sources of biofuels.

2.3 Other benefits and advantages of integrated microalgae cultures

Several research contributions discuss technical, economic and environmental advantages of dual purpose microalgae outdoor systems: nutrients removal in wastewater and biomass production for biofuel generation. Conventional nutrient removal methods in municipal wastewaters, such as aerobic activated sludge-based, nitrification-denitrification, chemical phosphorous removal and coagulating sedimentation, are facing challenges to meet the stringent nutrient discharge standards with high efficiency and low cost. Also, evident barriers for sustainable wastewater treatment are energy consumption, instability treatment effect, long process, carbon emissions, excess sludge discharge and recyclable resource wasting (Li et al., 2019). The activated sludge treatment process is the most common method and is considered economically and environmentally unsustainable, it consumes considerable amounts of fossil fuel-derived energy resulting in considerable anthropogenic greenhouse gas emissions. In addition, the processes themselves also generate potent greenhouse gases such as CH_4 and N_2O. (Sheik, Muller, & Wilmes, 2014). To improve the wastewater treatment, microalgae cultivation has been proposed as a sustainable solution for the biological remotion of pollutants.

As for energy savings in wastewater microalgae outdoor cultures, Díez-Montero *et al* observed that net ratio electricity energy of the outdoor microalgae process in wastewater treatment plants is over 1.32, it suggests that energy balance is largely positive being better in locations with high environmental temperatures. Results in this work confirm that local climate conditions have a great positive effect in energy balance, especially solar radiation and environmental temperature (Díez-Montero, Solimeno, Uggetti, García-Galán, & García, 2018). On the other hand, wastewater treatment processes are regularly established in outdoor conditions, it is therefore important to assessed microalgae cultures in environmental or semi-environmental conditions and also taking into account that solar irradiation can result in energy savings in the microalgae cultivation process. A cost-benefit analysis of a phytoremediation system using microalgae and receiving a wastewater

discharge of 150 m^3 d^{-1} (medium-scale industrial reactor) were evaluated, the net profit estimated was 0.31 US$ m^{-3} and a payback period of 14.8 years for a lifetime project of 15 years, implying that phytoremediation system became feasible in financial considering capital and operation costs (Ansari et al., 2019).

In discussing the water footprint and nutrient requirements, in microalgae cultures processes the freshwater consumption can be reduced in 90% when wastewater is used and also the nutrient consumption while advantages are added as the production of valuable algal biomass which can become biofuels feedstock (Bekirogullari et al., 2017) (Sukla, Subudhi, & Pradhan, 2019). In an example, a value report for footprint water in microalgae biodiesel production was approximately 3726 kg_{water} $kg_{biodiesel}^{-1}$, this quantity can be reduced to low as 373 kg_{water} $kg_{biodiesel}^{-1}$ if wastewater is used.

Most of the results related to microalgal biomass composition have been obtained in indoor conditions. Main challenges for scale-up are energy and nutrient consumption, authors report that large-scale microalgae processes can only be cheap if sunlight is used, however light acclimation will be very different from controlled laboratory conditions (González-Camejo et al., 2019) (Holdmann, Schmid-Staiger, & T. Hirth, 2019). According to Lozano-Garcia, suitable areas for microalgae outdoor production in México were identified in up to 26.8% of the country equivalent to 526,672 km^2. The states with the largest areas for highest possible production (around 9 million Ton yr^{-1}) are Jalisco, Oaxaca, and Veracruz. Morelos is one of the states with sites highly suitable for outdoor microalgae cultivation. Considering solar irradiation (5.9–6.27 kWh m^{-2} d^{-2}), evaporation (1085-1400 mm), temperature (26-29 °C), wastewater treatment plants proximity and other nutrient sources, dry biomass production can be approximately 2,244,288,640 Ton yr^{-1} (Lozano-Garcia et al., 2019).

Theoretical costs for microalgae cultivation in outdoor conditions could be between \$9 kg^{-1} to \$3.5 kg^{-1} for low-temperature regions (≈19 °C) and ranging between \$3 kg^{-1} to \$3.5 kg^{-1} by areas with high temperatures (≈25 °C) and long hours of sunshine over an extended period of the year, indicating that they may be more suitable for microalgae cultivation (Bello, Ranganathan, & Brennan, 2017). From a design perspective and to ensure commercial and economic success in microalgae cultivation in outdoor photobioreactors, geographic location plays a critical role and they need to be carefully considered.

2.4 Previous works

There are several works focused on the study of microalgal biomass production using waste-water as a culture medium and obtaining feedstock to biofuels production. From 2011, an increase in publications related to this topic was observed, being China (448), United States (329), India (259), Spain (209), Malaysia (140) and Brazil (132) the countries with highest contributions reported, México is among the first 15 ranked with 78 contributions reported (Scopus, 2019). Most of the consulted studies that have been evaluated in outdoor conditions and using closed photobioreactors are carried out with controlled culture media, axenic strains and, controlled aeration.

In 2019, in Valencia, Spain, two outdoor photobioreactors were operated to evaluate the effect of variable environment temperature on an indigenous microalgae-nitrifying bacteria culture dominated by *Chlorella*. The substrate used in this study was the effluent from the primary settler of a waste-water treatment plant. Four experiments were carried out in different seasons, days of experimentation were; 29, 14, 16, and 25 for Autumn, Winter, Spring, and Summer respectively; light intensity and temperature in Spring were 225 µmol m^{-2} s^{-1} and 28.8 ± 1.5 °C in average. Microalgae were cultivated in methacrylate flat-plates PBRs with working volumes of 550 L. The optimal temperature range for the growth of microalgae was 15-30 °C, in this interval no significant differences were found in microalgae cultivation performance, also, it was observed that microalgae viability was significantly reduced at temperatures over 30-35 °C (González-Camejo et al., 2019).

An experiment was conducted from August to October 2015 in outdoor conditions in Stuttgart, Germany. Holdmann et al evaluated the productivity of *Chlorella sorokiniana* in DSN culture medium with different initial biomass concentrations (1.5, 3, 6 and 9 g L^{-1}), in flat-plates PBRs with a volume of 28 L and south-north orientation, maintaining the maximum temperature at 30 °C. Light intensity during one day was calculated as the integral of the photon flux density. They reported that if a low initial biomass concentration was used, the productivity increased with an increasing light integral and the productivity of the cultures with biomass concentrations of 6 and 9 g L^{-1} seemed to be independent of the light integral (Holdmann et al., 2019).

In 2015 in Brisbane, Australia, ten pilot scale trials were conducted under subtropical conditions using 2 microalgae strains (*Chlorella sorokiniana* and *Chlorella sp.*) with TAP medium, in five different PBRs geometries; high-rate ponds, flat panel, and tubular PBRs. The performance of five

different production systems was evaluated in September and November in shade experimental reactors. Flat-plates were vertically illuminated in east-west orientation and temperatures were maintained at 35°C. For the pilot-scale trials, half-hourly incident total irradiation (direct and diffuse) and diffuse PAR were measured using a universal light meter. In flat-plates PBRs, biomass yield were <1 g L^{-1} and <0.5 g L^{-1} after 14 and 7 days respectively for both species, having similar final volumetric yields (Wolf et al., 2016).

Similar work was carried out in North Carolina USA, Feng et al investigated in 2011 the feasibility of culturing *Chlorella zofingiensis* outdoors for biodiesel production in BG-11 medium, effects of nitrogen limitation and initial cell concentration on growth and lipid accumulation of this alga were investigated in 60 L flat plate photobioreactors. PPDF in spring light intensity was usually lower than 1300 µmol m^{-2} s^{-1}. The average temperature of culture media in spring was higher than 20°C. The authors concluded that cells in the spring reached higher μ_{max} (d^{-1}) and biomass productivity (mg L^{-1} d^{-1}) (Feng, Deng, Hu, & Fan, 2011).

On the other hand, outdoor cultures that have been evaluated in wastewater are mainly developed in open ponds and/or non-axenic strain. It is difficult to maintain axenic strains in open PBRs under environmental conditions or in PBRs fed with non-sterile wastewater, due to wastewater is habitat for a wide variety of undesirable microorganisms which will be detrimental for algal growth by acting as competitors (other algae with low oil production or bacteria), parasites (virus, fungus or protozoans) or predators (protozoans, fungus or aquatic invertebrates) (Venkata Mohan et al., 2015).

In 2016, Novoveská et al designed and implemented a novel approach to wastewater treatment in which municipal wastewater is used to cultivate microalgae in modular offshore PBRs, this process was used to treat up to 50,000 gal d^{-1} of incoming raw wastewater, process removed 75% of total nitrogen, 93% of total phosphorus and 92% biochemical oxygen demand from influent wastewater. During one year of operation, microalgae composition shifted from dominance of *Scenedesmus dimorphus* to a diverse polyculture dominated by genus *Chlorella*, *Cryptomonas*, and *Scenedesmus*. In spring, PAR was between 400-600 µmol m^{-2} s^{-1} and temperature in 20-25°C, medium-high light intensity favored *Chlorella* species (Novoveská, Zapata, Zabolotney, Atwood, & Sundstrom, 2016).

There are two works carried out in indoor conditions for *Verrucodesmus verrucosus* specie. In 2018, Orantes used treated and sterile wastewater as a culture medium and evaluated lipid production, the efficiency of phosphate removal (PO_4^{3-}) and nitrates (NO^{3-}); the culture conditions were 12:12

light/dark photoperiods with a light intensity of 1,650 lumens, the temperature was maintained at 25°C. Results showed the final biomass values of 0.38 g L^{-1} and a lipid yield of 2.43%. Nutrient removal efficiency in wastewater was 98.73% for nitrates, and 83.44% for phosphates. It was concluded that the strain has capacity to remove nutrients from wastewater but the crude oil production is low (Orantes-Calleja, 2018).

Arenas in 2017, evaluated the remediation of municipal wastewater by *V. verrucosus* in indoor conditions. Arenas state that strain is capable of removing 90% of the ammonium and 80% of phosphorus present in the wastewater in the first 15 days, and can synthesize approximately 37.4% of lipids, whose composition is mainly fatty acids such as elaide (45%) and palmitic (22%). The author concluded that biomass presented favorable characteristics for obtaining biofuels such as biodiesel. The light intensity during the experiment was 160 µmol m^{-2} s^{-1} with a 12:12 photoperiod, and an environmental temperature of 22 °C (Guerrero, 2017).

CHAPTHER III
Research Objectives

3.1 GENERAL OBJECTIVE

- To evaluate the potential of mixed-culture microalgae as a raw material for biofuels and its influence in nutrient removal in wastewater.

3.2 SPECIFIC OBJECTIVES

- To compare the yield of microalgae biomass, crude lipids and starch production in conventional culture medium and sanitary wastewater in outdoor conditions.

- To evaluate the influence of the culture on physical-chemical characteristics and nutrient removal in sanitary wastewater.

CHAPTHER IV
Materials and Methods

4.1 Mixed culture inoculum

The original sample was collected in the temporary lake area of Ciudad Azteca in the Estado de México, México, and was donated by the Laboratory of Applied Phycology of the Universidad Autónoma Metropolitana Iztapalapa to the Bioenergy Laboratory of Instituto de Energías Renovables of the Universidad Nacional Autónoma de México (IER-UNAM) where culture was maintained. Mixed culture used as inoculum was propagated using 1 mL L^{-1} of Bayfolan® Forte foliar fertilizer as a growth medium, culture conditions were 19°C and light intensity of 150 µmol m^{-2} s^{-1} with a 12:12 h light: dark photoperiod.

4.2 Wastewater samples collection

Sanitary wastewater (SWW) used as a culture medium was obtained from the Wastewater Treatment Plant (WWTP) at the IER-UNAM in Temixco, Morelos, México. The sampling point was located in the high rate solid separator that follows the aeration bioreactors (Figure 7.1, supplementary information) since a preliminary test showed the best growth kinetic at this stage. Individual samples were collected at three different depths of the solid separator. Samples were passed through a homemade silica filter, details are described in supplementary information. The filtered wastewater was transported to the experimental site in 20-L polyethylene containers.

4.3 Experimental protocol

Outdoor experiments were carried out during the spring season from 9th to May 23rd, 2019. Six flat-plate bioreactors (FPB) made of commercial glass were supported by a metal structure and elastic safety tapes (Figure 4.1). Two simultaneous experimental blocks where performed: control and wastewater with three interspersed replicates. Control block FPB's were filled with filtered water enriched with 1 mL L^{-1} Bayfolan® Forte foliar fertilizer. The sterilization of FPB´s was performed using a UV sterilization portable lamp (iTrustech® U-60) in a closed alcohol cleaned area with an exposure time of 15 min. Wastewater FPB's were filled with filtered wastewater without sterilization.

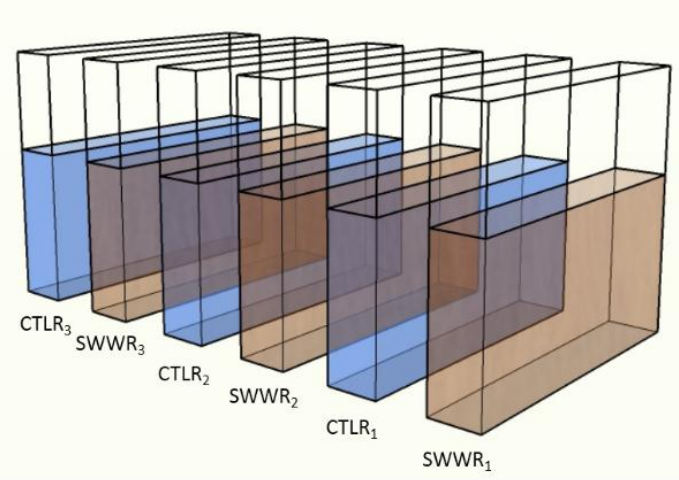


Figure 4.1 | Photobioreactors arrangement. SWW: Sanitary Wastewater; CTL: Control. R_1, R_2, R_3 are replicates.

Each FPB was filled at a total volume of 20 L and inoculated with 10% v/v fresh inoculum. Carbon dioxide was provided by air supply, aeration inside the FPB was carried out by 24“aquarium tubes (OxiKril®). Airflow was induced by a 6-outlet compressor with a cotton filter placed in the main outlet to prevent particles from the environment entering the culture system. A humidifier was used in each bioreactor to avoid water loss by evaporation.

4.4 Culture conditions and monitoring

The culture was maintained for 14 days according to a preliminary test conducted under small-scale outdoor conditions (Figure 7.2, supplementary information) and data reported by Arenas (2017), where it was observed that the stationary phase and maximum cell concentration values where reached at day 15. The FBRs were located under a polycarbonate roof of the Pilot Plant at IER-UNAM (18°50’22’’ N, 99°14’09’’ W) that allowed indirect irradiation. The monitoring of temperature inside each bioreactor was carried out every two days by taking the temperature of 50 mL sample immediately after extraction, with a digital submersible thermometer (HANNA® 08311). The environmental temperature was recorded in three different points 10 cm above of FBRs.

To estimate the light amount reaching each FBR, Photosynthetically Active Radiation (PAR) was measured with a quantum light-meter (FieldScout®) at five points on each side of the flat-plates every two days at 14:00 h, since photon flux density reaches its maximum value at noon (> 2000 µmol s^{-1} m^{-2}) (Holdmann et al., 2019). Microalgal growth was evaluated in terms of cell concentration, 1 mL samples of each FBR were taken every 2 days and cell concentration was

determined by direct counting with an optical microscope (40X) using the Neubauer camera method (Andersen, 2005). Maximum specific growth rate (μ_{max}) at the exponential phase was calculated according to $\mu_{max}= (ln\ X_2 - ln\ X_1)/ (t_2 - t_1)$, were X_2 and X_1 is the cell concentration (cell mL^{-1}) at time t_2 and t_1, respectively (Andersen, 2005).

4.5 Wastewater quality analysis

Physical-chemical wastewater quality parameters were monitored every two days. The same samples used for temperature monitoring were used for Chemical Oxygen Demand (COD), ammonia (NH_3^-), nitrate (NO_3^-), nitrite (NO_2^-), phosphates (PO_4^-), hydrogen potential and turbidity determinations. These samples were centrifuged at 4000 rpm for 10 min and the supernatant was collected and diluted as demanded each measured parameter. Nutrients and turbidity were measured following the Hach DR900 Colorimeter Manual (HACH, 2019) as follows: 8000 Reactor Digestion for COD; 8192 Powder Pillows LR Cadmium Reduction for NO_3^-; 8153 Colorimeter Powder Pillows HR Ferrous Sulfate for NO_2^-; 10031 Test 'N Tube HR Salicylate for NH_3^-; 8190 Persulfate Digestion (PhosVer 3®) for PO_4^{-3} and 8237 Colorimeter Absorptometric for turbidity. Nutrient Removal Percentage (RP) and nutrient Removal Rate (RR) were calculated by the following formulas: $RP(\%)= (C_0 - C_t)/C_0$, $RR(mg\ L^{-1}\ d^{-1})= (C_0 - C_t)/ (t - t_0)$, where C_t and C_0 are nutrient concentration at time t and at the beginning respectively (Lu, Wang, Wang, & Yuan, 2015).

4.6 Biomass, crude oil and starch productivity

For total biomass, crude oil, and starch yield after 14 days of culture, the harvest process was carried out for each FBR. First, aeration was stopped and liquid cultures were immediately transferred to clean 20 L polyethylene containers. Liquid cultures in sealed containers were transported to the Pilot Plant of the Instituto de Biotecnología-UNAM and kept in a refrigeration chamber at 4°C until further processing. Biomass separation was carried out by centrifugation with a tubular centrifuge (Mini Sharples® CL-I-1) fed at 9 L h^{-1}. Biomass pellet where lyophilized in a freeze dryer (Labconco® FreeZone 4.5) at a temperature of -50°C for 12 h. Final biomass dry weight was recorded for each FBR harvest. Biomass yield ($Y_{X/S}$) was calculated according to $Y_{X/S}= DW/V$, where DW is dry weight (g) at day 14 and V is the final volume of liquid culturing (19.593 L) (Andersen, 2005).

Lyophilized biomass was characterized in a laboratory belonging to the Biochemical and Bioprocess Engineering Group, in the School of Chemical Engineering and Analytical Sciences at the University

of Manchester, UK. Crude lipid was quantified by the soxhlet extracted lipids method using an automated extraction system (FOSS® ST 243 SOXTEC). Hexane (ACS spectrophotometric grade, ≥ 98.5 %, Sigma Aldrich, UK) was used as extracting solvent. Lyophilized cells were placed in cellulose extraction thimbles (26 x 60 mm, thickness 1.5 mm, Whatman®, UK) and positioned in the SOXTEC unit. The procedure followed to quantify crude lipid concentration was: boiling for 2 h, rising time was 40 min and solvent recovery was made for 20 min. Extracted lipids were measured gravimetrically (Bekirogullari et al., 2017).

The starch content of cells was quantified according to a Total Starch Assay Kit (Megazyme, 2019). The assay consists of a high-temperature two-stages (α- amylase and β-amyloglucosidase) enzymatic hydrolysis which solubilizes starch and releases free D-glucose. The concentration of free D-glucose was determined colorimetrically by measuring sample absorbance values at 508 nm against a D-glucose standard curve. Total starch concentration was then calculated by multiplying D-glucose concentration by 0.9 (162/180, a factor adjusting free D-glucose to anhydrous D-glucose) (Torres, 2018).

4.7 Statistical analysis

To determine the influence of culture medium in microalgae performance, results obtained for biomass, crude lipids and starch were analyzed and compared by two-sided F-ratio test and one-sided t-test assuming data normality. Two-sided F-ratio test was calculated to determine variance equality and differences between control (CTL) and the treatment (SWW) means values. One-sided t-test was carried out to assessed statistically significant differences ($p < 0.05$) (Verma, 2005).

CHAPTHER V
Contribution 1

CRUDE LIPIDS AND STARCH PRODUCTION OF MICROALGAE: COMPARISON OF WASTEWATER AND CONVENTIONAL MEDIUM IN OUTDOOR CONDITIONS.

ABSTRACT

To achieve sustainable low-cost processes for obtaining microalgae biofuels, two strategies have been proposed in recent years for microalgal biomass production: crops cultivated in outdoor conditions and wastewater valorization as culture medium. For this study, two simultaneous batch process was conducted in 20 L flat-plates bioreactors during the spring season in Morelos, México. Mixed cultures were evaluated under outdoor conditions: temperature, photosynthetically active radiation, and hydrogen potential were monitored during the experiment. Final biomass yield, growth rate, crude lipid, and starch production were compared for wastewater and conventional culture medium. Results showed that microalgae can grow faster than indoor cultures reported, and a significant difference in crude lipid production up to 50% was observed for biomass growth in wastewater. Similar values for biomass production can be achieved in wastewater compared with conventional culture medium, 0.253 g L^{-1} versus 0.312 g L^{-1}, respectively. Likewise, starch values were similar with 0.409% in control, and 0.546% in wastewater biomass.

5.1 INTRODUCTION

The production of microalgae on a large-scale is impeded by commercialization challenges such as deficiency of energy and cost-intensive processes for microalgae growth and harvesting, also a significant amount of nutrients are needed like nitrogen (N) and phosphorus (P) in conventional cultivation methods. Water and nutrient dependence in microalgae cultures has been a vital challenge to achieve sustainability in these processes. To minimize the cost of large-scale microalgae cultivation, the best approach is the utilization of wastewater as they contain varies

essential nutrients necessary for cultivation (Javed, Aslam, Rashid, & Shamair, 2019)(Gebremedhin, Mishra, & Mohanty, 2018).

When wastewater is used in microalgae cultivation, freshwater consumption can be reduce in 90%; nutrients consumption in 94% for nitrogen and 100% for sulfur, potassium, and magnesium; the costs process is cheaper and the net ratio energy utilization could be positive in hot locations (Javed et al., 2019) (Yang et al., 2011)(Díez-Montero et al., 2018)(Bekirogullari et al., 2017).

In this manner, the purpose of this chapter was to determine if there are significant differences between microalgal biomass characteristics growing in sanitary wastewater, and biomass obtained in a conventional culture medium (Bayfolan Forte® fertilizer) under outdoor conditions. An experiment was conducted in batch mode using flat-plate reactors from May 9 to 23, 2019. Six experimental bioreactors were inoculated with mixed culture microalgae. Environmental conditions and cell concentration were monitored for 14 days. Growth kinetic, final biomass, crude lipid, and starch yield were determined.

5.2 RESULTS AND DISCUSSION

5.2.1 Outdoor-conditions

All results are shown as mean ± standard deviation of the replicates. Two simultaneous batch experiments with a mixed culture were performed in six FPA reactors to evaluate the biomass yield, crude lipids, and starch productivity in outdoor conditions. The figure 5.1 and 5.2 shows culture outdoor-conditions; incident light intensity that reaches flat-plates, average temperature inside of experimental FPB´s and environmental temperature (TENV).

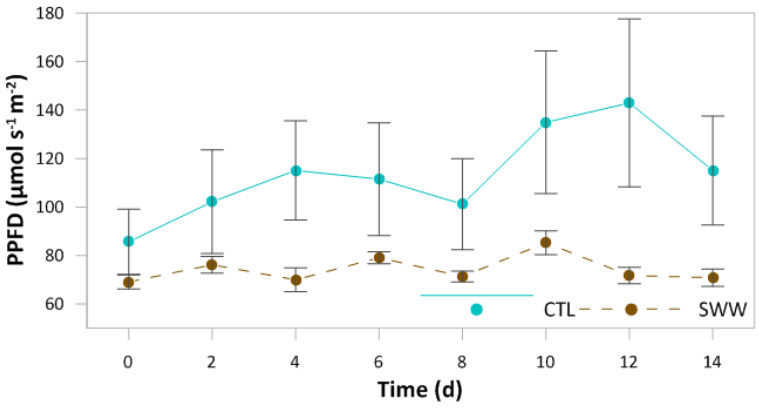


Figure 5.1 | Outdoor-conditions during the experiment: Photosyntetic Photon Flux Density (PPFD) in control (CTL) and wastewater (SWW) treatments.

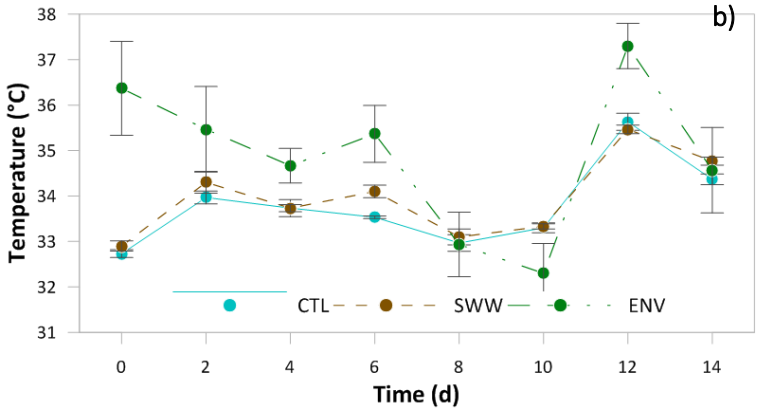


Figure 5.2 | Outdoor-conditions during the experiment: temperature in CTL, SWW and experiment surroundings (ENV).

It can see in figure 5.1 that PAR is different for CTL and SWW during the experiment, PAR is 114 µmol m^{-2} s^{-1} on average for CTL and 74 µmol m^{-2} s^{-1} for SWW. The difference in incident PAR may be due to the arrangement of experimental flat-plates, sun position could have favored CTL replicates because have east-west orientation and time PAR determination (14:00 h) where replicates could have been reached by indirect radiation, making it a discordant data that significantly influence the mean value in CTL.

Light irradiation in outdoor conditions is not a predictable source, it depends on the season and location of the site experiment, also the sunlight can fluctuate considerably during one day (Holdmann et al., 2019). The PAR average in this experiment is under data obtained in other works, authors reported different values in outdoor conditions depending on location such as in Foshan, China, where an experiment was carried out without shading, and values between 123–1418 µmol m^{-2} s^{-1} were reported. During the spring in Valencia, Spain, PAR value of 225 µmol m^{-2} s^{-1} was observed; in Stuttgart, Germany a 289.4 µmol m^{-2} s^{-1} value was reported and in Brisbane, Australia a 768 µmol m^{-2} s^{-1} value was observed. Those PAR values show that radiation surely depends on geographic location and results comparison can be difficult due to this difference.

According to authors, at photon flux densities of 463 µmol m^{-2} s^{-1}, no photoinhibition was observed for *Chlorella sorokiniana* in outdoor conditions, also for *Chlorella sp*. when PAR is around 2000 µmol m^{-2} s^{-1} biomass yield could below (≈ 0.5 g L^{-1}). At PAR average observed in CTL and SWW, light inhibition could be a behavior that does not occur (Lu et al., 2015), (González-Camejo et al., 2019), (Holdmann et al., 2019), (Wolf et al., 2016).

The figure 5.2 shows that the temperature value fluctuated in an interval of 32-36 °C inside bioreactors, reaching the maximum value on day 12 in both CLT and SWW (≈35°C). Average in T_{CTL} and T_{SWW} during the experiment were 33.78°C and 33.96°C respectively, fluctuation in both treatments had similar behavior. Optimal temperatures (T_{opt}) for many species are between 28 and 35°C, nevertheless, the optimal temperature is species-specific and values are often controversial. For *Chlorella* species optimal temperatures are widely reported. *Chlorella protothecoides* have their optimal growth at 30°C in primary effluent wastewater and 25°C in secondary effluent. For *C. pyrenoidosa* grown in synthetic wastewater T_{opt} was 38°C (González-Camejo et al., 2019), for *C. vulgaris* T_{opt} was 25°C. Flat-plate photobioreactors are very susceptible to overheating due to its thin layer structure and high light exposure, in ideal condition temperature should be around 25°C for this reason the FPBs must have a temperature control system (usually water spraying) (Jacob-lopes, 2018). It can be seen that temperature in CTL and SWW was not over T_{ENV} except in days when temperature was lower (days 10 and 12), results are opposed to data reported for an experiment in outdoor conditions where no shading was used during warm months (35.4°C), overheating in enclosed FPBs was minimal than surroundings (Novoveská et al., 2016).

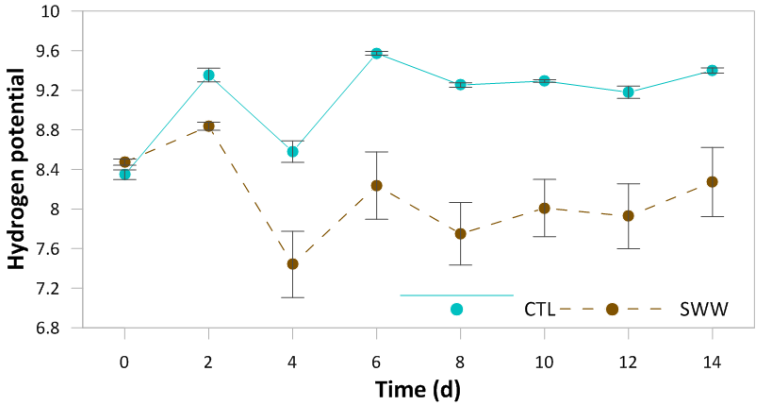


Figure 5.3 | Hydrogen potential in CTL and SWW during the experiment.

In figure 5.3 it can be seen the hydrogen potential in culture medium can affects the characteristics of the biochemical reactions in microalgae. The most favourable pH interval for microalgae growth varies between pH 7 and 9 being 8.2 and 8.7 the optimal value, also it can vary with different strains (Venkata Mohan et al., 2015). According to literature, the pH average in SWW is close to the optimal value for microalgae growth, while CTL is over it. Different metabolisms involved in microalgae growth could be distinguished according to pH changes; in photoautotrophic

metabolism, the pH tends to increase; in heterotrophic mode, pH can decrease; while in mixotrophic metabolism, changes are not significant (Chojnacka & Marquez-Rocha, 2004). Per that it was observed in CTL, that after 14 days pH increased from 8.35 to 9.4, this increase could indicate that metabolism is possible photoautotrophic, while in SWW where pH changes are not significant (8.35 to 8.47 from day 0 to 14 respectively) a mixotrophic metabolism can be occurring. Similar results have been observed in a mixed cultures growing in dairy wastewater (Woertz, Feffer, Lundquist, & Nelson, 2009).

5.2.2 Growth kinetic and biomass yield

The figure 5.5 shows the microalgae growth kinetics in cultures during the experiment. The adaptation phase is not observed for any experimental block, probably due to the bioreactors were aerated during one night before inoculation, or light and temperature were adequate to allow the exponential phase in the first two days of experimentation. A stationary phase is not clearly defined for CTL photobioreactors, it was observed that growth is occurring until day 14 despite the low counting in day 6. For SWW photobioreactors the exponential phase end after day 4, and cell concentration tend to decline.

Figure 5.4 | Growth of a mixed-culture microalgae in experiment stablished in outdoor conditions.

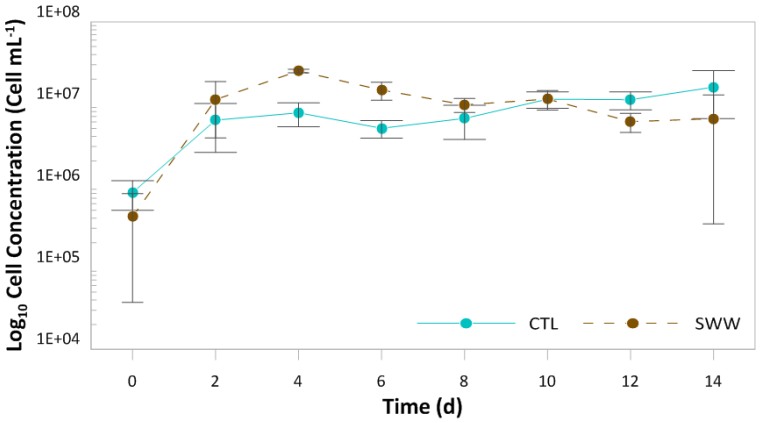


Figure 5.5 | Growth of a mixed-culture microalgae inoculated in 20 L flat-plate phoobioreactos under outdoor conditions at day 14 in sanitary wastewater (SWW) and conventional culture medium (CTL).

The table 5.1, shows the obtaining biomass yield, starch, and crude lipid production after 14 days. Comparing μ_{max} and $Y_{X/S}$ values obtained in this study with values reported for two species isolated from a mixed cultured in concentrated municipal wastewater, similar values can be observed for *Chlorella vulgaris,* which shows a biomass yield around 0.44 g L^{-1} and μ_{max} of 0.239 d^{-1}, and *Chlorella sp.* presents values of 0.45 g L^{-1} and 0.325 d^{-1} respectively (Zhou et al., 2011). The authors report higher µmax for *Chlorella zonfingiensis* during spring in outdoor conditions (0.415 d^{-1}) (Feng et al., 2011). This value is lower than the value shown in table 5 for SWW.

Table 5.1 | Microalgal biomass characterization of a mixed culture after 14 days.

Treatment	Biomass yield (g L^{-1})	Maximal growth rate (d^{-1})	Crude lipid content (%)	Starch content (%)
CTL	0.253 ± 0.031	0.3014	0.381 ± 0.146	0.409 ± 0.026
SWW	0.312 ± 0.104 **	0.5941	0.922% ± 0.389*	0.546 ± 0.279**

**means a statistically significant difference (t-test; α=0.05) compared to CTL*

***means no statistically significant diference compared to CTL*

Data for *Verrucodesmus verrucosus* specie cultivated in indoor conditions were reported. Arenas determinated a μ_{max} of 0.08 d^{-1} and $Y_{X/S}$ of 0.3497 g L^{-1} after 51 days of cultivation in municipal treated wastewater (Guerrero, 2017) on the other hand, Orantes observed a µmax of 0.23 d^{-1} and $Y_{X/S}$ of 0.6 ± 0.38 g L^{-1} in crude wastewater after 10 days of culture (Orantes-Calleja, 2018). Results obtained in this study indicate that mixed culture can grow faster under natural sunlight than in indoor conditions, and accumulate similar dry-biomass yield in less time of culture. Also, it has a similar biomass yield compared with other works carried out in different climatic conditions.

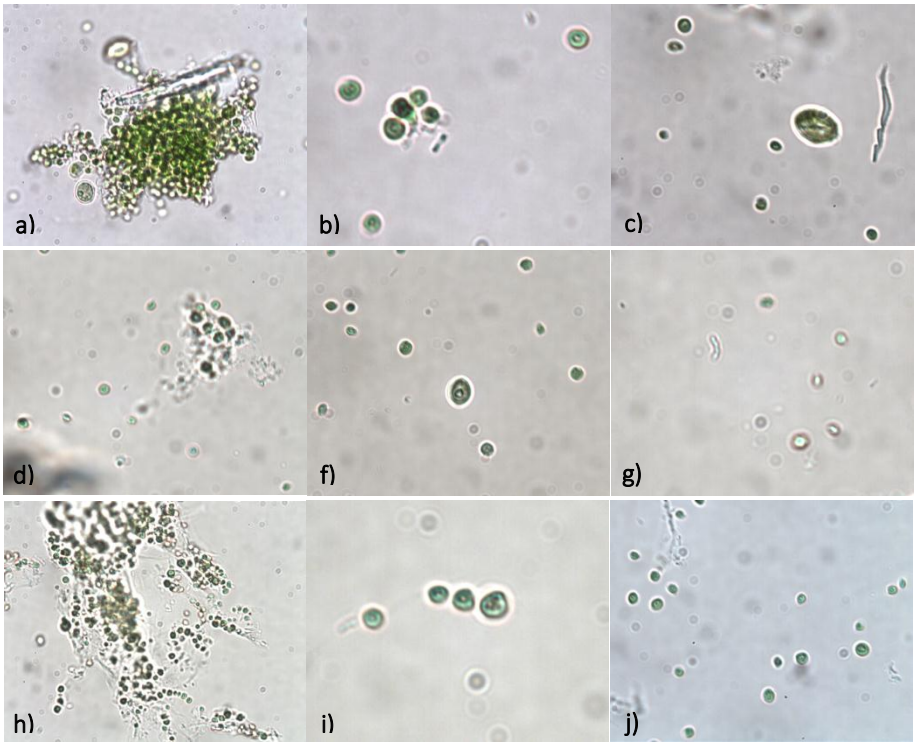


Figure 5.6 | Microalgae cultures viewed under optical microscope (100X). a) to c) correspond to inoculum cultures; d) to g) correspond to control FPB; h) to j) are sanitary wastewater FBR views.

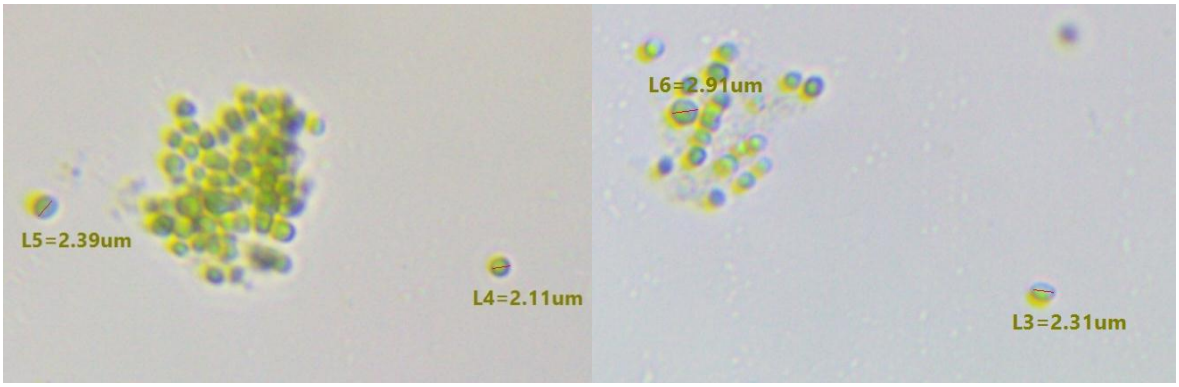


Figure 5.7 | Microalgae cell size viewed under optical microscope (40X) for cultures in wastewater at day 14.

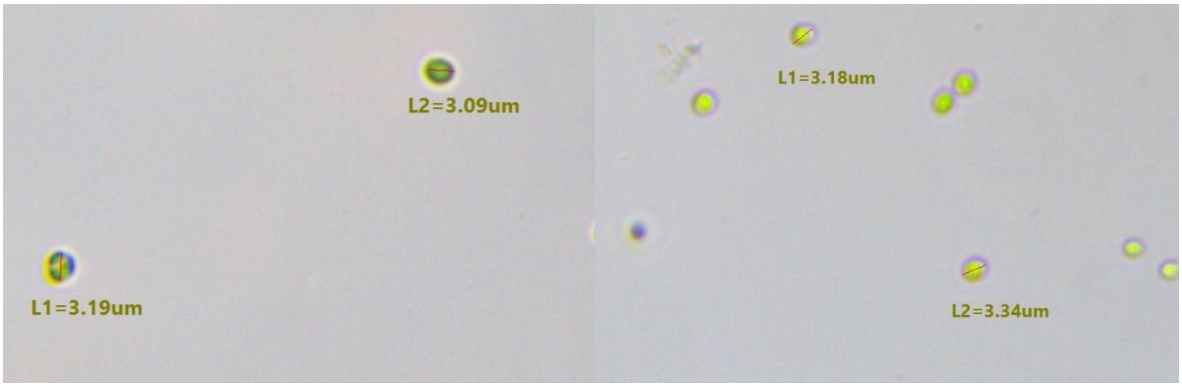


Figure 5.8 | Microalgae cell size viewed under optical microscope (40X) for cultures control at day 14.

In respect of the microalgae community in mixed culture, it was reported by López-Mendoza a phytoplankton screening made in the Xochimilco Lake area, this is the same body-water where the mixed-culture inoculum used in this study comes from. The author identified the possible species present in the lake area and its frequency. It was observed that *Chlorophyta* is the dominant division in body water, represented by *Desmodesmus*, *Pediastrum*, *Pseudopediastrum*, *Acutodesmus*, and *Coelastrum* species genera (Tavera, 2015)(Zhou et al., 2011). Nevertheless, is not possible to conclude by microscope views wich microalgae species are present in cultures, due to a detailed assessment is needed.

Some common microalgae species that could be found in a wastewater environments and have been studied as species for phytoremediation include *Botryococcus, Chlamydomonas, Chlorella, Phormidium, Haematococcus, Spirulina, Oscillatoria, Dunaliella, Desmodesmus, Arthrospira, Nodularia, Nostoc, Cyanothece, Scenedesmus*, etc. (Sanjay Kumar Gupta, f.a.).

In a bioprospection carried out by Zhou *et al*, a strain classification, cell size, growth rate, and biomass productivity in concentrate municipal wastewater were estimated. According to those characteristics reported, *Chlorella vulgaris* has similar characteristics compared with observations made in this work (Table 5.1), author report that *Chlorella vulgaris* has a cell size of 2-4 µm, a growth rate of 0.293 d^{-1}, 17.41% total lipid accumulation, and biomass concentration of 0.43 g L^{-1} (Zhou et al., 2011). Similar cell size was observed in both cultures, as is shown in figures 5.7 and 5.8.

5.2.3 Crude lipid and starch content

The starch and lipid contents in microalgae cells can increase in response to specific changes in the cultivation environment, particularly under stress conditions. The biomass crude lipid content in CTL and SWW blocks exhibits a significant difference shown in Table 5. A higher crude lipid percentage is observed in SWW after 14 days of experimentation, nevertheless, it is a low value compared with contents reported by many authors for wastewater microalgae biomass. For mixed cultures, lipid productivity can be 28.2% and 14-29% for mixotrophic and heterotrophic metabolism respectively (Venkata Mohan et al., 2015). In axenic cultures growing in wastewater, species as *Chlorella vulgaris* can accumulate 17.41% of total lipids, and *Chlorella sp.* shows values of 26.85% (Zhou et al., 2011). It is widely known that many microalgae species can store high lipid content when is exposed to low nutrient concentrations. According to Feng *et al*, high lipid content

in *Chlorella sp.* (54.3%) can be achieved in flat-plates under outdoor conditions when microalgae are growing in N limitation (NO_3^- < 29 mg L^{-1}), by contrast, lipid accumulation is low (27.3%) when NO_3^- is 729 mg L^{-1} (Feng et al., 2011). The NO_3^- concentration at t_0 in this experiment was 23.13 mg L^{-1}, at this value NO_3^- is limited and lipid accumulation would be expected, although in this case, NO_3^- is not the nitrogen specie most assimilated by microalgae, being NH_3 the preferred form.

For microalgae lipid extractions, the cell wall has to be disrupted properly. A pretreatment has to be applied to enhance lipid recovery efficiency (Ghasemi Naghdi, González González, Chan, & Schenk, 2016). The biomass samples were not subjected to a disruptive process during the lipid extraction, therefore, it is expected that lipid concentration shown in Table 3 could be higher. The extraction efficiency of lipids in microalgae biomass treated by the Soxhlet method is high when it is coupled with cellular disruption methods (45% of oil recovery)(Pragya, Pandey, & Sahoo, 2013), while without a pretreatment, efficiency can be lower than 2.5% (Menegazzo & Fonseca, 2019). Significance difference between crude lipids in CTL and SWW probably correspond to lipids presents in municipal wastewaters, that can be up to 41% of the total organic components, mainly triacylglycerides (TAGs) (Sheik et al., 2014). This could be contributing to having higher crude lipids percentage in SWW.

5.3 CONCLUSIONS

For biomass cultivated in sanitary wastewater, the yield production, crude lipid, and starch were evaluated. Significant differences in crude lipid content were observed in SWW biomass compared with CTL biomass, crude lipid was 41.32% higher in SWW. Results obtained in this work demonstrate that similar biomass yield expected for conventional culture medium, can be obtained when microalgae are grown in sanitary wastewater, also, a better yield of crude lipids can be achieved in wastewater biomass.

CHAPTHER VI
Contribution 2

INFLUENCE OF MICROALGAE CULTURE ON PHYSICAL-CHEMICAL CHARACTERISTICS AND NUTRIENT REMOVAL OF SANITARY WASTEWATER

ABSTRACT

Traditional biological wastewater treatments already use photosynthetic microorganisms for nutrient removal, nevertheless in recent years microalgae as wastewater bio-treatment microorganism have been widely studied. In this chapter, the main objective was to evaluate the influence of a mixed-culture microalgae on physical-chemical characteristics and nutrient removal of sanitary wastewater. The COD, NH_3, NO_2^-, NO_3^- and PO_4^{-3} concentrations and removal efficiency were measured and estimated after 14 days in SWW flat-plates under outdoor conditions. Also physical-chemical parameters as pH, temperature and turbidity were analysed. High efficiency removal values were obtained, better removal percentage was observed for ammonia with 81.11%, followed by COD (63.99%) and nitrates (50.5%). Nitrogen assimilation by microalgae, nitrification-denitrification and organic matter oxidation by bacteria are possible mechanisms for nutrients removal.

6.1 INTRODUCTION

Municipal wastewater discharged in the sewerage system represents 70-75% of freshwater consumption in communities or institutions. Inadequate wastewater management can result in a negative environmental impact on receptor water bodies. Inorganic substances as ammonia, nitrates, and phosphates can contribute to the eutrophication process by allowing the uncontrolled growth of aquatic species. High nutrients concentrations in discharged effluents can reduce oxygen demand in water bodies receptors, also can be toxic for aquatic life, risky for public health and could reduce reused wastewater potential. In consequence, nutrient control is the main issue in quality water management and treatment plant projects, and integrated this issue with

biomass production for biofuels obtention, is a task toward optimal use of renewable biological resources (Zabaniotou, 2018).

The role of microalgae in wastewater treatment is not a new founding, traditional wastewater treatments include the microalgae metabolism in conjunction with other species for treating wastewater. Microalgae are being intensively studied due to their potential at removal nutrients coupled with obtaining different bio-products potentially used in energy transformation. Studies showed positive results regarding the potential of utilizing microalgae to remove nitrogen, phosphorus, and other elements from wastewaters and at the same time, reduce up to 90% nutrient consumption in freshwaters cultures (Pittman et al., 2011).

Meanwhile, an advantage in microalgae wastewater cultures is the consortia microalgae-bacteria, which can make a symbiotic relationship that could enhance the assimilation of nutrients and results in higher biomass productivity. Generally, bacteria assimilate organic carbon for growth provide CO_2 which is more favorable for microalgae, while microalgae produce oxygen and other nutrients that could be utilized by bacteria (Del Rio-Chanona, Cong, Bradford, Zhang, & Jing, 2019)(Olguín, 2012)(Li et al., 2019).

The purpose of this chapter was to evaluate the influence of the mixed culture on physical-chemical characteristics, and nutrient removal in sanitary wastewater used as culture media for microalgae growth. Four nutrient species were evaluated to determinate its concentration during the experiment. Removal rate en removal percentage were estimated after 14 days. Chemical Oxygen Demand, turbidity, temperature and hydrogen potential were compared at the beginning and end of experiment.

6.2 RESULTS AND DISCUSSION

6.2.1 Nutrient variation in wastewater used as microalgae culture medium

Nutrient concentration in SWW flat-plates was quantified for 14 days. The figure 6.1 shows three nitrogen species variation; ammonia (NH_3), nitrates (NO_3^-) and nitrites (NO_2^-), figure 6.2 shows phosphates (PO_4^{-3}) and organic matter indirectly estimated by Chemical Oxygen Demand (COD).

In biological treatment processes, there are two mechanisms for nitrogen transformation: assimilation and nitrification-denitrification. In nitrogen assimilation microorganisms present in wastewater can assimilate ammoniacal nitrogen and incorporate it into cell mass.

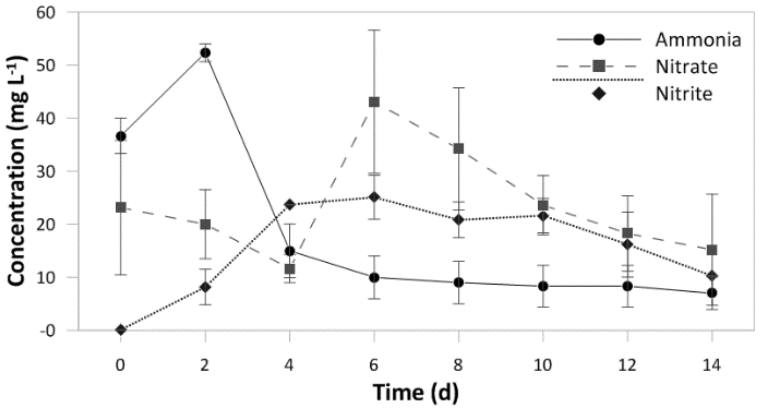


Figure 6.1 | Variation of nitrogen species concentration in wastewater use as microalgae culture medium in outdoor conditions.

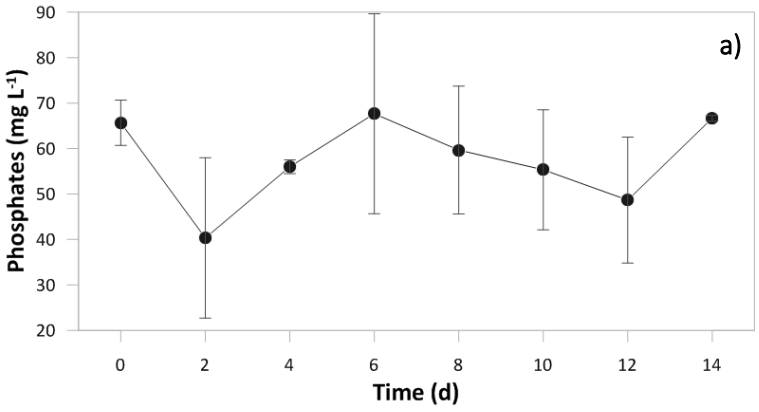
In nitrification-denitrification bacterias, as *Nitrosomas* and *Nitrobacter* oxidized NH_3 into NO_2^- (an intermediate product) and then NO_2^- is transformed into NO_3^-, however, at this step the nitrogen has only been transformed and not eliminated. In the second step, nitrate is converted into a gaseous product that is eliminated as N_2 (Metcalf, 2014). It is observed in figure 6.1 that at day 0 the highest nitrogen form is NH_3, while NO_2^- is the compound with the lowest concentration. Also, it can be seen that between day 2 and 4, the NH_3 concentration tend to decline and NO_2^- start to increase. From this behavior can be deducted that a nitrification-denitrification process could be occurring starting on day 4. Due to an inherent characteristic of crude wastewaters, composed of a complex biological population that includes bacterias, these organisms could be present in unsterilized wastewater samples used in the current experiment.

It is important to discuss that ammoniacal nitrogen can be present in wastewaters in two different forms: ammonia (NH_3-N) and ammonium (NH_4^+-N). The main factor that determines the presence of one or another is pH, with pH values over 6, NH_4^+ is extremely higher than NH_3. At initial values of pH and T in the experiment (Table 6.1), the percentage of ammonia in aqueous solution is around 18.77% (HACH, 2018), it could mean that ammoniacal nitrogen concentration shows in figure 6.1, could correspond mainly to NH_4^+. Ammonium is less toxic to aquatic life than ammonia and is the major assimilable nitrogen specie to microorganisms (Emerson, Russo, Lund, & Thurston, 1975). Ammonium can serve as a nitrogen source for microalgal growth due to is a precursor for amino acid production inside the cell. The direct uptake of ammonium takes place due to the energy requirement necessary for reduction and assimilation is reduced when ammonium is used (Kunar-Sinngh, Farooqi, Zainul-Addin, & Kumar, 2019). Ammoniacal nitrogen concentration decreases inversely to microalgal growth in the first 4 days, the same interval where the

exponential phase in microalgae growth is observed (figure 3.4). This behavior can indicate that microorganisms are using ammoniacal nitrogen for cell growth.

The ammonia reduction in cultivation of *Scenedesmus sp.* in primary and secondary wastewater shows similar behavior to that observed in figure 6.1. An experiment demonstrated the rapid removal of NH_3 after 8 days of culture in outdoor conditions (McGinn et al., 2011). Similar results were observed for *Chlorella sp.* growing in dairy diluted wastewater, ammonium was removed under undetectable values after 4 days of culture, reducing its concentration from 43.20 mg L^{-1} to 11.80 mg L^{-1} (Lu et al., 2015). Another study reports ammonium removal after 35 days when *Verrucodesmus verrucosus* were cultivated in indoor conditions using municipal wastewater, NH_4^+ concentration decrease from 63.05 mg L^{-1} to 0.35 mg L^{-1} (Guerrero, 2017). For the same strain growing in sterilized wastewater in indoor conditions, ammonia removal percentage (79.91%) after 45 days (Orantes-Calleja, 2018) were similar to results obtained in this experiment, however the time need for ammonium removal is considerably long, it may indicate that in sterilized wastewater there are no other microorganisms that could accelerate ammonium assimilation by microalgae.

The most frequently phosphorous species that could be found in aqueous solutions are orthophosphate, polyphosphates, and organic phosphates. Orthophosphates available for biologic metabolism are PO_4^{-3}, HPO_4^{-2}, $H_2PO_4^-$, and H_3PO_4, subsequent rupture is unnecessary. Polyphosphates are in the P_2O_7 form, hydrolysis of this type of phosphorous is a slow process that takes place in aqueous solution, and is transformed and recovered in orthophosphates. The polyphosphate and organic phosphorus constitute approximately 70% of phosphorus content in wastewater (Metcalf, 2014). Phosphates concentration decreases on the first day of experimentation and after day 2, concentration fluctuates and tends to increase.



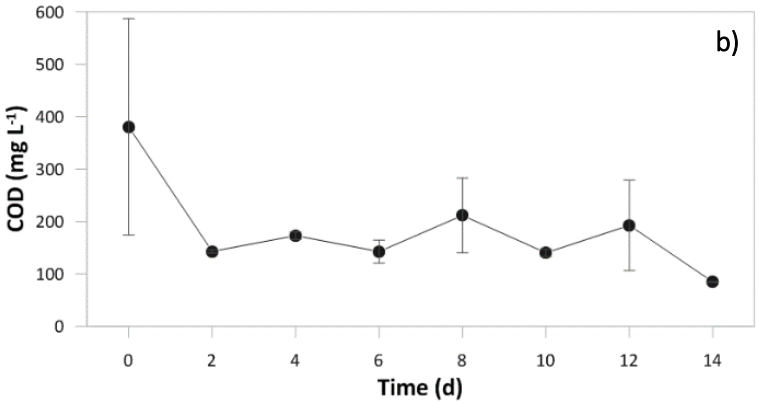


Figure 6.2 | Phosphate variation in wastewater use as microalgae culture medium in outdoor conditions, Chemycal Oxygen Demand variation in wastewater use as microalgae culture medium in outdoor conditions.

Two possible reasons for the increase of phosphate concentrations in SWW are polyphosphates transformation into phosphates by hydrolysis, and phosphorous liberation as a consequence of rupture cells, which spilling their P content into the culture medium (Martínez, Sánchez, Jiménez, El Yousfi, & Muñoz, 2000). It can be observed in figure 6.2, that increase in PO_4^{-3} concentration is indirectly proportional to COD decrease, it may explain the P liberation due to the organic matter oxidation.

Biodegradable organic matter in wastewater is composed of carbohydrates, proteins, and fats that can be measured in the function of chemical demand oxygen (COD). The COD concentration can be reduced by microorganisms metabolism (mostly by bacterias), which oxidize organic matter in the presence of oxygen to produce new cells, CO_2 and NH_3 are products of this reaction. It is observed in figure 6.2a that COD concentration decrease at the first two days of experimentation, on the other hand, the NH_3 concentration increases at the same time, it is possible to assume that an organic matter oxidization process is occurring.

6.2.3 Nutrient removal and physical-chemical characteristics in wastewater

Nutrient removal by microalgae in wastewaters will depend on the initial nutrient concentration, organism species, and culture conditions. The efficiency nutrient removal and physical-chemical characteristics in SWW flat-plates were estimated during the experiment. **Table 6.1** shows the nutrient removal rate (RR) and removal percentage (RP) after 14 days, as well as hydrogen potential, temperature, and turbidity at the beginning and the end of the experiment.

Table 6.1 | Initial and final characteristics and nutrient removal in SWW flat-plates during experiment.

Wastewater characteristics	Initial	Final	RR (mg L^{-1} d^{-1})	RP (%)
Ammonia (mg L^{-1})	36.67 ± 5.77	7.0 ± 5.29	2.12	81.11
COD (mg L^{-1})	380 ± 291.8	86 ± 1.3	21.14	63.99
Nitrate (mg L^{-1})	23.13 ± 21.99	15.2 ± 8.1	0.76	50.5
Nitrite (mg L^{-1})	0.1 ± 0.12	10.27 ± 10.6	0	0
Phosphate (mg L^{-1})	65.67 ± 10.3	66.7 ± 1.2	0	0
Temperature (°C)	32.9 ± 0.46	37.77 ± 0.35		
pH	8.47 ± 0.13	8.27 ± 1.41		
Turbidity (FAU)	60.67 ± 60.05	5.33 ± 1.53		

RR= Removal rate; RP= Removal percentaje; N/C= not calculated

The highest value for nutrient removal was achieved for ammonia with a removal percentage of 81.11% and a removal rate of 2.12 mg L^{-1} d^{-1}. The COD was the second parameter with high removal efficiency (63.99%) and removal rate (21.14 mg L^{-1} d^{-1}), nitrates were removed in 50% with a rate of 0.76 mg L^{-1} d^{-1}. Between days 0 to 4 great values in nutrient removal and cell concentration were observed, which could suggest that culture time can be shorter (around 5 days). According to some authors, similar values for nutrient removal rate and removal percentage were reported. For *Chlorella sp*. growing in dairy wastewater in outdoor conditions, the RR_{COD} and RP_{COD} where 41.31% and 54.82% respectively, lower than values reported in this study. For the ammoniacal nitrogen removal ratio were reported an RP of 72.70% after 4 days, it is lower value compared with the removal percentage observed in table 6.1 (Lu et al., 2015). Woertz *et al* reported that ammonium was the main nitrogen form in the initial characterization of wastewater. After algal growth organic nitrogen was predominant, and after 4 days of operation, a removal value of 84% was reached for ammonium (Woertz et al., 2009).

No phosphates removal process was observed during the experiment, high-efficiency values in phosphorous removal by microalgae are well established, RR and RP were 2.74 and 65.33% for phosphates after 4 days (Lu et al., 2015). In other study PO_4^{3-} was also removed until undetectable levels within 8 days of cultivation (McGinn et al., 2011). There are two mechanisms for phosphorus removal in wastewater including biomass assimilation and chemical precipitation, it last induced by the alkalinity of culture medium (Lu et al., 2015). According to Woertz *et al*, phosphates are

removed from wastewater in 99% after 15 days of experimentation using a mixed-culture in outdoor conditions, with initial PO_4^{3-} concentration of 2.6 mg L^{-1}.

On the other side, according to McGinn *et al*, typical concentrations of ammonia nitrogen and phosphates in secondary-treated wastewater fall into the ranges of 20–40 mg L^{-1} and 1–10 mg L^{-1} respectively, which are adequate to support high productivities from most microalgae strains (McGinn *et al*., 2011). Initial values in SWW (table 6.1) are adequate to promote microalgal activity and growth.

Among important physical wastewater quality parameters are temperature and turbidity. Turbidity compares light intensity disperse in a sample with a reference solution, colloidal matter present in wastewater impedes light transmission dispersing or absorbing light, however, there is not a relation between turbidity and suspended solids concentration. Turbidity is not a concluding parameter but can indicate water quality (Metcalf, 2014). Higher turbidity can affect the temperature in water due to suspended particles absorb more heat and it reduces dissolved oxygen concentration, also turbidity reduces light penetration in the water column and reduces the photosynthesis process, in consequence, microalgae activity (EPA, 2012). Turbidity achieve at final experiment time where reduce in, at this turbidity values wastewater quality can be high in physics terms, due to turbidity is under FAU value of 7.

6.3 CONCLUSSIONS

Ammoniacal nitrogen and phosphates concentration present in sanitary wastewater are adequate to promote microalgae biomass accumulation. In cultures that used wastewater as microalgae growth medium, symbiosis interaction between microalgae-bacteria could be present, nitrogen assimilation by microalgae and nitrification-denitrification by bacteria, are process that could be occurring in un-sterilized wastewater.

CHAPTHER VII
General conclusions

- In microalgae cultures in outdoor conditions where wastewater is used as a culture medium, similar biomass production can be obtained compared with conventional cultures, as well as a faster growth rate due to favorable conditions in radiation and temperature.
- In respect of starch and crude lipid contents, results are not conclusive due to is expected that these biomolecules are present in higher concentrations than those reports in present work. Nevertheless, it is important to note that in wastewater cultures, a significant crude lipid percentage is added due to the oily wastewater characteristic.
- The mixed-culture microalgae evaluated in the present work can improve the wastewater quality characteristic, by removing ammoniacal nitrogen, nitrates and organic matter from influent after 14 days of culture.
- It was observed a possible symbiotic consortium in microalgae-bacteria in wastewater culture, improving the nutrient removal that was not observed in the conventional culture medium.
- The wastewater used to biomass obtaining prove to be better for microalgal growth and biomass accumulation and demonstrates that the nutrient content in wastewater can be better than conventional cultures for outdoor crops.

Recommendations

- To improve crude lipid extraction it is recommended to evaluate the content by Soxhlet method complemented with a pre-treatment process, in example, by sonication.

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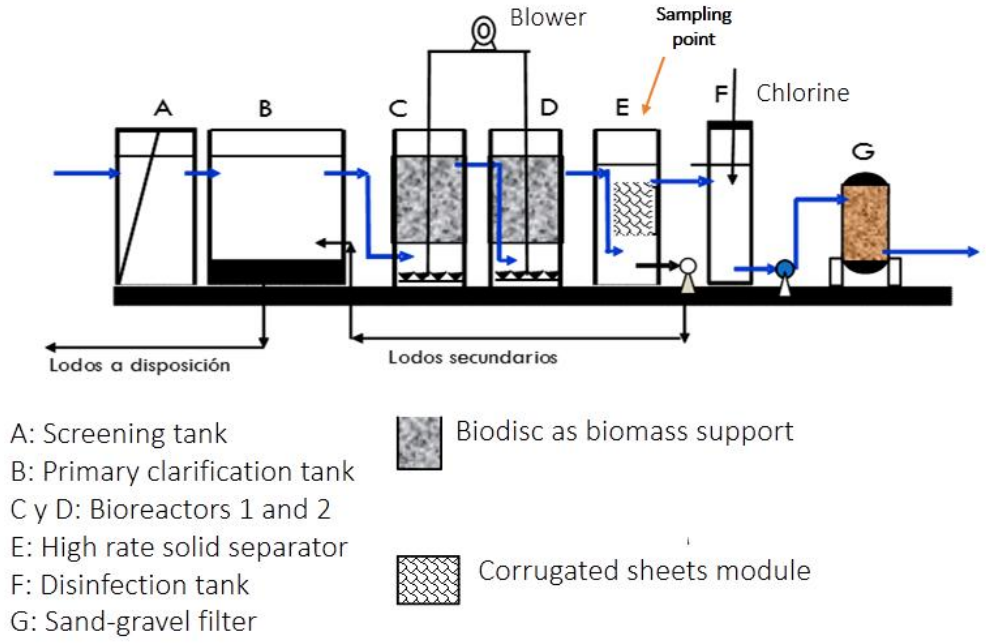


Figure 7.1 | Diagram of wastewater treatment system at IER-UNAM (Camargo-Rodríguez, 2011).

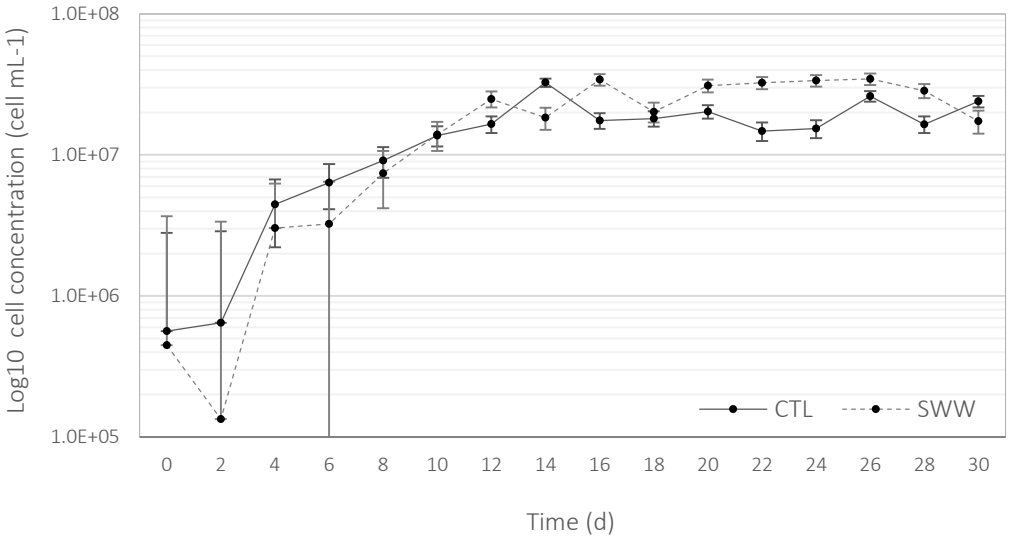
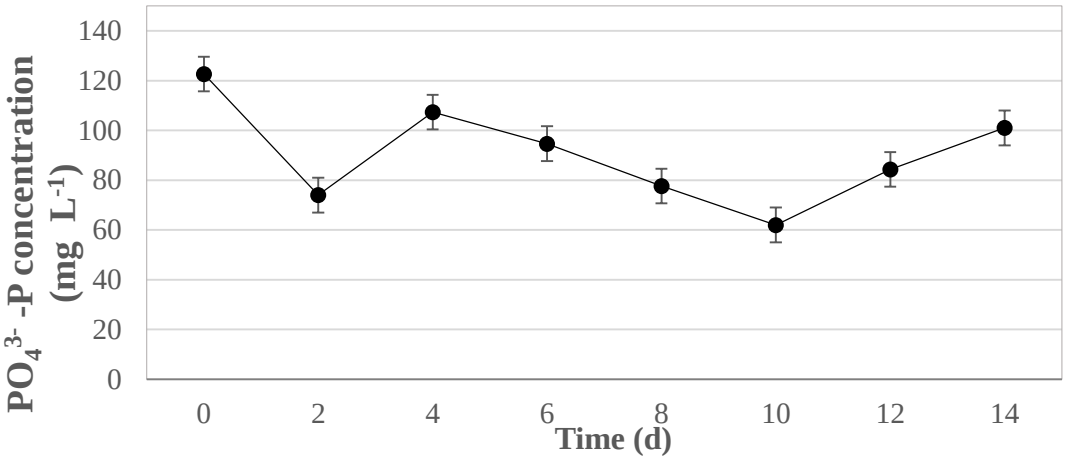
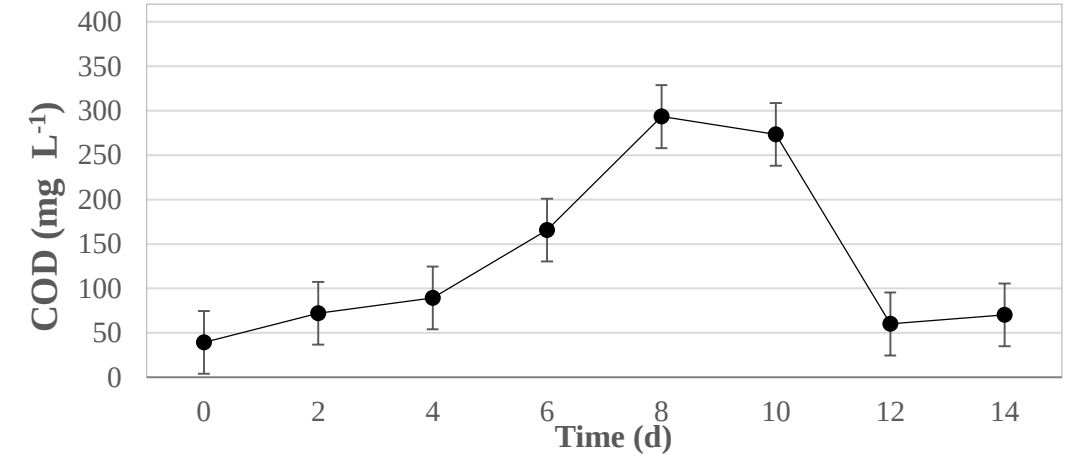


Figure 7.2 | Small scale preliminary test in outdoor conditions using six polyethylene bottles of 500 mL as bioreactors. Experiment were carried out from, average conditions were: PAR= 156.2 ± 10.8 µmol s^{-1} m^{-2}, T= 31.2 ± 0.37°C. The CTL correspond to sterile and filter freshwater with 1 mL L^{-1} Bayfolan® Forte foliar fertilizer. SWW correspond to sanitary wastewater taken in high rate solid separator in WWTP-IER-UNAM.





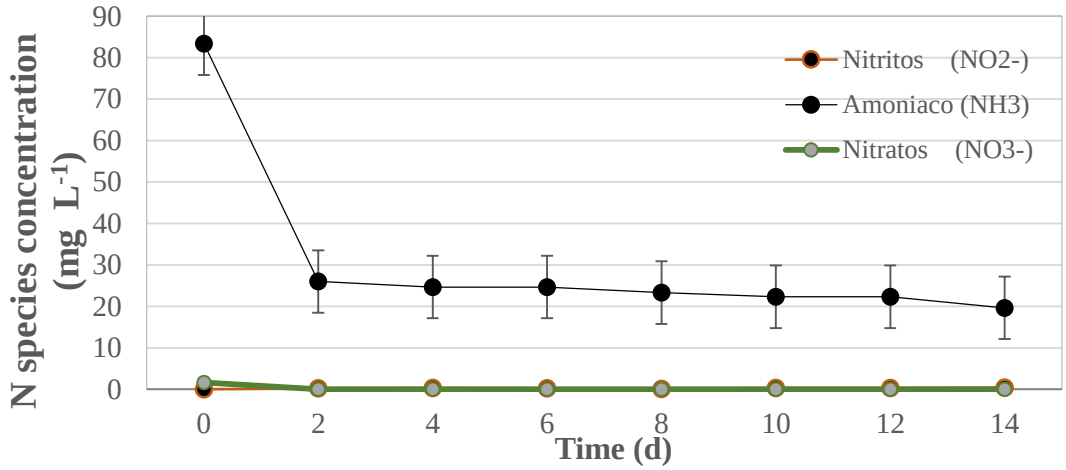


Figure 7.4 | Nutrient variation for nitrogen, phosphates and COD for CTL flat-plates during the experiment.

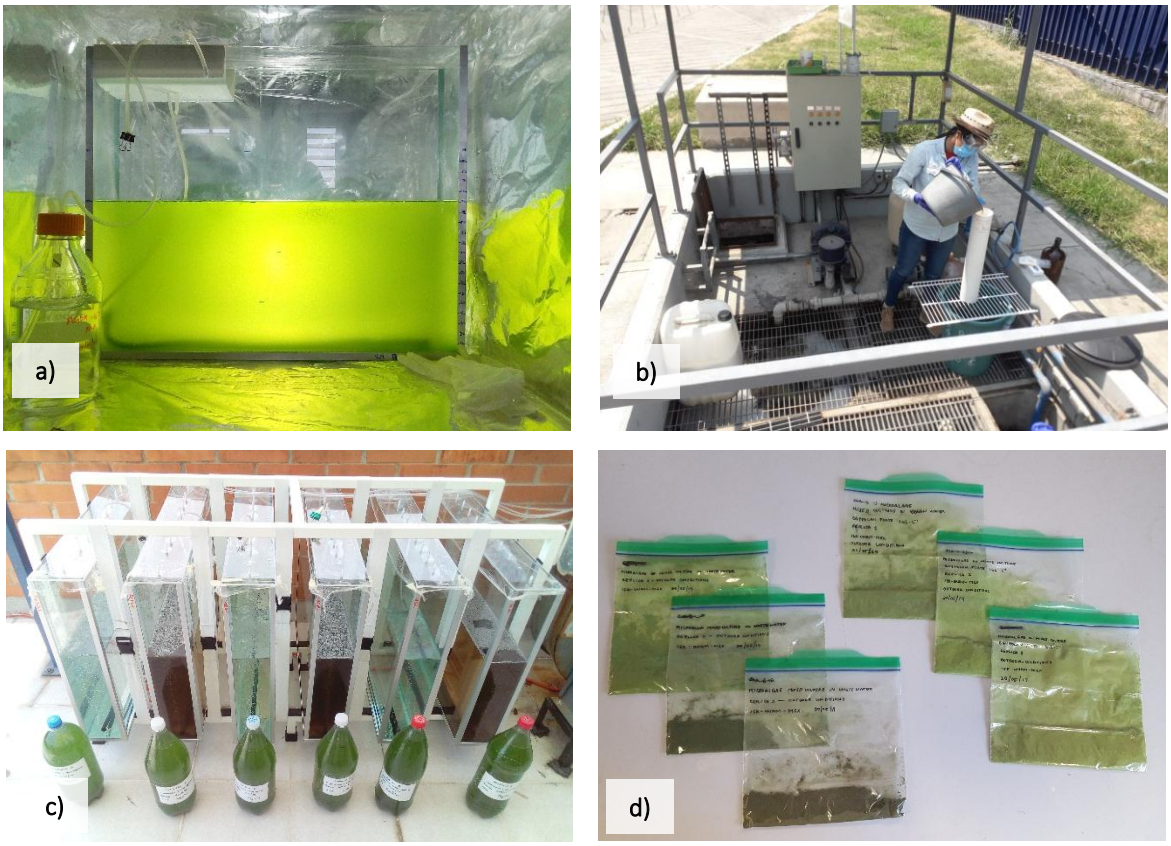


Figure 7.3 | Growth kinetic of microalgae mixed-culture in sanitary wastewater (SWW) and conventional culture medium (CTL).