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Microalgae-based livestock wastewater treatment and resource recovery: A circular bioeconomy approach

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Dedication

In memory of my father, who always truly loved me in his own way and taught me the meaning of being an engineer.

I dedicate the present thesis to my mother and brothers and thank them for their unconditional support. I´m especially grateful to my mother for her endless love, patience and resilience and for always listening to me.

I thank my boyfriend Sergio, my future husband, for his unconditional love and patience and for always giving me the strength and motivation to become a better person. The last steps of this journey would not have been possible without your support.

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Microalgae-based livestock wastewater treatment and resource recovery: A circular bioeconomy approach

by

Anaid López-Sánchez

Abstract

The livestock industry is a sector of great relevance worldwide. This sector accounts for 1.4% of the world's Gross Domestic Product (GDP) and is a source of livelihood for more than 1.3 billion people. Furthermore, thirty-nine percent of the worldwide protein demand is covered by this sector. However, this activity is one of the top polluting industries, accounting for 14% of the greenhouse gasses (GHG) originated from anthropogenic sources. Additionally, the livestock sector is the largest land user on earth, using 70% of the total agricultural land and 30% of Earth's land surface. One-third of the global cereal production is destinated for animal feed, of which some nutrients are retained by the animals and the rest is released to the environment without previous treatment, resulting in soil degradation, water and air pollution and, consequently, serious human health impacts.

Circular bioeconomy (CBE) has emerged as a potential driver towards the sustainability of livestock production systems. One of the main objectives of the CBE model within the livestock industry is the minimization of the usage of raw material resources through the recycling, reuse, and revalorization of waste and wastewater. Microalgae-based wastewater treatment (MbWT) is a potential solution aligned with the CBE principles, in which the nutrients contained in the livestock wastewater (LW) are recovered and transformed into high value-added products with a wide range of industrial applications.

The overall performance of MbWT (i.e., nutrient removal efficiencies and biomass production) is highly dependent on a wide range of factors, such as the microalgal strain and the composition of the wastewater. However, most of the existing studies that implemented MbWT have focused on a single LW type. Therefore, the main objective of this thesis is to treat a mixed effluent composed of the most common ADLW (from cattle, swine, and poultry), to understand the effects of the mixture of all three types of LW on cell growth and pollutant removal efficiencies of microalgal cultures (*Chlorella vulgaris, Haematoccocus pluvialis* and *Chlamydomonas spp*.). Through an evaluation of the mixture design, the optimal fraction of these different types of effluents (ADCW, ADSW, and ADPW) was analyzed to obtain maximum microalgal biomass productivity and pollutant removal rates (COD, TN and TP). Furthermore, these microalgae were tested in all possible combinations of mono-, bi-, and tri-cultures.

The first chapter of the present thesis consists of a thorough review of the literature to address the most significant factors affecting nutrient removal and biomass productivity in MbWT, including: (i) microbiological aspects, such as the microalgal strain used for

MbWT and the interactions between microbial populations; (ii) physical parameters, such as temperature, light intensity and photoperiods; and (iii) chemical parameters, such as the C/N ratio, pH and the presence of inhibitory compounds. Additionally, different strategies to enhance nutrient removal and biomass productivity, such as acclimation, UV mutagenesis, multiple microalgae culture stages (including monocultures and multicultures) are discussed.

The second chapter of this thesis presents the first study of MbWT using anaerobically digested swine, poultry and cattle wastewater (ADSW, ADPW and ADCW) mixtures. A centroid mixture design was used to determine the optimal mixture to promote higher cell concentrations and pollutant removal efficiencies of the microalgae *Chlorella vulgaris, Haematococcus pluvialis* and *Chlamydomonas* spp. cultured as mono-, bi-, and tri-cultures. Additionally, A redundancy analysis was performed to analyze the correlation between microalgal cultures and the removal efficiencies of the digestate pollutants.

The results herein show that *C. vulgaris* as a monoculture in a digestate mixture of 0.125:0.4375:0.4375 (ADSW:ADPW:ADCW) resulted in cell growth of 3.61×107 ± 2.81x106 cell mL-1, a total nitrogen removal of 85%±2%, a total phosphorus removal of 66%±3% and a chemical oxygen demand removal of 44%±7%. The specific composition of the effluents plays a key role in microalgal performance due to their respective nitrogen and phosphorus content. Furthermore, this study suggests that a mixture of the three most common digestates generated by livestock farms offers a promising alternative for the treatment and revalorization of LW, by taking advantage of the unique composition that each digestate possesses. Further studies are warranted to gain a deeper understanding of the interspecific microalgal interactions occurring in mixed cultures that may enhance or hinder the performance of MbWT.

The final chapter of this thesis delves into a research endeavor conducted in Jalisco, Mexico, aimed at delineating the spatial diversity of livestock waste (LW) generated across cattle, swine, and poultry farms. The investigation assesses the viability of an alternate management scenario involving anaerobic digestion in conjunction with microalgae-based wastewater treatment. The objective is to adhere to regulations, curtail greenhouse gas emissions, and yield protein-rich biomass for animal feed. Additionally, the paper underscores the obstacles necessitating resolution to foster more ecologically sound livestock production methodologies. The analysis provides insights into the hurdles of transitioning towards more sustainable livestock production techniques. The abatement of greenhouse gas emissions also presents an avenue for trading carbon credits in voluntary markets for Mexican livestock producers.

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Chapter 1. Introduction

1.1 Motivation

1.1.1 Livestock sector

The livestock sector is an essential part of the global economy, accounting for 1.4% of the world's gross domestic product (GDP). The average annual growth of this sector is 3.8% annually (Rojas-Ramírez & Vallejo-Rodríguez, 2016; Sakadevan & Nguyen, 2017a). Additionally, this sector covers 39% of the protein demand and is critical for the income of several regions in developing and developed countries, as it is the source of livelihood for around 1.3 billion people worldwide (McClelland et al., 2018; Rout & Behera, 2021; Sajeev et al., 2018; Sakadevan & Nguyen, 2017a).

In 2018, the global population of poultry, cattle, and swine was about 29.1, 1.6 and 1.4 billion heads, respectively, and the demand for these products is expected to increase by 70% by 2050 (Garmyn, 2021; McClelland et al., 2018). However, the Livestock industry is considered to be one of the major contributors to greenhouse gas emissions (GHG). This is a result of feed production, enteric fermentation and due to the waste management practices that are commonly used. Livestock activities account for more than 14% of the GHG originating from anthropogenic sources, of which 50 to 85% are due to feed production, and one-third of the global cereal production is dedicated to animal feed (Garcia-Launay et al., 2018; Rout & Behera, 2021; Swain et al., 2018). Additionally, the livestock industry is the largest land user on earth, accounting for about 70% of the total agricultural land and 30% of the Earth's land surface, which results in land degradation, soil erosion, loss of biodiversity, deforestation, among others (J. Liu et al., 2017; McClelland et al., 2018; Poore & Nemecek, 2018; van Wagenberg et al., 2017). Ten percent of the global anthropogenic water use is consumed by the livestock industry, and 60% of the global biomass harvested is used in animal breeding (Sakadevan & Nguyen, 2017a). While some of these nutrients are retained, metabolized and used for animal growth, the rest is released to the environment through the animal's manure, along with different products formed during metabolization (Rojas-Ramírez & Vallejo-Rodríguez, 2016).

Around 128 million tons of nitrogen (N) and 24 million tons of phosphorus (P) are released in livestock manure annually, and it has been projected that in 2050, the global livestock production will lead to global increases of 23 and 54% of N and P surpluses, respectively. Surpluses of nutrients happen when the amount of nutrients that the environment can absorb are exceeded. Most of the N surplus released to the environment goes through volatilization, denitrification, leaching to groundwater and runoff to surface waters. The majority of the P surplus is lost to waterways through leaching and surface runoff and transported toward coastal marine systems. Both N and P surpluses result in eutrophication (Sakadevan & Nguyen, 2017a). Around 64–97% of the eutrophication worldwide is attributed to the livestock industry (Garcia-Launay et al., 2018). This process occurs when waters are enriched with nutrients, such as N and P, causing the overgrowth of plants and aerobic microorganisms. Consequently, an increase in the biochemical oxygen demand (BOD) and chemical oxygen demand (COD) occurs, decreasing the dissolved oxygen in the water column, which is essential for survival of aquatic organisms. In addition, high levels of nitrate and nitrite are toxic to humans and livestock (Rojas-Ramírez & Vallejo-Rodríguez, 2016).

The livestock industry is associated with 10 out of 17 of the sustainable development goals (SDG), established by the UN. While the livestock industry has negative effects on environmental-related SDGs, it conversely has positive effects on poverty reduction, infrastructure investment and inequality-related SDGs (Mehrabi et al., 2020). Thus, there is an increasing demand to develop sustainable solutions to address the negative effects and to leverage the positive effects of the sector.

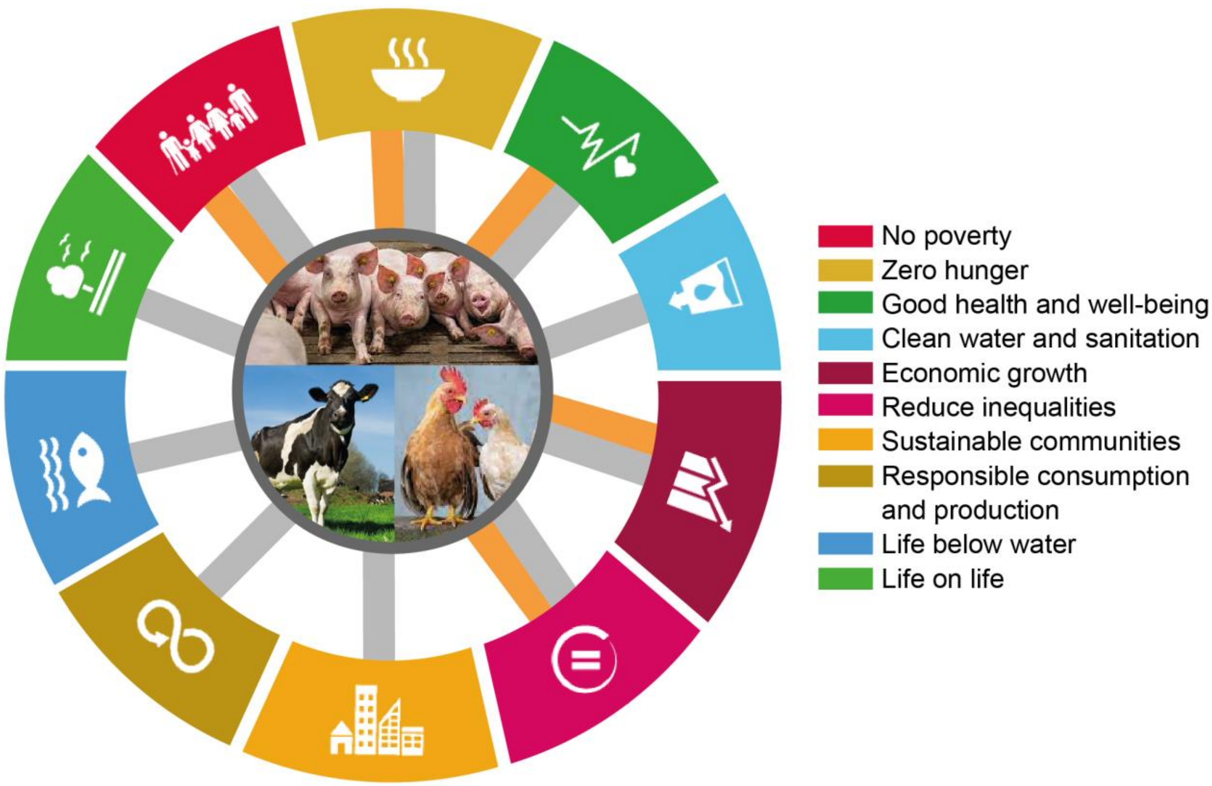


Fig. 1.1.1 Interactions between the livestock industry and the SDGs. Adapted from Mehrabi et al. (2020). The positive (orange lines) and negative (gray lines) impacts of livestock production are displayed.

Circular bioeconomy (CBE) has emerged as a potential driver of livestock production systems. According to the European Union (EU) (2018), CBE strategies are positively related to 12 of the 17 SDGs. CBE seeks to produce higher added-value products from reused or recycled material of biological sources, through innovative processes and principles, to maximize value and reduce biological waste (Ummalyma et al., 2021) Circularity refers to the reuse and recycling of inevitable by-products that must be reintroduced into value-chains to avoid the overexploitation of raw materials (Ronzon & Sanjuán, 2020; Venkatramanan et al., 2021).

The aims of circular bioeconomy applied in the livestock sector include: (1) minimizing the primary natural resources used throughout animal production, (2) avoiding unsustainable practices and (3) recycling, transformation and reuse the livestock waste to produce bioenergy, nutrients and biofertilizers (Paltaki et al., 2021).

Microalgae-based wastewater treatment (MbWT) is a promising biological treatment method that has gained special attention recently. From a CBE perspective, MbWT offers the possibility of carbon fixation and nutrient recovery for the synthesis of valuable bioproducts (Crini & Lichtfouse, 2019; Shahid et al., 2020). Microalgae are photosynthetic microorganisms that possess a high capacity to grow in harsh environments, such as those encountered in livestock wastewater. MbWT is considered to be a cost-effective treatment method that can be implemented in the livestock industry due to its low water usage, along with its high capacities for CO_2 fixation and biosorption of pollutants. MbWT additionally has great potential to produce valuable bioproducts (Ferreira et al., 2018; Hammed et al., 2016) that can be used for the health, cosmetics, biochemical, food and animal feed, biomaterials and biofuels industries. Fuentes-Grünewald et al. (2021) documented that the biomass produced through MbWT could be used for quality animal feed. The incorporation of microalgal biomass in livestock feed is frequently performed due to the benefits it produces, such as higher yields for milk production with elevated contents of omega 3, linolenic acid and DHA. It also allows farmers to lower the feed intake in swine breeding by enhancing their metabolism and producing higher growth yields with increasing contents of polyunsaturated fats (PUFAs) in the meat. In poultry breeding, microalgal supplementation enhances the metabolism of the animals and increases the DHA content in egg yolk as well as in meat, while also enhancing the immune systems of the poultry (Dineshbabu et al., 2019; Yaakob et al., 2014).

1.1.2 Livestock industry in Jalisco and its waste management practices

The livestock industry is an important sector for Mexico, which ranks 11th globally in livestock production. Mexico has an area of 196,437,500 ha, from which 55.44% of the total land area (108.9 million ha) is dedicated to livestock production purposes. The livestock population of the main farming species comes to 584.6, 35.2 and 18.4 million heads of poultry, cattle and swine (Rodríguez-Vivas et al., 2017; SIAP, 2020). Among the 32 states that comprise Mexico, Jalisco is the principal producer of swine, eggs and milk. Jalisco is known as the "agri-food giant" due to its contribution of 11.2% to the country's agricultural and livestock GDP (López-Sánchez, Luque-Badillo, et al., 2021). The livestock industry in Jalisco accounts for 3.3, 2.8 and 78.5 million heads of cattle, swine and poultry, respectively with a total livestock production above 5 Mton and a related income of 22 billion USD registered in 2020 (fig 1.1.2.1) (Díaz-Vázquez et al., 2020; SEMADET, 2021).

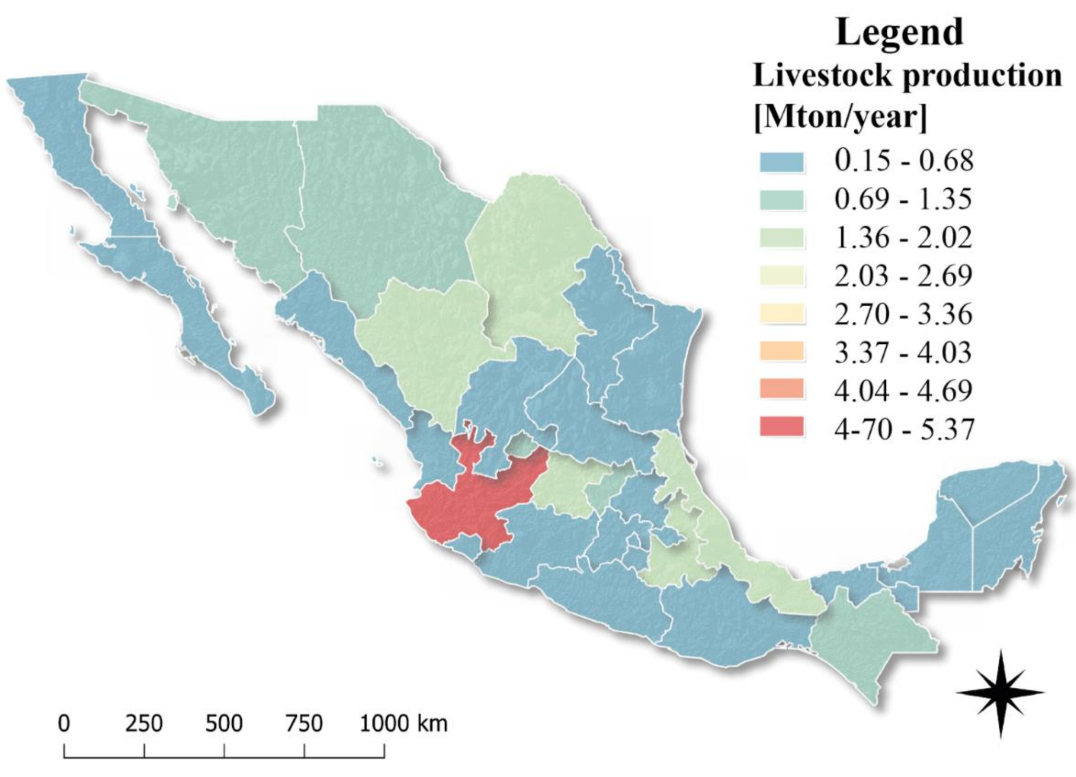


Fig. 1.2.2.1 Mexico's livestock production (SIACON, 2020)

However, high production rates of meat products are almost always associated with elevated rates of waste generation, and in Jalisco, only a few livestock producers have implemented infrastructure to treat the solid and liquid waste produced and to comply with the basic applicable environmental regulations (Rojas-Ramírez & Vallejo-

Rodríguez, 2016). According to Cervantes-Astorga et al. (2021), some bodies of water in Jalisco are already in an extreme hyper-eutrophication situation primarily due to direct waste discharges from livestock, agricultural and industrial activities.

Based on the livestock production inventory of Jalisco provided by SIACON, the three main livestock industry species (cattle, swine, and poultry) generate more than 64 Mton per year of LW in state (Table 1.1.2.1), where cattle, swine, and poultry account for 67.86%, 17.81% and 14.32%, respectively.

Table 1.1.2.1 Livestock waste generation in Jalisco in 2020.

Species	Heads[a]	Waste generation rate[b] [ton head^{-1} year^{-1}]	Generated waste [ton year^{-1}]
Cattle	**3,370,866**	**12.91**	**43,517,880.06**
Swine	**3,898,760**	**2.93**	**11,423,366.80**
Poultry	**131,210,547**	**0.07**	**9,184,738.29**
		TOTAL	**64,125,985.15**

a. According to historical records from (SIACON, 2020) (Consultation Agrifood Information System of Mexico)
b. According to Díaz-Vázquez et al. (2020)

Livestock wastewater (LW) generally contains high concentrations of biochemical and chemical oxygen demand (BOD, COD), nitrogen (N) and phosphorus (P). However, the proportion of each of these elements in the LW is highly influenced by the species generating the waste, their unique genetics and nutritional requirements, as well as the management practices carried out in the livestock unit (Park et al., 2018). Table 1.2.2.2 shows the minimum, mean and maximum values of fat, oil and grease (FOG), BOD, COD, total dissolved solids (TDS), total solids (TS), TP and TN of the manure produced by the principal livestock species (cattle, swine, and poultry), as recently documented by SEMADET (2021) through a Sate wide waste characterization. The swine wastewater (SW) tends to contain the highest concentrations of fat, oil and grease (FOG), BOD, COD and TP, whereas the highest maximum contents of TN, total dissolved solids (TDS) and total solids (TS) are found in poultry wastewater (PW).

Table 1.1.2.2. Physicochemical characterization of the collected samples grouped by species (SEMADET, 2021)

Animal Type		pH [upH]	FOG [mg/kg DM]	BOD [mg/kg DM]	COD [mg/kg DM]	TDS [mg/kg DM]	TS [mg/kg DM]	TP [mg/kg DM]	TN [mg/kg DM]
Cattle	**Minimum**	4.88	2,402.93	20,680.42	26,342.67	16,379.86	190,538.90	109.27	1,603.48
	Mean	6.64	7,590.02*	307,855.36	629,722.81	31,201.34	444,444.46	1,013.79**	10,328.82**
	Maximum	7.63	21,583.43	936,963.11	1,900,794.00	76,545.43	833,686.47	3,209.04	15,753.17
	Standard deviation	0.86	6,080.48	421,525.04	876,303.62	17,944.01	193,946.68	1,010.59	5,288.39
	Variation coefficient [%]	12.90	80.11	136.92	139.16	57.51	43.64	99.68	51.20
Swine	**Minimum**	5.77	3,741.16	24,448.58	39,849.29	16,193.22	38,101.69	286.22	3,596.73
	Mean	6.87	36,153.04*	292,473.73	610,943.67	207,925.95	405,143.43	4,969.38**	48,327.43**
	Maximum	7.44	88,923.99	1,319,004.93	3,665,909.68	1,061,946.90	1,092,288.24	23,457.65	234,800.25
	Standard deviation	0.53	30,280.57	405,206.86	1,108,876.87	317,239.02	287,524.04	7,105.74	72,595.88
	Variation coefficient [%]	7.74	83.76	138.54	181.50	152.57	70.97	142.99	150.22
Poultry	**Minimum**	6.22	316.22	34,174.13	49,608.16	26,702.79	100,135.46	23.90	7,837.27
	Mean	6.74	4,858.10*	210,560.80	422,108.88	322,568.57	475,643.25	2,574.89**	69,591.97**
	Maximum	7.87	13,609.25	341,322.77	767,672.88	1,183,815.03	1,216,184.97	13,151.45	332,203.47
	Standard deviation	0.77	5,805.62	120,411.12	272,170.10	574,202.32	508,073.36	4,747.23	116,625.50
	Variation coefficient [%]	11.41	119.50	57.19	64.48	178.01	106.82	184.37	167.58

*At least one mean is different with a significance level of 95%
** At least one mean is different with a significance level of 90%

As a result of inadequate waste management practices carried out in Jalisco, the mean estimated potential nitrogen, phosphorus and organic matter released were estimated to be 56, 4 and 288 thousand tons in 2020. These emissions contribute to the eutrophication of ground and surface waters. Additionally, these emissions are precursors for GHG, such as methane (CH_4) and oxide nitrogen (N_2O), which promote climate change (Herrero et al., 2011). The GHG estimated as a result of livestock waste management in Jalisco in 2020 was estimated to be around 1.46 Mton CO_{2eq}; nevertheless, this amount may be underestimated because of the poor reporting activities of the sector (SEMADET, 2021).

Reducing GHG emissions is gaining priority by governments worldwide because of the Kyoto Protocol and the Paris Agreement (Anjos et al., 2022; González et al., 2015). Carbon credits markets have been adopted by several countries and provinces to account for the negative externalities of GHG emissions. The carbon credits are called Certified Emission Reductions (CER) and are registered under the clean development mechanism by the United Nations. Each CER corresponds to one ton of reduced equivalent carbon dioxide and is issued to stakeholders that have reduced their GHG emissions (González et al., 2015). The global carbon market can be divided into two groups: voluntary markets and regulated or compliance markets. Currently, the Mexican compensation market is a voluntary market operated by free trade of CER to accomplish corporate goals and not for mitigation responsibilities with the Mexican government. In the voluntary market, one carbon credit issued in Latin America from the waste disposal category has a price of $3.62 USD (Ecosystem Marketplace, 2021).

1.2 Problem Statement and Context

According to the waste hierarchy principles, an appropriate waste management system must prioritize prevention, recycling, reuse and recovery (of material or energy) over the treatment or disposal of waste (Fig. 1.2.1) (Pires & Martinho, 2019). Nevertheless, due to the increasing consumption of animal-products worldwide and the associated waste generation, prevention is not always possible in the livestock industry. In Jalisco, more than 80% of the livestock producers apply the manure directly to croplands as fertilizer, which leads to serious environmental impacts, such as the eutrophication of water bodies (SEMADET, 2021). While anaerobic digestion is valued due to its pollutant removal efficiencies and its potential to produce biogas, the effluents from anaerobic digesters, referred to as digestates or anaerobically digested livestock wastewater (ADLW), are often still rich in N, P and other nutrients (Xu et al., 2015a).

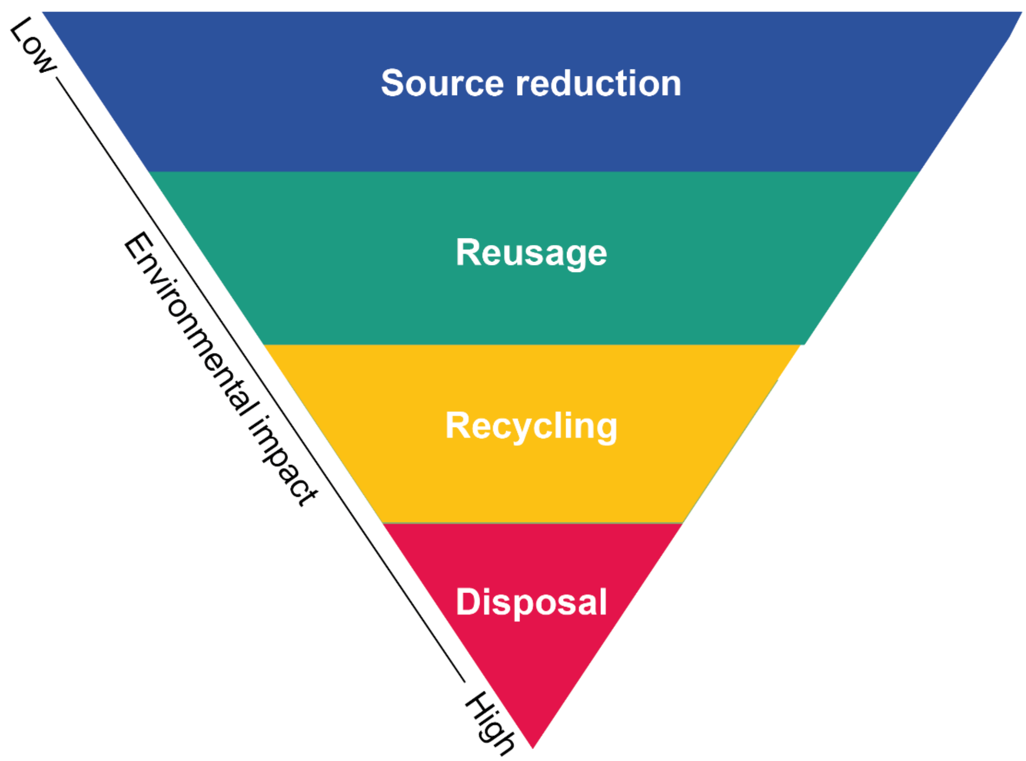


Fig. 1.2.1 Waste hierarchy principles

The livestock production sector in Jalisco follows a linear production model, which involves the subtraction of raw materials, their transformation and the disposal of waste (Fig. 1.2.2), recovering only a small fraction of the nutrients in croplands. Livestock manures possess primary nutrients, secondary nutrients and micronutrients necessary to improve agricultural production. However, poor management practices may lead to negative environmental impacts due to the runoff of nutrient surpluses that lead to the eutrophication of water bodies (Kumar et al., 2013).

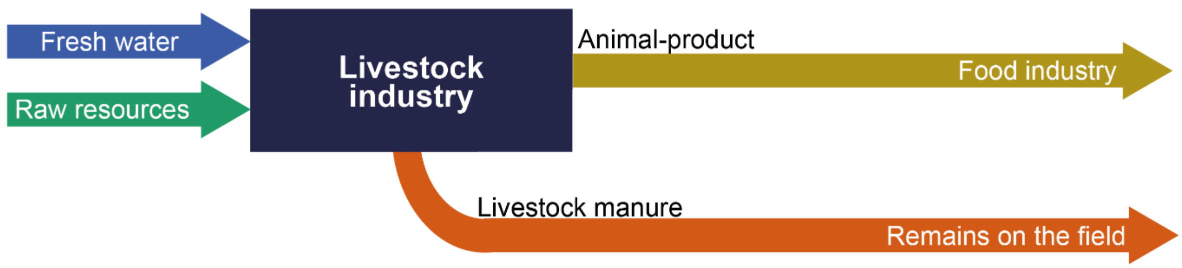


Fig. 1.2.2 Current linear livestock production model

MbWT has already been widely applied in the treatment of both LW and ADLW (Lv et al., 2018; Markou et al., 2016; Salama et al., 2017; Y. Wang et al., 2020). Through this CBE alternative, nutrients from the LW and ADLW are not only recovered but upgraded for the generation of high value-added products with different applications (Fig. 1.2.3). However, the existing studies have focused on the treatment of a single livestock wastewater source, even though most livestock production farms breed more than one animal species. Therefore, further research is needed to evaluate the MbWT using different livestock wastewater sources.

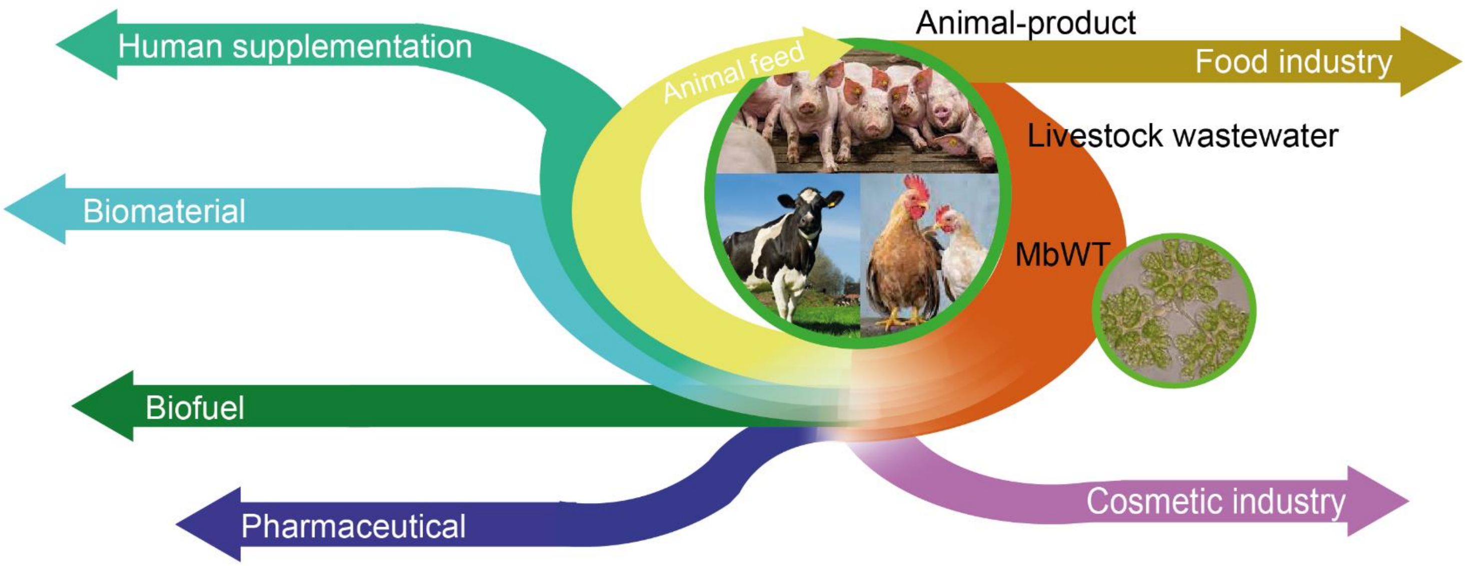


Fig. 1.2.3 Microalgae-based wastewater treatment applied to livestock sector from a circular bioeconomy approach.

1.3 Research Question

Considering the critical situation of the livestock industry in Jalisco and that MbWT offers great potential to help transition from the current linear livestock production model to a more circular approach, it is important to understand the principal internal and external factors that directly influence the overall performance of MbWT. Accordingly, several research questions are presented in this thesis:

How do the microbiological factors and physical and chemical parameters affect biomass productivity and pollutant removal efficiencies in MbWT? Which strategies could be followed to improve the overall performance of MbWT? Is it possible to find an optimal mixture of the three principal livestock wastewater sources (swine, poultry and cattle) through which microalgal biomass productivity and nutrient removal efficiencies could be optimized? Do microalgae perform better as mono-, bi- or tri-cultures in such optimal mixtures of livestock wastewater?

1.4 Solution overview and main contributions

A deeper understanding of the most significant factors that influence the overall performance of MbWT is required. Therefore, a systematic review of the literature was performed on the microbiological factors and the chemical and physical parameters that influence microalgal biomass productivity and nutrient removal rates in MbWT applied to the treatment of different types of LW (primarily and anaerobically digested swine, poultry and cattle wastewater). Additionally, the main strategies to improve MbWT is discussed herein.

An experimental study of MbWT using digestate mixtures of different animal species (swine, cattle, and poultry) was performed. A centroid mixture design was used to determine the optimal mixture to promote higher cell concentrations and pollutant removal efficiencies of the microalgae in monoculture and mixed cultures. A redundancy analysis was performed to analyze the correlation between microalgal cultures, the fractions of the ADLW types and the removal efficiencies of the digestate pollutants.

Followed by the experimental study was performed a characterization of the spatial variation of the LW generated in cattle, swine, and poultry farms in Jalisco, Mexico. Total nitrogen (TN), phosphorus (TP), and organic matter released from these production units were estimated, along with the associated GHG, considering the standard practice of uncontrolled release. An alternative management scenario using anaerobic digestion (AD) combined with microalgae-based wastewater treatment (MbWT) was evaluated by developing a software-based techno-economic analysis, showing that a centralized LW treatment system could represent a technical and economical feasible solution to comply the legislation while generating high-protein biomass for animal feed. Besides, the reduction in GHG represents an opportunity for carbon credits trading in voluntary markets for livestock producers in México.

1.5 Thesis Organization

Chapter one presents the motivation of this work, the problem statement, the research question, the solution overview and the main contributions.

Chapter two offers a review of the most significant factors that influence and enhance microbial growth and nutrient removal rates in MbWT applied to different types of LW (primary and anaerobically digested swine, poultry and cattle wastewater). This discussion covers the composition of different types of LW, its application in MbWT and the possible high value-added compounds obtained through this process, such as carotenoids and pigments, polysaccharides, proteins, lipids and metal nanoparticles. Additionally, microbiological factors are addressed, such as growth regimes of the microalgal species used in MbWT and the interactions within microbial populations (algae-bacteria and microalgae-microalgae interactions). Likewise, physical factors, such as temperature, light intensity and photoperiods, and chemical factors, like the C/N ratio, pH and the presence of inhibitory compounds, are reviewed. Moreover, strategies to enhance nutrient removal efficiencies and biomass productivity are discussed, such as acclimation, UV mutagenesis, effluent pretreatment and mixed effluents, immobilization of cultures and the implementation of multiple microalgal culture stages (including monocultures and mixed cultures). Finally, a circular bioeconomy approach is proposed, using a MbWT system for LW treatment coupled with the acquisition of high value-added compounds.

Chapter three contains an experimental study with an optimal mixture design testing different mixtures of all three types of LW and their effects on cell growth and pollutant removal efficiencies of microalgal cultures (*Chlorella vulgaris, Haematoccocus pluvialis* and *Chlamydomonas* spp.). Through an evaluation of the mixture design, the optimal fraction of these different types of effluents (ADCW, ADSW and ADPW) was determined, in order to enhance maximum microalgal biomass productivity and pollutant removal rates (COD, TN and TP). Furthermore, these microalgae were tested in all possible combinations of mono-, bi-, and tri-cultures. Prior to performing these experiments, gradual domestication and UV mutagenesis were applied to the microalgae to enhance their adaptability to the LW medium and, thus, cell growth and the removal of pollutants.

Chapter four delves into a research endeavor conducted in Jalisco, Mexico, aimed at delineating the spatial diversity of livestock waste (LW) generated across cattle, swine, and poultry farms. The investigation assesses the viability of an alternate management scenario involving anaerobic digestion in conjunction with microalgae-based wastewater treatment. The objective is to adhere to regulations, curtail greenhouse gas emissions, and yield protein-rich biomass for animal feed. Additionally, the paper underscores the obstacles necessitating resolution to foster more ecologically sound livestock production methodologies. The analysis provides insights into the hurdles of transitioning towards more sustainable livestock production techniques. The abatement of greenhouse gas emissions also presents an avenue for trading carbon credits in voluntary markets for Mexican livestock producers.

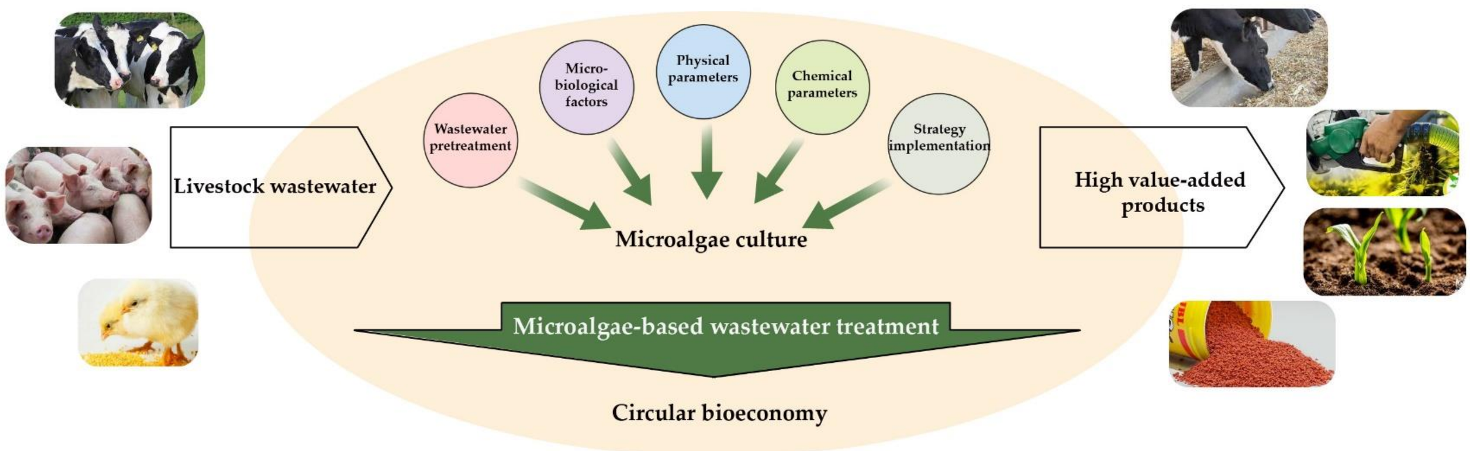
Chapter five presents the conclusions and perspectives of the present thesis. This includes the principal findings of this research in addition to future perspectives regarding the implementation of MbWT as a promising approach to mitigate the negative impacts related to the current inadequate waste management practices, from a circular bioeconomy perspective.

Complementary information about the initial and final concentrations measured in each run of the optimal mixture design is included in appendix A.

Chapter 2. Microalgae-based livestock wastewater treatment (MbWT) as a circular bioeconomy approach: enhancement of biomass productivity, pollutant removal and high-value compound production

Accepted manuscript in the Journal of Environmental Management.

Graphical abstract:



Abstract:

The intensive livestock activities that are carried out worldwide to feed the increasing population have led to significant environmental problems, such as soil degradation, as well as surface and groundwater pollution. Livestock wastewater (LW) contains high loads of organic matter, nitrogen (N) and phosphorus (P), which can promote cultural eutrophication of water bodies and poses environmental and human hazards. Therefore, humanity faces an enormous challenge not only to adequately treat LW but also to recover the valuable compounds contained in them, avoiding the overexploitation of natural resources through circular bioeconomy schemes. Circular bioeconomy aims to achieve sustainable production using biological resources as feedstock, such as LW, in innovative processes to produce biomaterials and bioenergy, while lowering the consumption of virgin resources. Microalgae-based wastewater treatment (MbWT) has recently received special attention due to its low energy demand, the robust capacity of microalgae to grow under different environmental conditions, and the possibility to transform wastewater pollutants into highly valuable bioactive compounds. Some of the high-value products that may be obtained through MbWT are biomass and pigments for human food and animal feed, nutraceuticals, biofuels, polyunsaturated fatty acids, carotenoids, phycobiliproteins and fertilizers. This review encompasses the recent advances on MbWT of LW (swine, cattle, and poultry wastewater). Additionally, the most significant factors affecting nutrient removal and biomass productivity in MbWT are addressed, including: (i) microbiological aspects, such as the microalgae strain used for MbWT and the interactions between microbial populations; (ii) physical parameters, such as temperature, light intensity, and photoperiods; and (iii) chemical parameters, such as the C/N ratio, pH and the presence of inhibitory compounds. Finally, different strategies to enhance nutrient removal and biomass productivity, such as acclimation, UV mutagenesis, multiple microalgae culture stages (including monocultures and multicultures) are discussed.

Keywords: Livestock wastewater; Microalgae; Microalgae-based wastewater treatment; Bioremediation; Circular bioeconomy

2.1 Introduction

Every year, 56 billion livestock animals are raised and slaughtered worldwide for human consumption, and this quantity is expected to double by 2050 to satisfy the needs of an increasing global population. Additionally, livestock needs large volumes of water for direct animal consumption, livestock barn maintenance (water services) and animal feed production. On average, 15,340 liters of water is required for the previously mentioned activities in order to produce about 200 kg of boneless beef (Hoekstra and Chapagain, 2007). Furthermore, 64% of the human population is expected to live in water-stressed basins by 2050, due to an increasing water demand (Ilea, 2009; Zhu and Hiltunen, 2016), and livestock manure is considered a key source of nutrients that significantly contributes to water pollution (Ilea, 2009), causing the eutrophication of water bodies (Díaz-Vázquez et al., 2020; Zhu et al., 2017).

Livestock wastewater (LW) may be defined as the mixture of effluents derived from livestock production, most commonly swine, cattle and poultry. The direct disposal of raw or partially treated LW in water sources is considered a human health and environmental hazard, because it frequently results in surface and groundwater pollution, pathogen dissemination and greenhouse gas (GHG) emissions (Chen et al., 2020; Tak et al., 2015). However, LW is increasingly gaining interest as a source of valuable materials and energy (van der Hoek et al., 2016). The recovery of the resources contained in LW, such as water, organic compounds and nutrients, is fundamental to promote a circular bioeconomy approach and to avoid the overexploitation of natural resources and the pollution of water sources (Hsien et al., 2019).

Several chemical, physical, biological and combined methods have been used for LW treatment (EPA, 1977). However, even though conventional LW treatment methods, such as coagulation/flocculation and precipitation, provide satisfactory levels of carbon, nitrogen and phosphorus removal, these treatments result in high energy consumption and nutrient loss; such technologies are complex and costly, and they require qualified management personnel and significant inputs of chemical reagents, making them economically non-viable (Bohutskyi et al., 2015; Crini and Lichtfouse, 2019; Pacheco et al., 2020; Sutherland and Ralph, 2019). Alternatively, biological treatment has also been utilized for the reuse and recycling of LW. Conventional biological treatment methods, such as aerobic, anaerobic and facultative lagoons, are relatively simple to operate. However, these processes are land-intensive, display low efficiencies in temperate regions and produce unpleasant odors. Conversely, microalgae-based wastewater treatment (MbWT) has gained significant interest (Agüera et al., 2020; Miller et al., 2011), as it is known to offer several advantages, such as atmospheric carbon fixation, removal of pollutants, like chemical oxygen demand (COD), total phosphorus (TP) and total nitrogen (TN), and an opportunity to develop valuable bioproducts from a circular bioeconomy perspective (Crini and Lichtfouse, 2019; Shahid et al., 2020). Additionally, MbWT can be achieved in a single stage (Beuckels et al., 2015) in contrast to physicochemical methods, which requires at least two stages (Oliveira et al., 2019).

Microalgae are photosynthetic microorganisms with high biotechnological importance due to their capability to grow in different environmental conditions, such as wastewater. LW has been regarded as a sustainable alternative to supply the nutrients that microalga requires for growth (Astroc et al., 2015; Lozano-Garcia et al., 2019). MbWT removes the nitrogen (N) and phosphorus (P) contained in LW with a low energy demand due to the microalgae's photosynthetic activity. Thus, it has shown a remarkable capacity to fix CO_2 from the atmosphere. Therefore, using the CO_2 by-product derived from industrial processes for the supplementation for microalgae cultures is an approach that has been highly investigated to reduce GHG emissions (Molinuevo-Salces et al., 2019). Furthermore, MbWT is a sustainable production process that results in highly valuable compounds of commercial interest, such as biomass and pigments for food and feed, nutraceuticals, fuels, polyunsaturated fatty acids, carotenoids, phycobiliproteins and fertilizers, among others (Arashiro et al., 2020; de Mendonça et al., 2018; Dineshkumar et al., 2018; Molino et al., 2018).

Microalgae breeding is considered a cost-effective technology for wastewater treatment (X. Li et al., 2020). Likewise, MbWT offers several advantages over conventional methods of treatment, such as lower water usage, reduction of CO_2 emissions, biosorption of toxic metals and the possibility to obtain biomass with high-value-added compounds (de Mendonça et al., 2018; Hammed et al., 2016). Additionally, the microalgal biomass recovered from the MbWT has the potential to be used in place of traditional animal feed protein sources and could also be used to extract different biological compounds, including lipids, peptides (amino acids), antioxidants and polysaccharides. Furthermore, the pigments extracted from this process have several applications in the industrial sectors, such as for food production, aquaculture, wastewater treatment, as well as the nutraceutical, pharmaceutical, cosmetic, and personal care industries (Acién Fernández et al., 2019; Singh et al., 2020).

While there are several studies that have implemented MbWT (Lv et al., 2018; Markou et al., 2016; Salama et al., 2017; Wang et al., 2015), information remains scarce on the specific biotic and abiotic parameters that influence nutrient removal in MbWT. This review aims to discuss the most significant factors that influence and enhance microbial growth and nutrient removal rates in MbWT applied to different types of LW (primary and anaerobically digested swine, poultry, and cattle wastewater) and considering a circular bioeconomy approach. This discussion covers the composition of different types of LW, its application in MbWT and the possible high value-added compounds obtained in this process, such as carotenoids and pigments, polysaccharides, proteins, lipids and metal nanoparticles. Also, microbiological factors are discussed, including growth regimes of the microalgal species used in MbWT, and its interaction with different microbial populations (algae-bacteria and microalgae-microalgae interactions). Additionally, physical factors, such as temperature, light intensity and photoperiods, and chemical factors, like the C/N ratio, pH and the presence of inhibitory compounds, are reviewed. Moreover, strategies to enhance nutrient removal efficiencies and biomass productivity are discussed, such as acclimation, UV mutagenesis, effluent pretreatment and mixed effluents, immobilization of cultures, and multiple microalgae culture stages (including monocultures and multicultures). Finally, a circular bioeconomy approach is proposed, using a MbWT system for LW coupled with the acquisition of high value-added compounds.

2.2 Wastewater composition and its suitability as a microalgae growth medium

LW is mainly composed of excrement, urine, feed residue and washing water (Hu et al., 2020). LW contains high concentrations of N, P and organic matter (expressed as chemical and biochemical oxygen demand, COD and BOD, respectively). However, the proportions between these pollutants in LW depends enormously on the animal species and age, the feed composition, housing methods, as well as environmental factors (Lv et al., 2018; Nagarajan et al., 2019). As shown in Table 2.2.1, the effluents from swine, poultry and cattle production display different concentrations of COD, TN, TP, NH_3-N and pH. Swine wastewater (SW) presents the highest COD concentrations, followed by the cattle wastewater (CW). As expected, the effluents resulting from the anaerobic digestion of LW contain lower COD, TN, and TP concentrations, but higher NH_3-N concentrations and pH values, in comparison to the raw effluents presented in Table 2.2.1. The NH_3-N concentrations observed in the raw LW tend to be lower compared to the anaerobically-digested livestock wastewater (ADLW), as a result of methanogenesis, by which this ion is produced (Kirchman, 2018). However, it is important to mention that a pH-dependent equilibrium between NH_4^+-N and NH_3-N is known to occur (Nagarajan et al., 2019). Therefore, in this review, NH_4^+-N and NH_3-N will simply be referred to as ammoniacal nitrogen (NH_3-N), without making a distinction between the two forms. The application of MbWT for the treatment of different types of LW (as shown in Table 2.2.1) is discussed in the following paragraphs.

Table 2.2.1 Typical values of water quality parameters found in the livestock wastewater types.

Type of livestock wastewater	**COD [mg/L]**	**TN [mg/L]**	**TP [mg/L]**	NH_3-N **[mg/L]**	**pH**	**References**
Swine	2,000.00-37,643.00	200.00-2,055.00	100.00-620.00	110.00-1650.00	7.97	(Cheng et al., 2019; López-Pacheco et al., 2019; Salama et al., 2017)
Poultry	320.00-4,000.00	49.00-80.00	7.60-55.00	122.7-150.00	8.7	(Carlini et al., 2015; Ferreira et al., 2018; Hülsen et al., 2018; Salama et al., 2017; T. Zheng et al., 2019)
Cattle	2,913-10,416.00	94.89-1,236.06	40.60-86.33	498.00-663.18	7.1-8.1	(de Mendonça et al., 2018; Lv et al., 2018)

Anaerobic digestate from swine	793.57-8,640.00	453.62-532.68	20.20-188.00	1,180.00-1,317.00	8.33-9.32	(P. Cheng et al., 2020a; Guo et al., 2017; Li et al., 2018; Mingzi Wang et al., 2016)
Anaerobic digestate from poultry	177.78-3,200.00	20.82-1,580.00	10.14-382.00	11.02-3,000.00	7.11-10.00	(Cai et al., 2013; Han et al., 2019; Li et al., 2017b; Moungmoon et al., 2020)
Anaerobic digestate from cattle	2,913.02	4.90-618.20	1.42-30.6	5.14-498.00	7.1-8.44	(de Mendonça et al., 2018; Köster et al., 2015; Walsh et al., 2018)

2.2.1 Swine wastewater

Pork is the second most consumed product in the meat industry. In 2018, the global pork meat production and the global pig population were approximately 118.8 million metric tons and 769,905 million, respectively (Tsai, 2018). Swine wastewater (SW) is a mixture of swine excrement and the water used for cleaning the pig sheds. The generation of wastewater per pig head is estimated at 1,300 ton per year, which is approximately 4-8 L of effluent per pig per day (García et al., 2017b; Zhang et al., 2017).

SW generally has a high concentration of NH_3-N, TP and COD in comparison with other types of raw LW. High NH_3-N concentration makes MbWT suitable for SW treatment due to the strong tolerance of microalgae to this compound (Wen et al., 2016).

Li et al. (2020) reported an operational MbWT system for SW treatment using a microalgae consortium that included *Microspora willeana Lagerh, Ulothrix ozonate, Rhizoclonium hieroglyphicum* and *Oedogonium* sp. This system was used to treat a loading rate of 0.4 L $m^{-2}d^{-1}$, and removal efficiencies of 98.0 and 76.0% were reported for TN and TP, respectively (X. Li et al., 2020). In a different study, the strain *Chlorella sorokiniana* reached a remarkable biomass concentration of 5.45 g L^{-1} when supplemented with 50% (v/v) untreated SW and displayed removal efficiencies of 90.1, 97.0 and 92.8% for COD, TN and TP, respectively (C.-Y. Chen et al., 2020). Besides *Chlorella* spp., some other microalgae strains, such as the *Desmodesmus* spp., *Parachlorella kessleri* and *Neochloris aquatica,* have been cultured in SW. These microalgae have displayed high tolerance to the ammoniacal nitrogen NH_3-N and pollutant removal rates higher than 80% for COD, TN and TP (Qu et al., 2020, 2019; Wang et al., 2017). Recently, Qu et al. (2020) reported a carbohydrate production rate of 944 mg L^{-1} d^{-1} by *Chlamydomonas* spp. and removal efficiencies of 81, 96 and 100% for COD, TN and TP, respectively, using non-sterilized and non-diluted SW as a medium. To accomplish these results, the authors focused on optimizing the culture light and temperature conditions and reached the highest biomass productivity and nutrient removal rates with a temperature and light intensity of 30°C and 500 µmol m^{-2} s^{-1}, respectively. According to the literature, the SW-cultivated microalgae that have shown the best performance in terms of biomass productivity are *A. platensis*, *Chlamydomonas* spp., *P. kessleri, C. sorokiniana, N. aquatica* and *C. vulgaris* with a maximum biomass concentration of 2.18, 9.90, 9.20, 8.08, 6.10 and 3.9 g L^{-1}, respectively (Table 2.5.1.2). Also, these microalgae achieved a maximum COD removal rate above 80% and TP and TN maximum removal rates above 90%. Taking this into consideration, to avoid prioritizing either the biomass productivity or the nutrient removal efficiency in a MbWT system, different strategies, such as mixed effluents, immobilized cultures, and co-cultures between different strains of microalgae or bacteria, among others, should be implemented. However, these topics are discussed in section 8.

2.2.1 Poultry wastewater

The global poultry meat production was 122 million metric tons in 2017, with a total of 68,785 million chicken heads. During this same year, poultry meat represented about 37% of global meat production, and it was projected to increase 24% over the next decade (FAO, 2020). Poultry wastewater (PW) is a mixture of feed residues, bedding material, feathers, broken eggs, manure and water used for cleaning. Manure, which is the main component of PW, is widely used to enrich and fertilize the soil and crops due to its N- and P-rich composition (Markou et al., 2016). The characteristics of PW depend largely on the species, age and health of the chickens, in addition to the practices carried out by the specific chicken production unit. It has been estimated that, for every 1,000 average chicken heads, 120 kg and 80 kg of wastewater are produced for laying hens and broiler chicken, respectively (Williams, 2013).

Anaerobic digestion has been implemented for PW. However, the low carbon: nitrogen (C: N) ratio and the high contents of organic nitrogen and ammonia (resulting from the uric acid present in PW) inhibit the anaerobic process (Bruni et al., 2013; Solovchenko et al., 2016). Therefore, MbWT has been considered a suitable biological alternative. Altunoz et al. (2017) cultivated

Neochloris oleabundans in PW under optimal culture conditions (pH = 7.3, light intensity = 120 µmol m^{-2} s^{-1}, temperature = 26°C and photoperiod of 8/16 h) and achieved significant microalgal growth rates ($8x10^6$ cell ml^{-1}), similar to those displayed when grown in commercial medium BG-11. The microalgae *Chlorella pyrenoidosa* was also reported to be efficient for PW treatment, achieving removal efficiencies of 84.2, 53.1 and 96% for TN, NH_3-N and TP, respectively (Mohan Singh et al., 2020). Solovchenko et al. (2016) used an artificial medium mimicking real PW to grow *Chlorella vulgaris* and, after three days, the microalgae removed 90% and 48% of the initial concentrations of TN and TP, respectively. Although several microalgal strains have been cultivated in PW, more research is needed to identify ideal strains that display optimal biomass growth rates, along with ideal breeding conditions to optimize nutrient removal in this type of effluent. The existing literature (Table 2.5.1.2) has shown that PW can be suitable for microalgae production. The microalgal bi-cultures *C.globosa–C.minutissima, Chlorella* sp.–*A. platensis* and *C. globosa*-*C.minutissima*-*S.bijuga* showed the highest biomass productivity found in the reviewed literature (Table 2.5.1.2), with a biomass concentration above 3 g $^{-1}$ (Bhatnagar et al., 2011; Wang et al., 2018). Regarding nutrient removal, from PW, only a few studies have reported the TN, TP, and COD removal efficiencies. *C. sorokiniana* is the strain that displayed the highest removal efficiency among the reported values in Table 2.5.1.2, achieving 95.8 and 84.5% of COD and TN removal, respectively in monoculture (Cui et al., 2020). L*eptolyngbya* *s*pp. and *Choricystis*-like chlorophyte (Trebouxiophyceae) in coculture, showed the highest removal efficiencies among the consortiums (Table 2.5.1.2), reporting values of 94%, 88.2% and 97.4% for COD, TN and TP, respectively (Patrinou et al., 2020).

2.2.2 Cattle wastewater

The cattle population worldwide was estimated at 1.625 billion heads in 2018 (Shen et al., 2018), and each head is estimated to produce 37 kg of wastewater per year. Cattle wastewater (CW) is a combination of excrement, urine and water used for cleaning the cattle stables (de Matos Nascimento et al., 2020). As for the application of MbWT to treat CW, Lv et al. (2018) evaluated the growth profile of five microalgae strains, *C. vulgaris, Chlorococcum* sp. GD, *P. kessleri* TY, *S. obliquus* and *S. quadricauda*, using undiluted CW. All of these strains grew without a lag phase and reached a concentration of 1.18, 1.21, 1.21, 0.93 and 1.03 g L^{-1} (starting from 0.1 g L^{-1}), respectively, after four days of cultivation. *C. vulgaris* showed the best removal efficiencies of COD, NH_3-N, NO_3- N and TP of 62.30, 81.16, 98.69

and 85.29%, respectively. Mendonça *et al*. (2018) studied the performance of *Scenedesmus obliquus* growing in vertical alveolar flat panel photobioreactors operated in batch and continuous mode for the treatment of CW. In continuous operation, the removal efficiencies and productivity displayed were lower than those displayed for the batch mode. After 12 days in batch mode, removal efficiencies of 65-70, 98-99 and 69-77.5% were achieved for COD, NH_4^+ and PO_4^{-3}, while in continuous operation, the removal efficiencies were 57-61, 94 -96 and 65-70%, respectively. The volumetric biomass productivities were 0.21-0.36 g L^{-1} d^{-1} and 0.13-0.18 g L^{-1} d^{-1} for batch and continuous mode, respectively. To our knowledge, only 5 microalgae strains have been tested in CW as monocultures (Table 2.5.1.2). From these strains, *C. vulgaris* has been the most studied and has displayed the highest removal efficiencies of COD, TN, and TP (92.17, 94.28 and 94.41%, respectively). In terms of biomass productivity, all 5 microalgal strains display similar biomass concentration (between 0.9 and 1.213 g L^{-1}). When comparing SW and PW (Table 2.5.1.2), the lowest biomass concentrations are reached when CW is used as the growing media. However, further studies that focus on this type of effluent as growing media for a wider variety of microalgal strains are needed to assess their performance and to study the potential of CW as an effective nutrient source for microalgal cultures.

2.2.4 Anaerobic digestate

Anaerobic digestion is frequently used to treat LW, with an additional advantage of biogas generation. However, the digestate resulting from this process is frequently still rich in N, P and other nutrients that may cause environmental problems if a secondary treatment is not applied before disposal (Xu et al., 2015). Compared to untreated LW, digestates generally have lower carbon concentrations because of the activity of the hydrolytic, acidogenic, acetogenic and methanogenic microorganisms in the anaerobic process (Cai et al., 2013; Chen et al., 2018). ADLW has been employed as agricultural fertilizer and animal feed (Raja et al., 2015) and as a nutrient source for microalgae breeding (Miao et al., 2016).

Chlorella vulgaris, Scenedesmus obliquus and *Spirulina platensis* have been reported to possess the highest growth rates and nutrient removal rates, among other microalgae, when cultured in anaerobically digested swine wastewater (ADSW) (Ayre et al., 2017; Kuo et al., 2015; Mingzi Wang et al., 2016; Xu et al., 2015). Also, *Dunaliella* spp. has been reported as a promising microalga to be used for ADSW treatment. This microalga has displayed satisfactory performance due to its ability to use NH_3-N as its sole nitrogen source. After 15 days of treatment, at 36 practical salinity units (psu) and using a light intensity of 200 μmol m^{-2} s^{-1}, the TN, TP and TOC removal efficiencies were of 63.8, 87.2 and 64.1%, respectively, and the biomass and β-carotene yields were 678 and 4.02 mg L^{-1}, respectively (Han et al., 2019).

MbWT has also been applied to treat ADLW using microalgal consortiums, which have shown an improvement in nutrient removal and biomass productivity in comparison to monocultures. Moungmoon et al. (2020) studied the growth of a consortium composed of *Leptolyngbya sp*. (30.4%), *Chlorella* sp. (16.1%) and *Chlamydomonas* sp. (52.2%), in undiluted anaerobically digested poultry wastewater (ADPW). The consortium showed total biomass productivity and removal efficiencies of NH_3-N and TP at 64.38 mg L^{-1} d^{-1}, 43.5 and 49.6%, respectively. The implementation of microalgae consortiums for MbWT is described further in section 5.

Compared with raw LW, ADLW has been more extensively explored using a MbWT approach (Table 2.5.1.3). To the best of our knowledge, a total of 16 microalgal strains have been studied in monoculture and 19 microalgal cocultures have been used for MbWT in ADSW, ADPW and anaerobically-digested cattle wastewater (ADCW. In monoculture, *Botryococcus braunii* reached an noteworthy biomass concentration of 8.5 g L^{-1} when cultured in ADSW; however, the removal efficiency of TN was 43% (An et al., 2003). In comparison, *A. platensis, C. vulgaris, Coelastrella* sp., *Desmodesmus* sp., *N. oculata, Scenedesmus* sp. and *S. obliquus* displayed biomass concentrations below 7 g L^{-1}, but the removal efficiencies were above 80% in at least two of the evaluated parameters (COD, TN and TP).

2.3 Microalgae species and their valuable products using livestock wastewater

Microalgae may be prokaryotic or eukaryotic photosynthetic microorganisms. As shown in Fig. 2.3.1, the eukaryotic domain is divided into 9 phyla, of which the phylum Chlorophyta has been the most extensively studied for wastewater treatment. These microorganisms are found in almost all the Earth's habitats and are widely recognized for their rapid growth rates and high lipid productivity when compared with field agricultural crops (Lu et al., 2020a).

DOMAIN	KINGDOM	PHYLUM	CLASS
Prokaryota	Eubacteria	Cyanophyta	Cyanophyceae Gloeobacterophyceae
Eukaryota	Protozoa	Euglenozoa	Euglenophyceae
	Chromista	Cryptista	Cryptophyceae
		Haptohyta	Coccolithophyceae Pavlovophycae
		Miozoa	Apicomonadea Dinophyceae
		Ochrophyta	Bacillariophyceae Bolidiophyceae Chrysophyceae Eustigmatophyceae Dictyochophyceae Pinguiophyceae Raphidophyceae Schizocladiophyceae Phaeophyceae Xantophyceae
	Plantae	Cercozoa	Chlorachnea
		Glaucophyta	Claucophyceae Cyanidiophyceae
		Chlorophyta	Chlorodendrophyceae Chlorphyceae Pedinohyceae Tebouxiophyceae

Fig. 2.3.1 Microalgae taxonomic classification. Adapted from Borowitzka et al. (2018)

The biochemical composition of microalgae depends on the species, light, temperature, and growth stage/conditions. Nonetheless, their proportions can be altered by varying the culture environment, such as by placing them in nitrogen starvation conditions, which promotes lipid accumulation, or by increasing the temperature or light intensity, which induces carotenoid synthesis, among others. In general, the most abundant organic microalgal macromolecule is protein, followed by carbohydrate (and fiber) or lipids, depending on the growth culture conditions (Matos, 2017; Matos et al., 2016). If the production of high added-value compounds is an objective for MbWT, then the microalgae strain should be chosen depending on the bioactive compound of interest. The principal molecules synthesized by different microalgae phyla as well as their applications are shown in Fig. 2.3.2.

Bioactive compounds / Phylum (Species)	Chlorophyta 6,714	Rhodophyta 7,263	Cyanobacteria 4,722	Glaucophyta 25	Cryptista 220	Haptophyta 764	Heterokontotphyta 15,821	Possible applications
Phycobiliproteins		●	●	●	●			A,B,C,E
Carotenoids	●							A,B,C,D,E
Chlorphylls	●							A,B,C,E
Polysaccharides	●	●	●					B,E,F,H,J
Proteins	●		●					C,D,E,F,G,H,I
Sterols	●					●	●	C,E,H,J,K
Vitamins	●					●	●	E
Polyphenols	●		●				●	C,E,H,I
PUFAs					●	●	●	C,H,J,K

A) Dyeing in food, pharmaceutic or cosmetic industries; B) Immune stimulant in pharmaceutical or cosmetic industries; C) Anti-inflammatory pharmaceutical or cosmetic industries; D) Photoprotective in pharmaceutical or cosmetic industries; E) Antoxidant in nutraceutics, pharmaceutical or cosmetic industries; F) Rheological agent in food, pharmaceutical or cosmetic industries; G) Collagen stimulant in cosmetic industry; H) Anti-chronic diseases in pharmaceutical industry; I) Antimicrobial/viral in pharmaceutical or cosmetic industries; J) Anti-lipidemic/ glucemic cachetic; K) Anti-degenerative in pharmaceutical industry

Fig. 2.3.2 Main bioactive compounds synthesized by microalgae and their biotechnological applications. Adapted from Levasseur et al. (2020)

2.4 High value-added products

2.4.1 Carotenoids and pigments

High amounts of carotenoids, such as astaxanthin, β-carotene, canthaxanthin and phycobiliproteins, are synthesized by microalgae. A carotenoid biomass composition range between 0.69% and 14% has been reported depending on the microalgal species (Koyande et al., 2019; Matos, 2017). These carotenoids are used to produce natural colorants because of their non-allergic composition (as opposed to synthetic colorants), as well as to produce nutrient supplements and functional foods for human and animal nutrition due to their antioxidant activity (Khoo et al., 2019b; Sathasivam et al., 2019; Zanella and Vianello, 2020). The synthesis of carotenoids is a typical response of microalgae to different abiotic stresses, including light intensity, salinity, or nutrient starvation. For example, *Chlorella* and *Scenedesmus* strains were cultivated in diluted ADLW and were found to increase the biogenesis of carotenoids by phosphate and sulfate limitation (Zuliani et al., 2016). Likewise, *Dunaliella* FACHB-558 was cultured using a two-stage process to maximize the production of β-carotene accumulation. At the first stage, this strain was cultivated in ADPW to maximize the production of biomass, while at the second it was cultivated in a modified BG-11 medium to optimize the production of β-carotene. During the ADPW cultivation process, nutrients were used and transformed into microalgal biomass, leading to an increase in β-carotene production yield of 42.4% in the second stage when compared to the first step. The accumulation of β-carotene was mainly attributed to salinity levels (Han et al., 2019).

Astaxanthin is a ketocarotenoid that is well known for its “super antioxidant” activity, stronger than that of other carotenoids, such as β-carotene, lutein or canthaxanthin (Khoo et al., 2019b). Astaxanthin is also known for its capacity to inhibit lipid peroxidation and sequester free radicals, and it has anti-inflammatory and anti-apoptotic properties (Gwaltney-Brant, 2016; Kamalanathan and Quigg, 2019). The main microalgal producers of astaxanthin are *H. pluvialis, C. zofingiensis, C. nivalis, B. braunii, C. vulgaris, C. striolata, Monoraphidium* sp.*, Chlamydocapsa sp., Neospongiococcum sp., Chlorococcum sp.* and *S. obliqus* (Khoo et al., 2019a). *H. pluvialis* is the richest microalgal source of astaxanthin and can contain up to 5% of this pigment in dry weight. This microalgal strain has been successfully cultured in SW and primary-treated SW. The primary treatment consists of a physicochemical or chemical process that takes place after a pre-treatment, which in this case included a membrane bioreactor with a four-stage Bardenpho system and an intermediary clarifier (pre-anoxic, oxic, clarifier, post-anoxic, oxic, ultrafiltration) (Crini and Lichtfouse, 2019; Shah, 2019). When grown in SW, this microalga displayed a biomass production of 1.31 g L^{-1} and removal efficiencies of 99% and 98% for TN and TP, respectively. (The total astaxanthin production was not reported). When grown in primary-treated SW, it displayed a biomass production of 1.43 g L^{-1}, 100% removal of TN and TP and an astaxanthin production of 83.9 mg L^{-1} (Shah, 2019).

β-carotene is the precursor of retinol (vitamin A), an essential vitamin for pregnant women and children. This compound offers protection to the cornea, has anti-aging and anti-cancer properties, modulates the immune system and helps to prevent cardiovascular diseases (Barkia et al., 2019). This vitamin is used in animal feed additives and as a colorant in food industries (Hu, 2019). The major microalgae species that produce β-carotene are *D. salina, D. tertiolecta, D. bardawil, B. braunii, C. nivalis, C. acidophila, Chlorococcum* sp., *Chlamydocapsa* sp., *Tetraselmis* sp., *C. sorokiniana, P. obovate* and *C. striolata* (Sathasivam et al., 2019). *D. salina* is known to produce the highest amount of β-carotene and can account for as much as 10% of the cell weight (Kalra et al., 2020). When cultivating *D. salina* in ADPW, previously treated by ultrafiltration, removal efficiencies of TN, TP and TOC of 63.8, 87.2 and 64.1%, respectively, were reached, in addition to a biomass production of 0.678 g L^{-1} and a β-carotene yield of 4.02 mg L^{-1} (Han et al., 2019).

2.4.2 Polysaccharides

Polymers include distinct types of polysaccharides, such as cell wall polysaccharides and extracellular polymers (EPS). The former play a crucial role in microalgal structure and resistance, and the latter can be found solubilized in the medium or surrounding the microalgal cell. The microalgal production of extracellular polymers occurs through a light-dependent mechanism that is enhanced by high-intensity and continuous light conditions. Besides acting as auto-flocculate agents, EPS display antibacterial, antifungal, antioxidant, anticancer, immunomodulatory, anti-inflammatory, anticoagulant, antitussive, antiglycemic, antilipidemic and antiaging activities (Prybylski et al., 2020). Despite the many potential applications of EPS synthesized by microalgae, few studies have actually been carried out to characterize the EPS production using LW as a growing medium for microalgae.

One of the major challenges in MbWT is the low recovery efficiencies of the microalgae biomass due to its intrinsic characteristics, such as small size, negative charged surface and similar density to water (Cheng et al., 2020a). The production of EPS confers the capacity to create multicellular structures, which increases the harvesting efficiency due the formation of cell aggregations in the medium that results in self-flocculation (Bernaerts et al., 2018). *C. vulgaris* JSC-7, *S. obliquus* AS-6-1, *Ankistrodesmus falcatus* SAG202-9, *Ettlia texensis* SAG79.80 and *B. braunii* have been reported as self-flocculating microalgae (Ummalyma et al., 2017). In a study performed by Cheng et al. (2020a), four microalgae, *C. zofingiensis, B. braunii, Synechocystis* sp., *Tribonema* sp., were initially tested for their capacity to self-flocculate and the latter two were then further examined due to their high recovery efficiencies (74.3% and 78.2%, respectively) in comparison to *C. zofingiensis* (72.6%) and *B. braunii* (39.5%). This result was attributed to the self-flocculating capabilities of *Synechocystis* sp. and *Tribonema* sp. In another part of this study, the authors evaluated the pollutant removal efficiency of *Synechocystis* sp. and *Tribonema* sp. cultured in SW as a nutrient source. After 5 days of cultivation, *Tribonema* sp. reached removal efficiencies of 89.9%, 55.6% and 72.7% for NH_3-N, COD and TP, respectively, while *Synechosystis* sp. displayed removal efficiencies of 75.8%, 68.6% and 71.4% of NH_3-N, COD and TP, respectively.

Another interesting polymer synthesized by microalgae is poly-β-hydroxybutyrate (PHB). This compound is a promising material to produce biodegradable plastic due to its similarities with synthetic plastics, such as polyethylene and polypropylene (Koutra et al., 2018). *Nostoc muscorum, S. platensis, Aulosira fertilissima* and *Synechocystis* sp. have been reported to accumulate PHB under mixotrophic growth and nutrient-limited conditions (Koutra et al., 2018). Kovalcik et al. (2017) evaluated the growth of *Synechocystis salina* in digestate from the anaerobic digestion process of a biogas production plant as a low-cost media for microalgal growth. After 21 days of cultivation, *Synechocystis salina* was reported to reach a 6.6% PHB content in dry weight. The thermal and rheological properties of the PHB synthesized by *S. salina* were tested and reported to be like those showed by commercial PHB. However, further research is needed to increase thermal stability before polymer processing, and innovative strategies are warranted to increase the recovery rate (Kovalcik et al., 2017).

2.4.3 Proteins

A well-known characteristic of microalgae is their high protein content, which varies between 6 and 71% of the dry matter composition depending on the microalgal species (Koyande et al., 2019). Microalgal strains produce glycoproteins, phycoerythrins and mycrosporine-like amino acids (Amador-Castro et al., 2020; Fuentes-Tristan et al., 2019). The latter are widely used in the personal care industry (Orfanoudaki et al., 2019; Sathasivam et al., 2019). Additionally, microalgae synthesize several enzymes with industrial applications, such as phytases, α-galactosidase, protease, laccases, lipase, cellulases, carbonic anhydrase, amylolytic enzymes and antioxidant enzymes (Brasil et al., 2017).

The cultivation of protein-rich microalga in LW is a promising option to produce animal feed sources. As seen in Table 2.4.3.1, the protein content of different species of microalgae cultivated in LW has been evaluated. Protein contents above 50% in dry weight has been reached by *A. platensis* growing on different types of LW (Li et al., 2017b; Rui et al., 2017). In contrast, *C. minutissima, C. sorokiniana* and *S. bijuga* have been reported to reach protein contents between 37.1% and 40.1% in dry weight (Singh et al., 2011).

Koutra et al. (2018) performed two experiments using ADSW as a nutrient source for microalgae. Two local microalgal strains, UMN 231 (Lake Johanna, west side) and UMN 271 (Loon Lake, Waseca), were grown and reached a protein content of 46% and 40% of total biomass, respectively, while *Chlorella* (PY-ZU1 strain) reached a protein content of 46% of the total biomass (Koutra et al., 2018). These experiments proved that protein-rich biomass cultivated in digestates can be comparable to traditional protein sources for animal feed (de Medeiros et al., 2021). However, achieving a high protein content in microalgae biomass during MbWT may be a challenge due the heterogeneous compositions of the different LW. Some trace elements present in these effluents can inhibit the microalgal protein synthesis. Li et al. (2018) cultured *Coelastrella* sp. in ADSW with different Cu (II) concentrations and demonstrated a high sensitivity of this microalga to this element. In the absence of Cu (II), the microalgal protein content reached was 70.2%, and only 29.7% when the concentration of Cu (II) was above 0.10 mg L^{-1}. Additionally, the NH_3-N removal efficiency decreased from 80% (in the absence of Cu (II)) to 38.6% (with a concentration of 3.0 mg L^{-1}) (Li et al., 2018).

Table 2.4.3.1 Protein production and nutrient removal efficiencies by different microalgal species during Microalgae-based Wastewater Treatment (MbWT)

Species	Wastewater source	Biomass production or productivity	Protein content or productivity	Nutrient removal	Reference
Arthrospira platensis	ADSW	4.7 g m^{-2} day^{-1}	59.1% DW	Nitrates: 61.2% TP: 68.1%	(Li et al., 2017b)
		45.2 - 64.7 g m^{-2} day^{-1}	> 50.0% DW	TN: 80-93% TP: 84-98%	(Rui et al., 2017)
Chlorella minutissima	ADPW	25.0 - 75.0 mg L^{-1} day^{-1}	38.7 – 40.9% DW	TN: 46-79 mg L^{-1} TP: 1.6 – 5 mg L^{-1}	(Singh et al., 2011)
Chlorella sorokiniana	ADPW	0.066 g L^{-1} day^{-1}	37.1 – 39.6% DW	NR*	(Singh et al., 2011)
Consortium of *Chlorella* sp. and	ADSW	2.20 g m^{-2} day^{-1}	39.2% DW	COD: 5.83 g m^{-2} d^{-1}	(Moheimani et al., 2018)

Scenedesmus sp.				NH_3-N: 1.97 g m^{-2} d^{-1}	
Desmodesmus sp.	SW	0.88 g L^{-1}	0.51 g L^{-1}	NH_3-N: 78.5% TP: 91.7%	(HaiXiang et al., 2017)
Consortium of *Chlorella* sp. and *Scenedesmus* sp.	ADSW	0.47 g L^{-1}	0.194 g L^{-1} Day $^{-1}$	NH_3-N: 0.2 g L^{-1} d^{-1} TN: 0.12 – 0.19 g L^{-1} d^{-1} TP: 6 – 11 g L^{-1} d^{-1}	(Luo et al., 2019)
Scenedesmus bijuga	ADPW	0.031- 0.076 g L^{-1} day^{-1}	37.2 – 40.6% DW	TN: 44 – 71 mg L^{-1} TP: 1.2 – 4.9 mg L^{-1}	(Singh et al., 2011)

* NR: Not reported

Phycobiliproteins are another valuable compound produced by microalgae. These are highly fluorescent protein-pigments used as labeling reagents in flow cytometry, fluorescence immunoassays, immunohistochemistry and other biomedical science activities (Brasil et al., 2017; Chew et al., 2019; Manirafasha et al., 2016). *Spirulina platensis* has been regarded as an excellent source of phycobiliproteins and is particularly rich in c-phycocyanin, which is a blue pigment (Pan-utai and Iamtham, 2019). These compounds have been commercialized as natural colorants in the food industry and nutraceutical products (Hu, 2019). Narindri Rara Winayu et al. (2021) examined the production of phycobiliproteins, specifically c-phycocyanin, phycoerythrin and allophycocyanin, growing the cyanobacterium *Thermocynechococcus* sp. in SW with two different pretreatments, an anoxic treatment (SW A) and a combination of anoxic and aerobic treatments (SW B). When the cyanobacterium was grown on the SW A conditions, c-phycocyanin was the dominant phycobiliprotein type, reaching 13% content in dry weight. In contrast, when the cyanobacterium was grown on the SW B conditions, allophycocyanin was dominant, also reaching 13% in dry weight (Narindri Rara Winayu et al., 2021). A deeper understanding of the influences of the medium composition and the microalgal species employed for the protein synthesis is crucial to reach the objectives of the MbWT.

2.4.4 Lipids

Microalgae contain high concentrations of non-polar lipids, polar lipids and structural lipids, saturated fatty acids (SFAs), poly-unsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs), glycolipids or phytosterols and waxes (Santos-Sánchez et al., 2016). Lipid concentrations have been reported between 2 and 40% of the biomass composition, although these values depend on the microalgal species, and under certain conditions, may be as high as 85% in dry weight (Koyande et al., 2019; Matos, 2017). PUFAs are of high nutritional interest for humans due to their antioxidant, antibacterial, antiviral, and detoxifying capacities, which help prevent hypercholesterolemia, improve brain function, and stimulate the immune system (de Morais et al., 2015; Roy and Pal, 2015). One factor that influences the microalgal lipid production is the microalgal biomass concentration in the medium. Low biomass concentration facilitates light uptake per microalgal cell, which triggers lipid storage and nutrient removal from the medium (Mandotra et al., 2016; Zhu et al., 2017). Therefore, microalgae with low initial biomass concentration tend to accumulate more lipids.

The production of microalgal lipids is classified as a third generation ‘feedstock’ of high interest due to its potential use to produce renewable energy like biofuels. Microalgal lipid productivity per unit of dry mass is 15-300 times higher than oil-bearing crops like corn, sunflower, soybean, and palm (S. Y. Lee et al., 2020; Poh et al., 2020)

2.4.5 Lipids in the context of biofuels

First-generation biofuels are obtained from oilseeds and food crops, and the process only involves a simple pressing of oil-bearing biomass (Correa et al., 2017). Nevertheless, the escalation of biofuel production from these raw materials has been associated with food insecurity, water scarcity, soil degradation, deforestation and biodiversity loss (Correa et al., 2017; Elshout et al., 2019). Second-generation biofuels are obtained from residual plant tissues or agricultural residues, wood residual wastage and, to a lesser extent, from used cooking oils, restaurant grease, animal fats, beef tallow and pork lard (Ben Hassen Trabelsi et al., 2018; Bryngemark, 2019). However, second-generation fuels have some disadvantages, such as variation in feedstock’s composition, increase in viscosity due to high loads of substrate, cost of enzymes involved in enzymatic hydrolysis, high content of saturated fatty acids derived from animal fats and low performance in cold temperatures due to an increase in the oil’s viscosity, which results in coking and trumpet formation on the engine injectors after long-term use, as well as carbon deposits, thickening and gelling of the lubricant and oil ring sticking (Alalwan et al., 2019; Binod et al., 2019; Mahmudul et al., 2017). Therefore, there is still a need for the development of more efficient technologies to make the implementation of second-generation biofuels feasible and economically competitive. Alternatively, microorganisms are regarded as the third-generation 'feedstocks' for biofuel production (Alalwan et al., 2019). Microalgae species are the most promising microbial oil source for biofuel production due to their high content of lipids and, in contrast to second-generation fuels, they possess a lower requirement of water and land to grow under specific conditions. However, currently, the cost of algae-based biofuel is higher than fuel generated from other sources due the lack of efficient large-scale technologies (Shah et al., 2018).

MbWT can contribute to overcoming our dependence on fossil fuels and to mitigate environmental pollution by generating energy from LW and sunlight (Jaiswal et al., 2020). The high microalgal biomass, starch and lipid production rates make MbWT ideal for biodiesel production. Moreover, other strategies have been applied to recover and produce biofuels from microalgae, such as biogas, bio-hydrogen, biodiesel, bioethanol and other value-added products (Javed et al., 2019; Khalid et al., 2018; Maaz et al., 2019). *Chlorella, Botryococcus, Scenedesmus, Dunaliella, Neochloris* and *Nannochloris* are highly recognized for their capacity to remove nutrients from LW and for their lipid accumulation capacity, especially the latter two, which can accumulate up to 50% lipid of their total dry weight biomass under nitrogen starvation conditions (Kadir et al., 2018). The lipid production of *Chlorella vulgaris* has been reported to increase by 300% when cultured in ADLW rather than digestate derived from municipal wastewater (MW) (Zuliani et al., 2016).

However, the production of microalgae-based biofuels still faces two main challenges: (1) the improvement of biomass and lipid productivity and quality, and (2) the development of cost-effective downstream technologies (S. Y. Lee et al., 2020). Some recent studies have focused on promoting a better fatty acid composition to improve biodiesel stability (reducing the SFAs and PUFAs content and increasing the MUFAs content) by changing cultivation factors and parameters such as medium composition (G. Li et al., 2020; Sonkar and Mallick, 2018), temperature (Chaisutyakorn et al., 2018), pH, CO_2 addition (Qiu et al., 2017) and stress conditions (Kwak et al., 2016).

2.4.6 Metal nanoparticles

Metallic nanoparticles (MNPs) have multiple applications in cosmetics, food packaging, textiles and technology industries (Arévalo-Gallegos et al., 2018). These compounds are currently mainly synthesized by physical methods, which are energy intensive and time consuming, and chemical methods, which generate large volumes of environmentally hazardous wastewater (Iravani et al., 2014). Considering these limitations, biological synthesis has gained attention as an environmentally sustainable alternative with lower costs and nontoxicity (Arya et al., 2018; Salem and Fouda, 2021).

The biosynthesis of MNPs through microalgae breeding has recently gained wide interest. *Chlorella pyrenoidosa, Botryococcus braunii* and *Gelidium amansi* have been tested for the synthesis of silver MNPs (Arévalo-Gallegos et al., 2018; Kusumaningrum et al., 2018; Pugazhendhi et al., 2018). *Galdiera* sp. has been tested for the synthesis of silver, iron and zinc MNPs (Çalışkan et al., 2020), *Chlorella kessleri, Dunaliella tertiolecta* and *Tetraselmis suecica* have been tested for the synthesis of copper MNPs (Salas-Herrera et al., 2019), and *B. braunii* has also been used to synthesize palladium, platinum, copper or zinc MNPs (Arya et al., 2020, 2018). However, to the best of our knowledge, Subramaniyam et al. (2016) performed the first experiment using a MbWT approach to synthesize iron MNPs. These authors performed the study in two stages. Firstly, the strain *Chlorella* sp. MM3 was cultured in brewery wastewater, and then, the biomass was harvested and incubated with a solution of $FeCl_3$ to produce the iron MNPs. After four days of cultivation, TN, TP and TOC were completely removed from the medium and the synthetized iron MNPs were found to display similar chemical and morphological characteristics compared to iron MNPs obtained using synthetic medium (Subramaniyam et al., 2016). In this sense, using LW could mean a promising low-cost and eco-friendly solution to synthetize MNPs.

To enhance the economic feasibility of MbWT, it is crucial to optimize operational strategies to maximize the rates of microalgal growth, the removal of pollutants and the accumulation of valuable compounds in SW. Previous studies have demonstrated that microalgal growth and cellular composition can be significantly affected by various culture parameters (e.g., temperature, light intensity) (Jiang et al., 2018; Qu et al., 2020; Wu et al., 2017). Different microbiological, physical, and chemical factors and strategies to optimize the nutrient removal and production of high value-added products through MbWT are discussed in the following sections.

2.5 Effects of microbiological factors on the microalgal performance in wastewater treatment

2.5.1 Microalgal species and growth regimes used for MbWT

One of the most important factors to consider for the design of a MbWT process is the selection of the microalgal species or consortium, even more than the culture conditions (Lv et al., 2018). The control of the microalgal population can be challenging in MbWT due to potential contamination from other microbial communities. The presence of other microbial populations, such as bacteria and protozoa, generates competition to the microalgal population for the available nutrients in LW (Dahmani et al., 2016). Thus, the identification of robust and fast-growing microalgae, capable of outgrowing competitors, is a research priority (Khalid et al., 2018).

It is estimated that 72,500 microalgal species exist, but only about 44,000 have been widely described (Chu, 2017). Microalgal species vary enormously in their characteristics, such as their nutrient removal efficiency, their tolerance to toxic compounds, adaptability and cell composition (Lu et al., 2020a). As shown in Figure 2.5.1.1, there are three categories of microalgal growth, depending on their carbon source: (1) Autotrophic, (photosynthetic growth), (2) Heterotrophic, through the consumption of organic and inorganic carbon sources contained in the medium and (3) Mixotrophic, which involves different metabolic pathways (both autotrophic and heterotrophic) at different points in time, depending on the medium conditions (Hammed et al., 2016; Zhan et al., 2017). In mixotrophic growth, photosynthetic activity is promoted in the presence of high CO_2 concentrations, while the utilization of organic carbon sources is diminished. On the hand, in the absence of light during dark cycles, the utilization of the carbon sources present in the medium is promoted, which is a fundamental characteristic to achieve efficient COD removal from the LW (Hammed et al., 2016). Mixotrophic cultivation is desirable because the sunlight and organic sources are not limiting factors. Compared to autotrophic and heterotrophic cultivation, mixotrophic cultivation can allow for higher biomass production because the exponential phase is prolonged and the photooxidative damage by the accumulated O_2 is prevented (Patel et al., 2020). These advantages maximize the possibility to obtain specific compounds synthesized by microalgae and to promote pollutant removal, which are the main objectives of MbWT.

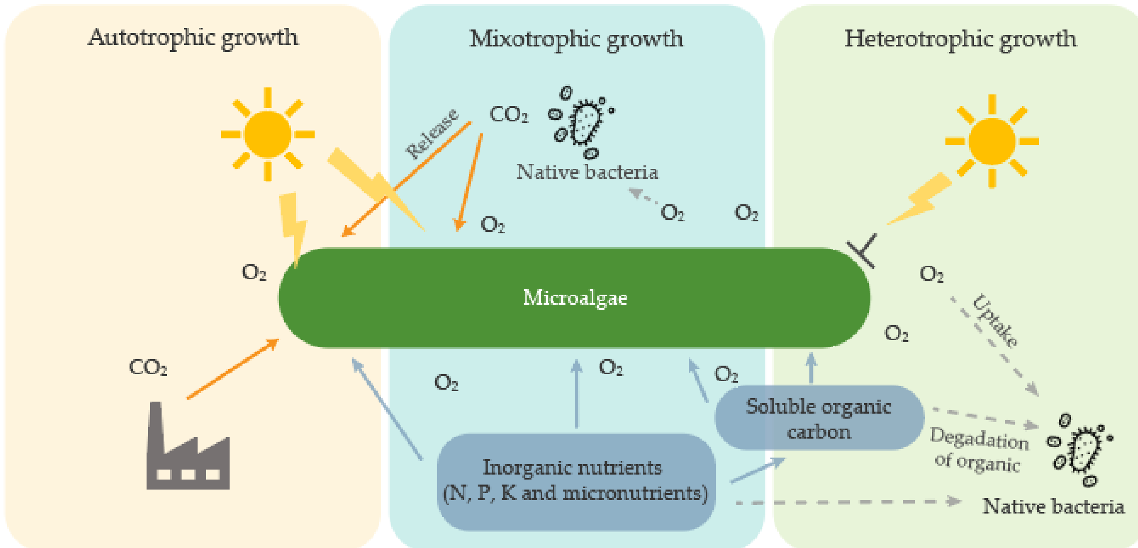


Fig. 2.5.1.1 Microalgal growth regimes under different environmental conditions. Adapted from Hammed et al. (2016)

The microalgal genera that are known to exhibit a mixotrophic capacity are shown in Table 2.5.1.1. This capacity makes them ideal for MbWT of LW. Among all these genera, the specific species that have shown better biomass production and organic carbon uptake are *C. vulgaris, C. regularis, S. platensis, H. pluvialis* and *Euglena gracilis* (Patel et al., 2020).

Table 2.5.1.1 Microalgae genera suitable for livestock wastewater treatment due their mixotrophic capacity

Genera	Reference	Genera	Reference
Chlorella	(Lee et al., 2017; Zanette et al., 2019)	*Tetraselmis*	(Patel et al., 2020)
Chlamydomona	(Lee et al., 2017; Zhan et al., 2017)	*Synechosystis*	(Patel et al., 2020)
Nannochloropsis	(Lee et al., 2017; Zanette et al., 2019; Zhan et al., 2017)	*Pseudochlorococcum*	(Patel et al., 2020)
Haematococcus	(Pang et al., 2019)	*Planothidium*	(Patel et al., 2020)
Scenedesmus	(Pang et al., 2019; Zanette et al., 2019)	*Eskeletonema*	(Patel et al., 2020)

Nannochloris	(Pang et al., 2019)	*Microcystis*	(Patel et al., 2020)
Arthrospira	(Pang et al., 2019; Zanette et al., 2019)	*Selenastrum*	(Patel et al., 2020)
Tetradesmus	(Pang et al., 2019; Zanette et al., 2019)	*Anabaena*	(Patel et al., 2020)
Desmodesmus	(Pang et al., 2019)	*Nitzschia*	(Patel et al., 2020)
Acutodesmus	(Lacroux et al., 2020)	*Oscillatoria*	(Patel et al., 2020)
Dunaliella	(Zanette et al., 2019)	*Prototheca*	(Patel et al., 2020)
Neochloris	(Zanette et al., 2019)	*Pleurochrysis*	(Patel et al., 2020)
Nostoc	(Patel et al., 2020)	*Euglena*	(Patel et al., 2020)

Besides the microalgal growth mechanism, another important aspect to consider for the selection of the microalgal population or consortium to be employed for MbWT is their tolerance to high concentrations of NH_3-N. As previously mentioned, LW contains high concentration of this ion, which may vary from 5.14 to 3,000.00 mg L^{-1} (Table 2.2.1), depending on the type of LW (type of animal) and if the effluent has been pretreated or not. Microalgae from the Chlorophyceae class have shown tolerance to NH_3-N concentrations as high as 1,600.00 and 702.00 mg L^{-1}, respectively. An optimal concentration of 136.80 mg L^{-1} of NH_3-N has demonstrated to have a positive effect on the biomass productivity of these microalgae (Ayre et al., 2017; Nagarajan et al., 2019). The *Chlorella* sp., *Chlamydomona* sp. and *Dunaliella* sp. genera, from the Chlorophyta phylum, have gained particular attention because of their ability to grow in LW with NH_3-N concentrations above 1,600 mg L^{-1} and to reduce up to 63.7% of the NH_3-N, while reaching a biomass productivity of 17.4 mg L^{-1} d^{-1} (Ayre et al., 2017; Khalid et al., 2018; Qu et al., 2020).

The Cyanophyta phylum has been well-studied for the MbWT of LW. However, it has been reported to display a lower tolerance to NH_3-N concentrations in comparison to the Chlorophyta phylum. Resistance has been documented at concentrations of up to 234 mg L^{-1} of NH_3-N, with an optimal concentration of 41 mg L^{-1} (Nagarajan et al., 2019).

The best-known genera of the Cyanophyta phylum used for MbWT of LW are *Anabaena*, *Phormidium, Oscillatoria, Synechocystis, Nostoc* and *Spirulina* (Cepoi et al., 2016). Cheng et al. (2020) tested 12 microalgae of three different phylums (Chlorophyta, Cyanophyta and Euglenophyta) for the MbWT of ADSW. These authors found that the *C. pyranoidosa* microalgae*,* from the Chlorophyta phylum, displayed a value of optical density of 1.12 at 680 nm (OD_{680}), whereas the other microalgae displayed OD_{680} values below

0.7. The OD measurements are performed under the premise that the OD value obtained is proportional to the cell number (Stevenson et al., 2016). Therefore, *C. pyranoidosa* displayed the highest biomass production and tolerance to ADSW. On the other hand, the growth of *S. platensis* was unsatisfactory, as ADSW presented poisonous effects on this cyanobacterium.

Table 2.5.1.2 summarizes the experiments carried out using SW, PW and/or CW. As shown in this table, *C. vulgaris* has been studied extensively due to its capacity to grow in LW and remove pollutants (COD, TN, and TP), showing maximum removal efficiencies of 99% in the case of SW, 89% in the case of PW, and 94% in the case of CW. Other strains from the same genera, such as *C. pyrenoidosa, C. sorokiniana* and *C. zofingiensis,* have been reported to be promising for MbWT due to their high biomass production and nutrient removal efficiencies (C.-Y. Chen et al., 2020; Godos et al., 2010; Wang et al., 2012; Zhu et al., 2013). Further research is needed to evaluate their performance in other types of effluents. Besides *Chlorella* spp., the strains *Chlamydommona* QWY37*, Neochloris aquatica, Parachlorella kessleri* and the consortium of *C. sorokiniana*, *Coelastrella* sp. and *Acutodesmus nygaardii* displayed a COD removal of 81%, 81.7%, 88% and 92%, respectively, making these microalgae strains the most suitable for MbWT due to their higher nutrient removal efficiencies.

More research has been conducted regarding MbWT of ADSW, ADPW and ADCW (Table 2.5.1.3). Due to the lower concentrations of COD, TN and TP in these types of LW, compared with raw LW, most of the microalgae strains tested have displayed TN and TP removal efficiencies above 80%, with *C. vulgaris* being the microalgae that displayed the highest nutrient removal efficiencies. Similarly, the consortium of *S. obliquus* FACHB-12 and *C. vulgaris* FACHB-8 used for MbWT of ADSW displayed high removal efficiencies. Is important to point out that most of the studies using cocultures were performed using ADSW and only one study used ADPW. Thus, it is necessary to consider the utilization of these three kinds of ADLW (ADSW, ADPW, and ADCW) separately and in combination to explore the technical and economic feasibility of the MbWT, considering that livestock producers commonly generate these three effluents in the same production unit.

The results observed in Table 2.5.1.2 and Table 2.5.1.3 may serve as a general guide for the selection of the microalgae for MbWT, depending on the wastewater being treated. Prior to the selection of a microalgal strain, it is necessary to determine, in each case, if the LW will be pretreated first. Some of the pretreatment methods can positively influence microalgal growth and pollutant removal efficiencies. However, the implementation of additional unitary processes would increase the costs and resources needed to implement MbWT at a larger scale and, thus, affect the feasibility of MbWT from an economic and sustainability perspective (Guo et al., 2018; Gupta et al., 2019).

Table 2.5.1.2 Summary of maximum biomass concentration (BC) and chemical oxygen demand removal (CODr), total nitrogen removal (TNr) and total phosphorus removal (TPr) from different microalgae species reported using primary livestock wastewater as a medium.

Microalgae species	Swine wastewater				Poultry wastewater				Cattle wastewater				References
	BC [g/L]	CODr [%]	TNr [%]	TPr [%]	BC [g/L]	CODr [%]	TNr [%]	TPr [%]	BC [g/L]	CODr [%]	TNr [%]	TPr [%]	
Single-strain performance													
Arthrospira platensis (Spirulina platensis)	1.27 - 2.18	33.7 - 88.6	74.44 - 91.4	41.6 - 98.6	1.72 - 1.79	NR	NR	94.6	0.99 - 1.26	NR	NR	NR	(Çelekli et al., 2016; Gantar et al., 1991; Lu et al., 2020a; Markou et al., 2016; Mezzomo et al., 2010)
Botryococcus braunii 765	0.94	-	40.8	93.3	-	-	-	-	-	-	-	-	(Liu et al., 2013)
Chlamydomonas sp. QWY37	4.1 - 9.9	71 - 81	86 - 96	100	-	-	-	-	-	-	-	-	(Qu et al., 2020)
Chlamydomonas mexicana GU732420	0.56 - 0.92	-	62 - 63	28 - 62	-	-	-	-	-	-	-	-	(Abou-Shanab et al., 2013; Salama et al., 2017)
Chlamydomonas reinhardtii	-	42	32.3 - 52.4	78 - 93	-	-	-	-	-	-	-	-	(Qi et al., 2017)
Chlamydomonas globosa	-	-	-	-	0.0764 - 1.278	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
Chlorella minutissima	-	-	-	-	0.170 - 2.599	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
C. pyrenoidosa	0.175 - 0.289	36.5 - 57.6	54.7 - 74.6	31.0 - 77.7	0.80 - 2.52	NR	61.84 - 80.19[a]	83.43 - 88.57	-	-	-	-	(Mohan Singh et al., 2020; Wang et al., 2012)
C. sorokiniana	5.45 - 8.08	83.5 - 94	88.6 - 98.4	20 - 99.5	0.0079 - 0.0388	42.7 - 95.8	36.3 - 84.5	70.1 - 79.2	-	-	-	-	(P. Cheng et al., 2020a; Cui et al., 2020; Godos et al., 2010)

C. vulgaris	0.49 - 3.96	25 - 99	50 - 98.30	41 - 95	1.756 – 1.869	45 – 82	NR	89	1.183	62.30 - 92.17	81.16 - 94.28[b]	85.29 – 94.41	(Abou-Shanab et al., 2013; Fallowfield and Garrett, 1985; Lv et al., 2018; Markou et al., 2016; Salama et al., 2017; Wang et al., 2015; Wen et al., 2017; T. Zheng et al., 2019)
C. zofingiensis	1.063 - 2.962	65.81 - 79.84	68.96 - 82.70	85.00 - 100	-	-	-	-	-	-	-	-	(Zhu et al., 2013)
Chlorococcum sp. GD	-	-	-	-	-	-	-	-	1.208	46.99	73.38 [b]	81.39	(Lv et al., 2018)
Chodatella sp.	1.406	-	48.6 [a]	75.9	-	-	-	-	-	-	-	-	(Li et al., 2014)
Coelastrella sp. QY01	0.103 - 0.625	-	(38.9 - 95) [b]	68.0 - 94	-	-	-	-	-	-	-	-	(X. Li et al., 2020; Luo et al., 2016)
Desmodesmus sp.	0.33 - 7.5	2.5 - 41.8	9.4 - 98.3	47.6 - 93.2	-	-	-	-	-	-	-	-	(Z. Chen et al., 2020; Cheng et al., 2013, 2013; G. Li et al., 2020; Qu et al., 2020)
Haematococcus pluvialis	0.78 - 1.43	19 - 26.4	0.71 - 100	7 - 100	-	-	-	-	-	-	-	-	(Kang et al., 2006; Ledda et al., 2016; Shah, 2019)
Micractinium reisser	0.35	-	13.4	49	-	-	-	-	-	-	-	-	(Abou-Shanab et al., 2013)
Neochloris aquatica CL-M1	3.7 - 6.1	44.2 - 81.7	10.3 - 96.2 [b]	46.3 - 100[c]	-	-	-	-	-	-	-	-	(Wang et al., 2017)
Nitzschia cf. pusilla	0.37	-	19.5	53	-	-	-	-	-	-	-	-	(Abou-Shanab et al., 2013)
Ourococcus multisporu	0.34	-	23	53	-	-	-	-	-	-	-	-	(Abou-Shanab et al., 2013)
Parachlorella kessleri	3.3 - 9.2	47 - 88	58 - 95	100	-	-	-	-	1.213	25.95	73.23 [b]	80.69	(Lv et al., 2018; Qu et al., 2020, 2019)
Scenedesmus bijuga	-	-	-	-	0.264 - 2.194	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
S.obliquus	0.53 - 0.77	42*	61	27 - 65	-	-	-	-	0.928	20.77	11 - 71.19	62.37 - 78	(Abou-Shanab et al., 2013; Godos et al., 2010; Lv et al., 2018; Salama et al., 2017; Xia et al., 2020)

S. quadricauda	-	-	80.1 - 100	51.5 - 100	-	-	-	-	1.033	55.18	77.23	81.51	(Gantar et al., 1991; Lv et al., 2018)
Thermosynechococcus sp. CL-1	0.828 - 1.001	NR	NR	NR	-	-	-	-	-	-	-	-	(Narindri Rara Winayu et al., 2021)
Consortium performance													
Chlamydomonas globosa – Scenedesmus bijuga	-	-	-	-	0.227 - 1.593	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
C. globosa – Chlorella minutissima	-	-	-	-	0.170 - 3.017	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
C. globosa – C. minutissima– S.bijuga	-	-	-	-	0.229 - 3.144	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
C. minutissima – S. bijuga	-	-	-	-	0.216 - 2.299	NR	NR	NR	-	-	-	-	(Bhatnagar et al., 2011)
Chlorella sorokiniana and an acclimated activated sludge	1.070 - 2.870[e]	61[d]	94 - 100	70 – 90	-	-	-	-	-	-	-	-	(de Godos et al., 2009)
Chlorella sorokiniana, Coelastrella sp. *and Acutodesmus nygaardii*	NR	92	90 [b]	100	-	-	-	-	-	-	-	-	(Lee et al., 2021)
Chlorella sp., *Scenedesmus* sp., *Desmodesmus* sp. and *Monoraphidium* sp.	0.619[e]	70.6	44.2	76 [c]	-	-	-	-	-	-	-	-	(Arango et al., 2016)
Chlorella sp. and *Spirulina platensis*	-	-	-	-	3.84	7 – 72[d]	24 - 74	17 – 83	-	-	-	-	(Wang et al., 2018)
Chlorella vulgaris, *Scenedesmus obliquus* and aerobic bacteria	NR	NR	58.2 - 94.8 [b]	NR	-	-	-	-	-	-	-	-	(González-Fernández et al., 2011)
Chlorella vulgaris, Scenedesmus	0.72 - 1.25[e]	NR	96 - 100[b]	82 - 100[c]	-	-	-	-	-	-	-	-	(Molinuevo-Salces et al., 2016)

obliquus and *Chlamydomonas reindhardtii*													
L*eptolyngbya s*p. (95%) and a green coccoid alga (*Choricystis*-like chlorophyte (Trebouxiophyceae))	-	-	-	-	0.313 - 1.1	51.5 – 94.0[f]	36.4 – 88.2	42 – 97.4[c]	-	-	-	-	(Patrinou et al., 2020)

NR: Not reported. a, Total Kjeldhal nitrogen. b, Reported as ammonia removal. c. Reported as PO_4-P, d. Reported as a total organic carbon. e. Volatile suspended solids. f. dissolved oxygen demand.

Table 2.5.1.3 Summary of maximum biomass concentration (BC) and chemical oxygen demand removal (CODr), total nitrogen removal (TNr) and total phosphorus removal (TPr) from different microalgae species reported using anaerobic digested livestock wastewater as a medium.

Microalgae species	Anaerobically digested swine wastewater				Anaerobically digested poultry wastewater				Anaerobically digested cattle wastewater				References
	BC [g/L]	CODr [%]	TNr [%]	TPr [%]	BC [g/L]	CODr [%]	TNr [%]	TPr [%]	BC [g/L]	CODr [%]	TNr [%]	TPr [%]	
Single-strain performance													
Arthrospira platensis	1.23 - 1.9	50	80 – 93	84 – 98	0.829 - 1.627	75	99[b]	96	-	-	-	-	(Liu et al., 2015; Lu et al., 2020b; Olguín et al., 2003)
Ankistrodesmus gracilis	1.36[h]	NR	NR	NR	-	-	-	-	-	-	-	-	(Kim et al., 2014b)
Botryococcus braunii UTEX 572	7.5 - 8.5[h]	NR	43	NR	-	-	-	-	-	-	-	-	(An et al., 2003)
Chlorella sp.	3.98 - 4.81	79	73[b]	95 - 99.6	0.283[h]	86.34[f]	91.24	29.67	-	-	-	-	(Cheng et al., 2015; Li et al., 2017a; Wang et al., 2018)
Chlorella minutissima	-	-	-	-	0.340 - 0.386	NR	34	70 - 100	-	-	-	-	(Singh et al., 2011)
C. sorokiniana	-	-	-	-	0.313 - 0.387	NR	23 - 41	70 - 100	0.150 - 0.280	NR	85.46 - 88.65	61.31 - 64.98	(Kobayashi et al., 2013; Singh et al., 2011)
C. vulgaris	0.71 - 1.5	74.21 - 83.99	89.52 - 94.22	57 - 96.9	0.105 - 1.526	75	20 – 100[b]	39.3 - 99.3	-	-	-	-	(Cao et al., 2018; Gao et al., 2018; Markou, 2015; Salama et al., 2017; Mengzi Wang et al., 2016; Mingzi Wang et al., 2016; Wang et al., 2015)
Chlorococcum sp.	0.66 - 0.85	NR	NR	NR	-	-	-	-	-	-	-	-	(Montero et al., 2018)
Coelastrella sp.	0.0848 - 0.248	NR	38.6 - 80.0[b]	12.6 - 84.9	-	-	-	-	-	-	-	-	(Li et al., 2018)

Desmodesmus sp.	0.15 - 1.039	NR	12.86 - 94.2	35.51 - 90	-	-	-	-	-	-	-	-	(Ji et al., 2015, 2014; G. Li et al., 2020; M. Wang et al., 2020)
Dunaliella FACHB-558	-	-	-	-	0.678	64.1[d]	63.8	87.2	-	-	-	-	(Han et al., 2019)
Nannochloropsis oculata	2.38 - 3.22	NR	64.7 - 86.4	99.6 - 99.8[c]	-	-	-	-	-	-	-	-	(Wu et al., 2013)
Scenedesmus sp.	0.197 - 1.34	NR	91.28	88.72	-	-	-	-	4.65	90	90	83	(Jia et al., 2016; Kim et al., 2007; Luo et al., 2019; Prandini et al., 2016)
S. accuminatus	0.656 - 3.13[h]	-	-	-	-	-	-	-	-	-	-	-	(Kim et al., 2014b)
S. bijuga	-	-	-	-	0.329 - 0.377	NR	17 - 49	70 - 100	-	-	-	-	(Singh et al., 2011)
S. obliquus	1.07 - 7	61.6 - 93.1	58.39 - 90	60 – 88.79	-	-	-	-	1.92 - 3.70	25 - 35	96 – 99[b]	65 - 77.5[c]	(de Mendonça et al., 2018; Gao et al., 2018; Kim et al., 2016; Mingzi Wang et al., 2016; Xu et al., 2015)
S. quadricauda	1.19 - 3.35[h]	NR	NR	NR	-	-	-	-	-	-	-	-	(Kim et al., 2014b, 2014a)
Consortium performance													
Chlorella minutissima, C. sorokiniana and *S. bijuga*	-	-	-	-	0.329 - 0.371	NR	16 - 26	70 - 100	-	-	-	-	(Singh et al., 2011)
Chlorella vulgaris and *Bacillus licheniformis* sp	NR	62.3	55.7	79.7	-	-	-	-	-	-	-	-	(Y. Wang et al., 2020)
C. vulgaris and *Exiguobacterium* sp.	NR	64.6 - 86.3	57.9 - 78.5	78.4 - 87.7	-	-	-	-	-	-	-	-	(Y. Wang et al., 2020)
C. vulgaris, Exiguobacterium sp. and *Bacillus licheniformis* sp	NR	64.4 - 86.4	53.3 – 78.2	81.4 - 83.9	-	-	-	-	-	-	-	-	(Y. Wang et al., 2020)

C. vulgaris and *Ganoderma lucidum* (fungi)	3.69 - 4.77	66.36 - 79.74	65.27 - 74.28	69.23 - 85.37	-	-	-	-	-	-	-	-	(Guo et al., 2017)
Chlorella vulgaris, Scenedesmus obliquus and aerobic bacteria	NR	NR	64.4 - 93.9[b]	NR	-	-	-	-	-	-	-	-	(González-Fernández et al., 2011)
Chlorella sp. and *Scenedesmus* sp.	1.63 - 2.95	39 - 44	98 - 69[b]	NR	-	-	-	-	-	-	-	-	(Raeisossadati et al., 2019)
Chlorella pyrenoidosa and *Rhodotorula glutinis*	3.96 - 5.35	56.25 - 85.44	15.76 - 82.65	33.2 - 53.51	-	-	-	-	-	-	-	-	(Li et al., 2019)
Chlorella sorokiniana and aerobic sludge	NR	62.3	82.7[b]	58[g]	-	-	-	-	-	-	-	-	(Hernández et al., 2013)
C. vulgaris with activated sludge mod	NR	79.86	80.23	89.37	-	-	-	-	-	-	-	-	(Gao et al., 2018)
C. vulgaris with fungi	NR	77.62	79	83	-	-	-	-	-	-	-	-	(Gao et al., 2018)
Desmodesmus sp. CHX1 and nitrifying-bacteria	0.00547	NR	50.37	100	-	-	-	-	-	-	-	-	(M. Wang et al., 2020)
Desmodesmus sp. CHX1 and organic degrading bacteria	NR	NR	25.86	39.33	-	-	-	-	-	-	-	-	(M. Wang et al., 2020)
Desmodesmus sp. CHX1, organic degrading bacteria and nitrifying-bacteria	NR	NR	50.37	82.85 - 100	-	-	-	-	-	-	-	-	(M. Wang et al., 2020)
Pseudokirchneriella subcapitata and *Ganoderma lucidum* (fungi)	3.21 - 4.23	63.09 - 72.09	62.55 - 70.18	67.52 - 82.41	-	-	-	-	-	-	-	-	(Guo et al., 2017)

Scenedesmus obliquus with activated sludge mod	NR	77.35	80	89	-	-	-	-	-	-	-	-	(Gao et al., 2018)
S.obliquus FACHB-12 and *C.vulgaris* FACHB-8	1.8	75.85 - 97.99	90	80	-	-	-	-	-	-	-	-	(Cao et al., 2018; Gao et al., 2018; Salama et al., 2017; Mingzi Wang et al., 2016)
S. obliquus and *Ganoderma lucidum* (fungi)	3.01 - 3.62	65.48 - 70.33	63.28 - 69.75	66.74 - 80.91	-	-	-	-	-	-	-	-	(Guo et al., 2017)
S. obliquus with fungi	NR	77.36	80	88	-	-	-	-	-	-	-	-	(Gao et al., 2018)

NR: Not reported. a, Total Kjeldhal nitrogen. b, Reported as ammonia removal. c. Reported as PO_4-P, d. Reported as a total organic carbon. e. Volatile suspended solids. f. dissolved oxygen demand. g. Soluble phosphorus. h. Dry weight.

2.5.2 Algae-bacteria interactions

MbWT involves the interaction between microalgae and other microorganisms. These interactions can be beneficial or detrimental to the growth of specific microalgal populations, and thus to MbWT (Fig. 2.5.2.1). In mutualistic interactions between bacteria and microalgae, the former may synthesize beneficial compounds for microalgal growth, such as micronutrients, siderophores, growth stimulants and antibiotics, all of which can protect microalgae from pathogenic microorganisms. Moreover, the bacterial respiration process releases carbon dioxide, which is used by microalgae for the process of photosynthesis (Fig. 2.5.2.1.A). Additionally, bacteria can improve microalgal growth during the acclimatization process by enhancing the development of a microbial system in order to overcome environmental fluctuations and other species invasions (Gonçalves et al., 2017; Hu et al., 2019).

The microalgae-bacteria mutualistic interactions are species-dependent. Certain bacterial species are regarded as microalgal growth-promoting bacteria. For example, the growth of *Dunaliella* sp., *Lobomonas rostrata*, *Thalassiossira rotula* and *Phaeodactylum tricornutum* is stimulated when co-cultured with the bacteria *Alteromonas* sp. And *Muricauda* sp., *Mesorhizobium loti*, *Roseobacter* sp. And *Hyphomonas* sp., *Alphaproteobacteria* sp., respectively (Liu et al., 2020). These growth stimulation findings were supported through different methods, such as optical measurements, microalgal cell densities and growth rates in cocultured, axenic microalgal cultures treated with spent bacterial medium to evaluate the influence of extracellular bacterial molecules in microalgal growth, extracellular metaproteome analysis to demonstrate that protein secretion may depend on interactions between both organisms, mathematical models that describe the interdependence between the two organisms, among others. However, it has also been proven that some bacteria have universal compatibility with microalgal species, such as *Rhizobium*, which can enhance the growth of several microalga, including *Chlorella vulgaris*, *Chlamydomonas reinhardtii*, *Scenedesmus* sp. and *Botryococcus braunii* (Liu et al., 2020).

Despite these benefits, other bacteria commonly present in LW can affect microalgal growth through antagonistic or amensalistic interactions, which happens when one member has a positive or neutral impact during the interaction, while the other has a negative impact (Gradilla-Hernández et al., 2020). Nitrifying bacteria, for instance, transform the NH_3-N into nitrite (NO_2) and nitrate (NO_3), which is not easily assimilated by microalgae. Additionally, if the CO_2 is limited, a competition between nitrifying bacteria and microalgae can occur (García et al., 2017a; Liu et al., 2017). Furthermore, these bacteria can inhibit microalgal growth due to the excretion of secondary metabolites, such as algicides, or due to the degradation of the microalgal cells through direct contact (Fig. 2.5.2.1.B). Some bacteria excrete Streptomycin, which affects the microalgal photosynthetic mechanisms by blocking off the electron transport, causing a negative impact on the growth rate, which results in an accumulated biomass reduction (Perales-Vela et al., 2016). Likewise, some microalgal species release chlorellin to the medium, which is a metabolite that has a bactericidal effect against Gram-negative and Gram-positive bacteria (Gonçalves et al., 2017). A deeper understanding of the specific interaction mechanisms between bacteria and microalgae is needed because there are some conflicting results present in the literature. For instance, *Flavobacterium* sp. cultured in SW mixed with MW (1:10) have been reported to cause a detrimental effect on the microalgal cell wall of *Scenedesmus* sp., thereby affecting its growth (Utomo et al., 2020). Conversely, other studies suggest that the same bacteria cultured in SW with *Scenedesmus* sp. may result in a mutualist relationship, because when the flavobacteriales were found at its highest proportion, the highest TP removal efficiency was observed. However, this approach could be associated with bacteria capable of eliminating TP and not precisely with mutualistic interactions (Sánchez Zurano et al., 2020).

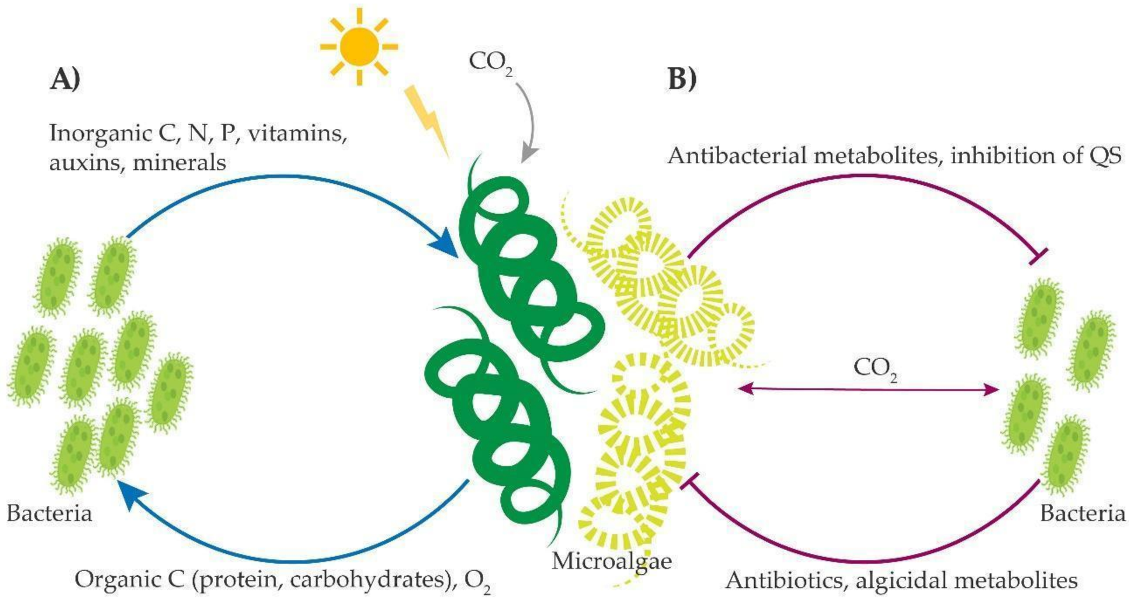


Fig. 2.5.2.1 (a) Mutualist relationships in microalgae-bacteria systems; (b) Competitive and antagonistic relationships in microalgae-bacteria systems. *Quorum sensing. Adapted from Liu et al. (2017).

Some microalgae tested in coculture with bacteria in LW have shown improvements in their growth and nutrient removal efficiencies. Recently, Wang et al. (2019) observed that the bacteria *Pseudomonas alcaliphila, Exiguobacterium, Bacillus amyloliquefaciens, Bacillus subtilis* and *Bacillus tequilensis* display a synergic relationship with *C. vulgaris* for the treatment of aquaculture wastewater, which has a similar TN, TP and COD composition to that of ADLW (Wang et al., 2019). These authors reported that the axenic culture of this microalgae displayed

TP, TN and COD removal efficiencies of 50, 30 and 25%, respectively, while the co-culture of *C. vulgaris, P. alcaliphila, Exobacterium* and *Bacillus* sp. displayed removal efficiencies of 90%, 63% and 67% of TP, TN and COD, respectively.

Wang et al. (2020) tested *C. vulgaris* in the presence of two bacteria (*Exiguobacterium* and *Bacillus licheniformis)* growing in sterilized ADPW. The experiment was carried out to test the performance of *C. vulgaris* as a monoculture, in biculture with *Exiguobacterium* and *Bacillus licheniformis* separately, and in triculture including all three microorganisms. The biomass production of *C. vulgaris* in triculture and biculture with *Exiguobacterium,* was 84.9% higher than the growth observed for *C. vulgaris* in monoculture, and the TN and COD removal efficiencies were 1.9 times and 2.3 higher than those of the axenic culture. The authors observed that the improvements in growth rates and removal efficiencies were attributed to synergistic effects, such as gas exchange. They reached this conclusion by measuring the net photosynthetic activity (NPA), the dissolved oxygen (DO) and the total inorganic carbon (TIC), which displayed an increase of 70.8%, 172.4% and 71.0%, respectively. Additionally, this gas exchange maintained the system at a lower pH in comparison to the axenic culture. The enzymatic activities related to nitrogen transformation of the microalgal cells were determined by a nitrate reductase assay. A *C. vulgaris* and *Exiguobacterium* consortium exhibited improved activities of 94.2%, 57.5%, 58.6% and 79.4% for nitrate reductase, nitrite reductase, glutamine synthetase and glutamate synthetase, respectively, suggesting that *Exiguobacterium* might secrete small molecule metabolites or signal molecules into the culture system, such as enzyme activators, which could enhance the removal of nitrogen by microalgae (Wang et al., 2019; Y. Wang et al., 2020).

The indigenous bacteria present in unsterilized LW has been examined for antagonistic or mutualistic interactions with microalgae. Qu et al. (2020) used both sterile and unsterilized SW to grow *P. kessleri* and observed that the biomass productivity, as well as the TN and COD removal efficiencies, were enhanced when SW was not sterilized (from 550 to 775 mg L^{-1} d^{-1}, from 244 to 350 mg L^{-1} and from 287 to 369 mg L^{-1}, respectively). The authors suggested that common bacteria found in LW, such as Verrucomicrobium, Firmicutes and Proteobacteria, display a mutualistic relation with microalgae (Qu et al., 2020; Toyama et al., 2018). Furthermore, other studies performed to treat MW and ADLW suggested that the co-culture of microalgae with *Achromobacteria, Alcaligenes, Bacillus, Hydrogenophaga* or *Pseudomonas* enhance the nutrient removal efficiencies, mainly TP and TN, by taking in large amounts of phosphorus and accumulating it intracellularly, and by carrying out heterotrophic nitrification and aerobic denitrification, through which NH_3-N is transformed to nitrogen gas and ammonium inhibition is avoided (Chen et al., 2015; Liu et al., 2017; Xie et al., 2018).

All these studies provide valuable information needed for the development of strategies to increase the technical and economic feasibility of MbWT through the implementation of microalgae-bacteria consortiums, which offer important advantages, such as: (1) simplification of the process, as the nitrification and denitrification can take place simultaneously, (2) increase of the microalgae survival rate after an acclimation process due to the development of a robust system and (3) reduction of costs associated with the sterilization of the LW. Nevertheless, the information available about the effects of indigenous and nonindigenous bacteria on microalgae cultures in LW is still limited. Therefore, it is necessary to study the specific mechanisms by which microalgal-bacteria mutualistic interactions occur considering different types of LW and several culture conditions that could enhance these beneficial interactions.

2.5.3 Microalgae-Microalgae interactions

Studies regarding the interactions occurring within microalgal communities are even less common. However, the available information suggests that microalgae consortia cultured in LW may display superior growth rates and nutrient removal efficiencies compared with axenic microalgal cultures. A microalgal consortium offers two main advantages: (1) robustness of the consortia and tolerance to environmental fluctuations and (2) compensation of eventual losses of specific strains due to changes in the culture conditions by the growth of other strains (Qin et al., 2016).

Choudhary et al. (2016) tested 9 native microalgal consortia isolated from two wastewater sources (sewage treatment plant and slaughterhouse effluent) to analyze their potential biomass production and pollutant removal efficiencies when cultivated in LW used as a nutrient source. The microalgae genera involved in the consortia were *Scenedesmus, Chroococcus, Merismodepdia, Chlorella, Photmidium* and *Dycyospherium.* Nine microalgal consortia were paired depending on the source of the isolation medium and then on the biomass they produced in BG-11 medium. Five of the nine consortia were selected for further studies. The selected consortia were: (1) *Scendesmus/ Chroococcus*, (2) *Chrocooccus/ Phormidium*, (3) *Chlorella/ Phormidium,* (4) *Chroococcus/ Chlorella* and (5) *Chlorella/ Dictyospherium*. The consortium that achieved the highest biomass concentration was that of *Chlorella* (as dominant strain) and *Phormidium*. The final biomass concentration was 1.9 ± 0.28 g L^{-1}, and the COD and TP removal efficiencies were 80 and 85%, respectively, which were reached after 12 days of cultivation (Choudhary et al., 2016).

Although *Chlorella* sp. has demonstrated superior removal efficiencies of pollutants in MbWT compared to other microalgae in pure cultures, microalgal consortia including *Chlorella vulgaris* have displayed even higher nutrient removal efficiencies. Wang et al. (2016), for example, used undiluted ADSW to culture *C. vulgaris* and *S. obliquus,* separately and in coculture, to evaluate the COD removal efficiency. In the case of the axenic cultures, *C. vulgaris* and *S. obliquus* showed a COD removal of 83.99% and 73.98%, respectively, while the consortium displayed a COD removal of 97.99%. Similarly, Qin et al. (2016) tested four consortia to treat CW: (1) *Chlorella* sp./ *C. zofingiensis*; (2), *Scenedesmus* spp./*C. zofingiensis*; (3), *Chlorella* sp./ *Scenedesmus* spp.; and (4), *Chlorella* sp./*Scenedesmus* spp./ C. *zofingiensis*. They observed COD and TP removal efficiencies above 55% and 80%, respectively, for all the consortia, higher than those of the axenic culture of *Chlorella* sp., which displayed a removal of 40 and 77%, respectively. Regarding TN removal, no significant differences were observed among all consortia and axenic culture, reaching a TN removal rates above 80%. Although these studies evaluated the performance of the microalgae consortia, the interactions between microalgal members have not been fully studied. Different approaches have been proposed for characterizing microbial interactions with the implementation of neural networks that could be implemented for quantitatively assess microbial interactions occurring within microalgal communities used for MbWT (Gradilla-Hernández et al., 2020). Additionally, the combination of unicellular microalgae with filamentous microalgal strains have been shown to enhance the nutrient removal efficiencies and biomass recovery rates (Zhu et al., 2019). However, to the best of our knowledge, no studies have been conducted using LW.

The implementation of microalgal consortia for MbWT represents an opportunity to achieve more robust treatment systems that are resistant to adverse environments, to increase removal efficiencies and to reduce biomass recovery process costs. Nevertheless, as mentioned previously, the interactions occurring between microalgal populations for MbWT have been poorly documented and further studies are needed regarding the mechanisms behind the cooperative and competitive interactions within microalgal communities.

2.6 Effects of physical parameters on the microalgal performance in wastewater treatment

2.6.1 Temperature

Temperature is a relevant parameter that must be considered in order to obtain optimal microalgal development for MbWT (Fig. 2.6.1.1). In general, the optimal temperature for growth is between 15°C and 30°C. However, this can vary depending on the microalgal strain. Temperatures below this range affect the kinetics of cellular enzymatic processes, which translate into a low biomass production. On the contrary, temperatures above this range deactivate some proteins involved in photosynthesis, reduce the metabolic rates of microalgae and trigger oxidative damage that may lead to cell death (Cheng et al., 2019; Qu et al., 2020; Singh et al., 2020). Additionally, optimal temperatures positively influence the light intensity resistance of microalgae (González-Camejo et al., 2019).

Besides the microalgal metabolism, the solubility of CO_2 has an inverse relationship with the temperature; when the temperature increases, the CO_2 solubility in the medium decreases, and thus, the pH-value (Binnal and Babu, 2017; Xu et al., 2019; Yin et al., 2020).

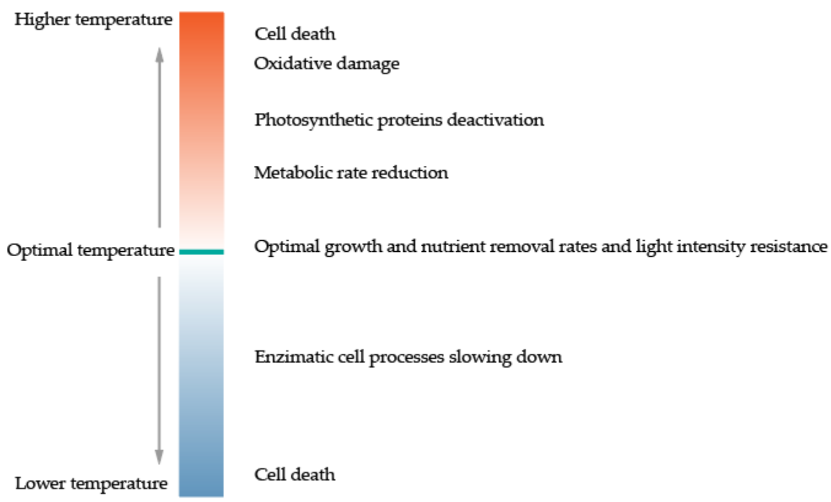


Fig. 2.6.1.1 Graphical illustration of the general temperature effect on microalgae.

Previous reports have indicated that the suitable temperature range for microalgal growth in SW is approximately 20–27°C (Cheng et al., 2019). However, some strains display higher growth rates at other temperature ranges. Lee et al. (2017) explored the optimal growth conditions of *C. sorokiniana* cultured in ADSW and reported an optimal temperature at 40°C. At this temperature, the highest specific growth rate was achieved (0.53 d^{-1}) and COD and NH_3-N removal rates of 105 and 17.8 mg L^{-1} d^{-1} were reported. Within a 36-h cultivation period, >99% and >88% of the COD and NH_3-N contents were removed from the medium. A clear correlation between the temperature and NH_3-N removal was observed. The highest NH_3-N removal rate (0.777 h^{-1}) was observed at a higher temperature (40°C), while the NH_3-N removal rate decreased to 0.015 h^{-1} at a lower temperature (<20°C) (Lee et al., 2017).

However, temperature is not an independent variable, other factors, such as medium composition, may influence the optimal temperature. As shown in Table 2.6.1.1, only few studies have been focused on determining this influence. Sforza et al. (2014) reported an optimal growth temperature of 30°C in unsterilized domestic wastewater (DW) using *Chlorella protothecoides.* At this temperature, they found the highest specific growth rate achieved was above 2 d^{-1} and the TN and TP removal efficiencies were 87 and 99%, respectively. When using the same strain to treat DW, Binnal and Babu (Binnal and Babu, 2017) reported the highest biomass concentration of 1.23 g L^{-1} and removal efficiencies of COD, TN and TP of 54.23, 68.38 and 81.31%, respectively, at a temperature of 25°C. While at 30°C, the biomass concentration decreased to 0.31 g L^{-1} and the removal efficiencies of the same parameters decreased to 28.12, 49.37 and 58.47%, respectively. These last studies did not use LW, but they illustrate the different optimal temperatures reported for the same microalgal strain that may occur in response to the medium composition.

Table 2.6.1.1 Microalgae optimal temperature reported in artificial and Livestock wastewater mediums.

Specie	T min	T opt	T max	T opt reported in LW	References
Scenedesmus obliquus	10 °C	25 °C	40°C	29.55 °C	(Calhoun et al., 2021; Christov et al., 2001; Hodaifa et al., 2010)
Chlorella vulgaris	10°C	25 °C	42°C	30 °C	(Kessler, 1985; Ma et al., 2014; Mayo, 1997; Serejo et al., 2021; Shukla et al., 2013)
Chlamydomonas reinhardtii	17 °C	25 °C	30 °C	27 °C	(Carr et al., 2017)

An evaluation of the optimal temperature for the specific strain and medium composition should be performed to achieve the highest nutrient removal and biomass production in every process implemented for MbWT. Likewise, the cost implications of a temperature control system must be considered due to its significant impact on the overall operational costs to assure the economic viability of the MbWT process (Zhu and Hiltunen, 2016). Nonetheless, a common problem of MbWT implemented in photobioreactors directly exposed to sunlight is the uncontrolled rising of temperature. It has been documented that, during summertime, the temperature inside an open or a closed photobioreactor may rise 10-30°C above the ideal temperature (Yeo et al., 2018). In this case, a robust cooling strategy must be planned to maintain the optimal temperature in order to enhance biomass production and nutrient removal efficiencies.

Natural geographical variations of temperature and light intensity can be used to decrease the expenditure of microalgae cultivation. Geographic Information Systems (GIS) are spatial models that can be used to identify areas with high potential for microalgae production. Recently, a model was built to identify ideal areas for MbWT facility locations in Mexico, using multiple criteria, such as land use, temperature, solar radiation, water and CO_2 sources, location of cities and roads, natural protected areas, and geological faults, among others. Large portions of the country were found to have appropriate environmental conditions that tend to be relatively stable and are favorable for microalgae development. Furthermore, these authors estimated that the potential microalgal biomass production was between 3.8-24 billion tons of dry biomass per year using synthetic media (Lozano-Garcia et al., 2019). The implementation of GIS in the development of a MbWT design may help overcome the high operational costs related with artificial light supply and temperature control systems. Additionally, with the aid of GIS, it is possible to consider other important factors, such as the transportation distances of the nutrient's sources (in this case, LW), the microalgae biomass and the type/quantity of high-value compound production that may be used for commercialization.

2.6.2 Light intensity, cycle, and wavelength

Light intensity is probably the most important parameter to consider in microalgae breeding. It can be supplied continually or in light-dark cycles. The light intensity at which microalgae reach the maximum photosynthetic activity is called the point of light saturation, and it is specific to the microalgae strain and the medium composition. This point can be determined from the photosynthetic parameters of the light response curve (photosynthesis versus irradiance) as the intersection point of the maximum photosynthetic rate and the initial slope of the light curve (Elisabeth et al., 2021; Jazzar et al., 2016). Light intensity below the point of light saturation translates into poor cell growth (especially in autotrophic microalgae). On the other hand, light intensity above the point of light saturation may be deleterious for microalgae, by the disruption of the chloroplast lamellae, as well as for the production of pigments, carbohydrates, and proteins (Andersen, 2005; Fan et al., 2020). The minimal requirement of light for mixotrophic microalgae is in the range of 10-15 µmol m^{-2} s^{-1}. Below this rate, the microalgal growth sharply decreases (Patel et al., 2019).

Providing the optimal light requirement is one of the challenges of MbWT because LW contains large amounts of suspended solids and turbidity that minimizes the penetration of light into the medium, interfering with microalgal growth. This reduction on light penetration negatively affects the photosynthetic rate, especially for microalgae that are not able to float on the surface. A solution to this could be a pretreatment, such as flocculation, or the development of turbulence within the LW media. An example of the latter is the use of raceway ponds (Mohd Udaiyappan et al., 2017).

Qu et al. (2020) cultured the microalga *Parachlorella kessleri* in SW using different light intensities (from 200 to 800 µmol m^{-2} s^{-1}). The highest biomass productivity (1.15 g L^{-1} d^{-1}) was achieved at a light intensity of 600 µmol m^{-2} s^{-1}, and the COD and TN removal efficiencies were found to be 88 and 95%, respectively. At a light intensity of 800 µmol m^{-2} s^{-1}, the biomass productivity decreased to 0.913 g $L^{-1}d^{-1}$ and the removal efficiencies of COD and TN were 65% and 70%, respectively. In a similar experiment performed by the same authors, the microalga *Chlamydomonas* sp. QWY37 was grown in SW, and light saturation was found to occur at 500 µmol m^{-2} s^{-1}. At this value, the biomass productivity was 1.23 g L^{-1}, and the COD and TN removal efficiencies were 71 and 86%, respectively. Below and above this point, the overall performance decreased. At a light intensity of 250 µmol m^{-2} s^{-1}, the biomass productivity decreased to 0.8 g L^{-1}, while the COD and TN removal efficiencies decreased to 54% and 61%, respectively. The corresponding values at 750 µmol m^{-2} s^{-1} were 1.1 g L^{-1}, 62% and 76%, respectively (Qu et al., 2020).

In addition to the light intensity, the implementation of a light-dark cycle is extremely important for microalgal cells, as photosynthesis comprises two phases, the photochemical phase (light phase) and the biochemical phase (dark phase). The first phase is light dependent as light is used to synthesize ATP and NADPH, whereas, in the dark phase, these molecules are used to synthesize metabolites. The variation of the light-dark cycle results in a variation of the cellular content of proteins, carbohydrates, and lipids (G. Li et al., 2020). Some experiments have been carried out to study the influence of the light-dark cycle in the overall microalgae performance for MbWT of LW. Lv et al. (2018) cultivated *C. vulgaris* in undiluted CW using a light intensity of 55.50 µmol m^{-2} s^{-1} and a light-dark cycle of 14h:10h, resulting in removal efficiencies of COD and TP between 91–92% and 91–94%, respectively. Moreover, when a coculture of *C. vulgaris* and activated sludge grown in SW was subjected to a light-dark ratio of 12h:12h and a photosynthetic photon flux density of 200 µmol m^{-2} s^{-1}, removal efficiencies of 80%, 80%, and 89% were observed for COD, TN and TP, respectively (Gao et al., 2018). Similarly, *Scenedesmus* sp. was cultivated in ADCW combined with MW, using white fluorescent light at 5000 lux and light-dark cycles of 12h:12h, and it reached a biomass production of 4.65 g L^{-1} and a COD removal efficiency above 90% (Luo et al., 2019).

In general, the light-dark cycle tends to enhance biomass production, as reported by Luo et al. (2019) however, the reviewed studies using continuous illumination have reported slightly higher COD and TN removal efficiencies than those using a light-dark cycle, with certain exceptions (Gao et al., 2018; Luo et al., 2019; Lv et al., 2018; Qu et al., 2020, 2019). Nonetheless, not only the removal efficiencies and biomass productions must be considered when deciding on the light regime, but also the cost of providing illumination continuously. Open ponds are an economic wastewater treatment method where the chosen microalgal cultures withstand the changes in illumination during the day-night cycle (Nagarajan et al., 2020). Models have been developed and validated to predict biomass productivity in outdoor ponds and photobioreactors under diurnally fluctuating light intensities and water temperatures, for three different species: *Chlorella sorokiniana*, *Nannochloropsis salina* and *Picochlorum* sp. However, these predictive models were developed and validated for microalgae growing on artificial mediums. Thus, there is a need for the development of predictive models for microalgal growth on LW as a promising tool to reduce the requirement for costly outdoor pond tests. In addition, there is likewise a need to integrate the parameters of light and temperature in order to achieve maximum biomass productivity and economic feasibility of the MbWT in open ponds and photobioreactors (Huesemann et al., 2016).

The light wavelength at which the microalgae is exposed is another important parameter to enhance microalgal performance in MbWT. Efficient illumination is obtained by the implementation of light-emitting diodes with specific wavelengths or by the manipulation of solar wavelengths through special filters. Blue light-emitting diodes have a low cost and have proven to be the most efficient light source. Looking at the typical absorption spectra of algae, the maximum peak of absorbance is in the range of blue light, while a lesser peak is in the range of red (Kang et al., 2018).

The implementation of a blue (436 nm), red (665 nm) and a red-blue (1:3) LED light combination at a rate of 120 µmol photons m^2 s^{-1}, for the culture of *Neochloris oleoabundans* grown in PW resulted in a 14.42% increase in cell concentration compared to a culture provided with white light at the same conditions. Additionally, the content of *Chlorophyll a*, which is the most abundant pigment in all photosynthetic organisms, displayed a steady concentration during a 6-day application period of the blue LED light, whereas the white and the red-blue illumination resulted in a decrease in its concentration after four and two days, respectively. Contrarily, *chlorophyll b*, which is the second most abundant pigment in green microalgae, displayed the highest concentration with the red-blue light application (Altunoz et al., 2017). Studies have also demonstrated that red light enhances biomass production and accumulation of carbohydrates, while blue light increases the lipid content, protein synthesis and enzymatic activation (Altunoz et al., 2017; Choi et al., 2015; Izadpanah et al., 2018). *Acutodesmus obliquus* cultivated in BG-11 medium presented an increase in growth and fatty acid production, in comparison to the control, when exposed to diverse frequencies of intermittent LED flashing light (blue and red lights). The frequency of light flashing rate was adjusted to 120, 10, 5, 3.75, and 1 times per minute (Choi et al., 2015). Similarly, three different strains of *C. sorokiniana,* cultivated in DW were subjected to white, red, and blue light spectrums with a light regime of 16h:8h light-dark. The highest lipid density was found to occur using the blue light, while the maximum biomass productivity was achieved under red light (Izadpanah et al., 2018). The use of LED offers other advantages, in comparison with conventional fluorescent lamps, such as low power consumption, longer lifetime, low heat generation, and high conversion efficiency (power input to light output) (Singh et al., 2016). The use of LED and fluorophores for improving microalgal growth has shown promising results. However, these strategies need to be investigated further from a perspective of nutrient removal efficiencies in large-scale microalgal cultures using LW as the growing media.

Light intensity, regime, and wavelength are important parameters to consider for the enhancement of microalgal performance in MbWT. Nevertheless, when deciding the light regime, not only the removal efficiencies and biomass productions must be considered, but also the balance between the cost of providing illumination continuously in a large-scale system along with the potential to commercialize valuable metabolic byproducts that can assure the economic sustainability of the MbWT. Still, more investigation is needed regarding cultivation strategies that would allow for optimal penetration of light in highly turbid waters, and the impact that the light wavelength has on nutrient removal efficiencies to assure the feasibility of large-scale MbWT of LW.

2.7 Effects of physical parameters on the microalgal performance in wastewater treatment

2.7.1 Relation C/N

MbWT applied to LW generally results in low biomass yields because of the low carbon/nitrogen (C: N) ratio found in LW, which translates into an insufficient carbon source for microalgal growth. Previous studies have shown that a proper C: N ratio is necessary to improve microalgal cell viability, biomass concentration and nutrient removal (Lu et al., 2016; Ma et al., 2016; Zheng et al., 2019; Zheng et al., 2018). Zheng et al. (2019) compared cell viability and biomass productivity of *C. vulgaris* when provided different C: N ratios (5:1, 25:1 and 125:1) against sterilized SW with a C: N ratio of 17:20. This microalga displayed its highest cell viability and biomass concentration at a C: N ratio of 25:1. The increased C: N ratio reduced the ammonia concentration, and its toxicity, which promoted cell division and stimulated ammonia absorption. A potential strategy used to increase the C: N ratio in LW is the addition of cheap carbon sources, such as sodium acetate (Lv et al., 2019) or other effluents rich in carbon like brewery wastewater (Zheng et al., 2018) or nejayote wastewater (López-Pacheco et al., 2019). Table 2.7.1.1 summarizes significant improvements achieved with the addition of a carbon source to LW.

Table 2.7.1.1 Summary of recent studies in which a carbon source was added to LW to improve the microalgae growth and nutrient removal.

Base wastewater	Carbon source addition	C: N rate	Improvements	Reference
Filtrated cattle wastewater	Sodium acetate	6-8:1	Biomass production and nutrient removal rates were enhanced. Moreover, the lipid accumulation from microalgal cells grown in municipal wastewater and cattle farm wastewater were 33.2% and 20.9% biomass.	(Lv et al., 2019)
Simulated wastewater	Glucose	24-30:1	Biomass productivity increased 6.6 times. Nutrient removal was 1.45 times higher, reaching a removal <99% of TN and TP.	(Gao et al., 2019)
Secondary SW	Nejayote wastewater	NR*	Cellular growth was enhanced with a combination of 25% nejayote, 25% swine and 50% water.	(López-Pacheco et al., 2019)
Manure-free SW	Biodiesel-derived glycerol	25:1	Resulted in improved biomass concentrations (3.83 g L^{-1}), cell viability (97%) and nutrient removal rates (>95%) for all parameters tested (TP, TN, COD and NH_4^+-N).	(H. Zheng et al., 2019)
Centrifugated and sterilized SW	Brewery wastewater	7.9:1	Enhanced nutrient removal efficiency (>90% for all parameters) and reduced production costs.	(Zheng et al., 2018)
Sedimented and filtrated SW	Sodium acetate	NR*	Biomass concentration was close to that in fresh Zarrouk's medium (2.30 g L^{-1}). Nutrient removal was higher than 85% for all parameters.	(Lu et al., 2020b)
	Sodium bicarbonate	NR*	Significantly higher biomass concentration (1.70 g L^{-1}). Nutrient removal was higher than 87% for all parameters.	

*Not reported.

The adjustment of the C: N ratio of LW with other effluents is a cost-effective strategy to enhance microalgal growth and nutrient removal efficiencies during MbWT. However, in some of the experiments presented in Table 2.7.1.1, the addition of the carbon source to a C:N proportion of 1:1 or higher was necessary. Nevertheless, this strategy can only be considered as viable if the carbon and LW sources are geographically close in order to avoid a sharp increase in operational costs due the transportation of the carbon source to the MbWT unit. If this is not the case, the addition of the carbon source may be a disadvantage in terms of an economic or logistical perspective.

2.7.2 pH

pH is an important parameter to be considered for MbWT due to its direct relation with microalgal growth. In most cases, microalgae grow at pH values close to 7, but some exceptions have been reported. For instance, *Dunaliella salina* has been found to grow at a pH close to 11.5, whereas *D. acidophila* can grow at a pH between 0 and 3 (Sakarika and Kornaros, 2016). Thus, microalgae may prefer an atypical pH at which their intracellular functions are performed optimally depending on the environment where they naturally grow (Galès et al., 2020). Alkaline conditions decrease contamination risks, thus increasing the feasibility of large-scale outdoor MbWT systems (Lu et al., 2020a).

In addition to the influence on microalgal intracellular functions, pH affects the medium composition. As mentioned before, LW generally has a high concentration of NH_3-N, which is the nitrogen source preferred by microalgae due to the lower energy required for its assimilation (Zuccaro et al., 2020). Nevertheless, as previously mentioned, when NH_4^+-N reacts in the presence of water, a pH dependent equilibrium is formed between NH_4^+-N and NH_3-N at a pH close to 7. pH values higher than 9.2 favor NH_3-N formation, which is highly volatile and highly toxic to some microalgae strains. However, the volatilization of NH_3-N could become a disadvantage when there is no other nitrogen source available in the medium, resulting in nitrogen limitations to microalgae growth (Lu et al., 2020a). At the same time, pH values above 9.2 favor the formation of CO_3^{2-} and HCO^{3-}, which negatively influence the uptake of organic carbon, preventing the microalgae from performing active transportation, which needs more energy than diffusion and results in a slower growth rate (Zuccaro et al., 2020).

Additionally, pH can impact the bioavailability of nutrients. Paquette et al. (2020) evaluated the bioavailability of C, N, P, S, Mg, S, Ca and Fe, among others, at an alkaline pH (pH >10.4) and concluded that all of these nutrients were in a bioavailable form for microalgal uptake at this range, with the exception of Ca^{2+}, Fe^{3+} and Mg^{2+}, which precipitated out of solution as carbonates and hydroxides. The absence of Mg^{2+} could be deleterious for microalgae, as this ion is part of the active center of *chlorophyll a* and plays an essential role for microalgal growth. pH values below 9 increase the bioavailability of Mg^{2+} and PO_4^{-3} ions, while pH values lower than 6 suppress photosynthesis, which results in a low biomass concentration (Qian et al., 2020). Thus, a pH control between 6 and 9 is important to assure the bioavailability of these ions and to avoid low biomass concentrations.

The highest nutrient removal efficiencies achieved through MbWT have been reported for pH values between 6 and 8 (Nie et al., 2020). This behavior was observed in a study where *C. vulgaris* was cultivated in SW, and it displayed removal efficiencies of 97%, 94% and 99% for TN, TP, and COD, respectively, at a pH of 7, while at pH of 5, the respective removal efficiencies were 85%, 81% and 87%. The lowest removal efficiencies were found at a pH of 9, with respective values of 56%, 60% and 58%. Furthermore, a decrease in cell viability was reported at pH values other than 7. At a pH of 7, cell viability was 96%, while at pH values of 5 and 9, cell viability decreased to 81% and 68%, respectively. These results were attributed to the effect of pH on the enzymes related to photosynthesis and nutrient uptake (H. Zheng et al., 2019). Nevertheless, the nutrient removal efficiencies reported by Ma et al. (2016) for *C. vulgaris,* cultivated in a synthetic wastewater and supplemented with pretreated waste glycerol generated from biodiesel production at alkaline conditions (pH=9), were 92% for TN, 91% for TP and 98% for COD, similar to those reported at a pH of 7 (95%, 95% and 98%, respectively). Acidic conditions (pH=5) were found to be lethal to *C. vulgaris* at later growth stages.

Controlling the pH for MbWT of LW could be both an opportunity and a challenge. Higher pH values reduce contamination risks while increasing the potential of large-scale outdoor systems, which in turn promote the volatilization of NH_3-N and increase the bioavailability of some nutrients. However, higher pH values also reduce the bioavailability of Mg^{2+}, resulting in growth inhibition and in a promotion of inorganic carbon uptake, instead of organic carbon, for microalgae metabolism. On the other hand, lowering the pH enhances the bioavailability of the Mg^{2+} ion for microalgal uptake, while reducing biomass concentrations caused by the depression of photosynthesis. Extreme conditions, whether acidic or alkaline, negatively affect microalgal growth and pollutant removal. Therefore, it is important to identify a suitable pH to enable maximum biomass production and nutrient removal efficiencies of specific microalgal populations of consortia using different types of LW.

2.7.3 Inhibitory compounds

LW contains significant amounts of sodium, calcium, potassium, chlorine, sulfur, phosphate, bicarbonate, NH_3-N and heavy metals, along with organic compounds, which makes for a complex environment and affects microbial growth and activity (H.-H. Cheng et al., 2020).

2.7.3.1 Ammonia

Microalgal toxicity can be caused by NH_3-N and NH_4^+-N. NH_4^+-N is the most abundant form, but the relative concentration of both is highly dependent on the pH and, to a lesser extent, on the temperature and the salinity. The most toxic form for microalgae is NH_3-N because it presents a high lipid solubility, which allows it to permeate the membrane. However, this molecule is gaseous and highly volatile to the atmosphere. At a pH of 9.2, NH_3-N and NH_4^+-N exist in equilibrium, while above this pH, NH_3-N is the dominant form, and NH_4^+-N dominates at pH values lower than 9.2 (Nagarajan et al., 2019).

Although microalgae generally display a higher tolerance to NH_3-N than other microorganisms, NH_3-N concentrations above their specific tolerance range (Table 2.7.3.1) is directly related to the inhibition of the photosynthetic process, leading to a decrease in biomass productivity and nutrient assimilation. This inhibition is associated with different mechanisms, such as the damage of the oxygen-evolving complex of the photosystem II and the disruption of the ΔpH component of the thylakoid proton gradient due to its accumulation inside the membranes. Additionally, photosystem I and the dark respiration rates are negatively affected by high concentrations of NH_3-N (136 mg L^{-1} approximately), but these mechanisms are still not well understood (Rossi et al., 2020).

Significant differences between microalgae of the same class have been reported in terms of their NH_3-N tolerance. Regarding some species of the oleaginous class, inhibiting concentrations of ammonia have been reported to be 2.3 and 3.3 mg L^{-1} NH_3-N for *N. oleoabundans and D. tertiolecta*, respectively, at a of pH 8. However, the growth rates of *C. sorokiniana* and *N. oculata* remained unaffected by ammonia at the complete concentration range tested (10–1000 mg L^{-1} NH_3Cl) (Gutierrez et al., 2016). In Table 2.7.3.1**,** a summary of the optimum, inhibitory, and toxic ammonium concentrations among microalgal classes are displayed. Chlorophytes are the most ammonium-tolerant class, followed by Cyanophytes.

Table 2.7.3.1 Mean ± SD of the optimal, inhibitory, and toxic ammoniacal nitrogen (NH_3-N) concentration for microalgae growth in batch cultures. Retrieved from Collos and Harrison (Collos and Harrison, 2014)

Class	Optimal Concentration [mg L^{-1}]	Inhibitory Concentration [mg L^{-1}]	Toxic Concentration [mg L^{-1}]
Chlorophyceae	128.72 ±129.52	403.886 ±440.67	666.10 ±1,020.42
Cyanophyceae	42.26 ±26.69	112.47 ±110.74	220.69 ±224.84
Diatomophyceae	5.73 ± 6.95	12.32 ±14.26	60.77 ±80.60

Dinophyceae	1.87 ± 1.30	5.51 ±4.81	20.50 ±44.89
Prymnesiophyceae	24.34 ±20.35	16.29 ±21.10	41.47 ±47.72
Raphidophyceae	4.47 ±5.64	10.79 ±10.61	44.53 ±69.17

Zheng et al. (2019) evaluated the influence of the NH_3-N concentration on the nutrient removal efficiency and cell viability of *C. vulgaris* growing in SW. The optimal NH_3-N concentration was reported to be 110 mg L^{-1}, producing a cell viability of 89% and removal efficiencies of 96% for COD, 100% for NH_3-N and 91% for TP. When a higher concentration (220 mg L^{-1}) was used, the cell viability decreased to 61% and the removal efficiencies were around 50% for all cases. Although *C. vulgaris* belongs to the Chlorophyceae class, the inhibitory effect of NH_3-N was observed at a lower concentration than reported by Collos and Harrison (Collos and Harrison, 2014). As described in Table 2.2.1, the NH_3-N concentration may vary from 5.14 to 3,000.00 mg L^{-1}, depending on the source of LW. Different strategies must be implemented to avoid this inhibition and to assure adequate removal efficiencies, such as dilution or the addition of a carbon source to reach an appropriate C: N ratio.

A novel strategy to avoid NH_3-N inhibition and to increase its uptake by microalgae, is the application of nitrogen-starved microalgae cells for MbWT. When these cells are re-exposed to a nitrogen-rich medium like LW, the cells are triggered to uptake more nitrogen than necessary to grow (Xie et al., 2017). Ran et al. (2021) used a nitrogen-starved inoculum of *C. vulgaris* for the MbWT of ADSW. After 6 days of cultivation, the biomass production increased 1.7 times (from 1.33 to 2.56 g L^{-1}) and the removal efficiency of NH_3-N increased from 77.5% to 99.1%, in comparison with non-starved microalgae. The TP removal efficiencies, however, were found to be similar, 95.5% for non-starved microalgae and 96.5% for the nitrogen-starved microalgae.

2.7.3.2 Copper

Copper (Cu) is an important trace element regularly added to animal feeds and commonly found in LW, due to of its low absorption rate. More than 80% of the Cu (II) contained in the animal feed is known to remain in the livestock's excrement (S. Li et al., 2020; Scott et al., 2018). In SW, common Cu (II) concentrations vary between 0.6 to 21 mg L^{-1}, which may become an important obstacle to achieve optimal microalgal growth and nutrient removal in MbWT (Hu et al., 2020). Although Cu (II) is an essential element, high concentrations of Cu (II) could be toxic to microalgae, as it induces oxidative damage by the production of reactive oxygen species, such as O_2, H_2O_2 and OH (Li et al., 2018). In the presence of high concentrations of Cu (II) (>20 mg L^{-1}), microalgae are not only affected by changes to their shape, but also by their capability to perform physiological and biochemical processes, such as cell division, growth, photosynthesis, respiration and regeneration of cellular organelles. High secretion of polysaccharides may be another microalgal response to high Cu (II) concentrations, which causes conglomerations (Wan Maznah et al., 2012). This effect could be positive if one goal is to increase the content of polysaccharides.

Li et al. (2018) cultured *Coelastrella* spp. in several samples of ADSW containing different Cu (II) concentrations (0.10, 0.50, 1.0, 2.0 and 3.0 mg L^{-1}). The ADSW initial concentrations of NH_3-N, TP, and Cu (II) were 1,317, 20.2 and 7.3 mg L^{-1}, respectively. A previous 10% dilution using distilled water was performed to obtain the desired Cu (II) concentrations. The authors found that after the first 4 days, microalgae biomass and nutrient removal efficiencies decreased with Cu (II) concentrations above 1.0 mg L^{-1}. The highest TP removal efficiency was 84.9% at a Cu (II) concentration of 0.5 mg L^{-1}, and only 12.6% of the TP was removed at the highest concentration of 3.0 mg L^{-1}. Additionally, the authors examined the physiological stress caused by the increasing concentrations of Cu (II) through the evaluation of the enzymes, malondialdehyde and superoxide dismutase, which are considered injury biomarkers of oxidative stress. The content of malondialdehyde significantly increased from 0.7 to 5.7 nmol mg $prot^{-1}$ at concentrations of 0.50 and 3.0 mg L^{-1} of Cu (II), respectively. A high concentration of malondialdehyde is known to cause cell damage due to the peroxidation induced by Cu (II) ions. Regarding the superoxide dismutase evaluation, its concentration also increased with higher Cu (II) concentrations, but it was not enough to prevent damage by reactive oxygen species (ROS). According to these results, Cu (II) concentrations above 1 mg L^{-1} could be deleterious for microalgal cells, resulting in a challenge for MbWT, as common concentrations of Cu (II) in LW vary from 1.02 to 7.3 mg L^{-1} (P. Cheng et al., 2020c; Li et al., 2018; Zhu and Hiltunen, 2016), which requires either dilution or chemical precipitation to avoid toxicity to microalgal cells and impairment of MbWT. However, Cu (II) tolerance is species-dependent, and it is necessary to evaluate the tolerance capacity of the most common microalgae employed for MbWT, such as *C. vulgaris, S. obliquus, H. pluvialis,* among others, prior to dilution to avoid the use of large volumes of fresh water.

2.7.3.3 Zinc

Zinc (Zn) is another metal supplemented in swine feed to reduce diseases and to accelerate the pig's growth rate. Nevertheless, only 10-20% of this compound is absorbed and the rest is excreted by the swine. Previous studies reported a concentration between 431 and 471 mg kg^{-1} in dry excrement, and between 2 and 22 mg L^{-1} in SW (X. Li et al., 2020). Although Zn (II) acts as an important enzyme cofactor for microalgal metabolism and replication, high concentrations of Zn have been reported to be toxic to microalgae (Monteiro et al., 2011). Li et al. (2020) assessed the inhibitory response of *Coelastrella* sp. to different Zn (II) concentrations (0.0, 0.50, 1.0, 2.0, 4.0, 6.0 and 8.0 mg L^{-1}) in SW with initial concentrations of 1,550 and 35 mg $L^{-1}$for NH_3-N and TP, respectively. A Zn (II) concentration of 0.5 mg L^{-1} produced the highest NH_3-N removal (62.3%), while at Zn (II) concentrations above 1 mg L^{-1}, the removal efficiency significantly decreased to values between 55% and 38.9%. In contrast, the highest TP removal efficiency (77.6%) was achieved with a Zn (II) concentration of 8 mg L^{-1}, attributed to a chemical reaction between Zn and P that promoted TP sedimentation ($Zn_3(PO_4)_2 \cdot 2H_2O$) and not microalgae metabolism. With the highest concentrations of Zn (II) (8.0 mg L^{-1}), the biomass production was inhibited by 54% (from 0.190 to 0.103 g L^{-1}) but the TP precipitation was promoted. Nonetheless, it is important to mention that the protein content increased from 0.097 to 0.2017 g g^{-1} in presence of high concentrations of Zn (II) (X. Li et al., 2020).

According to Cheng et al. (2020) and Li et al. (2020), the concentration of Zn (II) in LW varies from 0.8 to 22 mg L^{-1}, resulting in a potential issue with obtaining high biomass production and nutrient removal efficiency by MbWT, due to the fact that high concentrations of Zn(II) might become toxic to microalgae by reducing cell division rates, chlorophyll content and adenosine triphosphate activity, even though the protein content could be increased. In this sense, it is important to establish a Zn (II) tolerance range for specific microalgae strains in order to optimize their performance on MbWT.

2.8 Strategies to enhance nutrient removal efficiencies and biomass productivity in MbWT.

There are several studies regarding the use of LW as a medium for microalgae growth, however, most of these have used different strategies to enhance the performance of MbWT, such as the pretreatment of LW (e.g., sterilization) and the implementation of acclimation stages for microalgae to improve their resistance to LW composition, among others (Ayre et al., 2017; Kim et al., 2016; Nagarajan et al., 2019; Qu et al., 2020). The most relevant strategies followed by researchers using a MbWT system are discussed below.

2.8.1 Acclimation

Extensive laboratory trials have demonstrated that microalgal growth and nutrient removal depends on the specific characteristics of the microalgae species. Factors, such as adaptability and biomass elemental composition, which substantially vary between species, can directly impact the success of the MbWT (Khalid et al., 2018).

An acclimation process involves random genetic transformations over multiple generations for a specific microorganism to survive. This process is also known as domestication. The domestication of microalgae allows for the adaptation of a wild strain in an environment with non-optimal conditions, such as those found in wastewater (Mingzi Wang et al., 2016). LW is an environment that may be toxic for some microalgal strains, especially those that have never been exposed to these effluents (Osundeko et al., 2014). In the acclimation period, the microalgae are exposed to gradually increasing concentrations of LW to enhance the development of specific genetic mutations that allow them to survive these conditions (Ayre et al., 2017).

Wang et al. (2016) reported the gradual domestication of *C. vulgaris* and *S. obliquus,* separately and in coculture, in medium with an increasing concentration of ADSW (25, 50, 75 and 100%, ADSW: water, v/v). A 20% (v/v) microalgal inoculum was added to the 25% ADSW solution and after 10 days of cultivation. When the microalgae were in an exponential growth phase, they were transferred into the 50% ADSW solution. This procedure was then repeated until the microalgae were able to grow in undiluted ADSW (100% ADSW) for at least 6 generations. The results presented by the authors indicated that these strains can be rapidly domesticated to secure and accelerate their growth in undiluted ADSW. The axenic cultures of *C. vulgaris* and *S. obliquus* displayed COD removal of 83.99% and 73.98%, respectively, while the coculture displayed a COD removal efficiency of 97.99%. However, to the best of our knowledge, few studies have been performed in order evaluate the effectiveness of a previous acclimation prior to the MbWT process. As shown in Table 2.8.1.1, there are different objectives for implementing an acclimation process for MbWT: (1) to obtain robust microalga strains resistant to environmental fluctuations, (2) to reach higher nutrient removal efficiencies, (3) to avoid the utilization of large amounts of fresh water, (4) to avoid expensive pretreatments such as sterilization and (5) to reduce culture times by the shortening of the lag phase. Although the acclimation process is time consuming, it is performed only on one occasion and its benefits are superior.

Table 2.8.1.1 Acclimation strategies for microalgae growth for nutrient removal in different types of wastewaters.

Wastewater source	Acclimation methodology	Microalgae strains acclimated	Improvements	Reference
Modified basal bold medium (similar composition of	Microalgae were acclimated for seven days. The strains with a robust growth were selected and inoculated in	*Chlorella protothecoides, Scenedesmus obliquus* and *Chlorella vulgaris* and a	The acclimated strains adapted better in wastewater from the meat processing industry and achieved	(Hu et al., 2019)

livestock wastewater)	wastewater for another seven days. Selected microalgae were reinoculated in wastewater to run the experiment.	consortium with the 3 strains.	significantly (P>0.05) higher biomass production. 91%, 67% and 69% of COD, TN and TP, respectively were removed with the acclimated consortium.	
Palm oil mill wastewater	Serial microalgae cultivation was carried out until non-significant changes were observed in growth parameters.	Native and commercial Chlorella *sorokiniana* strains	After three cycles of acclimation, the microalgae were able to reduce their lag time from eight to two days and their stress tolerance of CS-N improved in palm oil mill effluent.	(Khalid et al., 2018)
Livestock wastewater	Six microalgae strains were cultivated in 50% Jaworski's medium (JM) and 50% autoclaved wastewater. After, 1 ml of the cultures were diluted and 20 µl of this mix were cultured in agar plates for 14 days. Subcultures in untreated wastewater were carried out for 7 days to select the most tolerant strains. Another stage of subcultures was performed for 8 weeks.	*Chlamydomonas debaryana, Chlorella luteoviridis, Chlorella vulgaris, Desmodesmus intermedius, Hindakia tetrachotoma* and *Parachlorella Kessler*	The acclimated strains showed higher growth rate and biomass production. Most of the microalgae increased their photosynthetic activity. Also, this process was correlated with a higher accumulation of carotenoid pigments and higher ascorbate peroxidase activity.	(Osundeko et al., 2014)
Secondary treated municipal wastewater	For 14 days, the microalgae were cultured in secondary-treated municipal wastewater. Daily, 1 L of the volume was renewed to ensure the nutrient source.	*Chlorella vulgaris*	The acclimated strain displayed significantly higher chlorophyll content and the nutrient removal rate, increased at least 20% for the TN and TP, reaching a removal rate of 86% and 70% respectively.	(Lau et al., 1996)
Saline sewage	First, a rapid preliminary survival test was performed to select specific microalgal strains. These strains were kept in the exponential growth phase by transferring the cells to a new medium every week for 5 generations.	*Dunaliella tertiolecta*	After 8 days, 65% of overall nutrient removal rate was achieved. 80% Orthophosphate and 74% of the nitrate were removed. After acclimation the microalgae grew successfully in unsterilized saline preliminary sewage.	(Wu et al., 2015)

2.8.2 UV mutagenesis

Mutagenesis is an attractive approach to achieve an improvement in the breeding process of microalgal strains of interest. Mutagenesis can be induced by UV irradiation or by the application of chemicals, such as hydrogen peroxide, ethyl methanesulfonate and nitrosomethyl guanidine. Generally, UV irradiation is preferred over the application of chemicals due to the higher mutation rates that can be accomplished and to the simple manipulation and control of this method, which helps avoid contamination (Sivaramakrishnan and Incharoensakdi, 2017).

UV mutagenesis has been applied successfully to improve cell growth and lipid accumulation of several microalgal strains (Chu, 2017; Liu et al., 2015; Sivaramakrishnan and Incharoensakdi, 2017). The fatality rate is the proportion between disrupted cells and the viable cells, measured after the UV exposure. A fatality rate range of 75% to 90% is used as the criterion to select the best-mutated microalgal strains. Wang et al. (2016) applied UV irradiation with a 253.7-nm wavelength lamp placed at 15 cm above the culture to grow *C. vulgaris* and *S. obliquus* in undiluted and autoclaved SW with raw COD, TN and TP concentrations of 745, 289 and 23 mg L^{-1}, respectively, at a pH of 8.5. After an acclimation process, a UV exposure was implemented from 3 to 21 minutes every three minutes. The treated microalgae were rapidly domesticated to grow in undiluted SW, which they could not withstand before the two-stage method (acclimation and UV exposure). A biomass production of 1.80 g L^{1} was achieved when cultured in undiluted SW on the 6th day by *S. obliquus* and the 10th day by *C. vulgaris*, a value comparable with that obtained in BG-11 medium (1.83 g L^{1}). In addition, outstanding COD, TN and TP removal efficiencies were achieved, with values over 73%, 90% and 80%, respectively, in both microalgal species.

The induction of mutagenesis by UV irradiation could represent an alternative to improve the microalgal survival and nutrient removal efficiencies in LW. Nevertheless, this could mean an increase in the initial costs of MbWT. Therefore, further research is needed to evaluate its impact at a larger scale and to assess its viability.

2.8.3 Effluent pretreatment

Some microalgal strains are largely inhibited by the presence of various compounds that exist in LW, such as ammonia, volatile organic loads, salts and heavy metals, as discussed in previous sections. This inhibition could lead to low pollutant removal efficiencies and a limited biomass productivity (Franchino et al., 2016). Thus, the most common practice is to dilute the effluent before using it as culture media for microalgae. Xia et al. (2020) evaluated the nutrient removal efficiencies of *S. obliquus* using CW at different dilution ratios (1/3, 1/5, 1/10, 1/15 and 1/20). They observed that the highest growth was achieved at dilution ratios between 1/5 and 1/15. Lower dilution ratios were reported to cause inhibition associated with high concentrations of NH_3-N, while higher dilution ratios caused inhibition due to of the low concentration of TP, which is essential for the synthesis of intracellular metabolites (Xia et al., 2020). Although dilution represents a potential opportunity to enhance biomass productivity at a laboratory-scale, using fresh water is not a suitable strategy for MbWT at a larger scale, as it would require large water volumes, thus compromising the sustainability of the process.

Suspended solids also represent a huge challenge, as their presence hinders light penetration and reduces the photosynthetic activity of microalgae. Filtration is a common pretreatment method employed for LW, even more common than autoclaving or applying UV, in order to reduce the content of suspended solids prior to the MbWT and to enhance microalgal productivity. Salama et al. (2017) performed a study using *Chlorella* sp. 227 to treat MW. They found that, after filtration with a 0.45 µm membrane or after UV radiation with a dose of 1,600 mJ cm^{-2}, the microalgae biomass production was comparable to that of autoclaved wastewater (500 mg L^{-1}). However, a filtration with a 0.20 µm membrane resulted in a biomass production above 600 mg L^{-1}, whereas the biomass production decreased below 200 mg L^{-1}, with a UV irradiation below 540 mJ cm^{-2}, as this was not enough to inactivate indigenous bacteria and protozoa (Cho et al., 2011). This experiment shows that filtration can serve as an alternative to other pretreatments, such as autoclaving, to assure economic feasibility at a larger scale.

Table 2.8.3.1 Advantages and disadvantages of different pretreatments in Microalgae-based wastewater treatment using livestock wastewater on a larger scale.

Pretreatment	Advantages	Disadvantages	References
Dilution	Decrease turbidity and promote microalgae light absorption. Reduction of high organic concentrations that cause substrate inhibition for microalgae. Reduction of feedstock costs in microalgal culture when LW is diluted with other wastewaters.	Operational costs and environmental impacts are increased when fresh water is used.	(Xia et al., 2020; Xie et al., 2019)
Autoclaving	Elimination of competitive microorganisms that could present antagonistic relations with microalgae. Removal of suspended solids, debris, and colloidal particles of LW that might block the penetration of light.	Not economically feasible in a large-scale system, since it is energy intensive and time consuming.	(Sandefur et al., 2016)

	Can significantly improve substrate bioavailability.		
Filtration	Removal of physical, microbial, chemical pollutants and suspended solids that might avoid penetration of light. Efficient membranes are characterized by high pollutant rejection rates, great durability, high permeate flux, low maintenance cost and high resistance to chemicals. Converts concentrated LW into profitable byproducts.	Fouling and high energy consumption. The clarified liquid could lack sufficient nutrients for microalgal growth, depending on the type of filter used.	(Gupta et al., 2019; Sandefur et al., 2016)
UV irradiation	Decomposes many kinds of antibiotics, encouraging antibiotic-sensitive microalgae, like cyanobacteria, to grow. Facilitates subsequent treatment processes, such as biodegradation and mineralization. Used as disinfection in order to inactivate microorganisms that could affect the microalgae growth.	Energy feasibility needs to be investigated in order to evaluate its application from lab-scale to full industrial scale use. Highly increases the operational cost and its complexity when artificial UV light is applied.	(Ding et al., 2020; Gupta et al., 2019; Nagarajan et al., 2019; Zheng et al., 2021)
Sedimentation	Separates materials such as grease and oil. Mitigation of membrane fouling in filtration. Removal of some organic compounds. Leaves most of the nutrients that microalgae need to grow, like P and N.	Limited pollutant removal that could inhibit microalgae growth. Only removes solid coarse materials but does not degrade pollutants. Limited amount of primary sludge if not complemented with other methods like flocculation.	(Cho et al., 2018; Sandefur et al., 2016)
Centrifugation	Removal of competitive microorganisms that could affect the microalgae growth. Removal of suspended solids, debris, and colloidal particles of LW that might avoid penetration of light. Effective for wastewater with high concentrations of suspended solids, like LW, coupled with autoclaving.	Not economically feasible in a large-scale system since it is energy intensive and time consuming	(Gupta et al., 2019)
Flocculation/ coagulation	Improves sedimentation and filtration performance. Typically, effective in high-turbidity water like LW. Mitigation of membrane fouling in filtration. Could decrease COD concentrations in LW, a parameter that not all microalgae are able to remove. A cost-effective way to remove most of the suspended solids from LW.	Highly dependent on pH and temperature changes and increases operational costs.	(Amenorfenyo et al., 2019; Guo et al., 2018; Gupta et al., 2019)
Chemical flocculants	Present better performance than natural coagulants.	The sediment tends to be toxic.	(Kurniawan et al., 2020)

	Highly proven and effective in large-scale operations.	Induces a great pH decrease, therefore it needs to be modified before microalgae cultivation. Toxic trace materials can accumulate, having a genotoxicity impact or even lethal effects on some microalgae by direct DNA damage.	
Biocoagulants/ bioflocculants	No chemical residue produced. Creates non-harmful, biodegradable sludge, with high nutritional value for microalgae culture. Can have heavy metal removal properties. Applicable in remote areas. Lower toxic effects on microorganisms in anaerobic digestion and will thus not interrupt their performance. Safer, eco-friendly, and low-cost.	Produces five times lower volumes of sludge compared with inorganic salts. Increases the concentration of organic matter in the water. Mostly conducted at the research stage and laboratory scale. Has not been successfully commercialized due to the lack of scientific proof of their working mechanism and efficiency. Dependent on the abundance of raw materials.	(Kurniawan et al., 2020)

As is shown in Table 2.8.3.1, all the pretreatment methods in combination and individually display advantages and drawbacks that need to be overcome to allow their large-scale implementation. Currently, the most used pretreatment process is dilution, but this technique compromises the sustainability of the MbWT if fresh water is used. As a consequence, the authors proposed the dilution of LW with other wastewater effluents with lower nutrient concentrations, such as MW and brewery wastewater. This strategy presents three main advantages: 1) the maintenance of the process sustainability, 2) the dilution of compounds presents in LW that may inhibit microalgae growth and 3) the addition of other nutrients that are scarce in LW (e.g., adding brewery wastewater to increase C/N ratios). On the other hand, if the other wastewater sources are not geographically close to the microalgae unit, this strategy could represent a threat to achieving economic feasibility due the additional costs of effluent transport.

2.8.4 Mixed effluents

As mentioned above, dilution is an effective method to reduce the toxicity of LW. However, using freshwater for dilution is not a sustainable option because of the scarcity of this natural resource. An alternative for dilution is mixing wastewater from different sources to achieve an optimal medium for microalgal development in MbWT (Cui et al., 2020).

Lu et al. (2016) increased the biomass yield of *C. vulgaris* from 1.32 to 2.66 g L^{-1} after mixing dairy wastewater with slaughterhouse wastewater (1:1). The former had lower NH_3-N concentration (48 mg L^{-1}) than the latter (307.5 mg L^{-1}). When these types of wastewaters were mixed, a NH_3-N concentration between 151.3 and 172.3 mg L^{-1} was obtained (Lu et al., 2016). Also, Cui et al. (2020) followed this strategy to mitigate excessive NH_3-N concentrations, they observed that *C. sorokiniana* was not able to grow in undiluted PW but reached a biomass yield of 45.9 mg L^{-1} with the dilution of PW with MW at a ratio of 1:3. This dilution decreased the ammonia concentration from 636.1 to 318.3 mg L^{-1}.

A different study performed with the same microalga (*C. sorokiniana*), but using SW mixed with MW, reached a biomass yield of around 1 g L^{-1}, with average removal efficiencies of dissolved inorganic carbon (DIC), PO_4^{-3} and NH_3-N of 46-56%, 40-60% and 100%, respectively (Leite et al., 2019). Yao et al. (2015) carried out a similar study, using a mixture of SW and MW at a 1:3 ratio as a medium to grow *C. sorokiniana* and *Desmodesmus communis.* The SW had TN and TP concentrations of 632.98 and 61.53 mg L^{-1}, and after mixing with MW, these concentrations decreased to 188.61 and 15.77 mg L^{-1}, respectively. The biomass final concentrations were 1.22 g L^{-1} for *C. sorokiniana* and 0.84 g L^{-1} for *D. communis*. The removal efficiencies of TN were 88.05% for *C. sorokiniana* and 83.18% for *D. communis,* while for the TP removal efficiencies were above 99.5% for both microalgae (Yao et al., 2015).

As shown in previous examples, MW has been widely utilized for LW dilution due to its relatively low nutrient concentration. However, other types of effluents have been explored. Zheng et al. (2018) enhanced nutrient removal and microalgal growth in SW by mixing it with brewery wastewater (1:5) to grow *C. vulgaris*. As previously mentioned, an important parameter to consider for MbWT is the C: N ratio, and, in this case, the addition of the brewery wastewater modified the C: N ratio of the SW from 1.0 to 7.9 and enhanced the performance of the microalga for the removal of the nutrients (Zheng et al., 2018). López-Pacheco et al. (2019) cultured microalga, *A. máxima* and *C. vulgaris,* separately in a mixture of SW, nejayote wastewater (effluent from corn nixtamalization) and freshwater. A significant reduction of pollutants was reported. *A. maxima* reached a cell concentration of 32×10^4 cell mL^{-1}, and removed 92%, 75% and 96% of TN, TP and COD, respectively. *C. vulgaris* reached a cell concentration of 128×10^6 cell mL^{-1} and removed 91%, 85% and 96% of TN, TP and COD. Furthermore, different mixtures of SW and wine wastewater (WW) (20:80, 50:50, 80:20, 100:0 and 0:100, SW: WW, v/v) were tested for MbWT with *Chlorella.* The TN and TP removal efficiencies were 41% and 3%, respectively, for the culture grown at 100% SW, while the culture with the best results was grown at 20:80 (SW: WW) and displayed TN and TP removal efficiencies of 90% and 56%, respectively, after 10 days of culture (Ganeshkumar et al., 2018).

Mixing different wastewater sources represents a sustainable alternative to ensure high nutrient removal efficiencies and biomass productivity of microalgae by improving several parameters, such as an optimal NH_3-N concentration and C: N ratio. However, the transport of large volumes of wastewater for MbWT can result in tangible disadvantages for large-scale application. This option only becomes sustainable when the mixing wastewater source is geographically near the LW source. To overcome this challenge, geographical and spatial factors, such as transportation distances, road infrastructure and environmental features, must be taken into consideration for the establishment of large-scale MbWT units.

2.8.5 Immobilized cultures

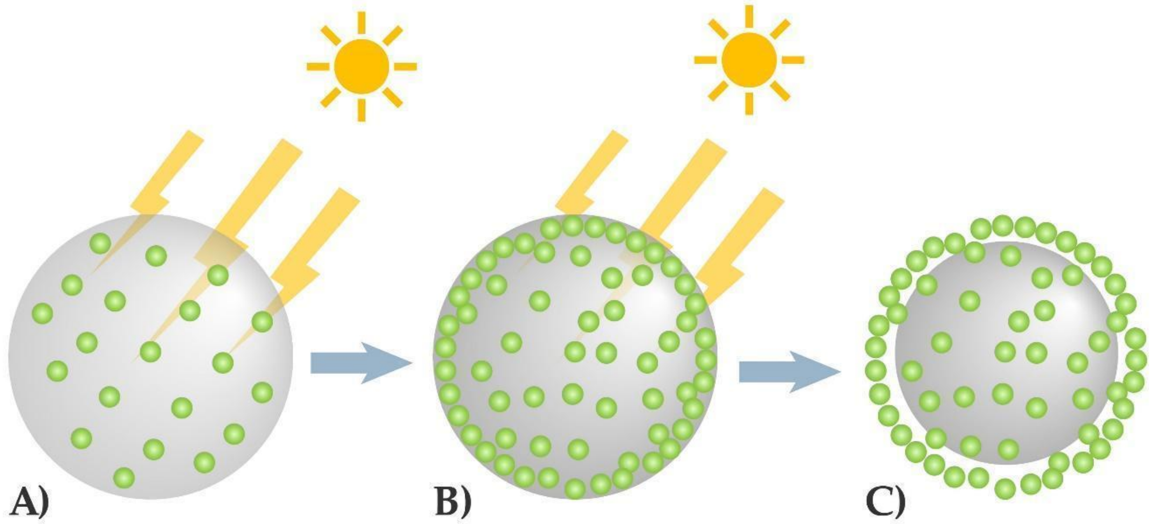
Cell immobilization offers a solution for the harvesting process, which involves an important fraction of the total MbWT costs and enhances nutrient removal efficiencies. The most common matrixes used for microalgae immobilization are alginate and carrageenan polymers, and, to a lesser extent, materials such as agar, chitosan, acrylamide, polyurethane and polyvinyl (Cuellar-Bermudez et al., 2017).

The enhancement of nutrient removal efficiencies with immobilized microalgae is attributed to the chemical interactions between the alginate matrix and the nutrients in the medium. Alginate presents a high affinity for divalent cations, such as Pb (II), Cu (II) and Cd(II), among others, due to its carboxyl group (Shengye Wang et al., 2016). Additionally, the physical adsorption of NH_3-N and PO_4^{-3} ions to the matrix is a step that facilitates microalgal assimilation. Furthermore, immobilized systems have been reported to be less sensitive to external conditions, like temperature and pH, and more tolerant to microbial contamination. Another advantage of immobilized microalgae is the delay of pheophytinization. This phenomenon is presented under stress conditions and is related to the replacement of the Mg^{2+} ion found in the chlorophyll molecule with H^+ ions, resulting in acidification of the system, which leads to a decrease in biomass growth and chlorophyll concentrations (Prathima Devi and Venkata Mohan, 2012). In research conducted with *Euglena gracilis*, pheophytinization occurred in 7 days. Conversely, when immobilized with calcium alginate matrix, the phenomenon presented after 3 months (Banerjee et al., 2019; H. Lee et al., 2020; Tamponnet et al., 1985).

The nutrient removal efficiencies reported when using alginate-immobilized microalgae bead systems are higher than those observed for microalgal-free suspension cultures. In a study performed by Banarjee et al. (2019), increases on the NH_3-N and TP removal efficiencies of 15% and 31% were observed when *C. vulgaris* was immobilized, compared with the same strain in free suspension culture. Another study, using the same immobilized strain for the MbWT of MW, reported removal efficiencies of 95% for NH_3-N and 99% for PO_4^{-3}, while the culture in free suspension displayed removal efficiencies of 50%

and 40%, respectively (Banerjee et al., 2019). The immobilization of a microalgae-bacteria consortium was also explored to enhance nutrient removal in wastewater effluents. The consortium involved the bacteria *Pseudomonas putida* and the microalga *C. vulgaris* immobilized in alginate, and it proved to enhance cell growth and nutrient removal levels. The co-immobilized consortium displayed higher cell concentrations (6.65×10^{6} cells mL^{-1}) in comparison to the free-suspended microalgae consortium (5.70×10^{6} cells mL^{-1}) and the single immobilized treatment (5.688×10^{6} cells mL^{-1}). Additionally, the COD removal efficiency was also higher in co-immobilized treatment (97%) than in the free-suspended treatment (92%) (Shen et al., 2017).

The physical adsorption of NH_3-N and PO_4^{-3} is dependent on the alginate concentration in the matrix. Banerjee et al. (2019) observed that the optimal alginate concentration to enhance nutrient removal was 3% (w/v %) when using *C. vulgaris* to treat MW. In contrast, other studies reported that the immobilization of microalgae can result in growth limitations due the shortage of space and light in the matrix. To avoid the effects of the formation of a shading layer (Fig. 2.6.1.1), the diameter of the beads should be defined for specific microalgal strains. The biomass productivity with immobilized *C. vulgaris* in alginate beads of a 2 mm diameter was not affected by the formation of a shading layer (Cao et al., 2020). In contrast, Lee et al. (2020) reported an optimal alginate bead size of 3.5 mm to improve nutrient removal using *C. vulgaris* and *Chlamydomonas reinhardtii.* TP removal increased from 17.3% (displayed by the suspension culture) to 65.3% (displayed by the alginate bead size of 3.5 mm). Similarly, the TN removal increased from 19% (suspension culture) to 55.5% (alginate bead size of 3.5 mm). These authors observed that, above the optimal alginate bead size (5 mm), a shading layer (Fig. 2.8.5.1.B) is formed creating an empty zone at the center of the bead, which affects microalgal growth. Chlorophyll *a* (an indicator of cell activity) decreased below the optimal alginate bead size (2 mm).



A) Initial stage of immobilized bead, no microalgae cells are released, and light has optimal penetration inside the bead; B) Formation of shading layer and some microalgal cells begin to release into the medium; C) Softening of immobilizing matrix and most microalgal cells release to the medium.

Fig. 2.8.5.1 Shading layer formation inside immobilizing matrix and microalgae release. Adapted from Banerjee et al. (2019).

Immobilizing matrices promote biomass recovery, which is one of the major challenges for the large-scale application of MbWT. Nevertheless, the beneficial and deleterious interactions between microalgae, the immobilizing matrix and the LW medium are still unknown. There is a need for further studies using LW to establish the optimal immobilizing material and its concentration, bed size and its influence on microalgal growth for the escalation of MbWT to save operational costs by avoiding other recovery techniques, such as centrifugation or flocculation.

2.8.6 Multi-stage processes

Multi-stage cultivation processes have been implemented focusing mostly on the enhancement of lipid accumulation in microalgae (Nagappan et al., 2019; Singh et al., 2016). However, the implementation of additional cultivation stages for MbWT treatment can also enhance nutrient removal. This strategy can be implemented to take advantage of the principal characteristics of different microalgal strains. As mentioned before, *C. vulgaris* displays a high tolerance to NH_3-N (125-1300 mg L^{-1}), while *Spirulina* spp. is often used to treat effluents with low ammonia concentrations (< 40 mg L^{-1}). Because of these characteristics, both microalgae were used sequentially in a two-stage cultivation system, where *C. vulgaris* was used in the first stage to remove nutrients (especially NH_3-N) from ADPW, and *S. platensis* was cultured in the second stage to metabolize the remaining nutrients and produce biomass. The initial concentrations of pollutants in the ADPW were 179, 3131 and 1058 mg L^{-1} of TP, TN and COD, respectively. Prior to the first cultivation stage, the ADPW was diluted to adjust the NH_3-N concentration to values tolerated by *C. vulgaris* (125-1300 mg L^{-1}). At the end of the second stage, the removal efficiencies of TOC, NH_3-N and TP were 72%, 100% and 83%, respectively, higher than those of the first stage (55%, 19% and 17%, respectively). The biomass accumulation of *S. platensis* was 10 times higher than that of *C. vulgaris.* Overall, with this two-stage system, a biomass production of 3.84 g L^{-1} was reached, and the removal efficiency was the one obtained in the second stage (Wang et al., 2018).

Lv et al. (2018) treated undiluted CW using a two-stage cultivation process, where two different processes (A and B) were performed. Process A consisted of a biological treatment where *C. vulgaris* was cultured in both stages. Whereas, in process B, the first stage consisted of the biological treatment via *C. vulgaris* followed by physical adsorption by activated carbon. Process A drastically reduced nutrients from CW on the 1st day, but few nutrients were removed on the 2nd day, resulting in the second stage where the effluent was used to cultivate fresh *C. vulgaris* again. Removal efficiencies of 92%, 94%, 98% and 94%, were achieved for COD, NH_3-N, NO_3 and TP, respectively, in 5 days. In process B, higher nutrient removal efficiencies are associated with higher dose of granular activated carbon (except the removal efficiency of TP). In general, after 3 days of treatment via the process B, 91%, 83%, 98% and 91% of COD, NH_3-N, NO_3 and TP were removed when the dose of granular activated carbon was 80 g L^{-1}. In contrast, a single stage MbWT system usually takes from 10 to 30 days to reach similar results.

In a recent study, the same two-stage cultivation strategy was implemented for the treatment of anaerobically digested municipal wastewater (ADMW), using *C. sorokiniana* in the first stage and a floating macrophyte, *Lemma minor,* in the second stage. The microalgae completely removed COD and partially removed TN and TP nutrients, while *L. minor* contributed mainly to nitrogen removal. The partial removal of TN by the microalgae was attributed to the depletion of TP, for a high N:P ratio (20:1) in MW was observed in comparison with the reported ideal mass ratio of N:P for microalgae (5:1). On the other hand, ammonium nitrogen is the favorite form of nitrogen for *Lemna minor*, a duckweed plant that significantly improved the TN removal. With a hydraulic retention time of 3 days per stage, they removed, on average, 99% of COD, 90% of both NH_3-N and TN, and 91% of the PO_4^{-3} (Kotoula et al., 2020).

The implementation of a multi-stage cultivation process could overcome the main challenges of MbWT, such as high retention times and the dilution required to optimize nutrient removal and biomass productivity. Retention time tends to decrease with the implementation of additional stages, using microalgal strains or even macrophytes, because the second stage complements the first, as seen in the previous examples. However, this strategy has been tested using MW, which has lower concentrations of TN, TP and COD in comparison to LW. Further research on multi-stage cultivation processes is needed to assess the economic and technical viability of MbWT to treat LW.

2.9 Circular bioeconomy

The availability of freshwater is a challenging factor associated with the production of microalgal biomass because environmental concerns are raised regarding the scarcity of this resource. In addition, the nutrients supplemented for microalgal biomass production also affects the feasibility and sustainability of microalgae-based products. Studies have confirmed that the production of 1 ton of microalgal biomass needs ~0.05 ton of N, 0.01 ton of P and 1.88 ton of CO_2 (Renuka et al., 2021). Although N is available in abundance in the atmosphere, P is a finite resource, and due to its high demand, it is expected that in 2070 there will be a deficit of P with a high possibility of depletion within 100 years from now (Díaz-Vázquez et al., 2020; Nagarajan et al., 2020). Among the microalgae-based products, biofuels have gained popularity because of their high productivity and quality, with the potential for large-scale operations (Renuka et al., 2021). However, microalgae-based biofuels are not economically competitive to fossil fuels (Díaz-Vázquez et al., 2020). Literature has reported that the feedstock cost for microalgal culture directly influences the economic feasibility of biocrude production, and this cost can be reduced by 35–86% with the use of nutrients from waste sources, such as like LW (Renuka et al., 2021). LW is rich source of water and nutrients, which is frequently released without the proper treatment, or recovery, into the environment (Nagarajan et al., 2020). Around 50–80% of the wastewater generated globally is discharged into water sources, and, on average, only 40% of the wastewater generated worldwide is treated with a variation of 8% and 70%, when comparing low-income and high-income countries, respectively, which results in several environmental issues that were previously discussed here (health problems in humans and wildlife, eutrophication and greenhouse gas emissions, among others) (Renuka et al., 2021).

Circular bioeconomy is the accomplishment of a sustainable production by using biological resources as feedstock, such as LW, in innovative processes like MbWT to produce biomaterials, bioenergy and other bioproducts, while optimizing the resources needed in the system in a closed loop, to lower the consumption of virgin resources (Leong et al., 2021; Nagarajan et al., 2020; Ubando et al., 2020). Additionally, the biorefinery approach, from a perspective of circular bioeconomy, refers to an infrastructure facility wherein several conversion technologies (thermochemical, biological, biochemical, and mechanical) are integrated to produce sustainable bio-based products, such as biofuels, biochemicals, biomaterials, bioenergy and other high-valued compounds (Leong et al., 2021). This model aims to mitigate the effects of climate change by recovering or recycling renewable carbon sources (biomass) and nutrients, while creating business and employment opportunities (Ubando et al., 2020). MbWT has gained attention for its potential as a circular bioeconomy approach due to several advantages, such as the fixation of CO_2, high biomass growth rates and the ability of microalgae to store carbon in both carbohydrate and lipid forms for the recovery of biofuel and value-added products (Rajesh Banu et al., 2020).

Furthermore, Fuentes-Grünewald *et al*., 2021 (Fuentes-Grünewald et al., 2021) demonstrated that producing microalgae biomass through the bioremediation of excess nutrients in wastewaters is, at the same time, a reliable and consistent way to ensure a quality suitable for animal feed. These authors applied a two-step approach, where autotrophic and mixotrophic growth were combined with the goal of producing a high concentration of microalgal biomass with an improved profile of macromolecules. In this manner, a biorefinery approach was implemented, where wastewater was valorized for several applications, including the production of biofuels and high value-added products. In addition, several advantages were achieved, such as nutrient recycling and clean water acquisition, mitigation of environmental footprints related to conventional wastewater treatment, maintenance of ecological balance in aquatic systems and CO_2 sequestration (Renuka et al., 2021). (Leong et al., 2021; Nagarajan et al., 2020; Renuka et al., 2021; Leong et al., 2021)).

Therefore, the biorefinery scheme proposed consists of using MbWT for LW, to consequently extract valuable compounds from the biomass obtained, such as pigments, polysaccharides, proteins, lipids or metal nanoparticles that can then be used in the health, cosmetics, biochemical, food and animal feed, biomaterials, and biofuels industries, as previously detailed in this document.

However, the production of biomass through MbWT, and its utilization as a renewable carbon source for bio-based product streams, is limited by its supply and conversion yields, which in turn depends on the LW´s variable composition, as well as on the microalga strain used and its adaptability and several physical, chemical, and environmental factors. Process optimization must be implemented to enhance nutrient recovery and biomass and compound production and for cost reduction.

2.10 Conclusions and future perspectives

MbWT of LW represents a sustainable solution to remove pollutants from LW at a low cost while generating valuable products. Recent advances for the enhancement of biomass productivity, pollutant removal and production of high-value compounds were discussed in this review.

There is wide variation between microalgal species, and each microalga possess specific characteristics that make them uniquely suitable depending on the goal. The mixotrophic microalgae, specifically from the phylum Chlorophyta, such as *C. vulgaris, C. regularis* and *H. pluvialis*, have demonstrated to be highly efficient at nutrient removal from LW, while still producing high-added value products for the market (e.g., biomass rich in protein, pigments and carotenoids, among others). Most of the available studies have been performed using monocultures. However, from a large-scale perspective, it is difficult to maintain sterile conditions, especially in open-ponds systems. Hence, the examination of microalgae consortia with other microorganisms is a promising study area that has not been explored with the same depth as monocultures. More investigation is thus needed regarding cocultures used for MbWT, for these consortia with different individual capabilities can complement each other, resulting in better treatment efficiency and more robust systems, which are resistant to a wide variety of physicochemical conditions.

Additionally, the modulation of physical and chemical factors and the implementation of different strategies are required to ensure microalgal growth and pollutant removal efficiencies in different types of LW sources. Not only do the nutrients profile vary between different types of animal effluents, but feed composition, housing methods and several regional environmental factors can also be determinants, which may negatively affect the implementation of MbWT for LW using a large-scale system. To overcome these challenges, different strategies have been implemented, such as acclimatization, UV light supplementation and effluent pretreatment, among others. Furthermore, the design and development of simulation programs for the optimization of MbWT systems for LW, including the different variables presented herein, such as the microbial, physical and chemical factors, could help to increase the cost-effectiveness of the process.

Some efforts have been conducted to assure the technical feasibility for MbWT, such as modifications in temperature, light intensity, pH and C: N ratio, among others. However, most of these studies were performed under highly controlled environments in laboratories and few studies have been developed from a holistic perspective, where all those parameters are evaluated simultaneously in a real, working MbWT. Beyond the specific parameters that guarantee an adequate performance at the laboratory scale, the applicability of a MbWT at a larger scale could depend on external factors, such as environmental or local-specific aspects. Studies on those factors, especially the latter, are the next step to make the MbWT a mature technology. For the maturation of MbWT, future studies should focus on: 1) large-scale trials applied under biorefinery approaches, 2) downstream design, 3) different strategies of biomass harvesting, such as immobilized cultures and 4) socio-cultural factors affecting the implementation of MbWT.

Chapter 3. Microalgae-mediated bioremediation of cattle, swine and poultry digestates using mono- and mixed-cultures coupled with an optimal mixture design

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Abstract

Microalgae-based wastewater treatment (MbWT) has been proposed as a promising approach to revalorize anaerobically digested effluents (digestates) from the livestock sector, resulting in the production of high added-value products, while reducing pollutants in the digestates. This study is the first to address MbWT using digestate mixtures of different species (swine, cattle, and poultry). A centroid mixture design was used to determine the optimal mixture to promote higher cell concentrations and pollutant removal efficiencies of the microalgae *Chlorella vulgaris, Haematococcus pluvialis* and *Chlamydomonas* sp. cultured as mono-, bi-, and tri-cultures. The best results, obtained from the mixture design, were achieved using *C. vulgaris* as a monoculture in a digestate mixture of 0.125:0.4375:0.4375 (ADSW:ADPW:ADCW), which resulted in a cell growth of $3.61\times10^{7}\pm 2.81\text{x}10^{6}$ cell mL^{-1}, a total nitrogen removal of 85%±2%, a total phosphorus removal of 66%±3% and a chemical oxygen demand removal of 44%±7%. The optimal mixture design, using the global performance index (GPI), suggested that using a mixture of 0.125 ADSW, 0.200 ADPW and 0.675 ADCW will promote higher cell growth and pollutant removal efficiencies using *C. vulgaris* as a monoculture. Additionally, a redundancy analysis was performed to analyze the correlation between microalgal cultures and the removal efficiencies of the digestate pollutants. The results herein suggest that the specific composition of the effluents plays a key role in microalgal performance due to their respective nitrogen and phosphorus content. Furthermore, this study suggests that a mixture of the three most common digestates generated by livestock farms offers a promising alternative for the treatment and revalorization of LW, by taking advantage of the unique composition that each digestate possesses. Further studies are warranted to gain a deeper understanding of the interspecific microalgal interactions occurring in mixed cultures that may enhance or hinder the performance of MbWT.

Keywords: Microalgae-based wastewater treatment, livestock wastewater, mixture design, *Chlorella vulgaris, H. pluvialis, Chlamydomonas* sp.

3.1 Introduction

The global population is expected to grow to almost 10 billion by 2050, resulting in increased pressure on the agricultural and livestock production systems to satisfy the heightened demand. Furthermore, it has been projected that low- and middle-income countries will display economic growth and, thus, a dietary transition towards higher consumption of animal-derived products is expected to double the current consumption by 2050 (FAO, 2018; Madeira et al., 2017). The livestock industry contributes approximately 14.5% of the anthropogenic greenhouse gas (GHG) emissions. GHG emissions from this sector represent 9% of global carbon dioxide emissions, 37% of global methane emissions, and 65% of global nitrous oxide emissions (Fu et al., 2021). Additionally, the livestock industry occupies 40% of the arable land, and 10% of the global anthropogenic water use is consumed by the livestock industry (Mottet et al., 2017; Sakadevan & Nguyen, 2017b; Weindl et al., 2017). Furthermore, livestock wastewater (LW) can significantly contribute to diminished surface water quality and the eutrophication of water bodies (Díaz-Vázquez et al., 2020).

LW is a mixture composed mainly of excrement, urine, feed residues and sewage from the cleaning of the stables (López-Sánchez et al., 2021). The disposal of these effluents without adequate treatment represents a serious human health and environmental hazard due to their high concentrations of organic matter and pathogens (Chen et al., 2020; Tak et al., 2015). Amongst the most common methods employed for LW treatment, anaerobic digestion is valued due to its efficient pollutant removal and its potential to produce biogas. However, the effluents from anaerobic digesters, referred to as digestates or anaerobically digested livestock wastewater (ADLW), are often still rich in nitrogen (N), phosphorus (P) and other nutrients (Xu et al., 2015b). The most common sources of LW are swine, cattle and poultry effluents, due to their high production densities worldwide. Therefore, the composition of ADLW is highly influenced by these sources. While the anaerobically digested swine livestock wastewater (ADSW) tends to display a higher concentration of chemical oxygen demand (COD), the anaerobically digested poultry wastewater (ADPW) generally displays higher concentrations of total phosphorus (TP) and total nitrogen (TN) (Cai et al., 2013; Cheng et al., 2020; Köster et al., 2015).

Considering the nutrients available in the ADLW, microalgae-based wastewater treatment (MbWT) has been widely accepted as a promising treatment method, which is rooted in a circular bioeconomy approach. Circular bioeconomy seeks to produce higher added-value products from reused or recycled material of biological sources, through innovative processes and principles, in order to maximize value and reduce biological waste (Ummalyma et al., 2021). Microalgae are microorganisms that display high photosynthetic efficiency and rapid growth without the need for arable land. These microorganisms can use natural light to fix CO_2, and they can use nutrients, such as N and P, from ADLW for their metabolism (Li et al., 2021). Moreover, they can produce high added-value products, such as polysaccharides, biopeptides, biopolymers, antioxidants and pigments (Moreira et al., 2021).

Mixing different types of LW is an approach that has been used to enhance the growth medium for microalgal breeding. For instance, Lu et al. (2016) reported an improvement in the biomass growth yield of *Chlorella vulgaris* of almost 130% when cultured in a mixed media (1:1) composed of dairy wastewater (48 mg L^{-1} of NH_3-N) and slaughterhouse wastewater (307.5 mg L^{-1} of NH_3-N), compared with the same microalga cultured only in dairy wastewater. By mixing these effluents, a concentration of NH_3-N between 151.3 and 172.3 mg L^{-1} was obtained, and nitrogen inhibition was avoided. Although effluents from cattle, swine and poultry are frequently generated in the same livestock production unit, to the best of our knowledge, the study of a blend of these effluents using a MbWT approach has never been addressed. As contribution to the literature, this is the first study in which MbWT has been implemented to treat a mixed effluent composed by the most common ADLW (from cattle, swine, and poultry). Hence, the present study aims to understand the effects of the mixture of all three types of LW on the cell growth and pollutant removal efficiencies of microalgal cultures (*Chlorella vulgaris, Haematoccocus pluvialis* and *Chlamydomonas* spp.). Through an evaluation of the mixture design, the optimal fraction of these different types of effluents (ADCW, ADSW, and ADPW) was analyzed, in order to obtain maximum microalgal biomass productivity and pollutant removal rates (COD, TN, and TP). Furthermore, these microalgae were tested in all possible combinations of mono-, bi-, and tri-cultures. *C. vulgaris* and *Chlamydomonas* spp. were used in this study because, according to the literature, these strains have shown an intrinsic tolerance to ADLW (Cao et al., 2018; Escudero et al., 2014; Marjakangas et al., 2015; Salama et al., 2017; M. Wang et al., 2016b; Y. Wang et al., 2015). *H. pluvialis*, on the other hand, was used due to its ability to synthesize astaxanthin, a highly valuable pigment; yet, before the work herein, this alga had only been tested using untreated LW (Ledda et al., 2016b; Shah, 2019) Prior to performing these experiments, gradual domestication and UV mutagenesis were applied on the microalgae to enhance their adaptability to LW medium and, thus, cell growth and the removal of pollutants.

3.2 Materials and methods

3.2.1 Microalgal species and pre-culture conditions

Axenic microalgae cultures of *Chlorella vulgaris* (CV) and *Haematoccocus pluvialis* (HP) were obtained from the 'Algae Bank S.A. de C.V. ' in Guadalajara, Jalisco, Mexico (Algae Bank, 2021). *C. vulgaris* was pre-cultured in BBM medium composed of 0.25 g $NaNO_3$, 0.075 g $MgSO_4 \cdot 7H_2O$, 0.025 g $CaCl_2 \cdot 2H_2O$, 0.175 g KH_2PO_4, 0.075 g K_2HPO_4, 0.025 g NaCl, 0.00498 g $FeSO_4 \cdot 7H_2O$, 0.01 g Na_2EDTA, 8.05 mg H_3BO_3, 1.81 mg $MnCl_2 \cdot 4H_2O$, 0.222 mg $ZnSO_4 \cdot 7H_2O$, 0.079 mg $CuSO_4 \cdot 5H_2O$, 0.390 mg $NaMoO_4 \cdot 5H_2O$ and 0.0494 mg $Co(NO_3)_2 \cdot 6H_2O$ (per liter) (Wu et al., 2012). *H. pluvialis* was grown in NIES medium with the following composition: 0.15 g $Ca(NO_3)_2$, 0.10 g KNO_3, 0.05 g β-glycerophosphoric acid disodium salt pentahydrate, 0.04 g $MgSO_4 \cdot 7H_2O$, 0.50 g Tris-aminomethane, 0.01 mg thiamine, 3.00 mL PIV metal solution, 0.10 µg biotin and 0.10 µg vitamin B12. One liter of PIV metal solution consists of 1.0 g Na_2EDTA, 0.196 g $FeCl_3 \cdot 6H_2O$, 36.0 mg $MnCl_2 \cdot 4H_2O$, 22.0 mg $ZnSO_4 \cdot 7H_2O$, 4.0 55 mg $CoCl_2 \cdot 6H_2O$, and 2.5 mg $Na_2MoO_4 \cdot 2H_2O$ (per liter) (Fitriana et al., 2021).

The strain *Chlamydomona* spp. (Chl) was obtained from 'Proteína Animal S.A. de C.V.' in Lagos de Moreno, Jalisco, México. This microalga was pre-cultured in the BBM medium, as described above. All the microalgae were initially grown as monocultures in transparent 500 mL Erlenmeyer flasks containing their respective medium (300 mL). Prior to cultivation, all media and Erlenmeyer flasks were sterilized (121°C for 21 min). The cultivation conditions were as follows: white fluorescent light illumination at an intensity of 150± 1 µmol photons m^{-2} s^{-1}; continuous light regimen; a temperature of 25°C ± 2; an air supply rate of 0.5 L/min.

3.2.2 Composition and pretreatment of livestock wastewater

ADSW, ADCW and ADPW used in this study were collected from 'Proteína Animal S.A. de C.V.' (***PROAN*, 2021**), the biggest farm of laying hens in Latin America, located in Lagos de Moreno, Jalisco, México. The samples were stored at 4°C while in transit to the laboratory. Once in the lab, samples were then stored at -20 ºC for less than 2 days prior to their processing. The samples were analyzed by the Analytical and Metrologic Services Unit (USAM) of the 'Centro de Investigación y Asistencia Tecnológica del Estado de Jalisco' (CIATEJ) based on methods established by the American Public Health Association **(Eaton & Franson, 2005)** to determine the concentrations of biochemical oxygen demand (BOD), fat, oil and grease (FOG), sedimentable and total suspended solids (TS, TSS, respectively), total Kjeldahl nitrogen (TKN) and total phosphorus (TP). Prior to the culture experiments, the non-soluble solids were removed by sedimentation and subsequent filtration through a nylon filter cloth. The pH of the supernatant was adjusted to 7.0 using a HCl solution and then sterilized (121° for 21 min). According to previous studies, the presence of native microorganisms present in LW tends affect the metabolism of the microalgal cultures employed for MbWT, thus, a sterilization process at 121°C for 21 min was performed prior to further experimets (Covarrubias et al., 2011; Tejido-Núñez et al., 2019).

3.2.3 Experimental design

3.2.3.1 Gradual domestication and UV mutagenesis

For gradual domestication, the microalgae were inoculated on LW mixtures with the volumetric fractions shown in Table 3.2.3.1. An inoculum of 15% (v/v) was employed in each level of this process, for each microalgal strain, when each was in the exponential phase. The inoculum for level 1 were taken directly from the precultures described in section 2.1, and the inoculums for levels 2 through 7 were taken from the previous levels. In level 5 of the acclimation process, the UV mutagenesis method was employed to improve the adaptive response of the microalgae to the ADLW. During the acclimation, the ADLW was composed of all three anaerobically digested effluents (ADSW, ADCW, ADPW) in a ratio of 1:1:1.

Table 3.2.3.1 Volumetric fractions of the artificial medium, water and anaerobically digested livestock wastewater (ADLW) used for the acclimation process.

Level	Artificial medium[a]	Distilled water	ADLW[b]
1	0.5	0.3	0.2
2	0.3	0.4	0.3
3	0.2	0.4	0.4
4	0.0	0.5	0.5
5	0.0	0.3	0.7
6	0.0	0.0	1

a. For the strains *C. vulgaris* and *Chlamydomona* sp., BBM medium was used, and for *H. pluvialis*, NIES medium was employed.
b. The ADLW was composed of ADSW:ADCW:ADPW (1:1:1).

Methodology proposed by Wang et al. (2016a) was followed for the UV mutagenesis process. Before performing UV mutagenesis, samples of the microalgal cultures in the exponential phase were aliquoted into sterile petri dishes (in triplicate) and placed under a UV lamp using a wavelength of 253.7 nm (UVC) at 15 cm and an exposure time of 12 min. The cell density was counted using a Neubauer chamber before and after exposure to the UV light to determine the fatality rate. The fatality rate (FR) was calculated using Equation 1, where $\boldsymbol{y_0}$ is the cell concentration before the UV exposure and $\boldsymbol{y_1}$ is the cell concentration after the UV mutagenesis has been implemented. A FR between 75 and 90% was chosen to indicate substantial mutagenic modifications. The microalgae that displayed an adequate FR were placed in the dark for 5 h to avoid light repair after the UV light exposure (Liu et al., 2015).

$$\boldsymbol{FR}\ [\%] = \frac{(\boldsymbol{y_0} - \boldsymbol{y_1})}{\boldsymbol{yo}} * \mathbf{100}$$

Fatality rate calculation (1)

After the UV mutation process, the microalgae were recovered from the Petri dishes to be cultured in triplicate following levels 4, 5 and 6 of the acclimation processes (Table 3.2.3.1) (at ratios of 50%, 70%, and 100% ADLW: distilled water). The dilution level that showed the highest cell concentration after 7 days of culture was further implemented for the optimal mixture design experiments.

3.2.3.2 Mixture design

Seven mixtures (combining all three types of LW) were prepared in duplicate according to a centroid mixture design of experiments with three factors (fractions of ADSW, ADCW and ADPW, respectively; Table 3.2.3.2). This design does not consider pure blends; it instead includes different combinations of all three types of LW ranging from a fraction of 0.125 to 0.750 for each effluent. Considering the results of the gradual domestication and the UV mutagenesis process, all the runs of the optimal mixture design were diluted with 30% of distilled water. Subsequently, these mixtures were inoculated with all three microalgae and a total of 98 runs were performed to test all possible combinations of the microalgae (*Chlamydomonas* sp., *C. vulgaris* and *H. pluvialis*) in mono-, bi- and tri-culture. Four response variables were considered: (1) cell growth (cell mL^{-1}), (2) total nitrogen removal (TN; %), (3) total phosphorus removal (TP; %) and (4) chemical oxygen demand removal (COD, %). An initial sample (day 0) and final sample (day 18) were used to compute each variable, as described in section 2.5.

Table 3.2.3.2 Anaerobically digested livestock wastewater fractions used for the mixture design

Run ID	ADSW	ADPW	ADCW
1	0.7500	0.125000	0.125000
2	0.1250	0.750000	0.125000
3	0.1250	0.125000	0.750000
4	0.4375	0.437500	0.125000
5	0.4375	0.125000	0.437500
6	0.1250	0.437500	0.437500
7	0.3333	0.333333	0.333333
8	0.7500	0.125000	0.125000
9	0.1250	0.750000	0.125000
10	0.1250	0.125000	0.750000
11	0.4375	0.437500	0.125000
12	0.4375	0.125000	0.437500
13	0.1250	0.437500	0.437500
14	0.3333	0.333333	0.333333

3.2.3 Sample collection and data analysis

For every given run (98 in total) using the optimal mixture design, 10 mL samples were collected in triplicate both on the initial day (day 0) and on the final day (day 18) from each flask. An aliquot was then taken from each sample for the determination of cell density using a Neubauer chamber, and the remainder of the sample was centrifuged in a Gyrozen 1580R at 4,000 rpm for 30 min. The resulting supernatant was recovered, properly diluted, and analyzed according to the Hach DR 5000 Spectrophotometer protocol (HACH, DR 5000) to compute the removal efficiencies of TN, TP and COD (TNr, TPr and CODr, respectively).

To calculate the cell growth (CG), the difference in the cell density between the final day (day 18) and the initial day (day 0) was calculated for any given mixture of ADLW (Eq. 2). The experiments were terminated on the 18th day because in previous runs the exponential phase ended, on average, after 15 to 18 days, similarly to other studies (Sanjeev et al., 2014;Tejido-Núñez et al., 2019).The TNr, TPr and CODr efficiencies were obtained using equations 3, 4 and 5, respectively. The initial and final cell and pollutant concentrations are shown in Appendix A.

$$\boldsymbol{CG}\ [\%] = \boldsymbol{CD_o} - \boldsymbol{CD_1}$$

Cell growth equation (2)

$$\boldsymbol{TNr}\ [\%] = \frac{(\boldsymbol{TN_0} - \boldsymbol{TN_1})}{\boldsymbol{TN_0}} * \mathbf{100}$$

Total nitrogen removal equation (3)

$$\boldsymbol{TPr}\ [\%] = \frac{(\boldsymbol{TP_0} - \boldsymbol{TP_1})}{\boldsymbol{TN_0}} * \mathbf{100}$$

Total phosphorus removal equation (4)

$$\boldsymbol{CODr}\ [\%] = \frac{(\boldsymbol{TCOD_0} - \boldsymbol{TCOD_1})}{\boldsymbol{TN_0}} * \mathbf{100}$$

Total chemical oxygen demand removal equation (5)

3.2.4 Global performance index

The performance of each mixture design experimental run was evaluated in terms of overall CG and pollutant removal efficiencies (TNr, TPr and CODr). To appraise the overall performance with a single value, a weighted global performance index was used (*GPI*; Equation 6), which integrates the four response variables into a normalized index (0-1 range). To normalize the cell growth (CG_N), the final cell concentration of each experimental run was divided by the maximum cell concentration obtained within all 98 experimental runs. Since the pollutant removal responses were already expressed as a fraction from 0 to 1, a normalization procedure was not necessary. For the index integration, equal weights were assigned to all four response variables (W=0.25), since, for the purpose of this study, the potential revalorization of the microalga biomass and the removal efficiencies of COD, TN and TR are equally valued. However, these weights could change for other studies depending on the specific goals stablished for the MbWT. For instance, If the application of the MbWT is focused on biomass recovery, a higher weight must be given to the CG response, on the contrary, if the main goal is to reduce the pollutant concentration, higher weights must be assigned to the removal performance.

The GPI determined for each experimental run is reported in Appendix A.

$$\boldsymbol{PGI = (CG_N + TNr + TPr + CODr) * 0.25} \tag{6}$$

Global performance index equation

3.2.5 Statistical analyses

The mixture design and the contour plots were analyzed using Minitab 19.2020.1.
The analyses of variance (one-way ANOVAs) were performed on the four response variables after 18 days of cultivation and using the global performance index to compare experimental runs. A redundancy analysis (RDA) was performed to analyze the correlation between microalgal cultures (i.e., all mono-, bi- and tri-cultures) and the LW components (ADCW, ADSW and ADPW), as well as between the response variables measured (CG, TNr, TPr, and CODr). A significant value of 0.05 was used in all statistical tests employed in this research. Correlation triplots were used to extract the RDA results. A correlation triplot consists of two superimposed biplots that include quantitative explanatory and response variables (represented by vectors) and observations (represented by points) (Zuur et al., 2007). The statistical analysis was performed using R software version 4.0.2, applying the scales and vegan packages. Graphics were made with the ggplot2 package.

3.3 Results and Discussion

3.3.1 Livestock digestate characterization

The pH of all three digestates was approximately 7.7, as the pH tends to rise in digestates due to the removal of CO_2 and the formation of $(NH_4)_2CO_3$ during anaerobic digestion, a result of the mineralization process of raw proteins (Makádi et al., 2012; Möller & Müller, 2012). Regarding the contents of FOG, the ADCW exhibited more than double the concentration displayed by the other digestates evaluated (ADSW and ADPW). The FOG determined in the LW is the fat not accumulated or digested by the animal, which goes to the excreta. Fat accumulation in the animal is influenced by its genetics (breed, sex, heritability), management (weaning age, castration, age, and environment) and nutritional factors (fat metabolism, digestion and absorption, glucose/starch availability, dietary energy, protein and vitamin levels, nutritional programming, and stage-specific feeding systems). However, according to FAO, the lipid content of cattle manure is lower than swine manure; thus, the higher content of FOG and ADCW must be attributed to management practices from the livestock unit (Park et al., 2018). The ADPW displayed a concentration of suspended solids (SS), which was 55 and 80 times higher than the ADSW and the ADCW, respectively. This is consistent with the literature reporting total solids (TS) concentrations in poultry manure as high as 20-25 [% w/w] (Iyappan et al., 2011). Additionally, the ADPW had a concentration of TKN which was 1.5 times higher than those of the ADSW and the ADCW due to higher protein concentrations contained in poultry manure (2,500 mg protein L^{-1}), which displays delayed degradation due to the higher concentration of carbohydrates in poultry manure; this leads to an incomplete digestion of protein (Alejo et al., 2018; Breure et al., 1986; Lu et al., 2019). The ADSW exhibited a TP concentration higher than those of the ASCW and the ADPW. The absorption of P in swine is lower than in other animals, as TP concentrations have been previously reported as high as 29.1 g kg^{-1} in swine manure, higher than those in cattle (6.7 g kg^{-1}) and poultry (18.0 g kg^{-1}) (Barnett, 1994). Furthermore, the ADSW and the ADCW contained similar contents of BOD, with slightly higher values than the ADPW, due to their initial concentration prior to anaerobic digestion, which tends to be higher in cattle and swine manure (80.90% DM) than in poultry manure (45.30% DM) (Song et al., 2015).

Table 3.3.1.1 Physicochemical composition of the anaerobically digested swine (ADSW), cattle (ADCW) and poultry wastewater (ADPW).

Physicochemical parameter	**Unit**	**ADSW**	**ADCW**	**ADPW**
pH	upH	7.70	7.70	7.74
Fat, oil and grease	mL L^{-1}	45.71	100.00	36.84
Sedimentable solids	mg L^{-1}	1.00	0.70	55.00
BOD_5	mg L^{-1}	25,531.91	23,100.30	19,452.89
Total suspended solids	mg L^{-1}	728.57	3,660.00	2,358.33
Kjeldahl nitrogen	mg L^{-1}	152.59	150.55	230.92
Total phosphorus	mg L^{-1}	49.67	2.73	3.97

3.3.2 UV mutagenesis and gradual domestications

UV irradiation produces both useful and useless mutations in the DNA of microorganisms, and its combination with a gradual domestication approach not only can save much effort in the screening of the desired mutations, but it can also accelerate the adaptation of microalgae to ADLW (Chu, 2017; S. Liu et al., 2015; Sivaramakrishnan & Incharoensakdi, 2017). The assessment of these mutations is achieved by the fatality rate (FR), with a FR between 75 and 90% being considered an indication of positive mutations that may increase microalgal tolerance to harsh conditions, such as those displayed by ADLW. As shown into Table 3.3.2.1, the microalgae that displayed the highest FR were chosen for further acclimation steps. The highest FR percentages for each microalga were 85%, 76% and 80% for *Chlamydomonas* sp., *C. vulgaris* and *H. pluvialis*, respectively.

Table 3.3.2.1 Fatality rate (%) after exposure to 12 minutes of UV light at 253.7 nm from a 15-W UV lamp. The highest FR percentages for each microalga are indicated in bold.

	Replicate #	*Chlamydomonas* sp.	*C. vulgaris*	*H. pluvialis*
Fatality rate [%]	1	72	**76**	67
	2	70	68	**80**
	3	**85**	60	54

Once the FRs were determined, each microalga was cultured in ADLW composed of a mixture of ADSW: ADCW: ADPW (1:1:1) at concentrations of 50%, 70% and 100% (v/v) (the 50% and 70% ADLW were diluted with distilled water). Even though the microalgal strains were not capable of surviving in undiluted ADLW before the UV exposure, a cell growth above 1×10^{7}cell mL^{-1} was achieved by all three strains in undiluted ADLW after UV exposure. Nevertheless, as shown in Fig. 3.3.2.1, the highest cell growth was found in diluted ADLW at 70% (v/v), except for *C. vulgaris*, which exhibited a higher cell growth at a dilution of 50% (v/v). Thus, the 70% ADLW concentration was subsequently used for the mixture design. Similar results were achieved before using swine wastewater (SW) as a medium source, as Wang et al. (2016a) applied UV exposure to *C. vulgaris* and *Scenedesmus obliquus* for 12 minutes, resulting in a biomass concentration of 1.80 g L^{1}, which was comparable to that obtained in BG-11 medium (1.83 g L^{1}). The mutations are observed phenotypically through the improvement of the adaptative response of microalgae, nevertheless, the confirmation of these mutations using UV mutagenesis in LW has never been addressed, thus, the implementation of gene mapping techniques is recommended for future studies.

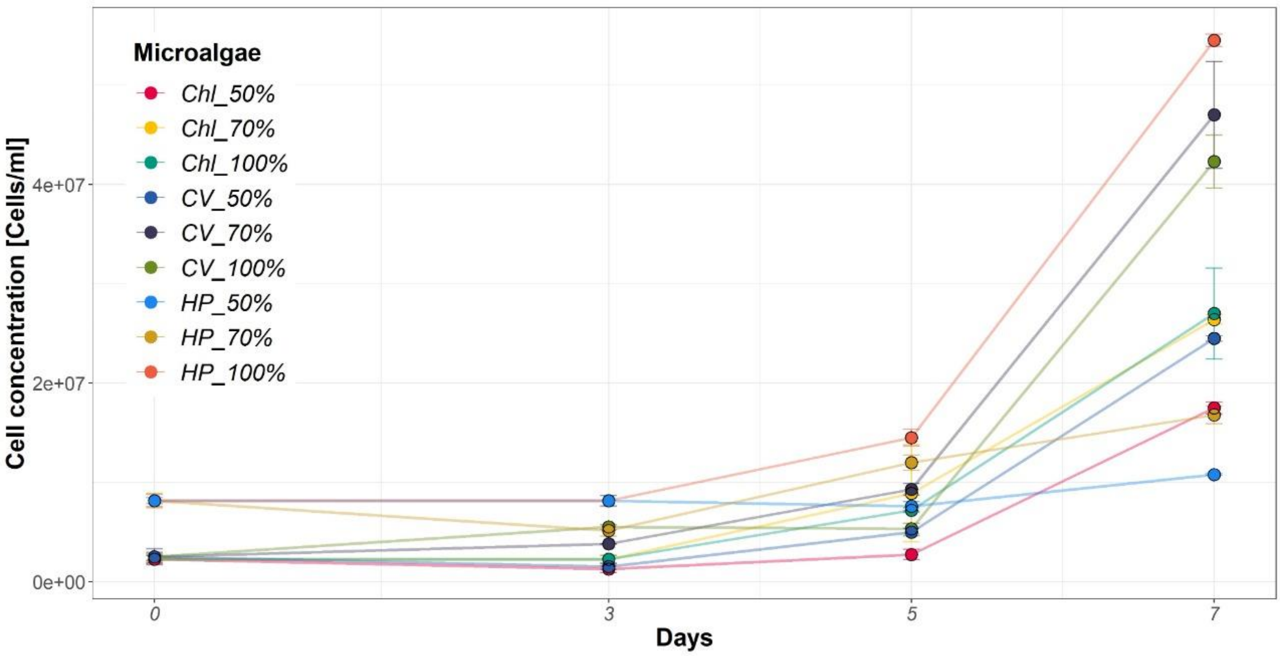

Fig. 3.3.2.1 Microalgae growth after UV exposure to different digestate concentrations. Chlamydomonas sp. (Chl) with 50%, 70% and 100% digestate. *Chlorella vulgaris* (CV) with 50%, 70% and 100% digestate. *Haematococcus pluvialis* (HP) with 50%, 70% and 100% digestate. The cultures were diluted with distilled water.

Additionally, Fig. 3.3.2.1 displays a prolonged lag phase attributed to the harmful substances contained in the ADLW, such as ammonium, and to the opacity of this effluent, which exerts negative effects on the photosynthesis processes. However, the lag phase observed in pilot runs was reduced from 8 to 5 days with the application of the UV mutagenesis and domestication process,. Khalid et al. (2018) suggested that the microalgae should be cultured in the same medium until the lag phase is avoided in acclimation processes.. Thus, a longer acclimation process could result in the elimination of the lag phase. Furthermore, some strategies such as the administration of CO_2, have resulted in an accelerated microalgal growth without a lag phase when treating LW (Cheng et al., 2015).

3.3.3 Optimal mixture design

3.3.3.1 Effects of ADLW mixtures on the response variables

In this section, the main findings regarding the influence of the ADLW composition (i.e. the fractions of ADCW, ADPW and ADPW) on cell growth and pollutant removal efficiencies (CG, TNr, TPr and CODr) are discussed. The main results, summarized by descriptive statistics, are shown in Table 3.3.3.1.

Table 3.3.3.1 Response variables (Cell growth, Nitrogen, Phosphorus, and Chemical oxygen demand) grouped by ADLW mixtures

	CG [cell mL^{-1}]				TNr [%]				TPr [%]				CODr [%]			
Mixture (ADSW: PW: CW)	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**
1. 0.75: 0.125: 0.125	1.04E+07	1.95E+07	-2.43E+06	6.91E+07	0.65	0.18	0.23	0.96	0.01	0.15	-0.37	0.22	0.40	0.16	0.13	0.65
2. 0.125: 0.75: 0.125	3.37E+06	5.98E+06	-2.21E+06	1.66E+07	0.66	0.10	0.49	0.87	0.39	0.16	0.14	0.63	0.41	0.16	0.09	0.66
3. 0.125: 0.125: 0.75	6.15E+06	1.06E+07	-6.11E+06	3.58E+07	0.70	0.23	0.00	0.91	0.38	0.17	0.07	0.71	0.47	0.21	0.08	0.77
4. 0.4375: 0.4375: 0.125	6.31E+06	7.45E+06	-3.47E+06	2.13E+07	0.63	0.14	0.34	0.84	0.23	0.12	0.03	0.44	0.43	0.20	-0.03	0.81
5. 0.4375: 0.125: 0.4375	7.67E+06	1.10E+07	-3.60E+06	3.94E+07	0.62	0.24	0.15	0.91	0.25	0.20	-0.09	0.62	0.48	0.26	-0.07	0.94
6. 0.125: 0.4375: 0.4375	5.51E+06	1.05E+07	-5.90E+06	3.61E+07	0.65	0.22	0.13	0.89	0.34	0.25	-0.22	0.72	0.42	0.21	-0.10	0.72
7. 0.333: 0.333: 0.333	8.33E+06	1.37E+07	-6.15E+06	4.02E+07	0.61	0.18	0.26	0.86	0.28	0.24	-0.22	0.66	0.41	0.20	0.00	0.70

In general, the different LW mixtures used as microalgal growth medium induced cell growth. The highest mean cell growth ($1.04 \times 10^7 \pm 1.95 \times 10^7$ cell ml^{-1}) was observed for mixture 1 (0.75:0.125:0.125 ADSW:ADPW:ADCW) considering all microalgal cultures (for mono-, bi- and tri-cultures). It is important to point out that the high standard deviation displayed in Table 3.3.3.1 is attributed to the fact that for each ADLW mixture, the results obtained from each microalgae species were grouped into one single category, thus leading to a high variability. The rest of the mixtures (2-7) displayed cell growths ranging from 3.37×10^6 to 8.33×10^6 cell mL^{-1}, as shown in Fig. 3.3.3.1. A Although mixture 1 had the highest cell growth, it also possessed the lowest mean TPr ($1 \pm 15\%$) (Fig. 3.3.3.1.C), which was significantly lower than those displayed by the rest of the mixtures ($p<0.05$). Furthermore, some of the experimental runs resulted in negative TPr and CODr values, especially for the former (Table 3.3.3.1). In general, when these negative values were obtained, the culture also had a low CG, meaning that these results may be due to the release of organic matter and phosphoric compounds via cell lysis. This was likely a result of exposure to harsh environments and can also be attributed to the presence of recalcitrant compounds (Xia & Muprphy, 2016). Nevertheless, some TPr values were below 0%, while the CG was above 1×10^5. In these cultures, the observed microalgae displayed a smaller cell size, which could lead to a low TPr, as suggested by Powell et al. (2011), who documented that microalga typically contain approximately 1% P by dry weight, indicating that TPr is dependent of the cell size. Furthermore, 29 - 77% of the TPr is thought to be due to a chemical precipitation induced by the microalgae; however, at low aqueous calcium concentrations, precipitated calcium phosphates may also be redissolved into the medium, increasing the P concentration (Larsdotter et al., 2010; Su, 2021). In addition to the smaller size observed, this caused a final concentration of P that was higher than the initial concentration. Thus, an increase in cell concentration is not always proportional to pollutant removal efficiencies. The mean TNr were similar for all LW mixtures, ranging from 61 to 70%.

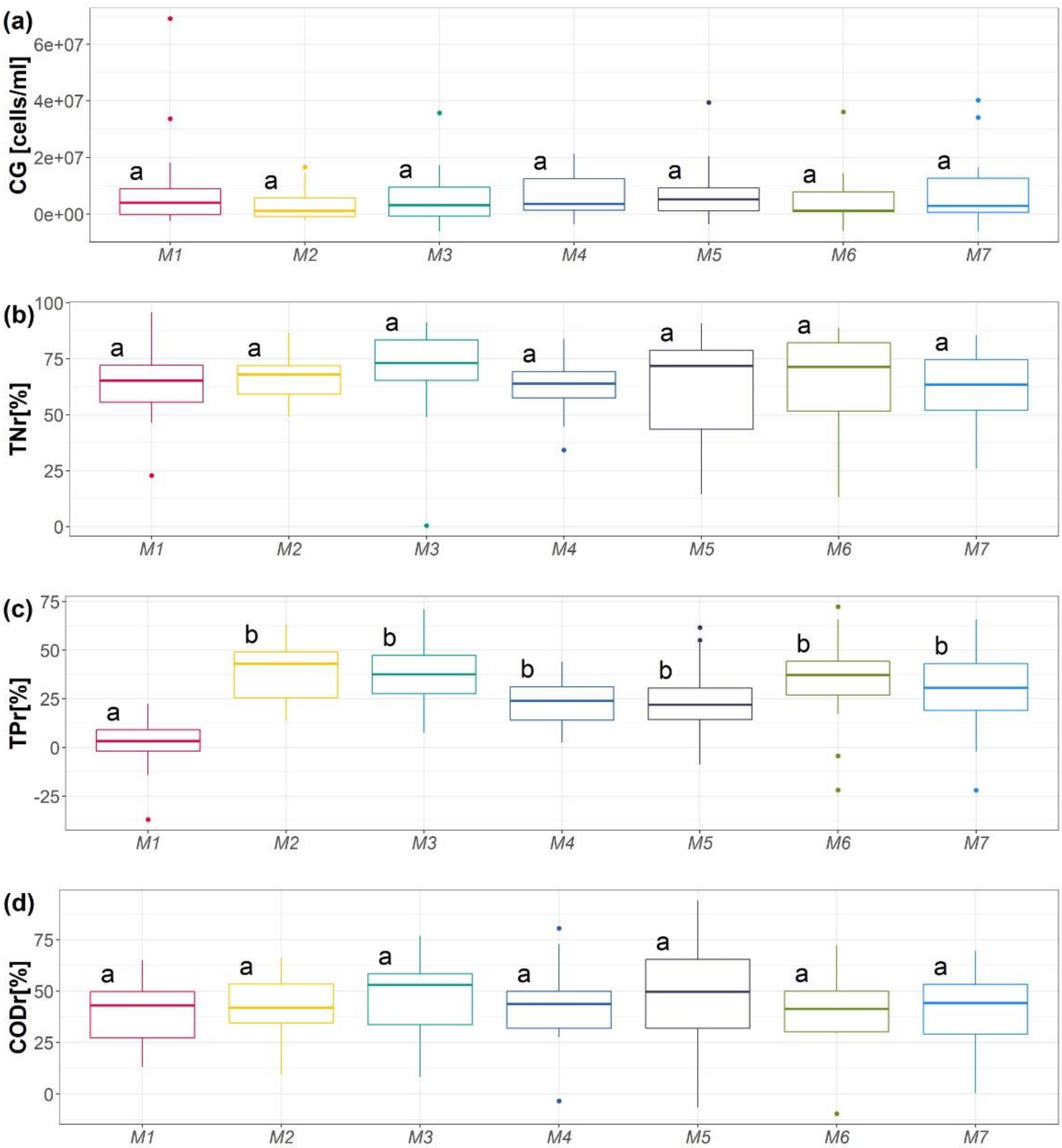

Fig. 3.3.3.1 Box plot with the principal results grouped by mixture fractions of ADLW. A) Cell growth (CG), B) Total nitrogen removal (TNr), C) Total phosphorus removal (TPr), D) Total chemical oxygen demand (CODr). M 1-7, stands for the mixture levels used in the mixture design.

3.3.3.2 Effects of microalgae strains in the response variables

Considering the mean growth of each microalgal culture in all LW mixtures, the highest mean cell growth was observed in *C. vulgaris* in monoculture ($2.81\times10^7 \pm 1.67\times10^7$ cell mL^{-1}), followed by the consortia of *C. vulgaris* with *Chlamydomonas* spp. ($5.99\times10^6 \pm 6.68\times10^6$ cell mL^{-1}) and *H. pluvialis* ($4.62\times10^6 \pm 6.29\times10^6$ cell mL^{-1}), both as bi- and tri-cultures

($6.33\times10^6 \pm 5.81\times10^6$ cell mL^{-1}). *Chlorella* is a genus widely recognized for its simple life cycle, high growth potential and photosynthetic machinery, which is similar to higher plants. This translates into an ability to rapidly uptake and assimilate carbon dioxide and nutrients from waste streams, such as livestock wastewater (Richmond, 2008). The mean cell growth displayed by *Chlamydomonas* spp. in monoculture ($1.20\times10^5 \pm 1.77\times10^6$ cell mL^{-1}) increased by almost 49 times when cultured with C. *vulgaris* as a biculture ($3.54\times10^6 \pm 2.77\times10^6$ cell mL^{-1}), and by 52 times ($6.33\times10^6 \pm 5.81\times10^6$ cell mL^{-1}) when cultured in tri-culture, which suggests a potentially synergistic relationship. Microalgal consortia are reported to offer two main advantages: (1) higher tolerance to environmental fluctuations, and (2) compensation of eventual losses of specific strains due to changes in the (López-Sánchez et al., 2021). Bhatnagar et al. (2011) established four consortia for poultry wastewater treatment: (1) *Chlamydomonas globosa* - *Scenedesmus bijuga*, (2) *Chlamydomonas globosa* - *Chlorella minutissima*, (3) *C. minutissima* - *S. bijuga* and (4) *C. globosa* - *C. minutissima* - *S. bijuga*. In monoculture, *C. globosa*, *C. minutissima* and *S. bijuga* displayed a maximum biomass concentration of 1.278, 2.599 and 2.194 g L^{-1}, respectively. Furthermore, while the second consortia displayed a similar maximum biomass concentration of 3.017 g L^{-1}, the triculture (consortium 4) displayed the highest maximum biomass of 3.144 g L^{-1}. Several more authors have reported high pollutant removals and biomass productivity using microalgal consortia (Choudhary et al., 2016; Qin et al., 2016; Wang et al., 2016b). However, the specific interactions between microalgae have not been fully elucidated and further research is needed to evaluate the mechanisms behind the microalgal interactions that affect the overall performance on microalgal mixed cultures.

As shown in Table 3.3.3.2 and Fig. 3.3.3.2, the mean cell growth of *C. vulgaris* ($2.81\times10^7 \pm 1.67\times10^7$cell mL^{-1}) was the highest between all three monocultures, while *H. pluvialis* displayed a reduction in its initial cellular concentration, resulting in a mean negative growth ($-9.74\times10^5 \pm 27.9\times10^6$ cell mL^{-1}) (Fig. 3.3.3.2.A). Furthermore, the bi and tri-cultures displayed a higher cell growth than the *Chlamydomonas* spp. and *H. pluvialis* monocultures, potentially due to mutualistic interactions between these microalgae. Particularly, *Chlamydomonas* spp. displayed a cell growth of $1.20\text{x}10^5 \pm 1.77\times10^6$ cell mL^{-1} in monoculture, while mean cell growth values of $5.99\times10^6 \pm 6.68\times10^6$ and $6.33\times10^6 \pm 5.81\times10^6$ were observed for this microalga in bi-culture with *C. vulgaris* and in tri-culture (*Chlamydomonas* sp, *C. vulgaris* and *H. pluvialis*). *H. pluvialis* had a negative mean cell growth of $-9.74\text{E}\times10^5 \pm 2.79\times10^6$ cell mL^{-1} in monoculture, which increased to a mean cell growth of $5.99\times10^6 \pm 6.68\times10^6$ in bi-culture with *C. vulgaris*, and $6.33\times10^6 \pm 5.81\times10^6$ in tri-culture. Morphologically, the structure between these microalgae is similar due to their proximate taxonomy. Therefore, it was not possible to differentiate between them using optical microscopy, and further studies using molecular techniques should be implemented to assess the growth of each microalga separately to study the specific interactions occurring within the mixed cultures.

Table 3.3.3.2 Response variables (Cell growth, Nitrogen, Phosphorus and Chemical oxygen demand) grouped by microalgae strains.

	CG [cell mL^{-1}]				TNr [%]				TPr [%]				CODr [%]			
Strain	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**	**mean**	**sd**	**min**	**max**
Chl[A,B]	1.20E+05	1.77E+06	-2.92E+06	2.92E+06	0.66	0.19	0.34	0.96	0.42	0.22	-0.02	0.72	0.55	0.16	0.29	0.72
CV[A]	2.81E+07	1.67E+07	8.00E+05	6.91E+07	0.63	0.22	0.15	0.85	0.43	0.24	-0.14	0.66	0.50	0.21	0.13	0.94
HP[C]	-9.74E+05	2.79E+06	-6.15E+06	1.49E+06	0.43	0.23	0.00	0.72	0.20	0.18	-0.37	0.35	0.60	0.11	0.37	0.77
Chl + CV[B,C]	5.99E+06	6.68E+06	-2.75E+06	1.49E+07	0.65	0.12	0.45	0.90	0.30	0.15	-0.01	0.49	0.29	0.28	-0.10	0.76
Chl + HP[B,C]	3.54E+06	2.77E+06	-6.10E+05	9.93E+06	0.66	0.13	0.46	0.85	0.30	0.14	0.06	0.53	0.48	0.05	0.43	0.59
CV + HP[C]	4.62E+06	6.29E+06	-2.43E+06	1.66E+07	0.72	0.08	0.55	0.84	0.06	0.19	-0.22	0.47	0.27	0.06	0.13	0.36
Chl+ CV + HP[B,C]	6.33E+06	5.81E+06	-6.11E+06	1.82E+07	0.76	0.13	0.55	0.91	0.18	0.14	-0.09	0.42	0.32	0.06	0.16	0.43

In terms of TNr, almost all of the microalgal cultures had a mean removal efficiency in the range of 65 to 76% in all the LW mixtures employed as growth medium, except for *H. pluvialis* in monoculture, which displayed a TNr of 43%±23% (Fig. 3.3.3.2.B). The uptake of TN by microalgae has been related to higher cell concentrations, where the cell growth is sustained until TN concentration is depleted (Ledda et al., 2016a). However, in some cases, the microalgal cell concentration does not increase as the TN content of wastewater decreases gradually [cell mL^{-1}], but the biomass concentration does [g L^{-1}]. This phenomenon was described by (Lee & Lee, 2002) as these authors observed that nitrogen uptake is proportional to the average cell size. They attributed this phenomenon to a possible suppression of cell division as well as to the promotion of cell aggregation, causing an increase in total biomass but not in cell concentration. In this sense, nitrogen uptake is linked to cell growth but is not always proportional.

In contrast, the highest TPr was displayed by the monocultures of *C. vulgaris* (43%±24%) and by *Chlamydomonas* sp. (42%±22%), but not by *H. pluvialis* (20%±18%). The bi-cultures and the tri-culture had a lower TPr, which ranged from 6 - 30%, from which the lowest growth was observed in the bi-culture of *C. vulgaris* and *H. pluvialis* with a mean TPr 6%±19% (Fig. 3.3.3.2.C). Therefore, interspecific microalgal interactions may not enhance TPr, especially for the cultures where *H. pluvialis* is present. As mentioned before, these low TPr efficiencies could be attributed to the resuspension of TP from the precipitate and the small cells observed, which leads to a low phosphoric internalization (Larsdotter et al., 2010; Powell et al., 2011).

Regarding CODr, as shown in Fig. 3.3.3.2.D, there were significant differences ($p<0.05$) among microalgal cultures. *H. pluvialis* displayed the highest mean CODr with a removal rate of 60%±11%, whereas the bi-culture of *C. vulgaris* and *H. pluvialis* had the lowest removal rate at 27.60%±6%. In general, the CODr rates of *C. vulgaris* as mono-, bi- and tri-cultures were lower than previously reported in the literature (74 – 84%) (López-Sánchez et al., 2021). Though it should be mentioned that most previous studies were performed using LW, which contains lower concentrations of recalcitrant compounds than ADLW. This difference is mainly attributed to the thermal pre-treatment usually applied in anaerobic digestion that increases the formation of soluble recalcitrant compounds in ADLW (Ortega-Martínez et al., 2021). The high CODr presented by *H. pluvialis* suggests an elevated tolerance and affinity of this microalga to the organic carbon sources found in the digestates. This ability of *H. pluvialis* to grow under mixotrophic conditions has been widely assessed, as this growth regime has been associated with higher overall performance and also with higher synthesis of astaxanthin, a high-valued pigment (Arashiro et al., 2020; Bauer & Minceva, 2021; Wen et al., 2019).

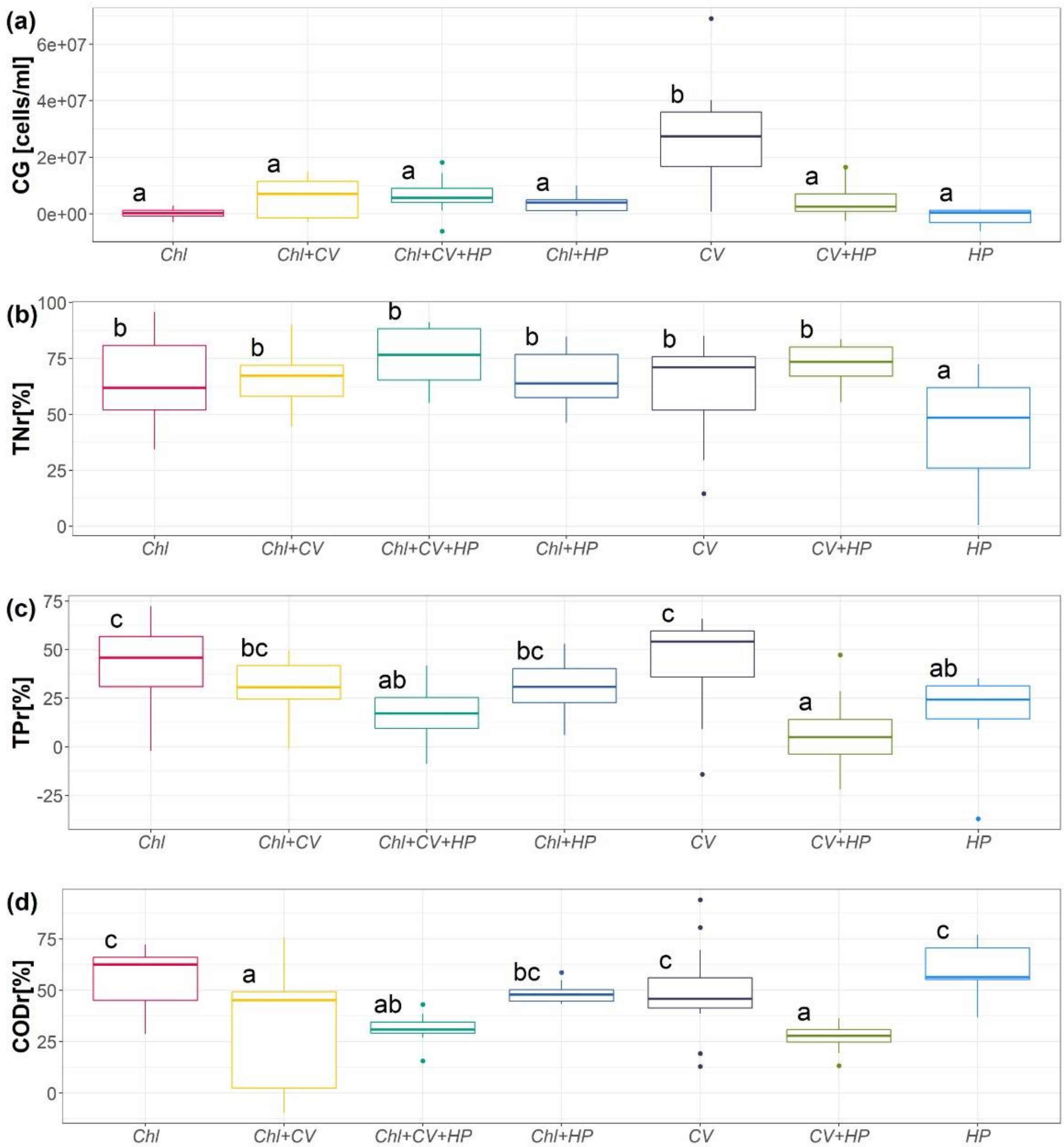

Fig. 3.3.3.2 Graphical summary of the principal results grouped by microalgae strains in mono-, bi- and tri-culture. A) Cell growth (CG), B) Total nitrogen removal (TNr), C) Total phosphorus removal (TPr), D) Total chemical oxygen demand (CODr).

3.3.4 Global performance index (GPI) and response optimization

Based on the overall GPI results (Appendix A), the culture of *C. vulgaris* in monoculture with a LW mixture of 0.125:0.4375:0.4375 ADSW:ADPW:ADCW displayed the best overall performance with the highest GPI (0.6171). A summary of the results of this culture are presented in Table 3.3.4.1.

The culture of *C. vulgaris* as monoculture displayed a cell growth of $3.61\times10^{7}\pm 2.81\times10^{6}$ cell mL^{-1}. Additionally, removal efficiencies of 83%, 40% and 59% were achieved for TN, TP, and COD, respectively. Similarly, (Gu et al., 2021) cultured *C. vulgaris* in ADSW in a mixed culture with indigenous bacteria and achieved TNr, TPr and CODr efficiencies of 75.00, 63.83, and 57.14%, respectively. In this study, the highest GPI was displayed by the monoculture of *C. vulgaris* in a mixture of 0.125:0.4375:0.4375 ADSW:ADPW:ADCW and with a N:P ratio of 11.78. These conditions also significantly enhanced the pollutant removal efficiencies and CG. However, a proximate analysis to determine the biochemical composition of the biomass should be performed to evaluate its revalorization potential.

This GPI, considered equal weights for all the response variables, as mentioned in section 3.2.4, since there was an equal interest on biomass production and pollutant removal in the present study, however, distinct weights could be assigned to the response variables depending on the specific amis of MbWT. Furthermore, different weights could be assigned to different microalgal species depending on the metabolites they produce and their technical and economic feasibility, since the downstream processes to recover different compounds may considerably increase the operation costs (Molinuevo-Salces et al., 2019).

Table 3.3.4.1 Summarized initial conditions from the culture with the highest GPI. The culture of C. vulgaris in monoculture with a LW mixture of 0.125:0.4375:0.4375 ADSW:ADPW:ADCW

	Cell density [cell mL^{-1}]	**TN [mg l^{-1}]**	**TP [mg l^{-1}]**	**COD [mg l^{-1}]**
Initial concentration	$4.53\times10^{6}\pm2.41\times10^{6}$	333.33 ± 25.17	28.47 ± 2.11	680.00 ± 19.50
Final concentration	$4.06\times10^{7}\pm2.42\times10^{6}$	50.00 ± 0.00	9.76 ± 0.15	381.11 ± 53.89
Response variables obtained in the mixture design	**Cell growth [cell mL^{-1}]**	**TNr [%]**	**TPr [%]**	**CODr [%]**
	$3.61\times10^{7}\pm2.81\times10^{6}$	$0.85\pm.02$	$0.66\pm.03$	$0.44\pm.07$

Once the best performing microalgal culture was identified (*C. vulgaris* in monoculture with a LW mixture of 0.125:0.4375:0.4375 ADSW:ADPW:ADCW) in terms of the highest GPI, a contour plot was constructed to evaluate the relationship between the cell growth and the pollutant removal efficiencies with the fractions of all three types of ADLW in the LW mixture. Regarding this microalga, the highest cell growth in the contour plot is predicted for higher volumetric fractions of ADSW (0.750:0.125:0.125; ADSW:ADPW:ADCW), while the lower cell growth is predicted in the lower left side of the contour plot, where ADPW is the predominant effluent (0.125:0.750:0.125; ADSW:ADPW:ADCW). This is possibly due to the N:P ratio of the LW mixture.

Based on the average molar ratio of microalgae ($C_{106}H_{181}O_{45}N_{16}P$), a N:P ratio of around 16 to 1 is typically used (Choi & Lee, 2014; Su, 2021). However, this ratio varies among microalgal strains and is critical for nutrient removal efficiencies and biomass productivity. In a recent study, when *C. vulgaris* was used for the treatment of dairy wastewater with a N:P ratio of 16:1, it displayed a TN removal efficiency of 98% and reached a higher biomass protein content (21.92% wet mass) compared to the control culture in BBM (12.58% wet mass) (Rodrigues-Sousa et al., 2021). Final biomass composition is a very important consideration when the MbWT is used to obtain bio-based products, such as high-protein animal feed supplements, biofuels, biochemicals, biomaterials and other high-valued compounds (Leong et al., 2021). Additionally, (Choi & Lee, 2014) evaluated the impact of the N:P ratios in biomass production and nutrient removal efficiencies of *C. vulgaris* growing in domestic wastewater. These authors reported that increasing N:P ratios, up to 10, resulted in an increased in biomass production and, above this point, the biomass production decreased gradually, reaching a constant value around an N:P ratio of 30:1. In the present study, the N:P ratio of ADPW was around 58:1, which according to the results of Choi & Lee (2014), is a ratio that is almost 6 times higher than the optimal conditions for biomass production. The N:P ratios displayed by each type of ADLW used in this study were 3, 35 and 58 for ADSW, ADCW and ADPW, respectively (Table 3.2.3.1). Additionally, the microelements present in the media also have a significant influence on the overall performance of the microalgae. The microelements Mn, Zn, Cu, Ca and Fe are directly linked to the photosynthetic process in microalgae (Shah, 2019). The presence of these microelements is essential for microalgal metabolism, but high concentrations may be detrimental for the microalgae. For example, Cu is a common supplement that is added to animal feed. However, more than 80% is not metabolized and remains in the livestock excreta, especially in swine waste, where the concentration of Cu varies from 0.6 to 21 mg L^{-1} (Hu et al., 2020). Wan et al. (2012), documented those high concentrations of Cu (above 20 mg L-1) negatively affect physiological and biochemical processes on microalgae. Therefore, the implementation of a mixed effluent to adjust for macro- and micro-elements is key to enhancing biomass productivity without needing to include additional supplements to satisfy specific microalgal needs. As a result, this would decrease operational costs.

According to Fig. 3.3.4.1.B, TNr is enhanced with higher concentrations of ADPW (0.125:0.750:0.125; ADSW:ADPW:ADCW), since a predicted TNr above 70% is observed on the lower left side of the contour plot. The ADPW used in this study contained a lower concentration of organic matter (compared to either the ADCW or

ADSW), which may lead to a promotion of cell growth and, thus, an increase in TNr efficiency. Patrinou et al. (2020), reported that high concentrations of COD (above 2,500 mg L^{-1}) may be detrimental to cell growth. In this study, as previously mentioned, a dilution of 30% was necessary to reduce the high concentration of organic matter that was present in all three of the livestock wastes sources (above 19,000 mg L^{-1}). The predicted TPr and CODr are not enhanced with higher concentrations of ADPW. Accordingly, higher fractions of ADCW may result in TPr (0.25:0.25:0.500; ADSW:ADPW:ADCW) and CODr (0.375:0.1875:0.4375; ADSW:ADPW:ADCW) values above 60%. The ADPW contains elevated concentrations of ammonium, which, above the threshold of microalgal tolerance induces an inhibition of the photosynthetic process, leading to a decrease in biomass productivity and nutrient assimilation. This inhibition has been related to direct damage of the chloroplasts, specifically to the thylakoid (Lu et al., 2020; Rossi et al., 2020). The optimal concentration of ammonium for the Chlorophyceae class (to which all three microalgae used in this study belong) is 128.72 ±129.52 mg L^{-1}; above this concentration, the overall performance of this microalgal class tends to decrease (Collos & Harrison, 2014). The ADPW used in this study had a concentration of 230 mg L^{-1}, which correlates to the finding that higher concentrations of the digestate resulted in decreased cell growth.

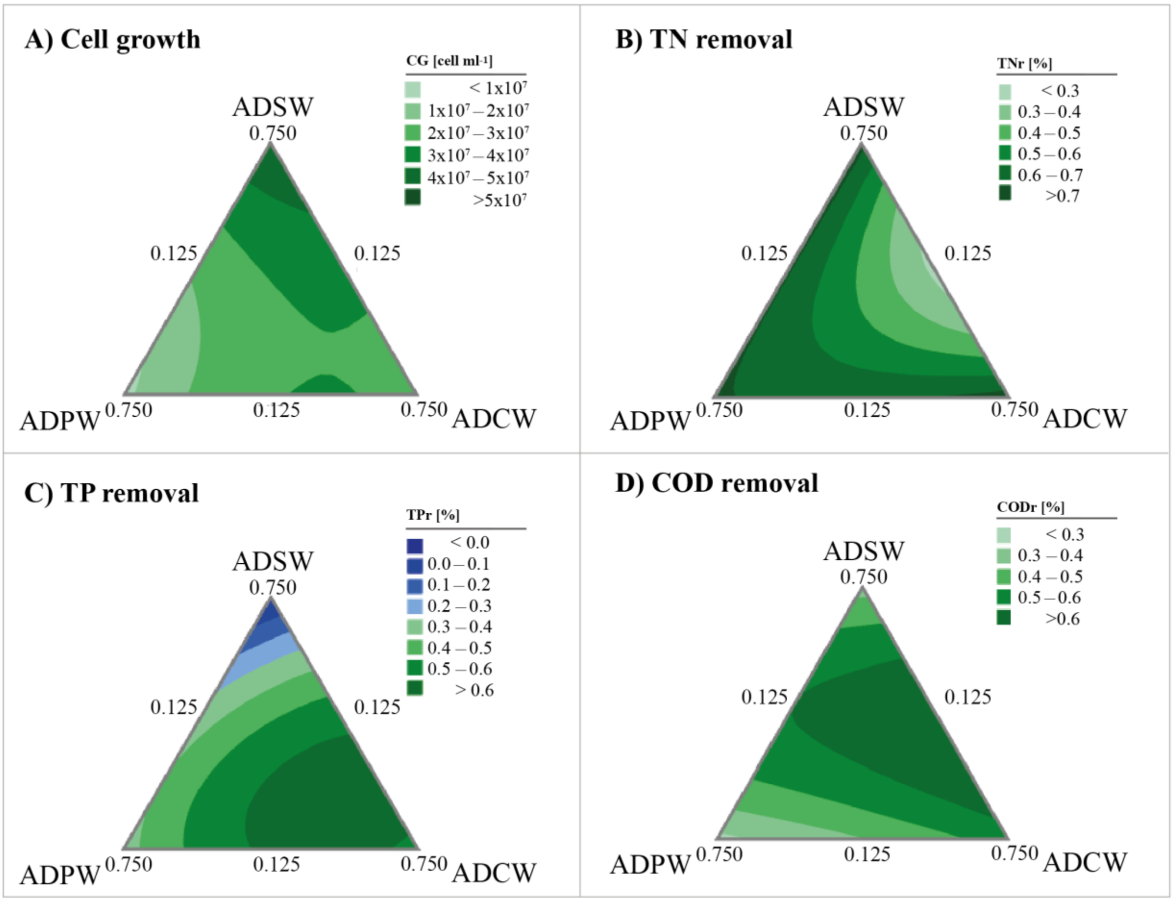

Fig. 3.3.4.1 Contour plots of cell growth (A), total nitrogen removal (B), total phosphorus removal (C) and chemical oxygen demand (D) of the culture of *C.*

vulgaris in monoculture with a LW mixture of anaerobically digested swine, poultry and cattle wastewater (ADSW:ADPW:ADCW).

By evaluating the GPI in the contour plot (Fig. 3.3.4.2), an optimal mixture consisting of 0.125 ADSW, 0.200 ADPW and 0.675 ADCW was determined, with a predicted GPI above 0.6. An optimal GPI of 1 may not be possible to achieve considering that the individual performance of each parameter (CG, TNr, TPr and CODr, as shown in Fig. 3.3.4.1) is enhanced by different compositions of ADLW. However, with the optimal mixture, a CG between 2×10^7 and 3×10^7 cell mL^{-1} and TNr, TPr and CODr efficiencies of 60-70%, >60% and 50-60%, respectively, are expected using *C. vulgaris*, which should be experimentally tested in further studies. The optimal mixture may present TN and TP concentrations of 166.91 and 8.84 mg L^{-1}, respectively (based on initial concentrations reported in Table 3.3.1.1), resulting in a N:P ratio of 18.87, which concur with what has been reported in literature (Choi & Lee, 2014; Su, 2021). The ADLW composition presents an intrinsic variability due to the species-specific physiological processes, thus, before the implementation of MbWT, a characterization of the different types of LW to be treated must be performed to adjust the volumetric fractions of the mixture in order to meet the specific micro and macronutrients requirements of the microalgae to be employed to MbWT. Additionally, the implementation of other effluents from other sectors, such as those from the food industry, must be explored.

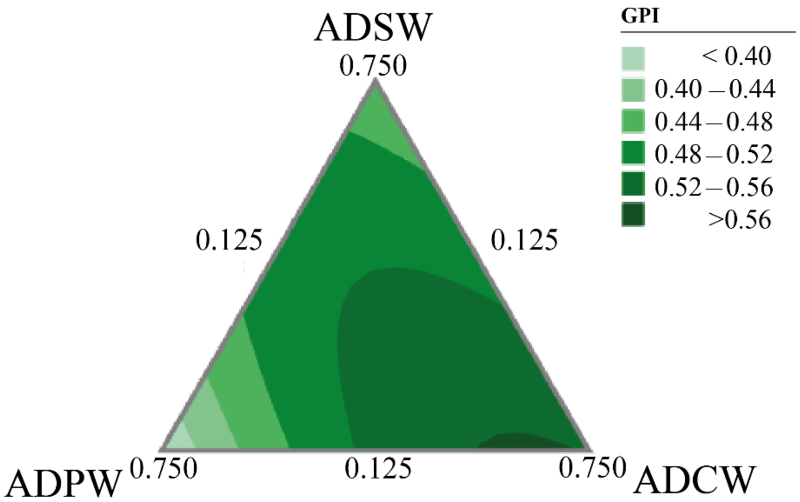

Fig. 3.3.4.2 Contour plots of the global performance index (GPI) with a LW mixture of anaerobically digested swine, poultry and cattle wastewater (ADSW:ADPW:ADCW).

3.3.5 Redundancy analysis (RDA)

An RDA was performed to analyze the correlation between microalgal cultures (i.e., all mono-, bi- and tri-cultures) and the LW components (ADCW, ADSW and ADPW), as well as between the response variables measured (cell growth, TNr, TPr and CODr). The two main redundancy components explained 99.9% of the total variability. Fig.3.3.5.1 shows the RDA correlation triplot that explains the correlation between the LW components and the response variables, and microalgal cultures are represented by a colored dot. The angles between the vectors of LW components (ADCW, ADSW and ADPW) and angles of the vectors of response variables (cell growth, TNr, TPr and CODr) represent correlations between these parameters, where the more similar an angle is to another, the greater the correlation is between them (Fig.3.3.5.1). Additionally, points representing microalgal cultures can be projected perpendicularly on the vectors of families or the vectors of the physicochemical parameters and give an indication of their corresponding values in such observations. The origin represents the mean value, projections in the same direction as the vector indicate values above average, and projections in the opposite direction represent values below average.

Fig.3.3.5.1 Redundancy analysis between biomass growth, medium concentration (ADCW, ADSW and ADPW), and nutrient removal of Chemical Oxygen Demand (COD), Total Nitrogen (TN) and Total Phosphorus (TP).

A positive correlation is displayed in the lower right quadrant between TNr, CODr and increasing fractions of ADCW. The experimental observations of *C. vulgaris* (gray dots in Fig.3.3.5.1) are also positively correlated with the TNr and CODr vectors in this quadrant. *C. vulgaris* achieved the highest TNr and CODr efficiencies with higher concentrations of ADCW. Similarly, there is a positive correlation between higher TPr efficiencies and the increasing fractions of ADCW and ADPW. This is observable in Fig. 3.3.4.1.C, where the highest TPr efficiencies are predicted for the higher ADCW fraction in the LW mixtures and near the central zone of the contour plot, where the mixture proportion is almost equal among the digestates. The triplot shows a positive correlation between *Chlamydomonas* spp. and TNr, and to a lesser magnitude, with the TPr and CODr, which is confirmed by the results presented in Table 3.3.3.2. The microalgal cultures that displayed a negative correlation with pollutant removal efficiencies were the monoculture of *H. pluvialis*, the bi-culture (*C. vulgaris* and *H. pluvialis*.) and the tri-culture (*C. vulgaris, H. pluvialis* and *Chlamydomonas* sp.). Other studies have suggested that *H. pluvialis* is less adaptable to harsh environments compared with other microalgae, such as *C. vulgaris.* For example, Wang et al., (2016c) studied the biomass production and the TNr and TPr of *C. vulgaris, H. pluvialis, Chlorella pirenoidosa* and *Scenedesmus obliquus* cultured in 25% swine wastewater. The lowest biomass production was displayed by *H. pluvialis*, with a dry cell weight of 0.3 g L^{-1} after 14 days culture, while the other microalgae ended with a dry cell weight above 1.0 g L^{-1} for the same period of time. Regarding nutrient removal, *H. pluvialis* resulted in TNr and TPr rates of 50% and 83%, respectively, while *C. vulgaris* displayed a TNr rate of 90% and a TPr rate of 98%. This lower performance of *H. pluvialis* can be attributed to a preference for a distinct optimal N:P ratio. As previously mentioned, higher cell density and nutrient removal efficiencies have been displayed by *C. vulgaris* with a N:P ratio of around 16:1, while the optimal N:P ratio documented for *H. pluvialis* in Basal Bold medium has been reported as less than 1:1 (Tocquin et al., 2011; Zhu et al., 2021). However, the N:P ratio is dependent on the medium source, as some studies have suggested an appropriate N:P ratio around 5 for BBM and over 30 for secondary municipal wastewater (Liu, 2018). Even though *H. pluvialis* displayed a lower overall performance in this study, it is still a promising microalga due to its capacity to synthesize astaxanthin, a high-value compound. Further studies need to be conducted to evaluate the optimal conditions that enhance both the pollutant removal efficiency and the biomass as well as the astaxanthin productivity of this microalga.

Increasing fractions of ADSW are negatively correlated with TPr efficiency, and to a lesser magnitude, with the TNr and the CODr efficiencies. These negative correlations are attributed to the lower N:P displayed by the ADSW used in this study (3:1::N:P) Nevertheless, this lower content of TN (frequently in the form of ammonia) avoids the nitrogen inhibition usually displayed by microalgae growing in ADPW, which is reflected by the negative correlation between cell growth and the increasing concentrations of ADPW, shown in Fig.3.3.5.1.

3.4 Conclusion

A centroid mixture design was used to optimize the fraction of three different anaerobically digested wastewater sources in a microalgae culture medium. Among the microalgal cultures evaluated, *C. vulgaris* obtained the highest global performance

(GPI=0.6171). This microalgal culture displayed a cell growth of $3.61\times10^7\pm 2.81\times10^7$ cell mL^{-1}, a total nitrogen removal of 85%±2%, a total phosphorus removal of 66%±3%, and a chemical oxygen demand removal of 44%±7%. The global solution of the mixture optimization for *C. vulgaris,* using a digestate mixture composed of 0.125:0.200:0.675 of ADSW:ADPW:ADCW, predicted a cell growth of 2×10^7-3×10^7 cell mL^{-1} and TNr, TPr and CODr efficiencies of 60-70%, >60% and 50-60%. Mixtures with higher fractions of ADSW were found to cause lower pollutant removal efficiencies possibly due to a low N: P ratio. Additionally, higher fractions of ADPW were found to be detrimental to the microalgal cell growth due to a high concentration of TN that may cause nitrogen inhibition.

The performance of the monocultures of *H. pluvialis* and *Chlamydomonas* sp. was improved when cultured in consortium, suggesting a potential mutualistic relationship. However, these interactions must be further studied and assessed quantitively. A mixing effluent approach is nevertheless a promising strategy to obtain a media source with optimal macro- and micronutrient concentrations in order to enhance biomass productivity and pollutant removal efficiencies without additional medium supplementation. However, it is necessary to perform a more detailed characterization of these effluents to assess the optimal concentrations of microelements to maximize the results obtained through MbWT. Additionally, this strategy offers a holistic opportunity to revalorize the most common livestock wastewater effluents generated by farms, where the breeding of different animal species (like swine, cattle and poultry) is often carried out simultaneously.

Chapter 4. Valorization of livestock waste through combined anaerobic digestion and microalgae-based treatment in México: A techno-economic analysis for distributed biogas generation,animal feed production, and carbon credits trading

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Abstract

A large percentage of world's eutrophication is attributed to the livestock industry, which is responsible for producing over 14% of the global anthropogenic greenhouse gasses emissions (GHG). In Mexico, very few livestock producers have the necessary infrastructure to treat their livestock waste (LW) and most fail to comply with environmental regulations. The treatment of LW through a circular bioeconomy approach can mitigate these negative environmental impacts, while simultaneously producing value-added products. The present work aimed to characterize the spatial variation of the LW generated in cattle, swine, and poultry farms in Jalisco, México. Total nitrogen (TN), phosphorus (TP), and organic matter released from these production units were estimated, along with the associated GHG, considering the standard practice of uncontrolled release. An alternative management scenario using anaerobic digestion (AD) combined with microalgae-based wastewater treatment (MbWT) was evaluated by developing a software-based techno-economic analysis, showing that a centralized LW treatment system could represent a feasible solution to comply the legislation while generating high-protein biomass for animal feed. Besides, the reduction in GHG represents an opportunity for carbon credits trading in voluntary markets for livestock producers in México. Insights are provided regarding the economical, technical, and sociocultural challenges that must be overcome to transition towards more sustainable livestock production practices in Jalisco, México, and other developing regions around the globe.

4.1 Introduction

The livestock sector is an essential component of the global economy, accounting for 1.4% of the world's gross domestic product (Ramírez and Rodríguez, 2016; Sakadevan and Nguyen, 2017). This sector covers 39% of the worldwide protein demand and is the source of livelihood for 1.3 billion people (McClelland et al., 2018; Rout and Behera, 2021). In 2018, the global poultry, cattle, and swine populations were 29.1, 1.6, and 1.4 billion heads, respectively (Garmyn, 2021), which are all expected to increase by 70% by 2050 (McClelland et al., 2018). While there is no doubt of the economic and social importance of this sector, livestock activities also account for more than 14% of global anthropogenic greenhouse gas (GHG) emissions. The mismanagement of livestock waste (LW), which is mainly composed of excrement, urine, feed residue, and washing water, is a significant environmental problem affecting the health of soil and surface and groundwater ecosystems, specially under uncontrolled release, which is the main practice in developing countries (Hu, 2019; Díaz-Vázquez et al., 2021).

Around 128 and 24 million tons of nitrogen (N) and phosphorus (P) are annually released into the environment from LW, and surpluses are projected to increase by 23 and 54%, respectively by 2050 (Sakadevan and Nguyen, 2017). Nutrient surpluses occur when the nutrient's load that the environment can naturally absorb is exceeded, resulting in their accumulation in water and soil, along with antibiotics, heavy metals, and pathogens in LW. This accumulation results in increased toxicity of soil-grown food products, direct phytotoxicity, and reduced soil fertility and productivity (Ramírez and Rodríguez, 2016; Díaz-Vázquez et al., 2020; Garcia-Launay et al., 2018; Leclerc and Laurent, 2017). Most of the N surpluses released to the environment are volatilized, denitrified, and leached into groundwater, while P is internalized into surface water sources and groundwater through leaching and surface runoff causing several environmental impacts (Sakadevan and Nguyen, 2017). Around 64%–97% of eutrophication and 14.5% of all anthropogenic GHG emissions worldwide is attributed to nutrient surpluses from the livestock industry (Garcia-Launay et al., 2018; Jiang et al., 2020).

Recovery of the resources contained in LW, such as water, organic compounds, and nutrients, through a circular bioeconomy approach can help to avoid the overexploitation of natural resources and the pollution of water and soil sources from nutrients surpluses, as well as mitigate the GHG preventing the uncontrolled released of LW (Tan and Lamers, 2021). The circular bioeconomy model aims to integrate the biological recovery of organic resources and nutrients from waste into the value chain (Sherwood, 2020). In this context, a LW treatment using anaerobic digestion (AD) coupled with microalgae-based wastewater treatment (MbWT) has gained special attention due to the high potential of biogas generation in AD process and the high metabolic efficiency of microalgae along with their capacity to grow using nutrient-rich wastewaters (Adamczyk et al., 2016; López-Sánchez et al., 2022b; Shi et al., 2018).

Although the maturity level of AD coupled with MbWT has significantly increased in the last decade, a profitability analysis based on biogas generation, microalgae biomass production, added-value products (e.g., biodiesel, metabolites), or carbon credits (generated from methane emissions reduction and livestock enteric digestion improvement) is required to demonstrate the technical and financial sustainability of the process' application to treat LW, specially aiming to encourage private investment in developing countries. The trend towards voluntary or mandatory carbon markets represents an opportunity to potentialize the feasibility of the proposed LW treatment scheme by trading issued carbon credits due to GHG emissions and climate change mitigation (Patel et al., 2020).

Reducing GHG emissions is gaining priority by governments worldwide because of the Kyoto Protocol and the Paris Agreement (Anjos et al., 2022; González et al., 2015).

Carbon credits markets have been adopted by several countries and provinces to account for the negative externalities of GHG emissions. The carbon credits are called Certified Emission Reductions (CER) and are registered under the clean development mechanism by the United Nations. Each CER corresponds to one ton of reduced equivalent carbon dioxide and is issued to stakeholders that have reduced their GHG emissions (González et al., 2015). The global carbon market can be divided into two groups: voluntary markets and regulated or compliance markets. Currently, the Mexican compensation market is a voluntary market operated by free trade of CER to accomplish corporate goals and not for mitigation responsibilities with the Mexican government. In the voluntary market, one carbon credit issued in Latin America from the waste disposal category has a price of $3.62 USD (Ecosystem Marketplace, 2021).

The present work aims to characterize the composition and distribution of the LW generated in cattle, swine, and poultry farms located in a region with intensive livestock production systems in the State of Jalisco, México and to estimate the current release of total N (TN), total P (TP), and organic matter into the environment, along with the GHG emissions. These baseline estimations were derived from the current LW management practices of uncontrolled release and were based on a mean composition weighted by the representation of each livestock producer segment (small, medium, or large). Additionally, a system comprising AD coupled with MbWT was evaluated from a techno-economic perspective as a circular bioeconomy alternative approach to the current LW management scenario. To this date, there is a specific protocol in Mexico to generate CER (CAR, 2010a) that only considers methane destruction after biogas generation by LW treatment and does not account for secondary GHG reductions by other operations such as MbWT. This protocol is mainly focused on the implementation of biogas control systems related to AD technologies, but it also allows the measurement of non-anaerobic treatments (limited to their listed technologies and practices, thus not considering MbWT or similar processes). The environmental benefits, i.e., the mitigation of TN, TP, and biological oxygen demand (BOD) release and CO2 fixation, along with the potential production of energy from AD, high-protein biomass from MbWT, and the potential CER trade in the voluntary market were accounted as the co-benefits for producers that implement a centralized circular bioeconomy treatment scheme for LW. Finally, insights into the economical, technical, and sociocultural challenges that must be overcome to implement MbWT for LW management in developing regions are provided.

4.2 Materials and methods

4.2.1 Study site and sampling scheme

The livestock sector is an important part of Mexico's economy, as Mexico ranks 7th in global cattle production (Rodríguez-Vivas et al. 2017). In 2020, Mexico produced 3.5, 2.0, and 1.6 million ton of poultry, cattle, and swine meat, respectively (SIACON 2020). Jalisco tops the list among the 32 states of Mexico, reporting 78.5, 2.8, and 3.3 million heads of poultry, cattle, and swine (Díaz-Vázquez et al., 2020) (Figure 1). It is considered an agri-food giant of Mexico due to its high agricultural and livestock production, which brings in 11.3% of the livestock GDP for the country (SADER 2017). However, only a small percentage of the livestock producers within the State have implemented infrastructure to treat solid and liquid LW, and most producers fail to comply with environmental regulations (Adeyi, Omidiran, and Osibanjo 2014; Grossi et al. 2019; Ramírez and Rodríguez 2016).

This study considered a stratified LW sampling scheme including small, medium, and large producers of swine, cattle, and poultry animal species (Table S1 of supplementary material). This segmentation was done based on the categorization proposed by Delgado et al. (2005), for cattle producers in developing countries. A livestock unit conversion coefficient was used for the segmentation of swine and poultry producers (CAPDR 2006; EUROSTAT 2022).

An initial meeting with Jalisco's Ministry of Environmental and Territorial Development (SEMADET) took place to discuss the scope of this study. The authorities provided information on the livestock producers that fulfilled the criteria shown in Table S1 and that could potentially participate as volunteers. The producers were contacted, and face-to-face meetings were arranged on their respective farms. These meetings were carried out from June to November 2021. A total of 28 LW samples were collected from farms located in the most intensive livestock clusters within the State (INEGI 2020) (as shown in Figure 1). 9, 10, and 9 of these farms corresponded to CW, SW, and PW, respectively, including small, medium, and large producers (Supplementary material Table S2).

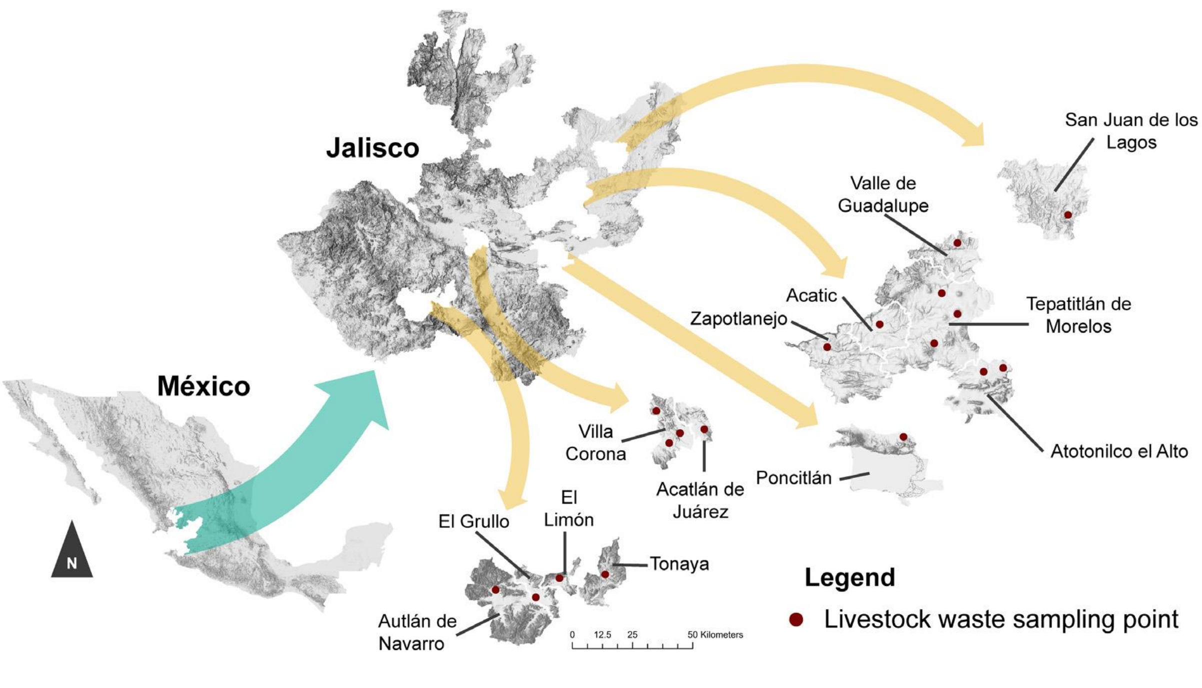

Figure 4.2.1.1. Livestock units sampled in 13 municipalities within the state boundaries of Jalisco, México.

The LW samples (approximately 200 g each) were collected in plastic bags and kept at 4 ºC for less than 5 hours during transportation. Samples were then stored at -20 ºC for less than 2 days prior to processing and characterization. The samples were obtained directly from the livestock shed in solid or liquid form, depending on the practices carried out on each farm. Consequently, only a fraction of the LW samples (approximately 50 g) was used to determine the humidity content.

4.2.2 Livestock waste characterization

A dilution of the LW samples (approximately 150 g) was prepared with 4 L of distilled water to determine the COD (chemical oxygen demand), BOD (biochemical oxygen demand), FOG (fat, oil, and grease) content, TN (total nitrogen), TP (total phosphorus), TS (total solids) and TSS (total soluble solids). These parameters were analyzed according to the Federation, W. E., & American Public Health Association (Eaton, Franson, and Water Environment Federation 2005). All physicochemical determinations were performed in triplicate.

The results were then adjusted (as shown in Eq. 1) to be expressed as mg kg^{-1} of dry waste.

$$\boldsymbol{Y_{ij,DM} = Y_{ij} * M * \left(\frac{1}{1-H_{ij}}\right) * 1000} \quad (1)$$

$Y_{i,j,DM}$ is the concentration of each parameter (COD, BOD, FOG, TN, TP, TS, and TSS) measured in the undiluted LW samples, expressed in dry base (mg kg_{DM}^{-1}) per species (*i*) and producer size (*j*), Y_{ij} is the parameter concentration measured in the diluted samples (mg L^{-1}), *M* is the ratio of distilled water added to the solids samples (L g^{-1}) (this factor was omitted in the case of the liquid samples). H_{ij} represents the humidity content in the sample. The mean, minimum, maximum, standard deviation, and variability coefficient were computed for each parameter. Moreover, a weighted mean of the LW composition was calculated for each parameter, weighing the representation of each livestock-producer segment (small, medium, or large). According to the National Institute of Statistics and Geography (INEGI 2020), the agricultural and livestock sector of Mexico is composed of 66.0% small, 32.52% medium, and 1.48% large producers. Hence, the weighted mean of manure composition was calculated using these fractions.

4.2.3 Territorial distribution of livestock waste generation

A livestock inventory provided by the Ministry of Environment and Territorial Development of the State of Jalisco (SEMADET 2022) was used to determine the territorial distribution of livestock production units. This database includes official georeferenced records on the location and productive activities of the livestock producing units per municipality, as well as the animal heads per unit. However, due to the low regulatory compliance of the sector, this inventory does not contain the complete list of livestock production units in Jalisco. Compared to the SIACON dataset, this inventory only contains around 15% of the total livestock units. Therefore, the data gathered from the SEMADET's inventory were extrapolated to the SIACON dataset to evaluate the microalgal potential on a municipal scale for Jalisco. Employing both inventories, a geographic information system (GIS) was used to assess the potential of each municipality and to determine those with the highest livestock density, and thus the highest potential for the implementation of AD coupled with MbWT in clusters for distributed generation. Data processing and analysis were performed using ArcGIS pro 3.0.2.

4.2.4 SuperPro Simulation

A conceptual treatment scheme comprising an AD unit coupled with a MbWT, and other operating units to optimize the process, was modeled and evaluated through a techno-economic feasibility analysis using SuperPro Designer v12 software. The simulation aimed to estimate the material and energy requirements, process equipment sizing, and specifications, and ultimately aimed to develop a techno-economic analysis of the whole process. For this alternative potential scenario, the inlet stream to the system represents the total production of LW in the state of Jalisco whose physicochemical properties were modeled in terms of carbohydrates, proteins, and water to match the experimental characterization values of COD, BOD_5, TN, TP, and TS based on the results of the stratified sampling (Tables S1 and S2 of supplementary material). This process is focused on obtaining flocculated biomass from *Chlorella* sp., this microalga was selected a robust strain able to grow in the presence of fat, oil, and grease, as well as in the conditions of the digestate studied herein. The microalgae biomass price ranges from 6 to 660 USD/kg (Fernández et al. 2021) depending on the strain and the process. However, according to search in the current market an average *chlorella* sp. biomass price was found to have an a of $20 USD kg^{-1} , this price was used for the simulation but it can vary depending on the purpose of the biomass. Moreover, the biogas produced through the AD unit was assumed to be upgraded and converted to energy through a gas turbine generator. Revenue from the energy production was modeled as savings from the energy amount required for the process assuming that the produced energy was recycled internally into the process. The treated water stream was given an economical attribution based on the costs of treating water to comply with the local regulations (0.1 USD ton^{-1}). Additionally, the potential revenue from CER was also considered for the economic analysis. The proposed methodology for CER estimation is described in detail in section 2.6. The capital and operational costs of the process, as well as profitability analysis were estimated based on the equations and indexes embedded in the software.

4.2.5 Environmental impact estimation

Two scenarios were considered for the estimation of the environmental impacts associated with LW management in Jalisco. (i) As a first management (baseline) scenario, the TN, TP, and BOD current release, as well as the associated GHG emissions, were estimated considering uncontrolled release of the LW into the environment, which is currently the most common practice in Jalisco (Ramírez and Rodríguez 2016). (ii) As an alternative management scenario, a system combining AD and MbWT for LW treatment was considered for its potential to reduce the release of TP, TN, and BOD, thus reducing GHG emissions. The concentration of these parameters after the full treatment process was assessed through the simulation described in the previous section.

Additionally, CO_2 fixation through microalgal photosynthesis was considered for the estimation of the global carbon footprint of this alternative scenario. The CO_2 consumption rate of 53.12 mg L^{-1} d^{-1} was used to compute the CO_2 fixation potential (Mousavi et al. 2018). The potential organic matter (BOD) and nutrient (TN and TP) release, as well as the greenhouse gas emissions (GHG) derived from the livestock production units in Jalisco, under both management scenarios described herein were estimated considering the livestock production unit inventory in the Consultation Agrifood Information System (SIACON) (SIACON 2020). According to the literature, the mean average waste generation per species (W_i) is 12.911, 2.933, and 0.068 ton head^{-1} year^{-1} for cattle, swine, and poultry, respectively (Díaz-Vázquez et al. 2021). The weighted mean of TN_i, TP_i, and BOD_i concentrations determined for the LW samples (Section 2.1) was used to compute a mean total nitrogen potential release (TN_{PR}), total phosphorus potential release (TP_{PR}), and total organic matter potential release (BOD_{PR}) under these scenarios.

The TN_{PR} was determined according to Eq. 2, in which H_i is the total animal heads per species (i) in the State, W_i (ton head^{-1} year^{-1}) represents the mean LW generation per species, and TN is the weighted mean total nitrogen measured in the LW samples, expressed as dry mass. TP_{PR} and BOD_{PR} were determined in an analog manner.

$$\boldsymbol{TN_{PR} = \Sigma H_i * W_i * TN_i} \qquad (2)$$

The weighted mean GHG emissions (ton $CO_{2,\,eq}$ year^{-1}) were calculated according to Eq. 3 adapted from the IPCC (Intergovernmental Panel on Climate Change) Guidelines for National Greenhouse Gas inventories based on specific factors (IPCC 2019). The weighted mean TN_{PR} and BOD_{PR} from the initial and secondary scenarios were used.

$$\boldsymbol{GHG = (B_0 * MCF * BOD_{PR} * CF_{CH4}) + \left(TN_{PR} * EF * \frac{44}{28} * CF_{N2O}\right)} \qquad (3)$$

B_0 (0.6) and *MCF* (0.11) are dimensionless factors; B_0 represents the maximum fraction of organic matter that can be transformed into CH_4 independent of the degradation process and its conditions, and *MCF* indicates the methane correction factor used to estimate the conversion of the organic matter into methane under specific degradation conditions (direct discharge into aquatic environments). The *EF* dimensionless factor (0.016) represents the fraction of nitrogen converted into N_2O and released into the atmosphere. The factor $\frac{44}{28}$ is for the conversion of kg N_2O-N into kg N_2O. Finally, the CF_{CH4} and CF_{N2O} dimensionless factors are the conversion factors used to estimate the transformation of CH_4 (25) and N_2O (279) into CO_{2eq}, respectively.

4.2.6 Certified Emissions Reductions (CER) estimation

The protocol established by CAR (2010a) for LW treatment for Mexico only considers methane and carbon dioxide emissions missions, integrating them in the following equation:

$$\begin{aligned} \textbf{\textit{Total GHG reductions}} \\ &= (\boldsymbol{CH_4}\ \textbf{\textit{baseline emissions}} - \boldsymbol{CH_4}\ \textbf{\textit{project emissions}}) \\ &+ (\boldsymbol{CO_2}\ \textbf{\textit{baseline emissions}} - \boldsymbol{CO_2}\ \textbf{\textit{project emissions}}) \end{aligned} \tag{4}$$

The first term of the equation can be defined as the destroyed methane according to the measurement in the biogas control system. The current methodology is limited to CH_4 reduction to CO_2, which is calculated as CO_{2eq} using emission factors. The full treatment proposed in this paper combining AD and MbWT aims for reducing atmospheric CO_{2eq} besides reducing emissions, thus creating a scenario that could be measured by the following equation:

$$\textbf{\textit{Total GHG reductions}} = \boldsymbol{CH_{4\,destroyed}} + \boldsymbol{CO_{2eq\,removed}} \tag{5}$$

Where the CH_4 destroyed is calculated by the same equation used by CAR (2010) as shown in equation 4 (CH_4 baseline emissions – CH_4 project emissions), and CO_{2eq} removed is calculated by the following:

$$\boldsymbol{CO_{2eq\,removed}} = (\ \boldsymbol{\Sigma FQ} *\ \boldsymbol{EF_{CO2}}) - \boldsymbol{GHG} \tag{6}$$

Where *FQ* is the fuel quantity used in the process and EF_{CO2} the specific emission factor for the emitted CO_2, while *GHG* corresponds to equation 3 and represents the CO_{2eq} removals.

However, these calculations could have additionality of GHG reductions by the use of the products in another productive activity. Also, the organic matter fixed in their biomass requires waste management that guarantees that GHG is not released to the atmosphere in the short term. As a future perspective biomass could be integrated through a circular economy scheme in a livestock production system as a feed improver, a practice that could also cause GHG reductions under the protocol VM0041 for the reduction of enteric methane emissions from ruminants using feed ingredients (Verra 2021). Besides, it could be used as a source for biochar synthesis or even used as fuel.

These reductions should be considered in the same process, being the final component of the circularity process and assuring the permanency of GHG reductions, thus allowing for the traceability of the CO_{2eq} removed by microalgae, e.g., using microalgae as livestock food. According to Verra (2021), net emissions for the enteric fermentation are calculated by the following:

$$\boldsymbol{ER_{enteric_i}} = \sum_{i=1}^{n}[\boldsymbol{BE_{enteric_i}} - \boldsymbol{PE_{enteric_i}}] \tag{7}$$

Where $ER_{Enteric_i}$ is the Total GHG emission reductions due to the project activities during the monitoring period (ton CO_{2eq}), $BE_{Enteric_i}$ is the total baseline enteric CH_4 emissions from livestock enteric fermentation on farm *i* during the monitoring period (ton CO_{2eq}) and $PE_{Enteric_i}$ is the total project enteric CH_4 emissions from livestock enteric fermentation on farm *i*, and from the production, transport and application of the ingredient used during the monitoring period. Hence, the total GHG reduction could be calculated as follows:

$$\boldsymbol{CO_{2,removed}} = (\boldsymbol{\Sigma FQ} * \boldsymbol{EF_{CO2}}) - \boldsymbol{GHG} + \boldsymbol{ER_{enteric_i}} \tag{8}$$

Once these GHG emissions are calculated, the total GHG reductions can be calculated as proposed in equation 4, thus estimating a value that quantifies the amount of CER to be available for compensation.

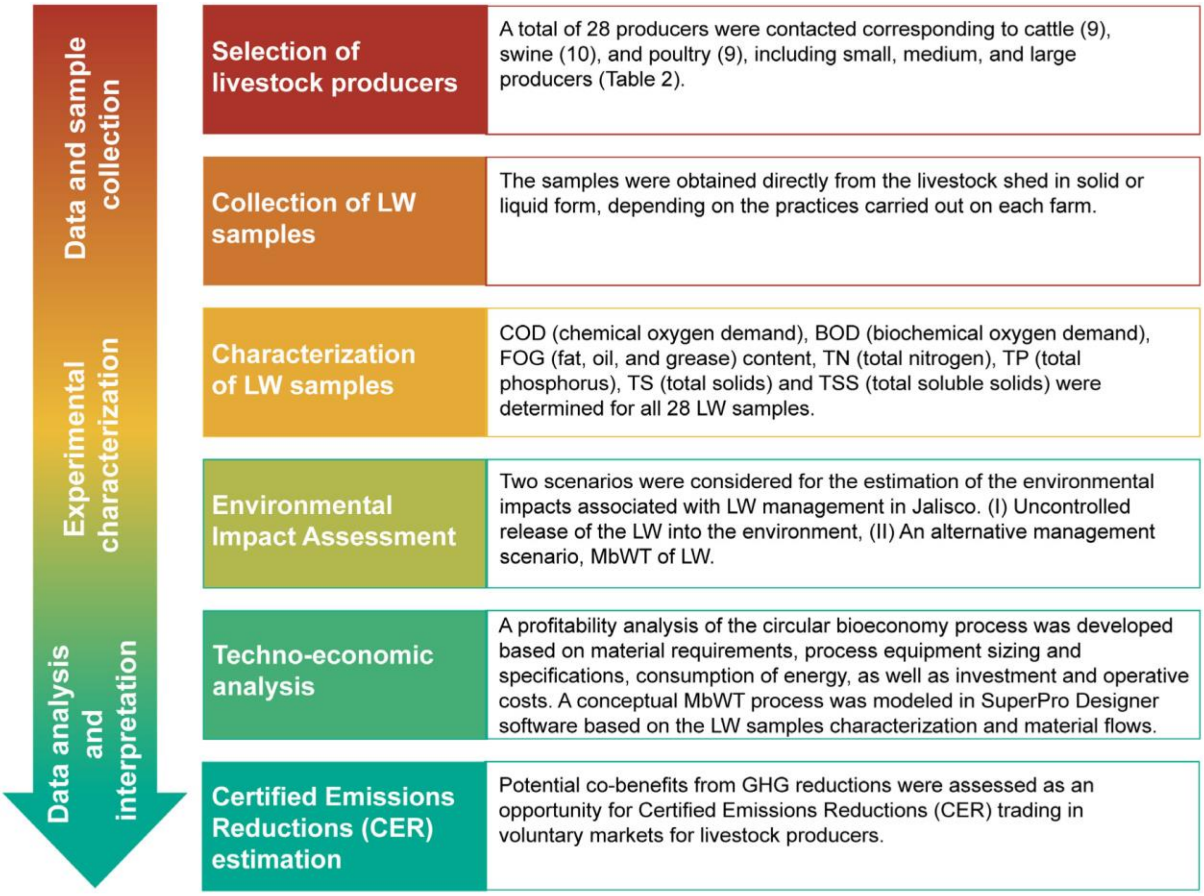

Figure 4.2.6.1. Methodological approach of this study.

4.3 Results and discussion

4.3.1 LW generation and physicochemical characterization

Based on the livestock production inventory of Jalisco provided by SIACON, the three main livestock industry species (cattle, swine, and poultry) generate more than 64 Mton per year of LW in state (Table 1), where cattle, swine, and poultry account for 67.86%, 17.81% and 14.32%, respectively.

Table 4.3.1.1. Livestock waste generation in Jalisco in 2020.

Species	Heads[a]	Waste generation rate[b] [ton head^{-1} year^{-1}]	Generated waste [ton year^{-1}]
Cattle	3,370,866	12.91	43,517,880.06
Swine	3,898,760	2.93	11,423,366.80
Poultry	131,210,547	0.07	9,184,738.29
		TOTAL	64,125,985.15

c. According to historical records from (SIACON, 2020) (Consultation Agrifood Information System of Mexico)
d. According to Díaz-Vázquez et al. (2020)

The physicochemical characterization of the LW generated by these three types of livestock is summarized in Figure 3. In general, LW displayed elevated COD levels (Figure 3b), ranging from 113,571.74 to 1,866,260.12 mg kg^{-1} DM, respectively. CW displayed the highest mean concentration of BOD and COD (307.86 and 629.72 g kg^{-1} DM, respectively). High values of these parameters may cause a significant drop in dissolved oxygen in water sources, which is critical for aquatic life (Ramírez and Rodríguez 2016). The mean biodegradability ratio (BOD_5/COD) found in this study was 0.46 (Figure 3a and 3b), which is a ratio that has been reported to have a high biodegradability potential (Gupta, Pandey, and Pawar 2016).

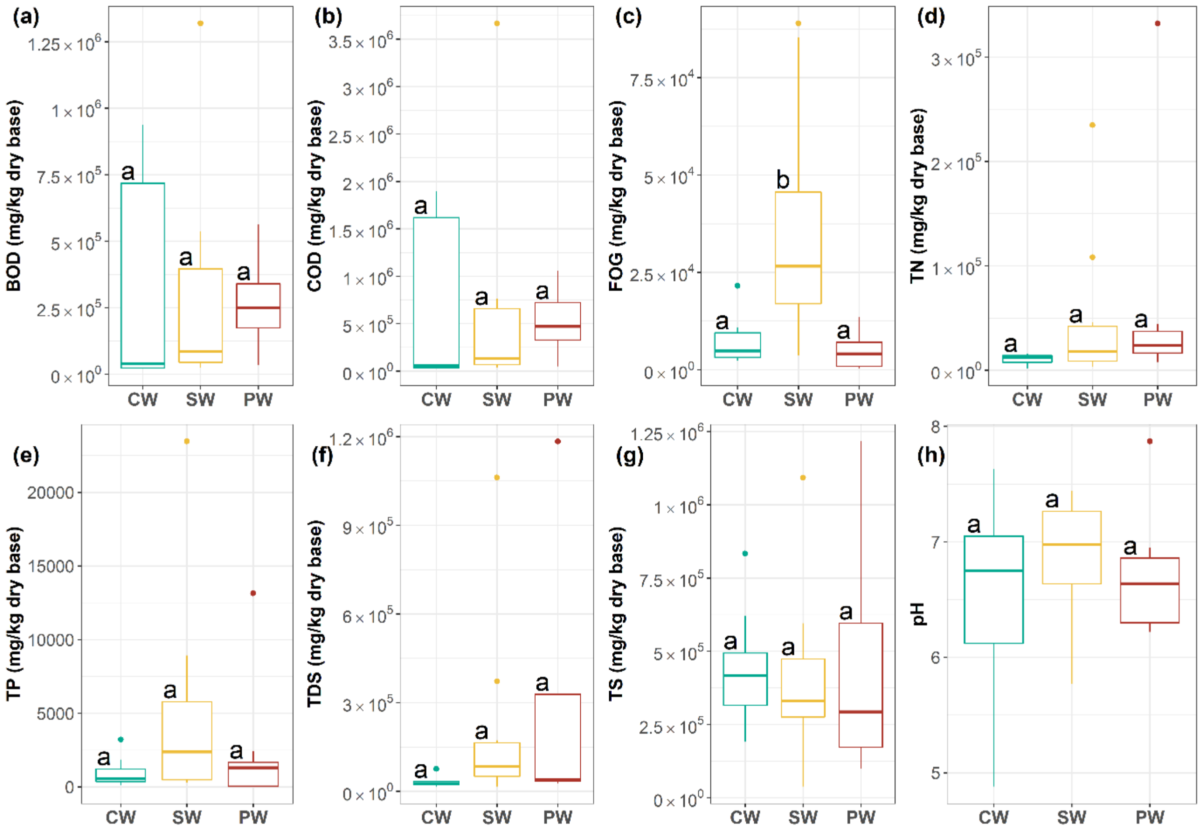

Figure 4.3.1.1 Physicochemical characterization of LW samples grouped by animal species.

The TP and TN contents were different among animal sources. The highest TP mean concentration (4.97 g kg^{-1} DM) was observed in SW (Figure 3e), whereas the highest TN mean concentration (Figure 3d) (69.59 g kg^{-1} DM) was observed in PW. AD has been successfully implemented to treat LW resulting in remaining effluents with significant amounts of nutrients, such as N and P, and a lower C/N ratio, suitable for microalgae growth (Li et al. 2021).

Using all 28 LW samples, an average C/N ratio of almost 50 was estimated, which is higher than in previous studies where C/N ratios ranged from 11.3 to 38.9 (Calderón et al. 2004). The optimal C/N ratio for microalgal growth varies between 5 to 25, depending on the strains used, the culture conditions employed, as well as other factors (Dang et al. 2022; López-Sánchez, Silva-Gálvez, Aguilar-Juárez, et al. 2022). An optimal C/N ratio can enhance microalgal metabolism to effectively absorb TN and TP from LW, while low or high C/N ratios can restrict nutrient removal performance due to low microalgal growth (Dang et al. 2022), therefore, the previous treatment of LW through AD and other unit operators must be performed to assure optimal conditions for microalgal growth. In previous studies, the implementation of the digestate as culture media for microalgae were carried out without the addition of additional carbon sources, nevertheless, further studies need to be performed in large scale to observe this parameter over time in a semi-continuous process . A C/N ratio higher or lower than expected can be controlled with the dilution of the digestate or the addition of nitrogen or carbon sources (Al-Mallahi and Ishii 2022; López-Sánchez, Silva-Gálvez, Zárate-Aranda, et al. 2022; Prajapati et al. 2014; Shi et al. 2018). Considering that the proposed system includes an anaerobic digestor, the biogas can be recirculated into the microalgae pond to adjust the C/N ratio, since this effluent tends to contain between 4 and 15% of CO_2 (Chong et al. 2022).

The highest concentration of FOG (Figure 3c) was observed in SW (36.15 g kg^{-1} DM), in comparison to CW (7.59 g kg^{-1} DM) and PW (4.86 g kg^{-1} DM). This difference in FOG content is attributed to fat digestibility, which depends on dietary sources (Cera, Mahan, and Reinhart 1988). When released into the environment, FOG can affect the physical properties of the soil by inhibiting water infiltration, restricting soil permeability, and resulting in pore-clogging, which causes deficiencies in soil aeration, eventually affecting plant growth (Klamerus-Iwan et al. 2015). For microalgal growth, FOG can only be assimilated by a few microalgal strains (A. Patel et al. 2022), and it inhibits the growth of some strains when present in high concentrations (Panahi et al. 2019). Nonetheless, microalgal strains that can assimilate FOG content can convert low-quality raw materials contained in FOGs into biodiesel, where the overall biodiesel yield may be even higher. Coupling a previous LW treatment through AD prior to microalgal growth could result in a more efficient microalgal metabolism due to FOG hydrolysis in the AD unit (Frkova et al. 2020; A. Patel et al. 2022; Vieira de Mendonça et al. 2021).

The mean pH of all the samples ranged from 6.02 to 7.38, as shown in Figure 3h, which is close to the optimal pH value of 7 for microalgae growth (López-Sánchez, Silva-Gálvez, Zárate-Aranda, et al. 2022). Finally, PW displayed the highest TS (1,216,184.97 mg kg^{-1} DM) and TDS (1,183,815.03mg kg^{-1} DM) concentrations (Figures 3f and 3g). The high TS content in PW that has been previously reported ranges from 20% to more than 60% (Nie et al. 2015; K. Wang et al. 2014). In addition, high solid content has been associated with high turbidity (Vasistha and Ganguly 2020), which may inhibit microalgal growth in the MbWT scenario, as the microalgae cannot perform photosynthesis. Therefore, pretreatment is mandatory for MbWT with LW. As mentioned before, AD could be used to lower the turbidity of LW and obtain the optimal C/N and BOD_5/COD ratios for microalgal growth. Hence, the MbWT coupled with anaerobic digestion were chosen to perform the techno-economic analysis simulation mentioned in section 2.4. Additionally, the implementation of AD opens the possibility to access carbon credits, according to international protocol that considers methane destruction after biogas generation by LW treatment.

4.3.2 Territorial analysis of the microalgal potential in Jalisco

In addition to the circular bioeconomy potential to recover nutrients and organic matter, the natural conditions of the region also favor microalgal cultivation. For example, (Lozano-Garcia et al. 2019) made a GIS model to identify areas in Mexico with high potential for microalgae production, considering land use, topographic slope, temperature, evaporation, solar radiation, vegetation, water, and CO_2 sources, wastewater treatment plants, rivers and lakes, cities and roads, natural protected areas, historical sites, Ramsar sites, airport locations and geological faults. From this analysis, the state of Jalisco displayed the largest areas suitable for microalgal cultivation and highest biomass production capacities, with a high number of cultivation sites in the country (> 600 sites with an area ≥ 4 km) because this region concentrates optimal environment conditions (a mean annual temperature of 26.01-29.5°C and a solar radiation of 5.90-6.2 kWh $m^{-2}d^{-1}$). As observed in Figure 4A, the largest clusters of livestock producers are in the northeast region of Jalisco, which concentrates 75% of the total livestock producers in Jalisco. In these clusters, the Jalisco's regions of Altos Norte, Altos Sur and Ciénega, are identified in which a mean temperature variation from 19 and 29°C and a mean photoperiod of 12.7 hours is presented, favoring the AD operation and the microalgae growth.

Additionally, a wide variety of livestock producers of swine, poultry and bovine are found in these regions, which can help to overcome the challenge of generating a mix of substrate optimal for MbWT. Considering the different concentrations, specifically of organic matter, TP, and TN, of each effluent, a substrate with a tolerable organic content and optimal N/P ratio can be achieved. For *C. vulgaris,* it presents a mean tolerance of COD concentrations of 2,500 mg L^{-1}, above this quantity it starts to reveal a growth inhibition; and it present higher growth rates with a N/P ratio of 16/1when cultured in LW(López-Sánchez, Silva-Gálvez, Aguilar-Juárez, et al. 2022).

However, most of the livestock units in this region are classified as small livestock producers (INEGI 2020), which cannot invest in technologies, like MbWT, or assure their continuous operation. Hence, due to the geographical proximity of these livestock production units in Jalisco, this region could become a strategic and suitable area to apply large-scale centralized systems combining AD and MbWT. This model would reduce energy consumption, individual investment, and transportation costs, and simplify the logistics necessary for treating LW for several producers, assuring a continuous LW supplementation to the system (Díaz-Vázquez et al. 2020, 2021). In the present work, several volumes of LW were simulated using the methodology described in section 2.4. Bigger inputs enhanced the economic feasibility of the model. However, a centralized model requires close coordination between the public, private and academic sectors to succeed. Other studies should be consulted regarding challenges in the implementation of centralized systems.

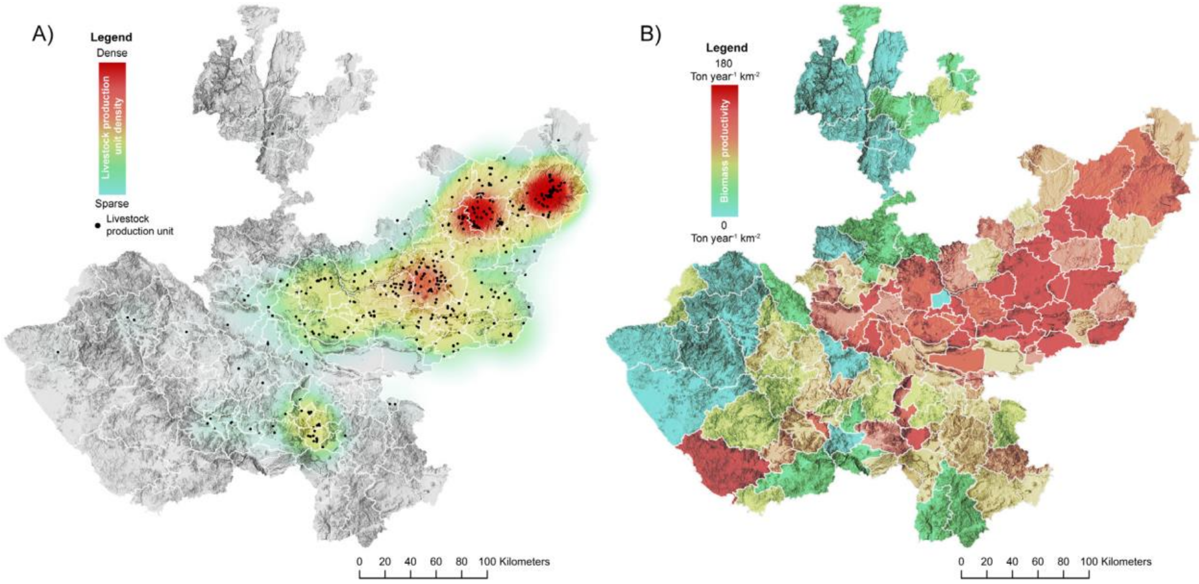

Figure 4.3.2.1 (A) Density of livestock producers according to SEMADET (2019). (B) Livestock waste generation per municipality in Jalisco based on the inventory of SIACON (2020).

4.3.3 Techno-economic analysis of MbWT process

The simulation of the process is shown in Figure 5. The treatment system consists of operating units that improve the quality of anaerobically digested LW (ADLW) for microalgal performance. The LW is directly fed into an anaerobic bio-digester with a vessel volume of 2,213.98 m^3, producing biogas and digestate (working to a vessel volume ratio of 85%) and with an HRT of 240 h. The ADLW is fed into the microalgae raceways (3775.16 m^3) for MbWT. The biomass of *Chlorella* sp. has a yield of 1.26 g of biomass per liter of digestate (Z. Wang et al. 2022), thus producing 2034.4 tons biomass yr^{-1} after the downstream process. It should be noted that the turbidity of the digestate has been reported to hinder the microalgae growth, which has been typically overcome through dilution with water or using different streams. However, one of the limitations of this simulation is the unavailability of options to simulate the negative effect of turbidity on microalgae growth. Hence, the effect of turbidity and dilution of the digestate prior to the raceway ponds were not simulated in this study. The culture media containing microalgae was used to upgrade the biogas by absorbing CO_2 and H_2S in a stripping unit before heading into the bio-flocculation unit to recover the biomass. As harvesting can contribute up to 15% of the total production cost for microalgal biomanufacturing, flocculation was chosen as it is potentially a cost-effective approach (Butler et al. 2021). The bio-flocculation was selected over conventional flocculation to present toxicity and structural deformities to microalgae (Pandey et al. 2019), while chitosan has proven to be an effective bio-flocculant that can be consumed (Hadiyanto et al. 2021). This component has been widely used for microalgae harvesting due to its properties. The negative charges and the possession of amino groups contained in the chitosan surface allows an effective harvest of the microalgae. Even though this bio-flocculant is more expensive than the conventional one, it possesses other advantages that makes it economical feasible to its application (as further described in the economic analysis section) such as its non-toxicity and biodegradability, that avoid an additional process to remove it (Yin et al. 2021), nevertheless, the chitosan were choose because of the objective to produce biomass as supplement, if this objective changes, the election of the flocculant can vary to lower operational costs. Finally, the process culminates when the recovered microalgal biomass is dried in tray-drying units, while the upgraded biogas is burned in a gas turbine generator to produce electric energy.

This process allowed obtaining high protein microalgal biomass that contributes to the profitability of the treatment scheme as the main product. Additionally, two more resources are generated, the power generated by the combustion of 3451.42 L h^{-1} of methane (93.70% methane content) and the treated water, which can both be used reused in the process, thus making MbWT a circular economy strategy for LW remediation.

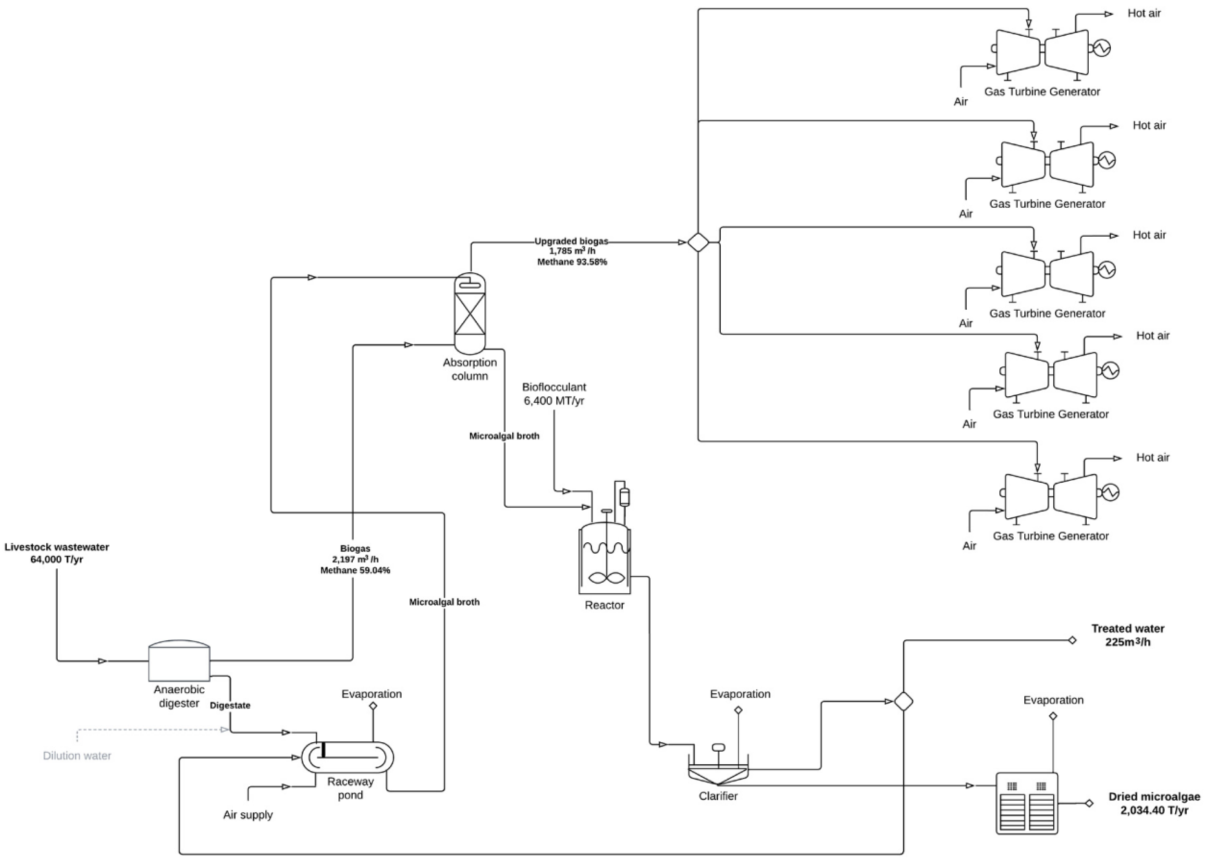

Figure 4.3.3.1. Circular bioeconomy treatment of livestock waste modeled in SuperPro Designer v12 software.

The permissible daily average pollutant concentration for disposal into rivers, streams, canals, drains, reservoirs, lakes, and lagoons is shown in Table 2. The final pollutant concentration of the effluent obtained in the proposed treatment system meets the conditions established in the norm, except for TOC and TS. Therefore, further optimization must be considered for its application, which may include coagulation-flocculation or dissolved air flotation units.

Table 4.3.3.1. Effluent composition after the treatment.

Parameters	Final concentration (Simulation)	Removal (%)	Maximum allowable volumes in Mexican standard: (DOF 2022)
TOC (mg C L^{-1})	69.13	96.73	53
COD (mg O L^{-1})	100.99	91.71	210
BOD5 (mg O L^{-1})	42.41	98.40	-
TKN (mg N L^{-1})	19.71	98.87	35
TP (mg P L^{-1})	14.22	72.70	21
TS (mg Solids L^{-1})	757.35	88.44	84

4.3.4 Environmental impact assessment considering the current and alternative scenarios.

The weighted means for TN, TP, and BOD in SW, PW and CW determined herein, along with the total animal heads per species and mean waste generation per species, was used to assess the environmental impacts. The potential release of pollutants (TN_{PR}, TP_{PR}, BOD_{PR}) and GHG emissions were estimated considering two scenarios of LW management: (i) LW management under the current dominant practices of uncontrolled TN, TP, and BOD release into the environment, which was used as a baseline for conditions where LW is not generally treated, and (ii) LW under a circular bioeconomy approach considering LW treatment through AD coupled with MbWT, minimizing the release of nutrients and reducing GHG emissions, while enhancing CO_2 fixation via microalgal photosynthesis. N_2O emissions were assumed to be avoided in this second scenario because microalgae consume the nitrogen present in LW that otherwise would be transformed into N_2O when released into bodies of water (Singh et al. 2021). Table 3 summarizes the environmental impacts of both scenarios. It is important to mention that in this section a net balance of the GHG emissions were computed considering the microalgal CO_2 fixation, independently that these savings cannot be considered for CER acquisition.

The current practices of uncontrolled release would potentially account for 34% of Jalisco's total GHG emissions (10,765,453 ton CO_2 eq year^{-1}). GHG emissions in Jalisco were estimated at 30,798,268 ton CO_2 eq year^{-1}, and livestock cultivation was ranked as the number two activity (only after transportation) for emitting GHGs (SEMADET 2019). Alternatively, AD coupled with MbWT is a sustainable approach for achieving carbon neutrality, as microalgae can simultaneously treat LW while assimilating the pollutants present in these residues to be used for biomass synthesis (Xiaogang et al. 2020). In this study, the potential LW management through AD and MbWT would result in mean GHG emissions of 5,105 ton CO_2 eq year^{-1}, which is 99% lower than those under uncontrolled LW dumping in the current scenario.

The majority of the GHG reduction is presented by avoiding the uncontrolled release of LW to the environment, preventing the formation of methane and nitrous oxide. Up to 99% reduction of the nutrients TP, TN and BOD are presented with this treatment model as can be observed in table 3.

Table 4.3.4.1 Environmental impact assessment under both scenarios.

	Nitrogen	Phosphorus	BOD_5	Treatment process direct emissions	CO_2 fixed	Total GHG	Biomass potential
	[ton year^{-1}]	[ton year^{-1}]	[ton year^{-1}]	[ton year^{-1}]	[ton year^{-1}]	[ton CO2eq year^{-1}]	[ton year^{-1}]
Scenery 1	238,716.40	15,105.27	5,509,631.29	-	-	10,765,453.09	-
Scenery 2	1,005.54	70.55	1,263.86	15,168.64	19,201.87	5,105.86	2,034.40
Reduction (%)	99.58	99.53	99.98	-	-	99.95	-

The proposed circular bioeconomy scenario could reach a potential GHG emission of 15,186 ton CO_2 eq year^{-1} for direct emissions of the process (energy consumption and a 5% methane), while the CO_2 fixation by microalgae under autotrophic growth could reach up to 19,210 ton year^{-1}.

4.3.5 Economical analysis

The initial investment of the proposed facility \$90,379,000 USD with an annual operating cost of \$18,546,000 USD and achieving a total microalgal (*Chlorella* sp.) biomass production of 2,034.40 T per year, considering a yield of 1.26g L^{-1} (Z. Wang et al. 2022) (Table S3 in supplementary material). Microalgal biomass production increases economic viability through the production and commercialization of protein-rich biomass. The unit production cost of one kilogram of dried microalgal biomass is \$8.67 USD, while microalgal biomass, as reported in section 2.4, was considered to have a market selling price of \$20 USD kg^{-1}. Thus, the biomass produced annually could generate \$42,792,000 USD in profit. Furthermore, when cultured in LW, certain strains of microalgae (*C. vulgaris*) have displayed a protein content between 44.57 – 58.8% (Sun, Sun, and Chen 2018; Vuppaladadiyam et al. 2018), which could display higher protein content than that of existing commercial microalgal biomass products

(22%) (Phycom 2022). In this circular bioeconomy approach scheme, \$4,826,560.00 USD are saved, as the electricity generated in the treatment system can be reintroduced into the process (lowering fossil fuels consumption), while the cost of treating the water externally is avoided (\$3,375.00 USD). Besides, the generation of CER for the voluntary market could be profitable if accredited by a standard recognized by the UNFCCC or other legislations. The potential CER estimated is close to 10 $MtCO_2eq$ (according to this exploratory data), resulting in an income of \$38,952,456.97 USD (Table S3 in supplementary material).

The generation of CER for the voluntary market could be profitable if accredited by a standard recognized by the UNFCCC or other legislations. The potential CER estimated is close to 10 $MtCO_2eq$ (according to this exploratory data), resulting in an income of \$38,952,456.97 USD. This represents almost 1/9 of the projected reduction goal for Mexico through nature-based solutions, low carbon mobility, and better industrial practices for 2030 (SEMARNAT 2022). While Mexico has not implemented the operative phase of its emissions trade system, compensation by CER as a flexibility mechanism for the emissions rights compliance for the national market makes CER trade unattractive for the obligated companies, but the CER could be sold in the international market (DOF 2019).

Finally, an executive summary of the economic considerations of the circular bioeconomy potential scenario for LW management is presented in Table 4. These numbers show a great economic viability for this technological transition. However, economic viability is greatly dependent on the input amount of LW; bigger inputs result in greater economic viability. If the input of LW is less than 3,458 ton yr^{-1}, the Gross Margin of the treatment system becomes negative, which means that the system is no longer economically viable. Therefore, a large-scale centralized system must be implemented to achieve profitability of the system. Even more, GHG reduction only considers CO_{2eq} removals due to the AD-MbWT system and does not consider reduction permanency. Also, biomethane potential (BMP) assays yield the amount of biogas produced by the anaerobic digestion of organic matter in terms of liters per kilogram of volatile solids, whereas in our study we considered the amount of volatile solids of livestock manure to be 40% based on literature values (Kafle and Chen 2016; Raju et al. 2012) Similarly, the BMP value considered for this study was 680 L kg VS^{-1}, which is in the range of biogas production obtained from cattle, swine, and poultry manure (Johannesson et al. 2020; Kaur and Kommalapati 2021). Further considerations of the profitability analysis of the circular bioeconomy treatment are depicted in supplementary material (Table S3).

Table 4.3.5.1 Executive summary of the circular bioeconomy treatment of livestock manure.

Parameter	Value	Unit
Total Capital Investment	90,379,000	USD$
Operating Cost	18,546,000	$/yr

Principal Revenue (dried biomass)	42,791,562	$/yr
Other revenues (treated water, energy saving and CER)	43,779,017	$/yr
Total Revenues	86,570,579	$/yr
Net Unit Production Cost	8.67	$/kg MP
Gross Margin	78.58	%
Return On Investment	65.47	%
Payback Time	1.53	yr
IRR (After Taxes)	46.51	%
NPV (at 7.0% Interest)	334,286,000	$
Project development to achieve CER	10,000	$
Project registration to the standard	500	$
Standard commissions for account creation and management	500	$
Initial and subsequent verifications	10,000	$/yr

*MP: main product, i.e. dried microalgae biomass.

Comparting the current linear scenario of LW management in Jalisco with the alternative circular bioeconomy scenario proposed herein focusing on MbWT, 64 Mton of LW year^{-1} is produced per year in Jalisco, which, if untreated, can produce 5.5 Mton year^{-1}, 238,716 ton year^{-1}, and 15,105.27 ton year^{-1} of BOD, TN and TP, respectively that are potentially released to the environment with GHG emissions as high as 10.76 Mton CO_2 eq year^{-1} (Figure 5A). Additionally, 19 Mton year^{-1} of CO_2 would be fixed from the environment into value-added byproducts, which could potentially be 2 Mton year^{-1} of *Chlorella* sp. biomass rich in protein. However, these numbers should be analyzed with caution, as the circular bioeconomy scenario represents the optimal case in the application of MbWT, considering ideal conditions and nutrient uptake.

The main advantage of CERs generation is the existence of the CAR (2010) methodology specific for Mexico and the quantity of GHG removals proposed by this study makes this alternative attractive to implement MbWT. Moreover, the infrastructure, logistics and technical knowledge among producers to assure system's operation and all the specifications required by a CER protocol represents a major concern, thus penalties to project developers could engage system's economic viability. Besides the lack of technical capacities for system's management that could cause sanitary risks for livestock systems. However, the benefits by CER trade in Mexico are going to growth due the national carbon market (DOF 2019) and Jalisco's local market implementation (Gobierno de Jalisco 2022; Gobierno del Estado de Jalisco 2019) that could bring the necessary investment to infrastructure and equipment if financial runs are correct. Nevertheless, the lack of logistics and technical preparation of producers and the lack of verification bodies for this methodology could derive in the misoperation of the system could also cause penalties and project loses. A strong safeguards protocol should be implemented to protect projects of possible

CO_2 reversals, as said by CAR (2010) and the limited options by CAR (2010) protocol to get CER by the LW treatment presents the main difficulty to get CER's income by MbWT.

From an economic perspective, the high initial investment of a system AD coupled with MbWT ($90,379,000USD) for livestock producers is the largest challenge for small producers, which could be overcome by implementing a centralized system further discussed in Political and Legal aspects (Liu and Hong 2021). Nonetheless, after the initial investment, AD and MbWT have low operational costs, as the natural condition in Jalisco favors their operations, especially the temperature, that promotes a mesophilic operation of the AD and higher microalgal growth rates without external energy inputs. Additionally, the CO_2 and sunlight (photoautotrophic growth), and the nutrients available in the environment and ADLW favors the MbWT performance without additional supplementation (Hussain et al. 2021). Moreover, the production of microalgal biomass can increase economic viability through the production and commercialization of value-added compounds, such as biomass-based food supplements rich in protein. In recent years, the microalgal biomass market, including microalgal based supplements, has been growing substantially, achieving a market price estimated at $3.4 billion USD and projected to reach $4.6 billion USD by 2027 (Loke Show 2022). However, the production of value-added compounds through MbWT requires the implementation of expensive downstream processes to extract the desired product from the biomass. Therefore, the initial investment and operational costs could increase when considering the biorefinery. For example, the initial investment and operating costs of the proposed biorefinery system is $90,379,000and $18,546,000, respectively, which is higher in comparison with the system without the biorefinery ($24,247.00 and $4,620.00, respectively). However, the total revenues by just treating the water is null, while the biorefinery approach could potentially generate $42,791,562USD annually. However, the legislation for the consumption and application of microalgae and microalgal-based products in Mexico is nonexistent. The lack of acceptance of LW-derived products (i.e., feed supplements) is a potential threat to the commercialization of the value-added compounds produced, as there are lack of studies demonstrating their safety for consumption (Koutra et al. 2018; Rumin et al. 2021). Furthermore, the market at which these microalgal supplement products should be directed is unclear. However, there is a growing worldwide interest in microalgae-related products from MbWT of LW, along with the consumption of related food supplements (Rumin et al. 2021). Therefore, more research regarding target markets is warranted. This is a field in which progress must be made to reduce the risks associated with consumption, so that the resulting biomass may be used as a supplement for livestock or humans.

Besides the biomass generation, the CO_{2eq} fixation capacity of microalgae represents an important opportunity for CER trading, however, standards should include the methodology for this alternative to measure reductions as CER to promote the microalgae technology for wastewater remediation and not only AD and other listed technologies as proposed by CAR. Moreover, the capacity of microalgae to grow 10 times faster than terrestrial plants and produce up to 300 times more oil per acre in comparison to conventional crops (soybean, palms, among others) makes them a potential feedstock for biofuel production, both biodiesel and biogas (Giri et al. 2022; Kowthaman et al. 2022; Thanigaivel et al. 2022) and represent an opportunity to complete the circular bioeconomy approach to the system and for the accredit reductions permanency. In 2020, the energy independence index of Mexico, which shows the relationship between national energy production and consumption, was 0.87, implying that energy consumption was greater than what was produced in the country. Therefore, renewable energy in Mexico has gained much attention, with an increase of 25.64% in the electric power generation matrix in 2020 (SEMADET 2019). In contrast, the oil production in Mexico has continuously decreased, recording in 2019 the lowest production since 1979 (U.S. Energy Information Administration 2020). Consequently, this represents an opportunity for Jalisco, the agri-food giant of Mexico, to produce clean energies using livestock residues not only for the potential biodiesel production from MbWT, but the energy generation through methane combustion from AD, thus, achieving energetic independence.

From a technical and infrastructural perspective, open-air raceway ponds, which are the most common configuration for large-scale microalgal cultivation due their economic feasibility, have a high risk of contamination by native microbiota from LW and microbes present in the environment that could affect or even displace the microalgae (Ferro et al. 2020). However, specific microbial consortia, including microalgae and bacteria, have been reported to be less sensitive to fluctuations in environmental conditions and more resistant to contamination (Nguyen et al. 2020). In this regard, further research is required to understand the consortia that perform best for MbWT. Moreover, MbWT can be coupled with AD, to treat ADLW or purify the produced biogas (methane enrichment) for energy usage through an absorption unit (Nasir, Mohd Ghazi, and Omar 2012). Additionally, even better methane yields could be achieved including microalgae biomass or hydropyrolysed microalgae of a fraction of the biomass gathered to recirculate to the AD system (Choudhary, Malik, and Pant 2020; Prajapati et al. 2014), these strategies could help to adjust the C/N in the systems. Depending on the goals and the market conditions for the potential products, the proposed system can be adapted to improve revenues. Furthermore, harvesting accounts for up to 20–30% of the total production costs (Kim et al. 2022), however, these costs are lowered by implementing a bio-flocculation operating unit, which has been reported to display high biomass recovery (Butler et al. 2021; Hadiyanto et al. 2021).

High evaporative losses from MbWT systems may decrease the water volume and thus increase pollutant concentrations (i.e., suspended solids and turbidity), while the high turbidity of LW can reduce light penetration for autotrophic and mixotrophic growth, inhibiting microalgal growth (Torres-Franco et al. 2020). In those cases, strategies can be applied to reduce the evaporative losses, such as the geometric manipulation of open raceways ponds and the application of thin-film monolayers, or to reduce the turbidity by implementing a flocculation (or bio-flocculation) stage employing locally available natural flocculating agents that are non-toxic and biodegradable (Al-Mallahi and Ishii 2022; Ganeshkumar et al. 2018; Poddar et al. 2022; Torres-Franco et al. 2020)

Moreover, the land necessary to implement AD combined with raceway ponds for MbWT may be considered as wasted land to livestock producers, since it could be applied to a more secure business, such as crop cultivation. Nonetheless, a centralized AD + MbWT system could mitigate these threats.

From a political perspective, the State´s Government has presented a carbon tax initiative and a proposal for a local carbon market based in its own carbon pricing methodology., This makes an important opportunity to use MbWT as a source of CER if the methodology is recognized by CAR (2021) methodology. The resulting CER could compensate for the emissions in the state and represent an additional income for producers and provide funds to the state through transaction commissions to develop additional mitigation projects.

4.4 Conclusion

The aim of this work was to characterize the composition of the LW generated in the cattle, swine, and poultry farms located in the livestock-intensive region. The LW composition indicated a high biodegradability potential. However, the C/N ratio was far from optimal, and the high turbidity may inhibit biomass growth. Therefore, a previous anaerobic digestion was proposed before its application as microalgal culture media. The treatment of LW through a circular bioeconomy scenario using AD coupled with MbWT would represent a significant decrease in GHG emissions, TP, TN, and organic matter compared to the current LW management. The centralized clusters model for LW treatment and distributed generation seems to be technically and economically feasible considering the generation of energy and high-protein biomass from AD and MbWT, respectively. Additionally, the CER acquisition from methane combustion for energy generation represents an important additional revenue for the system to promote sustainable LW management and the biomass obtained should be used for another emissions reduction scheme, being enteric methane reduction or reductions for the use of biofuels potential alternatives to guarantee the reductions permanency. While CER trade is a possible co-benefit for its implementation. However, there is no validated methodology for emissions reduction using MbWT. The cost-benefit for the MbWT considers both economic and environmental impacts, as well as the necessity to guarantee the permanency of CO_{2eq} removed from the atmosphere.

Limitations of this study

This work was an exploratory study in which the LW sample number should be increased to lower the variability in the LW characterization. The alternative scenario represents an ideal case for the application of MbWT according to previous research, nevertheless, more pilot cases must be developed to assure the technical feasibility. Additionally, to achieve this potential scenario many economic, sociocultural, legal, and political challenges need to be overcome. Optimal conditions (i.e., nutrient and micronutrient concentration and bioavailability, sun irradiation, temperature, presence/absence of other synergistic/pathogenic microorganisms) were considered for the potential assessment of microalgal growth estimation. With the software used in the simulation it is not possible to simulate the negative effect of turbidity on microalgal growth. Hence, the effect of turbidity and dilution of the digestate prior to the raceway ponds were not simulated in this study. Also, further research is required regarding the downstream processing of biomass, the acclimation of microbial consortia in the LW, and the safety of LW-derived products for consumption. The use of biofuels produced by MbWT could also accredit CER by the methodologies of Verra VM0002 and VM0019, but additionality of those reductions could be analyzed in further research.

Chapter 5. Conclusions and perspectives

The livestock industry is one of the most important production sectors for all of humanity. It significantly contributes to attaining some of the SDGs related to poverty, zero hunger, health and well-being and economic growth. Nevertheless, current production and waste management practices are resulting in negative human health and environmental impacts. Thus, there is a great challenge to preserve livestock production in large geographical areas where intensive livestock practices are carried out, such as Jalisco, while mitigating the negative impacts.

MbWT of LW represents a sustainable approach to remove pollutants from these effluents with the potential of generating valuable products. Recent studies focused on the enhancement of biomass productivity, pollutant removal and the production of high-value compounds suggest that the modulation of physical and chemical factors and the implementation of different strategies, such as multistage processes, are required. One of the main factors that affects microalgal metabolism is the high content of COD, TN (normally found in its ammonium form), Cu and Zn, which are typically found in LW. Conversely, high contents of TP have been related to higher biomass productivity. The composition of the effluents used for microalgal breeding vary depending on animal species, its diet, housing methods and regional environmental factors. Thus, for the applicability of MbWT on a large-scale, different effluents from the regions in question must be implemented as mixtures to optimize MbWT growth conditions.

An effluent mixing approach is a promising strategy to obtain a growth media with the optimal macro- and micronutrient concentrations to enhance biomass productivity and pollutant removal efficiencies without additional medium supplementation. However, it is necessary to perform detailed characterizations of these effluents to assess the optimal concentrations of microelements. Additionally, this strategy offers a holistic opportunity to revalorize the most common livestock wastewater effluents generated within farms, where the breeding of different animal species (like swine, cattle and poultry) is often carried out simultaneously.

Using an effluent mixing strategy, *C. vulgaris* in monoculture, cultured in LW media composed of 0.125 ADSW, 0.4375 ADPW and 0.4375 ADCW, displayed a cell growth of 3.61×107± 2.81x107 cell mL-1, a total nitrogen removal of 85%±2%, a total phosphorus removal of 66%±3% and a chemical oxygen demand removal of 44%±7%, considering the initial concentrations of 333.33, 28.47 and 680 mg L^{-1} of TN, TP and COD, respectively. Using a centroid mixture design, an optimal mixture was determined for *C. vulgaris* composed of 0.125:0.200:0.675 (ADSW:ADPW:ADCW). Nevertheless, further studies using this mixture should be performed to assess the effects on the growth and pollutant removal performance of *C. vulgaris.* In Jalisco, this optimal mixture could represent a convenient solution, since almost 70% of the total livestock effluents come from cattle and dairy wastewater. However, the optimal mixture highly depends on the MbWT goals and, in this study, biomass production and pollutant removal efficiency were equally weighted to evaluate the overall performance.

Additionally, the interactions within microalgal consortia play an important role in MbWT. The performance of the monocultures of *H. pluvialis* and *Chlamydomonas* sp. was improved when cultured in a consortium, suggesting a potential mutualistic relationship, potentially improving the robustness and tolerance of the microalgal cultures to high pollutant concentrations encountered in LW. Furthermore, the interactions between the microalgae and the native microorganisms within LW must be assessed to quantitatively assess their effects on the overall performance of MbWT to avoid the sterilization of these effluents to lower the operational costs for future applications at large scale. However, these interactions must be further studied and assessed quantitively through molecular techniques to elucidate the specific interactions between the microalgae that remain unclear. The implementation of microalgal consortia for MbWT represents an opportunity to obtain more robust treatment systems that are resistant to adverse environments.

The centralized clusters model for LW treatment and distributed generation seems to be technically and economically feasible considering the generation of energy and high-protein biomass from AD and MbWT, respectively. Additionally, the CER acquisition from methane combustion for energy generation represents an important additional revenue for the system to promote sustainable LW management and the biomass obtained should be used for another emissions reduction scheme, being enteric methane reduction or reductions for the use of biofuels potential alternatives to guarantee the reductions permanency. While CER trade is a possible co-benefit for its implementation. However, there is no validated methodology for emissions reduction using MbWT. The cost-benefit for the MbWT considers both economic and environmental impacts, as well as the necessity to guarantee the permanency of CO_{2eq} removed from the atmosphere.

Beyond the specific parameters that guarantee an adequate performance at the laboratory scale, is necessary to perform further research on the applicability of MbWT at a large-scale focusing on: 1) large-scale trials applied under biorefinery approaches, 2) downstream design, 3) different biomass harvesting strategies, such as immobilized cultures and 4) socio-cultural factors affecting the implementation of MbWT.

Appendix A

Table A.1 Initial and final concentrations of the response variables of the mixture design and global performance index (GPI) (cell growth, total nitrogen removal, total phosphorus removal and chemical oxygen demand removal).

						Initial concentration [mg/L]				final concentration [mg/L]					Nutrient removal [%]			
Strain	Mix	ADSW	ADPW	ADCW	Initial cell density [cell/ml]	TN	TP	DQO	final cell density [cell/ml]	TN	TP	DQO	Cell growth [cell/ml]	Norm_ cell growth	TN	TP	DQO	GPI
Chl	1	0.750	0.125	0.125	3.67E+06	658.89	25.38	692.22	1.32E+06	28.33	25.95	242.44	-2.35E+06	-0.03	0.96	-0.02	0.65	0.39
Chl	2	0.125	0.750	0.125	6.83E+06	696.67	26.49	880.00	4.62E+06	93.33	9.80	298.00	-2.21E+06	-0.03	0.87	0.63	0.66	0.53
Chl	3	0.125	0.125	0.750	6.05E+06	422.22	20.63	934.44	3.13E+06	41.67	5.97	333.56	-2.92E+06	-0.04	0.90	0.71	0.64	0.55
Chl	4	0.438	0.438	0.125	4.18E+06	566.67	29.39	738.89	7.10E+06	106.67	16.43	348.89	2.92E+06	0.04	0.81	0.44	0.53	0.46
Chl	5	0.438	0.125	0.438	3.32E+06	407.78	21.52	1044.44	3.55E+06	85.00	9.99	294.67	2.30E+05	0.00	0.79	0.54	0.72	0.51
Chl	6	0.125	0.438	0.438	3.43E+06	536.67	21.43	1007.78	4.77E+06	108.33	5.92	279.33	1.34E+06	0.02	0.80	0.72	0.72	0.57
Chl	7	0.333	0.333	0.333	3.63E+06	466.67	22.20	1017.78	5.43E+06	170.00	9.41	348.44	1.80E+06	0.03	0.64	0.58	0.66	0.47
Chl	1	0.750	0.125	0.125	2.13E+06	430.00	27.56	646.67	1.65E+06	205.00	23.63	253.33	-4.80E+05	-0.01	0.52	0.14	0.61	0.32
Chl	2	0.125	0.750	0.125	2.32E+06	670.00	30.76	1026.67	3.43E+06	285.00	16.13	710.00	1.11E+06	0.02	0.57	0.48	0.31	0.34
Chl	3	0.125	0.125	0.750	2.58E+06	434.44	21.90	913.33	4.82E+06	221.67	11.13	421.11	2.24E+06	0.03	0.49	0.49	0.54	0.39
Chl	4	0.438	0.438	0.125	2.67E+06	600.00	31.81	730.00	3.55E+06	238.33	22.40	419.33	8.80E+05	0.01	0.60	0.30	0.43	0.33
Chl	5	0.438	0.125	0.438	3.68E+06	464.44	23.02	864.44	3.35E+06	305.00	19.23	271.33	-3.30E+05	0.00	0.34	0.16	0.69	0.30
Chl	6	0.125	0.438	0.438	3.65E+06	523.33	24.79	976.67	2.75E+06	268.33	14.81	679.33	-9.00E+05	-0.01	0.49	0.40	0.30	0.30
Chl	7	0.333	0.333	0.333	3.27E+06	413.33	25.82	887.78	3.62E+06	198.33	16.77	632.67	3.50E+05	0.01	0.52	0.35	0.29	0.29
CV	1	0.750	0.125	0.125	3.43E+06	292.22	22.94	643.33	7.25E+07	56.67	26.18	313.33	6.91E+07	1.00	0.81	-0.14	0.51	0.54
CV	2	0.125	0.750	0.125	5.25E+06	418.89	27.39	593.33	6.05E+06	113.33	23.39	351.33	8.00E+05	0.01	0.73	0.15	0.41	0.32
CV	3	0.125	0.125	0.750	4.33E+06	258.89	24.36	860.00	4.01E+07	61.67	11.39	424.00	3.58E+07	0.52	0.76	0.53	0.51	0.58
CV	4	0.438	0.438	0.125	5.20E+06	344.44	29.27	633.33	2.65E+07	55.00	18.52	360.44	2.13E+07	0.31	0.84	0.37	0.43	0.49
CV	5	0.438	0.125	0.438	4.57E+06	71.67	25.21	6170.00	4.40E+07	43.33	11.33	372.89	3.94E+07	0.57	0.40	0.55	0.94	0.61
CV	6	0.125	0.438	0.438	4.53E+06	333.33	28.47	680.00	4.06E+07	50.00	9.77	381.11	3.61E+07	0.52	0.85	0.66	0.44	0.62
CV	7	0.333	0.333	0.333	5.02E+06	392.22	27.53	1380.00	3.91E+07	98.33	14.07	418.67	3.41E+07	0.49	0.75	0.49	0.70	0.61
CV	1	0.750	0.125	0.125	3.83E+06	316.67	23.76	500.00	3.75E+07	103.33	21.60	404.00	3.37E+07	0.49	0.67	0.09	0.19	0.36
CV	2	0.125	0.750	0.125	2.70E+06	428.89	29.28	570.00	1.93E+07	131.67	12.72	496.00	1.66E+07	0.24	0.69	0.57	0.13	0.41
CV	3	0.125	0.125	0.750	3.03E+06	341.11	28.37	1103.33	2.04E+07	88.33	11.41	467.33	1.74E+07	0.25	0.74	0.60	0.58	0.54
CV	4	0.438	0.438	0.125	2.53E+06	415.56	25.11	2090.00	1.73E+07	180.00	16.18	407.33	1.48E+07	0.21	0.57	0.36	0.81	0.49
CV	5	0.438	0.125	0.438	3.20E+06	302.22	27.87	830.00	2.36E+07	258.33	10.70	441.56	2.04E+07	0.30	0.15	0.62	0.47	0.38

CV	6	0.125	0.438	0.438	3.78E+06	380.00	25.43	710.00	1.82E+07	188.33	10.49	434.67	1.44E+07	0.21	0.50	0.59	0.39	0.42
CV	7	0.333	0.333	0.333	2.82E+06	260.00	29.93	720.00	4.30E+07	183.33	10.22	395.33	4.02E+07	0.58	0.29	0.66	0.45	0.50
HP	1	0.750	0.125	0.125	5.15E+06	245.56	21.76	601.11	5.82E+06	90.00	29.82	301.33	6.70E+05	0.01	0.63	-0.37	0.50	0.19
HP	2	0.125	0.750	0.125	5.80E+06	250.00	27.47	757.78	6.28E+06	120.00	17.83	336.00	4.80E+05	0.01	0.52	0.35	0.56	0.36
HP	3	0.125	0.125	0.750	4.93E+06	235.56	24.40	933.33	6.42E+06	65.00	15.93	414.00	1.49E+06	0.02	0.72	0.35	0.56	0.41
HP	4	0.438	0.438	0.125	4.70E+06	347.78	29.50	663.33	5.92E+06	105.00	22.33	330.67	1.22E+06	0.02	0.70	0.24	0.50	0.37
HP	5	0.438	0.125	0.438	4.27E+06	244.44	25.13	824.44	5.58E+06	75.00	21.30	362.00	1.31E+06	0.02	0.69	0.15	0.56	0.36
HP	6	0.125	0.438	0.438	5.75E+06	166.67	25.09	1041.11	6.82E+06	75.00	16.99	368.00	1.07E+06	0.02	0.55	0.32	0.65	0.38
HP	7	0.333	0.333	0.333	4.18E+06	261.11	30.04	1096.67	5.53E+06	143.33	19.67	337.33	1.35E+06	0.02	0.45	0.35	0.69	0.38
HP	1	0.750	0.125	0.125	5.83E+06	278.89	29.24	496.67	7.17E+06	215.00	26.59	314.22	1.34E+06	0.02	0.23	0.09	0.37	0.18
HP	2	0.125	0.750	0.125	6.45E+06	375.56	28.23	828.89	4.78E+06	158.33	22.47	358.00	-1.67E+06	-0.02	0.58	0.20	0.57	0.33
HP	3	0.125	0.125	0.750	6.18E+06	234.44	24.33	2000.00	4.40E+06	233.33	17.46	462.00	-1.78E+06	-0.03	0.00	0.28	0.77	0.26
HP	4	0.438	0.438	0.125	6.45E+06	263.33	30.01	1836.67	2.98E+06	173.33	26.31	497.33	-3.47E+06	-0.05	0.34	0.12	0.73	0.29
HP	5	0.438	0.125	0.438	6.35E+06	216.67	25.48	1410.00	2.75E+06	160.00	21.90	362.67	-3.60E+06	-0.05	0.26	0.14	0.74	0.27
HP	6	0.125	0.438	0.438	8.60E+06	226.67	23.80	1414.44	2.70E+06	196.67	17.77	408.00	-5.90E+06	-0.09	0.13	0.25	0.71	0.25
HP	7	0.333	0.333	0.333	8.52E+06	270.00	26.47	1242.22	2.37E+06	200.00	19.97	558.00	-6.15E+06	-0.09	0.26	0.25	0.55	0.24
Chl + CV	1	0.750	0.125	0.125	7.65E+06	210.00	29.13	682.00	1.77E+07	90.00	29.40	344.00	1.01E+07	0.15	0.57	-0.01	0.50	0.30
Chl + CV	2	0.125	0.750	0.125	9.93E+06	345.00	30.20	732.67	1.80E+07	103.33	15.27	372.67	8.07E+06	0.12	0.70	0.49	0.49	0.45
Chl + CV	3	0.125	0.125	0.750	9.43E+06	215.00	21.87	1872.00	2.14E+07	70.00	12.63	458.00	1.20E+07	0.17	0.67	0.42	0.76	0.51
Chl + CV	4	0.438	0.438	0.125	6.00E+06	220.00	29.70	706.00	1.96E+07	120.00	20.40	358.00	1.36E+07	0.20	0.45	0.31	0.49	0.36
Chl + CV	5	0.438	0.125	0.438	6.63E+06	235.00	18.73	846.00	1.59E+07	60.00	14.40	423.33	9.27E+06	0.13	0.74	0.23	0.50	0.40
Chl + CV	6	0.125	0.438	0.438	5.90E+06	275.00	20.17	802.00	1.99E+07	70.00	11.00	420.67	1.40E+07	0.20	0.75	0.45	0.48	0.47
Chl + CV	7	0.333	0.333	0.333	5.73E+06	230.00	19.80	755.33	2.06E+07	110.00	13.90	391.33	1.49E+07	0.22	0.52	0.30	0.48	0.38
Chl + CV	1	0.750	0.125	0.125	6.47E+06	305.00	28.43	661.33	1.17E+07	100.00	27.10	378.67	5.23E+06	0.08	0.67	0.05	0.43	0.31
Chl + CV	2	0.125	0.750	0.125	9.07E+06	255.00	29.00	393.00	6.90E+06	70.00	16.00	357.33	-2.17E+06	-0.03	0.73	0.45	0.09	0.31
Chl + CV	3	0.125	0.125	0.750	1.31E+07	205.00	17.73	464.33	1.16E+07	20.00	12.40	425.33	-1.50E+06	-0.02	0.90	0.30	0.08	0.32
Chl + CV	4	0.438	0.438	0.125	9.22E+06	235.00	29.07	351.00	7.90E+06	130.00	22.90	362.67	-1.32E+06	-0.02	0.45	0.21	-0.03	0.15
Chl + CV	5	0.438	0.125	0.438	7.10E+06	180.00	17.87	368.00	1.33E+07	60.00	12.70	392.00	6.20E+06	0.09	0.67	0.29	-0.07	0.25
Chl + CV	6	0.125	0.438	0.438	1.03E+07	220.00	21.30	437.00	7.55E+06	70.00	12.70	478.67	-2.75E+06	-0.04	0.68	0.40	-0.10	0.24
Chl + CV	7	0.333	0.333	0.333	7.75E+06	205.00	21.73	397.00	6.10E+06	80.00	14.90	395.33	-1.65E+06	-0.02	0.61	0.31	0.00	0.23
Chl + HP	1	0.750	0.125	0.125	4.83E+06	275.00	41.85	674.00	4.22E+06	100.00	32.50	361.00	-6.10E+05	-0.01	0.64	0.22	0.46	0.33
Chl + HP	2	0.125	0.750	0.125	4.90E+06	315.00	31.03	688.00	6.03E+06	160.00	18.20	384.33	1.13E+06	0.02	0.49	0.41	0.44	0.34
Chl + HP	3	0.125	0.125	0.750	4.05E+06	240.00	23.13	892.00	9.15E+06	73.33	18.20	425.33	5.10E+06	0.07	0.69	0.21	0.52	0.38
Chl + HP	4	0.438	0.438	0.125	3.53E+06	260.00	35.60	720.00	5.30E+06	93.33	27.20	396.00	1.77E+06	0.03	0.64	0.24	0.45	0.34
Chl + HP	5	0.438	0.125	0.438	4.80E+06	165.00	23.43	836.00	5.93E+06	73.33	21.90	416.00	1.13E+06	0.02	0.56	0.07	0.50	0.28
Chl + HP	6	0.125	0.438	0.438	5.18E+06	335.00	24.60	871.33	6.22E+06	180.00	16.80	433.00	1.04E+06	0.02	0.46	0.32	0.50	0.32
Chl + HP	7	0.333	0.333	0.333	7.20E+06	220.00	29.50	746.00	8.58E+06	80.00	21.67	408.00	1.38E+06	0.02	0.64	0.27	0.45	0.34

Chl + HP	1	0.750	0.125	0.125	6.92E+06	205.00	34.80	715.33	1.19E+07	110.00	32.67	405.00	4.98E+06	0.07	0.46	0.06	0.43	0.26
Chl + HP	2	0.125	0.750	0.125	6.77E+06	330.00	31.13	862.00	1.33E+07	120.00	14.65	388.00	6.53E+06	0.09	0.64	0.53	0.55	0.45
Chl + HP	3	0.125	0.125	0.750	5.67E+06	410.00	26.50	1048.00	1.56E+07	70.00	15.77	433.00	9.93E+06	0.14	0.83	0.40	0.59	0.49
Chl + HP	4	0.438	0.438	0.125	3.30E+06	396.67	36.60	685.33	7.47E+06	130.00	25.35	379.33	4.17E+06	0.06	0.67	0.31	0.45	0.37
Chl + HP	5	0.438	0.125	0.438	3.40E+06	330.00	27.00	778.00	7.62E+06	50.00	18.59	393.00	4.22E+06	0.06	0.85	0.31	0.49	0.43
Chl + HP	6	0.125	0.438	0.438	2.83E+06	395.00	27.97	776.00	6.82E+06	70.00	16.90	393.67	3.99E+06	0.06	0.82	0.40	0.49	0.44
Chl + HP	7	0.333	0.333	0.333	3.90E+06	435.00	33.27	692.00	8.63E+06	90.00	18.03	390.33	4.73E+06	0.07	0.79	0.46	0.44	0.44
CV + HP	1	0.750	0.125	0.125	7.50E+06	351.67	23.93	442.00	5.07E+06	116.67	27.04	383.00	-2.43E+06	-0.04	0.67	-0.13	0.13	0.16
CV + HP	2	0.125	0.750	0.125	8.62E+06	368.33	27.73	616.00	7.28E+06	126.67	14.65	402.00	-1.34E+06	-0.02	0.66	0.47	0.35	0.36
CV + HP	3	0.125	0.125	0.750	9.35E+06	350.00	22.53	747.33	1.16E+07	130.00	16.34	501.00	2.25E+06	0.03	0.63	0.27	0.33	0.32
CV + HP	4	0.438	0.438	0.125	5.18E+06	305.00	20.27	566.00	7.13E+06	56.67	19.72	408.67	1.95E+06	0.03	0.81	0.03	0.28	0.29
CV + HP	5	0.438	0.125	0.438	8.57E+06	295.00	25.30	608.00	9.85E+06	70.00	18.03	444.33	1.28E+06	0.02	0.76	0.29	0.27	0.33
CV + HP	6	0.125	0.438	0.438	9.58E+06	261.67	17.10	724.00	1.03E+07	116.67	20.84	506.00	7.20E+05	0.01	0.55	-0.22	0.30	0.16
CV + HP	7	0.333	0.333	0.333	9.58E+06	315.00	18.23	622.00	8.40E+06	83.33	18.59	448.00	-1.18E+06	-0.02	0.74	-0.02	0.28	0.24
CV + HP	1	0.750	0.125	0.125	8.82E+06	265.00	26.47	504.00	1.20E+07	70.00	25.91	380.00	3.18E+06	0.05	0.74	0.02	0.25	0.26
CV + HP	2	0.125	0.750	0.125	9.52E+06	285.00	18.30	676.00	1.25E+07	73.33	15.77	430.67	2.98E+06	0.04	0.74	0.14	0.36	0.32
CV + HP	3	0.125	0.125	0.750	9.70E+06	245.00	20.70	760.00	1.79E+07	40.00	19.15	567.00	8.20E+06	0.12	0.84	0.07	0.25	0.32
CV + HP	4	0.438	0.438	0.125	7.52E+06	280.00	22.97	582.00	2.37E+07	90.00	19.72	401.00	1.62E+07	0.23	0.68	0.14	0.31	0.34
CV + HP	5	0.438	0.125	0.438	8.72E+06	345.00	20.67	595.33	2.12E+07	60.00	18.59	474.00	1.25E+07	0.18	0.83	0.10	0.20	0.33
CV + HP	6	0.125	0.438	0.438	7.48E+06	390.00	17.27	718.67	1.13E+07	70.00	18.03	506.00	3.82E+06	0.06	0.82	-0.04	0.30	0.28
CV + HP	7	0.333	0.333	0.333	5.75E+06	255.00	18.47	548.00	2.23E+07	80.00	22.53	441.00	1.66E+07	0.24	0.69	-0.22	0.20	0.23
Chl + CV + HP	1	0.750	0.125	0.125	7.02E+06	230.00	23.37	589.67	1.24E+07	20.33	23.66	431.33	5.38E+06	0.08	0.91	-0.01	0.27	0.31
Chl + CV + HP	2	0.125	0.750	0.125	6.70E+06	305.00	20.40	750.00	9.12E+06	99.00	15.77	427.00	2.42E+06	0.04	0.68	0.23	0.43	0.34
Chl + CV + HP	3	0.125	0.125	0.750	8.77E+06	260.00	22.90	793.67	1.28E+07	92.00	16.90	507.00	4.03E+06	0.06	0.65	0.26	0.36	0.33
Chl + CV + HP	4	0.438	0.438	0.125	5.52E+06	230.00	22.17	668.00	1.06E+07	91.00	20.28	436.33	5.08E+06	0.07	0.60	0.09	0.35	0.28
Chl + CV + HP	5	0.438	0.125	0.438	7.80E+06	220.00	18.13	707.33	1.69E+07	20.00	19.72	484.67	9.10E+06	0.13	0.91	-0.09	0.31	0.32
Chl + CV + HP	6	0.125	0.438	0.438	6.52E+06	313.33	18.37	802.00	1.56E+07	35.00	15.21	493.33	9.08E+06	0.13	0.89	0.17	0.38	0.39
Chl + CV + HP	7	0.333	0.333	0.333	8.50E+06	400.00	20.23	671.67	1.27E+07	58.00	19.15	467.00	4.20E+06	0.06	0.86	0.05	0.30	0.32
Chl + CV + HP	1	0.750	0.125	0.125	7.50E+06	320.00	25.70	560.67	2.57E+07	143.67	22.53	399.67	1.82E+07	0.26	0.55	0.12	0.29	0.31
Chl + CV + HP	2	0.125	0.750	0.125	7.77E+06	430.00	18.33	732.33	2.22E+07	134.67	12.11	480.00	1.44E+07	0.21	0.69	0.34	0.34	0.39
Chl + CV + HP	3	0.125	0.125	0.750	1.52E+07	340.00	19.33	647.00	9.09E+06	30.00	11.27	545.67	-6.11E+06	-0.09	0.91	0.42	0.16	0.35
Chl + CV + HP	4	0.438	0.438	0.125	8.67E+06	300.00	22.93	592.67	1.79E+07	108.33	19.72	424.00	9.23E+06	0.13	0.64	0.14	0.28	0.30
Chl + CV + HP	5	0.438	0.125	0.438	1.12E+07	345.00	22.07	690.33	1.75E+07	77.00	17.46	460.00	6.30E+06	0.09	0.78	0.21	0.33	0.35
Chl + CV + HP	6	0.125	0.438	0.438	1.16E+07	295.00	19.93	660.00	1.28E+07	38.33	12.96	461.00	1.20E+06	0.02	0.87	0.35	0.30	0.38
Chl + CV + HP	7	0.333	0.333	0.333	9.78E+06	350.00	20.40	642.67	1.59E+07	85.00	16.90	449.00	6.12E+06	0.09	0.76	0.17	0.30	0.33

Appendix B

Table B.1. Livestock producer stratification by animal.

	Segmentation [heads farm^{-1}]		
Animal	Small	Medium	Large
Cattle[a]	< 14	15-140	> 140
Swine[b]	< 100	101-1,000	>1,000
Poultry[c]	< 1,000	1,001-20,000	>20,000

a. According to cattle producers' segmentation in developing countries (Delgado et al., 2005).
b. Using a livestock unit coefficient conversion of 0.014 (EUROSTAT, 2022).
c. Using a livestock unit coefficient conversion of 0.14 (CAPDR, 2006).

Table B.2. Livestock waste (LW) samples collected.

	Livestock producer segmentation			
Animal	Small	Medium	Large	Total
Cattle	3	4	2	9
Swine	2	4	4	10
Poultry	3	2	4	9
Total	8	10	10	28

Table B.3. Profitability analysis of the circular bioeconomy treatment of livestock manure.

Parameter	Value	Unit
A. Direct Fixed Capital	85,873,000	$
B. Working Capital	212,000	$
C. Startup Cost	4,294,000	$
D. Up-Front R&D	0	$
E. Up-Front Royalties	0	$
F. Total Investment	90,379,000	$
G. Investment Charged to This Project	90,379,000	$
H. Revenue/Savings Rates		
Dried microalgal biomass (Main Revenue)	2,139,578	kg/yr
Treated wastewater	33,754	T/yr
Energy generated	48,232,140	kW-h/yr
Total GHG reductions	10,760,347.23	Tons /yr
I. Revenue/Savings Price		
Dried microalgal biomass (Main Revenue)	20.00	$/kg
Treated wastewater	0.10	$/T
Energy generated	0.10	$/kW-h
CER	3.62	$/CER
J. Revenue/Savings		
Dried microalgal biomass (Revenue)	42,791,562	$/yr
CER (Revenue)	38,952,457	$/yr
Treated wastewater (Savings)	3,375	$/yr
Energy generated (Savings)	4,823,185	$/yr
Total Revenues	86,570,579	$/yr
K. Annual Operating Cost		

Bio-flocculant	925,000	$/yr
Labor-Dependent	195,000	$/yr
Facility-Dependent	16,182,000	$/yr
Laboratory/QC/QA	29,000	$/yr
Utilities	1,215,000	$/yr
Total Annual Operating Cost	18,546,000	$/yr
L. Carbon credits transaction (additional costs)		
Total of project development costs	10,500	$
Total of project management costs	10,500	$
M. Unit Production Cost/Revenue		
Net Unit Production Cost	8.67	$/kg MP
Unit Production Revenue	40.46	$/kg MP
N. Gross Profit	68,023,000	$/yr
O. Taxes (25%)	17,006,000	$/yr
P. Net Profit	59,175,000	$/yr
Gross Margin	78.58	%
Return On Investment	65.47	%
Payback Time	1.53	yr

Table B.4. SWOT analysis with economic (EC), technical and infrastructural (TI), political and legal (PL), and sociocultural (SC) factors.

Strengths		Weaknessess	
Jalisco is the agri-food giant of Mexico, producing a great quantity of residues that could be used as feedstock for the AD-coupled MbWT system.	EC	High initial investment of MbWT for livestock producers that small producers cannot afford, and elevated costs in downstream processing of microalgal biomass.	EC
The implementation of a biorefinery has great economical profitability with a large quantity of residues.	EC	Low financial guarantees regarding MbWT.	EC/ PL
Low operational costs after the implementation of MbWT, as natural conditions in Jalisco are optimum for microalgal growth.	EC /IT	High risk of contamination by native microbiota from LW.	IT
Carbon emissions and pollutants from LW can be captured by microalgae and upgraded into scaleable value-added products, alongside neutral or even negative emissions.	IT	High evaporative losses and turbidity that could inhibit microalgal growth.	IT
Jalisco has shown a clear commitment to mitigating climate change through the State Strategy for Climate Change of Jalisco.	PL	Insufficient coordination between the stakeholders (academic, public and private sectors).	PL
The largest clusters of livestock producers are in the northeast region of Jalisco, concentrating 75% of the total livestock producers.	PL	Poor surveillance mechanisms to comply with the regulations.	PL
Jalisco is committed to making a 90% reduction in GHG emissions and to increasing resources needed for renewable energy.	PL	Lack of management logistics that guarantee system's sanitary safety	IT
		MbWT is not recognized by any standard as an alternative to generate CER	PL
Opportunities		**Threats**	
Increased global microalgal biomass market price over the years.	EC	MbWT is still an immature technology that has elevated costs in the downstream processing and will need to compete with mature markets.	EC
Jalisco is the agri-food giant of Mexico, producing great quantity of residues that could be used as feedstock for the AD-coupled MbWT system.	EC	High pollutant concentration and turbidity of ADLW could hinder MbWT application.	IT

		Misoperation of MbWT system and the lack of capacitated verify bodies could discourage its application to get CER	IT
CER generation by MbWT and trade in the local or national market	EC	Legislation for the consumption and application of microalgae in Mexico is nonexistent.	PL
The biomass generated could be used to produce biofuels.	EC /IT	Jalisco's livestock producers deal with the dilemma of generating wealth and jobs or fully complying with environmental regulations.	SC
Robust consortium between species could display less sensitivity to fluctuations in environmental conditions and more resistence to contamination.	IT	Livestock producers believe that the land used for the raceway ponds for the application of the MbWT system is wasted.	SC
MbWT could be coupled with AD to decrease the nutrient concentration of ADLW or to purify biogas (methane enrichment) for energy use and further environmental impact mitigation	IT	Lack of social acceptance due to cultivation media and unclear market of the biomass produced.	SC
There is a worldwide increasing consumer interest in microalgae-related products.	SC	The target market at which these microalgal supplement products should be directed is unclear.	SC

Economic (EC), technical and infrastructural (TI), political and legal (PL), and sociocultural (SC) factors.

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Curriculum Vitae

Anaid López-Sánchez was born in Guadalajara, Jalisco, Mexico on May 29, 1995. She graduated from the Biotechnology Engineering program at Instituto Tecnológico y de Estudios Superiores de Monterrey, Guadalajara Campus, with a specialization in Bioprocesses. Anaid has participated in several consulting projects including the following: (1) Causal analysis of the proliferation of mosquitoes in the Guadalajara International Airport, and (2) Diagnosis of food loss and waste across the food supply chain in the State of Jalisco, Mexico. Her research is focused on the biotechnological revalorization of agro-food waste. She is author/coauthor of three published or submitted articles on JCR (Q1/Q2) indexed journals.

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