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Modeling growth kinetics and community interactions in microalgal monocultures and co-cultures for bioremediation of anaerobically digested swine wastewater

A thesis presented by

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Dedication

I would like to dedicate my work to,

My dad, thanks to you I found the love of science, from my early years when you took me to the Chapa de Mota planetary to see Saturn rings in the telescope. You gave me Carl Sagan books when I was just a little girl and you taught me about genes, atoms and evolution. I owe you my love to science. You taught me always, to question things. You showed me the hard but liberating path of finding the truth. Not only have you been a great example of hard work and discipline to me, but you also gave me excellent moments that I treasure in my heart forever. Thank you for providing me the resources and support to achieve my goals through my infancy, as well. You have always been my inspiration of life.

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―Hay que ser valiente para preferir ser auténtico que estar cómodo” M. Fuster Volio

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Modeling growth kinetics and interactions in microalgal monocultures and co-cultures for bioremediation of anaerobically digested swine wastewater

by

María Fernanda Palafox Sola

Keywords: microalgal-based wastewater treatment; swine wastewater; *Chlorella vulgaris*; *Scenedesmus acutus*; mathematical modeling

ABSTRACT

The pork production industry generates high annual volumes of swine wastewater that increase proportionally to the global demand for pork meat. Incorrect handling and discharge of swine wastewater causes the eutrophication of water bodies as well as soil and air pollution. Microalgal-based wastewater treatment has been proposed as a cost-effective alternative to traditional treatment methods. It also possess several environmental benefits and offers the opportunity to harvest valuable biomass, thus making wastewater treatment a biocircular economy process. Additionally, microalgae can be used in either primary or secondary wastewater treatments, as they allow for the simultaneous removal of nutrients (TN - total nitrogen and TP - total phosphorus) and organic matter. Most of the existing research is based on laboratory cultures under highly controlled conditions and with previous modification of the substrate, either by applying dilution or sterilization. However, these practices make full-scale implementation complex and cost-elevated. Three microalgal strains, *Chlorella vulgaris*, *Scenedesmus acutus* and *Arthrospira maxima,* were monocultured and co-cultured in raw (undiluted, non-sterelized) anaerobically digested swine wastewater. An overall performance index showed that all of the treatments that included *C. vulgaris* were the most efficient in terms of biomass production, along with COD and nutrient removal. The co-culture of *C. vulgaris* and *S. acutus* achieved the highest OPI of 0.68, displaying 9 biomass folds, a production of 2.97 ± 0.36 gL^{-1}, as well as 89%, 56% and 67% removal efficiencies for COD, TN and TP, respectively. Additionally, three mathematical models were used to calculate relevant growth kinetic parameters, including the specific growth rate, lag-phase duration, interspecific interaction, affinity constant and biomass productivity. *C. vulgaris* monoculture kinetics were adjusted using a double Gompertz model, showing a maximum growth rate (μ_2) of 0.89 $days^{-1}$ and a lag phase (λ_2) of 9.69 days. The Lotka-Volterra model was used to assess interactions between both strains in the co-culture, showing a commensalistic relationship between *C. vulgaris* and *S. acutus*, as denoted by the interspecific paramaters $\beta_{cs} = 1.99 \pm 0.92$ and $\beta_{sc} = -0.007 \pm 0.008$. Finally, the growth kinetics as a function of the three substrates (COD, TN and TP) were adjusted to the monod model, and the resulting parameters were used in a dynamics simulation of the inside of a continuously stirred reactor tank. A SOWT (strenghts, opportunities, weaknesses and threats) analysis was developed to assess the feasibility of MbWT implementation at industrial level. MbWT is an efficient solution for the treatment of SWW, however, further research in pilot or full-scale systems is still required to move towards full-scale industry implementation. Therefore, the results of the present work presented herein should be applied to eventually make MbWT a viable circular bioeconomy solution to SWW management in Mexico and other developing countries around the world.

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Abbreviations and acronyms

AD	Anaerobic digesters
ADSWW	Anaerobically digested swine wastewater
BF	Biomass folds
BOD5	Biological oxygen demand
CD	Cellular density
COD	Chemical oxygen demand
CSTR	Continuous stirred tank reactor
C1	Co-culture of *C.vulgaris* and *S. acutus*
C2	Co-culture of *S. acutus* and *A.maxima*
C3	Co-culture of C.vulgaris and *A.maxima*
D	Dilution rate (flow/ volume)
DNN	Differential neural network
DW	Dry weight
FAO	Food and Agriculture Organization of the United Nations
MA	Microalgae
MbWT	Microalgal based wastewater treatment
M1	Monoculture of *C.vulgaris*
M2	Monoculture of *S. acutus*
M3	Monoculture of *A.maxima*
ODE	Ordinary differential equation
OECD	Organization for Economic Co-operation and Development
OL	Oxidation lagoons
OPI	Overall performance index
pH	Hydrogen potential
SCOD	Soluble biodegradable COD
SWOT	Strengths, weaknesses, opportunities and threats
SWW	Swine wastewater
TKN	Total Kjeldahl nitrogen
TN	Total nitrogen
TP	Total phosphorus
TS	Total solids
TSS	Total suspended solids
WW	Wastewater

Chapter 1
Introduction

1.1 Motivation

1.1.1 Water as an essential resource

Water is an essential resource for human life as well as for all of earth's ecosystems, and it is likewise vital for economic and social development. Nevertheless, population growth and economic development have caused the degradation of both surface water and groundwater sources. These issues are exacerbated in developing countries due to poor urban, industrial, agricultural and livestock wastewater (WW) management, which has compromised the future of a continuous safe water supply for humans and for other species (Li et al., 2020b); UN (2021). In Mexico, it is estimated that 42% of the municipal WW and 62% of industrial WW is discharged untreated into lakes and rivers, or is directly reused for agriculture and, in some cases, for human consumption, which can result in serious health problems (CONAGUA 2018; Ronderos-Lara et al., 2020). Minimizing the generation of WW and adequately managing the WW that is produced are crucial steps towards finding a solution to this significant world problem. Furthermore, when WW is correctly managed, it can be a source of water for irrigation, energy, nutrients and other recoverable materials (UN, 2021).

1.1.2 The production of swine meat and its environmental impacts

Pork is the most consumed meat in the world, accounting for 36% of the global meat demand (FAO, 2021b). Its excellent flavor, cost-effectiveness, and nutritional properties are some of the characteristics that explain its high consumption (FAO, 2015; OECD, 2019). In 2019, the annual world consumption of pork was estimated to be 120 million tons. The Organization for Economic Co-operation and Development (OECD) forecasted a continuous global annual increase in pork meat demand, which is predicted to reach 140 thousand tons by 2029 (OECD, 2020).

Mexico ranks thirteenth in the world for swine production, with more than 20 million individual pigs raised as livestock in 2020 alone (SADER, 2020). Furthermore, the Food and Agriculture Organization of the United Nations (FAO) reported a steady increase in the number of swine produced in Mexico during the last decade (fig 1.1.2 a).

Fig 1.1.2 (b) shows a map of the following six Mexican states that account for 70% of the pigs raised in the country: Jalisco (20.56%), Sonora (17.55%), Puebla (11.10%), Veracruz (9.46%), Yucatán (8.83%) and Guanajuato (8.02%) (SIACON, 2020). Of those states, Jalisco is the top swine producer of the country, where 3.5 million pigs were raised in 2018; this translates to 321,736 tons of pork meat with a value of 638 million USD (SADER, 2019).

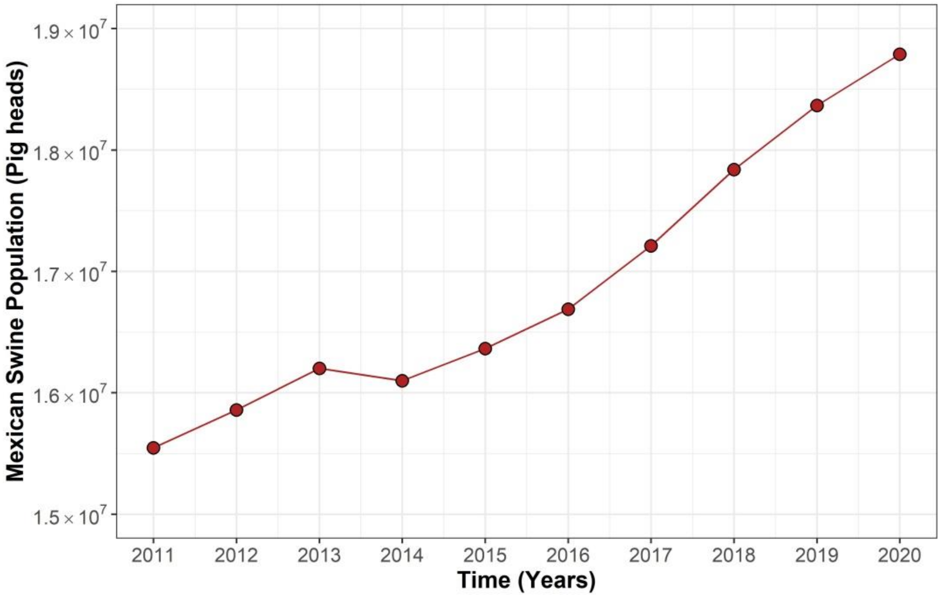

Figure 1.1.2 (a) Mexican swine inventory (pig heads). Source: (FAO, 2021a).

Figure 1.1.2 (b) Mexican swine production by state. Source: (FAO, 2021a; SIACON, 2020).

The Mexican pork market produces revenue of more than 62 million pesos per year and generates around 350 thousand direct jobs. Furthermore, 176 thousand tons of pork meat were exported in 2019 (SAGARPA, 2019). Therefore, the Mexican pork industry is very important for the country's economy and development and is an important food source.

Swine wastewater (SWW), a co-product of pig farming activities, contains swine manure and urine as well as water and any cleaning products used for the maintenance of the pens. When SWW is discharged into the environment without previous treatment, it can cause several types of environmental problems, such as water eutrophication, soil degradation, and greenhouse gas emissions (Jongbloed & Lenis, 1998). It can also pose a threat to human health (Cheng et al., 2020a).

The composition of SWW can vary depending on factors such as climate and humidity, as well as swine diet, age, and sex (Chynoweth et al., 1999). However, nearly all SWW is characterized by high levels of total suspended solids (TSS) and high concentrations of chemical oxygen demand (COD) (5,000- 30,000 mg L^{-}1), biological oxygen demand (BOD$_5$) (2,000– 30,000 mg L^{1}), total nitrogen (TN) (200–2,055 mg L^{-1}), and total phosphorus (TP) (100–620 mg L^{-1}) (Cheng et al., 2019a; Ding et al., 2017). A major problem posed by SWW is its high TN content in the form of ammonium, which is toxic to many types of aquatic life (Li et al., 2020a; Yousefi et al., 2020). SWW can also contain elevated levels of antibiotics (Han et al., 2020; Tong et al., 2009), steroidal hormones (Cheng et al., 2020a), pathogens (i.e. *Escherichia coli, Listeria monocytogenes, Citrobacter freundii, Salmonella, Campylobacter* and *Giardia)* (Guan & Holley, 2003) and metals, which accumulate in crops and soil (Sosa-Rodríguez et al., 2020) and represent a significant risk to human health (Zhang et al., 2017). Since a single pig produces an average of 6 L of SWW per day (Lory & Zulovich, 2018) and up to 25 L per day (Vanotti & Hunt, 2000), it is imperative that swine farms adopt an active role in the treatment of the SWW they produce. Such actions will protect the health of both humans and the environment, as untreated SWW can result in ecological imbalances in lakes, rivers, and oceans. Eutrophication, for example, is the gradual increase in phosphorus and nitrogen concentrations, as well as other nutrients found in SWW, in a water body. This causes excessive algal blooms that hinder light penetration and deplete dissolved oxygen, affecting various types of aquatic life (Britannica, 2019; Smith & Schindler, 2009).

1.2 Problem Statement, Context and Proposed Solution

Feeding the global population in a sustainable manner is one of the world‘s main priorities for the coming decades (Vågsholm et al., 2020). Because the pork industry will continue to grow in the coming years, pig farmers, therefore, must find cost-effective alternatives to reduce their environmental impacts.

The United Nations defined 17 sustainable development goals for 2030, with the main objectives being to end extreme poverty, to reduce inequality, and to protect the planet. The need for new approaches for the treatment of SWW is strongly linked to several of these objectives, including: zero hunger, clean water and sanitization, affordable and clean energy, industry innovation, climate action, life below water, life on land, and responsible consumption and production (UN, 2015).

1.2.1 The Most common SWW treatments in Mexico

In Mexico, WW that is discharged directly into surface water sources is regulated by the official norm NOM-001-SEMARNAT-1996, while WW intended to be reused for crop irrigation (or any public service) is regulated by the norm NOM003-SEMARNAT-1997 (CONAGUA, 1996, 1997). Although these official regulations exist, very few farms actually comply with these regulations and, thus, many are subject to penalty fees from the national water commission (CONAGUA) (Garzón-Zúñiga & Buelna, 2014; Herrera Piñuelas et al., 2021; Mojarro et al., 2020). Furthermore, the surveillance system that CONAGUA has on livestock farms is already operating near capacity (Ávalos, 2020).

According to CONAGUA, at the end of 2019 there were 4,698 industrial WW treatment plants operating in the country. Primary treatment accounts for 28.4% of the treatment plants, while secondary treatment represents 68% and tertiary treatment accounts for 1.7%. The remainders are non-specified treatments. Primary WW treatments, which are commonly performed with sedimentation ponds, are only focused on the removal of suspended materials and pH adjustments (SINA, 2019). Oxidation lagoons (OL) and anaerobic digesters (AD) are among the secondary treatments available in the country. While there is no specific data on treatment plants within the agro industry, industrial data provides a clear perspective of the most common WW treatment plant configurations in the country (SINA, 2019). Although significant efforts have been made worldwide to improve these methods (Craggs et al., 2008; Domingues et al., 2021; Loughrin et al., 2012), they still have drawbacks, such as the need for large spaces and trained personal to adequately operate them. Furthermore, since Mexico is a developing country, WW treatment plants including secondary or tertiary treatments are generally too expensive to implement and operate for most pig farmers, and this is especially true for small producers (Garzón-Zúñiga & Buelna, 2014). For these reasons, the resulting effluents often still contain high contents of TN, TP, COD and BOD_5, which are subsequently discharged into rivers and lakes.

OL, which can be classified as aerobic, anaerobic, facultative or maturation according to their specific oxygen concentration (Cortés-Martínez et al., 2015), normally display low removal efficiencies of organic matter (Techio et al., 2011). For example, poor COD removal efficiencies ($\leqslant$50%) have been reported in the effluents of facultative OL (Calderon et al., 2021; Garzón-Zúñiga & Buelna, 2014) as well as high nutrient loads due to inadequate oxygenation conditions (Vanotti & Hunt, 2000). Additionally, intense odors, insect problems, and human health risks have been also associated with OL (Garzón-Zúñiga & Buelna, 2014). Furthermore, aerobic treatments strongly depend on oxygen diffusion by mechanical aeration, which can be costly. Despite these drawbacks, OL are the most commonly used treatments because their operation is simpler and investment costs are lower compared to traditional bioreactors (Cortés-Martínez et al., 2015; Nadafi et al., 2009). Moreover, when properly operated, OL can become efficient in treating WW. For instance, Pombo et al. (2011) reported removal efficiencies of 80%-90% for TSS, 65%-80% for COD, and 50%-95% for BOD_5. Another advantage of OL is that they can be used to homogenize flow and concentrations of diverse pollutants, which is useful when treating SWW, since a large part of its pollutants are particles, and their sedimentation can significantly lower the TSS and COD concentration. (Garzón-Zúñiga & Buelna, 2014).

AD require high initial investments, which can be a substantial obstacle for livestock producers, especially for small producers (Barua & Kalamdhad, 2019; Daneshvar et al., 2022b). Additionally, these systems require long retention times, often greater than 60 days, to obtain good quality effluents (O'Flaherty et al., 2010), and a thorough understanding of the bio-digestion process is required to operate them efficiently. In developing countries, this expertise might not be available, while in developed countries, the high cost of employing people with the necessary skills can be restrictive (Daneshvar et al., 2022a).

The biggest advantage of AD is the production of valuable biogas and bio-fertilizers (O'Flaherty et al., 2010). However, when AD is used to treat SWW as the only treatment stage, high loads of organic matter and nutrients may remain in the effluent (Bernet et al., 2000). Hence, subsequent treatment is required before the final effluent can be released into water bodies.

1.2.2 SWW treatment in Jalisco

The environmental and territorial development secretary of Jalisco (SEMADET) reported a total of 389 pig farms in the Mexican state of Jalisco in 2019 (fig. 1.2.2). These records show that the most common treatment for SWW was OL (68%), followed by AD (28%), and only 3% of the farms used a combination of treatments (OL and AD). Bioremediation of WW using microalgae (MA) was the least popular treatment, used by only 0.3% of the farms. This novel treatment, which is the focus of the present thesis, is discussed within the following sections.

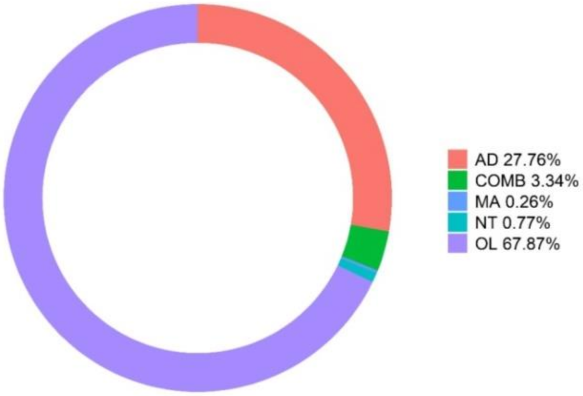

Figure. 1.2.2 Common SWW treatments used in Jalisco's swine farms. AD: anaerobic digesters, COMB: combination of lagoons and AD; MA: micro algal treatments; NT: no treatment; OL: oxidation lagoons. Data provided by SEMADET 2019.

1.2.3. Microalgal based wastewater treatment for SWW

The use of MA to bioremediate SWW offers many environmental benefits and valuable market opportunities, as discussed in the subsequent paragraphs. Fig 1.2.3 shows a diagram of the complete approach to microalgal-based waste water treatment (MbWT).

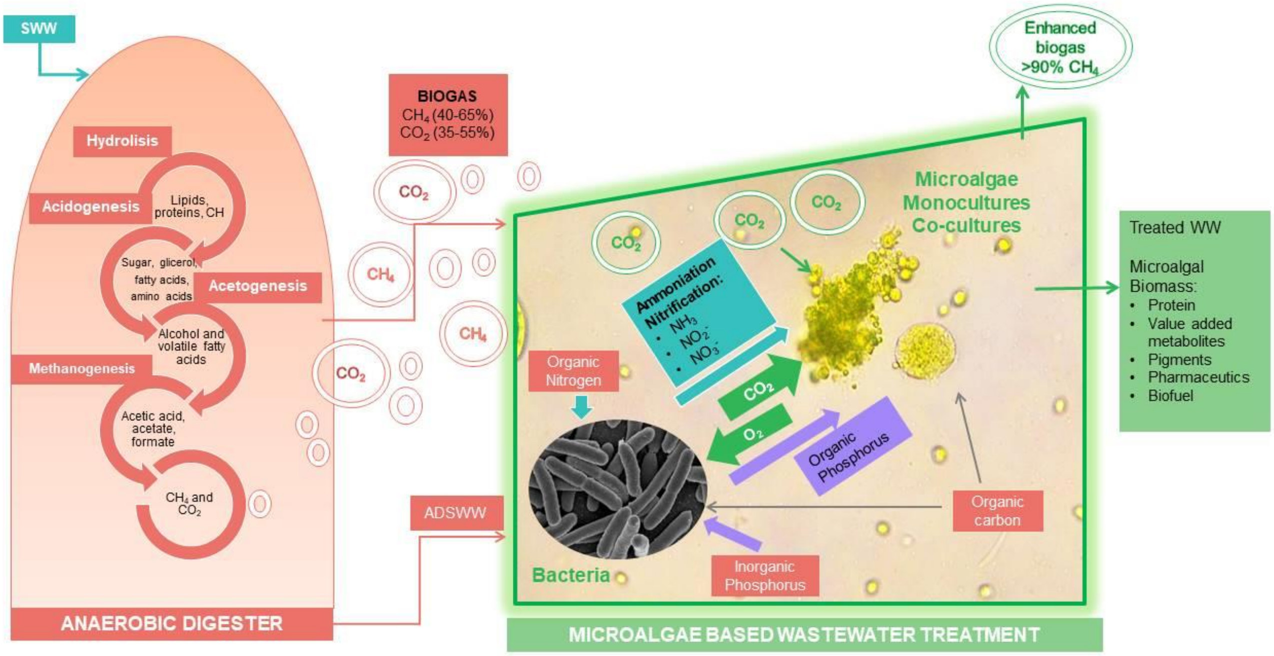

Fig 1.2.3 Diagram of the process of MbWT with ADSWW. SWW: Swine wastewater, ADSWW: anaerobically-digested swine wastewater. Adapted from: Prajapati et al. (2018); Mu et al. (2021) and Sial et al. (2021).

1.2.3.1 High nutrient removal rates and COD

MA are unicellular photosynthetic organisms ranging from a several to a few hundred micrometers, depending on the species (Kumar & Bera, 2020). They offer an excellent capacity to remove pollutants, owing largely to their nutrient requirements that are three times higher than traditional agricultural crops (Elser et al., 2000). Therefore, they display elevated TN and TP recovery (da Fontoura et al., 2017; Lu et al., 2015).

Microalgal-based wastewater treatment can use microalgae (MA), cyanobacteria, or mixed-microbial consortiums to reduce the TN, TP, and organic matter contents within the effluents of OL and AD, thus decreasing their negative impacts on the environment (Arashiro et al., 2019; Jiang et al., 2020) (Chawla et al., 2020; Chen et al., 2020; Reno et al., 2020). In mixed cultures, the oxygen produced by MA can be used by bacteria to oxidize carbon in SWW, thereby enhancing organic matter removal. This positive interaction can significantly reduce expensive aeration costs and lower the CO_2 emissions associated with SWW treatments (Liu et al., 2020). Additionally, bacterial production of nitrous oxide (N_2O), which is a greenhouse gas produced via the metabolic pathways of nitrification and denitrification, can be reduced by the action of MA, which can uptake high concentrations of nitrogen (Liu et al., 2020).

Many researchers have cultured MA using SWW, which is rich in nutrients and carbon, as a cost-effective way of producing biomass and reducing pollutant loads. For example, a MA co-culture of *Chlorella vulgaris* and *Scenedesmus dimorphus* was used by Zhu et al. (2019) to remove up to 74% of the TN in SWW. Likewise, Praveen et al. (2018) used the effluent SWW of an AD to culture *Chlorella vulgaris* and removed 87%, 100% and 77% of the COD, NH_4^+-N and PO_4^{-3} respectively. Furthermore, different species of the *Chlorella* and *Scenedesmus* genera have been cultured in diverse types of SWW because they have shown to be highly tolerant to ammoniacal nitrogen (Krichen et al., 2019) and are able to consume high amounts of phosphorous (de Lourdes et al., 2017).

1.2.3.2 *Biomass Revalorization*

MbWT has been proposed as a circular bioeconomy approach since the nitrogen and phosphorous removed from SWW can be recovered in the useful form of MA biomass (Liu et al., 2020). Some types of MA are edible and can be consumed by humans or animals as rich protein sources (Chojnacka et al., 2018). Therefore, existing research has explored diverse applications for the resulting microalgal biomass, including using it as protein source for food supplements or animal feed as well as for biofuel development (Abubackar et al., 2012; Cheng & Stomp, 2009; Colombo et al., 2017; Raheem et al., 2018).

Additionally, MA biomass has gained recent interest because it can be harvested to obtain a wide range of valuable molecules, such as proteins, enzymes, lipids, pigments, vitamins, food additives, antioxidants and sterols to produce cosmetics, drugs, food, and biofuels (Mascia et al., 2017; Petrushkina et al., 2017; Pourkarimi et al., 2020; Rajendran et al., 2020). For instance, *Nannochloropsis oceanica, Porphyridium cruentum* and *Chlamydomonas reinhardtii* have been cultured to extract carbohydrates like cellulose, starch, and simple sugars for bioethanol and bio-plastic production (da Maia et al., 2020; Kim et al., 2017; Mathiot et al., 2019). Lipids, such as triacylglycerols, glycolipids, and phospholipids, are other frequently targeted molecules from microalgal biomass. These molecules usually form around 3-20% of the dry biomass. Amino acids, peptides, and proteins are likewise valuable constituents, which can be used as feed or food supplements. Some essential amino acids contained in microalgal biomass are threonine, methionine, isoleucine, valine, leucine, lysine, and histidine (Daneshvar et al., 2022a). Species like *Spirulina maxima, Spirulina platensis, Chlorella vulgaris, and Scenedesmus obliquus* are known to have high protein contents of 60–71%, 46–63%, 51–58%, 50–56%, respectively (Becker, 2007).

Despite these facts, the commercial applications of MA have been limited by the high costs associated with MA cultivation, due to the need to input large amounts of nutrients and water. Thus, the utilization of anaerobically digested swine wastewater (ADSWW) offers a solution to this problem. MA can grow in autotrophic, heterotrophic or mixotrophic modes, as they can use different sources of energy or carbon. For this reason, they are ideal candidates to grow in turbid substrates like SWW, utilizing efficiently the carbon present even when deprived of light (Kumar & Bera, 2020). Nevertheless, there is some controversy as several reports argue that AD effluents must be diluted before being used as a culture medium for MA (Ganeshkumar et al., 2018; Koutra et al., 2018; Liu & Hong, 2021). However, other studies have aimed to attain high biomass production and pollutant removal efficiencies when treating undiluted effluents, with the main purpose of making MbWT feasible for large-scale use in the agro-industry by simplifying operations and lowering costs. For example, Sánchez-Zurano et al. (2021), proposed the use of software to adjust the depth of the raceway ponds in order to minimize WW dilution ratios for the cultivation of *Scenedesmus almeriensis*. Similarly, Wang et al. (2016), achieved TN and TP removal efficiencies of over 85% when treatingundiluted SWW through the domestication and UV mutagenesis of several MA strains (*C. vulgaris, C. pyrenoidosa, Haematococus pluvialis* and others) cultured in raceway ponds. Particularly, *Chlorella spp*. have been cultured in WW because they have been reported by many authors as highly efficient removing nutrients, they achieve high growth rates, are able to survive in severe WW environments and present high metabolic plasticity (photoautotrophic, heterotrophic and mixotrophic growth) (Safi et al., 2014; Sousa et al., 2021; Znad et al., 2018).

1.2.3.3 Renewable energy: Biofuels

MA biomass can also be used to produce biofuels, which are thought to reduce emissions of particulate matter, carbon monoxide (CO), hydrocarbons and sulfur oxides (SOx) in comparison to fossil fuels (Barnwal & Sharma, 2005). Additionally, microalgal biodiesel production is considered a ‗carbon neutral‘ process because the carbon dioxide (CO_2) released during combustion is in part sequestered from the atmosphere for the growth of the MA. MA are indeed one of the most promising sources for biodiesel production, as they have lower land requirements for cultivation than other crops and are predicted to have lower costs per yield (Stephens et al., 2010). Although the extraction of algal oil can be expensive, research is moving rapidly towards simpler, cheaper and more efficient methods (Kirrolia et al., 2013).

1.2.3.4 Environmental solutions: Carbon footprint reduction

A variety of energy-intensive carbon capture techniques have been proposed to remove CO_2 from the environment (Barati et al., 2021). Biological CO_2 sequestration using microalgae has been long recognized as one of the more effective carbon sequestration methods (Alami et al., 2021). Furthermore, CO_2 can be transferred to MA cultures as flue gas after combustion of carbon-based materials. (Daneshvar et al., 2022b). Chlorella has been referred to as the most promising species for CO_2 capture, as it can grow in an atmosphere containing 40% (v/v) CO_2 with a CO_2 fixation rate of 0.77 to 2.22 g L^{-1} day^{-1} (Brennan & Owende, 2010; Cheah et al., 2015)

1.2.3.5 *Government Incentives*

Governments throughout the world have taken an active role prioritizing the implementation of solutions that protect the environment (Barnwal & Sharma, 2005; Neal, 2011). Programs approving tax breaks and incentives to producers that apply sustainable solutions to their wastes have been implemented in many countries, including the USA, Indonesia, Japan, and others (Agarwal et al., 2015; Kollikkathara et al., 2009; Okuda & Thomson, 2007). Unfortunately, though, compliance with environmental regulations is not always as expected in Mexico (Garzón-Zúñiga & Buelna, 2014). Therefore, in order to increase compliance in developing countries like Mexico, local governments are actively supporting the implementation of sustainable solutions, which involve low-cost infrastructure systems that can be accessible to small producers.

1.2.4 Contaminant removal mechanisms through MbWT

Although MA have the potential to decrease levels of antibiotics, heavy metals, hormones and pathogen concentrations from SWW (Al-Gheethi et al., 2017; Daneshvar et al., 2018; Heubeck et al., 2007; Zeraatkar et al., 2016), the focus of this study is on the removal of TN, TP and organic matter contents from SWW effluents treated using AD. The following sections briefly describe the uptake mechanisms of MA that reduce these pollutants through MbWT.

1.2.4.1 Carbon

MA have the ability to grow autotrophically, heterotrophically or mixotrophically, depending on the species and strain.

Autotrophic organisms can fix inorganic carbon from CO_2 into complex organic molecules through photosynthesis using light as the energy source. On the other hand, heterotrophs obtain carbon from organic molecules and are unable to produce organic molecules from inorganic ones, while mixotrophic organisms can use multiple sources of energy and carbon. It has been shown that heterotrophic and mixotrophic microalgal cultures grow at higher rates compared to autotrophic cultures (Chojnacka & Zielińska, 2012).

MA in MbWT display mixotrophic growth using two main sources of carbon: inorganic carbon sources in gaseous forms and organic molecules present in the SWW (Markou et al., 2014; Msanne et al., 2020). For this reason, the organic molecules present in SWW can be used successfully by MA as a source of energy and/or carbon (Perez-Garcia et al., 2011).

Carbon is the main component of microalgal biomass (at around 65% dry weight). Microalgal organic carbon uptake occurs by diffusion, active transport and phosphorylation (Perez-Garcia et al., 2011). The most common organic molecules that have been tested for in MA cultures are monosaccharides, volatile fatty-acids, glycerol and urea (Chen & Zhang, 1997; Heredia-Arroyo et al., 2011; Hsieh & Wu, 2009). SWW is an important source of acetic, propionic, and isobutyric acids, while other volatile fatty-acids, such as n-butyric, n-valeric, iso-valeric, and n-caproic have been found in smaller amounts (Tenuta et al., 2002). Using volatile fatty acids as a carbon source for the culture of MA produces biomass with high amounts of valuable molecules like omega-3 fatty acids (Chalima et al., 2017). Additionally, when organic molecules are reduced by algal assimilation, COD concentrations are reduced.

COD is an indicator of the amount of oxygen that is needed to oxidize the organic matter contained in WW. COD can be divided into four fractions according to its solubility and degradability: 1) soluble biodegradable COD (SCOD), 2) non-soluble COD that is slowly biodegradable, 3) dissolved COD that cannot be biodegraded and 4) non-biodegradable particulate COD (Lee et al., 2019). SCOD is the most common form contained in SWW. Bacterial based treatments have been used for decades to treat WW removing COD (Cydzik-Kwiatkowska & Zielińska, 2016; Saravanan et al., 2021). While such treatments can remove high concentrations of COD, they have a limited capability for removing N and P. Similarly, previous studies on MbWT exposed a common problem, which is the increase in COD after a certain amount of time (Lee et al., 2016; Mujtaba & Lee, 2017). These elevated COD levels are likely caused by MA releasing organic compounds to the medium during the stationary growth phase, while they decrease SCOD in other growth stages (Lee et al., 2019). These problems can be solved using algal-bacterial consortia, which develop symbiotic interactions where the oxygen produced by MA during photosynthesis is in turn utilized by bacteria to oxidize COD and nitrogenous compounds. While bacterial nitrification is enhanced by microalgal oxygen production, the CO_2 released from bacteria is consumed by MA to synthesize nitrogen for their biomass (Munoz & Guieysse, 2006). Therefore, mixed cultures of bacteria and MA can enhance SWW treatment (Kouzuma & Watanabe, 2015).

Photosynthesis is the process that occurs inside microalgal cells through which inorganic carbon and light energy is converted into organic matter. This process of carbon fixation happens through the Calvin cycle inside the chloroplasts, where the enzyme Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) catalyzes a reaction transforming CO_2 into glucose ($C_6H_{12}O_6$). MA can use CO_2 and the anion HCO_3^- for photosynthesis but not the carbonate anion CO_3^{2-} (Price et al., 2007). Therefore, pH levels are highly important to maintain carbon forms adequate for efficient microalgal uptake.

When CO_2 dissolves in water, a weak acid-base buffer system forms, represented by the following equation (2):

$$CO_{2(aq)} + H_2O \xleftrightarrow{pK_1 \approx 3.6} H_2CO_3 + CO_{2(aq)} \xleftrightarrow{pK_2 \approx 6.3} HCO_3^- + H^+ \xleftrightarrow{pK_3 \approx 10.3} CO_3^{-2} + 2H^+ \quad \text{(eq.1)}$$

HCO_3^- is the most common form of carbon found in aqueous systems because the pH mostly ranges between 6.5 and 10. At high pH levels around pK_3, the dominant form of carbon will be CO_3^{-2}; which can only be uptaken by certain alkaliphilic cyanobacteria (Mikhodyuk et al., 2008).

The dissolution of CO_2 gas into liquids is a mass transfer process affected by many factors. However, the contact area between the two phases is extremely important. In MA production cultures, CO_2 is pumped directly into the medium, while the bubble size is controlled, and the rising speed of the bubbles helps to increase the mass transfer area (Rubio et al., 1999; Suh & Lee, 2003). The most commonly used methods of supplying CO_2 are pumping in air or concentrated CO_2 or adding bicarbonate salts. Supplementation of bicarbonate salts is a great option since they possess a higher solubility compared to CO_2 (>90 and 1 g L^{-1} at 1 atm and 25ºC, respectively) (Chi et al., 2011).However, all of these options are costly processes (around 50% of the production operation cost) (Chisti, 2013; Rubio et al., 1999). Because SWW is rich in carbonates and bicarbonates, it represents an excellent option for culturing MA. Furthermore, a more circular approach would be to use an AD-treated effluent as a medium to grow the MA and to pump the CO_2 produced in the AD system into the medium (Fig1.2.3). During anaerobic digestion, a fraction of the organic carbon is converted into CH_4 and CO_2, and the CO_2 produced may be pumped back to the medium so the operating costs can be lowered (Markou & Georgakakis, 2011).

MA employs passive and active mechanisms for CO_2 transport. Passive CO_2 diffusion is preferred by the cell over active transport since active transport happens through pump membrane transporters and is very energy-intensive (Markou et al., 2014). Therefore, CO_2 uptake presents a more favorable energetic process for the cell. On the other hand, HCO_3^- can only be pumped into the cell actively when MA produce carbonic anhydrase metalloenzyme to convert HCO_3^- to CO_2 (Badger & Spalding, 2004). This conversion process occurs intracellularly, but some MA have developed the ability to excrete carbonic anhydrase so that the conversion to CO_2 occurs extracellularly and diffusion can subsequently take place (Amoroso et al., 1998; Huertas et al., 2000).

1.2.4.2 Nitrogen

Nitrogen is the second most abundant element in MA, and its availability is a determining factor for the synthesis of proteins, enzymes, chlorophyll, genetic material, and energy-transfer molecules (ADP and ATP) (Rimmer & Shorttle, 2019). MA can uptake nitrogen from either organic or inorganic forms. Nitrogen oxides and ammoniacal nitrogen are commonly available in SWW, as well as more complex organic forms, such as urea or purines (Sommer & Husted, 1995b). Mixotrophic MA may switch their growth mechanism from autotrophic to heterotrophic depending on the nitrogen sources available. Different forms of nitrogen lead to variations in assimilation rates by the microalgal cell (Hellebust & Ahmad, 1989). Figure 1.2.4.2, shows the different sources of nitrogen that can be taken up by the microalgal cell.

Regarding inorganic nitrogen, three main forms can be uptaken: 1) atmospheric molecular nitrogen (N_2), 2) ammonium (NH_4^+) and 3) nitrogen oxides (NO_2^- and NO_3). Only certain cyanobacteria, however, can assimilate molecular nitrogen (N_2) as their sole nitrogen source. Such is the situation for diatoms (i.e., Rhizosolenia and Hemiaulusare), which cooperate with cyanobacterial symbionts that, in turn, help the diotom fix atmosphere nitrogen into usable forms like ammonia.

Ammonium is the preferred source of nitrogen for MA because its uptake requires little energy and, thus, MA do not consume other inorganic sources until the ammonium is exhausted (Perez-Garcia et al., 2011). Ammonium can also control or inhibit the assimilation of other nitrogen forms by repressing uptake genes (Franco et al., 1988).

Ammonium in an aqueous solution ionizes as shown in the following equation (eq2):

$$NH_3 + H_2O \overset{pk=9.25}{\longleftrightarrow} NH_4^+ + OH^- \quad \text{(eq.2)}$$

The behavior of ammonia in aqueous solution must be considered as it can affect the growth of MA. When ammonia gas (NH_3) is dissolved, it reaches equilibrium and forms ammonium ions (NH_4^+) in a reversible reaction at pH of 9.25. Thus, at room temperature and pH values above 9.2, the dominant form will be free ammonia. Ammonia can act as a weak base accepting protons, and it coexists with ammonium ions depending on three main parameters: pH, temperature, and ionic strength. Although the ammonium ion is the preferred source of nitrogen for MA, ammonium ions at higher concentrations (>2 mM) can cause cell death (Azov & Goldman, 1982; Li et al., 2019) by damaging the photosystem II complex or by disrupting the proton gradient of the thylakoid membrane (Drath et al., 2008; Gutierrez et al., 2016).

MA and cyanobacteria uptake ammonium through active transportation mechanisms, which allow them to control intracellular concentrations. However, ammonia diffuses passively into cells, which is why MA have little control over the intracellular concentration of free ammonia and are subject to its toxic effects at high levels (Drath et al., 2008). Once inside the cell, ammonium is assimilated through two main pathways: the glutamine synthetase and glutamate synthetase enzyme pathway (GS-GOGAT), which require carbon flow from the Krebs cycle (Miflin & Lea, 1982), and the glutamate dehydrogenase pathway (GDH). The GS-GOGAT pathway is the most common in photosynthetic organisms, and it provides carbon for catabolic activities (Nagarajan et al., 2019). The GDH pathway occurs only under heterotrophic cultivation conditions where catabolic processes provide important nitrogenous amino acids, such as aspartate, glutamate, and glutamine (Perez-Garcia et al., 2011). The assimilation of other inorganic nitrogen forms, such as nitrites and nitrates, depends on the MA capacity for nitrification and denitrification. The uptake of these inorganic forms is regulated by permeases, which can be blocked by ammonium (Florencio & Vega, 1983). MA uses these enzymes to reduce nitrate to nitrite and then to ammonium, which is finally assimilated by the GS-GOGAT pathway (fig 1.1). Nitrate reduction is affected by the absence of carbon; therefore, the carbon to nitrogen ratio (C:N) is an essential factor that moderates biomass yield in a culture system (Barros et al., 2017).

MA can also uptake different organic forms of nitrogen, such as purines, urea, pyrimidines and amino acids, using different specialized transporters (such as the ABC transporter) and permease enzymes (Flores & Herrero, 2005). However, all types of organic nitrogen are reduced to NH_4^+ and then integrated into the algal carbon skeleton by the GS-GOGAT pathway (Flores & Herrero, 2005; Muro-Pastor et al., 2005).

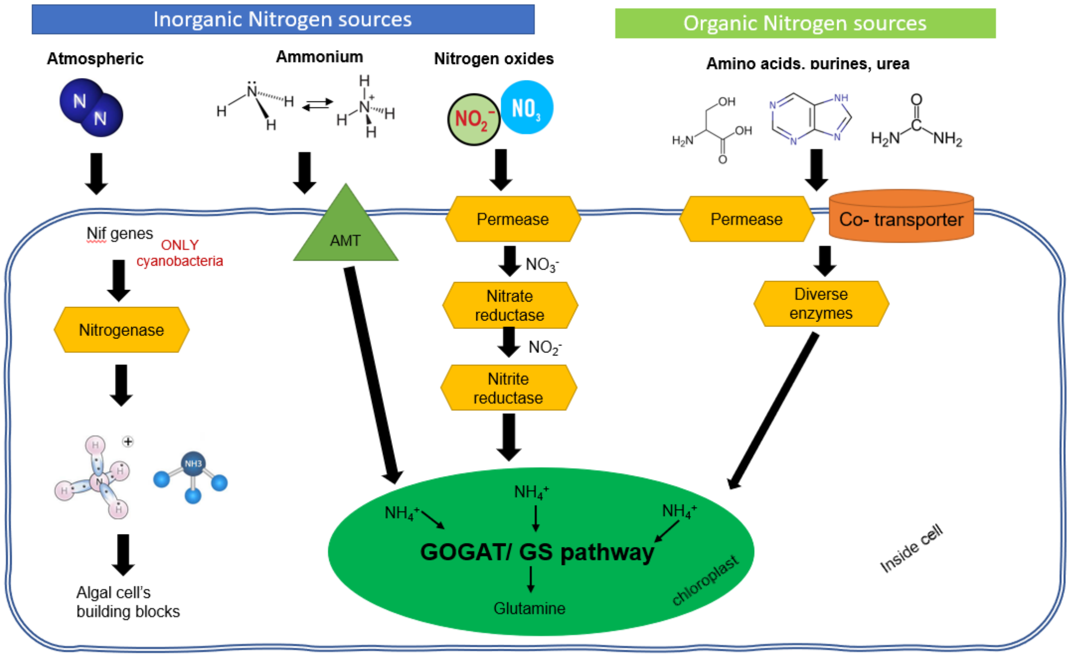

Figure 1.2.4.2 Different sources of nitrogen that enter the microalgal cell. Adapted from Kumar and Bera (2020).

1.2.4.3 Phosphorus

About 0.05% to 3.30% of the microalgal dry weight is phosphorous, which is an essential element (Grobbelaar, 2004). Phosphorous is a component of important biomolecules, such as phospholipids and nucleic acids (Merchant & Helmann, 2012). Phosphorus in SWW can exist in diverse forms, such as orthophosphates, polyphosphates, and metaphosphates, but it is usually measured either as total phosphorous (TP) or as phosphates (PO_4^{-3}). MA can utilize organic and inorganic phosphorous in different dissolved or insoluble chemical forms but orthophosphates are the most easily assimilated (Huang & Hong, 1999; Whitton et al., 2005). The uptake rate of phosphorous by MA is affected by cell conditions and several environmental factors, such as light, pH, temperature, salinity, and the availability of ions such as K^+, Na^+ and Mg^{2+}, that can be controlled during MbWT to obtain higher removal rates (Cembella et al., 1982; Correll, 1998; Rigby et al., 1980).

Most of the dissolved organic phosphorus can be assimilated actively into cells but certain compounds need to be first mineralized externally by enzymes (Dyhrman & Ruttenberg, 2006). Inorganic phosphorous, other than orthophosphates, must also be converted first to orthophosphate to be suitable for MA up-take. Orthophosphates enter the cell mostly by active transport, but a small fraction of them can enter passively by diffusion (Cembella et al., 1982). Phosphate enzymes, which act intracellularly, extracellularly, and sometimes attached to the cell walls, enable phosphorous conversion. These enzymes are affected by several factors, such as pH, light, temperature or the presence of inhibitors (Whitton et al., 2005). Furthermore, enzyme production could be enhanced when cells are phosphorous-limited (Dyhrman & Ruttenberg, 2006).

There are two main mechanisms of phosphorous assimilation: 1)the overshoot mechanism, which takes place when phosphorous accumulates after a starvation period and 2) the luxury uptake mechanism, which takes place after polyphosphate accumulates in the absence of a previous starvation period. The later mechanism has been observed for both MA and cyanobacteria (Bolsunovskii & Kosinenko, 2000). ‗Luxury uptake' is the preferred method when culturing MA in SWW due to the high concentrations of orthophosphates contained in this kind of substrate, and because a starvation period might can cause irreversible damage to cells, rendering the resulting biomass useless for harvest (Nagarajan et al., 2019). However, it is not yet clear if phosphorous stress makes any difference in MA development and growth since many kinds of MA contain intracellular phosphorous reserves, such as polyphosphate granules (Kornberg et al., 1999). Furthermore, MA can efficiently respond to phosphorous stress by increasing uptake transporters and by recycling intracellular phosphates with the help of different enzymes (Dyhrman, 2016). However, as photosynthesis increases the pH of the medium, cations like calcium and magnesium can precipitate out with phosphates, reducing the elements availability (Cembella et al., 1982).

1.2.4.4 The importance of nutrient balance to enhance biomass production and pollutant removal

The Universal Redfield C:N:P molar ratio (106:16:1) has been proposed as an ideal phytoplankton elemental composition, which provides all elements in adequate and bioavailable quantities in the culture medium, thus avoiding growth limitations (Spaargaren, 1996). However, previous studies have shown that MA has the ability to adapt to different substrates and their elemental ratios (Choi & Lee, 2015; Woertz et al., 2009).

Regarding MbWT, the N:P ratio largely depends on the source of the SWW. Municipal WW tends to have a 10:1 N:P ratio, while SWW can reach N:P ratios up to 40:1 (Kumar et al., 2010). High N:P ratios, such as 30:1, suggest a potential P limitation for MbWT, whereas low ratios, like 5:1, suggest a potential N limitation (Choi & Lee, 2015).

Several authors have experimented and proposed different N:P ratios to enhance nutrient removal and biomass production for specific microalgal strains, as summarized in table 1.2.4.4. Although there are inconsistencies between these studies, interesting conclusions can be drawn from their research.

All authors who cultured *Chlorella* spp. achieved significant TP and TN removals (>85%). These authors consistently reported that the uptake of phosphorous occurred during the first few days (within 4-6 days), with almost complete removal, while the uptake of nitrogen required 5-10 additional days. The various microalgal strains of this genus may be highly adaptable to different ratios, as there are several optimal removal ratios reported by these authors. Only Fernandes et al. (2017) reported 100% removal efficiencies for TP and TN with all tested ratios, even when they were above 15. However, the remainder of the authors agreed that the optimal removal N:P ratio was between 7 and 11 and that ratios above 20 considerably hindered removal efficiencies.

The rate of biomass production significantly varied between authors, possibly due to the different culture conditions implemented, such as the type of bioreactors, the use of artificial illumination, the volume cultured, or variations in the experimental and harvesting methodologies. Regardless, these studies have made it evident that, generally speaking, ratios larger than 18 (phosphorous limitating conditions) or smaller than 5 (nitrogen limitating conditions) directly affect the growth of MA. However, Filamentous MA, such as *Tribonema* spp. and *spirulina spp.*, need higher ratios (>20) to grow, which can be attributed to their elevated nitrogen requirements resulting from their larger physiology. These two strains displayed a higher removal of TN (>96%) but only a partial removal of TP (40-75%) with N:P ratios that ranged from 10-50. According to the literature, *Scenedesmus spp.* displays a similar behavior to C*hlorella spp.*, uptaking phosphorous during the first days and then nitrogen. For example, Vasconcelos Fernandes et al. (2015) used concentrated sewage water with N:P ratios of 20-30 and showed that *Chlorella sorokiniana* was able to remove all TP after 4 days but took 8 additional days to remove 100% of the TN. This is usually because microalgal intracellular nitrogen and phosphorous concentrations are often imbalanced relative to the WW stoichiometry, and generally TP is removed rapidly but TN is removed more slowly (Fernandes et al., 2017)

Fernandes et al. (2017), cultured *Chlorella sorokiniana* in anaerobically digested black water (concentrated toilet water) with different initial N:P ratios (15, 17, 20, 23, and 26), which were prepared by adding PO_4^{-3} to the WW. These authors reported that nitrogen uptake occurred only after phosphorous had been depleted from the medium, with removal efficiencies of 75% and 100% for TN and TP, respectively. This period of P-sufficiency to P-depletion was completed in 4 days. After that, the rate of nitrogen uptake decreased significantly, showing that WW with higher N:P ratios (>15) are a great challenge for MbWT due to the delayed removal of nitrogen relative to phosphorous. It is important to keep in mind that this removal delay reflects the need for larger storage capacities when scaling-up to an industrial level.

The rate at which nutrients are removed from the medium is also directly related to microalgal physiological traits and requirements, such as nutrient demand and growth rate. MA with higher growth rates display increased nutrient uptake rates (Geider & La Roche, 2002b). Screening for microalgal species with N:P ratios resembling that of the WW is essential to optimize N:P removal and recovery. However, removal efficiencies could be enhanced by culturing MA communities, since the different species may have complementary N:P uptake ratios (Cardinale, 2011). Using a multi-species culture can thus optimize removal of both TN and TP, if the microalgal community collectively exhibits balanced competitive abilities for TN and TP uptake (Cardinale, 2011).

In their experiments, Choi and Lee (2015) cultured *Chlorella vulgaris* in domestic WW and assessed various N:P ratios with the objective of analyzing the effect of the N:P ratio on biomass production and nutrient uptake. The main outcomes of their study indicate that biomass production is highly dependent on the initial N:P ratios. The initial N:P ratios (which ranged from 1-80) that were tested were prepared by the addition of $NaNO_3$ and $NaH_2PO_4 \cdot H_2O$ solutions to WW. These authors reported optimal N:P ratios between 1-10, reaching maximum productivities of 2 g L^{-1} d^{-1}. However, ratios above 10 affected biomass production, with productivities gradually declining to as low as 0.43 g L^{-1} d^{-1} at a N:P ratio around 30. Regarding nutrient uptake, a N:P ratio of 11-15 resulted in maximum TN (84%) and TP (88%) removal efficiencies. This work is consistent with the work of others, such as Choi et al. (2013), Fu et al. (2008), and Fernandes et al. (2017), who all show that biomass production and removal efficiencies of TP and TN are highly dependent on the initial N:P ratios of the medium.

Alketife et al. (2017) cultured *Chlorella vulgaris* in MLA medium with different concentrations of TN (0 - 56 mg/l) and TP (0 - 19 mg/L) and reported that initial TP concentrations higher than 3 mg L^{-1} favored biomass production, while cultures with lower initial TP concentrations negatively affected biomass production. The maximum biomass production (of 1.6 g L^{-1}) was displayed in cultures with initial ratios ranging from 4 to 10, and the authors concluded that a N:P ratios between 7-10 were optimum for enhancing the growth rate, as well as TN and TP removal efficiencies, similarly to the results reported by Choi and Lee (2015). Karapinar Kapdan and Aslan (2008) reported an optimum N:P ratio of 8, which is still within the range mentioned by the previous authors.

All of these studies make it evident that TP and TN uptake by MA is not always in stoichiometric proportion with the WW. Furthermore, removal efficiencies are highly affected by other factors, such as intracellular phosphorous concentration, existing chemical forms of both elements in the medium, light intensity, pH, and temperature.

Table 1.2.4.4 N:P ratio effect in biomass production, phosphorous and nitrogen removal in MbWT.

				Biomass		**Phosphorous**		**Nitrogen**		
Source	**Microalgae**	**Culture Media**	**N:P Experimented ratios**	**Yield**	**N:P ratio**	**Removal (%)**	**N:P ratio**	**Removal (%)**	**N:P ratio**	
Fernandes et al. (2017)	*Chlorella sorokiniana*	Concentrated toilet water	15, 17, 20, 23 and 26	14 *	15-17	100 TP	all tested ratios	100 TN	all tested ratios	**MAX**
				10-12*	20-26					**MIN**
Choi and Lee (2015)	*Chlorella vulgaris*	Domestic WW	1 to 80	2.75 ¤	1-10	88 TP	1-10	84 TN	11-20	**MAX**
				0.43 ¤	>41	34 TP	>41	73 TN	> 41	**MIN**
Alketife et al. (2017)	*Chlorella vulgaris*	MLA medium	0 to 58 (mass)	1.58 *	4-10	100 TP	7-10	100 TN	7-10	**MAX**
				0.5 *	<3 or >20	53 TP	<3 or >20	27 TN	<3 or >20	**MIN**
Molazadeh et al. (2019)	*Chlorella vulgaris*	Effluent of lagoons of municipal treatment plant	10 and 18 (mass)	0.79 *	10	95 TP	10	95 TN	10	**MAX**
				0.47 *	18	73 TP	18	82 TN	18	**MIN**
Doan et al. (2020)	*Spirulina sp*	Anaerobically diggested piggery WW	15,19,22 and 25 (mass)	0.5 ¤	22	55 PO_4^{-3}	15	>99.7 $N\text{-}NH_4$	all tested ratios	**MAX**
				0.24 ¤	15	40 PO_4^{-3}	25			**MIN**
Huo et al. (2020b)	*Tribonema sp*	Anaerobically digested liquid swine manure	1 to 50 (mass)	2.04 *	30	70- 75 TP	10-20	96-98 TN	40-50	**MAX**
				0.8-0.9 *	10	70- 73 TP	30-50	80-90 TN	10-20	**MIN**
Xin et al. (2010)	*Scenedesmus sp*	Modified BG11 medium	2,4,8,12,20 (N weight); 5,10,20,50, 100 (P weight)	1.34 •	20	>99 TP	all tested ratios	100 TN	2.5-20	**MAX**
				0.26 •	100			39 TN	100	**MIN**

Arbib et al. (2013)	*Scenedesmus obliquus*	*Urban WW treatment plant effluent*	1,3,5,9,13,22 and 35	2.56 *	>13	100 TP	9-35	>90% TN	3-13	**MAX**
				1.58 *	<9	<50 TP	1-5	<60% TN	22 & 35	**MIN**

Ratios are molar when not specified as mass ratios. Ratios were adjusted in all the experiments by the addition of NO_3 or PO_4^{-3} salts. *Production (g L^{-1}), ¤Productivity (g L^{-1} d^{-1}), • Production (X10^6 cell mL^{-1} d^{-1}). NR: Not reported

1.2.5 Bacterial- micro algal interactions

The interactions between bacteria and MA are synergistic in most cases and can be used to enhance the treatment of SWW. Rapidly growing MA can be used as O_2 suppliers for bacteria. In addition to their contribution as nutrient consumers, MA also consume the CO_2 that bacteria release through respiration. Moreover, some studies have shown the enhancement of bacterial activity and nutrient removal due to metabolites excreted by MA (Liang et al., 2015; Wolfaardt et al., 1994). Likewise, bacteria can release growth-promoting factors that enhance microalgal metabolism as reported by Fukami et al. (1997) and, Gonzalez and Bashan (2000).

However, not all microbial interactions are positive. Competition between heterotrophic MA and heterotrophic bacteria tends to be negative for MA, as they often exhibit lower specific growth rates than bacteria (Semple et al., 1999). Moreover, Schumacher et al. (2003) reported that the excretion of inhibitory metabolites, alkalization of the medium, and increase in dissolved inorganic carbon are some of the detrimental effects that MA can exert on bacteria. For this reason, it is highly important to choose bacteria and MA that are native to the waste substrates (and that exhibit favorable interactions with one another) when designing a WW treatment system (Cooper & Smith, 2015; Sison-Mangus et al., 2014).

1.3 Hypothesis

This dissertation hypothesized that concentrations of the main contaminants present in SWW (COD, TN, and TP) can be significantly reduced if raw (undiluted and unsterilized) ADSWW is used as a culture medium for three microalgal strains (*Chlorella vulgaris, Scenedesmus acutus* and *Arthrospira maxima*, as monocultures and co-cultures) while obtaining efficient biomass production.

1.4 Objectives

1.4.1 General objective

The main objective of this thesis is to characterize the removal of TN, TP, and COD as well as biomass production in raw (undiluted, unsterilized) ADSWW through MbWT using monocultures of *Chlorella vulgaris*, *Scenedesmus acutus* and *Arthrospira maxima*, as well as combinations of these three strains in co-cultures.

1.4.2 Specific objectives

The specific objectives of the present thesis are as follows:

1. To evaluate the performance of monocultures and co-cultures of *Chlorella vulgaris, Scenedesmus acutus* and *Arthrospira maxima* based on their ability to remove nutrients (TN, TP, and COD) and to produce biomass, when applied for the MbWT of raw ADSWW.
2. To evaluate mathematical models that satisfactorily describe the growth kinetics of the microalgal monocultures and co-cultures in raw ADSWW, as well as to determine kinetic parameters from experimental data.

3. To determine the interaction parameters between MA strains in co-cultures, as expressed by the Lotka-Volterra model.
4. To adjust the experimental data to the the Monod equation in order to analyze the growth rates of the MA based on their affinity to the different substrates (COD, TN and TP).
5. To apply model‘s parameters to simulate MbWT dynamics inside a bioreactor.

1.5 Nobel contributions of this work to the boarder scientific community

There are several previous studies where different types of MA have been cultured in various types of WW (Franchino et al., 2016; Karapinar Kapdan & Aslan, 2008; Wang et al., 2015). Many of these studies have shown removal efficiencies above 95% for TN and TP and greater than 80% for COD (Alketife et al., 2017; Fernandes et al., 2017; Lee & Lee, 2001). Additionally, high biomass production rates have been reported by authors, such as Fernandes et al. (2017). Table 1.5 summarizes some of the most recent studies regarding MbWT for SWW.

Although these studies lay the foundations for the bioremediation of SWW, most of the existing reports have included nutrient addition, artificial illumination, SWW sterilization, the use of complex bioreactors or intensive SWW dilution to achieve the desired nutrient ratio. Each of these aforementioned factors adds complexity and extra costs to the processes, making them unviable for large-scale use in the agro-industry. Laboratory conditions, such as continuous artificial illumination and previous substrate autoclaving, would be impossible to scale to an industrial level due to the high costs and complexity related to these operations. Moreover, diluting WW or adding salts to balance the nutrient ratio compromises additional fresh water. The work of Sutherland et al. (2020), however, stands out from the rest, as it was conducted in an existing MbWT plant. Their work focused on finding the ideal operation conditions of the ponds to optimize nutrient removal without adding previous treatments or diluting the WW. They tested different hydraulic retention times and operated their reactors in two ways: parallel and series.

To advance the literature related to MbWT, the present work focused on testing three microalgal species for the treatment of raw ADSWW (undiluted and unsterilized). Since most of the WW in the state of Jalisco is treated by AD or OL and these effluents generally contain high pollutant loads (Díaz-Vázquez et al., 2020a; Wentzel, 2018; Zurita-Martínez et al., 2011), this proposal aims to validate an efficient and low-cost treatment approach that could be directly applied in Jalisco (and at other similar operations). This thesis focused on determining MA cultures (mono or co cultures) that could grow in the raw effluents of ADSWW, with the intention of exploring the feasibility of full-scale MbWT implementation in the agro-industry. The experiments herein were performed in simple cylindrical glass reactors using the natural photoperiod as the sole illumination. There was no modification of the ADSWW through dilution or through salt addition for the adjustment of nutrients concentration. To attain the goal of this study, three different MA were tested: *Chlorella vulgaris*, *Scenedesmus acutus* and *Arthrospira maxima* (Spirulina), in mono and co-cultures, which were characterized for both growth and removal capacities of TN, TP, and COD in the raw ADSWW.

Table 1.5 MbWT recent research.

Source	Microalgae	WW type	Highlights	Drawbacks	Implications when scaling up
Li et al. (2020b)	*Coelastrella sp.*	SWW	62.3% N-NH3 removal 77.6% TP removal	SWW was diluted to 10% with ultrapure water 20 days culture time	Compromising of extra fresh water Long retention times= huge storage pits
Aketo et al. (2020)	*Chlorophyceae, Trebouxiophyceae, Prasinophyceae, Bacillariophyceae, Cyanobacteria*	Livestock WW	99%TN removal 82% TP removal Undiluted, Non- autoclaved	130 mmol/m2 /s of continuous illumination Sterile air with 2% CO_2 (0.8 L L^{-1} min^{-1})	Costly and complex operations
Cheng et al. (2020d)	*Chlorella zofingiensis, Synechocystis sp., Tribonema sp. Botryococcus braunii*	AD SWW	SWW diluted 3 times with fish wastewater 89.9% N-NH_3 removal 68.38% COD removal 73% TP removal	Previous sterilization of substrate TiO_2 and intense pulse light as photocatalysts	Costly and complex operations
Sutherland et al. (2020)	*Desmodesmus, Micractinium, Microcystis, Mucidosphaerium, M. aeruginosa*	Industrial WW	WW treatment plant- Pond system 23 kg N removal per day 4 days retention time		
Fan et al. (2020)	*Spirulina platensis, Scenedesmus obliquus*	Domestic WW	99% TP removal 94% TN removal	Previous sterilization of the substrate Agitation provided with pumped filtered air (0.22 µm membrane) Temperature controlled room at 25°C (±1 °C) Light source supplied by 28 W fluorescent lamps	Costly and complex operations

Tejido-Nuñez et al. (2020)	*Chlorella vulgaris, Scenedesmus obliquus*	Aquaculture WW	Laboratory and pilot scale test Sterilized and non- sterilized substrate MA monocultures and co-cultures	19 days culture time Lab assay: 25 °C, 2% CO_2 atmosphere, 115 rpm, constant illumination (warm white LEDs, 3500 K) Pilot scale: pure CO_2 injected, phosphate supplementation for N:P ratio adjusting (KH_2PO_4)	Long retention times= huge storage pits Costly and complex operations Application of salts affect water conductivity and salinity
Nguyen et al. (2020)	*Chlorella vulgaris*	Activated sludge-Municipal WW	Process combination of aerobic membrane and membrane microalgal bioreactors 4.0 ± 1.1 g m^{-3} d^{-1} NO_3^- uptake 1.5 ± 0.9 g m^{-3} d^{-1} PO_4^{3-} uptake Flocculation and separation technique capable of recovering 98% microalgal biomass	18 days culture time A larger Microalgal bioreactor (37 times the aerobic bioreactor) is needed at the obtained growth rate for scaling-up	Long retention times= huge storage pits Costly and complex operations
Huo et al. (2020a)	*Tribonema sp*	BG11 Medium	Effects of bacteria in MA- *Rhizobium rosettiformans, Hydrogenophaga intermedia*, *Sphingopyxis terrae* 98.34% TP removal 94.64% TN removal	Cultures controlled at 28 ± 1 °C, light intensity of 300 $\mu mol m^{-2} s^{-1}$ (white fluorescent light tubes) 5% carbon dioxide (CO_2) with 0.50 vvm flow rate	Costly and complex operations

1.6 Thesis Organization

Chapter one describes the motivation, problem statement, context, hypothesis, specific and general objectives, as well as the main contributions of this work.

Chapter two presents an experimental approach focused on the growth kinetics of *Chlorella vulgaris*, *Scenedesmus acutus* and *Arthrospira maxima* monocultured or co-cultured in raw ADSWW focusing on pollutant removal efficiencies and biomass production. For this chapter, mathematical models were developed to analyze the growth kinetics and microalgal interactions in the co-cultures.

Chapter three presents the conclusions and future perspectives of the thesis. Future perspectives are presented in the form of a SWOT (strengths, weaknesses, opportunities and threats) analysis in order to analyze the scalability of MbWT in the agro-industry in Jalisco.

Appendix A contains supplementary figures.

Chapter 2

Modeling growth kinetics and community interactions of microalgal monocultures and co-cultures for bioremediation of anaerobically digested swine wastewater

2.1 Introduction

Food demand continuously increases with world population, as well as with the environmental footprint associated with food production. Pork is the most consumed meat, accounting for 36% of the global demand (FAO, 2021b).

Intensive swine farming is coupled with significant volumes of swine wastewater (SWW), which can pose serious environmental problems and human health risks when handled incorrectly (López-Pacheco et al., 2021). SWW contains manure and urine as well as water and any cleaning products used in the pen´s maintenance. It is one of the most concerning agroindustrial wastes due to its high content of nutrients, such as nitrogen, phosphorus and potassium, as well as the presence of heavy metal ions (Zheng et al., 2020), organic matter, suspended solids, and antibiotics (Zhang et al., 2016). From an environmental point of view, SWW can cause several problems, such as water source eutrophication, soil and air pollution, and greenhouse gas emissions (Jongbloed & Lenis, 1998). The composition of SWW may vary as a result of the diet, sex and age of the pigs, as well as other factors like reproductive stage and stress, but common concentrations of chemical oxygen demand (COD), biological oxygen demand (BOD_5), total nitrogen (TN), and total phosphorus (TP) can be as high as 100,000 mg/L, 30,000 mg/L, 2,000 mg/L, and 600 mg/L, respectively (Cheng et al., 2019a; Cheng et al., 2020b). In countries ranked among the highest for global pork production, such as the EU, USA, China, Brasil, Rusia, Vietnam, Canada, and Mexico (FAO, 2021a), anaerobic digesters (AD) and oxidation lagoons (OL) are the most common processes for SWW treatment (Córdova et al., 2022; Dan et al., 2020; Nagarajan et al., 2019; Sui et al., 2019; Zárate - Guzmán et al., 2021). While ADs are efficient for organic matter removal, their effluents usually contain high nutrient concentrations (Ji et al., 2014; Kimura et al., 2019) and do not always meet the quality standards to be discharged into water bodies (Al Seadi & Lukehurst, 2012; Thompson et al., 2020).

Using SWW to culture microalgae (MA) is a cost-effective treatment solution that has many advantages over traditional treatment systems (Cheng et al., 2020d). The ability of MA to grow using autotrophic, heterotrophic, or mixotrophic modes makes them ideal candidates for use in SWW as a culture medium to remove pollutant loads (e.g. COD, TN and TP) while simultaneously producing high amounts of biomass (Zheng et al., 2020). Furthermore, MA tolerate high levels of ammonia nitrogen (NH_3-N), which is commonly present in SWW and highly toxic to other organisms (Hu et al., 2019). Additionally, microalgal based wastewater treatment (MbWT) offers a low-cost solution that entails many benefits and market opportunities. MA are highly adaptable to polluted wastewater and have been cultured along with bacteria to successfully treat SWW (Cheng et al., 2019b; Li et al., 2020b; Qu et al., 2021). Furthermore, when photosynthesizing, MA can uptake high amounts of CO_2 (Esteves et al., 2020) and their biomass can be harvested to obtain potentially valuable molecules for pharmaceutics, fuels, cosmetics, and food (Gupta et al., 2019; Nagarajan et al., 2019; Oishi et al., 2019).

As the benefits of MbWT are gaining appreciation, the need to test these systems at a full-scale level intensifies. Given that most of the existing research has been carried out in laboratories with highly controlled conditions, it is necessary to understand the challenges that full-scale systems present (López-Sánchez et al., 2022). For instance, it is crucial to understand the interactions that could exist between bacteria and MA, including both positive associations as well antagonistic and competitive behaviors (Di Caprio, 2020; Lam et al., 2018). Additionally, the turbidity of SWW can significantly affect the growth of MA in full-scale systems by reducing light penetration, thus interfering with the photosynthesis (Wang et al., 2019b). For these reasons, conducting larger-scale laboratory research using raw WW and realistic, straightforward conditions is an approach that could help lead to full-scale MbWT implementation. Several studies have cultured MA in SWW and reported removal efficiencies as high as 80-90% for COD and ≥90%, for TP and TN, respectively. However, most of these studies have applied high dilutions or sterilization prior to the microalgal treatment (Li et al., 2021). For example, Zhao et al. (2022) optimized the removal percentages of COD, TN and TP to 86.9%, 60.75% and 96.13% respectively, when culturing *Scenedesmus sp*. in 10-fold diluted SWW. Nutrient supplementation and flocculation are likewise common pretreatments in existing research (Arana & Gude, 2018; Cheng et al., 2020c). However, these practices make MbWT less affordable for small-scale producers and more complex to scale up for larger farms.

Therefore, the main objective of this study is to evaluate MbWT performance when applied to the treatment of anaerobically digested swine wastewater (ADSWW) under raw conditions (undiluted and unsterilized). Hence, the initial nutrient concentrations were not modified, and no pretreatments were applied. Culture experiments were conducted using three different strains of MA (*Scenedesmus acutus*, *Chlorella vulgaris* and *Arhtrospira maxima* [spirulina]) in anaerobic digested swine wastewater (ADSWW) in both mono and mixed cultures, resulting in six microalgal treatments that were tested for biomass production and pollutant removal (COD, TP, and TN).

Mathematical models were then used to adjust the experimental data to describe microalgal growth kinetics and community interactions. The following three models were selected based on parameter interpretability: 1) a double Gompertz model to express the growth kinetics of the *C.vulgaris* monoculture, 2) the generalized Lotka-Volterra model to characterize the strength of interaction between co-cultures of *C.vulgaris* and *Scenedesmus acutus*, and 3) the Monod model to describe the growth rate of *C.vulgaris* as a function of nutrient and COD concentrations.

2.2 Materials and methods

2.2.1 Microalgae cultures

Chlorella vulgaris, Arthrospira maxima and *Scenedesmus acutus* were obtained from ‗Centro Universitario de Ciencias Biológicas y Agropecuarias' (CUCBA, 2022). *Chlorella vulgaris* and *Scenedesmus acutus* were cultured in an initial volume of 10 mL, doubling in volume every 8 days in modified Bristol‘s medium with a pH of 7.5. The Bristol‘s medium was prepared with the following mass per liter of distilled water (L^{-1} dH_2O): $NaNO_3$ (1 g); $CaCl_2 \cdot 2H_2O$ (0.025 g); $MgSO_4 \cdot 7H_2O$ (0.075 g); K_2HPO_4 (0.075 g); NaCl (0.025 g); KH_2PO_4 (0.075 g); and trace elements solution (5 mL) (Fujita, 1972). The trace elements solution contained (L^{-1} dH_2O): H_3BO_3 (2.8 g); $MnCl_2 \cdot 4H_2O$ (1.8 g); $ZnSO4 \cdot 7H_2O$ (0.222 g); $NaMoO_4 \cdot 2H_2O$ (0.39 g); $CuSO_4 \cdot 5H_2O$ (0.79 g); and Co $(NO_3)_2 \cdot 6H_2O$ (0.049 g). *Arhtrospira maxima* was cultured in 10 mL, doubling in volume twice every 8 days in Zarrouk‘s medium (Zarrouk, 1966) with a pH of 9, containing (L^{-1} dH_2O): $NaHCO_3$ (16.8 g); NaCl (1 g); $NaNO_3$ (2.5 g); $CaCl_2 \cdot 2H_2O$ (0.04 g); $MgSO_4 \cdot 7H_2O$ (0.2 g); $C_{10}H_{16}N_2O_8$ (0.08 g); $FeSO_4 \cdot 7H_2O$ (0.01 g); K_2HPO_4 (0.05 g); K_2SO_4 (1 g); and 1 mL of the trace element solution previously described. All media were sterilized according to standard procedures. All cultures were placed under natural illumination (sunlight) with a photoperiod of 13/11 h at 30°C ± 5, with the goal of eliminating artificial light costs and increasing the potential industrial scalability of MbWT. The intensity of the natural sunlight was measured for 30 consecutive days, twice a day at 12:00 pm and 6:00 pm. The mean values of natural sunlight illumination were 97 ± 13 μmol photons m^{-2} s^{-1} at 12:00 pm and 3 ± 5 μmol photons m^{-2} s^{-1} at 6:00 pm. An air supply rate of 0.5 L/min was used. An image of all three MA strains is shown in Fig 2.2.1.

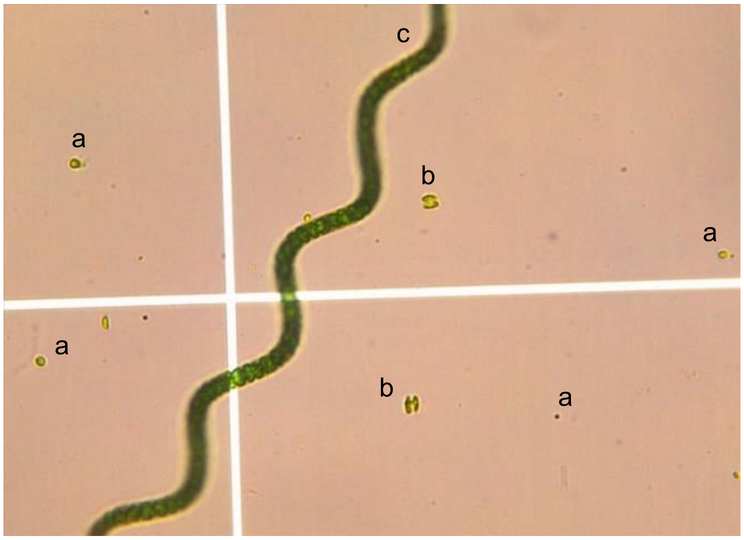

Figure 2.2.1. 40X Microscopic image of MA obtained with a Leica ICC50 microscope camera. a) *Chlorella vulgaris*, b) *Scenedesmus acutus* and c) *Arthrospira maxima* (spirulina).

2.2.2. ADSWW sampling and characterization

A 40 L sample of ADSWW was obtained from an anaerobic digester at ‗Proteína Animal, S.A de C.V.‘ (PROAN, 2022), which is a large livestock producer in Jalisco. This anaerobic digester receives SWW from approximately 60,000 pig heads and operates with a load of 60 tons of SWW. The ADSWW sample was stored at 4°C and was characterized within 48 hours of receipt. Insoluble solids were removed prior to MbWT by decantation and filtration through a 0.45 μm nylon membrane. Undiluted and non-sterilized ADSWW was used as the culture medium for every treatment, and no pH adjustments or chemical pretreatments were performed. Hydrogen potential (pH), total solids (TS), total suspended solids (TSS), BOD_5, COD, total Kjeldahl nitrogen (TKN), TN, and TP were determined according to the Federation, W. E. & American Public Health Association methods (APHA, 1992).

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2.2.3 *Experimental design*

The inoculation of monocultures was performed using 30% v/v of the MA culture in the corresponding medium and 70% v/v of the undiluted ADSWW in 1 L flasks. Co-cultures were prepared in 1L flasks, inoculated with 15% v/v of each of the two mixed MA in their corresponding media and the remaining volume was filled with the undiluted ADSWW, as shown in table 2.2.3. All experiments were performed in triplicate to test the between-run reproducibility.

Table 2.2.3 Volumetric fractions of MA *vs*. ADSWW for each treatment used.

Treatment		Strain	MA (v/v)	ADSWW (v/v)
M1	Monoculture 1	*C. vulgaris*	0.3	0.7
M2	Monoculture 2	*S. acutus*	0.3	0.7
M3	Monoculture 3	*A. maxima*	0.3	0.7
C1	Co-culture1	*C. vulgaris* *S. acutus*	0.15 0.15	0.7
C2	Co-culture 2	*S. acutus* *A. maxima*	0.15 0.15	0.7
C3	Co-culture 3	*A. maxima* *C. vulgaris*	0.15 0.15	0.7

2.2.4 *Analytical methods*

To assess biomass production and pollutant removal, 1 mL samples were taken and homogenized with a vortex for cell counting using a Neubauer chamber and a Leica ICC50 microscope. 10 mL samples were also collected every third day for COD and nutrient (TN, TP) monitoring. These samples were centrifuged at 3000 rpm for 10 min using a Gyrozen 1580R centrifuge and the supernatant was analyzed according to the Hach DR 5000 Spectrophotometer protocol (HACH, DR 5000). TN, TP, and COD were determined using the 10072 (Hach persulfate digestion), the 10127 (Hach molybdovanadate with acid persulfate digestion) and the 800 (USEPA Reactor Digestion) methods, respectively. pH was monitored daily using a standard pH meter.

Dry weight was measured by centrifuging 50 mL samples in previously weighed falcon tubes. The liquid was decanted to recover the biomass pellet, which was then dried in a conventional oven at 60°C until the weight was constant. Dry weight was computed using the difference between final and initial weights. All measurements were made in triplicate to test the within-run reproducibility. A linear regression model was proposed to express the dry weight (DW) of each MA culture as a function of the cellular density (CD). The coefficients were estimated using the least squares methodology. R^2, goodness of fit, and t-test analyses were used to evaluate the performance of the linear regression model (Maulud & Abdulazeez, 2020). For the validation of the linear model, assumptions of normality, homogeneity and independence were corroborated and the Shapiro-Wilk and Breusch-Pagan tests were used (Gareth James, 2013; Kwak & Park, 2019).

The following equations (3-6) were used to compute the biomass folds (BF), the COD removal percentage (%rCOD), the TN removal percentage (%rTN) and the TP removal percentage (%rTP) for each experimental run.

$$BF = CD_{max}/CD_0 \qquad \text{(eq. 3)}$$

$$\%rCOD = \frac{COD_0 - COD_f}{COD_0} * 100 \qquad \text{(eq.4)}$$

$$\%rTP = \frac{TP_0 - TP_f}{TP_0} * 100 \qquad \text{(eq.5)}$$

$$\%rTN = \frac{TN_0 - TN_f}{TN_0} * 100 \qquad \text{(eq.6)}$$

Where CD_{max} is the maximum cell density (cell mL^{-1}) observed during the experiment and CD_0 is the cell density (cell mL^{-1}) on the initial day of the experiment. COD_0 is the COD concentration on day 0 (mg L^{-1}) and COD_f (mg L^{-1}) is the COD concentration on the final day. TN_0 and TN_f correspond to the TN concentration on day 0 (mg L^{-1}) and on the final day, respectively. TP_0 (mg L^{-1}) is the TP concentration on day 0 and TP_f (mg L^{-1}) is the TP concentration on the final day.

2.2.4.1 Overall Performance Index

An overall performance index (OPI) was computed to compare all six microalgal treatments. The OPI integrates all four response variables (BF, %rCOD, %rTN and %rTP) normalized on a 0-1 scale. All four response variables were equally weighted when calculating the OPI, as shown in Equation 7.

$$OPI = (BF + \%rCOD + \%rTN + \%rTP) * .25 \qquad \text{(eq.7)}$$

2.2.5 Growth kinetic models

2.2.5.1 Double Gompertz model

The modified Gompertz model was used to describe the sigmoidal microalgal growth. Growth kinetics was only modeled for *C. vulgaris* (M1) since this strain displayed the best results for all the response variables collectively. This model was used as proposed by Tattershall et al. (2021) for double-peaked curves. For this study, the Gompertz model was employed as follows:

$$C(t) = \alpha_1 e^{-e^{\mu_1(\lambda_1 - t)}} + (\alpha_2 - \alpha_1) e^{-e^{\mu_2(\lambda_2 - t)}} \qquad \text{(eq. 9)}$$

Where C(t) corresponds to biomass folds, α_1 and α_2 are the two asymptotes of each of the two peaks, respectively, and represent the maximum growth in BF displayed by the MA. μ_1 and μ_2 are the specific growth rates during peak 1 and peak 2, respectively, and t is the time (days^{-1}). λ_1 and λ_2 represent the lag phases of each stage (days).

2.2.5.2 Lotka -Volterra Model

The generalized Lotka-Volterra equation (eq. 10) was used to model the growth kinetics of *C. vulgaris* and *S. acutus* as a function of the microalgal specific growth rate and the interactions among microalgal populations.

$$\frac{dX_i(t)}{dt} = X_i(t)\left(\alpha_i + \sum_{j=1}^{n} \beta_{ij}\, X_j(t)\right) \qquad \text{(eq. 10)}$$

This model has been used to explain interactions between microalgal populations in previous studies by interpreting its parameter β_{ij} (Tevatia et al., 2014; Zhang et al., 2013). It can be expressed in the following terms for the purpose of the present study.

$$X_c'(t) = \alpha_c X_c(t) + \beta_{cc} {X_c}^2(t) + \beta_{cs} X_c(t) X_s(t) \qquad \text{(eq.11)}$$

$$X_s'(t) = \alpha_s X_s(t) + \beta_{ss} {X_s}^2(t) + \beta_{sc} X_s(t) X_c(t) \qquad \text{(eq.12)}$$

Where α_c and α_s (day $^{-1}$) coefficients denote the growth rates for *C. vulgaris* and *S. acutus,* respectively, without the influence of the other strain in the co-culture. The interaction parameters between strains are expressed as $\beta_{cc,}$ β_{ss}, β_{cs}, and β_{sc} (mL cell^{-1} day^{-1}). β_{cc} and β_{ss} are parameters that describe intraspecific interactions. In this case, β_{cc} describes the interaction that *C. vulgaris* exerts on other members of *C. vulgaris.* β_{cs} and β_{sc} account for the interspecific interactions between populations. For instance, β_{cs} describes the effect that *S. actus* has on *C. vulgaris* while β_{sc} refers to the effect that *C. vulgaris* has on *S. acutus*. X_c and X_s represent the cell density of *C. vulgaris* and *S. acutus,* respectively, and are expressed as cells per mL^{-1}.

The first derivatives of the Lotka-Volterra model were estimated using a nonparametric differential neural network (DNN), which has been shown to be an effective way to approximate the behavior of nonlinear systems describing the growth of mixed microbial cultures (Gradilla-Hernández et al., 2018). In the derivative ($\frac{dX(t)}{dt} \in \mathbb{R}^2$), the components of the vector correspond to the cellular concentration of each of the microalgal strains. Equation (13) presents the DNN identifier, where $\hat{X}(t) \in \mathbb{R}^2$ stands for the identified vector. The linear term of the model (Hurwitz matrix) is represented by $A \in \mathbb{R}^{2\times2}$. The adaptation to nominal dynamics is presented as $W_1(t) \in \mathbb{R}^{2\times2}$. The activation vector function is represented by $\sigma \in \mathbb{R}^2$ (Gradilla-Hernández et al., 2020).

$$\frac{d\hat{X}(t)}{dt} = \overline{f}(\hat{X}(t), |W_1(t)) = A\hat{X}(t) + W_1(t)\left(\sigma(\hat{X}(t))\right) \qquad \text{(eq.13)}$$

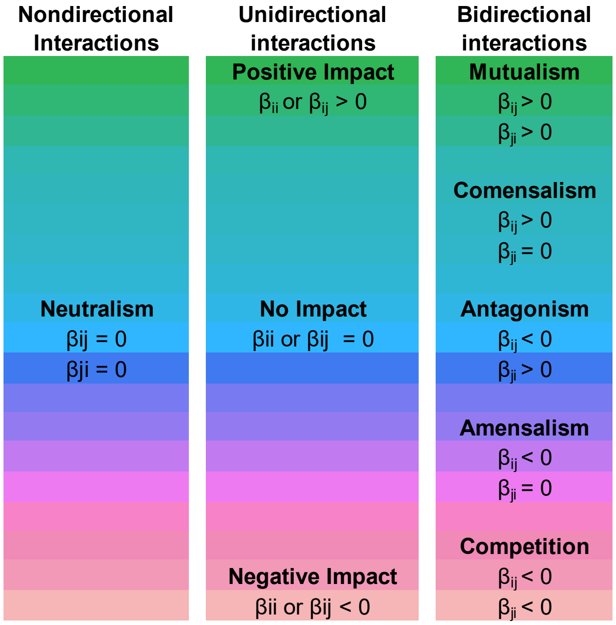
Equation (14) is an optimization problem, established to obtain α_c, α_s, β_{cc}, β_{ss}, β_{cs} and β_{sc} and solved with the Nelder-Mead algorithm.

$$k^* = \underset{\alpha_c,\alpha_s,\beta_{cs},\beta_{sc},\beta_{ss},\beta_{cc}}{argmin} \left\| \bar{f}(\hat{X}(t), |W_1(t)) - X'(t) \right\|_2^2 \qquad \text{(eq. 14)}$$

where k^* is the vector of the estimated parameters as reported by (Gradilla-Hernández et al., 2020).

In order to fit the concentration curves to the parameters, MATLAB R2020a software was used. Curve Fitting, Simulink toolboxes and the 'fminsearch' function was used to run the DNN algorithm and to determine parameters.

Figure 2.2.5.2 shows the interpretation of Lotka-Volterra's equation parameters defined as interactions among the two strains, *i* and *j*, participating in co-cultures. The interactions between microorganisms can be positive, negative or neutral in a bidirectional, unidirectional or nondirectional way (Gradilla-Hernández et al., 2020).



Nondirectional Interactions	**Unidirectional interactions**	**Bidirectional interactions**
	Positive Impact	**Mutualism**
	β_{ii} or β_{ij} > 0	β_{ij} > 0
		β_{ji} > 0
		Comensalism
		β_{ij} > 0
		β_{ji} = 0
Neutralism	**No Impact**	**Antagonism**
βij = 0	βii or βij = 0	β_{ij} < 0
βji = 0		β_{ji} > 0
		Amensalism
		β_{ij} < 0
		β_{ji} = 0
		Competition
	Negative Impact	β_{ij} < 0
	βii or βij < 0	β_{ji} < 0

Figure 2.2.5.2 Interpretation of Lotka-Volterra's interaction parameters.

2.2.5.3 Monod Model

To model the assimilation of contaminants by the different monocultures and co-cultures, a mass balance equation was developed where Monod's kinetic equation was used to model the substrate consumption (equation 15).

$$\frac{dS(t)}{dt} = -\frac{X}{Y^{X}/_{S}} \frac{\mu_{max}\, S(t)}{K_S + S(t)} \qquad \text{(eq.15)}$$

In this equation, S (mg L^{-1}) represents the evaluated substrate (COD, TN and TP) concentration, X is the biomass concentration (mg L^{-1}), μ_{max} (day^{-1}) corresponds to the maximum growth rate, K_s represents the substrate-specific affinity constant (mg L^{-1}) and $Yx_{/_S}$ describes the biomass productivity per consumed substrate. This parameter is nondimensional.

The DNN was again used to determine the non-parametric values of the derivative ($\frac{dS(t)}{dt} \in \mathbb{R}^3$), where each component of the vector corresponds to a specific substrate concentration (Gradilla-Hernández et al., 2020). The DNN identifier is presented in equation 16, where $\hat{S}(t) \in \mathbb{R}^3$ is the state vector and $\hat{S}(t)$ values at t time were used to determine the three parameters, μ_{max}, K_s and$Yx_{/_S}$.

$$\frac{d\hat{S}(t)}{dt} = \overline{f}(\hat{S}(t), |W_1(t)) = A\hat{S}(t)+W_1(t)\big(\sigma(\hat{S}(t))\big) \qquad \text{(eq. 16)}$$

The Hurwitz matrix, linear term of the equation is represented by $A \in \mathbb{R}^{3\times3}$ The adaptation to nominal dynamics is presented as $W_1(t) \in \mathbb{R}^{3\times3}$ and $\sigma \in \mathbb{R}^3$ represents the activation vector function.

The optimization problem established to obtain the three parameters is shown in equation 17, where k* is the vector of the estimated parameters. Finally, the Nelder-Mead algorithm was used to solve the optimization problem.

$$k^* = \underset{\mu_{max},\ K_s,\ Yx_{/_S}}{argmin} \left\|\bar{f}(\hat{S}(t), |W_1(t)) - S'(t)\right\|_2^2 \qquad \text{(eq.17)}$$

In order to fit the concentration curves to the parameters, MATLAB R2020a software was used. Curve Fitting, Simulink toolboxes and the ‗fminsearch‘ function were used to run the DNN algorithm and to determine Monod‘s model kinetic parameters.

For the simulation of the continuous stirred tank reactor (CSTR), the differential mass balances were solved using the built-in Ordinary Differential Equation (ODE) solver in MATLAB® (Mathworks, R2017b). The ode23tb method was used to solve stiff differential equations by means of the trapezoidal rule and backward differentiation formula.

2.2.6 Statistical analysis

Analysis of variance (ANOVA tests) were performed for the three response variables (COD, TN and TP) with a significance value of $\alpha = 0.05$; thus the null hypothesis was rejected for $p < 0.05$. Tukey‘s tests were performed to establish differences between every pair of microbial treatments. Statistical analyses were performed using R software version 4.0.2. Graphics were made with ggplot2 package

2.2.7 Experimental diagram

The following diagram (fig 2.2.7) briefly summarizes the complete research methodology.

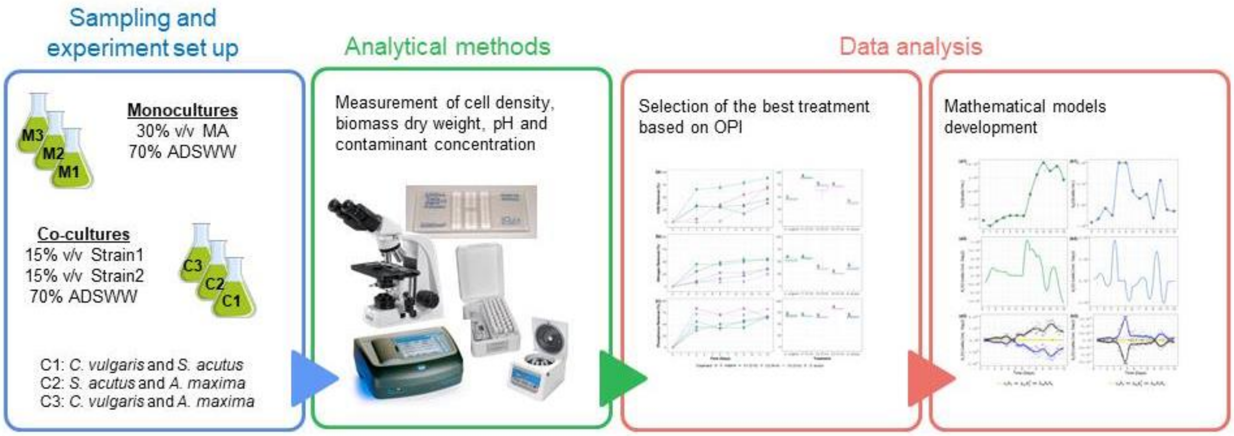


Figure 2.2.7 Research methodology diagram.

2.3. Results and Discussion

2.3.1 ADSWW characterization

The physicochemical characterization of ADSWW is summarized in table 2.3.1. All of the physicochemical parameters were within the allowable ranges for SWW pollutants (Cheng et al., 2019a; Huang et al., 2014; Ye et al., 2020). BOD_5 was slightly above the maximum limit established by the Mexican regulatory body for wastewater discharges, as well as the EPA permissible limits for centralized waste discharges (CONAGUA, 1996; EPA, 2021). Total suspended solids were also found above the limits established by international regulations (EPA, 2021). However, the Kheldahl nitrogen and TP concentrations significantly surpassed local limits. The BOD_5/COD ratio displayed by the ADSWW was 0.26, which indicates high biodegradability (Samudro & Mangkoedihardjo, 2010). The mean pH was 7.87 which is expected due to the formation of ammonium carbonate $(NH_4)_2CO_3$ (Georgacakis et al., 1982; Webb & Hawkes, 1985) and the removal of CO_2 in the carbon equilibrium that results in CO_3^{-2} and $2H^+$ (Möller & Müller, 2012; Sommer & Husted, 1995a).

Table 2.3.1 Physicochemical characteristics of ADSWW and Mexican legislation limit values

Parameter	Unit	Value	Mexican legislation (NOM-001)	Permissible limits established by EPA guidelines for centralized waste treatment point discharges
pH		7.87	5-10	6-9
Sedimentable solids	mg L^{-1}	1.00	1	-
COD	mg L^{-1}	808.00	-	-
BOD5	mg L^{-1}	212.77	200	163
Total suspended solids	mg L^{-1}	220.00	200	74.1
Kjeldahl nitrogen	mg L^{-1}	808.08	60	-
Total phosphorus	mg L^{-1}	70.96	30	-

*Current acceptable ranges established by Mexican legislation, as obtained from CONAGUA (1997).
**Permissible limits in the USA, as obtained from the EPA (2021)

2.3.2 Overall comparison of microalgal treatments

Three different microalgal strains were cultured in raw ADSWW to evaluate their potential for pollutant removal and biomass production (expressed as BF). Six different microalgal treatments were performed, including monocultures (M1-M3) and co-cultures (C1-C3). The ADSWW was not previously autoclaved so that indigenous bacteria created a native microenvironment for the inoculum. The substrate was not modified with the application of extra nutrients or by dilution.

Table 2.3.2 presents the initial conditions of each treatment (pH, N:P ratio, cellular density and pollutant concentrations) and the four response variables (BF, %rCOD, %rTP and %rTN).

Table 2.3 Initial culture conditions and overall results

T	MA	Initial culture conditions					pH		Maximum Biomass Production		Final concentration (mg L^{-1})				Contaminant removal (%)			OPI
				Concentration (mg L^{-1})														
		N:P ratio	Cell Density (Cell mL^{-1})	COD	TN	TP	Initial	Assay mean	Cell mL^{-1}	g L^{-1}	COD	TN	TP	BF	rCOD	rTN	rTP	
M1	*C. vulgaris*	8:1	1.36E+07 ± 5.29E+05	860 ± 43.59	540 ± 39.05	66 ± 7.77	7.0 ± 0.14	6.6 ± 2.0	1.32E+08 ± 1.99E+07	1.51 ± 0.22	464± 23.86	249 ± 35.80	22 ± 2.56	10	46 ± 2.77	54 ± 6.63	67 ± 3.86	0.67
M2	*S. acutus*	8:1	2.67E+04 ± 2.08E+04	860 ± 43.59	540 ± 39.05	66 ± 7.77	6.9 ± 0.14	7.3 ± 0.3	7.33E+04 ± 5.77E+03	0.06 ± 0.00	533 ± 27.3	343 ± 30.55	24 ± 2.80	3	38 ± 3.17	36 ± 5.66	64 ± 4.22	0.42
M3	*A. maxima*	8:1	7.50E+03 ± 0.00	860 ± 43.59	540 ± 39.05	66 ± 7.77	9.87 ± 0.02	9.3 ± 0.4	CD	CD	CD	CD	CD	CD	CD	CD	CD	0.00
C1	*C. vulgaris* *S. acutus*	13:1	4.32E+07 ± 1.21 E+07 1.17E+05 ± 2.89E+04	613 ± 47.26	620 ± 10.00	48 ± 1.53	7.56 ± 0.01	7.9 ± 0.2	2.54E+08 ± 1.22E+07 4.00E+05 ± 5.00E+04	2.88 ± 0.35 0.09 ± 0.01	70 ± 18.00	270 ± 10.00	16 ± 0.58	6 3	89 ± 2.93	56 ± 1.61	67 ± 1.21	0.68
C2	*S. acutus* *A. maxima*	13:1	5.00E+04 ± 0.00 4.17E+03 ± 1.44E+03	613 ± 47.26	620 ± 10.00	48 ± 1.53	8.98 ± 0.02	9.0 ± 0.1	8.33E+04 ± 2.89E+04 CD	0.06 ± 0.00 CD	215 ± 70.00	400 ± 17.32	17± 0.71	2 CD	67 ± 11.41	35 ± 2.79	65 ± 1.48	0.47
C3	*C. vulgaris* *A. maxima*	13:1	2.68E+07 ± 2.93E+06 4.17E+03 ± 1.44E+03	613 ± 47.26	620 ± 10.00	48 ± 1.53	9.01 ± 0.01	9.1 ± 0.1	2.46E+08 ± 7.04E+07 CD	2.79 ± 0.79 CD	175 ± 15.00	463 ± 15.28	8 ± 1.00	9 CD	71 ± 2.45	25 ± 2.46	83 ± 2.10	0.7

T= treatments, CD= cell death, M= monoculture, C= Co-culture, BF= Biomass Folds. All results are mean values. In Co-cultures biomass folds is calculated for each strain at the time it displayed its maximum production

2.3.2.1 Culture N:P ratios

The initial N:P ratios in monocultures and co-cultures were 8:1 and 13:1, respectively. These ratios were lower than those reported by Kumar et al. (2010), as is characteristic of WW containing animal manure (40:1). The ratios observed in this study (<30) reflect nitrogen limitation (Zhang & Hu, 2011). The limited initial TN concentrations are indicative of the effectiveness of the anaerobic digestion as well as ammonia stripping and volatilization (Arriagada et al., 2017; Li et al., 2020b; Lotti et al., 2019). Conversely, AD generally displays low phosphorous removal, especially for inorganic phosphates (Campos et al., 2019; Morse et al., 1998) and, therefore, TP concentrations can be as high as 100–1400 mg L^{-1} in AD effluents (Wang et al., 2019a).

The stoichiometric elemental formula for an average algal cell is $C_{106}H_{263}O_{110}N_{16}P$ (Redfield, 1963), which closely matches the empirical formula of *C.vulgaris* proposed by Huang et al. (2021). Therefore, the theoretical N:P mass ratio for *C.vulgaris* is 7.24:1. Accordingly, the monocultures possessed an N:P ratio that favored both strains growth compared to the co-cultures. While the N:P ratio of the co-cultures (13:1) was higher than the theoretical N:P ratio, it was still within the range of 7:1 - 15:1, which is reported as appropriate for microalgal growth and nutrient removal (Choi & Lee, 2015; Gonçalves et al., 2017; Rhee, 1978; Zhang & Hu, 2011). Moreover, the Redfield ratio (16:1) for phytoplankton is considered ideal for enhancing nutrient removal when culturing MA in WW (Geider & La Roche, 2002a). However, assimilation of TP and TN resembling the Redfield ratio only occurs when both nutrients are abundant, and several authors have reported the ability of MA to adjust their stoichiometric N:P biomass ratios to the media in which they are growing (Choi & Lee, 2015; Geider & La Roche, 2002a; Molazadeh et al., 2019).

SWW dilution is a common practice to adjust unfavorable N:P ratios in the substrate and to promote the effectiveness of the MbWT. However, to make MbWT a feasible, environmentally sustainable option in agro industry, this practice is not attractive to livestock producers who are not willing to input extra freshwater. For these reasons, screening for microalgal strains or co-cultures with N:P ratios resembling that of the SWW is essential to optimize simultaneous nitrogen and phosphorus removal as well as valuable biomass growth.

2.3.2.2 Biomass folds

C. vulgaris displayed 10 BF in monoculture (M1) and 9 BF in co-culture with *A. maxima* (C3) (table 2.3), despite the potential substrate changes that the death of *A. maxima* may have induced. The maximum biomass production of *C. vulgaris* was 2.88 ± 0.35 g L^{-1} in C1, which is also within the range reported by other authors who cultured *C. vulgaris* in ADSWW. For instance, Deng et al. (2018) reported a maximum biomass concentration of 1.68 - 3.47 g L^{1} by *C. vulgaris* using ADSWW in a system with substrate recirculation. However, Xie et al. (2022) tested *C. vulgaris* cultured in SWW in outdoor open raceway ponds with 3% CO_2 supply and observed a lower biomass production of 0.478 g L^{-1} .

In the present study, *S. acutus* displayed a biomass growth of 3 BF in both M2 and C1, and only 2 BF in C2. This slight decrease in BF can be attributed to the early death of *A. maxima* in C2. The biomass production displayed by *S. acutus* in monoculture was within the range of 0.06-0.09 g L^{-1}, which is lower than is reported in the literature. However, in most of these works, SWW was diluted or sterilized. For instance, Xu et al. (2015) cultured *Scenedesmus obliquus* in sterilized SWW diluted to five different COD concentration levels (2200, 1600, 1200, 800, and 400 mg L^{-1}) and observed the highest production (2.3 mg L^{-1}) in the 1600 mg L^{-1} COD concentration. Similarly, Wang et al. (2016) obtained 0.8 g L^{-1} of *Scenedesmus obliquus* biomass using 25% v/v SWW diluted in water as their media.

While higher growth was expected in monocultures rather than in co-cultures, due to N:P ratios of 8:1 that were closer to the microalgal stoichiometry, the growth of both strains was not drastically affected under the co-culture ratio of 13:1 (Choi & Lee, 2015; Geider & La Roche, 2002b; Redfield, 1963).

Although *A. maxima* displayed cell death within the first three days in co-cultures C2 and C3, these cultures were monitored for 12 days like the rest of the treatments to characterize the growth and nutrient removal of the strains that survived. Nevertheless, *A. maxima*'s cellular death may have affected the surviving strain by modifying the substrate in the following ways: 1) an alteration of the nitrogen, carbon and phosphorus chemical forms available as a result of lysed cells (Deng et al., 2019); 2) a rise in COD concentration caused by the extra load of organic matter to be biodegraded (Wang et al., 2015); and 3) a release of toxins or harmful metabolites from damaged *A. maxima* cells (Toh et al., 2012). However, the strain that survived (C2 - *S. acutus* and C3 - *C.vulgaris*) was able to survive these possible substrate modifications as both showed adequate cell growth during the remainder of the experiment.

The cell death of *A. maxima* may have been caused by an intolerance of high NH_3 concentrations that appear at pH values above 9. Certain microalgal strains are sensitive to high NH_3 concentrations, which uncouple the electron transport in photosystem II and compete with H_2O in the oxidation reactions leading to O_2 generation (Arun et al., 2019; Pfeiffer et al., 2018). For instance, Mezzomo et al. (2010) concluded that undiluted SWW with high ammonia concentrations affected the growth of *Arthrospira platensis*. While authors like Markou et al. (2016) and Belkin and Boussiba (1991) reported that *Arthrospira* species are more tolerant to NH_3 than other strains, other authors reported that undiluted SWW cannot be used to culture *Arthrospira* species. López-Pacheco et al. (2019) observed a productivity of *A. maxima* of 1.25E +04 cell $mL^{-1}day^{-1}$ using a composite substrate of 25% nejayote, 25% SWW and 50% water. However, biomass production was affected in treatments with 90% SWW, 10% water and 0% nejayote (< 5.0E+04 cell mL^{-1}). Further experimentation is required to identify the nitrogen species present in the ADSWW at different pH levels affecting the growth of different strains of *Arthrospira* species.

2.3.2.3 pH effects on microalgal cultures

pH values were not modified but only monitored during the 12 days of the experiment (figure 2.3.2.3) to keep the culture conditions as simple as possible. All treatments displayed an initial pH within the range of 6.9 ± 0.14 - 9.87 ± 0.02. Higher pH values (M3 - 9.87 ±0.02, C2 - 8.98 ±0.02 and C3 - 9.01 ±0.01) occurred only in the treatments containing *A. maxima*, whereas the rest of the treatments maintained a pH in the range of 6.6 - 7.9 throughout the experiment. This was expected due to the Zarrouk's medium (pH of 8.5 or above) that was used to culture *A. maxima*.

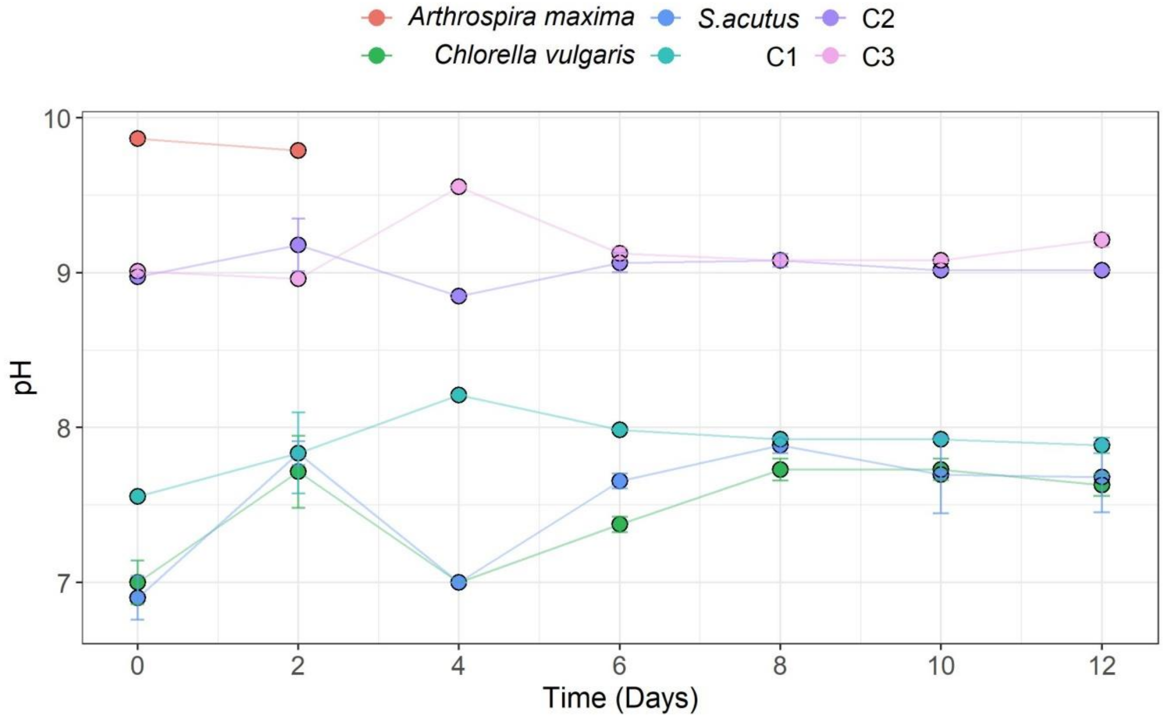


Figure 2.3.2.3 pH monitored during the microalgal treatments. C1: co-culture of *C .vulgaris* and *S. acutus*; C2: co-culture of *S. acutus* and *A. maxima*; C3: co-culture of *A. maxima* and *C. vulgaris.*

The optimum pH for the growth of most MA is between 6-10 (Gerardi, 2015; Rastogi et al., 2017). Most MA tend to alkalize their culture media as a result of photosynthetic reactions that convert bicarbonate to CO_2 with the help of anhydrase enzymes, releasing OH^- ions (Badger & Spalding, 2004). In the present work, there was no substantial alkalization observed during the twelve days in any of the treatments. This could be due to the low experimental volumes or the experimental retention time of twelve days. These results suggest that the cultures employed can perform for at least 12 days without pH alternations. However, controlling the media pH is suggested for industrial implementation, especially in open systems like raceway ponds. Open systems tend to change their pH as a result of environmental factors and are susceptible to contamination from external microorganisms, which can alter pH due to their metabolic activities (Koley et al., 2019; Ranganathan et al., 2017). Maintaining an optimum pH is extremely important for MbWT, as alterations affect many biochemical process, such as $NH_3 \leftrightarrow NH4^+$ equilibrium and carbon availability (López-Sánchez et al., 2022). Moreover, an alkaline pH could increase the feasibility of large-scale outdoor systems, as it reduces the risk of contamination.

2.3.2.4 COD and nutrients removal

The initial concentrations of COD and nutrients were different between monocultures and co-cultures (Table 2.3). The largest difference was observed in COD concentrations and can be attributed to the time elapsed between the experiments. As a result of avoiding ADSWW sterilization, it had a high content of indigenous bacteria and microorganisms acquired from the previous AD process. However, the OPI (discussed in section 2.3.2.5) is calculated in terms of removal percentages to avoid bias when comparing the treatments. Moreover, in the search for scalability potential of MbWT, it is relevant to consider that the effluents of an AD will not always have the same concentrations of COD and nutrients, but they will instead fluctuate within a reasonable range (Brito-Espino et al., 2021; Giwa et al., 2019; Schmidt et al., 2019). Figure 2.3.2.4 shows the contaminant removal percentage graphs as well as the box plots for the total removal results. The treatments were grouped using the Tukey mean-difference test. The results of COD removal confirm that a higher COD removal can be expected by co-cultures compared to monocultures (Fito & Alemu, 2018; Kong et al., 2022; Qin et al., 2016), as C1 was the treatment that displayed the higher COD removal efficiency (89%) and was statistically different from the rest of the treatments.

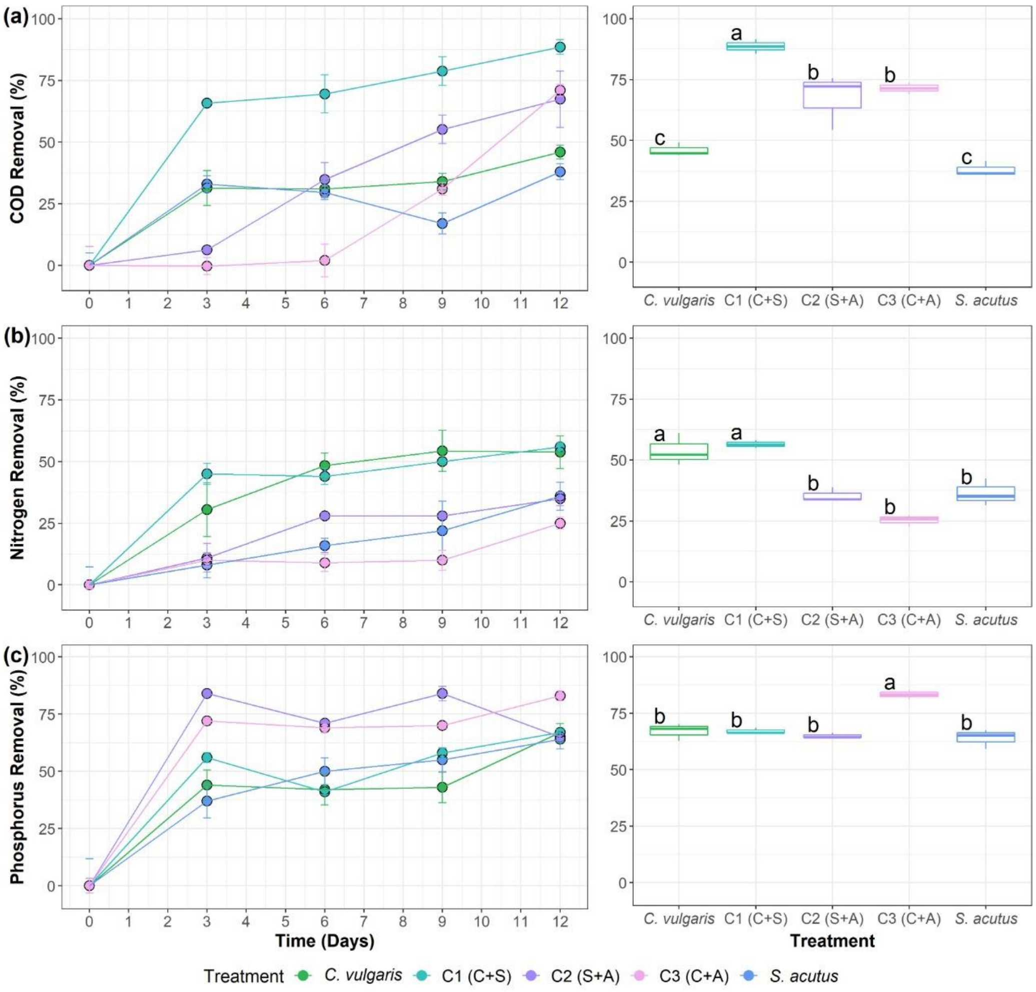


Figure 2.3.2.4 Contaminant removal percentages and box plots with Tukey grouping. a) %rCOD, b) %rTN and c) %rTP.

C.vulgaris and *S. acutus* viably co-existed in C1 during the entire experimental time. Therefore, it can be inferred that the synergy among them and native bacteria in ADSWW enhanced carbonic matter oxidation and assimilation resulting in higher COD removal. Similarly, Qin et al. (2016) reported that 57–63% of the COD content could be removed by a microalgal consortia (*Scenedesmus spp*. and *Chlorella zofingiensis*), higher than the removal displayed by a *Chlorella* sp. monoculture of 45%. Synergy in assimilation of carbon in co-cultures may be caused by cross-feeding among the two strains, specific enzyme production, or complementary assimilation of different forms of carbon available in the substrate (Kong et al., 2022). However, further investigation into microalgal metabolomics is required to understand the synergies of carbon assimilation.

Although co-cultures C2 and C3 behaved as monocultures due to the early death of *A. maxima,* they both displayed higher COD removal efficiencies compared to the either monoculture of the two strains (M1 and M2). This can be attributed to a difference in available carbon forms present in the original ADSWW and those released from *A. maxima*'s lysed cells. *C. vulgaris* and *S. acutus* likely benefited from the chemical forms of carbon released by *A. maxima* to the extent that they were able to assimilate the excess carbon that was produced by the decaying *A. maxima* cells. Both co-cultures displayed a delay in COD uptake within the first 4-6 days of the experiment, potentially indicating that the death of *A. maxima* increased levels of organic matter in the ADSWW only for a period of time, and thereafter the remaining microalgal strain together with bacteria were able to oxidize and uptake the excess of carbonic matter (Ghernaout & Elboughdiri, 2020; Kong et al., 2019; Toh et al., 2012). However, in full-scale MbWT, the cell death effect of *A. maxima* on COD concentrations is unknown and may even be harmful for the surviving strain, as larger volumes are treated. Therefore, until this is further understood, *A. maxima* should not be used in any type of culture to treat ADSWW.

The assimilation rate of COD by the *C. vulgaris* monoculture was highest during the first 3 days and then a second phase of rapid uptake was observed after the seventh day. This trend suggests that the consumption of simple carbohydrates occurred during the first growth phase and the more complex forms were oxidized and hydrolyzed to be assimilated during the second phase.

All treatments achieved higher TP removal (⩾ 64%) than TN removal (⩽ 56%), possibly due to the phosphorus ‗luxury uptake' behavior that is common in MA growing in phosphorus-rich media. This ability to uptake more phosphorus than necessary has been reported to depend on the following three main variables: 1) phosphate concentration in the substrate, 2) light intensity, and 3) temperature (López-Vázquez et al., 2008) (Solovchenko et al., 2019).

The C3 co-culture of *A. maxima* and *C. vulgaris* displayed the highest %rTP of 83%. However, it only removed 25% of the TN, which was the lowest %rTN among all the treatments. This superior %rTP can be attributed to the cellular death presented by *A. maxima*, which could have modified the existing N:P ratios by increasing nutrient concentrations through the release of cellular content when lysing. In addition, cyanobacterial cells, such as *A. maxima,* are well known to enhance bioavailability of phosphorus through solubilizing organic phosphorus by phosphatase enzymes, which is subsequently released into the substrate when damaged and dying cells lyse, thus facilitating its uptake by *C.vulgaris* (Kumar & Singh, 2020). Although C3 is the culture that removed the most TP, it cannot be suggested that it is the most suitable for treating ADSWW, since the primary objective of this research is to screen for the treatment that simultaneously removes both COD and nutrients. In this case, the higher phosphorus removal strongly compromised the removal of nitrogen.

These results suggest that *C. vulgaris* and *S. acutus* were viable candidates to grow in undiluted ADSWW with N:P ratios that range from 8-13. However, nutrient assimilation by *S. acutus* was slightly lowered under these ratios. The N:P ratios reported herein are within the N:P ratio ranges published by Arbib et al. (2013), where *S. obliquus* assimilated 100% of the TP and more than 90% of TN. The present results also suggest that there was no complementarity of the two strains (*C. vulgaris* and *S. acutus*) in the assimilation of nutrients, since the removal rates of C1 and M1 were not found to be statistically different. Rather, it can be inferred that *C. vulgaris* was responsible for the uptake of nutrients due to its higher growth rate compared to that of *S. acutus*. Because phosphorus is an essential nutrient for micro algal growth and cell division, higher uptakes would correspond to higher biomass volumes. Furthermore, microalgal growth and phosphate uptake have been reported to be linearly proportional (Chu et al., 2013; Yaakob et al., 2021).

2.3.2.5 Overall performance index

Overall, the treatments that contained *C. vulgaris* displayed higher OPI values, which suggest higher biomass production and higher removal efficiencies for COD and nutrients collectively. The C1 co-culture (*C. vulgaris* and *S. acutus*) displayed the best performance of all treatments (OPI = 0.68) closely followed by the monoculture of *C. vulgaris* (M1) and the co-culture of *C. vulgaris* with *A. maxima* (C3), both with an OPI of 0.67. This co-culture displayed a biomass production of 6 and 3 BF for *C. vulgaris* and *Acutus*, respectively. Additionally, this co-culture displayed removal efficiencies of 89%, 56% and 67% for COD TN and TP, respectively.

These results support the conclusion that *C. vulgaris* cultures were more efficient than *S. acutus*, maximizing biomass production, COD and nutrient removal. Overall performance of *C. vulgaris* was not affected by the type of culture or by the initial N:P ratios. Its cellular growth and nutrient removal results were not statistically different from the monoculture *vs*. when it was co-cultured with *S. acutus*.

2.3.3 Microalgal growth kinetics

The microalgal growth curves of each treatment are presented in figure 2.3.3(a). *C. vulgaris* presented a two-peak sigmoidal growth curve in all treatments. Additionally, it always displayed a prolonged adaptation phase that reached exponential growth between days 5 and 8. Furthermore, the growth rates of *C. vulgaris* in monoculture (specific μ=0.89 days^{-1}) and in co-culture with *S. acutus* (mean μ= 0.81 ± 1.29 days^{-1}) were very similar (table 2.3.3).

S. acutus attained exponential growth sooner than *C. vulgaris* (day 3) but for a shorter period. The unusual growth curve observed for *S .acutus* could be attributed to the experimental method used for cell counting. This strain is typically found in coenobiums (Contreras-Angulo et al., 2019) and hence the accuracy on the count process may be compromised. Alternative cell counting methods may present a better way to count microalgal strains that form coenobiums, such as a flow cytometer, optical density or dry weight determination (Di Caprio et al., 2021; Maeng et al., 2018).

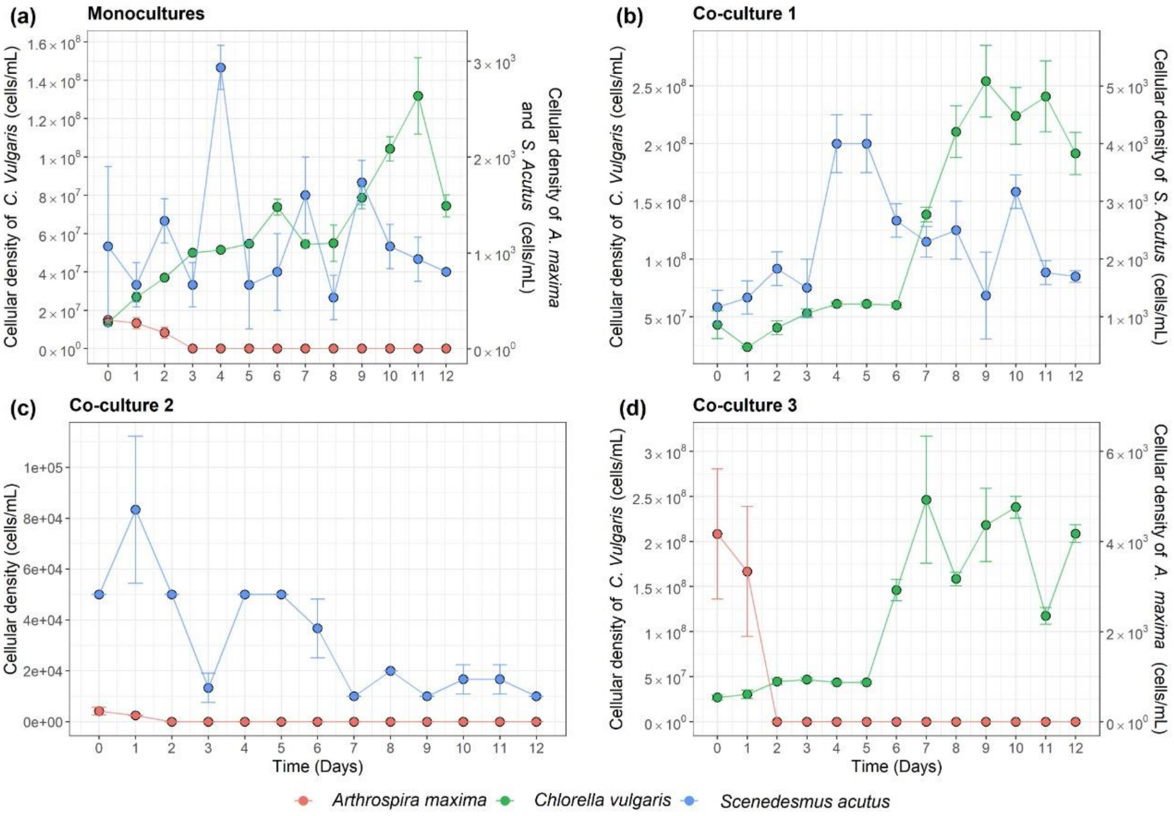


Figure 2.3.3 (a) Microalgal growth curves for every treatment. a) Monocultures of: *C. vulgaris* (M1), *S. acutus* (M2) and *A. maxima* (M3). b) C1: co-culture of *C. vulgaris* and *S. acutus*. c) C2: co-culture of S*. acutus* and *A. maxim*a. d) C3: co-culture of *A. maxima* and *C. vulgaris.*

Table 2.3.3 Kinetic parameters for Gompertz, Lotka-Volterra and Monod models.

	Gompertz Model						Lotka-Volterra Model						Monod Model			
	Max BF		Specific growth rate (days^{-1})		Lag phase (days)		Mean growth rate (days^{-1})		Interaction Parameters (mL cell^{-1} day^{-1})					Max growth rate (days^{-1})	Affinity constant (mg L^{-1})	Biomass productivity (mg biomass mg contaminant^{-1})
Treat.	α_1	α_2	μ_1	μ_2	λ_1	λ_2	α_c	α_s	β_{cc}	β_{ss}	β_{cs}	β_{sc}	Substrate	μ_{max}	K_s	$Y_{x/s}$
M1	4.50	11.56	0.63	0.89	0.71	9.69							COD	0.037 ± 0.06	2.27 ± 0.07	0.75 ± 0.09
													TN	0.031 ± 0.04	2.28 ± 0.05	0.76 ± 0.05
													TP	0.006 ± 0.01	2.31 ± 0.01	0.79 ± 0.02
M2													COD	-	-	-
													TN	0.166 ± 0.04	2.11 ± 0.05	0.55 ± 0.08
													TP	0.047 ± 0.03	2.28 ± 0.06	0.73 ± 0.05
C1							0.81 ± 1.29	0.58 ± 1.37	-0.006 ± 0.005	2.23 ± 1.31	1.99 ± 0.92	-0.007 ± 0.008	COD	0.032 ± 0.05	2.28 ± 0.06	0.75 ± 0.07
													TN	0.021 ± 0.04	2.28 ± 0.04	0.77 ± 0.06
													TP	0.002 ± 0.006	2.30 ± 0.009	0.79 ± 0.01

The modified Gompertz model was used to further assess the growth kinetics of *C. vulgaris,* either in monoculture or co-culture, because it was the strain with the best performance in ADSWW. The growth of *S. acutus* was not modeled due to the atypical behavior shown by its growth curve. Additionally, this strain did not show the best performance based on the response variables of the OPI.

The graphical representation of the modified Gompertz model (eq. 9) is shown in figure 2.3.3 (b), where the two terms of the equation are shown separately and as a total. The experimental observations of *C. vulgaris* in monoculture, represented as dots, fit the modified Gompertz model satisfactorily (R^2=0.9722).

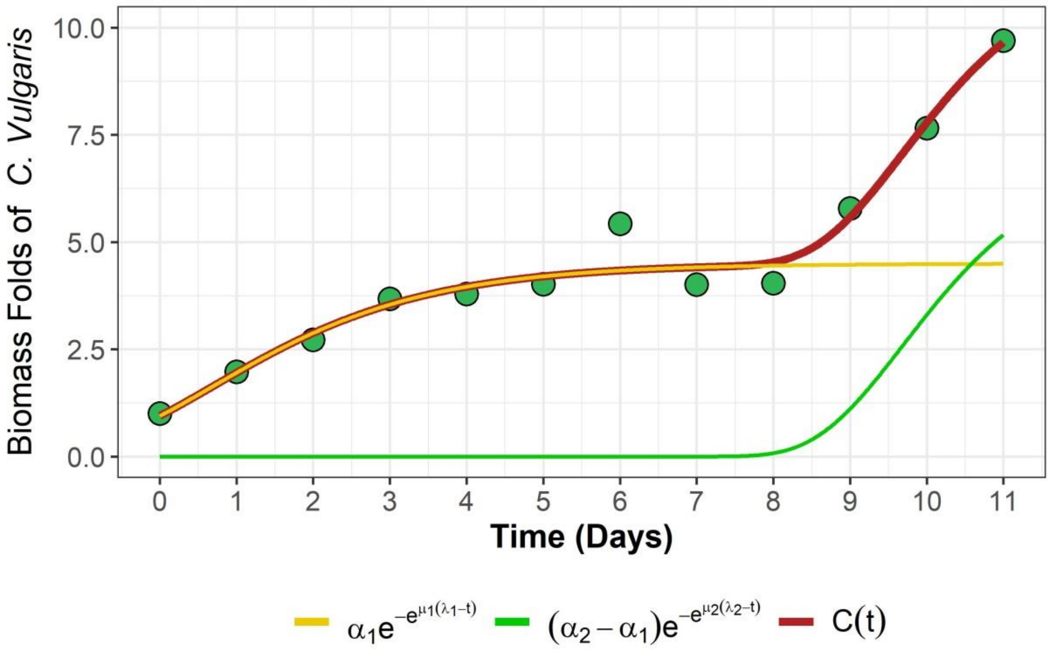


Figure 2.3.3 (b) *C. vulgaris* growth represented by the modified Gompertz model.

The first growth phase of *C. vulgaris* began immediately after the inoculation day, showing a very short lag phase (λ_1 = 0.71 days). Hence, this strain requires little adaptation to the ADSWW. In this phase, C. *vulgaris* grew at a specific growth rate of 0.63 days^{-1}, reaching the first asymptote which denotes the maximum value of biomass production (α_1) of 4.5 BF. Afterwards, *C. vulgaris* remained in a stationary phase that lasted until day 9 (λ_2 = 9.69), where a second phase of exponential growth occurred. In this second phase of exponential growth, *C. vulgaris* possessed a biomass production of 11.6 BF at a higher growth rate of 0.89 days^{-1}. Empirically, our results show that *C. vulgaris* achieved 10 BF, which agrees closely with the model. This two-phase growth behavior is common for *C. vulgaris*, as reported by other authors who previously cultured *C. vulgaris* in different types of WW (de Macedo Dantas et al., 2020; Raposo et al., 2010). These results indicate that *C. vulgaris* rapidly consumed a simpler source of nutrients during the first phase until depletion, and after a short stationary growth phase, the MA underwent a second growth phase when it likely encountered a second, more complex source of nutrients that allowed it to reactivate its productivity at a higher growth rate.

ADSWW is a fairly complex source of nutrients and carbohydrates that changes constantly depending on several factors, such as the metabolism of the population of microbes found in the mixed culture, oxygen levels, and pH (Haaning Nielsen et al., 2004; İrdemez et al., 2006; Zhang et al., 2020). Hence, the availability of nutrients required for microalgal growth may be favorable at different stages of the culture. MA, bacteria, and other microorganisms present in ADSWW can easily assimilate and degrade soluble organic matter of low molecular weight, such as simple sugars. However, organic polymers and more complex organic material must be attacked first by extracellular enzymes that release low molecular weight compounds to be further assimilated (Grady Jr et al., 2011). The time difference between the first and second lag phases (0.71 and 9 days) could have been enough for the formation of nitrogen and phosphorus compounds more assimilable by the MA (Lage et al., 2021; Su, 2021), triggering a productive second growth phase.

The parameters obtained by this model are highly valuable when designing a bioreactor for a full-scale MbWT. For instance, λ_1 and λ_2 show that the hydraulic retention time in the bioreactor must be extended to 9 days in order to reach the second exponential growth phase where *C. vulgaris* is more productive. After the ninth day, however, productivity declines significantly, suggesting that there is no need to continue the culture time past this timepoint.

2.3.3.1 Microalgal interactions in co-cultures

The generalized Lotka-Volterra model was used to evaluate microalgal interactions in the co-culture of *C .vulgaris* and *S. acutus*. This model allows for the estimation of growth rates and microalgal interaction parameters (Gradilla-Hernández et al., 2020).

Each step of the parameter determination process is depicted in Figure 2.3.3.1 (a)**.** Graphs a1 and b1 show the growth curves of both microalgal populations. Graphs a2 and b2 display the first derivative of the biomass concentration (cell mL^{-1}) as a function of time. The analysis of the most relevant inflection points in *C. vulgaris* (a2) reveals the two periods of maximum growth acceleration. The first phase appeared around 0.5 days and displayed a productivity of around 2.5 $x10^7$ cell mL^{-1} day^{-1}, whereas the second growth phase increased in productivity to over 1.0 $x10^8$ cell mL^{-1} day^{-1}, which is ten times greater than in the first growth phase. During the first stationary phase, between the fourth and sixth day, the derivative decreased to zero. A similar behavior can be observed in the derivative during the last days of the culture, from day nine, where the derivative value finally became negative, signaling a decline in the microalgal population.

The derivative of the growth curve for *S. acutus* (b2) also revealed two phases of exponential growth. However, for this strain, the first phase was more productive than the second one. Furthermore, the difference in maximum productivity of both phases was not as evident as it was for the growth dynamics of *C. vulgaris.* The derivative significantly increased only for periods of one day, which were shorter than those of *C. vulgaris* at 4-5 days. In addition, at many points in time, the derivative displayed negative results, corresponding to periods in which biomass production decreased and growth rates stagnated.

The last row of the figure panel (a3 and b3) shows the behavior of all three terms that make up the derivative of the Lotka-Volterra model. As can be seen in a3, the terms $\beta_{cc}X^2_c$ and $\beta_{cs}X_cX_s$ cancel each other during the experimental time, despite the great difference of magnitude in microalgal concentrations (X_c order 10^8 and X_s of 10^5), maintaining $\alpha_c X_c$ as the only term that influences the derivative result. The behavior is the same for b3, where $\beta_{ss}X^2_s$ and $\beta_{sc}X_cX_s$ cancel each other, thus leaving α_sX_s, as the only significant term of the model. From these results , it can be concluded that the population growth of both strains in co-culture is only dependent on their specific growth rates and not on the interactions established among them.

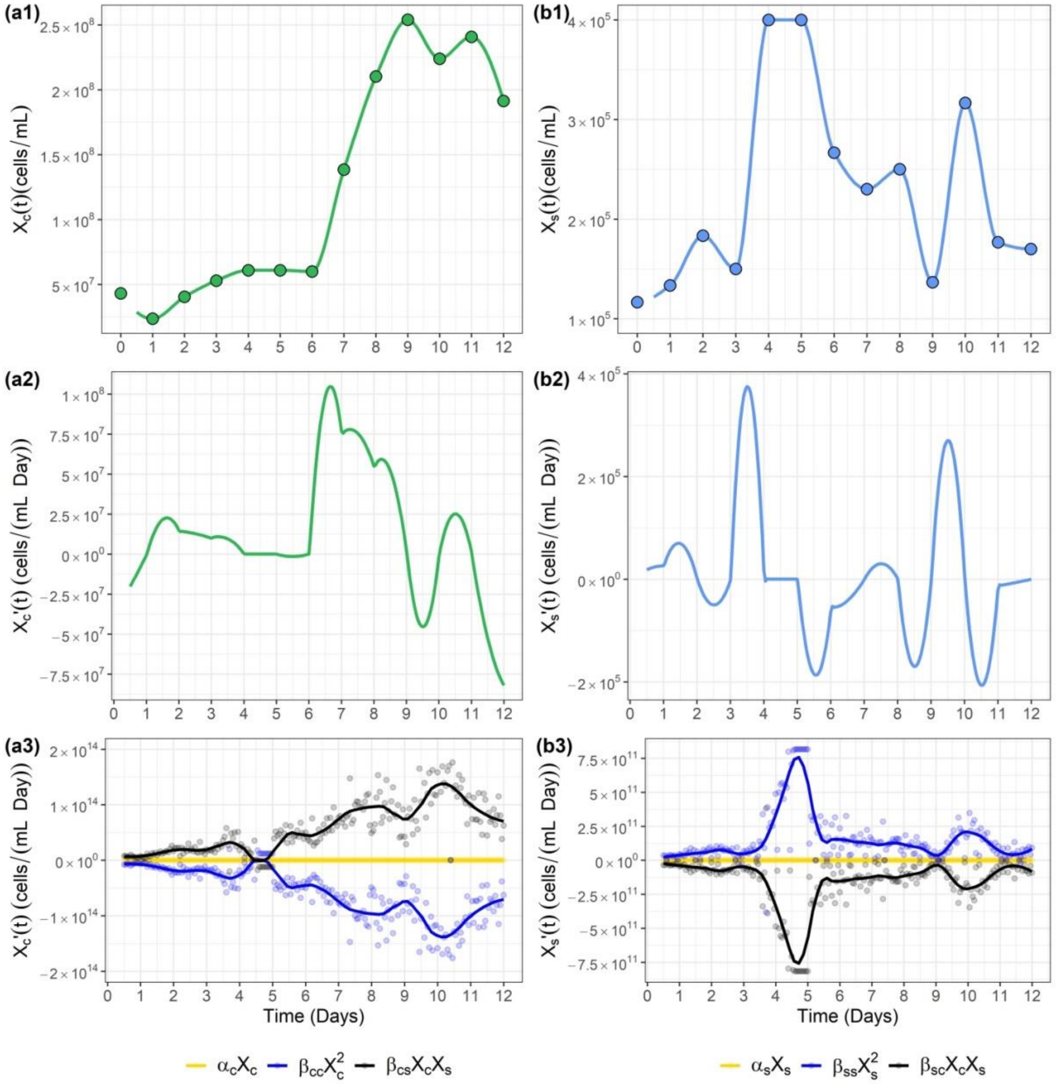


Figure 2.3.3.1(a) Growth curves of *C. vulgaris* and *S. acutus* with their derivatives and Lotka-Volterra equation terms. A = *C. vulgaris*, b = *S. acutus.*

Table 2.3.3 shows the mean values for the strains' specific growth rates as well as the intraspecific and interspecific interaction parameters. The mean growth rate for *C. vulgaris* was 0.81 ± 1.29 days^{-1}, whereas *S. acutus* displayed lower growth rate of 0.58 days^{-1}. The intraspecific parameters are those that describe the type of interaction that a strain establishes with itself. Parameter β_{cc}, which describes the interaction type among *C. vulgaris* members in the co-culture, was -0.006 ± 0.005. Since this parameter is a mean value of the time series and it is negative but close to zero, it can be inferred that the interaction was neutral. On the contrary, the β_{ss} parameter was 2.23 ± 1.31, meaning that a positive interaction occurred among *S. acutus* population members. Interspecific parameters must be analyzed in pair to show the relationships that were established among the two microalgal strains in the co-culture. The parameter results show a commensalistic interaction, where *S. acutus* acted positively on *C. vulgaris* (β_{cs} = 1.99 ± 0.92), while a neutral effect was exerted on *S. acutus* by *C. vulgaris* (β_{sc} = -0.007 ± 0.008). The graphical representation of these parameters over time is shown in figure. 2.3.3.1 (b). The graphic panel displays the behavior of the three parameters for each strain (a = *C. vulgaris* and b =*S. acutus*). The first row shows the behavior of two parameters, α_c and α_s, which denote the growth rates without influencing the interaction of the other strain in the co-culture. *C. vulgaris* displayed a faster growth rate compared to *S. acutus,* which remained stable at a value above 1 during almost all of the experimental time. The growth rate of *S. acutus* remained below 1 and only increased above 2.5 during its first more productive period of exponential growth. As the second exponential growth phase was much lower than the first, a small increase in the parameter can be observed in the graph starting from the tenth day. Additionally, an important decrease in the growth rate can be observed in the final two days, where the second stationary phase was reached.

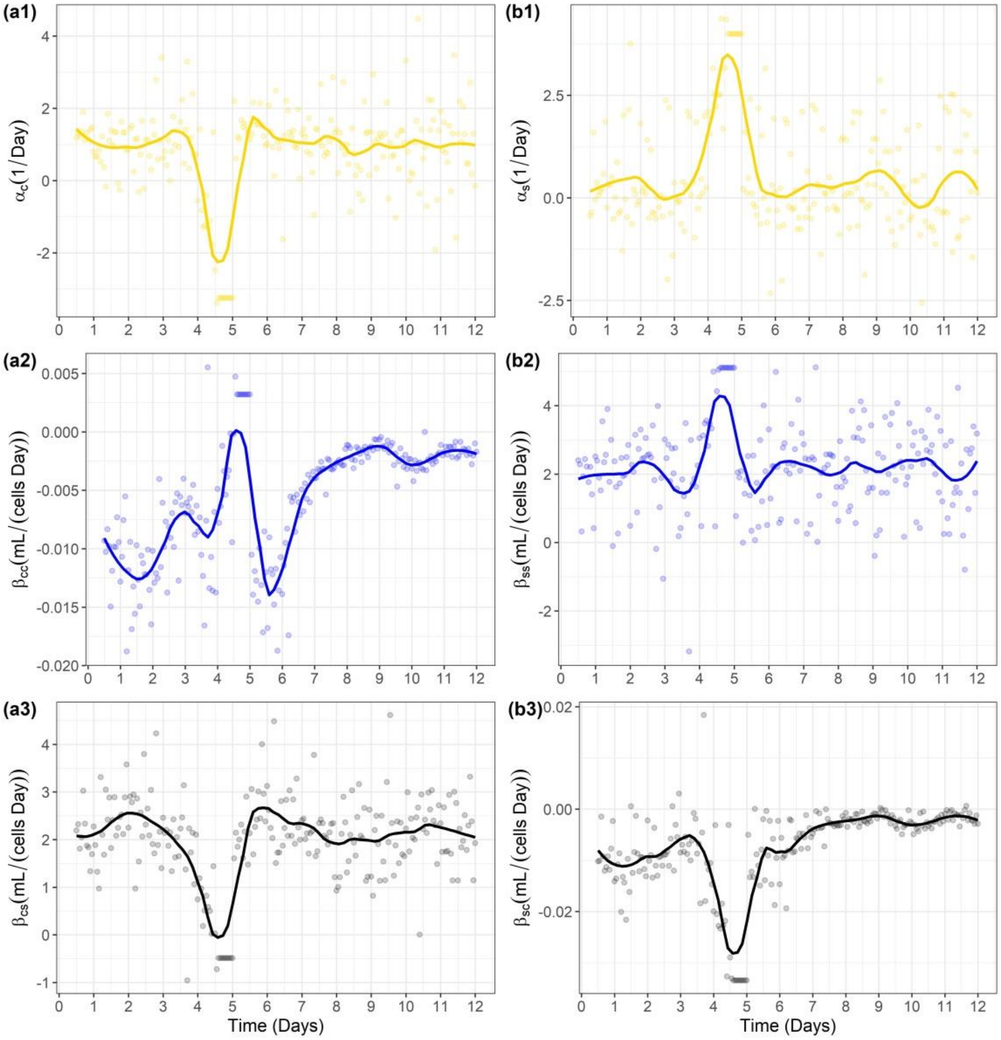


Figure. 2.3.3.1 (b) Lotka-Volterra's model parameters over time.

The second row displays the intraspecific interaction parameters for each strain (β_{cc} and β_{ss}). The intraspecific parameter, β_{cc}, revealed a neutral interaction among *C. vulgaris* population members (graph a2). Because *C. vulgaris* achieved the highest growth rates in the co-culture, it can be assumed that the neutral, slightly negative relationship between members of the same strain might result from their competition for nutrients and space (Knillmann et al., 2012). The value of the parameter approached zero during the stationary phases.

Regarding interactions between members of *S. acutus* (graph b2), the β_{ss} value remained stable and positive throughout the experiment. This parameter increased twice in its mean value during the period of maximum growth, showing that intraspecific competition for nutrients and space was not as strong as for *S. acutus* as it was for *C.vulgaris*. Therefore, the nutrients contained in the ADSWW were in excess of the *S. acutus* population requirements, which were always much lower than those of *C. vulgaris*. The third row (graphs a3 and b3) corresponds to the evolution time of the interspecific interaction parameters (β_{cs} and β_{sc}). β_{cs} represents the effect that *S. acutus* displays on *C. vulgaris*. As the graph shows, this parameter was positive throughout the entire experimental time, with a strong decrease during the period when *C. vulgaris* went through its first stationary phase and a slight decrease starting on day 11, when both populations reached the stationary phase for the second time. However, the effect of *C. vulgaris* on *S. acutus,* as denoted by $\beta_{sc,}$ was close to zero during all the entire experimental time. Thus, the analysis of the behavior of these parameters reveals a commensalistic interaction of *C. vulgaris* on *S. acutus*, due to the cell concentration and growth rates of *C. vulgaris* that surpass those of *S. acutus*. As *C. vulgaris* was always the dominant strain in the co-culture, *S. acutus* was only able to reach its maximum growth periods when *C. vulgaris* went through its stationary phases. Similarly, *Chlorella sp.* outcompeted *Scenedesmus quadricauda* in all cultures under conditions of phosphorus limitation in a study reported by Grover (1991). Another factor that might be causing this competitive behavior could be the production of harmful secondary metabolites, as is common in biological systems between different taxonomic groups and even between different strains (Żak & Kosakowska, 2016). For instance, in a study by Żak and Kosakowska (2016), *C. vulgaris* was strongly inhibited by exudates from one strain of *Oscillatoria* species. However, further research is needed regarding the secondary metabolites produced by the MA used herein.

2.3.3.2 Monod model: growth kinetics as a function of substrate

The parameters of the Monod model based on the three substrates, COD, TN and TP, for each of the treatments (*C .vulgaris*, *S. acutus* monocultures *vs*. C1) are reported in table 2.3.3. COD parameters for *S. acutus* are not reported because they did not accurately fit the model since COD removal by *S. acutus* was not always positive and increased between days 4-9 (figure 2.3.4). This increase in COD may be due to microalgal release of organic compounds into the medium during the stationary growth phase (Lee et al., 2016).

The growth rates adjusted to the model were very similar for all experimental treatments (0.002 ± 0.006- 0.047 ± 0.03) except for that displayed by *S. acutus* using TN as substrate, which was higher (0.166 ± 0.04 days $^{-1}$). Moreover, of all the treatments, *S. acutus* also displayed the highest affinity towards TN (2.11 ± 0.05 mgL^{-1}). Figure 2.3.3.2 (a) shows a graphical representation of μmax over time for *S. acutus* with TN as a substrate. During the first 5 days the μ_{max} maintained a steady mean value, however, after day 6 it displayed a major drop and increased considerably after the ninth day. During this period (days 6-9), *S. acutus* showed a limited growth phase with high fluctuation in biomass production, which was potentially caused by changes in the chemical forms of nitrogen available in the ADSWW.

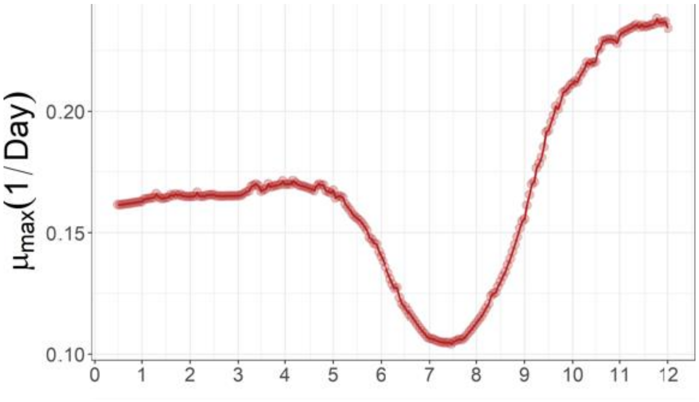


Figure 2.3.3.2(a) Monod's μ_{max} over time for *S. acutus* with TN as a substrate.

The kinetic parameters displayed by C1 were similar to those of M. Therefore, it can be inferred that *C. vulgaris* led the growth kinetics of the co-culture. This behavior is considered normal, as *C. vulgaris* biomass is in a higher order (10^8) when compared to *S. acutus* (10^5).

Biomass productivity was similar between all treatments considering each of the substrates, except for *S. acutus,* which showed a lower productivity of 0.55 ± 0.08 $mg_x\ mg_{TN}^{-1}$ for TN. Leesing and Kookkhunthod (2022) cultured *Chlorella sp.* under heterotrophic conditions using molasses as a carbon substrate and yeast extract as nitrogen source. The maximum productivities ($Y_{x/s}$) that they reported were 0.906 and 0.945 using a concentration of $1gL^{-1}$ of yeast extract and 15 gL^{-1} of molasses, respectively. Both values are higher than those reported herein for *C .vulgaris*, which were 0.76 ± 0.05 and 0.75 ± 0.09, for TN and COD, respectively.

Adjusting the parameters to the Monod model is key for the full-scale implementation of MbWT, since the design of the bioreactor highly depends on these variables. To demonstrate this point, a CSTR with a constant volume was simulated using the following mass balance equation (eq. 18).

$$\frac{dX}{dt} = X(\mu - D) \quad \text{eq.18}$$

Where the change in biomass over time depicted by $\frac{dX}{dt}$ is given by the biomass production, Xμ, minus the biorreactor outputs, which are given by the dilution rate times the biomass concentration at *t* time, DX_t. D at a constant volume is calculated as flow over volume. This expression does not consider biomass reactor inlets or cellular death. Using the monod equation to solve for μ, (eq. 19), the mass balance for each of the substrates is expressed by equations 20-22 for COD, TN and TP, respectively.

$$\mu = \mu_{max}\left[\left(\frac{[COD]}{K_{COD}+[COD]}\right)\left(\frac{[TN]}{K_{TN}+[TN]}\right)\left(\frac{[TP]}{K_{TP}+[TP]}\right)\right] \quad \text{eq.19}$$

$$\frac{d[COD]}{dt} = D([COD]_{in} - [COD]) - \frac{\mu X}{Y_{X/COD}} \quad \text{eq.20}$$

$$\frac{d[TN]}{dt} = D([TN]_{in} - [TN]) - \frac{\mu X}{Y_{X/TN}} \quad \text{eq. 21}$$

$$\frac{d[TP]}{dt} = D([TP]_{in} - [TP]) - \frac{\mu X}{Y_{X/TP}} \quad \text{eq.22}$$

For the purpose of this simulation, the initial experimental conditions (table 2.3) as well as the adjusted Monod parameters were considered for *C. vulgaris* in monoculture. Figure 2.3.3.2(b) displays the results of these differential equations.

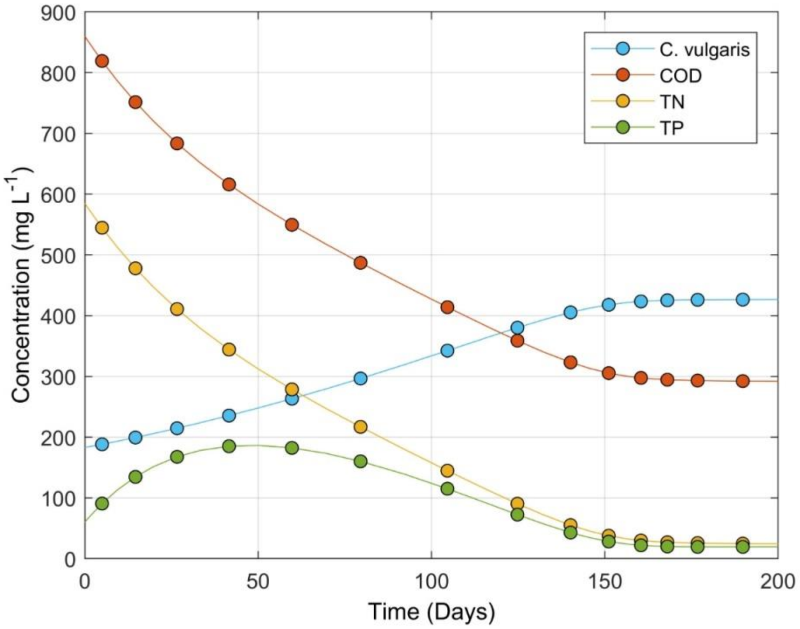


Figure 2.3.3.2 (b) Mass balance equations for a CSTR using *C. vulgaris* monoculture.

These results were obtained assuming a D of 0.03, which optimized the biomass production, thereby avoiding wash-out. The graph displayed TP as the limiting substrate in the ADSWW. During the experimental time of 12 days, the phosphorous source was depleted, stopping *C. vulgaris* growth and limiting its maximum capacity for biomass production. Therefore, phosphorous supplementation would be required to extend the operation time to 200 days with an inlet concentration of 100 mg L^{-1}.

Continuous operation processes involve several advantages for the scalability of MbWT, such as higher productivity, product uniformity, capital cost reduction and the need for smaller bioreactors (Van et al., 2020; Van Sonsbeek et al., 1993). Moreover, when a steady-state is reached, biomass production becomes self-sufficient, thus avoiding the need to continuously inoculate (Mantzaris & Daoutidis, 2004).

A large disadvantage evidenced by the CSTR simulation is that a steady-state is not reached until 150 days. However, a main objective of MbWT is to remove contaminants, and considerable removals can be achieved in the period prior to 150 days, with 65%, 91% and 82% of COD, TN and TP, respectively removed.

Chapter 3

Future perspectives and conclusions

The swine livestock sector is one of the most profitable worldwide and contributes significantly to nourishment of the global population. Some developing countries, including Mexico, contribute significantly to this market, ranking among the main pork producers in the world. However, one of the principal consequences of massive pig farming is the generation of swine wastewater (SWW), which, when incorrectly disposed, can cause serious harm to the environment, such as the degradation of freshwater sources. While developed countries use sophisticated SWW treatment methods, such as anaerobic digesters (AD), developing countries often use simpler methods like oxidation lagoons (OL) as well as physical and chemical treatments, such as precipitation and flocculation. However, most of these processes, including AD, still produce effluents with high levels of contaminants, including chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP). Thus, the proper disposal of SWW has become a major challenge, especially in developing countries.

MbWT offers a sustainable way to remove pollutants from ADSWW through a circular bioeconomy approach, converting waste into microalgal biomass with a high market potential. However, the implementation of MbWT at full-scale or even pilot-scale levels in the agro-industry is still rare (López-Sánchez et al., 2022). Additionally, the vast majority of the existing literature focuses on microalgal cultures under highly controlled laboratory conditions, including sterilizing or diluting the AD effluents, nutrient supplementation, the use of expensive bioreactors, among others. These practices, however, are costly and complex to implement at a full-scale level. The dilution of SWW is not attractive to swine producers because it requires the addition of large volumes of fresh water. Furthermore, sterilized microalgal monocultures are far from being a reality under the operating conditions that currently prevail in developing countries for SWW treatment systems, where, in the absence of resources and sophisticated equipment, many producers simply implement open ponds or raceways (Yang et al., 2011). Although the existing literature lays the foundations for the full-scale implementation of MbWT, the aforementioned challenges still need to be addressed. For these reasons, the present work is focused on screening microalgal strains (mono- and co-cultures) that perform better in raw ADSWW, thus reducing the need for complex pre-treatments or modification of the effluents.

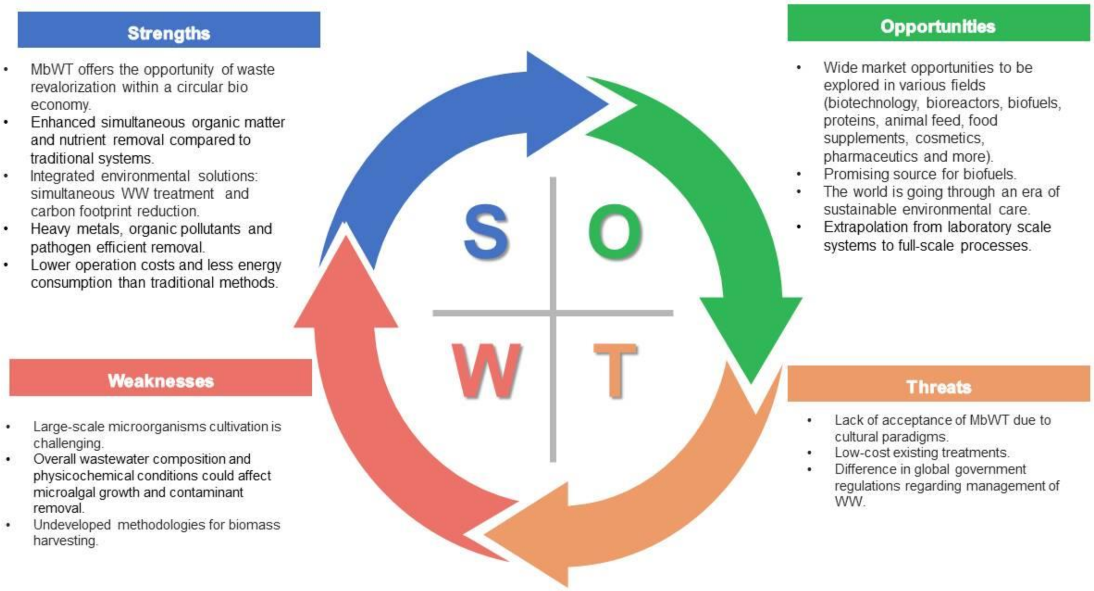
This study presents evidence supporting the treatment of raw anaerobically digested swine wastewater (ADSWW) using monocultures and co-cultures of *C. vulgaris and S. acutus.* Based on an overall performance index (OPI) integrating four response variables (%rCOD, %rTP, %rTN and BF), from all of the treatments tested herein, the co-culture of *C. vulgaris* and *S. acutus* was selected as the best treatment for raw ADSWW. This co-culture displayed the simultaneous removal of COD, TN and TP at 89 ± 2.93%, 56 ± 1.61% and 67 ± 1.21%, respectively. In this co-culture, *C. vulgaris* and *S. acutus* possessed growth rates of 6 biomass folds (BF) and 3BF, respectively, producing a total maximum biomass of 2.97 ±0.36 gL^{-1}. The *C. vulgaris* monoculture was close in results to those of the co-culture, suggesting that this strain was the more adaptable one to the ADSWW with initial N:P ratios that ranged from 8-13. Additionally, the results suggested that the COD removal efficiency was enhanced by the co-culture of the two proliferating strains, as they may display complementarity in assimilation of different forms of carbon available in the ADSWW. Furthermore, co-culture C3 (with *C. vulgaris* and *A. maxima*) was the treatment with the highest TP removal efficiency and the lowest TN removal of all treatments, showing that simultaneous assimilation of nutrients is affected when

MA are uptaking phosphorus. *A. maxima* is not recommended for ADSWW treatment since this strain underwent cell death within the first three days for every treatment condition.

The growth kinetics pattern of *C. vulgaris* was mathematically modeled with the modified Gompertz model and the growth of *C. vulgaris* and *S. acutus* was modeled with the Lotka-Volterra general equation to assess community interactions. The parameters obtained from the modified Gompertz model showed that *C. vulgaris* went through two exponential growth phases. The first one started immediately after a lag phase of 0.71 days with a specific growth rate of 0.625 days^{-1}, while the second one did not occur until almost the tenth with a growth rate of 0.89 days^{-1}. However, the second phase was much more productive than the first one, as the model suggests a maximum production of 11.6 BF, in contrast to that of the first phase of 4.5BF. The Lotka-Volterra's model includes the following four interaction parameters: two intraspecific parameters (β_{cc} and β_{ss}), which describe the interaction between members of the same strain, and two interspecific parameters (β_{cs} and β_{sc}), which describe interactions among the different strains in the co-culture. The intraspecific parameters revealed that the *S. acutus* members maintained an interaction close to neutral through time (β_{cc}= -0.006 ±0.005). On the other hand, β_{cs} was equal to 1.99 ± 0.92, meaning that *S. acutus* exerted a positive relationship towards the members of *C. vulgaris*, and, in contrast, β_{sc} was -0.007 ± 0.008, suggesting that *C. vulgaris* members acted negatively towards *S. acutus* members. These interactions imply an antagonistic relationship. Furthermore, the adjusted model showed mean growth rates of 0.81 ± 1.29 and 0.58 ± 1.37 for *C. vulgaris* and *S. acutus*, respectively.

The parameters adjusted to the Monod model showed a higher affinity of *S. acutus* for TN as well as the highest μ_{max}, at 0.166 ± 0.04. The simulation of the CSTR allowed for the identification of the optimal process conditions using the initial experimental conditions, a D of 0.03 and supplementing 100 mgL^{-1} of extra TP, as it was detected as the limiting substrate.

Even though MbWT appears to be a promising method for the treatment ADSWW, there are still many challenges to overcome to implement it at a full-scale level, and optimal operation conditions must be determined to maximize biomass production and nutrient removals. Therefore, figure 3 summarizes a SWOT analysis, where the principal strengths, weaknesses, opportunities, and threats of MbWT are identified. Moreover, future perspectives for MbWT laboratory investigation and pilot-scale implementation are considered below.



Strengths

- MbWT offers the opportunity of waste revalorization within a circular bio economy.
- Enhanced simultaneous organic matter and nutrient removal compared to traditional systems.
- Integrated environmental solutions: simultaneous WW treatment and carbon footprint reduction.
- Heavy metals, organic pollutants and pathogen efficient removal.
- Lower operation costs and less energy consumption than traditional methods.

Opportunities

- Wide market opportunities to be explored in various fields (biotechnology, bioreactors, biofuels, proteins, animal feed, food supplements, cosmetics, pharmaceutics and more).
- Promising source for biofuels.
- The world is going through an era of sustainable environmental care.
- Extrapolation from laboratory scale systems to full-scale processes.

Weaknesses

- Large-scale microorganisms cultivation is challenging.
- Overall wastewater composition and physicochemical conditions could affect microalgal growth and contaminant removal.
- Undeveloped methodologies for biomass harvesting.

Threats

- Lack of acceptance of MbWT due to cultural paradigms.
- Low-cost existing treatments.
- Difference in global government regulations regarding management of WW.

Figure 3 MbWT SWOT analysis

3.1 MbWT strengths

The growing importance that has been placed worldwide on a circular bioeconomy is the greatest strength of MbWT (McCormick & Kautto, 2013; Stegmann et al., 2020). A circular bioeconomy focuses on the sustainable and circular valorization of biomass in integrated production chains, while making use of residues and wastes (Stegmann et al., 2020). In MbWT, there is no need of huge land space or costly substrates in order to obtain valuable biomass because nutrients and oganic waste are revalorized. Moreover, phosporous is a finite resource that has a high possibility of depletion within 100 years from now (Díaz-Vázquez et al., 2020b), but it can be recovered into microalgal biomass through MbWT. In contrast, traditional WW treatment processes generate many wastes (sludge, chemical precipitates) that are difficult to dispose of and that cannot be re circulated within the same process or revalorized (Mantis et al., 2005; Shi et al., 2018).

The opportunity to revalue wastes in MbWT is given through enhanced simultaneous removal of organic matter and nutrients from WW due to their effective assimilation, which is promoted in bacterial-microalgal mixed cultures. In conventional WW treatments, carbonic matter is oxidized to CO_2, nitrogen volatilized to the atmosphere as ammonia or N_2 and phosphorus is precipitated and disposed as waste sludge (Adav et al., 2008). Microalgae (MA) releases O_2 during photosynthesis, which is used by bacteria to degrade organic matter into CO_2, soluble phosphorus and inorganic nitrogen forms that can be easily assimilated by MA (Molinuevo-Salces et al., 2019).

Additionaly, CO_2 capture is necessary to mitigate global climate change (Zhou et al., 2017). Microalgae play a vital role as photosynthetic organisms in this process by sequestering CO_2 (Cuellar-Bermudez et al., 2015). In this way, MbWT is considered to be a ‗carbon neutral‘ process because the CO_2 released during the process had been sequestered from the atmosphere for the growth of MA (Shi et al., 2000).

MA are able to accumulate heavy metals from WW when their concentrations do not reach levels that could result toxic to them by inhibiting their photosynthesis process (Molinuevo-Salces et al., 2019). Heavy metals are assimilated by MA by different mechanisms such as adsorption, ion exchange, covalent bonding or precipitation (Chojnacka et al., 2005; Ozturk et al., 2014). Moreover, several commercial biofilms have been developed using MA to accumulate heavy metals. For instance BV-SORBEX™, produced by BV Sorbex, Inc. (Canada), is able to recover 99% of the WW metals. Likewise, MbWT can contribute to the removal of parasites in the WW since their photosynthesis process increases levels of dissolved oxygen in the media, which produces photo-oxidative damage of cells (Molinuevo-Salces et al., 2019). Other pathogens can be inhibited by microalgal metabolism which increases media pH levels and by the excretion of inhibitory metabolites in their attempt to over compete bacteria (Natrah et al., 2014; Posadas et al., 2015).

Considering the limited resources that many pig producers face, especially in developing countries, the search for affordable solutions must be a priority. This way, lower operational costs can be achieved through MbWT by using raw SWW effluents, screening for ideal microalgal strains, and maintaining a simple, cost-effective process (Jia et al., 2016; Ledda et al., 2016; López-Sánchez et al., 2022).

3.2 MbWT weaknesses

Biological processes can be efficient but challenging because they depend on external factors that are hard to control. Related to this, the following list gives some examples of situations where MbWT would be especially challenging: 1) contaminant levels that surpass the adequate ranges for MA to survive (Wang et al., 2018); 2) harsh environmental conditions, such as lack of adequate sun irradiance or extreme temperatures that can affect microalgal growth (Haines et al., 2022; Pérez-López et al., 2017); 3) lack of operational knowledge (especially in developing countries) (Gao et al., 2020; Habe et al., 2020); and 4) culture contamination with exogenous microorganisms that prey on microalgae in open systems. As research expands into the field of bioreactor design, better solutions might be generated to overcome these problems. Bioreactors offer various advantages, such as less fluctuation in the effluent quality, better control of physicochemical parameters (pH, temperature, light), low space requirements, and optimization of hydraulic retention times (Asante-Sackey et al., 2022; Posten, 2009; Xu et al., 2009). However, as previously mentioned, the agro-industry is searching for low-cost WW treatment solutions, with operation simplicity and low resource needs. Unfortunately, bioreactors have been associated with high initial investments and operational costs as well as high energy demand, despite all the operational benefits that they offer (Judd, 2008). Therefore, the development of simple and cost-affordable bioreactors is undoubtedly a key element that will turn MbWT into a feasible practice in the agro-industry.

Additionally, overall composition of the SWW can affect microalgal growth and nutrient removal. It is well known that several factors affect the efficiency of this treatments such as N:P ratios (Zhang & Hu, 2011), concentrations of heavy metals and emergent contaminants (Sutherland & Ralph, 2019) or physicochemical characteristics of the substrate (López-Sánchez et al., 2022) . Thus further research should be focused on screening for the adequate strains that adapt to the SWW composition, finding microalgal consortia that display contaminant assimilation synergies or mixing different waste effluents (López-Sánchez et al., 2022) rather than appealing to complex substrate pretreatments thus mantaining MbWT cost affordable and simple.

Biomass harvesting is another important challenge to be addressed. An economic evaluation of a pilot-scale treatment plant for dairy effluents, as performed by Kumar et al.

(2020), demonstrated that harvesting was the most cost-incurring part of the process. Nevertheless, several alternatives are currently being explored, such as microbial aggregation and flocculation by enhancing excretion of bacterial and microalgal extracellular polymeric substances (Zhang et al., 2021) or the use of immobilized cultures with polymeric matrixes of alginate and carrageenan (Cuellar-Bermudez et al., 2017). These alternatives could be a promising solution to this problem. Furthermore, immobilized cultures have been shown to enhance nutrient removal (Kube et al., 2018).

3.3 MbWT opportunities

The weaknesses that MbWTs entail can be transformed into research opportunities and could generate new markets that have not yet been explored. Biotechnology is one of the most promising fields that could benefit from MbWT. For instance, synthetic biology could be used to enhance MA and maximize biomass production yields, production of molecules of high commercial value, and contaminants uptake (MU et al., 2019). For example, Chang et al. (2020), mutated *Tetraslemis sp*. using CRISPR-Cas9, which resulted in a 2.7-fold higher lipid productivity compared to the wild type MA. Likewise, Lin et al. (2018) successfully overexpressed an exogenous carbonic anhydrase in *Chlorella Sorokiniana* and *C. vulgaris* and were able to achieve higher lipid productivity and acceleration of carbon capture and fixation. Other existing literature reports show promising research in this area, including microalgal recombinant proteins and biopharmaceuticals, photo-bioreactor engineering and design, as well as development of harvesting technologies (Barolo et al., 2020; Kadir et al., 2018; Kirnev et al., 2020; Specht et al., 2010).

MA culturing has been identified as one of the most promising techniques for biofuel production. Since one of the major challenges today is the need to replace fossil fuels with renewable energy and mitigate CO_2 release to the atmosphere, MA culturing offers a carbon neutral process to produce biofuel. Moreover, MA does not compete for agricultural land and their simple morphology makes lipid extraction easier as compared with traditional crops (Gilmour, 2019). Alternatives of culturing MA, such as using solar energy and WW, represent an environmentally friendly solution for the production of biofuels like biogas, bio-hydrogen, bioethanol and biodiesel (Jia et al., 2016; Sivaramakrishnan & Incharoensakdi, 2018).

With the United Nations declaring a climate emergency in 2020, complex challenges must be adressed to increase the world's desire for sustainable, ecofriendly, and circular economies. The 17 goals for sustainable development defined by the United Nations are guideliness that ensure technologies like MbWT are in a ―boom era," in which resources and research will be extensive (UN, 2015).

MbWt research has been extensive but laboratory encased. Hence, extrapolationg laboratory-scale systems to pilot-scale processes is one of the most urgent identified needs. Ilot-scale systemes will open up new research opportunities and life cycle assessments are key to successful implemtentation of MbWT in industry.

3.3 MbWT threats

Several external factors were identified, including threats to implementation, acquisition of resources, and acceptance of MbWT. One of the greatest threats that was here could be a lack of acceptance of MbWT in the agro-industry, due to economic restrictions and cultural paradigms (John et al., 2020; López-Sánchez et al., 2022; Moondra et al., 2021). Ignorance of the functionality of these treatments, lack of regulation in some countries or deficient surveillance capacity from the authorities, may be strong paradigms that affect the acceptance of MbWT in the industry (Ávalos, 2020; Ray et al., 2021; Shoushtarian & Negahban-Azar, 2020). In most developing countries, agro-industrial WW treatments are confined to OL, activated sludge and anaerobic treatments, which tend to be inefficient for contaminant removal, but are commonly accepted even though they demonstrate poor results (Cheng et al., 2018; López-Sánchez et al., 2022). However, Moondra et al. (2021) revealed that MbWT can offer a promising technique for developing countries and can overcome the limitations of the commonly used treatments in an eco-friendly and cost-effective way.

Governments around the world bear different regulations towards the disposal and management of WW. However, many developing countries lack proper governmental surveillance and adequate regulations for agro-industrial WW management and reuse (Ray et al., 2021; Shoushtarian & Negahban-Azar, 2020). Therefore, the work presented herein should be used as jumping point to eventually make MbWT a viable circular bioeconomy solution to SWW management in Mexico and other developing countries around the world.

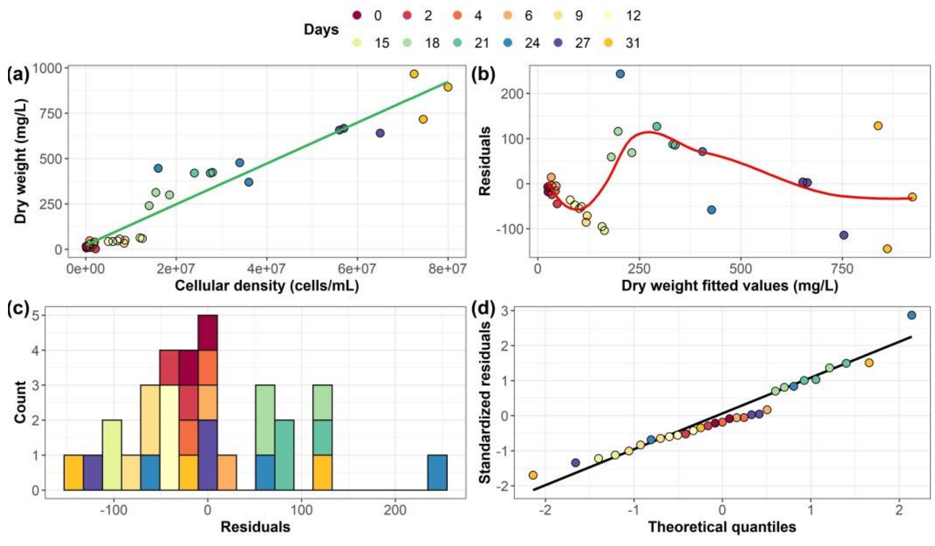
Appendix A

For the linear model, coefficients α and β that maximized R^2, were estimated finding the relationship between the variables DW and CD for *C. vulgaris* and *S. acutus*. The assumptions of normality and constant variance were first confirmed using the Anderson-Darling ($p < 0.05$) and Levene ($p<0.05$) tests. The obtained linear regression equations can be observed in table 2.3.3. Both coefficients were positive in the case of the two MA, indicating DW increase with respect to CD progression. *S. acutus* shows a higher coefficient that can be explained because its cell size is superior compared to *C.vulgaris* cells which can result in weight rise. In the case of *S. acutus* linear regression, R^2 is not as close to 1 meaning that the exactitude with the linear model is slightly compromised. However, both coefficients were significant, thus the obtained linear regression gives us a fairly accurate estimate for the purpose of this study. Appendix A contains the linear regression graphs for MA, as well as the scatterplots, histograms and QQ plots for the residuals.

Table 2.3.3. DW and CD correlation equations

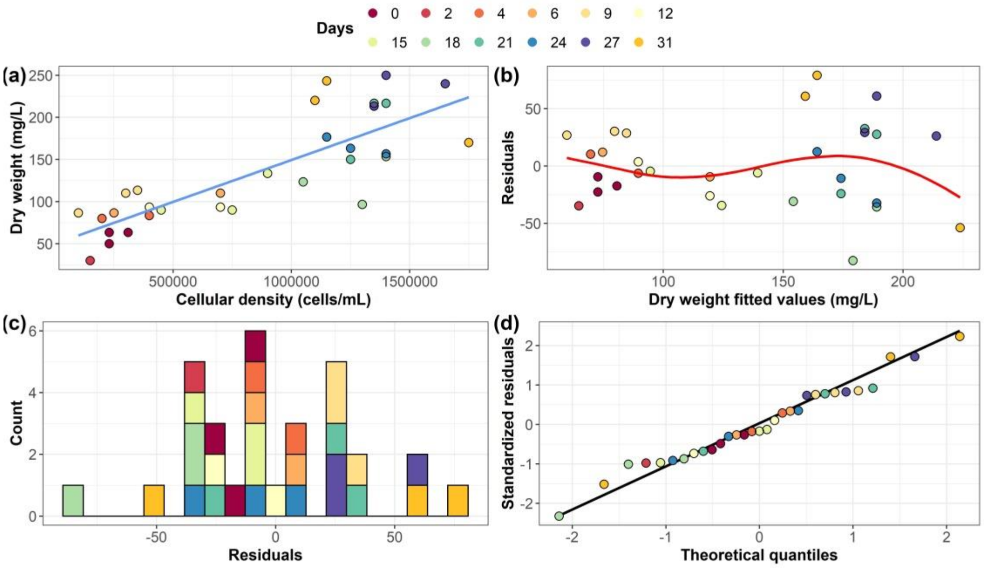
MA	Equation	R^2
C. vulgaris	$W_C = 1.124 \times 10^{-5} (CD_C) + 23.12$	0.9152
S. acutus	$Ws = 1.012 \times 10^{-4} (CDs) + 48.5$	0.7622

Figure A1. Linear regression for *C.vulgaris*



a)Linear regression for dry weight and cellular density. b) Residuals scatterplot. C) Residuals histogram. d) Residuals QQplot

Figure A2. Linear regression model for *S.acutus*



a)Linear regression for dry weight and cellular density. b) Residuals scatterplot. C) Residuals histogram. d) Residuals QQplot

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