BIOREMEDIATION BY *Chlorella vulgaris*: POTENTIALS FOR TREATMENT OF MUNICIPAL, AGRICULTURAL, AND INDUSTRIAL WASTEWATER SOURCES

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ABSTRACT

Developing countries such as the Philippines suffer from a lack of policy development and implementation on wastewater treatment and discharge. *Chlorella vulgaris* is a microscopic green algae that has been employed in other countries for WWT due to its ability to simultaneously reduce pollutants and produce valuable biomass. However, challenges in technology adaptation such as differential efficiency depending on the location and wastewater types were encountered. Three liquid wastes of different origins—municipal (public market), agricultural (piggery effluent), and industrial (meat processing plant) wastewaters, were used to gauge the remediation potentials of *C. vulgaris* in a simple photobioreactor setup. Treated samples from municipal, agricultural, and industrial wastewaters showed microalgal growth rates of 0.2685, 0.1527, and 0.1809, respectively, along the 6-day treatment period. Post-intervention comparisons of treated vs. untreated samples revealed a lower electrical conductivity, total dissolved solids, chemical oxygen demand, nutrients (nitrate, ammonia, phosphate), and fecal coliform (MPN/100 mL) on treated samples. Moreover, all treated samples demonstrated relatively higher dissolved oxygen concentrations, denoting the photosynthetic activity by the microalgae. Therefore, *Chlorella vulgaris* could be harnessed for the remediation of different wastewaters in Nagcarlan, Laguna, Philippines to circumvent issues in water reclamation and degradation.

Keywords: *Chlorella vulgaris*, bioremediation, wastewater, photobioreactor, water reclamation

CHAPTER I

INTRODUCTION

As the primary sources of water for domestic, agricultural, and industrial usage, freshwaters serve as a foundation for a wide range of human needs and activities (United States Environmental Protection Agency [US EPA], 2020). The over-exploitation of freshwaters and its incapacity for replenishment leads to water shortages worldwide, hence, it is fundamental to utilize all available water sources. In this fight against water crises, the recycling of wastewater is critical since it is an integral part of the value chain in all sectors of life, making it an absolute alternative water source (Obotey Ezugbe & Rathilal, 2020). However, wastewater requires proper treatment before discharge since it contains organic and inorganic pollutants, as well as pathogenic microorganisms that may cause degradation of receiving water bodies and can be detrimental to public health and safety (Bensig *et al.*, 2014; Coelho *et al.*, 2015).

Conventional techniques for wastewater treatment are expensive and uneconomical (Aung & Swe, 2019). To address this, environmentally friendly wastewater treatment technologies, such as those utilizing microalgae, are considered promising alternatives since they remove pathogens, heavy metals, nutrients, and other pollutants in water (Abdulredha *et al.*, 2021; Ahmad *et al.*, 2014). *Chlorella* is genus of microscopic green algae that have been employed in wastewater treatment because of their high removal efficiencies of pollutants (Mathew *et al.*, 2022; Wei *et al.*, 2008; Zhou *et al.*, 2012). However, there are several challenges in utilizing *Chlorella* and other microalgae for wastewater biotreatment. This includes high dependency on dynamic environmental conditions (Zouboulis & Moussas, 2019), low cell density (Yuvraj *et al.*, 2016), and variable efficiency from location to location (Wang & Tam, 2019).

The present study aims to solve the iterated difficulties through the introduction of *Chlorella vulgaris* to three wastewater sources in Nagcarlan, Laguna. This study specifically aims to: 1) assess the differences in physico-chemical parameters of municipal, agricultural, and industrial wastewaters; 2) gauge the effectiveness of *Chlorella vulgaris* in inhibiting fecal coliforms; 3) determine the percentage reduction of chemical oxygen demand (COD), total dissolved solids (TDS), total suspended solids (TSS) and percentage removal of nitrate-N (N), ammonia-N (A), and phosphate (P) after treatment; and 4) assess the overall quality of wastewaters after intervention. Moreover, the researchers aim to contribute to the accomplishment of the UN Sustainable Development Goals 3 (Good Health and Well-Being), 6 (Clean Water and Sanitation), 7 (Affordable and Clean Energy), 13 (Climate Action), and 14 (Life Below Water) for the betterment of our environment and society in retrospect.

Background of the Study

Wastewaters are generated after deliberate processes and applications and can therefore be categorized depending upon their main source—municipal, agricultural, or industrial (Plöhn *et al.*, 2021). Organic and inorganic contaminants are present in these waters (Ahmad *et al.*, 2014). In the Philippines, only 10% of wastewater is treated and barely 5% of the population is connected to a sewer network. Discharge of untreated or poorly treated wastewaters is destructive since it ultimately pollutes the receiving environment, which eventually pose threats to the health and safety of the general public (Ansa *et al.*, 2015; World Health Organization, 2015).

Municipal wastewater—water released from communities—often contains high amounts of organic carbon, nitrogen, and phosphorus, which are the major causes of eutrophication. Consequently, the agricultural sector is the largest producer of wastewater (Mateo-Sagasta *et al.*, 2013). Agricultural wastewater is extremely rich in nitrogen and phosphorus, coupled with herbicides, pesticides, and antibiotics used for agricultural practices (Plöhn *et al.*, 2021). Industrial wastewaters, on the other hand, generally contain much smaller amounts of nitrogen and phosphorus, but higher concentrations of different carbon sources.

The application of *Chlorella* spp. in wastewater bioremediation is prevalent in literature due to its ease of cultivation, rapid development, resilience to harsh growth conditions, high nutrient value, and numerous biologically active chemicals (Lv *et al.*, 2022). However, it does not meet the expected scale of cultivation due to various abiotic, biotic, and other operational factors (Chowdury *et al.*, 2020; Loftus & Johnson, 2017). Moreover, previous literature is not “fit-for-all” since the efficacy of microalgal bioremediation varies from location to location. This leads to the low cell densities on some studies following adaptation of existing methodologies (Wang & Tam, 2019; Yuvraj *et al.*, 2016). The ever-changing environmental and atmospheric conditions are also one of the most critical factors that influence growth and degradation rates of *Chlorella* and other microalgae species (Zouboulis & Moussas, 2019).

Continuous deterioration of water quality and quantity is becoming a more pronounced societal problem, yet policy implementation to deal with wastewater has been poor, and only expensive ways of treatment have been devised. To mitigate these problems, the researchers aim to assess the potential of microalgae *Chlorella vulgaris* in the construction of a greener and more sustainable system for wastewater remediation. The findings of this study will help formulate an efficacious methodology of feasible treatment of municipal, agricultural, and industrial wastewater sources in Nagcarlan, Laguna, Philippines.

Theoretical Framework

The conventional biological wastewater treatment (WWT) is defined as a method that focuses on the removal of suspended solids, but drawbacks such as high energy consumption, greenhouse gas emissions, recyclable resource wastage, and excessive solid landfilling pose a challenge to developing a sustainable waste management solution for wastewater treatment and disposal (Al-Jabri *et al.*, 2020; Wollmann *et al.*, 2019).

One such alternative option to treat these wastewaters could be the use of microalgae, which was first proposed by Oswald and Golueke as early as 1950 (Al-Jabri *et al.*, 2020; Oswald & Golueke, 2008). Compared to conventional systems, microalgae-based wastewater treatment does not only manage to treat human sewage, livestock, agro-industrial, and industrial wastes but also uses the nutrients in wastewater to produce algal biomass. Therefore, maintaining microalgae growth in wastewaters is more sustainable and cost-effective (Abdel-Raouf *et al.*, 2012; Amenorfenyo *et al.*, 2019; Liu & Hong, 2021).

Microalgae have the potential to harness sunlight as an energy source for growth and concurrently eliminate contaminants, making them a viable option for remediating sewage from municipalities, agriculture, and industries (Al-Jabri *et al.*, 2020; Liu & Hong, 2021; Merlo *et al.*, 2021). Microalgae's ability to perform photoautotrophic, mixotrophic, or heterotrophic metabolism makes them flexible organisms for treating a variety of wastewater sources (Wollmann *et al.*, 2019). The use of microalgae in WWT serves two purposes: 1) direct uptake or transformation of water contaminants; and 2) improving the purification performance of bacterial systems (microalgae-bacteria aggregates) by providing additional oxygen from photosynthesis, thereby lowering the total energy costs of direct or indirect oxygen supply (Quijano *et al.*, 2017; Wollmann *et al.*, 2019).

The majority of microalgal species are pollution-tolerant, and the use of wastewater rather than freshwater for microalgal cultures significantly lowers the cost of nutrient addition, since they remove nutrients readily present in wastewater. Further, microalgae produce useful biomass applicable for biofuel production and other high-value by-products (Chiu *et al.*, 2015; Polizon *et al.*, 2015). Hence, the concept of using microalgae for wastewater treatment is the foundation of the present study.

Conceptual Framework

The conceptual framework is presented in the form of an independent variable-dependent variable (IV-DV) model. The research paradigm of the study is shown in Figure 1, constituting the independent variable *Chlorella vulgaris* biomass. These factors may affect the dependent variables: the physico-chemical parameters of municipal, agricultural, and industrial wastewaters.

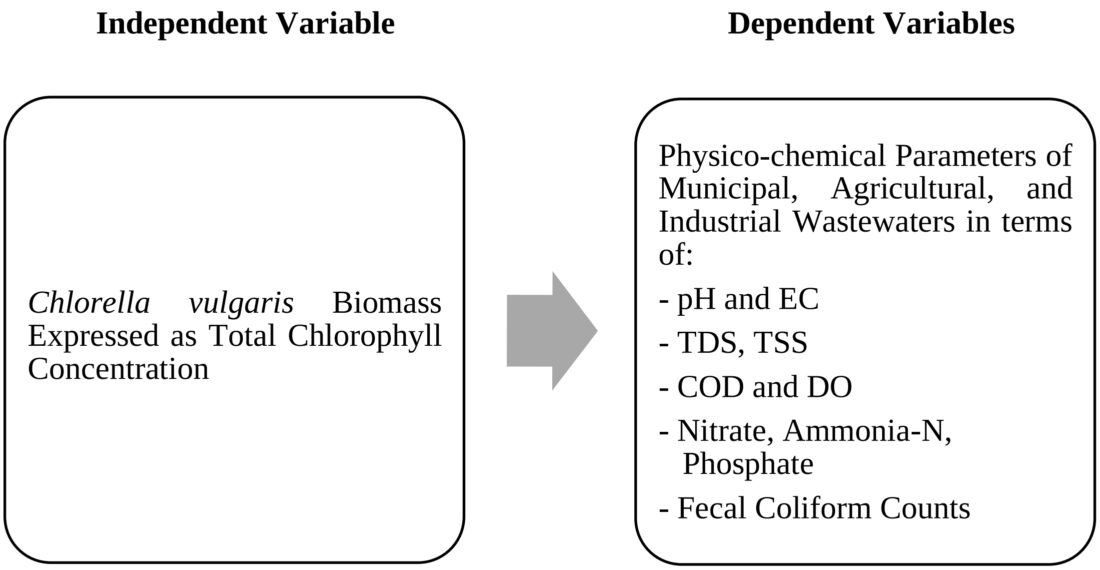


Figure 1. Research Paradigm of the Study.

Statement of the Problem

This study aims to assess the various physico-chemical parameters of three different wastewaters in Nagcarlan, Laguna with and without *Chlorella vulgaris* intervention. It seeks to highlight the efficiency of *C. vulgaris* to assimilate nutrients and reduce contamination to mitigate contemporary problems in water degradation. In this regard, the study seeks to answer the following questions:

1. What are the implications of the different physico-chemical parameters on municipal, agricultural, and industrial wastewaters in terms of:

    1.1 pH
    1.2 electrical conductivity (EC)
    1.3 total dissolved solids (TDS)
    1.4 total suspended solids (TSS)
    1.5 chemical oxygen demand (COD)

1.6 dissolved oxygen (DO)
1.7 nitrate-N (N)
1.8 ammonia-N (A)
1.9 phosphate (P)
1.10 fecal coliform (FC)

2. What is the daily trend in pH, electrical conductivity (EC), and total dissolved solids (TDS) induced by *Chlorella vulgaris*?

3. What is the efficiency of *C. vulgaris* in increasing dissolved oxygen (DO) concentrations on all three wastewaters?

4. What is the efficiency of *C. vulgaris* in the reduction of chemical oxygen demand (COD), total dissolved solids (TDS), and total suspended solids (TSS) on all three wastewaters?

5. What is the efficiency of *Chlorella vulgaris* in the assimilation and removal of nutrients nitrate-N (N), ammonia-N (A), and phosphate (P) on all three wastewaters?

6. What is the efficiency of *Chlorella vulgaris* in the inhibition of fecal coliforms (FC) on all three wastewaters?

Research Hypothesis

There is no significant monotonic relationship between the growth of *Chlorella vulgaris* (expressed as total chlorophyll concentration) and the physico-chemical parameters tested daily (pH, EC, TDS) in municipal, agricultural, and industrial wastewaters. As the value of total chlorophyll increases, the values of the parameters neither increase nor decrease. Furthermore, there is no significant difference between the treated groups and control groups in terms of the parameters tested post-intervention only (N, A, P, COD, DO, TSS, FC). Therefore, *Chlorella vulgaris* has no potential in wastewater bioremediation.

Significance of the Study

This study will provide significant contributions to the field of ecology, waste management, and conservation. The findings of this study could be highly significant and beneficial to the following. Government and affiliated agencies may acquire substantial techniques on wastewater treatment that may then be employed, reformulated, and/or fused with existing treatment methods. The expansive utilization of *Chlorella vulgaris* in wastewater treatment will provide a less costly and more efficacious way of water treatment in local areas and communities. Industrial companies may also benefit from the subsequent exploitation of microalgae in wastewater treatment, as this will serve as an additional or alternative treatment process to expensive chemicals and bacteria used by industrial companies. The present study will also provide sufficient data for wastewater treatment suitable for agricultural reuse to combat the adverse effects of untreated wastewater to soil and crops. This study will also serve as a foundation that will be instrumental in rectifying the paucity of phycology-related studies in the province. Furthermore, the methods and results obtained in this paper will provide supplementary data for future researchers interested in microalgae, wastewater management, and bioremediation.

Scope and Limitation of the Study

This study applies a comprehensive understanding of the efficiency of *Chlorella vulgaris* as an alternative natural wastewater treatment. A total of three sets of wastewater samples from municipal, agricultural, and industrial wastewaters (n = 9) were subjected to multiple tube fermentation technique at NASAT Labs in Cabuyao, Laguna, in order to assess the level of fecal contamination with and without algal treatment. Furthermore, other physico-chemical parameters (e.g., COD, DO, TSS) were tested post-intervention by the researchers in the Ecosystems Research and Development Bureau (ERDB).

The investigations mainly focused on species under the genus *Chlorella*. With considerations from various studies, *Chlorella* is found to be highly effective in regulating a number of pollutants and can successfully adapt to many types of wastewaters, hence, is critical in wastewater treatment (Plöhn *et al.*, 2021). Limitations also encompass varying data sources. Prior studies have emphasized the variable efficacy of *C. vulgaris* in assimilating nutrients and inhibiting fecal coliforms. Due to expensive materials, reagents, and testing, the researchers devised plans and modified some protocols to minimize costs without tampering the credibility of the results. However, due to financial constraints, the proponents were only able to test several parameters on a daily basis; hence, the Posttest-Only Control Group Design. Moreover, the lack of available and comprehensive related studies, and the paucity of step-by-step methodologies and ofttimes-clashing methods of several authors contribute to the limitations encountered.

Definition of Terms

The following terminologies are defined based on how they are used in the study.

Bioremediation. This refers to the process of using *Chlorella vulgaris* to reduce the concentration of organic and inorganic pollutants in wastewaters.

***Chlorella vulgaris*.** This refers to unicellular freshwater microalgae that can be used in sewage treatment to remove nutrients and promote fecal coliform inhibition.

Dissolved oxygen (DO). This refers to the level of oxygen present in wastewater that influences the organisms present in it, i.e., fecal coliforms. It is an important parameter in water quality assessments.

Electrical conductivity (EC). This refers to the capacity of wastewater to carry an electrical current and it assesses if the water quality has changed or varied in any way due to *Chlorella vulgaris* induction.

Fecal coliform (FC). This refers to microorganisms that are generally harmless but its die-off is used as an indicator of the action of *Chlorella vulgaris* in pathogen inhibition.

Multiple tube fermentation technique. This refers to a procedure used in detecting fecal coliform (MPN/100 mL) in wastewaters.

Nitrates, Ammonia, and Phosphates (N, A, P). This refers to limiting nutrients that are assimilated by *Chlorella vulgaris*, removing them in wastewater.

Total dissolved solids (TDS). This refers to dissolved organic matter and inorganic salts in wastewater. It is an essential indicator of the capability of *Chlorella vulgaris* in removal of organic and inorganic pollutants.

Total suspended solids (TSS). This refers to a water quality parameter of the total solids present in the wastewater samples that are trapped by filters (including algal biomass).

Wastewater. This refers to used water that contains elements from municipal, agricultural, and industrial origins.

CHAPTER II

REVIEW OF RELATED LITERATURE

The increasing amount of waste discharged untreated into water bodies and the lack of emphasis and policies on wastewater treatment have kept wastewater a major global problem in restoring water quality. However, with continued research and innovation, an inexpensive yet eco-friendly technique called bioremediation which uses microorganisms to remove pollutants, has been used for effective wastewater treatment. To provide a continuous awareness and in-depth understanding regarding bioremediation, the researchers provide a comprehensive overview of related literature and studies solely focused on the ecological characteristics of *Chlorella vulgaris,* the implications of three wastewater sources and their role in the microalgae species' bioremediation potentials, as well as its associated physico-chemical parameters. Overall, this section addresses knowledge that proves or disproves the findings of previous research studies following the results of the current condition of treating three wastewater sources in Nagcarlan, Laguna using *C. vulgaris* and subsequently offers new knowledge to identify research gaps.

Ecological Characteristics of *Chlorella vulgaris*

The study of algae in bioremediation started in 1960, although there are some naturally-occurring algal flora in sewage, only a few selective and effective strains might be employed to treat wastewater. Among microalgal strains, *Chlorella* leaves a distinguishable impact on bioremediation, especially *Chlorella vulgaris* (Dasgupta *et al*., 2019; Plöhn, *et al.,* 2021; Podder & Majumder, 2017; Sen *et al*., 2013). Their ecological characteristics become an intriguing topic for research in terms of their economic significance in wastewater treatment which has been specifically highlighted in the succeeding paragraphs.

Chlorella vulgaris, even at its small size, has a myriad of distinct characteristics compared to other green microalgal species. It undergoes asexual reproduction, in which a mother cell can yield four daughter cells that take about 19 hours to complete their division. It has high yields of proteins, lipids, carbohydrates, minerals, pigment (β-carotenes), and vitamins (vitamin C, Vitamin B1, B2, B6, and B12) that are used for photosynthesis, respiration, and protection against adverse environmental stressors, including bacteria, viruses, fungi, and various contaminants. Due to their primary and secondary metabolites, *C. vulgaris* can live under autotrophic, heterotrophic, and mixotrophic conditions in freshwater, marine, and terrestrial environments in which their energy is neither dependent on light nor organic carbon sources. Furthermore, they can survive from 28° to 35° C with a pH range from 10.0 to 10.5, and in some cases, they can rapidly fix their DNA when it breaks (Coronado-Reyes *et al.,* 2020; Ru *et al.,* 2020).

The ability of *C. vulgaris* to consistently survive and rapidly grow in a wide range of unfavorable conditions becomes one of the crucial factors in instigating different methods of mass production between open systems and closed systems. The open systems offer the simplest and most inexpensive approach to cultivating microalgae in a large pond under natural environmental conditions. However, a lack of control regarding its abiotic factors such as light, temperature, CO_2, water loss, and the existence of other organisms that could contaminate the culture limits the circulation of nutrients, thus resulting in reduced growth of algae. Therefore, a closed system such as photobioreactors has been used to control the cultivation conditions and minimize the contamination of microalgae. It results in high yields of biomass production but is comparatively costly due to the various nutrients requirements for the media. Thus, extensive research has been conducted to propose alternative inexpensive nutrient sources for biomass production (Coronado-Reyes *et al.,* 2020; Paddock, 2019; Ravindran *et al.,* 2016).

The introduction on the concept of microalgae-based wastewater treatment gives a dual advantage for economic cultivation and wastewater treatment systems. It is based upon the usage of wastewater for microalgal culture while simultaneously lowering the cost of nutrient addition, removing nitrogen and phosphorus, pathogens, and other types of contaminants, while upscaling the growth of microalgae (Abdel-Raouf *et al.,* 2012). They have a capacity to uptake inorganic nutrients and remove heavy metals due to their cell wall having alginate compounds and ligands that can form bonds with various metal groups (Abdel-Raouf *et al.,* 2012; Kumar *et al.,* 2015; Manzoor *et al.,* 2019). Furthermore, research has shown that some heavy metals, such as iron and manganese (for photosynthesis), chrome (for metabolism), zinc (affecting the performance of chlorophyll and proteins), and cobalt (for intracellular reactions and production of essential vitamins), are essential for the normal functioning of microalgae (Coronado-Reyes *et al.,* 2020).

The disclosure of the characteristics of microalgae addresses an integral role in investigating and extending the capabilities of *Chlorella vulgaris* in biotechnology, besides its application in cosmetics, pharmaceuticals, therapeutics, aquaculture, agriculture, biofuel production, and the food industry (Park *et al.,* 2022). It paves the way to become a novel biological wastewater treatment compared to conventional, which produces secondary pollution and waste sludge contributing to 3% of the total anthropogenic greenhouse gas emissions (Gangaraju *et al.*, 2021). The mechanisms of this microalgae facilitate a more detailed understanding and evidence to investigate the different components of municipal, agricultural, and industrial wastewater, being an alternative nutrient source for algal biomass production and potential for wastewater treatment.

Implications of Three Wastewater Sources: Municipal, Agricultural, and Industrial Origins

Through the years, these three sectors—municipal, agricultural, and industrial—significantly influenced the lifestyles within society, economy, and the environment. Their contribution to developing fields of science and technology faces significant challenges due to the varying strength and volume of wastewater contaminants released untreated into various aquatic and terrestrial systems. Several methods and technological practices have been proposed to treat wastewater, yet problems continually increase. Therefore, to better understand the sources of these problems, the succeeding paragraphs highlighted the presence of various components in municipal, agricultural, and industrial wastewaters, specifically waste from the public market, piggery lagoon, and effluent from a meat processing industry, along with their impacts on the environment and the society. Furthermore, it discloses reports that these contaminants could be harnessed as valuable nutrients for *Chlorella vulgaris* to remodel new methods in wastewater treatment.

Municipal wastewater has relatively small amounts of suspended and dissolved organic and inorganic solids that could come from commercial sources like private and public markets which are typically built near rivers and estuaries to ease the disposal of solid and liquid waste (Abdullah, 2017; Pescod, 1992). A substantial amount of waste that being discharged untreated to natural waterways comes from various activities such as meat and chicken slaughtering, seafood entrails, rotten fruits and vegetables, food preparation and consumption, public restroom waste, and waste from cleaning the stalls and market streets (Apandi *et al.*, 2018; German Cooperation, 2016). These heavy wastes could clog into the sewage system and release surface run-off giving more serious hygiene diseases due to the accumulation of microbial diseases from pathogenic bacteria, viruses, protozoa, and helminths derived from the high concentrations of organic solids, COD, BOD, and heavy metals (Al-Gheethi *et al*., 2021; Apandi *et al*., 2018; Pescod, 1992). Al-Gheethi *et al*. (2021) also emphasized that the interference of microorganisms with organic compounds stimulates oxygen depletion, which results in anoxic conditions in water bodies, thus, disrupting aquatic life. More so, both anionic and cationic surfactants used in cleaning and disinfection contribute to the total increase of nitrogen and phosphorus in wastewater.

In another study, Loehr (1978), as cited in Gaur *et al*. (2020) discussed that the piggery wastewater produced by the agricultural sector contributes to a substantial amount of overall wastewater sources and faces controversial issues about human health and the environment. In the Philippines, almost 80% are still using backyard piggery farming which constitutes 80% of wastes discharged directly into creeks and rivers. Catelo *et al.*, (2016) assessed the affected surface waters in 91 pig farms in

Majayjay, Laguna, and found that the entire pig population had produced 6,900 tons of manure per year which deteriorated the water quality; this did not meet the criteria imposed by the Department of Environment and Natural Resources (DENR). These solid, liquid, and slurries produced high concentrations of nitrogen, phosphorus, heavy metals (copper, lead, zinc, and arsenic), and more than 500 volatile organic compounds (VOCs) caused by pig's digestion processes and consumption of nutritional additives or any veterinary medications (Catelo *et al.*, 2016; Cheasley, 2015; Loehr, 1978). Substantially, nitrogen and phosphorus have the most detrimental impact on the environment, and it was found that 50% of nitrogen from animal manure increased from 1930 to 2012 (Chrisman, 2022). In 1997, approximately 100 million L of swine urine and feces had been released on the coastline of North Carolina, resulting in the existence of the toxic microorganisms *Pfiesteria piscicida* that killed most of the fish (Catelo *et al.*, 2016). Furthermore, a concentration of just five ppm of nitrates in drinking water shows an increased rate of various cancers and infant problems due to blue baby syndrome (Chrisman, 2022), while exposure to high concentrations of hydrogen sulfide and ammonia could result in skin or eye irritation and nausea (Cheasley, 2015). More so, animal wastes are carriers of pathogens; drinking contaminated water could result in gastrointestinal and respiratory diseases due to *Campylobacter, Giardia, E. coli, Salmonella, Cryptosporidium, Chlamydia,* and *Streptococcus* (Catelo *et al.*, 2016).

In industrial wastewater, US EPA reported that the effluents from the meat processing industry in many countries generate a significant volume of wastewater with contaminant contents five to ten times stronger than municipal wastewater, and around

80% of it had been directly discharged into the river (Huun, 2021; Latiffi *et al.*, 2019; Yapıcıoğlu, 2018). The wastewater effluent produced during meat processing, packaging, and storing (Djogo *et al.*, 2016) contained significant levels of COD and BOD due to the high concentration of animal fat, blood, and mucosa (Sam, 2022). Latiffi *et al*. (2019) revealed that the resulting data on the average reading of physico-chemical parameters of effluents had a high concentration of COD (2 350 mg/L), BOD (1,070 mg/L), TSS (1,400 mg/L), total phosphorus (62.86 mg/L), orthophosphate (47.37 mg/L), total nitrogen (317 mg/L) and total organic carbon (493.82 mg/L) which were contrary to the discharge of the acceptable effluent imposed by the Department of Environment (DOE). It worsens the situation due to the use of corrosive chemicals for cleaning processes or wastewater treatment systems, as it contains heavy metals such as copper, molybdenum, zinc, chromium, nickel, arsenic, titanium, and vanadium (De Sena *et al.,* 2009; Djogo *et al.*, 2016). Djogo *et al*. (2016) also confirmed that there was a substantial amount of indicator bacteria such as fecal coliform, fecal streptococcus, and total coliform found in meat processing wastewater.

Beyond the adverse impact of wastewater contaminants, it serves as valuable nutrients for microalgae that give them potentials for bioremediation. Analysis of selected parameters was carried out in various studies utilizing *Chlorella vulgaris*, and results revealed a high removal percentage efficiency for every WWT quality parameter tested. Nguyen *et al*. (2022) cultivated *C. vulgaris* in a membrane photobioreactor (MPBR) with diluted piggery wastewater and obtained a COD (65.85%) and PO_4^{3-} (70.20%) removal efficiency. Choi (2016) observed an 88%, 82%, and 54% reduction in BOD, N, and P, respectively. Experimental research of *Chlorella* *vulgaris* usage in WWT processes was also performed by Vovk *et al*. (2020), wherein the effect of treatment varied for BOD (95.67%), COD (83.73%), ammonium nitrogen concentration (95.93%), phosphates (96.92%), and suspended solids (96.84%) reduction efficiency within the 10.5° to 20°C temperature range of wastewater. Lastly, an analysis of all selected parameters carried out by Ahmad *et al.*, (2014) results in a maximum reduction percentage of almost 100%; for BOD (100%), COD (99.9%), NO3 (99.98%), PO4 (99.96%) and total coliform (TC) (100%). The environmental engineers even commended *C. vulgaris* for its ability to remove inorganic materials from wastewater, even in the absence of either nitrogen or phosphorus oppositely with other algae that could not function without the presence of both (Water Technology, 2019).

These studies on the implications of various wastewater contaminants reveal two opposing consequences in the environment and economy and science and technology. First, its adverse environmental impacts hindered the growth of other organisms in aquatic and terrestrial systems while stimulating various pathogenic microorganisms, which could lead to scarcity of potable water, worsening health, poverty crises, and declining economic growth. However, due to the efforts of scientists and researchers, it was justified that wastewater contaminants could be the source of valuable nutrients for microalgae growth which may lead to an eco-friendly marketing scheme for wastewater treatment. Furthermore, understanding these wastewater components gives observation to the effect of physico-chemical parameters in controlling the growth and levels of pathogenic microorganisms, as well as *Chlorella vulgaris*, and its impact on intensifying the strength of contaminants on various wastewater types.

Physico-chemical Parameters of Wastewater

Issues associated with varying volumes and strength of wastewater depend on negligence in conducting tests on the physicochemical parameters of water or noncompliance with the effluent discharge standards for wastewater treatment. Rahman *et al*. (2021) implied that monitoring the physico-chemical parameters of water plays a significant role in evaluating the aquatic system and restoration of water quality and the ecosystem—that it is directly proportional to the physical and chemical properties of water. Therefore, the succeeding paragraphs go over the role of various parameters: pH, electrical conductivity, total dissolved solids, total suspended solids, dissolved oxygen, chemical oxygen demand, nitrate, ammonia, phosphate, and fecal coliform in wastewater systems, along with standard methods.

pH

Water chemistry is influenced by the concentration of hydrogen ions, which is associated with pH for being acidic or alkaline. It has a pivotal role in determining the biological activity and solubility of chemical constituents in wastewater treatment processes, as metals become more toxic at lower pH in a way that the toxic compounds bind with other ions (Akcin *et al.*, 2006; Brandt *et al.*, 2017). Therefore, the proposed pH value in wastewater required for microalgal growth is 6.5 to 7.0, or those close to neutrality (Ihnken *et al*., 2014; Wang *et al*., 2015). Microalgae induce an increase in the pH of the media as they photosynthesize (Larsdotter, 2006). This could be monitored through a colorimeter or pH meter. However, inaccurate pH measurements could be caused by a disconnection of the pH sensor from a meter, a polluted pH glass electrode, a broken probe, and an expired calibration buffer (Akcin *et al.*, 2006).

Electrical Conductivity

The dependency of the increasing EC values on heightened temperatures causes a standard of conductivity at only 25°C (Choo-in, 2019). EC is a type of parameter that monitors the amount of salinity (Schellenberg *et al*., 2020) that procures from water's capability to conduct electricity from ion concentrations of inorganic compounds and dissolved solids (i.e., nitrate, chloride, phosphate, and sulfate anions or iron, calcium, magnesium, aluminum, and sodium cations) within the water (US EPA, 2012). O'Donnell (2022) reported that a high EC content in water denotes high amounts of contaminants, which was always evident in wastewater (Schellenberg *et al.*, 2020) as more ionic compounds dissolve in increased conductivity. The conductivity probe measures the electric current that flows between electrons and provides a conductance measurement in micro siemens per centimeter (μS/cm) or micrometers per centimeter (μmhos/cm) (Choo-in, 2019). After determining the conductivity, an appropriate treatment procedure could be employed since it can be used to gauge other wastewater treatment processes that cause changes in electrical conductivity, i.e., nitrogen and phosphorus (Levlin, 2010).

Total Dissolved Solids

The amount of organic and inorganic materials (minerals, salts, ions, and metals) with a size of less than 2 microns dissolved in water are referred to as total dissolved solids (TDS). High concentrations of TDS are associated with turbidity, water hardness, and toxic contaminants (manganese, bromide, arsenic, iron, and sulfate), thus, infers to high risk of water contamination (Hancock, 2022; Murphy, 2007). According to the US EPA and Bureau of Indian Standards (BIS), the maximum

TDS level of water is 500 mg/L; however, the proposed TDS level by WHO is 300 mg/L. Nevertheless, the TDS value should neither exceed 1000 mg/L, since it is unsafe to consume, nor 2000 mg/L since the filtration system has a filtering limit (Woodard, 2021). Woodard also (2021) suggested that the easiest and most convenient way to measure TDS is by using a TDS meter, as it detects the conductivity of solution from dissolved ionized solids (Carollo, n.d.). However, the TDS meter does not address the type of contaminants present in wastewater, thus, restricting the declaration of water safeness (Woodard, 2021).

Total Suspended Solids

Total suspended solids (TSS) play a vital role in wastewater treatment facilities and aquatic systems as they measure all the suspended organic or inorganic solids unable to pass through filtration due to their larger size. The disposal of TSS influences the waterways by increasing the turbidity and water temperature, thus lowering the amount of dissolved oxygen and photosynthetic activity (Hern *et al*., 2014). Furthermore, it is associated with increased microbiological contamination as these solids provide a surface area for adhesion (Schellenberg *et al.*, 2020). Therefore, the TSS test becomes an important wastewater quality control in wastewater treatment facilities. Specifically, the TSS concentration in industrial wastewater can contain up to 30,000 mg/L, and if left unnoticed, it would exacerbate the disinfection processes and cost high energy demand (Rocker, 2023). The gravimetric method is used to determine the TSS level, especially since it applies to surface waters and industrial and domestic wastes with a TSS determination of 4-20,000 mg/L. It measures the residues from collected cellulose nitrate after filtration and drying at 103-105° C (EPA 160.2).

Dissolved Oxygen

Electrical conductivity and pH influence the content level of dissolved molecular oxygen within the water (Akcin *et al.*, 2006). Dissolved oxygen (DO) is an essential parameter in wastewater treatment plants as well as in aquaculture (Li *et al*., 2022). It can influence other water parameters including BOD, the presence of microorganisms, turbidity, taste, and odor (Norvill *et al*., 2016). Pierce (2019) emphasized that the DO below 4.5 mg/L indicates heavily-contaminated water. In raw domestic wastewater, a low level of DO stems from a high concentration of organic matter which accelerates some biological activity of various microorganisms by consuming more oxygen (Žitnik *et al*., 2019). Therefore, modern methods (e.g., electrochemical) were proposed to monitor the amount of DO in the wastewater, and an example was the galvanic electrode method and the polarographic method belonging to the membrane electrode method. These methods utilize electrodes to determine the amount of dissolved oxygen that pass through the membrane. The electrodes produce an electric current that is proportional to the DO concentration in the sample, and uses a unit of mg/L.

Chemical Oxygen Demand

The analysis of chemical oxygen demand (COD) determines the concentration of oxidizable contaminants and correlates to biological oxygen demand (BOD) in wastewater, and thus, can tell the effect of wastewater disposal on the environment and the efficiency of wastewater treatment (Khaldi *et al*., 2017). Akcin *et al.*, (2006) emphasized that COD amounts to all the oxygen consumed from the chemical oxidation between organic matter (including those toxic compounds and nonbiodegradable substances) and strong oxidants (potassium dichromate, potassium permanganate, or potassium iodate) in a water sample. Furthermore, it evaluates those wastes which are excessively toxic for BOD, which provides more accurate and higher results value within a short period of analysis (2-3 hours) than a five-day BOD test (Merck, n.d.). Ecologix Systems (2021) reported that the required levels of COD for effluent before disposal ranges from 500- 1 000 mg/L; to determine it, the open reflux method and the closed reflux method could be used (Merck, n.d.). However, the closed reflux method is more economical and requires only a small amount of hazardous waste compared to the open reflux method. It is done by using ampoules and placing the sample in cultured tubes with premeasured reagents (Kumar, 2012).

Nitrogen

Nitrate is a compound consisting of one nitrogen atom and three oxygen atoms that are present in ground and surface water (Real Tech Inc., 2022; YSI, 2009;). A small level of nitrate does not pose any danger (Akcin *et al.,* 2006); however, the high content level of it causes eutrophication and health hazards, especially in infants due to blue baby syndrome. Because of this, identifying high levels of nitrate becomes a priority in monitoring water quality, specifically that it is evident in wastewater (Real Tech Inc., 2022) that stems from agricultural, industrial, and domestic waste. Furthermore, the transformation of one compound in the nitrogen cycle leads to another form, such as ammonia converted to nitrites and then into nitrates, the final form of oxidation. Therefore, nitrates become an indicator to calculate the oxygen fraction of nitrogen contamination (Pierce, 2019). Koceba (2021) noted that the Total Kjeldahl Nitrogen method is extensively used and recommended standard for nitrogen measurement which accounts for the total content level of organic nitrogen and nitrogen in ammonium and ammonia. Despite that, previous investigations reported that nitrate waste standards are uncommon; therefore, most wastewater treatment facilities do not have a denitrification stage to remove nitrates (Ni *et al*., 2017).

Phosphorus

Phosphorus could be monitored using either manual or automated colorimetry (Korostynska *et al*., 2012). In its analysis, it is essential to distinguish the orthophosphate, poly- and metaphosphates, and phosphorus compounds in a sample because it provides the value of the total phosphorus in wastewater (Akcin *et al.,* 2006). It becomes an indicator of water quality since domestic and industrial wastewater accounts for a higher amount of phosphorus, and similar to nitrates, it causes an adverse environmental impact (Malairajan & Namasivayam, 2021). Previous reports confirmed that the municipal and residential wastewater contains 5-20 mg/L of total phosphorus and 30-50% phosphorus, respectively. Then, 50-70% polyphosphate compounds account for the extensive use of detergents. Mostly these forms of phosphorus, including organic phosphate, polyphosphate, and orthophosphate, are water-soluble; therefore, the precipitation method only removes a small amount (Akcin *et al*., 2006; Ruzhitskaya & Gogina, 2017). To circumvent this, the federal government issues an increased fund for wastewater treatment and limits the total phosphorus effluent discharge from 1.5 mg/L in critical areas (Pierre *et al.*, 2021).

Fecal Coliform

Fecal coliform (FC) is a group of bacteria that is not pathogenic, but rather an indicator of waterborne pathogenic microorganisms. They primarily live in the intestinal tract of warm-blooded animals (Murphy, 2007) and could enter surface waters through point and nonpoint sources. Under favorable conditions, they could multiply quickly and increase the bacterial concentrations within the water up to 100,000 MPN/L. Furthermore, they could survive for a few hours up to several days in the water yet could live longer up to months in sediments as they adhere to surface solids, which have a higher level of organic carbon (USDA/Agricultural Research Service, 2011). The indication of a higher concentration of FC over 200 MPN/100 mL of water sample increases the risks of developing diseases (e.g., hepatitis, gastroenteritis, typhoid fever, dysentery) from pathogens (Avigliano & Schenone, 2015; Azizullah *et al.*, 2010). As a result, the acceptable value of FC was only 100 MPN/100 mL (Mayuga, 2021). The multiple-tube fermentation technique is used to conduct coliform testing, which estimates the coliform density (MPN). The results provide the best assessment of the efficiency of wastewater treatment (US EPA, 2015).

Overall, the cruciality of wastewater is at risk when it exceeds the standard values set by government agencies on water resource management. It influences other physico-chemical properties of water, resulting in dramatic fluctuations of pollutants. It alters biological activities of organisms in aquatic systems and the rates of chemical processes on various compounds. The analysis of water parameters gives insights into the effectiveness of wastewater treatment, thus, helps in the employment of better techniques utilizing bioremediation agents and/or facilitating wastewater treatment.

CHAPTER III

RESEARCH METHODOLOGY

This chapter systematically explains the methodologies used in the conduct of the study. Procedures for the collection, presentation, and analysis of pertinent data were presented henceforth in order to address the research statements and objectives. Justifications for the research design, research instruments, data collection techniques, data presentation techniques, and analytical methods used were also stated.

Research Design

The researchers used the Posttest-only Control Group Design under True Experimental Research Design. Subjects are randomly assigned to either a control group that will not be exposed to any intervention or an experimental group that will be exposed to treatment. The outcome of interest is measured after the intervention to ascertain its effect on the subject. This research design is chosen over the Pretest-Posttest Research Design due to temporal and financial constraints. Nevertheless, the Posttest-only Control Group Design is deemed appropriate since both treatment and control groups are equivalent at baseline. Moreover, control groups regulate external factors, making it a reliable metric in assessing the potentials of *Chlorella vulgaris* in wastewater treatment.

Subject of the Study

The present study focuses on the wastewater bioremediation potentials of *Chlorella vulgaris*, a species of microscopic green algae under the Division Chlorophyta. They are characterized by their spherical, subspherical, or ellipsoid shape, size ranging from 2-10 μm, nonmotility, and single cup-shaped chloroplast with or without visible pyrenoids. Their chloroplasts contain the photosynthetic pigments chlorophyll-a and -b. They either appear as single cells or in colonies such as in the figures presented below. *C. vulgaris* reproduces by means of production of asexual autospores. These microalgae species have been commonly used in wastewater treatment due to their high growth rates and remarkable nutrient uptake capabilities (Yu *et al.*, 2019).

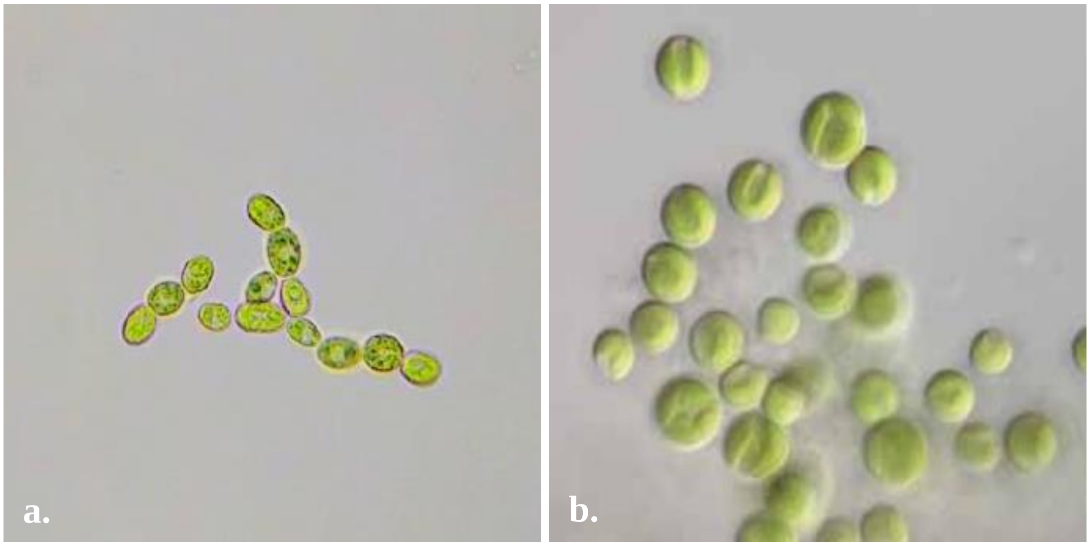


Figure 2. Microscopic image of *Chlorella vulgaris*. (a) Microscopic observation by the researchers (1000x); (b) Reference image from Serediak and Huynh (2011).

Research Instrument

The researchers employed both qualitative and quantitative observation throughout the span of the study. The former was employed in inspecting the microalgal assemblage present in the sampling site. The latter was extensively used to systematically measure the efficiency of *Chlorella vulgaris* in wastewater treatment, which is the prime focus of the study. Quantitative observation allowed the researchers to collect scientifically-sound data through the monitoring of set parameters.

Research Procedure

A five-stage procedure was conducted for the experimental setup of the study. The first part includes the cultivation and identification of native microalgal species from the sampling site. It is shortly followed by the isolation trials (i.e., single cell isolation and serial dilution method) and acquisition of pure *Chlorella vulgaris*. The third stage tackles the setup of the photobioreactor, sampling of wastewaters, and microalgal treatment induction. Growth of *C. vulgaris* was then quantified spectrophotometrically using the trichromatic method of chlorophyll determination. Finally, analytical methods for the assessment of wastewater quality parameters: pH, electrical conductivity, total dissolved solids, total suspended solids, dissolved oxygen, chemical oxygen demand, nitrate, ammonia, phosphate, and fecal coliforms, are aptly presented.

Cultivation and Identification of Microalgal Assemblage

For the initial microalgae cultivation, the researchers used the protocol of Omoni and Abu (2014) as the baseline of the experiments. One gallon of sample was collected at 11:00 am in San Diego River, Nagcarlan, Laguna, Philippines (14°08'10.9"N 121°24'58.3"E) using grab sampling method. The researchers moved toward the midstream and faced upstream to collect the sample. The sampling bottle was fully submerged to a depth of ~0.2 meters below the surface, loosely capped, and placed in a cool box prior to transport to the laboratory. The sample name, date, time, and other notable observations about the site were recorded. This would serve as the growth medium.

A synthetic medium was prepared by mixing specific quantities (mg/L) of nutrients in 1 L distilled water: potassium nitrate (0.132), sodium silicate (0.066), monosodium phosphate (0.03), and ethylene diamine tetraacetic acid (0.066). The outcome solution was calibrated to pH 7.3 and was autoclaved at 121°C for 15 minutes. After media sterilization, the synthetic medium was allowed to cool inside the laminar flow hood to minimize contamination while waiting for the growth medium (river water). Microalgal growth was initiated by introducing 80:20 (v/v) of river water and synthetic medium in 250 mL Erlenmeyer flasks. Afterward, the culture bottles were incubated for 14 days under natural sunlight, ensuring they are not directly hit by the sun's rays, since excessive lighting can have an inhibitory effect on microalgae (Raqiba & Sibi, 2019). The flasks were shaken thrice daily at irregular intervals to enhance the growth by preventing sedimentation of the microalgae, avoid thermal stratification, ensure the equal exposure of cells to light and nutrients, as well as to improve gas exchange between the culture medium and air. A total of three samplings, media formulation, and 14-day proliferation were performed on separate sampling dates. Weather and atmospheric conditions on these dates were also retrieved from https://weatherspark.com/ © 2022 by Cedar Lake Ventures, Inc.

After each two-week cultivation period, the samples were transferred to the laboratory for determination of native microalgae species present. The species were identified morphologically using the algae identification guides by Janse van Vuuren *et al*. (2006) and Serediak and Huynh (2011). Furthermore, they were counterchecked using the Freshwater Algae Identification Guide © Manaaki Whenua – Landcare Research. Comparisons were drawn from the microscopy of samples from the different sampling dates in correlation with atmospheric data in Nagcarlan, Laguna during those time periods.

Isolation Trials and Acquisition of Pure Chlorella vulgaris

The researchers performed two techniques with the aim of obtaining a pure culture of *Chlorella*: single-cell isolation and serial dilution method. For single-cell isolation, the researchers first prepared a CHU-10 medium in the form of stock solutions following the chemical composition of HiMedia Laboratories (Table 1), then adjusting the pH to 6.4. A physiological saline solution was also prepared by dissolving 8.5 g of NaCl in distilled water. Both solutions were autoclaved for 15 minutes at 121°C. The formulated CHU-10 medium was stored in the refrigerator for at least 24 hours. Following microscopy, aliquots of the cultures were sucked out using a pipette and transferred to a watch glass containing 1 mL of physiological saline. Additional drops of physiological saline were put in the glass as needed to separate the cells. Using a different pipette, the prospective isolated *Chlorella* cells were sucked out, transferred to a small culture vessel, and introduced to the CHU-10 medium.

After the iterated method yielded undesirable results, i.e., culture contaminated by other microalgae species, the researchers performed serial dilution with the hopes of successful isolation. Aliquots were transferred from the culture flasks into test tubes containing media (1:10). A total of 5 dilutions were performed, all of which were subjected to microscopy and identification. However, the cultures have shown the presence of similar-looking microalgae (*Chlorococcum* species), making the isolation more challenging. With this, the researchers adopted a procedure done by similar studies (Lekshmi *et al.,* 2015; Zhou *et al.,* 2014) where pure microalgae species are procured to yield results befitting the aim and purpose of the study. Pure live *Chlorella vulgaris* was procured from Juan Algae (Algacon Aquafeeds Manufacturing), in the form of a concentrated algal paste containing 3.735 x 10^8 cells per mL. The paste was stored in the refrigerator under chilled condition of 5°C until further use.

Table 1. The Components of Chu's Medium Number 10.

Chemicals*	**Stock Solution (mg/L)**
Calcium nitrate ($Ca(NO_3)_2$)	40.0
Magnesium sulphate ($MgSO_4$)	25.0
Dipotassium hydrogen phosphate (K_2HPO_4)	5.0
Sodium carbonate (Na_2CO_3)	20.0
Sodium silicate (Na_2SiO_3)	25.0
Iron (II) chloride ($FeCl_2$)	8.0

Wastewater Sampling, Photobioreactor Setup, and Treatment Induction

The researchers collected wastewaters from Nagcarlan Public Market, a piggery wastewater lagoon, and effluent from a meat processing plant wastewater treatment facility, categorized as municipal, agricultural, and industrial wastewaters, respectively (Appendix C). The wastewater samples were stored in an ice box and transferred to the laboratory where they were filtered using a fine mesh net to discard solids, scums, and visible biota (e.g., worms). Simultaneously, 100 g of *C. vulgaris* paste was diluted to 10 L of fresh water, filtered using a fine mesh net, and activated by putting aeration for 15 minutes.

A photobioreactor is a cultivation system for growing microalgae using artificial light sources to facilitate photosynthesis. The researchers set up a photobioreactor in a room by placing a 40-watt fluorescent light with 2,600 luminance flux and a color temperature of 6,200 K (Figure 3). Silicone air hoses were fixed to a high-power aerator (Hailea$^{®}$ Model ACO-9610) with a pressure of 0.015 MPa (2.18 psi), and each terminal hose were inserted inside the bottles via a small puncture on the container shoulder. As per manual instruction, the aerator was elevated to level with the height of the containers.

For the experimental setup, 1000 mL of each filtered wastewater type were transferred to the 4 L culture bottles (three setups per wastewater category). Two out of the three bottles per setup were inoculated with 1000 mL activated *Chlorella vulgaris* cells, constituting a 50:50 (v/v) ratio of wastewater to microalgae. One culture bottle per wastewater type served as the control, i.e., with no treatment. The samples were placed in the photobioreactor for 6 days.

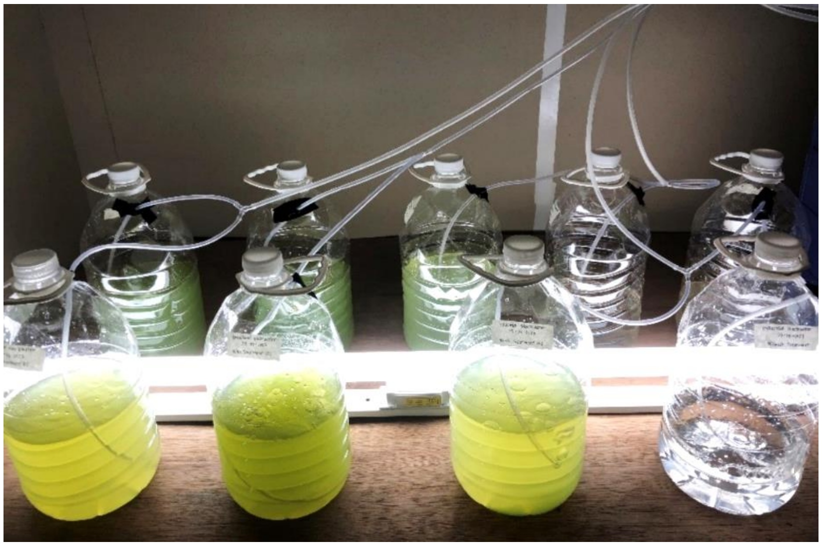


Figure 3. Experimental setup in the photobioreactor.

Spectrophotometric Determination of Chlorophyll a and b Concentration

To estimate microalgal biomass, the concentration of photosynthetic pigments is often quantified (Picazo *et al.*, 2013). Aliquots were transferred to small vessels, wrapped with aluminum foil, and transferred to the laboratory. The researchers employed the spectrophotometric determination of chlorophyll as stipulated in the Standard Methods for the Examination of Water and Wastewater (10200 H).

Photosynthetic pigments were first extracted under subdued light in order to avoid degradation. The researchers transferred 10 mL aliquots to centrifuge tubes and subjected them to a vortex mixer for 1 minute each so as to achieve a consistent and complete pigment extraction. The samples were then concentrated through centrifugation at 500 rpm for 20 minutes (Allegra X-30R). After discarding the supernatant, 10 mL of 90% aqueous acetone solution was added to the centrifuge tubes. Each tube was vortexed for 1 minute and were clarified by centrifuging at 500 g (relative centrifugal force, RCF) for 20 minutes.

After pigment extraction, the researchers performed the trichromatic method for determination of chlorophylls a and b. Sample extracts were transferred to quartz cuvettes, and the optical density was measured at 750, 664, 647, and 630 nm using a spectrophotometer (Jenway Model 3000). OD readings at 750 nm were subtracted from each of the pigment OD values before substituting them to the equations below. OD750 serve as the correction for turbidity as this wavelength is significantly beyond the range of chlorophyll contents, thus, minimizing error caused by these pigments (Badar *et al*., 2017).

Chlorophyll a and b concentrations were then calculated using the following equations:

$$C_a = 11.85\ (OD664) - 1.54\ (OD647) - 0.08\ (OD630)$$

$$C_b = 21.03\ (OD647) - 5.43\ (OD664) - 2.66\ (OD630)$$

where:

C_a and C_b = chlorophyll a and b concentrations in mg/L; and

OD = optical densities at various wavelengths

Furthermore, growth rates of *Chlorella vulgaris* on the three wastewaters were calculated using the equation:

$$\mu = \frac{\ln C_t - ln\ C_0}{t_t - t_0}$$

where:

C_t is the algal density at time t_t

C_0 is the initial algal density

Analytical Methods

Several physicochemical parameters were measured every day throughout the treatment period namely, pH, EC, and TDS. After the 6-day duration, the waters in each flask were divided into separate smaller vessels (depending upon the laboratory requirement), labelled, and placed in ice boxes (<8°C) until analysis. Sample vessels for FC counts and COD were transported to NASAT Labs in Cabuyao, Laguna. Concurrently, the remaining samples were transported to the Chemistry Laboratory of DENR-ERDB, where the researchers tested for TSS, DO, nitrate, ammonia-N, and phosphate of the samples.

1. In situ (pH, Electrical Conductivity, Temperature)

After calibration of the pH meter (Bante Model 920), the researchers measured the pH values of aliquots from the photobioreactor setup. Electrical conductivity measurements were also done daily using a handheld meter. Temperature of all samples were monitored everyday so as to assure that they conform with the optimal range of 25-28°C (Ma *et al*., 2015). However, temperature is not included in the results and discussion section as there was no observed deviation from the optimal range.

2. Solids (Total Dissolved Solids, Total Suspended Solids)

Total suspended solids in mg/L were quantified daily using a handheld meter. Conversely, due to sample size difficulties and complex procedures, total suspended solids were only measured after the six-day treatment induction. TSS in mg/L were determined using a gravimetric and drying test method (EPA 160.2). The researchers prepared and labeled 10 petri plates, one for each sample plus the field blank.

Afterward, Whatman™ membrane filters were individually weighed using a pre-calibrated analytical balance. The weight of each filter was recorded in grams. Using forceps, the filters were then carefully transferred onto the petri plates.

The filtration apparatus was set up, wherewith filters were inserted and a vacuum was applied. The filter was wet with a modest amount of distilled water in order for it to seat. Afterward, three 20 mL volumes of field blank, allowing full drainage between each washing. Suction was continued for three minutes after filtration. The filter was transferred to the petri plate where it was initially placed. After the field blank, 400 mL aliquots of each wastewater sample were subsequently filtered using the apparatus. Each filter was carefully transferred on their assigned petri plates every after complete filtration. Afterward, the laboratory in-charge and personnel transferred the filters to a baking sheet and placed it into an oven set to 104 ± 1ºC and dried for not less than an hour. The filters were removed from the oven and were transferred to a desiccator to cool at room temperature. Sample filters were then weighed and the Oven Dry Weight (ODW) in grams were recorded. TSS of all 9 samples were calculated using the equation:

$$TSS = \frac{A - B}{V}\ x\ 1000000$$

where:

A = weight of the filter + dried residue in grams;

B = weight of the filter (tare weight) in grams; and

V = volume of the sample filtered in mL

3. Dissolved Oxygen and Chemical Oxygen Demand

The researchers placed aliquots of the samples on separate vessels depending upon the requirement of the laboratory, as prescribed by the reference methods. The dissolved oxygen values present in the samples were measured by the laboratory in-charge and personnel of the Chemistry Laboratory of DENR-ERDB using membrane electrode method. After transporting the samples to NASAT Labs, the chemical oxygen demand (COD) values were measured by the laboratory analysts using a closed reflux and colorimetric method, following the methods in SMEWW 5220-D.

4. Nutrients (Nitrate, Ammonia-N, Phosphate)

In testing for nitrate present in the experimental and control wastewater samples, the researchers transferred 20 mL of each into test tubes (n = 9). One level spoonful (spoon included in the kit) of Nitratest Powder and one Nitratest tablet were added to each tube. The tubes were tightly capped and subjected to a vortex mixer for 1 minute each. Afterward, the samples were allowed to stand for 1 minute and inverted for 3-4 times to aid in flocculation. To ensure complete settlement, the tubes were allowed to stand for 3 more minutes. After the settlement period, the tubes were uncapped, and the mouth of the tubes were gently wiped off with a clean tissue. The researchers decanted the clear solutions to different test tubes, filling up to the 10 mL mark. One Nitricol tablet was added per tube, crushed, and mixed thoroughly until dissolved. For full color development, the tubes were allowed to stand for 10 minutes. Afterward, the researchers filtered the samples using a filter paper and a funnel until all liquid components were transferred onto the new test tubes. Phot 23 was selected on the Palintest® Photometer 3000 and the dilution was set to x1. Following the usual photometer instructions, the field blank was transferred to a tube and is used to blank the photometer. Afterward, the nitrate readings (mg/L) of each sample were performed.

Ammoniacal nitrogen (Ammonia-N) testing was performed by first filling the test tubes with the samples up to the 10 mL mark (n = 9). One Ammonia No. 1 tablet and one Ammonia No. 2 tablet were added to each tube, crushed, and mixed until dissolved. The tubes were allowed to stand for 10 minutes to allow color development. Phot 4 was selected on the photometer and the dilution was set to x1. The usual photometer readings were done in the usual manner (see previous paragraph).

Phosphate levels in the wastewater samples (n = 9) were quantified using the photometer method which is based on the vanadomolybdate method. 10 mL aliquots of the samples were transferred to test tubes. One Phosphate HR tablet was added, crushed, and mixed into each test tube. Similarly, the samples were allowed to stand for 10 minutes for color development. Afterward, the researchers filtered each sample using a Whatman™ filter device with 0.2 μm pore size. Phot 29 was selected on the photometer and the usual readings were performed.

5. Fecal Coliform

150 mL aliquots of the wastewater samples were transferred to the pre-sterilized glass vessels, cotton-plugged, secured, and placed in an ice box prior to transport to NASAT Labs in Cabuyao, Laguna. The laboratory performed thermotolerant (fecal) coliform procedure (multiple tube fermentation technique), as stipulated in SMEWW 9221-E.

Table 2. Summary of Analysis and Test Methods Employed.

Analysis	Unit	Test Method	Reference Method
Temperature	°C	In situ	-
pH	-	In situ	-
Electrical conductivity	µS/cm	In situ	-
Total dissolved solids	mg/L	In situ	-
Total suspended solids	mg/L	Gravimetric, drying	EPA 160.2
Dissolved oxygen	mg/L	Membrane electrode	-
Chemical oxygen demand	mg/L	Closed reflux, colorimetric	SMEWW 5220-D
Nitrate - N	mg/L	NED hydrochloride	SM 4500 NO^{-}_3 Method E
Ammonia - N	mg/L	Nessler	SM 4500 NH_3 Method C
Phosphate	mg/L	Ascorbic acid, colorimetric	EPA 365.1
Fecal coliform	MPN	MTFT	SMEWW 9221-E

Data Analysis

To investigate the relationship between *Chlorella vulgaris* (independent variable) and the different physico-chemical parameters (dependent variable), various statistical tools were used. Prior to correlational analysis, the Doornik-Hansen Test for Multivariate Normality using an r-programming software was used in order to test for assumptions. Since not all assumptions were met, the non-parametric counterpart of Pearson's *r*, the Spearman's Rank-Order Correlation (Spearman's ρ) is used to assess the strength and direction of the monotonic relationship between total chlorophyll and the daily parameters. For parameters that were only measured post-intervention, the values were deliberately compared with standard values from existing literature.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter is concerned with the presentation, analysis and interpretation of data gathered from the methodologies employed to assess the wastewater bioremediation capabilities of *Chlorella vulgaris*.

Comparison of Microalgal Assemblage on Different Sampling Dates

Table 3 presents the different species of microalgae observed in the sampling site at three different sampling times. In the first sampling, the cultures were dominated by green microalgae, specifically by *Chlorella* and *Chlorococcum* species. These two species have similar morphological features which made their characterization difficult. There are no diatoms (Bacillariophyceae) observed on all cultures from the first sampling. Consequently, the cultures from the second sampling were dominated by diatoms, with sparse distribution of green microalgae *Scenedesmus*, *Ankistrodesmus*, *Chlorella*, and the yellow-green microalgae *Closterium*. The cultures from the third sampling showed an abundance of *Chlorella* with a dispersed presence of diatoms. Although microalgae are unique to ecological sites of isolation (Lloyd *et al*., 2021), previous surveys of microalgal genera showed the high domination potentials of *Chlorella* (Lloyd *et al*., 2021; Palmer, 1974 as cited by Abdel-Raouf, 2012; Severes, 2018). However, the varying results may be attributable to the local geographical, climatic, and ecological conditions (Ramachandra *et al*., 2011). Nevertheless, *Chlorella* species was proven to be native on the sampling site.

Table 3. Microalgal Assemblage Observed in San Diego River, Nagcarlan on Three Sampling Dates.

Taxa	Sampling 1	Sampling 2	Sampling 3
Chlorophyta [a]			
Trebuxiophyceae			
Chlorella sp.	+	+	+
Chlorophyceae			
Chlorococcum sp.	+	-	-
Ankistrodesmus sp.	-	+	-
Scenedesmus sp.	+	+	-
Ulvophyceae			
Ulothrix sp.	-	+	-
Charophyta [b]			
Zygnematophyceae			
Closterium sp.	-	+	-
Xanthophyta [c]			
Xanthophyceae			
Tribonema sp.	-	+	-
Ochrophyta			
Bacillariophyceae [d]			
Nitzschia sp.	-	+	-
Synedra sp.	-	+	+
Navicula sp.	-	+	+

[a] green microalgae with a variety of forms
[b] green microalgae that resemble land plants
[c] yellow-green microalgae
[d] diatoms, brown microalgae

Table 4 presents the weather and atmospheric conditions in Nagcarlan on all three sampling dates. The microalgal taxa observed is attributable to the varying weather and atmospheric conditions on these dates. Temperature posed a significant effect on the microalgal assemblage. The average temperature in Nagcarlan during the first sampling is 28°C, with an average surface water temperature of 28-29°C. The microalgal cultures from this sampling time had shown dominance of green microalgae, with no presence of diatoms. In elevated temperatures, Mei *et al*. (2022) noted a dominance of planktonic microalgae over the denser species (e.g., diatoms) due to the reduction of light intensity at the deeper water column. Green microalgae such as *Chlorella* are competitive species and have the ability to dominate especially in favorable conditions (Sugoro *et al*., 2022).

Furthermore, the relatively high wind speed and mixing during the second and third sampling dates (4.3 m/s and 4.7 m/s, respectively) promoted the dominance of diatoms. Severe wave action and surface agitation impede the microalgal growth (Severes *et al*., 2018). However, diatoms, which are relatively heavier and tend to sink down the water column, prefer mixing since it gets them in the photic zone, dominating the algal assemblage (Dell'Aquila *et al*., 2017).

All in all, the differential weather and atmospheric conditions on all three sampling dates are attributable to the varying microalgal assemblage observed by the researchers. Fluctuations in temperature are one of the major bottlenecks in the mass cultivation of *Chlorella* and other microalgal species (Yuvraj *et al*., 2016). It is therefore important to consider various abiotic factors when sampling for they greatly impact species distribution.

Table 4. Atmospheric Conditions in Nagcarlan, Laguna on the Three Sampling Dates.

Parameters	Sampling 1	Sampling 2	Sampling 3
Ave. temperature	28.3°C	27.5°C	26.3°C
Ave. cloud cover	15% (clear)	26% (mostly clear)	47% (partly cloudy)
Ave. precipitation	5%	5.2%	5.8%
Ave. wind speed	2.9 m/s	4.3 m/s	4.7 m/s
Ave. surface water temperature	28°C-29°C	27°C-28°C	26°C-27°C

Source: https://weatherspark.com/ © 2022 by Cedar Lake Ventures, Inc.

Spectrophotometric Determination of Chlorophyll a and b Concentrations

Figure 4 shows the growth of *Chlorella vulgaris* on the three wastewater types across the treatment period. Agricultural wastewater showed the highest total chlorophyll on the first day of treatment induction (0.7927 mg/L), followed by municipal wastewater (0.1923 mg/L), and industrial wastewater (0.18946 mg/L). Liquid wastes from agricultural sources typically have high nutrient concentrations which are favorable for microalgae (Jia & Yuan, 2016). However, *C. vulgaris* showed the highest growth rate on the municipal wastewater (0.2685), followed by industrial wastewater (0.1809), and agricultural wastewater (0.1527), respectively. It is therefore conjectural that even though *Chlorella vulgaris* in the agricultural wastewater were able to efficiently utilize the nutrients on the very first day of treatment, the growth rate on the municipal wastewater was more consistent and sustainable. Lakaniemi *et al*. (2012) showed a similar trend in their study, where specific growth rates of *C. vulgaris* in various photobioreactor setups are significantly higher on the initial days.

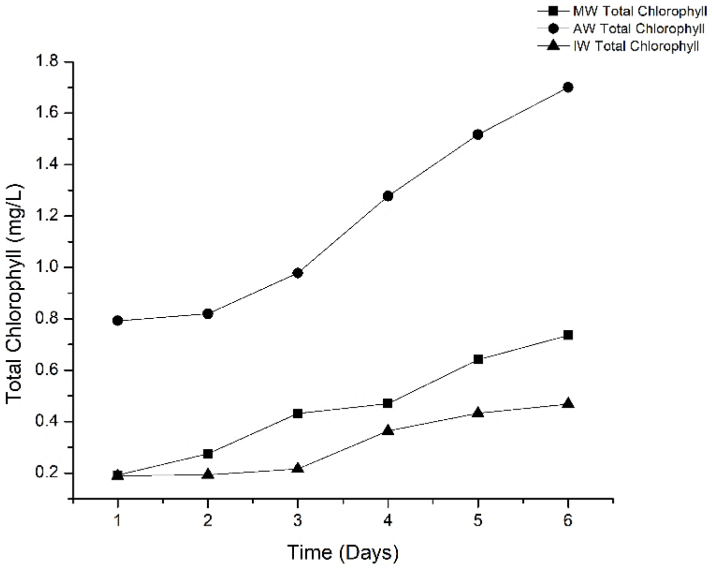


Figure 4. Growth curve of *Chlorella vulgaris* in the three wastewater samples.

Analytical Parameters

1. In situ (pH, Electrical Conductivity, and Total Dissolved Solids)

Figure 5a shows the pH fluctuation of the wastewater samples along the 6-day treatment period. Treated municipal wastewater showed the lowest mean pH level on the first day of treatment (7.683) followed by agricultural wastewater (7.733) and industrial wastewater (8.2035). All samples with treatment displayed a daily increase in pH, ultimately rising up to 7.921, 7.9505, and 8.4535 for municipal, agricultural, and industrial wastewaters, respectively. *Chlorella vulgaris* grows best at an initial pH of 6.5 to 7.0, or those close to neutrality since relatively little energy is required to maintain homeostasis (Ihnken *et al*., 2014; Wang *et al*., 2015). Regardless, Goldman *et al*. (1982), as cited in Ihnken *et al*. (2014), stated that *Chlorella* can grow efficiently up to a pH of 10.5. This increasing pH is due to the photosynthetic CO_2 assimilation of the microalgae (Ramanan *et al*., 2016). Consequently, since there is no *C. vulgaris* introduced in the control samples, the pH values were shown to be stable or have dropped significantly, i.e., no trend.

Figure 5b shows the electrical conductivity (EC) of the treated and untreated wastewater samples along the treatment period. On the first day, treated samples had a mean value of 531 μS/cm, 541 μS/cm, and 2120 μS/cm for municipal, agricultural, and industrial wastewaters, respectively. The high initial conductivity in the industrial wastewater sample is attributable to the chemicals used in treatment facilities (Jia & Yuan, 2016). A decline in EC levels on the three wastewaters occurred on the second day, and eventually the levels continued to decline until it dropped to 449 μS/cm, 399 μS/cm, and 1760 μS/cm on the final day of treatment. Consequently, a study by Cheng *et al.* (2021) emphasized the capacity of *Chlorella vulgaris* to collect metals naturally, which may cause a decrease in electrical conductivity of water. The levels of electrical conductivity in untreated wastewaters were higher due to the presence of impurities, pH, cation exchange capacity, organic carbon, and organic matter. More so, even small amounts of contaminants can lead to higher conductivity levels in wastewater (Guadie *et al*., 2021). The decrease in levels of electrical conductivity in the wastewater samples by *Chlorella vulgaris* shows a positive effect after the treatment period.

As shown in Figure 5c, TDS for municipal, agricultural, and industrial wastewaters treated with *Chlorella vulgaris* showed a decreasing trend. On the first day, treated municipal wastewater had a value of 272 mg/L, but on the sixth day, the value had been reduced to 246 mg/L. Additionally, treated agricultural wastewater was 254.5 mg/L initially, but dropped to 223 mg/L. Lastly, the value of the industrial wastewater after treatment decreased from 1124.24 mg/L to 947.50 mg/L on the last day. These results reflect with the study of Vinodhini and Soundhari (2019), and Singh *et al*. (2021), where they concluded that uptake of dissolved nutrients by microalgae for their growth and development may cause a decrease in TDS. On the contrary, among the control groups, industrial wastewater contained the highest TDS concentration on the final day with a value of 1689 mg/L. Industrial wastewaters are prone to be highly saline due to the presence of high concentrations of dissolved minerals. Binu (2020) mentioned that the presence of hazardous ions and higher concentrations of potassium, chloride, and sodium contributes to high levels of TDS.

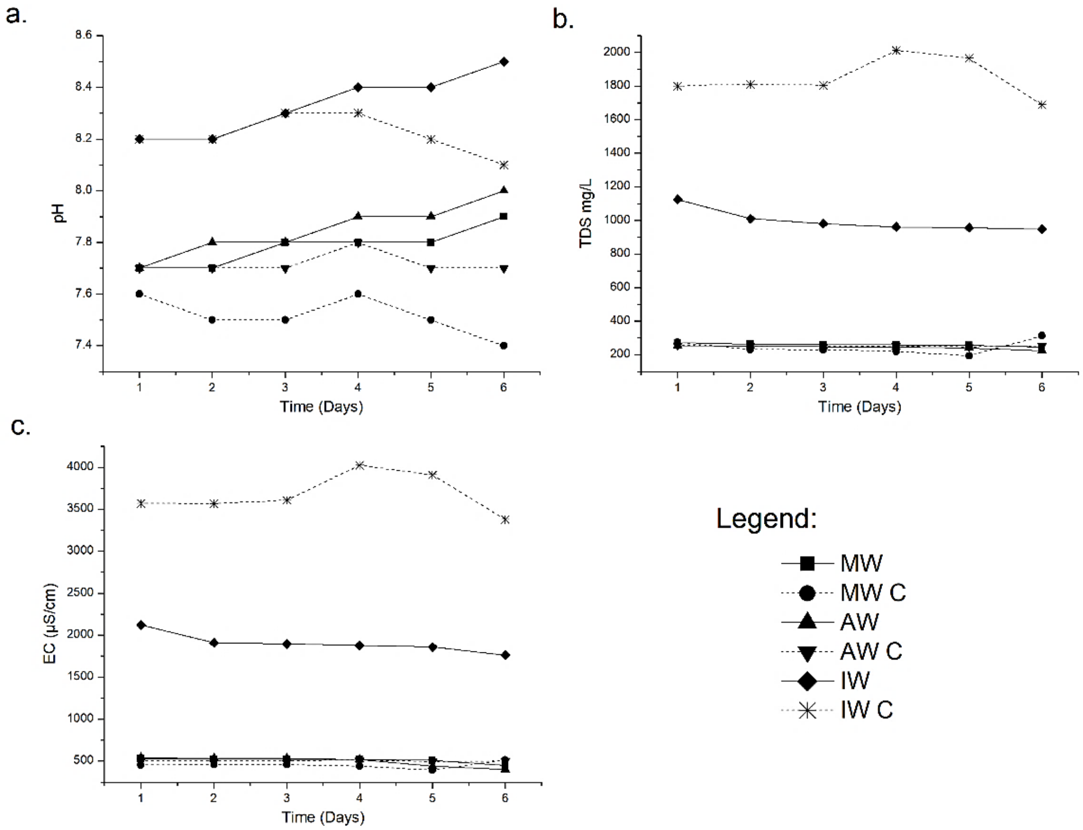


Figure 5. Daily fluctuations of (a) pH, (b) total dissolved solids, and (c) electrical conductivity in the wastewater samples.

Table 5 shows the results of the Doornik-Hansen Test for Multivariate Normality. The distributions of pH and total chlorophyll, with a *DH* statistic of 9.838924 (*p-value* = 0.04323018) is not multivariate normal. The same is true for EC (µS/cm) and total chlorophyll, with a *DH* value of 16.88872 (*p-value* = 0.002031581), and TDS (mg/L) and total chlorophyll, with a *DH* of 17.56913 (*p-value* = 0.001497796). Therefore, the non-parametric equivalent of Pearson *r*, which is the Spearman correlation (ρ), was employed to test the significant relationships among the variables.

Table 5. Test for Multivariate Normality Between Total Chlorophyll and In Situ Parameters Using the Doornik-Hansen Test.

	DH	**p-value**
pH and Total Chlorophyll	9.838924	0.04323018
EC and Total Chlorophyll	16.88872	0.002031581
TDS and Total Chlorophyll	17.56913	0.001497796

Note: p – value $\leq$ 0.05 is significant

Table 6 shows that there is a significant, strong negative monotonic relationship between EC and Total Chlorophyll (*Spearman's rho* = -0.7093, *p – value* = 0.00098) and TDS and Total Chlorophyll (*Spearman's rho* = -0.9236, *p – value* = <0.00001) in the municipal, agricultural, and industrial wastewaters. This means that as the concentration of total chlorophyll increases, the EC and the TDS decreases. However, there is no significant monotonic relationship between pH and Total Chlorophyll (mg/L) (*p – value* = 0.56397). This means that as the concentration of total chlorophyll increases, the pH concentration sometimes decreases and sometimes increases—the values fluctuate. Overall, this data suggests that there is sufficient statistical evidence to reject the hypothesis of this research for EC and Total Chlorophyll, and TDS and Total Chlorophyll, since there is an observed significant monotonic relationship between the growth of *Chlorella vulgaris* (expressed as total chlorophyll concentration) and the iterated physico-chemical parameters in municipal, agricultural, and industrial wastewaters. However, there is no sufficient statistical evidence to reject the null hypothesis on the monotonic relationship between pH and Total chlorophyll.

Table 6. Monotonic Relationship Between Total Chlorophyll and In Situ Parameters on All Treated Wastewaters.

	Spearman's rho	**p-value**
pH and Chlorophyll	-0.1457	0.56397
EC and Chlorophyll	-0.7093	0.00098***
TDS and Chlorophyll	-0.9236	<0.00001***

Note: * p < .05, ** p < .01, *** p < .001 is significant. The strength of r is interpreted as follows: (0, 0.2) – very weak; (0.2, 0.4) – weak; (0.4, 0.6) – moderate; (0.6, 0.8) – strong; (0.8 ,1) – very strong.

2. Total Suspended Solids

Figure 6 shows the varying concentrations of total suspended solids (TSS) between the wastewater samples. The treated groups have shown a greater concentration of TSS as opposed to the control groups. Municipal wastewater treated with *Chlorella vulgaris* (MW) displayed a mean TSS of 46 mg/L, while the untreated sample (MWC) showed a concentration of 13.25 mg/L. Similarly, treated agricultural wastewater (AW) displayed a TSS of 56.5 mg/L, compared to the 16 mg/L of the untreated sample (AW C). Industrial wastewater also showed a parallel trend—16.125 mg/L and -3 mg/L for the treated (IW) and untreated (IW C) samples, respectively. The observed increase in total suspended solids is attributable to the high microalgal density in the treated samples (Hill, 2020). Moreover, turbidity is also often assumed to be a surrogate for TSS (Fondriest Environmental, Inc., 2019). It is generally true that the higher the TSS, the more particles are expected in suspension, and the higher the level of turbidity. Turbid water could indicate the elevated presence of algae, which could be an indicator of higher photosynthetic activity and effective waste removals (Branigan, 2013).

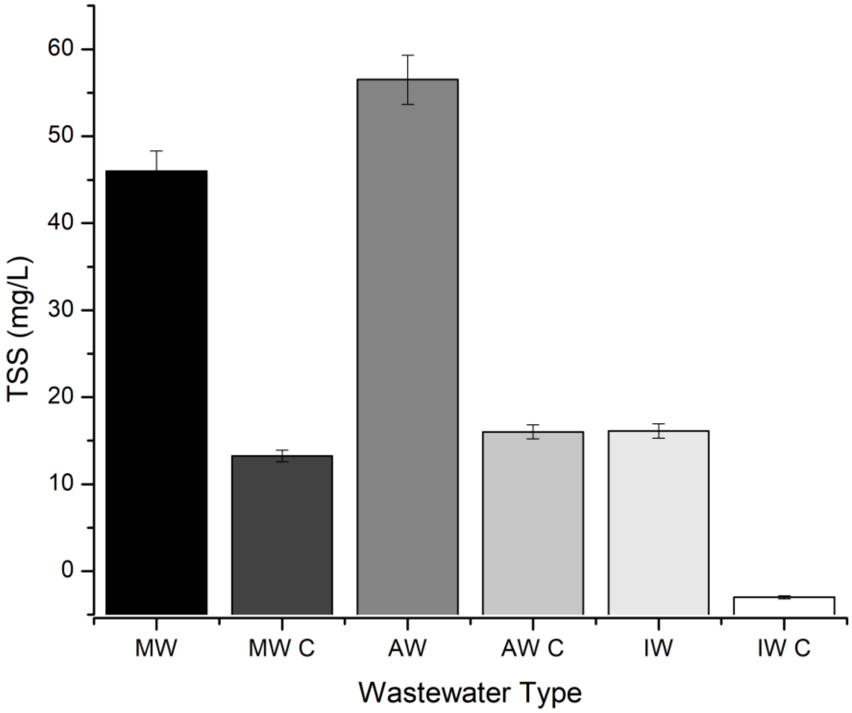


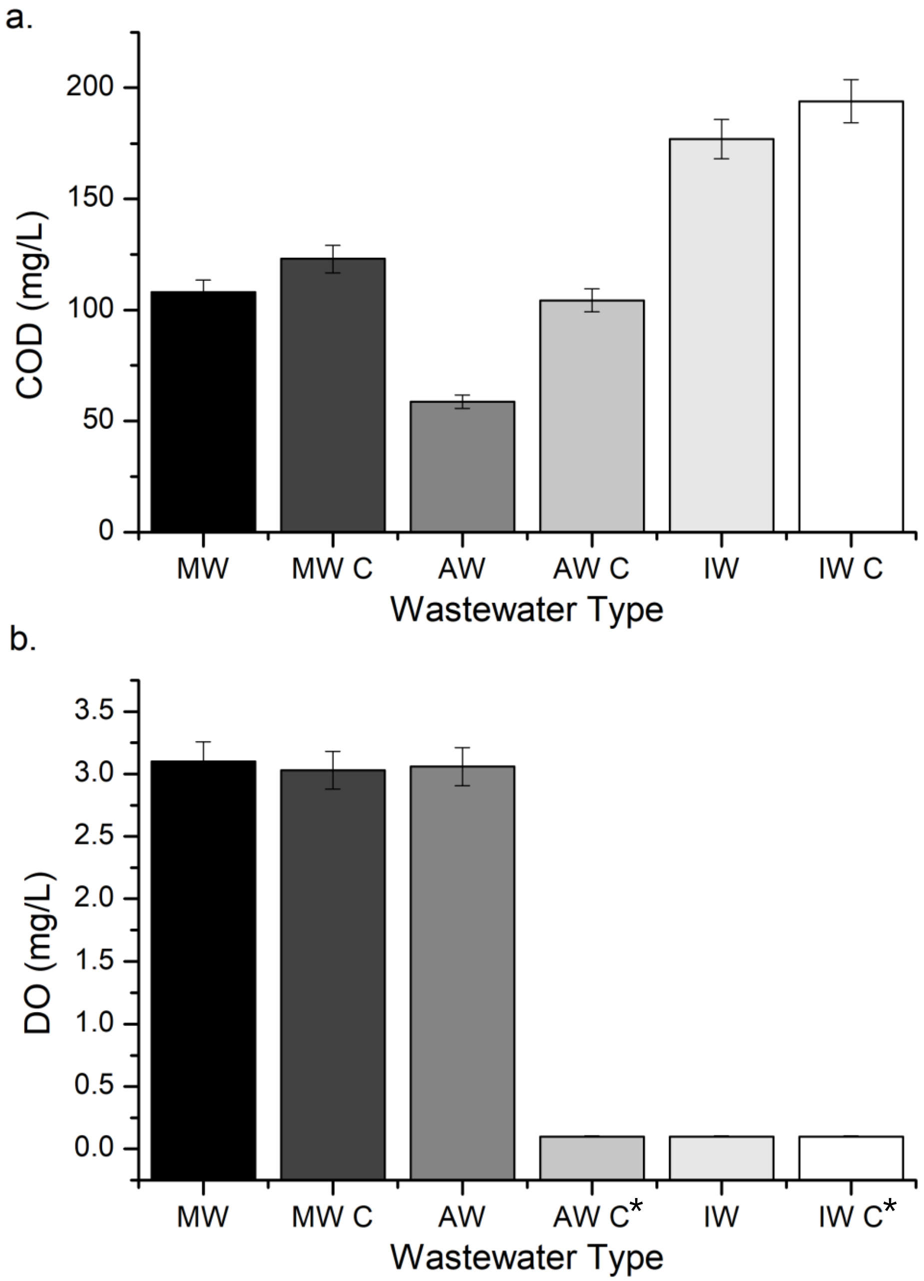
Figure 6. Comparison of total suspended solids on all wastewater samples.

3. Chemical Oxygen Demand and Dissolved Oxygen

Figure 7a shows the different concentrations of chemical oxygen demand (COD) between treated and untreated wastewater samples. The untreated municipal, agricultural, and industrial wastewater samples accounted for high COD concentrations of 122.96 mg/L, 104.34 mg/L, and 139. 89 mg/L, respectively. Among these, the industrial wastewater (IW) had the highest level of COD; this reflects the report of Latiffi *et al.* (2019) that effluent from meat processing facilities contain a significant COD level due to the high accumulation of animal fat, blood, and mucosa. Burns (2021) noted that the high level of COD indicates a greater amount of oxidizable organic material, which had an integral role in the metabolism of microalgae. Lee *et al.* (2022)

reported that the uptake of organic carbon was much faster than inorganic carbon by photosynthesis; this explains the low COD concentration in all treated wastewater samples. The reported results indicate a COD removal efficiency of *C. vulgaris* in the following percentages: 11.76%, 43.74%, and 8.75% for municipal, agricultural, and industrial wastewaters, respectively. However, the study of Rani *et al.* (2021) on the COD removal efficiency of *Chlorella* sp. in the effluent was 25%, 67.2% in domestic wastewater treatment (Abdel-Raouf *et al.*, 2012), and 60-70% in piggery wastewater (Nguyen *et al.*, 2022). These differences in their removal efficiency may vary depending on the concentration of COD and specific type and origin of wastewaters.

Figure 7b shows the different concentrations of dissolved oxygen (DO) between treated and untreated wastewater samples. All the untreated wastewater samples had a report of below detectable limits except for municipal wastewater, which had 3.03 mg/L. The low concentration of DO was directly proportional to the level of COD and salinity in wastewater samples, in which an increased COD level denotes an increased demand for oxygen due to the high concentrations of organic material (Burns, 2021). It stimulates the biological activity of different microorganisms that negatively affect aquatic life (Pierce, 2019). Meanwhile, the increased level of DO denotes a decreased level of salinity in wastewater due to the nutrient uptake of *Chlorella* (Bhuyar *et al.*, 2020). More so, Bhuyar *et al*. (2020) also emphasized that the photosynthetic activity of *C. vulgaris* produces oxygen, resulting in an increased level of dissolved oxygen in treated wastewater samples. The reported results from the dissolved oxygen of treated municipal and agricultural wastewaters were 3.095 mg/L, and 3.06 mg/L, respectively, while industrial wastewater showed values that are below the detectable limits.



Note:

** below detectable limits*

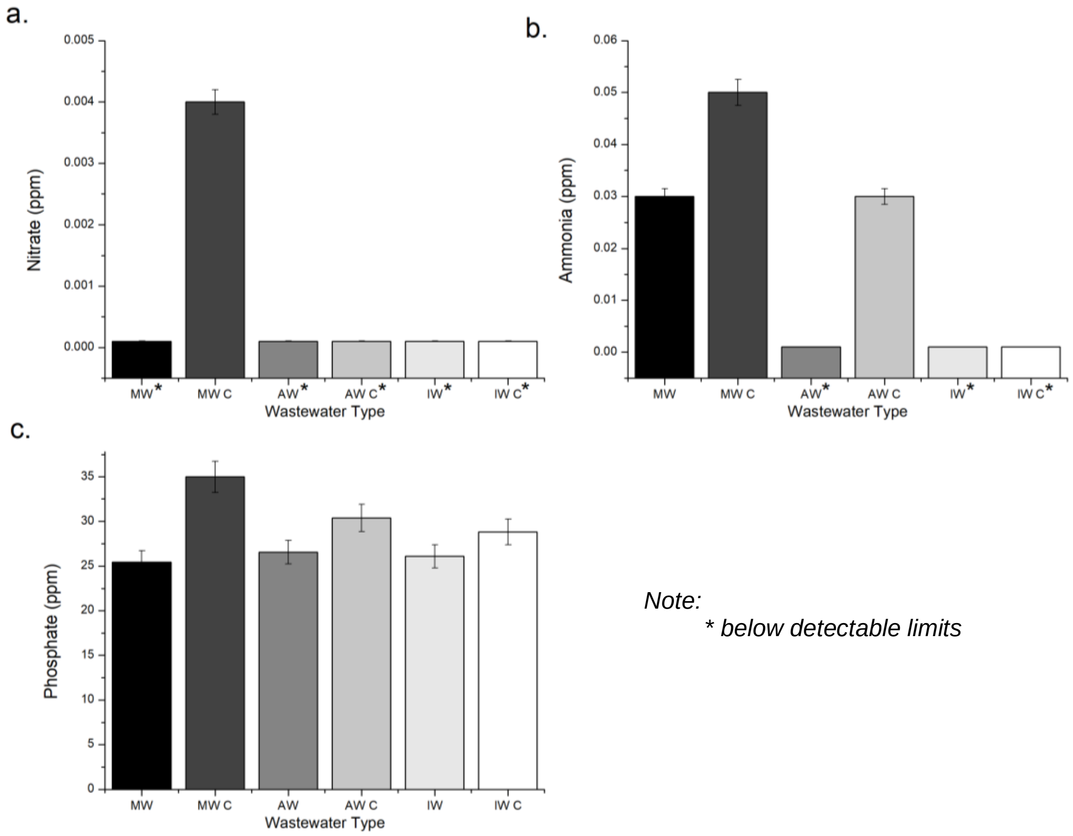
Figure 7. Comparison of (a) chemical oxygen demand, and (b) dissolved oxygen concentrations on all wastewater samples.

4. Nutrients (Nitrate-N, Ammonia-N, Phosphate)

Figure 8a shows the nitrate-nitrogen (N) concentration on different wastewaters both in the treatment and control setups. Levels of N on the treated wastewater samples were reported to be below detectable limits, as well as in the control setup for agricultural wastewater and industrial effluent—while municipal wastewater has a reported value of 0.004 ppm. As compared to the appropriate reference levels of water quality on total N which range from 0.12 to 2.2 ppm; in the effluent of wastewater treatments plants, which can range up to 30 mg/L (U.S. EPA, 2002), the concentrations of nitrate which the researchers measured were significantly lower. This indicates that the introduction of *Chlorella vulgaris* in wastewater resulted in an increased uptake of nitrogen compounds in the form of nitrates and revealed a high removal efficiency rate of almost 100%. Studies revealed that too much level of nitrates in water will cause algae to grow faster than the ecosystem can handle, which may harm the overall water quality (U.S. EPA, 2022). Furthermore, this uptake efficiency is directly proportional to the increase of pH (Bhuyar *et al.*, 2020). Municipal wastewater, particularly the control sample, has the lowest mean pH (7.6) value as compared to agricultural (pH 7.9) and industrial wastewater (pH 8.3), which accounts for the 0.004 ppm of detected nitrate-N level.

Figure 8b shows the ammonium nitrogen (A) concentration on different wastewaters both in the treatment and control setups. Levels of ammonia (NH_4^+) on the treated wastewater samples were reported to be below detectable limits, except in municipal wastewater (MW) which has a reported ammonia concentration of 0.03 ppm. Elevated levels of 0.05 ppm and 0.03 ppm ammonia in raw wastewaters—municipal and agricultural wastewaters, respectively—are attributed to the presence of ammonia-based solutions in household sewages such as human waste, municipal effluent discharges and the excretion of nitrogenous wastes from animals such as in piggery lagoon (Oregon Department of Human Services, 2000; US EPA, 2022). The reported 0.03 ppm ammonia concentration in treated municipal wastewater, still falls under the required environmental limits for ammonia stated by the US, which ranges from 0.25 to 32.5 ppm. However, it is important to note that water quality objectives for ammonia vary from region to region. The undetectable ammonia-N values in industrial effluent may be attributed to the initial treatment of the wastewater by different bacteria and compounds from the wastewater treatment facility. From the stated results, it was found that NH_4-N was almost completely removed by *C. vulgaris* in treated samples; this is because ammonium nitrogen is one of the most energy-efficient nitrogen sources for algal metabolism (Ruan & Giordano, 2017; Wang *et al.*, 2015).

Figure 8c shows the concentration of phosphate on different wastewaters, both in the treatment and control setups. In terms of the control group, municipal wastewater showed the highest level of phosphate (35 ppm), followed by agricultural wastewater (30.4 mg/L ppm) and industrial wastewater (28.8 mg/L ppm). According to Cleary *et al*. (2008), wastewaters typically contain about 1 to 5 mg/L of phosphate concentrations; however, effluent of lower quality, i.e., higher phosphate concentration can be monitored. The reported phosphate level in this study may result from poor agricultural practices, human and animal wastes, leaking septic systems, or polluted discharges from treatment plants where these wastewaters were collected and sampled. The low phosphate assimilation may be attributable to the N/P ratio, where low nitrogen content in wastewater would limit the phosphorus uptake of microalgae. This data explains why only a little portion of phosphates were removed from wastewaters during treatment with *Chlorella vulgaris.* The phosphate removal efficiency of *Chlorella* is reported as follows: 27.29% (from 35 mg/L to 25.45 mg/L), 12.66% (from 30.4 mg/L to 26.55 mg/L), and 9.37% (from 28.8 mg/L to 26.1 mg/L) in municipal, agricultural, and industrial wastewaters, respectively. In addition, the low uptake level of phosphates by *C. vulgaris* in the treatment samples may account for the relatively low rate of chlorophyll a & b concentrations with the following mean values of 0.49626 mg/L, 1.16945 mg/L, and 0.39772 mg/L for MW, AW, and IW, correspondingly, but is approximately enough to sustain microalgal growth.



Note:
** below detectable limits*

Figure 8. Comparison of (a) nitrate, (b) ammonia, and (c) phosphate concentrations.

5. Fecal Coliform

Figure 9 shows the variations in fecal coliform counts of all wastewater samples after the treatment period. All treated samples have shown a low number of FC as opposed to the control groups. Untreated samples displayed $3.5x10^6$, $5.4x10^5$, and $5.4x10^4$ MPN/100 mL for municipal, agricultural, and industrial wastewaters, respectively. The comparatively lower FC count on the industrial wastewater is attributable to the primary treatment the facility performed. Experimental groups showed a lower level of FC: $8.8x10^5$, $2.6x10^5$, and <7.4 MPN/100 mL for municipal, agricultural, and industrial wastewaters, accordingly. This relatively lower FC count in the samples treated with *Chlorella vulgaris* is ascribable to various microalgal characteristics. Ansa *et al*. (2012) stated that as microalgae induce an increase in pH and DO concentration, the environment becomes hostile to FC. Furthermore, light attenuation, starvation, sedimentation, and release of exudates by microalgae inhibit the coliform growth, and ultimately, pathogenic microorganisms (Ravindran *et al*., 2016). FC inhibition efficiency of *C. vulgaris* was highest on industrial wastewater (99.986%), followed by municipal (74.857%), and agricultural (51.85%). Existing literature showed similar percentage removal of fecal coliforms using microalgae (Ahmad *et al*., 2014; Ansa *et al*., 2012; Wollman *et al*., 2019). The lower FC removal efficiency of *C. vulgaris* in agricultural wastewater may be due to the high turbidity in swine effluent that can block light and decrease photosynthetic efficiency despite the notable increase in chlorophyll concentration (Jia & Yuan, 2016). Moreover, the type of microorganisms present and their interactions with *Chlorella vulgaris* may be a factor in the lower FC removal in agricultural wastewater (Amaro *et al*., 2023).

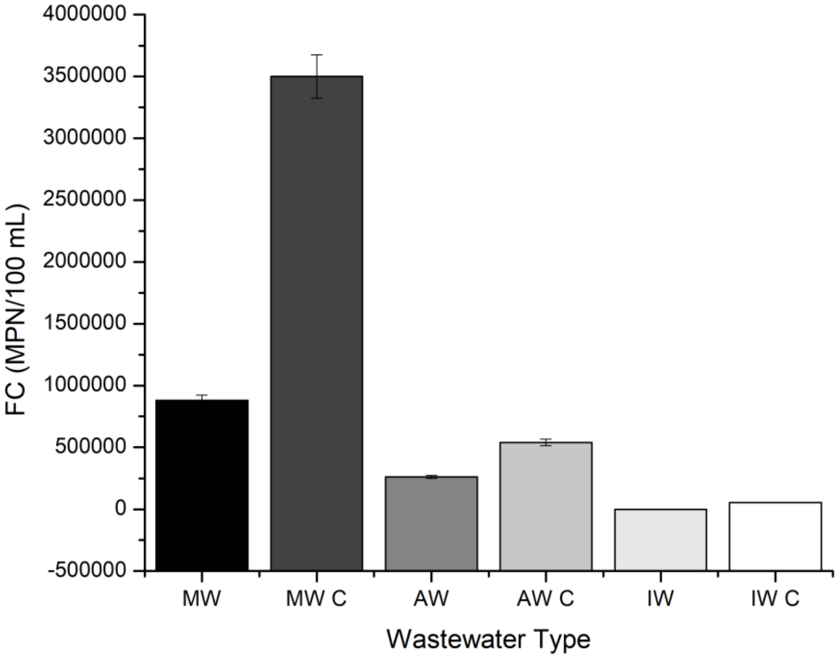


Figure 9. Comparison of fecal coliforms (MPN/100 mL) on all wastewater samples.

CHAPTER V

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

This chapter summarizes the findings from the experiments conducted utilizing the green microalgae *Chlorella vulgaris* for bioremediation of municipal, agricultural, and industrial wastewaters. It also provides the conclusions and recommendations of the study.

Summary

This study was conducted in order to alleviate problems in water scarcity and quality that stems from the poor policy development and implementations on wastewater treatment and discharge. The viability of the process was demonstrated in photobioreactors, and critical parameters were tested and analyzed to instigate its correlation with *Chlorella vulgaris*.

Municipal, agricultural, and industrial wastewaters from Nagcarlan, Laguna, were found to be variable in the ten (10) physico-chemical parameters observed. Industrial wastewater with no treatment showed the highest values for electrical conductivity, total dissolved solids, and chemical oxygen demand, which are attributable to the chemicals and reagents used in the meat processing plant. Raw agricultural wastewater demonstrated the highest total suspended solids, ascribable to the large particles and solids present in the piggery discharge. Untreated municipal wastewater showed the highest concentrations of nutrients and fecal coliforms, followed by agricultural, and industrial liquid wastes, respectively.

During the treatment period, all treated wastewater samples displayed an increase in pH, and a decrease in electrical conductivity and total dissolved solids. Consequently, total suspended solids were relatively higher in experimental groups compared to the control groups, which is attributable to the aggregation of cells trapped by the filter. All treated samples showed a relatively lower chemical oxygen demand and higher dissolved oxygen concentrations as opposed to the untreated ones. Nutrient assessments revealed a successful assimilation and uptake by *Chlorella vulgaris* on all three wastewater types. Fecal coliforms were also significantly lower on the treated samples, owing to the inhibition characteristics of the microalgae. This is indicatory of the parallel die-off of pathogens present in the wastewaters. The findings of the study demonstrate the potentials of *Chlorella vulgaris* in bioremediation of wastewaters of different origins.

Conclusions

The following conclusions were made based on the findings of this study. Using Spearman's Rank-Order Correlation and intensive comparisons with existing literature and set standards, *Chlorella vulgaris* was found to effectively assimilate nutrients and harness them for growth and proliferation, as observed on the daily increase in biomass (expressed as total chlorophyll) on all three liquid waste types. The ability of *C. vulgaris* in light attenuation, starvation, sedimentation, and toxin release account for the observed reduction of wastewater pollutants and inhibition of pathogen-indicator fecal coliforms. Therefore, the data and statistical evidences presented in this study were sufficient to reject the research hypothesis. All in all, *Chlorella vulgaris* was proven to be effective in remediating wastewaters from municipal, agricultural, and industrial wastewaters, which may be critical in policy development and implementation given the contemporary problems of water scarcity and degradation in the Philippines.

Recommendations

The small-scale setup and short-term treatment period carried out by the researchers resulted in insufficient algal growth and biomass production. For that reason, close monitoring of the light intensity, temperature, and pH are of utmost importance since these are factors that greatly influence the microalgal-based wastewater treatment process. Different *Chlorella* strains demonstrate a diverse range of photoperiod regimes and various light intensities. An illumination cycle of 16 h light: 8 h dark at 2,600 flux light intensity was carried out in this experiment, however, it resulted in a relatively low microalgal biomass production as opposed to those present in related literature. Therefore, the researchers recommend that *C. vulgaris* be cultivated at a light intensity of 7,000 luminance flux and light: dark ratio of 24:0 in the photobioreactor to obtain high biomass productivity, as suggested by You *et al.* (2023). More so, observation of various temperature ranges that are most suitable for *Chlorella vulgaris* proliferation and biomass production are advised.

Despite the impressive ability of *Chlorella vulgaris* to simultaneously uptake nutrients and reduce wastewater pollutants, the researchers recommend an upscale of the microalgae cell concentration inoculated in the wastewater samples. Based on the trends observed, this increase in cell count will ultimately help in the complete removal of contaminants in wastewater. However, this will bring forth the need for mass cultivation, and therefore, a relatively higher cost which, in a sense, may beat the strive for sustainability and cost-effectiveness. Regardless, microalgal-based wastewater treatment procedures are substantially more feasible and sustainable as opposed to conventional treatment processes such as those utilizing chemicals and sludge.

The researchers tested a total of ten (10) physico-chemical parameters. However, due to temporal and financial constraints, only the first three were measured daily and the remaining variables were only quantified post-intervention. As a result, differences in the statistical analysis made data interpretation rather difficult. Statistical observation of the monotonic relationship and the measurement of significance only accounts for chlorophyll concentration and the iterated parameters. Aside from regular monitoring of the other parameters, the proponents recommend to test for the efficiency of *Chlorella vulgaris* in reducing other pollutants such as heavy metals and different microbial strains, i.e., common pathogens in wastewater.

The prime focus of this study is the wastewater remediation potentials of *Chlorella vulgaris*. However, the integration of microalgae is not limited to wastewater treatment. *Chlorella vulgaris* may also be beneficial in CO_2 sequestration and production of biofuels and other high-value by-products in the pharmaceutical, food, and feed industries. Furthermore, the treated wastewaters may be reused for water reclamation and reutilization which are of paramount importance in order to circumvent challenges on water scarcity in both developing and developed countries.

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APPENDIX A

Cultivation and Isolation Trials

Figure A1. Sampling in San Diego River, Nagcarlan.

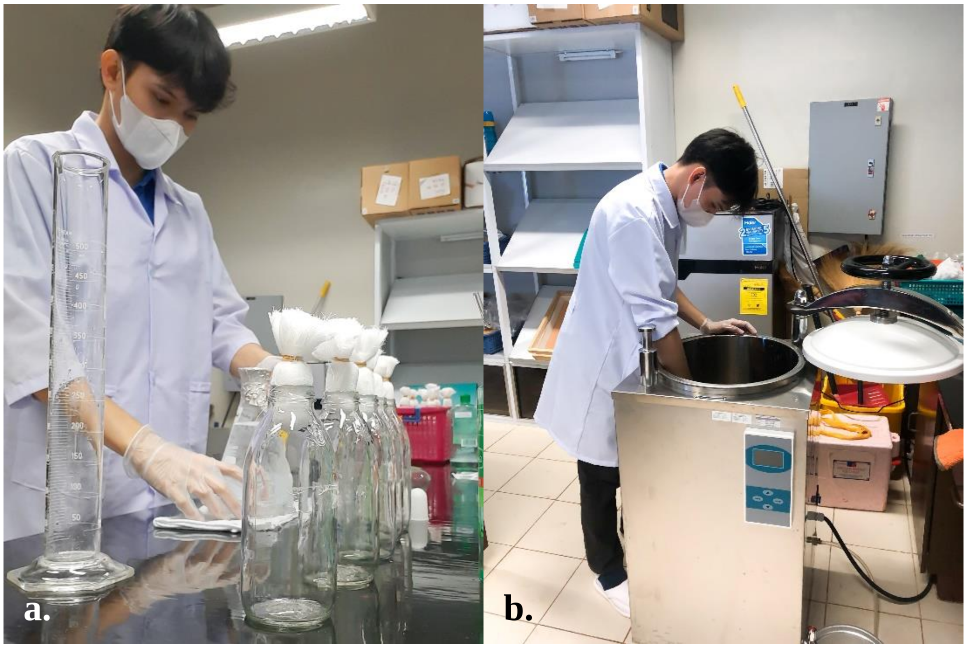

Figure A2. Formulation of culture media: a) dispensing into vessels; b) sterilization using autoclave.

APPENDIX A

Cultivation and Isolation Trials (*cont.*)

Figure A3. Cultures after 14-day cultivation: a) first sampling; b) second sampling; and c) third sampling.

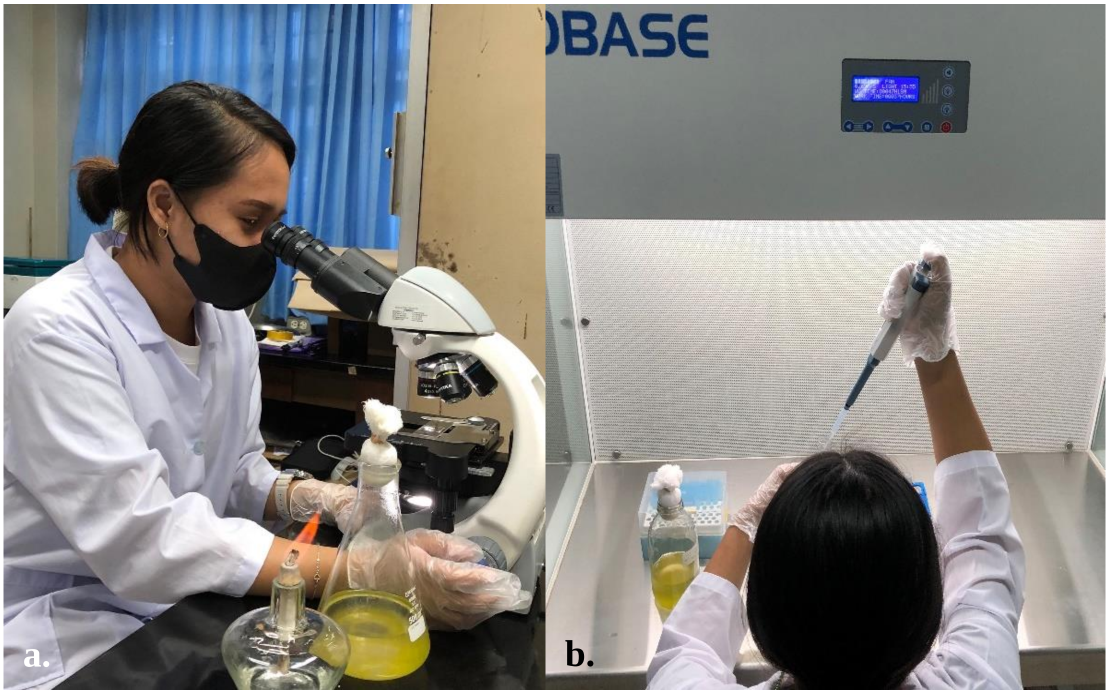

Figure A4. Isolation trials: a) manual cell picking; b) serial dilution method.

APPENDIX B

Morphological Determination of Microalgal Assemblage

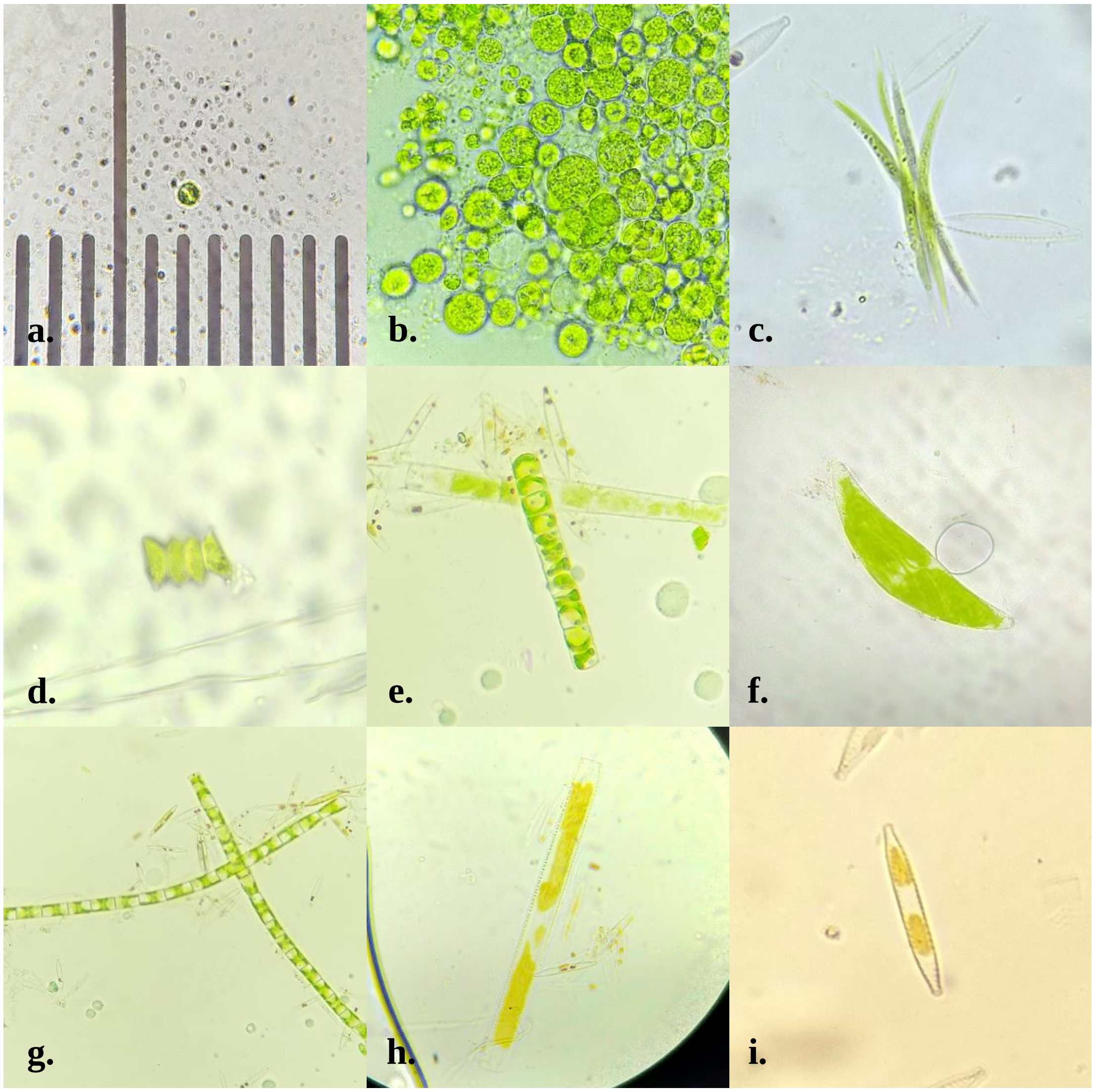

Figure B. Microalgae species observed in the sampling site: a) *Chlorella*; b) *Chlorococcum*; c) *Ankistrodesmus*; d) *Scenedesmus*; e) *Ulothrix*; f) *Closterium*; g) *Tribonema*; h) *Nitzschia*; i) *Synedra*.

APPENDIX C

Collection of Wastewater Samples

Figure C1. Map plotting and coordinates of the wastewater collection sites.

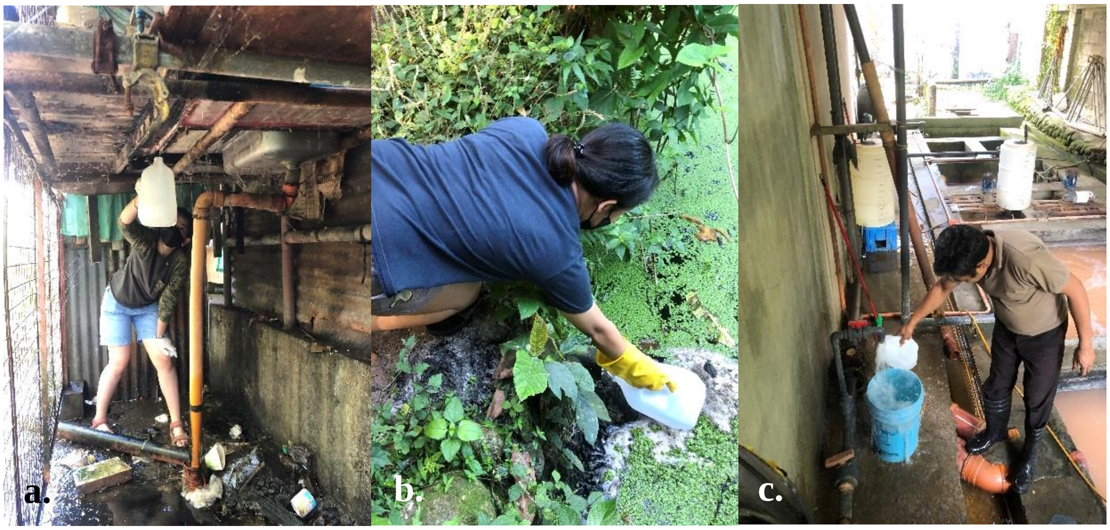

Figure C2. Wastewater collection sites: a) municipal wastewater; b) agricultural wastewater; c) industrial wastewater.

APPENDIX D

Trichromatic Method of Chlorophyll Determination

Figure D1. Pigment extraction: a) vortex mixing; b) centrifugation; c) 90% aqueous acetone addition.

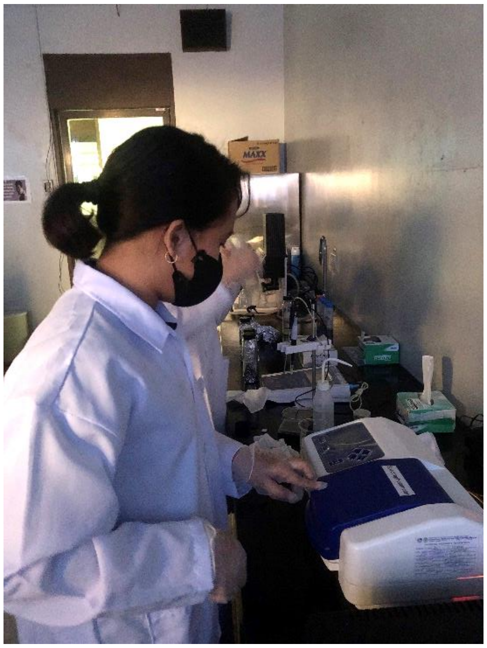

Figure D2. Optical density reading at 750, 664, 647, and 630 nm.

APPENDIX E

Analytical Methods

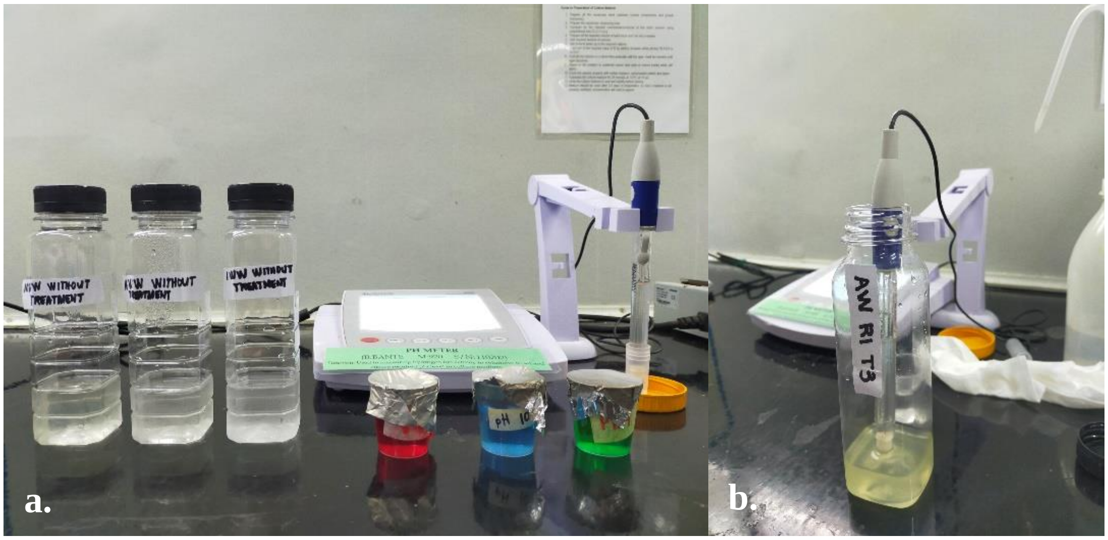

Figure E1. pH measurement: a) meter calibration; b) probe immersion.

Figure E2. Total dissolved solids and electrical conductivity measurement.

APPENDIX E

Analytical Methods *(cont.)*

Figure E3. Total suspended solids measurement: a) straining reagent residues; b) filtration apparatus; c) Whatman™ membrane filters after filtration.

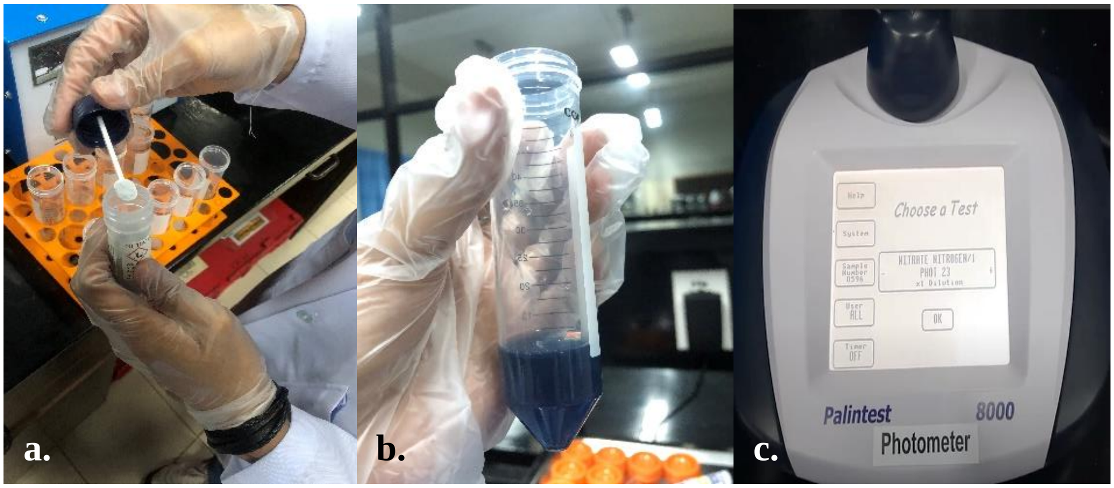

Figure E4. Nitrate-N measurement: a) addition of reagents; b) color development; c) photometer test selection (Phot 23).

APPENDIX E

Analytical Methods *(cont.)*

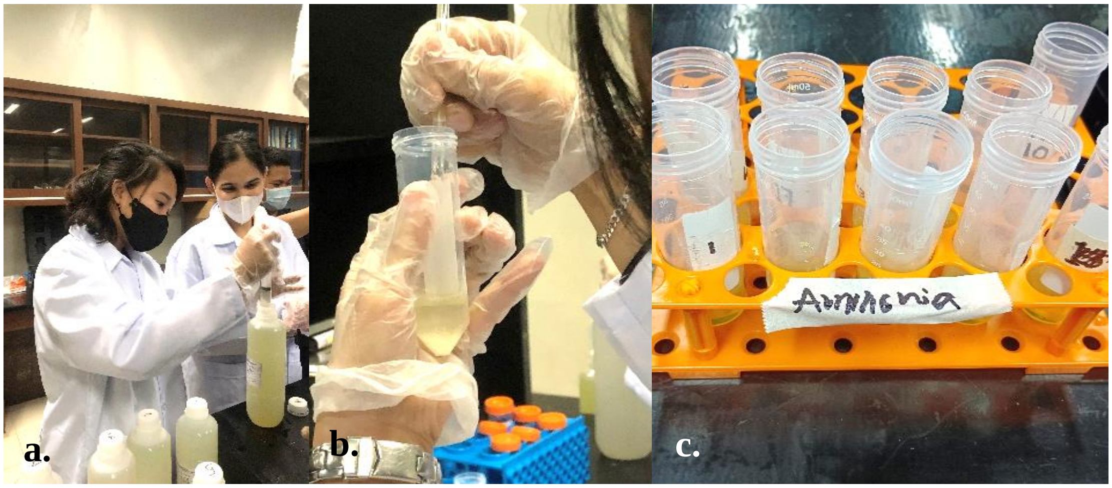

Figure E5. Ammonia-N measurement: a) transferring of aliquots; b) addition and crushing of reagents; c) set-up while inducing color development.

Figure E6. Phosphate measurement. (a) addition and crushing of reagents; (b) inversion and mixing of tubes; (c) color development.