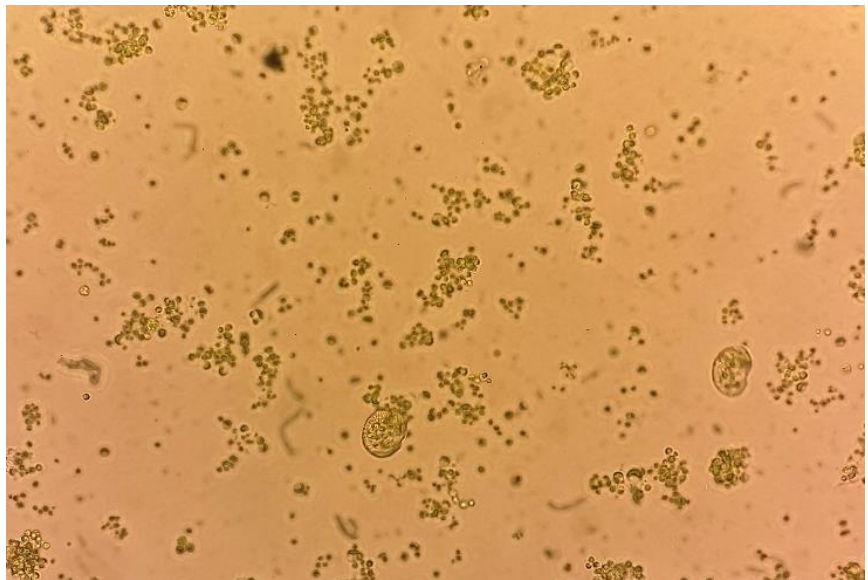# Integrating microalgae in wastewater treatment

Nutrient recovery and biomass production using *Chlorella vulgaris* in anaerobically digested black water treatment

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Integrating microalgae in wastewater treatment

Nutrient recovery and biomass production using *Chlorella vulgaris* in anaerobically digested black water treatment

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Acknowledgement

Abstract

With the growing need for sustainable wastewater management solutions, this master's thesis explored the use of microalgae, specifically *Chlorella vulgaris*, in the treatment of anaerobically digested black water (AnBW). The study assessed the viability of using AnBW as a growth medium for microalgae to recover nutrients and produce biomass. Through controlled laboratory experiments, the effects of different dilutions of AnBW, CO2 enrichment, and the necessity of adding trace elements on the growth and nutrient uptake efficiency of *Chlorella vulgaris* were investigated. These findings suggest that by utilising this waste stream, microalgae could substantially decrease the nutrient load on wastewater treatment plants while providing a sustainable source of biomass for various applications. An external input of carbon, such as CO_2, is essential for growth while the addition of trace elements needs to be investigated further, although results indicate an overall improvement of the performance specifically in phosphorus reduction. The study highlights the potential of integrating microalgal treatment in wastewater management to promote sustainability and resource recovery. Future research should focus on harvesting the biomass, scaling up the process, and exploring the economic viability of large-scale applications.

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Abbreviations

AnBW	Anaerobically digested blackwater
BOD	Biological oxygen demand
BW	Blackwater
COD	Chemical oxygen demand
DI	Distilled water
DW	Dry weight
FA	Free ammonia
HCl	Hydrochloric acid
HRT	Hydraulic retention time
NaOH	Sodium hydroxide
NH_3-N	Ammonia-nitrogen
NH_4^+-N	Ammonium-nitrogen
OD	Optical density
PBR	Photobioreactor
PE	Population equivalent
SRT	Sludge retention time
TE	Trace elements
TN	Total nitrogen
TOC	Total organic carbon
TP	Total phosphorus
WWTP	Wastewater treatment plant

1 Introduction

As in most industries, wastewater management is facing the challenge of adapting to climate change and a circular economy. However, the perception of wastewater is shifting from being a societal problem to an emerging resource. In this transition, one must allow for new technologies to be tested and developed to build resilience and meet increasingly stringent requirements. RecoLab is a research facility in Helsingborg, Sweden, that enables this kind of development. Here, wastewater is collected source-separated, where black water, grey water, and food waste arrive at the plant in three separate pipes. This allows for targeted treatment and better recovery of nutrients. Taking advantage of this, we are testing microalgae's capabilities in wastewater treatment, a nature-based solution that has received increasing attention recently and could prove to be an important part of the green transition.

Microalgal species exist up to several hundreds of thousands (Guiry, 2012). They produce nearly 50% of all oxygen and being one of the earliest species on the planet, they have evolved to thrive even in the harshest conditions. Phycoremediation, using algae for wastewater treatment, is not a novel idea and several researchers have developed techniques for using microalgae for this purpose (Gonçalves et al., 2017). Although current wastewater treatment plants (WWTP) can successfully remove substances such as nitrogen (N) and phosphorus (P) from the water column, preventing toxic enrichment of nutrients in surface water, the process is energy inefficient with low opportunities for nutrient recovery (Gao et al., 2014). Microalgae can effectively assimilate N and P, but also macro- and micronutrients from the wastewater. The feedstock produced is gaining more interest for the economic possibilities it constitutes, as the recovered substances can be further refined into valuable products such as biodiesel, bioplastics, dyes, and chemicals (Li, Y. et al., 2022, p147). Using microalgae in wastewater treatment also has a clear advantage over the conventional use of bacteria; it produces oxygen out of atmospheric carbon dioxide.

This report will focus on the microalgal treatment of anaerobically digested black water (AnBW), rich in the nutrients essential to microalgae growth. Traditionally AnBW is recycled back to the main treatment, and while the

influence on the hydraulic load is of minor importance, the rejected nitrogen load accounts for a substantial part of the load on the process (Meyer and Wilderer, 2004). By using AnBW for the cultivation of microalgae, the nutrient load on the treatment plant will decrease at the same time as the waste stream's potential for nutrient recovery is unlocked. Although previous research has investigated the potential of using microalgae in the treatment of black water, and even AnBW (Eshetu Moges et al., 2018; Segovia Bifarini et al., 2020), this report will bring additional understanding of doing so in nutrient-rich, vacuum-collected AnBW, and the effects of adding trace elements.

1.1 Aim

This master's thesis will explore the combined impacts of cultivating microalgae in municipal black water. It seeks to assess the viability of using AnBW as a growth medium for microalgal biomass production, while also implementing a sustainable treatment for this specific waste stream. Through laboratory experiments, it will assess nutrient removal and biomass production for the species *Chlorella vulgaris,* in different dilutions of AnBW. It will also investigate the necessity of adding external carbon and trace elements, as well as testing the efficiency of microalgae-bacteria consortia, which will serve as the foundation for the discussions on large-scale applications.

1.2 Research questions

- How does *Chlorella vulgaris* perform in terms of nutrient removal and biomass production when cultivated in AnBW?
- What is the optimal dilution ratio of AnBW for maximising the growth and nutrient uptake efficiency of *Chlorella vulgaris*?
- How does CO2 enrichment affect growth and nutrient uptake when cultivated in various dilutions of AnBW?
- How does external input of trace elements affect the growth and nutrient removal of *Chlorella vulgaris* when cultivated in AnBW?
- How does AnBW compare to synthetical growth mediums in terms of biomass production?
- How does *Chlorella vulgaris* perform in terms of nutrient removal and biomass production when cultivated in consortia with bacteria?

1.3 Delimitations and scope

The study is conducted within a laboratory setting, utilising specific controlled environmental conditions including temperature, light intensity, and photoperiod, which may not replicate external environmental conditions. The AnBW used is from a specific wastewater treatment facility, and results may vary with wastewater of different compositions or from different geographical locations. The scalability of results is considered theoretically but not tested in real-world, large-scale applications.

The scope of the experiments is limited to testing the effects of different dilutions of AnBW and the addition of CO2 and trace elements on the growth and nutrient removal performance of the microalgae. Other potential variables such as different light conditions, varying CO_2 concentrations, or alternative nutrient sources have already been extensively researched and are not explored further in this thesis (Marazzi et al., 2017). Only photoautotrophic growth will be explained in detail. It will mention, but not investigate, other metabolic pathways.

The focus on *Chlorella vulgaris* is due to its well-documented efficiency in nutrient uptake and robustness in various growing conditions, as outlined by previous research. The findings may not be directly applicable to other microalgae species, which might react differently in similar conditions.

This research contributes to the field by exploring the synergistic effects of microalgae cultivation using AnBW as growth medium, and especially by increasing the understanding of external addition of trace elements.

2 Theoretical background

2.1 Conventional wastewater treatment and regulations

Municipal wastewater treatment plants typically involve several steps to remove contaminants from wastewater to make it suitable for discharge or reuse. Although the process can differ, a general approach is as follows. A primary treatment separates solids from the wastewater through screening and sedimentation. Following is a secondary, often biological, treatment that uses microorganisms to decompose organic matter and reduce the levels of nutrients (i.e. nitrogen and phosphorus). Conventionally, bacteria are used due to their rapid growth and excellent nitrogen reduction in the process of nitrification-denitrification. If applied, a third step further refines the wastewater by removing residual nutrients and pathogens, often through chemical precipitation or advanced filtration. (Sonune and Ghate, 2004)

European wastewater treatment plants (WWTPs) are required to adhere to the standards set by the Council Directive 91/271/EEC (1991). It regulates the allowed levels of chemical and biological oxygen demand, nitrogen, and phosphorus in the effluents. The directive is currently being updated to more stringent requirements and will most likely include new parameters such as pharmaceutical residues and energy efficiency (European Council, 2024).

2.2 Microalgae in wastewater treatment

Substantial amounts of nitrogen and phosphorus are required for microalgal growth. This makes them suitable for the uptake of these nutrients from wastewater. Microalgae have already been showing high efficiencies (80-100%) in removing nitrogen and phosphorus from different streams of wastewater, such as municipal, industrial, and agricultural (González et al., 1997; Li et al., 2011; Zhu et al., 2013). The studies have been conducted on monocultures as well as polycultures, but also on microalgae-bacteria consortia. Combining different organisms with different metabolic pathways and environmental adaptations has been found to increase resilience to a diversity of environmental conditions and nutrient loads (Gonçalves et al., 2017).

Both academia and industry have engaged in the topic over the last decades. In 2016, an EU-funded (9.8 million €) project started to investigate the techno-economic feasibility of using microalgae in the treatment of saline wastewater, primarily from the food industry. *SaltGae* was a response to the problem induced by conventional treatment methods not being able to remediate the high salinity wastewater from some food and dairy industries. As certain species of microalgae are naturally found in high salinity waters, the project used these in three demonstration sites and found that algae-bacteria consortia can effectively treat these waste streams. It found that integrating microalgae-based systems could offer a sustainable solution and reduce the life cycle cost and environmental impacts compared to current practices. The project ran over three years. (Coutiño et al., 2018)

In Broadwindsor, UK, the municipal treatment plant (480 PE) installed a microalgae tertiary treatment in 2021 to enhance phosphorus removal. Using photobioreactors (PBRs) with LED lamps and aeration with atmospheric air, it is designed to remove phosphorus to levels below 0.5 mg/L. (I-PHYC, 2021) Another system has been developed by GWT, USA, that targets wastewater streams from both food and beverage industries, as well as municipal tertiary treatment and anaerobic digestate. Instead of using PBRs, microalgae is grown on the surface of vertical conveyor belts that use sunlight and CO_2 when in contact with the atmosphere and consume nitrogen and phosphorus when submerged (Gross-Wen Technologies, 2023).

2.2.1 Operational limitations

Despite the operational advantages and environmental benefits, several challenges hinder the widespread adaptation of using microalgae in wastewater treatment. Among the most profound obstacles are the difficulties of efficient harvesting of microalgal biomass and the large footprint compared to conventional methods, as basins must be shallow if sunlight is to be used as the energy source. When grown photoautotrophic, the turbidity of the wastewater can also inhibit photosynthesis, thereby reducing the efficiency of the treatment. (Wang et al., 2016)

2.3 Introduction to microalgae

Microalgae constitute a diverse group of organisms that are pivotal to aquatic ecosystems. The term "algae" is not a taxonomic term but rather a name of convenience when referring to primitive, plant-like organisms which contain chlorophyll a, are usually able to photosynthesise, and are not specialised land plants (Borowitzka, 2016a). The many species of microalgae, with estimations in the range of tens of thousands to hundreds of thousands, are currently represented by four kingdoms: Bacteria, Plantae, Chromista, and Protozoa (Guiry, 2012). A more common way of referring to species is by their phylum, such as green algae, cyanobacteria (or blue-green algae), and diatoms. Microalgae are unicellular, although some species can be filamentous which could be considered a simple form of multicellularity (Zachleder et al., 2016).

As for all life, microalgae are dependent on carbon. The metabolic pathways for utilising carbon can differ amongst species and be categorised into photoautotrophic, heterotrophic and mixotrophic. In photoautotrophic metabolism, microalgae utilise light energy to fix inorganic carbon such as carbon dioxide or bicarbonate or into organic compounds through photosynthesis, whereas heterotrophic growth allows microalgae to grow in the absence of light by utilising organic carbon sources for energy and biomass production. Mixotrophic metabolism combines photoautotrophic and heterotrophic metabolic pathways, allowing microalgae to simultaneously utilise inorganic and organic carbon. (Daliry et al., 2017)

This chapter serves to introduce the most important factors for the growth of microalgae, as well as present the extreme variations that can be found in this group of organisms.

2.3.1 Macronutrients and trace elements

Three main elements are needed for microalgae growth: carbon, nitrogen, and phosphorus. Additionally, low concentrations of trace elements are also required (Andersen, 2005). This section further develops the importance of these nutrients, and in which forms they can be utilised. The ratio between carbon, nitrogen, and phosphorus is known as the C:N:P ratio and must be in the optimum range for the specific species to not be inhibited by one element or another. A common stochiometric benchmark is the Redfield ratio, which is

approximately 106:16:1 (Islam et al., 2019). In a study looking at the influence of different N:P ratios on the freshwater alga *Scenedesmus obliquus*, they found that optimum biomass production and nutrient removal were achieved at N:P ratio between 9 and 13 (Arbib et al., 2013).

The uptake rate of a specific nutrient by the algal cell is influenced by the concentration gradient across the cell membrane and the diffusion rates through the cell wall. A thick boundary layer with unstirred water immediately adjacent to the cell wall results in reduced diffusion rates. Therefore, to improve the mass transfer it is important to maintain turbulence. (Mostert and Grobbelaar, 1987)

Carbon source

Carbon is the primary structural component, and in a photoautotrophic culture system CO_2 or HCO_3^- compounds serve as the sole carbon source (Daliry et al., 2017). Through photosynthesis, light energy is converted to chemical energy, which is thereafter used in the Calvin cycle to convert CO_2 to sugars according to the overall formula:

$$6H_2O + 6CO_2 + light \rightarrow C_6H_{12}O_6 + 6O_2$$

When HCO_3^- is used as a carbon source, it needs to be converted to CO_2 through the enzyme carbonic anhydrase (Su, 2021). CO_2 that is dissolved in water enters the carbonic acid equilibrium, shifting towards CO_3^{2-} at high pH. As this form of carbon cannot be utilised by microalgae, pH has a strong influence on the inorganic carbon availability.

Some algal species can utilise organic carbon, such as organic acids, sugars, or glycerol, in heterotrophic growth. This allows microalgae to grow in the absence of light and could potentially lead to higher biomass concentrations and a smaller area footprint when shadowing and light deficiency do not need to be considered. Heterotrophic growth is however often associated with higher production costs and is more prone to biological contamination. (Carone et al., 2019)

A common practice to elevate the carbon content in the culture media is to inject CO_2. This can be done using pure CO_2 or by aerating with CO_2-enriched

air. A study by Patil and Kaliwal (2017) found that not only did additional CO_2 enhance biomass production on the freshwater microalgae *Scenedesmus bajacalifornicus* BBKLP-07 when grown in BG-11 medium, but different levels of CO_2 enrichment changed the biochemical composition of the cell. Maximum biomass productivity was found with 15% CO_2, but the content of carbohydrates and lipids was highest with 25% CO_2. Chaudhary et al. (2018) instead achieved optimum biomass productivity and nutrient reduction (nitrogen and phosphorus) at 5% CO_2 enrichment, when grown in municipal wastewater.

Nitrogen
Comprising more than 10 % of microalgal biomass, nitrogen (N) is essential for growth and productivity and is fundamental in synthesising amino acids, proteins, RNA, and DNA. Nitrogen can exist in many forms, and the most common nitrogen compounds used by microalgae are ammonium (NH_4^+), nitrate (NO_3^-), and occasionally nitrite (NO_2^-) or urea ($CO(NH_2)_2$). Nitrate and nitrite, the more oxidised forms of nitrogen, are reduced in the cell to ammonium before the synthesis into organic molecules. Ammonium, on the other hand, can be directly assimilated, making it the preferred source for many microalgae due to its lower energy requirement for assimilation. (Su, 2021) Cyanobacteria are also capable of using the amino acids arginine, glutamine and asparagine as a source of nitrogen, and some species can fix nitrogen gas (N_2), although this is the most energy-demanding type of nitrogen fixation (Bhaya et al., 2002).

High levels of nitrogen, especially in the form of free ammonia (NH_3, FA), have been identified as inhibitory to the growth and productivity of microalgae. Jiang et al. (2021) discuss the many different theories of FA toxicity to microalgal cells, but highlight the important factor that it can freely diffuse through the cell membrane where it damages the oxygen-evolving complex of the PS II, the photosynthetic process where light energy is converted into chemical energy. Whether nitrogen is present in the water as ammonium or the more toxic ammonia is pH-dependent, where an increase in pH shifts the equilibrium towards ammonia. Reversely, the pH will also be dependent on the form of nitrogen consumed. When microalgae are grown on ammonium, the pH will decrease due to the release of H^+ into the growth medium. Where

nitrate is the main nitrogen source, pH will instead increase due to the release of OH⁻ ions. (Collos and Harrison, 2014)

Nitrogen becomes inhibitory to microalgae at levels that are species-dependent, and as mentioned above: pH-dependent. Collos and Harrison (2014) compiled the effects of high ammonium concentrations on the different phylum of microalgae, and found that the mean optimal concentrations ranged from 137 mg/L to 1.8 mg/L for the green algae (e.g. *Chlorella sp.*) and the dinoflagellates respectively, with toxic levels (no growth) at 704 mg/L and 22 mg/L. A study by Jiang et al. (2021), which tested the growth of *Chlorella Vulgaris* at different ammonium concentrations and different pHs found that at a pH of 6.5, the algae grew regardless of initial concentration (50 to 500 mg/L), but with a pH of 7.5, they saw an obvious inhibition at 500 mg/L. Only the cultures growing in the lowest concentration (50 mg/L) maintained good growth at a pH of 9.5.

Microalgae can achieve high removal of nitrogen from wastewater. Vasconcelos Fernandes et al. (2015) reported 100% removal from AnBW after 12 days under optimal conditions, and Slompo et al. (2020) reached a 66% removal from AnBW without filtering the medium or adding CO_2. Another way to reduce ammonium from water samples is through a process called ammonia stripping. Through adjustment of the pH to alkaline conditions (11-11.5), ammonium is converted to gaseous ammonia which then can be removed from the water sample through aeration. Although not as effectively, this process occurs already at a lower pH (>9.25). (Wu and Vaneeckhaute, 2022)

Phosphorus
Acting as a key component in adenosine triphosphate (ATP, energy transfer), nucleic acids (genetic information storage) and phospholipids (cell membrane structure), phosphorus (P) is essential for microalgal growth. The primary form of phosphorus used by microalgae is inorganic phosphate (PO_4^{3-}), although some species can utilise organic phosphorus compounds by enzymatic degradation. When grown in conditions with low levels of phosphorus, microalgal cells contain around 1% of phosphorus in the dry weight of the cells. However, when exposed to high concentrations, phosphorus can be absorbed and stored in large amounts through a process known as "luxury

uptake", and reach cellular phosphorus content up to 4-6 % of dry weight. (Yu et al., 2024)

In a review on phosphorus removal from microalgae-based wastewater treatment by Yu et al. (2024), it is shown that microalgae can extensively remove the phosphorus from the wastewater. They reported 100 % removal rates for species such as *Chlamydomonas sp., Scenedesmus obliquus* and *Chlorella Vulgaris* at initial concentrations around 18-20 mg/L phosphate. They also saw that *Chlorella Vulgaris* was able to remove 85.6 % of phosphorus in a high concentration sludge centrate. However, phosphorus removal from wastewater does not only occur through absorption by the cell but also by precipitation. This process is dependent on pH, where alkaline environments enhance phosphorus precipitation. Maintaining pH is therefore crucial. (Rott et al., 2017)

Trace elements

Trace elements play a critical role in the growth and development of microalgae. Some elements are essential for various metabolic processes, such as photosynthesis, respiration, and nutrient assimilation. Other elements can, if levels are too high, be inhibitory to the growth of microalgae (Liu et al., 2024). The composition of trace elements in the growth medium is also important, as it can affect the composition of species because of the large differences in requirements among species (Sunda et al., 2005, p.36). Understanding the importance and the impact of trace elements on microalgae growth is therefore essential when optimising cultivation conditions and evaluating new growth mediums. de Oliveira et al., (2020) discuss in their article 30 different elements essential for microalgae growth, many of them being trace elements. This section will only briefly touch upon the most important.

Ferric iron (Fe^{3+}) is used as an electron acceptor in the photosynthetic processes. The electron transfer is essential for the synthesis of the energy-carrying molecules ATP and NADPH, ultimately enabling the conversion from light to energy. It has also been shown to play an important role in nitrogen fixation and other processes. Adding Fe^{3+} could increase the efficiency of photosynthesis and therefore increase the carbon flow towards microalgae. (Liu et al., 2024) The cellular requirement of iron varies with both species and

ambient conditions. It increases with decreasing light intensity and shorter photoperiods and is highly dependent on nitrogen sources. It is higher when nitrate is in abundance over ammonium. (Sunda et al., 2005, p.37)

Copper (Cu^{2+}) is required for photosynthetic electron transport, as it is essential for the synthesis of the protein cytochrome oxidase. Too high concentrations can inhibit growth, and even cause death of cells due to oxidative stress and change of chlorophyll absorption spectra. (Liu et al., 2024; Sunda et al., 2005, p.38)

Zinc (Zn^{2+}) is found in many metabolic functions. One of its uses is in carbonic anhydrase, the enzyme responsible for CO_2 fixation. The need for zinc is therefore increased in conditions with lower levels of CO_2 (Sunda et al., 2005). Toxicity has been found due to the destruction of protein when testing for high levels of zinc (Liu et al., 2024).

Magnesium (Mg^{2+}) is an important atom in the skeleton of the chlorophyll molecule. It is also required by many enzymes, such as RNA polymerase, ATPase, and phosphatase amongst others. Deficiency of magnesium will therefore hinder cell division and chlorophyll synthesis, resulting in a decrease in photosynthetic rate. (Liu et al., 2024) It is also a cofactor in fatty acid synthesis, and increased levels of the element can enhance lipid and protein content. A study that investigated the effect of Mg^{2+} on *Chlorella Vulgaris* found that the optimal concentration for biomass production was 5 mg/L and for protein and carbohydrate production 15 mg/L. (Salman et al., 2023)

Other trace elements are manganese, cobalt, calcium, molybdenum, and silicon. Manganese (Mn^{2+}) is a component of the water-splitting mechanisms in photosynthesis that produces electrons. It is also present in enzymes that remove toxic superoxide radicals (Sunda et al., 2005). Cobalt (Co^{2+}) is a component of vitamin B_{12} (Quigg, 2016), but could also substitute zinc in carbonic anhydrase (Sunda et al., 2005). Calcium (Ca^{2+}) is toxic at high levels but required in low concentrations for nutrient uptake and pollutant removal. A slight increase in calcium may decrease pollutant removal rate, but promote flocculation which can be beneficial for harvest. (Liu et al., 2024) Molybdenum is used in the reduction of nitrate and nitrite to ammonium, with requirements strongly correlated with nitrogen sources (Quigg, 2016). Silicon

is generally only acquired in the cultivation of diatoms, as it is the foundation for the formation of their cell walls (Borowitzka, 2016b).

This section showcases the complex interaction of the requirements of trace elements with cultivating conditions and microalgae species. It does not have the intention of presenting the full understanding of these systems, but rather to highlight the importance of being aware of their existence.

2.3.2   Other factors influencing microalgal growth

Besides the chemical composition of the water, the physical environment, such as pH, light, and temperature, plays an important role in the success of growing microalgae.

pH

The effect of pH has already been discussed in terms of ammonia inhibition, phosphorus precipitation, and carbon availability, but the importance of pH to microalgae cultivation stretches further than so. As microalgae grow, pH naturally fluctuates by up to one unit between light and dark periods, which can cause inactivation of coliform bacteria (Slompo et al., 2020).

The majority of microalgae have been found to favour neutral pH, with species-specific adaptation allowing them to withstand either acid or alkaline environments (Berge et al., 2012). Khalil et al. (2010) reported that *Chlorella ellipsoidea* could tolerate both acidic (pH 4) and alkaline (pH 10) conditions, but also that the composition of the algae changed with pH. Production of biomass and carbohydrate content were largest in slightly alkaline conditions but with optimum protein production at pH 4. In another study by Yu et al. (2022), they measured ammonium reduction and bacterial competition when a polyculture of microalgae was cultivated in anaerobic digestion effluent at different pH. Although growth was present down to pH 3, it was highest in the cultures maintained between 7 and 8. This pH also returned the highest ammonium removal. In terms of bacterial competition, controlling the pH below 8 favoured the growth of microalgae. Increasing the pH could be of benefit for harvesting, as pH-induced flocculation can make the otherwise costly separation of the biomass easier (Wu et al., 2012).

Light

Microalgae, being photosynthetic organisms, require light as an energy source for photosynthesis. The intensity and photoperiod (duration of light exposure) play a significant role in the growth rate and productivity of microalgae. Understanding the impact of light and photoperiod is essential for optimising growth and maximising nutrient removal. Not only are requirements different amongst species but also amongst processes. Longer light periods seem to benefit nutrient removal, whereas longer dark periods benefit carbon removal (Li et al., 2019). One study found that *Chlorella vulgaris* performed best at 12:12h (light:dark h) in terms of COD removal, but had an optimum ammonium removal under continuous light (Ardo et al., 2024). A second study found the highest growth rate at 16:8 for the same species (Kendirlioglu et al., 2015), while a third did so under continuous light (Anyanwu et al., 2022). While photoperiod has proven to be an important parameter, it is affected by others such as the intensity and colour of the light (Christwardana et al., 2022). Many species can also thrive under a wide range of light conditions (Maltsev et al., 2021).

As essential as light is, there are also toxic effects of photoinhibition above light saturation, where dark periods have been found to counteract these effects. Too long photoperiods or too strong light intensity can cause damage to the cells. However, microalgae can repair these cells during dark period (Liu et al., 2024). Microalgae have also been shown to schedule processes that are sensitive to UV, like cell division, RNA and DNA synthesis during dark periods. When microalgae and bacteria are cultivated in consortium, a longer dark period can enhance the growth of bacteria. (Su, 2021)

Temperature

Temperature is one of the most important environmental factors in the cultivation of microalgae, influencing their growth rate, lipid accumulation, and overall biomass productivity, As with most biological reactions, the turnover of the microalgal cell cycle increases with temperature until optimal conditions have been reached (Zachleder et al., 2016). Research has demonstrated that microalgae such as *Scenedesmus sp.* and *Chlorella sp.* can thrive across a broad temperature range (10-30°C), but optimal growth often occurs at specific temperatures. *Scenedesmus sp.* shows optimal lipid and

biomass productivity around 20°C (Xin et al., 2011) and *Chlorella sp.* 25-30°C (Choi and Lee, 2011).

2.4 Anaerobically digested black water

Blackwater (BW), as distinct from greywater (showers, laundry, etc) and food waste (from kitchen food grinder), refers to the wastewater that originates from toilet flushes, containing faeces, urine, toilet paper and flush water. The flush water can have a large diluting effect depending on the system. The chemical composition is one of the primary differences among these waste streams, particularly in the carbon, nitrogen, and phosphorus ratios. The incoming blackwater is much more nutrient-dense than greywater and food waste, with concentrations 25 times higher than total domestic wastewater if collected with a vacuum system (de Graaff et al., 2011).

AnBW, or anaerobically digested black water, refers to the centrate of the blackwater which has been treated using an anaerobic digestion process, commonly to produce biogas. The separated centrate from the sludge is less turbid, with most of the nutrients kept in the water phase. However, the process yields a large reduction in COD and carbon to nitrogen/phosphorus ratio. (Zhou et al., 2020) AnBW has successfully been used as a growth medium for microalgae in previous research (Slompo et al., 2020).

2.5 Biomass valorisation

When microalgae are used for the treatment of wastewater, large quantities of biomass are produced. The harvest from such a system can be converted into a variety of valuable products, as well as generating income for the WWTP. Microalgae synthesise phycobiliprotein, chlorophyll, and carotenoid pigments which have been studied as a promising alternative for textile dyes (Mutaf-Kılıc et al., 2023). It has also been heavily studied for its prospects within the field of bioenergy, both for biomethane and biodiesel, with higher yields per unit land area compared to terrestrial plants (Singh and Dhar, 2011).

3 Methodology

The research design for this thesis involves a literature review followed by controlled laboratory experiments. The strategy is selected to explore the capabilities of *Chlorella vulgaris* in treating AnBW. The literature review sets the foundations by offering insights into previous studies, identifying gaps in the current understanding, and justifying the need for further exploration.

The core of the study is the laboratory work, which provides insight into various variables, such as cultivating algae in different dilutions of AnBW and the impact of growth and nutrient uptake when adding external carbon and trace elements. These experiments are designed to optimise conditions that enhance the efficiency of microalgae in wastewater treatment under specific, replicable settings.

3.1 Experimental design

The experiments consisted of two tests, denoted *phase I* and *phase II.* The first aimed to find the optimal dilution of AnBW concerning NH_4^+-N concentration, and the effects of adding CO_2. The second investigated the need for an external input of trace elements when cultivating microalgae in BW, and its performance when cultivated in microalgae-bacteria consortia. All experiments were carried out in 500 mL glass flasks (SILEX) with a working volume of 450 mL and an inoculation ratio of 10% *Chlorella Vulgaris.* The experiments were conducted in climatic chambers (KK750, POL-EKO) at 27°C and 16:8 light:dark period with a light intensity of 7000 lux by white fluorescent lights, as suggested by Kendirlioglu et al. (2015). All batches were performed in duplicates under non-aseptic conditions, with inlet and outlet fitted with cotton to minimise the risk of contamination.

Phase I – Dilution and CO_2-enrichment
This part of the experiment aimed to find the optimal ratio of AnBW to water, and the effect of CO_2-enrichment. Based on the NH_4^+-N levels in the AnBW, four tests were conducted with different dilutions of AnBW with distilled water (DI), i.e., 100% AnBW, 50% AnBW, 30% AnBW and 10% AnBW. These ratios are intended is to cover the upper limits of NH_4^+-N inhibition as well as

levels below optimum. A duplicate of this set-up was used to test the effects of CO_2 enrichment. While both set-ups were aerated at a rate of 0.3 L/min, the latter was also enriched with 5-15% CO_2. As mentioned in section 2.3.1, small changes in CO_2-enrichment can generate large differences in growth performance and biochemical composition. However, instruments did not allow for more accuracy.

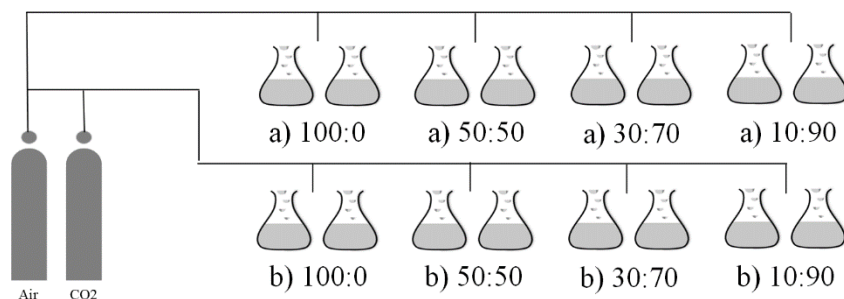Figure 1 shows a schematic set-up of the experiment.

Figure 1: Scheme of dilution of AnBW to DI (AnBW:DI). a) is grown in ambient CO_2 concentrations, and b) is enriched with 5-15% CO_2.

3.1.1 Phase II – Trace element and bacterial consortia

Using the optimal dilution from *phase I,* the second experiment tested the need for external input of trace elements (TE) when growing microalgae in AnBW. It also investigated the performance of microalgae-bacteria consortia by cultivating microalgae in unsterilised AnBW. Additionally, two references were set up for the results to be compared against. The first cultivated microalgae in synthetic Z8 growth medium, to assess the potential of biomass production in AnBW. The second reference used the same dilution of AnBW but without inoculation of microalgae, to test for non-algal nutrient reduction. The cultivating conditions were the same as for *phase I.*

Using unsterilised blackwater is not only interesting for experiments on bacterial competition, but will also test scale-up potential, where sterilisation of larger quantities of blackwater might not be suitable.

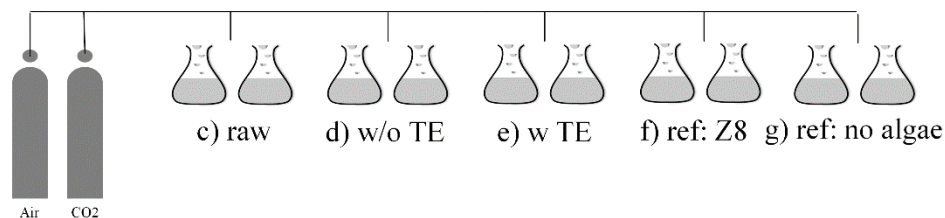Figure 2 shows a schematic set-up of the experiment.

Figure 2: Set-up for phase II. c) unsterilised, testing bacterial competition and scale-up potential, d) and e) testing the effects of adding trace elements, f) reference where industrial growth medium (Z8) is used, and g) a reference testing for non-algal reduction of nutrient, in which no microalgae was inoculated.

3.2 Selection of microalgae species

Chlorella vulgaris has been identified as a particularly effective species for wastewater treatment. This microalga is extensively researched and has demonstrated a high rate of nutrient uptake as well as a short cell cycle. Its robustness allows it to be cultivated in a broad range of both pH and temperature. (Jiang et al., 2021) Moreover, *Chlorella vulgaris* has a strong tolerance to ammonium (Collos and Harrison, 2014) and can be cultivated in both phototrophic and mixotrophic conditions (Slompo et al., 2020).

The strain (NIVA-CHL 108 *Chlorella vulgaris*) was purchased from the Norwegian culture collection of algae.

3.3 Growth mediums

Z8

Z8 is used as the standard growth medium for blue-green algae at the Norwegian culture collection of algae and was purchased together with the microalgae strain. Its chemical composition is for 1 L: 4.67 mg $NaNO_3$, 0.59 mg $Ca(NO_3)_2 \cdot 4H_2O$, 0.25 mg $MgSO_4 \cdot 7H_2O$, 0.31 mg K_2HPO_4, 0.21 mg Na_2CO_3, 2.8 mg $FeCl_3 \cdot 6H_2O$, 3.61 mg EDTA-Na_2, 0.033 mg $Na_2WO_4 \cdot 2H_2O$, 0.088 mg $(NH_4)_6Mo_7O_24 \cdot 4H_2O$, 0.12 mg KBr, 0.083 mg KI, 0.287 mg $ZnSO_4 \cdot 7H_2O$, 0.155 mg $Cd(NO_3)_2 \cdot 4H_2O$, 0.146 mg $Co(NO_3)2 \cdot 6H_2O$, 0.125 mg $CuSO_4 \cdot 5H_2O$, 0.198 mg $NiSO_4(NH_4)_2SO_4 \cdot 6H_2O$, 0.041 mg $Cr(NO_3)_3 \cdot 9H_2O$, 0.089 mg V_2O_5, 0.474 mg $KAl(SO_4)_2 \cdot 12H_2O$, 31.0 mg H_3BO_3, 1.6 mg $MnSO_4 \cdot H_2O$.

Blackwater collection and characterisation

The AnBW was sampled from the effluents from the UASB reactor at RecoLab, Helsingborg. The BW is collected and transported to the facility through a vacuum system that minimises dilution from flush water. After collection, the samples were immediately cooled down to 4°C to promote stability and thereafter filtered through two subsequent granular activated carbon (GAC) filters to reduce colouration for better light penetration. The filters were constructed according to Figure 3, with inspiration from Eshetu Moges et al. (2018). Both filters were cylindrical with the dimensions 60 x 350 mm and 45 mm x 250 mm respectively and constructed as up-flow filters to ensure saturation. The GAC was made from coal in the range of 0.4 mm – 1.7 mm and was thoroughly washed before use. The hydraulic retention time (HRT) in the filters was between 1 to 2.5 hours, and the turbidity was reduced from 195 to 107 NTU in the raw sewage. For the second experiment, a final filtration through a 10 µm filter was added to remove fine particles. After filtration, the AnBW was diluted with distilled water in 500 ml flasks according to the design of the experiments and autoclaved at 121°C at 15 psi for 20 minutes.
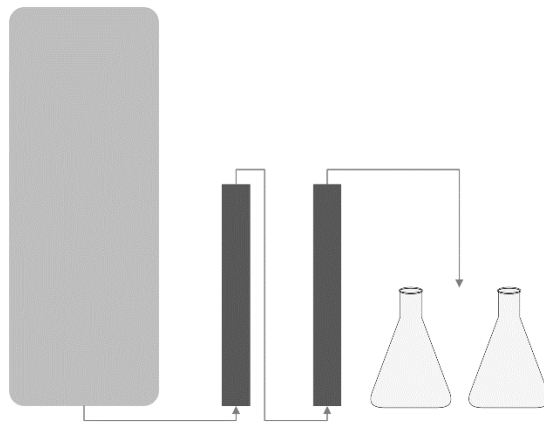

Figure 3: Two upflow GAC filters in series to reduce colour and turbidity.

The initial nutrient conditions were measured before and after the GAC filters, as well as in the 100% and 30% batches, see Table 1. Concentrations for 50% and 10% were interpolated from those of 100%. The initial batch of the 30% AnBW was diluted incorrectly, and a new batch of AnBW had to be filtered.

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As the HRT could not be precisely controlled, the initial concentrations of this batch differ somewhat from the otherwise linear dilution of nutrients.

To present the important stoichiometric C:N:P ratio, as discussed in 2.3.1 above, the concentrations of carbon, nitrogen, and phosphorus are converted by their molar mass. COD is not a direct measurement of carbon, but rather a quantification of the total amount of oxygen required to oxidise both biodegradable and non-biodegradable substances. However, a linear relationship between TOC (total organic carbon) and COD for influent wastewater was found by Dubber and Gray (2010):

$$TOC = \frac{COD - 49.2}{3.00}$$

In their study, however, they only included data from municipal wastewater plants with COD concentrations up to 500 mg/L. Using this equation, where TOC is assumed equivalent to carbon, could therefore carry sources of error, although it is considered best practice. TIC (total inorganic carbon) was not measured but would increase the accuracy if done so.

Table 1: Characterisation of BW and AnBW. BW$_{RecoLab}$ and AnBW$_{RecoLab}$ are calculated with data from July 2021 to January 2024 with values presented as average ± standard deviation. AnBW$_{inc}$ and AnBW$_{filt}$ are laboratory-produced values. C:N:P in molar ratio. " – " represents errors in measurement.

Parameter	Unit	BW$_{RecoLab}$	AnBW$_{RecoLab}$	AnBW$_{inc}$	AnBW$_{filt}$	Phase I				Phase II		
						100%	50%	30%	10%	50%	50%$_{raw}$	30
COD	mg/L	8957 ± 3150	1928 ± 1335	-	-	1800	900	769	180	205	103	123
TN	mg/L	1526 ± 527	1631 ± 601	819	705	501	250	165	50	260	380	156
NH$_4^+$-N	mg/L	1161 ± 450	1278 ± 436	640	609	474	237	140	47	185	316	111
TP	mg/L	122 ± 27	107 ± 22	55	42	42	21	17	4.19	17	20	10
C:N:P*	M	63:28:1	13:30:1			36:26:1	36:26:1	36:21:1	36:26:1	8:34:1	2:35:1	8:34:1

* Molar carbon concentrations are derived from COD measurements.

Phase II tested if an external input of trace elements could yield higher growth and nutrient reduction. Trace metal mix A5 with CO (92949, Merck) was used, with the following composition per litre: 2860 mg H_3BO_3, 1810 mg $MnCl_2 \cdot 4H_2O$, 222 mg $ZnSO_4 \cdot 7H_2O$, 390 mg $Na_2MoO_4 \cdot 2H_2O$, 79 mg $CuSO_4 \cdot 5H_2O$, and 49 mg $Co(NO_3)_2 \cdot 6H_2O$. According to the recommendations, 1 mL per L of growth medium was added before inoculation.

3.4   Data collection methods

Estimation of biomass

The growth of microalgae in *phase I* was monitored using a spectrophotometer (HACH DR3900) at 680 nm, with the different growth mediums as blanking. A calibration curve was established relating optical density and dry weight, see Figure 8. Dry weight was calculated according to standard protocol (APHA et al., 1998) where 30 mL culture of *Chlorella Vulgaris* was prepared in five serial dilutions. 10 mL was used for measurements of absorbance and 20 mL of each dilution was filtered through a 0.45 µm membrane filter under a vacuum of 5 kPa. The filters were pre-washed in 50 mL distilled water, dried in a hot-air oven (BINDER) at 60°C overnight, and then left to cool in a desiccator before they were weighed. After the filtration of samples, the funnel and filters were washed with 50 mL of distilled water and dried in a hot-air oven at 60°C overnight, and then cooled to room temperature in a desiccator before final weighing. Dry weight was then calculated according to the equation below.

$$Dry\ weight\ (mg/mL) = \frac{initial\ weight\ of\ filter\ (mg) - final\ weight\ of\ filter\ (mg)}{volume\ filtered\ (mL)}$$

Due to unwanted interference with optical density, the biomass growth for the second experiment was determined by chlorophyll-a concentration using the AlgaeLabAnalyser (bbe-moldaenke). Instead of measuring the absorbance of light, as is the case for the spectrophotometer, the AlgaeLabAnalyser uses coloured LEDs to excite chlorophyll-a within the algae and measures the resulting fluorescence emission. To account for disturbance due to the turbidity of the growth medium, each reading was blanked with the non-inoculated growth medium.

Initial and final levels of total nitrogen (TN), ammonium nitrogen (NH_4^+-N), total phosphorus (TP) and chemical oxygen demand (COD) were analysed using cuvette tests (HACH) and spectrophotometer (HACH DR3900). TN was determined using cuvette tests LCK438, LCK338 and LCK238. NH_4^+-N was determined using LCK302 and LCK303. TP was determined using LCK348 and LCK350. Finally, COD was determined using LCK114 and LCK314.

pH

pH was monitored using pH1100L (VWR) and maintained around 7 with 0.1M NaOH or 0.1M HCl. Due to a delayed delivery of HCl, Citric acid was used for pH regulation in *phase I.*

3.5 Analytical methods

The statistical significance among the different batches was determined using a two-tailed Student's t-test, with a 95% confidence interval.

4 Results and discussion

4.1 Microalgae growth and general observations

The growth of microalgae in various dilutions of AnBW was monitored over a period of 14 days in *phase I*. The experimental set-up included dilutions of 100%, 50%, 30%, and 10% AnBW, with and without CO_2 enrichment. Although the results from using OD_{680} to estimate biomass was not desirable due to the large background noise, Figure 4 shows the visual results by colour, with successful growth in *50% AnBW w. CO₂, 30% AnBW w. CO₂, 10% AnBW w. CO₂*, and one of the two batches *10% AnBW w/o CO₂.*

Figure 4: Phase I, day 14. The dilution of AnBW:DI from left to right (in pairs): 100%, 50%, 30%, and 10%. The bottles at the lower row are enriched with CO2.

The darkest green, indicating the highest biomass, was found in *50% AnBW w. CO₂.* This was therefore the dilution that was used in *phase II*. With an initial concentration of 237 mg/L NH_4^+-N, it is almost twice as high as the optimum range found by Collos and Harrison (2014), which instead would suggest that 30% AnBW would yield the highest biomass. However, another visual observation is that the batches *30% AnBW w. CO₂* seemed to have its peak in biomass around day 10. At this stage, 30% *AnBW w. CO₂* and *50% AnBW w. CO₂* had the same dark green colour. While the intensity of the green stayed throughout the experiment for *50% AnBW w. CO₂,* it faded for *30% AnBW w.*

CO_2. Due to the uncertainties in biomass production between these two batches, a control batch of *30% AnBW w. CO_2* was added to the second experiment.

The high initial ammonium concentration probably inhibited the absence of growth in the 100% batches. If pH had been more closely monitored and kept around 7, growth would not necessarily be inhibited. As described in 2.3.1 above, *Chlorella vulgaris* did successfully grow even when ammonium levels reached 500 mg/L if the pH was continuously maintained around 7 (Jiang et al., 2021).

Figure 4 also shows that some batches saw significant evaporation, most likely due to complications with regulating the individual airflow. However, no significant difference was found in nutrient reduction between the batches where the evaporation differed the most (i.e. *50% AnBW w. CO_2* and *10% AnBW w. CO_2*). For *phase II* the problem was partly solved by installing flow valves at the inlet of each bottle, see Figure 5. A future experiment could either use perforated plastic film or larger working volumes to tackle problems with evaporation.

Figure 5: Phase II, day 14. From upper left: 30% AnBW, ref: Z8, and ref: no algae (contaminated). From lower left: 50% AnBW w/o TE, 50% AnBW w TE, and 50% AnBW unsterilised.

Figure 5 also shows that the reference without inoculation of microalgae was contaminated, as microalgal growth was observed from day 10. The reduction can therefore not be derived from non-algal processes, and these results are excluded from the study.

Differences in growth behaviour of the unsterilised batches can be seen in Figure 5, and even more pronounced in Figure 6, with flocculation of microalgae unseen in the other batches. A study by Lee et al. (2013) found that bacteria play a significant role in the flocculation and sedimentation of microalgae. The presence of bacteria enhanced flocculation of *Chlorella vulgaris* from 2% to 94%, and the study suggests that it is the bacterial extracellular polymeric substances (EPS) that enable this process. The result from this study supports those of Lee et al. Given that harvesting microalgal biomass is one of the largest bottlenecks in microalgal production, looking further into these findings could be of great value to the field.
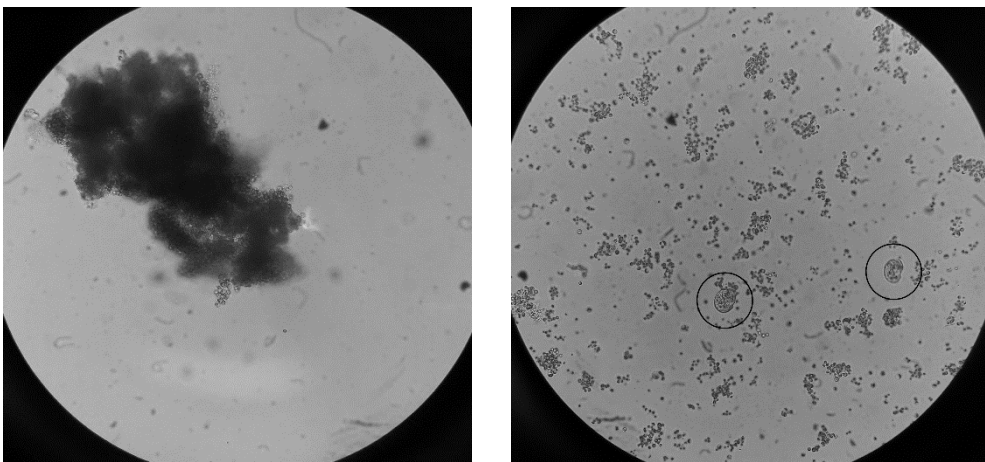

Figure 6: Flocculation in unsterilised batch (left) compared to dispersed algae in sterilised batch (right). Protozoa were present in the latter (marked with circles), but not in the unsterilised batch. Viewed under microscope at x40 (Olympus, model unknown).

4.1.1 CO_2 enrichment

Without the enrichment of CO_2, only one of the 10% batches had microalgae growing at day 14 in *phase I*. This indicates that the AnBW itself is carbon-limited and that even if growth seems to be possible, the low C:N/P ratio is creating a fragile system. With the enrichment of CO_2, all batches except 100% AnBW saw successful growth of *Chlorella vulgaris*. This supports the

necessity of maintaining the culture's C:N:P ratio close to that of the Redfield ratio (106:16:1).

In this experiment, CO_2 was added continuously, even during the dark period. As it can only be utilised during photosynthesis, a real-world example should optimise CO_2 consumption by scheduling enrichment to light period only.

4.1.2 Contamination

The experiments were conducted in a non-aseptic environment. Microbial contaminations are therefore possible and even likely. Figure 6 shows contamination of protozoa in a sterilised sample (*50% AnBW w. TE*), but not in the unsterilised (*50% AnBW unsterilised*). However, bacterial contamination was not possible to detect at this magnification (x40), why its presence cannot be excluded. Further discussion on contamination will follow in 4.3.

4.1.3 pH

pH fluctuates naturally because of microalgal growth. During photosynthesis, they consume CO_2 and release oxygen, which leads to an increase in pH. The reverse can be seen during dark periods when microalgae are respiring. The addition of CO_2 should then compensate for this increase and could be used for pH regulation. This is partly reflected in Figure 7, presenting the pH levels over the two experiments. The first experiment showed a quite large variation in pH regardless of the enrichment of CO_2 or not. However, the large increases on day 7 and day 10 can be explained by the CO_2 containers running dry. The variations in pH for the experiments with CO_2 enrichments are most likely connected to the physical properties of CO_2 and poor mixing with air. The CO_2 was added only by a connection to the air hose, not in a mixing chamber. CO_2 is substantially heavier than air, so the turbulence in the air hose might not be sufficient to mix the gases, and the different batches were therefore not exposed to the same concentrations of CO_2. This calls for the importance of having a thorough mixing infrastructure when conducting these kinds of experiments.
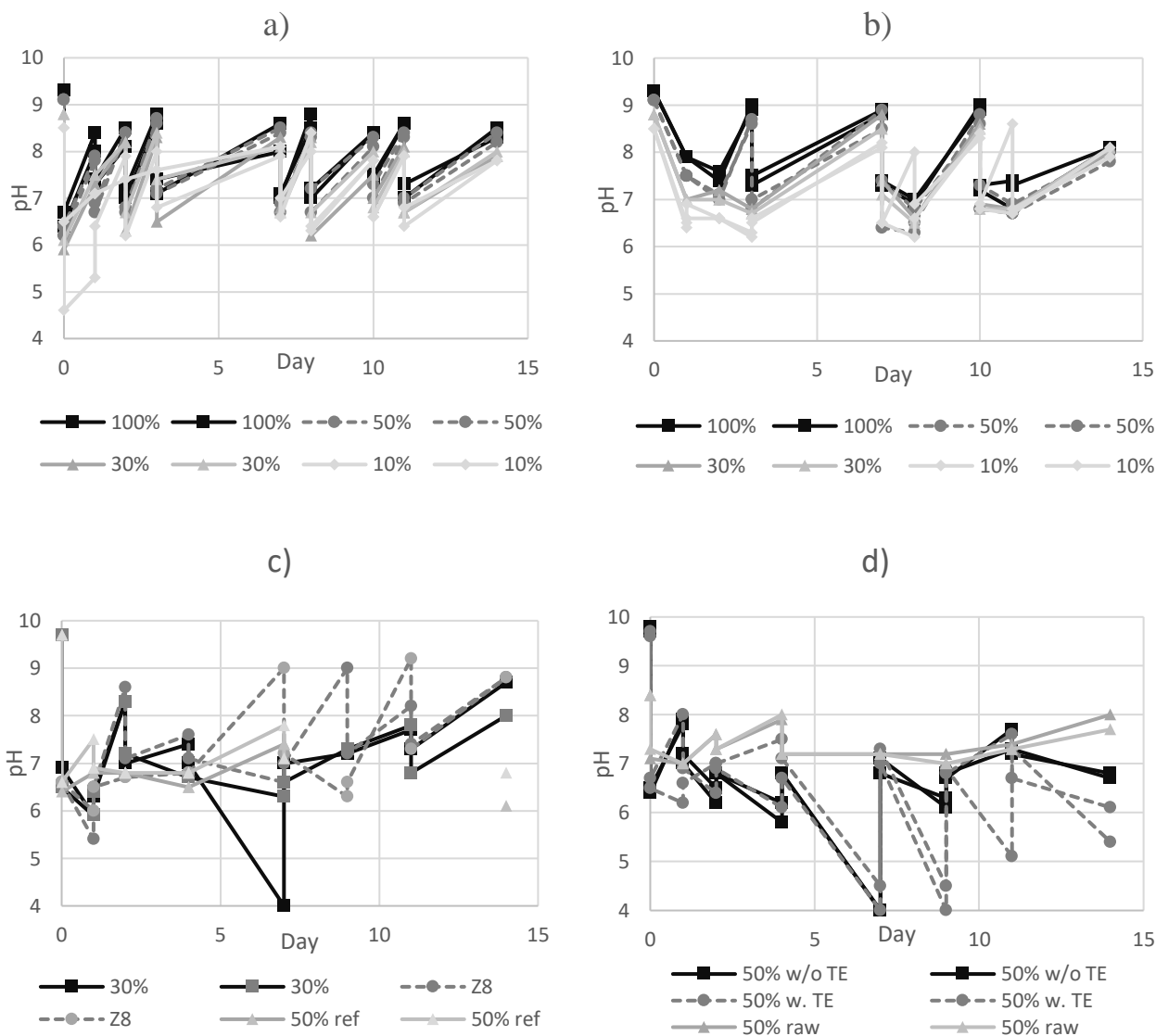
Figure 7: Fluctuation of pH during the experiments. a) phase I without enrichment of CO_2, b) phase I with enrichment of CO_2, c) and d) phase II

Why pH is increasing in the batches even without microalgae growing, Figure 7 a), could indicate a high buffering capacity of the AnBW. *Phase II* saw instead both an increase and decrease in pH, see Figure 7 c) and d). The main difference in the AnBW in the different experiments is initial COD levels, which were substantially lower in the second experiment. To enable a precise

pH adjustment, a thorough analysis of the alkalinity of the raw AnBW would be beneficial.

4.2 Biomass production

Phase I and *phase II* used different approaches in estimating growth and biomass production. The first used OD_{680nm} and the second measurements of chlorophyll-a. These two approaches are discussed in section 4.2.1 and 4.2.2 respectively. This is then followed by a discussion on the use of AnBW as a growth medium in 4.2.3.

4.2.1 Phase I - Optical density

In theory, optical density is a convenient indirect method for the estimation of biomass concentrations. By correlating OD readings with samples of known DW, the correlation curve can be used for a quick and easy understanding of growth development. This method does not lead to water losses due to sampling, as is the case for measurements of DW which could introduce problems in laboratory experiments when batches often hold small volumes. Figure 8 shows a strong correlation between OD_{680nm} and DW, with an R^2 value of 0.9941.

Calibration curve

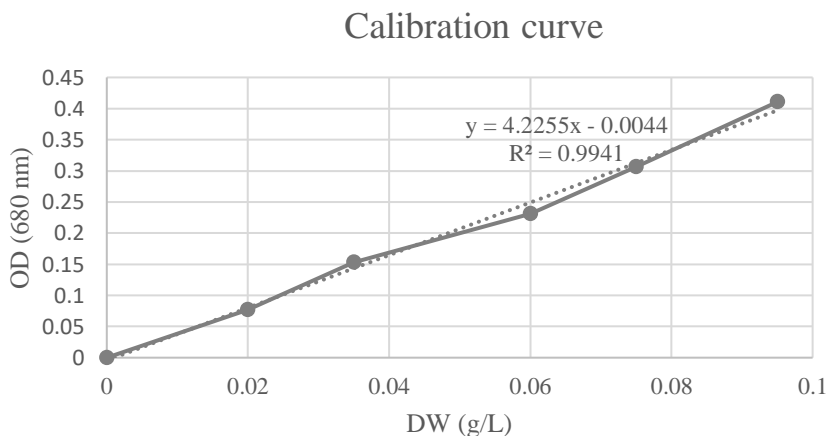$y = 4.2255x - 0.0044$
$R^2 = 0.9941$

Figure 8: Calibration curve between OD (680 nm) and DW (g/L).

Looking at Figure 9, one can see an increase in biomass concentration over time in all batches. Knowing that there was only successful growth in seven out of sixteen batches in *phase I,* as mentioned in 4.1, something else is causing

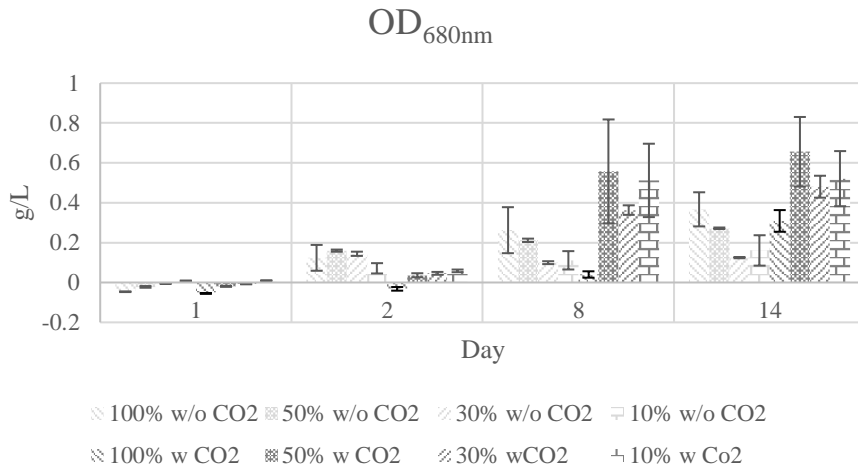readings of OD_{680nm}. This could potentially be contamination of bacteria, protozoa, or other microorganisms.

Figure 9: Estimation of biomass in phase I using OD_{680}.

The results show that using OD as a measurement for the estimation of microalgae growth should be done with caution. It seems to only be valid if the growth media is transparent or not susceptible to change of background turbidity, where one also can ensure a sterile environment with no contamination.

4.2.2 Phase II – Chlorophyll

A different approach for estimating growth was used in *phase II,* which measured the fluorescence of chlorophyll rather than absorbance. Figure 10 shows the highest final yield (7945±169 µg/L) in *50% AnBW w. TE*, although the difference was not statistically significant compared to *50% AnBW w/o TE* (7210±811 µg/L). The cultures cultivated in a conventional growth medium, Z8, had its peak around day 11 (8242±1395 µg/L).

In *phase I*, a visual observation of a peak at day 10 followed by a decline was made for *30% AnBW w. CO₂*. This was not seen in *phase II,* where the experiment was replicated. However, it did enter the stationary phase earlier than *50% AnBW w. CO₂*.

The graph also shows an initial lag phase, with exponential growth from day 4. Having such a long lag phase would require extensive HRT in real-world

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applications, with high associated costs. To counteract this, one should focus future research on plug-flow reactors instead of batch reactors, acclimatisation of algae culture before inoculation, and larger inoculation ratio.
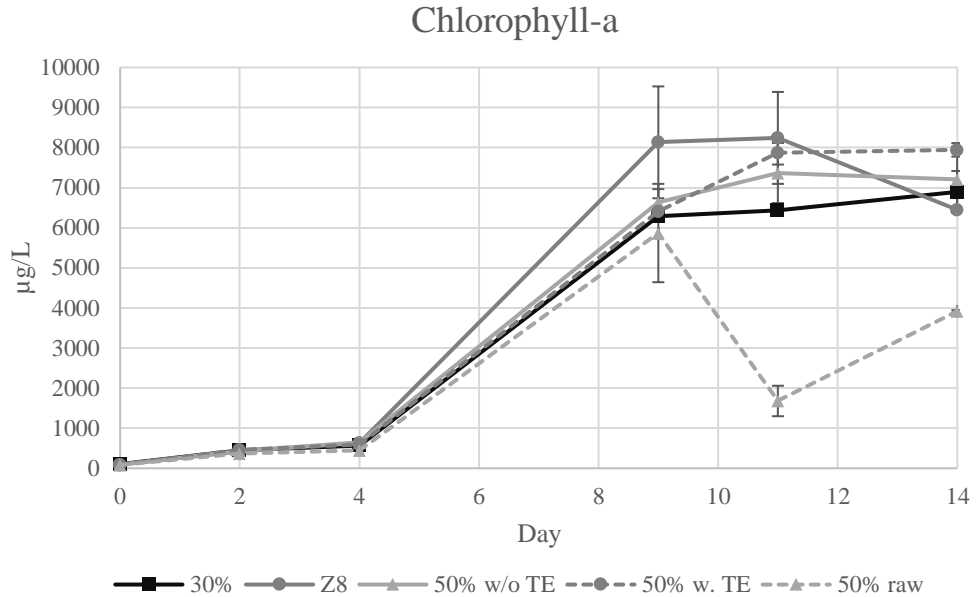

Figure 10: Chlorophyll-a for estimation of growth in phase II, error bars represent std deviation.

No measurements were made on day 7, due to the limitations of the machine to measure in high turbidity. After this point, the samples were diluted to below 200 NTU (dilution: x15) as per recommendation from the manufacturer. This method is not suitable for cultures with large flocculation, so the results from the unsterilised batches should be dismissed.

4.2.3 AnBW as a growth medium

The findings from *phase I* and *phase II* suggest that AnBW could successfully replace synthetical growth mediums in some applications if external carbon is added. *Phase II* compared the growth of *Chlorella vulgaris* in AnBW to a conventional growth medium, Z8, and saw no statistical differences in the biomass produced from batches grown in AnBW to those grown in Z8. All batches did also produced yields considerably higher than in a study where *Chlorella vulgaris* was grown for biomass production in municipal sludge run-off, which achieved a concentration of 2378 µg/L (Pacheco et al., 2021).

Another study, aiming to optimise different synthetic growth mediums for biomass production, achieved growth only somewhat above, between 10 000 and 20 000 µg/L for the same period (Ilavarasi et al., 2011).

4.3 Performance of microalgae-based wastewater treatment

Microalgal reductions to levels that adhere to regulatory requirements have been found across many studies, as discussed in chapter 2. Although large reductions of nitrogen and phosphorus can be seen in some batches of the experiments conducted in this study, the variations are large, and the conclusions drawn can not support microalgal treatment as the only process step before discharge. However, if the water is continually directed back to the main treatment, it could bring the benefits of significantly decreasing the nutrient load on the treatment plant while simultaneously producing valuable biomass.

The chapters below will describe the removal of COD, nitrogen, and phosphorus.

4.3.1 COD removal

Figure 11 presents the reduction of chemical oxygen demand for the different experimental set-ups in *phase I.* For *50% AnBW w/o CO₂* enrichment and all *10% AnBW* batches, the test reagents (LCK114 and LCK314) turned green under digestion, indicating incorrect measurements. Doing the tests again returned the same error, and these results are therefore not included in the report.

COD reduction was observed in all batches where measurements were successful. The reduction was stable between 42-49%, see Figure 11, and no significant difference can be seen between those with successful growth of microalgae and those without. Therefore, no microalgal reduction of COD can be derived. This opposes previous studies that have seen a large reduction of COD through microalgal treatment of wastewater (Nagarajan et al., 2020) but could be explained by a sufficient rate of CO_2 enrichment covering the carbon needs of *Chlorella vulgaris.* The enrichment, and consumption, of CO_2 does not affect the COD. The results indicate that the microalgae only grew

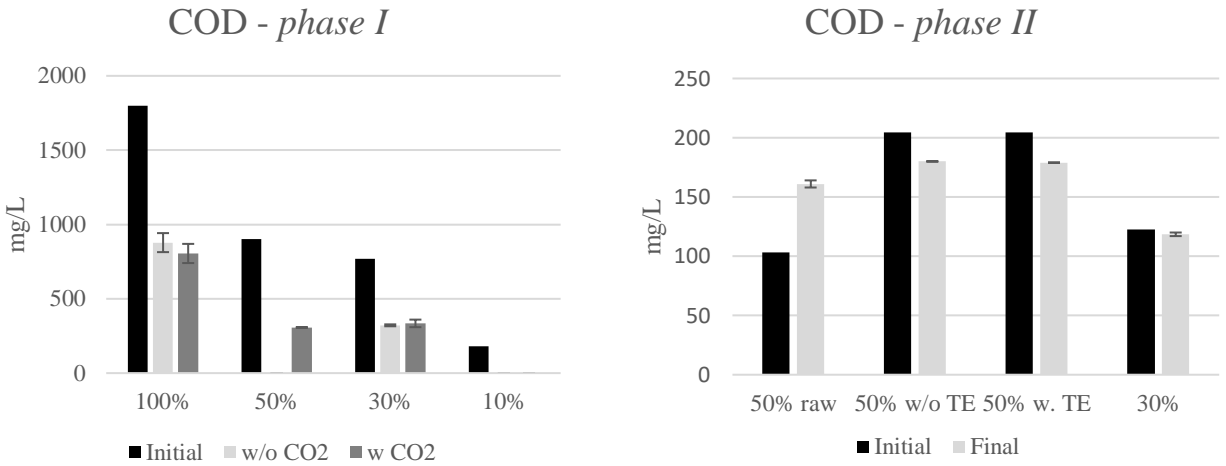photoautotrophic, as heterotrophic growth utilises organic carbon and would lead to a reduction of COD.

COD - *phase I*

COD - *phase II*

Figure 11: COD reduction over the two experiments. Phase I, without measurements for 50% w/o CO₂ and 10% w and w/o CO₂ due to technical issues and phase II.

Although the reduction of COD could be explained by contamination of other microorganisms, it could also be a result of methane (CH_4) evaporation. When the raw BW is digested, it is saturated with CH_4 in the process. Due to the low solubility in water, the CH_4 will be released during aeration. The stable reduction across all batches strengthens the hypothesis of an underlying physiological process rather than a biological process responsible for COD reduction.

The biggest difference in initial AnBW characteristics between the two experiments is found in the concentrations of COD, with a difference of almost 77%. Both experiments used the same sample of AnBW, but the second experiment started three weeks after the first. During this time, the raw AnBW was stored at 4ºC in a closed container, filled to one-third, before being filtered and autoclaved. This substantial drop in COD during shelf time was surprising. One reason for this could be due to sedimentation, as COD is mostly bound to organic particles, or further evaporation of CH_4.

Furthermore, Figure 11 illustrates only a small reduction of COD in the second experiment (3-12%). It also shows unlikely behaviour for *50% AnBW raw*,

which has an increase of COD over the experiment. This is more likely to be a result of an unknown interfering substance from the GAC filters on the measurement, which were later neutralised during autoclaving.

4.3.2 Nitrogen removal

As can be seen in Table 1, most nitrogen in the AnBW is in the form of ammonium nitrogen (NH_4^+-N), especially in *phase I* (84-95%, compared to 71-83% in *phase II*). Figure 12 shows that reductions can be seen in all batches, but microalgae only seem to provide a significant reduction in the 50% and the 10% batches. Why this is not seen in the 30% batches, which also saw successful microalgal growth with the enrichment of CO_2 but not without, could potentially be explained by an earlier peak in biomass concentration, which resulted in decaying organic matter that releases nitrogen back to the water column. This should be further investigated in future research as it, if correct, highlights the importance of a controlled sludge retention time (SRT) and harvest to the peak of biomass concentration.

For NH_4^+-N, all batches saw a reduction between 49% – 56%, except *50% AnBW w. CO_2* and *10% AnBW w. CO_2* which had a reduction of 99% and 93% respectively. TN had an overall reduction between 35% and 58%, with the reduction increasing to 88% and 71% for the *50% AnBW w. CO_2* and *10% AnBW w. CO_2*. Why such a large reduction can be seen in all batches could be explained by bacterial contamination or by the physical process of ammonia stripping. Figure 7 shows that the fluctuations in pH during *phase I* occasionally approach 9, meaning that ammonia stripping can occur. Although ineffective at this low pH, it could still cause large nitrogen reduction given the two-week exposure.
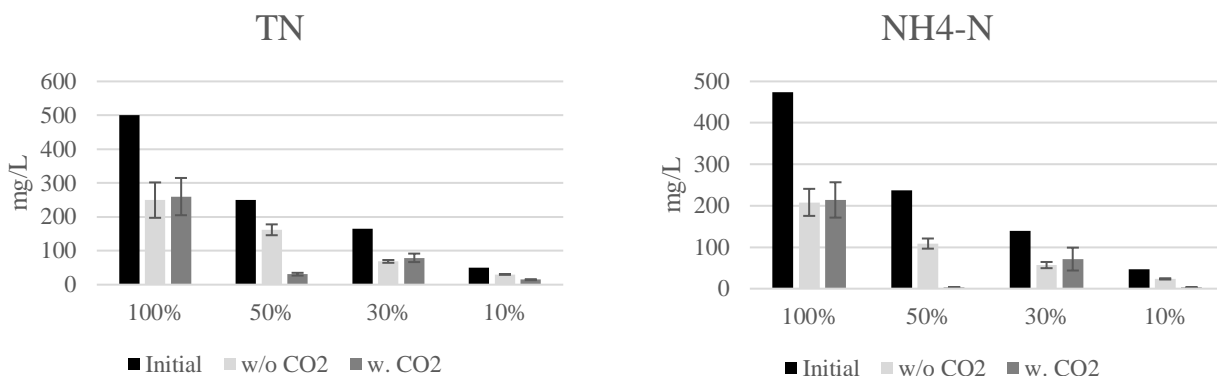
Figure 12: Nitrogen removal in phase I.

The presence of bacterial contamination is indicated by an increase of non-ammonium nitrogen (TN minus NH_4^+-N) in all but the 30% batches. While TN is the combined concentrations of nitrogen, such as NH_4^+-N, organic nitrogen compounds (proteins, amino acids, etc), nitrite (NO_2^-) and nitrate (NO_3^-), it is the two latter forms that are most likely to increase under aerobic conditions due to the bacterial process of nitrification. The 100%, 50%, and 10% batches saw an increase in non-ammonium nitrogen of 27.5 mg/L to 43.7±2.3 mg/L, 13.7 mg/L to 40.3±12.9 mg/L, and 2.7 mg/L to 9.2±2.5 mg/L respectively. The same result for the 30% batches was, however, reversed with a decrease from 25.7 mg/L to 9.8±2.0 mg/L. Worth noting is that this batch had an initial lower NH_4^+-N to TN ratio compared to the other (84% to 95%).

The reductions of nitrogen in *phase II* are considerably lower than in *phase I*. Figure 13 shows a reduction of NH_4^+-N between 40-65%, and for TN between 40-64%. Although a somewhat larger reduction of nitrogen for *50% AnBW w. TE* in relation to *50% AnBW w/o TE* (50% and 40%), the reduction was not statistically significant. That was however the reduction of nitrogen in *50% AnBW raw* compared to the two others. It shows that microalgae-bacteria consortia could be beneficial for nitrogen reduction.
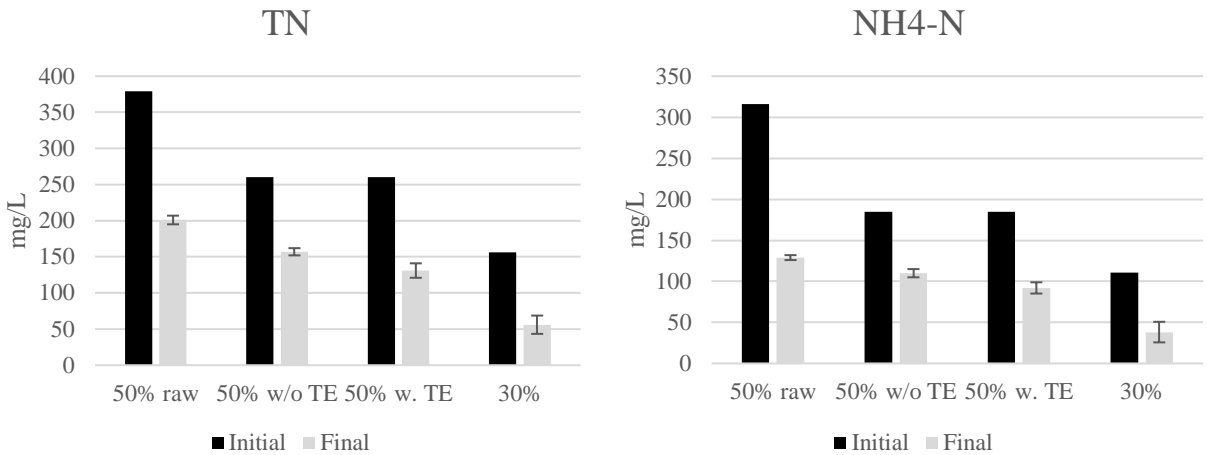
Figure 13: Nitrogen removal in phase II.

The lower reduction in *phase II* could be connected to pH, which was generally maintained well below 9 and therefore not as susceptible to ammonia stripping. It could also be an effect of a very high N:P ratio. As discussed in 2.3.1, removal efficiencies decreased above a ratio of 13. While both experiments were conducted on N:P >13 (see Table 1), the second experiment had a substantially higher N:P ratio. In future studies, the addition of phosphorus should be considered. One way to do so is to use the phosphorus-rich greywater for dilution of the AnBW.

The overall results are somewhat below expected, as previous research has found an almost complete reduction (Nagarajan et al., 2020). However, most studies have been made on wastewater with substantially lower nitrogen content.

4.3.3 Phosphorus removal

Figure 14 shows the phosphorus reduction in *phase I, with a n*oticeable reduction only be found in the batches where microalgae were successfully growing. However, no statistical significance is found for the microalgal reduction of phosphorus due to the large variance within the groups. This could be a result of poor filtering with residual microalgal biomass in the water phase which would overestimate readings of total phosphorus. Previous research suggests phosphorus removal up to 99% (Nagarajan et al., 2020).
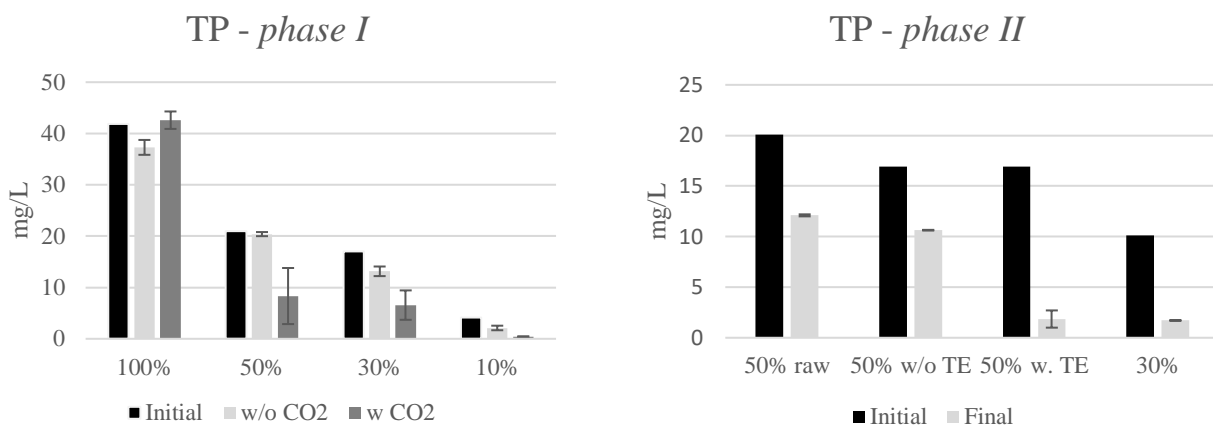
Figure 14: TP removal in phase I and phase II.

Figure 14 further illustrates the phosphorous reduction in *phase II.* Interestingly, here is where the largest impact of external input of trace elements saw effect. For *50% AnBW raw* and *50% AnBW w/o TE*, the reduction reached 40% and 37% respectively, while the reduction when TE was added reached 89%. A similar reduction was, however, achieved by *30% AnBW*, which reduced 83% of total phosphorus.

In the batches without successful microalgae growth, there was almost no phosphorus reduction. Microbial contamination, bacterial or protozoan, also requires phosphorus to grow. The result does not exclude contamination but suggests that non-algal growth is minimal.

4.4 External input of trace elements

Phase II of the experimental part of this study investigated the necessity of adding external input of trace elements to the AnBW, and if supplementing TE could enhance growth and nutrient removal efficiency. The results showed that the addition of trace elements generally led to better performance compared to the control group without trace elements. However, the most notable improvement was observed in phosphorus reduction, which was the only parameter to show a statistically significant difference.

Despite the enhanced performance in phosphorus reduction, the results for nitrogen removal were not as conclusive. Although there was a slightly better

performance in nitrogen reduction in the batches with added TE, the differences were not statistically significant. The reduction of NH_4^+-N varied between 40-65% across all batches, with a marginally higher reduction in the *50% AnBW w. TE* compared to *50% AnBW w/o TE* (50% vs. 40%). This suggests that while TE does contribute to nutrient uptake, its impact on nitrogen removal may not be as pronounced under the conditions tested.

For the experiment, *Trace metal mix A5 with CO* (Merck) was used, designed for the maintenance of microalgal cultures. Although covering a wide spectrum of elements, other TE could still prove beneficial for microalgal growth.

4.5   Synergies, implementation, and challenges

Using the reject water from AnBW when cultivating microalgae offers several synergetic effects that enhance both economic and environmental sustainability. These advantages include lower operational costs, reduced nutrient loads on treatment plants, efficient nutrient recovery, and contributions to a circular economy.

One of the largest operational costs associated with the cultivation of microalgae is the production of synthetic growth medium (Ezea and Ogbonna, 2023). AnBW is rich in the nutrients essential for microalgae to grow, and this report shows that this waste stream has the possibility of producing microalgal biomass in the same range as synthetic growth mediums. By coupling the microalgal and wastewater industries, both parts could benefit from reducing costs and generating new income. The operational expenses connected to microalgal production could be severely reduced, and the WWTP's nutrient load would decrease in tandem. This could lead to a potential reduction of HRT and chemical usage of the conventional treatment plant. Section 2.5 explored the potential valorisation that can be done from microalgal biomass.

The revision of the Urban Wastewater Treatment Directive (UWWTD) emphasises reducing greenhouse gas emissions and achieving energy neutrality by 2045. A conventional treatment method generates CO_2 emissions due to bacterial respiration. Applying microalgal treatment to certain waste streams could prove to be an efficient way to reduce overall emissions, as microalgae can store large amounts of greenhouse gas during photosynthesis.

Using AnBW to produce biomass has a clear disadvantage in terms of availability. It is almost always mixed with other waste streams before reaching the treatment plant. RecoLab in Helsingborg is the only facility in Sweden that collects the blackwater separated from different streams. However, other actors in society also collect blackwater separately due to convenience. Trains, planes, and campsites often collect blackwater in a separate unit before it is released to the municipal collection network. This could be a valuable resource for microalgal biomass production, enabling the production to be placed in many parts of the country.

4.5.1 Limitations and challenges

One of the most prominent challenges of using microalgae for the treatment of wastewater is harvesting the biomass. In line with previous studies (Lee et al., 2013), this report suggests enhanced flocculation and sedimentation when cultivation is performed as a microalgae-bacteria consortium. If this process can be controlled, it does not only solve the harvesting bottleneck. A review on the topic by Gonçalves et al. (2017) found that the use of consortiums, or polycultures, can lead to symbiotic interaction enhancing the overall nutrient uptake and more resilient systems. Furthermore, the energy-intensive and costly process of sterilisation could be removed.

Another common critique of microalgae-based treatment of wastewater is the large footprint it requires. AnBW has the benefit of having a low hydraulic load but is exceptionally rich in nutrients. By combining conventional methods for the main treatment, and microalgal treatment for certain waste streams, benefits can be harvested even when space is a limiting factor.

This study was conducted in a laboratory with almost full control of the growing conditions. Future research should focus on the applicability of the technique on a larger scale. These applications should consider using reused water from the WWTP, or even greywater when diluting the AnBW, rather than distilled water as in this study. Neither is a continuous enrichment of CO_2 necessary, as enrichment during the dark period will only serve to acidify the water and evaporate CO_2 into the atmosphere.

5 Conclusions

The implementation of microalgae-based treatment of anaerobically digested black water (AnBW) presents a range of opportunities and challenges. This study has demonstrated the viability of using AnBW as a growth medium for the species *Chlorella vulgaris*, showing promising results in nutrient recovery and biomass production. The controlled laboratory experiments revealed that CO_2 enrichment is crucial for microalgae growth, while the addition of trace elements notably improves phosphorus removal efficiency. However, the high nitrogen-to-phosphorus ratio in AnBW indicates phosphorus limitation and suggests the need for external additions for optimised growth and nutrient uptake. Although the results from this study do not support microalgae-based treatment as the only process step before discharge, integrating microalgae in black water treatment could substantially decrease the nutrient load on treatment plants and contribute to a circular economy. Future research should focus on optimising the harvesting process, exploring the potential of microalgae-bacteria consortia, and evaluating the economic feasibility of large-scale applications. These findings highlight the potential of microalgae not only as a sustainable solution for wastewater treatment but also as a valuable resource for biomass production.

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