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Abstract

Microalgal biotechnology has been growing and expanding tremendously in recent years in various sectors. Being metabolically flexible, microalgae can adapt to demanding and even extreme growth conditions. Multiple applications for microalgae itself and value-added products, obtained from algal biomass, are described in the literature. Meanwhile, costly nutrients for algal growth media make it economically inefficient and commercially not viable for scale-up production. To mitigate the economic impact of the cultivation stage on the end-product cost, wastewater from different industries can be used as a substitution for synthetic nutrient media.

The current research aimed towards the possibility to use ACWW as an alternative nutrient media for microalgae cultivation. Three microalgae species (*Scenedesmus*, *Chlorella*, and *Nostoc* spp.) were cultivated in nutrient media variants with different ACWW fractions (100%, 75%, 50%, and 25%) diluted with BBM. Growth parameters like optical density, biomass accumulation, and specific growth rate were monitored with different frequency in accordance with research goals. The highest biomass concentration under both constant and limited aeration was observed during cultivation of *Scenedesmus* sp. and was quantified as 0.991±0.073 g/L and 0.637±0.014 g/L respectively.

Simultaneously, wastewater treatment efficiency was monitored and evaluated in all cultivated samples by means of TN and TP removal. The peak TN and TP removal efficiency was observed while cultivating *Scenedesmus* sp. under constant aeration and amounted to 92.9% and 99.3% respectively. Relatively high nutrient removal results were obtained during *Chlorella* sp. cultivation under limited aeration which amounted to 40.4% and 98.2% for TN and TP respectively.

Subsequently, harvested microalgal biomass were subjected to the quantification of essential biocomponents (carbohydrates, proteins, and lipids) for further utilization in various applications. The results showed that dominating accumulated fraction in all the biomass samples was carbohydrates ranging from 54.2±3.6% to 62.3±6.4% in *Scenedesmus* sp. cultivated under limited aeration and from 29.4±3.0% to 44.5±18.7% in *Nostoc* sp. cultivated under limited aeration.

Overall, the results obtained through this approach provide the basic information towards the future applications of the biomass in various sectors such as biopolymer production.

Abbreviations

ACWW	Aquaculture Wastewater
ATP	adenosine triphosphate (organic compound that provides energy)
BBM	Bold's Basal Medium
BMP	biomass production
BOD	Biological Oxygen Demand
BSA	Bovine Serum Albumin
COD	Chemical Oxygen Demand
CSTR	Continuous Steered-Tank Reactor
DCW	Dry Cell Weight
dH_2O	distilled water
DNA	deoxyribonucleic acid (polymeric molecule: development, functioning, growth & reproduction of almost all known living forms on Earth)
LED	Light-Emitting Diode
N	Nitrogen
NIVA	Norsk institutt for vannforskning / Norwegian institute for waterscience
OD	Optical Density
OECD	The Organization for Economic Co-operation and Development
P	Phosphorus
PHAs	Polyhydroxyalkanoates
RAS	Recirculating Aquaculture System
RNA	ribonucleic acid (polymeric molecule: coding, decoding regulation & expression of genes)
SDG	Sustainable Development Goals
SS	Settleable Solids
TN	Total Nitrogen
TP	Total Phosphorus
TS	Total Solids
TSS	Total Suspended Solids
TVS	Total Volatile Solids
UiS	Universitetet i Stavanger / University of Stavanger
UV	ultraviolet, form of electromagnetic radiation
VSS	Volatile Suspended Solids
WW	Wastewater
WWT	Wastewater Treatment

Symbols

%	percentage
&	and
°C	centigrade
€	Euro
±	sign used for adding standard deviation values
≈	approximately
abs	absorbance
atm.	atmosphere
day^{-1}	1 per day
μ	specific growth rate
μL	microliter
μm	micrometer
μmol/m^2/s	micromole per square meter per second
g	gram
×g	relative centrifugal force
g/g	gram per gram
g/L	gram per liter
g/L/day	gram per liter per day
kg	kilogram
L	liter
mg/g	milligram per gram
mg/L	milligram per liter
mg/mL	milligram per milliliter
mL	milliliter
min	minute
N	normal
nm	nanometer
NOK	Norwegian Krones
rpm	revolutions per minute
sp./spp.	specie / species
USD	United States dollar
v/v	volume per volume

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1. Introduction

Nowadays humanity is facing several challenges that can potentially lead to serious consequences both for human health and the environment. Constantly growing population and utilization of various products in daily life results in gradually growing volumes of liquid and solid wastes. Clean and fresh water becomes one of the most demanding and valuable natural resources, while lack of food raw materials is contributing to limitations of food supply worldwide (Ummalyma et al., 2022). At the same time, greenhouse gases emissions leads to global warming, creating high demand for progressive and sustainable technologies to provide complex and efficient solutions towards resolving abovementioned challenges (Farooq et al., 2021).

The aquatic environment is found to be one of the most vulnerable among the others. Sooner or later everything that we produce, consume, excrete, or pollute ends up in the water (Lee Ii et al., 2008; Samadi et al., 2022). In 2020-2021, the evaluation for fresh water resources have been provided due to its unequal distribution and value worldwide (United Nations, 2021). The United Nations concluded that lack of political attention to this problem leads to unsustainable use and degradation of water supplies. Immediate actions should be applied to ensure water preservation and safety for further use.

At the same time, rapid population growth and a limited amount of food resources constantly increase seafood consumption on a global scale as a quickly derivable and recoverable protein source. Thus, aquaculture sector is growing along with demand for it, reaching an annual average of 4.5% (Cardoso et al., 2021). Currently, aquaculture industry provides around 50% of the world's food supply (Hawrot-Paw et al., 2019). Along with the extensive growth of seafood production, waste management issues are becoming a more relevant threat and require immediate action.

It is known that release of untreated or poorly treated aquaculture wastewater leads to an increase of nutrient concentration in natural water bodies (Hawrot-Paw et al., 2019). Pathogenic microflora in these conditions is likely to grow and affect the health of aquatic species and humans in a longer perspective. At the same time, uncontrolled proliferation of microalgae will lead to eutrophication and hypoxia in affected areas, eventually increasing fish mortality. Lakes and river estuaries in this regard are the most endangered due to low or limited dispersion rates (Viegas et al., 2021).

Another undoubtedly major challenge for our society is an overwhelming volume of petroleum-based plastics. Annual world demand is about 140 million tons of plastics to sustain population habits. Being non-biodegradable or partially biodegradable, plastics and its derivatives accumulate in the environment, especially in its aquatic part, when 8 million tons of plastics leak into the ocean per year (Rajpoot et al., 2022). As a result, carbon capture ability of phytoplankton is decreased, and aquatic species are seriously affected through ingestion, entanglement, and suffocation (Nanda & Bharadvaja, 2022). There is a desperate need for alternatives that will provide safe use of the environment and, at the same time, sustain the population's habits. Therefore, research and industrial institutions are looking for biopolymers that will attain properties similar to conventional plastics while having lower environmental impact and thus will be able to replace it on a large industrial scale (*Figure 1*).

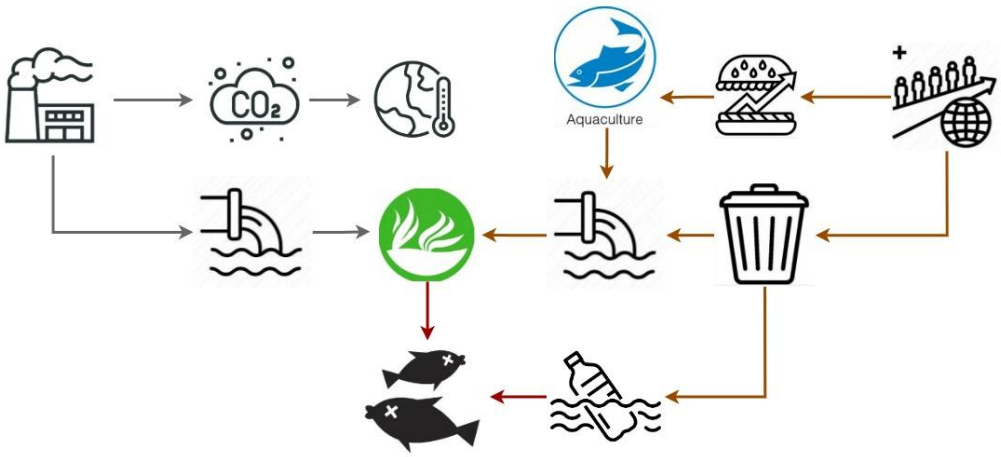

Figure 1. Major environmental and social challenges resulting in aquatic pollution. Main environmental hazards and their consequences are illustrated.

Within the last decade, the interest in using microalgae in wastewater treatment increased significantly due to efficient nutrient removal properties without addition of chemicals, O_2 generation and CO_2 mitigation during the growth process (Lutzu et al., 2021; Mohsenpour et al., 2021; Oviedo et al., 2022; Wang et al., 2017). Microalgae are also studied as a treatment unit in aquaculture WWT (*Figure 2*) (Cardoso et al., 2021; Tom et al., 2021; Viegas et al., 2021). In the present thesis, an attempt to cultivate different microalgae species on ACWW as a nutrient medium to compare its sustainability and biorefinery aspects has been performed. This study will potentially make it possible to create a nexus between water, food, environment, and energy chain.

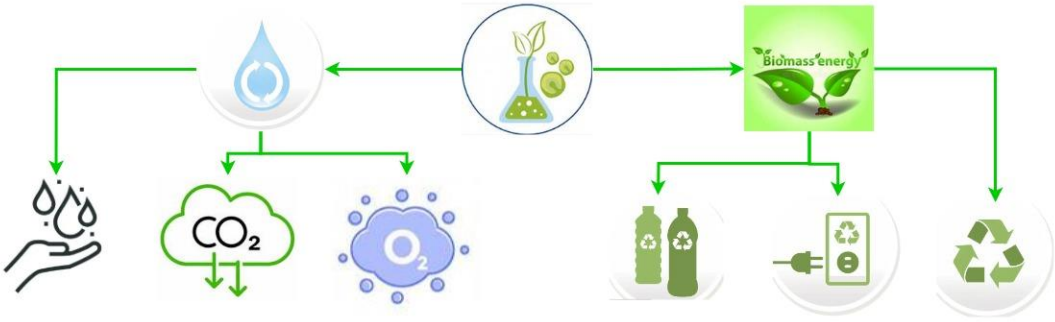

Figure 2. Remediation and biorefinery perspectives of microalgae biomass. Water quality improvement possibilities and biorefinery approaches are suggested.

A possible scenario could include the growth of land-based aquaculture industry which can contribute to solving a food deficiency problem but at the same time creating challenges for the environment through increasing eutrophication risks through its WW effluents, solid wastes generation, and decreasing water quality (Cardoso et al., 2021; Kashem et al., 2023). Including microalgae cultivation step in ACWW treatment could result in significant nitrogen and phosphorus depletion in the effluent. In addition, microalgal biomass can be harvested and turned into value added products such as biofuels or even bioplastics that potentially will contribute to plastic pollution mitigation.

1.1. Scope of work

As it was mentioned in the Introduction chapter, there are several challenges regarding marine pollution that might be resolved with the use of microalgae cultivation in secondary or even primary effluent from land-based aquaculture plants. Simultaneously, current research can more or less contribute to Sustainable Development Goals (SDG) achievement, namely: Zero hunger (2nd goal), Clean water and sanitation (6th goal), Affordable and clean energy (7th goal), Sustainable cities and communities (11th goal), Responsible consumption and production (12th goal), Climate action (13th goal), and Life below water (14th goal).

The current study can become a part of the solution for the eutrophication hazard connected to effluent release from land-based aquaculture facilities through implementation of microalgae facilitated WWT. The pretreated or even raw ACWW can be used as an alternative nutrient media for microalgae cultivation. Biomass accumulated during the cultivation can be utilized for value-added products obtainment. Before algal WWT can be implemented in aquaculture industry with perspectives for utilization of biomass within circular economy ambitions, it is

necessary to identify the most efficient microalgal strains with high nutrient removal efficiency and rich biochemical composition.

1.2. Main objective

The main objective of the current research is to test ACWW as an alternative nutrient media for microalgae cultivation and to select microalgae species that will be the most suitable for efficient downstream application in bioplastic production.

1.3. Specific objectives

- Provide a characterization of aquaculture wastewater, collected at the land-based aquaculture plant, Tyslandsvik Aqua AS.
- Test feasibility of ACWW (25%, 50%, and 75% dilution ratios with BBM) as a nutrient medium for microalgae cultivation via biomass accumulation potential.
- Study the growth dynamics of selected microalgae species (*Scenedesmus*, *Chlorella*, and *Nostoc* spp.).
- Check microalgae based WWT efficiency and biochemical characteristics of harvested biomass.
- Suggest various applications towards biorefinery aspects.

2. Theoretical background

In this chapter we will discuss the main reasons for eutrophication in freshwater bodies, the potential of different wastewaters to be a substrate for microalgae cultivation, as well as conventional and microalgae-facilitated WWT. Together with that, microalgae itself will be described as a biological unit with its basic characteristics, growth requirements, cultivation techniques, and biorefinery perspectives. A short characterization of chosen for the experiment species will be provided.

2.1. Nitrogen and Phosphorus eutrophication and its environmental impact

Nitrogen (N) and Phosphorus (P) are known as essential macronutrients for life processes on Earth. These elements participate in protein and DNA synthesis, cellular growth, primary production, and reproduction. At the same time, the excess of N & P in marine environments can become a global problem, called eutrophication, due to chiefly the same reasons. Before extensive industrialization, the main sources of N & P were environmental erosion and leakage of dissolved elements in their organic forms, which sustained low concentrations in rivers and lakes. However, global anthropogenic activity has dramatically increased levels of Nitrogen and Phosphorus in water bodies through increased production and usage of synthetic fertilizers and industrial pollution (Shen et al., 2020).

Eutrophication causes algae to bloom, hypoxic and anoxic conditions, acidification in oceans which in its turn facilitate dead zones appearance, water quality reduction, increase in fish mortality, production of exotoxins, biodiversity decrease, habitat degradation, food chains alteration, losses of recreation amenities. In addition, the risk to human health increases, tourism possibilities become limited in affected areas which leads to economic losses (Ngatia et al., 2019).

2.1.1. Nitrogen

Nitrogen is an essential element for all life forms, but at the same time it is a limiting nutrient because not all the forms of N are bioavailable: nitrite, nitrate, and ammonium limit primary production in coastal aquatic environments. The nitrogen cycle contains both gaseous and non-gaseous dissolved and particulate forms, which allows the exchange with the atmosphere (Ngatia et al., 2019). Currently, the main source of nitrogen in the environment is anthropogenic

activity, synthetic N fertilizers, fossil fuels combustion, and manure application (Choi et al., 2004).

2.1.2. Phosphorus

Phosphorus is another essential and limiting nutrient for living organisms. This element is introduced in soil and aquatic environments in a range of organic and inorganic forms with remarkably diverse bioavailability (Condron et al., 2005). The pH is a key parameter that regulates P solubility and bioavailability for organisms. Unlike N, P does not exist in volatile forms which limits its distribution to neighboring ecosystems. However, the excess of P fertilizer application can result in pollution of freshwater bodies which will remain there in dissolved or particulate form. Main P pollution sites are industrial discharges, urban areas, construction sites, runoff from agriculture, and sewage (Ngatia et al., 2019).

2.2. Wastewater as a potential growth media for algae and water footprint

Any kind of water from a process or human activity that has been released and unused is called wastewater. The literature sources report that about 80% of produced WW are discharged without prior treatment worldwide (Oviedo et al., 2022), and in some countries, this percentage is as high as 90% (Lee et al., 2015). Such high volumes of unthreatened wastewater cause social, environmental and health problems for population and fauna (United Nations, 2021).

The United Nations World Water Development Report (2021) and Xiaogang et al. (2022) divided cumulative water consumption into several categories. According to their publications, the agricultural sector uses 69-70% of global freshwater resources, 19-22% of freshwater supply belongs to different industries, and domestic utilization of water occupies 8-12%.

At the same time, food production industry discharge 2-73 L of wastewater per 1 kg of produced unit (Ummalyma et al., 2022), while municipal wastewater generation variate between 265-375 L per capita per day and depends on several factors such as year season, weather, contribution from industry etc. (Lawton et al., 2014). In other literature sources the overall productional water footprint has been reported. Thus, water footprint for diary production can variate within 0.2-1 L water per 1 L of milk (Ummalyma et al., 2022), paper industries create water footprint as high as 10-50 L per 1 kg of paper (Gentili, 2014), while textile industries discharge about 200 L of WW per 1 kg of product per day (Khandare & Govindwar, 2015).

Composition of wastewater, originating from different industrial process, can variate significantly, and depends on the type of industry, its intensity, and operational processes (Ummalyma et al., 2021). Typical compounds of WW include different types of solid suspensions, organic and inorganic compounds, microorganisms, and other persistent organic pollutants (Amaro et al., 2023).

Industrial streams are known to contribute to environmental pollution, harm ecosystem and endanger human life. Main pollutants in most of the industrial effluents are nitrogen, phosphorus, and heavy metals (Morsi et al., 2022; Ummalyma et al., 2021, 2022; Xiaogang et al., 2022). Conventional water treatment process is usually costly. For example, Norwegian municipalities' annual costs for wastewater treatment in 2020 totaled 10 billion NOK, including both capital and operating expenditures (Berge & Onstad, 2021).

2.2.1. Aquaculture wastewater

The seafood industry is one of the largest industries in Norway. Within the last decade, the country has increased its export of fish and fish products by 72% (from 2008). In 2018 the production volumes were around 4 million tons with a value of USD 10814.6 million (OECD, 2021). This industry comprises both capture fisheries and aquaculture farming, and encourages significant investments and implementation of innovative technical solutions into the production process (Enwereuzoh et al., 2021). Seafood industry covers almost 50% of the world's food demand (Hawrot-Paw et al., 2019). At the same time, the wastewater from the aquaculture system can endanger the marine ecosystem. Uncontrolled inflow of effluents with high content of essential nutrients (N & P) can result in misbalancing of trophic levels and affect species diversity (Zhang et al., 2021).

Aquaculture wastewater typically contains uneaten food, residues of medicines and cleaning chemicals, fish feces, and other excretes. Dissolved compounds like ammonia, nitrate, phosphate, and organic matter create conditions that are very likely to harm the environment. Suspended solids affect water turbidity and organic deposits formation on the bottom of recipient waters (Lui, 2022; Pietros et al., 2000). Already in 2003 extremely high waste generation volumes from aquaculture production have been reported (Suzuki et al., 2003): on average, 0.8 kg of N and 0.1 kg of P per 1 ton fish, which was equivalent to waste generation by 73 people per day. Therefore, sustainable aquaculture industry intensification where

production volumes increase without impact on land area and environment needs to be implemented (Dauda et al., 2019).

2.2.2. Conventional wastewater treatment systems and their flow

Wastewater treatment aims to improve water quality and significantly reduce organic substances contents as COD and BOD and the most essential for receiving water bodies inorganic compounds (N & P) prior the discharge of effluent into the receiving ecosystems. The effluent discharge limits are set by the Urban Wastewater Treatment Directive for COD at 125 mg/L, for TP at 1-2 mg/L, and for TN 10-15 mg/L (European Comission, 2016).

The essential wastewater treatment phases are primary and secondary WWT, combined with preliminary (mechanical screens and grits) followed by tertiary treatment if the facility allows it (*Figure 3*). Physical, chemical, and biological methods of WW treatment are combined within these two main phases. Sedimentation, filtration, and primary screening are the physical methods, while addition of the chemicals creates conditions for coagulation-flocculation or precipitation mechanisms for contaminants removal. Microorganisms are used for degrading biodegradable organic matter and removing N and P (Burton et al., 2013). Tertiary, or advanced, treatment is usually represented by advanced oxidation processes, the use of membranes, filtration, ozonation, chlorination, UV irradiation, addition of activated carbon. However, the use and implementation of tertiary treatment technologies lead not only to economic disadvantage, but also can initiate formation of toxic and even carcinogenic by-products (Zahmatkesh et al., 2022).

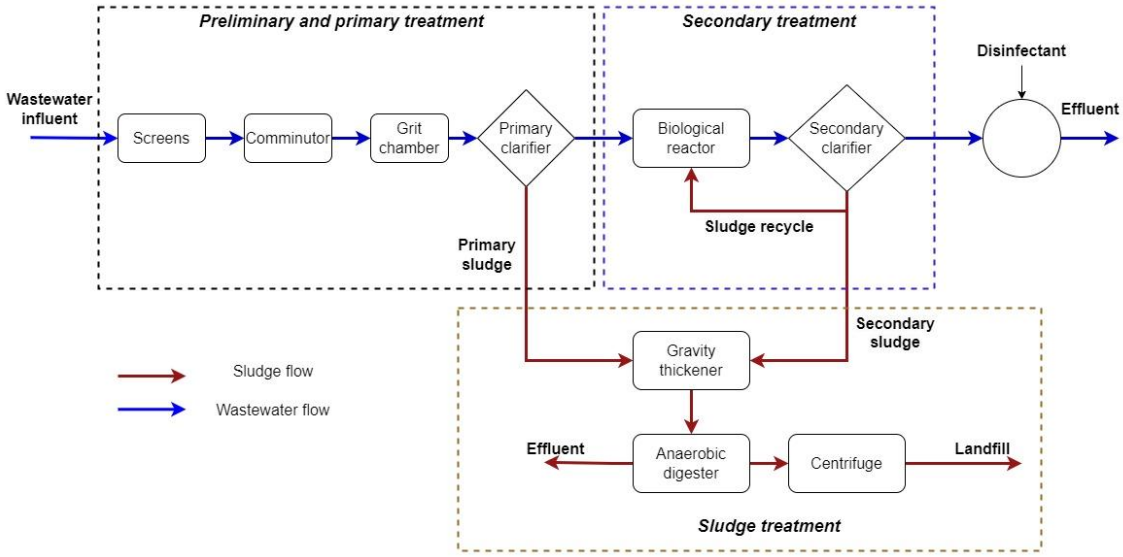

Figure 3. Schematic diagram of conventional wastewater treatment system (Burton et al., 2013).

Despite significant reduction of targeted compounds during WWT process, there are concerns that even acceptable concentrations of N and P under constant feed conditions into the small inland water bodies will lead to eutrophication (Mohsenpour et al., 2021). Another concern considers the economic feasibility and environmental impact of conventional WWT systems. As an example, high energy consumption (only mechanical aeration takes up to 50-90% of total energy demand), greenhouse gas emissions (mainly CO_2 release) and the use of chemicals which may generate harmful by-products are the key factors to look for alternative or additional WWT approaches. Moreover, the majority of WWT plants are not able to utilize antibiotic residues allowing their release into the environment (Amaro et al., 2023; Oviedo et al., 2022).

2.2.3. Microalgae-facilitated wastewater treatment

Microalgal systems can remove up to 99% of conventional pollutants (TP, TN), 90% of COD, and biodegrade antibiotics dissolved in WW with the efficiency of 42-93% depending on the antibiotic group and microalgae species. Effective tertiary treatment can be acquired due to ability of microalgae to remove hazardous bacteria (Amaro et al., 2023).

There is a number of published research that consider microalgae cultivation for various effluents treatment: municipal, agricultural, brewery, refinery, industrial, and some others (Asadi et al., 2020; Castellanos-Estupiñan et al., 2022; Gumbi et al., 2021; Morsi et al., 2022; Rani & Ojha, 2021; Ratomski & Hawrot-Paw, 2021; Sendzikiene & Makareviciene, 2022; Shanthi et al., 2021). Both monoculture, polyculture, and microalgae-bacterial consortia are used for WWT, which also results in different treatment efficiencies (Amaro et al., 2023).

Microalgae-based WWT, unlike conventional, requires only a single-step treatment stage to facilitate inorganic N and P removal and thereby reduces the complexity and energy demand for the process. It also does not generate toxic by-products and may have biorefinery potential. On the other hand, relatively long treatment time and energy consumption for aeration and pumping systems remain to be a challenge (Amaro et al., 2023; Mohsenpour et al., 2021).

The use of algal biotechnology for wastewater remediation improves the environmental impact of conventional WWT methods, maintaining its efficiency and reducing sludge formation (Satpal, 2016). At the same time, it offers the highest atmospheric CO_2 fixation rate (Shahid et al., 2020), providing real opportunity for solving CO_2 sequestration problematics (1.83 kg CO_2 used for 1 kg biomass produced) (Enwereuzoh et al., 2021).

2.3. Microalgae

Microalgae are photosynthetic procaryotic and eucaryotic cellular organisms with a size ranging from a few µm to approximately 200 µm. It is a large and diverse group of microorganisms that contain around 200 000 species (Bleakley & Hayes, 2017). Microalgae are able to cover up to 50% of Oxygen demand on Earth in addition to synthesizing various bioproducts including proteins, lipids, polysaccharides, pigments, vitamins, bioactive compounds, and antioxidants (Zuccaro et al., 2020).

Due to high diversity microalgae species attain their own unique characteristics and properties (J. S. Tan et al., 2020). There are different approaches to differentiate and classify microalgae, but the most common classification is based on optimal growth temperature and metabolic pathway (*Table 1*).

Table 1. Microalgae classification with a focus on optimal growth temperature and utilized energy source.

Growth requirement	***Classification***	***Optimal growth conditions***	***References***
Temperature	Psychrophile	Growth temperature under 15°C	(Zuccaro et al., 2020)
	Mesophile	Growth temperature 15-50°C	
	Thermophile	Growth temperature over 50°C	
Energy source	Photoautotrophic	Illumination, inorganic carbon, and minerals dissolved in the nutrient media.	(Enamala et al., 2018)
	Heterotrophic	Absence of light, organic carbon is used through aerobic respiration.	(Perez-Garcia & Bashan, 2015)
	Mixotrophic	Organic carbon is assimilated in the light simultaneously with CO_2 fixation. Organic compounds accounted as a major energy source.	(Chew et al., 2018; Kang et al., 2004)
	Photoheterotrophic	Microalgae require light while utilizing organic compounds as a carbon source. Light is serving as a major energy source.	(Chew et al., 2018)

Microalgae are not only metabolically flexible but also attain features that favorably distinguish them from similar biomass producers. As an example of such features, the nutrient requirements are very simple, light and CO_2 are easily accessible, and the whole biomass is actively participating in photosynthesis. Algae have a short doubling time, which makes production extremely effective. At the same time microalgae can grow in different aquatic

environments, including saline water and even WW, and it does not require fertile land to grow, which make them more environmentally sustainable compared with plants (Benedetti et al., 2018). It is possible to select certain species to grow in specific and even demanding conditions (Benedetti et al., 2018; Nagappan et al., 2021), which will influence their biochemical composition (Dolganyuk et al., 2020).

2.3.1. Growth requirements and parameters

There are two groups of factors that affect the growth of microalgae: physiological factors and operational parameters. The first group includes light, temperature, nutrient availability, pH, salinity, gas exchange rates, and cell density. Operational parameters affect the growth of microalgae through mixing, dilution rate, culture depth, harvest frequency, fluid and hydrodynamic stress (Bartosh & Banks, 2007). In this chapter, we will closely discuss the importance and meaning of nutrient availability.

Temperature directly influences physiological and morphological responses of microalgal culture growth, including photosynthesis and carbon fixation rates (Zuccaro et al., 2020). The optimal growth temperature ranges are described in *Table 1*.

Light intensity is another factor that has a direct effect on photosynthesis and, as a result, on biochemical composition and biomass yield (Zuccaro et al., 2020). The majority of microalgae has an optimal light intensity range within 100-400 μmol/m^2/s (Schuurmans et al., 2015). However, the optimal light intensity needs to be evaluated in each experiment separately.

Essential macronutrients, trace elements, and vitamins are required for microalgal growth. The literature sources are referring to Redfield C:N:P ratio of 106:16:1 (Zuccaro et al., 2020). At the same time, the cultivation on different nutrient media compositions shows that microalgae adapt their metabolic processes to available macronutrient ratios and therefore allow us to look for cheaper and more environmentally favorable media solutions (Arrigo, 2005).

Carbon is the main constituent of microalgal biomass that can reach 50-65% of DCW. The uptake of the element happens through photosynthesis (autotrophic metabolism) and its mechanism depends on pH, alkalinity, temperature, atmospheric pressure, light availability, etc. At the same time, many species can grow on organic substrates performing heterotrophic and mixotrophic metabolism via diffusion, active transportation, and phosphorylation (Zuccaro et al., 2020).

Nitrogen is an essential biochemical component of DNA, RNA, proteins, and pigments, and its content in microalgal biomass usually varies between 1% and 14% of DCW. Inorganic nitrogen is consumed as NO_3^-, NO_2^-, NO, NH_4^+ forms while organic nitrogen – as amino acids or urea. All available forms of nitrogen require different energy levels for their consumption, and some of them are preferable (NH_4^+) than others. Nitrogen is commonly taken up by the cell through the transportation mechanism (Zuccaro et al., 2020).

Phosphorus is a limiting nutrient for microalgae and its content in biomass is 0.05-3.3% of DCW. Phosphorus is a part of RNA, DNA, ATP molecules, and membrane phospholipids. Orthophosphate is absorbed into the cell through enzymatic activity and depends on available light, pH, temperature, ionic strength, and ions of K^+, Na^+, and Mg^{2+}. The excess of phosphorus can be stored inside the cell, and this property of microalgae is used for WWT (Zuccaro et al., 2020).

Trace elements, widely used in nutrient media recipes, are Magnesium, Sulfur, Calcium, Iron. These elements function as enzyme activators (Mg), constituents of amino acids, lipids, vitamins, and other secondary metabolites (S), affect morphogenesis (Ca), participate in oxygen metabolism, electron transfer, nitrogen assimilation, chlorophyll synthesis (Fe), enable flocculation of microalgal biomass (Mg) (Zuccaro et al., 2020).

2.3.2. Cultivation systems

There are two main cultivation systems: raceway open ponds and enclosed photobioreactors (Xiaogang et al., 2022). A hybrid cultivation system can be considered as an additional type (Ummalyma et al., 2022). The advantages and disadvantages of the abovementioned systems are listed in *Table 2*.

Table 2. Comparison of different microalgae cultivation systems ((Narala et al., 2016; Xiaogang et al., 2022).

Cultivation system	***Advantages***	***Disadvantages***
Raceway open pond	• Easy to maintain. • Does not require a lot of energy for mixing. • Low setup cost.	• High contamination risk. • Requires a lot of space. • High evaporation losses. • Low CO_2 sparging efficiency. • Difficult to maintain continuous exponential phase. • Low biomass quality.
Photobioreactor	• Relatively small space requirements. • Low evaporation losses. • High CO_2 sparging efficiency. • Low contamination risk. • High biomass quality.	• Difficult to maintain. • High energy input required for mixing. • High setup costs. • Difficult to maintain continuous exponential phase.
Hybrid system	• Relatively small evaporation losses. • Relatively easy to maintain. • Low contamination risk. • High quality biomass. • Relatively low energy input required for mixing. • Easy to maintain.	• High space requirements. • Relatively low CO_2 sparging efficiency. • Relatively high setup costs.

All available cultivation systems have both advantages and disadvantages. When choosing one of these systems, several factors usually should be considered, and the choice is supposed to be made based on economic efficiency for the targeted goal.

2.3.3. Microalgae as a potential remediation and biorefinery agent.

The majority of microalgae species have a well-balanced combination of important macromolecules (proteins, lipids, carbohydrates) and amino acids (lysine, methionine, threonine, and tryptophan), which increase their potential use as a promising low-cost remediation agent (Nagappan et al., 2021; Nitsos et al., 2020; Viegas et al., 2021). Commercially important products also include cellulose, various polymers, and high-value functional bioactive compounds (Lutzu et al., 2021). These compounds are widely used in medicine, pharmacology, cosmetology, fish farming, agriculture, feed and functional food production, chemical industry (Calijuri et al., 2022; Dolganyuk et al., 2020; Viegas et al., 2021; Xia et al., 2021).

Despite demonstrating a bio-stimulating effect on seed germination, high concentrations of applied biomass can demonstrate a toxic effect on vegetation. Biomass can be used for the adsorption of some dyes, but there are some downsides like irreversibility of the adsorption

process, economic inefficiency, and formation of other contaminated effluents (Viegas et al., 2021). Addition of microalgae into the animal feed improves their immune response and gut function by increased antiviral and antibacterial protection, stimulates reproductive system, weight gain, and quality of meat and dairy products in cattle (Camacho et al., 2019; Madeira et al., 2017). Prebiotics, obtained from algae, have not been widely exploited in aquaculture yet. Only a few are used as immunostimulants, implemented in feed for some aquatic farm species. At the same time, the composition and possible usage of marine-derived saccharides should be explored in the future (Camacho et al., 2019; Kusmayadi et al., 2021).

The biomass harvested at the end of microalgae cultivation can be converted to biofuels, biochar, anaerobically digested for biogas production, or biofertilizers without any additional costs (Amaro et al., 2023). Microalgal biomass, obtained as a product of WWT coupled with microalgae growth, has promising usage for biopolymer production (*Figure 4)*.

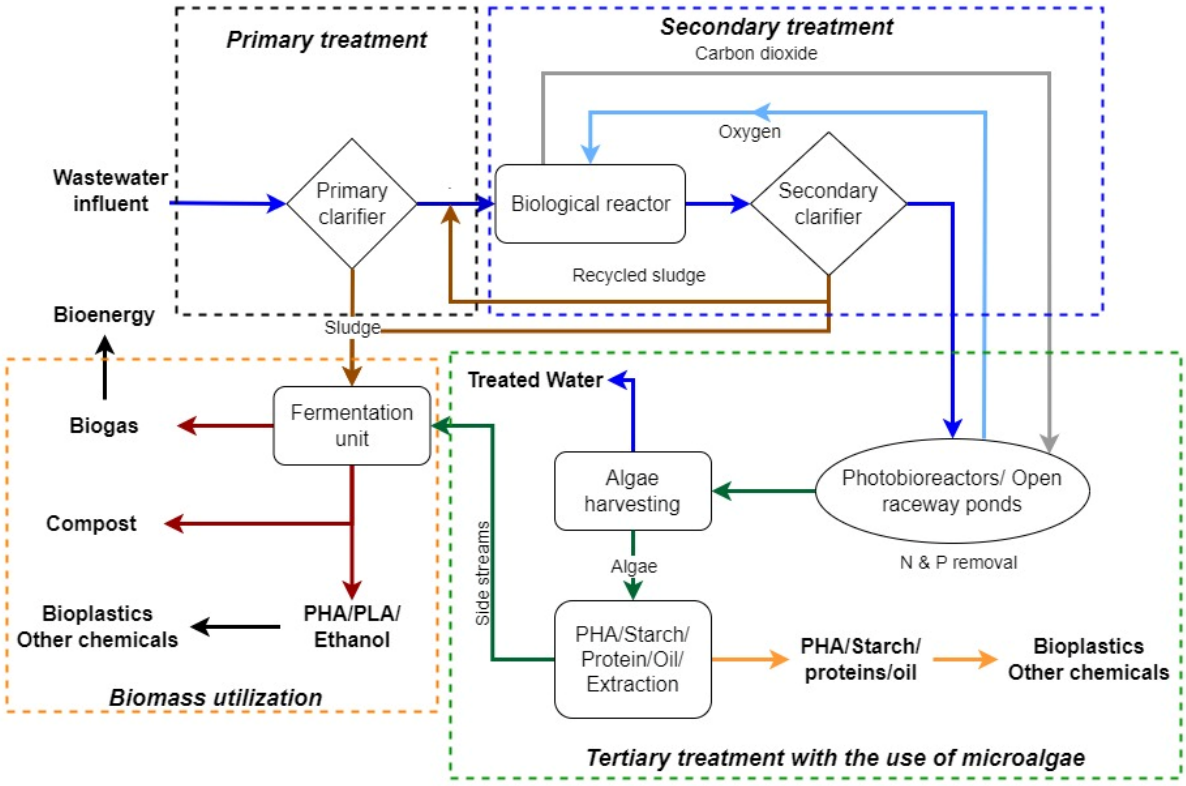

Figure 4. Example of a possible wastewater treatment process integrated with microalgae cultivation and bioplastic production ((Lutzu et al., 2021).

Polyhydroxyalkanoates (PHAs), recovered from microalgae, are considered as high-value products due to their similar physical properties with fossil-based plastics. PHAs can

potentially replace conventional plastics on a production scale and become a permanent solution to plastic waste problem (Lutzu et al., 2021; Mastropetros et al., 2022; Nanda & Bharadvaja, 2022; Rajpoot et al., 2022).

2.3.4. Species and strains suitable for wastewater treatment

Rapid growth is one of the most desirable properties of microalgae grown in WW. In addition, they need to have high nutrient requirements and be able to reduce low nutrient concentrations to even lower levels (Wang et al., 2017). Small, single-cell organisms are growing faster and less exposed to structure destruction than colonial and filamentous microalgae. However, the last ones are easier to harvest. In this section, the basic information on three microalgae species selected for the current experiment is provided.

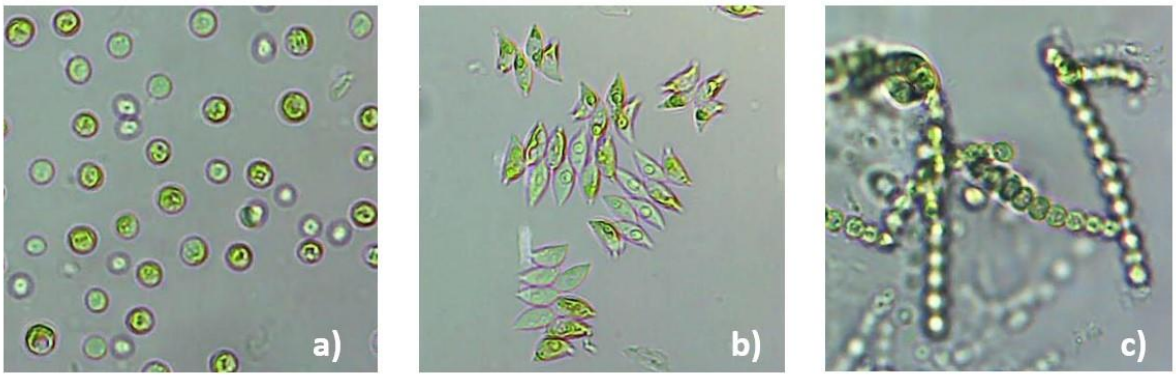

Figure 5. Microscopic pictures of studied microalgae species: a) Chlorella sp., b) Scenedesmus sp., c) Nostoc sp.

Chlorella species (*Figure 5 a*) is one of the most studied species that can be successfully cultivated in wastewater from seafood industry. *Chlorella* sp. is a freshwater microalga. It is a unicellular green alga, has spherical form and thick cell wall (100-200 nm) which provides protective function. Being one of the most studied and often used for waste treatment (Sydney et al., 2014), *Chlorella* sp. grows autotrophically or heterotrophically under various conditions and with a different composition of carbon source. It reported to be robust microalgae (Mulyawati et al., 2021).

It was observed that *Chlorella* doesn't need extra addition of nutrients and can be used for biodiesel and protein-rich feed production (Trivedi et al., 2019). In addition, high general nutrient removal efficiency was reported by several authors (Cardoso et al., 2021; Holanda et al., 2022; Venugopal & Sasidharan, 2021; Viegas et al., 2021). At the same time, biological oxygen demand in wastewater can be reduced due to oxygen release in a photosynthesis process (Hawrot-Paw et al., 2019).

Scenedesmus species is another common freshwater algae that are known to be tolerant to growing on wastewaters (Chew et al., 2018; Schoeters et al., 2023). It is not commonly used species in WWT studies, but its biochemical composition is reported to be favorable for different applications, including biofuels (Conde et al., 2021; Y. H. Tan et al., 2023). *Scenedesmus* sp. belongs to green algae and often found in multinucleate nonmotile chains coenobium of four or eight cells, arranged in a row (*Figure 5 b*). The formation of coenobiums depends on light intensities, temperature (Lurling, 1999).

Both, *Chlorella* and *Scenedesmus* spp. are robust to grow on different organic streams and adapted to stressful growth conditions (Sánchez-Zurano et al., 2021; Schoeters et al., 2023). At the same time, lipid yield in *Scenedesmus* sp. can reach up to 60% DCW under optimized conditions (Mandal & Mallick, 2009), and CO_2 utilization efficiency is higher than other algae species reported (Yoo et al., 2010).

Nostoc species is a genus of blue-green multicellular algae (*Figure 5 c*) that can fix atmospheric nitrogen when combined nitrogen is depleted. The filaments of *Nostoc* sp. are covered with large protective gelatinous matrix and have both photosynthetic cells and N_2-fixing heterocysts (Celis-Plá et al., 2021; Sand-Jensen, 2014). The diversity of metabolic pathways allows microalgae to adapt to varied environmental conditions, and even extremes such as hot springs and glaciers (Nowruzi et al., 2018). At the same time, there are certain difficulties connected to outdoor mass cultivation of *Nostoc* sp.: filamentous are sensitive to friction caused by rotating installations, limited light availability due to depth of cultivation ponds, wide range of photo accumulation responses (Celis-Plá et al., 2021).

Nostoc sp. is known as a species with high lipid content and is a promising source as a feedstock for biofuel (Damacena et al., 2020; Shetty & Krishnakumar, 2023) and other products that can be obtained from lipids. Other bioactive compounds that can be produced by *Nostoc* sp. are amino acids, fatty acids, polysaccharides, and carotenoids with their specific antimicrobial, antioxidant, and antibacterial activities (Nowruzi et al., 2018).

3. Materials and methods

Detailed information on experimental strategies (*Figure 6*), setup settings, methods used for ACWW characterization, WW treatment efficiency analysis, and biocomponents quantification methods will be provided in this chapter. ACWW was collected at Tyslandsvik Aqua AS, a land-based aquaculture plant, where recirculating aquaculture system (RAS) is implemented, and subjected to the abovementioned studies. Chemicals and equipment, used in the study is described in *Table 7* and *Table 8* in Appendixes A & B.

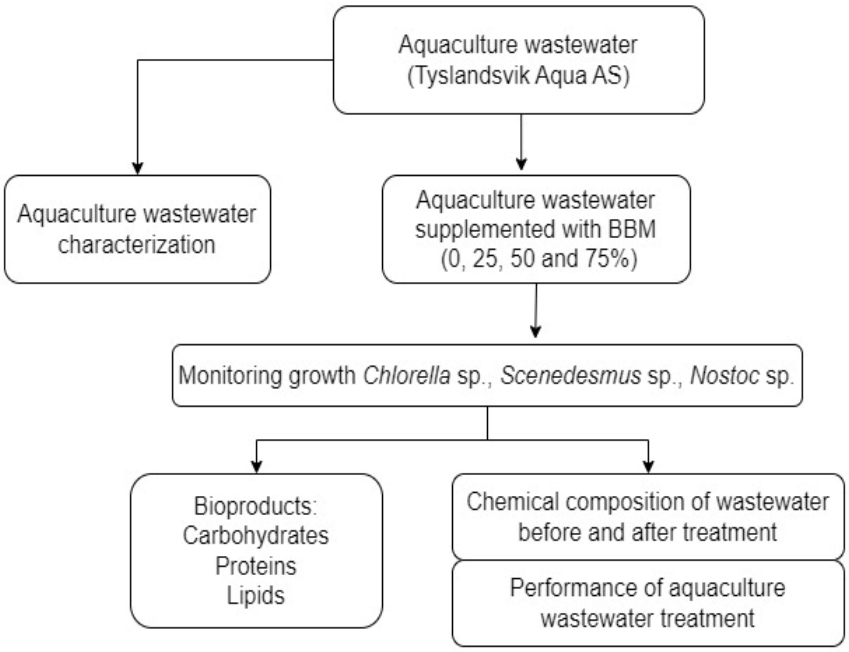

Figure 6. The experimental strategy for Scenedesmus, Chlorella, and Nostoc spp. cultivation in ACWW.

3.1. Aquaculture wastewater characterization

Aquaculture wastewater was subjected to basic wastewater characterization which included quantification of particulate and dissolved constituents, volatile and fixed solids, particulate and dissolved COD, TP, and TN in raw and filtered WW. In the *Figure 7* the approach for particulates and solids characterization is shown.

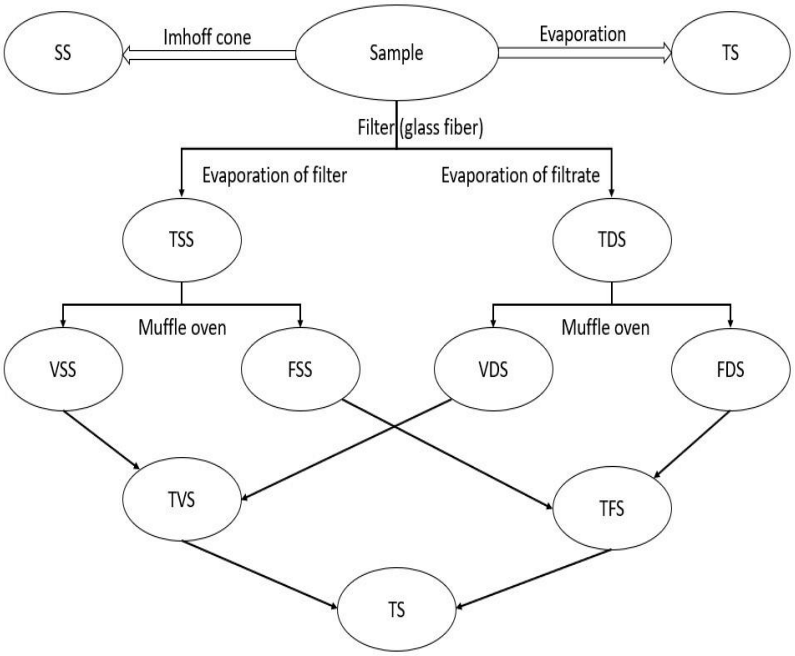

Figure 7. Fractionation and classification of solids in wastewater (Tchobanoglous & Schroeder, 1987). SS = settleable solids; TS = total solids; TSS = total suspended solids; TDS = total dissolved solids; VSS = volatile suspended solids; FSS = fixed suspended solids; VDS = volatile dissolved solids; FDS = fixed dissolved solids; TVS = total volatile solids; TFS = total fixed solids

3.1.1. Procedure for settleable solids analysis

A clean Imhoff cone was filled with 1000 mL of raw ACWW and left for settling for 45 minutes. The supernatant was gently stirred to prevent the settlement of solids on the glass walls and left for another 15 minutes. The settled solids volume was read from the Imhoff cone measuring scale. Further solids were decanted and the remaining supernatant was stirred and sampled (200 mL) for TSS determination.

3.1.2. Procedure for TS and TVS determination

Pre-ignited and cooled porcelain dish was weight before the test. 200 mL of raw homogenized ACWW was transferred into the dish and left in the oven at 95-97°C overnight for evaporation. The following day the evaporated residual was dried at 103-105°C for 1 hour, cooled in a desiccator to room temperature, and weighted. TS was calculated using the following formula:

$$TS\left[\frac{mg}{l}\right] = \frac{m_{dish+residual} - m_{dish}}{V_{sample}} \quad (1)$$

Later the porcelain dish was placed into a muffle oven at 550°C, combusted for 30 minutes, cooled in a desiccator, and weighed. TVS was calculated using the following formula:

$$TVS\left[\frac{mg}{l}\right] = TS - \frac{m_{dish+ignited\ residual} - m_{dish}}{V_{sample}} \quad (2)$$

3.1.3. Procedure for TSS and VSS determination

For TSS and VSS analysis pre-dried GF/C filters with a diameter of 47 mm were used. Filters were cooled in a desiccator, weighed, and fixed to the filtering device. 200-230 mL of raw homogenized ACWW were filtered while applying vacuum to the filtering device. The filter was gently removed and placed on the supportive dish in the oven at 105°C for 2.5 hours. After that, it was cooled in the desiccator and weighed. TSS was calculated using the following formula:

$$TSS\left[\frac{mg}{l}\right] = \frac{m_{filter\ +\ solids} - m_{filter}}{V_{sample}} \quad (3)$$

Later the filter with solids on it was placed into a muffle oven at 550°C together with a clean pre-weighed filter, combusted for 25 minutes, cooled in a desiccator, and weighed. VSS was calculated using the following formula:

$$VSS\left[\frac{mg}{l}\right] = TSS - \frac{m_{filter\ +\ ignited\ residual} - m_{filter} + m_{filter\ loss}}{V_{sample}} \quad (4)$$

3.1.4. Procedure for COD measurement

The COD analysis shows the amount of oxygen that reacts with the oxidizable substances contained in the sample. The reaction is catalyzed by silver sulfate, where hot sulfuric solution of potassium dichromate oxidizes the water sample, and the concentration of green Cr^{3+} -ions is then measured photometrically (Merck, 2021a).

2 mL of each sample was transferred into the reaction cell and mixed vigorously. After that cells were digested in the thermoreactor at 148°C for 120 minutes. After cooling to room temperature measurements were read in the photometer.

3.1.5. *Procedure for TN measurement*

The product of the reaction that can be measured photometrically is 4-nitro-2,6-dimethylphenol. It forms as a result of acidification of nitrate in the presence of sulfuric and phosphoric acids when it reacts with 2,6- dimethylphenol (DMP). Nitrate, in its turn, is derived from organic and inorganic nitrogen compounds in the water sample as a result of treatment with an oxidizing agent in a thermoreactor (Merck, 2021b).

For the digestion purpose, 1 mL of sample was transferred into a clean empty cell together with 9 mL of dH_2O and mixed. Reagents N-1K (1 level blue micro spoon) and N-2K (6 drops) were added one by one and mixed after each addition. Cells were digested in the thermoreactor for 1 hour at 120°C.

1 mL of cooled sample was transferred into the reaction cell followed by the addition of 1 mL of reagent N-3K. Reaction cells were carefully mixed and left for the reaction to proceed for another 10 minutes. Quantification was performed photometrically.

3.1.6. *Procedure for TP measurement*

Orthophosphate ions react with molybdite ions in a sulfuric solution and form molybdophosphoric acid which is reduced to phosphomolybdenum blue (PMB) by ascorbic acid. PMB can be measured photometrically (Merck, 2021c).

For total phosphorus determination, the digestion step was required: 1 mL of sample was added to the reaction cell together with one dose of reagent P-1K, mixed until the total dissolution of reagent, and digested in a thermoreactor at 120°C for 30 minutes.

After cooling the sample to room temperature, 5 drops of reagent P-2K and 1 dose of reagent P-3K were added to the reaction cell and mixed after each addition. Reaction cells were left for 5 min (reaction time) and followed by photometrical measurements.

3.2. Experimental setup and cultivation conditions

During the current study, four batches of three microalgal species were cultivated: two of *Scenedesmus* sp., one of *Chlorella* sp., and one of *Nostoc* sp. Cultivation of *Scenedesmus* sp. had to be repeated due to certain difficulties with the setup installation that emerged one week after launching the first experiment. More information on setup failure is given in the following chapters (Chapters 4.2.3 & 5.5).

The cultivation was performed in 250 mL conical flasks with a working cultivation volume of 200 mL. Initial inoculum concentration was 10% (v/v): to 180 mL of tested nutrient media 20 mL of previously grown on BBM (Appendix C) microalgal culture were inoculated in aseptic conditions (see Chapter 3.2.1). Flasks were covered with rubber stoppers, connected to air spargers, adapted for daily sampling, and sealed with parafilm. Conical flasks were placed on magnetic stirrers under continuous stirring in a chamber isolated from external insolation. Controlled light conditions inside the chamber were provided using LED stripes placed on the ceiling, and automatic switch controlled the light regime. The applied light intensity was somewhat different for all 4 batches, but on average amounted to 96.8±3.7 µmol/m^2/s. More detailed information on the cultivation conditions of all 4 batches is collected in *Table 3*.

Table 3. Overview of the microalgae cultivation conditions for different batches during the experiment.

Batch number	Species cultivated	Cultivation time, days	Light intensity, µmol/m^2/s	Light/dark regime, hours/hours	Magnetic stirring speed, rpm	Air sparging, hours/day	Average cultivation temperature, °C in chamber
Batch 1	*Scenedesmus* sp.	10	96.8±3.7	12/12	150	24	24.4±2.5
Batch 2	*Chlorella* sp.	12	98.9±2.8	12/12	140	3	24.0±1.7
Batch 3	*Scenedesmus* sp.	12	99.2±4.6	12/12	140	3	23.9±2.3
Batch 4	*Nostoc* sp.	12	92.4±3.7	12/12	140	3	24.2±2.8

Every batch consisted of 15 conical flasks placed on the magnetic stirrer simultaneously: 4 variants of alternative nutrient media (ACWW in dilutions) in triplicates and a control triplicate (BBM). The air temperature inside the chamber and water temperature in a glass, placed in the chamber, was measured several times per day and used for representation of the average cultivation temperatures. Some of the experimental setup installations are shown in *Figure 35* in Appendix D.

3.2.1. Equipment preparation

All the equipment used during the experiment was carefully washed with non-phosphate detergents and disinfected prior to inoculation. Autoclaving at 120°C at 1 atm. for 30 min was the primary disinfecting method used for conical flasks, magnets, pipette cones, and measuring cylinders. Rubber stoppers, and pipes for air sparging and culture sampling were washed with the 96% Ethanol and subjected to UV exposure for 10-12 min. Measuring pipettes and additional glass beakers (for waste and discharge) were exposed to UV radiation prior inoculation the batches.

3.2.2. Microalgae strains and their origin

For the current experiment, three algal strains were chosen: *Scenedesmus* sp., *Chlorella* sp., and *Nostoc* sp. The first two strains are polyculture with a clear dominance of one of the species (further called *Scenedesmus* sp. and *Chlorella* sp. by the name of the dominant one) that were selected from the water sample of the local fjord (Hafrsfjord, Rogaland). Water samples were inoculated in BBM and cultivated at a temperature of 22°C, light intensity 100 µmol/m^2/s, and light/dark regime 12h/12h under continuous mixing on magnetic stirrers. Every 10^{th} day samples were observed under a microscope and subcultured until the clear dominating of one of the species. In the performed selection two clearly dominating cultures (*Chlorella* and *Scenedesmus* spp.) were obtained after 3^{rd} subculturing.

The *Nostoc sp.* was ordered at NIVA algae bank and adapted for cultivation on BBM at UiS by continuous subculturing.

3.2.3. Growth media variants

ACWW was used as an alternative nutrient medium for microalgae cultivation. Collected WW was subjected to WWT at the facility, which included the following steps: mechanical filter (40 µm pore size), biofilter, CO_2-stripping/aeration, ozone addition, and pH adjustment with lime. At the laboratory raw wastewater was filtered through a glass microfiber filter and autoclaved at 120°C at 1 atm for 20 min to minimize bacterial contamination in the liquid. An additional filtration step was applied to remove particulate COD from the WW and prevent these particles to influence DCW analysis results while biomass quantification.

Four dilutions of ACWW were prepared: 100%, 75%, 50%, and 25%. Wastewater was diluted with BBM, and pure BBM was used as a reference nutrient medium for cultivation of all 3 microalgae species grown in this experiment. BBM was prepared one day before each batch inoculation following the recipe that can be found in Appendix C.

3.2.4. OD measurements of cell growth

Optical density (OD) measurements are often used as a rapid method for cell density estimation. A suitable standard curve can be used for precise quantification of biomass density. But while applying this method for microalgae growth measurements it is important to avoid interference with absorbance of algal pigments which can significantly influence the results (Borowitzka & Moheimani, 2012; Griffiths et al., 2011).

The number of cells in the sample is usually determined by the amount of light absorbed by the algal cell. In our experiment the absorbance at the wavelengths 680 and 750 nm was measured and correlated with the dry cell mass, obtained through DCW analysis of microalgae culture. For OD analysis, 1 mL of homogenized microalgae suspension was collected with the syringe, transferred into glass UV cuvettes, mixed 1:1 with dH_2O, and analyzed once a day by measuring the absorbance in the spectrophotometer. Obtained results were afterwards used for the specific growth rate calculations:

$$\mu \left[\frac{1}{day}\right] = {(\ln N(f) - lnN(i))}/{t(f) - t(i)} \quad (5)$$

μ = specific growth rate
N(i) = initial OD values, abs;
N(f) = final OD values, abs;
t(i) and t(f) = initial and final time respectively, day

3.2.5. Algal quantification based on DCW analysis

Another biomass quantification approach that was used during our experiment was DCW analysis. From all the conical flasks used under the experiment, 5 mL of the culture were sampled every 3rd day of cultivation and subjected to the DCW analysis. 4 mL of each sample in triplicates were filtered through GF/C filters (47 mm) on the filtering device, moved into the drying oven at 105°C for at least 2.5 hours. Cooled filters were weighed and the DCW was calculated using the following formula:

$$DCW \left[\frac{mg}{L}\right] = \frac{m_{filter\ +\ solids} - m_{filter}}{V_{sample}} \quad (6)$$

Obtained results were correlated with the OD measurements and used for average biomass production (BMP) calculations. The following formula was used for this purpose:

$$BMP \left[\frac{mg}{L/day}\right] = {DCW}/{SV}/{(t(f) - t(i))}, \quad (7)$$

DCW – dry cell weight, mg
SV – sample volume, L
t(i) and t(f) = initial and final time respectively, day

3.3. Wastewater treatment efficiency measurements

The WWT efficiency was estimated based on three significant parameters: COD, TP, and TN. All these parameters were analyzed for all 5 variants of nutrient media before the experiment and every 3rd day of the cultivation in accordance with the procedures described in Chapters 3.1.4. – 3.1.6.

Due to low sample volumes and limited availability of analytical equipment, mixed samples for every cultivation condition were collected and analyzed. For every nutrient media variant, nutrient depletion charts were established and discussed.

The nutrient removal efficiency for COD, TN, and TP was determined with the use of the following formula:

$$Removal\ efficiency\ (\%) = \left(\frac{Ci - Cf}{Ci}\right) \times 100 \quad (8)$$

Ci = initial concentration, mg/L;
Cf = final concentration, mg/L

3.4. Analytical methods

After cultivation of three microalgal strains, the biocomponent composition of obtained biomass becomes a target of our interest. Total carbohydrate, total protein, and total lipid percentage were estimated and compared during the analytical part of the current project.

3.4.1. Biomass preparation before the analysis

At the end of every batch cultivation, microalgal biomass was harvested by centrifugation at 5000 rpm for 10 min, washed two times with dH_2O to eliminate residues of nutrient media, and stored in the freezer until analysis performance. Prior to the analytical experiments, the biomass was transferred into separate tubes and dried at the temperature of 90-95°C, cooled to room temperature, and weighed. The dry weight of microalgal biomass was considered for quantification of all the biocomponents.

3.4.2. Calibration curves establishment for total protein and total carbohydrate analysis

Prior to the carbohydrate and protein quantification analysis, the standard calibration curves were established. These curves are shown in *Figure 36* in Appendix D.

The standard curve for total carbohydrate analysis (*Figure 36 A*) in microalgal biomass was prepared with the use of 1 g/L glucose solution: 0.1 g of Glucose was dissolved in 100 mL of dH_2O. Five dilutions (20, 40, 60, 80, and 100 mg/L) were prepared and followed the testing procedure described in the following chapter (Chapter 3.4.3). Obtained results were plotted in the standard curve which was used for total carbohydrate quantification in microalgal biomass.

The standard curve for total protein analysis (*Figure 36 B*) in microalgal biomass was prepared with the use of 2 mg/mL BSA solution (part of total protein test kit). Six dilutions (0.1, 0.2, 0.3, 0.5, and 1.0 g/L) were prepared and followed the testing procedure described in the following chapter (Chapter 3.4.4). Obtained results were plotted in the standard curve which was used for total protein quantification in the studied microalgal biomass.

3.4.3. Total carbohydrate content determination

The total carbohydrate estimation was performed in accordance with the manual established by HiMediaLaboratories for its quantitative kit (HiMediaLaboratories, n.d.) using Phenol-Sulfuric carbohydrate quantification method in triplicates or duplicates. Prior to the analysis, all the samples were subjected to acid hydrolysis in 2*N* solution of Hydrochloric acid (HCl). 1 mL of HCl was added to pre-dried and weighed biomass and placed in the water bath at 95°C for 1 hour. After cooling, samples were diluted with dH_2O until concentration ≈ 0.2 - 0.3 g/L.

For the analysis itself 1 mL of hydrolyzed sample (or 1 mL of dH_2O for blank) was moved into a clean glass tube followed by addition of 1 mL 5% phenol solution and mixing with a vortex mixer. 5 mL of concentrated Sulfuric acid were added to the same tube, capped, and mixed vigorously. After 5 min the test tubes were placed in a water bath at 30°C for 25 minutes.

The UV spectrophotometer was adjusted to a «zero» with the blank sample at the wavelength 490 nm and absorbance values were read for each sample. The carbohydrate concentration was obtained by plotting obtained absorbance values against the carbohydrate standard curve (*Figure 36 A*). Carbohydrate content in % was estimated by using the following formula:

$$Carbohydrate\ \left(\%\frac{w}{w}\right) = \frac{CVD}{m} \times 100\% \qquad (9)$$

C = carbohydrate concentration, mg/L;
V = volume of HCl used for hydrolysis, mL;
D = dilution factor;
m = mass of biomass, mg.

3.4.4. Total protein content determination

The total protein analysis was performed based on the modified Lowry's Method (Miles, n.d.) with some corrections connected with equipment issues in triplicates or duplicates. Prior to the analysis, samples were subjected to high-temperature alkaline hydrolysis. Into the dried biomass 1 mL of 2*N* solution of NaOH was added, vortexed, and placed in the water bath at 95°C for 1 hour. After cooling, samples were diluted with dH_2O until the concentration reached ≈ 2 – 2.5 g/L.

Prior to use, all test kit components were warmed to room temperature. Reagent A was also prepared in advance. Each sample or standard required 510 µL of Reagent A. Basing on that, the total volume of Reagent A was estimated, and 20 µL of Reagent D (part of total protein test kit) to each mL of Copper solution were added. The obtained mixture was gently mixed to prevent foaming and stored at 4°C. Prior to use, the observed precipitate in Reagent D was dissolved by placing it into the warm water bath at 37°C.

For the analysis itself, 100 µL were pipetted into 15 mL plastic centrifuge tubes followed by addition of 500 µL of «UPPA^{TM} – I» to each tube. Tubes were vortexed and incubated at room temperature for 1 minute. Another 500 µL of «UPPA^{TM} – II» were added, vortexed again, and centrifuged at 4332×g for 15 min (original centrifuge regime is 15000×g for 3-5 min). Obtained supernatant was decanted and 510 µL of the Reagent A were added to each tube and vortexed. After that, 4 mL of Folin's reagent were added to each sample, vortexed immediately, and left for incubation at room temperature for another 15 min.

The UV spectrophotometer was adjusted to a «zero» with dH_2O at the wavelength 750 nm and absorbance values were read for each sample. Protein concentration was obtained by plotting measured absorbance values against the protein standard curve (*Figure 36 B*). Protein content in % was estimated by using the following formula:

$$Protein\ \left(\%\frac{w}{w}\right) = \frac{CVD}{m} \times 100\% \qquad (10)$$

C = protein concentration, mg/L;
V = volume of NaOH used for hydrolysis, mL;
D = dilution factor;
m = mass of biomass, mg.

3.4.5. Total lipids content determination

The lipid extraction was performed in accordance with the method described by Bligh and Dyer (1959) with small-scale modifications to it (Olmstead et al., 2013). All the samples were studied in duplicates due to limited biomass availability. 15 mL plastic centrifuge tubes with screw-tops were used as a biomass storage and as analysis tare.

The lipid quantification analysis in short: 3 mL of Methanol and 1.5 mL of Chloroform were added to reaction tubes with dried microalgal biomass, vortexed, and left on a rotating shaker for approximately 24 hours. The next day 1.5 mL of Chloroform and 1.5 mL of dH_2O were added. Samples were vortexed one more time and centrifuged at 1,400×g for 5 min. After phase separation, the Methanol layer was removed from the top and discarded. The Chloroform layer was carefully pipetted from the bottom and moved to glass tubes.

Another 3 mL of Methanol and 1.5 mL of Chloroform were added to the remaining biomass, the mixture was vortexed and incubated at room temperature and rotation for 2 more hours. The procedure was repeated until the biomass lost its color (2-5 or even 6 cycles depending on the initial biomass weight and lipid content in it). The extracted Chloroform was then dried in pre-weighed aluminum cups, and the amount of total lipids estimated using the gravimetric method. Lipid content in % was determined using the following formula:

$$Lipid\ content\ = \frac{M-M(0)}{DCW} \times 100\%, \quad (11)$$

M – weight of tare with oil
M(0) – weight of tare
DCW – dry cell weigh of used biomass

4. Results and Observations

Results of all the performed analyses during the experiment are mentioned in this chapter. Short observations from tables and figures are included and are briefly discussed.

4.1. Aquaculture wastewater characterization

The ACWW was tested for its suitability as a nutrient medium for cultivation of microalgae. Therefore, the basic wastewater characterization tests have been applied to it. Particulate and dissolved solids fractions were determined together with particulate and dissolved COD, Phosphorus and Nitrogen. Filtered ACWW was also autoclaved, and basic chemical analysis (COD, TN, and TP) was performed to check if sterilization influences nutrient composition in the WW. Results of performed characterization are reported in *Table 4*.Table 4. Aquaculture wastewater basic characterization results.

Table 4. Aquaculture wastewater basic characterization results.

Parameter	Pretreatment	Unit	Average Value	Standard error
SS-fraction	*raw ACWW*	%	0.015	0.005
TS		mg/L	3021.0	42.5
TVS			746.2	51.0
TSS			8.21	0.60
VSS			5.69	0.34
COD	*raw ACWW*	mg/L	257.0	41.0
	filtered		186.0	6.0
	filter + autoclave		180.0	12.0
TN	*raw ACWW*	mg/L	99.0	2.0
	filtered		98.5	1.5
	filter + autoclave		102.5	2.5
TP	*raw ACWW*	mg/L	4.75	0.05
	filtered		4.60	0.00
	filter + autoclave		4.55	0.05

From the basic characterization of received ACWW we assume that this water can be considered as a suitable nutrient medium for microalgae cultivation. The WW showed high

transparency, had yellowish color and no recognizable odor. The volumetric fraction of settleable solids was only 0.015±0.005 % which is practically insignificant value, and it provides high transparency of the liquid. TS and TVS values were 3021.0±42.5 and 746.2±51.0 mg/L respectively. The total volatile solids amounted to ≈25% of total solids, which evidence that most of the solids in the aquaculture WW are attributed to inorganic compounds. The standard deviation values are under 10% and therefore deviation is acceptable.

The TSS and VSS contents amounted to 8.21±0.60 mg/L and 5.69±0.34 mg/L respectively. The volatile suspended solids amounted to almost 70% of all suspended solids, showing high suspended organic content of the WW. The standard deviation in both cases was lower than 10% which is an acceptable deviation.

Analysis for COD, TN, and TP was performed on 3 samples of ACWW: pure raw water, raw water filtered through a microfiber glass filter, and the same WW both filtered through a filter and autoclaved. The filtration pretreatment step eliminated particulate COD, N, and P from the wastewater, so only soluble fractions of these compounds were available. Filtrate was also autoclaved to check how sterilization step influences the presence of abovementioned compounds since autoclaved WW will be used for growing the microalgae.

Results from COD analysis showed that particulate fraction of COD was little bit lower than 30% (27.7%), and autoclaving did not significantly influence the soluble COD content in the WW. The standard deviation, on the other hand, was highest at raw WW (±41 mg/L), which can be explained by unequal content of particulate COD fractions in different test cells. The filtration did not influence TN and TP content of studied ACWW, which is the evidence that all the particulate N and P were removed prior to discharge. At the same time, autoclaving step slightly increased TN concentration in the WW and did not affect total Phosphorus content. Increase in TN after autoclaving was insignificant (4%) and therefore was not regarded as a major issue in this study.

4.2. Start of the experiment: growth and biomass accumulation

Microalgae were grown in pure aquaculture wastewater and its different dilutions with BBM: 75% ACWW+25% BBM, 50% ACWW +50% BBM, and 25% ACWW + 75% BBM. Pure Bold's Basal Medium was used as a control medium, since all the studied species (*Scenedesmus*, *Chlorella*, and *Nostoc* spp.) were previously adopted to it. The optical density values were monitored daily at the wavelengths 680 nm and 750 nm and used for further

analysis, while a wider range of tests was performed every 3rd day of cultivation. There was no significant difference between OD values at different wavelengths, therefore the results from one set of measurements were used for the growth curve establishment, while results from the other sets of measurements were used for specific growth rate calculations and curve establishment.

The biomass accumulation curves were established based on the results of DCW analysis performed every 3rd day of cultivation. The concentrations obtained during this analysis were dependent on the amount of liquid evaporated from the single sample and were compatible with measured OD values. The DCW analysis performance for *Chlorella* and *Scenedesmus* spp. can be seen in *Figure 37 B & C* in Appendix D.

In addition, microscopic investigation was performed on all the cultivated batches prior the harvesting to estimate contamination of the samples and dominance of microalgae species. Several pictures of the microscopic investigation of *Chlorella* and *Scenedesmus* spp. can be observed in *Figure 39* and *Figure 40* as well as *Scenedesmus* sp. biomass harvested from different nutrient media variants (*Figure 37 A*).

4.2.1. Chlorella sp. cultivation during limited aeration

For *Chlorella* sp. cultivated under limited aeration conditions (3 hours/day) the batch was observed every 3rd day of cultivation that made it possible to trace the growth progress of this strain. In *Figure 8* triplicates of *Chlorella* sp. are placed in the following order of nutrient media from left to right: BBM (control), 25% ACWW, 50% ACWW, 75% ACWW, and 100% ACWW.

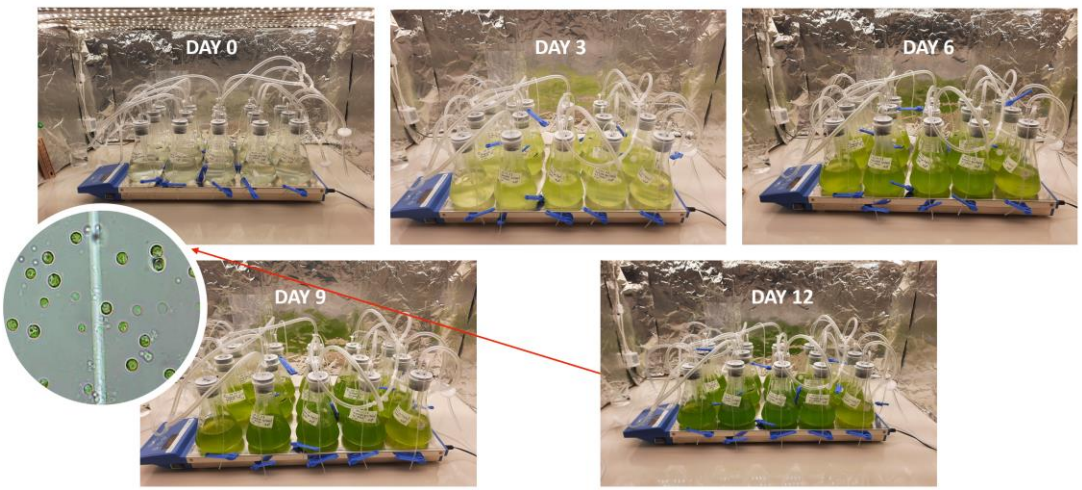

Figure 8. Observation of Chlorella sp. cultivation progress on every 3rd day of cultivation, limited aeration.

The graduate decrease in transparency in all the conical flasks was observed while cultivation progressed. At the same time, it needs to be mentioned that color was not similar in all the samples. Already from day 3 this difference begins to be noticed: the rightmost and the leftmost triplicates have higher transparency than the others. On days 6 and 9 the transparency decreased proportionally in all the samples but still was higher in triplicates with the control medium (BBM) and 100% ACWW. On day 12 the transparency noticeably decreased only in the triplicate with BBM, while no significant changes in other samples were observed.

Together with the visual observation of the growth other essential parameters like OD and DCW were measured and plotted in charts, showing the OD values change (daily), biomass accumulation (once in 3 days), and changes in specific growth rates (daily). The plots are demonstrated in *Figure 9*.

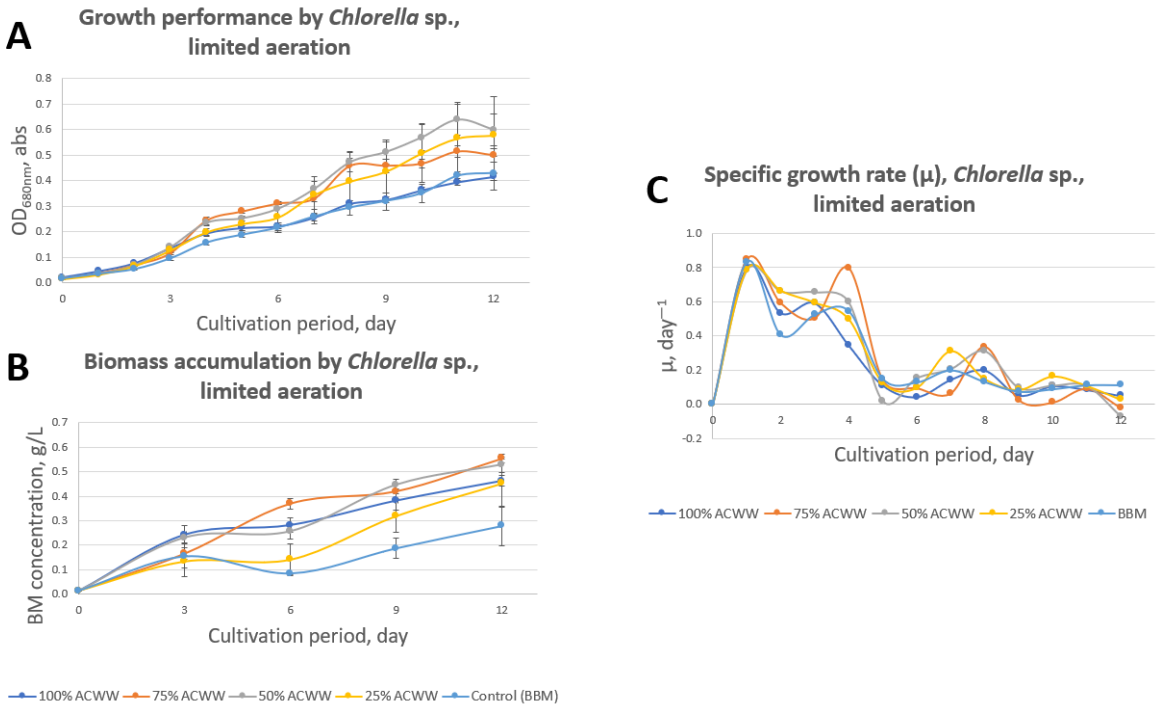

Figure 9. Growth performance by Chlorella sp. under limited aeration: A) absorbance measurements at wavelength 680 nm; B) biomass accumulation measured by DCW method; C) calculated specific growth rate. Error bars represent the standard deviation from the mean (n=3).

In *Figure 9 A* the average values of absorbance at 680 nm are plotted against the time and show the growth dynamics of *Chlorella* sp. in different nutrient media compositions. It was observed that all the nutrient compositions supported different growth intensity of *Chlorella* sp. The best performance was observed with 50% and 25% ACWW. The lowest OD increase was registered for BBM and 100% ACWW triplicates. At the same time, biomass accumulation study showed

that growth trends of OD values not fully corresponded to biomass concentration in the samples (Figure 9 *B*). Nutrient media variants containing 75% and 50% ACWW showed the highest final biomass concentration (0.554±0.018 and 0.529±0.33 g/L respectively), followed by 100% (0.463±0.022 g/L) and 25% ACWW (0.453±0.099 g/L), while control nutrient media (BBM) demonstrated the lowest biomass concentrations (0.279±0.080 g/L). The error bars in both parts of the *Figure 9* (*A & B*) represent the standard deviation from the mean (triplicates) value.

The specific growth rate was calculated in accordance with the formula, mentioned in *Chapter 3.2.5*. The OD values, measured at the wavelength 750 nm were used for these calculations. Immediate high growth rate (*Figure 9 C*) was observed during the first 3 days (4 days for 75% ACWW) of cultivation, followed by equally rapid decrease during the next days. On the 7^{th} – 9^{th} days of cultivation the specific growth rate slightly increased for all triplicated with the highest values for 75%, 50%, and 25% ACWW at 0.34, 0.31, and 0.31 day^{-1} respectively.

4.2.2. Scenedesmus sp. cultivation during limited aeration

The *Scenedesmus species*, cultivated under limited to 3 h/day aeration conditions was also subjected to visual color and transparency evaluation of the samples *(Figure 10*). Triplicates of *Scenedesmus* sp. are placed from left to right in the following order of nutrient media in *Figure 10*: BBM (control), 25% ACWW, 50% ACWW, 75% ACWW, and 100% ACWW.

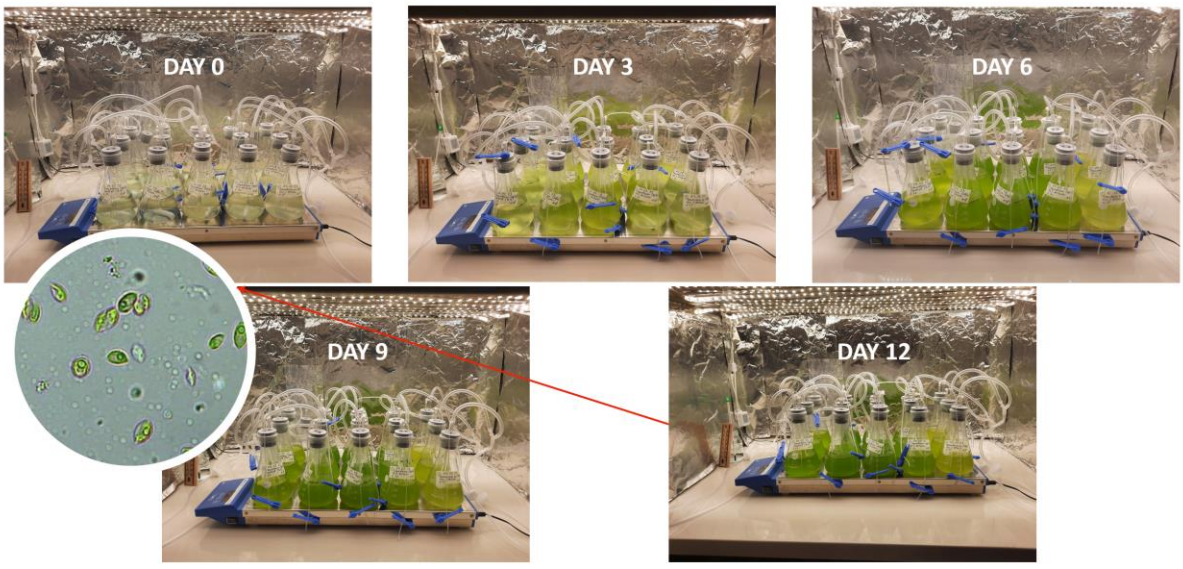

Figure 10. Observation of Scenedesmus sp. cultivation progress on every 3rd day, limited aeration.

The initial transparency of the batch with *Scenedesmus* sp. was lower than for *Chlorella* sp. due to slightly higher initial inoculate concentrations (0.016 and 0.014 g/L respectively) and

possibly higher content of pigment in the microalgal cells. On the 3rd day of cultivation transparency decreased more or less equally in all the grown samples, but on the 6th day in the samples with BBM and 100% ACWW higher transparency was observed. On days 9 and 12 only in triplicates with 100% ACWW higher transparency compared to all the other samples was observed, where the color intensity was similar.

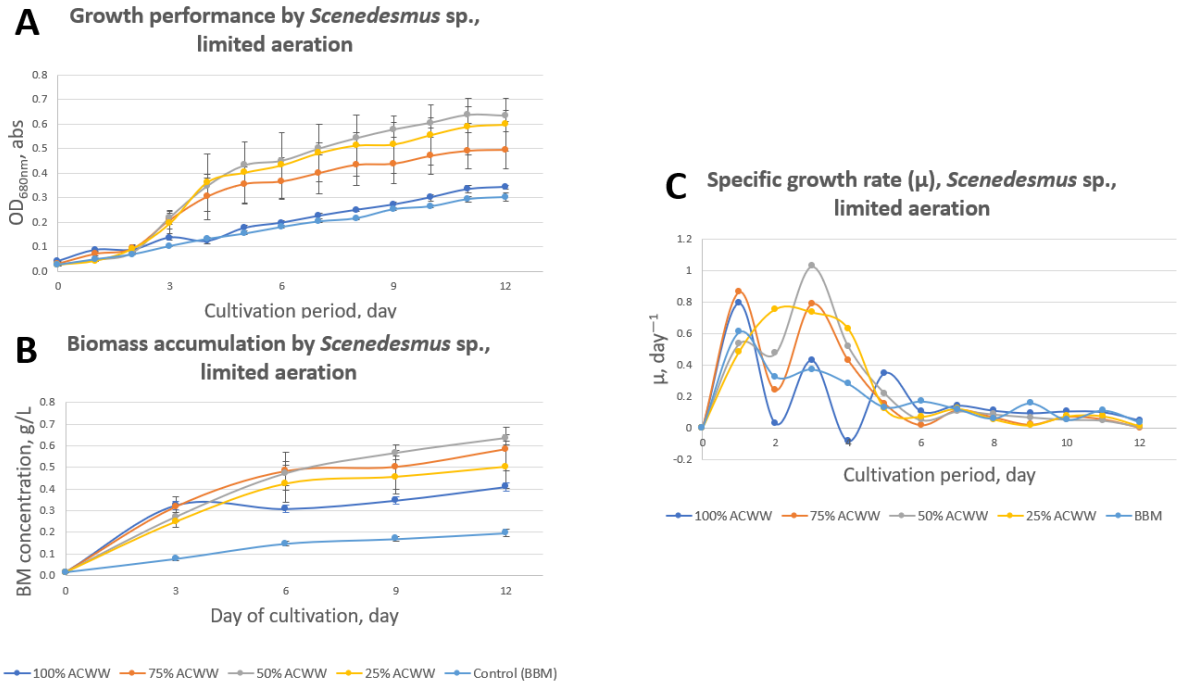

Figure 11. Growth performance by Scenedesmus sp. under limited aeration: A) absorbance measurements at wavelength 680 nm; B) biomass accumulation measured by DCW method; C) specific growth rate. Error bars represent the standard deviation from the mean (n=3).

The optical density at the wavelengths 680 nm and 750 nm was monitored daily and DCW parameters were obtained once in 3 days. The growth curve for *Scenedesmus* sp. under limited aeration was established based on the average OD values at λ=680 nm (*Figure 11 A*).

The *Scenedesmus* sp., similarly to *Chlorella* sp., demonstrated different growth intensity in all the ACWW dilutions. The highest OD values reported while the growing in 50%, 25%, and 75% ACWW were 0.64±0.03 abs, 0.60±0.1 abs, and 0.49±0.07 abs respectively on the 11th – 12th day of cultivation. The absorbance values for BBM and 100% ACWW samples were almost twice as low as the highest values obtained from the batch (around 0.3 abs). The biomass accumulation plot (*Figure 11 B*) almost perfectly corresponds to the previously described data. From the nutrient media variants containing 50%, 75%, and 25% of ACWW the final biomass concentration was reported to be 0.637±0.014 g/L, 0.585±0.101 g/L, and 0.503±0.099 g/L respectively. The triplicates with 100% ACWW contained 0.411±0.019 g/L of biomass, and

control triplicates (BBM) – only 0.197±0.016 g/L. Unlike with the *Chlorella* sp. cultivation, the error bars on both curves (*Figure 11 A & B*) for BBM, 100% ACWW, and 25% ACWW were almost unnoticeable due to very close measurement results. The error bars for the other 2 variants of nutrient media were significantly lower than while *Chlorella* sp. cultivation.

The specific growth rate curve for *Scenedesmus* sp. (*Figure 11 C*) shows significant amplitude during the first 4 days of cultivation for all the nutrient media variants with pick values on days 1 and 3, and significant decrease on day 2 for all variants and day 4 for 100% ACWW variant. These rapid changes in growth rate can be explained by partially unstable cultivation conditions when the aeration rates are not measured and maintained at the same pace, unequal illumination for every separate sample due to limited lightning conditions and limited space in the cultivation chamber. At the same time, analyzing data for the triplicate with 25% ACWW allows us to distinguish a clear sigmoidal growth curve with exponential growth, stationary, and decline phases. After day 5, all the samples entered the decline phase demonstrated in the growth curve chart.

4.2.3. Scenedesmus sp. cultivation during constant aeration

The current cultivation batch of *Scenedesmus* sp. significantly differs from the other 3 batches because of constant aeration conditions applied. During the cultivation it has been noticed that by day 6 ≈15-20% of the working volume evaporated from all the conical flasks, which significantly influenced optical density and other important parameters. Therefore, it was decided to stop aeration immediately and terminate the experiment after 10 days of cultivation. The biomass was harvested and analyzed to check how the aeration for 6 days influenced targeted experimental objectives. The observation of transparency was performed only twice, on the 3rd and 6th days (*Figure 12*), and DCW analysis was performed on day 7 and 10 before harvesting the batch. The OD values were measured daily during all 10 days of cultivation. Triplicates of *Scenedesmus* sp. are placed in the following order of nutrient media from left to right in *Figure 12*: BBM (control), 25% ACWW, 50% ACWW, 75% ACWW, and 100% ACWW.

Figure 12. Observation of Scenedesmus sp. cultivation progress, constant aeration.

The initial concentration of microalgal inoculate was 0.010 g/L which provided high initial transparency on day 0. By day 3 the transparency significantly decreased in all the samples, but color was slightly lighter in a triplicate with BBM. On day 6 the color of all the samples was dark green and nutrient media totally lost its transparency. In addition, a significant amount of liquid evaporated which made it impossible to continue the experiment within planned terms.

Nevertheless, we continued cultivation until day 10 and kept measuring the optical density which made it possible to create the growth curve based on the OD values measured at λ=680 nm (*Figure 13 A*).

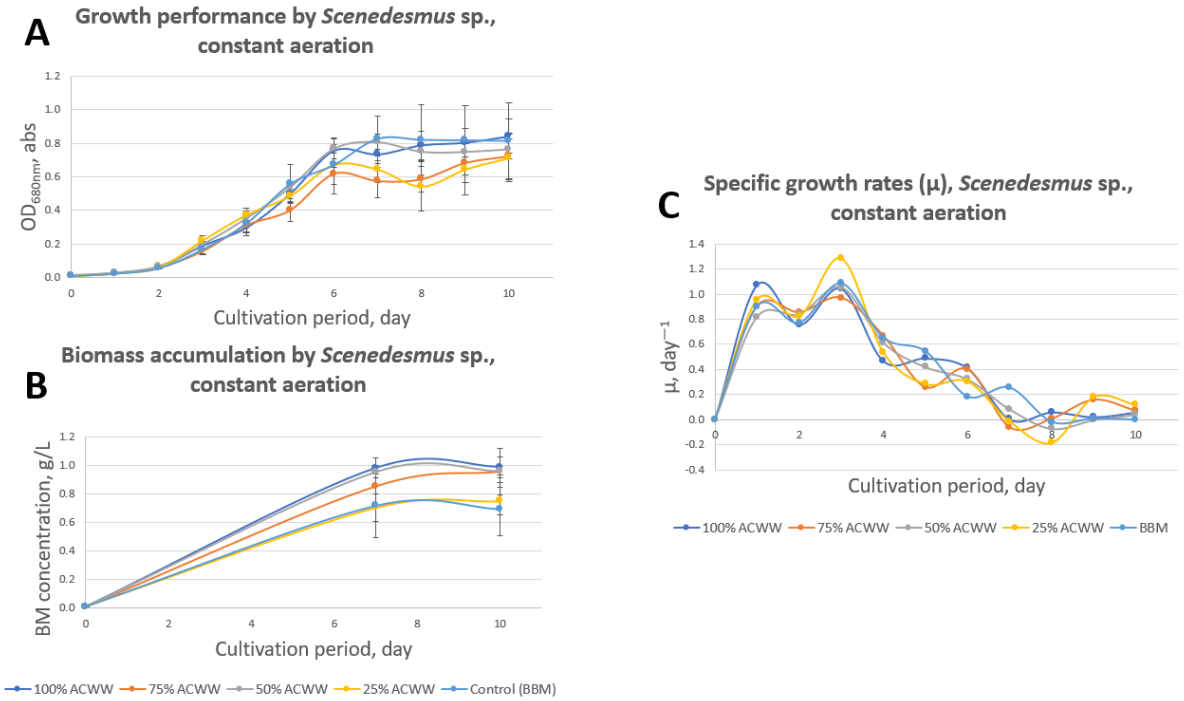

Figure 13. Growth performance by Scenedesmus sp. under constant aeration: A) absorbance measurements at wavelength 680 nm; B) biomass accumulation measured by DCW method; C) specific growth rate. Error bars represent the standard deviation from the mean (n=3).

Unlike in previously described growth curves, *Scenedesmus* sp. with constant air supply did not show significant differences in OD values. In this batch the highest OD values were observed in the samples containing 100% ACWW, BBM, and 50% ACWW (0.842±0.101 abs, 0.815±0.228 abs, and 0.766±0.059 abs respectively), followed by 75% (0.723±0.140 abs) and 25% (0.713±0.144 abs) ACWW. The biomass accumulation monitoring was not performed sufficiently in this batch. Therefore, it is impossible to describe the correspondence of curve patterns, but the biomass concentrations were measured on the 7th and 10th day of cultivation using DCW method (*Figure 13 B*). The highest average final biomass concentrations were measured in the samples containing 100%, 50%, and 75% ACWW with 0.991±0.073 g/L, 0.963±0.031 g/L, and 0.959±0.164 g/L respectively. The samples containing BBM and 25% ACWW accumulated significantly lower biomass concentrations (0.694±0.185 g/L and 0.752±0.094 g/L respectively). The standard deviation bars in both graphs (*Figure 13 A & B*) are significantly higher than in previously described batches. It can be explained by the different evaporation percentage in each sample within the triplicates. It was noticed that the most affected triplicates in this regard were triplicates, containing BBM and 75% ACWW. Disproportion of evaporated fractions can also explain high average OD values and low average biomass concentrations in triplicates containing BBM: lower volume at equal biomass concentration provides higher optical density.

Regarding the specific growth rate curve (*Figure 13 C*) plotted based on the OD values measured at 750 nm, this curve demonstrated the most similar sigmoidal pattern in results for all the samples in the batch. The growth and stationary phases are observed within days 0 and 3, followed by decline phase from day 4 reaching almost a «zero» values by day 10.

4.2.4. Nostoc sp. cultivation during limited aeration

The *Nostoc* sp. was studied as a WWT agent during this experiment. Same as in the previously cultivated batches, the OD values at same wavelengths were measured daily and DCW analysis together with visual transparency evaluation were performed every 3rd day. The changes in transparency are demonstrated in *Figure 14.* Triplicates of *Nostoc* sp. are placed in the following order of nutrient media from left to right in *Figure 14:* BBM (control), 25% ACWW, 50% ACWW, 75% ACWW, and 100% ACWW.

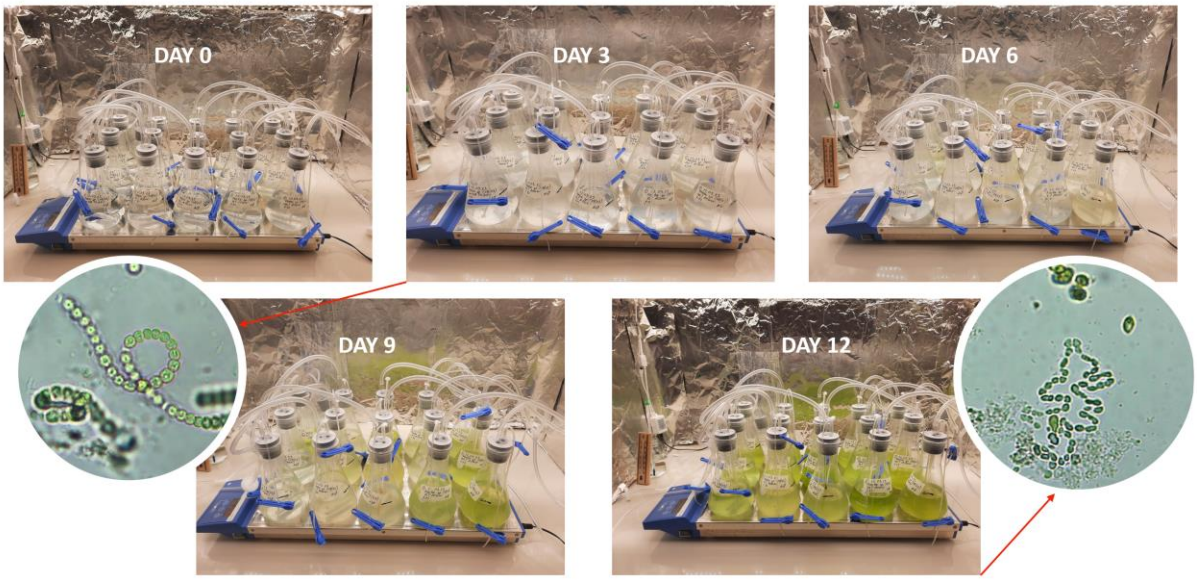

Figure 14. Observation of Nostoc sp. cultivation progress on every 3rd day of cultivation, limited aeration.

Compared to the other cultivated batches, *Nostoc* sp. inoculate had the lowest initial biomass concentration, only 0.004 g/L (2,5-4 times lower than previously described). Therefore, the highest transparency for the whole experiment was registered at the day 0, no coloring was observed in any sample. By day 3 the transparency had decreased in all the samples containing any percentage of ACWW but was slightly higher in triplicates with BBM. By day 6 the transparency further decreased and light green coloring appeared in 1 sample of each triplicate containing BBM, 100%, and 50% ACWW. By day 9 approximately half of the samples acquired different intensity of green coloring coupled with the decreasing transparency. By the end of cultivation (day 12) all the samples had a green color and significantly decreased transparency compared to the beginning of this batch cultivation.

Based on daily OD measurements, the growth curves for *Nostoc* sp. cultivated in different nutrient media variants were created and demonstrated in *Figure 15 A*.

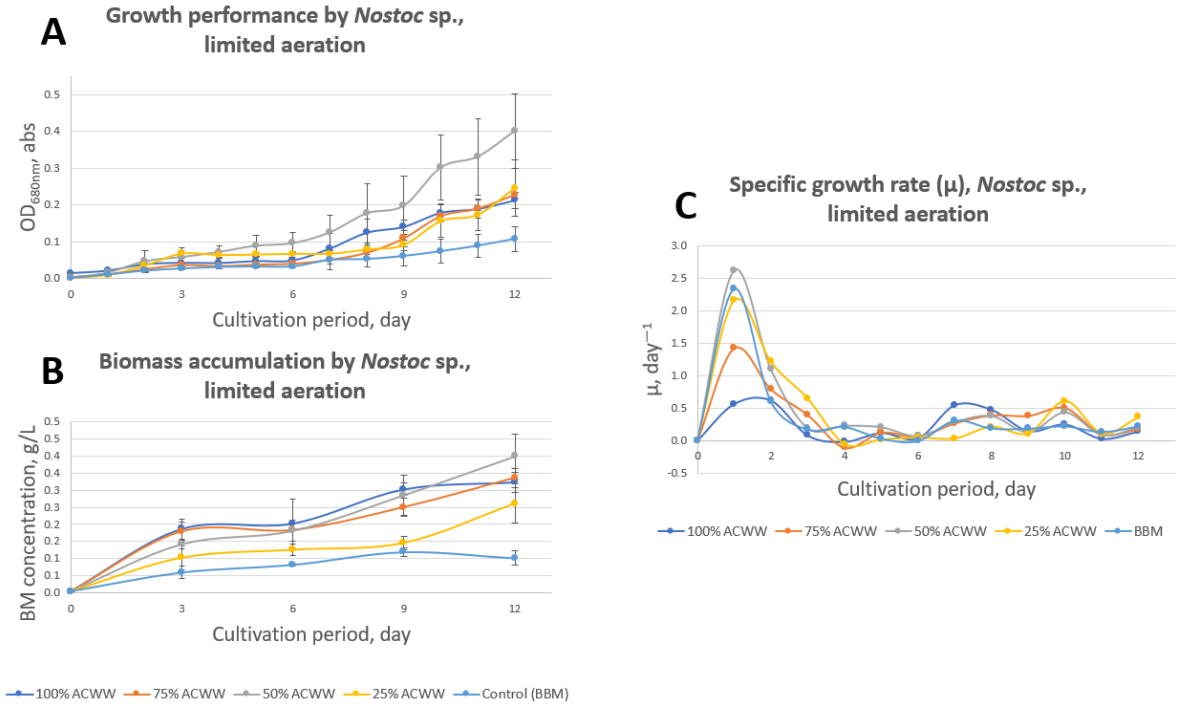

Figure 15. Growth performance by Nostoc sp. under limited aeration: A) absorbance measurements at wavelength 680 nm; B) biomass accumulation measured by DCW method; C) specific growth rate. Error bars represent the standard deviation from the mean (n=3).

During the first 6 days there was no significant change in OD values for all the nutrient media variants. A slight increase was observed by day 6 of cultivation reaching its maximum of 0.096±0.029 abs. From day 7 an unequal increase in OD values was observed in all the samples. The highest average OD values were reported for triplicates containing 50%, 25%, 75%, and 100% ACWW as 0.400±0.101 abs, 0.246±0.076 abs, 0.227±0.008 abs, and 0.212±0.022 abs respectively prior harvesting while the triplicates with BBM had the lowest OD values (0.108±0.034 abs).

Microalgal biomass concentration measured with the use of DCW method demonstrated that biomass accumulation progression did not correspond with the OD values (*Figure 15 B*), unlike in other cultivated batches. Thus, on day 3 significant increase in TSS values compared to initial one was already observed, and final average biomass concentration in the nutrient media variants containing 50%, 75%, 100%, and 25% ACWW was measured to be 0.398±0.065 g/L, 0.336±0.028 g/L, 0.323±0.030 g/L, 0.261±0.056 g/L respectively. The triplicates with BBM had the lowest concentration of biomass: only 0.102±0.020 g/L. The standard deviation bars in *Figure 15 A* are bigger than in *Figure 15 B* more likely due to unequal and uncontrolled color appearing in different samples of the batch. Contamination with bacteria and another microalgal species led to outcompeting the *Nostoc* sp. in this batch. It is worth mentioning that

on days 9 and 12 mixed samples of all the triplicates were subjected to microscopic investigation, and obtained results will be mentioned in the following chapter (Chapter 4.2.5).

Analyzing the specific growth rate curve (*Figure 15 C*) we can see that the highest specific growth rate was observed on day 1 of cultivation for all the studied triplicates with different intensity. Thus, samples containing 50%, 25% ACWW, and BBM had the highest specific growth rate: 2.61, 2.16, and 2.33 day^{-1} respectively, followed by 75% ACWW with 1.42 day^{-1} and 0.56 day^{-1} for 100% ACWW. From day 2 there was no significant increase in the specific growth rate, the highest value reached 0.603 day^{-1}.

4.2.5. Additional data from cultivation experiments and experiences

During cultivation of 4 batches of microalgae several additional parameters were analyzed to make an evaluation of how cultivation conditions influenced biomass production and general cultivation characteristics. In this regard the percentage of liquid evaporated during the cultivation was calculated and plotted towards cultivated bathes, accounting for the volumes collected during samplings (*Figure 16 A*). There is a clear difference between batches cultivated under limited aeration and a batch where aeration was provided constantly. Under limited aeration the evaporation percentage was reported from 1.5% to 3.8% in different batches with standard deviation varying from 0.2 to 2.3%. These values are incomparably lower than those reported from the batch with constant aeration applies: 12.3% to 24.9% and significantly higher standard deviations (from 2.4 to 10.2%). The standard deviation bars are very much higher under the constant aeration conditions caused most likely by unequal and not measured constant air frow into the cultivated samples.

On the other hand, the constant aeration while cultivation of *Scenedesmus* sp. stimulated the highest average biomass production (*Figure 16 B*), registered during the experiment from 46.51 g/L/day for triplicates containing BBM and up to 77.56 g/L/day for the triplicates containing 75% of ACWW. A reasonably good average biomass production was registered while cultivation of *Scenedesmus* sp. under limited aeration condition in nutrient media containing 50% and 75% ACWW which was accounted for 51.75 and 47.44 mg/L/day respectively. The least performance of the average biomass accumulation was observed while cultivating *Nostoc* sp. under limited aeration conditions when the highest values of BMP was registered in samples with 50% ACWW (32.83 mg/L/day) and the lowest – in samples with BBM (only 8.11

mg/L/day). Summarized information on final biomass concentration, average biomass productivity, and evaporated fraction is collected in *Table 9* in Appendix E.

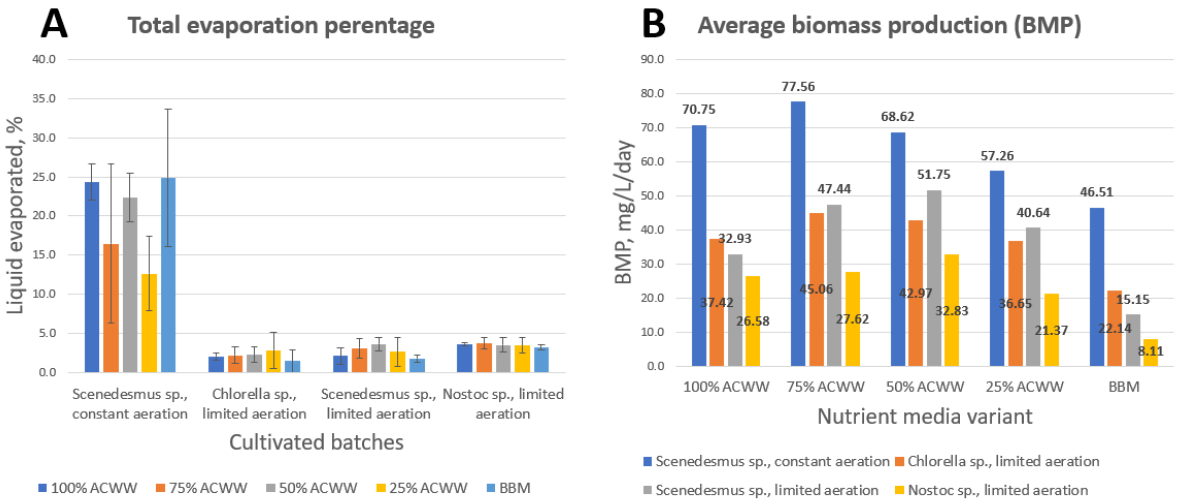

Figure 16. Combined figure for additional cultivation data: A) Percentage of evaporated liquid in studied batches. Error bars represent the standard deviation from the mean (n=3); B) Average biomass production in different nutrient media by 3 algae strains at limited and constant aeration.

Another important study that was applied to all the microalgal batches was ***Microscopy*** of mixed samples from every triplicate at the end of the cultivation. The samples were investigated mainly under 10-fold and 40-fold multiplications and evaluated for microalgal cell density, bacterial contamination, dominating culture, microalgal cell morphology. The pictures of performed microscopy can be found in *Figure 39* and *Figure 40* in Appendix D.

Based on microscopic investigation of mixed samples from every batch it was found that *Scenedesmus* sp. under both limited and constant aeration conditions can maintain dominance of the specie, reached high cell density, was exposed to insignificant bacterial contamination in few mixed samples, and mostly kept microalgal cell morphology. In some samples *Scenedesmus* sp. cells did not form coenobiums, were placed as single cells, and changed cell morphology to more round form.

The microscopy of *Chlorella* sp. demonstrated similar to previously described experimental results: species dominance was maintained, high cell density was achieved during the cultivation, single mixed samples contained insignificant amounts of bacteria and single cells of other microalgal species, morphology of cells was maintained. Single cells achieved bigger size though.

On the contrary, cultivation of *Nostoc* sp. turned out to be the most challenging in terms of maintaining all the abovementioned parameters. Several microscopy investigations were performed while cultivating this batch. It was observed that first coloring in single samples and afterwards in all of them appeared because *Nostoc* sp. was contaminated and gradually outcompeted by *Scenedesmus* sp., which has higher robustness and better growth properties in ACWW. In addition, some samples were significantly contaminated with bacteria and formed medium size clogging. Dominance of *Nostoc* sp. was not maintained during the cultivation and *Scenedesmus* sp. became the dominant specie in the batch (*Figure 41 C* and *Figure 42 C* in Appendix D). The ell density of *Nostoc* sp. was not enough to assume that it was responsible for nutrient depletion or even increase in biomass concentration after the 6th day of cultivation. Furthermore, even cell morphology was changed in some samples: cells acquired more round form and became bigger in size.

4.3. Nutrient removal properties

Microalgal wastewater treatment efficiency was studied during the experiment through analyzing selected basic wastewater parameters such as COD, TN, and TP. All these parameters were analyzed prior to inoculation and every 3rd day of cultivation until harvesting the batch with the use of analytical cell kits. Obtained values were plotted against the cultivation time and removal dynamics were discussed.

All the figures in this chapter, both for COD, TN, and TP concentration monitoring in every cultivated batch, have *A & B* parts. The initial concentration of COD content charts is taken as a «zero» (figure's *A* parts), and positive and negative values represent either increase or decrease in an actual concentration value respectively. For TN and TP figures, the initial values are accounted for 100%, and depletion dynamics are mainly observed. On *B* charts the actual concentration values in mg/L with standard deviation bars are depicted.

4.3.1. Nutrient removal properties of Chlorella sp. under limited aeration

In this subchapter, COD, TN, and TP concentration profiles for *Chlorella* sp. grown under limited aeration are presented in three following figures: *Figure 17, Figure 18, Figure 19*. Both percentage content and actual concentration in mg/L are plotted against the cultivation time.

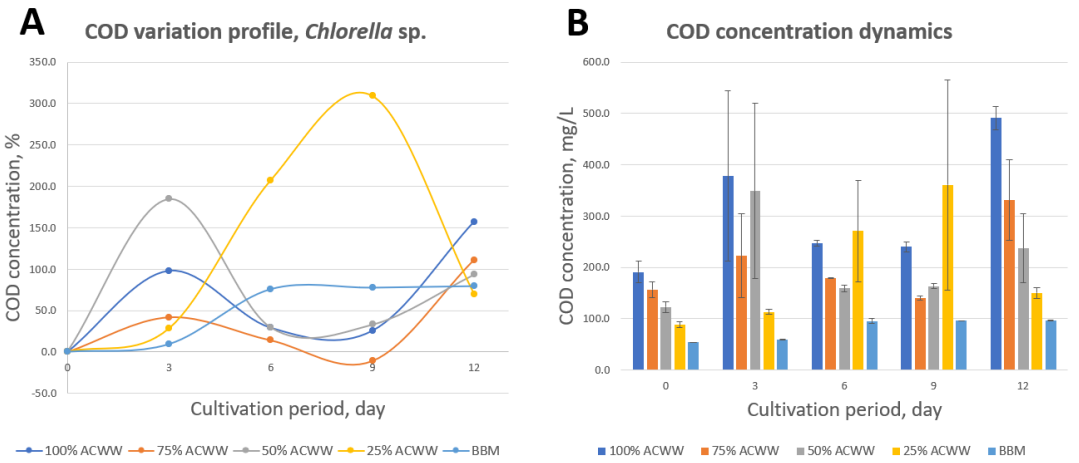

Figure 17. COD content dynamics while cultivating Chlorella sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

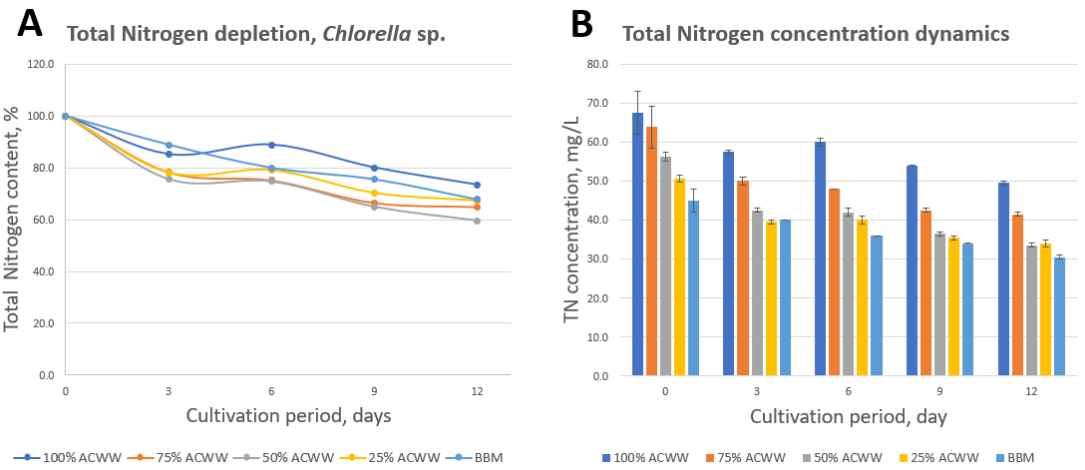

Figure 18. TN content dynamics while cultivating Chlorella sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3)

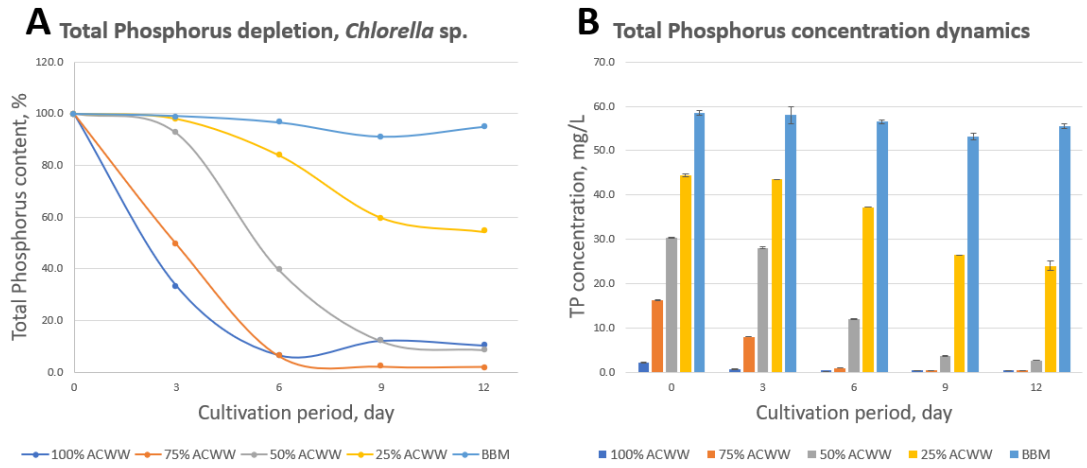

Figure 19. TP content dynamics while cultivating Chlorella sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

On the *Figure 17 A* the COD concentration in percentage for *Chlorella* sp. cultivation is demonstrated. The COD monitoring showed a general tendency to increase its content during all the cultivation period in all variants of nutrient media. Thus, on the 3rd day of cultivation the COD values increased in all the triplicates from 5 mg/L in BBM to 349 mg/L in 50% ACWW which is almost 3-fold from the initial average value in this triplicate. This rapid increase did not follow similar dynamics for all the triplicates and showed both increase and slight decrease during following cultivation days. All the triplicates were harvested with the increased COD values such as 491.0±23.0 mg/L, 331.0±79.0 mg/L, 237.0±67.0 g/L, 150.0±10.0 mg/L, and 97.0±1.0 mg/L in triplicates containing 100%, 75%, 50%, 25% ACWW, and BBM respectively. Regarding actual concentration values for COD, the highest values were measured on day 12 for 100% ACWW (491.0±23.0 mg/L), 75% ACWW (331.0±79.0 mg/L), BBM (97.0±1.0 mg/L), on day 3 for 50% ACWW (349.0±171.0 mg/L), and on day 9 for 25% ACWW (361.0±205.0 mg/L). The standard deviation bars are unacceptably high in many samples, which can be partially explained by the low number of test replications, and for more reliable results the experiment should be repeated.

The TN monitoring within *Chlorella* sp. cultivation, demonstrated in *Figure 18*. Both, *A* and *B* parts of the *Figure 18*, show clear decrease in Nitrogen concentrations in the nutrient media. Thus, on day 3 of cultivation all the TN values in all the triplicates decreased within ≈11-24%, followed by slight increase on day 6 in all the triplicates containing ACWW. At the same time, decrease in TN values in BBM followed almost linear trend (*Figure 18 A*). At the end of experiment, the TN content decreased by 26.7%, 35%, 40.7%, 32.8%, and 32.2% in triplicates with 100%, 75%, 50%, 25% ACWW, and BBM respectively. Initial and final average concentrations of TN can be observed in the *Table 10* in Appendix E. The standard deviation values are way below 5% and therefore results can be evaluated as trustworthy.

The TP monitoring is demonstrated in *Figure 19*. TP depletion is observed on both parts of the *Figure 19, A & B*. Way larger percentage fractions of TP compared with TN were depleted: from 46.0% to 98.2% in 25% ACWW and 75% ACWW respectively (*Figure 19 A*). At the same time, a very low depletion percentage was observed in triplicates containing BBM, only 5.1%. From the data represented in *Figure 19 B* and *Table 10* in Appendix E, we can see clear differences in initial values for BBM and different dilutions of ACWW. The results of this test can be considered noteworthy due to the low standard deviation for TP concentration in all the

checked cases. Phosphorus removal efficiency in general has an inverse relationship to its initial concentration.

4.3.2. Nutrient removal properties of Scenedesmus sp. under limited aeration

While growing *Scenedesmus* sp. under limited aeration, COD, TN, and TP concentration profiles were established and graphically represented in *Figure 20, Figure 21,* and *Figure 22.* Actual concentration in mg/L and percentage content were analyzed for all the three parameters.

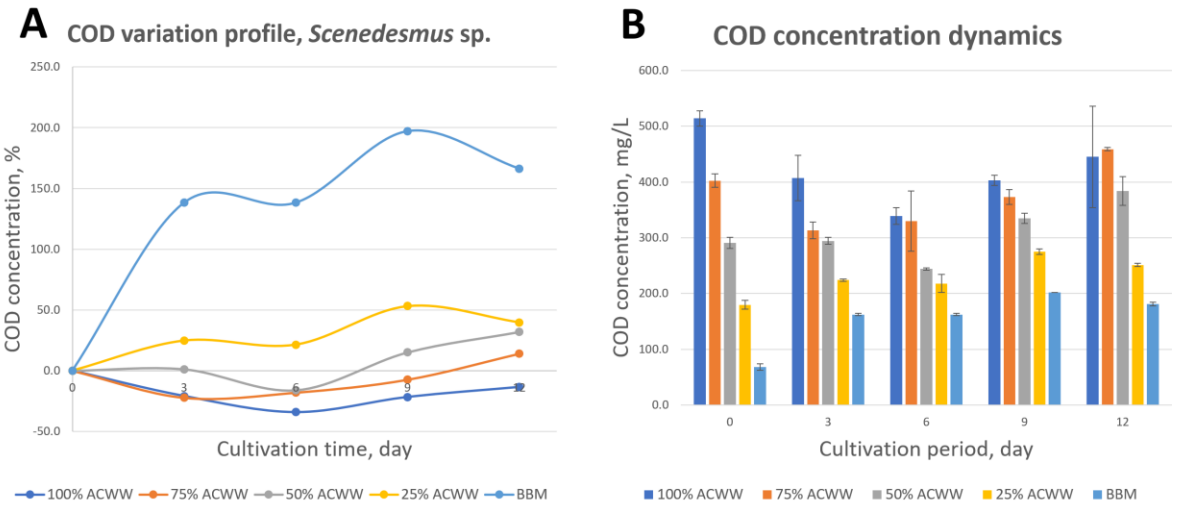

Figure 20. COD content dynamics while cultivating Scenedesmus sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

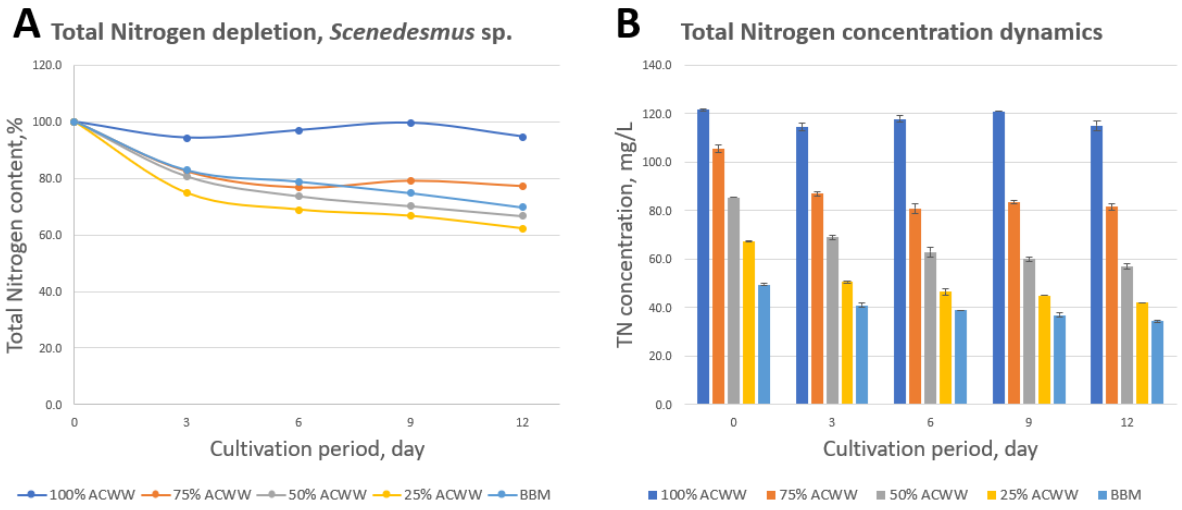

Figure 21. TN content dynamics while cultivating Scenedesmus sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3)

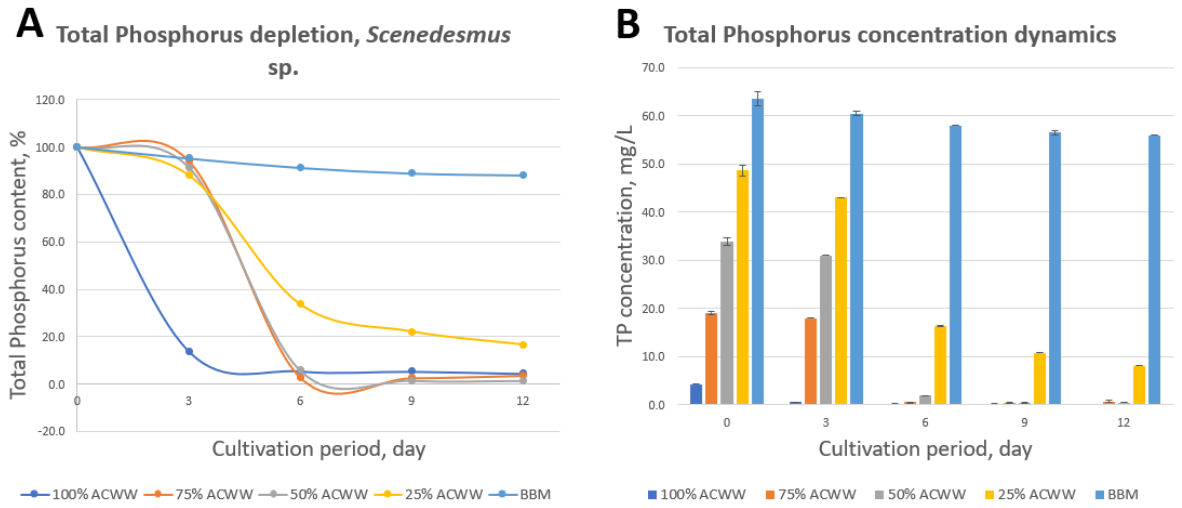

Figure 22. TP content dynamics while cultivating Scenedesmus sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3)

The COD variation profile (*Figure 20*) while *Scenedesmus* sp. cultivation was slightly different to the one previously described for *Chlorella* sp. Thus, the concentration dynamics in percentage (*Figure 20 A*) showed small rise, from 14% (75% ACWW) to ≈40% (25% AWW), and even actual depletion of the final COD values by 13.4% in triplicates with 100% ACWW. But at the same time, almost a 3-fold increase in COD values was observed in triplicates containing BBM as a nutrient media. The first 6 days of cultivation differed from the previously described batch with actual depletion of COD content in samples with 100%, 75%, and 50% ACWW, when average concentrations fall to 339.0±15.0 mg/L, 330.0±54.0 mg/L, and 244.0±2.0 mg/L respectively. It is also important to highlight that initial COD concentration in WW (*Figure 20 B*) was almost 2.5 times higher than in batch with *Chlorella* sp. (*Table 10*) which was most probably caused by usage of the effluents collected on different day. The standard deviation bars were significantly smaller than those described in the previous chapter, which can assure more reliable results of perforemed test.

The TN concentration chart (*Figure 21 A*) demonstrated similar to *Chlorella* sp. TN removal efficiency but it was slightly lower for the triplicate with 75% ACWW and significantly lower for the triplicate with 100% ACWW. At the end of experiment 37.8%, 33.3%, 30.3%, 22.7%, and 5.3% of TN were removed from the samples containing 25%, 50% ACWW, BBM, 75%, and 100% ACWW respectively. The lowest TN concentrations were reported on day 12 for triplicates with BBM, 25%and 50% ACWW (*Table 10*), while the lowest average concentrations for 100% (114.5±1.5 mg/L) and 75% (81.0±2.0 mg/L) ACWW were observed

on days 3 and 6 respectively (*Figure 21 B*). Insignificant error bars indicate high reliability of the obtained results.

Analyzing TP depletion profile in *Figure 22*, we can see that already on day 3 86.0% of TP was removed from the triplicate with 100% ACWW, and by the 6th day 97.0%, 94.1, and 66.3% of TP was removed from samples with 75%, 50%, and 25% ACWW respectively (*Figure 22 A*). The lowest Phosphorus removal efficiency was observed from triplicates with BBM, which reached only 11.8% on day 12. The inverse relationship to average TP concentration in mg/L, similarly to TP removal efficiency by *Chlorella* sp., is observed in this experiment (*Figure 22 B*). The standard deviation values justify the noteworthy results of TP cell test for this batch.

4.3.3. Nutrient removal properties of Nostoc sp. under limited aeration

The main WWT chemical parameters were also analyzed for *Nostoc* sp. under limited aeration, and results for COD, TN, and TP removal efficiencies are reported in *Figure 23*, *Figure 24*, and *Figure 25* both in percentage and actual concentrations in mg/L.

Like in previously described batches, the COD content has increased on the 3rd day of cultivation, but this increase was not exceeding 56% as the highest increase percentage in triplicate with 50% ACWW (*Figure 23 A*). During further cultivation the COD concentrations have been both increased and decreased not following any easily explainable patterns, but ended up by relatively small increases in COD concentrations compared with the other batches (*Table 10*). At the same time, the standard errors do not exceed 10% limit and represent reliability of the obtained results (*Figure 23 B*).

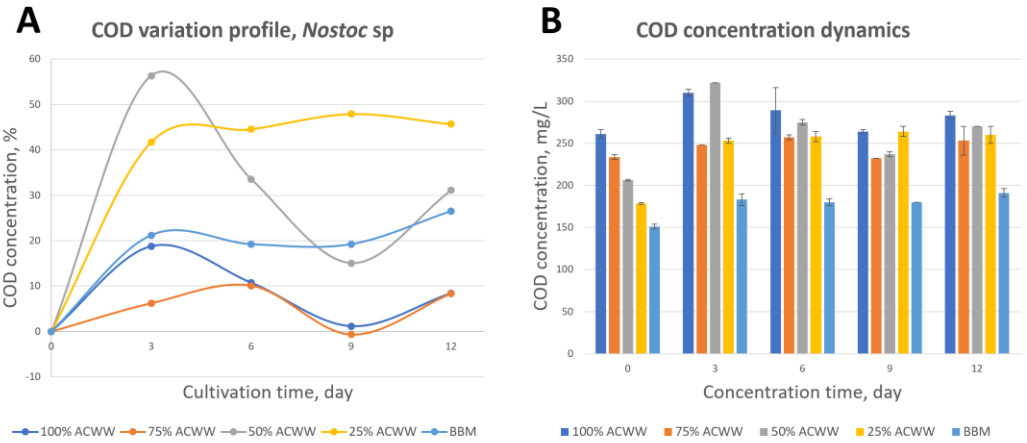

Figure 23. COD content dynamics while cultivating Nostoc sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

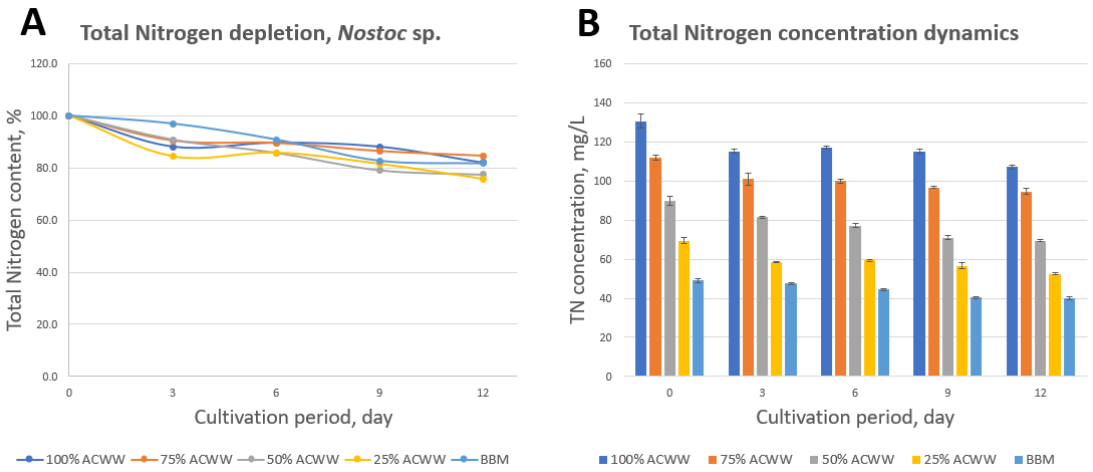

Figure 24. TN content dynamics while cultivating Nostoc sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3)

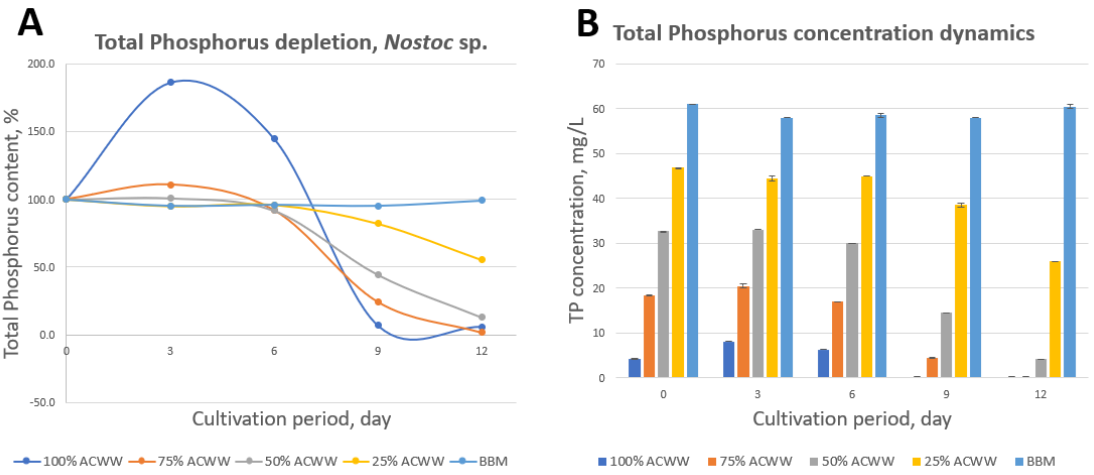

Figure 25. TP content dynamics while cultivating Nostoc sp. under limited aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3)

The TN was gradually taken up by microalgae that grew in the samples, but overall removal efficiency was lower (*Figure 24*) compared to *Chlorella* and *Scenedesmus* spp. under the similar aeration conditions. The highest percentage of removed TN was 25.4% in the triplicate contained 25% ACWW followed by 50% ACWW, BBM, 100%, and 75% ACWW with removal efficiency of 22.6%, 18.4%, 18.0%, and 15.4% respectively (*Figure 24 A*). Both initial and final values for TN concentrations can be found in *Table 10* in Appendix E, and on the *Figure 24 B* its graphical representation is shown. At the same time, the small standard deviation values indicate high accuracy of obtained results.

The TP depletion charts demonstrated different nutrient removal profile (*Figure 25*). Unlike in two previously described profiles, nutrient uptake was not observed until the 9th day of

cultivation. Moreover, almost 2-fold increase (86.0%) was observed on day 3 in triplicates with 100% ACWW (*Figure 25 A*), followed by somewhat decrease on day 6 which amounted in overall accumulated TP (+44.8%), and almost complete TP depletion by day 9 (93.1%). Small increase (+10.7%) in TP concentration was also observed in triplicates with 75% ACWW on day 3, not changed TP concentration in 50% ACWW, and ≈5% TP removal for both 25% ACWW and BBM. Noticeable TP depletion was observed only on the 9th day of cultivation in the samples containing all dilutions of ACWW, but not with BBM. In *Table 10* the actual initial and final concentrations of TP can be found, but the overall nutrient removal performance by *Nostoc* sp. showed the worst results. The initial values of TP were increasing with the increase of BBM fractions in the nutrient media variants, and the standard deviation values were insignificantly small which indicated high accuracy of the test results (*Figure 25 B*).

4.3.4. Nutrient removal properties of Scenedesmus sp. under constant aeration

The COD, TN, and TP concentration profiles for *Scenedesmus* sp. grown in constant aeration conditions are presented in three following figures: *Figure 26, Figure 27,* and *Figure 28.* Like in the previous three subchapters, both percentage content and actual concentration in mg/L are plotted in 2 charts. But the data set represented in these figures is incomplete and it is unfortunately not possible to follow the nutrient removal progress and observe on which day the most of TN and TP were depleted by microalgae. The nutrient quantification tests were performed only on the 7th and 10th day of cultivation after which the batch was harvested. On the other hand, the final results are different from all the others, and it is valuable for the experiment to present this information as well.

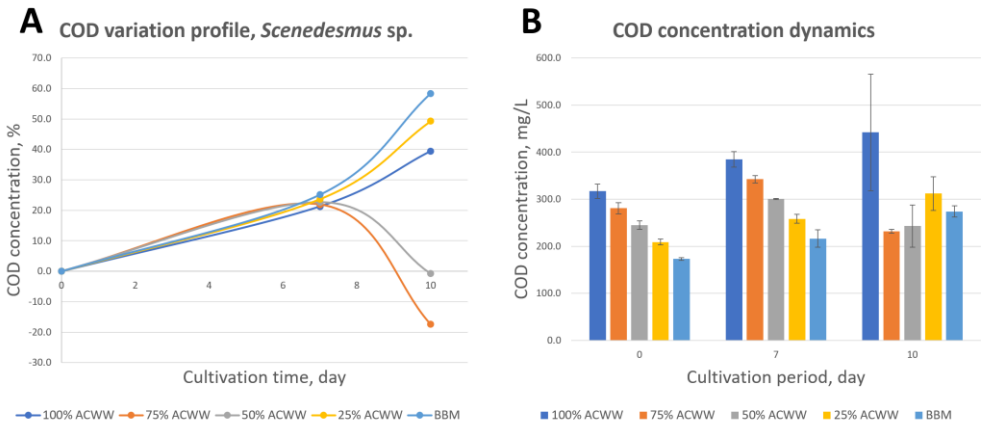

Figure 26. COD content dynamics while cultivating Scenedesmus sp. under constant aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

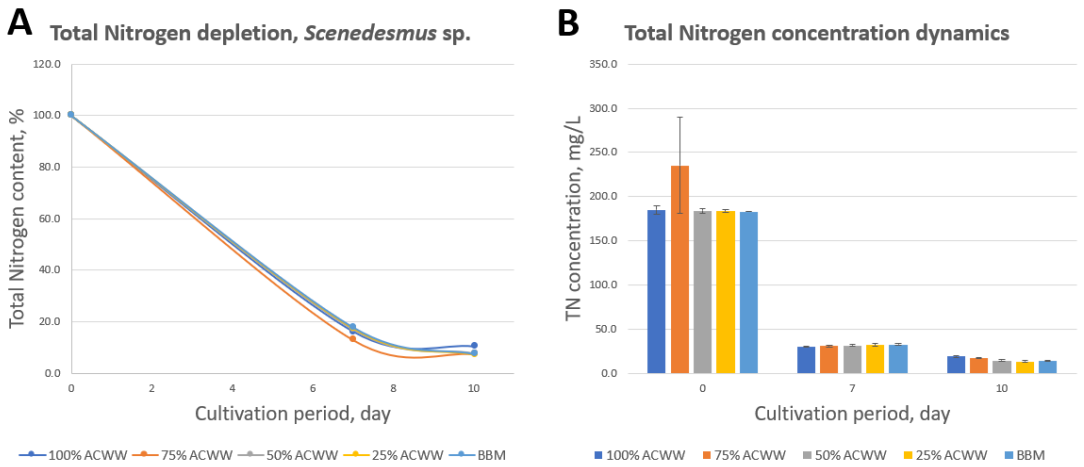

Figure 27. TN content dynamics while cultivating Scenedesmus sp. under constant aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

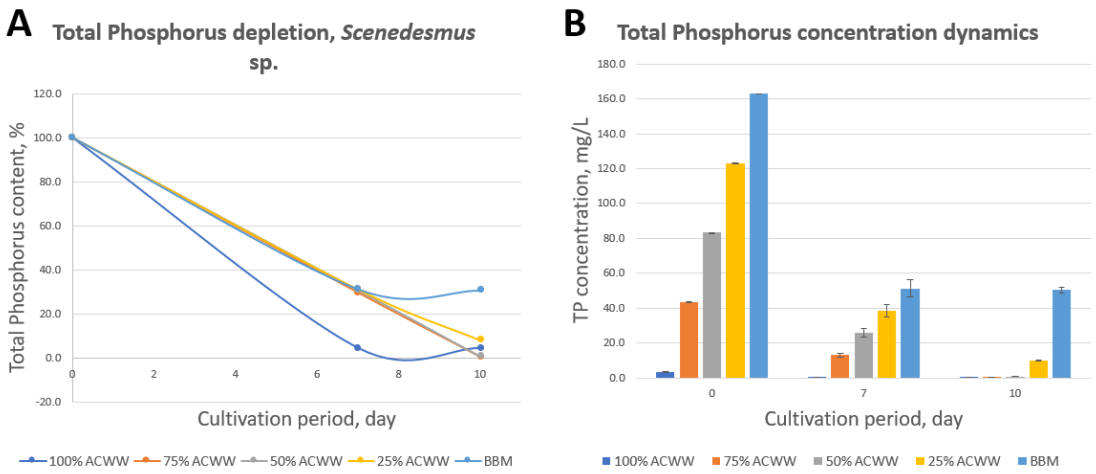

Figure 28. TP content dynamics while cultivating Scenedesmus sp. under constant aeration in: A) %, and B) mg/L. Error bars represent the standard deviation from the mean (n=3).

The COD concentration profile for *Scenedesmus* sp. cultivated under constant aeration (*Figure 26*), demonstrated a graduate increase of oxygen demand ranging within 21.3 – 25.1% for all the samples (*Figure 26 A*) by day 7. Cultivation during another 3 days resulted in both increase and decrease of COD concentrations which have elevated for triplicates with BBM (274.0±12.0 mg/L), 25% (312.0±36.0 mg/L), and 100% ACWW (442.0±19.0 mg/L), and have lowered for the samples containing 50% and 75% ACWW when values of 243.0±45.0 and 232.0±4.0 mg/L respectively were measured (*Figure 26 B*). The standard deviation values did not exceed 10% but only in triplicates with 100%. 50%, and 25% ACWW on 10th day amounted to 13%, 18%, and 11% respectively. Compared with the initial COD concentration, oxygen demand was even removed from the samples, containing 75% and 50% ACWW (*Table 10*).

Unlike the results obtained during the cultivation of batches under limited aeration, constant aeration conditions supported the highest TN removal efficiency (*Figure 27*) observed during the experiment. Thus, by the 7th day of cultivation from 82.2% to 87.1% of TN was removed from all the samples (*Figure 27 A*). There was no significant change in TN concentration by the 10th day (*Figure 27 B*). Values that demonstrate the overall nitrogen removal progress are noted in *Table 10*. The standard deviation values were reported to be under 10% with the only one exception at day of inoculation (day 0) in triplicates with 75% ACWW. This exception might be addressed to human error while performing the TN cell test.

The TP removal efficiency is demonstrated in *Figure 28* where concentration of this element is presented both in actual concentration in mg/L and in percentage expression. By day 7 the highest (95.0%) TP removal efficiency was registered in triplicates with 100% ACWW (*Figure 28 A*) followed by 68.5 – 70.2% in all the other samples. On day 10 TP was removed from all the triplicates with any dilution of ACWW, and 69.2% was removed from BBM samples. The final TP concentrations can be found in *Table 10* in Appendix E and graphically observed in *Figure 28 B*. It also can be pointed out that the standard deviation values do not exceed 10% and indicate comparable results.

4.4. Characterization of microalgal biomass

Another significant part of the experiment was dedicated to analytical quantification of essential biocomponents in harvested microalgal biomass: total carbohydrates, total proteins, and total lipids. In the *Figure 29*, there is united representation of biocomponent composition of all the batches cultivated during the experiment. In addition to total carbs, proteins, and lipids fraction there is a fraction of ashes that has been calculated by the simple extraction of the summed biocomponents form 100%.

All the biomass samples are characterized by different biocomponent compositions with certain similarities. Thus, total carbohydrates were the dominating biocomponent in almost all the cases (exception is *Figure 29 B*, BBM). The highest carbs accumulation was observed in *Scenedesmus* and *Chlorella* spp. biomass under the limited aeration from 54.2 – 62.3% to 35.8 – 55.7% respectively.

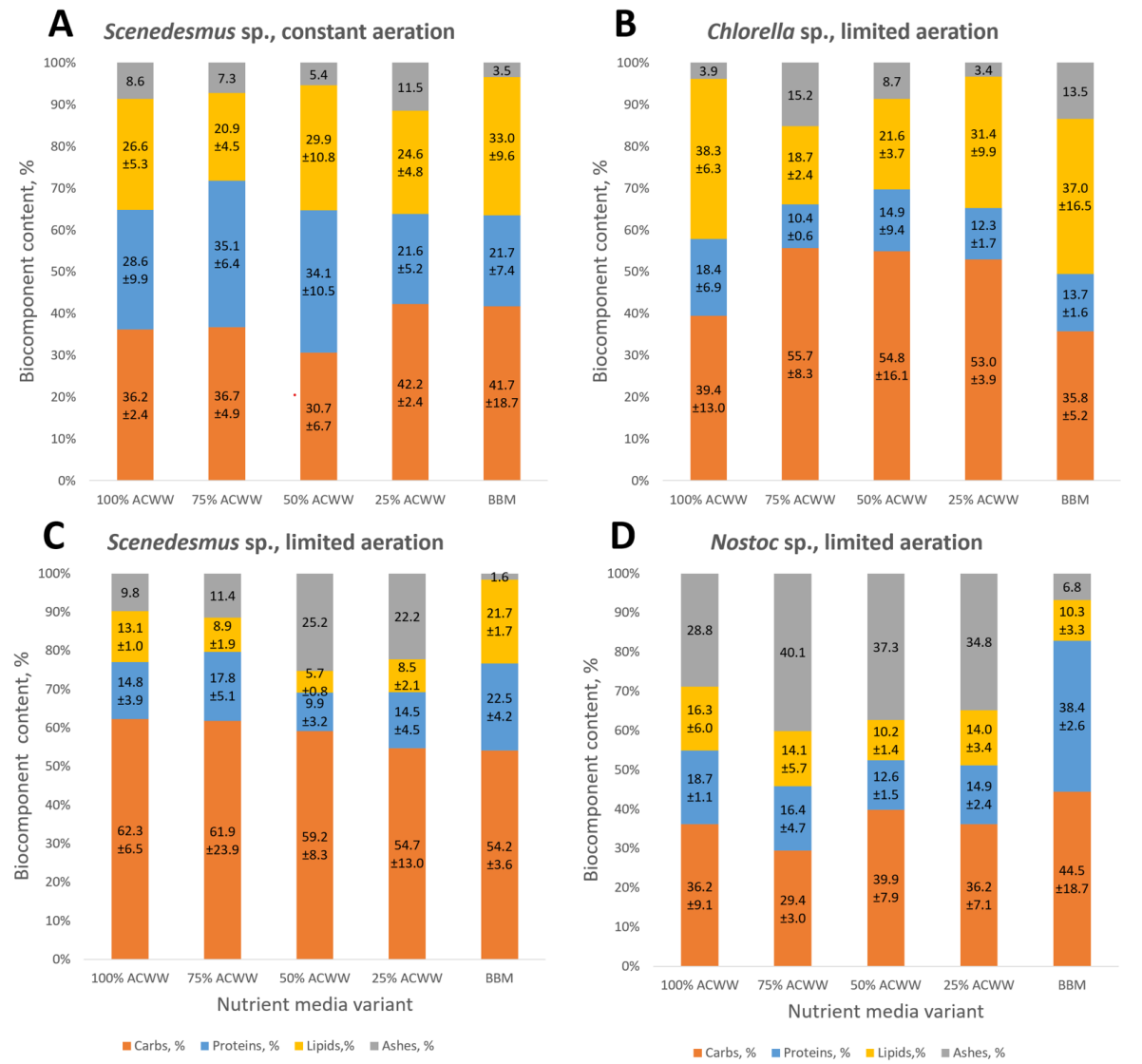

Figure 29. Results of analytical methods performance for carbohydrate, protein, and lipid quantification: A) Scenedesmus sp. at constant aeration; other species at limited aeration: B) Chlorella sp., C) Scenedesmus sp., D) Nostoc sp. Error numbers represent the standard deviation from the mean (n=2 or 3).

The second dominating compound varied within species and aeration conditions during the cultivation. For example, in some biomass under limited aeration the total lipid content exceeded total proteins (*Figure 29 B*), and in others – total protein content was higher than total lipids (*Figure 29 C* & *Figure 29 D*). On the other hand, providing constant aeration to *Scenedesmus* sp. provided almost equal accumulation of all the major biocomponents. It has increased accumulation of lipids and proteins (*Figure 29 A*), and simultaneously reduced carbohydrates in the biomass compared to the same species under limited aeration.

Besides analytically quantified fractions of all the known biocomponents, there was an unidentified fraction of constituents, called "ashes" in this research. Biomass of all the species

grown on BBM had generally the lowest ashes content (usually under 10%), with one exception (13.5% in *Chlorella* sp. biomass). The highest fraction of ashes was observed in *Nostoc* sp. biomass, grown in different dilutions of ACWW: from 28.8% in 100% ACWW to 40.1% in 75% ACWW. Relatively high fractions of ashes were found in *Scenedesmus* sp. biomass grown under limited aeration: 22.2% and 25.2% in triplicates with 25% and 50% ACWW respectively.

4.4.1. Carbohydrate content

The total carbohydrate fraction obtained during analysis was the most dominating or equally dominating in all the biomasses. But at the same time carbohydrate content varied in biomasses grown in different nutrient media variants within the same species (*Figure 30*). Thus, the highest carbohydrate concentration was accumulated in *Scenedesmus* sp. biomass grown under limited aeration in all the variants of nutrient media, followed by *Chlorella* sp. biomass. In *Nostoc* sp. biomass (limited aeration) relatively high concentration of carbs (in mg/g) was accumulated, but these values were significantly lower compared to other species. In the *Scenedesmus* sp. biomass (constant aeration) concentrations of total carbohydrates (in mg/g) were high compared to other carbs concentrations, but within the batch it was not exceeding 422.3±23.9 mg/g BM which corresponds to 42.2%. More information on the actual biomass concentrations can be found in *Table 11* in Appendix E.

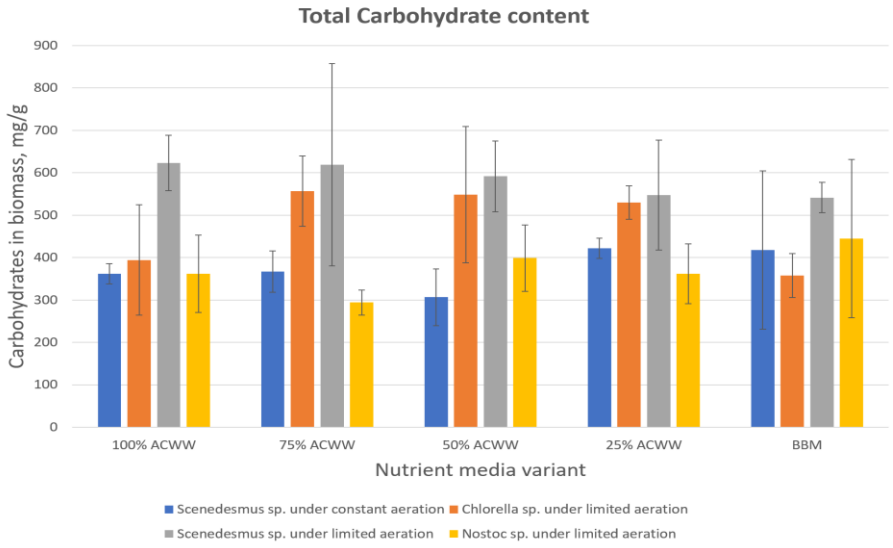

Figure 30. Total carbohydrate content in harvested microalgal biomasses harvested from different nutrient media variants. Error numbers represent the standard deviation from the mean (n=3).

The standard deviation bars in *Figure 30* are high and in some cases exceed acceptable 10% limits. It happens most likely because we analyzed every single sample from the triplicate and

afterwards calculated the average value based on 7-9 samples and did not study samples separately which could provide more relevant deviation. It is important to emphasize that the standard deviation within studied triplicates of every single sample did not exceed 5%.

4.4.2. Protein content

The total protein fraction obtained during the experiment was significantly lower than carbs fraction with 2 exceptions (*Figure 31*) for *Scenedesmus* sp. (from 216.0±52.2 mg/g to 350.5±64.5 mg/g) biomass under constant aeration and *Nostoc* sp. grown in BBM (383.9±26.4 mg/g BM). The lowest total protein concentration was observed in *Scenedesmus* sp. biomass (limited aeration) grown in 50% ACWW and in *Chlorella* sp. biomass (limited aeration), grown in 75% ACWW and was found to be 99.3±31.6 mg/g BM and 103.9±6.3 mg/g BM respectively.

Regarding not mentioned combinations of microalgae species with nutrient media variants, the average concentrations of total proteins varied between 122.6±17.1 mg/g and 183.8±68.5 mg/g in *Chlorella* sp. biomass (limited aeration), from 144.7±45.0 mg/g to 225.5±42.0 mg/g in *Scenedesmus* sp. biomass (limited aeration), and from 125.7±14.5 mg/g to 186.6±10.6 mg/g in *Nostoc* sp. biomass (limited aeration). Detailed information on average total protein concentrations in mg/g can be found in *Table 11* in Appendix E.

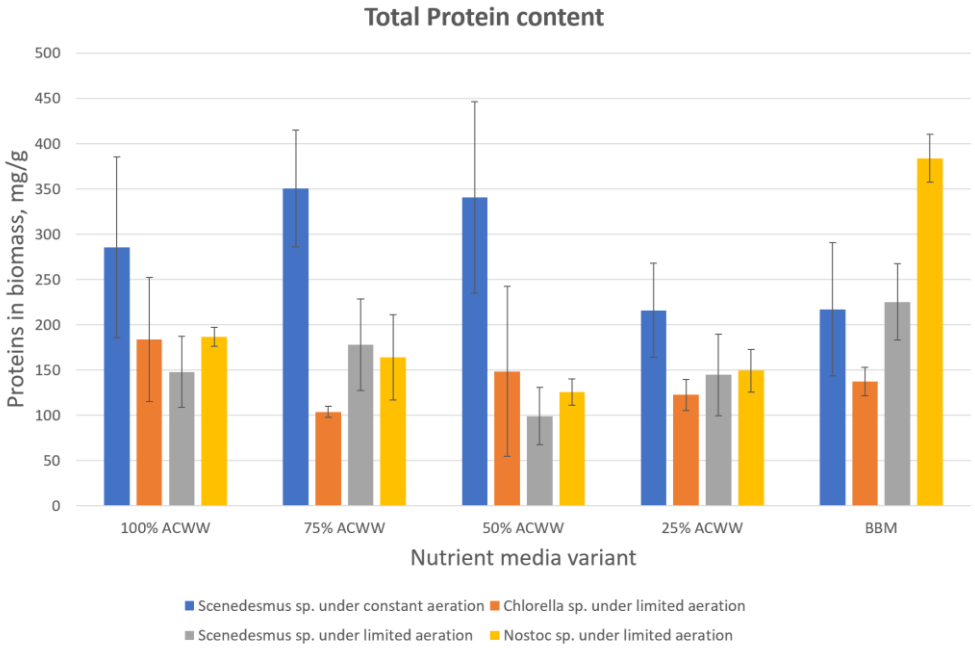

Figure 31. Total protein content in harvested microalgal biomasses harvested from different nutrient media variants. Error numbers represent the standard deviation from the mean (n=3).

The standard deviation bars in total protein content analysis are also high, similarly to those in total carbs content analysis. The reason in this case is the same: triplicates from the single sample were analyzed and afterwards shown in *Figure 31* as an average value for 3 samples. The standard deviation in this case was estimated within 7-9 samples of each sample combination «microalgae species + nutrient media variant». The standard deviation within the triplicates of one sample did not exceed 2.5%, and the highest value observed was 4.3%.

4.4.3. Lipid content

The total lipid content was studied during the analytical part of the experiment (*Figure 32*). *Chlorella* sp. under limited aeration and *Scenedesmus* sp. under constant aeration have highly performed on total lipids accumulation reaching 383.3±63.3 mg/g at the pick and 187.1±23.9 mg/g as the lowest concentrations. Two other species grown under the limited aeration (*Scenedesmus* and *Nostoc* spp.) resulted in accumulation of low total lipid concentrations. The concentration values varied between 56.9±8.3 mg/g and 216.6±17.4 mg/g for *Scenedesmus* sp., and between 102.3±13.7 and 163.4±60.0 mg/g for *Nostoc* sp. Just like for the other biocomponent fractions, detailed information on the average concentrations of total lipid fractions can be found in *Table 11* in Appendix E.

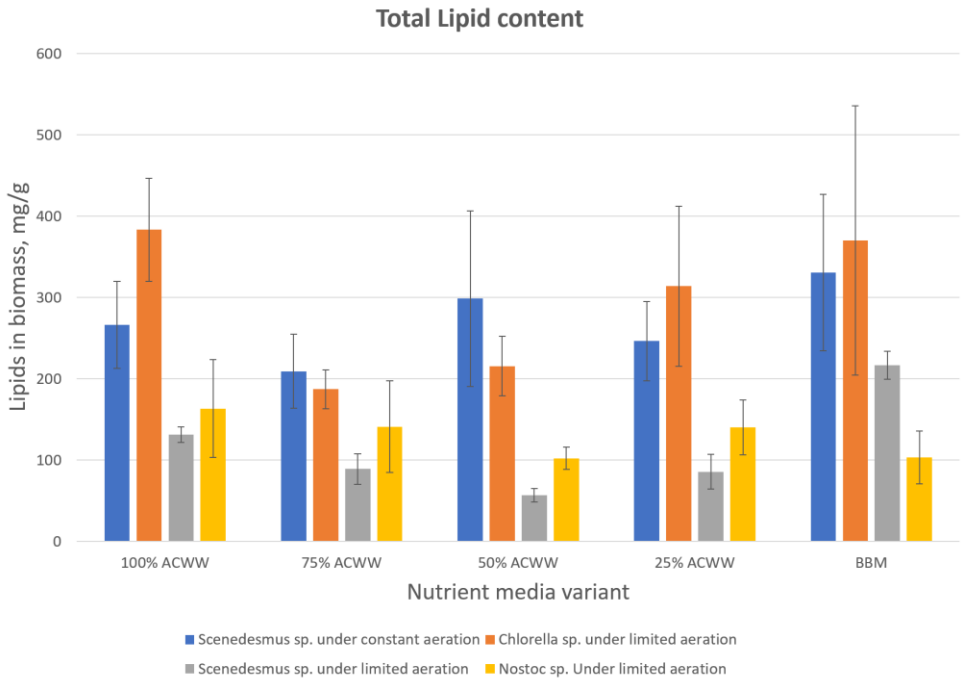

Figure 32. Total lipid content in harvested microalgal biomasses harvested from different nutrient media variants. Error numbers represent the standard deviation from the mean (n=2).

The standard deviation bars for total lipid fractions were also high due to the reasons described previously. In contrast to the other two biocomponent fractions quantified, the total lipids were studied only in duplicates due to limited biomass available (one sample weight need to be at least 8-10 mg). Standard deviation within duplicates of one sample usually did not exceed 5.0%, but the higher values were also observed in samples with low weight of biomass used for analysis (between 11.0 – 13.0%).

Deficiency of biomass turned to be the main challenge during this analysis which resulted in certain observations (*Figure 38*), when different volumes of chloroform was extracted from samples with different initial weight of biomass. At the same time, significant difference in color of extracted chloroform with lipid fraction was noticed between different microalgae species.

5. Discussions

In this chapter general trends and common reasons for obtained results will be represented and discussed. Major connections between different experiment parts will be drawn, and biomass utilization possibilities will be highlighted. At the same time, we discuss challenges that appeared during the experiment performance and adjustments that have been applied to overcome them.

5.1. Aquaculture wastewater basic characterization

Performed analysis of collected aquaculture wastewater from Tyslandsvik Aqua AS (pretreated at the facility) showed that this water can be used as an alternative nutrient media for microalgae cultivation. First, it has very low turbidity and a practically unnoticeable fraction of settleable solids, which ensures undisturbed growth of the microalgal culture. No extreme colorings are present in the WW that can potentially affect light availability and consequently growth performance. At the same time, the absence of odor allows algae to grow in open ponds without disturbing neighboring facilities.

Secondly, the basic chemical composition of used ACWW satisfies core requirements for microalgal growth: carbon, nitrogen, and phosphorus are available. The ratios though are different from those, described in the literature as an ideal (Zuccaro et al., 2020) but levels of bioavailability are expected to be higher than in artificial nutrient media due to straight relation to an aquatic ecosystem through various food chains (Santhanam et al., 2023). At the same time, volatile fractions of the TSS and TS are found to be 69,3% and 25% for VSS and TVS respectively. It means that solids mainly consist of inorganic compounds, but organics are the prevailing in suspended solids fraction.

Before the cultivation, two additional pretreatment methods were used on wastewater: filtration through a glass microfiber filter (1.2 µm particle retention) only and coupled with autoclaving at 120°C for 20 minutes. Both liquids together with raw ACWW were analyzed for COD, TN, and TP content and compared afterward. The only significant change was observed in COD values for raw and filtered WW with and without autoclaving. This difference can be easily explained by the retention of particulate COD on a microfiber filter. TN and TP values indicate an absence of particulate N and P in the WW which makes both elements more bioavailable for microalgae. Insignificant increases in TN value after autoclaving were not seen as a major issue in this case because the increase was lower than 5% and was within an acceptable

deviation range. This small change might arise due to phase change by some nitrogen species under pressure combined with a high temperature in the autoclave (Spaulding et al., 2014).

5.2. Cultivation of microalgae species

This chapter was divided into smaller subchapters to emphasize the discussion of every considered parameter.

5.2.1. Selection of microalgae strains

To provide the best solution for the objectives targeted in this study, three microalgae species were chosen based on their cultivation properties and sufficient biochemical composition. Thus, *Chlorella* and *Scenedesmus* spp. are widely mentioned in the literature as bioremediation agents in different wastewater effluents, including aquaculture wastewater (Aditya et al., 2022; Fallahi et al., 2021; Ji et al., 2015; Nagarajan et al., 2020; Oliveira et al., 2019; Oviedo et al., 2022; Sánchez-Zurano et al., 2021). The biochemical composition of these 2 species is also reported to be suitable for further utilization for various applications, including PHA production (Aditya et al., 2022; Asadi et al., 2020; Bohutskyi et al., 2016; Kumari et al., 2022). On the other hand, there was very limited information on *Nostoc* sp., cultivated on ACWW, and only one study regarding this was just recently published (Kashem et al., 2023). At the same time, *Nostoc* sp. was cultivated in municipal wastewater effluents (Silambarasan et al., 2021), and have been reported to accumulate high amounts of lipids (Shetty & Krishnakumar, 2023), and even up to 78% of biopolymers (Bhati & Mallick, 2015).

Based on the abovementioned information, *Chlorella*, *Scenedesmus*, and *Nostoc* spp. were cultivated in ACWW effluent from salmon smalt production. In the case of *Nostoc* sp., this research can be accounted as a new attempt, because in the publication (Kashem et al., 2023) this microalgae specie was cultivated on ACWW effluent from tilapia production.

5.2.2. Growth observations

Microalgal growth characteristics in a specific wastewater stream were studied and growth kinetics were established for different algal strains. In addition to it, observations of transparency and color change were performed and analyzed. Thus, similar transparency change dynamics were observed for *Scenedesmus* sp. under constant aeration, and for *Chlorella* sp. and *Scenedesmus* sp. under limited aeration (*Figure 8, Figure 10, Figure 12*). At the same time, certain differences in colors were observed which are more likely to be explained by

variations in *chlorophyll* content that depend on age and culturing conditions (Griffiths et al., 2011). Another reason for different transparency dynamics within 4 batches can be addressed as variation in the initial concentration of microalgal inoculate which was 0.010 g/L, 0.014 g/L, 0.016 g/L, and 0.004 g/L in *Scenedesmus* sp. (constant aeration), *Chlorella* sp. (limited aeration), *Scenedesmus* sp. (limited aeration), and *Nostoc* sp. (limited aeration) respectively.

The biggest differences in transparency and color intensity are observed while cultivating *Nostoc* sp. During the first 6 days of microalgal growth together with decreasing transparency no significant coloring was noticed (*Figure 14*) which can be explained by lower concentrations of chlorophyll in the biomass (Baracho & Lombardi, 2023). But after the 6th day, light green coloring was observed in a gradually increasing number of samples until all of them attained green color of different intensity by day 12. Comparing color intensity and its manifestation in *Figure 14*, *Figure 10*, and *Figure 8*, it can be seen that there are similarities between *Figure 10* and *Figure 14*, leading to an assumption that *Nostoc* sp. was contaminated and gradually outcompeted by *Scenedesmus* sp. The current assumption was eventually proven by microscopic investigation of *Nostoc* sp. samples and documented with pictures shown in *Figure 41* and *Figure 42* (Appendix D) where both high microbial and microalgal contamination is observed.

5.2.3. OD monitoring

Optical density is one of the simplest and most available methods of microalgal growth indication (Chioccioli et al., 2014). However, variations in pigment content during growth can lead to its unreliability (Griffiths et al., 2011). Therefore, it is essential to establish a relationship between actual biomass accumulation and absorbance. Daily change in OD at wavelength 680nm and 750 nm during microalgal growth was monitored and afterward compared to biomass accumulation curves, built on DCW analysis results obtained on every 3rd day of cultivation. The following data were collected and plotted in graphs, as a function of time, for all the cultivated batches (*Figure 9 A & B, Figure 11 A & B, Figure 13 A & B, Figure 15 A & B*).

Analysis of the abovementioned figures shows a high correspondence between measured OD values and biomass accumulation measurements while cultivation of *Scenedesmus* sp. at constant aeration, and *Chlorella* sp. and *Scenedesmus* sp. at limited aeration. At the same time, the OD increase in one nutrient media variant not equally corresponded to the DCW values

increase, but this can be explained by the interference of OD measurements with the *chlorophyll* in the microalgae (Griffiths et al., 2011), proving that OD values monitoring not always representative for biomass accumulation. Low correspondence between OD and the DCW values is observed for *Nostoc* sp. (*Figure 15 A & B*) when a noticeable increase in the OD values starts from the 6th day of cultivation while the TSS values are increased already on the 3rd day.

5.2.4. Biomass accumulation and specific growth rates

Biomass accumulation dynamics and OD measurements evidenced that better microalgal growth was provided in nutrient media variants, containing ACWW. Cultivation in BBM showed the lowest growth results in all the batches. Despite higher initial concentrations of essential nutrients in BBM (*Table 10*), ACWW more likely contained N & P in better bioavailable forms which provided better growth in corresponding samples.

The specific growth rate curves (*Figure 9 C, Figure 11 C, Figure 13 C, Figure 15 C*) in all the batches have similar sigmoidal form, but without detected lag phase, which is most probably absent because all the species was adapted to growth conditions and lag phase duration was not longer than few hours and therefore was not detected by the analysis. Another remarkable feature observed on specific growth rate curves for 3 first batches was that after an immediate increase of the μ on day 1 slight decrease in growth on day 2 was detected, followed by another increase the following day. From the 4th day of cultivation specific growth rates decreased over time. These variations in growth rates can be explained by the possible adaptation for uptake of nutrients with different bioavailability followed by a general decrease of growth rates with increasing nutrient depletion.

On the other hand, specific growth rates of *Nostoc* sp. did not follow similar patterns: after a rapid increase of μ on day 1 gradual decrease in its value was observed. This can be evidence that the current ACWW type is not very suitable for *Nostoc* sp. cultivation. But contradiction appears when growth rates in BBM followed the same pattern. In this case, bacterial contamination of the samples can be a possible reason for such growth rate behavior. After the sixth-day slight increase appeared again which was most probably connected to contamination by *Scenedesmus* sp. and its growth since green coloring in samples was observed at the same time (*Figure 14*).

Two specific growth rate curves for *Scenedesmus* sp. under constant and limited aeration (*Figure 11 C, Figure 13 C*) can be compared as well. Under constant aeration, the higher growth rate was sustained over a longer time whereas limited aeration conditions caused unstable μ dynamics at lower amplitude.

Another observation requiring explanation is the relatively high standard deviation bars in the *A & B* parts of relevant figures. The first reason for that is graphs plotting based on average OD and DCW values, obtained from triplicates of different nutrient media variants. Experimental setup installation was not considered for universal aeration supply in every single sample which could be higher or lower within the same triplicate. In addition, light intensity was not unified for a single sample as well. Some samples received slightly higher light intensities than others. All in all, it created cultivation conditions that were slightly different for all the samples in the batch which resulted in average values with high standard deviation values.

Overall, good, or acceptable growth intensities and biomass accumulation dynamics were observed in the studied species. At the same time, a sufficient amount of biomass was harvested for biochemical composition study.

5.3. Nutrient removal efficiency

To evaluate microalgae-based nutrient removal efficiency from the wastewater, initial and final concentrations of COD, TN, and TP were considered and plotted as a function of time in *Figure 17–Figure 28*. Additional measurements of these parameters were performed every 3rd day of cultivation and nutrient removal dynamics were also analyzed.

Several publications have also report remediation of ACWW with microalgal and summary of information from the articles compared to the current study is demonstrated in *Table 5*.

Table 5. Rate of remediation and biomass production for cultivated strains compared with published data (Kashem et al., 2023).

Strain name	TN removal (%)		TP removal (%)		Biomass yield (g/L)	
	ACWW-1	*ACWW-2*	*ACWW-1*	*ACWW-2*	*ACWW-1*	*ACWW-2*
Chlorella sp.	26.7±0.1	92.3±2.7	89.7±0.0	75.6±3.6	0.463±0.022	0.494±0.053
Scenedesmus sp.	5.3±0.2	91.2±2.1	95.6±0.0	78.7±2.4	0.411±0.019	0.344±0.024
Scenedesmus sp.*	89.7±0.1		95.4±0.0		0.991±0.073	
Nostoc sp.	18.00±0.1	46.6±3.3	94.0±0.0	94.2±0.1	0.323±0.030	0.349±0.029

Scenedesmus sp. – limited aeration
*Scenedesmus sp.** – constant aeration
ACWW-1 – aquaculture WW from salmon smolt production (current experiment)
ACWW-2 – aquaculture WW from the tilapia fish farm *(Kashem et al., 2023)*

5.3.1. COD concentration profile

In most of the samples, the COD concentration increased reaching elevated values from 8.3% to 166.2%. However, in some exceptional cases, actual COD removal from 1% to 17.4% was registered. There was no clear pattern of COD dynamics, and no patterns were established or observed (*Figure 17, Figure 20, Figure 23, Figure 26*). The major question in this situation was why COD mainly increased and has not been removed while microalgal growth.

There are several possible reasons for COD increase during microalgal cultivation. Published studies reveal that the N/P ratio plays an important role in COD removal efficiency. Thus, the maximum COD removal efficiency by *Scenedesmus* sp. (up to 89.5±1.2%) was obtained with N_{250}/P_5 ratio (Debeni Devi et al., 2023). The closest to that ratio was registered while cultivating *Scenedesmus* sp. in 75% ACWW under constant aeration (N_{235}/P_{43}), and this combination provided the highest COD removal efficiency (17.4%) in our experiment. There are grounds to assume that different N/P ratios will influence COD dynamics in various ways. To conclude on this assumption, more studies need to be performed aiming for a certain purpose.

Other authors reported stable levels or insignificant increase of COD concentrations together with high TN and TP removal efficiency due to CO_2 sparging which reduces the potential of microalgae to take-up the carbon material from WW through stimulating autotrophic or mixotrophic metabolism and oppression heterotrophic metabolism (Mohsenpour et al., 2021). In our experiment the system was aerated with air and the CO_2 concentration in it was not significant, but account considering the above-mentioned publication, it is fair to assume that this factor also participated in the total COD increase.

In another publication, the COD rise was explained by reactor system choice and by the duration of cultivation itself (Ramaraj et al., 2015). The author mentions that in CSTR all ages of algae exist in the same volume due to low circulation of liquid, and partially decomposed microalgal cells can contribute to COD increase. In our experiment, a batch reactor has been used, which makes the circulation of liquids inside impossible. Therefore, this scenario seems to contribute the most to the overall COD increase.

5.3.2. TN concentration profile

Nitrogen can be assimilated by microalgae in different forms like ammonium, nitrate, nitrite, and sometimes organic nitrogen, like urea (Wang et al., 2017). ACWW is usually characterized by significant levels of nitrates. At the same time, the most bioavailable inorganic form of nitrogen is NH_4^+, other nitrogen species need to be reduced by enzymes to bioavailable ammonia (Cardoso et al., 2021).

In all the samples tested for TN depletion, its concentration has been reduced: in the batches with limited aeration within 15.4 – 40.4%, and up to 92.9% in the batch with constant aeration (*Figure 18, Figure 21, Figure 24, Figure 27*). Considering the significant difference in TN removal efficiency it is reasonable to compare batches in terms of «the same aeration conditions + different microalgal species», and «the same microalgal species + different aeration conditions».

Under limited aeration, all 3 species demonstrated different TN removal efficiencies when the lowest removed percentage was observed while cultivation of *Scenedesmus* sp. in 100% (5% TN removed) and 75% (14% TN removed) ACWW. On average approximately 26 – 29% of TN have been removed from samples through a gradual decrease in TN concentration that was monitored.

Different TN rates have been observed while cultivating *Scenedesmus* sp. under different aeration streams. By the 7th day under constant aeration over 80% of TN was depleted, but due to limited data from this batch cultivation, it is impossible to evaluate at which time point the highest Nitrogen removal was happening. At the same time under limited aeration, the highest Nitrogen depletion rate was observed by day 3 of cultivation.

The most possible explanation for such a difference in nitrogen uptake can be the composition of ACWW itself: not all nitrogen species are bioavailable and only a limited amount of TN can be consumed without enzymatic activities. Aeration is reported to enhance nitrogen removal

(Izadi et al., 2021), therefore, continuous air sparging most likely initiated the reduction of nitrate and nitrite to ammonia (Kumar & Bera, 2020; Tang et al., 2008) which can easily be taken up and assimilated by microalgal cells.

5.3.3. TP concentration profile

Phosphorus is a limiting nutrient for growing cells, including microalgae (Ren et al., 2017). TP in WW usually present in its dominating form, phosphate (PO_4^{-3}) which is bioavailable for microalgae (Wang et al., 2017) and assimilated by them for important cell functions. Microalgae are capable to regulate the P uptake by rapid accumulating the excess of the nutrient and slow utilizing it during the subsequent life cycle (Ren et al., 2017).

Analysis of TP concentration trends in *Figure 19, Figure 22, Figure 25*, and *Figure 28* shows high nutrient depletion in all the cultivated batches, especially for samples, containing ACWW. Triplicates cultivated in BBM reduced TP at much lower rates, and a general observation concluded that TP utilization rates fall with the increase of BBM fraction in the nutrient media variant. Thus, TP removal by *Chlorella* and *Scenedesmus* spp. was very similar and provided 89.7 (46.0) – 98.2% and 83.4 – 98.5% decrease in phosphorus concentrations in samples with ACWW, and only 5.1 % and 11.8% in BBM respectively.

Nostoc sp. demonstrated different removal rates compared to the other 2 species under limited aeration when TP concentrations in 2 samples increased and were almost totally removed by the end of batch cultivation (*Figure 25*). Literature sources reported that TP values can increase due to phosphorus release from bacterial cells when those undergo the lysis process (Mine et al., 2021). In the present case, this explanation can make sense because when the batch was inoculated in triplicates with 100% ACWW the highest turbidity was observed which was most likely due to bacterial contamination. When *Nostoc* sp. started biomass accumulation microalgae managed to remove certain P from the nutrient media (TP decreased by day 6). At the same time, contamination of significant amounts of samples with *Scenedesmus* sp. was detected. At this point, the dominating sp. most probably took the initiative in TP removal efficiency, and by day 9 from 55.6% to 93.1% of TP was removed in 3 out of 5 triplicates. Triplicate with BBM did not demonstrate significant dynamics in TP concentration. The reason for it could be the too-low density of *Nostoc* sp. cells in inoculate that was not able to initiate phosphorus removal, insignificant microbial contamination, and contamination with other microalgae sp. late in the cultivation process.

The highest TP removal rates were observed in the batch with constant aeration applied. Thus, all the samples with ACWW fractions showed 91.8 – 99.3% removal efficiency, while in triplicate with BBM, 69.2% TP was removed. Higher phosphorus removal under aeration was most likely initiated by higher aeration rates during microalgae cultivation (Izadi et al., 2021).

5.4. Biochemical composition of microalgal biomass

The biochemical composition of harvested microalgal biomass was analyzed and compared to understand alternatives for its downstream applications. Carbohydrates, in general, represented the highest fraction of biocomponents followed by protein (*Nostoc sp*. under limited aeration and *Scenedesmus* sp. under both limited and constant aeration) or lipid (*Chlorella* sp.) fractions (*Figure 29*). The ash content was significant in some cases (*Nostoc sp*. under limited aeration), but chiefly was calculated within 1.6 – 11.5% of total microalgal biomass composition.

A similar biochemical composition study was performed on *Chlorella* and *Scenedesmus* spp. biomass cultivated in the concentrated (100%) effluent, produced by brown crab production (Viegas et al., 2021). In *Chlorella* sp. biomass cultivated in 100% ACWW fractions of carbs, proteins, and lipids was 39.4±13.0%, 18.4±6.9%, and 38.3±6.3% respectively which differed from crab production effluent with 34.8%, 17%, and 18.6% of corresponding biocomponents. Biomass grown in the control nutrient media (BBM in our experiment and F/2 in the one described in the article) had also different fraction percentages: biomass in BBM was higher on lipids and carbs when biomass in F/2 accumulated more protein and ashes. *Scenedesmus* sp. under limited aeration was subjected to a similar comparison, and it was concluded that biomass cultivated in our laboratory accumulated much higher fractions of essential biocomponents while 54.1% of ashes was detected in biomass cultivated in effluent from crab production. Fractions of biocomponents in control nutrient media in both experiments were similar with the domination of carbohydrate fraction, followed by almost equal amounts of proteins and lipids. Only 9.8% and 8.4% of ashes in *Scenedesmus* sp. biomass grown in BBM and F/2 were calculated respectively in *Table 6*.

The biochemical composition of *Nostoc* sp. biomass cultivated in BBM was compared to another study where *Nostoc* sp. was cultivated in BG11 nutrient media (Baracho & Lombardi, 2023). It was found that both biomasses had similar biocomponent fraction distribution: proteins dominated with 57.7±7.8% and 38.4±2.6% in BG11 and BBM respectively (*Table 6*), lipids fractions represented by 10.5±1.6% and 10.3±3.3%. Carbohydrate content, on the

contrary, significantly differed within biomass composition: 12.5±1.9% was obtained in biomass cultivated in BG11, and 44.5±18.7% carbs were detected in *Nostoc* sp. biomass during our experiments.

Table 6. The summary table for biocomponents accumulated in the biomass during the cultivation in the current experiment and reported in publications (Baracho & Lombardi, 2023; Viegas et al., 2021).

Microalgae spp.	Nutrient media	Carbs, %	Proteins, %	Lipids, %
Chlorella sp.	ACWW*	39.4	18.4	38.3
	ACWW**	34.8	17.0	18.6
	BBM	35.8	13.7	37.0
	F/2	26.6	37.5	14.4
Scenedesmus sp.	ACWW*	62.3	14.8	13.1
	ACWW**	23.6	9.5	12.8
	BBM	54.2	22.5	21.7
	F/2	48.0	21.0	22.6
Nostoc sp.	BBM	44.5	38.4	10.3
	BG11	12.5	54.7	10.5

ACWW* – aquaculture WW from salmon smolt production (current study)
ACWW** – aquaculture WW from brown crab production (Viegas et al., 2021)
F/2 – referent nutrient media used in reported study (Viegas et al., 2021)
BG11 – referent nutrient media used in reported study (Baracho & Lombardi, 2023)
BBM – referent nutrient media (current study)

Comparison between actual results obtained in the laboratory with published reports leads us to the conclusion that nutrient media composition has a direct influence on biocomponents that are going to be accumulated in microalgal biomass. Similarities between accumulated compounds in control nutrient media evidence that quantitative analytical methods were applied to biomass accordingly and can be reliable.

Overall observations lead to the conclusion that constant aeration initiates approximately equal biocomponent fractions accumulation and insignificant amounts of ashes in *Scenedesmus* sp. biomass grown in all nutrient media variants. *Chlorella* sp. under limited aeration demonstrated very good carbohydrate and lipid accumulation properties with very low amounts of ashes presented. *Scenedesmus* sp. cultivated in 100%, 75% ACWW, and BBM demonstrated relatively good carbohydrate accumulation properties, whereas cultivation in 50% and 25% ACWW resulted in higher amounts of ashes in the biomass (25.2% and 22.2% respectively).

Nostoc sp. cultivation in ACWW showed the worst accumulation properties regarding carbs, proteins, and lipids fractions, whereas cultivation in BBM resulted in the accumulation of close to previously reported fractions of essential biocomponents. A possible explanation for such a high percentage of ashes in biomass can be that other valuable biomolecules like PHAs, which were not targeted during the current study, were accumulated as well. *Nostoc* sp. is reported to be able to accumulate PHAs in the form of intracellular inclusions in the presence of excess carbon (Costa et al., 2018). At the same time, the low *Nostoc* sp. cell density and biomass contamination (microbial and microalgal) cast doubt on the abovementioned explanation.

5.5. Challenges connected to experimental performance

During the experiments, we met certain challenges connected both with the experimental setup itself and complications during the growth of microalgal cultures. The experimental setup was planned similarly to the experiment described in one of the articles, where microalgae were used as a remediation agent for aquaculture wastewater with certain adjustments to available equipment and conditions (Cardoso et al., 2021). The challenge with the setup itself was that high volumes of evaporated nutrient media affected OD measurements and prevented us from sampling for further analysis. The problem was first noticed on the 4th day of cultivation but didn't seem to have a serious influence on the experiment flow. On the 6th day volumes of evaporated liquid in several samples exceeded 15% after which aeration was stopped immediately. Measurements were still taken daily, but the duration of the experiment was reduced by 2 days.

The most probable reason for such a setup failure is the too high air sparging rate which forced high volumes of nutrient media to evaporate. Therefore, we had to adapt the planned setup to newly established circumstances and adjust the experimental plan. Unfortunately, the air sparging installation was not uniform for all the samples, the air sparging rate varied from sample to sample, and it was not possible to maintain that value at the same level. For the next batches, the aeration period was limited to 3 hours/day, sampling for OD measurements was performed daily, and nutrient depletion monitoring and TSS analysis – every 3rd day of cultivation.

Another major challenge that appeared during the experiment was connected to the different intensities of microbial contamination of the samples. It was observed during microscopic study during the harvesting, and in the case of the 4th batch – during the cultivation as well.

Contamination issues arose because aseptic conditions were not 100% maintained during daily samplings. Sterile syringes were used for collecting the growing microalgae, but a small risk for external contamination was present. The highest microbial contamination was observed during *Nostoc* sp. cultivation, and by the 6th day of cultivation it was complicated with contamination by another microalgal species.

To avoid contamination issues better conditions for aseptic sampling needed to be provided in the laboratory. On the other hand, while the real application of microalgae cultivation on wastewater happens, it is impossible to maintain even close to aseptic conditions. Therefore, this exact difficulty turned out to be an advantage. It gave us a possibility to test how studied microalgae species deal with different contaminations and if they can sustain the domination of species in challenging cultivation conditions.

The overall problem for all the cultivated batches consisted of not unified cultivation conditions for all the samples due to different aeration rates caused by installation issues and the inability to control and sustain aeration flow rates at a constant pace. In addition, the light intensity obtained by samples was not unified as well because of mechanical obstacles connected to the setup installation.

One more challenge that we met during the experiment was connected to limited resources and special equipment availability. To solve this problem, we adjusted the setup itself, and sampling frequency, reduced studied volumes and several probes, and at the same time, maintained the comparability of the tests.

5.6. Microalgal biorefinery towards circular economy

Almost all the existing microalgal species has well-balanced combination of proteins, lipids, carbohydrates, and amino acids (Nagappan et al., 2021). These compounds have been used in medical industry, cosmetology, aqua- and agriculture, feed and food production, chemical industry, and biofuels as value-added products (Dolganyuk et al., 2020; Xia et al., 2021). Various biological applications have been utilized for microalgal biomass such as the production of pigments and antibiotics, nanoparticles and bio-flocculants in the wastewater treatment process (Baldev et al., 2021; Sung & Sim, 2022).

Special attention to products of algal biorefinery can be given to biopolymers like polyhydroxyalkanoates (PHA). Being biodegradable, they attained similar mechanical and physical properties as synthetic plastics (Nanda & Bharadvaja, 2022). These biopolymers can

become a feasible solution to the plastic waste problem, and in a certain way minimize pollution (Rajpoot et al., 2022).

The PHA synthesis process is schematically demonstrated in *Figure 33*. Polymers are accumulated inside the microbial or microalgal cell as a product of sugars and lipids fermentation under stress conditions, nutrient depletion (N & P), and carbon excess. The extraction process is usually carried out by cell lysis and further separation of targeted compounds (Abraham et al., 2021; Ali et al., 2022; Costa et al., 2018).

Different microalgae species can accumulate from 0.04 to 80% of their biomass dry weight as biopolymers (Costa et al., 2018; Mastropetros et al., 2022; F. H. P. Tan et al., 2022). At the same time, obtained bioplastics can be widely used in medicine, agriculture, food packaging, 3-D printing material, and in other industries such as natural textiles, fibers, fillers, and even thermoplastics. In this regard, microalgae can be considered a promising source of bioplastics with high biodegradability (Lutzu et al., 2021; Rajpoot et al., 2022).

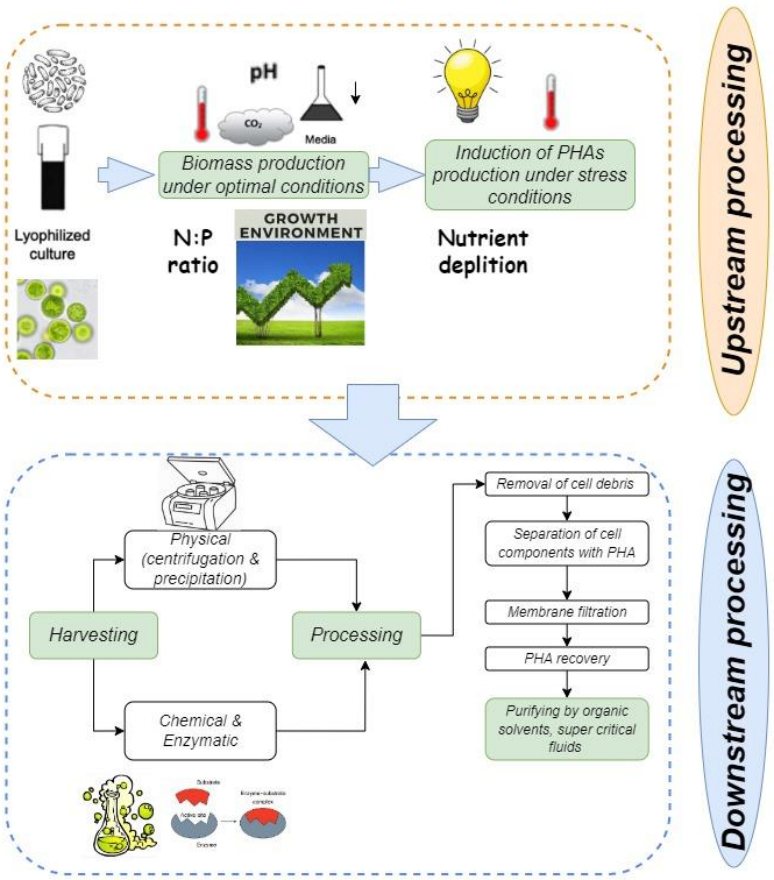

Figure 33. Complete PHA synthesis process from naturally or genetically engineered microorganisms or microalgae (Ali et al., 2022; Naser et al., 2021).

Bioplastics, obtained from microalgal biomass, can potentially replace fossil-based feedstock providing undoubted environmental benefits. However, high productional costs (2.5 – 6 €/kg) remain to be a challenge in preventing mass production of microalgae-based plastics. But the integration of microalgal biomass production with WWT may reduce associated costs. There is a potential for production efficiency improvement through systems with repeated cycles with adapted nutritional conditions aimed at PHA production. On the other hand, the overall costs of conventional WWT methods can be reduced through microalgae-based bioplastics production (Lutzu et al., 2021).

The circularity concept within microalgal biorefinery can be realized as it is demonstrated in *Figure 34,* where sunlight together with CO_2, N, and P is utilized by microalgae for biomass production. Essential for biopolymer production biocomponents like carbohydrates and lipids, or even biopolymers themselves, are accumulated inside microalgal cells.

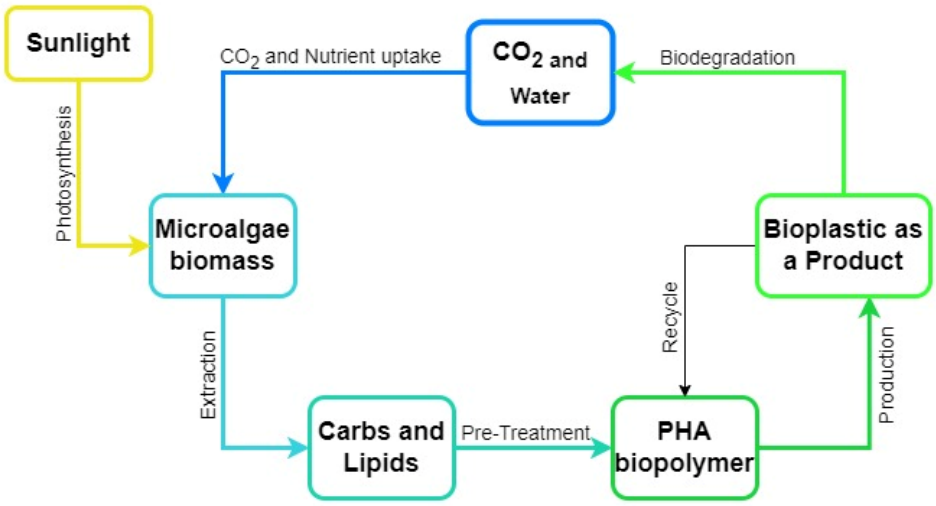

Figure 34. Carbon cycle while microalgae biopolymers production (Rajpoot et al., 2022).

The fermentation process through enzymatic activity converts carbs and lipids into PHAs. Bio-composites suitable for bioplastic production are made by the compression of fermented microalgal biomass, with the use of different solvents and following drying, or by a combination of several methods (Rajpoot et al., 2022). The industry will use produced biopolymers which can be either recycled or biodegraded with CO_2 and water released fulfilling the cycle.

6. Concluding remarks

The results of aquaculture wastewater basic characterization show that it can be used as an alternative and at the same time cost-effective nutrient media for microalgae cultivation. ACWW has acceptable physical and chemical properties and contains essential nutrients to sustain the growth of microalgae as demonstrated through this study. Filtration through a fiberglass filter as an additional pretreatment step significantly reduces particulate matter in the WW, preventing high errors while biomass accumulation measurements using both OD and DCW methods. At the same time, sterilization of ACWW by autoclaving does not significantly affect its chemical composition (COD, TN, and TP), and therefore can be applied before inoculation aiming prevention of microbial contamination.

The experiment demonstrated that at least 2 microalgae species (*Scenedesmus* and *Chlorella* spp.) can grow in aquaculture effluent from salmon smolt production with different growth performance and final biomass characteristics. *Nostoc* sp. growth was not satisfactory during the current study due to microbial and microalgal contamination, and low nutrient removal efficiency. Additional experiments need to be performed to formulate the conclusion about the ability of this microalgae sp. to grow on a certain ACWW effluent. It was observed that higher aeration rates stimulate biomass accumulation, maintain a higher specific growth rate, increase TN and TP removal efficiency, and balance essential biocomponents (carbs, lipids, proteins) accumulation in biomass minimizing ashes content in it. At the same time, it seems that microalgal biomass accumulation was limited by low TP concentration in ACWW and did not reach its potential maximum.

Microalgal WWT efficiency was evaluated by comparing initial and final COD, TN, and TP values in mg/L. TN removal rates demonstrated dependence on aeration rates and increased proportionally to it. TP was efficiently removed from all the nutrient media variants, containing fractions of ACWW in all the batches. However, TP removal efficiency from BBM was the lowest most likely due to the lower bioavailability of this limiting nutrient. *Nostoc* sp. cultivation demonstrated that bacterial contamination could be a possible reason for TP increase during the cultivation, and contamination by *Scenedesmus* sp. initiated the nutrient removal process.

Unexpected results were obtained during the COD concentration monitoring which ended up in COD increase in almost all the samples cultivated during the experiment. Literature sources

suggested that the N/P ratio plays an important role in COD removal efficiency, and CO_2 sparging might initiate the transition from heterotrophic metabolism to autotrophic, reducing COD removal efficiency. These factors together with the closed type of cultivation reactor used in our research have most likely created conditions resulting in a monitored COD increase.

Experimental setup installation created unique cultivation conditions inside every single sample, which influenced all the monitored parameters like OD values, biomass concentration, nutrient removal efficiencies, and accumulation of essential fractions of microalgal biomass (proteins, lipids, and carbohydrates). Biochemical composition analysis demonstrated clear dominance of carbohydrate fraction in all the batches cultivated under limited aeration conditions. *Chlorella* sp. accumulated high concentrations of lipids and reasonable amounts of proteins keeping the ashes fraction under 10%. Constant aeration of *Scenedesmus* sp. growth facilitated a balanced accumulation of all the essential fractions and an insignificant percentage of ashes was obtained.

Considering a circular economy approach, the 100% ACWW can be used as a cost-efficient nutrient medium due to its ability to maintain reasonably high growth rates and microalgal biomass accumulation for *Scenedesmus* sp. and *Chlorella* sp. BBM supplement into the ACWW does not largely influence the abovementioned parameters but significantly increases additional costs through expensive nutrient media compounds required. *Chlorella* sp. and *Scenedesmus* sp. biomass is high in carbohydrates, and *Chlorella* sp. has high lipid content in addition which makes it possible to study biomass further for economically feasible applications like, for example, PHA production. *Scenedesmus* sp. grown under constant aeration conditions has a very balanced biocomponent composition, therefore different biomass utilization approaches can be tested for this biomass.

Overall, microalgae-facilitated WWT seems to be technologically and environmentally feasible. The high cultivation costs can be considered as installation costs, similar to conventional WWT systems, which will make the system economically competitive. Microalgal nutrient removal properties do have the potential to operate with lower energy and less carbon footprint.

7. Future research directions and recommendations

During the current experiment several important assumptions were not clarified such as exact aeration rates and their influence on biomass production and its biochemical composition. Due to the limited experiment time, the optimal cultivation conditions were not sufficiently established for the targeted biocomponent accumulation. For example, the feast and famine nutrient availability conditions during microalgae cultivation can instantiate PHA accumulation inside the microalgal cells (Afreen et al., 2021; Lutzu et al., 2021; Rajpoot et al., 2022). Different experimental strategies and conditions such as light intensity, light cycles, temperature, CO_2 sparging at different rates, etc. can also be tested regarding PHA accumulation or even pigments and vitamins markets in Norway.

Possibly, several other microalgae species, preferably indigenous, with high potential for PHA accumulation can be studied in a similar experiment. Upscaling the reactors can open the possibility of monitoring the dynamics of biocomponents accumulation and finding the optimal cultivation time with optimized conditions for achieving targeted value-added products.

Pilot testing the process with integration of microalgae cultivation systems into the land-based plants with RAS should be approached. It may open practical abilities to evaluate economic feasibility and contribution to circularity within microalgal biotechnology coupled with ACWW treatment.

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Appendixes

Appendix A. Equipment

Table 7. Equipment used during the study.

Equipment	Producer
250 mL coneical flasks	VWR
Autoclave SX-700E	TOMY
Digital microscope: VisiScope BL254 T1	VWR
Thermoreactor: Spectroquant TR 620	Merck
Photometer: Spectroquant Prove 300	Merck
UV mini-1240 spectrophotometer	Shimadzu
15 ml centrifuge tubes	VWR
Vortex mixer Stuart SA8	VWR
Eppendorf Centrifuge 5804 R	VWR
Drying oven Dry-Line	VWR
Vacuum pump	VWR
Multi-position magnetic steerer RO15	IKA
Shaking machine Unimax 2010	Heidolph
Airpump air400	EHEIM
Analytical balances XP205 Delta Range	Mettler Toledo
Water bath	VWR

Appendix B. Chemicals

Table 8. Chemicals used during the study.

Chemials	Producer
Spectroquant COD cell test (range: 100-1500 mg/L COD)	Supelco
Spectroquant Phosphate Cell Test (range: 0.5-25.0 mg/L PO4-P)	Supelco
Spectroquant Total Nitrogen Cell Test (range: 10-150 mg/L N)	Supelco
Hydrochloric acid, 20%	VWR
Sodium Hydroxide (NaOH)	Supelco
Whatman Glass microfiber filter, 1.2 µm particle retention. Grade GF/C	VWR
96% Sulfuric acid	Supelco
Phenol, crystals	VWR
CL Protein Assay with BSA Standard test kit	G-Biociences
Chloroform	VWR
Methanol anhydrous for analysis	Supelco
D-Glucose anhydrous	VWR
96% Ethanol	Supelco

Appendix C. Bold's Basal Medium (BBM) recipe.

Stocks		**per 400 ml**
(1)	$NaNO_3$	10.0 g
(2)	$MgSO_4 \cdot 7H_2O$	3.0 g
(3)	NaCl	1.0 g
(4)	K_2HPO_4	3.0 g
(5)	KH_2PO_4	7.0 g
(6)	$CaCl_2 \cdot 2H_2O$	1.0 g
		per liter
(7)	Trace elements solution (autoclave to dissolve):	
	$ZnSO_4 \cdot 7H_2O$	8.82 g
	$MnCl_2 \cdot 4H_2O$	1.44 g
	MoO_3	0.71 g
	$CuSO_4 \cdot 5H_2O$	1.57 g
	$Co(NO_3)_2 \cdot 6H_2O$	0.49 g
(8)	H_3BO_3	11.42 g
(9)	EDTA	50.0 g
	KOH	31.0 g
(10)	$FeSO_4 \cdot 7H_2O$	4.98 g
	H_2SO_4 (conc.)	1.0 ml

Medium	**per liter**
Stock solutions 1 – 6	10.0 ml each
Stock solutions 7 – 10	1.0 ml each

Make up to 1 liter with glass distilled or deionized water.

Appendix D. Additional graphical data.

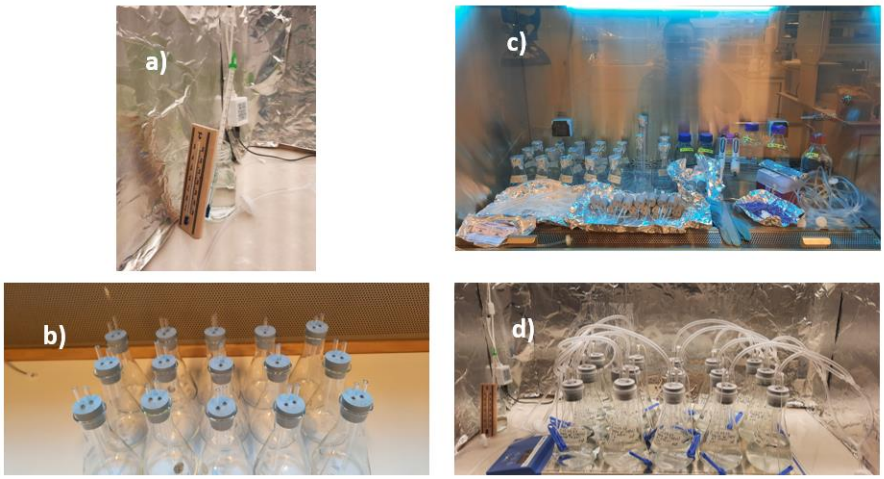

Figure 35. Experimental setup preparations: a) an approach for temperature measurements; b) conical flasks, prepared for inoculation; c) clean bench preparation for aseptic inoculation of microalgal culture; d) launched experiment where flasks are placed on magnetic stirrer and connected to aeration pumps.

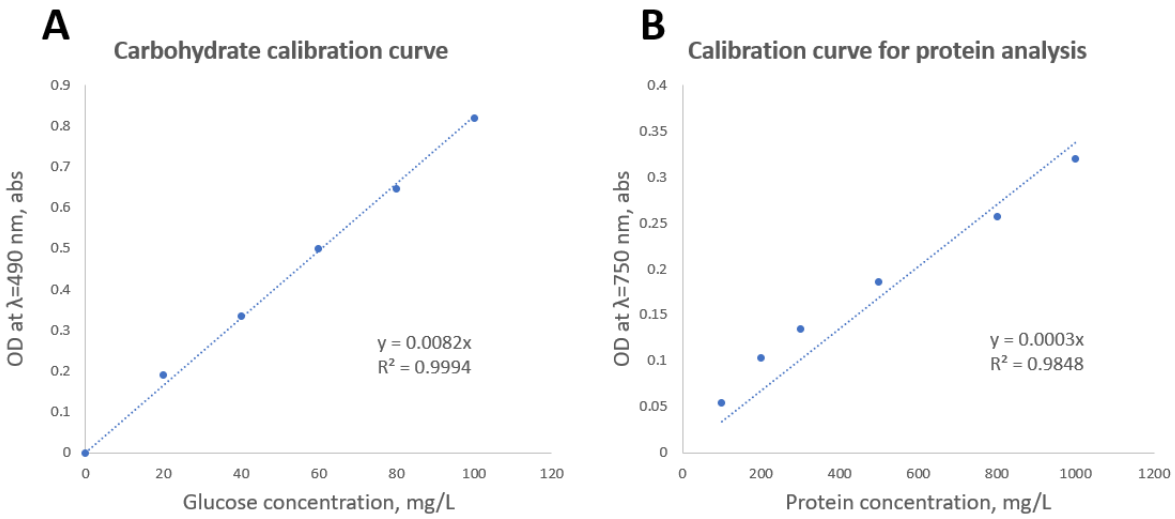

Figure 36. Established calibration curves for A) carbohydrate and B) protein analysis.

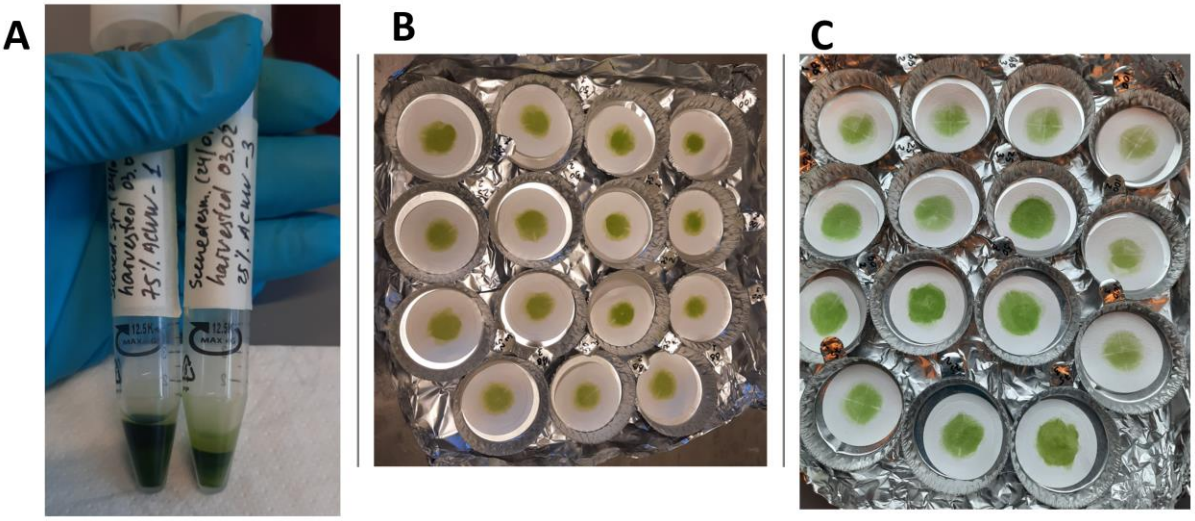

Figure 37. Biomass harvesting and DCW analysis performance: A) Difference in accumulated Scenedesmus sp. under constant aeration biomass in samples containing 75% (left) and 25% (right) ACWW; B)TSS analysis of Chlorella sp. under limited aeration; C) TSS analysis of Scenedesmus sp. under limited aeration.

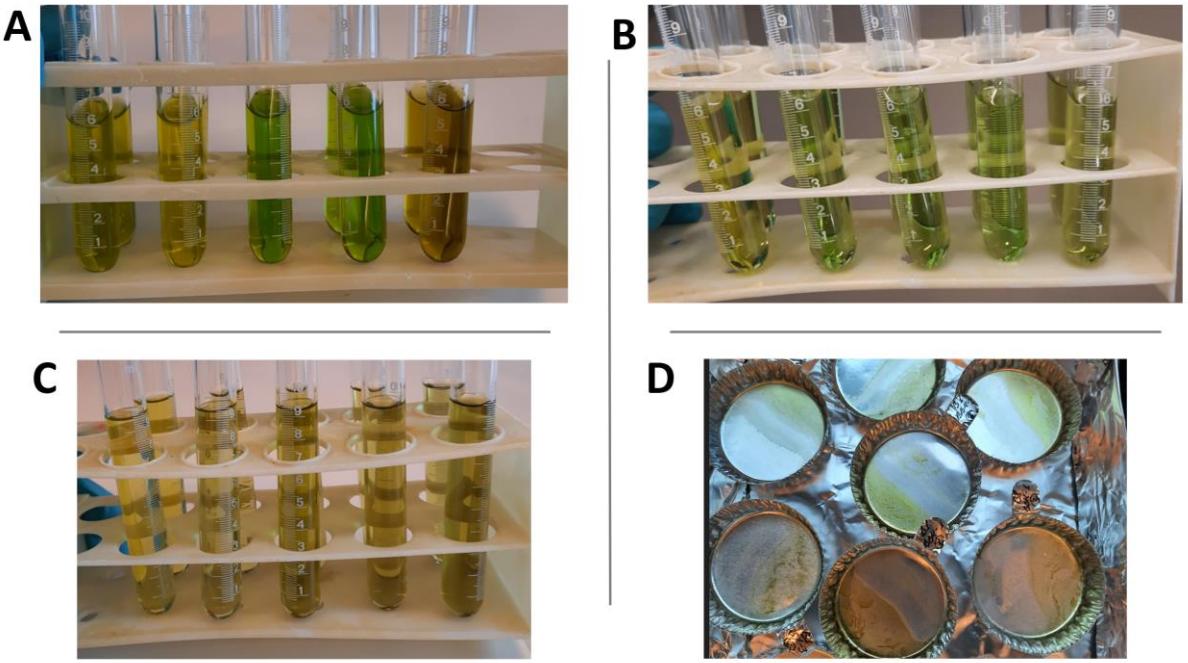

Figure 38. Observation of different stages during the lipid extraction: A), B), and C) Chloroform solution with extracted lipids in it; duplicates of different species are studied simultaneously; clear observation of difference in color of each duplicate; D) dried chloroform solution; fat fractions are observed on aluminum dishes.

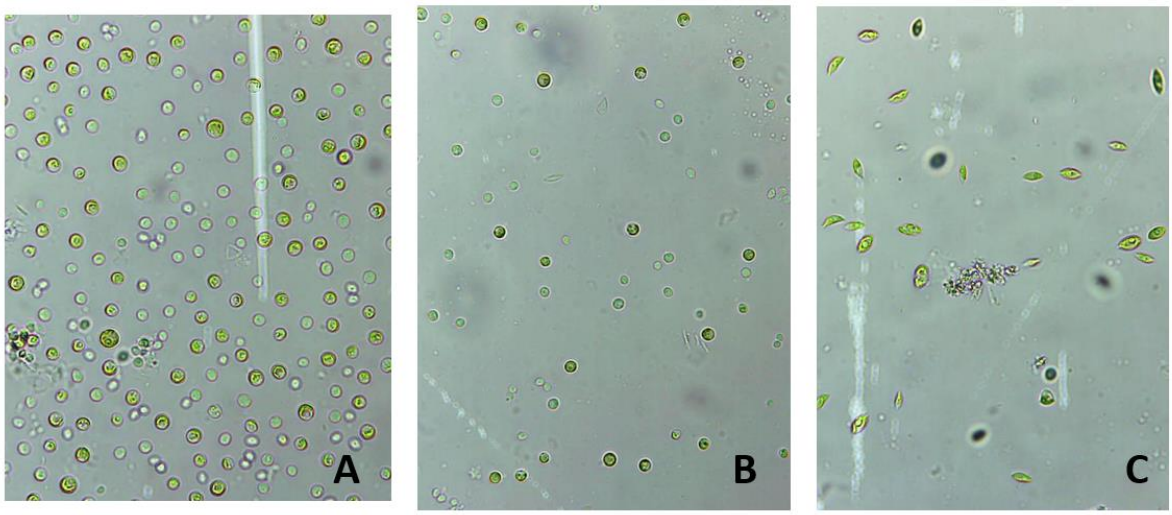

Figure 39. Microscopic investigation of Chlorella and Scenedesmus spp.: A) & B) Chlorella sp. cultivated in different triplicates; C) Scenedesmus sp. with insignificant microbial contamination.

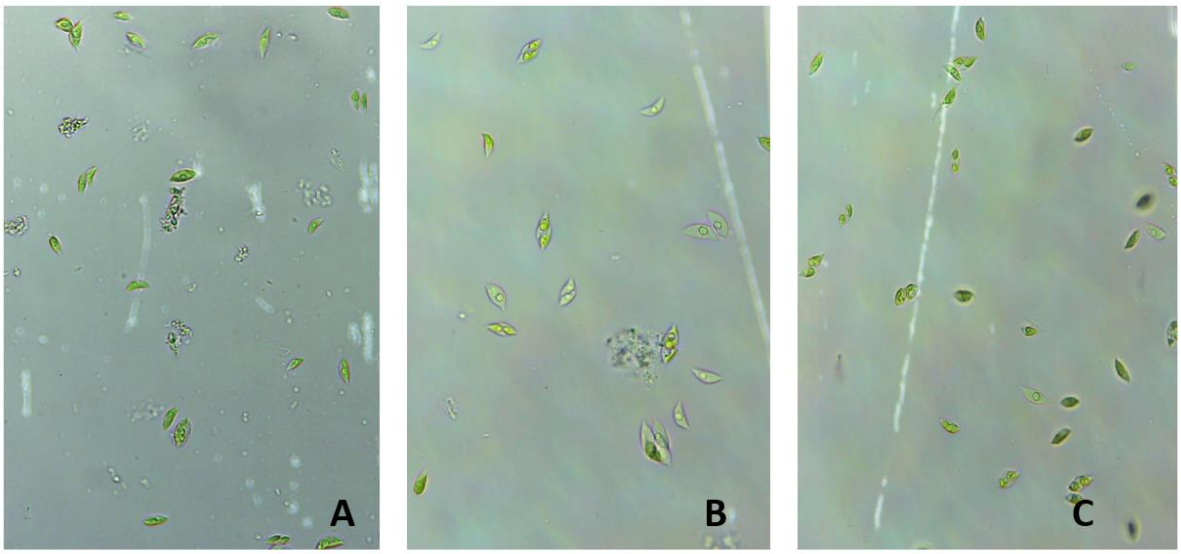

Figure 40. Microscopic investigation of Scenedesmus sp.: A) & B) insignificant microbial contamination can be observed; C) Scenedesmus sp. when coenobium formation is absent.

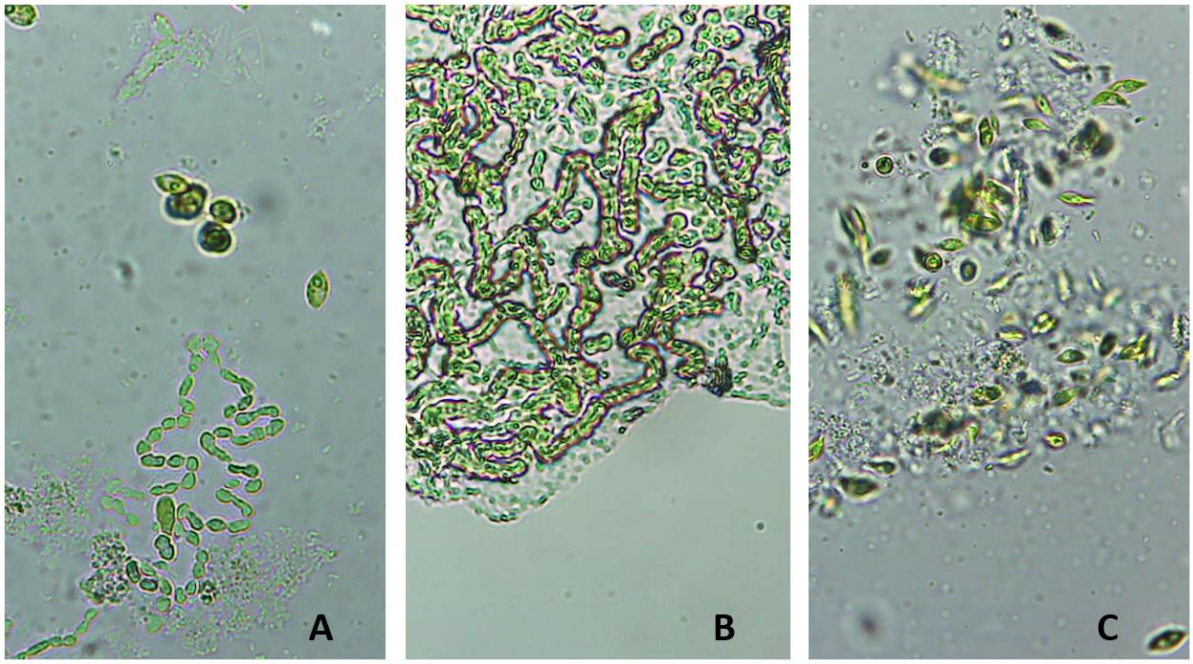

Figure 41. Microscopic investigation of Nostoc sp. under limited aeration, 40x multiplication: A) Nostoc sp. chains with visible bacterial contamination and single Scenedesmus sp. cells; B) Flocks, formed by Nostoc sp. with visible bacterial cells around microalgal cells; C) Dominating of Scenedesmus sp. over Nostoc sp. coupled with bacterial contamination.

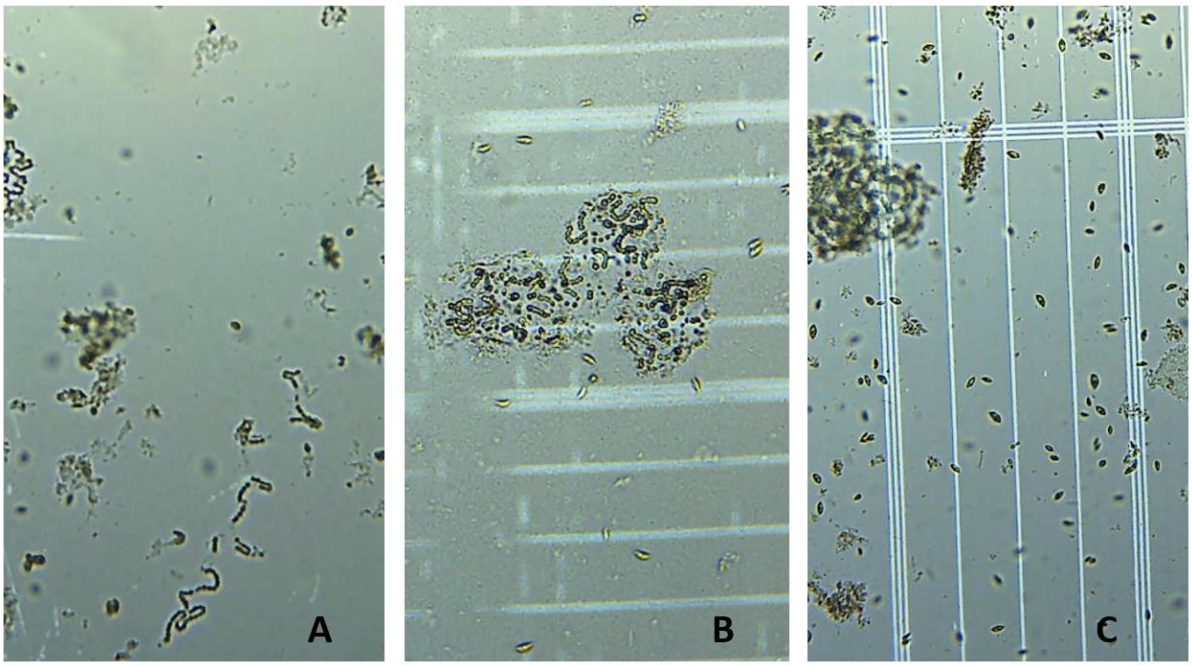

Figure 42. Microscopic investigation of Nostoc sp. under limited aeration, 10x multiplication: A) Nostoc sp. with visible bacterial contamination and single Scenedesmus sp. cells; B) Flock formed by Nostoc sp. with visible bacterial cells around microalgal cells, single Scenedesmus sp. cells are observed; C) Dominating of Scenedesmus sp. over Nostoc sp. coupled with bacterial contamination.

Appendix E. Additional numerical data.

Table 9. Harvesting parameters, obtained from all the cultivated batches during the experiment: average values of final biomass concentration, average biomass productivity, and evaporation percentage.

Batch number	***Nutrient media variant***	***Final biomass concentration***	***Average biomass productivity****	***Evaporation***
		g/L	*mg/L/day*	*%*
Batch 1. *Scenedesmus sp, constant aeration*	100% ACWW	0.991±0.073	70.7	24.3±2.4
	75% ACWW	0.959±0.164	77.6	16.5±10.2
	50% ACWW	0.963±0.031	68.6	22.3±3.1
	25% ACWW	0.752±0.094	57.3	12.7±4.7
	BBM	0.694±0.185	46.5	24.9±8.8
Batch 2. Chlorella *sp, limited aeration*	100% ACWW	0.463±0.022	37.42	2.1±0.4
	75% ACWW	0.554±0.018	45.06	2.3±1.0
	50% ACWW	0.529±0.033	42.97	2.3±1.0
	25% ACWW	0.453±0.099	36.65	2.9±2.3
	BBM	0.279±0.080	22.14	1.5±1.5
Batch 3. *Scenedesmus sp, limited aeration*	100% ACWW	0.411±0.019	32.9	2.2±1.0
	75% ACWW	0.585±0.101	47.4	3.2±1.2
	50% ACWW	0.637±0.014	51.8	3.7±0.9
	25% ACWW	0.503±0.099	40.6	2.7±1.8
	BBM	0.197±0.016	15.2	1.8±0.5
Batch 4. Nostoc *sp, limited aeration*	100% ACWW	0.323±0.030	26.6	3.7±0.2
	75% ACWW	0.336±0.028	27.6	3.8±0.7
	50% ACWW	0.398±0.065	32.8	3.6±0.9
	25% ACWW	0.261±0.056	21.4	3.5±1.0
	BBM	0.102±0.020	8.1	3.3±0.4

* - the average biomass productivity is reported without standard deviation values

Table 10. Initial and final concentrations of COD, TN, and TP (average values) in all the samples, cultivated during the performed study.

Batch number	Nutrient media variant	COD (chemical oxygen demand)		TN (total Nitrogen)		TP (total Phosphorus)	
		Initial	Final	Initial	Final	Initial	Final
		mg/L		mg/L		mg/L	
Batch 1. *Scenedesmus* sp., constant aeration	100% ACWW	317±15	442±124	185±5	19±1	3.52±0.17	0.16±0
	75% ACWW	281±12	232±4	235.25±54.5	17.5±0.5	43.4±0.1	0.3±0
	50% ACWW	245±9	243±45	184±2.5	14±1	83.3±0.1	0.7±0
	25% ACWW	209±6	312±36	183.5±1.25	13±1	123.1±0	10.1±0.1
	BBM	173±3	274±12	183±0	13.5±0.5	163±0	50.25±1.75
Batch 2. *Chlorella* sp., limited aeration	100% ACWW	191±21	491±23	67.5±5.5	49.5±0.5	2.13±0.13	0.22±0
	75% ACWW	157 ±16	331±79	63.9±5.4	41.5±0.5	16.2±0.02	0.3±0
	50% ACWW	123±11	237±67	56.25±1.25	33.5±0.5	30.5±0.2	2.6±0
	25% ACWW	88±5	150±10	50.6±0.9	34±1	44.4±0.3	24±1
	BBM	54±0	97±1	45±3	30.5±0.5	58.5±0.5	55.5±0.5
Batch 3. *Scenedesmus* sp., limited aeration	100% ACWW	514±14	445±91	121.5±0.5	115±2	4.3±0	0.19±0
	75% ACWW	402.5±12	459±3	105.4±1.6	81.5±1.5	19.1±0.4	0.7±0.2
	50% ACWW	291±10	384±26	85.5±0	57±1	33.9±0.75	0.5±0
	25% ACWW	179.5±8	251±3	67.5±0.25	42±0	48.7±1.1	8.1±0
	BBM	68±6	181±3	49.5±0.5	34.5±0.5	63.5±1.5	56±0
Batch 4. *Nostoc* sp., limited aeration	100% ACWW	261±5	283±5	130.5±3.5	107±1	4.35±0.05	0.26±0
	75% ACWW	233.5±3	253±17	111.75±1.25	94.5±1.5	18.5±0.04	0.32±0
	50% ACWW	206±1	270±0	89.75±2.25	69.5±0.5	32.7±0.02	4.2±0
	25% ACWW	178.5±1	260±10	69.4±1.6	52.5±0.5	46.8±0.01	26±0
	BBM	151±3	191±5	49±1	40±1	61±0	60.5±0.5

Table 11. Essential biocomponents concentration obtained from the quantitative analyzis of microalgal biomass.

Batch number	***Nutrient media variant***	***Total carbohydrates***	***Total proteins***	***Total lipids***
		mg/g	*mg/g*	*mg/g*
Batch 1. *Scenedesmus* sp., constant aeration	100% ACWW	361.8±23.8	285.6±99.9	266.3±53.4
	75% ACWW	367.2±48.8	350.5±64.5	209.2±45.4
	50% ACWW	306.6±66.6	340.6±105.8	298.6±108.1
	25% ACWW	422.3±23.9	216.0±52.2	246.3±48.9
	BBM	417.5±186.7	217.1±73.5	330.5±96.0
Batch 2. *Chlorella* sp., limited aeration	100% ACWW	394.1±130.0	183.8±68.5	383.3±63.3
	75% ACWW	556.6±83.1	103.9±6.3	187.1±23.9
	50% ACWW	548.5±160.9	148.7±93.6	215.6±36.7
	25% ACWW	529.7±39.4	122.6±17.1	313.8±98.5
	BBM	357.5±51.8	137.3±15.9	370.1±165.4
Batch 3. *Scenedesmus* sp., limited aeration	100% ACWW	622.9±65.2	148.0±39.1	131.3±9.7
	75% ACWW	618.6±238.5	178.1±50.6	89.1±18.8
	50% ACWW	592.0±83.4	99.3±31.6	56.9±8.3
	25% ACWW	547.5±130.0	144.7±45.0	85.5±21.3
	BBM	541.8±35.9	225.2±42.0	216.6±17.4
Batch 4. *Nostoc* sp., limited aeration	100% ACWW	362.2±91.4	186.6±10.6	163.4±60.0
	75% ACWW	294.3±29.6	164.0±46.8	141.0±56.5
	50% ACWW	398.9±78.5	125.7±14.5	102.3±13.7
	25% ACWW	361.9±70.6	149.5±23.5	140.4±33.8
	BBM	445.0±186.6	383.9±26.4	103.4±32.5