Thesis for Doctor of Philosophy

in Graduate School of Environmental Science

The University of Shiga Prefecture

"**Biochemical compositions in two *Arthrospira* species cultivated in anaerobic digestion effluent of water hyacinth, which is growing excessively worldwide.**"

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Keywords

Arthrospira, water hyacinth, fatty acids, beta-glucans, nutrient removal, anaerobic digestion effluent, phycocyanin, gamma-linolenic acid, poly-unsaturated fatty acid, dietary fibers.

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CHAPTER 1

General Introduction

1.Wastewater treatment and microalgae cultivation

The growth in global population has resulted in increased agricultural, industrial, and urban activities. Consequently, this has also caused a significant rise in waste production and environmental pollution (Ruiz et al., 2013). As a result, one of the most pressing environmental issues people face today is the need for sustainable and effective management of urban wastewater (Geremia et al., 2021). Untreated wastewater containing excessive nutrients such as nitrogen (N) and phosphorus (P) can lead to eutrophication of aquatic environments and represent a severe threat to water bodies (Liu et al., 2020). It is, therefore, necessary to implement appropriate treatment plans for reducing and removing substances such as N and P. Several methods are available to reduce and mitigate the risks associated with pollutants while discharging wastewater. These methods include adsorption, membrane filtration, coagulation, flocculation, oxidation, biological treatment, and so on (Xu et al., 2018).

However, wastewater treatment cost can be significant since it is necessary to effectively remove pollutants from the water to achieve cleanliness and reusability. Khan et al. (2020) noted that most wastewater treatment techniques are designed to meet effluent characterization and purification requirements without considering their impact on overall treatment performance and the environment. Additionally, Yahya et al. (2018) stated that conventional treatment methods can only partially remove pollutants and may concentrate or degrade them into another phase. On the other hand, the biological process with activated sludge is the most commonly used method in wastewater treatment (Su et al., 2012a). Nevertheless, the disposal of large amounts of sludge remains a significant challenge, which has led researchers to explore alternative technologies.

In this regard, microalgal cultivation is an excellent option for removing nutrients from wastewater. The utilization of microalgae in wastewater treatment has been a subject of interest since the 1960s (Golueke and Oswald, 1962; Lau et al., 1995; Tam and Wong, 2000) because

they possess the unique ability to absorb nutrients as they grow, producing bioenergy. This bioenergy can serve various purposes, including biofuel (Cai et al., 2013), dietary supplements for humans, and additives for animal feeds (Depraetere et al., 2013; Markou et al., 2021).

2. Anaerobic digestion effluent from overgrown aquatic macrophytes

Sustainable management of aquatic ecosystem is an immense global challenge. The challenge is to manage ecosystems in such a way that it can sustainably deliver an optimal combination of ecosystem services both today and into the future, with resilience to environmental and social advancement. Aquatic macrophytes is a vital biotic element of freshwater ecosystem, and play a role for stabilizing water quality, binding nutrients, providing spawning and nursery grounds for various fishes colonizing the littoral zone (Ban et al., 2019), and as a shelter for many birds and aquatic organisms. Conversely, the overgrowth of aquatic macrophytes, such as water hyacinth (*Eichhornia crassipes*), creates severe ecological, economic, and social problems worldwide (Milicevic, 2023; O'Sullivan et al., 2010). Water hyacinth is considered one of the world's worst invasive aquatic macrophytes (Holm et al., 1977) that grows and spreads rapidly in lakes, ponds, canals, and rivers (Gundupalli et al., 2023). Such overgrowth of macrophytes hinders fishery activities and boat traffic, decreases biodiversity, reduces dissolve oxygen level, produces filthy odors, and displays unpleasant landscapes for residential people and visitors (Ban et al., 2019).

Conserving of a healthy freshwater aquatic ecosystem, it may be important to know how to manage the appropriate biomass of the macrophytes in a system. It is also important to know which effective economical method is to select for controlling the macrophyte biomass because of several methods have been proposed, and evaluated (Hussner et al., 2017; Lishawa et al., 2017; Xu et al., 2014). Some previous studies also have been focused on mechanical harvesting, cutting the submerged vegetation, or finding appropriate timing for eradicating the macrophytes from the aquatic ecosystem (Xu et al., 2014). However, these techniques are

expensive and carry some risks of spreading the macrophyte distribution by producing a lot of vegetative fragments and interrupting the bottom sediments.

In this perspective, anaerobic digestion (AD) is one possible effective and relatively low-cost procedure for treating the macrophytes harvested. The AD process utilizes microorganisms to break down organic waste and generate methane-rich biogas as an energy source, along with nutrient-rich digested fluid as a liquid fertilizer (Koyama et al., 2014). Morover, the AD process requires low energy input and operation cost, which makes this process sustainable and effective for a nutrient recycling system with macrophytes, and subsequently microalgae cultivation. AD effluent (ADE) has relatively lower amount of carbon compared to agricultural, municipal, and industrial wastewater, because microbial actitivities involve converting the carbon to methane and carbon dioxide gas during the digestion process (Wang et al., 2010). Additionally, the ADE also contains a large amount of nutrients such as N and P, trace elements, metals, which are needed for algal growth (Liu et al., 2020).

Recently, several studies have been focused on nutrients removal from ADE by microalgal cultivation (Li et al., 2019; Singh et al., 2011). However, some studies showed that nutrient removal and algal biomass were limited by available magnesium (Mg) for algal growth in the ADE (Kimura et al., 2019). Mg^{2+} is a crucial element for the growth and division of algal cells because it is present in the active center of chlorophyll *a* (Masojídek et al., 2013). Mobility of Mg was inhibited by dissolved organic carbon (DOC) in wastewater because of increasing bound as DOC complexes (Christensen and Christensen, 2000). Since the ADE contain large amount of Mg but not available for algal growth, Liu et al. (2020) found that the maximum algal biomass and nutrient removal can be obtained by lowering pH of ADE at 6.5 using a flow-through cultivation system. Qian et al. (2021) provided an evidence for the binding available Mg^{2+} with dissolved organic matter in the ADE of aquatic macrophytes through photolysis with UV radiation.

3. Microalgae used for wastewater treatment

Several microalgae species, including cyanobacteria and green algae, can efficiently remove nutrients from wastewater, thus making them a valuable asset in wastewater treatment. Their ability to absorb N and P during their growth is a significant factor in this process (Renuka et al., 2015). However, nutrient removal efficiency and biomass yield may depend on different species or even different strains of the same species (Su et al., 2012b). Olguín (2003) reported that cyanobacterium, *Phormidium* sp., with a high tolerance to extreme temperatures was an efficient strain for tertiary wastewater treatment. Other studies exhibited that green algae, *Chlamydomonas* and *Chlorella* species played significant roles during long-term piggery wastewater treatment (Godos et al., 2009; Kong et al., 2010). *Scenedesmus* included suitable species for industrial wastewater treatment and biodiesel production (Di Termini et al., 2011; Martínez, 2000; Zhang et al., 2008).

On the other hand, finding a cost-effective harvesting method is one of the biggest obstacles in producing algal biomass (Nurdogan and Oswald, 1995). Although physical separation methods like filtration or centrifugation have been used, they are not practical for large-scale production (Mohn, 1988). Chemical flocculation followed by sedimentation or flotation can cause secondary pollution and increase effluent salinity, making them less than ideal (Imase et al., 2008; Oh et al., 2001). Alginate, carrageenan, or chitosan immobilization systems are available but expensive and inefficient over the long term (Chevalier and de la Noüe, 1985; Gonzalez and Bashan, 2000). Godos et al. (2010) suggested that when selecting algal species for wastewater treatment, it is crucial to consider nutrient removal efficiency, growth rate, lipid content in cells, and good algal biomass settleability. In these perspectives, the large size of *Arthrospira* filament provides a practical advantage over other single-celled green algae, such as *Chlorella* spp., as it can be easily harvested using a mesh (Anny et al., 2024) or low-cost settlement method (Depraetere et al., 2015). This microalga contains

numerous valuable biomolecules, such as proteins, carbohydrates, pigments, minerals, vitamins, and antioxidants (Markou et al., 2021). Overall, microalgae-based wastewater treatment has the potential to address environmental concerns and offer promising solutions to various industries.

4. Microalgae used for valuable biomass and functional food production

Currently, researchers have taken a keen interest in cultivating microalgae because of their ability to produce various biologically active substances, grow biomass rapidly, and adapt to diverse environments. Additionally, microalgae biomass production methods are eco-friendly as they do not pollute the environment. They use carbon dioxide while generating oxygen, consume a relatively small amount of water, and can be cultivated on land that is inappropriate for crop cultivation. Microalgae are considered a valuable resource for two main purposes: biomass production and the cultivation of biologically active substances. These substances include various compounds such as proteins, polysaccharides, lipids, polyunsaturated fatty acids, vitamins, pigments, phycobiliproteins, enzymes, and so on. These biologically active compounds found in microalgae have been shown to have various beneficial effects, such as antioxidant, antibacterial, antiviral, antitumor, regenerative, antihypertensive, neuroprotective, and immunostimulating effects (Markou et al., 2021). These compounds are in high demand in numerous fields, including pharmacology, medicine, cosmetology, chemical industry, fish farming, energy, and agriculture, particularly in producing feed and functional foods (Bhattacharjee, 2016).

4.1 Microalgae for polysaccharides production

Liu et al. (2019) developed a novel method for synthesizing a type of polysaccharide from *Arthrospira platensis* which could be a potential glucose source for industrial application. They cultivated the microalga in open reservoirs under nitrogen-deficient conditions, and achieved a maximum productivity of 27.5 g/m^2/day for biomass and 26.2 g/m^2/day for

polysaccharides. Similarly, El-Naggar et al. (2020) identified and extracted water-soluble polysaccharides from *Chlorella vulgaris* for their potential use as plant growth stimulants. Ai et al. (2023) revealed *Spirulina platensis* as a safe and nutritious source of bioactive polysaccharides, suitable for functional foods and dietary supplements. Recently, the bioactive properties of β-glucans from *Arthrospira* cells have gained attention in the pharmaceutical, cosmetics, and nutraceutical industries. Markou et al. (2021) revealed that in P limited condition, *Arthrospira* cells can accumulate more carbohydrates than P-replete medium. β-glucans from microalgal cells have been found to possess immunomodulatory, anti-aging, antioxidant, antitumor, serum cholesterol-reducing, and obesity-preventing properties (Jin et al., 2021; Murphy et al., 2020). According to a research report by Business Communications Company in 2018, the global β-glucan market is expected to reach $576.28 million by 2025, growing at an annual rate of 7.3% from 2017 (Dana Byrtusová, 2020).

4.2 Microalgae for lipid production

Microalgae can produce different kinds of lipids, including triacylglycerols, phospholipids, glycolipids, and phytosterols. These lipids consist of fatty acids that range from C12 to C24, with mono- and polyunsaturated fatty acids (PUFA) of C16 and C18 being the most common (Dolganyuk et al., 2020). The lipid content in microalgae usually varies from 20% to 50% of algal dry mass. These lipids can perform various functions, such as serving as energy storage and structural components of the cell membrane. Moreover, they play a vital role in metabolic processes like signal transduction, transcriptional and translational control, intercellular interactions, and secretion and transfer of vesicles (Bellou et al., 2014). Among the different kinds of PUFAs, ω-3 and ω-6 fatty acids are important, as they cannot be produced inside the human body and must be consumed through diets (Calder, 2017). Foods containing ω-6 fatty acids, such as γ-linolenic acid, have been shown to provide cardiometabolic benefits in blood lipids and glycemia markers (Wu et al., 2019). Additionally, gamma-linolenic acids

can help prevent diabetes, obesity, atherosclerosis, and inflammatory-autoimmune diseases (Simopoulos, 2016). However, depending on the cultivation conditions and the composition of nutrient in the medium, the profile of fatty acids may vary even in the same species of microalgae (Dolganyuk et al., 2020). Previous studies have found that the production of microalgal biomass and lipid contents are often contradictory. While nutrient depletion can inhibit the production of microalgal biomass, it can enhance the lipid content under the same cultivation condition. Therefore, the highest lipid content does not necessarily result in high total lipid production (Xin et al., 2010).

A. platensis is a microalgal species known for being rich in protein and low in lipid content (Hena et al., 2018). This microalga is easy to be cultivated and harvested in a large scale, and highly resistant to contamination. Despite its low-fat content, *A. platensis* can still be considered a potential candidate for biodiesel production due to its highly productive biomass. Hena et al. (2018) reported that the fatty acid profile of the extracted lipid from *A. platensis* cultivated in dairy wastewater is suitable for biodiesel production. However, it is worth noting that the linolenic acid content was slightly higher compared to the control.

4.3 Microalgae for pigment contents

Arthrospira, a type of blue-green algae, contains bioactive pigment compounds that are gaining increasing attention in the health and food industries. Phycocyanin and carotenoids are two such bioactive pigments that have been found to have various health benefits, such as antioxidant, anti-inflammatory, anti-cancer, and antiviral properties (Dewi et al., 2018; Romay et al., 2003). Phycocyanin, a blue colorant, is of particular interest due to its limited natural sources for blue pigments and its potential as a natural alternative to commonly used artificial dyes (Tavanandi and Raghavarao, 2020). It is used in the food industry to produce various attractive infant foods such as candies, desserts, ice creams, chewing gums, and yogurts (da Silva Faresin et al., 2022). Additionally, due to its fluorescent nature, cyanobacterial

phycocyanin is crucial for molecular and pharmaceutical diagnostics as a phycofluoro probe (Li Sun et al., 2003). A recent market research report has predicted that the global phycocyanin market will grow annually by 9.6% from 2021 and could be worth $409.8 million by 2030 (Park et al., 2022). These findings suggest that bioactive pigments from *Arthrospira*, especially phycocyanin, have enormous potential for various applications in the health and food industries.

5. The purpose and scientific approach of the study

The ultimate goal of this study is a consavation of aquatic ecosystems through sustainable utilization of excessively growing macrophytes, e.g. water hyacinth, using two modern tequnologies, i.e. AD and mass-culturing of micro-algae, e.g. *Arthrospira* (Fig. 1-1). In this study, I confirmed ADE from water hyacinth as suitable medium for growth of two cyanobacterial species having large trichome, and then determined biochemical compositions, including valuable biomolecules like ß-glucans, in the algal cells cultivated in the ADE comparing with those in the standard media, in order to achieve sustainable utilization of the over-grown macrophytes.

I firstly cultured the two cyanobacterial species, *A. platensis* and *Arthrospira fusiformis*, in ADE of water hyacinth (*E. crassipes*) with different dilutions, to select suitable dilution treatments for the growth of both algal species. Secondly, I measured algal biomass, biochemical components, such as total protein, carbohydrates, lipids, dietary fibers, total glucans, and α and β-glucans. I mainly focused on enhancing β-glucan production in cultivating *A. platensis* and *A. fusiformis* using the water hyacinth's P-limited ADE.

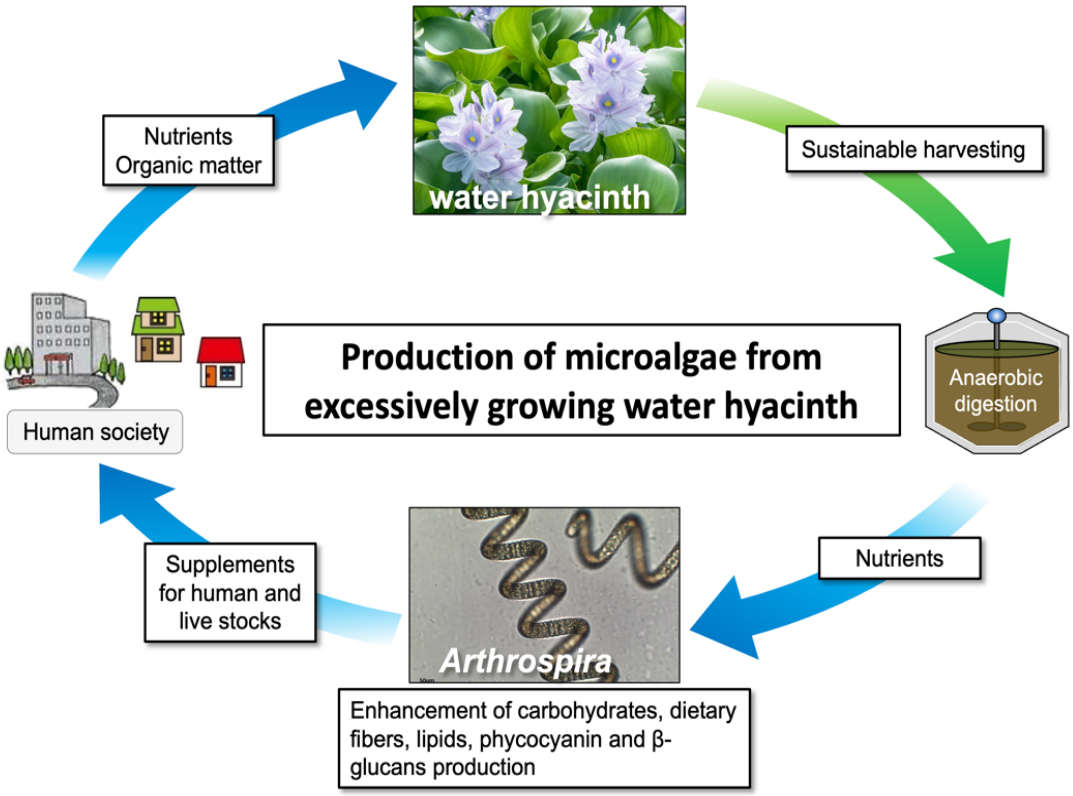

Fig. 1-1. Schematic diagram for conserving healthy aquatic ecosystems through sustainable utilization of overgrowing water hyacinth (*Eichhornia crassipes*) using anaerobic digestion (AD) and mass-culturing of two *Arthrospira* species by utilizing the AD effluent.

Thirdly, I determined nutrient removal efficiency from the ADE using the two *Arthrospira* species. Additionally, I did not only measure the Carbon (C), N, and P contents in the algal cells cultivated in the ADE, but also contents of heavy metals, such as aluminum (Al), chromium (Cr), nickel (Ni), selenium (Se), lead (Pb), copper (Cu), zinc (Zn), mercury (Mg), cadmium (Cd), and manganese (Mn) to confirm safety for human and animal consumption for using the algal product.

Finally, in Chapter 3, I determined the essential fatty acid contents of the two *Arthrospira* species cultivated in the ADE. I also measured the pigment contents, i.e. chlorophyll *a* and phycocyanin. Based on my findings, I proposed that cultivated *Arthrospira*

could be used to produce functional food. My research demonstrates that cultivating *Arthrospira* using the ADE from overgrown aquatic weeds has a potential to produce valuable biomolecules, such as β-glucan, essential fatty acids, dietary fibers, and phycocyanin, through sustainable utilization of aquatic weeds and nutrient recycling.

The thesis is structured as follows:

Chapter 1: General introduction.

Chapter 2: Enhancement of β-glucan production in two *Arthrospira* species cultivating in anaerobic digestion effluent of water hyacinth.

Chapter 3: A comparison of major fatty acid compositions and pigment contents of the two *Arthrospira* species culturing in anaerobic digestion effluent of water hyacinth.

Chapter 4: General discussion.

CHAPTER 2:

"Enhancement of β-glucan production in two *Arthrospira* species cultivating in anaerobic digestion effluent of water hyacinth"

This section is mainly based on the manuscript

"Enhancement of β-glucan production in two *Arthrospira* species cultivating in anaerobic digestion effluents of water hyacinth" by Most Fahima Ahmed Anny, Taiabur Rahman Tuhin, Xin Liu, Kohei Yoshiyama, Akizuki Shin-ichi, Tatsuki Toda, Syuhei Ban" published on-line in **Biochemical Engineering Journal** on 26 December 2023. https://doi.org/10.1016/j.bej.2023.109206.

1. Introduction

Macrophytes are a critical part of freshwater ecosystems, significantly stabilizing water quality, binding nutrients, and providing spawning and nursery grounds for numerous fish species (Ban et al., 2019). However, the overgrowth of aquatic macrophytes, such as water hyacinth (*Eichhornia crassipes*), creates severe ecological, economic, and social problems worldwide (Milicevic, 2023; O'Sullivan et al., 2010). Water hyacinth is one of the world's worst invasive aquatic macrophytes (Holm et al., 1977) that grows and spreads rapidly in lakes, ponds, canals, and rivers (Gundupalli et al., 2023). The overgrowth of this macrophyte hinders fishery activities and boat traffic, decreases biodiversity, reduces dissolved oxygen levels, produces unpleasant odors, and creates unsightly landscapes (Milicevic, 2023).

Anaerobic digestion (AD) is an eco-friendly and cost-effective bioenergy-generating method of recycling organic waste from macrophytes harvested from water bodies (Liu et al., 2020). The macrophytes as organic matter can be converted into biogas and nutrient-rich liquid digestate through AD process (Zhang et al., 2014).This process helps to reduce the volume of waste material and provides valuable resources that can be utilized for agriculture and energy production (Maroušek and Marouškovά, 2021). Overall, AD can be a sustainable approach to utilize the biomass of overgrown macrophytes. However, the AD effluent (ADE) contains a high concentration of nutrients (Kimura et al., 2019; Park et al., 2010), which can contribute to eutrophication in aquatic environments if not correctly treated before discharge (Depraetere et al., 2013). Microalgal cultivation is an excellent option for removing nutrients from wastewater, including ADE, while producing algal biomass (Liu et al., 2020). Microalgae are highly effective in consuming nutrients during their growth. Their biomasses can be used to produce biofuel (Cai et al., 2013), as food supplements for human and animal feeds (Depraetere et al., 2013; Markou et al., 2021).

Arthrospira (commonly known as *Spirulina*) is a photosynthetic, edible, and prokaryotic microalgal genus with a high potential for use in high-nutrition foods. The size of *Arthrospira* filament also provides a practical advantage over other single-celled green microalgae, such as *Chlorella* spp., as it can be easily harvested using a mesh. This microalga contains numerous valuable biomolecules, such as proteins, carbohydrates, pigments, minerals, vitamins, and antioxidants (Markou et al., 2021). However, the chemical composition of the cultivation medium and the cultivation conditions, e.g. temperature, salinity, and nutrient availability, play a crucial role in composition and biomass production of microalgae produced (Papadopoulos et al., 2022).Bioactive properties of β-glucans from *Arthrospira* cells have recently gained attention in the cosmetics, nutraceutical, and pharmaceutical industries (Markou et al., 2021).

β-glucans are polysaccharides primarily composed of D-glucose monomers linked together by β-D-glycosidic bonds. β-glucans from different sources have varying structures, degrees of branching, branching patterns, and molecular weights (Zhu et al., 2016).The properties of β-glucans include immunomodulation, antiaging, antioxidation, and antitumor activities, serum cholesterol reduction, and obesity prevention (Jin et al., 2021; Murphy et al., 2020). Based on a Business Communications Company research report in 2018, the global β-glucan market will be worth $576.28 million by 2025, growing at 7.3% annually from 2017 (Dana Byrtusová, 2020).The biotechnology sector is undertaking extensive research to identify new sources to meet the increasing demand for this immunostimulatory polysaccharide in advancing immunotherapy treatments (Dana Byrtusová, 2020).

The primary sources of β-glucans studied are yeast, mushrooms, cereals, and bacteria (Jin et al., 2021; Zhu et al., 2016). There is a dearth of research on β-glucans from cyanobacteria and green algae (Markou et al., 2021; Vogler et al., 2018). Furthermore, no studies have yet

explored the production of β-glucan by cultivating *Arthrospira* using wastewater or ADE from macrophytes.

In this chapter, *Arthrospira platensis* and *A. fusiformis* were cultivated using ADE of water hyacinth and standard algal media. Biochemical compositions, especially carbohydrates, dietary fibers, and β-glucan production, in the algal cells, and nutrient removal efficiencies from the culture media were determined for evaluating the ADE as algal media, in order to achieve sustainable use of overgrown water hyacinth through AD and mass-culturing of microalgae.

2. Materials and Methods

2.1 Preparation of anaerobic digestion effluent

ADE used for all the experiments was obtained from an invasive macrophyte, *Eichhornia crassipes*, collected from Lake Ibanei (35°11'22.7" N 136°08'25.1" E), Shiga Prefecture, Japan, on 27 August 2021. The whole water hyacinth plants were shredded and compressed using milling (RSCz-3500, R-ing Co. Ltd., Japan) and a screw dewatering machine (Dash-1, R-ing Co. Ltd.). The water hyacinth juice was then filtered through a 106-μm mesh and stored in a refrigerator at 4 °C. Mesophilic anaerobic sludge was collected from a full-scale anaerobic digester that treated dewatered sewage sludge at the Hokubu Sludge Treatment Center in Yokohama City, Kanagawa Prefecture, Japan. Then, the sludge was stored in a temperature-controlled room at 37 °C for a few days to remove residual organic compounds from the sludge.

An up-flow anaerobic digester (internal diameter 100 mm) with an effective volume of 7.8 L was used as the reactor. First, 7.8 L of mesophilic sludge was added to the reactor. After that, water hyacinth juice as substrate was continuously fed from the lower part of the reactor, starting with a constant hydraulic retention time of 7.2 days. The ADE was passed through a U-shaped pipe connected to the upper portion of the reactor and was stored in an effluent

storage tank. The operational time for the reactor was around 250 days. The reactor temperature was maintained at 37 ± 1 °C in a temperature-controlled laboratory.

2.2 Chemical analysis of ADE

To analyze the chemical content of the ADE, it was first filtered using a 0.2-µm pore-size membrane filter (model no. A020A142C, ADVANTEC, Tokyo, Japan). The concentrations of ammonium nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), phosphate phosphorus (PO_4^{3-}-P), and several metal ions such as Na^+, K^+, Ca^{2+}, and Mg^{2+} in the filtrate were measured (Table 2-1). The concentrations of the nutrients were determined using a continuous flow system autoanalyzer (AACS-II, Bran + Luebbe, Norderstedt, Germany). NH_4^+-N was analyzed by the Berthelot reaction method according to (Koroleff, 1970). NO_2^--N and NO_3^--N concentrations were determined with a cadmium reduction column where nitrate was quantitatively reduced to nitrite (Armstrong et al., 1967). PO_4^{3-}-P concentration was determined by the phosphomolybdenum blue method (Murphy and Riley, 1962). Total P concentration was also determined after autoclaving 10 ml of the samples with potassium peroxydisulfate at 121°C for 30 min to convert all the P into PO_4^{3-}. Dissolved organic carbon (DOC) concentration was measured using a Total Organic Carbon analyzer (SHIMADZU, TOC-5000A, Kyoto, Japan). Metal concentrations were determined by inductively coupled plasma-atomic emission spectroscopy (NexION 1000 ICP-MS, Waltham, MA, USA). Before the analysis, 50 µL of nitric acid (61%, Wako, Osaka, Japan) was added to 1 mL of the sample solution to prevent metal adsorption (Kimura et al., 2019).

2.3 Algal strains and preculture conditions

A. platensis (NIES-39) was precultured in 100-mL Erlenmeyer flasks containing Spirulina Ogawa Terui (SOT) medium (Table 2-1), using a shaker at 80 rpm with 360 µmol/m^2/s light intensity and a 12-h light:12-h dark cycle at 22 °C in a growth cabinet (MLR-350, Sanyo, Osaka, Japan). *A. fusiformis* (UTEX LB2721) was precultured in 100-mL

Erlenmeyer flasks containing *Spirulina* medium (Table 2-1) at 25 °C using a shaker at 75 rpm with light intensity 400 µmol/m^2/s and a 12-h light:12-h dark cycle.

2.4 Cultivation of *Arthrospira* in ADE

For the preparation of inoculum, each of the two species of *Arthrospira* reared to the logarithmic growth phase was filtered by a 20-µm nylon mesh, washed twice, and resuspended in 0.6 mL of autoclaved distilled water. Then, the algal suspension was transferred to a 100-mL flask containing 100 mL of culture medium, and, finally, the initial culture density was adjusted to optical density (OD) at 730 nm = 0.1. To determine the optimal dilution for the growth of microalgae, ADE was firstly filtered with a 0.2-µm pore-size membrane filter to remove particles including bacteria and microalgae, and then diluted at two-, four-, six-, eight-, and ten-fold with autoclaved distilled water and used as an algal culture medium. Undiluted ADE and standard media (SOT or *Spirulina* medium) were also prepared. After adding the same amount of $NaHCO_3$ (0.2 mol/L) as in the standard medium, the pH of each treatment was adjusted to 8.5 with 1 M NaOH. All experimental cultures were incubated in identical conditions to the precultures in triplicate, and optical density was monitored daily at 730 nm by using a spectrophotometer (SP-300, OPTIMA, Tokyo, Japan) for six days from the start of the experiment.

2.5 Calculation of nutrient removal rate

To determine nutrient removal rates, NH_4^+-N and PO_4^{3-}-P were measured on the initial and final days of the experiment using the method described in Section 2.1. The culture media were filtered using a 0.2-µm pore-size membrane filter (ADVANTEC, DISMIC25 AS, Tokyo, Japan) to remove suspended materials before the measurement. The nutrient removal rate (R, %) was calculated as follows:

$$R = 100 \times (C_i - C_f)/C_i,$$

where C_i and C_f are, respectively, the concentrations of NH_4^+-N or PO_4^{3-}-P on the initial and final days of the experiment. PO_4^{3-}-P is a most relevant P form that is directly utilized by algae (Liu et al., 2020), and just it was measured as P source of nutrients.

2.6 Analysis of biochemical compositions

2.6.1 Proximate biochemical compositions of algal biomass

At the end of the cultivation period, the algae were filtered with a precombusted and preweighed GF/C glass fiber filter (Cytiva, model no. 1822-047, Tokyo, Japan) and rinsed with distilled water to remove the salts from the culture medium. Then, the filter was dried in an oven (DV-600, YAMATO, Tokyo, Japan) at 60 °C until constant weight was reached (*ca*. 24 h). The dry mass of the algae was measured by using a microbalance (AW220, SHIMADZU, Tokyo, Japan) at the precision of 0.1 mg. Apart from this, for biochemical analysis, algal cells grown for six days were collected with a 20-µm nylon mesh, washed three times with distilled water, and then lyophilized in a freeze-drying system (Taitec VD-800F, Taitec Co., Saitama, Japan) and stored at −80 °C until further analysis, except for measuring protein content, for which fresh algal cells were used.

The protein content was obtained from cells by extracting it using 0.5 M NaOH, and its measurement was performed using the method developed by (Lowry et al., 1951). Briefly, 1.5 mL of fresh algal sample was centrifuged at 3,000 × *g* for 15 min and the supernatant was removed. The pellet was resuspended in 1.5 mL of 0.5 M NaOH and incubated on a hotplate at 100 °C for 20 min. Then, an aliquot of 100 µL from the treated solution was added to a mixture of 100 µL of 5% sodium dodecyl sulfate and 1 mL of 2% Na_2CO_3 in 0.1 M NaOH. After 15 min, it was added to 100 µL of 1 M Folin–Ciocalteu reagent and left for 30 min in the dark. Finally, the OD of the final solution was measured at 750 nm using a spectrophotometer (SP-300). Bovine serum albumin was applied as a standard.

Total carbohydrates were measured by a modified phenol-sulfuric acid method (Kochert, 1978). Briefly, 100 mg of freeze-dried algae was extracted with 80% ethanol and boiled at 100 °C for 15 min. Then, 10 µL of 90% phenol and 1.25 mL of 96% H_2SO_4 were added to 0.1 mL of the extracted sample. Finally, the OD of the solution was measured at 485 nm with a spectrophotometer. D-glucose was used as a standard (Markou et al., 2021).

A modified sulfo-vanillin method was employed to determine the amount of total lipids in a sample (Izard and Limberger, 2003). Total lipids were first extracted from 1 g of freeze-dried algae using 2:1:0.2 chloroform: methanol: MilliQ water (v:v:v). In brief, 20 µL of the lipids extracted (approximately 150–500 mg/L lipid) was incubated at 80 °C to evaporate the chloroform. After the addition of 0.4 mL 96% sulfuric acid, the mixture was boiled in a water bath for 10 min. Then, it was cooled for 15 min at room temperature, 1.0 mL of phosphoric acid/vanillin solution was added, and the mixture was incubated at 37 °C for 15 min. Finally, the OD was measured at 530 nm with a spectrophotometer. Corn oil was used as a standard (Markou et al., 2021).

2.6.2 Measurement of total dietary fiber content

Total dietary fiber content was determined according to Markou et al. (2021). A 200-mg aliquot of freeze-dried algae was put into a 300-mL beaker containing 25 mL of distilled water and stirred for *ca*. 30 min. After placing it at 37 °C without stirring for 90 min, 100 mL of 95% ethanol was added, and the mixture was left at room temperature for 1 h. The precipitate was collected on a preweighed ashless filter (Whatman no. 41, cat. no. 1441-070) and washed twice with 20 mL of 78% ethanol, 10 mL of 95% ethanol, and 10 mL of acetone, in order. The residue on the filter was dried at 105 °C in an oven (DV-600) to constant weight, and it was weighed with a microbalance to the precision of 0.1 mg. After combusting the dried material in a muffle furnace (FUL220FB, Tokyo, Japan) at 525 °C for 5 h, the dry weight of dietary

fiber was calculated as the difference between the dry weight of the material before and after combustion.

2.6.3 Fourier-transform infrared spectroscopy

Fourier-transform infrared (FTIR) spectroscopy is a widely used technique capable of rapidly detecting various functional groups present in the main components of biomass (Guo et al., 2018; Markou et al., 2021). It is also a reliable method for analyzing biomolecules such as proteins, carbohydrates, and lipids (Fadlelmoula et al., 2022). The range 1200 to 950 cm^{-1} in FTIR spectra is called the carbohydrate "fingerprint" region. This part of the spectrum is essential for identifying chemical groups in polysaccharides because the position and intensity of the bands are distinct for each polysaccharide (Guo et al., 2018). In this study, 100 mg of freeze-dried algal material was first homogenized with an agate pestle and mortar and powdered. The powdered material was sieved with a 35-µm nylon mesh. Then, 2 mg of the sieved powder was placed on an FTIR spectrometer (FT/IR-4100, Jasco, Tokyo, Japan) and pressed with a gripper to ensure the best possible contact with the crystal surface. Finally, the range 4000–650 cm^{-1} was scanned.

2.6.4 Measurement of β-glucans

1,3:1,4-β-D-Glucan was measured using a commercially available K-BGLU kit (Megazyme Ltd., Wicklow, Ireland) following the manufacturer's procedure. Briefly, a 100-mg aliquot of fine-powdered freeze-dried algae (<0.5 mm) was put into a 20-mL screw-capped test tube. Then, a sequence of procedures was performed, such as gelatinization, lichenase depolymerization of β-glucan, pH adjustment and incubation with β-glucosidase, and glucose determination. Finally, the OD of the solution was measured at 510 nm against the reagent blank using a spectrophotometer. Total glucans and α-glucans were measured by using a K-YBGL kit (Megazyme Ltd.). Briefly, a 90- or 100-mg aliquot of the fine-powdered freeze-dried algae (for measuring total or α-glucans, respectively) was put into a 20-mL screw-capped test

tube. Afterward, a series of processes were carried out, including solubilization and partial hydrolysis, incubation with β-glucosidase, and glucose measurement. The OD of the solutions was measured at 510 nm for both total glucans and α-glucans and compared with a reagent blank. Finally, 1.3:1.6-β-glucan was calculated using the manufacturer's online Mega-calc tool as the difference between the total glucans and α-glucans amounts.

2.6.5 Measurement of intracellular carbon, nitrogen, and phosphorus

The Total C and N contents of freeze-dried algae were determined using a CHN elemental analyzer (NCH-22A, SUMIGRAPH, Tokyo, Japan). The total P content of freeze-dried algae was measured using a modified thermal acid digestion method described by (Yao et al., 2013). Briefly, 5 mg of freeze-dried algal sample was placed into a glass tube containing 6 M HNO_3, digested with $K_2S_2O_8$ solution (5:1 v:v), and autoclaved at 120 °C for 30 min. Finally, the P content in the solution was quantified as phosphate as described above (see Section 2.1).

2.7 Statistical analyses

The effects of differences between culture media, including diluted ADE, on biomass production, growth rate, chemical content, elemental concentration and composition, and removal rates of NH_4^+-N and PO_4^{3-}-P were tested by one-way analysis of variance (ANOVA). When ANOVA showed a significant difference, a Tukey-Kremer *post-hoc* test was performed at $p < 0.05$. All statistical analyses were performed using R-studio ver. 4.0.3 (2020).

3. Results and discussion

3.1 Chemical characteristics of ADE

The ADE derived from water hyacinth had low organic matter content but still contained enough essential nutrients, except for PO_4^{3-}-P, for the growth of *Arthrospira* spp.

(Table 2-1). The ADE included just 7% of the PO_4^{3-}-P concentration of both SOT and *Spirulina* medium. Although the NH_4^+-N concentration in the ADE was slightly lower than the NO_3^--N concentrations in the standard media, the values were comparable. ADEs derived from traditional feedstocks, such as animal manure, food waste, and municipal solid wastes, contain high amounts of DOC, e.g., 12 g/L (Watanabe et al., 2009), NH_4^+-N, e.g., 1400–2650 mg/L (Massa et al., 2017), and PO_4^{3-}-P, e.g., 24–1119 mg/L (Kobayashi et al., 2013; Massa et al., 2017). In this study, just the liquid part of water hyacinth was used to prepare the ADE, which explains the lower organic load (DOC concentration of 40 mg/L) and lower concentrations of inorganic nutrients such as PO_4^{3-}-P and NH_4^+-N.

The ADE also contained a relatively low Na^+ concentration (4838 mg/L; 241 mg/L before $NaHCO_3$ addition), 70%–80% of those in the standard media (Table 2-1). The K^+ concentration was notably high in the ADE, 2220 mg/L, approximately triple that in the standard media (Table 2-1). Both Ca^{2+} and Mg^{2+} concentrations were seven to nine times those in the standard media.

Table 2-1 Major nutrient and metal compositions in the undiluted anaerobic digestion effluent (ADE) of water hyacinth (*Eichhonia crassipes*) and the standard media used in this study (SOT and *Spirulina* medium). Hyphens represent components that are not included in the medium. DOC, dissolved organic carbon; NH_4^+-N, ammonium; NO_3^--N, nitrate nitrogen; NO_2^--N, nitrite nitrogen; TP, total phosphorus; PO_4^{3-}-P, phosphate phosphorus.

Component	ADE	SOT	*Spirulina* medium
		(mg /L)	
DOC	40.0	–	–
NH_4^+-N	345.7	–	–
NO_3^--N	1.1	411.8	411.8
NO_2^--N	2.1	–	–
TP	10.0	–	–
PO_4^{3-}-P	6.6	89.0	89.0
Na	241*	5677.5	6542.7
K	2220.0	671.5	671.5
Ca	83.0	10.9	10.9
Mg	184.0	19.7	19.7

*After the addition of 0.2 mol/L $NaHCO_3$, the Na concentration in the ADE was 4838 mg/ L.

3.2 Optimal dilution of ADE for microalgal growth

Cell yields of *A. platensis* varied significantly among treatments after day 2 and throughout the remainder of the experimental period (Fig. 2-1a). The mean cell yield at the end of the experiment was highest in the SOT medium, followed by those in the six- and eight-fold diluted ADE, 91% and 88% of that in the SOT medium, respectively. However, these three were not significantly different. The cell yields in undiluted, and two-fold diluted ADE were significantly lower, just 28% and 30% of that in the SOT medium, respectively. The cell yields in the remaining two dilutions (four- and ten-fold diluted ADE) were slightly lower than in the SOT medium, 79% and 81%, respectively, while significantly higher than those in lesser dilutions.

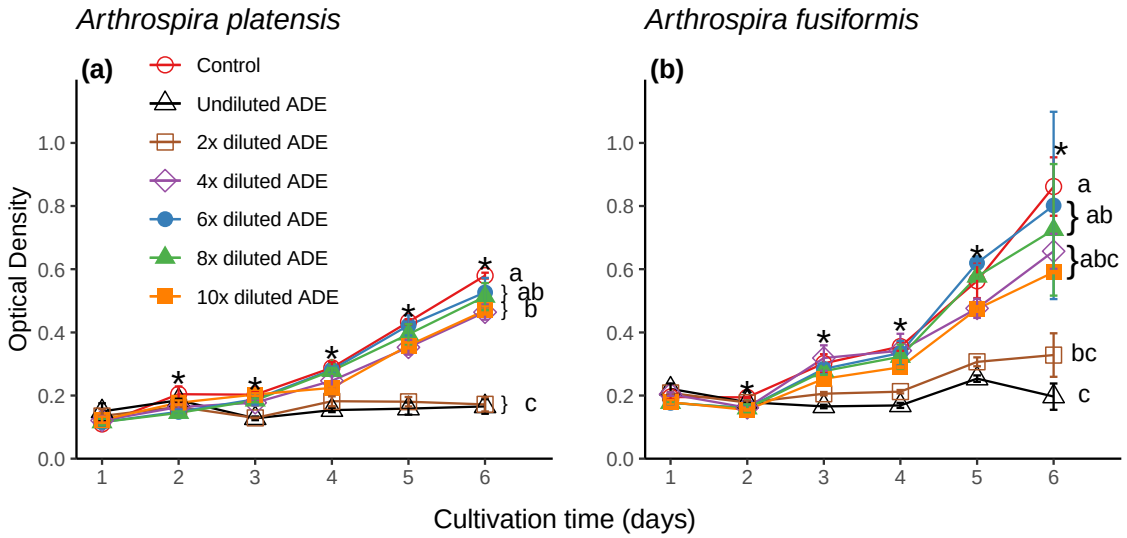

Fig. 2-1. Growth curves of *Arthrospira platensis* (a) and *A. fusiformis* (b) in undiluted, 2×, 4×, 6×, 8×, and 10× diluted anaerobic digestion effluent (ADE) of water hyacinth (*Eichhornia crassipes*) and SOT (a) or *Spirulina* medium (b) as a control. Symbols and vertical bars represent average and standard deviation values of triplicates. Asterisks denote significant differences among treatments by analysis of variance at $p < 0.05$. Different letters on the right side of each graph indicate significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$ on the last day of the experiment.

For *A. fusiformis*, cell yields at the end of the experiment were highest in *Spirulina* medium, followed by those in the six-, eight-, four-, and ten-fold diluted ADE. The cell yields were not significantly different among the most diluted ADE-based media and the *Spirulina* medium. However, in two-fold and undiluted ADE, they were only 38% and 22% of that in *Spirulina* medium, respectively (Fig. 2-1b).

Growths of both *A. platensis* and *A. fusiformis* were limited in undiluted and two-fold diluted ADE, probably because of the high concentrations of NH_4^+-N and K^+, while the algae grew well in ADE that was diluted more (Fig. 2-1). This suggests that diluted ADE can be used

as a cost-effective medium for cultivating the two *Arthrospira* spp. tested in this study. A high concentration of NH_4^+-N hinders algal growth by increasing oxidative stress and disrupting the electron transport system (Lu et al., 2018). Belkin and Boussiba (1991) showed that the growth of *A. platensis* was inhibited by 50% when the NH_4^+-N concentration reached 140 mg/L at pH 10, compared with that in lower ammonium concentrations. Jiang et al. (2015) reported that the lethal concentration of NH_4^+-N for *Spirulina subsalsa* was ≥90 mg/L. This concentration was comparable to those in the undiluted and two-fold diluted ADE in the present study, but not in the remaining treatments. Parker et al. (1997) showed K^+ inhibition of the growth of *Microcystis* spp. with no growth of *Microcystis* at > 2.8 mmol/L (equivalent to 53.2 mg/L) K^+. The two *Arthrospira* spp. used in this study are more tolerant of high K^+ than *Microcystis* because the K^+ concentrations in the standard media are > 670 mg/L. However, that in two-fold diluted ADE was 1110 mg/L, 1.6 times that in the standard media. The two algae grew well in the four-fold diluted ADE (555 mg/L of K^+, comparable to that in the standard media). Therefore, K^+ inhibition may occur for the two *Arthrospira* spp. in ADE diluted less than four-fold, but not in more diluted ADE.

Although the cell yields were similar in ≥ 4-fold diluted ADE for both *A. platensis* and *A. fusiformis*, to save freshwater, four-, six-, and eight-fold diluted ADE were chosen for the following biochemical analyses. Based on the goal of microalgal cultivation using ADE of water hyacinth through sustainable utilization of the macrophyte for maintaining healthy lake ecosystem, it is important to be concerned about the global scarcity of clean water.

3.3 Biomass production and proximate biochemical composition

The biomass productions of *A. platensis* and *A. fusiformis* after the 6-day experimental period are shown in Fig. 2-2. Although there was no large variation in optical densities among the dilution treatments in growth monitoring (Fig. 2-1), the biomass production of both algae

in the four-fold diluted ADE was significantly the lowest, just 42% and 41% of those in SOT and *Spirulina* medium, respectively. For *A*. *platensis*, though the biomass productions in the six- and eight-fold diluted ADE were significantly higher than that in the standard medium, the difference was small, <0.7% (Fig. 2-2a).

For *A*. *fusiformis*, the biomass productions in the six- and eight-fold diluted ADE were also similar and not significantly different from that in the standard medium (Fig. 2-2b). These results suggest that the low PO_4^{3-}-P concentration in the medium, even at eight-fold dilution (0.82 mg P/L), can support the same algal growth (*ca*. 620 mg/L) as that in the standard medium on the experimental timescale. These values are comparable to the biomass production of *ca*. 400–600 mg/L when *A*. *platensis* was reared in brewery wastewater for 6 days (Papadopoulos et al., 2022), but lower than the biomass of 1700 mg/L when *A*. *platensis* was reared with a semi-continuous culture at 5 days of hydraulic retention time in a modified Zarrouk medium (Markou et al., 2021).

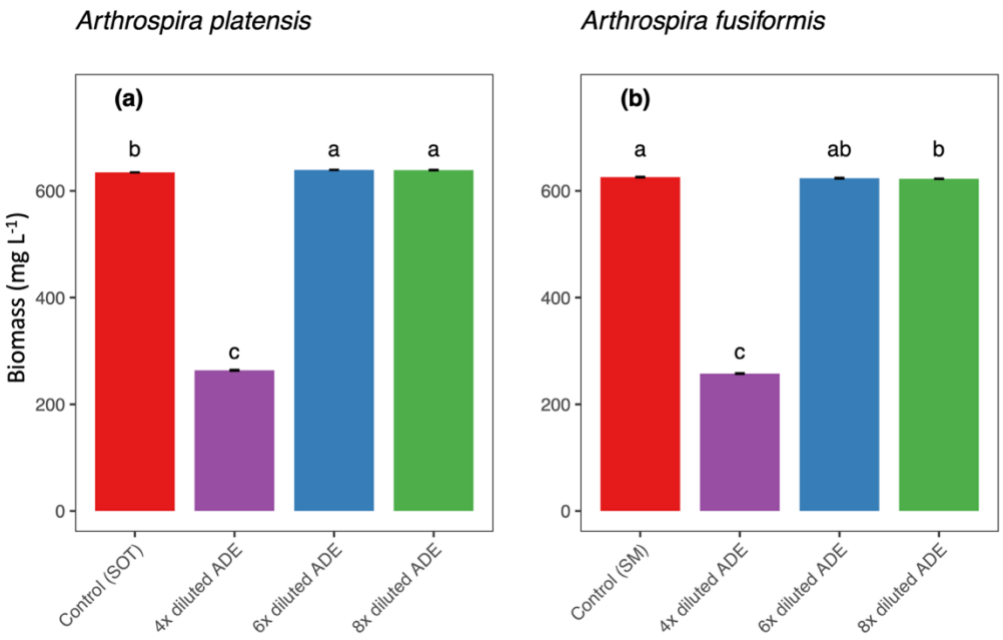

Fig. 2-2. Biomass production of *A. platensis* (a) and *A. fusiformis* (b) cultivated in 4×, 6×, and 8× diluted ADE of water hyacinth (*E. crassipes*) and SOT or *Spirulina* medium (SM) as a control during a 6-day experiment. Columns and error bars represent the average and standard deviations of triplicates. Different letters above columns indicate significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$.

Low biomass production in the four-fold diluted ADE may be attributable to dark-colored ADE that could impede cell growth (Depraetere et al., 2013), i.e., leaving insufficient light for photosynthesis of the culture overall, even though there was enough PO_4^{3-}-P for growth. Therefore, the four-fold diluted ADE was excluded from the following biochemical analyses.

Table 2-2 Biochemical composition (total carbohydrates, proteins, and lipids) as percentages of algal dry mass in *Arthrospira platensis* and *A. fusiformis* cultivated in six- and eight-fold diluted ADE of water hyacinth (*E. crassipes*) and SOT or *Spirulina* medium, and results of analysis of variance (ANOVA). Different superscripts within a column for a given alga represent significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$. The values are the average ± standard deviation of triplicates.

Algal sp. and culture medium	**Carbohydrates (%)**	**Proteins (%)**	**Lipids (%)**
A. platensis			
6x diluted ADE	50.71 ± 0.77^{b}	16.39 ± 2.44^{c}	7.04 ± 0.70^{b}
8x diluted ADE	59.32 ± 0.62^{a}	21.86 ± 0.95^{b}	7.43 ± 0.22^{ab}
SOT	23.42 ± 0.36^{c}	40.07 ± 1.78^{a}	8.55 ± 0.58^{a}
Results of ANOVA	df = 2, 6, F = 2837.6, $p < 0.001$	df = 2, 6, F = 137.99, $p < 0.001$	df = 2, 6, F = 6.2683, $p = 0.034$
A. fusiformis			
6x diluted ADE	45.52 ± 0.80^{a}	14.06 ± 0.36^{b}	5.83 ± 0.08^{b}
8x diluted ADE	46.84 ± 0.67^{a}	14.51 ± 1.90^{b}	6.50 ± 0.34^{a}
Spirulina medium	21.39 ± 0.82^{b}	44.30 ± 1.26^{a}	5.71 ± 0.28^{b}
Results of ANOVA	df = 2, 6, F = 1050.99, $p < 0.001$	df = 2, 6, F = 508.44, $p < 0.001$	df = 2, 6, F = 8.3431, $p = 0.019$

The biochemical compositions of *A. platensis* and *A. fusiformis* reared in the diluted ADE (six- and eight-fold) and the standard media showed significant differences in terms of proteins and carbohydrates, but no significant difference in terms of lipids (Table 2-2). In *A. platensis*, total carbohydrate content in the six- and eight-fold diluted ADE accounted for 50%–60% of the algal dry mass, which was significantly higher than that in the standard medium

(23%), while the total protein content was significantly lower (16%–22%) than that in the standard medium (40%).

In *A. fusiformis*, the total carbohydrate contents were also significantly higher in the six- and eight-fold diluted ADE (45%–46% of algal dry mass) than in the standard medium (22%), while the total protein contents were significantly lower in diluted ADE (14%) than in the standard medium (44%). These differences may be attributed to P limitation in the diluted ADE (Markou, 2012), though the biomass productions were almost the same. Several studies have reported that the storage of carbohydrates increased in microalgae reared in P-deficient media (Cade-Menun and Paytan, 2010; Dean et al., 2008a). It has also been shown that carbohydrates accumulate more in algal cells during stress conditions, such as in saline (Warr et al., 1985) and N-limited media (Sassano et al., 2010).

Cyanobacteria such as *Arthrospira* store carbohydrates mainly as glycogen (e.g., α-1,4 linked glucan) (Markou, 2012).Generally, in cyanobacteria and green algae, the process of glycogen synthesis is regulated by ADP-glucose pyrophosphorylase, which is activated by 3-phosphoglycerate and inhibited by inorganic P (Gómez-Casati et al., 2003). If the inorganic P in the medium is deficient, glycogen production might be stimulated (Markou, 2012). This explains why PO_4^{3-}-limited conditions induce the accumulation of more carbohydrates in the cells (Heldt et al., 1977; Markou, 2012), and why more carbohydrates accumulate in *Arthrospira* spp. In the diluted ADE in this study, conditions in which PO_4^{3-} was limited.

Total lipid contents of *A. platensis* and *A. fusiformis* reared in the six- and eight-fold diluted ADE and the standard media ranged from 7.04% to 8.55% and 5.71% to 6.5%, respectively; the lipid content was significantly lowest in *A. platensis* in six-fold diluted ADE, while it was significantly highest in *A. fusiformis* in eight-fold diluted ADE (Table 2-2). Markou et al. (2021) showed that total lipid contents decreased for *A. platensis* reared in a P-deficient medium (1.5 mg P/L) compared with those in a P-replete medium. Contrastingly, it

has been shown that *Anabaena flos-aquae* accumulates more lipids under P limitation (Dean et al., 2008b). Responses to P deficiency may vary among species because of differences in P demand.

3.4 Fourier-transform infrared (FTIR) spectroscopy analyses

Fourier-transform infrared (FTIR) spectroscopy analyses confirmed that the stress induced by PO_4^{3-} limitation caused an increase in carbohydrate accumulation (Markou et al., 2021) (Fig. 2-3). Guo et al. (2018) described that the spectral range 1200 to 900 cm^{-1} (known as the carbohydrate fingerprint region) is important for identifying chemical groups in polysaccharides. FTIR spectra were measured from 4000 to 650 cm^{-1} in *A. platensis,* and *A. fusiformis* reared in the six- and eight-fold diluted ADE and the standard media. The highest absorbance peaks for all treatments were observed at 1080, 1015, and 1005 cm^{-1} (mainly from polysaccharides and carbohydrates) and 1150 cm^{-1} (mainly from carbohydrates and/or lipids). All the peaks within the range 1200 to 900 cm^{-1} showed higher absorbance values in both algae reared in six- and eight-fold diluted ADE than in the standard media (Fig. 2-3).

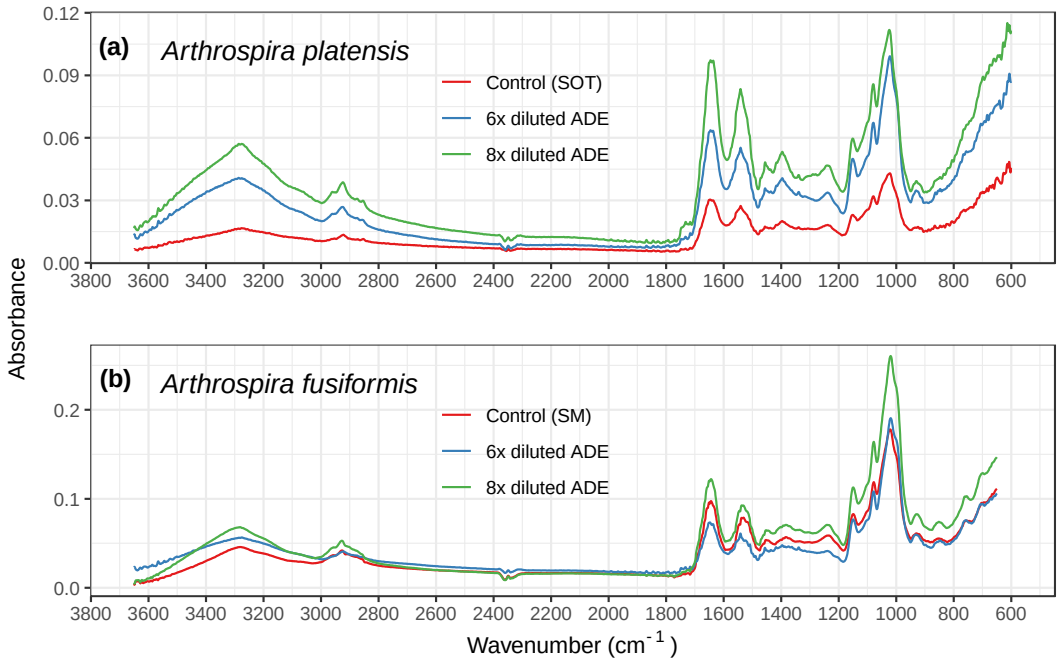

Fig. 2-3. FTIR spectra of (A) *Arthrospira platensis* and (B) *A. fusiformis* samples grown in six- and eight-fold diluted anaerobic digestion effluent of water hyacinth and control media. The highest absorbance peaks for all treatments were observed at 1080, 1015, and 1005 cm^{-1} (mainly from polysaccharides/carbohydrates) and at 1150 cm^{-1} (mainly from carbohydrates and/or lipids). The possible assignments of the vibration modes were reported by (Markou et al., 2021).

3.5 Contents of total dietary fiber

In both *Arthrospira platensis* and *A. fusiformis*, the total dietary fiber contents were significantly higher in the six- and eight-fold diluted ADE than in the standard media (Fig. 2-4). In previous studies, the total dietary fiber content of *A. platensis* generally ranged from 3.6% to 8.5% (Markou et al., 2021; Raczyk et al., 2022). However, (Markou et al., 2021) found that *A. platensis* strain SAG 21.99 accumulated a greater amount of total dietary fiber (20%–32% of the algal dry mass) when it was cultured in a P-limited medium compared with a P-

replete medium. In the present study, the higher carbohydrate content of the two algae reared in P-limited ADE-based media may be attributed to higher total dietary fiber content.

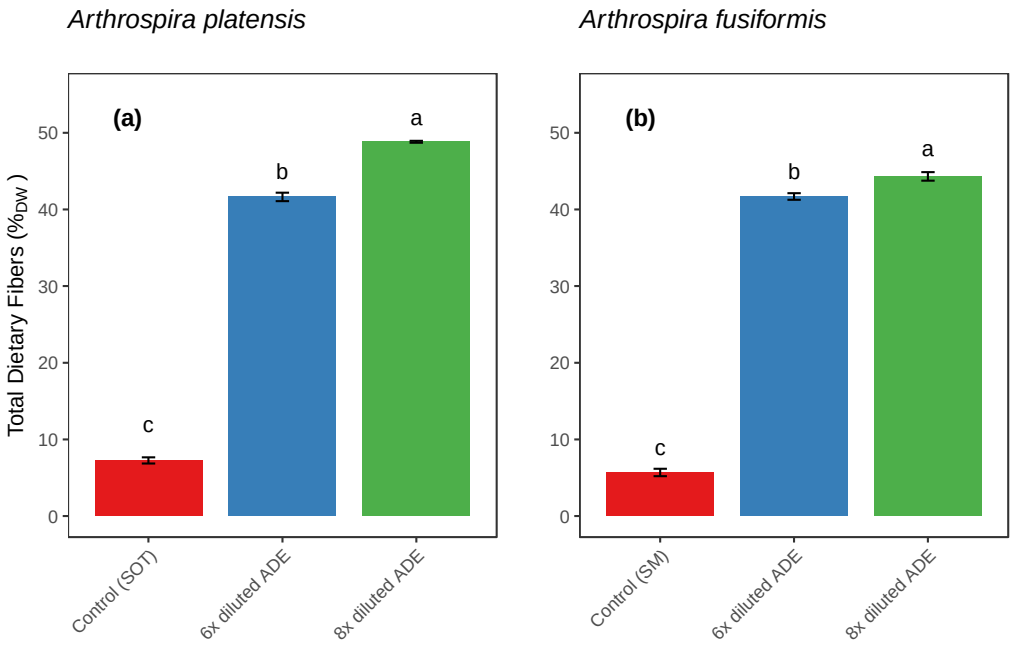

Fig. 2-4. Composition of total dietary fiber in dry mass of *A. platensis* (a) and *A. fusiformis* (b) cultivated in 6× and 8× diluted ADE of water hyacinth (*E. crassipes*) and SOT (a) or *Spirulina* medium (SM) (b) as a control. Columns and error bars represent the average and standard deviations of triplicates. Different letters above columns indicate significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$.

3.6 β-glucan content

For *A. platensis* and *A. fusiformis*, 1.3:1.4-β-glucans, very common in cereals (Zhu et al., 2016), were not detected following culture in any medium. At the same time, α-glucans and 1.3:1.6-β-glucans, which often occur in mushrooms, yeast, and bacteria (Stier et al., 2014), were significantly increased in the six- and eight-fold diluted ADE compared with standard media (Table 2-3). The 1.3:1.6-β-glucans in the two *Arthrospira* species cultivated with diluted

ADE was produced at 4–18% of the algal dry masses, being 1.5–7 times higher than those in the standard media.

Table 2-3 Percentage compositions of α- and β-glucans in the dry mass of *A. platensis* and *A. fusiformis* cultivated in six- and eight-fold diluted ADE of water hyacinth (*E. crassipes*) and standard media (SOT or *Spirulina* medium), and results of ANOVA. Different superscripts within a column for a given alga represent significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$. The values are the average ± standard deviation of triplicates. Hyphens denote a value below the detection limit.

Algal sp. and culture medium	**Total Glucans (α+β) (%)**	**1.3:1.6-β-Glucans (%)**	**1.3:1.4-β-Glucans (%)**	**α-Glucans (%)**
A. platensis				
6x diluted ADE	24.15 ± 0.23^{a}	9.03 ± 0.22^{a}	-	15.12 ± 0.07^{a}
8x diluted ADE	12.91 ± 1.34^{b}	4.05 ± 0.15^{b}	-	8.86 ± 1.33^{b}
SOT	8.66 ± 0.33^{c}	2.64 ± 0.25^{c}	-	6.02 ± 0.43^{c}
Results of ANOVA	df = 2, 6 F = 294.26 $p < 0.001$	df = 2, 6 F = 770.35 $p < 0.001$	-	df = 2, 6 F = 99.729 $p < 0.001$
A. fusiformis				
6x diluted ADE	28.53 ± 1.14^{a}	17.51 ± 0.96^{a}	-	11.02 ± 0.19^{b}
8x diluted ADE	24.81 ± 1.73^{b}	9.06 ± 1.59^{b}	-	15.75 ± 0.76^{a}
Spirulina medium	18.36 ± 0.02^{c}	2.49 ± 0.26^{c}	-	15.89 ± 0.29^{a}
Results of ANOVA	df = 2, 6 F = 55.434 $p < 0.001$	df = 2, 6 F = 145.46 $p < 0.001$	-	df = 2, 6 F = 99.192 $p < 0.001$

In *A. platensis*, the total glucan contents were significantly highest in the six-fold diluted ADE (24.2% of algal dry mass), followed by the eight-fold diluted ADE (12.9%) and the standard medium (8.7%). In *A. fusiformis*, the total glucan contents were 28.5%, 24.8%, and 18.4% in the six- and eight-fold diluted ADE and standard medium, respectively. Markou et al. (2021) reported that the accumulation of β-glucan content in *A. platensis* increased in P-deficient cultures. Jin et al. (2021) showed that genes for carbohydrate synthesis in the green alga *Chlorella sorokiniana* were upregulated, while those for protein and lipid synthesis were downregulated in response to P-limitation.

3.7 Elemental compositions of algae

In both *A. platensis* and *A. fusiformis*, C contents were higher following culture in the diluted ADE, ranging from 452 to 467 mg/g. In contrast, N and P contents were lower, ranging from 69 to 85 mg/g and 7 to 9 mg/g, respectively, compared with those in the standard media (Table 2–4). These findings were consistent with the high carbohydrate and low protein contents in the algal cells (Table 2–2).

Table 2-4 Carbon (C), nitrogen (N), and phosphorus (P) contents of the algal dry mass of *A. platensis* and *A. fusiformis* cultivated in six- and eight-fold diluted ADE of water hyacinth (*E. crassipes*) and standard media (SOT or *Spirulina* medium), and results of ANOVA. Different superscripts within a column for a given alga represent significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$. The values are the average ± standard deviation of triplicates.

Algal sp. and culture medium	C	N	P
	(mg/g)		
A. platensis			
6x diluted ADE	452.73± 4.10^{a}	74.87± 1.84^{b}	6.90± 0.75^{b}
8x diluted ADE	459.20± 4.29^{a}	69.11± 1.03^{c}	8.74± 1.15^{b}
SOT	425.81± 6.15^{b}	97.92± 1.07^{a}	11.66± 1.23^{a}
Results of ANOVA	df = 2, 6	df = 2, 6	df = 2, 6
	F = 38.575	F = 374.11	F = 15.250
	$p < 0.001$	$p < 0.001$	$p = 0.004$
A. fusiformis			
6x diluted ADE	466.77± 6.98^{a}	71.83± 3.29^{c}	7.88± 0.79^{b}
8x diluted ADE	464.88± 4.34^{a}	85.02± 0.85^{b}	7.61± 0.91^{b}
Spirulina medium	426.88± 6.97^{b}	101.09± 2.58^{a}	10.02± 0.66^{a}
Results of ANOVA	df = 2, 6	df = 2, 6	Df = 2, 6
	F = 39.237	F = 106.33	F = 8.2415
	$p < 0.001$	$p < 0.001$	$p = 0.019$

Arthrospira is considered one of the best food supplements and safe food for people of all ages (Al-Dhabi, 2013). However, it can accumulate certain metals (Arunakumara and Zhang, 2008), some of which can be toxic to humans, such as Cd, Hg, Pb, Ni, and As. Although some heavy metals like Cu, Zn, and Cr are essential for human nutrition, they become hazardous when they exceed certain levels (Muys et al., 2019). In this study, the heavy metal contents of both *A. platensis* and *A. fusiformis* cultivated in the six- and eight-fold diluted ADE were all below the levels specified in the safety standards of the United Nations World Health

Organization and Food and Agriculture Organization, and by the European Union (Al-Dhabi, 2013; Muys et al., 2019). (see appendex 1). The contents of Al, Cr, Mn and Se in all of the samples tested were within the daily intake level for humans (Al-Dhabi, 2013; Anderson, 1997; Cuciureanu et al., 2000; Kipp et al., 2015).

3.8 Nutrient removal efficiency

At the end of the experiment, in *A. platensis*, the PO_4^{3-}-P removal rate was almost 100% in the eight-fold diluted ADE and 95.3% in the six-fold diluted ADE (Fig.2-5a), being significantly higher than that in the standard medium (80.2%). In *A. fusiformis*, the PO_4^{3-}-P removal rates were much higher in both the six- (93%) and eight-fold diluted ADE (97%) than in the standard medium (85%) (Fig. 2-5b).

The NH_4^+-N removal rates were almost 100% for *A. platensis* reared in six- or eight-fold diluted ADE, while those for *A. fusiformis* were 92.0% and 97.2% in six- and eight-fold diluted ADE, respectively (Fig. 2-5c, d). At the end of the experiment, the pHs of all culture media were > 8.5. Because NH_4^+ can be volatile from culture media with pH > 8 (Escudero et al., 2014) such NH_4^+-N removal might include volatilization loss during the cultivation.

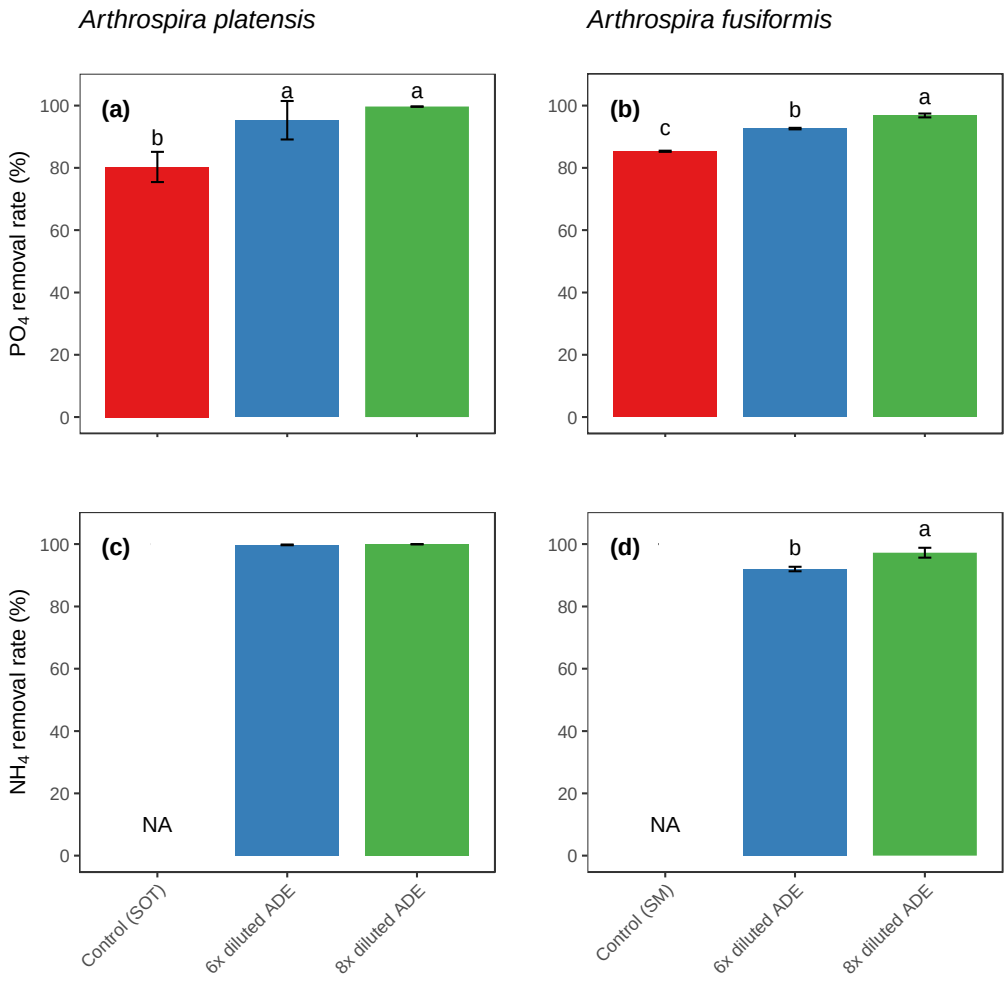

Fig. 2-5. Average removal rates of PO_4^{3-}-P (a, b) and NH_4^+-N (c, d) from the six- and eight-fold diluted ADE of water hyacinth (*E. crassipes*) and SOT or *Spirulina* medium (SM) by *A. platensis* (a, c), and *A. fusiformis* (b, d). Columns and error bars represent the average and standard deviations of triplicates. Different letters above columns indicate significant differences by the Tukey-Kremer *post-hoc* test at $p < 0.05$. 'NA' denotes the absence of that component from the medium.

4. Conclusion

The cultivation of two species of *Arthrospira* in an inexpensive medium derived from water hyacinth, which is otherwise considered as a nuisance, resulted in the production of more carbohydrates, dietary fiber, and β-glucan compared to the algae cultivated in the standard PO_4^{3-}-P-replete media. This finding would open up the possibility of mass-culturing *Arthrospira* species with the ADE of water hyacinth, which can be a potential economic source for producing valuable biomolecules such as β-glucans and also removing nutrients from the ADE, thus benefiting both environment and economy. Further studies are needed to determine the effects of various culture conditions that influence β-glucans production and nutrient removal.

CHAPTER 3:

"A comparison of major fatty acid compositions and pigment contents of the two *Arthrospira* species culturing in anaerobic digestion effluent of water hyacinth"

1. Introduction

The demand for functional food is surging worldwide, reflecting the developing awareness of the role of food in maintaining good health. It is crucial to ensure that diets are rich in bioactive properties to help prevent and even treat chronic diseases (Fithriani and Sinurat, 2019). According to the Functional Food Center (FFC), "Functional food" is a natural or processed food containing biologically active compounds in specific, non-toxic amounts. They provide clinically proven health benefits, promote optimal health, reduce the risk of chronic/viral diseases, and manage their symptoms (Gur et al., 2018). Recently, research involving functional foods has gained attention, and developing this kind of product has strongly influenced the global food market. Based on a market research company report in 2022, the global functional food market will be worth $586.1 billion by 2030, growing at 8.5% annually from 2022 (Grand View Research, 2022). Therefore, exploring new functional food sources that are sustainable and eco-friendly is crucial. One such alternative is microalgae biomass, which has been identified as a promising source of bioactive compounds for producing functional food (Bortolini et al., 2022).

Arthrospira is a genus of cyanobacteria that are filamentous, photosynthetic, and edible. It was previously classified as *Spirulina* (Nowicka-Krawczyk et al., 2019). This microalga has a high potential for use as functional foods, as it contains a large number of various bioactive compounds such as proteins, carbohydrates, pigments, minerals, vitamins, and antioxidants (Markou et al., 2021). *Arthrospira* also can produce essential polyunsaturated fatty acids (PUFAs), such as γ-linolenic acids and linoleic acid (Cohen and Vonshak, 1991; Golmakani et al., 2012). PUFAs are necessary for various cellular activities in human body, such as cell signaling, structure integrity, cell membrane fluidity, regulation of blood pressure, glucose level, nervous system function, inflammatory reactions, and hematic clotting (Kapoor et al., 2021). These essential fatty acids are used as a nutrient to treat non-alcoholic fatty liver (Lee

et al., 2017), and in specific diets for lipid metabolism disorders (Li et al., 2019). Among the different kinds of PUFAs, ω-3 and ω-6 fatty acids are crucial, as they cannot be produced inside the human body and must be consumed through diets (Calder, 2017). Foods containing ω-6 fatty acids, such as γ-linolenic acid, have been shown to provide cardiometabolic benefits in blood lipids and glycemia markers (Wu et al., 2019). Furthermore, γ-linolenic acids can help prevent diabetes, obesity, atherosclerosis, and inflammatory-autoimmune diseases (Simopoulos, 2016).

On the other hand, phycocyanin and carotenoids are another type of bioactive pigment compounds found in *Arthrospira* that are used as antioxidant (Romay et al., 2003), anti-cancer, anti-inflamatory, antiviral and as natural dyes in the food industries (Dewi et al., 2018). Phycocyanin, which is a blue colorant, can be an excellent alternative to commonly used artificial dyes as there are limited natural sources of blue pigments (Tavanandi and Raghavarao, 2020). This colorant is often used to make attractive infant foods such as candies, ice creams, desserts, yogurts, and chewing gums (da Silva Faresin et al., 2022). Additionally, due to its fluorescent nature, cyanobacterial phycocyanin is vital for molecular and pharmaceutical diagnostics as a phycofluoro probe (Li Sun et al., 2003). Recently, a market research report predicted that the global phycocyanin market will grow at a rate of 9.6% annually from 2021 and will be worth $409.8 million by 2030 (Park et al., 2022).

Although *Arthrospira* has many commercial uses, its high cultivation cost has made it difficult to be widely available. One way to minimize the cost of cultivating this alga is to increase the production of the most desired valuble products. Since *Arthrospira's* metabolism is significantly influenced by environmental factors such as temperature, light, pH, salinity, and nutrient availability (Papadopoulos et al., 2022), optimizing these factors can lead to an enhancement of a specific product, such as lipids or pigments. Another promising solution to cut down on cultivation expenses is to use wastewater as microalgae cultivation media (Wang

et al., 2016). Some previous studies demonstrated that using different types of wastewaters, such as dairy and carbon rich wastewater (Pereira et al., 2019; Shayesteh et al., 2023), can result in an elevated amount of essential fatty acids or phycocyanin in cultivated *Arthrospira*. Furthermore, it is vital to continue exploring new algal strains for specific type of wastewater (Hena et al., 2018), and developing the most efficient and cost-effective cultivation method to make *Arthrospira* products more accessible to consumers.

In this chapter, two species of *Arthrospira* were cultivated using anaerobic digestion effluent (ADE) obtained from water hyacinth and the standard algal media, and the accumulation of essential fatty acids and phycocyanin in the algal cells were determined, to evaluate the ADE as algal culture medium for mass-culturing Arthrospira and producing the bioactive compounds, i.e. PUFAs and pigments, through sustainable utilization of overgrown water hyacinth as described in chapter 1.

2. Materials and methods

2.1 Reagents and chemicals

FAME standards, chloroform, methanol were obtained from Sigma Aldrich (Tokyo, Japan). Methylation catalysts and purification kits were obtained from NACALAI TESQUE, INC. (Kyoto, Japan). The chemicals used in all experiments were analytical grade reagents from Wako, Japan.

2.2 Preparation of anaerobic digestion effluent

Anaerobic digestion effluent (ADE) used for this study was obtained from an over growing invasive macrophyte, *Eichhornia crassipes*, collected from Lake Ibanei (35°11'22.7" N 136°08'25.1" E), Shiga Prefecture, Japan. This macrophyte is commonly known as water hyacinth. The preparation of the ADE was followed as Chapter 2.

2.3 Microalgal strains and pre-culture conditions

Arthrospira platensis (NIES-39) and *Arthrospira fusiformis* (UTEX LB2721), were obtained from the National Institute for Environmental Studies (NIES) in Japan and The University of Texas, USA, respectively. The *Arthrospira platensis* was grown in 100-mL Erlenmeyer flasks with Spirulina Ogawa Terui (SOT) medium (Table 3-1) under the following conditions: a shaker with a speed of 80 rpm, a light intensity of 250 µmol/m^2/s, and a 12-hour light/dark cycle at 22°C in a growth cabinet (MLR-350, Sanyo, Osaka, Japan). However, the *A. fusiformis* was grown in 100-mL Erlenmeyer flasks with Spirulina medium (Table 3-1) at 25°C under the following conditions: a shaker with a speed of 75 rpm, a light intensity of 250 µmol/m^2/s, and a 12-hour light/dark cycle. Overall, these conditions were selected to ensure each microalga's optimal growth for obtaining the accurate results.

2.4 Chemical characterization of ADE

The undiluted ADE derived from water hyacinth was capable of entirely inhibiting the growth of *Arthrospira* as described in Chapter 2. However, since the current study aims to produce a larger biomass enriched with valuable bioactive compounds like essential fatty acids and pigments, 8-fold diluted ADE has been selected in all experiments in this study. The chemical composition of the 8-fold ADE used in all the experiments was determined by diluting it eight times with sterile distilled water and then filtering through a 0.2-µm pore size membrane filter (model no. A020A142C, ADVANTEC, Tokyo, Japan). The concentrations of various elements such as phosphate phosphorus (PO_4^{3-}-P), ammonium nitrogen (NH_4^+-N), nitrate nitrogen (NO_3^--N), nitrite nitrogen (NO_2^--N), and metal ions like Na^+, K^+, Ca^{2+}, and Mg^{2+} were measured and shown in Table 3-1.

The nutrient concentrations were determined using an AACS-II continuous flow system autoanalyzer manufactured by Bran + Luebbe, Norderstedt, Germany. NH_4^+-N was analyzed

using the Berthelot reaction method, according to Koroleff (1970). NO_2^--N and NO_3^--N concentrations were determined with a cadmium reduction column where nitrate was quantitatively reduced to nitrite (Armstrong et al., 1967). PO_4^{3-}-P concentration was determined by the phosphomolybdenum blue method (Murphy and Riley, 1962). The dissolved organic carbon (DOC) concentration was estimated by a Total Organic Carbon analyzer (TOC-5000A) from SHIMADZU, Japan. The concentrations of metals in ADE were measured using inductively coupled plasma-atomic emission spectroscopy (NexION 1000 ICP-MS, Waltham, MA, USA). Before the analysis, 50 µL of nitric acid (61%, Wako, Osaka, Japan) was added to 1 mL of the sample solution to prevent metal adsorption, as suggested by Clesceri (1999).

2.5 Cultivation of *Arthrospira* in ADE

Each species of the two *Arthrospira* species cultivated to the exponential phase of growth was filtered by a 20-µm nylon mesh, washed three times, and resuspended in 0.8 mL of autoclaved distilled water. Then, the algal suspension was transferred to a 100-mL flask containing 100 mL of culture medium, and, finally, the initial culture density was adjusted to 0.1 optical density (OD) at 730 nm wavelength. To assess the growth performance of the microalgae, 8-fold diluted ADE solution was filtered through a 0.2-µm pore-size membrane filter to eliminate particles, and then used it as the algal culture medium. SOT and *Spirulina* medium were also prepared as standard control media. After adding the same amount of $NaHCO_3$ (0.2 mol/L) as in the control medium, pH of each treatment was adjusted to 8.5 with 1 M NaOH. All experimental cultures were incubated in triplicate under the same conditions as the precultures. Optical density was measured daily at 730 nm using a spectrophotometer (SP-300, OPTIMA, Tokyo, Japan) for six days from the start of the experiment.

2.6 Analytical methods

2.6.1 Proximate biochemical compositions

At the end of the cultivation period, the cultured algae were filtered with a precombusted and preweighed GF/C glass fiber filter (Cytiva, model no. 1822-047, Tokyo, Japan) and washed with sterile distilled water to remove the salts from the culture medium for measuring dry mass of the algae tested. Then, the filter was dried in an oven (DV-600, YAMATO, Tokyo, Japan) at 60 °C until constant weight was acheived (*ca*. 24 h). The dry biomass of the algae was determined using a microbalance (AW220, SHIMADZU, Tokyo, Japan) at the precision of 0.1 mg. On the other hand, for biochemical analysis, the algal cells grown for six days cultivation period were collected with a 20-µm nylon mesh, washed three times with sterile distilled water, and then lyophilized in a freeze-drying system (Taitec VD-800F, Taitec Co., Saitama, Japan) and stored at −80 °C until further analysis, except for determining total protein content, for which fresh algal cells were used.

The total protein content was obtained from the algal cells by extracting it with 0.5 M NaOH, and its measurement was done using the method developed by Lowry et al. (1951). Precisely, a fresh algal suspension of 1.5 mL was centrifuged at 8,000 × g for 15 min. The supernatant was discarded, and the remaining pellet was mixed with 1.5 mL of 0.5 M NaOH. The mixture was incubated at 100 °C for 20 min. A 100 µL aliquot from the resulting solution was added to a mixture of 100 µL of 5% sodium dodecyl sulfate and 1 mL of 2% Na_2CO_3 in 0.1 M NaOH. After it had been left for 15 min, 100 µL of 1 M Folin–Ciocalteu reagent was added, and the solution was left for 30 min in darkness. Finally, the OD of the final solution was measured at 750 nm using a spectrophotometer (SP-300). Bovine serum albumin was applied as a standard.

On the other hand, a modified sulfo-vanillin method was used to determine the total amount of total crude lipids in a sample (Izard and Limberger, 2003). First, total lipids were

extracted from 1 g of freeze-dried algae using 2:1:0.2 chloroform: methanol: MilliQ water (v:v:v). Then, 20 µL of the extracted lipids (approximately 150–500 mg/L lipid) was incubated at 80°C until the chloroform evaporated. After 0.4 mL of 96% sulfuric acid was added, the mixture was boiled in a water bath for 10 min. After cooling for 15 min at room temperature, 1.0 mL of phosphoric acid/vanillin solution was added, and the mixture was incubated at 37°C for 15 min. Finally, the OD was measured at 530 nm using a spectrophotometer. Corn oil was used as the standard (Markou et al., 2021).

2.6.2 Preparation of Fatty Acid Methyl Esters (FAMEs)

Lipid extraction procedure was made to determine the total crude lipid content of *Arthrospira* (see section 2.6.1). Then, the lipid phase obtained from the extraction was concentrated using the flow of nitrogen gas (*ca*. 3 h) and used to analyze fatty acid composition. In this study, commercial kits for methylation and purification of fatty acid methyl esters (fatty acid methylation kit and methylated fatty acid purification kit, Nacalai tesque, Kyoto) were used. The fatty acids were converted to fatty acids methyl esters (FAME) by the method developed by manufacturer. Briefly, a 100 µL of extracted lipid mixed with 900 µL of methanol, and then added 0.25 ml of reagent A (52: 48 mixtures of toluene and methanol) and 0.25 ml of reagent B (7% methanol solution of undisclosed chemicals) to the vial, and mixed it thoroughly. After the mixture was kept at 37 °C for 5 min, 0.25 ml of reagent C (30 wt. % boron trifluoride methanol solution) was added to the vial, mixed it for 30 sec using a voltex mixer, and then kept it at 37 °C for 20 min. Subsequently, 0.5 ml of extraction reagent and MilliQ water were added to the vial, mixed it for 3 min with a voltex mixer at 4000 rpm, and then incubated it for 3 min at room temperature. After that, the organic layer was transferred to a new vial.

To purify the methylated fatty acid (fatty acid methylated esters; FAME), silica gel cartridges of the kit were used. Prior to use, the cartridge was thoroughly cleaned with 3 ml of n-hexane. Subsequently, the layer containing FAME in n-hexane was introduced into the

cartridge. The cartridge column was treated with 3 ml of washing solvent (n-hexane) and 3 ml of eluent solution (2% ethyl acetate in n-hexane). The resulting eluted liquid, containing FAMEs, was collected and later diluted to a suitable concentration for a gas-chromatography (GC) analysis.

2.6.3 Instrumental analysis of FAMEs

Gas chromatography-mass spectrometry (GC-MS) was utilized to measure FAMEs. The Agilent GC system 7890B/ JEOL MS was used in EI-mass mode and equipped with a CP-Sil 88 column (100 m x 0.25 mm; w 0.25, Agilent Technologies). Helium gas was used as the carrier gas, with a flow rate of 1.2 mL/min. The GC column was injected splitless at 250 °C. The oven temperature was initially set at 50 °C for 1 min, then it was increased from 50 °C to 175 °C at 25 °C/min, further increased from 175 °C to 230 °C at 4 °C/min, and held at 230 °C for 10 min. A standard mixture of FAME (Supelco 37-compound FAME Mix, Sigma, USA) was analyzed using the same method for characterizing and quantifying FAME using retention times and peak areas. Finally, all methylated fatty acids were analyzed by GC/MS, and their mass spectrum was checked for identification. The fatty acid concentrations were calculated using the specific fatty acid concentrations provided by the manufacturer.

2.7 Pigment analysis

2.7.1 Chlorophyll *a* analysis

To determine chlorophyll *a* (Chl *a*) concentration in the samples tested, *ca*. 25 mg of freeze-dried algal powder was mixed with 10 mL of 90% acetone and kept in the dark at 4℃ overnight. After that, it was filtered through a GF/C filter (Cytiva, model no. 1822-047, Tokyo, Japan) and centrifuged at 3000 rpm for 15 min. The supernatant liquid was collected, and the absorbance of the extract liquid was measured using a spectrophotometer (V-530 UV/VIS, Jasco, Tokyo, Japan) at 630, 645, 663, and 750 nm, and the Chl *a* concentration was determined using the UNESCO formula (Han et al., 2018):

Chl *a* (μg/ L) = (11.64E_{663} − 2.16E_{645} + 0.10E_{630}) × *b*/*VL*,

where, E_{663} is value obtained by subtracting the absorbance at a wavelength of 750 nm from the absorbance at a wavelength of 663 nm; E_{645} is value obtained by subtracting the absorbance at a wavelength of 750 nm from the absorbance at a wavelength of 645 nm, E_{630} is value obtained by subtracting the absorbance at a wavelength of 750 nm from the absorbance at a wavelength of 630 nm; *V* is sample volume filtered (L), and *b* is total amount of acetone (mL); *L* is length of absorption cell of spectrophotometer (cm).

2.7.2 Phycocyanin analysis

To determine phycocyanin concentration, 25 mg of freeze-dried algal powder was mixed with 5 mL of 10 mM phosphoric acid (Wako, Tokyo, Japan). The mixture was then kept in the dark at 4 ℃ for 24 hours. After that, the extracted liquid was filtered through a GF/C filter (GF/C, Whatman, Maidstone, England). The absorbance of the filtrate was measured at 650 nm (A_{650}), 620 nm (A_{620}), and 565 nm (A_{565}) using a UV-visible spectrometer (V-530, Nihonbunko, Tokyo, Japan). Then, phycocyanin concentration (PC) was calculated using these absorbance values and the following equation (Saito et al., 2014):

PC (μg/L) = (0.918A_{620} − 0.133A_{650} − 0.00190A_{565}) × *cL* / *V*;

Where, *c* is volume of phosphoric acid (mL), *V* is volume of phycocyanin (L), and *L* is length of the light path (cm).

2.8 Statistical analyses

The effects of differences between culture media, including diluted ADE, on biomass production, growth rate, fatty acid compositions, and pigment contents were tested by one-way analysis of variance (ANOVA). All statistical analyses were performed using R-studio ver. 4.0.3 (2020).

3. Results and discussion

3.1 Chemical composition of ADE and algal growth

In this study, 8-fold diluted ADE of water hyacinth contained enough essential nutrients for the growth of *Arthrospira* spp. As shown in Table 3-1, PO_4^{3-}-P concentration of 8-fold diluted ADE was 1.0 mg/L, which is lower than the PO_4^{3-}-P concentration of the standard SOT medium (89 mg/L), being a P-limited medium. However, NH_4^+-N concentration of the ADE was 43.21 mg/L and still sufficient for *Arthrospira* growth, being even ten times lower than the NO_3^--N concentrations of the standard media (412 mg/L). Depraetere et al. (2013) reported that N concentration of > 35 mg/L is not a limiting factor for *Arthrospira* growth in 10-day cultivation. Additionally, concentrations of K^+, Ca^{2+}, and Mg^{2+} in the ADE were similar to those in the standard media, and Na^+ concentration was 4627 mg/L after adding 0.2 mol/L $NaHCO_3$ (see Table 3-1). The growth curves of both *A. platensis* and *A. fusiformis* in 8-fold diluted ADE during 6 days cultivation were almost the same as those in the standard SOT and *Spirulina* medium (Fig.3-1).

Table 3-1 Chemical compositions in the 8-fold diluted anaerobic digestion effluent (ADE) from water hyacinth (*Eichhonia crassipes*) and the standard algal media (SOT and *Spirulina* medium). Dashes represent components that are not included in the medium. DOC, dissolved organic carbon; NH_4^+-N, ammonium nitrogen; NO_3^--N, nitrate nitrogen; NO_2^--N, nitrite nitrogen; TP, total phosphorus; PO_4^{3-}-P, phosphate phosphorus.

Component	8x diluted ADE	SOT medium	*Spirulina* medium
		(mg/L)	
DOC	10 ± 21	–	–
NH_4^+-N	43.21± 6.1	–	–
NO_3^--N	0.2 ± 0.03	411.8	411.8
NO_2^--N	0.3 ± 0.05	–	–
PO_4^{3-}-P	1 ± 0.3	89.0	89.0
Na	30.12* ± 5.5	5677.5	6542.7
K	278 ± 12.3	671.5	671.5
Ca	10 ± 6.4	10.9	10.9
Mg	23 ± 3.3	19.7	19.7

*After addition of 0.2 mol /L $NaHCO_3$, the Na concentration in the ADE was 4627 mg/L.

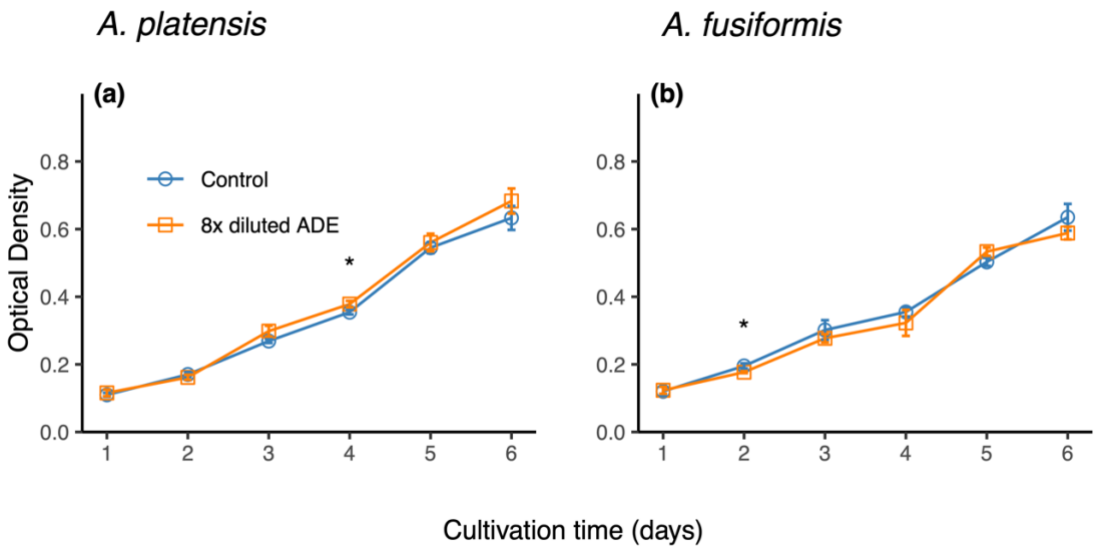

Fig. 3-1. Growth curves of *Arthrospira platensis* (a) and *A. fusiformis* (b) in 8× diluted anaerobic digestion effluent (ADE) of water hyacinth (*Eichhornia crassipes*) and SOT (a) or *Spirulina* medium (b) as a control. Symbols and vertical bars represent average and standard deviation of triplicates. Asterisks denote significant differences among treatments by analysis of variance at $p < 0.05$.

3.2 Biomass production and proximate biochemical composition

The results of the biomass productions of *A. platensis* and *A. fusiformis* after the 6-day experimental period are depicted in Fig. 3-2. No significant differences in optical densities were observed between the 8-fold dilution treatment and the standard algal media during the growth monitoring experiment (Fig. 3-1). For *A. platensis*, the biomass productions in the eight-fold diluted ADE were slightly higher than in the standard medium, but the difference was only about 0.63%. Similarly, for *A. fusiformis*, the biomass production in the eight-fold diluted ADE were similar and not significantly different from that in the standard medium (Fig. 3-2). These results suggest that the low PO_4^{3-}-P concentration of the medium, even at eight-fold dilution (1.0 ± 0.3 mg P/L), can support the same algal biomass production (ca. 620 mg/L) as that in the standard medium during the experimental timescale. The biomass productions in

the two species were quite similar, but that in *A. platensis* was a bit larger than that in *A. fusiformis*.

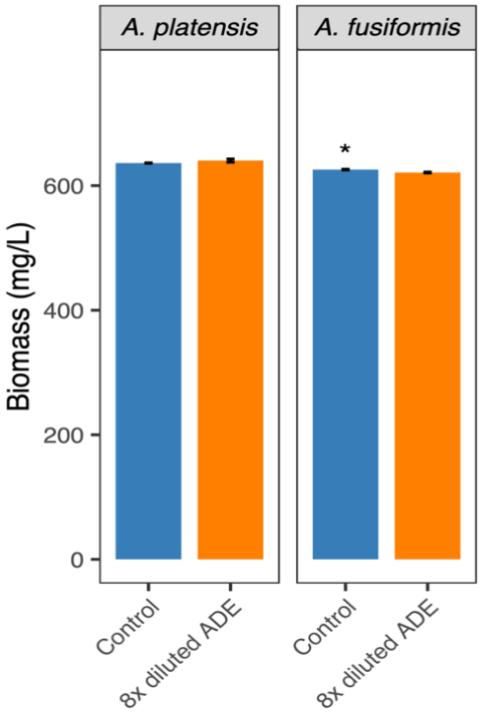

Fig. 3-2. Biomass production of *A. platensis* and *A. fusiformis* cultivated in 8× diluted ADE of water hyacinth (*Eichhornia crassipes*) and SOT or *Spirulina* medium (SM) as a control during a 6-day experiment. Columns and error bars represent the average and standard deviations of triplicates. Asterisk denote significant difference between the treatments with ANOVA at $p<0.05$.

In *A. platensis*, total crude protein content was significantly lower (23.51%) in 8-fold diluted ADE than that in the standard medium (40.30%). Similarly, in *A. fusiformis*, the total protein contents were also significantly lower in 8-fold diluted ADE (25.45%) than that in the standard medium (35.22%) (Table 3-2). These differences may be attributed to P limitation in the diluted ADE, though the biomass productions were almost identical.

Markou (2012) described that P plays a crucial role in producing adenosine triphosphate (ATP), a molecule that facilitates the transfer of cellular energy. In case of a P shortage,

microorganisms' energy production strategy is impacted, leading to a decline in the synthesis of energy-consuming proteins. This results in the accumulation of carbohydrates and lipids, which explains the absence of any significant difference in biomass production but a noticeable difference in the protein content of the treatments.

In *A. platensis*, total lipid content was significantly higher (7.55%) in 8-fold diluted ADE than that in the standard medium (7.05%). Likewise, in *A. fusiformis*, the total lipid content was considerably higher at 6.90% in 8-fold diluted ADE compared to the standard medium at 5.06%, as shown in Table 3-2. However, it has been reported that P limitation can significantly contribute to lipid accumulation in *A. platensis* algal cells, as observed by Baunillo et al. (2012). Kamalanathan et al. (2015) showed that the green alga, *Chlamydomonas reinhardtii*, can accumulate more lipids under P limited conditions. Whereas, *Anabaena flos-aquae* was found to accumulate more lipids under P limitation (Dean et al. 2008b). It's important to note that the response to P deficiency can differ among species because of variations in their P demand.

Table 3-2 Biochemical compositions as percentages of algal dry mass in total lipids, and proteins in *Arthrospira platensis* and *Arthrospira fusiformis* cultivated in batch mode with the 8-fold diluted anaerobic digestion effluent (ADE) of water hyacinth and the standard control media, and results of analysis of variance (ANOVA). The figures represent average ± standard deviation in triplicates.

Algal species	**Culture medium**	**Lipid content (%)**	**Protein content (%)**
A. platensis	8x diluted ADE	7.55 ± 0.34	23.51± 1.26
	SOT medium	7.05 ± 0.28	40.30 ± 1.90
A. fusiformis	8x diluted ADE	6.90 ± 0.08	25.45 ± 0.25
	Spirulina medium	5.06 ± 2.90	35.22± 0.66

3.3 Fatty acid profile and relative composition

The CP-Sil 88 capillary column gave a clear separation of long chain PUFA from other short chain fatty acid (see Fig. 3-3, Fig.3-4, and Fig.3-5; Table 3-3). Relative fatty acid compositions in *A. platensis* and *A. fusiformis* cells cultivated in 8-fold diluted ADE from water hyacinth and control media are illustrated in Fig. 3-6. The composition of the major fatty acid class in *A. platensis* cultivated in 8-fold diluted ADE was almost the same as that in the SOT medium. PUFA accounted for 45 - 46%, followed by saturated fatty acids (SFAs) at 43%, and monounsaturated fatty acids (MUFAs) at 12%. This result on SFA and PUFA agree with earlier studies. Diraman et al. (2009) revealed that *Spirulina platensis* (= *A. platensis*) contained both SFA and PUFA, with varying percentages between 33.7% and 66.8% and 28.2% and 47.8%, respectively. However, Ljubic et al. (2018) reported that *A. platensis* contained PUFA at 42-45% of total fatty acids when it was cultivated in Zarrouk medium supplemented with 25% of industrial wastewater.

A. fusiformis cells cultivated in 8-fold diluted ADE accumulated higher levels of health-beneficial PUFA compared to that in the control. The compositions of PUFA were 55% and 0.9%, respectively. On the contrary, *A. fusiformis* cells cultivated in 8-fold diluted ADE contained less amounts of SFA (41%) than that in the control (81%). In *A. fusiformis* cultivated in 8-fold diluted ADE, MUFAs composition was quite low, just 2% of total lipid, compared to control medium (18%) (Fig. 3-6).

Table 3-3 Name of the fatty acids of 37 component FAMEs standard mixture. 'n' and 't' represents the position of the carbon-carbon double bonds (C=C) in fatty acid chain and trans fatty acid, respectively.

Peak No.	Fatty acid	Name	Peak No.	Fatty acid	Name
1.	C4:0	Butyric acid	20.	C18:2n6t	Linolelaidic acid
2.	C6:0	Caproic acid	21.	C18:3n6	γ-linolenic acid (ω-6)
3.	C8:0	Caprylic acid	22.	C18:3n3	α-linolenic acid (ω-3)
4.	C10:0	Capric acid	23.	C20:0	Arachidic acid
5.	C11:0	Undecanoic acid	24.	C20:1n9	cis-11-Eicosenoic acid
6.	C12:0	Lauric acid	25.	C20:2	cis-11,14-Eicosadienoic acid
7.	C13:0	Tridecanoic acid	26.	C20:3n6	cis-8,11,14-Eicosatrienoic acid
8.	C14:0	Myristic acid	27.	C20:3n3	cis-11,14,17-Eicosatrienoic acid
9.	C14:1	Myristoleic acid	28.	C20:4n6	Arachidonic acid
10.	C15:0	Pentadecenoic acid	29.	C20:5n3	cis-5,8,11,14,17-Eicosapentaenoic acid
11.	C15:1	cis-10-Pentadecenoic acid	30.	C21:0	Henicosanoic acid
12.	C16:0	Palmitic acid	31.	C22:0	Behenic acid
13.	C16:1	Palmitoleic acid	32.	C22:1n9	Erucic acid
14.	C17:0	Heptadecanoic acid	33.	C22:2	cis-13,16-Docosadienoic acid
15.	C17:0	cis-10-Heptadecenoic acid	34.	C22:6n3	cis-4,7,10,13,16,19-Docosahexaenoic acid
16.	C18:0	Stearic acid	35.	C23:0	Tricosanoic acid
17.	C18:1n9c	Oleic acid	36.	C24:0	Lignoceric acid
18.	C18:1n9t	Elaidic acid	37.	C24:1n9	Nervonic acid
19.	C18:2n6c	Linoleic acid			

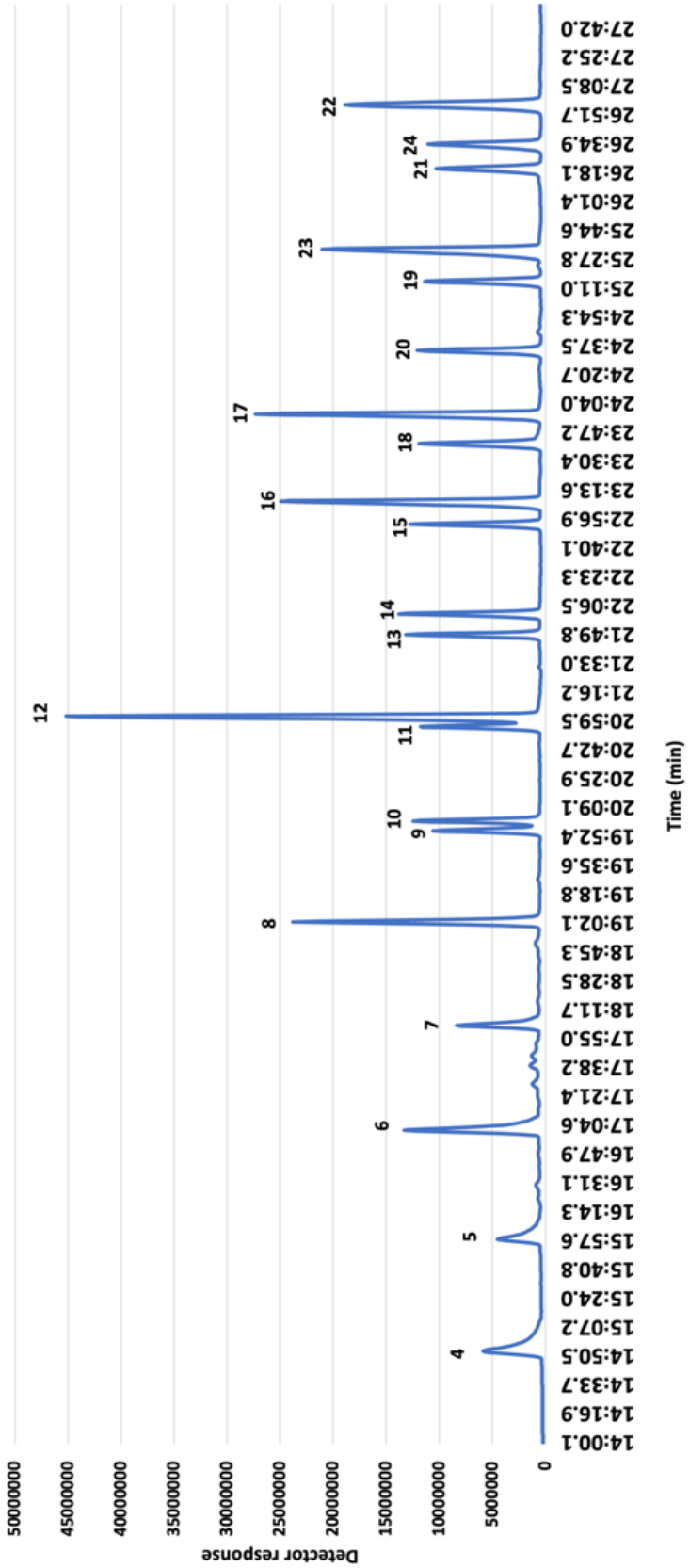

Fig. 3-3. GC/EI chromatogram of 37-component FAMEs standard mixture on a CP-Sil 88. Peak no indicate the different fatty acids.

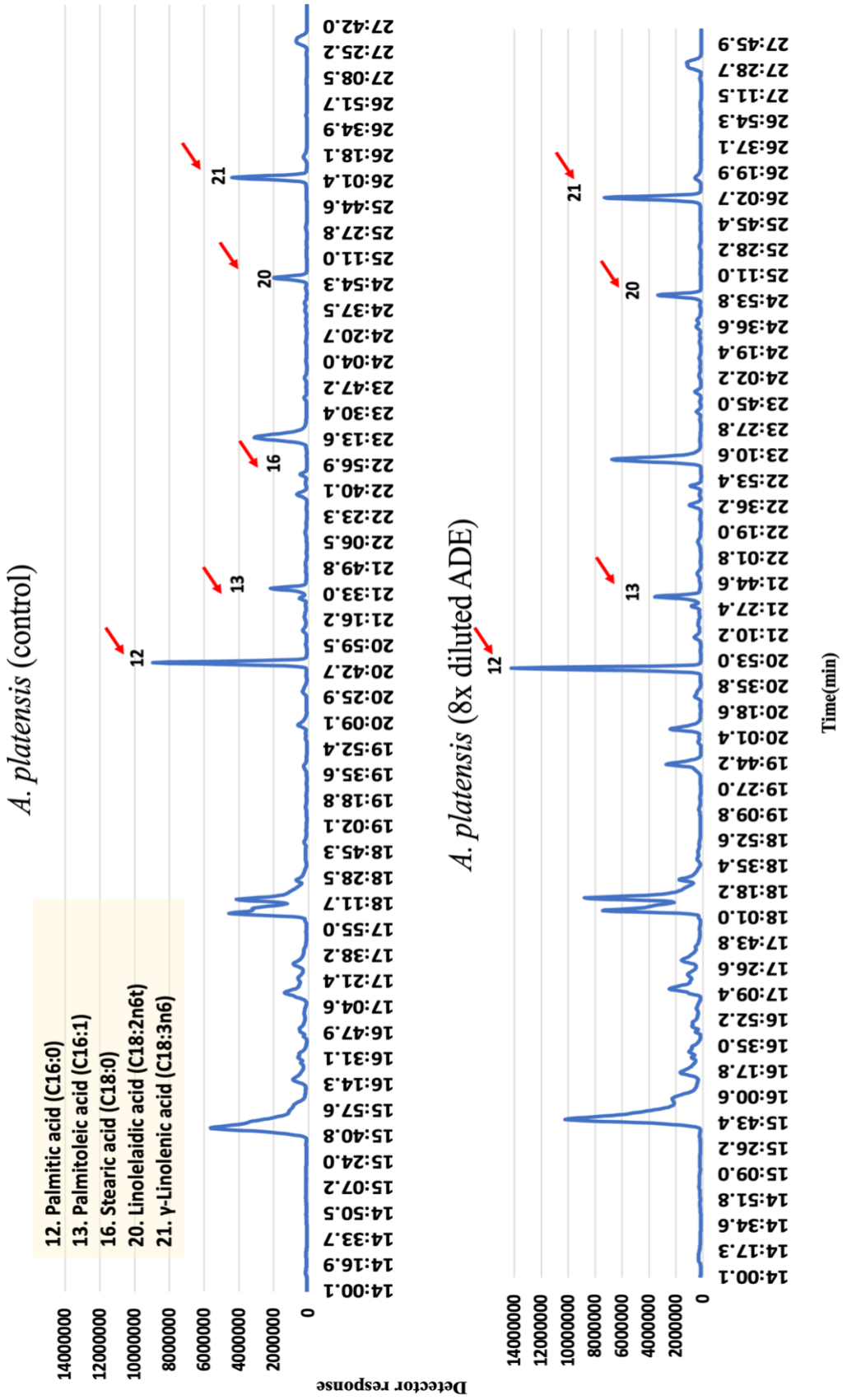

Fig. 3-4. GC/EI chromatogram of *A. platensis* cultivated in diluted anaerobic digestion effluent of water hyacinth and standard SOT medium on a CP-Sil 88 column. Peak no indicate the different fatty acids.

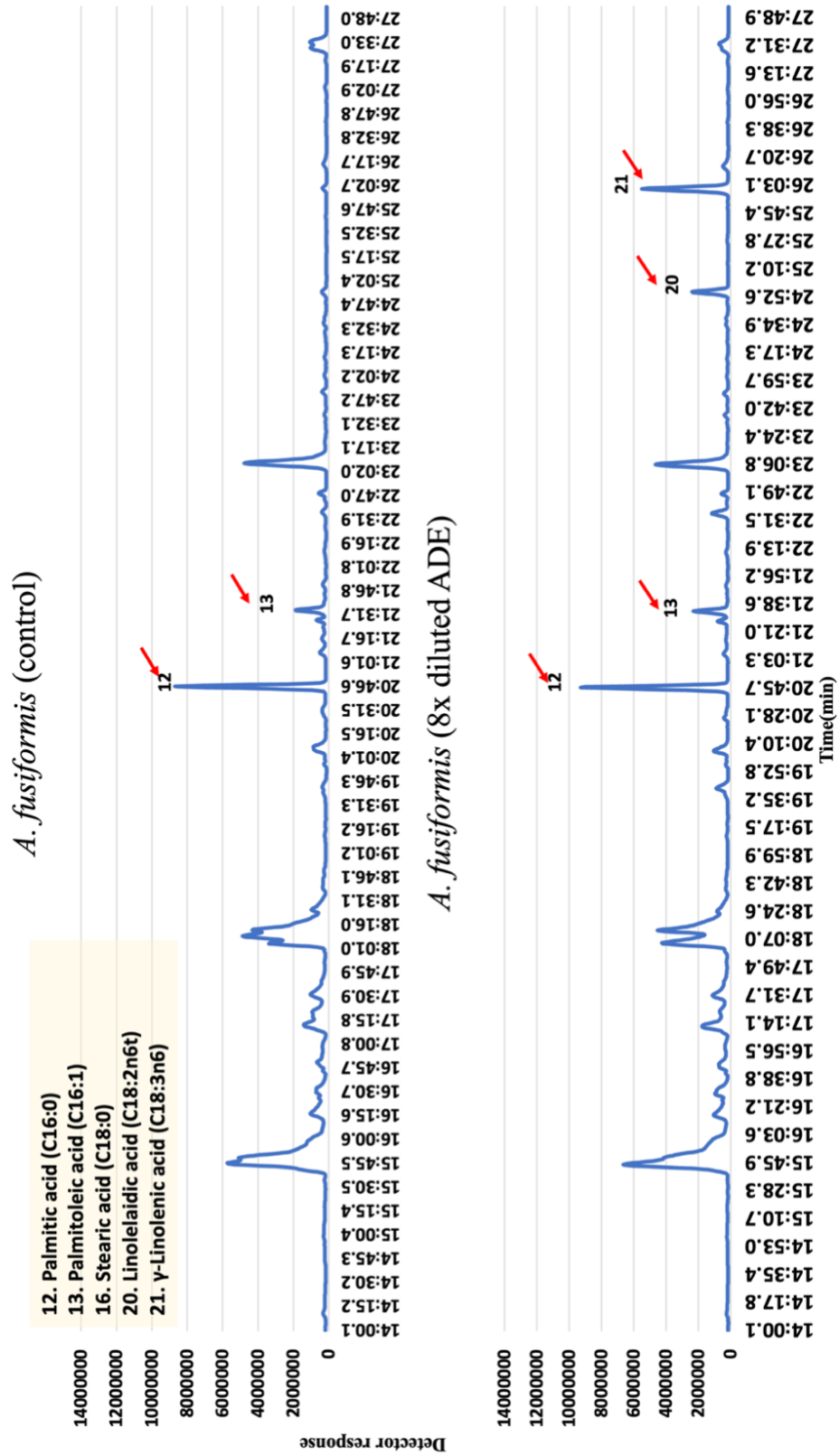

Fig. 3-5. GC/EI chromatogram of *A. fusiformis* cultivated in diluted anaerobic digestion effluent of water hyacinth and standard spirulina medium on a CP-Sil 88 column.

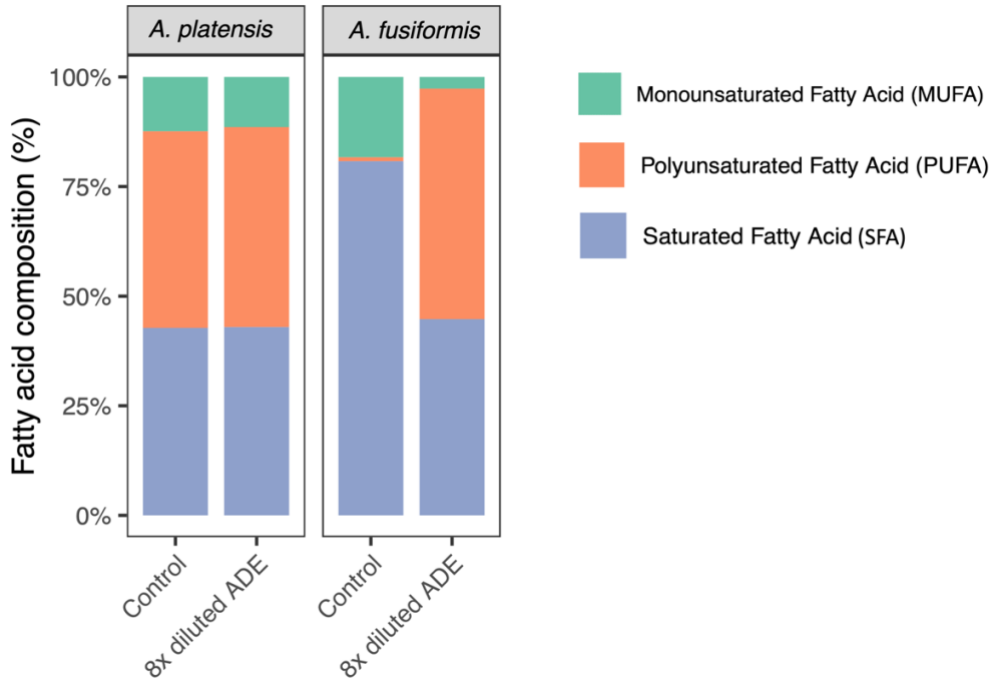

Fig. 3-6. Relative fatty acid composition (%) of *Arthrospira platensis* and *A. fusiformis* cultivated in 8× diluted ADE of water hyacinth (*E. crassipes*) and SOT or *Spirulina* medium (SM) as control during 6-day experimental period.

3.4 Concentration of major fatty acids

Fig. 3-7 shows the concentrations of major fatty acid classes of the two species of *Arthrospira* cultivated in 8-fold diluted ADE and standard control media (SOT or *Spirulina* medium). *A. platensis* grown in 8-fold diluted ADE contained all fatty acid classes at higher amounts than those in the SOT medium. On the other hand, in *A. fusiformis*, SFA contents were considerably higher in the 8-fold diluted ADE than those in the control medium, while MUFA contents were substantially lower in the 8-fold diluted ADE than those in the control medium (Fig. 3-7). *A. fusiformis* cultivated in *Spirulina* medium (control) contained no PUFAs.

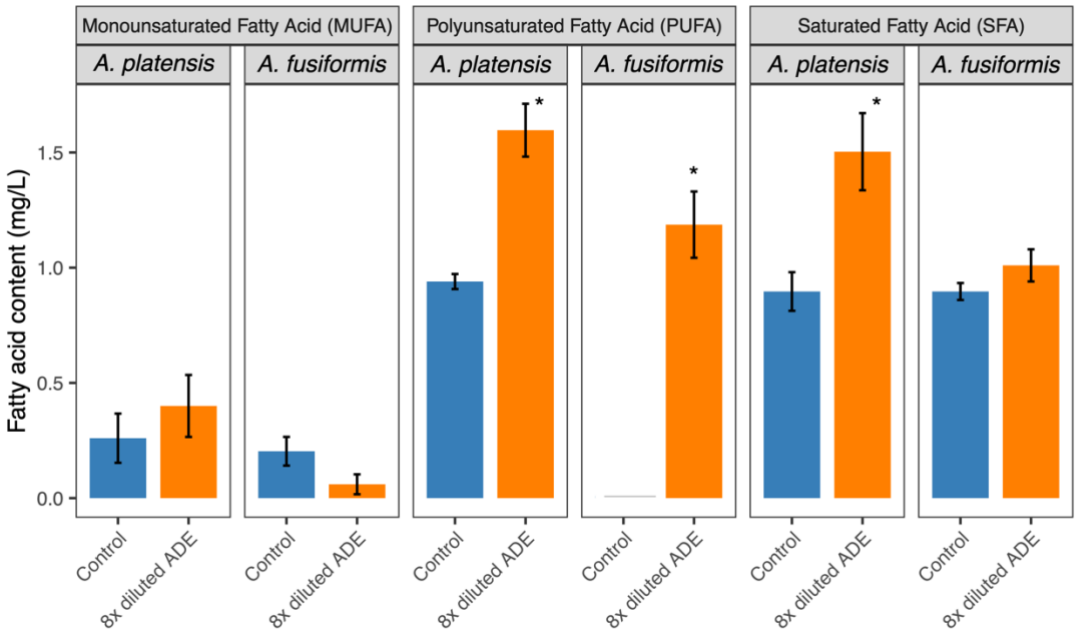

Fig. 3-7. Concentration of major fatty acid classes of *Arthrospira platensis* and *A. fusiformis* cultivated in 8× diluted ADE of water hyacinth (*E. crassipes*) and SOT or *Spirulina* medium as control during 6-day experimental period. Columns and error bars represent the average and standard deviations of triplicates. The concentration of fatty acid was calculated using the Supelco 37 FAMEs concentration provided by the manufacturer, and the fatty acid concentrations were measured per liter of solvent (mg/L).

Table 3-4 shows the individual fatty acid contents (per 100 g of algal dry mass) of *A. platensis* and *A. fusiformis* cells cultivated in 8-fold diluted ADE and control media. Palmitic acid (C16:0) as SFA, palmitoleic acid (C16:1) as MUFA, and linoleic acid (C18:2) and γ-linolenic acid (C18:3) as PUFA were found at higher amounts. Only trace amounts of stearic acid (C18:0) were detected in *A. platensis* grown using the SOT medium. Among these fatty acids, palmitic acid (C16:0) was found at the hightest concentration in all algal biomss tested. Both two *Arthrospira* species cultivated in 8-fold diluted ADE contained considerable higher amounts of palmitic acid (C16:0) than in control media (Table 3-4). Prates et al. (2018) reported that palmitic acid (C16:0) was the main component of the fatty acid profile of *Spirulina*

platensis (= *A. platensis*) . Palmitic acid has been known to be various applications in industries such as cosmetics, pharmaceuticals, and surfactants (Batista et al., 2017).

On the other hand, both *Arthrospira* species cultivated in 8-fold diluted ADE contained a considerably higher amount of γ-linolenic acid, with 1200 mg/100g in *A. platensis* and 880 mg/100g in *A. fusiformis*. However, Guldas et al. (2021) reported more higher amount of γ-linolenic acid, 1866.27 mg/ 100g of dry mass of *S. platensis*.

Furthermore, both *Arthrospira* species had different concentrations of MUFA, with only palmitoleic acid (C16:1) in all samples. Specifically, the *A. platensis* cultivated in 8-fold diluted ADE exhibited a higher concentration of palmitoleic acid (400 mg/100 g algal dry mass) when compared to the control media (260 mg/ 100 g algal dry mass). Meanwhile, in *A. fusiformis*, notable decrease in palmitoleic acid (C16:1) contents was found in 8-fold diluted ADE compared to those in the control, from 400 mg/100 g to 60 mg/100 g of dry mass (Table 3-4).

Table 3-4 Individual fatty acid contents (per 100 g of algal dry mass) in the two species of *Arthospira* biomass, cultivated in 8-fold diluted anaerobic digestion effluent (ADE) of water hyacinth and the standard control media. The figures represent average ± standard deviation in triplicates. 'n.d.' represents 'not detected' in the sample. The results of the fatty acid profile are expressed in mg of fatty acid per 100 g of dry algal sample.

Algal sp.	Culture medium	C16:0	C16:1	C18:0	C18:2	C18:3
		(mg/100 g of algal dry mass)				
A. platensis	8x diluted ADE	1500 ± 22.91	400 ±10	n.d.	400 ±13.23	1200 ±12.66
	SOT medium	940 ±13.23	260 ± 30.41	0.01±.02	250 ± 10	690 ±15.50
A. fusiformis	8x diluted ADE	1010 ± 15	60 ± 8.66	n.d.	310 ± 4.58	880 ± 15.50
	Spirulina medium	900 ± 16.80	200 ± 18.03	n.d.	n.d.	n.d.

C16:0 = Palmitic acid; C16:1 =Palmitoleic acid; C18:0 = Stearic acid; C18:2 = Linoleic acid; C18:3 = γ-linolenic acid (nomenclature by Sigma catalogue).

3.5 Chlorophyll *a* content

Chlorophyll *a* contents in *A. platensis* cells cultivated in both 8-fold diluted ADE and control media were considerably lower than those in control media, though the Chlorophyll *a* contents were slightly higher in *A. fusiformis* than those in *A. platensis*, being 7.2 mg/g and 6.5 mg/g of algal dry mass, respectively, reared in 8-fold diluted ADE (Fig. 3-8a). Papadopoulos et al. (2022) showed that *A. platensis* cultivated in undiluted brewery waste contained 6.19 ± 1.02 mg/g algal dry mass as Chlorophyll *a*. This is the similar Chlorophyll *a* content in this study. Roopnarain et al. (2014) has shown that P limitation can negatively affect chlorophyll synthesis for culturing *Isochrysis galbana*. Similarly, Fan et al. (2014) found that lower P content in *Chlorella pyrenoidosa* cells decreased chlorophyll *a* contents from 28 to 18 mg/g of algal dry mass during 7 days of cultivation period. Theodorou et al. (1991) reported that had a 67% reduction of chlorophyll *a* content of *Selenastrum minutum* cells cultured in P limited

medium compared those in P sufficient control medium. da Silva Ferreira and Sant'Anna (2017) explained that microalgae could not synthesize adenosine triphosphate (ATP) under P limited conditions, which might need any cellular processes including chlorophyll synthesis. In this study, 8-fold diluted ADE included lower P content than control media did. This could be the reason for the lower chlorophyll level in the cells cultured in the diluted ADE compared to those in control.

3.6 Phycocyanin content

A. platensis cells cultured in 8-fold diluted ADE contained significantly lower phycocyanin than those in the SOT medium (Fig. 3-8b). On the other hand, *A. fusiformis* cells cultured in the 8-fold diluted ADE contained significantly higher phycocyanin than those in the *Spirulina* medium. The maximum phycocyanin contents observed in this study was 17 mg/g DW in *A. fusiformis* reared with diluted ADE. This value is quite lower than that in *A. platensis* cultivated in brewery wastewater supplemented with 1 g/ L NaCl and 5 g/ L $NaHCO_3$, 22.35 ± 1.34 mg/g DW (Papadopoulos et al., 2022).

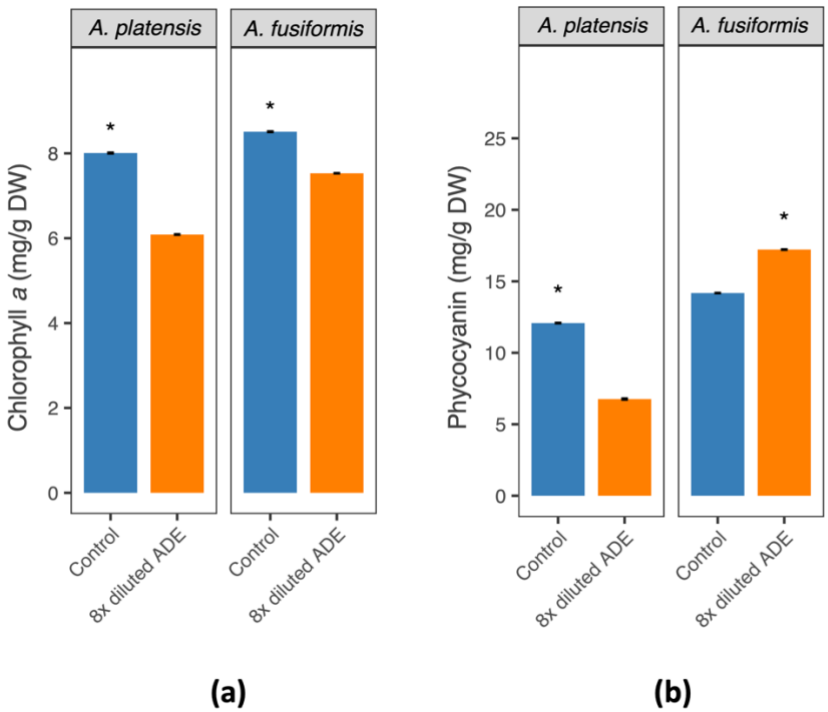

Fig. 3-8 Contents of chlorophyll *a* (a) and phycocyanin (b) of *Arthrospira platensis* and *A. fusiformis* cultivated in 8× diluted ADE of water hyacinth (*E. crassipes*) and SOT or *Spirulina* medium (SM) as control during 6-day experimental period. Columns and error bars represent the average and standard deviations in triplicates. Asterisks denote significantly different with analysis of variance at $p<0.05$.

Chapter 4:

General Discussion

Arthrospira has gained global recognition for its potential health benefits. It is rich in essential nutrients, including proteins, vitamins, and minerals, which make it a valuable ingredient in the production of health foods and therapeutic supplements (de la Jara et al., 2018). Its commercialization has increased significantly due to its potential therapeutic properties, and researchers continue to explore its potential uses in treating various health conditions. The biochemical compositions of *Arthrospira* play a crucial role for determining its potential applications in downstream industries. For instance, biomass with high phycocyanin content can be utilized for developing natural food colors or other phycocyanin products. On the other hand, if the biomass contains high carbohydrate, it can be a promising source for generating biofuel or producing active polysaccharides (Markou et al., 2021; Wu et al., 2021). However, the chemical composition of the cultivation medium and the cultivation conditions, e.g. temperature, salinity, and nutrient availability, play a crucial role in composition and biomass production of microalgae produced (Papadopoulos et al., 2022).

Cultivating microalgae using ADEs poses several challenges, including inappropriate nutrient concentrations, high turbidity, competing biological contaminants, ammonia toxicity, and metal toxicity (Chong et al., 2022). This study proved that two *Arthrospira* species could be grown using ADE derived from water hyacinth, which contained enough essential nutrients despite having a very low concentration of PO_4^{3-}-P. While NH_4^+-N concentration in the ADE was also slightly lower than the NO_3^--N concentrations in the standard media, the values are comparable. Furthermore, water hyacinth-derived ADE had a low DOC concentration, making it a practical option for an *Arthrospira* culture medium. Traditional feedstocks for AD process, like animal manure, food waste, and municipal solid wastes, usually contain high levels of DOC, NH_4^+-N, PO_4^{3-}-P, and heavy metals (Kobayashi et al., 2013; Massa et al., 2017; Watanabe et al., 2009). This study used only the liquid part of water hyacinth to prepare the

ADE, resulting in a lower organic load and lower concentrations of inorganic nutrients such as PO_4^{3-}-P and NH_4^+-N.

Lu et al. (2018) described that a high concentration of NH_4^+-N hinders algal growth by increasing oxidative stress and disrupting the electron transport system. Belkin and Boussiba (1991) exhibited that the growth of *A. platensis* was inhibited by 50% when the NH_4^+-N concentration reached 140 mg/L at pH 10, compared with that in lower ammonium concentrations. Jiang et al. (2015) reported that the lethal concentration of NH_4^+-N for *Spirulina subsalsa* was ≥ 90 mg/L. This concentration was comparable to those in the undiluted and two-fold diluted ADE in the present study but not in the remaining dilution treatments.

The ADE used in this study contained a relatively low PO_4^{3-}-P concentration which had beneficial effect on the higher accumulation of carbohydrates, β-glucans, dietary fibers, and γ-linolenic acid in the cells of *Arthrospira* species. Several studies have described that the storage of carbohydrates increased in microalgae reared in P-deficient media (Cade-Menun and Paytan, 2010; Dean et al., 2008a). Markou (2012) narrated that cyanobacterium such as *Arthrospira* stored carbohydrates mainly as glycogen (e.g., α-1,4 linked glucan). Generally, in cyanobacteria and green algae, the process of glycogen synthesis is regulated by ADP-glucose pyrophosphorylase, which is activated by 3-phosphoglycerate and inhibited by inorganic P (Gómez-Casati et al., 2003). If the inorganic P in the medium is deficient, glycogen production might be stimulated. Recent research conducted by Markou et al. (2021) has revealed that *A. platensis* strain SAG 21.99 shows a significant increase in the accumulation of total dietary fiber (20%–32% of algal dry mass) when grown in a P-limited medium compared to a P-replete medium. These findings are consistent with the results of this study.

Additionally, the ADE used in this study had a lower concentration of Na^+. To optimize the medium composition for *Arthrospira* culture, I added 0.2 M of $NaHCO_3$, which resulted in a perfectly balanced medium. On the other hand, the K^+, Ca^{2+} and Mg^{2+} concentration was

notably high in the ADE. Preparation of 6- and 8-fold diluted ADE solved this unbalance metal condition. Parker et al. (1997) showed K^+ inhibition of the growth of other cyanobacterium, *Microcystis* spp. with no growth of *Microcystis* at >2.8 mmol/L (equivalent to 53.2 mg/L) K^+. The two *Arthrospira* spp. used in this study are more tolerant of high K^+ than *Microcystis* because the K^+ concentrations in the standard media are >670 mg/L.

Furthermore, it's worth noting that anaerobic digestion effluents may contain biological contaminants, such as bacteria, zooplankton, pathogens, viruses, and other algae. These contaminants can pose a threat to a target microalgal growth through interactions such as competition, parasitism, predation, and mutualism (Chong et al., 2022; Di Caprio, 2020; Xia and Murphy, 2016). The high turbidity of the ADEs due to suspended materials may also impede algal growth by hindering light transmission through lowering photosynthesis (Marcilhac et al., 2014). However, this study successfully addressed these issues using a 0.2-µm membrane filter and simple dilution techniques. The other advantage of this study is the culturing of *Arthrospira* in higher pH (> 8.5) which makes it safer from common bacterial contaminants.

Cultivation systems is one of the important factors for the successfully producing microalgae. To increase the efficiency of microalgal production and reduce its costs, it is essential to adopt large-scale culture systems in the industrialization process. *Arthrospira* is mainly produced commercially in open pond systems that are affordable and convenient to use, utilizing solar radiation as a cost-effective energy source. However, they have certain limitations in achieving optimal temperature levels, which results in lower biomass productivity (Casazza et al., 2022). Therefore, they are only suitable for use in tropical and subtropical regions. On the other hand, closed systems are becoming increasingly popular as they offer significant advantages over open cultivation systems. These benefits include sterility,

enhanced control over cultivation conditions such as light intensity, carbon dioxide and nutrient levels, temperature, and greater biomass productivity.

Future perspectives

This study is a fundamental approach with bench scale experiments and attempts to make sustainable management of the freshwater ecosystem by effectively utilizing excessively growing aquatic macrophytes, such as water hyacinth, using biotechnology approaches such as anaerobic digestion and algal mass-culturing with the ADE. The results of this research demonstrated the potentials of two *Arthrospira* species reared with the ADE from water hyacinth to yield significant amounts of valuable biomolecules, such as β-glucan, γ-linolenic acids, and dietary fibers. This finding would open up the possibility of mass-culturing *Arthrospira* species with the ADE of water hyacinth, which can be a potential economic source for producing valuable biomolecules and also removing nutrients from the ADE, thus benefiting both environment and economy. However, these findings are promising, there are still many researches and practical questions that need to be solved for expanding to industrial and large-scale culture as described below.

• For optimal growth of algae, it is suggested to ensure that the ADE of water hyacinth contains essential inorganic nutrients but does not contain heavy metals. Therefore, one of the important procedures for expanding the scale of cultivation might be to determine just how much dilution of the ADE for reducing ammonium toxicity and maximizing algal production would be needed. A shortage or limitation of some nutrients, e.g. P, can positively affect the production of valuable biomolecules like β-glucans in the microalgal cells rearing with the ADE, though algal biomass production would not necessarily enhance. This suggests that the ADE originated from water hyacinth would be used as a suitable and cost-effective algal medium.

• To increase productivity, it is suggested to focus on one or two factors at a time, such as light, temperature, media pH, photoperiod, and so on. Although the procedure and the development of system components should be simplified, high temperature and light intensity should be need for enhancing the algal production. Lowering pH could enhance concentrations of trace metal ions and algal production as well (Qian et al. 2020). These effects should be necessarily evaluated prior to expanding the algal culture scale.

• To ensure successful microalgae production, it is essential to have a comprehensive understanding of downstream processes such as harvesting, extraction, and purification. Identification of possible contaminants such as bacteria and fungi is also crucial. This approach may improve the quality and quantity of microalgae produced by addressing these risks.

• The design of the photobioreactor is a one of the important issues for achieving high algal production. It is recommended to develop a new outdoor closed-system bioreactor that can be prepared with the round-shaped transparent PVC pipe and attached pump, allowing it to be a continuous culture system (Novoveská et al., 2023). This design would also help to enhance photosynthesis and efficiently eliminate potential contaminants, which is a common issue in open-pond systems.

Summary:

In this study, I determined the biochemical compositions of *Arthrospira platensis* and *A. fusiformis* cultivated in phosphorus-limited anaerobic digestion effluent (ADE) obtained from water hyacinth, which excessively grows in various water bodies and consequently causes significant environmental issues in various countries around the world. Even with six- and eight-fold diluted ADE, which contained a limited amount of phosphate, the algal biomass production during a 6-day experiment reached 0.62 g/L, including higher carbohydrate but lower protein and lipid contents than cells cultivated in standard algal growth media. β-glucan was produced at three to seven times the amount in standard algal media. Phosphate and ammonium removal efficiencies in the cultivation were > 96%. These results suggest that cultivating *Arthrospira* using the ADE from excessively growing water hyacinth has great potential to produce valuable biomolecules such as β-glucan through sustainable nutrient recycling.

Next, I measured the essential fatty acid contents of this two *Arthrospira* species cultivated in eight-fold diluted ADE with a gas chromatography-mass spectrometry (GC-MS) system equipped with a capillary CP-Sil 88 column. However, the ADE obtained from water hyacinth had a low amount of phosphorus, which caused the accumulation of health-beneficial γ-linolenic acid in the cells of the cultivated *Arthrospira*. Additionally, I measured the contents of pigments, including chlorophyll *a* and phycocyanin. *A. fusiformis* cultivated in ADE contained higher amount of phycocyacin than that in standard algal medium. Based on my findings, I proposed that growing *Arthrospira* could lead to functional food production and the sustainable management of nuisance water hyacinths.

References:

Ai, X., Yu, P., Li, X., Lai, X., Yang, M., Liu, F., Luan, F., Meng, X., 2023. Polysaccharides from *Spirulina platensis*: Extraction methods, structural features and bioactivities diversity. Int J Biol Macromol 231, 123211. https://doi.org/10.1016/j.ijbiomac.2023.123211

Al-Dhabi, N.A., 2013. Heavy metal analysis in commercial *Spirulina* products for human consumption. Saudi J Biol Sci 20, 383–388. https://doi.org/10.1016/j.sjbs.2013.04.006

Anderson, R.A., 1997. Chromium as an Essential Nutrient for Humans. Regulatory Toxicology and Pharmacology 26, S35–S41. https://doi.org/10.1006/rtph.1997.1136

Anny, M.F.A., Tuhin, T.R., Liu, X., Yoshiyama, K., Akizuki, S., Toda, T., Ban, S., 2024. Enhancement of β-glucan production in two *Arthrospira* species cultivating in anaerobic digestion effluent of water hyacinth. Biochem Eng J 203, 109206. https://doi.org/10.1016/j.bej.2023.109206

Armstrong, F.A.J., Stearns, C.R., Strickland, J.D.H., 1967. The measurement of upwelling and subsequent biological process by means of the Technicon Autoanalyzer® and associated equipment. Deep Sea Research and Oceanographic Abstracts 14, 381–389. https://doi.org/10.1016/0011-7471(67)90082-4

Arunakumara, K.K.I.U., Zhang, X., 2008. Heavy metal bioaccumulation and toxicity with special reference to microalgae. Journal of Ocean University of China 7, 60–64. https://doi.org/10.1007/s11802-008-0060-y

Ban, S., Toda, T., Koyama, M., Ishikawa, K., Kohzu, A., Imai, A., 2019. Modern lake ecosystem management by sustainable harvesting and effective utilization of aquatic macrophytes. Limnology (Tokyo) 20, 93–100. https://doi.org/10.1007/s10201-018-0557-z

Batista, A.P., Niccolai, A., Fradinho, P., Fragoso, S., Bursic, I., Rodolfi, L., Biondi, N., Tredici, M.R., Sousa, I., Raymundo, A., 2017. Microalgae biomass as an alternative ingredient in cookies: Sensory, physical and chemical properties, antioxidant activity and in vitro digestibility. Algal Res 26, 161–171. https://doi.org/10.1016/j.algal.2017.07.017

Baunillo, K.E., Tan, R.S., Barros, H.R., Luque, R., 2012. Investigations on microalgal oil production from *Arthrospira platensis*: towards more sustainable biodiesel production. RSC Adv 2, 11267. https://doi.org/10.1039/c2ra21796a

Belkin, S., Boussiba, S., 1991. High internal pH conveys ammonia resistance in *Spirulina platensis*. Bioresour Technol 38, 167–169. https://doi.org/10.1016/0960-8524(91)90149-E

Bellou, S., Baeshen, M.N., Elazzazy, A.M., Aggeli, D., Sayegh, F., Aggelis, G., 2014. Microalgal lipids biochemistry and biotechnological perspectives. Biotechnol Adv 32, 1476–1493. https://doi.org/10.1016/j.biotechadv.2014.10.003

Bhattacharjee, M., 2016. Pharmaceutically valuable bioactive compounds of algae. Asian Journal of Pharmaceutical and Clinical Research 9, 43. https://doi.org/10.22159/ajpcr.2016.v9i6.14507

Bortolini, D.G., Maciel, G.M., Fernandes, I. de A.A., Pedro, A.C., Rubio, F.T.V., Branco, I.G., Haminiuk, C.W.I., 2022. Functional properties of bioactive compounds from *Spirulina* spp.: Current status and future trends. Food Chemistry: Molecular Sciences 5, 100134. https://doi.org/10.1016/j.fochms.2022.100134

Cade-Menun, B.J., Paytan, A., 2010. Nutrient temperature and light stress alter phosphorus and carbon forms in culture-grown algae. Mar Chem 121, 27–36. https://doi.org/10.1016/j.marchem.2010.03.002

Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renewable and Sustainable Energy Reviews 19, 360–369. https://doi.org/10.1016/j.rser.2012.11.030

Calder, P.C., 2017. Omega-3 fatty acids and inflammatory processes: from molecules to man. Biochem Soc Trans 45, 1105–1115. https://doi.org/10.1042/BST20160474

Casazza, A.A., de Souza Oliveira, R.P., Silva, M.F. da, Solisio, C., Farias, C.B.B., Sarubbo, L.A., Converti, A., 2022. *Arthrospira platensis* Cultivation in a Bench-Scale Helical Tubular Photobioreactor. Applied Sciences 12, 1311. https://doi.org/10.3390/app12031311

Chevalier, P., de la Noüe, J., 1985. Wastewater nutrient removal with microalgae immobilized in carrageenan. Enzyme Microb Technol 7, 621–624. https://doi.org/10.1016/0141-0229(85)90032-8

Chong, C.C., Cheng, Y.W., Ishak, S., Lam, M.K., Lim, J.W., Tan, I.S., Show, P.L., Lee, K.T., 2022. Anaerobic digestate as a low-cost nutrient source for sustainable microalgae cultivation: A way forward through waste valorization approach. Science of The Total Environment 803, 150070. https://doi.org/10.1016/j.scitotenv.2021.150070

Christensen, J.B., Christensen, T.H., 2000. The effect of pH on the complexation of Cd, Ni and Zn by dissolved organic carbon from leachate-polluted groundwater. Water Res 34, 3743–3754. https://doi.org/10.1016/S0043-1354(00)00127-5

Clesceri, 1999. Standard Methods for the Examination of Water and Wastewater.

Cohen, Z., Vonshak, A., 1991. Fatty acid composition of *Spirulina* and *spirulina*-like cyanobacteria in relation to their chemotaxonomy. Phytochemistry 30, 205–206. https://doi.org/10.1016/0031-9422(91)84125-C

Cuciureanu, R., Urzică, A., Voitcu, M., Antoniu, A., 2000. Assessment of daily aluminum intake by food consumption. Rev Med Chir Soc Med Nat Iasi 104, 107–12.

da Silva Faresin, L., Devos, R.J.B., Reinehr, C.O., Colla, L.M., 2022. Development of ice cream with reduction of sugar and fat by the addition of inulin, *Spirulina platensis* or phycocyanin. Int J Gastron Food Sci 27, 100445. https://doi.org/10.1016/j.ijgfs.2021.100445

da Silva Ferreira, V., Sant'Anna, C., 2017. Impact of culture conditions on the chlorophyll content of microalgae for biotechnological applications. World J Microbiol Biotechnol 33, 20. https://doi.org/10.1007/s11274-016-2181-6

Dana Byrtusová, I., 2020. Production of beta-glucans and other polysaccharides by yeast and microalgae. Brno University of Technology.

Dean, A.P., Nicholson, J.M., Sigee, D.C., 2008. Impact of phosphorus quota and growth phase on carbon allocation in *Chlamydomonas reinhardtii* : an FTIR microspectroscopy study. Eur J Phycol 43, 345–354. https://doi.org/10.1080/09670260801979287

Dean, Estrada, B., Nicholson, J.M., Sigee, D.C., 2008. Molecular response of *Anabaena flos-aquae* to differing concentrations of phosphorus: A combined Fourier transform infrared and X-ray microanalytical study. Phycological Res 56, 193–201. https://doi.org/10.1111/j.1440-1835.2008.00501.x

de la Jara, A., Ruano-Rodriguez, C., Polifrone, M., Assunçao, P., Brito-Casillas, Y., Wägner, A.M., Serra-Majem, L., 2018. Impact of dietary *Arthrospira* (*Spirulina*) biomass consumption on human health: main health targets and systematic review. J Appl Phycol 30, 2403–2423. https://doi.org/10.1007/s10811-018-1468-4

Depraetere, O., Foubert, I., Muylaert, K., 2013. Decolorisation of piggery wastewater to stimulate the production of *Arthrospira platensis*. Bioresour Technol 148, 366–372. https://doi.org/10.1016/j.biortech.2013.08.165

Depraetere, O., Pierre, G., Deschoenmaeker, F., Badri, H., Foubert, I., Leys, N., Markou, G., Wattiez, R., Michaud, P., Muylaert, K., 2015. Harvesting carbohydrate-rich *Arthrospira platensis* by spontaneous settling. Bioresour Technol 180, 16–21. https://doi.org/10.1016/j.biortech.2014.12.084

Dewi, E.N., Kurniasih, R.A., Purnamayati, L., 2018. The Application of Microencapsulated Phycocyanin as a Blue Natural Colorant to the Quality of Jelly Candy. IOP Conf Ser Earth Environ Sci 116, 012047. https://doi.org/10.1088/1755-1315/116/1/012047

Di Caprio, F., 2020. Methods to quantify biological contaminants in microalgae cultures. Algal Res 49, 101943. https://doi.org/10.1016/j.algal.2020.101943

Di Termini, I., Prassone, A., Cattaneo, C., Rovatti, M., 2011. On the nitrogen and phosphorus removal in algal photobioreactors. Ecol Eng 37, 976–980. https://doi.org/10.1016/j.ecoleng.2011.01.006

Diraman H, Koru E., Dibeklioglu H, 2009. Fatty acid profile of *Spirulina platensis* used as food supplement.

Dolganyuk, V., Belova, D., Babich, O., Prosekov, A., Ivanova, S., Katserov, D., Patyukov, N., Sukhikh, S., 2020. Microalgae: A Promising Source of Valuable Bioproducts. Biomolecules 10, 1153. https://doi.org/10.3390/biom10081153

El-Naggar, N.E.-A., Hussein, M.H., Shaaban-Dessuuki, S.A., Dalal, S.R., 2020. Production, extraction and characterization of *Chlorella vulgaris* soluble polysaccharides and their applications in AgNPs biosynthesis and biostimulation of plant growth. Sci Rep 10, 3011. https://doi.org/10.1038/s41598-020-59945-w

Escudero, A., Blanco, F., Lacalle, A., Pinto, M., 2014. Ammonium removal from anaerobically treated effluent by *Chlamydomonas acidophila*. Bioresour Technol 153, 62–68. https://doi.org/10.1016/j.biortech.2013.11.076

Fadlelmoula, A., Pinho, D., Carvalho, V.H., Catarino, S.O., Minas, G., 2022. Spectroscopy to Analyse Human Blood over the Last 20 Years: A Review towards Lab-on-a-Chip micromachines Fourier Transform Infrared (FTIR) Spectroscopy to Analyse Human Blood over the Last 20 Years: A Review towards Lab-on-a-Chip Devices. https://doi.org/10.3390/mi13020187

Fan, J., Cui, Y., Wan, M., Wang, W., Li, Y., 2014. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. Biotechnol Biofuels 7, 17. https://doi.org/10.1186/1754-6834-7-17

Fithriani, D., Sinurat, E., 2019. Utilization of *Spirulina* as functional food: Phytosterol and amino acid profiles study. IOP Conf Ser Earth Environ Sci 278, 012028. https://doi.org/10.1088/1755-1315/278/1/012028

Geremia, E., Ripa, M., Catone, C.M., Ulgiati, S., 2021. A Review about Microalgae Wastewater Treatment for Bioremediation and Biomass Production—A New Challenge for Europe. Environments 8, 136. https://doi.org/10.3390/environments8120136

Godos, I. de, Blanco, S., García-Encina, P.A., Becares, E., Muñoz, R., 2009. Long-term operation of high rate algal ponds for the bioremediation of piggery wastewaters at high loading rates. Bioresour Technol 100, 4332–4339. https://doi.org/10.1016/j.biortech.2009.04.016

Godos, I. de, Vargas, V.A., Blanco, S., González, M.C.G., Soto, R., García-Encina, P.A., Becares, E., Muñoz, R., 2010. A comparative evaluation of microalgae for the degradation of piggery wastewater under photosynthetic oxygenation. Bioresour Technol 101, 5150–5158. https://doi.org/10.1016/j.biortech.2010.02.010

Golmakani, M., Rezaei, K., Mazidi, S., Razavi, S.H., 2012. γ-Linolenic acid production by *Arthrospira platensis* using different carbon sources. European Journal of Lipid Science and Technology 114, 306–314. https://doi.org/10.1002/ejlt.201100264

Golueke, C.G., Oswald, W.J., 1962. The Mass Culture of *Porphyridium cruentum*. Appl Microbiol 10, 102–107. https://doi.org/10.1128/am.10.2.102-107.1962

Gómez-Casati, D.F., Cortassa, S., Aon, M.A., Iglesias, A.A., 2003. Ultrasensitive behavior in the synthesis of storage polysaccharides in cyanobacteria. Planta 216, 969–975. https://doi.org/10.1007/s00425-002-0949-4

Gonzalez, L.E., Bashan, Y., 2000. Increased Growth of the Microalga *Chlorella vulgaris* when Coimmobilized and Cocultured in Alginate Beads with the Plant-Growth-Promoting Bacterium *Azospirillum brasilense*. Appl Environ Microbiol 66, 1527–1531. https://doi.org/10.1128/AEM.66.4.1527-1531.2000

Grand View Research, 2022. Functional Foods Market Size Worth $586.1 Billion By 2030. Grand view Research. URL https://www.grandviewresearch.com/press-release/global-functional-foods-market (accessed 12.22.23).

Guldas, M., Ziyanok-demirtas, S., sahan, Y., yildiz, E., gurbuz, O., 2021. Antioxidant and anti-diabetic properties of *Spirulina platensis* produced in Turkey. Food Science and Technology 41, 615–625. https://doi.org/10.1590/fst.23920

Gundupalli, M.P., Tantayotai, P., Chuetor, S., Cheenkachorn, K., Joshi, S., Bhattacharyya, D., Sriariyanun, M., 2023. Improvement of Water Hyacinth Bioconversion by Different Organic and Mineral Acid Pretreatment and the Effect of Post-pretreatment Washing. Bioenergy Res 16, 1718–1732. https://doi.org/10.1007/s12155-022-10528-9

Guo, Q., Ai, L., Cui, S.W., 2018. Fourier Transform Infrared Spectroscopy (FTIR) for Carbohydrate Analysis. pp. 69–71. https://doi.org/10.1007/978-3-319-96370-9-9

Gur, J., Mawuntu, M., Martirosyan, D., 2018. FFC's Advancement of Functional Food Definition. Functional Foods in Health and Disease 8, 385. https://doi.org/10.31989/ffhd.v8i7.531

Han, J., Jeon, B.S., Mizuno, K., Yoshida, K., Park, H.D., 2018. Influences of small-scale oscillations on growth inhibition and ultrastructural changes of *Microcystis* cells. J

Environ Sci Health A Tox Hazard Subst Environ Eng 53, 1161–1166. https://doi.org/10.1080/10934529.2018.1530329

Heldt, H.W., Chon, C.J., Maronde, D., Herold, A., Stankovic, Z.S., Walker, D.A., Kraminer, A., Kirk, M.R., Heber, U., 1977. Role of Orthophosphate and Other Factors in the Regulation of Starch Formation in Leaves and Isolated Chloroplasts. Plant Physiol 59, 1146–1155. https://doi.org/10.1104/pp.59.6.1146

Hena, S., Znad, H., Heong, K.T., Judd, S., 2018. Dairy farm wastewater treatment and lipid accumulation by *Arthrospira platensis*. Water Res 128, 267–277. https://doi.org/10.1016/j.watres.2017.10.057

Holm, L.G., Plucknett, D.L., Pancho, J. V., Herberger, J.P., 1977. The world's worst weeds. Distribution and biology. The world's worst weeds. Distribution and biology.

Hussner, A., Stiers, I., Verhofstad, M.J.J.M., Bakker, E.S., Grutters, B.M.C., Haury, J., van Valkenburg, J.L.C.H., Brundu, G., Newman, J., Clayton, J.S., Anderson, L.W.J., Hofstra, D., 2017. Management and control methods of invasive alien freshwater aquatic plants: A review. Aquat Bot 136, 112–137. https://doi.org/10.1016/j.aquabot.2016.08.002

Imase, M., Watanabe, K., Aoyagi, H., Tanaka, H., 2008. Construction of an artificial symbiotic community using a *Chlorella*–symbiont association as a model. FEMS Microbiol Ecol 63, 273–282. https://doi.org/10.1111/j.1574-6941.2007.00434.x

Izard, J., Limberger, R.J., 2003. Rapid screening method for quantitation of bacterial cell lipids from whole cells. J Microbiol Methods 55, 411–418. https://doi.org/10.1016/S0167-7012(03)00193-3

Jiang, L., Pei, H., Hu, W., Ji, Y., Han, L., Ma, G., 2015. The feasibility of using complex wastewater from a monosodium glutamate factory to cultivate *Spirulina subsalsa* and accumulate biochemical composition. Bioresour Technol 180, 304–310. https://doi.org/10.1016/j.biortech.2015.01.019

Jin, X., Gong, S., Yang, B., Wu, J., Li, T., Wu, Hualian, Wu, Houbo, Xiang, W., 2021. Transcriptomic analysis for phosphorus limitation-induced β-glucans accumulation in *Chlorella sorokiniana* SCSIO 46784 during the early phase of growth. Algal Res 54, 102208. https://doi.org/10.1016/j.algal.2021.102208

Kamalanathan, M., Gleadow, R., Beardall, J., 2015. Impacts of phosphorus availability on lipid production by *Chlamydomonas reinhardtii*. Algal Res 12, 191–196. https://doi.org/10.1016/j.algal.2015.08.021

Kapoor, B., Kapoor, D., Gautam, S., Singh, R., Bhardwaj, S., 2021. Dietary Polyunsaturated Fatty Acids (PUFAs): Uses and Potential Health Benefits. Curr Nutr Rep 10, 232–242. https://doi.org/10.1007/s13668-021-00363-3

Khan, A.H., Khan, N.A., Ahmed, S., Dhingra, A., Singh, C.P., Khan, S.U., Mohammadi, A.A., Changani, F., Yousefi, M., Alam, S., Vambol, S., Vambol, V., Khursheed, A., Ali, I., 2020. Application of advanced oxidation processes followed by different treatment technologies for hospital wastewater treatment. J Clean Prod 269, 122411. https://doi.org/10.1016/j.jclepro.2020.122411

Kimura, S., Yamada, T., Ban, S., Koyama, M., Toda, T., 2019. Nutrient removal from anaerobic digestion effluents of aquatic macrophytes with the green alga, *Chlorella sorokiniana*. Biochem Eng J 142, 170–177. https://doi.org/10.1016/j.bej.2018.12.001

Kipp, A.P., Strohm, D., Brigelius-Flohé, R., Schomburg, L., Bechthold, A., Leschik-Bonnet, E., Heseker, H., 2015. Revised reference values for selenium intake. Journal of Trace Elements in Medicine and Biology 32, 195–199. https://doi.org/10.1016/j.jtemb.2015.07.005

Kobayashi, N., Noel, E.A., Barnes, A., Watson, A., Rosenberg, J.N., Erickson, G., Oyler, G.A., 2013. Characterization of three *Chlorella sorokiniana* strains in anaerobic digested effluent from cattle manure. Bioresour Technol 150, 377–386. https://doi.org/10.1016/j.biortech.2013.10.032

Kochert, 1978. Carbohydrate determination by the phenol-sulfuric acid method. Handbook of phycological methods, Physiological and biochemical methods. 95.

Kong, Q., Li, L., Martinez, B., Chen, P., Ruan, R., 2010. Culture of Microalgae *Chlamydomonas reinhardtii* in Wastewater for Biomass Feedstock Production. Appl Biochem Biotechnol 160, 9–18. https://doi.org/10.1007/s12010-009-8670-4

Koroleff, 1970. Direct determination of ammonia in natural waters as indophenol blue. Information for techniques and methods for sea water analysis.

Koyama, M., Yamamoto, S., Ishikawa, K., Ban, S., Toda, T., 2014. Anaerobic digestion of submerged macrophytes: Chemical composition and anaerobic digestibility. Ecol Eng 69, 304–309. https://doi.org/10.1016/j.ecoleng.2014.05.013

Lau, P.S., Tam, N.F.Y., Wong, Y.S., 1995. Effect of algal density on nutrient removal from primary settled wastewater. Environmental Pollution 89, 59–66. https://doi.org/10.1016/0269-7491(94)00044-E

Lee, J., Kim, Y., Friso, S., Choi, S.-W., 2017. Epigenetics in non-alcoholic fatty liver disease. Mol Aspects Med 54, 78–88. https://doi.org/10.1016/j.mam.2016.11.008

Li, G., Bai, X., Li, H., Lu, Z., Zhou, Y., Wang, Y., Cao, J., Huang, Z., 2019. Nutrients removal and biomass production from anaerobic digested effluent by microalgae: A review. International Journal of Agricultural and Biological Engineering 12, 8–13. https://doi.org/10.25165/j.ijabe.20191205.3630

Li Sun, Shumei Wang, Lixue Chen, Xueqin Gong, 2003. Promising fluorescent probes from phycobiliproteins. IEEE Journal of Selected Topics in Quantum Electronics 9, 177–188. https://doi.org/10.1109/JSTQE.2003.812499

Li, T.-T., Tong, A.-J., Liu, Y.-Y., Huang, Z.-R., Wan, X.-Z., Pan, Y.-Y., Jia, R.-B., Liu, B., Chen, X.-H., Zhao, C., 2019. Polyunsaturated fatty acids from microalgae *Spirulina platensis* modulates lipid metabolism disorders and gut microbiota in high-fat diet rats. Food and Chemical Toxicology 131, 110558. https://doi.org/10.1016/j.fct.2019.06.005

Lishawa, S.C., Carson, B.D., Brandt, J.S., Tallant, J.M., Reo, N.J., Albert, D.A., Monks, A.M., Lautenbach, J.M., Clark, E., 2017. Mechanical Harvesting Effectively Controls Young Typha spp. Invasion and Unmanned Aerial Vehicle Data Enhances Post-treatment Monitoring. Front Plant Sci 8. https://doi.org/10.3389/fpls.2017.00619

Liu, Q., Yao, C., Sun, Y., Chen, W., Tan, H., Cao, X., Xue, S., Yin, H., 2019. Production and structural characterization of a new type of polysaccharide from nitrogen-limited *Arthrospira platensis* cultivated in outdoor industrial-scale open raceway ponds. Biotechnol Biofuels 12, 131. https://doi.org/10.1186/s13068-019-1470-3

Liu, X., Fujiwara, M., Kodera, T., Watanabe, K., Akizuki, S., Kishi, M., Koyama, M., Toda, T., Ban, S., 2020. Conditions for continuous cultivation of *Chlorella sorokiniana* and nutrient removal from anaerobic digestion effluent of aquatic macrophytes. Int Biodeterior Biodegradation 149, 104923. https://doi.org/10.1016/j.ibiod.2020.104923

Ljubic, A., Safafar, H., Holdt, S.L., Jacobsen, C., 2018. Biomass composition of *Arthrospira platensis* during cultivation on industrial process water and harvesting. J Appl Phycol 30, 943–954. https://doi.org/10.1007/s10811-017-1332-y

Lowry, OliverH., Rosebrough, NiraJ., Farr, A.L., Randall, RoseJ., 1951. Protein measurement with the Folin phenol reagent. Journal of Biological Chemistry 193, 265–275. https://doi.org/10.1016/S0021-9258(19)52451-6

Lu, Q., Chen, P., Addy, M., Zhang, R., Deng, X., Ma, Y., Cheng, Y., Hussain, F., Chen, C., Liu, Y., Ruan, R., 2018. Carbon-dependent alleviation of ammonia toxicity for algae cultivation and associated mechanisms exploration. Bioresour Technol 249, 99–107. https://doi.org/10.1016/j.biortech.2017.09.175

Marcilhac, C., Sialve, B., Pourcher, A.-M., Ziebal, C., Bernet, N., Béline, F., 2014. Digestate color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem. Water Res 64, 278–287. https://doi.org/10.1016/j.watres.2014.07.012

Markou, G., 2012. Alteration of the biomass composition of *Arthrospira* (*Spirulina*) *platensis* under various amounts of limited phosphorus. Bioresour Technol 116, 533–535. https://doi.org/10.1016/j.biortech.2012.04.022

Markou, G., Eliopoulos, C., Argyri, A., Arapoglou, D., 2021. Production of *Arthrospira* (*Spirulina*) *platensis* Enriched in β-Glucans through Phosphorus Limitation. Applied Sciences 11, 8121. https://doi.org/10.3390/app11178121

Maroušek, J., Marošková, A., 2021. Economic Considerations on Nutrient Utilization in Wastewater Management. Energies (Basel) 14, 3468. https://doi.org/10.3390/en14123468

Martínez, M., 2000. Nitrogen and phosphorus removal from urban wastewater by the microalga *Scenedesmus obliquus*. Bioresour Technol 73, 263–272. https://doi.org/10.1016/S0960-8524(99)00121-2

Masojídek, J., Torzillo, G., Koblížek, M., 2013. Photosynthesis in Microalgae, in: Handbook of Microalgal Culture. Wiley, pp. 21–36. https://doi.org/10.1002/9781118567166.ch2

Massa, M., Buono, S., Langellotti, A.L., Castaldo, L., Martello, A., Paduano, A., Sacchi, R., Fogliano, V., 2017. Evaluation of anaerobic digestates from different feedstocks as growth media for Tetradesmus obliquus, *Botryococcus braunii*, *Phaeodactylum tricornutum* and *Arthrospira maxima*. N Biotechnol 36, 8–16. https://doi.org/10.1016/j.nbt.2016.12.007

Milicevic, D., 2023. Modeling water hyacinth growth dynamics. Arch Biol Sci 75, 165–185. https://doi.org/10.2298/ABS230222014M

Mohn, F.H., 1988. Harvesting of micro-algal biomass. Micro-algal biotechnology. Cambridge University Press, Cambridge, pp.395-414.

Murphy, E.J., Rezoagli, E., Major, I., Rowan, N.J., Laffey, J.G., 2020. β-Glucan Metabolic and Immunomodulatory Properties and Potential for Clinical Application. Journal of Fungi 6, 356. https://doi.org/10.3390/jof6040356

Murphy, J., Riley, J.P., 1962. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27, 31–36. https://doi.org/10.1016/S0003-2670(00)88444-5

Muys, M., Sui, Y., Schwaiger, B., Lesueur, C., Vandenheuvel, D., Vermeir, P., Vlaeminck, S.E., 2019. High variability in nutritional value and safety of commercially

available *Chlorella* and *Spirulina* biomass indicates the need for smart production strategies. Bioresour Technol 275, 247–257. https://doi.org/10.1016/j.biortech.2018.12.059

Novoveská, L., Nielsen, S.L., Eroldoğan, O.T., Haznedaroglu, B.Z., Rinkevich, B., Fazi, S., Robbens, J., Vasquez, M., Einarsson, H., 2023. Overview and Challenges of Large-Scale Cultivation of Photosynthetic Microalgae and Cyanobacteria. Mar Drugs 21, 445. https://doi.org/10.3390/md21080445

Nowicka-Krawczyk, P., Mühlsteinová, R., Hauer, T., 2019. Detailed characterization of the *Arthrospira* type species separating commercially grown taxa into the new genus *Limnospira* (Cyanobacteria). Scientific Reports 2019 9:1 9, 1–11. https://doi.org/10.1038/s41598-018-36831-0

Nurdogan, Y., Oswald, W.J., 1995. Enhanced nutrient removal in high-rate ponds. Water Science and Technology 31, 33–43. https://doi.org/10.1016/0273-1223(95)00490-E

Oh, H.-M., Lee, S.J., Park, M.-H., Kim, H.-S., Kim, H.-C., Yoon, J.-H., Kwon, G.-S., Yoon, B.-D., 2001. Harvesting of *Chlorella vulgaris* using a bioflocculant from *Paenibacillus* sp. AM49. Biotechnol Lett 23, 1229–1234. https://doi.org/10.1023/A:1010577319771

Olguín, E., 2003. Phycoremediation: key issues for cost-effective nutrient removal processes. Biotechnol Adv 22, 81–91. https://doi.org/10.1016/S0734-9750(03)00130-7

O'Sullivan, C., Rounsefell, B., Grinham, A., Clarke, W., Udy, J., 2010. Anaerobic digestion of harvested aquatic weeds: water hyacinth (*Eichhornia crassipes*), cabomba (*Cabomba Caroliniana*) and *salvinia* (*Salvinia molesta*). Ecol Eng 36, 1459–1468. https://doi.org/10.1016/j.ecoleng.2010.06.027

Papadopoulos, K., Economou, C., Markou, G., Nicodemou, A., Koutinas, M., Tekerlekopoulou, A., Vayenas, D., 2022. Cultivation of *Arthrospira platensis* in Brewery Wastewater. Water (Basel) 14, 1547. https://doi.org/10.3390/w14101547

Park, J., Jin, H.-F., Lim, B.-R., Park, K.-Y., Lee, K., 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga *Scenedesmus* sp. Bioresour Technol 101, 8649–8657. https://doi.org/10.1016/j.biortech.2010.06.142

Park, J., Lee, H., Dinh, T.B., Choi, S., De Saeger, J., Depuydt, S., Brown, M.T., Han, T., 2022. Commercial Potential of the Cyanobacterium *Arthrospira maxima*: Physiological and Biochemical Traits and the Purification of Phycocyanin. Biology (Basel) 11, 628. https://doi.org/10.3390/biology11050628

Parker, D.L., Kumar, H.D., Rai, L.C., Singh, J.B., 1997. Potassium Salts Inhibit Growth of the Cyanobacteria *Microcystis* spp. in Pond Water and Defined Media: Implications for

Control of Microcystin-Producing Aquatic Blooms. Appl Environ Microbiol 63, 2324–2329. https://doi.org/10.1128/aem.63.6.2324-2329.1997

Pereira, M.I.B., Chagas, B.M.E., Sassi, R., Medeiros, G.F., Aguiar, E.M., Borba, L.H.F., Silva, E.P.E., Neto, J.C.A., Rangel, A.H.N., 2019. Mixotrophic cultivation of *Spirulina platensis* in dairy wastewater: Effects on the production of biomass, biochemical composition and antioxidant capacity. PLoS One 14, e0224294. https://doi.org/10.1371/journal.pone.0224294

Prates, D. da F., Radmann, E.M., Duarte, J.H., Morais, M.G. de, Costa, J.A.V., 2018. *Spirulina* cultivated under different light emitting diodes: Enhanced cell growth and phycocyanin production. Bioresour Technol 256, 38–43. https://doi.org/10.1016/j.biortech.2018.01.122

Qian, J., Liu, X., Ban, S., Fujiwara, M., Kodera, T., Akizuki, S., Toda, T., 2020. pH treatments in continuous cultivation to maximize microalgal production and nutrient removal from anaerobic digestion effluent of aquatic macrophytes. J Appl Phycol 32, 3349–3362. https://doi.org/10.1007/s10811-020-02196-z.

Qian, J., Shimotori, K., Liu, X., Ban, S., Akizuki, S., Fujiwara, M., Kodera, T., Toda, T., Imai, A., 2021. Enhancement of algal growth by Mg^{2+} released from anaerobic digestion effluent of aquatic macrophytes through photolysis. Biochem Eng J 172, 108065. https://doi.org/10.1016/j.bej.2021.108065

Raczyk, M., Polanowska, K., Kruszewski, B., Grygier, A., Michałowska, D., 2022. Effect of *Spirulina* (*Arthrospira platensis*) Supplementation on Physical and Chemical Properties of *Semolina* (*Triticum durum*) Based Fresh Pasta. Molecules 27, 355. https://doi.org/10.3390/molecules27020355

Renuka, N., Sood, A., Prasanna, R., Ahluwalia, A.S., 2015. Phycoremediation of wastewaters: a synergistic approach using microalgae for bioremediation and biomass generation. International Journal of Environmental Science and Technology 12, 1443–1460. https://doi.org/10.1007/s13762-014-0700-2

Romay, Ch., Gonzalez, R., Ledon, N., Remirez, D., Rimbau, V., 2003. C-Phycocyanin: A Biliprotein with Antioxidant, Anti-Inflammatory and Neuroprotective Effects. Curr Protein Pept Sci 4, 207–216. https://doi.org/10.2174/1389203033487216

Roopnarain, A., Gray, V.M., Sym, S.D., 2014. Phosphorus limitation and starvation effects on cell growth and lipid accumulation in Isochrysis galbana U4 for biodiesel production. Bioresour Technol 156, 408–411. https://doi.org/10.1016/j.biortech.2014.01.092

Ruiz, J., Arbib, Z., Álvarez-Díaz, P.D., Garrido-Pérez, C., Barragán, J., Perales, J.A., 2013. Photobiotreatment model (PhBT): a kinetic model for microalgae biomass growth and nutrient removal in wastewater. Environ Technol 34, 979–991. https://doi.org/10.1080/09593330.2012.724451

Saito, Y., Takano, K., Kobayashi, F., Kobayashi, K., Park, H.-D., 2014. Development of a UV laser-induced fluorescence lidar for monitoring blue-green algae in Lake Suwa. Appl Opt 53, 7030. https://doi.org/10.1364/AO.53.007030

Sassano, C.E.N., Gioielli, L.A., Ferreira, L.S., Rodrigues, M.S., Sato, S., Converti, A., Carvalho, J.C.M., 2010. Evaluation of the composition of continuously-cultivated *Arthrospira* (*Spirulina*) *platensis* using ammonium chloride as nitrogen source. Biomass Bioenergy 34, 1732–1738. https://doi.org/10.1016/j.biombioe.2010.07.002

Shayesteh, H., Laird, D.W., Hughes, L.J., Nematollahi, M.A., Kakhki, A.M., Moheimani, N.R., 2023. Co-Producing Phycocyanin and Bioplastic in *Arthrospira platensis* Using Carbon-Rich Wastewater. BioTech 12, 49. https://doi.org/10.3390/biotech12030049

Simopoulos, A., 2016. The FTO Gene, Browning of Adipose Tissue and Omega-3 Fatty Acids. Lifestyle Genom 9, 123–126. https://doi.org/10.1159/000448617

Singh, M., Reynolds, D.L., Das, K.C., 2011. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Bioresour Technol 102, 10841–10848. https://doi.org/10.1016/j.biortech.2011.09.037

Stier, H., Ebbeskotte, V., Gruenwald, J., 2014. Immune-modulatory effects of dietary Yeast Beta-1,3/1,6-D-glucan. Nutr J. https://doi.org/10.1186/1475-2891-13-38

Su, Y., Mennerich, A., Urban, B., 2012a. Synergistic cooperation between wastewater-born algae and activated sludge for wastewater treatment: Influence of algae and sludge inoculation ratios. Bioresour Technol 105, 67–73. https://doi.org/10.1016/j.biortech.2011.11.113

Su, Y., Mennerich, A., Urban, B., 2012b. Comparison of nutrient removal capacity and biomass settleability of four high-potential microalgal species. Bioresour Technol 124, 157–162. https://doi.org/10.1016/j.biortech.2012.08.037

Tam, N.F.Y., Wong, Y.S., 2000. Effect of immobilized microalgal bead concentrations on wastewater nutrient removal. Environmental Pollution 107, 145–151. https://doi.org/10.1016/S0269-7491(99)00118-9

Tavanandi, H.A., Raghavarao, K.S.M.S., 2020. Ultrasound-assisted enzymatic extraction of natural food colorant C-Phycocyanin from dry biomass of *Arthrospira platensis*. LWT 118, 108802. https://doi.org/10.1016/j.lwt.2019.108802

Theodorou, M.E., Elrifi, I.R., Turpin, D.H., Plaxton, W.C., 1991. Effects of Phosphorus Limitation on Respiratory Metabolism in the Green Alga *Selenastrum minutum*. Plant Physiol 95, 1089–1095. https://doi.org/10.1104/pp.95.4.1089

Vogler, B.W., Brannum, J., Chung, J.W., Seger, M., Posewitz, M.C., 2018. Characterization of the *Nannochloropsis gaditana* storage carbohydrate: A 1,3-beta glucan with limited 1,6-branching. Algal Res 36, 152–158. https://doi.org/10.1016/j.algal.2018.10.011

Wang, L., Li, Y., Chen, P., Min, M., Chen, Y., Zhu, J., Ruan, R.R., 2010. Anaerobic digested dairy manure as a nutrient supplement for cultivation of oil-rich green microalgae *Chlorella* sp. Bioresour Technol 101, 2623–2628. https://doi.org/10.1016/j.biortech.2009.10.062

Wang, Y., Ho, S.-H., Cheng, C.-L., Guo, W.-Q., Nagarajan, D., Ren, N.-Q., Lee, D.-J., Chang, J.-S., 2016. Perspectives on the feasibility of using microalgae for industrial wastewater treatment. Bioresour Technol 222, 485–497. https://doi.org/10.1016/j.biortech.2016.09.106

Warr, S.R.C., Reed, R.H., Stewart, W.D.P., 1985. Carbohydrate accumulation in osmotically stressed cyanobacteria (blue-green algae): interactions of temperature and salinity. New Phytologist 100, 285–292. https://doi.org/10.1111/j.1469-8137.1985.tb02779.x

Watanabe, T., Masaki, K., Iwashita, K., Fujii, T., Iefuji, H., 2009. Treatment and phosphorus removal from high-concentration organic wastewater by the yeast *Hansenula anomala* J224 PAWA. Bioresour Technol 100, 1781–1785. https://doi.org/10.1016/j.biortech.2008.10.006

Wu, Hualian, Li, T., Lv, J., Chen, Z., Wu, J., Wang, N., Wu, Houbo, Xiang, W., 2021. Growth and Biochemical Composition Characteristics of *Arthrospira platensis* Induced by Simultaneous Nitrogen Deficiency and Seawater-Supplemented Medium in an Outdoor Raceway Pond in Winter. Foods 10. https://doi.org/10.3390/foods10122974

Wu, J.H.Y., Micha, R., Mozaffarian, D., 2019. Dietary fats and cardiometabolic disease: mechanisms and effects on risk factors and outcomes. Nature Reviews Cardiology 2019 16:10 16, 581–601. https://doi.org/10.1038/s41569-019-0206-1

Xia, A., Murphy, J.D., 2016. Microalgal Cultivation in Treating Liquid Digestate from Biogas Systems. Trends Biotechnol 34, 264–275. https://doi.org/10.1016/j.tibtech.2015.12.010

Xin, L., Hong-ying, H., Ke, G., Ying-xue, S., 2010. Effects of different nitrogen and phosphorus concentrations on the growth, nutrient uptake, and lipid accumulation of a freshwater microalga *Scenedesmus* sp. Bioresour Technol 101, 5494–5500. https://doi.org/10.1016/j.biortech.2010.02.016

Xu, H., Yang, B., Liu, Y., Li, F., Shen, C., Ma, C., Tian, Q., Song, X., Sand, W., 2018. Recent advances in anaerobic biological processes for textile printing and dyeing wastewater treatment: a mini-review. World J Microbiol Biotechnol 34, 165. https://doi.org/10.1007/s11274-018-2548-y

Xu, W., Hu, W., Deng, J., Zhu, J., Li, Q., 2014. Effects of harvest management of *Trapa bispinosa* on an aquatic macrophyte community and water quality in a eutrophic lake. Ecol Eng 64, 120–129. https://doi.org/10.1016/j.ecoleng.2013.12.028

Yahya, N., Aziz, F., Jamaludin, N.A., A. Mutalib, M., Ismail, A.F., W. Salleh, W.N., Jaafar, J., Yusof, N., A. Ludin, N., 2018. A review of integrated photocatalyst adsorbents for wastewater treatment. J Environ Chem Eng 6, 7411–7425. https://doi.org/10.1016/j.jece.2018.06.051

Yao, C.-H., Ai, J.-N., Cao, X.-P., Xue, S., 2013. Characterization of cell growth and starch production in the marine green microalga *Tetraselmis subcor*

diformis under extracellular phosphorus-deprived and sequentially phosphorus-replete conditions. Appl Microbiol Biotechnol 97, 6099–6110. https://doi.org/10.1007/s00253-013-4983-x

Zhang, C., Su, H., Baeyens, J., Tan, T., 2014. Reviewing the anaerobic digestion of food waste for biogas production. Renewable and Sustainable Energy Reviews 38, 383–392. https://doi.org/10.1016/j.rser.2014.05.038

Zhang, E., Wang, B., Wang, Q., Zhang, S., Zhao, B., 2008. Ammonia–nitrogen and orthophosphate removal by immobilized *Scenedesmus* sp. isolated from municipal wastewater for potential use in tertiary treatment. Bioresour Technol 99, 3787–3793. https://doi.org/10.1016/j.biortech.2007.07.011

Zhu, F., Du, B., Xu, B., 2016. A critical review on production and industrial applications of beta-glucans. Food Hydrocoll 52, 275–288. https://doi.org/10.1016/j.foodhyd.2015.07.003

Appendix 1 Heavy metal contents in dry mass of *A. platensis* and *A. fusiformis* cultivated in diluted anaerobic digestion effluent of water hyacinth and standard media (SOT or SM), and safety standard regulations for food supplements of the United Nations World Health Organization (WHO)/Food and Agriculture Organization (FAO) and the European Union (EU). 'nd' represent no detection. Dashes denote no data, because FAO/WHO and EU do not set the safety levels of these elements.

Algal sp., culture medium, and organization	Heavy metal contents of the algal dry mass (mg g^{-1})									
	Al	Cr	Ni	Se	Pb	Cu	Zn	Hg	Cd	Mn
A. platensis										
6× diluted ADE	0.36000	0.03000	0.00010	0.01000	0.00010	0.00020	0.01000	nd	0.00001	0.02000
8× diluted ADE	0.60000	0.10000	0.00010	0.00100	0.00010	0.00010	nd	0.00001	0.00001	0.00012
SOT	0.70000	0.10000	0.00020	0.00010	0.00020	0.00020	nd	0.00001	0.00001	0.00011
A. fusiformis										
6× diluted ADE	0.22000	0.03000	0.00020	0.01000	0.00010	0.01000	0.12000	nd	0.00001	0.01000
8× diluted ADE	0.50000	0.03000	0.00010	0.01000	0.00010	0.00012	0.07000	nd	0.00001	0.02000
SM	0.23000	0.03000	0.00020	0.01000	0.00012	0.01000	0.00012	nd	0.00001	0.02000
FAO / WHO	–	–	0.06790	–	0.00030	0.07330	0.09940	–	0.00020	–
EU	–	–	–	–	0.00300	–	–	0.0001	0.00100	–