UTS UNIVERSITY OF TECHNOLOGY SYDNEY

Application of microalgae and wastewater as plant nutrients and stimulants in hydroponic technology

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Certificate of original authorship

I, Swaminathan Palanisami declare that this thesis, is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Civil and Environmental Engineering, Faculty of Engineering and Information and Technology at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Thesis abstract

Nitrous oxide is 300 times stronger than carbon dioxide in causing climate change, and 80% of global nitrous oxide is from nitrogen fertilisers used in soil-based agriculture. Finding ways to minimise the carbon footprint related to the production of nitrogen-based agricultural fertiliser, and reusing waste nutrients from wastewater, will benefit parallelly in saving the energy expenses of wastewater treatment and in producing fertilisers. This research proposes utilising high concentration wastewater, such as desalination brine, blended with secondary treated domestic wastewater in the optimal proportion to produce microalgae growth media. Cultivating nitrogen-fixing microalgae in blended wastewater yields a dual solution for wastewater nutrient recovery and obtaining biologically fixed nitrogen. The nitrogen-fixing microalgae remove (exhaust) all the nitrogen in wastewater and, for its further growth, fix (produce) nitrogen. The nitrogen produced by the microalgae is a usable form of plant nutrient. Microalgae are known to produce plant hormones; the acid-digested algal biomass (extract) can be used as a source of nutrients and plant stimulants to grow plants in hydroponics. Growing plants in hydroponics minimise nitrogen nutrient loss (as in soil-based agriculture) and nitrous oxide evolution. This study used non-hazardous sources of wastewaters to demonstrate the possibility of producing microalgae biomass using blends of high and low-concentration wastewaters and assessed its nutrient recovery rates. Applied algal biomass extract as a source of nitrogen and whole nutrients to grow plants in hydroponics and added effort to profile the plant hormones in the microalgal biomass.

The aims of this research focused on (i) using different wastewater nutrient concentrations as a source to attain an optimal microalgal growth media for biomass production and nutrient recovery from wastewater; (ii) comparing the efficacy of microalgal biomass extract-based hydroponic nutrients with other commercial hydroponic nutrients. Consequently, the objectives are (1) to develop a wastewater blending method for algal cultivation and nutrient recovery, (2) to examine the feasibility of producing hydroponic nutrients and stimulants from algal biomass, (3) to compare the growth efficiency of plant in algal extracts nutrients and other available hydroponic-nutrients. The comparative study of algal biomass extract-based hydroponic nutrients with other commercial products showed clear evidence

that the microalgal biomass-based hydroponic nutrients have commercialisation potentials. Further product improvement by using different algal species can yield high and robust nutrients and stimulants.

Chapter 1

Introduction

1.1. Background

Water usage for agricultural purposes requires standard maintenance of its quality concerning environmental safety and agrarian productivity. Globally, 70% of freshwater is used for agriculture, and the percentage is constantly increasing in parallel to population growth (Khokhar, 2020). In Australia, from 2019 to 2020, 3.8 million megalitres were applied to crops which are 67% of all water applied (Australian bureau of statistics 2021). Agricultural land use and food production in Australia have evolved in resonance with environmental and economic frameworks which considerably fluctuate in terms of inadequate resource, productivity rates, policy to reduce greenhouse gas, climate change, national and global demands (Grundy et al., 2016). Amid these disparities, the government systems balance the food need of the growing population and maintain food security. 'Food security' refers to the availability of food and the possibility that people have the resources and opportunity for consistent access to food (Maxwell, 1996). It is common in any functional system; the rise of new impediments causes an imbalance, with examples such as nitrous oxide and climate change (Li et al., 2014). Unawareness of greenhouse gases other than carbon dioxide to the general public and political societies is masking the reality and the severity of the issue (Ahmed et al., 2017). Nitrous oxide (N_2O) is a greenhouse gas that is 300 times stronger than carbon dioxide in causing climate change (Daelman et al., 2013). Land for agricultural development is inadequate (Pardey et al., 2014), especially in countries like Australia (Bryan et al. 2013), Japan (Hoshino 2001) and the Middle East (Sowers, 2011); the competition of land uses for other purposes impact on the existing land base. Many challenges impend the future agricultural productivity of Australia, especially climate change, water shortage, and degradation of the available natural resources, especially water.

Nitrous oxide emissions occur mainly from soil-based agriculture (Table 1.1), a significant portion of which is contributed from crops using nitrogen-based fertilisers (De Klein et al., 2001). Soil-based conventional agriculture needs land and water and also causes a range of adverse impacts on the environment, such as surface runoffs (Barbosa et al., 2015a). Conventional agricultural methods include growing crops in soil with irrigation, cultivation in open-air, application of fertiliser-based nutrients, herbicides and pesticides (Ammann,

2005). These traditional processes use a large land area, and inefficient water use paves the way to runoff containing high concentrations of pesticides and nutrients (Nakano et al. 2004). Within the limitations described above, the soil-based modern agriculture technologies are also ever-changing, incorporating innovations and farming practices that support farmers in increasing efficiency and reducing the quantity of natural resource requirements. This includes applying methods that ensue efficient water spraying, crop rotation in alignment to seasons/weather, and eco-friendly biologically sourced renewable pest and weed control strategies (Nadykto 2019).

Table 1.1 Summarized sources of nitrous oxide emission globally (Shankman 2021)

Emission source	**Percentage (%)**
Agricultural soil	74
Power plant combustion	8
Industries	6
Manure	5
Transportation	5
Other	2

Hydroponics is the method of growing plants without soil by using mineral nutrient solutions (Sardare, 2013). Various benefits (Table 1.2) in hydroponic agriculture, including higher yields and efficient water use in a controlled environment, can support the continuous production of the desired crop throughout the year (Barbosa et al. 2015b). The IBIS World Industry Research Report of Australia's under-cover vegetable growing hydroponic farming states the annual growth of the hydroponic industry in Australia from 2014 to 2019 is 7.8%, and the projected yearly increase based on industry research statistics from 2019 to 2024 will be 2% (Anon 2020) (Https://Www.Ibisworld.Com/Au/Industry/under-Cover-Vegetable-Growing/2055/).

The degradation of groundwater quality and eutrophication prevail in Australia as the consequences of the application of a higher rate of fertilisers by the farms targeting to boost agricultural productivity and pasture growth (Melland et al. 2008).

Table 1.2 Comparison of applicable cultivation features between hydroponic mode of cultivation and conventional soil-based crop cultivation (Mohammed 2018)

Hydroponic cultivation (greenhouse)	**Conventional soil-based cultivation**
Less space/area occupant	Require more space
Year-round cultivation opportunity	Confined to a particular season in a year
Less water usage	Requires periodical watering
Significant weed control	Prone to weed
Controllable pest management	Less potential pest control
High nutrient use efficiency	High nutrient loss possibilities
Can change nutrient composition as needed	Not 100% changeable
Root harvest possible as needed	Not feasible to replant after root harvest
Can optimise/control light as needed to the plant	Impossible
Effective pesticide application	Higher possibility of loss and inactivation

The transformation of soil based agriculture to hydroponic production for crops that pertinent to hydroponic mode of cultivation benefits the reduction of nutrient requirements and reduced nutrient loss due to surface runoff (Bugbee, 2004). Appropriately formulated nutrients (liquid) circulating in the hydroponic systems are more easily available for plants, as the formulation contains optimised levels of ready–absorbable ingredients and the roots are in direct contact (Da Silva, 2018). Therefore, they allow obtaining higher yields, much faster than in the case of plants cultivated in soil. Importantly, regardless of weather or climatic conditions, crops can be harvested throughout the year. Such a scientific approach fully supports governmental policies, e.g. the suggested options from the Department of Primary Industries and regional development of the Government of Western Australia (Government of Western Australia, 2019) to reduce soil-based nitrous oxide emissions are:

● Using less nitrogen fertiliser while implementing soil testing and tissue testing, and visual signs to manage nitrogen fertiliser rates.

● Employing split applications of nitrogen fertilisers, to increase the efficiency of use by plants, allowing less nitrogen loss to the atmosphere or leach. This option is particularly suited to waterlogged sites.

● Using legume crops or pastures in the rotation instead of nitrogen fertiliser. More of the nitrogen is in the form of organic matter which is released more slowly and is used more effectively by growing plants.

● Using minimum tillage for cropping, this minimises organic matter breakdown and the release of nitrous oxide and nitrogen gas.

● Prevention of waterlogging. Under waterlogged conditions, nitrate can be denitrified by soil bacteria to form nitrous oxide and nitrogen gas.

● Application of nitrification inhibitors. These work by reducing nitrification, which reduces nitrate leaching and the production of nitrous oxide. These inhibitors can be mixed with nitrogen fertilisers or applied separately.

● The European Commission suggests options to optimise fertiliser application rates and emphasise managing practices of agricultural soils that emit nitrous oxide from manure spread on soil surfaces (Comission 2018). United Nations report propose routes to manage animal husbandry and application procedures of inorganic fertilisers (Pierre J. Gerber 2013).

The hydroponic approach can be another unattended option, with which a variety of leafy vegetables (Kimura and Rodriguez-Amaya 2003), fruits (Chow 1992), and even bananas (Patel et al. 2019) can be grown. This research will assist in the development and applications of sustainable hydroponic methods to alleviate land, water use, most importantly, curtailing nitrous oxide emissions.

Biological means of nitrogen fixation and simultaneous carbon dioxide usage is possible with prokaryotic microalgae (also known as cyanobacteria) (Krishnakumar et al. 2013). Since cyanobacteria fix nitrogen from atmosphere (Tsygankov 2007), they do not need externally supplied nitrogen for their growth. The nitrogen fixed by the cyanobacteria is present in the cytoplasm or cytosol (cellular liquid). Usually, cyanobacteria grow in alkaline pH, or change the pH to the alkaline range upon growth (Pandey et al. 2005), and the cell wall disrupts if exposed to sudden pH changes. It is hypothesised that the fixed nitrogen inside the cyanobacterial cells can thus be extracted by breaking the cells through osmotic shock, for example, using edible grade organic vinegar or a hydrothermal process and used as a source of nitrogen nutrient in the hydroponic nutrient solution. This would enable a natural means of nitrogen production from atmospheric air. The chemical contents of secondary treated wastewater, nutrient-deprived hydroponic wastewater (Richa et al. 2020a), aquaculture wastewater (Gao et al. 2016), and secondary treated domestic wastewater

(Gómez-Serrano et al. 2015), desalination brine (Shirazi et al. 2018a) devoid of toxic chemicals, are an ideal nutrient source for microalgal cultivation and the produced microalgal biomass can be used as hydroponic nutrients (Figure 1.1). This multidimensional approach would yield benefits in the utilisation of wastewater, paving the way for direct disposal of wastewater to natural water bodies or reuse of the water for subsequent microalgal cultivation or hydroponics by topping up nutrients. Microalgal biomass is known to have nitrogen content from 1% to 14% (typically around 5-10%) of dry weight; phosphorus content varies from 0.05% to 3.3%, sulphur 0.15% to 1.6% (Markou, Vandamme, and Muylaert 2014b), potassium 1.2% to 1.5%, magnesium ranges between 0.35% and 0.7%, calcium 0.2% to 1.4% (Tokucsoglu and Üunal 2003).

Hence, this research is targeting at the production of nitrogen from hydroponic wastewater, desalination brine, and residential wastewater using the biological machinery of nitrogen-fixing cyanobacteria (Benemann 1979) and other microalgae. Microalgal biomass produced from wastewater can be used either as a source of nitrogen for hydroponic nutrient solution, or as a whole nutrient. The cytoplasm has all the necessary nutrients as well as metabolites, such as vitamins (Tarento et al., 2018; Bonnet et al., 2010) and free amino acids (Cermakova et al., 2017) to enrich the hydroponic nutrient solution, yielding an microalgae-based complete nutrient solution. Notably, microalgae are known for producing plant hormones (Tarakhovskaya et al. 2007), which are small molecules that regulate plant's life cycle in different stages, exclusively increase the growth, fruiting, accumulation of nutrients/biomolecules that enrich the nutraceutical value of the target produce. The cytosol extraction process from microalgal biomass, if performed by vinegar (osmotic shock) method, will result in a solution that is 100% organic, making it an organic hydroponic product. Usually, the hydroponic nutrient solution is maintained at acidic pH in which the roots absorb nutrients optimally (Koehorst et al. 2010).

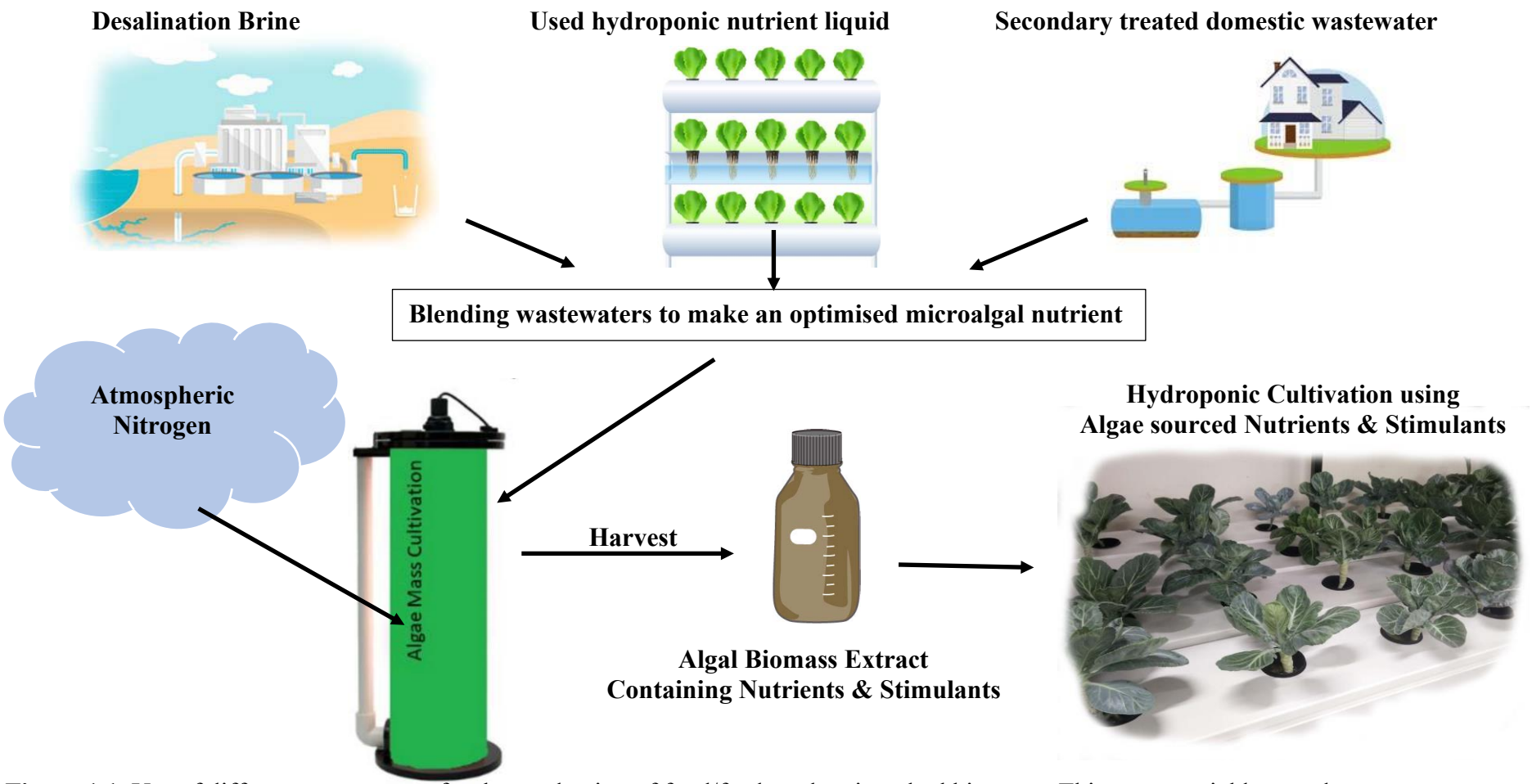


Figure 1.1. Use of different wastewaters for the production of food/feed grade microalgal biomass. This process yields a ready absorbable form of biologically sourced plant nutrients, high-value stimulants (plant hormones) combining an energy/cost-effective wastewater treatment allowing the direct disposal of wastewater without further treatment process.

In general, hydroponic cultivation requires a considerable volume of acid to maintain the acidic pH of nutrient solution (Tyson et al. 2008). After nutrient extraction from microalgal biomass grown in wastewater, the remaining microalgal debris can be processed through hydrothermal extraction (Li et al. 2017) and liquefied as a (hydroponic) nutrient solution concentrate, enabling 100% use of microalgal biomass without any no remnant at the end of the process. This microalgae-based system of nutrient/nitrogen bio-factory can be used as a means of hydroponic nutrient production, as well as a continuous running system mounted aside if the hydroponics system uses fertiliser grade chemicals, which produce post cultivation waste drain of nutrients. The nutrient-depleted hydroponic solution will have a concentration of minerals unabsorbable by plant but suffice microalgal growth.

1.2. Research Aims and Objectives

The overall aim of this project is to test the hypothesis of growing microalgae in different wastewaters for simultaneous biomass production and nutrient recovery, a cost-effective means of biomass harvest, extraction of nutrients from microalgal biomass, investigating its chemical qualities and testing the extract in the hydroponic system. In order to achieve the aim, the following objectives were set:

- To investigate the feasibility of using different wastewater to cultivate microalgae.
- To test the chemical contents and plant hormones of microalgal biomass for its use as a nutrient and stimulant for hydroponic cultivation.
- To study the use of microalgal extract in the growth of Pak Choy (*Brassica rapa*) and Collards (*Brassica oleracea*), its nutrient viability compared with other commercial hydroponic nutrients.

1.3. Knowledge gaps

Microalgal biomass from different species are characterised for their nutrient element contents and identified the presence of various plant hormones. No research so far has tried the use of biologically fixed microalgal nitrogen as a source of nutrient in hydroponic cultivation as well, never used the acid digested microalgal biomass as a source of the whole nutrient and plant stimulant for hydroponics. This is one of the reasons that many nitrogen-fixing prokaryotic microalgae are toxin-producing;

therefore, researchers restricted to use of microalgal biomass in food production (hydroponics) for human consumption. It is a fact that not all microalgae are toxin-producing; such non-toxin-producing microalgae can be used as a source of nitrogen/nutrient and plant hormone bio factory. This study demonstrated the potential of algal biomass as a source of nutrients and stimulants for growing plants in hydroponics.

This research contributes to the proof of concept that the microalgae fixed nitrogen can be used as a source of nitrogen nutrients in hydroponic cultivation. Microalgal biomass extract can be applied as a sole nutrient source, comprising both macro (nitrogen, phosphorus, potassium, sulphur) and micronutrients (copper, zinc, cobalt, molybdenum, manganese, magnesium) needed for hydroponic cultivation.

1.4. Thesis Synopsis

This thesis has six chapters. Chapter 1 is the introduction to the thesis. Chapter 2 provides a literature review of the research topic. The first part of literature review is, different wastewaters to source nutrients for microalgal cultivation. This is to understand better how the high and low nutrient content wastewaters can be mixed and used to favour algal growth mediated nutrient recovery, thus enabling, reducing the chemical method of wastewater treatment. The second part of the literature review engrosses renewable and eco-friendly sources of hydroponic nutrients and plant bio-stimulants, signifying a way to microalgal biomass as a sustainable source to enhance hydroponic crop productivity. In the first part of the chapter, wastewater sourced micro and macronutrients for microalgal growth and plots of its usage were reviewed. The compensational aspect of each nutrient element from different wastewater sources that can replenish microalgal nutritional requirements was analysed to explain how they impact the microalgal cultivation. Additionally, wastewater chemical resource-based microalgal technology was reviewed to lend insight into the potential production of enriched plant stimulants from algal biomass, micro and macronutrients.

Chapter 3 investigated the use of different wastewater blends for algal cultivation. Twelve different wastewater mixtures were prepared from; nutrient-deprived hydroponic wastewater, desalination brine, nitrified human urine, secondary treated domestic wastewater and direct human urine for testing the nutrient recovery and growth efficiencies of microalgae. As the (real) wastewater contain algal flora of their own, it is observed the dominance of wastewater contained microalgae and also the survival of the species of interest. Nutrient Stoichiometry and meta-analysis identified the specific retention timeline, which day which nutrient element can recover to its maximum and least recovering elements. Overall within the tested twelve different blends, irrespective of wastewater nutrient complexity, a particular nutrient element is recovered to its maximum on a specific timeline by algal biomass. From the meta-analysis profiles, within the given wastewater nutrient mixture, a specific nutrient element that highly controls or influences the recovery of other elements (interlinks or selective control) was evident. In the given wastewater nutrient mixture, it is possible to pinpoint a key nutrient element that controls or influence the recovery of other elements, as well the specific nutrient element(s) that are not influenced the recovery of other elements.

Chapter 4 presented an investigation of the role of algal strain selection that favoured the cost-effective biomass harvest method and its feasibility. Large scale production of microalgae using 1200-L photo-bioreactor and optimising customised sedimentation based harvest method was conducted. The extraction and quantification of hydroponic nutrients and stimulants (plant hormones) from microalgal biomass showed viable micro, macro nutrients and two plant hormones detected from the microalgal biomass.

Chapter 5 studied the growth responses of pak choy and collards in microalgae-based hydroponic nutrients. Initially, the growth performance of pak choy compared in algal extract hydroponic nutrients and other commercial hydroponic nutrients. Based on the results of plant height and plant fresh biomass weight, it was identified the macronutrients, nitrate, and phosphate are insufficient. Therefore, the growth responses were tested in a combination of microalgal extract + nitrified human urine using collards.

Chapter 6 summarised the key findings from this thesis. As a result of the evidence and applicability of algal biomass as nutrient and stimulants in hydroponic cultivation, the simultaneous algal cultivation and nutrient recovery process from different wastewater blends, recommendations to future research directions and improvements to enable practical application of algae-based hydroponic nutrients and their commercialisation potentials were described.

Chapter 2

Literature review

2.1. Literature review

2.1.1. Nitrous oxide and global warming

Since the beginning of the industrial revolution, the concentration of greenhouse gases has significantly increased in the atmosphere. In addition to carbon dioxide, the other most important greenhouse gasses are methane and nitrous oxide, which are 25 and 289 times more potent in causing global warming. Most nitrous oxide emissions are from soil-based agricultural activities that use nitrogen fertilisers in the soil (Syakila and Kroeze 2011). From the solar radiation, two-thirds reach the earth by passing through the atmosphere and being absorbed by the earth's surface; the remaining are reflected back to space. This absorbed radiation becomes emitted back as longwave radiation in the form of infrared rays. A massive amount of this energy received by the atmosphere and re-emitted back to the earth's surface is known as the greenhouse effect; without this mechanism, the planet's temperature will be below the water freezing point and non-conducive for many life forms (Signor and Cerri 2013). Water vapour and carbon dioxide are greenhouse gases; additionally, other known gases such as methane, nitrous oxide, ozone, halocarbons, and aerosol are also involved in the phenomenon of increasing atmosphere temperature (Le Treut et al. 2005). Though water vapour is a greenhouse gas in the atmosphere, it is meagrely influenced by human activities (Prather and Holmes 2017), while the problematic greenhouse gases altered by anthropogenic activities are carbon dioxide, methane and nitrous oxide. Each greenhouse gas absorbs the infrared radiation and emits it back as heat; this enables increasing the atmosphere temperature, known as global warming potential. The global warming potential is distinctive of each greenhouse gas, and it is given as a function of its lifetime in the atmosphere and rated in relation to carbon dioxide since carbon dioxide is the most abundant greenhouse gas in the atmosphere (Snyder et al. 2009). Therefore, considering a time horizon of 100 years, methane has 12 years of life and a global warming potential of 25, nitrous oxide has a lifetime of 114 years, and a global warming potential of 298 (Signor and Cerri 2013).

2.1.2. Nitrous oxide from soil-based agriculture

Amongst the anthropogenic activity mediated emissions of nitrous oxide, agricultural soils are measured to produce 2.8 Tg nitrous oxide per year and thus the main source of nitrous oxide (Hénault

et al. 2012). Compared to the other greenhouse gases, nitrous oxide is estimated to contribute to 8% of the radiative forcing globally; amongst human activities, agriculture is estimated to contribute to 14% of the radiative forcing. The magnitude of nitrous oxide emissions from various agricultural nitrogen sources under aerobic conditions and emission of nitrous oxide was more significant with urea than with the other forms of (ammonium sulphate, ammonium nitrate, and calcium nitrate) nitrogen-based fertilisers (Tenuta and Beauchamp 2003). Australian farmers use 1.9 million tonnes of urea per year, study on the effects of urea formulations, application rates and crop residue retention on nitrous oxide emissions from agricultural fields in Australia revealed application of 150 kg nitrogen ha^{-1} as urea evident with annual emissions of up to 3.6 kg nitrous oxide ha^{-1} (Wang et al. 2016). Apart from agricultural fertilisers, pasture-based emissions are another major contributor of nitrous oxide emissions. In grazed pasture-based systems, cattle habitually consume more nitrogen than they need for their growth and productivity. As a result, the extra consumed is excreted in the urine, ensuing a small area of cattle field soils concentrate large amounts of urinary nitrogen (de Klein et al. 2020), and act as a key source of nitrous oxide emissions in grazed pastoral systems (Krol et al. 2016). The production of nitrous oxide from soils majorly involves biological processes. Though, small quantities of nitrous oxide are produced through non-biological means through chemo-denitrification or chemical decomposition of nitrite and hydroxylamine (Bremner 1997). When considering biological processes, there are a number of groups of microbes involved in the production of nitrous oxide (Conrad 1996); however, in general, biological nitrification is the commonly occurring process over chemical conversion. The classical biological process of nitrification is the ammonia oxidation resulting in nitrite to nitrate in aerobic conditions. It is a known process that nitrification by autotrophic bacteria; for example, *Nitrosomonas* oxidises ammonium to nitrite, and *Nitrobacter* oxidises nitrite to nitrate (Bremner 1997). Studies identified ammonia oxidation by *Crenarchaea* in soil (Leininger et al. 2006); these microbes, apart from the production of nitrite and nitrate, can also release nitrous oxide (Blackmer, Bremner, and Schmidt 1980; Molinos-Senante, Hernández-Sancho, and Sala-Garrido 2010). The aforesaid mechanisms act synergistically with other nitrogen metabolising microbial groups and instigate the release of nitrous oxide.

2.1.3. Pollution evading advantages of hydroponic cultivation

Since the industrialisation, the basic natural resources that assist human survival, the fundamental factor of food production that is soil and water, has been tainted by human activities. natural water is being Significantly polluted.; and annually 87% of freshwater is globally used for the purpose of agricultural food productions (Postel 2001). This pollution causes direct and indirect influence in the human system in terms of absorbing these polluted entities into the plant parts that are being used as food. To feed the human population also sustaining with such a polluted environment in the usage of water causes doubling of expenses in purifying the water used for agriculture and the treatment of water after the agricultural process. In the current global climate change scenario, the impact of even a minimal level in the energy expenses are significantly reflected in the advent of the whole picture. Therefore, curtailing every level of energy expenses and carbon footprint is essential. Nutrient production of fertilisers its carbon footprint, and the in terms of nutrient uptake efficiencies; hydroponics is comparatively the best for the curtailment of nutrient waste, efficient water use and carbon footprint (Grewal, Maheshwari, and Parks 2011). Studies have proven that hydroponic techniques in terms of nutrient delivery are superior and result in higher yields than soil-based agriculture (Majid et al. 2021).

In terms of food demand and global climate change, economic viability and eco-friendliness of cultivation systems are considered sustainable farming systems in future. In terms of crop harvest timeline, hydroponic conditions favour the reduction of the number of days stipulated for the cultivation and harvest cycles, also allow possibilities for multiple crops in a year, which are commercially fulfilling to producers benefits. Hydroponic cultivation increases the opportunity to minimise water consumption and increase the yield and profitability of production. Additionally, producing food around the year, especially locally without engulfing arable lands, is an added advantage, representing the hydroponic mode of cultivation systems as a sustainable way of food production in the future.

2.1.4. Wastewater nutrients and their environmental impact

The concept of waste-based economy has been a long-term strategic plan. Due to the pressure of climate change, recently, a paradigm shift happened from waste treatment to the trend of resource recovery (Lin et al. 2016). The waste-based economy approach shifted the focus towards environmental sustainability and opened up investment opportunities into waste markets, which are currently gaining attraction. Here are the wastewater chemical contents, their environmental impacts and consideration of the nature of their nutrient as resource for algal cultivation.

Usually, nitrogen pollution in the environment occurs through the release of nitrogen in three common forms: nitrate, urea, and ammonium (Leong et al. 2004). Generally, they are known to cause eutrophication. In recent decades, these nutrient pollution issues revealed how nitrogen intrusion into the environment is seeping to the level of food safety concerns. Nitrogen intrusion into the environment causes toxic algal blooms is a major concern to the aquaculture industry (Granéli, Weberg, and Salomon 2008). In addition to nitrogen, phosphorus also contributes to the toxic algae bloom formation (Granéli et al. 2008). The major source of nitrogen and phosphorus wastes is from agricultural runoff (Arheimer and Lidén 2000); the other sources of these nutrients are tabularised in Table 2.1. Domestic wastewater contains organic and inorganic sulphur compounds that act as the sulphur source, but the primary source of sulphur in wastewater is dissolved inorganic sulphate (Rabbani et al. 2015). Reports show data that 52% of the inorganic sulphate in wastewater are from the coagulant chemical aluminium sulphate added during the water supply purification; this denotes the source of sulphur in sewer systems mostly from the coagulants than other sources (Pikaar et al. 2014). Sulphur pollution cause serious damage to biological systems, and it is highly toxic to aquatic organisms (Zhang et al. 2008). Long-term exposure of aquatic organisms to sublethal concentrations results in the imbalance of intestinal and immune enzyme activities and their gene expression, cause inflammation and distort the immune system and instigate oxidative stress (Duan et al. 2017). Winery wastewater contains a high concentration of potassium, and studies have shown that winery wastewater pollution is harmful to soil structural stability (Liang et al. 2021). Magnesium is an essential nutrient to the plant kingdom as it contributes the chlorophyll synthesis; however, at concentrations higher than the required, magnesium will yield a toxic effect (Venkatesan and

Jayaganesh 2010); mining industries are the major contributors of magnesium pollution (Canham et al. 2020). Sodium carbonate manufacturing industry (Farmanbordar, Kahforoushan, and Fatehifar 2016) and mining process release calcium wastes. High calcium is a severe threat to both human and animal systems, causing hormonal and vitamin imbalance leading to the renal system's damage and acting as a facilitator of cancerous formation (Endres 2012). High calcium contents in the water cause growth inhibition in microalgae (Gollnisch et al. 2021); this algal growth inhibition manifest changes in the ecology of water bodies as they are primary producers.

Copper from the electroplating industry (Ilyas et al. 2018) leads to environmental contamination, leading to bioaccumulation in crops and cyclically reaching the human and animal systems (Becker and Asch 2005). Vehicle tire-wear particles, mining and smelting are the sources of zinc to the environment (Councell et al. 2004; Zhang et al. 2012), zinc causes lung-related ailments in the human and animal system (Sahu et al. 2013). Mine tailing wastes are the sources of molybdenum and manganese (Lian et al. 2013); exposure of molybdenum and manganese at above the normally required levels cause acute psychosis with visual and auditory hallucinations, seizures (Momcilovic 1999), cardiac, liver, and reproductive systems ailments. Textile, electroplating and tannery wastes and their dust are the sources of cobalt (Muhammad 2013); exposure to such wastes occurring as a contaminant in the environment causes lung diseases (Lison 1996). Such wastes are toxic to the ecosystem; however, they can be used as a resource of algal nutrients if made into an optimal blend that supports algal growth. After repeated reuse, till the level of exhausting the nutrients to direct disposal concentration levels, the post cultivation water can be released to the natural water bodies without treatment.

2.1.5. Real wastewater for algal cultivation and hydroponics

Using synthetic wastewaters for research studies helps understand the overall responses of the intended process (Ak and Gunduz 2013). The advantage of using the real wastewater samples for experimental purposes reveals the actualities, practical difficulties and also yield information on many hidden factors that can result either as an enhancer or hindrance to the targeted task. Many studies involving biological and physical/chemical wastewater treatment methods used synthetic and real samples

simultaneously and concluded with hidden factors that affected the targeted process (Hadavifar et al. 2014). Especially when employing microbes in wastewater treatment and nutrient recovery processes, it is crucial to consider the consequences of associated factors and various matters present in wastewaters leading to synergistic effects either in the immediate initial step or in the intermittent stage of the intended process.

Phosphate removal from synthetic and real wastewater using steel slags showed that calcium release from the slag was insufficient to maintain the optimal calcium concentrations to enable phosphate precipitation, particularly when slags were used to remove phosphate from synthetic wastewater (Ebner 2014). The experiments on phosphate removal from real wastewater revealed that wastewater calcium acted as an additional source of calcium ions that were available for phosphate precipitation, consequently increasing the efficiencies of phosphate removal. Photocatalytic decolourisation of synthetic and real textile wastewater containing benzidine-based azo dyes showed the decolourisation of water contaminated with a benzidine-based azo dye was achieved using solar driven photo-Fenton process (Bandala et al. 2008). In synthetic wastewater, complete decolourisation occurred when using 1mM of iron and 50mM of hydrogen peroxide in 60 minutes of light exposure. In real wastewater containing azo dye and organic matters, decolourisation decreased to 56%; additionally, in the same reaction, increased COD removal was achieved (Bandala et al. 2008). Decolourisation of synthetic and real textile wastewater containing benzidine-based azo dyes using dark solar driven photo-Fenton process and Fenton reaction; showed 90% decolourisation efficiency in synthetic wastewater and 56% in real wastewater. It is identified the influence of organic matter in the reaction (real wastewater) contributed to the effectiveness of decolourisation (Bandala et al. 2008). Microbial community greatly contributes to the content of organic matter matters in the wastewaters (Wang et al. 2017). The diversity of microbial flora in wastewaters varies depending on the industrial process that originates wastes and batch to batch variations. A study on the laccase-catalysed conversion of natural and synthetic hormones from municipal wastewater revealed pH significantly influenced the catalysis process (Auriol et al. 2007). pH variations occur through various factors, especially in the scenario of diverse microbial communities and the environment of dynamic chemistry change circumstances. In

such a facet, investing funds and resources for devising methods will be fruitful if the experimental testings are done using real wastewater of interest.

2.1.6. Strategies for wastewater as a source of nutrients for algal cultivation

Studies have demonstrated the use of untreated raw wastewater for the cultivation of microalgae for biomass and bioproducts (Gupta, Pandey, and Pawar 2016)(Choi, Jang, and Kan 2018); (Lu et al. 2015). Nutrient elements required for algae growth are of two groups; micro and macronutrients (Saha et al. 2013). Amongst, nitrogen, phosphorus and potassium (NPK) are essential elements indispensable for algal growth (Kumari et al. 2014). The chemical complexity of industrial wastewater varies depending on the manufacturing stages and processes involved in the production. Wastewaters can be selected for the blend based on the available type of nutrients to create a mixture that supports algal growth. **Table 2.1** describes the compositions of algal media BG11 (Habibi et al. 2019) widely used for the cultivation of freshwater microalgae, and the type of industries from these nutrient elements discharged as wastewater. Using suitable methods (Michalski 2018) (Baysal, 2013), quantifying the composition of micro and macronutrients of these (Table 2.1) wastewaters would benefit in crafting a blend to reach nutrient levels matching BG 11 so that to attain an ideal effluent-amalgam for algal growth. Similarly, using the different type of wastewater (as nutrients) enacting a fed-batch cultivation method in a way to maintain the nutrient levels and biomass harvesting ratio-balance would help in limiting excess nutrient retention in the cultivation system. This technique would favour the direct disposal of post cultivation water to natural water bodies without pre-treatment. The effective operation of blend-based algal cultivation requires a one-time investment on analytical instrumentation and construction of waterways to transport effluents (Table 2.2) to the blending site. Industries investing in such canal or pipeline constructions would worth for establishing a sustainable assemblage of energy and cost-effective waste utilisation systems. When comparing the overheads of individual wastewater treatments, undoubtedly, this blend assemblage yields a cost-effective method.

Investing efforts in the development of dual-purpose technology would allow access to a tangible, cost-effective wastewater treatment/reuse method; however, this requires a thorough understanding and strategic standardisation of the chemical composition of the water to be treated. Notably, the nutrient contents in the wastewaters (Shi et al. 2014), concentrations (Ziemba et al. 2018), decay time, chemical form/valency changeability (nitrate ⇌ nitrite ⇌ ammonia or sulphate ⇌ sulphite ⇌ sulphide), and formation of a complex with other chemicals are important attributes. Phosphorus is a major nutrient that required for algal growth (Singh et al. 2018). Normally in wastewaters, phosphorous occur as organic compounds or phosphate, upon the growth of algae, due to the prevalence of high oxidative conditions, it will become oxidised to phosphate (Acién et al. 2018). The problem associated with the phosphorus utilisation in microalgae cultivation is the calcium phosphate precipitation that takes place in alkaline conditions (Morales-Amaral et al. 2015). To overcome this, calcium pH and concentration in the blend have to be maintained through out the cultivation system (Posadas et al. 2015a). Algal biomass cultivated from dairy and swine manure wastewater shown to apply as slow-release phosphorus and nitrogen bio-fertiliser (Mulbry, Kondrad, and Pizarro 2007). In such schemes of phosphorus removal from wastewater using microalgae, the occurrence of phosphorous precipitate becomes an advantage; since the formed phosphorous precipitate will get harvested together with the biomass, which favours the purpose and the mode of biomass utilisation as slow-releasing fertiliser (Mulbry et al. 2005).

Considering the micronutrients, it is a great possibility of beitng able to choose candidate strains that does not required vitamins for their growth. Preferably, selecting algal species capable to grow in BG11 would be appropriate, or otherwise, strains with BG 11 adaptable traits benefit their growth in wastewater blends. While blending wastewater sourced micronutrients, the occurrence of more than one trace element in the wastewater may lead to direct interactions between ions (Ting et al. 1991). It is critical to predict their chemical interactions, because, they are not only influenced by their concentrations, valency of metal ion, regimes of algal cultivation/operation pattern, and nature of the influent, but also reliant on the prevailing bio-materials and the sequence in which the elements are

added (Burgess et al. 1999). Therefore, a clear design is essential in blending trace metal micronutrients.

Table 2.1 Industrial wastewaters that can contribute nutrients matching BG 11 composition.
(The chosen wastewater representing a source of particular nutrient may also contain other nutrient elements)

	BG 11	Nutrient element	Wastewater/Effluent source	Reference
1	$NaNO_3$	Nitrogen	Navy - ship boiler tube cleaning	(Arquiaga and Canter 1993)
2	K_2HPO_4	Phosphorus, potassium	Meat – slaughterhouse	(Couillard 1989)
3	$MgSO_4.7H_2O$	Magnesium	Sodium carbonate manufacturing industry	(Farmanbordar et al. 2016)
4	$CaCl_2$ $2H_2O$	Calcium	Sodium carbonate manufacturing industry	(Farmanbordar et al. 2016)
5	Ammonium ferric citrate green	Iron	Iron and steel industry	(Das et al. 2018)
6	$EDTA.Na_2$	Chelating agent	Pulp industry	(Eklund 2002) (PRICE et al. 1988)
7	Na_2CO_3	Inorganic carbon	Sodium carbonate manufacturing industry	(Farmanbordar et al. 2016)
8	H_3BO_3	Boron	Polariser manufacturing wastewater	(Tsai and Lo 2015)
9	$MnCl_2 \cdot 4H_2O$	Manganese	Mine water	(He, Yang, and He 2010)
10	$ZnSO_4 \cdot 7H_2O$	Zinc	Steel processing plant	(Falayi and Ntuli 2018)
11	$Na2MoO_4$ $2\ H_2O$	Molybdenum	Mine tailing effluents	(Lian et al. 2013)
12	$CuSO_4 \cdot 5\ H_2O$	Copper	Electroplating industry	(Ilyas et al. 2018)
13	$Co(NO_3)2.\ 6\ H_2O$	Cobalt	Textile, electroplating, tannery	(Muhammad 2013)

2.1.7. Importance of hydraulic retention time

Cost-effective implementation of sustainable nutrient recovery from wastewater requires a timely control of processes which is essential in the strategy of energy-efficient methods. High rate algal ponds (shallow raceway ponds that circulate wastewater via a low-power paddle wheel) are the recent interest in the majority of research and investments that focused on nutrient removal from wastewater using microalgae and symbiotic bacteria (Christenson and Sims 2011); when using microalgae as nutrient recovery agents studies have shown that the hydraulic retention time in large scale ponds ranges from 4 to 10 days (Craggs et al. 1992). As hydraulic retention time is a component of the harvest method, in an large-scale biomass cultivation, amongst the various considered parameters, the impact of the selection of microalgal species with a quick settling rate is essential in the energy-efficient harvest. This study has fixed a maximum of 15 days of hydraulic retention time to test the nutrient recovery. The hydraulic retention time will also influence the removal of nutrients and organic matter; this reflects the quality of biomass and biomass processing (for example, digestibility) for further desired product production (Arcila and Buitrón 2016), some of the microalgae produce more extracellular polysaccharide upon the culture gets (old) matured (González-Hourcade et al. 2020).

There are various stratagem of factors that influence determining the retention time for an efficient wastewater nutrient recovery. The algal extracellular polysaccharide matrix tend to quickly absorb the wastewater nutrients and then release them slowly (Zhuang et al. 2020); algal species with such trait exhibit pseudo nutrient recovery or lead to cell toxicity due to the high concentration of the accumulated nutrient. The nutrient binding affinity to the polysaccharide is relevant to the chemistry of polysaccharides (Arumugam et al. 2021), which is linked to the growth phase (age) of the algal cells. Usually, matured cells produce more polysaccharides, and young cells are prevalent if adequate nutrients are available in the growing niche (Parwani, Bhatt, and Singh 2021). Optimised hydraulic retention time varies to different intends and plays role in the balance of nutrient recover, product recovery and the robustness of the algal cells.

2.1.8. Wastewater chemicals as inducers for mixed algae and bacterial flora

Several studies have shown the addition of bicarbonate into the cultivation system enhances microalgal biomass and biochemical content (Kim et al. 2017; Pancha et al. 2015; Umetani et al. 2021; Zhai et al. 2020). Soybean wastewater contains rich ammonium bicarbonate (Song et al. 2019). To provide nitrogen nutrient in the form of ammonium, adjust the pH of the cultivation system, and reducing the danger of ammonia release, soybean wastewater is an ideal source of inducer and nutrient inducer. Monoculture based cultivation methods are sensitive to the risk of contamination and loss of product. In the context of using wastewater for algal cultivation, maintaining monoculture throughout the cultivation process will not be practically feasible; as it is unsterile wastewater. There will be mixed algal flora and wastewater contained bacterial community cumulatively act on the nutrient recovery. The nutrient recovering functions of mixed algae or algae-bacteria consortiums have advantages in synergistically enhanced recovery and limiting undesired contamination/invasions. When blending wastewater for algal cultivation, the natural selection or development of microalgae-microalgae or microalgae-bacteria consortium takes place in six phases; [i] quorum sensing, [ii] establishing the relationship, [iii] protection from unwanted partner and invaders, [iv] segregation of synergism, [v] assigning functions between partners, and [vi] progression, maturation and evolution (Padmaperuma et al. 2018). All the six stages described above are susceptible (changeable) to physical, chemical, and biological influences and show their impacts on biomass or nutrient recovery. Amongst these, inducers used to enhance biomass or bioproducts fall into the category of chemical factors, which express their effects in various strata of cellular functions both in algae and bacteria partners.

Quorum sensing is the first stage in the consortia propagation, in which primarily the establishment of communication occurs within and between microbial groups, as depicted in **Figure 2.1**. This includes [i] prokaryotic-&-prokaryote, [ii] prokaryote-&-eukaryote, [iii] eukaryote-&-eukaryote, [iv] phototrophic-&-heterotrophic, and [v] heterotrophic-&-heterotrophic, [vi] phototrophic-&-phototrophic. Biological factors influencing the quorum-sensing alters the affinity and receptor preference of signalling molecules (Lesouhaitier et al. 2009) within the six (Figure 4C) microbial interactions. The chemical factors influencing the quorum-sensing bring modifications to the inducer (chemical) destined to the enhancement of growth or bioproduct accumulation. Inducers present in

the cultivation milieu tend to change the physiology of both the algae and bacterial partners. Chemical factors also influence the mechanism of quorum sensing by producing quorum-signal-inhibitors (Das and Bengal 2019) and by the presence of quorum quenching bacteria (Rehman and Leiknes 2018) that produce enzymes to degrade the communicating molecules of the milieu which cause changes in the scenario of communication. Physical factors influencing the quorum sensing include light, temperature and pH. The temperature of the milieu impacts the bacteria produced "quorum-sensing-enzyme-inhibitors" that affects the (optimal) enzyme activity. Light influences the algal photosynthesis and pH, as algae raise pH at its active growth.

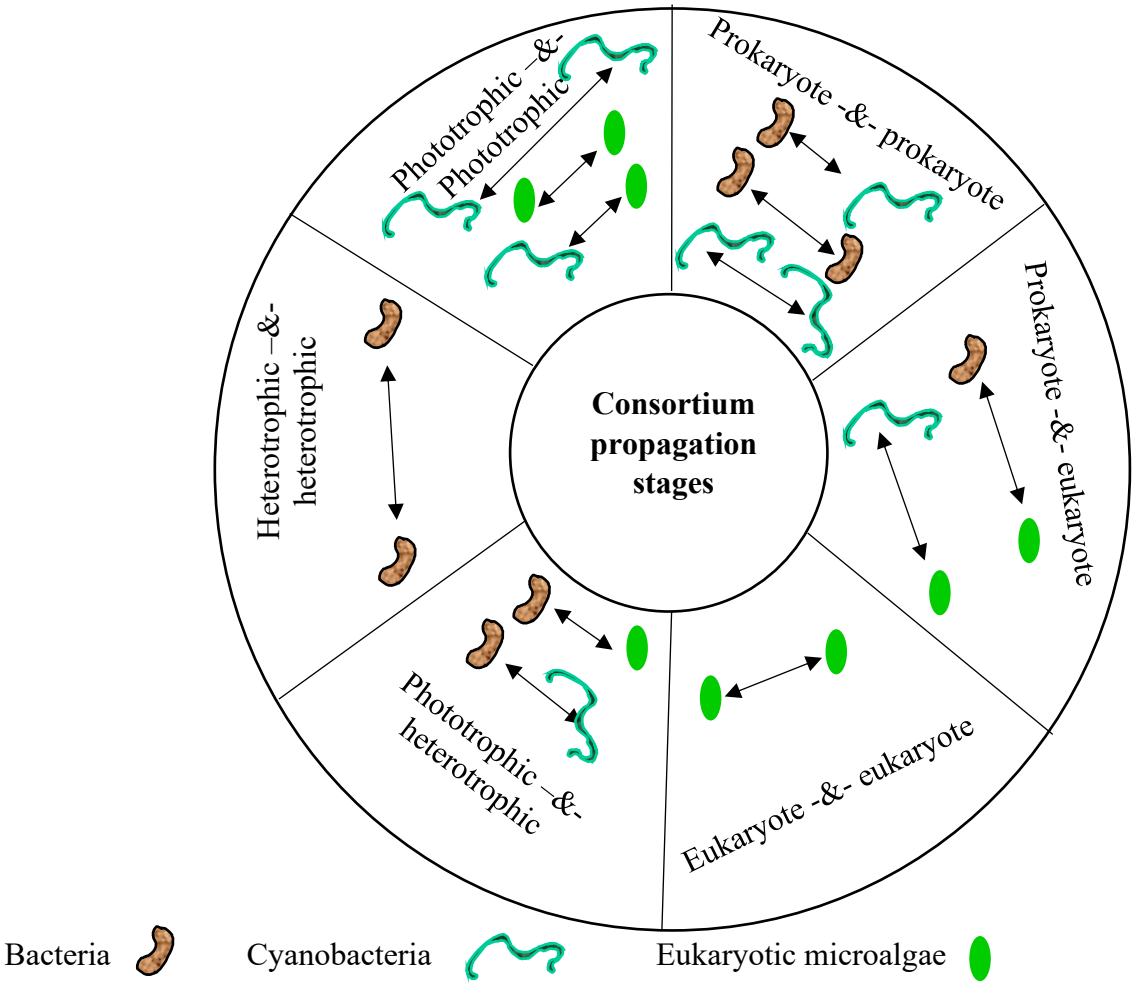


Figure 2.1 Microalgae and bacteria quorum communication within and between microbial groups

2.1.9. Tailoring algal technology based on wastewater nutrients

The actual intention is to use the algal biomass grown using wastewater as a source of nutrients and stimulants (plant hormones of microalgae) for hydroponic cultivation. In addition to the biomass and stimulants for hydroponic nutrient extraction, to make the system cost-effective, there are other products and processes produced as an allied product from microalgae; a few are detailed here. These processes help compensate the net production cost and help the maximum and quick recovery of nutrients and chemicals in the wastewater. The use of wastewater chemicals for subjugating the algal physiology; by steering the biosynthesis of the target product is worth for the sustainable blend based

algal-bio-refinery. Rare earth elements (REEs) are among the best representatives of wastewater-containing chemicals that can steer the algal biosynthetic mechanisms (Goecke et al. 2017). Due to their diverse chemical, electrical, optical, metallurgical, magnetic, and catalytic properties REEs contribute in the manufacturing sectors ranging from fluorescent lamps, batteries, lasers, super-magnets, futuristic high-temperature superconductivity, information storage, conservation and transport of energy kind of devices (Barros et al. 2019). These industries produce contents of REEs in their waste. REEs are challenging to remove from the environment, they are non-biodegradable, and their influence is resilient by their characteristic accumulation throughout the food chain (Anastopoulos et al. 2016). At lower concentrations, REEs have shown to be beneficial for plant growth; they improved the yield and quality of several kinds of crops (Pang et al., 2001).

Microalgae have a variety of applications in agriculture; they facilitate increased nutrient availability, upholding the organic carbon and soil fertility, as well as enhance the growth and crop yields through stimulating the activity of soil microbiota (Ronga et al., 2019). The pollution-free methods of crop production used algal biomass as such, as bio-fertiliser, as well as de-oiled biomass after the biofuel extraction (Nayak et al. 2019). A variety of cyanobacterial species provide nitrogen enrichment through biological nitrogen fixation and enzymatic activities connected to nitrogen mobilisation and interconversions of different nitrogen forms (Abinandan et al. 2019). Algal biomass with bio-accumulated REEs has the potential to increase crop yields by slow-releasing REEs into the soil. The foresaid agricultural benefits are also applicable in hydropnics with algae biomass produced from wasterwater.

There is a variety of REEs containing wastewater ranging from the outlet of acid mine drainage (Nogueira et al. 2019) to urban sewage sludge (Yessoufou et al. 2017), these waste resources could be a potential source for wastewater blend for algal bio-refinery. Optimised culturing conditions with REEs showed enhanced growth of microalgae, lipid and pigment productions (Goecke et al. 2017), as well it was evident that the use of REEs to alleviate the effects of micronutrient (metal) deficiency showed possible substituting effect in their nutrient requirements (Goecke et al. 2015). Microalgae are known for metal bioaccumulation (Suresh Kumar et al. 2015), bio-mining of REEs from red mud (red mud is a by-product of alumina production; contain REEs) using microalgae demonstrated the

REE bioaccumulation/growth potentials, as well showed the replacing (replenishing) effects of REEs in place of micro-elements nutrients, evident by the cultivation of microalgae cultivated in an incomplete nutrient solution without added micro-element nutrients (Čížková et al. 2019). Seagrass revealed selective accumulation of REEs and its affinity to specific REE (Ramasamy et al. 2019), identification of similar mechanisms from microalgae could be explored in blends with mixed REEs for targeted recovery (Giese 2020). Process optimisation for microalgae-based selective REE accumulation has potential on applying the technology in REE wastewater blends, which can also support the replacement or alleviation of micro-nutrient deficiencies of the cultivation milieu (Čížková et al. 2019). Additionally, selective REE(s) accumulated algal biomass can be used as a plant growth stimulator along with the slow nutrient release algal bio fertiliser. REEs indicated limiting effects on toxic-metal accumulation in plant; it has been hypothesised that the application of REEs to soil might improve the yields and inhibit cadmium uptake in foliage (McDowell, Catto, and Orchiston 2015). Commonly, wastewater-based algal biofilm (immobilisation) technologies focus on nutrient removal and often disregarded the aspects of growth, biomass production and bioproduct accumulations (Kesaano and Sims 2014). As a venture of further development of immobilisation technologies, wastewater blends can be utilised in a three-phased method, where the first phase can assign to nutrient removal, the second confine to biomass production, and the third phase dedicates to REE or precious metal recovery. From the outlet of the immobilisation system, further bioproduct accumulation can be induced in suspended cultivation modes in an open pond or closed bioreactors as appropriate.

2.1.10. Monetary worth of wastewater and algal-products

Strategies of conventional wastewater treatment are reliant on high energy and cost for treating wastewater contained chemicals. Cultivation of microalgae in wastewater provides various merits and advantages on economic and environmental deeds, as well yields a sustainable means to produce algal biomass and high-value products. Use of wastewater curtails the need and competition for fresh water; they are rich in nutrients; spares the cost for supplying micro and macronutrient also acts as a means of treatment process through the assimilation of inorganic and organic contents, pollutants and toxicants, furthermore limit the (CO2) carbon emissions and energy expenses that linked to the

wastewater treatment (Samorì et al. 2013) (Chinnasamy et al., 2010; Wang et al., 2010). In terms of the financial value, organic carbon, nitrogen and phosphorus from the cassava processing wastewater generate a value of US $13.7/m3; this validates the potential of its use as nutrient feedstock (Francisco et al. 2015). Studies have demonstrated if the wastewater treatment combined with algal cultivation, the production cost of 1 ton of algal biomass can get reduce from US $808.79 to US $231.59 (Olguín 2012). Without the integration with wastewater treatment algae-based biofuels exceeds US $400/barrel, the combination of algae cultivation with wastewater treatment can lower the production cost less than $30/barrel (Silva et al. 2015). Worldwide, a significant amount of agricultural fertilisers are lost untreated or in the form of partially treated wastewater. The value of agrarian fertiliser grade urea is about US $190.5/ton (Produce 2022), diammonium phosphate $1114 (Index 2022) and potash $562 (Mundi 2022), respectively, henceforward, use of wastewater in algal cultivation considerably saves the cost on nutrients requirements. Algal biomass grown in wastewaters converts the nutrients and pollutants into valuable biomass which is of economic importance and a potent bio fertiliser, they have an advantage of its ability to hold moisture in the soil, and slow discharge of nitrogen and phosphorus as needed by the crop (Coppens et al. 2016).

Depending on the different wastewater treatment methods and mode of algal cultivation yields a variety of quality of biomass or bioproduct from wastewater nutrients. The operational cost estimation for membrane photobioreactors showed around 0.113 US $/m^3, based on a treatment capacity of 5520 m^3/day, which showed advantageous cost comparison rates to regular photobioreactors that usually reach about 0.65–0.96 $/m^3 (Sheng et al. 2017). In wastewater treatment, the hydraulic retention time is important which determines the efficiency of the process in terms of manpower overheads, carbon footprint and other energy expenses. Technologies showed 98–100% removal of ammonium, 70–80% of organic matter, 60–70% of total nitrogen and 40–60% of phosphate removal rates respectively; occurred in high rate algal ponds at a hydraulic retention time of 3–4 days (Posadas et al. 2015b). High rate algal ponds can reduce wastewater treatment costs from 0.22 $ m^{-3} (in activated sludge processes) to 0.17 $ m^3 and even to 0.15 $ m^{3}if the technology combined with the commercialisation of microalgae as biofertiliser (Qi et al. 2013). There is a rapidly growing market for algae-based bio-

production of molecules with health benefits, such as functional foods and nutraceuticals (Borowitzka 2013), worldwide carotenoid market is expected to increase from US $1.24 billion (in 2016) to more than US $1.53 billion by 2021 (Barkia, Saari, and Manning 2019). Eicosapentaenoic acid (EPA) sells for US $2,194/kg (99% purity) and US $185/kg (50–70% purity) (Komolafe et al. 2014). The prices are US $ 2500-7000/kg for astaxanthin is, $ 300-1500/kg for β-carotene, $ 80-160/kg for Omega-3 fatty acids, $ 44/kg for *Chlorella* biomass, and $ 42/kg for *Arthospira* biomass (Barkia et al. 2019). The price differences are due to the biomass quality, content of pigment concentration on particular algal biomass, and type/expenses of required extraction methods. Blend based algal cultivation allows flexibility to determine the quality enhancement of biomass and aiming to specific bioproduct synthesis and accumulation deeds.

2.1.11. Benefits of blended wastewater nutrient recovery

Blending wastewater has various benefits, especially high concentration wastewaters, in which both the chemical and physical conditions will not be supportive for the growth of algae due to high concentration levels of nutrients and other chemical constituents. To overcome the growth-suppressive effect of these high concentration wastewater chemicals, wastewater with low nutrient levels can be used as a kind of diluent to mix with the high concentration wastewater to bring the nutrient levels optimal for algal growth. An example of low concentration wastewater is sewage water with a chemical oxygen demand concentration lower than 1000 mg/L or a biochemical oxygen demand concentration lower than 500 mg/L, mainly sewage and diluted industrial wastewater (Kang et al. 2017). Due to the lack of nutrient sources, low concentration wastewater will not be conducive to microbial growth. It is also possible to use low nutrient wastewater resources by blending with nutrient-rich sources like nitrified urine (Wilde et al. 2019). Nitrified urine contains all micro and macronutrient required for algal growth (Martin et al. 2020; Volpin et al. 2020a). Unprocessed direct human urine is also a good source of nutrients; however, the likelihood of using the produced algal biomass in food or feed grade bioproducts is constrained, due to its contents of pharmaceutical products, as urine is the excretory system for pharmaceutical categories of chemical compounds from the human system (Behera et al. 2020). The production of nitrified urine is a process where the

biological nitrification makes the complex form of nutrient sources into a simple absorbable form; for example, conversion of urea, ammonium to nitrate, this process also renders in the alterations of the chemical nature of excreted pharmaceutical compounds if any (Volpin et al. 2020b).

Highly concentrated wastewater does not allow microbial growth because of the presence of a high concentration of nutrients and toxic substances, which will be toxic to many microbial groups; very few of these species have the stability to survive in high nutrient concentrations and succeed to grow (Zhou et al. 2011). The characteristic, as mentioned earlier, is an advantage; the high concentration wastewater can function as a mode of restraining the undesired microbial groups if the wastewater nutrients are intended to use for the monoculture algal cultivation. Usually, the pathogenic form of bacteria is sensitive to hypertonic nutrient environments (Barzily and Kott 1991). Algal growth increases the pH of the growing medium, which will not be suitable for the survival of pathogenic forms of bacteria; therefore, the algal cultivation itself acts as a decontaminating mode when blending wastewater with pathogenic bacteria (Mezrioui et al. 1994).

Understanding the growth phenotype and physiology of carbon requirement of algae is an opportunity to improve the particular species of interest in large-scale cultivation. Bicarbonate is a known algal biomass enhancer; wastewater with a high concentration of bicarbonate can be used as an inducer (Zhou et al. 2011). Microalgae that has a characteristic trait for the selective preference of bicarbonate as carbon source can be enhanced using the blend of the right proportion of bicarbonate containing wastewater. Microalgae are photosynthetic, and the availability of light is essential in the case of dark effluent or colour wastewater, for example, textile dye wastewater (Palanisami and Lakshmanan 2010). Low and high concentrations of wastewater can be used to dilute dark wastewater to facilitate the light availability for active photosynthesis of microalgae. Wastewater with toxic chemicals can also be used as a diluent in the right proportion; it can act as a factor of Hormesis (Palanisami and Lee 2014) which is the stimulatory effect of a low concentration of toxic chemicals in the organismal metabolism (Teeguarden et al. 2000). Therefore, wastewater with toxic chemicals is an advantage in the blend-based wastewater nutrient recovery technology. Microalgae can simultaneously remove organic and inorganic nutrients from the wastewater (Mujtaba et al. 2015). The phenotype of different algal groups has uptake capacity to different categories of organic and inorganic forms of nutrients

accustomed naturally by the wastewater contained chemicals. Therefore blending wastewater with the high organic nutrient content allows the opportunity to recover nutrients from the blend based nutrient recovery method. Effluent polishing is a process in wastewater treatment meant to remove additional suspended solids; usually, effluent polishing is performed in the physiochemical method of wastewater treatment using filters that can filter fine particles. Microalgae are known to be used in the process of affluent polishing (Sheng et al. 2017b), which minimises the use of filter materials and enables renewable green technology.

2.2.1 Safe source of wastewater for food grade biomass production

This research intends to use wastewater as a source of nutrients for the cultivation of microalgae and then use the algal biomass as a nutrient source for hydroponic cultivation. Therefore, the process involves food or feed grade biomass production, which should not fetch toxic chemical entities from wastewater. For this reason, blending wastewaters are selected based on the safety aspect, both in the form of pathogenic bacteria and harmful chemicals.

Post cultivation nutrient-deprived hydroponic wastewater can be used as a source of nutrients for microalgae cultivation (Richa et al. 2020b). In general, the nutrient water in hydroponics forms is recycled; however, the reused water should be released after some point due to the accumulation of nutrients and other remaining matters not absorbed by the plants (Hultberg, Carlsson, and Gustafsson 2013). Therefore, wastewaters of hydroponic farms need treatment before being released into the natural environment (Delrue et al. 2021). Among the various treatment methods of hydroponic nutrient wastewaters, microalgae-based treatment is most efficient for the treatment and simultaneous nutrient recovery (Richa et al. 2020b). Recently, research attention shifted towards cultivating microalgae in the waste nutrient solution of hydroponics as a secondary and tertiary treatment mode (Matos et al., 2017). This algae-based method uses leftover hydroponic nutrients to limit its release of natural water bodies that lead to eutrophication.

The demand for clean water to the growing population and industrial activities led to the desalination of seawater successfully implemented in many countries. Though the technology of desalination is well established and contributes positively to water needs, many associated environmental concerns

exist with the desalination plant, especially the concentrated waste from the desalination process (Zhou et al. 2013). The adverse effect of desalination brine on aquatic organisms is its high salinity/salt concentration (Ahmad and Baddour 2014). Even in small quantities, the desalination brine causes harmful effects to marine organisms not only in the short term but there are also long-term environmental effects to the marine life forms that are well studied and recognised (Matsumoto and Martin 2008). The desalination concentrate has high nitrogen content, and blending other wastewater containing phosphorus, will be suitable for microalgae cultivation (Sánchez and Matos 2018). Reports render evidence that blending treated urban wastewater with reverse osmosis desalination concentrate can be sued for agricultural irrigation (Bunani et al. 2015); the same will be suitable for microalgae cultivation.

Nitrified human urine is an excellent source of microalgal nutrients; the bioreactor based nitrification process involves actions of the bacterial community (Volpin et al. 2020b) that has the potentials to remove other pharmaceutical pollutants from urine, making it safe for the use of food and feed grade biomass. Removing pharmaceutical compounds from nitrified human urine is possible (Almuntashiri et al. 2021); however, the process is not authentic in the complete removal of undesired chemical compounds that quality food grade biomass. Secondary treated domestic wastewater is an excellent source of algal nutrients; however, the concentration of nutrients varies from batch to batch (Ben-David et al. 2021). There are several research evidence showing the possibility of using mere secondary treated domestic wastewater for the cultivation of microalgae (Fernández-Linares et al. 2017; Gómez-Serrano et al. 2015; Sydney et al. 2011). In the case of blend-based wastewater nutrient recovery use of high concentration wastewater with the secondary treated domestic wastewater yields optimal biomass.

2.3. Microalgae harvesting technology

Microalgal biomass harvest refers to the separation of grown algae from its growing nutrient solution. Principally, the method of harvest depends on the features of the selected microalgae and the nature of the targeted bio-product; this includes the culture density, cell size stipulations of the final product and amiability for reusing post-harvest nutrient liquid. The various algae harvesting methods are mechanical, chemical, biological and electrical-based methods. The mechanical mode of microalgal

biomass harvesting methods is the most commonly used and reliable for intact harvest (Molina Grima et al. 2003). When planning for the harvest of biomass for its use as a source of hydroponic nutrients, especially in the context of this project, the cytosolic content is important, which contains both the fixed nitrogen, other nutrients and plant hormones. There are various methods that include coagulation/flocculation, flotation, electrical-based processes, filtration, centrifugation. There are various advantages and disadvantages considered to selecting a strategy that fit the requirements. Coagulation/flocculation is a fast and easy technique that can be successfully used for large-scale cultivation systems, cause minor cell damage, and applicable for various species, less energy requirements, and is inexpensive.

The disadvantages of coagulation/flocculation are that coagulating or flocculating chemicals have to be applied to facilitate the coagulating process, and difficult or extra steps have to be implemented to separate coagulant from the harvested biomass, influence the reuse of post-harvest nutrient liquid, high risk of secondary contamination or pollution (Molina Grima et al. 2003). Electrical based processes apply to all unicellular and filamentous species, with no chemicals required; however, the used metal electrode cause metal contamination and interfere in the cell intactness (Chen et al. 2015). Filtration is efficient for high biomass recovery, no chemical process and low energy consuming especially low shear stress to the cells resulting in favour of the maintenance of cell intactness, but, the process will be slow, not suitable for small-sized algal species fouling or clogging of filter mesh and the replacement of filter increases operational costs and maintenance, high energy consuming (vacuum) process. Centrifugation is a fast and effective method with a high biomass recovery rate. However, it is an expensive method requiring high energy expenses, time-consuming and not feasible for large scale systems, particularly more chances of cell disruption (Dassey and Theegala 2013). Settling/flotation is a suitable method for large scale biomass systems, require low operational space and cost and quick operating time; also, the selected species *Anabena circinalis* had the character of fast settling trait.

2.4. Microalgal biomass as hydroponic nutrients

The modern agricultural method is highly dependent on fertilisers for crop production; the more extensive use of chemical-based inorganic fertilisers become a severe threat to the environment and human health (Timilsena et al. 2015). To overcome this, biologically sourced bio-fertilisers are used for the safer and long-standing sustainable agricultural process (Mukhuba et al. 2018). Biofertilisers are organic and inorganic nutrient compounds from live microorganisms for plant growth; this may be single or microbial consortia supplying essential nutrients and stimulants or extracted products from microbes (Win et al. 2018). Microalgae, both eukaryotic and prokaryotic (cyanobacteria), has agricultural applications; they facilitate increasing nutrient availability. The use of prokaryotic microalgae (Bharti et al. 2019; Salamah et al. 2019) and eukaryotic (Zhang et al. 2017) in hydroponic cultivation is reported as a co-cultural method. The nutrient solution of hydroponics has micro- and macro-nutrients that support the growth of microalgae; the microalgae will yield organic nutrients, and primarily cyanobacteria are known for fixing nitrogen from atmospheric air, supporting the plant nitrogen needs (Bharti et al. 2019). The use of microalgal extract in hydroponics is rare, to the best of our knowledge, no reports are available; therefore, this method was patented (Palanisami 2021). This study used the acid digested prokaryotic microalgal biomass as a source of hydroponic nutrients and stimulants (plant hormones).

Nitrogen is the second most abundant chemical element present in the microalgal biomass; its content ranges from 1% to 14%; this is equivalent to around 5–10% of the dry weight that constitutes nitrogen (Markou et al. 2014b). Nitrogen is present in the biomass biochemicals in cellular components such as amino acids (proteins), nucleic acids (genetic materials), and pigments such as phycocyanin and chlorophyll (Gupta and Mathews 2010). Strong acid digestion of biomass yields hydrolysed form of nitrogen from the foresaid biomolecules (Doneen 1932). Usually, nutrient solutions in hydroponics are maintained in acidic pH (Koehorst et al. 2010). The low pH is to facilitate the roots for optimal nutrient absorption. Since the nutrient solution contains rich micro and macronutrient components, microalgal contaminants are constantly encountering it. Usually most algae grow at pH above neutral; the acidic pH of hydroponic nutrient solution creates an unfavourable algal growth environment; also, hydroponic systems often require concentrated or dilute sulphuric acid for maintenance of pH (Taylor

et al. 2021). Therefore, the use of concentrated sulphuric acid will be feasible and applicable for the digestion of algal biomass and use it as a nutrient source. The hydrolysed organic form of biomass contained nutrients is an advantageous for the hydroponic roots, as they have affinity to absorb amino acids and other organic forms of nutrients (Chen GuiLin, Gao XiuRui 2002). Phosphorous is one of the most important nutrients for microalgae, and its biomass content varies from 0.05% up to 3.3% (Markou et al. 2014b). The potassium content of microalgal biomass varies from 1.2% to 1.5% (Tokuşoglu 2003); however, it could be as high as 7.5% (Markou et al. 2014b). The magnesium content in microalgae biomass ranges around 0.35% and 0.7%; however, a content as high as 7.5% can be found in some species (Markou et al. 2014b). Sulphur content in biomass ranges from 0.15% to 1.6%, and calcium content in microalgal biomass varies from 0.2% to 1.4% (Kay and Barton 1991).

In addition to macronutrients, micronutrients that include copper, zinc, cobalt, manganese, molybdenum are also present in microalgal biomass. Moreover, microalgae are known for bioaccumulation of these metal nutrients in excess if when exposed to high concentrations of metals. Therefore, the acid digest of algal biomass has a complete set of nutrients required for plant growth. Prokaryotic microalgae are known for their nitrogen fixation physiology; it is the emphasis of this study, nitrogen nutrients from microalgae. Additionally, the algae fixed nitrogen in its cytosol will be existing in different nitrogen nutrient forms; nitrite, ammonium, and urea. Hydroponically growing plants are known for the use of different forms of nitrogen sources, ammonium (Savvas et al. 2006), urea (Luo et al. 1993) and nitrite (Hoque et al. 2008).

2.5. Microalgae as hydroponic growth stimulants

Microalgae naturally inhabit agricultural areas, as the farming fields are rich in nutrients, these inhabited algae involve in biological nitrogen fixation (Alvarez et al. 2021), help in phosphate solubilisation, improving mineral conversions, and facilitating release of absorbable forms of nutrient ions that promote fertility and crop production (Kumsiri et al. 2021). In addition to naturally fertilising and plant nutrient mineral balancing, microalgal groups are known to release a variety of biologically active substances that include proteins, vitamins, carbohydrates, amino acids, polysaccharides and

phytohormones; function as elicitors to promote plant growth and also contribute to the process to fight against abiotic and biotic stress (Singh 2014). Attention to plant biostimulants has increased over the past decade, motivated by the growing interest of scientists, extension specialists, industries, and agricultural growers in using these products in various eco-friendly sustainable methods to warrant improved production and stable crop yields (Rouphael and Colla 2018). According to the recent European Union regulation (2019/1009), plant biostimulants are 'fertilising products able to stimulate plant nutrition process independently of the products nutrient content' (Colla and Rouphael 2020). Based on this regulation, plant biostimulants are referred through claimed agronomic effects, such as improved efficiency of use nutrients, crop quality and tolerance to abiotic stressors. This description includes numerous organic and inorganic substances and/or microorganisms such as humic acids, hydrolysate proteins (Paul et al. 2019), carbohydrates (Rachidi et al. 2021), extracts of seaweed (Mukherjee and Patel 2020), mycorrhizal fungi, bacteria, eukaryotic microalgae and algae that fix nitrogen. Amongst these direct (nutrient metabolism stimulation) and indirect (nutrient uptake and root morphology modulation) mechanisms by which microbial and non-microbial plant biostimulants improve nutrient quality, plant output and physiological status, resistance to environmental stressors, and stimulate plant microbiomes, this work is confined to plant hormones (phytohormones) from microalgae. There are few macroalgae/seaweed-based plant bio stimulants available in the market, but there is no successful product from microalgae to date. The products that are available in the market are not have the claim of the content of phytohormones. Therefore, as an effort, this study selected this area to understand the possibilities of its source from microalgae.

Several microalgae species belonging to the family of *Charophyceae, Chlorophyceae, Trebouxiophyceae*, and *Ulvophyceae* were reported for their phytohormone activities including, auxins, cytokinins, gibberellins, abscisic acid, and brassinosteroids (Ronga et al. 2019). Brassinosteroids are polyhydroxy steroids, they have a common a-cholestane skeleton, and their varieties vary from the types and the orientation in the rings and side-chain (Vardhini et al. 2006). These are the new class of plant hormones with the potential to improve crop productivity. Reports are scant about the microalgal brassinosteroids, and little is known about the physiological role of brassinosteroids in algae; so far, the presence of brassinosteroids detected in 25 algal species (Hayat

et al. 2019). All the available reports on brassinosteroids are from eukaryotic microalgae; there are no reports on the existence of procaryotic microalgae.

Microalgal extracts are used in seed germination and crop cultivation since they contain growth-promoting substances and their stimulating effects on the ability of crops to endure abiotic stresses, such as high salinity, hot temperatures, mineral deficiency and drought (Bello et al. 2021). However, these studies used either a foliar spray (Godlewska et al. 2019) or soil or solid substrate-based cultivation. A recent study showed Spirulina (prokaryotic microalgae) extract as a stimulant in hydroponic systems; however, the study does not investigate the nutrient contribution from algal biomass to hydroponic plants (Leonard et al. 2021).

2.6. Contemplation of harmful algal species in hydroponic systems

Hydroponic growing systems with nutrient solution recirculation are rich in nutrients and a perfect nutrient environment for microalgal invasion and growth; however, it is unclear whether harmful algae will adversely impact the hydroponic crop (Schwarz and Gross 2004). Reports are scant, and to the best of our knowledge, there are no reports available on the intrusion of toxic microalgae in hydroponic systems and its harmful effect detected in the plant system. On the other hand, a variety of prokaryotic (Bharti et al. 2019; Spiller and Gunasekaran 1991) and eukaryotic (Christenson and Sims 2011) microalgae are used along with the growing plants in hydroponic systems as a co-culturing method. It is reported that the release and accumulation of toxic root exudates from the hydroponically growing plants excretes in the nutrient solution leads to autotoxicity of plants (Richa et al. 2020a), (autotoxicity is a phenomenon whereby a species inhibits growth or reproduction of other members of its same species). These substances are sugars, amino acids, and organic acids; most are organic substances from the plant root released in huge quantities (Farrar et al. 2003). From our speculation that these released root substances are reported to cause autotoxicity of plants (Richa et al. 2020a), as microalgae are also microscopic sized plants there may be an functional inhibition of toxin producing microalgae. Studies on *Acorus calamus* indicated that roots extracts could inhibit harmful toxin-producing cyanobacteria, and it is suggested that the extract of the root can act as a natural agent to manage harmful cyanobacteria (Microcystis) blooms (Zhang and Benoit 2019; Zhang, Zhang, and Li 2016).

Additionally, it is evident that herbaceous plant extracts have the antialgal property (Sinang et al. 2019).

2.8. Treatment and reuse of hydroponic nutrient solution

Among the various benefits of hydroponic cultivation, the round the year production is a striking advantage that balances the market need and supply, the other benefits include less grow time, pest and disease incidence, and no weeding compared to conventional growing (Sharma et al. 2018). One of the other important benefits is the well-defined use of nutrients (Grewal et al. 2011). Though the nutrients are used efficiently, many hydroponic growers reuse them for several grow batches by topping them up with nutrients. However, at some point, a particular nutrient element accumulates and becomes non-conducive for roots to absorb or become toxic, eventually resulting in less crop productivity. Globally, tomato and cucumber are popular in hydroponic production (Sabir and Singh 2013). Monthly loss of nutrients (kg ha^{-1}) during tomato and cucumber cultivation in hydroponic cultivation systems is shown in Table 2.2; remarkably, the loss of secondary nutrients, potassium, calcium and magnesium was high, yet, their recovery received less attention compared to nitrogen and phosphorus (Richa et al. 2020a).

Table 2.2 Maximum monthly losses of nutrients (kg ha^{-1}) during cultivation in hydroponics (Richa et al. 2020a)

Nutrient element	**Tomato** (kg ha^{-1})	**Cucumber** (kg ha^{-1})
Nitrogen	240	231
Phosphorus	54	41
Potassium	413	337
Calcium	178	106
Magnesium	57	44
Sodium	33	51
Chlorine	10	34
Sulphur	101	90

There are various methods adopted for the treatment and reuse of used hydroponic nutrient wastewater:

(i) Denitrification-based treatment of hydroponic waste nutrient solution (Rodziewicz et al. 2019). The concentrated hydroponic wastewater with nitrate and minerals subjected to biological

denitrification is an efficient process that removes nitrogen from hydroponic wastewater using facultative anaerobic microorganisms (van et al. 2006).

(ii) Constructed wetlands-based treatment of hydroponic waste nutrient solution. Constructed wetlands are planted with common reed (*Phragmites australis*) or common bulrush (*Scirpus lacustris*), which remove nitrogen and phosphorus by directly incorporating them into plant biomass (Zubair et al. 2020).

(iii) Microalgae-based treatment of hydroponic waste nutrient solution.

Microalgae-based wastewater treatment is the most prominent method for the treatment and advanced nutrient recovery from hydroponic wastewater (Li et al. 2019).

(iv) Activated carbon-based treatment for root exudates removal. Activated carbon method, especially for the removal of root exudates from hydroponic nutrient water. This method is advantageous as it possesses a high affinity for low molecular weight organics, low energy requirements and low operating cost (Mwakabole et al. 2020).

(v) Advanced Oxidation Processes have attracted attention for treating used hydroponic nutrient water because of its effective decomposition of organic substances (Liu et al. 2019).

2.9. Conclusions and knowledge gaps

In this literature review, an overview on the use of microalgal biomass as plant nutrients and stimulants, their nutritive constituents, possibilities of algal biomass production using different safe wastewater sources, connotation on the mixed algae and bacteria contained in the wastewater in the context of biomass production and nutrient removal, wastewater contained chemicals as an enhancer, benefits of making wastewater blend, contemplation of toxic are harmful algae, possible reuse of hydroponics nutrient solution has been provided. The specific conclusions of this literature review are as follows:

● The demand of food production to the growing population and the parallel issue of balancing climate change requires a shift in the agricultural production methods that meet the market demands and the stipulations of the climate change criteria. Especially in terms of the emission of nitrous oxide. Hydroponics is the method that has the potential to reduce nitrous oxide emissions compared to

conventional soil-based agriculture. Therefore, shifting to the hydroponic way of food production to all the maximum possible crops that can be grown using hydroponic systems.

● Hydroponics will also cause pollution in terms of the release of waste nutrients post cultivation. Biological based nutrient sources can be a solution to overcome the nutrient pollution from the hydroponic mode of cultivation.

● Production of microalgae-based nutrient and stimulant help in the reduction of carbon footprint related to the industrial production of plant nutrients and stimulants; additionally, the production of microalgal biomass for such purposes from wastewater nutrients is the three-way benefit that

(i) treats the wastewater, thus saving energy and expenses of water treatment,

(ii) utilise the wastewater nutrient resource, and

(iii) supply nutrients and stimulants for hydroponic cultivation.

● Blend-based wastewater utilisation helps limit the use of physicochemical methods needed for the treatment of concentrated wastewater. This enables benefits to both sides, limiting the resource required for the wastewater treatment of concentrated wastewater and utilising the wastewater contents as a resource for useful product production.

● It is evident from the literature that the algal biomass itself acts as a source of nitrogen, phosphorous, potassium, sulphur, copper, manganese, zinc, cobalt, molybdenum.

From this literature review, the following gaps in research have been found in relation to the use of algal biomass as a source of hydroponic nutrients and stimulants:

→ Usually, hypersaline marine microalgal species are used in the bioremediation of seawater desalination brine wastewater. Use of freshwater species has not been investigated.

→ Cyanobacteria (prokaryotic microalgae) are recognised for their nitrogen fixation abilities. The use of algae biomass as nitrogen bio-factories for hydroponics cultivation yet to be investigated.

→ Microalgal biomass (cytosol and cellular components) contain nutrient minerals. The use of algal biomass extract as a sole source of nutrients to hydroponic roots has not been effectively quantified and investigated.

→ Occurence of plant hormones in microalgae are well studied, but their biosynthetic profile differences in nitrogen and nitrogen less large scale cultivation have not been analysed.

Chapter 3

Blending different wastewaters for algal cultivation and nutrient recovery

3.1. Introduction

Water is a natural resource essential for life, and available in various sources such as lakes, rivers, streams, and oceans. During the last century, the explosion of human populations and activities has caused a drastic reduction in clean and safe freshwater (Keiser and Shapiro 2018). Disposal of waste originated as a cause of human activities in reservoirs poses a serious threat to the environment that affects all layers of organisms in the ecosystem (Mukherjee and Ghosh 2016). To conserve water bodies, a clear understanding is required about sources of wastewater and their components that cause pollution. The three sectors, domestic, industry and agriculture, are the three most important sources of wastewater (Wang et al. 2020). This wastewater is rich in organic and inorganic components (nutrient elements). When such nutrients are released excessively into the water, they increase the minerals and organic nutrients, resulting in plants and algae growth, therein eutrophication (Preisner et al. 2021). These waters can be used or reused as a source of nutrients if subjected to a strategic nutrient removal process. First, it is necessary to know the contents of wastewater, its chemical characteristics, the safety of its use in a feed or food-grade production process so that suitable technologies can be adopted for wastewater treatment before they are released into natural water bodies or recycled. Notably, a method that can yield useful products and the process that allows the direct release of the water into natural water bodies is needed for the current criteria and stipulations of waste disposal policies.

With current attention on global warming and resource recovery from waste, there is a trend of utilising eco-friendly wastewater treatment methods to replace conventional treatment systems (Khiewwijit et al., 2015). It is projected that in 2030, the world will face a 40% water scarcity which will bring an overwhelming situation in social and economic development and wellbeing of human beings (Sun et al., 2016). Such water scarcity may emerge as a cause of growing water demand, contamination of water resources, and technological deficiencies of the appropriate mode of reclaiming used water of varied chemistry and characteristics. Large volumes of wastewater generated by industrial processes, agricultural and urban activities with excess nutrients would inevitably create eutrophication in the aquatic environments. Factual analysis of the prediction of such water scarcity with an example of

populated China, reveals the root causes being rapid industrialisation, urbanisation, and anthropogenic activities exerted significant pressure on natural resources and the environment's health (Meng et al. 2021). Nitrogen, organics, and phosphorus have contaminated almost 80% of China's rivers and waterbody to different degrees(Qu and Fan 2010). Transiently, the same scenario manifests in other countries given its population growth. With the current technological capacities of wastewater treatment, only 16.6% of municipal and domestic wastewaters were reused; amongst these, the nutrient recovery rates of nitrogen, organics, and phosphorus are 35.8%, 35.8% and 35.7%, respectively, representing 70% utilisation potential and treatment rate (Sun et al., 2016). This lucidly indicates impending room for improvement of wastewater nutrient recovery and reuses. Conventional wastewater nutrient removal methods, such as sedimentation, coagulating, activated sludge, nitrification, denitrification, and chemical mode of phosphorus removal, are facing challenges to satisfy the strict standards of nutrients discharge. Especially maintaining low process costs combined with good nutrient recovery efficiencies is not easily achieved in the conventional methods (Kumar and Pal 2015). Furthermore, other disadvantages on conventional methods, such as carbon emission, high energy consumption, long process, excess sludge discharge, instability treatment effect, recyclable resource-wasting, are also drawbacks to opt them as a sustainable method of wastewater treatment with evident low energy/carbon requiring and resource recycling (Li et al. 2019).

Reusing wastewaters from any known industrial processes allows the opportunity to design treatment methods that can use the waste as a resource to its fullest capacity of utilising every individual chemical entity of the wastewater. Conversely, the treatment of mixed industrial wastewater is challenging due to its high chemical complexity (Nidheesh et al. 2020). In general, medium and small scale industries spend more operational costs on wastewater disposal and treatment. Usually, developed countries have organised criteria and policies for establishing and constructing an industrial set-up that includes disposal plans of the produced waste. In many developing countries, small industries are unaffordable to install individual treatment plants due to inadequate space and specialised operational human resource skills. In such a position, they organise for a common effluent treatment plant (Popat et al. 2019). In such common effluent treatment plants dealing with mixed

wastewaters applying eco-friendly and cost-efficient methods is essential to curtail secondary pollution and sustain human and environmental health.

The conventional physiochemical method based wastewater treatment can directly operate with raw and highly contaminated wastewater. Biological process or using wastewater for microalgal cultivation requires safe wastewater; this includes biologically safe or non-toxic water for the microbe that remediates the wastewater or the algae assigned to grow using such wastewaters. As the microalgae are photosynthetic, concentrated and coloured wastewaters impact algae's light quality. Microalgae based wastewater treatment methods are known for their low energy consumption, biologically utilising the sludge (Baek et al. 2010), consistent treatment process, generation of recyclable resources and biomolecules (Hussain et al. 2021). Worldwide, bio-engineering of microalgae-based bioenergy and high-value bioproduct productions rigorously working on reducing the net production cost. The mission of reducing the production cost targets effort on reducing the investments in the production process and use of less expensive materials and apply methods of reusing renewable resources like wastewater nutrients (Yao et al. 2015). Incorporating such reusable resources paves the way to reduce production costs. When it comes to a cost-effective method of nutrient recovery from wastewaters, it is essential to consider whether the process can yield food-grade nutrients or biomolecules that are feasible to apply or reuse in animal feed or human food-grade materials (Gopinatha et al. 2019). The yield of food and feed grade wastewater recovered resources, either nutrient element or bio-molecules, compensate to higher degrees in the net production cost of bioproduct. Therefore, production methods that can yield food and feed grade biomass and bio-products are of great importance in wastewater green technology. Based on the approach described above, this study used safe wastewaters not to be concerned about pathogenic microbes or chemical pollutants present in the wastewater.

Raw industrial wastewaters have no universally applicable treatment process because of their complex chemical composition and toxic contents from varied sources and process stages (Li et al. 2019). In the meantime, wastewater sources such as secondary treated domestic wastewater contain less

nutrients than most industrial wastewater (Gómez-Serrano et al. 2015). Heavy concentrate wastewaters like desalination brine are mostly rich in nutrient contents (nitrogen and phosphorus) rather than toxic chemicals (Zarzo et al. 2014). Wastewater sources such as used or post cultivation hydroponic farm wastewater and nitrified urine (Feng et al. 2008) contain essential nutrients for microalgae growth. Designing a microalgae-based wastewater treatment process would be assessed case by case based on wastewater's physical and chemical characteristics. Through microalgae growth, wastewaters are usually considered as micronutrients (copper, zinc, cobalt, manganese, molybdenum) and macronutrients (nitrogen, phosphorus, potassium, calcium, magnesium, sulphur). Some specific microalgae species could remove toxic heavy metals and high concentrations of nutrients in wastewater, while others are sensitive to even low concentrations of metal elements. When considering micronutrients, are actually metal elements, higher nutrient concentration in wastewaters can be used to blend with wastewaters with low nutrient concentration to make it optimal for algal growth.

Every nutrient element has its characteristic chemical property, for example, redox potential (Droop 1961) and biologically essential or non-essential in the physiological role. Depending on their concentration, either low, optimal or higher concentrations instigate cellular changes in microalgae. Some nutrient elements, merely as an element, react and form complexes or attain an unavailable state to the process of microalgal nutrient uptake. It is shown in prokaryotic microalgae; nutrient availability is significantly influenced by other ions, such as calcium and phosphate, which alter the zinc physiology (Cavet et al. 2003). As well, every species of microalgae has its characteristic trait of preferring nutrients in different growth stages. It is unpredictable in mixed (blend) wastewater; the influence of the concentration of every metal element instigate varied effects on the algal system. Identifying the specific nutrient element that pronounces impact on the uptake of other nutrient elements or some nutrient element acting as a 'key' entity facilitates the utilisation of other nutrients in the wastewater.

This chapter deals with the growth efficiencies and nutrient recovery of inoculated cyanobacteria and innate wastewater microflora in different wastewater blends. Also, the selective control of micro and macronutrient uptake was plotted to identify the most influencing nutrient element and the key nutrient element that influences the uptake of other nutrients in the given nutrient content of a wastewater blend and the overall nutrient uptake patterns. The aim is –

- To utilise different wastewaters as a source of nutrients for algal cultivation, (ii) to use low and high nutrient concentration wastewaters as a diluent to attain favourable algal growth and nutrient recovery, and
- To test the influence of retention timelines on the different nutrient recovery profiles.

These aims manifest in the objective of (i) green technology means of wastewater utilisation, (ii) evading the obsessive use of the energy-expensive chemical method of pre-treatment of high concentration effluents, (iii) effort on devising method for targeted (chemical) nutrient element recovery. Phycological terminology - Microalgae is a collective term that includes both prokaryotic and eukaryotic types of algae in microscopic size. Cyanobacteria are prokaryotic microalgae (Aketo et al. 2020; Borowitzka 2020; Diaconu 2020; Guedes et al. 2013). This study used real wastewater samples (unsterilized) to grow nitrogen-fixing cyanobacteria. Upon growth on a stipulated time, algal community shit occurs as the unsterile wastewater has its microbial/algal flora. Therefore, the contents in this thesis mentioned microalgae without specifying prokaryote or eukaryote.

3.2. Materials and methods

3.2.1.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma and used without any further purification. Dibasic potassium phosphate (98%), Magnesium sulphate heptahydrate (98%), Calcium chloride dehydrate (99%), Citric acid (99.5%), Ammonium ferric citrate green (99%), Ethylenediaminetetraacetic acid disodium salt (99%), Sodium carbonate (99%), Boric acid (99%), manganese(II) chloride tetrahydrate (98%), Zinc sulphate heptahydrate (99%), Sodium molybdate dehydrate (99.5%), Copper Sulfate Pentahydrate (98%), Cobalt(II) nitrate hexahydrate (99.9%), Bacteriological agar, *Agilent ICP-MS (*Inductively Coupled Plasma Mass Spectrometry*)* environmental calibration standard (5183-4688), ACR (Australian Chemical Reagents) Ion Chromatography Nitrate, Phosphate, Sulphate standards,

3.2.1.2. Selection of Algal strain

Nitrogen-fixing cyanobacteria (prokaryotic microalgae) was selected to cultivate in wastewater. The following characteristic features are stipulated for the selection: [i] good recovery of nutrients from wastewater, [ii] fast settling (sedimenting) species, and [iii] non-toxin-producing species of microalgae. *Anabaena circinalis* CS-533/02 is the strain obtained from the University of Technology Sydney, Faculty of Science, Environmental Science Laboratory, which was originally obtained from The Commonwealth Scientific and Industrial Research Organisation *(*CSIRO*),* Castray Esplanade, Hobart, Tasmania, 7000, Australia.

3.2.1.3. Algal media

Table 3.1 Micro and macronutrient composition of nitrogen-free freshwater microalgal media 'BG11'

Stocks	**Per 500ml**
(1) K_2HPO_4	2.0 g
(2) $MgSO_4.7H_2O$	3.75 g
(3) $CaCl_2.2H_2O$	1.80 g
(4) Citric acid	0.30 g
(5) Ammonium ferric citrate green	0.30 g
(6) $EDTANa_2$	0.05 g
(7) Na_2CO_3	1.00 g
(8) **Micronutrient solution:**	**Per litre**
H_3BO_3	2.86 g
$MnCl_2.4H_2O$	1.81 g
$ZnSO4.7H_2O$	0.22 g
$Na2MoO4.2H_2O$	0.39 g
$CuSO4.5H_2O$	0.08 g
$Co(NO3)2.6H_2O$	0.05 g

Freshwater microalgal media BG11 [Blue Green medium] were prepared using the composition of micro and macronutrients (Stanier et al. 1971). This experiment aims to study the nitrogen utilising and fixing efficacies of inoculated *Anabaena circinalis* CS-533/02 and wastewater innate microalgae; therefore, nitrogen-free media was prepared, the original BG11 (Stanier et al. 1971) contains sodium nitrate as one of the ingredients. Algal media was prepared by mixing 10 ml of stock solution [1 to 7] and 1 ml of stock solution 8, and made up to 1 L with deionised water. The media was adjusted to pH 7.1 with 1M NaOH or HCl and then autoclaved at 15 psi for 15 minutes.

3.2.1.4. Maintenance of stock culture, seed inoculum and experimental algal culture

BG11 agar plates were prepared by adding 20 g per L of bacteriological agar, autoclaved at 15 psi for 15 minutes, and aseptically poured into Petri plates. After cooling down and solidification, the agar algal culture was inoculated using a disposable plastic inoculation loop. The cultures were incubated at 25°C ± 2 °C under 1500 LUX (Pandey et al. 2007) white fluorescent lights of 12 hours day/night cycles. The stock culture was periodically subcultured every ten weeks into the fresh solid medium. The purity of the culture was monitored by light microscopic observation (Figure 1). To prepare the seed inoculum, cultures were grown in a 500 mL conical flask with 200 mL of liquid BG11 media.

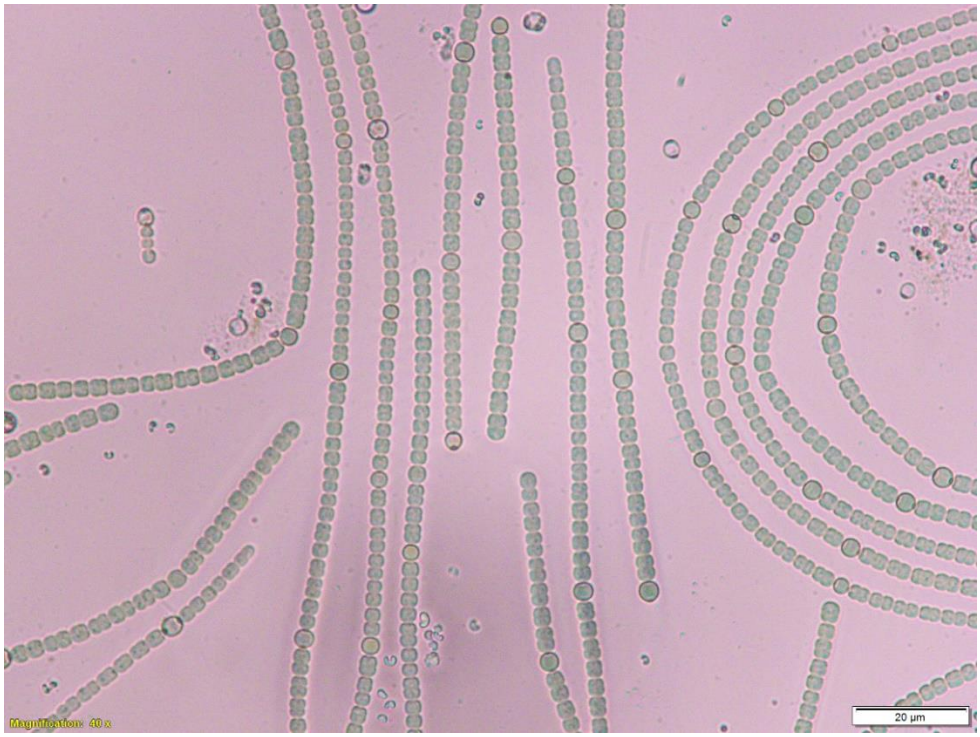


Figure 3.1a. Light microscopic image of *Anabaena circinalis* CS-533/02.

From the seed inoculum, cultures were grown in 1-L conical flasks with 400mL of liquid BG11. Mid-log-phase cultures were harvested by centrifugation at 9000 rpm for 5 minutes using Sigma 1-16KL centrifuge, the supernatant was discarded, and the pellet was resuspended in Milli-Q water, vortexed, and the Milli-Q resuspension/washing of pellet was repeated five times to ensure the algae cleaning

up of BG11 nutrient contents. The cells were observed under a microscope for checking centrifuge shear-induced cell damage. The final pellet was brought into a thick (high cell density) inoculum.

3.2.1.5. Experimental conditions

Post cultivation hydroponic farm wastewater was obtained from Hurlstone Agricultural High School, Roy Watts Rd, Glenfield NSW 2167, Australia. Secondary treated domestic wastewater was collected from the wastewater treatment plant of Sydney Central Park, Chippendale NSW 2008 (located opposite to University of Technology Sydney – Building 1). Desalination brine was collected from one of the desalination plants in Australia. Nitrified human urine (Volpin et al. 2020a) was obtained from the Centre for Technology in Water and Wastewater, School of Civil and Environmental Engineering, University of Technology Sydney. Raw human urine was collected from female and male student volunteers who had not been on any medication since before the collection week.
With the selected five wastewaters – [i] post cultivation hydroponic waster, [ii] secondary treated domestic wastewater, [iii] desalination brine, [iv] nitrified human urine and [v] raw human urine, only the hydroponic wastewater and secondary treated domestic wastewater was used undiluted. The rest of the wastewaters are diluted with secondary treated domestic wastewater in different percentages, as shown in Table 3.2. BG11 media was maintained as a control.

Table 3.2 Wastewaters combinations used for the microalgae-based nutrient recovery and biomass production.

#	Wastewater blends used for algal cultivation
1	BG11
2	Hydroponic Farm water (H)
3	Secondary treated Domestic STD
4	Desalination Brine (DB) 5% in STD
5	Desalination Brine 10% in STD
6	Desalination Brine 15% in STD
7	Desalination Brine 20% in STD
8	Unprocessed human urine (U) 10% in STD
9	Urine 20% in STD
10	Urine 30% in STD
11	Mixed blend 5% of H, DB, and U in STD
12	Mixed blend 10% of H, DB, and U in STD
13	Urine concentrate 5% in STD

An equal amount of known concentration of processed (3.2.1.4) experimental algal suspension (22.57±0.36 mg/L) was inoculated into a final volume of 300ml of thirteen different wastewater conditions (Table 3.2). From the prepared 300ml experimental culture, 15 ml of culture was transferred into eighteen 50 ml polystyrene tissue culture flasks making triplicates for six sampling points, i.e. day 0 (initial), day 3, day 6, day 9, day 12, and day 15 (final). The cultures were incubated at 25°C ± 2 °C under 1500 LUX (Pandey et al. 2007) white fluorescent lights of 12 hours day/night cycles.

3.2.2.1. Sample processing and analysis of micro- and macro-nutrients

Triplicate samples were collected at the interval of every three days and centrifuged at 9000rpm for five minutes; the cell-free supernatant was filtered through a sterile 0.4-µm filter. Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Agilent 7900 with an auto sampler was used to quantify the concentrations of copper, Zinc, Manganese, Magnesium, Molybdenum, Calcium, Iron, Potassium, Cobalt, and Sodium. In order to derive the calibration curve, the Agilent environmental calibration standard (5183-4688) was diluted to five different concentrations (1.25 to 10 µg/L) according to the nutrient concentration of different sample sets. Every sample injection will be made in triplicates, and

the Agilent MassHunter software gives result outputs from the average and standard deviation of triplicates. Nitrate, phosphate and sulphate were quantified using Dionex Integrion RFIC Ion Chromatography with Thermo Fisher Scientific Dionex AS-AP auto-sampler. Five increasing concentrations of ACR nitrate, phosphate and sulphate standard was diluted to different concentration (0.1 to 100 mg/L) according to the nutrient concentration of different wastewater samples.

3.2.2.2. Quantification of algal biomass

Every three days, starting from day 0 (initial) triplicate samples were collected, thoroughly mixed, the algal cells attached to the sides were scrapped using a teasing needle, and the biomass was filtered through pre-weighed glass microfiber filters. The filters were then incubated at 80°C overnight, and the biomass was measured by subtracting the empty filter mass.

3.2.3.1. Nutrient recovery calculations

For the tested thirteen different wastewater blends (Table 3.2), the following calculations are done from the values of every three days sampling/measurements of ICP-MS (metal/ micronutrients) and ion chromatography (macronutrients), to derive Nutrient Recovery (NR); NR_{max}, NR_{min}, $NR_{x0D\text{-}3D}$, DNR_{xmax}, DNR_{xmin}, $NR\%_{x}$, $NR\%_{xincrease}$, $NR\%_{xdecrease}$, NR_{xnull}. These emphasising calculation factors for the particular aspects are labelled and their descriptions for each factor are detailed below and their calculations are done further.

NR_{max} - nutrient element recovered to the maximum on the given blend within the 15 days

NR_{min} - nutrient element recovered to the minimum/least on the given blend within the 15 days

$NR_{x0D\text{-}3D}$ - Nutrient Recovery rate for every three-day interval, where *x* is the nutrient element measured

DNR_{xmax} - the Day in which highest Nutrient Recovery (DNR) of an individual nutrient element (*x*) occurred on the given blend within the 15 days

DNR_{xmin} - the day in which lower recovery of an individual nutrient element (x) occurred on the given blend within the 15 days

$NR\%_x$ - Nutrient Recovery percentage of individual nutrient element (x) on a given blend within the 15 days

$NR\%_{xdecrease}$ - Nutrient Recovery percentage of decrease of individual nutrient element (x) on a given blend within the 15 days

$NR\%_{xincrease}$ - Nutrient Recovery percentage of increase of individual nutrient element (x) on a given blend within the 15 days

NR_{xnull} - 'no recovery' point (day) of an individual nutrient element (x) within the 15 days of retention

The measured micro and macronutrients are copper (x=Cu), zinc (x=Zn), manganese (x=Mn), magnesium (x=Mg), molybdenum (x=Mo), calcium (x=Ca), iron (x=Fe), potassium (x=K), cobalt (x=Co), sodium (x=Na), nitrate, (x=NO3), phosphate (x=PO4) and sulphate (x=SO4).

(A) NR_{max} and NR_{min}

The nutrient recovery (NR_x) for every individual tested nutrient element (x) on the given wastewater blend should be calculated by subtracting the initial amount of nutrients measured on 'day 0' (N_{x0}) from 'day 15'(N_{x15}).

$$\text{i.e } NR_x = N_{x0} - N_{x15} \qquad \text{(Equation 3.1)}$$

Then the values NR_{Cu}, NR_{Zn}, NR_{Mn}, NR_{Mg}, NR_{Mo}, NR_{Ca}, NR_{Fe}, NR_K, NR_{Co}, NR_{Na}, NR_{NO3}, NR_{PO4}, NR_{SO4} were arranged in increasing or decreasing order to identify which nutrient element recovered to the maximum (NR_{max}) and minimum/least (NR_{min}).

(B) NR_{x0D-3D}

To measure the nutrient recovery rate for every three-day interval (NR_{x0D-3D}) for each tested nutrient element (x) amount of nutrients measured on 'day 0'(N_{x0}) is subtracted from 'day 3'(N_{x3}).

$$\text{i.e } NR_{x0D-3D} = N_{x3} - N_{x0} \quad \text{(Equation 3.2)}$$

Similarly

$$NR_{x3D-6D} = N_{x6} - N_{x3} \quad \text{(Equation 3.3)}$$

$$NR_{x6D-9D} = N_{x9} - N_{x6}$$

$$NR_{x9D-12D} = N_{x12} - N_{x9}$$

$$NR_{x12D-15D} = N_{x15} - N_{x12}$$

(C) DNR_{xmax} and DNR_{xmin}

The day in which higher (DNR_{xmax}) and the day in which lower (DNR_{xmax}) recovery of an individual nutrient element (x) occurred can be found from the nutrient recovery rate for every three-day interval values NR_{x0D-3D}, NR_{x3D-6D}, NR_{x6D-9D}, $NR_{x9D-12D}$, $NR_{x12D-15D}$ by arranging these in increasing or decreasing order.

(D) $NR\%_x$, $NR\%_{xincrease}$, $NR\%_{xdecrease}$

The Nutrient Recovery percentage ($NR\%_x$) of individual nutrient element (x) on a given blend can be calculated as,

$$NR\%_x = \{[(N_{x15} - N_{x0})] / N_{x0}\} \times 100 \quad \text{(Equation 3.4)}$$

(E) NR_{xnull}

The 'no recovery' point (day) (NR_{xnull}) of an individual nutrient element (x) within the 15 days of retention can be found from the nutrient recovery rate for every three-day interval values NR_{x0D-3D}, NR_{x3D-6D}, NR_{x6D-9D}, $NR_{x9D-12D}$, $NR_{x12D-15D}$ by finding the difference between them when the value of difference is “Zero” that’s the NR_{xnull}.

3.2.3.2. Selective nutrient recovery calculation

This calculation was devised to identify the [i] specific nutrient element that acts as a 'key' (element) in the recovery of other micro/macronutrients of the given blend and [ii] the highly 'influencing' nutrient element in the recovery of other nutrients. Using Matlab R2018b, correlation coefficients were derived for each blend, containing a set of measured thirteen micro- and macro-nutrients sampled

in the interval of every three days (Table 3.3). From the derived correlation coefficients (Table 3.3), the values 0.7 and above was shortlisted. The shortlisted correlation values were arranged in a vertical table, and then the nutrient elements having correlation interlinks were segregated (Table 3.4). From the segregated interlinks, the nutrient element with the highest number of interlinks is the highest influencing nutrient element on the given nutrient blend. In this considered example Table 3.4 (secondary treated domestic wastewater), molybdenum is the highly influencing nutrient element. In some cases, two highly influencing nutrients are identified with the same number of interlinks; ranking is implemented based on the 'sum' of correlation coefficient values. Sulphate, cobalt and sodium are the nutrient elements not involved in the interlinks (Table 3.5).

Table 3.3 Matlab output of correlation coefficients derived for the secondary treated domestic wastewater (the colour codes represents the increasing intensity for higher numeric values)

	Days	NO3	PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo
Days	1.000	-0.712	-0.455	0.467	0.707	-0.794	-0.830	-0.436	-0.717	-0.873	-0.320	-0.783	-0.696	-0.795
NO3	-0.712	1.000	0.742	-0.163	-0.821	0.817	0.617	0.458	0.993	0.744	-0.426	0.781	0.990	0.957
PO4	-0.455	0.742	1.000	-0.501	-0.764	0.323	0.060	0.355	0.684	0.324	-0.457	0.350	0.762	0.568
SO4	0.467	-0.163	-0.501	1.000	0.176	-0.028	0.017	0.159	-0.090	-0.005	-0.391	-0.034	-0.125	-0.032
Na	0.707	-0.821	-0.764	0.176	1.000	-0.548	-0.464	-0.505	-0.800	-0.724	0.232	-0.552	-0.837	-0.819
Mg	-0.794	0.817	0.323	-0.028	-0.548	1.000	0.927	0.601	0.867	0.907	-0.086	0.983	0.819	0.910
K	-0.830	0.617	0.060	0.017	-0.464	0.927	1.000	0.529	0.678	0.933	0.236	0.906	0.611	0.797
Ca	-0.436	0.458	0.355	0.159	-0.505	0.601	0.529	1.000	0.526	0.703	-0.201	0.727	0.571	0.560
Mn	-0.717	0.993	0.684	-0.090	-0.800	0.867	0.678	0.526	1.000	0.794	-0.418	0.838	0.991	0.976
Fe	-0.873	0.744	0.324	-0.005	-0.724	0.907	0.933	0.703	0.794	1.000	0.080	0.918	0.767	0.895
Co	-0.320	-0.426	-0.457	-0.391	0.232	-0.086	0.236	-0.201	-0.418	0.080	1.000	-0.084	-0.458	-0.270
Cu	-0.783	0.781	0.350	-0.034	-0.552	0.983	0.906	0.727	0.838	0.918	-0.084	1.000	0.807	0.880
Zn	-0.696	0.990	0.762	-0.125	-0.837	0.819	0.611	0.571	0.991	0.767	-0.458	0.807	1.000	0.955
Mo	-0.795	0.957	0.568	-0.032	-0.819	0.910	0.797	0.560	0.976	0.895	-0.270	0.880	0.955	1.000

Table 3.4 Shortlisted correlation coefficient values 0.7 and above were then segregated from highest to lowest

		Correlation Coefficient	
1	Mo	0.976391	Mn
2	Mo	0.956892	NO3
3	Mo	0.954671	Zn
4	Mo	0.910327	Mg
5	Mo	0.895425	Fe
6	Mo	0.880473	Cu
7	Mo	0.796644	K
1	Zn	0.991293	Mn
2	Zn	0.990022	NO3
3	Zn	0.819012	Mg
4	Zn	0.807265	Cu
5	Zn	0.766913	Fe
6	Zn	0.76185	PO4
1	Cu	0.983351	Mg
2	Cu	0.917894	Fe
3	Cu	0.906194	K
4	Cu	0.837723	Mn
5	Cu	0.781178	NO3
6	Cu	0.727053	Ca
1	Fe	0.703389	Ca
2	Fe	0.744453	NO3
3	Fe	0.794006	Mn
4	Fe	0.906659	Mg
5	Fe	0.932549	K
1	Mn	0.993301	NO3
2	Mn	0.866626	Mg
1	K	0.926761	Mg
1	Mg	0.817085	NO3
1	PO4	0.741877	NO3

Table 3.5. Some of the tested thirteen nutrient elements are not involved in the interlinks

	Tested nutrients	Nutrient elements involved in the interlinks
1	Ca	Ca ✓
2	Co	Not involved
3	Cu	Cu ✓
4	Fe	Fe ✓
5	K	K ✓
6	Mg	Mg ✓
7	Mn	Mn ✓
8	Mo	Mo ✓
9	Na	Not involved
10	NO3	NO3 ✓
11	PO4	PO4 ✓
12	SO4	Not involved
13	Zn	Zn ✓

Table 3.6 Columns and rows arranged with interlinking nutrient elements to identify the 'key' element

	Nutrients	Mo	Zn	Cu	Fe	Mn	K	Mg	PO4	Number of Interlinks
1	Ca			Ca	Ca					2
3	Cu	Cu	Cu							2
4	Fe	Fe	Fe	Fe						3
5	K	K		K	K					3
6	Mg	Mg	Mg	Mg	Mg	Mg	Mg			6
7	Mn	Mn	Mn	Mn	Mn					4
10	NO3	NO3	NO3	NO3	NO3	NO3		NO3	NO3	7
11	PO4		PO4							1
13	Zn	Zn								1
	Number of Interlinks	7	6	6	5	2	1	1	1	

3.3.3.3. Identification of maximum recovery points of an individual nutrient element in stipulated retention

Within the recovery rates of different sampling intervals; identifying the maximum recovery points of an individual nutrient element. From the measured highest recovery of the individual nutrient element, tabulation was made using colour codes to represent different sampling points. Yellow = day15, green = day12, red = day9, blue = day6, and pink = day3 (Table 3.7).

3.3.3.4. Method to gauge retention timelines of targeted nutrients

This calculation reveals which are all nutrient elements recovered to their maximum on the given retention timelines. Alternatively, it can be referred to as identifying the timeframe when the particular nutrient will recover to its maximum. From the identified maximum recovery points of thirteen different nutrient elements in the stipulated retention (Table 3.7), nutrient elements were sorted and identified for nutrient elements (majority) recovered to their maximum on a particular period. As this testing measured thirteen different nutrient elements, a cut off of five was maintained to identify the

major nutrients that got recovered. For example, a set of five or above nutrient elements recovered in a given blend in a particular timeframe; those sets are recognised nutrients that get recovered on a stipulated time (Table 3.8).

3.3.3.5. Overall nutrient recovery pattern profiling

To profile the overall nutrient recovery, within the tested thirteen blends/nutrient conditions, maximum recovery days of individual nutrients were sorted (Figure 3.9). The total count of maximum recovery points of every nutrient element was totalled for individual nutrient elements (Figure 3.11). Within the tested thirteen blends/nutrient conditions, a cut off of five was maintained for selecting the day at which the maximum recovery of a particular nutrient (Figure 3.11). For example, amongst the tested thirteen nutrient elements, within the tested fifteen days of retention maximum recovery of iron happened at day3.

3.3.3.6. Method to gauge retention timelines of unfavourable nutrient recovery

This calculation reveals the least recovered group of nutrient elements on the given retention timelines. From the identified least recovery points of thirteen different nutrient elements in the stipulated retention, they were sorted and grouped for the nutrients (majority) recovered to their least during a particular period. As this testing measured thirteen different nutrient elements, a cut off of five was maintained to identify the major nutrients that got recovered to the least levels. For example, a set of five or above nutrient elements recognised in a given blend and all thirteen conditions in a particular timeframe (Table 3.8).

Table 3.7 Highest recovery points of different nutrient elements at the given retention time, the colour code represents;

yellow = day15, green = day12, red = day9, blue = day6, and pink = day3

	Blend Combinations	NO3	PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo
1	BG11													
2	Hydroponic Farm water - H													
3	Secondary treated Domestic wastewater - STD													
4	Desalination Brine(DB) 5% in STD													
5	Desalination Brine 10% in STD													
6	Desalination Brine 15% in STD													
7	Desalination Brine 20% in STD													
8	Human Urine (U) 10% in STD													
9	Human Urine 20% in STD													
10	Human Urine 30% in STD													
11	Mixed blend 5% of H, DB, and U in STD													
12	Mixed blend 10% of H, DB, and U in STD													
13	Nitrified Urine 5% in STD													

Table 3.8 Representation of nutrient groups recovered to its maximum in a particular timeframe **yellow = day15**, **green = day12**, **red = day9**, **blue = day6**, **and pink = day3** The right end column "timeframe / nutrient group" shows the colour code of the particular day (timeframe) in which the maximum recovery occurred, and the total number of elements (in bracket).

	Blend Combinations	NO3	PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo	Timeframe/Nutrient groups
1	BG11														(5) NO3, SO4, Na, Cu, Zn
2	Hydroponic Farm water - H														(7) Na, Mg, K, Ca, Co, Cu, Zn
3	Secondary treated Domestic wastewater - STD														(7) Na, PO4, Mg, Mn, Cu, Zn, Mo
4	Desalination Brine(DB) 5% in STD														(6) PO4, Na, Mg, Ca, Cu, Zn
5	Desalination Brine 10% in STD														No pattern observed
6	Desalination Brine 15% in STD														(5) PO4, Na, K, Fe, Mo
7	Desalination Brine 20% in STD														(5) PO4, K, Fe, Zn, Mo
8	Human Urine (U) 10% in STD														(7) NO3, PO4, SO4, Na, Mg, Ca, Co
9	Human Urine 20% in STD														(5) NO3, K, Fe, Cu, Mo
10	Human Urine 30% in STD														(7) NO3, K, Mn, Fe, Cu, Zn, Mo
11	Mixed blend 5% of H, DB, and U in STD														(7) PO4, K, Mn, Fe, Cu, Zn, Mo
12	Mixed blend 10% of H, DB, and U in STD														(7) PO4, Na, Mn, Fe, Cu, Zn, Mo
13	Nitrified Urine 5% in STD														(6) SO4, K, Mn, Fe, Zn, Mo

Table 3.9 Depiction of values sorted within the identified maximum recovery days of different nutrient elements (colour codes **yellow = day15**, **green = day12**, **red = day9**, **blue = day6**, **and pink = day3**). Among the tested thirteen blend/nutrient conditions, nutrient elements with the same recovery peak point – having five and above counts are sorted and totalled.

	Blend Combinations	NO3	PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo
1	BG11 [Freshwater algal nutrient]						1		1	1				1
2	Hydroponic Farm water - H									2	1			2
3	Secondary treated Domestic wastewater - STD				1		2		2			1	1	3
4	Desalination Brine(DB) 5% in STD			1	2					3	2			4
5	Desalination Brine 10% in STD			2	3	1		1	3	4	3	2		5
6	Desalination Brine 15% in STD			3	4	2	3	2			4	3		
7	Desalination Brine 20% in STD			4			4		4		5			
8	Human Urine (U) 10% in STD			5			5		5	5	6	4		
9	Human Urine 20% in STD			6	5					6	7	5		6
10	Human Urine 30% in STD			7				3	6	7	8	6	2	7
11	Mixed blend 5% of H, DB, and U in STD			8		3		4	7	8	9	7	3	8
12	Mixed blend 10% of H, DB, and U in STD			9		4		5	8	9		8	4	9
13	Nitrified Urine 5% in STD				6	5			9	10			5	10
		NO3	PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo
	Total	X	X	9	6	5	5	5	9	10	9	8	5	10

Table 3.10 Depiction of values sorted within the identified least recovery days of different nutrient elements (colour codes **yellow = day15, green = day12, red = day9, blue = day6, and pink = day3**). Among the tested thirteen blend/nutrient conditions, nutrient elements with the same least recovery point – having five and above counts are sorted and totalled for the vertical and horizontal axis. The vertical axis count reveals the overall majority of least recovered nutrients, and the horizontal count yields the group of nutrient elements within the tested blend condition.

	Blend Combinations	NO3	PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo	Least Recovery
1	BG11 [Freshwater algal nutrient]				1					1		1		1	No Pattern
2	Hydroponic Farm water - H		1	1			1		1	2		2	1		No Pattern
3	Secondary treated Domestic wastewater - STD		2		2										No Pattern
4	Desalination Brine(DB) 5% in STD			2			2		2	3	1	3		2	Day12 [5 out of 13]
5	Desalination Brine 10% in STD			3			3		3	4				3	No Pattern
6	Desalination Brine 15% in STD			4			4			5	2	4			No Pattern
7	Desalination Brine 20% in STD											5		4	No Pattern
8	Human Urine (U) 10% in STD			5							3		2		Day15 [5/13]
9	Human Urine 20% in STD			6	3							6	3		No Pattern
10	Human Urine 30% in STD			7	4				4		4		4		Day3 [5/13]
11	Mixed blend 5% of H, DB, and U in STD		3	8	5				5		5		5	5	Day15 [6/13]
12	Mixed blend 10% of H, DB, and U in STD		4	9			5		6	6		7	6		No Pattern
13	Nitrified Urine 5% in STD		5							7			7	6	No Pattern
			PO4	SO4	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo	
			5	9	5		5		6	7	5	7	7	6	
											5				

3.3. Results

3.3.1. Characteristics of *Anabaena circinalis* CS-533/02 culture

To obtain non-toxin-producing nitrogen-fixing algal strain, search throughout Australian universities and institutes ended up in the option of *Anabaena circinalis* CS-533/02. The selected strain was catalogued (CSIRO - Australian National Algae Culture Collection [ANACC] as 'toxin below detection'. In 2013, the Environmental Research group at the Faculty of Science in University of Technology Sydney purchased *Anabaena circinalis* CS-533/02 from CSIRO- ANACC and maintained the culture by periodical subculturing in BG11 medium. From the 16th of December 2015, the Australian Water Quality Centre announced the name change of several algal strains, including *Anabaena circinalis is now known as Dolichospermum circinale* (WAQC 2015). As this research demonstrates the proof of concept of nitrogen-fixing microalgae's ability to fix nitrogen and the fixed nitrogen act as a nutrient and stimulant (plant hormones) (Lu and Xu 2015) in hydroponic cultivation, it is conceded to use *Anabaena circinalis* CS-533/02 for the experiments. Another trait of this species is its ability to sediment or settle faster, which facilitates the study's planned biomass harvest method.

3.3.2. Algal growth in different wastewater blends

Among the tested wastewater blends, 5% nitrified urine in STD showed the highest algal growth/biomass production. Comparing to the 5% nitrified urine in STD, the next highest was hydroponic farm wastewater, which showed 92.7% growth. Algal growth was observed in different degrees in the following order, desalination brine 15% in DW [70.4%] > desalination brine 10% in DW [48.2%] > desalination brine (DB) 5% in DW [28.8] > mixed blend 5% of H, DB, and U in DW [26%] > Mixed blend 10% of H, DB, and U in DW [22.8%] > secondary treated Domestic wastewater – DW [22.5%] > Desalination Brine 20% in DW [11.4%] > BG11 [11.3%] > Human Urine (U) 10% in DW [10.6%] > Human Urine (U) 20% in DW [10.5%] > Human Urine (U) 30% in DW [9.3%], respectively. The BG11 produced less biomass comparing to wastewater conditions and were comparable with previous study (Patel Akash Kumar et al. 2017).

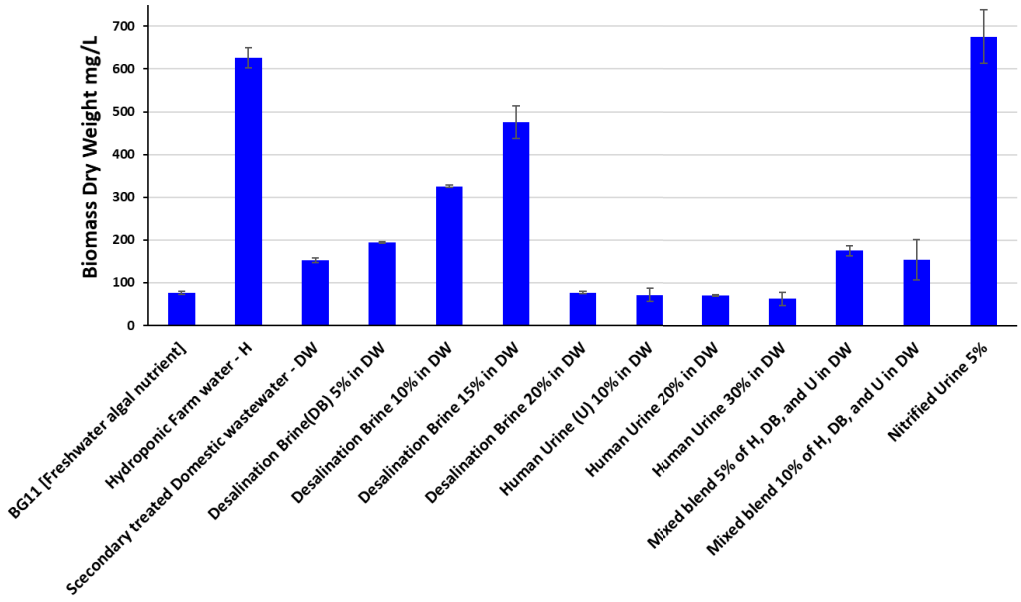


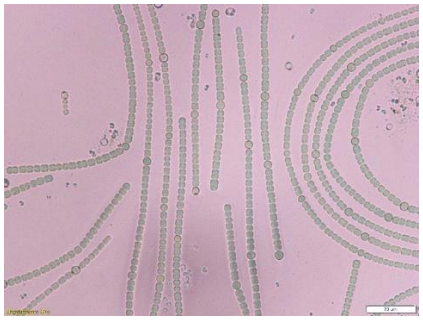
Figure 3.1b Growth in terms of dry biomass weight after 15 days of retention (results represented average of triplicates)

The three tested concentrations of unprocessed urine (10%, 20% and 30% in STD) showed the least growth rates comparing the other wastewater blends. These results are analogous with other studies (Canizales et al. 2021); the presence of urea has not pronounced an inhibitory effect; however, it caused physiological changes that resulted in unfavourable growth. The subsequent better growth was noticed in four wastewater blends (hydroponic wastewater, desalination brine, and unprocessed urine in STD). Both the 5% and 10% four wastewater blends showed low biomass production; comparing the 5%, there was a 12% decreased growth observed in the 10% blend. Amongst the tested four different increasing concentrations of desalination brine (5%, 10%, 15% and 20%) in STD showed gradual increase in biomass/growth upon the increasing proportion of brine in the blend, and there was a sudden decline noticed at 20% brine. Comparing the 15% brine (in STD), the 10% brine showed a 31% decline in biomass, and when comparing it to 10% brine, a 40% decrease in biomass was observed in 5% brine.

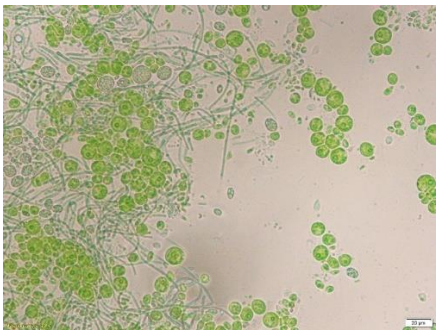
3.3.3. Growth of inoculated *Anabaena circinalis* CS-533/02 and wastewater contained flora

Light microscopic observation of experimental blends revealed different degrees of dominance of wastewater algal flora. The BG11 (filter sterilised) was observed with 100% *Anabaena circinalis* CS-533/02 (Figure 3.2a). Throughout all the sampling points, no protozoan grazers or fungal domination was noticed in the tested blends. The was no wastewater chemical mediated colour change or cloudiness observed within the stipulated retention time. It is observed in all the tested blend conditions the dominated wastewater algal flora are unicellular organisms; no detection of filamentous eukaryotic or prokaryotic

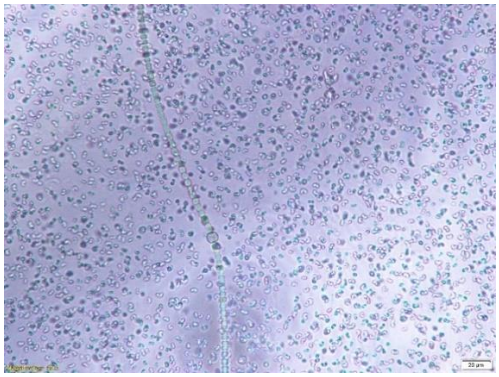
algae (Figure 3.2 b to m) except the inoculated *Anabaena circinalis* CS-533/02. No algal growth was detected in the three tested concentrations of unprocessed human urine (Figure 3.2 h, i and j); moreover, it is evident with disintegrated cells debris of inoculated *Anabaena circinalis* CS-533/02. Based on the cell morphology, one dominating species was observed in all the blend conditions, except nitrified urine, which showed two distinct types of unicellular microalgal species (Figure 3.2 m).



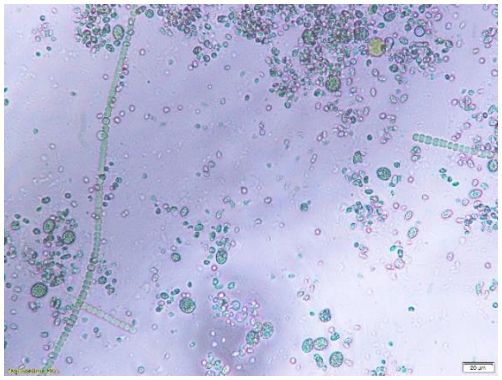
a BG11



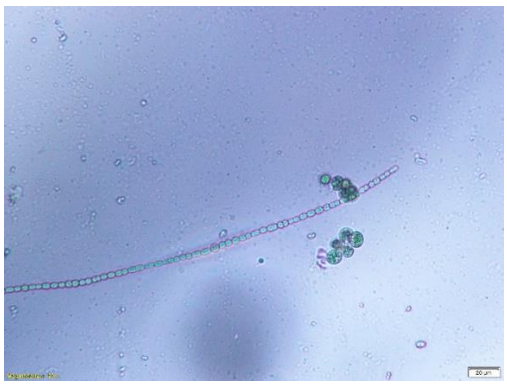
b Hydroponic wastewater



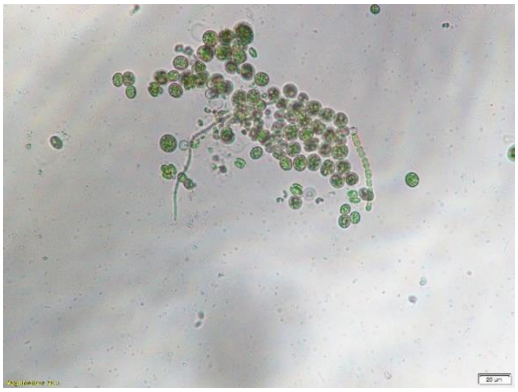
c Secondary treated domestic wastewater



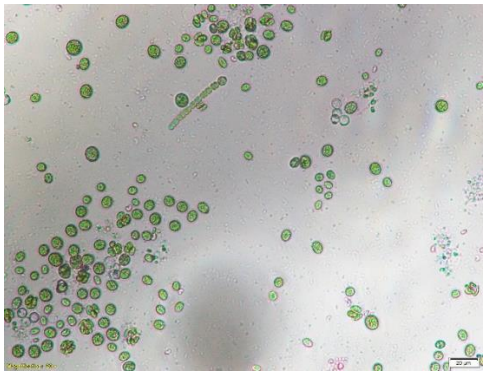
d 5% Desalination brine



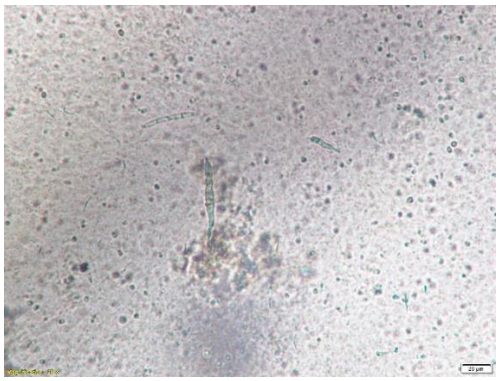
e 10% Desalination brine



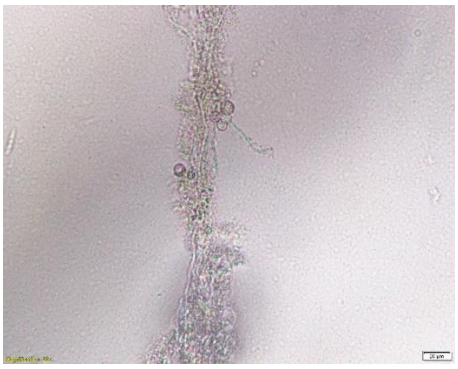
f 15% Desalination brine



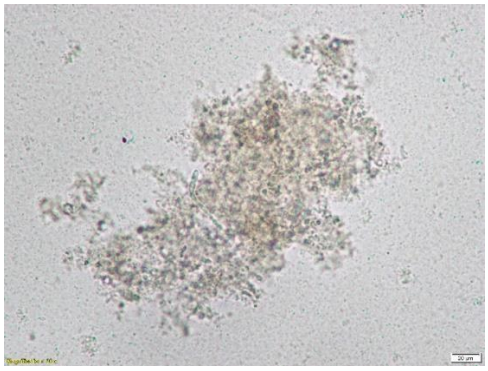
g 20% Desalination brine



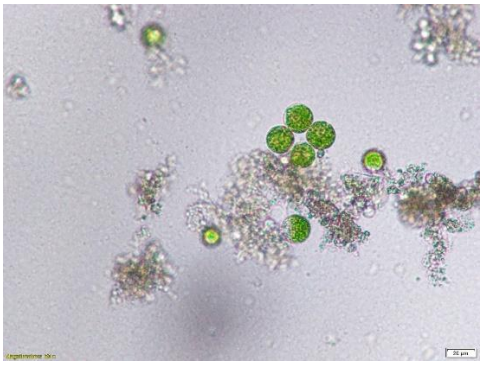
h 10% Unprocessed human urine



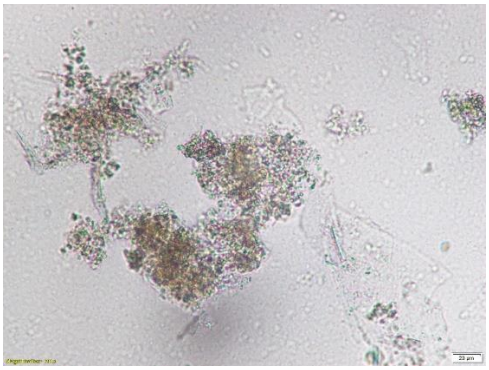
i 20% Unprocessed human urine



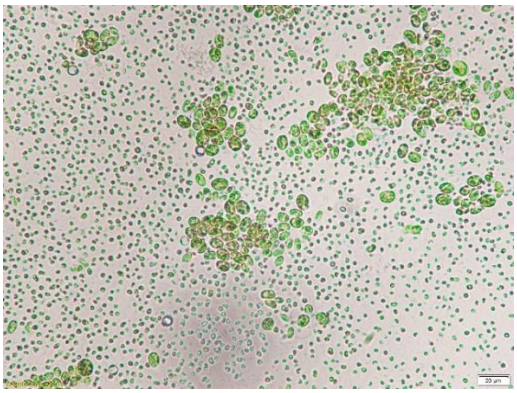
j 30% Unprocessed human urine



k 5% Mixed blend



l 10% Mixed blend



m Nitrified urine

Figure 3.2 (a to m) Algal community change in the given retention time

3.3.4. Nutrient recovery rates of different sampling intervals and the maximum/least recovery points

Irrespective of nutrient regimes and algal flora, a clear recovery pattern was observed among the tested thirteen nutrient conditions/blends (Figure 3.1). Within the tested thirteen conditions, maximum recovery of iron and molybdenum occurred on the third day in ten blend conditions. Maximum recovery of manganese, sulphate and cobalt ensued at nine different nutrient conditions. Within these, the recovery timeframe varied, cobalt and sulphate recovery on day six and manganese on day three (Figure 3.3). Based on the maximum number of recovery conditions (irrespective of nutrient regimes), the nutrient elements are sequentially grouped as follows, (Mo, Fe [10]) > (SO_4^{2-}, Co, Mn [9]) > (Cu [8]) > (Na [6]) > (K, Mg, Ca, Zn [5]). The order of the above shown sequentially grouped profile divulges the information that irrespective of the characteristics of individual algal nutrient preference or uptake, iron ad molybdenum can be expected to recover at the first instance. Based on the timeline of recovery, nutrients are grouped as (Mo, Fe, Mn, Cu, Zn)-day3 > (Ca, Mg)-day6 > (Na, K)-day9 > (SO4, Co)-day12. Following the similar method of scoring the maximum recovery points of individual nutrient elements, the "least" recovery points within a given blend and overall amongst the thirteen nutrient conditions were plotted (Table 3.10). Scoring within the given blend showed results only for four nutrient conditions, (i) desalination brine 5% in STD, (ii) 10% human urine in STD, (iii) 10% human urine in STD, and (iv) mixed blend 5%. Fitting to the criteria of five and above cut off, the rest of the nine have not shown any least recovery points.

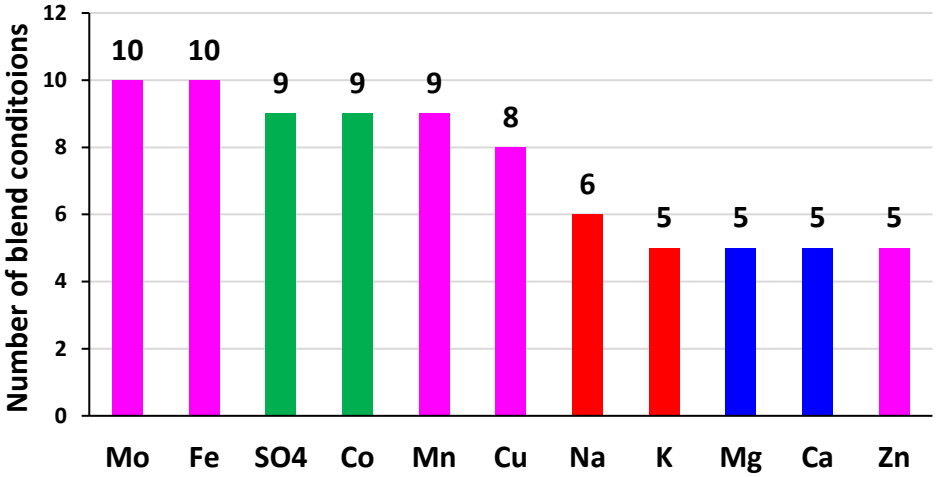


Figure 3.3 Profile of maximum nutrient recovery on different wastewater blends/nutrient conditions (nutrient regimes), colour codes **green = day12**, **red = day9**, **blue = day6**, **and pink = day3**.

3.3.5 Nutrient recovery stoichiometry

Within the given retention time (15 days), phosphate and nitrate were entirely recovered from six tested wastewater conditions (Table 3.11). Nitrate, phosphate, iron, magnesium, cobalt, and manganese were the six nutrients recorded to recover to maximum levels among the tested thirteen different nutrient conditions/blends and thirteen micro and macronutrients. The recovery were in the following order phosphate (in 5 nutrient conditions) > nitrate (3) > iron (2) > magnesium, molybdenum, cobalt. All four desalination brine containing blends were found to recover 100%phosphate (Table 3.11). 100% recovery of nitrate occurred in hydroponic farm wastewater and secondary treated domestic wastewater. Human Urine 30% in DST showed 99% nitrate recovery > nitrified urine 5% in STD showed 97% molybdenum recovery > Mixed blend 10% of H, DB, and U in STD showed 93% phosphate recovery > Mixed blend 5% of H, DB, and U in STD showed 91% iron recovery, BG11 showed 89% cobalt recovery > human urine 10 % in STD showed 76% iron recovery > human urine 20 % in STD showed 76% magnesium recovery.

Table 3.11 Percentage of nutrients recovered in the given 15 days of retention, calculated by considering Day0 as '100'

	Blend Combinations	Highest Recovered Nutrient	Highest Percentage Recovered (%)
1	BG11	Cobalt	89
2	Hydroponic Farm water - H	Nitrate	100
3	Secondary treated Domestic wastewater - STD	Nitrate	100
4	Desalination Brine(DB) 5% in STD	Phosphate	100
5	Desalination Brine 10% in STD	Phosphate	100
6	Desalination Brine 15% in STD	Phosphate	100
7	Desalination Brine 20% in STD	Phosphate	100
8	Human Urine (U) 10% in STD	Iron	76
9	Human Urine 20% in STD	Magnesium	76
10	Human Urine 30% in STD	Nitrate	99
11	Mixed blend 5% of H, DB, and U in STD	Iron	91
12	Mixed blend 10% of H, DB, and U in STD	Phosphate	93
13	Nitrified Urine 5% in STD	Molybdenum	97

3.3.6. Selective control nutrient recovery plot

Recovery of a particular nutrient will be influenced by one or more other nutrient elements present in the medium, in a relationship called interlinks. Amongst the tested conditions, the number of interlinks ranged between 30 to 11 (Table 3.12). Based on the scoring, interlink forming nutrient conditions were as follows, BG11 (30 interlinks) > secondary treated domestic wastewater (29) > hydroponic farm water (24) > Nitrified Urine 5% in STD (21) > human urine 20% in STD (15) > human urine 30% in STD (14) > desalination brine 5% in STD (13) > mixed blend 10% of H, DB, and U in STD (13) > desalination brine 15% in STD (12) > desalination brine 20% in STD (11) > human urine 10% in STD (11) > mixed blend 5% of H, DB, and U in STD (13). Calcium, copper, molybdenum, phosphate, and zinc are the identified highly influencing nutrient elements in interlink formation. Human urine 20% in STD, mixed blend 10% of H, DB, and U in STD, and desalination brine 15% in STD have had calcium as their influencing element. Human urine 10% in STD had copper as their influencing element; hydroponic farm water, desalination brine 20% in STD, desalination brine 10% in STD, mixed blend 5% of H, DB, and U in STD had molybdenum as their influencing element; BG11 had phosphate as its influencing element; secondary treated domestic wastewater, human urine 30% in STD, desalination brine 5% in STD, nitrified urine 5% in STD had zinc as its influencing element.

Sodium, potassium, nitrate, phosphate, and zinc are the identified key interlink forming elements. Phosphate and zinc are the elements identified as both highly influencing and key interlinking element. Human urine 20% in STD, mixed blend 5% of H, DB, and U in STD, human urine 30% in STD have had potassium as their key influencing element in interlink formation. Hydroponic farm water, desalination brine 10% in STD, desalination brine 15% in STD, desalination brine 20% in STD, mixed blend 10% of H, DB, and U in STD have had sodium as their key influencing element in interlink formation. Nitrate acted as a key element for secondary treated domestic wastewater, desalination brine 5% in STD, human urine 10% in STD. For nitrified urine 5% in STD, phosphate and for BG11, zinc acted as a key interlinking element.

Copper, zinc, sodium, molybdenum, phosphate are identified as highly influencing and key interlink forming nutrient elements; they are also found in the group of nutrient elements that are not involved in interlink formation. This study used nitrogen-free BG11 as a positive control for algal growth. Clear evidence observed from the interlink plot that nitrate was not involved in the interlink formation group (Table 3.12). It is evident in every place where sodium is identified as 'key' interlinking nutrient, the two nutrients 'sulphate and cobalt' were not involved in interlink formation. Similarly, a profile revealed between sodium and cobalt; there were five nutrient conditions (mixed blend 10% of H, DB, and U in STD, desalination brine 10% in STD, hydroponic farm water, desalination brine 15% in STD, desalination brine 20% in STD) identified with sodium as a key interlinking element, amongst three conditions (hydroponic farm water, desalination brine 15% in STD, desalination brine 20% in STD) showed no involvement of cobalt in interlinking.

Table 3.12 Selective control nutrient recovery plot; representing nutrient elements that influence the recovery of one or more other nutrient elements present in the medium and the key nutrient element involved and not involved in such interlink formations

#	Blend Combinations	Number of interlinks	Highly influencing element in interlink formation	Key interlinking nutrient element	Nutrients elements that are NOT involved in interlinks
1	BG11	30	PO_4^{3-}	Zn	Na^+, NO_3^-
2	Hydroponic Farm water - H	24	Zn	Na	Co, Mn, SO_4
3	Secondary treated Domestic wastewater - STD	29	Mo	NO3	Co, Na, SO_4
4	Desalination Brine(DB) 5% in STD	13	Zn	NO3	Ca
5	Desalination Brine 10% in STD	11	Ca	Na	Cu, PO_4
6	Desalination Brine 15% in STD	12	Zn	Na	Co, Mo
7	Desalination Brine 20% in STD	12	Zn	Na	Co, Mn, Mo, SO4
8	Human Urine (U) 10% in STD	11	Ca	NO3	Co, PO4, Zn
9	Human Urine 20% in STD	15	Ca	K	Mn
10	Human Urine 30% in STD	14	Mo	K	PO4
11	Mixed blend 5% of H, DB, and U in STD	11	Mo	K	Co
12	Mixed blend 10% of H, DB, and U in STD	13	Cu	Na	SO4
13	Nitrified Urine 5% in STD	21	Mo	PO4	Co

3.4. Discussion

This study focused on demonstrating the proof of concept; blending wastewaters of high and low nutrient levels to produce algal biomass and envisage to extract hydroponic nutrients and stimulants from algal biomass. In this chapter, nutrient recovery attributes from wastewater are emphasised because microalgae-based wastewater nutrient recovery is an incentive for reducing the net production cost. The large scale production of algal biomass, harvesting, and extraction of nutrients are covered in the next chapter (chapter 4). The essence of this chapter is to show evidence of the proof of concept. This investigation aims to produce biologically fixed nitrogen to reduce the carbon footprint associated with the production of agricultural nitrogen (nutrient). Additionally, plant stimulants (plant hormones) production is an added advantage as microalgae is known for producing phytohormones (Lu and Xu 2015). Therefore the nitrogen-fixing *Anabaena circinalis* CS-533/02 is selected to investigate the nitrogen-fixing efficacy in wastewater based nutrients. *Anabaena circinalis* CS-533/02 perfectly aligned with the expected fast settling or sedimentation trait, the characteristic feature anticipated to implement on the energy-efficient biomass harvest. Many cyanobacteria have gas vacuoles, a cellular organelle that provides buoyancy; these gas-vacuolated organisms regulate their buoyancy and control their vertical movements in the water column, settling and surface flotation (Oliver and Walsby 1984). Regulating gas vesicles helps the species to respond to light-dependent buoyancy. This is an advantage of the aimed harvesting method (dark settling) *Anabaena circinalis* CS-533/02 had natural settling trait within few hours. Amongst the microbe based high value bio-product production system, bacteria are highly diverse and potential bio-product yielding bio-source. Bacteria are well known to produce an array of phytohormones (Primo et al. 2015). However, investigation on the comparative assessment of the efficacy of bacterial and cyanobacterial phytohormones in plant tissue culture revealed, cyanobacteria sourced phytohormones are more efficient in the induction physiology of both root and shoot systems (Hussain and Hasnain 2012). Additionally, the genus *Anabaena* is renowned for phytohormones of various kinds (Hashtroudi et al. 2013).

Nitrogen-fixing microalgae produce more vegetative cells and grow faster in the presence of combined nitrogen in the media (nitrogen in the form of nitrate or ammonium) (Torres-Sánchez, Gómez-

Gardeñes, and Falo 2015). The observed higher growth rate in the wastewater conditions (Figure 3.1b) comparing to the nitrogen-free BG11 is clear evidence that *Anabaena circinalis* CS-533/02 grow faster in wastewater with nitrogen. Also, it is apparent that the absence of combined nitrogen in the BG11 medium (nitrogen-free) did not support the advent of more vegetative cells, thus resulted in reduced growth. As evident (Figure 3.1b), the nitrified urine yielded higher biomass in the given retention time (fifteen days). Hence, for this reason, nitrified urine was not used in the mixtures of four wastewaters blend; only the desalination brine, hydroponic wastewater, and unprocessed human urine (5% and 10%) were used in the blends. Incorporating nitrified urine will support the algal growth, but the study aims to investigate how it works when blending low growth yielding wastewaters (desalination brine, unprocessed human urine and hydroponic wastewater). Human urine is a good source of urea (Ray, Perreault, and Boyer 2019), a nitrogen source preferred by cyanobacteria (Erratt 2017). Previous studies demonstrated the differences in chemical content and algal growth between male and female human urine (Tuantet et al. 2014); therefore, this study was designed to compare urine from both male and female volunteers.

Additionally, it is observed (Figure 3.1b) that the low growth rate in conditions with different concentrations of unprocessed human urine in DST is because of the insufficient absorbable forms of copper, iron, manganese and zinc required for algal growth (Tuantet et al. 2014). Studies have demonstrated that bloom-forming cyanobacteria species *Microcystis aeruginosa, Dolichospermum flos- aquae,* and *Synechococcus* sp. grow abundantly in the presence of urea, as well; it is evident that the three species mentioned above primarily prefer urea as a nitrogen source comparing to inorganic nitrogen nutrients; additionally, it is shown the prokaryotic microalgae (cyanobacteria) render a competitive advantage over eukaryotic algae when growing in the presence of urea (Erratt 2017). Every microalgal species differs in its nutrient preference, especially in the uptake of different nitrogen nutrients (Salbitani and Carfagna 2021). There are various factors involved in the uptake preference. The presence of other nutrients in excess or deficient in the growing media influences the uptake of a particular nutrient element. This portrays the inoculated species *Anabaena circinalis* CS-533/02 and the innate wastewater species that have occurred on the three unprocessed urine blends that are not compatible with growing in the media with urea.

The four mixture showed stunted growth (Figure 3.1b); this could be an effect of the presence of unprocessed urine. The observed 12% reduced growth difference in the 10% blend comparing the 5% is clear evidence the increasing content of urea brings unfavourable growth. In general, it is known that urea will get decomposed into ammonia (Yim et al. 2004). Studies clearly demonstrated the inhibitory effects of ammonia in wastewater-treating systems involving microalgae and cyanobacteria, as well it is evident that cyanobacteria wastewater media are more sensitive to ammonia than eukaryotic microalgae (Rossi et al. 2020). Though the advantage of low molecular weight favouring its biochemical incorporation of ammonia into amino acids, preference of ammoniacal nitrogen forms by microalgae and ammonia toxicity differs to individual algal species (Gutierrez et al. 2016).

Usually, the bioremediation or utilisation technology of desalination brine are implemented with marine prokaryotic (Sánchez, Nogueira, and Kalid 2015) and eukaryotic microalgae (Ahamefule et al. 2021). In this study, we envisioned using freshwater microalgae because the produced biomass was intended to be used as a nutrient source in hydronic crops. In case of using marine microalgae complete dewatering of harvested biomass is required as it renders salinity. Previous studies conducted in *Anabaena doliolum* showed evidence that there will be a decrease in vegetative cells and nitrogen fixation efficiency upon increasing salinity concentrations (Rai and Abraham 1993). Though the desalination brine is a good source of mineral nutrients for microalgal cultivation (Shirazi et al. 2018b), the salinity tolerance plays role in the vegetative cells and nitrogen-fixing efficiencies. From 5% desalination brine in STD up to 15%, the gradual increase of algal growth upon the increasing concentration of desalination brine is the clear evidence of nutrients from brine enhanced the growth, the sudden decrease in 20% brine is the salinity stress. Hormesis is defined as the stimulatory effect of low concentrations of toxic chemicals or detrimental/stress factors in the organismal growth; in this context, salinity stress also pronounces hormetic effect (Jäger and Krupa 2009). Optimising the Hormetic salinity concentration that induces hormesis is an opportunity to use desalination brine to cultivate freshwater microalgae.

Many reports have shown that hydroponic wastewater can are useful for the cultivation of microalgae (Bertoldi, Sant'anna, and Barcelos-Oliveira 2009; Richa et al. 2020a; Salazar et al. 2021). Usually, hydroponic nutrient waters are reused to use the nutrients to their fullest possible for plant cultivation. After repeated nutrient topping up and reuse of hydroponic wastewater, at some point, there will be an imbalanced micro and macronutrient composition will occur and not compatible with the root uptake physiology. However, microalgae can utilise such residual nutrients of hydroponic wastewater that are low in root uptake concentrations or incompatible with root uptake physiology. During algal growth, if a particular micro or macronutrient is exhausted by a specific dominated algal species, the next algal species that feel optimal for its growth dominate the medium (Felisberto, Leandrini, and Rodrigues 2011). Within these species domination switches; at some point, nitrogen-fixing microalgae dominate when nitrogen is completely exhausted in the growing medium. By this repeated cycle of domination of different algal species, all nutrients are utilised entirely to a point where there will not be treatment required and ready to release directly to the natural water bodies. Thus, leading to energy-efficient wastewater treatment with the advantage of producing algal biomass.

Studies have demonstrated the effectiveness of algal growth in nitrified human urine, comparing the growth efficiencies on direct human urine (Feng et al., 2007). The absorbable form of nitrogen source is the critical factor determining the algal growth in the urine-based medium. Usually, nitrogen-fixing cyanobacteria grow slower than non-nitrogen fixers (Alya Raphael and Wittig 2003; Reddy et al. 1993); besides, if the medium is amended with combined nitrogen, nitrogen-fixers gain faster growth (Torres-Sánchez et al. 2015). Wastewater nitrogen content is the causative of eutrophication; when using the wastewater blending technique, nitrified urine can be applied as an initial stimulant for the induction of biomass. Further, the fully grown algal biomass goes into nitrogen-fixing mode and exhausts other micro and macronutrients, thus enabling complete nutrient removal.

This testing aims to understand the possibility of using different wastewater blends, focusing on their nutrient contents and recovery. Furthermore, it intended to approach an application-oriented method that enunciates the feasibility of its implementation – a blend-based nutrient cocktail (De Bhowmick,

Sarmah, and Sen 2019). Therefore, the samples were not subjected to the process of sterilisation; it is also known that the wastewater containing algal flora dominate and algal community change occurs upon utilisation of different micro and macronutrients. Within the given retention time (15 days), the phenomenon of algal community change is unavoidable. However, the microscopic observation was performed at every sampling point (every three days) to monitor the other intruding factor, like, protozoan, which grazes the algae (Day, Gong, and Hu 2017). Most microalgae screening studies used sterilised wastewater which is challenging and unfeasible on a large scale (Bohutskyi et al. 2015). The endogenous microbial flora of wastewater significantly impacts algal growth, nutrient removal efficiencies and overall biomass productivity (Palanisami et al. 2014). Therefore, to understand the reality, in this study, the wastewater was used unsterilised. The retention time reflects the energy-efficient process of any wastewater treatment (Borzooei et al. 2019); therefore, the retention time, two weeks (15 days), was set to study the recovery and biomass profiles. Urea can cause oxidative stress, leading to peroxidation and triggering microalgal cells' death (Sakamoto, Delgaizo, and Bryant 1998). Every microalgal species have a different physiological and withstanding trait of different nutrient elements; the microscopic observation of disintegrated cells in all the unprocessed human urine blend conditions revealed the used concentration instigated a detrimental effect on algal cells. Biomass production and nutrient recovery are the two potentials considered for the venture of wastewater treatment using microalgae. Though the efficiencies of biomass production and nutrient recovery differ in every filamentous and unicellular organism, studies have shown the advantages and shortcomings of unicellular species and filamentous microalgae, comparing unicellular filamentous forms support harvest methods (Rearte et al. 2021). All the tested wastewater conditions (Figure 3.2 b to m) showed domination of unicellular algae, on one side it is an advantage in terms of faster nutrient recovery and challenging for biomass harvest (Rearte et al. 2021).

It is a known phenomenon that prokaryotic and eukaryotic microalgae are diverse in their trait of nutrient uptake physiology (Amaral, Bonilla, and Aubriot 2014; Berman-Frank et al. 2007). Especially proportion of the availability of micro and macronutrient makes a significant difference in

their growth and other physiological activities (Saha et al. 2013). However, there will be an essential common trait in their nutrient requirement applicable to all microalgae categories cyanophyta, chlorophyta, haptophyta. Irrespective of nutrient regimes and algal flora, a clear recovery pattern was observed among the tested thirteen nutrient conditions/blends (Figure 3.11). Within the tested thirteen conditions, maximum recovery of iron and molybdenum occurred on the third day in ten blend conditions (Figure 3.11). Cyanobacterial photosynthetic electron transport chains, respiration, and nitrogen metabolism enzymes involve a high iron content; hence, comparing to other microbial groups, cyanobacterial iron requirements are much higher (González et al. 2018). The chemical form of bio-availability in the medium is essential for the recovery (uptake); the nutrient recovery result (Figure 3.11) clearly shows that the used wastewaters have high iron content in the algal oriented bioavailable form are quickly absorbed by the algae in the first three days. Although iron is a vital nutrient element in microalgae and cyanobacterial physiology, an excess of free intracellular iron is deleterious; through Fenton reactions, iron catalyses the formation of reactive oxygen species and cause oxidative stress (Kranzler et al. 2013). As observed in the recovery results, maximum recovery on the third day yields a safe environment which lessens the opportunity of iron-mediated oxidative stress. Molybdenum is another nutrient element that showed maximum absorption on day three (Figure 3.11). Molybdenum is an essential micronutrient, particularly nitrogen assimilation in microbes; molybdenum exists in nitrogenase, an enzyme that performs nitrogen fixation and nitrate reductase, which contributes the first step in nitrate assimilation, reduction of nitrate to nitrite (Glass et al. 2012). Over fifty enzymes in microalgae with molybdenum in their active sites and based on genomic and bioinformatic analysis, more gene products have been annotated as putative molybdenum-containing proteins (Hille, Hall, and Basu 2014). As this study used nitrogen-fixing algae *Anabaena circinalis* CS-533/02 as the initial inoculum, it is obvious the molybdenum uptake was rapid in the initial few days and satisfied their requirement. This rapid quench of molybdenum makes the cells grow healthy and, consequently, recover other nutrients efficiently. At its lower concentrations, iron acts as an essential nutrient element for algal growth and at higher concentrations, they are toxic. Algae mediated iron bioremediation is extensively studied; however, iron toxicity, bioavailability and speciation play a key role in its removal by microalgae (Subramaniyam et al. 2016). From the obtained results (Figure 3.3),

it is evident that iron and molybdenum is expected to recover at the first instance, which is conducive for not creating chances for speciation mediated toxicity with interaction with other metal nutrient elements.

Grouping of nutrient elements based on the recovery timeline (Mo, Fe, Mn, Cu, Zn)-day 3 > (Ca, Mg)-day 6 > (Na, K)-day 9 > (SO4, Co)-day 12; reveals, maximum removal of sulphate and cobalt expected to occur at day 12. This timeline-based plots of maximum nutrient recovery point greatly help in designing target specific nutrient recovery method. It also helps in arranging maps for the retention timeline of particular nutrient elements in mixed wastewaters. Application of targeted nutrient recovery with a planned recovery timeline yields an opportunity to devise methods for energy-efficient nutrient recovery. Also, this method will help determine blend proportions of different wastewaters.

Nutrient recovery profiles and plots of selective control nutrient recovery can contribute to the in-depth planning of wastewater utilisation and nutrient recovery strategies. Additionally, the derived least recovery profiles (sampling points/timelines at which least recovery of nutrients) (Table 3.10) provide a clear timetable layout when less recovery occurs. These least recovery profiles can be used in planning strategies to cut off or add on processes that require energy and expenses. Nutrient availability (recovery) can be significantly influenced by the concentrations of other ions, such as calcium and phosphate change the zinc physiology in microalgae (Cavet et al. 2003). Correlation coefficient based identification of nutrient recovery interlinks serves as a layout for blend composition decisions and can yield higher recovery in a short retention period. Some groups of nutrient elements will not influence the recovery of other nutrients; those sets of nutrient groups were tabulated in Table 3.12. When making blends with high and low wastewater nutrient concentrations, nutrient elements that will not participate in interlink formations can be mapped depending on their initial nutrient concentration profiles.

3.5. Summary

The specific conclusions drawn from this work are as follows:

- Mixing (blending) wastewaters of high and low nutrient concentrations can effectively be used for the production of algal biomass which is indisputably evident from the outcomes of different proportions of desalination brine mixed with secondary treated domestic wastewater. Also, it is lucid that the optimised range of marine wastewater (desalination brine) can be utilised as a source of algal nutrients in the cultivation of freshwater microalgae. This method offer opportunity for the utilisation of freshwater species in place of marine microalgae, marine algal biomass at some point require an extra step of salinity removal in biomass harvest and processing when using the acquired biomass for the manufacturing of food or feed grade commodities.
- Blending nitrified urine with nutrient depleted wastewaters suggests the prospect of the use of human urine, which is a massive waste source used for the production of food feed grade materials. As previous studies definite the lacuna of using human urine as it is an excretion path of pharmaceuticals, which will be removed from biological nitrification, then the blending of nitrified urine with other wastewater is a double-positive yield in terms of bio-material production and saving expenses pertain to waste remediation.
- Use of the nutrient stoichiometry calculation reveals the blueprint of the (i) total recovery of individual nutrients in the stipulated retention timeline, (ii) recovery rate of specific nutrients at specific intervals, (iii) forecast of highest recovery can be attained at the given timeline, (iv) highest and least recovery percentage of individual nutrient element (v) no recovery timelines of the particular nutrient element. The aforesaid factors are relevant to any composition of wastewater blends, this is reliable because the derivation was plotted on the basis of naturally dominated wastewater microflora (not species-specific) and the recovery profile occurred as a cumulative/overall nutrient uptake trait of a variety of wastewater algal flora.
- Irrespective of different blend composition, a clear recovery pattern was observed. Therefore, this selective control nutrient recovery plot is helpful for designing (i) targeted' nutrient (specific) recovery method, is an opportunity of utilising wastewaters with a high concentration of specific nutrient element, (ii) trategic planning of 'Retention Time lines' that yields devising schemes for 'Energy Efficient Recovery Process', leading to curtailing water treatment process and expenses.

Chapter 4

Microalgal biomass as hydroponic nutrients and stimulants

4.1. Introduction

The use of prokaryotic and eukaryotic microalgae as a food source and food supplements has been well-known for centuries (Wells et al. 2017). Worldwide, Australia, Asian countries, the United States, and European countries, have the practice of cultivating microalgae for human consumption for several decades (Vigani et al. 2015). Marine based resources are raising great expectations in the bio-economy; specifically, microalgae are attractive as a source of various bioproducts and high-value molecules of diversified utilisation potentials (Pulz and Gross 2004). In context to direct application of feed and food-grade bio-product, comparing the marine and freshwater microalgae is feasible for the cost-effective production, as the marine microalgal biomass require an extra step of the downstream process in the removal of (marine) salt concentrations. Especially when the algal product is intended for use in agricultural productions, freshwater algal biomass is practical for direct use. Though the research based evidence on microalgal nutrients is very assuring, in actuality, the currently available algae based products on the market for daily use are still in infancy. There are two categories of food-grade products that can possibly be produced from microalgae; the first category is dried algae with high nutrient content, especially high value bio-molecules; fatty acids, pigments and vitamins. Dried algal products can be sold directly as dietary supplements and can be added in bulk products as a base source of carbohydrates and proteins. The second category is extracted and isolated bio-products from the algal biomass used as food and feed to enhance their nutritional value. Overall, the bio-energy, cosmetic and pharmaceutical industries are utilising the bioresource potentials of microalgae, however, the reality is the nutrition segment appears to potentially benefit more from microalgae technologies (Vigani et al. 2015), especially the use of the algal products in agriculture.

Nitrogen-fixing cyanobacteria are used in rice fields as a bio-fertiliser to enhance crop productivity (Iniesta et al. 2021). To feed the growing human population and food demand, advancements of food security developed high yielding types of crop cultivars to achieve maximum production in a short duration. Compared to the low yielding cultivar crops, these high yielding cultivars acquire a higher quantity of nitrogen fertilizer for growth and development. Plants consume nitrogen nutrients in the form of nitrate and ammonium ions (Fagodiya et al. 2017), and 50% to 70% of total applied nitrogen,

not absorbed by the plants and lost in the form of nitrous oxide (Malyan et al. 2016). This process causes 30% to 50% reduction in nitrogen use efficiency by the crops (Kumar et al. 2019); on the other hand, the released nitrous oxide enhances global warming (Malyan et al. 2020). Direct feeding of nitrogen nutrients to plants by supplying the nitrogen directly to the root through hydroponics is an alternative for efficient nutrient use. To implement this, changing from soil-based cultivation to all possible food crop types appropriate to grow in hydroponics is the way to efficient nutrient use without loss and a pathway to limit nitrous oxide production. This shift enables reducing climate change. This is the first time using nitrogen-fixing algal biomass as a source of nitrogen for hydroponic crops. Microalgae produce an array of plant hormones, which can act as plant biostimulants (Singh 2014). This is an added advantage of using algal biomass as a nutrient source in hydroponic cultivation.

Globally, the interest in plant biostimulants has increased over the past decade, driven by the increasing interest of scientists, extension specialists, private industry, and growers incorporating these products into various environmentally sustainable methods to ensure improved crop yields production and stability of yields (Rouphael and Colla 2018). According to the recent European Union regulation (2019/1009), plant biostimulants are 'fertilising products able to stimulate plant nutrition process independently of the products nutrient content' (Colla and Rouphael 2020). Based on this specification, plant biostimulants are referred through claimed agronomic effects, such as improved efficiency of use nutrients, crop quality and tolerance to abiotic stressors. This description includes numerous organic and inorganic substances and/or microorganisms such as humic acids, hydrolysate proteins (Paul et al. 2019), carbohydrates (Rachidi et al. 2021), extracts of seaweed (Mukherjee and Patel 2020), mycorrhizal fungi, bacteria, and microalgae.

Cost-effectiveness is essential for sustainable microalgae-based agricultural bioproduct productions. The drawback of many algae-based bio-products is their high production cost (Rafa et al. 2021), especially a significant portion occupying the harvesting cost. A study on the low-cost harvesting processes reveals that harvesting engulfs 30% of biomass production costs (Barros et al. 2015); additionally, other extraction and finishing steps engross further expenses. Therefore, reducing the harvesting method's expenses is critical in developing a sustainable production method (Christenson

and Sims 2011). This study ventured a natural gravity settling based inexpensive harvest method, additionally, used phototaxis mechanism for efficient settling and harvest. As this study intends to use the harvested biomass for food production (hydroponic cultivation), experimentations are planned in such a way as to avoid chemical-based flocculation or other processes causing cell damage or disruption. Biomass with intact cells is essential to prevent loss of cytosolic (fixed nitrogen) nutrients and plant hormones. Natural gravity settling makes a rapid, inexpensive, relatively simple, and less cell disruptive than other harvest methods (Singh and Patidar 2018). This chapter aims to demonstrate the potential of microalgal biomass as a source of nutrients and stimulants.

4.2. Materials and methods

4.2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma, Australia, and used without any further purification. Dibasic potassium phosphate (98%), magnesium sulphate heptahydrate (98%), calcium chloride dehydrate (99%), citric acid (99.5%), ammonium ferric citrate green (99%), ethylenediaminetetraacetic acid disodium salt (99%), sodium carbonate (99%), boric acid (99%), manganese chloride tetrahydrate (98%), zinc sulphate heptahydrate (99%), sodium molybdate dehydrate (99.5%), copper sulfate pentahydrate (98%), cobalt(II) nitrate hexahydrate (99.9%), bacteriological agar, Agilent ICP-MS (*Inductively Coupled Plasma Mass Spectrometry*) environmental calibration standard (5183-4688), ACR (Australian Chemical Reagents) Ion Chromatography Nitrate, Phosphate, sulphate standards. Plant hormone standards - 3-Indoleacetic acid, 2-cis-4-trans-Abscisic acid, benzoic acid, gibberellic acid, gibberellin A4, indole-3-acetic-2,2-d2 acid, indole-3-carboxylic acid, jasmonic acid, salicylic acid, trans-cinnamic acid, trans-cinnamic-d7 acid, zeatin, (±)-dihydrojasmonic acid, 12-oxo phytodienoic acid.

4.2.2. Algal culture – maintenance of stock culture and seed inoculum

As this project used sedimentation based algal harvest, the algal species selection was based on the criteria of fast settling rate. BG11 media prepared as in section 3.2.1.3, BG11 agar plates were prepared by adding 20 g/L bacteriological agar, autoclaved at 121°C, 15 psi for 15 minutes, and

aseptically poured into Petri plates. After cooling, and solidification of agar algal culture was inoculated using a disposable plastic inoculation loop. The cultures were incubated at 25°C ± 2 °C under 1500 LUX (Pandey et al. 2007) white fluorescent lights of 12 hours: 12 hours(day: night) cycles. The stock culture was periodically sub-cultured every ten weeks into the fresh solid medium. The purity of the culture was monitored by light microscopic observation (Figure 1). To prepare the seed inoculum, cultures were grown in a 500 ml conical flask with 200 ml of liquid BG11 media.

4.2.3. Experimental algal culture

From the seed inoculum, cultures were grown in 1-L conical flasks with 400 mL of liquid BG11. Mid-log-phase cultures were harvested by centrifugation at 9000 rpm for 5 minutes using Sigma 1-16KL centrifuge, the supernatant was discarded, and the pellet was resuspended in Milli-Q water, vortexed, and the Milli-Q resuspension/washing of pellet was repeated five times to ensure the algae cleaning up of BG11 nutrient contents. The cells were observed under a microscope for checking centrifuge shear-induced cell damage. The final pellet was brought into a high cell density inoculum.

4.2.4. Large scale growth experiments

Large scale cultivation was performed on 1200-L capacity photobioreactor (Industrial Plankton, Canada). The photobioreactor has inbuilt sensors for pH, temperature, and relative density (optical density). The sensors were programmed to read measurements every minute, and the day/night light shift was set for twelve hours, from 7 am to 7 pm. The total capacity of the reactor is 1200 litres; ten times diluted nitrogen-free BG11 media was used to grow algal biomass. The intensity of the LEDs light can be set between 10% to100% (values below 10% turn off lights). 100% is ~1200 $\mu Em\text{-}2S^{-1}$. The reactor was filled with 400 litres of ten times diluted BG11 and inoculated with *Anabaena circinalis* CS-533/02. The light intensity was maintained at 50% for five days to allow the initial pick up of biomass; then, the light intensity was changed to 100%. The cultures were allowed to grow until reaching active mid-log phase cultures (21 days). The first set was cultivated in nitrogen-free media; after harvesting the biomass, the cultures were drained completely, and a fresh inoculum was made

along with 1mg/ L sodium nitrate; the same growth cycle was followed for ten times diluted BG11 (with 1mg/ L sodium nitrate).

Figure 4.1a 1200-L photobioreactor used for the large scale cultivation of algal biomass to demonstrate the fast settling trait, biomass cultivated in a 12 litres aquarium tanks using nitrogen free BG11. The cultures were mixed with frying pan spoon, image capture after two hours and overnight; to prove the fast settling characteristic of *Anabaena circinalis* CS-533/02.

4.2.4. Algal biomass harvest

The cultures were transferred to 200 litre plastic barrels using the sampler pump (Figure 4.1b). The barrels were then covered with their black polythene bag (Figure 4.1c) to avoid light and

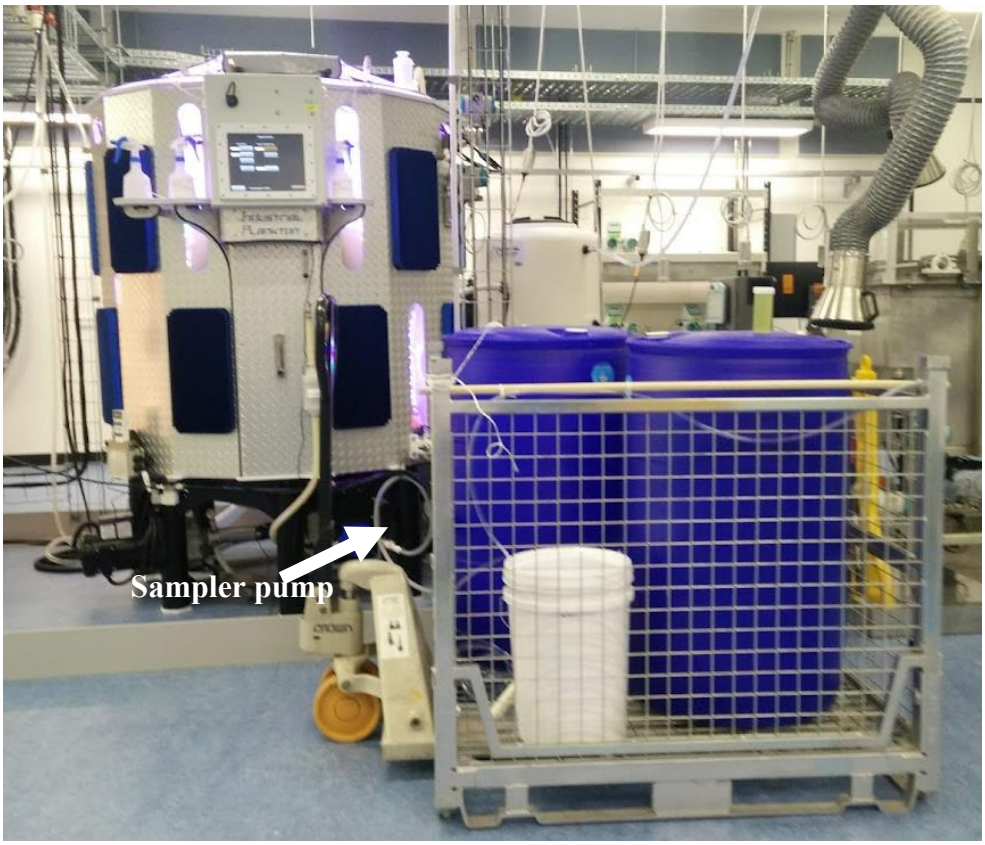


Figure 4.1b Transferring culture from the bioreactor to 200 litter plastic barrels using two way (in/out) sampler pump

allowed to settle (sediment) overnight. The harvest was scheduled in alignment with the day/night light cycles; the top liquid layer was carefully transferred back to the reactor using the sampler pump. The harvested thick biomass was centrifuged at 9000 rpm at 25 °C for 5 minutes.

4.2.5. Acid digestion of algal biomass

The 1 kg of harvested thick paste of algal biomass was transferred into a two-litre glass (Schott Duran) reagent bottle. The algal biomass was pre-chilled by placing the container inside an ice bath; concentrated sulphuric acid was slowly added along the sides of the container and intermittently mixed thoroughly to dissolve the biomass. Acid was added to the level of saturation until the biomass was completely dissolved. To adjust the pH, potassium hydroxide pellets were added until reaching pH 6. The digested biomass was then stored at 4 ^{o}C until further use.

Figure 4.1c After transferring the culture from the bioreactor to 200 litter plastic barrels, the barrels were covered with a black plastic bag to inhibit light exposure.

4.2.6. Phytohormones profiling sample extraction

Algal biomass cultivated from the first set (refer to section 4.2.4) was used to extract hydroponic nutrients; from that, a portion of biomass was saved to study the phytohormone profile difference between the algae grown in nitrogen and without nitrogen. The phytohormones profiling was done following (Cao et al. 2017). For each sample, algae grown with and without nitrogen; 100 mg of freeze dried biomass were weighed into a 2 mL polypropylene screw-capped tubes. The tube was prewashed using 70% methanol. Then, 1 mL of 70% methanol containing 5 µl internal standard working solution (3-Indoleacetic acid, 2-*cis*-4-trans-Abscisic acid, Benzoic acid, Gibberellic acid, Gibberellin A4, Indole-3-acetic-2,2-d2 acid, Indole-3-carboxylic acid, Jasmonic acid, Salicylic acid, trans-Cinnamic acid, trans-Cinnamic-d7 acid, Zeatin, (±)-Dihydrojasmonic acid, 12-oxo Phytodienoic Acid) was added to the sample. Samples were then homogenized using a cryomill coupled to a cryolys cooler set to -10°C (6,800 rpm, 3 × 30 s, 30 s break) followed by shaking for 30 min at 900 rpm at 4°C. Then, the samples were centrifuged at 15900 rpm at 4°C for 5 min. The supernatant was transferred to a 2 mL tube and dried using a vacuum concentrator under full vacuum at 30°C. The dried extract was reconstituted in 50 µl of starting mobile phase 5% acetonitrile with 10 mM ammonium acetate (NH_4Ac) and successively sonicated for 10 min until the dried extract was dissolved completely. The extract was centrifuged at 15900 rpm at 4°C for 15 min prior to transfer to amber vials with glass insert. Samples were stored at -80°C until Liquid Chromatography-Mass Spectrometry (LC-MS) analysis.

4.2.7. Liquid chromatography-mass spectrometry (LC-MS) analysis of phytohormones

Phytohormones were separated on a Phenomenex Kinetex C_{18} reversed-phase column (2.1 mm × 100 mm, 1.7 µm) maintained at 45°C using TSQ Quantum Access with U3000 UHPLC. The mobile phases and gradient were as follows: mobile phase A: 10 mM NH_4Ac in deionized water; mobile phase B: 10 mM NH_4Ac in ACN. Flow rate: 0.4 mL min^{-1}. The programmed step gradient was: 5% B over 0.5 min, 5–35% B over 4 min, 35–55% B over 1 min, 55–75% B over 2 min, 75–100% B over 0.1 min, followed by a clean-up step: isocratic elution at 100% B for 2 min, 100% to 5% B over 0.1 min

and column wash for 2.5 min. Mass Spectrometry parameters (for positive and negative ionization, respectively): gas temperature: 100°C; gas flow: 11 L min^{-1}; nebulizer: 40 psi; sheath gas temperature: 400°C; sheath gas flow: 12 L min^{-1}; capillary: ±3500 V; nozzle voltage: ±300 V; high pressure radiofrequency: +120 V, -140 V; low pressure radio frequency: +80 V, -100 V.

4.2.8. Quantification of nutrients from the algal extracts

Triplicate samples were collected at the interval of every three days and centrifuged at 9000rpm for five minutes; the cell-free supernatant was filtered through a sterile 0.4-µm filter. Inductively Coupled Plasma Mass Spectrometry (ICP-MS), Agilent 7900 with an auto sampler was used to quantify the concentrations of copper, Zinc, Manganese, Magnesium, Molybdenum, Calcium, Iron, Potassium, Cobalt, and Sodium. In order to derive the calibration curve, the Agilent environmental calibration standard (5183-4688) was diluted to five different concentrations (1.25 to 10 µg/L) according to the nutrient concentration of different sample sets. Every sample injection will be made in triplicates, and the Agilent MassHunter software gives result outputs from the average and standard deviation of triplicates. Nitrate, phosphate and sulphate were quantified using Dionex Integrion RFIC Ion Chromatography with Thermo Fisher Scientific Dionex AS-AP auto-sampler. Five increasing concentrations of ACR nitrate, phosphate and sulphate standard was diluted to different concentration (0.1 to 100 mg/L) according to the nutrient concentration of different wastewater samples. The above mentioned method is the same as section 3.2.2.1 (in chapter 3).

4.3. Results

4.3.1. Fast settling algal species

To implement a settling (sedimentation) based harvest method, algal species with quick settling trait was selected. Figure 4.2 represents the fast settling trait of the selected species *Anabaena circinalis* CS-533/02; when the culture was allowed to stand overnight, complete biomass settling occurred. Biomass settles faster in two hours, and a complete settling occurs when allowed to stand overnight.

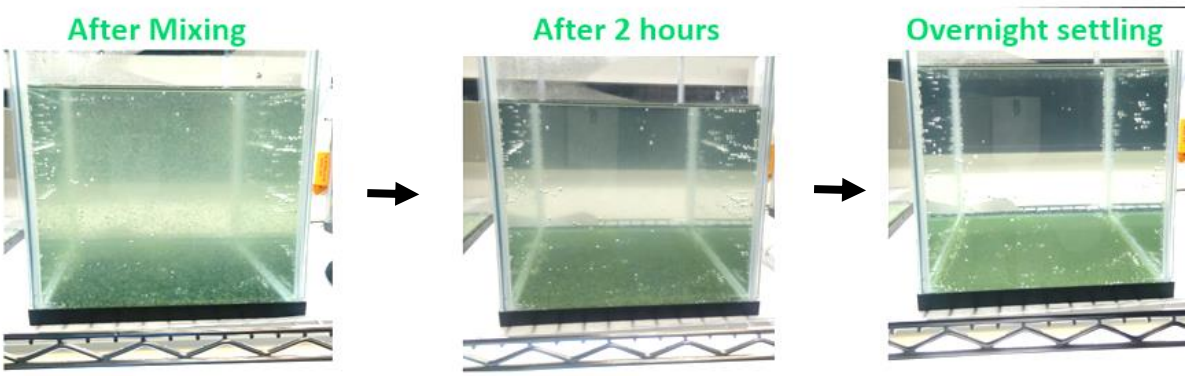


Figure 4.2 *Anabaena circinalis* CS-533/02 showing biomass settles faster in two hours of time and a complete settling occurs when allowed to stand overnight

4.3.2. Large scale biomass harvest efficiency

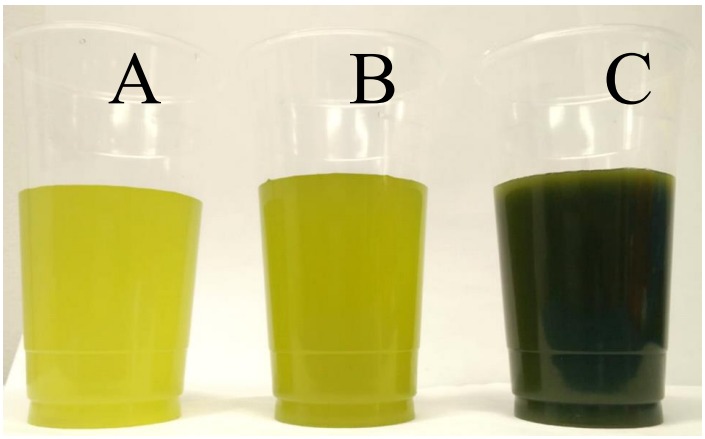


Figure 4.3 Visual differences in the biomass from (A) top layer liquid after settling, (B) bioreactor (actual culture), and (C) settled (sediment) thick biomass.

To understand the harvest efficiency (Figure 4.1c), algal biomass was quantified in terms of dry weight from the (i) bioreactor, (ii) top layer liquid after settling, and (iii) settled (sediment) thick biomass (Figure 4.3). In terms of dry weight, the culture was sixteen times concentrated in the sedimented biomass (Table 4.1).

Table 4.1 Algal biomass measured in terms of dry weight

Sample	Dry Biomass (mg/L)
Supernatant	51.62±3.42
Actual Culture	155.67±7.07
Harvested sludge	1867.43±60.82

4.3.3. Nutrient content in algal biomass extract

Among the thirteen measured nutrient elements, potassium was the highest concentration in the biomass extract, followed by sulphate (Table 4.2). The concentration of potassium and sulphate ranged to grams/litre; sodium, phosphate, calcium, nitrate, and magnesium were ranged in milligrams, copper, cobalt, iron, manganese, molybdenum, and zinc were in micrograms/litre. The nutrient contents were in the order of highest to lowest as follows; potassium > sulphate > sodium > phosphate > calcium > nitrate > magnesium > manganese > zinc > iron > molybdenum > copper > cobalt.

Table 4.2 Nutrient element contents in the acid-digested algal biomass extract

	Nutrient Element	Algal Extract working solution (5 mL of extract diluted to 1 L)	Concentration in original acid-digested algal extract
1	Nitrate	0.86±0.02 mg/L	172 mg/L
2	Phosphate	4.56±0.2 mg/L	912 mg/L
3	Sulphate	1.38±0.002 g/L	276 g/L
4	Sodium	9.35±0.24mg/L	187g/L
5	Magnesium	0.3±0.00mg/L	60mg/L
6	Potassium	2.54±0.01g/L	508g/L
7	Calcium	2.28±0.01mg/L	456mg/L
8	Manganese	74.75±0.81µg/L	14.95mg/L
9	Iron	32.87±0.79µg/L	6.574mg/L
10	Cobalt	0.994±0.06µg/L	198.8µg/L
11	Copper	1.18±0.004µg/L	236µg/L
12	Zinc	52.68±0.37µg/L	10.53mg/L
13	Molybdenum	2.98±0.06µg/L	596µg/L

Table 4.3 List of fourteen plant hormone standards used for their detection in microalgal biomass extract, occurrence in microalgae, and plant biological functions

	Tested Plant Hormones	Reported algal species	Biological function
1	2-cis-4-trans-Abscisic acid	No report from microalgae so far	Abscission of leaves and dormancy in buds and seeds (Ueda and Tanaka 1977)
2	3-Indoleacetic acid	*Emiliania huxleyi* (Labeeuw et al. 2016)	Altering cell orientation, organ development, fertility, and cell elongation (Fu et al. 2015)
3	Benzoic acid	*Calothrix* sp. (Renuka et al. 2018)	Flowering (Fujioka et al. 1985)
4	Gibberellic acid	*Scytonema hofmanni* (Rodríguez et al. 2006)	Cell division and elongation and has been used to manipulate flowering and fruit development (Kevin Lacey 2019)
5	Gibberellin A_4	*Chlorella sorokiniana* (Do et al. 2020)	Stem elongation, germination, dormancy, flowering, flower development, and leaf and fruit senescence (Cerny-Koenig, Faust, and Rajapakse 2005)
6	Indole-3-acetic-2,2-d2 acid	No report from microalgae so far	cell division, differentiation, and vascular bundle formation (Bianco et al. 2014)
7	Indole-3-carboxylic acid	*Planktothricoides (Duong et al. 2021)*	Cell elongation and cell division (Jiang et al. 2016)
8	Jasmonic acid	*Microcystis aeruginosa* (Zhao et al. 2020)	Signalling pathway (Ruan et al. 2019)
9	Salicylic acid	*Microcystis aeruginosa* (Zhao et al. 2020)	Endogenous signal mediating local and systemic plant defence responses against pathogens (Rivas-San Vicente and Plasencia 2011)
10	trans-Cinnamic acid	*Oscillatoria* sp (Babaoglu Aydaş, Ozturk, and Aslim 2013)	Leaf expansion (Kurepa and Smalle 2019)
11	trans-Cinnamic-d7 acid[1]	No report from microalgae so far	Abiotic stress response and leaf expansion (Kurepa and Smalle 2019)
12	Zeatin	*Scenedesmus* (Kurepa and Smalle 2019)	Plant development and defence responses to pathogen (Schäfer et al. 2015)
13	(±)-Dihydrojasmonic acid	No report from microalgae so far	Drought stress stabilisation (Merlaen, De Keyser, and Van Labeke 2020)
14	12-oxo Phytodienoic Acid	*Chlorella sorokiniana* (Khasin et al. 2017)	Growth control, flower and fruit development, senescence (Koeduka et al. 2015)

4.3.4. Phytohormone content in algal biomass extract

Amongst the tested fourteen plant hormones (Table 4.3), only 3-Indoleacetic acid and Indole-3-carboxylic acid were in the detectable range (Figure 4.4). In both the hormones (3-Indoleacetic acid and Indole-3-carboxylic acid) measured in the detectable levels, the culture grew with nitrogen free BG11medium found to produce lesser quantities of hormone. In case of 3-Indoleacetic acid cultures grown with nitrogen produced a fivefold higher hormone concentration than nitrogen free cultivation. There is almost forty-five percentage of increased Indole-3-carboxylic acid production in cultures supplied with nitrogen.

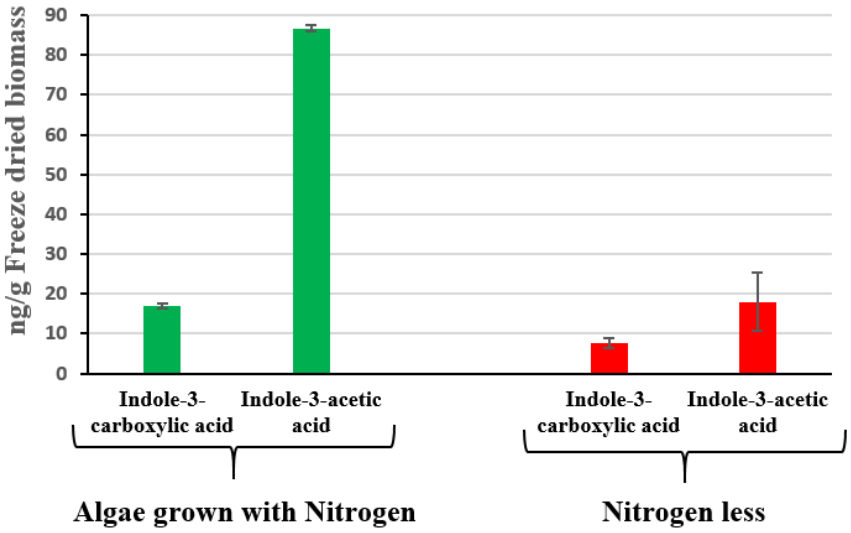


Figure 4.4 Quantification of plant hormones in algal biomass grown with and without nitrogen shows a higher hormone production rate in cultures supplied with nitrogen (results represented average of triplicates).

4.4. Discussion

Studies have demonstrated the use of BG11 medium to enrich the microalgal species isolated from environmental sources, specifically, samples isolated from wastewater (Gatamaneni, Orsat, and Lefsrud 2018), suggesting BG11 enrich various kinds of strains isolated from natural water bodies and environmental samples. Furthermore, BG11 is supportive for culturing various microalgae species,

including blue-green microalgae (cyanobacteria) under photoheterotrophic and chemoheterotrophic modes by addition with glucose (McKinley and Wetzel 1979). In this study, the selected algal species *Anabaena circinalis* CS-533/02 is capable of adapting to BG11. This work aims at wastewater conditions, which will not have all enriching factors like a standard algal growth medium. Most microalgal growth mediums other than BG11 have vitamins in their composition (Verma et al. 2020), which will not be available in wastewater derived nutrients. Studies have used nitrogen free BG11 media in large scale photobioreactors (Lara-Gil, 2016). The actual composition of BG11 is formulated to supply full strength nutrients that can serve a few growth cycles. As this study intends to grow algae in mixed wastewater and estimate the biologically fixed nitrogen for the cultivation of plants, BG11 was diluted to ten times to simulate a wastewater situation.

Many microalgae species produce gas vesicles, giving buoyancy and allowing movement to optimal light intensities in the dwelling water surfaces (Walsby 1994). Principally, the buoyancy of microalgae is balanced with the cell mass resulting from the production of photosynthetic carbohydrates and other cell constituents (Kinsman, Ibelings, and Walsby 1991). The sedimentation phenomenon is the gravitational forces that create the separation liquid or solid particles to separate from a liquid of different densities. Sedimentation can be explained by Stokes' Law, "the sedimentation velocity is proportional to the square of the radius of the cells and the difference in densities between the microalgal cells and the liquid medium as shown below (Milledge and Heaven 2013):

$$\text{Setting velocity} = \frac{2}{9} g \frac{r^2}{\eta} (\rho_s - \rho \quad \text{(Equation 4.1)}$$

where r is cell radius, η is fluid dynamic viscosity and ρ_s and ρ_l are the solid and liquid densities.

The microalgal cell density is near that of the density of water and salt water at 1,025 kg m^{-3} (Lepple 1972); there is a small density difference that functions on microalgal settlement. Different group of microalgae have different densities; the cytoplasm density of marine eukaryotic microalgae range between 1,030 and 1,100 kg m^{-3}, the prokaryotic microalgae (cyanobacteria) is between 1,082 and 1,104 kg m^{-3} (Kromkamp and Walsby 1990). Marine diatom and dinoflagellates have density range

between 1,030 and 1,230 kg m^{-3} and the freshwater eukaryotic microalgae (*Chlorococcum*) between 1,040 and 1,140 kgm^{-3} (Van Ierland and Peperzak 1984). The settling velocity of microalgae is very much relevant to the type of microalgae growing in the cultivation medium, either prokaryotic or eukaryotic marine or freshwater type of species; additionally the water quality influence the sedimentation rates. The selected candidate species study *Anabaena circinalis* CS-533/02 is a prokaryotic freshwater microalgae (cyanobacteria). Modelling or mapping the growing media's water quality in alignment with algal growth stages allows species-dependent harvest and can be used to evade toxic algal forms in circumstances if they dominate the cultivation system.

The harvesting efficiency of biomass is important in large scale microalgae production. In this study, the settling-based harvest method was originally designed to directly use sedimented biomass for acid extraction. During the method optimization, upon a few trials, it was evident that more volume of concentrated acid is required for the digestion; if the harvested biomass is in sludge-like consistency instead of paste. More the dewatering proportionally reduced the consumption of acid volume for attaining an (acid) saturated biomass digest. To improve the efficacy of biomass harvest in the perspective of achieving a reduced production cost, centrifugation can opt as a final dewatering method preceded by settling based biomass harvest (Singh and Patidar 2018). Large scale centrifugation based algal biomass harvest identified to have high efficiency with low energy expenses; studies involved in algal oil extraction for bioenergy have shown the harvesting costs of US$ 0.864/L algae oil (Dassey and Theegala 2013).

Depending on the nature of the targeted bioproduct, the biomass harvest method has to be optimised. Irrespective of the adopted harvesting method, the overall process should overcome the difficulties associated with separating biomass grown from the growing media suspension (Ferreira et al. 2020). The algae fixed nitrogen, cytosolic/cellular nutrients, and stimulants are the target bio products in this context. In this 1200 litres pilot-scale (study) biomass production, though the settling based harvest was successful (Figure 4.3) by the optimised method (Figure 4.3), the final dewatering was attained by centrifugation. As is evident from studies (Dassey and Theegala 2013) and feasible to implement

for large scale (Singh and Patidar 2018), optimised centrifugation method will be an idyllic option for the extraction of nutrients and stimulants from algal biomass.

The use of nitrogen fixed by microalgae as a source of nitrogen nutrient to hydroponic crops in the form of acid digested algal biomass is a novel approach. This study has shown the algal biomass grown in nitrogen free BG11 media produced 0.86±0.02mg/L (Table 4.2) of nitrate in the working solution, which is prepared by diluting 5ml of the original acid digested biomass. Nitrogen and phosphorus are the major nutrients that cause wastewater-related pollution and damage natural water bodies and ecosystems (Sengupta, Nawaz, and Beaudry 2015). Ammonia is one of the most harmful pollutants that disrupts the environment through eutrophication (Darwish et al. 2016). Ammonium nitrogen is toxic to many organisms, specifically plants and other oxygenic photosynthetic types of microorganisms (Markou, Vandamme, and Muylaert 2014a). The microalgal physiology evolved to directly assimilate ammonium nitrogen, incorporating them into amino acids via the enzymes glutamate synthase and glutamine synthetase (Wu et al. 2016). In the case of utilising nitrate as a nitrogen source, first, it gets reduced to nitrite in the cytosol, then to ammonium (nitrogen) in plastids or chloroplasts (Terrado et al. 2015). The obtained results (Table 4.2) signify the potential of practically applying algal biomass-based nitrogen nutrients to hydroponic crops.

Phosphorus is one of the vital nutrient elements in the physiology of living beings. Conventional agricultural fertilization dominated the usage of phosphorus resources; currently, though phosphorus has substantial other applications, over 90% of current phosphorus resources and >80% of fossil phosphorus resources are used as fertilizers (Chowdhury et al. 2014). Fossil phosphorus resources have been depleting at an increasing rate (Reijnders 2014). The current rate of phosphorus use emerges negative environmental impacts by contaminants from industrial, agricultural processes, and fossil phosphorus resources, also leading to possible adverse effects on human health. Recycling and reusing phosphorus is important in the current global phosphorus demand and climate change situations that require reducing carbon footprint from industrial productions of phosphorus products.

Batch variation in the wastewater chemistry will be helpful for blend based wastewater nutrient recovery. These batch variations of phosphorus content can be used as a tool for biological phosphate

recovery using microalgae. In microalgae, “luxury uptake”is a phenomenon, refers to the ability to take up more phosphorus than the required concentration for growth and metabolism (Solovchenko et al. 2019) and also this absorbed phosphorus are produced as polyphosphate granules. Every algal species have different optimal conditions for luxury phosphorus uptake; understanding and mapping the conditions that trigger the luxury uptake is helpful to explore the algal system as a biofactory for the application of phosphorus recovery and reuse. For example, *Scenedesmus* spp trigger luxury uptake in optimal light intensity and temperature (Powell et al. 2011). Microbes produce polyphosphate granules; they are characterized by high phosphorus content in the form of polyphosphate (Docampo 2006). In eukaryotic microalgae, *Scenedesmus* spp, increased temperatures elevate the biosynthesis of polyphosphate, and the increased light intensity lowers the production of polyphosphate (Powell et al. 2009). Unlike eukaryotic microalgae, in the case of prokaryotic microalgae, the scenario was different, *Plectonema boryanum* polyphosphate biosynthesis or luxury uptake is controlled by changes in phosphate concentrations in the living niche in a manner of “feast and famine” (Yang et al. 2017). Phosphate-starved *Plectonema boryanum* produced polyphosphates when grown the growing medium enriched with phosphate. Continuous availability of high phosphorus concentrations in the growing medium will not trigger luxury uptake, whereas, immediately next to a phase of phosphorus starvation, microalgae significantly increase polyphosphates in the biomass (Wu et al. 2012). Consequently, alterations in the phosphate concentration in a mode of “famine and feast” is a key for luxury uptake of phosphorus. Batch variations in the wastewater can be used as a tool to operate a famine and feast mode off nutrient supply to steer the algal physiology for the production of polyphosphate.

Cyanobacteria are known for the production of variety of plant hormone (Shariatmadari et al. 2013). This study clearly shows nitrogen content in the growing media influenced the production of plant hormones in *Anabaena circinalis* CS-533/02 (Figure 4.4). Studies have clearly demonstrated that various biochemical and environmental factors influence cyanobacteria production of plant hormones (Tan et al. 2021). *Planktothricoides raciborskii* produced 0.67 µg/gram fresh weight of indole 3 carboxylic acid and 5.51 µg/gram fresh weight of indole 3 acetic acid, which was grown in BG11

media with nitrogen (Duong et al. 2021). Compared to *Planktothricoides raciborskii*, *Anabaena circinalis* CS-533/02 produced (Figure 4.4) less quantity of indole 3 carboxylic acid and indole 3 acetic acid, as it is known that every individual microalgal's genotypic and phenotypic trait; in addition to that the external physicochemical factors significantly determines the producing capacity of different bio-materials in the microalgal system (Chen et al. 2017). The aromatic amino acid tryptophan is a precursor for the biosynthesis of indole 3 acetic acid (phytohormone), and microalgae are known to synthesize indole 3 acetic acid in the presence of tryptophan (Mazhar et al. 2013). It is clearly evident that when *Anabaena* sp grown with nitrate as the nitrogen source; will not produce heterocysts and nitrogenase activity; in such cultures growing on nitrogen-free medium, the production of heterocysts and nitrogenase can be induced by supplying tryptophan (Baalen et al. 1980). His is the clear evidence that nitrogen and biosynthesis of indole 3 acetic acid have a direct link. The study led by Hashtroudi and Prasanna (Hashtroudi et al. 2013; Prasanna et al. 2010) concluded that in *Anabaena* without the presence of tryptophan, biosynthesis of indole 3 acetic acid would be negligible but not impossible, this finding indicating the activated state of the tryptophan-independent pathway.

This study has not used inducers (tryptophan) to produce indole 3 acetic acid and clearly showed that the supply of nitrogen in the cultivation system enhances phytohormone production (Figure 4.4). For material safety reasons, the real wastewater was not used in the (1200 litres photobioreactor) large-scale cultivation; instead, ten times diluted nitrogen free BG11 was used to mimic the wastewater chemistry. Nitrogen-fixing cyanobacteria can grow in the presence and absence of nitrogen nutrients in the growing medium by switching on and off heterocyst producing mechanisms and nitrogen fixation (Baalen et al. 1980). This study aims for simultaneous nutrient recovery and biomass production that can act as a nutrient and stimulant for hydroponic plants. When using real wastewater, initially, the presence of nitrogen will support growth, and later, when the nitrogen exhausts, switching to nitrogen-fixing mode will not affect the production of phytohormones. As it is evident from the results that nitrogen-free conditions will also produce plant hormones. Both pro and eukaryotic microalgae release various organic substances upon growth and maturation; this includes amino acids and sugars (Leloup et al. 2013). It is a common phenomenon that phytohormones production in

cyanobacteria was observed more prominent at the stationary phase than the early growth stage. This is because the available organic substances and inducers (tryptophan) in the medium were initially used as a source of nitrogen and nutrient, especially when growing in the nitrogen-free medium (Sergeeva et al. 2002).

4.5. Summary

The specific conclusions drawn from this work are as follows:

● Biomass of nitrogen-fixing microalgae grown in nitrogen free media can act as a source of hydroponic nitrogen-nutrient. Especially, the quantified nitrate from this study were the same as hydroponic media.

● Contents of other micro and macronutrient elements in the algal biomass extract affirm that it can supply all nutrient requirements for plant growth. Additionally, there is clear evidence and scope for the modulation of attaining desired biomass chemical quality when using wastewater blends for large scale biomass production; as microalgae are known for the bioaccumulation of metal nutrients, nutrients such as – copper, zinc, cobalt, manganese, magnesium, molybdenum, and iron.

● Gravity settling combined with large scale centrifugation can effectively be applied for concentrating algal biomass. When cultivating algae on a large scale using wastewater blends, the wastewater algal microflora can be eluded by finely optimising the settling method in alignment with the existing water status of water quality (nutrient contents) of the growing medium.

● Using wastewater blends, it is possible to apply nutrient feeding modulation method; adding limiting nutrients as needed in coherence with the nutritional requirements of particular growth stage that trigger the plat hormone biosynthesis.

Chapter 5

Growth responses of pak choy and collards in acid digested microalgal extract as hydroponic nutrients

5.1. Introduction

The contemporary scenario of global pollution, multi-layer manifested impacts of climate change, freshwater scarcity, and the insistent need for growing global food demand necessitates finding a solution and a balancing factor that controls the current requirements. Hydroponics, a soilless method of plant cultivation, assures quality, healthfulness, freshness, pollutant residue-free fruits and vegetables, especially; yields an option to produce locally Khan et al., 2020 (Khan et al. 2020). The fundamental principle for vegetable production in hydroponic systems is to supply all required nutrients to the plant. Principally in hydroponics, plants are cultivated in enriched nutrient water, oxygenated, in substrata without soil. Perfect nutrient solution management is the key to successful hydroponic cultivation (Sato et al. 2006). The ultimate purpose of hydroponic nutrient solution is to provide the roots with water, mineral elements and oxygen in a soluble and root absorbable form. There are seventeen nutrient elements required for the optimal growth of plants in hydroponics (Arnon 1938). Of different nutrients, nine elements (sulphur, phosphorus, calcium, magnesium, potassium and nitrogen) are referred to as macronutrients which are needed in large quantities. The eight remaining nutrient elements are known as micronutrients, which are iron, zinc, copper, manganese, boron, chlorine, cobalt and molybdenum required in small quantities (Sato et al. 2006). The majority of the hydroponic nutrient solution formulations are produced based on the composition of Hoagland and Arnon 1938 (Arnon 1938).

Factually, the quality of different hydroponic nutrients available in the market differs depending on the purity of the chemical ingredient, solubility, and cost. Small scale operation farms/growers generally purchase ready-made nutrient mix formulations; it will be in the form of just diluting it and applying. Larger scale growers usually formulate their own nutrient composition aligned with the standard (Hoagland) hydroponic nutrients or slightly modified formulations (Khan et al. 2020). The fundamental requirement for hydroponic production is to deliver all the nutrients needed for the plant. If higher yields are expected, specific nutrient elements essential for the plant's growth must be supplied accordingly. Strategic designing of optimal formulation and managing the hydroponic

nutrient solutions is vital for successful hydroponic production (Sato et al. 2006). An optimal hydroponic nutrient solution is characterised to supply the roots with a soluble form of nutrient elements and oxygen without hindrance in all growth stages and harvest conditions.

In an ideal hydroponic system, nutrient absorption is mainly relative to the concentration of available nutrients in the solution-connecting the roots. Various factors influence the root nutrient uptake, such as nutrient solution's pH/conductivity, oxygenation, temperature, and salinity (Wortman 2015). Within the plant, every individual macro and micronutrient has a characteristic physiological function, and if it is deficient or occurs in excess, it causes characteristic symptoms of deficiency or toxicity (Domingues et al. 2012). The susceptibility and resistance of these anomalies in nutrient solutions degrade the productivity of cultivation. This study ventured on designing a calculation method that can facilitate the development of automated water and nutrient reuse system based on the existing ionic strength of the nutrient solutions at a given cultivation stage.

The post cultivation hydroponic nutrient solution contains high nitrogen and phosphorus concentrations without organic matters (Gagnon et al. 2010), thus causing a large quantity of point source pollution (Kumar and Cho 2014). This study envisions the circular economy-based green technology method of the post cultivation nutrient water from hydroponic systems applied to microalgae cultivation (Guedes et al. 2013), then the produced algal biomass used as a source of nutrients and stimulants to hydroponic plants. Studies have used the microalgal biomass extract as a foliar spray (Godlewska et al. 2019), algal extract as an antioxidant source in pot cultivation (Guedes et al. 2013), as a growth stimulant (Leonard et al. 2021), and simultaneous co-cultivation of microalgae in hydroponic nutrient solution added with alkaline extract of agro biomass waste (Barone et al. 2019). To the best of our knowledge, this is the first approach to use acid digested algal biomass as a source of nutrients and stimulants for hydroponic cultivation. There are methods technologies in which human excreta has been processed for food-grade commodity production and applied for hydroponic cultivation, for example, nitrified human urine (Volpin et al. 2020a). To support the nutrient content and study the potential, along with acid digested microalgal extract, nitrified human urine was also combined and applied to test the plant growth efficiencies. Hydroponic cultivation engrosses many factors that control the shoot growth, nutrient absorption of the root region; these factors include light

quality and light exposure time, the flow rate and time-length of nutrient solution, temperature, and humidity (Halbert-Howard et al. 2021). The intention and study focus of this chapter is to test the proof of concept of using the microalgal extracts as hydroponic nutrients and stimulants; thus, the algal extract compared with other commercially available hydroponic formulations and standard Hoagland hydroponic nutrients (Arnon 1938), in a simple hydroponic testing system. The second target is to test selective nutrient uptake and nutrient interlink calculation methods, as hydroponic roots differ and modulate the nutrient uptake physiology in accordance with the level of available micro and macronutrients at different growth stages (Carmassi et al. 2003).

5.2. Materials and Methods

5.2.1. Chemicals

Unless otherwise stated, all chemicals were obtained from Sigma and used without any further purification. These include ammonium dihydrogen phosphate (98%), potassium nitrate (99%), calcium nitrate tetrahydrate (99%), magnesium sulphate hepta hydrate (99.5%), boric acid (99.5%), manganese(II) chloride tetrahydrate (98%), zinc sulfate heptahydrate (99%), copper(II) sulphate pentahydrate (98%), sodium molybdate dehydrate (99%), potassium hydroxide (90%), ethylenedinitrilotetraacetic acid (99.4%), and iron(II) sulphate hydrate (99%).

5.2.2. Seed germination

Collards- Champion (*Brassica oleracea*) seeds purchased from Rangeview Seeds, Australia and Mr Pak Choi (Brassica rapa) seeds from Fothergill's Seeds, Bunnings, NSW, Australia. The seeds were placed in a wet tissue paper in a 17.5cm length, 11.8cm width and 5.3cm height plastic container. The container was loosely covered with cling wrap in a way to allow aeration and kept in the dark for five days. Every 48 hours, the tissue paper was sprayed with Milli-Q water to maintain the moisture (Millington 2018). The sprouts ranging from 4.5 to 6 cm were used for the experiments.

5.2.3. Hydroponic cultivation

5.2.3.1. Cultivation of Pak Choi (*Brassica rapa)*

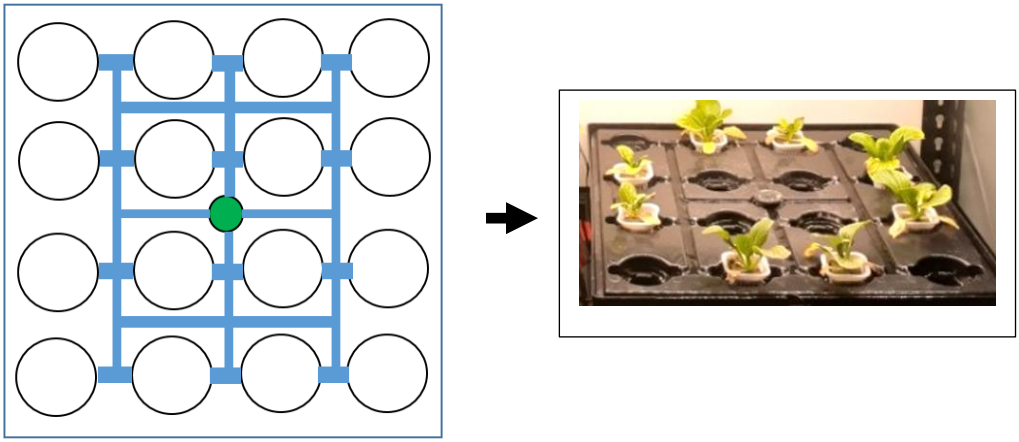


Figure 5.1 Indoor hydroponic growing system with four lanes containing four plant slots per lane. The internally mounted pump located at the centre (green circle) pump the nutrient solution, which will travel through the lanes (blue lines) and reach the rockwool.

The indoor hydroponic growing system of 50cm x 50 cm; from 'Shenzhen keisue technology' was used for this study. The growing system had four lanes containing four plant slots per lane, an 8 cm gap between plant slots (Figure 5.2). The germinated sprouts were implanted in rockwool grow cubes (3.6cm). The light was mounted to a distance that can yield 350 µmol/m^2/s light intensity at canopy level.

The efficiency of acid digested algal biomass nutrients was compared with other commercially available hydroponic nutrients and stimulants. This comparative study was conducted in the research and development facility of Invertigro – a hydroponic company located at Dunning Ave, Rosebery NSW 2018. Different nutrient conditions were tested as shown in Table 5.1.

Table 5.1 Different nutrient conditions used for comparing the growth efficiency of pak choy

	Nutrient conditions
1	Leafy Green Mix [Control1]
2	Leafy Green Mix ½ + Kreotec
3	Leafy Green Mix ½ + Amino acid Hydrolysate
4	Kreotec + Kreostim
5	Leafy Green Mix ½ [Control2]
6	Microalgal Extract

1. The nutrient condition (1) 'Leafy Green Mix' is the composition customised by INVERTIGRO basically macro and micro nutrient formuaiton (chemical composition is INVERTIGRO's intellectual property protected). The nutrient condition (1) is considered as a control.
2. The nutrient condition (2) is half the concentration of nutrient condition (1) + Kreotec from 'ThinkBio' (https://thinkbio.com.au/product/kreotec-kreostim-combo/) is a bio fertiliser microbial composition comprising *Bacillus velezensis*, *Azospirillum brasilense* and *Herbaspirillum seropedicae*.
3. The nutrient condition (3) is half the concentration of nutrient condition (1) + amino acid hydrolysate sourced from seaweed formulated by INVERTIGRO.
4. The nutrient condition (4) is the combination of Kreotec + Kreostim, where Kreostim is the natural starch-based chelators, saccharides plus other organics, phyto-proteins, vitamins and plant hormones; containing organically held nitrogen, phosphorus and potassium and minor calcium, magnesium, sulphur, chlorine and trace iron, manganese, zinc, silica, copper, boron, molybdenum and cobalt and ultra-trace selenium, iodine, chromium, nickel, fluorine, tin, lithium and vanadium elements.
5. The nutrient condition (5) is half the concentration of nutrient condition (1), nutrient condition (5) is considered as the second control.
6. Acid digested microalgal biomass, refer to section 4.3.3 in chapter 4 for its chemical composition.

5.2.3.2. Cultivation of *Collards- Champion* (*Brassica oleracea*)

Customised nutrient film technique [NFT] hydroponic frame with four lanes containing ten plant slots per lane was used for the cultivation of collards (Figure 5.2). The germinated sprouts were implanted in rockwool grow cubes (3.6cm). Two Apollo 6 LED grow lights - true watt 209W were mounted on the frame, and the fixture height was calibrated to a distance that can yield 200 µmol/m^2/s light intensity at canopy level.

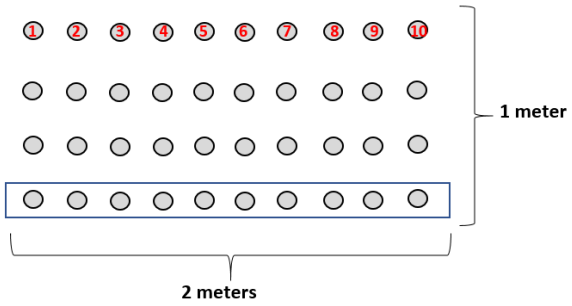


Figure 5.2 Nutrient film technique [NFT] hydroponic frame with four lanes containing ten plant slots per lane

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Four nutrient conditions were set up in the NFT, they are (i) Hoagland nutrient solution [control], (ii) acid digested algae extract [5mL/L], (iii) acid digested algae extract [5mL/L] + nitrified human urine [10mL/L], (iv) nitrified human urine [10mL/L].

Hoagland nutrient solution (Arnon 1938)–

The following stock solutions were prepared and the quantities used are indicated below:

(1) 1M Ammonium dihydrogen phosphate, with 1 mL/L of nutrient solution used

(2) 1M Potassium nitrate, with 6 mL/L of nutrient solution used

(3) 1M Calcium nitrate tetrahydrate used 4 mL/L of nutrient solution

(4) 1M Magnesium sulphate heptahydrate used 2 mL/L of nutrient solution

(5) Micronutrient stock solution was prepared by combining the following amount of salts in a total volume of one litre of milliQ water, and then 1 mL/L was used for the working nutrient solution.

5. 2.86 gm Boric acid

1.81 gm Manganese (II) chloride tetrahydrate

0.22 gm Zinc sulphate heptahydrate

0.08 gm Copper sulphate pentahydrate

0.06 gm Sodium molybdate dihydrate

(6) Iron stock: 100 mL of stock solution was prepared using the following composition of ingredients *ferrous sulphate* heptahydrate 4.98g, ethylenediaminetetraacetic acid 5.22g, and potassium hydroxide

3.8g. The pH was adjusted to 7.1, the solution appeared wine red in colour, and the solution was stored in the dark. 0.25 mL of iron stock was added for one litre of nutrient solution.

15 L of each of the aforementioned four nutrient solutions were loaded to four individual nutrient tanks pertaining to four NFT lanes. The laboratory temperature was maintained at 22 ± 2° C, and the nutrient solution flow rate was set to one litre per minute condition. The lights were set to 7 am to 7 pm day/night cycle. Initially, the sprouts were grown in a commercial 'Liquid Grow Science' Hydroponic Nutrient Liquid until the plants attained ten centimetres height. Then, along with roots, carefully the plants were moved from NFT, and the NFT system was washed thoroughly with deionised water. After a thorough wash, the plants were placed back to NFT and allowed to run for 48 hours in deionised water; every eight hours, individual nutrient tanks are replaced with new deionised water to make sure the plants are not carrying residual 'Liquid Grow Science' hydroponic nutrients.

5.2.4. Growth measurement

For pak choi (*Brassica rapa*), sixteen replicate plans were maintained for each different nutrient condition. Plant height was measured every seven days for up to twenty-one days, and the biomass was measures by sacrificing four replicate plants at every seven days sampling points. The whole shoot system without root was weighed, and the average with standard deviation was recorded. For collards, shoot height and leaf width was measured as an indicator of plant productivity.

5.2.5. Nutrient measurements

The following nutrient elements were considered for measurement at respective sampling points (pak choy – starting from day '0' every seven days upto 21 days; collards – starting from day '0'10^{th} and 20^{th} day); the nutrient elements are *c*opper, zinc, manganese, magnesium, molybdenum, calcium, iron, potassium, cobalt, sodium. nitrate, phosphate and sulphate. Both for pak choy and collards, before sampling, the nutrient tanks were topped up with milliQ water to the respective volumes; pak choy was maintained with twelve litres and collards 15 litres. Micro and macro nutrients were measured using Inductively Coupled Plasma Mass Spectrometry *(ICP-MS) and Dionex Integrion RFIC Ion*

Chromatography with Thermo Fisher Scientific Dionex AS-AP auto-sampler following the same method described in section 3.2.2.1 – chapter 3.

The following nutrient elements were considered for calculating the uptake rates at respective sampling points (pak choy – starting from day '0' every seven days upto 21 days; collards – starting from day '0'10th and 20th day); copper, zinc, manganese, magnesium, molybdenum, calcium, iron, potassium, cobalt, and *sodium. nitrate, phosphate and sulphate. The nutrient* uptake rates were calculated following the method described in section 3.2.3.1 – chapter 3, and the selective nutrient recovery calculations following 3.2.3.2.

5.2.6. Selective nutrient uptake plot

The same method described in section 3.3.3.3 (chapter 3) was followed to identify the maximum nutrient uptake of individual nutrient elements; in the given sampling intervals. Following the method described in section 3.3.3.5, the overall nutrient uptake profile patterns were derived. The above-stated calculation methods that are followed for nutrient recovery stoichiometry described in chapter 3 was in context to "nutrient RECOVERY", here in pak choy; the same calculation applied in the context of "nutrient UPTAKE".

More number of sampling points helps in deriving more accurate trends and profiles of nutrient stoichiometry and selective nutrient uptake plots. As the collards had only two sampling points (day10 and day20), nutrient stoichiometry and selective nutrient uptake plot were derived only for pak choy.

For the tested six different nutrient conditions, the following calculations are done from the values of every seven days of sampling/measurements of ICP-MS (metal/ micronutrients) and ion chromatography (macronutrients), to derive (Nutrient Uptake-NU) NU_{max}, NU_{min}, $NU_{x0D\text{-}7D}$, DNU_{xmax}, DNU_{xmin}, $NU\%_{x}$, $NU\%_{xincrease}$, $NU\%_{xdecrease}$, NU_{xnull}. These emphasising calculation factors for the particular aspects (maximum, minimum, zero) are labelled and their descriptions for each factor are detailed below and their calculations are done further.

NU_{max} - nutrient (element) uptake to the maximum on the given nutrient condition within the 21 days

NU_{min} - nutrient (element) uptake to the minimum on the given nutrient condition within the 21 days

NU_{x0D-7D} - Nutrient Uptake rate for every seven-day interval, where *x* is the nutrient element measured

DNU_{xmax} - the Day in which the highest Nutrient Uptake (DNU) of an individual nutrient element (*x*) occurred on the given nutrient condition within the 21 days

DNU_{xmin} - the day in which the lowest uptake of an individual nutrient element (*x*) occurred on the given nutrient condition within the 21 days

$NU\%_{x}$ - Nutrient Uptake percentage of individual nutrient element (*x*) on a given nutrient condition within the 21 days

$NU\%_{xdecrease}$ - Nutrient Uptake percentage of decrease of individual nutrient element (*x*) on a given nutrient condition within the 21 days

$NU\%_{xincrease}$ - Nutrient Uptake percentage of increase of individual nutrient element (*x*) on a given nutrient condition within the 21 days

NU_{xnull} - 'no uptake point (day) of an individual nutrient element (*x*) within the 21 days of growth

The measured micro and macronutrients are copper (*x*=Cu), zinc (*x*=Zn), manganese (*x*=Mn), magnesium (*x*=Mg), molybdenum (*x*=Mo), calcium (*x*=Ca), iron (*x*=Fe), potassium (*x*=K), cobalt (*x*=Co), sodium (*x*=Na), nitrate, (*x*=NO3), phosphate (*x*=PO4) and sulphate (*x*=SO4).

(A) NU_{max} and NU_{min}

The nutrient uptake (NU_x) for every individual tested nutrient element (*x*) on the given nutrient condition should be calculated by subtracting the initial amount of nutrients measured on 'day 0'(N_{x0}) from 'day 21'(N_{x21}).

$$\text{i.e } NU_x = N_{x0} - N_{x21} \qquad \text{(Equation 5.1)}$$

Then the values NU_{Cu}, NU_{Zn}, NU_{Mn}, NU_{Mg}, NU_{Mo}, NU_{Ca}, NU_{Fe}, NU_{K}, NU_{Co}, NU_{Na}, NU_{NO3}, NU_{PO4}, NU_{SO4} were arranged in increasing or decreasing order to identify which nutrient element uptaken to the maximum (NU_{max}) and minimum/least (NU_{min}).

(B) NU_{x0D-7D}

To measure the nutrient uptake rate for every seven-day interval (NU_{x0D-7D}) for each tested nutrient element (x) amount of nutrients measured on 'day 0'(N_{x0}) is subtracted from 'day 7'(N_{x7}).

$$\text{i.e } NU_{x0D-7D} = N_{x7} - N_{x0} \qquad \text{(Equation 5.2)}$$

Similarly

$$NU_{x7D-14D} = N_{x14} - N_{x7}$$
$$NU_{x14D-21D} = N_{x21} - N_{x14} \qquad \text{(Equation 5.3)}$$

(C) DNU_{xmax} and DNU_{xmin}

The day in which the highest (DNU_{xmax}) and the day in which the lowest (DNU_{xmax}) uptake of an individual nutrient element (x) occurred can be found from the nutrient uptake rate for every seven-day interval values NU_{x0D-7D}, $NU_{x7D-14D}$, $NU_{x14D-21D}$ by arranging these in increasing or decreasing order.

(D) $NU\%_{x}$, $NU\%_{xincrease}$, $NU\%_{xdecrease}$

The Nutrient Uptake percentage ($NU\%_{x}$) of individual nutrient element (x) on a given nutrient condition can be calculated as,

$$NU\%_{x} = \{[(N_{x21} - N_{x0})] / N_{x0}\} \times 100 \qquad \text{(Equation 5.4)}$$

(E) NU_{xnull}

The 'no recovery' point (day) (NU_{xnull}) of an individual nutrient element (x) within the 21 days of retention can be can be found from the nutrient recovery rate for every three-day interval values NU_{x0D-7D}, $NU_{x7D-14D}$, $NU_{x14D-21D}$ by finding the difference between them. When the value of difference is "Zero" that's the NU_{xnull}.

Note - Pak Choy (*Brassica rapa*) was selected for the experiments as a choice of the collaborator company, Invertigro, NSW, Australia, test species. Collards (*Brassica oleracea*) was selected for the choice of leaf sturdiness helps in measuring the leaf width.

5.3. Results

5.3.1. Growth responses of pak choy

Amongst the tested nutrient conditions, the leafy green mix (INVERTOGRO's formulation) showed maximum growth (Figure 5.3). Based on the net growth measured after 21 days, that is, the plant height measured on day 21 was subtracted by the height of day '0' was in the order of leafy green mix [control1] > leafy green mix ½ + kreotec > kreotec + kreostim > leafy green mix ½ + amino acid hydrolysates > microalgal extract > leafy green mix ½ [control2] (Table 5.2).

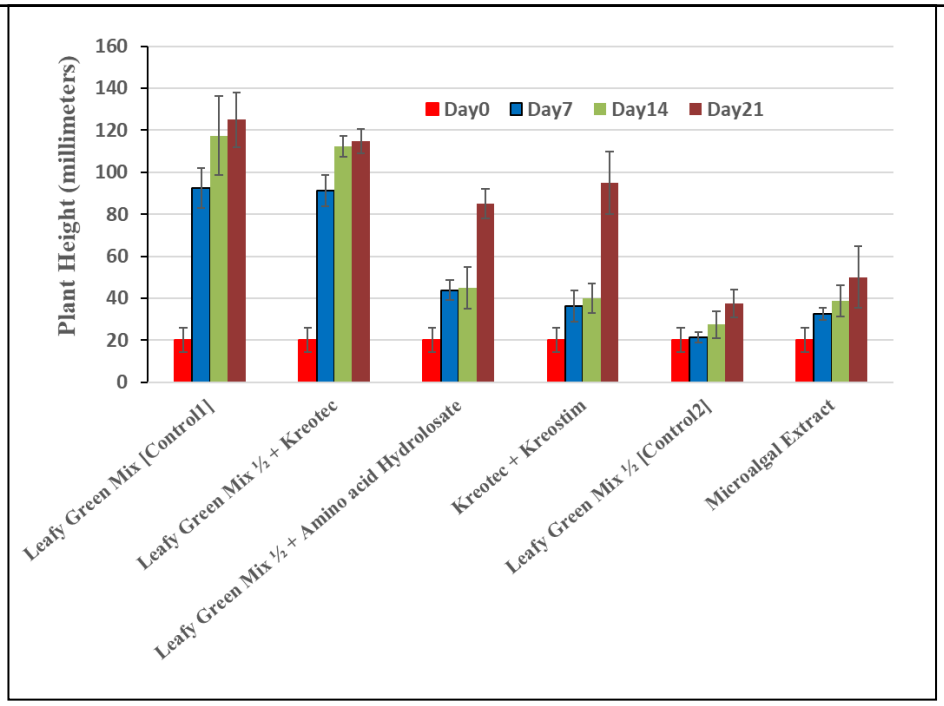

Figure 5.3 Growth comparison of pak choy in algal extract with other commercial hydroponic nutrient formulations (results represented average of triplicates)

Table 5.2 Growth profile of pak choy after 21 days, the plant height pertaining to different nutrient conditions sorted from highest to lowest growth (height day 21 subtracted by day 0)

Nutrient conditions	Plant height (mm)
Leafy Green Mix [Control1]	105
Leafy Green Mix ½ + Kreotec	95
Kreotec + Kreostim	75
Leafy Green Mix ½ + Amino acid Hydrolysate	65
Microalgal Extract	30
Leafy Green Mix ½ [Control2]	17.5

Microalgal extract ranked fifth among the tested six conditions in this comparative growth profile (Table 5.2). The growth rates in between sampling points from the measured height differences between day '0' and day '7', '7' and day '14' and '14' and day '21' showed different nutrient conditions dominated growth at different sampling points (day7, day14 and day21). The day '7' sampling revealed leafy green mix [control1] in the first place, leafy green mix ½ [control2] in the last (sixth) place and microalgal extract in the fifth place. The order of growth rate was as follows; leafy green mix [control1] > leafy green mix ½ + kreotec > leafy green mix ½ + amino acid hydrolysates > kreotec + kreostim > microalgal extract > leafy green mix ½ [control2]. The day '14' sampling revealed leafy green mix [control1] in the first place, leafy green mix ½ + amino acid hydrolysates in the last (sixth) place and microalgal extract in the third place. The order of growth rate was as follows; leafy green mix [control1] > leafy green mix ½ + kreotec > microalgal extract > leafy green mix ½ [control2] > kreotec + kreostim > leafy green mix ½ + amino acid hydrolysates. The day '21' sampling revealed kreotec + kreostim in the first place, leafy green mix ½ + kreotec in the last (sixth) place and microalgal extract in the third place (Table 5.3). The order of growth rate was as follows; kreotec + kreostim > leafy green mix ½ + amino acid hydrolysates > microalgal extract > leafy green mix ½ [control2] > leafy green mix [control1] > leafy green mix ½ + kreotec.

Table 5.3 Growth rate of pak choy, height measured between day 14 and day 21, the plant height pertaining to different nutrient conditions sorted from highest to lowest growth

Nutrient conditions	Plant height (mm)
Kreotec + Kreostim	55
Leafy Green Mix ½ + Amino acid Hydrolysate	40
Microalgal Extract	11.2
Leafy Green Mix ½ [Control2]	10
Leafy Green Mix [Control1]	7.5
Leafy Green Mix ½ + Kreotec	2.5

The highest to lowest sorted measurements of plant productivity in terms of fresh weight (Table 5.4) revealed a different pattern within day 14 and day 21. The sorted order for day14 was as follows, Leafy Green Mix [Control1] > Leafy Green Mix ½ + Kreotec > Leafy Green Mix ½ + Amino acid Hydrolysate > Microalgal Extract > Kreotec + Kreostim > Leafy Green Mix ½ + Amino acid Hydrolysate > Leafy Green Mix ½ [Control2], and on day 21 was in the order of Leafy Green Mix [Control1] > Leafy Green Mix ½ + Kreotec > Leafy Green Mix ½ + Amino acid Hydrolysate > Kreotec + Kreostim > Microalgal Extract > Leafy Green Mix ½ [Control2]. Microalgal extract ranked four on day 14, and on day 21 it ranked five. Within the tested fresh weight-based biomass productivity, between day 14 and day 21, except for Kreotec + Kreostim and microalgal extract, other tested nutrient conditions remained in the same ranking. When comparing the profiles of plant growth in terms of plant height and biomass fresh weight, the ranking of plant height and fresh weight varied entirely (Table 5.5). The pH of the tested different nutrient solutions showed a gradual increase in Leafy Green Mix [Control1], Leafy Green Mix ½ + Kreotec, and Kreotec + Kreostim (Table 5.6). Leafy Green Mix ½ + Amino acid Hydrolysate, Leafy Green Mix ½ + Amino acid Hydrolysate, and Leafy Green Mix ½ [Control2] showed a slight decrease in pH.

Table 5.4 Pak choy growth measured in terms of fresh weight (g)

Nutrient conditions	Day14 (g)	Day21 (g)
Leafy Green Mix [Control1]	22.6±5.4	48.6±21.7
Leafy Green Mix ½ + Kreotec	11.8±1.5	22.2±8.6
Leafy Green Mix ½ + Amino acid Hydrolysate	1.9±0.3	9.9±4.3
Kreotec + Kreostim	0.8±0.08	8.5±3.6
Leafy Green Mix ½ [Control2]	0.4±0.1	1.3±0.3
Microalgal Extract	1.6±0.5	2.7±0.9

Table 5.5 Comparative plant productivity in terms of plant height and fresh weigh (results sorted highest to lowest) -

Day 21 Growth in terms of plant height	Day 21 Growth in terms of fresh weight
Kreotec + Kreostim	Leafy Green Mix [Control1]
Leafy Green Mix ½ + Amino acid Hydrolysate	Leafy Green Mix ½ + Kreotec
Microalgal Extract	Leafy Green Mix ½ + Amino acid Hydrolysate
Leafy Green Mix ½ [Control2]	Kreotec + Kreostim
Leafy Green Mix [Control1]	Microalgal Extract
Leafy Green Mix ½ + Kreotec	Leafy Green Mix ½ [Control2]

Table 5.6 The pH profile of different tested nutrient conditions (pak choy cultivation)

Nutrient conditions	Day0	Day 7	Day 14	Day 21
Leafy Green Mix [Control1]	6	6.1	6.1	6.3
Leafy Green Mix ½ + Kreotec	4.6	6.1	6.1	6.9
Leafy Green Mix ½ + Amino acid Hydrolysate	7.3	7.1	6.7	6.7
Kreotec + Kreostim	3.8	4.4	5.6	6.6
Leafy Green Mix ½ [Control2]	6.4	6.2	6	6.2
MicroalgaL extract	6.6	6.6	6.7	5.9

5.3.2. Growth responses of pak choy and collards

Among the tested four nutrient conditions, the Hoagland solution (control) showed maximum growth (Figure 5.4a). Based on the net growth measured after 20 days, that is, the plant height measured on

day 20 was subtracted by the height of day '0' was in the order of Hoagland solution [control] > microalgal extract > microalgal extract + nitrified urine > nitrified urine. The plant height trend mentioned above was the same on both the sampling points (day 10 and day 20); on day 20, the height difference between the nitrified urine and combination of microalgal extract + nitrified urine appeared with similar measurements. Growth measurements in terms of leaf width were in the order as follows, Hoagland solution [control] > microalgal extract + nitrified urine > nitrified urine > microalgal extract (Table 5.4b). The pH was gradually increased in all the tested four conditions, except the nitrified urine found decreased pH 0.4 on day 20 (Figure 5.5).

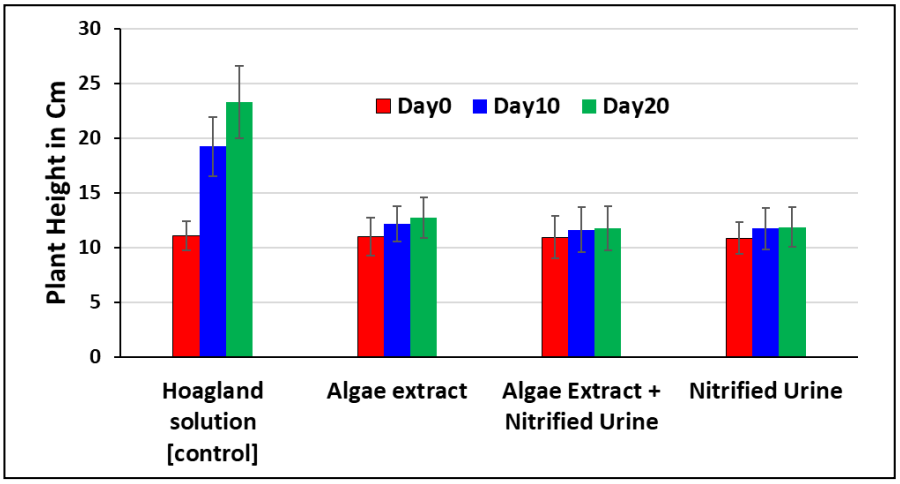

Figure 5.4 (a) Growth comparison of collards grown in different nutrient conditions (results represented average of triplicates)

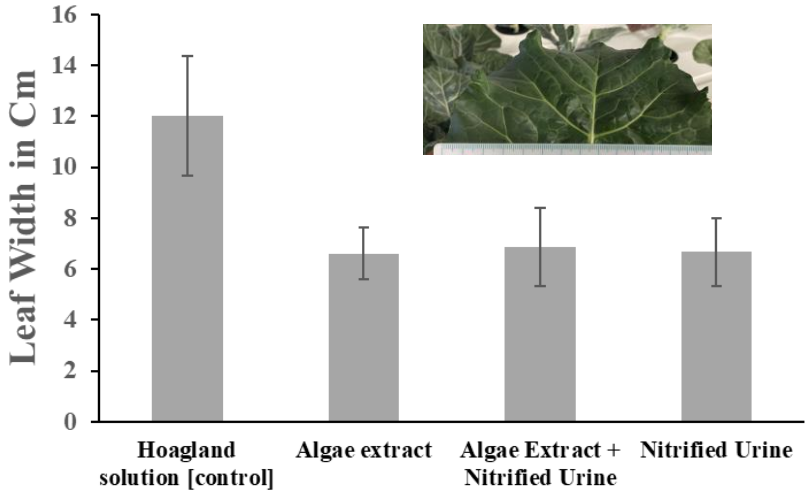

Figure 5.4b Growth comparison of collards in algal extract against nitrified human urine, with Hoagland hydroponic nutrient solution as a control (results represented average of triplicates).

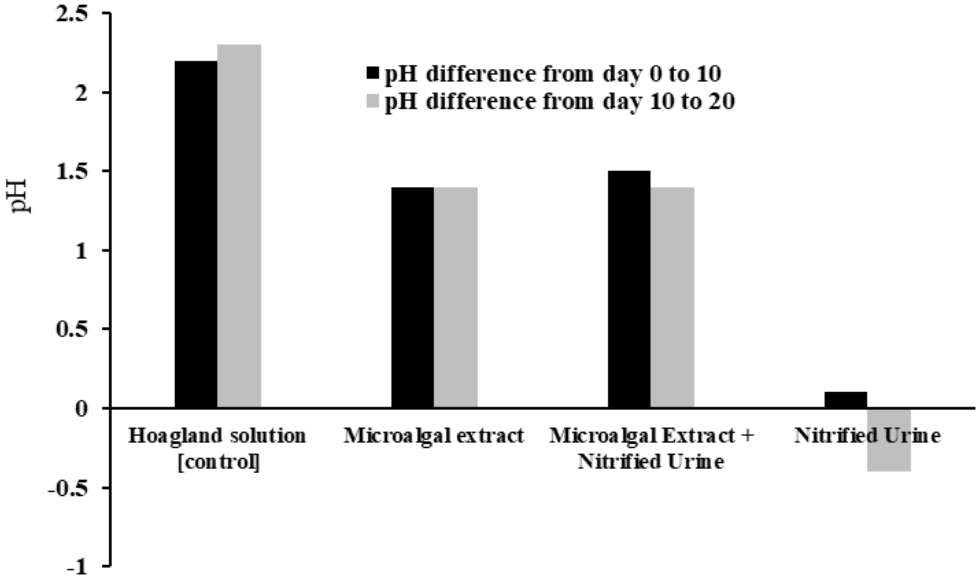

Figure 5.5 pH profile of different tested nutrient conditions (collards cultivation) (results represented average of triplicates)

5.3.3. Identification of maximum nutrient uptake and interlink points of pak choy

Nutrient uptake rates of thirteen micro and macronutrient elements (copper, zinc, manganese, magnesium, molybdenum, calcium, iron, potassium, cobalt, *sodium. nitrate, phosphate and sulphate*) were calculated by subtracting the nutrient contents of the previous sampling point, for example, subtracting the nutrient content values of Day 0 with Day 7, Day7 – Day14, and Day14 – Day21, and identifying which day the maximum uptake occurred. In this calculation, Leafy Green Mix [Control1], Leafy Green Mix ½ + Kreotec, and Leafy Green Mix ½ [Control2] showed day 14 as the maximum nutrient element uptake points. Microalgal extract and Kreotec + Kreostim on day 21, and Leafy Green Mix ½ + Amino acid Hydrolysate showed maximum absorption rates on day 7 & 21. Irrespective of different nutrient regimes (though the nutrient concentrations and composition are different in the tested six different nutrient conditions), pak choy showed maximum copper absorption on day 7 (Table 5.8). In this profiling, the pak choy supplied with microalgal extract as nutrients was an exception; the maximum copper absorption occurred on day 21.

The terminology 'interlink' used here in the context that denotes the influencing effect of the uptake of other nutrient elements; for example, copper influence the nitrogen nutrient uptake in pak choy (Wang et al. 2009). Amongst the tested six nutrient combinations (Table 5.9), four nutrient conditions contained ingredients of leafy green mix, amongst the four leafy green mix (Leafy Green Mix [Control1], Leafy Green Mix ½ + Kreotec, and Leafy Green Mix ½ [Control2]) three showed nitrate as key interlinking element. Amongst the tested six nutrient combinations, three nutrient combinations showed molybdenum as their highest interlinking element (Table 5.9). It has to be noted that amongst the six, only two conditions contain kreotec, and both the two kreotec was identified as molybdenum as their highest interlinking element (Table 5.9).

Table 5.8 Pak choy - within the nutrient uptake rates of different sampling intervals identifying the maximum uptake points o individual nutrient element

Nutrient condition	NO_3^-	PO_4^{3-}	SO_4^{2-}	Na	Mg	K	Ca	Mn	Fe	Co	Cu	Zn	Mo	Maximum Uptake
Leafy Greens Mix (LGM) [control1]	14	14	14	21	14	14	14	14	14	14	7	21	14	Day14 (10 out of 13)
LGM ½ + kreotec	7	21	21	14	14	14	14	14	14	14	7	21	14	Day14 (8/13)
LGM ½ + amino acid hydrolysate	7	21	21	14	14	21	14	7	7	7	7	21	21	Day7&21 (5/13 each)
kreotec + kreostim	21	14	21	14	21	21	21	7	7	7	7	7	21	Day21 (6/13)
Control 2 (LGM½)	14	21	14	21	14	14	14	14	14	21	7	21	14	Day14 (8/13)
Microalgae Extract	21	21	14	7	21	7	21	7	21	7	21	14	7	Day21 (6/13)

Table 5.9 Selective control nutrient uptake plot of pak choy

Nutrient Conditions	Number of interkinks	Highest interlinking Element	Key interlinking Element	Nutrients not involved in interlinks
Leafy Greens Mix [LGM] [Control 1]	46	Zn	NO3	Co, Cu
LGM ½ + kreotec	29	Mo	Mg	Co
LGM ½ + amino acid hydrolysate	19	Fe	NO3	All tested elements Involved in interlinks
kreotec + kreostim	22	Mo	Mn	K, Na, PO4
LGM ½ [Control 2]	42	Mo	NO3	All tested elements Involved in interlinks
Microalgal Extract	18	Cu	Na	Mn, NO3, SO4

5.4. Discussion

Every plant species vary in the uptake pattern of nutrient elements and their transfer to various plant parts, leaves, tissues, fruits. Particularly the micronutrients, when it occurs at slight high concentrations, nutrient elements will get bio-accumulated; this phenomenon ensues as a cause of particular selective nutrient absorption traits of the given type of plant species (Abou-Hadid et al. 1996). These kinds of plant nutritional traits and phenomena result in differences in yield and plant productivity. Therefore, these variations in plant growth, physiology, or yield in hydroponic cultivation systems may result in a concentration or dilution effect on cation and anion constituents of the nutrient solution (Kirkby and Mengel 1967). The growth rates in between sampling points that the measured height differences from day '0' to day '7' sequentially up to day 21 showed different nutrient conditions dominated growth at different sampling points (day7, day14 and day21). These growth differences pronounce due to the change in nutrient composition of nutrient solution of that particular growth stage, the leftover nutrient composition; (this refers to) the altered nutrient complexity leading to or instigating selective nutrient uptake that may enhance or suppresses the growth.

The comparative growth rate measurements of the day '7 and 14' revealed leafy green mix [control1] in the first place, and in day 21 kreotec + kreostim was found to be in the first place. The kreotec + kreostim contains three bacterial plant growth promoting bacterial species *Bacillus velezensis*, *Azospirillum brasilense* and *Herbaspirillum seropedicae.* The application of plant growth-promoting bacteria facilitates the availability of nutrients and absorption, especially in unfavourable conditions (Hamad et al. 2015); in soil-based cultivation, inoculants of plant growth-promoting bacteria have been shown to contribute to nutrient cycling and increased growth (Singh et al. 2011). In the perspective of growth stages and root nutrient uptake physiology, studies have attempted to use controlled-release fertilisers; this facilitates the slow release of micro and macronutrients, curtails over absorption and bioaccumulation of particular nutrient elements and supply nutrients in balanced concentration upon gradual growth (Trientini and Fisher 2020). However, the study concluded that the application of controlled-release fertilisers was not suitable for a hydroponic type of cultivation system because the rate of nutrient release was not matched with the plant uptake requirements, and

nutrient ratios were deficient in the nutrient solution. The sudden change (increase) in the growth at kreotec + kreostim on day 21 is the possible mechanism of plant beneficial bacterial content facilitating nutrient uptake upon the progression of days.

Initially, on day '7', the growth difference was not distinct and significant; therefore, fresh weight samples (plant sacrifice) was collected from day 14 (no sampling on day 7). In hydroponic cultivation, as the plant grows and uptake nutrients, there will be changes occurring in the nutrient concentrations due to absorbing (uptaking) nutrients. Thus the altered nutrient concentrations manifest changes and show characteristic symptoms in every part of the plant; this includes root, flower, fruits and leaf (Ding et al. 2018). As the different tested six nutrient formulations had different compositions of micro and macronutrients, within the plant height-based measurements (Tables 5.2 and 5.3), the tested two controls with full strength and half strength, that is. Leafy Green Mix [Control1] and Leafy Green Mix ½ [Control2] showed different growth rates. This is a shred of clear evidence to attest to the phenomenon that, though the micro and macronutrient compositions are similar, they exert deficiency or toxicity if they vary in concentrations (full strength and half strength).

Reports have shown the acidification effect in hydroponic cultivation systems and identified it as a cause of released acidic root exudates, which measured a decrease of pH from 5.9 to 3.2 upon three weeks of growth (Loffredo et al. 1997). Especially in pak choy, the variation in the concentration of nutrient composition occurring in the nutrient tanks, particularly alterations in the different forms of nitrogen nutrients, instigates pH decrease (Pelayo Lind et al. 2021). A similar phenomenon might have occurred in Leafy Green Mix ½ + Amino acid Hydrolysate, Leafy Green Mix ½ + Amino acid Hydrolysate, and Leafy Green Mix ½ [Control2] where a gradual decrease in pH upon the cultivation days progress (Table 5.6). This clearly shows the other tested nutrients (Leafy Green Mix [Control1], Leafy Green Mix ½ + Kreotec, and Kreotec + Kreostim) yielded root conditions that can buffer the pH of the nutrient solution. Optimal physicochemical and biological conditions allow the maximum yields and in the enrichment of plant parts that are nutritious to the human system, mainly the leaf and fruit, which are often used as food (Conn et al. 2013). Studies have demonstrated that optimised growth conditions increased collards' leaf width (Gagne 2019).

Studies have shown the increased uptake of copper in hydroponically grown plants related to increased ammonium and nitrate ratio (Pelayo Lind et al. 2021). As it is evident from the observed results (Table 5.8) pak choy uptake maximum concentration of copper in the initial growth stage (day 7) indicates the connectivity of rich nitrogen contents on the nutrient solution at the initial days of cultivation facilitates copper uptake. As well, it is evident that pak choy grown in microalgal extract was an exception for this (Table 5.8) maximum copper uptake on day 7, however, the selective control nutrient uptake plot of pak choy (Table 5.9) showed copper is the identified maximum interlink forming nutrient element. Studies demonstrated the nitrogen sources supplied to hydroponically grown pak choy in the form of ammonium nitrogen and glutamine nitrogen was not converted into nitrate-nitrogen (Wang et al. 2009), the obtained results (Table 5.8 and Table 5.9) evidently shows the interlinks or instigating connectivity of nitrogen metabolism. With reference to the derived selective control nutrient uptake plot of pak choy and maximum uptake chart of individual nutrients, modulating the copper concentration/supply can initiate the pathway necessary to utilise different forms of nitrogen nutrients. The phenomenon, as mentioned earlier, is a potential hydroponic nutrient management tool helpful in limiting nutrient pollution and saving waste treatment costs by increasing the number of reuse of hydroponic nutrient solutions by topping up specific nutrient elements deprived at the calculated growth stage using the derived plots. The two nutrient conditions that contained 'kreotec + kreostim and leafy green mix ½ + kreotec' (Table 5.9) showed molybdenum as their highest interlinking nutrient element. Kreotec is the cobinaito of biofertiliser microbial composition comprising *Bacillus velezensis*, *Azospirillum brasilense* and *Herbaspirillum seropedicae*. Studies have shown the phenotypic features and analysis of *B. velezensis* revealed genes sets of molybdenum cofactor synthetase (Quach et al. 2021). Notably, reports have demonstrated the involvement of *A. brasilense* in the interlinking of molybdenum with nitrogen metabolism in the cultivation of maise (Picazevicz et al. 2017) and soya bean (Galindo et al. 2020). *H. seropedicae* shown to involve in the metabolism of nitrogen under iron-deficient conditions (Klassen et al. 2003). Molybdenum is essential in nitrogen metabolism; primarily, it acts as a cofactor for nitrogenase enzymes to catalyse the redox reaction to convert elemental nitrogen into ammonium ions (Alam et al. 2015) and all the three bacteria (*B. velezensis*, *A. brasilense* and *H. seropedicae*). are nitrogen fixers.

The plots and derivation proposed in this chapter; (i) within the uptake rates of different sampling intervals identifying the maximum and least uptake points of individual element (Table 5.8), (ii) identifying the key interlinking and highly influencing nutrient element, (iii) selective control nutrient uptake plots (Table 5.9) are helpful in devising an optimised method, especially an artificial intelligence method for hydroponic nutrient management. For example, suppose a hydroponic grower intends to grow plants in his own nutrient formulation. In that case, the first step is to generate base data by growing a known number of plants and plotting the maximum and least uptake chart, interlink chart and selective control nutrient uptake charts. The charts derived from the base data can be extrapolated for the desired number of plants to forecast and identify the deprivation points of a particular nutrient element. This method helps formulate nutrient solutions that will not allow the accumulation of specific nutrient elements upon continual reuse of nutrient water. This strategic planning of (specific) nutrient top-up helps in the complete utilisation of nutrients to an environmentally safe level. The post cultivation water, after repeated reuse, can be drained out without the treatment process, thus saving energy, water treatment costs and carbon footprint.

Devising systems for the 'energy-efficient nutrient utilisation process' in a circular economy method is a current global interest (Elvanidi et al. 2020). Digital agriculture and designing sustainable agricultural systems, e-digital agriculture is the current growing are, therefore it is essential to have an automated artificial intelligence-based technology in hydroponic farming (Basso and Antle 2020). As a cause of distraction and tiredness, it is common for human beings to end up in errors while working or engaged in certain activities. The application of automated robotic mechanisms in production control reduces the chances of error and save from adverse causes, financial losses, and suffering (Domingues et al. 2012). The rapid development of electronics and software, merged with the vast expansion of the market, had allowed access to state-of-the-art technologies and tools that were previously only accessible to higher-end and well-equipped labs and research organisations. Agricultural engineering and artificial intelligence played a significant role in these technological advancements, both in the ventures of new equipment development and in modifying or integrating

the existing technology used in agriculture production or from other sectors (Verola et al. 2007). Greenhouse and hydroponic producers in Australia have room to improve their cultivation method and have great potential to expand water and nutrient use efficiencies because currently, a very limited number of growers recycle water and nutrients (Grewal et al. 2011). Currently, the runoff and post cultivation nutrient waters are not recycled or reused by most of the hydroponic greenhouses of Australia; the reasons for the aversion to recycling is the fear of reduced yields and the risk of plant disease (Parks et al. 2009). When considering nutrient water reuse, an appropriately formulated and balanced nutrient solution prepared for the circulation in the hydroponic systems create an optimal chemical environment to root systems that leverage easy absorption and facilitate availability to plants. Essentially the nutrient element formulation should contain optimised levels of a ready biologically-absorbable form of nutrient ingredients to the roots (da Silva Cuba Carvalho et al. 2018). Application of methods as plotted in Table 5.9; (i) predicting the interlink formation amongst the concentration and composition of nutrients contained in the nutrient feeding tank, (ii) predicting the possible number of interlinking nutrients that influence the uptake of other nutrient elements, (iii) forecast of the particular nutrient element that can form the highest number of interlinks (iv) specific nutrient element that act as a key in the formation interlinks, (v) nutrient elements that are no likely involve in the interlinks, are an opportunity to combine with artificial intelligence method for the increase of confidence in framers for the reuse of nutrients with the top of identified key nutrient elements especially at the right.

5.5. Summary

The main conclusions drawn from this work are as follows:

- In hydroponic cultivation, the root exudates are an essential factor through which the exposed physical conditions of the shoot are balanced by root, therefore acting as a interconnect, interacting, communicating and controlling their growing environment, especially in cooperating with the chemistry of nutrient solutions. The current study intends to increase the repeated reuse of nutrient solutions to save water and residual nutrients as a reuse base and top up with the required nutrient

elements by applying nutrient uptake plots. Accumulation of root exudates upon repeated reuse negatively influence plant productivity. However, the method implemented in Chapter 3, blending different wastewaters for the cultivation of microalgae, can facilitate the successful use of post cultivation water. The reuse is possible in one or the other way; use the water for algal cultivation for up to one batch of growth cycle, once the nutrients and exudates are utilised by algae then resend again to hydroponic systems.

- Acid digested microalgal extracts are rich in hydrolysed biomolecules; consequently, when the algal extracts are added to the nutrient tanks, there will be possible bacterial action that facilitates the process of nutrient release in a positive mode. The plant height growth rate results showed that pak choy supplied with microalgal extract as hydroponic nutrients was fifth among the tested six nutrient conditions and progressed to third place on day 14. This ascertains the evidence that the acid digested microalgal extract can act as a slow-release fertiliser.
- The precision of selective control nutrient uptake plot is evident from the identified molybdenum as the highest interlinking nutrient element in both the kreotec added nutrient conditions containing Bacillus velezensis and *Azospirillum brasilense* and *Herbaspirillum seropedicae* are nitrogen-fixing bacteria that apparently utilise more molybdenum which is required for the synthesis of nitrogen fixation cofactor. This attests to the factual reality of the application of selective control nutrient plot as a tool for the reuse of post cultivation water and nutrients.

Chapter 6

Conclusions and recommendations for future work

6.1. Conclusions

This research targeted limiting the greenhouse gas nitrous oxide evolved from nitrogen fertilisers used in soil-based agriculture. Planned strategies to reduce the carbon footprint associated with the production of nitrogen fertilisers and wastewater treatment by utilising wastewater nutrients to cultivate microalgae contribute to energy saving , carbon and N_2O emissions. The plotted methods allow the use of the different wastewater without additional pre-treatment in the optimum quantity to cultivate nitrogen-fixing microalgae,. This ensures benefit for nutrient recovery from wastewater and simultaneously generating a biologically fixed ready usable organic form of plant nitrogen nutrient through the nitrogen-fixing attribute of microalgae. The cultivated algal biomass can be used as a source of nutrients and plant stimulants to grow plants in hydroponics, as microalgae are known to produce plant hormones, and the acid digested algal biomass extract can yield mineral nutrients and hydrolysed biomolecules. Switching to the hydroponics mode of crop cultivation helps minimise nitrogen nutrient loss and soil-based agriculture that evolves nitrous oxide. The foresaid plans led to addressing knowledge gaps and demonstrated the potential of using wastewater as a nutrient source for producing microalgal biomass that yields plant hormones and nutrients.

It is necessary to ensure that the mixed wastewater used for the algal bio-production does not contain toxic chemical substances, algal predators, or pathogenic microbes. One way of leveraging this is to develop appropriate systems to adopt a method that can source wastewater in a stage of containing non-toxic entities. The other way is a catalyst based non-residue making pre-treatment method to present conducive wastewater to microalgae, such as pretreatment by the Fe-Cu process for enhancing biological degradability of the mixed wastewater (Fan and Ma 2009). An alternative of avoiding such pollution using microalgae is possible with a two-step process. The first step can be considered algal cultivation in wastewater to primarily remove toxic chemicals, harvest the biomass, and use non-food or feed processes like biogas production (Passos et al. 2016). The second step of cultivation can be used as a source of biomass for hydroponic nutrient extraction.

Investigation on the possible application of nutrient stoichiometry attempted in this study revealed prominent evidence. Irrespective of nutrient regimes, a clear recovery pattern was observed in the

tested thirteen different nutrient conditions. Australia is robustly investing and moving towards digital agriculture.

The current study revealed from the testing of a proof of concept "algal biomass as hydroponic nutrients and stimulants" that replacing the nitrogen fertiliser with biologically fixed nitrogen nutrients in hydroponic cultivation. This method is practically possible and has potential opportunities to scale up in green technology.

6.2. Recommendations for future work

The outcomes of this study have yielded direction to the identification of several research topics that could be investigated in the future. These areas are detailed as follows:

- The intention of developing this algae-based hydroponic nutrient method primarily focused on reducing the carbon footprint generated in the production of nitrogen fertilisers. The application of such nitrogen fertilisers instigates the evolution of nitrous oxide, a potent greenhouse gas. The other objective is to reduce the energy expenses and carbon footprint generated in wastewater treatment using algae which replenish the nitrogen fertiliser needed for food production by nitrogen fixation. In general, nitrogen-fixing microalgae are slow growers, which makes the process unappealing as the long retention time consumes more energy expenses due to slow growth. It is one of the facts that the unavailability of fast-growing nitrogen-fixing microalgae. There are several non-nitrogen fixing microalgae grow faster than nitrogen fixing types (Jiang, Li, 2021). It will be beneficial if investigations are oriented to match a perfect synergistic pairing of fast-growing non-nitrogen-fixing microalgae, which quickly absorb the wastewater nitrogen and allow the nitrogen-fixing partner to dominate and produce nitrogen using the other nutrients available in the wastewater.

- Vitamins from microalgae can be a valuable stimulant. This study primarily focused on plant hormones produced by microalgae. Devising methods for the non-chemical extraction of microalgal cytosols, such as edible grade organic vinegar, allows microalgae to release cytosol. This mild acid-based extraction or using marine microalgae freshwater exposure to the harvested biomass generates osmotic shock mediated cytosol leak. Unlike the method used in this study using strong acid for the digestion

of biomass, use of any mild treatment that ensues the cytoplasmic rupture facilitates the release of intact or mild acid-tolerant algal vitamins viable and available to plants.

- The current study has not attempted the use of nitrified urine in wastewater blends. Secondary treated wastewater is an excellent safe wastewater source to dilute concentrated or raw wastewaters/effluent. Secondary treated wastewater will always not be supportive for algal growth as it has mid nutrient loads. In such a case, nitrified urine can be used as a blend, enrich nutrients and supply all nutrient elements required for algal growth.

- The current study identified that unprocessed human urine is not favourable for algal growth as the ammonia evolution inhibits algal growth. There are ammonia tolerant microalgae, use of ammonical tolerant microalgae (Lin et al. 2007) can be a way of primary pre-treatment.

- Speciation studies of metal nutrients. Every metal nutrient behaves differently in its oxidised or reduced states; changes in the redox potential or alteration in valency changes the algal tolerance limit; for example, hexavalent chromium is more toxic than trivalent chromium. Investigation on the metal speciation helps to understand different hidden mechanisms on the selective control nutrient recovery/uptake and interlink forming characteristics and plots. Correspondingly, fractionation-based profiling and proportion of different nitrogen forms (nitrate, nitrite, ammonia) and sulphur forms (sulphate, sulphite, sulphide) should be conducted.

- High throughput based screening using micro well plate or microfluidic device to narrow down the testing concentration of desalination brine and other types of wastewaters making more blends proportions paves the way for the identification of optimal nutrient composition yield high-value products from wastewater nutrients.

- Improving retention time by selecting dominating and fast-growing algal species. Fast-growing microalgae absorb the waster nutrients in a short time and produce higher biomass, thus dually accomplishing the purpose of cleansing and biomass production in a short retention time. It is also essential the fast-growing species should dominate the other wastewater microflora.

- The current study identified that different nutrient elements influence the uptake and recovery of other elements in wastewater and hydroponic nutrient solution. Particular nutrient element that influence the recovery of more number of elements. Investigation of externally adding such specific nutrient element, to optimise the fast recovery, also this phenomenon can also be used as a tool for enhancing the recovery of nutrient of interest.

- The acid digested microalgal biomass contains nutrients as well hydrolysed form of simple absorbable form of fatty acids, amino acids and sugars. Further investigation on discriminating these effects reveals that plant growth stimulation is a cause of algal phytohormones or synergistic enhancing functions of hydrolysed simpler units of biomolecules.

- Hydroponic plant roots secrete various organic acids and compounds known to inhibit plant growth, and reports show evidence it arrests algal growth. Reusing the post cultivation water with plant exudates are difficult. Meanwhile, studies have demonstrated the use of post cultivation hydroponic nutrient water for algal cultivation. Diluting the post cultivation hydroponic water with other high concentration wastewater, blending to the level of inactivating the growth-inhibiting effects of root exudates. The blend can be used for algal cultivation and then connected back to hydroponic systems.
- Various physiochemical factors influence the settling character of microalgae. Investigations against the different algal growth stages and nutrient regimes allow further optimisation and fine-tuning to attain a more rapid settling-based biomass harvest.

- The core objective of this research is the repeated reuse of water and residual nutrients in the post-cultivation wastewater as a base for the top-up and replenishment of the successive cultivations without hindering productivity. Further investigation on repeated reuse is needed, especially involving the feature of the accumulation of root exudates upon repeated reuse of nutrient solution.

- The algal biomass extract has not given competitive plant growth comparing the chemical fertilisers, further work in this area is needed for nutrient improvement.

- This research focused on minimising the carbon footprint associated with wastewater treatment, fertilizer, and crop stimulant productions. As a contribution to greenhouse gas mitigation, systematic lifecycle analysis and the modelling of nitrous oxide emission when using the algae-based nutrients and stimulants in hydroponics help, the policy-making agencies understand the effectiveness of this technology.

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