Potential of tropical filamentous cyanobacteria for low-cost bioremediation and bioproduct synthesis

Thesis submitted by

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Chapter No.	Details of publication on which chapter is based	Nature and extent of the contribution of each author, including the candidate
1	Not Applicable	Initial draft was made by **Velu** and the draft was revised with editorial input by Heimann
2	**Velu, C.,** Cirés, S., and Heimann, K., (*ready to submit*). Biomass & Bioenergy	**Velu** collected the data, developed tables and figures and drafted the paper, which was revised with editorial input by Cirés and Heimann. Language correction was offered by Bielig.
3	**Velu, C.,** Karthikeyan, OP., Cirés, S., and Heimann, K., (*in prep*). Algal Research	Experiment was designed by **Velu**, Cirés and Heimann. Data collected and table and figures developed by **Velu** and verified by Heimann**. Velu** and Karthikeyan drafted the paper, which was revised with editorial input by Cirés and Heimann.
4	**Velu, C.,** Cirés, S., Brinkman, D., and Heimann, K., (2019). Effect of CO_2 and metal-rich waste water on bioproduct potential of the diazotrophic freshwater cyanobacterium, *Tolypothrix* sp. Heliyon, 5(4), e01549.	Experiment was designed by **Velu**, Cirés and Heimann. Data collected and analysed statistically, table and figures developed by **Velu** and verified by Heimann**. Velu** and Heimann drafted the paper, which was revised with editorial input by Cirés and Heimann.
5	**Velu, C.,** Cirés, S., Alvarez-Roa C and Heimann, K., (2015). First outdoor cultivation of the N_2-fixing cyanobacterium *Tolypothrix* sp. in low-cost suspension and biofilm systems in tropical Australia J Appl Phycol 27(5):1743-1753.	Experiment was designed by **Velu**, Cirés and Heimann. Data collected by **Velu** and Alvarez-Roa C. Data analysed statistically, table and figures developed by Cirés and **Velu**. Cirés and **Velu** drafted the paper, which was revised with editorial input by Cirés and Heimann.
6	**Velu, C.,** Cirés, S., and Heimann, K., (2020). Bioproduct potential of outdoor cultures of *Tolypothrix* sp.: effect of carbon dioxide and metal-rich wastewater. Front. Bioeng. Biotechnol. 8:51.	Experiment was designed by **Velu**, Cirés and Heimann. Data collected and analysed, table and figures developed by **Velu** and verified by Heimann. **Velu** drafted the paper, which was revised with editorial input by Cirés and Heimann.
7	**Velu, C.,** Karthikeyan, OP., Brinkman, D., Cirés, S., and Heimann, K., (2020). This chapter submitted to Chemosphere and reviewers recommended to publish after minor revisions.	Experiment was designed by **Velu**, Karthikeyan and Heimann. Data collected and analysed statistically, table and figures developed by **Velu** and verified by Karthikeyan and Heimann. **Velu** drafted the paper, which was revised with editorial input by Karthikeyan, Cirés and Heimann.
8	Not Applicable	Initial draft was made by **Velu** and the draft was revised with editorial input by Heimann

Abstract

Global greenhouse gas (GHG) emissions, particularly energy-related CO_2 emissions, have been associated with a 0.9^oC increase in surface temperature, resulting in climatic instability, documented to negatively affect agriculture and food production. At the same time, predicted world population growth to 9.8 billion people by 2050 places increased demands on energy, food and water supplies, exacerbated by decreasing arable land, soil fertility and freshwater availability. To maintain adequate food production on a reducing arable land footprint, an increased use of fertilisers is evident. Continuous use of chemical fertilisers, however, has been connected to soil infertility and fertilisation costs are high. In an agricultural context, continued potable water supplies are also a main challenge under changing climatic conditions, particularly in arid areas. In an economical context, cost-effective and environmentally friendly water purification methods need to be developed, as existing physical and chemical methods are too expensive. At present guaranteeing energy-security demands production of energy from fossil fuel supplies, i.e. coal, in many countries, resulting in increased CO_2 emissions and large amounts of contaminated wastewaters. Solving the challenges that the modern world is confronted with is a priority for world economies and demands reduction of CO_2 emissions, reclamation of wastewaters and guaranteeing improved agricultural production.

Like plants, aquatic photosynthetic microorganisms use sunlight and CO_2 for biomass growth, making them suitable for CO_2 sequestration and wastewater remediation, whilst the biomass produced could potentially yield

biofertilisers. CO_2 sequestration at industrial sites is possible with eukaryotic micro- and macroalgae and prokaryotic cyanobacteria (the focus organisms of this thesis), but the eukaryotic microalgae can only be used if the wastewater produced is enriched with nitrogen and phosphate. CO_2 remediation at coal-fired power stations, the focus of this thesis, is possible, but ash dam wastewaters that need to be used for biomass production are typically metal-enriched and lack adequate nitrogen and phosphate levels, which would ensue expensive fertilisation costs, if plants or eukaryotic algae would be deployed. Some cyanobacteria can fix atmospheric nitrogen significantly reducing fertilisation costs and many produce exocellular polymeric substances (EPS), composed of negatively charged polysaccharides ideal for metal sorption. Application of cyanobacteria and microalgae as biofertilisers has been shown to improve soil carbon, increasing soil water retention and cation exchange capacity, which improve fertility of weathered agricultural soils. Therefore, the use of such diazotrophic cyanobacteria offer an ideal pathway for the remediation of coal-fired power station CO_2 and metal-rich wastewater, theoretically yielding valuable biofertiliser as a co-product. Cyanobacteria also produce high amounts of the high value anti-oxidant pigments phycocyanin and phycoerythrin, offering a potential to significantly offset remediation costs. Anaerobic digestion of cyanobacterial biomass, on the other hand, yields biogas as an energy product on site, which can be used to offset energy requirements for biomass production, while the anaerobic digestate can be applied as a fertiliser. Alternatively, the biomass hydrolysate could also be fermented to bioethanol, as a liquid on site fuel, as cyanobacterial biomass is rich in carbohydrate. A potential problem that could result in techno-economic

infeasibility are low biomass productivities under the cultivation conditions on site, as biomass productivities are often not sustained in N_2-fixing cyanobacteria when cultivated in the absence of inorganic nitrogen.

Therefore, the aims of my PhD research were to

1. Evaluate the effect of ash dam wastewater on growth, metal remediation capacity and bioproduct synthesis by the freshwater cyanobacteria *Tolypothrix* sp. *Limnothrix* sp. and a mixed consortium of these two species (chapter 3),
2. Determine the effect of CO_2 and metal-rich wastewater on bioproduct potential of *Tolypothrix* sp. (chapter 4),
3. Determine metal removal capacity and biomass- and bioproduct productivities of *Tolypothrix* sp. cultivated as biofilms and in traditional bubble column suspension systems under outdoor conditions (chapters 5 and 6), and
4. Examine the biogas production potential of *Tolypothrix* sp. biomass through anaerobic digestion (chapter 7).

Chapter 3 compared growth of the N_2-fixing strain *Tolypothrix* sp. (NQAIF319) to the non N_2-fixing strain *Limnothrix* sp. (NQAIF306) and the potential use of a *Tolypothrix/Limnothrix* consortium (NQAIF319 + NQAIF306) grown in aerated 2 L batch cultures in three different media, simulated ash-dam water (SADW), BG11 and BG11 without nitrogen (BG11(-N)). The simulated ash dam wastewater was formulated to contain the maximum levels of metals determined in ash dam water of a Queensland, Australia coal-fired power station. The purpose of using the consortium was to examine if the N_2-fixing

NQAIF319 would provide sufficient nitrogen for the growth of non N_2-fixing high-value product synthesising cyanobacterium NQAIF306. Al, As, Cu, Fe, Mo, Ni, Se, Sr and Zn were analysed in all chapters to investigate metal removal capacity of *Tolypothrix* sp.. Cultures grown in BG11(medium with nitrogen) achieved highest biomass yields of 3.5, 2.2 and 2.0 g DW L^{-1} for NQAIF319, NQAIF306 and the consortium, respectively compared to growth in SADW as well as in BG11(-N). The biomass productivity of NQAIF319 (0.139 g L^{-1} d^{-1}) was 46 and 58% higher than NQAIF306 (0.075 g L^{-1} d^{-1}) and the consortium (0.059 g L^{-1} d^{-1}) grown in BG11, respectively. On the other hand, NQAIF319 growth was 37 and 48% higher than NQAIF306 and the consortium grown in SADW and BG11(-N), respectively. NQAIF306 did not grow in nitrogen-deficient media in the consortium approach, suggesting that no inorganic nitrogen was secreted by NQAIF319 cultivated in BG11(-N). Compared to NQAIF306, NQAIF319 removed more metals and percentage removal was also higher for NQAIF319. Phycoerythrin productivity was higher in NQAIF319 (8 -11 mg g^{-1} DW d^{-1}) compared to NQAIF306 (1.5 – 2.5 mg g^{-1} DW d^{-1}) in all media tested. In contrast, phycocyanin productivities were comparable for NQIAF306 and NQAIF319 (9-16 and 9-13 mg g^{-1} DW d^{-1}), respectively. Metal removal of Al: 90%, As: 81%, Fe: 99%, Ni: 44%, Se: 93%, Sr: 38 and V: 79% were achieved with NQAIF319, whereas NQAIF306 removed Al: 98%, As: 37%, Fe: 99%, Ni: 53%, Se: 31%, Sr: 53 and V: 26% from SADW medium, supplemented with nitrogen for NQAIF306. In summary, NQAIF319 without nitrogen fertilisation grew well, showed similar phycocyanin productivity and higher phycoerythrin productivity and for most elements higher metal removal percentages than NQAIF306. The consortium approach was not suitable for production of

bioproducts, such as pigments, biofertiliser, fatty acids, carbohydrate, protein and lipids. Therefore, *Tolypothrix* sp. NQAIF319 was selected for research conducted in chapter 4 to investigate the effect of CO_2 and SADW on bioproduct potential.

Aerated 2 L batch cultures of *Tolypothrix* sp. were grown in two different media, SADW and BG11 without nitrogen (BG11(-N) controls) (chapter 4). Supplied air was supplemented with either 15% CO_2 (v/v) or not (non-CO_2 controls). CO_2 supplementation resulted in 2.4 and 3.3 times higher biomass productivities and 1.3 and 1.2 times higher phycocyanin and phycoerythrin contents compared to non-CO_2 controls. Metals Al, Cu, Ni and V were more efficiently removed (50 to 90%) when cultures were supplemented with CO_2, while As, Mo, Se and Sr removal was higher (30 to 87%) for non-CO_2 controls, and no significant CO_2-effect was detected for Zn and Fe removal. CO_2 supplementation increased carbohydrate content by 35% whilst protein and lipid contents were not affected. However, these results were obtained in small-scale laboratory experiments under limiting light conditions. To take the research to the next level in order to investigate commercial applicability, NQAIF319 was grown in different cultivation systems outdoors under natural sunlight supplemented with air (chapter 5).

To investigate the effect of cultivation system, impact of CO_2 supplementation and outdoor conditions on metal removal capacity and biomass and bioproduct productivities, *Tolypothrix* sp. NQAIF319 was cultivated as biofilms and in traditional bubble column suspension systems using SADW as a cultivation medium at James Cook University, Queensland, tropical

Australia (chapter 6). *Tolypothrix* sp. was grown outdoors in simulated ash dam wastewater (SADW) in 500 L vertical bag suspension cultures and as biofilms in modified algal-turf scrubbers. The cultivation systems were aerated with air containing either 15% CO_2 (v/v) or not. CO_2-supplementation resulted in ~1.2- and 1.4-fold higher biomass productivities in biofilms and in suspension cultures, respectively. Biomass productivities of biofilms were 3.6 and 2.7 g m^{-2} d^{-1} with and without CO_2-fertilisation, respectively, whereas they were 39.2 and 37 g m^{-2} d^{-1} in the bubble column suspension cultures. CO_2-supplementation resulted in ~40 and 27% increased phycocyanin and phycoerythrin contents for biofilm and suspension cultures, respectively. CO_2 had no effect on removal of Al, As, Cu, Fe, Sr and Zn, while Mo removal increased by 37% in both systems. In contrast, Ni removal was reduced in biofilm systems, while Se removal increased by 73% in suspension cultures. In conclusion, based on carbon footprint, bubbled column suspension systems supplemented with CO_2, are the growth system of choice for biomass and phycobilin production, whereas biofilm cultivation is better suited for bioremediation of heavy metals when supplemented with CO_2. Based on biomass yields and biochemical data obtained, net present value (NPV) and sensitivities analyses used four bioproduct scenarios: 1) phycocyanin sole product, 2) biofertiliser sole product, 3) 50% phycocyanin and 50% biofertiliser, and 4) 100% phycocyanin and 100% biofertiliser (residual biomass) for power station co-located and not co-located 10 ha facilities over a 20-year period. Economic feasibility for the production of food-grade phycocyanin either as a sole product or with co-production of biofertiliser was demonstrated for CO_2-enriched vertical suspension cultures raised without nitrogen-fertilisation and co-location with power stations

significantly increased profit margins. Biomass produced from this chapter was utilised for biogas production by anaerobic digestion in chapter 7, as an essential pathway to utilise the biomass in a bio-refinery approach.

Biogas production potential of *Tolypothrix* sp. NQAIF319 biomass through anaerobic digestion was investigated in chapter 7, comparing different pre-treatment conditions on methane (CH_4) production potential. Five different pretreatments – thermal, hydrothermal, microwave, sonication and freeze and thaw cycles were investigated. Thermal, hydrothermal and sonication pretreatments supported high solubilisation of organic compounds and higher CH_4 recovery of 126 for thermal and 118 mL CH_4 g^{-1} volatile $solids_{removed}$ for hydrothermal and sonication pretreatments. Actual CH_4 yields, however, were ~55% lower than theoretical yields, potentially due to high amounts of volatile fatty acids and/or ammonium-nitrogen as a consequence of low carbon/ nitrogen ratios, suboptimal pH and oxygen reduction potential. In conclusion, thermally hydrolysed *Tolypothrix* sp. NQAIF319 biomass is suitable for CH_4 production, but a distinctive mismatch between theoretical and actual CH_4 yields demands further investigation to better understand changes in physico-chemical parameters over the course of the anaerobic digestion process, which is essential for process improvements (e.g. addition of carbon-rich co-feeds) and increased CH_4 yields.

In summary, *Tolypothrix* sp. NQAIF319 remediates CO_2 and metals when cultivated in SADW without N-fertilisation. Based on nitrogen fertilisation requirements of wheat, it was demonstrated that accumulated metal levels (calculated for the biomass of NQAIF319 in outdoor experiments) are unlikely to

be toxic to wheat, hence enabling provision of minerals to crops without toxicity. Toxicity threshold of different metals to wheat are as follows Al: 20-50, As: 80-100, Cu: 55-500, Ni: 55-500, Se: 5-10, V: 250 and Zn: 70 mg kg^{-1} soil. Based on N fertiliser requirement in the wheat belt area in Western Australia, to produce 2.69 t ha^{-1}, 97.63 kg N is required ha^{-1}. 1.3 tonnes of NQAIF319 should be applied per hectare to meet the N requirement. If 1.3 tonnes of NAQIAF319 were applied, the final concentrations of metals in soil would be as follows Al: 0.048, As: 0.006, Cu: 0.001, Fe: 0.281, Ni: 0.003, Se: 0.016, Sr: 0.110, V: 0.101 and Zn: 0.006 mg kg^{-1} soil, which is much lower than the toxicity threshold to wheat. . In conclusion, based on biomass productivities achieved in outdoor cultivation systems, 117.5 t biomass ha^{-1} y^{-1} could be produced; the phycocyanin content of *Tolypothrix* sp. grown in this study is 8.8% (w/w) on average and food-grade phycocyanin (A620nm/A280nm: 0.7) yield is 6.9 t ha-1 y-1 (Chaiklahan et al. 2018). Additionally, this research aimed to develop a framework for an integrated bio-economic model for co-locating cyanobacterial cultivation with coal-fired power station based on a biorefinery approach to evaluate the economic feasibility of high value products from *Tolypothrix* sp.. According to this analysis, 10.8 t food-grade phycocyanin ha^{-1} y^{-1} can be sold at US$ 500 kg^{-1}, given an extraction/purification efficiency of 67%, producing a value of US$ 3,448,155 ha^{-1} y^{-1}. 117.5 t biomass dry weight of biofertiliser/biochar can be sold at the rate of US$ 500 t^{-1} DW, valued at US$ 58,750 ha^{-1} y^{-1} or if 50% biomass alone can be sold as biofertiliser, the selling value will be US$ 29,375 ha^{-1} y^{-1}. A sensitivity analysis demonstrated that production of *Tolypotrhix* sp., whether co-located with coal-fired power plants or not, would still be economically viable at a quarter of the sales price for food-

grade phycocyanin, while production of biofertiliser alone would not be financially attractive.

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Refereed papers

1. **Velu, C.,** Cirés, S., Alvarez-Roa C and Heimann, K., First outdoor cultivation of the N_2-fixing cyanobacterium *Tolypothrix* sp. in low-cost suspension and biofilm systems in tropical Australia J Appl Phycol 27:1743-1753.
2. **Velu, C.,** Cirés, S., Brinkman, D., and Heimann, K., (2019). Effect of CO_2 and metal-rich waste water on bioproduct potential of the diazotrophic freshwater cyanobacterium, *Tolypothrix* sp. Heliyon, 5(4), e01549.
3. **Velu, C.,** Cirés, S., Brinkman, D. and Heimann, K., (2020). Bioproduct potential of outdoor cultures of *Tolypothrix* sp.: effect of carbon dioxide and metal-rich wastewater. Front. Bioeng. Biotechnol. 8:51. doi: 10.3389/fbioe.2020.00051.

Contributed paper at National and International conferences

1. **Velu C**, Cirés S and Heiman K (2016) Effect of carbon dioxide on wastewater remediation and bioproduct synthesis by a tropical filamentous nitrogen-fixing cyanobacterium. ASIA-PACIFIC Conference on Biotechnology for Waste Conversion 2016. Hong Kong, 6-8 December 2016. (oral)
2. Heiman K, Parthiba Karthigeyan O, Cirés S, Chidambarampadmavathy K, **Velu C** (2016) Greenhouse gas mitigation and bio-product development. ASIA-PACIFIC Conference on Biotechnology for Waste Conversion 2016. Hong Kong, 6-8 December 2016. (oral)
3. **Velu C**, Cirés S and Heiman K (2016) Bio-product and waste water/CO_2 remediation potential of a nitrogen-fixing cyanobacterium. Bioenergy Australia 2016 Conference. The Mercure Brisbane Hotel, 14 - 15 November 2016. (poster)
4. **Velu C**, Cirés S, Alvarez-Roa C and Heiman K (2015) Low cost suspension and biofilm cultivation of the N_2-fixing cyanobacterium *Tolypothrix* sp. for bioenergy and-biofertiliser production. Bioenergy Australia 2015 Conference. Hotel Grand Chancellor Launceston, Tasmania, 30 November - 2 December 2015. (poster)
5. **Velu C**, Cirés S, Alvarez-Roa C and Heiman K (2014) Mass production of native N_2-fixing cyanobacteria for bio-fertilisers and bio-products: a pilot study in tropical Australia. 5th International Congress of the International Society for Applied Phycology (ISAP) 2014, 22 - 27 June 2014. (oral)
6. **Velu C**, Cirés S, Alvarez-Roa C and Heiman K (2014) Bioproduct synthesis by the tropical N_2-fixing cyanobacterium *Tolypothrix* sp. in low-cost outdoor bioreactors. School of Marine and Tropical Biology Postgraduate Student Conference in JCU**,** 12 - 13 June 2014. (oral)
7. Cirés S, Álvarez-Roa C, Loza V, Huerlimann R, **Velu C**, Karthikeyan O.P, Heimann K (2013) From mine ventilation air to bioenergy and bioproducts: Strain selection of diazotrophic cyanobacteria to bioremediate greenhouse gases in Australian coal mines. Annual conference of the Australasian Society of Phycology and Aquatic Botany, Sydney, Australia, 27- 29 November 2013. (poster)

Dedicated to the supreme power of the Universe, the Nature.

And

Dedicated to the ones I love.

To my parents, supervisors, spiritual mentors & spiritual family,

siblings and their family

To my beloved wife and my daughter

Chapter 1: General Introduction to the Thesis

1.0 Preface

The global population is expected to grow to 9.8 billion people by 2050, requiring more food and clean water resources. Food production is challenged, as arable land is diminishing globally, and the current climate is becoming increasingly unpredictable. Rising greenhouse gas emissions place enormous challenges on the food-water-energy nexus. Appropriate research in this field, the focus of this thesis, can help to transform these challenges into opportunities, whilst, at the same time, building environmentally and economically sustainable societies, especially in underprivileged regions. Such research could focus on developing cost-, water- and energy-smart biomass production platforms, which will be transferrable to industry, if overall production costs are equal or lower than the original systems.

1.1 Greenhouse emissions and global warming

Greenhouse gases are the major contributor and driving force of climate change. Australia's greenhouse gas (GHG) emissions are continuing to rise and the federal government's target of 26% GHG emission reduction to the 2005 level, set for 2030, may not be met and is not aligned with the Paris climate targets (CC 2018). According to the IPCC, the global mean surface temperature increased by 1°C since the middle of the 19th century. At the current rate of emissions, a rise of 3°C is likely to occur between 2030 and 2052 (IPCC 2018). Limiting global warming to 1.5°C rather than 2°C is crucial, as several hundred million people are expected to be exposed to climate-related risks and poverty

by 2050 at a 2°C rise (IPCC 2018). Adoption of the Australian GHG emission targets globally would result in an increase of temperature over 3°C and up to 4°C, higher than pre-industrial levels with severe consequences; i.e. extreme temperatures, risk of floods and droughts, sea level rise and inundation of low lying areas, and permanent loss of sensitive ecosystems (Table 1.1) (CC 2018; IPCC 2018; Schleussner et al. 2016).

Various sources contribute to greenhouse gas emissions. Among them, fossil fuel used for energy production continue to be a major source of CO_2 emissions. Globally, the primary sources of energy in 2011 were oil, coal, natural gas, biofuel, nuclear and hydro energy etc. among these, oil and coal alone contributed with more than 60% and coal was ~29%. In 1970, the global consumption of coal was around 3,000 million tons, increasing to >7,500 million tons in 2012 (IEA 2013a). In 2011, coal-fired power station generated ~41% of required electricity (IEA 2011).

Table 1.1 The effect of global temperature rises between 1.5°C and 2°C

Impact of warming	**1.5°C warming**	**2°C warming**
Heatwaves duration (months)	Up to 1.1	Up to 1.5
Freshwater availability (lower by)	9%	17%
Heavy rainfall (increase in intensity)	5%	7%
Crop yields in tropical regions		
Wheat (lower by)	9%	16%
Maize (lower by)	3%	6%
Soy (rise by)	6%	7%
Rice (rise by)	6%	6%
Sea level rise by 2100 (cm)	40	50
Coral bleaching from 2050 onwards	90%	98%

Source- (Schleussner et al. 2016)

Australia is the 5th largest coal producer in the world with annual production of 421 million tons, accounting for 1.4% of the global GHG emissions and representing the highest per capita GHG emission world-wide; with the primary cause being coal-fired power generation (78% of energy) and mining. In 2016, 24 coal-fired power stations operated in Australia (AEC 2016). Electricity generation from black and brown coal accounts for one third of Australia's total emissions (CEC 2015), releasing 0.288 kg and 0.363 kg CO_2 MJ^{-1}, respectively (AGEIS 2016).

Typical flue gas emitted from coal-fired power plants has a temperature of 140-160°C and contains 10 to 15% of CO_2 and others gases such as sulphur dioxide (SO_2), nitrogen oxide and oxygen at an atmospheric pressure of one (Artanto et al. 2014). Various methods have been employed to reduce the CO_2 content of flue gas such as amine-based chemical absorption, CO_2 capture and storage (CCS), chilled ammonia process, membrane separation and cryogenic technologies (Baker and Lokhandwala 2008). CO_2 capture and storage (CCS) at coal-fired power plants is predicted to negatively affect electricity generation by up to 40% (Goto et al. 2011). In addition, coal-fired power stations produce large quantities of wastewater enriched with heavy metals (Saunders et al. 2012). Consequently, to make fossil fuel-derived energy more environmentally sustainable, this research investigated the potential of a diazotrophic cyanobacterium, *Tolypothrix* sp. to bioremediate ash dam wastewater and growth and bio-product potential under CO_2-supplemented conditions (Chapters 4 to 7), whilst an in depths review is provided in Chapter 2 and species-selection, i.e. the advantage of nitrogen-fixing vs non-nitrogen fixing

cyanobacteria, as well as potential opportunity and benefits of co-cultivation is explored in chapter 3. The economical advantage of adopting such an approach is evaluated in Chapter 8.

1.2 Ash dam wastewater

Globally 80% of wastewater is not treated properly (UNESCO 2017). Discharge of untreated wastewater into watersheds can cause eutrophication, may be a health risk, and release GHGs like nitrous oxide and methane (IWA 2018). In thermal power plants, water is consumed to generate steam, for cooling of exhaust steam and flue gas, and disposal of ash. In Australia, thermal power plants use nearly 1.4% of Australia's water consumption (Radcliffe 2018). Thus, coal-fired electricity generation produces large volume of wastewater with above discharge-level concentrations of arsenic, molybdenum, vanadium and selenium and other metals and metalloids based on the source of the coal (Alberts et al. 1985; Saunders et al. 2012). For instance, the ash dam wastewater collected from Tarong coal-fired power station contains more than 21 different trace metals which necessitates that the wastewater must be treated before release into the environment (Saunders et al. 2012).

Entry of these untreated ash dam wastewater into natural waterways such as ground water, lakes and ponds can damage ecosystems and pose a potential risk to humans (Rai 2010). Heavy metals entering the environment can accumulate in the food web, potentially affecting consumers at every level through biomagnification (Chary et al. 2008; Vijayaraghavan et al. 2004). Depending on exposure dosage, vanadium can cause eye and lung irritation, selenium can induce nausea, diarrhoea, and hair loss in humans (MacFarquhar

et al. 2010) and molybdenum impairs reproduction and foetal development in animals (Vinceti et al. 2001; Zhai et al. 2013). Other metals like cadmium can impair kidney function, weaken bones and adversely affect the nervous system (ATSDR 2007; ATSDR 2012). In plants, metal toxicity causes oxidative stress which in turn inhibits photosynthesis (Baumann et al. 2009). In general, toxic effects of heavy metals are acute when exposed to higher concentrations or low concentrations for prolonged periods (Förstner and Wittmann 2012).

Thus, ash dam wastewaters cannot be directly discharged and are stored in ash dams without any treatment which may pose a threat to the environment, in particular water reservoirs, due to overflow in strong rain periods (Roberts et al. 2013b). Globally, 1200 new coal fired power stations are under construction with a combined capacity of 1.5 million Mega Watts (Yang and Cui 2012). These new power stations will produce up to 750 billion L of additional ash dam wastewater annually, effectively doubling the annual global production of ash dam wastewater in the next decade (Roberts et al. 2015). Therefore, this research investigated cyanobacteria species-selection in this context in Chapter 3.

1.3 Fertiliser consumption and contribution to CO_2 generation

The success of the agriculture industry depends on the supply of fertilisers. The global NPK (nitrogen, phosphorus and potassium) fertiliser consumption was ~187 million tons in 2016, up by 1.4% from 2015 (FAO 2017). Australia's fertiliser consumption was >5 million tons in 2012 due to favourable cultivation conditions. The use of nitrogenous fertiliser alone accounts for ~60%

of the total fertiliser demand. Synthesis of nitrogenous fertilisers results in significant emissions of CO_2 and nitrous oxide (Wood and Cowie 2004).

Demand for fertilisers is increasing, due to the challenging situation of having to produce more food on less arable land. Fertilisers can pollute the environment essentially in two ways: (i) on-site pollution during production and (ii) off-site pollution due to leaching and run off into water bodies (Kremser and Schnug 2002). Fertiliser production is also energy-intensive, requiring electricity and petroleum products (Woods et al. 2010). Wet application of nitrogenous fertiliser releases nitrous oxide and application of excess fertiliser may leach from soils, potentially polluting ground water and surrounding water bodies causing eutrophication. Even in ideal conditions, plants absorb a maximum of 50% of applied nitrogenous fertiliser with between 2 and 10% leaching into ground water (Savci 2012). Use of potassium and sodium fertilisers adversely affects the soil, soil structure and pH through acid formation, resulting in reduced soil fertility (Savci 2012). Finding suitable biofertilisers that will adequately promote agricultural productivity is necessary for sustainable and eco-friendly agriculture crop cultivation. In addition to rhizobia, nitrogen-fixing (N_2-fixing) soil bacteria that form a symbiosis with legumes (Bomfeti et al. 2011), some cyanobacteria, a group of photosynthetic aquatic bacteria, can also fix atmospheric nitrogen (Kulasooriya 2011).

In the context of this thesis, these cyanobacteria could be grown in ash dam wastewater and be provided with CO_2 from flue gas, generating a nutrient-rich biomass for value-add bioproduct development. This would be an ideal choice for bioremediation and abatement of pollution potential of the wastewater

and flue gas coupled with biomass production. Although various eukaryotic microalgae are widely used in different wastewater bioremediation scenarios, ash dam wastewaters contain little or none of the required macro-nutrients such as nitrogen and phosphate, requiring macro-nutrient fertilisation for production of eukaryotic algae at such industries. Thus, N_2-fixing cyanobacteria could potentially serve the need of the hour in an even more sustainable way (Chapters 4, 6 and 7).

1.4 Cultivation systems – advantages of biofilm cultivation

Microalgae and cyanobacteria are presently being cultivated primarily photoautotrophically in various cultivation systems. Since algae utilise sun light and CO_2 from the atmosphere and other nutrients from the aquatic systems, enhanced optimal cultivation conditions need to be provided to maximise productivities (Kannah et al. 2018). The main phototrophic cultivation systems are open ponds, closed photobioreactors and biofilm cultivation systems (Heimann 2016). There are pros and cons for each cultivation strategies with specifications being based on climate conditions, cost of land, water, energy and harvesting cost (Borowitzka 1997; Borowitzka 1999; Kannah et al. 2018). Traditionally microalgae and cyanobacteria are mass-produced in suspension cultivation systems, such as raceways, high-rate oxidation ponds, and tubular- or flat panel photobioreactors, which have several disadvantages such as lack of light penetration, dewatering/ harvesting cost and operation cost (Heimann 2016). A novel and modern cultivation strategy of microalgae is biofilm cultivation which avoids dewatering/harvesting cost, as the solid content of the

produced biomass is similar to biomass obtained by centrifugation (Heimann 2016).

A biofilm is defined as an “aggregate of microorganisms in which cells are frequently embedded within a self-produced matrix of extracellular polymeric substance (EPS) adhere to each other and/or to a surface”. Biofilms may contain non-microscopic microorganisms such as fungi, macroalgae or higher organisms in natural environments but is usually dominated by microalgae or cyanobacteria (Berner et al. 2015). Additionally, bacteria are also mostly present and considered to be essential for microalgal biofilm formation (Berner et al. 2015).

Biofilm cultivation has several advantages (Table 1.2) over traditional suspension cultivation systems as follows (i) simple biomass harvest by scraping which avoids the harvesting and dewatering cost; (ii) biofilms can use light and CO_2 more efficiently, as light and CO_2 easily penetrate the surface; (iii) higher biomass concentration reduces water content and cultivation medium requirement and lowers transportation costs; (iv) differentiation of hydraulic retention time (HRT) and solid retention time (SRT). In suspension systems, harvesting/dewatering infrastructure is needed to separate the biomass from the water, as the cells are homogeneously suspended. In contrast, in biofilm systems, biomass attaches to the surface and water can either be run over the surface or delivered through perfusion from below or behind the biofilm. This results in significantly lower water requirements, as the same water can be reused (Berner et al. 2015; Gross et al. 2015). Biofilm cultivation of microorganisms is traditionally used for wastewater treatment (Heimann 2016).

Interestingly, some cyanobacterial species such as *Tolypothrix* sp. showed no difference when cultivated either as a biofilm or in suspension cultures, due to their self-flocculating behaviour (Velu et al. 2015). This makes *Tolypothrix* an outstanding candidate for wastewater treatment of ash dam water and to investigate the ideal growth system (biofilm *vs* suspension culture) in this context (Chapters 5 and 7).

1.5 Cyanobacteria – potential problem solvers

Cyanobacteria are free-living, prokaryotic photosynthetic organisms which can thrive in a wide range of environments from terrestrial to aquatic, freshwater to saline, and basic to highly acidic conditions (Stal 2007). Some cyanobacteria form symbiotic relationships with other higher organisms and fungi (Stal 2007). They are a major group of primary producers in the phytoplankton in marine and freshwater environments (Short and Suttle 2005). Many species thrive in conditions previously thought to be inhabitable, tolerating desiccation, high temperatures, extreme pH, high salinity and pesticides, illustrating their capacity to acclimate to extreme environments (Stal 2012). Morphologically, cyanobacteria range from single cells (e.g. *Chrococcus*) and colony formers (e.g. *Nostoc*) to filamentous genera (e.g. *Spirulina/Arthrospira*[1], *Anabaena*).

[1] This thesis refers to information on *Spriulina* sp. or *Spirulina* spp as *Spriulina*/ *Arthrospria*, because lack of detailed taxonomic identification does not allow to validate genus identity. For *Spriulina*/ *Arthrospria maxima*, the most current classification is applied, *Limnospira maxima* (Nowicka-Krawczyk et al. 2019).

Table 1.2 A typical comparison of biofilm and suspension cultivation system

Parameters	Biofilm	Suspension system
Energy		
Resuspension	Not required	Required
Dewatering	Much less than for traditional suspension systems	Required
CO_2 delivery	Optional	Required
Water		
Volume	Small	Large
Gas exchange		
Oxygen	Good	Poor
CO_2 delivery	Diffusion	Bubbling
System cost		
Open	Low	Low
Closed	Expensive[1]	Very expensive[1]
Biomass harvesting	Scraping	Technical
Biomass transport	Less water	High water to transport
Dry biomass	12 – 20%	~1.5%

[1]The terms are relative to the costs of raceways
Source - (Berner et al. 2015; Gross et al. 2015)

Cyanobacteria like *Anabaena*, *Oscillatoria*, *Trichodesmium* and *Tolypothrix* are called diazotrophs, as they are capable of N_2-fixing with the help of the nitrogenase enzyme complex (Stal 2012). As N_2-fixation is metabolically expensive, requiring 16 moles of ATP for fixing 1 mole of nitrogen (Kustka et al. 2003), the preferred source of nitrogen is ammonium, followed by nitrate and then elemental nitrogen (Kustka et al. 2003). Nitrogen fixation is catalysed by the nitrogenase enzyme complex, composed of two different a metalloproteins, the molybdenum iron protein and another Fe protein (Kim and Rees 1994). Consequently, presence of oxygen inhibits the activity of the nitrogenase complex, and therefore some filamentous cyanobacteria produce specialised anoxygenic cells called heterocysts for N_2-fixation (Lindblad and Guerrero 1993).

Free-living cyanobacteria in rice fields fix 20 to 30 kg N ha^{-1} $year^{-1}$ and produce several plant growth regulating substances such as auxin (indole acetic acid) and gibberellic acid, while the symbiotic relationship of *Azolla* and *Anabaena* produces up to 600 kg N ha^{-1} $year^{-1}$ (Bhat et al. 2015; Vaishampayan et al. 2001). On average and on a dry biomass basis, cyanobacterial biomass contains between 2 and 10% nitrogen along with other nutrients and trace elements. Thus, cyanobacterial biomass serves as fertiliser also providing plant growth substances to agricultural crops (Bhat et al. 2015). Additionally, cyanobacteria can utilise the CO_2 in the medium with higher efficiency compared to eukaryotic microalgae, e.g *Spirulina/Arthrospira* cultures had a CO_2 fixation efficiency of 38% (Chiu et al. 2008). Potential of cell growth and ability of CO_2 metabolism may be a reason for observed species-dependent CO_2 removal or fixation rates (Chiu et al. 2008). Similarly, the N_2-fixing *Anabaena variabilis* could be successfully cultivated in medium supplied continuously with 20% CO_2 and showed increased nitrogenase activity (De Morais and Costa 2007; Yoon et al. 2002). These data provide evidence that cyanobacteria have the potential for CO_2 remediation, could be cultivated in macro-nutrient poor ash dam water if a N_2-fixing cyanobacterium is selected, and have biofertiliser potential, but the complex metal mixtures in ash dam water could either retard growth and/or adversely affect biofertiliser potential through bioaccumulation of high levels of these metals.

1.5.1 Metal remediation

Several studies have shown that algae could be ideally used for heavy metal absorption from industrial and domestic wastewaters. However, often

non-living algal biomass was used for metal adsorption and only a few studies used live materials (Lamai et al. 2005; Zeraatkar et al. 2016). Characterisation of wastewater is essential for selection of species, as fertilisation requirements for growth needs to be considered (Komolafe et al. 2014; Rawat et al. 2011). All photosynthetic organisms require nitrogen and phosphorus along with other micro-nutrients such iron, molybdenum, magnesium, calcium, copper and zinc. Ash dam wastewaters are typically devoid of macro-nutrients such as nitrogen and phosphorus (Saunders et al. 2012), restricting the choice of organism to more macro-nutrient self-sufficient species. Diazotrophic cyanobacteria are a suitable choice in this scenario, as they can fix atmospheric nitrogen to sustain growth. Additionally, some cyanobacteria also store phosphate intracellularly in granules for utilisation under phosphate-limiting conditions. These features are comparatively unique for diazotrophic cyanobacteria (Stevens et al. 1981), however, providing phosphate is an essential fertilisation requirement for cyanobacterial growth, and phosphate storage occurs only when initial phosphate supplementation exceeds growth requirements (Mukherjee et al. 2015; Velu et al. 2015).

Cyanobacteria possess certain features that assist in metal remediation. The cyanobacterial cell wall is made up of peptidoglycan which is much thicker and more highly cross-linked than in other gram-negative bacteria (Hoiczyk and Hansel 2000). An exopolysaccharide layer, which is called capsule, sheath or slime layer based on their chemical and physical properties, is external to the cell wall (Hoiczyk and Hansel 2000). Presence of heteropolymeric exopolysaccharides with uronic acid provides a strong anionic character to the

cyanobacterial cell surface and these features aid in adsorption of metals present in polluted wastewaters (Bhunia et al. 2018; Singh et al. 2019).

1.5.2 Value adding by-products from the cyanobacterial biomass

Integration of anaerobic digestion (AD) with microalgae or cyanobacteria biomass production is predicted to support economic and environmental development of commercial-scale cultivation efforts (Gonzalez-Fernandez et al. 2015). In this scenario, the biomass produced, or a portion thereof is hydrolysed and then subjected to AD to produce biogas. AD is defined as “a highly sophisticated process in which a variety of microorganisms play various roles in the decomposition of organic material and production of methane and CO_2.”(Nishio and Nakashimada 2013). AD converts cyanobacterial biomass into biogas by various biochemical processes under anaerobic conditions using bacteria. The AD process is characterised by four stages; hydrolysis, acidogenesis, acetogenesis and methanogenesis (Astals et al. 2015). Complex organic molecules, such as carbohydrate, protein and lipids, are hydrolysed by extra-cellular bacterial enzymes into amino acids, sugars and free fatty acids (FFA). The hydrolysed molecules are converted to volatile fatty acids, hydrogen and carbon dioxide during acidogenesis and acetogenesis. Acetogenic bacteria degrade FFA into acetate and hydrogen. The methanogenic archaea are involved in the final stage of methanogenesis, where acetate, hydrogen and CO_2 are the main substrates for methanogenic microorganism growth (Gonzalez-Fernandez et al. 2015). Total ammonia-nitrogen concentrations above 3 g L^{-1} at a pH >7.4 will inhibit methanogenesis, reducing methane production (Parimi et al. 2015).

Alternatively, cyanobacterial biomass provides species-specific bioactive compounds and numerous by-products, which are viable alternatives to various chemically and artificially synthesised products. Many species of cyanobacteria produce antagonistic chemical molecules, known as allelochemicals, made of alkaloids, terpenoids, peptides and volatile organic compounds (Singh et al. 2005). Some cyanobacteria secrete compounds like calothrixin A, nodularin, microcystins and saxitoxins, which have cytotoxic effects, but they also have potential anti-bacterial, anti-fungal and algicidal activities (Gupta et al. 2013; Wiegand and Pflugmacher 2005). For example, microcystin from *Microcystis aeruginosa* is a cyclic peptide with potential activity against bacterioplankton and zooplankton, while nodularin from *Nodularia* sp. is also a peptide with antiviral and antifungal activities (Gupta et al. 2013). UV-absorbing mycosporine-like amino acids are found in N_2-fixing cyanobacteria (Matsunaga et al. 1993; Sinha et al. 2001). For example, cyanovirin-N (CV-N) is a unique, 101 amino acid long, 11 kDa protein discovered in *Nostoc ellipsosporum*. CV-N inactivates and inhibits all immunodeficiency viruses including HIV-1, HIV-2, SIV and FIV irreversibly. CV-N inhibits the fusion of viruses with CD4 cell membranes by interacting in an unusual manner with the viral envelope (Singh et al. 2005).

Cyanobacterial photosynthetic pigments such as carotenoids and phycobiliproteins are most sought after in the medical and food industry (Yen et al. 2013). Phycobiliproteins consist of phycocyanin, phycoerythrin and allophycocyanin which account for about 20% of dry biomass and are found only in cyanobacteria, red algae, Glaucocystophytes, a small group of

freshwater eukaryotic microalgae, and Cryptophytes (e.g. *Rhodomonas*) (Cuellar-Bermudez et al. 2015; Heimann and Huerlimann 2015b). Carotenoids act as antioxidants and boost the immune system, while phycocyanin showed anti-inflammatory and hepatoprotective activity in animal cells (Wu et al. 2016). In *Anabaena*, phycobiliproteins can account for 50% of total protein, which are used as natural colouring agents in ice cream, candies, beverages and dairy products and phycocyanin is a naturally occurring blue pigment used in the cosmetic industry (Kumar et al. 2014; Nayak et al. 2007). One of the photosynthetic pigments with a β-subunit called C-phycocyanin has various applications in the pharmacological industry, including anti-inflammatory and anticancer activities (Wang et al. 2007). An *in vitro* study reported that recombinant C-PC/β inhibited proliferation and induced apoptosis (Wang et al. 2007). Glyceraldehyde-3-phosphate dehydrogenase mRNA levels decreased significantly due to the interaction of the recombinant protein with membrane-associated β-tubulin and glyceraldehyde-3-phosphate dehydrogenase. These properties revealed that the C-PC/β has potential for cancer prevention or as a therapeutic agent (Singh et al. 2011; Wang et al. 2007). Another study showed that C-phycocyanin selectively inhibited cytochrome oxidase-2 (COX-2), with hepatoprotective and anti-inflammatory effects (Reddy et al. 2000). COX-2 is over-expressed in breast cancer (Singh et al. 2005). Moreover, cyanobacteria are a rich source of protein, vitamins and omega fatty acids which are used as food and animal feeds (Gupta et al. 2013).

One of the diazotrophic cyanobacteria, *Tolypothrix* has the above mentioned advantages but its potential has not been investigated in this

context. *Tolypothrix* sp. is filamentous with rod-shaped cells and belongs to the order of Nostocales. It has a gelatinous sheath outside the cell wall and possesses heterocysts for N_2-fixation. When grown in suspension culture, it exhibits self-flocculating characteristics for cost-effective harvesting of biomass and is also amenable for biofilm cultivation (Silva and Silva 2007). Cytotoxins, such as tolytoxin and thiamazoles produced by *Tolypothrix* sp., are toxic to humans as well as animals and have antifungal and antimicrobial activity against several pathogens (Gupta et al. 2013; Rastogi and Sinha 2009).

1.6 Synchronisation with United Nation's sustainable development goals

In 2015, world countries envisioned 17 Sustainable Development Goals (SDG) and set the agenda for 2030. The 17 SDG goals are no poverty, zero hunger, good health and well-being, quality education, gender equality, clean water and sanitation, affordable energy, decent work and economic growth, industry, innovation and infrastructure, reduced inequality, sustainable cities and communities, reasonable consumption and production, climate action, life below water, life on land, peace and justice, and strong institutions and partnerships to active the goal (UN 2015). Clean water and sanitation, affordable and clean energy, climate action, life below water and life on land are most crucial goals to be considered by scientists. These goals should be achieved by 2030 by several actions such as (i) clean water and sanitation by providing safe water to all, and developing wastewater treatment, recycling and reuse technologies; (ii) affordable and clean energy by increasing and sharing sustainable renewable energy in the global energy mix and facilitating the

accessibility to clean energy research and technology and upgrading these into least developed countries; (iii) climate action by educating and rising awareness for humans to increase institutional capacity on climate change mitigation, adaptation, impact reduction and early warning; (iv) life below water by preventing and significantly reducing marine pollution from land based activities including nutrient pollution; and (v) life on land by restoring degraded land and soil which has been affected by desertification, drought and floods (UN 2015).

Keeping this in view, enlisting the environmental services of diazotrophic cyanobacteria for the remediation of CO_2 and metals from flue gas and ash dam water of coal-fired power plants, respectively, whilst producing biofertilisers and other value-add bio-products directly addresses these core sustainable development goals (Chapters 4, 6, 7 and 8).

1.7 Knowledge gaps and bottlenecks

The ability of cyanobacteria to remove nutrients and metals from wastewaters is beyond doubt. However, most previous studies have been conducted in the laboratory, with few testing the actual growth and remediation performance in meso- or large-scale outdoor systems. In the particular case of metals, most research has focused on the use of immobilisation agents (e.g. polyethylemine beads, polysulfone resin) to attach the cyanobacterial biomass (De Philippis et al. 2011), the feasibility of which at large-scale is debatable. This PhD explored the direct use of cyanobacterial biofilms attached to surfaces without the need of immobilisation agents as a low cost and environmentally friendly alternative.

To date, bioremediation and biofertiliser/bioproduct synthesis studies have been performed in pure cultures of cyanobacteria, or in consortia of cyanobacteria/eukaryotic algae or cyanobacteria/non-photosynthetic bacteria naturally present in wastewaters (Abed et al. 2009; Markou and Georgakakis 2011). Although results are reasonably good for waters rich in nitrogen and phosphate, metal-rich but macro-nutrient-poor waters from coal mines or mining industries require additional fertilisation, which reduces the technical and economic feasibility at large scale. Hypothetically, the use of consortia combining cultures of native N_2-fixing and non N_2-fixing filamentous cyanobacteria may reduce fertilisation requirements, increase the resilience of the biofilm to biological contamination and build exopolysaccharide-rich matrixes ideal for the biosorption of metals. Testing these hypothetical advantages, which have been insufficiently explored in both the laboratory and, especially, outdoors, have been one of the main goals of my PhD research.

Microalgal/cyanobacterial biomass production has been assessed in 2 types of systems: open ponds (raceways ponds) and closed photobioreactors (Chisti 2007) To date, only open systems have proven economically and energetically feasible for outdoor mass productions (Parmar et al. 2011). However, their susceptibility to biological contamination restricts their use to cyanobacterial species tolerant to high salinity or high pH, like the non-nitrogen fixer *Arthrospira*/*Spirulina*. The lack of ammonium and nitrate in the water source could be another restrictive condition, potentially minimizing biological contamination while broadening the list of species suitable for mass production to freshwater N_2-fixing cyanobacteria. This theoretical possibility, which has not

been critically addressed to date, has been evaluated in the present PhD. Furthermore, the evaluation of low cost bioreactors, easy to handle and maintain, may turn growing cyanobacterial nitrogen biofertiliser into an actual option for certain rural areas.

Commercial bioproduct development from cyanobacteria, except from *Arthrospira*, is not yet a reality due to high fertilisation (nitrogen, phosphate), and biomass harvesting and dewatering costs (Chisti 2007; Parmar et al. 2011). Growing biofilms of biofilm-forming and/or self-settling native cyanobacteria, including nitrogen-fixers, would reduce fertilisation and centrifugation costs drastically. This possibility, and especially the use of filamentous tropical strains able to cope with high temperatures and light irradiances potentially achieving sufficient biomass production all year round, is still poorly explored at meso and large scale. Moreover, most previous studies have focused on the production of biofuels and/or a small number of bio-products (Gupta et al. 2013). Even though few species of cyanobacteria have been studied for anaerobic biogas production, no studies have been undertaken using N_2-fixing cyanobacteria, despite enormous cost reduction potential for biomass production, due to savings on nitrogen fertilising cost. To date, no study investigated pre-treatment conditions for effective combined biogas and biofertiliser production from *Tolypothrix* sp. or other N_2-fixing cyanobacteria *via* anaerobic digestion. In this context, this PhD critically evaluated the effect of different pretreatment conditions for biogas production and different bioproduct extraction pathways from the environmental (carbon footprint) and economic perspectives, in order to maximise the profitability of the process while ensuring its sustainability.

1.8 Thesis structure

The overreaching aim of this PhD research was to develop cost-effective processes for wastewater remediation, biofertiliser and bioproduct synthesis by native tropical filamentous cyanobacteria. A detailed literature review on this topic is presented in chapter 2, which laid the foundation for the experimental approaches chosen in the actual data chapters of the thesis. In this context, this study evaluated the growth of native cyanobacteria in simulated ash dam wastewater and examined the effect of CO_2 and metals on the growth, biochemical profile (carbohydrate, protein, lipids, fatty acids and pigments) and elemental composition (carbon, hydrogen, nitrogen, sulphur, potassium and phosphorus). Additionally, this research determined the effect of different pretreatments for biogas production from N_2-fixing cyanobacterium *Tolypothrix* sp..

The data chapters in this thesis are divided into five chapters. The aims of each chapter are as presented below:

Chapter 3: Bioremediation and bioproduct potential of native filamentous cyanobacteria. The aims were to evaluate the growth of natural freshwater N_2-fixing cyanobacterium *Tolypothrix* sp., the non N_2-fixing cyanobacterium *Limnothrix* sp. and the consortium of these two-species grown in simulated ash dam wastewater. This chapter evaluated the effect of heavy metals on growth and biochemical profiling of above species.

Chapter 4: Effect of CO_2 and metal-rich wastewater on bioproduct potential of the diazotrophic cyanobacterium, *Tolypothrix* sp.. The aims

were to determine the effect of 15% CO_2 and metal-rich wastewater on the growth of *Tolypothrix* sp. and its bioproduct potential in indoor suspension cultures.

Chapter 5: First outdoor cultivation of the N_2-fixing cyanobacterium *Tolypothrix* sp. in low-cost cultivation systems in tropical Australia. The aims were to evaluate the biomass productivity and biochemical profile of the N_2-fixing cyanobacterium *Tolypothrix* sp. grown in nitrogen-free medium in outdoor suspension and biofilm cultivation systems in tropical Australia.

Chapter 6: Bioproduct potential in outdoor cultures of the *Tolypothrix* sp.: effect of CO_2 and metal-rich wastewater. The aims were to evaluate the effect of 15% CO_2 and metal-rich wastewater on the growth and bioproduct productivities of *Tolypothrix* sp. grown as biofilms and in traditional suspension cultivation system under outdoor conditions.

Chapter 7: Pre-treatment of *Tolypothrix* for methane production. The aim of this chapter was to determine the effect of pre-treatment on biogas production from *Tolypothrix* sp. through anaerobic digestion.

The outcomes of the individual data chapters are summarised and based on biomass yields and biochemical data obtained, net present value (NPV) and sensitivities analyses discussed the general discussion of this thesis (Chapter 8).

Chapter 2: Review on coupled benefits of diazotrophic cyanobacteria cultivation for bioremediation and bioenergy / bioproduct development[2]

2.1 Abstract

Coupling bio-energy production with bioremediation is considered a promising solution for cost-effective biofuel production. Cyanobacteria are prokaryotic, oxygenic photosynthetic organisms that have an ability to colonise a wide range of aquatic and terrestrial habitats including extreme environments such as hot springs, wastewater, mining tailings and acidic bogs. Their adaptation to a diversity of environmental habitats is attributed to specialised cells, one being akinetes, a resting cell that enables them to survive extreme conditions, while heterocysts enable some species to fix atmospheric nitrogen. These unique characteristics make them potential candidates for bioremediation of wastewaters and production of bio-fertilisers for agricultural industries. Diazotrophic cyanobacterial species, such as *Tolypothrix* sp., *Nostoc* sp. and *Anabaena* sp. are effective candidates for remediating metals in wastewaters, because of the metal binding capacity of their extracellular polysaccharides. Cyanobacterial biomass productivities are comparable to that of eukaryotic algae and many species produce sufficient amounts of carbohydrates for conversion to bioethanol. Additionally, cyanobacteria produce pigments and fatty acids, which have commercial applications in pharmaceutical,

[2] This review chapter is ready to submit to Biomass and Bioenergy journal.

nutraceuticals and food industries. Cyanobacteria also produce intracellular bioactive molecules with potential pharmaceutical applications against bacteria, fungi, virus and algae. This review considers the prospects and challenges in using diazotrophic cyanobacteria species for bioremediation, bio-energy production and bio-product synthesis.

2.2 Introduction

Globally, energy demand has risen continuously ever since the industrial revolution in line with population growth. The human population is expected to increase from 7.6 billion in 2017 to 9.8 billion in 2050 and 11.2 billion in 2100 (UN 2017). Economic - and population growth have resulted in increased pressures on natural and environmental resources. World energy consumption is estimated to increase by ~22% from 575 quadrillion (one quadrillion is equal to 1,000 trillion) British thermal units (Btu) in 2015 to 663 quadrillion Btu by 2030 and then to 736 quadrillion Btu by 2040 (EIA 2017). Satisfying the food requirements of the growing population is facing two challenges: supply of unpolluted water and increasing production on diminishing arable land. Continuous utilisation of freshwater and discharge of untreated wastewater can lead to ecosystem degradation (Tyagi et al. 2014). Clean water is already a scarce commodity in arid countries (Schewe et al. 2014) and the increased use of fertilisers places additional pressures on agricultural productivity through diminishing soil fertility (Asif and Muneer 2007). This problem is exacerbated by rising energy requirements, which, if produced from fossil resources, has been shown to lead to climatic instability (Lotfalipour et al. 2010)

Clean water and reduction of greenhouse gas emissions are at the heart of the food – energy nexus. While it is possible to recycle industrial wastewaters, existing processes are expensive, energy-intensive and can release toxic by-products (IEA 2008). Around 85% of the world population lives in the driest regions on earth, which incidentally are also the regions experiencing fastest population increases. (UNESCO 2013). In addition to water scarcity, depletion of fossil fuels and greenhouse gas emission-induced climatic instability also demands to limit emissions whilst implementing renewable, efficient, cost-effective energy alternatives (Emodi et al. 2019).

A myriade of first generation and second-generation feedstock exist for bioenergy -, including biofuel production (Carriquiry et al. 2011). The production of biofuel from energy crops such as canola, agave, giant seed etc. demands arable land and adequate freshwater supply (López-Bellido et al. 2014), sharpening the food vs. energy controversy. An environmentally friendly solution for generating renewable energy exists through deployment of environmental services of photosynthetic cyanobacteria for industrial water remediation and CO_2 emission abatement. Their ability to tolerate high metal concentrations and their capacity to biodegrade aromatic pollutants by enhancing the breakage of aromatic rings and the remediation of calcium chloride from industrial wastewater and factory effluent make them ideal candidates for biomass production in industrial wastewaters (Kiran et al. 2008). In addition, a wide range of temperature, pH and CO_2 levels can be tolerated, although this is strongly species-dependent (De Philippis et al. 2011). Furthermore, they are a rich source of bioactive compounds with various

applications in food and pharmaceutical industries. They produce a number of secondary metabolites including toxins, vitamins and enzymes, which are useful in the pharmaceutical industry. Some cyanobacteria are also capable of producing hydrogen gas which can be utilised as a future source of renewable and CO_2 emission-free energy (Hallenbeck 2012).

The increasing global population depends on adequate food supplies where fertilisers play a crucial role in improving crop yields and areal productivity, which, in addition to high energy demands for production and transport, has adverse effects on water quality and soil fertility (Edmeades 2003; Tilman et al. 2002). Some cyanobacteria are able to fix atmospheric nitrogen (N_2), improving soil fertility through encouraging proliferation of soil microorganisms which in turn facilitates vegetation growth (Xu et al. 2019). Thus, these cyanobacteria are a valuable source of biofertilisers. Capacity of N_2-fixation is an additional advantage over traditionally used eukaryotic photosynthetic aquatic organisms (micro-/ macroalgae and plants). If cyanobacteria can be used in these industrial applications, their value as an alternative energy source may be exploited.

In this context, this review provides a brief overview of global challenges and then discusses the coupled benefit of diazotrophic cyanobacteria for bioremediation of wastewater and utilisation of the produced biomass as a potential source for cost-effective bioenergy production and bioproduct development. To take bioremediation and bioenergy synthesis to the next level, the effects of various factors affecting biochemical properties, such as

carbohydrate, protein, lipids, pigments and elemental composition, of diazotrophic cyanobacteria is also considered.

2.3 Global challenges

2.3.1 Rising energy demand

Between 1995 and 2017 global energy demand grew 2.1% per year. The global energy demand is predicted to grow 1.3% per year from ~13,000 million tonnes in 2017 to over ~17,000 million tonnes oil equivalent in 2040 with developing countries being the largest energy consumers (BP 2019). According to the IEA (2018b), fossil fuels will remain the principal sources of energy, accounting for more than 75% (20% coal, 32% oil and 23% gas) of the projected increase in demand in 2035 and 66% (14% coal, 29% oil and 22% gas) in 2050 (IEA 2018b; Tryggestad et al. 2019). These unprecedented global demands for energy are mainly due to the rapidly increasing human population coupled to an energy-intensive life style (Dorian et al. 2006; van Vuuren et al. 2012). According to the World Health Organisation, more than 8% of health problems are due to air pollution caused by energy generation from fossil fuels (Smith et al. 2013; van Vuuren et al. 2012). By 2050, annual warming of the earth's surface is predicted to range between 0.8 °C to 1.8 °C (Bailis et al. 2005; Emodi et al. 2019). Thus, decarbonisation of the energy sector is a core recommended action to mitigate and avert predicted catastrophic consequences of climate instability (Bailis et al. 2005). To cultivate oil crops such as Jatropha, Pongamia and palm, arable land and freshwater are required and this competes with resource requirements for the production of food crops. In contrast, cultivation of carbon-neutral photosynthetic organisms, like micro-

and macroalgae or cyanobacteria, is independent of these resources and would therefore be ideal for the production of liquid biofuel and biogas (Sun et al. 2018).

2.3.2 Water scarcity and water pollution

Nearly 97.5% of water on earth is saline while the remaining 2.5% is freshwater of which 1.5% is in the form of permanent ice or glaciers in polar regions and less than 1% is available for human consumption (Sivakumar 2014). As stated previously, 85% of the world's population inhabit the driest regions, with Australia being one of the driest inhabited continents on earth (UNESCO 2013). About 1.8 billion people are predicted to be living with absolute water scarcity in 2025 (UN-Water 2019). Untreated wastewater not only pollutes freshwater systems, it also threatens the availability of safe drinking water for human consumption. Globally, 3 in 10 or 2.1 billion people are lacking to access safe and readily available water at home and over 8 million people including 361,000 children under 5 years old die annually from water-borne diseases and consumption of polluted water (IEA 2008; UNESCO 2013; WHO 2017). In Australia, coal mines and coal-fired power stations produce large amounts of wastewater characterised by high electrical conductivity, total dissolved solid (TDS) and suspended solid (SS), giving the water a reddish brown to blackish tint**.** The presence of moderate concentrations of various minerals in water is harmful to humans (Roberts et al. 2015). Large quantities of mine water are discharged into the Fitzroy river basin in Queensland and coal mines operating in the Hunter Valley, NSW are close to agricultural land, raising concerns over potential pollution of nearby water bodies (Bartley et al. 2012).

Other than mining, agriculture also adds to water pollution issues, e.g. through application of surplus chemical fertilisers causing nitrate and phosphate contamination of ground water and inland and coastal water systems. Total water consumption in Australia during 2016-17 was 16,558 gigalitres (GL) of which about 63% (10,504 gigalitres) was consumed by agriculture, forestry and the fishing industry, 9% (1,483 gigalitres) for water supply, sewerage and drainage services, and 16% (2,662 gigalitres) by all other industries, and 12% (1,909 gigalitres) by households. The total water consumption increased by 3% from 2015-16, primarily due to use for irrigation in agriculture (ABS 2019). While Australia uses only 5% of its total freshwater resources and the availability of freshwater for human consumption is secured even under projected population growth, current water use is not ecologically sustainable. Efficient treatment of water used in industries and wastewater generated by human consumption also provides significant challenges especially in developing countries due to lack of infrastructure, technical and institutional capacity and financing (UNESCO 2017).

Mine wastewater that is unsuitable for discharge into the inland and coastal water system requires treatment prior to disposal into natural water bodies (Costello 2003; Thiruvenkatachari et al. 2011). Various physio-chemical technologies employed for wastewater treatment are energy-intensive and require sophisticated mechanical instrumentation and use of chemicals such as ammonia, limestone, magnesium hydroxide, sodium carbonate and sodium hydroxide, which results in sludge generation requiring disposal (Costello 2003; Nedved and Jansz 2006). Bioremediation of wastewater with photosynthetic

cyanobacteria will not only reduce pollution loads but also provide biomass that can be used for the generation of value-added by-products (Renuka et al. 2015).

2.3.3 Fertiliser demand

Population growth and arable land scarcity increase pressures on crop production by 70-100% in order to feed the globe in 2050 (McKenzie and Williams 2015). Sustainable population growth relies on secured food supplies. According to IFA (2018), total global fertiliser consumption in 2017/18 was 187 million tonnes of which 68% were used by developing countries, an increase of 1.3% per annum from 2015/16. Nitrogen, phosphate and potassium (N, P and K) are the main fertilisers used with an annual growth rate of 1.0, 1.4 and 1.8 % respectively. Global N, P and K fertiliser consumption is predicted to be 200 million tonnes in 2022/23 from 187 million tonnes in 2018 (IFA 2018). In Australia, (N, P, and K) fertiliser consumption increased from around 0.69 million tonnes in 1983 to 1.7 million tonnes in 2004 and it reached 4.8 million tonnes in 2011 (FIFA 2011). The N, P and K fertiliser consumption demand further increased to 5.1 million tonnes in 2015/16 with ammonium phosphate continued to be the most widely used fertiliser (ABS 2017). However, fertiliser consumption decreased by 4% from 5.1 to 5 million tonnes from 2015/16 to 2016/17 (ABS 2018). Although application of inorganic fertilisers increases crop productivity, the amount of energy spent in the form of natural gas and electricity for the production and fossil fuels for transport releases substantial amount of greenhouse gases (FIFA 2011; Ryan 2010). For example, in 2011, Fertiliser Industry Federation of Australia (FIFA)-registered companies alone

generated 772,285 tonnes of CO_2 from energy used in the manufacturing process, using 10.51 million kilolitres of water of which only 1% was recycled (FIFA 2011).

Another ecological problem resulting from inorganic fertiliser usage is eutrophication of fresh- and marine water ecosystems. In many parts of Australia, concentrations of greater than 10 mg L^{-1} of nitrate were found in ground water and at some places concentrations were above 50 mg L^{-1}. In the case of the Eastern Darling Downs, the probable source of this contamination is over-application of inorganic nitrogen fertilisers (Schult 2016).

Australia is one of the driest continents, characterised by loose sandy soils with low soil organic carbon (SOC) contents of 0.9% to 1%. The required amount of SOC for cultivation of wheat is 2.4% (Griffin 2003). Wheat is predominantly grown in Western Australia (WA), where the SOC content is only 1% (Griffin 2003). Micro-nutrients required for plant growth such as copper, zinc and molybdenum are often also scarce in these soils (Chen et al. 2008a). Increasing SOC contents does improve soil fertility, water holding - and ion exchange capacity and reduces soil erosion (Cann 2000). In South Australia, the water holding capacity of the soil is very low which ultimately leads to land degradation and loss of productivity affecting 2 million ha (Cann 2000). To improve soil quality in South Australia, clay was added and the results showed promise, but mixing of clay is problematic at a large scale. In this scenario, cyanobacterial-based fertilisers represent an attractive option to provide macro - and micro nutrients to the soil together with organic carbon (Cann 2000).

2.4 Cyanobacteria

2.4.1 General characteristics

Cyanobacteria possess the same characteristics as macro- and microalgae, making them a potential cost-effective source for renewable energy and bioproduct synthesis. The ability of some strains to perform different types of metabolism, such as oxygenic photosynthesis and heterotrophic and mixotrophic growth are the same as reported for microalgae (Subashchandrabose et al. 2013). Bioactive compounds produced by cyanobacteria possess antibacterial (Bhadury and Wright 2004; Dahms et al. 2006; Jaki et al. 2000) antifungal, antiviral, anticancer, algacidal and immunosuppressive activity (Griffiths et al. 2016). Additionally, some cyanobacteria are potential producers of hydrogen gas, which can be utilised as a renewable source of biofuel (Krishnakumar et al. 2013). *Anabaena* and *Nostoc* sp, produce significant amounts of hydrogen gas under different culture conditions (Yoshino et al. 2007). Alternatively, cyanobacterial biomass can be anaerobically digested to produce biogas. Anaerobic digestion of cyanobacterial biomass degrades the organic carbon into organic acids and biogas. The digestate after anaerobic digestion can potentially be used as a fertiliser (Passos et al. 2014). Anaerobic digestion of algal biomass requires hydrolysis to break down organic materials for better digestion. Alternatively, cyanobacterial hydrolysates could be fermented to bioethanol, as cyanobacterial biomass is rich in carbohydrates (45 to 65) (Möllers et al. 2014). Biomass of cyanobacteria can also be converted to biochar through slow pyrolysis (Roberts et al. 2013b) to form a carbon-rich product characterised by a

high pH which can be used to improve the pH of acidic soils (Bird et al. 2012). Additionally, the high content of nitrogen, phosphorus and inorganic elements can improve soil fertility (Bird et al. 2012). The ability of some strains to fix atmospheric nitrogen and conversion to ammonium results in improved fertility of desert soils and permits soil reclamation (Garcia-Pichel and Pringault 2001). Cyanobacterial species such as *Anabaena, Arthrospira* (formerly *Spirulina)* (Jiménez et al. 2003a) and *Nostoc* are an excellent source of nutrients reported to assist digestion in humans and are used as food supplements. They are rich in protein (more than 60% of protein, Vitamin B_{12}, thiamine, ß-carotene, riboflavin) and fibre which play a vital role in physiological and nutritional quality of human food (Fuhrman 2003). Some species tolerate extreme environmental conditions like hot springs, deserts, permafrost zones, bare rocks, high UV irradiances, and high salt concentrations and are capable of harnessing solar energy and assimilating significant amounts of CO_2 (Fuhrman 2003). Cyanobacteria are also capable of tolerating high levels of toxic metals, as they use metal-binding proteins (phytochelatin) for detoxification (Gaur and Rai 2001). Compared to eukaryotic microalgae, cyanobacteria are of particular interest in bioremediation and for bioenergy production, due to tolerance to environmental extremes, capacity to grow on limited nutrient supplies through fixation of atmospheric nitrogen, and capacity to store phosphorus as polyphosphate granules (Wijffels et al. 2013).

2.4.2 Photosynthetic capacity

As photosynthetic prokaryotes, cyanobacteria are capable of utilising light for photosynthesis at low photon flux densities. They can use H_2S as an

electron donor for photosynthesis and they are also tolerant of free sulphide (Miller and Bebout 2004). Low concentrations of inorganic carbon are sufficient for their photosynthetic CO_2 reduction activity. Like in eukaryotic algae and plants, photosynthesis involves two reaction stages namely, the light-dependent stage, resulting in the production of $NADPH+H^+$ and ATP (photosynthesis), and the light-independent stage (CO_2-fixation), yielding carbohydrates (Masojídek et al. 2004). Like photosynthetic eukaryotes, cyanobacteria assimilate CO_2 into organic matter via the enzyme 1, 5-bis-phosphate carboxylase/oxygenase (RubisCO). RubisCO exhibits a higher affinity for O_2 over CO_2, a problem counteracted by cyanobacteria and microalgae by using a Carbon Concentration Mechanism (CCM) (Beardall and Giordano 2002).The microalgal CCM elevates CO_2 concentration around RubisCO by at least four moles of inorganic carbon uptake. These uptake systems accumulate HCO_3^- in the cytosol of the cell, which is utilised by the RubisCO-containing carboxysome (Badger and Price 2003).

The dissolution of CO_2 in water generates carbonic acid, resulting in the acidification of the medium, although this effect is partially counter-acted by the production of OH^- in photosynthetic reactions. Thus, there are certain thresholds in the tolerance of cyanobacteria to CO_2 concentration (Jansson and Northen 2010). Some reports claim that *Synechococcus elongatus* can tolerate concentrations as high as 60% CO_2 (v/v) (Salih 2011). However, most studies indicate that optimal biomass production and fixation rates occur only up to 12-18% CO_2 (v/v) (Markou and Georgakakis 2011), i.e., within the level of up to 15% CO_2 (v/v) usually found in industrial flue gases.

CO_2 removal efficiencies have been reported as being up to 60% for *Arthrospira platensis* at concentrations of up to 12% of CO_2, with a fixation efficiency of 0.413 g CO_2 L^{-1} d^{-1} at a cell concentration of 3.5 g dry cell biomass L^{-1} final culture density (Wang et al. 2008). In contrast, *Anabaena* sp. ATCC 33047 fixed up to 1.45 g CO_2 L^{-1} d^{-1} at the maximum biomass productivity of 0.31 g L^{-1} d^{-1}. CO_2 fixation was increased to 3.0 g CO_2 L^{-1} d^{-1} under outdoor cultivation conditions (González López et al. 2009), equating to 57 g m^{-2} day^{-1} (Ugwu et al. 2005). Based on this information, removal capacity of up to 74 tons CO_2 ha^{-1} y^{-1} could be achievable.

2.4.3 Cultivation factors

Cultivation of cyanobacterial biomass has gained a lot of attention recently, because some are easy to grow, since their basic survival needs (sunlight, water, CO_2 and a few nutrients) are very simple and commonly available. Cyanobacteria do not require any arable land for their production (Benemann 1990), and can be efficiently grown commercially (Soto Ayala and Luque de Castro 2001). They provide non-edible feedstock resources for various applications (Bandyopadhyay et al. 2010) and can grow in a variety of water sources (Benemann and Oswald 1994). Yet, like for the cultivation of other photosynthetic organisms, factors such as available nutrients, pH and alkalinity, light and culture cell density, temperature and contamination by other microorganism affect the physiology and biology of cyanobacteria (Markou and Georgakakis 2011). In order to maximise biomass productivity, these aspects of cyanobacteria growth and development must be understood.

2.3.3.1 Nutrient and light requirements and contamination control

Carbon is the most important nutrient for the cultivation of cyanobacteria, which can utilise both CO_2 and HCO_3^- (Markou et al. 2014b). Diazotrophic cyanobacteria are capable of using elemental nitrogen as their sole nitrogen source because of their capacity to reduce N_2 to NH_4^+ and consequently, this facilitates protein production (Benemann 1979; Markou et al. 2014b). Phosphorus is an important macro-nutrient for biomass cultivation, and while it is not required in large amounts, it may be a growth limiting factor. Like eukaryotic algae and plants, other macro-nutrients such as sulphur, calcium, magnesium and potassium and micro-nutrients such as molybdenum, iron, nickel, copper, zinc, cobalt, boron, manganese and chloride are also required for the growth (Markou et al. 2014b). Light is the vital energy source for the production of photosynthetic biomass, affecting photosynthetic efficiency and other metabolic processes. Cyanobacterial pigments absorb light in the range of 400-500 nm and 600-700 nm wavelength in the electromagnetic spectrum (Janssen 2002). At high light and cell densities, the photosynthetic capacity of cyanobacteria decreases resulting in decreased lipids, fatty acids and protein content (Agel et al. 1987; Chen et al. 2011a).

Like in the cultivation of micro- and macroalgae, contamination by bacteria, fungi, yeasts and other aquatic protists affects the growth of cyanobacterial biomass (Masojídek et al. 2004; Wu and Pond 1981). Effective ways to eliminate such contamination include growing cyanobacteria at high pH and ammonium concentrations (Belkin and Boussiba 1991), to provide optimal conditions (Canizares-Villanueva et al. 1994). The unique ability of

cyanobacteria to grow at low water potential, i.e. in deserts and hyper saline ponds, provides an advantage over other microorganisms (Thajuddin and Subramanian 1992). Cultivated cyanobacterial biomass can be utilised for bioproduct synthesis through applying a biorefinery concept, a process also in discussion for cost-effective bioproduct synthesis from micro/macroalgal biomass (Meixner et al. 2018).

2.4.4 Nitrogen fixation

Nitrogen is, together with phosphate, one of the essential macro-nutrients required for the growth of photosynthetic organisms and a limiting factor in most terrestrial and aquatic ecosystems (Grobbelaar 2004). Most photosynthetic microorganism uptake nitrogen either as nitrate (NO_3^-) or as NH_4^+, which is far less abundant (Flores et al. 2005; Kirchman and Wheeler 1998). In addition to such sources of nitrogen, diazotrophic cyanobacteria are capable of atmospheric nitrogen (N_2) fixation (Eq. 2.1), which poses an evolutionary advantage over other competitors and represents the only form of entry of new nitrogen into a number of terrestrial and aquatic ecosystems, while organic nitrogen is being recycled through nitrification and denitrification (Fay 1992; Kuhlbusch et al. 1991).

$$N_2 + 8e^- + 16\ ATP + 8H^+ \rightarrow 2NH_3 + 16\ ADP + 16\ P_i + H_2 \text{----------Eq- 2.1}$$

The enzyme complex nitrogenase is responsible for N_2 conversion to NH_4^+, a process strongly inhibited by the presence of O_2 generated in copious amounts in oxygenic photosynthesis, and, generates H_2 as a by-product (Eady

et al. 1978; Fay 1992). Therefore, N_2-fixing cyanobacteria have developed a series of adaptations to separate photosynthesis and nitrogen fixation. Physical separation of photosynthesis and N_2-fixation occurs in differentiated cells (thick-walled heterocysts) that protect the nitrogenase from O_2, as photosystem II – where O_2 evolution occurs as a by-product of the water splitting reaction – is absent and from O_2 entering the cell from the environment. In contrast, no heterocysts are made in species in which N_2-fixation is temporally separated, occurring only during the night under aerobic conditions or under increased respiratory rates (microaerobic conditions). Since fertilisation (addition of nitrogen, phosphate and micro-nutrients) accounts for a large part of the cost for mass cultivation of photosynthetic microorganisms, the use of N_2-fixing cyanobacteria has been proposed as a possible solution to replace energy-intensive chemical fertilisers (Benemann 2013). For example, *Anabaena-azollae,* a cyanobacterial symbiont of the water fern, can fix up to 600 kg of N_2 ha^{-1} $year^{-1}$ (Bhat et al. 2015; Vaishampayan et al. 2001). Results of some studies suggest that N_2-fixing cyanobacteria represent a significant source of bioproducts, with reduced cultivation costs. *Anabaena siamensis* grown in a WAVE™ bioreactor exhibited increased phosphate consumption and 37 to 70% higher CO_2 fixation rates than conventional suspension batch cultures (Cirés et al. 2015). Another advantage is that during N_2-fixation, hydrogen gas is released which can be used as a non-CO_2 generating renewable fuel (Krishnakumar et al. 2013).

Some species of cyanobacteria such as *Anabaena, Aulosira, Calothrix, Cylindrospermum, Gloeocapsa, Nostoc, Rivularia, Scytonema* and *Tolypothrix*

grow well in waterlogged rice fields (Roger et al. 1987). They are used as natural fertilisers for rice cultivation in many countries (Pereira et al. 2009). For producing an effective cyanobacterial biofertiliser, *Tolypothrix tenuis* has been mixed with *Nostoc* sp. (Silva and Silva 2013). Heterocysts are more numerous in symbiotic forms rather than free living cyanobacteria (West and Adams 1997), suggestive of higher rates of N_2-fixation. Usage of cyanobacteria on crops such as barley, oats, tomato, radish, cotton, sugarcane, maize, chilli and lettuce has shown beneficial effects, as they increase soil fertility (Kaushik and Venkataraman 1979). This enables the production of biofertilisers, which add fertility to the soil and assist in soil reclamation without adverse environmental impacts.

2.5 Bioremediation of wastewaters by cyanobacteria

Phycoremediation is defined as the use of eukaryotic algae or cyanobacteria to remove contaminants present in urban and agro-industrial wastewaters, including inorganic pollutants (N and P sources and metals) and organic carbon sources. N_2-fixing cyanobacteria such as *Nostoc, Phormidium* and *Tolypothrix* have been the subject in research on wastewater remediation, given their natural presence in wastewater, their well-known metabolic flexibility, and their tolerance to harsh conditions (Heimann and Cires 2015). Additionally, large-scale cultivation of cyanobacteria requires mineral nutrients to support growth: for example, the mineral nutrients used account for 50% of the biomass production cost for the non-nitrogen fixer *Arthrospira* (Venkataraman et al. 1982). Thus, the use of wastewaters rich in mineral nutrients has been

proposed as a sustainable option to combine phycoremediation and production of biomass for commercial purposes (Li et al. 2010).

Wastewater is characterised by an enormous range of chemical components, depending on its origin which may be urban, agriculture/livestock or industrial (Markou and Georgakakis 2011). Features of various wastewaters are given in Table 2.1. Paper mill effluents contain ~562 mg N L^{-1} and ~68 mg P L^{-1}, while municipal wastewaters contain only 15-90 and 5-20 mg L^{-1} N and P, respectively. Animal wastewaters contain 63-4,165 mg N L^{-1} and 14-1,195 mg P L^{-1}, depending on the source, e.g. s diary, poultry, swine, beef feedlot and piggery wastewater. On the other hand, industrial wastewaters contain 1.1-273 mg N L^{-1} and 0.6-182 mg P L^{-1} based on the source, such as textile, winery, tannery, paper, olive mill, and carpet industry. The effluents from anaerobic digestion contain 125-3,456 mg N L^{-1} and 18-382 mg P L^{-1}, which is almost similar to animal wastewater. Generally, agro-industrial wastewaters contain 71-4,200 mg N L^{-1} and 100-3,800 mg P L^{-1} (Table 2.1). Microalgae require a minimum of 72 mg N and 25 mg P per gram biomass for cultivation (Chen et al. 2011b; Peccia et al. 2013), whereas N_2-fixing cyanobacteria require only 3 mg P g^{-1} biomass and no nitrogen may be required for the cultivation of some strains (Velu et al. 2015). As N_2-fixing cyanobacteria require lower amounts of phosphate and do not require ammonium- or nitrate-nitrogen, they can be grown in various types of wastewaters. Additionally, high nutrient-containing wastewaters, such as animal wastewater, anaerobic digestion effluent and agro-industry wastewater, may not support microalgae or cyanobacterial growth, as these wastewaters are characterised by high ammonium, turbidity,

high organic matter, encouraging bacterial growth, which may restrict growth of the desired species (Table 2.1) (Cai et al. 2013; Olguín 2012)

The cultivation of cyanobacterial strains in diverse agro-industrial wastewaters has received increasing scientific attention (Tsolcha et al. 2018). Some studies presented evidence of high remediation efficiencies, for example the reduction of 98% chemical oxygen demand (COD), 99.9% ammonia and 99.4% phosphate with a biomass productivity of 144 mg L^{-1} d^{-1} achieved by *Arthrospira* cultures in starch-rich agro-industry wastewater (Phang et al. 2000). The remediation of metals (e.g., Co, Cd, Cr, Cu, Fe, Hg, Mn, Ni, Pb, Sn and Zn) in synthetic metal mixtures has been confirmed in a number of cyanobacterial genera, including non-N_2-fixers (mostly *Arthrospira*, *Lyngbya* and *Oscillatoria*) and nitrogen-fixers (mainly *Anabaena*, *Calothrix*, *Nostoc*, *Tolypothrix*) (Gupta et al. 2013). Removal efficiencies varied, largely depending on the species used and target metal, e.g., (El-Sheekh et al. 2005) grew *Nostoc muscorum* and *Anabaena subcylindrica* in different types of wastewaters, obtaining a reduction of the amount of Cu, Co, Pb, and Mn of between 12.5 and 81.8%; 11.8 and 33.7%; 26.4 and 100%; 32.7 and 100% of the initial concentration, respectively.

Table 2.1 Nutrient levels of different types of wastewaters

Wastewater sources	Nutrient levels (mg L^{-1})		CO_2 level	Inhibitors	References
	N	P			
Paper mill effluents	562	68.3	Low	Cl compounds, Sx and NOx	(Nanda et al. 2010)
Municipal wastewater	15 – 90	5 – 20	-	Heavy metals	(Cai et al. 2013)
Animal wastewater					
Diary	185 – 2,636	30 – 727	-	-	(Cai et al. 2013; Markou and Georgakakis 2011)
Poultry	802 – 1,825	50 – 446			
Swine	1,110 – 3, 213	310 – 987			
Beef feedlot	63 – 4,165	14 – 1,195			
Piggery wastewater	1,931	58.5			
Industrial wastewater					
Textile	21-57	1.0-9.7	low	Heavy metals	(Cai et al. 2013; Chinnasamy et al. 2010; Olguín 2012)
Winery	110	52			
Tannery	273	21			
Paper	1.1-10.9	0.6-5.8			
Olive mill	532	182			
Carpet industry	17.6-45.9	5.5-35			
Anaerobic digestion effluent					
Dairy manure	125 – 3,456	18 – 250	low	High ammonium concentration and turbidity. Bacterial growth	(Cai et al. 2013)
Poultry manure	1,380 – 1580	370 – 382			
Sewage sludge	427 – 467	134 – 321			
Food waste and dairy manure	1,640 – 1,885	296 – 302			
Sewage effluent	83.7	3.2	-	-	(Renuka et al. 2013)
Agriculture wastewaters	1,700-2,550	125-183		Turbidity High organic matter Content	(Olguín 2012)
Soybean processing	71-140	-			
Sugarcane vinasses	600-4,200	100-3,800			
Sugarcane stillage	2,975	432			

Cyanobacterial species used in the bioremediation of wastewaters are listed in Table 2.2 and the target chemical compounds are shown. Cyanobacterial genera such as *Anabaena, Arthrospira, Aphanothece, Chroococcus, Fischerella, Lyngbya, Limnothrix, Nostoc, Oscillatoria* and *Phormidium* were used to remove various nutrients NO_3^-, NH_3, $PO_4^{3-}$and metals Cd, Co, Cr, Cu, Ni, Pb and Zn from different wastewaters such as ground water, domestic and industrial sewage, synthetic -, plating industry -, urban -, swine -, agro industrial - and animal wastewater (Table 2.2). However, there is no adequate literature for using a single organism to remove various metals collectively from a single wastewater source.

Metal biosorption mechanisms are not yet fully understood in cyanobacteria, although most studies agree that the highly negatively charged EPS plays a major role (Li and Yu 2014). Cyanobacterial EPS are commonly referred to as sheaths (thick layers with high mechanical and physico-chemical stability), capsules (gelatinous layer associated with cell surface) and slime (amorphous mucilaginous material loosely dispersed around the microorganism) and the complex chemical composition of the EPS seems to be strain-dependent (De Philippis et al. 2011). Hypothetically, metal sorption occurs via binding of cationic metals to the negative charges of the EPS polysaccharides surface, mainly via carboxylic groups especially at low pH with a minor role of other functional groups (e.g., sulfonate and amino groups, particularly at high pH) (Heimann and Cires 2015). Other than the EPS, intracellular polyphosphate granules (De Philippis et al. 2011) and metallothionines (metal-chelating proteins) are also involved in the

sequestration of metals by cyanobacteria (Maclean et al. 1972; Mallick and Rai 1990).

Although cyanobacteria are capable of remediating different wastewaters and metal solutions, no single strain can optimally reduce and assimilate all compounds contained in wastewaters. Recent studies suggest that cyanobacterial consortia may show a bioremediation capacity superior to that of single-species cultures, both for the removal of nutrients (Renuka et al. 2013) and metals (Burgos et al. 2013). This, together with the fact that pure cultures are easily contaminated in open outdoor bioreactors, indicates the need to further investigate the use of cyanobacterial strain mixtures as a feasible option for wastewater remediation.

2.6 Cyanobacterial biorefinery

The term “biorefinery” appeared in the 1990’s. A biorefinery may be defined as “a facility that integrates biomass conversion processes and equipment to produce fuels, power, materials and/or chemicals from biomass” (Olguín 2012). The biorefinery concept is comparable to fuel refineries, where various chemical conversion processes and infrastructure are integrated to produce multiple fuels and products from fossil oil (Zhu 2015). The biorefinery concept has been explored as the most economical way for developing biomass-based industries. Existing and recent technologies have to be, however, re-evaluated, if the biorefinery concept is used to transform biomass into biofuels and other bio-products (Trivedi et al. 2015). The biggest drawback of a biorefinery approach is a possible reduction in the exploitability of other fractions when extracting one fraction (Chew et al. 2017).

Fig.2.1 illustrates the process and applications of an integrated cyanobacterial biorefinery model. Photosynthetically produced cyanobacterial biomass using wastewater and flue gas CO_2 could be directly applied as a soil conditioner/fertiliser or pass through a biorefinery approach to extract various bioproducts. At the first stage, phycobiliproteins can be extracted from the biomass and the residual biomass can either be hydrolysed or fractionated to separate more bioproducts.

The hydrolysed biomass can be either anaerobic digested to produce biogas or fermented to produce bioethanol. The residual biomass from anaerobic digestion and fermentation can be used as a biofertiliser. On the other hand, carbohydrate, protein, lipids and other secondary metabolites can be extracted through fractionation. At the end, the carbohydrate can be fermented to bioethanol, protein can be used as an animal feed supplement and other metabolites can be utilised to produce more bioproducts and cosmetics (Chew et al. 2017; Suganya et al. 2016). Various bioproducts and co-products can be obtained from cyanobacterial biomass in a biorefinery approach as explained below.

Table 2.2 Cyanobacteria used for bioremediation of different types of wastewaters

Types of wastewater	Applied cyanobacteria	Chemical compounds	References
Synthetic heavy metal solution	*Aphanothece halophytica*	Zn	(Gupta et al. 2013)
	Lyngbya taylorii	Cd, Pb, Ni, Zn	
	Phormidium laminosum	Cu, Ni, Zn, N and P	
	Oscillatoria anguistissima	Zn, Cu, Co	
Agro industrial wastewater Synthetic wastewater	*Phormidium laminosum*	NO_3 and PO4	(Gupta et al. 2013)
	Anabaena sp.*	CH_3, NO^- and NH	
	Arthrospira maxima	NH_3–N and total phosphorus	
Industrial sewage water	*Nostoc linckia, Calothrix sp.* *Anabaena subcylindrica** *Phormidium* sp. *Limnothrix* sp. *Westiellopsis* sp. *Fischerella* sp.	Cd, Co, Cu, Mn, Pb & Zn	(Cai et al. 2013; Gupta et al. 2013; Renuka et al. 2013)
Industrial wastewater	*Nostoc PCC7936**	Cr(VI)	(Gupta et al. 2013)
Domestic sewage effluent	*Oscillatoria* sp.	NO_3 and PO_4	(Gupta et al. 2013)
Ground water	*Synechococcus* sp. *PCC 7942*	NO	(Gupta et al. 2013)
Urban wastewater	*Phormidium* sp. *Oscillatoria* sp.	NO_3^- and PO_4^{3-}	(Cai et al. 2013; Gupta et al. 2013)
Synthetic wastewater	*Arthrospira* sp,	Hexamethylenediamine-tetrakis	(Gupta et al. 2013)
Swine-wastewater	*Arthrospira maxima*	NH_3–N and total phosphate	(Gupta et al. 2013)

* Nitrogen fixing cyanobacteria

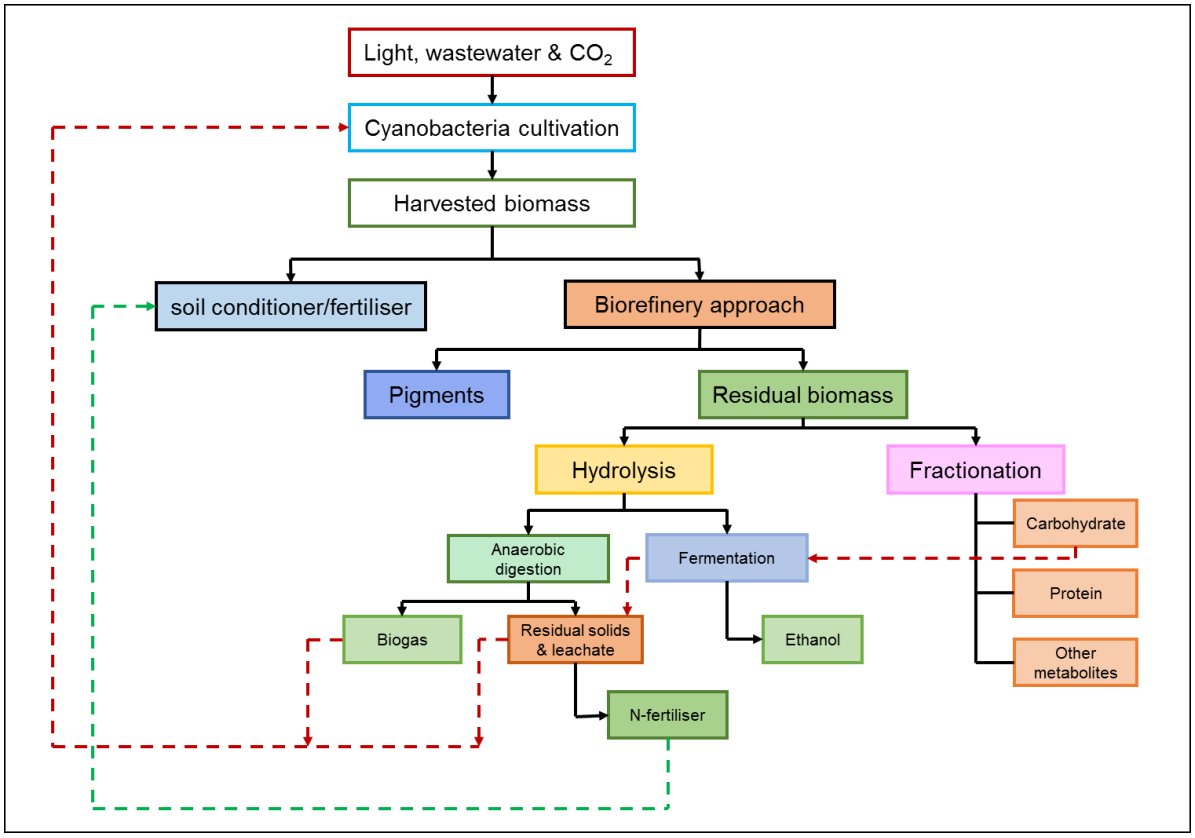


Figure 2.1 Schematic process diagram for a cyanobacterial biorefinery model

2.6.1 Bioenergy from cyanobacteria

N_2-fixing cyanobacteria may play an important role in increasing the yield of biofuel and bioenergy (Heimann and Cires 2015). Lipids and carbohydrates have a high energy content which can be used to produce biodiesel and bioethanol as an alternative for fossil fuels (Feinberg 1984). It has been suggested that hundreds to thousands of gram of dry biomass could be produced in a single growth cycle (Schopf 2000), suitable for several uses including protein production and biofuel applications. Technically, fractional pyrolysis of natural cyanobacteria could be proposed to be an effective method to develop a potential solution for obtaining high quality bio-oil (Li et al. 2014).

An advantage of using cyanobacterial biomass in the bioenergy sector is that they can be cultured cost-effectively; grow well with a limited supply of basic nutrients and have higher photosynthesis levels and growth rates when compared to other microalgae and higher plants. These unique features make them a viable source for bioenergy generation (Rittmann 2008). The natural production of hydrocarbons (alkanes and alkenes) by at least 13 N_2-fixing cyanobacterial genera has also been documented (Coates et al. 2014). Some of the hydrocarbons, for example penta-decane, could be directly transesterified to biodiesel. In addition, the feedstock-agnostic process of hydrothermal liquefaction is opening promising avenues for the use of wet cyanobacterial biomass to produce jet biofuel economically (Heimann and Cires 2015).

2.6.1.1 Bioethanol

Ethanol can be produced from renewable resources. The ethanol can be mixed with existing diesel and used without modification in existing diesel engines (Quintana et al. 2011). At present, agricultural crops, such as sugarcane and corn, are being used to produce bioethanol by fermentation, but ethanol production from agricultural crops is not an alternative to fossil fuel, as it negatively affects food supplies (Quintana et al. 2011). Ethanol production from cyanobacterial biomass, however, is sustainable, as cultivation does not require arable land and hence food supplies are not impacted(Quintana et al. 2011). In addition to the green microalgal species *Chlamydomonas reinhardtii*, several cyanobacterial species, such as *Cyanothece* PCC 7822, *Microcystis aeruginosa* PCC 7806, *Oscillatoria limosa*, *Oscillatoria* sp., and *Spirulina platensis* (now *Arthrospira platensis*) can produce ethanol photosynthetically (Luo et al. 2010).

Presently, large-scale production of bioethanol is achieved by fermentation of agricultural crops, mainly sugarcane in Brazil (Goldemberg 2007).The main carbon-storage product in cyanobacteria is starch-based carbohydrate, which is ideal for the production of bioethanol, as cellulosic complex sugars are absent (Packer 2009; Subhadra and Edwards 2010). It needs to be born in mind though, that, in order to have access to market, bioethanol production from cyanobacterial biomass must meet a sales price comparable to the subsidised bioethanol produced from sugar cane, which has not been demonstrated in large-scale cultivation to date. It is more likely, that a biorefinery approach to biomass fractionation will be required to offset production costs and to subsidise the low market value through production of a high-value co-product.

2.6.1.2 Hydrogen

Hydrogen is the most abundant element on the planet and combustion leads to the formation of water and NO_x. Unlike hydrocarbons, use of hydrogen for energy production yields clean water (Momirlan and Veziroglu 2005). Hydrogen can be separated from water and can be used as a gaseous and carbon-free fuel, thus could play a vital role in future clean energy production (Meher Kotay and Das 2008). Hydrogen has three times the amount of energy (122 kJ g^{-1}) of traditional hydrocarbon fuels, however its production to date is not cost-effective (Balat 2008). Cyanobacteria are being used as cell factories for producing biological hydrogen and production includes processes such as dark-fermentation, photo-fermentation and bio-photolysis (Hosseini et al. 2015). Bio-photolysis is used by cyanobacteria and green algae to produce hydrogen. In this process, water acts as a sole electron donor (Pfromm et al. 2010). It has

several advantages over other photo-electrochemical or thermochemical processes (Levin et al. 2004; Masukawa et al. 2010; Tamagnini et al. 2007), such as its eco-friendly nature, efficiency, renewability and the absence of CO_2 emission during its production and utilisation (Lindblad 1999).

For biological hydrogen production, two natural pathways are used in cyanobacteria. Hydrogen is produced as a by-product during N_2-fixation and it is directly produced by bidirectional hydrogenases (Angermayr et al. 2009). Hydrogenases do not require ATP for hydrogen production, hence the process is considered to be high turnover and efficient. Large-scale production of biological hydrogen is achieved by a variety of processes using various types of bioreactors such as vertical column reactors, tubular types and flat panel photo-bioreactors (Dutta et al. 2005). The diazotrophic cyanobacterium *Cyanothece* sp. ATCC 51142, has the capacity to produce high levels of hydrogen (465 μmol mg^{-1} chlorophyll h^{-1}) under aerobic conditions (Bandyopadhyay et al. 2010). Ananyev et al. (2012) reported that hydrogen is produced by a [NiFe]-hydrogenase in the cyanobacterium *Limnospira maxima* (formerly *Spriulina* and *Arthrospira maxima* (Nowicka-Krawczyk et al. 2019)) during auto-fermentation of photosynthetically accumulated glycogen under dark anaerobic conditions. The production of bio-hydrogen with applications for transport and electricity has been described in cyanobacterial genera such as *Anabaena, Aphanocapsa, Calothrix, Chroococcidiopsis Cyanothece, Gloeobacter, Microcoleus*, *Microcystis, Nostoc, Oscillatoria,* and *Synechococcus* (Lambert and Smith 1977; Masukawa et al. 2001; Sveshnikov et al. 1997), including N_2-fixing

cyanobacteria (Heimann and Cires 2015). *Anabaena cylindrica* synthesised the highest amount of hydrogen, 30 mL H_2 L^{-1} hr^{-1} (Jeffries et al. 1978).

2.6.2 Bioproducts

Cyanobacteria are an excellent source of biologically active compounds that possess antiviral, antibacterial, antifungal and anticancer properties and many of them have been commercialised as given in Table 2.3. These products have various applications in diverse fields such as aquaculture, wastewater treatment, as food additives, fertilisers, and production of secondary metabolites including exopolysaccharides, vitamins, toxins, enzymes and pharmaceutical products. Phycobiliproteins are the most essential products, present in all cyanobacteria. The estimated global phycocyanin market value is US $ 10-50 million annually, with the price ranging between US$ 130 – 5,000 kg^{-1}, depending on the grade (Querques et al. 2015). The value of C-phycocyanin has been reported to range from US$ 500 to 100, 000 kg^{-1}, depending on purity. The purity of C-phycocyanin is generally determined by the absorbance 420/280 nm ratio, i.e. ~ 0.7 is regarded as food-grade, ~ 3.9 as reagent grade and ~ 4.0 as analytical grade (Borowitzka 2013). Earthrise Farms and DIC Corporation, Japan are major producers of phycobiliproteins (Borowitzka 2013) (Table 2.3). β-carotene is also present in almost all cyanobacteria with the global market value of US$ 270 million, with natural β-carotene estimated to hold about 20 – 30% of the market (Borowitzka 2013). Four producers namely, Koor Foods (Nature Beta Technology) in Israel, Western Biotechnology Ltd and Betatene Ltd in Australia, and Nutralite in the USA started the β-carotene production in the 1980s. Two of these companies continue production today

(the Israeli plant is now owned by Nikken Shohonsha Co, and the Australian plants are both now owned by BASF) are major producers of β-carotene (Borowitzka 2013) (Table 2.3). ω-3 and ω-6 fatty acids are being produced from various cyanobacteria such as *Anabaena, Arthrospira*, *Calothrix, Nodularia,* and *Nostoc* (Spolaore et al. 2006). The wholesale market price for algae ω-3 is about US$ 140 kg^{-1}, ranging from US$ 80 – 160 kg^{-1} depending on purity (Borowitzka 2013) (Table 2.3).

Despite these obvious applications, genetic engineering of specific metabolic pathways in cyanobacteria is under continuous development to improve product productivities and lower costs of production. Currently, more than 20 different bioactive compounds, including 40.2% lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides, 9.4% amides and others, are being derived from cyanobacterial strains (Singh et al. 2011).

Cyanobacteria also play a major role in human diets as a supplement. Particular strains of *Anabaena* and *Nostoc* can be used as human food, especially *Nostoc* which is fibre- and protein-rich (Spiller 2001). Furthermore, N_2-fixing cyanobacteria offer an attractive alternative for production of biomass and bioproducts with potentially low cultivation and harvesting costs. The N_2-fixing cyanobacterium *Tolypothrix* sp. NQAIF319 produced 45 – 49 g dry weight m^{-2} day^{-1} (Velu et al. 2015).

2.6.2.1 Pigments – phycocyanin and phycoerythrin

Cyanobacteria are a viable source for producing pigments with various applications in the bio-industry. These pigments include chlorophyll *a*, carotenoids, and phycobiliproteins (Rastogi et al. 2014). Carotenoids especially

β-carotene has the ability not only to be converted into vitamin A but also possesses antioxidant properties and reduces gastric cancer risk by neutralizing DNA-damaging free radicles which are generated by various factors such as chronic *Helicobacter pylori* infection (Larsson et al. 2007; Tatsuta et al. 1999). Phycobiliproteins such as phycocyanin, phycoerythrin and allophycocyanin are used as natural food colourants (candy, ice creams, dairy products and soft drinks). Pigments are being used in drugs and cosmetics, which are high-value commercial products. Phycocyanin from *Phormidium valderianum* (food-grade) is used in the field of diagnostics as a phycofluor, a blue natural colourant (Griffiths et al. 2016). Buffered extracts of phycocyanin can be used in cosmetic products, for example, eye shadow, eyeliner and lipsticks.

C-phycocyanin is one of the photosynthetic pigments with a β-subunit which has various applications in the pharmacological industry, including anti-inflammatory and anticancer activities (Wang et al. 2007). In *in vitro* tests, recombinant C-PC/β inhibited proliferation and induced apoptosis. Glyceraldehyde-3-phosphate dehydrogenase mRNA levels decreased significantly due to the interaction of recombinant protein with membrane-associated β-tubulin and glyceraldehyde-3-phosphate dehydrogenase. These properties revealed that the C-PC/β has potential as a cancer prevention or therapy agent (Singh et al. 2011; Wang et al. 2007). C-phycocyanin has been shown to selectively inhibit cytochrome oxidase-2, with hepatoprotective and anti-inflammatory effects (Reddy et al. 2000).

Table 2.3 Potential applications of cyanobacterial bioproducts and annual global market size and product values

Product	Use	Cyanobacterial producers (some)	Producers	Price US$/kg	References
Phycobiliproteins	Biomedicine (fluorescent markers), Food colouring Pharmaceuticals Cosmetics	Present in all cyanobacteria	Earthrise Farms DIC corporation, Japan	130 - 100,000	(Borowitzka 2013)
β-carotene	Neutraceuticals, Food colorants	Present in all cyanobacteria	Cognis Nutrition and Health, Beta technologies & Betatene Ltd in Australia	-	(Borowitzka 2013)
Fatty acids (ω-3 & ω-6)	Neutraceuticals Animal feed (aquaculture)	*Anabaena* sp., *Aphanizomenon flos-aquae, Calothrix, Nodularia* sp., *Spirulina (*now *Arthrospira)*	Spectra stable Isotopes	80 -140	(Borowitzka 2013)
Bioplastics (PHAs)	Substitute for non-biodegradable petrochemical-based plastics	*Gloethece* sp., *Nostoc muscorum*, *Arthrospira* (*A. platensis*) *Limnospira maxima*, *Synechocystis* sp., Sy*nechococcus* sp., *Trichodesmium thiebautii*	-	-	(Philip et al. 2007)
Metallic nanoparticles	Chemical industry (catalysis) Environmental remediation Biomedicine (gene therapy, biomarkers)	*Plectonema boryanum, Anabaena* sp., *Calothrix* sp., *Leptolyngbya* sp., *Arthrospira platensis, Lyngbya majuscula*	-	-	(Lengke et al. 2006)
Ultraviolet sunscreens	New generation sunscreens (anti-inflammatory, anti-proliferative) and cosmetic applications	*Anabaena*, *Aphanizomenon*, *Aphanothece*, *Calothrix, Synechococcus*, *Chlorogloeopsis*, *Gloecapsa, Gloethece, Lyngbya, Microcoleus, Microcystis*	-	-	(Griffiths et al. 2016)
Secondary metabolites & toxins	Anti-fungal, anti-tumor, anti-viral & anti-HIV drugs	*Stigonematales*, *Nostoc*, *Oscillatoria, Lyngbya lagerheimii*	-	-	(Griffiths et al. 2016)

2.6.2.2 Biofertilisers

Crop productivity relies on sufficient amounts of the soil macro-nutrients N, P, K, Ca, Mg, and S and micro-nutrients including B, Cu, Fe, Mn, Mo, and Zn. The biomass of cyanobacteria, particularly of N_2-fixing species, contains relevant amounts of those elements and thus may be suitable as biofertiliser to replace energy-consuming inorganic fertilisers.

N_2-fixing cyanobacteria are naturally present in paddy -, sugarcane - and maize fields in various parts of the world, including free-living and symbiotic cyanobacteria such as *Anabaena, Aulosira, Nostoc* and *Tolypothrix* (Silva and Silva 2007). N_2-fixation rates of up to 1.6 to 2 kg N ha^{-1} d^{-1} and 140 - 312 kg ha^{-1} $year^{-1}$ have been observed (Brocke et al. 2018). Whitton (2000) documented that cyanobacterial abundances strongly correlated with pH and P concentration. Besides providing nitrogen to the soil, N_2-fixing cyanobacteria improve soil structure due the effect of the EPS. Certain species also contain phytohormones such as indole-3-acetic acid and gibberellin-like compounds, which lead to increased grain yields and reduced NaCl stress in rice (Grewe and Pulz 2012). It is thus not surprising that cyanobacteria are extensively used as natural biofertilisers for rice cultivation in Asian countries (Pereira et al. 2009). In South America, a combination of *Tolypothrix tenuis* with *Nostoc* sp. have been used for the production of powdered cyanobacterial biofertiliser in Argentina (Silva and Silva 2013).These studies took advantage of the ability of certain N_2-fixing cyanobacteria to re-grow and inoculate soils after being subjected to gentle drying. As an alternative to the direct inoculation with viable cyanobacteria, the biomass can be pyrolised to biochar at 450°C for 2 h under

low oxygen concentrations then added to the soil as biofertilisers and soil amendments (Castine et al. 2013). Experiments with microalgal mixtures (Table 2.4) showed that biochar contains lower carbon and nitrogen amounts than non-pyrolysed biomass, but similar proportions of other macro- (P, K, Ca, Mg) and micro-nutrients (B, Cu, Fe, Mn, Mo, Zn) (Castine et al. 2013). The biochar generated after biofuel production by hydrothermal carbonisation (HTC) and hydrothermal liquefaction (HTL) of cyanobacterial biomass appears to retain sufficient amounts of N and micro-nutrients for use as a soil amendment (Levine et al. 2013; Roberts et al. 2013b). It needs to be considered though that the exact chemical composition is feedstock-dependent and is still being investigated in depth to design combined biofuel-biofertiliser production pathways (Levine et al. 2013; Roberts et al. 2013b).

Table 2.4 Chemical composition of N_2-fixing cyanobacteria and microalgae relevant to their use as plant biofertilisers

Parameter	N_2-fixing cyanobacteria	Microalgal biomass	
		Pre-pyrolysis	Post-pyrolysis
Carbon (%)	37 - 48	25 - 30	14 - 22
C: N ratio	4 – 7	5 – 6	6 – 7
Macro-nutrients			
N (%)	7 – 11	5	2 -4
P (%)	0.4 – 1.2	0.8 – 0.9	1
Others (K, Ca, Mg, S) (%)	0.2 – 2.6	0.8 – 1.3	0.7 – 2.0
Micro-nutrients (Cu, Fe, Mn, Zn) (%)	< 0.01 – 0.2	ND – 0.09	ND – 0.1

Sources: (Castine et al. 2013; Cires et al. 2013; Heimann and Cirés 2015; Roger et al. 1986; Sigee et al. 1999).

2.6.2.3 Bioplastics

Cyanobacteria have the capacity to store many materials like glycogen, sulphur, polyamino acids, polyphosphate, and lipid. Especially, lipoidic material, such as polyhydroxyalkonates (PHA) are produced and stored under carbon-enriched conditions (Anderson and Dawes 1990). PHA is a crystalline biodegradable thermoplastic similar to polypropylene. It has gained a lot of attraction, as it is a promising alternative for non-biodegradable petrochemical-based plastics and biodegradation of PHA yields water and CO_2 (Philip et al. 2007). Another advantage is the use of PHA in the field of biomedical and biopharmaceuticals. (Sudesh 2004; Williams et al. 1999). These biodegradable PHAs are widely used as bone plates, osteosynthetic materials and surgical sutures. PHA fibres are used to make swabs and dressing materials for surgery (Philip et al. 2007). The most common type of PHA generated by cyanobacteria is polyhydroxybutyrate (PHB) (Griffiths et al. 2016), a polymer used in surgeries for internal sutures, which require degradation with time into harmless constituents (Sudesh 2004; Williams et al. 1999).

2.6.2.4 UV-Protectant

Excessive UV-radiation has been linked to skin cancer (Stein et al. 1989). Cyanobacteria counteract the DNA-damaging UV effects using three different mechanisms of protection: (i) stress avoidance by gliding mechanisms, (ii) stress defence by synthesis of UV-absorbing compounds such as mycosporine-like amino acids (MAAs) and scytonemin, antioxidants and extracellular polysaccharides, and (iii) repair mechanisms including DNA repair and resynthesis of UV-sensitive proteins (Ehling-Schulz and Scherer 1999;

Fleming and Castenholz 2007). Certain species of cyanobacteria such as *Anabaena, Aphanizomenon, Aphanothece, Calothrix, Chlorogloeopsis, Gloecapsa, Gloethece, Lyngbya, Microcoleus, Microsystis, Synechococcus* and *Scytonema* are excellent sources of UV-protectants. They are used in pharmaceuticals as anti-inflammatory and anti-proliferative medicines and in cosmetics.

2.6.2.5 Anti-cancerous and other bioactive molecules

Lyngbya majuscula (Burja et al. 2001) is a well-known cyanobacterium for the production of chemicals such as nitrogen-containing compounds, polypeptides, lipopeptides, cyclic peptides and many other compounds (Shimizu 1993). These compounds act as protein kinase C activators and tumour promoters, inhibitors of microtubule assembly, antimicrobial and antifungal compounds and sodium-channel blockers (Bloor and England 1991). Some cyanobacterial species have broader applications, for example, *Phormidium* sp. has useful antibacterial and antifungal activities (Bloor and England 1991). Members of some orders such as *Stigonematales, Nostocales* and *Oscillatoriales* synthesise toxins such as fisherellin A, hapalindole, carazostatin, phytoalexin, tolytoxin, scytophycin, toyocamycin, tjipanazole, nostocyclamide and nostodione, which possess antifungal activity (Dahms et al. 2006). Cyanobacteria such as *Oscillatoria raoi* aid in the production of medicinally important bioactive compounds that exhibit antiviral or anti-HIV activity (Reshef et al. 1997). For example spirulan from *Arthrospira platensis* (Hayashi et al. 1996), *Lyngbya lagerhaimanii* and *Phormidium tenue* (Jha and Zi-rong 2004) has antiviral activity. *Arthrospira platensis* and *Spriulina*/ *Arthrospira* sp. are rich

in γ-linolenic acid (GLA), which has been implemented in lowering blood pressure and in lipid metabolism (Singer et al. 1986).

2.6.2.6 Metal nanoparticles

Cyanobacteria are also capable of synthesising nanoparticles, which have potentially significant applications in chemical industries, bioremediation and in biomedicine as biomarkers and in gene therapy. Six strains of cyanobacteria, *Plectonema sp., Anabaena sp., Calothrix sp., Leptolyngbya sp., Arthrospira platensis* and *Lyngbya majuscule* are able to generate gold-nano particles (Lengke et al. 2006). Gold-nanoparticles have several applications in electronics, catalysis in biology, pharmaceutical and medical diagnosis and therapy. The gold-nanoparticles are being utilised to enable the specific interactions between anticancer drugs and DNA (Sadowski 2010). Additionally, these gold-nanoparticles are also used in the biorecovery of gold from gold(III)-chloride solutions and this application can also be applied in mining (Lengke et al. 2006).

2.7 Challenges

Although cyanobacteria are potentially useful in bioremediation and bioproduct synthesis, several applications have not been fully explored on a commercial scale (Heimann and Cires 2015). There are many challenges of biomechanical, ecological and economic nature, that are yet to be addressed, in order to find an effective means for exploiting their full potential.

The major consideration in cyanobacterial biomass production is cultivation and extraction. In terms of biomass production, light is a critical

parameter. Too much light may cause photoinhibition, while too little results in light-limitation, yet light distribution inside photobioreactors, e.g. the suspension mechanism-induced periodic light/dark circulation of cells and filaments remains poorly understood. This has been one of the major biotechnical challenge in designing bioreactors (Kumar et al. 2011). Not all species/strains of cyanobacteria can be cultivated in open ponds (Jiménez et al. 2003b), with less competitive species requiring cultivation in closed type bioreactors, to reduce contamination by other microorganisms, the design of which is a challenge (Chisti 2007).

The energy demand for harvesting of cyanobacterial biomass can also be very high, if it requires centrifugation, while sedimentation or filtration are more cost-effective but time efficiency is a potential issue (Harun et al. 2010). Additionally, the energy-intensive complete dewatering and drying requirement in harvesting would be a considerable constraint. Flocculation is a low energy technique, but it has to date not been successful applied, because operation specifics are strongly influenced by strain behaviour (Vandamme et al. 2013). Another challenge is the extraction of lipids from cell walls, which requires advanced techniques and special equipment, meaning the process is highly cost-intensive (Hejazi and Wijffels 2004).

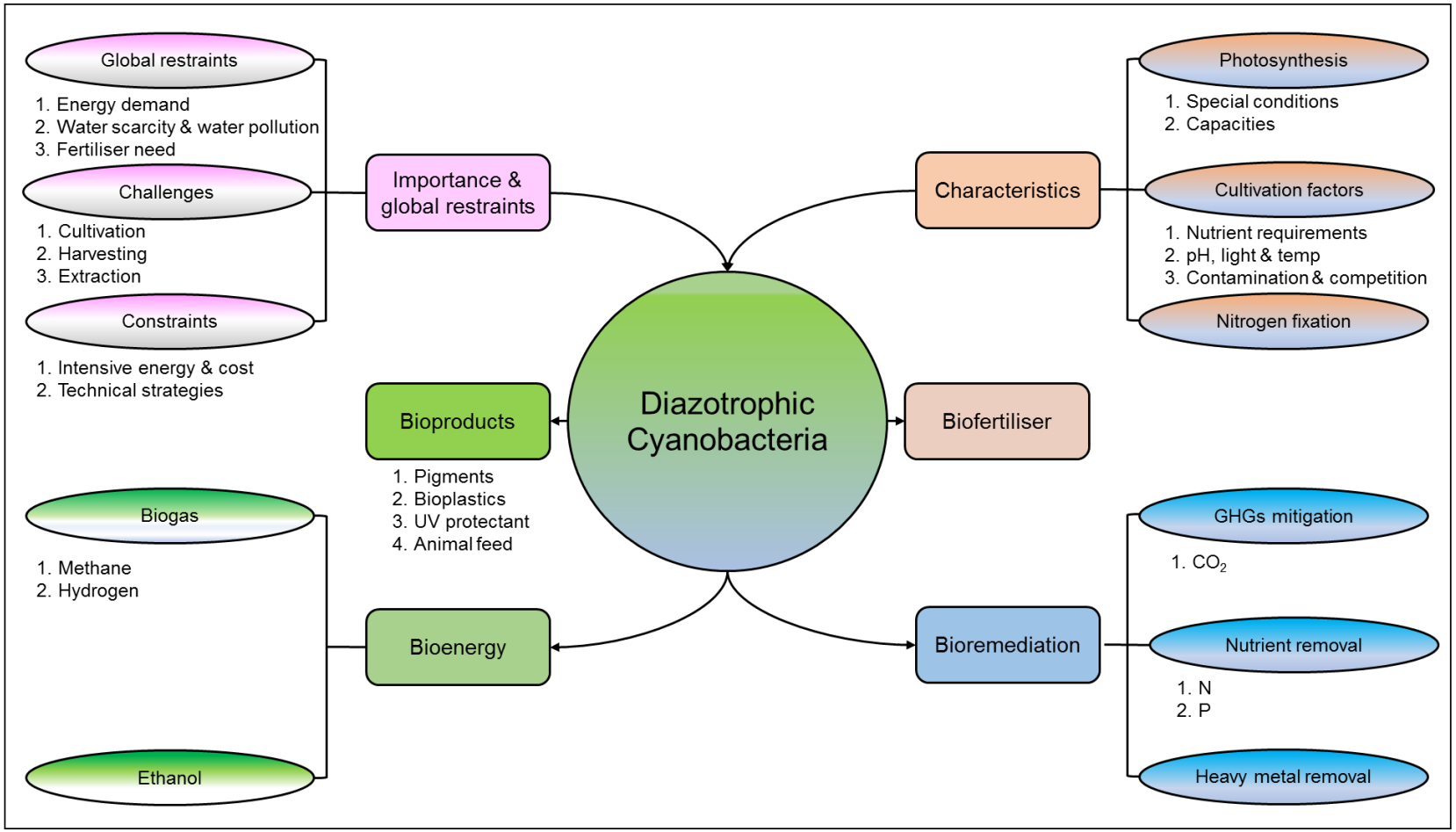
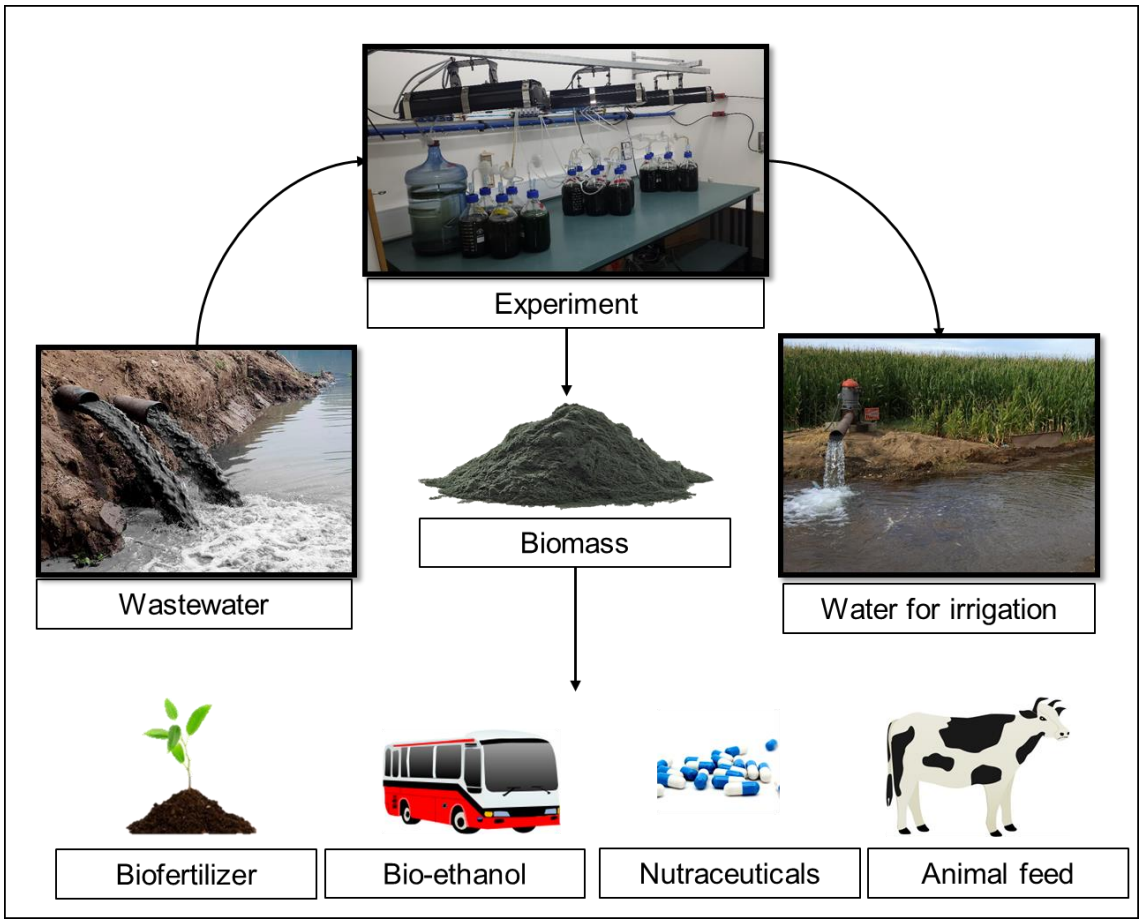


Figure 2.2 Summary diagram for cyanobacteria implementation and achievement

2.8 Conclusion

The prospect of using cyanobacteria for coupled bioremediation, biomass production and bioproduct synthesis to overcome global restraints to economic growth and development is theoretically viable. Several applications can be theoretically combined to provide a product pipeline, yet some require application of unprocessed biomass, which from a cost-benefit perspective are the least preferred options (Fig. 2.2). Strain-specific biomass production criteria must, however, be optimised, as well as overcoming growth system related challenges and current constraints, to realise the commercialisation of biofuel from cyanobacteria (Fig. 2.2). A holistic approach is needed to explore which species could be effectively applied for the coupling of biomass production with phytoremediation and in particular bioenergy generation. Although cyanobacteria are capable of remediating different wastewater and metal solutions, no single strain can optimally reduce and assimilate all compounds contained in wastewater. Nonetheless, recent studies suggest that cyanobacterial consortia may show a bioremediation capacity superior to that of single-species cultures, both for the removal of nutrients and metals, which should additionally benefit bioproduct synthesis potential, bringing us one step closer to a circular economy.

Chapter 3: Bioremediation and bio-product potential of native filamentous cyanobacteria[3]



[3] This chapter is in preparation to submit to Algal Research

3.1 Abstract

Cyanobacteria produce bioactive compounds and pigments with various applications in food, pharma- and nutraceuticals industry sectors. Cyanobacteria are photosynthetic Gram-negative prokaryotic microorganisms and some can fix atmospheric nitrogen, reducing nitrogen fertilisation cost for cultivation, while some are self-flocculating, reducing dewatering cost. Additionally, the high density of negative charges of the extracellular polymeric substances on the surface of the cell wall make them ideal candidates for metal remediation. In this study, the nitrogen-fixing cyanobacterium *Tolypothrix* sp. and the non-nitrogen fixing cyanobacterium *Limnothrix* sp. and the consortium of these two species were grown in simulated ash dam wastewater, simulated urban wastewater, cyanobacterial culture medium BG11 with and without nitrogen enrichment. *Tolypothrix* sp. achieved ~36 and 42% higher biomass growth than *Limnothrix* sp. and the consortium. Cumulative metal removal from SADW medium was ≥ 90% for Al, Fe and Se followed by As (81%), V (79%), Ni (44%) and Sr (38%) by *Tolypothrix* sp. whereas it was ≥ 90% for Al and Fe followed by ~52% for Ni and Sr, As (37%), Se (31%) and V (26%) by *Limnothrix* sp.. Cumulative metal removal was slightly lower for the consortium; Fe (99%), ≥80% for Al and Se, followed by Ni (67%), Sr (40%), As (31%) and V (26%). *Tolypothrix* sp. had a high carbohydrate content (40-48%), while *Limnothrix* sp. was rich in protein (~42%). The phycocyanin content was 2-fold higher in *Limnothrix* sp. (224 mg g^{-1} DW) than in *Tolypothrix* sp. (117 mg g^{-1} DW) when cultivated in SADW, suggesting an effect of metal on PC synthesis. Carbon and nitrogen contents were ~ 42-44% and ~6.5% in *Tolypothrix* sp. biomass while

~46% and ~11% in *Limnothrix* sp. biomass. The high carbohydrate content of *Tolypothrix* sp. biomass renders it suitable for bioethanol production, while the high protein content of *Limnothrix* biomass makes it ideal for animal feed production, in addition to C-PC product potential for both species. In the context of bioremediation of metals from macro-nutrient poor ash dam wastewater, however, based on biomass growth, nitrogen fixation and self-flocculation, cultivation of *Tolypothrix* sp. has distinctive advantages over *Limnothrix* sp. for bioremediation and bio-product synthesis.

3.2 Introduction

Biomass is a primary resource to develop a circular, sustainable and smart economy. Biomass can be categorised into four major groups: (1) forest biomass (e.g. wood), (2) waste biomass (e.g. urban organic waste), (3) energy crop biomass (e.g. oil seed crops) and (4) aquatic biomass (e.g. micro- and macroalgae, cyanobacteria, aquatic plants (e.g. *Lemna* (duck weed)) (Starikov et al. 2016). Global biomass demand is expected to double by 2030 (108 exajoules (EJ)) compared to in 2010 (53 EJ) (IRENA 2014), requiring freshwater and energy for production, the latter contributing to climate change via CO_2 emissions, as energy is primarily derived from fossil fuels (Global Energy Trend 2018; Karthikeyan et al. 2017). Together, these factors create a water-energy-biomass nexus. Therefore, water-wise and close to carbon-neutral production of energy are the key priorities in the 21st Century. Developing an integrated bio-refinery concept with the aim of (a) re-use of carbon, nitrogen and other macro- and micro-nutrients from wastewater; (b) production of high-value bio-products or bio-fuels from biomass; (c) reduction of

greenhouse gas emissions; and/or (d) removal of toxic pollutants or metals, are considered a reliable approach to produce biomass and value-add through bio-product generation (Garcia-Nunez et al. 2016).

Cyanobacteria are robust, aquatic, oxygenic, photosynthetic organisms, exhibiting significant growth and have high- and low-value bio-product potential. Like plants and micro- and macroalgae, all cyanobacteria fix CO_2 and the biomass can yield high-value products, such as pigments, vitamins and enzymes, as well as medium and low-value products, such as biofuel precursor molecules, bioplastics and biofertilisers (Priyadarshani and Rath 2012; Singh et al. 2011). Some cyanobacteria can fix atmospheric nitrogen (N_2), making their cultivation independent of nitrogen fertilisation and some can grow in extreme conditions such as high temperature (45-70^{o}C), a wide range of pH (4-10) and salinity levels (freshwater to marine) (Rosgaard et al. 2012; Singh et al. 2016). Some species, such as *Nostoc* and *Anabaena* are already being used as biofertilisers, improving soil nitrogen levels and producing plant growth promoting hormones such as auxin, while gibberellins are produced by *Cylindrospermum* and cytokinins by *Anabaena*, plant pathogen-inhibiting toxins by *Calothrix* and *Fischerella*, and some genera have been explored for their ability to absorb toxic metals from industrial wastewaters (e.g. *Nostoc*) (Singh et al. 2016). *Calothrix*, *Lyngbya*, *Microcystis*, *Oscillatoria* and *Scytonema* have been shown to accumulate linoleic acid, an essential polyunsaturated fatty acid (Sharathchandra and Rajashekhar 2011). A major drawback associated with the cultivation of microalgae and cyanobacteria is freshwater demand, e.g. 3,726 kg of water are required to produce 1 kg microalgae based biodiesel

(Yang et al. 2011), while bacterial contamination can also be prevalent, if selected species cannot be grown under relatively harsh cultivation conditions (Rosgaard et al. 2012).

Provision of industrial wastewater/urban wastewater for cultivation can reduce freshwater usage, but wastewater characteristics could adversely impact biomass growth and/ or associated bio-product suitability (Delrue et al. 2016; Menger-Krug et al. 2012). A ~10-fold reduction of urban wastewater toxicity effects was achieved with *Leptolyngbya* sp., in addition to removal of trace metals and nitrogen and phosphate (Singh et al. 2016). Similarly, *Oscillatoria* sp. and *Anabaena* sp. appear to be hyper-accumulators of pollutants from mine-wastewater (Bwapwa et al. 2017). An efficient mat-cultivation system of co-existing bacteria and cyanobacteria has been developed for efficient treatment of acid mine-wastewater (Sheoran and Bhandari 2005), and bacterial/cyanobacteria co-cultivation appears to be generally suitable for wastewater treatments (Karaca et al. 2018; Pires et al. 2013). Therefore, cyanobacterial biomass production (with or without contaminants) will be a feasible and reliable approach. The selection of suitable cyanobacteria is, however, not straight forward, as biomass and bio-product need to be taken into account simultaneously with evaluating adaptability to the type of wastewater to be used for production. As a consequence, a three step selection approach for screening for robust species for commercialisation has been proposed: (i) efficiency and ability to grow in urban wastewater; (ii) ability to treat specific wastewater; and (iii) ability to treat/remove specific pollutants (Delrue et al. 2016). This approach, however, does not consider any limitation of biomass use

imparted by wastewater-derived accumulation of contaminants for specific product development.

Even though a number of studies applied N_2-fixing cyanobacteria for wastewater treatment and bio-product development, only very few compared biomass growth and productivities for single species and co-cultivated non-nitrogen and nitrogen-fixing species in different types of wastewater. The present study aimed to systematically characterise remediation efficiency of and adaptability to simulated urban wastewater and simulated ash- dam water of a coal-fired power station of *Tolypothrix* sp. NQAIF319 (N_2-fixer), *Limnothrix* sp. NQAIF306 (non-N_2 fixer) and the consortium in the context of biomass production and bio-product yields.

3.3 Methods

3.3.1 Culture collection and strain characterisation

The filamentous cyanobacteria *Limnothrix* sp. NQAIF306 and *Tolypothrix* sp. NQAIF319 were isolated in 2012 from a seasonal creek in Townsville (19.3264° S, 146.7573° E; Queensland, tropical Australia). Stock cultures were maintained at the North Queensland Algal Identification and Culturing Facility (NQAIF, James Cook University, Townsville, Australia) as aerated suspension batch cultures in BG11 medium for NQAIF306 and nitrogen-free BG11(-N) medium for NQAIF319 (Rippka et al. 1979) at 28 °C under a 12:12 light/dark photoperiod at a photon flux density of 30 µmol photons m^{-2} s^{-1}. Cyanobacterial cultures were subcultured every 28 days.

3.3.2 Culture media and synthetic wastewater preparation

Two standard cyanobacterial culture media, nitrogen-containing BG11 and nitrogen free BG11(-N), as well as two modified simulated wastewaters, simulated ash-dam wastewater (SADW) and simulated urban wastewater (SUWW) were used in this experiment. The detailed composition of these four media is given in Appendix Table A.1. SADW was prepared in BG(-N) supplemented with salts to simulate the concentrations of the main metals B, Zn, Mo, Fe, Sr, V, Al, Se, As and Ni present in ash-dam water at Tarong Power Plant (26.7809° S, 151.9125° E), Queensland, Australia based on (Saunders et al. 2012). SUWW was prepared by supplementing BG11 medium with NH_4^+ and organic carbon sources (glucose and peptone) to simulate the average composition of urban wastewaters (Johnson and Admassu 2013).

3.3.3 Experimental setup, growth and biomass productivities

Three independent cultures (n=3) were set up by inoculating 1 g L^{-1} fresh biomass of NQAIF306, NQAIF319 and a 1:1 consortium of NQAIF306 and NQAIF319 into 2 L aerated bottles with working volume of 1.8 L containing the four-different media SADW, BG11 (-N), SUWW and BG11. BG11 and BG11(-N) were used as controls. The experiment was carried out for 19 days at 28 °C at a photon flux density of 100 µmol photons m^{-2} s^{-1}. Culture bottles were aerated with 0.1 L min^{-1} of air. The biomass was harvested on day 19 using centrifugation at 8000×g, for 20 min (Beckman, Avanti® J-26XP, Australia), the biomass pellets were freeze-dried (Dynavac freeze dryer model Fd12, Australia) and stored in darkness at −80 °C (Sanyo ultra-low temperature freezer (MDF-U33V), Japan) until analysis for the contents (mg g^{-1} dry weight) of pigments

(phycocyanin and phycoerythrin), fatty acids (fatty acid methyl esters, FAMEs), total lipids, alkane/alkenes, protein, carbohydrate and carbon-hydrogen-nitrogen-sulphur (CHNS) following the analytical procedures detailed below (Section 3.3.6). Biomass productivity (g L^{-1} day^{-1}) was calculated from dry weight (g DW L^{-1}) determined by gravimetric analysis (von Alvensleben et al. 2013) of 40 mL culture samples taken on days 0, 3, 7, 11,15 and 19. Biomass specific growth rate (μ) , doubling rate (k) and doubling time (T2) were calculated based on (Gour et al. 2014)

3.3.4 Nutrient Analysis

Nitrate (NO_3^-), nitrite (NO_2^-) and phosphate (PO_4^{3-}) concentrations in the culture media were determined on days 0, 3, 7, 11, 15 and 19 for nitrogen-enriched media while PO_4^{3-} only was analysed for nitrogen-free media on the respective days. The supernatant remaining after dry weight analysis was used. 15 mL of supernatant was filtered using a 0.22 μM syringe filter (hydrophilic PTFE, MicroAnalytix Pty Ltd, Australia) to remove any suspended particulate material. From this, 1.25 mL was used for nitrate (NO_3^-), 1.25 mL for nitrite (NO_2^-) and 200 μL for phosphate (PO_4^{3-}) determination. Deionised water (Elix® 5, Millipore, Australia) was used to dilute the samples, when PO_4^{3-} concentrations exceeded 2.5 mg L^{-1} to ensure accuracy of the assay. Nitrate and nitrite were determined spectrophotometrically (PerkinElmer EnSpire® Multimode plate reader, USA) in triplicate using a method adapted from (Carvalho et al. 1998) to suit a 96-well plate (Ultraviolet start®, Greiner Bio-One GmbH) format. Nitrite (λ = 540 nm) and nitrate+nitrite (λ = 220 nm) concentrations were calculated by comparison of absorbances with serial

dilution linear regression calibration curves (R^2>0.99), generated previously from known nitrate and nitrite concentrations (0-32.21 and 0-43.38 µM sodium nitrate and sodium nitrite, respectively). Nitrate concentrations were calculated by subtracting the nitrite concentration from the total nitrate+nitrite concentration. Similarly, phosphate was determined spectrophotometrically (λ = 610 nm) in triplicate following standard methods adapted from (van Veldhoven and Mannaerts 1987) and R&D Systems, Malachite Green Phosphate Detection Kit (Cat. Number: DY996, www.RnDSystems.com) to suit a 96-well plate format. Phosphate concentrations were then calculated from serial dilution linear regression calibration curves (R^2>0.99), generated previously from known phosphate concentrations (0-50 µM).

3.3.5 Metal analysis

SADW samples of 0.22 µm-filtered (hydrophilic PTFE, MicroAnalytix Pty Ltd, Australia) culture supernatants were collected on days 0, 3, 9, 11and 19. Collected samples were analysed on a Varian 820-MS inductively coupled plasma mass spectrometer (Melbourne, Australia) for Al, As, Fe, Ni, Se, Sr and V at the Advanced Analytical Centre (AAC), James Cook University (JCU). Indium acted as the internal standard to correct for the matrix effects and instrument drift, and for quantification, a series of multi-element standard solutions were used to calibrate the instrument (Taylor et al. 1998). An independent multi-element standard was used as the quality control, the accuracy achieved is typically within 5%.

3.3.6 Biochemical analysis

Freeze-dried biomass of NQAIF306, NQAIF319 and the consortium (NQAIF306 and NQAIF319) was analysed for total lipid, carbohydrate, protein, FAME and pigment (phycocyanin and phycoerythrin) contents and by analysis of carbon, hydrogen, nitrogen and sulphur.

3.3.6.1 Total lipid content

Total lipid content was determined gravimetrically following a direct transesterification procedure adapted from (Lewis et al. 2000) and modified following (Rodríguez-Ruiz et al. 1998) and (Cohen et al. 1988). Briefly, 30 ± 0.1 mg of freeze-dried biomass was weighed into 8 mL glass vials and 2 mL of freshly prepared methylation reagent (HPLC-grade methanol: acetyl chloride, 95:5 (v/v)) and 1 mL hexane were added. To achieve phase separation, after heating (100˚C, 60 min), 1 mL MilliQ purified water was added to the samples and samples were centrifuged at 1,800 g for 5 min at 4˚C (Eppendorf® 5810R, VWR, USA). The hexane layer was collected, and the pellet was suspended with 1 mL hexane and centrifuged twice as above between washes to extract all lipids into the organic phase. The combined 3 mL hexane extract was collected in a pre-weighed glass vials and dried through evaporation under a gentle stream of nitrogen gas and weighed to determine total lipids (mg).

3.3.6.2 Carbohydrates

Total carbohydrate content was determined using the phenol-sulphuric acid method (DuBois 1956). Prior to analysis, lyophilised algal samples were lysed in MilliQ-purified water with a bullet Blender bead beater (ZrO_2 beads, 0.5

mm diameter) (Next Advance, Lomb Scientific Pty Ltd, New South Wales, Australia) to collect homogenous sub-sample for extraction.

3.3.6.3 Proteins

Total protein content was determined using the Lowry method based on González López et al. (2009) using a kit (Sigma, total protein kit, Micro Lowry, Peterson's Modification, TPO300, Sydney, Australia). Briefly, 20 mg freeze-dried biomass was weighed into microfuge tubes (Eppendorf®, New South Wales, Australia) and 0.9 mL lysis buffer and 0.1 mL SDS were added. Then, the samples were bead beaten with a Bullet Blender bead beater (ZrO_2 beads, 0.5 mm diameter) and centrifuged at 1,800 g for 10 min at 4˚C. 1 mL of sample supernatant was transferred to fresh 15 mL Falcon tubes (Eppendorf®, New South Wales, Australia) and 1 mL Lowry reagent was added and incubated for 20 min at room temperature and contents were immediately and rapidly mixed. 0.5 mL Folin and Ciocalteu's phenol working solution was added to each tube and incubated for 30 min. 250 µL of the sample reaction mixture was transferred to a 96-well micro-titre plate and read at λ = 750 nm. The spectrophotometric absorbances were converted to protein concentration using a calibration curve performed with bovine serum albumine (BSA) as standard (R^2=0.99). Precent protein content was determined by equation 3.1. (eq. 3.1)

$$Protein\ (\%, w/w)\ = \frac{CVD}{M} x100 \text{ (eq….3.1)}$$

Where,

C : Concentration of protein (µg L^{-1})

V : Volume (L) of lysis buffer

D : Dilution factor

M : The amount of biomass (mg)

3.3.6.4 Fatty acid extraction, transesterification and analysis

Fatty acid methyl ester (FAME) were analysed at the Sustainable Coastal Ecosystems and Industries in Tropical Australia, Australian Institute of Marine Science, (AIMS, Townsville, Australia). FAME analyses were performed as detailed in (von Alvensleben et al. 2013). Briefly, 30 ± 0.1 mg freeze-dried biomass was weighed into Teflon-capped 8 mL glass vials. 2 mL freshly prepared methylation reagent (methanol: acetyl chloride, 95:5, (v/v)) and 300 µL internal standard (nonadecanoic acid (C19:0), cat # 72332-1G-F, Sigma Aldrich, Sydney, Australia) 0.2 mg L^{-1} in methanol were added to the sample. Then, samples were heated at 100°C for 1 h and allowed to cool before adding 1 mL hexane. Samples were heated at 100°C for 1 min to form a single methanol/hexane phase. To ensure phase separation, 1 mL de-ionised water (MilliQ, Millipore) was added to the cooled samples and the hexane layer containing FAMEs was collected and filtered through a 0.22 µm PTFE syringe filter into FAME vials (Mastelf Solutions, China). 1 mL Butylated-hydroxy-toluene (BHT) (0.01%: 6.6 mg BHT to 100 mL HPLC grade hexane) was added as an antioxidant during the extraction.

FAME profiles were analysed using gas chromatography (GC) (Agilent 7890B GC-Agilent 5975C) equipped with a DB-23 capillary column (60m x 0.25mm x 0.15 µm) and flame ionisation detector (FID). Split injection mode was used at 1/50 split ratio, temperature was 250°C and FID inlet temperature was 270°C. High purity nitrogen gas was used as carrier gas. Fatty acid quantities were determined against calibration curves of external standards (C8-C24, Sigma Aldrich) and were corrected for recovery of the internal standard

nonadecanoic acid (C19:0), added prior to transesterification. Total fatty acid content (mg g^{-1} DW) was determined as the sum of all FAMEs. Alkanes and alkenes were analysed using an Agilent GC-MSD system (6890/5973N) in scan/SIM mode (m/z 50-500 scan; m/z 57, 71, 83, 85, 97 SIM) with a Restek Rxi-5Sil MS fused silica column (30m x 0.25mm x 0.25µm) and ultra-high purity helium as the carrier gas (1 mL min^{-1} constant flow). Samples (1 µL) were injected into the inlet in pulsed split-less mode (280°C; pulse pressure 25 psi for 2 min) and the column temperature was programmed to initially hold at 50°C for 1 min, increase from 50 °C to 110 °C at 5 °C min^{-1}, then 100 °C to 310 °C at 30 °C min^{-1}. Alkanes/alkenes were quantified using TIC (SIM) peak area data against calibration curves of external standards (C8-C40 even carbon number alkane mixture (Novachem Pty Ltd, South Yarra, Australia)) and docosene (C22:1), as the internal standard. Alkanes/alkene concentrations were corrected for percent recovery of the internal standard (C22:1).

3.3.6.5 Pigment analysis

Phycocyanin and phycoerythrin contents were determined spectrophotometrically (SpectraMax® M2, USA) according to (Lawrenz et al. 2011). Briefly, 10 mg freeze-dried biomass was dissolved in 5 mL phosphate buffer (pH = 6.0; 0.2 M K_2HPO_4 and 0.2 KH_2PO_4) and sonicated using a rod sonicator (brief 5 s, 8-W pulses over 30s) (Misonix, XL-2000, New York, United States). The extracts were kept at 5°C for 48 h. The extract was centrifuged (Eppendorf® 5810R, VWR, USA) at 10,870 g for 5 min and the recovered supernatant was read at λ = 545 nm for phycoerythrin, 620 nm for phycocyanin and 750 nm for turbidity.

Phycobiliprotein concentrations were determined as per equation 3.2 (eq…3.2).

$$C = (A/\varepsilon d)x(MW)x(V_{buffer})x(V_{sample})x10^6 \quad \text{(eq…. 3.2)}$$

Where,

C : Phycobilin concentration (μgL^{-1})

A : Absorbance at 650 or 545 nm minus absorbance at 750 nm

$\boldsymbol{\varepsilon}$: Molar extinction coefficient (phycocyanin,1.9 x 10^6 L mol^{-1} cm^{-1} phycoerythrin, 2.41 x 10^6 L mol^{-1} cm^{-1}

d : Path length of the cuvette (1 Cm)

MW : Molecular weight of the phycobilins (phycocyanin: 264,000 g mol^{-1}; phycoerythrin: 240,000 g mol^{-1})

V_{sample} : Volume of the sample

V_{buffer} : Volume of phosphate buffer

3.3.6.6 Elemental analysis

Carbon-hydrogen-nitrogen-sulphur contents (CHN) (w/w, %) of the samples was determined by OEA Labs Ltd. Callington, Cornwall (UK) using an EA-1110 elemental analyser (CE Instruments Ltd, Italy) set up in CHNS mode.

3.3.7 Statistical analysis

Statistical significance of experimental results was evaluated by two-way ANOVA or one-way ANOVA (SADW elemental analyses), with a significance level (α) of 5%, using Statistica v13.2. Normality was assessed using P-P plots and homogeneity of variances were assessed using the Cochran-Bartlett test. Data were log-transformed, if ANOVA assumptions of homogeneity of variance or normality were not met. Tukey HSD tests were used for two-way ANOVAs to

determine the factor driving significance. Grubb's outlier test with 0.01 significance level was calculated for all the test results for comparison.

3.4 Results

3.4.1 Effect of media composition on growth of *Tolypothrix* sp. NQAIF319, *Limnothrix* sp. NQAIF306, and Consortium

Over a 19-day time course, *Tolypothrix* sp. achieved the highest dry weight biomass (DW) yield of 3.54 g DW L^{-1} grown in BG11, followed by cultivation in SADW, BG11(-N), with 3.18 and 3.29 g DW L^{-1}, respectively (Fig 3.1a). In contrast, ~36 and 42% lower biomass yields were obtained for *Limnothrix* sp. and the consortium (Figs. 1b and c, respectively), but all cultures showed highest growth in BG11 medium, while no growth was achieved in SUWW. The latter could be due to high-concentrations of nitrate and bacterial overgrowth due to enrichment with organic carbon sources (Ernst et al. 2005; Flores et al. 2005; Ohashi et al. 2011).

Achieved biomass productivities were statistically significantly different for species and highest for *Tolypothrix* sp. (0.139, 0.127 and 0.119 g L^{-1} d^{-1} grown in BG11, SADW and BG11(-N), respectively), followed by *Limnothrix* sp. (0.075 and 0.073 g L^{-1} d^{-1} grown in BG11 and SADW+N, respectively) and were lowest for the consortium (0.059, 0.050 and 0.040 g L^{-1} d^{-1} grown in BG11, SADW and BG11(-N), respectively (Fig. 3.2, $p < 0.0001$). This was also reflected in specific growth rate (μ1), doubling rate (k) and doubling times (t2), with average μ1 of 0.131, 0.076 and 0.077 per day for *Tolypothrix* sp., *Limnothrix* sp., and the consortium, respectively (Table 3.1). Irrespective of

culture media composition used, doubling times for *Limnothrix* sp. and the consortium were 1.6 – 1.4 times lower (i.e., ~ 8-12 days) for BG11 and 1.4 – 2 times lower in SADW compared to *Tolypothrix* sp. (i.e., ~ 5-6 days).

3.4.2 Removal of nutrients and metals by *Tolypothrix* sp., *Limnothrix* sp., and the consortium

3.4.2.1 Phosphate removal:

Media t_0-phosphate concentrations were ~18 – 19.5 mg L^{-1} (Fig. 3.3A - C). PO_4^{3-}-removal was rapid (50-60 and 85-87%) and media(metal)-independent for the first 3-days *Tolypothrix* sp. (Fig 3.3A) and *Limnothrix* sp. over the first 8 days (Fig 3.3B). PO_4^{3-}-removal by the consortium was affected by medium, as BG11-grown cultures removed ~50-60% within 3 days, but it took 12 days to achieve this in SADW and BG11(-N)-grown cultures (Fig. 3C). At the end of the 19-day time course, 88-98% PO_4^{3-} was removed from the different culture media (Fig3.3A-C). Maximum PO_4^{3-}-uptake rate were between 2 and 2.5 mg g^{-1}DW d^{-1} for *Tolypothrix* sp., grown in different culture media on day 3, dropping to <0.1 mg g^{-1}DW d^{-1} as the media became PO_4^{3-}-deplete (Fig 3.3 A_1). In contrast, PO_4^{3-}-uptake was affected by medium and was ~35% lower for *Limnothrix* sp., grown in BG11 compared to SADW (~2.5 mg g^{-1}DW d^{-1}) on day 3 (Fig 3.3B_1). The consortium grown in BG11 had similar PO_4^{3-}-uptake rates to *Tolypothrix* sp. and *Limnothrix* sp (grown in SADW+N), while they were less than half when grown in BG11(-N) and SADW (Fig 3.3C_1).

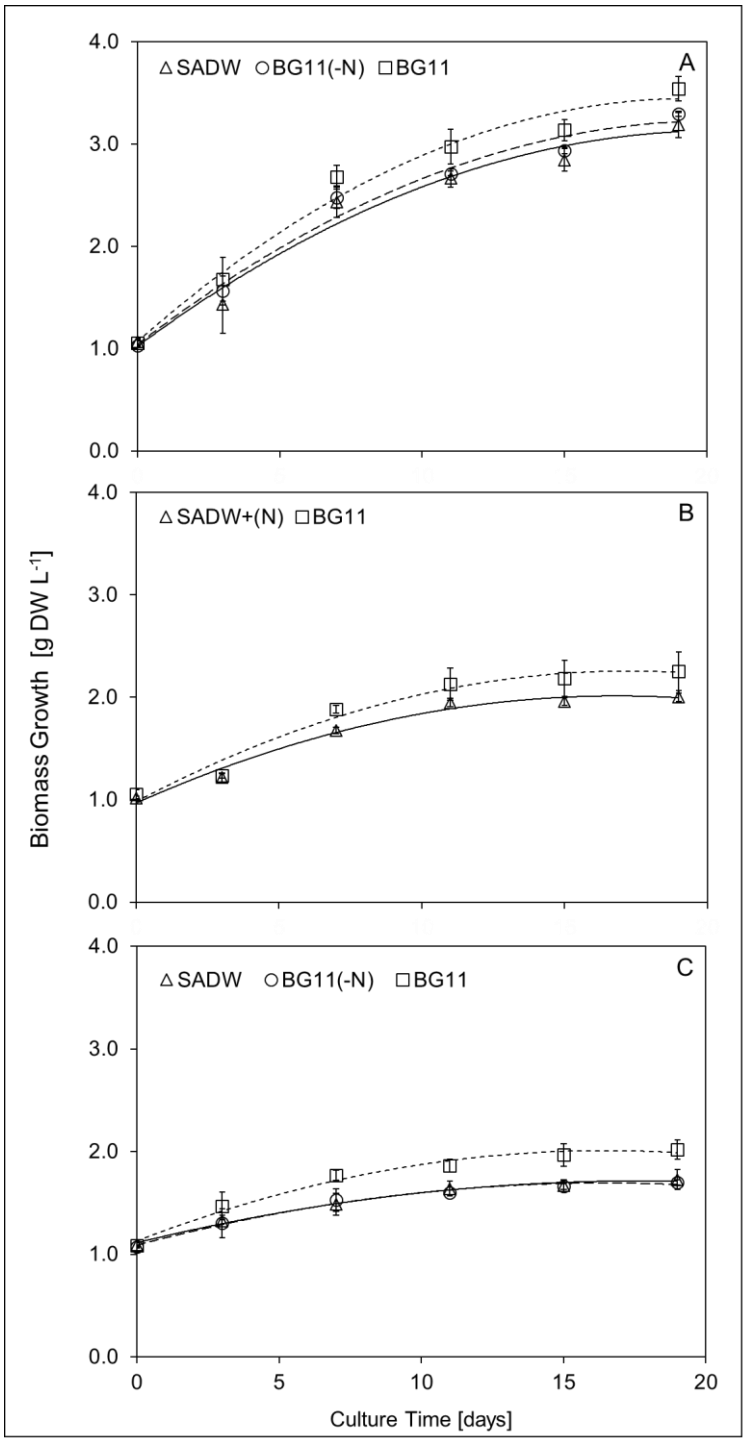


Figure 3.1 Growth of cyanobacterial culture in different media. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen. A: *Tolypothrix* sp. B: *Limnothrix* sp. and C: Consortium

Table 3.1 Effect of culture medium on growth of *Tolypothrix* sp., *Limnothrix* sp., and the consortium. Results are expressed as mean ± standard deviation (n =3 independent cultures). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Cyanobacteria	Parameters	Media		
		SADW	BG11(-N)	BG11
Tolypothrix sp.	Specific growth rate (μ_1) [d^{-1}]	0.118 ± 0.012	0.142 ± 0.025	0.133 ± 0.007
	(μ_2) [d^{-1}]	0.023 ± 0.007	0.039 ± 0.009	0.023 ± 0.004
	Doubling rate (k) [d^{-1}]	0.170 ± 0.018	0.204 ± 0.036	0.192 ± 0.011
	Doubling time (t_2)	5.925 ± 0.580	4.992 ± 0.835	5.211 ± 0.295
Limnothrix sp.	Specific growth rate (μ_1) [d^{-1}]	0.071 ± 0.003	-	0.081 ± 0.005
	(μ_2) [d^{-1}]	0.015 ± 0.002	-	0.015 ± 0.007
	Doubling rate (k) [d^{-1}]	0.103 ± 0.004	-	0.120 ± 0.006
	Doubling time (t_2)	9.723 ± 0.360	-	8.348 ± 0.360
Consortium	Specific growth rate (μ_1) [d^{-1}]	0.064 ± 0.020	0.065 ± 0.003	0.102 ± 0.035
	(μ_2) [d^{-1}]	0.017 ± 0.006	0.015 ± 0.004	0.015 ± 0.005
	Doubling rate (k) [d^{-1}]	0.092 ± 0.029	0.094 ± 0.004	0.147 ± 0.051
	Doubling time (t_2)	11.83 ± 4.550	10.59 ± 0.427	7.345 ± 2.484

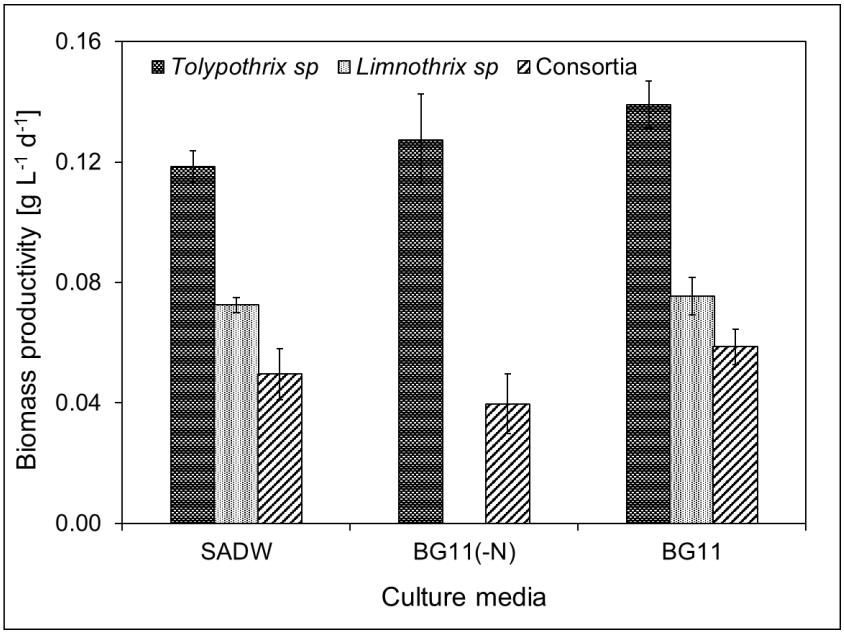


Figure 3.2 Biomass productivity of *Tolypothrix* sp., *Limnothrix* sp. and the consortium grown in SADW, BG11(-N) and BG11 Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

3.4.2.2 Nitrate removal:

Over the 19-day time course, nitrate-removal was linear for all cultures (Fig. 3.4A – C), but *Tolypothrix* sp. and the consortium removed only 45% (Fig 3.4A and C), while *Limnothrix* sp. removed ~70% (3.4B), when grown in BG11 medium. These data suggest that *Tolypothrix* sp. supplemented its nitrogen requirement through N_2-fixation when on its own and may indicate that the consortium was dominated in abundance by *Tolypothrix* sp.. In contrast, *Limnothrix* sp. grown in SADW(+N) also only removed ~49%, suggesting an effect of metals. Nitrate uptake rates per gram DW biomass did not correlate with the nitrate removal rates, since peak uptake rates were determined for the first 3-days of cultivation (similar to PO_4^{3-}-uptake) for *Tolypothrix* sp. (~5.9 mg g^{-1} DW d^{-1}) and *Limnothrix* sp. (~ 7.6 mg g^{-1}DW.d^{-1}) grown in BG11 (Figs 3.4A$_1$

and B_1). Uptake rates dropped to 0.6 and 1.5 mg g^{-1}DW d^{-1} for *Tolypothrix* sp. (Fig 3.4A_1) and *Limnothrix* sp. (Fig 3.4B_1), respectively. In contrast to *Tolypothrix* sp., nitrate uptake rates plateaued for *Limnothrix* sp. between days 3 and 12 in BG11 and SADW (+N) culture media (~4.2 – 5.9 mg g^{-1}DW d^{-1}) (Fig. 3.4B1) and patterns were similar for the consortium, but uptake rates were 2-fold lower (Fig 3.4C_1)

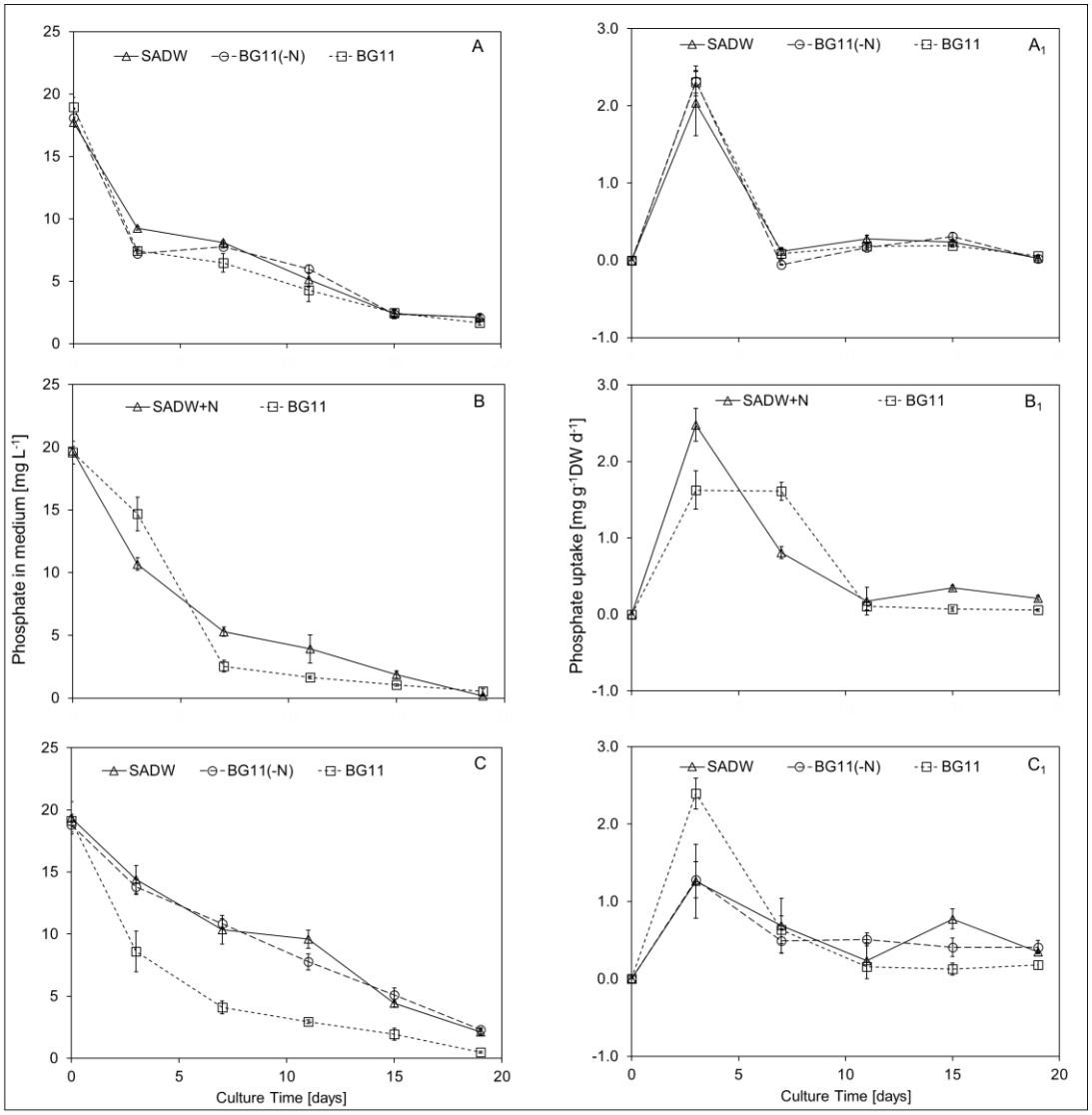


Figure 3.3 Time course of culture media phosphate levels and phosphate uptake rates. A, A_1: *Tolypothrix* sp., B, B_1: *Limnothrix* sp. C, C_1: Consortium. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

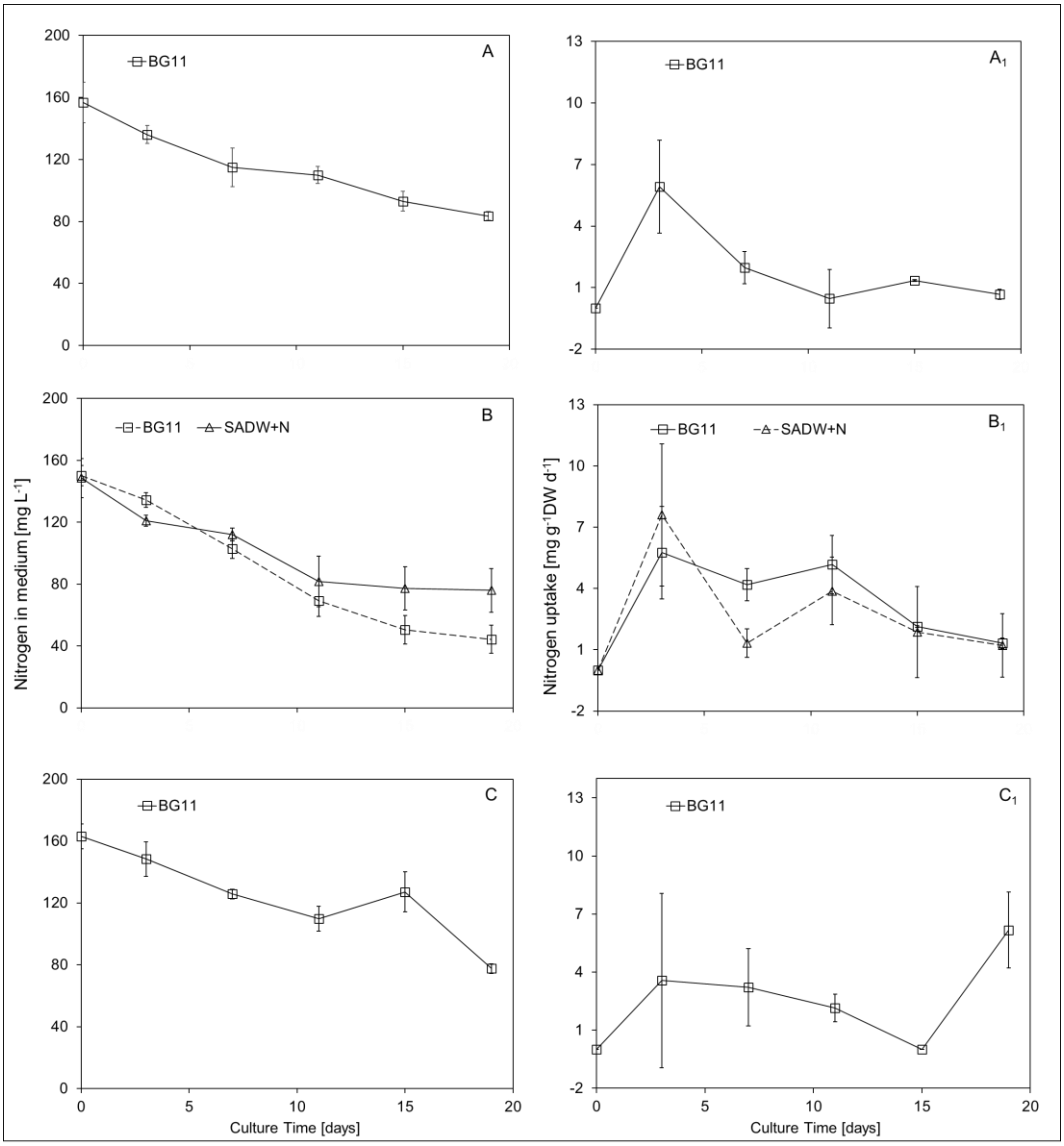


Figure 3.4 Time course of culture media nitrate levels and uptake rates. A, A_1: *Tolypothrix* sp., B, B_1: *Limnothrix* sp. C, C_1: Consortium. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

3.4.2.3 Heavy metal removal:

Cumulative metal removal from SADW in the 19-day time course was highest for Fe (>99%) for all three species, followed by Al (>86%) (Table 3.2). *Tolypothrix* sp. removed ~2.2 – 2.6 and 3-times more As and V (81.2 and 79.9%, respectively) than *Limnothrix* sp. and the consortium and also 3-times more Se (~93%) than *Limnothrix* sp. (Table 3.2) Se removal capacity of *Tolypothrix* sp. was almost matched by the consortium (~85%) (Table. 3.2). In contrast, *Limnothrix* sp. and the consortium achieved 1.2 and 1.6-fold higher removal of Ni than *Tolypothrix* sp. (~44%), while removal of Sr was 1.4-fold higher by *Limnothrix* sp. (~53%) compared to *Tolypothrix* sp. and the consortium (Table 3.2).

Based on metal concentrations present in the uninoculated SADW, ~1,320 µg Fe g^{-1} DW biomass was absorbed by the consortium, followed by *Limnothrix* sp., and *Tolypothrix* sp. within the first 24 h following inoculation. High 24 h V uptake (~112 µg g^{-1} DW biomass) also occurred for *Limnothrix* sp., while maximum uptake (~118 µg g^{-1} DW biomass) for *Tolypothrix* sp. took 11 days (Figs 3.5A_1 and B_1). In contrast, a gradual removal of Sr occurred for *Tolypothrix* sp. and the consortium (Figs 3.5A and C), while no Sr removal was evident for the first 11 days of cultivation for *Limnothrix* sp. but occurred rapidly between days 11 and 19 (Fig. 3.5B). In contrast, As and Ni uptake was not significantly different between *Tolypothrix* sp., *Limnothrix* sp., and the consortium (Figs 3.5A_1-C_1) over the time course and similarly, Al uptake was gradual (Figs 3.5A-C). Selenium uptake rates sharply increased between days

1 and 11 for *Tolypothrix* sp and the consortium (Figs 3.5A1 and C1), while the opposite trend was observed for *Limnothrix* sp. (Fig. 3.5B$_1$).

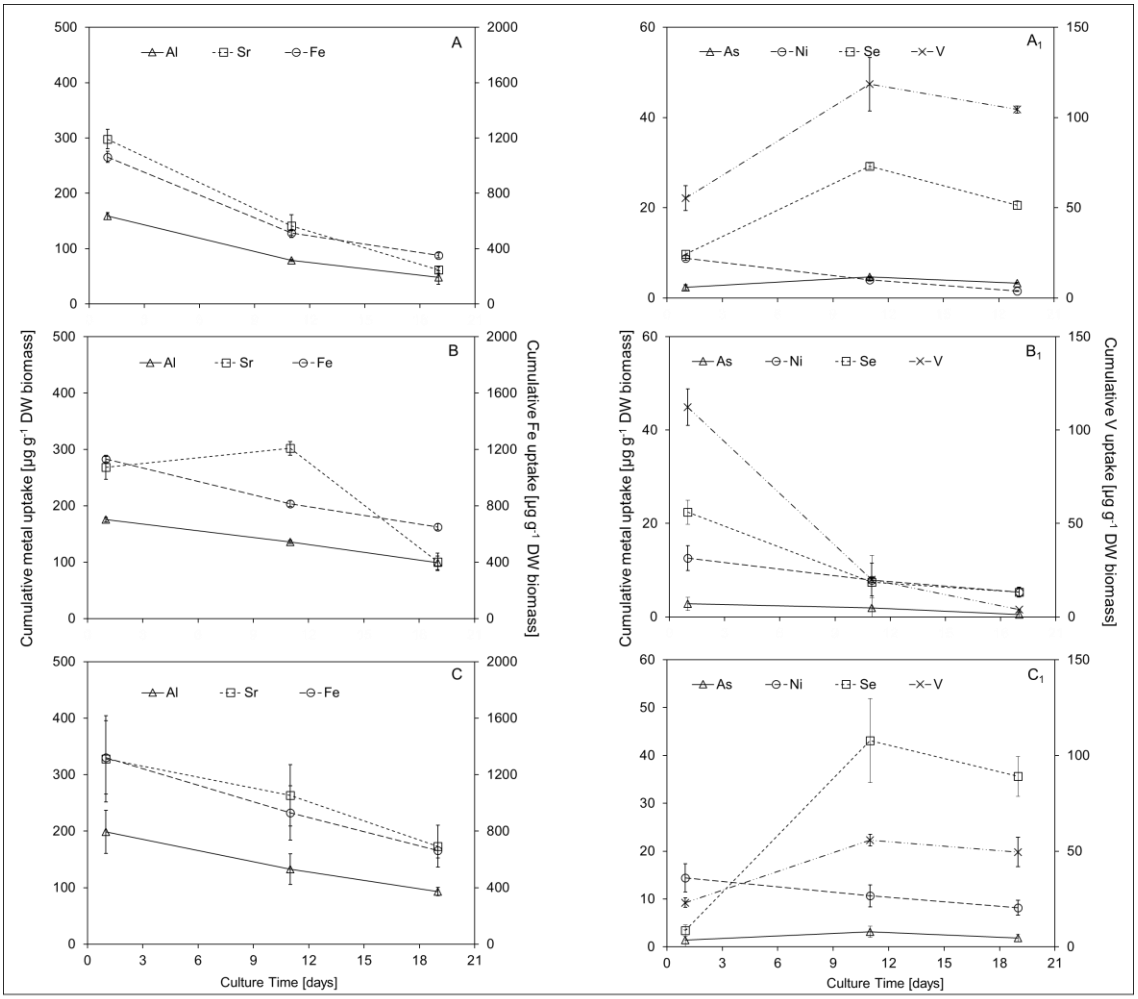


Figure 3.5 Metal uptake from SADW medium. A, A$_1$: *Tolypothrix* sp. B, B$_1$: *Limnothrix* sp. and C, C$_1$: Consortium. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water.

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Table 3.2 Metal removal potential of *Tolypothrix* sp. *Limnothrix* sp. and the consortium. Results are expressed as mean ± standard deviation (n =3).

Metals	Initial medium concentration [µg L^{-1}]	Cumulative metal removal [%]		
		Tolypothrix sp.	*Limnothrix* sp.	Consortium
Al	200 ± 1.0	90.61±3.16	98.55±1.00	86.64±0.61
As	13 ± 0.2	81.20±1.13	37.37±0.12	31.05±0.50
Fe	1184 ± 3.0	99.49±1.26	99.55±0.01	99.31±0.06
Ni	21 ± 0.4	44.28±1.25	53.74±4.93	67.53±7.86
Se	73 ± 1.0	93.04±0.81	31.19±4.27	85.34±4.14
Sr	831 ±12.0	38.85±4.82	52.58±1.20	40.14±1.20
V	434 ± 4.0	79.91±6.03	26.38±2.87	26.34±9.74

3.4.3 Bio-products from *Tolypothrix* sp., *Limnothrix* sp., and Consortium

3.4.3.1 Carbohydrate, Protein and Lipids

Culture media/metals had no effect on biomass classes (protein, carbohydrate, and lipids of *Tolypothrix* sp. and *Limnothrix* sp. (Figs 3.6A and B). *Tolypothrix* sp. biomass was dominated by carbohydrate (40-48%) ($p<0.0002$), protein (~23%) ($p<0.0001$) and lipids (~13%) ($p<0.0001$) (Fig. 3.6A), while protein content (~ 40-42%) was dominated in *Limnothrix* sp., followed by carbohydrate (~26%) and lipids (13%) (Fig. 3.6B). In contrast, medium significantly affected carbohydrate and protein contents of the consortium (Fig. 3.6C). Biomass of the consortium grown in nitrogen-deficient media (SADW and

BG11(-N) had higher carbohydrate contents (~29%), followed by protein (~18%) and lipid (10%) (Fig. 3.6C) and were dominated by *Tolypothrix* sp. towards the end of the time course experiment (Appendix Figs A.1A and B). In contrast, biomass of the consortium grown in BG11 had a high protein content (~37%) and *Limnothrix* sp. gradually gained dominance of the consortium over the cultivation time (Appendix Fig. A.1C).

3.4.3.2 Phycobiliproteins

Phycocyanin (PC) and phycoerythrin (PE) productivities and contents of *Tolypothrix* sp. biomass were not greatly affected by the culture media/metals (Fig. 3.7A, A_1). Highest PC and PE productivities (~15 and 12 mg g^{-1} DW d^{-1}) were obtained for biomass raised in BG11(-N), followed by BG11 and SADW (Fig 3.7A, $p<0.0001$ for PC and PE) and PC and PE contents ranged from 84 – 117 and 64 – 79 mg g^{-1} DW, respectively (Fig. 3.7A_1). In contrast, *Limnothrix* sp. biomass had much higher PC than PE productivity (~16 and 2.7 mg g^{-1}DW d^{-1}, respectively) and contents (~120 – 224 and ~37 mg g^{-1} DW , respectively (Fig. 3.7B and B_1), with PC contents being up to 2-fold higher in SADW-cultured *Limnothrix* sp. compared to *Tolypothrix* sp. PC and PE productivity and contents of *Limnothrix* sp. were significantly affected by medium, i.e. 1.5-fold higher in SADW than in BG11. PC and PE productivities and contents of the consortium (Fig 3.7C&C_1) reflected medium-induced shifts in dominance profiles, i.e. dominance of *Limnothrix* sp. (Appendix Fig A.1) in BG11-grown consortium cultures is clearly evidenced by the high PC content (Fig. 3.7C_1).

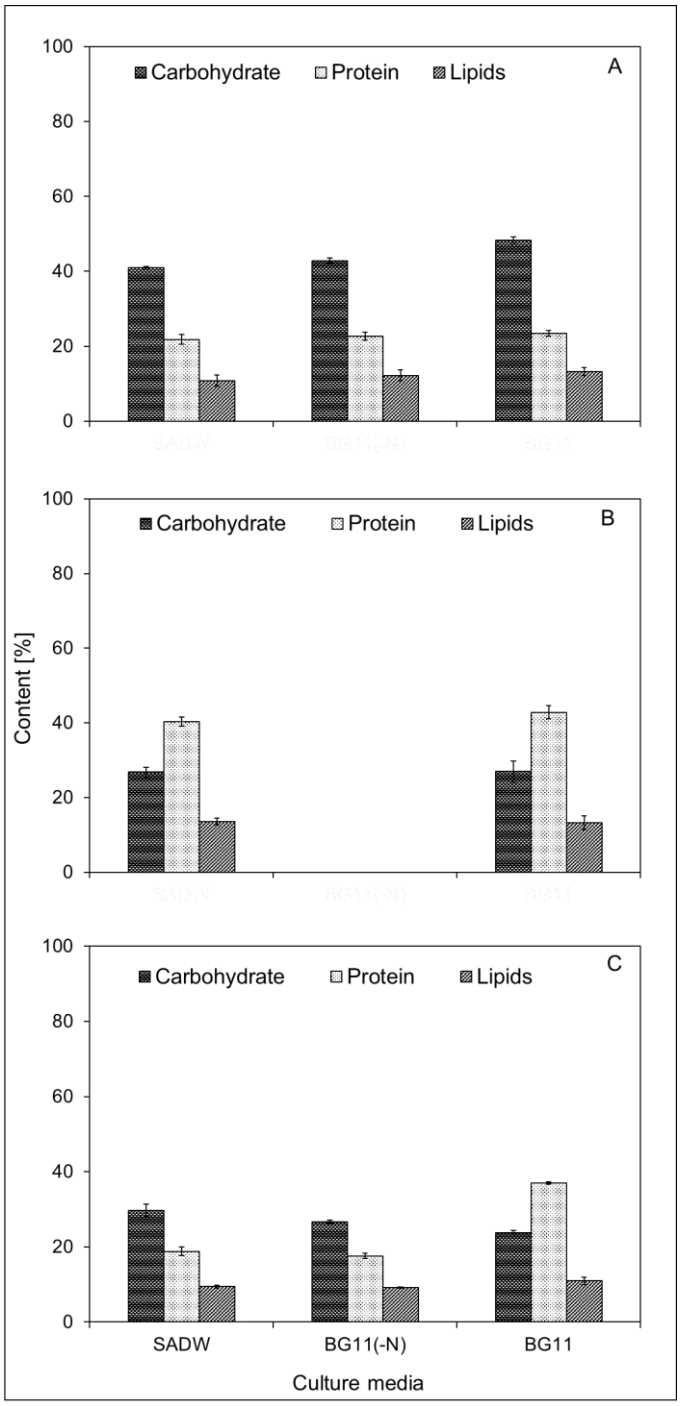


Figure 3.6 . Carbohydrate, protein and lipid contents of cyanobacterial culture grown in SADW, BG11(-N) and BG11 A: *Tolypothrix* sp., B: *Limnothrix* sp. C: Consortium. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

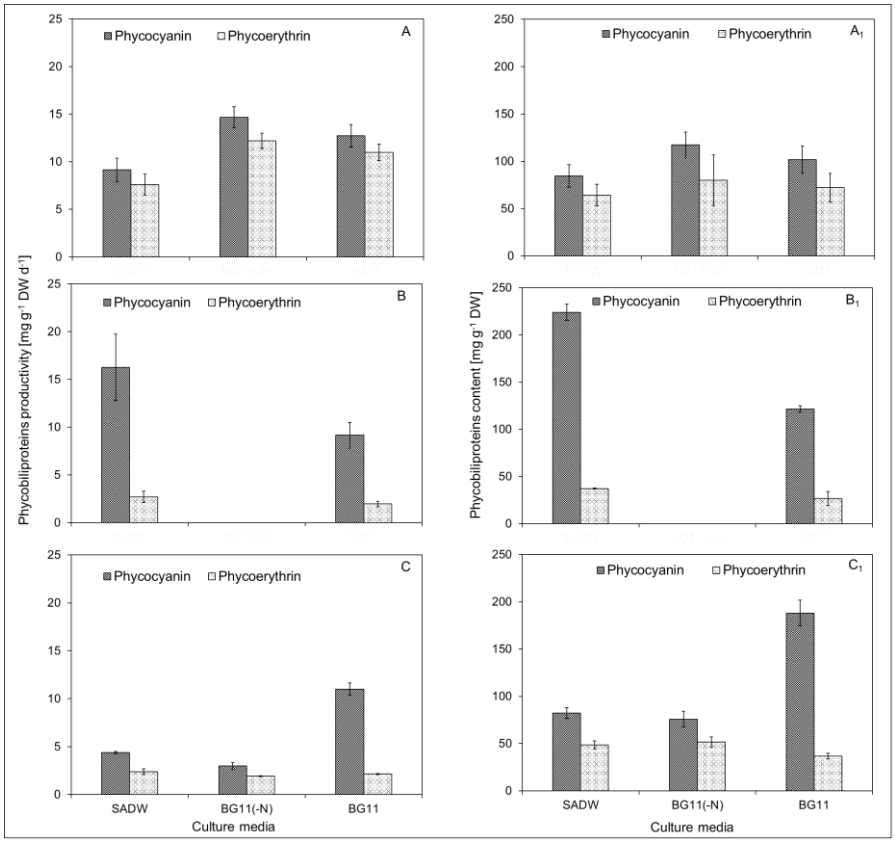


Figure 3.7 Phycobiliproteins productivity and content of cyanobacterial culture grown in SADW, BG11(-N) and BG11 A, A_1: *Tolypothrix* sp., B, B_1: *Limnothrix* sp. C, C_1: Consortium. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Table 3.3 Effect of culture medium on FAME profiles of *Tolypothrix* sp., *Limnothrix* sp. and the consortium Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Fatty Acids	*Tolypothrix* sp.			*Limnothrix* sp.		Consortium		
[mg g^{-1} TFA]	SADW	BG11(-N)	BG11	SADW+N	BG11	SADW	BG11(-N)	BG11
C13:0 (cis-9)	1.06 ± 0.04	1.14 ± 0.04	1.00 ± 0.03	1.48 ± 0.16	1.54 ± 0.09	0.99 ± 0.04	1.00 ± 0.03	1.32 ± 0.04
C14:0	0.57 ± 0.01	0.57 ± 0.01	0.55 ± 0.01	5.84 ± 0.49	5.76 ± 0.47	4.11 ± 0.15	5.16 ± 0.08	4.30 ± 0.09
C14:1 (cis-9)	0.65 ± 0.01	0.66 ± 0.05	0.61 ± 0.01	7.98 ± 0.78	7.98 ± 0.54	3.45 ± 0.01	3.97 ± 0.18	6.06 ± 0.12
C15:0	-	-	-	0.64 ± 0.06	0.67 ± 0.03	-	-	-
C15:1	0.88 ± 0.02	0.94 ± 0.01	0.86 ± 0.01	1.06 ± 0.07	1.12 ± 0.01	0.77 ± 0.03	0.79 ± 0.01	1.02 ± 0.04
C16:0	11.90 ± 0.51	13.03 ± 0.19	11.39 ± 0.38	9.69 ± 0.75	9.62 ± 1.06	10.67 ± 0.07	10.66 ± 0.49	9.90 ± 0.21
C16:1	1.71 ± 0.06	1.83 ± 0.04	1.71 ± 0.08	9.05 ± 0.64	9.55 ± 0.42	4.72 ± 0.15	5.06 ± 0.26	7.92 ± 0.09
C17:1 (Cis-10)	0.61 ± 0.02	0.64 ± 0.02	0.59 ± 0.00	-	-	-	-	-
C18:0	0.72 ± 0.03	0.72 ± 0.04	0.77 ± 0.04	-	-	0.70 ± 0.06	0.77 ± 0.02	0.68 ± 0.02
C18:1 (Cis-9) ω-9	2.18 ± 0.12	2.17 ± 0.08	2.51 ± 0.04	-	-	1.17 ± 0.04	1.11 ± 0.04	0.98 ± 0.06
C18:2 (cis-9,12) ω-6	2.84 ± 0.17	2.92 ± 0.09	2.87 ± 0.12	-	-	1.48 ± 0.11	1.33 ± 0.08	0.96 ± 0.06
C18:3 (cis-6,9,12) ω-6	0.80 ± 0.08	0.99 ± 0.06	0.72 ± 0.03	-	-	-	-	-
SUM SFA	14.25 ± 0.59	15.46 ± 0.27	13.71 ± 0.45	17.65 ± 1.47	17.58 ± 1.65	16.47 ± 0.33	17.59 ± 0.62	16.20 ± 0.36
SUM MUFA	6.04 ± 0.25	6.25 ± 0.20	6.28 ± 0.15	18.09 ± 1.49	17.58 ± 1.65	10.09 ± 0.23	10.93 ± 0.49	15.68 ± 0.32
SUM PUFA	3.65 ± 0.24	3.90 ± 0.16	3.60 ± 0.15	-	-	1.48 ± 0.11	1.33 ± 0.08	0.96 ± 0.32
TFA [mg g^{-1} DW]	23.94 ± 1.08	25.60 ± 0.63	23.58 ± 0.75	35.75 ± 2.96	36.23 ± 2.62	28.04 ± 0.67	29.84 ± 1.19	32.84 ± 0.74
Productivity [mg g^{-1} DW day^{-1}]	2.84 ± 0.15	3.26 ± 0.06	3.28 ± 0.20	2.56 ± 0.31	2.72 ± 0.24	1.39 ± 0.21	1.18 ± 0.15	1.93 ± 0.18

3.4.3.3 FAME Profile

Total fatty acid (TFA) contents were not affected by the media composition of mono- or mixed cultures of *Tolypothrix* sp. and *Limnothrix* sp (Table 3.3). Highest TFA contents were determined for *Limnothrix* sp. (~35 mg g^{-1} DW) being 30% higher than in *Tolypothrix* sp. (~24 mg g^{-1}DW, $p<0.0001$)). For the consortium grown in BG11, TFA content was 15% higher than in *Tolypothrix* sp. ($p<0.0001$). FAME productivity and profiles differed between *Tolypothrix* sp. and *Limnothrix* sp., but culture media composition did not alter the FAME profiles. Highest FAME productivity was observed for *Tolypothrix* sp. biomass raised in BG11 (with and without N) (~3.20 mg g^{-1} DW d^{-1}), followed by *Limnothrix* sp. (~2.6 mg g^{-1}DW d^{-1}) and was 2-fold lower than for the consortium (1.18 to 1.93 mg g^{-1} DW d^{-1}).

The FAME profile of *Tolypothrix* sp. was dominated by palmitic acid (up to 50% C16:0, 11-13 mg g^{-1}), while myristic acid (C14:0, ~5.8 mg g^{-1}), myristoleic acid (C14:1cis-9, ~7.9 mg g^{-1}), palmitic acid (C16:0, ~9.5 mg g^{-1}) and palmitoleic acid (C16:1, ~ 9mg g^{-1}) were the dominant FAMEs contributing up to 90% of TFA contents in *Limnothrix* sp. Therefore, total MUFA and SFA contents were relatively higher in *Limnothrix* sp., when compared to *Tolypothrix* sp. Since, the consortium was dominated by *Limnothrix* sp. when raised in BG11, the distribution of FAMEs were similar, but unexpectedly the consortium displayed a mix of fatty acid dominance irrespective of medium, showing high concentrations of C14:0, C14:1 and lack C18:3 characteristic of *Limnothrix* sp. biomass and C17:1, C18:0, C18:1, C18:2 as determined for *Tolypothrix* sp. biomass but not *Limnothrix* sp., despite the consortium showing clear

dominance shifts in nitrogen-deplete vs. nitrogen-replete BG11 (Appendix Fig. A.1).

Table 3.4 Effect of culture medium on elemental composition of *Tolypothrix* sp. *Limnothrix* sp. and the consortium. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Cyanobacterial culture	Elementals	SADW	BG11(-N)	BG11
Tolypothrix sp.	Carbon (C)	44.02 ± 0.43	44.48 ± 0.24	42.97 ± 0.19
	Hydrogen (H)	6.96 ± 0.11	6.80 ± 0.04	6.56 ± 0.10
	Nitrogen (N)	6.52 ± 0.15	6.80 ± 0.04	6.50 ± 0.04
	C/N ratio (C: N)	6.76 ± 0.15	6.54 ± 0.07	6.61 ± 0.03
**Limnothrix* sp.	Carbon (C)	45.02 ± 0.40	-	46.25 ± 0.64
	Hydrogen (H)	6.76 ± 0.02	-	6.82 ± 0.07
	Nitrogen (N)	10.40 ± 0.16	-	10.51 ± 0.13
	C/N ratio (C: N)	4.39 ± 0.03	-	4.40 ± 0.04
Consortium	Carbon (C)	43.49 ± 0.13	43.36 ± 0.33	44.94 ± 0.30
	Hydrogen (H)	6.74 ± 0.02	6.73 ± 0.13	6.71 ± 0.06
	Nitrogen (N)	5.56 ± 0.14	5.46 ± 0.18	9.17 ± 0.11
	C/N ratio (C: N)	7.83 ± 0.18	7.94 ± 0.31	4.60 ± 0.08

3.4.3.4 Elemental Composition:

Culture medium and species had no effect on carbon and hydrogen, but nitrogen contents were almost 2-fold higher in *Limnothrix* sp. compared to *Tolypothrix sp.* ($p<0.0001$), affecting also C/N ratios (Table 3.4). Likewise, a 2-fold higher N content was observed in the consortium grown in BG11 compared to cultivation in SADW and BG11(-N), leading to a lower C/N ratio. The nitrogen content of the consortium reflected nitrogen-induced dominance shifts (Appendix Fig. A.1).

3.5 Discussion

Cyanobacterial biomass production at large scale is constrained by freshwater usage and nutrient supplements, which could be tackled using industrial or domestic wastewaters under bio refinery/bioremediation concepts. On the surface, this appears to be a feasible undertaking, but the characteristics of the wastewater to be used is the key for biomass production and product development potential. Therefore, understanding the impacts of wastewater on selected strains and their bioremediation potential are of prime importance before commercialisation concepts can be developed. This present study evaluated the use of *Tolypothrix* sp., *Limnothrix* sp., and a consortium of the two species for bioremediation of nutrients (phosphorus and nitrogen) from SUWW and metals from SADW and examined impacts on growth and biochemical profiles to determine product potential.

Neither *Tolypothrix* sp., nor *Limnothrix* sp., or the consortium grew in SUWW. This is likely due to bacterial overgrowth enabled by the richness of organic carbon and/or ammonium toxicity in SUWW (Ernst et al. 2005; Flores et al. 2005; Ohashi et al. 2011). It has been reported that dissolved organic matter limits photosynthesis by inhibiting the protein-pigment complex of photosystem II (PSII), resulting in poor or no-growth of cyanobacteria (Keren and Krieger-Liszkay 2011; Laue et al. 2014). This photoinhibition was alleviated by reducing growth temperature (from 37^{o}C to 27^{o}C) and light intensity (from 200 to 100 µmol photons m^{-2} s^{-1}) for *Synechococcus* sp. strain PCC 7002 grown in organic-rich medium (Korosh et al. 2018). Although not investigated, it is unlikely that choice of a lower temperature and light regime would have

reversed the outcomes for the investigated species, as the growth temperature and light regime were within the range reported to prevent organic-rich medium-induced photoinhibition of *Synechococcus*.

Culture media had an effect on biomass production, particularly evident for *Limnothrix* sp. and the consortium. In general, biomass growth and productivities were higher in cyanobacterial culture media BG11 than SADW, suggestive of some minor metal toxicity (El-Sheekh et al. 2005). In contrast, metal-rich wastewater increased biomass growth of *Nostoc muscorum* and *Anabaena subcylindrica* (El-Sheekh et al. 2005). In a 7-day time course, maximal biomass production for *Limnothrix* sp. was 0.88 g DW L^{-1} after deducting the inoculum, which is ~80% higher than achieved previously for *Limnothrix* sp. (70-145 mg DW L^{-1}) (Economou et al. 2015). For N_2-fixing cyanobacteria, maximal biomass growth of *Nostoc muscorum* and *Anabaena subcylindrica* raised in industrial wastewater were reported as 0.430 and 0.540 g DW L^{-1}, respectively (El-Sheekh et al. 2005), which was ~66-73% lower than realised here for *Tolypothrix* sp. grown in SADW after 11 days. The biomass productivity for both *Tolypothrix* sp. (119 mg L^{-1} d^{-1}) and *Limnothrix* sp. (75 mg L^{-1} d^{-1}) grown in SADW were also ~55 and 10% higher than reported for the green algae *Oedogonium* (~45 mg L^{-1} d^{-1}), *Rhizoclonium* (~65 mg L^{-1} d^{-1}), and *Hydrodictyon* (~60 mg L^{-1} d^{-1}) grown in ash dam water enriched with f/2 medium (Saunders et al. 2012). In comparison to the study by Saunders et al. (2012), which is in terms of metal mix most similar to the research presented here, *Tolypothrix* sp. produced higher biomass and removed more metals even without nitrogen fertilisation, hence *Tolypothrix* sp. could be a potential

candidate for bioremediation and biomass production using nutrient-poor ash dam wastewater.

With increasing cultivation time, metal removal was positively correlated with biomass increase. In nitrogen-limited media, the mixed consortium was dominated by *Tolypothrix* sp., after 7 days, which is also reflected in changes in V and Se removal from SADW (Fig 3.5C$_1$). Complete removal of all test metals was achieved within 19 days, which highlights the suitability of the test species for effective remediation of metal-rich wastewater i.e., SADW. Metal bio-remediation was more efficient with *Tolypothrix* sp., than *Limnothrix* sp. or the consortium. The metal binding capacity for microalgae was reported to be 10-50 mg g^{-1}, while the absorption and adsorption kinetic for varies with metal species (Freitas et al. 2008). In this study, overall metal removal was <2 mg g^{-1} for the species tested, as concentrations of metals in SADW were based on concentrations typically found at Tarong power station, a coal-fired power station in Queensland, which are comparatively low (Appendix Table, A.1). Metal removal rates decreased gradually from day 1 to day 19 for the species tested due to saturation of growth by nutrient availability. In the presence of multiple metal ions, regulation may be positively influenced by regulating wastewater pH, which aid to alter surface charges of the biomass thereby influencing the metal adsorption and uptake rates (Israel et al. 2005; Murphy et al. 2008). As the cultures were not supplemented with CO_2, culture pH increased due to utilisation of dissolved CO_2 in carbon-fixation, enhancing interactions of positively charged metals with the organisms' surfaces.

Metal removal patterns were common and in the order of Fe<Se<Al<As<V<Ni<Sr for *Tolypothrix* sp., while Fe<Al<Ni<Sr<As<V for *Limnothrix* sp. and Fe<Al<Se<Ni<Sr<As<V for the consortium. Fe is an essential micro-nutrient required for the many redox reactions, is a co-factor involved in nitrogen assimilation/fixation and has other major regulatory functions *in-situ* (Weisany et al. 2013). It is thus not surprising that Fe was most readily taken up and 98% was removed from the medium within 24 h. Removal of other metal ions also occurred within 24 h, except for V and Se for *Tolypothrix* sp., Sr for *Limnothrix* sp., and Se for the consortium. Metal removal within 24 h could be due to biosorption, which is passive binding by either dead or living biomass and has been described as a cost-effective approach to metal removal from industrial wastewater (Gavrilescu 2004).

Culture media composition had no effect on carbohydrate, protein and lipid content of the cyanobacterial cultures. Carbohydrate content was higher (40-48%) in *Tolypothrix* sp. biomass, making it an ideal candidate for bioethanol production (Möllers et al. 2014), while protein content was higher (~40-42%) in *Limnothrix* sp. biomass, which is therefore an ideal choice for animal feed production (Kovač et al. 2013). It is, however, undetermined whether the higher protein content of Limnothrix sp. was due to differences in extraction efficiencies between the two species. To determine this, residual biomass must be inspected for remaining protein contents, which was not done in the presented research. There was no significant difference in lipid content between the two species. However, lipid content was very low in both species, as is the case for all cyanobacteria, and thus cyanobacterial biomass is not suitable for production

of lipid-based biofuel or for targeting lipid-based product development, unless a biorefinery concept is applied.

Pigments reduce oxidative stress and are important for light energy transfer in photosynthesis. Pigment productivity is largely influenced by nutrients such as phosphate, nitrate, chloride and sulphate or salinity of culture medium (Hifney et al. 2013; Kathiresan et al. 2007).Cyanobacteria are very promising sources of high-value pigment products, such as phycocyanin and phycoerythrin with various applications in the field of food, biomedical and pharmaceutical industries (Liu et al., 2014). Phycocyanin (PC) and phycoerythrin (PE) are the two major phycobiliproteins produced by *Tolypothrix* sp. as well as *Limnothrix* sp.. PC content in *Limnothrix* sp., was 2-fold higher than in *Tolypothrix* sp. but PC productivity was not very different. In contrast to *Tolypothrix* sp., culture medium significantly affected phycobiliprotein productivity in *Limnothrix* sp., which was 1.5-fold higher in SADW than in BG11. As PC and PE are nitrogen-containing water soluble pigments, the higher content of phycocyanin of *Limnothrix* sp. could be due to nitrogen availability, as SADW was not enriched with nitrogen for *Tolypothrix* sp. (Eriksen 2008). This is, however, highly unlikely, as the nitrogen-content of BG11 medium and SADW medium were the same, yet phycocyanin content was only half of that in BG11 compared to SADW, pointing to species-specific differences, perhaps in terms of extraction efficiencies, between *Tolypothrix* sp. and *Limnothrix* sp. and an enhancing effect of metals for *Limnothrix* sp. grown is SADW. To determine whether extraction efficiency differences exist between the species, biomass needs to be analysed for residual pigment content, which was not carried out in

this study. Phycocyanin from *A. platensis* has been used as food and cosmetic colourant, PC also possesses antioxidant, anti-inflammatory, anti-viral, anti-cancer and cholesterol-lowering effects as described in chapter 2. Purified C-PC has nutraceutical and pharmaceutical applications. The antioxidant and radical scavenging activities of C-PC from different cyanobacteria are well studied, as C-PC is bleached during scavenging of peroxyl radicals. Research also suggests that C-PC inhibits cell proliferation, creating interest in C-PC for anti-carcinogenic applications (Eriksen 2008). The market value of C-PC is between US$500 to 100,000 kg^{-1} depending on the purity (Borowitzka 2013). Hence, coupling wastewater remediation with cyanobacterial phycocyanin production could represent a lucrative business.

Total fatty acids were ~30% higher in *Limnothrix* sp. compared to *Tolypothrix* sp.. Saturated fatty acid contents were similar (~17 mg g^{-1} TFA), while mono-unsaturated fatty acids were ~65% higher in *Limnothrix* sp. (~18 mg g^{-1} TFA) compared to *Tolypothrix* sp. (~6 mg g^{-1} TFA). In contrast, no polyunsaturated fatty acids were observed in *Limnothrix* sp. under these culture conditions. Cyanobacterial species investigated in this culture conditions are not suitable for fatty acids production and related applications.

Nitrogen content of biomass is a crucial criterion for nitrogen-based bio-fertiliser applications. Cyanobacterial biomass has nitrogen-based bio-fertiliser potential, as nitrogen concentration (8 to 12% of DW) were higher than reported for green algae (3 to 8%) (Benemann 1979). Results obtained here demonstrate that *Tolypothrix* sp. and *Limnothrix* sp. both have biofertiliser application potential as carbon and nitrogen contents in the *Tolypothrix* sp. and *Limnothrix*

sp. were ~ 44-45% and ~7-11%, respectively. Fertilisers from these two species would increase soil organic matter and water holding capacity, which is attributed to the high carbon and nitrogen content of the biomass applied (Uysal et al. 2015).

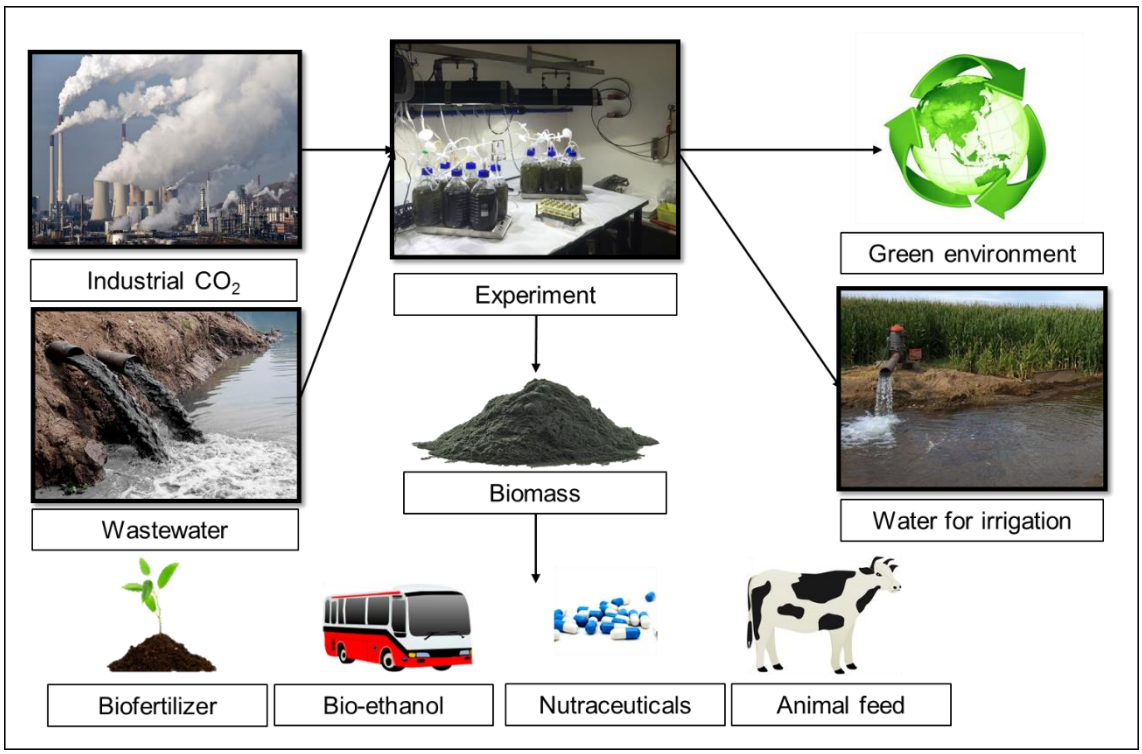
Overall, the results obtained in this study highlight the advantages and disadvantages of nitrogen-fixing and non-nitrogen fixing cyanobacteria for bioremediation and bio-product development using macro-nutrient-poor metal-rich wastewaters. The N_2-fixing cyanobacterium *Tolypothrix* sp. appears to be better suited for the purpose based on higher biomass growth, carbohydrate content, pigment productivities, metal removal and self-flocculating ability without nitrogen fertilisation.

3.6 Conclusion

This study demonstrated the potential of native filamentous cyanobacteria for bioremediation of ash dam wastewater and bio-product synthesis, while the species did not prove suitable for urban wastewater treatment. The consortium approach was not successful, as N_2 fixed by *Tolypothrix* sp. did not secrete assimilated nitrogen into the medium, therefore not providing a nitrogen source to support growth of *Limnothrix* sp.. *Tolypothrix* sp. produced high biomass with a high carbohydrate content, while phycocyanin and protein contents were higher in *Limnothrix* sp., the former only when cultivated in SADW+N. Both species efficiently removed heavy metals from SADW, making them both suitable bioremediators. *Limnothrix* sp., however, should only be employed, if such wastewaters also provide adequate nitrogen contents to support its growth, as nitrogen fertilisation is otherwise required,

increasing remediation costs and limiting profitability. Based C and N content of the biomass, both species can be utilised for biofertiliser applications, but *Tolypothrix* sp. biomass could also yield bioethanol based on high carbohydrate contents, while *Limnothrix* sp is more suitable for high value C-PC production, fatty acid-based products due to high amounts of SFA and MUFA, and high protein animal feeds. *Tolypothrix* sp. was, however, chosen for the subsequent research chapters based on better growth, the unique characteristics of self-flocculation and nitrogen fixation, as this research aimed to develop cost-effective biomass production platforms and to investigate bio-product potential in a biorefinery approach.

Chapter 4: Effect of CO_2 and metal-rich wastewater on bioproduct potential of the diazotrophic cyanobacterium, *Tolypothrix* sp[4].



[4] Part of the research from this chapter was invited for oral presentation at ASIA-PACIFIC conference Hong Kong in 2016.

Velu C, Cirés S and Heiman K (2016) Effect of carbon dioxide on wastewater remediation and bioproduct synthesis by a tropical filamentous nitrogen-fixing cyanobacterium. ASIA-PACIFIC Conference on Biotechnology for Waste Conversion 2016. Hong Kong, 6-8 December 2016.

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Velu, C., Cirés, S., Brinkman, D. L., & Heimann, K. (2019). Effect of CO_2 and metal-rich waste water on bioproduct potential of the diazotrophic freshwater cyanobacterium, *Tolypothrix* sp. Heliyon, 5(4), e01549. doi:https://doi.org/10.1016/j.heliyon.2019.e01549

4.1 Abstract

Continued economic growth is reliant on stable, affordable energy, requiring at present fossil fuel-derived energy production. Coal-fired power stations produce metal-rich but macro-nutrient-poor wastewaters and emit flue gas, containing ~10% CO_2. Algae and cyanobacteria remediate metals and CO_2 but use of N_2-fixing (diazotrophic) cyanobacteria can reduce nitrogen-fertilisation costs. The resulting biomass represents a promising source for biofuel and bio-product development. This study investigated the effect of CO_2- and trace metals on growth, biochemical profiles and metal content of the freshwater diazotrophic cyanobacterium *Tolypothrix* sp. to assess bioproduct potential. Aerated 2 L batch cultures were grown in simulated ash-dam water (SADW) and BG11 without nitrogen (BG11(-N) controls). Supplied air was supplemented with either 15% CO_2 (v/v) or not (non-CO_2 controls). CO_2 supplementation resulted in 2.4 and 3.3-fold higher biomass productivities (92.2 ± 6.72 and 100.6 ± 5.1 mg DW L^{-1} d^{-1}) and 1.3 and 1.2-fold higher phycocyanin and phycoerythrin (~99 ± 8.3 and 78 ± 7.4 mg g^{-1} DW) contents, whilst metals (media) had no effect. Al, Cu, Ni and V were more efficiently removed (50 to 90%) with CO_2-addition, while As, Mo, Se and Sr removal was higher (30 to 87%) for non-CO_2 controls. No significant effect on Zn and Fe removal was evident. Calculated biomass metal concentrations, at quantities required to meet N-requirements of wheat, suggests no metal toxicity when applied as a mineral-nitrogen biofertiliser. With a carbohydrate content of 50%, the biomass is also suitable for bioethanol production. In summary, *Tolypothrix* sp. raised in ash dam wastewater supplemented with flue gas CO_2 could yield high-value

phycobiliproteins, bioethanol or biogas, and mineral-rich nitrogen fertiliser which would offset remediation costs and improve agricultural productivity.

4.2 Introduction

The Earth's average surface temperature increase of 0.9^{o}C (NASA 2016) since 1880, is largely attributable to higher concentrations of atmospheric greenhouse gases (GHGs) (IPPC 2013). Global energy-related CO_2 emissions rose by 1.4% in 2017, an increase of 460 million tonnes (MT) to a high of 32.5 giga tonnes (GT) (IEA 2018a). Climatic instability induced by GHG-increases negatively affects food production (Backlund et al. 2008; McMichael and Haines 1997; McMichael et al. 2007). Reduction of CO_2 emissions is thus an emerging priority for world economies (Keith 2009; Moss et al. 2010). Despite known large contributions to GHG emissions, combustion of coal is still the main global energy source (IEA 2013b). Since the world economy relies on guaranteed energy supplies, novel technologies and strategies to minimise CO_2 emissions need to be developed (Byers et al. 2018; Pielke Jr 2009).

Similarly, a general increase in global and regional populations increases demands on freshwater resources (Renuka et al. 2013). Climatic instability and water scarcity have interactive effects that are expected to worsen in the coming decades (Moss et al. 2011). In addition to being a major source of GHG emissions, coal-fired power generation is water-intensive, giving rise to large amounts of metal-rich wastewater (ash dam water (ADW)). A 1,000 MW power station generates up to 2.5 GL of ADW per year (Smart and Aspinall 2009). Depending on the coal deposit, diverse metals leach from the ash into the water, resulting in ADW containing high concentrations of metals, which do not

meet water quality standards for discharge (Roberts et al. 2015). Therefore ADW is generally stored in ash dams, threatening watersheds in severe rain events (Roberts et al. 2015). As of 2015, 1,200 coal-fired power stations were under construction globally, with a projected ADW generation of 750 billion liters annually, effectively doubling annual ADW production (Yang and Cui 2012). Considering ADW is a legacy contaminant whose threat persists following the decommissioning of a power station, it is surprising that few options for treating ADW have been proposed (Oman et al. 2002).

A potential approach to bioremediation of ADW and CO_2 is the use of live microalgae or cyanobacteria to sequester waste gas and wastewater constituents (Roberts et al. 2015). The ability of microalgae, cyanobacteria or plants (Welch and Shuman 1995) to biosorb and bioaccumulate metals present in ADW (Al, As, B, Cu, Fe, Mo, Ni, Se, Sr, V and Zn) depends on concentrations present (Chojnacka 2010; Mehta and Gaur 2005). A large number of cyanobacteria produce exocellular polymeric substances (EPS), a surface coat typically rich in negatively charged polysaccharides, providing a high binding capacity for metal ions (De Philippis and Vincenzini 1998) and an ideal pathway for ADW remediation. In addition, CO_2 supplementation of algal cultures with flue gas overcomes carbon-limitation to growth and the resulting low pH of the water improves metal bioavailability (Aslam et al. 2019).

Algal-based bioremediation could be more attractive, if the resulting biomass could be applied as feedstock in the manufacture of bioproducts (Shurin et al. 2013). For example, algae-derived pigments are a lucrative market and commercial production has been realised for astaxanthin from

Haematococus pluvialis and ß-carotene from *Dunaliella salina* (Mostafa 2012). The production of high-value phycobiliproteins (PBPs, water-soluble supramolecular protein aggregates) is restricted to cyanobacteria and red algae, where they may constitute as much as 40-60% of the total soluble protein (Kumar et al. 2014). Phycocyanin (PC) is used as colorant in food and cosmetics in Japan, Thailand and China and has anti-oxidant and anti-inflammatory properties (Qureshi et al. 1996). Algae and cyanobacteria can be used efficiently for the synthesis of bioethanol, biodiesel, and biohydrogen (Jones and Mayfield 2012). Biodiesel production, however, demands the use of oil-rich strains, which due to high levels of polyunsaturated fatty acids are very valuable for the omega-3 nutraceutical market (Islam et al. 2013; von Alvensleben et al. 2013). Alternatively, microalgal and cyanobacterial biomass can be anaerobically digested to produce biogas. The digestate after anaerobic digestion (AD) can be used as a soil conditioner (Passos et al. 2014) or the hydrolysed biomass could be fermented to bioethanol, as cyanobacterial biomass is rich in carbohydrates (45 to 65%) (Möllers et al. 2014). Biomass of microalgae and cyanobacteria can also be converted to biochar through slow pyrolysis to form a carbon-rich product characterised by a high pH (Roberts et al. 2015) which can be used to improve the pH of acidic soils Additionally, the high content of nitrogen, phosphorus and inorganic elements can improve soil fertility (Bird et al. 2012).

Regardless of the product choice, microalgal biomass production is carbon-limited when produced on sites without CO_2 access. While integrated cultivation at power stations could overcome carbon limitation, other fertiliser

requirements, such as nitrogen and phosphate, for biomass growth must also be met, elements that are low to absent in ADW (Saunders et al. 2012). Microalgae require large amounts of costly phosphate and nitrogen fertilisation for optimal biomass productivity, while diazotrophic cyanobacteria can sustain growth based on uptake and conversion of atmospheric nitrogen (N_2). In particular, sustained growth in the absence of nitrogen-fertilisation and capacity for CO_2 remediation was recently demonstrated for the nitrogen-fixing, non-toxic freshwater cyanobacterium *Tolypothrix* sp. Based on biochemical profiles, *Tolypothrix* sp. has bioproduct potential for high-value phycocyanin and as a carbohydrate-based feedstock (Heimann and Cires 2015; Velu et al. 2015).

This study evaluated the effect of CO_2-supplementation and simulated ash dam water (SADW) on *Tolypothrix* sp. growth and biochemical profiles to determine bioproduct potential. It also investigated metal removal capacity from nitrogen- and phosphate-poor SADW to determine suitability of the biomass as biofertiliser.

4.3 Methods

4.3.1 Strain and medium

Aerated batch stock cultures of *Tolypothrix* sp. NQAIF319 (Velu et al. 2015) were maintained at the North Queensland Algal Identification and Culturing Facility (NQAIF, James Cook University, Townsville, Australia) in nitrogen-free BG11(-N) medium (Rippka et al. 1979) at 28 °C, a photon flux density of 30 µmol photons m^{-2} s^{-1} and a 12:12 light/dark photoperiod. Cultures were sub-cultured every 28 days.

To investigate the effect of trace metal-rich ADW, *Tolypothrix* sp. was grown in simulated ash-dam wastewater (SADW: BG11(-N) supplemented with highest concentrations of trace metals (Al, As, B, Cu, Fe, Mo, Ni, Se, Sr, V and Zn) present in ADW at the Tarong coal-fired power plant (26.7809° S, 151.9125° E), Queensland, Australia (Appendix Table B.1, Initial concentration in SADW [µg L^{-1}]) (Saunders et al. 2012). BG11(-N) cultures served as non-treatment controls.

4.3.2 Experimental setup, growth and biomass productivities

2 L aerated (100 mL min^{-1}) SADW and BG11(-N) suspension batch cultures were inoculated with 0.3 g dry weight (DW) of *Tolypothrix* sp. and cultivated for 25 days at a photon flux density of 100 µmol photons m^{-2} s^{-1} and 28 °C. Culture media were supplemented with CO_2-enriched air (15% v/v) or air (non-CO_2 controls) (n=3). On day 25, the biomass was harvested by centrifugation (8,000 g, 20 min; Beckman, Avanti® J-26XP, Australia). The biomass pellets were freeze-dried (Dynavac freeze dryer model Fd12, Australia) and stored at −80 °C (Sanyo Ultra-Low Temperature Freezer (MDF-U33V), Japan) until analysis. Biomass productivity (g DW L^{-1} day^{-1}) was determined gravimetrically (von Alvensleben et al. 2013) using 40 mL culture samples taken on days 0, 3, 6, 9, 12,15, 18, 21 and 25. Biomass-specific growth rates ($\mu_{1\text{-}3}$), doubling rate (k) and doubling time (T2) were calculated as per (von Alvensleben et al. 2013).

4.3.3 Phosphate analysis

Medium phosphate (PO_4^{3-}) concentrations were determined in triplicate spectrophotometrically (PerkinElmer EnSpire® Multimode plate reader, USA) on days 0, 3, 6, 9, 12,15, 18, 21 and 25 at 610 nm in 96-well microtitre plate

(Ultraviolet Star®, Greiner Bio-One GmbH) as described in section 3.3.4 (von Alvensleben et al. 2013).

4.3.4 Metal analysis

Culture supernatants were collected on days 0, 3, 6, 9, 12,15, 18, 21 and 25 and filtered (0.22 µm hydrophilic PTFE, Micro Analytix Pty Ltd, Australia). Filtrates were analysed for Al, As, B, Cu, Fe, Mo, Ni, Se, Sr, V and Zn as described in 3.3.5

4.3.5 Biochemical analyses

Freeze-dried biomass of *Tolypothrix* sp. was analysed for total lipid, carbohydrate, protein, fatty acids, phycocyanin, phycoerythrin, and elemental carbon, hydrogen, nitrogen, sulphur, phosphorous and potassium (CHNSPK) as described in 3.3.6.

4.3.5.1 Total lipid and carbohydrate contents

Total lipid content was determined gravimetrically following a direct transesterification procedure as described in section 3.3.6.1 and carbohydrate content was analyzed using the phenol-sulphuric acid method as described in section 3.3.6.2.

4.3.5.2 Protein content

Total protein content was determined using the Lowry method based on González López et al. (2009) using a kit (Sigma, Total Protein Kit, Micro Lowry, Peterson's Modification, TPO300, Sydney, Australia) as describer in section 3.3.6.3.

4.3.5.3 Fatty acid extraction, transesterification and alkane/alkene analysis

Fatty acids were analysed at the Australian Institute of Marine Science, Townsville, Australia. Fatty acid extraction, transesterification and quantification was performed as detailed in 3.3.6.4.

4.3.5.4 Pigment analysis

Phycocyanin and phycoerythrin contents were determined spectrophotometrically (SpectraMax® M2, USA) using freeze-dried samples according to (Lawrenz et al. 2011) and (Velu et al. 2015) as explained in 3.3.6.5.

4.3.5.5 Elemental analysis

Biomass carbon, hydrogen, nitrogen, sulphur, phosphorous and potassium contents (CHNSPK) (mg g^{-1} DW) of the samples were determined by OEA Labs Ltd. Callington, Cornwall (UK) using an EA-1110 elemental analyser (CE Instruments Ltd, Italy) set up in CHNS mode.

4.3.6 Statistical analysis

Statistical significance of experimental results was evaluated by two-way ANOVA or one-way ANOVA (SADW elemental analyses), with a significance level (α) of 5%, using Statistica v13.2. Normality and homogeneity of variances were determined using P-P plots and the Cochran-Bartlett test, respectively. Data were log-transformed, if ANOVA assumptions of homogeneity of variance or normality were not met. Tukey HSD tests were used to determine the factor driving significance.

4.3.7 Reagents and chemicals

All chemicals and solvents were obtained from Sigma-Aldrich, Sydney, Australia.

4.4 Results

4.4.1 Effect of CO_2 and heavy metals on growth and phosphate uptake of *Tolypothrix* sp.

Over a 25-day time course, CO_2-supplementation resulted in cultivation medium-independent significantly enhanced growth of *Tolypotrix* sp. (Figs.4.1A and B; $p < 0.001$) with 2.4 and 3.3-fold increased final biomass yields of 2.4 ± 0.1 and 2.5 ± 0.1 g DW L^{-1} (Fig. 4.1A) and 2 – 3-fold improved biomass productivities (92.2 ± 6.72 and 100.6 ± 5.1 mg DW L^{-1} d^{-1}) (Fig. 4.1B). The effects medium and CO_2 fertilisation had significant interaction on biomass productivity ($p<0.005$, Appendix Table B.5), due to a marginal effect of medium on non-CO_2 controls. Based on specific growth rate (μ), three phases of growth were distinguishable (μ_{1-3}) (Table 1); μ_1 was ~2-fold higher for CO_2-supplemented cultures irrespective of culture medium used (0.26 ± 0.04), while μ_2 was ≥ 2-fold lower being similar for all culture conditions. A distinctive μ_3 phase was not discernible for SADW-grown non-CO_2 controls and decreased further to no appreciable growth for CO_2-supplemented cultures. Doubling rates (k) for the entire growth period were also ≤ 2-fold higher for CO_2-supplemented cultures than for non-CO_2 controls, resulting in doubling times (t_2) of ~2.5 days compared to 5 and 6 days for non-CO_2 controls (Table 4.1). Low biomass growth parameters of non-CO_2 controls were likely due to carbon-limitation.

Despite effects of CO_2 fertilisation on biomass productivity, phosphate removal from the medium showed no large differences (Fig. 4.1C). Growth of *Tolypothrix* sp. was phosphate-limited as of days 15 and 18 for CO_2-supplemented cultures and non-CO_2 controls, respectively (Fig. 4.1C). Phosphate uptake rates was rapid for the first 3 days (3 – 4 mg PO_4^{3-} g^{-1} DW d^{-1}), halved over the next 3 days and halved again for the following 3 days, showing steady low uptake rates of approximately 0.5 mg PO_4^{3-} g^{-1} DW d^{-1} from day 9 for the remainder of the cultivation period (Fig. 4.1C).

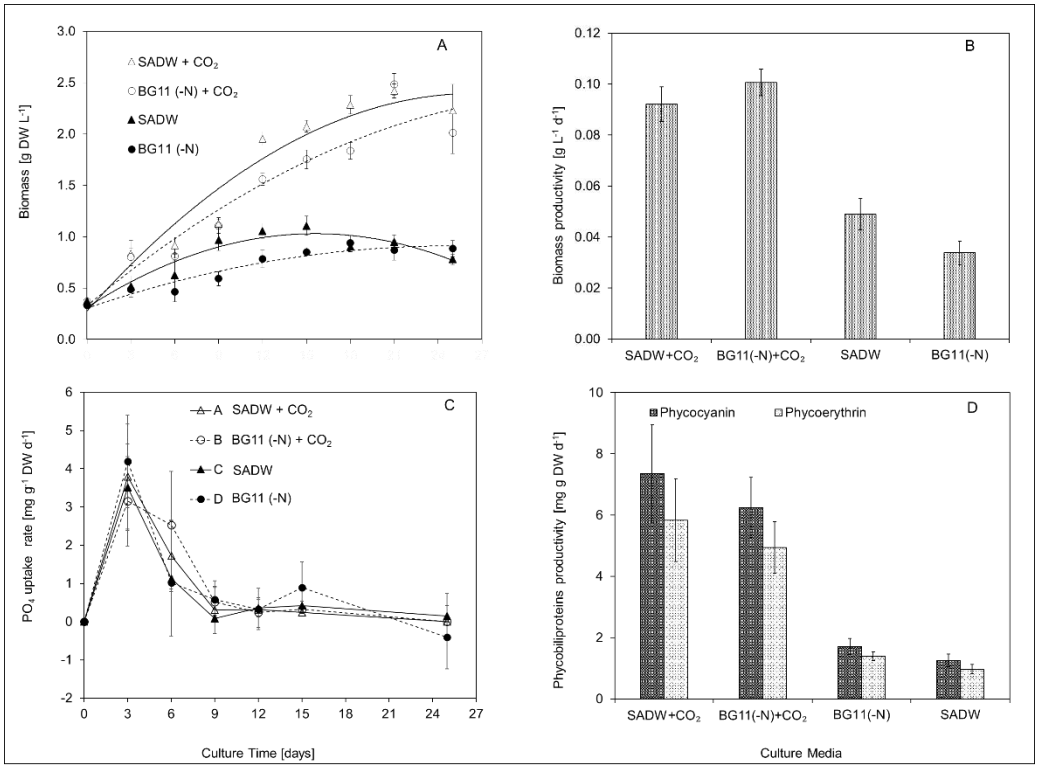


Figure 4.1 Effect of CO_2 and heavy metals on (A): biomass growth; (B): biomass productivity rates; (C): PO_4 uptake rate and (D): phycobiliproteins productivity by *Tolypothrix* sp. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Table 4.1 Effect of CO_2 and heavy metals on growth of *Tolypothrix* sp. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Parameters	Media			
	SADW+CO_2	**SADW**	**BG11(-N) +CO_2**	**BG11(-N)**
Specific growth rate (μ_1) [d^{-1}]	0.26 ± 0.04	0.14 ± 0.02	0.26 ± 0.04	0.13 ± 0.04
(μ_2) [d^{-1}]	0.10 ± 0.02	0.08 ± 0.03	0.07 ± 0.02	0.06 ± 0.02
(μ_3) [d^{-1}]	0.02 ± 0.00	-	0.05 ± 0.00	0.01 ± 0.01
Doubling rate (k) [d^{-1}]	0.38 ± 0.06	0.20 ± 0.04	0.37 ± 0.05	0.18 ± 0.05
Doubling time (t_2)	2.71 ± 0.46	5.15 ± 0.85	2.73 ± 0.41	5.94 ± 2.02

4.4.2 Effect of CO_2 supplementation and heavy metals on the biochemical profile of *Tolypothrix* sp.

4.4.2.1 Carbohydrate, protein, lipid, phycocyanin and phycoerythrin contents

A medium-independent significant positive effect of CO_2-supplementation was observed for carbohydrate, protein and lipid contents (Tables B.10, B.15 and B.20), with largest effects of ~35 and 4-6% increase for carbohydrate and lipid contents ($p < 0.0001$ and 0.0001), respectively. In contrast, although an effect of CO_2-supplementation on protein content ($p < 0.003$) was observed, BG11(-N) CO_2-supplemented cultures were not significantly different to non-CO_2 controls in SADW (Table B.16). Maximal yields of carbohydrate, protein and lipids (~50 ± 1.5, 23 ± 1.3 and 16 ± 0.3 % (w/w), respectively) were achieved for *Tolypothrix* sp. grown in SADW with CO_2-supplementation (Fig. 4.2A).

Similarly, PC and phycoerythrin (PE) contents increased by ~23% under these conditions irrespective of culture medium, yielding maximal contents of ~99 ± 8.3 and 78 ± 7.4 mg g^{-1} DW, respectively (Fig. 4.2B). A medium-independent significantly positive effect of CO_2-fertilisation on PBP-productivity was demonstrated (Tables B.25, B.30, B.26 and B.31), leading to a 5.9- and 3.6-fold increase for PC- and PE-productivity, respectively (Fig. 4.1D).

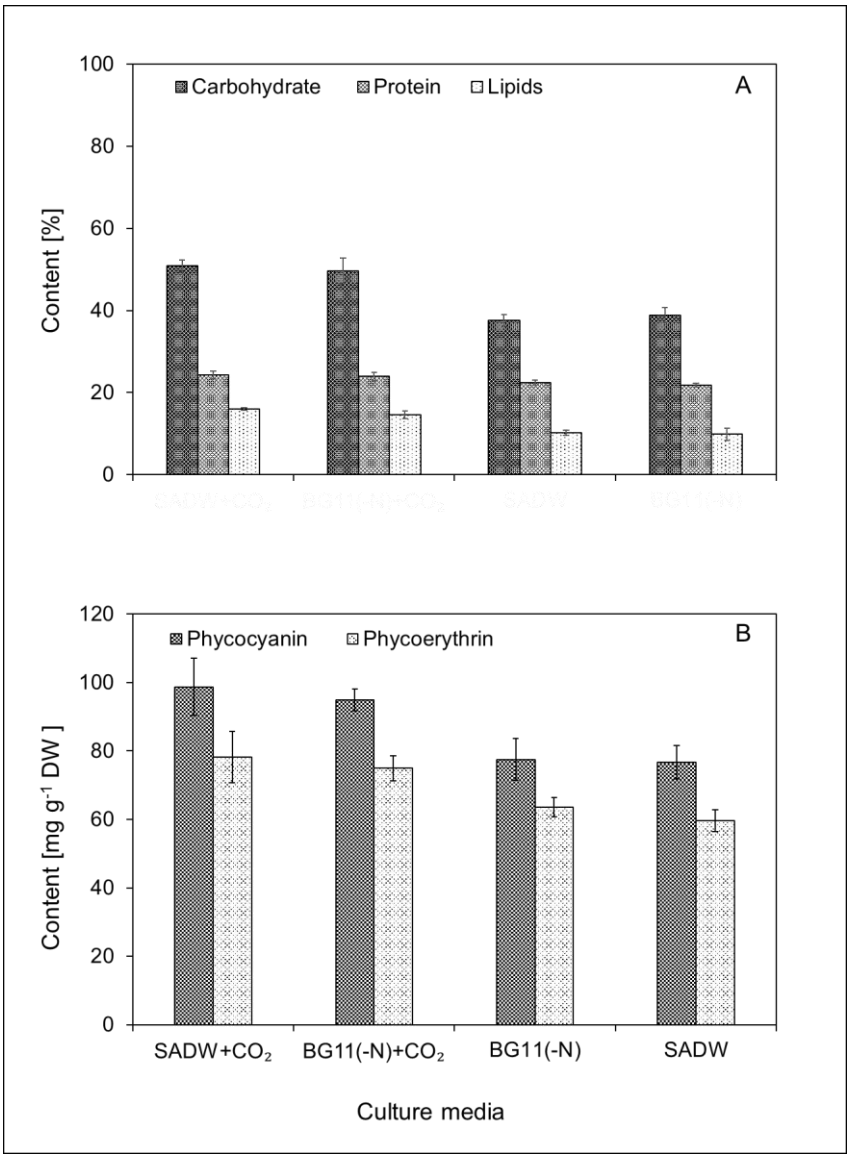


Figure 4.2 Effect of CO_2 and heavy metals on (A) carbohydrate, protein and lipid content; (B) Phycobiliprotein contents. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

4.4.2.2 Fatty acid and elemental compositions

As growth, lipid and PBP contents were significantly increased by CO_2-supplementation but not affected by heavy metals (medium), potential effects on fatty acid profiles and elemental composition (N, P, K and C/N ratio) was investigated. As for total lipids, CO_2-fertilisation resulted in higher total fatty acid (TFA) contents and productivity (Table 4.2; Appendix Tables B.35-36)

The fatty acid (FA) profile of *Tolypothrix* sp. was dominated by saturated fatty acids (SFA) and a similarly high content of polyunsaturated fatty acids (PUFA), with monounsaturated fatty acids (MUFA) representing less than half the amounts of either SFA or PUFA (Table 4.2). SFA, MUFA, and PUFA contents were higher in SADW- than in BG11(-N)-grown *Tolypothrix* sp. irrespective of CO_2-supplementation with highest amounts measured in CO_2-supplemented SADW grown biomass (~22 ± 3, 9 ± 2, and 22 ± 5 mg g^{-1} TFA) (Table 2), but differences were not significant (Appendix Tables B.40, B.44 and B.48). The most abundant FAs in *Tolypothrix* sp. biomass were palmitic acid (C16:0, ~40% of TFA), γ-linoleic acid (C18:3(cis 6, 9, 12), ~27% of TFA) and linoleic acid (C18:2 (cis/trans 9, 12), oleic acid (C18:1, ~11% of TFA) and the ω-3 fatty acid α-linolenic acid (C18:3(cis 9, 12, 15)) (Table 4.2). CO_2-supplementation of SADW-grown *Tolypothrix* biomass showed increased contents of all FAs compared to BG11(-N)-grown CO_2-supplemented biomass and non-CO_2 controls (Table 4.2).

Heavy metals and CO_2-supplementation did not result in large differences in C, H, N, S, P, K contents (Table 3). CO_2-supplementation resulted in slightly higher contents of C (~45 % (w/w) and significantly higher levels of K (~0.825 % w/w; $p < 0.0001$, Appendix Tables B.56-57), while H (7.28 ± 0.38) was highest in SADW and not affected by CO_2-supplementation (Table 4.3).

Significant effects of CO_2-supplementation were also detected for N (7.48 % w/w, $p < 0.02$, Appendix Table B.52) and consequently C/N ratios, which also showed a significant effect of medium, due to SADW grown cultures,

which had the lowest N content (6.88 ± 0.15% w/w) but there was no significant interaction (Appendix Table B.58). Higher P-contents (~0.94% w/w, Table 4.2) in SADW-non-CO_2 controls and BG11(-N)-CO_2 treatments suggested a significant interactive effect of medium*CO_2 fertilisation, although individual treatments had no significant effects (Appendix Table B.54). This was, however, not supported in a Tukey's post hoc analysis (Appendix Table B.55). Cultures with the highest P-content also had the highest sulphur contents (~0.43% w/w, Table 4.3) but treatments were not significant and no significant interaction was determined.

Table 4.2 Effect of CO_2 and heavy metals on fatty acids profiles of *Tolypothrix* sp. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen. *p<0.002.

Fatty Acids [mg g^{-1} TFA]	SADW+CO_2	SADW	BG11(-N) +CO_2	BG11(-N)
14:1 (cis-9)	0.28 ± 0.22	0.19 ± 0.14	0.19 ± 0.07	0.14 ± 0.05
14:0	0.26 ± 0.08	0.38 ± 0.16	0.36 ± 0.11	0.23 ± 0.04
16:1 (cis-9)	2.64 ± 0.62	3.52 ± 0.90	3.07 ± 0.50	2.15 ± 0.03
16:0	21.32 ± 3.42	20.55 ± 3.74	18.95 ± 2.09	17.31± 0.88
18:3 (cis-6,9,12)	14.08 ± 3.55	12.65 ± 2.53	11.69 ± 3.19	9.66 ± 2.33
18:3 (cis-9,12,15)	4.83 ± 1.68	3.46 ± 1.65	2.31 ± 1.23	3.33 ± 0.22
18:2 (cis/trans-9,12)	2.67 ± 0.03	2.60 ± 0.56	1.98 ± 0.65	1.88 ± 0.29
18:1 (cis/trans-9)	6.09 ± 1.64	4.02 ± 0.54	3.63 ± 0.64	3.88 ± 0.14
18:0	0.54 ± 0.05	0.23 ± 0.04	0.28 ± 0.04	0.48 ± 0.03
SUM SFA	22.12 ± 3.55	21.17 ± 3.88	19.59 ± 2.21	18.02 ± 0.91
SUM MUFA	9.00 ± 1.96	7.72 ± 1.56	6.89 ± 1.19	6.17 ± 0.19
SUM PUFA	21.58 ± 5.24	18.71 ± 4.61	15.98 ± 4.85	14.87 ± 2.37
TFA [mg g^{-1} DW]	52.70 ± 9.95	47.60 ± 9.86	42.46 ± 7.89	39.07 ± 2.99
FA productivity [mg g^{-1} DW day^{-1}]	3.97 ± 1.30	0.783 ± 0.22*	2.83 ± 0.84	0.87 ± 0.17*

Table 4.3 Effect of CO_2 and heavy metals on elemental composition of *Tolypothrix* sp. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Elemental Analysis [%]	SADW+CO_2	SADW	BG11(-N) +CO_2	BG11(-N)
Carbon (C)	45.28 ± 0.34	44.68 ± 0.68	45.43 ± 0.11	44.48 ± 0.41
Hydrogen (H)	7.28 ± 0.03	7.28 ± 0.03	7.21 ± 0.03	7.05 ± 0.07
Nitrogen (N)	[1]6.88 ± 0.15	7.48 ± 0.20	7.27 ± 0.35	7.44 ± 0.14
Sulphur (S)	0.35 ± 0.05	0.41 ± 0.03	0.46 ± 0.03	0.38 ± 0.05
Phosphorous (P)	0.56 ± 0.02	0.97 ± 0.12	0.91 ± 0.28	0.71 ± 0.19
Potassium (K)	0.85 ± 0.12	[2]0.49 ± 0.03	0.80 ± 0.11	[2]0.45 ± 0.04
C/N ratio (C: N)	6.58 ± 0.10	[2]6.11 ± 0.08	6.46 ± 0.05	[2]5.93 ± 0.11

[1]$p<0.05$ and [2]$p<0.002$.

Table 4.4 Effect of CO_2 on cumulative metal removal from SADW medium by *Tolypothrix* sp. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

Metals	Initial medium concentration [µg L^{-1}]	SADW+CO_2 [%]	SADW [%]
Al	200 ± 1.0	91 ± 0.3	69 ± 6.9
As	13 ± 0.2	67 ± 0.7	72 ± 2.1
Cu	7 ± 0.2	75 ± 5.6	79 ± 4.4
Fe	1184 ± 3.0	92 ± 0.0	92 ± 0.0
Mo	750 ±11.0	7 ± 1.1	7 ± 1.8
Ni	21 ± 0.4	58 ± 0.5	58 ± 0.2
Se	73 ± 1.0	87 ± 0.3	87 ± 1.8
Sr	831 ±12.0	51 ± 6.8	37 ± 1.4
V	434 ± 4.0	90 ± 1.2	55 ± 5.8
Zn	31 ± 2.6	64 ± 0.7	73 ± 4.6

4.4.3 Effect of CO_2 on metal removal from SADW by Tolypothrix sp.

Removal of metals from SADW, containing concentrations typically occurring in ash dam water of coal-fired power stations, was investigated in 25-day time course experiments (Appendix Table B.1). Cumulative metal removal from SADW medium was ≥ 90% for Al, Fe, and V, followed by Se (~87%), Cu (~75%), Zn (~64%), As (~67%), Ni (~58%), Sr (~51%) and Mo (~7%) in CO_2-supplemented cultures (Table 4.4). Metal removal was the same for Fe, Mo, Ni, and Se with or without CO_2-fertilisation, while they were higher for As, Cu, and Zn in non-CO_2 controls despite lower biomass contents (Table 4.4). Surprisingly, Mo, an essential metal for the nitrogen-fixing nitrogenase complex, was removed at much lower rates of only ~7 % (Table 4.4).

To investigate the effect of CO_2 on metal removal, metal uptake was calculated for time course experiments and values were standardised for biomass [µg metal g^{-1} DW] (Fig. 4.3). Time periods required for metal removal from SADW did not correlate with cellular function. Maximal biomass-standardised uptake was observed within the first 24 h of cultivation for Al, Cu, Fe, Ni and Zn, whilst As, Mo, Se, Sr and V were removed gradually over the cultivation period, with highest removal of V observed on day 25 (Fig. 4.3). In contrast, boron (B) was the only element not removed, irrespective of CO_2 supply (data not shown). Calculated biomass-standardised metal uptake gradually decreased over the time course for metals where highest uptake occurred after 24 h (Fig. 4.3).

CO_2-supplementation had no significant effect on maximal metal uptake for Fe and V (~2,862 ± 131 and 66 ± 7 *vs* 2,914 ± 133 and 60 ± 14 µg g^{-1} DW d^{-}

^{1} for non-CO_2 controls, respectively; Appendix Tables B.65 and B.70), while the removal rate of Zn was negatively affected (40 ± 10 *vs* 62 ± 3 µg g^{-1} DW d^{-1} for non-CO_2 controls; Appendix Table B.71). In contrast, a significant effect of CO_2-fertilisation was determined for Al, As, Cu, Mo, Ni, Se and Sr (Appendix Tables B.62-64 and B.66-69). Biomass-standardised uptake of Al and V was ~25 and 9% higher when supplemented with CO_2 (477.2 ± 22.9 and 65.8 ± 6.9 µg g^{-1} DW, respectively; Fig. $3A_1$ *vs* $3B_1$), as well as for Cu and Ni (11.8 ± 1.0 and 32.7 ± 1.8 µg g^{-1} DW; Fig.$4.3A_3$ *vs* $4.3B_3$). In contrast, highest maximal uptake was recorded for As, Mo, Se and Sr in SADW-non-CO_2 controls (Fig. $4.3B_{1-3}$ *vs* $4.3A_{1-3}$). Maximal Mo, Se, and Sr uptake occurred on day 3 and was 65%, 41% and 66% higher for non-CO_2 controls (Mo and Se: 111 ± 53 and 111 ± 53 *vs* 39 ± 22 and 49 ± 10 mg g^{-1} DW; Fig. $4.3B_2$ *vs* $4.3A_2$ and Sr 604 ± 153 *vs* 227 ± 58 mg g^{-1} DW; Fig. $4.3B_1$ *vs* $4.3A_1$, respectively), while the significance was marginal for As (Fig. $4.3B_3$ *vs* $4.3A_3$).

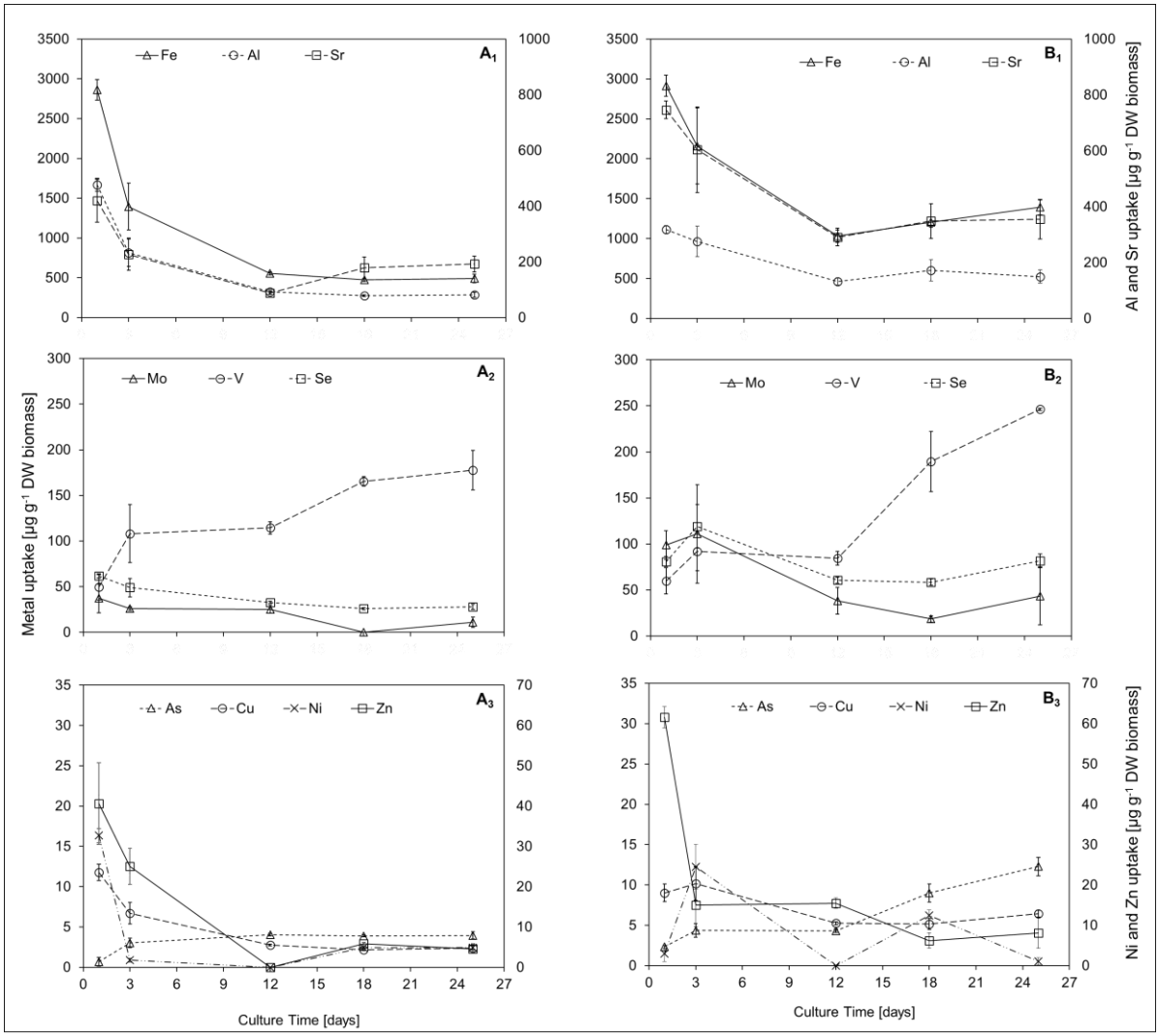


Figure 4.3 Effect of CO_2 on metal uptake from SADW medium by *Tolypothrix* sp. (A) SADW with CO_2 enrichment (B) SADW without CO_2 enrichment. Results are expressed as mean ± standard deviation (n =3). SADW: Simulated ash dam water, BG11(-N): Cyanobacterial growth medium without nitrogen and BG11: Cyanobacterial growth medium with nitrogen.

4.5 Discussion

The exploitation of environmental services of micro- and macroalgae, i.e. abatement of industrial CO_2 emissions and the cleaning of wastewaters, demand sufficiently high biomass productivities and remediation efficiencies (Farrelly et al. 2013). Bioremediation is more attractive, if the biomass produced can yield economically sustainable bio-products. The growth, biomass biochemical contents and profiles, metal removal capacity and biomass-standardised metal uptake of the diazotrophic, non-toxic cyanobacterium *Tolypothrix* sp. is discussed in this context.

Supplementation with 15% CO_2 (v/v) resulted in strongly increased biomass productivities (92.2 ± 6.72 and 100.6 ± 5.1 mg DW L^{-1} d^{-1}) and yields (2.4 ± 0.1 and 2.5 ± 0.1 g DW L^{-1}), indicating that non-CO_2 controls were carbon-limited. Increased biomass productivities and yields were comparable to those observed for 10% CO_2-fertilised cultivation of the diazotrophic cyanobacterium *Anabaena siamensis* under normal batch culture and in a novel WAVE™ bioreactor, respectively (Cirés et al. 2015). In contrast, nitrogen- and 10% CO_2-supplemented cultures of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* achieved 42 to 43% higher biomass yields (Tang et al. 2011). Growth studies are difficult to compare, as inoculation density, light regime and – quality and – intensity, fertilisation conditions, cultivation period and strains, singly or combined affect growth (von Alvensleben et al. 2016). Despite this difficulty, biomass productivity of *Tolypothrix* sp. was comparable to average raceway productivities of 10-15% CO_2-supplemented *Nannochloropsis oculata* (20 g DW m^{-2} d^{-1}, equates to 80 mg L^{-1} d^{-1}) (De Morais and Costa 2007). It was,

however, 5- and 3.7-fold lower than for *N. oculata* biomass produced semi-continuously in a photobioreactor with 2 and 15% CO_2 and 3-times higher photon flux density of 300 µmol photons m^{-2} s^{-1} (Chiu et al. 2009).

Biomass productivities varied from 0.026 to 2.47 g L^{-1} d^{-1} in 2 – 60% CO_2-supplemented microalgal and cyanobacterial species, ie. *Chlorella vulgaris* (Yoo et al. 2010)*, S. obliquus (Kumar et al. 2011), Botrycoccus braunii (Kumar et al. 2011), Spirulina* (*Arthrospira*) sp.(De Morais and Costa 2007), *Anabaena* sp. ATCC 33047 (González López et al. 2009), and *Phaeodactylum tricornutum* (Mazzuca Sobczuk et al. 2000). Highest biomass productivity of 2.47 g L^{-1} d^{-1} was reported for *P. tricornutum* in an airlift photobioreactor with 60% CO_2 (Kumar et al. 2011), growth conditions that are not comparable to those for *Tolypothrix* sp. here (0.335 g L^{-1} d^{-1}). Under near similar culture conditions, biomass productivities of *B. braunii, C. vulgaris, and Scenedesmus sp.* were lower (0.026, 0.105 and 0.217 g L^{-1} d^{-1}) (Yoo et al. 2010) and CO_2 concentrations >10% led to decreased growth (Chiu et al. 2011). In contrast, reported biomass yields for *Spirulina* (*Arthrospira*) sp., supplemented with 12% CO_2 in a photobioreactor, were 1.4-fold higher than for *Tolypothrix* sp. (De Morais and Costa 2007). This is likely due to differences in inoculum size (0.15 g L^{-1} *vs* 0.3 g L^{-1} this study), which could have resulted in light limitation of the *Tolypothrix* sp. cultures, and/or pre-adaptation of *Spirulina* (*Arthrospira*) sp. to CO_2, (De Morais and Costa 2007), as the latter yielded higher biomass productivities (Lee et al. 2002). In summary, biomass yields of *Tolypothrix* sp. with CO_2-fertilisation are adequate, and even superior to other cultures and systems, including *N. oculata* cultured in raceway ponds. Initial fast uptake rates

of phosphate observed for *Tolypothrix* sp. are characteristic for inoculation with phosphate-deplete mother cultures, requiring filling of the intracellular phosphate stores (von Alvensleben et al. 2016), while subsequent low uptake rates indicate limitation by either light or depletion of available external phosphate concentrations (von Alvensleben et al. 2016). Light is one of the key limiting factors for algal biomass productivity as dense cultures induce self-shading (Kumar et al. 2015). As such, light-limitation could have affected biomass productivities and yields in dense cultures of *Tolypothrix* sp. towards the end of the cultivation period (Velu et al. 2015).

In the context of ADW metal remediation, low concentrations of nitrogen and phosphate present are inadequate to sustain growth (Saunders et al. 2012), requiring supplementation, adding significant costs to the process (von Alvensleben et al. 2016). The ability of *Tolypothrix* sp. to completely satisfy nitrogen requirement for growth through fixation of atmospheric nitrogen, significantly reduces cultivation cost. In terms of phosphate utilisation, phosphate-replete cultures of *Tolypothrix* sp. require ~3 to 4 mg PO_4^{3-} g^{-1} DW d^{-1} to maintain biomass productivity, unless light or other factors become limiting, whereas other microalgae require 20 to 90% more phosphate. For example, to produce 1 kg DW of *C. vulgaris*, *H. pluvialis*, *Nannochloropsis* and *Dunaliella tertiolecta* requires 9 – 13, 25 – 30 (Handler et al. 2012) and 4 – 23 g PO_4^{3-} (Chen et al. 2011b), respectively. Significantly lower phosphate fertilisation costs make *Tolypothrix* sp. an ideal candidate for ADW remediation.

A key factor in the production of nitrogen fertilisers is the nitrogen content of the candidate organisms. Generally, diazotrophic cyanobacteria exhibit

significantly higher nitrogen concentrations (8 to 12% of DW) compared to green algae (3 to 8%) (Benemann 1979). Diazotrophic cyanobacteria of diverse genera, including *Tolypothrix*, *Anabaena*, *Nostoc*, and *Aulosira* are being used as inoculants in paddy agriculture in both lowland and upland conditions (Abed et al. 2009; Priyadarshani and Rath 2012). Application of microalgae-based fertilisers led to an increase in soil organic matter and water holding capacity, which was attributed to the high carbon and nitrogen content of the biomass applied (Uysal et al. 2015). *Tolypothrix* sp. biomass contained 45% carbon and 7.5% nitrogen, resulting in a high C/N ratio of 6.58, making it a suitable biofertiliser candidate, in particular in regions were coal-fired power stations are located near agricultural production, as is the case for Tarong Power Station in this study. Quantitative approaches, using QUEFTS (Quantitative Evaluation of Fertility of Tropical Soils), modeled N, P, and potassium (K) requirements for producing 1 tonne of wheat as 23.1, 3.5, and 28.5 kg, respectively (Pathak et al. 2003). Likewise, on-farm experiments conducted in North China to determine optimum N application rates for *Triticum aestivum* L. (winter wheat) estimated the optimal range for N application to be 12 - 22 kg N ha^{-1} per tonne (Cui et al. 2010). Translating this to the potential of *Tolypothrix* sp. as an N-fertiliser would require the application of 0.17 t ha^{-1} – 0.30 t ha^{-1} of *Tolypothrix* sp..

The utilisation of *Tolypothrix* sp. biomass as an N-fertiliser could raise concerns regarding metal toxicity to plants when using metal-containing ADW biomass production. Although trace metals are naturally present in soils and are required for plant health and function, increased concentrations can be harmful to both animals and plants (Chibuike and Obiora 2014). The minimum

permissible levels (mg kg^{-1} of soil) of context-relevant trace metals in agronomic crops are As: 0.43; Cr: 2.30, Ni: 67.90; Cu: 73.30; and Zn: 99.40 (Al-Othman et al. 2016). Based on metal uptake by *Tolypothrix* sp. from SADW and applied to the above nitrogen application requirements of wheat, calculated trace metal levels in ADW-raised *Tolypothrix* sp. biomass would be below thresholds and unlikely to elicit toxicity effects in wheat (Al: 0.048, As: 0.006, Cu: 0.001, Ni: 0.003, Se: 0.016, Sr: 0.110, V: 0.101 and Zn: 0.006 mg kg^{-1} soil).

Cyanobacteria are a prolific natural source of high-value bioproducts e.g. PBPs (PC and PE), carotenoids, and mycosporine-like amino acids) with applications in the food, biomedical and pharmaceutical industries (Liu et al., 2014). Cultivation in ADW would require a biorefinery approach to extract and purify high-value compounds and to overcome the problem of metal contamination. This approach is feasible for the high-value PBP market worth $60 million per year (Cirés et al. 2015). The observed CO_2-induced increase in PBP-productivity and content of *Tolypothrix* sp. could be attributable to enhanced light harvesting requirements in the much denser cultures (Zeng et al. 2012). Compared to *Tolypothrix* sp., an 80% higher PBP-content and higher productivities were reported for the non-nitrogen fixing cyanobacterium *Spirulina platensis* (now *Arthrospira platensis*) (Jiménez et al. 2003a). PBP production in other diazotrophic cyanobacteria such as *Anabaena* sp. was about 70% higher than reported here for *Tolypothrix* sp. (Moreno et al. 2003; Velu et al. 2015), but both cyanobacterial genera were grown in outdoor conditions under natural sunlight. Nitrogen-fixation could limit PBP accumulation, as PBP stores can serve as a nitrogen reservoir available to cells under nitrogen-limiting growth

conditions (Kromkamp 1987). To substantiate this hypothesis will require comparison to *Tolypothrix* sp. biomass raised in nitrogen-containing BG11 under identical cultivation conditions and with the same strain. Although PC production by *Tolypothrix* sp. was lower than reported for other species, in the context of reducing remediation costs, it is worthwhile considering a biorefinery approach, as the market value of the pigment is US$ 3,000 kg^{-1}.

In the present study, CO_2- supplementation did not significantly increase protein content, but carbohydrate and lipid levels increased significantly. This suggests that carbon could be diverted to storage as carbohydrates and to a certain extent lipid in cultures approaching stationary growth phase. Achieved protein content of *Tolypothrix* sp. was 2-3-fold lower than reported for the commercially produced *Spirulina* (*Arthrospira*) sp., *Chlorella* (Tokuşoglu and Üunal 2003) and *Scenedesmus* (Apandi et al. 2017), but comparable to the commonly used aquaculture-feed microalga *Isochrysis* (Tokuşoglu and Üunal 2003). The relatively low protein content achieved in *Tolypothrix* sp. in metal-rich SADW under the cultivation conditions does not seem warrant a biorefinery approach as a value-add product, unless bioactive peptides of high value can be recovered.

In contrast, carbohydrate content of *Tolypothrix* sp. was comparable to those reported for other microalgae such as *Nannochlororopsis* sp. (15 – 50%), *Porphyridium cruentum* (40 – 57%) *Isochrysis zhangjiangensis* (48%) and *Scenedesmus* (42 – 53%) (González-Fernández and Ballesteros 2012), but the use of metal-rich SADW would limit exploitability as a product in its own right.

As the primary carbohydrate storage form in cyanobacteria is easily fermentable starch (Möllers et al. 2014), fermentation to bioethanol could be considered.

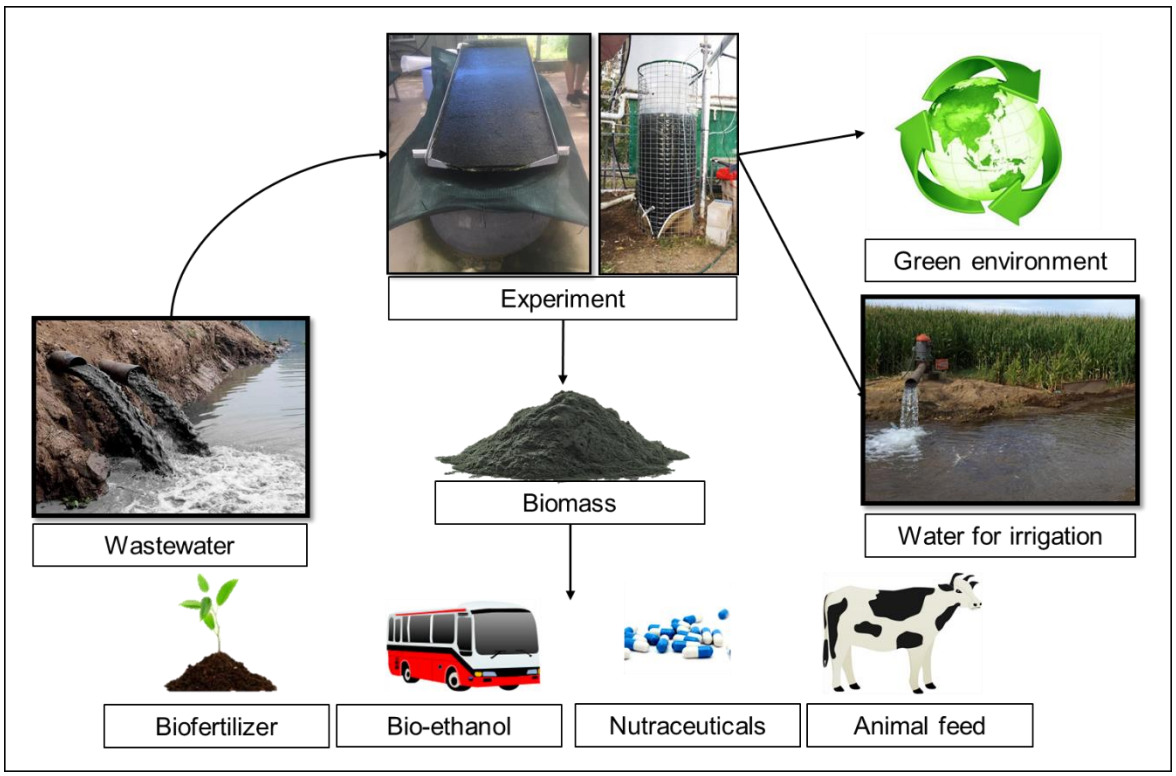
SADW with CO_2 supplementation yielded highest amounts of TFA (5.2% of DW) in *Tolypothrix* sp., dominated by SFA and PUFA, with MUFA contents being half of these. Unlike negative responses to CO_2-fertilisation reported for *C. vulgaris* (Tsuzuki et al. 1990), no significant were determined for *Tolypothrix* sp.. Considering their proclaimed health benefits, PUFAs, including γ-Linolenic acid (C18:3 ω-6; an ingredient in cosmetics) and α-Linolenic acid (C18:3 ω-3), are intensively investigated (Ryckebosch et al. 2012). γ-Linolenic acid content of *Tolypothrix* sp. was 27% of TFA (4.83 mg g^{-1} TFA), being 1 – 1.5-times higher than reported for *Spirulina* (*Arthrospira*) spp. (11-16%) (De Oliveira et al. 1999) In a biorefinery context, however, it would not be sufficiently high to warrant the extra costs for biomass drying and processing.

4.6 Conclusion

This study demonstrated excellent growth responses to CO_2 and metal removal capacity of *Tolypothrix* sp. when cultivated in SADW without nitrogen fertilisation, making *Tolypothrix* sp. an outstanding candidate for bioremediation of CO_2 and metals at freshwater-utilizing coal-fired power stations. Based on growth data obtained, an estimated 1.98 tonne ha^{-1} biomass can be produced in a year set at 300 days of cultivation. While the cultivation conditions, as well as low protein and lipid contents prohibit use of *Tolypothrix* sp. as a nutraceutical or the application of a full biorefinery fractionation approach, a limited biorefinery approach for value-adding product synthesis is advisable. Based on obtained data, 5.88 kg PC could be produced ha^{-1} Y_{prod}^{-1}, worth ~US$ 17,640.

The residual biomass could be either hydrolysed or anaerobically digested. With a 50% primarily starch-based carbohydrate content, hydrolysed biomass could be fermented to produce bioethanol with an estimated production of ~ 548 L ha^{-1} Y_{prod}^{-1} worth ~US$ 816.52. Alternatively, ~792 L CH_4 d^{-1} could be produced via AD. Based on mineral content of biomass raised under these conditions, *Tolypothrix* can provide mineral-rich biofertiliser to plants without metal toxicity. Based on N-content of *Tolypothrix* sp., ~0.149 t N fertiliser ha^{-1} Y_{prod}^{-1} can be expected, worth US $74.25.

Chapter 5: First outdoor cultivation of the N_2-fixing cyanobacterium *Tolypothrix* sp. in low-cost cultivation systems in tropical Australia[5]



[5] Part of the research from this chapter was invited for oral presentation at ISAP conference Sydney, Australia in 2014.

This chapter presented as published in Journal of Applied Phycology except for changing the format to match that of the rest of this thesis.

Velu, C., Cirés, S., Alvarez-Roa C and Heimann, K., First outdoor cultivation of the N_2-fixing cyanobacterium *Tolypothrix* sp. in low-cost suspension and biofilm systems in tropical Australia J Appl Phycol 27:1743-1753.

5.1 Abstract

Tropical N_2-fixing cyanobacteria offer an attractive alternative for production of biomass and bioproducts with potentially low cultivation and harvesting costs. The present study evaluated the biomass productivity of the N_2-fixing cyanobacterium *Tolypothrix* sp. NQAIF319 grown in nitrogen-free medium in outdoor suspension and biofilm prototype cultivation systems in tropical Australia (Queensland). One-week cycles yielded maximum biomass productivities -estimated based on ground area occupied by single systems- of 45-49 g dry weight m^{-2} day^{-1} (suspension) and 1.0-1.2 g dry weight m^{-2} day^{-1} (biofilm) with minimal biological contamination (*Tolypothrix* sp. biomass representing 94-98 % of the photosynthetic community). Moderate productivities of the pigments phycocyanin/ phycoerythrin (0.1-2.8 g m^{-2} day^{-1}), fatty acids (0.1-2.0 g m^{-2} day^{-1}) and nitrogen stored in the biomass (0.1-5.9 g m^{-2} day^{-1}) were reached in biofilm and suspension systems, respectively, opening avenues for production of low-value commodities with potentially big markets (nitrogen-rich biofertilisers and aquaculture feed) and higher-value chemicals (phycobiliproteins and fatty acids). Simulated multi-system arrangements yielded theoretical overall areal productivities 4-6 times lower than those in single systems thus highlighting the need for future tests fine-tuning inter-system separation to minimise shadowing while maximizing the efficiency in land use in larger-scale production plants. Biofilm and self-flocculated biomass showed 80-fold and 53-fold reduced extracellular-water contents compared to suspension cultures, respectively, which will need to be considered for techno-economic and water/carbon footprint evaluation of each of the possible

bioproduct synthesis pathways. In conclusion, the flexible and simple prototypes developed together with the good properties of *Tolypothrix* sp. represent a promising platform for low cost production of cyanobacterial bioproducts in tropical regions using low nitrogen-containing water sources.

5.2 Introduction

Since the 1950´s, controlled mass production of photosynthetic microorganisms ("microalgae farming") has been explored as a sustainable "green" bioenergy and bioproducts source not competing for arable land (Benemann 2013). Despite its promising start, the bulk of commercial production of microalgal bioproducts is currently restricted to certain high-value chemicals (i.e., pigments – phycocyanin, β-carotene and astaxanthin – and essential fatty acids –Eicosapentaenoic acid, EPA, and Docosahexaenoic acid, DHA –) (Borowitzka 2013)and a few genera of eukaryotic microalgae: *Chlorella* (4000 t biomass year^{-1}) *Dunaliella* (1000 t year^{-1}) and *Haematococcus* (200 t year^{-1}) (Benemann 2013). The contribution of the photosynthetic prokaryotic cyanobacteria is almost entirely restricted to the filamentous non-nitrogen fixer *Spirulina* (*Arthrospira*) (10,000 t biomass year^{-1}) (Benemann 2013) for the production of phycocyanin and health food supplements rich in amino acids and ω-3/ω-6 fatty acids (Sili et al. 2012).

Mass production of microalgae has been traditionally performed in suspension cultures grown in open, closed (photobioreactors, PBRs), and hybrid systems. Closed and hybrid systems can theoretically provide the highest productivities, but their current use is mostly restricted to scientific and pilot studies, due to their very high building and maintenance costs together

with additional problems like high shear stress, oxygen build-up, overheating and, especially, fouling (Singh and Ahluwalia 2013). Open systems (e.g. raceway ponds) have proven cost effective and are hence the selected system for most commercial production applications (Borowitzka and Moheimani 2013). However, suspension-based open systems face a series of challenges compromising the economic feasibility of most potential products, such as: excessive cultivation costs due to nutrient provision and mixing; contamination by exogenous algae different from the target species; and, especially, prohibitive dewatering costs (e.g. by centrifugation, filtration or chemical flocculation) that may contribute up to 20-30% of the total biomass production costs for certain applications (Molina Grima et al. 2003).

N_2-fixing cyanobacteria are photosynthetic prokaryotes offering a promising alternative to address some of these challenges. These organisms can be cultivated under low nutrient concentrations due to their ability to fix atmospheric N_2 and their optimized phosphorus uptake (e.g. by either accumulating phosphorus into phosphate granules or use of organic phosphorus) (Mateo et al. 2010). Due to their remarkably flexible metabolism, N_2-fixing cyanobacteria are a prolific source of bioproducts including high-value pigments (phycocyanin and phycoerythrin), moderate-value omega-3 and omega-6 fatty acids for nutraceuticals or aquaculture feed, and co-products with low value yet potentially broad markets like biofertilisers (i.e. as a source of nitrogen newly incorporated from the atmosphere) (Sharma et al. 2014). Furthermore, strains of certain N_2-fixing cyanobacteria, e.g. *Tolypothrix tenuis*, are renowned for their self-flocculating ability (Silva and Silva 2007) that could

potentially reduce dewatering costs in suspension culture. Additionally, as usual components of biofilms naturally present in waters and soils of all latitudes (Whitton 2012), cyanobacteria offer a source for exploring biofilm cultivation as an alternative to produce biomass with lower content of extracellular water than suspension cultures (Bruno et al. 2012; Ozkan et al. 2012).

Biofilm systems have been scarcely studied for mass production of photosynthetic microorganisms aiming at bioproducts, with most focussing on green microalgae (e.g. *Botryococcus braunii*) (Ozkan et al. 2012) and less frequently on cyanobacteria (Bruno et al. 2012). However, there is extensive knowledge on the use of biofilm-based indoor and outdoor systems for remediation of a variety of wastewaters (Kesaano and Sims 2014), with the "algal turf scrubber" being one of the most widely used systems. The Algal Turf Scrubber®-ATS™ technology from Hydromentia (http://www.hydromentia.com) consists of a sloped flow way (e.g. fabricated with a plastic liner covered by nylon netting (Mulbry et al. 2008)) across which polluted water is pulsed in waves allowing growth of algal turfs or dense mats of algae to remediate nitrogen and phosphorus and use the biomass for compost. Such a simple and flexible growth principle invites to combine ATS with different inexpensive, reusable and low-weight substratum materials. Of the six materials tested, polystyrene foam proved to be the most effective substratum for growth of small-scale biofilms (Johnson and Wen 2010). Inoculation with organisms (e.g. N_2-fixing cyanobacteria) other than the eukaryotic microalgae-dominated films traditionally described in ATS studies was also successful (Kesaano and Sims 2014).

Among the diversity of N_2-fixing cyanobacterial taxa (e.g. more than 100 heterocystous genera described in a recent compilation by Komárek (2013)), the present study focused on a strain of *Tolypothrix* sp. isolated from Queensland, tropical Australia. The strain *Tolypothrix* sp. NQAIF319 was selected based on tests conducted prior to this study that evidenced moderate-high productivities in nitrogen-free medium $BG11_0$ and satisfactory attachment to polystyrene foam (Samuel Cirés, personal communication). The aim of the present study was to evaluate the productivities of biomass and bioproducts (pigments, fatty acids and biofertilisers) by *Tolypothrix* sp. NQAIF319 grown outdoors in nitrogen-free medium in two low-cost meso-scale suspension and biofilm-based mass production prototypes. Results will provide initial insight into the conditions necessary for the future development of larger scale systems for production of cyanobacterial biomass in tropical areas using nitrogen-poor water sources (e.g. contaminated groundwater or metal-rich ash dam waters from coal mines).

5.3 Methods

5.3.1 Strain characterisation

The N_2-fixing filamentous cyanobacterial strain *Tolypothrix* sp. NQAIF319 was isolated in 2012 from a seasonal creek in Townsville (Queensland, tropical Australia). In order to assess the suitability of *Tolypothrix* sp. NQAIF319 for mass production and commercial purposes, the absence of the most common cyanotoxins (anatoxin-a, cylindrospermopsin, microcystins and saxitoxins) was proven using commercial ELISA kits and molecular methods (PCR of *mcy*, *ana*,

cyr and *sxt* genes involved in toxin biosynthesis) following protocols detailed in (Cirés et al. 2014) (data not shown).

Stock cultures were maintained at the North Queensland Algal Identification and Culturing Facility (NQAIF, James Cook University, Townsville, Australia) as bubbled suspension batch cultures in nitrogen-free medium $BG11_0$ (Rippka et al. 1979) at 28°C under 12:12 light: dark photoperiods at a light intensity of 30 μmol photons m^{-2} s^{-1}. Biomass productivity and bioproduct content (%w/w) of the strain were characterised in 2-L air-bubbled suspension batch cultures grown in triplicate for 4 weeks in $BG11_0$ medium at 28 °C under two light intensities (30 μmol photons m^{-2} s^{-1} and 100 μmol photons m^{-2} s^{-1}). Additionally, bubbled suspension batch cultures were grown in triplicate in the same medium and temperature conditions (BG11(-N), 28 °C) with high light intensity (100 μmol photons m^{-2} s^{-1}) and supplied with 0.1 L min^{-1} of 10% CO_2 (v/v) as inflow gas. Biomass productivity (g L^{-1} day^{-1}) was calculated from dry weight (g DW L^{-1}) determined by gravimetric analysis (von Alvensleben et al. 2013) in 40 mL culture samples taken on days 0 and 28. Additionally, culture samples taken on day 28 were centrifuged (8000 x *g*, 20 min), and the pellets were freeze-dried and stored in darkness at -80°C until analysed for the contents (mg g^{-1} dry weight) of pigments (phycocyanin, phycoerythrin, chlorophyll *a*), fatty acids (fatty acid methyl esters, FAMEs), and carbon-hydrogen-nitrogen (CHN) following the analytical procedures detailed below.

5.3.2 Outdoor experimental setup

Outdoor experiments were carried out in two meso-scale open bioreactor prototypes designed and assembled at James Cook University, Australia

(Appendix Fig. C1 and C2, Appendix Tables C1 and C2) For the experiments, the two systems were located in 50%-shaded open culture areas at the Marine and Aquaculture Research Facilities Unit (MARFU, James Cook University) in Townsville, Australia (Latitude 19.33 S, Longitude 146.76 E). The ATSs were 2.2 m long, 1 m wide with a slope of 7% consisting of plastic trays with a polystyrene surface. Water flow conditions were continuous with 0.6 m s^{-1} delivered from a sump with a 600 L fill volume beneath the ATS (Appendix Table C2, Figs. C1). The 400 L vertical bubbled suspension cultures systems were constructed from PVC bag material contained in a wire cage with a plastic keeled footing of 0.3 m^2 (Appendix Figs C1A-B). A tap was fitted to the bottom corner of the vertical bag for harvesting, while aeration was provided by a suspended diffuser delivering 0.05 L of air per L of culture per minute from the main compressor supplying aeration to all systems at MARFU (Appendix Table C1).

Laboratory cultures of *Tolypothrix* sp. NQAIF319 were grown in aerated 20 L sterile plastic carboys under the same culturing conditions detailed above (BG11(-N) medium, 28°C, 100 µmol photons m^{-2} s^{-1}) until reaching stationary phase. For inoculation of the vertical bag system, the cultured biomass was centrifuged (8000 x *g*, 20 min) and an adequate volume of the cell pellet was resuspended into 400 L of fresh $BG11_0$ medium to reach an initial biomass concentration of 0.1 g dry weight L^{-1}. For biofilm inoculation, another aliquot of the centrifuged biomass was spread homogeneously over the polystyrene foam surface up to an initial biomass of 5 g dry weight m^{-2}. The water flow was turned off overnight to facilitate biomass attachment to the polystyrene surface, after

which water flow resumed upon start of the experiment. 2-week growth experiments were simultaneously performed in both systems in two consecutive runs, during March 2014 (run 1) and May 2014 (run 2). Temperature and pH were monitored continuously by NTC030HP03 plastic temperature sensors (CAREL, Australia) and WQ201 pH sensor (Global Water, USA) submerged in the culture media. Irradiance was measured at the time of sampling using a LI-250A photosynthetic active radiation (PAR) light meter (LI-COR Biosciences, USA). Culture samples for monitoring growth, biochemical profiles and bioproducts were taken from a tap located at the bottom of the vertical bag (Fig. S1). In the modified turf scrubber, samples for the same purposes were taken by scraping the biomass contained in 100 cm^2 (10 cm x 10 cm) biofilm squares using a silicone rubber cell scraper (IWAKI, Japan).

5.3.3 Growth and Tolypothrix sp. dominance in outdoor cultures

Growth was monitored as dry weight (DW) (g L^{-1} in suspension -; g cm^{-2} in biofilm cultures) determined in 40 mL suspension culture samples (vertical bag) and 100 cm^2 biofilm samples (modified algal turf scrubber) taken every second day during the two-week growth period. In the suspension system, areal biomass (g m^{-2}) was calculated from dry weights (g L^{-1}) by considering the volume of culture medium (400 L) and the surface of the system´s base (0.28 m^2). In the turf scrubber, the dry weight (g cm^{-2}) was directly extrapolated to g m^{-2}.

Tolypothrix sp. dominance was evaluated by light microscopy in 15 mL culture samples (vertical bag) and 100 cm^2 biofilm samples that were taken on days 7 and 14, fixed with acidic Lugol and kept in darkness at 4 °C until

analysed. 1 mL aliquot of the fixed samples was mounted in a Sedgewick-Rafter Chamber and analysed under an Olympus CKX41 inverted microscope equipped with an Olympus SC-30 camera. The surface (μm^2) of all filaments and cells of cyanobacteria and microalgae present in the whole chamber were measured using the software CellSens Standard version 1.6 (2011). The relative abundance of *Tolypothrix* sp. was then calculated as percentage surface by dividing the surface of *Tolypothrix* sp. (μm^2) over the total surface of cyanobacteria and microalgae (μm^2).

5.3.4 Self-flocculation test in outdoor suspension cultures

To evaluate the potential use of self-flocculation to harvest *Tolypothrix* sp. biomass in future experiments, triplicate 1-L culture samples were taken after 1-week growth in both runs of the suspension cultures (vertical bag). The culture samples were introduced into a 1-L glass measuring cylinder and left undisturbed for 2 minutes (Appendix Fig C3). Dry weights ($g\ L^{-1}$) of the original suspension and the biomass accumulated at the bottom of the cylinder were measured separately. The concentration factor (Cf) was calculated as the ratio between the dry weight ($g\ L^{-1}$) of the biomass pellet and the dry weight of the initial suspension ($g\ L^{-1}$).

5.3.5 Biochemical profile and bioproducts

Samples from laboratory and outdoor cultures were analysed for their basic biochemical profile (carbohydrate -, protein - and total lipid contents), the content of bioproducts (pigments –phycocyanin, phycoerythrin, chlorophyll *a*- and fatty acids) as well as the content of nitrogen (from carbon-nitrogen-hydrogen contents) and phosphorus (total phosphorus) accumulated in biomass

as described in section 3.3.6. During the outdoor experiments, 2-L samples taken from the vertical bag on days 7 and 14 were centrifuged (8000 x *g*, 20 min) and the pellets were freeze-dried and stored in darkness at -80°C until analysed. For the modified algal turf scrubber, three 100 cm^2 biofilm samples scraped as described above were pooled, centrifuged (8000 x *g*, 20 min) and the pellets were freeze-dried and stored in darkness at -80°C until analysed (Velu et al. 2015).

5.4 Results

5.4.1 Characterisation of *Tolypothrix* sp. laboratory cultures

Aerated *Tolypothrix* sp. cultures were characterised for their biomass productivity and bioproduct contents under two light intensities of 30 µmol photons m^{-2} s^{-1} (the intensity at which stock cultures were maintained in the laboratory) and a higher light intensity of 100 µmol photons m^{-2} s^{-1} (closer to the lowest values of irradiances available outdoors Appendix Table C3 in the Supplementary material). The higher light intensity induced a significantly higher biomass productivity being 1.7-fold of the productivity at 30 µmol photons m^{-2} s^{-1}, with increased N contents but with a 1.4-2.3-fold reduction in pigment contents ($p<0.05$, t-tests) (Table 5.1). At 100 µmol photons m^{-2} s^{-1}, aerated cultures produced 41.1 mg dry weight L^{-1} day^{-1}, containing 0.8-2.0% w/w pigments, and 8.2 % w/w nitrogen (Table 5.1). Under 100 µmol photons m^{-2} s^{-1}, supplementation of cultures with 10% (v/v) CO_2 generated an additional 2.4-fold increase in biomass productivity, but significant decreases of phycocyanin, phycoerythrin and nitrogen contents ($p<0.05$, t-test). In contrast, chlorophyll *a* content was not significantly affected by the addition of CO_2 ($p>0.05$, t-test)

(Table 5.1). Carbon and fatty acids contents were not significantly affected by either light intensity or CO_2 and remained stable representing 45.5-46.8% and 3.8-4.7% of the dry weight, respectively (Table 5.1)

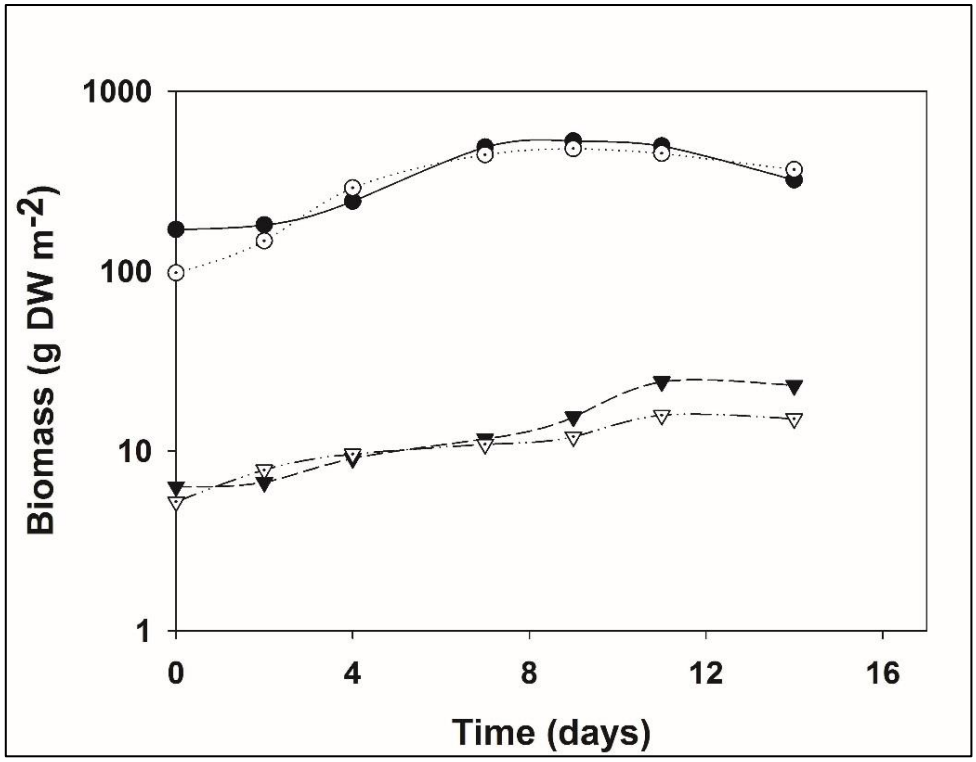


Figure 5.1 Growth of *Tolypothrix* sp.-dominated outdoor cultures grown in suspension and biofilm mass production systems during two consecutive 14-day runs. Biomass growth (g DW m^{-2}) calculated based on ground area occupied by a single system. Growth results (g dry weight m^{-2}) in vertical bag (suspension) cultures are represented by black circles (first run) and white circles (second run). Growth results (g DW m^{-2}) in modified turf scrubber (biofilm) cultures are represented by black triangles (first run) and white triangles (second run). Y axis is in logarithmic scale.

5.4.2 Growth, biomass productivities and *Tolypothrix* sp. dominance in outdoor systems

Two-week growth curves of outdoor cultures are shown in Figure 5.1. Within each culture system, the two runs showed similar growth trends (Fig. 5.1). In suspension cultures, the areal biomass yield (g m^{-2}) peaked on day 7, remained stable for 4 days and started to decline on day 11. Biofilm growth

was slower but more sustained, with the peak reached on day 11 followed by a slight decrease until day 14.

The growth trends were mirrored by the daily biomass productivity (g m^{-2} day^{-1}) behaviour of both systems (Fig. 5.2). In suspension, the average daily biomass productivities were clearly higher for the first week than for the overall two weeks in both runs. In fact, biomass productivities during the first week (45.4-49.2 g m^{-2} day^{-1}) were 2.6-4.2-fold those of the overall bi-weekly period (10.9-19.2 g m^{-2} day^{-1}). Biofilm systems were characterised by lower and more stable biomass productivities reaching 1.0-1.2 g m^{-2} day^{-1} in run 2 and run 1, respectively, with slight differences (<1.5-fold) between the weekly and the bi-weekly productivities (Fig. 5.2).

Microscopic observations confirmed that both types of outdoor cultures contained not only *Tolypothrix* sp. but also variable quantities of eukaryotic microalgae and, in some samples of the biofilm, minor amounts of filamentous oscillatorial cyanobacteria (data not shown). Small bacterial cells were also observed, but concentrations were negligible compared to the biomass represented by photosynthetic organisms. *Tolypothrix* sp. clearly dominated the photosynthetic community during the first week in both systems and runs, representing 94.8% (first run) to97.9% (second run) of the microalgal-cyanobacterial community in suspension cultures and 98.5 (first run) to99.2% (second run) in biofilms (Fig. 5.3).

Table 5.1 Laboratory characterisation of the *Tolypothrix* sp. used to inoculate the outdoor cultivation systems. Results are expressed as mean ± standard deviation (n =3). Chla: chlorophyll a; C, carbon; N, nitrogen

Species	Light (μmol photons m^{-2} s^{-1})	Gas	Biomass (mg DW L^{-1} d^{-1})	PC (% w/w)	PE (% w/w)	Chla (% w/w)	Fatty acids (% w/w)	C (% w/w)	N (% w/w)
Tolypothrix sp.	30	Air	23.8 ± 5.3	3.5 ± 0.5	3.2 ± 0.5	1.8 ± 0.1	3.8 ± 0.8	46.8 ± 0.9	7.3 ± 0.01
	100	Air	41.1 ± 9.1	2.0 ± 0.3	1.4 ± 0.2	0.8 ± 0.1	4.1 ± 0.9	45.5 ± 0.8	8.2 ± 0.02
	100	CO_2	99.9 ± 22.2	1.2 ± 0.2	1.0 ± 0.1	0.9 ± 0.1	4.7 ± 1.0	45.5 ± 0.8	7.4 ± 0.01

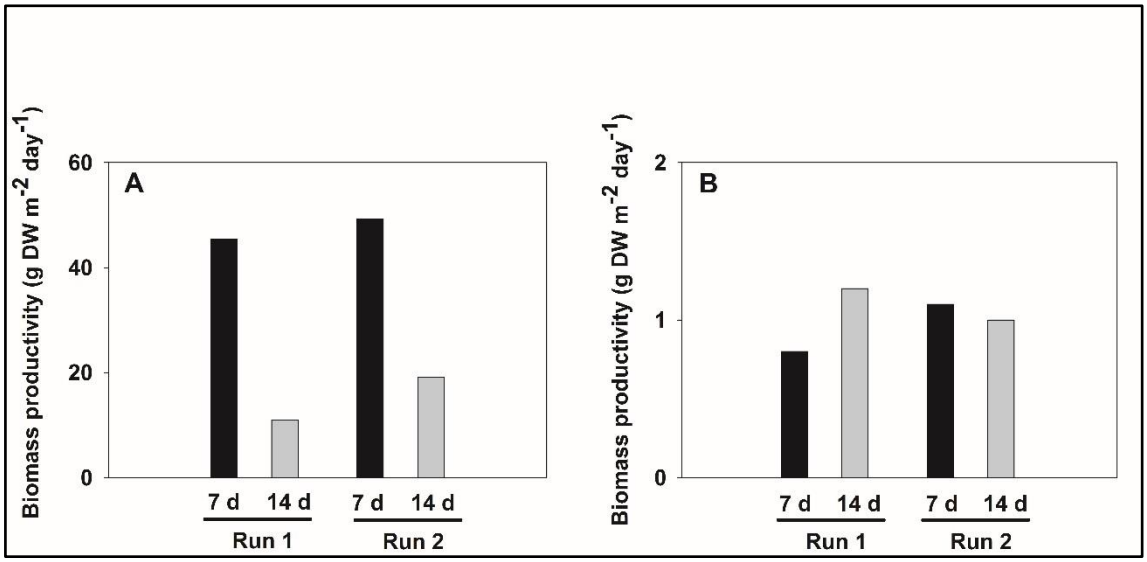


Figure 5.2 Biomass productivity of *Tolypothrix* sp.-dominated outdoor cultures grown in suspension (a) and biofilm (b) mass production systems during two consecutive 14-day runs. Areal productivity (g DW m^{-2} d^{-1}) calculated based on ground area occupied by a single system. 7 d: average daily productivity over 7-day growth; 14 d: average daily productivity over 14-day growth.

However, samples taken on day 14 were characterised by a decrease in *Tolypothrix* sp. dominance in both systems along with sharp differences between the two runs. In the first run, green algae subdominated the biofilm community and clearly dominated the suspension community, with *Tolypothrix* sp. population representing 52.5% in biofilms (Fig. 3B) and a very low 5.4% in suspension cultures (Fig. 5.3A) after 14 days. Contrastingly, during the second run *Tolypothrix* sp. remained dominant on day 14, representing 91.9% and 75.9% of the suspension culture and biofilm communities, respectively.

Table 5.2 Performance of cyanobacterial-based open cultivation systems. Areal productivities based on ground area occupied by single systems. 1 *Synechococcus* sp., *Pseudanabaena* sp., *Phormidium* sp.; 2 Culture medium without nitrogen with composition detailed in (Moreno et al. 1995); 3 Several N-containing media with compositions detailed in (Borowitzka and Moheimani 2013) and references therein; 4 Country abbreviations: SP, Spain; IS, Israel; USA, United States of America; CH, Chile; IT, Italy; TH, Thailand; ME, Mexico; AU, Australia.

Culturing mode	System	Species	Culture medium	Location [4]	Biomass productivity (g m^{-2} day^{-1})	References
Suspension	Raceway pond	*Anabaena* sp.	Detailed in [2]	Outdoors (SP)	9.4-23.5	(Moreno et al. 2003)
		Gloeotrichia natans	$BG11_0$	Outdoors (IS)	14.7-18.1	(Querijero-Palacpac et al. 1990)
		Spirulina (*Arthrospira*) spp.	Several (with N) [3]	Outdoors (SP, IS, USA, CH, IT, TH, ME)	8.2-27	Reviewed in (Borowitzka and Moheimani 2013)
	Aerated vertical bag	*Tolypothrix* sp.	$BG11_0$	Outdoors (AU)	10.9-49.2	This study
Biofilm	Flow-lane incubator with polycarbonate slides	Mixed culture (cyanobacteria [1] and green algae)	Modified BG11	Indoors	0.02-2.9	(Guzzon et al. 2008)
	Flow-lane incubator with polycarbonate slides	*Phormidium autumnale*	Modified BG11	Indoors	3.3	(Bruno et al. 2012)
		Anabaena augstumalis	$BG11_0$		3.3	
		Trichormus varibilis	$BG11_0$		1.1	
	Algal turf scrubber with polystyrene foam sheets	*Tolypothrix* sp.	$BG11_0$	Outdoors (AU)	0.8-1.2	This study

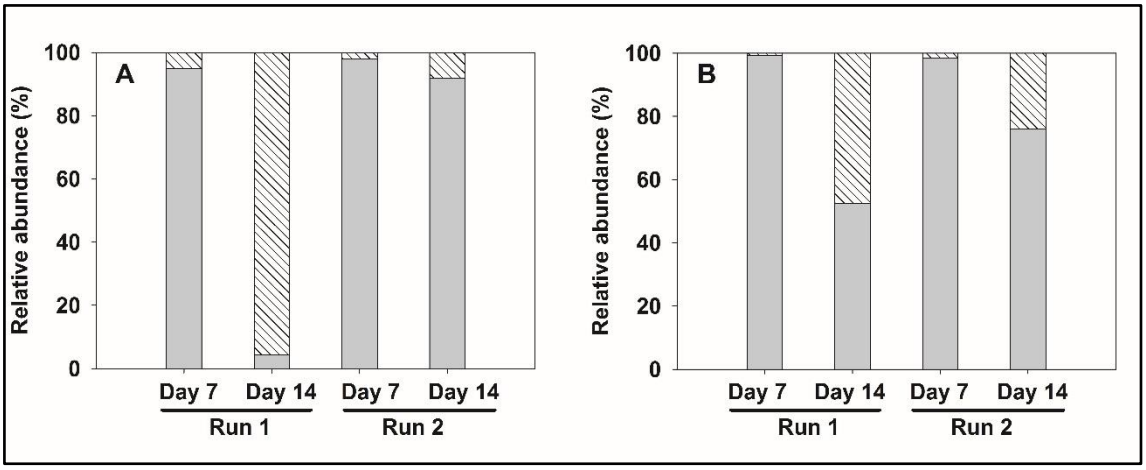


Figure 5.3 Tolypothrix sp.-dominance in outdoor cultures grown in suspension (A) and biofilm (B) mass production systems during two consecutive 14-day runs. Vertical bars represent relative abundance (% area) for *Tolypothrix* sp. (grey bars) and other photosynthetic cyanobacteria/microalgae (striped white bars) on days 7 and 14.

5.4.3 Self-flocculation test and concentration factors in outdoor systems

Fig. 5.4 shows the maximum values of dry weights recorded in suspension (0.35 g DW L^{-1} on 7/03/2014) and biofilm (29.1 g DW L^{-1} in the sample taken on 07/03/2014) systems throughout the outdoor experimental setup. Maximum biomass recorded in the biofilms was 83 times more concentrated than that of the suspension cultures (Fig. 5.4).

Very interestingly, a punctual self-flocculation test in 1-week old suspension samples showed that the biomass settled naturally at average sinking rates of 12.5-13.5 cm min^{-1}. After 2 minutes, a thick cell pellet was formed containing 78-85% of the biomass in the original suspension culture (data not shown) with a dry weight of 21.1 g DW L^{-1} being 53 times more concentrated than the original suspension culture (Fig. 5.4). Although this self-flocculation capacity of *Tolypothrix* sp. opens interesting avenues to be explored

in future studies, it has to be clarified that all results on growth and biochemical properties shown in the present manuscript refer to biomass collected from a tap at the bottom of the vertical bag which was homogenously distributed in the suspension culture due to the intense continuous aeration (see materials and methods section, Fig. S1 and Table S1) and thus not influenced by self-flocculation processes.

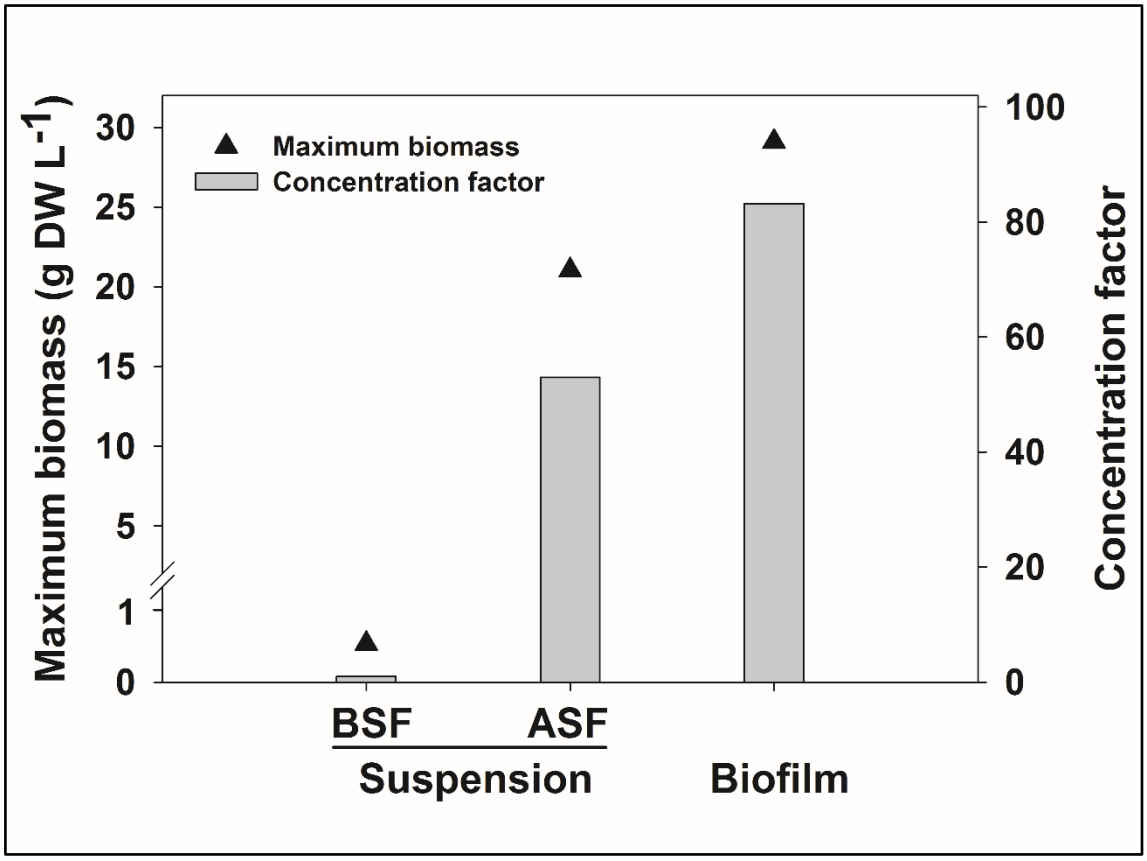


Figure 5.4 Maximum dry weight (g L^{-1}) recorded and concentration factor of *Tolypothrix* sp.-dominated cultures grown in suspension and biofilm mass production systems. Concentration factor was calculated respective to the maximum dry weight (g L^{-1}) of suspension cultures. BSF: suspension culture before self-flocculation; ASF: suspension culture after self-flocculation.

5.4.4 Biochemical profiles and bioproducts in outdoor systems

This section will focus on describing the biochemical and bioproduct characterisation of the biomass during the first week of growth, since

characterised by the highest maximum biomass productivity combined with a clear dominance of *Tolypothrix* sp. (Fig. 5.2 and Fig. 5.3).

On day 7, the biomass was predominantly composed of protein in both systems and runs, representing maxima of 54.8-56.8% of the dry weight in suspension and biofilm cultures, respectively. Carbohydrates accounted for a relevant 33.3-43.5% of dry weight in suspension cultures, being slightly higher than that in biofilms (25.5-34.8% w/w). Lipids were low in both systems and runs, representing less than 8% of the dry weight. Ash content was clearly higher in the biofilms (12.2-13.6%) than in suspension cultures (5.5-7.9%).

The biomass collected on day 7 from outdoor experiments was also analysed for the productivity of bioproducts (Fig. 5.5) in suspension (Fig. 5.5A-C) and biofilm systems (Fig. 5D-F).Potential applications explored include high-value chemicals (phycocyanin, phycoerythrin) (Fig.5.5A and 5.5D) nutraceuticals and/or aquaculture feed (fatty acids) (Fig.5.5B and 5.5E) and biofertilisers (nitrogen and phosphorus content) (Fig.5.5C and 5.5F). Suspension systems showed higher areal productivities (g m^{-2} day^{-1}) for the three types of bioproducts, being slightly higher during the second run. In the suspension system, phycocyanin was the most abundant pigment (2.5-2.8 g m^{-2} day^{-1}) among the three analysed, closely followed by phycoerythrin (2.3-2.6 g m^{-2} day^{-1}) and with one order of magnitude lower productivities for chlorophyll *a* (0.2-0.4 g m^{-2} day^{-1}) (Fig. 5.5A). Fatty acid productivities reached 2.0 g m^{-2} day^{-1}, with the saturated fatty acid C16:0 being most abundant (e.g. representing 42.1 % of total fatty acids in run 1, data not shown) and important amounts of

C16 and C18 polyunsaturated fatty acids (40-42-% of fatty acid productivity) (Fig. 5.5B).

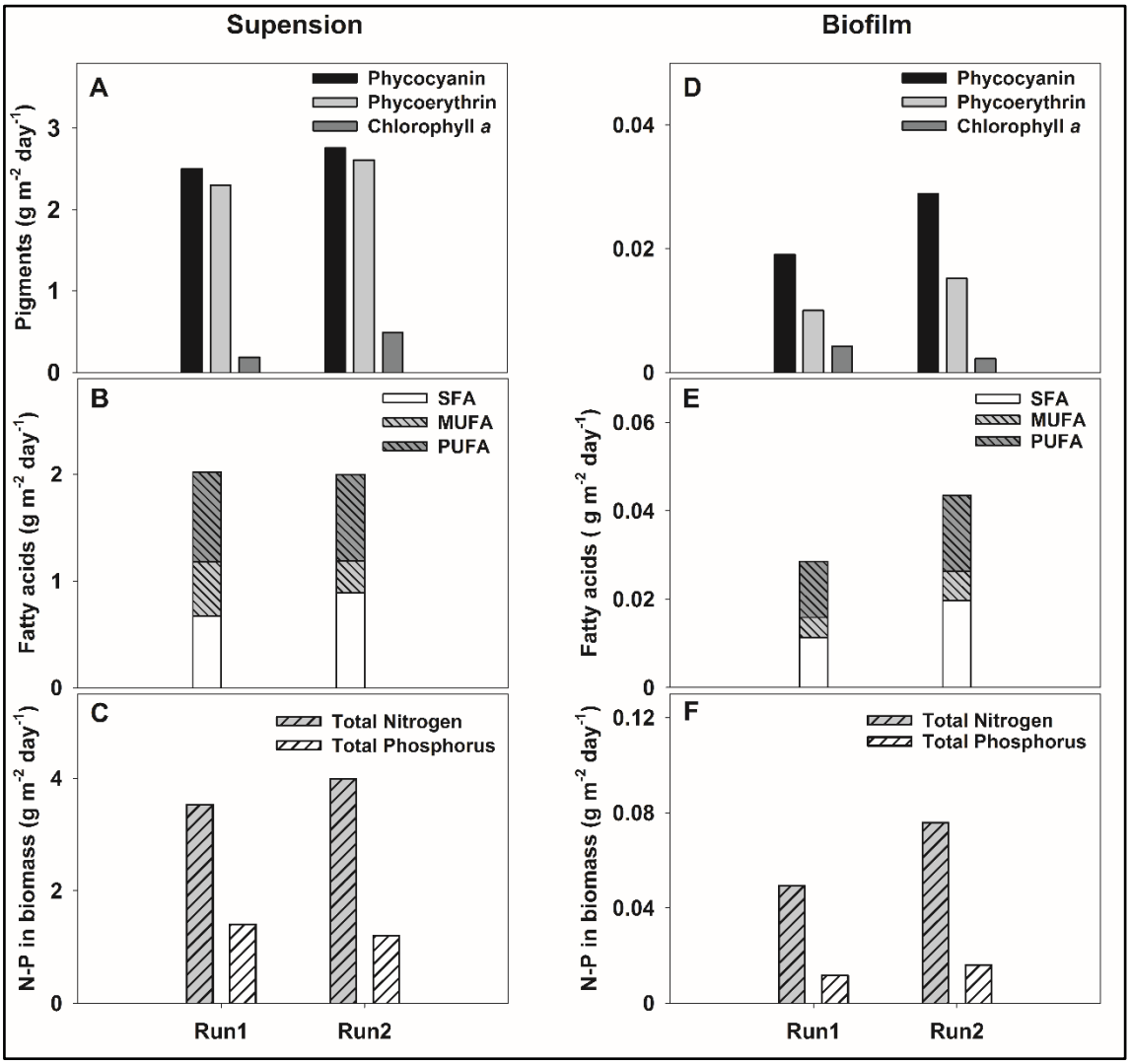


Figure 5.5 Bioproduct synthesis by *Tolypothrix* sp.-dominated outdoor cultures grown in suspension (A-C) and biofilm (D-F) mass production systems during two consecutive 14-day runs. Results obtained from the biomass harvested after 7-days growth. Areal productivity (g m^{-2} day^{-1}) calculated based on ground area occupied by a single system. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids

Stearidonic acid (C18:4n-3) (SA) and Gamma-linoleic acid (C18:3n-6) (GLA) were the most abundant ω-3 and ω-6 fatty acids in both runs, representing maxima of 8.2% (SA, run 1) and 8.3 % (GLA, run 1) of the total fatty acid productivity, respectively. Nitrogen stored in the biomass accounted

for 5.3-5.9 g m^{-2} day^{-1} whereas phosphorus productivities were 2.5-3.3-fold lower (1.2-1.4 g P m^{-2} day^{-1}). The biofilm was characterised by lower areal productivities than suspension cultures, reaching maxima of 0.08 g N m^{-2} day^{-1} (run 2) (Fig. 5C), 0.04 g fatty acids m^{-2} day^{-1} (run 2) (Fig. 5.5B) and 0.03 g phycocyanin m^{-2} day^{-1} (run 2) (Fig. 5.5A). The general bioproduct composition was similar to that of the suspension cultures but with the biofilm producing higher proportions of nitrogen (e.g., TN:TP ratios of 4.2-4.7 in biofilm *vs* 2.5-3.3 in suspension) and slightly lower phycoerythrin proportions (e.g., phycoerythrin: phycocyanin ratios of 0.5-0.6 in biofilm *vs* 0.8-0.9 in suspension).

Table 5.3 Comparison of areal productivities (range) of *Tolypothrix* sp. NQAIF319 measured in single mass production systems *versus* simulated productivities based on multi-system arrangements. Simulated multi-system productivities calculated considering east-west oriented rows with inter-system spacing avoiding shadowing between systems according to two arrangements of bubble tank farms proposed in the literature.

System	Measured productivity of single system (g m^{-2} day^{-1})	Simulated productivity of multi-system arrangements (g m^{-2} day^{-1})	
		Arrangement A	Arrangement B
Vertical bag	10.9-49.2	1.7-7.6	2.5-11.3
Modified algal turf scrubber	0.8-1.2	0.6-1.0	0.7-1.1

5.5 Discussion

In the present study, outdoor cultures of the tropical cyanobacterium *Tolypothrix* sp. reached areal biomass productivities of 0.8-1.2 g dry weight m^{-2} day^{-1} and 10.9-49.2 g dry weight m^{-2} day^{-1} in single open systems of biofilm and suspension-based prototypes, respectively. On a single system basis, maximum productivities from this study were higher than those reported in open suspension-based cultures (raceway ponds) with N_2-fixing strains (*Anabaena*

sp, and *Gloeotrichia echinulata*) grown in nitrogen-free media and even with *Spirulina* (*Arthrospira*) spp. grown in media containing nitrogen (Table 5.2). However, the areal productivities measured in our study would be likely reduced in larger-scale setups including multi-modular arrangements due to the effect of shadowing by neighbouring systems. This aspect was comprehensively evaluated in two prior studies (Sánchez M. et al. 1999; Zittelli et al. 2006) considering column plants ("tank farms") comprised of 38-45 2 m-tall bubble columns, very similar in shape and size to the vertical bag columns used in our study. The estimations we made based on the two non-shadowing column arrangements proposed by these authors (Table 5.3) suggested a 4-6-fold decrease in areal productivity in hypothetical multi-module arrangements of our suspension systems and a proportional increase in land needs to host the entire facility. Furthermore, the fact that, unlike vertical columns, the horizontal biofilm systems may be located wall to wall without lateral shadowing smoothed the differences between suspension and biofilm productivities observed in single system measurements (Table 5.3). Therefore, optimisation of inter-system and inter-row separation will definitely need to be explored by further studies in order to set up multi-system facilities for large scale commercial applications. In any case, the promising preliminary results obtained in our study can be considered a stepping stone for future development and optimisation of the suspension-based prototype tested herein.

Maintaining sufficiently high productivities along the annual cycle is a key aspect to ensure a profitable mass production of microalgae and cyanobacteria. This raises questions about the feasibility of algae farming in temperate/cold

climates with long winter seasons, and points to subtropical and tropical regions as the most suitable for microalgal/cyanobacterial mass production projects (Table 5.2). However, important seasonal differences have been also observed under climates with mild temperatures, as illustrated by the differences in biomass productivity (9 g m^{-2} day^{-1} in winter and 20 g m^{-2} day^{-1} in summer) described in outdoor cultures of *Anabaena* sp. in the Mediterranean climate in Southern Spain. In our study, the two runs performed in the tropical climate of NE Australia yielded similar biomass productivities but very different degrees of contamination by microalgae and cyanobacteria. The highest biological contamination was observed during the first run in March, coinciding with the end of the wet season in this area of tropical Australia. Under these conditions, high air humidity could have acted as a potential vector for microalgae and cyanobacterial cells from neighbouring areas (Després et al. 2012). In contrast, lower levels of humidity might restrict airborne invasions during the second run performed at the beginning of the dry season. Invasion of exogenous microalgae and cyanobacteria is frequent in open systems being often addressed by the use restrictive culture conditions (e.g. high pH and salinity) only tolerated by the target species. Our results suggest that the use of nitrogen-free medium may be only effective for short periods (1 week) after which invading organisms are able to survive in the system using the nitrogen-containing compounds released from decaying cells of N_2-fixing cyanobacteria.

Areal productivities of *Tolypothrix* sp. in the outdoor biofilm system from our study (0.8-1.2 g m^{-2} day^{-1} measured in single systems; simulated overall areal productivities of 0.6-1.1 g m^{-2} day^{-1}) were in the range of low productivities

of 0.02-3.3 g m^{-2} day^{-1} previously reported in biofilms of pure cyanobacterial cultures (*Anabaena*, *Trichormus* and *Phormidium*) and mixed consortia of green algae and cyanobacteria (*Synechococcus*, *Phormidium*, *Pseudanabaena*) (Guzzon et al. 2008) under controlled laboratory conditions (Table 5.2). No reliable data could be found in the literature regarding productivities of cyanobacterial-dominated biofilm systems in outdoor conditions. Besides the relatively simple open biofilm-based systems in Table 2, much more sophisticated designs like the closed membrane photobioreactor developed by Kremer et al. (2006) exist. Using a thermophilic cyanobacterium much higher productivities of up to 55 g m^{-2} day^{-1} were reached, but the system was economically unfeasible (Kremer et al. 2006). Indeed, the very simple prototype from our study demonstrates that biofilm growth can be carried out using inexpensive, low-weight (thus transportable) and easy to produce materials (polystyrene) with the potential advantage of enhanced re-growth by the inoculum remaining attached to polystyrene after harvesting (Johnson and Wen 2010) to be explored in future studies. In addition, the reduced extracellular water content of biofilms compared to suspension cultures (e.g. 1 kg dry weight in a biofilm would require 50 L of biofilm volume compared to 4000 L of non-concentrated suspension culture required to obtain the same biomass) may potentially reduce transportation costs of biomass towards facilities for further processing steps (e.g. centrifugation or spray-drying facilities for production of algal paste/algal pellets for aquaculture feed) or final product applications (e.g. wet biofertiliser in remote agricultural areas). Indeed, the natural concentration factor of 80 in biofilms compared to suspension cultures (Fig. 5.3) is similar to those achieved by using energy-consuming centrifugation and/or expensive

chemical flocculation (Pahl et al. 2013). The reduction in dewatering costs of biofilms, if properly taken into account in techno-economic and carbon footprint analyses of the different product possibilities, might likely change the vision of biofilm potential based exclusively on areal productivity criteria (Ozkan et al. 2012).

Besides the reduction of water content through biofilm production, our study briefly explored the capacity of *Tolypothrix* sp. NQAIF319 for self-flocculation as an alternative to reduce extracellular water in suspension cultures and optimise production pathways in future experiments. Prior studies already described self-flocculation ability in strains from the same genus, i.e. *Tolypohtrix tenuis*, and highlighted the importance of nutrient status on self-flocculation efficiency (Silva and Silva 2007). Therefore, the satisfactory preliminary results achieved in 1-week old biomass in our study (85% of biomass concentrated in cell pellets after 2 minutes, equivalent to a sinking rate of 13 cm min^{-1} and concentration factor of 53) need to be explored in more detail in the context of nutrient concentrations to evaluate the most appropriate moment for biomass harvesting by self-flocculation. Overall areal productivities of *Tolypothrix* sp. NQAIF319 simulated for suspension cultures in our study (1.7-11.3 g $m^{-2}day^{-1}$) were in the range of the 3-9 g m^{-2} day^{-1} reported in an Argentinian *Tolypothrix tenuis* strain grown in theoretically more productive (and sophisticated) closed photobioreactors (Silva and Silva 2013), indicating the flexibility of this genus for outdoor mass production. Together with their flocculation ability, *Tolypothrix* spp. strains seem characterised by a biochemical profile suitable for low-cost production of nitrogen-rich biofertilisers (e.g., 0.1-4 g

nitrogen m^{-2} day^{-1} in our study), as cells might retain viability and N_2-fixation capacity for 15 months even after freeze-drying (Silva et al. 2007). Low-cost production of high-value products in biomedical/nutraceutical applications like phycocyanin/phycoerythrin (0.1-2.8 g m^{-2} day^{-1}) and the omega-6 fatty acid gamma-linoleic acid (up to 0.2 g m^{-2} day^{-1} in suspension cultures, data not shown) is also possible. All these characteristics, together with recent findings of novel bioproducts with markets still in development such as blood clotting agents produced by *Tolypothrix tenuis* (Bhatnagar et al. 2014), suggest that the genus *Tolypothrix* will be of increasing importance in the field of cyanobacterial/microalgal bioproducts in the near future.

In summary, the simple prototypes developed in our study can be considered a feasible alternative for low-cost biomass production in tropical areas with low-nutrient waters. One-week inoculation-harvesting cycles particularly during the dry season are recommended in order to maximise productivity and minimise biological contamination by non-target microalgal and cyanobacterial species. Among the varied potential applications, biofilm systems may be recommended for inexpensive production of low-value commodities such as biofertiliser or aquaculture feed, either *in situ* in agricultural or farming areas, or transport to more remote areas, as there is no need for dewatering prior to transformation or use. The suspension system, in turn, may also be used for those applications due to self-settling capacity of the strain but also for high value products (e.g., phycocyanin/phycoerythrin) especially if self-flocculation harvesting is fully optimized. Further tests may include evaluating the long-term behaviour of the systems (typically 3-12

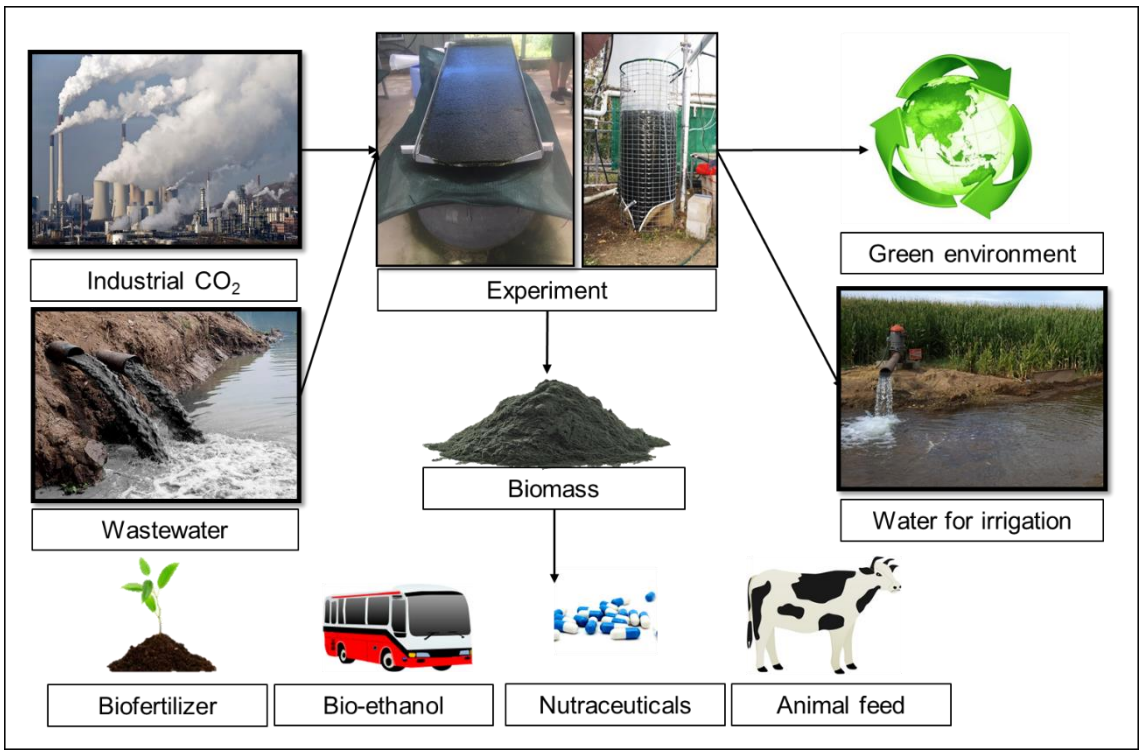
months duration as most of the experiments summarised in Table 5.2) and the scalability to higher culturing volumes or multi-modular configurations. For the latter, it is essential to find the optimal separation between systems to minimise shadowing while maximizing efficient land use. Further improvements to be explored may also include optimisation of growth conditions to enhance productivity (e.g., maximizing the growth rate by using semi-continuous culturing instead of batch or testing different water flow rates in the biofilm system to reduce shear stress) and minimise contamination by, for instance, increases of medium salinity given preliminary results on *Tolypothrix* sp. indicating tolerance to 36 ppt salinity (Kirsten Heimann, personal communication). The simplicity and flexibility of the prototypes from our study together with the well-known metal bioremediation capacity of many filamentous cyanobacteria (Philippis et al. 2011) invites for future tests coupling bioremediation of nutrient-poor waters (e.g. ash dam water from coal-fired power plants in Queensland, Australia, see (Saunders et al. 2012)) with bioproducts synthesis in order to maximise the economic and environmental sustainability of mass production of N_2-fixing cyanobacteria.

5.6 Conclusion

This study revealed that N_2 fixing freshwater *Tolypothrix* sp. NQAIF319 has potential to grow in outdoor cultivation in tropical Australia. The simplicity of the prototypes developed in this study with the bioproduct potential of *Tolypothrix* sp. NQAIF319 indicates a promising cultivation platform for low-cost bioproduct synthesis. The effect of heavy metals and CO_2 on bioproduct

synthesis of *Tolypothrix* sp. NQAIF319 under outdoor cultivation system in tropical Australia will be discussed in the next chapter.

Chapter 6: Bioproduct potential of outdoor cultures of *Tolypothrix* sp.: effect of carbon dioxide and metal-rich wastewater[6]



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[6] **Velu, C**., Cirés, S., Brinkman, D. and Heimann, K., (2020). Bioproduct potential of outdoor cultures of *Tolypothrix* sp.: effect of carbon dioxide and metal-rich wastewater. Front. Bioeng. Biotechnol. 8:51. doi: 10.3389/fbioe.2020.00051.

6.1 Abstract

Rising CO_2 levels, associated climatic instability, freshwater scarcity and diminishing arable land exacerbate the challenge to maintain food security for the fast-growing human population. Although coal-fired power plants generate large amounts of CO_2 emissions and wastewater, containing environmentally unsafe concentrations of metals, they ensure energy security. Nitrogen (N_2)-fixation by cyanobacteria eliminate nitrogen fertilisation costs, making them promising candidates for remediation of waste CO_2 and metals from macronutrient-poor ash dam water and the biomass is suitable for phycocyanin and biofertiliser product development. Here, the effects of CO_2 and metal mixtures on growth, bioproduct and metal removal potential were investigated for the self-flocculating, N_2-fixing freshwater cyanobacterium *Tolypothrix* sp.. *Tolypothrix* sp. was grown outdoors in simulated ash dam wastewater (SADW) in 500 L vertical bag suspension cultures and as biofilms in modified algal-turf scrubbers. The cultivation systems were aerated with air containing either 15% CO_2 (v/v) or not. CO_2-fertilisation resulted in ~1.25- and 1.45-fold higher biomass productivities and ~40 and 27% increased phycocyanin and phycoerythrin contents for biofilm and suspension cultures, respectively. CO_2 had no effect on removal of Al, As, Cu, Fe, Sr and Zn, while Mo removal increased by 37% in both systems. In contrast, Ni removal was reduced in biofilm systems, while Se removal increased by 73% in suspension cultures. Based on biomass yields and biochemical data obtained, net present value (NPV) and sensitivities analyses used four bioproduct scenarios: 1) phycocyanin sole product, 2) biofertiliser sole product, 3) 50% phycocyanin and

50% biofertiliser, and 4) 100% phycocyanin and 100% biofertiliser (residual biomass) for power station co-located and not co-located 10 ha facilities over a 20-year period. Economic feasibility for the production of food-grade phycocyanin either as a sole product or with co-production of biofertiliser was demonstrated for CO_2-enriched vertical suspension cultures raised without nitrogen-fertilisation and co-location with power stations significantly increased profit margins.

6.2 Introduction

Anthropogenic emissions of carbon dioxide (CO_2) account for 68% of total emissions (Ho et al. 2011), posing a threat to the global climatic equilibrium. At present, coal-powered electricity generation is still required in Australia and globally to meet energy requirements and security (Stock 2014). Flue gas from coal-fired power plants contain 10-15% CO_2 (v/v) and generate wastewater enriched with heavy metals (Artanto et al. 2014). Biological fixation of CO_2 and absorption of nutrients/metals from wastewaters by photosynthetic organisms such as microalgae and cyanobacteria are gaining industrial interest, as the biomass produced can yield a variety of high- and low-value renewable products (Wang et al. 2008). Wastewater generated at coal-fired power plants (ash dam water) cannot be discharged due to its potential toxicity and is therefore stored in ash dams (Roberts et al. 2015; Velu et al. 2015). The ash dam water contains metals, many of which serve as micronutrients important for plant growth, but macro-nutrients, such as nitrogen and phosphate, are lacking (Saunders et al. 2012). As the cultivation of eukaryotic photosynthetic organisms requires nitrogen and phosphate for growth, ash dam water needs to

be supplemented with these macro-nutrients, increasing the cost of bioremediation and biomass production (Velu et al. 2019). In contrast, the cultivation of diazotrophic cyanobacteria does not require nitrogen fertilisation, as these organisms can fix atmospheric nitrogen (N_2), making them an ideal choice for bioremediation of metals from nitrogen-limited wastewaters (Markou and Georgakakis 2011). This is a clear advantage, as globally 85 million tons of nitrogenous fertiliser were used in 2000 for food production. Synthetic nitrogenous fertilisers are projected to not meet the demands of the ever-growing human population in the near future (Singh et al. 2016). In addition, the current exploitation of chemically derived fertilisers have been shown to contribute to environmental problems, such as pollution, reduced soil fertility and adverse impacts on the ozone layer (Benemann 1979; Singh et al. 2016). In an Australian context, agricultural productivity is declining in regions with marginal/leached soils, as microbial consortia, essential for soil fertility, are negatively affected by declining soil carbon contents. Soil fertility cannot be improved by provision of nitrogen without the addition of large amounts of carbon (QLD 2016). Despite the realisation of adverse impacts of synthetic nitrogenous fertilisers, ~5.3 million tonnes of chemical fertilisers were used on 49.1 million ha agricultural land in Australia between 2014-2015 (ABS 2015). Thus, there is a pressing need for the sustainable production of innovative fertilisers that are effective, renewable, environmentally friendly, cost-efficient, and improve soil fertility to ensure food security in the future. In this context, fertilisers derived from biological nitrogen fixation and through recycling and re-use of nitrogen contained in various wastewaters offer great potential benefits (Benemann 1979; Singh et al. 2016).

Nitrogen fixation is carried out by the oxygen-sensitive, iron and molybdenum-containing nitrogenase complex (Abed et al. 2009), resulting in higher iron and molybdenum requirements, elements that are present at elevated concentrations in ash dam wastewater (Saunders et al. 2012). While excessive metal concentrations can retard cyanobacterial growth (Pereira et al. 2011), species such as *Anabaena subcylindrica*, *Aphanocapsa* sp., *Calothrix* sp., *Microcystis* sp., *Oscillatoria salina*, *Plectonema terebrans*, and *Synechococcus* sp. can be used for the treatment of domestic and industrial wastewater (Dubey et al. 2011). Biominerelisation of metals by cyanobacteria occurs via intracellular bioaccumulation and/or passive biosorption, the latter is mediated by the presence of an exopolysaccharide layer (EPS) on the outside of many cyanobacterial species (Pereira et al. 2011). The EPS consists of complex heteropolysaccharides on a glucosamine backbone, providing an accumulation of negative charges that play an essential role in the chelation of metal ions (De Philippis et al. 2011). On a dry weight basis, *Calothrix scopulorum* and *C. marchica* chelated 0.7 and 6.4% of lead (Ruangsomboon et al. 2007; Weckesser et al. 1988). *Nostoc muscorum* chelated 22.5, 11.8, 26.4 and 32% of copper, cobalt, lead and manganese, while *Anabaena subcylindrica* performed much better (81.8. 33.7, 100 and 100%, respectively) (El-Sheekh et al. 2005). The large differences in the biosorption of metals indicates that the choice of species is an important criterion to consider, especially when the reutilisation of large volumes of wastewater is an important aspect of the industrial process. As CO_2-fertilisation enhances cyanobacterial growth (Velu et al. 2015; Velu et al. 2019), EPS content will increase simultaneously, which should enhance metal chelation capacity of the cultures and, hence,

remediation capacity. The presence of complex metals mixtures in industrial wastewaters may, however, result in competition for the same binding sites, which can result in reduced adsorption efficiencies (Pereira et al. 2011). The diazotrophic filamentous freshwater cyanobacterium, *Tolypothrix* sp. has been used for treatment of domestic and industrial wastewaters and *T. ceytonica* achieved an 86 and 64.4% efficiency for the removal of zinc and total suspended solids (El-Bestawy 2008).

In addition to the exploitation of environmental services (CO_2 and metal remediation), the prokaryotic cyanobacteria show additional advantages for biotechnological applications, such as strain-dependent wide environmental tolerances, e.g. marine to freshwater, acid and/or alkaline conditions (Gupta et al. 2013), rapid growth and high photosynthetic activities (Hall et al. 1995). Furthermore, the produced biomass has multiple commercial applications through bioproduct development. In general, potential algal bioproducts include medicinal compounds, food and feed supplements (restricted to CO_2 enriched grown species without inclusion of metal- or other potentially toxic compound-containing wastewater treatment), pigments (e.g. β-carotene, astaxanthin, fucoxanthin, lutein, phycocyanin, phycoerythrin, the latter two from cyanobacteria), protein, carbohydrate, biofuel and biohydrogen, and biofertilisers (Setta et al. 2017). Specific cyanobacterial bioproducts could be protein, mineral and unsaturated fatty acid supplements and the pigments phycocyanin and phycoerythrin from *Arthrospira platensis* or *Limnospira maxima* (formerly *Spirulina platensis* and *S. maxima*), where the protein content of the biomass can reach 74% (Cohen 1997), which can be extracted through

biorefining (Borowitzka 2013). It might be argued that cyanobacterial biomass produced using metal-rich wastewater is not suitable for high-value phycocyanin product development. Indeed some binding of iron and mercury, the latter not present in ash dam water of coal-fired power plants used in this study, and less efficient binding of some other metals to phycocyanin has been described (Bermejo et al., 2008; Gelagutashvili and Tsakadze, 2013; Bhayani et al., 2016). It is, however, unclear how much binding would occur and how irreversible the binding would be. In addition, affinity of the metals present in ash dam water can be expected traditional metal chelating proteins, such as metallothioneins, and the highly negatively charged EPS, both present in cyanobacterial including Tolypothrix sp. biomass, would be more efficient binding sites. In addition, as proposed in this study, phycocyanin extracted from Tolypothrix sp. biomass will be purified to upgrade the product to food-grade phycocyanin to obtain a higher sales price, which would further remove any metals bound to phycocyanin. It will nonetheless be essential to analyse the final product for metal contents for quality assurance.

While light, temperature and CO_2 supplies can be easily controlled at laboratory scale, therefore producing best biomass yields, biomass productivities are typically reduced in large volume suspension-based systems, due to light and carbon limitation, particularly in raceway pond cultivation (Pierobon et al. 2018). Under outdoor large-scale cultivation conditions, improved solar and carbon supplies can be achieved in closed bioreactors, but this adds energy and infrastructure costs, limiting suitability to high-value product development (Pierobon et al. 2018). In contrast, cyanobacterial biofilm

reactors are better suited for cost-effective biomass production and are frequently used for wastewater treatment (Hoh et al. 2016). Cyanobacterial biofilm cultivation requires minimal water supplies, gas exchange (CO_2 absorption and O_2 venting) is more efficient and harvesting is energy-efficient (Heimann 2016).Recently developed porous substrate biofilm reactors show efficient light – , carbon – and water utilisation and scale-up of this technology is easily possible, making them a promising technology for economical microalgal/ microbial biomass production (Pierobon et al. 2018) . For example, cyanobacterial biomass productivity was greater in rotating biofilm reactors without aeration or additional CO_2-supplementation compared to suspension reactors (Gross and Wen 2014), but the adhesion process for mat establishment is sensitive to shear forces, and species- and substrate-dependent.

Integrated biomass production with wastewater and CO_2 emission-generating industries has many advantages, i.e. use of non-arable land, non-potable water and provision of trace metals, and CO_2 to support biomass and bioproduct productivities (Aslam et al. 2019; Moheimani 2016; Roberts et al. 2015). Nonetheless, economic feasibility still needs to be demonstrated on a case-by-case basis, as outcomes are dependent on the value of the bioproduct(s) and yields. In addition, in the case of ash dam water generated at coal-fired power plants, metal toxicity may occur, reducing yields and application potential of generated bioproducts (Velu et al. 2019). Previous research established that the diazotrophic *Tolypothrix* sp. (isolated from tropical Australia) efficiently self-flocculates, reducing energy requirements for

harvesting/ dewatering of biomass by 90% (Heimann et al. 2013; Velu et al. 2015). Furthermore, no metal toxicity was observed for *Tolypothrix* sp. biomass production in simulated ash dam water (SADW) and growth was independent of nitrogen supply, yet costs for phosphate fertilisation are incurred (Velu et al. 2019). Figure 6.1 illustrates the integrated production of *Tolypothrix* sp. biomass and potential bioproducts when co-located at a coal-fired power plant.

Therefore, this study used the Australian isolate of *Tolypothrix* sp. to contrast biomass productivities, metal removal capacity and bioproduct potential for biomass cultivated in simulated ash dam water (SADW) between a traditional bubble column reactor and a modified algal turf scrubber with and without CO_2 supplementation under outdoor conditions. Additionally, the economic feasibility for bioproduct development was estimated, considering four scenarios: 1) production of food-grade phycocyanin as a sole product, 2) biofertiliser as a sole product, 3) use of half the biomass for biofertiliser and food-grade phycocyanin production and 4) biorefining of the high-value phycocyanin with the residue being used as biofertiliser. This study modeled net present value (NPV) and sensitivity analysis for these four scenarios under conditions of co-location with coal-fired power plants and traditional cultivation (not co-located) for a 10 ha plant using suspension bubble columns for biomass production.

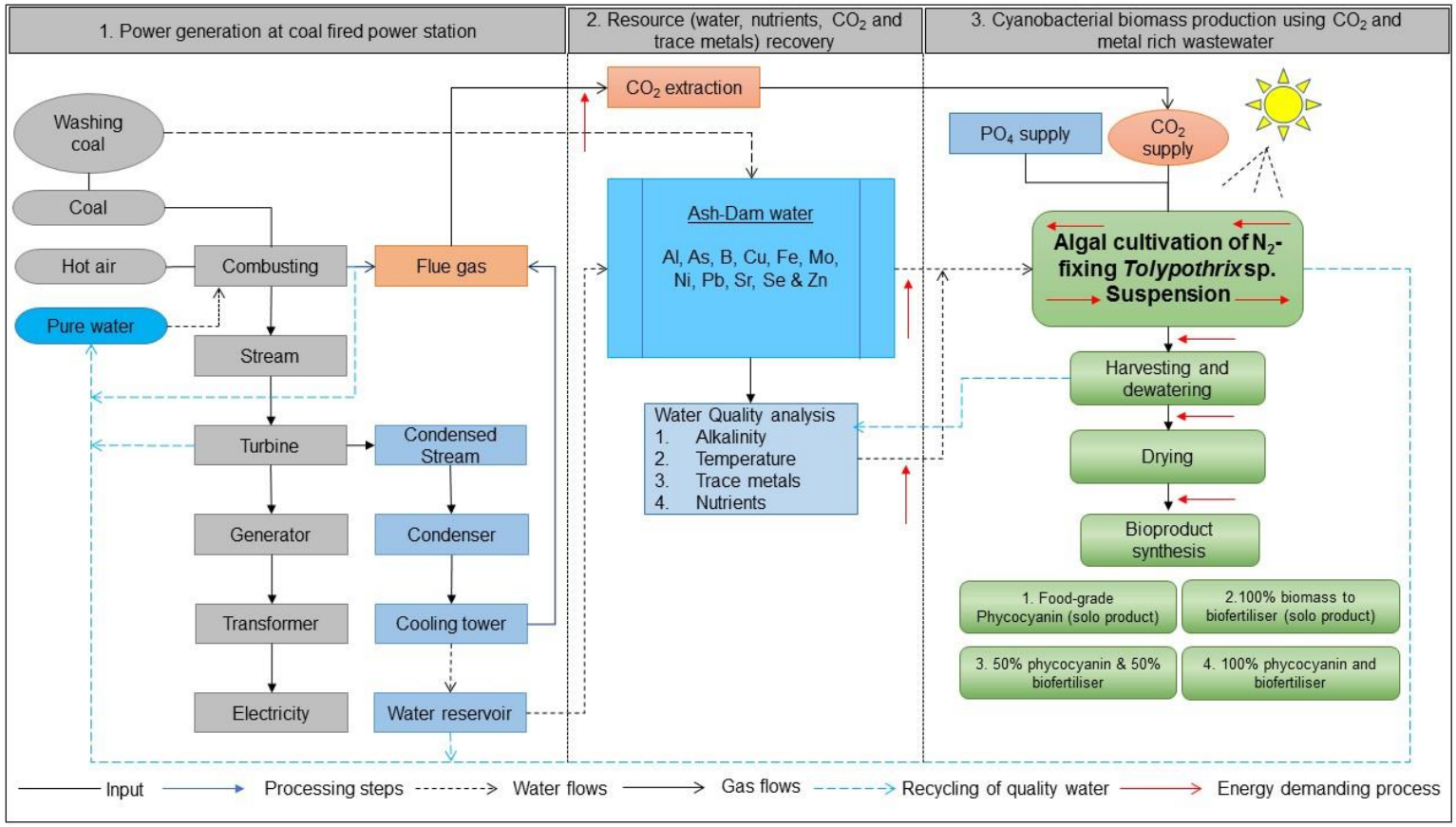


Figure 6.1 Framework for an integrated bio-economic model for co-locating cyanobacterial cultivation with coal-fired power stations based on a biorefinery approach

6.3 Methods:

6.3.1 Culture collection and strain characterisation

The N_2-fixing filamentous cyanobacterial strain *Tolypothrix* sp. NQAIF319 was isolated and maintained as described in section 3.3.1 (Velu et al. 2015).

6.3.2 Synthetic wastewater preparation

To investigate effect of trace metal-rich ash-dam wastewaters on the growth of the selected cyanobacterium, the simulated ash-dam wastewater (SADW) was prepared as described in section 3.3.2 based on (Saunders et al. 2012).

6.3.3 Outdoor experimental setup, growth and biomass productivities

Outdoor experiments were carried out in four meso-scale open bioreactor prototypes (two algal turf-scrubber (ATS) of 2.2 m^2 each (2.2 m long x 1 m wide), two suspension vertical bags of 500 L each with a 0.3 m^2 area footprint) designed and assembled at James Cook University, Australia (Appendix Fig. D.1 and Fig. D.2, Appendix Tables D.1 and D.2). The cultivation area was shaded with a UV-shade cloth (Coolaroo, 3.66 m wide, 60% shading) to control photon flux density between 500 and 900 µmol m^{-2} s^{-1} at the Freshwater Compound at James Cook University, Townsville, Australia (19.33 S, 146.76 E). The plastic trays of the ATSs were lined with polystyrene for attachment of *Tolypothrix* sp. and had a slope of 7%. Water flow conditions were continuous at 66 L min^{-1} delivered from a sump with a 500 L fill volume beneath the ATS. The 500 L vertical bubbled suspension cultures systems were constructed from PVC bag material contained in a wire cage with a plastic

keeled footing of 0.3 m^2. A tap was fitted to the bottom corner of the vertical bag for harvesting, while aeration was provided by a suspended diffuser delivering 0.05 L air L^{-1} min^{-1} from the main compressor.

The two ATS tanks and two suspension vertical bags were filled with 500 L SADW. Laboratory cultures of *Tolypothrix* sp. NQAIF319 were grown in aerated 20 L sterile plastic carboys in BG11(-N) medium, at 28 °C, 100 µmol photons m^{-2} s^{-1} until cultures reached stationary phase. For inoculation of the vertical bag system, the cultured biomass was centrifuged (8,000×g, 20 min) (Beckman, Avanti® J-26XP, Australia) and an adequate volume of the cell pellet was resuspended into 500 L of fresh SADW medium to reach an initial biomass concentration of 0.1 g dry weight L^{-1}. The ATS were inoculated by spreading the centrifuged *Tolypothrix* sp. biomass to an initial concentration of 5 g DW m^{-2} without water flow. After an overnight attachment period, water was supplied to the top of the system via a baffle. 16-day growth experiments were simultaneously performed in both systems in two consecutive runs, during September 2016 (run 1) and October 2016 (run 2). In both runs, one set of each cultivation system was supplemented with CO_2-enriched air (15% v/v), while the other sets were supplied with air at atmospheric CO_2 levels (non-CO_2 controls). CO_2-enriched air and air were baffled in both cultivation systems. Gases were 99.9% pure, ISO certified and supplied by BOC, a member of the Linde Group, Townsville, Australia. Temperature and pH were monitored twice daily with a WP-81 water-proof conductivity / TDS-pH / MV-temperature meter (TPS instruments, Australia). Irradiance was measured at the time of sampling using a LI- 250A photosynthetic active radiation (PAR) light meter (LICOR

Biosciences, USA). The incident sun light at sample time varied between 500 to 700 µmol photons m^{-2} s^{-1}. Culture samples for monitoring growth and nutrients were taken every four days from a tap located at the bottom of the vertical bag, while samples from the ATS were taken by scraping the biomass contained in 100 cm^2 (10 × 10 cm) biofilm squares using a silicone rubber cell scraper (IWAKI, Japan). Water sample for nutrient and metal analysis were collected from the tank and from the supernatant of the centrifuged samples of the vertical bags. The biomass was harvested on day 16. The biomass pellets were freeze-dried (Dynavac freeze dryer model Fd12, Australia) and stored in the dark at −80°C (Sanyo Ultra-Low Temperature Freezer (MDF-U33V), Japan) until analysis for pigment (phycocyanin and phycoerythrin)-, fatty acid (fatty acid methyl esters, FAME)-, total lipid-, alkane/alkene-, protein-, carbohydrate- and carbon-hydrogen-nitrogen-sulphur-phosphorous-potassium (CHNSPK) contents following the analytical procedures detailed at section 3.3.6. Biomass growth (g DW m^{-2}) and productivity (g DW m^{-2} day^{-1}) was determined gravimetrically (von Alvensleben et al. 2013) on days 0, 4, 8, 12 and 16. Biomass-specific growth rates (μ_{1-3}) , doubling rate (k) and doubling time (T2) were calculated as per Gour et al. (2014) and von Alvensleben et al. (2013).

6.3.4 Phosphate analysis

Phosphate concentrations in the culture medium (µg PO_4^{3-} L^{-1}) were determined as described in section 3.3.4 on days 0, 4, 8, 12 and 16 using 15 mL of 0.22 µm-filtered (PTFE, Micro Analytix Pty Ltd, Australia) culture supernatants.

6.3.5 Metal analysis

SADW samples of 0.22 µm-filtered (hydrophilic PTFE, Micro Analytix Pty Ltd, Australia) culture supernatants were collected on days 1, 4, 8, 12 and 16. Metal (Al, As, B, Cu, Fe, Mo, Ni, Se, Sr, V and Zn) concentrations were determined using a Varian 820-MS inductively coupled plasma mass spectrometer (ICP-MS) (Melbourne, Australia), as described in section 3.3.5 based on Montaser (1997).

6.3.6 Biochemical analyses

Freeze-dried biomass of *Tolypothrix* sp. was analysed for total lipid-, carbohydrate-, protein-, FAME-, alkane/alkene-, and pigment (phycocyanin phycoerythrin)-, carbon-, hydrogen-, nitrogen-, sulphur-, phosphorous- and potassium contents.

6.3.6.1 Total lipid content

Total lipid content was determined gravimetrically following a direct transesterification procedure adapted from Lewis et al. (2000) and modified as described in section 3.3.6.1.

6.3.6.2 Carbohydrate content

Total carbohydrate content was determined using the phenol-sulphuric acid method (DuBois 1956), as described in section 3.3.6.2.

6.3.6.3 Protein content

Total protein content was determined using the Lowry method based on González López et al. (2009), as described in section 3.3.6.3 using a kit (Sigma,

Total Protein Kit, Micro Lowry, Peterson's Modification, TPO300, Sydney, Australia).

6.3.6.4 Fatty acid extraction, transesterification analysis

Fatty acids were analysed after extraction and transesterification at Australian Institute of Marine Science, Townsville, Australia by gas chromatography (GC) (Agilent 7890B GC-Agilent 5975C) equipped with a DB-23 capillary column (60 m x 0.25 mm x 0.15 µm) and flame ionisation detector (FID), as detailed in von Alvensleben et al. (2013), but temperature was 250 °C and FID inlet temperature was 270 °C, as detailed in section 3.3.6.4

6.3.6.5 Pigment analysis

Phycocyanin- and phycoerythrin contents were determined spectrophotometrically (SpectraMax® M2, USA) using freeze-dried samples according to Lawrenz et al. (2011), as described in section 3.3.6.5

6.3.6.6 Elemental analysis

Biomass carbon-, hydrogen-, nitrogen-, sulphur-, phosphorous- and potassium contents (CHNSPK) (%) of the samples were determined using an EA-1110 elemental analyzer (CE Instruments, Italy) set up in CHN mode as described in section 3.3.6.6.

6.3.7 Gas bottles, reagents and chemicals

Gasses for calibrations (99.9% pure CO_2, CO_2 1-20% with air) were supplied by BOC, a member of the Linde group, Townsville, and all were ISO certified. All chemicals and solvents were obtained from Sigma-Aldrich, Sydney, Australia.

6.3.8 Net present value (NPV) and sensitivity analyses

A techno-economic cost assessment was used to evaluate the economic feasibility for production of food-grade phycocyanin and biofertiliser from *Tolypothrix* sp. biomass, assuming either co-location with a coal-fired power plant or traditional cultivation, which is classified here as biomass production solely for bioproduct development without co-location with either an industrial wastewater producer and/or CO_2 emitter. The following boundaries were set: 1) The algae cultivation plant has a 10 ha cultivation area and employs suspension-based open cultivation systems with an annual biomass production period of 300 days year^{-1}. 2) The operating life was set to 20 years. 3) Production data generated in this study in vertical suspension culture systems in SADW and supplied with 15% CO_2 (v/v) formed the basis of the analyses. 4) All costs were adjusted to increase by 5% annually, while production sale prices were not adjusted. 5) Food-grade phycocyanin production assumed an extraction/purification efficiency of 67% (Chaiklahan et al. 2018) and a sale price for total phycocyanin of US$ 500 kg^{-1} (Querques et al. 2015), while the sale price for biofertiliser was estimated to be US$ 500 t^{-1}.

The NPV was calculated by difference between the present value of cash income and the present value of all cash expenditures following the equation (6.1):

$$NPV = \sum_{t=1}^{r} \frac{C_t}{(1+r)^2} - C_0$$ ---------- eq 6.1

where C_t is the net cash flow during the period t, C_0 is the total initial investment cost, t is the number of time periods (years) and r is the discount rate.

The weighted average costs of capital (WACC) was set to 10% and sensitivity analyses were performed for two scenarios: 1) a reduced biofertiliser price of 25% of the current value (US$ 125 t^{-1}) and 2) a reduced sales price for food-grade phycocyanin at 25% of today's price (US$ 125 kg^{-1}).

6.4 Results

6.4.1 Effect of culture system and CO_2-supplementation on growth and phosphate uptake of SADW-grown *Tolypothrix* sp.

CO_2-supplementation enhanced growth of *Tolypothrix* sp. in both types of outdoor cultivation systems. A 1.15- and 1.26-times higher final biomass yield of 34 and 42 g DW m^{-2} was obtained for biofilm cultures (Figs 6.2$A_{1,2}$), while a 1.2- and 1.3-times higher yield in vertical suspension-based systems achieved a final biomass yield of 870 and 1310 g DW m^{-2} for runs 1 and 2, respectively (Figs 6.2$B_{1,2}$). Growth over the 16-day time course could be divided into two phases of specific growth rate ($\mu_{1, 2}$) (Table 6.1). μ_1 was not affected by CO_2-supplementation for any of the cultivation systems, whereas μ_2 was ~21 and 30% higher for biofilm cultures and ~11 and 32% higher for vertical bag suspension cultures for runs 1 and 2, respectively, when CO_2 was supplied. CO_2-supplementation had no effect on doubling rates (k) in either cultivation system, but doubling time was generally reduced by 15% for suspension-grown *Tolypothrix* sp..

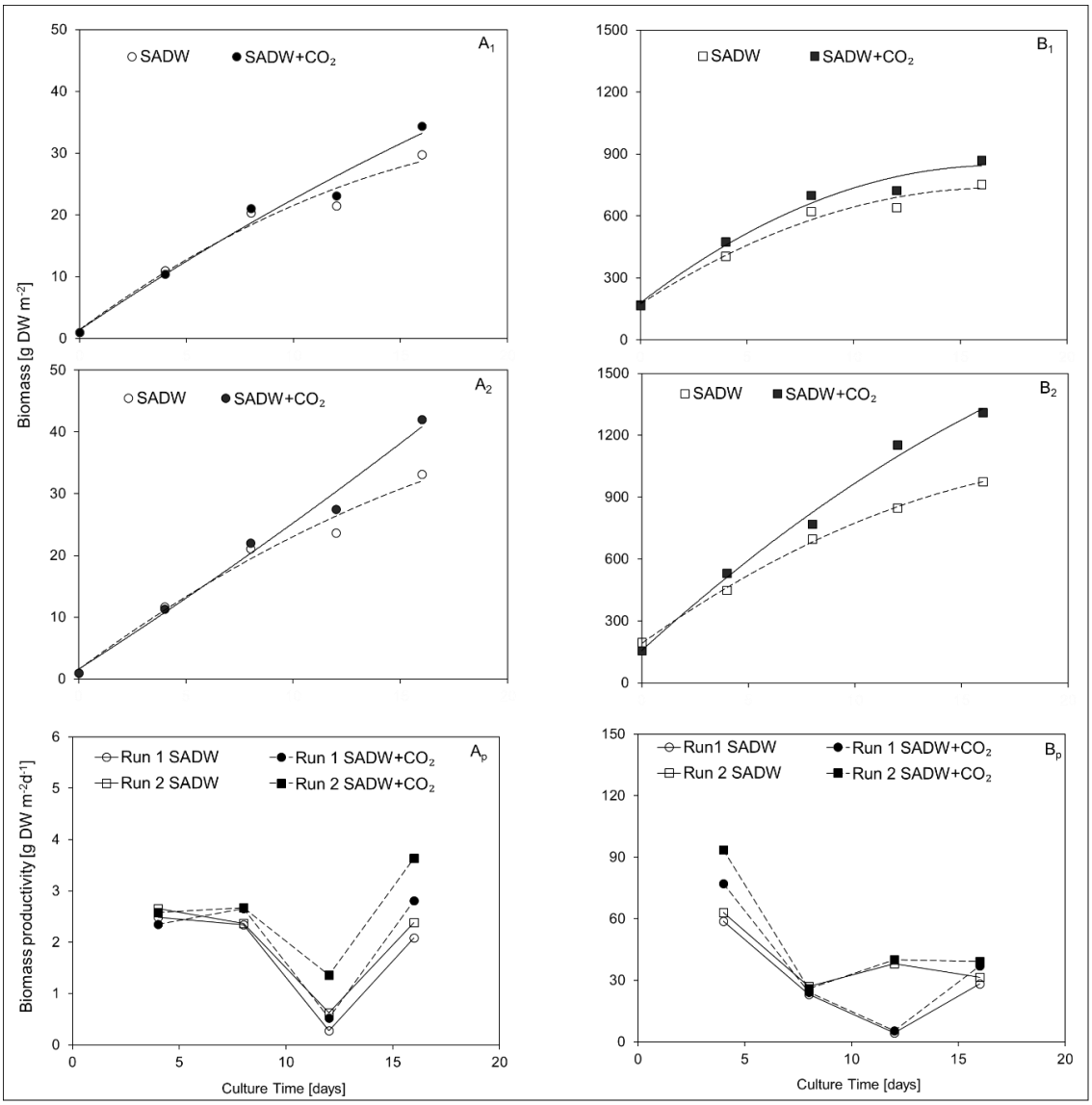


Figure 6.2 Effect of heavy metal and CO_2 on *Tolypothrix* sp. growth in outdoor cultivation A_1:ATS run 1, A_2: ATS run 2, A_p: ATS biomass productivity, B_1: V. Bag run 1, B_2: V. Bag run 2, B_p: V. Bag biomass productivity

Despite positive outcomes of CO_2-fertilisation on biomass productivity (Figs 6.2A_p, 6.2B_p) and system-dependent differences in growth performance, phosphate removal from the medium were slightly higher when supplemented with CO_2, showing rapid uptake for the first three days of cultivation (Figs 6.3A, 6.3B). In contrast, phosphate removal rates (mg PO_4^{3-} g^{-1} DW d^{-1}) were higher for CO_2-supplmented suspension cultures, while no trends were discernible for biofilms. Growth of *Tolypothrix* sp. was phosphate-limited from day 8, and systems were devoid of phosphate from days 16 and 12 for CO_2-supplemented cultures and non-CO_2 controls of biofilm and suspension cultures, respectively. Biomass-standardised phosphate uptake rates were 0.2 to 0.3 mg PO_4^{3-} g^{-1} DW d^{-1} for biofilms, but only 0.0065 to 0.0074 mg PO_4^{3-} g^{-1} DW d^{-1} for suspension cultures and uptake rates were much lower for both cultivation systems from days 8 to 16, reflecting phosphate depletion from the SADW medium (Figs 6.3A_1, B_1).

6.4.2 Effect of culture system and CO_2-supplementation on the biochemical profile of SADW-grown *Tolypothrix* sp.

6.4.2.1 Carbohydrate, protein, lipid, phycocyanin and phycoerythrin contents

CO_2-supplementation increased carbohydrate and lipid contents of *Tolypothrix* sp. by 16 and 25% for biofilms and 26 and 38% for suspension cultures for runs 1 and 2, respectively (Figs 6.4A_1, 6.4B_1). In contrast, effects of CO_2-supplementation on protein contents were marginal (Figs 6.4A_1, 6.4B_1). Maximal carbohydrate, protein and lipid contents were ~49.2, 25.1 and 12.4% of cell dry weight (DW) for *Tolypothrix* sp. biofilms and ~54.7, 26.0 and 14.8% of

DW for suspension cultures when fertilised with CO_2 (Figs 6.4A_1, 6.4B_1). Similarly, fertilisation with 15% CO_2 (v/v) increased phycobiliprotein (phycocyanin, phycoerythrin) contents (% w/w) by ~40 and 27% for *Tolypothrix* sp. biofilms and suspension cultures, respectively. Maximal phycocyanin and phycoerythrin productivities were 0.3 and 0.2 and 3.6 and 2.9 g m^{-2} d^{-1} for biofilms and suspension cultures, respectively (Figs 6.4A, 6.4B).

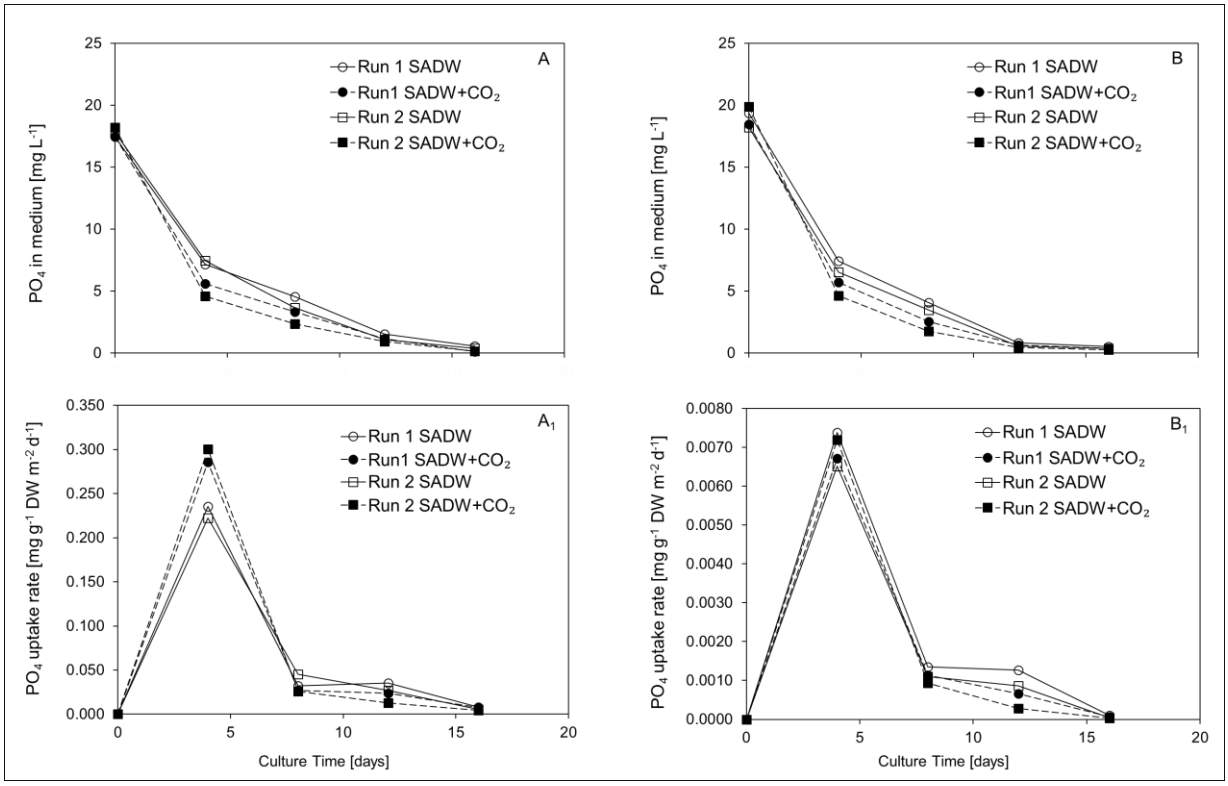


Figure 6.3 Effect of heavy metal and CO_2 on phosphate levels in media and uptake rate of *Tolypothrix* sp. in outdoor cultivation A, A_1: ATS and B, B_1: V. Bag

Table 6.1 Effect of CO_2 and heavy metals on growth of *Tolypothrix* sp. in outdoor cultivation.

	Outdoor cultivation system							
	Algal turf-scrubber				Vertical Bag			
	SADW		SADW+CO_2		SADW		SADW+CO_2	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Specific growth rate (μ_1) [d^{-1}]	0.377	0.381	0.381	0.386	0.162	0.159	0.180	0.199
(μ_2) [d^{-1}]	0.048	0.057	0.061	0.081	0.024	0.045	0.027	0.066
Doubling rate (k) [d^{-1}]	0.543	0.550	0.549	0.558	0.234	0.229	0.260	0.287
Doubling time (t_2)	1.841	1.818	1.820	1.793	4.268	4.371	3.840	3.482

6.4.2.2 Fatty acid and elemental compositions

As growth, lipid and phycobiliprotein contents were increased under CO_2-fertilisation, potential effects on fatty acid profiles and elemental composition (C, H, N, S, P, and K) and C/N ratios were investigated. Total fatty acid (TFA) contents and TFA productivities were ~19 and 12% higher under CO_2 supply for *Tolypothrix* sp. biofilms and suspension cultures, respectively. Maximal TFA yields were ~75 mg g^{-1} DW for biofilms and ~38 and 47 mg g^{-1} DW for runs 1 and 2, respectively (Table 6.2).

A positive effect of CO_2-supplementation on saturated – (SFA), mono-unsaturated – (MUFA) and polyunsaturated fatty acid (PUFA) contents was noticeable for suspension-grown *Tolypothrix* sp., where the fatty acid profile was dominated by SFA, followed by PUFA and MUFA (Table 2). In contrast, responses to CO_2-fertilisation varied between both runs for *Tolypothrix* sp. biofilms, especially for MUFA and PUFA (Table 6.2), possibly due to variations in co-habiting bacterial communities, which are present and required for biofilm establishment and stabilisation. The most abundant fatty acids were palmitic (hexadecanoic) acid (C16:0), followed by the ω-3-group of fatty acids α-linolenic acid (C18:3 (cis 9. 12. 15)), myristoleic acid (C14:1), the SFA myristic acid (C14:0), linoleic acid (C18:2 (cis/trans 9, 12)), the ω-9 oleic acid (C18:1) and the ω-6 γ-linolenic acid (C18:3 (cis 6, 9, 12)) (Table 6.2).

CO_2-fertilisation increased contents of C14:1, palmitoleic acid (C16:1), α-linolenic acid (C18:3 (cis 9. 12. 15)), C18:2 and C18:1 by 33, 31, 45, 72 and 48% in *Tolypothrix* sp. biofilms, respectively, while contents of C16:0 and C18:0 were unaffected. In contrast, CO_2-supplementation increased C16:0, C18:3,

C18:2 and C18:1 by 25, 57, 33 and 32% for *Tolypothrix* sp. suspension cultures, respectively (Table 6.2).

Culture system and CO_2-supplementation did not result in large differences in C, H, N, S, P, and K contents. Carbon (~45 and 47, and ~47% (w/w)), K (~0.79 and 0.99 and 0.68 and 0.79% (w/w)), and S (0.5 and 0.7, and 0.7% (w/w)) were higher when supplemented with CO_2 for *Tolypothrix* sp. biofilms and suspension cultures in runs 1 and 2, respectively (Table 6.3). A small positive effect of CO_2-fertilisation on nitrogen content and C/N ratios was also evident for *Tolypothrix* sp. biofilms, but not for suspension cultures (Table 6.3). In contrast, CO_2 supply had a positive effect on P content of suspension cultures, but not biofilms (Table 6.3).

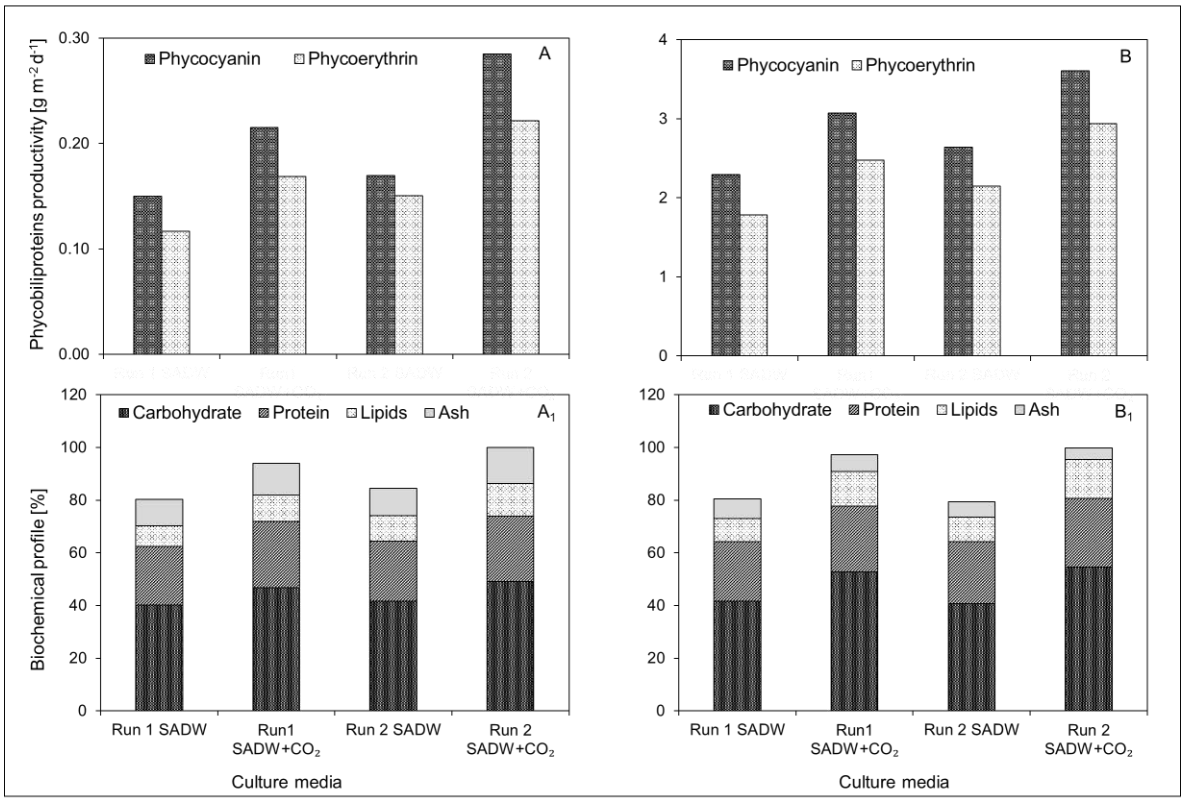


Figure 6.4 Effect of heavy metal and CO_2 on pigments and biochemical profile of *Tolypothrix* sp in outdoor cultivation A, A_1: ATS and B, B_1: V. Bag

Table 6.2 Effect of CO_2 and heavy metals on fatty acid profiles of *Tolypothrix* sp. in outdoor cultivation

Fatty acids [mg g^{-1} DW]	Outdoor cultivation system							
	Algal turf-scrubber - biofilm				Vertical Bag			
	SADW		SADW+CO_2		SADW		SADW+CO_2	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
14:1 (cis-9)	8.6	6.0	9.6	12.2	1.4	1.7	1.0	1.0
14:0	6.0	7.5	0.7	8.6	1.6	1.5	0.9	1.9
16:1	9.8	9.5	7.7	20.1	3.9	3.7	0.9	0.9
16:0	13.0	14.2	11.8	13.1	8.0	11.0	10.9	14.9
18:3 (cis-6, 9,12)	2.2	4.9	0.2	0.2	1.2	1.7	1.5	1.5
18:3 (cis-9,12,15)	10.1	7.1	18.5	13.0	1.4	4.7	6.1	8.1
18:2 (cis/trans-9,12)	1.0	1.6	8.9	0.4	2.6	3.3	4.4	4.5
18:1 (cis/trans-9)	3.0	2.7	7.6	3.4	3.0	5.0	4.9	6.9
18:0	0.2	0.2	0.5	0.2	1.2	1.3	1.2	1.8
SFA [mg g^{-1} FA]	20.7	22.9	14.9	23.8	13.5	17.5	15.1	20.0
MUFA [mg g^{-1} FA]	25.8	18.5	23.1	36.6	8.7	10.7	7.4	9.4
PUFA [mg g^{-1} FA]	13.4	14.6	33.7	13.5	8.9	12.8	14.8	17.9
Total Fatty acids [mg g^{-1} DW]	59.9	60.1	74.6	73.9	31.0	41.0	38.3	47.3
FA productivity [g g^{-1} DW m^{-2} d^{-1}]	0.2	0.1	0.2	0.3	1.1	1.3	1.4	1.9

Table 6.3 Effect of CO_2 and heavy metals on elemental composition of *Tolypothrix* sp. in outdoor cultivation

Elements [%]	Outdoor cultivation system							
	Algal turf-scrubber - biofilm				Vertical Bag - suspension			
	SADW		SADW+CO_2		SADW		SADW+CO_2	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Carbon (C)	41.21	44.38	45.12	47.0	43.17	44.53	47.43	47.47
Hydrogen (H)	6.42	7.18	7.07	7.03	6.99	7.21	7.10	7.13
Nitrogen (N)	6.63	6.88	7.18	7.01	7.18	7.33	7.05	7.06
Sulphur (S)	0.25	0.37	0.46	0.65	0.41	0.47	0.69	0.67
Phosphorous (P)	2.48	2.56	2.41	2.57	0.70	0.98	1.53	1.74
Potassium (K)	0.46	0.71	0.80	0.99	0.44	0.51	0.68	0.79
C/N ratio (C: N)	6.21	6.45	6.28	6.70	6.01	6.08	6.73	6.72

6.4.3 Metal removal

16-day time course experiments investigated the effect of cultivation system and CO_2 on metal removal from SADW, containing metals and concentrations typically found in ash dam water of coal-fired power plants, by *Tolypothrix* sp. (Table 6.4). Cultivation system and CO_2-fertilisation had no effect on maximal cumulative metal removal (Al, Sr, and Zn (≥90%), followed by Cu and Fe (~70-80%), and As (~65-75%)) for both biofilms and suspension cultures. In contrast, CO_2-supplementation increased Mo removal by 37% in both cultivation systems, but an additional cultivation system effect was evident under CO_2-fertilisation, i.e. maximal Mo removal was ~98% for biofilms but only ~60% for suspension cultures. Conversely, a cultivation system effect was evident under CO_2 supply for Se removal, with a 73% increase but a slight decrease for suspension-cultivated *Tolypothrix* sp. and biofilms, respectively. An even stronger cultivation effect was observed for Ni, where CO_2-supplementation negatively affected removal in biofilm cultures, but not suspension cultures of *Tolypothrix* sp..

Table 6.4 Effect of CO_2 on cumulative metal removal from SADW medium by *Tolypothrix* sp. during 16 days of growth

Metals	Initial concentration [µg L^{-1}]	Metal removal in outdoor cultivation system [%]							
		Algal turf-scrubber - biofilm				Vertical Bag - suspension			
		SADW		SADW+CO_2		SADW		SADW+CO_2	
		Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Al	200.00	99.8	99.8	99.8	99.8	99.8	99.8	99.8	99.8
As	31.60	70.0	68.4	69.0	69.0	67.4	63.5	75.7	75.7
Cu	38.20	83.2	82.3	79.5	74.3	76.2	72.5	77.2	77.2
Fe	1110.00	81.0	79.5	75.7	65.5	72.4	70.8	79.7	76.9
Mo	1040.00	63.6	58.9	98.9	98.9	38.9	27.5	60.3	60.3
Ni	22.90	80.1	79.1	57.5	57.5	69.6	63.7	68.3	54.6
Se	174.00	82.5	81.7	77.8	77.7	23.2	12.3	84.2	84.2
Sr	830.97	99.2	99.1	98.6	98.4	98.8	98.8	99.3	99.0
Zn	90.70	94.0	93.5	90.8	90.6	100.0	87.00	100.0	98.0

6.4.4 Economic viability assessment of bioproduct commercialisation derived from SADW-produced *Tolypothrix* sp. biomass – effect of co-location with coal-fired power plants

6.4.4.1 Direct and indirect capital and operational costs

Based on the biomass productivities and biochemical profiles achieved with CO_2 supplementation of SADW-grown *Tolypothrix* sp. in vertical bubble column suspension cultures, NPV analyses assessed the economic viability of four bioproduct scenarios under co-location with coal-fired power stations and traditional cultivation (no co-location) in raceway ponds, commonly used for production at commercial scale. The four bioproduct scenarios modelled were: 1) food-grade phycocyanin as the sole product, 2) biofertiliser as the sole product, 3) 50% of biomass used each for food-grade phycocyanin and biofertiliser production, and 4) biorefining of phycocyanin (100%) and use of the extracted biomass as a biofertiliser (Table 6.5). All capital costs and operating costs for *Tolypothrix* sp. cultivation (Table 6.5) were derived from published data for production of microalgal biomass (Davis et al. 2011; Griffin and Batten 2013; Heimann et al. 2015; Heimann and Huerlimann 2015a; Schenk 2016) Direct and indirect capital costs (engineering fees set at 15% of capital and contingency set at 5% of capital) for building a 10 ha production facility and some operational expenses were considered to be unavoidable. The co-location scenario considered savings on capital costs (land acquisition) and operational costs for maintenance and insurance, water (ash dam wastewater) and CO_2 supply (flue gas) (Table 6.5), but did not apply potential income generated through CO_2 emission reduction and wastewater treatment, as these were

deemed covered by the savings made. Irrespective of location scenario, the lack of nitrogen-fertilisation and the benefits of the self-settling properties of *Tolypothrix* sp. on energy saving for harvesting were considered by applying no costs for nitrogen fertilisation and a 90% saving on dewatering costs (Table 6.5). Accordingly, total capital costs for constructing the 10 ha facility not co-located with a coal-fired power plant were estimated at US$ 853,442 for pond construction, CO_2 -, nutrient supply -, and water-recirculation -, and harvest/ dewatering systems and land acquisition. The only direct capital cost avoided by co-location was for land acquisition (US$ 147,526), reducing the direct capital cost to US$ 705,916 (Table 6.5). Standardised indirect capital costs for engineering fees (including land acquisition), set at 15% and applying a 5% contingency were estimated to be US$ 170,688 for a not co-located facility, of which US$ 29,505 were avoided through free land provided in the co-location scenario, reducing indirect capital costs to US$ 141,183 (Table 6.5). A working capital of 5% of the total costs did not consider any of the benefits derived through co-location. Total annual operating costs for a not co-located facility was estimated to be US$ 2,440,633 for the 10 ha facility and included cost for salaries, maintenance and insurance, phosphate fertiliser and water requirements, CO_2, energy for cultivation, dewatering and drying, and pigment extraction and purification (Table 6.5). Co-location resulted in significant operational savings of ~70%, particularly through avoiding water and CO_2 supply costs and a 90% saving on maintenance and insurance, and energy expenditure; the latter considered that energy would be purchased from the power station at 10% of the sales price to ordinary customers, and maintenance/insurance costs would also be 10% of ordinary costs. Therefore,

annual operational costs were estimated to be US$ 758,241 when co-located (Table 6.5).

6.4.4.2 Bioproduct-generated income and net present value and sensitivity analyses

Bioproduct yields were estimated from biomass productivity and bioproduct productivities obtained in this study. Based on this, it is estimated that a 10-ha facility could produce 117.5 t *Tolypothrix* sp. biomass ha^{-1} y^{-1} for a 300-day production period. The average phycocyanin content of *Tolypothrix* sp. used in this study is 8.8% (w/w), although as shown here, higher yields are possible. In order to put the NPV on a realistic footing, the average yields were used to calculate the yields of food-grade phycocyanin (A620nm/A280nm: 0.7), which can be extracted and purified with an efficiency of 67% (Chaiklahan et al. 2018). This equates to a production of 10.8 t unpurified phycocyanin ha^{-1} y^{-1}, which yields 6.9 t food-grade phycocyanin ha^{-1} y^{-1}, valued at US$ 3,448,155 ha^{-1} y^{-1} based on a sales price at US$ 500 kg^{-1} (Table 6.5). The final purified product will require metal analysis for quality assurance, which has not been considered as a cost, as these costs are expected to be absorbed by the coal-fired power plant the production would be collocated with. Furthermore, production of the food-grade phycocyanin produced when not collocated would not be subject to such analyses, i.e. in this scenario no metal analyses costs would be incurred. Biofertiliser/biochar can fetch a sales price of US$ 500 t^{-1}, which, based on biomass productivities achieved here, equates to US$ 58,750 ha^{-1} y^{-1} (Table 6.5). Based on this, the predicted values for the modelled bioproduct scenarios are highest for co-located production for phycocyanin

extraction in a biorefinery approach and conversion of the extracted biomass to biofertiliser (scenario 4, US$ 548,313,095), closely followed by producing food-grade phycocyanin as the sole product (scenario 1, US$ 538,038,679) (Table 5). Net predicted values were 50% lower based on assumed tax and distribution costs (Table 6.5).

In order to decide whether a project remains commercially viable, sensitivity analyses are essential. Accordingly, the weighted average costs of capital (WACC) is 10% and the sensitivity analyses modelled two scenarios: 1) reduction of the biofertiliser price to 25% of the current value (US$ 125 t^{-1}) and 2) a food-grade phycocyanin price to 25% of the current value due to market saturation (US$ 125 kg^{-1}). This showed that producing biofertiliser as the sole product (scenario 2) is not of commercial interest even when the production facility is co-located. It is assumed that income for environmental services provided at the coal-fired power plant would not provide a strong business incentive. In contrast, producing phycocyanin as the sole product (scenario 1) remains to be of commercial interest (Table 6.5).

Table 6.5 Microalgal culturing facility capital and operating costs, product income potential, net profit value and sensitivity analyses

Size of *Tolypothrix* facility [ha]	10		
Culturing facility costs			
Direct unavoidable capital costs	**Units**	**Cost (USD) ha^{-1}**	**Cost (USD) 10 ha^{-1}**
Open raceway pond construction	$ ha^{-1}	35,436	354,368
CO_2 feed system	$ ha^{-1}	6,717	67,170
Water and nutrient system	$ ha^{-1}	15,832	158,321
Harvesting and dewatering system	$ ha^{-1}	12,606	126,057
Total unavoidable direct capital cost	**$**		**705,916**
Direct capital costs avoided by co-location			147,526
Land acquisition Co-located (assumed land owned by partner industry)	$ ha^{-1}	14,753	
Total avoidable capital cost	**$**		**147,526**
Grand total of all capital cost	***$***		***853,442***
Standardized indirect capital costs			
Engineering Fees - unavoidable	15% capital		105,887
Land acquisition fees - avoidable	15% capital		22,129
Engineering fees - total	**15% capital**		**128,016**
Contingency - unavoidable	5% capital		35,296
Contingency - avoidable	5% capital		7,377
Contingency - total	5% capital		**42,673**
Total indirect costs - unavoidable	**$**		**141,183**
Total indirect costs - avoidable	**$**		**29,505**
Total Indirect costs	***$***		***170,688***
Working capital - all unavoidable	**5%capital**		**51,206**
Operational costs			
Operational costs not co-located	**Units**		

Labour-plant manager/supervisor	1 person ($)		81,072
Labour-Engineer	1 person ($)		60,333
Labour-Lab analyst	2 persons ($)		90,499
Labour-Administration	1 person ($)		43,364
Labour-Technician/pond operator	2 persons ($)		71,645
Maintenance and insurance	10%[B]		84,710
Phosphate input	$ t^{-1}		35,757
Water requirement-avoidable if co-located with waste water industry	$	1,217,646	1,217,646
CO_2 purchase - unpaid able if co-located with coal fired power stations	$	397,127	397,127
Energy demand cultivation, dewatering and drying	$ y^{-1}	10,980	10,980
Costs for pigment extraction and purification[A]	$ t^{-1} biomass		347,500
Total annual operating costs when not co-located	$		**2,440,633**

Operational costs when co-located			
Labour - plant manager/supervisor	1 person ($)		81,072
Labour-Engineer	1 person ($)		60,333
Labour-Lab analyst	2 persons ($)		90,499
Labour-Administration	1 person ($)		43,364
Labour-Technician/pond operator	2 persons ($)		71,645
Maintenance and insurance	10%[B]		17,703
Phosphate input	$ t^{-1}		35,757
Ash dam water	$		0
Flue gas	$		0
Energy demand for flue gas supply (at 10% of supply charge to customer)	$ y^{-1}	9,270	9,270
Energy demand cultivation, dewatering and drying (at 10% of supply charge to customer)	$ y^{-1}	1,098	1,098
Costs for pigment extraction and purification[A]	$ t^{-1} biomass		347,500
Total annual operating costs when co-located	**$**		**758,241**

			Bioproduct income
Biomass derived potential income			

Food-grade phycocyanin 10.8 t ha^{-1} y^{-1} (US$ 500 kg^{-1}) at an extraction/purification efficiency of 67%[A]		$ ha^{-1} y^{-1}	3,448,155	34,481,550
Biofertiliser/ Biochar (US$ 500 t^{-1} DW) (117.5 t biomass dry weight)		$ ha^{-1} y^{-1}	58,750	587,500
Biofertiliser from 50% biomass ((US$ 500 t^{-1} DW) (58.57 t biomass dry weight)		$ ha^{-1} y^{-1}	29,375	293,750
Weighted average costs of capital (WACC)		**%**		**10**
Net profit value analyses				
Profit Value (PV) scenarios over a 20-year period			**Co-located**	**Not co-located**
1)	Food-grade phycocyanin (sole product)	$	538,038,679	491,380,90
2)	100% of biomass converted to biofertiliser (sole product)	$	9,384,962	9,384,962
3)	50% of biomass converted to biofertiliser + 50% food-grade phycocyanin	$	274,601,274	251,458,27
4)	100% food-grade phycocyanin and biofertiliser yields	$	548,313,095	501,841,258
Sales tax and distribution costs for PV scenarios 1-4				
1)	Assumed tax & distribution costs including transport as % sales value [50%]	$	269,019,340	245,690,40
2)	Assumed tax & distribution costs including transport as % sales value [50%]	$	4,692,481	4,692,481
3)	Assumed tax & distribution costs including transport as % sales value [50%]	$	137,300,637	125,729,148
4)	Assumed tax & distribution costs including transport as % sales value [50%]	$	274,156,548	250,920,69
Net profit value for scenarios 1-4				
1)	Food-grade phycocyanin (sole product)	$	269,019,340	245,690,40
2)	100% of biomass converted to biofertiliser (sole product)	$	4,692,481	4,692,481
3)	50% of biomass converted to biofertiliser + 50% food-grade phycocyanin	$	125,729,148	125,729,18
4)	100% food-grade phycocyanin and biofertiliser yields	$	250,920,629	250,920,69
Sensitivity Analyses for NPV scenarios 1-4				
Biofertiliser price at 25% (125 USD t^{-1})				
1)	Food-grade phycocyanin (sole product)	$	269,019,340	245,690,40
2)	100% of biomass converted to biofertiliser (sole product)	$	1,173,120	1,173,120
3)	50% of biomass converted to biofertiliser + 50% food-grade phycocyanin	$	135,540,957	123,969,48

4)	100% food-grade phycocyanin and biofertiliser yields	$	$270,637,187	247,401,28
Food-grade phycocyanin price at $125 kg^{-1}				
1)	Food-grade phycocyanin (sole product)	$	59,067,104	35,738,244
2)	100% of biomass converted to biofertiliser (sole product)	$	4,692,481	4,692,481
3)	50% of biomass converted to biofertiliser + 50% food-grade phycocyanin	$	32,324,519	20,753,030
4)	100% food-grade phycocyanin and biofertiliser yields	$	64,204,311	40,968,393

[A](Chaiklahan et al. 2018); [B] Total direct and indirect capital.

6.5 Discussion

6.5.1 Effect of CO_2-supplementation on biomass production

Successful cultivation of microalgae and cyanobacteria in wastewaters arising from energy -, mining - and mineral-processing industries would provide for a sustainable platform for production of algal biomass and bioproducts, whilst simultaneously providing for efficient bioremediation of potentially harmful nutrients and metals (Roberts et al. 2013a). Significant growth challenges, hampering overall productivities and economics when using wastewater for production, must be addressed first for the realisation of the true commercial potential.

This study demonstrated that CO_2 supplementation significantly improved final phycobiliprotein and biomass yields, as well as biomass productivities of the diazotrophic cyanobacterium *Tolypothrix* sp. grown in SADW under outdoor conditions as biofilms of suspension cultures. Similar increases of up to 60% have been reported for biomass productivities using the non-diazotrophic cyanobacterium *Arthrospira platensis* for cultures supplemented with 1% CO_2 (v/v) (Ravelonandro et al. 2011). In contrast, growth of *Tolypothrix* sp. was reduced by 60% under outdoor (this study) compared to indoor suspension cultivation under the same CO_2-supplemented and wastewater conditions (Velu et al. 2019). Growth of outdoor cultures is often generally lower due to difficulties in controlling cultivation and environmental conditions, such as hydrodynamics, temperature, UV irradiation and irradiance within optimal ranges (Chen et al. 2011a). Light intensity is one of the major factors affecting cyanobacterial and microalgal growth. The optimal light

intensity for growth of *Tolypothrix* sp. is ~500 µmol photons m^{-2} s^{-1}, however, observed light intensities at noon varied from 500 to 900 µmol photons m^{-2} s^{-1} (Table 6.6). This could suggest that periodic photoinhibition might have occurred, especially in younger cultures with low cell concentrations. It must be emphasised that the reported CO_2-supplemented growth was comparable to other microalgae and cyanobacteria grown in various cultivation systems but was achieved without nitrogen-fertilisation (Table 6.7). In addition, biomass growth was phosphate-limited after ~3 to 8 days of cultivation. It is therefore conceivable that phosphate fertilisation at appropriate intervals should improve biomass yields and productivities further. High temperature and irradiance experienced in the ATS were the most likely factors impeding growth of biofilms of *Tolypothrix* sp. (Chen et al. 2011a; Del Campo et al. 2007); a conclusion supported by higher growth of indoor-cultivated microalgal biofilms (Table 6.7).

The observed higher growth of *Tolypothrix* sp. when supplemented with CO_2 could be attributable to positive effects on nitrogen – and photosynthesis-linked carbon fixation. For example, supplementation of cultures of the marine diazotrophic cyanobacterium *Trichodesmium* sp. with large amounts of CO_2 resulted in a 20% increase in nitrogen fixation rates (Levitan et al. 2007). In addition, CO_2 supplementation could have allowed for the reallocation of energy required for inorganic carbon (Ci) uptake and scavenging of O_2. For instance, CO_2-supplementation reduced the energy requirements for Ci uptake in *Trichodesmium* sp. by suppressing the energy-intensive carbon concentrating mechanism (CCM), which are employed under carbon-limiting conditions, freeing up this energy for the fixation of atmospheric nitrogen, another metabolic

pathway with large energy requirements (Levitan et al. 2007). In contrast, despite a large effect of CO_2-supplementation on biomass productivity, no effect was observed for phosphate requirements in biofilm and suspension-grown *Tolypothrix* sp., which was also observed for indoor-cultivated suspension cultures (Velu et al. 2019). This could represent a direct result of phosphate limitation after large uptake over the first three days of the growth period for replenishing internal phosphate stores, providing sufficient energy for CO_2 fixation. In the context of deploying industrial cultivation of *Tolypothrix* sp. for the bioremediation of macronutrient-poor ash dam wastewaters at coal-fired power plants, the ability of sustained growth without requirements for nitrogen-fertilisation offers a distinctive economical advantage. For example, nitrate provision for large-scale production of the non-diazotrophic *Arthrospira platensis* (synonym *Spirulina platensis*) was estimated to account for 50% of the overall production costs (Vonshak and Richmond 1988). Energy savings provided by the self-settling ability of *Tolypothrix* sp. is another significant advantage for wastewater-utilising large-scale cultivation, as costly dewatering infrastructure and energy requirements, that apply to the commonly used microalgal genera *Chlorella* spp and *Scenedesmus* spp (Silva and Silva 2007), is abolished. For example, biofilms and self-flocculated biomass of *Tolypothrix* sp. were 80- and 53-fold more concentrated than the original suspension culture (Velu et al. 2015).Taken together, these properties reduce the need for finite chemical fertilisers and improve the overall economics of cultivation in macronutrient-limited ash dam wastewaters.

Table 6.6 Environmental parameters during the study at the freshwater compound, James Cook University, Australia. (Parameters refers to the noon time)

Culture type	System	Media	Run	Dates	Water temperature (ºC)	pH	Light irradiance (µmol photons m^{-2} s^{-1})
Suspension culture	Vertical bag	SADW	1	September 2016	25 -30	7.0 – 9.0	500-900
		SADW+CO_2			25 -30	6.0 – 9.0	500-900
		SADW	2	October 2016	25-32	7.0 – 9.0	500-900
		SADW+CO_2			25-32	6.0 – 9.0	500-900
Biofilm	ATS	SADW	1	September 2016	25 -30	7.0 – 9.0	500-900
		SADW+CO_2			25 -30	6.5 – 9.0	500-900
		SADW	2	October 2016	25-32	7.0 – 9.0	500-900
		SADW+CO_2			25-32	6.5 – 9.0	500-900

Table 6.7 Effect of CO_2 on the performances of cyanobacterial and microalgal based cultivation systems

Culturing mode	System	Species	Culture medium	CO_2 [%]	Location	Cultivation system working volume/area	Biomass productivity [g m^{-2} d^{-1}]	References
Suspension	Vertical flat-plate photobioreactor (PBR)	*Synechocystis aquatilis* SI-2	Modified SOT inorganic medium	10	Outdoor[1]	24 L	31 – 45	(Zhang et al. 2001)
				40		9 L	18.3	(Zhang et al. 1999)
	Bubble column PBR	*Anabaena* sp. ATCC 33047	Detailed in[a]	NG	Indoor[2]	9 L	81	(López et al. 2009)
	Acrylic cylindrical tank	*Phormidium valderianum*	TDS effluent[b]	NG	Outdoor[3]	550 L	0.03	(Dineshbabu et al. 2017)
	PBR	*Anabaena sp.* CH1	Arnen medium	15	Indoor[4]	5 L	~31	(Chiang et al. 2011)
	PBR	*Chlorella* sp. MM-2	Modified f/2	5	Indoor[4]	0.8 L	~51	(Kao et al. 2012)
	PBR	*Chlorella* sp. MM-2	Swine wastewater	20*	Outdoor[4]	50 L	~36	
	Column PBR	*Spirulina* sp. (*Arthrospira*)	Zarrouk medium	6 & 12	Indoor[5]	1.8 L	27 & 22	(De Morais and Costa 2007)
		Scenedesmus obliquus	MC medium			1.8 L	12 & 17	
	Aerated vertical bag	*Tolypothrix* sp.	SADW[c]	15	Outdoor[6]	500 L	25 - 95	This study
Biofilm	Single layer PBR	*Botryococcus braunii*	Autotrophic nutrient medium	1	Indoor[7]	0.08 m^{-2}	6.5	(Cheng et al. 2013)
	Multi-layer PBR					0.08 m^{-2}	49.1	
	Twin-Layer PBR	*Halochlorella rubescens*	LSBM[d]	3&5	Indoor[8]	3 m^{-2}	~30 & 24	(Schultze et al. 2015)
	PBR	*Pseudochlorococcum* sp.	BG11	10	Indoor[7]	0.00025 m^{-2}	~5.5	(Ji et al. 2014)
	Algal turf-scrubber	*Tolypothrix* sp.	SADW	15	Outdoor[6]	2.2 m^{-2}	1.36 – 3.63	This study

[1]Japan; [2]Spain; [3]South India;[4]Taiwan;[5] Brazil; [6]Queensland, Australia; [7]China;[8]Germany.

[a] Culture medium detailed in (Moreno et al. 1998); [b] Seawater and ossein effluent from gelatin manufacturing industry

[c] Simulated ash-dam wastewater; [d] Large Scale Brackish Medium prepared based on Bold's Basal medium NG- CO_2 percentage not given but CO_2 was used

6.5.2 Effect of CO_2-supplementation on biochemical profiles, metal removal capacity and bioproduct potential

CO_2-fertilisation of outdoor-grown biofilms and suspension cultures of *Tolypothrix* sp. resulted in increased total carbohydrate and lipid contents, as has also been reported for indoor cultivated suspension cultures (Velu et al. 2019). Similarly, an increase in CO_2 supply from 5 to 25% (v/v) increased total carbohydrate and lipid contents of *Scenedesmus bajacalifornicus* by up to 20 and 10%, respectively (Patil and Kaliwal 2017). Elevated CO_2 supplies typically result in increased carbohydrate contents in microalgae and are likely a result of enhanced photosynthetic efficiencies (Giordano 2001) or CO_2-induced low pH stress (Dragone et al. 2011). In contrast, 15% CO_2 (v/v) supplementation led to an increase in pH from 6.0 to 9.0 and 7.0 to 9.0 for CO_2-supplemented and non-CO_2 controls under outdoor cultivation of *Tolypothrix* sp. biofilms and suspension cultures (Table 6.6), suggesting strongly that cultures were still carbon-limited (Coleman and Colman 1981). Accounting for the fact that nitrogen-requirements for growth had to be met solely through nitrogen fixation, it is not surprising that CO_2-supplementation had no effect on protein content for biomass cultivated in either system, which differs from other reported outcomes, where CO_2-supplementation has been shown to correlate with improved nitrate uptake and thus higher protein production (Xia and Gao 2005). As demonstrated by significant CO_2-induced increases in the nitrogen-containing pigments phycocyanin and phycoerythrin, nitrogen supply through nitrogen fixation must have been sufficient under outdoor cultivation of *Tolypothrix* sp.. The increase in the content of these pigments could have been also responsible

for improved growth under CO_2-supplementation, as they are accessory pigments for the capture of light in the light harvesting complexes of the photosystems and protect the photosynthetic apparatus from excess light and reactive oxygen damage (Chakdar and Pabbi 2016).

As for phycobiliproteins, such as phycocyanin and phycoerythrin, CO_2-supplementation resulted in 19 and 12% higher TFA contents in *Tolypothrix* sp. biofilms and suspension-grown biomass, which is similar to results obtained in indoor cultivation (Velu et al. 2019) and with eukaryotic microalgae (Tsuzuki et al. 1990), but no significant effect on MUFA or PUFA content was evident. α-(C18:3 ω-3) and γ-Linolenic acid (C18:3 ω-6) are important dietary supplements with critical health benefits, and the latter is also an ingredient in cosmetics (Ryckebosch et al. 2012). Similar to indoor suspension cultivation (Velu et al. 2019), *Tolypothrix* sp. produced 25% C18:3 ω-6 or 18.5 mg g^{-1} TFA, which is higher than reported for *Arthrospira* (*Spirulina*) sp. (11-16%) (De Oliveira et al. 1999). In contrast to phycocyanin, yields of (C18:3 ω-6), however, remained insufficient for consideration as a main target product in a biorefinery approach, due to low TFA contents characteristic for cyanobacteria.

Irrespective of cultivation system and CO_2-fertilisation, metal bioremediation of *Tolypothrix* sp. from macronutrient-poor ash dam wastewater, containing concentrations of Al, As, Cd, Ni and Zn that exceed the ANZECC guidelines (Roberts et al. 2013a), showed that levels were lowered to acceptance thresholds at the end of the cultivation period. This demonstrated that *Tolypothrix* sp. is a suitable organism for the bioremediation of metals from complex mixtures, under macronutrient-limiting conditions. The produced

Tolypothrix sp. biomass was rich in carbon (45%) and nitrogen (7%), resulting in a C/N ratio of 6.58, similar to results obtained in indoor cultivation (Velu et al. 2019). In addition, the elemental composition and concentrations were comparable to those found in other cyanobacteria, previously reported as suitable for biofertiliser applications (Osman et al. 2010). Importantly, biomass of *Tolypothrix* sp. remained suitable at application rates required for the fertilisation of wheat, supplying in addition to nitrogen and carbon also essential trace elements, such as Cu, Fe, Mo, and Zn (Velu et al. 2019). Diazotrophic cyanobacteria, such as *Tolypothrix* sp., are natural and renewable sources of biological nitrogen, contributing up to 30 kg N ha^{-1} and providing large quantities of organic matter and important plant hormones (i.e. gibberellin, auxin and cytokines) to soils, thereby improving soil fertility (Issa et al. 2014), supporting plant development and protecting against pathogens (Singh et al. 2016). Diazotrophic cyanobacteria, such as *Tolypothrix* sp., are commonly employed as biofertilisers in rice fields (Karthikeyan et al. 2007) and applications of *Tolypothrix* sp. specifically resulted in a 25% increase in crop yields in poorly drained rice fields (Watanabe et al. 1951). Long-term applications improved nitrogenous fertility of soils, attributable to the increase in soil carbon and nitrogen through accumulation of decomposing and live biomass, respectively (Watanabe 1962). In summary, the above application potential, together with CO_2-enhanced growth responses and phycocyanin yields, makes *Tolypothrix* sp. biomass production for use as a biofertiliser a real potential in regions, where agricultural production is located near freshwater-using coal-fired power plants, as is the case for the Tarong power station in Queensland, Australia.

To test and substantiate the commercial viability of *Tolypothrix* sp.-derived food-grade phycocyanin and biofertiliser, net present value and sensitivity analyses evaluated four bioproduct scenarios for the production of *Tolypothrix* sp. biomass under coal-fired power plant co-location and non-colocation of the production facility. Production in traditional raceway ponds was chosen, as no published data on the construction of large-scale production facilities using bubble columns could be found and a comparison of average microalgal and cyanobacterial maximal biomass productivities was not strongly influenced by cultivation system when operated under outdoor conditions (Heimann et al., 2015). In addition, biofilm cultivation was not considered in the modelled scenarios for several reasons. 1) Systems used in the study have a very large areal production footprint. 2) The areal productivity is low. 3) The systems are more prone to contamination by other microalgae when used for extended periods under outdoor conditions (Velu et al., 2015). 4) Establishment costs for large-scale production cannot be applied with any certainty, as biofilm cultivation systems vary significantly in design (Heimann, 2016).

The modelled NPV and sensitivity analyses showed that the production of food-grade phycocyanin is advantageous for commercial viability, whether or not the facility would be co-located, whereas biofertiliser production as a sole product provided very a low incentive for investment. Outcomes for biofertiliser production income were similar to the commercial production of *Azospirillum*, a nitrogen-fixing bacterium, simulated for liquid biofertiliser production in Cuba, but the production scale for the plant was 4-fold larger in terms of product volume (Segura Silva and Pérez Sánchez 2018) than for the *Tolypothrix* sp.

plant in the presented study. In that analysis, salary costs accounted for >50% of the production cost, as the process is labour-intensive, requiring 29 employees to man 24 h shifts (Segura Silva and Pérez Sánchez 2018), while production of *Tolypothrix* sp. biomass represented only 10% of the overall production costs. An NPV analysis for the commercial production of dried microalgal biomass (US$625 t^{-1}) using dairy effluent as a nutrient and water supply also concluded that the process is commercially feasible for a plant size treating 1 million litre of dairy effluent over a 20-year period (Kumar et al. 2020). The sensitivity analyses using one quarter of today's food-grade phycocyanin sales price demonstrated that facilities producing phycocyanin as a sole product or phycocyanin and biofertiliser remain commercially viable whether co-located or not. Instead of using product sales prices, reduction of biomass yields are an alternative parameter in sensitivity analyses. Reduction of biomass yields to one quarter of the original tonnage therefore had a comparable effect on NPV outcomes (Kumar et al. 2020). An obvious worst-case scenario for commercial production would be reduced yearly biomass yields and reduced product sales prices. Applying this situation to the *Tolypothrix* sp. production scenario proposed here over the entire 20-year period determined that production of food-grade phycocyanin as a sole product, 50% of biomass extraction for each food-grade phycocyanin and biofertiliser, and 100% food-grade phycocyanin with the residual biomass converted to biofertiliser (100%) remain commercially feasible. With regards to a decision whether co-location offers significant benefits, the profit difference predicted here would be as large as ~US$ 23 million over 20 years for production of 100% food-grade phycocyanin at a quarter of today's sale price with 100% co-production of biofertiliser. This

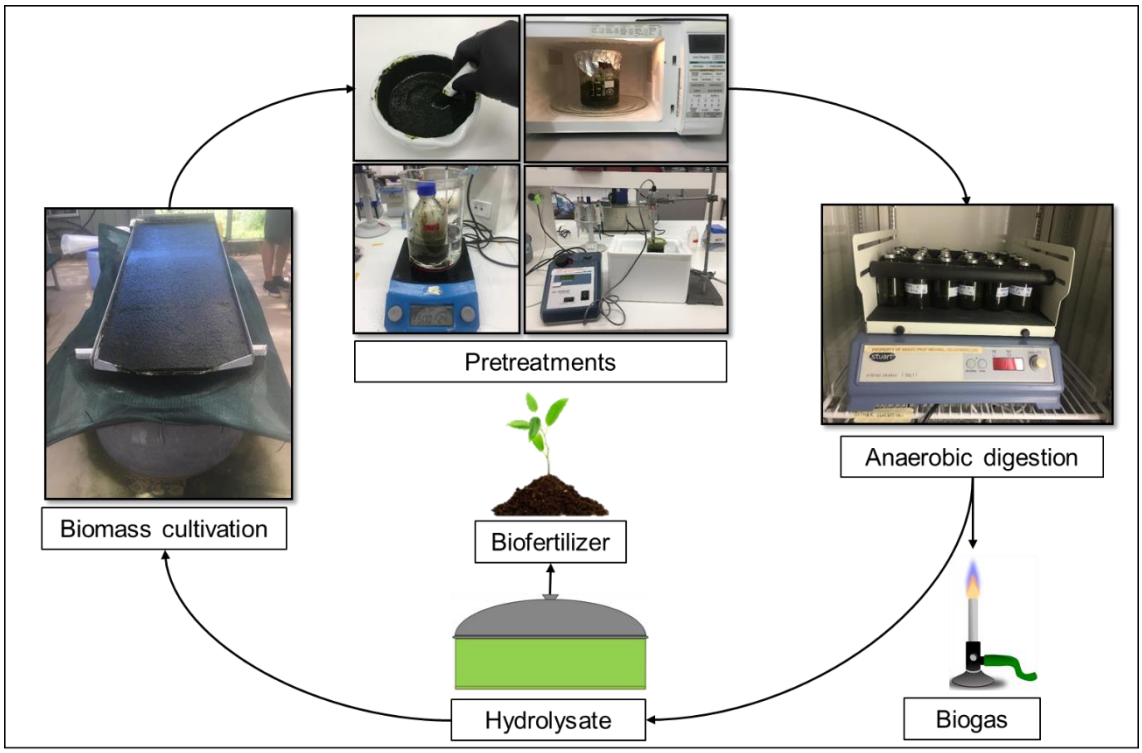
provides a significant incentive for co-locating production facilities with CO_2 – polluting and metal-rich wastewater generating industries, for the simultaneous application of the environmental services of diazotrophic cyanobacteria and bioproduct development.

6.6 Conclusion

This study demonstrated significantly enhanced biomass and phycocyanin yields and productivities in response to CO_2-fertilisation and excellent metal removal capacity of *Tolypothrix* sp. cultivated under outdoor conditions in meso-scale systems without supply of nitrogen fertilisation. This makes *Tolypothrix* sp. an outstanding candidate for bioremediation of CO_2 and metals at freshwater-utilising coal-fired power plants. Obtained growth suggests that 10.91 and 117.5 t dry biomass ha^{-1} can be produced in a year set at 300 days of cultivation in biofilms and vertical bag suspension cultures, respectively. The NPV and sensitivity analyses performed with production data obtained in this study, taking the organism's self-settling ability into account, demonstrated that co-location with coal-fired power plants was not essential for commercial viability, but significantly increased achievable net present values for all modelled product scenarios, making it an attractive proposition, if freshwater-utilising plants are in close proximity to agricultural land. The most profitable scenario was production of food-grade phycocyanin (100%) coupled with co-production of biofertiliser (100%), followed by food-grade phycocyanin as the sole product, and 50% each of phycocyanin and biofertiliser production. In contrast, production of biofertiliser as a sole product was not commercially attractive and not viable under the worst modelled scenario of 20 years of

reduced biomass yields and biofertiliser costs. Based on the above, cultivation of *Tolypothrix* sp. in vertical suspension cultures with CO_2 supply, but without nitrogen-fertilisation is recommended for the production of food-grade phycocyanin either as a sole product or with co-production of biofertiliser.

Chapter 7: Pre-treatment of *Tolypothrix* sp. for methane production[7]



[7] Part of the results from this chapter was invited for oral presentation and presented at ASIA-PACIFIC conference Hong Kong in 2016.

This chapter submitted to Chemosphere and reviewers recommended to publish after minor revisions

7.1 Abstract

Tolypothrix, a self-flocculating, fast growing, CO_2 and nitrogen-fixing cyanobacterium, can be cultivated in nutrient-poor ash dam waters of coal-fired power stations, additionally converting CO_2 emissions into organic biomass. Therefore, the biomass a promising source for bio-energy and bio-fertiliser production through anaerobic digestion (AD). The aim of this study was to evaluate effectiveness of pre-treatment conditions and subsequent methane (CH_4) production potential of *Tolypothrix* under out-door cultivation conditions. The effectiveness of pre-treatment conditions on biogas and methane production investigated for *Tolypothrix* biomass were (1) thermal at 95°C for 10 h, (2) hydrothermal by autoclave at 121°C at 1013.25 hPa for 20 min, using a standard moisture-heat procedure, (3) microwave at an output power of 900 W and an exposure time of 3 min, (4) sonication at an output power of 10 W for 3.5 h at 10 min intervals with 20s breaks and (5) freeze-thaw cycles at -80°C for 24 h followed by thawing at room temperature. Thermal, hydrothermal and sonication pre-treatments supported high solubilisation of organic compounds up to 24.40 g L^{-1}. However, higher CH_4 recovery of 126 mL $CH_4.g^{-1}$ volatile $solids_{removed.}$ was achieved for thermal pre-treatment. High N- and lower C-content of the *Tolypothrix* biomass affected CH_4 recovery, while pre-treatment-accelerated production of volatile acids (15.90 g L^{-1}) and ammonia-N-accumulation (1.41 g L^{-1}) lead to poor CH_4 yields. Calculated theoretical CH_4 yields based on the elemental composition of the biomass were higher than actual yields. This highlights the complexity of interactions during AD which are not adequately represented by elemental composition.

7.2 Introduction

The global population is predicted to reach 9.8 billion by 2050 (UN 2017), resulting in higher energy demands which are predicted to rise by 48% (i.e., 549 British thermal units (Btu) in 2012 to 815 Btu in 2050) in 2050 (IEO 2016). Carbon dioxide (CO_2) emissions are also forecasted to increase by 65% (i.e., 33.6 Gt y^{-1} in 2015 to 55.5 Gt y^{-1} in 2050) (Canadell and Schulze 2014; OECD 2011) due to fossil fuel combustion and transport (EPA 2016). Increased CO_2 emissions are forecasted to raise the global temperature by 4 to 6°C by 2050 (Rowlands et al. 2012), with negative flow-on effects envisaged for the food-energy-water nexus. Therefore, there is an immediate need to reduce CO_2 emissions, which can be achieved through development of alternative fuel resources capable to at least partially replace fossil fuel consumptions (Santos-Ballardo et al. 2016). Biological approaches to energy generation use waste organic material or purposefully produced biomass (e.g. microalgae/ cyanobacteria) in processes such as anaerobic digestion (AD), which yields biogas and biofertilisers (Bird et al. 2012), or fermentation, which yields bio-ethanol (Möllers et al. 2014), and through other hydrothermal liquefactions approaches (Roberts et al. 2013b). Therefore, developing a win-win approach that could effectively reduce CO_2 emissions, remediate any organic/ inorganic contaminants from point sources linked with energy recovery/ savings will be a promising future approach that will provide more economic incentives compared to conventional processes (Roberts et al. 2013b).

Cyanobacteria, a group of fastest growing photosynthetic microorganisms, are highly resilient to high concentrations of CO_2 (up to 70%)

and produce 30 to 180 tons dry weight biomass ha^{-1} y^{-1} (Moreno et al. 2003). Some cyanobacteria can also be grown under extreme environmental conditions of -18 to 55°C, withstand desiccation, pH ranges of 3 to 10, salinities of 10 g L^{-1} NaCl , light intensities of 80 to 1700 µmol m^{-2} s^{-1}, and require low nutrient addition, although these characteristics are strongly species-dependent (Chevalier et al. 2000; Markou and Georgakakis 2011; Sand-Jensen and Jespersen 2012; Vaishampayan et al. 2001). Also, a few cyanobacterial species are being effectively used for bioremediation of organic (e.g. P and N) and inorganic (e.g. metals such as Al, As, B, CU, Fe, Mo, Ni, Se, Sr, V and Zn) contaminants from industrial wastewaters (Gupta and Diwan 2017; Queiroz et al. 2007). Photosynthetic fixation of CO_2 by cyanobacteria of 100 to >200 tons CO_2 ha^{-1} y^{-1} has been reported under outdoor cultivation conditions in open ponds, raceway ponds, photobioreactors and attached growth bioreactors (Cirés et al. 2015; Moreno et al. 2003; Ugwu et al. 2005). For example, *Synechocystis aquatilis* fixed 138.7 to 277.4 tons CO_2 ha^{-1} y^{-1} under winter and summer conditions, respectively (Ugwu et al. 2005). Some strains, such as *Anabaena, Nostoc, Tolypothrix, Cylindrospermum*, *Scytonema* and *Aulosira* are extensively used in rice fields, as they can fix atmospheric nitrogen (N_2) at a rate of 20-30 kg N ha^{-1} $season^{-1}$, significantly improving organic content and soil fertility (Issa et al. 2014). This reduces fertilisation costs; an important criterion for industrial-scale productions of these species for bio-product/ bio-energy recovery. Therefore, selection and consideration of use of particular cyanobacterial species for specific applications requires prior evaluation and scenario analysis for cost-effectiveness.

Unlike many cyanobacterial species, *Tolypothrix* sp., a freshwater cyanobacterium, is filamentous and forms aggregates that self-flocculate, making it very easy to harvest from suspension cultures, which reduces dewatering costs up to 90% (Velu et al. 2015). *Tolypothrix* sp. is also a nitrogen fixing (N_2-fixing) strain which stores phosphorus in phosphate granules, reducing nitrogen and phosphorus fertilisation costs. In the context of metal-rich but otherwise nutrient-poor wastewater remediation, the ability of atmospheric nitrogen fixation, in particular, is an enormous advantage over eukaryotic microalgae and non-nitrogen-fixing cyanobacteria (Velu et al. 2015). These specific features make such process-derived *Tolypothrix* biomass a promising source for bio-energy and bio-fertiliser production through anaerobic digestion (AD) (Quesada et al. 1997; Silva and Silva 2007).

AD, a low-cost energy-generating technology, could be an ideal process, which in addition to biogas yields bio-fertiliser. *Spirulina* (*Arthrospira*) is a cyanobacterial genus most widely studied with high reported methane (CH_4) production potential. According to calculated theoretical CH_4 yields, AD of *Spirulina* sp. (*Arthrospira* sp.), *Spirulina maxima* and *Spirulina platensis* (now *Limnospira maxima* and *A. platensis*, respectively) can produce CH_4 yields of ~ 0.26 – 0.32, ~ 0.63 – 0.74 and 0.47 – 0.69 L CH_4 g^{-1} volatile solids (VS) (Sialve et al. 2009). The broad range of CH_4 yields reported indicate that hydrolysis pre-treatment of the biomass, as well as bioreactor performance, strongly affect obtainable actual CH_4 yields. Hence, pre-treatment of cyanobacterial biomass prior to AD has been proposed to improve bioconversion efficiency and chemical oxygen demand (COD), increase total solids (TS) and VS contents

and CH_4 recovery, whilst reducing digestion time (Sialve et al. 2009). Although a few species of cyanobacteria have been used for anaerobic biogas production, no studies have been undertaken using N_2-fixing cyanobacteria, despite enormous cost reduction potential for biomass production, due to savings on nitrogen fertilising cost. Despite the mentioned advantages of growing *Tolypothrix* sp. for biofertiliser and potentially AD purposes, the thick fibrous gelatinous sheath enclosing the cell wall presents a disadvantage, requiring appropriate pre-treatment methods (Gantt and Conti 1969). To date, no study investigated pre-treatment conditions for effective combined biogas and biofertiliser production from *Tolypothrix* sp. or other N_2-fixing cyanobacteria *via* AD.

Therefore, the present study aimed to test the effectiveness of thermal (Th), hydrothermal (HTh), microwave (μWAVE), sonication (sonic) and freeze and thaw (F&T) pre-treatments to evaluate biomethane potential of the robust cyanobacterial species *Tolypothrix* sp. grown as a water- and energy-saving biofilm for future commercialisation.

7.3 Methods

7.3.1 Outdoor cultivation of N_2-fixing cyanobacterium *Tolypothrix* sp.

Tolypothrix sp. biomass was cultivated as a biofilm under outdoor cultivation conditions in two meso-scale semi-horizontal algal turf scrubbers (ATS) designed and assembled at James Cook University, Australia. ATS set up, inoculation, culture maintenance, light and growth measurements were

carried out as described previously (Velu et al. 2015). Biomass was harvested using a scraper.

7.3.2 Pre-treatment of *Tolypothrix* sp. biomass for anaerobic digestion

Harvested and pre-characterised *Tolypothrix* sp. biomass was pre-treated prior to AD to improve the digestibility and biogas recovery potential. Approximately 1,200 g of wet biomass was homogenised using a mortar and pestle. The ground slurry biomass was refrigerated at 4 ˚C for 24 h for passive leaching of soluble sugars and other organic compounds. Then, the biomass slurry was subjected to five different pre-treatments, which had been reported as ideal and optimised for different types of microalgal biomass in the literature. Pre-treatment of biomass was carried out using 200 g of pre-ground biomass per treatment replicate (n = 3).

i. **Th:** The biomass slurry was kept in a water bath under continuous stirring at 95˚C for 10 h (Passos et al. 2015);
ii. **HTh:** The biomass slurry was autoclaved using a standard moisture-heat procedure of 121°C at 1013.25 hPa for 20 min (Tomy, VWR International, Murarrie, QLD 4172, Australia) (Passos et al. 2015; Razaghi et al. 2016);
iii. **µWAVE:** The biomass slurry was treated with an output power of 900 W and an exposure time of 3 min using a household microwave (Passos et al. 2015);
iv. **SONIC:** The biomass was treated with a rod sonicator at an output power of 10 W for 3.5 h at 10 min intervals with 20s breaks to avoid process heat transfer to the biomass (Passos et al. 2015), and

v. **F&T:** The biomass slurry was frozen (Sanyo ultra-low temperature freezer, MDF-U33V) at -80°C for 24 h followed by thawing at room temperature (Carrère et al. 2010).

Activated sludge without biomass or hydrolysed biomass addition served as an inoculum control for contribution by the microbial consortia to the various measured parameters. Physico-chemical characteristics of the pre-treated biomass were analysed after inoculation with activated sludge prior to digestion (day 0, Table 7.1).

7.3.3 Anaerobic digestion

7.3.3.1 Inoculum

Anaerobically digested activated sludge, used for inoculation, was collected from Cleveland Bay Water Purification Plant (19.2590° S, 146.8169° E), Townsville, Queensland, Australia. Sample aliquots were collected for pre-characterisation following standard procedures (detailed in sub-section 7.3.4). Total - and volatile solid contents of the sludge were 6.22 and 3.75 mg L^{-1}, respectively.

7.3.3.2 Anaerobic digestion set up

Anaerobic digestion was carried out in triplicate in 120 mL serum bottles with a working volume of 100 mL. Fifty grams of pre-treated biomass and 50 g of anaerobic sludge were mixed, the bottles were sealed with butyl rubber stoppers and incubated at 35°C (Panasonic Versatile Environmental Test Chamber, MLR-352) for 21 days. Activated sludge without cyanobacterial biomass served as CH_4 production baseline controls. Total biogas volume was

measured using the gas displacement method using an air-tight syringe. Physico-chemical properties and biochemical profiles of digested slurries was carried out on the day of inoculation (day 0) and after 21 days of AD.

7.3.3.3 Theoretical methane potential calculation

Theoretical methane yields were calculated based on C, H, N, O composition using the following equation (eq. 7.1; (Nielfa et al. 2015))

$$BMP_{thAtC} = \frac{22.4\left(\frac{n}{2}+\frac{a}{8}-\frac{b}{4}-\frac{3c}{8}\right)}{12n+a+16b+14c} \quad \text{---------- (eq.7.1)}$$

Where, n- carbon; a- hydrogen; b- oxygen; c-nitrogen

7.3.4 Analytical methods

7.3.4.1 Physico-chemical properties

All physico-chemical analyses were carried out in triplicates and results are given as mean values plus/minus standard deviation. Total solids (TS) and volatile solids (VS) were determined gravimetrically. pH was analysed with a WP-81 water-proof conductivity / TDS-pH / MV-temperature meter (TPS instruments, Australia). Oxygen reduction potential (ORP) was measured using a HM Digital ORP-200 ORP Meter. Samples were analysed by TropWater's analytical services (James Cook University, Australia) for COD, electrical conductivity (EC), total volatile fatty acids (VFAs) and ammonia-N. Carbohydrate was determined spectrophotometrically (Enspire 2300, Perkin Elmer, Waltham, Massachusetts, USA) using the sulphuric acid-UV method as described in (Albalasmeh et al. 2013).

7.3.4.2 Biochemical profiling

Biomass, biomass slurries and sludge were freeze-dried prior to biochemical analysis. FAME and alkane/alkene were analysed at Sustainable Coastal Ecosystems and Industries in Tropical Australia, Australian Institute of Marine Science, Townsville, Australia. Fatty acid extraction and transesterification (fatty acid methyl ester; FAME) were performed as detailed in (von Alvensleben et al. 2013). Briefly, FAME profiles were analysed using a gas chromatograph (GC) (Agilent 7890B GC-Agilent 5975C) equipped with a DB-23 capillary column (60m x 0.25mm x 0.15 µm) and flame ionisation detector (FID). Split injection mode was used at 1/50 split ratio, temperature was 250°C and FID inlet temperature was 270°C. High purity nitrogen gas was used as carrier gas. Fatty acid quantities were determined against calibration curves of external standards (C8-C24, Sigma Aldrich) and were corrected for recovery of the internal standard nonadecanoic acid (C19:0), added prior to transesterification, and total fatty acid content (mg g^{-1} dry weight (DW)) was determined as the sum of all FAMEs. Alkanes and alkenes were analysed using an Agilent GC-MSD system (6890/5973N) in scan/SIM mode (m/z 50-500 scan; m/z 57, 71, 83, 85, 97 SIM) with a Restek Rxi-5Sil MS fused silica column (30m x 0.25mm x 0.25µm) and ultra-high purity helium as the carrier gas (1mL min^{-1} constant flow). Samples (1µL) were injected into the inlet in pulsed split-less mode (280°C; pulse pressure 25 psi for 2min) and the column temperature was programmed to initially hold at 50°C for 1 min, increase from 50°C to 110°C at 5°C min^{-1}, then 100°C to 310°C at 30°C min^{-1}. Alkanes/alkenes were quantified using TIC (SIM) peak area data against calibration curves of external standards

(C8-C40 even carbon number alkane mixture (Novachem) and docosene (C22:1), as the internal standard. Alkanes/alkene concentrations were corrected for percent recovery of the internal standard (C22:1).

7.3.4.3 Elemental analysis

Carbon-hydrogen-nitrogen-sulphur content (CHNS) (mg g^{-1} DW) of the samples was determined by OEA Labs Ltd. (UK) using an EA-1110 elemental analyser (CE Instruments, Italy) set up in CHNS mode.

7.3.5 Statistical analysis

The statistical significance of experimental results was evaluated by one- and two-way ANOVA and Tukey HSD test, with a significance level (α) of 5%, using Statistica (StatSoft v13.2). Normality was assessed using p-plots and homogeneity of variances were assessed using the Cochran, Hartley and Bartlett test. Data were square root-transformed if normality or homogeneity of variances assumptions were not met.

7.4 Results and Discussion

7.4.1 Characteristisation of *Tolypothrix* sp.

The protein, carbohydrate and lipid content of *Tolypothrix* sp. biomass was ~24, ~51, and 16%, respectively. FAME contents were lower than 5% of total dry weight. FAME contents for cyanobacterial biomass is typically lower than reported for eukaryotic microalgae (e.g. *Dunaliella tertiolecta*: 20 to 25% of total dry weight), as the main carbon storage is in the form of carbohydrates and some amino acids (van den Hoek et al. 1996). The most abundant fatty acids were Palmitic acid (C16:0) and α-linolenic acid (C18:3 ω-3 and ω-6 fatty acids),

which are also common in other N_2-fixing cyanobacteria (e.g. *Nostoc* sp. and *Anabaena* sp. (Liu et al. 2005; Vargas et al. 1998)). The elemental composition was C-45%, H-7%, N-7% and S-0.5%. The theoretical CH_4 potential was, ~ 324 mL g^{-1} $VS_{removed.}$, which was similar to that of *Spirulina* sp. (*Arthrospira* sp.) (González-Fernández et al. 2012; Sialve et al. 2009). Alkane/alkene content was low in *Tolypothrix* sp. and no changes in alkane/alkene profile were recorded following AD. Most abundant alkanes were pentadecane (C15:0), heptadecane (C17:0) and methylheptadecane (C18:0). The total alkane/alkene concentrations varied from 0.5 to 3.0 mg g^{-1} DW.

7.4.2 Theoretical CH_4 yields

Based on the elemental composition, theoretical methane yield was calculated for each test mix prepared from different pre-treatment conditions using a traditional approach (Prajapati et al. 2014). Theoretically achievable CH_4 yields for *Tolypothrix* sp. biomass was ~324 mL g^{-1} $VS_{removed}$. Theoretical CH_4 yields (Suppl. Figure S1) were highest for Th and F&T pre-treatments (~295 and 282 mL CH_4 g^{-1} $VS_{removed}$, respectively, Table 7.1) followed by SONIC (~158 mL CH_4 g^{-1} $VS_{removed}$), yet actual yields were lowest in F&T pre-treatments. A two-way ANOVA determined a significant effect of pre-treatment and time on VS removal ($F_{(5,24)}$=27.56; $p < 0.001$), which also showed significant interaction (Appendix Table E.7). A Tukey HSD analysis determined that the significant difference between the treatments was driven by controls on day 21, which were different to all other treatment, except for µWAVE pre-treatment (Appendix Table E.8). Although calculations on theoretical CH_4 yields are based on elemental analysis, i.e. C and N contents, C/N ratios were lower

than those reported to be optimal for AD (20-30) (Yen and Brune 2007) and cessation of actual CH_4 production towards the end of the AD period (Fig. 7.1) could be due to low C/N ratios (Table 7.1).

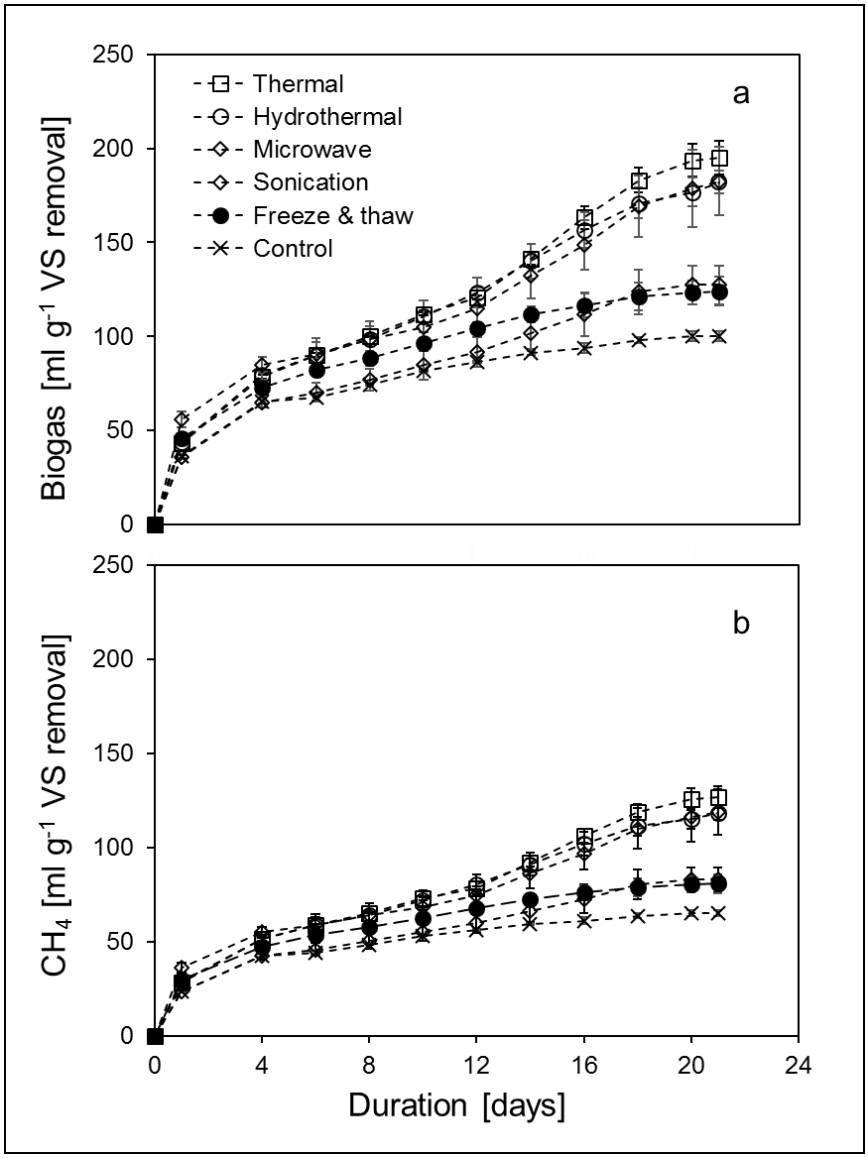


Figure 7.1 Time-course of (a) biogas and (b) methane production of hydrolysates of *Tolypothrix* sp. biomass and activated sludge controls. Results are expressed as mean ± standard deviation (n =3).

Table 7.1 Physio- and biochemical characteristics of anaerobically digested hydrolysates of biomass of the nitrogen fixing cyanobacterium *Tolypothrix* sp. Data are presented as average ± standard deviation (n=3)

Parameters	Time	Thermal	Hydrothermal	Microwave	Sonication	Freeze & Thaw	Sludge Control
pH	Day 0	6.21 ± 0.03	6.18 ± 0.01	6.22 ± 0.01	5.97 ± 0.02	6.06 ± 0.02	6.54 ± 0.03
	Day 21	6.50 ± 0.34	6.18 ± 0.78	6.70 ± 0.31	7.36 ± 0.45	6.52 ± 0.11	7.50 ± 0.51
Electrical cond. [mS cm^{-1}]	Day 0	0.22 ± 0.02	0.22 ± 0.02	0.14 ± 0.01	0.14 ± 0.01	0.21 ± 0.02	0.26 ± 0.02
	Day 21	1.26 ± 0.23	1.41 ± 0.18	1.46 ± 0.10	1.54 ± 0.10	1.27 ± 0.07	1.28 ± 0.16
ORP	Day 0	-44.33 ± 0.58	-33.00 ± 0.00	81.67 ± 0.58	36.67 ± 0.58	125.6 ± 0.58	-321.3 ± 1.15
	Day 21	-212.3 ± 16.66	-185.6 ± 33.01	-214.0 ± 12.17	-213.3 ± 38.73	-155.7 ± 7.77	-280.7 ± 10.02
Biogas [mL g^{-1} $VS_{removed}$]	Day 21	195.00 ± 9.17	182.00 ± 6.08	127.33 ± 10.21	182.67 ± 18.45	124.07 ± 7.52	100 ± 2.65
CH_4 [mL g^{-1} $VS_{removed}$]	Day 21	126.75 ± 5.96	118.30 ± 3.96	82.77 ± 6.64	118.73 ± 11.99	80.64 ± 4.89	65.00 ± 1.72
The. CH_4 [mL g^{-1} $VS_{removed}$]	Day 0	295.00 ± 27.00	93.00 ± 18.00	73.00 ± 22.00	158.00 ± 14.00	282.00 ± 40.00	-
VS removal [%]	Day 21	61.81 ± 4.88	73.18 ± 21.02	31.82 ± 9.08	61.11 ± 10.83	43.88 ± 1.48	21.74 ± 3.87
COD [g L^{-1}]	Day 0	8.01 ± 0.56	8.23 ± 0.58	7.02 ± 0.49	15.02 ± 1.06	13.21 ± 0.93	14.15 ± 3.29
	Day 21	16.98 ± 1.13	15.08 ± 1.87	20.33 ± 1.21	24.40 ± 0.86	19.72 ± 1.30	6.92 ± 1.54
C: N ratio	Day 0	6.32 ± 0.46	6.48 ± 0.73	6.15 ± 0.40	6.33 ± 0.34	5.82 ± 0.38	9.94 ± 1.25
	Day 21	9.31 ± 0.95	7.81 ± 1.45	9.80 ± 0.95	8.74 ± 0.69	9.64 ± 0.94	10.76 ± 1.82
Carbohydrates [g L^{-1}]	Day 0	4.95 ± 0.41	4.68 ± 0.42	3.20 ± 0.26	9.48 ± 0.77	6.84 ± 0.75	5.04 ± 1.49
	Day 21	3.41 ± 0.75	3.09 ± 1.25	7.14 ± 1.20	4.50 ± 0.62	4.60 ± 0.93	2.59 ± 1.60
VFA [g L^{-1}]	Day 0	2.06 ± 0.16	2.05 ± 0.15	3.11 ± 0.23	3.82 ± 0.29	2.38 ± 0.18	1.33 ± 0.10
	Day 21	9.57 ± 0.83	7.98 ± 1.45	11.18 ± 1.07	15.90 ± 0.95	12.11 ± 1.01	1.33 ± 0.09
Ammonia [g N L^{-1}]	Day 0	0.08 ± 0.01	0.08 ± 0.01	0.07 ± 0.01	0.11 ± 0.01	0.08 ± 0.01	0.10 ± 0.01
	Day 21	1.08 ± 0.06	1.05 ± 0.32	1.24 ± 0.20	1.41 ± 0.13	1.29 ± 0.07	1.00 ± 0.09

7.4.3 Methane potential of *Tolypothrix* sp.

Biogas production ceased within 20 days and pre-treatment condition affected maximal biogas and CH_4 production periods and yields (Fig. 7.1(a) and (b)). Carbon and nitrogen concentrations generally decreased, except for carbon concentrations for HTh hydrolysates (Figure 2(a) and (c)). One-way ANOVAs determined a significant effect of pre-treatment on cumulative biogas ($F_{(5,12)}$=47.48; $p < 0.001$) and methane ($F_{(5,12)}$=408.1; $p < 0.001$) production (Appendix Table E.1 and E.3). For biogas production, a Tukey HSD analysis determined that Th, HTh and SONIC-pre-treatments did not differ significantly from each other, but differed significantly from µWAVE, F&T and controls which were similar to each other (Appendix Table E.2). In contrast, for CH_4 production, only HTh and SONIC-pre-treatments and µWAVE and F&T were not significantly different to each other, but Th and controls were different to each other and the other treatments (Appendix Table E.4). Maximum biogas/ CH_4 yields were observed between days 12 to 18 for all treatments, whereas production slowed after day 6 in F&T pre-treatments showing a similar trend to activated sludge controls. Biogas production gradually slowed after day 18. Among the different pre-treatments, Th pre-treatment resulted in maximal actual biogas production of ~ 195 mL g^{-1} $VS_{removed}$ and CH_4 ~ 126 mL g^{-1} $VS_{removed}$ (Table 7.1), similar to yields of Th pre-treated *Arthrospira platensis* (~ 203 mL g^{-1} $VS_{removed.}$ (Markou et al. 2013). Compared to Th yields, SONIC and HTh pre-treatments of *Tolypothrix* sp. resulted in 7 to 8% lower biogas and CH_4 yields. F&T and µWAVE pre-treatment of *Tolypothrix* sp. biomass obtained lowest biogas and CH_4 yields (Figure 7.1, Table 7.1) (Kinnunen et al. 2014), which was

still 2-fold higher than for the sludge control. In general, Th pre-treated *Tolypothrix* sp. resulted in slightly higher CH_4 yields than reported for mechanically macerated biomass of the cyanobacterium *Spirulina maxima* (now *Limnospira maxima* (Nowicka-Krawczyk et al. 2019)) and for whole cells of the green microalga *Scenedesmus* sp. (Inglesby et al. 2015).

Except for HTh pre-treatments, CH_4 yields correlated with the percent volatile solid (VS) removal, which was significantly affected by pre-treatment with a maximum of ~73% in HTh (Table 7.1). A one-way ANOVA (Suppl. Table S5) determined a significant effect of pre-treatment on VS removal ($F_{(5,12)}=32$; $p < 0.001$). Lowest percent VS removal was achieved with µWAVE pre-treatments, Th and SONIC pre-treatments showed similar percent VS removal of ~ 61%, with similar results reported for Th-treated *Spirulina maxima* (now *Arthrospira maxima*) (González-Fernández et al. 2012; Yuan et al. 2010).

In conclusion, pre-treatment methods significantly affected CH_4 yields of *Tolypothrix* sp. in declining order of Th > HTh > SONIC > µWAVE > F&T. Improved CH_4 yields are commonly reported for Th or HTh pre-treatments of different types of biomass, including microalgae and cyanobacteria and energy requirements were reported to be positive for Th pre-treatment compared to the other pre-treatments (Passos et al. 2015). Actual biogas/CH_4 yields were ~55 and ~67% lower than theoretical CH_4 yields for individual pre-treatments (Appendix Fig E.1) for Th and F&T pre-treatments, respectively, which might be due to process inhibition and complex sugar formation (Chen et al. 2008b). Increased mineralisation of nitrogen and phosphorous may also impede high CH_4 yields (Kinnunen et al. 2014). In contrast, actual CH_4 yields were only

slightly lower for SONIC and marginally higher for HTh and μWAVE (Appendix Fig. E.1), which could be explained by the formation of more soluble simple sugars in these pre-treatments (Table 7.1).

7.4.4 Characterisation of pre- and post-digestion slurries

7.4.4.1 System pH:

Near neutral pH of 6.8-7.3 is more suitable for CH_4 production, while more acidic or alkaline pH inhibits the process (Chen et al. 2008b). The initial (i.e., day 0) pH of the digestion slurry for different pre-treatment conditions were slightly acidic i.e., 6.1 – 6.3 and pH did not reach pH 6.8 for Th, HTh, μWAVE and F&T pre-treatments, while SONIC pre-treatment exceeded the maximum range by 0.6 pH units after 21 days, being identical to control sludge (Table 7.1). The higher pH for the latter pre-treatment could be due to highest amounts of ammonia-N , recorded (Table 7.1), but ammonium content cannot be the sole explanation, as amounts were also high in F&T and μWAVE pre-treatments, yet pH was 6.5 and 6.7, respectively (Table 7.1). A two-way ANOVA (Suppl. Table S9) determined that pH was significantly affected by pre-treatment ($F_{(5,24)}$=5.16; p =0.0023), cultivation period (days) ($F_{(1,24)}$=29.32; p <0.001), and pre-treatment showed a significant interaction with days ($F_{(5,24)}$=3.14; p =0.0175). A Tukey HSD analysis determined that SONIC pre-treatment and sludge control were the drivers of the significance (Appendix Table E.10).

7.4.4.2 Oxidation-reduction potential:

The ORP reflects the net value of complex reactions that occur in the digestion process. The initial ORP, after inoculation on day 0 for the 1:1 mixed

Tolypothrix biomass: hydrolysates, was -321.3 and negative values were also recorded for Th and HTh pre-treatments, but positive initial ORPs were recorded for F&T, μWAVE and SONIC pre-treatments (+126, +82, +32 mV, respectively) (Table 7.1). Final values observed on day 21 ranged from -155.7 (F&T) to -214 (μWAVE) (Table 7.1). A two-way ANOVA determined a significant effect of pre-treatment ($F_{(5,24)}$=216.85; p <0.001), cultivation period (days) ($F_{(1,24)}$=1157.01; p <0.001) on ORP, and pre-treatment showed significant interaction with days ($F_{(5,24)}$=88.38; p <0.001) (Appendix Table E.11). A Tukey HSD analysis confirmed the significant difference between each treatment. (Appendix Table E.12). There is no consent on optimal ORP for high rates of methanogenic activity with reported values extending from <-350 mV (Wang et al. 2012) to ranges of -150mV to -250 mV (Nghiem et al. 2014). The latter ORPs are in the range achieved with the pre-treatments here on day 21. Sub-optimal ORPs at the time of inoculation could have significantly delayed methanogenesis for F&T and μWAVE pre-treatments, partially explaining that lowest CH_4 yields were achieved in these systems. In general, more negative ORPs close to the reported optimum could improve actual CH_4 yields. This could be achieved by increasing activated sludge inoculum/ hydolysate ratios for all pre-treatments and intermittent buffer addition to allow for a more homogenous colonisation of all organic particles by methanogens.

7.4.4.3 Electrical conductivity:

Electrical conductivity varied between 138 μs cm^{-1} and 262 μs cm^{-1} for day 0 across pre-treatments, increasing considerably during the cultivation period for all pre-treatments (Table 7.1). This is common due to solubilisation of

more conductive ions (Schwede et al. 2013). A two-way ANOVA determined that there was no significant effect of pre-treatment ($F_{(5,24)}$=0.855; p =0.525) on conductivity, but cultivation period (days) ($F_{(1,24)}$=1094; p <0.001) and the interaction of pre-treatment*days ($F_{(5,24)}$=3.361; p =0.019) were significant (Appendix Table E.13). A Tukey HSD analysis determined that the significant difference between each treatment was driven by cultivation time, as electrical conductivities were not significantly different on day 0 or day 21 between pre-treatments. (Appendix Table E.14).

7.4.4.4 Soluble organics:

Total COD [g L^{-1}] is a measure of soluble organics, which was high for SONIC and F&T pre-treatments (13-15 g L^{-1}) and lowest for μWAVE (7 g L^{-1}) on day 0 (Table 7.1). Total CODs increased 1.5-3 fold for all hydrolysates, which suggests effective solubilisation of organic compounds (Karthikeyan et al. 2016). Maximal COD concentrations were measured for SONIC pre-treatments (~ 24 g L^{-1}), followed by μWAVE and F&T (~20 g L^{-1}), Th (~17 g L^{-1}) and HTh (~15 g L^{-1}) on day 21 (Table 7.1). A two-way ANOVA determined a significant effect of pre-treatment ($F_{(5,24)}$=211.2; p <0.001), cultivation period (days) ($F_{(1,24)}$=1965; p <0.001) on COD, and pre-treatment significantly interacted with days ($F_{(5,24)}$=75.5; p <0.001) (Appendix Table E.15). A Tukey HSD analysis determined that HTh and μWAVE were significantly different to all other treatments on day 0 and μWAVE and F&T were significantly different to all other treatments on day 21 (Appendix Table E.15). High CODs are common for high organic loading rates and/or low inoculum to substrate ratios (Karthikeyan and Visvanathan 2013) and increases are commonly observed for microalgal and

cyanobacterial hydrolysates (Passos et al. 2015). CODs were lower than inhibitory concentrations for methanogenesis (25 g L^{-1}), thus poor methane yields could be a result of the interaction of lower and higher than optimal pH and ORP values, respectively.

7.4.4.5 Volatile fatty acids and Ammonium-N:

Initial volatile fatty acids (VFAs) contents were low (Table 7.1) and concentrations increased 3 to 5-fold. VFAs constituted 53-65% of soluble organics (Table 7.1) and are major intermediates formed during AD, and therefore followed the same trend as observed for COD. A two-way ANOVA determined a significant effect of pre-treatment ($F_{(5,24)}$=34.90; p <0.001) and cultivation period (days) ($F_{(1,24)}$=846.85; p <0.001) on VFA), and a significant interaction of pre-treatment and days ($F_{(5,24)}$=12.29; p <0.001) (Appendix Table E.17). A Tukey HSD analysis determined that on day 0 the significant difference was driven by SONIC pre-treatments and controls, being significantly different to all other, which showed no significant difference amongst them. On day 21, the significant effect was driven by SONIC being significantly different to all pretreatments (Appendix Table E.18). VFAs indicate the degree of acidification (Karthikeyan et al. 2016). Highest VFAs contents, with values similar to sludge control, were observed in SONIC (~16 g VFA L^{-1}), F&T (~12 g VFA L^{-1}) and μWAVE (~11.18 g VFA L^{-1}), followed by Th (~ 9.57 g VFA L^{-1}) and HTh (~7.98 g VFA L^{-1}), the latter indicative of a better conversion of soluble organics to CH_4. High VFA content, however, will adversely affect the conversion rate since the system was not buffered.

Similarly, a Tukey HSD analysis showed that differences in initial (day 0) ammonium-N concentrations were not significant (Table 7.1, Appendix Table E.20), indicative that pre-treatments hydrolysed primarily carbohydrates, but a two-way ANOVA determined a significant effect of pre-treatment ($F_{(5,24)}$=19.14; p <0.001), cultivation time (days) ($F_{(1,24)}$=5949.43; p <0.001) on ammonium-N, and pre-treatment showed significant interaction with days ($F_{(5,24)}$=21.59; p <0.001) (Appendix Table E.19). A Tukey HSD analysis determined that significance was driven by Th pretreatments and controls on day 21, being different amongst themselves and to all other treatments. (Appendix Table E.20). Ammonium-N concentrations reached moderately inhibitory levels (i.e. 0.5 to 1.5 g L^{-1}; (Karthikeyan and Visvanathan 2013)) for all hydrolysates, but another study reports inhibitory concentrations in an up to 2-fold high range of 1.5 to 3.0 g L^{-1} (Akunna et al. 1992). After 21 days of AD, C/N ratios increased due to lower elemental nitrogen levels (Fig. 7.2), but, paradoxically, ammonium-N concentrations increased (Table 7.1). High ammonium concentrations are typically the result of mineralisation of organic nitrogen compounds, such as protein and nucleic acids, with levels easily becoming toxic to methanogens (Fricke et al. 2007).

SONIC and F&T pre-treatments had the highest ammonium-N concentrations (~1.4 and ~1.3 g N L^{-1}, respectively), resulting in low CH_4 yields for F&T pre-treatments, as expected. Paradoxically, SONIC pre-treatments, with the highest concentration of ammonium-N, had CH_4 yields comparable to levels achieved for HTh pre-treatments with low ammonium-N (~1 g N L^{-1}) concentrations (Table 7.1). This could indicate that more than one factor

synergistically or antagonistically affect CH_4 production, as reported for the inhibitory effect of high concentrations of ammonia-N and VFA (Chen et al. 2008b; Montingelli et al. 2015). It is, however, equally possible that concentrations of the different parameters changed faster or slower for particular hydrolysates, which could have led to the same outcomes. Given that reported inhibitory concentrations vary 2-fold for ammonium-N and also for ORP, time course experiments or better still pulse-chase experiments and identification of concentration ranges of VFAs are required to resolve whether methanogenesis was inhibited or rates were affected by delays in the onset (Wang et al. 2009).

7.4.4.6 Biochemical profiling:

Biochemical characteristics of *Tolypothrix* sp. for carbohydrates, FAME profile and alkane/alkene content were described above, and FAME data after digestion are presented in Fig. 7.3, which includes contributions by methanogens and heterotrophic bacteria in addition to the hydrolysates. Initial carbohydrate concentrations were highest for SONIC pre-treatments (~9.5 g L^{-1}), followed by F&T (6.8 g L^{-1}) and were lowest for µWAVE (~3.2 g L^{-1}) (Table 1). Carbohydrate concentrations were reduced following 21 days of AD, but reliable statistical analysis was not possible due to large variances between replicates. Nonetheless, reduction of carbohydrate concentrations indicates the utilisation by methanogens and heterotrophic bacteria (Table 7.1). SONIC showed highest percent reduction of carbohydrate ~53%, while reductions of 30-33% were achieved for HTh, Th and F&T hydrolysates. Interestingly for µWAVE hydrolysates, carbohydrate concentrations increased (Table 1), which might

indicate that *Tolypothrix* biomass was solubilised by a heterotrophic bacteria-dominated microbial consortia, which would also explain the low biogas/CH_4 yields achieved with these hydrolysates (Fig. 7.1).

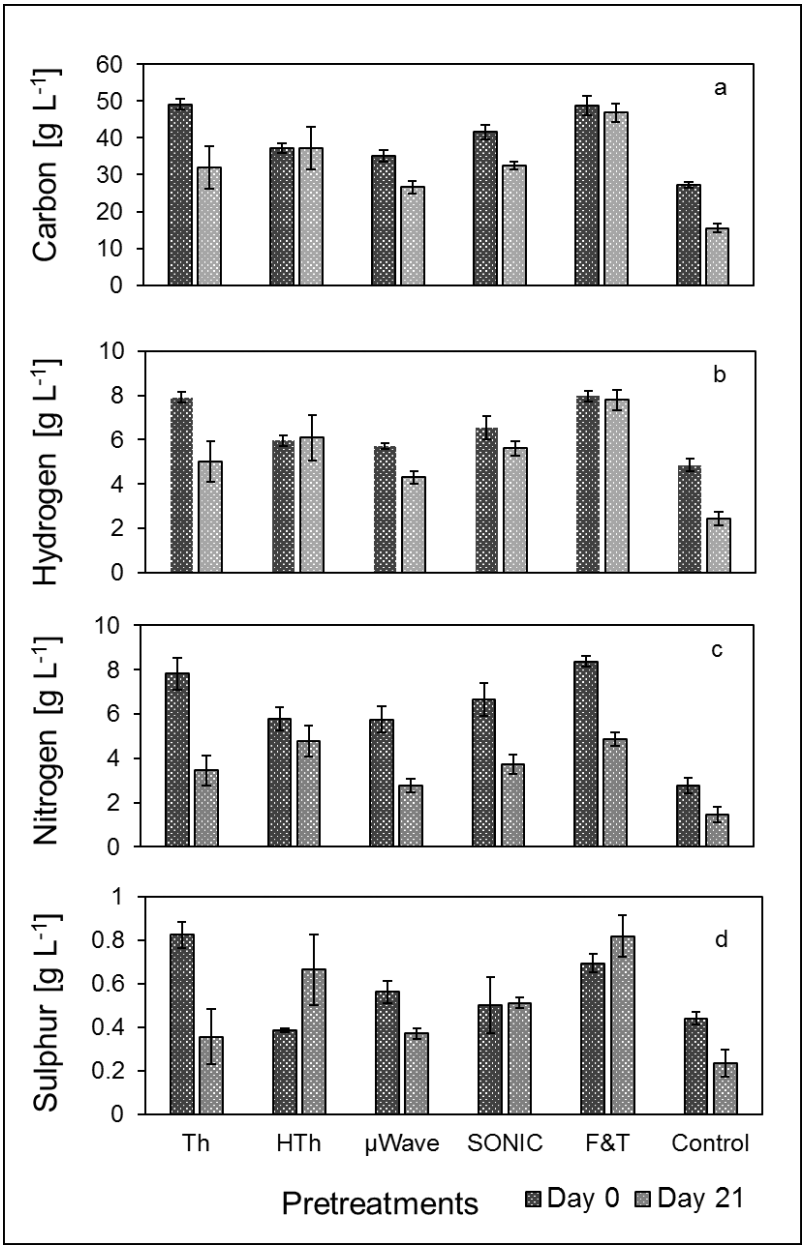


Figure 7.2 Elemental analysis of hydrolysates of *Tolypothrix* sp. biomass and activated sludge controls on day 0 and 21 days after anaerobic digestion. (a) Carbon, (b) Hydrogen, (c) Nitrogen and (d) Sulphur. Results are expressed as mean ± standard deviation (n =3)

The FAME profiles of *Tolypothrix* sp. hydrolysates were analysed before and after AD, the latter containing the AD microbial consortia in addition to the residual cyanobacterial hydrolysates. SONIC pre-treatment solubilised the highest amount of total FAME (~88 mg g^{-1} DW), whereas concentrations ranged between ~56 and 67 mg g^{-1} DW for all other treatments (Fig. 7.3(a)). A two-way ANOVA determined that the effect of pre-treatment ($F_{(5,24)}$=0.461; p =0.800), and cultivation time (days) ($F_{(1,24)}$=0.775; p =0.387) on total FAME profile was not significant, however, pre-treatment*days showed significant interaction ($F_{(5,24)}$=3.559; p =0.015) (Appendix Table E.21). A Tukey HSD analysis confirmed the non-significance of pre-treatment and cultivation time (Appendix Table E.22). Highest amounts of polyunsaturated fatty acids (PUFA) were solubilised in SONIC and μWAVE pre-treatments, while amounts of saturated (SFA) and monounsaturated fatty acid (MUFA) contents were not affected by pre-treatment (Fig. 7.3(a)). The FAME profile of activated sludge inoculum was characterised by a very high content of saturated fatty acids (SFA) and low MUFA and PUFA contents (Fig. 7.3(a)). After 21 days of AD, total FAME concentrations were up to 41% lower for SONIC pretreatments, but only 0.5 to 13% for F&T and μWAVE, while an increase of ~7 and 29% was observed for Th and HTh, respectively (Fig. 7.3(b)). The observed increase could represent contribution of a large population of methanogens and heterotrophs to the FAME, as the SFA profile also increased significantly (Fig. 7.3(b)), correlating with high CH_4 yields (Table 7.1). After 21 days of AD, all FAME profiles closely resembled the activated sludge profile, with a 2-fold increase of SFA being observed for all hydrolysates, irrespective of CH_4 yields, and MUFA and PUFA contents being reduced by 14 to 67% and 40 to 81%, respectively (Fig. 7.3(b)).

Taken the significant changes of SFA contents into account, more in detail fatty acid profiling could be useful to determine dominance of non-methanogens within the anaerobic consortia, if a particular marker fatty acid could be determined.

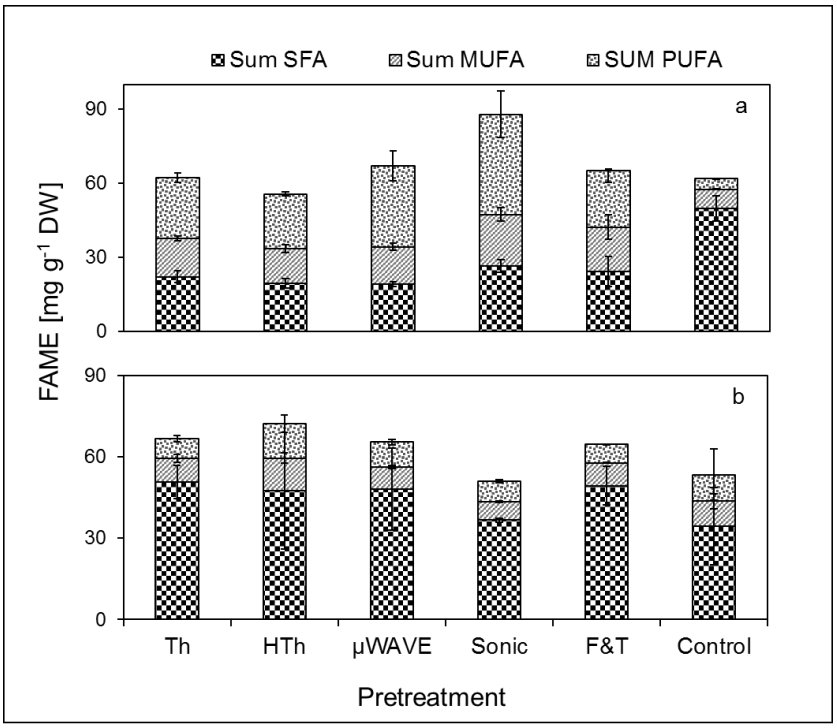


Figure 7.3 Total saturated -, mono-unsaturated – and poly-unsaturated fatty acid (SFA, MUFA, and PUFA) contents of *Tolypothrix* sp. hydrolysates and activated sludge control (a) and the hydrolysate/microbial consortia mix and activated sludge/microbial mix after 21 days (b). Results are expressed as mean ± standard deviation (n =3)

7.5 Conclusion

Anaerobic digestion of *Tolypothrix* sp. biomass hydrolysates produced moderate biogas/ CH_4 yields, despite low C/N ratios, high VFA and ammonium-N contents, sub-optimal pH and ORP. Thermal pre-treatment, reported to be energy positive, gave maximal actual CH_4 yields. Discrepancies between actual and theoretical CH_4 yields, reported here and in the literature, highlight that

complexity of interactions during AD are not adequately represented by elemental composition.

Chapter 8: General Discussion

8.1 Background

The research presented in this thesis was part of the AMCRC-funded research project (grant number 2.3.4) entitled “Bioremediation of methane from mine ventilation air”, established by my supervisor A/Prof. Kirsten Heimann (Heimann et al. 2013). As highlighted in section 1.6, this research is linked to 5 global challenges of the 17 SDG of United Nations (UN 2015). The five challenges were (i) clean water and sanitation, (ii) a6ffordable and clean energy, (iii) climate action (iv) life below water and (v) life on land (UN 2015). In this context, the overall aim of this research was to develop a cost-effective process for wastewater and CO_2 remediation, bioproduct and biofertiliser production by native tropical filamentous cyanobacteria. This research evaluated cost-, water-, and energy-smart biomass production platforms in the aquaculture, agriculture industrial context.

The main outcomes and conclusions discussed in each chapter are briefly restated and explained in the larger context with methodological considerations. The overall knowledge gained from this research is summarised, limitations and future research for bioenergy, environmental and agriculture applications and a brief technoeconomic analysis based on data obtained are presented.

8.2 Novel Research Outcomes

There were several novel contributions of this research to the research field, which are as follows:

Although several studies investigated mixed microbial culture conditions for bioremediation previously, the particular consortium approach in this thesis between a N_2-fixing cyanobacterium and non N_2-fixing cyanobacterium identified that the assimilated nitrogen is not released into the medium and therefore the non-nitrogen fixing cyanobacterium cannot thrive in nitrogen-deplete conditions (Burberg et al. 2018).

The species *Tolypothrix* sp. and *Limnothrix* sp. have not been used before for low-nutrient and metal-rich wastewater remediation combined with bioproduct synthesis. The research provided novel insights for this research field, as it identified that nitrogen fixed by *Tolypothrix* sp. is not shared with the non-nitrogen fixer *Limnothrix*. Therefore, a co-cultivation approach for the remediation of nutrient-poor industrial wastewaters is not recommended.

The effect of CO_2 combined with heavy metals on cyanobacterial biomass growth and bioproduct synthesis presented a novel approach to bioremediation of complex mixtures of heavy metals and CO_2. This research also determined that cultivation of *Tolypothrix sp.* NQAIF319 significantly lowered phosphate fertilisation requirements/costs, making it an ideal candidate for phosphate-limited ADW remediation (Saunders et al. 2012).

The modified algal turf-scrubber is a novel algal cultivation system combined with CO_2 presents the first study in tropical Australia (Velu et al. 2015) for bioremediation of complex heavy metal mixtures and bioproduct synthesis..

The effect of pre-treatment conditions for effective combined biogas and biofertiliser production from *Tolypothrix* sp. was not investigated before. The research identified that thermal hydrolysis is optimal for high CH_4 yields from

Tolypothrix sp. biomass, while sonication showed highest solubilisation of organic compounds. Furthermore, low C/N, high VFA and ammonium-N were confirmed to negatively impact CH_4 yields and, importantly, identified that theoretical estimates of biogas yields based on C/N ratios lacked accuracy.

This research determined at outdoor pilot scale, that the biorefinery approach combined with bioremediation, biofertiliser and pigment production is economically feasible with the N_2-fixing cyanobacterium *Tolypothrix* sp. Additionally, the economic feasibility for bioproduct development was estimated, taking four scenarios into account: 1) production of food-grade phycocyanin as a sole product, 2) biofertiliser as a sole product, 3) use of half the biomass for biofertiliser and food-grade phycocyanin production and 4) biorefining of the high-value phycocyanin with the residue being used as biofertiliser. This study modeled net present value (NPV) and sensitivity analysis for these four scenarios under conditions of co-location with coal-fired power plants and traditional cultivation (not co-located) for a 10 ha plant using suspension bubble columns for biomass production. These important research outcomes provide the knowledge for informed decision for bioremediation approaches for treating metal-rich but nutrient-poor wastewaters and CO_2 emissions, generating an income that easily offsets investment.

8.3 Major findings of the research

This research investigated the potential of native filamentous cyanobacteria in the context of developing an integrated bio-economic model for co-locating cyanobacterial cultivation with wastewaters based on a biorefinery approach for low-cost bioremediation coupled with biofertiliser and

bioproduct synthesis. To investigate this, in chapter 3, the effect of wastewater nature (i.e. nutrient-rich but metal poor (SUWW) (Johnson and Admassu 2013) and nutrient-poor and metal-rich (SADW) (Saunders et al. 2012)) on the growth and biochemical characteristics of the biomass of a non N_2-fixing filamentous cyanobacterium *Limnothrix* sp. NQAIF306 and the N_2-fixing filamentous cyanobacterium *Tolypothrix* sp. NQAIF319 were investigated. In addition, the research investigated whether N_2-fixation by *Tolypothrix* sp. NQAI 319 could support the growth of the non-nitrogen fixer *Limnothrix* sp. NQAIF306 in mixed cultures.

High organic carbon and nitrate loads present in urban wastewater can enhance bacterial growth, supressing algal growth (Ernst et al. 2005; Flores et al. 2005; Ohashi et al. 2011). On the other hand, heavy metals present in many industrial wastewaters can also be toxic to algae, inhibiting growth (Kochoni and Fortin 2019). Hence, *Limnothrix* sp. NQAIF306 and *Tolypothrix sp.* NQAIF319 were grown in SUWW and SADW to examine the tolerance of these organisms to these growth conditions. Eventually, no growth was observed in SUWW medium due to high bacterial growth enabled by the richness in organic carbon sources and high concentrations of nitrate (Ernst et al. 2005; Flores et al. 2005; Ohashi et al. 2011). *Tolypothrix* sp. NQAIF319 produced 36 and 43% higher biomass than *Limnothrix* sp. NQAIF306 and the consortium, respectively.

The consortium approach did not perform well in nitrogen-deficient medium, clearly indicating that N_2 fixed by *Tolypothrix sp.* NQAIF319 was not secreted into the medium and was therefore unavailable for the growth of *Limnothrix* sp. The low protein content of *Tolypothrix* sp. biomass suggests that

N_2-fixation by *Tolypothrix* sp. NQAIF319 was only adequate to support basic growth. Even though, *Tolypothrix* sp. NQAIF319 is capable of N_2-fixation, it assimilated available nitrogen from the media and both cyanobacterial species performed well in metal removal.

In the commercial interest of bioproduct synthesis from cyanobacteria, the biomass was investigated for carbohydrate, protein, lipids, fatty acids, pigments and elementals availability in the biomass. Carbohydrate content was higher (40-48%) in *Tolypothrix* sp. biomass which can be converted to bioethanol through fermentation (Möllers et al. 2014), which could be a feasible product route, should biomass metal contents limit the production of other products from ash dam water raised biomass. Ethanol production from cyanobacterial biomass is in principle sustainable, as cultivation does not require arable land and hence food supplies are not impacted (Quintana et al. 2011). Direct photosynthetic ethanol production (without need for biomass fermentation) has been observed in several cyanobacterial species, such as *Cyanothece* PCC 7822, *Microcystis aeruginosa* PCC 7806, *Oscillatoria limosa*, *Oscillatoria* sp., and *Spirulina platensis* (now *Arthrospira platensis*) (Luo et al. 2010). Hence, it would be an interesting option to explore whether *Tolypothrix* is able to produce ethanol photosynthetically. Protein content was higher (~40-42%) in *Limnothrix* sp. biomass than in *Tolypotrhix* sp.. Although higher protein content biomass is ideal for animal feed (Kovač et al. 2013), *Limnothrix* sp. from this research is not recommended for animal feed due to metal toxicity hazards for biomass raised in metal-rich waters (Dunn et al. 2013).

Based on the elemental composition C (42-46%) and N (7-10%), both species can be used for biofertiliser applications (Velu et al. 2019). The phycocyanin (PC) content was 2-fold higher in *Limnothrix* sp. NQAIF306 (~224 mg g^{-1} DW) compared to *Tolypothrix sp.* NQAIF319 (~117 mg g^{-1} DW), which could be the reason for high protein content in *Limnothrix* sp. NQAIF306 (Boussiba and Richmond 1980; Colyer et al. 2005). On the other hand, the phycoerythrin (PE) content was 2-fold higher in *Tolypothrix sp.* NQAIF319 (~80 mg g^{-1} DW) compared to *Limnothrix* sp. NQAIF306 (~38 mg g^{-1} DW). However, there was no significant difference between two species in terms of pigment productivities (g L^{-1} d^{-1}). The higher PC content (~188 mg g^{-1} DW) in the consortium grown in BG11 medium points to dominance of *Limnothrix* sp.

Total fatty acids were ~30% higher in *Limnothrix* sp. NQAIF306 compared to *Tolypothrix sp.* NQAIF319, while the saturated fatty acid contents were similar (~17 mg g^{-1} TFA). Mono-unsaturated fatty acids were ~65% higher in *Limnothrix* sp. NQAIF306 (~18 mg g^{-1} TFA) compared to *Tolypothrix sp.* NQAIF319 (~6 mg g^{-1} TFA), whereas no polyunsaturated fatty acids were observed in *Limnothrix* sp. NQAIF306 under these culture conditions. While the high protein, C-PC, SFA and MUFA contents of *Limnothrix* sp. NQAIF306 make it ideal for candidate for biorefinery-based products of this nature, ther requirement for nitrogen fertilisation is a strong disadvantage for production in ash dam water at coal-fired power plants. Therefore and based on biomass growth, abolishment of nitrogen fertilisation requirements and fastest growth, *Tolypothrix* sp. NQAIF319 was selected for further studies on the effects of CO_2

and complex metal mixtures on growth, biochemical profile and metal removal capacity.

In order to utilise the *Tolypothrix* sp. for bioremediation application, *Tolypothrix* sp. should be a metal and CO_2 tolerant species. Hence, the effect of CO_2 and metal-rich simulated ash dam wastewater (SADW) on biomass growth and bioproduct potential were determined for *Tolypothrix sp.* NQAIF319 under indoor cultivation conditions as described in section 4.3.2 (chapter 4). 15% CO_2 (v/v) supplementation increased biomass growth 3.3-fold and biomass productivity 3-fold compared to non-CO_2 supplemented cultures. This study was comparable to the 10% CO_2-fertilised diazotrophic cyanobacterium *Anabaena siamensis* grown in normal batch culture and in a novel WAVE^{TM} bioreactor (Cirés et al. 2015). On the other hand, nitrogen- and 10% CO_2-supplemented *Scenedesmus obliquus* and *Chlorella pyrenoidosa* achieved 42 to 43% higher biomass yields (Tang et al. 2011). In another study, a photobioreactor grown *Arthrospira* (*Spirulina*) sp. with 12% CO_2 supplementation showed 1.4-fold higher biomass yields compared to *Tolypothrix sp.* NQAIF319 (De Morais and Costa 2007). The inoculum size (0.15 g L^{-1} *vs* 0.3 g L^{-1} this study) could have resulted in light limitation of the *Tolypothrix sp.* NQAIF319 cultures, and/or the pre-adaptation of *Arthrospira* (*Spirulina*) sp. to CO_2, is also likely to play a significant role (De Morais and Costa 2007), as the latter yielded higher biomass productivities (Lee et al. 2002). In summary, CO_2 supplementation is a good option to enhance growth in several species of tolerant cyanobacteria though growth studies are difficult to compare, as there are many factors such as differences in inoculation density, light regime, light quality and intensity,

fertilisation conditions, cultivation period and strains, singly or combined may affect growth (von Alvensleben et al. 2016).

CO_2 supplementation increased carbohydrate and lipid content about ~ 35 and 4 – 6%, whereas there was no significant effect on protein content. This clearly indicated that photosynthetically fixed carbon could be diverted to storage as carbohydrate and to a certain extend lipid. The carbohydrate content present in *Tolypothrix* sp. NQAIF319 (50-55%) was comparable with *Nannochlororopsis* sp. (15 – 50%), *Porphyridium cruentum* (40 – 57%) *Isochrysis zhangjiangensis* (48%) and *Scenedesmus* (42 – 53%) (González-Fernández and Ballesteros 2012). Obtained protein contents in this study were, however, 2 – 3-fold lower compared to *Arthrospira* (*Spirulina*) sp., *Chlorella* (Tokuşoglu and Üunal 2003) and *Scenedesmus* (Apandi et al. 2017), but comparable to the commonly used aquaculture-feed microalga *Isochrysis* (Tokuşoglu and Üunal 2003).

Similarly, PC and PE contents were also increased by ~23% with highest yields of ~99 and 78 mg g^{-1} DW, respectively, irrespective of culture medium. PC and PE productivities were also increased by 5.9- and 3.6-fold due to 15% (v/v) CO_2 supplementation. Compared to *Tolypothrix sp.* NQAIF319, an 80% higher PBP-content and higher productivities were achieved for the non-nitrogen fixing cyanobacterium *Arthrospira* (*Spirulina*) *platensis* (Jiménez et al. 2003a). PBP production in other diazotrophic cyanobacteria such as *Anabaena* sp. was ~70% higher than reported here for *Tolypothrix sp.* NQAIF319 (Moreno et al. 2003), but both cyanobacterial genera were grown in outdoor conditions under natural sunlight. However, even though PC production from *Tolypothrix*

sp. NQAIF319 was lower than other species, the biorefinery approach would be worth to consider, as *Tolypothrix sp.* NQAIF319 reduces remediation cost and the market value of the pigment is US$ 500 to 1,500 kg^{-1}.

Total fatty acids content was increased by ~7 – 9% in cultures supplemented with 15% CO_2 (v/v). CO_2-supplemented SADW-grown *Tolypothrix* biomass showed increased contents of all FAs by ~19% compared to BG11(-N)-grown CO_2-supplemented biomass. Similarly, 15% CO_2 (v/v) supplementation slight increased carbon (C) and potassium (K) contents of *Tolypothrix sp.* NQAIF319, whereas a slight decrease in nitrogen (N) and sulphur (S) contents were observed, but no significant effect on hydrogen (H) and C/N ratio was detected. CO_2 supplementation slightly decreased P contents of *Tolypothrix sp.* NQAIF319 grown in SADW but increased in BG11(-N) cultivated cultures. CO_2 fertilisation also increased the cumulative removal rates for Al, Sr and V, whereas removal rates were higher for As, Cu and Zn in non-CO_2 controls and CO_2 had no effect on cumulative removal of Fe, Mo, Ni and Se.

To investigate growth under outdoor cultivation, *Tolypothrix* sp. was grown outdoors in simulated ash dam wastewater (SADW) in 500 L vertical bag suspension cultures and as biofilms in modified algal-turf scrubbers. The cultivation systems were aerated with air containing either 15% CO_2 (v/v) or not. CO_2-fertilisation resulted in ~1.25- and 1.45-fold higher biomass productivities and ~40 and 27% increased phycocyanin and phycoerythrin contents for biofilm and suspension cultures, respectively. Maximal biomass yields achieved were 34.4- and 42- and 870.5- and 1310- g DW m^{-2} d^{-1} in two independent runs of

biofilm and suspension cultures, respectively. Similar results were noted for the non-diazotrophic cyanobacterium *Arthrospira platensis* with 1% CO_2 fertilisation (Ravelonandro et al. 2011). *Tolypothrix* sp. NQAIF319 cultivated in suspension culture in this experiment with CO_2 enrichment resulted in higher biomass production compared to other microalgae and cyanobacteria, but outcomes from biofilm were low or equal at best (Table 6.7). CO_2 supplementation significantly increased carbohydrate and lipid content of *Tolypothrix sp.* NQAIF319 by ~16 and 25% for biofilms and ~26 and 38% for suspension culture but the effect on protein content was marginal. Maximal carbohydrate, protein and lipids contents were ~49.2, 25.1 and 12.4% DW for *Tolypothrix* biofilms and ~54.7, 26.0 and 14.8 for suspension cultures with CO_2-supplementation. The green microalga, *Scenedesmus bajacalifornicus*, showed similar results with 20 and 10% increase in carbohydrate and lipid contents when fertilised with 5 – 25% CO_2, respectively (Patil and Kaliwal 2017).

CO_2 had no effect on removal of Al, As, Cu, Fe, Sr and Zn, while Mo removal increased by 37% in both systems. In contrast, Ni removal was reduced in biofilm systems, while Se removal increased by 73% in suspension cultures. As explained in previous chapters, the SADW used in this project was low at macro-nutrients (nitrogen and phosphate) which must be provided through fertilisation for the growth of microalgae. There is a promising cost-effective advantage of cultivating diazotrophic cyanobacteria, such as *Tolypothrix sp.* NQAIF319, on such wastewaters over other commonly used cyanobacterial and microalgal species, such as *Arthrospira* (*Spirulina*), *Chlorella* sp. and *Scenedesmus* sp., which are unable to fix atmospheric nitrogen.

Nitrogen fertilisation accounted for ~50% of the total costs for large-scale production of the non-N_2 fixing cyanobacterium *Arthrospira* (*Spirulina*) (Vonshak and Richmond 1988). Another advantage of *Tolypothrix* over other microalgae is its self-flocculation ability which would reduce harvesting and dewatering costs (Silva and Silva 2007). *Tolypothrix sp.* NQAIF319 cultivated as biofilms as well as self-flocculated biomass were 80- and 53-fold more concentrated than the original suspension culture (Velu et al. 2015). This uniqueness not only reduced the need of finite chemical fertilisers but also improved the overall economics of algal cultivation in such nutrient-limited ash dam wastewaters.

The global population demands more nitrogenous fertilisers which is unlikely met by synthetic fertilisers (Singh et al. 2016). In addition, synthetic fertilisers cause several environmental and health problems such as pollution, soil infertility and significant changes to the ozone layer (Benemann 1979; Singh et al. 2016). Hence, there is need for sustainable, environmentally friendly and cost-effective and efficient innovative fertilisers to satisfy the present and future requirements. Fertilisers obtained from biological N_2-fixation and through recycling and re-use of nitrogen contained in various wastewaters offers great potential benefits (Benemann 1979).

Tolypothrix sp. NQAIF319 biomass is rich in carbon (45% w/w) and nitrogen 7% w/w, resulting in a high C/N ratio of 6.58, irrespective of cultivation system or CO_2 supplementation. Indoor grown CO_2-supplemented *Tolypothrix sp.* NQAIF319 suspension cultures showed similar compositions (Chapter 4), thus demonstrating the suitability of *Tolypothrix* sp. NQAIF319 for application as an organic fertiliser for the growth of various crops.

To test and substantiate the commercial viability of *Tolypothrix* sp. food-grade phycocyanin and biofertiliser potential, net present value and sensitivity analyses evaluated four bioproduct scenarios for the production of *Tolypothrix* sp. biomass under coal-fired power plant co-location and non-colocation of the production facility. These analyses showed that the production of food-grade phycocyanin is advantageous for commercial viability, whether or not the facility would be co-located, whereas biofertiliser production as a sole product provided a very low incentive for investment. Outcomes for biofertiliser production income were similar to the commercial production of *Azospirillum*, a nitrogen-fixing bacterium, simulated for liquid biofertiliser production in Cuba, but the production scale for the plant was 4-fold larger in terms of product volume (Segura Silva and Pérez Sánchez 2018) than for the *Tolypothrix* sp. plant in the presented study. In that analysis, salary costs accounted for >50% of the production cost, as the process is labour-intensive, requiring 29 employees to maintain 24 h shifts (Segura Silva and Pérez Sánchez 2018), while production of *Tolypothrix* sp. biomass represented only 10% of the overall production costs. An NPV analysis for the commercial production of dried microalgal biomass (US$625 t^{-1}) using dairy effluent as a nutrient and water supply also concluded that the process is commercially feasible for a plant size treating 1 million litre of dairy effluent over a 20-year period (Kumar et al. 2020). The sensitivity analyses using one quarter of today's food-grade phycocyanin sales price demonstrated that facilities producing phycocyanin as a sole product or phycocyanin and biofertiliser remain commercially viable whether co-located or not. Instead of using product sales prices, reduction of biomass yields is an alternative parameter in sensitivity analyses. Reduction of biomass yields to one

quarter of the original tonnage therefore had a comparable effect on NPV outcomes (Kumar et al. 2020). An obvious worst-case scenario for commercial production would be reduced yearly biomass yields and reduced product sales prices. Applying this situation to the *Tolypothrix* sp. production scenario proposed in the present study over the entire 20-year period determined that production of food-grade phycocyanin as a sole product, 50% of biomass extraction for food-grade phycocyanin and 50% biofertiliser and 100% food-grade phycocyanin with the residual biomass converted to biofertiliser (100%) remain commercially feasible. With regards to a decision whether co-location offers significant benefits, the profit difference predicted here would be as large as ~US$ 23 million over 20 years for production of 100% food-grade phycocyanin at a quarter of today's sale price with 100% co-production of biofertiliser. This provides a significant incentive for co-locating production facilities with CO_2-polluting and metal-rich wastewater generating industries, for the simultaneous application of the environmental services of diazotrophic cyanobacteria and bioproduct development.

In chapter 7, the biomass of *Tolypothrix* sp. NQAIF319 grown in SADW+15% CO_2 under outdoor conditions was anaerobically digested to produce biogas and biofertiliser. This chapter evaluated the effect of various pre-treatments on biogas production from *Tolypothrix* sp. NQAIF319 biomass. Of the five pre-treatment conditions, high solubilisation of organic compounds up to 24.40 g L^{-1} were achieved by thermal, hydrothermal and sonication pre-treatments. However, thermal pre-treatment achieved highest CH_4 yield of 126 mL CH_4 g^{-1} volatile solids removed, which were slightly higher than reported for

mechanically macerated biomass of the cyanobacterium *Limnospira* (*Spirulina/ Arthrospira*) *maxima* and for whole cells of the green microalga *Scenedesmus* sp. (Inglesby et al. 2015). Actual biogas/CH_4 yields were ~55 and ~67% lower than theoretical CH_4 yields for individual pre-treatments for Th and F&T pre-treatments, respectively, which might be due to process inhibition and complex sugar formation (Chen et al. 2008b). Moderate biogas/ CH_4 yields were achieved, despite unfavourable conditions, such as low C/N ratios, high VFA and Ammonium-N contents.

8.4 Nutrient utilisation and limitations to growth and implications for bioproduct synthesis

Microalgae and cyanobacteria utilize several essential macro and micro nutrients such as carbon, nitrogen, phosphorus, sulphur, potassium and iron along with solar energy during photosynthesis for their growth (Markou et al. 2014b). Among these nutrients, nitrogen and phosphorus are very fundamental for microalgae and cyanobacteria growth and biomass production. They require high amounts of nitrogen and phosphorus for protein, phospholipids and nucleic acids synthesis. Hence, microalgae and cyanobacteria cultivation is considered suitable for nutrient removal from wastewater sources and conversion to useful biomass-derived products (Lodi et al. 2003; Markou et al. 2014b). In order to utilise cyanobacteria or microalgae for bioremediation purposes, their potential for nutrient uptake/removal needs to be established for local conditions.

Nitrogen and phosphate utilisation of *Tolypothrix* sp. was analysed in this PhD study. Almost ~90% of PO_4^{3-} was utilised from BG11, BG(-N) and SADW within 3 days with maximum uptake rate between 2 - 2.5 mg g^{-1}DW d^{-1} by

Tolypothrix sp. (Section 3.4.3.1). In contrast, only ~50% of nitrate was utilised by *Tolypothrix* sp. from BG11 nitrogen-containing medium with maximum removal rate of ~5.9 mg g^{-1} DW d^{-1}(chapter 3: section 3.4.3.2). On the other hand, phosphate uptake rates were 3 – 4 mg PO_4^{3-} g^{-1} DW d^{-1} within 3 days when BG11(-N) and SADW were enriched with 15% CO_2 (v/v), halved over the next 3 days and halved again for the following 3 days, showing steady low uptake rates of approximately 0.5 mg PO_4^{3-} g^{-1} DW d^{-1} from day 9 for the remainder of the cultivation period. Growth of *Tolypothrix* sp. was phosphate-limited as of days 15 and 18 (chapter 4: section 4.4.1). The biomass standardised nutrient removal rate was even lower in outdoor cultivation system with maximum removal rate of 0.2 to 0.3 mg PO_4^{3-} g^{-1} DW d^{-1} for biofilms, but only 0.0065 to 0.0074 mg PO_4^{3-} g^{-1} DW d^{-1}. However, the cultivation systems were phosphate depleted on days 8 to 16.

Almost 80-90% of phosphate was removed within 3 and 8 days cultivation time from indoor and outdoor experiments, respectively. The phosphate removal within 3 days was likely due to biological assimilation and most likely accompanied by luxury update by *Tolypothrix* sp. as its growth continued in linear manner. The biomass growth and phosphate removal efficiency of *Tolypothrix* sp. from this study was about 50% lower than *Arthrospira platensis*. *Arthrospira platensis* removed ~ 2.5 mg P g^{-1} biomass with a biomass growth of ~3.5 g L^{-1} at the concentration used (8.4 mg P L^{-1}) in the medium (Markou et al. 2012). On the other hand, *Tolypothrix* sp. from this research removed 0.6 – 1.3 mg P g^{-1} biomass with the biomass growth of 3.5 g L^{-1} at the concentration used (5.4 mg P L^{-1}) in the medium. Although *Tolypothrix*

sp. was P-limited and uptate rate was halved from day 9, that the culture continued to grow up to 18 days could be due to assimilation of P from stored phosphate in phosphate granules. Phosphate stores in microalgae and cyanobacteria can be 3% of their cell weight, which can be utilised in phosphate-limiting conditions (Silva-Benavides and Torzillo 2012).

In general, phosphate can be removed either biotically (assimilation by the micro-organisms) or abiotically by precipitation. Abiotic phosphate removal is enhanced by photosynthesis-induced increase of pH (Lodi et al. 2003). Biological removal of phosphorus occurs during the biomass growth phase of young cultures, whereas chemical precipitation occurs when biomass concentration reaches a threshold value (Lodi et al. 2003). As the pH of the culture in this research increased to 9.00, phosphate removal through precipitation could occur, but since phosphate contents of the media used were low compared to other cultivation media and reduced during the most active phase of growth, it is more likely that removal was primarily due to biological uptake. The elemental P composition of *Tolypothrix* sp. biomass was analysed and P concentration ranged from ~ 0.5 – 0.97 mg g^{-1} DW biomass (Table 4.3) and P removal rate of from that experiment was ~ 0.97 – 1.3 mg P g^{-1} DW biomass. As the P concentration in the biomass at the end of the experiment was less than the maximum phosphate removal rate, some phosphate abiotic phosphate removal could have occurred. P elemental composition of *Tolypothrix* sp. was not analysed for all chapters due to lack of facility and funding availability. The lower growth of *Tolypothrix* sp. in indoor experiments was due to phosphorus depletion after 3 days of culture time.

Phosphate availability influences the biochemical composition of the biomass. Low phosphate availability generally increases carbohydrate contents, whilst decreasing biomass protein contents (Markou and Georgakakis 2011). *Tolypothrix* sp. biomass from this research was also high in carbohydrate (~50%) and low in protein (~23%) content (Fig. 4.3). *Anabaena variabilis* showed similar results under phosphate-depletion (Markou and Georgakakis 2011). Phosphate-limitation decreased protein and increased carbohydrates contents in *Arthrospira platensis (Markou et al. 2012)*. Additionally, phosphate-limitation has no effect on lipid content of *Arthrospira platensis* but increased lipid accumulation of *Anabaena (Markou et al. 2012)*. More research is, however, required to evaluate the effects of phosphate-limitations on biomass growth and biochemical composition of cyanobacteria for prevailing local conditions.

Biomass growth, photosynthesis, phosphate uptake/removal rate and biochemical composition are influenced by various factors such as light intensity, temperature, nutrient (C, P, N, Fe and other micro elements) availability, culture agitation/aeration and inoculum size. Phosphate-removal is influenced by biomass density, phosphate concentration, and light intensity. Hence, *Tolypothrix* sp. growth in the indoor study in this research was limited by phosphate-depletion. On the other hand, *Tolypothrix* sp. growth in the outdoor experiment in this research was affected by various factors such as carbon limitation due to less effective agitation/CO_2 aeration for the large volume culture, lower light intensity due to auto-shading, and phosphate-limitation (Lodi et al. 2003; Markou et al. 2012; Silva-Benavides and Torzillo 2012).

Results show that more research on this topic must be conducted to optimise the culture conditions for production of *Tolypothrix* sp. under outdoor conditions, in order to optimise bioproduct potential and yields and nutrient removal efficiency.

8.5 Limitations of this research and future directions

This research had a few limitations due to the time constraints of PhD research and available infrastructure. The limitations are as follows;

Cyanobacterial species used in this research did not grow in simulated urban wastewater due to bacterial overgrowth at high organic C loads (Zhang et al. 2012) and ammonium toxicity (Dai et al. 2008; Markou et al. 2014a). Axenic cultures could not be established, as the bacteria attached to the cyanobacterial filaments. To overcome this limitation, natural microbiome-based symbiotic relationships could be exploited for cyanobacteria of interest which could start with isolation of such species consortia from such high organic load containing wastewaters (Silaban et al. 2014).

It was not possible to calculate gas-analyzed-based CO_2-fixation rates, as leakage of gas was possible from the outlet of the Schott bottles. Air-tight bottles will be required for any experimental setup to determine actual CO_2-fixation rates. In addition, phospahte and metal concentration of the biomass could not quantified for all the experiments due to lack of facility and funding availability. The results obtained from this research clearly indicate that culture conditions used here limited biomass growth through phosphate-limitation, resulting in lower phosphate removal rates compared to other microalgae and cyanobacterial species. Hence, optimisation studies are required to evaluate the

effect of light intensity, temperature, and phosphate on growth, phosphate removal efficiency, biochemical composition of *Tolypothrix* sp. at different light intensities, inoculum, nutrient concentration, metal concentration, and CO_2 concentrations. Growth and photosynthesis analysis, nutrient availability, biochemical composition, metal concentration, elementals composition of *Tolypothrix* sp. biomass should be analysed in time-course experiments for each culture condition for each factor separately and in factorially designed experiments, taking into account that extraction efficacies of protein and phycobiliproteins need to be validated by determining remaining product levels in the extracted biomass (Lodi et al. 2003; Markou et al. 2012; Silva-Benavides and Torzillo 2012). Given the poor areal growth achieved in the algal turf scrubbers and the problem with invasion by biomass predators, the cultivation system is not ideal for long-term bioremediation, if product synthesis is an expected outcome. Too much light can inhibit the growth of the upper layer of the biofilm (photoinhibition), while photosynthesis in the lower self-shaded parts of the biofilm ceases (Kesaano and Sims 2014). Hence, the influence of light, temperature, nutrients (N & P) concentrations on biofilm cultivation of *Tolypothrix* sp. should be optimised and more pilot scale experiments are required to investigate the impact of harvesting/partial biofilm removal on productivities, biochemical composition and regrowth. Furthermore, constant production of laboratory inocula for biofilms is neither cost- not energy- efficient. To exploit the biofilm advantage, new systems and methods need to be researched.

This research was initially planned to produce biochar and pyrolysis biofuel after growing *Tolypothrix* sp. NQAIF319 in simulated ash dam wastewater in outdoor conditions. In addition to organic carbon and nitrogen, the biochar would also contain essential minerals, bioaccumulated from the ash dam wastewater. Biochar production is a carbon-negative technology which can be used for improving soil and carbon sequestration (Chaiwong et al. 2012). Unfortunately, the feasibility of this approach could not be investigated due to time constraints and lack of facilities, but future research should investigate this avenue as another bioproduct route for bioremediation-generated *Tolypothrix* sp. NQAIF319 biomass.

Unfortunately, a real life onsite pilot study at a coal-fired power station with actual ash dam wastewater and CO_2 to grow *Tolypothrix* sp. NQAIF319 could not be achieved (resource limitation, abolishment (The Clean Energy Legislation (Carbon Tax Repeal) Act 2014) of the Carbon Tax in Australia, resulted in loss of this industry partner in the project) and actual agricultural applications were also beyond the scope of this research, the latter due to time limitations. Such research must be undertaken in future to validate the performance of *Tolypothrix* sp. NQAIF319 in an actual remediation setting. Furthermore, the biomass generated at large-scale remediation sites must be tested in agricultural applications in order to validate application rates, verify non-soil and crop-toxicity in long-term applications and validate outcomes in terms of crop productivity and health improvements of crops and soils.

8.6 Conclusion

This research evaluated the bioremediation, biofertiliser and bioproduct synthesis potential of the native non-N_2-fixing filamentous cyanobacterium *Limnothrix* sp. NQAIF306 and N_2-fixing filamentous *Tolypothrix sp.* NQAIF319. Based on biomass growth, biomass productivity, self-flocculating and nitrogen fixation ability and other bioproduct synthesis potential, *Tolypothrix sp.* NQAIF319 was selected as the best candidate for low-nutrient metal-rich simulated wastewater and CO_2 remediation for biofertiliser and bioproduct synthesis at low-cost. This research determined the resilience of *Tolypothrix sp.* NQAIF319 against heavy metals and CO_2 and integrated bioproduct synthesis potential under indoor and outdoor conditions. This work also demonstrated that *Tolypothrix sp.* NQAIF319 can be cultivated in simulated ash dam water without nitrogen fertilisation, whilst remediating metals and CO_2. Bioaccumulated mineral levels would pose no metal toxicity to plants based on nitrogen-requirements of wheat. The cultivated biomass can be used as live soil conditioner, but the greatest financial gain can be achieved when subjecting the biomass to a biorefinery approach. Based on published data, application as live fertiliser or soil conditioner are predicted to transform structurally poor soils into healthier soils through building a healthy microbial flora, improving soil carbon content, increasing cation exchange and water retention capacity and providing fixed nitrogen to plants. Likewise, despite low C/N ratios, high VFA and ammonium-N contents, hydrolysates of *Tolypothrix sp.* NQAIF319 biomass produced moderate amount of biogas and CH_4 yields through anaerobic digestion, suggesting that biomass could be used for on-site energy generation

and solid and liquid biofertiliser production. In a biorefinery approach, phycocyanin can be extracted and the residual biomass can be used for either anaerobic digestion to produce biogas or be fermented to bioethanol. The residual solids after fermentation and anaerobic digestion can be utilised as mineral-rich biofertiliser. However, to implement this research in reality and to ensure economic viability of this application, a real time techno-economic analysis will be needed, based on a pilot study at a coal-fired power station in Australia, ideally where farmland is also nearby. The net present value (NPV) and sensitivity analyses performed with production data obtained in this study, taking the organism's self-settling ability into account, demonstrated that co-location with coal-fired power plants was not essential for commercial viability. However, it significantly increased achievable net present values for all modelled product scenarios, making it an attractive proposition, if freshwater-utilising plants are in close proximity to agricultural land. The most profitable scenario was production of food-grade phycocyanin (100%) coupled with co-production of biofertiliser (100%), followed by food-grade phycocyanin as the sole product and 50% phycocyanin and 50% biofertiliser production. In contrast, production of biofertiliser as a sole product was not commercially attractive and not viable under the worst modelled scenario of 20 years of reduced biomass yields and biofertiliser costs. Based on the above, cultivation of *Tolypothrix* sp. in vertical suspension cultures with CO_2 supply, but without nitrogen-fertilisation is recommended for the production of food-grade phycocyanin either as a sole product or with co-production of biofertiliser.

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Appendixes

Appendix A

Appendix Chapter 3

Table A.1. Elemental composition of culture media

	Concentration [mg L^{-1}]			
Component	**BG11**	**BG11(-N)**	**SADW**	**SUWW**
$MgSO_4 \cdot 7H_2O$	75	75	75	75
$CaCl_2 \cdot 2H_2O$	36	36	36	36
$NaNO_3$	1500	-	-	1500
$K_2HPO_4 \cdot 3H_2O$	40	40	40	40
$Na_2MgEDTA$	0.2	0.2	0.2	0.2
Ferric ammonium citrate ($C_6H_8O_7 \cdot xFe^{3+} \cdot yNH_3$)	6.0	6.0	6.0	6.0
Citric acid ($C_6H_8O_7$)	6.6	6.6	6.6	6.6
Na_2CO_3	20	20	20	20
Microelements				
H_3BO_3	2.86	2.86	12.74	2.86
$MnCl_2 \cdot 4H_2O$	1.81	1.81	1.81	1.81
$ZnSO_4 \cdot 7 H_2O$	0.222	0.222	0.57	0.222
$Na_2MoO_4 \cdot 2H_2O$	0.39	0.39	1.84	0.39
$CuSO_4 \cdot 5H_2O$	0.079	0.079	0.079	0.079
$Co(NO_3)_2 \cdot 6 H_2O$	0.05	0.05	0.05	0.05
HEPES ($C_8H_{18}N_2O_4S$)	600	600	600	600
Glucose ($C_6H_{12}O_6$)	-	-	-	170
Peptone	-	-	-	170
$(NH_4)_2SO_4$	-	-	-	63
$FeSO_4 \cdot 7H_2O$	-	-	1.4	2.2
$SrCl_2 \cdot 6H_2O$	-	-	2.5	-
$VOSO_4 \cdot 4H_2O$	-	-	1.8	-
$AlCl_3 \cdot 6H_2O$	-	-	0.7	-
Na_2SeO_3	-	-	0.1	-
$Na_2HAsO_4 \cdot 7H_2O$	-	-	0.04	-
$NiSO_4$	-	-	0.04	-

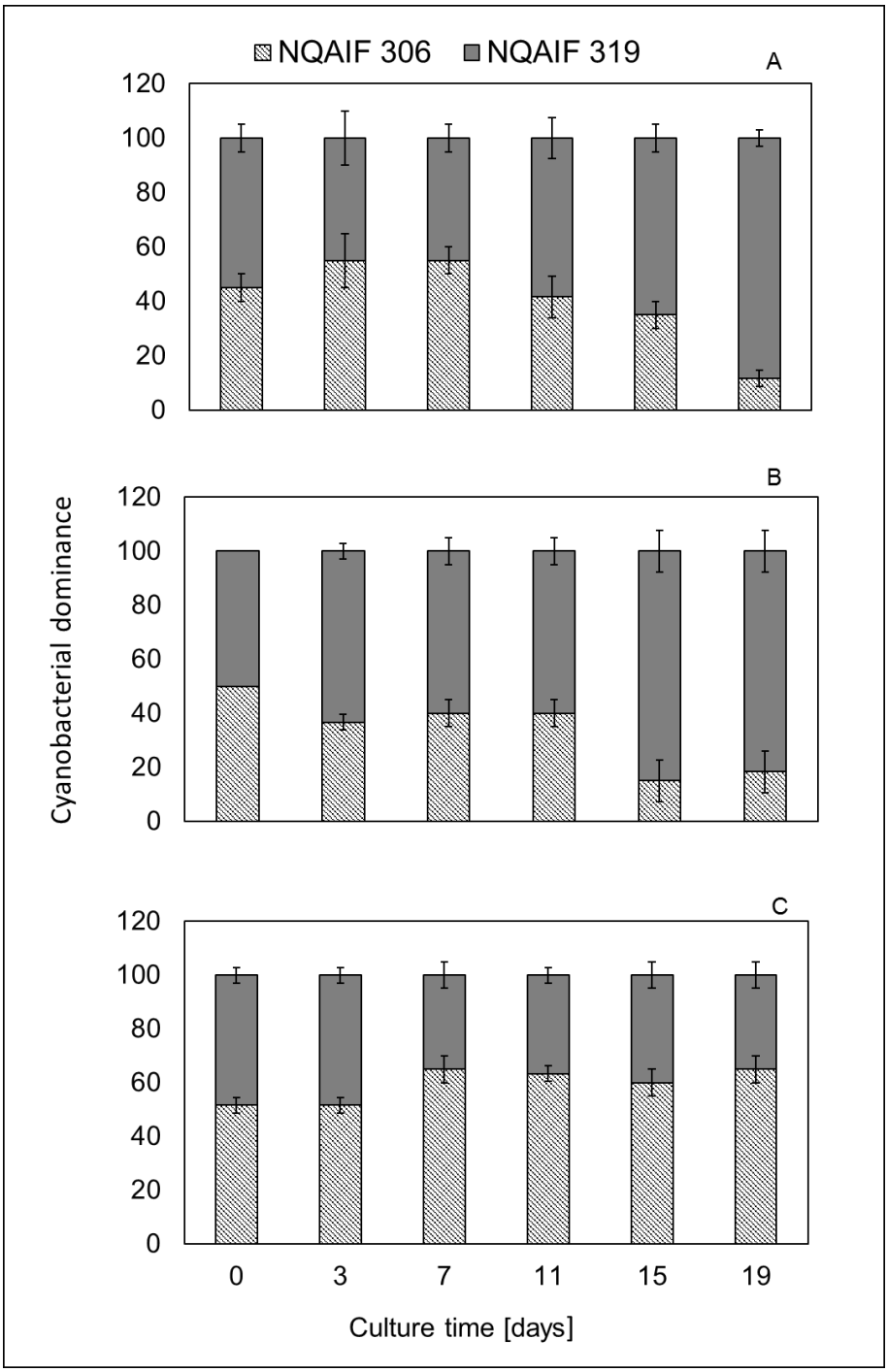

Fig A1 Community analysis of the consortium grown in A: SADW, B:BG11(-N) and C:BG11

Biomass productivity of *Tolypothrix* sp, *Limnothrix* sp. and consortium in BG11 medium

Table A.2. Tests of Homogeneity of Variances Effect: Cyanobacteria culture

	Hartley	Cochran	Bartlett	df	p
Sqrt. Biomass productivity [mg L^{-1} d^{-1}]	172.2400	0.694127	6.187256	2	0.045337

Table A.3. Univariate Tests of Significance for Sqrt. Biomass productivity [mg L^{-1} d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	779.2711	1	779.2711	4873.769	0.000000
Cyanobacteria culture	25.0029	2	12.5015	78.187	0.000050
Error	0.9593	6	0.1599		

Table A.4. Tukey HSD test; variable Sqrt. Biomass productivity [mg L^{-1} d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .15989, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000462	0.000249
2	*Limnothrix* sp.	0.000462		0.047071
3	Consortium	0.000249	0.047071	

Biomass productivity of *Tolypothrix* sp, and consortium in SADW, BG11(-N) and BG11 medium

Table A.5. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture*Media

	MS	MS	F	p
Sqrt of sqrt. biomass productivity [mg L^{-1} d^{-1}]	0.003146	0.000675	4.658321	0.013533

Table A.6. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
Sqrt of sqrt. biomass productivity [mg L^{-1} d^{-1}]	0.025192	0.002680	9.401162	0.007385

Table A.7. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
Sqrt of sqrt. biomass productivity [mg L^{-1} d^{-1}]	0.021966	0.003747	5.862403	0.013147

Table A.8. Univariate Tests of Significance for Sqrt of sqrt. biomass productivity [mg L^{-1} d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	161.6985	1	161.6985	34523.50	0.000000
Cyanobacteria culture	2.2937	1	2.2937	489.72	0.000000
Media	0.0748	2	0.0374	7.98	0.006246
Cyanobacteria culture*Media	0.0439	2	0.0220	4.69	0.031265
Error	0.0562	12	0.0047		

Table A.9. Tukey HSD test; variable Sqrt of sqrt. biomass productivity [mg L^{-1} d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00468, df = 12.000

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	*Tolypothrix* sp.	SADW		0.884232	0.460020	0.000159	0.000159	0.000161
2	*Tolypothrix* sp.	BG11(-N)	0.884232		0.961236	0.000159	0.000159	0.000159
3	*Tolypothrix* sp.	BG11	0.460020	0.961236		0.000159	0.000159	0.000159
4	Consortium	SADW	0.000159	0.000159	0.000159		0.180068	0.349766
5	Consortium	BG11(-N)	0.000159	0.000159	0.000159	0.180068		0.005699
6	Consortium	BG11	0.000161	0.000159	0.000159	0.349766	0.005699	

Table A.10. Tests of Homogeneity of Variances Effect: Cyanobacteria culture*media

	Hartley	Cochran	Bartlett	df	p
Sqrt. PC. Productivity [mg g^{-1} DW d^{-1}]	672.2671	0.394334	11.41080	5	0.043817

Table A.11. Tests of Homogeneity of Variances Effect: media

	Hartley	Cochran	Bartlett	df	p
Sqrt.PC. Productivity [mg g^{-1} DW d^{-1}]	73.68635	0.859370	15.40693	2	0.000451

Table A.12. Univariate Tests of Significance for Sqrt. PC. Productivity [mg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	150.6753	1	150.6753	17116.36	0.000000
Cyanobacteria culture	4.7987	1	4.7987	545.12	0.000000
Media	2.5751	2	1.2875	146.26	0.000000
Cyanobacteria culture*Media	2.8879	2	1.4439	164.03	0.000000
Error	0.1056	12	0.0088		

Table A.13. Tukey HSD test; variable Sqrt. PC. Productivity [mg g^{-1} DW d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00880, df = 12.000

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	Tolypothrix sp.	SADW		0.000159	0.000212	0.000159	0.000159	0.002055
2	Tolypothrix sp.	BG11(-N)	0.000159		0.008578	0.000159	0.000159	0.000368
3	Tolypothrix sp.	BG11	0.000212	0.008578		0.000159	0.000159	0.263058
4	Consortium	SADW	0.000159	0.000159	0.000159		0.004983	0.000159
5	Consortium	BG11(-N)	0.000159	0.000159	0.000159	0.004983		0.000159
6	Consortium	BG11	0.002055	0.000368	0.263058	0.000159	0.000159	

Table A.14. Tests of Homogeneity of Variances Effect: Cyanobacteria culture*media

	MS	MS	F	p
PE. Productivity [mg g^{-1} DW d^{-1}]	0.332538	0.104729	3.175216	0.046895

Table A.15. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
PE. Productivity [mg g^{-1} DW d^{-1}]	11.83702	0.638535	18.53780	0.000544

Table A.16. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
PE. Productivity [mg g^{-1} DW d^{-1}]	10.22169	0.366094	27.92098	0.000009

Table A.17. Univariate Tests of Significance for PE. Productivity [mg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	693.1927	1	693.1927	1514.788	0.000000
Cyanobacteria culture	296.4337	1	296.4337	647.777	0.000000
Media	14.0121	2	7.0060	15.310	0.000498
Cyanobacteria culture*Media	20.4434	2	10.2217	22.337	0.000090
Error	5.4914	12	0.4576		

Table A.18. Tukey HSD test; variable PE. Productivity [mg g^{-1} DW d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .45762, df = 12.000

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	Tolypothrix sp.	SADW		0.000173	0.000667	0.000161	0.000159	0.000160
2	Tolypothrix sp.	BG11(-N)	0.000173		0.310382	0.000159	0.000159	0.000159
3	Tolypothrix sp.	BG11	0.000667	0.310382		0.000159	0.000159	0.000159
4	Consortium	SADW	0.000161	0.000159	0.000159		0.954426	0.997439
5	Consortium	BG11(-N)	0.000159	0.000159	0.000159	0.954426		0.998389
6	Consortium	BG11	0.000160	0.000159	0.000159	0.997439	0.998389	

Total FAME of *Tolypothrix* sp, *Limnothrix* sp. and consortium in BG11 medium

Table A.19. Tests of Homogeneity of Variances Effect: Cyanobacteria culture

	Hartley	Cochran	Bartlett	df	p
TFA [mg g^{-1} DW]	155.2979	0.965070	8.788816	2	0.012346
TFA [%]	155.2979	0.965070	8.788816	2	0.012346

Table A.20. Univariate Tests of Significance for TFA [mg g^{-1} DW]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	8584.564	1	8584.564	3804.434	0.000000
Cyanobacteria culture	257.154	2	128.577	56.982	0.000125
Error	13.539	6	2.256		

Table A.21. Tukey HSD test; variable TFA [mg g^{-1} DW] Approximate Probabilities for Post Hoc Tests Error: Between MS = 2.2565, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000321	0.000869
2	*Limnothrix* sp.	0.000321		0.072711
3	Consortium	0.000869	0.072711	

Table A.22. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
SFA [mg g^{-1} FA]	0.991120	0.126755	7.819161	0.021320
MUFA [mg g^{-1} FA]	0.393496	0.046259	8.506449	0.017723

Table A.23. Univariate Tests of Significance for SFA [mg g^{-1} FA]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	2192.019	1	2192.019	2291.438	0.000000
Cyanobacteria culture	17.000	2	8.500	8.886	0.016080
Error	5.740	6	0.957		

Table A.24. Tukey HSD test; variable SFA [mg g^{-1} FA] Approximate Probabilities for Post Hoc Tests Error: Between MS = .95661, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.016543	0.047332
2	*Limnothrix* sp.	0.016543		0.658814
3	Consortium	0.047332	0.658814	

Table A.25. Univariate Tests of Significance for MUFA [mg g^{-1} FA]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	1649.459	1	1649.459	5317.310	0.000000
Cyanobacteria culture	250.288	2	125.144	403.422	0.000000
Error	1.861	6	0.310		

Table A.26. Tukey HSD test; variable MUFA [mg g^{-1} FA] Approximate Probabilities for Post Hoc Tests Error: Between MS = .31021, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000227	0.000227
2	*Limnothrix* sp.	0.000227		0.001683
3	Consortium	0.000227	0.001683	

TFA content of *Tolypothrix* sp, and consortium in SADW, BG11(-N) and BG11 medium

Table A.27. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
TFA [mg g^{-1} DW]	4.034670	0.429410	9.395838	0.007399

Table A.28. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
TFA [mg g^{-1} DW]	12.92115	0.319786	40.40564	0.000001

Table A.29. Univariate Tests of Significance for TFA [mg g^{-1} DW]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	13422.53	1	13422.53	33578.79	0.000000
Cyanobacteria culture	154.79	1	154.79	387.23	0.000000
Media	16.38	2	8.19	20.49	0.000135
Cyanobacteria culture*Media	25.84	2	12.92	32.32	0.000015
Error	4.80	12	0.40		

Table A.30. Univariate Tests of Significance for TFA [mg g^{-1} DW]

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	*Tolypothrix* sp.	SADW		0.062274	0.980300	0.000193	0.000159	0.000159
2	*Tolypothrix* sp.	BG11(-N)	0.062274		0.019687	0.005220	0.000176	0.000159
3	*Tolypothrix* sp.	BG11	0.980300	0.019687		0.000168	0.000159	0.000159
4	Consortium	SADW	0.000193	0.005220	0.000168		0.039607	0.000162
5	Consortium	BG11(-N)	0.000159	0.000176	0.000159	0.039607		0.001047
6	Consortium	BG11	0.000159	0.000159	0.000159	0.000162	0.001047	

Table A.31. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture*Media

	MS	MS	F	p
sqrt. FA Productivity [mg g^{-1} DW d^{-1}]	0.000838	0.000224	3.737545	0.028473

Table A.32. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
sqrt. FA Productivity [mg g^{-1} DW d^{-1}]	0.032263	0.001074	30.03500	0.000006

Table A.33. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
sqrt. FA Productivity [mg g^{-1} DW d^{-1}]	0.011571	0.002319	4.990458	0.040111

Table A.34. Univariate Tests of Significance for sqrt. FA Productivity [mg g^{-1} DW d^{-1}]

Effect	SS	Degr. of	MS	F	p
Intercept	40.46322	1	40.46322	30134.74	0.000000
Cyanobacteria culture	1.21036	1	1.21036	901.41	0.000000
Media	0.06951	2	0.03476	25.89	0.000044
Cyanobacteria culture*Media	0.06453	2	0.03226	24.03	0.000064
Error	0.01611	12	0.00134		

Table A.35. Tukey HSD test; variable sqrt. FA Productivity [mg g^{-1} DW d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00134, df = 12.000

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	*Tolypothrix* sp.	SADW		0.015362	0.045327	0.000159	0.000159	0.000160
2	*Tolypothrix* sp.	BG11(-N)	0.015362		0.985140	0.000159	0.000159	0.000159
3	*Tolypothrix* sp.	BG11	0.045327	0.985140		0.000159	0.000159	0.000159
4	Consortium	SADW	0.000159	0.000159	0.000159		0.106645	0.000896
5	Consortium	BG11(-N)	0.000159	0.000159	0.000159	0.106645		0.000165
6	Consortium	BG11	0.000160	0.000159	0.000159	0.000896	0.000165	

Carbohydrate, protein and lipids content of *Tolypothrix* sp, *Limnothrix* sp. and consortium in BG11 medium

Table A.36. Univariate Tests of Significance for Carbohydrate [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	9470.534	1	9470.534	1965.557	0.000000
Cyanobacteria culture	1089.936	2	544.968	113.105	0.000017
Error	28.909	6	4.818		

Table A.37. Tukey HSD test; variable Carbohydrate [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = 4.8182, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000258	0.000232
2	*Limnothrix* sp.	0.000258		0.105708
3	Consortium	0.000232	0.105708	

Table A.38. Univariate Tests of Significance for Protein [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	10481.38	1	10481.38	1894.762	0.000000
Cyanobacteria culture	514.17	2	257.09	46.474	0.000223
Error	33.19	6	5.53		

Table A.39. Tukey HSD test; variable Protein [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = 5.5318, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000381	0.003435
2	*Limnothrix* sp.	0.000381		0.017302
3	Consortium	0.003435	0.017302	

Carbohydrate, protein and lipids content of *Tolypothrix* sp. and consortium in SADW, BG11(-N) and BG11 medium

Table A.40. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
Sqrt. Protein [%]	0.333598	0.008614	38.72725	0.000001

Table A.41. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
Sqrt. Protein [%]	2.247252	0.053002	42.39932	0.000007

Table A.42. Univariate Tests of Significance for Sqrt. Protein [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	417.1021	1	417.1021	38736.96	0.000000
Cyanobacteria culture	0.0596	1	0.0596	5.54	0.036484
Media	3.8154	2	1.9077	177.17	0.000000
Cyanobacteria culture*Media	2.8831	2	1.4415	133.88	0.000000
Error	0.1292	12	0.0108		

Table A.43. Tukey HSD test; variable Sqrt. Protein [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .01077, df = 12.000"

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	*Tolypothrix* sp.	SADW		0.888832	0.347594	0.019363	0.001421	0.000159
2	*Tolypothrix* sp.	BG11(-N)	0.888832		0.893877	0.003428	0.000398	0.000159
3	*Tolypothrix* sp.	BG11	0.347594	0.893877		0.000780	0.000208	0.000159
4	Consortium	SADW	0.019363	0.003428	0.000780		0.586626	0.000159
5	Consortium	BG11(-N)	0.001421	0.000398	0.000208	0.586626		0.000159
6	Consortium	BG11	0.000159	0.000159	0.000159	0.000159	0.000159	

Carbon, hydrogen, nitrogen and CN ratio content of *Tolypothrix* sp. *Limnothrix* sp. and consortium in BG11 medium

Table A.44. Univariate Tests of Significance for Carbon [%]

Effect	SS	Degr. of	MS	F	p
Intercept	17998.76	1	17998.76	100525.2	0.000000
Cyanobacteria culture	16.34	2	8.17	45.6	0.000235
Error	1.07	6	0.18		

Table A.45. Tukey HSD test; variable Carbon [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .17905, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000392	0.003160
2	*Limnothrix* sp.	0.000392		0.021769
3	Consortium	0.003160	0.021769	

Table A.46. Univariate Tests of Significance for Hydrogen [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	403.5267	1	403.5267	71987.20	0.000000
Cyanobacteria culture	0.1050	2	0.0525	9.37	0.014279
Error	0.0336	6	0.0056		

Table A.47. Tukey HSD test; variable Hydrogen [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00561, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.012032	0.106145
2	*Limnothrix* sp.	0.012032		0.235186
3	Consortium	0.106145	0.235186	

Table A.48. Univariate Tests of Significance for Nitrogen [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	717.2207	1	717.2207	72093.04	0.000000
Cyanobacteria culture	27.2913	2	13.6456	1371.62	0.000000
Error	0.0597	6	0.0099		

Table A.49. Tukey HSD test; variable Nitrogen [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00995, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000227	0.000227
2	*Limnothrix* sp.	0.000227		0.000446
3	Consortium	0.000227	0.000446	

Table A.50. Univariate Tests of Significance for C/N ratio [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	243.6890	1	243.6890	78511.90	0.000000
Cyanobacteria culture	8.9538	2	4.4769	1442.37	0.000000
Error	0.0186	6	0.0031		

Table A.51. Tukey HSD test; variable CN ratio Approximate Probabilities for Post Hoc Tests Error: Between MS = .00310, df = 6.0000

Cell No.	Cyanobacteria culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.000227	0.000227
2	*Limnothrix* sp.	0.000227		0.011370
3	Consortium	0.000227	0.011370	

Carbon, hydrogen, nitrogen and CN ratio content of *Tolypothrix* sp. and consortium in SADW, BG11(-N) and BG11 medium.

Table A.52. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
sqrt. Nitrogen [%]	0.061035	0.000492	124.1362	0.000000

Table A3.53. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
sqrt. Nitrogen [%]	0.452614	0.008848	51.15481	0.000002

Table A.54. Univariate Tests of Significance for sqrt. Nitrogen [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	120.6074	1	120.6074	196236.7	0.000000
Cyanobacteria culture	0.0061	1	0.0061	9.9	0.008437
Media	0.5604	2	0.2802	455.9	0.000000
Cyanobacteria culture*Media	0.6590	2	0.3295	536.1	0.000000
Error	0.0074	12	0.0006		

Table A.55. Tukey HSD test; variable sqrt. Nitrogen [%] Approximate Probabilities for Post Hoc Tests
Error: Between MS = .00061, df = 12.000

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	Tolypothrix sp.	SADW		0.145442	0.999982	0.000160	0.000159	0.000159
2	Tolypothrix sp.	BG11(-N)	0.145442		0.113176	0.000159	0.000159	0.000159
3	Tolypothrix sp.	BG11	0.999982	0.113176		0.000161	0.000159	0.000159
4	Consortium	SADW	0.000160	0.000159	0.000161		0.905142	0.000159
5	Consortium	BG11(-N)	0.000159	0.000159	0.000159	0.905142		0.000159
6	Consortium	BG11	0.000159	0.000159	0.000159	0.000159	0.000159	

Table A.56. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture*Media

	MS	MS	F	p
CN ratio	0.016940	0.005111	3.314549	0.041299

Table A.57. Levene's Test for Homogeneity of Variances Effect: Media

	MS	MS	F	p
CN ratio	0.339882	0.022105	15.37572	0.000233

Table A3.58. Levene's Test for Homogeneity of Variances Effect: Cyanobacteria culture

	MS	MS	F	p
CN ratio	8.453419	0.171886	49.18048	0.000003

Table A.59. Univariate Tests of Significance for CN ratio Sigma-restricted parameterization Effective hypothesis decomposition

Effect	SS	Degr. of freedom	MS	F	p
Intercept	811.0619	1	811.0619	29352.94	0.000000
Cyanobacteria culture	0.1076	1	0.1076	3.89	0.071927
Media	11.0549	2	5.5274	200.04	0.000000
Cyanobacteria culture*Media	10.6208	2	5.3104	192.19	0.000000
Error	0.3316	12	0.0276		

Table A.60. Tukey HSD test; variable CN ratio Approximate Probabilities for Post Hoc Tests Error: Between MS = .02763, df = 12.000

Cell No.	Cyanobacteria culture	Media	[1]	[2]	[3]	[4]	[5]	[6]
1	Tolypothrix sp.	SADW		0.627584	0.882771	0.000196	0.000166	0.000159
2	Tolypothrix sp.	BG11(-N)	0.627584		0.995160	0.000161	0.000159	0.000159
3	Tolypothrix sp.	BG11	0.882771	0.995160		0.000164	0.000160	0.000159
4	Consortium	SADW	0.000196	0.000161	0.000164		0.947246	0.000159
5	Consortium	BG11(-N)	0.000166	0.000159	0.000160	0.947246		0.000159
6	Consortium	BG11	0.000159	0.000159	0.000159	0.000159	0.000159	

Metal removal [%]

The data for Al, As, Se, Sr and V could not be reliably analysed due to large variations of data.

Table A.61. Tests of Homogeneity of Variances Effect: Culture

	Hartley	Cochran	Bartlett	df	p
Fe removal [%]	495.4300	0.957218	10.13709	2	0.006292
Ni removal [%]	117.0702	0.962847	8.32188	2	0.015593

Table A.62. Univariate Tests of Significance for Fe removal [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	89010.84	1	89010.84	3099697	0.000000
Culture	0.09	2	0.05	2	0.275698
Error	0.17	6	0.03		

Table A.63. Tukey HSD test; variable Fe removal [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .02872, df = 6.0000

Cell No.	Culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.895581	0.456345
2	*Limnothrix* sp.	0.895581		0.270327
3	Consortium	0.456345	0.270327	

Table A.64. Univariate Tests of Significance for Ni removal [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	28336.11	1	28336.11	447.4049	0.000001
Culture	644.19	2	322.10	5.0857	0.051076
Error	380.01	6	63.33		

Table A.65. Tukey HSD test; variable Fe removal [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .02872, df = 6.0000

Cell No.	Culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.102588	0.056816
2	*Limnothrix* sp.	0.102588		0.887864
3	Consortium	0.056816	0.887864	

Highest metal uptake

The data for As, Ni and Sr could not be reliably analysed due to large variations of data.

Table A.66 Tests of Homogeneity of Variances Effect: Culture

	Hartley	Cochran	Bartlett	df	p
Se uptake rate [µg g^{-1} DW d^{-1}]	122.6873	0.864389	6.310776	2	0.042622
V uptake rate [µg g^{-1} DW d^{-1}]	62.4231	0.932462	6.419436	2	0.040368
Sqrt. Al uptake rate [µg g^{-1} DW d^{-1}]]	67.8746	0.953790	7.287628	2	0.026152
Sqrt. Fe uptake rate [µg g^{-1} DW d^{-1}]	151.7844	0.963087	8.655815	2	0.013195

Table A.67. Univariate Tests of Significance for Se uptake. Rate [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	1022.806	1	1022.806	25.73713	0.002281
Culture	186.741	2	93.370	2.34951	0.176369
Error	238.443	6	39.740		

Table A.68. Tukey HSD test; variable Se uptake rate [µg g^{-1} DW d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = 39.740, df = 6.0000

Cell No.	Culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.423332	0.717909
2	*Limnothrix* sp.	0.423332		0.160793
3	Consortium	0.717909	0.160793	

Table A.69. Univariate Tests of Significance for V uptake rate [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	27899.21	1	27899.21	26.67420	0.002085
Culture	3890.89	2	1945.44	1.86002	0.235206
Error	6275.55	6	1045.92		

Table A.70. Tukey HSD test; variable V uptake rate [µg g^{-1} DW d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = 1045.9, df = 6.0000"

Cell No.	Culture	[1]	[2]	[3]
1	*Tolypothrix* sp.		0.610725	0.636631
2	*Limnothrix* sp.	0.610725		0.211210
3	Consortium	0.636631	0.211210	

Table A.71. Univariate Tests of Significance for Sqrt. Al uptake rate [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. Of freedom	MS	F	p
Intercept	1591.323	1	1591.323	2485.329	0.000000
Culture	3.190	2	1.595	2.491	0.163096
Error	3.842	6	0.640		

Table A.72. Tukey HSD test; variable Sqrt. Al uptake rate [µg g^{-1} DW d^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .64029, df = 6.0000"

Cell No.	Culture	[1]	[2]	[3]
1	Tolypothrix sp.		0.614903	0.144819
2	Limnothrix sp.	0.614903		0.471725
3	Consortium	0.144819	0.471725	

Table A.73. Univariate Tests of Significance for Sqrt. Fe uptake rate [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	10500.04	1	10500.04	2414.878	0.000000
Culture	21.11	2	10.55	2.427	0.168875
Error	26.09	6	4.35		

Table A3.74. Tukey HSD test; variable Sqrt. Fe uptake rate [µg g-1 DW d-1] Approximate Probabilities for Post Hoc Tests Error: Between MS = 4.3481, df = 6.0000"

Cell No.	Culture	[1]	[2]	[3]
1	Tolypothrix sp.		0.819452	0.161912
2	Limnothrix sp.	0.819452		0.344353
3	Consortium	0.161912	0.344353	

Appendix B

Appendix for chapter 4

Table B.1: Initial SADW metal concentrations and effect of CO_2 on metal removal by *Tolypothrix* sp..

Media	Heavy metals	Initial concentration in SADW (µg L^{-1})	Metal concentration in media (µg L^{-1})					
			24 hrs.	48 hrs.	Day 3	Day 12	Day 18	Day 25
SADW+CO_2	Al	200.0 ± 1.0	19.3 ± 0.6	19.0 ± 0.0	19.3 ± 0.6	18.7 ± 0.6	20.0 ± 0.0	20.0 ± 0.0
	As	13.3 ± 0.2	13.0 ± 0.2	12.2 ± 0.1	10.9 ± 0.1	5.4 ± 0.6	4.4 ± 0.1	4.6 ± 0.0
	Cu	6.9 ± 0.2	2.5 ± 0.2	3.2 ± 0.2	1.7 ± 0.4	1.6 ± 0.1	2.0 ± 0.1	1.6 ± 0.1
	Fe	1184.0 ± 3.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	Mo	750.0 ± 11.0	741.0 ± 10.5	724.3 ± 2.5	721.0 ± 11.1	700.7 ± 8.1	751.0 ± 14.1	733.7 ± 21.0
	Ni	21.2 ± 0.4	8.8 ± 0.1	20.0 ± 0.4	19.8 ± 0.2	21.3 ± 2.3	10.0 ± 0.8	10.5 ± 1.0
	Se	73.3 ± 1.0	49.9 ± 1.2	47.5 ± 0.8	35.1 ± 0.8	9.3 ± 0.2	14.0 ± 0.2	11.5 ± 0.9
	Sr	830.9 ± 12.0	671.7 ± 31.7	683.0 ± 3.6	655.3 ± 8.1	658.3 ± 5.9	425.0 ± 70.1	404.3 ± 56.1
	V	434.1 ± 4.0	415.3 ± 5.7	384.3 ± 4.0	351.0 ± 7.0	210.3 ± 19.1	56.5 ± 6.7	41.7 ± 5.3
	Zn	31.4 ± 2.6	15.9 ± 4.4	11.2 ± 0.2	11.8 ± 1.2	43.6 ± 10.9	18.1 ± 0.8	21.0 ± 3.5
SADW	Al	184.8 ± 1.0	81.3 ± 2.3	73.3 ± 2.3	61.0 ± 13.9	61.0 ± 3.6	66.0 ± 2.8	82.7 ± 25.4
	As	13.3 ± 0.2	12.4 ± 0.1	12.1 ± 0.1	11.1 ± 0.2	8.7 ± 0.5	5.2 ± 1.1	3.7 ± 0.3
	Cu	6.9 ± 0.2	3.6 ± 0.3	3.0 ± 0.3	2.9 ± 1.5	1.4 ± 0.3	2.3 ± 0.4	2.0 ± 0.2
	Fe	998.0 ± 3.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
	Mo	750.0 ± 11.0	713.0 ± 7.5	703.3 ± 9.0	696.3 ± 13.3	710.3 ± 13.7	734.0 ± 55.7	738.0 ± 63.4
	Ni	20.6 ± 0.4	20.1 ± 0.8	20.0 ± 0.4	13.4 ± 7.8	21.3 ± 2.3	10.0 ± 0.8	20.4 ± 0.6
	Se	73.3 ± 1.0	43.2 ± 0.7	28.5 ± 1.3	13.3 ± 2.4	9.6 ± 1.3	20.6 ± 0.5	9.8 ± 1.3
	Sr	830.9 ± 12.0	553.3 ± 4.2	540.3 ± 3.2	529.3 ± 9.3	525.7 ± 11.7	518.0 ± 47.8	551.7 ± 69.0
	V	434.1 ± 4.0	412.0 ± 4.4	406.3 ± 1.5	388.0 ± 4.6	344.7 ± 14.0	263.3 ± 31.8	195.3 ± 68.7
	Zn	31.4 ± 2.6	8.5 ± 1.4	25.0 ± 0.2	24.0 ± 1.5	15.1 ± 1.3	25.8 ± 1.9	25.2 ± 2.4

Biomass productivity:

Table B.2: Test of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Square root transformed biomass Productivity [mg DW day^{-1}]	2.955467	0.353523	0.503246	3	0.918178

Table B.3. Test of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Square root transformed biomass Productivity [mg DW day^{-1}]	4.262038	0.809960	2.204523	1	0.137606

Table B.4. Test of Homogeneity of Variances: Effect: Medium

	Hartley	Cochran	Bartlett	df	p
Square root transformed biomass Productivity [mg DW day^{-1}]	2.510207	0.715117	0.930411	1	0.334756

Table B.5. Univariate tests of significance for square root-transformed biomass productivity [mg DW day^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	788.1682	1	788.1682	5808.027	0.000000
Medium	0.4219	1	0.4219	3.109	0.115860
CO_2 fertilisation	35.0417	1	35.0417	258.223	0.000000
Medium*CO_2 fertilisation	1.9571	1	1.9571	14.422	0.005254
Error	1.0856	8	0.1357		

Table B.6. Tukey HSD test; variable square root-transformed biomass productivity [mg DW day^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .13570, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.000306	0.018364	0.000247
2	SADW	15% CO_2	0.000306		0.000232	0.512413
3	BG11 (-N)	air	0.018364	0.000232		0.000231
4	BG11 (-N)	15% CO_2	0.000247	0.512413	0.000231	

Carbohydrate:

Table B.7 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Carbohydrate [%]	5.231840	0.550747	1.465120	3	0.690344

Table B.8 Tests of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Carbohydrate [%]	1.939619	0.659820	0.489882	1	0.483980

Table B.9 Tests of Homogeneity of Variances: Effect: medium

	Hartley	Cochran	Bartlett	df	p
Carbohydrate [%]	1.348602	0.574215	0.101262	1	0.750320

Table B.10 Univariate Tests of Significance for Carbohydrate [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	23478.67	1	23478.67	5328.647	0.000000
Medium	0.00	1	0.00	0.000	1.000000
CO_2 fertilisation	432.91	1	432.91	98.252	0.000009
Medium*CO_2 fertilisation	4.56	1	4.56	1.034	0.338883
Error	35.25	8	4.41		

Table B.11 Tukey HSD test; variable Carbohydrate [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = 4.4061, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.000441	0.886684	0.000688
2	SADW	15% CO_2	0.000441		0.000688	0.887020
3	BG11 (-N)	air	0.886684	0.000688		0.001226
4	BG11 (-N)	15% CO_2	0.000688	0.887020	0.001226	

Protein:

Table B.12 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Protein [%]	4.688267	0.412366	1.178003	3	0.758285

Table B.13 Tests of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Protein [%]	2.366524	0.702958	0.818386	1	0.365653

Table B.14 Tests of Homogeneity of Variances: Effect: medium

	Hartley	Cochran	Bartlett	df	p
Protein [%]	1.149278	0.534727	0.021980	1	0.882140

Table B.15 Univariate Tests of Significance for Protein [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	6406.318	1	6406.318	10087.64	0.000000
Medium	0.696	1	0.696	1.10	0.325783
CO_2 fertilisation	11.943	1	11.943	18.81	0.002490
Medium*CO_2 fertilisation	0.029	1	0.029	0.05	0.835573
Error	5.081	8	0.635		

Table B.16 Tukey HSD test; variable Protein [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .63507, df = 8.0000"

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.075106	0.809496	0.170959
2	SADW	15% CO_2	0.075106		0.021744	0.932822
3	BG11 (-N)	air	0.809496	0.021744		0.049010
4	BG11 (-N)	15% CO_2	0.170959	0.932822	0.049010	

Lipids:

Table B.17 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Lipids [%]	20.08853	0.627097	3.578306	3	0.310747

Table B.18 Tests of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Lipids [%]	1.108347	0.525695	0.012020	1	0.912699

Table B.19 Tests of Homogeneity of Variances: Effect: medium

	Hartley	Cochran	Bartlett	df	p
Lipids [%]	1.273750	0.560198	0.066369	1	0.796698

Table B.20 Univariate Tests of Significance for Lipids [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	1932.627	1	1932.627	2126.183	0.000000
Medium	2.167	1	2.167	2.384	0.161156
CO2 fertilisation	83.515	1	83.515	91.879	0.000012
Medium*CO2 fertilisation	0.816	1	0.816	0.898	0.371182
Error	7.272	8	0.909		

Table B.21 Tukey HSD test; variable Lipids [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .90897, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.000517	0.973195	0.002213
2	SADW	15% CO_2	0.000517		0.000412	0.355964
3	BG11 (-N)	air	0.973195	0.000412		0.001454
4	BG11 (-N)	15% CO_2	0.002213	0.355964	0.001454	

Phycocyanin

Table B.22 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Square root transformed phycocyanin productivity [mg g^{-1} DW day^{-1}]	9.344721	0.581957	2.778422	3	0.427064

Table B.23 Tests of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Square root transformed phycocyanin productivity [mg g^{-1} DW day^{-1}]	3.427858	0.774157	1.625463	1	0.202332

Table B.24 Tests of Homogeneity of Variances: Effect: medium

	Hartley	Cochran	Bartlett	df	p
Square root transformed phycocyanin productivity [mg g^{-1} DW day^{-1}]	1.773657	0.639465	0.368161	1	0.544008

Table B.25 Univariate Tests of Significance for square root-transformed phycocyanin productivity [mg g^{-1} DW day^{-1}]

Effect	SS	Degr. of Freedom	MS	F	p
Intercept	43.56615	1	43.56615	1246.927	0.000000
Medium	0.00031	1	0.00031	0.009	0.927082
CO_2 fertilisation	5.77535	1	5.77535	165.299	0.000001
Medium*CO_2 fertilisation	0.11653	1	0.11653	3.335	0.105232
Error	0.27951	8	0.03494		

Table B.26 Tukey HSD test; variable square root-transformed phycocyanin productivity [mg g^{-1} DW day^{-1}]
Approximate Probabilities for Post Hoc Tests Error: Between MS = .03494, df = 8.0000

Cell No	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.000243	0.629930	0.000283
2	SADW	15% CO2	0.000243		0.000276	0.555755
3	BG11 (-N)	air	0.629930	0.000276		0.000426
4	BG11 (-N)	15% CO2	0.000283	0.555755	0.000426	

Phycoerythrin

Table B.27 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Phycoerythrin productivity [mg g^{-1} DW day^{-1}]	22.84214	0.617687	4.529197	3	0.209701

Table B.28 Tests of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Phycoerythrin productivity [mg g^{-1} DW day^{-1}]	3.639762	0.784472	1.777789	1	0.182421

Table B.29 Tests of Homogeneity of Variances: Effect: medium

	Hartley	Cochran	Bartlett	df	p
Square root transformed Phycoerythrin productivity [mg g^{-1} DW day^{-1}]	1.886126	0.653515	0.450049	1	0.502312

Table B.30 Univariate tests of significance for square root transformed phycoerythrin productivity [mg g^{-1} DW day^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	34.58165	1	34.58165	1139.873	0.000000
Medium	0.00004	1	0.00004	0.001	0.971065
CO_2 fertilisation	4.52345	1	4.52345	149.101	0.000002
Medium*CO_2 fertilisation	0.11084	1	0.11084	3.654	0.092325
Error	0.24271	8	0.03034		

Table B.31 Tukey HSD test; variable square root transformed phycoerythrin productivity [mg g^{-1} DW day^{-1}]
Approximate Probabilities for Post Hoc Tests Error: Between MS = .03034, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.000249	0.544904	0.000307
2	SADW	15% CO_2	0.000249		0.000312	0.573926
3	BG11 (-N)	air	0.544904	0.000312		0.000570
4	BG11 (-N)	15% CO_2	0.000307	0.573926	0.000570	

Fatty acid productivity:

Table B.32 Tests of homogeneity of variances effect: medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Square root of fatty acids productivity [mg g^{-1} DW day^{-1}]	11.78627	0.523473	2.958017	3	0.398144

Table B.33 Tests of homogeneity of variances effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Square root of Fatty acids productivity [mg g^{-1} DW day^{-1}]	9.277458	0.902700	4.754654	1	0.029219

Table B.34 Tests of homogeneity of variances: effect: Medium

	Hartley	Cochran	Bartlett	df	p
Square root of fatty acids productivity [mg g^{-1} DW day^{-1}]	2.077961	0.675110	0.594787	1	0.440574

Table B.35 Univariate tests of significance for square root of Fatty acids productivity [mg g^{-1} DW day^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	22.28532	1	22.28532	464.1007	0.000000
Medium	0.04926	1	0.04926	1.0259	0.340775
CO_2 fertilisation	2.53277	1	2.53277	52.7459	0.000087
Medium*CO_2 fertilisation	0.09384	1	0.09384	1.9542	0.199676
Error	0.38415	8	0.04802		

Table B.36 Tukey HSD test; variable Sqrt fatty acids productivity [mg g^{-1} DW day^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = .04802, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.001432	0.992462	0.009718
2	SADW	15% CO_2	0.001432		0.001869	0.381015
3	BG11 (-N)	air	0.992462	0.001869		0.013812
4	BG11 (-N)	15% CO_2	0.009718	0.381015	0.013812	

Sum SFA [mg g^{-1} Total fatty acids]:

Table B.37 Tests of homogeneity of variances: effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Sum SFA [mg g^{-1} Total fatty acids]	18.00801	0.450493	3.037882	3	0.385821

Table B.38 Tests of homogeneity of variances effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Sum SFA [mg g^{-1} Total fatty acids]	1.046082	0.511261	0.002306	1	0.961698

Table B.39 Tests of homogeneity of variances effect: Medium

	Hartley	Cochran	Bartlett	df	p
Sum SFA [mg g^{-1} Total fatty acids]	3.743096	0.789167	1.850785	1	0.173692

Table B.40 Univariate Tests of Significance for Sum SFA [mg g^{-1} Total fatty acids]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	4908.574	1	4908.574	588.1584	0.000000
Medium	24.096	1	24.096	2.8872	0.127712
CO_2 fertilisation	4.763	1	4.763	0.5708	0.471615
Medium*CO_2 fertilisation	0.294	1	0.294	0.0352	0.855791
Error	66.765	8	8.346		

Sum of MUFA:

Table B.41 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Sum MUFA [mg g^{-1} Total fatty acids]	107.3835	0.498342	5.593466	3	0.133154

Table B.42 Tests of Homogeneity of Variances Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Sum MUFA [mg g^{-1} Total fatty acids]	2.021287	0.669015	0.551532	1	0.457693

Table B.43 Tests of Homogeneity of Variances Effect: Medium

	Hartley	Cochran	Bartlett	df	p
Sum MUFA [mg g^{-1} Total fatty acids]	4.076247	0.803004	2.080333	1	0.149208

Table B.44 Univariate Tests of Significance for Sum MUFA [mg g^{-1} Total fatty acids]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	665.1809	1	665.1809	343.5514	0.000000
Medium	10.0687	1	10.0687	5.2003	0.052038
CO_2 fertilisation	2.9786	1	2.9786	1.5384	0.250002
Medium*CO_2 fertilisation	0.2360	1	0.2360	0.1219	0.736016
Error	15.4895	8	1.9362		

Sum of PUFA:

Table B.45 Tests of Homogeneity of Variances Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Sum PUFA [mg g^{-1} Total fatty acids]	4.882511	0.352816	1.026031	3	0.794954

Table B.46 Tests of Homogeneity of Variances Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Sum PUFA [mg g^{-1} Total fatty acids]	1.960809	0.662254	0.505789	1	0.476967

Table B.47 Tests of Homogeneity of Variances Effect: Mediu

	Hartley	Cochran	Bartlett	df	p
Sum PUFA [mg g^{-1} Total fatty acids]	1.827668	0.646352	0.407134	1	0.523428

Table B.48 Univariate Tests of Significance for Sum PUFA [mg g^{-1} Total fatty acids]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	3796.216	1	3796.216	195.1133	0.000001
Medium	66.891	1	66.891	3.4380	0.100827
CO_2 fertilisation	11.904	1	11.904	0.6118	0.456633
Medium*CO_2 fertilisation	2.310	1	2.310	0.1187	0.739298
Error	155.652	8	19.456		

Elemental Analysis:

Table B.49 Tests of Homogeneity of Variances: Effect: Medium*CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Nitrogen [%]	5.7692	0.595754	1.832344	3	0.607922
Phosphorus [%]	200.6671	0.602140	6.925037	3	0.074326
Sqrt Potassium [%]	6.8222	0.462643	2.326667	3	0.507432
CN ratio	6.0051	0.403558	1.310729	3	0.726587

Table B.50 Tests of Homogeneity of Variances: Effect: CO_2 fertilisation

	Hartley	Cochran	Bartlett	df	p
Nitrogen [%]	4.255890	0.809737	2.200456	1	0.137970
Phosphorus [%]	1.708009	0.630725	0.321839	1	0.570505
Sqrt Potassium [%]	4.421760	0.815558	2.309131	1	0.128616
CN ratio	1.783016	0.640678	0.374863	1	0.540366

Table B.51 Tests of Homogeneity of Variances: Effect: Medium

	Hartley	Cochran	Bartlett	df	p
Nitrogen [%]	2.010787	0.667861	0.543557	1	0.460962
Phosphorus [%]	1.017312	0.504291	0.000335	1	0.985402
Sqrt Potassium [%]	1.028835	0.507106	0.000918	1	0.975826
CN ratio	1.212637	0.548051	0.042174	1	0.837288

Table B.52 Univariate tests of significance for Nitrogen [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	633.5730	1	633.5730	12581.80	0.000000
Medium	0.0946	1	0.0946	1.88	0.207685
CO_2 fertilisation	0.4396	1	0.4396	8.73	0.018293
Medium*CO_2 fertilisation	0.1359	1	0.1359	2.70	0.139091
Error	0.4029	8	0.0504		

Table B.53 Tukey HSD test; variable Nitrogen [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .05036, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.046812	0.997335	0.688397
2	SADW	15% CO_2	0.046812		0.061325	0.222540
3	BG11 (-N)	air	0.997335	0.061325		0.791483
4	BG11 (-N)	15% CO_2	0.688397	0.222540	0.791483	

Table B.54 Univariate tests of significance for Phosphorous [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	7.475367	1	7.475367	229.5565	0.000000
Medium	0.006948	1	0.006948	0.2134	0.656435
CO_2 fertilisation	0.032945	1	0.032945	1.0117	0.343950
Medium*CO_2 fertilisation	0.278697	1	0.278697	8.5583	0.019131
Error	0.260515	8	0.032564		

Table B.55 Tukey HSD test; variable Phosphorus [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .03256, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.090853	0.364495	0.979391
2	SADW	15% CO_2	0.090853		0.733585	0.155513
3	BG11 (-N)	air	0.364495	0.733585		0.556181
4	BG11 (-N)	15% CO_2	0.979391	0.155513	0.556181	

Table B.56 Univariate tests of significance for square root-transformed potassium [%]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	7.625506	1	7.625506	3509.868	0.000000
Medium	0.002384	1	0.002384	1.097	0.325460
CO_2 fertilisation	0.146726	1	0.146726	67.535	0.000036
Medium*CO_2 fertilisation	0.000003	1	0.000003	0.001	0.972421
Error	0.017381	8	0.002173		

Table B.57 Tukey HSD test; variable square root-transformed Potassium [%] – Effect of CO_2. Approximate Probabilities for Post Hoc Tests Error: Between MS =.00217, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.001898	0.888307	0.004379
2	SADW	15% CO_2	0.001898		0.000974	0.867584
3	BG11 (-N)	air	0.888307	0.000974		0.001997
4	BG11 (-N)	15% CO_2	0.004379	0.867584	0.001997	

Table B.58 Univariate tests of significance for CN ratio

Effect	SS	Degr. of freedom	MS	F	p
Intercept	471.7484	1	471.7484	59569.31	0.000000
Medium	0.0664	1	0.0664	8.39	0.020022
CO_2 fertilisation	0.7640	1	0.7640	96.48	0.000010
Medium*CO_2 fertilisation	0.0023	1	0.0023	0.29	0.606322
Error	0.0634	8	0.0079		

Table B.59 Tukey HSD test; variable C/N ratio – Effect of CO_2. Approximate Probabilities for Post Hoc Tests Error: Between MS =.00217, df = 8.0000

Cell No.	Medium	CO_2 fertilisation	[1]	[2]	[3]	[4]
1	SADW	air		0.000962	0.148885	0.005367
2	SADW	15% CO_2	0.000962		0.000284	0.397589
3	BG11 (-N)	air	0.148885	0.000284		0.000556
4	BG11 (-N)	15% CO_2	0.005367	0.397589	0.000556	

Table B.60 Tukey HSD test; variable C/N ratio. Approximate Probabilities for Post Hoc Tests Error: Between MS = .00792, df = 8.0000"

Cell No.	Medium	[1]	[2]
1	SADW		0.020204
2	BG11 (-N)	0.020204	

Metal analysis:

Table B.61 Tests of Homogeneity of Variances: Effect: CO_2 fertilization

	Hartley	Cochran	Bartlett	df	p
Al removed [µg g^{-1} DW d^{-1}]	2.74289	0.732826	0.391054	1	0.531746
As removed [µg g^{-1} DW d^{-1}]	1.07989	0.519205	0.002362	1	0.961235
Cu removed [µg g^{-1} DW d^{-1}]	1.12057	0.528428	0.005181	1	0.942620
Fe removed [µg g^{-1} DW d^{-1}]	1.03750	0.509202	0.000542	1	0.981425
Mo removed [µg g^{-1} DW d^{-1}]	11.75423	0.921595	1.985950	1	0.158765
Ni removed [µg g^{-1} DW d^{-1}]	1.09107	0.521776	0.003038	1	0.956046
Se removed [µg g^{-1} DW d^{-1}]	5.06946	0.835241	0.955218	1	0.328395
Sr removed [µg g^{-1} DW d^{-1}]	1.24647	0.554856	0.019376	1	0.889294
V removed [µg g^{-1} DW d^{-1}]	4.19159	0.807381	0.759530	1	0.383476
Zn removed [µg g^{-1} DW d^{-1}]	15.26607	0.938522	2.345977	1	0.125607

Table B.62 Univariate Tests of Significance for Al removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	950726.8	1	950726.8	2653.622	0.000001
CO_2 fertilization	37546.0	1	37546.0	104.796	0.000513
Error	1433.1	4	358.3		

Table B.63 Univariate Tests of Significance for As removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	21.38883	1	21.38883	570.3812	0.000018
CO_2 fertilization	1.12245	1	1.12245	29.9327	0.005430
Error	0.15000	4	0.03750		

Table B.64 Univariate Tests of Significance for Cu removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	648.0285	1	648.0285	583.4860	0.000017
CO_2 fertilization	11.3330	1	11.3330	10.2043	0.033077
Error	4.4425	4	1.1106		

Table B.65 Univariate Tests of Significance for Fe removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	50061960	1	50061960	2854.535	0.000001
CO_2 fertilization	3967	1	3967	0.226	0.659167
Error	70151	4	17538		

Table B.66 Univariate Tests of Significance for Mo removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	26466.14	1	26466.14	198.9304	0.000147
CO_2 fertilization	6320.16	1	6320.16	47.5050	0.002323
Error	532.17	4	133.04		

Table B.67 Univariate Tests of Significance for Ni removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	2504.357	1	2504.357	775.5934	0.000010
CO_2 fertilization	899.867	1	899.867	278.6867	0.000075
Error	12.916	4	3.229		

Table B.68 Univariate Tests of Significance for Se removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	30507.52	1	30507.52	1854.017	0.000002
CO_2 fertilization	559.60	1	559.60	34.008	0.004308
Error	65.82	4	16.45		

Table B.69 Univariate Tests of Significance for Sr removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	2702756	1	2702756	2458.050	0.000001
CO_2 fertilization	33838	1	33838	30.775	0.005165
Error	4398	4	1100		

Table B.70 Univariate Tests of Significance for V removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	23685.56	1	23685.56	190.7785	0.000159
CO_2 fertilization	53.18	1	53.18	0.4283	0.548534
Error	496.61	4	124.15		

Table B.71 Univariate Tests of Significance for Zn removed [µg g^{-1} DW d^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	15657.23	1	15657.23	288.2650	0.000071
CO_2 fertilization	658.99	1	658.99	12.1326	0.025279
Error	217.26	4	54.32		

Appendix C

Appendix for chapter 5

Figure C.1 Images of the low cost mass production systems used in this study. Left one- Cage used for the vertical bag (suspension culture); Right one- Vertical bag (suspension culture) with1-week old *Tolypothrix* sp. culture.

Fig C.2 Images of the low cost mass production systems used in this study: Top one- scheme of the design for the modified algal turf scrubber (biofilm) prototype; bottom one- algal turf scrubber with the biofilm developing.

Fig C.3 Images of the self-flocculation behaviour test of *Tolypothrix* sp.

Table C.1. Characteristics of the 2 low cost mass production systems

Parameter	Vertical bag	Modified turf scrubber
Construction material	Plastic (PVC)	Polystyrene foam
Volume (L)	400	600 [b]
Footprint (m^2)	0.3	2.2
Slope (°)	-	4
Aeration (L/L//min) [a]	0.05[a]	-
Flow delivery	-	Continuous (66 L min^{-1})
Water speed (m s^{-1})	-	0.6

[a] Refers to volume of air (L) per volume of medium (L) per minute.

[b] Refers to the volume of the sump situated underneath the polystyrene-foam surface for biofilm growth.

Table C.2. Comparison of design parameters of modified algal turf scrubber prototype designed at JCU and traditional Algal Turf Scrubber design (from (Mulbry et al. 2008)

Parameter	Modified ATS (this study)	ATS (Mulbry et al. 2008)
Cover material	Polystyrene	6 mm nylon mesh
Raceway dimensions (length x width, m)	2.2 x 1	30 x 1
Tank volume (L)	600	3700
Slope (%)	7	1-2
Pump flow (L min^{-1})	66	93
Flow delivery mode	Continuous	1 pulse each 8-15 s
Water speed (m s^{-1}) [1]	0.6	0.1
Water depth (cm)	~0.5	1-3
Estimated residence time (slope) (s)	4 (0.8%)	300 (11%)
Estimated residence time (tank) (s) [2]	450 (99.2%)	2387 (89%)

Table C. 3. Environmental parameters during the pilot study at MARFU, tropical Australia. Light irradiance range at noon (data in parenthesis refers to measurements in overcast days).

Culture type	System	Run	Dates	Water temperature (°C)	Light irradiance (µmol photons m^{-2} s^{-1})
Suspension	Vertical bag	1	March 2014	25-30	(200) 900-1500
		2	May 2014	24-31	(200) 900-1500
Biofilm	Modified algal turf scrubber	1	March 2014	25-30	(200) 900-1500
		2	May 2014	24-31	(200) 900-1500

Appendix D

Appendix Chapter 6

Figure D.1 Modified algal turf-scrubber used in this study for biofilm cultivation of *Tolypothrix* sp.

Figure D.2 Wire cage vertical bag systems used in this study for bubbled suspension culture of *Tolypothrix* sp.

Table D.1 Characteristics of the algal turf-scrubber (ATS) cultivation system

Parameters	ATS
Cover material	Polystyrene
Area (m^2)	2.2
Polystyrene thickness (cm)	~2.0
Raceway dimensions (length x with) (m)	2.2x1
Tank volume (L)	500
Slope (%)	7
Pump flow (L min^{-1})	66
Flow delivery mode	Continues
Water speed (m s^{-1})	0.6
Water depth (cm)	~0.5
Estimated residence time (slope) (s)	4 (0.8%)
Estimated residence time (tank) (s)	450 (99.2%)

Table D.2 Characteristics of the vertical bag cultivation system

Parameters	Vertical bag
Wire cage height (m)	1.9
Wire cage diameter (m)	2.0
Vertical bag height (m)	2.0
Vertical bag diameter (m)	1.9
Footprint area (m^2)	0.3
Bag volume (L)	500
Aeration	Continues
Air flow (L air L^{-1} min^{-1})	0.05
Tap height (m)	0.1

Table D.3 Environmental parameters during the study at the freshwater compound, James Cook University, Australia. (Parameters refers to the noon time)

Culture type	System	Media	Run	Dates	Water temperature (ºC)	pH	Light irradiance (µmol photons m^{-2} s^{-1})
Suspension culture	Vertical bag	SADW	1	September 2016	25 -30	7.0 – 9.0	500-900
		SADW+CO_2			25 -30	6.0 – 9.0	500-900
		SADW	2	October 2016	25-32	7.0 – 9.0	500-900
		SADW+CO_2			25-32	6.0 – 9.0	500-900
Biofilm	ATS	SADW	1	September 2016	25 -30	7.0 – 9.0	500-900
		SADW+CO_2			25 -30	6.5 – 9.0	500-900
		SADW	2	October 2016	25-32	7.0 – 9.0	500-900
		SADW+CO_2			25-32	6.5 – 9.0	500-900

Appendix E

Appendix chapter 7

Table E.1 Univariate Tests of Significance for Cumulative Gas [ml g^{-1} VS removal]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	411838.6	1	411838.6	4514.042	0.000000
Pretreatments	21660.4	5	4332.1	47.483	0.000000
Error	1094.8	12	91.2		

Table E.2 Tukey HSD test; variable Cumulative Gas [ml g^{-1} VS removal] Approximate Probabilities for Post Hoc Tests Error: Between MS = 91.235, df = 12.000

Cell No.	Pretreatments	[1]	[2]	[3]	[4]	[5]	[6]
1	Thermal		0.900433	0.000189	0.928055	0.000171	0.000159
2	Hydrothermal	0.900433		0.000289	0.999999	0.000229	0.000159
3	Microwave	0.000189	0.000289		0.000274	0.997945	0.054135
4	Sonication	0.928055	0.999999	0.000274		0.000221	0.000159
5	Freeze & Thaw	0.000171	0.000229	0.997945	0.000221		0.107657
6	Control	0.000159	0.000159	0.054135	0.000159	0.107657	

Table E.3 Univariate Tests of Significance for Sqrt. Cumulative CH_4 [ml g^{-1} VS removal]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	1704.258	1	1704.258	144573.6	0.000000
Pretreatments	24.054	5	4.811	408.1	0.000000
Error	0.141	12	0.012		

Table E.4 Tukey HSD test; variable Sqrt. Cumulative CH_4 [ml g^{-1} VS removal] Approximate Probabilities for Post Hoc Tests Error: Between MS = .01179, df = 12.000

Cell No.	Pretreatments	[1]	[2]	[3]	[4]	[5]	[6]
1	Thermal		0.029130	0.000159	0.000586	0.000159	0.000159
2	Hydrothermal	0.029130		0.000159	0.179670	0.000159	0.000159
3	Microwave	0.000159	0.000159		0.000159	0.982828	0.000168
4	Sonication	0.000586	0.179670	0.000159		0.000159	0.000159
5	Freeze & Thaw	0.000159	0.000159	0.982828	0.000159		0.000193
6	Control	0.000159	0.000159	0.000168	0.000159	0.000193	

Table E.5 Univariate Tests of Significance for sqrt of sqrt sqrted sqrt.VS removal [%] Sigma-restricted parameterization Effective hypothesis decomposition

Effect	SS	Degr. of freedom	MS	F	p
Intercept	29.33314	1	29.33314	199575.6	0.000000
Pretreatments	0.02350	5	0.00470	32.0	0.000002
Error	0.00176	12	0.00015		

Table E.6 Tukey HSD test; variable sqrt of sqrt sqrted sqrt.VS removal [%] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00015, df = 12.000

Cell No.	Pretreatments	[1]	[2]	[3]	[4]	[5]	[6]
1	Thermal		1.000000	0.001604	0.999982	0.104959	0.000205
2	Hydrothermal	1.000000		0.001652	0.999991	0.101513	0.000206
3	Microwave	0.001604	0.001652		0.002025	0.000172	0.290074
4	Sonication	0.999982	0.999991	0.002025		0.081033	0.000216
5	Freeze & Thaw	0.104959	0.101513	0.000172	0.081033		0.000159
6	Control	0.000205	0.000206	0.290074	0.000216	0.000159	

Table E.7 Univariate Tests of Significance for Theoretical CH_4 [L^{-1} g VS]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	4.920350	1	4.920350	2672.012	0.000000
Pre-treatments	0.253730	5	0.050746	27.558	0.000000
Days	0.059874	1	0.059874	32.515	0.000007
Pre-treatments*Days	0.037686	5	0.007537	4.093	0.007896
Error	0.044195	24	0.001841		

Table E.8 Tukey HSD test; variable Theoretical CH_4 [L^{-1}g VS] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00184, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.00	0.04	0.06	0.02	0.00	0.29	0.00	1.00	1.00	0.00	0.00
2	Thermal	21	0.00		0.90	0.85	0.99	0.94	0.35	1.00	0.00	0.00	1.00	0.00
3	Hydrothermal	0	0.04	0.90		1.00	1.00	0.16	1.00	1.00	0.05	0.14	0.39	0.00
4	Hydrothermal	21	0.06	0.85	1.00		1.00	0.12	1.00	0.99	0.07	0.18	0.32	0.00
5	Microwave	0	0.02	0.99	1.00	1.00		0.36	0.94	1.00	0.02	0.05	0.68	0.00
6	Microwave	21	0.00	0.94	0.16	0.12	0.36		0.02	0.66	0.00	0.00	1.00	0.09
7	Sonication	0	0.29	0.35	1.00	1.00	0.94	0.02		0.71	0.33	0.61	0.07	0.00
8	Sonication	21	0.00	1.00	1.00	0.99	1.00	0.66	0.71		0.01	0.02	0.92	0.00
9	Freeze & Thaw	0	1.00	0.00	0.05	0.07	0.02	0.00	0.33	0.01		1.00	0.00	0.00
10	Freeze & Thaw	21	1.00	0.00	0.14	0.18	0.05	0.00	0.61	0.02	1.00		0.00	0.00
11	Control	0	0.00	1.00	0.39	0.32	0.68	1.00	0.07	0.92	0.00	0.00		0.03
12	Control	21	0.00	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00	0.00	0.03	

Table E.9 Univariate Tests of Significance for pH

Effect	SS	Degr. of freedom	MS	F	p
Intercept	1518.921	1	1518.921	13953.54	0.000000
Pre-treatments	2.811	5	0.562	5.16	0.002346
Days	3.192	1	3.192	29.32	0.000015
Pre-treatments*Days	1.870	5	0.374	3.44	0.017560
Error	2.613	24	0.109		

Table E.10 Tukey HSD test; variable pH Approximate Probabilities for Post Hoc Tests Error: Between MS = .05108, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.91	1.00	0.88	1.00	0.31	0.97	0.00	1.00	0.85	0.80	0.00
2	Thermal	21	0.91		0.85	1.00	0.92	0.99	0.23	0.00	0.46	1.00	1.00	0.00
3	Hydrothermal	0	1.00	0.85		0.81	1.00	0.25	0.99	0.00	1.00	0.78	0.72	0.00
4	Hydrothermal	21	0.88	1.00	0.81		0.89	1.00	0.19	0.01	0.41	1.00	1.00	0.00
5	Microwave	0	1.00	0.92	1.00	0.89		0.33	0.97	0.00	1.00	0.87	0.82	0.00
6	Microwave	21	0.31	0.99	0.25	1.00	0.33		0.03	0.05	0.07	1.00	1.00	0.00
7	Sonication	0	0.97	0.23	0.99	0.19	0.97	0.03		0.00	1.00	0.18	0.14	0.00
8	Sonication	21	0.00	0.00	0.00	0.01	0.00	0.05	0.00		0.00	0.01	0.01	0.36
9	Freeze & Thaw	0	1.00	0.46	1.00	0.41	1.00	0.07	1.00	0.00		0.38	0.32	0.00
10	Freeze & Thaw	21	0.85	1.00	0.78	1.00	0.87	1.00	0.18	0.01	0.38		1.00	0.00
11	Control	0	0.80	1.00	0.72	1.00	0.82	1.00	0.14	0.01	0.32	1.00		0.00
12	Control	21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.36	0.00	0.00	0.00	

Table E.11 Univariate Tests of Significance for ORP

Effect	SS	Degr. of freedom	MS	F	p
Intercept	501500.0	1	501500.0	1894.041	0.000000
Pre-treatments	287080.1	5	57416.0	216.846	0.000000
Days	306362.3	1	306362.3	1157.054	0.000000
Pre-treatments*Days	117015.9	5	23403.2	88.388	0.000000
Error	6354.7	24	264.8		

Table E.12 Tukey HSD test; variable ORP Approximate Probabilities for Post Hoc Tests Error: Between MS = 264.78, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.00	1.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	Thermal	21	0.00		0.00	0.69	0.00	1.00	0.00	1.00	0.00	0.01	0.00	0.00
3	Hydrothermal	0	1.00	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	Hydrothermal	21	0.00	0.69	0.00		0.00	0.61	0.00	0.64	0.00	0.53	0.00	0.00
5	Microwave	0	0.00	0.00	0.00	0.00		0.00	0.08	0.00	0.09	0.00	0.00	0.00
6	Microwave	21	0.00	1.00	0.00	0.61	0.00		0.00	1.00	0.00	0.01	0.00	0.00
7	Sonication	0	0.00	0.00	0.00	0.00	0.08	0.00		0.00	0.00	0.00	0.00	0.00
8	Sonication	21	0.00	1.00	0.00	0.64	0.00	1.00	0.00		0.00	0.01	0.00	0.00
9	Freeze & Thaw	0	0.00	0.00	0.00	0.00	0.09	0.00	0.00	0.00		0.00	0.00	0.00
10	Freeze & Thaw	21	0.00	0.01	0.00	0.53	0.00	0.01	0.00	0.01	0.00		0.00	0.00
11	Control	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.15
12	Control	21	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.15	

Table E.13 Univariate Tests of Significance for E. Conductivity [µs/cm]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	22110779	1	22110779	1965.880	0.000000
Pre-treatments	48073	5	9615	0.855	0.525164
Days	12304591	1	12304591	1094.007	0.000000
Pre-treatments*Days	189022	5	37804	3.361	0.019261
Error	269934	24	11247		

Table E.14 Tukey HSD test; variable E. Conductivity [µs/cm] Approximate Probabilities for Post Hoc Tests Error: Between MS = 11247, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
2	Thermal	21	0.00		0.00	0.84	0.00	0.52	0.00	0.12	0.00	1.00	0.00	1.00
3	Hydrothermal	0	1.00	0.00		0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
4	Hydrothermal	21	0.00	0.84	0.00		0.00	1.00	0.00	0.94	0.00	0.90	0.00	0.92
5	Microwave	0	1.00	0.00	1.00	0.00		0.00	1.00	0.00	1.00	0.00	0.94	0.00
6	Microwave	21	0.00	0.52	0.00	1.00	0.00		0.00	1.00	0.00	0.61	0.00	0.66
7	Sonication	0	1.00	0.00	1.00	0.00	1.00	0.00		0.00	1.00	0.00	0.95	0.00
8	Sonication	21	0.00	0.12	0.00	0.94	0.00	1.00	0.00		0.00	0.15	0.00	0.17
9	Freeze & Thaw	0	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00		0.00	1.00	0.00
10	Freeze & Thaw	21	0.00	1.00	0.00	0.90	0.00	0.61	0.00	0.15	0.00		0.00	1.00
11	Control	0	1.00	0.00	1.00	0.00	0.94	0.00	0.95	0.00	1.00	0.00		0.00
12	Control	21	0.00	1.00	0.00	0.92	0.00	0.66	0.00	0.17	0.00	1.00	0.00	

Table E.15 Univariate Tests of Significance for sqrt. SCOD [$g^{-1}L$]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	128.1068	1	128.1068	223310.2	0.000000
Pre-treatments	0.6057	5	0.1211	211.2	0.000000
Days	1.1273	1	1.1273	1965.0	0.000000
Pre-treatments*Days	0.2165	5	0.0433	75.5	0.000000
Error	0.0138	24	0.0006		

Table E.16 Tukey HSD test; variable sqrt. SCOD [$g^{-1}L$] Approximate Probabilities for Post Hoc Tests Error: Between MS = .00057, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.00	1.00	0.00	0.24	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	Thermal	21	0.00		0.00	0.03	0.00	0.00	0.53	0.00	0.00	0.03	0.00	1.00
3	Hydrothermal	0	1.00	0.00		0.00	0.08	0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	Hydrothermal	21	0.00	0.03	0.00		0.00	0.00	0.87	0.00	0.93	0.00	0.00	0.08
5	Microwave	0	0.24	0.00	0.08	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	Microwave	21	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.80	0.00	0.00
7	Sonication	0	0.00	0.53	0.00	0.87	0.00	0.00		0.00	0.13	0.00	0.00	0.84
8	Sonication	21	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00
9	Freeze & Thaw	0	0.00	0.00	0.00	0.93	0.00	0.00	0.13	0.00		0.00	0.00	0.00
10	Freeze & Thaw	21	0.00	0.03	0.00	0.00	0.00	0.80	0.00	0.00	0.00		0.00	0.01
11	Control	0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00
12	Control	21	0.00	1.00	0.00	0.08	0.00	0.00	0.84	0.00	0.00	0.01	0.00	

Table E.17 Univariate Tests of Significance for VFA [$g^{-1}L$]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	1537.113	1	1537.113	2176.116	0.000000
Pre-treatments	123.267	5	24.653	34.902	0.000000
Days	598.181	1	598.181	846.854	0.000000
Pre-treatments*Days	44.827	5	8.965	12.692	0.000004
Error	16.953	24	0.706		

Table E.18 Tukey HSD test; variable VFA [$g^{-1}L$] Approximate Probabilities for Post Hoc Tests Error: Between MS = .70636, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.00	1.00	0.00	0.92	0.00	0.35	0.00	1.00	0.00	0.99	0.00
2	Thermal	21	0.00		0.00	0.49	0.00	0.47	0.00	0.00	0.00	0.04	0.00	0.03
3	Hydrothermal	0	1.00	0.00		0.00	0.92	0.00	0.35	0.00	1.00	0.00	0.99	0.00
4	Hydrothermal	21	0.00	0.49	0.00		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.91
5	Microwave	0	0.92	0.00	0.92	0.00		0.00	0.99	0.00	0.99	0.00	0.34	0.00
6	Microwave	21	0.00	0.47	0.00	0.00	0.00		0.00	0.00	0.00	0.96	0.00	0.00
7	Sonication	0	0.35	0.00	0.35	0.00	0.99	0.00		0.00	0.63	0.00	0.05	0.01
8	Sonication	21	0.00	0.00	0.00	0.00	0.00	0.00	0.00		0.00	0.00	0.00	0.00
9	Freeze & Thaw	0	1.00	0.00	1.00	0.00	0.99	0.00	0.63	0.00		0.00	0.92	0.00
10	Freeze & Thaw	21	0.00	0.04	0.00	0.00	0.00	0.96	0.00	0.00	0.00		0.00	0.00
11	Control	0	0.99	0.00	0.99	0.00	0.34	0.00	0.05	0.00	0.92	0.00		0.00
12	Control	21	0.00	0.03	0.00	0.91	0.00	0.00	0.01	0.00	0.00	0.00	0.00	

Table E.19 Univariate Tests of Significance for Ammonia [mg N g^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	15051643	1	15051643	7946.662	0.000000
Pre-treatments	181233	5	36247	19.137	0.000000
Days	11268718	1	11268718	5949.429	0.000000
Pre-treatments*Days	204445	5	40889	21.588	0.000000
Error	45458	24	1894		

Table E.20 Tukey HSD test; variable Ammonia [mg N g^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = 1894.1, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
2	Thermal	21	0.00		0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.05
3	Hydrothermal	0	1.00	0.00		0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00
4	Hydrothermal	21	0.00	0.01	0.00		0.00	0.16	0.00	0.18	0.00	0.87	0.00	0.00
5	Microwave	0	1.00	0.00	1.00	0.00		0.00	0.99	0.00	1.00	0.00	1.00	0.00
6	Microwave	21	0.00	0.00	0.00	0.16	0.00		0.00	1.00	0.00	0.96	0.00	0.00
7	Sonication	0	1.00	0.00	1.00	0.00	0.99	0.00		0.00	1.00	0.00	1.00	0.00
8	Sonication	21	0.00	0.00	0.00	0.18	0.00	1.00	0.00		0.00	0.97	0.00	0.00
9	Freeze & Thaw	0	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00		0.00	1.00	0.00
10	Freeze & Thaw	21	0.00	0.00	0.00	0.87	0.00	0.96	0.00	0.97	0.00		0.00	0.00
11	Control	0	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00	1.00	0.00		0.00
12	Control	21	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	

Table E.21 Univariate Tests of Significance for FAME [mg g^{-1}]

Effect	SS	Degr. of freedom	MS	F	p
Intercept	132817.6	1	132817.6	1295.806	0.000000
Pre-treatments	236.5	5	47.3	0.461	0.800913
Days	79.4	1	79.4	0.775	0.387491
Pre-treatments*Days	1824.1	5	364.8	3.559	0.015053
Error	2460.0	24	102.5		

Table E.22 Tukey HSD test; variable FAME [mg g^{-1}] Approximate Probabilities for Post Hoc Tests Error: Between MS = 102.50, df = 24.000

Cell No.	Pre-treatments	Days	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
1	Thermal	0		1.00	1.00	0.95	1.00	1.00	0.92	0.99	0.96	1.00	1.00	1.00
2	Thermal	21	1.00		0.85	1.00	1.00	1.00	1.00	0.75	0.61	1.00	1.00	0.89
3	Hydrothermal	0	1.00	0.85		0.46	1.00	0.91	0.40	1.00	1.00	0.94	0.99	1.00
4	Hydrothermal	21	0.95	1.00	0.46		0.88	1.00	1.00	0.35	0.24	1.00	0.98	0.52
5	Microwave	0	1.00	1.00	1.00	0.88		1.00	0.83	1.00	0.99	1.00	1.00	1.00
6	Microwave	21	1.00	1.00	0.91	1.00	1.00		1.00	0.83	0.70	1.00	1.00	0.94
7	Sonication	0	0.92	1.00	0.40	1.00	0.83	1.00		0.30	0.20	1.00	0.96	0.45
8	Sonication	21	0.99	0.75	1.00	0.35	1.00	0.83	0.30		1.00	0.87	0.97	1.00
9	Freeze & Thaw	0	0.96	0.61	1.00	0.24	0.99	0.70	0.20	1.00		0.75	0.91	1.00
10	Freeze & Thaw	21	1.00	1.00	0.94	1.00	1.00	1.00	1.00	0.87	0.75		1.00	0.96
11	Control	0	1.00	1.00	0.99	0.98	1.00	1.00	0.96	0.97	0.91	1.00		1.00
12	Control	21	1.00	0.89	1.00	0.52	1.00	0.94	0.45	1.00	1.00	0.96	1.00	