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# Effect of CO<sub>2</sub> and metal-rich waste water on bioproduct potential of the diazotrophic freshwater cyanobacterium, *Tolypothrix* sp.

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## 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 waste waters and emit flue gas, containing ~10% CO<sub>2</sub>. Algae and cyanobacteria remediate metals and CO<sub>2</sub>, but use of N<sub>2</sub>-fixing (diazotrophic) cyanobacteria can reduce nitrogen-fertilization costs. The resulting biomass represents a promising source for biofuel and bio-product development. This study investigated the effect of CO<sub>2</sub>- and trace metals on growth performance, 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<sub>2</sub> or not (non-CO<sub>2</sub> controls). CO<sub>2</sub> supplementation resulted in 2.4 and 3.3-fold higher biomass productivities and 1.3 and 1.2-fold higher phycocyanin and phycoerythrin contents, whilst metals (media) had no effect. Al,

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Cu, Ni and V were more efficiently removed (50–90%) with CO<sub>2</sub>-addition, while As, Mo, Se and Sr removal was higher (30–87%) for non-CO<sub>2</sub> 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 biofertilizer. With a carbohydrate content of 50%, the biomass is also suitable for bioethanol production. In summary, *Tolypothrix* sp. raised in ash dam waste water supplemented with flue gas CO<sub>2</sub> could yield high-value phycobiliproteins, bioethanol or biogas, and mineral-rich nitrogen fertilizer which would offset remediation costs and improve agricultural productivity.

Abbreviations: AD, anaerobic digestion; ADW, ash dam water; BSA, bovine serum albumin; DW, dry weight; EPS, exocellular polymeric substances; FA, fatty acids; GHG, greenhouse gas; MUFA, mono-unsaturated fatty acids; PBP, phycobiliproteins; PC, phycocyanin; PE, phycoerythrin; PUFA, polyunsaturated fatty acids; SADW, simulated ash dam water; SFA, saturated fatty acids; TFA, total fatty acids; Y<sub>prod</sub>, production year

Keywords: Agriculture, Biochemistry, Biotechnology, Plant biology

## 1. Introduction

The Earth's average surface temperature increase of 0.9 °C (NASA, 2016) since 1880, is largely attributable to higher concentrations of atmospheric greenhouse gases (GHGs) (IPPC, 2013). Global energy-related CO<sub>2</sub> emissions rose by 1.4% in 2017, an increase of 460 million tonnes (MT) to a high of 32.5 giga tonnes (GT) (IEA, 2018). 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<sub>2</sub> 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, 2013). Since the world economy relies on guaranteed energy supplies, novel technologies and strategies to minimize CO<sub>2</sub> 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 waste water (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<sub>2</sub> is the use of live microalgae or cyanobacteria to sequester waste gas and waste water 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<sub>2</sub> supplementation of algal cultures with flue gas overcomes carbon-limitation to growth and the resulting low pH of the water improves metal bioavailability (Roberts et al., 2013).

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 realized for astaxanthin from *Haematococcus pluvialis* and β-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 hydrolyzed biomass could be fermented to bioethanol, as cyanobacterial biomass is rich in carbohydrates (45–65%) (Möllers et al., 2014). Biomass of microalgae and cyanobacteria can also be converted to biochar through slow pyrolysis to form a carbon-rich product characterized by a high

pH (Roberts et al., 2015) 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).

Regardless of the product choice, microalgal biomass production is carbon-limited when produced on sites without CO<sub>2</sub> access. While integrated cultivation at power stations could overcome carbon limitation, other fertilizer 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 fertilization for optimal biomass productivity, while diazotrophic cyanobacteria can sustain growth based on uptake and conversion of atmospheric nitrogen (N<sub>2</sub>). Despite this advantage, care must be taken to select non-toxic strains, as some produce very potent toxins such as anatoxin-a, cylindrospermopsin, microcystins and saxitoxins (Chorus, 2012). *Tolypothrix* sp. is a non-toxic freshwater cyanobacterium (Velu et al., 2015), for which sustained growth performance in the absence of nitrogen-fertilization and capacity for CO<sub>2</sub> remediation was recently demonstrated (Velu et al., 2015). 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<sub>2</sub>-supplementation and simulated ash dam water (SADW) on *Tolypothrix* sp. growth performance 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 biofertilizer.

## 2. Material and methods

### 2.1. 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<sup>-2</sup> s<sup>-1</sup> 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 waste water (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 (Media metal concentrations (Table A.1, Initial concentration in SADW [μg L<sup>-1</sup>])) (Saunders et al., 2012). BG11(-N) cultures served as non-treatment controls.



## 2.2. Experimental setup, growth and biomass productivities

2 L aerated (100 mL min<sup>-1</sup>) 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<sup>-2</sup> s<sup>-1</sup> and 28 °C. Culture media were supplemented with CO<sub>2</sub>-enriched air (15% v/v) or air (non-CO<sub>2</sub> controls) (n = 3). On day 25, the biomass was harvested by centrifugation (8,000 g, 20 min; Beckman, Avanti<sup>®</sup> 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<sup>-1</sup> day<sup>-1</sup>) 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 (μ<sub>1-3</sub>), doubling rate (k) and doubling time (T<sub>2</sub>) were calculated as per von Alvensleben et al. (2013).

## 2.3. Phosphate analysis

Medium phosphate (PO<sub>4</sub><sup>3-</sup>) concentrations were determined in triplicate spectrophotometrically (PerkinElmer EnSpire<sup>®</sup> 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<sup>®</sup>, Greiner Bio-One GmbH) (von Alvensleben et al., 2013).

## 2.4. 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 on a Varian 820-MS inductively coupled plasma mass spectrometer (Melbourne, Australia) for Al, As, B, Cu, Fe, Mo, Ni, Se, Sr, V and Zn at the Advanced Analytical Centre (AAC), James Cook University (JCU). Indium served as the internal standard to correct for matrix effects and instrument drift. 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 for quality control; the accuracy achieved was typically within 5%.

## 2.5. 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).

### 2.5.1. Total lipid and carbohydrate contents

Total lipid content was determined gravimetrically following a direct transesterification procedure and carbohydrate content was analyzed using the phenol-sulphuric acid method as described in [von Alvensleben et al. \(2013\)](#).

### 2.5.2. Protein content

Total protein content was determined using the Lowry method based on ([González López et al., 2010](#)) using a kit (Sigma, Total Protein Kit, Micro Lowry, Peterson's Modification, TPO300, Sydney, Australia). Briefly, 20 mg freeze-dried biomass was lysed in 0.9 mL lysis buffer and 0.1 mL SDS using a Bullet Blender bead beater (ZrO<sub>2</sub> beads, 0.5 mm diameter; Next Advance, Lomb Scientific Pty Ltd, New South Wales, Australia). The lysed biomass was centrifuged at 1,800 g for 10 min at 4 °C. Collected supernatants received 1 mL Lowry reagent and, after 20 min incubation at room temperature, 0.5 mL Folin and Ciocalteu's phenol working solution, followed by 30 min incubation. Sample reaction mixtures were read at  $\lambda = 750$  nm in a 96-well micro-titre plate. Protein concentration was calculated from serial dilution linear regression BSA calibration curves ( $R^2 > 0.99$ ). Protein content was determined according to [González López et al. \(2010\)](#).

### 2.5.3. 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 [von Alvensleben et al. \(2013\)](#). Fatty acids profiles were analysed using gas chromatography (GC) (Agilent 7890B GC-Agilent 5975C) equipped with a DB-23 capillary column (60 m × 0.25 mm × 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.

### 2.5.4. Pigment analysis

Phycocyanin and phycoerythrin contents were determined spectrophotometrically (SpectraMax<sup>®</sup> M2, USA) using freeze-dried samples according to [Lawrenz et al. \(2011\)](#) and [Velu et al. \(2015\)](#).

### 2.5.5. Elemental analysis

Biomass carbon, hydrogen, nitrogen, sulphur, phosphorous and potassium contents (CHNSPK) (mg g<sup>-1</sup> 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.

## 2.6. Statistical analysis

Statistical significance of experimental results was evaluated by two-way ANOVA or one-way ANOVA (SADW elemental analyses), with a significance level ( $\alpha$ ) 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. The outcomes of the statistical tests are presented in Supplementary Materials: Appendix A: Statistical Analyses (Tables A.2–A71 and Figs. A.1–A.14).

## 2.7. Reagents and chemicals

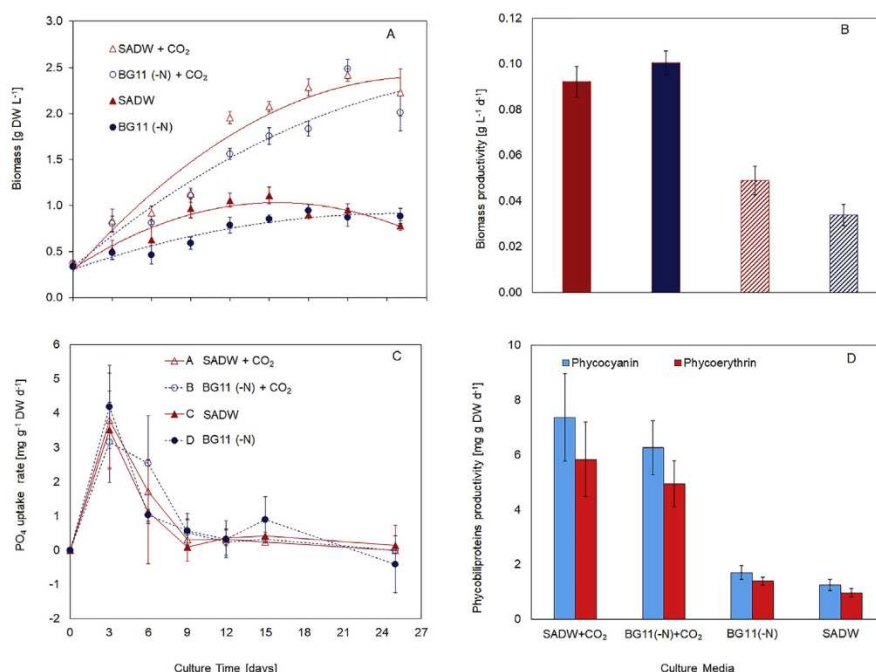
All chemicals and solvents were obtained from Sigma-Aldrich, Sydney, Australia.

## 3. Results

### 3.1. Effect of CO<sub>2</sub> and heavy metals on growth and phosphate uptake of *Tolypothrix* sp.

Over a 25-day time course, CO<sub>2</sub>-supplementation resulted in cultivation medium-independent significantly enhanced growth performance of *Tolypothrix* sp. (Fig. 1A and B;  $p < 0.001$ ) with 2.4 and 3.3-fold increased final biomass yields of  $2.4 \pm 0.1$  and  $2.5 \pm 0.1$  g DW L<sup>-1</sup> (Fig. 1A) and 2–3-fold improved biomass productivities ( $92.2 \pm 6.72$  and  $100.6 \pm 5.1$  mg DW L<sup>-1</sup> d<sup>-1</sup>) (Fig. 1B). The effects medium and CO<sub>2</sub> fertilization had significant interaction on biomass productivity (Statistical Analyses (Table A.5)), due to a marginal effect of medium on non-CO<sub>2</sub> controls (Statistical Analyses (Table A.6)). Based on specific growth rate ( $\mu$ ), three phases of growth were distinguishable ( $\mu_{1-3}$ ) (Table 1);  $\mu_1$  was ~2-fold higher for CO<sub>2</sub>-supplemented cultures irrespective of culture medium used ( $0.26 \pm 0.04$ ), while  $\mu_2$  was  $\geq 2$ -fold lower being similar for all culture conditions. A distinctive  $\mu_3$  phase was not discernible for SADW-grown non-CO<sub>2</sub> controls and decreased further to no appreciable growth for CO<sub>2</sub>-supplemented cultures. Doubling rates ( $k$ ) for the entire growth period were also  $\leq 2$ -fold higher for CO<sub>2</sub>-supplemented cultures than for non-CO<sub>2</sub> controls, resulting in doubling times ( $t_2$ ) of ~2.5 days compared to 5 and 6 days for non-CO<sub>2</sub> controls (Table 1). Low biomass growth parameters of non-CO<sub>2</sub> controls were likely due to carbon-limitation.





**Fig. 1.** Effect of CO<sub>2</sub> and heavy metals on (A) biomass growth; (B) biomass productivity; (C) PO<sub>4</sub><sup>3-</sup> uptake rate, and (D) phycobiliprotein productivity by *Tolypothrix* sp.

Despite effects of CO<sub>2</sub> fertilization on biomass productivity, phosphate removal from the medium showed no large differences (Fig. 1C). Growth of *Tolypothrix* sp. was phosphate-limited as of days 15 and 18 for CO<sub>2</sub>-supplemented cultures and non-CO<sub>2</sub> controls, respectively (Fig. 1C). Phosphate uptake rates was rapid for the first 3 days (3–4 mg PO<sub>4</sub><sup>3-</sup> g<sup>-1</sup> DW d<sup>-1</sup>), 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<sub>4</sub><sup>3-</sup> g<sup>-1</sup> DW d<sup>-1</sup> from day 9 for the remainder of the cultivation period (Fig. 1C).

**Table 1.** Effect of CO<sub>2</sub> and heavy metals on growth performance of *Tolypothrix* sp.

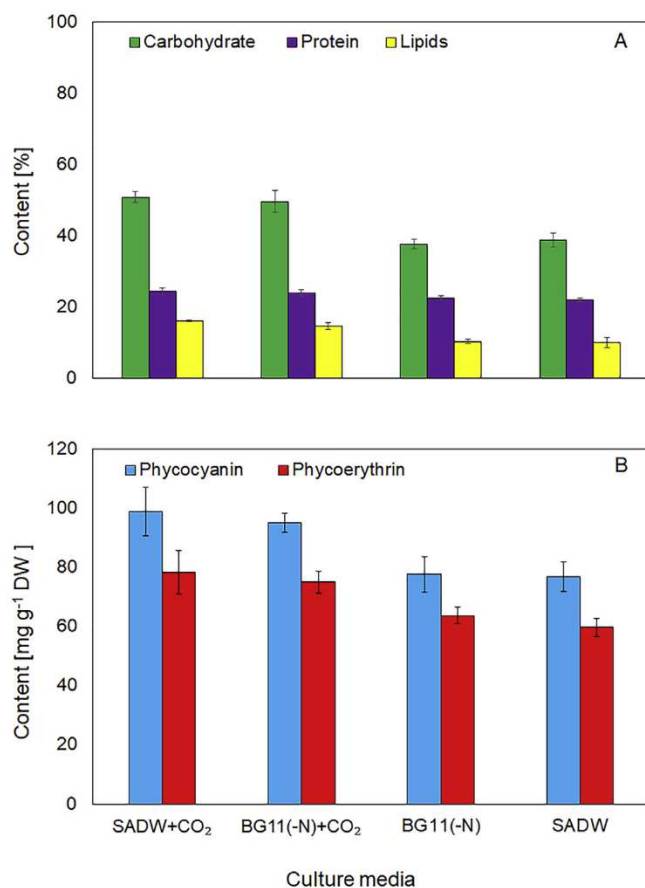
Parameters	Media			
	SADW + CO <sub>2</sub>	SADW	BG11(-N) + CO <sub>2</sub>	BG11(-N)
Specific growth rate ( $\mu_1$ ) [d <sup>-1</sup> ]	0.26 ± 0.04	0.14 ± 0.02	0.26 ± 0.04	0.13 ± 0.04
( $\mu_2$ ) [d <sup>-1</sup> ]	0.10 ± 0.02	0.08 ± 0.03	0.07 ± 0.02	0.06 ± 0.02
( $\mu_3$ ) [d <sup>-1</sup> ]	0.02 ± 0.00	-	0.05 ± 0.00	0.01 ± 0.01
Doubling rate (k) [d <sup>-1</sup> ]	0.38 ± 0.06	0.20 ± 0.04	0.37 ± 0.05	0.18 ± 0.05
Doubling time (t <sub>2</sub> )	2.71 ± 0.46	5.15 ± 0.85	2.73 ± 0.41	5.94 ± 2.02



### 3.2. Effect of CO<sub>2</sub> supplementation and heavy metals on the biochemical profile of *Tolypothrix* sp.

#### 3.2.1. Carbohydrate, protein, lipid, phycocyanin and phycoerythrin contents

A medium-independent significant positive effect of CO<sub>2</sub>-supplementation was observed for carbohydrate, protein and lipid contents (Statistical Analyses (Tables A.10, A.15 and A.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<sub>2</sub>-supplementation on protein content ( $p < 0.003$ ) was observed, BG11(-N) CO<sub>2</sub>-supplemented cultures were not significantly different to non-CO<sub>2</sub> controls in SADW (Statistical Analyses (Table A.16)). Maximal yields of carbohydrate, protein and lipids ( $\sim 50 \pm 1.5$ ,  $23 \pm 1.3$  and  $16 \pm 0.3\%$  (w/w), respectively) were achieved for *Tolypothrix* sp. grown in SADW with CO<sub>2</sub>-supplementation (Fig. 2A).



**Fig. 2.** Effect of CO<sub>2</sub> and heavy metals on (A) carbohydrate, protein and lipid content and (B) phycobiliprotein content of *Tolypothrix* sp.

Similarly, PC and phycoerythrin (PE) contents increased by ~23% under these conditions irrespective of culture medium, yielding maximal contents of  $99 \pm 8.3$  and  $78 \pm 7.4$  mg g<sup>-1</sup> DW, respectively (Fig. 2B). A medium-independent significantly positive effect of CO<sub>2</sub>-fertilization on PBP-productivity was demonstrated (Statistical Analyses (Tables A.25, A.30, A.26 and A.31)), leading to a 5.9- and 3.6-fold increase for PC- and PE-productivity, respectively (Fig. 1D).

### 3.2.2. Fatty acid and elemental compositions

As growth, lipid and PBP contents were significantly increased by CO<sub>2</sub>-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<sub>2</sub>-fertilization resulted in higher total fatty acid (TFA) contents and productivity (Table 2; Statistical Analyses (Tables A.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 2). SFA, MUFA, and PUFA contents were higher in SADW- than in BG11(-N)-grown *Tolypothrix* sp. irrespective of CO<sub>2</sub>-supplementation with highest amounts measured in CO<sub>2</sub>-supplemented SADW grown biomass

**Table 2.** Effect of CO<sub>2</sub> and heavy metals on fatty acids profiles of *Tolypothrix* sp.

Fatty Acids [mg g <sup>-1</sup> TFA]	SADW + CO <sub>2</sub>	SADW	BG11(-N) + CO <sub>2</sub>	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 <sup>-1</sup> DW]	52.70 ± 9.95	47.60 ± 9.86	42.46 ± 7.89	39.07 ± 2.99
Fatty acid productivity [mg g <sup>-1</sup> DW day <sup>-1</sup> ]	3.97 ± 1.30	0.783 ± 0.22*	2.83 ± 0.84	0.87 ± 0.17*

\*p < 0.002.

( $\sim 22 \pm 3$ ,  $9 \pm 2$ , and  $22 \pm 5$  mg g<sup>-1</sup> TFA) (Table 2), but differences were not significant (Statistical Analyses (Tables A.40, A.44 and A.48)). In contrast, fatty acid productivity was significantly higher in CO<sub>2</sub>-supplemented cultures, and highest in SADW with CO<sub>2</sub> fertilisation (Table 2).

The most abundant FAs in *Tolypothrix* sp. biomass were palmitic acid (C16:0,  $\sim 40\%$  of TFA),  $\gamma$ -linolenic acid (C18:3(cis 6, 9, 12),  $\sim 27\%$  of TFA) and linoleic acid (C18:2 (cis/trans 9, 12), oleic acid (C18:1,  $\sim 11\%$  of TFA) and the  $\omega$ -3 fatty acid  $\alpha$ -linolenic acid (C18:3(cis 9, 12, 15)) (Table 2). CO<sub>2</sub>-supplementation of SADW-grown *Tolypothrix* biomass showed increased contents of all FAs compared to BG11(-N)-grown CO<sub>2</sub>-supplemented biomass and non-CO<sub>2</sub> controls (Table 2).

Heavy metals and CO<sub>2</sub>-supplementation did not result in large differences in C, H, N, S, P, K contents (Table 3). CO<sub>2</sub>-supplementation resulted in slightly higher contents of C ( $\sim 45\%$  (w/w) and significantly higher levels of K ( $\sim 0.825\%$  w/w;  $p < 0.0001$ , Statistical Analyses (Tables A.56–57)), while H ( $7.28 \pm 0.38$ ) was highest in SADW and not affected by CO<sub>2</sub>-supplementation (Table 3). Significant effects of CO<sub>2</sub>-supplementation were also detected for N ( $7.48\%$  w/w,  $p < 0.02$ , Statistical Analyses (Table A.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 ( $6.88 \pm 0.15\%$  w/w) but there was no significant interaction (Statistical Analyses (Table A.58)). Higher P-contents ( $\sim 0.94\%$  w/w, Table 2) in SADW-non-CO<sub>2</sub> controls and BG11(-N)-CO<sub>2</sub> treatments suggested a significant interactive effect of medium\*CO<sub>2</sub> fertilization, although individual treatments had no significant effects (Statistical Analyses (Table A.54)). This was, however, not supported in a Tukey's post hoc analysis (Statistical Analyses (Table A.55)). Cultures with the highest P-content also had

**Table 3.** Effect of CO<sub>2</sub> and heavy metals on elemental composition of *Tolypothrix* sp.

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

\* $p < 0.05$ ; \*\* $p < 0.002$ .



the highest sulphur contents ( $\sim 0.43\%$  w/w, Table 3) but treatments were not significant and no significant interaction was determined.

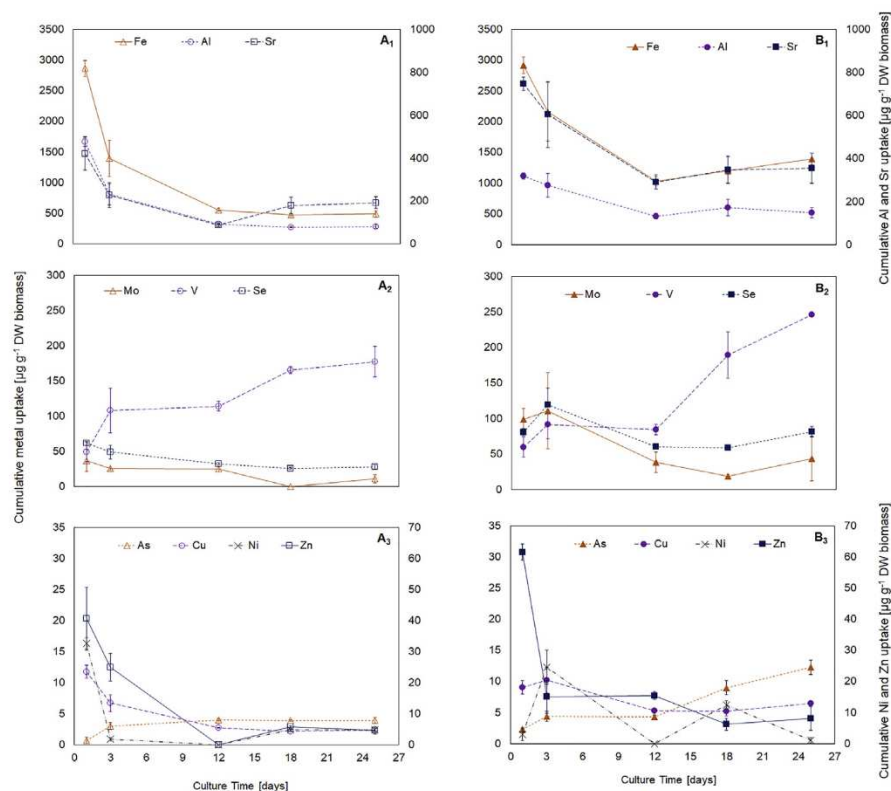
### 3.3. Effect of CO<sub>2</sub> on metal removal from SADW by *Tolypothrix* sp.

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 (Media metal concentrations (Table A.1)). Cumulative metal removal from SADW medium was  $\geq 90\%$  for Al, Fe, and V, followed by Se ( $\sim 87\%$ ), Cu ( $\sim 75\%$ ), Zn ( $\sim 64\%$ ), As ( $\sim 67\%$ ), Ni ( $\sim 58\%$ ), Sr ( $\sim 51\%$ ) and Mo ( $\sim 7\%$ ) in CO<sub>2</sub>-supplemented cultures (Table 4). Metal removal was the same for Fe, Mo, Ni, and Se with or without CO<sub>2</sub>-fertilisation, while they were higher for As, Cu, and Zn in non-CO<sub>2</sub> controls despite lower biomass contents (Table 4). Surprisingly, Mo, an essential metal for the nitrogen-fixing nitrogenase complex, was removed at much lower rates of only  $\sim 7\%$  (Table 4).

To investigate the effect of CO<sub>2</sub> on metal removal, metal uptake was calculated for time course experiments and values were standardized for biomass [ $\mu\text{g metal g}^{-1}$  DW] (Fig. 3). Time periods required for metal removal from SADW did not correlate with cellular function. Maximal biomass-standardized 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. 3). In contrast, boron (B) was the only element not removed, irrespective of CO<sub>2</sub> supply (data not shown). Calculated biomass-standardized metal uptake gradually decreased over the time course for metals where highest uptake occurred after 24 h (Fig. 3).

**Table 4.** Effect of CO<sub>2</sub> on cumulative metal removal from SADW medium by *Tolypothrix* sp.

Metals	Initial medium concentration [ $\mu\text{g L}^{-1}$ ]	SADW + CO <sub>2</sub> [%]	SADW [%]
Al	200 $\pm$ 1.0	91 $\pm$ 0.3	69 $\pm$ 6.9
As	13 $\pm$ 0.2	67 $\pm$ 0.7	72 $\pm$ 2.1
Cu	7 $\pm$ 0.2	75 $\pm$ 5.6	79 $\pm$ 4.4
Fe	1184 $\pm$ 3.0	92 $\pm$ 0.0	92 $\pm$ 0.0
Mo	750 $\pm$ 11.0	7 $\pm$ 1.1	7 $\pm$ 1.8
Ni	21 $\pm$ 0.4	58 $\pm$ 0.5	58 $\pm$ 0.2
Se	73 $\pm$ 1.0	87 $\pm$ 0.3	87 $\pm$ 1.8
Sr	831 $\pm$ 12.0	51 $\pm$ 6.8	37 $\pm$ 1.4
V	434 $\pm$ 4.0	90 $\pm$ 1.2	55 $\pm$ 5.8
Zn	31 $\pm$ 2.6	64 $\pm$ 0.7	73 $\pm$ 4.6



**Fig. 3.** Effect of CO<sub>2</sub> on cumulative metal uptake from SADW medium by *Tolypothrix* sp. (A) SADW with CO<sub>2</sub> enrichment and (B) SADW without CO<sub>2</sub> enrichment.

CO<sub>2</sub>-supplementation had no significant effect on maximal metal uptake for Fe and V ( $\sim 2,862 \pm 131$  and  $66 \pm 7$  vs  $2,914 \pm 133$  and  $60 \pm 14$   $\mu\text{g g}^{-1} \text{DW d}^{-1}$  for non-CO<sub>2</sub> controls, respectively; Statistical Analyses (Tables A.65 and A.70)), while the removal rate of Zn was negatively affected ( $40 \pm 10$  vs  $62 \pm 3$   $\mu\text{g g}^{-1} \text{DW d}^{-1}$  for non-CO<sub>2</sub> controls; Statistical Analyses (Table A4.71)). In contrast, a significant effect of CO<sub>2</sub>-fertilization was determined for Al, As, Cu, Mo, Ni, Se and Sr (Statistical Analyses (Tables A.62-64 and A.66-69)). Biomass-standardized uptake of Al and V was  $\sim 25$  and 9% higher when supplemented with CO<sub>2</sub> ( $477.2 \pm 22.9$  and  $65.8 \pm 6.9$   $\mu\text{g g}^{-1} \text{DW}$ , respectively; Fig. 3A<sub>1</sub> vs 3B<sub>1</sub>), as well as for Cu and Ni ( $11.8 \pm 1.0$  and  $32.7 \pm 1.8$   $\mu\text{g g}^{-1} \text{DW}$ ; Fig. 3A<sub>3</sub> vs 3B<sub>3</sub>). In contrast, highest maximal uptake was recorded for As, Mo, Se and Sr in SADW-non-CO<sub>2</sub> controls (Fig. 3B<sub>1-3</sub> vs 3A<sub>1-3</sub>). Maximal Mo, Se, and Sr uptake occurred on day 3 and was 65%, 41% and 66% higher for non-CO<sub>2</sub> controls (Mo and Se:  $111 \pm 53$  and  $111 \pm 53$  vs  $39 \pm 22$  and  $49 \pm 10$   $\text{mg g}^{-1} \text{DW}$ ; Fig. 3B<sub>2</sub> vs 3A<sub>2</sub> and Sr  $604 \pm 153$  vs  $227 \pm 58$   $\text{mg g}^{-1} \text{DW}$ ; Fig. 3B<sub>1</sub> vs 3A<sub>1</sub>, respectively), while the significance was marginal for As (Fig. 3B<sub>3</sub> vs 3A<sub>3</sub>).

#### 4. Discussion

The exploitation of environmental services of micro- and macroalgae, i.e. abatement of industrial CO<sub>2</sub> emissions and the cleaning of waste waters, 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 performance, biomass biochemical contents and profiles, metal removal capacity and biomass-standardized metal uptake of the diazotrophic, non-toxic cyanobacterium *Tolypothrix* sp. is discussed in this context.

Supplementation with 15% CO<sub>2</sub> resulted in strongly increased biomass productivities and yields, indicating that non-CO<sub>2</sub> controls were carbon-limited. Increased biomass productivities and yields were comparable to those observed for 10% CO<sub>2</sub>-fertilized 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<sub>2</sub>-supplemented cultures of *Scenedesmus obliquus* and *Chlorella pyrenoidosa* achieved 42–43% higher biomass yields (Tang et al., 2011). Growth performance studies are difficult to compare, as inoculation density, light regime and – quality and – intensity, fertilization conditions, cultivation period and strains, singly or combined affect growth performance (von Alvensleben et al., 2016). Despite this difficulty, biomass productivity of *Tolypothrix* sp. was comparable to average raceway productivities of 10–15% CO<sub>2</sub>-supplemented *Nannochloropsis oculata* (20 g DW m<sup>-2</sup> d<sup>-1</sup>, equates to 80 mg L<sup>-1</sup> d<sup>-1</sup>) (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<sub>2</sub> and 3-times higher photon flux density of 300 μmol photons m<sup>-2</sup> s<sup>-1</sup> (Chiu et al., 2009).

Biomass productivities varied from 0.026 to 2.47 g L<sup>-1</sup> d<sup>-1</sup> in 2–60% CO<sub>2</sub>-supplemented microalgal and cyanobacterial species, ie. *Chlorella vulgaris* (Yoo et al., 2010), *S. obliquus* (Kumar et al., 2011), *Botryococcus braunii* (Kumar et al., 2011), *Spirulina* sp. (De Morais and Costa, 2007), *Anabaena* sp. ATCC 33047 (González López et al., 2009), and *Phaeodactylum tricornerutum* (Mazzuca Sobczuk et al., 2000). Highest biomass productivity of 2.47 g L<sup>-1</sup> d<sup>-1</sup> was reported for *P. tricornerutum* in an airlift photobioreactor with 60% CO<sub>2</sub> (Kumar et al., 2011; Mazzuca Sobczuk et al., 2000), growth conditions that are not comparable to those for *Tolypothrix* sp. here (0.335 g L<sup>-1</sup> d<sup>-1</sup>). 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<sup>-1</sup> d<sup>-1</sup>) (Yoo et al., 2010) and CO<sub>2</sub> concentrations >10% led to decreased growth (Chiu et al., 2011). In contrast, reported biomass yields for *Spirulina* sp., supplemented with 12% CO<sub>2</sub> 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<sup>-1</sup> vs 0.3 g L<sup>-1</sup> this study), which could have



resulted in light limitation of the *Tolypothrix* sp. cultures, and/or pre-adaptation of *Spirulina* sp. to CO<sub>2</sub>, (De Moraes and Costa, 2007), as the latter yielded higher biomass productivities (Lee et al., 2002). In summary, biomass yields of *Tolypothrix* sp. with CO<sub>2</sub>-fertilization 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 utilization, phosphate-replete cultures of *Tolypothrix* sp. require ~3–4 mg PO<sub>4</sub><sup>3-</sup> g<sup>-1</sup> DW d<sup>-1</sup> to maintain biomass productivity, unless light or other factors become limiting, whereas other microalgae require 20–90% 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 (Chen et al., 2011) PO<sub>4</sub><sup>3-</sup>, respectively. Significantly lower phosphate fertilization costs make *Tolypothrix* sp. an ideal candidate for ADW remediation. Choosing strains that require low phosphate fertilization, such as *Tolypothrix* sp. is important for commercial production, as are phosphate recycling options to guarantee long-term food security, as phosphate supplies are ultimately limited y (Koppelaar and Weikard, 2013).

A key factor in the production of nitrogen fertilizers is the nitrogen content of the candidate organisms. Generally, diazotrophic cyanobacteria exhibit significantly higher nitrogen concentrations (8–12% of DW) compared to green algae (3–8%) (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 fertilizers 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 biofertilizer 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<sup>-1</sup> 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<sup>-1</sup> – 0.30 t ha<sup>-1</sup> of *Tolypothrix* sp.

The utilization of *Tolypothrix* sp. biomass as an N-fertilizer 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 (Chibuikwe and Obiora, 2014). The minimum permissible levels (mg kg<sup>-1</sup> 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<sup>-1</sup> 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<sub>2</sub>-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* (Jiménez et al., 2003). PBP production in other diazotrophic cyanobacteria such as *Anabaena* sp. was about 70% higher than reported here for *Tolypothrix* sp. (Moreno et al., 2003), 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<sup>-1</sup>.

In the present study, CO<sub>2</sub>- 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 lipids in cultures approaching stationary growth phase. Achieved protein content of *Tolypothrix* sp. was 2–3-fold lower than reported for the commercially produced *Spirulina* sp., *Chlorella* (Tokuşoglu and Ünal, 2003) and *Scenedesmus* (Apandi et al., 2017), but comparable to the commonly used aquaculture-feed microalga *Isochrysis* (Tokuşoglu and Ünal, 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 *Nannochloropsis* 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<sub>2</sub> 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<sub>2</sub>-fertilization reported for *C. vulgaris* (Tsuzuki et al., 1990), no significant were determined for *Tolypothrix* sp. Considering their proclaimed health benefits, PUFAs, including  $\gamma$ -Linolenic acid (C18:3  $\omega$ -6; an ingredient in cosmetics) and  $\alpha$ -Linolenic acid (C18:3  $\omega$ -3), are intensively investigated (Ryckeboesch et al., 2012).  $\gamma$ -Linolenic acid content of *Tolypothrix* sp. was 27% of TFA (4.83 mg g<sup>-1</sup> TFA), being 1.7–2.5-times higher than reported for *Spirulina* 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.

## 5. Conclusion

This study demonstrated excellent growth responses to CO<sub>2</sub> and metal removal capacity of *Tolypothrix* sp. when cultivated in SADW without nitrogen fertilization, making *Tolypothrix* sp. an outstanding candidate for bioremediation of CO<sub>2</sub> and metals at freshwater-utilizing coal-fired power stations. Based on growth data obtained, an estimated 1.98 tonne ha<sup>-1</sup> 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  $\text{ha}^{-1} Y_{\text{prod}}^{-1}$ , worth ~US\$ 17,640. The residual biomass could be either hydrolyzed or anaerobically digested. With a 50% primarily starch-based carbohydrate content, hydrolyzed biomass could be fermented to produce bioethanol with an estimated production of ~548 L  $\text{ha}^{-1} Y_{\text{prod}}^{-1}$  worth ~US\$ 816.52. Alternatively, ~792 L  $\text{CH}_4 \text{d}^{-1}$  could be produced via AD. Based on mineral content of biomass raised under these conditions, *Tolypothrix* can provide mineral-rich biofertilizer to plants without metal toxicity. Based on N-content of *Tolypothrix* sp., ~0.149 t N fertilizer  $\text{ha}^{-1} Y_{\text{prod}}^{-1}$  can be expected, worth US \$74.25.

## Declarations

### Author contribution statement

Chinnathambi Velu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Samuel Cirés: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Diane L. Brinkman: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Kirsten Heimann: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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### Competing interest statement

The authors declare no conflict of interest.

## Additional information

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