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Research article

Biomass Pre-treatments of the N₂-fixing cyanobacterium

Tolypothrix for co-production of methane

Chinnathambi Velu¹, Obulisamy Parthiba Karthikeyan^{**2}, Diane L. Brinkman³,
Samuel Cirés⁴, Kirsten Heimann^{*1,5}

¹College of Science Engineering, James Cook University, Townsville 4811, Queensland, Australia;

²Department of Civil and Environmental Engineering, University of Michigan, Ann Arbor, 48109, MI, USA;

³Australian Institute of Marine Science, Townsville, Australia;

⁴Department of Biology Autonoma de Madrid University, Madrid, ES-28049, Spain;

⁵Centre for Marine Bioproduct Development, Flinders University, Bedford Park, SA 5042

* Corresponding author email: kirsten.heimann@flinders.edu.au; Phone: +61 (0) 422 208 577

**Co-corresponding author email: opkens@gmail.com; Phone: +1 (734) 272 3855

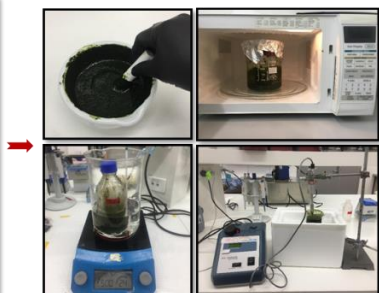
14 Highlights

- 15 • Pretreatment can improve biogas production especially for algae and
16 cyanobacteria.
- 17 • Sonication showed highest solubilization of organic compounds with a COD of
18 15.02 g L⁻¹.
- 19 • Thermal hydrolysis is optimal for *Tolypothrix* biomass giving high CH₄ yields
20 (126.27 ml g⁻¹ VS_{removed}).
- 21 • CH₄ yields were high despite low C/N 9.31, high VFA 9.57 g L⁻¹ and ammonium-
22 N 1.08 g L⁻¹ contents.
- 23 • AD system physico-chemical characteristics foiled investigated prediction
24 accuracy.

25 Graphical Abstract



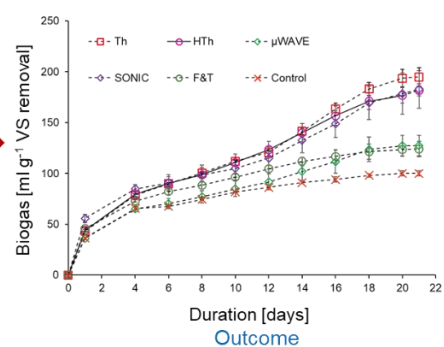
N_2 -fixing cyanobacterium
Tolypothrix sp.



Pretreatment



Anaerobic digestion



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Abstract

Tolypothrix, a self-flocculating, fast growing, CO₂ and nitrogen-fixing cyanobacterium, can be cultivated in nutrient-poor ash dam waters of coal-fired power stations, converting CO₂ emissions into organic biomass. Therefore, the biomass of *Tolypothrix* sp. is a promising source for bio-fertiliser production, providing micro- and macronutrients. Energy requirements for production could potentially be offset *via* anaerobic digestion (AD) of the produced biomass, which may further improve the efficiency of the resulting biofertiliser. The aim of this study was to evaluate the effectiveness of pre-treatment conditions and subsequent methane (CH₄) production of *Tolypothrix* under out-door cultivation conditions. Pre-treatments on biogas and methane production for *Tolypothrix* sp. biomass investigated 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 20 s 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 specific CH₄ production of 0.012 and 0.01 L CH₄.g⁻¹ volatile solids_{added}. was achieved for thermal and sonic pre-treatments, respectively. High N- and low C-content of the *Tolypothrix* biomass affected CH₄ recovery, while pre-treatment accelerated production of volatile acids (15.90 g L⁻¹) and ammonia-N-accumulation (1.41 g L⁻¹), leading to poor CH₄ yields. Calculated theoretical CH₄ yields based on the elemental composition of the biomass were ~55% higher than actual yields. This highlights the complexity of interactions during AD which are not adequately represented by elemental composition.

Keywords: Anaerobic co-digestion; horizontal algal turf scrubbers; volatile organics; methane; ash dam water.

1. Introduction

The global population is predicted to reach 8.5 billion in 2030, 9.7 billion in 2050, and 10.9 billion in 2100 (UN, 2019), resulting in higher food and energy demands, the latter is estimated to rise by 45% (i.e., 527,530 J in 2010 to 949,554 J) in 2050 (EIA, 2019). Carbon dioxide (CO₂) emissions are also forecasted to increase by 18% (i.e., 35.3 billion metric t y⁻¹ in 2018 to 43.2 billion metric t y⁻¹ in 2050) due to energy-related combustion (IEO, 2020). Increased CO₂ emissions are forecasted to raise the global temperature by 4 °C by 2050 (Dai et al., 2020), with negative flow-on effects envisaged for the food-energy-water nexus. Therefore, there is an immediate need to reduce CO₂ emissions, which can be achieved through development of microalgal production independent of arable land and capable of CO₂ assimilation of flue gas, preferably supporting energy requirements for production with 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; Passos et al., 2014). Alternatively, fermentation yields bio-ethanol (Möllers et al., 2014), while hydrothermal liquefaction is suitable for oil-based fuels (Roberts et al., 2013b). Therefore, development of a win-win approach that could effectively reduce CO₂ emissions, remediate any organic/ inorganic contaminants from point sources linked to energy recovery/ savings would be a promising future approach that will provide more economic incentives compared to conventional processes (Roberts et al., 2013a).

Cyanobacteria, a group of fastest growing photosynthetic microorganisms, are highly resilient to high concentrations of CO₂ (up to 70%) and produce 30 to 180 tons dry weight biomass ha⁻¹ y⁻¹ (Moreno et al., 2003; Velu et al., 2015). 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 $\mu\text{mol m}^{-2} \text{s}^{-1}$, and require low nutrient addition, although these characteristics are strongly species-dependent (Chevalier et al., 2000; Vaishampayan et al., 2001; Markou and Georgakakis, 2011; Sand-Jensen and Jespersen, 2012). 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 (Queiroz et al., 2007; Gupta and Diwan, 2017). Photosynthetic fixation of CO₂ by cyanobacteria of 100 to >200 tons CO₂ ha⁻¹ y⁻¹ has been reported under outdoor cultivation conditions in open ponds, raceway ponds, photobioreactors and attached growth bioreactors (Moreno et al., 2003; Ugwu et al., 2005; Cirés et al., 2015). For example, *Synechocystis aquatilis* fixed 138.7 to 277.4 tons CO₂ 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 (N₂) at a rate of 20-30 kg N ha⁻¹ season⁻¹, significantly improving soil organic content and 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 (Issa et al., 2014). Selection and consideration of use of particular cyanobacterial species for specific applications, however, requires prior evaluation and scenario analysis for cost-effectiveness (Velu et al., 2020).

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, reducing dewatering costs by up to 90% (Velu et al., 2015).

Tolypothrix sp. is also a nitrogen fixing (N₂-fixing) strain which stores phosphorus in phosphate granules, reducing nitrogen and phosphorus fertilization 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; Velu et al., 2020). These specific features make such process-derived *Tolypothrix* biomass a promising source for AD-derived bio-fertiliser and bio-energy production, but biomass metal concentrations could potentially interfere with the efficiency of the AD process.

Anaerobic digestion is a low-cost energy-generating technology compared to other ways of energy production such as coal combustion and can yield other value-added products such as bio-fertiliser in addition to biogas. *Spirulina* is a cyanobacterial genus most widely studied with high reported methane (CH₄) production potential. According to calculated theoretical CH₄ yields, AD of *Spirulina* sp., *Limnospira (Spirulina) maxima* and *Arthrospira (Spirulina) platensis* can produce CH₄ yields of ~ 0.26 – 0.32, ~ 0.63 – 0.74 and 0.47 – 0.69 L CH₄ g⁻¹ volatile solids (VS), respectively (Sialve et al., 2009). The broad range of reported CH₄ yields indicate that hydrolysis pre-treatment of the biomass, as well as bioreactor performance, strongly affect actual obtainable CH₄ yields (Sialve et al., 2009). The pre-treatment process significantly improves organic nutrient bioavailability and hence biodegradability of the organic matter by the anaerobic microflora (Sialve et al., 2009). Hence, pre-treatment of cyanobacterial biomass prior to AD has been proposed to improve bioconversion efficiency and CH₄ 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 N₂-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 biofertiliser and biogas production from *Tolypothrix* sp. or other N₂-fixing cyanobacteria raised in metal-containing ash dam water *via* AD.

Metal contents and the complexity of *Tolypothrix* sp. biomass raised in SADW could be expected to produce low methane yields. Therefore, the aim was to understand the effectiveness of pre-treatment, using thermal (Th), hydrothermal (HTh), microwave (μ WAVE), sonication (sonic) and freeze and thaw (F&T) for production of soil conditioner and mineral/N/P biofertiliser and biogas potential to offset energy requirements of the biomass production process. *Tolypothrix* sp. was grown as a water- and energy-saving biofilm in outdoor cultivation in simulated ash dam water (SADW) to obtain data relevant for future seasonally adjusted validation studies required for commercialization.

2. Materials and Methods

2.1. Outdoor cultivation of the N₂-fixing cyanobacterium *Tolypothrix* sp.

Tolypothrix sp. NQAIF319 biomass was cultivated in simulated ash dam wastewater (SADW) 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 (Fig. 1). ATS set up, inoculation, culture maintenance, light and growth measurements were carried out as described previously (Velu et al., 2015; Velu et al., 2020). Biomass was harvested on day 16 by scraping using a silicone rubber cell scraper (IWAKI, Co., Kyoto, Japan) with a yield of 42 g DW m⁻² and biomass was characterized as described in Velu et al. (2020).

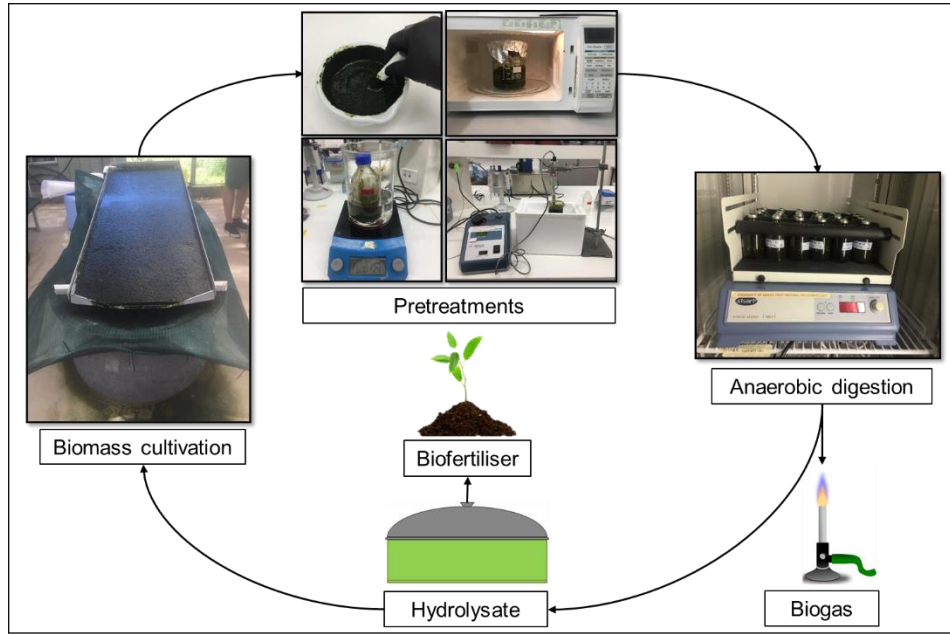


Fig 1. Schematic diagram of ATS-cultivation of *Tolypothrix* sp. biofilms, pretreatment and anaerobic digestion.

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

Harvested and pre-characterized *Tolypothrix* sp. biomass was pre-treated prior to AD to investigate biogas recovery potential. Approximately 5.0 kg of wet biomass was homogenised using a mortar and pestle. The ground biomass slurry 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, reported as ideal and optimized for different types of microalgal biomass. Pre-treatment of biomass was carried out using 200 g of pre-ground biomass per treatment replicate (n = 3).

Th: The biomass slurry was kept in a water bath under continuous stirring at 250 rpm at 95 °C for 10 h (Passos et al., 2015); Thermal treatment disintegrates the cell wall and aids in the release of sugars (Ometto et al., 2014).

HTh: The biomass slurry was autoclaved using a standard moisture-heat procedure of 121 °C at 1013.25 hPa (Passos et al., 2015; Razaghi et al., 2016) for 20 min (Tomy SX-

500E, VWR International, Murarrie, QLD 4172, Australia); Similar to Th, high temperature and pressure is expected to rupture cells and release and solubilise organic contents (Ometto et al., 2014).

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); The microwave radiation penetrates the biomass and continuous magnetic vibration of polar bonds generates heat. This leads to shock and disruption of the cells (Sridar, 1998).

SONIC: The biomass was treated with a rod sonicator (Fisherbrand Model 120, Thermo Fisher Scientific, Brisbane, Australia) at an output power of 10 W for 3.5 h at 10 min intervals with 20 s breaks to avoid process heat transfer to the biomass (Passos et al., 2015). The ultrasonic treatment is expected to create monolithic cavitations through physical and chemical changes, while generating heat and pressure inside the cells. The cavitation collapse produces reactive radicals (H^{\cdot} , OH^{\cdot} , HO_2^{\cdot}), that react with cellular components and disrupt the cells (Harris and McCabe, 2015).

F&T: The biomass slurry was frozen (Sanyo ultra-low temperature freezer, MDF-U33V, VWR International, Australia) at -80 °C for 24 h followed by thawing at room temperature (Carrère et al., 2010). Slow thawing of frozen biomass initiates the formation of ice crystals which rupture cell membranes and cell walls, leading to structural collapse and release of organic materials.

2.3. Anaerobic digestion

2.3.1 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-characterization following standard procedures (detailed in sub-section 2.4). Total - and volatile solid contents of the sludge were 6.22 and 3.75 g L⁻¹, respectively.

2.3.2 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 wet biomass (S) and 50 g of anaerobic sludge (I) were mixed using a shaker at the speed of 100 rpm, the bottles were sealed with butyl rubber stoppers and incubated at 35 °C (Panasonic Versatile Environmental Test Chamber, MLR-352, VWR International, Australia) for 21 days. Calculated S/I ratios [g COD / g VS] (Caillet et al., 2019) ranged from 1.9 for μ WAVE-pre-treated slurries to 4 for Sonic pre-treated slurries, while inoculum/substrate ratios (ISRs) based on g COD ranged from 0.94 for sonic pre-treatments to 2 for μ WAVE (Table 1). Activated sludge without cyanobacterial biomass served as CH₄ production baseline (control). Total biogas volume was measured using the gas displacement method using an air-tight syringe (Oliverio and Varlet, 2018).

Activated sludge without biomass addition served as an inoculum methane production baseline (control). Physico-chemical characteristics of the pre-treated biomass were analysed after inoculation with activated sludge prior to digestion and after digestion. Methane production of algal biomass without pre-treatment yielded no methane production within the incubation period of 21 days (data not shown), possibly due to high solid loads and substrate complexity.

2.3.3 Theoretical methane potential calculation

Theoretical methane yields were calculated based on C, H, N, O composition using the following equation (eq. 1; (Nielfa et al., 2015))

$$\text{BMP}_{\text{thAtC}} = \frac{22.4 \left(\frac{n}{2} + \frac{a}{8} - \frac{b}{4} - \frac{3c}{8} \right)}{12n + a + 16b + 14c} \quad \text{----- (eq.1)}$$

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

2.4. Analytical methods

2.4.1 Physico-chemical properties

Physico-chemical properties and biochemical profiles of digested slurries were determined on the day of inoculation (day 0) and after 21 days of AD. 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 (APHA, 2017). pH was analysed with a WP-81 water-proof conductivity / TDS-pH / MV-temperature meter (WP-81, TPS instruments, Brisbane, Australia). Oxygen reduction potential (ORP) was measured using a HM Digital ORP-200 ORP Meter (Brew Solutions, Murarrie, Queensland, Australia). Samples were analysed by TropWater's analytical services (James Cook University, Australia) for soluble 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).

2.4.2 Biochemical profiling

The harvested *Tolypothrix* sp. biomass was stored in the freezer (Sanyo ultra-low temperature freezer, MDF-U33V, VWR International, Australia) at -80 °C and freeze-dried using vacuum freeze drier (Dynavac Freeze Drier Model FD12, Dynavac vacuum and air solutions, Australia) prior to the following biochemical analysis.

Carbohydrate: An aqueous slurry of the freeze-dried *Tolypothrix* sp. biomass was lysed using a bullet blender (Next Advance, Lomb Scientific Pty Ltd, New South Wales,

Australia) with ZrO₂ beads (0.5 mm diameter). Carbohydrate contents were determined using the phenol-sulphuric acid method (DuBois, 1956).

Protein: For protein quantification, the freeze-dried biomass (20 mg) was lysed with 0.9 mL of lysis buffer containing 0.1 % of SDS (González López et al., 2009) and protein content of the lysates was determined using the Lowry method (Total Protein Assay Kit Micro Lowry TPO300, Peterson's Modification, Sigma-Aldrich, Sydney, Australia).

Fatty Acids: Biomass, biomass slurries and sludge were freeze-dried prior to biochemical analysis. Fatty acids contained in freeze-dried samples were simultaneously extracted and transesterified as detailed in von Alvensleben et al. (2013), and the fatty acid content was analysed by gas chromatography at Sustainable Coastal Ecosystems and Industries in Tropical Australia, Australian Institute of Marine Science, Townsville, Australia. Briefly, fatty acids profiles were analysed using a gas chromatography (GC) (Agilent 7890B GC-Agilent 5975C, Mulgrave, Victoria, Australia) equipped with a DB-23 capillary column (60 m x 0.25 mm 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 dry weight (DW)) was inferred from fatty acid profiles and determined as the sum of all fatty acids. 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 (30 m x 0.25 mm x 0.25 µm) 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, Heidelberg West, Victoria, Australia) and docosene (C22:1), as the internal standard. Alkanes/alkene concentrations were corrected for percent recovery of the internal standard (C22:1).

2.5. 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.

2.6. 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 was assessed using the Cochran, Hartley and Bartlett test. Data were square root-transformed if normality or homogeneity of variances assumptions were not met.

3. Results and Discussion

3.1. Characterisation of *Tolypothrix* sp.

Protein and carbohydrate contents of *Tolypothrix* sp. biomass were ~24 and ~51, respectively (Velu et al., 2020). Fatty acid contents were lower than 5% of total dry weight. Fatty acid contents of cyanobacterial biomass are typically lower than reported for

eukaryotic microalgae (e.g. *Dunaliella tertiolecta*: 20 to 25% of total dry weight), as the main carbon storage are carbohydrates and some amino acids (van den Hoek et al., 1996). The most abundant fatty acids were palmitic acid (C16:0) and α - and γ -linolenic acid (C18:3 ω -3 and ω -6 fatty acids), which are also common in other N₂-fixing cyanobacteria (e.g. *Nostoc* sp. and *Anabaena* sp. (Vargas et al., 1998; Liu et al., 2005)). The elemental composition of *Tolypothrix* biomass was C-45 \pm 0.5%, H-7 \pm 0.1%, N-7 \pm 0.2% and S-0.5 \pm 0.02%. The theoretical CH₄ potential calculated from the elemental composition was, ~ 324 mL g⁻¹ VS_{removed}, which was similar to that of *Spirulina* sp. (Sialve et al., 2009; González-Fernández et al., 2012). 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. Total metal removed from simulate ash dam water by ATS-cultivated *Tolypothrix* sp. NQAIF319 is complete in 24 to 48 h for most elements present, with biomass concentrations being suitable for biofertilizer applications for wheat cultivation, supplying many essential minerals in addition to nitrogen and phosphate (Velu et al., 2020).

3.2. Theoretical CH₄ yields

Based on the CHNS composition (Fig. 2), 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 CH₄ yields for *Tolypothrix* sp. biomass was ~324 mL g⁻¹ VS_{removed}. Theoretical CH₄ yields (Fig. 3) were highest for Th and F&T pre-treatments (~295 and 282 mL CH₄ g⁻¹ VS_{removed}, respectively, Table 1) followed by SONIC (~158 mL CH₄ g⁻¹ VS_{removed}), yet actual yields were lowest in F&T pre-treatments, while specific methane production (SMP) was lowest for μ WAVE and F&T pre-treatments,

achieving only 0.002 and 0.003 L CH₄ g⁻¹ VS_{added} (Table 1). This is not explained by differing S/I ratios and ISRs, which were 1.9 and 3.5 and 2.02 and 1.07, respectively, as Th pretreatments had a six-fold higher SMP, despite almost identical S/I ratios and similar ISRs (Table 1). One possible explanation for outcomes of μ WAVE pre-treatments could be enhanced extraction of cyanobacterial bioactives with antimicrobial properties in polar solvents, as demonstrated for the cyanobacterium *Arthrospira platensis* (Esquivel-Hernández et al., 2017). In contrast, F&T treatments yielded lowest quantities of VS, which together with unoptimised S/I ratios and ISRs, potentially limited methane production directly (Table 1). In contrast, SONIC with the least favourable S/I ratio (Caillet et al., 2019) and the second lowest ISR yielded the second highest SMP (Table 1), which could be explained by the pre-treatment conditions leading to the potential break-down of bioactives with antimicrobial activities (Rojas et al., 2020). These outcomes suggest that mass spectrometric analysis of digestates will be required to identify antimicrobials present and their concentrations, which should precede refinement of process parameters with regards to optimal ISRs and other process parameters.

A two-way ANOVA determined a significant effect of pre-treatment and time on VS removal ($p < 0.001$), which also showed significant interaction (Supplementary (Suppl.) Table S7). Although calculations on theoretical CH₄ 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 (20-25) (Yen and Brune, 2007) and cessation of actual CH₄ production towards the end of the AD period (Fig. 4) could be due to very low C/N ratios (Table 1). The typical C/N ratio of the mix was 6/1, which is too low for effective anaerobic digestion. Low C/N ratios can lead to accumulation of total ammonia nitrogen (TAN) which might inhibit the process. For these reasons, accumulation of volatile fatty acids (VFAs) was expected (Obulisamy et al., 2016;

Karthikeyan et al., 2013). These two factors also decreased methanogen activity and accumulation can completely inhibit anaerobic digestion (Yen and Brune, 2007). This could be avoided by co-digestion with materials with a high carbon content, which will aid to alleviate high ammonium accumulation (Yen and Brune, 2007)

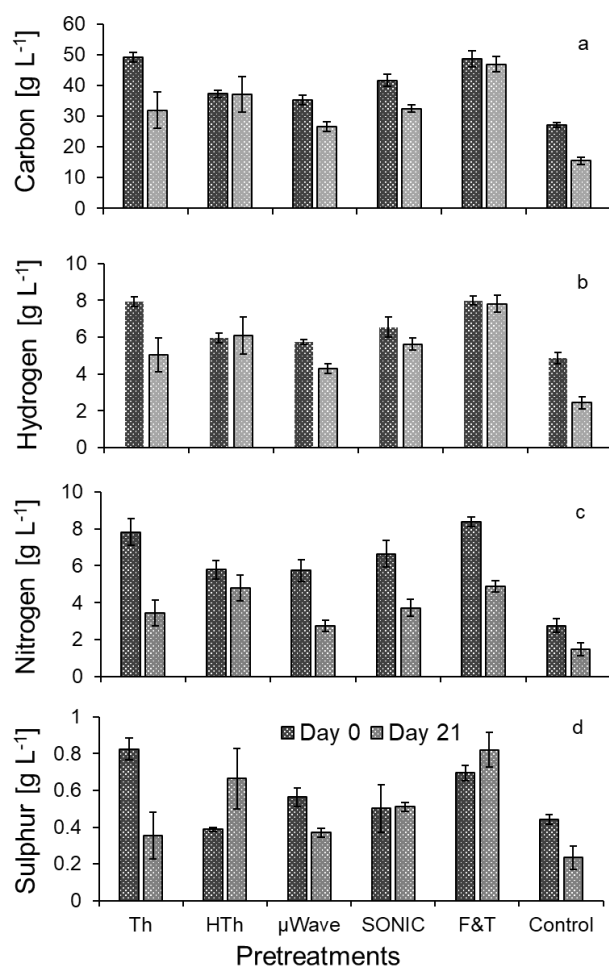


Fig. 2. Elemental analysis of hydrolysates of *Tolypothrix* sp. biomass and activated sludge baseline (control) on day 0 and 21 days after anaerobic digestion. (a) Carbon, (b) Hydrogen, (c) Nitrogen and (d) Sulphur.

Table 1. Physio- and biochemical characteristics of anaerobically digested hydrolysates of biomass of the nitrogen fixing cyanobacterium *Tolypothrix* sp.

Parameters	Time	Thermal	Hydrothermal	Microwave	Sonication	Freeze & Thaw	Sludge Baseline (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 ^a [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 ^a [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
SMP [L g ⁻¹ VS _{added}]	Day 21	0.012	0.009	0.002	0.010	0.003	-
CH ₄ ^b [mL g ⁻¹ VS _{removed}]	Day 21	61.75	53.3	17.77	53.73	15.64	-
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	-
Total VS [g]	Day 0	5.30 ± 0.11	5.86 ± 0.25	7.46 ± 1.04	5.23 ± 0.95	4.56 ± 0.66	2.36 ± 0.20
Total VS [g]	Day 21	2.03 ± 0.30	1.58 ± 1.28	5.15 ± 1.34	1.99 ± 0.39	0.55 ± 0.10	1.85 ± 0.24
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
Soluble 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
S/I ration [g COD/g VS]	Day 0	2	2	1.9	4	3.5	-
ISR [g COD/g COD]	Day 0	1.77	1.72	2.02	0.94	1.07	-
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	1.0 0.09

348 ^aContribution of CH₄ produced by the activated sludge inoculum are part of the total CH₄ and biogas yields

349 ^bCH₄ production corrected for contribution of CH₄ produced by the activated sludge inoculum

350 SMP: specific methane production

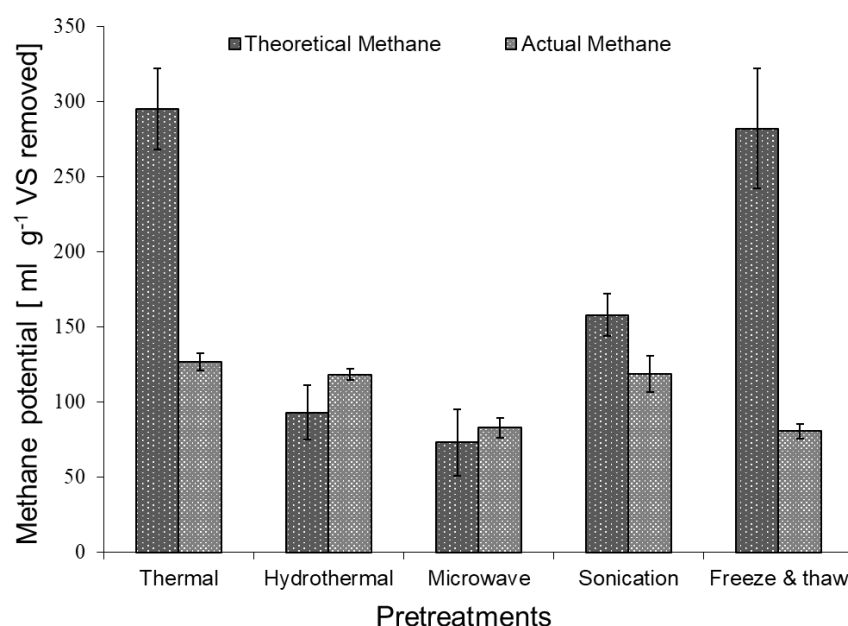


Fig. 3. Effect of pre-treatment condition of *Tolypothrix* sp. biomass on theoretical methane versus actual methane yields.

Methane potential of Tolypothrix sp.

Biogas yields ceased within 20 days and pre-treatment condition affected maximal biogas and CH₄ production (Figs 4(a) and 4(b)), with differences in S/I ratios and ISRs being contributing factors (Table 1). One-way ANOVAs determined a significant effect of pre-treatment on cumulative biogas ($p < 0.001$) and methane ($p < 0.001$) production (Suppl. Tables S1 and S3). 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 μ WAVE, F&T and activated sludge baseline (control) which were similar to each other (Suppl. Table S2) for reasons discussed previously. In contrast, for CH₄ production, HTh and SONIC-pre-treatments and μ WAVE and F&T were not significantly different to each other, but Th and activated sludge baseline (control) were different to each other and the other treatments (Suppl. Table S4). Among the different pre-treatments, Th pre-treatment resulted in maximal actual total biogas production of ~ 195 mL g⁻¹ VS_{removed} and CH₄ ~ 126 mL g⁻¹ VS_{removed} (Table 1), similar to yields of Th pre-

treated *Arthrospira platensis* ($\sim 203 \text{ mL g}^{-1} \text{ VS}_{\text{removed}}$. (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 yielded lowest biogas and CH_4 yields (Fig 3, Table 1) (Kinnunen et al., 2014), which was still 2-fold higher than for the sludge baseline yield (control). In general, Th pre-treated *Tolypothrix* sp. resulted in slightly higher CH_4 yields than reported for mechanically macerated biomass of the cyanobacterium *Arthrospira (Spirulina) maxima* and for whole cells of the green microalga *Scenedesmus* sp. (Inglesby et al., 2015).

Except for HTh pre-treatments, CH_4 yields correlated with percent VS removal, which was significantly affected by pre-treatment with a maximum of $\sim 73\%$ in HTh (Table 1). A one-way ANOVA (Suppl. Table S5) determined a significant effect of pre-treatment on VS removal ($p < 0.001$). Despite very similar S/I ratios to Th and HTh pre-treatments (Table 1), lowest percent VS removal was achieved with μWAVE pre-treatments, potentially due to enhanced extraction of antimicrobial compounds, Th and SONIC pre-treatments showed similar percent VS removal of $\sim 61\%$, with similar results reported for Th-treated *Arthrospira (Spirulina) maxima* (Yuan et al., 2006; González-Fernández et al., 2012).

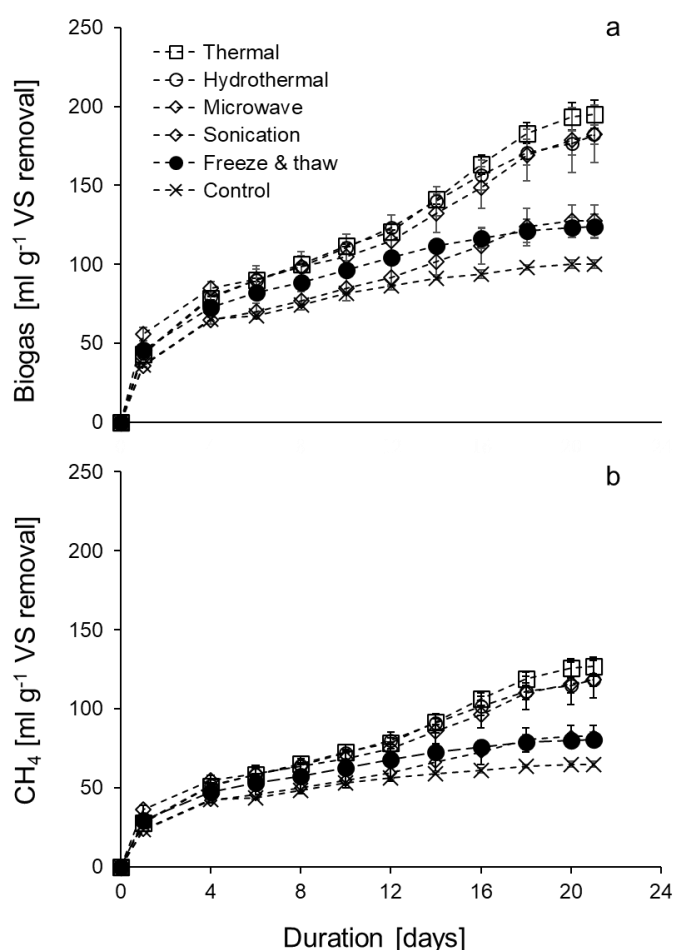


Fig. 4. Time-course of biogas and methane production of hydrolysates of *Tolypothrix* sp. biomass and activated sludge baseline (control).

In conclusion, pre-treatment methods in addition to S/I ratios and ISRs significantly affected CH₄ yields of *Tolypothrix* sp. in declining order of Th > HTh > SONIC > μ WAVE > F&T. Improved CH₄ 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; Patel et al., 2016; Ding et al., 2020). Actual biogas/CH₄ yields were ~55 and ~67% lower than theoretical CH₄ yields for individual pre-treatments (Fig. 3) for Th and F&T pre-treatments, respectively, which might be due to process inhibition and complex sugar formation (Chen et al., 2008). Increased mineralization of

nitrogen and phosphorous may also impede high CH₄ yields (Kinnunen et al., 2014). In contrast, actual CH₄ yields were only slightly lower for SONIC and marginally higher for HTh and μ WAVE (Fig. 4), which could be explained by the formation of more soluble simple sugars in these pre-treatments (Table 1). Although theoretical methane yields using the model BMP_{th}AtC are more accurate than COD-based estimates and provide the advantage of being applicable to different substrates, as well as yielding useful information on the elemental characteristics of the substrate, the model is limited by assuming that C, H, N, and O containing compounds are all suitable for bacterial metabolism, which is not always the case (Ali et al., 2018). If organic compounds cannot be consumed by the methanotrophs, the model overestimates theoretical methane yields. This might be the underpinning reason for the deviation of actual methane yields obtained for HTh pre-treatments and may contribute to observed differences for μ WAVE pretreatments as well, but the latter could also contain higher levels of antimicrobial compounds, which requires further investigation by hydrolysate characterization using mass spectrometry.

3.3. Characterization of pre- and post-digestion slurries

System pH: Near neutral pH of 6.8-7.3 is more suitable for CH₄ production, while more acidic or alkaline pH inhibit the process (Chen et al., 2008). The initial (day 0) pH of the digestion slurry for the different pre-treatment conditions were slightly acidic (6.1 – 6.3) and pH did not reach pH 6.8 for Th, HTh, μ WAVE and F&T pre-treatments, while SONIC pre-treatment exceeded the maximum range by 0.6 pH units after 21 days, being identical to baseline sludge production (control) (Table 1). The higher pH for the latter pre-treatment could be due to highest amounts of ammonia-N, recorded (Table 1), but ammonium content cannot be the sole explanation, as amounts were also high in F&T and μ WAVE pre-treatments, yet pH was 6.5 and 6.7, respectively (Table 1). A two-way ANOVA (Suppl.

Table S9) determined that pH was significantly affected by pre-treatment ($p=0.0023$) and cultivation period (days) ($p < 0.001$) (Suppl. Table S10).

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 mV and negative values were also recorded for Th and HTh pre-treatments, but positive initial ORPs were recorded for F&T, μ WAVE and SONIC pre-treatments (+126, +82, +32 mV, respectively) (Table 1). Final values observed on day 21 ranged from -155.7 (F&T) to -214 (μ WAVE) (Table 1). A two-way ANOVA determined a significant effect of pre-treatment ($p < 0.001$), cultivation period (days) ($p < 0.001$) on ORP, and pre-treatment showed significant interaction with days ($p < 0.001$) (Suppl. Table S11). A Tukey HSD analysis confirmed the significant difference between each treatment. (Suppl. Table S12). There is no consensus 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 μ WAVE pre-treatments, partially explaining lowest CH_4 yields 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/ hydrolysate ratios for all pre-treatments and intermittent buffer addition to allow for a more homogenous colonization of all organic particles by methanogens.

Electrical conductivity: Electrical conductivity varied between $138 \mu\text{S cm}^{-1}$ and $262 \mu\text{S cm}^{-1}$ for day 0 across pre-treatments, increasing considerably during the cultivation period for all pre-treatments (Table 1). This is common due to solubilization of more conductive ions (Schwede et al., 2013). A two-way ANOVA determined that there was no

significant effect of pre-treatment ($p = 0.525$) on conductivity, but cultivation period (days) ($p < 0.001$) and the interaction of pre-treatment*days ($p = 0.019$) were significant (Suppl. Table S13). 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. (Suppl. Table S14).

Soluble organics: Soluble COD [g L^{-1}] is a measure of total soluble organic carbon, which was high for SONIC and F&T pre-treatments ($13\text{--}15 \text{ g L}^{-1}$) and lowest for μ WAVE (7 g L^{-1}) on day 0 (Table 1). Soluble CODs increased 1.5-3 fold for all hydrolysates, which suggests effective solubilization of organic compounds (Obulisamy et al., 2016). Maximal soluble COD concentrations were measured for SONIC pre-treatment-digested samples ($\sim 24 \text{ g L}^{-1}$), followed by μ WAVE and F&T ($\sim 20 \text{ g L}^{-1}$), Th ($\sim 17 \text{ g L}^{-1}$) and HTh ($\sim 15 \text{ g L}^{-1}$) on day 21 (Table 1). High soluble COD 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).

Volatile fatty acids and ammonium-N: Initial volatile fatty acids (VFAs) contents were low (Table 1) and concentrations increased 3 to 5-fold. VFAs constituted 53-65% of soluble COD (Table 1) and are major intermediates formed during AD, and therefore followed the same trend as observed for soluble COD. A two-way ANOVA determined a significant effect of pre-treatment ($p < 0.001$) and cultivation period (days) ($p < 0.001$) on VFA, and a significant interaction of pre-treatment and days ($p < 0.001$) (Suppl. Table S17). A Tukey HSD analysis determined that on day 0 the significant difference was driven by SONIC pre-treatments and sludge baseline contents (control), being significantly different to all other treatments, which showed no significant difference amongst them. On day 21, the significant effect was driven by SONIC being significantly different to all pre-treatments (Suppl. Table S18). VFAs indicate the degree of acidification (Obulisamy et al.,

2016). Highest VFAs contents, with values similar to baseline sludge contents (control), were observed in SONIC ($\sim 16 \text{ g VFA L}^{-1}$), F&T ($\sim 12 \text{ g VFA L}^{-1}$) and μ WAVE ($\sim 11.18 \text{ g VFA L}^{-1}$), followed by Th ($\sim 9.57 \text{ g VFA L}^{-1}$) and HTh ($\sim 7.98 \text{ g VFA L}^{-1}$), 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. As the pH was high and remained near neutral in SONIC (~ 7.3) compared to other test conditions, the ammonia-N concentration could be buffering this system, as discussed earlier. However, further investigations are needed to understand why the biogas and methane yields were comparatively low in this test condition compared to the Th treatment.

Similarly, a Tukey HSD analysis showed that differences in initial (day 0) ammonium-N concentrations were not significant (Table 1, Suppl. Table S20), indicative that pre-treatments hydrolysed primarily carbohydrates, but a two-way ANOVA determined a significant effect of pre-treatment ($p < 0.001$), cultivation time (days) ($p < 0.001$) on ammonium-N, and pre-treatment showed significant interaction with days ($p < 0.001$) (Suppl. Table S19). A Tukey HSD analysis determined that significance was driven by Th pre-treatments and activated sludge baseline contents (control) on day 21, being different amongst themselves and to all other treatments. (Suppl. Table S20). 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 up to 2-fold higher in the 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. 2), but, paradoxically, ammonium-N concentrations increased (Table 1). High ammonium concentrations are typically the result of mineralization 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 $\sim 1.3 \text{ g N L}^{-1}$, 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 ($\sim 1 \text{ g N L}^{-1}$) concentrations (Table 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., 2008; Montingelli et al., 2015). 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).

Biochemical profiling: Biochemical characteristics of *Tolypothrix* sp. for carbohydrates, protein and fatty acid contents were described above, and fatty acid data after digestion are presented in Fig. 5, which includes contributions by methanogens and heterotrophic bacteria in addition to the hydrolysates. Initial carbohydrate concentrations were highest for SONIC pre-treatments ($\sim 9.5 \text{ g L}^{-1}$), followed by F&T (6.8 g L^{-1}) and were lowest for μWAVE ($\sim 3.2 \text{ g L}^{-1}$) (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 utilization by methanogens and heterotrophic bacteria (Table 1). SONIC showed highest percent reduction of carbohydrate $\sim 53\%$, 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 solubilized by a heterotrophic bacteria-dominated microbial consortium,

which would also explain the low biogas/CH₄ yields achieved with these hydrolysates (Fig 4).

The fatty acid 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 solubilized the highest amount of total fatty acids (~88 mg g⁻¹ DW), whereas concentrations ranged between ~56 and 67 mg g⁻¹ DW for all other treatments (Fig. 5(a)). A two-way ANOVA determined that the effect of pre-treatment ($p = 0.800$), and cultivation time (days) ($p = 0.387$) on total FAME profile was not significant, however, pre-treatment*days showed significant interaction ($p = 0.015$) (Suppl. Table S21). A Tukey HSD analysis confirmed the non-significance of pre-treatment and cultivation time (Suppl. Table S22). Highest amounts of polyunsaturated fatty acids (PUFA) were solubilized in SONIC and μ WAVE pre-treatments, while amounts of saturated (SFA) and monounsaturated fatty acid (MUFA) contents were not affected by pre-treatment (Fig. 5(a)). The fatty acid profile of the activated sludge inoculum was characterized by a very high content of saturated fatty acids (SFA) and low MUFA and PUFA contents (Fig 5(a)). After 21 days of AD, total fatty acid concentrations were up to 41% lower for SONIC pretreatments, but only 0.5 to 13% for F&T and μ WAVE, while an increase of ~7 and 29% was observed for Th and HTh, respectively (Fig. 5(b)). The observed increase could represent contribution of a large population of methanogens and heterotrophs to the fatty acid profile, as the SFA profile also increased significantly (Fig. 5(b)), correlating with high CH₄ yields (Table 1). After 21 days of AD, all fatty acid profiles closely resembled the activated sludge profile, with a 2-fold increase of SFA being observed for all hydrolysates, irrespective of CH₄ yields, and MUFA and PUFA contents being reduced by 14 to 67% and 40 to 81%, respectively (Fig. 5(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 consortium, if a particular marker fatty acid could be determined.

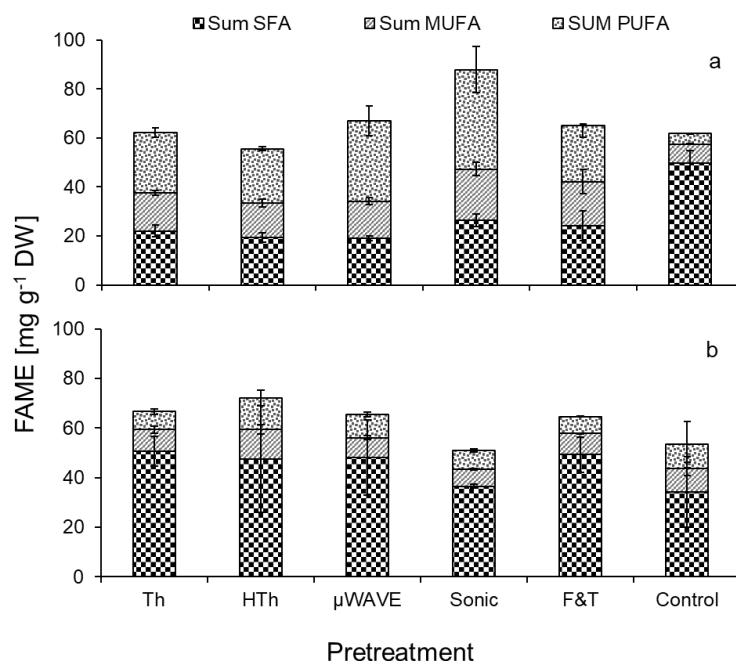


Fig. 5. Total saturated -, mono-unsaturated – and poly-unsaturated fatty acid (SFA, MUFA, and PUFA) contents of *Tolypothrix* sp. hydrolysates and activated sludge baseline contents (control) (a) and the hydrolysate/microbial consortia mix and activated sludge/microbial mix after 21 days (b).

4. Practical Application:

Tolypothrix sp., is a self-flocculating, nitrogen fixing cyanobacterium with excellent metal removal capacity from ash dam waste water (Velu et al., 2019, 2020). This research also determined that metal accumulation in the *Tolypothrix* sp. biomass does not exceed toxic levels for wheat cultivation when applied as a biofertilizer (Velu et al., 2020), potentially increasing product value when sold as a mineral and N/P fertilizer. Net present value analyses, however, determined that production of AD-generated energy and biofertilizer as sole products (sold at conventional prices) do not generate sufficient economic benefits for implementation at commercial scales (Velu et al., 2020). Economic

incentives could be greatly enhanced by either applying a charge for environmental services rendered (CO₂ and water remediation) and/or producing a biofertilizer that can be marketed as a mineral/N/P and simultaneous soil conditioner by promoting establishment and maintenance of beneficial soil microbial consortia. To bring commercialization a step closer to reality, this research explored the efficacy of pre-treatment strategies to recover CH₄ from *Tolypothrix* sp. for the purpose of offsetting energy requirements in the production process of the biomass. Due to the locality of the production site (at a coal-fired power station in Queensland, Australia), a non-optimised inoculum from a local WWTP was used. In addition, a lower than recommended ISR was used, as complete valorization would be counter-productive to the realization of a soil-conditioner as a higher value product. This should promote marketability of the resulting sludge as a biofertilizer and for supporting soil microbiomes required for healthy soils. Ash dam wastewater-derived metals could also interfere with efficient AD, but the data suggest that, in addition low TS content of the inoculum and differences in S/I ratios and ISRs, applied pre-treatments also significantly impacted on achieved CH₄ yields, presumably through more efficient extraction of bioactives with antimicrobial activities for μ WAVE- or destruction of antimicrobials in SONIC-intensified pretreatments. These possibilities need to be investigated via mass spectrometric compound profiling before system and parameter optimization can take place to improve CH₄ yields. If desired, VFAs present in the digested effluent could also be recovered and burned as an energy source, but the sludge would be a much higher product value if sold as a soil conditioner offering simultaneous mineral/N/P fertilisation. This applicability of this in practice and at scale will need to be further tested across seasons before sufficiently validated data are available for accurate techno-economic and life cycle analyses. In summary, development of an integrated process of ash dam wastewater treatment followed by biomass digestion to produce CH₄ to offset

energy costs of production, VFAs (soil conditioner) and mineral/N/P-fertilizer could help to reduce the environmental footprint of polluting industries, but techno-economic and life-cycle analysis and detailed field trials are also needed prior to implementation.

5. CONCLUSION

The growing population requires food security and energy demands of new industries should be met by alternative and renewable sources of energy. Solutions should ideally be coupled with wastewater and greenhouse gas remediation to improve sustainability.

Tolypothrix sp. is a promising source for cultivation, with a range of product potential such as usage of the sludge as a mineral/N/P soil conditioner and the potential use of AD-generated energy to offset energy requirements of the biomass production process. Due to its ability to use atmospheric nitrogen as a sole N-source for growth, production is likely more cost-effective compared to eukaryotic microalgae and non-nitrogen fixing cyanobacteria. Achieved SMPs ($0.012 \text{ L g}^{-1} \text{ VS}_{\text{added}}$) with Th pre-treatments were very low, due to a low C/N ratio 6.32, high VFA 9.5 g L^{-1} and ammonium-N 0.08 g N L^{-1} contents, sub-optimal pH 6.21 and ORP -44.33 mV , and sub-optimal ISRs and/or low TS content of the inoculum, but pre-treatments also impacted independently (μ WAVE) or contributed (F&T) to low methane production. Discrepancies between actual and theoretical CH_4 yields, reported here and in the literature, highlight that complex interactions of the process are not adequately captured by elemental composition alone.

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CREDIT AUTHOR STATEMENT

Chinnathambi Velu conducted the experiment, constructed the figures and wrote drafts of the manuscript

Obulisamy P. Karthikeyan designed the experiment, co-supervised the research and provided extensive feedback on initial manuscript drafts and contributed to the revision of the manuscript.

Samuel Cires co-supervised the research and provided critical comments on drafts of the manuscript.

Diane Brinkman conducted the FAME and alkane/alkene analyses

Kirsten Heimann co-designed the experiments, supervised the research, contributed to data analyses, provided extensive critical feedback on the manuscript content, led the revision of the manuscript and successfully obtained funding from the Advanced Manufacturing Cooperative Research Centre to support this project.

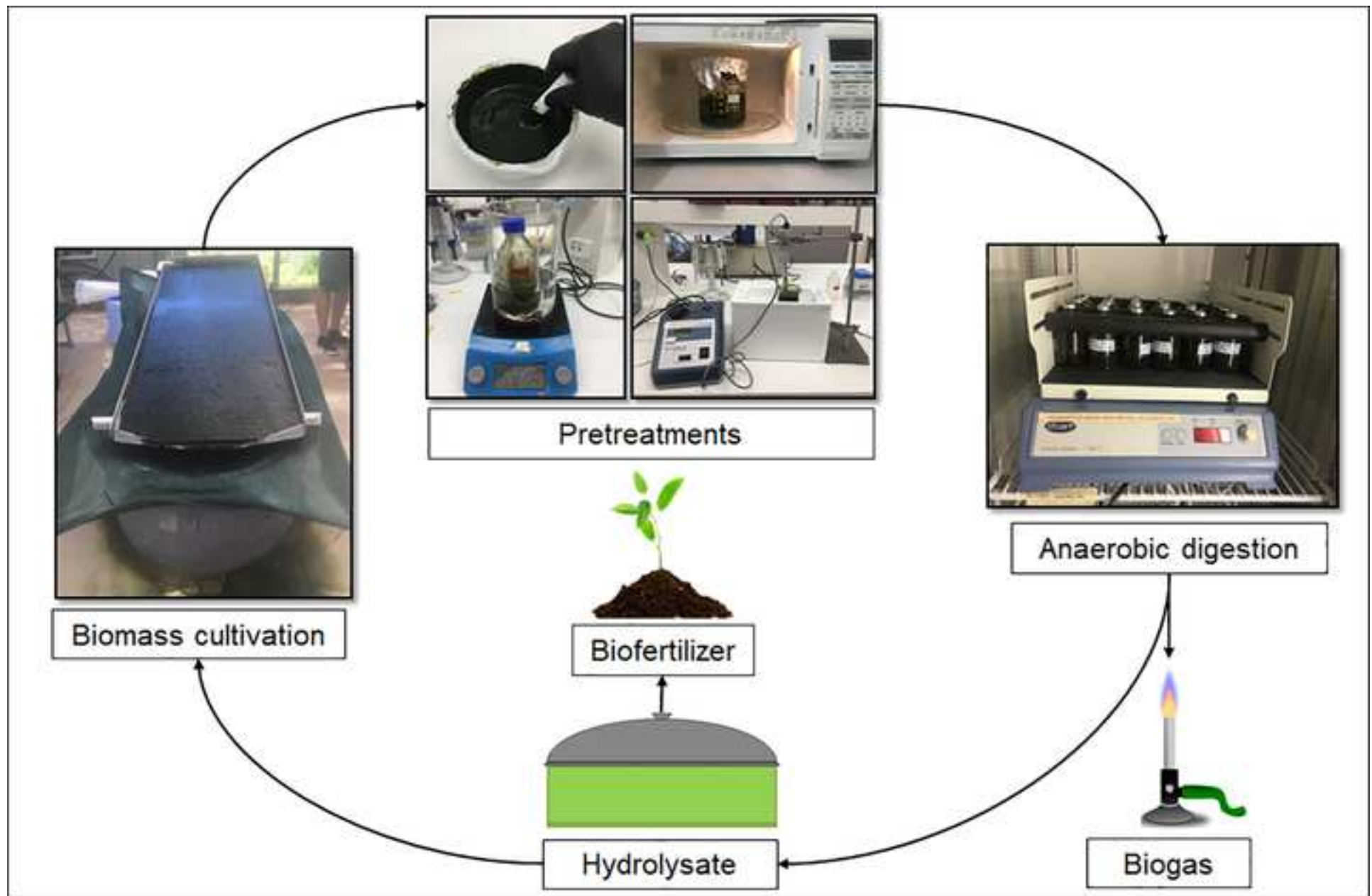


Figure 2

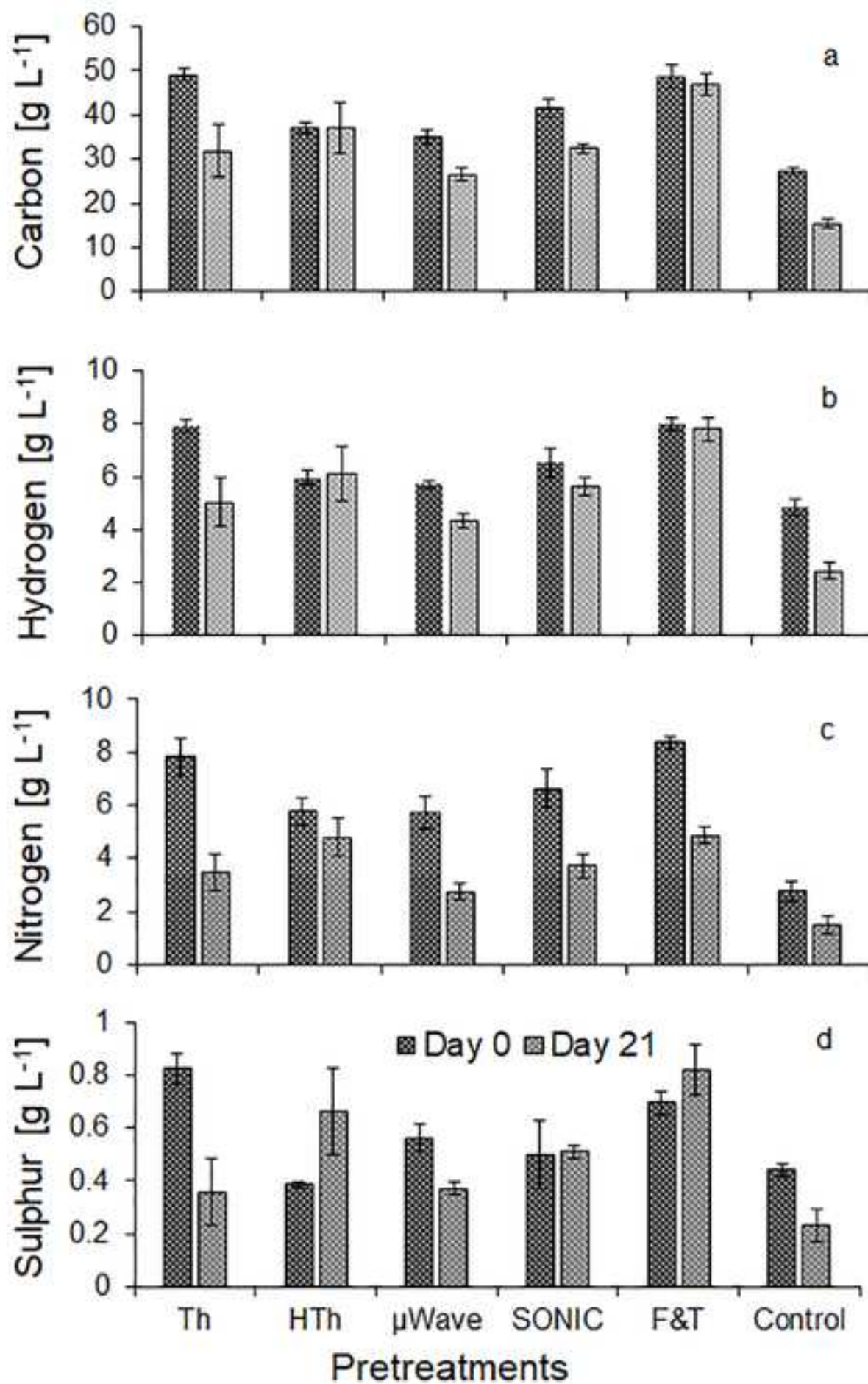
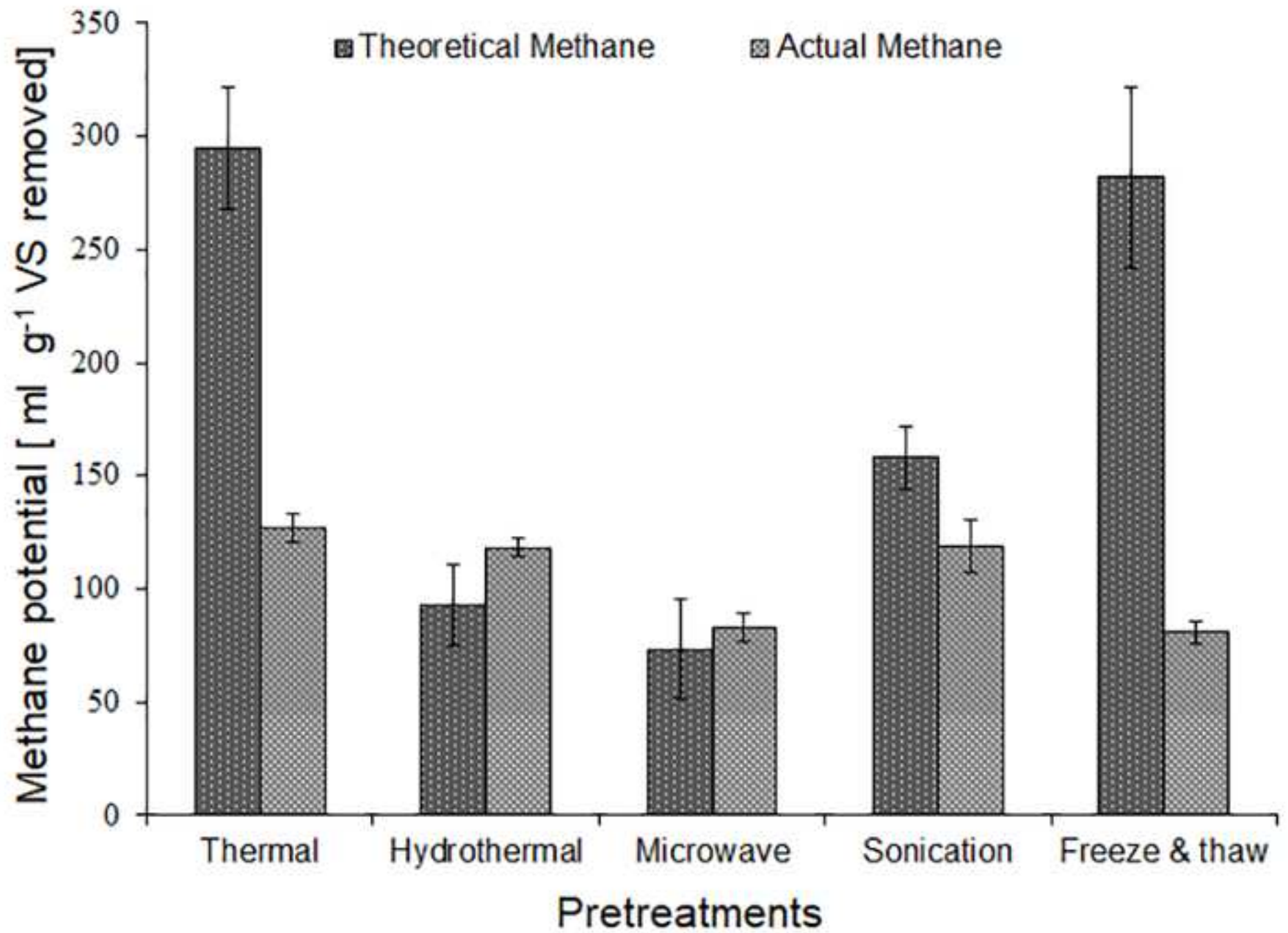
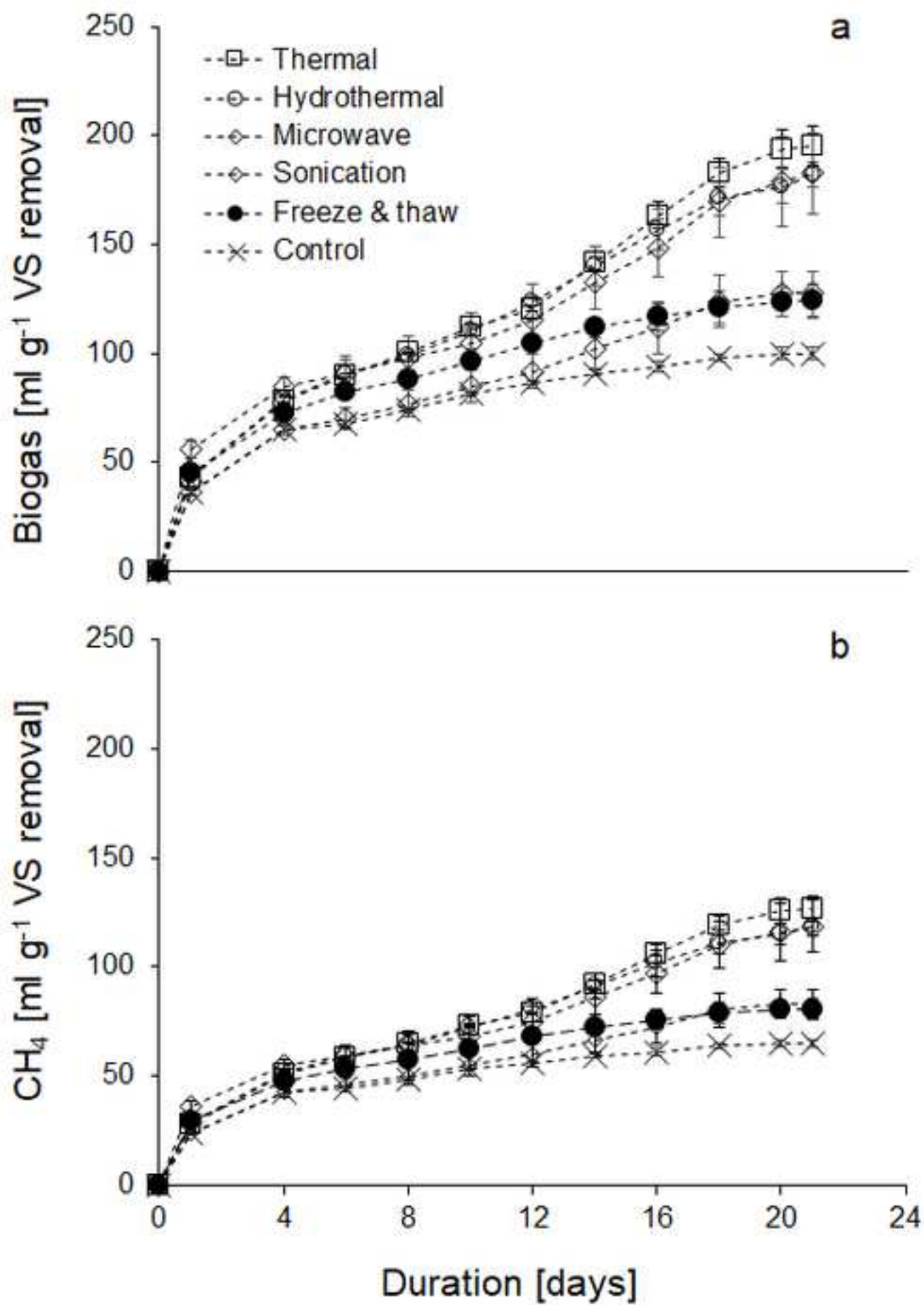
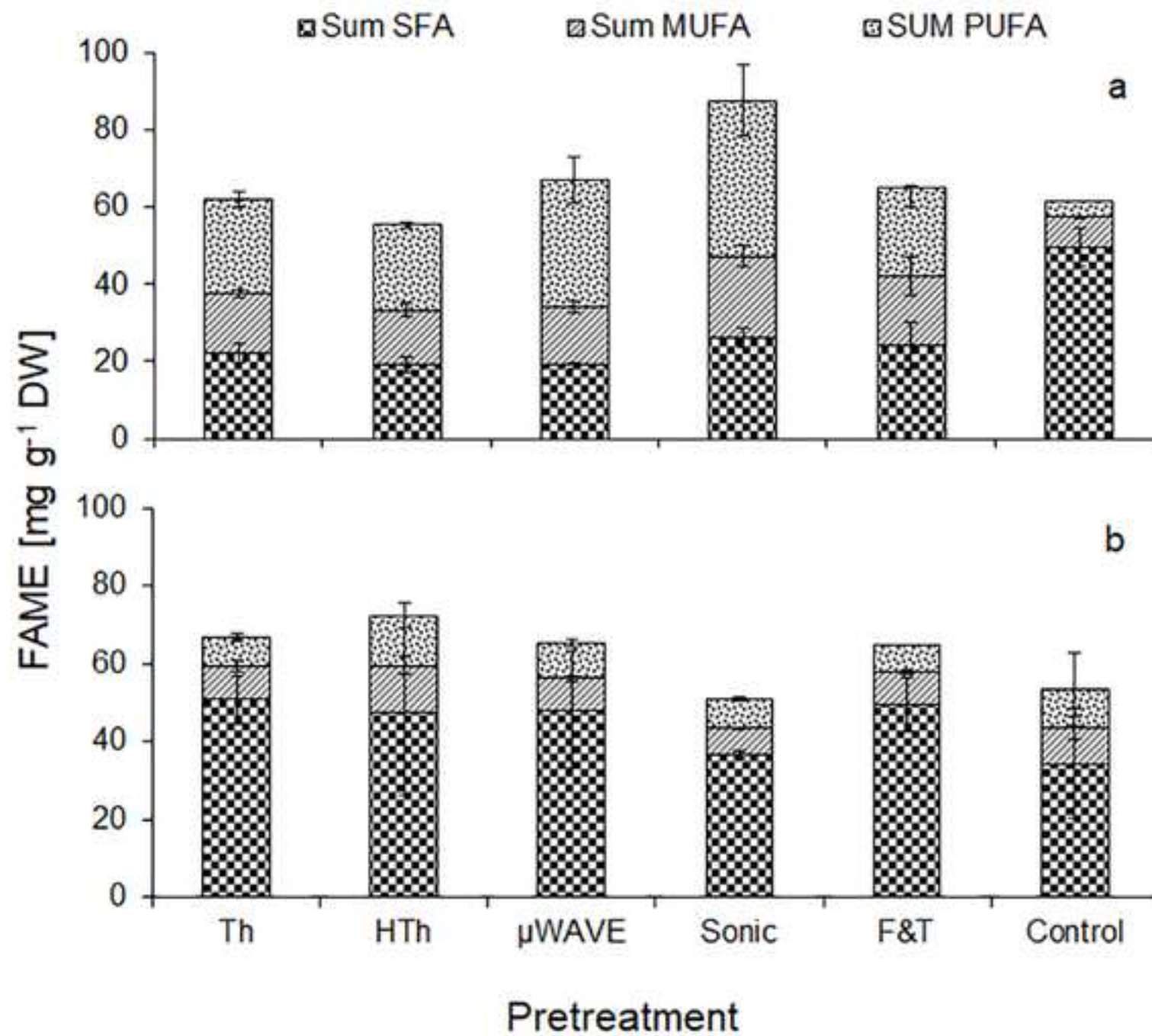
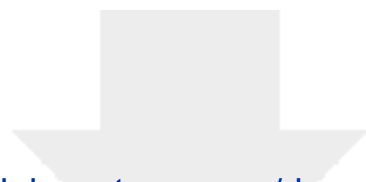


Figure 3









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Supplementary Material

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