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Research Article

Biopolymers made from methane in bioreactors

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Abbreviations: **GHG,** greenhouse gas; **CH_4,** methane; **CO_2,** carbon dioxide; **PHB**, polyhydroxybutyrate; **PHA,** polyhydroxyalkanoate; **MOC**, methane oxidation capacity; **LB**, landfill biomass; **CB**, compost biomass; **CSTRs,** continuously stirred tank reactors; **FAMEs**, fatty acid methyl esters; **pMMO,** particulate methane monooxygenase; **sMMO,** soluble methane monooxygenase; **DW,** dry weight; **OD,** optical density; **NMS,** nitrate mineral salt; **DNMS,** diluted nitrate mineral salt; **GC,** gas chromatography; **TCD**, thermal conductivity detector; **FID**, flame ionization detector;

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Practical application

A feasibility study for the production of bio-polymers (mainly polyhydroxybutyrate-PHB) from CH_4 in bioreactors was conducted. The project targeted major CH_4-emitting industries such as landfills, coal mine and anaerobic digestion units where CH_4 is collected-flared/simply oxidized. The PHB-accumulating, bacterial methanotroph consortia were enriched independently from landfill top-cover and compost soils and tested in continuously operating bioreactors. The project is expected to provide a common solution for three different environmental issues upon successful implementation/ demonstration, i.e. (i) a potential carbon sequestration method to reduce GHGs emissions; (ii) bio-polymers production from CH_4 for the potential reduction of usage of conventional plastic and fossil resources; and (iii) reduced usage of organic carbon sources for bio-polymer production.

Abstract

Methane (CH_4) is a potent greenhouse gas (GHG) and mitigation is important to reduce the global warming impacts. In this study, we aimed to convert CH_4 to polyhydroxybutyrate (PHB; a biopolymer) by enrichment of methanotrophic consortia in bioreactors. Two different methanotrophic consortia were established form landfill top-cover (landfill biomass [LB]) and compost soils (compost biomass [CB]), through cultivation under CH_4:CO_2:air (30:10:60) in batch systems. The established cultures were then used as inoculi (0.5 g LB or CB.L^{-1}) in continuous stirred tank reactors aerated with CH_4:CO_2:air at 0.25 L.min^{-1}. Under stable CSTRs operating conditions, the effect of spiking with 1:1 copper:iron (final concentrations of 5μM) was tested. Methane oxidation capacity (MOC), biomass dry-weight ($DW_{biomass}$), PHB and fatty acid methyl esters (FAMEs) contents were used as effect parameters. A maximum MOC of 481.9±8.9 and 279.6±11.3 mg CH_4.g^{-1} $DW_{biomass}$.h^{-1} was recorded in LB-CSTR and CB-CSTR, respectively, but PHB production was similar for both systems i.e., 37.7 mg.g^{-1} $DW_{biomass}$. Treatment with copper and iron improved PHB production (22.5 % of $DW_{biomass}$) in LB-CSTR, but a reduction of 13.6 % was observed in CB-CSTR. The results indicated that CH_4 to PHB conversion is feasible using LB-CSTRs and addition of copper and iron is beneficial.

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1 Introduction

Polyhydroxybutyrate (PHB) is a biopolymer and was the first characterized monomer of the polyhydroxyalkaonate (PHA) members [1-4]. It is a short chain length biopolymer containing four carbon atoms and a methyl-R functional group [3, 5, 6], with physical properties very similar to conventional plastics but completely biodegradable and biocompatible [2, 4, 7]. Therefore, it is widely used in industries for the production of bioplastics, biofuels and fine chemicals and in medical fields as implant materials and for drug deliveries, such as antibiotics [2, 3, 5, 6, 8-11].

PHB/PHA monomers are accumulated in number of bacteria, microalgae, cyanobacteria and yeasts derived from variety of carbon sources such as simple sugars, fatty acids and plant oils [1, 12-14]. However, the availability of carbon sources as feed-stock and provisions for the PHB/PHA producing microbes are limiting and cost-prohibitive for industrial mass production, i.e. 30 to 50% of the production cost is attributed to feed-stocks [15]. Therefore, recent research studies explored a number of unexploited carbon-based wasted resources, such as organic-rich domestic/industrial wastewaters, hydrolyzed solid wastes and industrial gases including methane (CH_4) and carbon-di-oxide (CO_2), as potential feed-stocks [16-25].

CH_4 is a prevailing greenhouse gas with global warming potential of 25 times higher than that of CO_2; contributing to 18 % (i.e. 0.509 $W.m^{-2}$) of the total atmospheric radiative forcing; and has an extended life span of 7 to 12 years in the atmosphere [7, 26-31]. According to a report by the Global Methane Initiative [32], anthropogenic CH_4 emissions were projected to reach 7,904 MMT-CO_{2eq} by 2020, which is 15 % higher than recorded 2010 emissions (6,875 MMT-CO_{2eq}). Therefore, it is vital to mitigate CH_4 emissions to combat global climate change. Mitigation could potentially be coupled with PHB/PHA production using methanotrophic bacteria [7, 22, 24], which would provide an economic incentive under the right environmental legislations.

Methanotrophs are gram-negative proteobacteria that utilize CH_4 as a sole carbon source for their growth/metabolism and convert it to the less potent GHG CO_2 [7, 33-35]. Among the three different methanotroph types classified, type II species can assimilate atmospheric nitrogen and are most widely reported to accumulate PHB/PHA (under nutrient stress) with lower CO_2 emissions (~30-50 %) compared to type I or type X strains [7, 10, 18, 20, 25, 36-39]. In addition, fatty acids with a

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chain length of 18 carbons (C_{18}) were reported as signature fatty acids for type II methanotrophs, while C_{16} dominance is characteristic for type I [39-42]. Type II methanotrophs generally rely on particulate – (pMMO) and soluble methane-monoxygenase (sMMO) for converting CH_4 to methanol in the first step of oxidation, which is a critical, highly energy-intensive step in the process [7, 35]. These two enzymes contain copper and iron centres, respectively, and it should therefore be possible to up-regulated their expression/activity through adequate provision of these two trace elements [43-46].

In addition, there are number of other factors, i.e. macro nutrients, other trace metals and environmental conditions which regulate CH_4 to PHB/PHA conversion (as detailed in [7]). Very few type II methanotrphos have been tested for PHB/PHA accumulation efficiencies as pure cultures [18] and results are variable for different species. Moreover, in an industrial setting, mono-culture systems are subjected to failure/contamination negatively affecting predictability of performance and yields [8, 24, 39, 47]. A few recent studies applied lab-scale bioreactors/biofilters (i.e. continuously stirred tank reactors, bubble columns, pressure bioreactors and fluidized packed beds), enriched with mixed methanotrophic consortia and tested for CH_4 to PHB/PHA conversion efficiencies [20, 22, 24, 25, 39, 48-52]. PHB/PHA accumulation potential by mixed methanotrophic consortium achieved in bioreactors under different nutrient-deplete/-replete conditions is detailed in Table 1. These studies, suggest that mixed-methanotrohic consortia are beneficial, (i) as the co-inhabiting bacteria improves physiological growth conditions of methanotrophic bacteria through removal of toxic and overproduced metabolites (e.g. methanol); (ii) essential vitamins and growth supplements are supplied by the excretion of accompanying bacteria; (iii) consortia remains stable for long period even in non-sterile conditions and (iv) higher PHB/PHA accumulations were achieved [8, 39, 47, 51]. However, there are number of unknowns and conflicting information exist in this budding research as detailed in our recent publication [7].

The present study aimed to enrich and test two different cultured methanotrophic consortia from two different soil sources (compost and landfill cover soil) to evaluate CH_4 to PHB/PHA conversion efficiencies in bioreactors. Both, compost and landfill top cover soils are reported to harbour active methanotrophic communities [53-58] and were therefore used in this study. First, cultures were

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enriched for both type I and II methanotrophs under nutrient-sufficient conditions with 20-25% CH_4 atmosphere. To enrich the mixed consortia for type II methanotrophs and to reduce the cost of cultivation and CH_4 to PHB/PHA production, nitrate was sequentially limited once every 5 days over 20 days, as type II methanotrophs have been reported to fix atmospheric nitrogen, and high CH_4 concentrations (30%) were used. The enriched cultures were used in bioreactors for continuous PHB/PHA production under nutrient limited conditions and continuous flow of CH_4 supplied at 0.25 $L.min^{-1}$. Cultures were then subjected to conditions that activate pMMO and/or sMMO activity (copper and iron (at 1:1 ratio; 5 µM final concentration) to improve CH_4 removal rates and PHB/PHA accumulation. In order to investigate the potential for an economic incentive for biological CH_4 remediation, the effect on PHB/PHA accumulation under stable operating conditions are discussed.

2 Materials and Methods

2.1 Preparation of methanotroph-enriched inoculi

Top cover soil of a 7 year-old landfill facility located in South Townsville, Queensland, Australia (lat. 19°15'0"S / long. 146°48'0"E), were auger drilled and samples were collected in an airtight pouch. Around 6 week-old compost was collected from a pile at McCahills landscaping supplies, Townsville. Slurries of 10 g soil with 200 mL of nitrate minimal salt medium (NMS [36]) were prepared in mini-bench top reactors (gas-wash bottle from Schott-Duran®, Germany) and purged with 20-25 % CH_4 using calibrated mass flow regulators in BioFlo®310 fermenter (New Brunswick, USA). The reactor bottles were incubated under 25°C and continuously stirred at 200 rpm by magnetic stirrer. Head space CH_4 was sampled after 24 h and concentrations were measured using gas chromatography equipped with thermal conductivity and flame ionization detectors (GC-TCD-FID, Varian-CP 3800, detailed in sub-section 2.4). Head space CH_4 was replenished every 24 h during the 20 days cultivation. CH_4 removal efficiencies ($[CH_{4in} - CH_{4out} / CH_{4in}]*100$) and biomass growth ($OD_{600}$; optical density at 600nm) were used for confirming activity of the mixed methanotroph consortia. After 20 days, a 10 mL inoculum was transferred into fresh NMS medium and cultivated under above growth conditions for further enrichment and the cycle was repeated for at least 4 sequential transfers to obtain healthy inoculi (these cultures are termed mother culture). Mother cultures were labelled as

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LB and CB to designate the soil type from which they were established from; LB: landfill top cover soil-derived biomass and CB: compost soil-derived biomass, respectively. Enriched methanotrohic communities from landfill and compost soil where characterized, by amplifying the V4 region of the 16S rDNA gene using the 515f and 806r primers (total read length ~ 300 bp) and sequenced using the Illumina Miseq platform (Karthikeyan et al. Chemosphere in review).

2.2 Nutrient stress and methane oxidation

After the above transfers and methanotroph enrichment cultivation of mother cultures, the LB and CB were subjected to nutrient stress. 50 mL of NMS medium was diluted with 50 mL of sterile de-ionized water every 5th day of the cultivation cycle to induce nutrient depletion until day 20th. For these cultures, the head space of the mini-bench-top reactors were purged with 30:10:60 of CH_4:CO_2:air every 24 h. This biomass was then used to seed cultures for continuous PHB/PHA production in bioreactors. All experiments were carried out in triplicate and samples were treated separately for analysis of dry weight ($DW_{biomass}$) and fatty acid methyl ester-FAMEs (detailed in sub-section 2.4). Negative controls without the inoculum were maintained to calculate the dissolution rate of gases in the NMS medium, which was found to be negligible.

2.3 Continuous PHB/PHA production from CH_4 in bioreactors

Two 15 L continuous stirred tank reactors (CSTR; BioFlo®310, New Brunswick, USA, Fig. 1) were used for PHB/PHA production from CH_4. 10 L diluted NMS medium (1:5 dilution with sterile de-ionised water (DNMS)) adjusted to pH 5.5 were inoculated with 0.5g $DW_{biomass}$ of methanotroph consortia cultures of LB and CB. Cultures were continuously aerated with CH_4:CO_2:air of 30:10:60 at 0.25 $L.min^{-1}$ and the outlet of the CSTRs were connected to an on-line quadrupole mass spectrometer (Cirrus-2, MKS instruments, Singapore), calibrated before the start of with the experiments using pure gases and mixtures.

Cultures in the CSTRs were grown at a constant temperature of 25 $^{\circ}$C and agitation of 200 rpm for 10 days. CH_4 removal was calculated ([CH_{4in} - CH_{4out} /CH_{4in}]*100) in percentile and biomass growth (OD_{600}) was measured every hour by the inline monitoring system. After 5 days, 2 L of cultures were harvested for PHB/PHA extraction (detailed in sub-section 2.4) and replenished with fresh DNMS followed by spiking with 1:1 copper:iron ratio (5 µM final concentration). The CSTRs were

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monitored for the next 5 days for CH_4 removal and final metal concentrations were measured at the end using inductively coupled plasma - optical emission spectroscopy (ICP-AES, Varian Liberty Series II; detailed in sub-section 2.4). Culture sub-samples taken on day 1, 5 and 10 from CSTRs were stored (-80°C) and analysed for $DW_{biomass}$, protein and lipid contents, as well as FAMEs profiles (detailed in sub-section 2.4) for sub-samples collected.

2.4 Analytical methods

2.4.1 Physico-chemical characterization of soil and biomass

Triplicate soil samples were characterized for total and volatile solids, pH (WP-81, German), carbon (total organic carbon analyzer, LECO, Germany) and nitrogen (APHA, [59]). The $DW_{biomass}$ were analyzed gravimetrically for both batches - (on days 1, 5, 10 and 15) and CSTR cultures (on days 1, 5, and 10). Biomass growth was monitored spectrophotometrically every day by measuring OD_{600} of the cultures (Enspire – 2300, PerkinElmer). Biomass protein was measured every two days using the Lowry method (TP0300, Sigma Aldrich).

2.4.2 Gas chromatographic (GC) analysis of CH_4

CH_4 was measured using a GC-TCD-FID fitted with a fused silica column (BR-Q PLOT; 30 m x 0.53 mm x 20 µm (Bruker Pty., Ltd., Australia) and helium at a flow rate of 1mL.min^{-1} was used as the carrier and make up gas . Column temperature was programmed 50 °C for 1 minute, followed by ramping to 250 °C at a rate of 20 °C.min^{-1} every five minutes. Injector temperature was set to 200 °C with a split ratio of 15. The GC was calibrated using standard CH_4 gas (10-50%) and a regression factor was calculated. The sample gas volume was 1000 µL, which was injected by an auto-sampler (Bruker, Australia).

2.4.3 CH_4 Oxidation potential

LB and CB, CH_4 oxidation capacities of both batch - and CSTR cultures were calculated using equation1 (eq. 1):

$$MOC = \frac{dCH_4}{dt} \times \frac{V_{system} \times MM_{CH_4} \times 10}{V_{molar\ gas} \times DW_{soil\ biomass} \times 24} \quad (eq. 1)$$

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Where, MOC = CH_4 oxidation capacity (μg CH_4. $g_{soil}^{-1}.h^{-1}$);

$\frac{dCH_4}{dt}$ = slope of change in CH_4 concentration (vol. %) per day

V_{system} = Culture system gas volume (L)

MM_{CH_4} = Molar Mass of CH_4 = 16 $g.mol^{-1}$

$V_{molar\ gas}$ = molar gas volume at given temperature (L)

$DW_{biomass}$ = dry weight of soil (g)

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2.4.4 PHB/PHA and FAMEs extraction and quantification

Sub-samples of 100 mL biomasses were aseptically collected and centrifuged (5810 R, Eppendorf AG, Germany) at 3220 × g for 20 mins at 24 °C. The supernatants were discarded and the biomass pellets were frozen at -80 °C and freeze-dried for PHB/PHA extraction (as detailed in [60]). In brief, 10 mg of freeze-dried biomass was weighed into clean screw cap extraction vials and extracted with 2 mL of acidified methanol (3% v/v of sulphuric acid) containing 1 $g.L^{-1}$ of benzoic acid and 2 $mL.L^{-1}$ of chloroform. The vials were agitated gently and extracted at 100 °C for 3.5 h. After cooling at room temperature, 1 mL of triple-distilled deionized water was added to each vial. The vials were vortexed for 30-60 s and allowed to stand for phase separation. The organic phase was collected (100 μL) and filtered (through 0.2 μm, PTFE membrane, Agilent) for gas chromatography-mass spectrometry (GC-MS, Agilent 7890). For trans-esterification of fatty acids, biomass was solvent-extracted as above and transesterified following von Alvensleben [61].

The GC-MS (Agilent 7890GC – 5975MS, Australia Pty Ltd.) was fitted with a DB-23 capillary column (0.15 μm cyanopropyl stationary phase, inner diameter - 60 m × 0.25 mm ID) and equipped with a flame ionisation detector (FID). The split ratio was 1/50 and helium was used as the carrier gas. Injector, FID inlet and GC column temperatures were programmed following David, Sandra [62]. For PHB/PHA analysis (Figure S1), a standard curve was prepared (range 0.1 - 6 mg) using pure PHB standards (Sigma-Aldrich, Australia). Fatty acids were quantified by comparison of peak areas of authentic standards (Sigma Aldrich, Australia). Benzoic acid and C19:0 was used as the internal standards for PHB/PHA and FAME analyses to correct for recovery and results were expressed in $mg.g^{-1}$ $DW_{biomass}$.

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2.4.5 Metal analysis using ICP-AES

The samples were pre-filtered using 0.2 μM PTFE membrane filter (Agilent) and diluted 10-fold with deionized water. The samples were acidified (2% nitric acid final concentration) and used in ICP-AES. 50 ppb concentration of high purity mixed standard (ICP-MSCS-M, Choice Analytical, Australia) were used for point calibration of the instrument.

2.5 Gas bottles and reagents

Gasses for calibrations (99.9% pure CH_4, 10-50 % CH_4 with air, CO_2 1-30 %) and compressed air (N_2-78.08 %; O_2-20.94 %) were supplied by BOC a member of the Linde group, Townsville and all were ISO certified. All chemicals and solvents were obtained from Sigma-Aldrich, Australia.

3 Results and Discussion

3.1 Physico-chemical characteristics of soil

pH of the landfill top-cover and compost soil samples were circum-neutral (7.4-7.8) with a similar moisture content of 16±0.9 and 18±0.2 %, respectively. Compost soil had a higher percentage of volatile solids and corresponding carbon of 41±2.6 % and 25.8±0.7 % compared to 5.7±0.2 % and 20±0.6 % of landfill top cover soil, respectively. Total nitrogen was also higher in compost soil than in landfill top-cover soil (10.60±0.28 *vs* 5.97±0.12 mg $N.g^{-1}$ soil, respectively). High volatile total solids, carbon and nitrogen indicates that the compost soil was not completely stabilized [63].

3.2 Methane oxidation capacities of soil and methanotrophic-enriched consortia

CH_4 removal efficiencies of both soil slurries reached a maximum after 24 h of incubation fluctuating between 40 and 70 % for the 20 day-enrichment period (data not shown). Similar CH_4 removal efficiencies have been reported for other soil types, where CH_4 removal efficiencies improved after an initial lag phase of 48 h [64, 65]. At the end of 20 day-enrichment period, the average (avg.) CH_4 removal efficiency for landfill top-cover soil was ≈ 60±1.2 % being slightly higher than for compost soil (i.e., ≈ 55±0.8 %). The average MOCs were also similar for the methanotroph-enriched consortia (i.e., 194.54±17 and 175.35±17 μg $CH_4.g_{soil\ type}^{-1}.h^{-1}$, respectively) and stabilized over the 20 days. Achieved MOCs reported here was higher than published values, which had a minimum of < 20 μg $CH_4.g_{sandy\ loam\ soil}^{-1}.h^{-1}$ for sandy loam soils and a maximum of 128 μg $CH_4.g_{bio\text{-}waste\ compost\ soil}^{-1}.\ h^{-1}$ (maximum) for organic-rich soil types [66-68]. The improved removal efficiencies and stable MOCs

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in this study could be due to maintenance of optimal temperature for methanotrophs (25 °C) [43, 64, 69] and/or improved CH_4 gas exchange due to continuous mixing of slurries [70]. Our result suggest that healthy methanotroph-enriched consortia established in mini-bench top reactors from the two different soil types at the end of the 20 day-enrichment period.

Next generation sequencing of enriched methanotrophic consortia from LB and CB showed similar community profiles. *Methylomicrobium (*96%) was dominant in LB and a presence of *Methylomarinum (2%)*, *Methylomonas* (1%), *Methylobacter (*<1%*), Methylosinus (*<1%*)* and *Methylosarcina* (<1%) at much lower abundances were recorded. *Methylomicrobium* (88%) were also dominant in CB, and low abundances of *Methylomonas* (9%), *Methylomarinum (1%)*, *Methylosarcina (1%) and Methylosinus* (1%) were also demonstarted. Apart from methanotrophs, methylotrophs such as *Methylophaga* and *Methylobacterium* along with heterotrophs (*Pseudomonas* and *Pseduoxanthomonas*) were present in high abundances in both LB and CB. Of the genera recorded *Methylosinus* sp., *Methylobacterium* sp*.,* and *Pseudomonas* sp*.,* has been shown to accumulate PHA/PHB [5, 18, 21, 25]. Enrichment of mother cultures under nutrient-deplete condition were expected to favour methanotrophic community shifts in LB and CB. FAME profiles (from Day 5 to Day 20; data not shown) changed to a dominance of C_{16} and C_{18}, indicative of community shifts changes to type II enrichment under nutrient-deplete conditions. However, the precise consortia composition requires further molecular characterization which is ongoing.

MOCs were 10-12 times higher in methanotroph-enriched cultures under nutrient-replete conditions than respective soil slurries (Fig. 2). MOCs of mother cultures further increased 2-2.5 times under nutrient-deplete conditions (Fig. 2). With regards to soil-specific methanotroph enrichment, CB had higher MOCs (2.10±0.48 and 5.76±0.54 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$ for mother cultures under nutrient-replete and - deplete conditions, respectively) than LB (1.83±0.46 and 3.85±0.24 mg $CH_4.g^{-1}DW_{biomass}.h^{-1}$). In line with published data, repeated sub-culturing under CH_4-rich conditions supported the selective growth of high-capacity, low-CH_4 affinity obligate methanotrophs [71, 72]. The observed differences in MOCs of LB and CB under nutrient-replete and -deplete conditions could be due to the differences in CH_4 concentrations (20 % *vs* 30 % CH_4, respectively) and/or

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community compositional shifts within the enriched consortia. Increasing CH_4 concentrations, as well as nutrient (e.g. nitrate)-induced community shifts have been reported to increase MOCs, the latter due to an increase in the ratio of type II/type I methanotrophs by 10-fold within 100 h [50, 71, 73, 74]. Changes in C_{16}:C_{18} ratios provide evidence of nutrient depletion- and CH_4 concentration-induced community shifts in LB and CB cultures. This was also observed by Bull et al. [41] and Helm et al. [39]. During the first 10 days, LB contained more of C_{16} than CB, indicating that the consortia were dominated by type I methanotrophs (Fig. 3). In contrast, CB contained C_{16} and C_{18} fatty acids (Fig. 3) indicating that both type I and type II methanotrophs were present in high abundances from the start of the enrichment period supporting the observed higher MOCs (Fig. 2).

3.3 PHB/PHA production in CSTRs

CH_4 removal efficiencies ranged from 3 to 21 % in both CSTRs under continuous operational mode. With addition of 5 μm of copper and iron on day 5, CH_4 removal peaked at 21 % on day 7 in both CSTRs. Although CH_4 removal efficiencies increased over time, no increase in biomass concentrations was recorded from day 5 to day 10 i.e., 0.40 to 0.43 g/L and 0.46 to 0.43 g/L measured in LB- and CB-CSTRs, respectively. Lower removal rates compared to other published data on mixed methanotrophic consortia could be due to high gas flow rates, which may have also affected biomass yields. Listenwnik [8] reported 25g/L biomass under non-sterile operation, however, operating conditions (high nutrients, pressurised bioreactors and 90% *Methylocystis* dominance) were chosen to favour biomass yields and CH_4 removal. In a natural setting, however, enriched consortia not derived from the soils present may not retain these set dominance profiles. As such, while natural consortia may not yield the same dominance profiles initially, they offer the opportunity to fine operational parameters to favour existing type II methanotrophs. We therefore believe that the data obtained in this study may provide more realistic performance data for unoptimised scale-up.

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3.3.1 MOCs and PHB/PHA production without copper and iron

Despite standardised initial biomass (0.5 g DW.10 L^{-1}; OD_{600} - 0.036 and 0.043; Day 1), the MOCs were higher in the LB-CSTR than in the CB-CSTR (260.09±25 and 165.35±15 mg $CH_4.g^{-1}DW_{biomass}.h^{-1}$, respectively) as were protein content (10.11±0.8 *vs* 7.38 ±0.4 µg.mL^{-1}) (Table 2). After 5 days of nutrient limitation, LB-CSTR and CB-CSTR biomass - (OD_{600} 0.27-0.28, respectively) and protein contents (100.8±2.2 and 112.3±1.8 µg protein.mL^{-1}, respectively) and increases in MOCs were similar (46 % *vs* 40 %, respectively) (Table 2). Yet, MOCs of LB-CSTR was almost 2-fold higher (481.95±22 *vs* 279.64±32 mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$) than for CB-CSTR, respectively (Table 2). Protein is reported to be a measure of active biomass in the system [75]. As the CB-CSTR contained more protein from day 1 and CB batch cultures showed better MOCs than LB systems, a higher MOC was expected for this system. The better MOCs of the LB-CSTR can be explained by a dominance of type I methanotrophs, as the ratio C_{16}:C_{18} (~11.88, Table 2) can be used as an indicator of type I/type II ratios within the methanotroph consortium. Reports that pMMO expressing cells (type I and type II) achieve steady state conditions and higher CH_4 removal rates (~30%) compared to sMMO expressing cells (type II) [43, 76] support our conclusion.

Detailed FAME profiles analyses for the LB-CSTR and CB-CSTR (Fig. 4a) also support the conclusion that the LB-CSTR was dominated by type I methanotrophs. Day 5-LB-CSTR was characterized by ~92 % of C_{16} methyl esters (67.73±3.1 mgFAME.g^{-1} $DW_{biomass}$; sum of $C_{16:0}$, $C_{16:1}$ cis-Δ^7, $C_{16:1}$ cis-Δ^9) and ~8 % of C_{18} esters ($C_{18:1}$ cis-Δ^9 and $C_{18:1}$ trans-Δ^{11}). In contrast, C_{16} and C_{18} contents were 78 % and 22 %, respectively in the CB-CSTR. For both consortia, $C_{16:1}$ cis-Δ^9 was the dominant FAME species, whereas $C_{18:1}$ cis-Δ^9 was not detected in biomass from LB-CSTR and the CB-CSTR biomass contained both $C_{18:1}$ cis-Δ^9 and $C_{18:1}$ trans-Δ^{11} (Fig. 4a).

Lower MOCs also resulted in lower PHB/PHA content for the CB-CSTR (Table 2). Total PHB/PHA content on day-5 was 37.03±2.3 and 25.28±1.4 mg PHB.g^{-1} $DW_{biomass}$ in LB-CSTR and CB-CSTR, respectively. Reported MOCs and PHB/PHA accumulation of mixed methanotrophic consortia were lower (~11%) than for pure cultures [25]. For example, pure cultures of the type II methanotroph *Methylocystis hirsute* had PHB/PHA contents ranging from 85 to 425 mg PHB.g^{-1} $DW_{biomass}$ (8.5 to 42.5 w%) in treatments with 20-80 % CH_4 in vertical loop bioreactors, with nitrogen limitation not

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leading to the expected improved accumulation (~135 mg PHB.g^{-1} $DW_{biomass}$) [20]. However Wendlandt [24] reported 51.3% PHB content in mixed methanotrophic consortia under nitrate-deplete condition. Apart from nitrate, Wendlandt [24] and Helm [22] studied the effect of phosphorus (P)-, potassium (K)-, sulfur (S)-, iron (Fe)- and magnesium (Mg)-limitation on PHB accumulation in a mixed consortium with dominance of *Methylocystis* sp. GB 25 DSM 7674 (86 to 90%) reared in 7 and 70 L pressure bioreactors under high pressure of methane ($p \leqslant 0.6$ MPa 20 to 25% CH_4) with a flow rate of 50-100 L.min^{-1}, and reported PHB contents of 46.8, 33.6, 32.6, 10.4 and 28.3% after 24 h of nutrient-deprivation, respectively. The high PHB content reported is likely due to dominance of *Methylocystis* species. Nitrate depletion should have resulted in high PHB contents, however, the low amount of PHB/PHA (3.7%) in our study could be due to the addition of CO_2 which increased the pH of the system at Day 5 (data not shown), favoring dominance of Type I methanotrophs which typically do not excel in PHB/PHA accumulation. Similar results were obtained by Lopez [23], when culture conditions were chosen to favour dominance of type I methanotrophs (PHB/PHA content of inoculum 12.6 % compared to biomass PHB/PHA content in biomass raised at 20 g CH_4 m^{-3} (0.002% CH_4 and nitrogen limiting conditions). In addition to strain-specific differences in PHB/PHA accumulation, cultivation system differences affecting gas residence time could also explain the higher PHB/PHA accumulation as vertical loop reactors, pressurised bioreactors, fluidised bioreactors dissolve CH_4 better than CSTRs [20, 24, 25, 50].

3.3.2 MOCs and PHB/PHA production with copper and iron

5 µM of copper and of iron was spiked on day-5 and monitored until day 10 (Table 2). Both copper and iron have been reported to regulate/improve pMMO and sMMO activities in methanotrophs thereby improving CH_4 removal [18, 22, 44, 46, 77-79]. As expected MOCs for the CB-CSTR increased by ~72 % following trace metal addition, while only a ~3 % increase was recorded for the LB-CSTR (Table 2). Differences in LB- and CB-CSTR biomass (OD_{600}) and protein content (Table 2) could explain the large increase in MOCs. However, the LB-CSTR had a slightly higher MOC compared to the CB-CSTR possibly due to community differences (based on C_{16}:C_{18} ratios, Table 2). Addition of copper has been reported to alter CH_4 affinity due to changes in species composition [43, 77]; 2-55-fold increases in MOC have been reported for pure and mixed cultures [43, 80]. For

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example, 1 µM of copper was reported to completely inhibit sMMO activity in pure isolates [81-83] and inducing pMMO expression [79]. Thus pMMO expressing methanotrophs show better MOCs and higher biomass yields [84]. As evident from the C_{16}:C_{18} ratio (Table 2), addition of 5 µM each of copper and iron did not shift the consortium composition of the CB-CSTR, thus the observed strong increase in MOC for the CB biomass could be interpreted as upregulation of pMMO. In contrast, copper and iron addition led to a strong shift in the C_{16}:C_{18} ratio of the LB-CSTR, i.e. the dominance of type I methanotrophs was abolished. These conclusions are supported by the shifts in FAME profiles in response to trace metal addition for both the CB- and LB-CSTRs (for details see below).

Previous studies have focused mainly on the individual effects of copper or iron [18, 22, 85]. Here, we investigated the combined effect of copper and iron under nitrate-deplete condition, the interactive effects of which may explain the shift in methanotroph type composition of the consortia. The 5-day copper uptake varied between LB-CSTR (36 %) and CB-CSTR (50 %), while the amount of iron uptake was the same for both cultures (~85 %) (data not shown). Iron, being a known cofactor for number of redox- reactions, plays a vital role in the regulation of cellular metabolism, and has been reported to increase sMMO activity [76, 81, 83, 86]. Based on this and the observed >2-fold increase in MOC in the CB-CSTR, we propose iron positively influenced sMMO activity (in parallel with copper influencing pMMO activity), and alleviated the inhibitory effect of copper on this enzyme.

sMMO activity requires high energy and reducing equivalents [87]. As CSTR cultures were grown under nutrient-limiting conditions, energy requirements to support MMO activities could have been met through oxidizing stored fatty acids and stored PHB/PHA [60, 79]. Pieja [18] also found that the addition of copper improved the PHB/PHA content by 25 % in pure cultures, which is comparable with our results for the LB-CSTR (23 %). In contrast, PHB/PHA content decreased by 13 % in the CB-CSTR, suggesting that PHB/PHA was oxidized to support MOC (Table 2), as has been reported for pure type II cultures [60, 79]. In contrast to CB-CSTR cultures, required reducing equivalents and energy needs for MOC were not fueled by PHB/PHA oxidation in the LB-CSTR. This is supported by a study where the addition of 30 µM copper increased PHB/PHA accumulation by 8.3 % [79].

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Total FAME contents (∑FAMEs) were reduced by 62 % and 25% after addition of copper and iron for the LB- and CB-CSTRs, respectively, whilst MOCs were not affected (Table 2), supporting the conclusion that energy and redox equivalent demands for sustaining MOCs were met by hydrolyzing fatty acids in both systems and additionally by PHB/PHA oxidation in the CB-CSTR. This conclusion is further supported when comparing FAME profiles before (Fig. 4a) and after copper and iron addition (Fig. 4b). 5-days after trace metal addition (day 10), the biomass of both CSTRs contained less C_{16} fatty acids, whilst C_{18} contents were unaffected. Whilst $C_{18:1}$ trans-Δ^{11} was absent in biomass from both CSTRs after trace metal addition, $C_{18:1}$ cis-Δ^{9} was present in high amounts (Fig. 4b). The appearance of type II-characteristic C_{18}-fatty acids supports the conclusion that copper addition (5μM) led to type II dominance, which is in agreement with other published data [18].
This could also suggest that copper and iron addition led to type II methanotroph-derived MOCs in both CSTRs. But the above hypothesis must be tested in future work through molecular analysis of consortia profiles, as a few type I and X methanotrophs have also been reported to contain sMMO [82, 88-91].

4 Concluding remarks

MOCs of nutrient-replete, enriched mother cultures were found to be 10-12 folds higher than in the unenriched respective indigenous soils, i.e. landfill top-cover and compost. Nutrient limitation further improved the MOCs due to selective enrichment of fast growing methanotrophic consortia. These enriched cultures were robust and accumulated C_{16} and C_{18} as the major FAMEs which can be of interest for biofuel factories. MOCs were lower in batch cultures than in continuous operating systems for both the biomasses (LB and CB), suggesting that for techno-economic analyses data obtained from batch culture systems should not be used.
From the bioreactor studies, the LB-CSTR was more robust in terms of MOCs, ∑FAMEs and PHB/PHA accumulation than the CB-CSTR. Based on C_{16} and C_{18} contents, community differences between LB-CSTR and CB-CSTR could be the key to the differences in performance, however, detailed molecular analyses are required to unequivocally demonstrate this (work in progress). Further addition of copper and iron at 5 μM each increased MOCs 2-fold in the CB-CSTR, but the ∑FAMEs and PHB/PHA accumulation were reduced. These results can be explained in two ways: (i)

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iron improved sMMO activity potentially by alleviating copper inhibition; and/or (ii) the stored PHB/PHA and FAMEs were used for delivering reducing equivalents for sMMO to accelerate CH_4 oxidation. Nonetheless the outcome suggests that continuous production of PHB/PHA under nutrient-limiting and high copper conditions is not feasible using compost biomass. On the other hand, sustained MOC and the 23 % increase in PHB/PHA content of the LB-CSTR under nutrient-limiting and high copper conditions suggests that landfill top cover soil-derived methanotroph consortia are suitable for continuous PHA/PHB production from waste CH_4 gas. However, compared to published data, the achieved PHB/PHA contents were low possibly due to low CH_4 residence time and nutrient-limiting conditions maintained in CSTRs, suggesting that further research should aim to improve PHB/PHA accumulation potential through improved system – and fertilisation regime designs.

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Table 1. Comparison of PHB/PHA content accumulation in biomass with different reactors

Seed culture/consortia	CH_4 Concentration and flow rate	Reactor volume	Conditions	PHB (%)	Reference
Methane-utilizing mixed culture (dominant species *Methylocystis* sp. GB 25 DSM 7674, >86% by biomass)	20% CH_4, 50–100 L.min^{-1}	70 L (Pressure bioreactor)	Nitrogen depletion	51.3	[24]
			Phosphorus depletion	46.3	
			Magnesium depletion	28.3	
Methane-utilizing mixed culture (dominant species *Methylocystis* sp. GB 25 DSM 7674, >86% by biomass)	25% CH_4, 50–100 L.min^{-1}	70 L (Pressure bioreactor)	Sulphur depletion	32.6	[22]
			Potassium depletion	33.6	
			Iron depletion	10.4	
Methane-utilizing mixed culture (dominant species *Methylocystis* sp. GB 25 DSM 7674)	107 Kg Natural gas.h^{-1}	40 m^3 (deep-jet fermenter)	-	70%	[8]
Methylocystis parvus & *Methylosinus trichosporium* OB3b	9–10 mg.L^{-1},	15.2 L (Fluidised Bed Reactor)	N_2 as the N-source, and low influent DO (2.0 mg/L)	6–10	[50]
Mixed consortium	2 and 20 gCH_4 m^{-3} 400 mL.min^{-1}	500 mL (Jacketed stirred tank reactors)	Nitrogen	12.6 and 1	[23]
Mixed consortium (Type I and type II)	30% CH_4 250 mL.min^{-1}	10 L (Continuous Stirred Tank Reactor)	Copper and Iron addition and Nitrogen deficiency	4.7 (LB) 2.1 (CB)	This study

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Table 2. Performance of LB-CSTR and CB-CSTR with and without copper and iron

Parameters	Unit	LB-CSTR			CB-CSTR		
		Day 1	**Day 5***	**Day 10**	**Day 1**	**Day 5***	**Day 10**
MOCs	mg $CH_4.g^{-1}$ $DW_{biomass}.h^{-1}$	260.09±25	481.95±15	495.02±18	165.34±1515	279.64±32	480.27±20
Biomass protein	$\mu g.mL^{-1}$	7.35±0.4	100.81±2.2	138.74±2.1	10.11±0.8	112.3±1.8	159.19±3.3
Biomass growth	OD_{600}	0.036	0.285	0.316	0.04	0.27	0.41
∑FAME	mg $FAME.g^{-1}$ $DW_{biomass}$	-	78±5.3	30±2.2	-	65±3.3	50±3.1
ratio of $C16:C18_{FAME}$	-	-	11.88	3.58	-	2.98	2.26
PHB/PHA content	$mg.g^{-1}$ $DW_{biomass}$	-	37.03±2.3	47.88±3.3	-	25.28±1.4	21.84±3.3

Note: MOC - Methane Oxidation capacity, FAME- Fatty acid methyl esters, PHB/PHA - Polyhydroxybutyrate/Polyhydroxyalkanoate. *5 µM of copper and iron (at 1:1 ratio) was spiked on day-5 and FAME profile, PHB/PHA content was measured on Day 10.

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Figure 1 Continuous stirred tank reactors used for cultivation of heterotrophic-methanotrophic consortia and for CH_4 remediation.

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Figure 2 MOCs of soil, mother culture under nutrient-replete and nutrient-deplete conditions for consortia cultured from landfill top cover soil and compost soil. *Note;* soil and mother cultures were enriched with 20:80 % CH_4:air gas mixture, while nutrient-deplete cultures received a gas mixture of 30:10:60 % CH_4:CO_2:air.

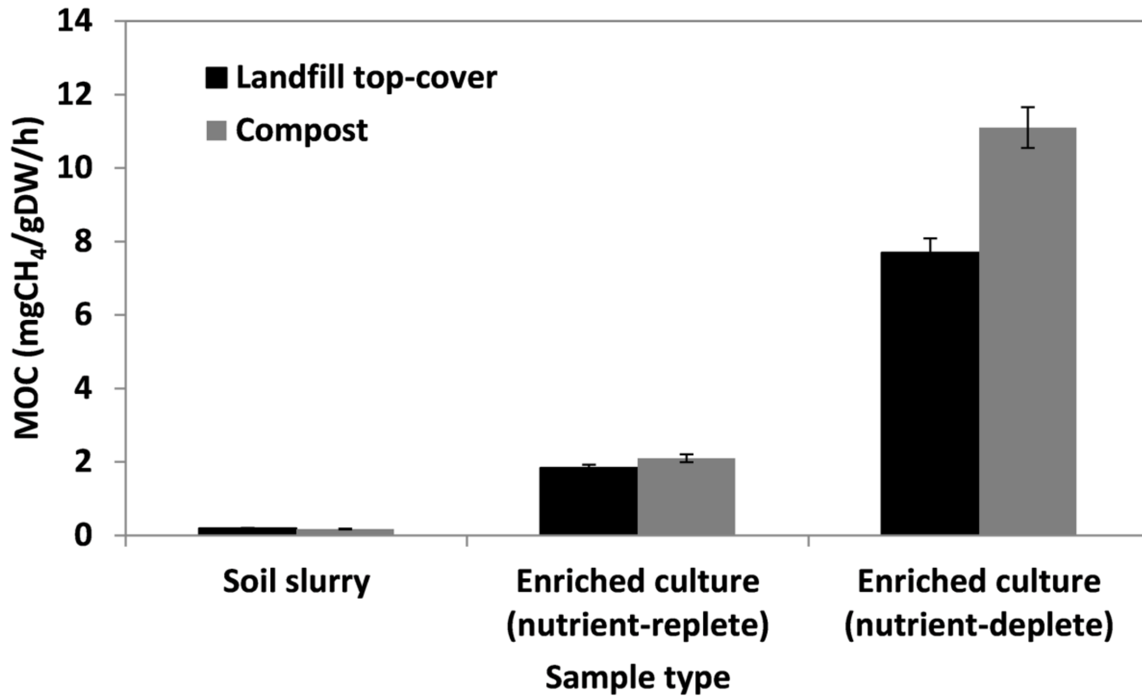

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Figure 3 C16:C18 profiles during enrichment of LB and CB under nutrient-deplete conditions.

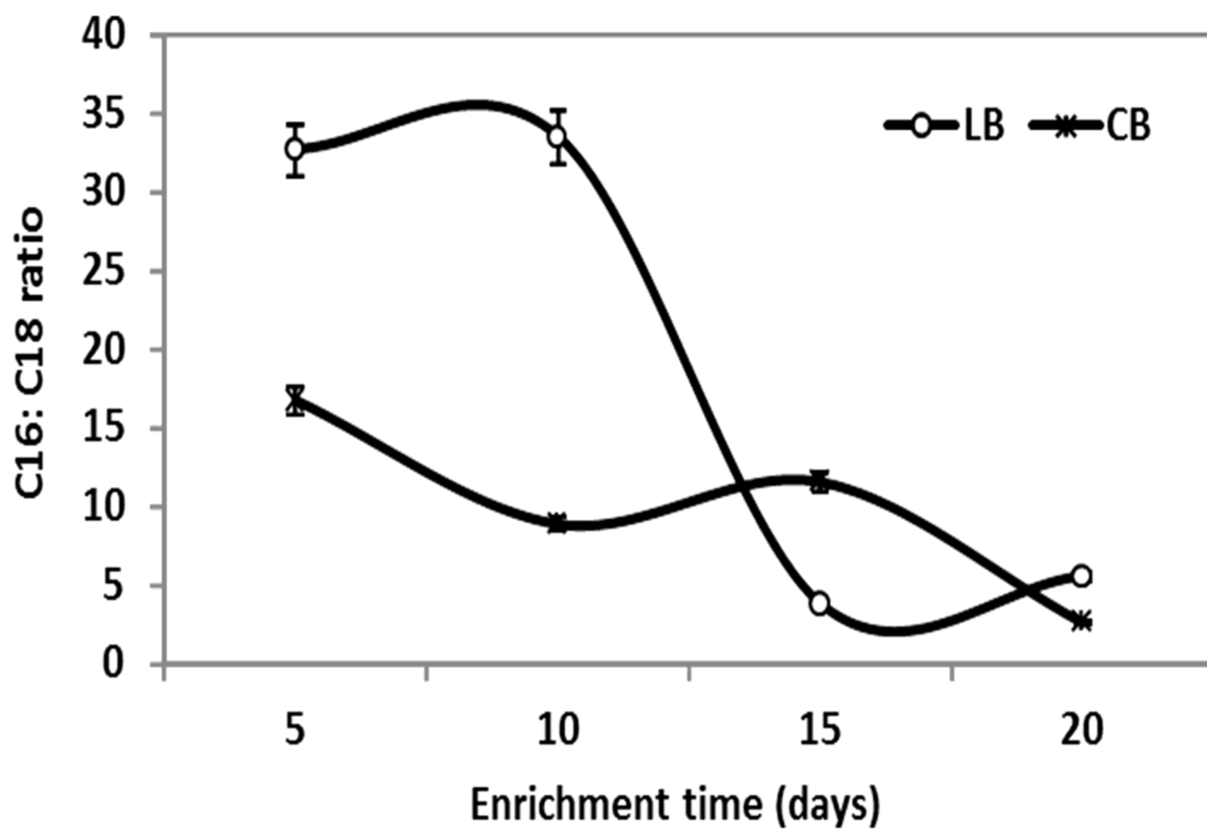

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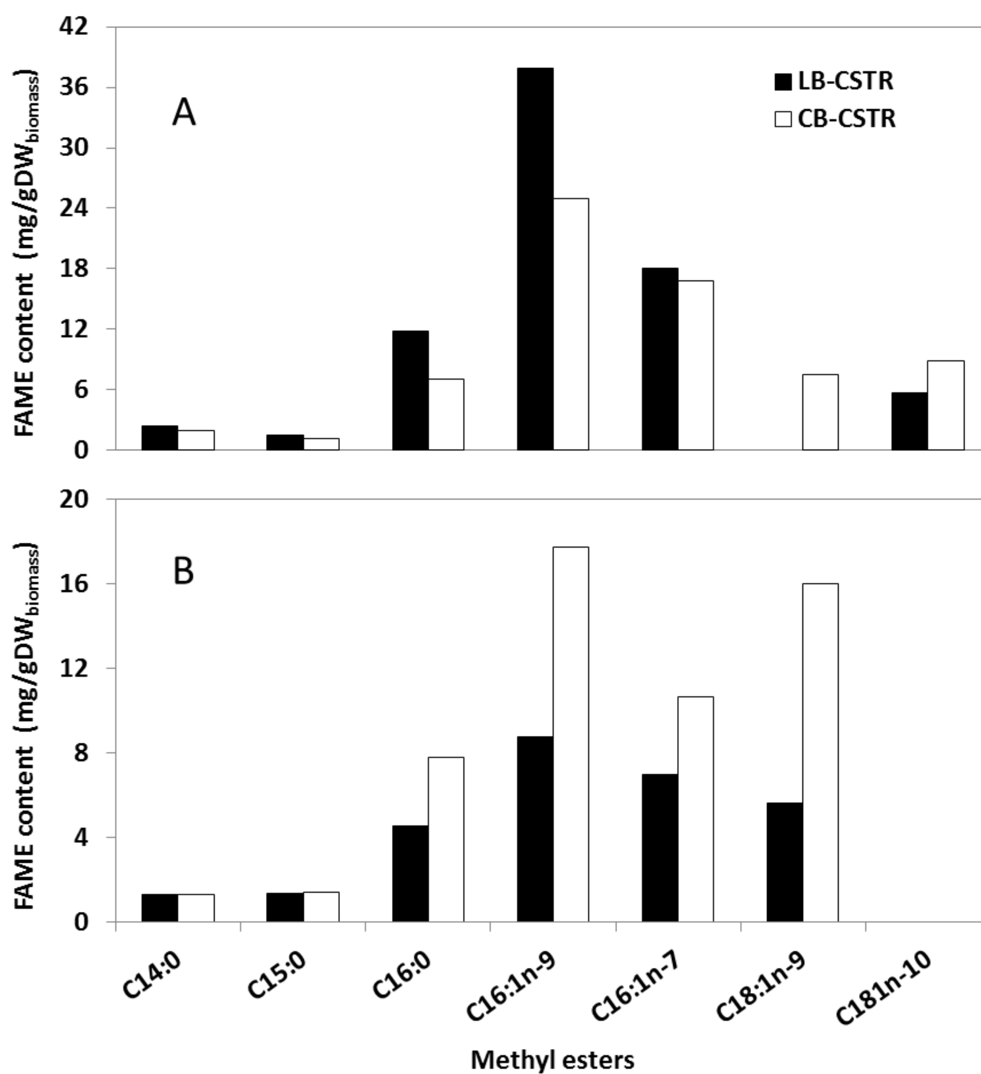
Figure 4 FAME profiles of biomass from LB- and CB-CSTRs (A) without copper and iron addition (day 5) and (B) with copper and iron spiking (day 10).



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