Supporting Information

Acetate production from anaerobic oxidation of methane via intracellular storage compounds

Chen Cai,[†,§] Ying Shi,[†,‡,§] Jianhua Guo,[†] Gene W. Tyson,[⊥] Shihu Hu,[*,†] and Zhiguo Yuan[*,†]

[†]Advanced Water Management Centre, The University of Queensland, St Lucia, Brisbane, Queensland 4072, Australia

[‡]School of Resource and Safety Engineering, Central South University, Changsha 410083, China

[⊥]Australian Centre for Ecogenomics, School of Chemistry and Molecular Biosciences, The University of Queensland, St Lucia, Brisbane, Queensland 4072, Australia

[§]These authors contributed equally to the work.

Number of pages: 9

Number of figures: 4

Extended materials and methods

Parent bioreactor operation and its performance

In this study, methane was supplied weekly (Figure S1) through flushing the parent bioreactor with a gas mixture of CH_4:N_2:CO_2 = 90:5:5% (Coregas, Australia). Nitrate and ammonium were supplied every 1-2 weeks (Figure S1) by injecting concentrated stock solutions (40 g NO_3^--N L^{-1} and 48 g NH_4^+-N L^{-1}, respectively). Bioreactor supernatant (~400 mL) was replaced every 1-2 months with fresh mineral medium consisting of KH_2PO_4 0.05 g L^{-1}, $CaCl_2 \cdot H_2O$ 0.3 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g L^{-1}, $FeSO_4$ 0.00625 g L^{-1}, and a trace elements solution (1.25 mL L^{-1})[1]. The medium was flushed with high purity nitrogen gas (99.999%, Coregas, Australia) to remove oxygen and placed in an anaerobic chamber for 24 h before use[2]. The bioreactor was operated in a temperature-controlled room at 22 ± 2 °C. pH in the bioreactor was controlled within the range of 7.0-7.5 through manually injecting 1 M HCl solution. Gas samples in the headspace were taken 3 times per week for methane measurement. Methane concentration in the liquid phase was calculated using the Henry's law. Liquid samples were taken 1-2 times per week for the analyses of nitrate, nitrite, ammonium, phosphate and VFAs. Biomass sample was taken for VSS analysis.

The performance of the parent bioreactor was stable during this study with an average methane consumption rate of 14 μmol g^{-1} VSS h^{-1}, concomitant with average ammonium and nitrate consumption rates of 42 and 56 μmol g^{-1} VSS h^{-1}, respectively (Figure S1). The observed stoichiometric ratio of 1:4 between methane consumption rate and nitrate consumption rate demonstrated that the bioreactor was dominated by nitrate-dependent AOM. Nitrite and acetate were not detectable in the bioreactor. The concentration of VSS in the parent bioreactor was approximately 1 g L^{-1}.

Microbial community analyses

16S rRNA gene amplicon profiling revealed that the microbial community in the parent bioreactor was dominated by *M. nitroreducens* at 80% with anammox bacterium *Candidatus* '*Kunenenia stuttgartiensis*' at a relatively low abundance of 2% (Figure S2). *Candidatus* '*Methylomirabilis oxyfera',* the only known bacterium capable of coupling AOM to nitrite reduction[3], was not detected in this co-culture. FISH results further supported the microbial populations with *M. nitroreducens* dominating the culture and anammox bacterium as a flanking community member (Figure S3). DNA extraction, 16S rRNA gene amplicon sequencing and FISH were conducted according to the methods described previously[4].

Chemical analyses

For ^{13}C-labelled acetate analysis, 1 mL liquid samples were filtered (0.22 μm, Millipore, USA) and stored in 2 mL glass bottles. The analysis of ^{13}C-labelled acetate was performed using an Isoprime Gas Chromatograph Combustion Isotope Ratio Mass Spectrometer (GC-C-IRMS) (Isoprime, UK). The GC-C-IRMS was equipped with a J&W DB-FFAP fused silica capillary column (30 m * 0.5 mm with 1 μm coating).

For PHA extraction and measurement, 50 mL biomass was taken from the batch reactor and centrifuged at a rate of 3,267 × *g* for 10 minutes. The supernatant was discarded, and the pellets were freeze-dried overnight. The PHA content in the dried samples was measured as described previously[5]. For glycogen analysis, 5 mL biomass was sampled into a 15 mL falcon tube, and centrifuged at a rate of 3,267 × *g* for 10 minutes. After discarding the supernatant, 3 mL 0.6 M HCl was added to the falcon tube, for digestion over 6 hours at 100°C. After cooling at room temperature for 3

hours, the sample was centrifuged, and 1 mL of the supernatant liquid was analyzed for glucose using High-Performance Liquid Chromatography (HPLC) (Bio-Rad, Hercules, CA)[6].

To fluorescently detect PHA during acetate production, biomass (0.6 mL) was mixed with 40 μL Nile red (32 mg L^{-1} dissolved in dimethyl sulfoxide), resulting in a final Nile red concentration of 2 mg L^{-1}. The suspension was incubated at room temperature for 30 min. Supernatant of the suspension was discarded after being centrifuged at 14,000 ×*g* for 5 min. The stained pellets were resuspended using 0.6 mL Milli Q water. Each sample of 0.2 mL was distributed to a 96-well microplate in triplicate. The fluorescence was read using The Reader Control Software in the CLARIOstar multi-mode, high-performance microplate reader (BMG LABTECH, Germany). The fluorescence intensity was assumed to be proportional to the PHA concentration[7].

References

1. Shi, Y.; Hu, S.; Lou, J.; Lu, P.; Keller, J.; Yuan, Z., Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane biofilm reactor. *Environ. Sci. Technol.* **2013,** *47*, (20), 11577-11583.

2. Hu, S.; Zeng, R. J.; Burow, L. C.; Lant, P.; Keller, J.; Yuan, Z., Enrichment of denitrifying anaerobic methane oxidizing microorganisms. *Environ. Microbiol. Rep.* **2009,** *1*, (5), 377-384.

3. Ettwig, K. F.; Butler, M. K.; Le Paslier, D.; Pelletier, E.; Mangenot, S.; Kuypers, M. M. M.; Schreiber, F.; Dutilh, B. E.; Zedelius, J.; de Beer, D.; Gloerich, J.; Wessels, H. J. C. T.; van Alen, T.; Luesken, F.; Wu, M. L.; van de Pas-Schoonen,

K. T.; Op den Camp, H. J. M.; Janssen-Megens, E. M.; Francoijs, K.-J.; Stunnenberg, H.; Weissenbach, J.; Jetten, M. S. M.; Strous, M., Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **2010,** *464*, (7288), 543-548.

4. Hu, S.; Zeng, R. J.; Haroon, M. F.; Keller, J.; Lant, P. A.; Tyson, G. W.; Yuan, Z., A laboratory investigation of interactions between denitrifying anaerobic methane oxidation (DAMO) and anammox processes in anoxic environments. *Sci. Rep.* **2015,** *5*.

5. Werker, A.; Lind, P.; Bengtsson, S.; Nordström, F., Chlorinated-solvent-free gas chromatographic analysis of biomass containing polyhydroxyalkanoates. *Water Res.* **2008,** *42*, (10), 2517-2526.

6. Oehmen, A.; Zeng, R. J.; Yuan, Z.; Keller, J., Anaerobic metabolism of propionate by polyphosphate-accumulating organisms in enhanced biological phosphorus removal systems. *Biotechnol. Bioeng.* **2005,** *91*, (1), 43-53.

7. Zuriani, R.; Vigneswari, S.; Azizan, M. N. M.; Majid, M. I. A.; Amirul, A. A., A high throughput Nile red fluorescence method for rapid quantification of intracellular bacterial polyhydroxyalkanoates. *Biotechnology and Bioprocess Engineering* **2013,** *18*, (3), 472-478.

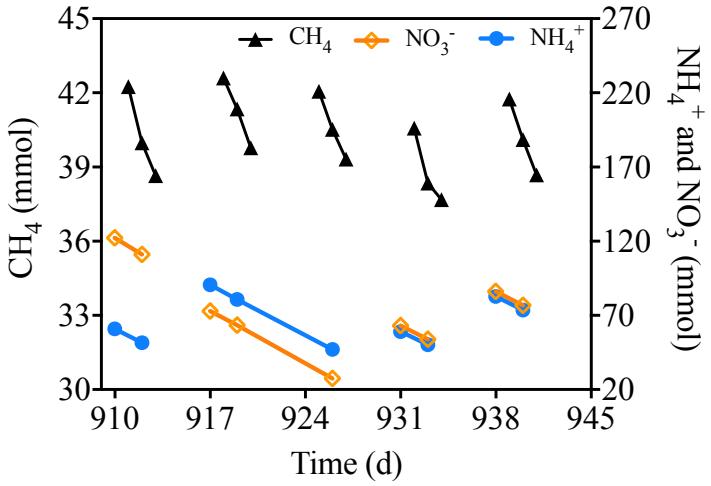

Figure S1. Performance data of the parent bioreactor during this study. Methane, ammonium and nitrate are consumed simultaneously. Nitrite or acetate was not detectable in the bioreactor. Biomass samples were taken on Day 940 for 16S rRNA gene amplicon and FISH analyses.

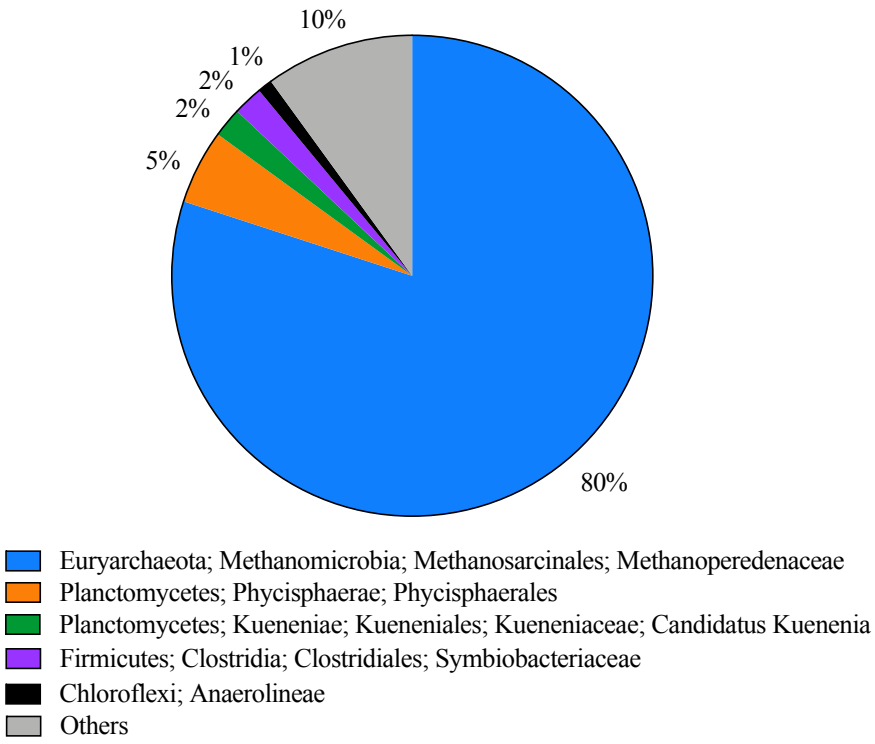

Figure S2. Microbial community composition in the parent bioreactor using 16S rRNA gene amplicon sequencing. Only operational taxonomic units (OTUs) over 1% relative abundance are shown and the remaining OTUs are grouped in 'others'.

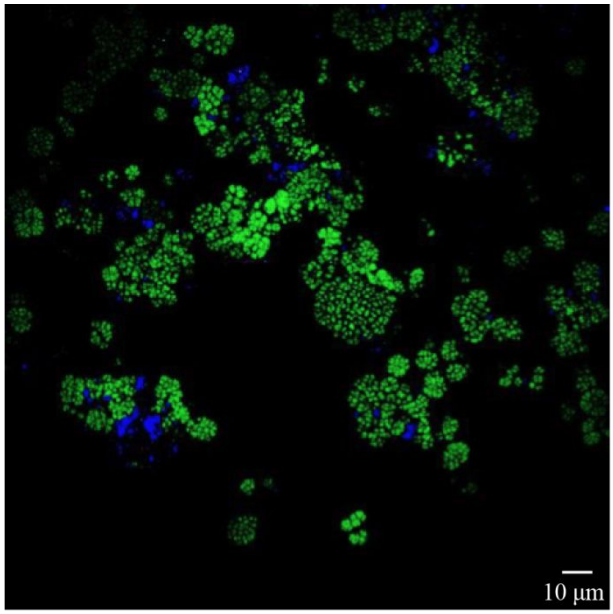

Figure S3. Fluorescence *in situ* hybridization micrograph of the parent bioreactor microbial community. The dominant population, *M. nitroreducens*, appears green (specific archaeal probe Darch-872), while anammox bacteria appear blue (anammox probe Amx-820). *M. oxyfera* was not detected (specific bacterial probe NC10-1151, red).

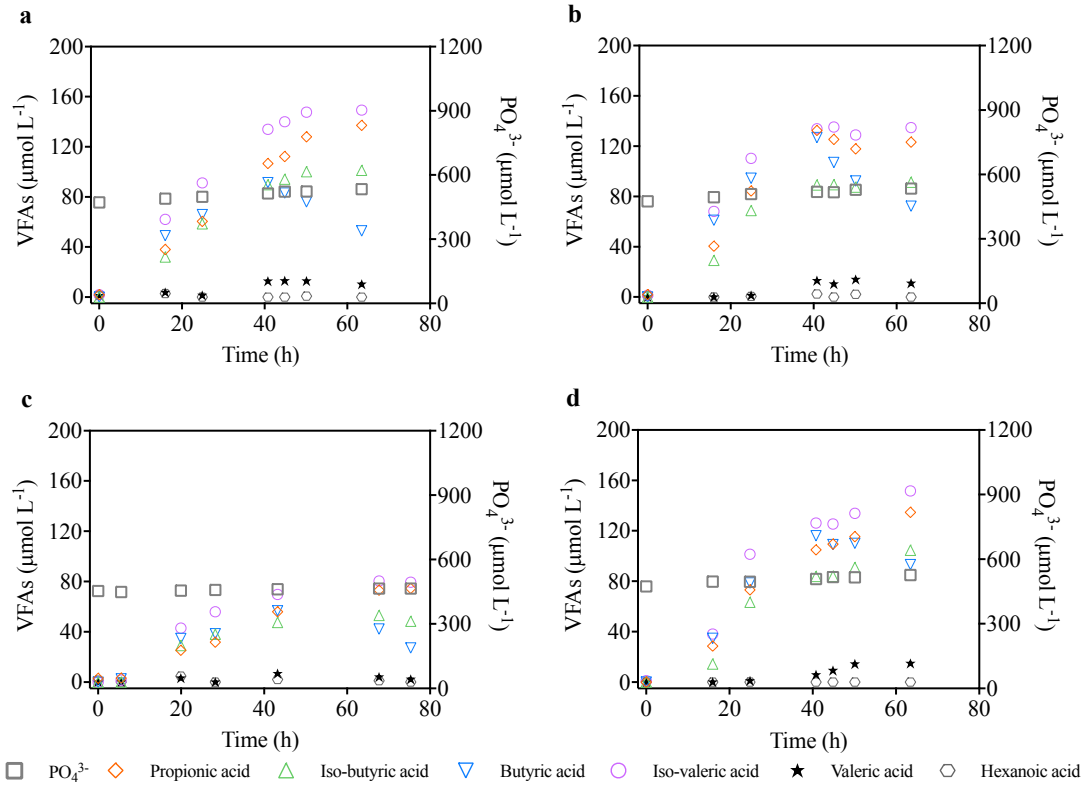

Figure S4. Concentration profiles of phosphate and VFAs other than acetate in Test A (a), B (b), C (c) and D (d).