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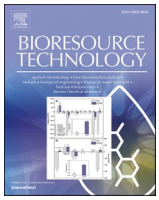

Short Communication

Metagenomic analysis of ethylene glycol contamination in anaerobic digestion

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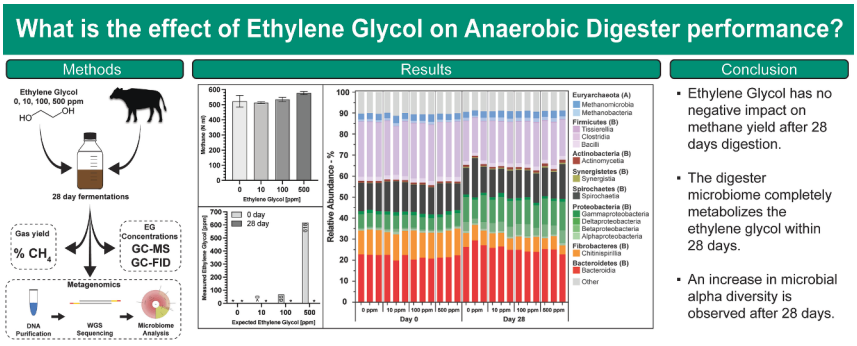
HIGHLIGHTS

- Ethylene Glycol has no significant effect on biogas yields.
- Digester microbiome completely metabolises the ethylene glycol within 28 days.
- An increase in microbial diversity is observed after 28 days fermentation.

GRAPHICAL ABSTRACT



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ABSTRACT

Anaerobic digestion is an established method for the biological conversion of waste feedstocks to biogas and biomethane. While anaerobic digestion is an excellent waste management technique, it can be susceptible to toxins and pollutants from contaminated feedstocks, which may have a detrimental impact on a digester's efficiency and productivity. Ethylene glycol (EG) is readily used in the heat-transfer loops of anaerobic digestion facilities to maintain reactor temperature. Failure of the structural integrity of these heat transfer loops can cause EG to leak into the digester, potentially causing a decrease in the resultant gas yields. Batch fermentations were incubated with 0, 10, 100 and 500 ppm (parts per million) of EG, and analysis showed that the EG was completely metabolised by the digester microbiome. The concentrations of EG tested showed significant increases in gas yields, however there were no significant changes to the digester microbiome.

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1. Introduction

Production of biomethane or renewable natural gas (RNG) by anaerobic digestion offers a sustainable, scalable solution for the generation of a chemically identical, fossil fuel equivalent biofuel (Molino et al., 2013). As governments and industries strive to achieve their net zero ambitions for the coming decades (DeAngelo et al., 2021), natural gas has been identified as a transition fuel (Safari et al., 2019) which may aid the global transition from conventional fossil fuels to low-carbon, renewable alternatives (Pääkkönen et al., 2019). Anaerobic digestion is an established method for the biological conversion of waste feedstocks, such as agricultural residues (Demirbas & Ozturk, 2005), animal manure (Hills & Roberts, 1981), sewage sludge (Duan et al., 2012) and municipal solid waste (Zamri et al., 2021) to biogas, a mixture comprising predominately of methane (CH_4) and carbon dioxide (CO_2) (Molino et al., 2013). Subsequent upgrading of the biogas, through a process separating the CH_4 from the CO_2, yields biomethane (Angelidaki et al., 2018). The resultant biomethane can be used to power on-site energy demands (Kaparaju & Rintala, 2013), fuel agricultural and heavy-duty vehicles (Savickis et al., 2020) or be piped into the natural gas grid to meet national energy demands (Cavana & Leone, 2022). Moreover, anaerobic digesters are scalable from micro-scale, on-site digesters processing 5 tonnes of feedstock per year (Walker et al., 2017) to large industrial scale digesters, capable of handling in excess of 30,000 tonnes of feedstock per year (Thiriet et al., 2020). This scalability is an appealing characteristic of anaerobic digestion for communities, farmers, industry and governments (Ackrill & Abdo, 2020). The European Biogas Association reported that as of 2022, 18,843 biogas facilities and 1,067 biomethane facilities were operational in Europe. Anaerobic digestion is an excellent waste management technique, however it can be susceptible to toxins and pollutants from contaminated feedstocks which may have a detrimental impact on a digester's efficiency and methane productivity (Chen et al., 2008). Ethane-1,2-diol, commonly termed ethylene glycol (EG), is a heat-transfer fluid frequently used as a coolant for internal combustion engines and air-conditioning systems (Rudenko et al., 1997}. Temperature control of anaerobic digesters is regularly performed via water and steam, however EG can also be utilised in heat-transfer loops to maintain a constant reactor temperature. Structural integrity failures of these heat transfer loops can cause EG to leak into the digester. This could have a detrimental effect on the digesters' microbiome, and therefore reduce the biomethane production from a facility. EG has been demonstrated to increase net gas productivity during anaerobic digestion (Battersby & Wilson, 1989) and was metabolised in the short-term in aerobic granular sludge reactors (Qi et al., 2020). However, long-term exposure was predicted to have a detrimental effect on the microbiome and productivity of the reactors (Qi et al., 2020). Accumulation of EG during anaerobic digestion can shift microbial metabolism to produce far greater yields of hydrogen than methane (Sołowski et al., 2021), by a process termed dark fermentation (Dzulkarnain et al., 2022). Propane-1,2-diol or propylene glycol (PG), similarly used commercially as a heat transfer fluid (JuGer & Crook, 1999), is a less toxic alternative to EG (West et al., 2014). However, PG was observed to reduce methane production during anaerobic digestion (Wang et al., 2021). Fundamental to anaerobic digestion is a diverse microbiome readily characterised by metagenomic analysis (Kim et al., 2022) that drives conversion of organic feedstocks to biogas via four main biological processes – hydrolysis, acidogenesis, acetogenesis and methanogenesis (Meegoda et al., 2018).

2. Materials and methods

2.1. Ethylene glycol concentrations

A representative commercial anaerobic digester with a volume of 11,090 m^3 has a heat transfer loop volume of 24 m^3, which would typically contain 25% (6 m^3) of ethylene glycol (EG). A complete failure of this heat transfer loop would result in 500 ppm of EG contaminating the digestate, with lower volume leaks simulated by 10 and 100 ppm concentrations.

2.2. Batch anaerobic digestion

Batch anaerobic digestion was performed in 500 ml reactors coupled to a Gas Endeavour System (BPC Instruments, Sweden). Each reactor contained 400 ml of matured manure inoculum with 5% total solids (TS) and 4% (wet weight) volatile solids (VS). EG was added to the reactors, in triplicate, to achieve final concentrations of 10, 100 and 500 ppm. After EG addition, the pH of the reactors was checked and ranged from 6.9 − 7.1. Three control reactors were run, each containing 4.5 g of cellulose and 0 ppm ethylene glycol. The reactor headspace was purged with nitrogen for 2 min prior to loading into the Gas Endeavor system. Reactors were incubated for 28 days at 38 ℃, with total gas and methane measured continuously. Data was analysed in GraphPad Prism v9.5.

2.3. Ethylene glycol analytical analysis of digestate

An Agilent 7890 Gas Chromatography (GC) with flame ionization detector (FID) (Agilent Technologies, Santa Clara, CA, USA) was used for EG analysis. Masshunter GC/MS (Agilent Technologies, Santa Clara, CA, USA) was used for both acquisition and data analysis. The system was equipped with a bonded Agilent DB-1 column (30 m × 0.25 mm × 0.25μm) (Agilent Technologies, Santa Clara, CA, USA). The number of injections was limited for aqueous samples. The temperature program started at 40 °C and increased to 250 °C at 8 °C min^{-1}, to ensure sufficient separation of EG from other components in the samples. The inlet was operated at 250 °C with an injection volume of 1 uL (10:1 split mode). Helium was employed as the carrier gas at 1 ml min^{-1}. FID was operated at 325 °C with 35 ml min^{-1} hydrogen flow, 350 ml min^{-1} air flow and 25 ml min^{-1} nitrogen makeup flow. Calibration was completed in the range of 3–600 ppm with an $R^2 > 0.99$. Slight shifting of retention time was observed due to the nature of the samples.

2.4. DNA purification and sequencing

DNA was purified from 500 mg digestate samples collected at day 0 and day 28 of the batch fermentation. DNA was purified using the Qiagen MagAttract PowerSoil Pro KF DNA extraction kit (Qiagen, USA) utilising a ThermoFisher KingFisher Flex liquid handling robot (ThermoFisher, USA) with a final elution volume of 100 μl. Purified DNA was quantified using the High-Sensitivity Qubit Assay (ThermoFisher, USA). Illumina whole genome shotgun (WGS) libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, USA) and prepared WGS libraries were sequenced using an Illumina MiSeq with a 2 × 300 paired end v3 flow cell.

2.5. Bioinformatic analyses

Illumina WGS DNA sequence reads were quality control trimmed using Trim_Galore (v0.6.4) and trimmed reads were taxonomically classified using DIAMOND (v2.0.13.151) against the NCBI nr database. DIAMOND taxonomic classifications were visualised in MEGAN (v6.24).

3. Results and discussion

Batch anaerobic digestion of manure yielded 522 N ml of methane after 28 days of fermentation (Fig. 1A). Manure feedstock was contaminated with increasing concentrations of ethylene glycol (EG) and produced 513 N ml of methane when incubated with 10 ppm of EG, 535 N ml with 100 ppm of EG, and 577 N ml during fermentation with 500 ppm EG (Fig. 1A). A one-way ANOVA determined a significant difference ($p = 0.03$) between the methane yields from each of the EG

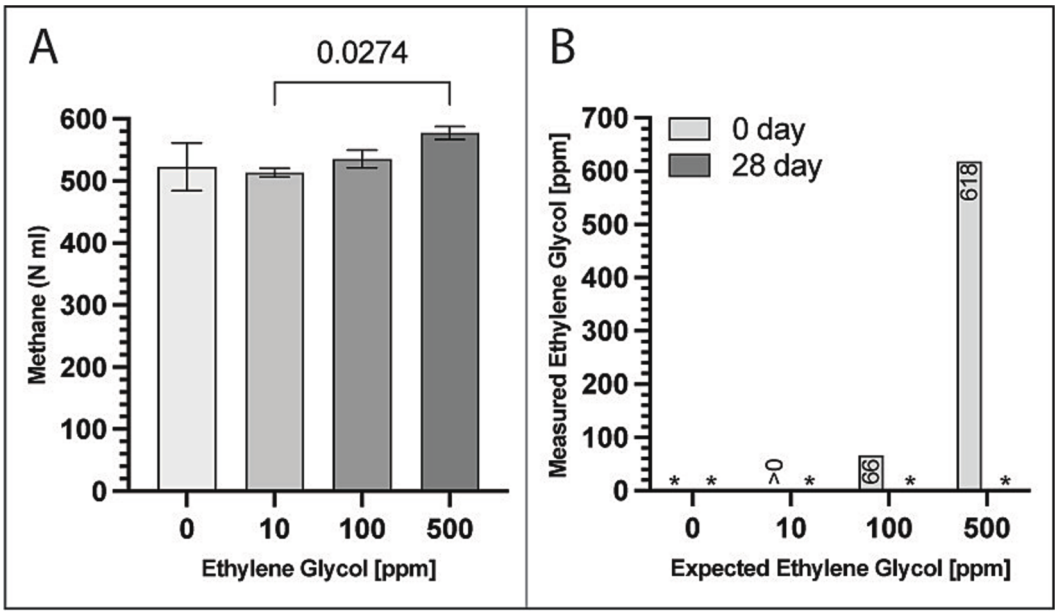

Fig. 1. Total Methane Production and Ethylene Glycol consumption **A**. Total methane production (N ml) after 28 days fermentation with 0, 10, 100 and 500 ppm ethylene glycol. Bars display mean (n = 3) volume of methane produced and error bars represent the standard deviation. A one-way ANOVA and subsequent Tukey multiple comparison tests were performed and p-values displayed for any significant differences. **B**. Concentration of ethylene glycol (ppm) in the digestate at day 0 (light grey) and day 28 (dark grey). Samples with concentrations below the limit of accurate quantification are shown by > 0 and samples with concentrations below the limit of detection are displayed as *.

fermentations. Furthermore, Tukey multiple comparison tests showed a significant difference (p = 0.03) between incubations with 10 ppm EG and 500 ppm EG (Fig. 1A). There was an increase of 55 N ml methane produced at 500 ppm compared to the control, which supports previous studies that EG is readily metabolised (Shin & Bae, 2019, Qi et al., 2020) and increases gas productivity (Battersby & Wilson, 1989). Consumption of EG was determined by GC-FID and showed that EG was fully consumed during fermentation (Fig. 1B) reducing to non-detectable for all samples after 28 days digestion.

Purified DNA from the batch fermentations was used to prepare whole genome shotgun (WGS) Illumina sequencing libraries. DNA sequence reads were classified against the NCBI nr database and visualised at Class taxonomic rank (Fig. 2A). The microbial community was dominated by an abundance of Bacteroidia and Clostridia, exhibiting between 19 and 22 % Bacterodia and ranging from 23 to 26 % Clostridia for day 0 samples across the EG concentrations. At day 28, the abundance of Bacterodia increased to 27 % for 0 ppm EG samples and 24 % for 500 ppm EG incubations. However, the abundance of Clostridia decreased to an average of 19 % across the concentrations of EG tested at day 28. Furthermore, the abundance of Beta- and Deltaproteobacteria increased after 28 days incubation, while the abundance of Alpha- and Gammaproteobacteria remained consistent between the timepoints. No change in the relative abundance of Spirochaetia, Synergistia, Actinomycteia and the Firmcutes classes Bacilli and Tissierellia was observed between day 0 and day 28. However, a decrease of Chitinispirillia was observed. The observed abundance of Archaea classes remained constant, with an average of 1 % Methanobacteria and 3 % Methanomicrobia measured across all concentrations of EG and time points. Alpha diversity, calculated by the Shannon diversity index at class taxonomic rank (Fig. 2B), shows consistent diversity index scores between 3.29 and 3.56 for all samples, with average values between 3.39 and 3.49 for the same samples. At each of the concentrations of EG tested, the average alpha diversity score increased after 28 days incubation. The largest difference was observed at 500 ppm, with a mean alpha diversity score of 3.40 at day 0 and 3.49 at day 28, suggesting that an increase in concentration of EG promotes an increase in bacterial diversity. There was a significant difference (p = 0.04) between the alpha diversity observed at day 0 and day 28, however a 2-way ANOVA demonstrated that there was no significant difference between the concentrations of EG and the measured timepoints. Principal Coordinate Analysis (PCoA) of the samples' Beta diversity was calculated using the Bray Curtis dissimilarity index for each of the communities (Fig. 2C) which revealed that microbiome samples from each individual timepoint clustered closely with one another. There was no apparent clustering of samples based upon the initial EG concentration at either day 0 or day 28 of the fermentations. A neighbour joining tree (Fig. 2D) further highlights the dissimilarity observed between samples from day 0 and day 28. The clustering observed is clearly driven by the sampling timepoint rather than the initial EG concentrations. Microbial consumption of EG has been observed in aerobic (Revitt & Worrall, 2003, Qi et al., 2020) and anaerobic conditions (McVicker et al., 1998, Carnegie & Ramsay, 2009). EG is a substrate for lactaldehyde reductase, encoded by the gene *fucO* (Panda et al., 2021) and is readily metabolised via glyoxylate to central metabolism (Boronat et al., 1983) and acetic acid, a precursor for methanogenesis (Dwyer & Tiedje, 1983). Consumption of EG has been identified in *Pseudomonas aeruginosa* and *Acinetobacter* (Haines & Alexander, 1975, Watson & Jones, 1977). Analysis at species taxonomic rank (see supplementary materials) reveals that *P. aeruginosa* abundance does increase at day 28. However, this increase is also observed in the 0 ppm EG fermentation, suggesting that if the *P. aeruginosa* is responsible for the metabolism of EG, the concentrations tested are insufficient in causing a significant increase in the taxa's growth.

4. Conclusion

Ethylene glycol, in concentrations that are comparable with a heating loop failure at a commercial anaerobic digestion facility, has no significant effect on inherent digester microbiome. Moreover, at concentrations >100 ppm, an increase in methane production may be observed. However, further investigations need to be performed to understand whether the digester microbiome is capable of metabolising continued input of EG and sustaining increased methane production.

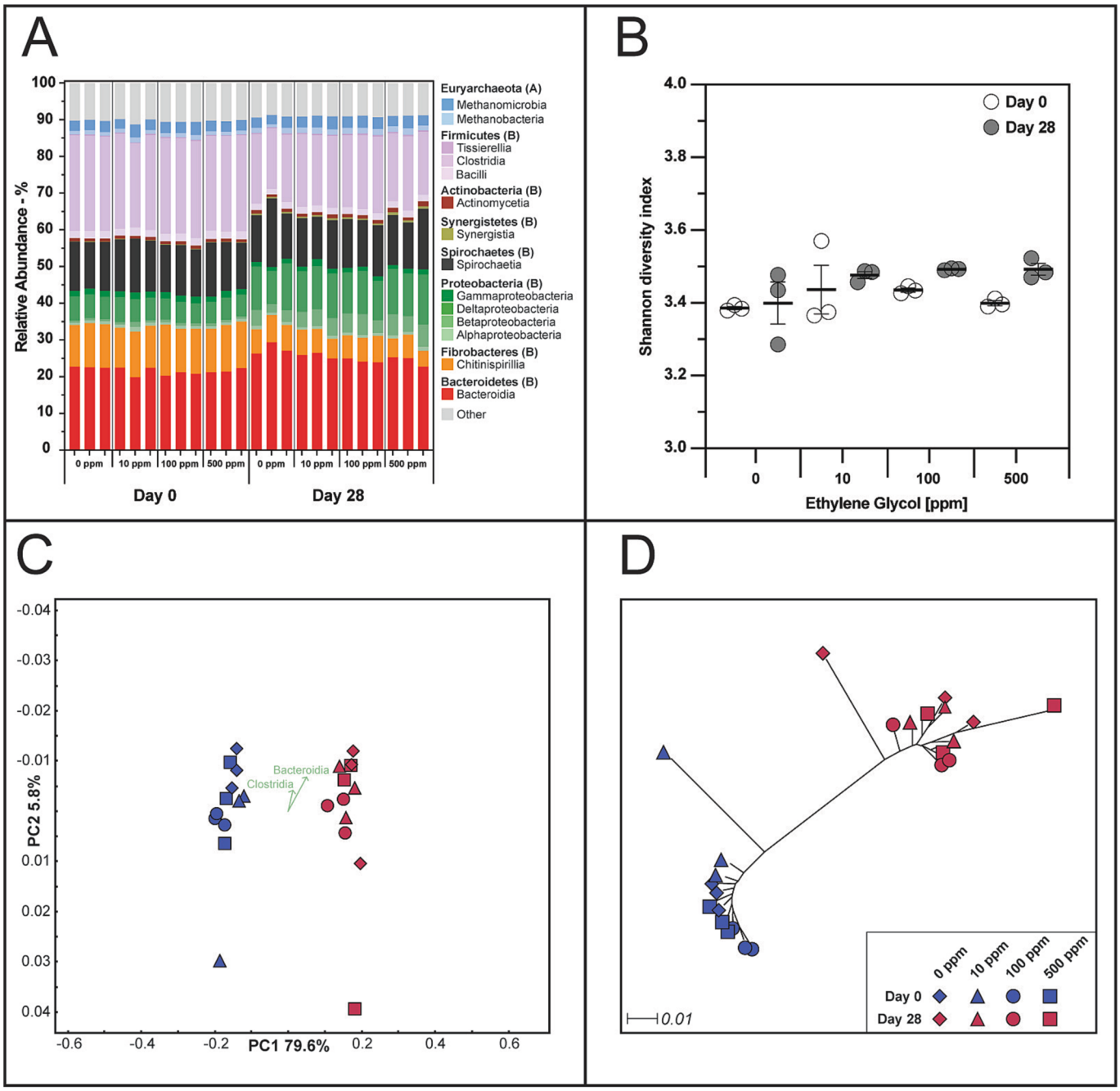

Fig. 2. Microbiome Analysis of Digestate **A.** Stacked bar chart representing the percentage relative abundance of taxa at Class taxonomic rank observed in the digester microbiome when incubated with ethylene glycol. Taxa observed at less than 1% relative abundance across all samples are summed to 'Other' and displayed in grey. Archaeal phyla are denoted by (A) and bacterial phyla are denoted with (B). **B.** Alpha diversity calculated using the Shannon diversity index at class taxonomic rank observed in the digester microbiome when incubated ethylene glycol at day 0 (white symbols) and day 28 (grey symbols). Bars represent the mean value (n = 3) and error bars denote the standard error of the mean. Principal Coordinate Analysis (**C**) and Neighbour Joining tree (**D**), displaying beta diversity calculated using the Bray Curtis dissimilarity index at class taxonomic rank.

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CRediT authorship contribution statement

Gabrielle R. Joslin: Investigation. **Daniel G. Barber:** Formal analysis. **Lindsay Aston:** Investigation. **Ping Liu:** Project administration, Writing – review & editing. **Olukayode Kuloyo:** Conceptualization, Methodology. **Kangsa Oentoro:** Methodology. **Jiayi Liu:** Methodology, Investigation. **Ashley V. Baugh:** Investigation. **Jeffrey R. Fedenko:** Conceptualization, Writing – review & editing. **Ioannis Melas:** Formal analysis. **Phillip G. Hamilton:** Funding acquisition, Supervision. **Damian J. Allen:** Conceptualization, Supervision, Project administration, Writing – review & editing. **Richard K. Tennant:** Formal analysis, Visualization, Supervision, Funding acquisition, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Illumina WGS DNA sequence reads have been deposited in the NCBI Sequence Read Archive under BioProject accession PRJNA982105.

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