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Research Article

LINKAGE OF OPERATIONAL PARAMETERS AND MICROBIOME IN ANAEROBIC CO-DIGESTION WITH GRAPHITE

Sevgi DEMİREL*[1], Öznur Begüm GÖKÇEK[2], Hamdi MURATÇOBANOĞLU[3]

[1]*Niğde Ömer Halisdemir University, Department of Environmental Engineering, NİĞDE;*
ORCID: 0000-0002-5329-591X
[2]*Niğde Ömer Halisdemir University, Department of Environmental Engineering, NİĞDE;*
ORCID: 0000-0003-1730-2905
[3]*Niğde Ömer Halisdemir University, Department of Environmental Engineering, NİĞDE;*
ORCID: 0000-0002-4720-8090

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ABSTRACT

In terms of renewable energy sources, studies on increasing the amount of methane produced per unit feeding materials have attracted attention in recent years. Many different methods like chemical, physical, thermal, and thermochemical processes have been applied to anaerobic digesters for enhancing biogas production efficiency. Recently, besides these processes, supporting anaerobic digestion (AD) system with conductive materials like graphite, magnetite, activated carbon, etc. is one of the trend topics. In this paper, the effect of graphite on biogas/biomethane production potential was investigated for co-digestion of food waste (FW) and cow manure (CM). Additionally, the relationship between the distribution of anaerobic microbial structure and operational conditions is examined using Redundancy analysis (RDA) revealed that *Clostridium* could enhance to methanogenesis process through conductive materials such as graphite. These findings will improve the understanding of the mutual relationship between operational conditions and community composition in anaerobic digestion of food waste and cow manure mixture. As a result, the analysis of the correlation between microbiome and operational parameters indicated that *Clostridium*, *Methanosaeta*, and *Methanosarcina*, all together could enhance to methanogenesis process with the graphite supplementation.
Keywords: Anaerobic digestion, microbial community, redundancy analysis, conductive material.

1. INTRODUCTION

Energy security concerns and environmental pollution are among the biggest challenges people face in recent years. Renewable energy sources are needed to reduce fossil fuel use, global warming and CO_2 emissions. Biomass energy, one of the low-cost energies, is one of the most attractive energy sources [1]. Anaerobic digestion (AD) is a technology that plays an important role in the removal of organic materials and renewable energy production [2-3].

AD is a complex and highly sensitive process to environmental conditions. Besides, this process contains many microorganisms including acetic acid-forming bacteria (acetogens) and methane-forming archaea (methanogens). The type and structure of the substrates used effect

* Corresponding Author: e-mail: sevgidemirel@ohu.edu.tr, tel: (388) 225 22 64

biogas and methane production efficiency. The organic materials are mainly composite of carbohydrates, proteins, lipids which can be degraded to simple compounds by microorganisms in an oxygen-free environment with the following process stages: hydrolysis, acidogenesis, acetogenesis and methanogenesis stage [4-5].

Food waste (FW) is one of the important raw materials used in anaerobic digestion due to its high methane potential. On the other hand, FW could lead to rapid acidification and low methane yield during the anaerobic digestion (AD) process [6]. Several researchers have investigated on co-digestion of food waste with different substrates in the literature [7-8]. Cow manure (CM) is also used in anaerobic digestion processes as it converts waste to bioenergy and reduces the amount of waste. However, the high carbon content of animal manure could hinder the optimum C/N ratio in the anaerobic digestion process, but it has good buffer capacity. Therefore, the co-digestion of FW and CM could be applied at an appropriate ratio to improve digestion performance [9-10]. The limited studies have been conducted on the performance of the co-digestion of FW and CM [11-14]. For example, Li et al. (2009) were examined co-digestion FW with CM in a batch digester and biomethane production boosted by 44% as compared to the mono-digestion of FW [6].

So far, various researchers have investigated the effect of conductive materials on AD performance and stability. Additionally, these studies indicate that supplementation of conductive materials such as biochar, active carbon, carbon nanotubes, stainless steel, and graphite could increase the performance of anaerobic digesters [15]. Compared with the conventional operation, biochar addition increased the methane production rate by 40.3% and enhanced VS removal efficiency. The enhancement in AD performance when conductive materials are added to the digester seems to be associated with the collaboration of microbiota of providing a direct route for electron transport between bacteria and methanogens species [14]. Therefore, to increase the production of biogas and methane, further investigations are needed to examine the relationship between operating conditions and microbiota in more detail and to explain the effect of conductive material on AD.

Determination of microbial identification and the functional mechanism is quite difficult in complex environmental samples such as digester environment with the traditional identification methods [16]. Recently, the increases in the number of cultures-independent molecular biology techniques have provided information about insight into the community structure and function of dominant populations in anaerobic digesters [17]. Unfortunately, it is not enough for the determination of the digester's microbial community alone to understand the AD phenomena. Complementary analysis and techniques are needed for the examination of the digestion process. However, there is a clear research gap that explains the interaction between performance parameters and microbiota with the conductive materials adding to the AD system. The innovation in this study is that it is the first to compare the effects of graphite on the AD performance and the shift of microbial communities using Redundancy Analysis (RDA).

RDA is widely used by scientists for better understanding and explanation for biological systems [18]. RDA analysis is an ordination technique that can be used on the analysis of any set of multivariate objects. There is a matrix of variables that often contains community structure data and a matrix of explanatory variables (e.g. operational conditions) in this analysis [19]. The present study evaluates the performance of the graphite addition on the anaerobic co-digestion (FW/CM). Additionally, the relationship between the distribution of anaerobic microbial structure and operational conditions is examined using RDA. The use of complementary RDA techniques will allow investigating the relationship between operational parameters and microbiome of sludge samples (FW, CM and FW/CM) in response to graphite added through 16S rRNA amplicon sequencing.

2. MATERIALS AN D METHODS

2.1. Anaerobic batch reactor design and operational parameters

FW, CM and their mixture (FW/CM) were used as the substrate for batch reactors. The FW was collected from Niğde Ömer Halisdemir University student (NOHU) canteen after lunchtime. CM was obtained from the breeding farm of NOHU Faculty of Agriculture. Once materials collected they have blended for 5 min to ensure diameter was less than 0.5 cm and kept at +4 °C for further usage. The mixture ratio of the FW/CM sample is 2:1 volatile solids (VS) based, this ratio was applied to reach the ideal carbon/nitrogen (C/N) ratio for AD i.e., 20 to 30 [20]. The inoculum was obtained from wastewater treatment plant (WWTP) (Adana Seyhan) sludge digester which is operated at mesophilic continuous stirred-tank reactor (CSTR) conditions. Some of the physical and chemical parameters of substrates and inoculum were shown in Table 1.

Table 1. Influent characterization of batch digesters

Parameters	FW	CM	FW/CM	Inoculum
pH	4.6	7.2	7.9	7.45
TS (%)	23.07	21.51	22.55	1.99
VS (%)	21.44	17.56	20.15	1.05
S/I	2.02	1.66	1.89	-

Batch experiments were carried out at mesophilic (35±1 °C) conditions via an incubator (Memmert, Germany). Serum bottles were used as batch reactors with 500 ml total volume. 300 ml effective volume was used for batch experiments. Graphite (Merck quality, particle size <50 µm (99.5%)) concentrations were 0.2, 0.5, 1, 1.5 g/L. In this paper, graphite concentrations are selected according to the results of preliminary studies. Substrate/Inoculum (S/I) ratio was 1.89 for FW/CM (2/1 VS based) samples. Before incubation, 300 ml inoculum and calculated amount of substrate added into the reactor and mixed well, the final volume of the reactor became 325 ml after addition CM (d=990 g/L) [21], FW (d= 760 g/L) [22] and FW/CM. Initial pH was measured with pH meter (WTW Inolab pH 7110, Germany) and before sealing with rubber stopper headspace purged with N_2 to strip O_2 (Figure 1). Daily biogas measurements made with a syringe with a capacity of 100 mL.

Figure 1. Anaerobic batch digesters

2.2. Analytical methods

The determination of Total Solids (TS) and Volatile Solids (VS) were carried out adapted from Standard Method 2540 D and 2540. The methane content of biogas was analyzed with a GC (Shimadzu GC-2010 plus, Japan) equipped with thermal conductivity detector (TCD) and capillary column (Restek Rxi-5ms 30m, 0.25mm ID 0.25µm). Temperatures of the injection port, column and detector were 50, 40, 230 °C respectively. The carrier gas is He (90 kPa) and the injected sample volume was 100 µl.

2.3. 16S rRNA amplicon sequencing and data analysis

Inoculum, FW, CM, FW/CM and graphite added FW/CM samples were collected from batch reactors at the end of the process (gas production nearly zero) to determine the microbial community diversity via pyrosequencing. The samples for DNA extraction were stored at -20 °C until use. Collected sludge samples washed three times with phosphate-buffered saline (pH 7.2) to avoid DNA contamination, then samples centrifuged (14,000 rpm) for 2 min at 4 °C.

DNA extraction was performed with DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions. Quality and concentration of extracted DNA were analyzed with nanodrop spectrophotometer (Q 5000, Quawell UV-VIS Spectrophotometer, USA). The purified PCR products were sequenced on 16S rRNA Illumina platform (Illumina, CA, USA). A polymerase chain reaction (PCR) targeting 16S rRNA genes was performed using the forward primer 16S Forward (TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 16S Forward Reverse (GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC) targeting the V3-V4 region.

OTU numbers were identified based on a 97% sequence similarity. Shannon and Simpson indexes of samples were determined using the R::vegan 7 package. Correlation between operational conditions and microbiome was assessed by Redundancy analysis (RDA) with the package easyCODA (v.0.31.1).

3. RESULTS AND DISCUSSION

3.1. Effects of graphite on co-digestion performance

Two of the key performance indicators in AD processes are biogas and CH_4 production rates. Cumulative biogas (Figure 2a) and CH_4 (Figure 2b) production rates are illustrated in Figure 1. Co-digestion of CM and FW has a positive effect on biogas and CH_4 production. Biogas production rates are 699, 943, and 1368 mL/g VS for CM, FW and FW/CM, respectively. Some similar values obtained at literature as 657 mL/g VS for CM [23] and 960 mL/g VS for FW [24]. Co- digestion improved biogas production 95% by CM and 45% by FW. The previous studies suggested that the use of conductive materials (such as biochar, granular activated carbon, magnetite) can enhance the efficiency of biogas/biomethane production, some of the recent waste activated sludge AD studies showed that; granular activated carbon enhances the methane yield by 17.4%, nano-carbon powder and nano-Al_2O_3 addition increase biogas production by 16.9% and 23.4% respectively [25-26]. Additionally, activated carbon has improved the efficiency of AD with different substrates such as waste activated sludge, food waste, and municipal solid waste [27-29]. Tian et al. showed that AD performance of glucose was improved by the addition of graphene during long-term anaerobic digestion under low temperature (10– 20 °C) [30]. Similarly, graphite addition also positively affects the biogas production, highest biogas production obtained from FW/CM+1 g/L sample with 1410 mL/g VS in this study. This value brings an additional 3% improvement in biogas production. Co-digestion and graphite addition improve biogas production up to %98 by CM and 48% by FW together.

Co-digestion and graphite addition also improve CH_4 production yield (Figure 2b). CH_4 production yields are 260, 212, and 425 mL/g VS for CM, FW and FW/CM, respectively. CH_4 production increment rates are 63% and 100% at CM and FW, respectively. The highest CH_4 production rate obtained from FW/CM+1.5 sample with 636 mL/g VS. The increase rate with co-digestion and graphite addition as a conductive material is 144% and 200% by CM and FW respectively. The addition of graphite as a conductive material is thought to support the direct interspecies electron transfer (DIET) metabolism between acetogens and methanogens. Therefore, the effect of graphite addition on CH_4 production is more pronounced. These results were consistent with the previous study that investigates the effect of magnetite-promoted DIET in anaerobic co-digestion of pig manure and wheat straw [31].

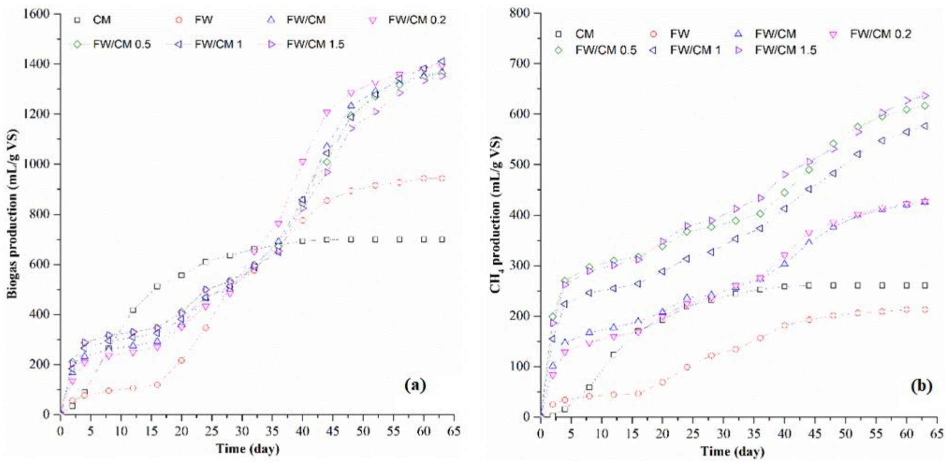

Figure 2. Cumulative biogas (a) and CH_4 (b) production rates for non-graphite added CM, FW, FW/CM and graphite added at different concentrations FW/CM samples.

3.2. Correlation between the microbial community and operational conditions

For evaluating the effects of adding graphite and co-digestion on digestion, the microbiome composition was analyzed using the Illumina sequencing. The structure of the microbiome (bacteria and archaea) in digesters with graphite addition are shown in Figure 3 and 5. The pyrosequencing results indicated that microbial community structure was changed depending on substrate type and graphite dosage in the batch digesters. *Actinobacilum* (9%), *Treponema* (7%), *Belliniea* (6%) were the most abundant bacteria of inoculum. In the mixture reactor, the relative abundance of the microbiome was observed as *Clostridium* (15%), *Selenomonas* (11%) and *Pedobacter* (7%), while it changed as *Clostridium* (12-18%), *Curvibacter* (7-9%) and *Selenomonas* (6-8%) with the graphite addition to the batch digesters (FW/CM+0.4, FW/CM+0.75, FW/CM+1 and FW/CM+1.5).

It was clear that the relative abundance of domain bacteria changed with graphite supplementation. This finding was similar to the results reported by Baek et al., (2017), community structure in magnetite added reactors changed significantly and resulted in high rate biomethanation [32]. In the present study, the supplementation of graphite (especially, 1.5 g/L) resulted in improved biomethane production rates (Figure 2b). These results suggest that the promotion effects occurred on biogas production due to abundances of *Clostridium* via DIET as presented in the previous study [33].

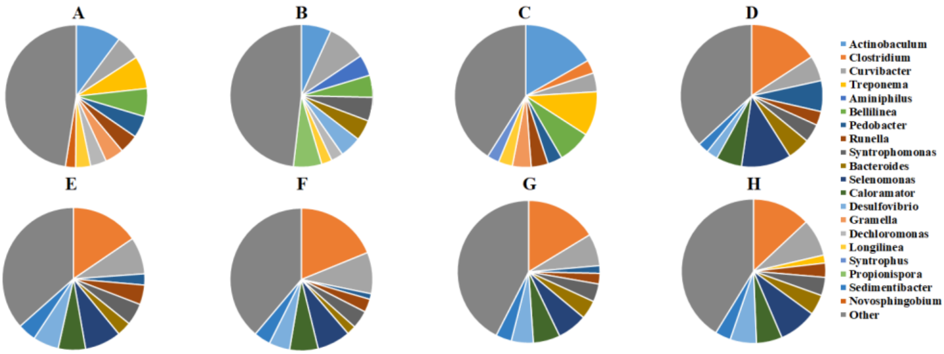

Figure 3. Relative abundance of bacterial community in batch reactors
(**A:** Inoculum; **B:** FW; **C:** CM; **D:** FW/CM; **E:** FW/CM+0.2; **F:** FW/CM+0.4; **G:** FW/CM+1; **H:** FW/CM+1.5)

The balance between acidogenesis and methanogenesis can result in a process promotion in anaerobic digestion of organic matters [34-35]. Recent studies have revealed that electrons can be directly transferred from acetogens to methanogens by conductive materials in anaerobic digestion (DIET) [36-37] (Figure 4). In this paper, *Methanosaeta* (77%), *Methanolinea* (11%), *Methanoculleus* (3%) were dominant methanogens in the inoculum. However, *Methanosaeta* (64%), *Methanosarcina* (17%), *Methanobacterium* (7%) were the dominant archaea in a non-graphite added mixture digester (FW/CM). The relative proportions of the dominant archaea were found *Methanosaeta* (36–81%), *Methanospirillum* (5–7%) (FW/CM +0.4 and FW/CM+0.75) and *Methanosarcina* (16–53%) (FW/CM+1 and FW/CM+1.5) in graphite supplemented digesters (Figure 5).

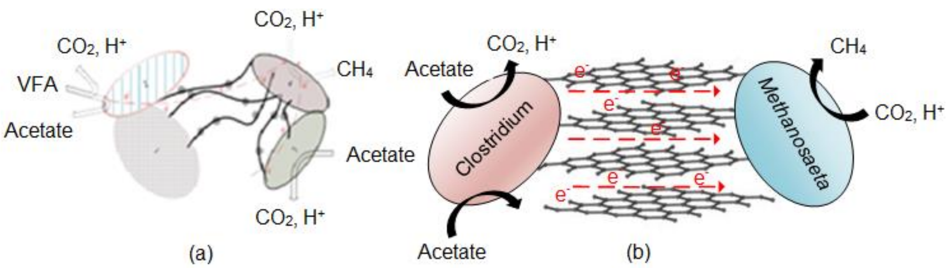

Figure 4. a) Biological DIET b) DIET via graphite[30][30]

With the gradual increase in the amount of graphite, the relative abundance of *Methanosarcina* has increased and as a result of leading to the highest biomethane production at 1.5 g of graphite addition (Figure 5). The similar community structures of digesters have been reported in various sources when used different conductive materials as a supplement [38]. For instance, Chen et al. (2014) investigated biochar for improving DIET kinetics between *Geobacter* and *Methanosarcina* [39]. Dang et al. (2016) observed that *Methanosaeta* and *Methanosarcina* species are capable of electron transfers through biological connections directly (DIET) [27].

Similarly, the presence of *Methanosarcina* on the AD biofilm structure shows that obtained suitable conditions to participate in DIET with the graphite in this study.

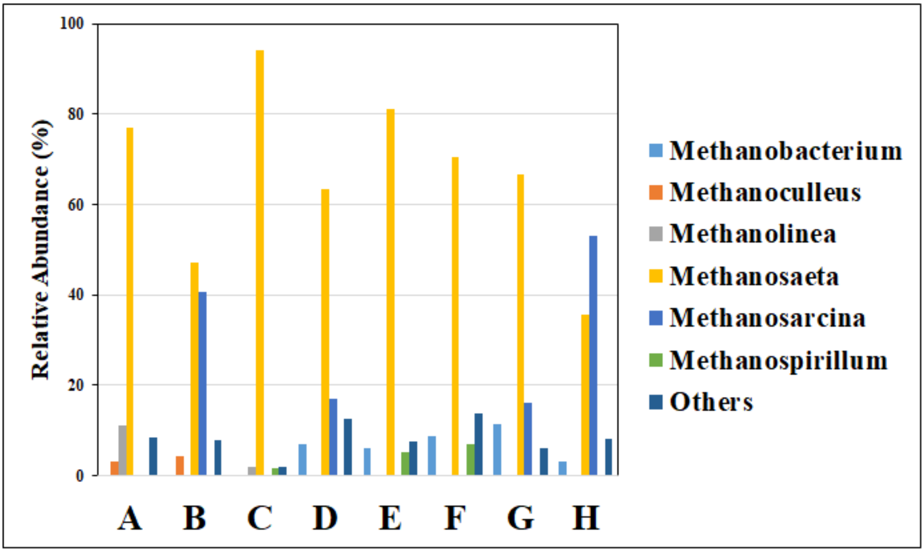

Figure 5. The relative abundance of the archaeal community in batch reactors

The diversity statistics of the microbiome are listed in Table 2. The results demonstrated that the operational taxonomic unit (OTU number) of inoculum was the highest (831) as compared to other digesters. On the other hand, the Shannon index was relatively higher after graphite supplementation, suggesting a higher bacterial diversity as compared to non-graphite digesters. These results show that graphite supplementation in mixture digesters was increased the species richness and diversity also contributing to improving anaerobic digestion performance. Therefore, higher index value and the number of species were found in graphite-added G, H reactors compared with non-graphite added digester. These results overlap with the value of biogas production from the anaerobic digesters.

Table 2. Diversity statistics of the community of anaerobic batch reactors with/without graphite addition

Substrate Type	Sample Number	Shannon Index	Simpson Index	OTU number
Inoculum	A	3,80	0,944335	831
FW	B	3,82	0,953374	639
CM	C	3,59	0,913455	650
FW+CM	D	3,79	0,944317	690
FW+CM (Graphite Added)	E	3,87	0,952135	613
	F	3,87	0,950631	655
	G	4,01	0,959203	697
	H	3,97	0,953155	760

The correlation between the microbiome and operational parameters was analyzed by Redundancy analysis (RDA). RDA was applied on four parameters belong to digesters including pH, biogas production, biomethane production and TS/VS with the 11 microorganisms from samples (10 genera of bacteria and 1 genus of archaea). The ordination axes explained 95.8% and 3.5% of the microbial community variations, respectively (Figure 6). According to Figure 6, the dominant microorganisms nearly located into two areas, the first part of the species has been seen related to TS/VS, and on the other hand, the second part of them has been seen related to pH, biogas, and biomethane production.

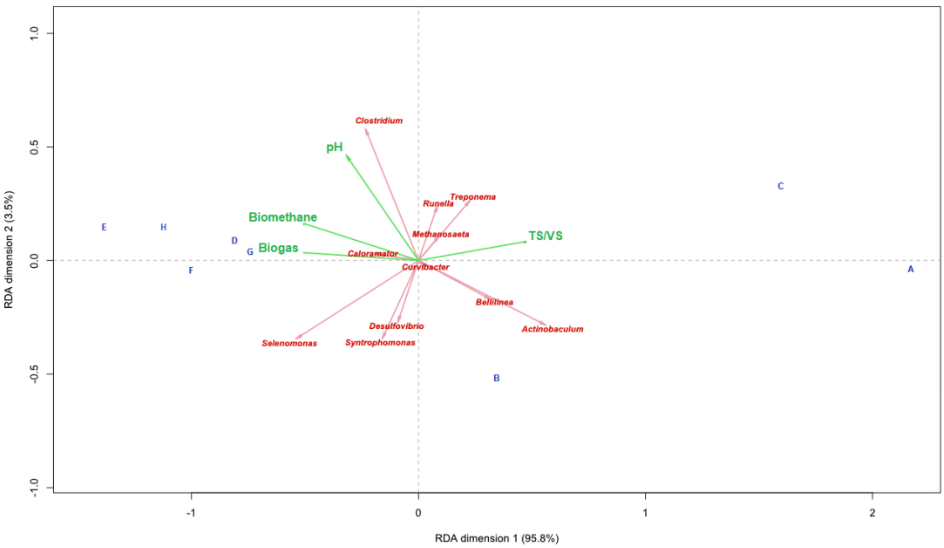

Figure 6. RDA of the relationship between the microbiome and operational conditionals for different reactors

RDA highlighted the presence of a strong relationship between *Methanosaeta* and biogas production. Additionally, *Clostridium* had a certain response relationship with pH and biogas/biomethane production [40]. Consequently, this correlation shows that there was a syntrophic association between *Clostridium* and methanogens leading to supporting methanogenesis. Ince et al. (2013) have observed a syntrophic relationship between *Clostridium* and methanogens by applying RDA [41]. Vanwonterghem et al. (2014) obtained a similar result in a study that conducted to explain how a process could respond to changes in operational conditions [16].

4. CONCLUSION

The results in the present study clearly showed that graphite supplementation was beneficial to improve methane yields at appropriate conditions. The highest biogas production obtained from FW/CM+1 g/L sample with 1410 mL/g VS and the highest CH_4 production rate obtained from FW/CM+1.5 sample with 636 mL/g VS. Besides, the pyrosequencing results indicated that microbial community structure was changed depending on substrate type and graphite dosage in the batch digesters. Additionally, redundancy analysis shows that *Clostridium* is the key microorganism of microbial community structure for improving digester performance in this study. The analysis of the correlation between microbiome and operational parameters represented that genera that belong to *Clostridium*, *Methanosaeta*, and *Methanosarcina*, all together could enhance to anaerobic digestion process with the graphite supplementation.

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