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Aged-engineered nanoparticles effect on sludge anaerobic digestion performance and associated microbial communities

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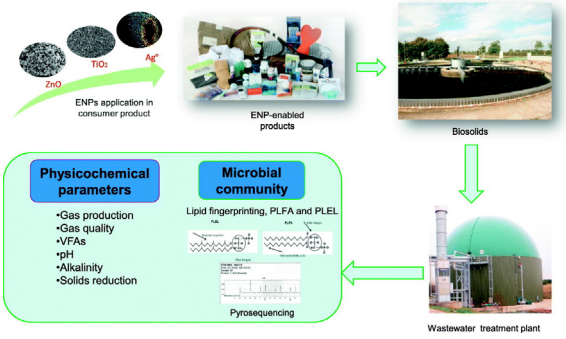
HIGHLIGHTS

- Aged engineered nanoparticles (ENP) did not affect significantly AD process and performance.
- Aged engineered nanoparticles (ENPs) lower H_2S and did not affect CH_4 production.
- *Actinobacteria* and *Fusobacteria* were competitively tolerant to ENP.
- *M. acetivorans* and *M. barkeri* are nano-tolerant methanogens in AD.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate the potential effect of aged engineered nanoparticles (a-ENPs) on sludge digestion performance, 150 L pilot anaerobic digesters (AD) were fed with a blend of primary and waste activated sludge spiked either with a mixture of silver oxide, titanium dioxide and zinc oxide or a mixture of their equivalent bulk metal salts to achieve a target concentration of 250, 2000, and 2800 mg kg^{-1} dry weight, respectively. Volatile fatty acids (VFA) were 1.2 times higher in the spiked digesters and significantly different ($p = 0.05$) from the control conditions. Specifically, isovaleric acid concentration was 2 times lower in the control digester compared to the spiked digesters, whereas hydrogen sulfide was 2 times lower in the ENPs spiked digester indicating inhibitory effect on sulfate reducing microorganisms. Based on the ether-linked isoprenoids concentration, the total abundance of methanogens was 1.4 times lower in the ENPs spiked digester than in the control and metal salt spiked digesters. Pyrosequencing indicated 80% decrease in abundance and diversity of methanogens in ENPs spiked digester compared to the control digester. *Methanosarcina acetivorans* and *Methanosarcina barkeri* were identified as nano-tolerant as their relative abundance increased by a factor of 6 and 11, respectively, compared to the other digesters. The results further provide compelling evidence on the resilience of *Fusobacteria*, *Actinobacteria* and the Trojan horse-like effect of ENPs which offered a competitive advantage to some organisms while reducing microbial abundance and diversity.

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1. Introduction

In recent years, engineered nanoparticles (ENPs) have been incorporated into many consumer products for their broad microbiostatic and biocidal properties (Suresh et al., 2010). Their extensive use and eventual release in the environment have raised concerned on the potential impacts on the environment and human health (Wiesner et al., 2009). Wastewater treatment plants have been identified as one of the main route (Gottschalk and Nowack, 2011), as most ENPs released from consumer products will sorb to biosolids and be transported to the wastewater treatment plant. Reported concentrations in biosolids vary from relatively low levels of 12–25 mg $kg^{-1}{}_{biosolids}$ and 7–39 mg/$kg_{biosolids}$ for zinc and silver respectively to higher values of 3–100 mg/$kg_{biosolids}$ for titanium (Blaser et al., 2008; Kiser et al., 2009; USEPA, 2009). In this context, ENPs has not only elicited concerns on their potential effects on humans and the environment but also on their influence on the biological processes occurring within wastewater treatment plants (Maurer-Jones et al., 2013). Microbiologically mediated processes can alter ENP core and surface functionality that will influence the fate, transport and toxicity of ENPs turning them into 'aged ENPs' (Barton et al., 2014; Eduok et al., 2015). It is important to remember that most of the published work on the biological effect of nanomaterials have been done at laboratory scale using pristine forms of ENPs and pure cultures of microorganisms; therefore caution should be taken in extrapolating the observed effects to microbial community in complex samples such as wastewater sludge (Eduok et al., 2013). The antimicrobial action of ENPs on aerobic wastewater processes has been well studied and documented (Zhang et al., 2016; Zheng et al., 2015; Li et al., 2014; Sun et al., 2013; Chen et al., 2012; Zheng et al., 2011; Brar et al., 2010; Choi and Hu, 2008) although there are still gaps in knowledge in relation to their impact on anaerobic processes, such as anaerobic digestion (AD) to generate biogas. Biogas production during AD process involves a complex multi-step sequence of substrate hydrolysis, fermentation and methanogenesis catalysed by diverse and unique microbial communities (Appels et al., 2008; Johnson et al., 2003; Schink, 1997) with different optimum requirements for growth and metabolism. To ensure efficient reactor performance it is important to maintain a subtle balance between the different process parameters and potential inhibitors, including heavy metals (Demirel and Yenigun, 2002). Heavy metals such as Zn, Cr, Cu, and Cd are able to hinder microbial activity in AD (Aquino and Stuckey, 2007). In contrast, their correspondent metal nanoparticles have not always exhibited the same physicochemical and toxicological effects. For example, EC_{50} concentration for inhibition of methane production by copper was attained at 10.7 mg L^{-1} for CuO nanoparticles and 129 mg L^{-1} for the correspondent bulk CuO (Luna-del Risco et al., 2011). Similarly, inhibition was recorded at 57.4 mg L^{-1} for ZnO nanoparticles and at 101 mg L^{-1} for the bulk correspondent ZnO (Luna-del Risco et al., 2011). Another author reported 18.3% and 75.1% methane inhibition at the concentrations of 30 and 150 mg ZnO/gTSS for the nanoparticle and the bulk respectively (Mu et al., 2011). In the case of silver (Ag) the reactors containing AgNPs treated sludge have not shown any significant difference in methane production compared to the controls at concentrations up to 40 mg L^{-1} (Yang et al., 2013). The performance of anaerobic digesters containing ENPs-enriched sludge depends on the antagonistic and/or synergistic interactions between bacteria and *Archaea*, although empirical data on the impact of aged-ENPs on Bacteria and Archaea in anaerobic process is still scarce. In this study, three parallel pilot-scale anaerobic digesters were used to assess the effect of aged-ENPs such as silver oxide (Ag°), titanium dioxide (TiO_2) and zinc oxide (ZnO) on the microbial community, their influence on volatile fatty acids (VFA) and biogas production.

2. Materials and methods

2.1. Pilot plant set up

Three parallel pilot-scale plants, each consisting of primary clarifier (180 L), secondary clarifier (~150 L), activated sludge tank (~300 L) and anaerobic digester (150 L) were used in the study (Fig. S1-S1 in supporting information) as previously described by Eduok et al. (2015). Identical conditions were maintained in the three plants with exception that treatment lines 1 and 2 were spiked with ENPs and bulk metal salts respectively, whereas treatment line 3 served as control (unspiked). The three ENPs used were chosen based on their wide application in many consumer products with particle size of 20 nm for Ag^0 and ZnO, and 21 nm for TiO_2. The ENPs are most likely to be transformed and accumulate in biosolids from wastewater treatment processes and ultimately in the soil. Silver was proprietary solution of Ag nanoparticles coated with polyvinylpyrrolidone (PVP). Zinc was a high purity and high quality zinc oxide nano-powder commercially known as Nanosun™. Titanium was high purity titanium oxide nano-powder commercially known as Aeroxide P25 (Degussa, Germany). The solution of mixed ENPs was made up of 0.01 mg L^{-1} Ag^0, 0.08 mg L^{-1} TiO_2, and 0.12 mg L^{-1} ZnO and the activated sludge was spiked at the rate of 0.14 mL min^{-1} (equivalent to 0.67 mL^{-1} L day^{-1}) for 315 days (details in supporting information). An equivalent concentration of bulk metal salts consisting of silver nitrate ($AgNO_3$), TiO_2, and anhydrous zinc nitrate ($Zn(NO_3)_2 \cdot 6H_2O$) and unspiked sludge (control) was used for comparison. The mixed ENPs and metal salt suspensions were maintained in dispersed state by continuous stirring at 200 rpm.

2.2. Anaerobic digester plants set up

The anaerobic digesters (AD) were batch-fed daily with freshly blended primary sludge (8 L) and thickened WAS (2 L) of 0.405 kg VS m^3 d^{-1} equivalent organic loading rate (OLR) without pre-treatment. The digester temperature was maintained with the aid of heating jacket at 35 ± 2 °C in a wet digestion condition. Prior to spiking with either ENPs or metal salt blended sludge, all digesters were operated at a fixed sludge retention time (SRT) of 15 days with a working volume of 121.5 L and subsequently operated over 3 SRT (45 days) to stabilize. ENPs-enriched waste activated sludge (WAS), thickened using Polygold® C420 coagulant (Goldcrest, Barnsley, UK) to 2 L and blended with 8 L settled primary sludge was spiked with 5 mg L^{-1} Ag^0, 40 mg L^{-1} TiO_2, and 56 mg L^{-1} ZnO and batch-fed into the digesters. To minimize the fluctuation in pH as a result of the high organic loading rate, the pH of blended sludge was adjusted with sodium bicarbonate anhydrous (Na_2CO_3, 99.5% pure) buffer to 7.2 ± 0.2. The targeted final concentration of the nanoparticles in the blended sludge (WAS + primary sludge) was 250, 2000, and 2800 mg kg^{-1} for Ag^0, TiO_2 and ZnO, respectively. Equivalent concentration of bulk metal salts comprising of titanium dioxide (TiO_2), silver nitrate ($AgNO_3$) and anhydrous zinc nitrate ($Zn(NO_3)_2.6H_2O$) and unspiked sludge (control) was used for comparison. The spiked concentrations of ENPs and metal salts represent the worst case scenario of the maximum allowable concentrations in sludge spread to agricultural land with reference to the Transatlantic Initiative on Nanoparticle and the Environment (TINE) project (NE/H01375X/1). Digestate circulation at a rate of 240 L h^{-1} was applied using a Mono pump (620S, Watson Marlow, UK) to ensure complete mixing of the digester content. 10 L of digestate was removed from each digester after HRT of 15 days and stored in holding tanks at 4 °C. The digestate produced from the 3 CE plants over 295 days of operation were dewatered in 200 L batches to 25% dry solid using the laboratory filter press. Prior to dewatering, 1.25 g L^{-1} solution of Polygold® C540 coagulant (Goldcrest, Barnsley, UK) was added to thicken the digestate and enhance solid-liquid separation. 10 g subsamples of dewatered digestate and filtrate were then stored at 4 °C until analysis.

2.3. Biogas production and physicochemical characterisation

The volume of biogas (CH_4, CO_2, O_2 and H_2S) produced by the anaerobic digesters was measured daily by the use of Geotech Biogas 5000 Portable Biogas analyser (Keison Products, Chelmsford, Essex, UK) connected to the digester (Fig. S1-S1 in supporting information). A thermostatically regulated heating jacket was used to control the operating temperature, whereas pH of the blended sludge fed into the reactors and digestate was measured by the use of Jenway Model 3540 Bench Combined conductivity/pH meter (Keison Products, Chelmsford, Essex, UK).

2.4. Measurement of volatile fatty acids (VFA)

To measure the changes in VFA composition when the reactors were stable, 100 mL duplicate digestate samples were centrifuged at 5000 rpm for 20 min and filtered through 0.45 μm and then 0.2 μm syringe filter. 10 μL of sulphuric acid was added to 9 mL of filtered sample to inactivate and stabilize the content, and frozen until analysis. VFA concentrations were determined by high performance liquid chromatography (HPLC) on Kontron 535 detector (Bio-Tek, Vermont, USA) with a Bio-Rad (California, USA) HPLC column for fermentation monitoring. The column was maintained at 60 °C with an eluent of 1 mM H_2SO_4 at a flow rate of 0.8 mL min^{-1}. VFA concentrations were detected with ultraviolet light at 208 nm.

2.5. Determination of ENPs and metal salts concentration

The residual concentration of ENPs and metal salts in form of Ag^{+}, Ti^{4+} and Zn^{2+} in the 60 digestate samples (10 duplicate samples) from each treatment per batch (Table S1-S1 in supporting information) was measured by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES, Perkin Elmer 4300DV). Batch 1 and 2 represents the timeline for attaining the targeted concentration of ENPs and metal salt in the digestate. Sample analysis was carried out externally by a commercial laboratory (Environmental Scientific Group, Straffordshire, UK). Briefly, the samples were digested prior to analysis using high purity nitric acid, hydrogen peroxide and hydrofluoric acid in sealed Teflon vessels with microwave assisted heating. After digestion, demineralized water having a resistivity of 18.2 MΩ·cm was used in making the samples into known volume. Ag^{+}, Ti^{4+} and Zn^{2+} concentrations were measured by ICP-AES calibrated using certified standards with 0.2, 0.5, 1.0 mg kg^{-1} and 0.03, 0.04 and 0.2 mg L^{-1} limit of detection for Ag^{+}, Ti^{4+} and Zn^{2+} in the digestate dry mass and filtrate respectively. 5.0 mg L^{-1} Ag, Ti and Zn prepared from an alternative source stock different from that used for the instrument calibration standard was measured with the samples as a quality control measure. A blank digestion vessel spiked with equivalent of 4.0 mg L^{-1} Ag Ti, and Zn was taken through the same procedure as a further quality control measure.

2.6. Phospholipid ether lipids (PLEL) analysis

For PLEL, total lipids were extracted from 40 g of freeze-dried digestate as previously described (Eduok et al., 2015). Briefly, 5 g of freeze-dried digestate samples was extracted using 0.8:1:2 (v/v/v) citrate buffer-chloroform-methanol, subjected to solid-phase fractionation followed by transesterification using mild alkaline methanolysis. Aliquots of the phospholipid fraction equivalent to ~12.5 g dry matter were subjected to PLEL analysis (Gattinger et al., 2003). Acid hydrolysis and methylated cleavage of the polar head group was performed using 2 mL of methanol: chloroform: 37% hydrochloric acid (10:1:1, v/v/v). The dried ether-linked isoprenoids were then reconstituted in 0.2 mL of hexane and analysed by gas chromatography coupled to mass spectrometry (GC–MS Agilent Technologies 6890 N) according to the operating conditions (Gattinger et al., 2003). 200 μL nonadecanoic acid methyl ester (Sigma, UK) was added as an internal standard to each sample after solid phase extraction. Archaeal biomass was estimated using a conversion factor of 5.9×10^{12} cells per 2.5 μmol PLEL (Bai et al., 2000).

2.7. Pyrosequencing analysis of the digestate samples

The microbial diversity and dynamics in the digesters was investigated by extracting total genomic DNA from 200 mg wet weight digestate samples in duplicate and applying the whole genome shotgun sequencing method of the Genome Sequencer FLX System (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. For amplification of bacterial 16S rRNA gene fragments (Hamady et al., 2008), PCR primers were adapted for 454 amplicon sequencing by attaching the M13 adapter (CACGACGTTGTAAAACGA) to the primer M13–16S-IA-FL (5′–CACGACGTTGTAAAACGACCATGCTG CCTCCCGTAGGAGT–3′), whereas the 25-mer Lib-L-specific sequence adapter B (CCTATCCCCTGTGTGCCTTGGCAGTC) was followed by the reverse template-specific primer sequence 16S-IA-RL (5′–CCTATCCCCTG TGTGCCTTGGCAGTCTCAGAGAGTTTGATCCTG-GCTCAG–3′). To aid multiplexing different samples, different barcodes were included in the M13 adapter using the 454 sequence adapter A (CCATCTCATCC CTGCGTGTCTCCGAC) and a 454 amplicon sequencing-specific 4-mer amplification key (TCAG) followed by a 10-mer barcode sequence (NNNN) (5′–CCATCTCATCCCTGCGTGTCTCCGACTCAGNNNNCACGAC GTTGT-AAAACGAC–3′). The amplification of the archaeal 16S rRNA gene fragment was carried out using primers ARC-344F (5′- ACGACG TTGTAAAACGAACGGGGYGCAGCAGGCGCGA) and ARC-915R (5′-CTAT CCCCTGTGTGCCTTGGCAGTCTCAGGTGCTCCCCCGCCAATTCCT-713′) which were adapted for multiplexing and 454 sequencing as described above. The PCR conditions were as described by Eduok et al. (2015). Quality control (QC) was carried out on the sequences using the Galaxy platform (Sequences with quality score lower <20 and shorter than 100 bp were removed). Further QC, chimera checking, OTU clustering, and taxonomic assignment with the Ribosomal database project (RDP) Bayesian classifier (http://rdp.cme.msu.edu/) was carried out using the CloVR-16S 1.0 pipeline (http://clovr.org/) according to White et al. (2011) as described in Eduok et al. (2015).

Prior to QC, there were 69,964 sequences; 51 low quality and 1365 short length sequences were removed. The dataset had 68,584 sequences that were grouped into 11 Operational Taxonomic Units (OTUs) representing the Archaeal community structure in the digesters. To identify the methanogenic *Archaea*, the different nucleotide sequences were blasted using the online National Centre for Biotechnology Information (NCBI) BLAST® tool. Nucleotide sequence length of 536–566 with >200 alignment and maximum identity match of 94–98% to the closest cultivable archaeal relative was obtained.

2.8. Statistical analysis

Kruskal-Wallis test was performed using *Statistica* software® version 11 (Statsoft, Tulsa, OK, USA). Values are presented as mean ± standard deviation with levels of significance maintained at 95% for each test. Biomass data based on the lipid and ether lipid profiles were log-transformed to reduce skewness in distribution, subjected to species-dependent hierarchical cluster analysis and non-metric multidimensional scaling (NMDS) ordination based on Bray-Curtis similarity measure using PRIMER version 6 (Clarke and Warwick, 2001).

3. Results and discussion

3.1. Biogas composition and production

Biogas production is used as an indicator of sludge digestion efficiency and rate of stable biosolids from AD process. The mean value of methane produced was 35 ± 15%, 26 ± 19% and 37 ± 19% in the control,

metal salts and ENPs spiked digesters respectively. A maximum of 73% (control), 71% (ENPs) and 70% (metal salts) methane content in the biogas was recorded at day 22, 106 and 187 days, respectively (Fig. 1) indicating there was a time lag for both microbial adaptation to the ionic concentrations of metal salts and ENPs compared to the control. The difference in methane content of the biogas from the three digesters was low but significant at $p < 0.05$. During the initial 120 days after spiking, the digestion process was unstable because of biomass washout which resulted in variable biogas production from the three digesters. From day 121 onwards, the average methane in the biogas increased substantially as the reactor operation stabilised, although between day 263 and day 295, methane in the biogas varied from 33 to 60% (control AD), 29 to 60% (metal salt spiked AD) and 24 to 50% in ENPs spiked AD.

The varying concentration of methane generated was in part influenced by lower digester temperature during feeding. For instance, the temperature fluctuation in excess of 0.6–1.0 °C day^{-1} can affect the anaerobic conversion process and methanogenic activity (Turovskiy and Mathai, 2006). In this study, digestate temperature fluctuated and slightly decreased from 35 ± 0.2 °C to 32 ± 2 °C during feeding suggesting temperature fluctuation probably influenced methane content in the biogas produced. At sub-optimum operational temperature in the digesters, anaerobic microbial growth and reaction rate were expected to decrease resulting in reduced formation of metabolic products. However, hydrogenotrophic methanogenesis can proceed at low temperature (Lettinga et al., 2001) and most likely was the metabolic pathways that methane composition of the biogas was generated when the digestate temperature fluctuated in our pilot scale experiment. Apart from temperature fluctuation, the presence of bicarbonates has been implicated in methane pathway shifts among hydrogenotrophic and aceticlastic methanogens. However, the pH of the control digestate was 7.6 ± 0.3, whereas 7.2 ± 0.7 and 7.5 ± 0.2 were obtained for the metal salt and ENPs spiked digestate respectively. Methanogens are sensitive to pH changes and are limited to a range of 7.0 to 7.6. (Lin, 1992). Apart from the slight fluctuation in the metal salt digestate, the pH of the other digestate were within range for optimum microbial activities in relation to the biogas produced because of the buffering effect. Overall, the result suggests that the digestate pH was not a factor that influenced the abundance of methanogen and volume of biogas produced.

Interestingly, hydrogen sulfide (H_2S) content of biogas from the three digesters differed substantially (Fig. 1). Mean gaseous H_2S concentration was 406, 208 and 186 ppm in the control, metal salt and ENPs spiked digesters, respectively. The result indicates that H_2S was at least 2 times lower in the ENPs spiked digester compared to the control and metal salt spiked digesters and suggest that ENPs influenced the activities of the sulfate-reducing bacteria (SRB) and thereby minimize risk of sour digester or digester failure. Although the concentration of H_2S in the digestate was not determined, its concentration in the gaseous phase suggests that the activities of SRB were negatively affected by the presence of ENPs and metal salts. The SRB are found mostly among the *Deltaproteobacteria* and *Firmicutes* and are responsible for generating H_2S, HS^- and S^2 in solution and H_2S in biogas from the reduction of sulfates (Wagner et al., 1998). It is undesirable for sulfide to accumulate in anaerobic digesters because the metabolic processes of aceticlastic methanogens are inhibited by approximately 50% at 50 mg L^{-1} and 100% at 200 mg L^{-1} of H_2S (Kroiss and Wabnegg, 1983). In addition, sulfide induces non-alkali metals which are essential for microbial growth to precipitate and thus reduce their availability for uptake with negative effect on the biogas production (Isa et al., 1986). Thus, ENPs inhibited SRB community, H_2S and complexes formed with wastewater component was unable to disrupt methane generated in ENPs spiked reactor.

3.2. Volatile fatty acids composition and concentration changes

Acidogenesis of glucose produces VFAs that are converted to methane during acetogenesis and methanogenesis. The mean VFA concentration varied across the 3 digesters (Fig. 2). The dominant VFA in the three digesters was acetic acid with a mean concentration of 1963.2 mg L^{-1}, 2447.1 mg L^{-1} and 2365 mg L^{-1} in the control, metal salt and ENPs

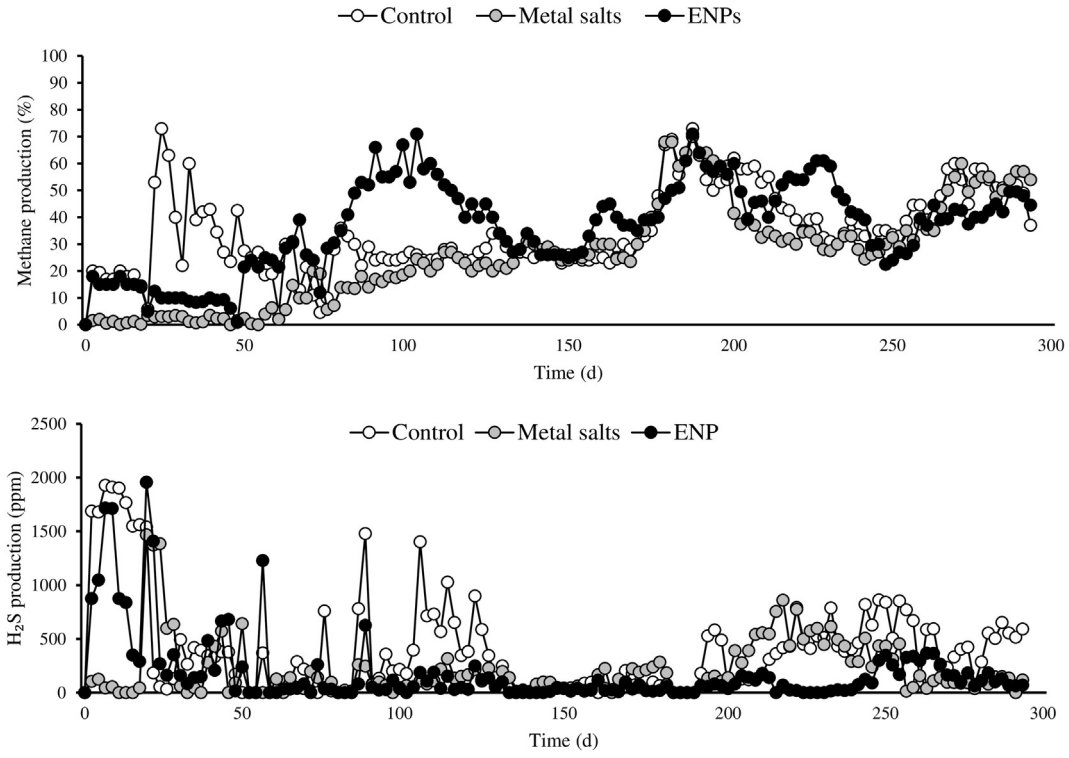

Fig. 1. Methane and hydrogen sulfide produced by the ENPs and metal salts spiked sludge from the pilot scale anaerobic digestion in relation to the control at 295 days of digester operation.

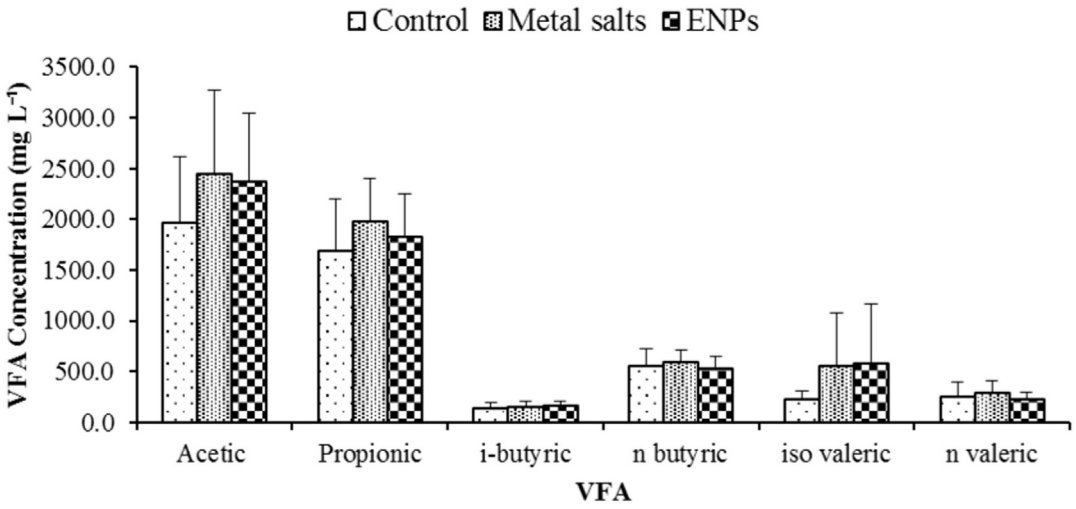

Fig. 2. Effect of mixed ENPs and metal salts on volatile fatty acid produced in the three digesters ($n = 60$). Accumulation of acetic acid suggests shifts in the metabolic pathway of the archaeal community from aceticlastic to hydrogenotrophic methanogens.

spiked digestate, respectively. The propionic acid content in the control digestate was 1692.6 mg L^{-1}, 1973.9 mg L^{-1} and 1828.1 mg L^{-1} in the metal salt and ENPs spiked digestate, respectively. The range of other VFAs across the three digestate were 138 to 156 mg L^{-1} (i-butyric acid), 530 to 592 mg L^{-1} (n-butyric acid), 229.8 to 583.4 mg L^{-1} (isovaleric acid) and 220 to 282.1 mg L^{-1} (n-valeric acid). The treatment increased total VFAs concentration by 1.2 and 1.1 times in the metal salts and ENPs spiked digestate, respectively compared to the control and this increase was statistically significant ($p < 0.05$). Low residence time as a result of biomass washout, slight temperature fluctuations during digester feeding and the digester feeding rate probably contributed to the variable VFAs concentration. The result is consistent with other studies in which increased acetic acid concentration was related to the metabolic pathway shift for methane production (Lin et al., 2013; Lettinga et al., 2001). Thus, the accumulation of acetic acid in the digesters indicates increased prevalence of hydrogenotrophic methanogens relative to acetoclastic methanogens.

Although the OLR and SRT resulted in organic overload and VFAs accumulation in the digesters, the propionic:acetic acid ratio was less than the value of 1.4 required to cause AD process instability (Hill et al., 1987). Several studies reported that high concentration of VFAs do not cause process imbalance especially in reactors with good buffering capacity where VFAs accumulation appears to exert no significant inhibitory effect on the methanogens (Nicol et al., 2004; Franke-Whittle et al., 2014). For instance, acetic and butyric acid concentrations of 2400 and 1800 mg L^{-1}, respectively, caused no significant inhibition of methanogenic activity when reactors have good buffering capacity (Wang et al., 2009). In contrast the methanogens were inhibited in reactor with poor buffering capacity at 900 mg L^{-1} propionic acid. Other studies showed that propionic acid concentration up to 2750 mg L^{-1} during anaerobic digestion did not influence the process performance and methanogenesis (Pullammanappallil et al., 2001). This is because VFAs have different and synergistic effect on AD microbial communities (Wang et al., 2009). Thus, in our overloaded but buffered digesters, the level of VFAs are considered normal under the experimental conditions that prevailed. This assertion is reasonable because the methane component of the biogas was continually generated and lowering of the pH due to the VFAs concentration was minimal. Although the propionic acid content in our digesters exceeded the reported 900 mg L^{-1} that can adversely affect methanogens, the concentrations were below 2750 mg L^{-1} and a near neutral pH maintained by the addition of the buffer minimized the potential negative effect on the organisms. Therefore, the effect on the methanogens was not as a result of pH fluctuations and varying VFAs concentrations in the digesters but mainly from the ions produced by the aged-ENPs.

Again, acid 3-methylbutanoic commonly called isovaleric acid, is a major end product of amino acid metabolism and is usually produced by microorganisms for post synthetic modification of structural molecules to increase membrane fluidity for growth under adverse conditions (Thierry et al., 2002; Kaneda, 1991). The isovaleric acid concentration was 229.8, 557.8 and 583.4 mg L^{-1} in the control, metal salt and ENPs-spiked digesters, respectively. The result indicates a 2.4 and 2.5 times lower level in the control than in the metal salt and ENPs spiked digesters, respectively but the difference was significant ($p < 0.05$) and suggest that the microbial cells in the control digester were not stressed compared to the organisms in the ENPs and metal spiked digesters. On the other hand, it is probable that most microbes in the ENPs and metal salt spiked digesters were in the stationary phase of growth, but produced isovaleric acid to increase membrane's resistance to the high concentration of toxic ions in the digestate (Kaneda, 1991). For example, members of *Methanosarcina* can reduce biomass growth and behave as resting cells while still producing methane (Welander and Metcalf, 2005). Indeed, among the methanogens recovered, *Methanosarcina* proliferated in the ENPs spiked digester and suggests that the inhibitory effect was selective and more pronounced on the other methanogens.

Notwithstanding, varying temperature can also influence the amount of isovaleric acid produced. For instance, at ≤24 °C more isovaleric acid is formed than at 30 °C (Kaneda, 1991; Hofherr et al., 1983). Thus the combined effect of ENPs or metal salt ions presence and the temperature that fluctuated during digester feeding probably induced the cells into a resting state and contributed to the high amount of isovaleric acid observed in the spiked digesters.

3.3. Residual concentrations of ENPs and metal salts in the digestate

The mean residual concentration of ENPs and metal salts in the three treatment plants are presented in Table 1 as ions (Ag^{+}, Ti^{4+} and Zn^{2+}) because of the difficulty of speciation between nano- and bulk forms of the metal oxides. The concentration of ions in the control digestate cake represents the baseline concentration of ENPs or metal salts ions in the sewage sludge. 122 mg kg^{-1} of Ag^{+} represents 49% of the spiked concentration (250 mg kg^{-1}) in the metal salt and ENPs spiked digesters, respectively was recovered. The 2482 and 2610 mg kg^{-1} of Ti^{4+} in the cake indicated 57% and 64%, whereas 1850 and 2128 mg kg^{-1} (66% and 77%) of Zn^{2+} was recovered in the ENPs and metal salts spiked

Table 1
Concentration of aged-ENPs and metal salts ions in filter-pressed anaerobic digestate cake.

	Average quantity in digestate cake (mg kg^{-1} dry weight)		
	Ag^{+}	Ti^{4+}	Zn^{2+}
Spiked concentration	250	2000	2800
Control AD	5.4 ± 2.2 (na)	1342 ± 203 (na)	757 ± 82 (na)
Metal salt spiked AD	122 ± 42 (49)	2610 ± 325 (64)	2128 ± 437 (77)
ENP spiked AD	122 ± 31 (49)	2482 ± 150 (57)	1850 ± 247 (66)
	Average quantity in digestate filtrate (mg L^{-1})		
Control AD	0.03 ± 0.0	0.07 ± 0.0	0.22 ± 0.12
Metal salt spiked AD	0.09 ± 0.12	0.75 ± 044	0.6 ± 0.6
ENP spiked AD	0.04 ± 0.02	0.4 ± 0.3	0.31 ± 0.01

Values are mean ± standard deviation, (%) of ENPs and metal salts partitioned into digestate cake (duplicate, $n = 60$). The limit of detection (LOD) in the filtrate was 0.03 mg L^{-1} (Ag^{+}), 0.04 mg L^{-1} (Ti^{4+}), 0.2 mg L^{-1} (Zn^{2+}) and 0.2, 0.5 and 1.0 mg kg^{-1} for Ag^{+}, Ti^{4+} and Zn^{2+} in the digestate cake respectively. Each value was corrected against the control (n.a = not applied).

digesters respectively. Indeed, the concentration of ions in ENPs spiked digestate cake was 1.12 to 1.17 times lower than in metal salt spiked digestate cake which indicates that the aged-ENPs mixture was either transformed, or soluble in the digestate and dissipated into the digestate liquor than the correspondent metal salts. It is known that ENPs can aggregate, adsorb, changing their oxidation state, precipitate or form complexes with ligands in wastewater (Xiu et al., 2011; Liu et al., 2010). Further to this, we suspect that the low concentration of aged-ENPs ions in the digestate was probably as a result of physicochemical transformation in the digestate in addition to the presence of solids which influenced ENPs behaviour and fate. For instance, in the ENPs spiked digestate, 57% of the spiked Ti^{4+} concentration compared to 64% of the metal salt ions, represents 1.12 times higher difference which was significant ($p < 0.05$). Thus, the result suggests that the solubility or sorption behaviour and potential fate of ENPs differed from the metal salt ion in anaerobic digestate.

3.4. Microbial community biomass, structure and dynamics

To understand the effect of ENPs and metal salts on anaerobic bacterial community structure and biomass, PLEL fingerprint analysis was carried out and four key biomarkers of fatty acid methyl esters (FAME) were identified including i15:0, i20:0, i20:1 (aceticlastic methanogens) and i40:0 (hydrogenotrophic methanogens). The FAME concentrations in the digestate ranged from 2 to 11.4 μg g^{-1}, 1.5 to 11.1 μg g^{-1} and 1.2 to 6.9 μg g^{-1} in the control, metal salts and ENPs spiked digesters, respectively (Fig. 3). The total FAME biomarkers in the ENPs spiked digester was 1.6 and 1.5 times lower than in the control and metal salt spiked digesters, respectively and the difference was significant at $p < 0.05$. The result indicates that methanogenic archaeal biomass was negatively affected by the presence of the aged-ENPs at the spiked concentration, and the reduced archaeal abundance can be directly correlated to the concentration of ENPs ions (Table 1). For instance, the residual concentration of Ag^{+}, Ti^{4+} and Zn^{2+} were 49%, 57% and 66%, respectively higher than the background concentration in the control. It is likely that inhibition of the organisms was exacerbated either by the synergistic, additive or potentiating effect of the ions produced by the ENPs. Furthermore, the FAME result indicates that ENPs ions were more soluble and bioavailable in the digestate which enhanced contact with the microorganisms to exert the damaging effects. In contrast, metal salts appeared less soluble and associated more firmly to the organic matter which therefore limited their interaction or contact with the microorganisms. The low abundance of methanogens in the ENPs spiked digester further demonstrates the potential effect of ions released by aged-ENPs and the interaction with anaerobic microorganisms. This is further indicated in Fig. S1-S2 in supporting document in which four discrete clusters at 70% similarity across the three digesters were observed with sub-clusters at 80% similarity and suggests a shift in the pattern of biomass concentration. The tight clustering of the different samples indicates that the methanogens were much less diverse or their taxonomic relatedness was almost similar in the three digesters. Overall, the reduced concentration of FAME biomarkers corroborates with the damaging effect of the ENPs, and is consistent with the scanning electron images of the microbial cells in the activated sludge exposed to the ENPs (Eduok et al., 2015).

3.5. Effect of treatment on anaerobic microbial community

The anaerobic bacterial phyla exposed to Ag^{+}, Ti^{4+} and Zn^{2+} are shown in Fig. 4. The closest cultivable relative and percentage similarity of the bacterial species indicates that *Firmicutes*, *Proteobacteria*, *Bacteriodetes* and unclassified bacteria were the most dominant phyla in all digesters. Overall, the presence of the ENPs exerted pronounced effect on the *Firmicutes*, *Proteobacteria* and *Bacteriodetes* compared to metal salt and control digesters because the relative abundance of all phyla with the exception of the unclassified bacteria was lower in the ENPs spiked digester. Specifically, *Firmicutes* abundance was 2 times lower in the ENPs spiked digester whereas the abundance of unclassified bacteria was 4 to 16 times higher at days 130, 160 and 190 (Fig. 4). Similarly, the relative abundances of *Proteobacteria* and *Bacteriodetes*

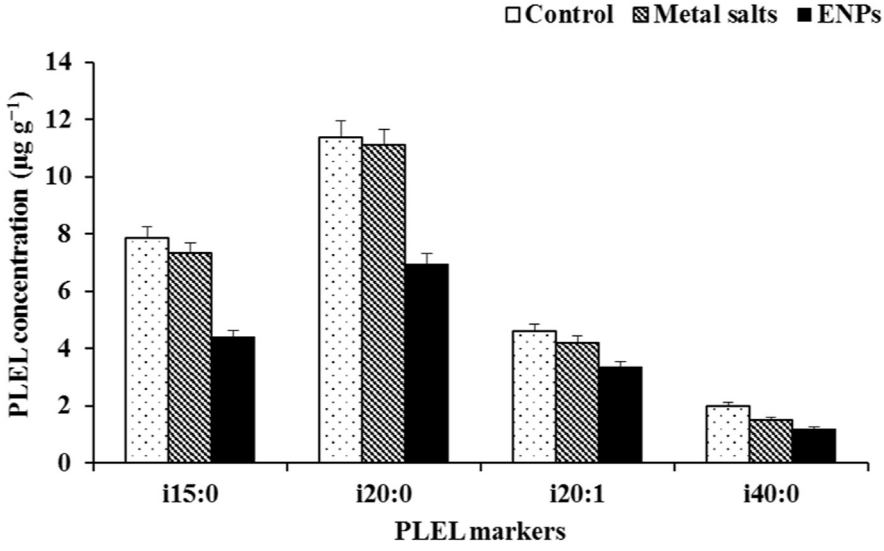

Fig. 3. Methanogenic Archaeal ether lipid fingerprint in the anaerobic digestate. Error bars representing standard deviation of FAME concentration in the digesters.

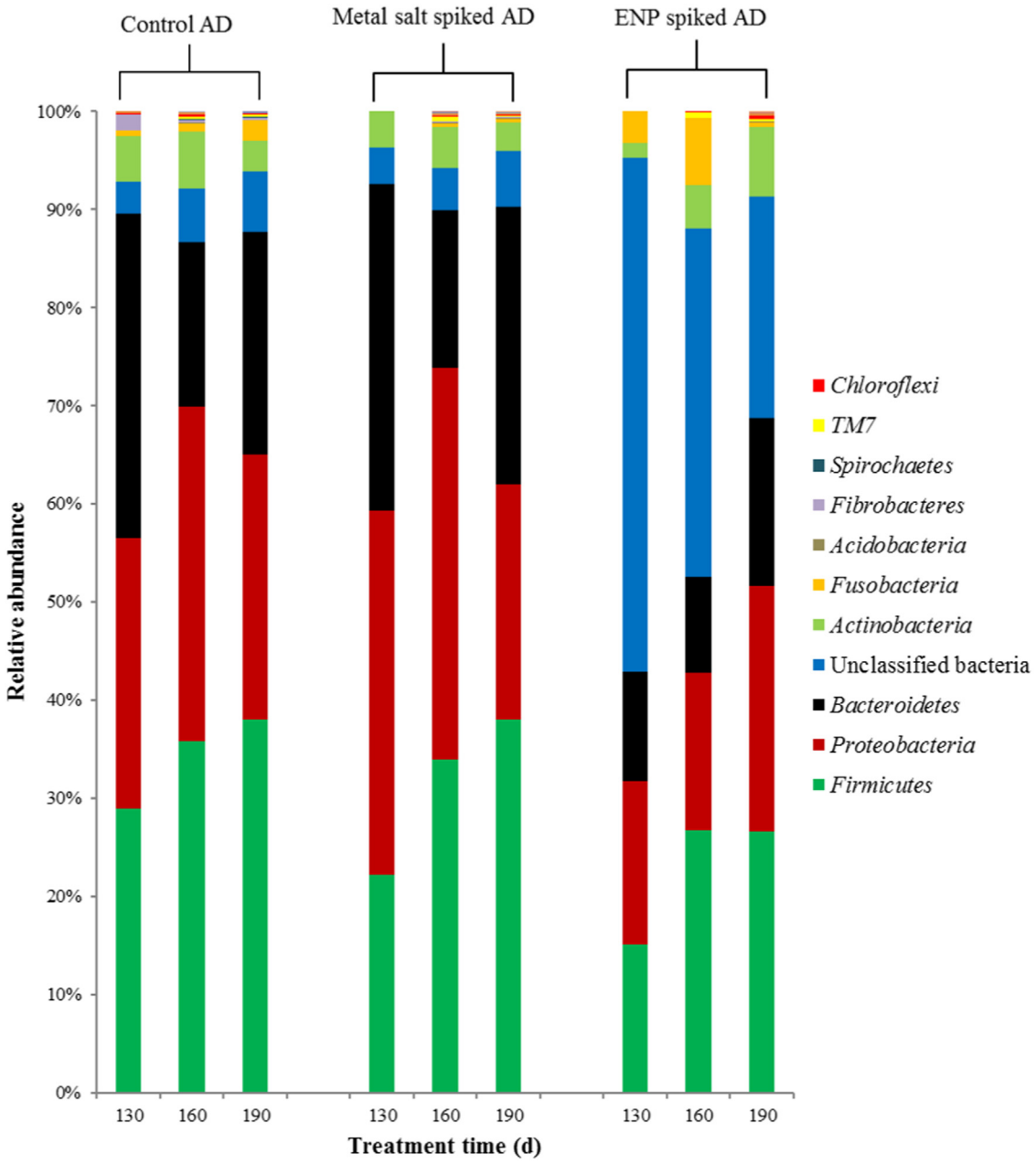

Fig. 4. Relative abundance and diversity of microbial community in AD between day 130 and 190 showing dominance of *Firmicutes* in the control, metal salts and ENPs spiked digestate.

were 3 times lower in the ENPs spiked digester compared to the control and metal salt spiked digesters. In contrast, *Fusobacteria* and *Actinobacteria* were about 8 and 2 times higher at days 160 and 190, respectively in the ENPs spiked digester. At day 130, the cluster of microbial community in the ENPs and metal salts spiked reactors was 80% similar in relation to the control (Fig. S1-S2 in supporting document). The tight clustering of the microbial community in the different samples at day 160 indicates that the organisms were less biodiverse or their taxonomic relatedness was almost similar in the three plants with a 50% shift in microbial community at day 190. The result indicates that initially there was inhibition followed by tolerance and a shift in microbial community composition.

Most members of the *Firmicutes* (Table S1-S1 in supporting information) are able to degrade organic molecules in a preferential and sequential way (Fonknechten et al., 2010). Their abundance in the digesters suggests that they played a key role in degrading complex materials during the digestion process. Members of the family *Peptococcaceae* belonging to class *Clostridia* such as *Desulfotomaculum*, *Desulfosprosinus* and *Desulfosporomusa* can reduce sulfate to H_2S (Sass et al., 2004). The low H_2S content in the biogas from the ENPs spiked digester can be attributed to the negative effect of the ions released from the aged-ENPs mixture on the SRB compared to the metal salt and control digesters. This is significant because accumulation of H_2S can inhibit the activities of methanogens and consequently reduce the digester performance, in addition corrode the digester.

Members of *Bacteriodetes* such as *Bacteriodia*, *Sphingobacteria* and *Flavobacteria* had their relative abundance reduced by up to 3 times in the in ENPs spiked digester. The results indicate that ions released by ENPs exerted a broad antimicrobial effect on the bacterial community structure and abundance. In spite of this, it is worth noting that the abundance of *Actinobacteria* in the ENPs spiked digester which was initially up to 3 times lower at day 130, increased over time and was up to 2.5 times higher than in the control and metal salt spiked digesters at day 190. This result indicates again, that the organisms were inhibited

at the initial exposure, but became tolerant of the ENPs effect and proliferated in the digester. Members of the *Propionibacterineae* capable of producing propionic acid were also identified in all digesters. Propionic acid is an intermediate product during AD process and about 30% of electrons required in methanogenesis are produced by microorganisms converting propionic acid to H_2 and acetic acid (Speece et al., 2006). This result suggests that propionic acid producing bacteria such as *Propionicimonas* and *Propionibacterium* species were not inhibited at the spiked concentrations of metal salt and ENPs.

Abundance of *Alphaproteobacteria and Betaproteobacteria* was up to 6 times lower in the ENP spiked digester. In contrast, the relative abundance of *Deltaproteobacteria* was <1% in the 3 AD plants. The relative abundance of unclassified bacteria belonging to different phyla in the ENPs spiked digester suggest that microbial diversity in a complex matrix such as anaerobic digestate is yet to be fully characterised.

Chloroflexi, the commonly known filamentous non sulfur bacteria was found in two-third (67%) of the metal salt and ENPs spiked digestate samples compared to the control samples (100%) which indicates that after the initial inhibition, *Chloroflexi* members were tolerant to the ionic concentration of ENPs and metal salts. Although the abundance of *Chloroflexi* in the digestate was low, members are able to degrade organic and cellular materials and nitrify (Kragelund et al., 2007). In synergy with other phylogenetic groups, the *Chloroflexi* probably converted ammonia to nitrate and offer competitive advantage for methanogens to thrive in the digesters.

3.6. Effects on methanogenic Archaea

To estimate the abundance and diversity of AD archaeal community, the identified 16S rRNA from high throughput 454-Pyrosequencing were grouped into 11 species level Operational Taxonomic Units (OTUs) representing the dominant microbial community structure with 0.01 dissimilarity clustering that spanned the v6 – v9 region. The methanogenic Archaea identified from the different DNA sequences from pyrosequencing were blasted using the online NCBI BLAST® tool. Nucleotide sequence length of 536–566 bp with >200 alignment and maximum identity match of 94–98% to the closest culturable archaeal relative was obtained. The methanogenic *Archaea* recovered belonged to the order Methanosarcinales, Methanobacteriales and Methanomicrobiales. The six closest species from five genera of methanogenic Archaea identified were *Methanobrevibacter acididurans* strain ATM, *Methanothermobacter thermoautotrophicus*, *Methanocorpusculum sinense*, *Methanosaeta concilii* GP6 strain, *Methanosarcina barkeri* strain HWS2.1, and *Methanosarcina acetivorans* C2A strain (Table 2). Despite the limited number of digestate samples analysed, sequences obtained when the pilot plant performance was stable in relation to biogas production indicate distinct temporal changes in the structure and relative abundance of the methanogens as a result of treatment. Indeed, a snapshot of data in the timeline of the anaerobic digesters at day 130, 160 and 190, provides insights on population dynamics of the methanogenic Archaea (Fig. S1-S3 and Table S1-S2 in supporting information). Here, only the genus *Methanosarcina* proliferated in the ENPs spiked reactor compared to the control and metal salt spiked reactor. This is significant because the three reactors were subjected to uniform conditions and suggests that the negative effect on the unrecovered methanogens was caused by the ENPs and its ions. The relative value of 80% similarity in abundance of the Archaea indicates high recovery of the methanogens (Fig. S1-S4 in supporting information). However, at day 130, the methanogenic community in the ENPs spiked digestate was 40% similar to the metal salt spiked digestate than to the control. The taxonomic similarity of the methanogens was less diverse and consistent with the result in Fig. 3. In the control AD, *Methanothermobacter* was not recovered whereas *Methanosaeta* (*Methanothrix*) was inhibited in the metal salts spiked AD (Table 2). Notably, *M. barkeri* and *M. acetivorans* were the only species identified in the ENPs spiked digester. *Methanosarcina* was tolerant to the spiked ENPs concentration thus positioning it as a key species in this study. Recent studies on the genome sequence of *Methanosarcina* reported that its genome consists of a complex organisation with uncommon features including large extrachromosomal material (Maeder et al., 2006) which possibly enabled *Methanosarcina* to adapt and repair damage by ENPs.

Besides the robust genome, *Methanosarcina* has a unique metabolic capability that enables the organism to grow on diverse substrate for methane production. For instance, members possess full complement of gene encoding enzymes for obligate reduction of CO_2 and methyl with H_2, aceticlastic conversion of acetate, metabolism of one carbon compounds (e.g. methylamine, dimethylsulfide, methanol, and methyl diols) through methylotrophic methanogenesis. Thus, *Methanosarcina* possesses all 4 methanogenic pathways required to produce methane in contrast to other methanogens with one pathway (Welander and Metcalf, 2005).

The conversion, stabilisation of organic matter and production of methane was maintained because *Methanosarcina* represents an adapted species able to competitively metabolize the available substrate in the presence of the toxic dose of mixed ENPs. It is common knowledge that based on the physicochemical properties, the presence of solids in the sludge can influence ENPs behaviour and fate by forming aggregate, adsorb, and change the oxidation state, precipitate or form complexes with ligands to mitigate toxic effect. In addition, antagonism of individual or mixed ENPs by substances in the sludge was a plausible factor in the reduced inhibitory effect. The low inhibitory effect of the ENPs on *Methanosarcina* has opened interesting questions for this keystone species in the digester that awaits further investigation, including what mechanism of tolerance made the organism to remain viable under the toxic exposure, and its potential use of ENPs as substrate. Furthermore, our results suggest that the threshold of ENPs inhibitory dose for an adapted biomass of methanogenic Archaea in a static-renewal test or flow-through reactor can be higher than predicted.

Although there are varying empirical evidences on the impact of different ENPs concentrations on AD microbial community in several mesophilic batch studies (Demirel, 2016), our data supports the view that the performance of flow-through pilot-scale anaerobic digester amended with mixed ENPs will depend on the resilience or susceptibility of the Archaea, the exposure concentration and operational conditions. Here, only the species of *Methanosarcina* were tolerant to the toxic dose of mixed ENPs in contrast to the species of *Methanobrevibacter*, *Methanothermobacter*, *Methanocorpusculum* and *Methanosaeta* that were susceptible. It therefore follows that the inhibitory or toxic effect of mixed and aged-ENPs on the methanogens was selective. Further to this, it suggests that interpretation made on a case-by-case approach based on dose-response or time-response relationships minimises bias. Overall, the result indicated that *Methanosarcina* was nano-tolerant and a strong candidate for the uptake, accumulation, use or conversion of ENPs.

Table 2
Percentage recovery of key methanogenic archaeal genera from anaerobic digestion of sludge.

Archaeal genus	Relative abundance of *Archaea* (%)			
	Control digestate	Metal salt spiked digestate	ENP spiked digestate	Total relative abundance
Methanobrevibacter	7	24	0	4.55
Methanothermobacter	0	4	0	0.41
Methancorpusculum	79	56	0	28
Methanothrix	4	0	0	1.24
Methanosarcina	9	16	100	65.7

ENPs adverse effect was more pronounced on the methanogens than the metal salts indicated by the non-recovery of four genera of the Archaea compared to the control and metal salt spiked digestate. The growth and proliferation of *Methanosarcina* suggests that the organism was able to adapt and tolerate the high concentration of the mixed ENPs and are referred to in this study as nano-tolerant species.

4. Conclusion

The presence of ENPs contributed to low H_2S concentration without significant decrease in methane production. Almost all key microbial phyla and their relative abundance decreased in the ENPs spiked digester compared to the metal salt spiked and control digesters. The most important findings were: (i) Reduced abundance of SRB which resulted in lower H_2S and therefore it was a positive outcome for AD performance (ii) *Actinobacteria* and *Fusobacteria* were competitively tolerant to the spiked ENPs concentration (iii) Increased abundance of *Methanosarcina* in presence of aged-ENPs provides strong evidence that *M. acetivorans* and *M. barkeri* are nano-tolerant methanogens. Although further research is still needed on the effect of ENPs on ecologically sensitive microbial species and microbial-mediated processes, our study provides empirical evidence at pilot scale that ENPs influenced the AD process performance and this was related to the shift in the microbial community structure especially the methanogenic community. Further to this, the impact of the bulk metal salts differed from the ENPs and therefore caution is needed when interpreting and extrapolating any allowable limits of ENPs in wastewater and or sludge.

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Appendix A. Supplementary data

The flow schematics of the pilot plant and details of ENPs effect on the bacterial and archaeal community by 454-Pyrosequencing are presented as supporting information. Supplementary data associated with this article can be found in the online version, at http://dx.doi.org/10.1016/j.scitotenv.2017.07.178.

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