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1 The effect of Fe_2NiO_4 and $\text{Fe}_4\text{NiO}_4\text{Zn}$ magnetic
2 nanoparticles on anaerobic digestion activity

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Abstract

Two types of magnetic nanoparticles (MNPs), i.e. Ni ferrite nanoparticles (Fe_2NiO_4) and Ni Zn ferrite nanoparticles ($\text{Fe}_4\text{NiO}_4\text{Zn}$) containing the trace metals Ni and Fe, were added to the anaerobic digestion of synthetic municipal wastewater at concentrations between 1-100 mg Ni L^{-1} in order to compare their effects on biogas (methane) production and sludge activity. Using the production of methane over time as a measure, the assays revealed that anaerobic digestion was stimulated by the addition of 100 mg Ni L^{-1} in Fe_2NiO_4 NPs, while it was inhibited by the addition of 1-100 mg Ni L^{-1} in $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs. Especially at 100 mg Ni L^{-1} , $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs resulted in a total inhibition of anaerobic digestion. The metabolic activity of the anaerobic sludge was tested using the resazurin reduction assay, and the assay clearly revealed the negative effect of $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs and the positive effect of Fe_2NiO_4 NPs. Re-feeding fresh synthetic medium reactivated the NPs added to the anaerobic sludge, except for the experiment with 100 mg Ni L^{-1} addition of $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs. The findings in this present study indicate a possible new strategy for NPs design to enhance anaerobic digestion.

KEYWORDS: anaerobic digestion; ferrite nanoparticles; resazurin; toxicity; stimulation

1. Introduction

As the technological benefits of nanotechnology begin to move rapidly from the laboratory to large-scale industrial application, release of nanomaterials to the environment is inevitable, and the major environmental receptors will be soil, sediment, and biosolids from wastewater treatment (Abhilash, 2010). Studies about the impact of nanoparticles (NPs) on the environment have become common in recent decades, and their potential effect on wastewater microorganisms has been thoroughly reviewed (Eduok et al., 2013), especially on anaerobic digestion (AD) (Chen et al., 2014). As one of the most commonly used biological processes for treating livestock manure, municipal wastewater solids, and even high strength industrial and hospital wastewater, AD breaks down organic waste in the absence of oxygen producing renewable energy (methane), biofertilizer and water (Stuckey, 2012). However, most of the studies examining the effect of NPs on AD are limited to a single metal, such as the NPs of Ag, Co, Ni, Fe, MgO, ZnO, and Fe₃O₄, and resulted in either a positive or negative impact on AD. For example, Abdelsalam et al. (2016) found that the addition of Fe NPs (20 mg L⁻¹) and Fe₃O₄ NPs (20 mg L⁻¹) significantly increased the biogas yield from the AD of cattle slurry and Juntupally et al. (2017) reported that addition of Fe₂O₃ NPs (12.5 mg L⁻¹) enhanced methane production from AD of cattle manure slurry. When studying the AD of waste activated sludge, Wang et al. (2016) found that the presence of 10 mg/g total suspended solids (TSS) nano zerovalent iron and 100 mg/g TSS Fe₂O₃ NPs increased methane production, whereas 500 mg/g TSS Ag NPs and 500 mg/g TSS MgO NPs generated lower levels of methane production. Mu and Chen (2011) found that 30 mg/g-TSS of ZnO NPs resulted in an 18.3% reduction in methane production in the AD of

waste activated sludge. In addition, it is well known that trace metals, such as Co and Ni, are essential constituents of cofactors and enzymes and their addition to anaerobic digesters has been shown to stimulate and stabilize biogas process performance (Chen et al., 2016; Mudhoo and Kumar, 2013). It was also found that the addition of Ni and Co NPs at 1 and 2 mg L⁻¹, respectively, stimulated biogas yield during AD (Abdelsalam et al., 2017).

Magnetic nanoparticles (MNPs) are a major class of nanoscale materials with the potential to revolutionize current clinical diagnostic and therapeutic techniques due to their interesting properties such as superparamagnetic behavior, a high surface area-to-volume ratio, and ease of separation using an external magnetic force (Sun et al., 2008). With a wide range of applications in the detection, diagnosis, and treatment of illnesses, such as cancer (Ferrari, 2005), cardiovascular disease (Wickline et al., 2007), and neurological disease (Corot et al., 2004), MNPs play a significant role in improving medical treatment for a variety of diseases. One important member of the MNP family, Ni Zn ferrite NPs, are found to have cytotoxicity against cancer cells, suggesting their potential application in cancer treatment (Al-Qubaisi et al., 2013a). Spinel ferrite NPs of the general formula AFe₂O₄ (NiFe₂O₄, CuFe₂O₄, CoFe₂O₄, and MnFe₂O₄) are another large group of nanomaterials with many applications, ranging from low wave-number to microwave and from low to high permeability including electronic devices, magnetic drug delivery, microwave devices, recording heads, and high density information storage (Gupta et al., 2017). In addition, nickel ferrite NPs were proposed as a potential adsorbent for the removal of the analgesic dipyrone from aqueous solution (Springer et al., 2016), indicating their potential applicability in the removal of pollutants.

With the wide use of MNPs, it is clear that these NPs will ultimately be released into the environment. To the best of the authors' knowledge, there has been no study reported in the literature about the effect of MNPs on AD. Hence, our present study investigated the effect of two types of MNPs, i.e. Ni ferrite nanoparticles (Fe_2NiO_4) and Ni Zn ferrite nanoparticles ($\text{Fe}_4\text{NiO}_4\text{Zn}$), on AD using methane production and the activity of anaerobic sludge as measures of their effects.

2. Materials and Methods

2.1 Chemicals

Resazurin (7-hydroxy-3H-phenoxazin-3-one 10-oxide), $\text{Fe}_4\text{NiO}_4\text{Zn}$ (<100 nm) and Fe_2NiO_4 (<50 nm) nano-powder were from Sigma-Aldrich, Singapore. Other reagents and chemicals used were of the highest available purity, and were obtained from Sigma-Aldrich, Singapore. Suspensions of $\text{Fe}_4\text{NiO}_4\text{Zn}$ and Fe_2NiO_4 NPs stock (2000 mg L^{-1}) were prepared by adding NPs to distilled water (pH 7.0), followed by 1 h of ultrasonication (40 kHz, 250 W). Dynamic light scattering (DLS) measurements were conducted using a Malvern Autosizer 4700 (Malvern Instruments, UK) to determine the average size of the NPs ($\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs: 89 ± 7.9 nm, Fe_2NiO_4 NPs: 46 ± 7.9 nm)

2.2 Anaerobic sludge

An anaerobic reactor was operated at $\sim 30^\circ\text{C}$ as the seed for use in testing the effects of NPs. The reactor was fed weekly with a synthetic medium consisting of glucose (1430 mg COD L^{-1}), peptone (430 mg COD L^{-1}), meat extract (140 mg COD L^{-1}), sodium bicarbonate (2000 mg L^{-1}), and K_2HPO_4 (80 mg L^{-1}) to give a final COD (chemical oxygen demand) of around 2 g L^{-1} .

2.3 Anaerobic digestion with nanoparticle addition and activity of anaerobic sludge

A certain volume of the $\text{Fe}_4\text{NiO}_4\text{Zn}$ and Fe_2NiO_4 NPs stock suspension was added to 125 mL serum bottles containing 100 mL of anaerobic sludge diluted with synthetic medium resulting in a final concentration of 1 g COD L^{-1} and a VSS of 1 g L^{-1} , and this resulted in 0, 1, 10 and 100 mg L^{-1} [Ni], and the corresponding [Fe], [Zn] and total NP concentrations are summarized in Table 1. The experimental setup (continuous mode) at each concentration had 3 replicates under anaerobic conditions; after 7 days, the sludge particles had settled and the medium above sludge particles was decanted and replaced by fresh feed (synthetic medium), resulting in 1 g COD L^{-1} . This length of time was considered adequate to indicate biogas inhibition compared to a control. After refeeding the experiments were continued for another two days.

Table 1

On day 7 and 8, 300 μL of anaerobic sludge was collected from the serum bottles for the resazurin reduction assay based on Chen et al.'s work (2015a), which has been used for studying the toxicity of chlorophenols (Chen et al., 2015b) and heavy metals (Chen et al., 2016; Ortiz et al., 2017). These 300 μL anaerobic sludge aliquots were transferred into a 96-well microplate with nitrogen gas purging, and resazurin was added to each well to result in a concentration of 25 ppm. The microplate was then sealed using sterile film, and triplicate experiments for each test were carried out at 35°C. The fluorescent signal was read every minute over half hour by an Infinite M200 Pro microplate reader (TECAN, Singapore) with TECAN i-control software at $\lambda_{\text{ex}}=530$ nm and $\lambda_{\text{em}}=590$ nm

with a gain of 30 (Rozzi et al., 2002). Resazurin, which is typically formulated into in-vitro toxicology assay kits (i.e. alamarBlue®), can be used as an oxidation-reduction indicator which results in colorimetric changes and a fluorescent signal in response to intercellular metabolic activity (Twigg, 1945) based on the reduction of resazurin to resorufin, and finally to dihydroresorufin (Scheme 1). The mechanism of resazurin reduction assay lies on that it is living cells but not medium that can reduce weakly fluorescent resazurin to highly fluorescent resorufin (Chen et al., 2018), and the more active the cells, the faster the reduction speed; hence, on the basis of the difference in kinetics of resazurin reduction, we can determine the activity of cells or active sludge (Chen et al., 2015b). Based on a pseudo-first order reaction rate, our previous study developed a model, $[B] = [A]_0 \frac{k_1}{k_2 - k_1} (e^{-k_1[M]t} - e^{-k_2[M]t})$, to describe the process of resazurin reduction in both pure culture, and mixed sludge under anaerobic conditions (Chen et al., 2016), where $[B]$ is the resorufin concentration linearly proportional to the fluorescent signal, $[A]_0$ is the initial resazurin concentration reacting in the cells, k_1 and k_2 are the reaction rate constants for resazurin to resorufin, and resorufin to dihydroresorufin, respectively, and $[M]$ is the total concentration of metabolites in the cells to reduce resazurin, which can represent the activity of the microorganisms.

By studying cumulative methane production and resazurin reduction, this extra two-day investigation after refeeding was to examine if the inhibition of anaerobic sludge by magnetic nanoparticles is temporary or permanent.

Scheme 1

2.4 Biogas production and composition

Biogas volumes from the serum bottles described above were measured daily by gas syringe. The composition of the biogas (methane- CH_4 , oxygen- O_2 , nitrogen- N_2 , and carbon dioxide- CO_2) produced was determined using a Shimadzu GC-TCD (2010 Plus) fitted with an Agilent J&W Column ($60 \text{ m} \times 0.53 \text{ mm} \times 30 \mu\text{m}$) and VICI Valcoplot column ($15 \text{ m} \times 0.53 \text{ mm} \times 20 \mu\text{m}$). The carrier gas was Helium set at a flow rate of 156 mL min^{-1} , while the column temperature was 35°C , detector temperature 100°C , and injector temperature was 40°C . The coefficient of variance (CV) for biogas composition for ten identical samples was within $\pm 3\%$.

2.5 Metal analysis

The concentrations of metals in the liquid phase were analyzed daily by MP-AES (Agilent model 4200). The anaerobic sample for metal analysis was first centrifuged at 10,000 rpm for 10 min, and then filtered through a $0.45 \mu\text{m}$ filter to remove any residual solids. After being acidified and digested with 1% (v/v) HNO_3 , the concentrations of Ni, Fe or Zn were analyzed by MP-AES. The detection limit was 0.01 mg L^{-1} , and the coefficient of variance (CV) for ten identical samples was within $\pm 1\%$. The metal content measured in this study was expressed as mg of metal/gTSS.

2.6 Data analysis

The fluorescent signals (RFU) obtained for activity of anaerobic sludge were converted to molar concentrations of resorufin by the linear calibration curve ($R^2=0.989$, $P<0.0001$), $\text{RFU}=6608.2[\text{RESORUFIN}]+1.76$, in the range of 0 to 0.1 mM. By fitting resorufin

concentrations over time to $[B] = [A]_0 \frac{k_1}{k_2 - k_1} (e^{-k_1[M]t} - e^{-k_2[M]t})$, k_1 and k_2 were obtained to show the kinetics of reduction of resazurin to resorufin and resorufin to dihydroresorufin, respectively. The values of k_1 for the each NPs addition setup on day 7 and 8 were then compared using t-Test. The concentrations of each metal in the liquid phase at each sampling time were compared among three NPs levels addition setup using t-Test.

3. Results

3.1 Effect of nanoparticles on gas production

Figure 1 illustrates the cumulative production of methane when substrates were treated with $\text{Fe}_4\text{NiO}_4\text{Zn}$ (Figure 1A) and Fe_2NiO_4 (Figure 1B) NP additives at 1, 10 and 100 mg Ni L^{-1} in comparison with the control (no NP addition). For the $\text{Fe}_4\text{NiO}_4\text{Zn}$ NP on 7 days, addition at 1, 10 and 100 mg Ni L^{-1} resulted in 47.2, 17.2 and 0.8 mL cumulative methane, respectively, which are all less than the control whose cumulative methane in 7 days was 50 mL, suggesting that the $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs containing Ni did not stimulate AD, but actually reduced the rate of gas production. This was especially noticeable at 100 mg Ni L^{-1} where there was permanent inhibition of AD, i.e. nearly no methane production recorded (∇ in Figure 1A). In contrast, for the Fe_2NiO_4 NP in 7 days, addition of NPs at both 1 and 10 mg Ni L^{-1} resulted in 50 mL cumulative methane which is same as that of control, suggesting that the Fe_2NiO_4 NPs did not stimulate or inhibit the AD process. However, the addition of 100 mg Ni L^{-1} of Fe_2NiO_4 NPs had a stimulatory effect on AD with 65 mL cumulative methane in 7 days, compared to only 50 mL in the control bottles. After refeeding the bottles with synthetic feed, cumulative methane production was

185 recorded in both the $\text{Fe}_4\text{NiO}_4\text{Zn}$ (1 and 10 mg Ni L⁻¹) and Fe_2NiO_4 (1, 10 and 100 mg Ni
186 L⁻¹) NP addition systems. However, $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs addition at 100 mg Ni L⁻¹ level had
187 no cumulative methane, indicating that the anaerobic sludge had been permanently
188 inactivated/killed, and it could not be reactivated with fresh feed.

Figure 1

189 3.2 Effect of nanoparticles on the activity of anaerobic sludge

190 It has been shown in the past that there is a strong correlation between the kinetics of
191 resazurin reduction and the activity of anaerobic sludge, suggesting that the faster the
192 resazurin reduction by anaerobic sludge, the higher the activity of the anaerobic sludge
193 (Chen et al., 2015a, 2015b). Figure 2 shows the activity of anaerobic sludge with two
194 types of MNPs addition analyzed by the resazurin reduction assay on day 7 and 8 (after
195 refeeding), and k_1 value of reaction rate constants for resazurin to resorufin are
196 summarized in Table 2. In the $\text{Fe}_4\text{NiO}_4\text{Zn}$ NP addition systems on day 7, the kinetics of
197 resazurin reduction by the sludge of control, 1 and 10 mg Ni L⁻¹ addition was similar
198 (Table 2), while the kinetics of resazurin reduction by the sludge of 100 mg Ni L⁻¹
199 addition (∇ in Figure 2A) was significant slower than other three situations (Table 2).
200 These observations suggest that the activity of the anaerobic sludge with 1 and 10 mg Ni
201 L⁻¹ addition was as same as the control on day 7, while anaerobic sludge with 100 mg Ni
202 L⁻¹ addition had very little activity on day 7. After one day of re-feeding (day 8), it can be
203 seen in Figure 2B that the anaerobic sludge with 1 and 10 mg Ni L⁻¹ addition, and the
204 control, were significantly reactivated based on their similar resazurin reduction kinetics

205 (Table 2), while the 100 mg Ni L⁻¹ addition showed a permanent inhibitory effect,
 206 keeping the same activity as that on day 7 (before refeeding) (Table 2, P=0.57). On day 7
 207 for the Fe₂NiO₄ NP addition system (Figure 2C), the kinetics of resazurin reduction by
 208 the sludge from the 1 and 10 mg Ni L⁻¹ NP addition systems was similar to that of control,
 209 while the kinetics of resazurin reduction by the sludge with 100 mg Ni L⁻¹ addition was
 210 faster than that of the control (Table 2). These findings reveal that the addition of
 211 Fe₂NiO₄ NPs at 1 and 10 mg Ni L⁻¹ had no effect on the activity of the anaerobic sludge
 212 exposed to them, while 100 mg Ni L⁻¹ addition enhanced the activity of the anaerobic
 213 sludge. After one day of re-feeding, it can be seen in Figure 2D that the kinetics of
 214 resazurin reduction by anaerobic sludge exposed to 1 and 10 mg Ni L⁻¹ addition, and the
 215 control, were similar to that of the 100 mg Ni L⁻¹ addition sludge, and all of 4
 216 experimental setups were significantly reactivated based on their similar resazurin
 217 reduction kinetics (Table 2). These results indicate that the addition of 1-100 mg Ni L⁻¹ in
 218 Fe₄NiO₄Zn NPs reduced the activity of anaerobic sludge, while Fe₂NiO₄ NPs did not
 219 result in any deactivation of the anaerobic sludge.

Figure 2

Table 2

220 *3.3 The partitioning of metals from nanoparticles into anaerobic sludge*

221 The concentration of the metals Ni, Fe and Zn in the liquid phase was monitored during
 222 the first 7 days of the batch experiments. Figure 3 shows that concentration profile of all

the metals present in the NPs over 7 days in liquid phase remained between 0.04-0.08 mg/gTSS. In the $\text{Fe}_4\text{NiO}_4\text{Zn}$ NP addition systems, the concentration of Zn in the liquid phase had no significant change among three NPs addition levels over 7 days (Figure 3A), and the concentration of Ni in 100 mg Ni L⁻¹ addition system is significantly higher than that in 1 mg Ni L⁻¹ addition system (Figure 3B), while the concentration of Fe in 100 mg Ni L⁻¹ addition system is significantly higher than that in both 1 and 10 mg Ni L⁻¹ addition system (Figure 3C). In the Fe_2NiO_4 NP addition systems, the same situation for the concentration of Fe was also obtained (Figure 3D), while the concentration of Ni in 100 mg Ni L⁻¹ addition system was nearly 10 times higher than 1 and 10 mg Ni L⁻¹ addition system, and increased over the experimental period (Figure 3E). These results indicate that in all cases, although the majority of the Ni, Fe and Zn remained in the sludge, the Ni present in Fe_2NiO_4 NPs at 100 mg Ni L⁻¹ was more bioavailable than in the other experiments with NPs treatment.

Figure 3

4. Discussion

Some trace metals (such as Fe, Co, Ni, Mn) are considered as “essential” elements for microbial growth in anaerobic digesters, functioning as protein stabilizers and osmotic balance controllers across various microbial membranes, and as catalysts for biochemical reactions (Oleszkiewicz and Sharma, 1990). Metals considered the most important in AD are from the iron family, i.e. Fe, Co, and Ni, which were proposed as essential for enzyme synthesis during the growth of methanogenic bacteria (Qiang et al., 2012). Besides playing a key role in factor F_{430} , which converts substrates irreversibly to

methane in all methanogens (Friedmann et al., 1991), Ni is also essential for the growth of acetoclastic organisms; both methane producers and sulfate reducing bacteria (Oleszkiewicz and Sharma, 1990). Stimulatory and inhibitory Ni concentrations in AD are summarized as being between 0.03 and 27 mg L⁻¹, 35 and 1600 mg L⁻¹ (50% inhibition), respectively (Lo et al., 2012), while optimal Ni concentration was determined as 10 mg L⁻¹ for stimulating methane production in AD (Chen et al., 2016). For iron, its often underestimated importance lies in its redox properties, and its involvement in energy metabolism through the cytochromes and ferredoxin (Takashima and Speece, 1989). When studying the effect of iron on both the acidogenic and methanogenic phases, it was reported that the addition of 10 mg Fe L⁻¹ could increase CH₄ production by 10.1% and 21.4%, respectively, with the stimulating effects on anaerobes (both catabolism and anabolism) (Hao et al., 2017). Although compared to their molecular or macrosized analogs, nanomaterials often exhibit significantly different physical and chemical properties due to their mesoscopic, quantum size and surface effects, and addition of iron or nickel NPs also shows a stimulatory effect on AD (Abdelsalam et al., 2017, 2016). The release of Ni and Fe ions into solution from the dissolution of NPs was believed to be responsible for the distribution of these ions in the slurry, and the increase in bacterial activity.

However, in contrast there are various mechanisms by which NPs can cause damage to organisms which are exposed to them, such as: damaging the cell membrane, proteins, and DNA; interrupting electron transport; releasing toxic components, or: releasing reactive oxygen species (ROS) (Klaine et al., 2008a). ROS production was suggested as being the primary mechanism for nanoparticle toxicity (Klaine et al., 2008b), and consist

of free radicals, such as the superoxide anion ($\bullet\text{O}_2^-$) and hydroxyl radical ($\bullet\text{OH}$). These are strong oxidants that can oxidize DNA, proteins, cell membranes, and other cell components. When present at high concentrations, ROS can cause oxidative stress, inflammation, and cell death, and the high reactivity of NPs can release large amounts of ROS; Fe_2O_3 and ZnO NPs can generate large amounts of ROS (Nguyen et al., 2015; Sadeghi et al., 2015). Zinc ferrite NPs, nickel ferrite NPs and nickel zinc ferrite NPs were all reported to induce cytotoxicity and oxidative stress in different cells due to ROS generation (Ahamed et al., 2011; Al-Qubaisi et al., 2013b; Alhadlaq et al., 2015). In addition, it has been shown that strong binding of nanoparticles to the outer bacterial membrane can prevent dehydrogenase enzyme function (McShan et al., 2014). These findings theoretically support our observation of the significantly negative effect of adding $\text{Fe}_2\text{NiO}_4\text{Zn}$ NPs to AD sludge in this present study.

Another important mechanism of toxicity of NPs is the release of metal ions caused by corrosion and dissolution of the NPs (Chen et al., 2014). However, if the ions released such as Ni^{2+} and Fe^{3+} reach the stimulatory concentration range, the addition of these corresponding NPs could result in the stimulation of AD. Total metal concentration is generally used to evaluate the stimulatory or toxic effects of metals in AD; however, it has been shown that the measurement of total metal concentration is not sufficient to determine its bioavailability (Amir et al., 2005), and trace metal bioavailability depends on speciation, which is controlled by the operating conditions of anaerobic reactors which can in turn alter their pH and the washout of the metals (Oleszkiewicz and Sharma, 1990; Thanh et al., 2017). However, there is still considerable confusion about the bioavailability of trace metals; although the total metal content which may be provided in

the feed should be theoretically sufficient, the metals may not be present in a bioavailable form that can be utilized by the microorganisms (Zitomer et al., 2008). Even full bioavailability does not mean that the required metals are all utilized by the bacterial population (Oleszkiewicz and Sharma, 1990). The bioavailability of trace metals in bioreactors depends on complex interactions between metals in the solid and liquid phases of the bioreactors, and the transport of free/complexed metal ions through the cell membrane is assumed to be the main mechanism for metal uptake by microorganisms (Thanh et al., 2015). However, sulfide is often present in anaerobic reactors and can precipitate the trace metals added, resulting in metal sulfides which are generally considered relatively non-bioavailable (Ketheesan et al., 2016). When studying the fate of ZnO NPs during the AD of wastewater, Lombi et al. (Lombi et al., 2012) found that, after adding ZnO NPs, a large proportion of the added Zn was converted to insoluble ZnS, while Zn in the aqueous phase was low ($0.70\text{--}1.40\text{ mg L}^{-1}$) but had already resulted in AD inhibition. In a study on various forms of zinc oxide, Zn^{2+} toxicity effects on activated sludge activity from the solubilisation of particles that create Zn^{2+} ions confirmed that Zn^{2+} is the most toxic, followed by nano-sized and then bulk-sized ZnO at same concentration (Liu et al., 2011). In the present study, the Zn concentration in the aqueous phase of the $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs addition assay was $0.04\text{--}0.06\text{ mg L}^{-1}$ over the 7 days without much change in concentration (Figure 3A), while the Ni concentration in the aqueous phase of the Fe_2NiO_4 NPs added at 100 mg Ni L^{-1} was $0.3\text{--}0.5\text{ mg L}^{-1}$ (Figure 3B), revealing that more Ni was available in the latter system, which could induce higher anaerobic sludge activities.

Figure 4

The structures of Fe_2NiO_4 (Perron et al., 2007) and $\text{Fe}_4\text{NiO}_4\text{Zn}$ (Job et al., 2014) NPs are shown in Figure 4. Addition of Fe_2NiO_4 NPs in the present study at 100 mg Ni L^{-1} stimulated AD, while at the same Ni concentration, the addition of $\text{Fe}_4\text{NiO}_4\text{Zn}$ NPs permanently inhibited AD. One possible explanation for this may lay in the structure of Ni ferrite NPs with a Zn^{2+} dopant which can be released into solution to result in toxicity. On the other hand, the Ni ferrite Fe_2NiO_4 NP (Figure 4B) adopts an inverse spinel structure with Ni^{2+} in the octahedral sites, and Fe^{3+} equally distributed between the octahedral and tetrahedral sites of the O^{2-} face-centered cubic cell. Considering the absence of Zn^{2+} cations in the Ni ferrite Fe_2NiO_4 , the amount of iron in both atomic sites would be equal and their contribution to the magnetic dipole moment would cancel out, and the formation of the magnetic dipole moment of the material would be the responsibility of the Ni^{2+} cations. Doping the Ni ferrite with Zn^{2+} cations, there is a migration of Fe^{3+} cations from the tetrahedral to octahedral sites, unbalancing the initial equality of the Fe^{3+} cations (Figure 4A). Therefore, substitution of the Zn^{2+} dopant into Ni ferrite can drastically affect the antiferromagnetic coupling interactions between the tetrahedral and octahedral interstices, and have been reported to achieve high and tunable nanomagnetism because both Fe^{3+} and Zn^{2+} cations contribute to the magnetic dipole moment of the material (Job et al., 2014). It is well known that in a magnetic material, the combination of all magnetic dipole moments produces a constant magnetic field as well (Yadav, 2006). Being exposed to a constant magnetic field area has been reported to have a significant impact of reducing the effectiveness of AD (Dębowski et al., 2016). The

enhanced magnetic field due to the substitution of the Zn^{2+} dopant in Ni ferrite could theoretically depress the activity of AD. Therefore, the structural differences between the two types of NPs in this present study could be another possible explanation for their different effects on AD. Compared to ROS and released metal ions, the extent of the contribution by NPs structure needs more study.

Nano-size is the cardinal property for interactions with biological systems since this determines the ability to penetrate cell membranes, thus facilitating the passage across biological barriers, interactions with the immune system, uptake, absorption, distribution and metabolism (Viscido et al., 2014). However, at the same time metal nanoparticles can theoretically affect anaerobic microorganisms via a multitude of other different mechanisms, which can both positively or negatively impact AD. Spiked by 1 and 10 mg $\text{Ni}^{2+} \text{ L}^{-1}$ in nickel salt format in the same experiment setup as present study, AD was found to start to be stimulated and to reach highest activity, respectively (Chen et al., 2016). At 100 mg $\text{Ni}^{2+} \text{ L}^{-1}$ concentration level, nickel salt showed permanent inhibition of AD (Chen et al., 2016) while in this present study, Fe_2NiO_4 NPs showed stimulation of AD. Besides the releasing ROS and metal ion, the explanation for this finding probably lies on the structure of Fe_2NiO_4 NPs as discussed above. Therefore, comparing with salt format of the metal, a proper structure of magnetic NPs could be better on controlling the generation of ROS and the release of metal ions, with an aim of trying to enhance AD efficiency.

The transformation of biomass into biogas is one of the possible solutions to some of the energy and environmental issues the world is facing nowadays: decrease consumption of fossil fuel reserves, a global increase in energy demand and the greenhouse effect, among

others. Many attempts to increase biogas production are not suited to industrial scale-up. However, essential metal NPs, such as Ni, Co and Fe NPs, have been proved to accelerate the AD of the sludge, thus making it more efficient in terms of duration and enhanced production of the biogas. Acceleration to enhance the efficiency of AD due to applying NPs could result in reduction in retention / residential time and energy consumption in AD plant. Besides, additive NPs can moderate the situation of pH decrease due to methanogens inhibited by high concentration of heavy metal in AD. So far, some commercial essential metal NPs have been successfully applied in cellulose and mud found in urban treatment plants; while the challenge lies in extrapolating the technology to digesters with capacity for hundreds of cubic metres ¹.

5. Conclusions

- At 100 mg Ni²⁺ L⁻¹ concentration level, Ni Zn ferrite nanoparticles (Fe₄NiO₄Zn) showed permanent inhibition of AD while Ni ferrite nanoparticles (Fe₂NiO₄) showed stimulation of AD.
- Resazurin reduction assay revealed the negative effect of Fe₄NiO₄Zn nanoparticles and the positive effect of Fe₂NiO₄ nanoparticles on AD activity.
- The effect of metal nanoparticle structure on AD needs further investigation with expectation of NPs structure design strategy to control the generation of ROS and release of metal ions to enhance AD efficiency.

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¹ <https://phys.org/news/2014-10-nanoparticle-technology-triples-production-biogas.html>

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523

524 **Table 1** The initial concentration of Ni, Fe, Zn and total NP in anaerobic digestion by
525 addition of Fe₄NiO₄Zn and Fe₂NiO₄ nanoparticles.

[Metal] \ NP	Fe ₄ NiO ₄ Zn			Fe ₂ NiO ₄		
[Ni] (ppm)	1	10	100	1	10	100
[Fe] (ppm)	3.8	38	380	1.9	19	190
[Zn] (ppm)	1.11	11.1	111	\	\	\
[total NP] (ppm)	7	70	700	4	40	400

526

527

Table 2 k_1 (reaction rate constants for resazurin to resorufin) in $\text{Fe}_4\text{NiO}_4\text{Zn}$ and Fe_2NiO_4 spiked systems with a control and P values of the t-Test comparing k_1 values between day 7 and 8.

$\text{Fe}_4\text{NiO}_4\text{Zn}$				
	Control	[Ni]-1ppm	[Ni]-10ppm	[Ni]-100ppm
k_1 (min^{-1}) -day 7	0.513±0.028	0.543±0.040	0.571±0.042	0.488±0.023*
k_1 (min^{-1}) -day 8	1.482±0.045	1.501±0.047	1.444±0.038	0.502±0.031*
P	2.89×10^{-5}	1.42×10^{-5}	1.27×10^{-5}	0.57**
Fe_2NiO_4				
	Control	[Ni]-1ppm	[Ni]-10ppm	[Ni]-100ppm
k_1 (min^{-1}) -day 7	0.577±0.034	0.581±0.028	0.571±0.030	0.632±0.018*
k_1 (min^{-1}) -day 8	1.598±0.037	1.591±0.042	1.611±0.029	1.630±0.029
P	4.17×10^{-6}	1.50×10^{-5}	6.22×10^{-6}	2.11×10^{-5}

* The significance of the bold number indicates the current result is significantly different by comparing k_1 values in the row according to the t-Test at $P < 0.05$.
 **The significance of the bold number indicates the current result is NOT significantly different by comparing k_1 values in the column according to the t-Test at $P < 0.05$.

535 **Figure and Scheme Captions:**

536 **Scheme 1** Conversion of resazurin to resorufin and dihydroresorufin.

537 **Figure 1** Effect of $\text{Fe}_4\text{NiO}_4\text{Zn}$ nanoparticles (**A**) and Fe_2NiO_4 nanoparticles (**B**) on
538 cumulative methane production in anaerobic digestion.

539 **Figure 2** Activity of anaerobic sludge analyzed by the resazurin reduction assay.
540 Experiment treated by $\text{Fe}_4\text{NiO}_4\text{Zn}$ addition on day 7 after feeding (**A**); and day 8 , i.e. 1
541 day after re-feeding (**B**); by Fe_2NiO_4 addition on day 7 after feeding (**C**); and day 8 , i.e. 1
542 day after re-feeding (**D**).

543 **Figure 3** The profile of free ions of Zn (A), Ni (B) and Fe (C) in the aqueous phase with
544 $\text{Fe}_4\text{NiO}_4\text{Zn}$ nanoparticles addition system, and Ni (D) and Fe (E) in the aqueous phase
545 Fe_2NiO_4 nanoparticles addition systems over the 7 day experiment in anaerobic digestion
546 bottles. * indicates that the metal concentrations are significantly different between these
547 two experimental setups.

548 **Figure 4** (**A**) Oxide nickel-zinc ferrite ($\text{Fe}_4\text{NiO}_4\text{Zn}$) representation with an inverse spinel
549 type structure. Octahedral sites FeO_6 or NiO_6 are presented in blue, and tetrahedral sites
550 FeO_4 or ZnO_4 are presented in red. (**B**) Fe_2NiO_4 inverse spinel unit cell. Oxygen atoms
551 have been added in order to complete the $\text{Ni}(\text{O}_h)$ and $\text{Fe}(\text{O}_h)$ first coordination shell (T_d :
552 tetrahedral sites; O_h : octahedral sites). Ni atoms are shown in blue, Fe atoms in green and
553 O atoms in red.

Scheme 1

Figure 1

557

Figure 2

558

559

Figure 3

Figure 4