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JES JOURNAL OF ENVIRONMENTAL SCIENCES

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Nanoparticles in mitigating gaseous emissions from liquid dairy manure stored under anaerobic condition

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ARTICLE INFO

Article history:
Received 28 September 2017
Revised 2 March 2018
Accepted 16 March 2018
Available online 24 March 2018

Keywords:
Nanoparticles
Greenhouse gas
Hydrogen sulfide
Anaerobic storage
Gaseous reduction

ABSTRACT

A number of mitigation techniques exist to reduce the emissions of pollutant gases and greenhouse gases (GHGs) from anaerobic storage of livestock manure. Nanoparticle (NP) application is a promising mitigating treatment option for pollutant gases, but limited research is available on the mode of NP application and their effectiveness in gaseous emission reduction. In this study, zinc silica nanogel (ZnSNL), copper silica nanogel (CuSNL), and N-acetyl cysteine (NACL) coated zinc oxide quantum dot (Qdot) NPs were compared to a control lacking NPs. All three NPs tested significantly reduced gas production and concentrations compared to non-treated manure. Overall, cumulative gas volumes were reduced by 92.73%–95.83%, and concentrations reduced by 48.98%–99.75% for H_2S, and 20.24%–99.82% for GHGs. Thus, application of NPs is a potential treatment option for mitigating pollutant and GHG emissions from anaerobically stored manure.

Introduction

Greenhouse gases (GHGs) such as methane (CH_4), carbon dioxide (CO_2), and nitrous oxide (N_2O) emitted from livestock production operations are contributing to climate change. The United States Agricultural sector contributes about 9% of the total US GHG emission, and the US livestock sector alone stands for ~28% of total CH_4 emission (USEPA, 2015; Johnson et al., 2007). GHGs are produced due to anaerobic digestion of manure and biomass (municipal solid waste, freshwater biomass, leaves, grasses, woods, weeds, fruit and vegetable solid wastes) (Gunaseelan, 1997; Kinsman et al., 1995). CO_2 is produced due to both aerobic and anaerobic digestion of manure. During the same digestion period process, organic nitrogen is converted into ammonia (NH_3), nitrite (NO_2^-), and nitrate (NO_3^-) through nitrification process. However, NO_3^- converts back to nitrogen (N_2) through denitrification process (Kinsman et al., 1995; Sommer et al., 2007). Additionally, hydrogen sulfide (H_2S) and other volatile organic compounds are also generated during anaerobic decomposition of manure (Abouelenien et al., 2009; Hobbs et al., 2004).

Worldwide, scientists and researchers are trying various treatment options including feed manipulation, implication of lifetime efficacy (Weiske et al., 2006), application of catalytic processes (Centi and Perathoner, 2012), addition of microbial additives (Rahman et al., 2011), anaerobic digestion (Clemens et al., 2006), and application of probiotics,

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https://doi.org/10.1016/j.jes.2018.03.014

acetogens, bacteriocins, and organic acids (Boadi et al., 2004) for mitigating GHG and volatile organic compounds (VOCs) from animal production facilities. Recently, nanoparticle (NP) application has shown promise in mitigating gaseous emissions from both industrial and animal wastes. NPs are used in the industrial sector for the removal of a trace amount of pollutants (Kaur and Gupta, 2009; Liu, 2009; Salata, 2004). Similarly, NP application is expected to bring solutions offering GHG mitigation not possible by using conventional methods such as household environmental mitigation and land application (Chinnamuthu and Boopathi, 2009). NPs in the agricultural sector (Arivalagan et al., 2011; Chinnamuthu and Boopathi, 2009), especially due to their presumed ability to mitigate GHGs (Gautam et al., 2016a, 2016b, 2016c) became an attractive part of research nowadays. Among the few studies performed on GHG mitigation, zinc oxide nanoparticles (nZnO) and copper oxide nanoparticles (nCuO) reportedly had inhibitory action concerning CH_4 production (Luna-delRisco et al., 2011; Mu et al., 2011). Depending on the nZnO dosage, a 19%–77% reduction of CH_4 was reported from waste activated sludge in comparison with a control (Mu et al., 2011). Other metal oxide NPs explored, such as titanium dioxide (TiO_2), silicon dioxide (SiO_2) and aluminum dioxide (Al_2O_3) did not show any effect (Mu et al., 2011).

Mixing of nZnO with swine manure slurry reduced the concentration of CH_4 and H_2S by 54% and 98%, respectively (Gautam et al., 2016a). nZnO used in a filter media reduced CH_4 and CO_2 concentrations by 14% and 18%, respectively, over the control (Asis, 2008). Asis (2008) also found that spraying tungsten oxide (WO_3) into the headspace gas from the manure slurry did not show any noteworthy response. nZnO compared to zirconium oxide NPs ($nZrO_2$) at application rates of 100, 250, 500 mg/L and 3 g/L in swine manure/dairy manure revealed that nZnO is much more effective than $nZrO_2$ in mitigating CH_4 and H_2S when compared to a control sample (Gautam et al., 2013).

In general, NPs are an effective means for mitigating or reducing gaseous emissions either by directly absorbing gases, by killing gas-producing microorganisms, or converting the contaminating chemical through a chemical reaction (Yang et al., 2013; Zhang et al., 2010; Ševců et al., 2011). However, it is not well understood if there are adverse environmental effects from NPs on aquatic ecosystems, plant uptake, and toxicity mechanisms (Fabrega et al., 2011; Ge et al., 2011; Navarro et al., 2008; Nowack and Bucheli, 2007). Therefore, researchers are continuously striving for new environment-friendly engineered NPs with intact active potentiality and minimal adverse environmental impact (Bolyard et al., 2013; Young and Santra, 2014). The behavior of such NP types (ZnO, TiO_2, Ag) has been analyzed for landfill leachate (Bolyard et al., 2013), seed germination (Das et al., 2015), and antibacterial efficacy (Young and Santra, 2014). The potential application of these NPs in livestock manure is limited although the research need has been identified (Gautam et al., 2016a). NPs can be applied either as a liquid, gel, or powder depending on the targeted treatment. However, to our knowledge, no study has been previously conducted to examine the efficacy of the liquid formulation of different NPs in reducing GHG emission. Therefore, the objective of this study was to compare the effectiveness of three NPs namely, zinc silica nanogel liquid (ZnSnL), copper silica nanogel liquid (CuSNL) and N-acetyl cysteine liquid (NACL) coated zinc oxide quantum dots (Qdots) in minimizing CO_2, CH_4, and H_2S emissions from liquid dairy manure stored under anaerobic conditions. Additionally, changes in the manure properties and gaseous reduction mechanisms in NPs treated manure were characterized.

1. Materials and methods

1.1. Manure collection and characterization

Dairy manure was collected from the dairy research unit of North Dakota State University (NDSU) to evaluate the effectiveness of three engineered NPs on manure properties, CO_2, CH_4, and H_2S gas production reduction, concentrations. Manure properties such as pH, conductivity, crude protein (CP), ash, total N (TN), ammonia (NH_3), and volatile fatty acids (VFAs) were determined both before and after the experimental period. Table 1 lists methods used for manure characterization.

1.2. Nanogel synthesization

1.2.1. Preparation of copper and zinc silica nanogel

All the materials required for the nanogel synthesization process were purchased and used unmodified from commercial vendors. CuSNL and ZnSNL were prepared as previously described (Young and Santra, 2014). Copper (II) sulfate pentahydrate (38.88 g) (CQ Concepts Inc., Ringwood, USA) or zinc sulfate monohydrate (27.5 g) (Fisher Scientific, Waltham, USA) was added to 1.9 mL of 1% hydrochloric acid (Fisher Scientific, Waltham, USA) in 660 mL of deionized water. After magnetic stirring (30 min) tetraethylorthosilicate (TEOS) (4.6 mL) (Gelest Inc., Morrisville, USA) was added drop-wise and stirred for 24 hr. The pH of the final solution was raised to 7.5 with 1 mol/L sodium hydroxide (NaOH) (Fisher Scientific, Waltham, USA) solution and then the nanogel was formed.

1.2.2. Preparation of N-acetyl cysteine (NAC) coated zinc oxide Qdot nanogel

The nZnO were synthesized using a modified sol–gel method described previously (Bang et al., 2006). N-acetyl cysteine (NAC) (14.97 g) (Acros Chemicals, Geel, Belgium) was dissolved in 600 mL of 95% ethanol at ~70°C (hot bath) with constant stirring in a glass beaker. Zinc acetate dehydrate (Case# 5970-45-6, Sigma, St. Louis, USA) (26.84 g) was added to this solution while in the hot-bath and allowed to dissolve completely. After 10 min of stirring, the beaker was transferred to an ice bath and cooled to 4–5°C. In a separate flask, 7.33 g of sodium hydroxide was dissolved in 200 mL of 95% ethanol added dropwise at the rate of 2–3 mL/min to the cooled zinc acetate and NAC solution to form the NAC coated nZnO.

1.3. Experimental setup and gas sampling

Twenty liters of raw dairy manure samples was kept in laboratory room conditions for about 6 hr to acclimate to the

Table 1 – Protocols followed to determine manure properties.

Parameters	Methods/protocols used	
pH	Environmental protection agency (EPA) SW-846, Method 9040	Mulkey (1999)
Redox	ASTM D1498-14 Standard Test Method for Oxidation–Reduction Potential	Batley and Simpson (2016)
Conductivity	ASTM D1125-14 Standard Test Methods for Electrical Conductivity	Marr and Heitkamp (2015)
Total nitrogen (TN)	Recommended Methods of Manure Analysis, A3769 Macro-Kjeldahl method (adapted from Kane, 1998)	Borhan et al. (2013)
Ammonia (NH_3)	Sigma Technical Bulletin #640. Sigma Diagnostics, St. Louis, MO 63178	Gautam et al. (2016b)
Crude protein (CP)	Official Methods of Association of Analytical Communities (AOAC) International (2005) 18th ED., AOAC International Gaithersburg, MD, USA, Official Method 2001.11 Run on the Kjeltec 2300, Foss NA, Eden Prairie, MN	Latimer (2012)
Ash content	Official Methods of Analysis of AOAC International (2005) 18th ED., AOAC International Gaithersburg, MD, USA, Official Method 942.05	Latimer (2012)
Volatile fatty acids (VFAs)	The method of Goetsch and Galyean (1983). Agilent 6890 N gas chromatograph with a FID (flame ionization detector) and the 7683 Series auto-injector and an auto sampler. The column used was the Supelco brand, NUKOL™ Fused Silica Column, 15 m × 0.53 mm × 0.5 um.	Gautam et al. (2016c)

experimental unit environment. Later on, raw manure sample was stirred thoroughly for homogeneous mixing before sub-sampling and initiating treatments with different nanogels. Four treatments were prepared including ZnSNL, CuSNL, NACL, and a control (no NPs added). Based on a previous study (Gautam et al., 2013), an application rate of 3 g/L was maintained for three NP based treatments and all four treatments were replicated three times. Thus, a total 12 Erlenmeyer flasks (4 treatments × 3 replicates) were used. All treatments were carried out in 1-L Erlenmeyer flasks with a working volume of 500 mL fitted with rubber stoppers. One end of steel tube (6 mm diameter × 100 mm long) was inserted to each flask through the rubber stopper for headspace gas collection into a 500 mL tedlar bag (SKC Gulf Coast Inc., Texas, USA) using a teflon tube. Before sealing the flask, residual oxygen in the headspace was driven out by flushing it with nitrogen to create an anaerobic environment (Fig. 1). After setting up all experiments, each treatment flask was mixed once again by shaking the flasks manually. The experiment continued until gas production was stopped (when no gas was collected in sampling bag) completely after 56 days.

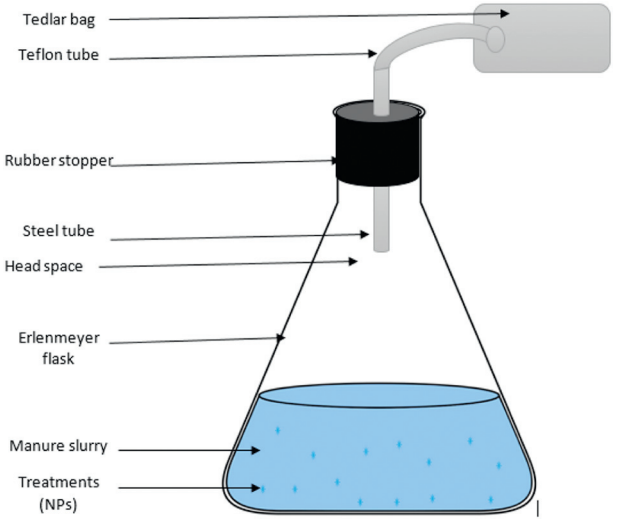

Fig. 1 – Schematic diagram of an experimental setup used in this study. NPs: nanoparticles.

1.4. Measurement of gas volume, GHGs, and hydrogen sulfide concentration

Headspace gas accumulated in the Tedlar bags was collected every 2 to 14 days during the entire experimental period. Gas was drawn out of the Tedlar bags by a graduated gas-tight syringe (SGE Syringe, 500 MAR-LL-GT, Trajan Scientific Americas Inc, USA) for measuring gas volume. A gas-tight syringe (5 mL, Luer-LokTM Tip Syringe, Franklin Lakes, NJ, USA) was used to collect headspace gas from Tedlar bags to measure gas concentration. This sample was diluted with pure nitrogen gas in different Tedlar bags to match the detection limit of the gas chromatograph (GC) and Jerome meter. This dilution was chosen based on gas concentrations in the headspace. The H_2S gas concentrations were measured with a Jerome meter (Jerome 631X, Arizona Instrument LLC, USA). Greenhouse gases (CH_4 and CO_2) were measured using a gas chromatograph (GC, 8610C, SRI instrument, USA) equipped with flame ionization detector (FID) and electron capture detector (ECD). Based on the pre-scheduled GC event program (method), 1 mL diluted gas mixture was injected in the sample loop. The FID and ECD detector temperatures were raised to 300 and 350°C respectively before the insertion of the gas sample into the GC. Additionally, before each measurement, the GC was calibrated using the research grade standard gasses (5, 10, 100 ppm for CH_4; 500, 1000, 3000 ppm of CO_2) and five to seven replications for each concentration levels were used. Estimated method detection limits of the GC for CH_4 and CO_2 were 87 and 109 ppm, respectively. Additional calibration and measurement processes are described in Rahman et al. (2013).

1.5. Microbial population density analysis

1.5.1. Bacterial cultivation and quantification

Plate counts were done to quantify the effect of different treatments on the aerobic *coliform* microbial population (*e.g.*, *coliform* and *Escherichia coli*) (Gautam et al., 2016a). Plate counts were carried out before and after the experimental period and reported as colony forming units (CFUs). All experimental plate count preparations of manure and reagents were performed in a sterile fume hood. Growth media for the microbial communities was prepared by placing a sterile membrane filter with an

absorbent pad (47 mm diameter, 0.45 μm pore size, cellulose nitrate type, Whatman Limited, Maidstone, England, UK) in a sterile petri-dish (Sterile Petri dishes, 60 mm diameter and 15 mm height, VWR, Radnor, USA). An M-Endo broth ampule (2 mL) (HACH LANCH GmbH, Willstatterstrasse 11, Dusseldorf, Germany) was poured evenly over the entire surface of the absorbent pad. Subsequently, 100 μL of the diluted environmental samples was added to the absorbent pad and spread evenly over the pad using a small sterile glass rod. To determine an optimum dilution level for better visibility and CFU counting, five ten-fold serial dilutions (10^3, 10^4, 10^5, 10^6, and 10^7) with duplicates from each treatment were used. Based on the initial test runs, a dilution level of 10^3 was found optimum for each treatment and the study continued using this dilution with three replicates. Petri dishes were incubated (24 hr, 35 ± 0.5°C), CFUs were counted using a manual darkfield colony counter with 1.5× magnification (Model-13,332,700, Reichert Inc. Depew, USA).

1.5.2. Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

Quantitative real time polymerase chain reaction (qRT-PCR) analysis was conducted to determine effects of NP treatment on the CH_4 producing methanogenic microbial community in the treated manure. The α-subunit of the methyl coenzyme M-reductase (mcrA) genes distinctive to methanogenic archaea was targeted. The deoxyribonucleic acid (DNA) copy numbers were used to understand the influence of NPs on methanogenic archaea (Freitag and Prosser, 2009; Ma et al., 2012).

1.5.2.1. Ribonucleic acid (RNA) extraction and complementary deoxyribonucleic acid (cDNA) synthesis.

To conduct the qRT-PCR analysis towards finding the NP effect on methanogenic community, ribonucleic acids (RNAs) were extracted from 0.25 g of liquid dairy manure samples using the PowerMicrobiome™ RNA isolation kit (MoBio Laboratories, Inc., Carlsbad, USA) and stored at −80°C for further cDNA synthesis. After RNA extraction and prior to cDNA synthesis, co-isolated or contaminating DNAs were removed from RNA samples using the Ambion DNA-free™ DNase Treatment & Removal Kit (Invitrogen, Carlsbad, USA). Isolated RNA was quantified using a spectrophotometer (NanoDrop 2000c, ThermoScientific, USA). Thereafter, approximately 80 ng of RNA was used to synthesize cDNA using the SuperScript® VILO™ cDNA synthesis kit (Invitrogen Carlsbad, USA). The synthesized cDNA was electrophoresed on a 1% agarose gel and was visualized using ultraviolet (UV) transilluminator paired with ethidium bromide dye that fluoresce under UV light to confirm the purity. Furthermore, NanoDrop spectrophotometer was used to quantify the synthesized cDNA. Simultaneously, the purity of RNA and cDNA were assessed based on the spread of the bands on an agarose gel and by measuring absorbance ratios at 260/280 nm and 260/230 nm in a spectrophotometer. Later on, cDNA samples were frozen at −80°C until quantitative polymerase chain reaction (qPCR) analyses.

1.5.2.2. Quantification of the methanogenic community.

Quantitative real time polymerase chain reaction (qRT-PCR) analysis was carried out in an ABI Prism™ 7500 (Model 7500, Applied Biosystems Inc, Foster City, CA, USA) real-time PCR system. Power SYBER Green PCR Master Mix (Cat. 4,368,577, Applied Biosystems Inc, Foster City, CA, USA) containing SYBR Green I dye was used for the reaction in a 96-well plate. Forward primer (MLF) and reverse primer (mcrA-rev) specific for mcrA genes were used to amplify genomic DNA and cDNA. MLF GGTGGTGTMGGATTCACACARTAYGCWACAGC-32 base pairs and mcrA-rev CGTTCATBGCGTAGTTVGGRTAGT-24 base pairs were the primer sequence synthesized by Integrated DNA Technologies (IDT) Inc. (IDT, Coralville, IA, USA) and were used to enumerate the α-subunit of mcrA gene (Luton et al., 2002; Narihiro and Sekiguchi, 2011). At a target reaction volume of 20 μL in each well in the plate used 10 μL of SYBER Green Master Mix, 1 μL of each template DNA (standard culture DNA and extracted cDNA, respectively), 0.4 μL of each forward and reverse primers when considered 200 nmol/L of primer concentration, and the remaining amount (8.2 μL) was HyPure ™Molecular Biology Grade Water (HyClone Laboratories, Inc., Logan, UT, USA). In this analysis, a total of 54 (((4 treatments (3 NPs + control) × 3 replications + 6 (5 dilutions of standard DNA + 1 blank)) × 3 replications) wells in a 96 well plate were used. The reactions in denaturing, annealing, and extension phases in the thermocycler were programmed for 10 min initial holding at 95°C; 45 cycles of denaturing (30 sec, 95°C); annealing (45 sec, 72°C) and extension (45 sec at 72°C). The dissociation step at 95°C for 15 sec and 60°C for 1 min was added at the end to check the specificity of the PCR outputs. Amplifications of five dilutions (10^2, 10^3, 10^4, 10^5, and 10^6) of the standard *Methanobacterium formicicum* Schnellen 1947 (DSM 1535; Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, 38,124 Braunschweig, Germany) was plotted against the real time threshold cycle (CT) to get the standard curve paired with amplification from dairy manure samples.

1.6. Statistical analysis

All treatments were replicated in triplicate and the averages reported. The analysis of variance (ANOVA) test was performed to find out the effect of treatments (*e.g.*, three nanogels) on CH_4, CO_2 and H_2S gas concentrations, pH, conductivity, ash, CP, TN, NH_3, VFAs, and microbial population. The averages of each variable among treatments were compared using programmed random occurrence (PROC) ANOVA procedure in SAS 9.3 software (SAS Institute Inc., Cary, NC, USA). The null hypothesis was treatments had equal impact on gas concentrations and other parameters at 95% ($p \leq 0.05$) significance level. Then, variables were separated using Duncan's multiple range test if the main effect (NP treatment) using *F*-test was significant at $p \leq 0.05$.

2. Results and discussions

2.1. Effects of NPs on manure properties

No significant differences in pH, conductivity, CP, TN, and ash content between pre- and post-treatment were seen in the untreated liquid dairy manure sample (no NPs). Nanogel treated manure pH values were significantly higher (7.20-7.91) than the control (6.47-6.64) (Table 2). Near neutral pH is expected for the anaerobic digestion, and higher onversion of VFAs towards CH_4 production is also evidential with neutral pH. In the present study, addition of NPs as treatment revealed an increase in

alkalinity by raising the pH. Higher pH in nanogel treated samples was likely due to the use of sodium hydroxide during nanogel formulation and further release of hydroxyl ions in the liquid dairy manure. This increase in pH was likely to lower the VFA accumulation in NP treated manure. Contrariwise, pH below the neutral range may ended up with higher VFAs in the control treatment (Rea, 2014).

Conductivities for the nanogel treated manures were higher (32%–52%) compared to the control (final) treatment. Conductivity of the liquid dairy manure treated with three nanogels were significantly different compared with control initial and final manure samples. The presence of metal NPs (zinc and copper) in the synthesized nanogel and their ionic release into the manure might have instigated higher conductivity values (14 to 16 μS/cm). However, no statistically significant difference was observed among the nanogel treated manures. Similar to pH and electrical conductivity, the ash, crude protein, total nitrogen, and NH_3 concentrations at the end of the experiment among the nanogel (ZnSNL, CuSNL, and NACL) treated liquid manure were similar but statistically significantly higher than those obtained in control manure (control final) except for NH_3 concentrations (Table 2).

No significant difference was observed between ash content levels of ZnSNL and CuSNL treatments. The ash content (%) of the NACL treatment is significantly different from the CuSNL treatments but not the ZnSNL treated manure. Ash content of all treated manure samples was significantly higher than the controls. Carbonaceous substances in the NP formulation could be the probable reason crude protein (CP) and total N were significantly higher (14.46%–17.76%) in all treatments compared with the final control. No statistically significant difference was found between the initial control and NP treatments. Utilization of CP and N by a large amount of microbial population in the control (final) treatment was the probable cause in this case. A lower microbial population in treated samples may result in higher CP and N because of the inhibitory effect of the NPs. Fecal NH_3 concentration was significantly lower (23.13%–111.29%) in all of the NP treated samples compared with control (Table 2).

2.2. Effect of NPs on gas production

The total gas produced during the experimental period is presented in Fig. 2. This study continued for 56 days since very little to no measurable gas production was happening thereafter. The cumulative gas production from 500 mL of dairy manure treated with control, ZnSNL, CuSNL, or NACL was approximately 1128, 82, 47, and 60 mL, respectively. The rate of gas production from 500 mL of dairy manure was 20.15, 1.46, 0.83, and 1.07 mL in a day for the control, ZnSNL, CuSNL, and NACL treatments, respectively. The experiment demonstrated that gas reduction from ZnSNL, CuSNL, and NACL treated manure was approximately 92.73%, 95.83%, and 94.68%, respectively. Differences in gas production reduction among NP treated manure were not statistically significant ($p \leq 0.05$) but they were significantly lower than the control treatment ($p \leq 0.05$). More than 90% reduction of gas production was observed in NP treated manure samples, compared to the control treatment which is much higher than previously published results where Gautam et al. (2013)) observed 60% total gas reduction using nZnO (10 nm to 50 nm size) application. Overall, CuSNL showed the highest total gas reduction potential (95.83%) among the NPs tested in this study, and ZnSNL showed the lowest (92.73%), but the reduction was not statistically significant among the NP treatments. Thus, any of the nanogel treatments were likely to reduce gaseous emissions.

In this study, the NP eradication or inhibition of microbial growth were the likely contributing factor to overall gas production reduction (Young and Santra, 2014). Additionally, the lower amount of cumulative gaseous emission from the NP treatments might be due to higher pH values in those treatments (Chen et al., 2005; Santra, 2012). Furthermore, absorption of the emitted gases by NPs within the manure slurry may also likely to reduce overall gas production. Therefore, all probable causes for gas reduction need to be determined to get a more detailed understanding of gas reduction chemistry.

2.3. Effects of NPs on CH_4 concentration

No statistically significant differences were observed among NP treatments ($p \leq 0.05$). All NPs treatment had statistically significantly less CH_4 compared with the control treatment ($p \leq 0.05$). Average CH_4 concentrations from the control treatment exhibited a sinusoidal pattern throughout the experimental period. CH_4 concentration increased gradually from 3.12% to 8.64% from 0 to 14 days, and then decreased to 4.94% after 42 days, thereafter it increased up to 7.42% until the end of 56 day experimental period (Fig. 3). In contrast, ZnSNL treatment exhibited CH_4 concentration reduction potential consistently. ZnSNL treatment showed an 87.52% CH_4 reduction at the end of day 2 and 98.84% CH_4 reduction at the end of 56 day experimental period. In contrast, the NACL

Table 2 – Properties of pre and post treated liquid dairy manure.

Treatments	pH	Conductivity (μS)	Ash (%)	Crude protein (%)	Total N (%)	Ammonia (mmol/L)
Control (initial)	6.64^{c}	9.84^{b}	18.47^{c}	15.00^{ab}	2.40^{ab}	96.56^{b}
Control (final)	6.47^{c}	10.91^{b}	17.45^{c}	13.33^{b}	2.13^{b}	121.68^{a}
ZnSNL	7.20^{b}	16.58^{a}	22.37^{ab}	15.54^{a}	2.49^{a}	78.42^{bc}
CuSNL	7.42^{b}	16.55^{a}	25.18^{a}	16.06^{a}	2.57^{a}	68.02^{c}
NACL	7.91^{a}	14.35^{a}	19.59^{bc}	16.20^{a}	2.59^{a}	57.59^{c}

N: nitrogen; ZnSNL: zinc silica nanogel liquid; CuSNL: copper silica nanogel liquid; NACL: N-acetyl cysteine liquid (NACL) coated zinc oxide quantum dots (Qdots).
Values followed by the same letter as a superscript (a, b and c) in a row are not significantly different at $p \leq 0.05$.
Control (initial) means the fresh manure collected from a source before starting the experiment.
Control (final) means the manure kept in a flask for 56 days without treating with nanoparticles (NPs).

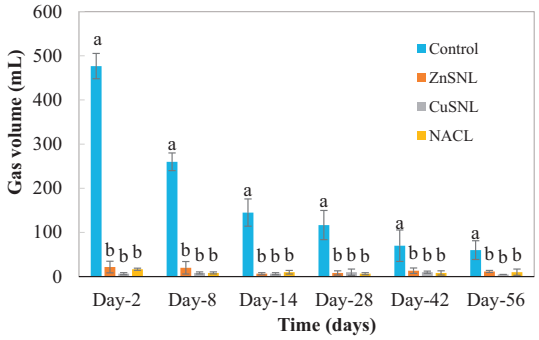

Fig. 2 – Gas production trends from dairy manure treated with different treatments and stored under anaerobic condition. Data presented are the means of three independent replications ± standard deviation. Values of each gas production form manure samples followed by different letters (a and b) above the bars indicate that the data points are significantly different at $p \leq 0.05$. ZnSNL: zinc silica nanogel liquid; CuSNL: copper silica nanogel liquid; NACL: N-acetyl cysteine liquid (NACL) coated zinc oxide quantum dots (Qdots).

treatment demonstrated a 98.42% to 99.82% reduction of CH_4 concentration compared to the control. This treatment showed a marginally better reduction potential than that of ZnSNL for the entire period of the experiment. CuSNL treated samples showed a similar CH_4 reduction trend as NACL and ZnSNL. CH_4 concentration reduction from the CuSNL treated samples varied 97.75% to 99.76% when compared with the control treatment.

The results were compared to a previous study (Gautam et al., 2013), where they observed the effect of nZnO impregnated sodium alginate beads on CH_4 concentration from anaerobic storage of manure and reported about 89% concentration reduction in comparison with the control treatment. All three NPs used in the present study showed a similar or better CH_4 concentration reduction (87.52% to 99.82%) over the entire experimental period where NACL exhibited the maximum reduction of 99.82% in CH_4 concentration although they are not statistically different. Reduction of CH_4 concentration was likely due to the antagonistic effect of the NPs on the methanogenic bacterial population because this environment along with the bacterial population is the driving force for CH_4 production (Van Elsas et al., 2006). Additionally, absorption of CH_4 within NP suspension over time was also likely a contributing factor to CH_4 concentration reduction (Swain et al., 2016).

2.4. Effects of NPs on CO_2 concentration

Carbon dioxide (CO_2) concentrations varied from 19.42% to 44.18%, 4.59% to 23.26%, 1.86% to 10.98%, and 1.52% to 6.87% for control, ZnSNL, CuSNL, and NACL treated samples, respectively (Fig. 4). Unlike CH_4 concentration, CO_2 concentration in the control treatment did not have a sinusoidal trend. Instead, it increased from 19.42% to 44.18% up to 28 days of the experimental period and then decreased and remained steady towards the end (29.16% to 31.54%). For all of the three nanogel treatments (ZnSNL, CuSNL, and NACL), the CO_2 concentration was lower at the beginning of the experimental period and marginally fluctuated up to day 28. Thereafter, CO_2 concentration increased gradually up to 21.28%. However, NACL treated manure showed the lowest CO_2 concentration compared with other two NP treatments and control. In the case of ZnSNL, within 28 days of the experiment CO_2 concentrations ranged 4.59% to 8.05% and then increased towards the end (23.26%). CO_2 reduction from the manure treated with ZnSNL ranged 20.24% to 85.33% compared with control during this 56 day and showed a maximum reduction at the end of day 28.

The CuSNL manure treatment resulted in 84.42% to 91.32% of CO_2 concentration reduction compared to the control treatment within the first 14 days. The CO_2 reduction was maximum at the end of day 8 (91.32%). Thereafter, it showed an increasing trend of CO_2 concentration and ended up with 62.34% reduction compared to the control. In comparison with the control treatment, overall CO_2 concentration reduction by CuSNL was 25.50% more than the reduction with ZnSNL

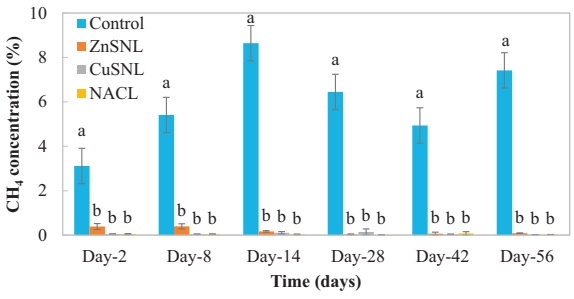

Fig. 3 – Methane (CH_4) concentration trends from dairy manure treated with different treatments and stored under anaerobic condition. Data presented are the means of three independent replications ± standard deviation. Values of each CH_4 concentrations form manure samples followed by different letters (a and b) above the bars indicate that the data points are significantly different at $p \leq 0.05$.

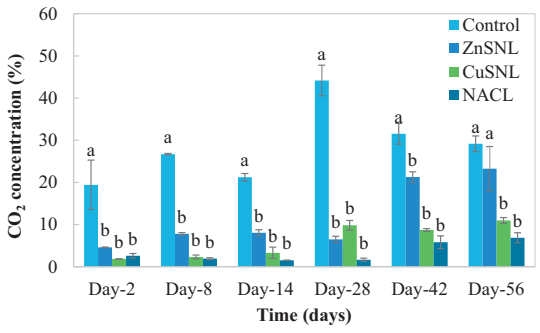

Fig. 4 – Carbon dioxide (CO_2) concentration trends from dairy manure treated with different treatments and stored under anaerobic condition. Data presented are the means of three independent replications ± standard deviation. Values of each CO_2 concentrations form manure samples followed by different letters (a and b) above the bars indicate that the data points are significantly different at $p \leq 0.05$.

treatment. In contrast, NACL treatment showed 76.44% to 96.28% reduction in comparison with control. However, compared to the control treatment, both ZnSNL and NACL treatment showed their maximum reduction potentiality by the end of the day 28. Additionally, at the end of the experimental period, NACL treatment exhibited overall 33.66% and 10.96% more CO_2 reduction than the amount reduced by ZnSNL and CuSNL treatments, respectively. Hence, among nanogels, NACL demonstrated the highest CO_2 reduction efficiency during the course of the experiment (Fig. 4). Moreover, NACL and CuSNL treatment showed the statistically significant amount of CO_2 reduction at $p \leq 0.05$ compared to other two (control and ZnSNL) treatments but not among them. Contrariwise, no statistically significant differences were found between ZnSNL and control treatments ($p \leq 0.05$).

As with total gas volume and CH_4 production, CO_2 generation is also dependent on the decomposition of organic matter in manure, and reduction in CO_2 generation might be an indication of the reduced amount of organic matter. Consequently, reduced activity of the microbial community due to the application of NPs is also likely to contribute to reduced CO_2 generation. Additionally, the conversion of most of the CO_2 to CH_4 through methanogenesis and absorption of CO_2 in the NP suspension are also likely causes towards CO_2 concentration reduction.

2.5. Effect of NPs on H_2S concentration

Hydrogen sulfide (H_2S) concentration in dairy manure treated with NPs was consistently lower than that of the control (Fig. 5). H_2S concentration was 2.45–156.00, 0.28–1.57, 0.17–2.30, and 0.27–2.58 ppm for control, ZnSNL, CuSNL, and NACL treatments, respectively. In the control treatment, apart from day 8, H_2S concentration increased up to day 28 and then decreased gradually. A higher activity of the sulfate reducing bacteria up to day 28 might have contributed towards higher H_2S concentration. In contrast, manure treated with ZnSNL exhibited a continuous reduction in the H_2S concentration until day 28 and then increased slightly. H_2S concentration from the ZnSNL treatment varied 48.98% to 99.75% throughout the experimental period. The other two NP treatments exhibited a sinusoidal trend of H_2S concentration within this period. No statistically significant differences were observed among the three NP treatments in terms of H_2S concentration reduction ($p \leq 0.05$), at day 42. Hence, H_2S concentration reduction was likely due to the biocidal effect of the NPs on the dissimilatory sulfite reductase (DSR) enzyme as well as reduced amount of substrate in the treatment since anaerobic bacteria use sulfur containing compounds to utilize sulfate as an electron acceptor to produce H_2S (Spence et al., 2008).

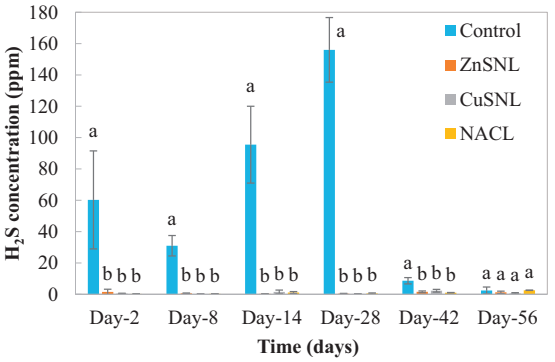

Fig. 5 – Hydrogen sulfide (H_2S) concentration trends from dairy manure treated with different treatments and stored under anaerobic condition. Data presented are the means of three independent replications ± standard deviation. Values of each H_2S concentrations form manure samples followed by different letters (a and b) above the bars indicate that the data points are significantly different at $p \leq 0.05$.

2.6. Effect of NPs on bacterial population

Total bacterial *coliform* counts in manure samples were as follows: initial untreated control 7.0×10^4 CFU/mL, 56 day untreated control 8.0×10^4 CFU/mL, ZnSNL 1.3×10^4 CFU/mL, CuSNL 1.1×10^4 CFU/mL, and NACL 8.0×10^2 CFU/mL (Fig. 6). The *coliform* counts were validated by similar total *coliform* bacterial counts of 5.8×10^4, 0.3×10^4, and 3.8×10^4 CFU/mL from untreated and treated liquid dairy manure reported by Gautam et al. (2016a). NACL treated manure exhibited the lowest *coliform* bacteria, whereas the control treatment showed the highest bacterial count compared to all other treatments. An approximately 15% increase in *coliform* bacteria count was observed between the initial control and final control treatments during the experimental period.

All three NP treatments exhibited 81.42% and 90.06% reduction in CFUs compared to the control (initial) and control (final) treatment, respectively ($p \leq 0.05$). CFUs among nanogel treated manure were not statistically significant but they were significantly lower than that with the control treatment ($p \leq 0.05$) (Fig. 6). Bactericidal action of the applied NPs is most likely the cause of the reduced bacterial count. However, the effect of the NPs on individual gas producing bacterial population under anaerobic storage condition needs to be evaluated to get the detailed knowledge on the mechanism and chemistry regulates gas volume and concentrations.

2.7. Effect of NPs on methanogen population

A standard curve representing qRT-PCR amplification of five 10-fold serial dilutions of standard (genomic DNA) from a pure

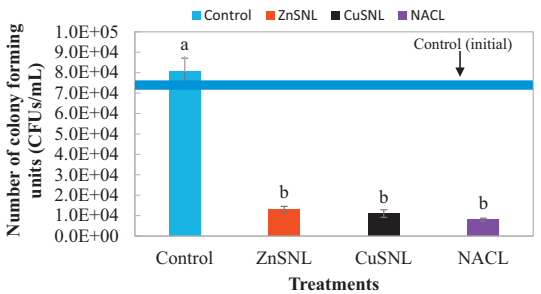

Fig. 6 – Effect of nanoparticles (NPs) on the colony forming units (CFUs) of *coliform* bacteria in liquid dairy manure treated with three NPs. Different letters (a and b) above bar indicate treatments are significantly different ($p \leq 0.05$) compared to control.

culture of *Methanobacterium formicicum* Schnellen had $R^2 > 0.99$ with the slope of −4.37 (Fig. 7). A qRT-PCR efficiency of >69% across all the standards were found from pre- and post-treated manure for quantification of a mcrA gene, which implied that more than 69% of the target sequences in the template genomic DNA were amplified in every cycle during the reaction process. Thereafter, amplifications of the genomic DNA were further investigated and specificity was found from the melting curve. Specific amplification of the standard mcrA gene was confirmed from the single sharp peak around 78°C in the melting curve (Fig. 8).

The presence of DNA copies (mcrA gene) in the environmental (treated manure) samples were in the same range (below or close to the lowest concentration of the standard chosen) as shown in the standard curves (Figs. 7 and 8). None of the mcrA genes from the treated and untreated manure samples were amplified. It is well known that the presence of polymerase inhibitors due to bile salts (cholic and deoxycholic acid) in human feces has a direct effect on the amplification efficiency of PCR with low detection limits and precision of the real-time qPCR quantification (Al-Soud et al., 2005; Lantz et al., 1997). The presence of urea in urine (Khan et al., 1991) and hemoglobin and heparin in a clinical blood samples (Vaughan-Shaw et al., 2015; Beutler et al., 1990; Brisson-Noel et al., 1991) were also recognized as inhibitors of PCR. Previously, it was also reported that humic and fulvic acid contaminants were extracted along with DNA during the DNA extraction process from soil, manure, and compost samples through inhibition of the amplicon production that limited amplification as PCR progressed (Al-Soud and Rådström, 1998; Fortin et al., 2004; Watson and Blackwell, 2000). In this research, a thorough and repeated optimization process was performed to optimize the PCR reaction parameters (denaturing, annealing, and extension temperatures and events) and concluded that some inhibitory contaminants were extracted along with DNA and were limiting the amplifications of mcrA gene from manure samples. Further studies are needed for a better understanding of the inhibition process and their removal strategies for getting better amplifications from the target gene sequence. However, a plate count was followed to quantify and to compare the total bacterial count in treated and non-treated samples.

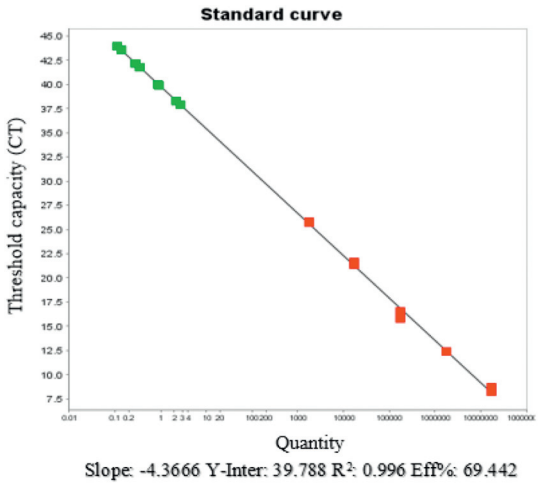

Fig. 7 – Standard curve for quantitative real time polymerase chain reaction (qRT-PCR) amplification of five ten-fold serial dilutions of pure culture *Methanobacterium formicicum* Schnellen extracted complementary deoxyribonucleic acid (c-DNA) samples. Eff%: percent efficiency. Red points indicate pure culture and the green points indicate environmental sample.

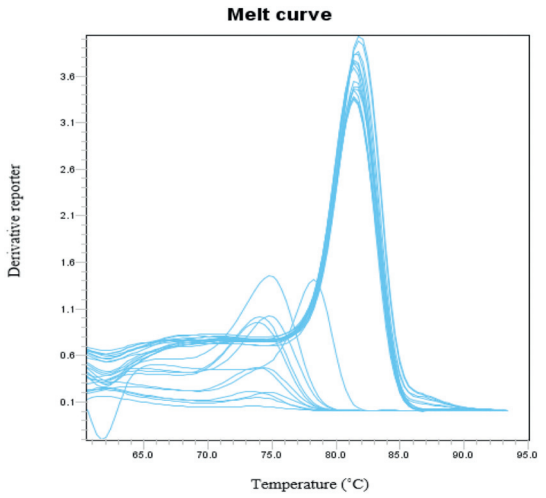

Fig. 8 – Melt curve for qRT-PCR amplification of five ten-fold serial dilutions of pure culture *Methanobacterium formicicum* Schnellen (peaks at ~82°C) and extracted c-DNA from environmental samples (peaks before 82°C).

2.8. Effects of NPs on VFAs

Total VFA (TVFA) concentrations in manure from the control and NP treatments ranged between 54.81 and 199.35 mmol/L (Table 3). A significantly higher TVFA concentration was observed with the control (final) compared to the initial control manure and NP treated manure (Table 3). ZnSNL and CuSNL NPs treated manure samples showed a significantly lower TVFA concentrations compared to the other treatment NACL ($p \leq 0.05$). The VFA concentrations between ZnSNL and CuSNL treated manure were similar ($p \leq 0.05$). Conversely, manure treated with NACL exhibited a higher amount of acetic acid compared to other two nanogel treatments. Acetic and propionic acid concentrations in ZnSNL, CuSNL, and NACL treatments were significantly higher in the control samples (initial and final).

Hill and Bolte (1989) and Lahav and Loewenthal (2000) reported that both acetic acid and propionic acid are substrates for bacterial CH_4 production. Hill and Bolte (1989) mentioned acetic acid as a substrate for methanogenic archaea and reported about 70% of CH_4 emission is from this substrate and bacterial combination under anaerobic storage condition. Hence, lower values of acetic acid from ZnSNL and CuSNL treated manure either revealed lower acetic acid production or conversion of most of the acetic acid to CH_4. Reduced amount of acetic acid conversion could be either by

Table 3 – VFAs from liquid dairy manure exposed and not exposed to nanoparticle treatments.

	VFAs (mmol/L)						
	Acetic acid	Propionic acid	Isobutyric acid	Butyric acid	Isovaleric acid	Valeric acid	Total VFA
Control (initial)	88.36[b]	32.58[a]	11.73[b]	31.98[a]	8.63[b]	2.74[b]	176.00[b]
Control (final)	104.47[a]	34.34[a]	14.93[a]	28.92[a]	10.66[a]	6.03[a]	199.35[a]
ZnSNL	39.51[d]	11.51[c]	2.8[d]	7.20[c]	1.32[d]	1.63[c]	63.98[d]
CuSNL	37.02[d]	6.92[d]	1.82[d]	6.44[c]	1.17[d]	1.45[c]	54.81[d]
NACL	80.71[c]	15.8[b]	6.37[c]	16.3[b]	4.36[c]	2.76[b]	126.30[c]

Values followed by the same letter as a superscript (a, b, c and d) in a row are not significantly different at $p \leq 0.05$.
Control (initial) is the initial sample of fresh manure not exposed to NPs.
Control (final) manure samples after 56 days incubation without NPs treatment.

an inhibition of fermentation or methanogenesis process in the anaerobic digestion pathway. However, a higher amount of acetic acid from the NACL treatment compared with other two NP treatments and lower amount of gas concentration from this treatment was likely to be an indication of reduced bacterial population from this treatment or adverse effect on any of the hydrolysis, acedogenesis or acetogenesis steps towards anaerobic digestion and hence gas production.

Generally, in the acidogenesis phase, acidogenic bacteria degrade the products of hydrolysis (sugar, amino acids, and fatty acids) into volatile fatty acids. During this process hydrogen (H_2), CO_2, and acetic acid are also produced and CH_4 is the main product of methanogenesis. CH_4 is mostly produced through acetate decomposition by aceticlastic methanogens and utilization of H_2/CO_2 by hydrogen-utilizing methanogens. Between these two ways of CH_4 production, two-third of the CH_4 gas is produced during anaerobic microbial conversion of acetate and about one-third is derived from H_2/CO_2 reduction (Bajpai, 2017). Since methanogens are susceptible to environment (depends mainly on optimum temperature, microbial population, and organic matter) and have a low metabolism rate that may have slowed down organic matter degradation process. Therefore, the acid balance between acidification and methanogenesis was broken as indicated accumulation of VFAs (Table 3). The accumulation of organic acids may further inhibited the metabolism of methanogens and even lead to a failure of anaerobic methanogenesis (Rea, 2014; Zhao et al., 2014).

Furthermore, low population in PCR studies (as not amplified) of methanogen microbial community, anaerobic digestion in room temperature (~22°C) (below optimum 25°C), and higher acetic acid contents with highest gas emission demonstrated that gas emissions were caused mostly from H_2/CO_2 reduction process. However, besides inhibition of bacterial population, relatively low VFAs (Table 3) and lower gas emissions (Fig. 2) in treated manure were likely due to probable chemical reaction between metal (Zn, Cu) NPs and acetate towards the formation of metal acetate (Brahmachari et al., 2010).

2.9. Possibilities and/or difficulties of applying the method

The present study was a proof of concept study for these applied NPs, and the principle objective of this study was to find the effectiveness of the applied NPs towards GHGs and pollutant gas emission mitigation. Direct application of such NPs by mixing with manure has revealed 92.73% to 95.83% reduction in cumulative gas volume paired with H_2S and GHG concentration reduction by 48.98% to 99.75%, and 20.24% to 99.82%, respectively. In contrast, such application method of NPs might have a number of environmental issues such as endemic bacterial death in the manure, soil, and neighboring ecosystems. The ultimate application of NP treated manure in the agricultural field presumptively a distress of heavy metal accumulation in the soil and hence residual toxicity. However, based on the annual pollutant loading rate (140 kg Zn/(ha·year) and 75 kg Cu/(ha·year)), and assumption of field application of the NP treated manure initial estimate with the current application rate of 3 g Zn/L has exposed with a final concentration of NPs in the soil which is within the allowable limit of 10–300 ppm for zinc and 2–100 ppm for copper in the soil (Balentine and Wilson, 1995; EPA Region VIII). Furthermore, part of the applied NPs might act as an essential micronutrient and may catalytically make fertilizer more readily available for uptake by plants as well. Alternatively, to avoid such environmental consequences, indirect application of NPs such as entrapment of NPs into porous polymer (Gautam et al., 2016a), and preparation of biofilters using the NPs can be done for further studies. Although application of entrapped NPs in manure management systems is very limited, entrapment of NPs in polymer is widely used in water and solid waste management area. However, indirect application of NPs might have ended up with reduced efficacy of the applied NPs. All of these warrant us a further study for the fate and transport of the applied NPs.

Besides, the NP formulation was prepared in a single pot using traditional wet chemistry. The process itself is industrially viable. In this proof-of-concept study, we have used reagent-grade chemicals which are expensive. We expect that adaptation of agriculture grade chemicals would drastically reduce the raw material cost. However, further research is needed to compare cost and efficacy of NP formulation using reagent-grade and agri-grade chemicals.

3. Conclusions

Compared with the control treatment, liquid dairy manure treated with three different NPs have exhibited increase in pH and consequently an decrease in VFA. All three nanogel

treatments reduced gaseous volume and gas concentration significantly. CuSNL outperformed other treatment in terms of total gas volume and H_2S concentration reduction, whereas, NACL treatment outperformed other treatments in terms of CH_4 and CO_2 concentration reduction. Reduction of GHGs and H_2S were likely due to microbial inhibition since NPs treated samples had lower CFUs than the controls. Further studies are needed to understand the amplification of inhibition process since none of the mcrA genes from the treated and untreated manure samples were amplified.

Acknowledgements

This was a collaborative initiation between North Dakota State University (NDSU) and University of Central Florida (UCF). This research was conducted using the corresponding author's discretionary funding. No specific funding was involved. Thanks to Debra Baer, Technical Communication Specialist, Agricultural and Biosystems Engineering Department, NDSU, for reviewing the manuscript.

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