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LONG AND SHORT TERM IMPACTS OF CuO, Ag AND CeO_2 NANOPARTICLES ON ANAEROBIC DIGESTION OF MUNICIPAL WASTE ACTIVATED SLUDGE

E. Kökdemir Ünşar [a], A.S. Çığgın [a], A. Erdem [a], N.A.Perendeci [a,]*

[a] Department of Environmental Engineering, Akdeniz University, 07058, Antalya–TURKEY

* Corresponding author

Department of Environmental Engineering, Akdeniz University, 07058, Antalya–TURKEY

E-mail: aperendeci@akdeniz.edu.tr

Tel: +90 242 310 63 34

Fax: +90 242 310 63 06

Environmental Impact Statement

Eventhough nanoparticles provides numerous advantages in industrial use due to their unique chemichal properties, they also create a great suspicion about their environmental impacts with the same reason. There are many studies investigating their impacts on aquatic and terrestiral organisms with mostly toxic findigs. Aerobic microorganisms are a research area for NPs as well. However, there are very limited number of studies concerning the environmental engineering application of waste activated sludge (WAS) treatment in terms of NPs impacts.

This study provides a comparison of short and long term impacts of various NPs on anaerobic digestion which is used for stabilizing WAS via biogas production, FISH analysis and modelling with both similar and novel findings to the literature.

Abstract

In this study, long and short term inhibition impacts of Ag, CuO and CeO_2 nanoparticles (NPs) on anaerobic digestion (AD) of waste activated sludge (WAS) were investigated. CuO NPs were detected as the most toxic NP on AD. As the CuO NP concentration increased from

5 to 1000 mg/gTS, an increase in the inhibition of AD from 5.8 to 84.0% was observed. EC_{50} values of short and long term inhibitions were calculated as 224.2 mgCuO/gTS and 215.1 mgCuO/gTS, respectively. Ag and CeO_2 NPs did not cause drastic impacts on AD as compared to CuO NPs. In long term test, Ag NPs created 12.1% decrease and CeO_2 NPs caused 9.2% increase on the methane production of WAS at the highest dosage. FISH imaging also revealed that the abundance of Archaea in raw WAS were similar in short and long term tests carried out with WAS containing Ag and CeO_2 NPs. On the other hand, CuO NPs caused inhibition on Archaea in long term test. Digestion kinetics of WAS containing Ag, CeO_2, CuO NPs were also evaluated with Gompertz, Logistic, Transference and First Order models. Hydrolysis rate constant (k_H) for each concentration of Ag and CeO_2 NPs and the raw WAS was 0.027745 d^{-1} while k_H of WAS containing high concentrations of CuO NPs was found as 0.001610 d^{-1}.

Keywords: Anaerobic digestion, Inhibition, Modeling, Nanoparticle, Sewage sludge

1. Introduction

The use of nanoparticles (NPs) in commercial products and industrial applications has increased greatly in recent years since they have eminent magnetic, electrical and optical properties with low cost and low energy needs. It is believed that products produced with NPs will have a significant role in future and improve the quality of life, so the amount of variety

and importance of these products are increasing continuously. As a natural consequence of this increase, NPs are released to the environment without any control. Their release can happen during the production, usage or disposal processes of the NP included products. Especially NPs usage in the hygiene and cosmetic industries increase their contact with water and cause an important concern for the wastewater treatment plants (WWTPs) with a biological treatment process. The contact of shower, laundry, cleaning and rain waters with the products containing NPs cause NPs to leave the product and follow the pathways of water to the WWTP. In the "*Nanotechnology Products Risk Assessment*" report [1] of European Union Scientific Committee, it is stated that NPs tend to aggregate and adsorb to organic materials and, therefore, accumulate in the solid part of wastewaters and reach to high concentrations. Similarly, there are studies presenting that NPs are removed from wastewaters to a large extend and accumulate in sludge [2 – 6]. This accumulation causes NPs to emerge in waste activated sludge (WAS) processing operations.

WAS management is a global environmental problem. Besides, WAS is also a quite destructive pollution source itself since most of the pollutants are sorbed onto suspended solids and as a result they are found in sludge through sedimentation occurring in primary and secondary clarifiers. It is stated that 53% of the sludge in EU-27 is used in agriculture directly or after composting and more than 40% of produced biosolids are applied to land in USA and Canada [7]. It is necessary to stabilize the sludge to prevent the application of pollutants accumulated in WAS to the soil. Anaerobic digestion (AD) is one of the most technically mature and cost-effective process to convert sludge into methane-rich biogas and, therefore, stabilize the sludge [8]. During AD, several biochemical pollutants such as surfactants and medicine residues accumulated in sludge can be biodegraded and, therefore, not become a threat to the application of sludge to the soil after AD [7]. Furthermore, many materials can

have inhibitory effects on AD and inhibition value varies according to the material itself and process conditions.

The potential risk of releasing NPs into the natural environment threatens human health and ecosystems [9]. However, compared to NP synthesis and applications, limited study has been focused on environmental and health impacts [10] suggesting that the fate and transport of NPs should be investigated thoroughly to minimize their toxic effects [11, 12]. Higher organisms as targets were used in most of the work performed on the toxicology of NPs for defining ecotoxicological effects [13]. Toxicity of NPs on aerobic microorganisms and aquatic organisms has been investigated in detail [14 – 25] however limited number of research [6, 10, 13, 26 – 33] addressed the impacts of NPs on AD. Therefore, it is important to determine the effects of NPs on microorganisms participate in WAS AD process [28].

Since available information and detailed scientific contributions of NPs effects on WAS AD are insufficient, any effort related to inhibition risk of NPs should help in the regulation of production and use of NPs in the industry. In this work, short and long term impacts of three different NPs on WAS AD were examined with acute and chronic impact tests. Inhibition amounts of NPs on AD and EC_{50} values were calculated from acute and chronic impact test results. Moreover, efforts have been made to explain the inhibition effects of NPs on AD process by the help of modeling of AD. Finally, FISH analysis was applied to determine the effect of NPs on methanogenetic diversity involved in WAS AD.

2. Materials and Methods

2.1. WAS characterization analyses

WAS is taken from the sludge centrifuge of a municipal WWTP with a design capacity of 31250 m^3/day. The anaerobic seed sludge was withdrawn from the WAS AD reactors of

another WWTP with a design capacity of 210000 m^3/day in Antalya. The analyses of dry matter (TS), organic matter (VS) and chemical oxygen demand (COD) were performed according to Standard Methods [34]. The contents of lignin, cellulose, hemicellulose and soluble matter were determined by Van Soest [35] using the Fiberbag system (Gerhardt). Total Kjeldahl nitrogen (TKN) was determined by TKN analyzer (Buchi Digest Automat K-438 and Buchi Auto Kjeldahl Unit K-370). Soluble carbohydrate concentration as glucose and soluble reducing sugar were determined by Anthrone [36] (Dreywood, 1946) and Dinitrosalicylic acid (DNS) methods [37] (Miller, 1959), respectively. Protein concentration and extractive matter including lipids were quantified by Lowry method [38] (Lowry et al., 1951) and soxhlet extraction [39] (Bridoux et al., 1994), respectively. Elemental analyses were performed by the CHNS analyzer (LECO, CHNS-932).

2.2. Preparing NP suspensions and determination of particle sizes

Ag, CuO and CeO_2 NPs were purchased from Alfa Aesar. The stock NP suspension of 2 g/L was prepared for BMP analyses [40, 41]. The stock NP suspension of 30 g/L was prepared for anaerobic inhibition test and then sonicated (25^{o}C, 30 W, 20 kHz, Sonics&Materials Vibra Cell) for 1 hour in order to break the NP aggregates in this suspension. Sodium dodecylbenzene sulfonate (SDBS) was added to the NP suspensions with a final concentration of 0.1 mM to prevent the aggregation of NPs for biochemical methane potential (BMP) and anaerobic inhibition tests. The final NP concentrations in BMP reactors were set to 5, 50, 150, 250 and 500 mgNP/gTS and final concentrations of 5, 50, 150, 250, 500, 750 and 1000 mgNP/gTS for each NP in the anaerobic inhibition test reactors were prepared from the stock suspensions. Lower concentrations of NPs were chosen to be environmentally relevant and to be able to compare to literature, however higher concentrations of NPs were chosen with consideration of a worst-case scenario such as an industrial spill and also to fill the lack of

investigation on the impacts of high concentrations to AD in literature as suggested by Nyberg et al [26] as well.

Particle size analysis for each stock NP suspension was performed by dynamic light scattering technique using a Malvern Zetasizer Nano ZS (Malvern Instruments, USA). The instrument has a 4 mW He-Ne laser with a wavelength of 633 nm, and a measurement angle of 173°.

It is a well-known phenomenon that when high concentrations of NPs are applied in solutions with high ionic strengths, NPs tend to agglomerate and to form larger sizes. Therefore, to control the concentration of NPs in the solutions for a better stability, the use of dispersants, pH adjustment or various mixing methods need to be applied [42]. In this study, a cationic surfactant SDBS was used and ultrasound treatment was applied to stabilize the suspensions, and the pH of the suspensions were adjusted to pH 7.5±0.5. Commercially determined particle sizes and average particle sizes after being used in stock suspensions are given in Table 1. Particle size results showed that SDBS addition and ultrasound treatment could not stop the agglomeration of NPs completely which might be due to the high ionic strength, high NP concentration, neutral pH of the stock suspensions and the consequential formation of aggregates [43-45]. The particle size results are consistent with the literature [28, 46].

2.3. Biochemical methane potential (BMP) test for long term inhibition

The long term impacts of NPs on AD of WAS are investigated by the measurement of methane production. The methane production was studied by batch BMP tests following the procedures established by Carrere et al. [40] to investigate the long term (chronic) inhibition impacts of different NP concentrations on WAS AD.

2.4. Anaerobic short term inhibition test

The test was performed according to the "*ISO 13641-1 Water Quality: Determination of inhibition of gas production of anaerobic bacteria*" standard [47] to observe the short term (acute) inhibition impacts of NPs on AD of WAS. A 400 mL of working volume containing WAS, anaerobic seed sludge, NP suspension, and distilled water was used in the 500 mL reactors. Total gas pressures in the reactors were measured for 72 hours in every 12 hours by Digitron 2085p handheld digital manometer. Inhibition values caused by different concentrations of each NP were calculated as a percentage (%) using the equation given in the standard.

EC_{10}, EC_{20} and EC_{50} concentrations were calculated by using EPA-TRAPv1.22 (Environmental Protection Agency - Toxicity Relationship Analysis Program Version 1.22) program. Model type and data analysis type were used as threshold Sigmoid and nonlinear regression, respectively.

2.5. Mathematical Modeling

The Gompertz equation [48], Logistic [49] and Transference functions [50] were used for the determination of the biogas production potential (P_M), the maximum methane production rate (R_M) and duration of lag phase (λ) for digestion of WAS containing Ag, CeO_2 and CuO NPs. Furthermore, first-order reaction kinetic model [51] was used to determine hydrolysis rate constant (k_H) and overall reaction rate constant (k_R). The models used for evaluating the AD of WAS are presented in Table 2. Model simulations and evaluations were performed using the AQUASIM 2.0 [52]. The parameters P_M, R_M and λ were estimated for each model by comparing calculated results with measured data, according to the best fit obtained between the experimental methane production and the model simulation. The parameters were estimated by the weighted least squares method. Model parameters were estimated by

minimizing the sum of the squares of the weighted deviations between measurements and predicted model results.

2.6. Fluorescence in situ hybridization (FISH) analysis

FISH was used to determine the inhibition impact of NPs on Archaea population in the WAS AD. FISH analyses were carried according to the Amann et al. [53]. Oligonucleotide probes were EUB338 mix for all bacteria [54], ARC915 for all Archaea [55], MX825 for *Methanosaeta spp.* [56], MSMX860 for *Methanosarcinales spp.* [56] and MC1109 for *Methanococcus spp.* [56]. The details of the oligonucleotide probes are presented in Table 3. Olympus BX51 fluorescent microscope was used for imaging and images were evaluated by ImageJ program (J 1.47 software) [57].

3. Results and Discussion

3.1. WAS characterization

The results of WAS characterization analyses are presented in Table 4. TS and VS were found as 182.60 gTS/kgSample (18.26%) and 124.86 gVS/kgSample (12.49%), respectively. Slightly lower results were reported for TS as 14.58% [58] and 13.8% [59] and for VS as 10.63% [58] and 11.8% [59]. Sludge centrifugation applied for dewatering in WWTP results in higher TS and VS values, compared to literature.

tCOD values were found as 1862.09 mgCOD/gVS and similar results were also reported by Chang et al. [60] as 1075 – 3399 mg/L and by Ji et al. [61] as 2080 mgCOD/gVS. However, relatively higher sCOD values of 198.36 mgCOD/gVS were found than the results given by Chang et al. [60] (as 5 – 159 mg/L) and Ji et al. [61] 28.1 mgCOD/gVS.

TKN was found as 96.35 mgTKN/gVS (1.3%) which is lower than the results (3.73 – 7.81%) in the literature [62, 63]. The composition of soluble fraction, cellulose, hemicellulose and

lignin contents of the WAS were found as 93.64, 0.76, 3.12 and 2.48%, respectively. Even though the results are lower than those of Mottet et al. [64], the findings are in the comparable distribution with the reported values for five different WAS samples. Sugar, protein and extractable material containing lipid content of WAS were determined as 98.30 mgGlu/gVS, 231.25 mgPro/gVS and 0.12%, respectively, and higher results were shown by Mottet et al. [64] as 170 – 300 mgGlu/gVS, 340 – 470 mgPro/gVS and 0 – 90 mg/gVS, respectively in WAS. The elemental analysis resulted as 32.59% C, 5.29% H, 5.31% N and 0.72% S showing that the results were consistent with the reported compositions by Heo et al. [65].

3.2. NPs long term impacts on AD of WAS

To determine the long term effects of NPs on WAS AD, 5, 50, 150, 250 and 500 mgNP/gTS (equal to 11, 110, 330, 550 and 1100 mgNP/L) concentrations of Ag, CuO and CeO_2 NPs were used in BMP tests. Methane produced by anaerobic seed sludge (31.26 $mLCH_4$/gVS) was subtracted from the samples' methane amount to normalize the BMP results. To determine the SDBS effect on methane potential, concentrations of 0.09, 0.86, 2.61, 4.34 and 8.68 mgSDBS/gTS which are equal to the SDBS concentrations added to the BMP reactors with NP suspensions were prepared as controls and their BMP tests were performed under same conditions. Low standard deviation (stdev) (2.78 $mLCH_4$/gVS) showed that SDBS had a negligible impact on methane production.

Methane productions of raw WAS, anaerobic seed sludge and WAS treated with 5 different concentrations of Ag, CuO and CeO_2 NPs are presented in Fig. 1 (a), (b) and (c), respectively. Methane production of the control sample (raw WAS) was measured as 72.3 $mLCH_4$/gVS. Concentrations of 150, 250 and 500 mgAg/gTS caused more than 5% inhibition on the methane production of WAS. The observed inhibitions are 6.5, 7.8 and 12.1%, respectively and the decreasing trend of biogas production started after the 17^{th} day of the BMP test. No

significant reduction in methane production was observed at 5 and 50 mgAg/gTS concentrations.

There are limited studies in literature evaluating impacts of NPs on AD. Low concentrations of Ag NPs have been mostly investigated and no impact was reported [6, 13, 30]. Barrena et al. [13], investigated the inhibition effects of 10, 16 and 18 mgAg/L NP concentrations on cellulose degradation during 21 days. It was demonstrated that Ag NPs do not have a significant effect on bacterial consortium since the biogas production was not significantly different ($p<0.05$) from the control test. Yang et al. [6] examined the inhibition effects of 10, 20 and 40 mgAg/L NP concentrations on WAS AD. Long term (28 days) WAS AD of control and Ag NP treated groups showed no significant difference of biogas production ($p=0.36$) in their study. Garcia et al. [30] employed a higher NP concentration of 170 mgAg/L on cellulose degradation. They also observed no statistical differences between control and Ag NP treated sample. Gonzalez-Estrella et al. [10] exposed anaerobic granular sludge to 1500 mgAg/L for 10 days. They indicated that Ag NPs did not pose a serious inhibitory effect to acetoclastic or hydrogenotrophic methanogenic assays with the normalized specific methanogenic activity value above 70%. The results of this study are consistent with the literature for the lower concentrations. However, when higher concentrations of Ag NPs (150, 250 and 500 mgAg/gTS) were used to simulate a worst case scenario, an inhibition more than 10% was observed. In contrast to Gonzalez-Estrella et al's 10 day study, we observed inhibition on biogas production for the higher concentrations of Ag NPs. On the other hand, low inhibition effects were observed after 17 days in this study. Yang et al. [32], determined the impact of Ag NPs on AD of municipal solid waste more than 200 days continuous operation. It is found that 10 mgAgNPs/kgWaste or higher concentrations inhibited methanogenesis in Yang et al. study. They explained inhibition with the silver bioavailability because of slow and long term silver release from nanosilver dissolution under long term AD.

Our results are consistent with the results of Yang et al. [32] in the context of longer exposure time. It is thought that the release of Ag ions creates the antibacterial impact of Ag NPs by destroying cell membrane or causing enzyme inactivation or protein dephosphorylation [66].

As seen in the Figure 1 (b), CuO NPs inhibited the methane production beginning from the 150 mgCuO/gTS concentration. Inhibition on methane production occurred at the beginning of the experiment and 150, 250 and 500 mgCuO/gTS concentrations showed severe inhibition. The toxic impact of CuO NPs should be associated with its heavy metal characteristic. There are a very limited number of studies [10, 29, 33, 67] searching the impacts of CuO NPs on AD in literature. Gonzalez-Estrella et al. [10], tested CuO NPs at high concentrations (250, 500 and 1500 mgCuO/L) with methanogenic assays during 10 days. Significant inhibitions were observed and CuO NPs affected the methanogenic activity decreasing the specific methanogenic activity to 79.9, 71.3 and 54.6% for the assays supplied with 250, 500 and 1500 mgCuO/L concentrations, respectively. Obtained results from this study are consistent with the work of Gonzalez-Estrella et al. [10], for the CuO NPs which is thought that copper has a similar inhibition impact mechanism associated with its solubility [29].

As seen from the Figure 1 (c), CeO_2 NP has a positive impact on WAS AD. CeO_2 concentrations of 150, 250 and 500 mg/gTS increased the methane production to a maximum of 18.8, 25.5 and 9.2%, respectively. There are also very limited studies [10, 30, 68] investigating the impacts of CeO_2 NPs to AD. Gonzalez-Estrella et al. [10], exposed anaerobic sludge to 1500 mg CeO_2 NP/L concentration for 10 days, and no inhibition was observed. Garcia et al. [30] found that CeO_2 NP concentrations <160 mg/L had no effect on methane production and inhibition impact reached to a maximum value when 640 mg/L concentration was applied. In the study of Garcia et al. [30], it is believed that the results were due to the production process of CeO_2 NP. CeO_2 NPs takes oxygen to its crystal structure

during its synthesis process and has the property of holding the oxygen or releasing it according to the environmental conditions. This property can cause disruption of the respiration mechanism of microorganisms and cause inhibition. Our findings present a different result than Garcia et al. [30] and Gonzalez-Estrella et al. [10] works and there is no literature to compare the findings of this study.

3.3. NPs short term impacts on AD of WAS

The impacts of 5, 50, 150, 250, 500, 750 and 1000 mgNP/gTS concentrations of CuO, Ag and CeO_2 NPs were investigated on WAS AD. SDBS was also added to the stock suspensions of NPs to determine the SDBS effect on anaerobic inhibition. Low stdev (11.3 mL biogas) showed that SDBS had a negligible impact on inhibition of AD. This result is consistent with the results of Mu et al. [28] on the impact of SDBS on AD.

Methane productions of WAS treated with 5, 50, 150, 250, 500, 750 and 1000 mgAg/gTS (equal to 30, 299, 896, 1494, 2987, 4481, 5975 mgAg/L) concentration, control sample without Ag NPs and reference DCP sample are given in Figure 1 (d). In long term tests, Ag NP caused lower inhibition on biogas production especially at higher concentrations. However, similar results are not observed in short term tests. Samples including Ag NPs did not present any significant difference on the biogas production than that of the control sample (Figure 1 (d)). Except WAS containing 1000 mg/gTS concentration of Ag NP, all of the Ag NP samples and control sample biogas productions were determined very close to each other. Stdev and average stdev are calculated as 12 and 9.1 mL, respectively. Therefore, it is concluded that 1000 mgAg/gTS sample has a very low inhibition impact on AD. Ag NPs lower inhibition impact was observed on biogas production as well as its lower positive impact. Inhibition calculations show that all of the impacts were below 10% except 1000 mgAg/gTS concentration. 5, 50, 250, and 500 mgAg/gTS concentrations increased biogas

pressure 2.5%, 1.9%, 4.1%, and 5.8%, respectively. 150 and 1000 mgAg/gTS concentrations decrease biogas pressure 3.73% and 11.3%, respectively. It can be seen from these results that Ag NP do not have a significant impact on AD.

CuO NP is determined as the most inhibitor NP both in BMP and short term inhibition tests. Methane productions of WAS containing 5, 50, 150, 250, 500, 750 and 1000 mgCuO/gTS (equal to 31, 310, 930, 1550, 3100, 4650, 6200 mgCuO/L) concentration, control sample without CuO NP and reference DCP sample are given in Figure 1 (e). As can be seen in the Figure 1 (e), all of the CuO NP concentrations had a negative impact on methane production and caused a production rate below the control reactor. According to the ISO13641-1 calculation, the inhibition amounts of 5, 50, 150, 250, 500, 750 and 1000 mgCuO/gTS concentrations are 5.8 30.8, 67.3, 70.5, 74.4, 81.3 and 84%, respectively.

Biogas and methane production dose-response curves during exposure to CuO NP of short and long term tests are presented in Figure 2 (a) and (b), respectively. EC_{50} values of short and long term inhibition tests were calculated as 224.2 mgCuO/gTS (≈1388.6 mgCuO/L) and 215.1 mgCuO/gTS (≈473 mgCuO/L), respectively. EC_{10} and EC_{20} values with the confidence 95% intervals are calculated as 134.18 and 161.30 mgCuO/gTS, respectively, for long term inhibition test.

To our knowledge, there are only a few works discussing short term impacts of CuO NPs on AD in the literature [29, 33]. Luna-delRisco et al. [29], applied ISO 13641 test for 7.5 – 480 mgCuO/L (0.8 – 53.3 mgCuO/gTS) concentrations. 15 mgCuO/L (1.7 mgCuO/gTS) concentration caused 30% of inhibition on biogas production compared to the control group without CuO NPs. The results of Luna-delRisco et al. [29] suggest lower inhibition concentrations than the findings of this study. It is thought that the differences between the results are due to the used concentration range for the assays. Gonzalez-Estrella et al. [10], calculated EC_{50} values for the BMP test lasted 10 days. EC_{50} values were found as 223

mgCuO/L and >1500 mg/L for the acetoclastic methanogens and hydrogenotrophic methanogens, respectively. The determined short term toxic impacts of CuO NP in this study are similar to the work of Gonzalez-Estrella et al. [10].

CeO_2 NP did not have a significant effect on the AD according to the ISO13641-1 test results. Methane productions of WAS containing 5, 50, 150, 250, 500, 750 and 1000 mgCeO_2/gTS (equal to 29, 284, 853, 1421, 2843, 4264, 5686 mgNP/L) concentration, control sample without CeO_2 NPs and reference DCP sample are presented in Figure 1 (f). Only a 5.5% of low inhibition impact was determined at the concentration of 1000 CeO_2/gTS. CeO_2 NP did not cause a significant decrease in gas production in neither short nor long term inhibition tests.

3.4. Modeling results

Methane production of raw WAS and WAS containing all concentrations of Ag and CeO_2 NPs were simulated with the regression coefficient (R^2) of > 0.93 using same kinetic parameters presented in Table 5. According to results, Ag and CeO_2 NPs did not have an impact on WAS AD. Since, Ag and CeO_2 NPs did not lead to any inhibition, hydrolyses were approximately completed in 3 days. k_H value was determined as 0.027745 for each concentration of Ag and CeO_2 NPs as well as the raw WAS with the R^2 of > 0.999.

Similarly, methane production from raw WAS and WAS containing 5 and 50 mgCuO/gTS were also modeled with relatively same kinetic parameters. As seen from Table 6, with the increasing concentration of CuO NPs, starting from 150 mgCuO/gTS, P_M and R_M values were decreased and λ values are increased. This means that CuO NPs inhibited the methanogenic consortium which resulted with low biogas production and spesific methane production rate. Furthermore, increased λ indicates inhibition and that anaerobic consortium needs more adaptation time for the production of biogas. According to results, WAS containing 250 and

500 mgCuO/gTS were simulated with the higher R^2 by Gompertz equation (Table 6), although first order modeling yielded best fit for the raw WAS and WAS containing 5, 50 and 150 mgCuO/gTS (Figure 3).

First order kinetic model was used to evaluate hydrolysis period of raw WAS and WAS treated with 5, 50, 150, 250 and 500 mgCuO/gTS concentrations (Table 7). Hydrolysis period was completed in 3 days for raw WAS and WAS containing 5 mgCuO/gTS. k_H of WAS started to decrease with the addition of 50 mgCuO/gTS and k_H reached to 0.001610 d^{-1} when WAS dosed with 500 mgCuO/gTS. On the other hand, hydrolysis period observed as 3 days with WAS containing 5, 50 and 150 mgCuO/gTS similar to raw WAS. Furthermore, hydrolysis period extended to 12 and 17 days when WAS contained 250 and 500 mgCuO/gTS, respectively, with the significant decrease in k_H. There is only one modeling work in literature performed by Doolette et al. [31]. Calculated k_H values of raw WAS and Ag NP dosed WAS were 0.13±0.020 and 0.12±0.014, respectively. Approximately similar k_H and methane potential values for raw WAS and WAS dosed with Ag NP were found. Modeling result supported that Ag NP have no inhibition effect on WAS AD. Since there is no reference to modeling of CuO NP inhibition on WAS AD in literature, modeling result from this study is the first confirmation of CuO NP inhibition of the anaerobic sludge degradability.

3.5. FISH results

The abundance of Archaea was observed in raw WAS (Figure 4a). Observed abundance of Archaea was similar in short and long term tests carried out with WAS containing Ag and CeO_2 NPs (Figure 4b and c). On the other hand, a small decrease in the amount of the total Archaea was observed after the short term test carried out with WAS containing 1000 mgCuO/gTS compared to raw WAS (Figure 4d). Additionally, a remarkable decrease occurred in the amount of Archaea after the long term test of WAS dosed with 500

mgCuO/gTS (Figure 4e) parallel to the observed inhibition of methane production in long term test of WAS containing CuO NP. This result suggests that Archaea population is not notably inhibited in short term with the CuO NP, but long term of CuO NP causes inhibition on Achaea.

The amount of *Methanosaeta spp.* population was reduced by exposure of CuO NP, although no change was observed in the amount of *Methanosaeta spp.* in the presence of other NPs at any concentration. With the addition of NPs, the small decrease was observed in the amount of *Methanosarcinales spp.* compared to raw WAS. This observation pointed out that *Methanosarcinales spp.* is not heavily affected by CuO NPs inhibition of WAS AD. On the other hand, *Methanococcus spp.* was detected in raw WAS and survived in WAS containing CuO NP while these species were not observed in the WAS dosed with Ag NP. The absence of *Methanococcus spp.* in WAS including Ag NP indicated the negative effect of Ag NP on this species.

4. Conclusions

CuO NPs were found to be the most toxic on biogas production both in long and short term inhibition tests. Ag NPs did not cause an inhibition impact in short term test, eventhough in long term tests, Ag NPs caused slight inhibition on biogas production, especially at higher concentrations which was interpreted that exposure time is as important as dosage. As opposed to Ag and CuO NPs, CeO_2 NP caused an increase in methane production in long term BMP test.

Ag and CeO_2 NPs and raw WAS k_H values were determined as same which revealed no inhibition impact on AD, caused from Ag and CeO_2 NPs. On the other hand, k_H values of CuO NPs decreased as the concentrations increase for the First Order model. Estimated P_M

and R_M decreased while the λ increased with the increase of CuO NPs concentration in Gompertz, Logistic and Transference models, which supports the experimental findings from long and short term inhibition tests.

FISH imaging revealed that the abundance of Archaea observed in raw WAS were similar in ISO and BMP tests carried out with WAS containing Ag and CeO_2 NPs. On the other hand, CuO NP caused decrease of Archaea.

The results of this study are an important contribution to published data related to inhibition of NPs on WAS AD. Additionally, the presented results emphasize the need for a deeper understanding of the interaction of NPs with the environment considering their rapid increase in industrial use and release to the environment.

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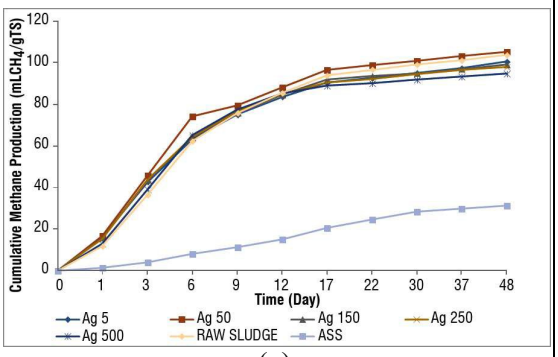
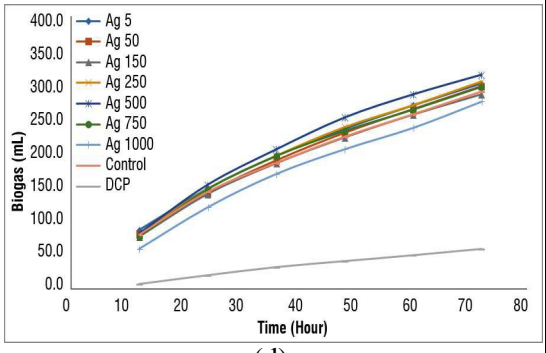
Supplementary File

Figure 1. FISH images for Raw WAS (a, c, e) and WAS dosed with 1000 mgCuO NP/gTS from ISO test (b, d, f). DAPI (blue), all bacteria (green) and (a, b) *Methanosaeta spp.* (red), (c, d) *Methanosarcinales spp.* (red), (e, f) *Methanococcus spp.* (red)

Figure 1. BMP test results for the concentrations of 5, 50, 150, 250, 500 mgAg/gTS, raw WAS and anaerobic seed sludge (ASS) (a), 5, 50, 150, 250, 500 mgCuO/gTS, raw WAS and ASS (b) and 5, 50, 150, 250, 500 mgCeO_2/gTS, raw WAS and ASS (c), Short term inhibition test results for the concentrations of 5, 50, 150, 250, 500, 750 and 1000 mgAg/gTS, control sample and reference DCP sample (d), 5, 50, 150, 250, 500, 750 and 1000 mgCuO/gTS, control sample and reference DCP sample (e) and 5, 50, 150, 250, 500, 750 and 1000 mgCeO_2/gTS, control sample and reference DCP sample (f)



(a)



(d)

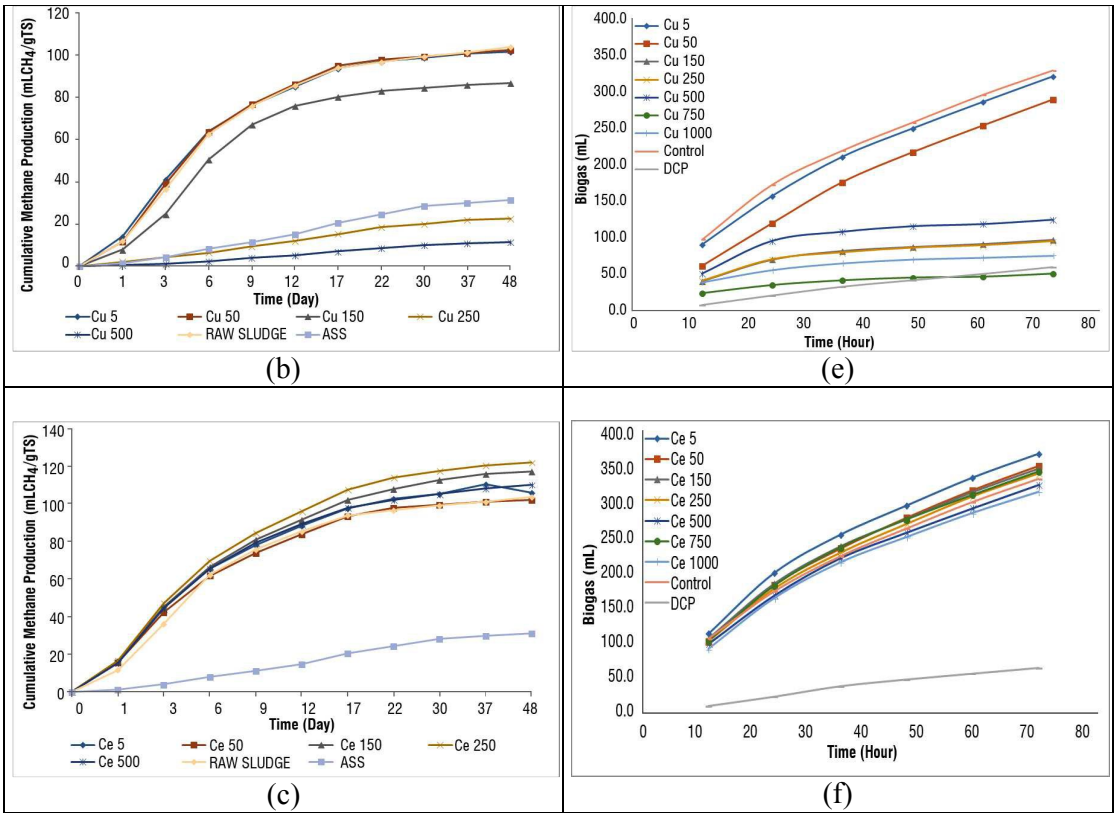


Figure 2. Dose-response curves during exposure to CuO NP of short term (a) and long term (b) AD

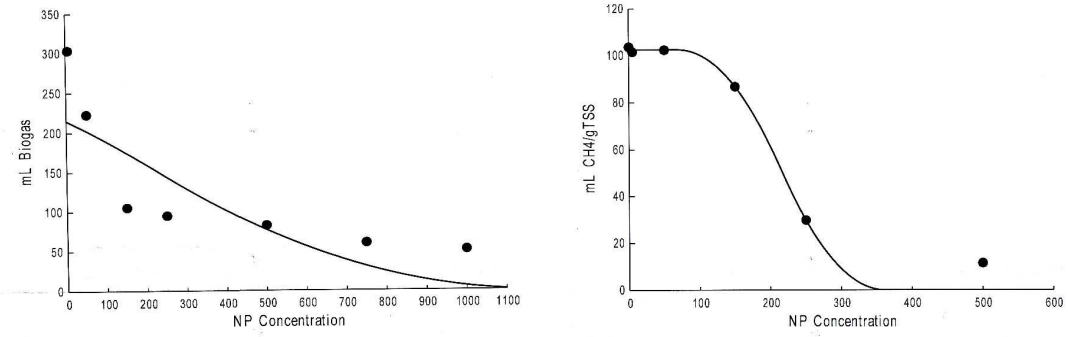


Figure 3. Model simulations of raw WAS (a), 5 mgCuO/gTS (b), 50 mgCuO/gTS (c), 150 mgCuO/gTS (d), 250 mgCuO/gTS (e), 500 mgCuO/gTS (f).

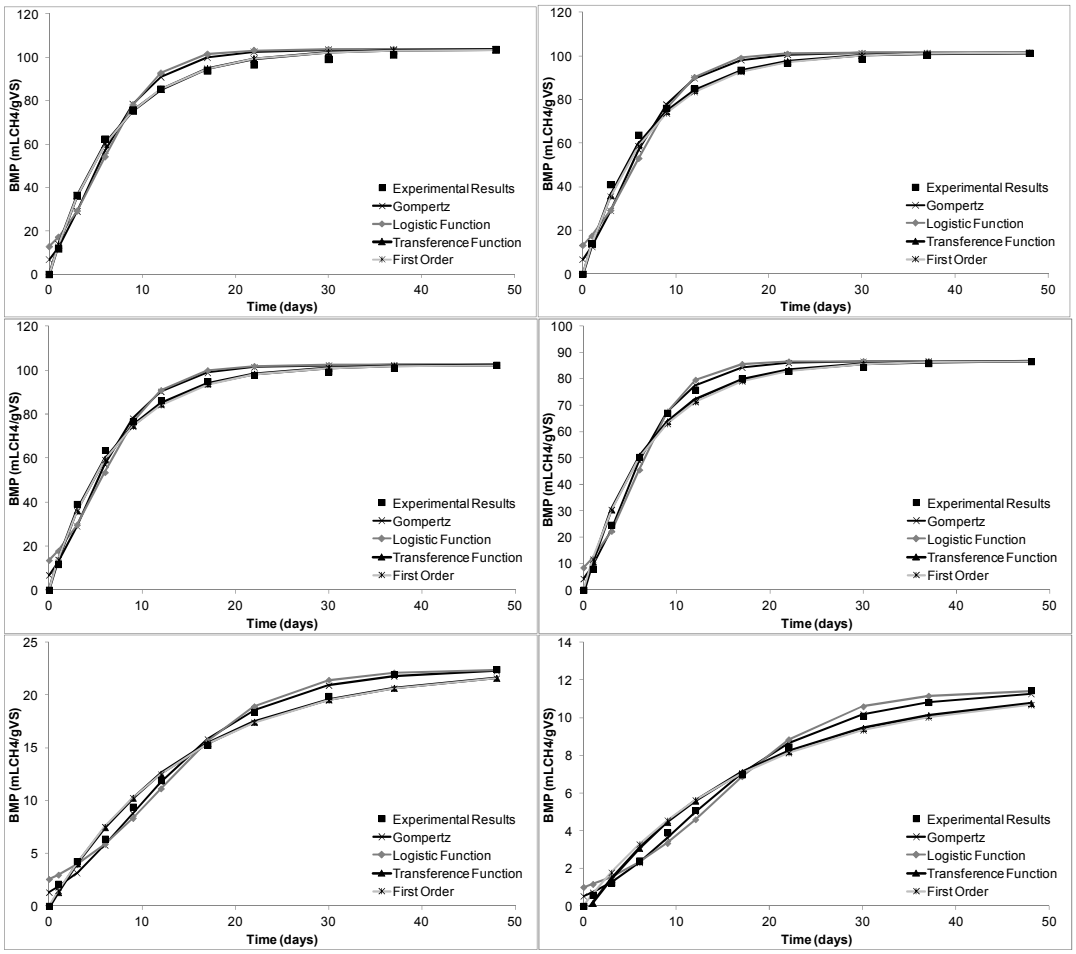
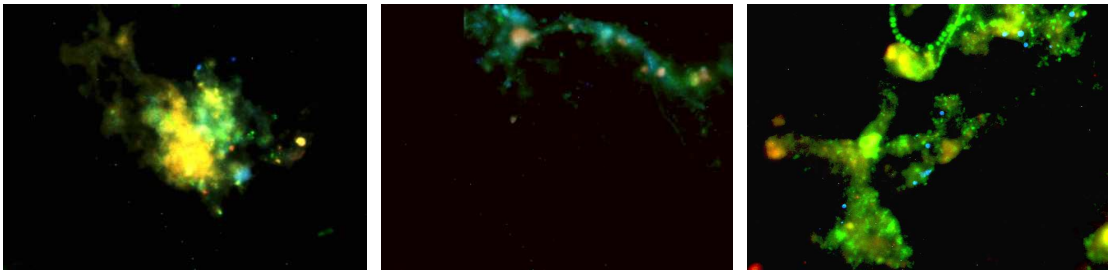
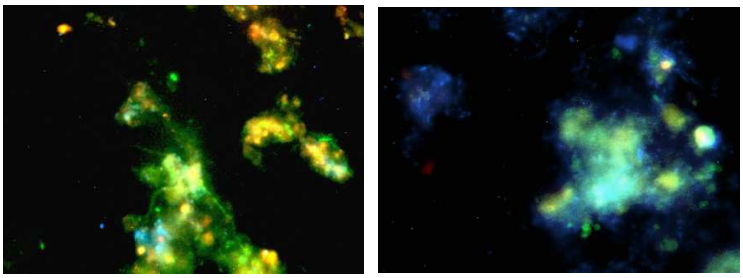


Figure 4. FISH images for DAPI (blue), all bacteria (green) and Archaea (red). Raw WAS (a), WAS containing 1000 mgAg/gTS from short term test (b) WAS containing 1000

mgCeO_2/gTS from short term test (c) WAS containing 1000 mgCuO/gTS from short term test (d) WAS containing 500 mgCuO/gTS from long term test (e)



(a) (b) (c)



(d) (e)

Nanoparticle	Reported commercial size (nm)	Size for long term (BMP) inhibiton test (nm)	Size for short term (ISO) inhibition test (nm)

Ag	20-40	75±30	45±5
CuO	30-50	499±40	320±20
CeO_2	15-30	220±10	162±20

Table 1. Particle sizes of NPs

Model	**Model equations**

Gompertz Model	$M_P = P_M \exp\left(-\exp\left[\frac{R_M \times e}{P_M}(\lambda - t) + 1\right]\right)$
Logistic Model	$M_P = \frac{P_M}{(1+\exp[4R_M(\lambda - t)/P_M + 2])}$
Transference Model	$M_P = P_M\left(1-\exp\left[-\frac{R_M(t-\lambda)}{P_M}\right]\right)$
First Order Model	$M_P = P_M(1-\exp[-k_h \times t])$

Table 2. Models used for the evaluation AD of WAS containing NPs

Probe	Target microorganism	Sequence	Fluorochrome	Reference

name	group			
EUB338	All bacteria	GCT GCC TCC CGT AGG AGT	FITC	Amann et al. 1990
$EUB338_{II}$	All bacteria	GCA GCC ACC CGT AGG TGT	FITC	Daims et al. 1999
$EUB338_{III}$	All bacteria	GCT GCC ACC CGT AGG TGT	FITC	Daims et al. 1999
ARC915	Archaea	5'- GTG CTC CCC CGC CAA TTC CT -3'	CY3	Stahl and Amann 1991
MX825	*Methanosaeta spp.*	5'- TCG CAC CGT GGC CGA CAC CTA GC -3'	CY3	Raskin et al. 1994
MSMX860	*Methanosarcinales spp.*	5'- GGC TCG CTT CAC GGC TTC CCT -3'	CY3	Raskin et al. 1994
MC1109	*Methanococcus spp.*	5'- GCA ACA TAG GGC ACG GGT CT -3'	CY3	Raskin et al. 1994

Table 3. Details of the oligonucleotide probes

Analysis	Unit	Value

TS		(g/kgSample)	182,60
VS		(g/kgSample)	124,86
TKN		(mg/gVS)	96,35
Total COD		(mg/gVS)	1862,09
sCOD		(mg/gVS)	198,36
Protein		(mgPro/gVS)	231,25
Total sSugar		(mgGlucose/gVS)	98,30
sRedSugar		(mg/gVS)	467,96
Extractable material and lipid		(%)	0,12
Soluble matter		(%)	93,64
Hemicellulose		(%)	3,12
Cellulose		(%)	0,76
Lignin		(%)	2,48
Elemental Analysis	Carbon (C)	(%)	32,59
	Hydrogen (H)	(%)	5,29
	Nitrate (N)	(%)	5,31
	Sulfur (S)	(%)	0,72

Table 4. WAS characterization results

Model	**R_M (mL/g VS.d)**	**λ (d)**	**k_R (1/d)**	**k_H (1/d)**

Gompertz	9.640546	0		
Logistic	9.037897	5.728832		
Transference	15.02649	0.039531		
First Order			0.144052	0.027745

Table 5. Modeling results of raw WAS and WAS containing Ag and CeO_2 NPs

Model	**P_M (mL/g VS)**	**R_M (mL/g VS.d)**	**λ (d)**	**R^2**

Raw WAS				
Gompertz	103.59	9.640546	0	0.9860
Logistic	103.59	9.037897	5.728832	0.9720
Transference	103.50	15.02649	0.039531	0.9983
5 mgCuONP/gTS				
Gompertz	101.47	9.640546	0.001385	0.9795
Logistic	101.47	8.590971	5.728832	0.9614
Transference	101.38	15.02649	0.039531	0.9975
50 mgCuONP/gTS				
Gompertz	102.29	9.640546	0.001385	0.9836
Logistic	102.29	8.590971	5.728832	0.9669
Transference	102.21	15.02649	0.039531	0.9976
150 mgCuONP/gTS				
Gompertz	86.66	8.794871	0.380276	0.9963
Logistic	86.66	8.590971	5.74176	0.9874
Transference	86.59	13.03323	0.127885	0.9940
250 mgCuONP/gTS				
Gompertz	22.26	1.017407	0.382337	0.9924
Logistic	22.39	1.037822	12.10004	0.9831
Transference	21.60	1.543438	0.129767	0.9918
500 mgCuONP/gTS				
Gompertz	11.25	0.458913	1.149593	0.9973
Logistic	11.39	0.544511	14.46818	0.9872
Transference	10.76	0.682365	0.769269	0.9869

Table 6. Modeling results of raw WAS and WAS containing CuO NP

Sample	**P_M**	**k_R**	**k_H**	**Hydrolysis**	**R^2 for k_R**	**R^2 for k_H**

	(mL/g VS)	(1/d)	(1/d)	period (day)		
Raw WAS	103.49	0.144052	0.027745	3	0.9982	0.9995
5 mgCuONP/gTS	101.37	0.144052	0.027745	3	0.9967	0.9999
50 mgCuONP/gTS	102.19	0.144052	0.002591	3	0.9970	0.9985
150 mgCuONP/gTS	86.57	0.144052	0.001669	3	0.9934	0.9997
250 mgCuONP/gTS	21.57	0.067803	0.001610	12	0.9924	0.9880
500 mgCuONP/gTS	10.67	0.056189	0.001610	17	0.9895	0.9985

Table 7. First order kinetic modeling results of raw WAS and WAS containing CuO NP

Supplementary Information

Figure 1. FISH images for Raw WAS (a, c, e) and WAS dosed with 1000 mgCuO NP/gTS from ISO test (b, d, f). DAPI (blue), all bacteria (green) and (a, b) *Methanosaeta spp.* (red), (c, d) *Methanosarcinales spp.* (red), (e, f) *Methanococcus spp.* (red)