CRANFIELD UNIVERSITY

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# EVALUATION OF THE IMPACT OF ENGINEERED NANOPARTICLES ON THE OPERATION OF WASTEWATER TREATMENT PLANT

SCHOOL OF APPLIED SCIENCES
DEPARTMENT OF ENVIRONMENTAL SCIENCE AND TECHNOLOGY

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# Evaluation of the impact of engineered nanoparticles on the operation of wastewater treatment plant

**Supervisors: Dr Frédéric Coulon and Dr Raffaella Villa**

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**Abstract**

The effect of engineered nanoparticles (ENPs) mixture consisting of silver oxide, ($Ag^0$, 20 nm), titanium dioxide, ($TiO_2$, 30-40 nm) and zinc oxide, (ZnO, 20 nm) compared with their bulk metal salts was evaluated against unspiked activated sludge (control) using 3 parallel pilot-scale treatment plants. The total concentration of the ionic species of $Ag^+$ Ti and $Zn^{2+}$ in the effluent of the ENP spiked activated sludge (AS) was below limits of detection and > 99% of the spiked ENP were found in the waste activated sludge (WAS), whereas 39 – 58 % of $Ag^0$, 51 – 63 % and 58 – 74 % of ZnO ion concentrations were recovered in the anaerobic digestate (AD) cake suggesting higher affinity of ENPs to WAS than to anaerobic digestate. ENPs induced a 2-fold increase of the microbial community specific oxygen uptake rate (SOUR) compared with the control and > 98 % of ammonia and 80 % of COD were removed from the AS suggesting that the heterotrophic biomass retained their ability to nitrify and degrade organic matter at the spiked ENP concentration. The floc size and cultivable microbial abundance was reduced in the ENP spiked AS with no apparent disruption of the overall AS process efficiency. However, scanning electron microscopic analysis clearly showed damage to specific microbial cells. The lipid fingerprint and 16S rRNA gene-based pyrosequencing evidenced the dominance of *Proteobacteria*, *Firmicutes*, *and Bacteriodetes* with a clear temporal shift in microbial community structure. The prominent nano-tolerant bacterial species identified were *Acidovorax*, *Rhodoferax, and Comamonas* whereas *Methanocorpusculum* and *Methanosarcina* were recovered in AS and were the dominant *Archaea* in the AD with 99 and 98 % similarities to the closest culturable relative. Their presence in the AS suggests tolerance to ENPs and oxygen-dependent respiration. *V. fisheri* activity was not sensitive to the ionic concentrations of the ENP or metal salt mixture in the digestate samples and illustrates the need to develop bioassay using indigenous wastewater microorganisms to detect the potential effect of ENP. Overall, unlike other xenobiotic compounds, ENPs can hasten the natural selection of microbial species in activated sludge and anaerobic digestion processes.

Keywords: Activated sludge, anaerobic digestion, engineered nanoparticles, metal salts, Bacteria, *Archaea*, potential effect, contaminant removal

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## List of abbreviations

| | |
|---|---|
| TINE | Transatlantic Initiative for Nanotechnology and the Environment |
| USEPA | United States Environmental Protection Agency |
| UK NERC | United Kingdom National Environmental Research Council |
| ENPs | Engineered nanoparticles |
| AD | Anaerobic digester/Anaerobic digestion |
| AS | Activated sludge |
| WAS | Waste activated sludge |
| RAS | Return activated sludge |
| $Ag^o$ | Silver oxide nanoparticle |
| $AgNO_3$ | Silver nitrate |
| $TiO_2$ | Titanium dioxide |
| ZnO | Zinc oxide |
| $Zn(NO_3)_2.6H_2O$ | Zinc nitrate (anhydrous) |
| CUDWTP | Cranfield University domestic wastewater treatment plant |
| PLEL | Phospholipid etherlipid |
| PLFA | Phospholipid fatty acid |
| NOAEL | No observed adverse effect level |
| LOAEL | Lowest observable adverse effect |
| OUR | Oxygen uptake rate |
| SOUR | Specific oxygen uptake rate |
| HRT | Hydraulic retention time |
| SRT | Sludge retention time |
| MLSS | Mixed liquor suspended solids |
| MLVSS | Mixed liquor volatile suspended solids |
| PVP | Polyvinypyrrolidone |

# Chapter 1: Introduction

The diversity and use of materials with diameter ranging between 1 – 100 nm generally referred to as engineered nanoparticles (ENPs) in industrial and consumer product manufacturing is increasing with seemingly limitless speed, whereas fears of potential adverse impact on microorganisms and the environment is gradually emerging but not well understood (Pan et al. 2010; Woodrow Wilson Database, 2011). With the array of consumer products in the health and fitness sector, pharmaceuticals, food, and textiles containing ENPs, it is already apparent that release of ENP-enriched waste into waste water treatment plant (WWTP) will sharply increase (Benn and Westerhoff, 2008; Mueller and Nowack, 2008). Once in wastewater, it is assumed that ENPs will associate with organic matter in the sludge, which would invariably pose a primary sink and source for waste containing ENPs (Brar et al., 2010; Choi et al., 2008; Kim et al., 2010; Liang et al., 2010). This scenario on the other hand entails that the environment will be flooded with large quantities of nanowaste gradually released from wastewater discharge (Clara et al., 2005; Kaegi et al., 2008; Kim et al., 2010; Liang et al., 2010; Kiser et al., 2010; Wang et al., 2012). Therefore, elucidation of any adverse effect of ENPs on microorganisms appears very significant due to the potential impact on the food web. Assuming different susceptibilities of microbial species to ENPs, selected microbes could serve as biomarker of response to ENP in wastewater, microbial interaction could transform and/or influence ENPs ecological transport and toxicity. The measurable response of these prokaryotic cells to ENPs toxic effect in turn might be extrapolated to eukaryotes (Weisner et al., 2006).

One of the main objectives of wastewater treatment is the disruption in the cycle of anthropogenic waste generation and environmental pollution. Biological processes provide the most efficient method of wastewater treatment due to the ability of microorganisms to rapidly utilize nutrients resulting in stabilization of the effluent physicochemistry for discharge into the environment (Wagner et al., 2010). Activated sludge (AS) and anaerobic digestion (AD) are examples of generally accepted biotechnological processes that rely on complex microbial communities for wastewater treatment. Accumulation of ENPs in sludge due to sorption to organic matter on the other hand has far reaching consequences due to the widespread use of sludge for thermal energy production, as bio-fertilizers and land reclamation material, or subjected

to anaerobic digestion (Kiser et al., 2009). The ENPs-sludge aggregates from soil amendment could on the other hand be spread by erosion and surface runoff to watercourses. Although knowledge about the effect of ENPs on microorganisms in wastewater (AS and AD) is limited, it is suspected that such contact could be detrimental to specific metabolic or physiological processes of microorganisms due to their established microbiocidal and inhibitory properties (Wen-Ru et al., 2010). A negative impact in turn would adversely affect the efficiency of the biological removal process in AS and biogas production during AD. On the other hand, toxicity might be mitigated by complexation of ENPs by ligands or microbial transformations. Given the lack of experimental evidence, the overall effect and its severity is difficult to predict at the moment (Kim et al., 2010).

The current paucity of information on the effects of the most widely used ENPs such as titanium dioxide ($TiO_2$), silver oxide ($Ag^0$) and zinc oxide (ZnO), copper oxide (CuO), gold ($Au^0$), fullerene ($C_{60}$) and nanofibres (Kim et al., 2010; Liang et al., 2010) might explain the lack of regulatory guidelines on ENP application and release due to risk-based policy formulation approach of 'no data, no regulation' (EPA, 2007; Woodrow Wilson Centre, 2007; Weisner et al., 2006). The ENP effects on microorganisms in complex environment such as wastewater are not clearly defined. As represented in Figure 1.1, the outcome of microorganisms interacting with pristine ENPs or wastewater components is common knowledge; however, there is not much information on how ENPs can influence microbial community in AS and AD.

To date, information available is only focused on the effect of pristine ENPs on pure cultures of different microbial species and does not allow to extrapolate the synergistic interactions in complex medium such as the AS and AD. Diverse microorganisms found in wastewater will respond in different ways to the type and concentration of ENPs. For example 0.5 mg $L^{-1}$ $Ag^0$ is toxic to the respiration of nitrifying bacteria (Choi et al., 2008) and $Ag^0$ concentrations of 10 and 50 µg $L^{-1}$ have been reported as inhibiting the growth of E. coli by 70 and 100% respectively (Sondi and Salopek-Sondi, 2004). In contrast, concentrations of 10 mg $L^{-1}$ ZnO only inhibit *E. coli* by 22% while the growth of *Bacillus subtilis* was inhibited by 90% (Adams et al., 2006). Other studies also showed polyvinylpyrollidone capped ZnO at a concentration of 0.3 mg $ml^{-1}$ can inhibit *Pseudomonas putida* (Febrega et al., 2009) and *Listeria monocyto*genes (Jin et al., 2009)

indicating that capping agents can enhance the toxic effect of ENPs on specific organisms. Based on the available information, it is plausible that ENPs can exhibit negative or positive effect on the AS and AD processes by disrupting microbial activity and structure or reduce abundance and diversity.

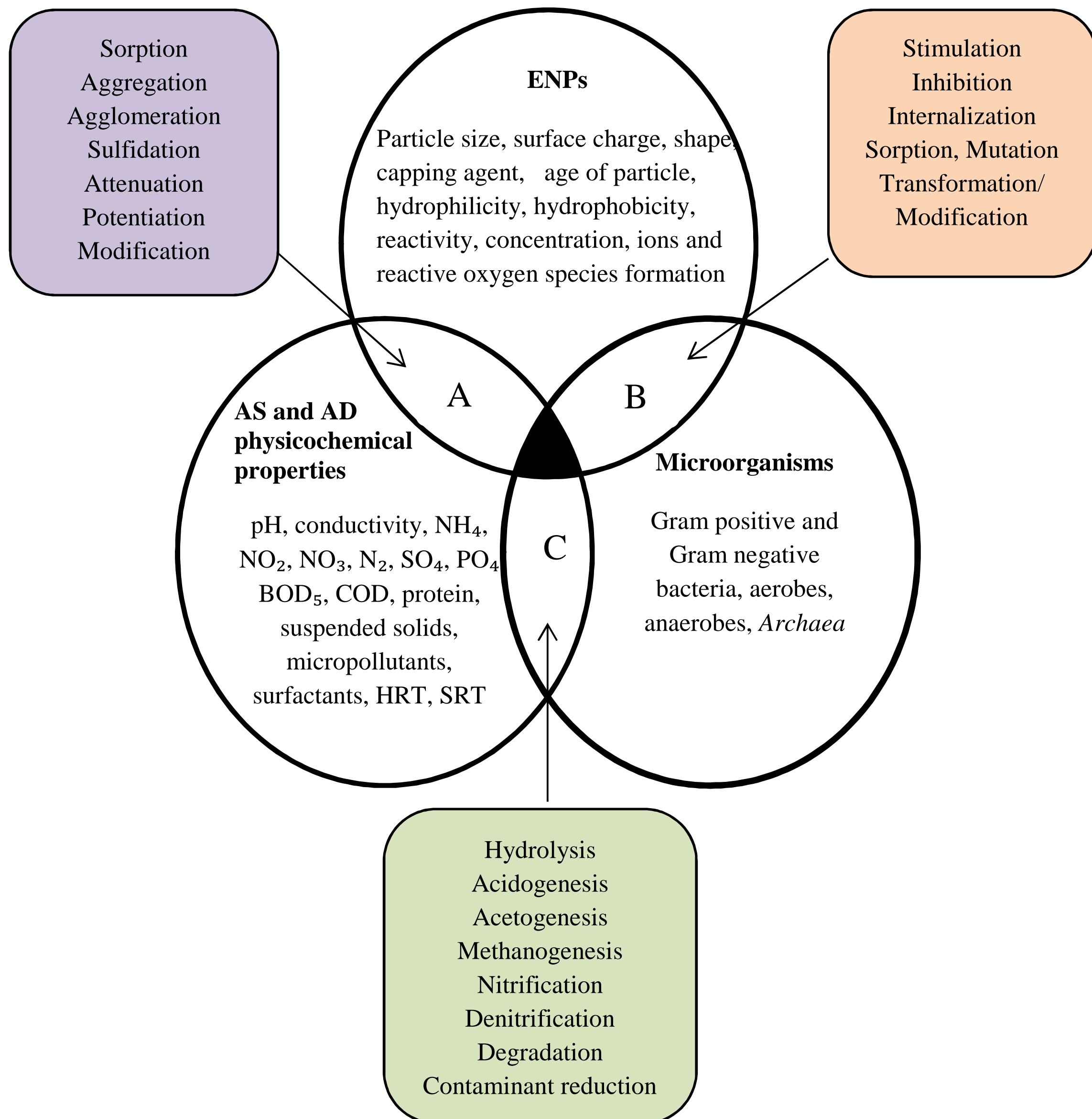



Figure 1.1 Venn diagram illustrating the chemical and biological interactions in AS and AD. The outcomes of interactions A, B and C enclosed in the coloured boxes are known. Gaps in knowledge and the need for more information to understand how ENPs interact with microorganisms influenced by AS/AD components, the focus of this study is represented by the dark area linking the three circles.

This study therefore proposes to have a holistic overview/understanding of the (i) effect on physicochemistry of activated sludge, treatment process efficiency and the

microbiological components of the two processes (ii) linking process efficiency to microbial community structure and dynamics (iii) understanding how the microbial communities respond to ENP and the potential effect on the process efficiency. The AS and AD processes however, are mediated by microbes which makes it essential to investigate ENPs effect on activated and digested sludge microbial community and process efficiency in removing contaminant. Also, information on ENPs-microbial interaction is important considering the functional roles of microorganisms in wastewater treatment plant (WWTP), soil, aquatic environment and the food chain. To provide insights on the current knowledge status, this study present empirical evidence of plausible long term effect of ENPs on AS and AD processes as a key biologically relevant environment for understanding ENPs-microbial interaction, the challenges associated with analysis, toxicity evaluation and data interpretation. Metal salts as parent compound closest to ENPs chemistry, behaviour, fate and transport and unspiked control were used for comparison.

### 1.2 Research hypotheses

The reactor performance of the pilot plant was planned to test specific hypotheses to ensure that the results will satisfy the aim and objectives of the research. The following hypotheses were tested:

**Hypothesis 1**: Biosolids in activated sludge and anaerobic digestion can remove aged-ENPs and metal salts and their ionic products from wastewater.

> The reactivity of ENPs are related to several factors including particle shape, size, surface charge, ionic concentration, aggregation, reaction with ligands and colloids in the environmental matrix. Wastewater treatment involves the degradation and removal of organic and offensive material to meet environmental disposal regulations though it is not clear if the treatment process can eliminate ENPs from the treated effluent and biosolids. Although little is known about the environmental fate and transport of ENPs, wastewater would serve as a sink for most of the nano-enabled waste and by-products. Depending on the wastewater composition and type of treatment, it is plausible that ENPs can associate with biosolids in AS/AD. The most likely mechanism of this association therefore would be sorption of ENPs to organic matter which can enhance ENPs removal from the treated wastewater at the point of clarification.

The physicochemical properties of the ENPs and metal salt spiked AS/AD compared to a reference sample will indicate the residual concentration and possible changes in the complex medium as a result of the presence of ENPs or metal salt.

**Hypothesis 2:** Engineered nanoparticles and metal salts are bioavailable in AS/AD biosolids and effluent to exert an observable effect on microbial cells and or biologically mediated processes

Most ENPs are colloids and likely to undergo complex reactions with waste water components, partitioning as a result of sorption to biosolids, aggregation and agglomeration influenced by pH during wastewater treatment. These processes are likely to affect ENPs solubility and dispersability, and as a consequence bioavailability will be reduced. Here, bioavailability refers to the ENP ionic concentration partitioned unto the WAS, effluent, digestate and filtrate that can exert an observed biological or quantifiable effect on microbial cells or community activity. The production of ions and reactive oxygen species (ROS) are the major mode of action by ENPs to exhibit inhibitory effect on microorganisms. Thus, ENPs contact and interaction with wastewater microorganisms will then be dependent on the concentration of free ions and ROS produced by aged-ENPs which are unaffected by factors such as change in pH, aggregation, or antagonistic reactions with the different and variable wastewater components. Though bioavailable data is primarily on ENPs pharmacokinetics and catalysis, it is plausible to assume that aged-ENPs and/or their ions and ROS in contact with microbial cell through adhesion to cell wall or uptake can exhibit the inhibitory or biocidal effect on target cell or component. Aggregation or dispersion of aged-ENP and their ions in AS and AD can attenuate or enhance the potential effect of the ENP mixture.

**Hypothesis 3:** Engineered nanoparticles and metal salts can impart competitive advantage on microorganisms to influence nitrification and degradation of organic matter in activated sludge, and biogas production in anaerobic digester.

ENPs have unique physicochemical properties such as the enlarged surface area that can increase their reaction rate and thus influence their interaction with AS/AD components. It is possible that the presence of an environmentally

relevant concentration of ENPs in activated sludge and anaerobic digesters can increase, inhibit or provide competitive advantage to microorganisms that are tolerant to the ENP effect. The microorganisms in wastewater contribute to vital ecosystem functions through bulk contaminant removal and biogas production because of their efficient metabolic abilities which can be augmented by the ENPs or metal salts catalysis. Although ENPs exert their antimicrobial effect through a wide range of mechanisms including the formation of reactive oxygen species, disruption of microbial physiology and metabolic processes, there is growing evidence that ENPs could also augment microbial-mediated processes in the ecosystem. The volume of biogas and stable biosolids production from sewage sludge anaerobic digestion can be significant in determining microbial tolerance, resilience and activity in the AS/AD spiked with ENPs.

**Hypothesis 4:** The key microbial groups such as the methanogens in wastewater treatment plants are not inhibited by ENPs and metal salts or their ions due to interaction with organic and inorganic components of AS and AD.

The diversity of microbial species in wastewater represents a balanced community with specific syntrophic interactions in the conversion of complex carbonaceous and nitrogenous material into utilizable forms for biomass production in addition to the release of biogas. The key microbial groups include heterotrophic, facultative and anaerobic bacteria of which the most important group are the methanogenic *Archaea* that produce methane. In a scenario that aged-ENPs and metal salts enriched biosolids inhibits the activities of methanogenic archaea, reduce reactor performance and biogas production, the result can be unstable biosolids production. Thus, an interruption of activated sludge and the methanogen-mediated final stages of anaerobic digestion during wastewater treatment processes by the ENPs may constitute a serious ecological problem when the unstable digestate is used for soil amendment or the discharge of effluent into surface water.

**Hypothesis 5:** Aged-engineered nanoparticles are not inhibitory to wastewater microorganisns or potentially toxic to bioassay due to interaction with natural organic matter and other wastewater components such as micropollutants and divalent cations and anions.

ENPs are widely used in a variety of consumer products but there are no empirical records of residual ENPs in environmental media which raise serious concerns on their potential accumulative impact on eco-sensitive aquatic and terrestrial bioreceptors. On the other hand, the inhibitory effect of ENPs can be attenuated by interacting with natural organic matter, micropollutants, divalent cations and anions, and other components in a complex medium such as wastewaster. At the same time, wastewater components can react in a synergistic or additive manner with the ENPs to increase their inhibitory effect on wastewater microorganisms. The exposure of microorganisms to ENPs in wastewater effluents and biosolids used in soil amendment could be detrimental to specific metabolic or physiological processes of ecoreceptors due to their established microbiocidal properties. Therefore, complexation reaction of the ENPs with organic and inorganic ligands, chlorides and sulfides or transformation could exert a positive, negative or no influence on its inhibitory effect to wastewater microorganisms and environmental biosensors.

## 1.3 Aims and objectives

This research evaluates the effects of ENPs on wastewater treatment and biological removal processes by characterising the physico-chemistry and microbial community structure, abundance and diversity in activated sludge and anaerobic digester.

**Objectives**

1 Understand the influence of ENPs on the AS physico-chemistry and biological removal of bulk contaminants using indicators such as $NH_3^-$, COD, TSS, floc size, and SVI.

2. Assess the effect of ENPs on AS microbial physiology and metabolism by electron microscopy, microbial abundance and oxygen uptake.

3. Assess ENPs bioavailability in digestate through the effect on by-product generation such as biogas/$CH_4$ content, VFA, bioluminescence, and sorption.

4. Understand and determine ENPs effect on AS and AD microbial community structure by lipid fingerprinting (PLFA/PLEL) and 454-pyrosequencing

## 1.4 Thesis structure

This thesis is organised in chapters formatted as manuscripts for publication. Apart from Chapters 1, 2 and 6, the other Chapters (3, 4 and 5) follow the pattern below:

- Abstract
- Introduction
- Objectives
- Methodology
- Results and discussion
- Conclusion
- References

Excerpts from Chapters 1 and 2 have been merged and peer-reviewed as a critical review paper titled: Evaluation of engineered nanoparticle toxic effect on wastewater microorganisms: Current status and challenges in *Ecotoxicology and Environmental Safety*, Eduok et al., 2013, 95:1 - 9.

### Chapter 1: Introduction

The context of the study with background information on ENPs, perspective and concerns on their use in consumer products, significance and potential effect against non-target microorganisms involved in wastewater treatment and plausible effect on wastewater treatment efficiency are highlighted. The relevant research questions and the aims/objectives that form the basis of the study are presented.

### Chapter 2: Evaluation of engineered nanoparticle toxic effect on wastewater microorganisms: Current status and challenges

This chapter presents a critical review on the development, properties and application of ENPs in domestic and industrial products. The release of free and bound ENPs during the life cycle of the products into wastewater which will serve as a sink and source of ENPs input into the environment. The specific concerns and key gaps in knowledge including the fate and transport, influence of natural organic matter on ENPs, its uptake and accumulation in microorganisms are illustrated with conceptual models. ENPs interaction with microbial community in AS / AD, influence of ENPs particle size and shape, ions and bacterial cell wall and the plausible operational problem in the short term and a long term ecological problem are discussed. Furthermore, the chapter specifically discusses the challenges in the evaluation of ENPs- microbial interaction, determination of inhibitory end-points, standards and guidance for toxicity evaluation,

uncertainties in the determination of ENP dosage and microbial contact. In addition, influence of pre-treatment on dosage, inadequacy of available bioassay for evaluation and interpretation of potential ENPs effect are highlighted.

**Chapter 3: Insights into the effect of mixed engineered nanoparticles on microbial communities in activated sludge**

In this chapter, the potential effects of aged-ENPs mixture compared with their bulk metal salts counterpart on microorganisms in activated sludge treatment process was evaluated by complementary approaches of physicochemical and microbiological analyses. The changes in physicochemical parameters of the AS such as organic and nitrogenous removal, sludge volume index and floc size of biomass due to the presence of ENPs were linked to the microbial activities such as bulk microbial community oxygen uptake rate and nitrification (Objectives 1 and 2). In addition, culture-dependent and independent evaluation of the microbial structure, abundance and diversity in the AS to understand the potential effect of ENP and its influence on the AS process efficiency is provided (Objectives 4).

**Chapter 4: Effects of engineered nanoparticles on activated sludge anaerobic digestion performance and associated microbial communities**

The relationship between aged-ENPs sorption to biosolids and its effect on AD by-products such as the volatile fatty acids (VFA) and biogas is established to broaden our understanding of the potential ENP effect in relation to AD reactor performance (Objectives 3 and 4). The chapter also attempts to provide answers to questions such as the most abundant phylogenetic taxa, temporal diversity/variation in phylogenetic structure across treatments as a result of microbial community exposure and contact with aged-ENPs and metal salts in AD. Overall, the chapter links the effect of treatment on microbial community response in AD and the implications on the microbial-mediated process.

**Chapter 5: Assessment of the potential ecological impacts of mixed engineered nanoparticle during activated sludge and anaerobic digestion processes**

The sorption of aged-ENPs to biosolids, the influence of variable factors in the AS and AD, the potential effect on microorganisms (Objective 3) is discussed in relation to the operational parameters of the reactors and context of the study. The use of available

toxicity bioassay (Microtox), the assessment, interpretation and ecological significance of the result are presented with suggestions for the use of indigenous wastewater microorganisms in ENP toxicity bioassay.

### Chapter 6: Summary and conclusion

An overview of the study, key findings and significance are presented in this chapter. The central focus also includes highlights on the knowledge gaps filled, analytical challenges and the contribution of the study to existing body of knowledge and ecological relevance. A schematic diagram to summarise the findings with implications on the AS and AD process efficiency is provided. Also, suggestions for further studies on specific and related areas of the study are enumerated.

# Chapter 2: Literature review

## 2.1 Fate and transport of ENPs

The novel physicochemistry of ENPs makes it essential to understand their fate when intentionally or accidentally released into wastewater to minimise adverse interactions with non-target and ecologically important organisms. Information on their mode of action, aging, interaction with other substances and biological systems in a complex matrix is rather limited though concerted effort is being made to determine the processes and properties governing their fate and transport. Once they are released from the consumer product into wastewater, it is suspected that their fate will vary and may include sorption to organic matter, biomass and/or extracellular polymeric substances (EPS), aggregation, reaction with other compounds or microbial conversion (Kiser et al., 2010; Weisner et al., 2009).

## 2.2 Influence of biomass extracellular polymeric substances (EPS) and natural organic matter (NOM) on ENPs

The transport of ENPs $< 100$ nm through porous media have been predicted to have high efficiencies of movement and attraction to surfaces influenced by Brownian diffusion (Dunphy Guszman et al., 2006; Locoanet and Wiesner, 2004) and affected by environmental conditions (Chen et al., 2012; Petosa et al., 2010). Although sorbed particles are expected to remain attached to media surfaces, recent findings have demonstrated that retained ENPs of 8 nm in low ionic strength solutions were released from saturated porous media against the prediction of Derjaguin-Landau-Verwey-Overbeek (DLVO) theory. Moreover, the assessment of ENPs retention and transport of different sizes using mass concentration (mg $L^{-1}$) and particle number concentration (particles/mL) resulted in divergent conclusions (Wang et al., 2012). Although transport and fate of ENPs is significantly influenced by NOM because of the tendency for sorption and aggregation (Kiser et al., 2010; Qu et al., 2010); sorption to biosolids in a wastewater context might be hindered in the presence of surfactants with increased environmental input of ENPs through effluent discharge (Christian et al., 2008; Domingos et al., 2009; Hyung et al., 2007; Kiser et al., 2010; Xie et al., 2011).

### 2.3 ENPs uptake and bioaccumulation

Bioavailable ENPs can penetrate cells or attach to the cell wall (Figure 2.1). They are not known to biodegrade and microorganisms have demonstrated *in vitro* adaptation to shock doses and contact (Lara et al., 2010; Martinez-Gutierrez et al., 2010). For instance, internalisation of ENPs and reductive deposition of palladium nanoparticles in the periplasmic space by *Shewanella oneidensis* (De Windt et al., 2006) indicates that ENPs presumably could bioaccumulate and may likely biomagnify along the food chain (Figure 2.2). On the other hand, accumulation of $Ag^0$ has been implicated in the resistance of *Pseudomonas stutzeri* AG259 to $Ag^0$ toxicity (Klaus et al., 1999). Recently, uniform, concentration-dependent uptake and internalisation of $TiO_2$ and ZnO have been reported in *Salmonella typhimurium* and *E.coli* with induced mutagenic effect. The mutagenic property may potentially be transferred to higher life forms (Holbrook et al., 2008; Unrine et al., 2010; Kumar et al., 2011).

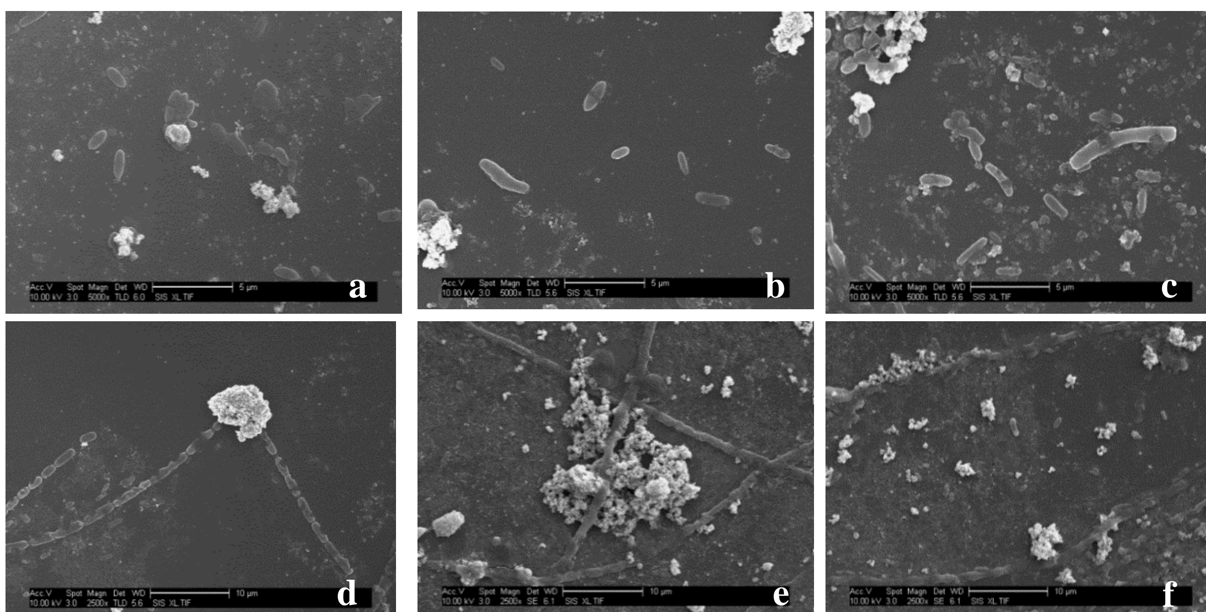



Figure 2.1 Scanning electron microscope (SEM) images showing ENPs sorption to cells (a,b), damage to microbial cell (c,d) and aggregation to biomass (e,f) in AS

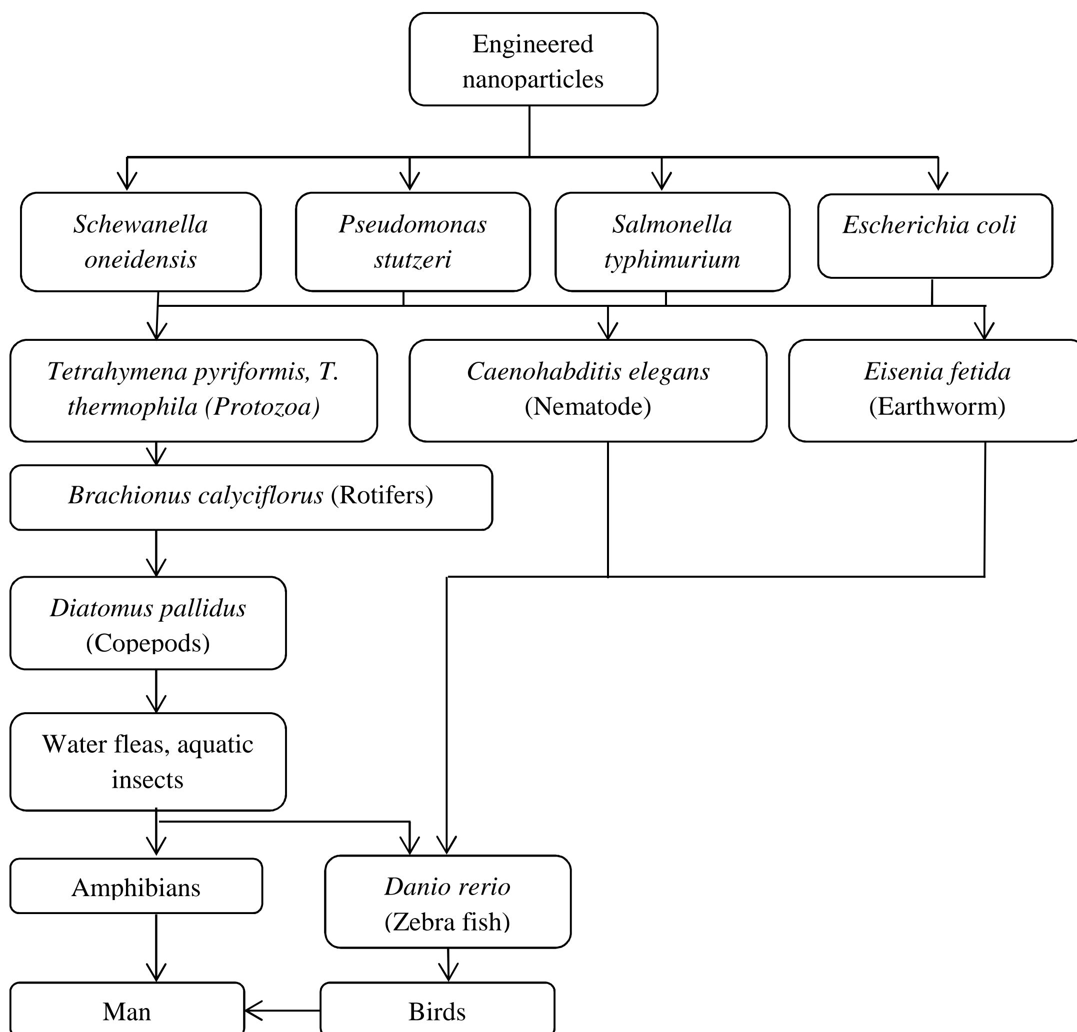



Figure 2.2 Conceptual model of ENP bioaccumulation in prokaryotes and trophic transfer to eukaryotes with extrapolated biomagnification in higher organisms

## 2.4 Effects of ENPs on microorganisms

Antimicrobial properties of ENPs is dependent on several factors including ENP physicochemistry, dose, contact time, type of organism, cultural conditions, and composition of growth medium which interact in synergy to injure or lyse the cell. (Aruguete and Hochella, 2010; Jaiswal et al., 2010). Interestingly, stabilizers and capping agents have also been shown to exert differential effect on microorganisms (Drogat et al., 2010; Jin et al., 2009). However, laboratory-controlled selective effect of size- and dose–dependent pristine ENPs as demonstrated by several authors (Figure 2.3)

does not suggest that the same size/concentration of ENPs in unaltered physicochemical state would be prevalent in wastewater to exert similar acute effect. In addition, most ENPs are colloidal and microorganisms lack uptake mechanism for colloidal and complex particulate materials therefore ENPs are suspected to exert their toxic effect by solubilized ions that enter the cell by oxidative disruption of the cell membrane (Kloepfer et al., 2005).

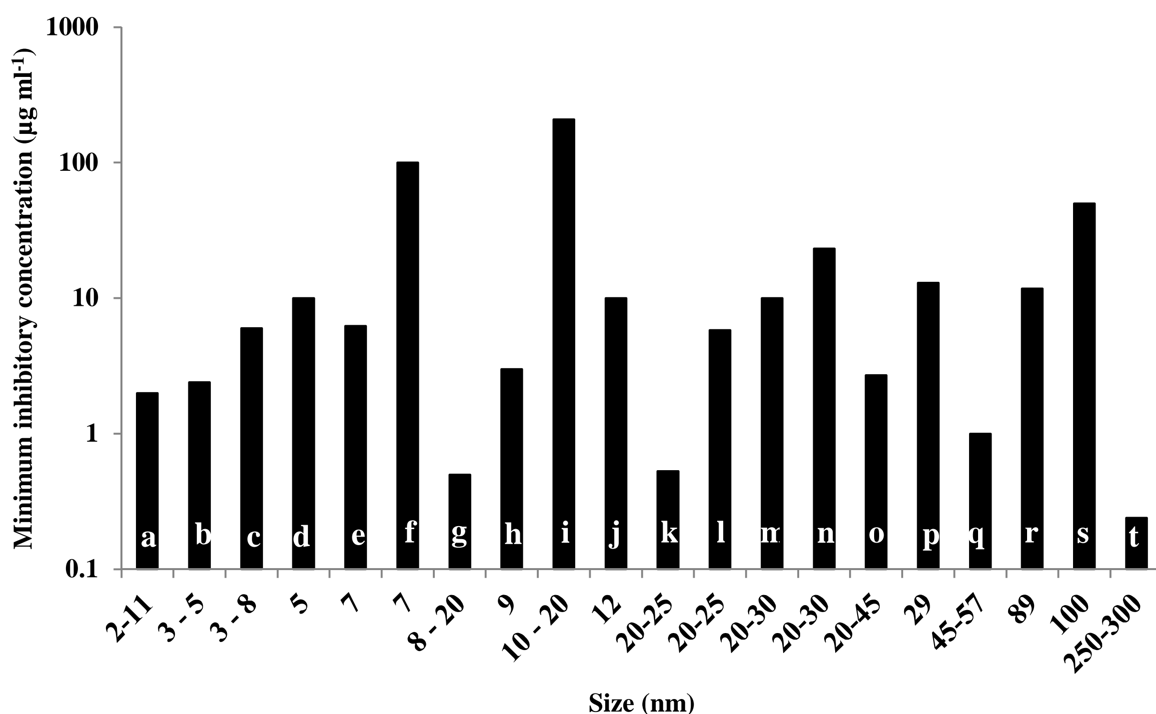



Figure 2.3 Size-dependent inhibitory effect of $Ag^0$ on pure cultures of *Escherichia coli*.

(a) Suresh et al., 2010 (b) Zhang and Chen, 2009 (c) Suresh et al., 2010 (d) Wen-Ru et al, 2010 (e) Martinez-Castanon et al., 2008 (f) Chudasama et al., 2010 (g) Choi et al., 2008 (h) Lok et al., 2007 (i) Malaiye et al., 2005 (j) Sondi and Salopek-Sondi, 2004 (k) Martinez-Gutierrez et al., 2010 (l) Verma et al., 2010 (m) Krishnaraj et al., 2010 (n) Sadhasivam et al., 2010 (o) Drogat et al., 2010 (p) Martinez-Castanon et al., 2008 (q) Vertelov et al., 2008 (r) Martinez-Castanon et al., 2008 (s) Lara et al, 2010 (t) Martinez-Gutierrez et al., 2010

Also, ENPs generate reactive oxygen species (ROS) such as free radicals ($OH^-$), singlet oxygen ($_1O^2$) and superoxides ($O_2^-$) which exerts several adverse effects on microorganisms including disruption of cell wall, damage of DNA/RNA, lipid peroxidation, oxidative stress, inhibition of exopolysaccharide and biofilm formation (Pelletier et al., 2010). The mechanism of action attributed to release of ions from $Ag^0$ was demonstrated with *E.coli* and found to be dependent on concentration and contact time. Adverse effects included the leaking of reducing sugars and proteins, enzyme

inhibition; cell disruption, and scattered vesicles which slowly dissolve thus inhibiting cellular respiration and cell growth (Wen-Ru et al., 2010).

An overview of antimicrobial properties of ENPs (Table 2.1) suggests potential adverse effect they could exert on wastewater microorganisms assuming conditions were similar. This has significant negative implications although at present, information on ENPs effect on wastewater microorganisms during AS and AD is rather limited (Batley et al., 2012; Krysanov et al., 2010). It is therefore difficult to make specific assertions regarding the toxic effect of ENPs on wastewater microorganisms. There is a possibility that ENPs in contact with microbial community may lead to reduced efficiency of AS and AD processes, complete failure of treatment and/or environmental pollution through discharge of contaminated effluent and use of biosolids for soil amendment (Hoffmann and Christoffi, 2001).

Although most studies address adverse effects, exposure to ENPs have also been associated with growth enhancement and increase in microbial reaction rates by ENPs which act as catalyst (Hilderbrand et al., 2009; 2008). Prominent examples include the temperature-dependent, anaerobic reduction of nitrate under batch conditions by integrated nanoscale zero-valent iron and microbial system (Shin and Cha, 2008), *Shewanella oneidensis*-palladium nanoparticle-mediated dechlorination of polychlorinated biphenyl (PCB) congeners in sediment matrices (De Windt et al., 2006) and stimulatory effect on dehydrogenase enzyme of soil microorganism (Cullen et al., 2011). Although the mechanism of action in ENPs stimulated processes is yet to be fully elucidated, the findings suggests that proper understanding and application of ENPs in environmental processes could enhance intrinsic metabolic potentials of indigenous microbial species. Further compilation of evidence that ENPs can have positive effect on biological processes appears interesting and could potentially be employed to augment wastewater treatment under certain conditions.

Table 2.1 Summary of different engineered nanoparticle effect and response of diverse microorganisms

| ENPs | Size (nm) | Test organism | Effect on microorganism | Reference |
|---|---|---|---|---|
| $Ag^0$ (*Shewanella oneidensis* synthsized) | ~2 - 11 | *Escherichia coli, Bacillus subtilis, Shewanella oneidensis* | Biogenic $Ag^0$ MIC of 2.0, 0.5, and 3.0 μg $mL^{-1}$ for *E.coli, B. subtilis, S. oneidensis* compared with colloidal $Ag^0$ (~3 - 8 nm) MIC of 6.0, 2.0, and 6.5 μg $mL^{-1}$, respectively. | Suresh et al., 2010 |
| $Ag^0$-β-CD, $Ag^0$ | 4 - 7, 17 | *Escherichia coli* ATCC 11229, *Pseudomonas aeruginosa* ATCC 27852, *Staphylococcus aureus* ATCC 25923 | Capped $Ag^0$ exhibited about 3.5-fold significant ($p < 0.05$) and higher antibacterial activity than the uncapped form. | Jaiswal et al., 2010 |
| $Ag^0$ (*Sesuvium portulacastrum* L. callus and leaf extract synthesized) | 5 - 20 | Bacteria (*P. aeruginosa, S. aureus, L. monocytogenes, Micrococcus luteus,Klebsiella pnuemoniae*), Fungi (*Alternaria alternaria, Penicillium italicum, Fusarium equisetti and Candida albicans*) | Antibacterial activity with inhibition zones that ranged from 8 mm (*M. luteus*) to 23 mm (*S. aureus*). Antifungal activity with inhibition zones from 12 mm (*C. albicans*) to 18 mm (P. *italicum*). | Nabikhan et al., 2010 |
| $Ag^0$ | 8.4, 16.1, 98 | *Streptococcus mutans* | MIC of 101 ± 72.03 , 145 ± 104.88 , and 320 ± 172.88 μg $mL^{-1}$ for the 8.4, 16.1, and 98 nm particle respectively, indicating particle size dependent toxicity. | Espinosa-Cristobal et al., 2009 |
| $Ag^0$ | ~ 9 | *E. coli* | Inhibition of $AgNO_3$ pre-exposed bacteria at 3 μM | Lok et al., 2007 |
| $Ag^0$ | 9 - 12 | Nitrifying bacteria | Inhibition of microbial growth at $EC_{50}$ of 0.14 mg $L^{-1}$ correlated positively with particle size < 5 nm in suspension. | Choi et al., 2008 |
| $Ag^0$ | 10, 30 - 40 and ~ 100 | Methicillin resistant *S. aureus* | $MIC_{90}$ and $MIC_{99}$ gave a corresponding 90% and 99% inhibition of growth and viability of bacteria at 1.35 mg $ml^{-1}$ dose, MBC value inhibited 100% of bacterial growth | Ayala-Nunez et al, 2009 |
| $Ag^0$ | ~ 12 | *E. coli* | 70% inhibition of bacterial activity with 10 μg $mL^{-1}$ | Sondi and Salopek-Sondi, 2004 |
| $Ag^0$ | 20-25 | *E. coli, P. aeruginosa, S. aureus* | *E. coli, P. aeruginosa, S. aureus* minimum inhibitory concentration (MIC) of 0.53, 0.37 and 0.74 μg $mL^{-1}$ compared with 0.83, 1.33 and 0.42 μg $mL^{-1}$ for gentamicin respectively. | Martinez-Gutierrez et al., 2010 |
| $Ag^0$ | 20 - 25 | *C. albicans* | MIC of 6 μg $mL^{-1}$ compared with 64 μg $mL^{-1}$ of fluconazole, and compared to 0.25 μg $mL^{-1}$ (amphotericin B) | " |
| $Ag^0$ | 20 - 25 | *Mycobacterium smegmatis* | MIC of 0.46 μg $mL^{-1}$ compared with 0.85 μg $mL^{-1}$ of rifampicin | " |
| $Ag^0$ | 20 - 25 | *Cryptococcus neoformans, C.albicans, Aspergillus niger* | MIC of 3, 6, and 25 μg $mL^{-1}$ for the three isolates respectively | " |
| $Ag^0$ (*Aspergillus clavatus* synthesized) | 20 - 25 | *E. coli, Pseudomonas fluorescens, C. albicans* | Average bacterial MIC of 5.83 μg $ml^{-1}$ and fungal MIC of 9.7 μg $ml^{-1}$. | Verma et al., 2010 |

Table 2.1 continued

| ENPs | Size (nm) | Test organism | Effect on microorganism | Reference |
|---|---|---|---|---|
| *$Ag^0$ (Streptomyces hygroscopicus* synthesized) | 20 - 30 | *B. subtilis, Enterococcus faecalis, E.coli, Salmonella typhimurium,C. albicans* | Broad spectrum growth inhibition zones of 17.3 ± 0.53, 14.2 ± 0.46, 17.2 ± 0.26, 11.4 ± 0.37, 17.7 ± 0.16 mm for the test isolates respectively at $10^{-3}$ (1 % v/v) | Sadhasivam et al., 2010 |
| $Ag^0$ (*Acalypha indica* leaf extract synthesized) | 20 - 30 | *E. coli, Vibrio cholerae* | MIC of 10 µg $ml^{-1}$ against the test isolates | Krishnaraj et al., 2010. |
| $Ag^0$-cellulose | 20 - 45 | *E. coli, S. aureus* | MIC of 2.7 and 5.4 µg $mL^{-1}$ for E. coli and S. aureus respectively | Drogat et al., 2010 |
| $Ag^0$-$TiO_2$ | 250 - 300 | *C. neoformans, C.albicans, A. niger* | MIC of 3.1, 6, and 12.5 µg $mL^{-1}$ for the three isolates respectively | Martinez-Gutierrez et al., 2010 |
| $Ag^0$-$TiO_2$ | 250 - 300 | *Uropathogenic E. coli* | MIC of 0.24 µg $mL^{-1}$ | " |
| Ag-$TiO_2$ | 26 - 56 | *Nonpathogenic E. coli* BL-21 | MIC of 25.46 µg/$cm^2$ | Thiel et al., 2007 |
| $Ag^0$ | 15 - 21 | *E. coli* PHL628-gfp | MBC of 38 and 10 mg $l^{-1}$ killing 99.9 % of planktonic and biofilm bacterial population indicating that biofilm cells are about 4 times more resistant | Choi et al., 2008 |
| $Ag^0$ | 7, 29, and 89 | *E. coli* ATCC 25922, *S. aureus* ATCC 25923 | MIC of 6.25, 13.02 and 11.79 µg $ml^{-1}$ (*E.coli*) and 7.5, 16.67 and 33.71 (*S. aureus*) for the different sizes respectively | Martinez-Castanon et al., 2008 |
| $Ag^0$ (Myramistin stabilized) | 10 ± 1.8 | *E. coli* ATCC 25922, *S. aureus* FDA 209P (St. 209) *strain, Leuconostoc mesenteriodes* VKPM B-4177, *Saccharomyces cerevisiae* RIA 259 *and A. niger* | MIC of < 1 µg $mL^{-1}$ (*E.coli*) and 5 µg $mL^{-1}$ (*S. aureus*) on liquid medium, 2.5 µg $l^{-1}$ on both liquid and agar plates. 5 µg $mL^{-1}$ for *L. mesenteriodes, S. cerevisiae* and *A. niger* | Vertelov et al., 2008 |
| $Ag^0$ | 13.83 | *E.coli* (ATCC 700926), *Bacillus subtilis* (ATCC 9372), *S. cerevisiae* (ATCC 26108) | 100 % inhibition of *E.coli* at 1.589 ppm after 1 hr, 2 orders of magnitude lower effect on *B. subtilis* than *E.coli*, and no observable inhibitory effect on *S. cerevisiae* | Lee et al, 2009 |
| $Ag^0$ | 100 | Estuarine Sediment bacterial consortia | No observable impact on the bacterial density and genetic diversity after 30 days exposure at 25 µg $L^{-1}$ or 1000 µg $L^{-1}$ concentration. | Bradford et al., 2009. |
| $Ag^0$-$TiO_2$ | < 5 | *E. coli* | Growth inhibition at 1.2 µg $mL^{-1}$ $Ag^0$ (98.8 %) and complete inhibition at 2.4 µg $mL^{-1}$ $Ag^0$ whereas < 3 nm Ag/$TiO_2$ of 3.9 wt % produced 99.9 % inhibition | Zhang and Chen, 2009 |
| $Ag^0$ | 10 - 20 | *E. coli, S. aureus* | MIC of 209 µg $mL^{-1}$ for *E.coli* and 433 µg $mL^{-1}$ for *S. aureus* | Malaiye et al., 2005 |
| $Ag^0$ | 100 | Multidrug-resistant *P. aeruginosa* (MRPA), ampicillin-resistant *E coli* 0157:H7 (AREC), erythromycin-resistant *Streptococcus pyogenes* (ERSP) | MIC of 66.7 ± 17.7 mM for ERSP, 83.3 ±16.7 for AREC and MRPA. 99.7%, 95.7%, and 92.8% MBC at 50 mM to ERSP, AREC and MRPA respectively. | Lara et al., 2010 |
| $Ag^0$ | 5 | *E.coli* ATCC 8739 | Complete inhibition of growth and viability at 10 µg $ml^{-1}$ for $10^7$ cfu $ml^{-1}$. Destruction of bacterial membrane integrity, lipid peroxidation, inhibition of respiratory chain dehydrogenases | Wen-Ru et al, 2010 |

Table 2.1 continued

| ENPs | Size (nm) | Test organism | Effect on microorganism | Reference |
|---|---|---|---|---|
| $Ag^0$-$TiO_2$ | 100 | *E. coli* WP2 trp uvrA | Complete photoactivated inhibition of growth and viability at 2.0 mg $L^{-1}$. Similar result for $Ag^0$ alone whereas $TiO_2$ alone had no observable effect | Pan et al., 2010 |
| $Ag^0$ (JS47N and SO-01) | 20 - 50 | *Microcystis aeruginosa* (cyanobacterium) | Selective cyanobacterial reduction in composition ratio of *M. aeruginosa* from 95.5% to 49 and 21% after 10 days incubation at 1 mg $l^{-1}$. Growth inhibition by 87 % | Park et al., 2010 |
| $Ag^0$ (Hydrophilic) | ~ 7 | *E. coli, Shigella sonnei, Bacillus megaterium, Proteus vulgaris, S. aureus* | MIC for *E. coli* (100 µg $mL^{-1}$), *Shigella sonnei* (215 µg $mL^{-1}$), *Proteus vulgaris* (275 µg $mL^{-1}$) *Bacillus megaterium* (300 µg $mL^{-1}$), *S. aureus* (350 µg $mL^{-1}$) were higher compared with commercially available antibacterial agent. | Chudasama et al., 2010. |
| *$Ag^0$ (Bacillus licheniformis* synthesized) | 50 | *P. aeruginosa, S. epidermidis* | 24 h exposure to 100 nM caused 95% and 98% disruption in extracellular polymeric substance/ biofilm formation in *P. aeruginosa, S. epidermidis* repectively whereas 50 nM resulted in 50% reduction. 12 ± 1.2 mm (*S. epidermidis* ) and 9.5 ± 0.9 mm (*P. aeruginosa* ) zones of inhibition | Kalishwaralal et al., 2010 |
| *$Ag^0$ (A. niger* synthesized) | 3 - 30 | *E. coli, S. aureus, A. niger, Bacillus species* | Growth inhibition with zone diameters of 1.2 cm (*A. niger* ), 0.9 cm (*S. aureus* ), 0.8 (*Bacillus* sp and *E. coli* ) | Jaidev and Narashimha, 2010 |
| $Ag^0$ (Banana peel extract synthesized) | 100 | *C. albicans (* BX and BH), *C. lipolytica* (NCIM 3589)*; Citrobacter koseri* (MTCC 1657), *Enterobacter aerogenes* (MTCC 111), E.coli (MTCC 728), *Klebsiella* sp, *Proteus vulgaris* (MTCC 426) *P. aeruginosa* (MTCC 728) | Inhibitory zones for *C. albicans* BX (1.2 cm), *C. albicans* BH (1.3 cm), *E. aerogenes* (1.3 cm), *E.coli* (1.4 cm), *Klebsiella* sp (1.7 cm); No inhibitory effect on *C. koseri, P. vulgaris, P. aeruginosa* | Bankar et al., 2010 |
| $Ag^0$, CuO, ZnO | 10, 33, and 50-70 repectively | *Pseudomonas putida* KT2440 | Dose-dependent bacteriostatic effect on light output of the biosensor at > 0.2 mg $L^{-1}$ $Ag^0$, 10 mg $L^{-1}$ CuO, < 7 mg $L^{-1}$ ZnO, whereas lower concentrations of ZnO had stimulatory effect on light production | Gajjar et al., 2009 |
| $Ag^0$- *Tribulus terrestris* synthesized | 16-28 | Mutidrug resistant *S. aureus, B. subtilis, E. coli, P. aeruginosa, S. pyogenes* | High bactericidal activity with 9.75, 9.25, 10.75, 9.25 and 10 mm zones of inhibition respectively | Gopinatha et al., 2012 |
| $Ag^0$ (citrate capped) | 10 | *E. coli* KACC10495*, B. subtils* KACC10111 | Colony forming rates of *E. coli* and *B. subtilis* exposed to 10 mg $l^{-1}$ were 0.5 % and 77.5 % respectively | Kim et al., 2011 |
| $Ag^0$-Graphene (GrO) hybrid | 5 - 25 | *E. coli, P. aeruginosa,* | Concentration-dependent growth inhibition of 7 to 26 mm at 60 µL of different $Ag^0$-GrO hybrids | Das et al., 2011 |
| $Ag^0$ | 15 - 21 | Autotrophic nitrifying bacteria | More than 80 % growth inhibition by 1 mg $l^{-1}$ | Choi et al., 2008 |
| Au, $Ag^0$, $Fe_3O_4$ | 10, 2 and 7 respectively | *Photobacterium phosphoreum* | No inhibition of luminescence at the dose of 28, 45, and 52 µg $mL^{-1}$ respectively | Berrana et al., 2009 |
| $AL_2O_2$ | < 50 | *E. coli* WP2 trp uvrA, | Exhibited no mutagenic effect or growth inhibition to test organism at the concentrations tested | Pan et al., 2010 |
| Alumina | 179 | *E.coli,* | Mild to moderate inhibition of microbial growth at a concentration range of 10 - 1,000 µg $mL^{-1}$ | Sadiq et al., 2009 |

Table 2.1 continued

| ENPs | Size (nm) | Test organism | Effect on microorganism | Reference |
|---|---|---|---|---|
| Boron | 10 - 20 | *V. fischeri* NRRL B-11177 | Toxic with EC50 of 55.85 to 65.98 mg $l^{-1}$ between 1 and 60 days of the solution age respectively | Strigul et al, 2009 |
| $CeO_2$ | 6.5 | *V. fischeri* | More than 80% inhibition of luminescence detected at MIC of 0.064 mg $ml^{-1}$ | Garcia et al., 2011 |
| $CeO_2$ | < 25 | Cyanobacteria *Anabaena* CPB43337 | 24 h $EC_{50}$ of 0.27 to 6.3 mg $l^{-1}$ with menbrane disruption and cell damage. | Rodea-Palomares et al., 2011 |
| $CO_3O_4$ | < 50 | *S. typhimurium* TA 97a and TA100, *E. coli* WP2 trp uvrA | Exhibited no mutagenic potential or growth inhibition on test organism at tested concentrations | Pan et al., 2010 |
| $C_{60}$ Fullerenes | Variable and dependent on medium | *E.coli, B. subtilis* | MIC of 0.5 to 1.0 mg $l^{-1}$ and 1.5 to 3.0 mg $l^{-1}$ for *E. coli* and *B. subtilis* respectively | Lyon et al, 2005 |
| CuO | < 50 | *S. typhimurium* TA 97a and TA100 | Exhibited low mutagenic potential to test organism at specific concentrations | Pan et al., 2010 |
| CuO | ~ 30 | *V. fischeri* | Toxic with 72 h $EC_{50}$ value of 79 mg $l^{-1}$, MIC value of 200 mg $l^{-1}$ | Heinlaan et al, 2008 |
| Cu-doped $TiO_2$ | 20 | *Mycobacterium smegmatis, S. oneidensis* MR-1 | High toxicity level compared with no observable effect at < 100 mg $L^{-1}$ of $TiO_2$ | Wu et al., 2010 |
| $Fe_3O_4$, Au | 8, 5 | *E.coli* | Dose-dependent microbiostatic effect by $Fe_3O_4$ (50 - 200 µg $mL^{-1}$); no observable effect by Au (25 - 100 µg $mL^{-1}$) | Chatterjee et al., 2011 |
| $Fe_3O_4$ | 6 | *V. fischeri* | $EC_{50}$ of 0.24 mg $ml^{-1}$ | Garcia et al., 2011 |
| Fe and Cu | 25, 25 | *Trametes versicolor* | Significant reduction in the production of lignocellulolytic enzymes; β-glucosidase, β-xylosidase, and cellobiohydrolase at 0.1 mmol/L after 24 h. Reduction in laccase production by copper nanoparticle. Production profile of enzymes not growth related | Shah et al., 2010 |
| $SiO_2$, $SiO_2/FeO_4$ and Au | 60 | *E.coli* | No inhibitory effect on bacterial growth at concentration 2.2 x$10^{-3}$ to 3.3 x $10^{-2}$ g $mL^{-1}$ of LB medium | Williams et al., 2006 |
| $TiO_2$ (81% anatase,19% rutile) and ZnO (100% zincite) | 15 - 20 and 20-30 | Soil microbial consortia | $TiO_2$ (0,0.5,1.0 and 2.0 mg $g^{-1}$ soil); ZnO (0.05, 0.1, and 0.5 mg $g^{-1}$ soil) in microcosm over 60 days exposure altered microbial diversity with ZnO exerting greater effect indicated by low DNA and high changes in bacterial composition at 0.5 mg $g^{-1}$ soil. | Ge et al., 2010 |
| $TiO_2$ | 15 - 30 | *E. coli, P. aeruginosa* | Particle size dependent bactericidal effect on test organism | Jang et al., 2007 |
| $TiO_2$, $SiO_2$ and ZnO | 330, 205, and 480 | *E.coli, B. substilis* | Photo-induced bactericidal activities. *B.subtilis* more sensitive than *E. coli* (*E.coli* - 44%, *B. subtilis* - 75%). $SiO_2$ least toxic | Adams et al, 2006 |

Table 2.1 continued

| ENPs | Size (nm) | Test organism | Effect on microorganism | Reference |
|---|---|---|---|---|
| $TiO_2$ | < 100 | *V. fischeri* | No toxic effect at a concentration of 1,000 mg $L^{-1}$ | Schaefer and Scott-Fordsmand, 2006 |
| $TiO_2$, Au-capped TiO2 | 12 - 18, 5 - 10 | *E. coli* DH 5α, *B. megaterium* QM B1551 | 60 - 100 % killing efficiency through photocatalytic formation of reactive oxygen species | Fu et al, 2005 |
| $TiO_2$ | 7.5 | *V. fischeri* | Only 21% inhibition of luminescence detected at a maximum concentration of 1.12 mg $ml^{-1}$ | Garcia et al., 2011 |
| $TiO_2$ and ZnO | 30 and 50 | *S. typhimurium* strain TA98, TA1537 and *E.coli* (WP2 uvrA) | Concentration-dependent uptake and internalization of nanoparticles with 8 and 80 ng $mL^{-1}$ after 60 min. Exhibition of weak mutagenic effect. | Kumar et al., 2011 |
| $TiO_2$ | 25 - 70 | *V. fischeri* | Not acutely toxic: $EC_{50}$ >20 g $l^{-1}$ even at 8 h exposure in the dark, MIC: >20 g $l^{-1}$ | Heinlaan et al, 2008 |
| $TiO_2$, CuO, ZnO and $Ag^0$ | 25, 30, 70 and < 100 repectively | Wild type *E.col* i AB1157 (pSLlux), *E.coli* sodABC strain AS391 (pSLlux), *E.coli* K12::soxRSsodAlux | $TiO_2$ inhibition of viability at > 4,000 mg $l^{-1}$ and stimulation of bacterial luminescence from superoxide sensing bacteria from 100 mg/l. 2 h $EC_{50}$ for CuO (8.1 and 2.0 mg $l^{-1}$), $Ag^0$ (46 and 3.1 mg $l^{-1}$), ZnO (4.5 and 54 mg $l^{-1}$) were toxic to the triple *sod* mutant than the wild type respectively whereas fullerene inhibited bioluminescence of *sod* triple mutant at 3,882 mg $l^{-1}$ but NOEC on wild type at 20,800 mg $l^{-1}$. | Ivask et al, 2010 |
| $TiO_2$ | < 100 | *E. coli* WP2 trp uvrA | Induced marginal mutagenesis to organism | Pan et al., 2010 |
| $TiO_2$, ZnO, CuO | 25 - 70, 50 - 70 and 30 respectively | *S. cerevisiae* | $TiO_2$ not toxic at $EC_{50}$ of > 20 g $l^{-1}$; 8 and 24 h $EC_{50}$ of 20.7 mg $l^{-1}$ CuO, 13.4 mg $l^{-1}$ CuO and 121-134 mg $l^{-1}$ ZnO and 131-158 mg $l^{-1}$ ZnO respectively | Kasemets et al, 2009 |
| $TiO_2$ | 79 | *E. coli* sodABC | Phototoxic effect by reactive oxygen species (ROS) formation | Brunet et al, 2009 |
| $TiO_2$ | 6 | *V. fischeri* NRRL B-11177 | EC50 ranged from 56 to 66 mg $l^{-1}$ and was dependent on the age of the TiO2 solution | Strigul et al, 2009 |
| $TiO_2$ | 6 - 40 | *V. fischeri* | 14 % loss of bioluminescence, low toxicity in aqueous medium LOEC of 500 - 1000 mg $l^{-1}$ ($EC_{50}$ of 650.6 and 940.6 mg $l^{-1}$) | Lee et al, 2009 |
| $TiO_2$, $Al_2O_3$, ZnO, $SiO_2$ | 50, 60, 20 and 20 respectively | *B. subtilis, E.coli, P. fluorescens* | Toxicity to the test organisms at 20 mg/l: ZnO (100 % toxic to all), $Al_2O_3$ (57, 36, and 70% respectively), $SiO_2$ (40, 58, and 70% respectively), $TiO_2$ (low toxic effect). Absence of light suspected as being responsible for the low toxicity of $TiO_2$, free radical formation enhancing toxicity of ZnO and $SiO_2$. | Wei et al., 2009 |
| $TiO_2$ | 21 | Phyllosphere *Bacillus cereus* 905 | Photo-induced dose-dependent change of microbial community structure at 0.02 mg $mL^{-1}$ during the 30 days exposure | Wang et al., 2009 |
| Zero-valent iron nanoparticles | 20 - 30 | *B. subtilis var niger, P. fluorescens* | Complete bacterial inactivation at 10 mg $ml^{-1}$ with agitation under aerobic condition. 95% and 80% inactivation at 0.1 and 1 mg $ml^{-1}$ respectively for *B. subtilis var niger* whereas *P. fluorescens* was completely inactivated at both concentrations | Diao and Yao, 2009 |

Table 2.1 continued

| ENPs | Size (nm) | Test organism | Effect on microorganism | Reference |
|---|---|---|---|---|
| ZnO | 20 | *S. oneidensis* MR-1, *E.coli* | Extracellular polymeric substance produced by *S. oneidensis* and *E.coli* prevented their growth inhibition even at high concentration (> 40 mg $L^{-1}$) in aquatic media whereas aerosolized particle of 480 nm and 20 nm was toxic to the cells | Wu et al., 2010 |
| ZnO | 20 - 45 | *S. aureus, E. coli* | Concentration-dependent enhancement of antibacterial effect of ciprofloxacin (27% and 22% increase in inhibition zones for *S. aureus* and *E.coli* respectively. | Banoee et al., 2010 |
| ZnO | 70 | *E. coli* 0157:H7 | Concentration-dependent growth inhibition with ≥ 12 mmol/l completely inhibiting microbial growth with membrane distortion/damage, leaking of intracellular content and cell death. | Liu et al., 2009 |
| ZnO | 480 - 4000 | *B. subtilis* CB310, *E. coli* DHSα | Growth inhibition by 90% at 10 mg $l^{-1}$ (*B. subtilis*), 48% at 1,000 mg $l^{-1}$ (*E.coli*) | Adams et al., 2006 |
| ZnO | < 100 | *E. coli WP2 trp uvrA* | Induced marginal mutagenesis to organism but no growth inhibition | Pan et al., 2010 |
| ZnO | 24 - 71 and 90 - 200 | *E. coli and V. fischeri* | Oxidative stress leading to membrane damage and death. MIC: 200 - 250 mg $l^{-1}$ and 100 mg $l^{-1}$ for *E. coli* and *V. fischeri* respectively. | Zhang et al., 2007 |
| ZnO | 100 - 150 | *Streptococcus agalactiae, S. aureus* | Inhibition of microbial growth through oxidative stress, disruption of cell and membrane by 0.12 M concentration | Huang et al, 2008 |
| ZnO | 50 - 70 | *V. fischeri* | Growth inhibition, bactericidal: 30 mins $EC_{50}$ of 1.1 - 1.9 mg $l^{-1}$ at 20 g $l^{-1}$ concentration, MIC value of 100 mg $l^{-1}$. | Heinlaan et al., 2008 |
| ZnO, ZnO-PVP, ZnO-PS | 4,5, 10 | *L. monocytogenes, E.coli* 0157:H7, *Salmonella enteritidis* | ZnO-PVP exhibited significant dose-dependent bactericidal effect at > 0.3 mg $ml^{-1}$ compared with the bacteriostatic nature of ZnO powder. No observable inhibitory effect with ZnO-PS film | Jin et al, 2009 |
| ZnO and ZnO-Brij-76 | 10.4 ± 1.2 and 12.7 ± 1.6 | *Anabaena flos-aquae* (cyanobacteria) | Size- and capping agent-dependent decrease in photosynthetic activities and cell death without nanoparticle internalization. ZnO and ZnO-Brij-76 stimulation of cyanobacterial photosynthetic activity after 10 days. | Brayner et al., 2010 |
| ZnO | ~ 30 | *Campylobacter jejuni, Salmonella enterica serovar enteritidis, E.coli* | MIC of 0.05 to 0.025 mg $ml^{-1}$ for *C. jejuni,* 0.4 mg $ml^{-1}$ for *S. enterica* and *E.coli*. Bactericidal effect due to membrane disruption and oxidative stress in *C. jejuni* | Xie et al., 2011 |

$Ag^0$ = Silver oxide, $TiO_2$ = Titanium dioxide, ZnO = Zinc oxide, $SiO_2$ = Silicon dioxide, $CeO_2$ = Cerium (iv) oxide nanoparticle, $EC_{50}$ = Effective concentration of substance that generate 50% reduction in bioluminescence

β-CD = β-cyclodextrin, TOPO = Tri-*n*-octylphosphine oxide, Brij-76 = Polyoxyethylene stearyl ether, PVP = Polyvinylpyrrolidone, PS = Polystyrene, MBC = Minimum bactericidal concentration

$CO_3O_4$ = Cobalt oxide, $Al_2O_2$ = Aluminium oxide, CuO = Copper oxide, $TiCl_4$ = Titanium tetrachloride, Au = Gold nanoparticle, LOEC = Lowest observed effect concentration, NOEC = No observed effect concentration

MIC = Minimum inhibitory concentration

### 2.5 ENPs – microbial interactions in wastewater treatment plant

Wastewater contains diverse microorganisms with different surface charges and sorption potentials. Significant factors likely to determine ENPs-microbial interactions in wastewater include solubility, bioavailability and bioreactivity. ENPs-microbial interaction is presumably reduced by aggregation which is likely to restrict the efficiency of cellular contact and thus reduces the bioavailable dose (Depledge, 2010; Grieger et al., 2010; Fabrega et al., 2011). For instance, $C_{60}$ have been found to be toxic to pure cultures of *B.subtilis* (Lyon et al., 2006 ) and *E. coli* (Chae et al., 2009), with no observable negative effect on bacterial soil communities and cellular respiration due to the influence of cultural conditions and soil NOM (Tong et al., 2007).

### 2.6 Interaction with activated sludge (AS) microbial community

The removal of nitrogenous material from wastewater during AS treatment could be negatively affected by ENPs. This is because $Ag^0$ has an inhibitory effect on bacteria and tends to bioaccumulate (Choi et al., 2010). The build-up of ammonia in AS due to ENPs effect on nitrifiers in turn will directly impact acetogenic and methanogenic organisms during AD treatment (Kayhanian, 1994). Although $Ag^0$ concentration reported in sewage ranged from 2 to 18 µg $l^{-1}$ (Blaser et al., 2008), it is not clear if this concentration could have a detrimental effect on microorganisms as a peak concentration of 0.75 mg $l^{-1}$ $Ag^0$ in activated sludge after 12 h shock load had no observable inhibitory effect on organic removal rate by heterotrophic bacteria (Liang et al., 2010).

As close contact with the cell surface is assumed to be important for achieving an effect of ENPs on microbes, a differential impact might be expected for planktonic and biofilm-associated cells. Cells within a biofilm matrix are typically embedded in a coat of extracellular polysaccharides restricting direct contact or lowering the effective dose of ENPs (Liu et al., 2007). In a recent study, NOMs and EPS have been found to hinder $C_{60}$-bacterial biomass interaction in AS (Kiser et al., 2010). ENP-microbial interaction may not be particle size-dependent alone and could vary in different cultural and environmental conditions especially when the organisms can synthesize EPS (Figure 2.4). On the other hand, interaction could be enhanced by proteins which promote disaggregation of ENP thus increasing bioavailability and contact (Karajangi et al., 2006; Neal, 2008; Wu et al., 2010).

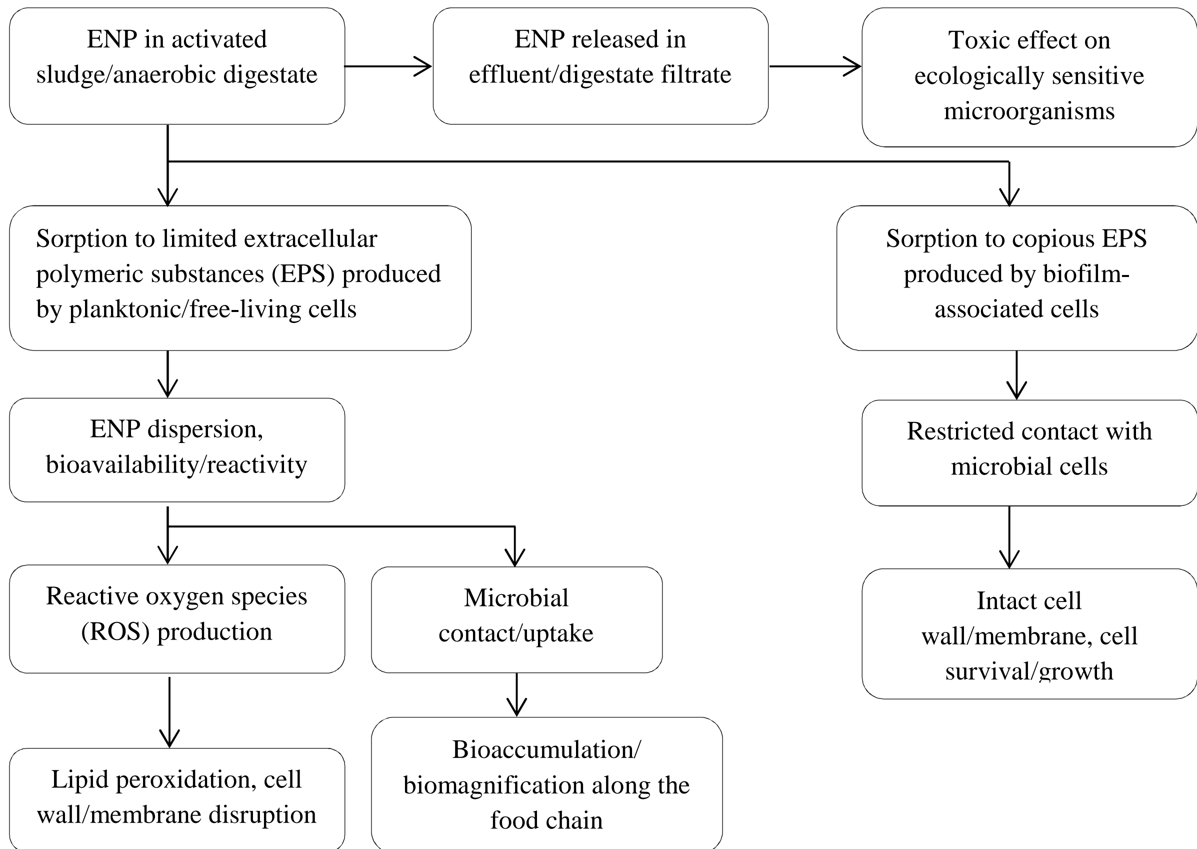



Figure 2.4 Conceptual interactions between ENPs and AS microbial biomass

**2.7 Interaction with AD microbial community**

The two key syntrophic groups involved in anaerobic digestion process are acetogenic and methanogenic microorganisms. Both are affected by accumulation of free ammonia (Kayhanian, 1994) and fatty acids (Wagner et al., 2010). The performance of anaerobic digesters amended with ENPs would be dependent on the resilience or susceptibility of archaeal cell wall to ENPs contact (Debabov et al., 2004). An adverse effect on any successive step of the anaerobic process will undoubtedly reduce reaction rate with increased accumulation of toxic metabolic products that may constitute a limiting step in the process. To date, not much information on the interaction of ENPs-microbial community during AS and AD is available (Kim et al., 2010).

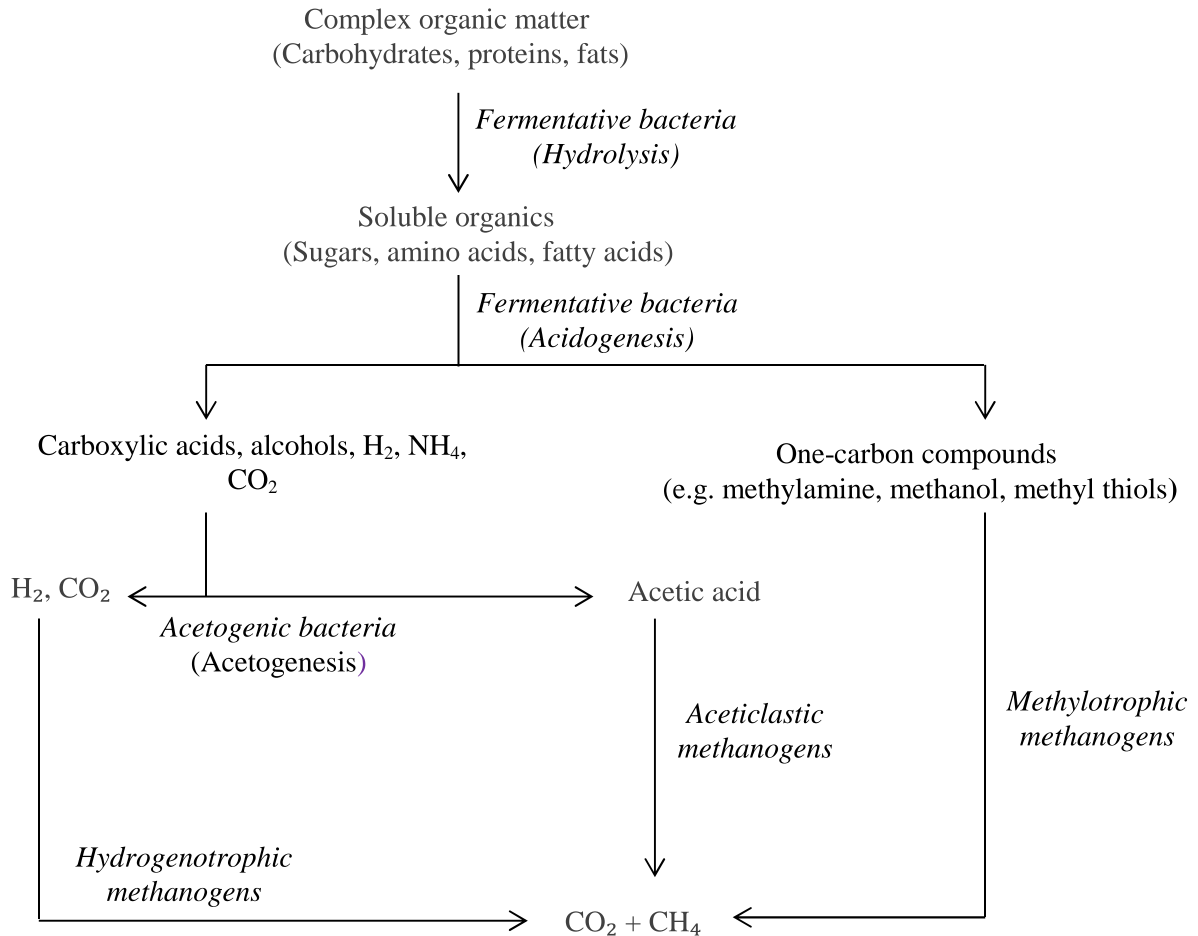



Figure 2.5 Schematic illustrations of the pathways of anaerobic digestion and methanogenesis

## 2.8 Influence of ENPs particle size and shape

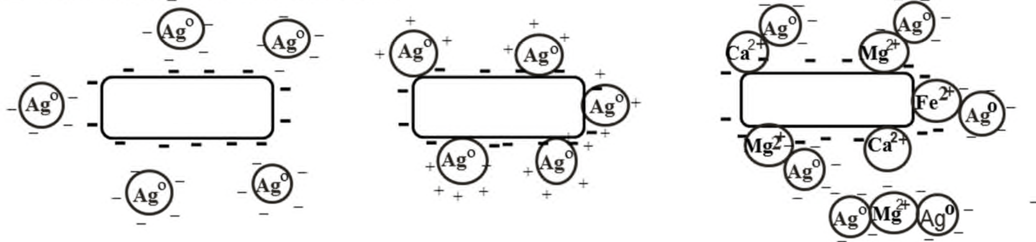
Particle size and shape are known to affect ENPs interaction in aquatic and terrestrial media (Ge et al., 2011; Pelletier et al., 2010). For instance, ENPs of < 30 nm was cytotoxic to *E. coli* and *S. aureus* (Martinez-Gutierrez et al., 2010) compared with 80 – 90 nm particle size (Martinez-Castanon et al., 2008). This suggests that $Ag^0$ of particle size greater than 30 nm could be non- inhibitory to microbial processes. Of particular interest is the size less than 5 nm in suspension capable of inhibiting nitrification in AS (Choi et al., 2010). Apart from particles size, their shape has been reported to play a role as shown for $Ag^0$ which can exist in a triangular, spherical or rod-shaped form. Comparing the effects of the three distinct shapes, the truncated triangular form of $Ag^0$ was found to exert the strongest bactericidal effect on *E. coli* in both agar plate and broth cultures (Pal et al., 2007). As previously mentioned, a direct extrapolation of this

observation from pure culture to complex wastewater is unclear because wastewater components can attenuate or enhance ENP contact and interaction with microbial cell.

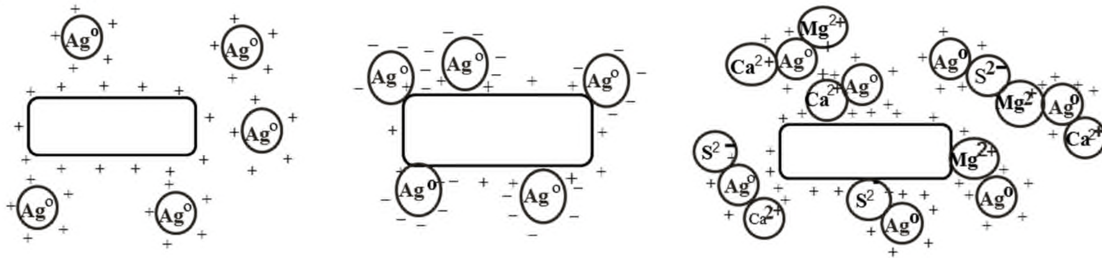
## 2.9 Influence of ions released by ENPs on bacterial cell

The effect of ions released by ENPs into wastewater on bacteria is not evident due to their low concentrations and complexation reactions with organic molecules (Febrega et al., 2009; Zhang et al., 2009; Gondikas et al., 2012). Bactericidal properties of $Ag^0$ through the release of ions ($Ag^+$) are dependent on the hardness and alkalinity of the medium and bacterial cell wall composition (Jin et al., 2009). For instance, teichoic acid contained in Gram positive bacterial cell wall is negatively charged and serves as a binding site (Kikuchi et al., 1997). Binding of ENPs to teichoic acid residues is in competition with divalent cations ($Mg^{2+}$, $Ca^{2+}$) resulting in reduction of $Ag^0$ toxicity to Gram positive organisms in the presence of divalent cations. In Gram negative cell walls, in contrast, it is lipopolysaccharides that restrict passage of toxic substances into the cell although the presence of divalent cations reportedly exacerbates bactericidal effect of $Ag^0$ especially in bicarbonate deficient medium (Kucerka et al., 2008).

**(1) Gram negative cell wall bacterium**




**(2) Gram positive cell wall bacterium**




(a) Absence of divalent cation: Electrostatic repulsion

(b) Electrostatic attraction between cell wall and Ag°

(c) Presence of divalent cation/anions: Adsorption to cell wall through cation /anion mediated ion bridge and/or competitive aggregation to ions

Figure 2.6 Schematic representation of the influence of divalent cations/anions on positive and negatively charged $Ag^0$ - bacterial cell wall interaction.

Apart from a mitigating effect of divalent cations, sorption to cells is in addition reduced by electrostatic repulsion (Stoimenov et al., 2002; Yamanaka et al., 2005). The repulsion is however overcome to some extent by ion bridges forming between negatively charged lipopolysaccharide surface molecules and negatively charged $Ag^0$. The distinct effect of divalent cations on ENP sorption efficiency is schematically shown in Figure 2.6. Sorption may result in conformational change of cell wall and uptake of ENPs (Sondi and Salopek-Sondi, 2004; Morones et al., 2005). Again, comparative studies have shown that $C_{60}$ are more firmly associated with *E. coli* than *B. subtilis* suggesting differences in sorption potential due to surface charges (Lyon et al., 2005). Similarly, sorption of a regular spatial pattern of $Ag^0$ on the surface of HIV-1 viruses suggests preferential binding to the viral glycoprotein spikes (Elechiguerra et al., 2005). However, there is not yet a common consensus on the interaction between bacterial cell wall and its influence on ENP effect. For instance, independent studies by Chudasama et al., (2010) and Jin et al., (2009) are of divergent opinion on the role of cell wall in reducing/enhancing ENPs-microbial interactions.

## 2.10 Challenges in the evaluation of ENPs-microbial interaction

At the moment, there is no generally accepted protocol for ecotoxicity tests and exposure characteristics of ENPs. A number of tests suggested for consideration in addition to the manufacturer's characterization for ENPs, includes measurements of concentration, surface area, zeta potential, primary particle size before dosing, impurities (if any), pre-treatment and analytical procedure, presence of NOMs, and divalent ions in test medium (Depledge, 2010; Grieger et al., 2010; Fabrega et al., 2011). Again, multiple interlinked transformation processes typically found in wastewater and environmental matrices such as the presence of sulfhydryl-containing ligands could alter reactivity and bioavailability of ENPs (Figure 2.6) and give a false-positive acute or chronic toxicity assessment (Gondikas et al., 2012).

## 2.11 Determination of ENPs inhibitory end-points could be misleading

ENPs are greatly influenced by their physicochemistry which in turn may affect standard test methods, octanol-water partition coefficients and bioaccumulation potential (Handy et al, 2008a). Thus, the measurable chemical endpoints such as lethal concentration (LC), lowest observed effect concentration (LOEC), inhibitory concentration (IC), effective concentration (EC) or no observed effect concentration (NOEC) may be inadequate to evaluate the effect of ENPs on microorganisms (Crane et al., 2008, USEPA, 1994). The estimation of 'safe' or 'no-effect concentration' of toxicant to microbes is usually extrapolated from quantitative measurement of cellular dysfunction. Determination of the end-points (NOEC, LOEC, LC, IC, EC) of toxicant concentration-microbial response using Point estimation technique or Hypothesis testing could be subjective, bias and misleading because concentrations below the limits of detection can exert biologically significant effect (Crane and Newman, 2000; Warne and van Dam, 2008).

Again, it is not clear if there is any relationship between ENPs physicochemistry and any of the biological end-points as particle size and surface area effect are not considered in most toxicological methods (Farre et al., 2009; Weisner et al., 2009). Apparently, LC, IC and EC values are estimates of toxicant adverse effect on test organisms, and it would be appropriate to determine 'safe' toxicant concentrations from a microbiological perspective rather than statistical tests. This presents a difficult task for microbiologist to determine the impact level that would be considered 'safe' for microbial activities in wastewater with variable composition. The problem is

exacerbated if the toxicants undergo transformation or react synergistically with other substances which could either enhance or attenuate the adverse effect. Similarly, the concept of hormesis, a biphasic response characterized by low-dose stimulation and high-dose inhibition which has generally been overlooked in toxicological studies (Calabrese and Baldwin, 2003; Cook and Calabrese, 2006) may play a significant role in determining the effect of ENPs on microbial cells in AS/AD.

Also, it is evident that the limited available EC data from research may not be comparable with predicted environmental concentrations (PEC) for a significant ENP toxicity assessment (Hund-Rinke and Simon, 2006; Tiede et al., 2009). The EU Directive on classification, packaging and labelling of dangerous substances (Council Directive 67/548/EEC) indicates that 10 – 100 mg $L^{-1}$ range of concentration without susceptibility to biodegradation should be classified as 'harmful to aquatic organisms; and may cause long term adverse effect in the aquatic environment'. The challenge is exacerbated due to a lack of methods of ENPs characterization (Tiede et al., 2009). Understanding ENPs behaviour and its potential toxicity to microorganisms in the presence of variable wastewater characteristics could be dependent on the nature, size and pre-treatment of ENPs (Brar et al., 2010). As a consequence of these uncertainties, models such as Mass balance partitioning (Mueller and Nowack, 2008) and Risk-ranking (Linkov et al., 2007) which may be used to predict ENPs effect are not performing adequately. However, Bayesian belief network can satisfactorily predict uncertainties in ENPs toxic effect estimations. The model provides a graphically robust and coherent framework for probabilistic evaluation of relationship between complex variables. It delineates cause and effect assumptions with complex causal chain linking actions to outcomes integrated into conditional relationships. Besides, each relationship can be independently assessed without significantly obscuring any variable unlike other models. (Borsuk et al., 2004). An effective interpretation of ENPs toxicity therefore would include an ENPs characterization and a model comparing *in vitro*, *in vivo,* acute and chronic, predictive and validated bioassay data with bioassay data from relevant environmentally aged-ENPs (Maynard, 2006; Fabrega et al., 2011; Puzyn et al., 2011).

### 2.12 Standards and guidance for ENPs toxicity evaluation are lacking

The lack of standardized guidance on dose metrics for ENP assessment exacerbates the uncertainty in toxicity data interpretation. For instance, documented *in vitro* minimum

inhibitory concentration (MIC) values for $Ag^0$ ranged from < 1 to 433 µg $mL^{-1}$ for a variety of organisms under different cultural conditions (Martinez-Gutierrez et al., 2010). Also, the exhibition of cytotoxic and mutagenic effect of different metal oxide ENPs (100 – 1600 µg $plate^{-1}$) on *E. coli* WP2 and *S. typhimurium* TA97 and TA100 followed a wide range of dose-dependent patterns (Warheit et al., 2008; Pan et al., 2010). The assessment of toxicity is closely related to the determination of bioavailable dose (Crane et al., 2008), therefore the interpretation of ENPs dose-contact may be problematic. Tests with some metals demonstrated that short term batch assays did not provide a true reflection of toxicity probably due to kinetics of internalisation, dosimetry and exposure (Liang et al., 2010; Fabrega et al., 2011). Again, there could be bias in evaluation of data from a continuous flow and batch systems due to different hydraulic loading rate (HLR), hydraulic retention time (HRT) and sludge retention time (SRT) which may increase or reduce ENP-microbial contact in wastewater (Wei et al., 2008). Furthermore, reliance on existing regulations and guidelines for metal salts to evaluate ENPs release from wastewater without appropriate ecological studies may constitute a risk because of ENPs bioreactivity (Farre et al., 2011; Wang et al., 2012; Holbrook et al., 2008; Powers et al., 2006; SCENIHR, 2007).

## 2.13 Uncertainties in determination of ENP dosage and microbial contact

The determination of effective ENP concentration available for contact with microorganisms in wastewater currently poses a severe challenge, and can greatly deviate from the overall ENP concentration. The dose defined as 'the amount of a particular agent that reaches a target organism, a system or (sub-) population in a specific frequency for a defined duration' (Leeuwen et al., 2007) is very important and critical to be precisely determined through characterisation of the ENPs (Oberdorster et al., 2005). The implication in wastewater scenario is that most concentrations may not be effective for contact and toxicity assessment (French et al., 2009; El Badawy et al., 2010). ENPs homogeneity has a strong influence on toxicity and the doses for toxicological tests are difficult to ascertain especially when the sample containing ENPs are not monodispersed (Crane et al., 2008). In addition, some ENPs are good absorbents due to their special structure and electronic properties, and could precipitate resulting in reduced bioavailability (Oberdorster et al., 2006; Nowack and Bucheli, 2007).

### 2.14 Influence of pre-treatment on ENPs dosage is uncertain

Analytical procedures and pre-treatment of the samples (e.g. drying for electron microscopy, autoclaving for sterility) in most cases would appear controversial due to the perceived alteration of ENP physicochemistry with influence on subsequent experimental result (Tiede et al., 2009). In the same way, the use of dispersants or filtration aids may denature ENP characteristics resulting in size/shape variation or change their final concentration (Handy et al., 2008). Again, toxicity could either be enhanced or minimized due to dispersant-ENPs interactions (Crane et al., 2008) with the possibility of re-aggregation and changes in ENPs chemical nature with sonication or prolonged stirring (Hassellov et al., 2008).

### 2.15 Inadequacy of available toxicity bioassay for evaluation

The different ENPs would require formulation of specific toxicity standards based on factors such as dose, shape/size-, elemental composition/functionalization-response to assess their impacts on biological systems (Harper et al., 2011; Maynard et al., 2006). The continual use of currently standardized ecotoxicological tests involving respirometry (Choi et al., 2008), *Daphnia magna*, bioluminescent and anaerobic toxicity test have been suggested especially when there is paucity of information on the substance (Gutierrez et al., 2002; Garcia et al., 2011). In comparison with 50 standardized microscale tests, Microtox® assay has been highly rated as a useful tool with 'environmental relevance' for toxicity testing (Ghirardini et al., 2009; Munkittrick and Power, 1989). Microtox assay is based on the exposure of the bacterium *V.fischeri* with subsequent measurement of bioluminescence. The use of this assay is however controversial because this marine species is not a representative member of sludge and requires 2 percent sodium chloride (NaCl). Such osmotic conditions obviously vary substantially from those in activated sludge suggesting that extrapolation of the resulting data to a wastewater context has to be interpreted with caution. Further to this, Ghirardini et al. (2009) and references therein generally agree on the ecological relevance and reliability of the Microtox® solid phase test due to its representativeness on diverse metabolic ability of microorganisms. However, they also highlighted the importance of confounding factors such as bacterial absorption, pH variation due to dilution, particle interference, redox potential which greatly influenced Microtox response to conditions tested. Therefore relying only on one bioassay test is not

sufficient and it can be inferred that an adequate evaluation of ENP toxic effect on AS or AD microorganisms would require the use of multiple bioassays employing species typically found in wastewater such as *E. coli*, ammonia- and nitrite-oxidising species and methanogens for comparative ENPs toxicity evaluation.

### 2.16 Interpretation of ENP toxic effect

ENPs effects on the cells are not fully understood and it is difficult to define a cellular target as a basis for toxicity measurement. The problem is made complicated due to the alteration in ENPs reactivity as a consequence of aggregation (Hassellov et al., 2008). The scarcity of this information makes it difficult to ascertain the safety or otherwise of ENPs released into the environment (Grieger et al., 2010). In addition, there are no standardized or reliable methods to determine or form definitive judgement on environmentally effective concentration, and complexation/aggregation reactions of ENPs (Nowack and Bucheli, 2007). Until recently, aggregation was not important in determination of ENPs toxicity (Hassellov et al., 2008). As a consequence, the effect of an ENP is rather determined based on the interpretation and judgement of individual researchers on the observable acute response of the test organism.

### 2.17 Conclusion

The inclusion of ENPs in consumer products has undoubtedly introduced a new and expanding group of xenobiotic compounds into the ecosystem. ENPs and their transformation products react differently from naturally-occurring substances which hinder microbial utilization/degradation with increased potential for accumulation and toxicity. The non-degradability and the resulting potential for bioaccumulation render ENPs as pollutant of more serious concern compared with other persistent organic pollutants. The lack of knowledge about their ecotoxicological impact and the non-existence of adequate analytical methods, guidelines and regulations add more uncertainty. At the moment, available information on ENPs-microbial interaction is restricted to varying acute effect of pristine forms, whereas aged-ENPs and wastewater microbial interaction is at best hypothetical with confounding variables. Thus, insights on ENPs effect on wastewater microbial community will require a case-by-case evaluation for understanding ENPs behaviour and environment-friendly management of nanowaste. This review demonstrates the dramatic lack of empirical evidence on what effect pristine and aged-ENPs have on wastewater microorganisms, AS/AD process efficiency, transport and fate. This knowledge gap is in great part caused by lack of

appropriate analytical tools and framework to elucidate factors that positively enhance or attenuate ENPs effect. Thus, a realistic correlation in data interpretation from available acute toxicity test without comparison with data from relevant environmental media could be subjective, with uncertainties and bias.

Therefore, future research needs would include development of relevant analytical technique for ENPs characterisation in complex environment. Correspondingly, experimental data from a pilot- and full-scale study as a relevant environmental condition and impact analysis of aged-ENPs in AS and AD would be greatly beneficial and provide comparative empirical evidence on the toxicological implications of ENPs on microbial community dynamics during wastewater treatment.

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## Chapter 3: Insights into the effect of mixed engineered nanoparticles on activated sludge performance


### Abstract

The effect of a mixture of engineered nanoparticles (ENPs) consisting of silver oxide, ($Ag^0$, 20 nm), titanium dioxide, ($TiO_2$, 30-40 nm) and zinc oxide, (ZnO, 20 nm) compared with their bulk metal salts was evaluated against unspiked activated sludge (control) using 3 parallel pilot-scale treatment plants. The introduction of both nanoparticles and bulk metals mixtures in the wastewater treatment plants induced a 2-fold increase of the microbial specific oxygen uptake rate (SOUR) compared with the control plant. The scanning electron microscopy (SEM) showed there was selective damage on some microbial cells. Further to this, activated sludge floc size was reduced in the presence of the ENPs while the sludge volume index (SVI) was unaffected. Despite these observed changes, the presence of ENPs barely affected the overall biological removal processes occurring in the activated sludge as > 98 % of ammonia and 80 % of COD were removed. Therefore the heterotrophic biomass retained its ability to nitrify and degrade organic matter. Further to this, > 99 % of the ENPs spiked into the AS were sorbed to the biosolids suggesting the strong affinity of ENP to AS biosolids. The lipid fingerprint and 454 pyrosequencing analyses showed a clear temporal shift in microbial community structure. The prominent nano-tolerant bacterial species identified were *Acidovorax*, *Rhodoferax, and Comamonas* whereas *Methanocorpusculum* and *Methanosarcina* were the dominant *Archaea* suggesting their tolerance to ENPs and oxygen-dependent respiration. Unlike other xenobiotic compounds, ENPs can hasten the natural selection of microbial species in wastewater and activated sludge.



Keywords: Activated sludge, engineered nanoparticles, microorganisms, contaminant removal, inhibitory effect

## 3.1 Introduction

The growing use of engineered nanoparticles (ENPs) in consumer and industrial products and the concerns surrounding their potential effects to the environment and human health cannot be dismissed. There is particularly a compelling need to investigate the effects, fate and transport of ENPs in wastewater treatment plants (WWTP) as they act as both primary sink and source for nano-enabled waste and aged-ENPs. Currently there are uncertainties in relation to ENPs effect on the biologically mediated contaminant removal processes occurring in WWTP. Although pristine ENPs have antimicrobial effects information on aged-ENPs effect on ecologically sensitive organisms in wastewater and the environment is still blurred (Liang *et al*., 2010).

The negative effects of bulk metal oxide salts on wastewater microorganisms (Hu et al. 2004; Tsa et al. 2006) as the parent material for ENPs suggest that metal oxide nanoparticles potentially can exert similar effect on wastewater organisms (Batley et al., 2012; Liang et al., 2010; Blazer et al., 2008). For instance, silver metal oxide in activated sludge (AS) can negatively affect specific growth rate of nitrifying bacteria resulting in reduced nitrification rate or failure of the organisms to nitrify (USEPA, 1993). ENPs in wastewater can lead to reduced efficiency or complete failure of nitrification during AS process and subsequent pollution of the environment during effluent discharge. For instance, silver nanoparticle ($Ag^0$) can inhibit the removal of nitrogenous material during wastewater treatment (Liang et al., 2010) and the effect can be magnified in the presence of other ENPs such as titanium dioxide ($TiO_2$) and zinc oxide (ZnO). A reduced activated sludge (AS) process efficiency due to the build-up of ammonia – a substance known to have adverse effect on anaerobic bacteria and archaea, can upset the delicate balance of acetogenic and methanogenic organisms in a subsequent anaerobic digestion (AD) (Kayhanian, 1994). Further to this, the use of ENP enriched biosolids for soil amendment can introduce aged-ENPs into the environment (Hoffmann and Christoffi, 2001).

It is suspected, however, that the potential effect of $Ag^0$, $TiO_2$ and ZnO nanoparticles in complex medium can vary due to their aqueous stability by natural organic matter (NOM) (Chen and Elimelech, 2007; Hyung et al., 2007), presence of sulphides (Levard et al., 2011), protein (Karajangi et al., 2006; Neal, 2008), and different rate of contact with biofilm embedded cells (Wu et al., 2010). ENPs can react with wastewater

micropollutants and surfactants to enhance or attenuate the potential antimicrobial effects which can differ based on the microbial groups and variable physicochemical component of AS. For instance, nitrification and microbial abundance can decrease in the presence of ENPs whereas organic matter removal remains unaffected (Sun et al., 2013; Kaegi et al., 2013). ENPs exert their negative via sorption to microbial cell wall, disruption of cell wall/membrane structure by lipid peroxidation, and alteration of cell permeability by producing reactive oxygen species and ions (Beer et al., 2012).

The empirical evidence for understanding aged-ENPs effect on wastewater microbial community is still limited although the effect on pure cultures of several organisms at laboratory-scale (see Chapter 2; Table 2.1) showed that factors such as contact time, cultural conditions, composition of growth medium, type of organism, ENPs dose, physico-chemistry and capping agent (Garcia et al., 2011; Martinez-Gutierrez et al., 2010) played important roles either to stimulate or inhibit viable cells, and disrupt cell structure and function. Apart from the antimicrobial effects, ENPs can enhance growth and increase microbial reaction rates (Huang et al., 2005; Hilderbrand et al., 2009). Notable examples include the dechlorination of polychlorinated biphenyl (PCB) congeners in sediment matrices by *Shewanella oneidensis*-palladium nanoparticle (De Windt et al., 2005), batch anaerobic reduction of nitrate by integrated nanoscale zero-valent iron and microorganisms (Shin and Cha, 2008) and enhanced dehydrogenase activity of soil microorganisms (Cullen et al., 2011). These compelling evidences suggest that understanding the mechanism of action and possible use of ENPs as nano-catalyst to augment the metabolic activities of indigenous microbial species involved in bulk contaminant removal and degradation during wastewater treatment can be achieved. Besides, ENP effect on microorganisms in complex environment such as wastewater is not clearly defined and at the moment, information on the long-term effect of mixed ENPs such as silver oxide ($Ag^0$), titanium dioxide ($TiO_2$) and zinc oxide (ZnO) released from consumer products into wastewater or present during AS treatment are unavailable.

To date, only studies at lab-scale on pure culture and limited studies in real environmental matrices such as AS where conditions are complex (presence organic matter, microbial groups and microbial interactions, mixture of metals and ENPs), are available. The AS process is mediated by complex groups of microbes interacting in a synergistic and antagonistic manner, thus the urgent need to investigate ENPs effect on

AS microbial community *in situ* and process efficiency in removing contaminant. Extrapolating the negative effect on monocultures to mixed population, to explain the probable behaviour of ENPs in AS poses a challenge because the different condition and synergistic activities of microorganisms can influence the result. It is plausible that ENPs can inhibit microbial activity, reduce the abundance and diversity or enhance AS process performance by stimulating the microbial communities.

The effects of silver oxide ($Ag^0$), Titanium dioxide ($TiO_2$) and Zinc oxide (ZnO) mixture on AS microbial communities in continuous flow pilot-scale treatment plant was investigated to understand how ENPs interact with (i) microbe morphology, (ii) microbial activity (respiration) (iii) microbial structure and diversity (adaptation, resistance, tolerance) and (iv) the implications on the AS process efficiency in removing contaminants and nitrification. Equivalent concentrations of bulk metal salts as parent compound closest to ENPs chemistry, behaviour, fate and transport and unspiked control were compared to interpret the effect of ENPs.

## 3.2Materials and methods

### 3.2.1 Pilot-scale activated sludge treatment plant

This study involved the running of three parallel pilot-scale plants, each consisting of primary clarifiers (180 L), secondary clarifiers (~ 150 L), and circular aeration tanks (~300 L) fitted with submerged diffusers for aerating and mixing the sludge (Figure 3.1). As a start-up material, return activated sludge (RAS) from a full-scale municipal waste water treatment works (Anglian Water, Cotton Valley, UK) and settled wastewater from the Cranfield University domestic wastewater treatment plant (CUDWTP) on a 50:50 ratio was fed into the aeration tanks (Figure 3.2). Thereafter, settled influent wastewater from CUDWTP was fed into the three primary clarifiers at a rate of 750 ml $min^{-1}$ (40 rpm) and maintained at 20 ± 5 $^0$C. Recirculation of RAS from the secondary clarifier into the aeration tank was done by the use of a peristaltic pump (505U, Watson and Marlow, UK) at the rate of 375 ml $min^{-1}$ (20 rpm). The plants were operated at a fixed hydraulic retention time (HRT) of 8 hours and sludge retention time (SRT) of 10 days. The plants were subsequently operated over 3 SRT (30 days) to stabilize. Identical conditions were maintained in the 3 plants with the exception that treatment lines 1 and 2 were spiked with ENPs and bulk metal salts respectively, whereas treatment 3 served as control (unspiked). The ENPs used in this study were

chosen based on their wide application in a variety of consumer products with particle size of 20 nm for $Ag^0$ and ZnO and 21 nm for $TiO_2$. Silver was proprietary solution of Ag nanoparticles coated with polyvinylpyrrolidone (PVP). Zinc was a high purity and high quality zinc oxide nano-powder commercially known as Nanosun™. Titanium was high purity titanium oxide nano-powder commercially known as Aeroxide P25 (Degussa, Germany). The solution of mixed ENPs was made up of 0.01 mg $L^{-1}$ $Ag^0$, 0.08 mg $L^{-1}$ $TiO_2$, and 0.12 mg $L^{-1}$ ZnO and the aeration tank was spiked at the rate of 0.14 ml $min^{-1}$ (equivalent to 0.67 ml L $day^{-1}$) for 315 days. An equivalent concentration of mixed metal salts comprising of silver nitrate ($AgNO_3$), $TiO_2$, and anhydrous zinc nitrate ($Zn(NO_3)_2.6H_2O$) and unspiked sludge (control) was used for comparison. The mixed ENP and metal salt suspensions were maintained in a dispersed state by continuous stirring at 200 rpm.

### 3.2.2 Sampling and analysis

Influent, effluent and mixed liquor suspended solids (MLSS) samples (1 L each) were collected into sterile wide mouth amber glass containers with Teflon-lined cap and stored at 4 $^0C$ until analysis. The influent, effluent and MLSS from the three reactors were sampled daily in duplicate for 20 days and subsequently on weekly basis for analysis (Table 3.1). Physicochemical analysis of the wastewater mixed liquor suspended solids (MLSS), influent and effluent was determined within 2 hours of sampling by measurement of the pH, suspended solids (SS), total volatile solids (TVS), sludge volume index (SVI) according to standard methods (APHA, 2005). The influent and effluent $NH_3^-$-N, $NO_2^-$-N, $NO_3^-$-N, total nitrogen, and COD were measured by Hach's vial methods (Camlab and Merck) adapted from the standard analytical method (APHA, 2005). Particle/floc size of biomass measurement was done using Malvern mastersizer 2000 (Malvern Instrument, Worcestershire, UK). Treatment efficiency (%) was calculated using the formula: (Influent – Effluent) (100) / Influent. The pilot plant experimental design was not for the removal of phosphate ($PO_4^{3-}$), sulphate ($SO_4^{2-}$) and denitrification because of the alternating aerobic (famine) and anaerobic (feast) conditions required. Here, the main focus was to produce enough activated sludge for use in anaerobic digestion although AS physicochemistry were characterised to provide information for understanding ENP fate, transport and effect on the AS process.

A = ENP spiked AS, B = unspiked control, C = Metal salts spiked AS, D = Primary clarifier, E = anaerobic digester, F= Secondary clarifier

Figure 3.1 Pilot-scale wastewater treatment plant set up. The reactors are standard Cranfield University design and made by Model Products Limited, Bedford, UK,

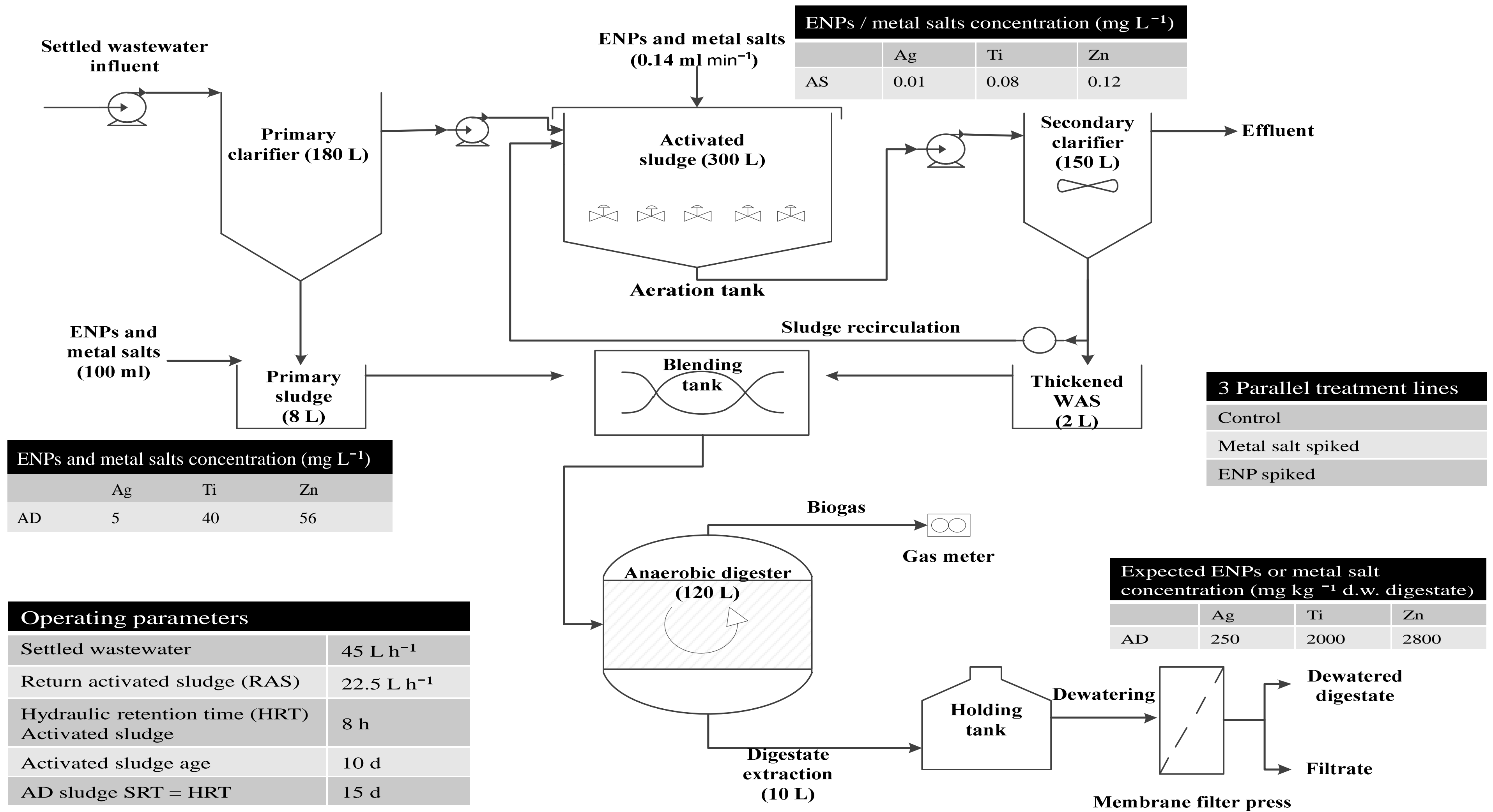



Figure 3.2 Flow schematic of the pilot-scale plant operation with the spiked and targeted ENPs and metal salt concentration in the digestate

Table 3.1 Summary of sampling regime for AS

| Parameter/ sample size | Time (d) | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 30 | 40 | 50 | 60 | 70 | 90 | 110 | 130 | 150 | 170 | 190 | 210 | 230 | 250 | 270 | 290 | 315 |
| Physicochemical analysis | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| PLFA (42 samples ) | | ○ | ○ | ○ | ○ | | ○ | | ○ | ○ | | | | | | | | |
| Pyrosequencing (21 samples) | | ○ | | | ○ | | ○ | | | ○ | | | | | | | | |
| ENP/metal salts | | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |

• = Influent and effluent, AS operated for 3 sludge retention time (30 days) to stabilise. ○ = MLSS.

### 3.2.3 Measurement of the residual concentration of ENPs and metal salts ionic forms

The ENPs and metal salts ($Ag^{+}$, $Ti^{4+}$, $Zn^{2+}$) residual concentration in the activated sludge and effluent at various times was measured by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES, Perkin Elmer 4300DV). Chemical analysis was carried out externally by a commercial laboratory (Environmental Scientific Group, Straffordshire, UK). Briefly, the samples were digested prior to analysis using high purity nitric acid, hydrogen peroxide and hydrofluoric acid in sealed Teflon vessels with microwave assisted heating. After digestion, demineralized water having a resistivity of 18.2 MΩ.cm was used in making the samples into known volume. The concentrations of $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ were measured by ICP-AES calibrated using certified standards. 5.0 mg $L^{-1}$ Ag, Ti and Zn was prepared from an alternative source stock from that used to prepare the instrument calibration standard and measured with the samples as a quality control measure. Procedural blanks spiked with equivalent of 4.0 mg $l^{-1}$ $Ag^{+}$, $Ti^{4+}$, and $Zn^{2+}$ was taken through the same procedure as a further quality control measure. The results of residual ENPs in the sludge and effluent are reported as ions of $Ag^{+}$, $TiO_2$, and $Zn^{2+}$ because it was difficult to distinguish the nano and bulk forms of the metal oxides in AS and effluent.. The concentration of residual ENPs and metal salts

recovered by analysis were corrected against the control using the formula: % recovery $= C_{spiked\ sample} - C_{unspiked\ sample} / C_{added}$ x 100, where C is the concentration.

### 3.2.4 Sludge Volume Index (SVI)

One litre MLSS sample was introduced into the settling column (1 L graduated cylinder). The solids were distributed by covering the top and inverting 3 times. The volume occupied by suspended solids was measured at 30 min intervals as the sludge volume (SV) and used in the determination of sludge volume index (SVI) as follows: SVI = SV x 1000 / SS where SV = sludge volume after 30 minutes of settling and SS = suspended solids concentration.

### 3.2.5 Determination of cultivable heterotrophs and coliforms

To assess the effect of ENP and/or metals on indigenous bacterial population of AS, a modified method of Coulon et al., (2010) was adopted to plate and count the colonies. Two ml of effluent samples from the reactors was vortexed for 30 seconds, sonicated for 1 min and allowed to stand for a further 2 minutes. 1.0 ml aliquot of the suspension was serially diluted using filter-sterilized AS water to the $10^{-10}$ dilution. 1.0 ml aliquot of appropriate dilution ($10^{-4}$ and $10^{-5}$) in each series was pour-plated in triplicate onto Plate count agar (Oxoid) to determine the heterotrophs and chromogenic *E.coli*/coliform selective medium (Oxoid) for *E.coli* and total coliforms. A set of duplicate sample dilutions were incubated at 35 ± 0.2 $^{o}$C for 24 h and thereafter the numbers of colony-forming units (CFUs) enumerated. Results are expressed as CFU $ml^{-1}$ of influent and effluent. *E. coli* was differentiated from other coliforms (pink colonies) by their typical purple colonies. Following the method of Dao and Yao (2009), the percentage survival (% S) of culturable bacterial density was determined using the log-transformed CFU/ml values and the formula:

$$\% S = CFU_{exposed} / CFU_{control} \times 100$$

where $CFU_{exposed}$ corresponds to either the colony forming units from the metal salts or ENP-dosed AS samples and $CFU_{control}$ is the total colony forming units from the control AS sample.

### 3.2.6 Effect of ENPs and metal salts on bacterial growth kinetics

Bacterial growth in the presence of ENPs was estimated spectrophotometrically from the optical density of the broth (Pal et al., 2007; Das et al., 2011). 1.0 ml of effluent

sample from each treatment line was vortexed for 30 seconds, sonicated for 1 min and allowed to stand for 2 min. The samples were inoculated onto 100 ml of Nutrient broth No. 2 (Oxoid) and incubated at 35 ± 0.2 $^0$C and constantly agitated at 170 rpm. At intervals of 1 hour, 3 ml of broth culture was withdrawn from each flask and optical density (OD) of two replicate inoculations measured at 600 nm for 8 h were used to determine effect on bacterial growth.

### 3.2.7 Measurement of oxygen uptake rate (OUR)

To measure the oxygen uptake rate of microbial community in AS, the Organization for Economic Co-operation and Development guidelines for the manometric respirometry test (OECD 301F) was employed. 100 ml of the control, ENPs and metal salts spiked mixed liquor suspended solids (MLSS) were shaken to saturate with oxygen and transferred to the reactor vessel in separate batches set up in the respirometer. The flasks were hermetically sealed, placed in a thermostatically regulated water bath maintained at 20 ± 0.2° C and magnetically stirred at 150 rpm. Oxygen uptake rate (OUR) was determined by measuring the quantity of oxygen (produced electrolytically) required to maintain constant gas volume in the respirometer flask. Evolved $CO_2$ was absorbed in a solution of 2 M NaOH. The resulting change in pressure attributed to consumption of only $O_2$ induced through the manometer an electrochemical generation of $O_2$ until the pressure was balanced. The flow of electrical current and changes in headspace pressure was continuously monitored and recorded for 24 hrs (O'Malley, 2006; Ricco et al., 2004 Reuschenbach et al., 2003). Specific oxygen uptake rate (SOUR) was calculated from the OUR and mixed liquor volatile suspended solids (MLVSS) normalized data using the formula: SOUR = OUR x 1000 / MLVSS.

### 3.2.8 Electron microscopy

To observe the structural changes on bacterial cell, microbial flocs from AS samples were screened using the scanning electron microscope (SEM) equipped with a charge-coupled device connected to a computer. A drop of unwashed AS suspension was placed on silicon square held onto aluminium stub by double sided carbon tape and allowed to dry without alteration in the floc morphology. The dried sample was sputter-coated with gold/palladium for 1 min to give a thin layer of about 2 – 3 nm, and examined using a scanning field emission gun electron microscope (FEI XL30 S-FEG).

### 3.2.9 Phospholipid fatty acid (PLFA) analysis

To characterise the changes in bacterial community structure of the influent, floc and effluent samples, PLFA extraction was performed using a modified method of Bligh and Dyer (1959) as described by Frostegard et al., (1993). Prior to analysis, samples for phospholipid fatty acid (PLFA) analysis were centrifuged at 5000 rpm; the biomass was frozen and freeze-dried, stored at -80 °C until analysis. Briefly, 5 g of freeze-dried activated sludge sample were selected as indicated in Table 3.1 and extracted using 0.8:1:2 (v/v/v) citrate buffer-chloroform-methanol, subjected to solid-phase fractionation followed by transesterification by mild alkaline methanolysis (Dowling et al., 1986) to obtain the fatty acid methyl esters (FAMEs). The dried FAMEs were resuspended in 0.2 ml of hexane and analysed by gas chromatography (GC) (Agilent Technologies 6890N) coupled to a flame ionization detector. Chromatography was performed as described by Pankhurst et al., (2012). PLFA composition was identified by comparing the retention times of the peak obtained with the 26 bacterial acid methyl ester (BAME) mix standard (Sigma-Aldrich Ltd., Dorset, UK). Nonadecanoic acid methyl ester (Sigma-Aldrich, UK) was added (200 µl) as internal standard to each sample after solid phase extraction. PLFA nomenclatures were as follows: X: YωZ, where X = number of carbon atom in the chain, Y = number of double bonds (unsaturation) in the aliphatic (ω) end of the carbon chain and Z = number of carbon atom from the methyl end of the molecule in relation to the first unsaturation. The suffices ‘c’ (*cis*) and‘t’ (*trans*) indicate the geometric isomers of the unsaturation and the prefixes ‘a’ (*anteiso*), ‘i’ (*iso*), ‘10me’ (*methyl* branch on the tenth carbon from the carboxyl end), ‘cy’ (*cyclopropyl*) and ‘Br’ (*branching* at unknown location) whereas hydroxyl ($OH^-$) substitutions is represented at the carbon atom where the substitution occurs (Macnaughton et al., 1999).

### 3.2.10 454-Pyrosequencing of activated sludge microbial community

Samples for PLFA and 454-pyrosequencing analyses were selected as indicated in Table 3.1. A 40 g aliquot of the AS samples were collected using aseptic techniques and stored at -80ºC until analysis. 200 mg of the samples were selected as indicated in Table 3.1 for DNA extraction using a MoBio Power Soil kit (MO BIO Laboratories, Inc, UK) and the quality of DNA was checked on 0.8% agarose gels. For the amplification of the bacterial 16S rRNA gene fragments, PCR primers were adapted for 454 amplicon sequencing by attaching the M13 adapter (**CACGACGTTGTAAAACGA**) to the

primer M13-16S-IA-FL (5'-**CACGACGTTGTAAAACGA**CCATGCTGCCTCCCGTAGGAGT-3'), whereas the 25-mer Lib-L specific sequence adapter B (CCTATCCCCTGTGTGCCTTGGCAGTC) was followed by the reverse template specific primer sequence 16S-IA-RL (5'-**CCTATCCCCTGTGTGCCTTGGCAGTC**TCAGAGAGTTTGATCCTGGCTCAG-3'). To aid multiplexing different samples, different barcodes were included in the M13 adapter using the 454 sequence adapter A (CCATCTCATCCCTGCGTGTCTCCGAC) and a 454 amplicon sequencing specific 4-mer amplification key (*TCAG*) followed by a 10-mer barcode sequence (NNNN) (5'-CCATCTCATCCCTGCGTGTCTCCGAC*TCAG*NNNN***CACGACGTTGTAAACGAC***-3').

Each 20 µL PCR mixture contained primers at 10 µM, 10 mM deoxynucleoside triphosphates and 0.2 µL of High fidelity polymerase (Phusion, Biolabs, New England, UK), 4 µL Phusion 5x buffer (Phusion, Biolabs, New England, UK) and 1.4 µL $MgCl_2$. Amplifications were performed using a Biorad C1000 Thermal cycler (BioRad) as follows: 95 °C for 5 min, followed by 35 cycles of denaturation at 98 °C for 20 sec, annealing at 57 °C for 20 sec and elongation at 72 °C for 30 sec. Cycling was completed by a final elongation at 72 °C for 10 min. Next generation sequencing (NGS) of all amplicons was completed using the GS FLX System (Roche). Emulsion PCR was carried out according to the manufacturer's instructions (Roche). Samples were multiplexed on a 1/8 section of the pyrosequencing plate. Sequencing resulted in a total of 17,022 bacterial sequences and an average sequence of 1067 in control, 590 in metal salt spiked AS and 775 in ENP spiked AS was obtained. The obtained sequence data were processed using the Galaxy platform (http://galaxyproject.org/). Sequences analysed were a minimum of 500 bp (mean length average 560 bp). Splitting of sequences into respective samples was carried out using respective barcodes.

### 3.2.11 Bioinformatics

The obtained sequence data were then processed using the CloVR-16S 1.0 pipeline (http://clovr.org/) according to White et al., (2011). Briefly, poor quality sequences were removed using the Qiime script "plit_libraries.py" (http://qiime.org) using the following parameters (minimum sequence length 100 bp, maximum sequence length 2000 bp, maximum homopolymer length 8, minimum quality score 25, and maximum ambiguous

bases 0). The Mothur script "unique.seqs" was then used to cluster unique sequences and a set of representative sequences was determined. The representative sequences were then searched against the "16S rRNA gold database" to identify putative chimeras using the default parameters. The chimeric sequences were then excluded from further analysis. Sequences were then clustered, aligned and classified using the Qiime workflow "pick_otus_throigh_otu_table.py". Sequences were clustered into operational taxonomic units (OTUs) with a 97 % nucleotide sequence identity threshold for all reads within a OTU using the Qiime script "pick_otus.py". Representative sequences for each cluster were then selected with "pick_otus.py". Representative sequences were then classified using the Ribosomal database project (RDP) Bayesian classifier (http://rdp.cme.msu.edu/) at phylum, class, order, and family with a confidence threshold of 0.5, with the script "assign_taxonomy.py". Results presented are the number of sequences assigned to OTUs identified at the respective taxonomic levels.

### 3.2.12 Statistical analysis

Kruskal-Wallis test was performed using *Statistica* software® version 12 (Statsoft, Tulsa, OK, USA) and values are presented as mean ± standard deviation with levels of significance maintained at 95% for each test. PLFA and 454 pyrosequencing data were log-transformed to reduce skewness in distribution, subjected to species-dependent hierarchical cluster analysis and non-metric multidimensional scaling (MDS) ordination based on Bray-Curtis similarities using PRIMER version 6 (Clark and Warwick, 2001).

## 3.3 Results and discussion

### 3.3.1 Residual concentration of aged-ENPs and metal salt ion in WAS, AS and effluent

Residual concentration of the spiked ENPs and metal salt characterised as metal ions ($Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$) in the WAS are presented in Figure 3.3. The trend in metals residual concentration in WAS from the three plants was Ti > Zn > Ag with the lowest concentration of the ions in the control. Concentrations reported for the WAS control samples are indicative of the ENPs or metal salts released from consumer products and sorbed to wastewater biosolids. In the ENP spiked AS, however, the concentration of 0.27 ± 0.09 mg Ag $L^{-1}$ sorbed to biosolids was 9 times higher than background concentration in control and 4 times than in the metal spiked AS. Similarly, the concentration of $Ti^{4+}$ (6.37 ± 1.39 mg $L^{-1}$) was 1.4 times higher than in control and 21

times in metal salt spiked AS whereas $Zn^{2+}$ ($2.33 \pm 0.35$ mg $L^{-1}$) was 3.2 times higher than in the control and 2 times higher than in metal salt spiked AS. The results suggest that ENPs were associated with biosolids and undetected concentration observed in the effluent compared to the metal salt in which a small fraction was detected in the effluent. Two plausible reasons are deduced for the low residual concentration of metal salts ions in the WAS: (i) metal salts are soluble in the aqueous WAS and most of them were not sorbed to biosolids (ii) release of concentration sorbed to AS biomass present in the effluent.

The $Ag^0$ concentration of 30 µg $L^{-1}$ in the control WAS was higher than concentrations reported by Blaser et al., (2009) which ranged between 2 and 18 µg $L^{-1}$. This indicates an increase in the release of $Ag^0$- enriched waste into CUDWTP. In addition, 2 times higher sorption of ENPs to biosolids than metal salts was obtained which is consistent with the tendency of ENPs to associate with natural organic matter. The result further suggest that wastewater biosolids is a porous medium for ENP sorption and removal from wastewater/AS and agrees with the findings of Kiser et al., (2010) in which 13 to 88 % of different ENPs such as fullerene, $Ag^0$, and $TiO_2$ were sorbed to the biosolids.

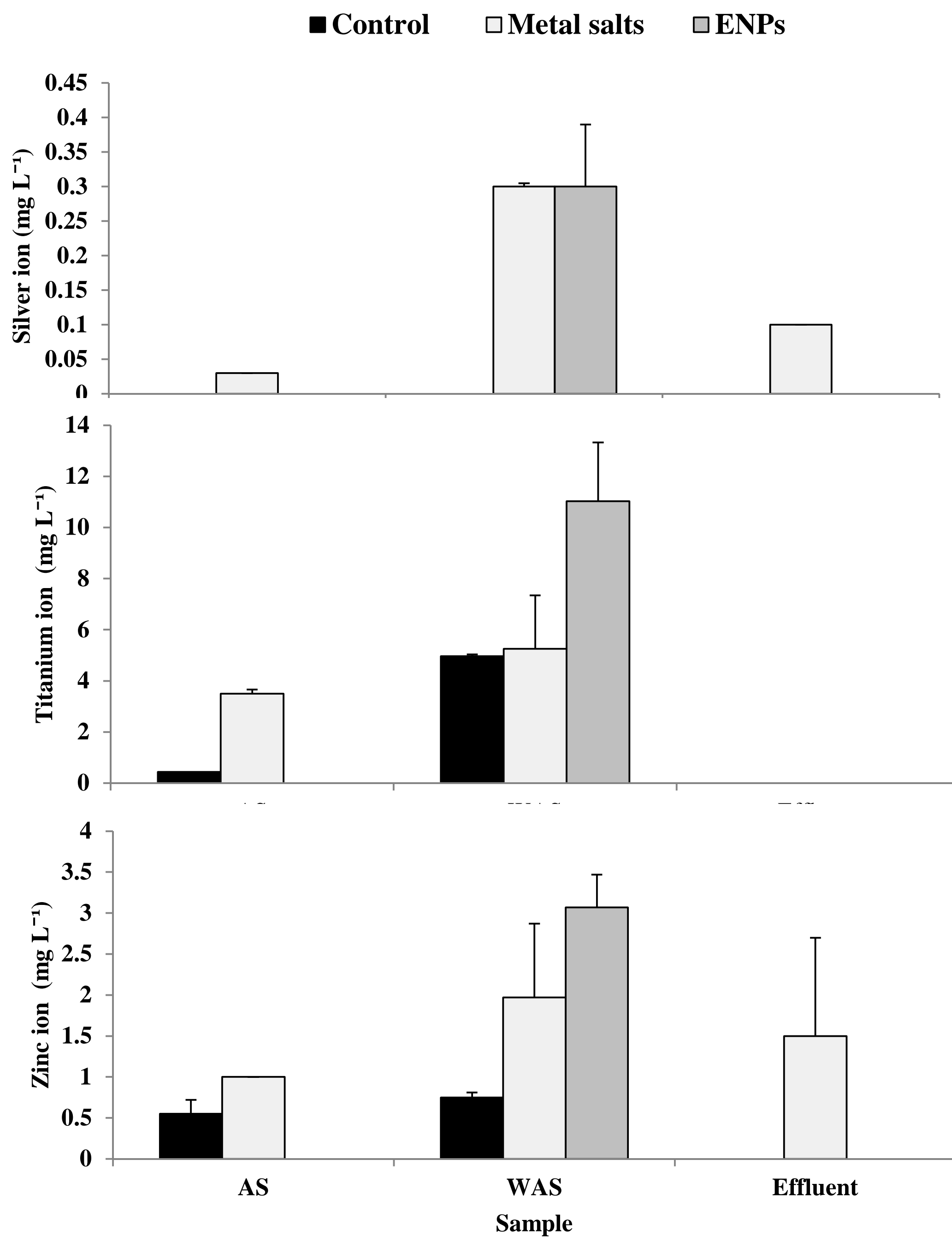



Figure 3.3 Residual concentrations of ENPs and bulk metal salt ions.
Values are mean ± standard deviation of ENPs and metal salts ions partitioned into AS, WAS and effluent from 315 days of treatment. Concentration of ENPs and metal salt in WAS were normalized against the control.

### 3.3.2 pH change

pH change between influent and effluent of each treatment line is shown in Figure 3.4 (b). A reduction of about 1.5 pH unit was observed between in the effluent and influent suggesting the effect of treatment on the AS process. The pH range in the influent and treated effluent samples is significant because ENPs can be partitioned to porous media such as AS biomass enhanced by alkaline pH (Kiser et al., 2010) and desorption can occur at acidic pH (Wang et al., 2012). It is reasonable to assume that the ENPs was adsorbed to the biomass and also desorbed considering the alkaline pH of the influent wastewater and the mildly acidic pH of the effluents. For instance, $Ag^0$ can dissolve under oxic conditions at pH of 5.68 (Liu et al., 2010). Although it was not investigated how $Ag^0$, ZnO and $TiO_2$ reacted with other contaminants in the AS, it remains unclear how pH influenced the interaction. It is suspected that $Ag^+$, $Zn^{2+}$ and $Ti^{4+}$ ions were released as key by-product of the dissolved ENPs. The influence of pH in the ENPs spiked AS is consistent with the study of Dobias and Bernier-Latmani (2013) in which aggregation and slow dissolution of the ENPs such as $Ag^0$ persisted and accumulated in the sludge although the pH values was within the 6 to 9 limit demand by EU.

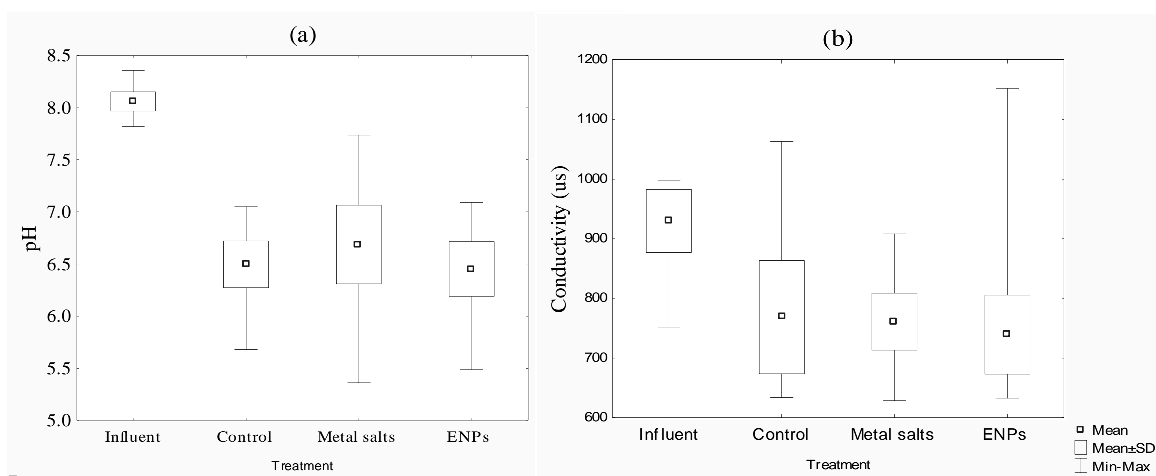



Figure 3.4 (a) pH and (b) conductivity for the influent and effluents of each treatment over 315 days. The pH and conductivity were measured every day for the first 30 days and thereafter twice a week.

### 3.3 3 Reduced electrical conductivity

As a general indicator of effluent quality, electrical conductivity measures the concentration of dissolved salts in the form of ionic content such as hydrogen ($H^+$), hydroxide ($OH^-$), phosphate ($HPO_4^-$), and nitrate ($NO_3^-$). Figure 3.4(b) shows the

influent-effluent conductivity variation during the 315 days of the pilot study. Conductivity of the influent was 929 µS whereas the conductivity of the effluents was 760 µS for the metal and ENP spiked AS and 730 µS for the control. The difference in conductivity between the samples was significant ($p < 0.05$) and indicates the presence of divalent ions such as $Ca^{2+}$ and $Mg^{2+}$ (Saleh et al., 2008; Chen et al., 2008). Divalent ions can form ion bridges which to some extent reduce electrostatic repulsion (Jin et al., 2010), mitigate ENPs adverse effect, reduce repulsion and increase sorption to microbial cell wall and biosolids.

### 3.3.4 Enhanced ammonia and nitrite oxidation

The oxidative conversion of ammonia ($NH_3^-$) to nitrite ($NO_2^-$) and nitrite to nitrate ($NO_3^-$) in the AS was not inhibited at the concentration of mixed ENPs or metal salts spiked into the reactors (Figure 3.5a). Mean residual $NH_3^-$ concentration of 0.2 ± 0.1 mg $L^{-1}$ (control AS), 0.8 ± 1.6 mg $L^{-1}$ (metal salts spiked AS) and 0.17 ± 0.9 mg $L^{-1}$ (ENP spiked AS) compared with 37 ± 6 mg $L^{-1}$ (influent) indicating that bulk ammonia removal was > 98 % in all conditions tested. An order of magnitude reduction in the $NH_4$ removal in the mixed ENPs spike AS compared to the control was observed whereas about 4 orders of magnitude $NH_3^-$ removal by the control compared with metal salts. It appears that there was no negative impact on nitrifying bacteria at the spiked ENPs and metal salt concentration compared with control during the oxidation of ammonia in the AS.

The result of $NO_2^-$ in Figure 3.5(b) indicates 10 to 13 times difference between the treated effluent and influent. At the moment, it is unclear what factors may have influenced the result in the metal salt spiked AS between days 261 to 306 in which the $NO_2^-$ concentration was above 1 mg $L^{-1}$. Nevertheless, nitrite concentration was < 1 mg $L^{-1}$ which suggest proper functioning of the AS process in the three reactors. Thus, ammonia was oxidised to nitrite and nitrite further oxidised to nitrate as expected and the spiked concentrations of ENPs and metal had no adverse effect on the nitrification process.

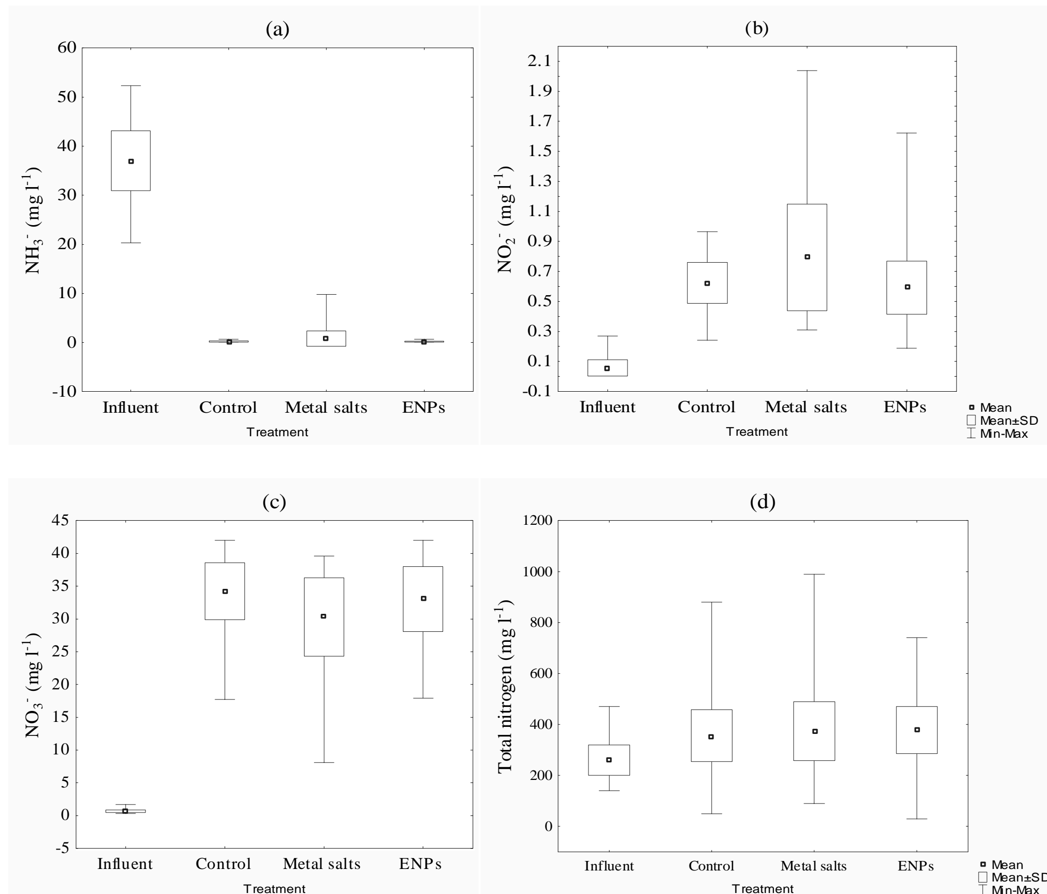



Figure 3.5 Effect of treatment on the bulk removal of nitrogenous compounds from the AS over 315 days of treatment.

### 3.3.5 Accumulation of nitrate and total nitrogen in the effluent

Nitrate concentrations in the effluents were about 45 to 51 times higher than the influent indicating that nitrate accumulated in the effluent. Although the pilot plants were not designed for denitrification as previously mentioned, the effect of treatment on nitrate reduction is shown in Figure 3.5c. The high concentration of $NO_3^-$ in the effluents implies that oxygen served as the primary terminal electron acceptor (Robertson and Kuenen, 1992) for microbial respiration in the AS. The concentration of total nitrogen, however, was about 1.5 times higher in the effluents than the influent with a significant ($p < 0.05$) difference observed. The concentration of total nitrogen in the effluents (Figure 3.5 d) was above the 10 to 15 mg $l^{-1}$ limit consent by EU for domestic effluent disposal and thus can harm receiving surface water. The accumulated total nitrogen observed in the AS is attributed to the operational design of pilot plants, thus, there is a need to investigate ENP effect on nitrogen and nitrate reduction in AS.

### 3.3.6 Reduction in chemical oxygen demand (COD)

The COD of the effluents decreased by 5 orders of magnitude compared to the influent mainly due to organic matter removal (Figure 3.6a). The COD concentration was below the 125 mg $l^{-1}$ limit specified by the EU for domestic effluents. Here, the 3 plants performed in the same way both in absence and presence of ENPs and metal salts with about 80 % COD removed. COD concentration between the treatments indicates that the concentration of ENPs and metal salts spiked into the AS had no negative influence on the heterotrophic microbial groups degrading organic matter. This finding is consistent with the study of Liang et al., (2010) in which no adverse effect was observed on the organic matter removed by heterotrophic bacteria by 0.75 mg $l^{-1}$ of $Ag^0$ in activated sludge after 12 h shock load.

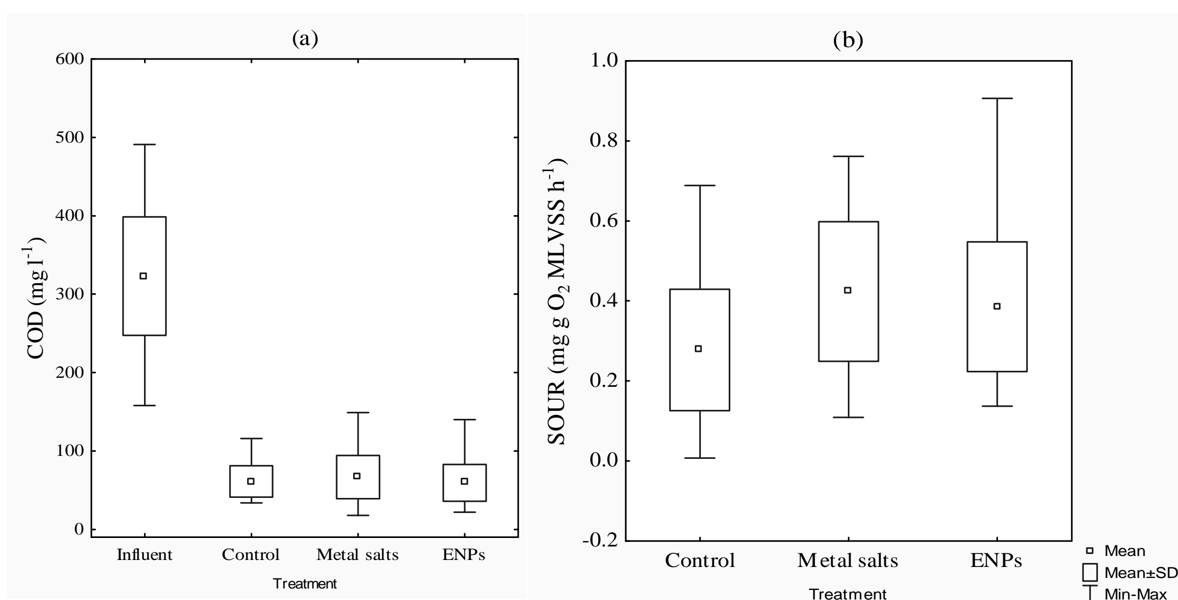



Figure 3.6 **(a)** Effect of mixed ENPs and metal salts COD removal and specific oxygen uptake of the activated sludge bacteria. The pilot plants performed in the same way with about 80 % efficiency in degrading organic matter over 315 days. **(b)** Influence of treatment on the specific oxygen uptake rate of AS microbial community. SOUR values are mean of triplicate determinations.

### 3.3.7 Effect of metals and ENPs on specific oxygen uptake rate (SOUR) of activated sludge bacteria (ASB)

The long-term effect of low-level ENP and metal exposure respectively on ASB assess by determining SOUR was measured every day for the first 30 days and subsequently twice a week over 200 days (Figure 3.6b). The mean SOUR of the ASB in the metal and ENP spiked AS was 1.5 times higher than in the control AS suggesting that ENP and

metal salts enhanced oxygen uptake of the microbial community. The increased oxygen uptake rate in the metal salts spiked AS can enhance metabolic process which contributed to the frequent AS bulking and foaming observed during the treatment. Thus, the respiration rate of AS microbial community indirectly indicates the ability of the organisms to degrade carbonaceous organic matter and build up biomass. Also, the unstable and different respiration rate reflects the resilient physiology of AS biomass or a shift in microbial community related to the ratio between *k* (slow growth rate) and *r* (fast growing) selection strategy in microbial groups due to available nutrients (Koronelli and Nesterova, 1990; Lemmer, 1986). Although it is not known at the moment how ENPs or metal salts interacted with wastewater components to stimulate SOUR, it is suspected that the organisms responded to the physiological stress by increased oxygen uptake. Overall, a mean difference of 1.8 times higher SOUR in ENP spiked AS and 2 times higher SOUR in metal salt spiked AS than the control was observed. Mean SOUR in metal salt spiked AS was 1.2 times higher than in the ENP spiked AS. However, mean difference in SOUR of the ASB in the control AS was approximately 2 and 3 times higher than in ENP and metal spiked samples respectively between day 69 and 102. This result is attributed to the varying wastewater physicochemistry which can either enhance or mitigate ENP and metal salt contact and interaction with the mixed population of the ASB.

### 3.3.8 Effect on activated sludge floc size, sludge volume index, MLSS and effluent suspended solids

Extracellular polymeric substances produced by microorganism influences the floc size formed by the biomass. Small or pin point floc reduces AS process efficiency at the point of clarification whereas large floc enhances efficient removal of biosolids. There was a decrease in mean floc size distribution in the ENP spiked AS compared with metal salts spiked AS and unspiked control (Table 3.2). At 50 % distribution, about 1.4 times reduction in the floc size of ENPs spiked AS compared with the control indicates significant ($p < 0.05$) effect on the biomass and the trend in floc size distribution was ENPs < metal salts < Control. Furthermore, the results demonstrate the heterogeneous nature of the particles in the three reactors. The small flocs, however, formed in ENPs spiked reactor compared with the control had minimal effect on the sludge volume index (SVI) which determines the ratio between liquid and solids (Figure 3.7a). Usually the settling of biosolids in activated sludge characterised by the SVI value between 50

and 150 mL $g^{-1}$ indicates proper functioning of the AS process in removing organic solids and prevent them from being released into receiving surface water.

Table 3.2 Particle size distribution of biomass flocs in AS treated with ENPs and metal salts for 315 days.

| Treatment | Mean particle size distribution (μm) | | | | | |
|---|---|---|---|---|---|---|
| | d(0.1) | d(0.2) | d(0.5) | d(0.9) | D[3,2] surface weighted mean | D[4,2] volume weighted mean |
| Control | 115 | 274 | 771 | 1472 | 249 | 787 |
| Metal salts | 133 | 269 | 696 | 1430 | 268 | 744 |
| ENPs | 104 | 193 | 568 | 1225 | 209 | 620 |

SVI value of 85 ± 27 mL $g^{-1}$ (control), 152 ± 68 mL $g^{-1}$ (metal salt spiked AS) and 100 ± 28 mL $g^{-1}$(ENPs spiked AS) demonstrates that settling characteristics in metal spiked AS was close to the optimal range. It is plausibly that the ENPs sorbed to the biosolids increased the specific density of the particles and thus enhanced their settling rate. The SVI was only an order of magnitude higher in ENPs spiked AS and about 2 orders of magnitude in metal salts spiked AS than the control, but the difference was significant ($p < 0.05$) indicating the treatment effect on the AS settling process. Suspended solids concentration in the AS is illustrated in Figure 3.7b. The effluent suspended solids in ENP spiked AS was 1.1 times higher than in the control and 1.5 times higher in the metal salt spiked AS, nevertheless, the difference was not significant indicating that ENPs at the spiked concentration barely influenced clarification process during the treatment.

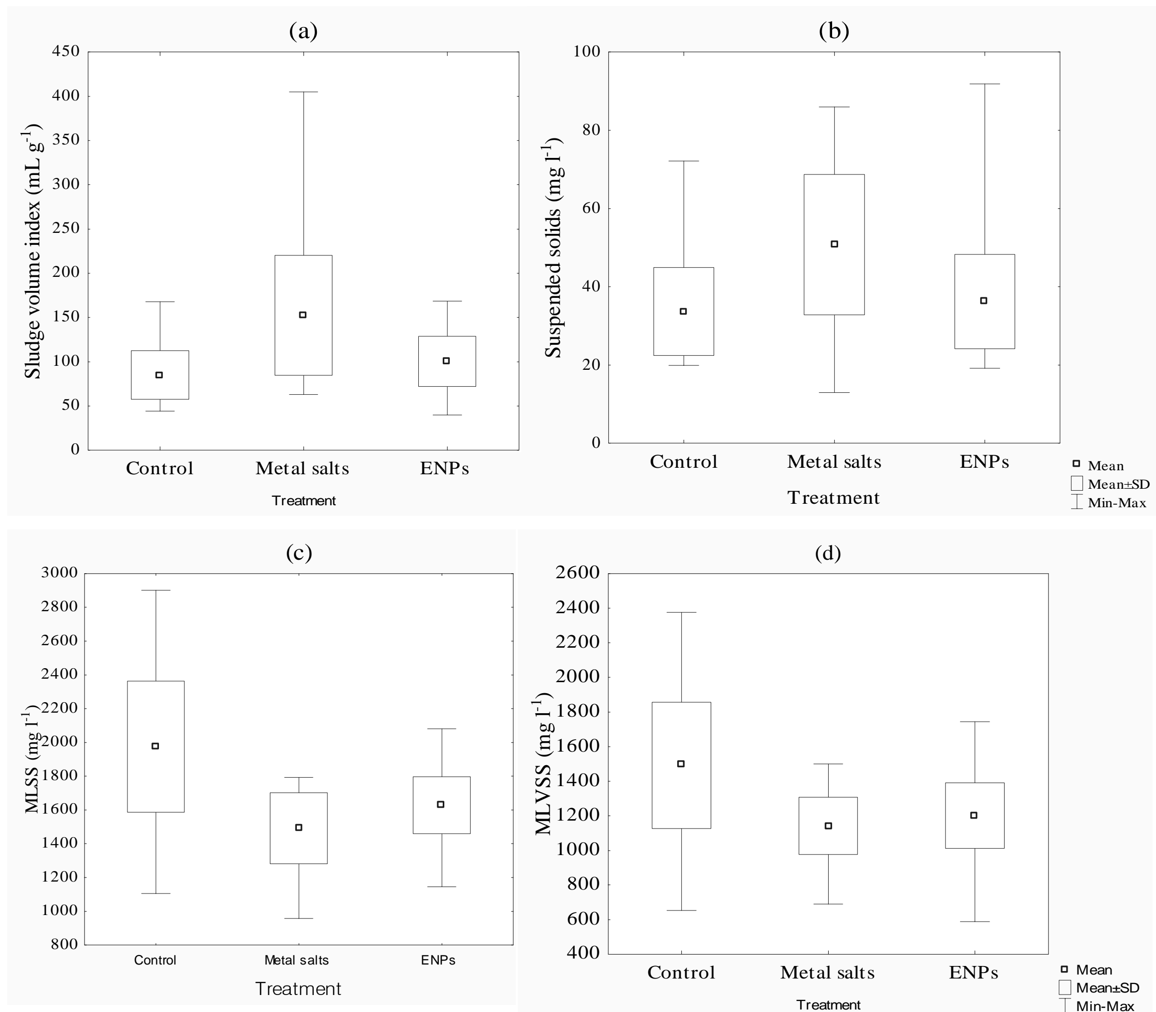



Figure 3.7 Effect of ENP and metal salt ions on the biosolids in pilot plant AS and effluent over 315 days of treatment

The MLSS and MLVSS (Figures 3.7c - d) concentration was 1.3 times lower in the metal salts spiked AS and 1.2 times in ENP spiked AS than the control which was significant at $p < 0.05$. The results suggest that there was more active biomass responsible for degrading organic matter in the control AS than the ENPs and metal salt spiked AS. The low MLSS and MLVSS in the metal salt and ENP spiked AS compared with the control AS were expected considering that ENPs and metal salt have antimicrobial properties which can reduce the synthesise of extracellular polymeric substances and floc formation (Liu et al., 2007).

### 3.3.9 Effect of mixed ENPs and metal salts on cultivable heterotrophs and coliforms

Abundance of *E. coli*, coliforms and heterotrophs decreased by an order of magnitude in the ENPs and metal salts spiked AS compared to the control (Figure 3.8). The

difference was significant ($p < 0.05$) and expected because of the established antimicrobial properties of both ENPs and metal salts which is desirable to kill pathogens in effluents before discharge. The microbial abundance, however, barely changed overtime and similar difference in abundance was observed throughout the experiment. Overall, the bacterial abundance trend was in the order: control > metal salt > ENPs (Figure 3.8).

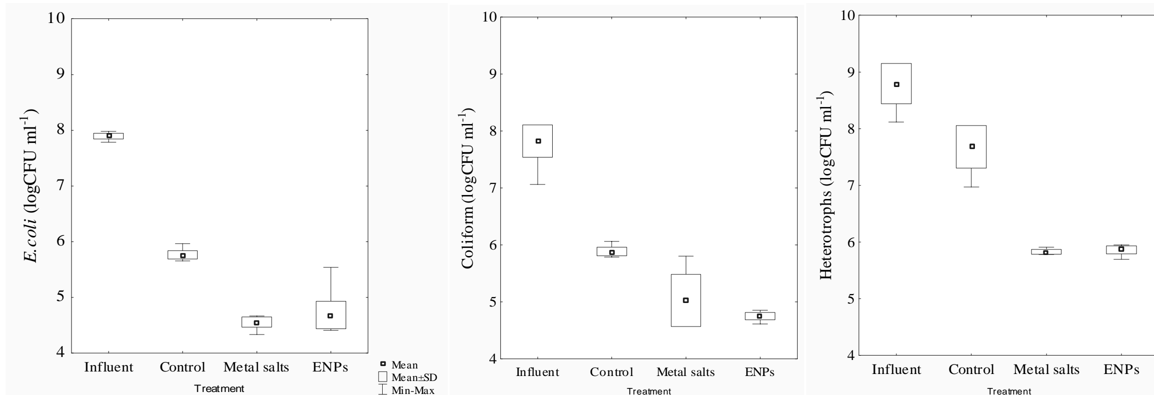



Figure 3.8 Box Whiskers plot of ENPs and metal salts effect on culturable microbial groups in activated sludge over 180 days of treatment

A survival rate of approximately 80 % of all cultivable groups investigated was observed. The trend in percentage survival of microbial groups (Figure 3.9) was in the order: heterotrophs < coliforms < *E.coli* for ENPs spiked AS and heterotrophs < *E.coli* < coliforms for metal salts spiked AS. Apart from the continuous flow design of the reactors, it is reasonable to infer from the result that *E. coli* persisted at low level throughout the treatment period probably because of pre-exposure to ENPs in personal care products and pharmaceuticals.

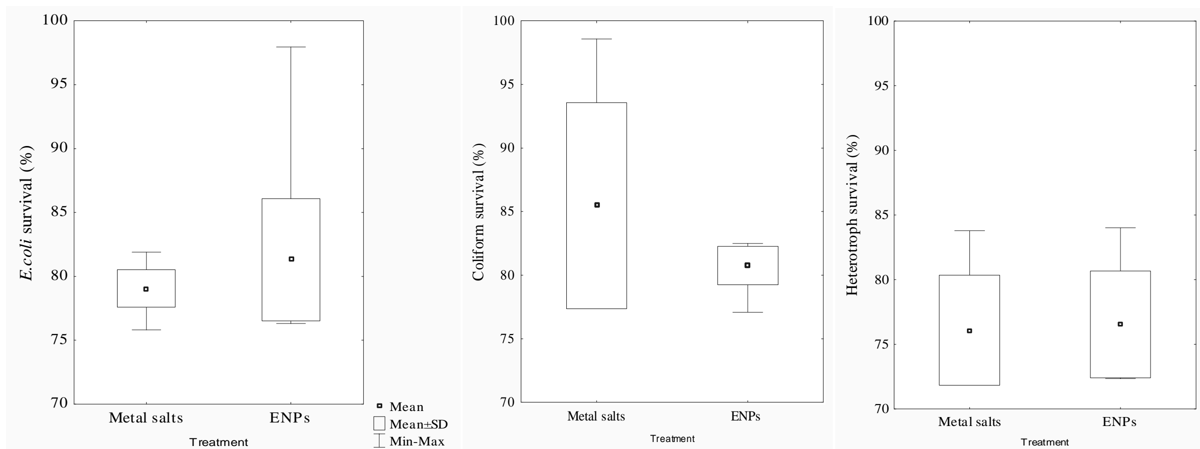



Figure 3.9 Box Whiskers plot of percentage survival of culturable microbial groups in activated sludge spiked with ENPs and metal salts over 180 days of treatment

**3.3.10 ENPs and /or metal salt ions influence on bacterial growth kinetics**

Temporal delay in microbial growth was evident in all the samples as a result of the presence of ENP or metal salts in the activated sludge compared with the control (Figure 3.10). Growth inhibition ranged between 2 - 85 % in ENP and 12 – 88 % in metal salt spiked AS. A mean of 57 % inhibition in metal salts and 58 % in the ENP spiked AS indicates that the treatment had similar effect on the microbial growth dynamics in the AS.

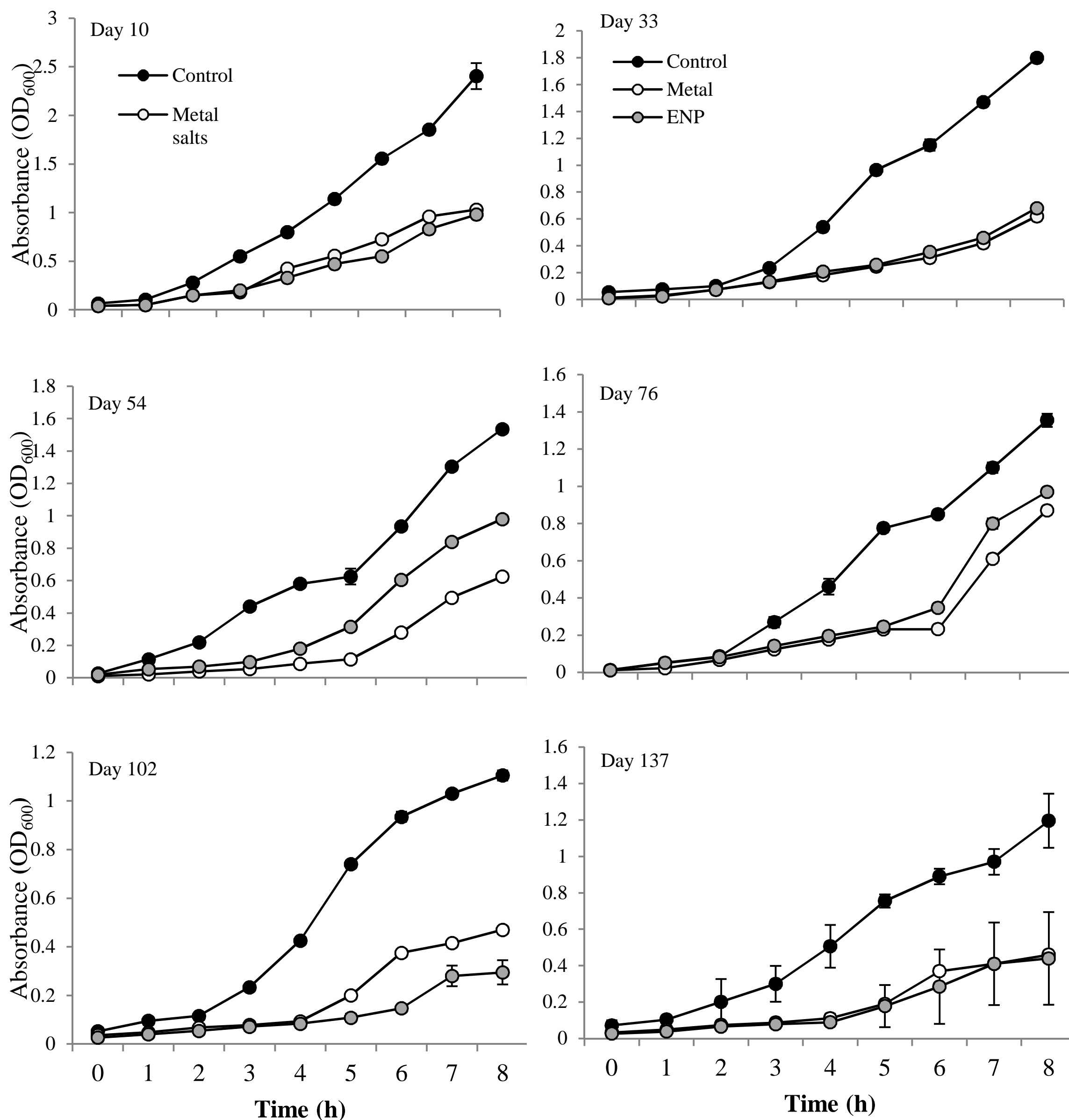



Figure 3.10. Effect of ENPs and metal salts on AS microbial community growth kinetics after 8 hours. Error bars represent the standard deviation of duplicate determinations.

### 3.3.11 Effect of mixed ENPs and metal salts on microbial cell wall structure

The structural integrity of the microbial cell wall and membrane to regulate transport and maintain osmotic balance are essential to keep the cell in a viable state. A disruption of these key functions as a result of cell wall damage is injurious to the cell and plausibly results in loss of viability and death for organisms without efficient repair mechanism. Scanning electron microscope (SEM) was used to observe and assess the effect of ENPs and metal salts on AS microbial structure. SEM images of AS biomass

exposed to mixed ENPs for 60 days evidenced visible and varying damage to some microbial cells (Figure 3.11). Mixed ENPs were adsorbed to some bacterial cell wall which disrupted cell wall/membrane integrity by forming pits, whereas some bacterial cells had no ENPs attached although the edges of the cell wall were distorted (Figure 3.11 panel k, l, m) This suggests that the rate at which the mixed ENPs were repulsed or attracted to the cells differed and may be as a result of charges on the bacterial cell wall influenced by wastewater components (Sondi and Salopek-Sondi, 2004). The observed pattern of how ENPs interact with cells support the notion that charges on Gram negative and Gram positive cell wall can be one of the underlying reasons for ENPs sorption to specific cells. The mixed ENP, however, exerts toxic effect on single, unsheathed and sheathed bacterial cell plausibly through the release of ions or by synergistic reaction with other micro-pollutants in the wastewater (Figure 3.11 panel i, l, p). Metal salts caused the congealing, shrinking and distortion of microbial cell wall, sheath, cytoplasmic material compared with formation of pits caused by ENPs (Figure 3.11 panels f, h vs panels i, j) although at the moment it is difficult to determine which ENPs or metal salts ions caused the damage and the specific microbial cell involved.

The formation of pits in the microbial cell wall can be enhanced by anions and cations in the AS, especially divalent ions such as $Ca^{2+}$ and $Mg^{2+}$ as the conductivity in the control, metal and ENPs was ≥ 740 µS (Saleh et al., 2008; Chen et al., 2008). Images in Figure 3.11 (i, j, k, l) indicates that the presence of ions influenced ENPs reactivity, and contact through the formation ion bridges was an important mechanism of ENPs toxic effect on the bacterial cells. Besides, pH of the wastewater influenced the negative ENP effect exerted on the wall/membranes. For instance, $TiO_2$ formation of reactive oxygen species (ROS) is enhanced at basic pH values (Kormann et al., 1991). In this study, influent pH of 8.06 ± 0.09 and HRT of 8 hours likely contributed to ROS formation before the lowering of pH in control effluent (6.5 ± 0.23), metal salt spiked AS effluent (6.69 ± 0.38) and ENPs spiked AS effluent (6.4 ± 0.27). At pH value of 5.68, complete oxidative dissolution of $Ag^0$ can occur under oxic conditions (Liu et al., 2010). It remains unclear what type of reaction the mixed ENPs consisting of $Ag^0$, ZnO and $TiO_2$ may have undergone or their transformed products. Therefore, the ENPs mechanism of action to exert the toxic effect most probably was the generation of $Ag^+$ and corresponding ions from ZnO and $TiO_2$.

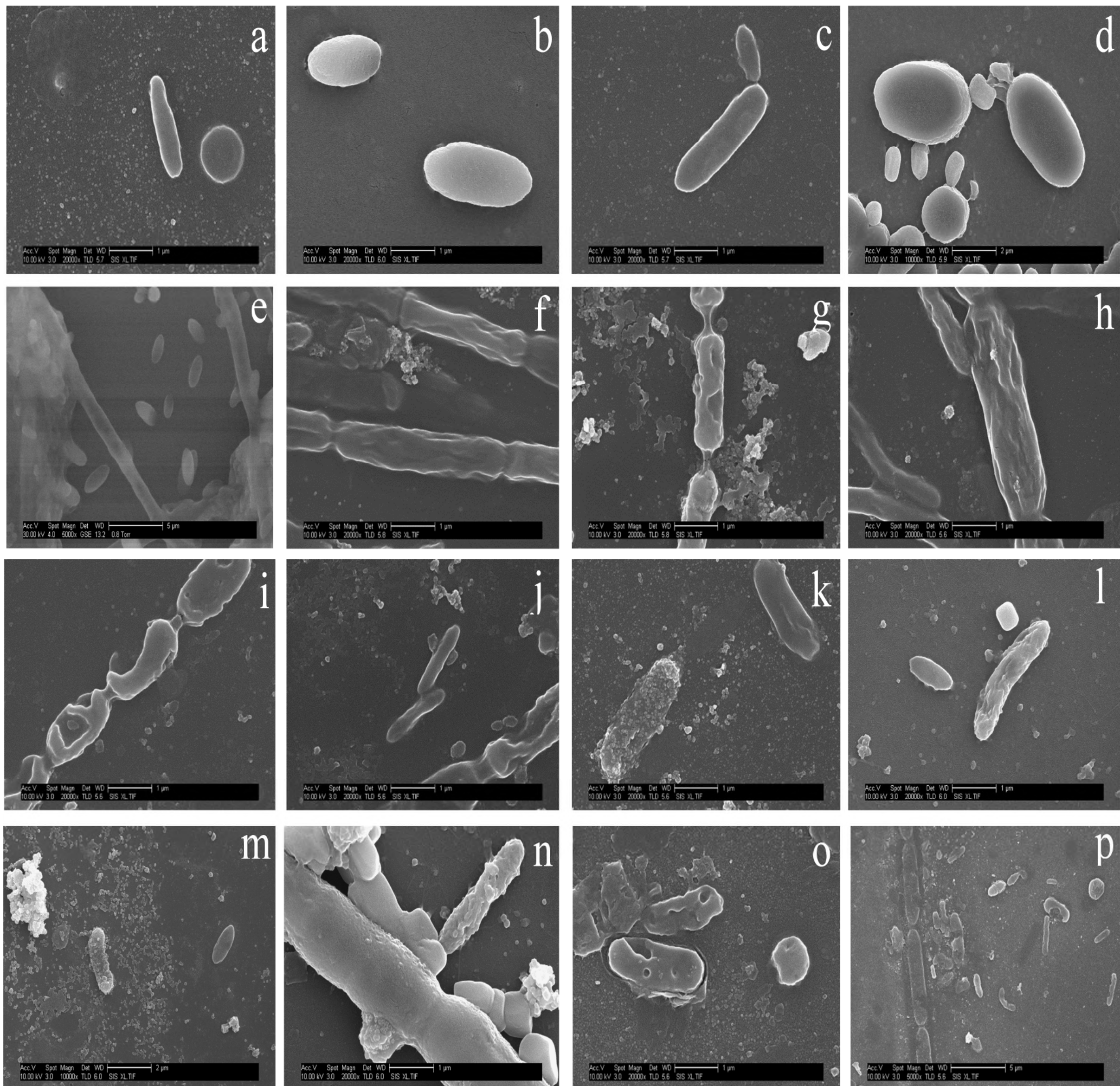



Figure 3.11 SEM images of ENPs and metal salts effect on AS microbial cells in relation to control. Samples contained diverse cell with varying effect on microbial cell structure after 60 days exposure. (**a,b,c,d,e**) Intact microbial cell structure in control **(f, g)** Distortion and shrinkage of microbial cell structure by metal salts. **(g)** Perforations of cell structure by metals salts. **(i, j)** cell wall perforation by ENPs. **(k)** selective adsorption of ENPs to microbial cells (**l**) selective cell structure degenerated by ENPs ions or ROS (**m**) selective adsorption to microbial cell and aggregation of ENPs (**n**) effect of ENPs on sheathed and unsheathed microbial cell with NOM stabilised ENPs, (**o**) cell wall perforations by ENPs (**p**) dissolved/hydrolysed microbial cell wall and sheath by ENPs.

The formation of ion and ROS (Morones et al., 2005; Sayes et al., 2005; Maness et al., 1999) are the most probable mechanism for ENPs toxic effect. Perforations on the microbial cell wall/membrane indicates damage to lipids in the membrane caused by reactive oxygen species (ROS) resulting in lipid peroxidation. Other possible

implications of ROS production include DNA damage as a result of nucleotide oxidation and strand breakage, proteins and amino acids oxidation (Cabiscol et al., 2000). Although the formation of ROS or ions by ENPs was not investigated in this study, perforated cell structure and dissolved cell wall/membrane as evidenced by SEM (Figure 3.11 i-p) raises the possibility that mixed ENPs in wastewater can produce ROS (singlet oxygen, $^1O_2$ and superoxide, $O_2^-$) and hydroxyl radicals ($OH^-$) to affect microbial membranes in AS. This assertion is consistent with the findings of Brunet et al., (2009) in which aggregates of $TiO_2$ generated hydroxyl radicals in ultrapure water and superoxides in minimal Davis microbial growth medium. Furthermore, the result implies that delayed toxic effect and cell damage was as a result of (i) additive, synergistic or multiplicative effect of ENPs with other micro-pollutants in wastewater (ii) production of ROS and hydroxyl free radicals, although it is not clear at what threshold concentration the effect became injurious to the cells.

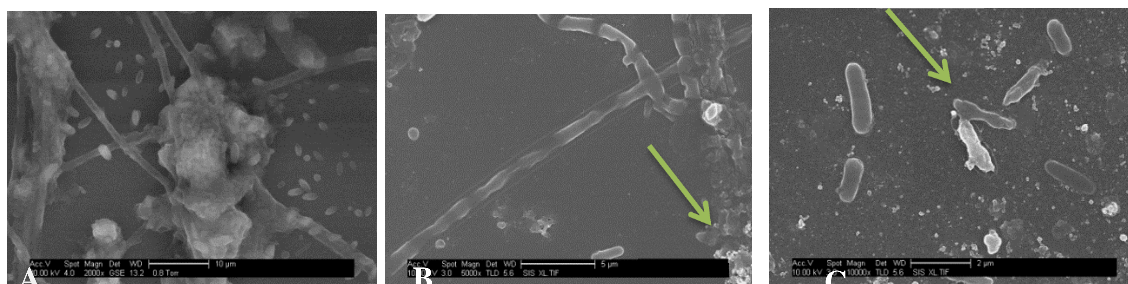



Figure 3.12 SEM image of AS showing microbial cells embedded in extracellular polymeric substance (EPS) and freely suspended cells. (A) Cells embedded in the floc core are shielded from direct contact with ENPs (B) Peripheral cell in EPS exposed to high concentration of ENPs due to sorption (C) Freely suspended bacterial cells exposed to bioavailable ENPs.

The bacterial cells able to tolerate the ENPs toxic effect were presumably protected by either exopolysaccharide (EPS) sheath which restricts contact with cell wall or charges on the cell wall that caused electrostatic repulsion (Figure 5.10 l, n). The images also reveal that the ENPs were insoluble and aggregated into flocs thus reducing availability and restricting contact with microbial cells. Since AS biomass are organised into flocs or freely suspended in the bulk liquid or both, it is suspected that microbial sensitivity to the mixed ENPs toxic effect was influenced by the spatial and temporal location of the cells (Sun et al., 2013). This is significant because peripheral cells located in the outer biomass floc adsorbed ENPs and in turn can act as a barrier against ENPs contact with

cells in the floc core (Figure 3.12). In addition, SEM images revealed sorption of mixed ENPs to biomass and disintegrated bacterial cell wall sheath. It is probable that ENPs sorbed to EPS released and delivered ions at high concentration to cells localized in the EPS matrix to disrupt the cell walls. Although, the synergistic or additive effect of ions generated by low concentration of mixed ENPs and other contaminants increased the metabolic rate of ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB) to remove $NH_3^-$ and $NO_2^-$ from the AS, damage to microbial cell wall/membrane and cytoplasmic organelles were observed. The most plausible explanation of this phenomenon is the Trojan-horse effect (Lubick et al., 2008; Park et al., 2010) in which the mixed ENPs in part enhanced microbial activity, and in part caused irreversible damage to the cell. Overall, the result suggests that the low concentration of ENPs and metal salts spikes can increase the removal of nitrogenous matter in a microbial mediated process and also inhibit specific groups of cells in the AS.

### 3.3.12 Changes in AS microbial community structure based on lipids and biomass analysis

13 lipid biomarker of AS microbial community were identified to represent bacterial PLFA (Figure 3.13). Ten biomarkers occurred at less than 100 μg $g^{-1}$ concentration with the exception of 16:0, 18:1ω7t and 16:1ω7c. The results indicate the dominance of *Proteobacteria*, *Bacteriodetes*, *Firmicutes* and *Actinobacteria* (Table 3.3). This pattern of dominance in the bacterial groups was maintained throughout the experiment indicating there was no rapid change in the bacterial response to the altered environmental conditions. Biomarker 16:0 is known to occur in more than one phylogenetic bacterial group such as *Proteobacteria* and *Actinobacteria* (Quezada et al., 2006; Lai and Shao, 2008; Cloete et al., 2003) which accounted for the high concentration and abundance in the AS. The dominance of Gram negative bacterial biomarker (18:1ω7t) over Gram positive indicates the enteric source of the bacterial community and corroborates the findings of Cloete et al., (2003).

Aerobic bacterial biomarker represented by 16:1ω7c biomarker occurred in higher concentration than other biomarkers and was expected because of the treatment conditions. However, the difference in 16:1ω7c biomarker concentration between the 3 AS treatments was not significant ($P < 0.01$). Usually the ratio of specific PLFAs such as cyclo/monounsaturated precursor (cy17:0/16:1ω7c and cy19:0/18:1ω7c) are used to indicate post synthetic modification of cell wall lipids by microorganisms under

starvation or physiological stress (Frostegard et al., 2010; Chang and Cronan, 1999). The result suggests there was no significant influence of metals or ENPs on the total bacterial biomass of the activated sludge after 150 days. However, the shift in proportions of the cy17:0 and 16:1ω7c markers (Figure 3.13) indicates either an altered bacterial cell wall composition or a shift in the species composition of the microbial community as a result of the treatment, although at the moment it is difficult to delineate which of the two outcomes occurred. Again, the shift in the PLFAs marker suggests that the stress on the bacterial community was more pronounced in the metal salt spiked AS than in the ENP spiked AS. This interpretation should be taken with caution because of the complex nature of wastewater and AS.

Recent studies (Frostegard et al., 2010; Fisher et al., 2010; Barcenas-Moreno and Baath, 2009) reported that an increase in the ratio of cy17/16:1ω7c can potentially be misinterpreted as stress condition instead of re-growing of the bacterial community. Comparison of the PLFA fingerprint between treatments over time (Figure 3.14) indicates a clear temporal shift in bacterial community structure. For instance, similarity in the three AS plants was 90 % at day 30 and thereafter, a shift in the bacterial community structure observed at day 40, 50 and 60 indicated 80 % similarity between the control and ENP and metal salt spiked AS. The result implies that the metal salt and ENP had effect on the AS bacterial community structure compared with the control. Again, 90 % similarity in the bacterial community was observed in the three plants at day 150 and suggests that the potential effect of ENP and metal salts was variable in the AS.

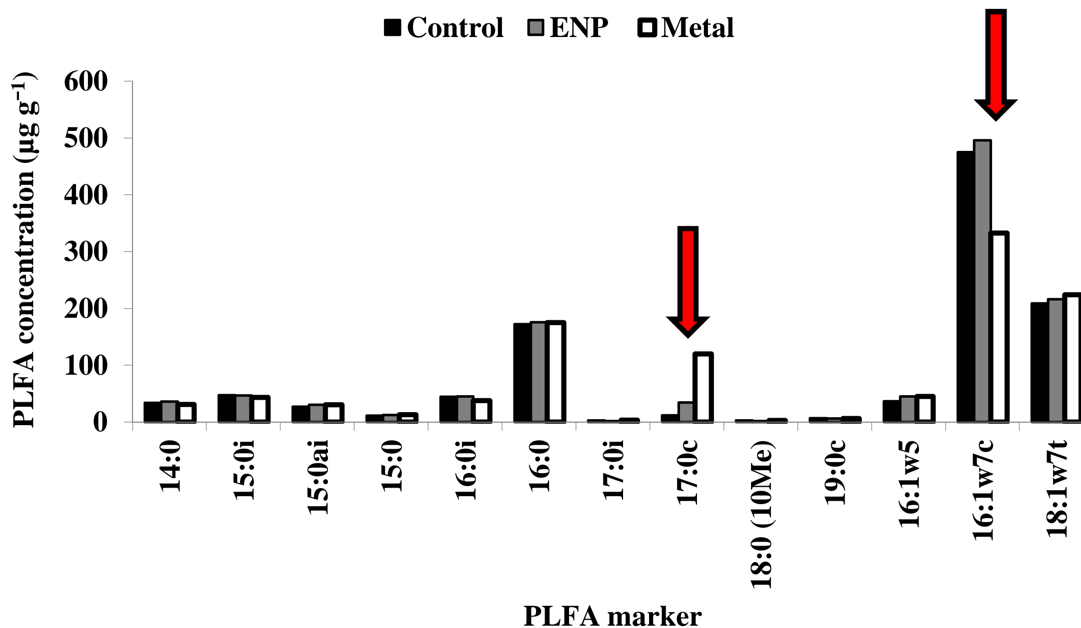



Figure 3.13 PLFA concentrations in AS microbial biomass exposed to chronic low concentrations of ENPs and metal salts

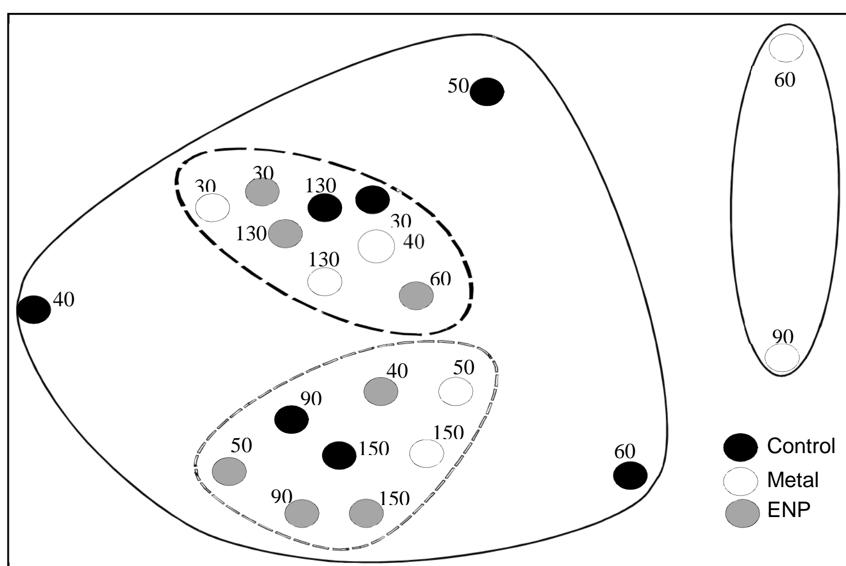



Figure 3.14 MDS plot of time related FAMEs distribution in AS microbial community. Similarity denoted by solid line (80%) and dashed lines (90%).

Table 3.3 Phospholipid fatty acid markers of selected microbial groups in the sludge

| **Lipid biomarker** | **Phylogenetic affiliation** | **Type** | **Related microbial group/genus/species** | **References** |
|---|---|---|---|---|
| Gram positive bacteria | | | | |
| 14:0, i15:0, 16:0, i16:0, i17:0, 16:1ω5, 18:1ω7, 19:0cy, 16:1ω7 | *Firmicutes, Proteobacteria Bacteriodetes* | Facultative anaerobe | *Streptococcus, Pediococcus, Enterococcus, Staphylococcus, Bacillus, Pectinatus* | Quezada et al., 2006; Oravecz et al., 2004; Cloete et al., 2003 |
| i15:0, i16:0, i17:0, 16:1ω5, 18:1ω7t | *Proteobacteria Actinobacteria* | Aerobe, Anaerobe, Aerotolerant | Sulfate-reducing, *Lactobacillus, Desulfobacter, Desulfovibrio desulfuricans, Streptomyces, Bacillus licheniformis,* | Lai and Shao, 2008; Quezada et al., 2006; Oravecz et al., 2004, Cloete et al., 2003; Richie et al., 2000 |
| i16:0, 10Me18:0 | *Actinobacteria* | Aerobe | *Corynebacterium, Gordonia, Rhodococcus, Norcardia, Arthrobacter, Tsukamurella, Micrococcus, Mycobacterium* | Lai and Shao, 2008; Quezada et al., 2006; Oravecz et al., 2004; Richie et al., 2000; Cloete et al., 2003 |
| Gram negative bacteria | | | | |
| 14:0, 17:0cy, 19:0cy | *Proteobacteria* | Facultative anaerobe | *Enterobacter, Vibrio* | Oravecz et al., 2004 |
| 16:0 | *Proteobacteria* | Aerobe | *Pseudomonas* | Haack et al., 1988 |
| ai15:0, 16:1ω5, 17:0cy, 19:0cy | *Proteobacteria* | Anaerobe | *Xanthomonas, Pseudomonas, Arthrobacter* | Oravecz et al., 2004; Haack et al., 1988 |
| 16:1ω5 | *Bacteriodetes, Firmicutes* | Aerobe | *Cytophaga, Flavobacterium* | Frostegard et al., 1993; Kelly et al., 1999; Oravecz et al., 2004 |
| 16:1ω5, 16:0 | Fungi/Bacteria | | *Arbuscular mycorrhiza* | Quezada et al., 2006; Oravecz et al., 2004; Richie et al., 2000; Olsson et al., 1995,Olsson, 1999; Kelly et al., 1999 |

### 3.3.13 AS bacterial community structure and diversity based on 454-pyrosequencing

To further understand the changes in AS bacterial community structure and diversity as a result of exposure to chronic low doses of ENPs and metal salts, 454 pyrosequencing was carried out. Bacterial distribution and diversity at the phylum level for each treatment at different times are summarised in Figure 3.15. A total of 17,022 sequences representing 25 classifiable phyla were obtained. 7468, 4130 and 5424 sequences were obtained for the control, metal salts and ENPs spiked AS respectively. The average classifiable sequence read length was 1067, 590 and 775 with a range of 98 - 2105 for control, 106 - 884 for metal salts spiked and 200 - 2146 for the ENP spiked AS. Most predominant phylotypes were members of the *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, unclassified Bacteria and *Actinobacteria* (Figure 3.15). The selected representative of the different phylogenetic affiliations of the bacterial species is shown in Table 3.4.

The dominant bacterial phyla in the ENPs and metal salts spikes AS responded by exhibiting a clear distribution pattern with temporal changes in diversity and relative abundance of the microbial community (Figure 3.16). The change in bacterial community response is indicative of specie-sensitive and specie-tolerant composition of the AS to ENPs effect. The relative abundance of *Bacteriodetes* in the control appeared stable throughout the experiment compared the ENP and metal salt spiked AS. In contrast, the *Firmicutes* decreased overtime in the three AS. Also, the relative abundance of unclassified bacteria varied largely probably because most microorganisms in environmental samples such as activated sludge are yet to be fully identified (Kragelund et al., 2008). Further to this, the variability among the samples exposed to ENPs and metal salt was greater than from the control (Figure 3.16). While the MDS plot showed a temporal variation, the overall the microbial community had 80 % similarity across the samples. Also, the trend indicates a temporal shift in higher taxa of bacterial community as a result of the change in AS composition with growth enhancing effect on members of the *Proteobacteria*.

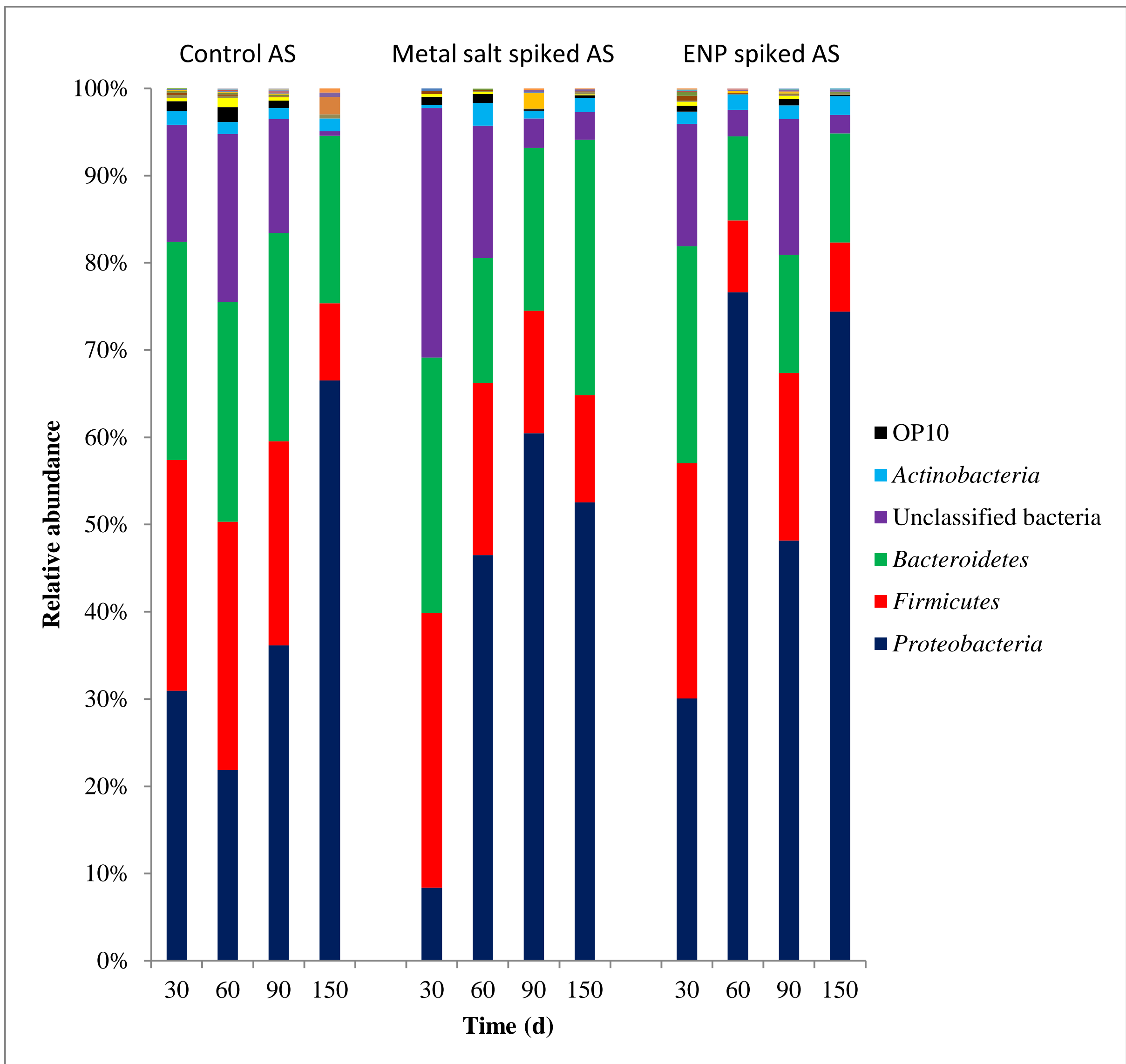



Figure 3.15 Dominant phylum of the activated sludge bacterial community based on based on 454 pyrosequencing of AS spiked with metals and ENPs compared to control. Phylogenetic group above 5 % abundance in the AS are shown with the dominance of *Proteobacteria*.

Table 3.4 Summary of the most dominant phylogenetic groups of bacteria in activated sludge based on 454 Pyrosequencing

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 112 | Proteobacteria | Betaproteobacteria/Rhodocyclales/Rhodocyclaceae/*Quatrionicoccus* | *Qautrionicoccus australiensis* strain Ben 117 | 99 | NR029035 |
| 306 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/*Comamonas* | *Comamonas* sp LYS1-1 | 97 | KC211010 |
| 359 | | Alphaproteobacteria | *Sphingopyxis* sp MC1 | 88 | JN940802 |
| 395 | | Betaproteobacteria/Burkholderiales/Incertae sedis 5/*Methylibium* | *Methylibium* sp BAC116 | 99 | EU130971 |
| 485 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Rhodobacter* | *Rhodobacter* sp TUT3732 | 93 | AB251408 |
| 502 | | Betaproteobacteria/Burkholderiales | *Acidovorax* sp BSB421 | 94 | Y18617 |
| 542 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/*Rhodoferax* | *Rhodoferax* sp B3 | 98 | DQ268771 |
| 564 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Rhodobaca* | *Rhodobacter* sp TCRI 3 | 94 | AB017796 |
| 649 | | Alphaproteobacteria/Rhizobiales | *Oligotropha carboxidovorans* | 90 | AB099659 |
| 727 | | Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/*Sphingopyxis* | *Sphingopyxis witflariensis* strain W-50 | 99 | NR028010 |
| 810 | | Deltaproteobacteria | *Dongia mobilis* strain LM22 | 83 | FJ455532 |
| 983 | | Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/*Novosphingobium* | *Novosphingobium hassiacum* strain W-51 | 99 | NR028962 |
| 1522 | | Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/Xylella | *Pseudoxanthomonas* sp E16 | 93 | AY488509 |
| 1579 | | Alphaproteobacteria/Rhizobiales/Hyphomicrobiaceae/*Hyphomicrobium* | *Hyphomicrobium* sp M3 | 97 | AF098790 |
| 1696 | | Gammaproteobacteria/Pseudomonadales/Moraxellaceae/*Acinetobacter* | *Acinetobacter iwoffii* strain JUN-5 | 97 | KF228924 |
| 1706 | | Gammaproteobacteria/Pseudomonadales/Pseudomonadaceae/*Flavimonas* | *Pseudomonas* sp HY-14 | 98 | EU620679.2 |
| 1773 | | Betaproteobacteria/Burkholderiales/Comamonadaceae | *Simplicispira metamorpha* strain DSM 1837 | 94 | NR044941 |
| 1997 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/*Curvibacter* | *Curvibacter* sp W2 09-301r | 95 | JX458451 |
| 2143 | | Betaproteobacteria | *Rhodoferax ferrireducens* | 90 | AF435948 |
| 2271 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/Giesbergeria | *Acidovorax ebreus* TPSY strain TPSY | 95 | NR074591 |
| 2410 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/*Acidovorax* | *Acidovorax defluvii* strain BSB411 | 93 | NR026506 |
| 2490 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Paracoccus* | *Paracoccus* sp BBTR62 | 98 | DQ337586 |
| 2592 | | Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/*Sphingosinicella* | *Spingosinicella microcystinivorans* | 95 | AB219940 |
| 2608 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Pseudorhodobacter* | *Rhodobacter* sp TUT3734 | 96 | AB251410 |
| 2739 | | Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/*Dokdonella* | *Dokdonella* sp LM 2-5 | 93 | FJ455531 |

Table 3.4 continued.

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 2823 | Proteobacteria | Alphaproteobacteria/Rhizobiales/Bradyrhizobiaceae/*Bosea* | *Bosea thiooxidans* strain E14 | 98 | AY488508 |
| 3277 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae | *Paracoccus aminophilus* JCM7686 | 95 | CP006650 |
| 3310 | | Betaproteobacteria/Burkholderiales/Incertae sedis 5/*Aquabacterium* | *Aquabacterium commune* strain B8 | 97 | NR024875 |
| 3597 | | Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae/*Thermomonas* | *Xanthomonas axonopodis* | 99 | AB101447 |
| 29 | Firmicutes | Clostridia/Clostridiales/Eubacteriaceae/*Eubacterium* | *Eubacterium* sp E-1 | 99 | AB470313 |
| 269 | | Bacilli/Lactobacillales/Lactobacillaceae/*Lactobacillus* | *Lactobaccillus vaginalis* strain DoxG3 | 97 | GQ422709 |
| 380 | | Clostridia/Clostridiales/Incertae Sedis XI/*Sedimentibacter* | *Sedimentibacter hongkongensis* strain K1 | 88 | AY571338 |
| 391 | | Clostridia | *Clostridium* sp strain Z6 | 83 | AY949859 |
| 475 | | Clostridia/Clostridiales/Syntrophomonadaceae | *Synthrophomonas curvata* strain GB8-1 | 89 | NR025752 |
| 530 | | Clostridia/Clostridiales/Ruminococcaceae | *Ruminococcus* sp 15975 | 84 | AJ308104 |
| 776 | | Clostridia/Clostridiales/Clostridiaceae/Clostridiaceae 4/*Caminicella* | *Proteocatello sphenisci* strain PPP2 | 94 | NR041885 |
| 844 | | Bacilli/Lactobacillales/Enterococcaceae/*Enterococcus* | *Enterococcus sp R-25205* | 94 | AM084029 |
| 845 | | Clostridia/Clostridiales | *Fecalibacterium prausnitzii* strain HTF-E | 82 | HQ457029 |
| 899 | | Bacilli/Bacillales/Bacillaceae/Bacillaceae 1/*Bacillus*/*Bacillus* h | *Bacillus* sp RCT10 | 90 | FJ755951 |
| 1147 | | Clostridia/Clostridiales/Incertae Sedis XI/Sedimentibacter | *Sedimentibacter* sp MO-SED | 90 | AB598275 |
| 1276 | | Clostridia/Clostridiales | *Clostridiales bacterium* JN18 A24 | 91 | DQ168655 |
| 1382 | | Clostridia/Clostridiales/Clostridiaceae/Clostridiaceae 1/*Clostridium* | *Clostridium sp* | 92 | X95274 |
| 1655 | | Clostridia/Clostridiales/Ruminococcaceae/*Ethanoligenens* | *Ethanoligens harbinense* strain CGMCC1152 | 90 | AY833426 |
| 1921 | | Clostridia/Clostridiales/Incertae Sedis XV/*Aminobacterium* | *Aminobacterium mobile* strain ILE-3 | 99 | NR024925 |
| 2477 | | Bacilli/Lactobacillales/Enterococcaceae/*Enterococcus* | *Enterococcus devriesei* strain LMG 13603 | 97 | DQ010644 |
| 2513 | | Clostridia/Clostridiales/Clostridiaceae/Clostridiaceae 1/*Clostridium* | *Clostridium thiosulforeducens* | 95 | AF317650 |
| 2976 | | Clostridia/Clostridiales/Ruminococcaceae | *Oscillibacter* sp G2 | 91 | HM626173 |
| 3227 | | Clostridia/Clostridiales/Peptostreptococcaceae/Peptostreptococcaceae Incertae Sedis | *Clostridium metallolevans* strain SN1 | 94 | EU887815 |
| 3305 | | Clostridia/Clostridiales/Lachnospiraceae | *Clostridium glycyrrhizinilyticum* | 92 | AB233029 |
| 3572 | | Clostridia/Clostridiales/Veillonellaceae/*Selenomonas* | *Selenomonas ruminantium* | 91 | AB198442 |

Table 3.4 continued.

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 43 | Bacteroidetes | Sphingobacteria/Sphingobacteriales/Flexibacteraceae/*Niastella* | *Niastella* sp Gsoil 221 | 88 | GQ339899 |
| 44 | | Sphingobacteria/Sphingobacteriales | *Myroides* sp XJ193 | 78 | GQ381279 |
| 62 | | Bacteroidales/Porphyromonadaceae/*Parabacteroides* | *Parabacteroides distasonis* ATCC 8503 | 89 | CP000140 |
| 362 | | Bacteroidales/Rikenellaceae/*Petrimonas* | *Proteiniphilum acetatigenes* strain TB 107 | 94 | NR043154 |
| 662 | | Bacteroidales | *Cytophaga sp* | 81 | X85210 |
| 1252 | | Bacteroidales | *Parabacteroides distasonis* strain JCM 5825 | 81 | EU136681 |
| 1344 | | Flavobacteria/Flavobacteriales/Flavobacteriaceae/*Flavobacterium* | *Flavobacterium columnare* strain E8 | 92 | AY488506 |
| 2155 | | Flavobacteria/Flavobacteriales/Flavobacteriaceae/*Flavobacterium* | *Flavobacterium swingsii* | 97 | AM934651 |
| 2259 | | Bacteroidales/Porphyromonadaceae/*Paludibacter* | *Paludibacter propionicigens* WB4 | 86 | CP002345 |
| 2464 | | Bacteroidales/Porphyromonadaceae/*Proteiniphilum* | *Proteiniphilum acetatigenes* strain TB 107 | 84 | NR043154 |
| 3214 | | Bacteroidales/Rikenellaceae/*Marinilabilia* | *Marinilabilia* sp AK2 | 87 | FN994992 |
| 3270 | | Bacteroidetes | *Bacteroides* sp strain Z4 | 90 | AY949860 |
| 3593 | | Bacteroidales/Porphyromonadaceae/*Parabacteroides* | *Parabacteroides johnsonii* DSM 18315 strain JCM 13406 | 86 | NR041464 |
| 281 | Actinobacteria | Actinobacteridae/Actinomycetales/Micrococcineae/Microbacteriaceae/*Leucobacter* | *Leucobacter komagatae* | 95 | DQ083486 |
| 565 | | Actinomycetales/Micrococcineae/Microbacteriaceae | *Microbacterium* sp RI 2 | 94 | AJ876685 |
| 1619 | | Actinomycetales/Propionibacterineae/Propionibacteriaceae/*Propionibacterium* | *Propionibacteriumsp* SV442 | 94 | AB264627 |
| 3130 | | Actinomycetales/Corynebacterineae/Mycobacteriaceae/*Mycobacterium* | *Mycobacterium aichiense* strain JS618 | 97 | AF498656 |
| 3625 | | Actinomycetales/Micrococcineae/Microbacteriaceae | *Microbacterium thalassium* | 98 | AM943052 |
| 2484 | Fusobacteria | Fusobacteriales/Fusobacteriaceae/*Fusobacterium* | *Fusobacterium* sp SRBBR5 | 99 | HM215007 |
| 883 | Chloroflexi | Chloroflexi/Anaerolineae | *Bacterium* JN18 A7 F* | 96 | DQ168648 |
| 883 | | Chloroflexi/Anaerolineae | *Levilinea saccharolytica* strain KIBI-1 | 87 | NR040972 |

sp = species

*Activated Sludge MDS Plot*

Transform: Log (X+1)
Resemblance: S17 Bray Curtis similarity

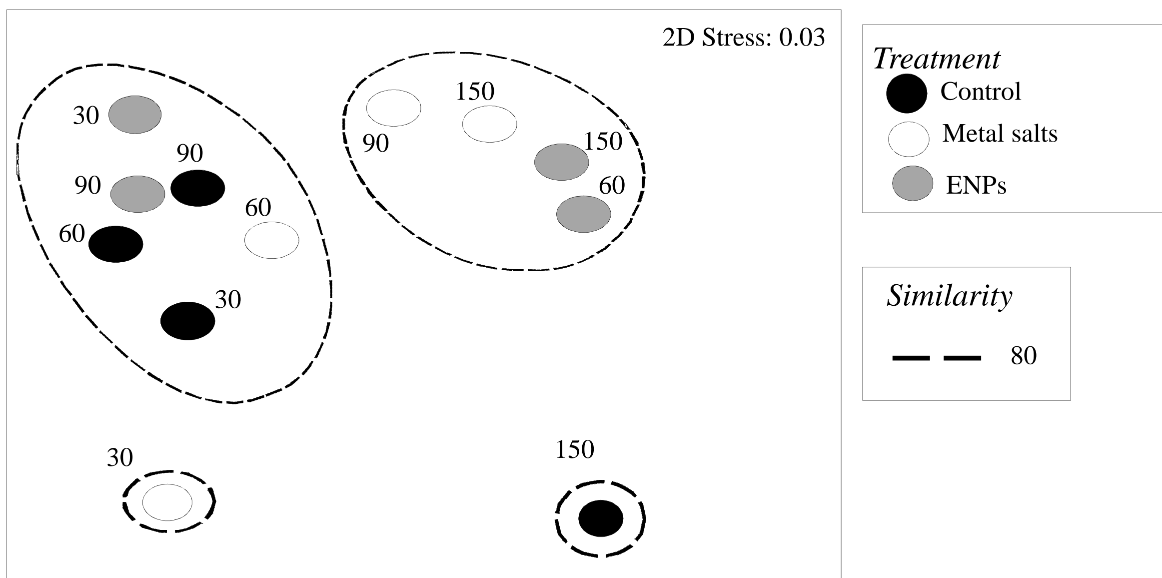


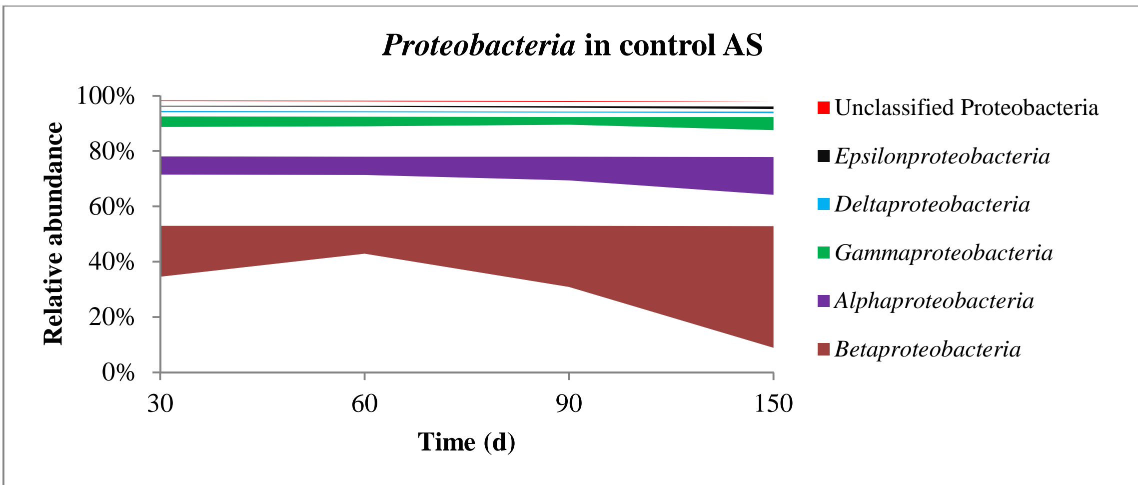

Figure 3.16 Non-metric 2 dimensional MDS of microbial community in AS by the Bray-Curtis similarity measure using log transformed data indicating 80% similarity level in relation to time.

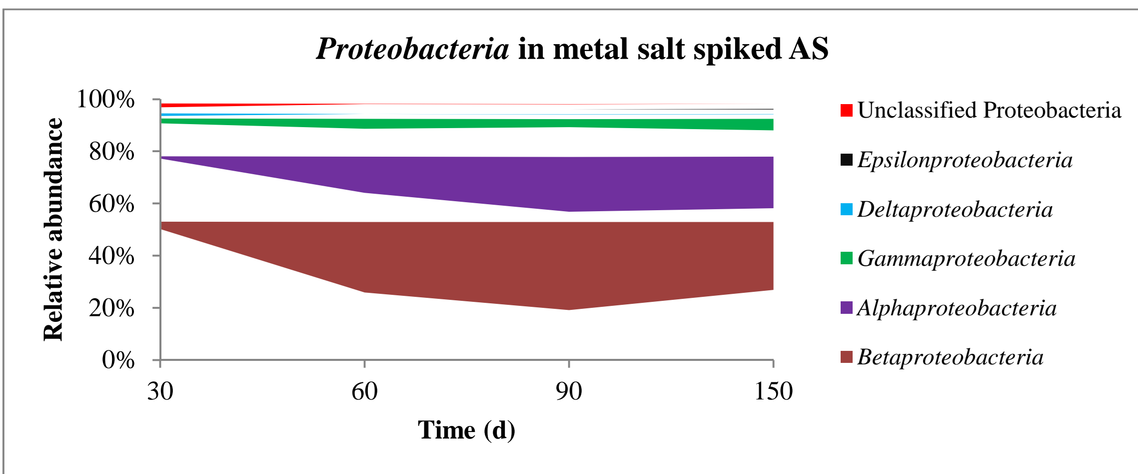
**3.3.14 Diversity and dynamics within the *Proteobacteria***

Members of the phylum *Proteobacteria* are usually Gram negative and include pathogenic and non-pathogenic species with ecological importance (Perez-Pantoja et al., 2012). The relative abundance of the dominating groups followed a similar pattern (Wilen et al., 2008; Klausen et al., 2004) regardless of the treatment (Figure 3.18). Members of the family *Comamonadaceae* (*Burkholderiales*) were identified as the most dominant and resilient bacterial species recovered in the AS. Members are strict aerobes, non-fermentative chemo-organotrophs, can accumulate polyhydroxybutyrate (Holt et al., 1994) in medium with deteriorating phosphate removal efficiency (Fang et al., 2002). Prominent genera of the Comamonadaceae recovered in the ENPs spiked AS were *Acidovorax*, *Rhodoferax*, *Comamonas*, *Curvibacter*, *Giesbergia*, *Hydrogenophaga*, *Ottowia*, and *Simplicispira* (Figure 3.18).

Compared with the control and metal salts spiked AS, *Acidovorax* was about 2 times higher in ENPs spiked AS suggesting that the ENP mixture had positive influence on metabolic activities and growth of the organism. The relative abundance and temporal increase of *Acidovorax* illustrates that the organism played a key role in the bulk

removal of contaminants in the reactors, possibly possesses a mechanism able to repair any damage to cellular structure and function by ENPs and metal salts. It is reasonable to assume that the mechanism may be resistant genes capable of being transferred to microorganisms targeted by the use of ENPs in consumer products. In addition to other metabolic versatility, *Acidovorax* can resist multiple metal salts toxic effect through horizontal gene transfer (Huang et al., 2012). AOB from both *Betaproteobacteria* and *Gammaproteobacteria* were not recovered in this study which illustrates that they were susceptible to the inhibitory effect of the ENPs and metal salts (Martinez-Gutierrez et al., 2010; Jin et al., 2009; Sterritt and Lester, 1980). Apart from the unclassified bacteria, members of the *Comamonadaceae* had a more robust growth indicated by the distinct distribution pattern compared with other dominant species in control and metal salt spiked AS.



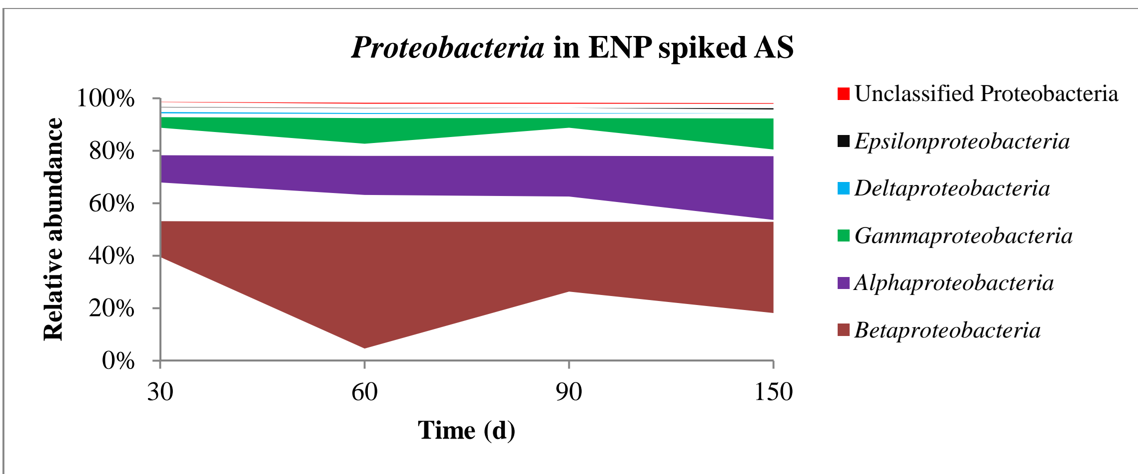
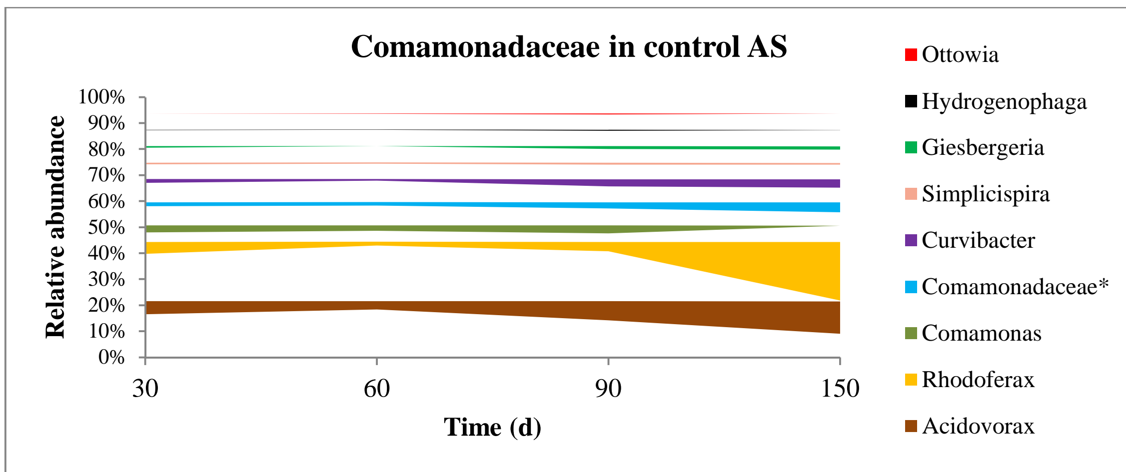
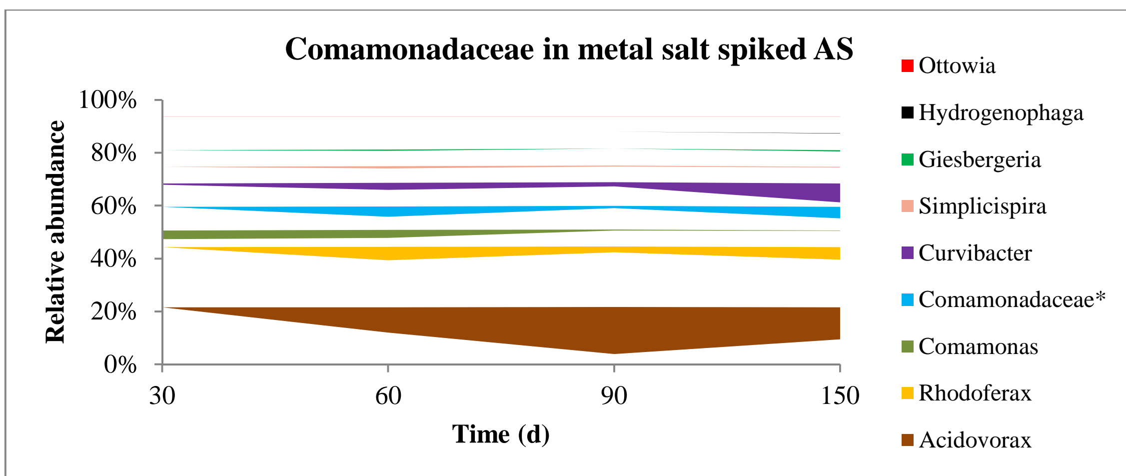




Figure 3.17 Relative abundance of *Proteobacteria* in AS indicating the dominance of members of the *Betaproteobacteria* and resilience of the Alphaproteobacterial subgroups to the effect of treatment compared with the control. Group above 1 % abundance in the AS are shown.



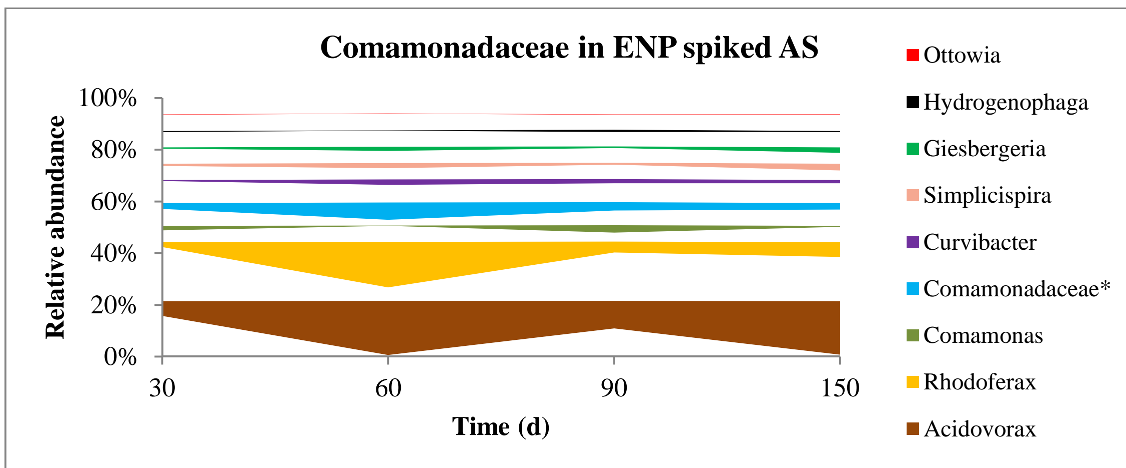
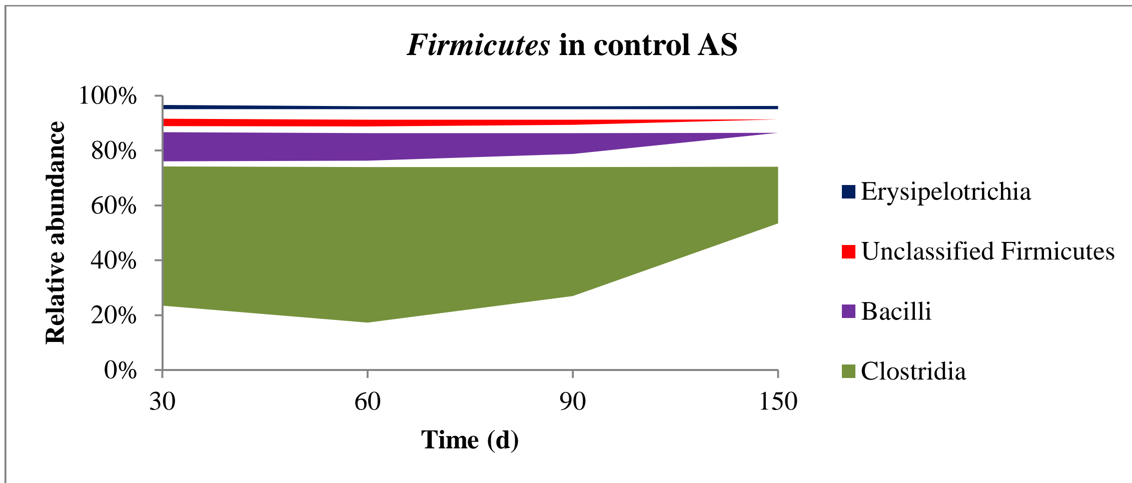
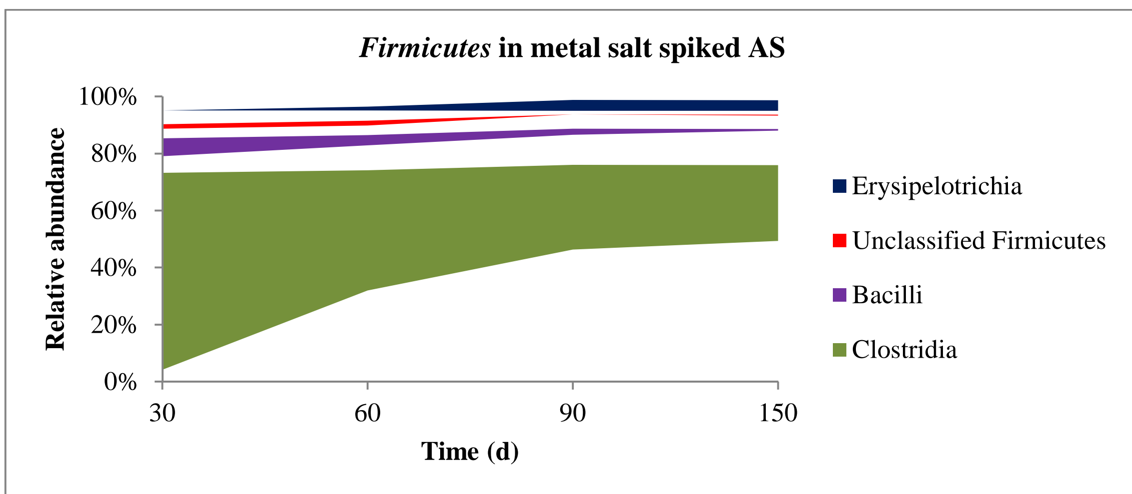




Figure 3.18 Relative abundance of members of the *Comamonadaceae* (β-*Proteobacteria*) showing *Acidovorax* and *Rhodoferax* with the highest percentage recovery in the subgroup and bacterial community. Species with at least 1 % abundance in the AS are indicated. * = Unclassified

### 3.3.15 Effects of metal salts and ENPs on *Firmicutes*, *Bacteriodetes* and *Actinobacteria*

The result in Figure 3.19 indicates clearly that *Firmicutes* were reduced in abundance overtime. For example in the control, members belonging to the class of *Clostridia* were most abundant, followed by *Bacilli* which both seem to be sensitive to the presence of metal salts or ENPs. A similar trend was observed for the *Bacteriodetes* (Figure 3.20) with the relative abundance of the clinically important anaerobic microbiota, *Bacteriodes* and *Parabacteroides* whose members are usually found in human faeces (Garrity, 2010; Hong et al., 2008). The filamentous members of the *Bacteriodetes* present in AS occasionally can cause bulking but are important in the overall filament index of the AS (Kragelund et al., 2008) and may have contributed to the frequent foaming incidence observed in the reactors. The abundance of *Actinobacteria* appeared to be relatively stable in the control and ENP spiked AS (Figure 3.15). In the metal salts spiked AS however, unclassified bacteria reduced in abundance while there was an increase in relative abundance of the *Proteobacteria*, *Actinobacteria* and *Bacteriodetes* such as *Flavobacteria* and *Sphingobacteria.* The relative abundance of unclassified bacteria, *Firmicutes* and *Bacteriodetes* in the three AS reactors decreased in favour of the *Proteobacteria*. More growth enhancing effect or competititive advantage on the *Proteobacteria* was exhibited by ENPs than metal salts when compared to the control.



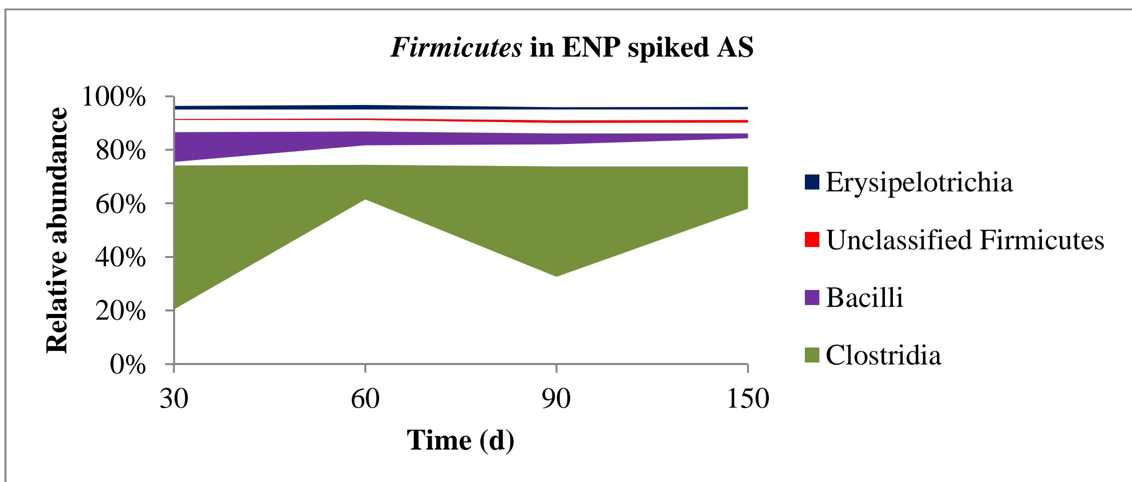
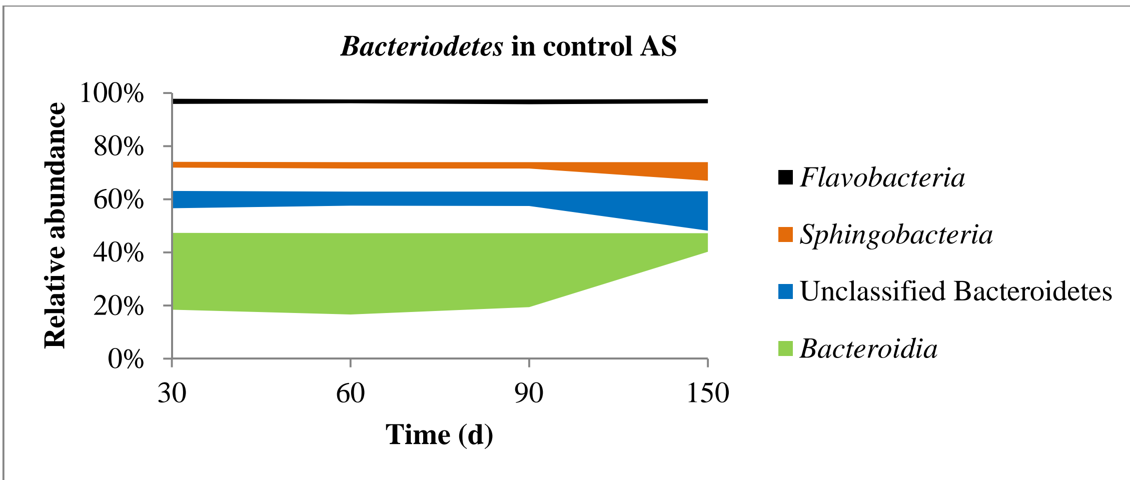
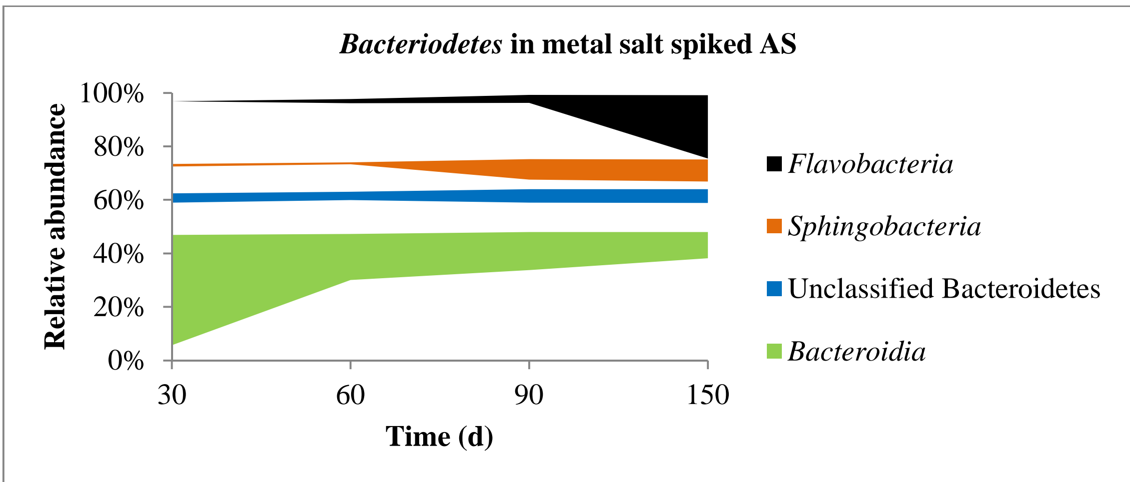




Figure 3.19 Effect of treatment on the relative abundance of the phylum *Firmicutes* in AS. Members of Class Clostridia were most abundant among the *Firmicutes*.



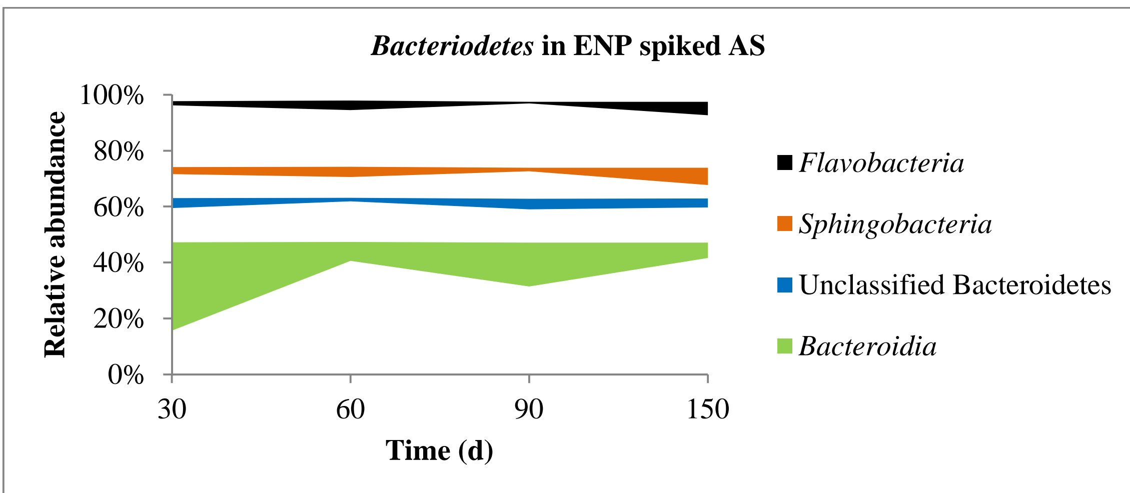




Figure 3.20 Effect of treatment on the relative abundance of the *Bacteriodetes* in AS.

### 3.3.16 Effect on the phylum *Chloroflexi* and other filamentous bacteria

Filamentous members of the phylum *Chloroflexi*, formerly known as the non-sulfur bacteria (Table 3.3) were present in 75 % of the control and metal salt spiked samples, and 50 % in the ENP spiked AS (Figure 3.15). Although it is difficult to make general statements regarding the physiology of the *Chloroflexi* and other filamentous bacteria in AS due to limited information (Wagner et al., 2002; Kragelund et al., 2007), the relative abundance of *Chloroflexi* in the AS was below 1 % except in the control. The result suggests that ENPs had adverse effect on the microbes compared to the metal salt and control. The *Chloroflexi* are important in removing organic carbon, nitrification and occasionally associated with bulking or foaming incidence in AS (Yoon et al., 2010; Kragelund et al., 2007). Thus, *Chloroflexi* although with low abundance, in part may have contributed to the frequent foaming observed in the control and metal salt spiked AS compared with the ENPs spiked AS, and in part were involved in nitrifying and removing of organic carbon in the AS.

### 3.3.17 Effect of treatment on members of the *Archaea*

The relative abundance of the 2 archaeal members were 4 % (*Methanocorpusculum*) and 96 % (*Methanosarcina*) in ENP spiked AS compared with 29 % and 71 % for *Methanocorpusculum* and *Methanosarcina* in metal salts spiked AS (Figure 3.21). The result indicates 1.3 times higher abundance of *Methanosarcina* in ENP spiked AS than in metal salt spiked AS. On the other hand, *Methanocorpusculum* was 6.7 times higher in metal salt spiked AS than in ENP spiked AS. Both organisms were absent in the control AS and the reason for this is unclear at the moment. The low level OTUs obtained in this study is consistent with the findings of Park et al., (2006) in which a range of 2 to 11 OTUs belonging to the *Archaea* were obtained in wastewater treatment plant bioreactor. Nevertheless, the *Archaea* were commonly known as obligate anaerobes and it is still debatable whether ammonia-oxidizing *Archaea* (AOA) is mixotrophic or heterotrophic organism (Tourna et al., 2011).

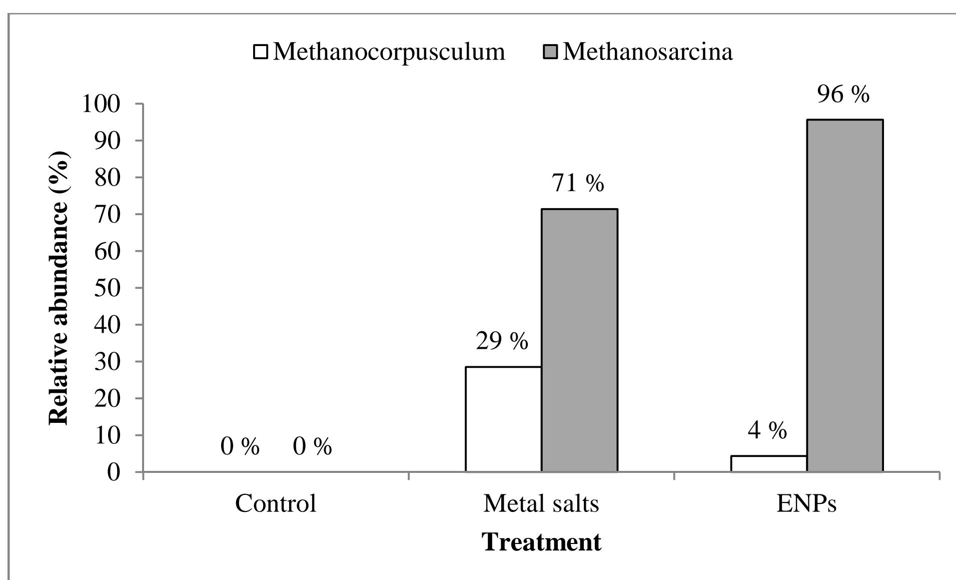



Figure 3.21 Relative abundance of the *Archaea* in activated sludge

Contrary to previously held notion, available evidence however, has shifted the weight of argument in favour of ammonia-oxidizing archaea in AS. Thus, it is reasonable to assume that the spiked concentrations of metal salts and ENPs altered the AS composition and created conditions that enhanced the growth of the *Archaea*. In the absence of the known AOB and NOB in the metal salt and ENP spiked reactors as previously mentioned, it is plausible that *Methanocorpusculum* and *Methanosarcina* were involved in oxidizing ammonia, thus contributing to the reduced ammonia concentration in the effluent.

### 3.3.18 Conclusion

The overall AS process efficiency based on the operational design of the pilot plants in this study to produce biosolids for anaerobic digestion was not reduced by the introduction of ENPs or metal salts at the spiked concentration of 0.01 mg $L^{-1}$ $Ag^0$, 0.08 mg $L^{-1}$ $TiO_2$ and 0.12 mg $L^{-1}$ ZnO. After 315 days of treatment, ability to nitrify and degrade organic matter was still retained by the AS biomass in the three experimental conditions investigated. However, significant differences were noticed in the floc size, MLSS and MLVSS whereas minor differences were observed in the $NH_3^-$, $NO_2^-$, $NO_3^-$, total N, SVI, SS and COD. The findings also suggest that the control AS and ENP spiked AS performed in almost the same way. It is suspected that the continuous flow system, presence of NOM and the low concentration of spiked ENPs and metal salts contributed to the no observed negative influence on bulk contaminant removal in the

reactors. Three plausible reasons can be deduced for the absence of adverse effect on the overall AS process (i) ENPs were rendered non-inhibitory by wastewater components (ii) spiked concentration was below the threshold to elicit an adverse effect due to dilution (iii) at the molecular level, the exposed organisms adapted and repaired any disrupted cellular component and therefore were able to cope with the ENPs or metal salt concentrations. Further to this, the positive influence of mixed ENPs on nitrifying microbial community to utilize organic matter and remove ammonia in wastewater can be exploited to enhance AS process efficiency.

This study has illustrated that ENP interacts with AS bacterial community in ways that differ from the bulk metal salts. The practical implication is that ENPs can augment microbial reaction rate and at the same time inhibit growth and abundance of most bacterial groups. For example, there was increase in SOUR and no observed inhibitory effect on nitrification, whereas growth and abundance of heterotrophic and coliform bacteria were delayed and or inhibited compared to the control. The cell wall and its charges, in addition to the protective exopolysaccharide sheath are critical factors that can increase or attenuate ENP-microbial contact and/or inhibitory effect. The disrupted bacterial cell wall suggests that the low concentration of ENPs reacted in an additive or synergistic manner with other micropollutants in the wastewater to exert inhibitory effect. Thus, aged-ENPs increased microbial reaction rate but with underlying harmful Trojan horse-like effect on bacterial cells influenced by confounding factors in wastewater. In addition, the change in cyclopropyl fatty acid to cis-monoenoic fatty acid indicates a conditional and post synthetic modification of the microbial cell wall under stress was more pronounced in the metal salt spiked AS. Given the metabolic versatility of the A*rchaea* and abundance of the *Comamonadaceae*, *Alphaproteobacteria*, *Bacteriodetes* and other filamentous bacteria, it is perceived that they played a key role in nitrification and removal of contaminant in the AS.

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## Chapter 4: Effects of engineered nanoparticles on activated sludge anaerobic digestion performance and associated microbial communities


### Abstract

To investigate the potential effect of engineered nanoparticles (ENPs) on activated sludge anaerobic digestion performance, three 150 L pilot scale anaerobic digesters (AD) were fed with a blend of waste activated sludge (WAS) and primary sludge spiked with a mixture of bulk metals (metal spiked AD) or metal oxide nanoparticles (ENP spiked AD). One of the three was fed with unspiked sludge (Control AD). The bulk metal and ENP mixtures consisted of 5 mg $L^{-1}$ silver (Ag), 40 mg $l^{-1}$ of titanium (Ti) and 56 mg $l^{-1}$ zinc to achieve a target concentration of 250, 2000, and 2,800 mg $kg^{-1}$ dry weight respectively in the digestate. The mixtures were added to the sludge on daily basis. In addition, the influence of ENP mixtures ranging between 5 and 2800 mg $L^{-1}$ on sludge biomethane potential (BMP) and volatile fatty acid (VFA) production was assessed in 100 ml mesophilic batch digesters for 42 days. The sludge BMP was 1.5 times higher in the presence of ENPs compared to the control sludge and no significant difference was observed with the metal spiked AD. VFA composition was not significantly ($p > 0.05$) different in the 3 pilot plants, the concentration of the isovaleric acid in the control AD was two times lower suggesting different metabolic state of the microbes. Besides, hydrogen sulphide ($H_2S$) concentration was at least 2 times lower in the ENP spiked AD than in control and metal salt spiked AD reactors. Based on the ether linked isoprenoid (PLEL) concentration, the abundance of methanogens was 1.4 times lower in the ENP spiked AD than in control and metal salt spiked AD. Pyrosequencing result indicated 80 % decrease in the abundance and diversity of methanogens in the ENP spiked AD in comparison to control AD. As a result, nano-tolerant species close to *Methanosarcina acetivorans* and *Methanosarcina barkeri* proliferated by a factor of 6 and 11 compared to the metal salt spiked and control AD. The result further provides compelling evidences on the resilience of *M. acetivorans*, *M. barkeri* and the Trojan horse-like effect of ENPs on microbial abundance and diversity.



**Keywords:** Sewage sludge, anaerobic digestion, volatile fatty acid, engineered nanoparticle, nano-tolerant *Archaea*, biomethane potential, inhibitory effect

## 4.1 Introduction

The environmental footprint of sewage sludge disposal has gained attention in effort to minimise environmental pollution, representing an operational cost of about 50% for water utilities (Appels et al., 2008). Among the methods used in sludge treatment for disposal, anaerobic digestion (AD) is generally preferred because of biogas and stabilised biosolids produced, pathogens destroyed and reduced odour (Appels et al., 2008; Johnson et al., 2003). Biogas production during AD process involves a complex multi-step sequence of substrate hydrolysis, acidogenesis, acetogenesis and methanogenesis catalysed by diverse and unique microbial community. The initial stage of the process is a rate-limiting step in which fermenting bacteria hydrolyse complex organic polymers such as carbohydrates, protein and fat into usable monomeric substrate and precursors for uptake and conversion to methane (Schink, 1997, Appels et al., 2008).

The final step of the process is catalysed by key methanogenic *Archaea* classified into four groups based on the pathway of substrate utilisation and product formation. These are the (i) $CO_2$ reduction pathway in which hydrogen serves as electron donor when carbon dioxide is reduced to methane (ii) methyl reduction pathway also involving hydrogen as electron donor in reducing methanol to methane with the transfer of the methyl group to coenzyme M (iii) Acetoclastic pathway where acetate is activated to acetyl-CoA, the carbonyl group oxidised to $CO_2$ and the methyl moiety transferred to tetrahydrosarcinapterin and then reduced to methane (iv) The methylotrophic pathway in which methane and $CO_2$ is produced from one-carbon compounds such as methylamines and methanol. Here, the substrate is oxidised to generate the reducing equivalents needed to reduce three molecules to methane (Welander and Metcalf, 2005). In addition, another pathway involving the oxidation of acetate to a mixture of $CO_2$ and formic acid coupled to methanol reduction exist for *Methanosarcina* lacking the *mtr* operon that code for the $N^5$-methyl-tetrahydrosarcinapterin ($CH_3$-$H_4$SPT): coenzyme M methyltransferase. It is unclear how the mtr bypass operates, but it is suspected that some steps in standard methylotrophic pathway are used by the organism. Most methanogens can use only one of these pathways although in members of the *Methanosarcina* such as *M. barkeri*, the four pathways are present and usually overlap each other (Welander and Metcalf, 2005).

AD microbial communities have different optimum conditions and therefore to ensure efficient reactor performance it is important to maintain a subtle balance between the different process parameters. An imbalance in microbial community abundance and diversity can upset and cause failure or instability of anaerobic digesters (Demirel and Yenigun, 2002). Specifically, the methanogens are sensitive to the accumulation of free ammonia and ammonia accumulation in digesters (Kayhanian, 1994), fatty acids (Wagner et al., 2010), pH, alkalinity, hydrogen, sodium, potassium, heavy metals, surfactants and other exogenous agents such as xenobiotic compounds (Appels et al., 2008). Apart from the intrinsic inhibitory substances of the substrate and their by-products, the release of xenobiotic compounds such as engineered nanoparticles (ENPs) from consumer products into wastewater is of concern as recent researches demonstrated that nanoparticles can inhibit biodegradation of organic compounds, nitrification and anaerobic digestion process (Maurer-Jones et al., 2013; Levard et al., 2012; Zheng et al., 2011).

ENPs are materials with unique characteristics which attract considerable interest for research and novel consumer product application. It is common knowledge that silver in the form of bulk metal oxide or nanoparticle ($Ag^0$) is used in many consumer products because of the biocidal effect of silver ion ($Ag^+$) and formation of reactive oxygen species (ROS). In recent years, other types of ENPs such as zinc oxide (ZnO) and titanium dioxide ($TiO_2$) have also been incorporated into diverse consumer products to enhance product function and efficiency. Another reason for the use of ENPs is the broad spectrum microbiostatic and biocidal properties (Suresh et al., 2010) which are adjudged more effective compared with most of the commercially available antibiotics. Once they are released from the consumer products, most of the ENPs will sorb to biosolids in wastewater and will be transported to the treatment plants. The ENP-enriched biosolids may then be subjected to anaerobic digestion which raises concern on the possible effect on non-target biologically sensitive groups and processes during wastewater treatment (Yang et al., 2012). However the potential impact of ENP on sludge treatment processes such as anaerobic digestion is still an area of research under investigated. In most studies (Table 2.1), pristine forms of ENPs and pure cultures of bacterial isolates were used and therefore caution should be taken in extrapolating the observed effects to any microbial community in real environmental samples such as sludge. It is suspected that the physicochemical properties of the wastewater and the

presence of solids can influence ENPs behaviour, fate and transport by either aggregating, absorbing, changing their oxidation state, precipitate or form complexes with ligands in wastewater to mitigate toxic effect (Xiu et al., 2011; Liu et al., 2010). For example, $Ag^0$ is known to react with chlorides, sulphide (Levard et al., 2011) and natural organic matter (Arvizo et al., 2010; Liu and Hurt, 2010) resulting in an attenuation of its toxic effect.

Other factors such as the size of ENPs (Sotiriou and Pratsinis, 2010), the presence of divalent cations/anions and surface charges (El Badawy et al., 2010; Li et al., 2010), capping agents which repel ENPs by electrostatic, steric or electrosteric forces to avoid forming aggregations (Phenat et al., 2008; Hotze et al., 2010) can influence ENPs effect. In addition, the bacterial cell wall composition and their charges (Jin et al., 2009) can either enhance or attenuate ENP bactericidal effect.

The two key mechanisms proposed for the ENPs toxic effect on microorganisms are the oxidation stress from reactive oxygen species (ROS) causing lipid peroxidation (Choi and Hu, 2008) and ions interacting with key biotic receptors such as cell membrane/wall, protein and DNA (Beer et al., 2012; Bottero et al., 2011). However, using $Ag^0$, ZnO and $TiO_2$ as model ENPs, there is no consensus yet on the relationship or influence between ENPs surface charge and charges on bacterial cell wall. For instance, Sondi and Salopek-Sondi (2004) argued that $Ag^0$ produces ions ($Ag^+$) that exert toxic effect on bacteria such as *E. coli* whereas Fabrega et al., (2009) demonstrated that the effect of $Ag^0$ in the presence and absence of fulvic acid with different pH ranges was not related to the presence of dissolve $Ag^+$. Also, Reinsch et al., (2012) showed that the aggregated $Ag^0$ form was inhibitory to *E. coli* than the dispersed one due to incomplete sulphidation of $Ag^0$ in the aggregate. This finding brings new insights on the effect of aged ENPs which can significantly alter predictions on the potential effect of nanoparticles. It has also raise further questions on the reactivity and behaviour of aged ENPs because about 90 % of reducing agents such as sulphides and chlorides in sewage sludge can react with $Ag^0$ to form stable non-soluble aggregates (Levard et al., 2012; Kaegi et al., 2011).

How anaerobic digester containing ENP-enriched sludge performs will depend on the interaction between bacterial and archaeal cells influenced by wastewater components. An adverse effect on any successive step of the anaerobic process (Figure 4.1) will

undoubtedly reduce reaction rate with increased accumulation of toxic metabolic products that may constitute a limiting step in the process. $Ag^0$ embedded in consumer products are usually released into wastewater (Yang et al., 2012) but empirical data on the impact of aged-ENPs on bacteria and archaea in anaerobic process is limited (Jin et al., 2009; Liu and Sun, 2009). To date, available information provides an unclear understanding on how ENPs interacts with the microbial community during anaerobic digestion process (Kim et al., 2010).

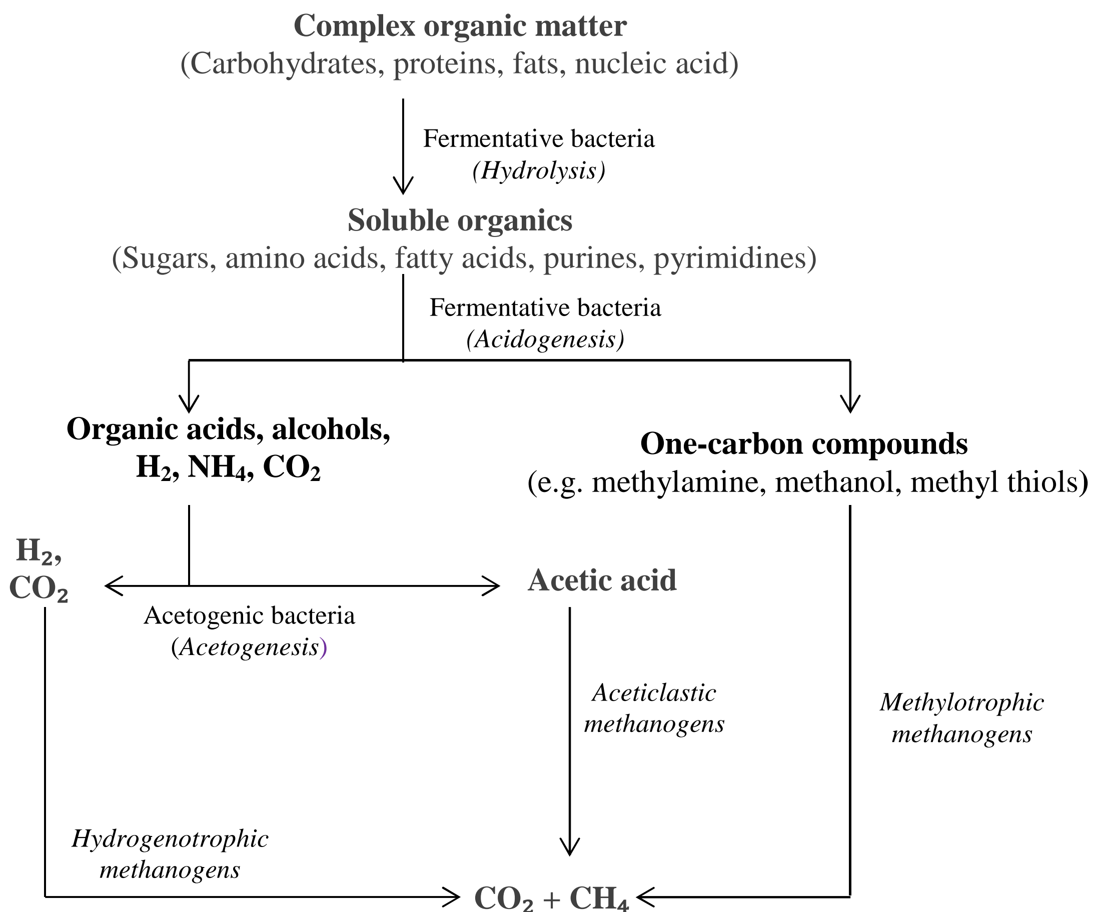



Figure 4.1 Schematic illustrations of the pathways of anaerobic digestion used by different microbial groups

To understand the effect of aged-ENPs on microorganisms and its influence on biological product formed during anaerobic digestion, laboratory batch test and 3 parallel pilot-scale anaerobic treatment plants were used to provide relevant environmental conditions. In addition, microorganisms were exposed to unspiked

sewage sludge (control) and equivalent concentrations of bulk metal salts spiked sludge for comparison. This study therefore, provides empirical evidence to evaluate the effect of $Ag^0$, $TiO_2$, ZnO on (i) indicators of AD by-products formed such as biogas, accumulated volatile fatty acid and implications on the key steps of anaerobic digestion (ii) microbial community structure, abundance and diversity in a pilot-scale anaerobic digestion.

## 4.2 Material and methods

### 4.2.1 Pilot plant operation

Three parallel mesophilic pilot-scale plants, each consisting of primary clarifiers (180 L), secondary clarifiers (~ 150 L), aeration tanks (~360 L) and anaerobic digesters (150 L) (Figure 4.2) were used in this study. 150 L of return activated sludge (RAS) from a full-scale municipal waste water treatment works (Anglian Water, Cotton Valley, UK) and 150 L of settled wastewater from Cranfield University domestic wastewater treatment plant (CUDWTP) was fed into the aeration tanks as a start-up material and maintained at 20 ± 2 $^0$C. Settled wastewater from CUDWTP was then fed into the 3 primary clarifiers at a rate of 750 ml min$^{-1}$ (40 rpm) using pumps (520S Watson Marlow, UK). RAS recirculation at the rate of 375 ml min$^{-1}$ (20 rpm) from the secondary clarifiers into the aeration tank was achieved by the use of peristaltic pumps (505U, Watson and Marlow, UK).

The plants were operated under identical conditions except that plant A was spiked with mixed ENPs solution of polyvinylpyrrolidone (PVP) capped silver oxide ($Ag^0$, 20 nm ), zinc oxide (ZnO, 20 nm), and titanium dioxide ($TiO_2$, 20 nm) Aeroxide P25 (Degussa, Germany). ENPs concentration spiked into the aeration tank was 0.01 mg L$^{-1}$ $Ag^0$, 0.08 mg L$^{-1}$ $TiO_2$, and 0.12 mg L$^{-1}$ ZnO at the rate of 0.14 ml min$^{-1}$ (206 ml day$^{-1}$). ENPs were chosen on the basis of their wide application in many consumer products. As basis for comparison, an equivalent mixed concentration of silver nitrate (> 99.9 % $AgNO_3$), titanium dioxide ($TiO_2$) and zinc nitrate ($Zn(NO_3)_2.6H_2O$, anhydrous) was spiked into plant B whereas plant C was unspiked (control). The chemicals were suspended in ultrapure water and used without any purification. The dispersion of mixed ENPs and metal salts suspension in amber glass bottles was maintained by continuous stirring at 200 rpm. Prior to spiking, the AS plant was operated at a fixed hydraulic retention time (HRT) of 8 hours and sludge retention time (SRT) of 10 days and 15 days SRT for the

anaerobic digester (AD). The AS and AD plants were subsequently operated over 3 SRT of 30 and 45 days respectively to stabilise.

ENP-enriched waste activated sludge (WAS) was thickened using Polygold® C420 coagulant (Goldcrest, Barnsley, UK) to 2 L and blended with 8 L settled primary sludge spiked with 5 mg $L^{-1}$ $Ag^0$, 40 mg $L^{-1}$ $TiO_2$, and 56 mg $L^{-1}$ ZnO; the targeted final concentration in the blended sludge (WAS + primary sludge) was 250, 2000, and 2,800 mg $kg^{-1}$ for $Ag^0$, $TiO_2$ and ZnO, respectively. The pH of the blended sludge was adjusted with sodium bicarbonate anhydrous ($Na_2CO_3$, 99.5% pure) to 7.2 ± 0.2 and batch-fed daily into the anaerobic digesters operated at mesophilic temperature of 35 ± 3 °C in a wet digestion condition. 240 L $h^{-1}$ (145 rpm) digestate circulation was achieved in the anaerobic digester using Mono pump (620S, Watson Marlow. UK). 10 L of the digestate was extracted from each digester and the equivalent amount of freshly blended primary sludge (8 L) and thickened WAS (2 L) fed into it daily. During the initial 120 days after spiking, the digestion process was unstable which resulted in variable biogas production from the three digesters. The aged-ENP and metal salt spiked digestate produced from the 3 plants over 295 days of operation was dewatered to 25 % dry solid. Prior to dewatering, 1.25 g $l^{-1}$ solution of Polygold® C540 coagulant (Goldcrest, Barnsley, UK) was added to thicken and enhance solid-liquid separation. The dewatered digestate and filtrate were analysed for residual ENPs and metal salt ions and also used for bioassay. Concentration of ENPs and metal salts spiked into the sludge and subjected to anaerobic digestion represents the worst case scenario of the maximum allowable concentrations in sludge spread to agricultural land with reference to the Transatlantic Initiative on Nanoparticle and the Environment (TINE) project.

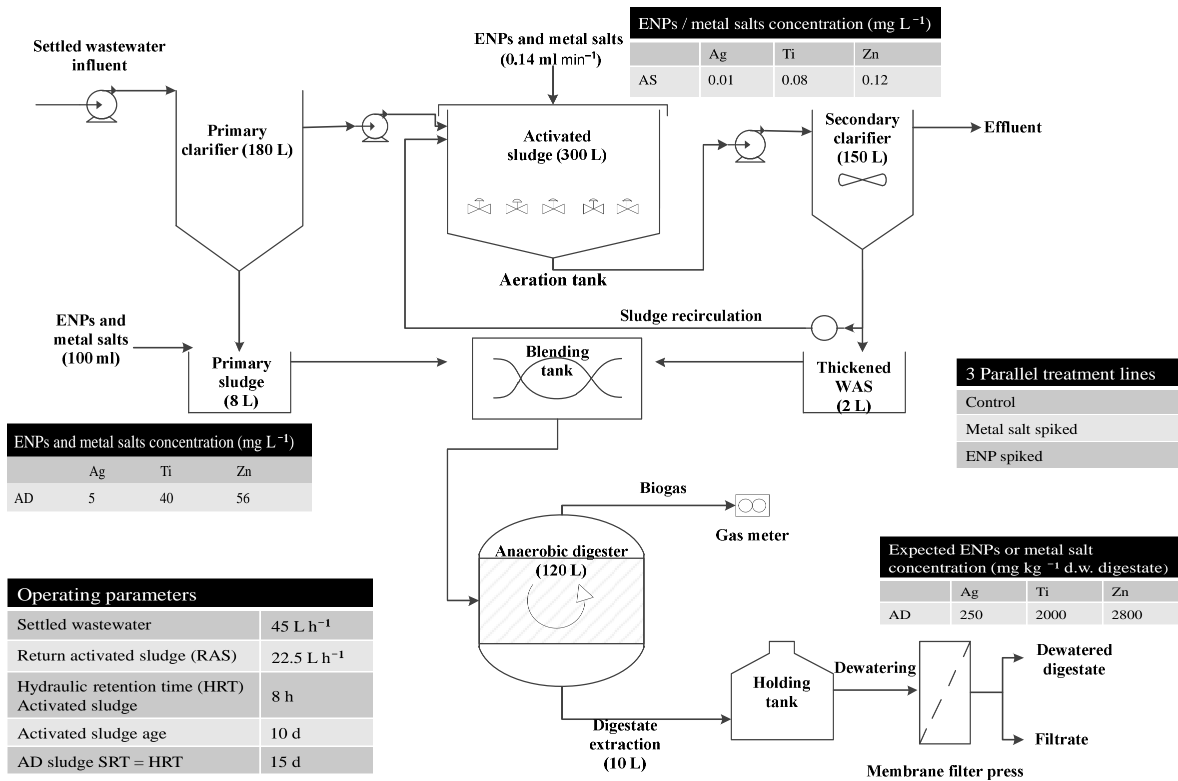



Figure 4.2 Schematic flow diagram of pilot-scale treatment plant operation

Table 4.1 Summary of sampling regime for AD

| Parameters/ sample size | Time (d) | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Batch 1 | | | | | | | | | Batch 2 | | | | | | | | |
| | | 45 | 60 | 75 | 90 | 115 | 130 | 145 | 160 | 175 | 190 | 205 | 220 | 235 | 250 | 265 | 280 | 295 |
| Chemical analysis (60 samples per batch) | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |
| PLFA/PLEL (30/84 samples) | | • | • | • | • | • | • | • | • | • | • | • | • | • | • | | | |
| Pyrosequencing (15 samples) | | | | | | | • | | • | | • | | | | | | | |
| VFA (150 samples) | | | | | | | • | • | • | • | • | • | • | • | • | • | • | • |
| Biogas | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • | • |

AD operated for 3 sludge retention time (45 days) to stabilise

### 4.2.2 Determination of residual ENPs and metal salts concentration

The residual concentration of ENPs and metal salts in form of $Ag^+$, $TiO_2$ and $Zn^{2+}$ ions in 60 digestate samples (10 duplicate samples) from each treatment per batch (Table 4.1) was measured by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES, Perkin Elmer 4300DV). Batch 1 and 2 represents the timeline for attaining the targeted concentration of ENPs and metal salt in the digestate. Sample analysis was carried out externally by a commercial laboratory (Environmental Scientific Group, Straffordshire, UK). Briefly, the samples were digested prior to analysis using high purity nitric acid, hydrogen peroxide and hydrofluoric acid in sealed Teflon vessels with microwave assisted heating. After digestion, demineralized water having a resistivity of 18.2MΩ.cm was used in making the samples into known volume. $Ag^+$, $Ti^{4+}$ and $Zn^{2+}$ concentrations were measured by ICP-AES calibrated using certified standards. 5.0 mg $L^{-1}$ Ag, Ti and Zn prepared from an alternative source stock different from that used for the instrument calibration standard was measured with the samples as a quality control measure. A blank digestion vessel spiked with equivalent of 4.0 mg $L^{-1}$ Ag Ti, and Zn was taken through the same procedure as a further quality control measure.

### 4.2.3 Phospholipid fatty acid (PLFA) analysis

Total lipids were extracted from 40 g aliquot of freeze-dried digestate using a modified version of Bligh-Dyer technique as described by Frostegard et al., (1996; 1993). Briefly, 5 g of freeze-dried activated sludge sample was extracted using 0.8:1:2 (v/v/v) citrate buffer-chloroform-methanol, subjected to solid-phase fractionation followed by transesterification by mild alkaline methanolysis (Dowling et al., 1986) to obtain the fatty acid methyl esters (FAMEs). The dried FAMEs were resuspended in 0.2 ml of hexane and analysed by gas chromatography (GC) (Agilent Technologies 6890N) coupled to a flame ionization detector. Chromatography was performed as described by Pankhurst et al., (2012). PLFA composition was identified by comparing the retention times of the peak obtained with the 26 bacterial acid methyl ester (BAME) mix standard (Sigma-Aldrich Ltd., Dorset, UK). Nonadecanoic acid methyl ester (Sigma-Aldrich, UK) was added (200 µl) as internal standard (ISTD) to each sample after solid phase extraction. Bacterial biomass was converted into number of cell equivalents using a conversion factor of $5.9 \times 10^{10}$ cells per µmol of PLFA (Kieft et al., 1994). PLFA nomenclatures were as follows: X: YωZ, where X = number of carbon atom in the chain, Y = number of double bonds (unsaturation) in the aliphatic (ω) end of the carbon

chain and Z = number of carbon atom from the methyl end of the molecule in relation to the first unsaturation. The suffices ‘c’ (*cis*) and‘t’ (*trans*) indicate the geometric isomers of the unsaturation and the prefixes ‘a’ (*anteiso*), ‘i’ (*iso*), ‘10me’ (*methyl* branch on the tenth carbon from the carboxyl end), ‘cy’ (*cyclopropyl*) and ‘Br’ (*branching* at unknown location) whereas hydroxyl ($OH^-$) substitutions is represented at the carbon atom where the substitution occurs (Macnaughton et al., 1999).

### 4.2.4 Phospholipid ether lipids (PLEL) analysis

Aliquots of the phospholipid fraction equivalent to ~12.5 g dry matter were subjected to PLEL analysis. Acidic hydrolysis and methylation cleavage of the polar head group to obtain ether core lipids was performed according to the method of Bai et al., (2000). After solvent removal the aliquot of the phospholipid fraction was dissolved in 2 ml of methanol: chloroform: 37% hydrochloric acid (10:1:1, v/v/v) and kept at 60 °C over night. After cooling, 4 ml water was added. The extraction of the ether lipids was carried out with 5 ml hexane. The organic phase was combined and dried with sodium sulphate (sodium sulphate was washed 3 times with hexane).

To release the etherlinked hydrocarbons (as alkyliodides) from ether lipids, hydriodic acid (HI) was used. The sample with the ether core lipids was transferred into a 50 ml tube and dried via nitrogen stream; 2.0 ml HI (57%) was added and shaken for 20 sec. The sample was incubated at 100 °C for 18 h. After cooling, 4 ml water was added to stop the reaction. The extraction was done with 3 x 5 ml hexane, the hexane phases was collected in a 100 ml separation funnel. The hexane extracts was washed with 4 ml water (15 sec shaking), 10 ml sodium carbonate 10% (30 sec shaking) and 10 ml sodium thiosulfate 50% (30 sec shaking). After 15 min the lower phase was discarded and the hexane phase dried with sodium sulphate.

Reductive dehalogenisation was achieved by adding 300 mg Zn to the dried sample of alkyliodides (generated above) in a centrifugation tube. 3 ml of acetic acid (100%) was added and the tube shaken for 20 sec. The sample was incubated at 100 °C for 18 h, after cooling the neutralisation was carried out with 5 ml 0.1 M sodium carbonate. The extraction was done with 3 x 7 ml hexane (centrifugation step 10 min), the hexane phases were combined in a 100 ml separation funnel and washed with 10 ml 0.1 M sodium carbonate (15 sec shaking being cautious of pressure balance), 2 x 8 ml water (30 sec shaking). After 15 min the lower phase was discarded and the hexane phase dried with sodium sulfate.

To the dried sample, 200 µl ISTD (Nonadecanoic acid methyl ester) was added, and the liquid transferred to GC vial, analysed and quantified by GC-MS (Agilent Technologies 6890N). Agilent G2070 ChemStation software for GC was used in identifying the peaks as previously described. Archaeal biomass was estimated using a conversion factor of 5.9 x $10^{13}$ cells per 2.5 µmol PLEL (Bai et al., 2000).

### 4.2.5 Sequencing of microbial community DNA in anaerobic digestate

To understand the diversity and dynamics in AD microbial community, total genomic DNA was extracted from 200 mg wet weight digestate samples using a MoBio Power Soil kit (MO BIO Laboratories, Inc, UK). The quality of DNA was checked on 0.8% agarose gels. For the amplification of the bacterial 16S rRNA gene fragments, PCR primers were adapted for 454 amplicon sequencing by attaching the M13 adapter (**CACGACGTTGTAAAACGA**) to the primer M13-16S-IA-FL (5'-**CACGACGTTGTAAAACGA**CCATGCTGCCTCCCGTAGGAGT-3'), whereas the 25-mer Lib-L specific sequence adapter B (CCTATCCCCTGTGTGCCTTGGCAGTC) was followed by the reverse template specific primer sequence 16S-IA-RL (5'-**CCTATCCCCTGTGTGCCTTGGCAGTC**TCAGAGAGTTTGATCCTGGCTCAG-3'). To aid multiplexing different samples, different barcodes were included in the M13 adapter using the 454 sequence adapter A (CCATCTCATCCCTGCGTGTCTCCGAC) and a 454 amplicon sequencing specific 4-mer amplification key (*TCAG*) followed by a 10-mer barcode sequence (NNNN)

(5'-CCATCTCATCCCTGCGTGTCTCCGAC*TCAG*NNNN***CACGACGTTGTAAAACGAC***-3'). The amplification of the archaeal 16S rRNA gene fragment was carried out using primers ARC-344F (5'-CACGACGTTGTAAAACGAACGGGGYGCAGCAGGCGCGA) and ARC-915R (5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGTGCTCCCCCGCCAATTCCT-3') which were adapted for multiplexing and 454 sequencing as described above.

Each 20 µL PCR mixture contained primers at 10 µM, 10 mM deoxynucleoside triphosphates and 0.2 µL of High fidelity polymerase (Phusion, Biolabs, New England, UK), 4 µL Phusion 5x buffer (Phusion, Biolabs, New England, UK) and 1.4 µL $MgCl_2$. Amplifications were performed using a Biorad C1000 Thermal cycler (BioRad) as follows: 95 °C for 5 min, followed by 35 cycles of denaturation at 98 °C for 20 sec,

annealing at 57 °C for 20 sec and elongation at 72 °C for 30 sec. Cycling was completed by a final elongation at 72 °C for 10 min. Next generation sequencing (NGS) of all amplicons was completed using the GS FLX System (Roche). Emulsion PCR was carried out according to the manufacturer's instructions (Roche). Samples were multiplexed on a 1/8 section of the pyrosequencing plate. As quality control (QC), base with quality $< 20$ and sequences $< 100$ bp were removed. Prior to QC, there were 69,964 sequences. 51 low quality and 1365 short length sequences were removed. Overall sequencing resulted in a total of 69,964 bacterial sequences and an average of 726 sequences per sample was obtained. The obtained sequence data were processed using the Galaxy platform (http://galaxyproject.org/). Sequences analysed were a minimum of 500 bp (mean length average 560 bp). Splitting of sequences into respective samples was carried out using respective barcodes

To estimate the abundance and diversity of AD archaeal community, the identified 16S rRNA from 454-pyrosequencing were grouped into 11 Operational Taxonomic Units (OTUs) representing the dominant microbial community structure. To identify the methanogenic archaea, the different nucleotide sequences from pyrosequencing were blasted using the online National Centre for Biotechnology Information (NCBI) BLAST® tool. Nucleotide sequence length of 536 – 566 with > 200 alignment and maximum identity match of 94 – 98 % to the closest culturable archaeal relative was obtained.

### 4.2.6 Biochemical methane potential (BMP) of ENP and metal salt spiked sludge

The effect of a mixture of engineered nanoparticles comprising polyvinylpyrrolidone (PVP) capped silver oxide ($Ag^0$), zinc oxide (ZnO) and titanium dioxide ($TiO_2$) was compared with equivalent concentration of silver (Ag), zinc (Zn), and titanium (Ti) ions added as silver nitrate ($AgNO_3$), zinc nitrate ($Zn(NO_3)_2.6H_2O$ anhydrous) and titanium dioxide ($TiO_2$). The measure of sample degradability was done by a modified approach of Owen et al., 1993 in which ENP or metal salts spiked complex and undefined sewage sludge, instead of chemically defined medium was used as substrate. The test was carried out in batch reactors of 100 ml serum bottle, in which 40 ml of blended activated and primary sludge from Cranfield University wastewater works was inoculated with 10 ml seed anaerobic sludge from Cotton Valley anaerobic treatment plant. The inoculated media were allowed to equilibrate for one hour at 35 °C before initiating the test.

Prior to inoculation, the seed inoculum was incubated at 35 °C for 5 – 10 days until no significant biogas generation was detected. The sludge-inoculum mix was homogenized and the pH adjusted with sodium bicarbonate to 7.2 ± 0.2 and transferred into the serum bottles. The substrate-inoculum mixtures were spiked with different concentrations of mixed ENPs and metal salts as reported in Table 4.2 to provide a range from non-inhibitory to acutely toxic effects. A control without ENPs or metal salts was also set up. The experiment was carried out in duplicate reactors and incubated at 35 ± 0.2 °C with continuous agitation at 120 rpm for 42days (Figure 4.3). Gas production was measured using a 10 ml glass syringe equipped with 20-gauge needle as described by Owen et al., (1979). Prior to sampling, the syringe was flushed with nitrogen. The syringe full of gas was removed and injected into Servomex 1440 gas analyser (Crowborough, UK) for measurement of methane content.

Table 4.2 Concentration of ENPs and metal salts spiked into the lab and pilot-scale AD. The concentrations are based on Transatlantic Initiative on Nanoparticles and Environment (TINE) projection of the worst case scenario of ENPs concentration in sewage sludge

| | Concentration (mg $L^{-1}$) of ENP or metal salts | | | | | |
|---|---|---|---|---|---|---|
| | A* | B | C | D | E | F* |
| Ag | 5.01 | 50 | 100 | 150 | 200 | 250 |
| Ti | 40.08 | 250 | 500 | 1000 | 1500 | 2000 |
| Zn | 56.12 | 500 | 1000 | 1500 | 2000 | 2800 |

*Series A (spiked) and F (expected) concentration in the pilot-scale anaerobic digestate

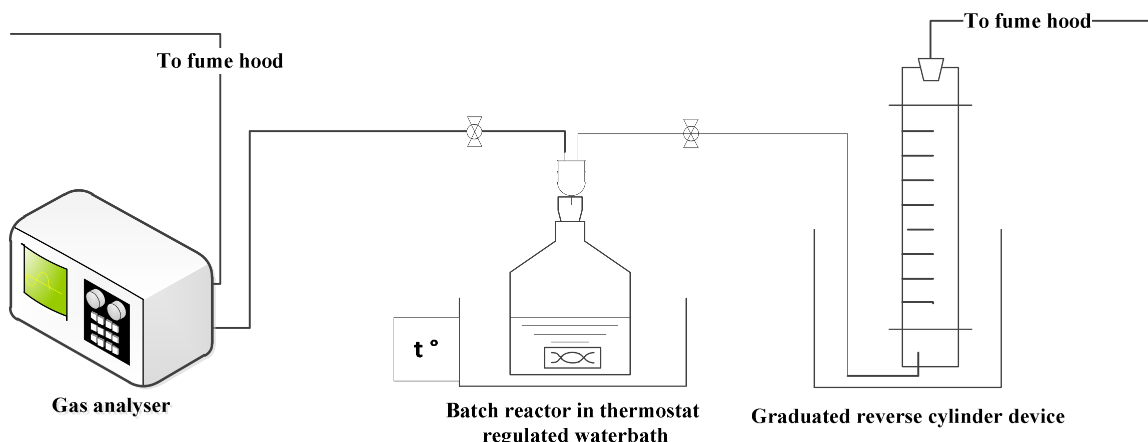



Figure 4.3 Schematic diagram of bio-methane potential test set-up with the batch reactor incubated at 35 ± 0.2 °C and stirred at 120 rpm for 42 days.

Gas volume was determined by volumetric method (Valcke and Verstraete, 1983). The reactor was connected to a graduated reverse cylinder device filled with water as a barrier solution and the liquid displacement measured and converted to biogas volume using the formula:

$$\text{Biogas [ml]} = \pi r^2 h * \frac{k+H-h}{k}$$

Where,

r = internal radius of the column (cm);

h = production of biogas as water level decreased in the column (cm);

H = working length of gas collection column (cm);

$k$ = standard atmospheric pressure (1033 cm water gauge).

Results are expressed as means of duplicate determinations from the experimental reactors.

### 4.2.7 Measurement of volatile fatty acids (VFA)

To measure the changes in VFA composition, duplicate digestate samples were centrifuged at 5000 rpm for 20 min and filtered through 0.45 µm and then 0.2 µm syringe filter. 10 µl of sulphuric acid was added to 9 ml of filtered sample and frozen until analysis. Volatile fatty acid (VFA) concentrations were determined by high performance liquid chromatography (HPLC) on Kontron 535 detector (Bio-Tek, Vermont, USA) with a Bio-Rad (California, USA) HPLC column for fermentation

monitoring. The column was maintained at 60 $^{o}C$ with an eluent of 1 mM $H_2SO_4$ at a flow rate of 0.8ml $min^{-1}$. VFA concentrations were detected with ultraviolet light at 208 nm.

### 4.2.8 Statistical analysis

Kruskal-Wallis test was performed using *Statistica* software® version 11 (Statsoft, Tulsa, OK, USA). Values are presented as mean ± standard deviation with levels of significance maintained at 95% for each test. PLFA and 454 pyrosequencing profiles of biomass data were log-transformed to reduce skewness in distribution, subjected to species-dependent hierarchical cluster analysis and non-metric multidimensional scaling (MDS) ordination based on Bray-Curtis similarities using PRIMER version 6 (Clark and Warwick, 2001).

## 4.3 Results and discussion

### 4.3.1 Residual ENPs and metal salt concentration in filtrate and digestate

The mean residual concentration of ENPs and metal salts from the three treatment plants are presented in Table 4.3 as ions of $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ because of the difficulty of speciation between nano- and bulk forms of the metal oxides. The concentration of ions in the control digestate cake represents the baseline concentration of ENP or metal salts ion released from ENP-enabled consumer products into the treatment plant. The concentration of ions in the ENPs spiked digestate cake was about 0.9 to 1.2 times lower than in the metal salt spiked AD cake and indicates that the aged-ENPs mixture was more soluble and in the oily tinged digestate than the metal salts. Although the octanol-water affinity coefficient of mixed ENPs and metal salts was not determined in this study, the result suggests that the sorption behaviour of ENP differs from the metal salt ion in anaerobic digestate. As a result, a mean of 57 % of the spiked concentration of ENP was recovered compared to 63 % of the metal salt ions.

Table 4.3 Concentration of ENPs and metal salts ions in the filter-pressed anaerobic digestate cake from pilot-scale plant

| Spiked concentration | Average quantity in digestate cake (mg $kg^{-1}$ dry weight) | | |
|---|---|---|---|
| | $Ag^+$ | $Ti^{4+}$ | $Zn^{2+}$ |
| | 250 | 2000 | 2800 |
| | **Batch 1 (1-160 days)** | | |
| Control AD | 5.4 ± 0.9 (n.a) | 1429 ± 234 (n.a) | 768 ± 60 (n.a) |
| Metal salt spiked AD | 84 ± 38 (34) | 1265 ± 517 (63) | 2563 ± 418 (92) |
| ENP spiked AD | 97 ± 23 (39) | 1023 ± 280 (51) | 2066 ± 217 (74) |
| | **Batch 2 (161-295 days)** | | |
| Control AD | 5.3 ± 3.4 (n.a) | 1255 ± 171 (n.a) | 745 ± 104 (n.a) |
| Metal salt spiked AD | 160 ± 46 (63) | 1271 ± 133.4 (64) | 1693 ± 455 (61) |
| ENP spiked AD | 146 ± 39 (58) | 1257 ± 20 (63) | 1633 ± 277 (58) |
| | **Average quantity in digestate filtrate (mg $l^{-1}$) (Batch 2)** | | |
| Control AD | 0.03 ± 0.0 | 0.07 ± 0.0 | 0.22 ± 0.12 |
| Metal salt spiked AD | 0.09 ± 0.12 | 0.75 ± 0.44 | 0.6 ± 0.6 |
| ENP spiked AD | 0.04 ± 0.02 | 0.4 ± 0.3 | 0.31 ± 0.01 |

Values are mean ± standard deviation, (%) of ENPs and metal salts partitioned into digestate cake (duplicate, n = 60). Each value was corrected against the control. Results are expressed as Ag+, $Ti^{4+}$ or $Zn^{2+}$ ion concentrations due to difficulty in speciation between ENPs or metal salts. Batch 1and 2 = timeline for achieving the spiked concentration in the reactor. n.a = not applied.

### 4.3.2 Effect of ENPs on pilot plant biogas production and quality

The rate of biogas production is an indicator of digestion efficiency and biosolids stabilisation during AD process. The differences in the biogas produced from the three digesters were low but significant at $p < 0.05$ (Figure 4.4).

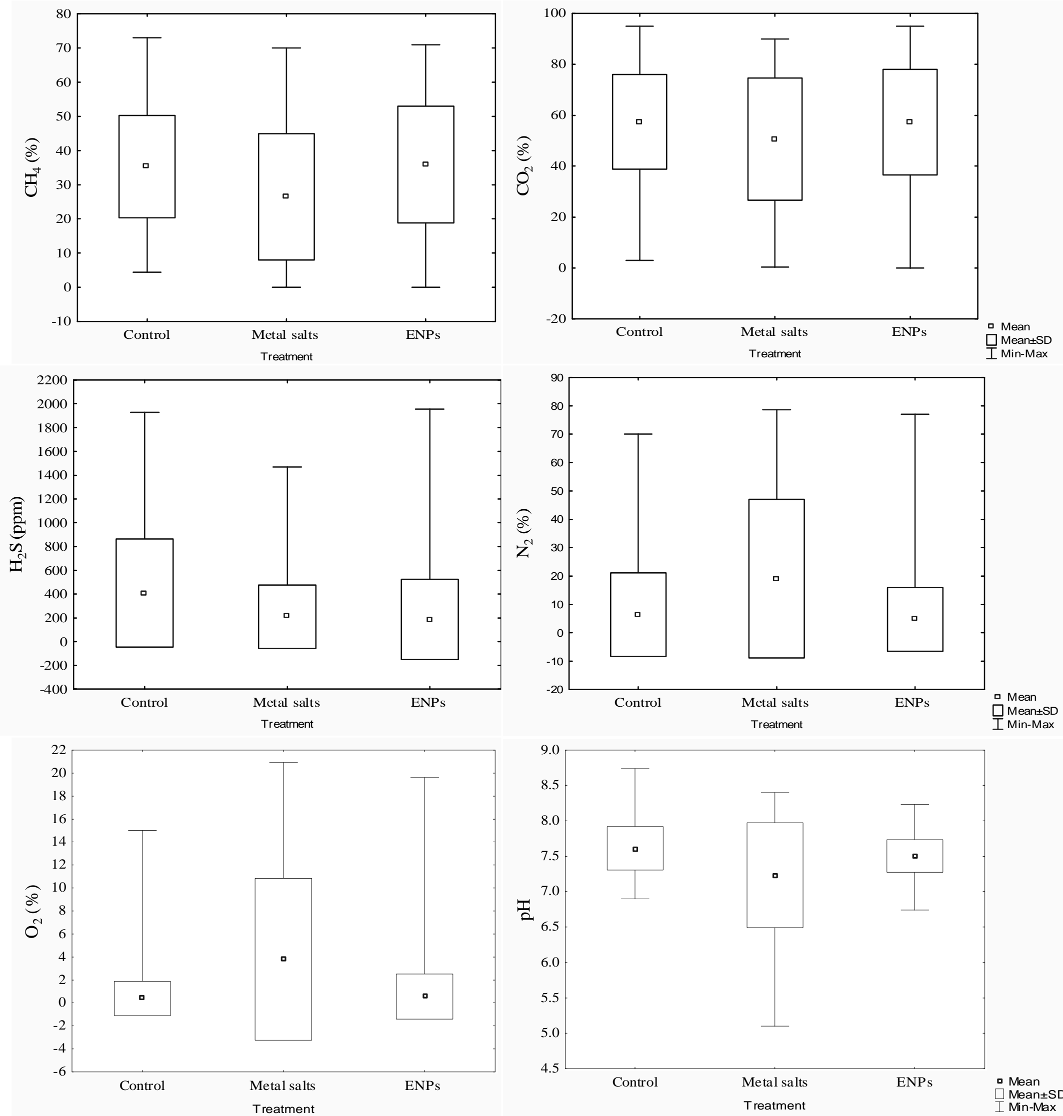



Figure 4.4 Treatment effect on pH and biogas production of the pilot-scale AD plants over 295 days of digestion.

A maximum of 73% (control), 70% (metal salts) and 71% (ENPs) methane production was recorded at day 22, 223 and 141 days, respectively (Figure 4.5) indicating there was a time lag for both microbial adaptation to the ionic concentrations of metal salts and ENPs formed in the reactors and to reach optimum methane production compared to the control. Between day 263 and day 295, $CH_4$ production varied from 33 to 60 % (control AD), 29 to 60 % (metal salt spiked AD) and 24 to 50 % in ENP spiked AD.

At the initial stage however, the digestion process was unstable which contributed to the variable biogas generated in the digesters. In addition, decreased methane production

was as a result of biomass washout, partial degradation of substrates, pulse disturbance due to substrate addition/digestate extraction and temperature fluctuations. It is common knowledge that anaerobic microbial substrate utilisation increases with a rise in temperature, thus, fluctuations can exert negative effect on the anaerobic conversion process and methanogenic activity. Indeed, digestate temperature fluctuations in excess of 0.6 – 1.0 °C $day^{-1}$, however, can affect anaerobic digestion process (Turovskiy and Mathai, 2006).

In this study, digestate temperature dropped to 10 ± 5 °C with fluctuations during feeding suggesting that temperature was one of the factors affecting methane production. At the sub-optimum operational temperature in the reactors, anaerobic microbial growth and reaction rate was expected to decrease resulting in reduced formation of metabolic products. It is suspected that hydrogenotrophic methanogenesis and acetate formation from hydrogen and bicarbonate that requires less energy and can proceed at low temperature (Lettinga et al., 2001) was probably one of the pathways to generate methane. From day 121 to 275 which was in the summer, the average methane production increased substantially as the reactor operation stabilized although with significant biomass washout. Overall, metal salts exerted more inhibitory effect on microbial activities in the AD indicated by the low methane production compared with the control.

Interestingly, hydrogen sulphide ($H_2S$) content of the biogas from the three reactors differed substantially (Figure 4.5). $H_2S$ was at least 2 times lower in biogas from ENP spiked AD compared to the metal salt spiked digestate suggesting that ENPs composites inhibited the activities of sulphate-reducing bacteria (SRB). The SRB are found mostly among the *Deltaproteobacteria* and *Firmicutes* and are responsible for generating $H_2S$, $HS^-$ and $S^2$ in solution and $H_2S$ in biogas from the reduction of sulphates (Wagner et al., 1998; Speece and Parkin, 1983). Although the presence of $H_2S$ in the digestate was not determined, its concentration in the gaseous phase suggests that the activities of SRB were negatively affected by the presence of ENPs. The accumulation of sulphide in the digester is undesirable because of the potentially negative effect on microbes and the digestion process. For instance, about 50 % and complete inhibition of aceticlastic methanogens by 50 and 200 mg $l^{-1}$ of $H_2S$ respectively have been reported (Kroiss and Wabnegg, 1983). In addition, the presence of sulphide can induce precipitation of nonalkali metals thus reducing their availability for use by microorganisms. Nonalkali

metals are required for microbial growth and therefore methane production can be negatively affected in their absence (Isa et al., 1986).

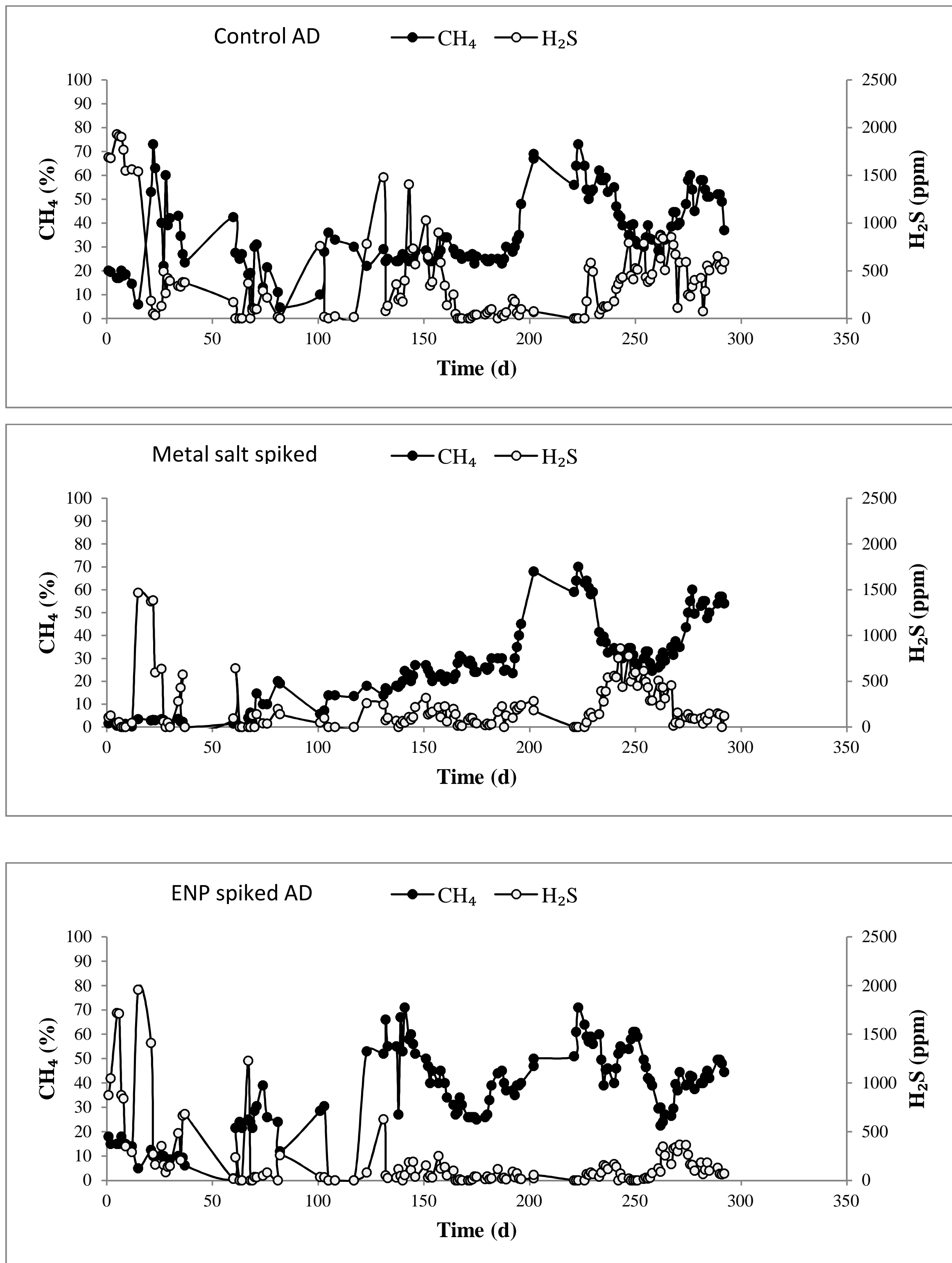



Figure 4.5 Influence of ENPs and metal salts on methane and hydrogen sulphide production during pilot scale anaerobic digestion of sewage sludge in relation to unspiked control

Another plausible reason for the low $H_2S$ production is the sulfidation reaction in the reactors. For example, $Ag^+$ ions are known to replace $Zn^{2+}$ ions in Zn-sulfide complexes plausibly because $Ag_2S$ is thermodynamically more stable than ZnS (Rozan et al., 2000). Again, sulfidation generally known to take place under anaerobic conditions may attenuate or enhance ENPs effect in AD. For instance, a recent study by Reinsch et al., (2012) illustrates the mitigating effect of $Ag^0$ sulfidation on the growth inhibition of *E. coli.* The authors suggest that sulfidation products of metal oxide ENPs such as $Ag^0$ released into the environment can mitigate their toxic potential on microbial growth. Also, heavy metal such as cobalt, copper, nickel lead and zinc can form precipitate with sulfate thus reducing their toxic effect (Chen et al., 2008; Kugelman and Chin, 1971; Lawrence and McCarty, 1965). It appears from the result (Figure 4.5) that the $H_2S$ concentration did not disrupt methane produced in the ENP spiked reactor, probably through a combined inhibiting effect on SRB and formation of complexes with ligands which attenuated the toxic effect of ENPs.

### 4.3.3 Effect of treatment on volatile fatty acid formation in the pilot-scale plant

The concentration of the total volatile fatty acid was highly variable across the 3 reactors (Figures 4.6). There was however, no significant difference in the VFA concentration between the treatments which may suggests that ENP or metal salts ions did not substantially affect VFA accumulation or degradation. At the same time, the variability of individual VFA concentration in the reactors does not indicate that microbial activities or digestion process was inhibited. The low residence time, biomass washout, temperature fluctuations and reactor feeding rate are probable reasons that contributed to the variable VFA concentration. The acetic acid accumulation in the reactors can be as a result of hydrogenotrophic methanogenesis taking place under low temperature (Lettinga et al., 2001).

3-methylbutanoic acid ($C_5H_{10}O_2$) commonly called isovaleric acid is a major end product of amino acid metabolism in different cultural conditions (Thierry et al., 2002). Isovaleric acid concentration in the control AD was about 2 times lower than in the ENP and metal salt spiked AD and can be attributed to the effect of treatment on the organisms involved in amino acid degradation.

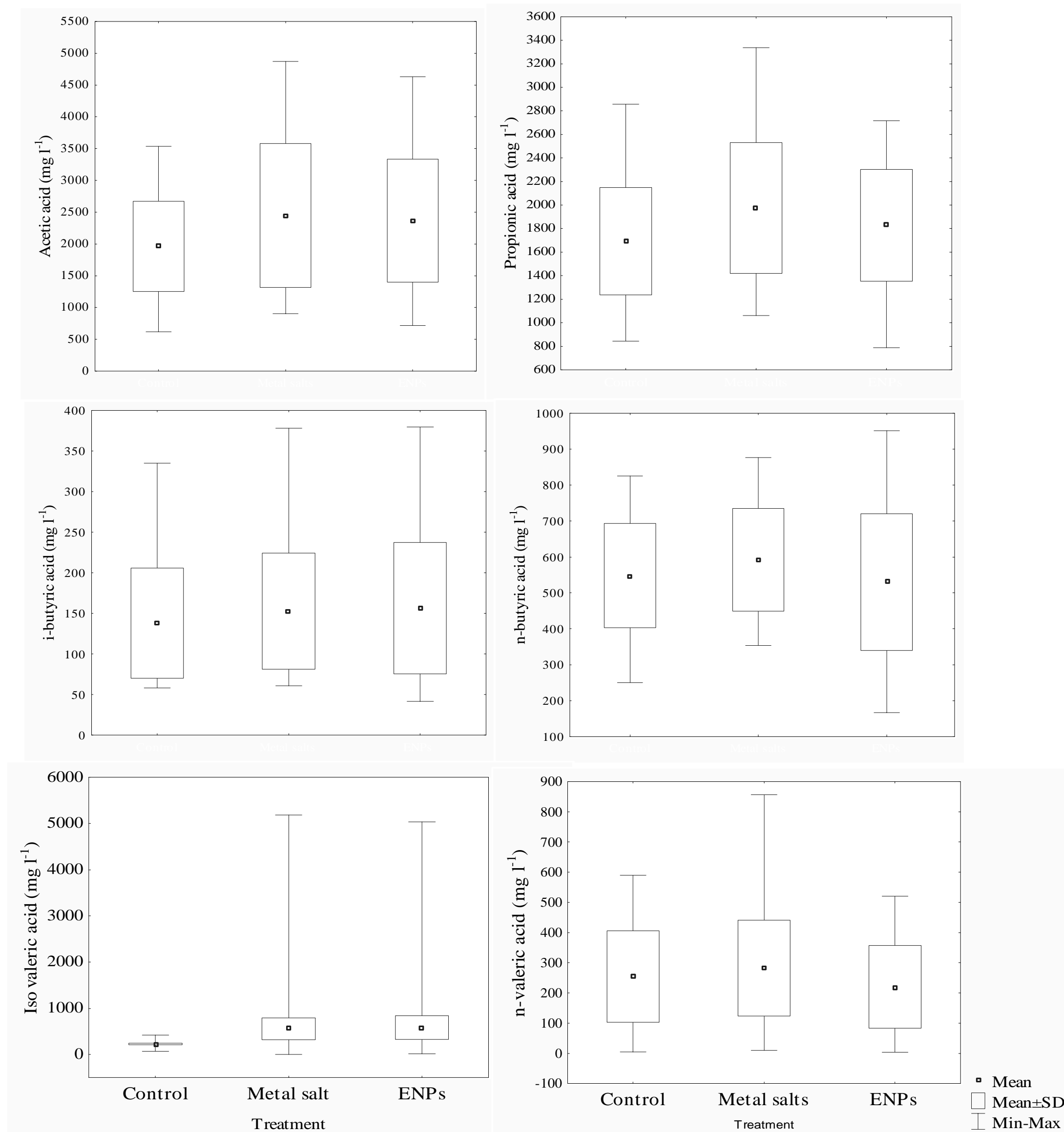



Figure 4.6 Effect of ENP and metal salts on the VFA production in the pilot plant AD plants

Usually, proteolysis by anaerobic organisms (Table 4.4) such as *Proteiniphilum acetatigenes* (Chen and Doug, 2005) and *Cloacibacillus evryensis* (Ganesan et al., 2008) results in the formation of amino acids regarded as precursors of glucose, fatty acids and ketones which can undergo transamination to α-keto acid and converted to isovaleric acid by multi enzymes complex found in most resting cells (Figure 4.7) (Thierry et al., 2002; Kaneda, 1991).

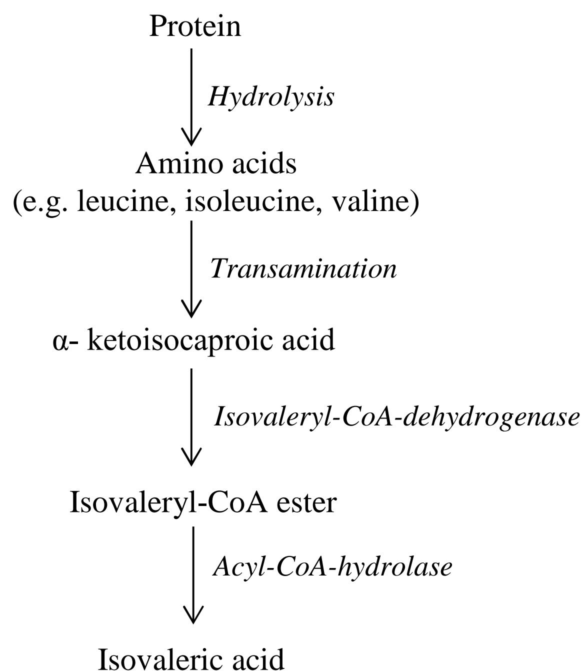



Figure 4.7 Schematic illustration of the pathway for isovaleric acid production by resting cells during anaerobic digestion of organic matter

During bacterial growth associated with high lytic activities in a complex medium however, low amounts of isovaleric acid are usually produced in contrast to the high amount produced by resting cells. Indeed, about 80 % of isovaleric acid is produced by cells during stationary phase of growth (Thierry et al., 2002). Isovaleric acid occurs in lipid and is produced by microorganisms to provide specific physical properties to structural molecules as they undergo post synthetic modification required to maintain membrane fluidity for growth under adverse conditions (Kaneda, 1991). Therefore, the low concentration of isovaleric acid in the control AD clearly shows that the cells were metabolically active compared to the resting state of most cells in ENP and metal spiked AD. On the other hand, the microbes in the ENP and metal salt spiked AD in their resting state plausibly were producing isovaleric acid to make the membranes impervious to the high concentration of toxic ions in the digestate. For example, members of *Methanosarcina* can reduce biomass growth and behave as resting cells while still producing methane (Welander and Metcalf, 2005). It is also plausible that the anaerobic saccharolytic and non-saccharolytic bacteria (Diaz et al., 2007 and references therein) in syntrophic association with hydrogenotrophic methanogens (Stams 1994;

Schink, 1997) that can degrade amino acid were stimulated by the concentration of ENPs and metal salts in the digestate.

In addition, temperature can influence the amount of isovaleric acid produced, for instance, at ≤ 24 $^{0}$C more isovaleric acid can be formed by various bacterial species than at 30 $^{0}$C (Hofherr et al., 1983; Kaneda, 1991; Klein et al., 1999). Also, more isovaleric acid can be produced in the presence of cofactors such as $NAD^{+}$ and NADH (Thierry et al., 2002; Christensen et al., 1999). It is thus plausible to assume that a combined effect of temperature fluctuations and inhibiting effect of ENPs and metal salt ions may have forced the cells into a state of rest and contributed to the high amount of isovaleric acid observed. Furthermore, ENP and metal salts ions may have induced a positive effect on the enzymes involved in the conversion by acting as cofactors in the production of isovaleric acid.

### 4.3.4 Effect of mixed ENPs on bacterial community structure based on PLFA fingerprint

To understand the effect of ENPs and metal salts on anaerobic bacterial community structure, phospholipid fatty acid fingerprint was carried out and their phylogenetic affiliations are shown in Chapter 3 (Table 3.2). Key PLFA biomarkers of the bacterial community identified in the AD are presented in Figure 4.8. The dominant PLFA markers were from members of the *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, *Actinobacteria* and unclassified bacteria.

The bacterial biomass in ENP spiked digester was approximately 3 times higher than in the control and metal salt spiked digestate indicating that the aged-ENPs concentration stimulated the growth of certain bacterial groups and or species in the digester. There was a temporal shift in the bacterial biomass structure in the digesters as a result of the treatment (Figure 4.8). The change in bacterial community structure was more pronounced in the presence of ENP than metal salts compared with the control at the initial stage of treatment. For instance, the similarity of ENP spiked sample was < 80 % in regards to the other samples (Figure 4.9). The 90 % similarity in bacterial PLFA (clusters 1, 2 and 3) suggests a homogenous PLFA distribution between the bacterial groups present in the reactors. The increase in bacterial abundance however, did not influence the biomass structure and diversity of the methanogens in the ENP spiked digester.

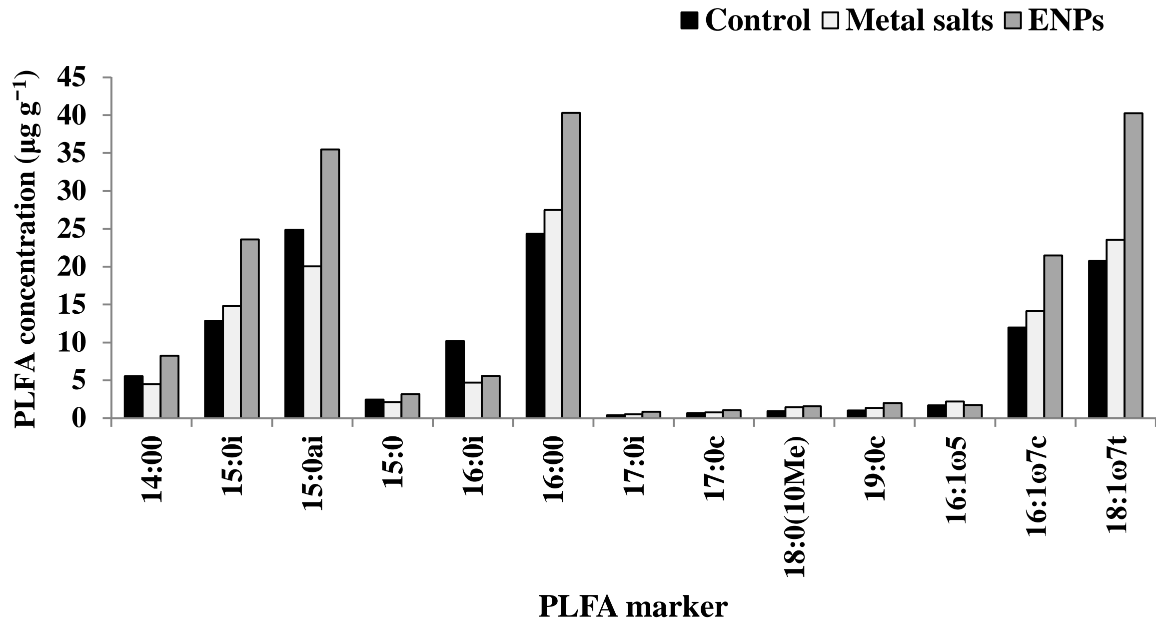



Figure 4.8 Diversity of bacterial phospholipid fatty acid fingerprint in anaerobic digestate over 115 days of treatment

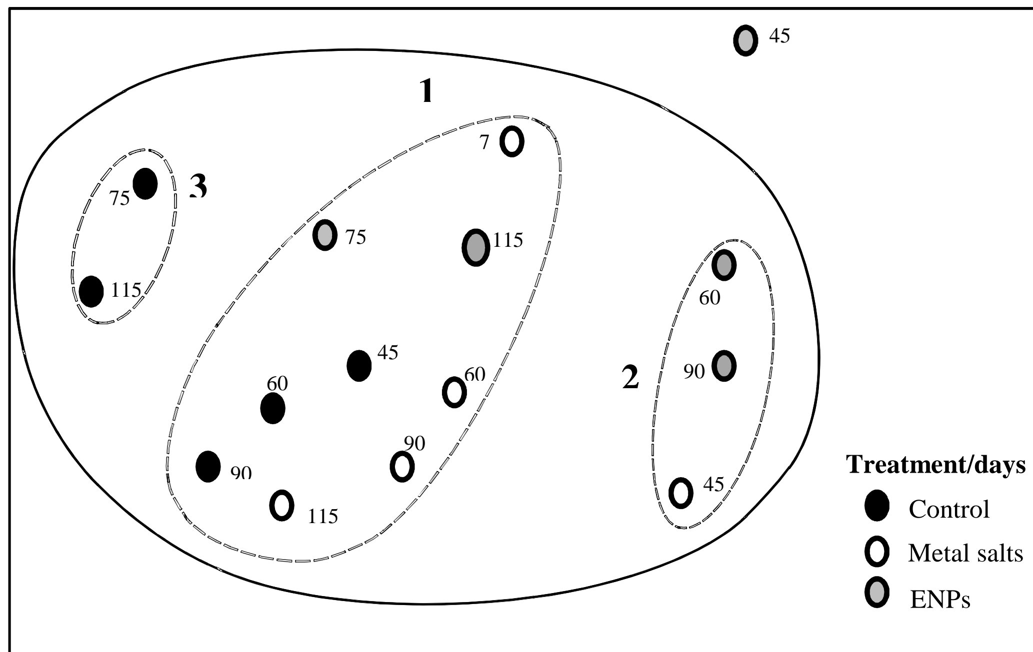
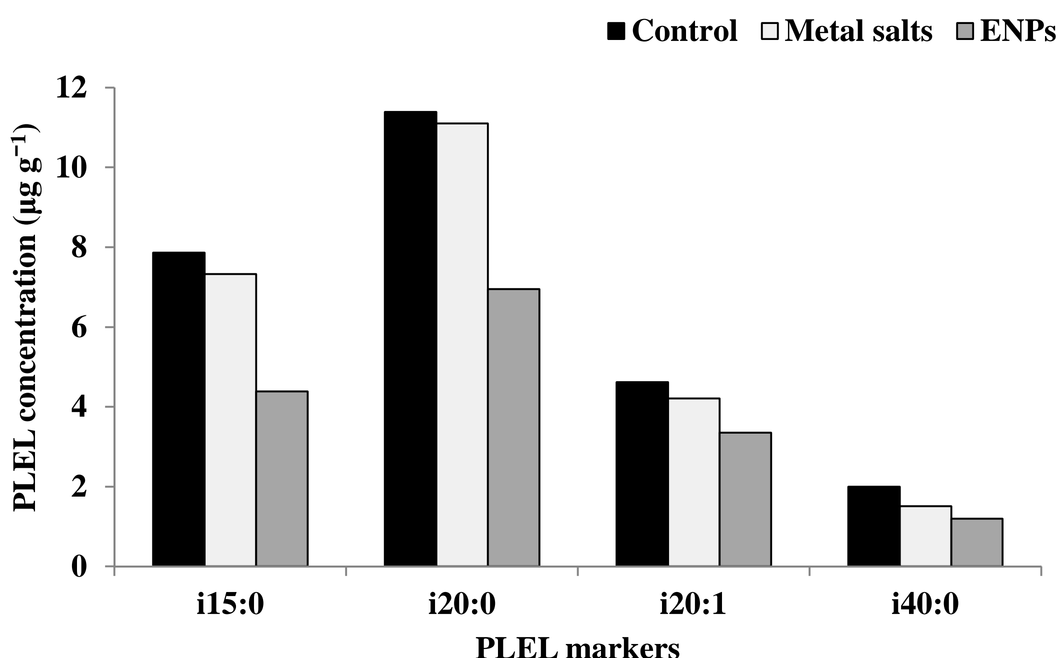



Figure 4.9 MDS plot of bacterial PLFA showing temporal shift in the community structure. Dashed lines represent 90 % similarity and the solid line represents 80 % similarity.

### 4.3.5 Inhibitory effect of mixed ENPs on anaerobic archaeal methanogens based on PLEL fingerprint

The PLEL concentration between day 45 and 250 is presented in Figure 4.10 The result shows 4 key PLEL biomarkers identified as i15:0, i20:0, i20:1 (aceticlastic methanogens) and i40:0 (hydrogenotrophic methanogens). PLEL biomarkers in the ENP spiked digestate were about 1.2 to 2 times lower than in the control and metal salt spiked digesters. The result clearly indicates that methanogenic archaeal biomass was negatively affected by aged-ENPs at the spiked concentration. This reduction in archaeal abundance can be related to the concentration of ENPs ions in the digestate (Table 4.3) suggesting that ENP were more soluble in the oily tinged digestate and hence contact with microorganisms was enhanced. In contrast, metal salts were less soluble and associated more firmly to the digestate and therefore were limited in their interaction or contact with the microorganisms.




Figures 4.10 Methanogenic Archaeal phospholipid etherlipid fingerprint in anaerobic digestate.

The reduction in abundance of methanogens in the ENP spiked AD compared with the control and metal salt spiked AD demonstrates the potential effect of ENP and microbial interaction with the increasing use, release and sorption of ENPs to biosolids in wastewater treatment plants. Biomass washout and inhibitory effect of ENP and metal salts on the anaerobic microbial biomass contributed to the low PLEL concentration in the samples. Further to this, the results in Figure 4.11 suggest that at 70 % similarity level, the samples were grouped into 4 discrete clusters across the three plants. The sub-clusters at 80 % similarity suggest a steady changing pattern in the biomass concentration. In addition, the tight clustering of the different samples indicates that the methanogens were much less biodiverse or their taxonomic relatedness was almost similar in the three plants. As will be seen later in the pyrosequencing result, a clearer insight into the nature of the methanogens is provided.

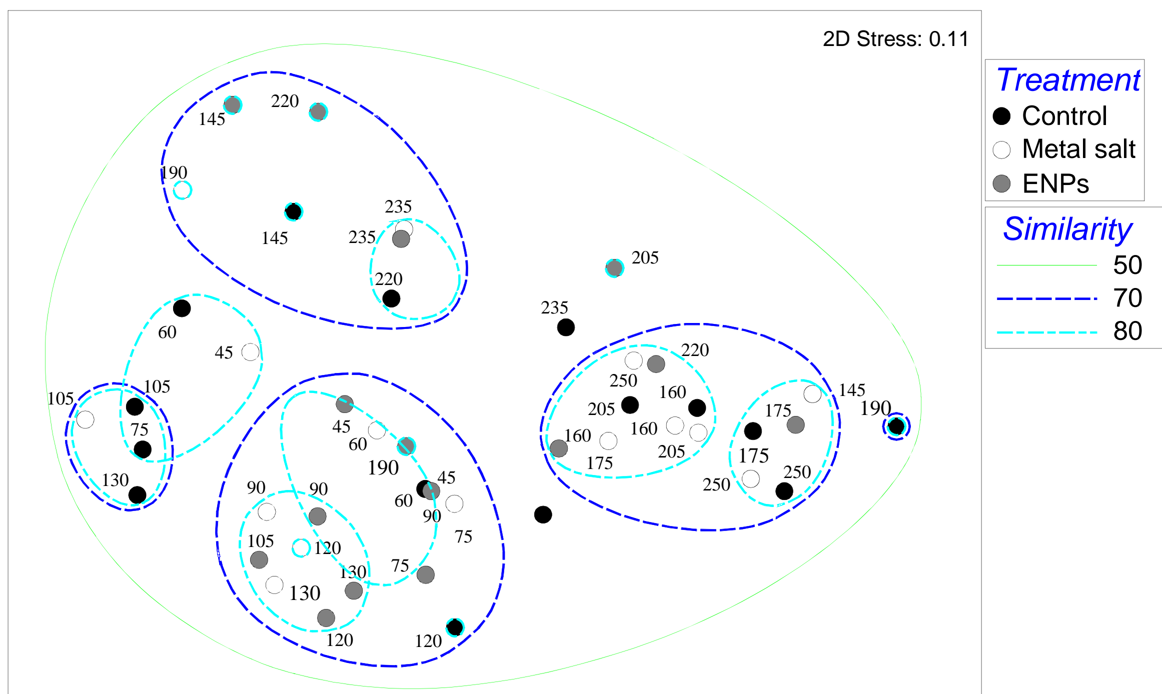



Figure 4.11 MDS plot of the PLEL concentration showing temporal shift in microbial community structure in the anaerobic digesters based on Bray-Curtis similarity

#### 4.3.6 Aged-ENPs effect on anaerobic bacterial community abundance and diversity based on 454-pyrosequencing

The response of the dominant anaerobic bacterial phyla to $Ag^0$, $TiO_2$ and ZnO during anaerobic digestion is shown in Figure 4.12. The closest cultivable relative and

percentage similarity of the bacterial species found in the AD is shown in Table 4.3. The *Firmicutes*, *Proteobacteria*, *Bacteriodetes* and unclassified bacteria were the most dominant phyla in all digesters.

*Firmicutes* relative abundance was 1.3 to 1.9 times lower in ENP spiked anaerobic digestate while unclassified bacteria abundance was 4 to 16 times higher compared to the control and metal salt spiked anaerobic digestate respectively (Figure 4.14). Similarly, the relative abundance of *Proteobacteria* and *Bacteriodetes* was about 1 to 3 times lower in the ENP spiked digestate compared to the control and metal salt spiked digestate. In contrast, *Fusobacteria* and *Actinobacteria* were about 0.2 to 18 and 0.34 to 2 times higher respectively in the ENP spiked. The results indicate that ENPs effect was more pronounced on the *Firmicutes*, *Bacteriodetes* and *Proteobacteria* compared to the metal salt spiked and control AD.

Most members of the *Firmicutes* such as *Clostridium sticklandii*, *C. difficile*, *C. botulinum*, *Alkaliphilus metalliredigens* are proteolytic species and can degrade organic molecules in a preferential and sequential way (Fonknechten et al., 2010). Their abundance in the digesters suggests that they played a key role in degrading complex materials during the digestion process. Members of the family *Peptococcaceae* belonging to class *Clostridia* such as *Desulfotomaculum*, *Desulfosprosinus* and *Desulfosporomusa* can reduce sulphate to $H_2S$ (Sass et al., 2004). The low $H_2S$ concentration in biogas from the ENP spiked AD (Figure 4.5) can be attributed to the negative effect of mixed ENP on the sulphate-reducing bacteria compared to the metal salt and control AD.

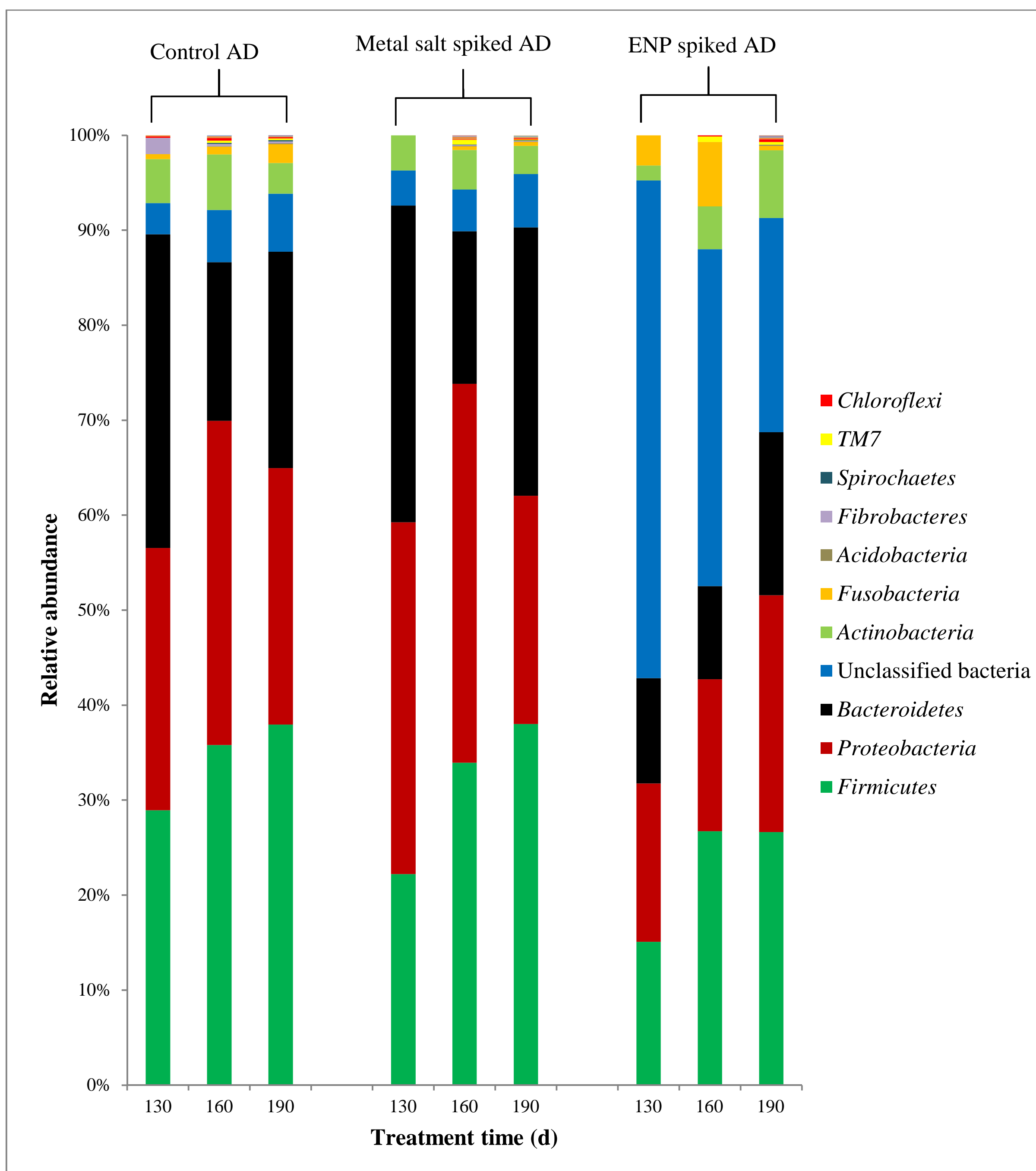



Figure 4.12 Relative abundance and diversity of microbial community in AD between day 130 and 190 with the dominance of *Firmicutes* in the control, metal salts and ENPs spiked digestate

Table 4.4 Summary of the most dominant OTUs of anaerobic bacterial community retrieved from anaerobic digestate

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 29 | **Firmicutes** | Clostridia/Clostridiales/Eubacteriaceae/*Eubacterium* | *Eubacterium* sp E-1 | 99 | AB470313 |
| 75 | | Clostridia/Clostridiales/Incertae Sedis XI/*Soehngenia* | *Clostridium ultunense* strain BS | 87 | NR026531 |
| 113 | | Clostridia/Clostridiales/Peptostreptococcaceae/*Peptostreptococcus* | *Peptostreptococcus russellii* strain RT-19A | 99 | AY167957 |
| 122 | | Clostridia/Clostridiales/Lachnospiraceae | *Lachnobacterium* sp G9 | 87 | AB730783 |
| 125 | | Clostridia/Clostridiales/Lachnospiraceae/*Lachnobacterium* | *Lachnobacterium* sp Wal 14165 | 88 | AJ518873 |
| 139 | | Clostridia/Clostridiales/Ruminococcaceae/*Ruminococcus* | *Ruminicoccus* sp 7L75 | 92 | AJ515914 |
| 142 | | Clostridia/Clostridiales/Incertae Sedis XV/*Anaerobaculum* | *Anaerobaculum mobile* strain NGA | 85 | NR028903 |
| 257 | | Clostridia/Clostridiales/Ruminococcaceae | *Clostridium orbiscindens* strain 17 | 83 | GU968170 |
| 269 | | Bacilli/Lactobacillales/Lactobacillaceae/*Lactobacillus* | *Lactobaccillus vaginalis* strain DoxG3 | 97 | GQ422709 |
| 283 | | Clostridia/Clostridiales/Incertae Sedis XIII/*Anaerovorax* | *Anaerovorax odorimutans* strain NorPut | 86 | AJ251215 |
| 284 | | Clostridia/Clostridiales/Lachnospiraceae | *Anaerostipes caccae* | 87 | AB243986 |
| 294 | | Clostridia/Clostridiales/Clostridiaceae/Clostridiaceae 1 | *Clostridium* sp L15 | 89 | AY337519 |
| 354 | | Clostridia/Clostridiales/Incertae Sedis XV/*Anaerobaculum* | *Anaerobaculum* sp OS1 | 94 | FJ862996 |
| 358 | | Clostridia/Clostridiales/Ruminococcaceae | *Hydrogenoanaerobacterium saccharovorans* strain SW512 | 84 | NR044425 |
| 380 | | Clostridia/Clostridiales/Incertae Sedis XI/*Sedimentibacter* | *Sedimentibacter hongkongensis* strain K1 | 88 | AY571338 |
| 385 | | Clostridia/Clostridiales/Incertae Sedis XV | *Aminomonas paucivorans* | 85 | AF072581 |
| 385 | | Clostridia/Clostridiales/Incertae Sedis XV | *Cloacibacillus evryensis* gen. nov. sp. nov | 86 | CU463952 |
| 445 | | Root/Bacteria/Firmicutes | *Clostridium spiroforme* | 82 | X73441 |
| 555 | | Clostridia/Clostridiales | *Cloacibacillus* evryensis gen. nov. sp. nov | 90 | CU463952 |
| 598 | | Clostridia/Clostridiales/Peptostreptococcaceae /Incertae Sedis | *Clostridium lituseburense* strain H17 | 95 | EU887828 |
| 648 | | Clostridia/Clostridiales/Lachnospiraceae | *Lachnobacterium* sp Wal 14165 | 88 | AJ518873 |
| 766 | | Clostridia/Clostridiales/Peptostreptococcaceae /Incertae Sedis | *Clostridium glycolicum* stain SN10 | 97 | EU887819 |
| 772 | | Firmicutes | *Clostridium difficile* 630 | 80 | AM180355 |
| 794 | | Firmicutes | *Lachnobacterium* sp Wal 14165 | 82 | AJ518873 |

Table 4.4 continued.

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 807 | **Firmicutes** | Clostridiaceae/Clostridiaceae 1/*Clostridium* | *Clostridium paraputrificum* G12 | 96 | AY343974 |
| 909 | | Erysipelotrichi/Erysipelotrichales/Erysipelotrichaceae/*Erysipelothrix* | *Erysipelothrix rhusiopathiae* strain 470/87 | 89 | EF050040 |
| 973 | | Erysipelotrichi/Erysipelotrichales/Erysipelotrichaceae/*Bulleidia* | *Bulleidia extracta* strain W1365 | 89 | Z36271 |
| 1147 | | Clostridia/Clostridiales/Incertae Sedis XI/*Sedimentibacter* | *Sedimentibacter* sp MO-SED | 90 | AB598275 |
| 1210 | | Clostridia/Clostridiales/Ruminococcaceae" | *Ruminococcus* sp YE78 | 86 | KF156793 |
| 1276 | | Clostridia/Clostridiales | *Clostridiales bacterium* JN18 A24 | 91 | DQ168655 |
| 1351 | | Clostridia/Clostridiales/Ruminococcaceae | *Ruminococcus* sp C047 | 82 | AB064904 |
| 1356 | | Clostridia/Clostridiales/Veillonellaceae/*Succinispira* | *Veillonella tobetsuensis* | 86 | AB679110 |
| 1447 | | Clostridia/Clostridiales/Veillonellaceae | *Veillonella tobetsuensis* | 88 | AB679110 |
| 1655 | | Clostridia/Clostridiales/Ruminococcaceae/*Ethanoligenens* | *Ethanoligens harbinense* strain CGMCC1152 | 90 | AY833426 |
| 1680 | | Clostridia/Clostridiales/Veillonellaceae | *Veillonela bacterium* WH074 | 99 | AB298734 |
| 2162 | | Firmicutes | *Clostridium saccharoqumia* strain SDG-Mt85-3Db | 83 | NR043550 |
| 2389 | | Clostridia | *Clostridium* sp U40A-5 | 83 | AB059480 |
| 2477 | | Bacilli/Lactobacillales/Enterococcaceae/*Enterococcus* | *Enterococcus devriesei* strain LMG 13603 | 97 | DQ010644 |
| 2489 | | Clostridia/Clostridiales/Incertae Sedis XV | *Cloacibacillus evryensis* gen. nov. sp. nov | 90 | CU463952 |
| 2499 | | Clostridia/Clostridiales/Ruminococcaceae/*Faecalibacterium* | *Fecalibacterium prausnitzii* strian S4L/4 | 84 | HQ457025 |
| 2653 | | Clostridia/Clostridiales/Clostridiaceae/Clostridiaceae 1/*Clostridium* | *Clostridium* sp | 90 | X95274 |
| 3070 | | Bacilli/Lactobacillales/Enterococcaceae/*Vagococcus* | *Vagococcus salimoninarium* | 97 | X54272 |
| 3081 | | Erysipelotrichi/Erysipelotrichales/Erysipelotrichaceae/*Turicibacter* | *Turicibacter sanguinis* strain PC909 | 99 | HQ428099 |
| 3227 | | Clostridia/Clostridiales/Peptostreptococcaceae /Incertae Sedis | *Clostridium metallolevans* strain SN1 | 94 | EU887815 |
| 3494 | | Bacilli/Lactobacillales/Carnobacteriaceae 1/*Carnobacterium* | *Carnobacterium* sp BBDP 71 | 93 | DQ337531 |
| 3572 | | Clostridia/Clostridiales/Veillonellaceae/*Selenomonas* | *Selenomonas ruminantium* | 91 | AB198442 |
| 287 | **Proteobacteria** | Gammaproteobacteria/Xanthomonadales/Xanthomonadaceae | *Pseudoxanthomonas* sp CHNTR 38 | 94 | DQ337597 |
| 359 | | Alphaproteobacteria | *Sphingopyxis* sp MC1 | 88 | JN940802 |

Table 4.4 continued

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 389 | **Proteobacteria** | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Amaricoccus* | *Amaricoccus kaplicensis* strain Ben 101 | 98 | NR029201 |
| 397 | | Alphaproteobacteria/Rhizobiales/Rhizobiaceae/*Kaistia* | *Kaista granuli* strain Ko04 | 94 | NR041362 |
| 469 | | Betaproteobacteria/Rhodocyclales/Rhodocyclaceae/*Shinella* | *Shinella zoogloeoides* | 99 | AB698675 |
| 485 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Rhodobacter* | *Rhodobacter* sp TUT3732 | 93 | AB251408 |
| 542 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/*Rhodoferax* | *Rhodoferax* sp B3 | 98 | DQ268771 |
| 564 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Rhodobaca* | *Rhodobacter* sp TCRI 3 | 94 | AB017796 |
| 602 | | Alphaproteobacteria/Rhizobiales | *Methylocella silvestris* BL 2 | 90 | CP001280 |
| 617 | | Gammaproteobacteria/Legionellales/Coxiellaceae/*Rickettsiella* | *Rickettsiella grylli* | 95 | U97547 |
| 649 | | Alphaproteobacteria/Rhizobiales | *Oligotropha carboxidovorans* | 90 | AB099659 |
| 727 | | Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/*Sphingopyxis* | *Sphingopyxis witflariensis* strain W-50 | 99 | NR028010 |
| 749 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Rhodobacter* | *Rhodobacter* sp TUT 3732 | 97 | AB251408 |
| 792 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae | *Rhodobacter* sp EMB 174 | 95 | DQ413163 |
| 983 | | Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/*Novosphingobium* | *Novosphingobium hassiacum* strain W-51 | 99 | NR028962 |
| 1046 | | Alphaproteobacteria/Rhizobiales/Phyllobacteriaceae/*Hoeflea* | *Hoeflea alexandrii* strain AMIV30 | 99 | NR042321 |
| 1123 | | Betaproteobacteria/Neisseriales/Neisseriaceae/*Uruburuella* | *Uruburuella suis* strain A51 | 99 | HQ259692 |
| 1206 | | Betaproteobacteria/Burkholderiales | *Herbaspirillum* sp | 91 | AJ012069 |
| 1579 | | Alphaproteobacteria/Rhizobiales/Hyphomicrobiaceae/*Hyphomicrobium* | *Hyphomicrobium* sp M3 | 97 | AF098790 |
| 1696 | | Gammaproteobacteria/Pseudomonadales/Moraxellaceae/*Acinetobacter* | *Acinetobacter iwoffii* strain JUN-5 | 97 | KF228924 |
| 1706 | | Gammaproteobacteria/Pseudomonadales/Pseudomonadaceae/*Flavimonas* | *Pseudomonas* sp HY-14 | 98 | EU620679.2 |
| 2143 | | Betaproteobacteria | *Rhodoferax ferrireducens* | 90 | AF435948 |
| 2410 | | Betaproteobacteria/Burkholderiales/Comamonadaceae/*Acidovorax* | *Acidovorax defluvii* strain BSB411 | 93 | NR026506 |
| 2490 | | Alphaproteobacteria/Rhodobacterales/Rhodobacteraceae/*Paracoccus* | *Paracoccus* sp BBTR62 | 98 | DQ337586 |
| 2592 | | Alphaproteobacteria/Sphingomonadales/Sphingomonadaceae/*Sphingosinicella* | *Spingosinicella microcystinivorans* | 95 | AB219940 |
| 2823 | | Alphaproteobacteria/Rhizobiales/Bradyrhizobiaceae/*Bosea* | *Bosea thiooxidans* strain E14 | 98 | AY488508 |

Table 4.4 continued.

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 2930 | **Proteobacteria** | Alphaproteobacteria/Rhizobiales/Bradyrhizobiaceae | *Rhodopseudomonas* sp R03 | 99 | EF219053 |
| 3001 | | Epsilonproteobacteria/Campylobacterales/Helicobacteraceae/*Sulfurimonas* | *Sulfurimonas*sp NS25-1 | 89 | AB175508 |
| 44 | **Bacteroidetes** | Sphingobacteria/Sphingobacteriales | *Myroides* sp XJ193 | 78 | GQ381279 |
| 62 | | Bacteroidales/Porphyromonadaceae/*Parabacteroides* | *Parabacteroides distasonis* ATCC 8503 | 89 | CP000140 |
| 151 | | Bacteroidales/Rikenellaceae/*Petrimonas* | *Petrimonas sulfuriphila* strain BSB411 | 94 | NR042987 |
| 362 | | Bacteroidales/Rikenellaceae/*Petrimonas* | *Proteiniphilum acetatigenes* strain TB 107 | 94 | NR043154 |
| 368 | | Bacteroidales/Prevotellaceae/*Prevotella* | *Prevotella shahii* strain EHS 11 | 85 | NR024815 |
| 871 | | Bacteroidales/Porphyromonadaceae/*Parabacteroides* | *Parabacteroides goldsteinii* strain JCM 13446 | 95 | EU136697 |
| 1684 | | Bacteroidales/Bacteroidaceae/*Bacteroides* | *Bacteroides uniformis* | 99 | AB215084 |
| 1684 | | Bacteroidales/Bacteroidaceae/*Bacteroides* | *Bacteroides uniformis* strain JCM5828 | 99 | EU136680 |
| 2017 | | Bacteroidales/Bacteroidaceae/*Bacteroides* | *Bacteroides intestinalis* | 92 | AB437413 |
| 2071 | | Bacteroidales/Rikenellaceae/*Petrimonas* | *Parabacteroides goldsteinii* | 90 | EU136697 |
| 2157 | | Bacteroidales/Prevotellaceae/*Prevotella* | *Prevotella* sp BV3C7 | 91 | JN809774 |
| 2157 | | Bacteroidales/Prevotellaceae/*Prevotella* | *Prevotella veroralis* strain F0319 | 87 | GQ131418 |
| 2170 | | Bacteroidales | *Bacteroides* sp CS24 | 81 | AB064911 |
| 2259 | | Bacteroidales/Porphyromonadaceae/*Paludibacter* | *Paludibacter propionicigens* WB4 | 86 | CP002345 |
| 2266 | | Bacteroidales/Bacteroidaceae/*Bacteroides* | *Bacteroides coprola* strain JCM 12979 | 91 | EU136688 |
| 2274 | | Bacteroidales | *Parabacteroides* sp NS31-3 | 83 | JN029805 |
| 2464 | | Bacteroidales/Porphyromonadaceae/*Proteiniphilum* | *Proteiniphilum acetatigenes* strain TB 107 | 84 | NR043154 |
| 2896 | | Root/Bacteria/Bacteroidetes | *Bacteroides* sp CJ47 | 82 | AB080887 |
| 3226 | | Bacteroidales/Porphyromonadaceae | *Parabacteroides goldsteinii* strain JCM13446 | 90 | EU136697 |
| 3593 | | Bacteroidales/Porphyromonadaceae/*Parabacteroides* | *Parabacteroides johnsonii* DSM 18315 strain JCM 13406 | 86 | NR041464 |
| 8 | **Actinobacteria** | Actinomycetales/Micrococcineae/Beutenbergiaceae | *Beutenbergia cavernae* DSM12333 | 91 | CP001618 |
| 212 | | Coriobacteriales/Coriobacterineae/Coriobacteriaceae/*Eggerthella* | *Eggerthella* sp CJ88 | 88 | AB080902 |
| 281 | | Actinobacteridae/Actinomycetales/Micrococcineae/Microbacteriaceae/*Leucobacter* | *Leucobacter komagatae* | 95 | DQ083486 |

Table 4.4 continued.

| Cluster | Phylogenetic affiliation | Order/Family/Genus | *Genus/Description* | % Match | Accession No. |
|---|---|---|---|---|---|
| 565 | **Actinobacteria** | Actinomycetales/Micrococcineae/Microbacteriaceae | *Microbacterium* sp RI 2 | 94 | AJ876685 |
| 972 | | Actinomycetales/Propionibacterineae/Nocardioidaceae/*Friedmanniella* | *Friedmanniella spumicola* strain Ben 107 | 93 | NR024907 |
| 1417 | | Actinomycetales/Micrococcineae | *Microbacterium* sp BBDP58 | 89 | DQ337517 |
| 1789 | | Actinomycetales/Propionibacterineae/Nocardioidaceae | *Propionicimonas paludicola* | 92 | AB078859 |
| 2520 | | Actinomycetales/Propionibacterineae/Nocardioidaceae | *Propionicicella superfundia* strain BL-10 | 94 | NR043609 |
| 3130 | | Actinomycetales/Corynebacterineae/Mycobacteriaceae/*Mycobacterium* | *Mycobacterium aichiense s*train JS618 | 97 | AF498656 |
| 3154 | | Actinomycetales/Propionibacterineae/Nocardioidaceae/*Micropruina* | *Micropruina glycogenica* strain Lg2 | 95 | NR024676 |
| 3179 | | Actinomycetales/Micrococcineae/Microbacteriaceae/*Microbacterium* | *Microbacterium* sp | 96 | JN196543 |
| 3625 | | Actinomycetales/Micrococcineae/Microbacteriaceae | *Microbacterium thalassium* | 98 | AM943052 |
| 3730 | | Actinomycetales/Corynebacterineae/Gordoniaceae/*Gordonia* | *Gordonia* sp IFM 0889 | 95 | AB476395 |
| 149 | **Chloroflexi** | Chloroflexi/Anaerolineae | *Levilinea saccharolytica* strain KIBI-1 | 88 | NR040972 |
| 596 | | Caldilineales/Caldilineacea/Caldilinea | *Chloroflexi bacterium* ET1 | 91 | EU875524 |
| 921 | | Caldilineales/Caldilineacea/Levilinea | *Longilinea arvoryzea* | 84 | NR041355 |
| 1280 | | Caldilineales/Caldilineacea/Caldilinea | *Chloroflexi bacterium* ET1 | 85 | EU875524 |
| 891 | **Fusobacteria** | Fusobacteriales/Fusobacteriaceae/*Sebaldella* | *Sebaldella termitidis* ATCC33386 | 99 | NR074413 |
| 2484 | | Fusobacteriales/Fusobacteriaceae/*Fusobacterium* | *Fusobacterium* sp SRBBR5 | 99 | HM215007 |

Sp = species

The results in Figure 4.13 indicate temporal changes in structure and relative abundance of the bacterial phyla according to the treatment conditions. The *Firmicutes*, *Proteobacteria*, *Bacteriodetes* and unclassified bacteria were the most dominant phyla in all digesters. The result indicates a temporal shift in relative abundance of the microbial community.

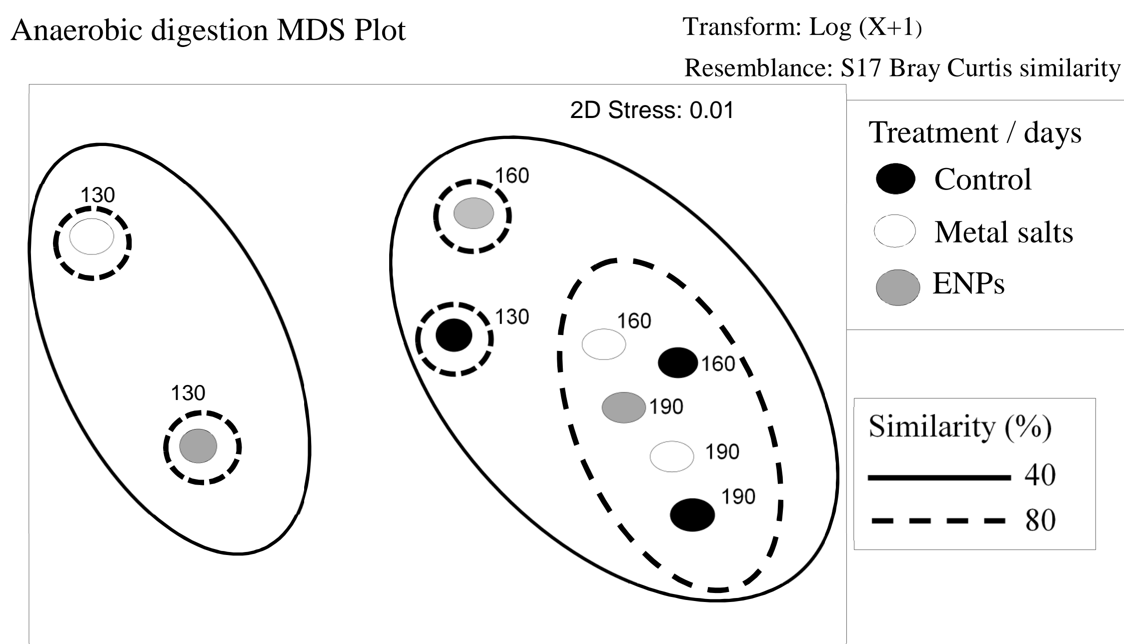



Figure 4.13 2-dimensional MDS configuration of similarity matrix between anaerobic microbial community at different exposure time and treatments based on 454-pyrosequencing. The numbers (130, 160, and 190) represent the samples/days.

Abundance of *Alphaproteobacteri* in the control AD was about 0.9 and 1.4 times higher than in metal salt and ENP spiked AD (Figure 4.15). Similarly, the *Betaproteobacteria* abundance was up to 1.2 times higher in the control AD plant than in the metal salt spiked AD and up to 6 times higher than in the ENP spiked AD. The relative abundance of *Deltaproteobacteria* was less than 1 % in the 3 AD plants (Figure 4.15). The result also suggest that the adverse effect on sulfate-reducing bacteria (SRB) belonging to the *Deltaproteobacteria* was more pronounced in the ENP than in the metal salt spiked and control AD and thus the lower $H_2S$ content in the biogas (Figure 4.5) of the ENP spiked digester.

The relative abundance of unclassified bacteria belonging to different phyla in the ENP spiked digestate suggest that most microorganisms in complex matrix such as the anaerobic digestate are yet to be characterised (Kragelund et al., 2008) and were simultaneously stimulated and inhibited in the presence of ENPs. It is however unclear at the moment which digestate components enhanced or attenuated the ENPs effect on the bacterial community, although the pattern of response by the bacterial groups to ENP effect differed from metal salt ions.

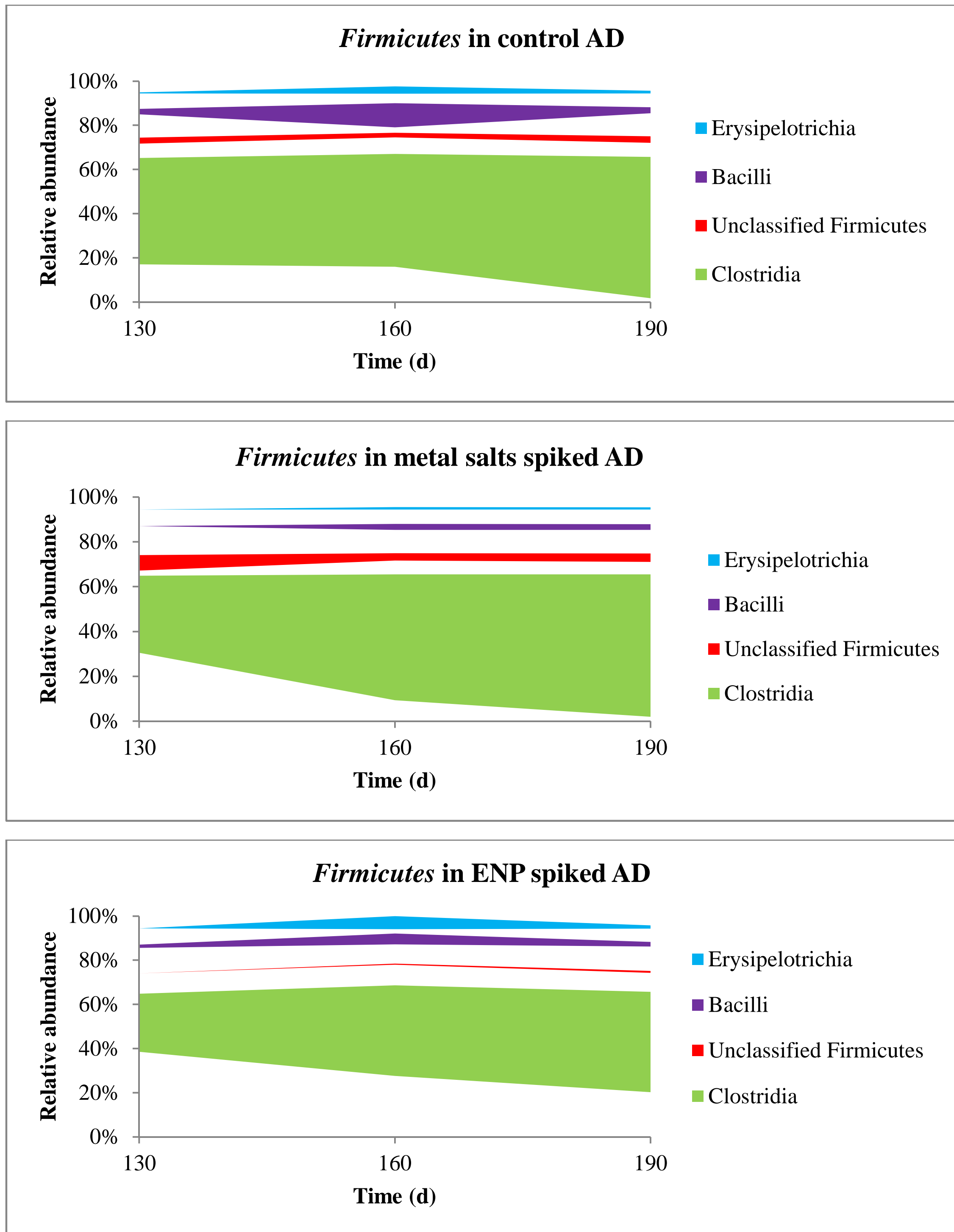



Figure 4.14 Effect of ENP and metal salts on the relative abundance of the *Firmicutes* in the anaerobic digesters

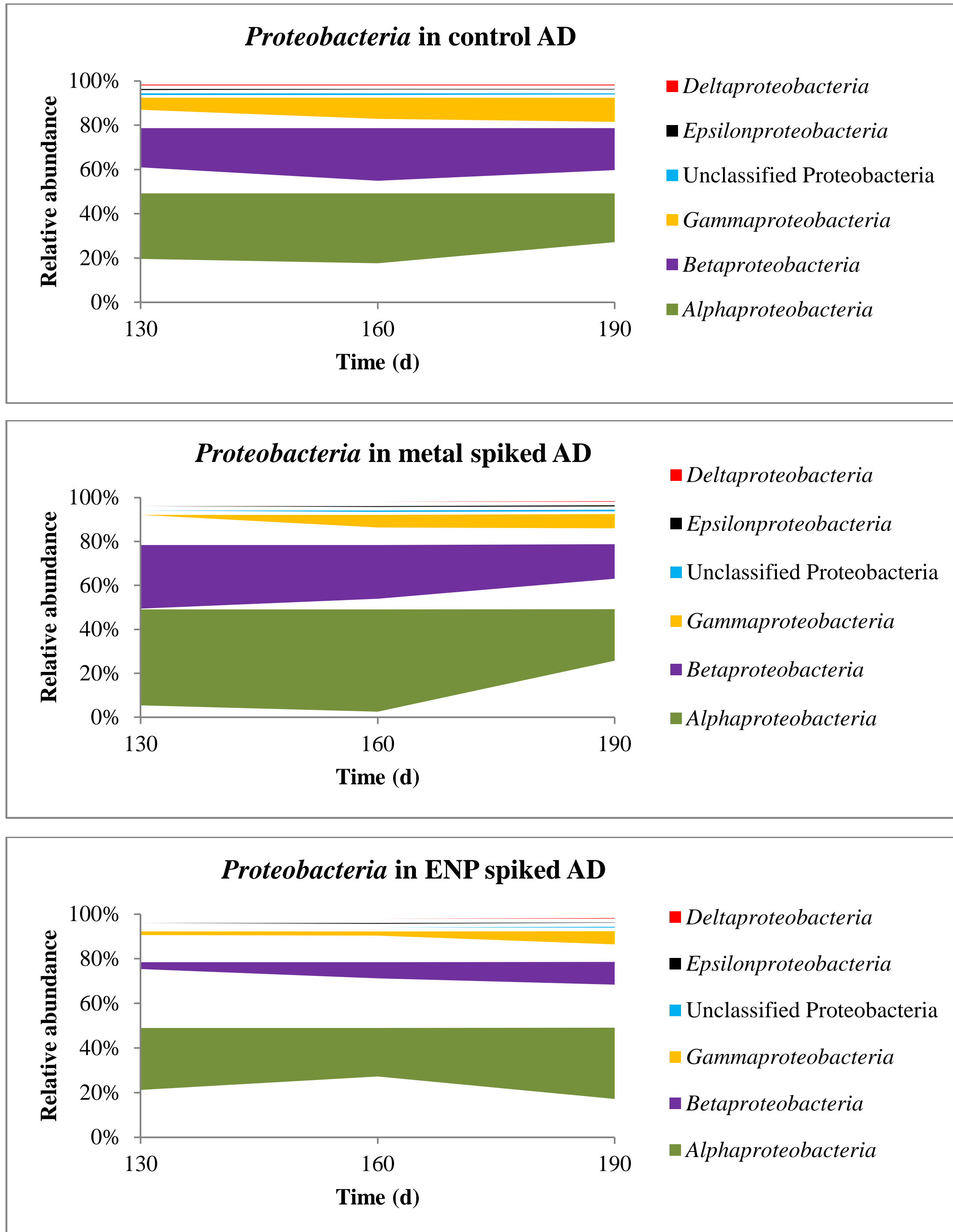



Figure 4.15 Effect of ENP and metal salts on the *Proteobacteria*. Sulphate-reducing members of the *Deltaproteobacteria* were more adversely affected by ENP than metal salts.

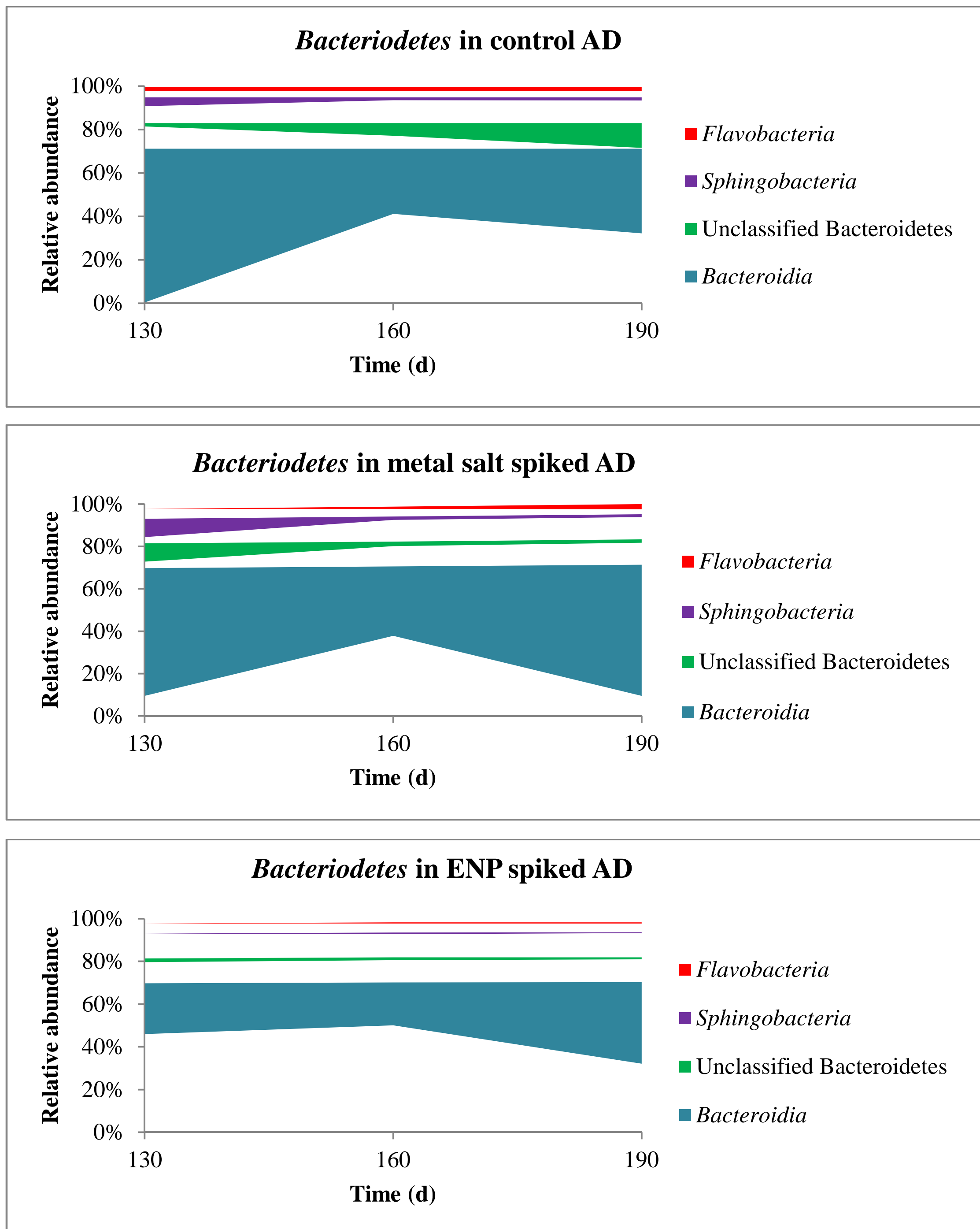



Figure 4.16  Relative abundance of the *Bacteriodetes* in the anaerobic digesters

A similar pattern of the ENP effect on the members of the *Bacteriodetes* was observed (Figure 4.16). Growth and abundance of members of the *Bacteriodia* in ENP spiked AD was 1.1 to 1.2 times lower than in the metal salt spiked and control AD whereas abundance of the *Sphingobacteria* was 1.3 to 2.2 times lower in ENP spiked AD than in

metal salts spiked AD and 2 to 3.2 times lower than in the control. Also, *Flavobacteria* in the ENP spiked AD was about 1.6 to 3.4 times lower in abundance compared to the metal salt spiked and control AD. Although the results indicate that ENPs can exerted a broad spectrum of effect on the AD bacterial community structure and abundance, accumulation of the primary product of fermentation such as acetic and propionic acids suggest that members of the *Bacteriodetes* were active in the digesters.

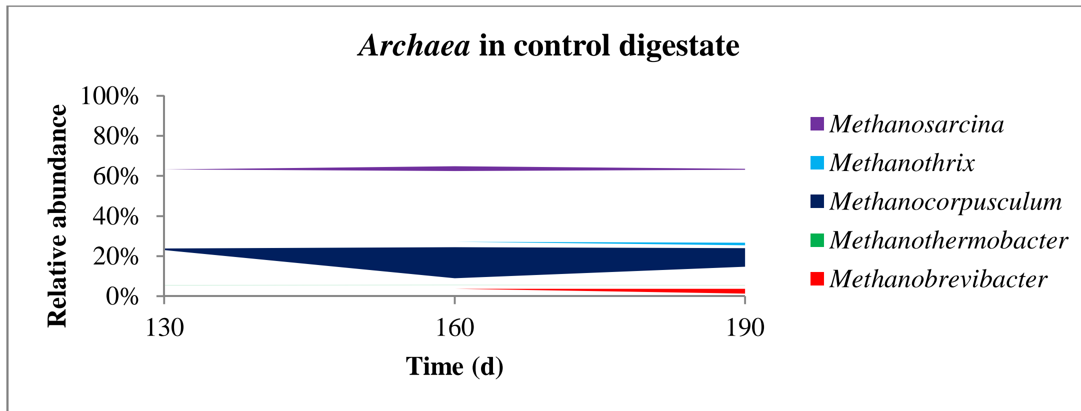
On the other hand, abundance of *Actinobacteria* in ENP spiked AD was 2 and 3 times lower than in metal salt spiked and control AD respectively on day 130 (Figure 4.12). The relative abundance of *Actinobacteria* in ENP spiked AD increased over time and was 2.2 and 2.5 times higher than in the control and metal salt spiked AD respectively on day 190. Members of the *Propionibacterineae* capable of producing propionic acid were found in the digesters. Propionic acid is an intermediate product during AD process and about 30 % of electrons required in methanogenesis are produced by microorganisms converting propionic acid to $H_2$ and acetic acid (Speece et al., 2006). The result indicate that propionic acid producing bacteria such as *Propionicimonas* and *Propionibacterium* species were not inhibited at the spiked concentrations of metal salt and ENPs.

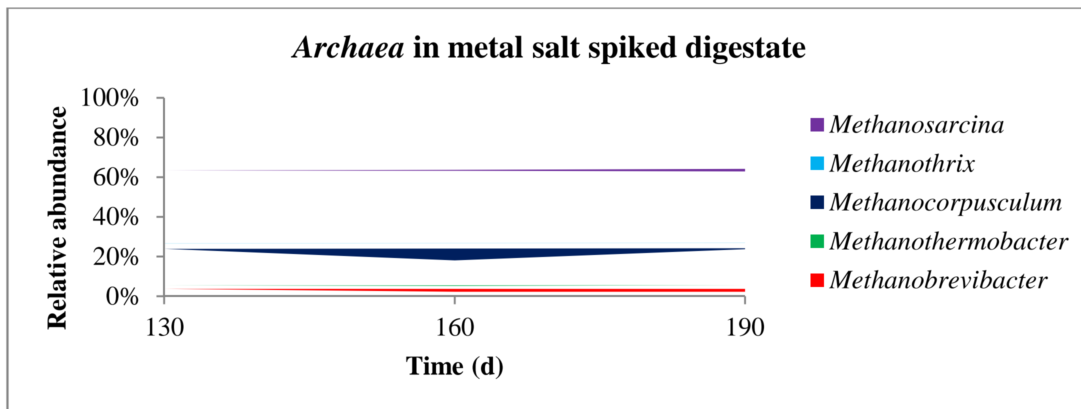
*Chloroflexi,* the commonly known filamentous non-sulfur bacteria was found in two-third (67 %) of the metal salt and ENP spiked AD samples compared with control (100 %) which indicates that after the initial inhibition, *Chloroflexi* were tolerant to the nanoparticles and/or metals (Figure 4.12). The evolutionary and environmental significance of the *Chloroflexi* as an important group of bacteria in wastewater treatment because of their key role in organic degradation is known (Bjornsson et al., 2002; Sekiguchi et al., 2001). Although low in abundance, the *Chloroflexi* can degrade organic and cellular materials; nitrify (Kragelund et al., 2007) and in synergy with the dominant phylogenetic groups are suspected to detoxify ammonia thus creating a favourable condition for the methanogenic archaea to thrive in the digesters. Further to this, disruption of bacterial cells in the AD during the hydrolytic and acidogenic stages of the digestion process (Tiehm et al., 2001; Appels et al., 2008) may have influenced the bacterial community diversity and abundance.

### 4.3.7 Aged-ENPs exerts inhibitory effect on methanogenic archaeal community abundance, diversity and distribution based on 454-pyrosequencing

The methanogenic archaea recovered belonged to three orders – Methanosarcinales, Methanobacteriales and Methanomicrobiales. The 6 closest species from 5 genera of methanogenic archaeal identified were *Methanobrevibacter acididurans* strain ATM, *Methanothermobacter thermoautotrophicus, Methanocorpusculum sinense, Methanosaeta concilii* GP6 strain, *Methanosarcina barkeri* strain HWS2.1, and *Methanosarcina acetivorans* C2A strain (Table 4.5). The percentage recovery of each archaeal genus from the treatments (Table 4.6) and temporal changes in relative abundance are presented in Figure 4.17. Despite the limited number of digestate samples analysed, sequences obtained indicate distinct temporal changes in the structure and relative abundance of the methanogens as a result of treatment. In the control AD, *Methanothermobacter* species was not found whereas *Methanosaeta* (*Methanothrix*) was inhibited in the metal salts spiked AD. Notably, *M. barkeri* and *M. acetivorans* were the only species recovered from ENPs spiked digestate. This result is of significance and indicates in part, 20 % competitive advantage for growth due to the presence and effect of ENPs ions on specific microorganism (*Methanosarcina*) in the digestate and in part, 80 % change in archaeal community type and inhibitory effect on the methanogens. As shown in Figure 4.17, ENPs enhanced the relative abundance of *Methanosarcina* by 74 and 65 times compared with the control and metal salt spiked digestate, respectively.

*Methanosarcina* was resilient, tolerant and able to proliferate which suggest that the organism possesses an efficient adaptive mechanism to mitigate the effect of ENPs during anaerobic sludge digestion making it a key species in this study. Furthermore, the results indicate that mixed ENPs at the spiked concentration (5 mg $L^{-1}$ $Ag^{o}$, 40 mg $L^{-1}$ $TiO_2$, and 56 mg $L^{-1}$ ZnO) was inhibitory to most methanogenic archaeal species during the digestion and gave organisms with versatile physiology and metabolism a competitive advantage and key role to play in the process.



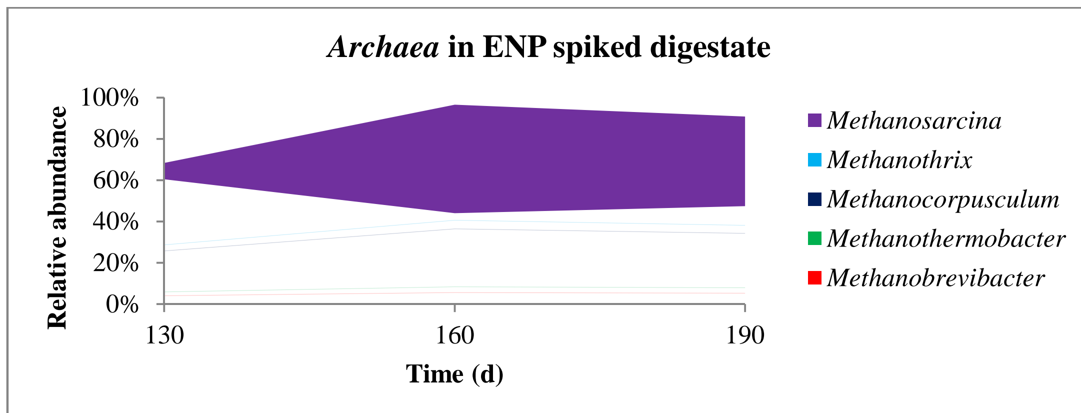




Figure 4.17 Effect of ENPs and bulk metal salts on the abundance and diversity of methanogenic *Archaea* during anaerobic digestion of sludge in relation to the control. Resilience and proliferation of *Methanosarcina* in the ENP spiked AD is indicative of a nano-tolerant species.

Detailed analysis of genome sequence of members of the genus *Methanosarcina* have been catalogued and consist of complex organisation with uncommon features including large extrachromosomal material, or plasmid (Meader et al., 2006). *Methanosarcina* possesses plasmids that replicate by the rolling circle method (Galagan et al., 2002)

which plausibly confers more metabolic and resistant genes to adapt and repair damage by the ENPs. Also, *Methanosarcina* exhibits a dichotomous morphology by growing as a large multicellular aggregate embedded in heteropolysaccharide matrix (methanochondroitin) which is composed of D-glucuronic acid and D-galactosamine (Kreisl and Kandler, 1986; Maeder et al., 2006).

Table 4.5 Phylogenetic tree of methanogenic microbial community in anaerobic digestate with closest culturable archaeal relative

| Phylum | Class | Order | Family | Genus | Species | % Match | Accession No. |
|---|---|---|---|---|---|---|---|
| Euryarchaeota | Methanomicrobia | Methanosarcinales | Methanosarcinaceae | *Methanosarcina* | *Methanosarcina bakeri* | 98 | JN243319 |
| | | | | | *Methanosarcina acetivorans* C2A | 94 | NR074110 |
| | | | Methanosaetaceae | *Methanothrix* | *Methanosaeta concilli* GP6 | 98 | NR102903 |
| | | Methanomicrobiales | Methanocorpusculaceae | *Methanocorpusculum* | *Methanocorpusculum sinense* | 99 | FR749947 |
| | Methanobacteria | Methanobacteriales | Methanobacteriaceae | *Methanobrevibacter* | *Methanobrevibacter acididurans* | 98 | NR028779 |
| | | | | *Methanothermobacter* | *Methanothermobacter thermoautotrophicus* | 97 | NR074260 |

Table 4.6 Percentage recovery of key methanogenic archaeal genera in anaerobic digestate

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

Besides the robust genome, *Methanosarcina* has a unique metabolic capability that enables the organism to grow on diverse substrate for methane production. For instance, members possess full complement of gene encoding enzymes for obligate reduction of $CO_2$ and methyl with $H_2$, aceticlastic conversion of acetate, metabolism of one-carbon compounds (e.g. methylamine, dimethylsulfide, methanol, and methyl diols) through methylotrophic methanogenesis. Thus, *Methanosarcina* possesses all 4 methanogenic pathways required to produce methane in contrast to other methanogens with one pathway (Galagan et al., 2002; Deppenmeir et al., 2002; Welander et al., 2005). Overall, the result indicates that the organism is a strong candidate for microbial uptake, use or conversion of ENPs.

### 4.3.8 Influence of ENP doses on bio-methane potential and VFA production

To understand and determine the effect of ENPs and metal salts on bio-methane potential of sewage sludge, the percentage production of methane from duplicate batch reactors exposed to different levels of ENPs was measured (Figure 4.18). Compared with the control, the mean difference in methane produced was about 23 times lower when metal salt concentration was 250, 2000 and 2800 mg $L^{-1}$ of $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ respectively (Panel F). Concentrations A and B (Table 4.2) barely affected the BMP and unlikely to be significant whereas a significant difference in BMP between conditions

was observed at concentration mixture D, E and F (Figure 4.18 panels D, E and F). This result was expected because the rates of biogas production in spiked and unspiked sewage sludge can differ probably as a result of impact on the microbial diversity or changes in substrate composition (Smith and Carliel-Marquet, 2008). At concentration C, a lag phase of about 15 days was observed and subsequently similar BMP was obtained.

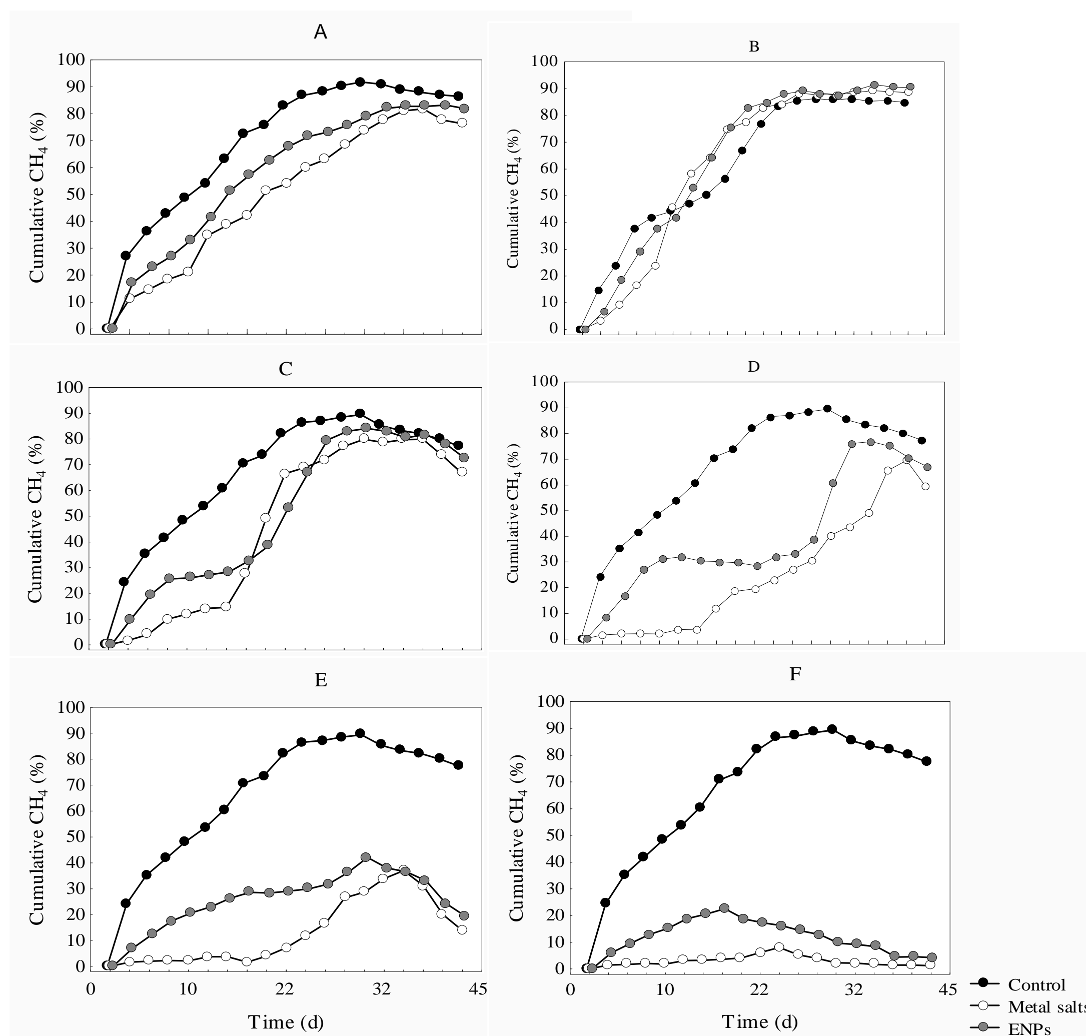



Figure 4.18 Methane productions in response to shock dose of different concentrations of composite ENPs and metal salts during a 42 days BMP test. Each value represents the mean from duplicate determinations. Compared with the control, lower percentages of methane were produced as follows by concentration series **A:** 10 and 8.6, **C:** 10 and 5.3, **D:** 20 and 13, **E:** 52.5 and 47.4, **F:** 81.3 and 67 for metal salt and ENP spiked digesters respectively

It should be noted however, that as the concentration of spiked ENP and metal salts increased, there was a corresponding reduction in methane production with a difference of about 10 % between metal salt and ENPs. The result demonstrates the concentration-dependent inhibitory effect of metal salts and ENPs on microorganisms associated with biogas production during anaerobic digestion of sewage sludge. Overall, reduced biogas volume was more pronounced in the metal salt than in the ENP spiked reactors plausibly because ENPs were transformed or adsorbed to the biosolids which attenuated the negative effect (Figure 4.19).

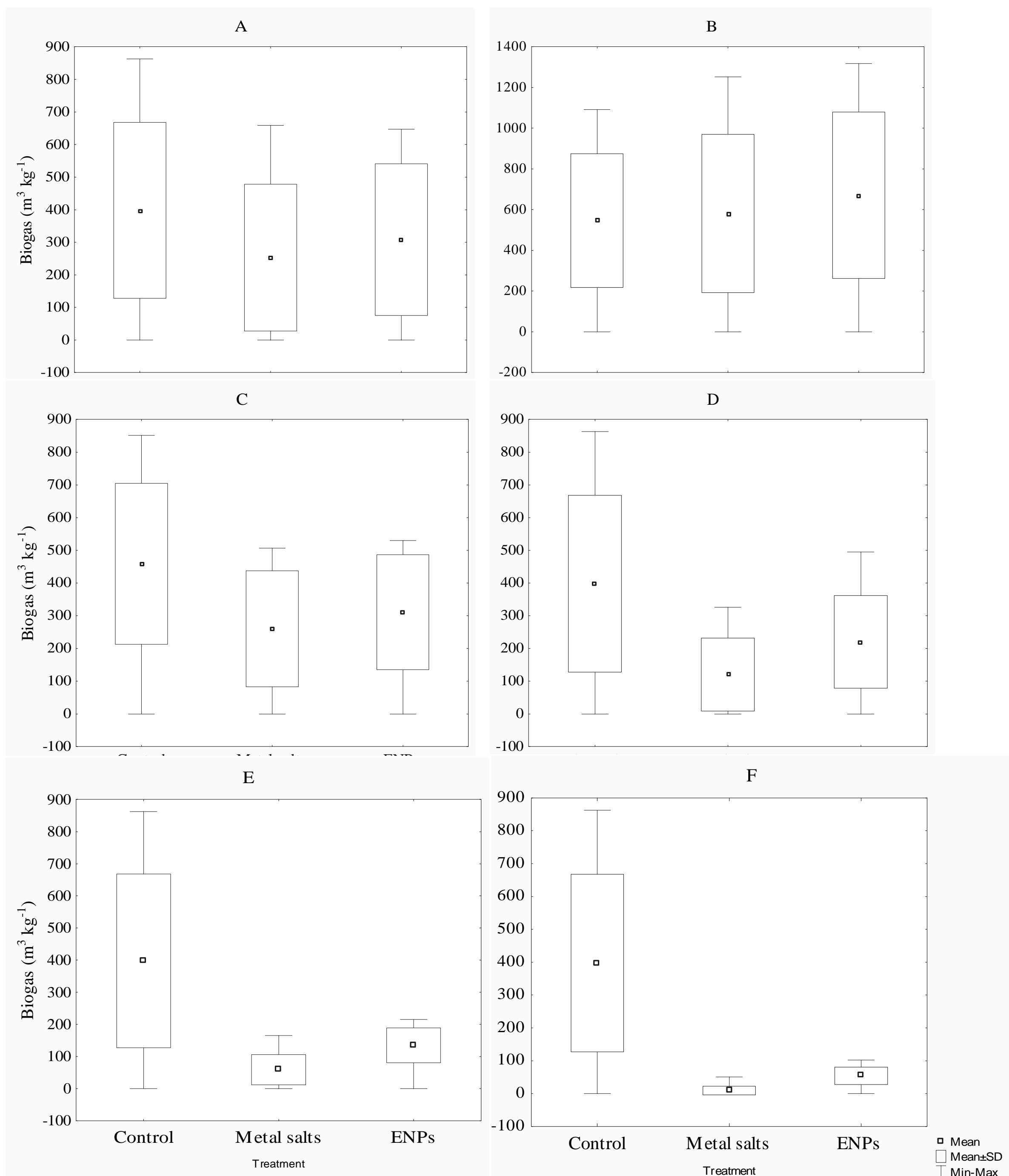



Figure 4.19 Box Whiskers plot of biogas volume from lab-scale sludge digestion.

The trend in the concentration of VFA (Figure 4.20) was a reverse of the pattern observed with the methane production (Figure 4.18). With increased concentration of metal salt and ENP in the reactors, a corresponding increase in VFA at the end of 42 days incubation was obtained suggesting that ENP and metal salts ions inhibited methanogenic activities in the batch digesters. Compared with the control digesters, VFA concentration was at least twice in the metal spiked digesters and at least 1.2 times

higher in the ENP spiked digesters with the exception of digesters spiked with 50, 100 and 500 mg $L^{-1}$ of $Ag^0$, $TiO_2$ and ZnO respectively (concentration B) where the VFA concentration was observed to be 2.5 times lower than in the control. The result suggests that ENP concentration series B may have enhanced the metabolic activities of methanogens in converting more VFA to biogas thus reducing their accumulation in the digester.

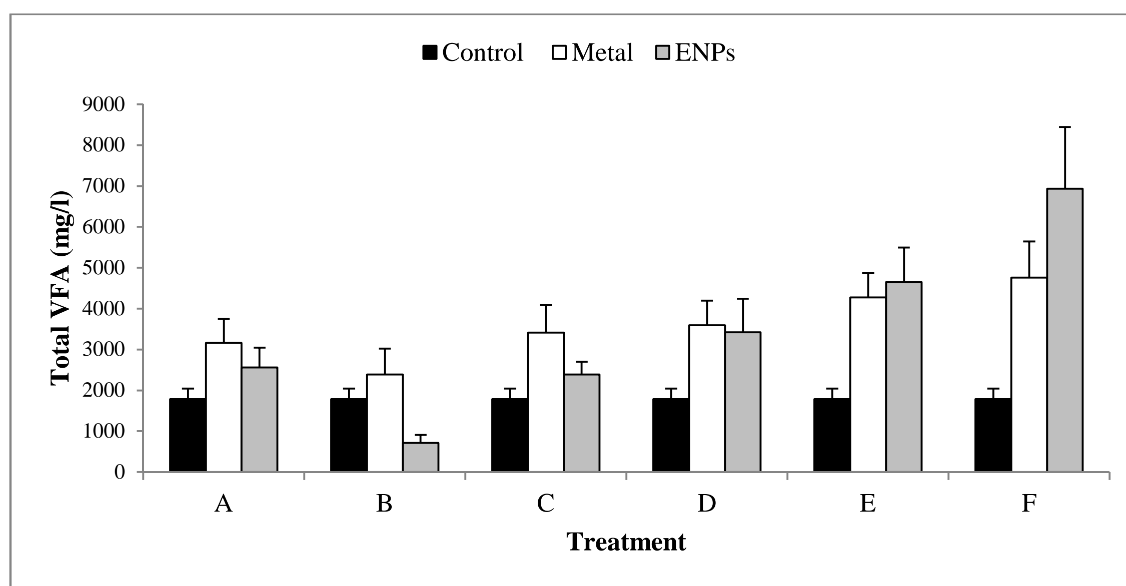



Figure 4.20 Effect of ENPs and metal salt concentrations on volatile fatty acid production in a batch anaerobic digestion of sewage sludge after 42 days incubation. Values are mean of duplicate determinations, error bars indicate standard deviation.

Compared with the control, about 1.2 to 3.4 times higher VFA accumulated in the digesters with increase in the concentration of metal salt or ENPs. 2.5 times lower VFA in ENP concentration series B than in the control. At the moment, it is unclear what factors may have caused the observed effect. Although the microbial community in the BMP test was not determined, it is plausible that higher concentration of ENP ions released into digesters E and F may have reduced the efficiency of VFA conversion by *Methanosarcina* (Table 4.5) compared to the synergistic interaction of 5 different species in the metal salt spiked digester. Furthermore, the result indicates that the trend of methane production in the laboratory-scale reactor was dependent on the concentration of ENPs and metal salts with time. Apparently, low concentrations of ENPs and metal salts stimulated methane production whereas concentration above 100 mg $L^{-1}$ Ag, 1000 mg $L^{-1}$ Ti and 2000 mg $L^{-1}$ Zn exhibited adverse effect on $CH_4$ production. It remains however difficult to compare the pilot scale results and the lab

scale results when the same concentrations of ENPs was used (Condition F) because of different experimental conditions such as pre-treatment of sludge, sludge retention time (SRT), hydraulic loading rate (HLR) and temperature variation. As a consequence, caution is exercised in extrapolating result from the lab-scale digesters to the pilot-scale plants in order to minimise bias in the interpretation of the result. Overall, microbial abundance and diversity was reduced in ENP-enriched biosolids indicating that ecologically beneficial processes dependent on syntrophic microbial interactions can be adversely affected from their long-term use and release into the environment.

## 4.4 Conclusion

The understanding of ENPs effect on microorganisms in complex medium such as wastewater continues to evolve as a result of increasing concern over the potential negative effect of ENPs on non-target biological system. The changes in microbial community structure, diversity and abundance observed in this study provide evidence on plausible effect on AD process performance. When methane produced in the laboratory and pilot-scale plants are compared, the result illustrates that ENPs exerted varying levels of stimulatory and inhibitory effects. It is difficult however, to make direct comparison between a batch and flow through/continuous fed reactors because of differences in experimental design, sludge retention times, biomass abundance and temperature variations. Although methane production in the BMP test was unaffected at low concentrations, the adverse effect of ENPs and metal salt ions increased with dosage. VFA accumulated in the BMP test indicating that the treatment had a negative effect on digestion process. In the pilot scale reactors, pulse disturbance during reactor feeding, digestate extraction and biomass washout contributed to the high VFA concentration. The result further suggests that the potential adverse effects were mitigated by sludge components in a continuously fed and spiked reactor than in a batch reactor.

Overall, the results demonstrate that the behaviour of mixed ENPs in AD differs when compared with the bulk metal forms. For instance, the reduced abundance and diversity of methanogenic archaea and SRB was more pronounced in ENPs than in the metal salt spiked AD. Although the chemical nature of mixed ENPs in wastewater is not certain at the moment, they can interact with wastewater components to selectively inhibit or stimulate most microorganisms based either on their genomic make-up or versatile metabolism or both. Thus, increase in the use and release of ENPs into wastewater can

become an hazard to the microbial community structure, abundance and diversity. ENPs spiked into and recovered from the digestate in this study can be regarded as a threshold concentration of toxicological concern for adverse effect on microbial community and processes in AD because of the negative and positive impact on key methanogenic archaea. Microbial abundance and diversity indicate that effect of ENPs from chronic exposure is different from its bulk metal forms despite the presence of organic materials and other wastewater components. Therefore, caution should be taken in the use of bulk metal salts as a basis to develop guidelines to regulate the release of ENPs because of the differences in behaviour which poses a risk to ecologically sensitive microbial species and processes. However, the growth of *Methanosarcina* in the presence of toxic ENP concentration provides strong evidence that *M. acetivorans* and *M. barkeri* are nano-tolerant methanogens.

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## Chapter 5: Assessment of the potential ecological impacts of mixed engineered nanoparticle during activated sludge and anaerobic digestion processes


### Abstract

This study evaluated the potential ecological impacts of a mixture of engineered nanoparticles (ENPs) consisting of silver oxide ($Ag^0$), titanium dioxide ($TiO_2$), and zinc oxide (ZnO) on the treatment processes of a wastewater treatment pilot plant (WWTP). Bulk metal salts as parent material and unspiked control were used for comparison. The total concentration of of the ionic species of Ag, $TiO_2$ and Zn in the activated sludge effluent of the ENP spiked WWTP were below limits of detection. ENPs concentration in the WAS was about 2 times higher than the metal salts ions suggesting higher affinity of the ENP to biosolids. > 99% of the spiked ENP were found in the WAS whereas in the digestate cake, 39 – 58 % of $Ag^0$, 51 – 63 % and 58 – 74 % of ZnO ion concentrations were recovered suggesting that sorption to WAS and anaerobic digestate differs. Concentration of ENP or metal salts ions in the AS and effluent of the three pilot plants exhibited hormetic responses, whereas the digestate filtrate exhibited varying toxic effect on the activity of *Vibrio fisheri.* 45 – 70 % inhibition and 100 – 189 % stimulatory effects were observed with detected and undetected ion concentrations in the ENP and metal salt spiked digestate filtrate. The results indicate that biologically significant responses occurred at concentration below the limit of detection probably from synergistic or antagonistic interactions of ENP or metal salts with digestate components. Concentration of ions in ENP spiked digestate cake was potentially not inhibitory to *V. fisheri* activity compared with > 99 % inhibition by the metal salt ions. Overall, the results shows that *V. fisheri* activity responded to other AS/AD components and was not sensitive enough to the ionic concentrations of the ENP or metal salt mixture in the liquid and solid phases of AS and AD. Thus, there is an urgent need to develop bioassay using relevant wastewater microorganisms.



Keywords: Activated sludge, anaerobic digestion, engineered nanoparticles, bioavailability, bioluminescence, potential effect

## 5.1 Introduction

There has been a remarkable development of nanotechnology and interest in the application of engineered nanoparticles (ENPs) in several products over the last decade. At the moment, it is estimated that more than 1500 products contains ENPs and the trend is expected to increase because of their novel physicochemical characteristics and notable antimicrobial effect (Sun et al., 2013; Woodrow Wilson Database, 2013). Although little is known about the environmental fate and transport of ENPs, there is increased concern for environmental health due to the perceived toxicological implications on microbial dependent processes in activated sludge (Sun et al., 2013; Zheng et al., 2013) and anaerobic digestion processes (Yang et al., 2012; Garcia et al., 2012). The release of ENPs by anthropogenic activities (Moore, 2006) will make wastewater a primary sink and point source of aged-ENPs pollution of the environment through effluent discharge and biosolids application to soil. This is important as it has been estimated that 55 % of sewage sludge is used for soil amendment and agriculture, 25 % for thermal energy generation and 20 % disposed in solid waste landfills (EEA, 2013). ENP-enriched biosolids introduced into the environment through any of these routes can have adverse effect on microbial cells and thus reduce/disrupt the normal functioning of ecological processes (Barrena et al., 2009).

Similarly, the use of ENPs such as titanium dioxide ($TiO_2$) (Mach, 2004) and iron nanoparticles (Lecoanet et al., 2004) in water treatment and land remediation introduces new hazards into the environment. Like most xenobiotic compounds, ENPs are not known to degrade suggesting persistence and a tendency to accumulate in environmental matrices which raises critical concerns about their fate, transport and effect in the environment (Barrena et al., 2009). For example, ENPs such as aluminium oxide ($Al_2O_3$) can reduce root growth as a result of disturbance of rhizosphere microbial composition (Yang and Watts, 2005), and zinc oxide (ZnO) can inhibit seed germination and root growth/elongation (Lin and Xing, 2007).

ENPs exert mainly antimicrobial effect through a wide range of mechanisms including the formation of reactive oxygen species, disruption of microbial physiology and metabolic processes. However, there is growing evidence that the ENPs could also augment microbial-mediated processes in the ecosystem (Cullen et al., 2011; Shin and Cha, 2008; Hilderbrand et al., 2008). At the moment, the need to understand the release

of ENPs into a complex environment such as wastewater and the interactions with microbial community during wastewater treatment process is a subject of intense research. Recent studies have shown that ENPs can inhibit different groups of microorganisms in activated sludge (Sun et al., 2013; Zheng et al., 2011) and during anaerobic digestion of sludge (Yang et al., 2012; Garcia et al., 2012). The activated sludge (AS) and anaerobic digestion (AD) processes are important in removing contaminants in wastewater, stabilizing the biosolids and generating biogas. The reduction in efficiency or inhibition of the AS and AD process can result in the accumulation and subsequent discharge of pollutants into the ecosystem (Yang et al., 2012).

It is also difficult to determine the effective dose or establish toxicological guidelines for ENPs in complex environmental matrix. The challenge is in part due to the variable nature of wastewater and in part to the inadequacy of the current analytical procedures to measure and differentiate the nano from the bulk forms of ENP parent material and their aggregation characteristics (Liu et al., 2013). Furthermore, the behaviour of ENPs and bulk metal salts differs and is influenced by the size and surface charge of ENPs making it difficult to predict how ENPs can react based on bulk metal salt properties. For instance, the Derjaguin-Landau-Verwey-Overbeek (DLVO) theory currently used in interpreting and understanding the interaction between colloidal size particle adsorption and desorption at the solid/liquid interfaces may not be useful for nanoparticles size < 10 nm (Liu et al., 2013; Feng et al., 2012).

In addition, ENPs can be transformed into a toxic or innocuous by-product (Kaegi et al., 2013; Levard et al., 2012) or associate with natural organic matter and divalent cations/anions in wastewater which can influence their behaviour and reactivity in a manner contrary to colloid chemistry (Liu et al., 2013). As a result of the concerns on the behaviour and potential effect of aged-ENPs on microorganisms, the bioavailability and effect of a mixture of ENPs comprising $Ag^0$, $TiO_2$ and ZnO were assessed in waste activated sludge, activated sludge effluent, anaerobic digestate cake and filtrate. Toxicity values obtained by Microtox® provide complementary information to the microbial activity and chemical analysis described in Chapters 3 and 4. They may be used to give information on the bioavailability of the nanoparticles and or metals present in sludge and digestate samples.

## 5.2 Materials and methods

### 5.2.1 Experimental design/Pilot plant operation

The experimental design of the pilot plant is as previously described in Chapters 3 and 4. ENP and metal salt present in form of ions were measured in the activated sludge, waste activated sludge (WAS) and anaerobic digestate cake (solid phase) as well as in the activated sludge effluent and anaerobic digestate filtrate (liquid phase) in order to assess the amount of ENP and metals sorbed to the solids. The extracted anaerobic digestate samples were stored and dewatered at different times, and samples selected for the Microtox® test are shown.

### 5.2.2 Determination of residual ENPs and metal salts concentration

The residual ENPs and metal salts ($Zn^{2+}$, Ti, $Ag^{+}$) concentrations in duplicate samples including WAS and effluent and anaerobic digestate cake and filtrate was measured by Inductively Coupled Plasma Atomic Emission Spectrometer (ICP-AES, Perkin Elmer 4300DV), in an external laboratory (Environmental Scientific Group, Straffordshire, UK). The samples were digested prior to analysis using high purity nitric acid, hydrogen peroxide and hydrofluoric acid in sealed Teflon vessels with microwave assisted heating. Demineralised water having a resistivity of 18.2 MΩ.cm was used in making the samples into known volume after digestion. $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ concentrations were measured by ICP-AES calibrated using certified standards. 5.0 mg $L^{-1}$ Ag, Ti and Zn prepared from an alternative source stock different from that used for the instrument calibration standard was measured with the samples as a quality control measure. As a further quality control measure, a blank digestion vessel spiked with equivalent of 4.0 mg $L^{-1}$ Ag Ti, and Zn was taken through the same procedure.

### 5.2.3 Standard Microtox® test –Liquid phase

To assess the potential ecological effect of residual ENPs and metal salt ionic concentration in AS effluent and digestate filtrate, the standard procedure described by Azur Environmental (1998) was adopted for the Microtox acute test. The test organism, lyophilized *Vibrio fischeri* NRRL B-11177 and all consumables for the test were supplied by Strategic Diagnostics Inc. (Pencader Drive, Newark, DE, USA).

Briefly, a solution of the luminescent bacterium was prepared by rehydrating a vial of freeze-dried *V. fischeri* in 1.0 ml of precooled *Reconstitution Solution* and incubated at 15 ± 0.2 $^{0}$C and time-zero luminescence was measured. The sample concentration was

prepared by adding 2.50 ml of AS effluent spiked with 0.01 mg $L^{-1}$ $Ag^0$, 0.08 mg $L^{-1}$ $TiO_2$, and 0.12 mg $L^{-1}$ ZnO and digestate filtrate spiked with of 250, 2000, and 2800 mg $kg^{-1}$ for $Ag^0$, $TiO_2$ and ZnO, respectively to 0.250 ml of Microtox osmotic adjustment solution (2 % NaCl), mixed and 0.75 ml was discarded. 1.0 mL of each sample concentration ranged from 5.63 to 45.00 mg $L^{-1}$ obtained by a series dilution sequence was added to the vial containing *V. fischeri* and incubated. Luminescence of individual vials was measured at 5, 15 and 30 min and sample toxicity determined on the basis of the decrease in light intensity of the luminescent bacteria and $EC_{50}$ (mg $L^{-1}$) calculated with 95% confidence limit using MicrotoxOmni software version 1.18. The effective concentration ($EC_{50}$) that inhibits 50 % of *Vibrio* activity was measured using Microtox® Model 500 Analyser (Azur Environmental).

### 5.2.4 Microtox® Solid Phase Test (SPT)

Approximately 7 g of dewatered digestate and WAS samples were thoroughly mixed in separate ~50 ml beaker. 3.5 ml of Solid-Phase diluent was added and magnetically stirred at 1000 rpm for 10 min and 400 rpm for 3 min. An oily tinge on the digestate slurry in SPT diluent was observed after stirring with aggregated particles that were not monodispersed. Subsample (1.5 ml) of this suspension was used for 1: 2 serial dilutions. The dilutions were allowed to equilibrate at 15 ± 0.2 °C for 10 min in water bath after which 20 µL of reconstituted *Vibrio fischeri* was transferred into each tube starting from the lowest concentration. The suspension was gently mixed, to disperse the cells and enhance contact, incubated at 15 °C for 20 min with shaking and 5 min without shaking. Separation of the bacteria and sample in each dilution was achieved using the SPT filter column and 0.5 ml subsample of filtrate transferred into the corresponding glass cuvettes in Microtox analyser Model 500 (Azur Environmental) and allowed to equilibrate for 5 min. Light emissions were measured and effective concentration ($EC_{50}$) on the basis of sample wet weight was determined as the concentration of the toxicant that causes a 50% reduction in bioluminescence at 5 min. Calculation of $EC_{50}$ with 95% confidence limit was done using MicrotoxOmni software version 1.18 provided with the test kits. The tests were carried out with two controls and 12 duplicate dilutions.

## 5.3 Results and discussion

### 5.3.1 Bioavailability of ENPs and metal salts in WAS and effluent, and anaerobic digestate cake and filtrate

To understand the level of ENPs sorbed to WAS and digestate compared with bulk metal salts, the ionic concentration in the AS, WAS, effluent, digestate cake and filtrate was determined using ICP-AES. As it was not possible to differentiate between nano and metal forms in the samples, the concentrations are reported as total $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ ionic species and normalised against the baseline concentration in the control sample. A summary of the bioavailable concentrations of ENPs and metal salts ions which caused discernible biological impact on microbial cells normalised against the control AS and AD is already presented in Chapters 3 and 4 (Figure 3.3. and Table 4.3.). The concentration of ions sorbed in the WAS of the ENP spiked wastewater treatment plant was about 2 times more than in the metal salt spiked WAS. The amount of ENPs or metal salts ions released from consumer products, sorbed to wastewater biosolids represented in the control AS and AD are regarded as the baseline concentrations.

No ionic species of $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ were detected in the AS effluent of the ENP spiked plant and control plant which suggest that > 99 % of the ENP were sorbed to biosolids. In the WAS, however, concentrations of ions released by ENPs was about 2 times higher than in the metal salts spiked WAS indicating greater affinity of ENP to the biosolids. Trace concentrations of ions detected in the metal salts spiked AS effluent further indicates that the sorption potential of ENPs and bulk metal salts differs. Sorption of the $Ag^{0}$ to the digestate in the first batch samples from day 1 – 160 (see Chapter 4, Table 4.3) was about 1.2 times higher than metal salts indicating that ENPs associated more firmly with biosolids. The result agrees with the work of Kiser et al. (2010) in which about 39% of $Ag^{0}$ was recovered in wastewater biosolids.

Although about 80 to 90 % of ENPs adsorbed to biosolids can be removed from wastewater (Wang et al., 2012b), the results obtained in the present study indicates that the scenario in AD is different with complex and synergistic acidogenic and hydrolytic reactions which plausibly resulted in desorption of the ENPs hence the low concentration in the digestate (Table 4.3). Fluctuations in pH can influence sorption and/or desorption of ENPs to digestate was lower than for metal salts. This finding suggest that ENPs were either more soluble in the digestate liquor or the low

concentration of ENP was presumably caused by desorption. For instance, the targeted $Ag^0$ concentration of 250 mg $kg^{-1}$ in the digestate sludge cake was not obtained probably because the $Ag^0$ was transformed (Levard et al., 2012; Kaegi et al., 2013) and/or desorbed (Wang et al., 2012) from the digestate.

Overall, the concentration of ions released by ENPs compared to bulk metal salts in the digestate differed slightly, although the difference was not significant except for the $Zn^{2+}$ in Batch 1 (Chapter 4, Table 4.3). Furthermore, complete sorption of ENP or metal salt to the digestate was not achieved. For instance, about 42 - 61 % of $Ag^+$, 37 – 49 % $Ti^{4+}$ and 26 – 42 % of $Zn^{2+}$ undetected in the digestate cake were potentially available in the digestate liquor. The available ENP or metal salt ion concentration in the digestate liquor was probably released during the filter pressing. It is also plausible that $Ag^0$ spiked into the AS and AD for instance was transformed to silver sulphide (Kaegi et al., 2013; Levard et al., 2012) resulting in reduced concentration of $Ag^+$ in the digestate. However, the transformed products of ENPs and metal salts were not measured in this study and therefore it is difficult to make any definitive statements on their concentrations or occurrence in the digestate. Similarly, it is difficult to determine if the sorbed ENPs were influenced by combined wastewater components, the sodium bicarbonate buffer and polygold coagulant used during treatment and processing of the sludge.

In addition, the result suggests that sorption and variation in the solubility/partitioning of ENPs in the aqueous WAS and oily tinged digestate matrix was probably due to different octanol-water affinity coefficients ($KA_{OW}$). The process determining affinity of ENPs to octanol or water phases ($KA_{OW}$) equivalent to octanol-water partitioning coefficient ($K_{OW}$) for organic molecules is influenced by the capping agent and can differ for each ENP. For instance, polyvinylpyrrolidone (PVP) and gum arabic capped ENPs have $KA_{OW}$ values close to 1 which suggest amphiphilic behaviour (Xiao and Wiesner, 2012). However, the $KA_{OW}$ values for $Ag^0$-PVP capped particles such as the one used in this study were larger than 1 and indicate hydrophobic material (Xiao and Wiesner, 2012). Moreover, the ability of a liquid to flow, commonly called viscosity measured in digested sludge supernatant centrifuged at 3000 rpm was more than 2 times that of water (Wang et al., 2011). Thus, it is plausible to assume that the ENPs used in the study has both hydrophobic and lipophilic properties and appeared to be

differentially sorbed to hydro- and lipophilic media which enhanced desorption, bioavailability and release from the digestate.

### 5.3.2 Effect of ENPs and metal salts ions on bioluminescence

The biphasic response usually characterised by low-dose stimulation and high-dose inhibition, commonly called hormesis, was exhibited by ENPs and metal salt ion concentrations in WAS, AS and effluent samples. The hormetic response is not shown because $EC_{50}$ values could not be calculated. Results presented in Figure 5.1 indicates that concentration of ions in the ENP-spiked digestate filtrate exhibited contrasting toxic and non-toxic effects at the initial and later stages of treatment compared with the ions in metal salt spiked filtrates. A threshold of inhibitory/toxic effect from the ENP or metal salt ion concentration on *V. fisheri* activity was obtained at 5 min for all the samples tested. There was no substantial difference in the 5 min $EC_{50}$ compared with 15 and 30 min time series in all the samples tested.

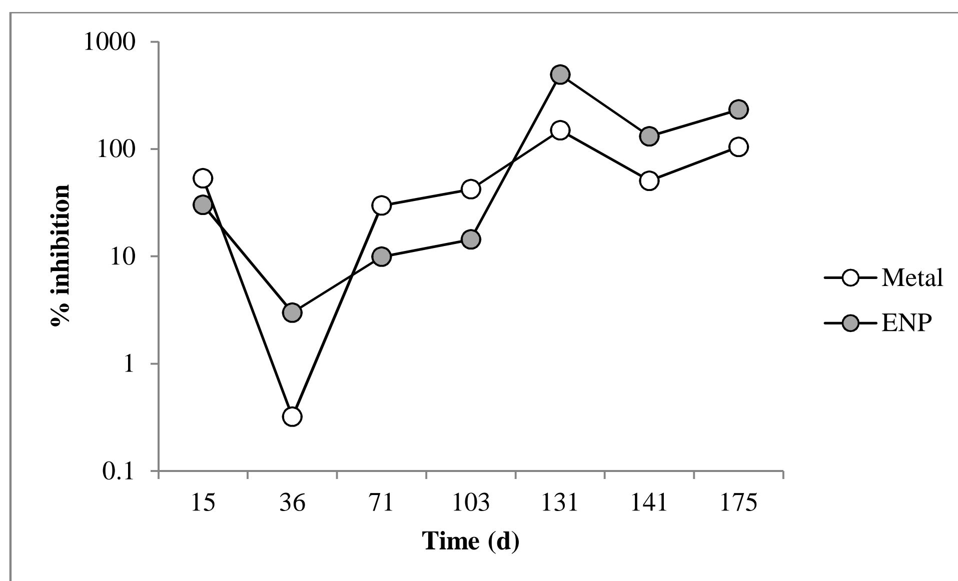


Figure 5.1 Percentage inhibitions based on $EC_{50}$ at 5 min by ENPs and metal salt ions in digestate filtrate

Toxicity decreases when the $EC_{50}$ value increases. Within the first 71 days, ENPs concentration that inhibited 50 % activity of *V. fischeri* was about 0.3 to 0.5 times higher than in metal salt spiked digestate filtrate except on day 36 in which metal salt ion was about 9 times higher than ENP. Although bioluminescence was inhibited at all

times, the trend however changed between 103 and 175 days as ENP was 2 to 3 times less toxic than the metal salt spiked filtrate. Factors such as sorption, interaction of ENPs with micropollutants, surfactants, natural organic matter and formation of complexes (Kiser et al., 2010), in addition to hydrolytic and fermentative stages of AD appeared to have changed ENPs redox potential and contributed to potentiation of the toxic effect.

Table 5.1 Percentage inhibition based on $EC_{50}$ at 5 min for ENPs and metal salt ions in digestate filtrate

| Time (d) | Treatment | Concentration in digestate filtrate (mg $l^{-1}$) | | | $EC_{50}$ (mg $l^{-1}$) | % Inhibition |
|---|---|---|---|---|---|---|
| | | $Ag^{+}$ | Ti | $Zn^{2+}$ | | |
| 251 | **Control** | - | - | 0.08 | 23 | n.a |
| 257 | | 0.03 | - | 0.4 | 17 | n.a |
| 263 | | - | - | 0.2 | 14 | n.a |
| 268 | | - | - | - | 29 | n.a |
| 282 | | - | 0.07 | - | 29 | n.a |
| 285 | | - | - | - | 28 | n.a |
| 289 | | - | - | - | 31 | n.a |
| 291 | | - | - | 0.2 | 38 | n.a |
| | **Metal salts** | | | | | |
| 251 | | 0.03 | - | 2.1 | 16 | 70 |
| 256 | | - | - | 0.05 | 17 | 100* |
| 262 | | 0.35 | - | 1.5 | 8 | 57 |
| 267 | | 0.05 | - | 0.1 | 13 | 45 |
| 284 | | 0.07 | 1.2 | 1.0 | 38 | 131* |
| 290 | | 0.09 | 0.44 | 0.9 | 41 | 146* |
| 297 | | - | - | 0.2 | 43 | 138* |
| 312 | | - | - | - | 28 | 100* |
| | **ENPs** | | | | | |
| 255 | | 0.05 | - | 1.1 | 15 | 65 |
| 262 | | - | - | 0.3 | 8 | 47 |
| 264 | | - | - | 0.04 | 20 | 143* |
| 269 | | 0.04 | 0.3 | - | 20 | 69 |
| 288 | | - | 0.4 | - | 30 | 103* |
| 291 | | 0.07 | 0.74 | 0.9 | 53 | 189* |
| 295 | | - | 0.2 | 0.3 | 20 | 65 |
| 298 | | 0.1 | 0.97 | - | 65 | 171* |

- = not detected, * = stimulating effect, n.a = not applicable

The concentration of ENPs and metal salts in digestate filtrate used for the test from day 251 to 312 are shown in Table 5.1. The $EC_{50}$ values from the three treatments were not

consistent with the concentration of ions in the samples. At undetected ENP or metal salt ion concentrations, toxic $EC_{50}$ values were observed and suggest that Microtox responded to the sludge components rather than the ENP or metal salt ions. The inability to estimate the true toxicity of aged-ENP ions in the anaerobic digestate is consistent with other studies in which antagonistic or synergistic interactions and the physicochemical properties prevalent in a complex medium had influenced the Microtox test (Li et al., 2007, Sherrard et al., 1996; Vasseur et al., 1986).

### 5.3.3 Bioavailable ENPs ions in digestate cake

The results presented in Table 5.2 shows that the ENP mixture in the digestate cake had no adverse effect on the activities of *Vibrio*. This was unexpected and suggests possible masking of ENPs effect by the digestate components. Also, it is plausible that the synergistic effect of diverse microbial activities including hydrolysis and fermentation transformed the ENPs to potentially innocuous by-products (Levard et al., 2012; Kaegi et al., 2011). Indeed, the response of Microtox to products of 8 weeks anaerobic degradation of vegetable oil by sediment microbial community indicated that toxicity decreased to near background level (Li et al., 2007).

Table 5.2 Effect of ENPs and metal salt concentrations on Microtox SPT

| Time (d) | Treatment (Digestate cake) | Concentration in digestate ($mg\ kg^{-1}$ dry wt) | | | 5 min $EC_{50}$ ($mg\ l^{-1}$) | Inhibition (%) |
|---|---|---|---|---|---|---|
| | | Ag | Ti | Zn | | |
| 251 | Control | 2.4 | 1600 | 760 | 56210 | n.a |
| 268 | | 1.0 | 1200 | 1000 | 61769 | n.a |
| 285 | | 7.4 | 1200 | 790 | 58963 | n.a |
| 263 | Metal salt spiked | 150 | 2200 | 2200 | 32.6 | > 99 |
| 284 | | 220 | 3000 | 3200 | 29.1 | > 99 |
| 290 | | 210 | 2800 | 3000 | 94.6 | > 99 |
| 262 | ENP spiked | 110 | 2500 | 2100 | 76215 | 136* |
| 269 | | 150 | 2500 | 2400 | 36302 | 59 |
| 289 | | 180 | 2600 | 2700 | 70196 | 119* |

*= stimulating effect

Surface charge and the release of ion are prominent mechanisms to exert toxic effect by ENPs and metal salts (Beer et al., 2012; El Badawy et al., 2012). Compared with the concentration of ions in metal salt spiked digestate, the result suggests that the effect of ions formed in the control and ENP spiked digestate were attenuated by organic matter and other sludge components. A change in the redox potential of ions in the digestate as a result of sequential anaerobic digestion process is a plausible reason for this outcome (Kaegi et al., 2011; Liu et al., 2013). On the other hand, the non-inhibition of *V. fischeri* bioluminescence was probably because ENPs were firmly bound to the oily tinged digestate cake and unavailable to make contact with the microbial cells or elicit any adverse effect. Besides, ENPs can exhibit amphiphilic behaviour and sorption to lipophilic components of the sludge can significantly reduce bioavailability in the aqueous medium used for the test. Again, it is also possible that the ions or reactive oxygen species usually formed by ENP to exert toxic effect may have been released from the digestate during filter-pressing. Thus, with only bound ENPs in the digestate

cake and unavailable free and active ionic $Ag^{+}$, $Ti^{4+}$ and $Zn^{2+}$ species, no toxic effect was observed for the digestate.

### 5.4 Conclusion

The concentration of ENPs and metal salts recovered in the WAS and effluent, and anaerobic digestate cake and filtrate illustrates the different sorption potential of the nano and bulk forms of similar substances influenced by wastewater components. Sorption to the AS and AD was influenced by wastewater components and plausibly by octanol-water affinity coefficient of the ENPs (Xiao and Wiesner, 2012) used in the study. Undetected levels of mixed ENPs or metal salt ions in the control and ENP spiked AS effluent evidenced the efficient removal of baseline and spiked ENPs through aggregation and sorption to wastewater biomass. Two times higher sorption/partitioning of the ENP than metal salts into WAS suggests significant difference in the behaviour of nano and bulk metal oxides. For instance, at the spiked concentration, traces of ions produced by metal salts were detected in the metal salt spiked AS in contrast to the ENP spiked AS. In the AD, bioavailable ENP ion concentration was higher in the digestate filtrate than in the cake compared with metal salts which further indicates different properties and affinities of the substances.

The use of Microtox can allow for indirect assessment of ENP bioavailability in AS and AD treatment products. The findings suggests that the response was a combined synergistic or antagonistic action of the mixed ENP and metal salts ions reacting with other wastewater component such as micropollutants which can either stimulate or inhibit *Vibrio* activity. In the Microtox SPT, however, ENPs were either in the bound form and unavailable for contact with the test organism and/or the free and active ionic species were plausibly lost during sludge dewatering thereby making the digestate appear potentially non-toxic. In relation to the filtrate ionic concentration, it was expected however, that toxic effect on bioluminescence can be exhibited by the concentrations in the digestate cake. Therefore, in a complex matrix such as the AS and AD, Microtox test does not seem to be sensitive to the presence of ENP ions and indicate the pressing need to develop bioassay using relevant wastewater microorganism.

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# Chapter 6 Integrated discussion

This research investigated the effects of a mixture of nanoscale materials referred to here as engineered nanoparticles (ENPs) comprising of $Ag^0$, $TiO_2$ and ZnO aged through activated sludge (AS) and anaerobic digestion (AD) processes. The guiding principle in the choice of the ENPs for the research was based on which ENPs were most used and or have the potential to be released into wastewater and interact and/or interfere with the microbial community functioning and activity within a wastewater treatment plant (WWTP). The results obtained were interpreted to provide insights and understanding on (i) the potential impact of ENPs on the biological processes during wastewater treatment (ii) contribute in bridging the existing knowledge gap and (iii) reducing the level of uncertainty in relation to the effect of ENP on microorganisms in complex matrix such as primary sludge, waste activated sludge (WAS), and anaerobic digestate. Activated sludge (AS) and anaerobic digestion (AD) are considered as important microbial-mediated biotechnological processes in the wastewater treatment which provided a relevant environmental condition for microbial interaction with aged-ENPs in real time.

Available information from most studies demonstrates the effects of pristine ENP on pure cultures of microbial cells and the potential interactions between ENPs and wastewater components (Figure 1.1). In this study, multiple physicochemical, microbiological and ecotoxicological methods were used as complementary approaches to investigate the effect of aged-ENPs in order to achieve the interlinked objectives (Figure 6.1). In this regard, the potential effect of aged-ENPs on microbial cells influenced by wastewater components in AS and AD were linked and evaluated to provide a holistic view of the interactions between them. The integrated evaluation of the three components comprising AS/AD, microorganisms and aged-ENPs is summarised in Figure 6.2.

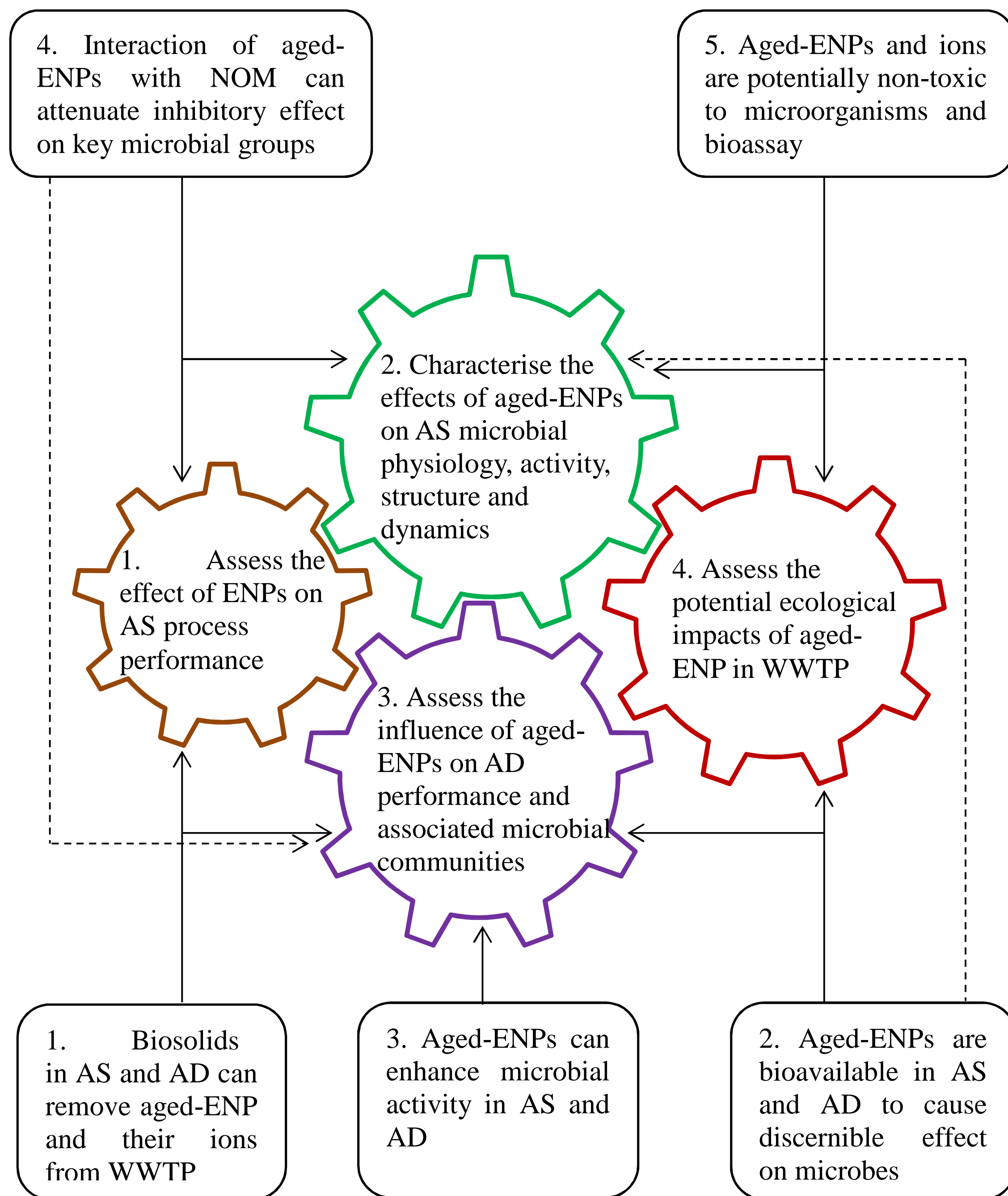



Figure 6.1 Schematic diagram of research hypothesis and the interlinked objectives Key: Hypotheses ▭ Objectives ⚙

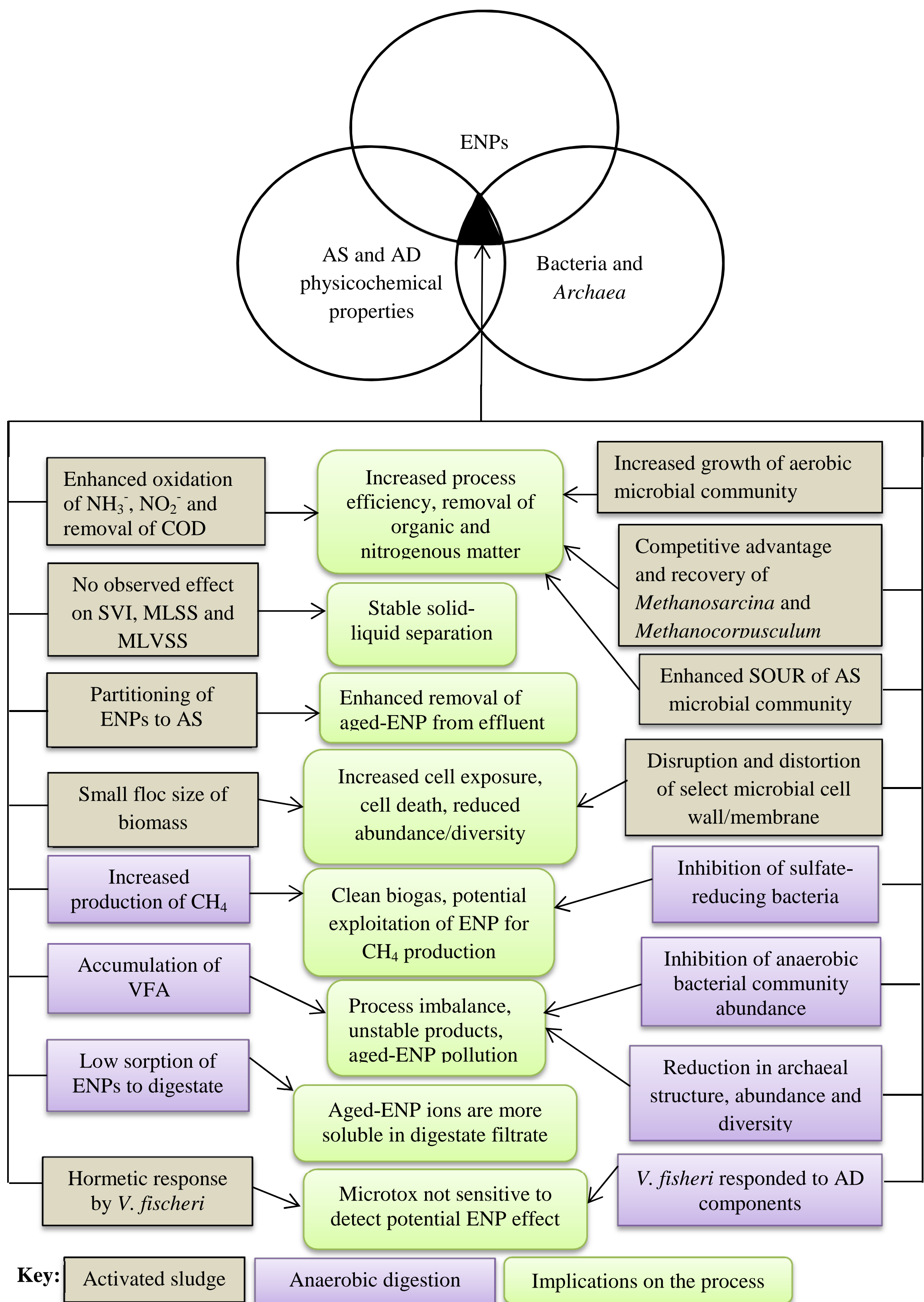



Figure 6.2 Schematic diagram of the interaction and potential impact of aged-ENP on AS and AD processes and microbial community

Data were generated based on the physicochemical characteristics of the sludge and digestate samples, the oxygen uptake rate of the sludge samples, the potential inhibitory effect/response of the nanoparticles using Microtox assay, and culture-dependent microbial growth response as well as the changes in microbial community structure, abundance and diversity using culture-independent molecular methods including lipid fingerprint analysis and 16S rRNA gene-based pyrosequencing analysis. Metal salts were used as a baseline parent compound of the ENPs and unspiked sludge (control) for comparison. The results were interpreted based on long-term (315 days) exposure of AS and AD microbes to aged-ENPs in a continuous flow pilot scale plants and 42 days cumulative effect of aged-ENPs on biomethane potential (BMP). In contrast to the effect of pristine ENPs on microbial cells in chemically defined medium, the results presented in Figure 6.1 suggest that the potential effect of aged-ENP can be stimulatory and/or inhibitory in a complex medium such as AS and AD.

## 6.2 Key findings in AS

The results presented in Chapter 3 relate to Objectives 1, 2 and 4 and illustrates the interaction between the physicochemical properties of the activated sludge and the microbial processes as well as evidence of the potential impacts as a result of exposure to nanoparticles. The aged-ENPs showed greater affinity to biosolids in aqueous medium than metal salts and as a result ions formed by ENPs were not detected in the effluent. The ENPs concentrations spiked into the AS induced a 2-fold increase of the specific oxygen uptake rate of the microbial community and stimulated the organisms to metabolise thereby removing organic and nitrogenous compound in the AS. *Nitrosomonas* and *Nitrospira* were inhibited by the spiked concentration of aged-ENPs mixture made up of 0.01 mg $L^{-1}$ $Ag^0$, 0.08 mg $L^{-1}$ $TiO_2$, and 0.12 mg $L^{-1}$ ZnO and spiked at the rate of 0.14 ml $min^{-1}$ (equivalent to 0.67 ml L $day^{-1}$) for 315 days. It is possible that ammonia and nitrite were oxidised by other microbial species which suggest that AS component can mitigate the potential inhibitory effect of aged-ENPs.

Further to this, there was no apparent disruption of biological removal processes in the AS by ENPs and metal salts although this does not mean there is no environmental risk. Indeed the electron microscopy (SEM) analysis of the sludge clearly showed damage to specific microbial. The results also pointed out that ions produced by aged-ENPs can reduce culturable microbial abundance, extracellular polymeric substances produced by cells and floc size of biomass without disrupting the overall process efficiency.

The 16S rRNA gene-based pyrosequencing analysis suggest that other chemolithoautotrophic bacterial species were involved in nitrification as a result of the steady substrate and product turnover in the ENPs and metal salts spiked AS. For instance, *Nitrospira* acknowledged as the most diverse and abundant nitrite oxidizing bacteria (NOB) in wastewater treatment plants (Daims *et al.*, 2001) had a 25 % occurrence in ENPs spiked AS compared with control (100 %) and metal salts spiked AS (0 %). Nitrification was not inhibited as a result of the absence of NOB and ammonia-oxidizing bacteria (AOB) in the ENPs and metal salts spiked AS which indicates that aged-ENPs ions can activate different organisms and/or metabolic pathway/enzyme of other microbial species to catalyse the degradation process.

ENPs induced a significant temporal shift in AS microbial community structure, abundance and diversity in which members of the *Betaproteobacteria* belonging to the *Comamonadaceae* such as *Acidovorax* and *Rhodoferax* were dominant nano-tolerant microbial species. Interestingly, members related to *Methanocorpusculum* sinense (99 % similarity), *Methanosarcina barkeri* (98 % similarity) and *M. acetivorans* (94 % similarity) retrieved from the ENP and metal salt spiked AS by pyrosequencing suggest that the organisms can use oxygen-dependent respiration and may have contributed to nitrogenous and organic carbon removal. Thus, aged-ENPs enhance the growth of ammonia-oxidizing archaea, *Methanocorpusculum* and *Methanosarcina* – organisms hitherto unidentified as having any presence or contribution in AS process. Nano-tolerant *Archaea* with oxygen-dependent respiration is reported here for the first time.

### 6.3 Key findings in AD

The findings in the AD corresponds to Objectives 3 and 4 achieved in Chapters 4 and 5. The results indicate that sorption of aged-ENPs to digestate cake in the AD differed from the metal salts. ENPs were more soluble in oily tinged and viscous digestate filtrate than metal salts which suggests different octanol-water affinity coefficients with plausible hydrophobic properties.  Results of the pilot-scale experiment suggest that the potential inhibitory effect of aged-ENP differs from their bulk metal salt forms. For example, about 80 % inhibitions of members of the *Archaea* suggest the potential effect of aged-ENP on key anaerobic organisms, although methanogenic activities of *M. barkeri* and *M. acetivorans* were not inhibited in the presence of aged-ENPs toxic dose. On the other hand, sulphate-reducing organisms were inhibited which resulted in low $H_2S$ production. As a result of process imbalance caused by frequent feeding and

biomass washout, volatile fatty acids (VFA) accumulated in the digesters although without any adverse effect observed on methane production. Isovaleric acid accumulation indicates microbes were subjected to a state of rest, delay of growth or inhibition by ENP and metal salt spike. Furthermore, the likelihood and severity of ENPs potential effect on AD microbial community increased with exposure time regardless of the organic material in the AD as reflected by the temporal changes in microbial community structure, abundance and diversity. Members of the *Archaea* belonging to the genus *Methanosarcina* were able to proliferate in digestate with toxic dose of aged-ENPs which suggests that *Methanosarcina acetivorans* and *M. barkeri* recovered in the digestate are nano-tolerant methanogenic *Archaea*.

Overall, the results suggest that the potential effect of aged-ENP in AS/AD is complex and multifactorial, and it can vary in response to the sludge and ENP physicochemical characteristics as well as the microbial physiology and metabolic ability. Methylotrophic and hydrogenotrophic methanogenesis appeared to be the preferred pathway of methane production in the presence of toxic concentration of ENP by the species of *Methanosarcina* which suggest that the organism can be exploited in AD process to enhance methane production in ENP-enriched sludge.

## 6.4 Knowledge gaps filled by the research questions and addressed in this study

The pilot plant and laboratory experiments provided key insights to fill the knowledge gaps raised from the research questions:

(i) Biosolids in activated sludge and anaerobic digester can act as porous media in the partitioning and removal of aged-ENPs during the wastewater treatment process. The mechanism of aged-ENP associating with the biosolids is perceived to be primarily due to sorption and agregation

(ii) Undetected concentrations of ions released by aged-ENP in AS effluent suggest that ENPs are unavailable in AS effluent probably as a result of sorption to the activated sludge, although were present in digestate filtrate. Therefore, aged-ENP partitioning into biosolids can differ in AS and AD influenced by the different physicochemical characteristics and the octanol-water affinity coefficient of the ENPs.

(iii) The growth of some microbial groups were enhanced in the presence of aged-ENP ions which suggest that the aged-ENPs mixture exerted beneficial

effects on their ability to use nitrogenous material, degrade organic matter in activated sludge and biogas production in anaerobic digester.

(iv) Varying growth enhancing and inhibitory effects of aged-ENP ions on the activities of the key microbial groups in AS/AD was observed in addition to cell wall/membrane damage in specific cells. The known AOB and NOB such as *Nitrosomonas* and *Nitrospira* were eliminated in the AS and members of the *Proteobacteria* and *Methanosarcina* can grow in the presence of aged-ENP.

(v) Synergistic and or antagonistic reaction of aged-ENPs with wastewater components resulted in masking of aged-ENPs potential impacts on AS and AD process efficiency with a Trojan horse-like effect on the microbial community.

The results of ion concentration detected in the control sample in part represent ions formed by the ENPs released into wastewater, and in part ions from bulk metal salts. Assuming the former is the case, the concentration in the control AS/AD evidenced the increasing use and release of ENPs from consumer products into wastewater and transport to wastewater treatment plant. In the AS pilot plants, however, processes such as oxidising of ammonia and nitrite, degrading of organic matter carried out by syntrophic action of diverse organisms was uninhibited at the spiked ENPs concentration, although an order of magnitude decrease in cultivable microbial growth was observed. In addition, ENPs and metal salts enhanced the AS microbial community specific oxygen uptake rate compared to the control. Despite the no observed adverse effect on biological removal process in AS, damage to select microbial cells by ENPs and metal salts was evident. The non-recovery of the common ammonia- and nitrite oxidising bacteria such as *Nitrosomonas*, *Nitrobacter* or *Nitrospira* in the ENP spiked AS suggests that ENPs can inhibit select organisms during AS process. The study shows that at the spiked concentration, aged-ENPs can exhibit biologically relevant characteristics (e.g., high surface area and reactivity) needed to inhibit microbes at below detected levels in the AS. At low ENPs concentration, it is plausible that through synergistic interactions or potentiation in contrast to a rapid change, aged-ENPs mixture exerted a slow build-up of negative effects on AS biomass. As a result, small flocs of biomass were formed which was an adverse effect on microbial cells producing

extracellular polymeric substances although the solid-liquid separation properties of the AS was barely affected.

In the anaerobic reactor, the frequency of feeding caused process imbalance during digestion, although the negative effect of accumulating VFA, $H_2S$, and other inhibiting substances including aged-ENPs on methane producing organisms were mitigated. For instance, ENP inhibited organisms that produce $H_2S$ and stimulated those that metabolise it resulting in the low $H_2S$ concentration in the digestate compared with the control and metal salt spiked AD. Similarly, ENPs may have formed complexes such a silver sulphide thus reducing the toxic effect. The differences in isovaleric acid concentration clearly suggests that most organisms in the metal salt and ENP spiked AD were in a state of induced rest.  On the other hand, the response of *V. fischeri* luminescence to ENPs ranged from hormetic effect in the AS and effluent to non-toxic and toxic effects in digestate cake and filtrate respectively. The response of *V. fischeri* to the detected concentrations in the digestate cake and filtrate were inconsistent indicating a discontinuous biological effect. Therefore, evaluating ENPs effect in AS and AD needs bioassay with indicator organisms from wastewater because varying wastewater components can influence *V. fischeri* to give false-negative result by responding as non-toxic to a concentration in the digestate that would be harmful to ecologically important microorganisms.

In regards to the shift in AS microbial community structure, abundance and diversity, members of the *Betaproteobacteria* notably *Acidovorax*, *Rhodoferax*, *Comamonas*, and the *Archaea* - *Methanocorpusculum* and *Methanosarcina* were identified as nano-tolerant species. Aged-ENPs in the both aerobic and anaerobic conditions tested did not induce any adverse effect on the growth and metabolic activities of *Methanosarcina* compared with other microbial groups. *Methanosarcina*, a key methanogen recovered in the digestate proliferated and produced methane in the presence of toxic dose of aged-ENPs demonstrates the tolerant nature of the organism which can be exploited in removing or converting ENPs to harmless substances. Methanogenesis was not inhibited in the ENP spiked digestate most probably because *Methanosarcina* through hydrogenotrophic pathway can scavenge $H_2$ from the digesters. Also, the versatile metabolic and genomic properties of *Methanosarcina* provided the organism with a competitive advantage over the other methanogens against the toxic effect of aged-ENPs in the AD.

The results presented here provide a basis to understand that aged-ENPs mixture can stimulate microbial-mediated processes, inhibit bioactivities or disrupt bacterial cells. In addition, wastewater components can react to increase or reduce the ENPs concentration available for contact to give a time-dependent Trojan horse-like effect on microbial community in both AS and AD. Therefore, ENPs effect on microbial community structure, abundance and diversity in AS/AD should be interpreted with caution based on the research context, experimental conditions, and parameters analysed. Further to this, quantum effect of ENPs can exert a discontinuous biological impact and their hazard potential to ecologically important microbial cells can increase or decrease in line with prevailing environmental conditions and biochemical reactions. Overall, the combined effect of aged-ENP mixture reacting with each other and wastewater components was expected to be greater than the effect of a single ENP and the AS/AD process efficiency can be extrapolated to ecosystem functioning with the understanding that aged-ENP in the long-term can affect microbial-mediated processes.

### 6.5 Significance and implications of the key findings of this research

This study offers multiple perspectives on the potential effect of aged-ENP mixture on key microorganisms in AS/AD which can be extrapolated to other complex environmental matrix e.g. sediment and soil to estimate the fate, effect and transport of aged-ENP mixture. Although wastewater biosolids is a primary sink and source for aged-ENPs released from consumer products, ENPs and bulk metal salts differed in their sorption to biosolids and potential effect on AS and AD microbial community structure, abundance and diversity. The results suggest that ions formed by aged-ENPs mixture are potentially inhibitory to specific microbial groups in AS/AD in a long-term exposure regardless of the presence of natural organic matter. It is suspected that wastewater components can enhance or attenuate ENPs contact and antimicrobial effect at different time of exposure. For instance, acute effect can be attenuated by natural organic matter and other wastewater components on short-term exposure. In a long-term exposure, however, the degradation of organic matter and the change in pH can result in desorption and release of aged-ENPs to reacts with micropollutants and exert antimicrobial effect.

The result also showed that microbial community response to aged-ENPs in AS/AD differs and is dependent on their physiology and metabolism whereas low ionic concentrations can stimulate certain microbial groups in AS/AD. However, the

synergistic effect of aged-ENP mixture and wastewater components can inhibit specific microbial activity in AS/AD which suggests negative implications for other microbial-mediated processes. For instance, culture-independent approaches such as the PLFA, PLEL and pyrosequencing results highlights the potential effect that aged-ENP can exert on the microbial community. The ENP spiked into the AS and AD can be considered as a threshold concentration of toxicological concern for microbial community as a result of the inhibitory effect on specific microbes in AS/AD.

Furthermore, nano-tolerant species of Bacteria and *Archaea* such as A*cidovorax* and *Methanosarcina* respectively have been identified. This finding suggest that aged-ENPs can hasten the natural selection of AS and AD microbial species. Also, there is a need to develop appropriate bioassay for ENP toxicity assessment using relevant indicator organisms in wastewater to complement existing approaches such as Microtox which was not appropriate or sensitive enough to detect effect of aged-ENP in digested sludge.

## 6.6 Challenges and limitations of the study

The detection and quantification of aged-ENPs in AS and AD requires new approaches in trace analysis with realistic limits of detection and quantitation which at the moment are not available. The implication is that undetected concentration of ions released by aged-ENP present in effluent or sorbed to the digestate can inadvertently be released into the environment which can potentially impact ecologically sensitive microbes. Also, it was not possible to differentiate between aged-ENPs and bulk metal salts in complex AS and AD matrices which made it difficult to establish effective dose or guidelines on toxic concentration as a result of varying wastewater components. In addition, the transformed products of aged-ENP or the complexes formed with ligands and micropollutants in the waste AS and AD were not characterised, which suggest that inadequate knowledge was obtained from the different biochemical reaction between the aged-ENP mixture in AS/AD influencing the microbes. On the other hand, the pilot plant anaerobic reactors were affected by temperature fluctuations especially during the winter period, frequent feeding of the reactors and digestate extraction which contributed to the AD process imbalance and biomass washout.

## 6.7 Suggestions for further studies

This study provides evidence of the potential impact of aged-ENP on microbial community structure, abundance and diversity in relation to AS and AD process efficiency although studies on case-by-case basis is required to delineate pathways or

mechanism of action by aged-ENPs. The interaction of aged-ENP mixture with microorganisms in AS/AD that needs further understanding listed below were beyond the scope of this study:

(i) Measurement of live/dead cell ratio, cellular uptake and accumulation of aged-ENPs by microorganisms

(ii) Transformation of ENPs by nano-tolerant bacteria and *Archaea* and in situ characterisation of the complexes formed in AS and AD.

(iii) Octanol-water affinity coefficient of mixed $Ag^0$, $TiO_2$ and ZnO in AS/AD and their potential influence on the microbial mediated nutrient removal

(iv) Development of bioassay using indigenous wastewater microorganisms to complement the existing method.

(v) Effect of ENPs on denitrification process, $PO_4^{3-}$ and $SO_4^{2-}$ removal in AS.

(vi) Interaction and influence of divalent cations and anions on aged-ENP mixture on the AS and AD processes.

(vii) Aged-ENPs mixture and bulk metal salt speciation in AS and AD.

(viii) Effect of pH and conductivity on the affinity and partitioning of aged-ENP mixture into AS and AD biosolids

(ix) Sequestration of aged-ENPs in AS and AD by nano-tolerant bacteria and *Archaea*

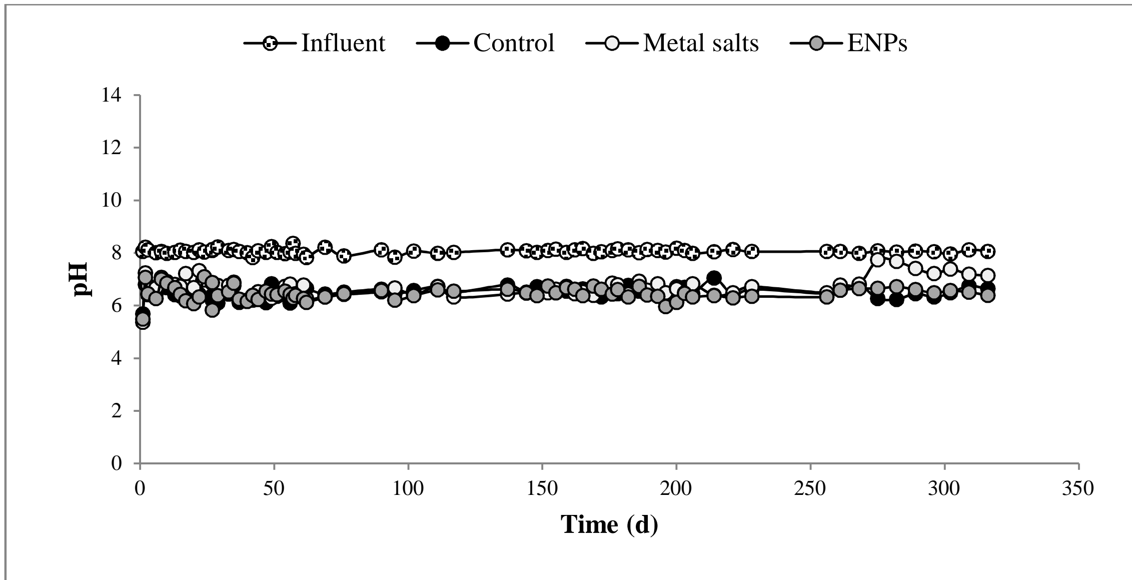
(x) Effect of aged-ENPs mixture on thermophilic anaerobic digestion of sewage sludge

(xi) Molecular basis of programmed cell death induced by aged-ENPs on the microbial community in AS and AD

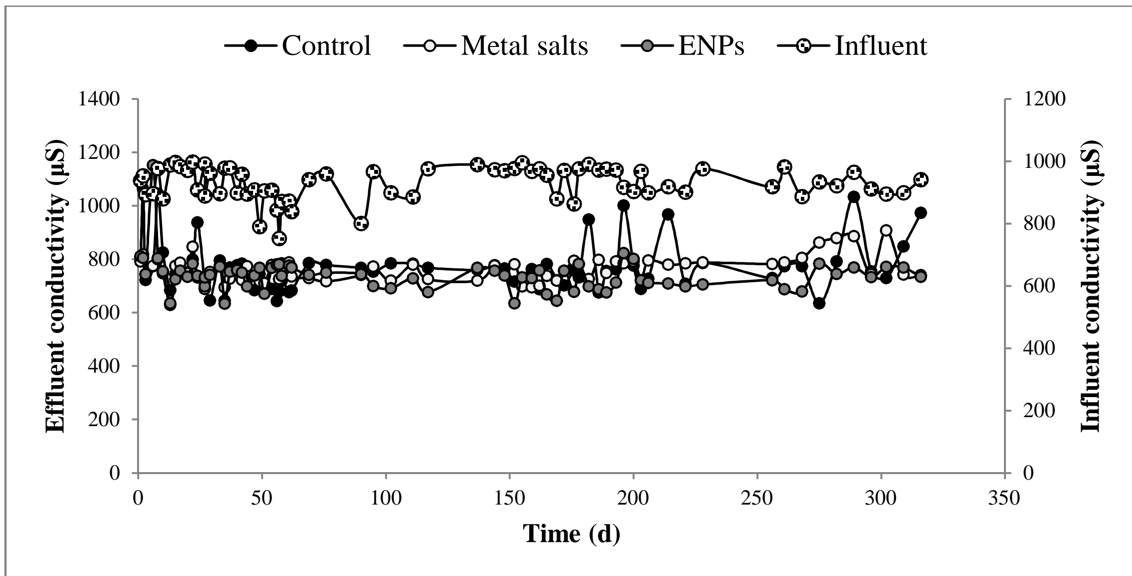
(xii) Influence of aged-ENPs mixture and their ions on bacterial and archaeal enzyme system

(xiii) Molecular basis for the tolerance of *Methanosarcina* to aged-ENP mixture in AS/AD

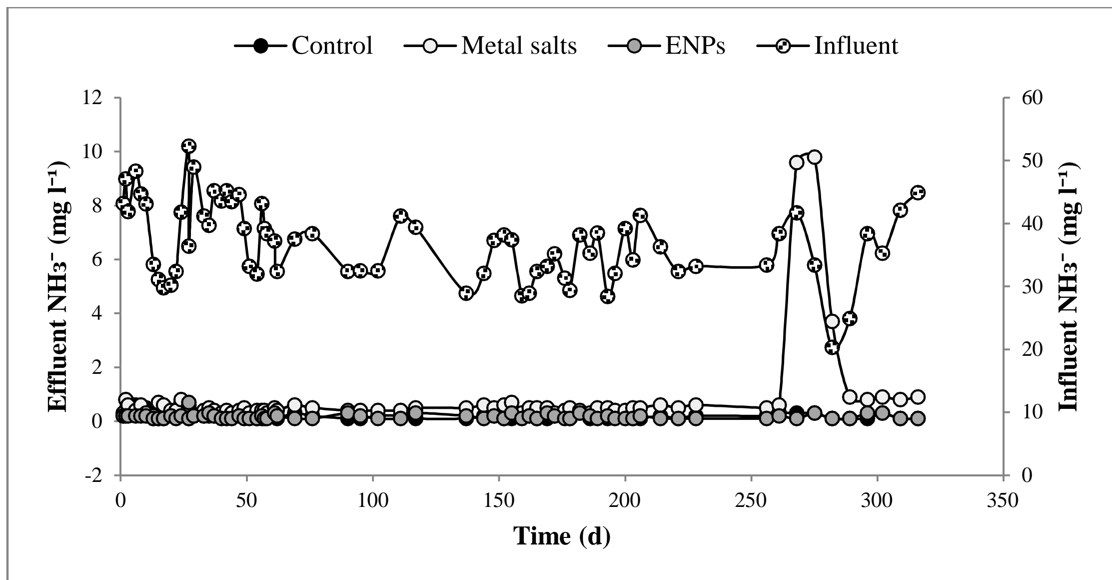
**Appendix**



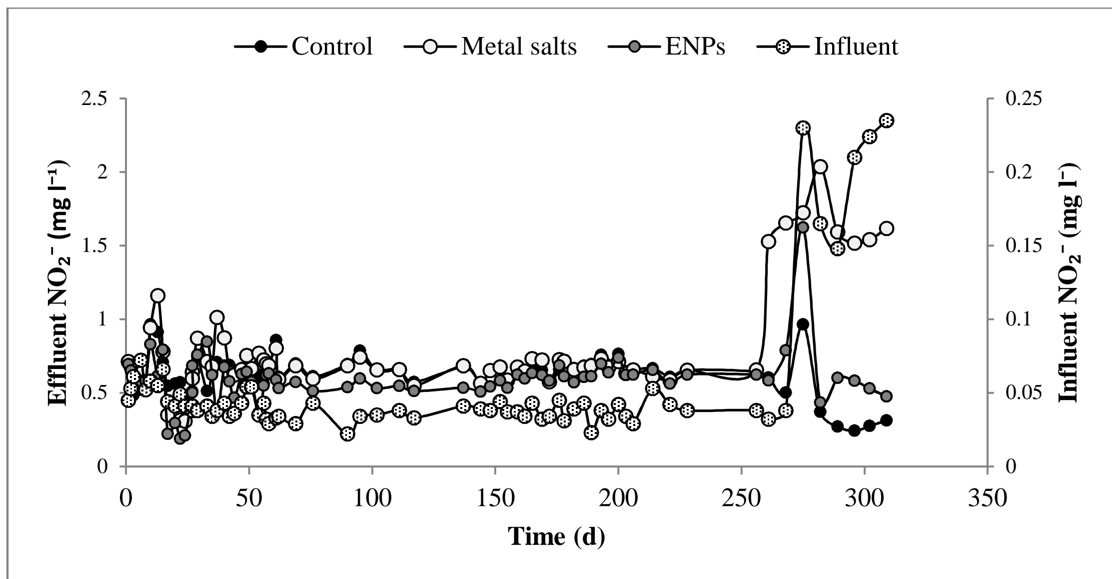

Influence of aged-ENPs and metal salt mixture on the pH of AS



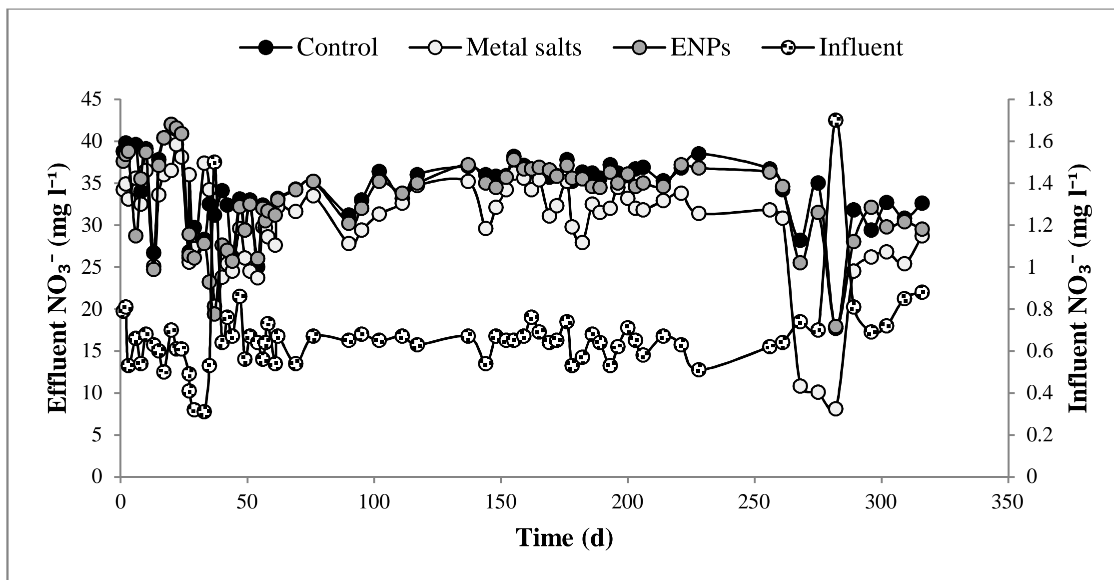

Influence of aged-ENP and metal salt mixture on AS conductivity



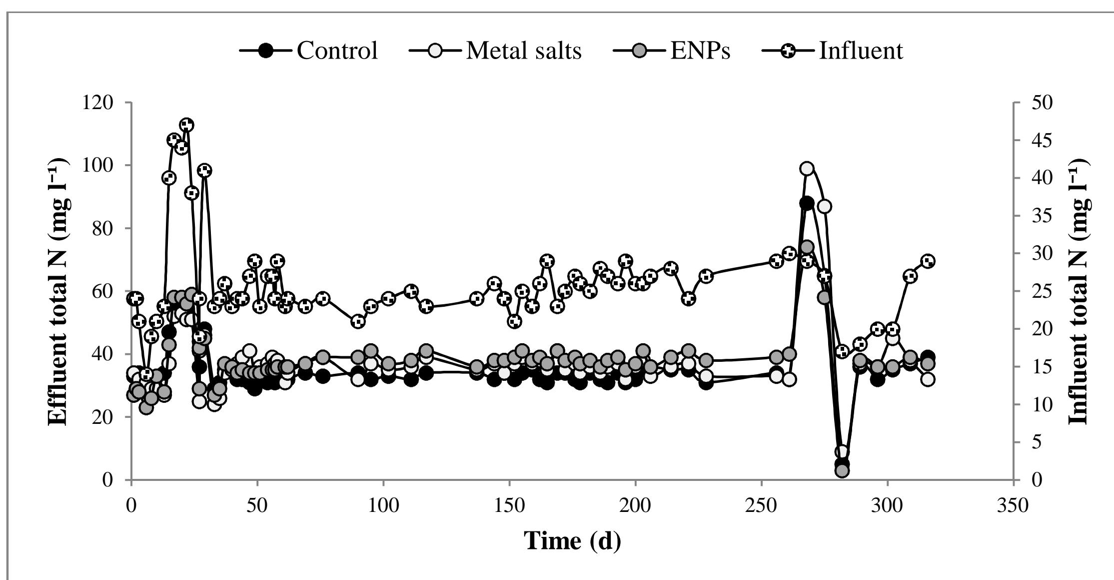

Effect of aged-ENP and metal salts on the bulk removal of ammonia in AS



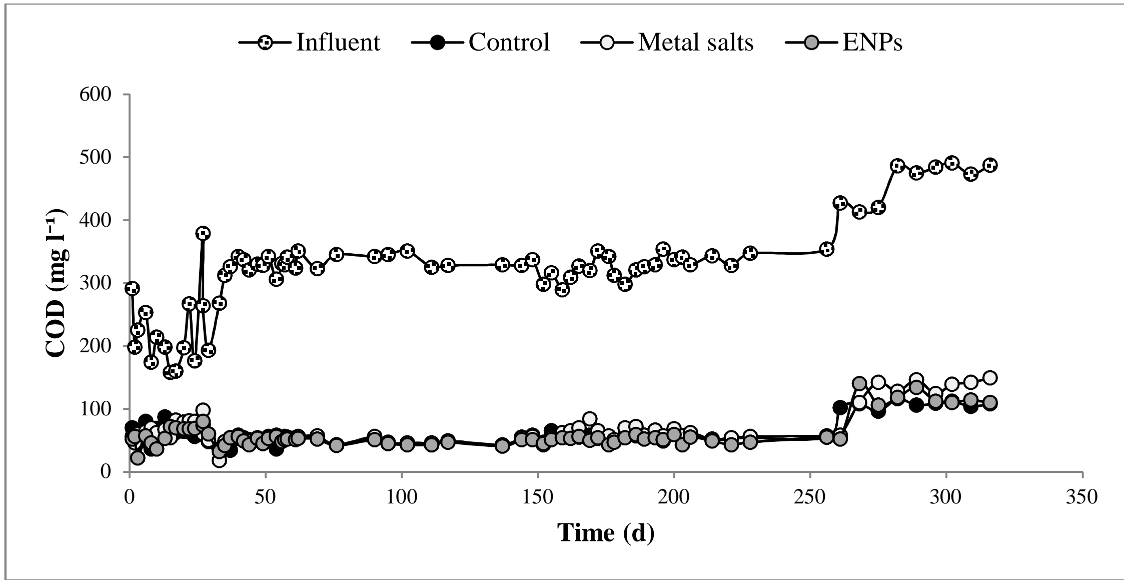

Effect of aged-ENP and metal salt on nitrite removal in AS



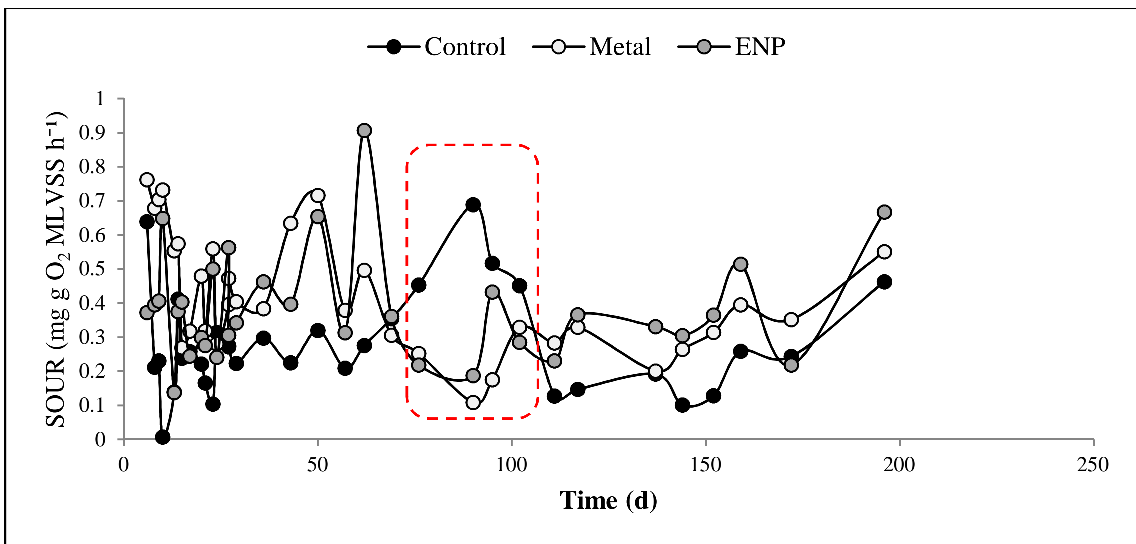

Effect of aged-ENPs and metal salts on nitrate reduction in the AS



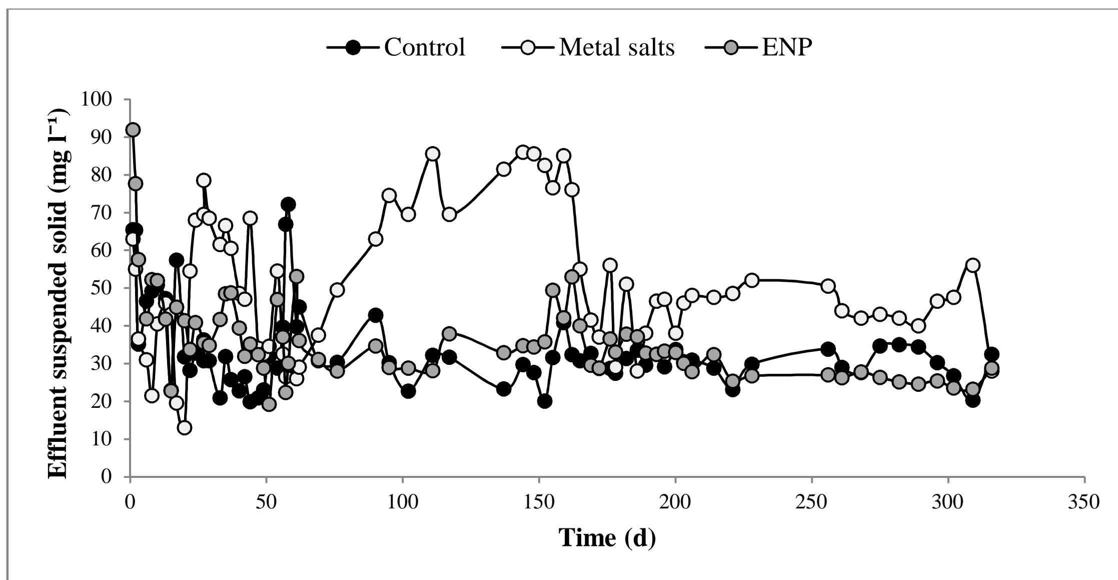

Effect of aged-ENP and metal salt on the total nitrogen in AS



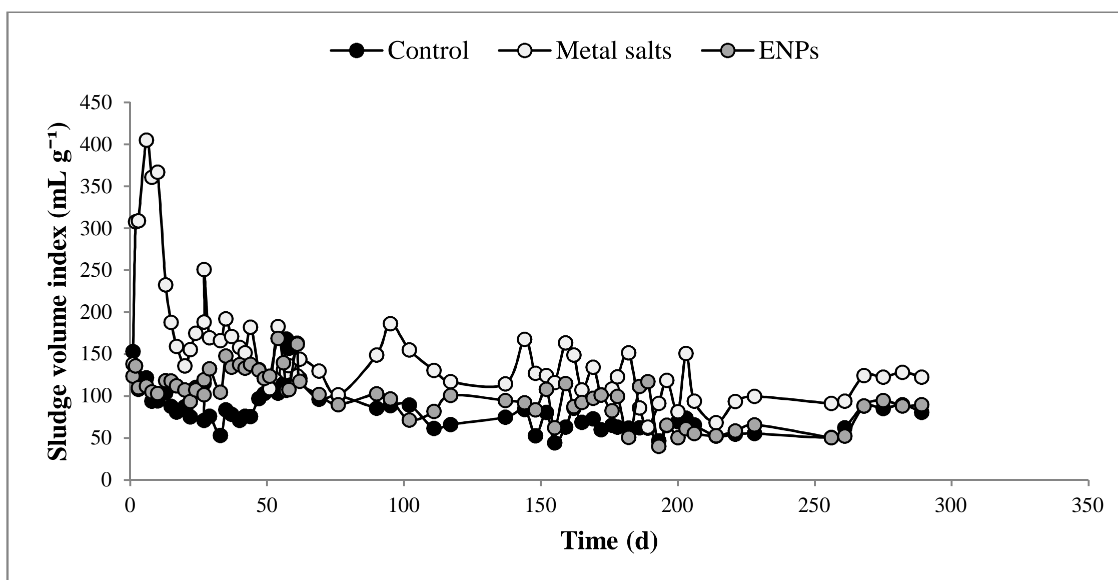

Effect of aged-ENP and metal salt on organic matter removal in the AS



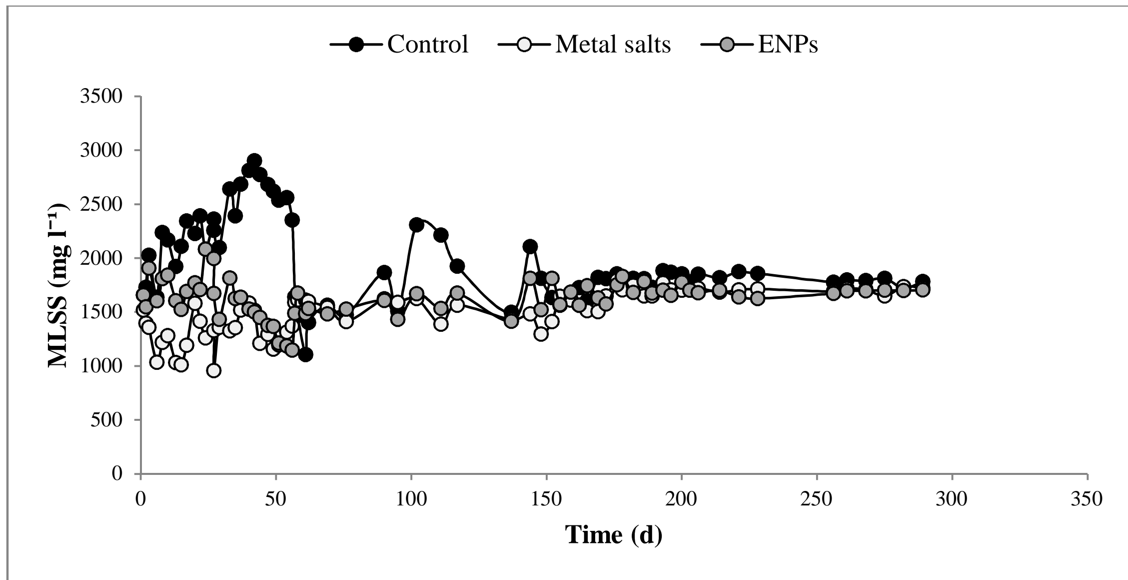

Effect of aged-ENP and metal salt on SOUR of AS microbial community



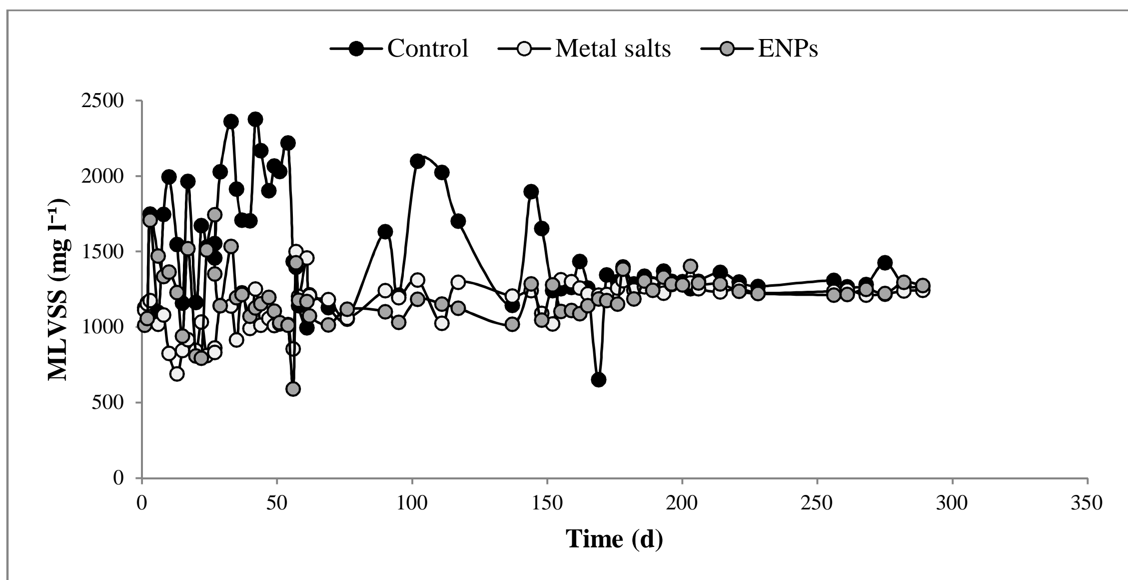

Influence of aged-ENP and metal salts on ffluent suspended solids




Effect of aged-ENP and metal salts on the sludge volume index




Aged-ENP and metal salt effect on mixed liquor suspended solids




Aged-ENP and metal salt effect on mixed liquor volatile suspended solids