Nanoparticles applied to membrane bioreactors: potential impact on reactor performance and microbial communities

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Abstract

Both aerobic and anaerobic membrane bioreactors (MBRs) are able to remove emerging contaminants from wastewater at high efficiencies. However, the main bottleneck of this technology is membrane biofouling. Coating heavy metal nanoparticles on the surface of membrane has been proposed as an effective antifouling strategy. Nevertheless, metal nanoparticles can potentially result in detrimental impact on the overall functionality of the MBRs. This review aims to understand how nanoparticles impact MBRs. To achieve this aim, the review starts off by illustrating the antibacterial mechanisms of nanoparticles. The review then critically evaluates past studies that illustrate the antibacterial effect of nanoparticles against pure bacterial cultures and biofilm-associated populations. Finally, the review discusses if the presence of nanoparticles would affect the overall performance of aerobic and anaerobic biological processes. Specifically, the impact of heavy metal nanoparticles on nitrogen and phosphorus removal process of aerobic MBRs were discussed. The effect on anaerobic MBR, which is comprised of hydrolysis, acidogenesis, acetogenesis and methanogenesis stages, was also reviewed.

Keywords: Heavy metals, nanoparticles, antibacterial, functional genes

Water impact

Nanoparticles have been widely applied to membrane modification for its excellent antibacterial and antifouling effects. Nevertheless, the unintended leaching of nanoparticles would be potentially detrimental to the biological activity in MBR, resulting in inadequately treated effluent. This review collates past studies and critically discuss the antibacterial mechanisms of nanoparticles and its potential impacts on aerobic and anaerobic biological processes.

1. Introduction

Over the last century, continued industrialization and population growth have resulted in a wastewater stream that is becoming increasingly complex with time. The presence of emerging contaminants (ECs) such as pharmaceuticals, illicit drugs, personal care products, antibiotic-resistant pathogens and antibiotic resistance genes in untreated wastewaters have been widely reported by many studies [1-4]. Heavy metals in influent of wastewater treatment plant located in Thuwal, Saudi Arabia was measured, and the result revealed heavy metal concentration to range from several ppb to more than 20000 ppb in the untreated wastewater [5]. Many of these ECs can detrimentally impact the natural ecosystems if left unremoved from the wastewaters. To illustrate, silver nanoparticles and silver ions can result in a significant decrease in marine biofilm volume and biomass, and perturb the major bacterial groups in a marine ecosystem [6]. Furthermore, as more countries pursue water reuse to mitigate water scarcity, the presence of ECs in the reclaimed water can impose public health concerns.

To remove ECs from wastewaters, processes including physicochemical treatment (e.g. activated carbon absorption), biological treatment (e.g. activated sludge) and advanced treatments (e.g. ultra-violet (UV) photolysis, and membrane filtration) [7] can be used. However, each process has its strengths and limitations. To exemplify, although activated carbon adsorb hydrophobic compounds and non-polar compounds effectively by up to 1 log, it is less effective against hydrophilic and polar compounds [8-10]. Activated sludge can remove a wide range of emerging contaminants by absorption and biodegradation, but is unable to remove recalcitrant compounds like polar ECs efficiently [11]. UV photolysis requires high UV intensity and contact time to remove ECs [12, 13], which makes it less cost-effective.

In contrast, membrane bioreactor (MBR) is able to remove ECs from wastewater at high log removal rates, usually reducing the concentrations by more than 1-2 log compared to the influent stream [14]. MBR systems couple microfiltration or ultrafiltration membranes to activated sludge and are able to achieve an efficient solid–liquid

separation. The result is a permeate stream of higher quality than that obtained from activated sludge tanks. Furthermore, MBR is typically operated with a longer sludge retention time and has higher sludge concentration which may improve the removal efficiency of ECs through adsorption and biodegradation. Despite MBR having these advantages, the main bottleneck of this technology is membrane biofouling.

Biofouling is the unwanted deposition of materials, particles, and bacteria on the membrane surface during the filtration process [15]. Soluble microbial products (SMP), bacteria and other colloidal particles first attach onto the membrane through adhesive forces. Nutrients and other trace metals which are highly abundant in wastewater streams then serve as substrates for the immobilized bacteria, resulting in the growth of bacteria and contributing to the biofilm formation. The inherent resistance associated with the biofilm layer would decrease water flux, increase energy consumption and operational costs, and shorten the lifetime of membranes [16].

Considering the problems caused by biofouling, devising antifouling strategies has become a widely researched topic. Examples of antifouling strategies include physical membrane maintenance (e.g. backwash, chemical wash, sonication) [17], chemical modification (e.g. nanoparticles coating, organic functional groups modification) [18] and biocidal control (e.g. quorum quenching, phage-based decomposition) [19, 20]. Among them, coating heavy metal nanoparticles on the surface of membrane has been widely applied to inhibit fouling layer. It is thought that antibacterial effects associated with heavy metals and nanoparticles [21, 22] would inhibit the initial attachment of bacteria to membranes. Although nanoparticles have been widely applied to control biofouling, the mechanism behind how nanoparticles decrease biofouling is relatively less understood at the molecular level. Furthermore, it should be brought to attention that heavy metal nanoparticles are themselves, a form of ECs. While coating membranes with heavy metal nanoparticles to create antifouling membranes can potentially delay or eradicate membrane biofouling, it may also result in unintended leaching of these ECs into the biological processes. The presence of heavy metal nanoparticles in the biological system

88 can impede biological activity and in turn cause a potential detrimental impact on the
89 overall treatment efficiency.

90 This review therefore aims to understand how nanoparticles can potentially impact
91 MBRs. To achieve this aim, the review starts off by illustrating how nanoparticles induce
92 antibacterial effect on microorganisms. Past studies that illustrate the antibacterial
93 effect of nanoparticles against pure bacterial cultures and biofilm-associated
94 populations were collated and critically assessed. Finally, this review evaluates if the
95 presence of nanoparticles in wastewater treatment systems would affect the overall
96 performance of aerobic and anaerobic biological processes. This is done by assessing the
97 gene expression profiles of keystone bacterial or archaeal populations that are
98 contributing to the overall functionality of those biological processes upon exposure to
99 heavy metal nanoparticles.
100

101 **2. Antimicrobial mechanisms of nanoparticles**

102 There are four main mechanisms to account for the antimicrobial effects associated
103 with nanoparticles. First, the production of reactive oxygen species (ROS) induces
104 oxidative stress on microorganisms. Second, metal ion toxicity directly kills or affect
105 microbial cell growth. Third, nanoparticles accumulate on cells' surfaces and induce
106 toxicity. Lastly, internalization of nanoparticles within the cells induce cell death [23-27] (Fig.
107 1).

108 *2.1 ROS induced damage*

109 The toxicity of nanoparticles is frequently attributed to oxidative stress induced by ROS
110 such as hydroxyl radicals, hydrogen peroxide, superoxide ions, and singlet oxygen [26-33].
111 Different nanoparticles produce ROS under different conditions. Some metal oxide
112 nanoparticles (i.e., ZnO and TiO_2 nanoparticles) produce ROS upon UV or visible light
113 illumination [34]. However, for sliver nanoparticles, it can spontaneously generate ROS
114 without illumination [35].
115

The molecular mechanisms of ROS damage has been widely studied. ROS could induce lipid peroxidation, which is a highly deleterious reaction. Furthermore, ROS can also oxidize membrane-bound proteins, and then alter the integrity of cellular components. Intracellular ROS in cytoplasm could lead to a series of damage to bacteria [36]. For example, superoxide and hydrogen peroxide (H_2O_2) produced by nanoparticles can oxidize 4Fe-4S clusters and other enzymes within the cytoplasm. In addition, hydrogen peroxide can directly react with cysteine residues while hydroxyl radicals can damage any biological molecules, including DNA, in a diffusion limited manner. These types of damage collectively result in metabolic defects and eventually cell death [36].

In order to protect themselves from oxidative stress induced by ROS, bacteria are equipped with an array of antioxidant defenses that range from small, oxidant-scavenging molecules to self-regulating gene networks [37]. ROS-induced damage would be regulated by a cascade of antioxidants, including superoxide dismutases, catalases, glutathione, peroxidase and some non-enzymatic antioxidants (i.e., α-tocopherol and ascorbic acid) [38]. A triple mutant *sod*ABC *E. coli* strain could not produce superoxide dismutases, and was very sensitive to silver nanoparticles [39], highlighting the role of this enzyme in protecting against ROS induced by silver nanoparticles. Similarly, Xie and co-authors showed that oxidative stress genes (i.e., *kat*A coding catalase and *ahp*C coding alkyl hydroperoxide reductase) were upregulated by 52- and 7-fold upon exposure to ZnO nanoparticles [40]. These studies highlight that one of the main mechanisms behind antimicrobial effect of heavy metal nanoparticles is through the production of ROS.

2.2 Metal ion toxicity

Heavy metals dissolute to release metal ions. When present in the ionic state, they induce toxicity due to their affinity for cellular components and biomolecules, and the subsequent formation of metal–biomolecule complexes. Metal ions can bind or block the functional groups in a biological molecule. It can also displace the essential metals in enzymes, and bind to the cellular thiol pool or participate in deleterious chemical reactions within the cell. Ultimately, metal ions damage proteins, DNA and biological

membranes, and interfere in enzyme function and cellular processes [41]. In addition, toxicity of metal ions is normally accompanied by ROS-mediated toxicity. Bondarenko and co-authors showed that Cu ions released from CuO nanoparticles was the key factor in triggering the ROS and DNA damage [42]. Ag nanoparticles can easily destroy the membrane, pass through the microbial body, then convert to silver ions in cytoplasm to damage the intracellular structure [43]. In one study, 19 different types of nanoparticles were examined and it was determined that ions released from nanoparticles were cytotoxic [44]. In another study, Zheng et al observed similar inhibitory effects on microbial denitrification by either Zn ions or ZnO nanoparticles, hence indicating that the toxicity of ZnO nanoparticlse was due to Zn ions release [45].

However, discerning the mode by which dissolved ions and nanoparticles lead to microbial cell death is difficult to prove experimentally and only few studies focused on this. To illustrate, Neal et al. compared the effects of exposing sublethal concentrations of ZnO nanoparticles and Zn ions to *Cupriavidus necator*, and results indicated nanoparticles mainly affected membrane-associated proteins. In contrast, zinc ions exposure affected metabolic processes, likely because Zn ions can act as important co-factor for a number of enzymes including DNA and RNA polymerases [46]. Similar result was observed when *Daphnia magna* was exposed to Ag nanoparticles and ions [47]. Ag nanoparticles could disrupt the major biological processes including protein metabolism and signal transduction, while the Ag ions disrupted developmental processes. It is likely that both metal ions and nanoparticles affect different metabolic pathways due to varying affinities towards certain biomolecules, but synergistically complement each other to result in cell death.

2.3 Nanoparticles accumulation on membrane surface

The interaction between microbial cell wall and nanoparticles may lead to toxicity. It was reported that nanoparticles accumulate on cell wall by complexing with lipopolysaccharide and lipoteichoic acid through hydrogen bonding and ligand exchange. This interaction can subsequently change protein structure [48]. Positively charged CeS_2

nanoparticles are electrostatically attached to the negatively charged bacterial membranes at neutral pH and display strong electrostatic attraction toward bacterial outer membrane [26]. Alternatively, the electrostatic interactions are attributed to negatively charged phospholipids in cell membrane absorbing to positively charged nanoparticles [30].

The toxicity of nanoparticles accumulation was reviewed by Djurisic et al. [23]. After nanoparticles attach onto the cell surface, nanoparticles facilitate redox reactions and result in oxidative stress. Hence the toxicity of nanoparticles accumulation can also be attributed to ROS generation. In addition, Ivask et al. pointed out that nanoparticles attachment may cause mechanical damage, such as change in cell morphology and deformation of membranes, disorganization or leakage of intracellular structures [38].

2.4 Internalization of nanoparticles

Normally, microorganisms have rigid cell wall structures which would inhibit the nanoparticles from entering into the cell. However, nanoparticles can still be internalized by non-specific diffusion [49]. It was observed that ZnO nanoparticles below 10 nm and Ag nanoparticles below 16 nm in diameter could enter into *E. coli* by non-specific diffusion [38]. For those nanoparticles larger in diameter, it is likely that non-specific diffusion through compromised membranes would play a more favorable role. To exemplify, previous injuries induced by ROS, metal ions and nanoparticles accumulation would possibly increase permeability of cell wall. Liu et al. found that ZnO nanoparticles could lead to destruction of lipids and proteins on membrane of *E. coli*, which then increase the amount of nanoparticles entering the cells [50].

3. Antibacterial effect of heavy metal nanoparticles against pure cultures

Based on the known mechanisms by which heavy metal nanoparticles induce cell death, many studies have hypothesized that gram-negative and gram-positive bacteria would exhibit different susceptibilities towards nanoparticles (Table 1). Unlike gram-positive

bacteria, gram-negative bacteria has a thinner peptidoglycan layer [51]. Besides, there is a layer of negatively-charged lipopolysaccharide (i.e., O-polysaccharide, core polysaccharide, lipid A, and proteins) at outer membrane layer of cell wall of gram-negative bacteria [23, 51], which may enhance the interaction between bacteria and nanoparticles. These differences of gram-positive and gram-negative bacteria can possibly result in different susceptibility to nanoparticles.

To evaluate this, Kim et al. tested the antibacterial effects of silver nanoparticles with less than 20 nm diameter against yeast, *E. coli* and *Staphylococcus aureus.* The authors showed that the minimum inhibitory concentration (MIC) of silver nanoparticles to *E. coli* was only 3.3 nM to 6.6 nM, while higher MIC of > 33 nM would be required for *S. aureus* [21]. Contrasting results were however obtained by Azam and co-authors, who evaluated the antibacterial effects of zinc oxide, copper oxide on both gram-positive (i.e., *Escherichia coli* and *Pseudomonas aeruginosa*) and gram-negative (i.e., *Bacillus subtilis* and *S. aureus*) bacteria. Results showed that gram-negative *E. coli* had a 24% and 28% smaller inhibition zone size than *B. subtilis.* Similarly, gram-negative *P. aeruginosa* had a 16% and 33% smaller inhibition zone size than *S. aureus* in the case of ZnO nanoparticles and CuO nanoparticles, respectively [22]. Nevertheless, these antibacterial effects were only evaluated by disk diffusion method and the inhibition zones can be affected by particles sizes and diffusion rate of nanoparticles or the dissolved ions in agar plate. Another study conducted by Suresh and his co-authors suggest that gram-negative bacteria (i.e., *E. coli* and *Shewanella oneidensis*) were more resistant to silver nanoparticles than gram-positive bacterium (i.e., *B. subtilis*). This is done based on several evaluation tests, including disk diffusion, minimum inhibitory concentrations, live/dead assays, and atomic force microscopy [52].

Bacteria could resist stress from environment by regulating associated genes. The reasons to account for these contradictory findings can be due to how different bacterial species upregulate genes to cope against the antimicrobial effects. For examples, nanoparticles can induce *S. aureus* to produce catalase, which could

neutralize the hydrogen peroxide and further decrease the toxicity of nanoparticles [53]. It should be noted that the coping mechanisms of bacteria is complex - even different bacterial strains in same species have different coping mechanisms under same environmental stress. To illustrate, Al-Jassim and her co-authors exposed two different *E. coli* strains to solar irradiation, and RNA-sequencing results indicated that these two strains had different expression and regulation of genes [54]. It is therefore likely that susceptibility towards nanoparticles is not simply governed by whether a bacterium is gram-negative or gram-positive, but instead, also attributed by other factors (e.g., genomic contents and the ability to upregulate genes related to oxidative stress response and repair mechanisms and so on).

4. Antibacterial effect of heavy metal nanoparticles against biofilm

Even though nanoparticles have demonstrated antibacterial activity against pure cultures of gram-positive and gram-negative bacteria, biofilm attached on membranes are comprised of complex microbial populations and extracellular polymeric substance (EPS), and therefore make it hard to predict the effect on biofilm by heavy metal nanoparticles. Generally, biofilm-associated bacteria should exhibit higher tolerance to heavy metal NPs compared to planktonic cells. To illustrate, it was reported that 1 mg Ag/L could inhibit most planktonic bacteria isolated from wastewater biofilm within 1 h. In contrast, 24 h of treatment on biofilm with 200 mg Ag/L was needed to achieve significant reduction of biofilm-associated bacteria [55]. In the case of anaerobic membrane biofilm, same results have been observed. Reyes et al. indicated that the growth rates and yields of *P. denitrificans* in biofilms exposed to Cu nanoparticles were 40-fold to 50-fold less inhibited than those in planktonic cells [56].

In recent years, membranes coated with nanoparticles have been applied to control biofouling in MBRs. For example, palladium nanoparticles on polysulfone membrane was used to minimize bacteria number and EPS content in bacterial monoculture (i.e., *P.*

aeruginosa) and to also minimize EPS content in complex biofilm (i.e., sludge) [18]. In another study, polyvinylidene fluoride membrane coated with MnO_2 nanoparticles could greatly reduce presence of bacteria and organic matter attached on the membrane, resulting in a significantly thinner cake layer [57]. Similarly, Maximous et al. immobilized ZrO_2 nanoparticles on polyethylsulfone membrane and then evaluated its antifouling effect. They successfully demonstrated that 5% weight fraction of ZrO_2 with polyethersulfone membrane has the lowest fouling rate [58]. Ag nanoparticles entrapped on polysulfone membrane can delay pore clogging of membrane and decrease EPS content in biofilm maxtrix, indicating an improvement in the antifouling effect [59]. The schematic representation of antifouling mechanism is shown in Fig. 2. However, these studies evaluated the potential antifouling effects by aerobic sludge, and it remains unknown whether the nanoparticles would achieve the same effect on anaerobic sludge.

Furthermore, most of the studies only evaluate the efficacy of heavy metals by measuring parameters related to the phenotypic characteristics of the biofilm and did not study the effect on biofilm formation at the molecular level. In the subsequent sections, we introduce genes and enzymes involved in biofilm formation so as to provide a basis for future studies to determine if these would be affected by nanoparticles.

4.1 Initial surface attachment

Biofilm formation relies on bacteria to first attach on a surface. Previous studies indicate that outer surface appendages (e.g. flagella, pili, curli) and outer membrane adhesion proteins can contribute to the initial biofilm attachment process [60]. To exemplify, the conjugation pilus acts as an adhesion factor for cell-cell and cell-surface interaction. It is widely accepted that initial bacterial adhesion is a key part of the biofilm development process [61]. However, in a high transmembrane pressure system like an MBR, the initial attachment is also contributed by filtration pressure and forces, and the subsequent adsorption of bacteria onto the inorganic or organic constituents in a cake layer present on the membrane.

4.2 Microcolony formation

After initial attachment, bacteria would form highly structured cell cluster, which is also called microcolony [62]. Two processes, namely bacterial motility and clonal growth contribute to microcolony formation. Bacterial motility are dependent on flagella, pili structures of bacteria, and chemotaxis. For example, type IV pilus of *Pseudomonas aeruginosa* is important for biofilm formation [63]. Type IV pilus could mediate twitching motility for the bacteria to move from one location to another. Similar to type IV pili, CupA fimbriae, encoded by three *cup* clusters, can also mediate cell-surface interactions [64].

In addition, EPS, the predominant "glue" in a biofilm matrix, is required for microcolony formation. Bacteria secrete exopolysaccharides. Using *P. aeruginosa* as an example, this bacterium could produce three kinds of polymers (i.e., alginate, Pel, and Psl), which are correspondingly regulated by *alg, pel and psl* genes [65]. *Staphylococcus aureus and Staphylococcus epidermidis* could also produce polysaccharides intercellular adhesion (PIA) which is encoded by PIA synthesis gene (*icaADBC* operon) [63]. It is likely that such production of polysaccharides are ubiquitous in most microorganisms. To illustrate, *E. coli* has the *pga* gene, which shares high sequence similarity with *ica* genes, and is found to be related to polymer production that facilitates biofilm formation [66]. *hms* genes, which shared high sequence homology to *ica* genes, have also been identified in *Yersinia pestis* [67].

4.3 Biofilm maturation

Once the bacteria attach and microcolony is initiated, the usual metabolic processes occur among the microorganisms since an MBR treating municipal wastewater is a nutrient-rich ecosystem. Bacterial cells are not substrate-limited and would reproduce to a high cell density. Microorganisms then perform cell-to-cell communication (i.e., quorum sensing). Gram-positive and gram-negative bacteria utilize quorum sensing communication circuits to regulate or coordinate certain traits and activities.

In *P. aeruginosa* and many other gram-negative bacteria, N-acyl homoserine lactones (AHLs) are produced. There are two AHLs signaling systems, *lac* and *rhl*, that are relatively well-studied. The *lac* system contains the signal synthase LasI and associated signal receptor LasR. Similarly, in *rhl* system, bacteria could synthesize signals by synthase RhlI and regulate associated genes expression by receptor RhlR [68]. It was reported that biosilver nanoparticles could reduce the levels of LaslR-RhllR and reduce AHLs production [69]. However, QS involves other signal molecules like autoinducer-2 (AI-2) and autoinducer peptides (AIP). Less is known about how heavy metal nanoparticules would affect cell-to-cell signaling via these other molecules.

5. Impacts induced by nanoparticles on performance of aerobic and anaerobic biological processes.

MBR has been widely applied to treat wastewater. Aerobic MBR couples a membrane-based filtration process with activated sludge to achieve nitrogen, phosphorus and COD removal [70]. Similarly, anaerobic MBR couples a membrane-based filtration process with anaerobic digester to convert organic carbon to biogas. Although coating heavy metal nanoparticles on polymer-based organic membrane could mitigate biofouling, one has to consider the potential impact on biological processes if the nanoparticles were to leach into the system. It should be taken into account that the nanoparticles are classified as one of the new emerging contaminants, and it would pose a challenge to the overall functionality of MBR [2]. Here, we will briefly introduce how the nanoparticles affect the biological nitrogen removal, biological phosphorus removal and anaerobic fermentation process.

5.1 Nitrogen removal

Untreated wastewater contains excessive nitrogen compounds which would cause adverse effects (e.g. eutrophication) if directly discharged into environmental waters.

342 The nitrogen compounds include ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and
343 other dissolved organic nitrogen. In biological aerobic processes, ammonium is oxidized
344 to hydroxylamine by ammonia-oxidizing bacteria via ammonia monooxygenase (*amo*),
345 and subsequently to nitrite (hydroxylamine oxidoreductase, *hao*) and nitrate (nitrite
346 oxidoreductase, *nxr*) by nitrifiers. The nitrate is then reduced to nitrite (nitrate
347 reductase , *nap* and *nar*), nitric oxide (nitrite reductase, *nirK* and *nirS*) and nitrous oxide
348 (nitric oxide reductases, *qNor* and *cNor*) and finally nitrogen gas (nitrous oxide reductase,
349 *nos*) via denitrifiers [71].

350 In the past years, many studies have reported the effects of nanoparticles on nitrogen
351 removal. To illustrate, it was reported that alumina nanoparticles would affect the
352 nitrogen removal after both short-term and long-term exposure [72]. The study found that
353 50 mg/L alumina nanoparticles would affect nitrification and denitrification during
354 short-term exposure. In contrast, long-term exposure only led to decrease in
355 denitrifying bacteria and denitrifying enzymatic activities (i.e., NAP, NAR and NIK). These
356 decreases then resulted in a deterioration of total nitrogen removal from 80.4% to
357 62.5%. Zheng et al. spiked TiO_2 nanoparticles into anaerobic-anoxic sequencing batch
358 reactor, and found that the total nitrogen removal also decreased from 80.3% to 24.4%
359 after 70 days of exposure. Further examination revealed TiO_2 nanoparticles decrease
360 the relative abundance of nitrifying bacteria, especially ammonia-oxidizing bacteria, and
361 simultaneously inhibited the activities of ammonia monooxygenase and nitrite
362 oxidoreductase [73]. Similarly, zinc oxide and copper nanoparticles also affected the
363 denitrification process and led to nitrous oxide accumulation via inhibitory effect on the
364 catalytic activities of key denitrifying enzymes after long-term exposure [45, 74].
365 Nevertheless, not all nanoparticles showed inhibitory effects to nitrogen removal. For
366 instance, 200 μg/L gold nanoparticles reported no influence on ammonia removal and
367 *amo* gene abundance in a sequencing batch reactor, even though such nanoparticles
368 changed microbial community in reactor [75]. It is likely that the types and concentrations
369 of nanoparticles affect the outcome on nitrogen cycle.

370 *5.2 Phosphorus removal*

371 Phosphorus can usually be removed from municipal wastewater by enhanced biological
372 phosphorus removal (EBPR) method. This process is operated in an anaerobic/aerobic
373 condition via the enrichment of phosphorus accumulating organisms (PAOs). In general,
374 PAOs are capable of storing organic compounds, such as short chain fatty acids (SCFA)
375 and glycogen as internal storage compounds (i.e., polyhydoxyalkanoates (PHA)) by
376 utilizing energy obtained from hydrolysis of the intracellular stored polyphosphates
377 (poly-P). Ortho-phosphate is released into wastewater under anaerobic condition.
378 However, in aerobic condition, PAOs are able to rapidly take in excess phosphorus and
379 store them as intracellular poly-P by using stored PHAs as the energy source. The net
380 phosphorus removal can be achieved by wasting excess sludge with high phosphorus
381 content because the aerobic phosphorus uptake is greater than the anaerobic
382 phosphorus release.

383 Similar to nitrogen removal, nanoparticles have been reported to affect phosphorus
384 removal. Hu et al. evaluated the effects of ZnO nanoparticles on phosphorus removal
385 based on one anaerobic-aerobic sequencing batch reactor, and results indicated long-
386 term exposure to 6 mg/L ZnO nanoparticles would significantly deteriorate phosphorus
387 removal via inhibitory effects on *Accumulibacter* and *Competibacter* [76]. Zheng and his
388 co-authors spiked 50 mg/L ZnO nanoparticles into anaerobic-anoxic sequencing batch
389 reactor. The phosphorus concentration in effluent increased from non-detectable to
390 16.5 mg/L, indicating a decrease in phosphorus removal efficiency. The authors
391 attributed the deterioration to inhibitory effects on PAOs , exopolyphosphatase (i.e.,
392 PPX, required to hydrolyze polyphosphates) and polyphosphate kinase (i.e., PPK,
393 required to convert orthophosphates to intracellular poly-P) [77]. Nevertheless, not all
394 nanoparticles cause deterioration in phosphorus removal. To illustrate, Chen and his co-
395 authors spiked alumina nanoparticles into anaerobic-anoxic sequencing batch reactor
396 and found that long-term exposure did not affect the phosphorus removal [72]. Similarly,
397 50 mg/L TiO_2 nanoparticles was spiked in the same kind of reactor, and no inhibition on

biological phosphorus removal was observed after 70 days of exposure. Furthermore, the PPX and PPK activities were also not affected [73]. Hence, similar to the nitrogen cycle, it is likely that the types and concentrations of nanoparticles would impact phosphorus removal in a different manner.

5.3 Anaerobic fermentation

Anaerobic membrane bioreactor (anMBR) couples a membrane-based filtration process with anaerobic fermentation, which has lower sludge production rates than activated sludge processes, and can achieve high COD removal. Anaerobic fermentation not only eliminates the need for aeration, but also generates methane as an energy source [78]. Anaerobic fermentation is mainly divided into four major steps, including hydrolysis, acidogenesis, acetogenesis, and methanogenesis [79]. Representative keystone bacterial and archaeal species of each step are shown in Fig. 3. In this section, we briefly introduce related genes involved in anaerobic fermentation and methanogenesis which would possibly be affected by nanoparticles.

5.3.1 Hydrolysis

During the hydrolysis process, a consortia of bacteria break down the complex compounds (e.g. carbohydrates, protein, lipids) into smaller fractions (e.g. fatty acids, amino acids, polysaccharides etc.). The microorganisms involved in hydrolysis include *Clostridium thermocellum, Bifidobacterium longum, Clostridium celluloliticum, Bacteroides thetaiotaomicron, Enterococcus faecalis, Bacteroides capillosus* [80]. There are four main classifications of hydrolases that would contribute to this step, namely lipases (EC 3.1.1), phosphatases (EC 3.1.3), glucosidases (EC 3.2.1) and proteases (EC 3.4). Hydrolysis is typically defined as the rate limiting step of the whole anaerobic fermentation since it is the first step of the whole process. This means increasing hydrolytic rate could improve the overall anaerobic fermentation rate. To illustrate, Yu and his coauthors enriched endogenous amylase and then added these enzymes into anaerobic digester. It was observed endogenous amylase could increase volatile fatty

acids (VFAs) concentration in supernatant after 7 h treatment, and could increase biogas production after 11 days of anaerobic digestion compared to the control [81].

In presence of nanoparticles, deleterious effect on the overall anaerobic fermentation process were observed because nanoparticles affected the hydrolytic rates. To illustrate, Mu and his co-authors showed that ZnO nanoparticle decreased the bovine serum albumin (BSA) and dextran degradation efficiencies by 37.5% and 12.3%, respectively. This suggests that nanoparticles can inhibit hydrolysis of protein and polysaccharide [82], both of which are important rate limiting steps in anaerobic fermentation.

5.3.2 Acidogenesis

Hydrolytic and acidogenic microorganisms are about ten times faster growing than methanogens [83]. Fermentative acidogenic microorganisms (e.g. *Slackia heliotrinireducens,* Candidatus *Cloacamonas acidaminovorans, Clostridium kluyveri, Clostridium acetibutilicum, Clostridium perfringens, Clostridium saccharolyticum, Caldanaerobacter subterraneus, Finegoldia magna, Enterococcus faecium, Lactobacillus helveticus*) could further convert the hydrolyzed products into VFAs (e.g. formic acid, acetic acid, propionic acid, butyric acid) [80]. Most of the VFAs are formed in the metabolism of glucose via the glycolytic Embden-Meyerhof-Parnas (EMP) pathway [83]. Hydrolyzed glucose is decomposed to produce pyruvic acid. The pyruvic acid fermentation then produces a number of VFAs. The production of VFAs by anaerobic bacteria is mediated by several kinds of enzymes. To illustrate, butyrate synthesis requires crotonase while alcohol synthesis requires alcohol dehydrogenase. Methylmalonyl-CoA mutase catalyzes the isomerization between methylmalonyl-CoA and succinyl-CoA, and are involved in propionate synthesis [84]. Certain nanoparticles were shown to inhibit acidogenesis process. It was reported that 150 mg/L ZnO nanoparticles could inhibit 40-80% acidogenesis efficiency of waste active sludge[85]. In addition, Cu nanoparticles added into fermentation system could inhibit hydrolysis and acidification and then result in VFAs reduction [86].

453

454 *5.3.3 Acetogenesis*

455 In a well-functioning anaerobic fermentation process, VFAs, especially long chain VFAs
456 do not accumulate but are instead converted into acetic acid, carbon dioxide and
457 hydrogen by the acetogenic microorganisms (e.g. *Carboxydothermus hydrogenoformans,*
458 *Morella thermoacetica, Pelotomaculum thermopropionicum*). Acetogenic
459 microorganisms are strict anaerobes since they utilize the acetyl coenzyme A pathway
460 that contains enzymes sensitive to oxygen. It was reported that phospho-transacetylase,
461 acetate kinase (AK) and acetyl-CoA synthase were involved in the acetogenesis step [84].
462 For example, acetate kinase (AK) is responsible for the transformation of
463 monosaccharides and amino acids into acetate. In addition, homoacetogens (a
464 subpopulation of acetogens) convert hydrogen and carbon dioxide to acetate [87].
465 Homoacetogens rely on pyruvate ferredoxin oxidoreductase, CO dehydrogenase/acetyl-
466 CoA synthase, tetrahydrofolate-dependent enzymes, methyltransferase (e.g. MeTr, AcsE)
467 and corrinoid iron sulfur protein (CFeSP, AcsCD), all of which are enzymes involved in
468 Wood-Ljungdahl pathway, to achieve this conversion.
469
470 It was reported that both Ag NPs and MgO NPs at the dosage of 500 mg/g total
471 suspended solids could inhibit acetogensis by significantly decreasing the AK activity [88].
472 In contrast, certain concentrations of NiO and CoO nanoparticles (i.e., 1.5 mg/L and 1.0
473 mg/L, respectively) could enhance the acetic acid and butyric acid concentration, and
474 finally increase the hydrogen yield by 1.51 and 1.67 fold respectively. This is achieved
475 through enhancing electron flow efficiency and enzyme activities in both acidogenesis
476 and acetogenesis processes [89].
477
478
479 *4.3.4 Methanogenesis*

480 In methanogenesis, *Methanosarcina barkeri, Methanosarcina acetivorans,*

Methanoculleus marisnigri, Methanoregula boonei, Methanosphaerula palustris, Methanospirillum hungatei, Methanoplanus petrolearius, Methanocorpusculum labreanum were reported to be involved in anaerobic digestion to produce methane [80]. The two distinct methanogenic pathways are from H_2/CO_2 to methane (hydrogenotrophic pathway) and from acetate to methane (aceticlastic methanogenesis). Hydrogenotrophic methanogenesis converts CO_2 and H_2 to methane. The H_2 and formate are the major electron donors. According to the KEGG-Orthology, there are seven genes involved in this process, including formate dehydrogenase (*fdh*, EC: 1.2.1.2), formylmethanofuran dehydrogenase (*fwdA/fmdA*, EC: 1.2.99.5), formylmethanofuran-tetrahydromethanopterin formyltransferase (*ftr*, EC: 2.3.1.101), methenyltetrahydromethanopterin cyclohydrolase (*mch*, EC: 3.5.4.27), oxidoreductases (*mtd*, EC: 1.5.99.9), coenzyme F420 hydrogenase (*frhB*, EC: 1.12.98.1) and coenzyme F420-dependent N5,N10-methenyltetrahydromethanopterin reductase (*mer*, EC: 1.5.99.11).

In aceticlastic methanogenesis, acetate is directly converted to methane. The enzymes involved include acetate kinase (*ackA*, EC: 2.7.2.1), phosphate acetyl-transferase (*pta*, EC: 2.3.1.8), acetyl-CoA decarbonylase/synthase complex (ACDS; composed of CdhA1, CdhB, CdhD, CdhE and CdhC), and acetyl-CoA synthetase (*acs*, EC: 6.2.1.1). Besides, the two modes of methanogenesis share the same enzymes and genes (i.e., Tetrahydromethanopterin S-methyltransferase (*mtr*, EC: 2.1.1.86), methyl coenzyme M reductase (*mcr*, EC: 2.8.4.1) and reductase heterodisulfide reductase (*Hdr*, EC: 1.8.98.1)), which are required to obtain methane as final product [90].

Despite knowing the genes and pathways involved in anaerobic fermentation process, studies that reported the effects of heavy metal nanoparticles on anaerobic MBR performance mainly rely on end point measurements of biogas and VFAs production but not on gene expressions. To illustrate, Luna-delRisco and the co-authors added 15 mg/L CuO nanoparticles and 120 mg/L ZnO nanoparticles to anaerobic digester of cattle manure for 14 days, and found that methane production was severely affected. The

authors further estimated that the half effective concentration (i.e., EC_{50}) for methane was 10.7 mg Cu/L and 57.4 mg Zn/L [91]. Mishra et al. indicated optimal concentration of NiO and CoO nanoparticles (i.e., 1.5 mg/L and 1.0 mg/L, respectively) could enhance hydrogen yield in anaerobic digestion of palm oil mill effluent, suggesting that methane production is also affected which would result in accumulation of hydrogen gas. The author attributed this to be due to nanoparticles enhancing catalytic effect of hydrogenases enzymes, but did not verify the effect on enzymes related to methanogenesis [89].

In another long-term exposure study, different concentrations of ZnO nanoparticles were spiked into anaerobic digester, and it was determined that 30 mg of ZnO and 150 mg of ZnO per gram of total suspended solids could inhibit about 18.3% and 75.1% of methane production, respectively, when compared to the control. It was further shown that the release of Zn ions from Zn nanoparticles may inhibit hydrolysis and methanogenesis steps of anaerobic digestion by decreasing the activities of protease and coenzyme F_{420} associated with archaeal methanogens [82] (Table 2).

Besides affecting activities of archaeal methanogens, exposure to heavy metal nanoparticles could also result in changes in the abundance of specific microbial groups. To exemplify, it was reported that when landfill solids were exposed to 10 mg Ag per kg of solids at an exposure time of 250 days, biogas production was reduced. These observations correlated with a decrease in the total copy numbers of 16S rRNA gene of methanogens from $1.97 \pm 0.21 \times 10^7$ to $5.79 \pm 2.83 \times 10^5$ [92]. However, this study determined abundance of methanogens by 16S rRNA genes, which may not relate to absolute abundance since some methanogens may contain > 1 copy of 16S rRNA gene per cell [93]. The decrease in methanogen-related 16S rRNA gene copies may be due to changes in specific methanogenic community in response to the nanoparticles.

Despite the earlier discussed studies, there still remain some contradictory observations on whether heavy metal nanoparticles would indeed affect methanogenesis. To exemplify, Garcia et al. reported that the Ag nanoparticles can lead to about 33%-50%

decrease in biogas production, while Au nanoparticles showed only slight or no inhibition to biogas production after 50 days exposure [94]. The impact of CeO_2-NPs on the acidogenesis and methanogenesis of anaerobic granule sludge and anaerobic flocculent sludge was also studied [95]. High concentrations of CeO_2 nanoparticles (i.e., 150 mg CeO_2-NPs/g-VSS) could inhibit 19% VFAs production in flocculent sludge and 35% VFAs production in granular sludge, but showed no inhibition to methane production. The toxicity of ceria nanoparticles to anaerobic sludge was attributed mainly to direct contact and interaction between nanoparticles with cell membrane. This finding indicated that the acidogenesis was more sensitive to CeO_2 nanoparticles than methanogenesis. Since acidogenesis is mediated by bacterial populations while methanogenesis is mainly contributed by archaeal populations, it is inferred that archaea is less susceptible to nanoparticles than bacteria when the main toxicity mechanism is nanoparticle-cell interaction, possibly due to the fact that archaea has a cell wall structure different from that of bacteria [51]. More studies detailing how heavy metal nanoparticles would impact anaerobic fermentation and methane production were listed in Table 2.

It should be noted that not all nanoparticles result in negative effects to anaerobic fermentation. This is especially so for Fe and other heavy metals introduced at sub-lethal dosage. For example, Fe nanoparticles can enhance methane production by enhancing immobilization of other metals, hence decreasing the potential toxicity of solubilized heavy metals on anaerobic fermentation process [96]. Alternatively, Fe acts as an electron donor to reduce oxidation/reduction potential (ORP) and as a buffer to control pH through reduction of protons to hydrogen gas, which would facilitate methanogenic activity. As such, the overall effect of heavy metal nanoparticles on MBR process would be dependent on the type of metal and the concentration exposed to the microbial consortium [97].

5. Conclusion

Heavy metal nanoparticles exhibit high antibacterial effects through production of reactive oxygen species (ROS), metal ion dissolution, accumulation on cell surfaces and internalization within cells. Therefore, nanoparticles can be coated on membranes surface to achieve antifouling effect in MBR. Many studies have applied the nanoparticles-coated membranes to lab-scale MBR, and demonstrated promising results in terms of high antibacterial effect and reduction in transmembrane pressures. However, nanoparticles from such coated membranes can leach into the system and impact the overall performance of MBR (e.g. biological nitrogen removal, biological phosphorus removal, and fermentation). A review on related papers revealed contradictory conclusions on how nanoparticles would affect these biological processes, especially anaerobic fermentation and/or anaerobic MBR. The contradictory outcomes reported by the various studies are possibly explained by the differences in heavy metals tested, concentrations of heavy metal nanoparticles spiked into the system and/or differences in the microbial consortium. Therefore, depending on the intended purpose of using heavy metal nanoparticles in MBR processes, for example if the purpose is for inhibiting membrane biofouling process while not deterimentally affecting the overall functionality of the reactor system, one should first determine the lethal dosage for that particular heavy metal nanoparticles and for that particular microbial consortium. Subsequently, it would be beneficial to understand the microbial consortium and map out the gene pathways affected by the nanoparticles so as to ensure that undesirable and unintended impact on the overall functionality of the reactor systems are minimized.

Conflicts of interest

There are no conflicts to declare.

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Table 1. List of studies demonstrating antibacterial effect of heavy metal nanoparticles.

Type of heavy metal	Form	Test methods	Bacteria	Outcome	References
Ag	Nanoparticles (NPs)	Cell density on Muller Hinton agar plates in the presence/absence of the NPs	*Escherichia coli* (gram –ve) *Staphylococcus aureus* (gram +ve)	MIC of *E. coli* > 3.3 nM MIC of *S. aureus* > 33 nm	21
ZnO CuO Fe_2O_3	Metal oxides NPs	Well-diffusion method on agar to observe for zone of inhibition; broth-dilution method and viable bacterial cell counts	*E. coli* *Pseudomonas aeruginosa* (gram –ve) *S. aureus* *Bacillus subtilis* (gram +ve)	Antibacterial activity (in ug/mL) and in decreasing order of efficacy: ZnO > CuO > Fe_2O_3; Based on zone of inhibition, observed higher tolerance against tested NPs for gram-ve vs. gram+ve	22
CoO	Metal oxides thin nanosheets	Broth cultivation and measurement of optical density at 600 nm to generate growth curves	*E. coli* *P. aeruginosa* *Micrococcus luteus* (gram +ve) *S. aureus*	More effective against gram +ve (MIC > 50 ug/mL) than gram –ve (MIC > 100 ug/mL)	98
ZnO TiO_2 CuO Cu_2O Ag	NPs	Well-diffusion method on agar to observe for zone of inhibition; activity and cationic assays (ATP, K+, H+)	*E. coli* *Enterococcus hirae* (gram +ve) grown anaerobically	Only Ag NPs showed antibacterial effect, most likely by affecting the proton and potassium ion fluxes	99
CuO	NPs	Broth cultivation and measurement of optical density at 600 nm to generate growth curves; Reactive oxygen species measurement; DNA damage and cell morphology characterization	*E. coli* *Proteus vulgaris* *M. luteus* *B. subtilis*	Sheet shaped CuO NPs were more effective on gram +ve; spherical shaped CuO NPs were more active on gram -ve	100
ZnO	NPs	Diffusion method on agar to observe for zone of inhibition; broth-dilution method and viable bacterial cell counts	*Salmonella enterica serotype Typhi* (gram –ve)	Antibacterial effect observed, activity increased by reducing size of NPs or by increasing the molarity of NPs	101
ZnO	NPs	Growth inhibition measured using colony counts and optical density at 600 nm; Reactive oxygen species measurement; DNA fragmentation, and membrane permeability	*Vibrio cholera* strains O395 and N16961 (gram –ve)	Antibacterial effect observed, ROS generation and induction of DNA damage are accounted as main antibacterial mechanisms; variation in response depending on the strain type	102
Fe_2O_3	NPs; functionalized with $–NH^{3+}$	Broth dilution method; cell viability assay using Live/Dead BacLight kit; crystal violet staining on biofilm	*B. subtilis*	Biofilm formation affected without detrimental impact on cell viability	103
Al_2O_3	NPs	Culture-based viability test; Reactive oxygen	Alga (*Pseudokirchneriella*	CuO and ZnO exhibit the highest toxicity (<1	104

Co_3O_4 CuO Fe_3O_4 MgO Mn_3O_4 Sb_2O_3 SiO_2 ZnO TiO_2 WO_3 Pd		species measurement	*subcapitata*), Bacteria (*V. fischeri, E. coli, S. aureus*), Protozoa (*Tetrahymena thermophila*)	mg/L)	

Table 2. List of studies detailing the effects of heavy metal nanoparticles on anaerobic fermentation

ZnO TiO_2	NPs	Cell viability; proteomics and biochemical assays	*B. subtilis*	130 µg/mL TiO_2 and 170 µg/mL ZnO stressed the cells and reduced viability by 50%; proteins related to central metabolism were affected by ZnO but not by TiO_2	105
Ag	NPs immobilized in PVDF	Well-diffusion method on agar to observe for zone of inhibition; Filtration based colony count and SEM observation.	*E. coli*	0.5%-1.5% Ag showed clear halo zones and inhibited bacteria. Few bacteria grew on the membrane surface.	106
Cu	NPs stabilized on acrylic polymer	Broth cultivation and measurement of optical density at 600 nm to generate growth curves, Atomic absorption spectroscopy applied to quantify copper ions release.	*Chlamydomonas* sp. strain CD1 Red. *Synechocystis* sp. PCC 6803, *Phaeodactylum tricornutum* CCMP 1327	All Cu NPs modified membrane exhibit good antibacterial activity.	107
Cu	NPs load on thin-film composite polyamide membrane	Viable cell attachment experiment: Cell viability measured using colony counts; SEM observed cells attached.	*E. coli* *P. aeruginosa* *S. aureus*	1 h contact decreased about 87% ± 0.2% *E. coil*, 96% ± 3% *P. aeruginosa* and 79.5% ± 13% *S. aureus* live cells attached on membrane.	108

Nanoparticles	NPs diameter (nm)	Dosage	Reactor type	Exposure time (days)	Impact on anaerobic digestion	Reference
Ag	29	40 mg/L	Anaerobic digestion	14	No negative effects in biogas and methane production. Dominant methanogens remained largely unchanged.	[109]
Ag	21	1 /10 mg Ag/kg TS	Landfill digestion	>250	High concentration Ag NPs reduce biogas production rate, accumulate volatile fatty acid (VFAs), decrease methanogens number.	[92]
Ag/Au	30/10	N.A	Anaerobic digestion	50	Ag NPs decreased biogas production	[94]
CeO_2	<25	5-150 mg/g VSS	Anaerobic granule/flocculent sludge	6	150 mg/g VSS CeO_2 NPs inhibited acidification process, resulting in less short-chain fatty acid through physical penetration and membrane reduction. However, there was no observed inhibition on methanogenesis. CeO_2 NPs has less influence on flocculent than granular sludge.	[95]
Cu	20-40	50 mg/L	Sequencing batch reactors followed with anaerobic digestion	>121	Releasing NPs through wastewater improved sludge solubilization, showed no effect on VFAs production. Directly adding NPs to anaerobic digestion inhibited hydrolysis and acidification, resulting in VFAs decreasing.	[86]
CuO	37	1.4 mg/L	Upflow Anaerobic Sludge Blanket	107	Short-term exposure caused minor inhibition to methanogenesis, while extended exposure caused severe toxicity and reduced 85% of the aceticlastic methanogenesis. >50% reduction in methane production.	[110]
Fe/ Fe_3O_4	50/20	N.A.	Anaerobic digestion	12	Both NPs improved biogas production.	[96]
Fe	119	1.25 g/L	Anaerobic batch reactor	10	Nanoscale zero valent iron increased 28% in methane production via directly decreasing reactor's oxidation/reduction potential.	[97]
NiO / CoO	14 / 16.8	0-3 mg/L	Anaerobic digestion	4	1.5 mg/L NiO and 1.0 mg/L CoO NPs enhanced VFAs accumulation and hydrogen yield, also increase the COD removal.	[89]
ZnO	<100	0.32 / 34.5 mg Zn/L	Upflow Anaerobic Sludge Blanket	165	High ZnO NPs concentration resulted in rapid deterioration of methane production and VFAs removal; Deterioration more gradual and partial in presence of low ZnO NPs concentration; aceticlastic methanogens severely inhibited	[111]
ZnO	140	1-150 mg/g TSS	Anaerobic digestion	>150	High concentration ZnO NPs inhibited methane production due to Zn^{2+} inhibiting hydrolysis and methanogenesis.	[82]

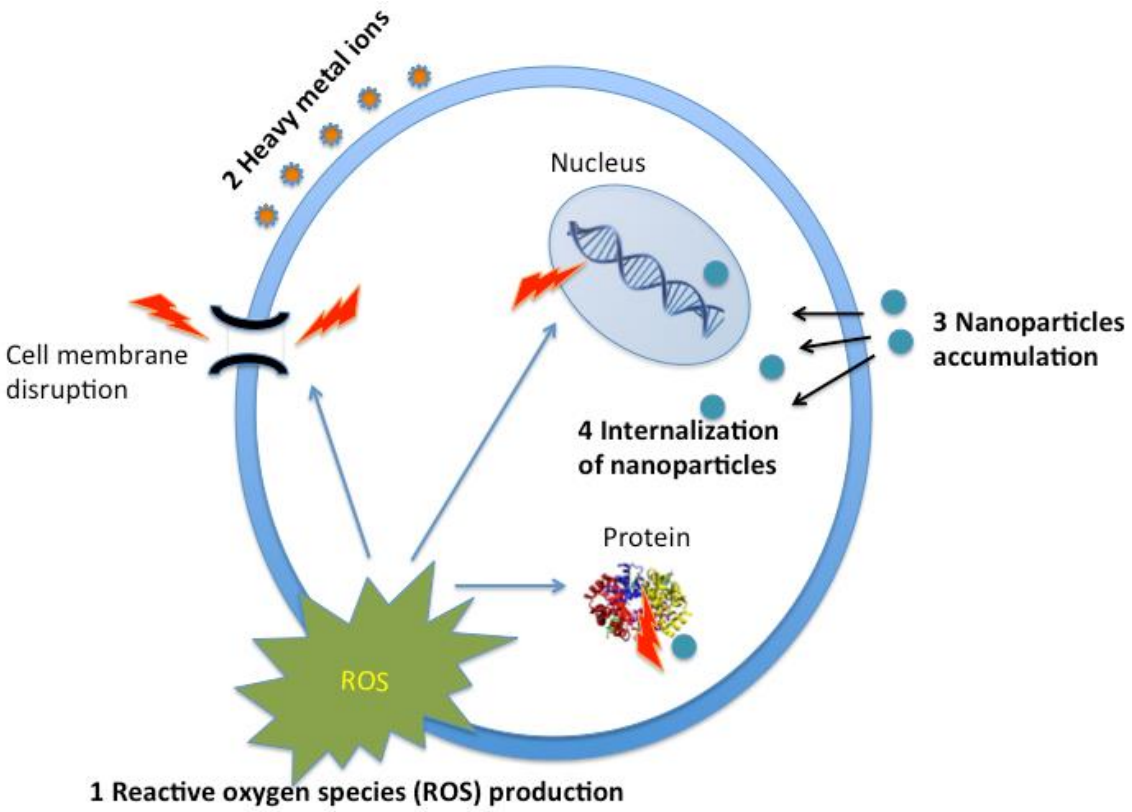

Fig. 1 Schematic representation of antibacterial effects induced by heavy metal nanoparticles.

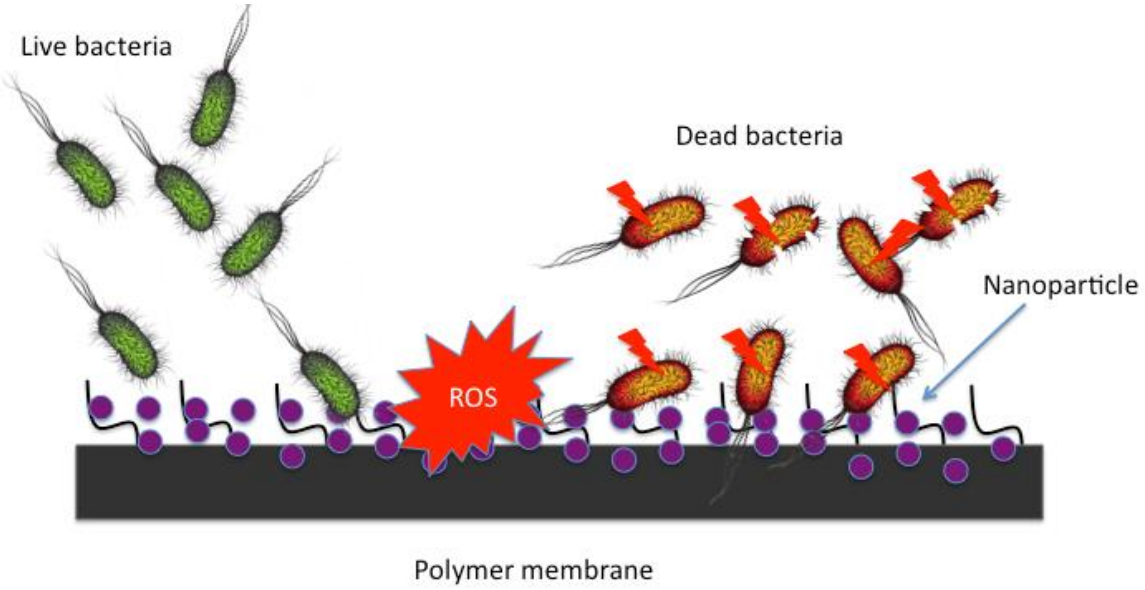

Fig. 2 Schematic representation of antifouling mechanism by heavy metal nanoparticles.

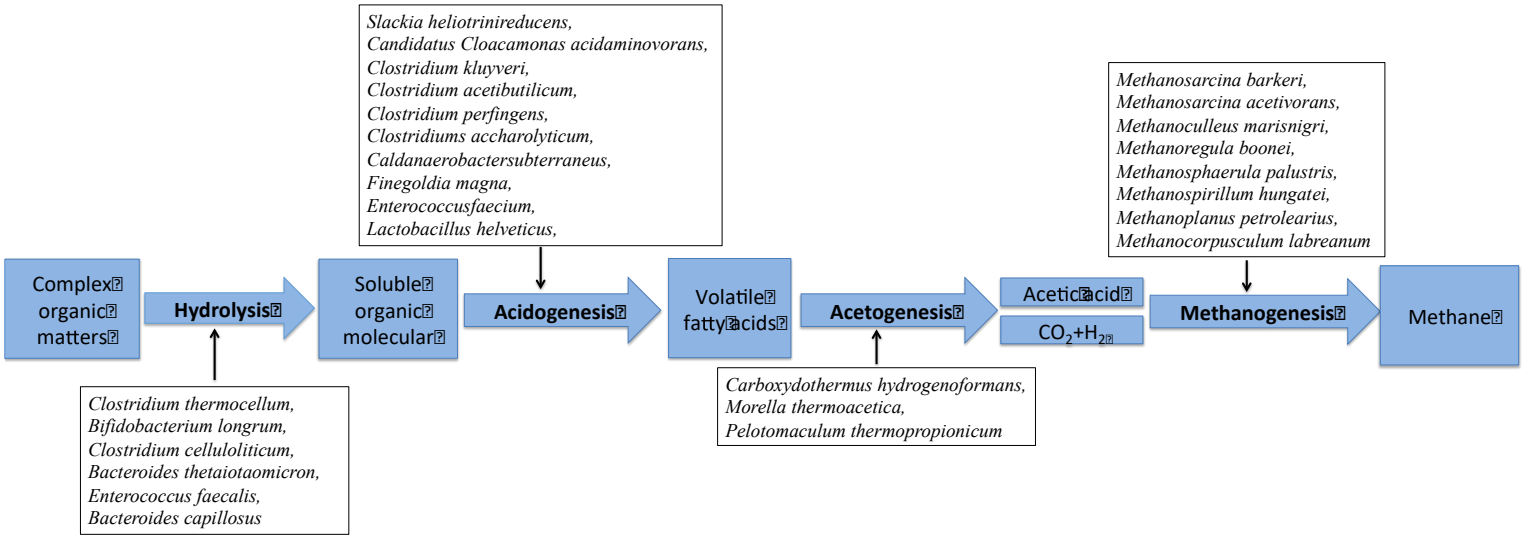

Fig. 3 Schematic representation of anaerobic fermentation process and the corresponding keystone microbial populations.

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