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

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Abstract

The use of engineered nanoparticles (ENPs) in a wide range of products is associated with an increased concern for environmental safety due to their potential toxicological and adverse effects. ENPs exert antimicrobial properties through different mechanisms such as the formation of reactive oxygen species, disruption of physiological and metabolic processes. Although there are little empirical evidences on environmental fate and transport of ENPs, biosolids in wastewater most likely would be a sink for ENPs. However, there are still many uncertainties in relation to ENPs impact on the biological processes during wastewater treatment. This review provides an overview of the available data on the plausible effects of ENPs on AS and AD processes, two key biologically relevant environments for understanding ENPs-microbial interactions. It indicates that the impact of ENPs is not fully understood and few evidences suggest that ENPs could augment microbial-mediated processes such as AS and AD. Further to this, wastewater components can enhance or attenuate ENPs effects. Meanwhile it is still difficult to determine effective doses and establish toxicological guidelines, which is in part due to variable wastewater composition and inadequacy of current analytical procedures. Challenges associated with toxicity evaluation and data interpretation highlight areas in need for further research studies.

Keywords: Engineered nanoparticles, Wastewater, Microorganisms, Toxicity

1. Introduction

The diversity and utilisation of engineered nanoparticles (ENPs) in industrial processes and consumer product manufacturing is rapidly increasing. Meanwhile concerns on their potential adverse effects on microorganisms and the environment are gradually emerging and are not yet 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-enabled waste into wastewater 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 act as a primary sink and source for waste containing aged ENPs (Brar et al., 2010; Kim et al., 2010; Liang et al., 2010). Likewise this scenario entails that the environment will be flooded with large quantities of nanowaste gradually released from wastewater discharge (Kaegi et al., 2008; Wang et al., 2012b).

To date, our knowledge on the effects of ENPs on biologically mediated processes such as activated sludge (AS) and anaerobic digestion (AD) in WWTP is scarce. Any 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 due to the presence of ligands or microbial transformation (Kim et al., 2010; Liang et al., 2010; Gondikas et al., 2012). Nevertheless, the overall effect and severity of the ENPs are difficult to predict at the moment given the lack of experimental evidence. The current paucity of information on the effects of the most widely used ENPs including titanium dioxide (TiO_2), silver oxide (Ag^0), zinc oxide (ZnO), copper oxide (CuO), gold (Au^0), fullerene (C_{60}) and nanofibres 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).

Clearly, there is a compelling need to characterise the fate, transport, behaviour and effects of ENPs in WWTP. The elucidation of any adverse effect on microbial species will certainly improve our understanding of the ENPs effects in WWTP and contribute to develop useful monitoring tool.

Here to shed more light on the current knowledge status, we present an overview of plausible effects of ENPs on AS and AD processes as two key biologically relevant environments for understanding ENPs-microbial interaction, the challenges associated with toxicity evaluation and data interpretation.

2. 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).

2.1. Influence of porous media and natural organic matter (NOM) on ENPs

The transport of ENPs with a particle size < 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 (Petosa et al., 2010). Although sorbed particles are

85 expected to remain attached to media surfaces, recent findings have demonstrated that
86 retained ENPs of 8 nm in low ionic strength solutions were released from saturated porous
87 media against the prediction of Derjaguin-Landau-Verwey-Overbeek (DLVO) theory.
88 Further to this, a recent study conducted by Wang et al. (2012a) on the assessment of ENPs
89 retention and transport of different sizes using mass concentration (mg/L) and particle
90 number concentration (particles/mL) resulted in divergent conclusions.
91 In the aqueous environment and soils, natural organic matter (NOM) plays a significant role
92 in the transport and fate of ENPs due to the tendency for colloidal absorption and aggregation
93 through hydrophobic reactions. However sorption to biosolids in a wastewater context might
94 be hindered in the presence of surfactants in the effluent discharge (Hyung et al., 2007; Kiser
95 et al., 2010).
96
97 *2.2. ENPs uptake and bioaccumulation*
98 Bioavailable ENPs can penetrate cells or attach to the cell wall (Figure 1). They are not
99 known to biodegrade and microorganisms have demonstrated *in vitro* adaptation to shock
100 doses and contact (Lara et al., 2010; Martinez-Gutierrez et al., 2010). For instance,
101 internalisation of ENPs and reductive deposition of palladium nanoparticles in the
102 periplasmic space of *Shewanella oneidensis* indicates that ENPs presumably could
103 bioaccumulate and may likely biomagnify along the food chain as illustrated in Figure 2 (De
104 Windt et al., 2006). On the other hand, accumulation of nanosilver has been demonstrated as
105 responsible of the resistance of *Pseudomonas stutzeri* AG259 to Ag^0 toxicity (Klaus et al.,
106 1999). Recently, concentration-dependent uptake and internalisation of TiO_2 and ZnO have
107 been reported in *Salmonella typhimurium* and *E.coli* with induced mutagenic effect which
108 may potentially be transferred to higher life forms (Kumar et al., 2011; Holbrook et al., 2008;
109 Unrine et al., 2010).

3. Effects of ENPs on microorganisms

Effects ENPs on microorganisms are 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 damage or lyse the microbial cells (Aruguete and Hochella, 2010). Illustration of these effects on microbial cell wall within activated sludge processes is shown in Figure 1. Interestingly, stabilisers and capping agents have also been shown to exert differential effect on microorganisms (Drogat et al., 2010; Jaiswal et al., 2010; Jin et al., 2009). However, laboratory-controlled selective effect of size- and dose–dependent pristine ENPs as demonstrated by several authors does not suggest that the same size/concentration of ENPs in unaltered physicochemical state would be prevalent in wastewater to exert similar acute effect. For illustration purpose, the size-dependent inhibitory effect Ag^0 is presented in Figure 3. 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 solubilised ions that enter the cell by oxidative disruption of the cell membrane (Kloepfer et al., 2005).

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 membrane leakage of 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).

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., 2008 and 2009). Prominent examples include (1) the temperature-dependent, anaerobic reduction of nitrate under batch conditions by integrated nanoscale zero-valent iron and microbial system (Shin and Cha, 2008), (2) the *Shewanella oneidensis*-palladium nanoparticle mediated dechlorination of polychlorinated biphenyl (PCB) congeners in sediment matrices (De Windt et al., 2006) and (3) the stimulatory effect on dehydrogenase enzyme of soil microorganisms (Cullen et al., 2011). Although the mechanism of action in ENPs stimulated processes is yet to be fully elucidated, these findings suggest that the use 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.

4. ENPs – microbial interactions in WWTP

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. A schematic overview of the ENPs-microbial interactions occurring in AS and AD is summarised in Figure 4. ENPs-microbial interactions are 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). For instance, fullerenes C_{60} water suspension has been found to be toxic to pure cultures of *B.subtilis* (Lyon et al., 2006) and *E. coli* (Chae et al., 2009), while no observable negative effect was noticed on bacterial soil communities and cellular respiration due to the cultural conditions and soil NOM (Tong et al., 2007).

160

161 *4.1. Interaction with AS microbial community*

162 The removal of nitrogenous material from wastewater during AS treatment can be inhibited
163 by nanosilver particles (Choi et al., 2010). The accumulation of ammonia in AS will have
164 then a negative effect on the key syntrophic microbial groups involved in AD (Kayhanian,
165 1994). Although Ag^0 concentration reported in sewage ranged from 2 to 18 µg/L (Blaser et
166 al., 2008), it is not clear if this concentration could have a detrimental effect on
167 microorganisms as a peak concentration of 750 µg Ag^0/L in activated sludge after 12 h shock
168 load had no observable inhibitory effect on organic removal rate by heterotrophic bacteria
169 (Liang et al., 2010).
170 As close contact with the cell surface is assumed to be important for achieving an effect of
171 ENPs on microbes, a differential impact might be expected for planktonic and biofilm-
172 associated cells. Cells within a biofilm matrix are typically embedded in a coat of
173 extracellular polysaccharides (EPS) restricting direct contact or lowering the effective dose of
174 ENPs (Liu et al., 2007). In a recent study, NOMs and EPS have been found to hinder C_{60}-
175 bacterial biomass interaction in AS (Kiser et al., 2010). ENP-microbial interaction may not be
176 particle size-dependent alone and could vary in different cultural and environmental
177 conditions especially when the organisms can synthesize EPS. On the other hand, interaction
178 could be enhanced by proteins which promote disaggregation of ENP thus increasing
179 bioavailability and contact (Karanjangi et al., 2006).

180

181 *4.2. Interaction with AD microbial community*

182 As mentioned above, the two key syntrophic groups involved in anaerobic digestion process
183 are acetogenic and methanogenic microorganisms. Both are affected by accumulation of free
184 ammonia 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 (Figure 4; Debabov, 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).

4.3. 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, nanosilver particles < 30 nm were cytotoxic to *E. coli* and *S. aureus* (Martinez-Gutierrez et al., 2010). This suggests that nanosilver particles greater than 30 nm could be non-inhibitory to microbial processes. Of particular interest is the interaction of nanosilver particles less than 5 nm in suspension capable of inhibiting nitrification in AS (Choi et al., 2010). Meanwhile the truncated triangular form of nanosilver particles which can also be spherical or rod-shaped form, was found to exert the strongest bactericidal effect on *E. coli* in both agar plate and broth cultures (Pal et al., 2007). However, caution should be taken when extrapolating these results from pure culture to complex microbial communities in wastewater.

4.4. Influence of ions released by ENPs on bacterial cell

The effect of ions released due to the presence of ENPs in wastewater on the microbial flora is not evident due to their low concentrations and complexation reactions with organic molecules (Fabrega et al., 2009; Zhang and Chen, 2009; Gondikas et al., 2012). Bactericidal properties of nanosilver 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,

210 teichoic acid contained in Gram-positive bacterial cell wall is negatively charged and serves
211 as a binding site (Kikuchi et al., 1997). Binding of ENPs to teichoic acid residues is in
212 competition with divalent cations (Mg^{2+}, Ca^{2+}) resulting in reduction of Ag^0 toxicity to Gram-
213 positive organisms in the presence of divalent cations. In Gram-negative cell walls, in
214 contrast, it is the lipopolysaccharides that restrict passage of toxic substances into the cell
215 although the presence of divalent cations reportedly exacerbates bactericidal effect of Ag^0
216 especially in bicarbonate deficient medium (Kucerka et al., 2008).
217 Apart from a mitigating effect of divalent cations, sorption to cells is in addition reduced by
218 electrostatic repulsion (Yamanaka et al., 2005). The repulsion is however overcome to some
219 extent by ion bridges forming between negatively charged lipopolysaccharide surface
220 molecules and negatively charged Ag^0. The distinct effect of divalent cations on ENP
221 sorption efficiency is schematically shown in Figure 5. Sorption may result in conformational
222 change of cell wall and uptake of ENPs (Sondi and Salopek-Sondi, 2004; Morones et al.,
223 2005). Again, comparative studies have shown that C_{60} are more firmly associated with *E.*
224 *coli* than *B. subtilis* suggesting differences in sorption potential due to surface charges (Lyon
225 et al., 2005). Similarly, sorption of a regular spatial pattern of Ag^0 on the surface of HIV-1
226 viruses suggests preferential binding to the viral glycoprotein spikes (Elechiguerra et al.,
227 2005). However, there is not yet a common consensus on the interaction between bacterial
228 cell wall and its influence on ENP effect. For instance, independent studies by Chudasama et
229 al., (2010) and Jin et al., (2009) are of divergent opinion on the role of cell wall in
230 reducing/enhancing ENPs-microbial interactions.
231
232 **5. Challenges in the evaluation of ENPs-microbial interactions**
233 Currently, there is no generally accepted protocol for ecotoxicity tests and exposure
234 characteristics of ENPs. A number of tests suggested for consideration in addition to the

manufacturer's characterisation 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). 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 and give a false-positive acute or chronic toxicity assessment (Gondikas et al., 2012).

5.1. Determining ENPs inhibitory end-points

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, 2008). 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). 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, biased 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 effects 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 microbiologists to determine the impact level that would be considered ‘safe’ for microbial activities in wastewater with variable composition. The problem is exacerbated if the toxicant undergoes transformation or reacts synergistically with other substances which could either enhance or attenuate the adverse effect. Similarly, the concept of hormesis, a biphasic response characterised by low-dose stimulation and high-dose inhibition has been generally overlooked in toxicological studies while it could play a significant role in determining the effect of ENPs on microbial cells in AS and AD processes (Calabrese and Baldwin, 2003; Cook and Calabrese, 2006).

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 substance concentration ranging between 10–100 mg/L 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 characterisation (Tiede et al., 2009). Understanding ENPs behaviour and their 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 very well. However, Bayesian belief network can satisfactorily predict uncertainties in ENPs toxic effect estimations (Borsuk et al., 2004). The model provides a graphically robust and coherent framework for probabilistic evaluation of the relationship between complex variables. It delineates cause and effect assumptions with

285 complex causal chain linking actions to outcomes integrated into conditional relationships.
286 Besides, each relationship can be independently assessed without significantly obscuring any
287 variable unlike other models (Borsuk et al., 2004). An effective interpretation of ENPs
288 toxicity therefore would include an ENPs characterisation and a model comparing *in vitro*, *in*
289 *vivo*, acute and chronic, predictive and validated bioassay data with those from relevant
290 environmentally aged-ENPs (Puzyn et al., 2011).
291
292 *5.2. Lack of standards and guidance for ENPs toxicity evaluation*
293 The lack of standardised guidance on dose metrics for ENP assessment exacerbates the
294 uncertainty in toxicity data interpretation. For instance, documented *in vitro* minimum
295 inhibitory concentration (MIC) values for Ag^0 ranged from < 1 to 433 μg/mL for a variety of
296 organisms under different cultural conditions (Martinez-Gutierrez et al., 2010). Besides the
297 cytotoxic and mutagenic effects of different metal oxide ENPs (100 – 1600 μg/plate) on *E.*
298 *coli* WP2 and *S. typhimurium* TA97 and TA100 showed a wide range of dose-dependent
299 patterns (Warheit et al., 2008; Pan et al., 2010).
300 The assessment of toxicity is closely related to the determination of bioavailable dose (Crane
301 et al., 2008), therefore the interpretation of ENPs dose-contact may be problematic. Tests
302 with some metals demonstrated that short-term batch assays did not provide a true reflection
303 of toxicity probably due to kinetics of internalisation, dosimetry and exposure (Liang et al.,
304 2010). Again, there could be bias in evaluation of data from a continuous flow and batch
305 systems due to different hydraulic loading rate (HLR), hydraulic retention time (HRT) and
306 sludge retention time (SRT) which may increase or reduce ENP-microbial contact in
307 wastewater (Wei et al., 2008). Furthermore, reliance on existing regulations and guidelines
308 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., 2012b; SCENIHR, 2007).

5.3. Uncertainties in determining ENP dosage and microbial contact

The determination of effective ENP concentration available for contact with microorganisms in wastewater currently is a challenge, and can greatly deviate from the overall ENP concentration. The implication 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).

5.4. 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 subsequent influence on the experimental result (Tiede et al., 2009). In the same way, the use of dispersants or filters 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 minimised 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).

5.5. Inadequacy of available toxicity bioassay

334 The different ENPs would require formulation of specific toxicity standards based on factors
335 such as dose, shape/size-, elemental composition/functionalization-response to assess their
336 impacts on biological systems (Harper et al., 2011). The use of standardised ecotoxicological
337 tests involving respirometry, *Daphnia magna*, bioluminescent and anaerobic toxicity tests
338 have been suggested especially when there is paucity of information on the substance
339 (Gutierrez et al., 2002; Choi et al., 2008; Garcia et al., 2011). In comparison with 50
340 standardised microscale tests, Microtox® assay has been highly rated as a useful tool with
341 'environmental relevance' for toxicity testing (Ghirardini et al., 2009; Munkittrick and
342 Power, 1989). Microtox assay is based on the exposure of the bacterium *V.fischeri* with
343 subsequent measurement of bioluminescence. The use of this assay is however controversial
344 because this marine species is not a representative member of sludge and requires 2% NaCl.
345 Such osmotic conditions obviously vary substantially from those in activated sludge
346 suggesting that extrapolation of the resulting data to a wastewater context has to be
347 interpreted with caution (Gutierrez et al., 2002). Further to this, Ghirardini et al. (2009) and
348 references therein generally agree on the ecological relevance and reliability of the
349 Microtox® solid phase test due to its representativeness on diverse metabolic ability of
350 microorganisms. However they also highlighted the importance of confounding factors such
351 as bacterial absorption, pH variation due to dilution, particle interference, redox potential
352 which greatly influenced Microtox response to conditions tested. Therefore relying only on
353 one bioassay test is not sufficient and it can be inferred that an adequate evaluation of ENP
354 toxic effect on AS or AD microorganisms would require the use of multiple bioassays
355 employing species typically found in wastewater such as *E. coli*, ammonia- and nitrite-
356 oxidising species and methanogens for comparative ENPs toxicity evaluation.
357
358 *5.6. Interpretation of ENP toxic effects*

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 convoluted due to the alteration in ENPs reactivity as a consequence of aggregation (Hassellov et al., 2008). To date, 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 standardised or reliable methods to determine and make definitive judgement on environmentally effective ENP concentration and complexation/aggregation reactions of ENPs (Nowack and Bucheli, 2007). Until recently, aggregation was not important in the 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.

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 utilisation/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. We report available information on ENPs-microbial interactions 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 effects 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 urgent need for further empirical evidences on the effects of pristine and aged-ENPs on wastewater microorganisms in AS and AD processes. This knowledge gap is in great part caused by the lack of appropriate analytical tools and framework to elucidate factors that positively enhance or attenuate ENPs effects. 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. 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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671

672 **Legend of the figures**

673 **Figure 1:** Scanning electroscopy microscope images showing ENPs sorption to cells (a,b),
674 damage to microbial cell (c,d) and aggregation to biomass (e,f) in AS (from Eduok S., PhD
675 study; data not published)

676

677 **Figure 2:** Illustration of ENP bioaccumulation in prokaryotes and trophic transfer to
678 eukaryotes and biomagnification in higher organisms.

679

680 **Figure 3:** Size-dependent inhibitory effect of nanosilver particles (Ag^0) on pure cultures of
681 *Escherichia coli*.

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

687

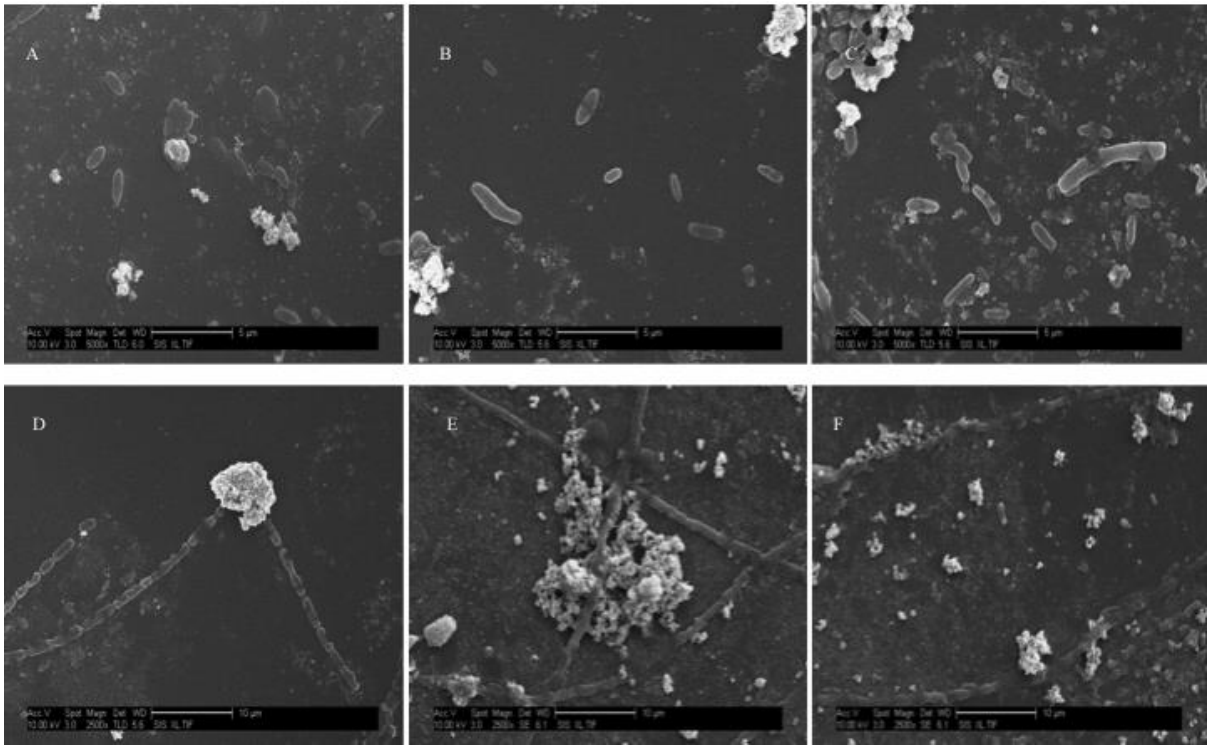
688 **Figure 4**: Schematic overview of the interactions occurring between ENP and microbial
689 biomass involved in activated sludge (AS) and anaerobic digestion (AD) processes

690

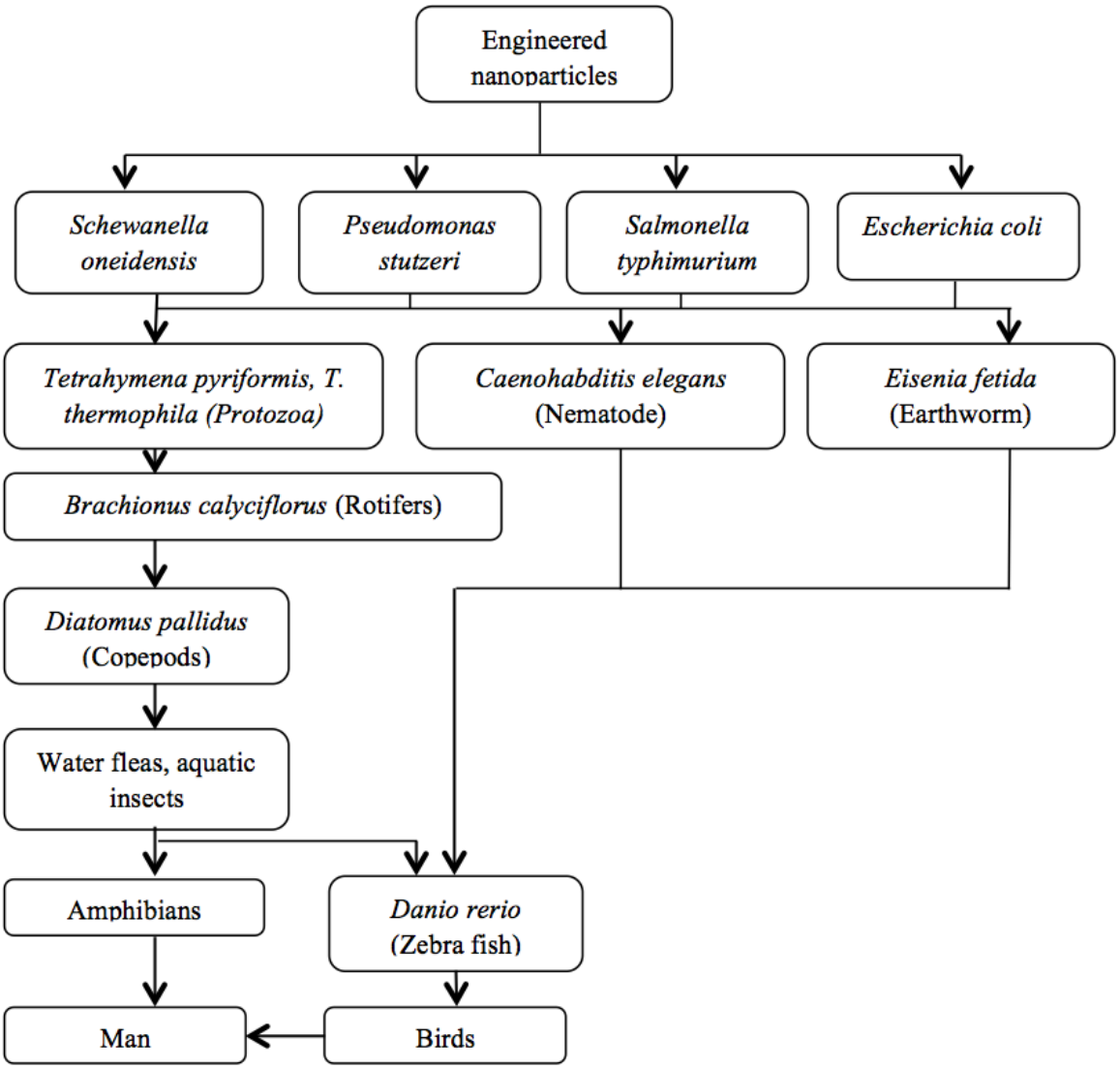
691 **Figure 5**: Representation of the influence of divalent cations in Ag^0-microbial cell wall
692 interaction

693

694

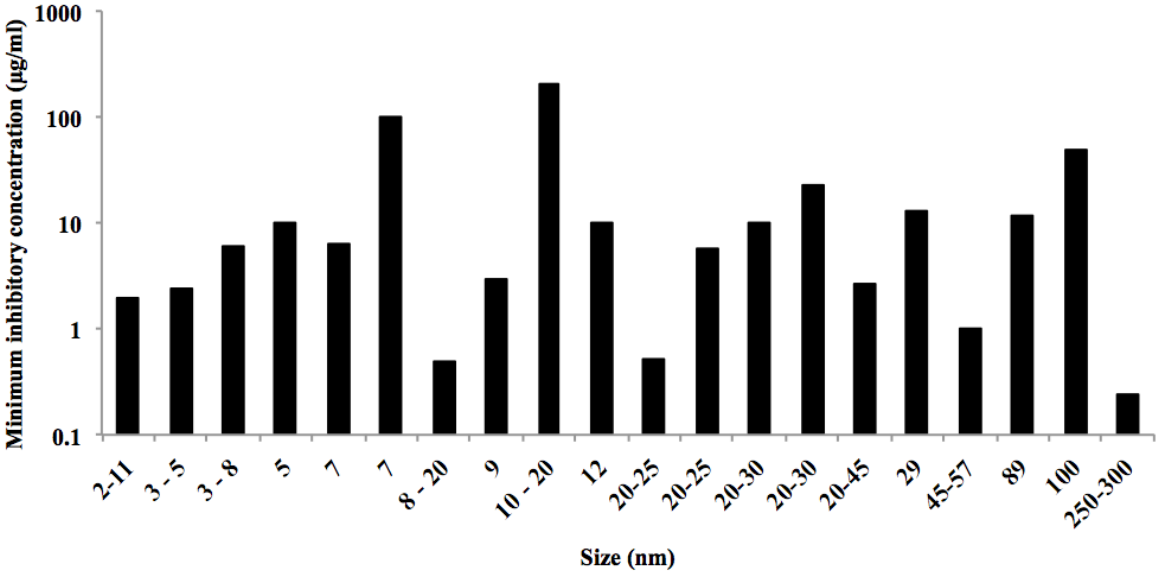
695 

696 Figure 1



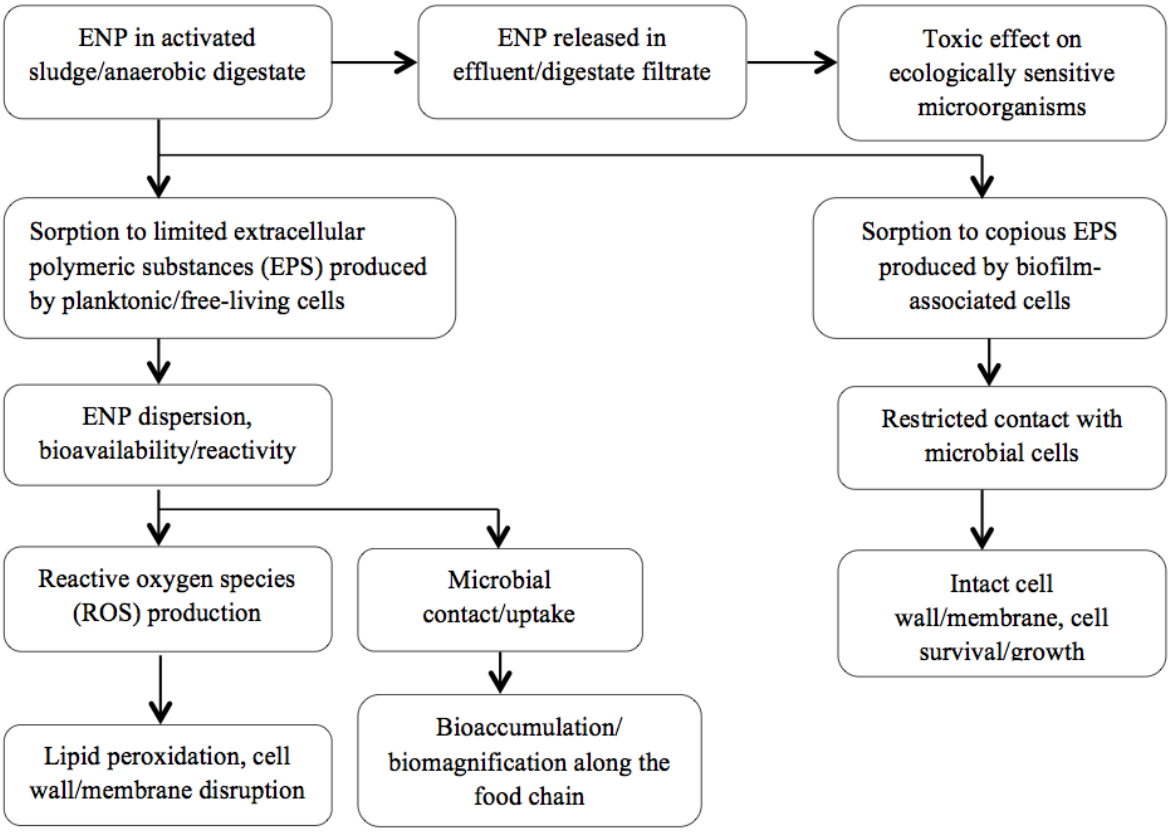
697

698 Figure 2



699

700 Figure 3

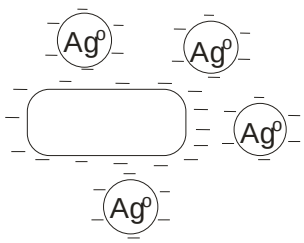


701

702 Figure 4

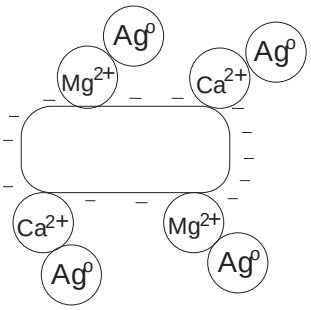
(1) Gram negative cell wall bacterium

(a)



Absence of divalent cations: Electrostatic repulsion of negatively charged cell wall and negatively charged Ag°

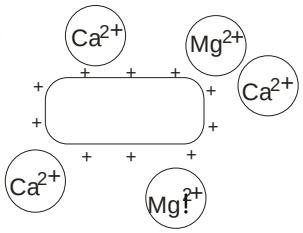
(b)



Presence of divalent cations: Facilitated adsorption/adhesion of Ag to cell wall through cation mediated ion bridges.

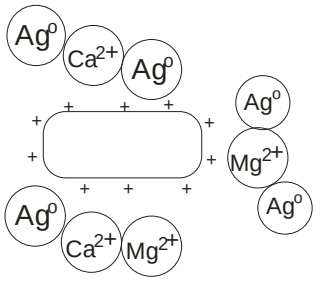
(2) Gram positive cell wall

(a)



Electrostatic repulsion of positively charged bacterial cell wall and divalent cations.

(b)



Competitive aggregation/inhibition of Ag° by divalent cations

703

704 Figure 5

705