Research paper

Bacterial exposure to ZnO nanoparticles facilitates horizontal transfer of antibiotic resistance genes

Xiaolong Wang[a], Fengxia Yang[a], Jing Zhao[a], Yan Xu[a], Daqing Mao[b], Xiao Zhu[a], Yi Luo[a,*], P.J.J. Alvarez[c,*]

[a] College of Environmental Science and Engineering, Ministry of Education Key Laboratory of Pollution Processes and Environmental Criteria, Nankai University, Tianjin 300071, China
[b] School of Medicine, Nankai University, Tianjin 300071, China
[c] Dept of Civil and Environmental Engineering, Rice University, Houston, TX 77005, USA

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ABSTRACT

Bacterial exposure to ZnO nanoparticles (nZnO) at sublethal concentrations (1 to 10 mg/l) for 24 h significantly increased the conjugative frequency of antibiotic resistance plasmid RP4. A 24.3-fold increase was observed in *E. coli* pure cultures, and an 8.3-fold increase occurred in a mixed culture of indigenous aquatic microbiota. In addition, nZnO increased by three fold the transformation efficiency of *E. coli* via the uptake of naked plasmid pGEX4T-1, which was confirmed by confocal fluorescence microscopy. Enhanced horizontal transfer of resistance plasmids was a nanoparticle-specific effect, since 5.3 mg/l Zn^{2+} (representing the maximum release of Zn^{2+} from 10 mg/l nZnO) did not enhance RP4 transfer or pGEX4T-1 uptake frequency. This phenomenon was attributed to increased bacterial cell permeability after exposure to nZnO (but not to Zn^{2+}), which was confirmed by flow cytometry with propidium iodide staining. Overall, this study forewarns that accidental or incidental release of nZnO may facilitate environmental dissemination and propagation of antibiotic resistance genes through horizontal gene transfer.

1. Introduction

With the issuance of the Antimicrobial Resistance Global Report on Surveillance 2014, there is no doubt that antibiotic resistance and the associated antibiotic resistance genes (ARGs) will continue to pose a major threat to public health in the foreseeable future (WHO, 2014). Horizontal gene transfer (HGT) by transduction, transformation or conjugation is widely recognized to facilitate the dissemination of ARGs (Dodd, 2012). For example, ARGs are often harbored in transmissible resistance plasmids, and plasmids belonging to the IncP-1 incompatibility group are noteworthy because of their stable replication in a wide range of Gram-negative bacteria and their efficient conjugative transfer to the wider range of bacterial taxa (Musovic et al., 2006). As members of the IncP group, RP4 plasmids are widely detected in the environment (Datta et al., 1971) and have been shown to facilitate HGT of ARGs across bacterial genera (Wang et al., 2015) in numerous environments, including biofilms (Ehlers and Bouwer, 1999), soils (De Rore et al., 1994), marine sediments and seawater (Combarro et al., 1992; Goodman et al., 1993), and agricultural drainage (Trevors et al., 1990). In addition, extracellular plasmid- or chromosomal-borne ARGs

can persists in sediments at higher concentrations than intracellular DNA and transform "competent" recipient bacteria that become resistant to antibiotics (Mao et al., 2014).

While considerable research has been devoted to understand environmental, genetic and physiological factors that affect HGT, limited attention has been paid to how different engineered nanoparticles that are released to the environment may affect ARG propagation. Some nanoparticles such as nanosized alumina and TiO_2 have been reported to promote horizontal transfer of plasmid-borne ARGs in pure bacterial cultures in nutrient rich LB medium, by damaging cell membranes and enhancing the expression of mating pair formation and DNA transfer and replication genes (Ding et al., 2016a; Qiu et al., 2015; Qiu et al., 2012). However, additional studies with other widely used nanomaterials under more realistic exposure conditions (e.g., lower nanoparticle concentrations in natural waters) are needed to understand the circumstances that favor nanomaterial-enhanced ARG propagation.

In this work, we consider ZnO nanoparticles (nZnO), whose widespread used in chemical industries, cosmetics and other applications result in a high potential for environmental release (Brayner et al., 2006; Espitia et al., 2012; Hajipour et al., 2012; Huang et al., 2008;
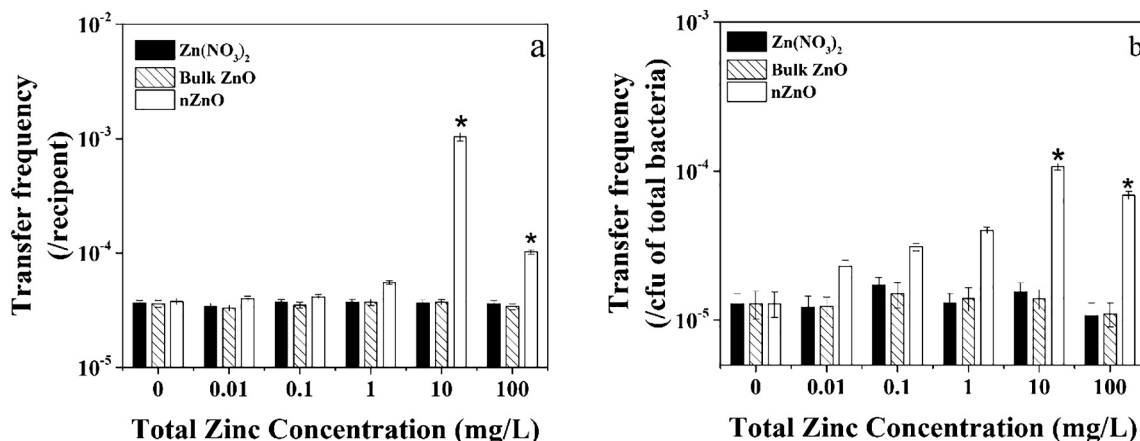
Fig. 1. Transfer frequency of plasmid RP4 in (a) *E.coli* and in (b) aquatic microbiota treated with various concentrations of nZnO, b ulk ZnO or Zn^{2+} ions after 12 h exposure. The concentration of nZnO had a significant effect on the transfer frequency of the plasmid RP4. Significant differences ($p < 0.05$) between each of the nZnO-exposed groups and the unexposed control were tested with two-tailed unpaired Student's *t*-test at the 95% confidence level, and are denoted by the asterisks (*). Error bars are standard deviations (n = 3).
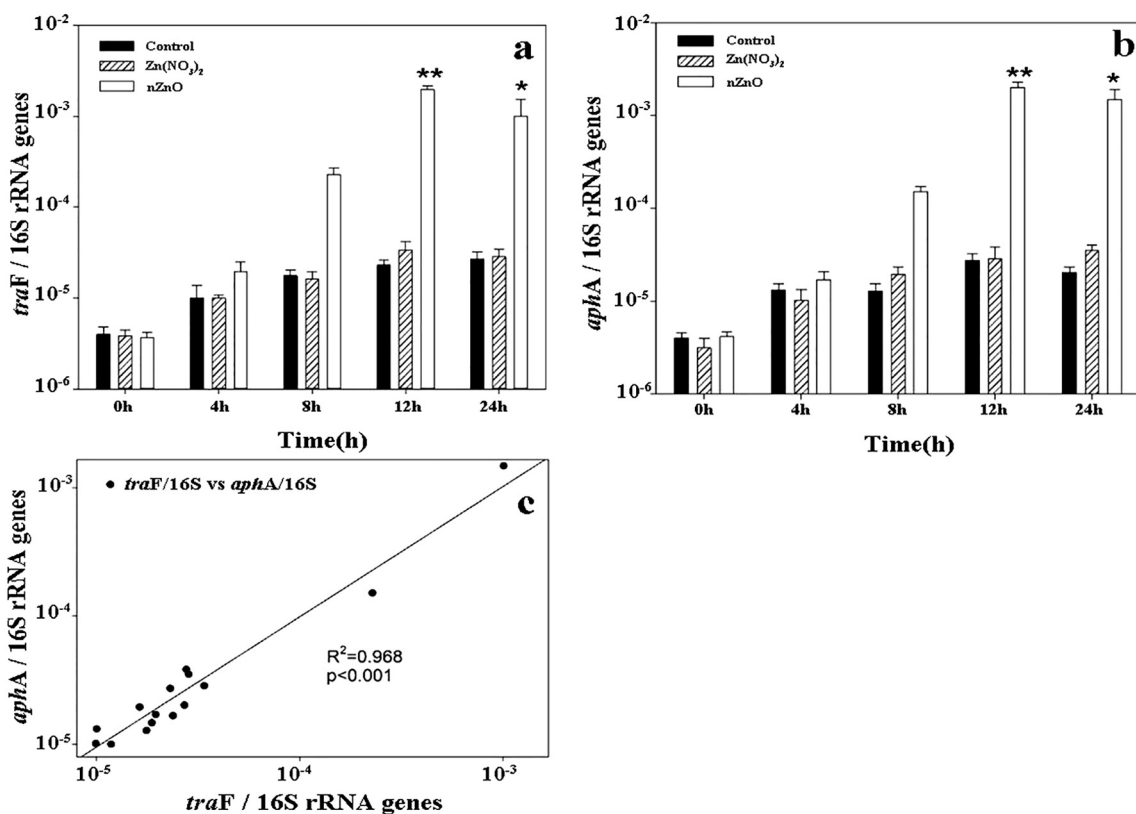

Fig. 2. Exposure of aquatic microbiota of sublethal nZnO concentrations (but not to $Zn(NO_3)_2$) significantly increased the relative abundance of (a) *tra*F and (b) *aph*A (normalized to 16S rRNA). A significant correlation was observed between *tra*F/16S rRNA and *aph*A/16S rRNA in aquatic microbiota (c). Note that *tra*F and *aph*A were not detected in the aquatic microbiota before adding the plasmid RP4. Error bars are standard deviations (n = 3).

Zhang et al., 2016). Bacterial exposure to nZnO has been reported to induce oxidative stress and damage the cell wall in both Gram-negative and Gram-positive bacteria (Feris et al., 2009; Jiang et al., 2009; Sinha et al., 2011). nZnO at 100 mg/l (which is an unrealistically high concentration for most aquatic systems) is reported to induce oxidative stress (Xie et al., 2011), and 405 mg/l nZnO is reported to induce cell wall damage (Sinha et al., 2011). However, no previous publications have considered how sublethal nZnO concentrations that would prevail in the environment may affect the dissemination and proliferation of ARGs. Here, we evaluate the potential for sublethal exposure to nZnO to enhance the horizontal transfer (conjugation and uptake) of plasmid-borne ARGs in both pure culture *E. coli* and a mixed cultures of aquatic

microbiota. We report that nZnO can increase ARG conjugative transfer frequency as well as facilitate bacterial uptake of naked resistance plasmid, as observed by confocal fluorescent labelling. Therefore, this study expands current understanding of the mechanisms and conditions under which released engineered nanomaterials may enhance the propagation of ARGs in aquatic environments.

2. Materials and methods

2.1. Samples, bacterial strains and plasmids

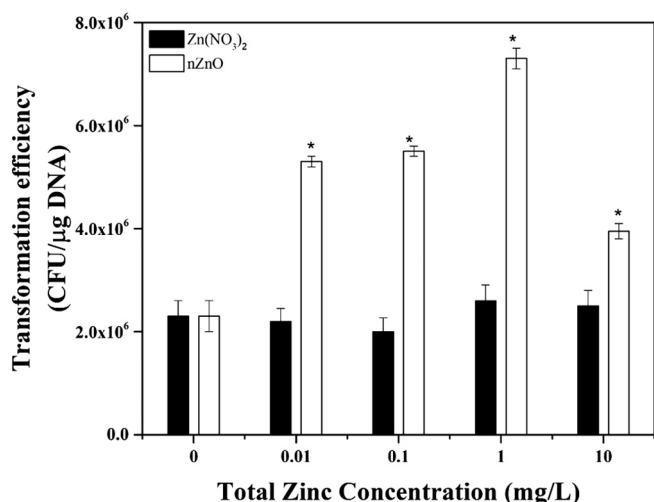Water samples were collected from a lake in Tianjin Water Park

Fig. 3. *E. coli* transformation efficiency (f_2) significantly increased by the presence of nZnO ($p < 0.05$) but not Zn^{2+}. Significant differences between each of the nZnO-exposed groups and the unexposed control were tested with two-tailed unpaired Student's *t*-test at the 95% confidence level, and are denoted by the asterisks (*). Error bars are standard deviations (n = 3).

(covering approximately 0.75 km²) in Northern China in June 2013. The water temperature of the sampling point was 28 °C. Water quality parameters are given in the Table S1 of the Supplementary Information (SI-1). These samples did not contain detectable levels of kanamycin- or ampicillin-resistant bacteria (or associated ARGs), or the *tra*F biomarkers of the RP4 plasmid (limit of detection 104 copies per ng DNA). A list of the bacterial strains and plasmids used in this study is given in the Table S2 of the Supplementary Information (SI).

2.2. Dispersion of nZnO

nZnO was purchased from Aladdin Co., Shanghai, China, with a nominal particle size of 50 ± 10 nm according to the manufacturer. The nZnO dispersed at 50 wt% in H_2O. The stock suspensions of 10 g/l nZnO were prepared by sonication (300 W) for 30 min in the corresponding filtration-sterilized water. The following concentrations were used in the present study: 0, 0.01, 0.1, 1, 10, and 100 mg/l, which were obtained by diluting the stock suspensions with filtration-sterilized water. These concentrations are representative of potential exposure in aquatic systems. For example, measured environmental concentrations of nZnO in surface water in the UK are as high as 0.1 mg/l (Boxall et al., 2007). Whereas higher concentrations might be encountered in the immediate vicinity of a release, regional-scale modeling suggests that volume-averaged nZnO concentrations in aquatic environment would be much lower (e.g., on the order of 10 ng/l may be found in surface waters and 430 ng/l sewage treatment effluents, compared to 1 ng/l for surface water and 300 ng/l sewage treatment effluents in the USA (Gottschalk et al., 2009).

The hydrodynamic diameter of suspended nZnO aggregates was determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK) equipped with a monochromatic coherent He-Ne laser with a fixed wavelength of 633 nm. Each value represents the average of three measurements consisting of 12 runs at 25 °C. The hydrodynamic diameter of the nZnO suspension in LB was 974 ± 29 nm, compared to 763 nm ± 19 nm in natural water (Fig. S1).

Samples were kept in a constant-temperature shaker incubator (150 rpm) at 25 °C overnight to reach a relative dissolution equilibrium (Hotopp, 2011). The concentrations of dissolved zinc released from different nZnO suspensions were then measured (Table S3). The details for measuring dissolved Zn concentrations are given in SI-2.
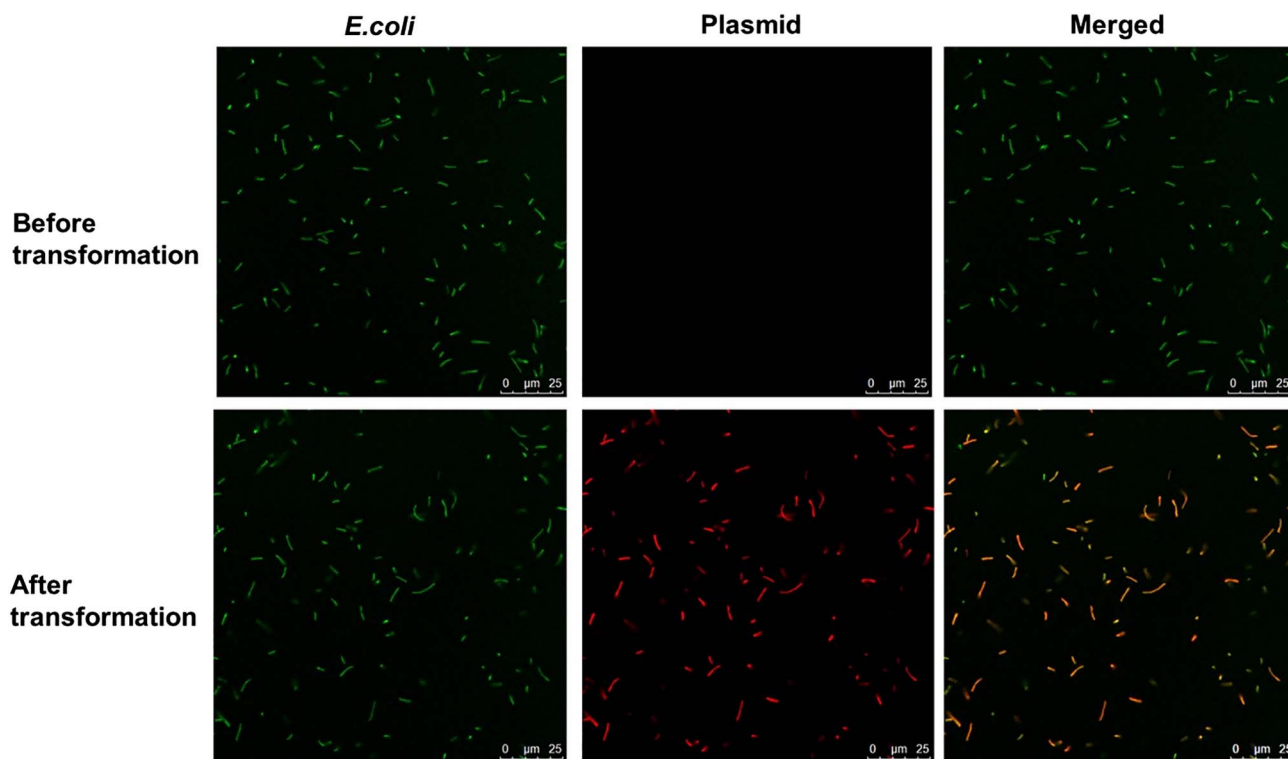

Fig. 4. Confocal fluorescent images showing plasmid uptake. The plasmid pGEX4T-1 and *E. coli* CV601 were respectively tagged with red and green fluorescent proteins. The merged graph shows both *E. coli* CV601 and plasmid. Scale bars correspond to 25 μm.
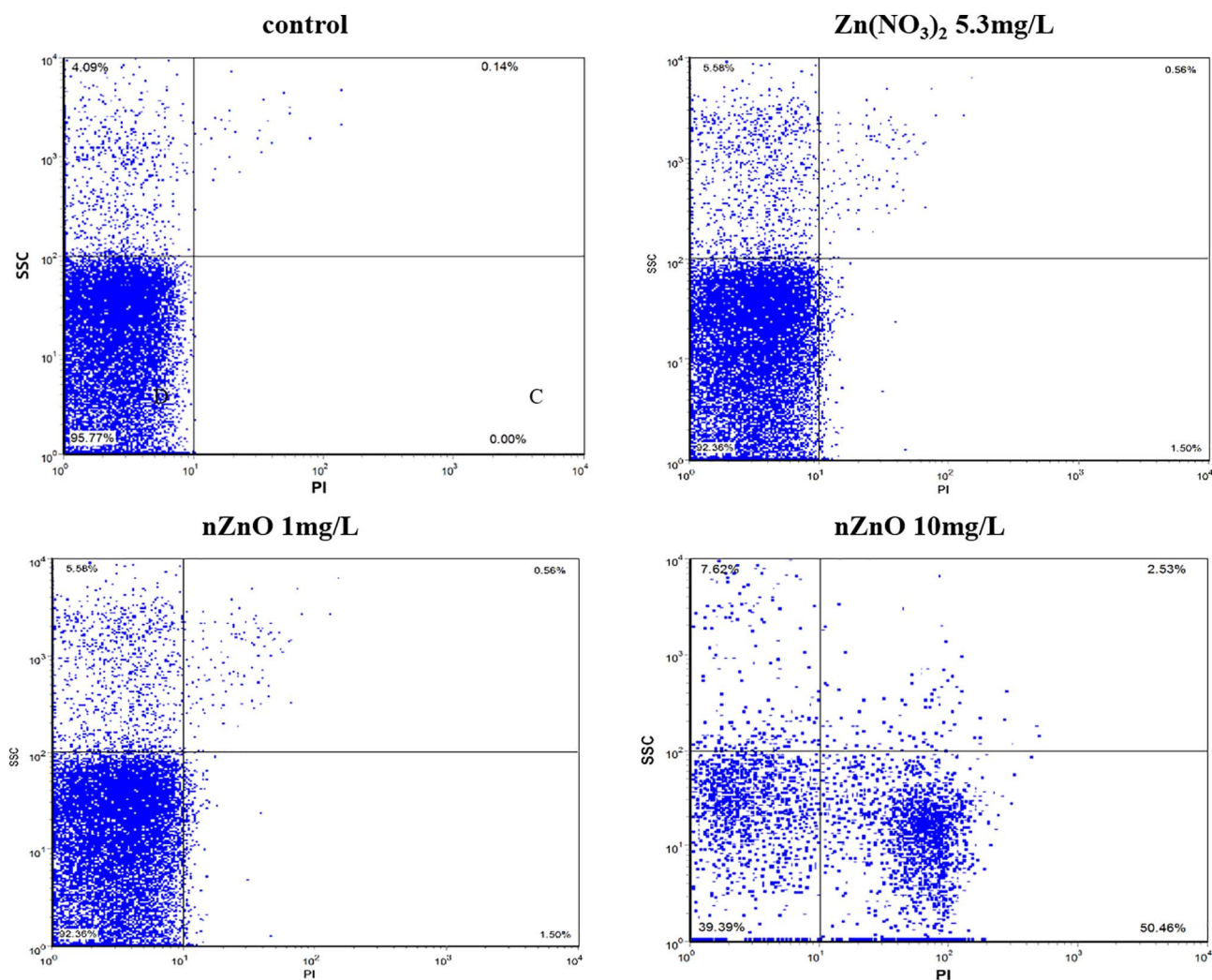
Fig. 5. Flow cytometry to quantify membrane permeability of control cells (0 g/l), and cells exposed to Zn(NO$_3$)$_2$ (5.3 mg/l) or nano-ZnO (1, 10 mg/l). Quadrant D: negative signal (normal cells); and Quadrant C: PI positive (cell membrane permeability was evidenced by a 53% increase in propidium iodide uptake exposure to 10 mg/l nZnO).

2.3. RP4 plasmid transfer in pure culture E. coli and aquatic microbiota

Experiments based on the OECD 308 test (Test, 2002) were used to discern the effect of nZnO on ARG plasmid transfer using both pure cultures of *E. coli* and a mixed culture of aquatic microbiota. These tests were repeated using bulk nZnO and Zn(NO$_3$)$_2$ (as a source of Zn^{2+}) as controls to discern nanoparticle-specific effects. *E. coli* DH5α-harboring RP4 plasmids—was used as the ARG donor in LB media and natural water samples. To enumerate resistant conjugants by plate counts, it was necessary to distinguish them from the original ARG donors. This was accomplished by adding streptomycin to the growth plates (30 mg/l), along with antibiotics to which RP4 confers resistance (i.e., kanamycin sulfate (50 mg/l), ampicillin (100 mg/l), and tetracycline (10 mg/l)). The RP4 plasmid does not confer resistance to streptomycin, which precludes growth of the ARG donor. Accordingly, streptomycin-resistant *E. coli* HB101 (str^R) (in LB-medium) was used as the RP4 recipient. A similar approach was used to enumerate indigenous conjugants in natural water samples, where streptomycin-resistant recipients of RP4 were isolated and counted. Cell numbers were adjusted to 10^8 for both plasmid donors and recipients based on previous CFU counts, and the initial ratio of donors to recipients was 1 to 1. Both media were spiked with well-distributed nZnO suspensions, with initial exposure concentrations of 0, 0.01, 0.1, 1, 10, and 100 mg/l. Each concentration was set up in triplicate in 500-ml flasks. All treatments (including aquatic microbiota and *E. coli*) were incubated for 24 h at

37 °C for the plasmid-transfer tests. The stability of nZnO in LB medium and natural water was shown in Fig. S2. It shows that the nZnO suspension was stable in both LB and water.

2.4. Calculation of the transfer frequency of RP4 plasmids

The concentration transconjugants was measured every 2 h as colony-forming units per milliliter of culture (cfu/ml), using LB plates containing streptomycin sulfate (30 mg/l), kanamycin sulfate (50 mg/l), ampicillin (100 mg/l), and tetracycline (10 mg/l). Plasmid recipients were counted on LB plates containing only streptomycin sulfate (30 mg/l) to similarly preclude growth of the RP4 plasmid donors. Controls were also prepared with only donors or recipients amended with nZnO to discern potential spontaneous mutations of the strains. PCR and 16S rRNA sequencing were used to confirm that the RP4 plasmid had been transferred into recipients, and details of transconjugants validation provided in the Supporting Information (SI-4).

The transfer frequency (f_1) in both the LB medium and natural water samples was calculated using the following formula:

$$f_1 = \frac{N_1}{N_2}$$

where N_1 is the number of transconjugants (CFU/ml), and N_2 is the total number of potential recipient bacteria (CFU/ml). N_2 was counted as the streptomycin resistant bacteria in the pure *E.coli* HB101 (str^R) culture,

and it was calculated as the total bacteria in the natural water samples.

2.5. DNA extraction and quantitative real-time PCR

Water samples (0.5 l) were vacuum-filtered (0.22 μm filters), and the filters were placed in extraction tubes provided in the Ultraclean Water DNA Kit (MoBio Laboratories, Inc.). DNA and plasmid DNA were extracted with the bacterial DNA Isolation Kit (OMEGA BioTek D3350-01) and plasmid DNA Isolation Kit (OMEGA BioTek D2156-01). An internal standard (E. coli DH5α cloned with the CESA9 gene) was used to determine the DNA extraction efficiency as detailed previously (Luo et al., 2010). The DNA extraction efficiency is shown in Table S4. The 16S rRNA, aphA, traF primers, and details of qPCR procedures are listed in the Supporting Information (SI-3) and in Table S5.

2.6. Flow cytometry detection of cell membrane permeability induced by nZnO

Flow cytometry (FCM, BD FACSCalibur, USA) was used to investigate whether increased ARG transfer in cells exposed to nZnO was related to increase cell permeability. A higher fluorescence intensity following propidium iodide (PI) uptake indicates enhanced cell membrane permeability. The FCM was equipped with an excitation wavelength of 488 nm. One mL of bacterial suspension (both nZnO-amended and the controls) was stained with 10 μl of PI (1 mg/ml, OMEGA, USA) and incubated in the dark for 8 min before measurement. The concentration of bacterial suspensions was always $< 10^6$ cells/ml. All data were processed with the CellQuest Pro.

2.7. Plasmid pGEX4T-1

Unconjugative plasmid pGEX4T-1 was purchased from Miaoling Bioscience & Technology Co., Ltd., China. Plasmid DNA was extracted using the E.Z.N.A. Plasmid Mini Kit I according to the manufacturer's instructions (OMEGA). The plasmid DNA was digested using the restriction enzymes BamHI and EcoRI (TAKARA). The red fluorescence protein was PCR amplified and cloned into the pGEX4T-1 plasmid using the one-step cloning kit (Vazyme Biotech). In addition, the construct was confirmed by PCR, enzyme digestion and sequencing.

2.8. Uptake of plasmid pGEX4T-1 into the recipient bacteria

E. coli CV601 was used as the recipient bacterium to uptake the constructed plasmid pGEX4T-1. The protocol for the preparation of competent cells is described by Kang et al. (Kang et al., 2013). For further details, please see SI-5 of the Supporting Information. A laser-scanning confocal microscope (Leica TCS SP8) was used to further confirm the uptake of pGEX4T-1 into the bacteria. The transformation efficiency (f_2) was calculated using the following formula:

$$f_2 = \frac{N_3}{D}$$

where N_3 is the number of transformants, which is the CFU grown in the presence of antibiotics (ampicillin, kanamycin, and rifampicin) in the LB plate, and D represents the plasmid DNA(1-μg).

2.9. Statistical analysis

All treatments were conducted in triplicate and data were analyzed with SPSS for Windows version 22.0. Statistical significance of differences between treatments and controls was assessed using two-tailed unpaired Student's t-test at the 95% confidence level.

3. Results and discussion

3.1. nZnO facilitates conjugative transfer of RP4 plasmids in pure E. coli culture and aquatic microbiota

Conjugative transfer is generally the principal mode for antibiotic resistance transfer via mobile genetic elements (Collard et al., 1994; Sorensen et al., 2005; Thomas and Nielsen, 2005). Conjugation is likely to occur between mated pairs of metabolically active donor and recipient bacterial cells in the presence of a high-density bacterial community and chemical stressors (Dodd, 2012). Surface water and wastewater, which are pools for antibiotic-resistant bacteria and ARGs (Rodriguez-Mozaz et al., 2015; Xu et al., 2016; Xu et al., 2017) and may also harbor a variety of engineered nanoparticles (Zhou et al., 2016; Boxall et al., 2007; Gottschalk et al., 2009), appear to contribute significantly to the spread of antibiotic resistance via conjugation (Gaze et al., 2008).

Fig. 1 shows an increase in the transfer frequency of the RP4 plasmid in pure culture E. coli and a mixed culture of indigenous aquatic microbiota exposed to nZnO (time course data shown in Fig. S3). Specifically, exposure to 10 mg/l nZnO for 12 h increased the transfer frequency of the RP4 plasmid by 24.3-fold in E. coli, and by 8.3-fold in aquatic microbiota relative to the unexposed control group. The higher transfer frequency for E. coli reflects that having a pure culture (with a higher density of compatible ARG donors and recipients) facilitates conjugative transfer. No significant enhancement of plasmid transfer was observed upon exposure to Zn^{2+} (Fig. 1) indicating that this was a nanoparticle-specific effect. The dissolved zinc ion concentration was much higher in LB medium than in fresh water medium because the LB medium has components such as PO_4^{3-}, citrate, and other organic ligands that promote nZnO dissolution (Li et al., 2011). Note that coating of nZnO could affect its bioavailability and potential impacts, but this was not monitored in our experiments. Nevertheless, this transconjugation analysis may conservatively underestimate the nanoparticle-specific enhancement of RP4 plasmid transfer frequency for the natural water microbiota (Fig. 1), because we considered only the streptomycin-resistant bacteria as recipients. Potential recipients that were susceptible to streptomycin were not counted.

Previous studies have shown that exposure to relatively high concentrations of nano-Al_2O_3 (> 610 mg/l) and nano-TiO_2 (> 40 mg/l) in LB growth medium can facilitate the propagation of ARGs in pure cultures of E. coli (Qiu et al., 2015; Qiu et al., 2012). Here, we show that exposure to nZnO under more realistic conditions (lower concentrations in natural water) can exert a similar effect also on the indigenous aquatic microbiota. The transconjugants that were selected from this aquatic microbiota were phylogenetically close (99% identical, 1465 matches) to Salmonella enterica subsp. enterica serovar (GenBank accession NO. FJ997268.1), Aeromonas salmonicida (GenBank accession NO. AF108136.1), Stenotrophomonas sp. (GenBank accession NO. AB646575.2), and Pseudomonas geniculata strain XJUHX-9 (GenBank accession NO. EU239471.1), showing that a variety of bacterial genera could serve as the recipient to the ARGs harbored by plasmid RP4.

The traF gene was used here as a biomarker to quantify the abundance of the RP4 plasmid. The relative abundance of traF in the aquatic microbiota (normalized to the total 16S rRNA abundance) significantly increased after exposure to 10 mg/l nZnO compared to the unexposed control (Fig. 2a), corroborating enhanced transfer of the RP4 plasmid. A significant increase in relative abundance was also observed for aphA, which is a gene in the RP4 plasmid that confer resistance to kanamycin sulfate (Fig. 2b). Positive correlations ($p < 0.01$) were observed between aphA/16S rRNA genes and traF/16S rRNA genes, which implies that increasing levels of antibiotic resistance genes (aphA) were primarily attributable to the enhanced transfer and replication of RP4 plasmids (indicated by the traF gene).

3.2. nZnO facilitates the transformation efficiency by naked plasmid pGEX4T-1

In addition to conjugative transfer, transformation (which is the acquisition of the extracellular DNA into bacterial strains (Chen and Dubnau, 2004)) plays an important role in the propagation of ARGs. Previous studies have shown that extracellular ARGs can persist in sediments at a higher concentration compared with intracellular ARGs and were transformed into indigenous bacteria (Mao et al., 2014). Furthermore, there is as much as 0.45 gigatons of extracellular DNA present in the deep-sea sediments (Dell'Anno and Danovaro, 2005), and various ARGs (e.g., *sul*I, *sul*II, *tet*T, *tet*W, *tet*Q, *tet*X, and *tet*O) have been found in extracellular DNA (Zhang et al., 2013).

The natural transformation frequency from external DNA to the recipient strain is lower than 10^3 transconjugants per µg DNA (Yoshida et al., 2001). However, the transformation efficiency significantly increases when the recipient bacterial strains are competent, especially in the presence of chemical stressors (Ding et al., 2016b). Recent studies reported that the transformation frequency increases in the presence of nano-Al_2O_3 (Ding et al., 2016b). Here, we used pGEX4T-1, which is a non-conjugative, synthetic resistance plasmid. Exposure to nZnO significantly increased the transformation efficiency of plasmid pGEX4T-1 (Fig. 3), and the highest transformation efficiency (7.5×10^6 CFU/µg) was observed with 1 mg/l nZnO. Fluorescence-labeled techniques and confocal fluorescence imaging verify that pGEX4T-1 was taken up by the *E. coli* CV601 (GFP-tagged; Fig. 4). However, Zn^{2+} did not enhance the transformation efficiency of pGEX4T-1 (Fig. 3), indicating that enhanced transformation was also a nanoparticle-specific effect.

3.3. nZnO increases cell membrane permeability

FCW analyses showed that the membrane permeability was significantly increased in cells treated with 10 mg/l nZnO (as evidenced by a 53% increase in propidium iodide uptake) compared to the untreated controls (Fig. 5). In contrast, the membrane permeability of the Zn$(NO_3)_2$-treated groups was not significantly increased compared to the untreated controls. Cell membranes are a selective, semi-permeable membrane and constitute an important barrier for the horizontal transfer of genetic information between aquatic microbiota of different species or genera (Alekshun and Levy, 2007; Thomas and Nielsen, 2005). Apparently, nZnO disorganizes and possibly damages the cell membrane, which leads to increased permeability (Hajipour et al., 2012) and this, in turn, increases HGT frequency (Achouak et al., 2001), which facilitates propagation of resistance plasmids. Note that nZnO does not enhance the growth of RP4 plasmid donors, potential recipients, or transconjugants (grown separately) relative to unexposed controls (Fig. S4). This rules out that transconjugants that arise may be more competitive in the presence of nZnO to explain the observed increase in the T/R ratio (Fig. 1), and corroborates that this result is due to nZnO increasing cell permeability, which facilitates HGT.

4. Conclusion

This study demonstrates that sublethal concentrations of nZnO can significantly facilitate the horizontal transfer of antibiotic resistance plasmid RP4 from *E. coli* DH5α to a wide variety of indigenous aquatic bacteria, which is conducive to increasing the resistome reservoir. HGT through transformation (i.e., bacterial uptake of naked resistance plasmid pGEX4T-1) was also enhanced by nZnO. Enhanced HGT increases the risk of ARG dissemination in an aquatic environment, which underscores the need for judicious use of nZnO and mitigation of incidental releases.

Acknowledgments

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