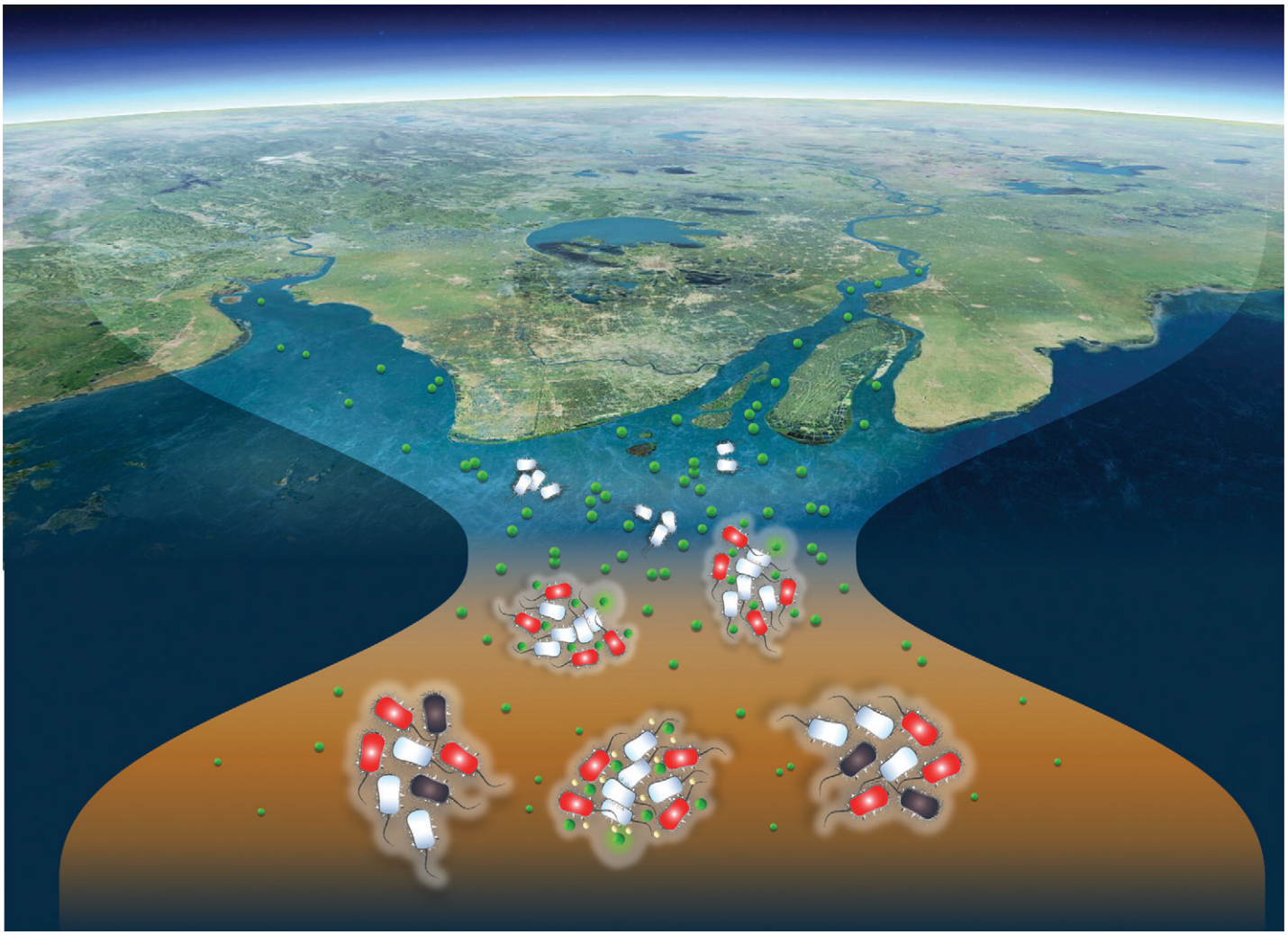

Featuring work from Prof. Yi Yang at State Key Laboratory of Estuarine and Coastal Research, East China Normal University, China. Prof Yang's group is working on nanoparticles in the environment: identification, behavior and environmental risk.

Impact of ZnO nanoparticles on the antibiotic resistance genes (ARGs) in estuarine water: ARG variations and their association with the microbial community

The occurrence of nanoparticles (NPs) in the environment may alter the behavior of antibiotic resistance genes (ARGs). It is for the first time reported that ZnO NPs as a selective pressure can significantly induce the enrichment and dissemination of ARGs in estuarine water, at environmentally relevant/low ZnO NP concentrations. The bacterial community was considered as the main driver for the changes in ARGs, followed by mobile genetic elements (MGEs) and dissolved Zn.

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Impact of ZnO nanoparticles on the antibiotic resistance genes (ARGs) in estuarine water: ARG variations and their association with the microbial community†

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Nanoparticles (NPs) promote the horizontal gene transfer (HGT) of plasmid-borne antibiotic resistance genes (ARGs) in pure bacterial culture, but the impact of NPs on ARG dissemination in the natural environment remains unknown. This study focused on the impact of ZnO NPs on the variation in ARGs and the association among ARGs, mobile genetic elements (MGEs) and microbial communities in an estuarine aquatic environment by taking the Yangtze Estuary as a representative area. The present study showed that the abundances of total ARGs increased after exposure to ZnO NPs, particularly at low doses (0.2 and 1 mg L^{-1}). Relatively low and environmentally relevant concentrations of ZnO NPs likely induced the selection of *sul*1, *tet*A, *erm*B and *qnr*S, whereas a high concentration (10 mg L^{-1}) of ZnO NPs was more selective for *sul*2 and *tet*W. After normalization of the abundances of ARGs exposed to NPs to those of the corresponding blanks, the ARGs in the DH samples with relatively high salinity and relatively low NP doses showed the highest normalized values by the end of the exposure period, which indicated the high dissemination potential of ARGs in relatively brackish water. A significant positive correlation was found between ARGs and MGEs (Tn*916*/*1545* and *int*I1), which indicated the high potential of ARG dissemination *via* HGT in estuarine waters after exposure to ZnO NPs. The response of the microbial community to ZnO exposure was dose- and time-dependent at both the phylum and genus levels. Although the absolute abundance of microbes showed a decreasing trend after exposure to ZnO NPs, the absolute abundance values normalized to the corresponding blanks generally showed a trend consisting of an initial inhibition followed by a rebound. A redundancy analysis showed that the microbial community contributed more to the variation in ARGs in estuarine waters after exposure to ZnO NPs than MGEs and dissolved Zn^{2+}. A network analysis evaluated the possible host bacteria for the detected ARGs, and the results revealed that some of these bacteria were associated with multiple ARGs and might pose a high risk for the dissemination of ARGs in estuarine environments.

Environmental significance

Up to now, knowledge of how NPs, including ZnO NPs, impact ARG profiles and their association with the microbial community in natural aquatic environments, especially in estuarine and coastal areas, is very limited. This study has, for the first time, reported that ZnO NPs as a selective pressure can significantly induce the enrichment and dissemination of ARGs in estuarine water, and this is especially true at environmentally relevant/low ZnO NP concentrations. The bacterial community was considered as the main driver for the changes in ARGs, followed by MGEs and dissolved Zn.

1. Introduction

Engineered nanoparticles (ENPs) have been manufactured for and used in a wide range of products (*e.g.*, cosmetics, paint, medicine, and textiles) and industrial processes (*e.g.*, agriculture, energy, and electronics) that take advantage of their novel properties, and inevitably, these nanoparticles have been discharged into the natural environment.[1] In particular, zinc oxide (ZnO) NPs are produced at a quantity of 550 t per year globally and thus rank third among metal NPs in terms

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of production.[2] It has been estimated that the concentrations of ZnO NPs in European natural surface water and treated wastewater are 0.01 and 0.43 mg L^{-1}, respectively.[3] Given the notable increases in the production and application of ZnO NPs, the potential ecological risks of their release into the environment and the subsequent impacts on human health have been explored.[4–6]

Antibiotic resistance has been encountered in the human body, particularly with respect to bacterial pathogens, and has become a great public health concern worldwide.[7,8] Although antibiotic resistance genes (ARGs) have existed along with natural antibiotics for billions of years, the overuse and abuse of antibiotics have resulted in environmental exposure to antibiotics and contributed to the selection of resistant strains and an increase in resistome elements.[9] The aquatic environment is an ideal pool for various contaminants, including antibiotics and ARGs.[10] Antibiotic-resistant bacteria (ARB) and ARGs accompanied by various pollutants from residential areas, industries, hospitals and livestock facilities can be discharged into rivers and streams and eventually accumulate in estuaries and coastal environments.[11] Through directional water transport, environmental contamination with antibiotics might exert pressure on the selection of inherent microbial communities that are capable of maintaining and spreading ARGs in the environment.[9,12] The dissemination of ARGs might occur in multiple ways in microbial communities; for example, (1) ARGs can be passed to the offspring of bacteria through self-replication,[13] (2) ARGs can be transferred to other bacterial species *via* horizontal gene transfer (HGT) (transduction or conjugation),[14] and (3) free ARGs can be acquired by competent bacteria *via* natural transformation.[13]

Recent studies, although limited, have found that NPs can promote the transfer of ARGs. For example, *E. coli* treated with alumina NPs (10.9 ± 3.5 nm) appear to more easily obtain exogenous genes.[15,16] It has been suggested that titanium dioxide (TiO_2) NPs (≈21 nm) promotes the horizontal transfer of the plasmid RP4,[17] and ZnO NPs (50 ± 10 nm) significantly increase the conjugative frequency of the antibiotic resistance plasmid RP4.[18] Notably, most of the relevant studies have focused on pure cultures of model bacteria, which do not represent the microbial community in the natural environment. Moreover, the impact of NPs on ARGs has mostly been studied in wastewater treatment plants (WWTPs), which are a quasi-natural environment. For example, Ag NPs stimulate a shift in the ARG profiles during wastewater treatment,[19] Au NPs with different morphologies impose a strong force in shaping the ARG profile in an activated sludge sequencing batch reactor,[20] and the bacterial community might be a major contributor to the variation in ARGs during sludge composting.[21]

Coastal marine environments and estuarine ecosystems in particular have important ecological service functions and receive large amounts of pollutant inputs from land-based sources *via* river runoff and sewage outfalls. Previous studies have frequently detected ARGs and found that these are ubiquitous in estuarine and coastal aquatic environments.[22,23] Although the concentration of ZnO NPs in the estuarine environment is currently unknown, it reaches 0.3 mg L^{-1} in WWTP effluents, and the concentration of ZnO NPs inevitably increases in the estuarine environment.[24] We hypothesized that ZnO NPs might place selective pressure and thus have a significant impact on ARG profiles and the inherent microbial community in the estuarine aquatic environment. However, the current knowledge on how NPs, including ZnO NPs, impact ARG profiles and their association with the microbial community in natural aquatic environments, particularly estuarine and coastal areas, is very limited.

In the present study, the Yangtze Estuary, which is located where the Yangtze River flows into the East China Sea past the city of Shanghai, one of the largest cities in the world, with a population of more than 24 million people, was taken as the representative study area. This vitally important estuary is severely influenced by anthropogenic activities, and pollutants, including polycyclic aromatic hydrocarbons (PAHs),[25] antibiotics,[23] and ARGs,[23] have been detected and pose significant environmental risk to this coastal ecosystem. The overall goals of the present study were to study the impact of ZnO NPs on the variations in ARG profiles in the estuarine aquatic environment and to determine the mechanisms underlying the selection of ARGs associated with mobile genetic elements (MGEs) and microbial community variations during NP exposure. The shifts in the ARG profile and the microbial community were revealed through quantitative PCR (qPCR) and Illumina MiSeq sequencing of the 16S rRNA gene. The specific objectives of this study were (1) to explore the impact of different doses of ZnO NPs on variations in the target ARGs, MGEs and metal resistance genes (MRGs) during a 24 h exposure time; (2) to characterize the variations in the composition of the microbial community at the phylum and genus levels in response to exposures to different concentrations of ZnO NPs for different times; and (3) to investigate the roles of the microbial community and MGEs in the propagation of ARGs under the selection of ZnO NPs.

2. Materials and methods

Estuarine water sample collection and analysis

In July 2017, water samples were collected along the Yangtze Estuary at three sampling sites with a salinity gradient (Fig. S1†): Shidongkou (SDK, close to a sewage outfall of a WWTP into the Yangtze River, salinity of 0.15%), Chaoyangnongchang (CY, tidal flat with a salinity of 1.44%) and Donghainongchang (DH, tidal flat with a salinity of 3.38%). All the samples were collected into glass barrels that were sequentially cleaned with tap water, filtration-sterilized ultrapure water and acetone and immediately transported to the laboratory. To characterize the water samples, the salinity, conductivity, pH, dissolve oxide (DO), and oxidation–reduction potential (ORD) were measured *in situ* using a portable water quality analyzer (HQ 40d, HACH, USA), and the dissolved organic carbon (DOC) was determined using a total

organic carbon automatic analyzer (SSM-5000A, Shimadzu, Japan). All these parameters are provided in Table S1.†

Characterization of ZnO NPs

Commercial ZnO NPs (99.9%, 60 nm, white powder) with no surface coating were purchased from Shanghai Chaowei Nanotechnology Co., Ltd. (Shanghai, China), and their morphology, grain size and element composition were investigated using a transmission electron microscope (TEM, JEM-2100F, JEOL, Japan) (Fig. S2†). Stock solutions (1000 mg L^{-1} in filtration-sterilized ultrapure water) were prepared and ultrasonically dispersed (100 W, 40 kHz) for 30 min before use.

Experimental setup

Once transported to the laboratory, the raw water samples were treated with ZnO NPs immediately to determine the response of the natural microbial community. The ZnO NP stock solution was added to a 1 L water sample in a conical flask to a final concentration of 0, 0.2, 1 and 10 mg L^{-1}. All the samples were maintained at 150 rpm and 25 °C under a 12 h light/12 h dark cycle. Based on the results of a related preliminary experiment (Fig. S3†), which showed the appearance of a significant time-dependent variation in the bacterial profile within 24 h, the impacts of ZnO on variations in ARGs and the microbial community were thus studied over a 24 h period, and samples were collected at 0, 3, 6, 12 and 24 h. Once collected, the samples were vacuum filtered through a 0.22 μm filter membrane (cellulose ester, SCBB-207, ANPEL, Shanghai, China) for the immediate collection of microbial cells. Moreover, the dissolved Zn^{2+} released from ZnO NPs was measured according to the method described by Guo *et al.*[23] Briefly, after filtration through a 0.45 μm syringe filter (polyethersulfone, SCAA-102, ANPEL, Shanghai, China), the water samples with 2% HNO_3 were analyzed using an inductively coupled plasma-mass spectrometer (ICP-MS, Perkin-Elmer, NexION 350D, USA). To better mimic the environmental conditions and to eliminate artificial disturbances, we did not add any nutrients to the samples during the experiments. In addition, three replicates were set up for each treatment in all the experiments.

DNA extraction and Illumina MiSeq sequencing

The microorganisms retained on the membrane were subjected to DNA extraction, and the total genomic DNA of the samples was extracted using an OMEGA Mag-Bing Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's recommended protocol. The V4–V5 region of the 16S rRNA gene was amplified to evaluate the overall diversity of the samples, and the details are presented in the SI. The purified amplicons were sequenced on an Illumina MiSeq platform at Personal Biotechnology Co., Ltd. (Shanghai, China), and the sequence data were mainly analyzed using QIIME software (v1.8.0).[26] After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity using UCLUST,[27] and OTUs containing less than 0.001% of the total sequences (the sum of the valid sequences of all the samples included in this study) were discarded.

Quantitative PCR (qPCR)

According to our previous study, 22 ARGs have been detected in water samples collected from the Yangtze Estuary (detailed in the ESI†), and these ARGs correspond to five antibiotic classes.[23] Only six among the 22 ARGs (*sul*1, *sul*2, *tet*A, *tet*W, *erm*B and *qnr*S), which represent four antibiotic classes, were abundant in the three estuarine water samples. Therefore, these six genes were selected for further study of the impact of NPs on the variations in ARGs in estuarine water samples collected from the same study area. These six ARGs together with two MGEs (Tn*916*/*1545* and *int*I1) and two MRGs (*znt*A and *znt*B) were subsequently quantified by qPCR using an ABI real-time PCR system 7500 (ABI, USA). The primers and annealing temperatures (Tables S2†) used in the PCR and qPCR analyses and the corresponding amplification efficiencies (Tables S3†) are summarized in the ESI.† Furthermore, the procedures used for PCR and qPCR amplification and copy number calculation are detailed in the ESI.† The absolute abundances were calculated as absolute copies per milliliter, and the absolute abundances normalized to the corresponding 16S rRNA gene copies are regarded as relative abundances. All the samples were analyzed in triplicate.

Data analysis

Principal component analysis (PCA) and redundancy discriminant analysis (RDA) were conducted using the R base packages. The significance of the differences in the microbiota structure among groups was assessed by PERMANOVA (permutational multivariate analysis of variance)[28] and ANOSIM (analysis of similarities)[29,30] using the R package "vegan". A network analysis based on a Spearman correlation analysis between ARGs and the microbial community (based on OTUs) as well as MRGs and MGEs was performed using Gephi. Comparisons of gene abundances at different sampling points were performed by one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test using SPSS 19.0 software (SPSS; Chicago, IL, USA). Differences were considered significant at $P < 0.05$, and Spearman's correlation coefficients were also calculated using SPSS 19.0.

3. Results and discussion

3.1 Response of ARGs, MGEs and MRGs to ZnO NPs in estuarine water

ARGs are ubiquitous in the Yangtze Estuarine aquatic system, and six ARGs (*sul*1, *sul*2, *tet*A, *tet*W, *qnr*S, and *erm*B) have been frequently detected at abundant levels.[23] Therefore, in the present study, these six ARGs were selected for investigating the impact of ZnO NPs on the variations in the ARG profile in estuarine water. The analysis of the original water

samples revealed that the absolute abundances of these six ARGs and 16S rRNA in the SDK samples were significantly higher than those in the CY and DH samples (Fig. S4,† $P < 0.05$), and this difference could be attributed to the influence of sewage outfall near the SDK site.[31] In particular, the absolute abundance of sulfonamide resistance genes was higher than that of the other three ARGs in the original water samples, and this difference might be attributed to the fact that sulfonamides are one of the most commonly used antibiotics.[32]

Response of total ARGs to ZnO NPs in estuarine water. A significant dose–response for both the absolute and the relative abundances of total ARGs was found after natural estuarine water was dosed with different concentrations of ZnO NPs, and this finding was particularly observed with the treatments with relatively low concentrations (0.2 and 1 mg L^{-1}) of ZnO NPs (Fig. 1). The absolute abundances of total ARGs in the three water samples dosed with 0.2 and 1 mg L^{-1} ZnO NPs were 4–63- and 2–77-fold higher than those in the corresponding blanks, respectively. In addition, the relative abundances of total ARGs in all the water samples treated with 0.2 and 1 mg L^{-1} ZnO NPs were 23–70- and 24–185-fold higher than those of the blank samples, respectively. However, the dose–response of the biomass, as indicated by the numbers of 16S rRNA gene copies, was generally not significant in the estuarine water samples dosed with 0.2 and 1 mg L^{-1} ZnO NPs (Fig. S5†). The decrease in the biomass in the water samples dosed with a relatively high concentration of ZnO NPs (10 mg L^{-1}) showed significant cytotoxicity, and this finding was observed in all the samples except the SDK samples. In contrast, in the SDK samples, cytotoxicity was evident at lower NP doses (0.2 and 1 mg L^{-1}), which indicated high acute cytotoxicity in the SDK samples after exposure to ZnO NPs. This finding is consistent with previous results, which indicated that 3 h can be used for observations of acute toxicity, and the LC50 values of the ZnO NPs in the SDK, CY and DH water samples were 0.66, 1.27 and 3.66 mg L^{-1}, respectively.[33] Interestingly, although the absolute abundance of total ARGs after exposure to 10 mg L^{-1} ZnO NPs did not show a significant difference compared with that in the blanks, their relative abundances were 4–20-fold higher than those of the corresponding blanks at each exposure time. The elevated relative abundance of total ARGs in the water samples after exposure to ZnO NPs suggested that ARGs could be enriched in certain microbes, even though the biomass could be reduced by ZnO NP-induced stress, which indicated that the dissemination risk of ARGs in microbes was enhanced in estuarine waters exposed to ZnO NPs.

Interestingly, the dose–response of ARGs was significant, particularly within 3 h of exposure, and this finding might be related to the hormetic response of microbes within 3 h of exposure to ZnO NPs. Similarly, a previous study revealed that weathered quantum dots significantly induce a multidrug resistance gene, *mar*C (3.6-fold), in *Pseudomonas aeruginosa*

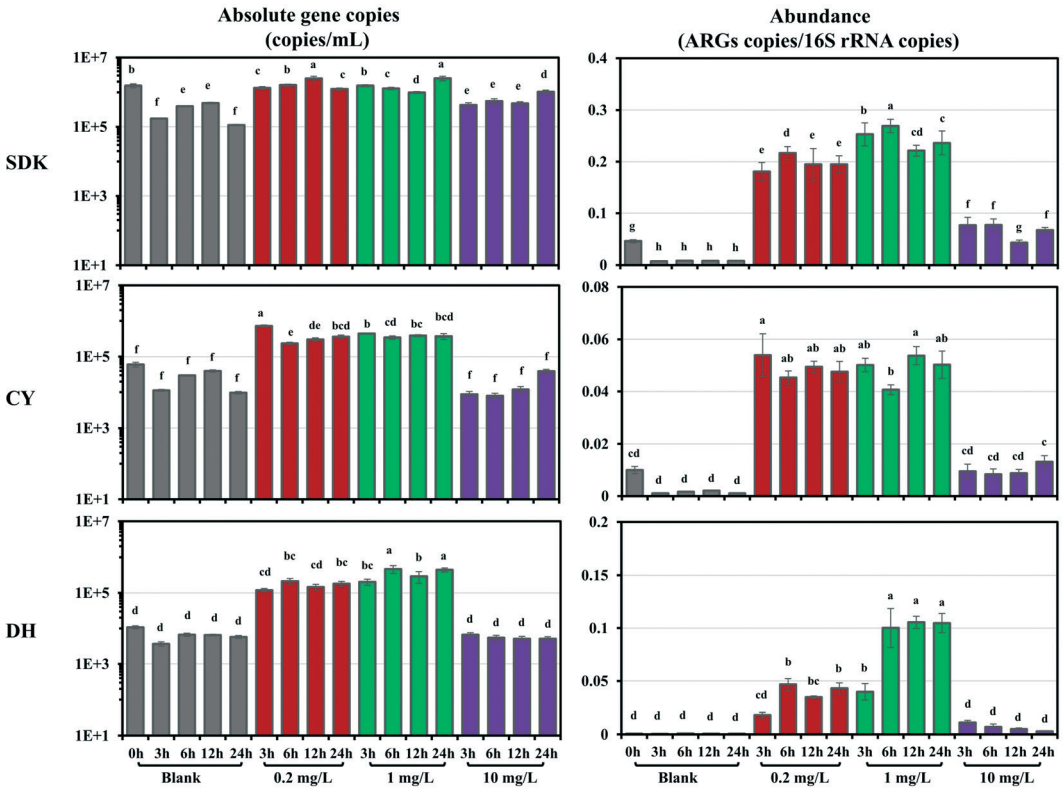

Fig. 1 Variations in the absolute gene copies and relative abundances of six detected ARGs (sum of *sul*1, *sul*2, *tet*A, *tet*W, *erm*B and *qnr*S) in different treatments during ZnO NPs exposure in SDK, CY and DH samples, respectively.

strain PAO1 within 4 h.[34] In addition, five (*sul*1, *sul*2,[35] *tet*A,[36] *erm*B,[37] and *qnr*S[38]) of the six detected ARGs are known to be plasmid-borne, and *tet*W might be located on either a plasmid or the chromosome.[39] Heavy metals reportedly drive the coselection of antibiotic resistance,[40] and plasmid DNA can transiently replicate within 1 h.[41] Thus, plasmid replication driven by ZnO NP-induced stress could also explain the significant increase in ARGs, regardless of the variation in bacterial abundance.

The time-dependent variation in both the absolute and relative abundances of total ARGs fluctuated over the 24 h period; however, ARGs showed a decreasing trend, particularly after 3 h, in the blank samples, which could be attributed to a decrease in biomass (16S rRNA gene copies). To reduce the influence of microbe self-variations over time, the relative abundance of total ARGs after exposure to ZnO for different times was normalized to the corresponding blanks (Fig. S6†). All the normalized values (NVs) were greater than 1, which indicated the enrichment of ARGs and their potentially enhanced dissemination in microbes after exposure to ZnO NPs. In addition, the NVs of ARGs showed trend consisting of an initial decrease followed by an increase in the SDK and CY samples, whereas the NVs showed an increasing trend in the DH samples exposed to 0.2 and 1 mg L^{-1} ZnO NPs, which indicated that microbes in estuarine water with relatively high salinity might be more readily enriched with ARGs than those in fresh water after exposure to ZnO NPs at environmentally relevant concentrations. Moreover, the abundances of ARGs in the DH samples treated with 1 mg L^{-1} ZnO NPs were significantly higher than those found after treatment with 0.2 mg L^{-1} ZnO NPs. As shown in Fig. S7,† the absolute values of the zeta potential decreased with increases in salinity. In addition, larger ZnO NP aggregates with lower surface charges formed the water samples with higher salinity, which could reduce the ZnO-cell interactions. However, we found that the ARGs in DH samples, which had a higher salinity, exhibited a more increasing trend, which could be attributed to the effect of salt. Similarly, previous studies reported that increased salinity levels could increase the antibiotic resistance of bacteria,[42,43] and this finding could be attributed to the salt-mediated activation of the multiple antibiotic resistance (Mar) operon, which is known to regulate the expression of a large number of genes (*e.g.*, *arc*AB efflux pump).[44]

Response of individual ARGs in estuarine water to ZnO NPs. To further explore the results, the responses of individual ARGs to exposure to different concentrations of ZnO NPs were analyzed (Fig. 2). Significant dose–responses were found for each ARG after treatment with 0.2, 1 and 10 mg L^{-1} ZnO NPs, and two dose–responses were generally observed, and these are denoted type A and type B. In the type A dose–response, the relative abundances of various ARGs, including *sul*1, *tet*A, *erm*B and *qnr*S, in all three water samples dosed with relatively low concentrations (0.2 and 1 mg L^{-1}) of ZnO NPs were significantly higher than those in the corresponding blanks, which was similar to the results found for the total ARGs. For example, the relative abundance of *sul*1, which was the most dominant ARGs, in the three water samples after exposure to 0.2 and 1 mg L^{-1} ZnO NPs was 18–103- and 25–461-fold higher, respectively, than those in the corresponding blanks, and these levels were significantly higher than those obtained after treatment with 10 mg L^{-1} ZnO NPs. In the type B dose–response, the abundance of ARGs, including *sul*2 and *tet*W, in all water samples dosed with relatively high concentrations (10 mg L^{-1}) of ZnO NPs was significantly higher than those in the corresponding blanks. For example, the abundance of *sul*2 was higher (3–15-fold higher than that in the corresponding blanks) in the samples treated with 10 mg L^{-1} ZnO NPs than in those treated with 0.2 and 1 mg L^{-1} ZnO NPs.

It has been reported that heavy metals can induce antibiotic resistance among various microbial species,[45] and a previous study confirmed that zinc ions at concentrations

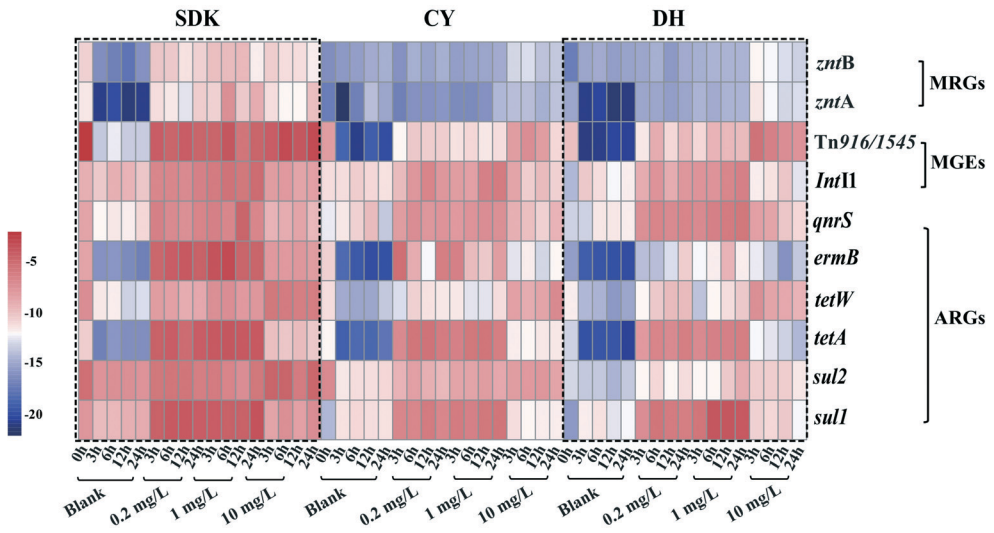

Fig. 2 Heatmap comparisons of the relative abundances of the 10 target genes (values were $\log_2$-transformed) in SDK, CY and DH samples. The redder, the higher the values, and the bluer, the lower the values.

ranging from 10.4 to 81.2 mg L^{-1} can induce tetracycline resistance in the bacterial strain LSJC7.[46] Notably, the results obtained in our studies suggested that relatively low and environmentally relevant ZnO NP levels (0.2 and 1 mg L^{-1}) in estuarine waters might be sufficient to induce bacterial ARGs, including *sul*1, *tet*A, *erm*B and *qnr*S. Moreover, the concentration of dissolved Zn^{2+} in the three estuarine waters tested in this study ranged from 0.07 to 2.6 mg L^{-1} (Fig. S8†), and these concentrations are notably lower than the concentration of Zn ions that induce LSJC7 resistance.[46] This finding might be related to the joint ecotoxicity induced by multiple pollutants in natural estuarine waters,[23] which could explain the induction of ARGs observed in our study. Additionally, the previous studies that found that Zn can promote antibiotic resistance were conducted using a single bacterial strain in culture medium with relatively high concentrations of $ZnSO_4{\cdot}7H_2O$.[47,48] Thus, these experimental parameters cannot be directly compared to those in the present study, which investigated the ARG variations in natural waters with lower nutrient levels, multiple contaminants and complex bacterial communities. Furthermore, the present study found significant positive correlations among the *sul*2 and *tet*W abundances and the Zn^{2+} concentrations in all three samples (Table 1). In addition, *sul*2 and *tet*W showed elevated abundances after exposure to relatively high concentrations of ZnO NPs, which suggested that the dissolved Zn^{2+} from ZnO NPs might be responsible for the increase in these two ARGs during exposure to ZnO NPs. Thus, the related antibiotic resistances could be induced by ZnO NPs through two possible mechanisms: (1) the dissolved Zn^{2+} can enhance the enrichment and growth of inherent ARBs in estuarine waters and (2) the ARGs in bacteria could be induced by dissolved Zn^{2+} in the aquatic environment.

The time-dependent variations in the abundance of individual ARG fluctuated over the 24 h exposure period in all of the samples with the exception of the DH samples, in which the ARGs showed an increasing trend during exposure to ZnO NPs, particularly over the first 3 h. The NVs of the individual ARGs fluctuated during the exposure period (Table S4†); for example, *sul*1 showed a trend consisting of an initial increase followed by a decrease in the three water samples during exposure to 0.2 mg L^{-1} ZnO NPs and a trend consisting of an initial decrease followed by recovery in the SDK and CY samples exposed to 1 and 10 mg L^{-1} ZnO NPs. The NVs of *tet*A and *tet*W showed an increasing trend only in the DH samples. Moreover, the highest NVs of *sul*1, *sul*2 and *tet*A were observed in the DH samples after 24 h of exposure to ZnO NPs, particularly at the relatively low concentrations (0.2 and 1 mg L^{-1}), which implied the enrichment and high disseminating potential of these ARGs in brackish water compared with fresh waters (SDK and CY waters with salinity levels of 0.15 and 1.44%). It has been reported that a relatively low salinity (approximately 5%) could induce typical ARGs (encoding efflux pump genes) and enhance the HGT of ARGs (by *int*I1) in bioelectrochemical systems.[49] The salinity of the DH samples (3.38%) might be one of the dominant factors controlling the enrichment of ARGs, particularly during exposure to environmentally relevant concentrations of ZnO NPs.

Response of MGEs and MRGs to ZnO NPs in estuarine water. Microbes can obtain ARGs *via* the HGT of MGEs to acquire resistance,[21] and thus, two MGEs (*int*I1 and Tn*916*/*1545*) were analyzed in this study. As shown in Fig. 2, *int*I1 showed a dose–response to ZnO NPs that was similar to that found for type A dose–response of ARGs, whereas the dose–response of Tn*916*/*1545* was similar to the type B dose–response of ARGs. Notably, in all the samples, the abundances of *int*I1 and Tn*916*/*1545* in all ZnO NP-treated groups were higher than those of the corresponding blanks at all tested exposure time, which indicated that the addition of ZnO NPs might be conducive to the enrichment of MGEs. The correlation coefficients between ARGs and MGEs are shown in Table 1. Significant positive correlations ($P < 0.01$) were found between *int*I1 and various ARGs, including *sul*1, *tet*A, *erm*B and *qnr*S, in all three samples, which suggested that *int*I1 might impact the dissemination of these four ARGs. This finding was consistent with those obtained in previous studies, which found that *int*I1 is related to several ARGs.[50,51] Therefore, *int*I1 is likely an indicator of the acquisition and spread of these four ARGs in bacteria in estuarine waters exposed to relatively low concentrations (0.2 and 1 mg L^{-1}) of ZnO NPs. In addition, Tn*916*/*1545*, which represents the common conjugative transposon, exhibited a significant correlation with the two other ARGs (*sul*2 and *tet*W) ($P < 0.01$), and

Table 1 Correlation coefficients among ARGs (gene copies/16S rRNA copies), MGEs (gene copies/16S rRNA copies) and dissolved Zn^{2+} (mg L^{-1}) in SDK, CY and DH samples, respectively

	SDK			CY			DH		
	*int*I1	Tn*916*/*1545*	Zn^{2+}	*int*I1	Tn*916*/*1545*	Zn^{2+}	*int*I1	Tn*916*/*1545*	Zn^{2+}
*sul*1	0.858^{b}	0.279	0.324	0.814^{b}	−0.287	0.047	0.944^{b}	0.272	0.413
*sul*2	0.265	0.772^{b}	0.625^{b}	0.252	0.797^{b}	0.500^{a}	0.502^{a}	0.770^{b}	0.736^{b}
*tet*A	0.966^{b}	0.252	0.468	0.877^{b}	0.341	0.363	0.787^{b}	0.402	0.330
*tet*W	0.348	0.806^{b}	0.843^{b}	0.267	0.831^{b}	0.684^{b}	0.037	0.890^{b}	0.771^{b}
*erm*B	0.880^{b}	0.304	0.395	0.620^{b}	0.270	0.221	0.669^{b}	0.498^{a}	0.562^{a}
*qnr*S	0.875^{b}	0.230	0.301	0.895^{b}	0.115	0.326	0.843^{b}	0.404	0.426
*znt*A	0.824^{b}	0.473	0.588^{a}	0.081	0.206	0.588^{a}	0.206	0.865^{b}	0.911^{b}
*znt*B	0.718^{b}	0.346	0.385	0.100	0.387	0.654^{b}	0.233	0.799^{b}	0.853^{b}

^{a} Significant at $P < 0.05$. ^{b} Significant at $P < 0.01$.

*tet*W has often been found to be related to the presence of Tn*916*/*1545*.[52] This finding suggested that during exposure to a relatively high concentration (10 mg L^{-1}) of ZnO NPs, Tn*916*/*1545* might have a significant impact on the variations in *sul*2 and *tet*W in estuarine waters. Furthermore, as shown in Table S5,† both MGEs were positively correlated with Zn^{2+} ($P < 0.05$) in the SDK samples, whereas only Tn*916*/*1545* showed a positive correlation with the Zn ion concentrations in the CY ($P < 0.05$) and DH ($P < 0.01$) samples. Haritha *et al.* reported that MGEs can be induced by 16.25 mg L^{-1} Zn^{2+},[53] and this concentration is substantially higher than the dissolved Zn^{2+} concentrations in the three estuarine water samples used in this study. In addition, the present study showed that Tn*916*/*1545* could be induced by the dissolved Zn^{2+} released from ZnO NPs during exposure to relatively high doses, and consequently promoted the dissemination of *sul*2 and *tet*W. The increased abundance of MGEs observed after exposure to ZnO NPs together with the significant correlations among ARGs, MGEs and Zn^{2+} found in the present study indicate the high potential of ARGs dissemination *via* HGT in estuarine waters during exposure to ZnO NPs.

The presence of zinc levels that surpass the cellular demand will activate the expression of the zinc efflux genes (*e.g.*, *znt*A and *znt*B).[54] In the three water samples investigated in the present study, the abundances of *znt*A and *znt*B after treatment with ZnO NPs were higher than those in the corresponding blanks and showed a similar dose–response to the type B dose–response found for ARGs (Fig. 2). The NVs of *znt*A showed a time-dependent trend (initial inhibition followed by recovery) that was similar to that of the ARGs, but the trend found for *znt*B consisted of an initial increase followed by a decrease (Table S4†). Furthermore, *znt*A and *znt*B showed significant positive correlations with Zn^{2+} (Table 1), which indicated that *znt*A and *znt*B were sensitive to the Zn^{2+} concentrations during exposure to ZnO NPs. It has been reported that selection by zinc could lead to the coselection of ARGs.[23] The abundances of *znt*A and *znt*B were generally positively correlated with ARGs in the three estuarine waters tested in the present study, particularly in the SDK samples (Table S6†). These results confirmed that ZnO NPs can exert selective pressures on microbes and increase antibiotic resistance in estuarine water samples.

3.2 Different taxa-level responses of the microbial community in estuarine water to ZnO NPs

The effect of ZnO NPs on the microbial community in estuarine water was analyzed through Illumina MiSeq sequencing. Table S7† summarizes the richness (ACE and Chao1) and diversity indices (Simpson and Shannon) of the microbial community in each sample, and the results suggested that the presence of ZnO NPs affected the microbial diversity and richness. According to the PCA analysis, the microbial communities in the three estuarine samples were scattered by exposure to different ZnO NP concentrations for different times (Fig. S9†). Thus, the microbial community structure was influenced by the addition of ZnO NPs, which coincided with the results from a previous study that demonstrated that the diversity and richness of the microbial community in an integrated OCO reactor changes during exposure to ZnO NPs.[55]

Phylum-level variations in the microbial community in estuarine water after treatment with ZnO NPs. A phylum-level analysis identified 26, 32 and 40 phyla in the SDK, CY and DH samples, respectively, and the dominant four phyla were *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes*, which accounted for 82.3–99.7% of the total composition of the microbial community in the water collected from the Yangtze Estuary (Fig. S10†). This result was in agreement with previous studies that found that these four phyla were usually dominant in aquatic microbial communities.[56–58]

The relative abundances of the four dominant phyla fluctuated during exposure to different concentrations of ZnO NPs (Fig. S10†). However, we considered that relative abundance data cannot provide direct information on the growth or decline in the profiles of microbes at different taxa levels during exposure to different ZnO NP doses. Therefore, we introduced the absolute abundance metric, which was calculated by multiplying the relative abundance of each microbe with the corresponding 16S rRNA level in each treatment sample. As shown in Fig. S10,† a significant dose–response was found for the four dominant phyla in estuarine waters during exposure to ZnO NPs at different concentrations. The absolute abundance of *Proteobacteria*, the most dominant phylum, generally decreased after the addition of ZnO NPs compared with its abundance in the corresponding blanks. In addition, the absolute abundances of *Proteobacteria* in the CY and DH samples treated with a high concentration (10 mg L^{-1}) of ZnO NPs were relatively low, whereas the absolute abundance of *Proteobacteria* in the SDK samples after exposure to this high concentrations of NPs was higher than those found after exposure to the low concentrations (0.2 and 1 mg L^{-1}) of ZnO NPs. Liu *et al.* also found a slight decrease in *Proteobacteria* in activated sludge after exposure to high ZnO NP concentrations (0.25–4 mg L^{-1}).[55] The *Firmicutes* was sensitive to ZnO NP exposure, as demonstrated by decreases in both their absolute and relative abundances in the samples after treatment with ZnO NPs, and similar results were observed in the gut microflora of worms (*Eisenia fetida*)[59] and the microbial community in an OCO reactor.[55] Additionally, the presence of ZnO likely causes an increase in the relative abundance of *Bacteroidetes*,[60] and a similar result was also observed in this study. As a known host for ARGs,[61,62] *Actinobacteria* are likely responsible for carrying and disseminating ARGs.[51,63] Notably, the relative abundances of *Actinobacteria* in the SDK samples after treatment with 0.2 and 1 mg L^{-1} ZnO NPs was 1.4–3.1- and 1.9–5.4-fold higher than those of the corresponding blanks, which is in agreement with the increasing trend obtained for the total ARG abundance during exposure to relatively low ZnO NP concentrations.

Interestingly, during the 24 h exposure period to 10 mg L^{-1} ZnO NPs, *Proteobacteria* showed a trend consisting of an

initial inhibition followed by a rebound at 24 h, and this phenomenon was particularly evident in the DH samples with high salinity. The absolute abundance of *Proteobacteria* in the DH samples during exposure to 10 mg L^{-1} NPs decreased sharply during the first 3 h and increased gradually throughout the rest of the exposure period. The absolute abundance of each phylum at each time point after each treatment was normalized to that of the corresponding blanks to eliminate microbe variations with exposure time. As shown in Fig. S11,† the NVs for all four phyla generally showed an evident time-dependent variation, *i.e.*, an initial inhibition followed by a rebound, and the most significant rebound was observed in the SDK samples after exposure to high ZnO NP doses. An exception was found for *Firmicutes* in the DH samples: although exposure to 0.2 mg L^{-1} ZnO NPs resulted in the same inhibition-rebound trend, all *Firmicutes* species were inhibited by exposure to higher NP concentrations (1 and 10 mg L^{-1}), which indicated that these microbes were sensitive to ZnO NPs in relatively brackish water.

Genus-level variations in the microbial community in estuarine water after treatment with ZnO NPs. The relative abundances of the 50 most abundant genera in each sample showed a significant dose–response during exposure to different concentrations of ZnO NPs, and the genera in the ZnO NP-treated groups that are marked in red in Fig. S12† represent those microbes that survived after exposure to relatively high abundances of ZnO NPs. In general, the absolute abundances of most genera in the SDK, CY and DH samples tended to decrease after treatment with different concentrations of ZnO NPs compared with that found in the corresponding blanks at 0 h, and this trend was more evident in the CY and DH samples treated with 10 mg L^{-1} ZnO NPs (Fig. 3). This finding could be attributed to the decrease in total biomass (16S rRNA gene copies). A Venn diagram was drawn based on the 50 most abundant genera to determine the similarities and differences among the three estuarine samples. As shown in Fig. S13,† among the 50 most abundant genera, 14 genera were found in all three samples, which could be attributed to the ubiquitous nature of these 14 genera due to interactions among these three sampling sites caused by hydrodynamics, such as tides.[31,64]

Most microbes belonging to the 14 genera found in all three samples showed a clear dose–response to ZnO NPs (Fig. 3). For example, compared with the values at 0 h, the absolute abundance of *Nocardioides* in the CY and DH samples increased after the addition of low concentrations (0.2 and 1 mg L^{-1}) of ZnO NPs, whereas a decreasing trend was observed in the SDK samples, and these values were higher than those obtained in the corresponding blanks. *Streptomyces* is the largest genus belonging to *Actinobacteria* and is an important antibiotic resistance genus.[65] The increase in the absolute abundances of *Streptomyces* in the three estuarine waters after exposure to relatively low doses (0.2 and 1 mg L^{-1}) of ZnO NPs compared with those in the corresponding blanks at 0 h likely contributes to the increase in ARGs. Furthermore, the absolute abundance of *Microbacterium* showed an increasing trend after exposure to elevated ZnO NP concentrations, which suggested that these microbes exhibit relatively high resistance to ZnO NPs, and this phenomenon was particularly true in the SDK and DH samples. The relatively high resistance of *Microbacterium* to ZnO NPs might be due to the following three facts: (1) *Microbacterium* species are reportedly capable of solubilizing ZnO,[66] which could result in reduced toxicity after direct contact with ZnO NPs; (2) *Microbacterium* species are able to synthesize ZnO NPs[67] and can thus exhibit resistance to ZnO NP exposure by maintaining zinc homeostasis; and (3) biosurfactants produced by *Microbacterium*[68] could protect the cells by functioning as a physical barrier and removing zinc.

As shown in Fig. S12† and 3, either the relative abundance or the absolute abundance of the top 50 genera showed variations depending on the ZnO NP exposure time in the three estuarine water samples. The NVs for the top 50 genera in all the samples generally showed a time-dependent variation (*i.e.*, a trend consisting of an initial inhibition followed by a rebound) that was similar to that found at the phylum level. For example, the NVs of *Nocardioides* in the three estuarine water samples showed a trend consisting of an initial inhibition followed by a rebound from 3 to 24 h after exposure to lower doses (0.2 and 1 mg L^{-1}) of ZnO NPs. These values gradually increased during exposure to high doses of ZnO NPs, particularly in the SDK samples, which had an NV value up to 3.7, and this finding indicated the acclimatization of the samples to ZnO NPs (Table S8†). In general, the microbial community in the estuarine water samples, particularly the SDK samples, showed robust performance during exposure to ZnO NPs. In fact, 17, 23 and 33 out of 50 genera with NVs $>$ 1 were found in the SDK samples after 24 h of exposure to 0.2, 1 and 10 mg L^{-1} ZnO NPs, respectively, which indicated that an increasing number of microbes in this sample acclimated to ZnO NP-induced stress with increases in the ZnO NP doses. In contrast, 24 h after exposure to 0.2, 1 and 10 mg L^{-1} ZnO NPs, 26 and 36 out of 50 genera showed NVs $<$ 1 in the CY samples, and 30, 39 and 40 genera had NVs $<$ 1 in the DH samples, respectively. These findings indicated that higher ZnO NP doses exhibited higher cytotoxicity, particularly in the samples with relatively high salinity. In addition, the microbial community in the DH samples was more vigorously disrupted by exposure to ZnO NPs than the two other freshwater samples, which may be due to synergistic cytotoxicity with higher salinity in DH water.[69]

3.3 Relationships between ARGs and microbial communities

To investigate the key factors that control the variation in ARGs during exposure to ZnO NPs at different concentrations, a RDA was conducted based on the ARGs, MRGs, MGE abundances, dissolved Zn^{2+} concentrations and the microbial community (relative abundance >1%). The results showed that the selected variables contributed 69%, 56% and 74% to the total variations in the ARG and MRG profiles in the SDK, CY and DH samples during exposure to ZnO NPs, respectively

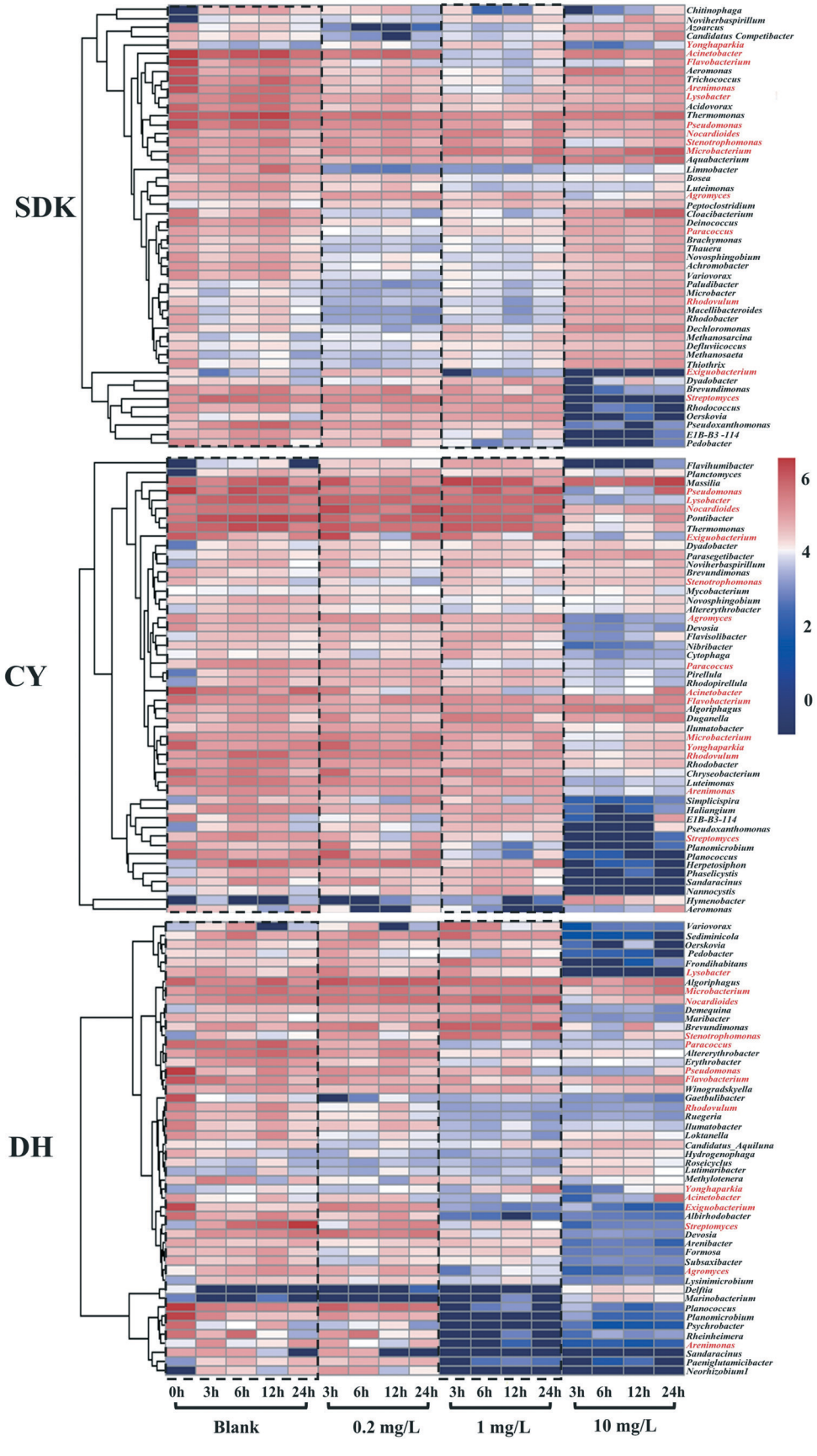

Fig. 3 Heatmap of absolute abundances of top 50 genera (values were $\log_{10}$-transformed) showing the evolution of the microbial community dosed with different concentrations of ZnO NPs at different exposure time. The redder, the higher the values, and the bluer, the lower the values. The genera marked in red are the common 14 genera in the three estuarine waters.

(Fig. 4). Among the selected variables, six phyla explained most of the variation, *i.e.*, 31.9%, 67.6% and 60.7% in the SDK, CY and DH samples, respectively, whereas MGEs explained 9.7%, 17.7% and 5.9% of the total variation, respectively, and the dissolved Zn^{2+} concentration accounted for 0.3%, 12.2% and 2.4% of the total variation, respectively. The enhanced abundance of *Actinobacteria* obtained after exposure to relatively low doses (0.2 and 1 mg L^{-1}) of ZnO NPs was most related to the increases in *sul*1, *tet*A, *erm*B and *qnr*S in the SDK water samples, whereas the *Planctomycetes* abundance was most positively related to the increase in these four ARGs in the CY and DH samples. Furthermore, the increase in *sul*2 and *tet*W after 10 mg L^{-1} ZnO NP treatment was most significantly correlated with microbes belonging to *Chloroflexi* in the SDK and DH samples, and *Proteobacteria* was related to the variations in *sul*2 and *tet*W in the CY samples. Recent studies have also revealed that *Proteobacteria* are involved in the dissemination of ARGs[70] and that *Planctomycetes* and *Chloroflexi* are significantly correlated with genes conferring resistance to sulfonamide, tetracycline and chloramphenicol in microbial communities in organically produced lettuce.[71] The results of our RDA analysis demonstrated that the main driver contributing to the changes in ARGs was the evolution of the microbial community induced by the addition of ZnO NPs. In addition, Tn*916*/*1545* together with the dissolved Zn^{2+} concentration could be responsible for the elevated concentrations of *tet*W and *sul*2 in estuarine water samples after exposure to 10 g mL^{-1} ZnO NPs, and this finding was particularly true in the SDK and CY samples. In contrast, *int*I1 was more related to the increase in the other four ARGs, including *sul*1, *tet*A, *qnr*S and *erm*B, after exposure to lower concentrations (0.2 and 1 mg L^{-1}) of ZnO NPs. This finding was consistent with the results discussed in the "Response of ARGs, MGEs and MRGs to ZnO NPs in estuarine water" section. Therefore, ZnO NPs might impact the ARG profiles mainly by affecting the abundance of potential host bacteria and enhancing the potential of ARG dissemination among microbial communities *via* HGT in estuarine water. A recent related study based on a single bacterium in culture revealed that nanoalumina can induce the conjugative transfer of the RP4 plasmid from *E. coli* to *Salmonella* spp. in PBS media, and this result was

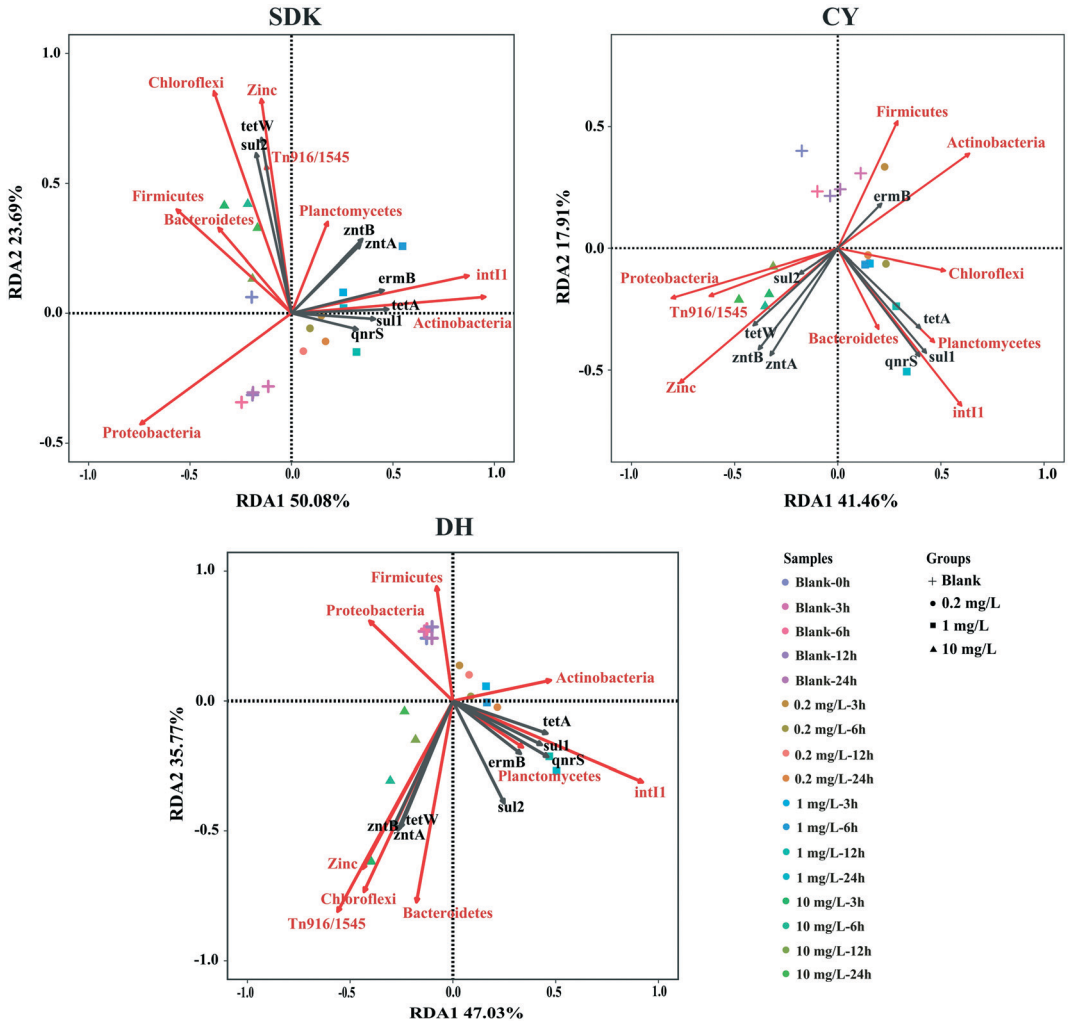

Fig. 4 RDA analysis of ARGs, MGEs, MRGs and microbial communities in SDK, CY and DH samples, respectively.

likely mainly due to the damage to bacterial membranes caused by oxidative stresses, an enhancement of the expression of conjugative genes and the repression of global regulatory factor genes involved in RP4 plasmid conjugation.[16]

A network analysis was conducted to obtain ARGs and coexisting bacterial genera in the SDK, CY and DH samples.[71,72] As shown in Fig. 5, tetracycline resistance genes (*tet*A and *tet*W) had the most potential hosts. Tetracycline resistance genes are reportedly present in *Streptomyces*,[73] and *Streptomyces* species were found to be negatively related to *tet*W in the SDK samples, which might have been due to the inhibition of these bacteria by ZnO NPs. Interestingly, *tet*A, which encodes tetracycline efflux proteins, is harbored by *Aeromonas*,[74] but the genus *Aeromonas* was found to be negatively related to *tet*A in the present study. A possible reason for this finding could be that the abundance of *Aeromonas* decreased during exposure to ZnO NPs, and the variation in *tet*A might be attributed to other identified hosts (*e.g.*, *Nocardioides*). Notably, the co-occurrence of MGEs and ARGs indicates a high dissemination potential of ARGs *via* HGT among microbial communities. Most of these potential hosts, such as *Nocardioides*, belong to the phylum *Actinobacteria*, which suggested that *Actinobacteria* played an important role in the variations in the ARG profile and the dissemination of ARGs during ZnO NP exposure. A recent study found that *Nocardioides* exhibits a significant positive correlation with sulfonamide and tetracycline resistance genes,[75] which suggests a potential risk of multiantibiotic-resistant *Nocardioides*. In the current study, *Nocardioides* was identified as a potential host for an MGE (*intI1*) and three ARGs (*tet*A, *erm*B and *qnr*S) and was distributed in all three water samples. In addition, as described in the previous section, the *Nocardioides* abundances were enhanced after exposure to ZnO NP at relatively low concentrations. Therefore, all the results indicate a severe

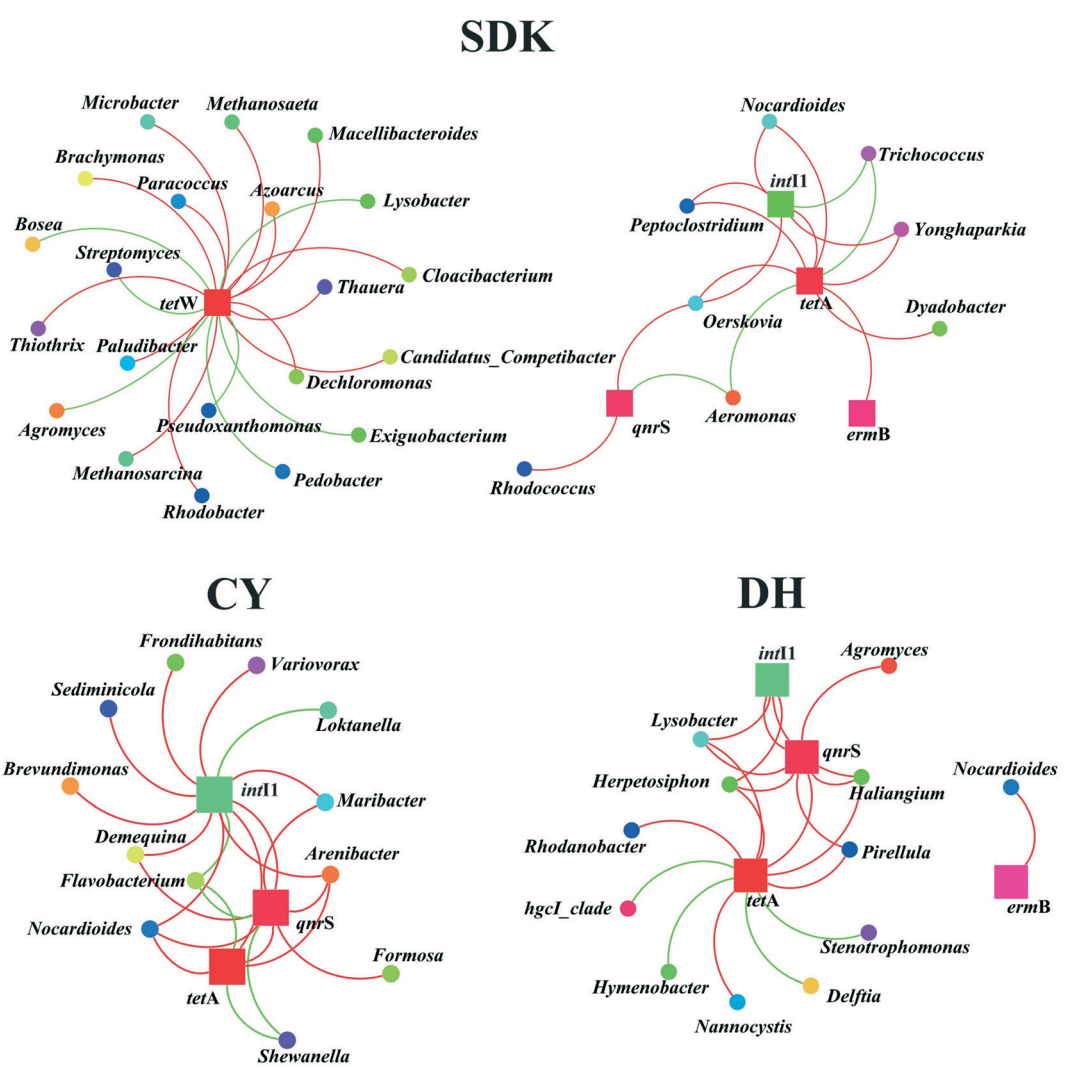

Fig. 5 Network analysis based on spearman correlation analysis of ARGs and their potential host bacteria in SDK, CY and DH samples, respectively. The connection between two nodes represents a significant positive correlation ($p < 0.01$), the red color means positive correlation and green color means negative correlation (for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

ARG risk related to the promotion of bacteria with multiantibiotic resistance potential induced by the release of ZnO NPs into the natural environment. Furthermore, potential host bacteria for *sul*1 and *sul*2 were not found in the present study, but these two ARGs were highly abundant. As described previously, the significant correlation between *sul*1 and *sul*2 and MGEs indicated the high mobility of these ARGs *via* HGT.[76] Therefore, in the present study, we concluded that although the changes in ARGs mainly resulted from the evolution of the microbial community driven by ZnO NPs, a high risk of ARG dissemination through HGT *via* MGEs could also be induced by ZnO NPs in estuarine waters. In addition, a previous study revealed that salinity impacts the potential hosts of ARGs in wastewater,[49] and the shifts in the potential hosts of ARGs in the DH samples observed in this study could also be driven by salinity. Some moderately halotolerant bacteria were identified in the DH samples, such as *Haliangium* species, which are known as extremophilic microbes, and these bacteria reportedly survive under many extreme conditions.[49] As a potential host for MGE (*int*I1) and ARGs (*qnr*S and *tet*A), *Haliangium* would highly enhance the risk of ARGs dissemination in the DH samples.

4. Conclusions

The ubiquitous distribution of NPs in the environment has caused scientific and public concerns, and the interaction between NPs and other environmental contaminants, such as ARGs/ARBs in the aquatic environment, is not fully understood. To the best of our knowledge, the present study constitutes the first effort to characterize the responses of ARGs and microbial communities in a complex estuarine environment to exposure to ZnO NPs at different concentrations. We found that ZnO NPs exert selective pressure and thereby induce the enrichment and dissemination of ARGs in estuarine water, and this finding was particularly observed with relatively low ZnO NP concentrations. Moreover, the ARG abundances in the DH water samples normalized to those of the corresponding blanks tended to increase after exposure to low ZnO NP doses, which indicated that microbes in estuarine water with relatively high salinity are more readily enriched in and more easily disseminate ARGs than those in fresh water during exposure to ZnO NPs at environmentally relevant concentrations. A correlation analysis showed that MGEs induced by ZnO NPs were significantly correlated with the abundance of ARGs during exposure to ZnO NPs, which indicated the high potential of ARG dissemination *via* HGT in estuarine waters during exposure to ZnO NPs.

According to the RDA analysis performed in the present study, the bacterial community can be considered the main driver for the changes in ARGs, followed by MGEs and dissolved Zn^{2+}. *Actinobacteria* and *Planctomycetes* are likely largely responsible for the enhanced abundances of *sul*1, *tet*A, *erm*B and *qnr*S in estuarine water samples observed after exposure to low doses (0.2 and 1 mg L^{-1}) of ZnO NPs. A network analysis determined the potential host bacteria for various ARGs. Although microbes were generally inhibited after exposure to ZnO NPs, dose- and time-dependent variations were detected. For example, the absolute abundances of bacteria, such as *Nocardioides*, which belong to *Actinobacteria*, normalized to that in the corresponding blanks showed a trend consisting of an initial inhibition following by a rebound, particularly after exposure to low ZnO NP doses. Furthermore, *Nocardioides* species were identified as hosts for multiple ARGs and MGEs. Therefore, the severe risk of ARGs dissemination might be related to the promotion of these type of bacteria induced by the release of ZnO NPs in the natural environment and the dissemination of multiple ARGs to different ecological niches. Furthermore, in the present study, we investigated the impact of ZnO NPs on the variations in ARG profiles in the estuarine aquatic environment and attempted to elucidate the mechanisms underlying the selection of ARGs by analyzing the relationship among the variations in ARGs, MGEs and the microbial community throughout the NP exposure period. However, due to the complexity of the natural aquatic environment, which involves various chemical and bacterial compositions, it is difficult to accurately elucidate the regulatory mechanism that controls the propagation of ARGs in a natural environment. Further studies in which single/multiple environmental factors of microbial communities are controlled are required to delineate the mechanism underlying the impact of NPs on ARGs and the microbial community.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgements

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