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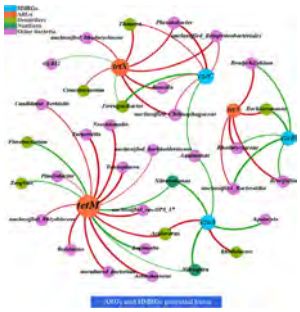
Evaluating responses of nitrification and denitrification to the co-selective pressure of divalent zinc and tetracycline based on resistance genes changes

Zichao Wang[a,*], Shengyu Yuan[a], Zhiwei Deng[b], Yuejing Wang[c], Shilong Deng[d], Youtao Song[a], Congting Sun[a], Naishun Bu[a], Xinruo Wang[e]

[a] College of Environment Science, Liaoning University, Shenyang, China
[b] College of Environment and Chemical Engineering, Dalian University, Dalian, China
[c] Shandong Provincial Yantai Eco-Environment Monitoring Center, Yantai, China
[d] College of Forestry, Northeast Forestry University, Harbin, China
[e] Liaoning Provincial Ecology and Environment Protection Science and Technology Center, Shenyang, China

GRAPHICAL ABSTRACT



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ABSTRACT

The responses of nitrification and denitrification to the divalent zinc (Zn(II)) and tetracycline (TC) co-selective pressure were evaluated in a sequencing batch reactor (SBR). The removal rates of organics and nitrogen, nitrifying and denitrifying enzymatic activity, and microbial diversity and richness at the Zn(II) and TC co-selective pressure were higher than those at the alone Zn(II) selective pressure, while were lower than those at the individual TC selective pressure. The Zn(II) and TC co-selective pressure induced the TC resistance genes abundance increase and the Zn(II) resistance genes levels decrease, and enhanced bacterial enzymatic modification resistance to TC and bacterial outer membrane resistance to Zn(II). The network analysis showed that the genera *Nitrospira* and *Nitrosomonas* of nitrifiers and the genera *Ferruginibacter, Dechloromonas, Acidovorax, Rhodobacter, Thauera, Cloacibacterium, Zoogloea* and *Flavobacterium* of denitrifiers were the potential hosts of antibiotics resistance genes (ARGs) and/or heavy metals resistance genes (HMRGs).

1. Introduction

Tetracycline (TC) and divalent zinc (Zn(II)) are widely applied in livestock breeding, human medicine, and other agri-industrial production, resulting in their frequent detection in biological wastewaters treatment systems. Investigations show that in different wastewater treatment systems, the concentrations of TC and Zn(II) in wastewaters could be as high as hundreds of mg L^{-1} and dozens of mg L^{-1},

* Corresponding author at: College of Environment Science, Liaoning University, No. 66 Chongshan Central Road, Shenyang, Liaoning Province 110036, China.
E-mail address: wangzichao10@sina.com (Z. Wang).

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respectively (Du et al., 2018; Li et al., 2018). TC and Zn(II) could inhibit the metabolism and growth of bacteria, which is harmful to the stability of biological wastewater treatment performance (Abdelrahman et al., 2018; Zhang et al., 2018). Nevertheless, some bacteria still keep normal metabolic activity at the selective pressure of TC or Zn(II), showing the bacterial tolerance to TC or Zn(II). This tolerance is associated with the formation of a class of coding genes that promote bacteria to form a defense mechanism against toxic substances, and these coding genes related to the antibiotics tolerance and the heavy metals tolerance are named antibiotics resistance genes (ARGs) and heavy metals resistance genes (HMRGs), respectively (Liu et al., 2019; Qian et al., 2017). These indicated that the pollutant removal in biological wastewaters treatment systems could be disturbed by the ARGs and HMRGs changes as their changes could affect bacterial metabolic activity.

Additionally, antibiotics could bind heavy metals through the interaction of electron-donor functional groups in antibiotics with heavy metal ions, which resulted in a difference in inhibiting microbial metabolism between the joint effects of antibiotics and heavy metals and the effect sum of antibiotics and heavy metals alone (Wang et al., 2018a; Tong et al., 2015). These suggested that the changes of defense mechanism of bacteria (e.g. ARGs and HMRGs) at the antibiotics and heavy metals co-selective pressure might be different from the superposition of their changes at the alone stress of antibiotics and heavy metals. Therefore, it is necessary to explore the resistance genes changes at the co-selective stress of antibiotics and heavy metals to understand the defense mechanism of bacteria against mixed antibiotics and heavy metals and the pollutant removal performance in biological wastewaters treatment systems. Zhang et al. (2020a) reported the response of partial nitrification to the combined stress of metal-oxide nanoparticles and antibiotics on microbial activity, community, ARGs and HMRGs, and found that the CuO nanoparticles and sulfamethoxazole combination caused the inhibition of partial nitrification process, the decrease of ammonia oxidizing bacteria (AOB) abundance and the improvement of ARGs (*copA* and *cusA*) and HMRGs (*sul1* and *sul2*) expression. Fan et al. (2020) illustrated the effects of ARGs on the denitrification process in an upflow anaerobic sludge blanket at the single stress of TC, and found that the inhibition of TC resistance genes (*tetG*) on the denitrification performance was achieved by affecting the conversion of nitrate to nitrite. Zhang et al. (2019a) investigated the impacts of cadmium on the partial nitrification performance, and found that the decrease of AOB and nitrite oxidizing bacteria (NOB) levels and the increase of microbial population harboring cadmium resistance genes (*merA*) caused the partial nitrification failure. Some researchers have reported the relations between nutrient removal, microbial community and resistance genes, nevertheless most studies were in progress at the single stress of antibiotics or heavy metals. No information has been carried out to explore the reasons of changes in nitrifying and denitrifying performance, enzymatic activity and microbial community in a biological wastewaters treatment system at the co-selective pressure of heavy metals and antibiotics from the perspective of resistance genes.

This study aimed to explore the changes of the nitrogen removal, nitrifying and denitrifying enzymatic activities, microbial community and the ARGs and HMRGs abundance in a SBR at the Zn(II) and TC co-selective pressure, to determine the links between ARGs and HMRGs and their potential hosts, and to investigate the effect mechanisms of ARGs and HMRGs on the nitrogen removal in a SBR in terms of the relations of the hosts from nitrifiers and denitrifiers with enzymatic activities.

2. Materials and methods

2.1. Reactor and wastewater

Three lab-scale plexiglass SBRs (R1, R2 and R3) with the same size and operation modes were operated in the study, and their size were illustrated in our previous study (Wang et al., 2018a). In this study, a cycle operation of SBR contained influent addition (0.05 h), aerobic stage (3.6 h), anoxic stage (1.8 h), settling (0.5 h) and effluent withdrawal (0.05 h), and it was operated 4 cycles in a day. The wastewaters in the aerobic and anoxic stages were mixed by a stirrer, and air was introduced by the air diffusers at the SBR bottom during the aerobic stage. The dissolved oxygen content in the aerobic and anoxic stages was above 2.0 mg L^{-1} and below 0.5 mg L^{-1}, respectively. The seeding sludge was obtained from a municipal wastewater treatment plant aerobic tank (Dalian City, China). The initial mixed liquor suspended sludge (MLSS) from R1, R2 and R3 was 3280, 3110 and 3440 mg L^{-1}, respectively. The synthetic influent composition was as below (mg L^{-1}): CH_3COONa, 530; NH_4Cl, 152; KH_2PO_4, 44; $ZnSO_4{\cdot}7H_2O$, 132 (corresponding to 30 mg L^{-1} Zn(II)) and/or TC, 30. The three reactors of R1 (the influent Zn(II) concentration: 30 mg L^{-1}), R2 (the influent TC concentration: 30 mg L^{-1}) and R3 (the influent Zn(II) concentration: 30 mg L^{-1}; the influent TC concentration: 30 mg L^{-1}) were operated steadily for 60 days under the above conditions. The activated sludge samples from R1, R2 and R3 and the seeding sludge sample were used to determine enzymatic activities, microbial community and resistance genes. The volume of activated sludge samples from R1, R2 and R3 taking on day 46, 53 and 60 was the same, and then every sample was divided into two equal volume parts. One part sample was used to determine enzymatic activities on day 46, 53 and 60, and the mean values of these test values above were calculated and used as the representative values for the analysis of enzymatic activities. The other part samples with the same volume taking on day 46, 53 and 60 were mixed to assess to the changes of microbial community and resistance genes.

2.2. Analytical methods

The standard methods were used to measure ammonia nitrogen (NH_4^+-N), nitrite nitrogen (NO_2^--N), nitrate nitrogen (NO_3^--N), chemical oxygen demand (COD) and MLSS (Chinese N.E.P.A., 2002). The ammonia monooxygenase (AMO), nitrate reductase (NAR), nitrite reductase (NIR) and nitrite oxidoreductase (NOR) activities were determined in accordance with the description of Deng et al. (2019). The DNA extraction of activated sludge samples were directly carried out with the PowerSoil DNA Isolation Kit according to Huang et al. (2020), and then they were sent to the Personalbio Company (Shanghai, China) for the measurements of ARGs, HMRGs, mobile genetic elements genes and microbial community. Microbial community and the gene abundance of ARGs, HMRGs and mobile genetic elements were determined by high-throughput sequencing (Di et al., 2020) and by quantitative polymerase chain reaction (Chen et al., 2020), respectively. The primers for target ARGs (*tetA*, *tetM* and *tetX*), HMRGs (*czcA*, *czcB* and *czcC*) and mobile genetic elements (Class I integron (*intI1*)) genes were showed in Supporting Information (E-Supplementary data for this work can be found in e-version of this paper online). Redundancy analysis (RDA) and network analysis were carried out by Canoco 5.0 and by Gephi 0.9 to analyze the potential links between ARGs and HMRGs and to determine the potential hosts of ARGs and HMRGs, respectively (Bastian et al., 2009; Chen et al., 2020).

3. Results and discussion

3.1. COD and nitrogen removal

Fig. 1 shows the changes of COD and nitrogen removal at the selective pressure of Zn(II) and/or TC. The influent comprised COD 400 mg L^{-1} and NH_4^+-N 40 mg L^{-1}, and did not comprise NO_2^--N and NO_3^--N. The average COD removal rates in R1 (the influent contained 30 mg L^{-1} Zn(II)), R2 (the influent contained 30 mg L^{-1} TC) and R3 (the influent contained 30 mg L^{-1} Zn(II) and 30 mg L^{-1} TC) were 62.63%, 78.33% and 72.92%, respectively, and the average NH_4^+-N

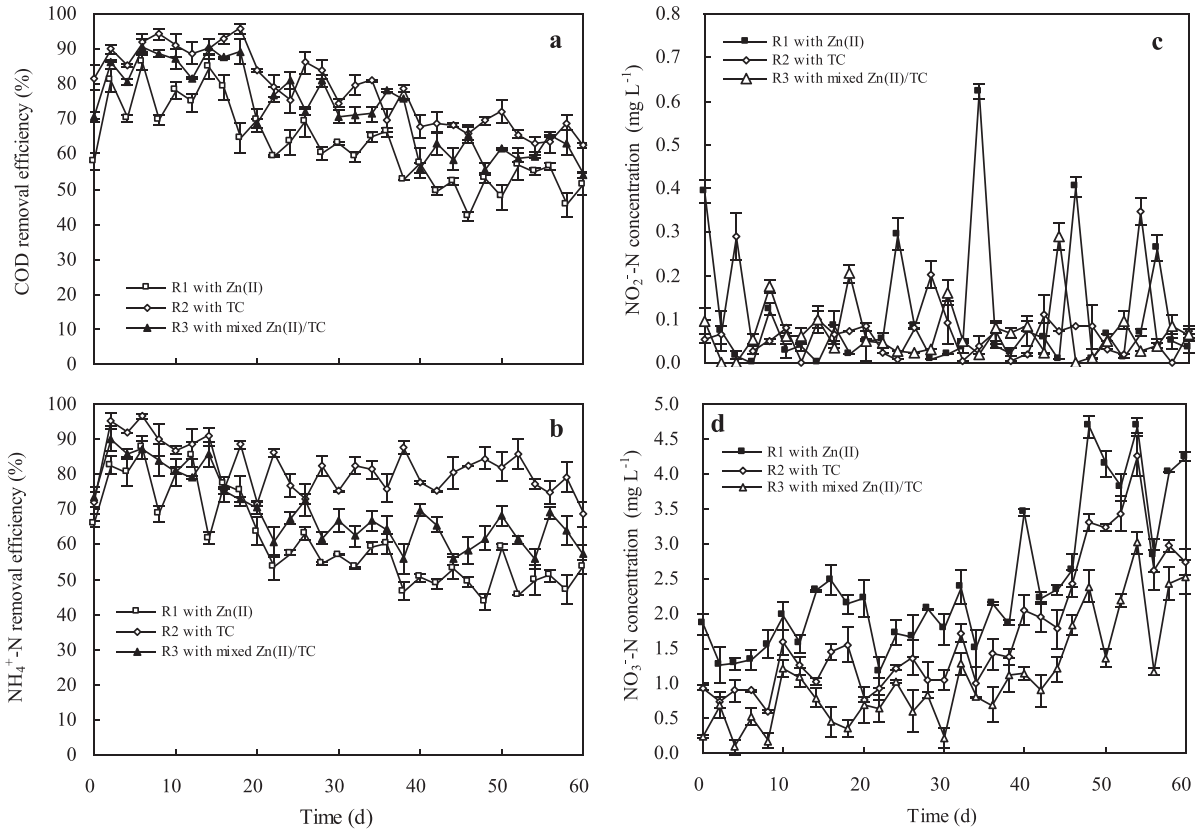

Fig. 1. Combined effects of Zn(II) and TC on the performance of SBR. (a) COD, (b) NH_4^+-N, (c) NO_2^--N and (d) NO_3^--N.

removal efficiencies in R1, R2 and R3 were 60.83%, 81.84% and 69.40%, respectively. In R1, R2 and R3, the average removal rates of COD and NH_4^+-N in R2 were the highest, and they in R1 were the lowest. The changes were connected with the antagonistic effects of Zn (II) and TC on the COD and NH_4^+-N removal due to the complexation of antibiotics with heavy metal ions (Wang et al., 2018a). The average effluent NO_3^--N concentrations in R1, R2 and R3 were 2.43, 1.73 and 1.09 mg L^{-1}, respectively. The average effluent NO_3^--N concentration in R3 at the mixed Zn(II)/TC stress was lower than those in R1 and R2, suggesting that the joint impacts of Zn(II) and TC on the NO_3^--N removal were antagonistic. The significant NO_2^--N accumulation in the effluent from R1, R2 and R3 was not observed.

3.2. Enzymatic activity

Fig. 2 shows the changes of enzymatic activities involved into nitrification and denitrification in activated sludge at the Zn(II) and/or TC selective pressure. The changes in the activities of AMO, NOR, NAR and NIR reflected the variations in the activities of AOB, NOB, nitrate reducing bacteria and nitrite reducing bacteria, respectively. The activities of AMO, NOR, NAR and NIR in seeding sludge without Zn(II) and TC were 0.50, 1.38, 0.98 and 6.48 μg N mg^{-1} protein min^{-1}, respectively, and they in R1 containing 30 mg L^{-1} Zn(II) in the influent decreased to 0.20, 0.51, 0.48 and 2.87 μg N mg^{-1} protein min^{-1}, respectively. Their decrease in R1 could be related to the certain enzymes composition and structure damage and the functional genes abundance decrease at the selective pressure of Zn(II) (Xu et al., 2019). Compared to the AMO, NOR, NAR and NIR activities in seeding sludge, they in R1 decreased by 60.20%, 63.04%, 51.02% and 55.74%, respectively, indicating that denitrifiers had a better resistance to Zn(II) than nitrifiers, and NOB was more easily inhibited by Zn(II) ions than AOB. Previous studies reported similar results that autotrophic nitrifiers had a lower

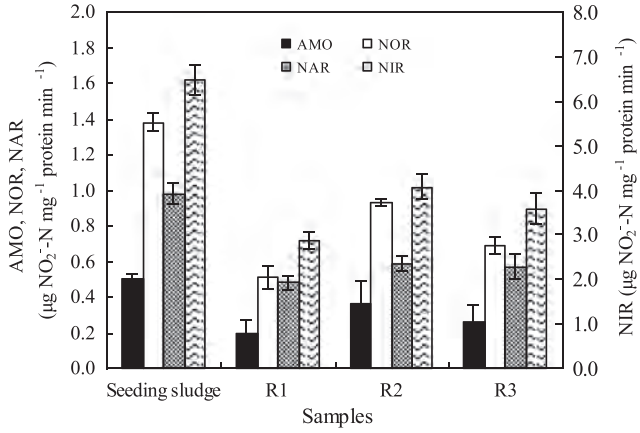

Fig. 2. Combined effects of Zn(II) and TC on the enzymatic activities involved into the processes of nitrification and denitrification in activated sludge. Seeding sludge: without Zn(II) and TC. R1: with 30 mg L^{-1} Zn(II). R2: with 30 mg L^{-1} TC. R3: with 30 mg L^{-1} Zn(II) and 30 mg L^{-1} TC.

resistance to heavy metals than heterotrophs, and heavy metals principally inhibited AOB, but not NOB (Ouyang et al., 2016).

In R2 containing 30 mg L^{-1} TC in the influent, the activities of AMO, NOR, NAR and NIR were 0.36, 0.93, 0.59 and 4.07 μg N mg^{-1} protein min^{-1}, respectively, and compared to seeding sludge, they decreased by 28.36%, 32.61%, 39.80% and 37.24%, respectively. Researchers reported that 20 mg L^{-1} TC could generate the damage in the integrity of cell membrane and inhibit some enzymes and functional genes synthesis associated with nitrifying and denitrifying processes (Tong et al., 2015; Deng et al., 2019), which explained why the AMO, NOR, NAR and NIR activities as the addition of TC in the influent. AMO and NOR activities had lower decreased degrees than NAR and NIR,

suggesting that nitrifiers had a greater ability to tolerate the disturbance of TC on bacterial growth and metabolism than denitrifying bacteria. Additionally, NOR activity had a higher decreased degrees than AMO activity in R2 at a selective pressure of 30 mg L^{-1} TC, suggesting that NOB was more easily inhibited by TC than AOB. Previous studies found similar results that denitrification was affected by antibiotics more obviously than nitrification, and antibiotics could produce more negative impacts on NOB than AOB (Prado et al., 2009; Yu et al., 2019). The decreased degrees in the activities of AMO, NOR, NAR and NIR in R1 were always more obvious than those in R2, suggesting that Zn(II) had a more significant inhibition on the activities of nitrifiers and denitrifiers than TC. This was similar to previous reports in which the IC_{50} of TC on the activities of partial nitration sludge was higher than that of Zn(II) (Xu et al., 2019).

In R3 containing 30 mg L^{-1} Zn(II) and 30 mg L^{-1} TC in the influent, the activities of AMO, NOR, NAR and NIR were 0.26, 0.69, 0.57 and 3.59 μg N mg^{-1} protein min^{-1}, respectively. Their activities in R3 were higher than those in R1, while were lower than those in R2. The results suggested an antagonistic impact of TC and Zn(II) on the nitrifiers and denitrifiers activities. TC had a large number of electron-donor functional groups that could bind metal ions (Wang et al., 2018a). This implied that the free TC or Zn(II) concentrations in R1 and R2 might be higher than those in R3, due to the formation of Zn(II) and TC complexes at the co-selective pressure of Zn(II) and TC. Zhang et al. (2017) found that the TC and heavy metals complexes bioavailability was lower than individual TC and heavy metals molecules, suggesting that individual TC and metals molecules had a higher toxicity to bacteria than the metals and TC complexes. Additionally, Zn(II) had a more significant inhibition on the activities of nitrifiers and denitrifiers than TC. These might explain why the enzymatic activities in R3 were higher than those in R1, while were lower than those in R2. The activities of AMO, NOR, NAR and NIR in R3 decreased by 48.26%, 50.00%, 41.84% and 44.64%, respectively, compared with seeding sludge, indicating that nitrifiers were more easily inhibited by mixed Zn(II)/TC than denitrifiers, and mixed Zn(II)/TC had a more significant inhibition on NOB than AOB. The changes of AMO, NOR, NAR and NIR activities at the co-selective pressure of Zn(II) and TC were similar with those at the alone selective pressure of Zn(II), while were different from those at the stress of TC alone. The results implied that the change in the ratio of Zn (II) content to TC content in the mixed Zn(II)/TC might affect the combined impacts of Zn(II) and TC to bacteria.

3.3. Microbial community

The microbial community richness and diversity variations at the selective pressure of Zn(II) and/or TC are depicted in Table 1. The indexes of Shannon and Simpson are related to microbial community diversity, and the Chao 1 and Observed species indexes reflect the changes of microbial community richness. The indexes of Chao1, Observed species, Shannon and Simpson in seeding sludge were 3306, 3297, 8.71 and 0.99, respectively. In R1, R2 and R3, the indexes of Chao1 and Observed species were 2089 and 1954 (R1), 2464 and 2353 (R2), as well as 2175 and 2043 (R3), respectively, and the Shannon and Simpson indexes were 5.44 and 0.80 (R1), 7.70 and 0.98 (R2), as well as 6.32 and 0.92 (R3), respectively. They in R1, R2 and R3 were lower than those in seeding sludge. Most bacteria could not survive at the selective pressure of Zn(II) and/or TC, causing the decrease of microbial community richness and diversity indexes in R1, R2 and R3 compared with seeding sludge. Among the three samples from R1, R2 and R3, the highest values of microbial community richness and diversity indexes were found in R2, while the lowest value were observed in R1. The results suggested an antagonistic impacts of Zn(II) and TC on microbial community richness and diversity.

Table 1
Combined effects of Zn(II) and TC on the microbial richness and diversity of activated sludge.

Samples	Concentration (mg L^{-1})		Richness indices		Diversity indices	
	Zn(II)	TC	Chao1	Observed species	Shannon	Simpson
Seeding sludge	0	0	3306	3297	8.71	0.99
R1	30	0	2089	1954	5.44	0.80
R2	0	30	2464	2353	7.70	0.98
R3	30	30	2175	2043	6.32	0.92

Fig. 3 shows the changes in the genus composition at the Zn(II) and/or TC selective pressure. There were 200 identified genera that their average relative abundance in the four samples from seeding sludge, R1, R2 and R3 were higher than 0.01%, and 50 genera with top abundance (accounting for above 95% in the total bacterial abundance) were used to analyze the genus composition shifts. In the 50 genera, 21 genera belonged to nitrifers and denitrifers. The genera *Nitrosomonas* and *Nitrospira* were nitrifers, and belonged to AOB and NOB, respectively (Wang et al., 2018a). The genera *Ferruginibacter, Dokdonella, Dechloromonas, Rubrivivax, Zoogloea, Thermomonas, Comamonas, Flavobacterium, Cloacibacterium, Acidovorax, Pseudomonas, Thauera, Rhodobacter, Bradyrhizobium, Aridibacter, Pseudoxanthomonas, Azoarcus, Hyphomicrobium* and *Hydrogenophaga* contributed to the nitrate and/or nitrite reduction (Wang et al., 2018a; Deng et al., 2019; Wang et al., 2016; Fang et al., 2020; Pishgar et al., 2019; Yuan et al., 2020; Liu et al., 2020; Wei et al., 2016; Obando et al., 2019). In the four samples from seeding sludge, R1, R2 and R3, the relative level sum of nitrifers accounted for 2.43%, 1.59%, 2.20% and 1.97% in the total bacteria, respectively, and the denitrifers relative abundance sum were 72.18%, 49.02%, 64.63% and 59.34%, respectively. In R1, R2 and R3, the nitrifers and denitrifers relative level sum in R1 was the lowest, while it in R2 was the highest. The results were in relation to the antagonistic interaction between Zn(II) and TC. Compared to seeding sludge, the nitrifers relative abundance sum in R1, R2 and R3 decreased by 34.49%, 9.60% and 18.95%, respectively, and the denitrifers relative abundance sum decreased by 32.09%, 10.47% and 17.80%, respectively. In R1 and R3, nitrifiers had higher decreased degrees of relative abundance than denitrifiers. However, the relative abundance decreased degree of nitrifers in R2 was lower than that of denitrifiers. The changes could explain why compared to the NAR and NIR activities, the AMO and NOR activities were more sensitive to Zn(II) and mixed Zn (II)/TC, while were more resistant to TC. The total nitrifiers and denitrifiers relative abundance in R1, R2 and R3 were 50.61%, 66.83% and 61.31%, respectively. Their relative abundance in R1 was the lowest, while was the highest in R2. This could be one of the reasons that the inhibition of Zn(II), TC and mixed Zn(II)/TC on the nitrifying and denitrifying enzymatic activities was in the order of Zn(II) > mixed Zn (II)/TC > TC.

The genus *Nitrosomonas* relative abundance in seeding sludge, R1, R2 and R3 were 0.97%, 0.70%, 0.90% and 0.83%, respectively, and the genus *Nitrospira* relative abundance in seeding sludge, R1, R2 and R3 were 1.46%, 0.89%, 1.29% and 1.14%, respectively. Compared to seeding sludge, the genus *Nitrosomonas* belonging to AOB relative abundance in R1, R2 and R3 decreased by 27.75%, 7.15% and 14.53%, respectively, and the relative abundance of genus *Nitrospira* belonging to NOB decreased by 38.99%, 11.23% and 21.89%, respectively. The decreased degree of genus *Nitrosomonas* relative level in R1, R2 and R3 was always lower than that of genus *Nitrospira*, suggesting that AOB was more sensitive to Zn(II), TC and mixed Zn(II)/TC than NOB. Compared with seeding sludge, the genera *Ferruginibacter, Zoogloea, Thermomonas* and *Flavobacterium* relative abundance in R1, the genera *Ferruginibacter, Dechloromonas, Zoogloea, Flavobacterium, Cloacibacterium, Acidovorax* and *Thauera* relative abundance in R2, and the genera *Zoogloea, Thermomonas, Flavobacterium, Cloacibacterium, Acidovorax* and *Thauera* relative abundance in R3 were always higher,

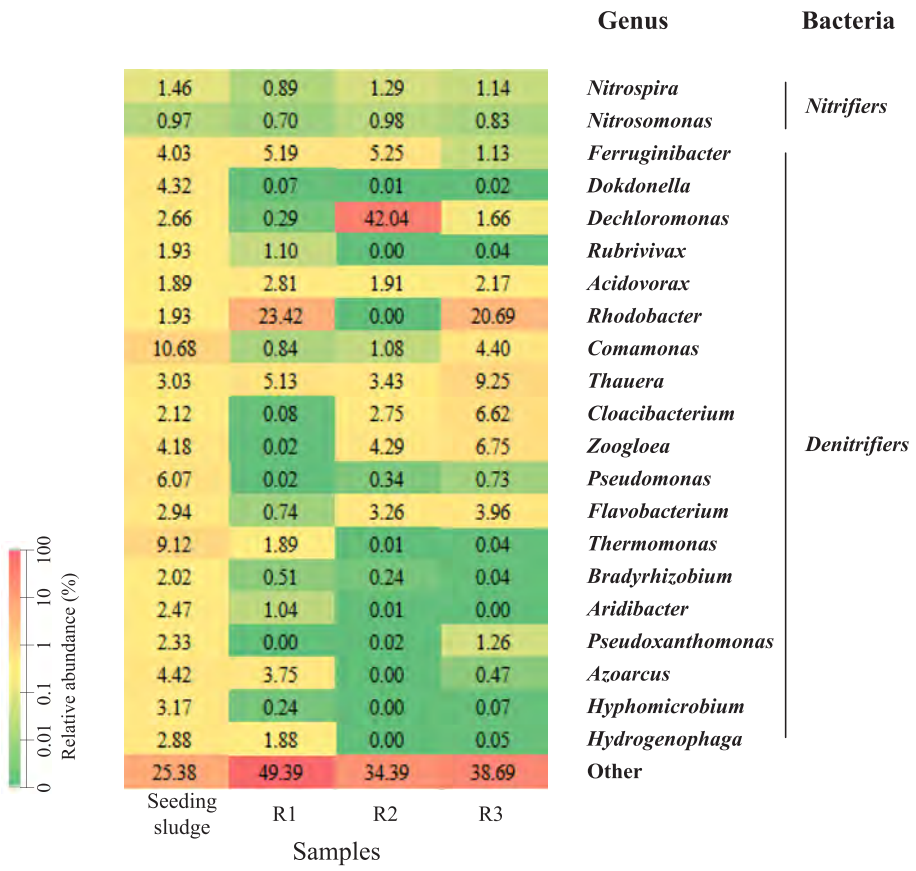

Fig. 3. Combined effects of Zn(II) and TC on the relative abundance of nitrifiers and denitrifiers at the level of genus in activated sludge. Seeding sludge: without Zn (II) and TC. R1: with 30 mg L^{-1} Zn(II). R2: with 30 mg L^{-1} TC. R3: with 30 mg L^{-1} Zn(II) and 30 mg L^{-1} TC.

indicating that these bacteria had strong tolerances to Zn(II) and/or TC and they were the main contributors to the removal of nitrogen.

3.4. Resistance genes

To analyze the reasons that bacteria could keep metabolisms at the selective pressure of Zn(II) and/or TC, the abundance of ARGs and HMRGs were quantitatively investigated (Fig. 4). In seeding sludge, the abundance of *tetA*, *tetM*, *tetX*, *czcA*, *czcB* and *czcC* genes were 8.25×10^7, 1.14×10^6, 9.38×10^8, 1.18×10^9, 1.64×10^5 and 9.30×10^6 copies g^{-1} MLSS, respectively, and the antibiotic resistance genes (*tetA*, *tetM* and *tetX*) accumulative level and the heavy metal resistance genes (*czcA*, *czcB* and *czcC*) accumulative abundance were 1.02×10^9 and 1.19×10^9 copies g^{-1} MLSS, respectively.

In R1, the genes *czcA*, *czcB* and *czcC* abundance were 3.05×10^9, 1.43×10^5 and 1.96×10^7 copies g^{-1} MLSS, respectively, and the total abundance of *czcA*, *czcB* and *czcC* genes was 3.07×10^9 copies g^{-1} MLSS. The *czcA*, *czcB* and *czcC* genes, encoding bacterial protein that located on inner membrane, membrane fusion cytoplasm and periplasm, and outer membrane, respectively, were HMRGs associated with Zn(II), and membrane fusion could connect inner membrane with outer membrane (Nesler et al., 2017). These proteins formed the efflux pump system of Zn(II) resistance-nodulation-cell division (RND), which exported Zn(II) ions from cytoplasm to periplasm via inner membrane and then from periplasm to outside of cell across outer membrane (Jeanvoine et al., 2019; Nesler et al., 2017). The total HMRGs level in R1 was higher than that in seeding sludge, which was related to the formation of bacterial Zn(II) resistance induced by Zn(II) ions. The abundance of *czcA* and *czcC* genes were 4 and 2 log unit higher than those of *czcB* gene, respectively, and the genes *czcA* and *czcC* abundance accounted for 99.36% and 0.64% of the accumulative levels of

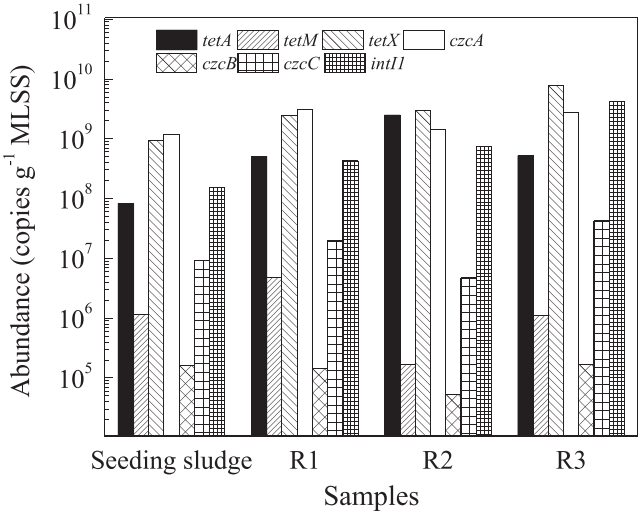

Fig. 4. Combined effects of Zn(II) and TC on the abundances of ARGs and HMRGs from activated sludge. Seeding sludge: without Zn(II) and TC. R1: with 30 mg L^{-1} Zn(II). R2: with 30 mg L^{-1} TC. R3: with 30 mg L^{-1} Zn(II) and 30 mg L^{-1} TC.

HMRGs in R1, respectively (E-Supplementary data for this work can be found in e-version of this paper online). Cha and Cooksey (1993) reported that an operon containing an amount of inner membrane protein genes but lacking one or more heavy metals resistance genes of other types could cause hyper-sensitivity and hyper-accumulation of heavy metals in cells. Therefore, based on the fate of genes *czcA*, *czcB* and *czcC* abundance in the total HMRGs level in R1, it might be inferred that an

amount of Zn(II) ions might be accumulated inside of cells, and few Zn (II) ions could be pumped out of cells. The fate of Zn(II) inside and outside of cells needs to be studied further in order to explore their relation with resistance genes. The results might be one of the reasons that Zn(II) caused the decrease of enzymatic activity at the selective pressure of 30 mg L^{-1} Zn(II) in this study. The genes *tetA*, *tetM* and *tetX* abundance in R1 were 5.02×10^8, 4.79×10^6 and 2.47×10^9 copies g^{-1} MLSS, respectively, and their sum was 2.97×10^9 copies g^{-1} MLSS, respectively. The genes *tetA*, *tetM* and *tetX* were the most frequent ARGs related to TC in activated sludge (Zhang et al., 2019b), and they belonged to genes of efflux pump, ribosomal protection and enzymatic modification, respectively, according to their resistance mechanisms (Zhang et al., 2009). The *tetA* gene controlled the process that extruded toxins (e.g. antibiotics and heavy metals, etc.) from the inside of cell to the external environment (Zhang et al., 2015). The *tetM* gene was associated with the synthesis of cytoplasmic protein that bound and protected ribosome from the TC action in vitro and in vivo (Roberts, 2003). The *tetX* gene could product cytoplasmic proteins that could destroy and modify antibiotics by appending chemical groups to antibiotic molecule vulnerable sites, which caused bacterial antibiotic resistance by preventing the bind of antibiotics to their target proteins due to steric hindrance (Blair et al., 2015). The total ARGs level in R1 was higher than that in seeding sludge, suggesting that Zn(II) ions alone could induce the formation of ARGs, and the efflux pump of TC might contribute to the extrusion of Zn(II) from cells. Previous researchers reported similar results that tetracycline resistance genes could be produce at the stress of Zn(II) ions alone, and the *tetA* gene was the porter of TC and Zn(II) in cells (Ding et al., 2019; Knapp et al., 2017).

In R2, the genes *tetA*, *tetM* and *tetX* abundance were 2.48×10^9, 1.65×10^5 and 3.01×10^9 copies g^{-1} MLSS, respectively, and their sum was 5.49×10^9 copies g^{-1} MLSS. The total ARGs level in R2 was higher than that in R1, indicating that TC had a more distinct effect on the formation of bacterial TC resistance genes than Zn(II). The levels of *tetA* and *tetX* genes were both 2 log unit higher than those of *tetM* gene, and the genes *tetA* and *tetX* levels accounted for 45.11% and 54.88% of the total level of ARGs in R2, respectively (E-Supplementary data for this work can be found in e-version of this paper online). The results suggested that the main TC resistance mechanisms of bacteria at a TC stress alone in the study were efflux pump (the extrusion of TC from the inside of cell to the external environment) and enzymatic modification (the breach or modification of TC by some enzymes). The genes *czcA*, *czcB* and *czcC* abundance in R2 were 1.43×10^9, 5.38×10^4 and 4.71×10^6 copies g^{-1} MLSS, respectively, and their sum was 1.44×10^9 copies g^{-1} MLSS. Their abundance sum was significantly lower than that in R1, while was similar with that in seeding sludge. The results implied that TC alone did not induce obviously bacteria to form the resistance of Zn(II). He et al. (2017) found similar results that the HMRGs levels were not correlated with antibiotics. The changes of ARGs and HMRGs in R1 and R2 showed that the selective pressure of 30 mg L^{-1} Zn(II) did not induce significantly efflux pump mechanism of bacterial Zn(II) resistance, while the efflux pump and enzymatic modification mechanisms of bacterial TC resistance were successfully induced by the selective pressure of 30 mg L^{-1} TC. The results might explain why the nitrifying and denitrifying enzymatic activities were more easily inhibited by Zn(II) than by TC.

In R3, the genes *tetA*, *tetM* and *tetX* abundance were 5.18×10^8, 1.09×10^6 and 7.82×10^9 copies g^{-1} MLSS, respectively, and their sum was 8.34×10^9 copies g^{-1} MLSS, respectively. The total ARGs level in R3 was the highest among R1, R2 and R3. The changes suggested that the co-selective pressure of Zn(II) and TC could enhance bacterial TC resistance. The results could be explained by Zn(II) ions were able to induce the formation of TC resistance genes (Knapp et al., 2017). The total ARGs level in R3 was lower than the sum of ARGs abundance in R1 and R2, which was related to an interaction of TC with Zn(II). Previous studies showed that free antibiotics and heavy metals had a significant positive relationship with the ARGs levels (Karkman et al., 2017; Sun et al., 2015). The interaction of TC with Zn(II) decreased the concentrations of free TC and Zn(II), causing that the total ARGs levels at the co-selective pressure of TC and Zn(II) were lower than the sum of total ARGs levels at the stress of TC and Zn(II) alone. In R3, the levels of *tetA* and *tetX* genes were 2 and 3 log unit higher than those of *tetM* gene, respectively, and the genes *tetA* and *tetX* levels accounted for 6.22% and 93.77% of the total level of ARGs, respectively (E-Supplementary data for this work can be found in e-version of this paper online). The results suggested that the TC resistance mechanisms of bacteria at the co-selective pressure of TC and Zn(II) were efflux pump and enzymatic modification, and enzymatic modification had a more important role than efflux pump. Compared to R2 at the selective pressure of TC, the percent of gene *tetA* (efflux pump genes) in the total ARG levels in R3 at the co-selective pressure of Zn(II) and TC was lower, while the gene *tetX* (enzymatic modification genes) percent in R3 was higher. The changes suggested that the co-selective pressure of TC and Zn(II) might promote the TC deactivation by destroying or modifying antibiotics. Wright (2011) found similar results that Zn(II) ions could induce the metallo-β-lactamases to attack on the β-lactam antibiotics centre, which resulted in the deactivation of β-lactam antibiotics. Although the percent of gene *tetA* in the total ARGs level in R3 at the co-selective pressure of Zn(II) and TC was significantly lower than that in R1 at the selective pressure of Zn(II), the abundance of gene *tetA* in R1 and R3 was not observed obvious difference. The results implied that the co-selective pressure of Zn(II) and TC did not affect the contribution of TC efflux pump to the extrusion of Zn(II) from cells. In R3, the genes *czcA*, *czcB* and *czcC* abundance were 2.71×10^9, 1.69×10^5 and 4.25×10^7 copies g^{-1} MLSS, respectively, and their sum was 2.75×10^9 copies g^{-1} MLSS, respectively. The total HMRGs level in R3 was higher than that in R2, which was related to the supplement of Zn (II) in the influent. However, the total HMRGs level in R3 was lower than that in R1, suggesting that the Zn(II) and TC co-selective pressure was not beneficial to induce the increase of HMRGs abundance compared to the Zn(II) selective pressure alone. These could be explained by the fact that the interaction of TC with Zn(II) decreased bioavailability of Zn(II), which was not beneficial for the HMRGs formation induced by free Zn(II) ions. The levels of *czcA* and *czcC* genes in R3 were 4 and 2 log unit higher than those of *czcB* gene, respectively, and the genes *czcA* and *czcC* levels accounted for 98.45% and 1.54% of the total levels of HMRGs, respectively. Compared to R1 at the selective pressure of Zn(II), the percent of gene *czcA* in the total HMRGs level in R3 at the co-selective pressure of TC and Zn(II) decreased by 11.20%, while the gene *czcC* percent in R3 increased by 117.28%. The changes suggested that at the co-selective pressure of TC and Zn(II), the addition of TC could promote the efflux of Zn(II) ions from the inside of cell. At the co-selective pressure of TC and Zn(II), the gene *tetX* percent was significantly higher than that at the pressure of TC, and the gene *czcC* percent was obvious increased compared to that at the pressure of Zn (II). The results implied that the Zn(II) and TC co-selective pressure enhanced bacterial enzymatic modification resistance to TC and bacterial outer membrane resistance to Zn(II), which might be one of reasons that the impact of Zn(II) and TC on bacterial activities was antagonistic.

The *intI1* gene was one of the major mobile genetic elements in the horizontal gene transfer (HGT) (Zhang et al., 2019c), which was responsible for the transfers of resistance genes between different bacterial species, and was the chief way of resistance genes spread in bacteria (Jang et al., 2018). The *intI1* gene abundance in seeding sludge, R1, R2 and R3 were 1.53×10^8, 4.25×10^8, 7.43×10^8 and 4.18×10^9 copies g^{-1} MLSS, respectively. Compared to seeding sludge, the supplement of Zn(II) and/or TC in the influent caused the increase of ARGs, HMRGs and *intI1* gene abundance, suggesting that HGT had contributions to the spread and diffusion of ARGs and HMRGs.

Fig. 5 showed the relations between ARGs, HMRGs and *intI1* gene based on redundancy analysis (RDA). The positive or negative correlations between *tetA*, *tetM* and *tetX* genes could be observed, while the

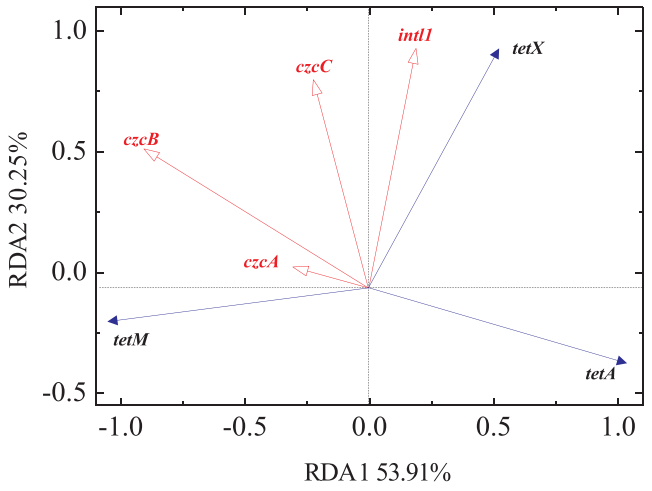

Fig. 5. The relations between ARGs and HMRGs based on RDA analysis.

correlations between *czcA*, *czcB* and *czcC* genes were positive. The changes indicated that the resistance mechanisms of bacteria to TC were more complicated than those to Zn(II). The *intI1* gene was significantly positive correlations with the *tetX* and *czcC* genes, suggesting that their transfers belonged to HGT, and were dependent on the same integron (Liu et al., 2019). The level of *tetX* gene in different samples was always higher than that of *tetA* and *tetM* genes, while the similar results that the *czcC* gene abundance was higher than that of *czcA* and *czcB* genes was only observed in R3. The results suggested that the formation mechanisms of the *czcC* gene and the *tetX* gene in some bacteria might be synergistic at the Zn(II) and TC co-selective pressure. This need be study in the future.

3.5. Relations between resistance genes and microbial community

To analyze the relations of ARGs and HMRGs with microbial community at the Zn(II) and/or TC selective pressure, a network analysis was conducted based on the Pearson's correlation ($|r| > 0.8$, $p < 0.05$) (Fig. 6). 34 genera from the 50 top abundant genera in the section 3.3 were significantly correlations with ARGs and/or HMRGs, suggesting that these bacteria were the potential hosts of ARGs and/or HMRGs (Zhang et al., 2020b). Among these potential hosts of ARGs and/or HMRGs, the genera *Nitrospira* and *Nitrosomonas* were nitrifiers, and the genera *Ferruginibacter, Dechloromonas, Acidovorax, Rhodobacter, Thauera, Cloacibacterium, Zoogloea* and *Flavobacterium* belonged to denitrifiers. Previous studies showed that the potential hosts positively correlating to resistance genes were the producers of resistance genes, and those negatively correlating to resistance genes were the contributors to the resistance genes removal (Liu et al., 2020).

The genera *Acidovorax, Rhodobacter* and *Thauera* were positively correlated to HMRGs, and their relative levels in R1 were higher than those in seeding sludge. The genera *Nitrospira*, *Nitrosomonas, Ferruginibacter* and *Dechloromonas* were negatively correlated to HMRGs, and their relative abundance in R1 were not always higher than those in seeding sludge. These implied that compared to the contributors to the resistance genes removal, the producers of resistance genes had a more prominent role in the removal of nitrogen. The *czcA* gene was strong correlated to the nitrifying bacteria genera *Nitrospira* and *Nitrosomonas* and the denitrifying bacteria genera *Acidovorax* and *Rhodobacter*, suggesting that these bacteria were the carriers of *czcA* gene. The section 3.4 discussed that when one or more of the other genes types were lacked, a high level of *czcA* gene was not beneficial to microbial metabolisms. In R1, all nitrifying bacteria harbored *czcA* gene, while the relative abundance sum of denitrifying bacteria genera harboring *czcA* gene accounted for 53.51% of denitrifying bacteria (E-Supplementary data for this work can be found in e-version of this paper online). The proportion of bacteria harboring *czcA* gene in nitrifying bacteria was higher than those in denitrifying bacteria, which might explain why nitrifying bacteria were more easily affected by Zn(II) than denitrifying bacteria.

Similar results were found in R2 in which the producers of resistance genes including the genera *Dechloromonas, Acidovorax, Thauera* and *Cloacibacterium* had higher relative abundance than those in seeding sludge, and the relative abundance of contributors to the resistance genes removal, including to *Nitrospira, Nitrosomonas, Ferruginibacter, Zoogloea* and *Flavobacterium*, were not always higher than those in seeding sludge. Additionally, all nitrifying bacteria harbored ARGs, while the relative abundance sum of denitrifying bacteria genera harboring ARGs accounted for 97.36% of denitrifying bacteria (E-Supplementary data for this work can be found in e-version of this paper online). The proportion of bacteria harboring *tetA*, *tetM* and *tetX* in nitrifying bacteria was higher than those in denitrifying bacteria, which might explain why nitrifying bacteria had a stronger tolerance to TC than denitrifying bacteria.

In R3, bacteria producing ARGs and HMRGs were the genera *Acidovorax* and *Thauera*, which belonged to denitrifying bacteria and were positive correlations with both ARGs and HMRGs. Bacteria involving with the removal of ARGs and HMRGs were the genera *Nitrospira*, *Nitrosomonas* and *Ferruginibacter,* which were negatively correlated to both ARGs and HMRGs. The genus *Dechloromonas* was positive correlations with ARGs, and was negatively correlated to HMRGs. These suggested that the genus *Dechloromonas* could produce ARGs and remove HMRGs. The genera *Nitrospira*, *Nitrosomonas*, *Ferruginibacter, Dechloromonas, Acidovorax* and *Thauera* were strong correlations with both ARGs and HMRGs, suggesting that they were multiple resistances to TC and Zn(II). The results were consistent with previous researches (Nõlvak et al., 2018; Huang et al., 2019; Ma et al., 2019; Wang et al., 2018b).

In R3, nitrifying bacteria genera *Nitrospira* and *Nitrosomonas* harbored *czcA* gene, and nitrifying bacteria harboring *czcA* gene were genera *Acidovorax* and *Rhodobacter*. Based on the relative abundance, the proportion of bacteria harboring *czcA* gene in nitrifying bacteria (100%) was higher than those in denitrifying bacteria (38.51%), which might explain why nitrifiers were more sensitive to the mixed Zn(II)/TC than denitrifying bacteria. The relative abundance sum of nitrifiers and denitrifiers with ARGs and/or HMRGs in R1, R2 and R3 were 39.27%, 65.13% and 54.19%, respectively, and they accounted for 77.60%, 97.46% and 88.39% in the relative abundance sum of nitrifying bacteria and denitrifying bacteria, respectively (E-Supplementary data for this work can be found in e-version of this paper online). Among R1, R2 and R3, the percent in R2 was the highest, while it in R1 was the lowest. The results might be one of the reasons that the order of nitrifiers and denitrifiers tolerance to Zn(II), TC and mixed Zn(II)/TC was TC > mixed Zn(II)/TC > Zn(II).

4. Conclusions

The impacts of Zn(II) and TC co-selective pressure on the organics and nitrogen removals, enzymatic activity, and microbial diversity and richness were antagonistic. Mixed Zn(II)/TC induced the ARGs abundance increase and the HMRGs levels decrease, and enhanced bacterial enzymatic modification resistance to TC and bacterial outer membrane resistance to Zn(II). The potential hosts of ARGs and/or HMRGs included the genera *Nitrospira* and *Nitrosomonas* of nitrifying bacteria, and the genera *Ferruginibacter, Dechloromonas, Acidovorax, Rhodobacter, Thauera, Cloacibacterium, Zoogloea* and *Flavobacterium* of denitrifying bacteria, and the genera *Nitrospira*, *Nitrosomonas*, *Ferruginibacter, Dechloromonas, Acidovorax* and *Thauera* were multiple resistances to TC and Zn(II).

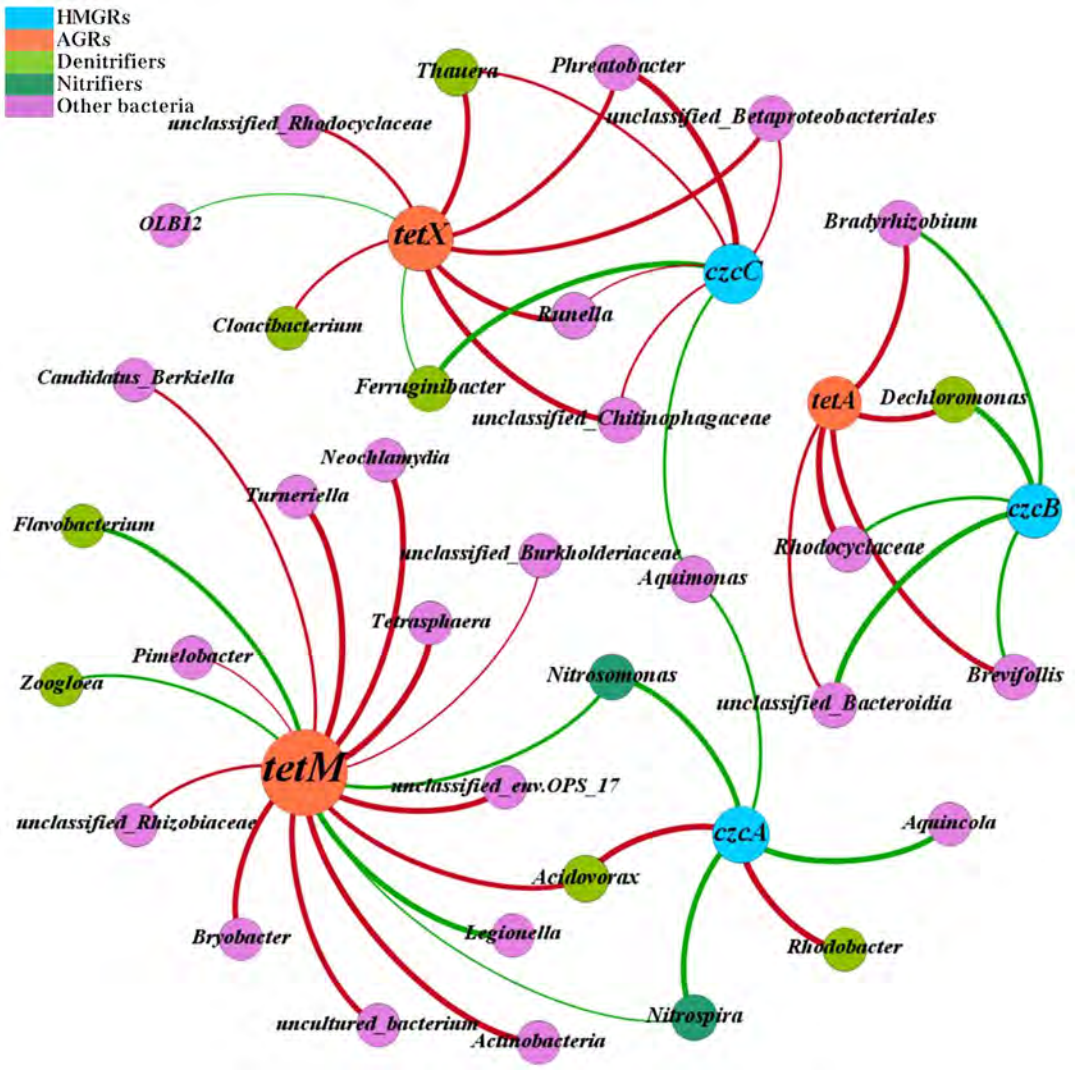

Fig. 6. Network analysis showed the co-occurrence patterns between ARGs, HMRGs and bacterial taxa (genus level). A node represented an ARG, HMRGs or bacterium, where the node size was proportional to the number of connections (degree). An edge represented a strong correlation between two nodes, and the red edges and the green edges represented positive and negative relations between two nodes, respectively. The edge thickness was proportional to Pearson's correlation coefficients (weight).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Zichao Wang: Writing - review & editing. **Shengyu Yuan:** Data curation. **Zhiwei Deng:** Data curation. **Yuejing Wang:** Software. **Shilong Deng:** Software. **Youtao Song:** Software. **Congting Sun:** Investigation. **Naishun Bu:** Investigation. **Xinruo Wang:** Software.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2020.123769.

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