Environmental Science
Processes & Impacts

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

rsc.li/process-impacts

Antibiotic resistance gene is considered as an emerging contaminant that imposes potential health risk on human health. The study evaluated the release of bacteria and genes resistant to six commonly used antimicrobials in a wastewater treatment plant (WWTP) over a whole year. The results indicated that a high prevalence of antibiotic-resistant heterotrophic bacteria was detected even after WWTP treatment. Sampling season greatly influenced the release loads of most antibiotic-resistant bacteria (ARB), while the antibiotic resistance gene (ARG) loads changed slightly over various seasons. A redundancy analysis implied that ARB and ARGs proportions were significantly related to wastewater quality and operation conditions in WWTP. These results may help us better understand and assess the fate of antimicrobial resistance in wastewater treatment plants.

Monitoring and assessing the impact of wastewater treatment on release of both the antibiotic-resistant bacteria and their typical genes in a Chinese municipal wastewater treatment plant

Qing-Bin Yuan, Mei-Ting Guo*, Jian Yang

State Key Laboratory of Pollution Control and Resources Reuse, College of Environmental Science and Engineering
Tongji University
200092, Shanghai, China

*Corresponding footnote:
College of Environmental Science and Engineering
Tongji University
Shanghai, 200092, China
Telephone: (86) 21-6598-4275
Fax: (86)21-6598-4275
E-mail: guomeiting@tongji.edu.cn

Abstract

Wastewater treatment plants (WWTPs) are important hotspots for the spread of antibiotic resistance. However, the release and impact factors of both antibiotic resistant bacteria and relevant genes over long periods in WWTPs have rarely been investigated. In this study, fate of bacteria and genes resistant to six commonly used antibiotics were assessed over a whole year.

In WWTP effluent and biosolids, a high prevalence of heterotrophic bacteria resistant to vancomycin, cephalexin, sulfadiazine and erythromycin were detected, each with a proportion over 30%. The corresponding genes (*vanA*, *ampC*, *sulI* and *ereA*) were all detected with proportions in the effluent of $(2.2 \pm 0.8) \times 10^{-10}$, $(6.2 \pm 3.2) \times 10^{-9}$, $(1.2 \pm 0.8) \times 10^{-7}$ and $(7.6 \pm 4.8) \times 10^{-8}$, respectively. The sampling season imposed considerable influence on the release of all ARB. High release loads of most ARB were detected in the spring, while low release loads were generally found in the winter. By comparison, the ARGs loads changed only slightly over various seasons. No statistical relevance was found between all ARB abundances and their corresponding genes over the long-term investigation. The inconsist behavior indicates that bacteria and genes should both be considered in exploring resistance characteristics in wastewater.

A redundancy analysis was adopted to assess the impact of wastewater quality and operational conditions on antibiotic resistance. The results indicated that most ARB and ARGs proportions were positively related to the COD and turbidity of the raw sewage, while negatively related to those of the effluent. The DO and temperature

exhibited strongly negative relevance to most ARB prevalence.

Introduction

Wastewater treatment plants (WWTPs) are considered important hotspots for the spread of antibiotic resistance. The prevalence and reductions of many kinds of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) in WWTPs are widely reported [1-5]. Huang et al. explored ARB abundances in a WWTP of Beijing; they found that the concentrations of penicillin-, ampicillin-, cephalothin-, and chloramphenicol-resistant bacteria were as high as 8.9×10^3 to 2.0×10^5 CFU/mL [6]. Borjesson et al. observed a removal of the methicillin-resistant *Staphylococcus aureus* gene (*mecA*) concentration ranging from 0.5 to 2.5 log in a WWTP [7]. Munir et al. reported that WWTPs treatment caused a removal by a log of 2.4–7.1 in bacteria and gene abundances resistant to tetracycline and sulfonamide [8]. Results of LaPara et al. indicated that the concentrations of typical antibiotic resistance determinants (*tetA*, *tetX*, *tetW*, *intI1*) were typically 20-fold higher in the tertiary-treated wastewater than in nearby surface water samples [28].

Nevertheless, the release load of ARB/ARGs and removal level in a long term period still lack of data. Seasonal change of the fate of antibiotic resistance is not clear so far.

Furthermore, numerous factors, such as the select pressure from antibiotics, operational conditions and wastewater quality, might significantly influence the fate of ARB and ARGs in WWTPs.

A few researchers explored the influence of a single abiotic condition. Threedeach et al. found that the proportions of bacteria resistant to most antibiotics

were higher under anaerobic operations than under semi-aerobic conditions [9]. Kim et al. investigated the fate of tetracycline-resistant bacteria as a function of the organic loading rate; results indicated that increases in organic loading and growth rates resulted in higher increased concentrations and production rates of tetracycline-resistant bacteria [10]. However, the methods used might not be suitable in evaluating the influence of abiotic conditions in WWTPs, since many factors might exhibit an interaction effect. Some multi-parametric analyses, such as those often used in ecology studies, might help to elaborate the influence more explicitly.

The objective of this study is to explore the release and its impact factors of antibiotic resistance in wastewater and biosolids treated by WWTP processes over a whole year. The heterotrophic bacterial abundances and prevalence in effluents and biosolids were investigated, as regards resistance to six classes of commonly used antibiotics [cephalexin (CEP), erythromycin (ERY), gentamicin (GEN), sulfadiazine (SD), tetracycline (TC) and vancomycin (VAN)]. One widely detected gene encoding resistance against antibiotic (*ampC*, *ereA*, *aacC1*, *sulI*, *tetA* and *vanA*) was selected and studied as representative of antibiotic resistance gene. To assess the influence of wastewater quality and operational conditions on the antibiotic resistance levels in the WWTP effluents, redundancy analyses were adopted for analysis.

Materials and methods

Sampling

The study was conducted in a secondary municipal wastewater treatment plant in Shanghai (in China), with a treatment capacity of 60,000 m^3/d. The treatment process

includes a grit chamber, an anaerobic-anoxic-oxic (A^2/O) biological process (total hydraulic retention time of 7.5 h) and a secondary settling tank. Ultraviolet (UV) disinfection is applied before discharging into a natural water stream.

Wastewater samples of the raw sewage (after the grit chamber), the effluent from the secondary settling tank and the final effluent were collected in sterile polyethylene bottles, refrigerated and transported to the lab within 2 h and analyzed within 12 h. Biosolid samples were also collected from the discharged excess sludge. In order to obtain seasonal data, samples were collected at the middle of every month over a whole year from October 2012 to September 2013.

Water quality analysis of wastewater samples

Wastewater samples from each month were analyzed to assess the influence of wastewater quality on the antibiotic resistance level. Regular analyses included the air temperature on each sampling date, chemical oxygen demand (COD), turbidity, ammonia nitrogen (NH_3-N), total nitrogen (TN), total phosphorus (TP) of raw sewage and final effluent, mixed liquor suspended solids (MLSS) and dissolved oxygen (DO) of the activated sludge according to national standard methods.

Antibiotic resistant bacterial analysis

Wastewater samples were analyzed for bacterial resistance using classical spread-plating techniques. Before plating, each sample was blended for 3 min to homogenize the culture. 1 mL of each homogenized sample was removed, serially diluted, and then plated in duplicate onto nutrient agar (beef extract 3 g/L, peptone 10 g/L, NaCl 5 g/L and agar 15 g/L, pH: 7.2 ±0.2) spiked with various antibiotics.

Concentrations of the six antibiotics in the agar were shown in the following (CEP: 16 mg/L; ERY: 8 mg/L; GEN: 16 mg/L; SD: 512 mg/L; TC: 16 mg/L; VAN: 32 mg/L). The added antibiotic concentrations for ARB in wastewater were defined as the maximum value of the Minimum Inhibition Concentration (MICs) for *enterobacter*, *enterococcus* spp. and *Staphylococcus* spp. resistant to that antibiotic [11, 12], since these three are the common species related to human health in wastewater.

The plates were then incubated at 37 ℃ for 24 h. All samples were processed by the standard count technique. Only dilutions with 20-300 CFU per plate were used for colony enumeration. Each sample was also incubated in nutrient agar with no antibiotic added to determine total heterotrophic bacterial count (HPC) levels.

Quantifications of ARGs through DNA extraction and Quantitative real-time PCRs

Each sample was filtered through a 0.45 μm micropore filter (Millipore, Billerica, MA). The filtrated volumes for raw sewage, effluent after secondary settling tank and final effluent were 20 mL, 300 mL and 300 mL, respectively. The filters were cut into small pieces and added directly to the extraction tubes. The extractions were conducted in duplicate using the FastDNA Spin Kit for Soil (MP Biomedicals, CA, USA) according to manufacturer's protocol. The extraction yield and the quality of the DNA were verified by agarose gel electrophoresis and spectrophotometry (NanoDrop 8000, NanDrop Technologies, Willmington, DE).

Six ARGs encoding resistance to each antibiotic were selected for quantitative detection using SYBR Green II Q-PCR. The primers of ARGs are listed in Table 1,

which have been developed and validated in previous studies. 16S rRNA genes were quantified using the method described by Muyzer et al. [16], so that the ARGs abundance could be normalized to the total bacterial community. The genes were cloned to plasmids to generate Q-PCR standard curves to determine the abundance per 100 mL in the filtered samples. In detail, the PCR product of each gene was purified by use of a PCR Production Purification Kit (Omega, USA) and cloned using pMD18-T Vector (TaKaRa, Japan). Plasmids carrying each ARG were extracted and purified using MiniBest Plasmid Purification Kit (TaKaRa, Japan). Plasmid concentrations were determined by Nano Drop and the abundance of each ARG per μL plasmid solution was calculated. Six-point calibration curve (*Ct* value versus log of initial ARGs copies) was generated for Q-PCR using 10-fold serial dilution of the plasmids. Based on the calibration curves, the *Ct* value of a test sample was used to calculate the abundance of each gene.

The Q-PCRs were conducted in 8 trip tubes with a final volume of 20 μL, containing 10 μL Power SYBR® Green PCR Master Mix (Tiangen Biotech, Beijing), plus 0.3 μL of each primer (10 μM) and 1 μL of the template DNA. Thermal cycling and fluorescence detection were conducted on an ABI 7500 with the software and fast real time PCR systems 2.0.5 (Applied Biosystems, USA), using the following protocol: 95℃ for 15 min, followed by 40 cycles of 95 ℃ for 10 s, annealing at defined temperatures for 20 s and 72 ℃ for 32 s. Each reaction was run in triplicate for each sample. The PCR efficiency of each gene ranged from 88% to 110% with R^2 values more than 0.99 for all calibration curves. In addition, 1×10^6 copies of the

plasmids carrying each gene were added in serial dilutions of environmental DNA to check for Q-PCR inhibition. The final concentration of template DNA in the reaction volume was controlled at < 0.25 ng/μL for all primers to avoid amplification suppression. The specificity of the Q-PCR products was further checked by melt curves and agarose gel electrophoresis.

Data analysis

The proportion of each ARB and ARG was determined as follows:

Proportion of ARB = count number of each ARB (CFU/mL) / total heterotrophic bacterial count (CFU/mL)

Normalization of ARGs to the total bacterial community = concentration of each ARG (Copies/100mL) / concentration of 16S rRNA (Copies/100mL)

The Ratio of ARB/ARGs in biosolids and in effluent was used to explain the distribution of ARB/ARGs in biosolids and effluent.

Ratio of ARB/ARGs= concentration of each ARB(ARG) in biosolids / concentration of each ARB(ARG) in effluent

Redundancy analysis (RDA, software package CANOCO version 4.5) was used to assess the ARB or ARGs proportions as a function of wastewater quality (COD, turbidity, NH_3-N, TN, TP, culturable bacteria abundance of raw sewage and final effluent) and operational conditions (air temperature of each sampling date, MLSS, DO, hydraulic loading rate). The raw data for multivariate analyses comprised proportions of ARB and ARGs (multiplied by 10^{12}) and other environmental variables. In the RDA analysis, all variables (abiotic conditions, ARB and ARGs proportion)

were included in the ordination. The significance of the relationship between species data (ARB and ARGs proportion) and the environmental data (wastewater quality and operation conditions) was tested by Monte Carlo permutations test (n = 199). Explanatory variables included in RDA analyses were selected by manual forward selection including the permutation test (Monte Carlo). Whenever only one explanatory variable was included in the ordination, no biplot was produced.

The student t-test (SPSS 19.0 for Windows) was used to assess statistically significant differences ($p < 0.05$) among the values of antibiotic resistance abundance or proportion. The null hypothesis that the ARB (or ARGs) concentration was not different between different samples was rejected at a p-value less than or equal to 0.05.

Results and discussions

Concentration reduction of ARB and their ARGs through wastewater treatment process

The removals of six kinds of ARB and relevant ARGs by the biological treatment process and UV disinfection were monitored and are shown in Fig. 1. The biological treatment process exhibited significant reduction for all ARB and ARGs concentrations in the WWTP, with log removals of 2.2 ± 0.1 and 1.8 ± 0.2, respectively. The effect of wastewater treatment on resistance level reduction was similar to that in other WWTPs in USA, Denmark and Spain (Table 2), although the treatment process varied greatly. The effective reduction possibly arises from the fact that most ARB has been transferred into biosolids through good settleability of

activated sludge. Actually much higher ARB/ARGs concentrations have been reported frequently in the biosolids compared to that in the effluents [8, 17].

The UV treatment inactivated ARB and ARGs significantly, with average inactivation of (0.8 ± 0.1) log and (0.3 ± 0.1) log, respectively. The reductions were at similar levels with total heterotrophic bacteria [(0.7 ± 0.1) log] and 16S rRNA [(0.3 ± 0.2) log], indicating that no tolerance to UV disinfection for all ARB existed during long-term investigation. Similar results were confirmed by Munir et al. [8], where they reported that UV disinfection contributed to a significantly lower reduction of TC- and SD-resistant bacteria/genes compared to biological treatment in five WWTPs. Significantly higher reductions of antibiotic resistance by the disinfection process were reported in previous bench-scale studies [12, 18]. It was considered that a low effective UV fluence (possible in WWTPs) might weaken the effect of UV treatment.

In addition, it was noticed that the WWTP treatment did not cause a significant difference concerning the total removal of various ARB/ARGs in a seasonal study ($p < 0.05$), with values maintained at (3.0 ± 0.1) log and (2.1 ± 0.2) log, respectively. In a single month, the reduction of ARB often varied greatly. It is possible that long-term investigation might cover short-term behaviors of various kinds of ARB. Actually no significant change of the ARB proportion was observed throughout the WWTP treatment process (Fig. S1). This result indicated that the WWTPs did not result in a selective reduction of antibiotic resistance over a long-term period. Previous reported observations that bacteria carrying some resistance genes were more difficult to be removed [19, 20], seemed to be occasional and transient.

Release of bacteria and genes resistant to antibiotics through the effluent and biosolids

Although more than 99% of ARB and ARGs were removed by biological treatment and UV disinfection, the WWTPs effluent still represents an important release source of antibiotic resistance, threatening the safety of receiving water body or water reuse. Besides, biosolids often carry much higher loads of antibiotic resistance because of the transfer of bacteria from wastewater. Therefore, the characteristics of ARB and the corresponding ARGs in the effluent and biosolids were also explored over a one-year period.

Six kinds of ARB were all detected in both the effluent and biosolids (Fig. 2). VAN- resistant bacteria were the most prevalent kind in the WWTP effluent, with an average concentration of $(3.6 \pm 2.6)\times10^3$ CFU/mL and a proportion over 67%. Similarly, a high prevalence of VAN-resistant bacteria was also detected in the biosolids, with an average concentration of $(8.8 \pm 5.1)\times10^5$ CFU/g and a proportion over 65%. An investigation in European WWTPs also reported that the bacterial proportion resistant to VAN was up to (19–62)% in WWTP effluents [21]. The ratio of VAN-resistant bacteria in biosolids and effluent is about 250, much higher than 1 (Fig. 3). It indicated that enrichment of ARB in biosolids is the distribution trend of ARB. In other words, most ARB moved into biosolids from effluent. Thus, biosolids became the main discharge channel of ARB.

CEP-, SD- and ERY-resistant bacteria were the other three dominant kinds with each proportion over 30% in both effluent and biosolids. Their prevalence was

considered to be relevant to wide application in China [22]. By comparison, low abundances of GEN- and TC- resistant bacteria were detected, with concentrations of (291 ± 236) CFU/mL and (160 ± 115) CFU/g, respectively, in the WWTP effluent. Their proportions were always below 10% in all effluent and biosolid samples.

However, the corresponding ARGs behaved differently. The gene of *vanA* was detected in the effluents and biosolids with quite low proportions [$(2.2 \pm 0.8)\times10^{-10}$ and $(2.3 \pm 1.6)\times10^{-11}$]. Very few studies had previously explored the occurrence of vancomycin resistance genes in the environment, although they had been detected in clinical treatment since 1986. Compared to high abundance of CEP-resistant bacteria, low gene prevalence of *ampC* was noticed with proportions of $(6.2 \pm 3.2)\times10^{-9}$ and $(5.0 \pm 1.9)\times10^{-10}$ in effluent and biosolids, respectively. So far, over 50 genes encoding resistance to *β*-lactam have been detected in the aquatic environment, and it was possible that *ampC* might not be the most prevalent class in this WWTP.

In this study, *sulI* was the most prevalent one among the six kinds of ARGs in both the effluent and biosolids. The average concentration in the effluent and biosolids were $(1.8 \pm 1.3)\times10^{7}$ copies/100mL and $(1.1 \pm 0.8)\times10^{9}$ copies/100g, respectively. *SulI* has been widely detected in the environment [14, 23]. In a case study of pathogenic *E. coli* from various livestock in Switzerland by Lanz et al. [24], about 70% of the sulfonamide-resistant isolates from pigs could be explained by the presence of sul(I) and sul(II). The *ereA* gene was also one of the most prevalent ARGs, with average concentrations of $(9.5 \pm 5.2)\times10^{6}$ copies/100mL and $(5.4 \pm 2.8)\times10^{8}$ copies/100g in the effluent and biosolids, respectively. This result is consistent with

our previous study [12], where the *ereA* concentration was found to be much higher than the other three ERY resistance genes (*ereB*, *ermA* and *ermB*). The genes of *aacC1* and *tetA* were also detected in the WWTP.

Although all ARB and corresponding ARGs were detected in all samples for each month, it was found that the ARB abundance in the WWTP effluent was quite different from the corresponding ARGs behaviors. Actually statistical analysis showed that there was no statistical relevance between all ARB abundances and their corresponding genes in the long-term investigation ($p > 0.05$). It was considered that not only one kind of genes encoding resistance to the same antibiotic probably caused the attempt to find the relevance between ARB and corresponding ARGs rather difficult. The investigation of other ARGs resistant to the antibiotic might help to better explore this relevance.

In general, significantly higher concentrations of all ARB and ARGs were detected in the biosolids compared to the effluent. This result points out that the potential health risk of antibiotic resistance through WWTP release, especially the biosolids part, needs more attention. Absolutely low abundance of ARB/ARGs in WWTP effluent did not indicate the 'reduction' of antibiotic resistance, as most of them was just transferred to the biosolids.

Seasonal change of release of bacteria and genes resistant to six antibiotics through the effluent and biosolids

The sampling season imposed significant influence on the release load for all ARB in the effluent and biosolids, shown in Fig. 4 (TC-resistant bacteria/genes as an

example) and Fig. S2. It was found that the distributions of ARB/ARGs in the WWTP effluent and biosolids were quite similar over the seasonal investigation. For all antibiotic resistant bacteria, much higher release loads were detected in the spring, with release loads of ($2.8 \times 10^{13} – 5.7 \times 10^{14}$) CFU/d. The ARB release load decreased significantly in the summer, with release loads of ($2.8 \times 10^{12} – 2.7 \times 10^{14}$) CFU/d. The releases of most ARB further decreased in the winter. Similarly, high release loads through the biosolids were detected in spring and autumn, while the values decreased in the winter. The release load was much higher than that in a US WWTP, where more than 10^{11} CFU/d of ARB were discharged through WWTPs effluents [8].

However, the release load for most ARGs changed only slightly in both the effluent and biosolids over different seasons (Fig. 4 and Fig. S2). The antibiotic resistance release was thought to be partially influenced by the sampling temperature. spring provided suitable conditions for bacterial growth, while low temperatures in the winter inhibited bacterial growth. By comparison, most ARGs exhibited more tolerance to temperature, which might partially be connected to gene transfer. The WWTP provides a suitable environment for horizontal gene transfer, making it possible that ARGs keep alive even when their host could not survive. Further explanation about the variation of antibiotic resistance release as a function of season needs to be explored.

Effect of wastewater quality and operational conditions on ARB and ARGs releases

The inconsist behavior between all ARB and ARGs should be noted, which indicates that bacteria and genes should both be considered in exploring resistance characteristics in wastewater. Therefore, both the ARB and ARGs proportion in the effluent were all assessed as a function of wastewater quality and operational conditions in the following.

According to multivariate analysis (RDA), most ARB and ARGs proportions were positively related to the COD and turbidity of the raw sewage (Fig. 5). The absolute inflow quality strongly affected the distribution of antibiotic resistant bacteria. It was expected as the organic load is a source of nutrients, and suspended solids are often the carrier of bacteria in wastewater. Complicated inflow quality often signified high abundances (HPCIN) and proportions of ARB in the raw sewage, and they were likely to maintain their prevalence during the treatment process. This result is supported by Novo et al. [25], who reported that the COD of the raw sewage greatly affected bacterial community diversity.

By contrast, the COD and turbidity of the effluent were negatively correlated to the proportion of ERY-, CEP-, TC- and GEN-resistant bacteria, as well as *vanA*, *ampC*, *tetA* and *aacC1* in the effluent. The reason might be related to the WWTP treatment efficiency. Actually a positive relationship between COD, turbidity and HPC abundance was observed in Fig. 5, indicating HPC value of the effluent might increase when COD and turbidity increased. Besides, data indicated that the increase of HPC value of the effluent was often accompanied with the decrease of most ARB proportion. Therefore, the decrease of ARB proportion was probably connected with

the increase of COD and turbidity of the effluent. DO imposed a strongly negative correlation with ARB proportion, except VAN-resistant bacteria. Most ARGs abundances were also inhibited by a high oxygen condition, except *ereA* and *sulI*. It was documented that high oxygen concentrations may weaken the prevalence of bacterial resistance. Threedeach et al. found that the proportions of isolates resistant to most antibiotics were higher in the anaerobic leachate than in the semi-aerobic leachate [9]. HØiby et al. also reported that a low oxygen condition was responsible for the development of tolerance to antibiotics [26].

Similar to the oxygen concentration, the temperature also had a negative correlation with most ARB and ARGs prevalence. This result is also supported by Fig. 4, where samples collected in the summer and autumn exhibited lower antibiotic resistance level compared to winter and spring. It was considered that bacteria possessed high activity and thus is difficult to settle in the secondary sedimentation tank. Large amount of bacteria still existed in final effluent, which then caused the decrease of ARB relative abundances, similar to the effect of high effluent turbidity.

In general, the results obtained in this study suggest that a relationship exists between environmental variables and antibiotic resistance in WWTP effluents and is rather complex. Some environmental variables are self-relevant and thus further promote the complexity. Further studies, involving other environment variables may help to elucidate this complex relationship.

Conclusions

Wastewater treatment process exhibited a significant reduction in the ARB and relevant ARGs concentrations in the WWTP. The biological treatment process contributed to a significantly higher reduction of antibiotic resistance level compared to UV disinfection. No significant difference on the total removal levels of various ARB/ARGs were detected in the seasonal study, indicating WWTPs did not result in a selection of antibiotic resistance reduction over a long-term period.

No statistical relevance between all ARB abundances and their corresponding genes was found over the long-term investigation. The inconsist behavior indicates that bacteria and genes should both be considered in exploring resistance characteristics in wastewater.

Antimicrobial resistance release seemed to be affected by the wastewater quality and operational conditions. RDA analysis implied that most ARB and ARGs proportions were positively correlated to the COD and turbidity of the raw sewage, while negatively related to the corresponding variables in the effluent, as well as DO and temperature. However, since many other environmental variables, such as environmental pressure and bacterial community, also impose complex effects on ARB/ARGs in WWTPs, a further exploration may still be needed.

Acknowledgements

This project was founded by Shanghai Natural Science Foundation (13ZR1443300) and National Natural Science Foundation of China (51308399). The authors would like to thank the engineers of WWTP for their assistance in obtaining the wastewater samples. The authors also express their gratitude to Prof. James Bolton

for writing improvement.

References

1. R. Szczepanowski, B. Linke, I. Krahn, K. H. Gartemann, T. Gutzkow, W. Eichler, A. Puhler and A. Schluter, *Microbiol.*, 2009, **155**(7), 2306–2319.
2. X. X. Zhang and T. Zhang, *Environ. Sci. Technol.*, 2011, **45**(7), 2598–2604.
3. M. Guo, Q. Yuan, J. Yang, *Water Res.*, 2013, **47**(16), 6388–6394.
4. C. Bouki, D. Venieri and E. Diamadopoulos, *Ecotoxicol. Environ. Saf.*, 2013, **91**(1), 1–9.
5. H. C. Su, G. G. Ying, L. Y. He, Y. S. Liu, R. Q. Zhang and R. Tao, *Environ. Sci. Process. Impacts*, 2014, **16**(2), 324–332.
6. J. J. Huang, H. Y. Hu, S. Q. Lu, Y. Li, F. Tang, Y. Lu and B.Wei, *Environ. Int.*, 2012, **42**, 31–36.
7. S. Borjesson, S. Melin, A. Matussek and P. E. Lindgren, *Water Res.*, 2009, **43**(4), 925–932.
8. M. Munir, K. Wong and I. Xagoraraki, *Water Res.*, 2011, **45**(2), 681–693.
9. S. Threedeach, W. Chiemchaisri, T. Watanabe, C. Chiemchaisri, R. Honda and K. Yamamoto, *Biores. Technol.*, 2012, **113**, 253–258.
10. S. Kim, J. N. Jensen, D. S. Aga and A. S. Weber, *Chemosphere*, 2007, **66**(9), 1643–1651.
11. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing: twenty-first informational supplement, 2011, 31(1).
12. M. Guo, Q. Yuan, J. Yang, *Chemosphere*, 2013, **93**, 2864–2868.

13. H. Heuer, E. Krögerrecklenfort, E. M. H. Wellington, S. Egan, J. D. van Elsas, L. van Overbeek, J. -M. Collard, G. Guillaume, A. D. Karagouni, T. L. Nikolakopoulou and K. Smalla, *FEMS Microbiol. Ecol.*, 2002, **42**, 289–302.

14. R. Pei, S. C. Kim, K. H. Carlson and A. Pruden, *Water Res.*, 2006, **40**(12), 2427–2435.

15. N. Woodford, D. Morrison, A. P. Johnson, V. Briant, R. C. George and B. Cookson, *J. Clinical Microbiol.*, 1993, **31**(3), 653–688.

16. G. Muyzer, G. C. De Waal and A. Uitterlinden, *Appl. Environ. Microbiol.*, 1993, **59**(3), 695–700.

17. T. R. Burch, M. J. Sadowsky and T. M. Lapara, *Front. Microbiol.*, 2013, **4**(17), 1–9.

18. J. J. Huang, H. Y. Hu, Y. H. Wu, B. Wei and Y. Lu, *Chemosphere*, 2013, **90**(8), 2247–2253.

19. G. Morozzi, R. Sportolari, G. Caldini, G. Cenci and A. Morosi, *Zbl. Bakt. Hyg. B*, 1988, **185**, 340–349.

20. S. R. Andersen, *Curr. Microbiol.*, 1993, **26**, 97–103.

21. A. R. Blanch, J. L. Caplin, A. Iversen, I. Kuhn, A. Manero, H. D. Taylor and X. Vilanova, *J. Appl. Microbiol.*, 2003, **94**, 994–1002.

22. P. Gao, D. Mao, Y. Luo, L. Wang, B. Xu and L. Xu, *Water Res.*, 2012, **46**(7), 2355–2364.

23. A. Pruden, R. T. Pei, H. Storteboom and K. H. Carlson, *Environ. Sci. Technol.*, 2006, **40**(23), 7445–7450.

24. R. Lanz, P. Kuhnert and P. Boerlin, *Microbiol.*, 2003, **91**, 73–84.

25. Novo, S. Andre, P. Viana, O. C. Nunes and C. M. Manaia, *Water Res.*, 2013, **47**(5), 1875–1887.

26. N. HØiby, T. Bjarnsholt, M. Givskov, S. Molin and C. Oana, *Int. J. Antimicro. Agents*, 2010, **35**, 322–332.

27. L. Guardabassi, D. M. Lo Fo Wong and A. Dalsgaard, *Water Res.*, 2002, **36**(8), 1955–1964.

28. T. M. Lapara, T. R. Burch, P. J. McNamara, D. T. Tan, M. Yan and J. J. Eichmiller, *Environ. Sci. Technol.*, 2011, **45**, 9543–9549.

Table 1 Primers of six ARGs and 16S rRNA gene.

Gene	Encoding resistance	Primer forward (5′–3′)	Primer reverse (5′–3′)	Fragment size /bp	Annealing Tempera-ture /℃	Reference
ampC	CEP	CCTCTTGCTCCAC ATTTGCT	ACAACGTTTGCTG TGTGACG	189	57	1
ereA	ERY	TCTCAGGGGTAA CCAGATTGA	TTATACGCAAGGT TTCCAACG	138	57	12
aacC1	GEN	TCATCAATCCCCT CAAGCAT	AAGTGCATCACTT CTTCCCG	130	58	13
sulI	SD	CGCACCGGAAAC ATCGCTGCAC	TGAAGTTCCGCCG CAAGGCTCG	163	56	14
tetA	TC	GCTACATCCTGCT TGCCTTC	CATAGATCGCCGT GAAGAGG	210	56	2
vanA	VAN	ATGGCAAGTCAG GTGAAGATGG	TCCACCTCGCCAA CAACTAACG	399	58	15
16S rRNA	—	CCTACGGGAGGC AGCAG	ATTACCGCGGCTG CTGG	193	58	16

Table 2 Removal of ARB in WWTPs of different countries or regions.

ARB	Environmental source	Treatment process	Log removal	Reference
TC- and SD-resistant bacteria/genes	Five WWTPs in Michigan	MBR, activated sludge process	2.4–4.6 (activated sludge); 2.6-7.1 MBR)	8
TC- and SD-resistant bacteria/genes	East Lansing WWTP in Michigan	activated sludge process	2–3	22
AMP-, GEN- and TC- resistant coliforms and *acinetobacter*	Two WWTPs in Denmark	activated sludge process	1–3	27
VAN- and ERY-resistant *enterococcus*	14 WWTPs in Sweden Portugal and Spain	activated sludge process	0.9–3.1	21
TC- resistant *E.coli*	Lab-scale process	UV disinfection or chlorination	4.1 (UV 10mJ/cm^2); 5.0 (10 mg Cl_2 min/L)	18
ERY- and TC-resistant bacteria	Lab-scale process	UV disinfection	1.9–3.0	12

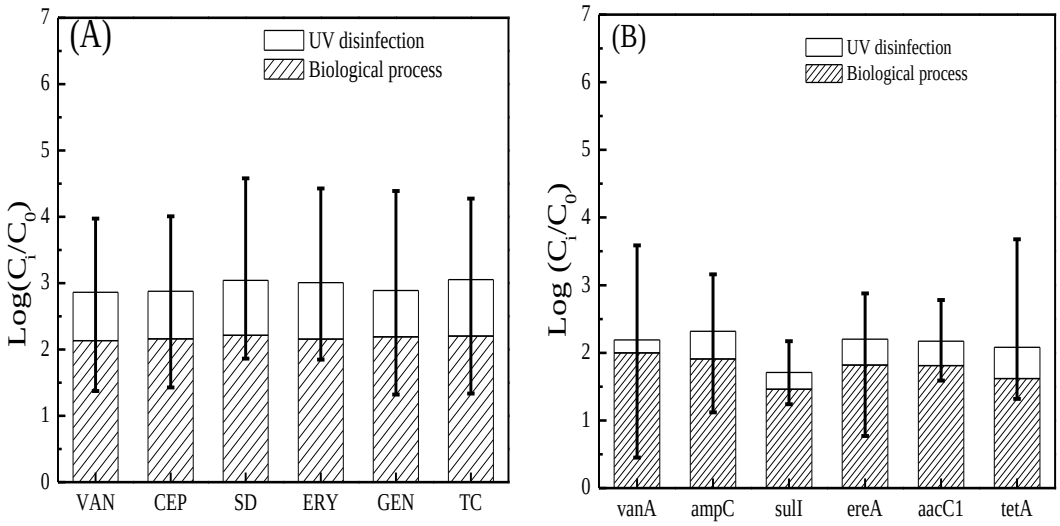


Fig. 1 Log reduction of six kinds of ARB (A) and relevant ARGs (B) concentrations through biological treatment process and UV disinfection over one year. For the biological process, C_i and C_0 represent ARB/ARGs concentrations in the effluent of the secondary sedimentation tank and raw sewage, respectively; for UV disinfection, C_i and C_0 represent ARB/ARGs concentrations in the final effluent and the effluent of secondary sedimentation tank, respectively. VAN, CEP, SD, ERY, GEN and TC represent total heterotrophic bacteria resistant to vancomycin, cephalexin, sulfadiazine, erythromycin, gentamicin and tetracycline, respectively. VanA, ampC, sulI, ereA, aacC1 and tetA represent genes encoding resistance to the six kinds of antibiotics. Error bars indicate the deviation of each ARB/ARG values over 12 months.

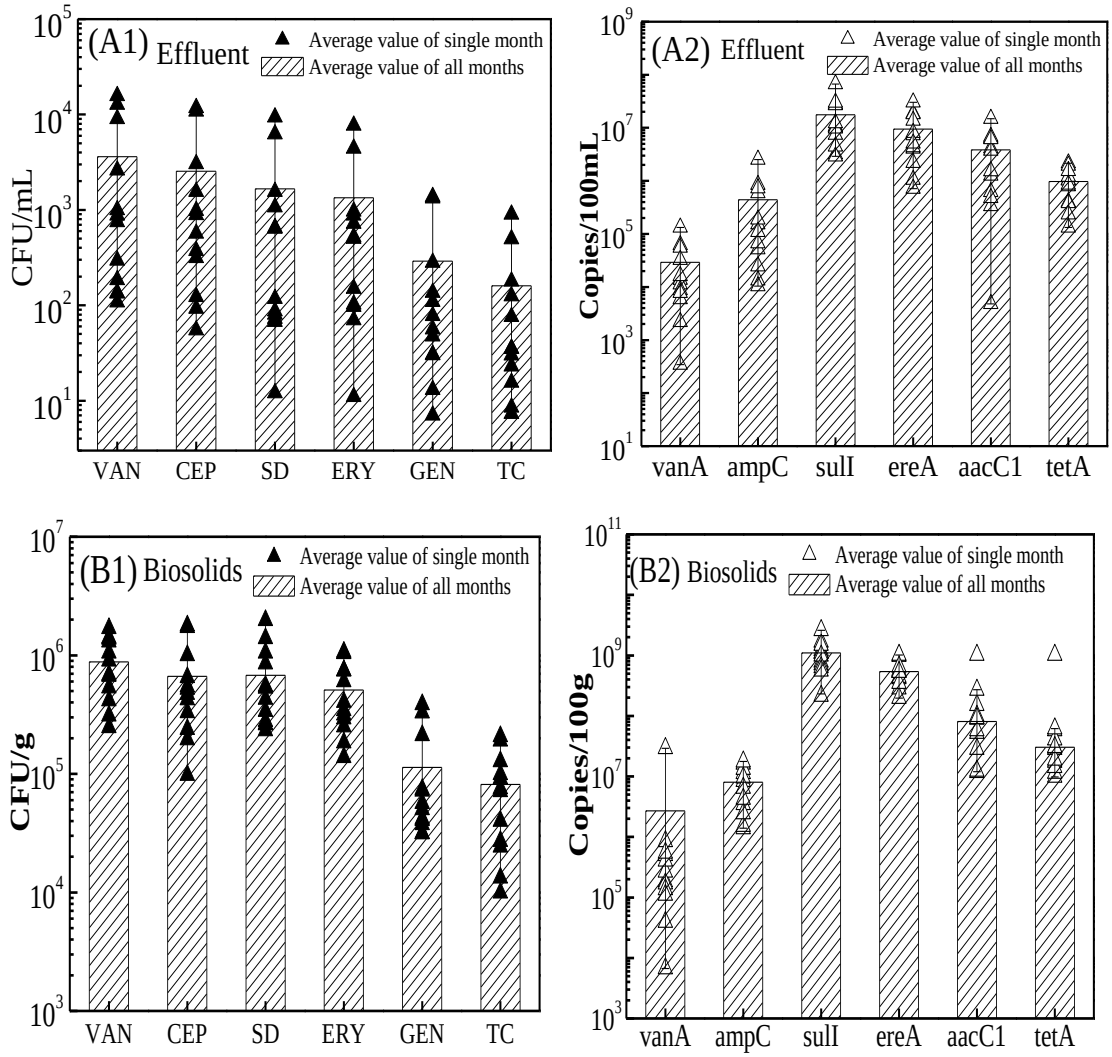


Fig.2 Concentration of six kinds of ARB and corresponding ARGs in the WWTP effluent (A1, B1) and biosolids (A2, B2).

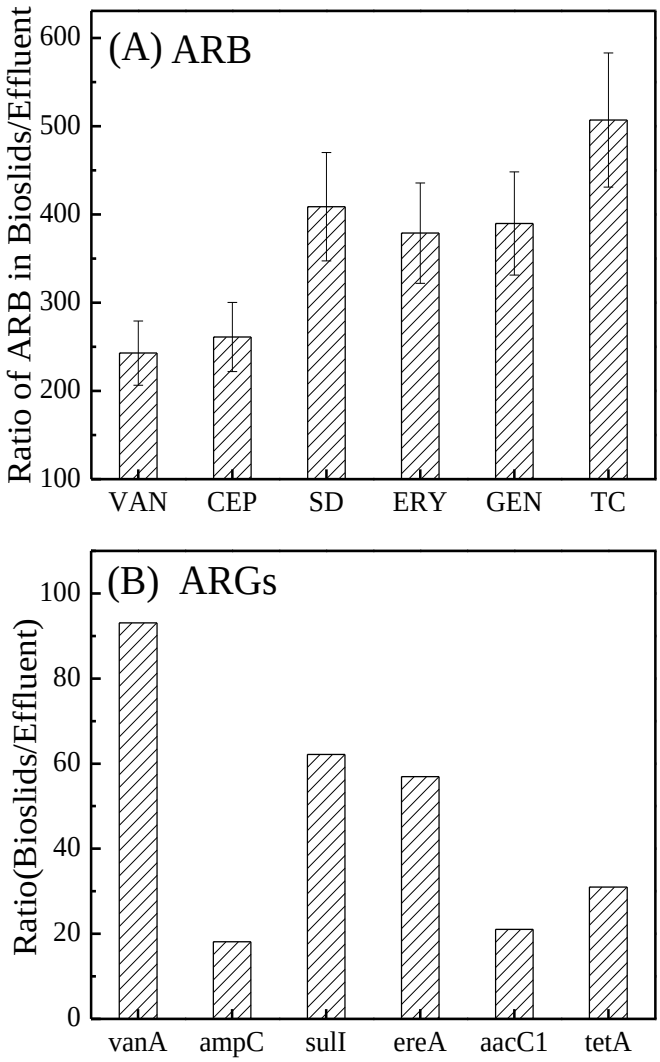
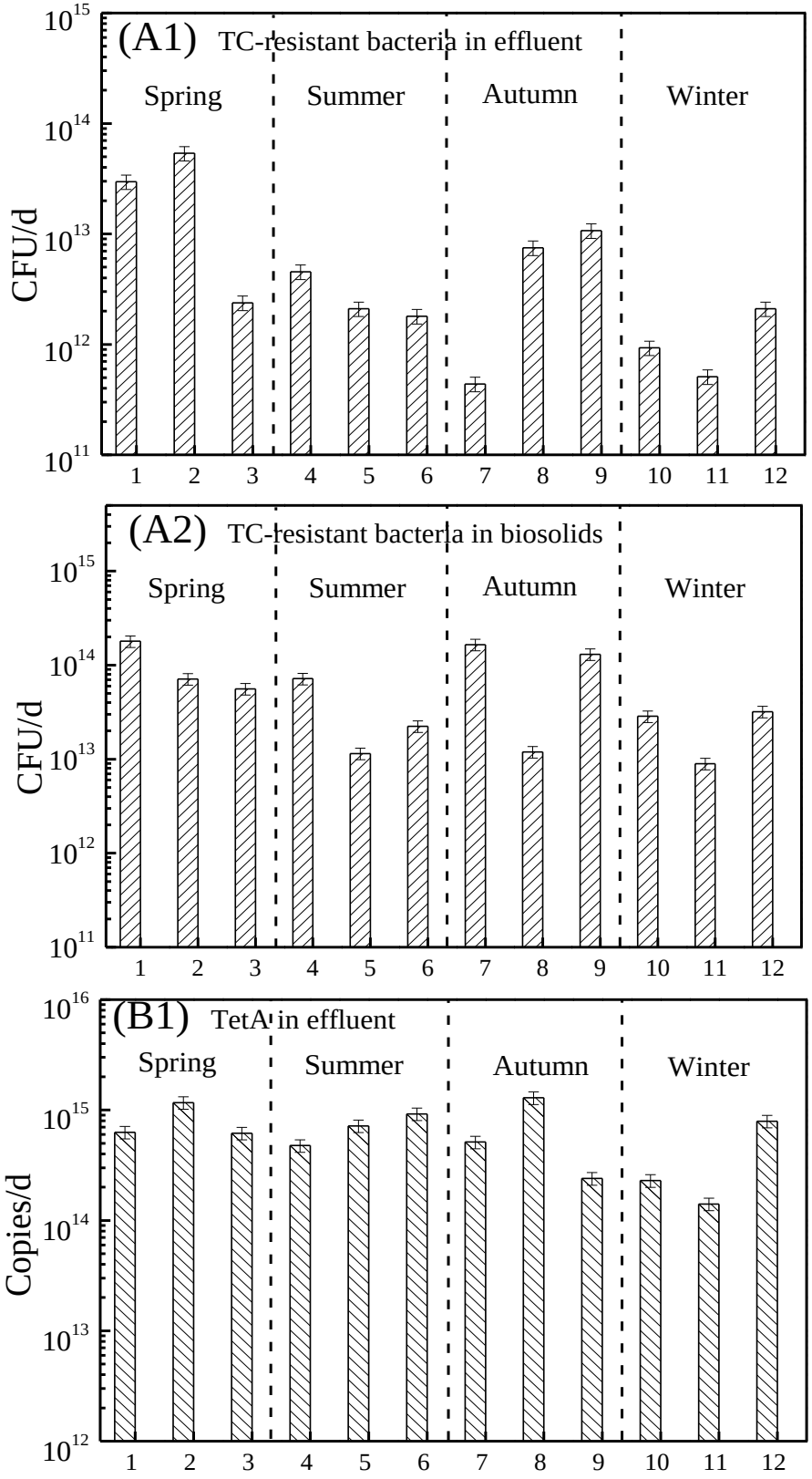


Fig. 3 The ratio between the concentration of six kinds of ARB (A) or ARGs (B) in biosolids and effluent.



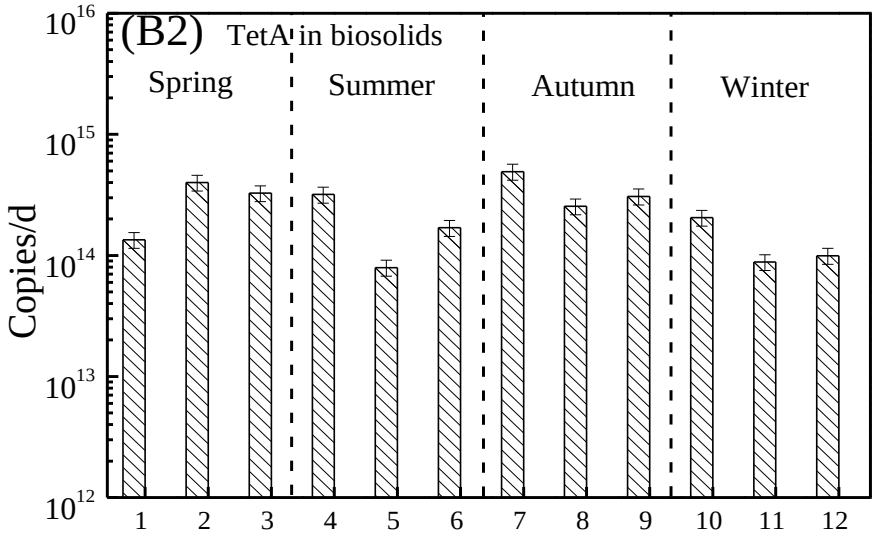


Fig.4 The release loads of TC-resistant bacteria (A1, A2) and tetA (B1, B2) through the effluent and biosolids over the whole year. The number in the abscissa represent the release load of ARB/ARGs in each month over the whole year (the spring represents Mar 2013 to May 2013; the summer represents June 2013 to Aug 2013; the autumn represents Oct 2012, Nov 2012 and Sep 2013; the winter represents Dec 2012 to Feb 2013).

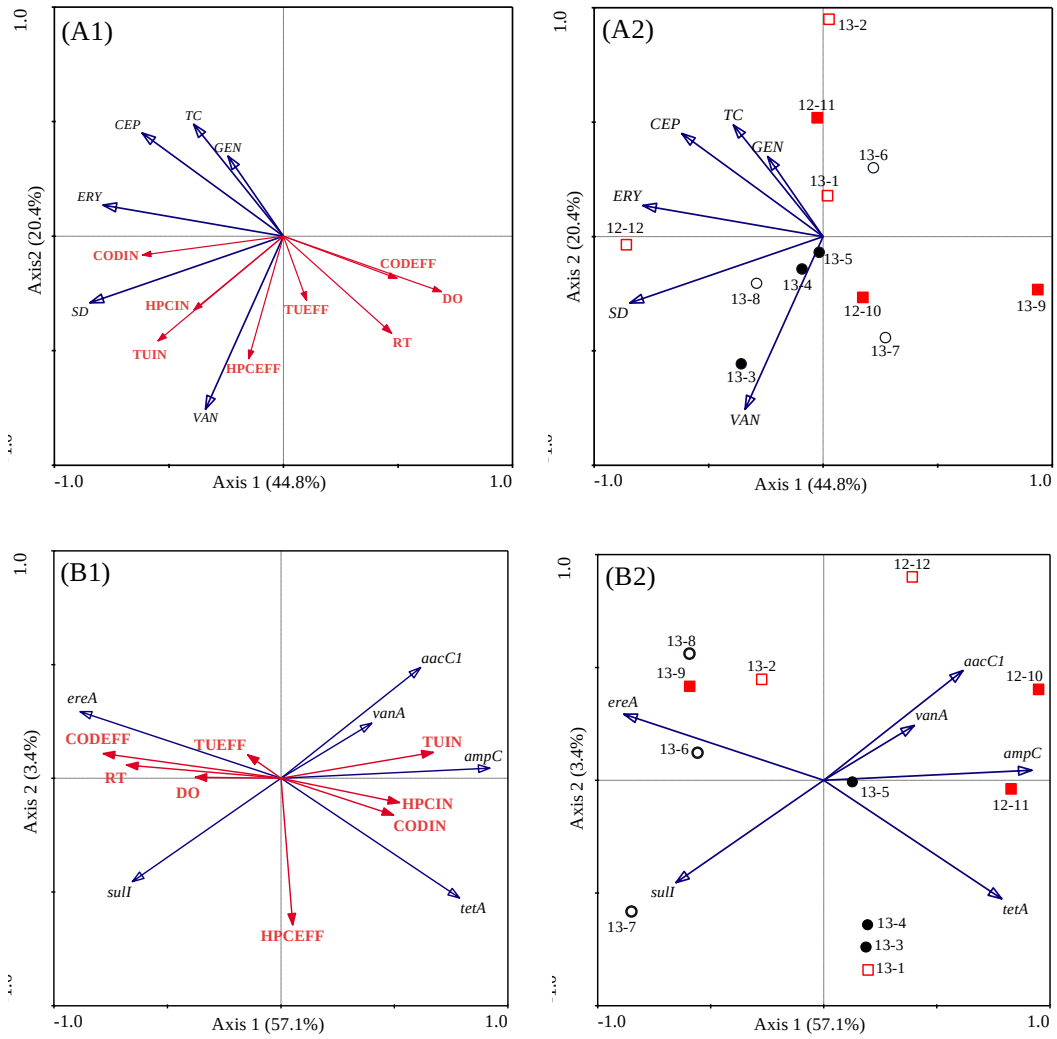


Fig. 5 Redundancy Analysis (RDA) of proportions of six kinds of ARB (A) and corresponding ARGs (B) in function of environmental variables, including the wastewater quality (air temperature, COD, turbidity and HPC abundance in raw sewage and final effluent) and operational conditions (MLSS, DO and hydraulic loading rate). A1 and B1: relations between ARB/ARGs proportions and environmental variables; A2 and B2: relations between ARB/ARGs proportions and samples. Only the variables significantly ($p < 0.05$) explaining ARB/ARGs proportion variation are shown and their inter-set correlation values are indicated. ARB/ARGs

proportions are represented by blue hollow arrows; environmental variables are represented by red solid arrows.