

# The Effect of Antibiotics on Consortium Structure in Effluent and Sludge from a Municipal Wastewater Treatment Plant

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**Abstract.** The distribution and diversity of antibiotic-resistance bacteria (ARB) from the excess sludge and effluent of Kaifeng wastewater treatment plant was studied using a culture-dependent method. In comparison with other phylum, microbial community analysis confirmed Proteobacteria was more susceptible to possess the ability to tolerate antibiotic pressure. With the addition of antibiotics from single erythromycin to three antibiotics, the relative abundance of Proteobacteria increased to 92.98%, 94.35%, 95.74% in water sample and 84.33%, 90.99%, 90.84% in sludge samples. *Acinetobacter* in Proteobacteria has stronger antibiotic resistance. This research identified that in the process of sewage treatment, attention should be paid to the removal of species in Proteobacteria to avoid potential resistance gene contamination in the receiving environments.

## 1 Introduction

Antibiotics have been widely used in veterinary and human medicine, as well as in agricultural disease control, due to their significant role in controlling microbial infections [1]. However, since the broad application of antibiotics, large amounts of these drugs are often overused and enter wastewater treatment plants (WWTPs) through the sewage collection system [2]. Multiple studies have reported high concentrations of antibiotics at the mg/L level within these environments [3,4]. The prolonged exposure to antibiotics creates selective pressure, leading to the widespread distribution of antibiotic resistant bacteria (ARB) and antibiotic resistance genes (ARGs) [5].

ARB are microorganisms that can withstand the effects of antibiotics. If a microorganism can resist three or more types of antibiotics, it is classified as a multidrug-resistant organism (MDRO). These genes are capable of replication and transfer. For example, Jin et al. [6] studied urban wastewater in China and found that sulfonamide-resistant bacteria had the highest proportion, and tetracycline and erythromycin resistant bacteria were also prevalent. Huang et al. [7] also detected a large number of multidrug resistant bacteria in the effluent of a sewage treatment plant.

In recent years, the occurrence of ARB has been extensively studied in WWTPs in developed countries and cities [8]. However, few studies have investigated the occurrence of ARB in underdeveloped areas and small cities [9]. Many small-scale sewage treatment plants have

focused only on the removal of biological oxygen demand (BOD) and nutrients (N and P), without incorporating tertiary treatment. Secondary treatment has been found to have a relatively low effectiveness in removing ARB and ARGs. Moreover, reports suggest that WWTPs may even promote the proliferation of ARBs and the horizontal transfer of ARGs [10,11]. Therefore, the effluent and excess sludge of small-scale sewage treatment plants are expected to contain large amounts of ARBs, which pose a significant environmental risk.

This research focuses on a small-scale municipal sewage treatment plant using oxidation ditch process to identify the potential for ARB pollution. Three antibiotics (erythromycin, tetracycline, and norfloxacin) were added to effluent and excess sludge samples to select for resistant communities. The primary objectives of this study were to (1) identify the ARB present in the activated sludge and effluent water, (2) describe the diversity and abundance of bacterial communities at the selected wastewater treatment plant.

## 2 Materials and Methods

### 2.1 Sample collection

Effluent water and excess sludge samples are collected from a small municipal wastewater treatment plant located in Kaifeng. The collected water samples were stored in a large plastic bucket, while excess sludge was wrapped in black plastic. Samples are immediately transported to the

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laboratory in an icebox and stored at 4°C until the experiments are completed.

## 2.2 Reagents

The standards of antibiotics about tetracycline (Te), erythromycin (Er), norfloxacin (No) were purchased from Sinopharm Chemical Reagent co., Ltd (Shanghai, China). The DNAiso Reagent, Taq DNA polymerase, RNAiso Plus, DNase I, cDNA synthesis kit and SYBR® Premix Dimer Eraser™ were purchased from TaKaRa (Dalian, China).

## 2.3 Cultivation of antibiotic-resistant microbial communities

The antibiotic resistant bacterial consortium were enriched into six flasks (500mL) by SSDM as previously described [12]. Three of these flasks were added with effluent water (5mL) and the other three were added with excess sludge (1g). In order to obtain antibiotic resistant bacteria consortium, erythromycin, tetracycline and norfloxacin were selected and added into these flasks respectively. Apart from these six flasks, two flasks without antibiotics addition were also settled. The settled treatments were shown in Table 1.

**Table 1** Treatment settling

Treatment	Name	Sample	Antibiotic
1	ew-D	Effluent water	None
2	ew	Effluent water	None
3	ew-e	Effluent water	Er
4	ew-e-t	Effluent water	Er+Te
5	ew-e-t-n	Effluent water	Er+Te+No
6	es-D	Excess sludge	None
7	es	Excess sludge	None
8	es-e	Excess sludge	Er
9	es-e-t	Excess sludge	Er+Te
10	es-e-t-n	Excess sludge	Er+Te+No

## 2.4 DNA extraction

The genome DNA were extracted from bacterial consortium ef-D, ef, ef-e, ef-e-t, ef-e-t-n, ex-D, ex, ex-e, ex-e-t, ex-e-t-n (The specific naming method is shown in Table 1). After transferred one time, using FsatDNATMSPIN Kit according to the manufacturer's instructions. The DNA was used for Illumia Miseq sequencing analysis with the V3-V4 region. The V3 and V4 region was amplified using forward primers containing the sequence "CCTACGRRBGCASCAGKVRVG AAT" and reverse primers containing the sequence "GGACTACNVGGGTWTCTAATCC" (TISINGKE Biological technology). The raw data was controlled and analyzed using QIIME (1.9.1). All reads were classified into operational taxonomic units (OTUs) at 97% similarity.

## 2.5 consortium structure analysis

The ACE, Chao1, Shannon index, and Simpson index were used to evaluate the diversity of consortiums structure. The ACE index was calculated according to the following formula using R programming language (4.2.0).

$$S_{ACE} = S_{Ab} + \frac{S_{ra}}{C_{ACE}} + \frac{F_1}{C_{ACE}} \gamma^2 \quad (1)$$

Among formula 1:

$S_{Ab}$  stands for the number of OTU above 10 reads

$S_{ra}$  stands for the number of OTU less than or equal to 10 reads

$F_1$  stands for the number of OTU with 1 reads

$$C_{ACE} = 1 - \frac{F_1}{S_{ra}}$$

$$\gamma^2 = \max \left[ \frac{N_{ra} \sum_{i=1}^{10} i(i-1)F_i}{C_{ACE} N_{ra} (N_{ra} - 1)} - 1, 0 \right]$$

The Chao1 index was calculated using formula 2:

$$S_{chao1} = S_{obs} + \frac{F_1(F_1 - 1)}{2(F_2 + 1)} \quad (2)$$

Among formula 2:

$S_{obs}$  stands for the number of OTUs with more than or equal to 1 reads

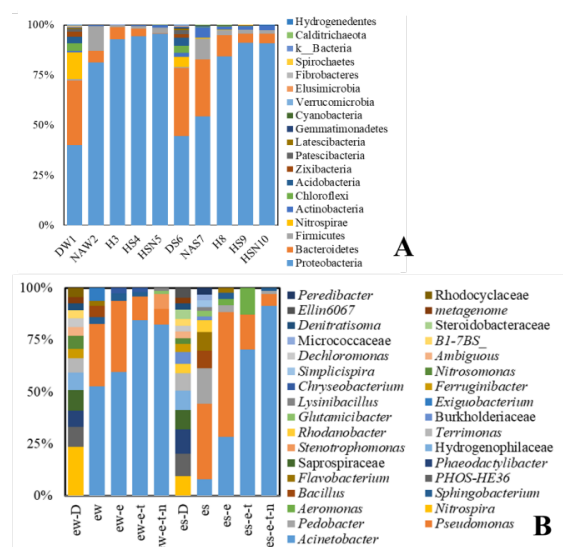
$F_1$  stands for the number of OTUs with 1 reads

$F_2$  stands for the number of OTUs with 2 reads

## 3 Results and Discussion

### 3.1 Compositions of microbial communities

In the case of adding of zero to three antibiotics, there was a significant change in the microbial communities in effluent samples and excess sludge samples, as depicted in Fig. 1. The figure shows the relative abundance of the different phyla (Fig. 1a) and the bacterial genus with an abundance of more than 1% (Fig. 1b), while abundances below 1% were classified as "others".



**Fig. 1** Composition of microbial community structure at phylum (a) and genus (b) levels

The analysis of effluent water samples (ew-D) revealed a high diversity of genera (Shannon diversity index of 7.536), with Nitrospira (Nitrospirae), Phaeodactylibacter (Phaeophyta), Saprospiraceae (Bacteroidetes), Hydrogenophilaceae (Proteobacteria), and Terrimonas (Bacteroidetes) as dominant genera. In the absence of antibiotics, the predominant phylum was Proteobacteria, accounting for approximately 81.29% of the total in ew, with *Acinetobacter* and *Pseudomonas* being the predominant genera (80.84%). However, with the addition of antibiotics, the relative abundance of Proteobacteria increased to 92.98%, 94.35%, and 95.74% in water samples from single erythromycin to three antibiotics (erythromycin, tetracycline, norfloxacin), respectively. On the other hand, the relative abundance of Bacteroidetes decreased from 5.72% to 0.02%, while the relative abundance of *Acinetobacter* increased. Additionally, *Stenotrophomonas* had the highest abundance in ew-e-t-n (7.14%).

In excess sludge samples (es-D), there was also a high diversity of genera (Shannon diversity index of 7.696), with the dominant genera being similar to those in ew-D. In the absence of antibiotics, the predominant phylum was Proteobacteria, accounting for approximately 54.33% of the total in es, with *Acinetobacter* and *Pseudomonas* being the predominant genera (36.94%). However, with the addition of antibiotics, the relative abundance of Proteobacteria increased to 84.33%, 90.99%, and 90.84% from single erythromycin to three antibiotics (erythromycin, tetracycline, norfloxacin), respectively. Conversely, the relative abundance of Bacteroidetes decreased, ranging from 28.45% to 4.78%. The relative abundance of *Acinetobacter* increased. Furthermore, *Aeromonas* had the highest abundance in es-e-t. Therefore, it is important to pay attention to the elimination of species belonging to the Proteobacteria in the oxidation ditch process of the wastewater treatment plant in order to avoid potential contamination of resistance genes in the receiving environment.

When multiple antibiotics were added to the effluent and sludge samples, the Nitrospirae phylum almost disappeared and the relative abundance of several bacterial phyla, including Firmicutes and Actinobacteria, significantly decreased. This may be attributed to the strong impact of the antibiotics on microbial structure.

### 3.2 Correlation analysis between microbial communities

Principal components analysis (PCA) is a powerful analytical tool for comparing differences between multiple samples. Based on the results presented in Fig. 2a, it can be observed that there is a significant similarity between the microbial communities of the effluent water and excess sludge samples after being cultured without antibiotics. The communities of the ew and es-e samples are almost identical, indicating that they share a high degree of taxonomic similarity. Moreover, the degree of overlap between the es-e-t and es-e-t-n samples is higher, indicating that the addition of two or more antibiotics has a minimal impact on the microbial community

composition of the excess sludge samples at the phylum level. The proximity of the es-e-t-n and ew-e-t-n, as well as the es-e-t and ew-e-t samples at the genus level (Fig. 2b), supports the conclusion that the addition of multiple antibiotics has no significant effect on the microbial community composition of both effluent water and excess sludge samples at the genus level. Notably, es are located at a significant distance from all other samples at both the phylum and genus levels, indicating a unique microbial community composition.

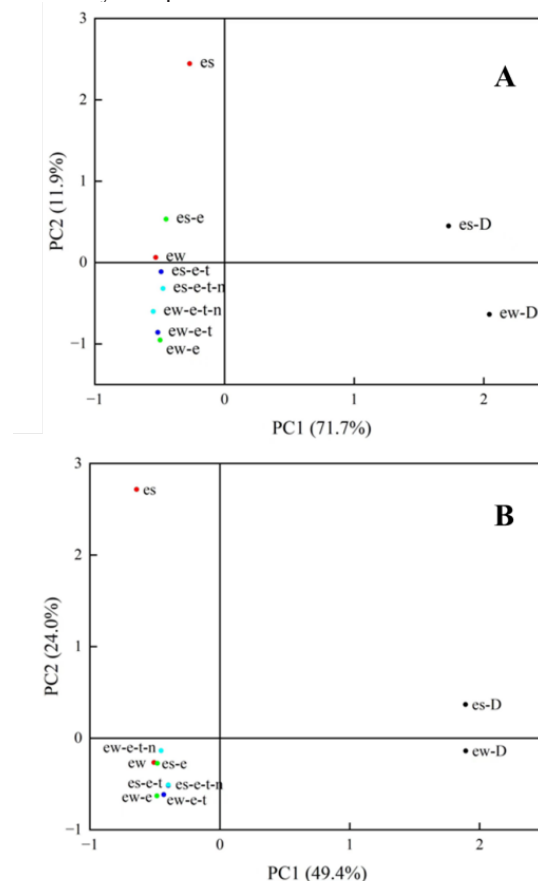


Fig. 2 The result of microbial communities PCA at phylum (a) and genus (b) levels

### 3.3 Diversity of microbial communities

To characterize the microbial community structures under different antibiotic pressures, Chao1, ACE, Shannon index of the community structures were calculated as shown in Table 2. As the values of these indices increase, so does the estimated total number of species and the diversity of the related consortium [13]. In this study, the addition of antibiotics caused a notable decrease in the ACE and Chao1 indices of both effluent and sludge samples. The bacterial sample of effluent water was more affected by the addition of antibiotics than excess sludge. It is believed that this is due to a higher concentration of antibiotics in the excess sludge, which could cause microbes to become less sensitive. The Shannon and Simpson indices, used to reflect the diversity of species within a community [14,15], showed a much greater decrease for effluent samples than for excess sludge samples when exposed to antibiotics. This suggests that the complexity of bacteria

and biomass of dominant bacteria in sludge samples is more moderately affected by the pressure of antibiotics.

**Table 2** Diversity estimation of bacterial communities in excess sludge and effluent water after biological treatment

Sample	ACE	Chao1	Shannon	Simpson
ew-D	<b>797.149</b>	<b>793.04</b>	<b>7.536</b>	<b>0.985</b>
ew	98.712	100	3.211	0.811
ew-e	50.494	50.333	2.644	0.739
ew-e-t	35.146	33.6	2.289	0.593
ew-e-t-n	43.146	35.25	1.612	0.425
es-D	<b>794.647</b>	<b>786.585</b>	<b>7.696</b>	<b>0.988</b>
es	614.33	600.786	5.166	0.917
es-e	588.127	578.714	4.269	0.867
es-e-t	515.434	499	4.533	0.914
es-e-t-n	538.972	535.962	3.403	0.765

Overall, regardless of whether it was water or excess sludge samples, the addition of SSDM medium caused the dominant functional bacteria to become more centralized. The diversity indices demonstrated a clear decline in the diversity of microorganisms after the addition of antibiotics, confirming the expectation that the selected antibiotics would disturb multiple members of the original microbial communities. Similar results were found as a significant decrease in microbial diversity emerged with the addition of antibiotics [16].

### 3.4 Microbial community structure

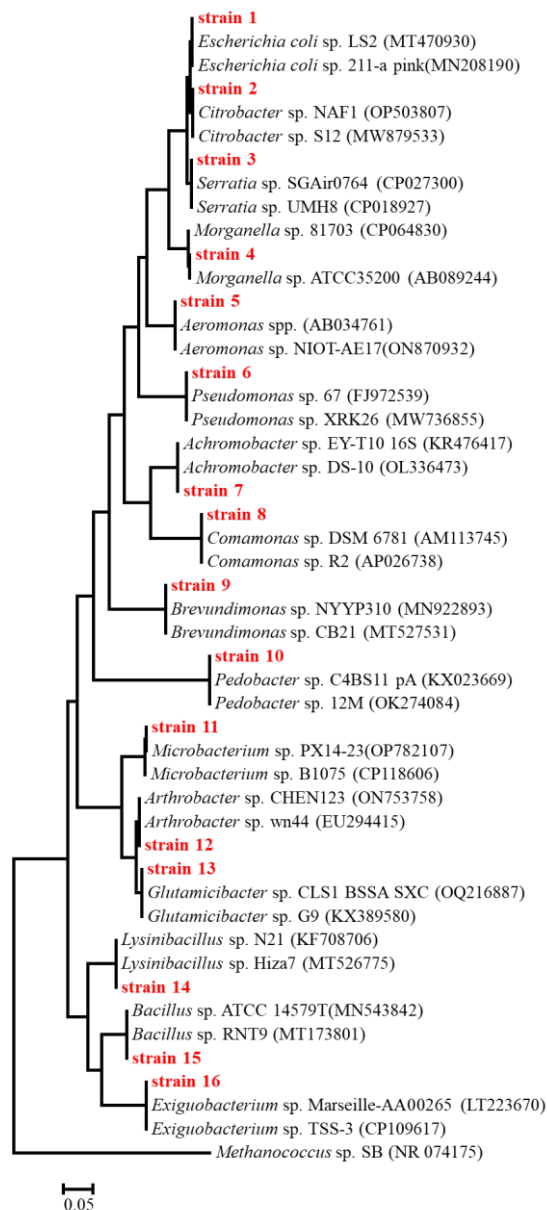
Proteobacteria was found to be the most dominant phylum in the studied microbial communities. The addition of multiple antibiotics caused an increase in the relative abundance and proportion of Proteobacteria. Most of the phosphorus-dissolving and nitrogen-fixing bacteria in sludge belong to Proteobacteria, which plays a crucial role in removing organic matter and nutrients from sewage.

Moreover, the metabolic diversity of Bacteroidetes has enabled them to thrive under the atypical conditions prevalent in wastewater treatment [17]. *Bacillus* exhibits remarkable versatility, including denitrification, organic matter degradation, and the maintenance of activated sludge granules. In this study, it represented the second largest proportion by relative abundance. However, in the presence of multiple antibiotics, its relative abundance sharply declined, consistent with previous findings.

Actinobacteria is associated with sludge swelling, while Firmicutes can utilize hydrolytic enzymes to decompose proteins and sugars in wastewater and sludge. Previous studies have identified four bacterial groups, Proteobacteria, Actinobacteria, Bacteroidetes, and Firmicutes, as major contributors to the microbial community structure of municipal wastewater treatment plant activated sludge [18], effluent [19], and excess sludge [20] samples.

At the genus level, *Acinetobacter*, known for its resistance to antibiotics, exhibited the highest concentration when exposed to antibiotics. Conversely, *Pseudomonas*, a widely recognized clinically resistant bacteria [21], experienced a decline in relative abundance following antibiotic treatment. The inhibitory effect of *Acinetobacter* secretions on *Pseudomonas* has been established [22], supported by our findings that

*Acinetobacter* increased while *Pseudomonas* decreased. Conversely, *Pseudomonas* can inhibit *Acinetobacter* [23]. It is pertinent to note that among the various groups, *Stenotrophomonas* had the highest relative abundance in ef-e-t-n (7.14%), potentially due to the presence of ARGs [24]. meanwhile, using plate coating method and line culture method, several bacteria species were isolated and identified able to tolerate the stress of mixed antibiotics. As shown in Fig. 3, *Escherichia coli* spp., *Citrobacter* spp., *Aeromonas* spp., *Achromobacter* spp., *Brevundimonas* spp., and other species from 16 genus were identified able to survive from the combined antibiotic stress.



**Fig. 3** Phylogenetic tree of isolated ARBs from the effluent and sludge

Additionally, *Stenotrophomonas* may have had a competitive advantage over *Pseudomonas* [25]. Furthermore, *Aeromonas* exhibited the highest relative abundance in ex-e-t. A study has reported that *Aeromonas* is resistant to 12 antibiotics, including erythromycin, tetracycline, neomycin, ampicillin, and ciprofloxacin, and

is a multidrug-resistant bacteria [26]. As can be seen, the relationship of these bacteria needs to be further explored based on the above contradictions and speculation.

### 3.5 Removal of microbial communities

In order to control the microbial risk of WWTP effluent and avoid negative effects of increasing disinfectant dosage on the receiving water body, it is necessary to reduce the biomass of ARB, especially Proteobacteria, to ensure the safety of the effluent water quality. Therefore, for this particular WWTP, the enhanced oxidation ditch process plays a crucial role in the removal of ARB. Oxidation ditch process enhances the contact between wastewater and sludge, leading to better treatment performance, and it is widely applied in urban wastewater treatment. However, the extended retention time of wastewater favors bacterial variation and proliferation, accelerating the horizontal transfer of ARGs, resulting in a significant increase in the variety of ARGs in the effluent. Activated sludge process mainly utilizes adsorption and interception principles to remove ARB from wastewater, through the physical or biological adsorption of the filtering medium and direct interception [27, 28]. Meanwhile, a thorough investigation of the influence and mechanism of hydraulic and water quality parameters on the transfer of resistance genes is needed, and by adjusting the operational parameters, the spread of resistance genes in conventional biological treatment systems can be minimized. The surplus sludge generated from the oxidation ditch process is enriched with a large number of ARB and ARGs, which can enter the soil environment through sludge reuse, posing a threat to human health. Therefore, the subsequent treatment of sludge in the activated sludge process is of great importance, and sludge digestion under high-temperature anaerobic conditions has shown significant removal effects on resistant bacteria and resistance genes [29].

## 4 Conclusions

The study revealed that antibiotic pressure significantly decreased the microbial diversity, leading to an altered composition and structure of the microbial community. Proteobacteria exhibited a higher ability to tolerate antibiotic pressure in comparison to other phyla. Additionally, the relative abundance of Proteobacteria increased to 92.98%, 94.35%, and 95.74% in water samples and 84.33%, 90.99%, and 90.84% in sludge samples upon the addition of antibiotics from single erythromycin to three (tetracycline, erythromycin, norfloxacin).

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