# Interaction between typical sulfonamides and bacterial diversity in drinking water

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## ABSTRACT

The abuse of antibiotics is becoming more serious as antibiotic use has increased. The sulfa antibiotics, sulfamerazine (SM1) and sulfamethoxazole (SMZ), are frequently detected in a wide range of environments. The interaction between SM1/SMZ and bacterial diversity in drinking water was investigated in this study. The results showed that after treatment with SM1 or SMZ at four different concentrations, the microbial community structure of the drinking water changed statistically significantly compared to the blank sample. At the genus level, the proportions of the different bacteria in drinking water may affect the degradation of the SM1/SMZ. The growth of bacteria in drinking water can be inhibited after the addition of SM1/SMZ, and bacterial community diversity in drinking water declined in this study. Furthermore, the resistance gene *sul*2 was induced by SM1 in the drinking water.

**Key words** | antibiotic resistance genes, bacterial diversity, drinking water, SM1, SMZ

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## ABBREVIATIONS

| | |
|---|---|
| SM1 | sulfamerazine |
| SMZ | sulfamethoxazole |
| ARGs | antibiotic resistance genes |
| WWTPs | wastewater treatment plants |
| UPLC | ultra performance liquid chromatograph |

## INTRODUCTION

Since their discovery in the 20th century, antibiotics have been extensively used in medicine and livestock husbandry. However, the abuse of antibiotics is becoming more serious as their use has increased, which has led to the presence of many drug-resistant pathogens (Chapin *et al.* 2005; Schmitt *et al.* 2006), accelerated the spread of antibiotic resistance genes, and increased the amount of antibiotic resistance genes in the environment (Peak *et al.* 2007). Antibiotic resistance genes (ARGs) are becoming recognized as environmental pollutants and action is being sought to preserve the efficacy of antibiotics (Zhu *et al.* 2013). Antibiotic resistance genes are present in wastewater treatment plants (WWTPs), livestock, and soil. They can enter surface water and underground water through rain or surface runoff, which leads to high levels of ARGs in the water environment (Wellington *et al.* 2013). Jones *et al.* reported that the high concentrations of antibiotics and ARGs remaining in surface water can't be treated effectively by traditional water treatment systems and might enter water distribution networks (Jones *et al.* 2005), and this increases the potential for antibiotic resistance pollution of drinking water (Xu *et al.* 2016). Drinking water treatment plants may affect the behavior of ARGs as they could increase the antibiotic resistance of surviving bacteria, particularly as the finished water from these treatment plants has been shown to contain ARGs (Xi *et al.* 2009; Guo *et al.* 2014). Antibiotic resistance genes can potentially enter the

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human body in a variety of ways, and could transfer between bacterial generations and different strains by cell division (Lindsey *et al.* 2001). This means that resistance strains that are non-pathogenic could transfer their resistance genes to the pathogenic strains, which would lead to the production of new drug-resistant pathogenic strains. Pathogenic strains with resistance will considerably reduce disease treatment efficacy. This means that to cure diseases and eliminate pathogenic strains, antibiotic doses must be increased, and, simultaneously, more effective antibiotics need to be invented, thus creating a vicious circle. This will lead to a decrease in the ability of the human body to resist disease as the numbers of resistance genes increase in the environment, which could considerably harm human health.

Sulfamerazine (SM1) is a sulfa antibiotic drug. It is widely used as a chemical drug treatment to prevent and treat bacterial and fungal infections in animal husbandry (Aitipamula *et al.* 2012; Zhou *et al.* 2016). The United States, Canada, and some European Union (EU) countries have conducted many studies on a variety of sulfonamide antibiotics in wastewater treatment plant effluent and the concentrations were found to be low (μg/L) (Pérez *et al.* 2005; Göbel *et al.* 2007). SM1 has been detected in some surface water, groundwater, and drinking water, because of its common use in livestock and aquaculture to prevent and treat diseases, but the detectable concentration in general is in ng/L (Nakada *et al.* 2007). Although it is only present in trace concentrations, the long-term use of drinking water polluted by SM1 will disturb human normal flora and can result in nausea, dizziness, vomiting, allergic reactions in the body, and drug resistance to many pathogenic bacteria.

Sulfamethoxazde (SMZ) is a synthesis of sulfa drugs. It is a type of antiphlogistic prescription drug, which is widely used in livestock and human medicine. When SMZ enters an animal or human body, it cannot be completely metabolized and about 15–25% of the SMZ is directly discharged without being metabolized (Ryan *et al.* 2011). Over recent years, the detection frequency of SMZ in the environment has risen by up to 73% (Watkinson *et al.* 2009). SMZ could persist in the environment for a long time, and may cause bacteria to become drug resistant and form new drug-resistant strains (Zhou *et al.* 2012). Even if SMZ is at a low concentration in the environment (ng/L), it can still cause slow genetic damage and even induce gene mutations (Zhang *et al.* 2010). SMZ is listed as one of the top 10 priority control drugs in the European PPCPs (pharmaceutical and personal care products) evaluation (Ter Laak *et al.* 2014). Therefore, SMZ removal has become a focus of researchers in China and abroad.

Many researchers have found that microorganisms in drinking water grow and reproduce rapidly due to the incomplete removal of organic nutrients in effluent, the inside roughness of pipe walls, pollution of secondary water supplies etc. Therefore, aquatic pathogenic microorganisms (mainly protozoa, viruses, and bacteria) play an important role in drinking water safety (Liu *et al.* 2013; Zhang *et al.* 2018). The demand for potable water increases as the population grows, and the study of pathogenic microorganisms that can spread in water has become more important.

The accumulation of SM1 or SMZ in the human body will harm human health, but there have been few studies on the interaction between SM1 or SMZ and bacteria in drinking water. In this study, the interaction between SM1 or SMZ and bacterial diversity in drinking water was investigated using high-throughput sequencing. Changes in bacterial community structure and microbial resistance genes after adding SM1 or SMZ to drinking water were also measured. The effects of different SM1 or SMZ concentrations on microbial community structure under common water quality conditions were analyzed.

## METHODS

Table 1 shows the concentration and detection frequency of SM1 and SMZ in the reservoir that is used to supply water for the detection area. Water samples were taken from drinking water distribution networks and residual chlorine was eliminated by adding ascorbic acid. The water samples were stored in 1,150 mL brown bottles (the bottles were sterilized by high-pressure steam). The SM1 or SMZ (purchased from J&K Scientific Ltd, Beijing, China) was added at 10 ng/L, 20 ng/L, 50 ng/L, or 100 ng/L, and there was a comparison blank control group. The experimental period was 20 days, and the water samples were collected in triplicate and analyzed immediately after collection. The pH value and

**Table 1** | Concentration and detection frequency of SM1/SMZ in nearby reservoirs (ng/L)

| Reservoir | Value | SM1 | SMZ |
|---|---|---|---|
| Panjiakou reservoir ($n = 40$) | Max | 3.80 | 7.23 |
| | Min | nd[a] | nd[a] |
| | Mean | 2.19 | 2.10 |
| | Median | 1.57 | 1.63 |
| | Frequency (%) | 57.50 | 75.00 |
| Yuqiao reservoir ($n = 18$) | Max | 3.33 | 1.83 |
| | Min | nd[a] | nd[a] |
| | Mean | 1.79 | 1.14 |
| | Median | 1.55 | 1.28 |
| | Frequency (%) | 83.33 | 83.33 |
| Daheiting Reservoir ($n = 15$) | Max | 3.90 | 3.40 |
| | Min | nd[a] | nd[a] |
| | Mean | 2.94 | 2.72 |
| | Median | 3.05 | 3.20 |
| | Frequency (%) | 93.33 | 66.67 |

[a]Not detected.

dissolved oxygen (DO) concentration were immediately measured using a portable Hach DO/pH/Eh meter (Hach SensION + DO6). $NH_3^-$-N was measured following standard methods (American Public Health Association [APHA] 2005); free chlorine was measured by a Hach PCII; and turbidity was measured by a Hach 2100N following their standard calibration and operational methods.

The samples were collected using a water grab sampler made with inert materials and stored in pre-cleaned amber glass bottles. Following collection, all samples were immediately sent to the laboratory, kept in the dark at 0–10 °C, and analyzed within 24 h. The water samples were filtered through 0.45 μm glass microfiber filters (Millipore, USA) to remove suspended particles. The samples were enriched by solid-phase extraction (SPE), which used Oasis HLB cartridges (6 mL/500 mg, Waters, USA). The eluate was collected in a test tube and was evaporated using nitrogen sparging. Finally, the sample was reconstituted to a final volume of 1 mL with 10% methanol (v/v) and transferred to an amber auto sampler vial for LC-MS/MS (liquid chromatography/tandem mass spectrometry) analysis. The chromatographic separation of the analyses was conducted using an ACQUITY ultra performance liquid chromatograph (UPLC) and the mass spectrometric measurements were performed on a Quattro Premier XE (Waters, USA) equipped with an electrospray ionization source. All samples were analyzed in duplicate to provide a 10% average coefficient of variation for the duplicated samples. To investigate the effects of tube wall adsorption of PPCPs in water samples, a tube wall adsorption experiment was performed, and the results showed that the effects of wall adsorption on PPCPs were very small and could be ignored.

Total bacterial DNA in the water samples was extracted using water DNA Kits D5525-02 (Omega, USA) following the manufacturer's protocol. Extracted genomic DNA was detected by 1% agarose gel electrophoresis and stored at −20 °C. The bacterial 16S rRNA (V3 + V4) genes were amplified, and bacterial diversity in the samples was detected by Illumina HiSeq 2000 and analyzed by Mothur software. Following genomic DNA extraction, PCR (polymerase chain reaction) was used to detect resistance genes *sul*1 and *sul*2. PCR was performed according to the method described by Selvam *et al.* (2012).

The means, standard deviation, and analysis of variance (ANOVA) were determined using SPSS software (PASW Statistics 18.0). The data in the figures are the mean values and standard errors of three samples.

## RESULTS

The drinking water quality without adding SM1/SMZ and the water quality standards (standards of drinking water quality, GB5749-2006) are shown in Table 2 (three parallel samples were monitored for each sample). No SM1/SMZ was detected in the drinking water by LC-MS/MS.

Total DNA was extracted from the drinking water samples without added SM1/SMZ, and the bacterial

**Table 2** | Water quality standards and the measured quality of the raw water

| Water quality parameters | Water quality standards | Raw water quality |
|---|---|---|
| Turbidity (NTU) | 1 | 0.6 |
| pH | $6.5 \leq pH \leq 8.5$ | 6.62 |
| Temperature (°C) | – | 15 |
| Free chlorine (mg/L) | $0.05 \leq FC \leq 0.3$ | 0.16 |
| DO | – | 8.01 mg/L |
| BDOC (biodegradable dissolved organic carbon) (mg/L) | – | 0.23 mg/L |
| Ammonia nitrogen (mg/L) | 0.5 mg/L | 0.403 mg/L |

diversity was analyzed. In total, 1,730,602 raw sequences and 1,575,006 high-quality reads were obtained for the identification of microbial communities from 10 samples, with an average length of ~458 bp. Then these gene sequences were assigned to conduct downstream analyses. Figure 1 shows the different levels in the bacterial community structure. The bacterial communities in drinking water at the genus level mainly included *Novosphingobium* spp., *Sphingomonas* spp., *Hyphomicrobium* spp., *Blastomonas* spp., and *Bradyrhizobium* spp., which accounted for 23%, 22%, 9%, 5%, and 3% of the total bacteria present, respectively. Other bacteria included *Achromobacter* spp., *Devosia* spp., *Bacillus* spp., *Lactococcus* spp., *Nitrosomonas* spp., *Methylobacterium* spp., etc.

SM1 or SMZ at four different concentrations was added to drinking water samples and sterilized ultrapure water. The SM1/SMZ concentrations and bacterial diversity were measured after 20 days. The detection results for SM1/SMZ are shown in Table 3. There were slight decreases in the SM1 and SMZ contents after 20 days. The total DNA in the water samples was extracted and bacterial diversity was analyzed at the genus level. HM1 to HM4 and HY1 to HY4 represent SM1 or SMZ added at 10 ng/L, 20 ng/L, 50 ng/L, or 100 ng/L, respectively. GW2 was a blank sample where no SM1 or SMZ had been added.

The genus composition of each sample and the different genera proportions in each sample are shown in Figure 2, which suggests that after treatment with SM1 at the four different concentrations, the microbial community structure of the drinking water changed considerably compared to the blank sample.

After treatment with SMZ at the four different concentrations, the *Bradyrhizobium* proportion increased considerably from about 2% to 40%. However, the *Lactococcus* percentage only increased slightly. The *Sphingomonas* proportion decreased substantially from 20% to 2%, and there were also declines in the *Blastomonas* and *Hyphomicrobium* proportions.

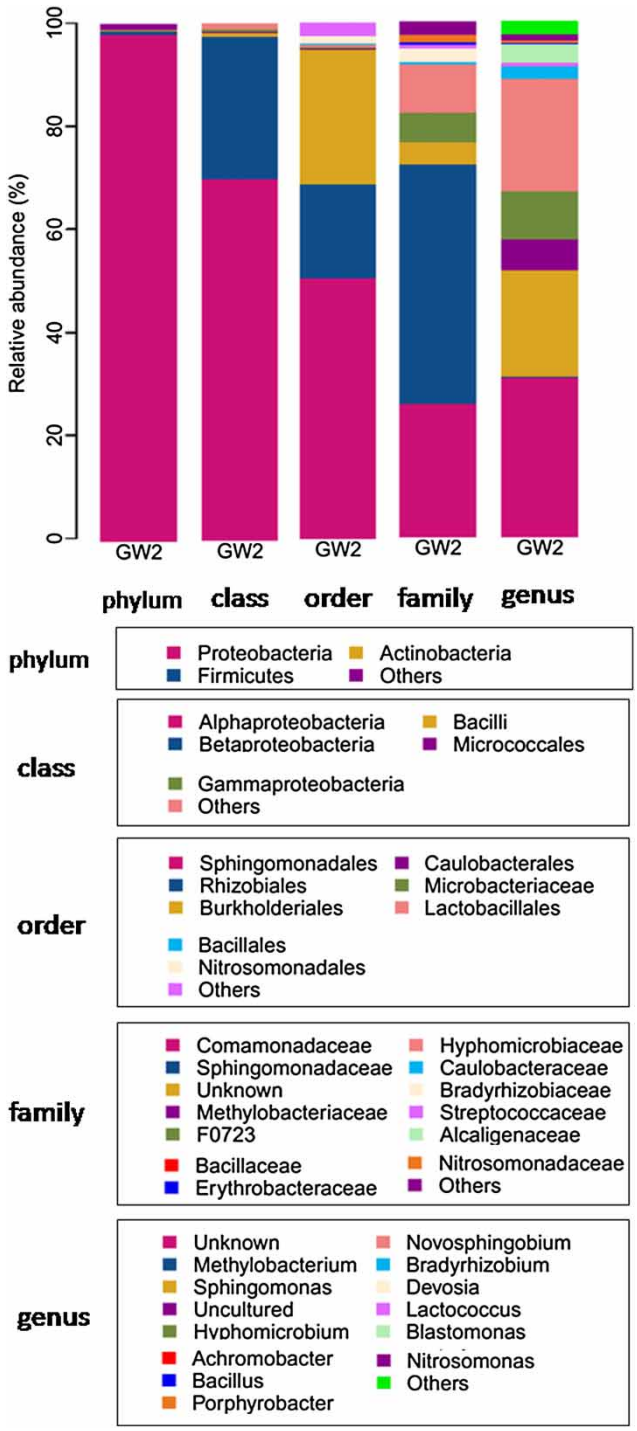


**Figure 1** | Bacterial diversity in the drinking water samples without added SM1/SMZ.

## DISCUSSION

Bacterial risk to drinking water is mainly caused by pathogenic bacteria in water, and pathogenic bacteria in drinking water, at even very small numbers, may cause human infections and disease. The results for the bacterial community in drinking water showed that the pathogenic

**Table 3** | Average concentration of SM1/SMZ in the drinking water samples (ng/L)

| Concentration of SM1/SMZ added | | 10 | 20 | 50 | 100 |
|---|---|---|---|---|---|
| SM1 | Sterilized ultrapure water | 8.6 | 16.8 | 45.1 | 94.0 |
| | Experimental sample | 7.1 | 15.0 | 43.2 | 93.1 |
| | Concentration difference | 1.5 | 1.8 | 1.9 | 0.9 |
| SMZ | Sterilized ultrapure water | 8.3 | 18.8 | 45.1 | 94.6 |
| | Experimental sample | 7.1 | 17.8 | 43.6 | 93.1 |
| | Concentration difference | 1.2 | 1.0 | 1.5 | 1.5 |

bacteria genus *Achromobacter*, *Lactococcus*, *Pseudomonas*, *Acinetobacter* spp., and *Staphylococcus* were present. The potential pathogenic bacteria can cause infection in all parts of the body, such as the central nervous system, respiratory tract, and urinary tract. They also cause endometrial inflammation, peritonitis, hepatic abscess, sepsis, and septicemia (Li *et al.* 2013; Kim *et al.* 2014; Rout *et al.* 2016). Pathogenic bacteria also show a wide range of resistance.

After being treated with SM1 at the four different concentrations, the microbial community structure of the drinking water was analyzed at genus level. The result showed that the *Bradyrhizobium* proportion increased dramatically, from about 3% to 30%. However, the *Sphingomonas* and *Blastomonas* percentages declined substantially from about 22% and 5%, respectively, to almost undetectable. *Hyphomicrobium* and *Nitrosomonas* were almost undetectable, accounting for 2% before and after SM1 or SMZ had been added. The *Methylobacterium* proportion decreased slightly and was almost unaffected by the change in SM1 concentration. The *Lactococcus* percentage increased slightly, but there were no differences as the SM1 concentration rose between HM1 and HM4.

The alpha diversity indexes for the samples at the different concentrations of SM1 or SMZ are shown in Table 4. The results show that the number of species in the bacterial communities of the drinking water samples decreased significantly after the addition of SM1, as did community richness and community evenness. However, the community structure did not change significantly when the different SM1 concentrations were compared. There was a slight decrease but the fluctuation was low. SM1 can inhibit the growth of bacteria and reduce bacterial community diversity in drinking water. It is very likely that some bacteria species developed resistant genes, which meant that the total number of species in the bacterial community did not vary as the SM1 concentration changed and that the bacteria sensitive to SM1 did not decrease further after an initial short period of time when they did decline. This meant that there was little change in the number of bacteria overall.

Table 4 and Figure 2 show that the proportion and abundance of *Bradyrhizobium* both increased, but the increase

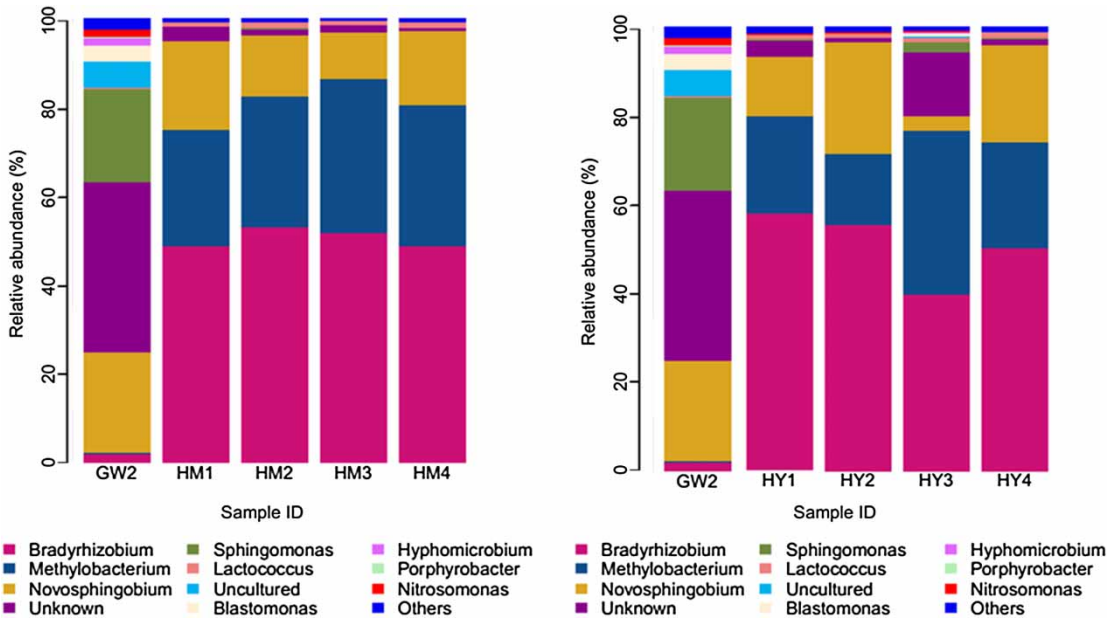


**Figure 2** | Community structure at the genus level in water samples with added SM1 (left) or SMZ (right).

**Table 4** | Alpha diversity indexes after adding SM1 or SMZ

| Sample ID GW2 | Sobs 108 | Ace 112.640 | Chao1 117.000 | Simpson 0.162 | Shannon 2.280 |
|---|---|---|---|---|---|
| SM1 | | | | | |
| HM1 | 64 | 68.067 | 68.000 | 0.345 | 1.281 |
| HM2 | 60 | 65.718 | 71.250 | 0.388 | 1.168 |
| HM3 | 61 | 64.907 | 63.333 | 0.399 | 1.112 |
| HM4 | 66 | 73.720 | 75.167 | 0.366 | 1.188 |
| SMZ | | | | | |
| HY1 | 68 | 71.977 | 74.000 | 0.404 | 1.218 |
| HY2 | 69 | 75.612 | 85.500 | 0.396 | 1.172 |
| HY3 | 83 | 90.159 | 96.200 | 0.299 | 1.665 |
| HY4 | 66 | 68.467 | 67.875 | 0.356 | 1.253 |

was not consistent. The proportion and abundance of *Sphingomonas* and *Blastomonas* decreased, but they increased slightly for *Lactococcus*. Therefore, the results show that the biological community in the drinking water was inconsistently inhibited as the SM1 concentration changed. SM1 considerably inhibited *Sphingomonas* and *Blastomonas*, but only inhibited *Hyphomicrobium* and *Nitrosomonas* to a smaller extent. However, the significantly increased proportion and abundance of *Bradyrhizobium* showed that it was not sensitive to SM1. Any inhibitory effect on *Methylobacterium* was not obvious.

The microbial community structure changes in the drinking water caused by SM1 and the increased proportion and abundance of the *Lactococcus* may increase drinking water security risk.

SMZ has the same effect on drinking water bacterial diversity as SM1. It can inhibit the growth of bacteria and reduce the diversity of the bacterial community in drinking water. However, increasing the SMZ concentration had no effects on changes to the bacteria community structure. SMZ has a clear inhibitory effect on *Sphingomonas* and *Blastomonas* and also inhibits the growth of *Hyphomicrobium* and *Nitrosomonas* to some extent. The results showed that *Bradyrhizobium* and *Methylobacterium* were not sensitive to SMZ. The increase in *Lactococcus* proportion and abundance may increase drinking water safety risk to some extent.

After the addition of SM1, total bacteria DNA in the water sample was extracted. Resistance genes *sul*1 and *sul*2 were detected by PCR. The results showed that there were no PCR *sul*1 resistance gene products in the water samples with or without SM1. The electrophoresis results for the *sul*2 resistance gene are shown in Figure 3. The numbers 1 to 4 represent water samples with SM1 concentrations of 10 ng/L, 20 ng/L, 50 ng/L, and 100 ng/L, and number 5 was the drinking water sample without added SM1. The target band for resistance gene *sul*2 was 296 bp.

Figure 3 shows that there was no *sul*2 target band in the PCR products from the drinking water samples without added SM1. This means that resistance gene *sul*2 was not present in the drinking water without added SM1. However, the target band did appear in the drinking water samples with SM1 added at the four different concentrations, which demonstrates that resistance gene *sul*2 was induced in specific bacteria after SM1 was added to the drinking water samples. Horizontal, or lateral, gene transfer (HGT) is commonly known for its role in the alarming spread of antibiotic resistance. For the past two decades, HGT has been recognized to play a more general role as an important force in the evolution of bacterial genomes (Amábile-Cuevas 2013; Van de Guchte 2017). The experiment was also performed in water samples where SMZ had been added, but no resistance genes were detected.

## CONCLUSIONS

The bacterial community in drinking water at the genus level and bacterial risk were analyzed. SM1 or SMZ at four different concentrations was added to drinking water samples and


**Figure 3** | Electrophoresis detection of resistance gene *sul*2.

sterilized ultrapure water, and slight decreases in the SM1 and SMZ contents were detected. The results showed that bacterial species decreased significantly after the addition of SM1 or SMZ, as did community richness and community evenness. The community structure did not change as the SM1 or SMZ concentrations increased. The detection results for the resistance genes showed that *sul*2 was induced in specific bacteria after SM1 had been added to the drinking water samples.

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