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# Alizarin enhancement of the abundance of ARGs and impacts on the microbial community in water

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## ABSTRACT

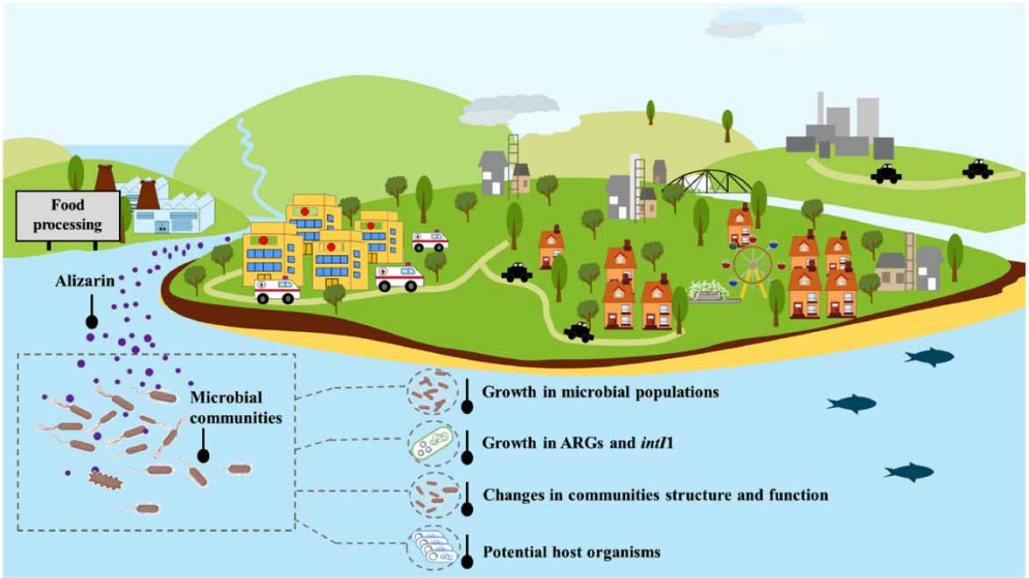
Alizarin, a dyestuff from herbs, showed effective inhibition effects on pathogenic bacteria, and thus has been frequently used in the world as the main alternative to antibiotics in the treatment of inflammations and pathogen infections. However, it was unclear whether alizarin played key a role in antibiotic-induced antibiotic-resistant gene (ARG) alterations and impacted microbial community shifts in aquatic environments. In this study, the effects of alizarin or co-exposure of alizarin with antibiotics on the fate of ARGs, class 1 integron-integrase gene (*intI*1), and microbial populations in lake water were investigated, and the potential hosts for ARGs were analyzed. The results showed that the absolute abundance of 16s rRNA gene, ARGs (*tetA*, *tetC*, and *qnrS*), and *intI*1 were increased during the treatment of alizarin. The combination of alizarin and antibiotics was superior to alizarin in its ability to promote population growth of bacteria and induce ARGs. Additionally, alizarin more significantly altered the community composition of microorganisms in water, which resulted in differences in bacterial communities and functions.

**Key words:** alizarin, antibiotic, antibiotic-resistant genes, antibiotic-resistant bacteria, bacterial community

## HIGHLIGHTS

- Relationships among ARGs, *intI*1, and microbial community were obtained.
- Exposure of alizarin promoted the growth of microbial populations.
- Alizarin boost the absolute abundance of ARGs and *intI*1 to a higher extent.
- Alizarin contributed obviously to changes in microbial communities and functions.

## GRAPHICAL ABSTRACT




## 1. INTRODUCTION

Antibiotics are defined as a class of natural, semi-synthetic, or synthetic compounds that exhibit antimicrobial activity against microorganisms (Kuemmerer 2009). As broad-spectrum antimicrobial agents, antibiotics are widely used for the treatment of infectious diseases in humans and animals, and are added to feeds to promote growth and development in animals (Qiao *et al.* 2018). Indeed, antibiotics that enter the human or animal body are difficult to be fully absorbed or degraded, leading to their detection in the environment (Wang *et al.* 2022). Depending on the antibiotic, sometimes very significant percentages of the dose are excreted (Kemper 2008). Antibiotic misuse is a significant contributor to selection pressure in the environment (Zainab *et al.* 2020). The emergence of antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) under antibiotic stress decreases therapeutic efficacy against both human and animal pathogens (Liu *et al.* 2022). A growing number of studies have reported antimicrobial resistance has been recognized as a significant global health threat, necessitating focused attention (Qiao *et al.* 2018).

Due to the development of drug resistance and the toxic side effects of antibiotics, it is imperative to explore alternatives to antibiotics. Herbal medicines, which are derived from natural plant materials, contain naturally occurring substances or secondary metabolites that possess antibacterial and antiviral effects, similar to antibiotics (Feng *et al.* 2018; Mitra *et al.* 2022). The use of herbal medicines is expanded rapidly throughout the world due to their stable nature, precise efficacy, and low toxicity (Ekor 2014; Zhu 2020). It was reported that approximately 80% of the global population relied on herbal medicine as the main source of treatment for diseases (Ekor 2014). In addition, the continuous use of antibiotics in aquaculture inevitably induces bacterial gene mutations and promotes horizontal ARG transmission as well as intergenerational transmission, highlighting the need for alternatives (Ning *et al.* 2022). Herbal medicines possess the potential to regulate immunity, prevent disease, and reverse drug resistance, making them ideal candidates for substitution for antibiotics in aquaculture as immunostimulants or antibacterial agents (Zhu 2020).

As the major component of the Chinese medicine *Rubia tinctorum L.*, 1,2-dihydroxyanthraquinone (also known as alizarin; $C_{14}H_8O_4$) is typically extracted from the roots of *R. tinctorum L.* (Lee *et al.* 2022). Due to its excellent medicinal value, alizarin has been utilized in the treatment of various diseases (Xu *et al.* 2019). Modern medical science has demonstrated that alizarin has a variety of pharmacological activities, such as anti-tumor, antioxidant, and anti-inflammatory properties (Wang *et al.* 2021a). In addition, alizarin has been found to effectively inhibit the growth of various bacteria, including *Staphylococcus aureus*, *Aspergillus*, *Escherichia coli*, and *Klebsiella*, as effectively as various antibiotics (Raj *et al.* 2022). Apart from medicinal value, alizarin is also widely used as a natural food coloring, plant dye, and staining reagent (Kaur *et al.* 2010). The demand for natural, green, and healthy products has resulted in an increased usage of alizarin (Wen *et al.* 2022).

However, no studies have reported the impact of alizarin exposure, alone or in combination with antibiotics, on antibiotic resistance and microbial communities in water.

In this study, we aim to investigate the effects of alizarin on the abundance of 16 s rRNA gene, ARGs, the class 1 integron-integrase gene (*intI*1), and microbial communities in lake water. Our objectives are to determine (1) whether alizarin has the benefit of enhancing the abundance of 16 s rRNA gene, ARGs, and *intI*1, (2) whether alizarin alters the community structure of microorganisms and abundance in lake water, (3) the relationship between ARGs, *intI*1, and the microbial community, as well as to identify potential host bacteria for ARGs. To approach this, we developed three experimental systems to study gene abundance and microbial community alterations. The absolute abundance of 16 s rRNA gene, ARGs, and *intI*1 was revealed using quantitative real-time PCR (qPCR). 16S rRNA gene amplicon sequencing was used to identify bacterial communities in water samples. The main goal of this study is to explore the effects of individual exposure to alizarin and co-exposure to alizarin and antibiotics on microbial resistance and community in lake water to improve our understanding of the potential environmental impacts of alizarin.

## 2. MATERIALS AND METHODS

### 2.1. Sampling area description and sample collection

The name of this sampling lake is Swan Lake (118°716501′N, 32°207933′E), which is located in the northern part of Nanjing City, Jiangsu, China. It is a typical lake in the urban landscape. The lake has a total surface area of approximately 0.02 $km^2$, with an average water depth of about 2 m. The lawn surrounds the lakeshore of this lake. Since there are no large factories or areas of agricultural production around it, the lake is not heavily impacted by agriculture or industry emissions, which makes its waters crystal clear and of excellent quality. The lake is mainly inhabited by Koi fish, goldfish, grass carp, etc. Fish in the lake are neither fed daily nor given antibiotics.

Surface water samples (12 L) were collected using a high-density polyethylene (HDPE) bucket. The sampling location was at a depth of approximately 15–30 cm below the surface of the water near the center of the lake (U.S. Geological Survey 2018). The HDPE bucket was first immersed in 75% ethanol for 60 min and subsequently washed three times with ultrapure water that had been sterilized at 121 °C for 40 min prior to sampling to ensure the complete elimination of any residual ethanol and other contaminants. The collected water was then immediately stored in a cool, light-tight location before being transported to the laboratory for further processing and analysis. The experimental systems were set up within 24 h of collection. The basic properties of lake water are presented in Supplementary material.

### 2.2. Construction of experimental systems

Three systems were established in this study to investigate whether exogenous input of antibiotics at environment-related concentrations (System-1), single exposure of alizarin (System-2), and the combined administration of alizarin with various antibiotics (System-3) could influence the abundance of ARGs and the structure of microbial communities in water. For the purposes of this study, to mimic the natural state of lake water in the environment, wide-mouthed glass beakers were covered with a low-density black polyethylene film and placed in a well-ventilated room temperature without direct exposure to sunlight.

In System-1, the volume of 1,000 mL of lake water and 0.4040 mg of antibiotic tetracycline (Tet) powder (>98% purity; Macklin, USA) or 0.004 mg of ciprofloxacin (Cip) powder (>98% purity; Macklin, USA) were evenly mixed to set up the simulation system for the exogenous supply of antibiotics to lake water. Previous studies reported that tetracycline at environmental concentration was about 0.4040 mg/L and ciprofloxacin at environmental concentration was about 0.004 mg/L (Singh *et al.* 2019). In order to provide better growth conditions for the microorganisms in the water, a stirrer (JJ-1, Sibo Instruments Co., Ltd, Nanjing, China) was used to stir water samples at a constant flow rate of 300 rpm for 5 min each day to ensure adequate aeration of the water. During the experiment, every 3 days, 10 mg of carbon sources (anhydrous glucose powder) (>99% purity; Macklin, USA) was added to water (Hou *et al.* 2020). Given that the half-life of tetracycline or ciprofloxacin in water was about 5 days (Liu *et al.* 2019), in this system, the antibiotic powder was dosed every 5 days.

For System-2, the experimental system was constructed following the previous System-1. In accordance with the previous design of the system, 200 mg of alizarin (Ali) powder (>96% purity, Sinopharm, China) was mixed into 1,000 mL of lake water. The concentration of alizarin was 200 mg/L that was below the minimum inhibitory concentration of alizarin (Lee *et al.* 2022). In addition, relevant studies found that anthraquinone compounds would not persist in water and that its half-life was less than 10 days (Schrader *et al.* 2003). In the natural environment, the biological functions of anthraquinone

compounds, including anti-inflammatory, antioxidant, anti-tumor, and antibacterial activities, decreased rapidly within about 5 days (Schrader *et al.* 2003). Therefore, alizarin powder was added to System-2 every 5 days to maintain the relative stability of the drug environment. Daily agitation of water samples and regular glucose dosing were then all performed as described in System-1.

System-3 involved adding both antibiotic (tetracycline or ciprofloxacin dosage referenced System-1) and alizarin to lake water following the same dosing and agitation methods as System-1 and System-2. The rules for regular dosing of drug and glucose as well as the method of daily water agitation were as described in System-1 and System-2.

Control groups without antibiotics were setup to monitor spontaneous changes in ARGs abundance and microbial community composition over time. The glucose dosing and water agitation methods in the control groups were identical to those in System-1 and System-2 to provide the microorganisms with the same growth environment.

### 2.3. DNA extraction and high-throughput quantitative PCR

Water samples (25 mL per sample) were collected from beakers in each of the systems on days 5, 15, and 30. Then, samples were harvested at 10,000 rpm for 5 min to obtain centrifugal matter. The total DNA from each precipitate sample was extracted in triplicate using the TIANNAMP Soil DNA Kit (TIANGEN, China). The quality of the DNA in each sample was checked by an ultra-micro spectrophotometer (Quawell Q3000, China). The concentration of the DNA in samples was presented in Supplementary material. Qualified DNA extracts were stored at −80 °C until further analyses.

qPCR was performed on a StepOnePlus™ Real-Time PCR system (Thermo, USA). A total of six target genes were quantified in this study, including three ARGs (*tet*A, *tet*C, *qnr*S), one class 1 integron-integrase gene (*intI*1), and a total of 16S rRNA gene. Detailed primer sequences and annealing temperatures were presented in Supplementary material. The detailed information of the reaction system and reaction conditions of qPCR was described as follows: for each 10 μL qPCR reaction, 5 μL of TB Green Premix Ex Taq II (2×), 0.4 μL of forward primer, 0.4 μL of reverse primer, 0.2 μL of 50 × ROX Reference Dye (50×), 1 μL of DNA template, and 3 μL of sterile double distilled water ($ddH_2O$) were used in the reaction. Each temperature profile consisted of a cycle at 95 °C for 30 s, followed by 40 cycles of the steps that consisted of 95 °C for 5 s and 60 °C for 30 s. The measured threshold cycle (CT) limit value of the target gene was set to 31. Then, CT value of the target gene was compared with the standard curve (regression coefficients, $R^2 > 0.990$) of this gene to obtain the original gene copy numbers. The relative and absolute abundance of target genes were calculated according to the previous method (Pu *et al.* 2018). All qPCR samples were performed in three technical replicates with negative controls. Genes were only considered to be successfully amplified in a sample if all three technical replicates yielded a positive result.

### 2.4. Bacterial 16S rRNA gene sequencing

Fluid samples (25 mL per sample) were also collected from each beaker over 5 days, 15 days, and 30 days, respectively. Total genomic DNA was extracted and verified as described in Section 2.3. The V3–V4 hypervariable regions of 16S rRNA genes were quantified with primers (314F: CCTAYGGGRBGCASCAG and 806R: GGACTACNNGGGTATCTAAT). Reaction conditions were the same with the previous assay (Sun *et al.* 2022). The PCR-purified products were then analyzed by high-throughput sequencing using Illumina HiSeq 2500 (Illumina, USA). The data of the raw reads were taken using Trimmomatic v 0.33 analysis software. Cutadapt 1.9.1 was used to remove primers from the raw reads. Double-ended reads were combined with longer reads by Usearch v 10. Chimeras were verified and removed using UCHIME v. 4.2 software to obtain final effective reads. The efficiency rates of effective reads in samples were all greater than 94%. The specific quality assessment data for sequenced genes was shown in Supplementary material. Subsequently, 16S rRNA gene sequences were analyzed by USEARCH 7.1, and the operational taxonomic units (OTUs) were determined based on the 97% sequence similarity of the genes. Representative sequences of OTUs were compared to data in the SILVA database. These sequences further were classified and was marked with biological classification information by Ribosomal Database Project (RDP) classifier. QIIME (v 1.9.1) was used to present the relative abundances of bacterial communities at the taxonomic levels.

### 2.5. Statistical analysis

Statistical analyses were performed using Origin 2023, R v 4.1.2. Histograms of the absolute abundance of the gene were performed using Origin 2023. Stacked bar diagrams of the composition and relative abundance of microbial populations were performed using the barplot package and the vegan package in R language. Principal coordinate analysis (PCoA) was based on the Bray–Curtis distance matrix. The non-metric multi-dimensional scaling (NMDS) analysis based on the chord distance matrix is used to look at differences between samples. The clustering heat map was drawn through the ggcor package and the

ggplot2 package in R language. Significant differences were analyzed using the independent-samples *t*-test. *p*-values of $<0.05$ were regarded as statistically significant.

## 3. RESULTS

### 3.1. Effects of tetracycline and alizarin on resistance in lake water

To test the effects of alizarin and tetracycline on the growth of microbial populations, we evaluated the absolute abundance of 16 s rRNA gene in lake water in the presence of the two drugs. Our results revealed that the absolute abundance of the 16 s rRNA gene was significantly increased in the presence of both drugs, with the combination of alizarin and tetracycline demonstrating the greatest enhancement ($p < 0.05$). For instance, the absolute abundance of 16 s rRNA gene in Ali-treated group, Tet-treated group, and Tet + Ali-treated group was 12.2-fold, 9.7-fold, and 52.0-fold higher than that of the control groups ($1.42 \times 10^6$ gene copies/mL) after 30 days of treatment, respectively (Figure 1(a), $p < 0.05$). During the experiment, alizarin more significantly facilitated the absolute abundance compared with tetracycline (Figure 1(a), $p < 0.05$). Notably, the absolute abundance of 16 s rRNA gene was most significantly increased when alizarin and tetracycline were input into water samples together ($p < 0.05$).

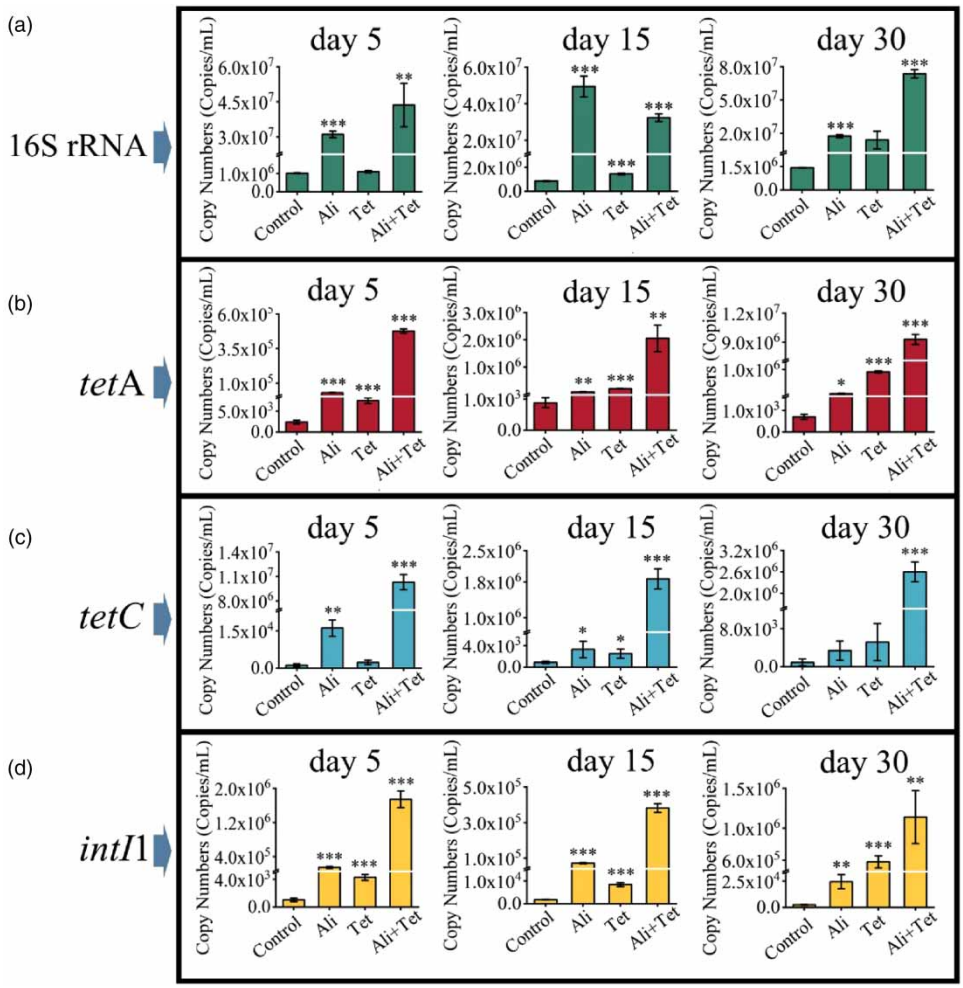



**Figure 1** | Absolute abundance distributions of 16S rRNA gene, two types of ARGs and one class 1 integron-integrase gene in the four water samples (the tetracycline experimental group) on days 5, 15, and 30, respectively. The absolute abundance of 16S rRNA (a), *tet*A (b), *tet*C (c), and *intI*1 (d) in water samples of drug-free, alizarin, tetracycline hydrochloride, and tetracycline hydrochloride combined with alizarin. 'Control' refers to the control group. 'Ali' refers to the experimental group with alizarin. 'Tet' refers to the experimental group with tetracycline. 'Tet + Ali' refers to the experimental group with tetracycline and alizarin. ('*' represents $p < 0.05$, '**' represents $p < 0.01$, '***' represents $p < 0.001$).

In addition, the effect of tetracycline and alizarin on the absolute abundance of ARGs (i.e., *tet*A and *tet*C) was also investigated (Figure 1(b) and 1(c)). The absolute abundance of ARGs had an obvious enhancement with the treatment of drugs ($p < 0.05$). Specifically, in the early stages of the experiment, the ability of antibiotics tetracycline to contribute to the absolute abundance enhancement was better than that of non-antibiotic alizarin (Figure 1(b) and 1(c)). Tetracycline exposure resulted in a stronger boost for ARGs absolute abundance with increased drug exposure time, whereas the co-presence of tetracycline and alizarin exhibited the most significant increase in absolute abundance of ARGs (e.g., *tet*A and *tet*C) compared to the control group (Figure 1(b) and 1(c), $p < 0.05$). For instance, the maximum absolute abundance of *tet*A and *tet*C, a 13,299.2-fold and 2,948.5-fold enhancement compared to the control group, was observed in Tet + Ali-treated group on day 30. Additionally, the relative abundance of ARGs was conducted as described in Section 2.3. As expected, the relative abundance of *tet*A also showed the same increasing trend on day 30 (Supplementary material, Figure S1(a)). However, as shown in Supplementary material, Figure S1(b), the relative abundance of *tet*C in Ali-treated group and Tet-treated group on day 30 was lower than the relative abundance in the control group.

Furthermore, our results revealed that the addition of tetracycline or alizarin into water samples significantly increased the absolute abundance of *intI*1 over 30 days. Also, the combined application of both drugs was most effective in promoting the growth of microbial populations and increasing the gene abundance in the first 15 days (Figure 1(d), $p < 0.05$). For instance, after treating water samples with tetracycline or alizarin for 15 days, the absolute abundance in Ali-treated group and Tet-treated group were 40.3 times and 4.7 times greater than that of the control group, respectively (Figure 1(d), $p < 0.05$). Additionally, in comparison to Ali-treated group and Tet-treated group, combined application of alizarin and tetracycline was seen to increase the absolute abundance most significantly (Figure 1(d), $p < 0.05$). For example, the combined application of drugs resulted in up to $1.14 \times 10^6$ gene copies/mL in Tet + Ali-treated group, which was more than 452.2, 46.7, and 2.0 times higher than the control group, Ali-treated group and Tet-treated group on day 30. However, relative abundance in Ali-treated group was lower than that in the control group on day 30 and relative abundance in Tet + Ali-treated group was lower than that in Tet-treated group (Supplementary material, Figure S1(c)).

Collectively, these results verified that alizarin, alone or in combination with tetracycline significantly, promoted the growth of microbial populations and increased the absolute abundance of ARGs and *intI*1. Compared with separate dosing of alizarin, the combination of alizarin and tetracycline was more effective.

### 3.2. Effects of ciprofloxacin and alizarin on resistance in lake water

The previous description indicated that tetracycline, alizarin, and the combination of both could increase the absolute abundance of 16 s rRNA gene. Thus, we hypothesize that quinolone antibiotic ciprofloxacin and non-antibiotic alizarin might have the same efficacy in promoting the growth of microbial populations. Our results demonstrate that the absolute abundance of 16 s rRNA gene significantly increased with the dosage of alizarin or co-dosing of alizarin and ciprofloxacin (Figure 2(a), $p < 0.05$). Although Ali-treated group showed a higher increase of gene absolute abundance in comparison with Cip + Ali-treated group in the middle of the experiment, the absolute abundance in Cip + Ali-treated group had a more noticeable increase with an observed maximum value on day 30, which showed a 69.2-fold change enhancement of gene abundance compared with Ali-treated group. However, the presence of ciprofloxacin resulted in a decrease of gene absolute abundance in Cip-treated group on days 15 and 30 (Figure 2(a)). For instance, the absolute abundance of 16 s rRNA gene was $9.01 \times 10^5$ gene copies/mL on day 30, which showed a 0.6-fold change compared to the control group.

To explore whether the combination of alizarin with quinolone antibiotics also enhanced the absolute abundance of ARGs (i.e., *qnr*S), ciprofloxacin was added in the corresponding system. The results demonstrate that the addition of alizarin or the combined addition of alizarin and ciprofloxacin significantly increased the absolute abundance, in all Ali-treated group or Cip + Ali-treated group compared with the control (Figure 2(b)). Furthermore, during a 30-day experiment, the absolute abundance of *qnr*S in Cip + Ali-treated group was all higher than Ali-treated group (Figure 2(b)). For example, the absolute abundance was $1.38 \times 10^7$ gene copies/mL in Cip + Ali-treated group on day 30, which showed 9.5-fold higher compared to Ali-treated group. As shown in Supplementary material, Figure S1(a), the results of the relative abundance of *qnr*S were consistent with those of absolute abundance. However, it was worth noting that the results of the qPCR assay did not reveal that the *qnr*S was detected in both the control group and Cip-treated group over 30 days (Figure 2(b)).

In addition, the absolute abundance of *intI*1 was still assessed to verify whether changes in the absolute abundance of *intI*1 was also associated with the addition of alizarin or the combined addition of alizarin and ciprofloxacin (Figure 3(b)). The results show that the absolute abundance was significantly increased under exposure to alizarin or combination of alizarin

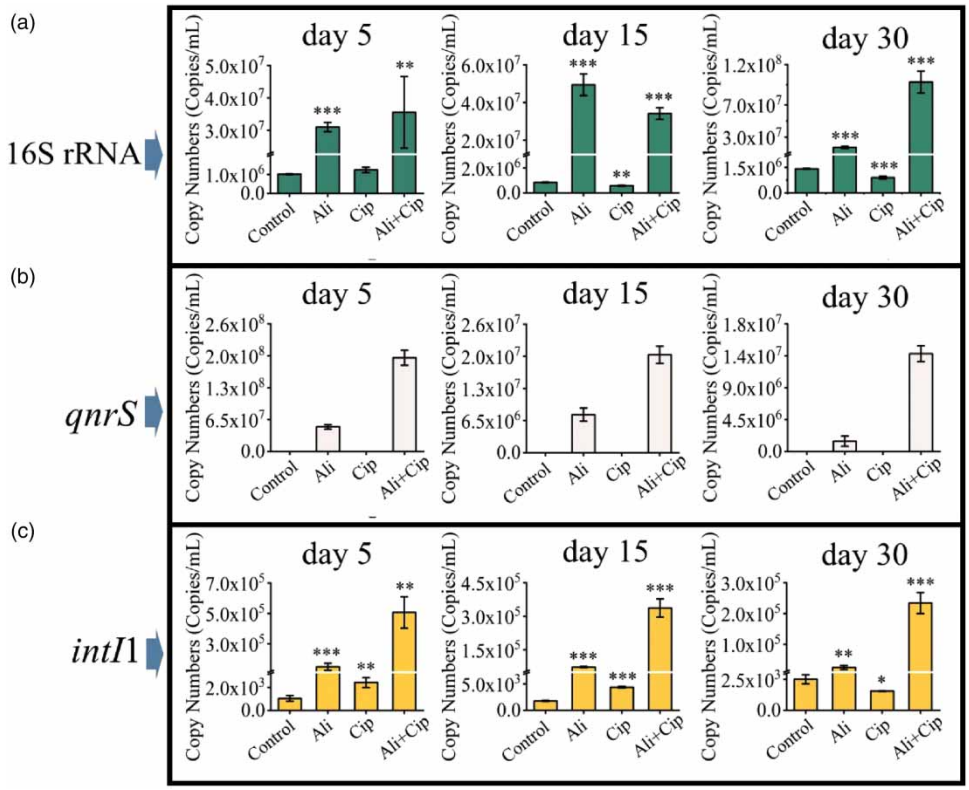



**Figure 2** | Absolute abundance distributions of 16S rRNA gene, one type of ARG and one class 1 integron-integrase gene in the four water samples (the ciprofloxacin experimental group) on days 5, 15, and 30, respectively. The absolute abundance of 16S rRNA (a), *qnr*S (b), and *intI*1 (c) in water samples of drug-free, alizarin, ciprofloxacin, and ciprofloxacin combined with alizarin. 'Control' refers to the control group. 'Ali' refers to the experimental group with alizarin. 'Cip' refers to the experimental group with ciprofloxacin. 'Cip + Ali' refers to the experimental group with ciprofloxacin and alizarin. ('*' represents $p < 0.05$, '**' represents $p < 0.01$, '***' represents $p < 0.001$).

and ciprofloxacin (Figure 3(b), $p < 0.05$). For instance, the absolute abundance of *intI*1 in Ali-treated group and Cip + Ali-treated group were up to 9.7-fold and 93-fold higher than that of the control on day 30. Moreover, the absolute abundance of *intI*1 always remained at maximum in Cip + Ali-treated group over 30 days, compared with the control group, Ali-treated group, and Cip-treated group. In particular, the absolute abundance under the exposure of the combination of alizarin and ciprofloxacin was up to 485-fold higher than that of the control on day 5 (Figure 3(b), $p < 0.05$).

Those findings suggest that alizarin can induce a variety of ARGs, and the combination of alizarin with different types of antibiotics may produce similar effects that promoted the growth of microbial populations and increase the absolute abundance of ARGs and *intI*1.

### 3.3. Effects of tetracycline and alizarin on microbial community structure in water

A total of 388,492 effective sequences were achieved from five samples with sequences per sample ranging from 38,600 to 102,436. These sequences were clustered into 843 OTUs at the 97% similarity level, with an average of 447 OTUs per sample. The stacked bar diagram of major phyla and genera was shown in Figure 3(a) and 3(b). The results revealed that Proteobacteria, Actinobacteria, and Bacteroidetes were dominant in microbial communities at the phylum level (Figure 3(a)). Furthermore, alizarin was found to have a significant impact on the composition of microbial communities at the phylum level, as demonstrated by changes in the relative abundance of Proteobacteria on day 30 of the experiment. Specifically, the relative abundance in the control and Ali-treated group was 53.70 and 91.21%, respectively. In contrast, the combination of tetracycline and alizarin failed to significantly affect the relative abundance of Proteobacteria on day 30. For instance, in comparison to Ali-treated group, the relative abundance of Proteobacteria increased from 91.21%, only up to 91.73% in Tet + Ali-treated group on day 30.

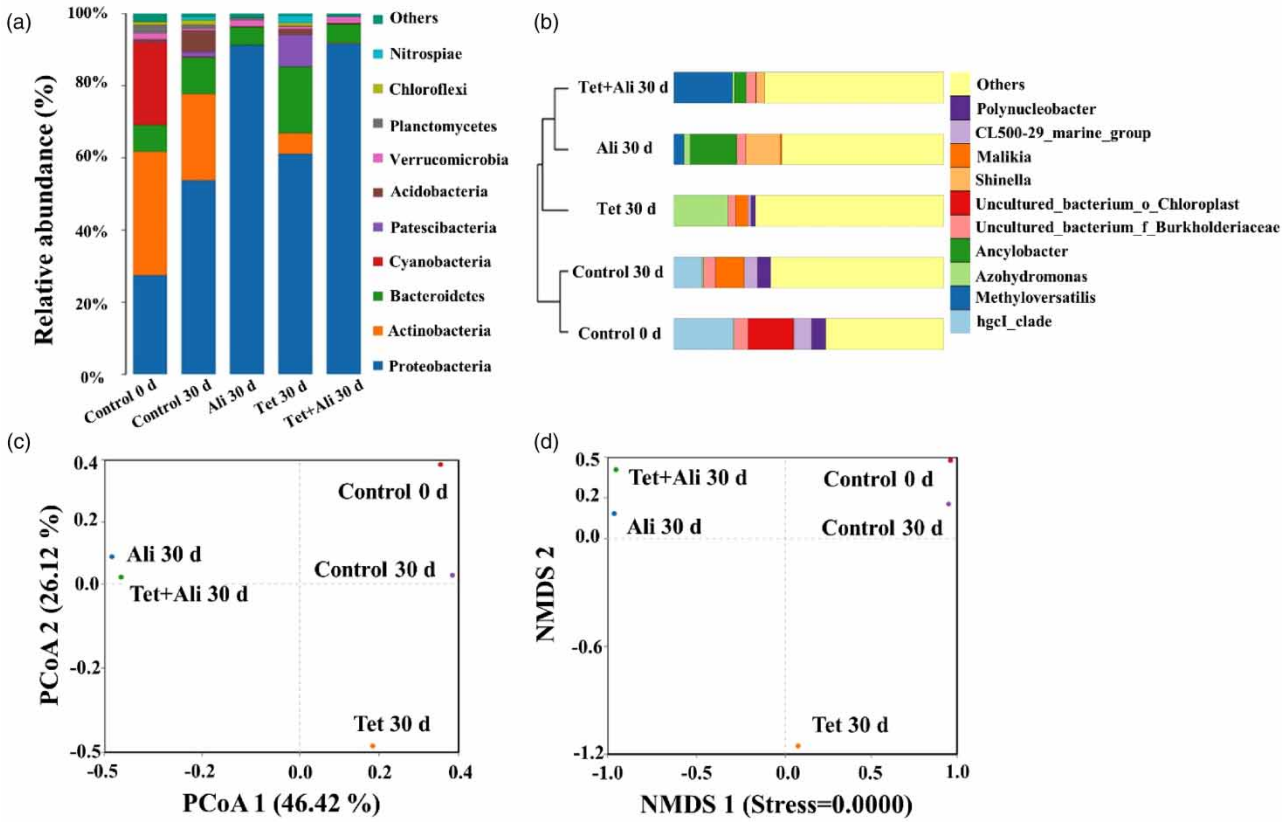



**Figure 3** | Composition and relative abundance of microbial populations in the five water samples (the tetracycline experimental group). (a) Composition of dominant phylum in microbial communities in lake water. (b) Similarity analysis of microbial populations at the genus level on samples. (c) Principal coordinate analysis plots of the genus level based on the Bray–Curtis distance matrix in water samples. (d) Non-MetricMulti-Dimensional Scaling plots of the genus level based on the chord distance matrix in water samples. 'Control 0 d' and 'Control 30 d' refer to the control group on day 0 and day 30, respectively. 'Ali 30 d' refers to the water samples collected on day 30 with alizarin. 'Tet 30 d' refers to the water samples collected on day 30 with tetracycline. 'Tet + Ali 30 d' refers to the water samples collected on day 30 with tetracycline and alizarin.

Additionally, the genus-level composition and relative abundances on water samples from the tetracycline experimental group were presented in Figure 3(b). The dominant genera, which accounted for 40% of the total bacterial 16S rRNA sequences, included *hgcI_clade*, *Methyloversatilis*, *Azohydromonas*, *Ancylobacter*, *uncultured_bacterium_f_Burkholderiaceae*, *uncultured_bacterium_o_Chloroplast*, *Shinella*, *Malikia*, *CL500-29_marine_group,* and *Polynucleobacter*. For the correlation between bacteria, at the genus level, *hgcI_clade* was negatively correlated with *Azohydromonas* (Supplementary material, Figure S3). Additionally, alizarin was found to significantly alter the relative abundances of bacterial compositions at the genus level, with the relative abundance of *Methyloversatilis* increasing from 0.007% in the control to 3.90% in the Ali-treated group on day 30 (Figure 3(b)). Conversely, the relative abundance of *hgcI_clade*, which belongs to the Actinobacteria, decrease from 10.51% in the control to 0.03% in Ali-treated group on day 30. Based on these findings, we speculated that alizarin played a more important role in varying the relative abundance of bacterial compositions at the genus level.

In addition, as shown in Figure 3(b), the results of the five samples were classified based on the microbial community at the genus level into three categories: group I (Tet-treated group 30 d and Tet + Ali-treated group 30 d), group II (Tet-treated group 30 d), group III (the control 0 d and the control 30 d). These findings suggest that the microbial community structure in water samples at the genus level could be significantly affected by alizarin, and the impact of alizarin on the microorganisms was greater than tetracycline. To further investigate whether alizarin was prominent for the alterations in the structure of microbial populations, compared to the tetracycline, the genus-level microbial community of five samples was explored by PCoA based on the Bray–Curtis distance matrix (Xiong *et al.* 2015) (Figure 3(c)). The PCoA of bacterial communities revealed that samples were clustered based on whether alizarin was added. Also, NMDS further confirmed that alizarin might more

readily facilitate altered microbial community structure compared with tetracycline at the genus level (Stress = 0.0000 < 0.2; Figure 3(d)).

### 3.4. Effects of ciprofloxacin and alizarin on microbial community structure in water

A total of 369,418 effective sequences were achieved from five samples with sequences per sample ranging from 38,600 to 102,436. These sequences were clustered into 840 OTUs at the 97% similarity level, with an average of 624 OTUs per sample. The stacked bar diagram of major phyla and genera was shown in Figure 4(a) and 4(b). At the phylum level, the average relative abundance of all seven bacterial phyla exceeded 1% (Figure 4(a)). Proteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria were dominant in the microbial community. Moreover, given that alterations in the microbial community were observed in the tetracycline experimental group, the microbial community in the ciprofloxacin experimental group should have been changed differently as well by alizarin. For instance, on day 30, in comparison with the control (53.70%), with exposure to this alizarin the relative abundance of Proteobacteria in Ali-treated group was significantly enhanced up to 91.21%. In addition, we observed that the relative abundance of Actinobacteria was 22.53 and 24.07% in the control and Cip-treated group respectively on day 30, whereas the combination of alizarin and ciprofloxacin dramatically decreased the relative abundance of Actinobacteria, down to 0.31%. These variations explained the more obvious effects of alizarin on the bacterial community at the phylum level.

In addition, at the genus level, significant alterations in microbial community structures were observed under exposure to alizarin over the course of a month (Figure 4(b)). The average relative abundance of 10 genera was above 2.0%, while the

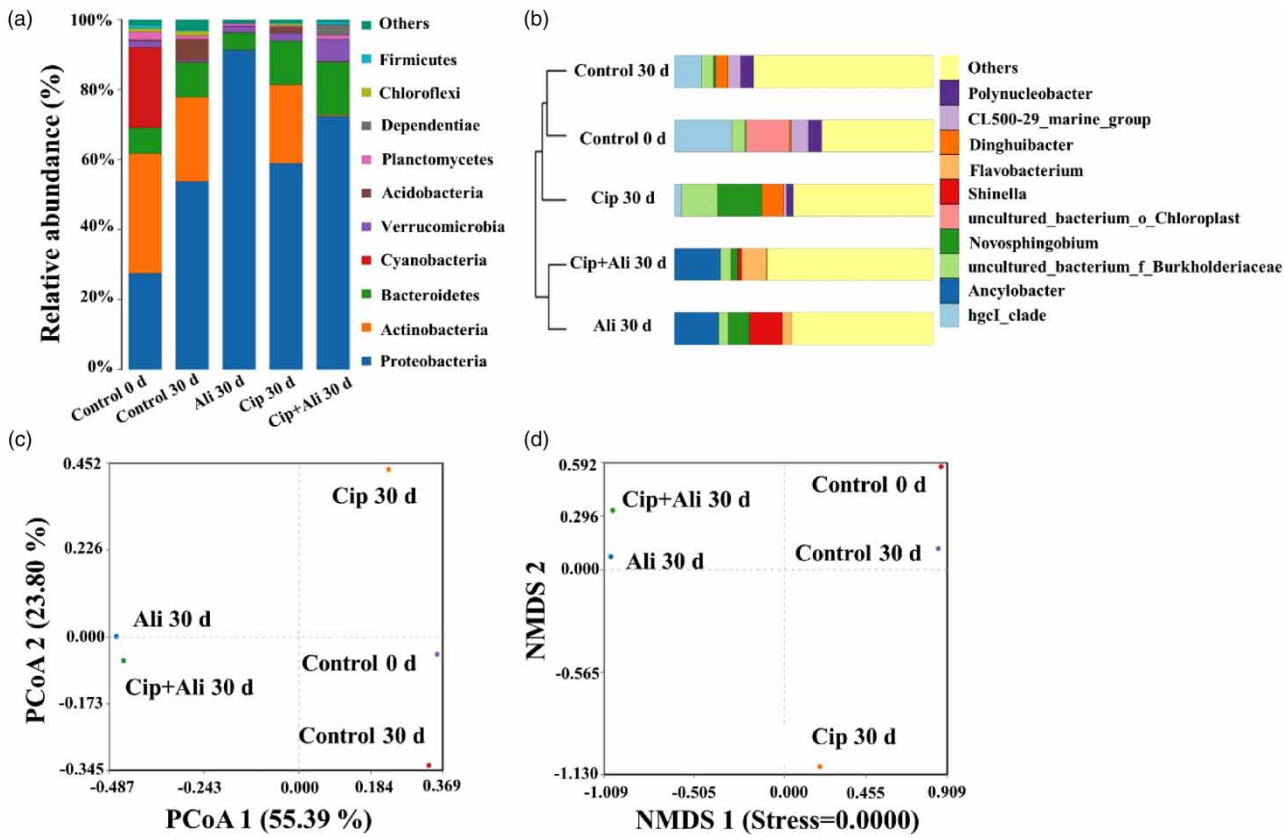



**Figure 4** | Composition and relative abundance of microbial populations in the five water samples (the ciprofloxacin experimental group). (a) Composition of dominant phylum in microbial communities in the lake. (b) Similarity analysis of microbial populations at the genus level on samples. (c) Principal coordinate analysis plots of the genus level based on the Bray–Curtis distance matrix in the water samples. (d) Non-MetricMulti-Dimensional Scaling plots of the genus level based on the chord distance matrix on the water samples. 'Control 0 d' and 'Control 30 d' refer to the control group on day 0 and day 30, respectively. 'Ali 30 d' refers to the water samples collected on day 30 with alizarin. 'Cip 30 d' denotes the water samples collected on day 30 with ciprofloxacin. 'Cip + Ali 30 d' denotes the water samples collected on day 30 with ciprofloxacin and alizarin.

remaining genera had an abundance of less than 2.0% and were classified as others. The dominant genera were *hgcI_clade*, *Ancylobacter*, *uncultured_bacterium_f_Burkholderiaceae*, *Novosphingobium*, *uncultured_bacterium_o_Chloroplast*, *Shinella*, *Flavobacterium*, *Dinghuibacter*, *CL500-29_marine_group* and *Polynucleobacter*. For the relative abundance of the above bacterial genera, bacterial genera in the relative abundance were also varied with the dosage of alizarin (Figure 4(b)). For instance, *Ancylobacter* was reported to contain two large plasmids with ARGs and can adapt to oligotrophic environments (Zlatkin *et al.* 2012). After 30 days of exposure to drugs, compared with the control (0.001%), the relative abundance of *Ancylobacter* was significantly increased to 17.19% under the alizarin treatment, and the relative abundance of those was only further increased to 17.64% under the co-exposure of alizarin and ciprofloxacin. Additionally, we found that *Polynucleobacter* was positively correlated with *CL500-29_marine_group*, while *hgcI_clade* was negatively correlated with *Shinella* (Supplementary material, Figure S4).

Furthermore, the five samples showed an obvious clustering analysis in the microbial community at the genus level by forming three divergent categories: the control 0 d and the control 30 d in group I; Cip-treated group 30 d in group II; Ali-treated group 30 d and Cip + Ali-treated group 30 d in group III (Figure 4(b)). It indicated alizarin more significantly altered the structural composition of the microbial community, compared to antibiotics ciprofloxacin. To further substantiate this conclusion, we analyzed the variability in microbial community structure represented by each sample site in the ciprofloxacin experimental group throughout NMDS (Gonzalez-Silva *et al.* 2016) (Figure 4(d)). NMDS analysis showed that samples containing alizarin clustered together and was separated from the other samples (Stress = 0.000 < 0.2). The PCoA showed that water samples were clustered based on the presence of alizarin, and obviously separated from Cip-treated group and the control, indicating that alizarin more significantly changed the microbial community (Figure 4(c)).

### 3.5. The function of bacterial communities, and relationship among ARGs, *intI*1, and microbial community

The functional overviews of bacterial communities in the tetracycline experimental group were forecasted based on the KEGG analysis (Figure 5(a)). Notably, variations in bacterial community functions were achieved in the water samples. After 30 days of culture, 30 functional predictions from Ali-treated group and Tet + Ali-treated group exhibited a higher level of similarity (Figure 5(a)). Specifically, the up-regulated in Ali-treated group and Tet + Ali-treated group included 'Drug resistance: antimicrobial', 'Membrane transport', and 'Cell growth and death', as compared with that on other samples.

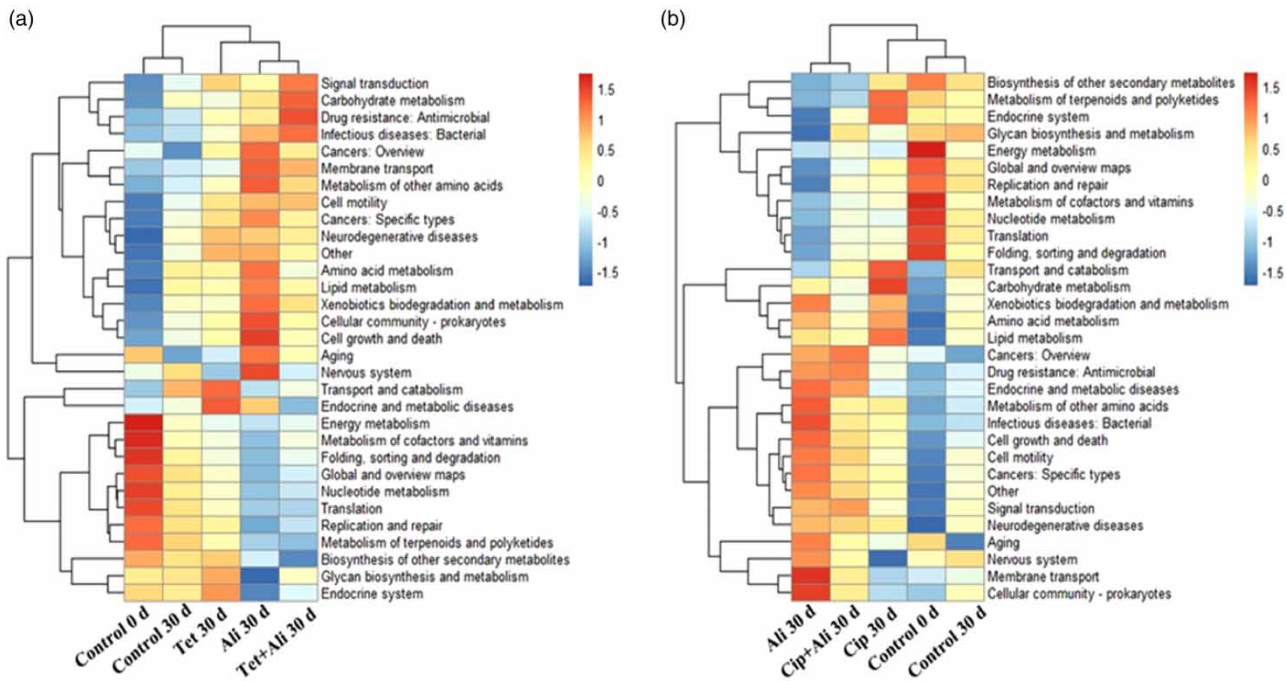



**Figure 5** | (a) The heatmap of functional profiles at level 2 KEGG on tetracycline hydrochloride and alizarin experimental group samples. (b) The heat map of functional profiles at level 2 KEGG on ciprofloxacin and alizarin experimental group samples.

Utilizing KEGG analysis, we were able to predict the functional profiles of the bacterial communities in the ciprofloxacin experimental group (Figure 5(b)). Ali-treated group and Cip + Ali-treated group demonstrate similar bacterial community functional predictions. For instance, 'Carbohydrate metabolism', 'Amino acid metabolism', and 'Lipid metabolism' were up-regulated in Ali-treated group and Cip + Ali-treated group compared with that in the control group.

To further investigate the relationship between microbial communities, *intI*1, and ARGs, we constructed a clustering heatmap using the absolute abundance of ARGs and *intI*1, as well as their potential host bacteria (based on the top 30 genera-level) (Zhang *et al.* 2022) (Figure 6). Bacteria genera that were significantly and positively associated with either ARGs or mobile genetic elements (MGEs) were regarded as their potential hosts (Guo *et al.* 2021). Among these genera, six (*Sphingopyxis*, *uncultured_bacterium_f_Xanthobacteraceae*, *Aeromonas*, *uncultured_bacterium_o_Rhodospirillales*, *Methyloversatilis*, *uncultured_bacterium_c_Gammaproteobacteria*) demonstrated significant positive correlations with two ARGs (i.e., *tet*A, *tet*C) and *intI*1 ($p < 0.05$). In addition, *qnr*S showed a significantly positive correlation with *Flavobacterium*, *Ideonella*, *uncultured_bacterium_o_Rhodospirillales*, *uncultured_bacterium_f_Saccharimonadaceae* ($p < 0.05$).

## 4. DISCUSSION

Antibiotic resistance has become a serious threat to the world's health and environment today. The misuse of antibiotics is a major driver of the emergence and spread of antibiotic resistance (Zhang *et al.* 2019). Alizarin, a non-antibiotic alternative to

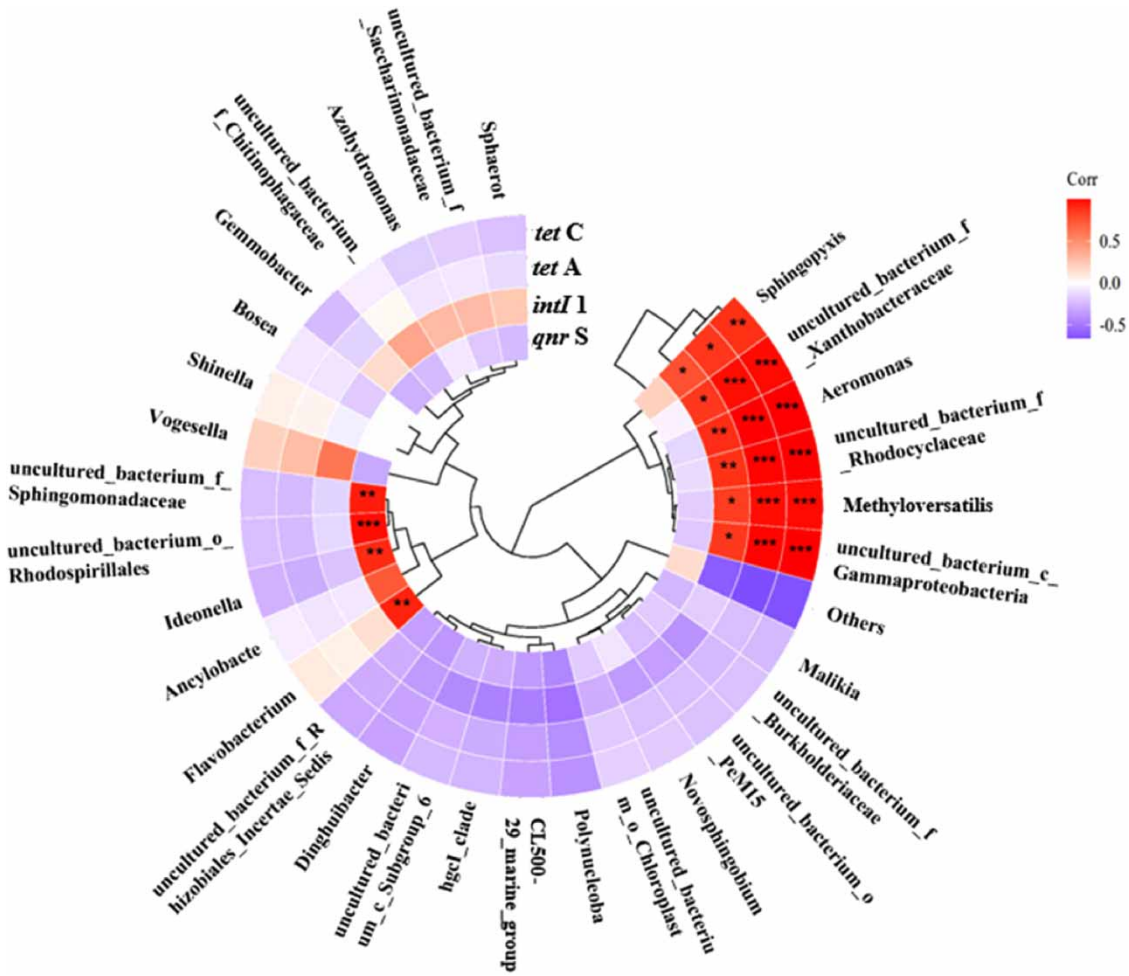



**Figure 6** | Network analysis between ARGs, *intI*1 of absolute abundance, and their potential host bacteria at water samples (inside to outside: *tet*C, *tet*A, *intI*1, *qnr*S). The color in this circular heat map represents the correlation coefficient among ARGs and potential host bacteria. The shade of the color represents the strength of the correlation and the color of the heatmap: red represents positive correlation and blue represents negative correlation ('corr' represents the correlation coefficient; '*' represents $p < 0.05$, '**' represents $p < 0.01$, '***' represents $p < 0.001$). Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2023.138.

antibiotics, has been intensively used and released into the water environment (Shan *et al.* 2016). Here, we found that alizarin under sub-MIC (e.g., 200 mg/L) could significantly increase the absolute abundance of 16 s rRNA gene. In addition, the combination of alizarin with antibiotics at environmentally relevant concentrations was able to further expand the absolute abundance of 16 s rRNA gene enhancement. These results all supported our conclusion that alizarin promoted the growth of microbial communities and that the combination of alizarin and antibiotics was more effective. The increase in the absolute abundance of the gene was likely due to the indirect long-term effects of alizarin or the combination of alizarin and antibiotics on bacteria in water, including selective resistance, genetic recombination, and mutation (Grenni *et al.* 2018).

A previous study indicated that alizarin, as anthraquinones, underwent a particular redox cycle to generate the superoxide radical anion ($\bullet O^{2-}$) and hydroxyl radicals (•OH) (Tian *et al.* 2020). Reactive oxygen species (ROS) caused damage of DNA in cells, inducing oxidative stress and SOS response (Wang *et al.* 2019; Yu *et al.* 2021). These responses were thought to drive gene repair, recombination, and mutation to adapt to new environments, which contributed to an increase in ARGs abundance (Jin *et al.* 2018). Our study further verified that non-antibiotic alizarin could increase the abundance of ARGs. In addition, it is well known that environmental stress from antibiotics promotes the production and spread of ARGs in water (Zainab *et al.* 2020). A synergistic action of alizarin and antibiotics might have further amplified this effect of enhancing the abundance of ARGs (Gonzalez-Pleiter *et al.* 2013). Thus, compared with a single antibiotic pharmaceutical, the combined administration of non-antibiotic alizarin and antibiotics had a more obvious effect to increase the abundance of ARGs in water. In addition, studies have suggested that the complex of anthraquinones and metal ions reduced the level of ROS (Hormann *et al.* 2018). Therefore, further research should be carried out to investigate the effect of environmental factors, such as heavy metals, on alizarin in water in order to explore the working mechanism of alizarin. In addition, the difference between the relative and absolute abundance of ARGs might be due to variations in the abundance of 16 s rRNA gene.

Given MGEs, such as *intI*1, contribute to interspecies transmission of ARGs and are positively associated with multiple antibiotics, qPCR was used to further study the abundance of *intI*1 (Gillings *et al.* 2015; Zhang *et al.* 2022). We found that alizarin could significantly increase the absolute abundance of *intI*1. This could be attributed to the fact that the dosage of alizarin led to elevate ROS generation levels and induced the bacterial SOS stress response (Zhang *et al.* 2021b). Cell membrane permeability might be improved by the production of ROS, which would facilitate horizontal gene transfer of MGEs (Wang *et al.* 2020, 2021b). Also, it is reported that the SOS response could cause the generation and release of MGEs (Zhang *et al.* 2021b). Notably, under the combination of alizarin and antibiotics, there was a more significant increase in the abundance of *intI*1 compared to that under the exposure of alizarin, likely due to a co-stressing of microorganisms by alizarin and antibiotics.

The phyla of Proteobacteria, Actinobacteria, and Bacteroidetes dominated the bacterial communities in water samples of tetracycline experimental group (Figure 3(a)), consistent with previous studies in antibiotic-contaminated receiving waters and livestock manure (Gao *et al.* 2018). Proteobacteria carried ARGs and were commonly found in antibiotic-contaminated wastewater, so the prevalence of Proteobacteria observed in our study is logical (Zhang *et al.* 2021a). Furthermore, our findings reveal that the phyla of Proteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria were dominant in the microbial communities in the ciprofloxacin experimental group (Figure 4(a)), which aligns well with previous studies assessing the impact of antibiotic application on the microbial composition of soils (Lin *et al.* 2016). The abundance of the major phyla in the alizarin-containing water samples was significantly different from that of the control group on day 30, indicating that alizarin was influencing microbial communities at the phylum level (Figures 3(a) and 4(a)). Additionally, the analysis of Unweighted Pair-group Method with Arithmetic Mean (U MA), PCoA, and NMDS also indicated that the microbial community was more significantly influenced by alizarin at the genus level (Figures 3 and 4). One reason for this result might be that alizarin strengthened selection pressure in the environment due to alizarin drug properties, leading to the death or growth inhibition of certain bacteria (Shan *et al.* 2016; Grenni *et al.* 2018).

Alteration of microbial community structure has been demonstrated as an important factor that affects the functionality of bacterial communities (Zhu *et al.* 2022). Our investigation showed that alizarin caused significant alterations to the microbial communities at the genus level (Figures 3(b) and 4(b)) and had a substantial impact on the functional changes of the microbial community (Figure 5). With the addition of alizarin, the functional characteristics of bacterial communities in Ali-treated group and Tet + Ali-treated group were more similar (Figure 5(a)). 'Drug resistance: antimicrobial', 'Membrane transport', and 'Cell growth and death' were up-regulated in Ali-treated group and Tet + Ali-treated group on day 30 compared with the control. This could be attributed to the microbial community's resistance to antibacterial drugs to cope with environmental stresses, which is manifested in the restructuring of the microbial community composition and abundance

(Liu *et al.* 2020). Moreover, previous studies have demonstrated that variations in metabolic pathways are useful in reducing biological toxicity in the environment, which is also supported by our findings (Zhang *et al.* 2021c). As shown in Figure 5(b), 'Carbohydrate metabolism', 'Amino acid metabolism', and 'Lipid metabolism' were up-regulated on Ali-treated group and Cip + Ali-treated group with the addition of alizarin, which is consistent with the results of prior research (Zhang *et al.* 2022).

In this study, the abundance of three ARGs and *intI*1 was significantly increased after alizarin treatment or the combination of alizarin and antibiotics treatment, compared with the control group ($p < 0.05$). Moreover, we observed a significant and positive correlation between ARGs, *intI*1, and ARB, suggesting the potential hosts for ARGs in the water samples (Figure 6). Specifically, *tet*A, *tet*C, and *qnr*S was significantly and positively correlated with six ARB as well as *intI*1 was also significantly and positively correlated with four ARB. These findings suggest that alizarin treatment could promote the proliferation and spread of ARGs, which could pose a significant risk to public health once transferred to human pathogens (Li *et al.* 2022). Our study also identified Flavobacterium as a potential host for qnrS (Figure 6), which is a known pathogenic bacterium associated with increased risk of cancer in humans (Kayani *et al.* 2021). These observations support the notion that alizarin could contribute to the generation and spread of ARGs, thereby exacerbating the risk of disease transmission.

## 5. CONCLUSION

This study demonstrated that alizarin, a non-antibiotic compound, had a significant effect on the growth of microbial populations and the abundance of ARGs and *intI*1 in water over a period of 30 days. When used in combination with antibiotics such as tetracycline or ciprofloxacin, alizarin was even more effective in promoting population growth and increasing the abundance of ARGs and *intI*1 than when used alone. Moreover, alizarin was found to alter both the composition structure and abundance of the bacterial community in water surpassing the effects of antibiotics. Specifically, genes such as *tet*A, *tet*C, and *intI*1 were positively correlated with potential hosts such as *Sphingopyxis*, *uncultured_bacterium_f_Xanthobacteraceae*, *Aeromonas*, *uncultured_bacterium_o_Rhodospirillales*, and *Methyloversatilis*.

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## AUTHORS CONTRIBUTIONS

H.F. investigated and wrote the original draft. L.T. investigated, visualized, and reviewed the study. N.Y. reviewed the study. S.Z. reviewed, supervised, and conceptualized the study, prepared the methodology, was involved in project administration, collected resources, and acquired funds.

## DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

## CONFLICT OF INTEREST

The authors declare there is no conflict.

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