4. Research report (Follow the guideline on the next page)

Title: Temporal and spatial variation in macrolide antibiotic concentrations in an urban river: resulting effects on periphytic communities

Baihuan Zhang[1], Hisato Iwata[2], and Jiahua Guo[1]

[1] Shaanxi Key Laboratory of Earth Surface System and Environmental Carrying Capacity, College of Urban and Environmental Sciences, Northwest University, Xi'an, 710127, China.

[2] Center for Marine Environmental Studies, Ehime University, Bunkyo-cho 2-5, Matsuyama, Ehime prefecture, 790-8577, Japan

1. Purposes

The aims of this study are: 1) to investigate the temporal and spatial distribution of macrolide antibiotic residues in river water; 2) to explore the resulting effects of macrolides in river on the structure of periphyton using 16S & 18S metabarcoding; 3) to clarify the periphytic photosynthesis and carbon metabolism process in response to macrolides in river.

2. Methods

2.1 Sampling sites and sample collection

The sampling campaign for water and periphyton was conducted in March (Spring), June (Summer), and September (Autumn) of 2023, with a total of 20 sampling sites chosen from upstream to downstream of the Zao River. Reference group A (Z1, Z2, and Z3) was situated approximately 50-80 m upstream of the STP E outlet, and a rubber dam with an elevation of 1 m used for regulating the water flow could partially prevent contamination from the discharged effluent. Other 17 sites, including groups B (Z4 – Z7, 20 m - 5 km away from STP E outlet), C (Z8 – Z15, 5 - 10 km away from STP E outlet), and D (Z16 - Z20, 10 - 15 km away from STP E outlet), were classified based on their distance away from STP E and were used for the characterization of impacts of effluents released from three STPs, respectively

2.2 Chemical analysis

Five macrolide antibiotics (azithromycin, anhydro erythromycin, clarithromycin, erythromycin, roxithromycin) concentrations in water and periphyton were determined by solid phase extraction (SPE) procedures and high performance liquid chromatography (HPLC-MS/MS, Agilent 1290 Infinity II for HPLC and Agilent 6470 triple quadrupole for MS/MS, USA) equipped with a tandem mass spectrometer.

2.3 Chlorophyll a fluorescence measurement

The Chl a fluorescence parameters were measured by AquaPen AP110-P chlorophyll fluorometer (Photon Systems Instrument, Czech). In brief, an aliquot of 0.1g wet weight periphyton after exposure was dissolved into 5 mL distilled water, and thereafter 4 mL solution was used to dilute to 16 mL. After 15 min of dark adaptation, a chlorophyll fluorometer was used to determine the OJIP parameters at 455 nm excitation wavelength.

2.4 Carbon utilization analysis

Carbon resource utilization experiment was conducted using Biolog EcoPlatesTM (Biolog Inc., Hayward, CA, USA). Specifically, an aliquot of 0.1g wet weight periphyton was dissolved into 5 mL distilled water and shaken at 200 rpm for 30 min at 25 ℃ in a shaker. 1 mL microbial solution was further taken and diluted to 15 mL in a centrifuge tube, where 150 μL was further pipetted into each well of EcoPlates. The microplates were incubated at 25 ℃ without light for 144 h. The AWCD value of each microplate was measured using an infinite 200Pro Microplate reader (Tecan Group Ltd., Männedorf, Switzerland) under 590 nm and 750nm.

2.5 DNA isolation, PCR amplification and next generation sequencing

DNA from the periphyton in each location was isolated and amplified. DNA was extracted and quantified on agarose gel electrophoresis by NanoDrop spectrophotometer (NC2000, Thermo Fisher Scientific, Waltham, USA). PCR amplicons were purified with Vazyme VAHTSTM DNA Clean Beads and quantified. The V3–V4 hypervariable regions of the 16S rRNA genes for prokaryotes and V4 hypervariable region of 18S rRNA genes for eukaryotes were amplified using the primer pairs 338F/806R (338F: ACTCCTACGGGAGGCAGCA; 806R: GGACTACHVGGGTWTCTAAT) and 547F/V4R (540F: CCAGCASCYGCGGTAATTCC; V4R: ACTTTCGTTCTTGATYRA), respectively [9,10]. Sequencing was performed using the Illlumina MiSeq platform with MiSeq Reagent Kit v3 at Shanghai Personal Biotechnology Co., Ltd (Shanghai, China). Details in data analyses can be found in the published paper.

3 **Results,**

3.1 Chemical analysis

After the STP E outlet was detected, an evident trend in the macrolide antibiotic residues in river water and periphyton was observed compared to group A, which had much lower pollution levels (**Figure 1**). Regarding temporal distribution, the highest overall antibiotic levels reached 2.19 μg/L, 1.70 μg/L, and 0.75 μg/L from March to September, respectively, where four parent compounds (ROX, AZI, ERY, and CLA) all exhibited a downward trend. The highest accumulation level in periphyton was 9.67 μg/g in Autumn, which was higher than in Spring (2.79 μg/g) and Summer (8.03 μg/g). In line with this, AZI and ROX occurred at 0.03 – 3.92 μg/g and 0.02 – 2.76 μg/g in Autumn, respectively. While hardly detected in March and June, the CLA level was1.23 μg/g in September. Surprisingly, ERY-H_2O was detected only in June. Concerning spatial distribution, the chemical concentration in water gradually decreased with the increase of distance from the sewage outlet of STP E, which was determined to be the hotspot of macrolide antibiotics among the downstream STPs. Although a similar trend was not detected for periphyton, the analysis demonstrated that high levels of these compounds in both water and periphyton were identified at a distance of 20 m - 5 km downstream of the sewage outlet (group B; **Figure 1**).

3.2 Composition of the periphytic community in Zao River

Here, the dynamic alteration of periphytic structures in the Zao River differed for prokaryotes and eukaryotes in all three seasons. In the case of prokaryotes, *Proteobacteria*

(19.7% – 86.4%, mean = 52.9%) and *Cyanobacteria* (0.05% – 59.1%, mean = 26.3%) occupied a dominant position in all sampling sites across three seasons. In particular, the proportion of *Cyanobacteria* in relative abundance in reference group A was 21.7 times higher than in group B (Z4 – Z7), with mean values of 24.7% and 1.52% in Spring, respectively. Concerning eukaryotes, Nematoda (0.01% – 97.1%, mean = 42.2%), Chlorophyta (0.36% – 98.6%, mean = 41.4%), and Rotifera (0% – 19.4%, mean = 2.31%) were the dominant phyla, comprising 85.9% of all eukaryotes (**Figure 2a**). For example, the relative abundance of Nematoda in group B (Z4 – Z7) diminished from 10.3% to 0.01%. In early sites in group C (Z8 - Z13), Nematoda diminished from 54.2% to 13.1% and was replaced by the growth of Chlorophyta in March, whereas its abundance gradually returned to the normal level of about 74.1% before entering the Wei River (e.g., from Z14 – Z18) (**Figure 2b**). Regarding the overall biological community index, the prokaryotic evenness in group B was 0.69, which was higher than that in group A (0.51). For eukaryotes, compared to 0.28 in group A, the evenness in groups B and C were significantly elevated to 0.49 and 0.51, respectively. Then, the evenness recovered to the normal level of 0.35 in group D. This result suggested that the STP E effluent increased the number of different species. Furthermore, in June, the prokaryotic richness increased significantly from 191 in group A to 556.8 in group D. In March, the eukaryotic richness increased from 73.7 in group A to 123 in group D. This evidence indicated a raised species diversity in periphyton.

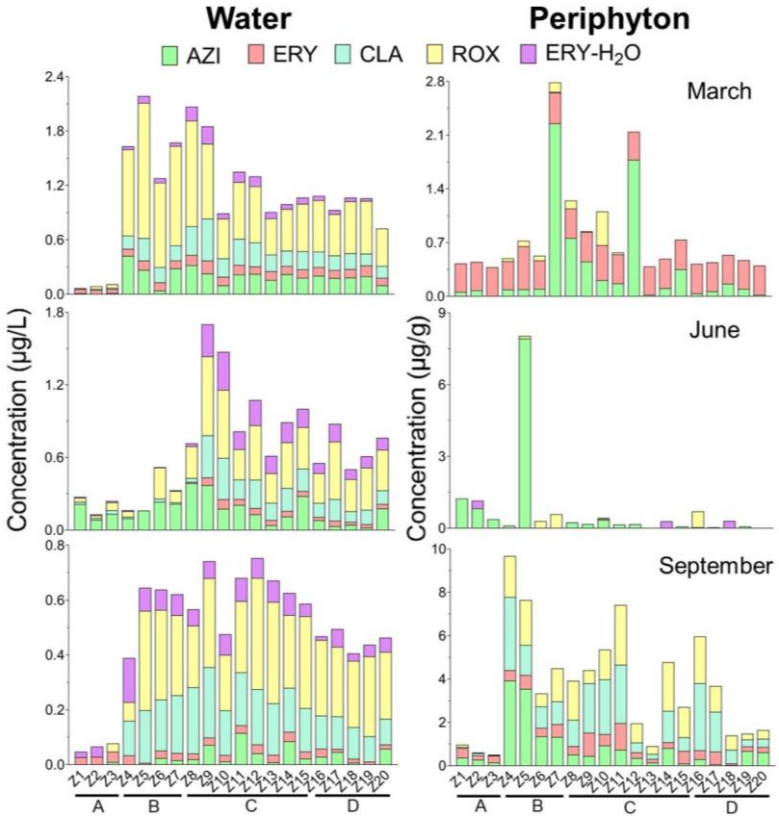

Fig.1 The relative abundance of prokaryotic and eukaryotic communities of all taxa at the phylum level. (a) is eukaryote; (b) is prokaryote. A = upstream group; B = 20 m - 5 km group; C = 5 – 10 km group; D = 10 -15 km group.

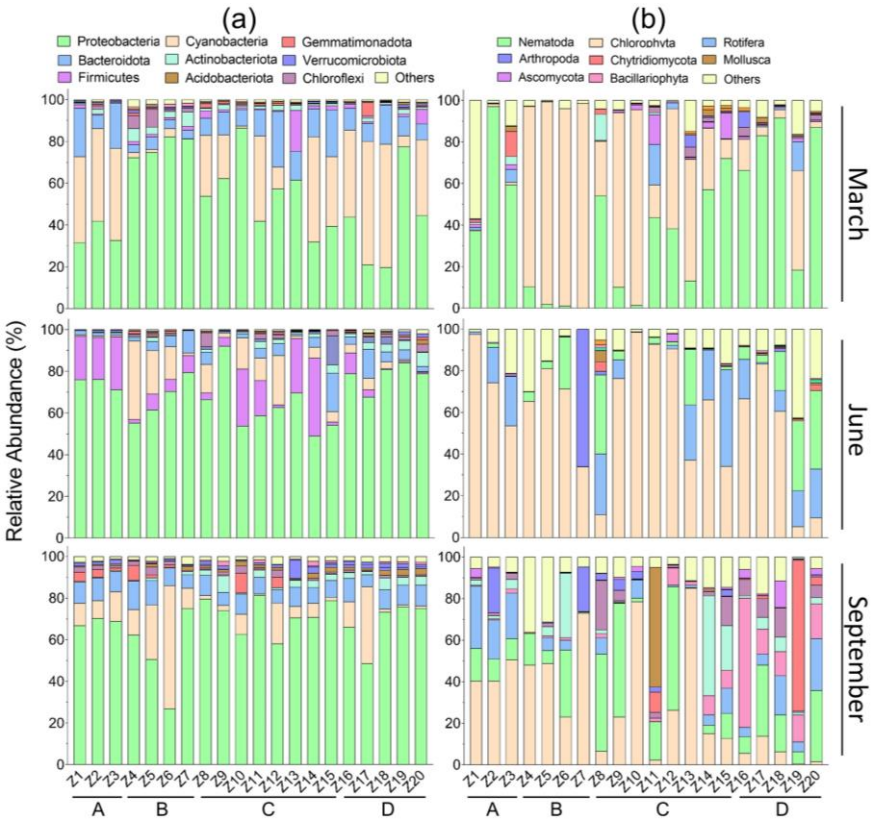

Fig.2 The relative abundance of prokaryotic and eukaryotic communities of all taxa at the phylum level. (a) is eukaryote; **(b)** is prokaryote. A = upstream group; B = 20 m - 5 km group; C = 5 – 10 km group; D = 10 -15 km group.

Owing to the copyright of publisher, other detailed results of correlation between chemical levels and the community composition, carbon metabolism and Chl a fluorescence measurement, and correlation between periphytic community composition and functions in three seasons can be found in the published paper (Zhang et al., 2014, *Environ. Pollut.*, 345: 123495, 2024).

4 Future challenges.

Our next plan is to investigate the antibiotic resistance genes and their hosts in periphyton of a macrolide antibiotics-contaminated river.