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

Title: Effects of azithromycin on river periphyton: Structure, meta-transcriptional process and ecological functions

Jiayi Liang[1], Hisato Iwata[2], Yulu Tian[1], 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 evaluate the inhibition effects of azithromycin on the photosynthesis and carbon metabolism processes in periphyton; 2) to investigate the effects of azithromycin on the periphytic composition using 16S & 18S metabarcoding; 3) to elucidate the molecular mechanism underlying the structural and functional changes using meta-transcriptomic analysis.

2. Methods

2.1 Periphyton culturing and testing

Pebbles that were gathered from pristine natural rivers (the Qinling reach of Taiyi River in Xi'an), China (109.0192◦ N, 34.0517◦E)) were selected as culture substrates. These pebbles were placed in a 2L beaker, immersed with BG11 medium and cultured in a light incubator at the following conditions for 21 days: 2800 lux light intensity, a 14 h/10 h light/dark circle, and a controlled temperature of 22 ± 2 °C. The culture medium was freshly prepared at an interval of 5 days. After 21 days of incubation, a sterile toothbrush was used to harvest the periphyton from the stones.

A total of twenty-one 250-mL Erlenmeyer flasks, which contain 0.1 grams (g) wet weight (ww) of periphyton and 150 mL of BG11 medium, respectively, were initially prepared and placed in the culture chamber. After 7 days of inoculation, the medium was replaced by a range of AZI test solutions, comprising 150 mL of 5 μg/L, 50 μg/L, 200 μg/L, 500 μg/L, 5,000 μg/L, and a control group (0 μg/L), which were all prepared in BG11 medium. A 50 μg/L treatment group without periphyton was prepared for the evaluation of potential abiotic degradation. All groups were in triplicates and the incubation lasted for 14 days, as the periphyton start decaying after 16 days in our earlier growth curve measurement. After that, 50 mL of periphyton samples from each group were subjected to filtration via a 0.22 μm membrane (Jintang Company, Tianjin, China), flash freeze with liquid nitrogen and kept in a -80 °C refrigerator before DNA metabarcoding and metatranscriptomic analysis.

2.2 Periphyton structure analysis

The taxonomic composition of the periphytic microbial community in response to AZI

exposure was revealed by DNA metabarcoding technology. Briefly, the DNA extraction was conducted for the filtered periphyton on day 14, and 16s & 18s rRNA sequencing were analyzed using the Illumina MiSeq platform of Personalbio Technology Co, Ltd (Nanjing, China).

2.3 Chlorophyll a fluorescence and carbon metabolism

The chlorophyll a fluorescence parameter, reflecting the activity and energy conversion of photosystem II (PSII) in autotrophic organisms of periphyton, was measured on day 14 using the AquaPen AP110-P (Photon Systems Instrument, Czech Republic). To evaluate the carbon utilization capacity for the heterotrophic organisms in periphyton, a Biolog EcoPlate™ (Biolog Inc., Hayward, CA, USA) test that contains 31 different sources of carbon, each with three replicates, was performed following the manufacturer's protocol.

2.4 The meta-transcriptomic analysis

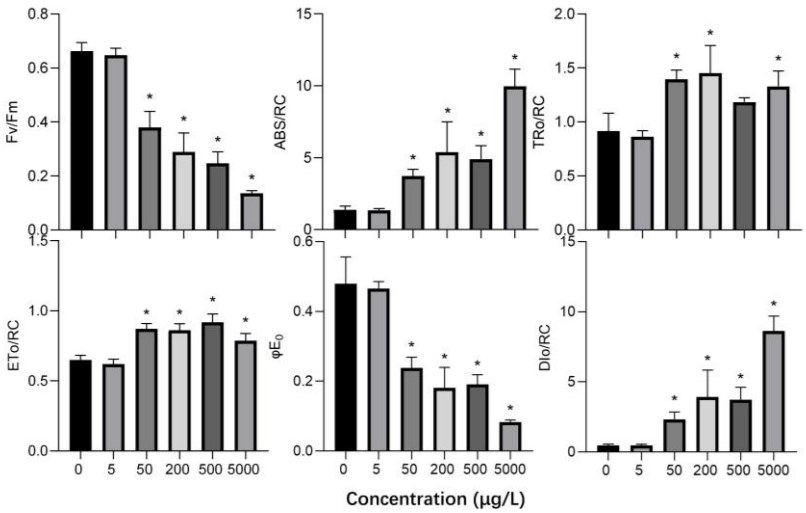
The total RNA was extracted and the purity and concentration were quantified by NanoDrop NC 2000 (Thermo Scientific, Wilmington, Delaware, US), followed by next-generation sequencing (NGS) using the Illumina HiSeq X Ten (Nanjing Personal Gene Technology Co, LTD, China). Detailed information for metatranscriptomic sequencing could be found in the published paper. The "DESeq" package was used to screen the differentially expressed genes (DEGs) in the treatment group based on the following criteria: adj *p* value < 0.05 and |log2 Fold Change (FC)| > 1. The enrichment of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was then analyzed to reveal the primary biological functions. The Pathview package of R software was used to visualize the metabolic pathways.

3. Results

3.1 Effects of AZI on periphytic photosynthesis

Exposure to AZI significantly inhibited PSII reaction centers involved in plant photosynthesis, especially the overall maximum photochemical quantum yield (Fv/Fm) of PS II that was decreased in a concentration dependent manner (Fig. 1). In particular, exposure to AZI at 5,000 μg/L resulted in a 63.64% decrease of Fv/Fm compared to the control group. In the 50 to 5,000 μg/L of AZI treatment, the ABS/RC, ETo/RC, TRo/RC, and DIo/RC levels involved in energy flux of PS II were promoted by AZI exposure compared with the control group, with the increased values of 63.00-86.16%, 16.09-29.35%, 19.55-36.55%, and 79.83-94.56%, respectively.

a



b

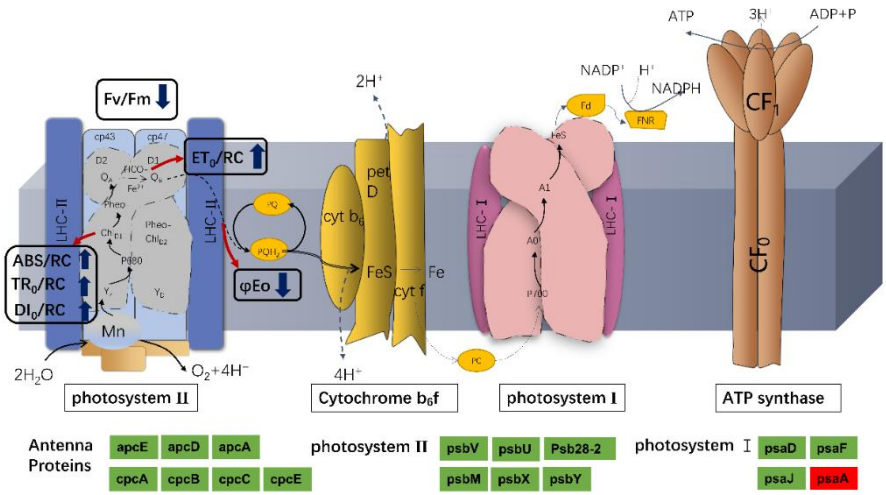
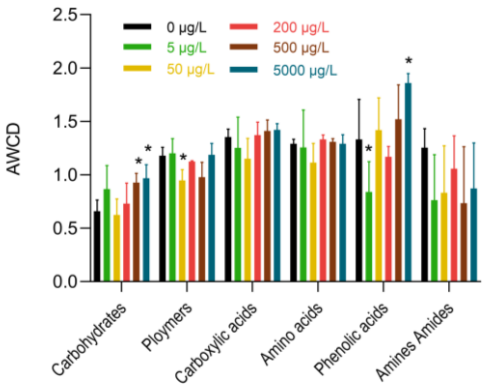


Fig.1 Effect of azithromycin on photosynthesis of periphyton. (a) OJIP parameter results of AZI exposure; (b) The photosynthetic process at the molecular and physiological levels.

3.2 Effect of AZI on periphyton carbon source utilization

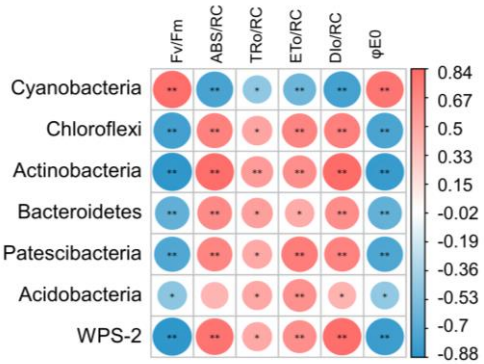
In the present study, the PCA analysis indicated that the periphytic capacity to utilize different carbon sources in medium (50 μg/L) and high (5,000 μg/L) treatments departed from controls (Fig. 2). Particularly, the metabolism of carbohydrates and phenolic acids were increased 46.70% and 39.54% in 5,000 μg/L AZI treatment, respectively, where no evident alteration was detected for other four carbon sources (Fig. 2). A positive correlation between the periphytic bacterial community and five carbon sources, in addition to polymers, was further detected (Fig. 2).

a.



b.

c.



d.

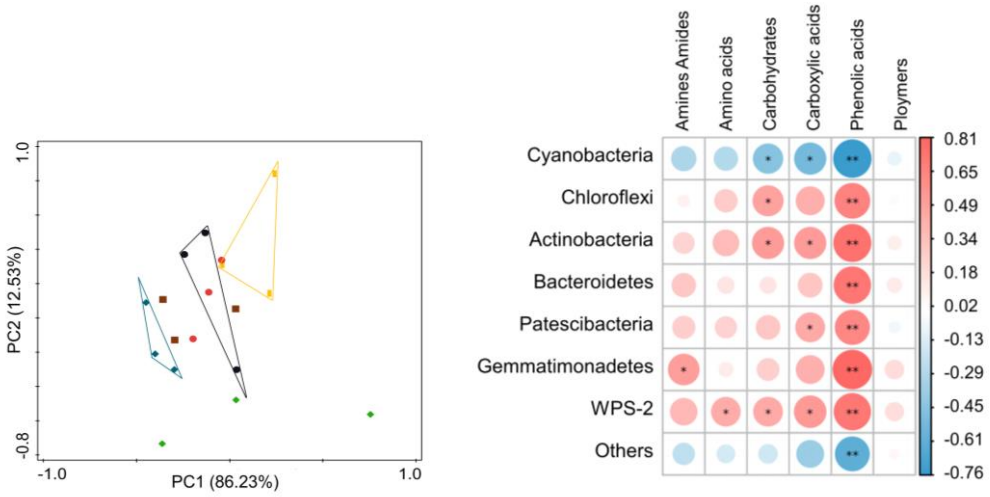


Fig. 2 Carbon source utilization capacity and correlation between periphytic composition and function analyses. (a) the utilization of six carbon sources in control and treatment groups; (b) PCA plots of AWCD; (c) Correlation between composition and Chl a fluorescence parameters; (d) Correlation between composition and carbon sources (*p < 0.05; **p < 0.01).

A total of 14 compounds and 46 pathways related to the carbon source utilization were significantly enriched, where energy and carbohydrate metabolism were the dominant pathways. These processes were in relation to the energy and carbon source supply for microbial activities, which were pivotal for the growth and metabolism of microorganisms. In the case of energy metabolism, the increase in the utilization of phenolic acids at AZI 5,000 μg/L group was mainly caused by the increased 4-Hydroxybenzoic acid, which was governed by the related metabolic pathways, including ubiquinone and other terpenoid-quinone biosynthesis (ko00030), benzoate degradation (ko00362), aminobenzoate degradation (ko00627) and folate biosynthesis (ko00790), in which all the DEGs involved were up-regulated. In the matter of carbohydrates, the elevated utilization of D-Galactonic acid -lactone, D-Cellobiose, i-erythritol, alpha-D-Glucose-1-phosphate, D-xylose, and N-Acetyl-D-glucosamine contributed to the enhanced carbohydrate consumption in 500 μg/L and 5,000 μg/L AZI treatment groups. The metabolism of these carbohydrates was primarily concerning the starch and sucrose metabolism (ko00500), ABC transporters (ko02010), galactose metabolism (ko00052), glycolysis/gluconeogenesis (ko00010), amino sugar and nucleotide sugar metabolism (ko00520), phosphotransferase system (ko02060), etc. where most of the DEGs were up-regulated.

Due to the copyright of publisher, other detailed results of the altered periphytic composition, KEGG secondary metabolic pathway composition, and the proposed transformation pathway of AZI can be found in the published paper (Liang et al., 2024, Water Res. 251: 121140, 2024)

4. Future challenges

Our next plan is to investigate the plasmid mediated conjugation transfer of ARGs in periphyton under azithromycin pressure.