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Metabolomic modulations in a freshwater microbial community exposed to the fungicide azoxystrobin

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ABSTRACT

An effective broad-spectrum fungicide, azoxystrobin (AZ), has been widely detected in aquatic ecosystems, potentially affecting the growth of aquatic microorganisms. In the present study, the eukaryotic alga *Monoraphidium* sp. and the cyanobacterium *Pseudanabaena* sp. were exposed to AZ for 7 days. Our results showed that 0.2–0.5 mg/L concentrations of AZ slightly inhibited the growth of *Monoraphidium* sp. but stimulated *Pseudanabaena* sp. growth. Meanwhile, AZ treatment effectively increased the secretion of total organic carbon (TOC) in the culture media of the two species, and this phenomenon was also found in a freshwater microcosm experiment (containing the natural microbial community). We attempted to assess the effect of AZ on the function of aquatic microbial communities through metabolomic analysis and further explore the potential risks of this compound. The metabonomic profiles of the microcosm indicated that the most varied metabolites after AZ treatment were related to the citrate cycle (TCA), fatty acid biosynthesis and purine metabolism. We thereby inferred that the microbial community increased extracellular secretions by adjusting metabolic pathways, which might be a stress response to reduce AZ toxicity. Our results provide an important theoretical basis for further study of fungicide stress responses in aquatic microcosm microbial communities, as well as a good start for further explorations of AZ detoxification mechanisms, which will be valuable for the evaluation of AZ environmental risk.

Introduction

With the development and intensification of agriculture, the application of pesticides is increasing rapidly. A large number of different pesticides are used to control agricultural diseases, pests and weeds, and some of them remain in the soil. With rainfall or irrigation runoff flowing into the nearby surface water, they seriously affect the water quality of rivers and ponds in the region (Lu et al., 2014), as evidenced by the high frequency of harmful cyanobacterial blooms (Huisman et al., 2018). Even so, the production of fungicides is increasing as agriculture relies on their efficiency. Azoxystrobin (AZ), the best-selling strobilurin (with worldwide sales of $1.2 billion in 2014), is a broad-spectrum fungicide that prevents as-

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comycetes, deuteromycetes, basidiomycetes, and oomycetes (Casida and Durkin, 2017). AZ impairs fungal growth by blocking the electron transfer of cytochrome bc1 complex between cytochrome b and cytochrome c1, thus ultimately preventing the production of ATP and inhibiting mitochondrial respiration and energy production (Bartlett et al., 2002; Balba et al., 2007).

The widespread use of AZ-containing formulations inevitably causes water pollution through the spraying of flotsam, surface runoff and leaching processes (Garanzini et al., 2015), and the concentration of AZ in aquatic systems has reached 0.01–29.70 μg/L in ponds, streams, lakes and groundwater in France, Denmark, Brazil, Germany, and the USA (Jorgensen et al., 2012; Liess and von 2005; Rodrigueset al., 2013). It is worth noting that fungicides have moderate lipotropy and a high adsorption potential for organic carbon. That means fungicides may adsorb into sediments and organic surfaces in aquatic systems and be continually released into water (Castillo et al., 2000; Kronvang et al., 2003; Smalling et al., 2013a, 2013b), persistently causing collateral damage to the aquatic system. Microorganisms, such as algae and cyanobacteria, play a pivotal role in nutrient cycling and the health of freshwater ecosystems and are sensitive indicators of various toxic substances (Navarro, et al., 2008; Du et al., 2019; Lu et al., 2020a; Zhang et al., 2018a). Garanzini and colleagues noted that a concentration of 50 μg/L AZ caused DNA damage and oxidative stress to the macrophyte *Myriophyllum quitense* (van Wijngaarden et al. 2014; Zubrod et al., 2015). AZ also changes the microorganism community composition in aquatic systems, leaves and soils (van Wijngaarden et al., 2014; Zubrod et al., 2015; Lu et al., 2019; Zhang et al., 2019). However, the toxicological mechanism of AZ remains unclarified.

Although AZ is used to control fungal pathogens, its general mode of action is not fungi-specific. Therefore, it may be potentially toxic to a wide range of non-target organisms in aquatic systems (Rodrigues et al., 2013). For a more comprehensive understanding of this mechanism, *Monoraphidium* sp. and *Pseudanabaena* sp., as representatives of eukaryotic algae and prokaryotic algae, respectively, were selected to investigate the effects of AZ on the growth, chlorophyll content, protein content and total organic carbon (TOC) content of such microorganisms. In addition, metabolomic analysis was conducted to determine the effect of AZ on the metabolism of the aquatic microorganism community. This study aims to provide insights into the potential toxic mechanism of AZ on the aquatic environment.

1. Materials and methods

1.1. Microalgae and growth conditions

The freshwater eukaryotic algae *Monoraphidium* sp. (FACHB-1853) and the cyanobacterium *Pseudanabaena* sp. (FACHB-2209) were obtained from the Institute of Hydrobiology at the Chinese Academy of Sciences (Hubei, China). Algae were cultured in 250-mL Erlenmeyer glass flasks containing 150 mL of BG-11 medium at 25 °C ± 0.5 °C under cool-white fluorescent light (46 μE/m^2/sec, with a photoperiod of 12 hr:12 hr light:dark). The medium containing macronutrients was autoclaved at 121 °C for 25 min. The trace metal solution was filter-sterilized beforehand and then mixed with macronutrients.

1.2. Measurement of microalgae cell yield after AZ treatment

According to a previous report, 0.5 mg/L AZ effectively inhibits the growth of *Chlorella pyrenoidosa* (Lu et al., 2018). Here, we chose the following concentrations of AZ: 0.1, 0.2 and 0.5 mg/L. The cell density of the culture was measured by spectrophotometry at 680 nm (OD_{680}) every 24 hr for 7 days. The initially cultured *Monoraphidium* sp. (a) and *Pseudanabaena* sp. (b) were enumerated microscopically to establish a linear regression equation between the number of cells ($y \times 10^5$ cells/mL) and OD_{680} (x). The number of cells was calculated based on the following equations: (a) $y = 94.0x + 1.1$ ($R^2 = 0.985$); (b) $y = 141.9x + 1.9418$ ($R^2 = 0.975$). The initial OD_{680} of each microalgae culture was adjusted to 0.01, where the initial algal cell density of *Monoraphidium* sp. and *Pseudanabaena* sp. were 2.04×10^5 and 3.36×10^5 cells/mL, respectively. All the cultures were stirred manually three times a day. The strobilurin fungicide AZ (purity 98.5%) was purchased from Aladdin (Shanghai, China), and the storage solution (50 g/L) was diluted with acetone. The data represent four replications of each biological experiment.

1.3. Determination of algal growth inhibition

The inhibition rates of *Monoraphidium* sp. and *Pseudanabaena* sp. growth in response to AZ treatment every 24 hr for 7 days were calculated using the following equation:

$$Inhibitionrate = (1 - N/N_0) \times 100\%$$

where N (cells/mL) and N_0 (cells/mL) are the cell density values in the treatment and control groups, respectively (Lu et al., 2018).

1.4. Aquatic microcosm set up and AZ exposure

Microcosm microbial community samples were collected in Nov. 2018 from the Meiliang Bay of Lake Taihu, the third largest freshwater lake in China (0°55′40″-31°32′58″N, 119°52′32″-120°36′10″ E). Surface water was collected at a depth of 0.5 m and then delivered to the laboratory within 3 hr. The water samples were filtered through a 0.45 μm fiber membrane to obtain the aquatic microbial community and then immediately inoculated into the culture medium. Considering that the N and P concentrations in the original BG11 were too high for the microcosm microbial community, the BG-11 medium was modified with 24.7 mg N/L and 0.54 mg P/L, which were only 10% of the N and P concentrations of the original medium (Lu et al., 2020b). The microcosm microbial community was cultured in 250-mL Erlenmeyer glass flasks under the same conditions used for the monoalgal culture, and the flask mouths were covered with absorbable sterile gauze to allow the exchange of gasses between the flask headspace and the atmosphere while preventing contamination of the culture by external microorganisms.

1.5. Determination of Chl-a content

All the samples of *Monoraphidium* sp., *Pseudanabaena* sp. and the microcosm microbial community in the experiment were collected for chlorophyll *a* (Chl-*a*) measurements after 3, 5, and 7 days of exposure using the method of Inskeep and Bloom (1985). In short, we determined the extinction coefficients of Chl-*a* in N, N-dimethylformamide (DMF) for wavelengths of 645 and 663 nm, using the following equations for quantifying Chl-*a*: $\text{Chl-}a = 12.70A_{663} - 2.69A_{645}$. Each treatment included four replicates.

1.6. Determination of total organic carbon (TOC) and protein contents

In the preliminary experiment, we observed the presence of flocculent sediments after AZ exposure in the microcosm

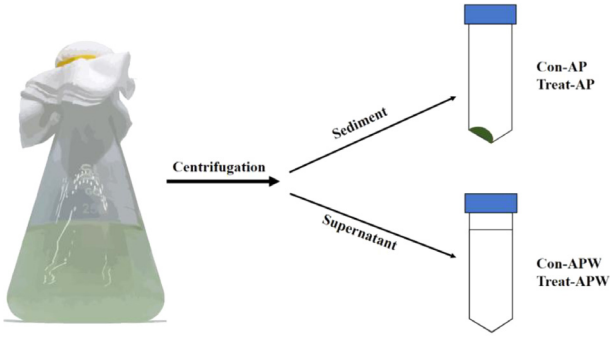

Fig. 1 – The method of sample collection.

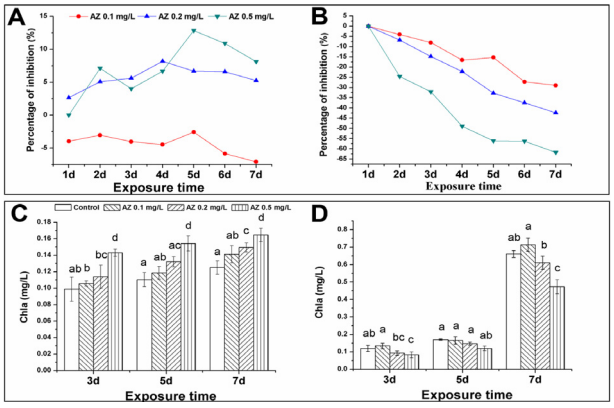

Fig. 2 – Growth inhibition of *Monoraphidium* sp. (A) and *Pseudanabaena* sp. (B) in response to azoxystrobin (AZ) treatments at different concentrations. Changes of Chl-*a* content in *Monoraphidium* sp. (C) and *Pseudanabaena* sp. (D) after AZ exposure. Different letters represent a significant difference between the two groups ($p < 0.05$).

microbial community, as well as in the monoalgal cultures. Then, we measured the TOC and protein content in the culture medium from both control and treated groups of *Monoraphidium* sp., *Pseudanabaena* sp. and the microcosm microbial community. The samples were centrifuged at 12,000 r/min for 10 min, and then the supernatant was filtered through a 0.45 μm fiber membrane (Xingya, Shanghai). The filtrate was poured into a transparent sample bottle to analyze the TOC concentration in a total organic carbon analyzer (TOC-LCPN, Shimadzu, Japan). The protein concentrations of the supernatant were measured using a bicinchoninic acid protein assay kit (Beyotime Biotechnology, Haimen, China). These data included four repetitions of each biological experiment.

1.7. *Metabolite analysis*

The metabolite analysis was performed with five replicate samples. After 7 days of incubation, floating flocculent samples and their culture medium were collected from both the 0.1 mg/L AZ-exposed group and the control group. The specific sampling method is shown in Fig. 1. Supernatant and sediment were collected after centrifugation. The supernatant was mainly medium, and the sediment was mostly floating flocculent, but it contained some algae. There were five replicates of each type of sample. A sample of approximately 1 mg floating flocculent was transferred into a 2-mL Eppendorf tube, and 450 μL of extraction liquid ($V_{Methanol}$:V_{H2O} = 3:1) was added. Then, 10 μL of L-2-chlorophenylalanine (1 mg/mL stock in dH_2O) was added as an internal standard. The mixture was swirled for 30 s. After the addition of porcelain beads, the mixture was processed in a 45 Hz grinder for 4 min and ultrasounded for 5 min (in an ice water bath). Meanwhile, a sample of 200 μL of culture medium was placed in a 2-mL Eppendorf tube. After the addition of 300 μL of methanol extract and 10 μL of L-2-chlorophenylalanine (1 mg/mL stock in H_2O), the mixture was swirled for 30 s and then was ultrasounded in an ice bath for 10 min. After that, the above processed samples were centrifuged for 15 min at 12,000 r/min, 4 °C. Approximately 200 μL (culture medium sample: 300 μL) of supernatant was extracted into a 1.5-mL Eppendorf tube. The extract was dried in a vacuum concentrator without heating, and 20 μL of methoxamine salt reagent methoxyamination hydrochloride (20 mg/mL in pyridine) was added to the dried metabolites. After being mixed gently, the sample was put into the oven at 80 °C and incubated for 30 min. A total of 30 μL of BSTFA (containing TMCS 1%, V/V) was added to each sample, and the mixture was incubated at 70 °C for 1.5 hr. All derivatized samples were analyzed by an Agilent 7890 gas chromatograph system combined with a Pegasus HT time-of-flight mass spectrometer (GC-TOF-MS). The mass spectrometer had an Agilent db-5 ms capillary column (30 m × 250 μm × 0.25 μm, J&W Scientific, Folsom, CA, USA). Finally, all MS data were analyzed using ChromaTOF software (V 4.3x, LECO).

1.8. *Statistical analyses*

One-way ANOVA (StatView 5.0, Statistical Analysis Systems Institute, Cary, NC, USA) was used to test the statistical significance of the biochemical and physiological measurement data. Differences were considered statistically significant when $p < 0.05$. The Leco-fiehn Rtx5 database, including mass spectrometry matching and retention time index matching, was used for qualitative analysis of metabolic data. Experiments were replicated four times, and metabolomic analyses were performed five times independently. Data were expressed as the mean ± standard error of the mean (SEM).

2. Results

2.1. *Growth and physiological reaction of* Monoraphidium*sp. and* Pseudanabaena*sp*

We measured the growth of microalgae after 7 days of AZ exposure and found that AZ slightly inhibited the growth of *Monoraphidium* sp. at concentrations of 0.2 and 0.5 mg/L, but not at the concentration of 0.1 mg/L. The peak inhibition was 12.9%, which appeared 5 days after exposure to 0.5 mg/L AZ (Fig. 2A). AZ exposure stimulated *Pseudanabaena* sp. at the tested concentrations. As shown in Fig. 2B, 0.1, 0.2 and 0.5 mg/L AZ treatments promoted the growth of *Pseudanabaena* sp. by nearly 30%, 40% and 60% at 7 days, respectively, compared to the control group.

Similarly, Chl-*a* was inhibited in *Monoraphidium* sp. but was stimulated in *Pseudanabaena* sp. Compared with the control group, the Chl-*a* contents of *Monoraphidium* sp. in the 0.5 mg/L AZ treatment were significantly reduced by more than 30% after 3, 5 and 7 days of exposure, while the Chl-*a* contents of *Pseudanabaena* sp. exposed to 0.5 mg/L AZ increased by approximately 30%-45%.

To verify whether AZ stimulated the secretion of EPS in microbes, TOC and protein concentrations were determined in *Monoraphidium* sp. and *Pseudanabaena* sp. culture medium. A marked increase in the TOC concentration was observed in

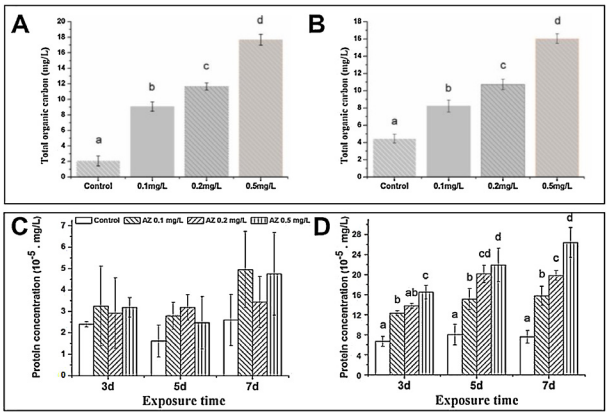

Fig. 3 – Changes of TOC content in the culture medium of *Monoraphidium* sp. (A) and *Pseudanabaena* sp. (B) after exposure to AZ on day 7; and the changes of protein content in the culture medium of *Monoraphidium* sp. (C) *Pseudanabaena* sp. (D) after exposure to AZ on day 3, 5, and 7; Different letters represent a significant difference between the two groups ($p < 0.05$).

Monoraphidium sp. culture medium, with 4.4 to 8.5 times the control after 7 days of exposure to different AZ concentrations (Fig. 3A), while the protein concentration did not increase dramatically (Fig. 3C). In *Pseudanabaena* sp. culture, TOC and protein concentrations were all significantly increased to nearly 2-fold of the control (Fig. 3B and D).

2.2. *Microbial community responses (chlorophyll, TOC, protein concentration)*

AZ had an interesting effect on the content of Chl-*a* in the microbial community and was increased after 3 days of AZ treatment (Fig. 4A); however, it decreased significantly after 5 and 7 days of AZ treatment.

The TOC concentrations in the microcosm medium all increased significantly during the measurement times (Fig. 4B). Notably, the TOC concentration in the microcosm medium exposed to high concentrations of AZ (0.5 mg/L) was more than 6 times greater than the TOC concentration of the control group. A high concentration of AZ also greatly stimulated the secretion of proteins by the microbial community, with values that were nearly 6 and 4 times higher than that of the control (Fig. 4C). There was also no significant difference in the protein and TOC concentrations in the microcosm medium treated with low concentrations of AZ (0.1 and 0.2 mg/L).

2.3. *Metabolite profile changes in the microbial community after exposure to AZ*

Metabolite analysis based on floating flocculants (Con-AP vs Treat-AP) mainly focused on insoluble extracellular polymers, while analysis of the culture medium (Con-APW vs Treat-APW) focused on soluble extracellular polymers. Volcano plots are provided to visualize changes in metabolites compared to the control groups. In the metabolomic analysis of the two treatments of floating flocculants, 507 metabolites were detected, 89 of which were upregulated and 5 of which were downregulated (Fig. 5a). In two microbial medium metabolomic analysis treatments, a total of 400 metabolites were detected in the 10 samples of culture medium, among which 4 metabolites were upregulated and 5 metabolites were downregulated (Fig. 5b).

First of all, the changes in the insoluble extracellular polymers were concerning. The results showed that AZ significantly boosted the contents of nucleotides, sugars, organic acids and amino acids in microbial intracellular and cell-surface polymers. The contents of purine riboside, citric acid, L-homoserine 1, elaidic acid, d-glucoheptose 1 and lysine sharply increased after AZ treatment (Fig. 6). In the group exposed to 0.1 mg/L AZ, the concentrations of these metabolites had increased by 3500%, 2251%, 1521%, 1280%, 1063% and 952% of that in control group, respectively. Only four metabolites, iminodiacetic acid, 1,3-diaminopropane, 3-hydroxybutyric acid, and ethanol, were decreased after AZ treatment; their concentrations were only 19.3%, 42.8%, 48.1% and 48.3% of that in control, respectively. Most amino acids and fatty acids increased in the exposed group, as did most secondary metabolites.

The cluster analysis method was used to regroup all independent samples according to the similarity between the distribution of metabolite abundances and the principal component analysis model. The second principal component (PC2) clearly separated Treat-AP from Con-AP (accounting for 17.49% of the total variance), confirming the effects of AZ on the plankton community. There were no significant differences between Con-APW and Treat-APW. To more reliably determine differences between groups of metabolites and the relevance of the experimental group information, we adopted the OPLS-DA (orthogonal projections to latent structures- discriminant analysis) statistical method to analyze the differences between the results. The analysis model showed that the clear separation between the two groups represented a significant difference in the metabolic fluxes between different treatments.

As shown in the experimental data, the clear separation between the two groups represents a significant difference in the metabolic fluxes with different treatments. Of the 94 significantly altered compounds (including 89 upregulated and 5 downregulated metabolites, $p < 0.05$), 56 were identifiable, and 27 metabolites were involved in the main metabolic pathways, including carboxylic acids and fatty acyls (Fig. 7). In response to AZ, the intermediates (ribose, glyceric acid) in the pentose phosphate pathway (PPP) were significantly increased (4- to 5-fold, $p < 0.05$), and the citrate cycle (TCA cycle) intermediates succinic, malic, citric, and fumaric acids were also obviously upregulated ($p < 0.05$). Inosine, guanosine and sulfuric acid in the purine metabolic pathway increased 6.24-, 5.54- and 2.35-fold after AZ treatment compared with the control group ($p < 0.05$). Similarly, glutamic acid, putrescine and proline were significantly upregulated (7.73, 4.28 and 2.33-fold, respectively, $p < 0.05$) after exposure to AZ. All the detected fatty acids, such as lauric acid, arachidonic acid, itaconic acid, linolenic acid, myristic acid, linoleic acid, oleic acid and palmitoleic acid, were significantly upregulated by nearly 2- to 9-fold of the control values ($p < 0.05$). The relationship between important metabolic pathways is shown in Fig. 8. The schematic diagram of metabolic pathways of the microbial community showed some key metabolites in the metabolic pathways of the microbial community, and the red font represents the increased pathways, while the blue font represents the decreased pathways.

The changes in extracellular soluble polymers of microorganisms after AZ treatment were not significant. Among the 400 detected metabolites, only 5 were upregulated, and 4 were downregulated, and none of the differentially expressed metabolites were involved in the main metabolic pathways.

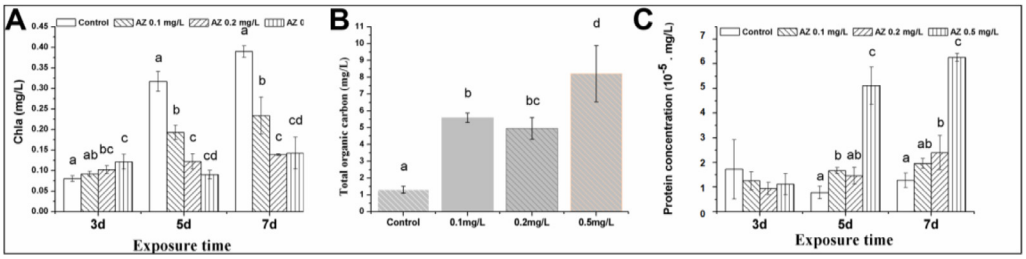

Fig. 4 – Changes in Chl-*a* content (A), TOC content (B) and protein content (C) in the microcosm microbial community after AZ exposure. Different letters represent a significant difference between the two groups ($p < 0.05$).

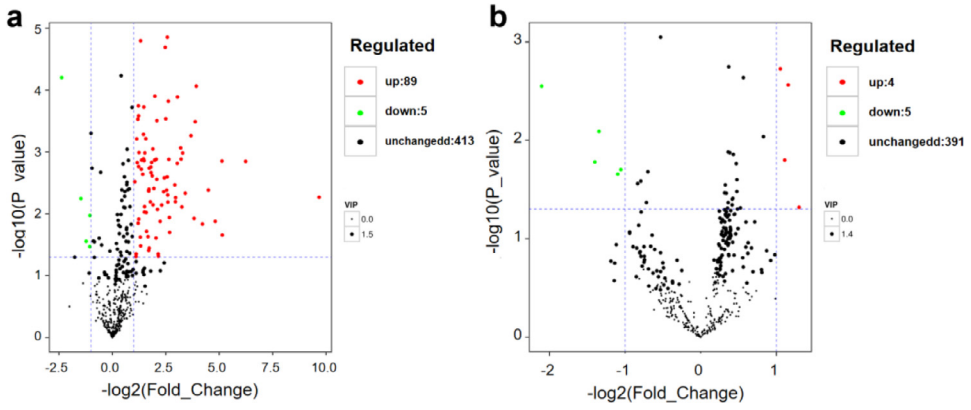

Fig. 5 – Volcano map visually shows that the changes of metabolites in floating flocculent (a) and culture medium (b) were significant after AZ treatment.

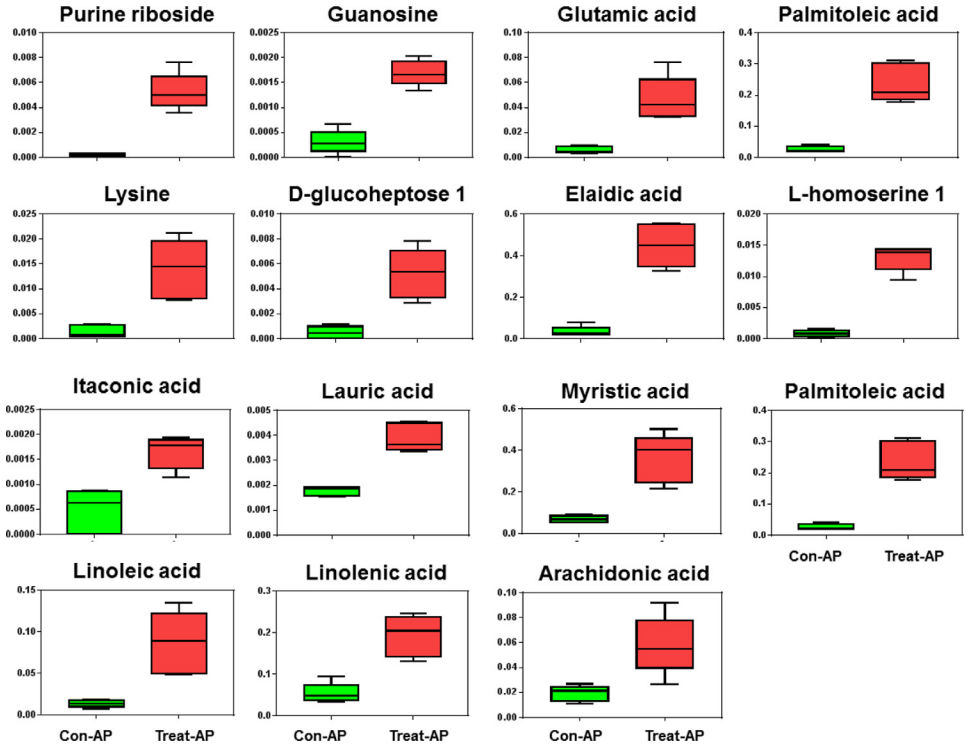

Fig. 6 – Relative abundance of significantly changed metabolites in a microbial community exposed to 0.1 mg/L AZ ($n = 5$). The Y-axis indicates absolute signal from GC-TOF-MS.

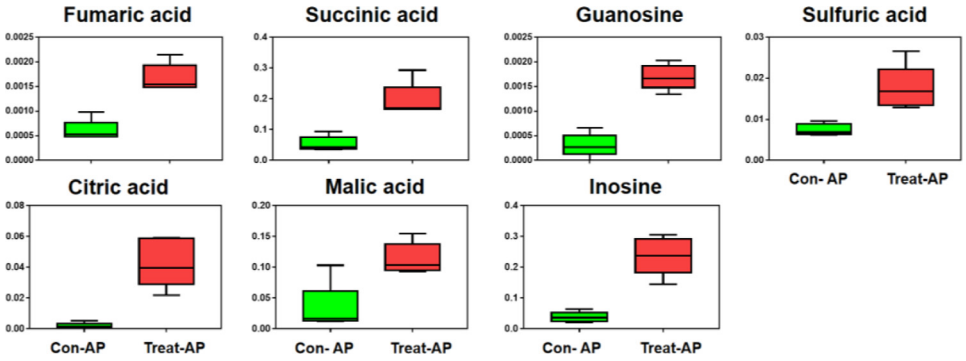

Fig. 7 – Relative abundance of significantly changed metabolites in microbes exposed to 0.1 mg/L AZ (n = 5). The Y-axis indicates absolute signal from GC-TOF-MS.

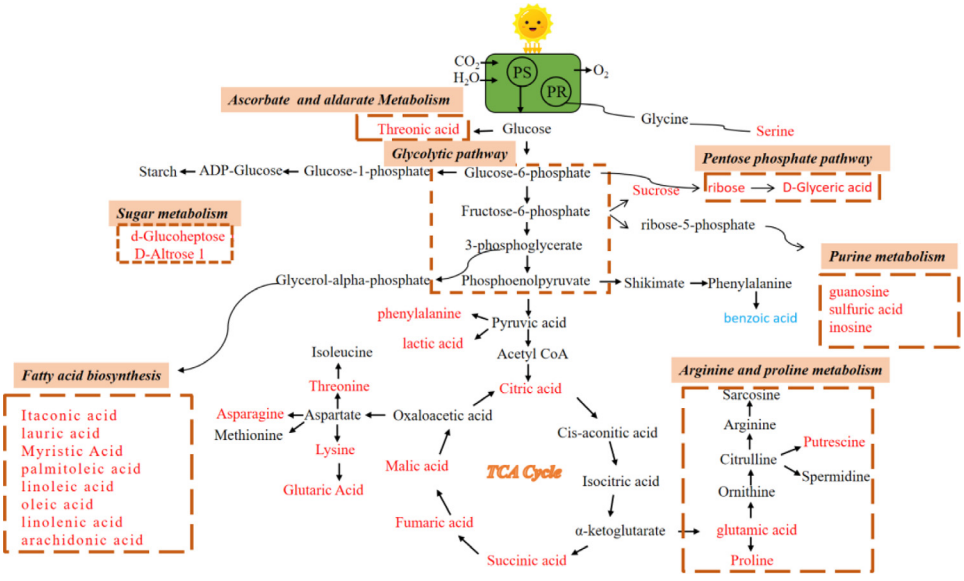

Fig. 8 – Schematic diagram of proposed metabolic pathways of the microbial community after AZ exposure for 7 days. Central metabolic pathways (glycolytic pathway, TCA cycle, arginine and proline metabolism and purine metabolism) and other metabolite biosynthetic pathways (sugar, amino acid and fatty acid) are shown. Red and blue typefaces indicate that the metabolites increased or decreased after AZ treatment, respectively.

3. Discussion

3.1. *Effects of AZ on* Monoraphidium *sp. and* Pseudanabaena *sp*

Increased use of AZ in chemical control of pathogenic fungi may disrupt aquatic biological systems. Photosynthesis is a common target of toxins affecting microalgae, and Chl-*a* can be used to indicate the capacity of algal photosynthesis (Qian et al., 2016). In this study, the Chl-*a* in *Monoraphidium* sp. and *Pseudanabaena* sp. was evaluated after exposure to AZ at 0.1, 0.2 and 0.5 mg/L. Both the results of the growth curve and the Chl-*a* changes indicated that AZ selectively inhibited algae growth, and eukaryotic algae were more sensitive than prokaryotic algae to AZ because AZ is active against almost all fungal pathogens (Anke et al., 1977). Similarly, a previous report also showed that 2.5 mg/L AZ significantly inhibited the growth of *Chlorella pyrenoidosa* but did not have an inhibitory effect on *Microcystis aeruginosa* growth even under treatments with high AZ concentrations (Lu et al., 2019). However, no report has clarified the reason why prokaryotes resist AZ treatment.

Interestingly, we observed flocculent sediment in the culture media when the microalgae were treated with a series of AZ concentrations, and we speculated that the microalgae reduced their sensitivity to AZ by secreting extracellular polymers. Microalgae can be as interfacial aggregates of microorganisms (such as flocs, sludge, mats, granules, or biofilms). The material that binds these cells together to form a heterogeneous matrix is excreted by the microalgae themselves. These excretions, also known as extracellular polymeric substances (EPS), are composed of a complex high-molecular-weight (HMW) mixture of biopolymers (Xiao et al., 2016). In fact, the secretion of EPS, mainly polysaccharides and proteoglycans, is a mechanism for protecting cells from adverse environmental conditions (Pereira et al., 2009). EPS is a multipurpose natural biological polymer that can be used as an anticoagulant, protect cells from dehydration and toxic substances, and be used as an energy and carbon sink to cope with pres-

sure (Frolund et al., 1996). Our results confirmed that both eukaryotic (*Monoraphidium* sp.) and prokaryotic (*Pseudanabaena* sp.) algae secrete EPS. However, the EPS secreted by *Monoraphidium* sp. contain high TOC, while the EPS secreted by *Pseudanabaena* sp. contain both high TOC and proteins. Our results indicated that different components of EPS played widely different functions in algae and caused the different effects of AZ on eukaryotic and prokaryotic algae.

3.2. *Effects of AZ on the growth of the microbial community*

Microbial communities from natural aquatic ecosystems include eukaryotes (algae), prokaryotes (cyanobacteria), and protozoa. We speculated that in the first 3 days of AZ treatment, the inhibitory effect of AZ on eukaryotic algae growth was less than the promotion degree of AZ on cyanobacterial growth, which caused an increase in the Chl-*a* content. However, with time, the eukaryotic microorganisms were dramatically inhibited by AZ, and the content of Chl-*a* decreased immediately after 5 and 7 days of AZ treatments.

The significant increase in TOC and protein concentrations also implied that microbial metabolites were altered under the stress of AZ. According to previous studies, after AZ treatment, the rhizosphere soil of plants had increased microbial abundances, while the amount of biofilm was also significantly increased (Gustafsson et al., 2010). However, few reports have focused on the effect of fungicides on EPS secretion in microbes.

3.3. *Metabolic effects of the microbial community after exposure to AZ*

The metabolic pathway assessment provides a basis for further understanding the impact of AZ on the microbial community. After AZ treatment, three critical molecules, ribose, glyceric acid and sucrose, increased significantly. Here, ribose is the raw material for nucleotide synthesis, and glyceric acid is phosphorylated to glycerate 3-phosphoric acid, which is involved in glycolysis and further in the synthesis of serine, cysteine and glycine (Igamberdiev et al., 2018). Sucrose, which is involved in starch/sucrose metabolism and is a soluble sugar, is known as an osmotic/phase solute (Sui et al., 2015), and its increase can be considered an effective way to resist stress. In the TCA cycle, AZ caused a 22-fold increase in citric acid, which is the next step in glycolysis. Similar trends were found in the levels of malic, succinic, and fumaric acids, suggesting promotion throughout the TCA cycle. The citrate cycle (TCA cycle) provides the necessary precursors for respiration, amino acid biosynthesis and general nitrogen metabolism, and it is also closely related to the biological stress response (Zhang et al., 2018b). This was also confirmed by the significant increase in the TOC content in the microbial community after AZ treatment, suggesting that the TCA cycle may play a key role in the stress defense by increasing the accumulation of intermediate products, as demonstrated in other stresses, such as herbicides (Szewczyk et al., 2015). The increase of glycolysis, the TCA cycle, and pentose phosphate metabolism demonstrate stimulating effects of AZ.

With the promotion of glycolysis and the TCA cycle, the biosynthesis of amino acids, nucleic acids and fatty acids has been improved (Sweetlove et al., 2010). The results of this experiment also confirmed the conclusion that glutamic acid, putrescine and proline were significantly upregulated. Glutamic acid is involved in the deamination and transamination reactions associated with the TCA cycle (Robinson et al., 1991). Putrescine is a precursor to the synthesis of spermidine and spermine (Wu et al., 2010). Heby et al. showed that high concentrations of putrescine and spermidine can increase the growth rate of cells (1975). Proline can be used not only as a compatible solute but also as a signal molecule to protect cells by removing reactive oxygen species (Ben Rejeb et al., 2014). The accumulation of proline in plants is thought to be a stress-induced response, as it reduces stress-induced cell acidification or primary oxidative respiration, providing energy for recovery (Hare and Cress, 1997). A series of previous studies have also reported that plant secondary metabolism associated with defense responses is stimulated by abiotic stress (Bennett and Wallsgrove, 1994; Schützendübel and Polle, 2002; Zhao et al., 2016). The results indicated that the increases in the above substances under AZ reflect chemical stress responses involved in adjusting some of the different pathways to maintain amino acid balances.

According to the result, inosine, guanosine and sulfuric acid in the purine metabolic pathway increased. Inosine is a key metabolite of purine metabolism that is oxidized and decomposed by hypoxanthine and xanthine to produce uric acid and can be the conversion product of adenosine produced by the TCA cycle (Jin et al., 1997). Allantoin, a derivative of uric acid, was reported to accumulate in plants during stress (Takagi et al., 2016). Cell metabolism involves a series of highly regulated successive biochemical reactions that produce the substrates required for basic cellular processes. The most abundant intracellular metabolites are the purines. The upregulation of these purine metabolites means that the microbial community reduces AZ damage by accelerating cell metabolism.

AZ also increased levels of several fatty acids. Fatty acids are one of the dominant components of the plasma membrane and are considered a mechanism to alleviate stress (Bai et al., 2016; Hu et al., 2008). The plasma membrane may be regarded as the first "living" structure that is the target of AZ toxicity. Fluctuations in fatty acid levels may lead to changes in membrane fluidity and permeability or be a mechanism by which cells face stress (Zhao et al., 2008). Under adverse environmental stresses, lipids in algal cells are not only traditional energy storage compounds but also act as antioxidants or protective molecules in the stress response, e.g., high pH inhibited Chlorella vulgaris growth and increased the lipid content of this species by promoting the synthesis of triacylglycerol (Guschina and Harwood, 2009). We inferred that AZ could also stimulate enzyme activities to synthesize lipids. In many microalgae cells, nitrogen deficiency is the most widely used means to initiate fat synthesis and the most important influencing factor (Rodolfi et al., 2009; Takagi et al., 2000). The data in this study showed that amino acids were generally upregulated, and lipid synthesis was at a disadvantage in the competition of nitrogen sources, so fatty acids were generally upregulated.

4. Conclusions

AZ slightly inhibited the growth of *Monoraphidium* sp. and induced the growth of *Pseudanabaena* sp. Furthermore, AZ treatment induced microalgae cells to secrete TOC and protein compounds to produce external polymers (mainly polysaccharides and proteoglycans) and protect the cells from adverse environmental effects. The results of the metabolomics data analysis showed that TCA, purine metabolism, arginine and proline and fatty acid synthesis metabolic pathways were regulated because several intermediate levels of metabolites were elevated. We hypothesized that the microbial community plays a role in the stress defense by increasing the accumulation of intermediate products in the TCA cycle. Meanwhile, lipids also act as antioxidants and protective molecules in the stress response. Although low concentrations of AZ

have little potential toxicity to the microbial community, the formation of a large amount of flocculent sediment can easily lead to the suspension of algae in the aquatic environment, further aggravating the outbreak of cyanobacteria. Therefore, AZ is a potential ecological threat to the aquatic environment.

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