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Critical roles of cyanobacteria as reservoir and source for antibiotic resistance genes

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ABSTRACT

The widespread occurrence of antibiotic resistance genes (ARGs) throughout aquatic environments has raised global concerns for public health, but understanding of the emergence and propagation of ARGs in diverse environmental media remains limited. This study investigated the occurrence and spatio-temporal patterns of six classes of ARGs in cyanobacteria isolated from Taihu Lake. Tetracycline and sulfonamide resistance genes were identified as dominant ARGs. The abundance of ARGs in cyanobacteria was significantly higher in the bloom period than in the non-bloom period. The contribution and persistence of ARGs were higher in extracellular DNA (eDNA) than in intracellular DNA (iDNA) from cyanobacteria. Cyanobacteria-associated eDNA carrying ARGs was more stable at lower temperature. The relative abundances of ARGs in *Microcystis* and *Synechococcus*, the dominant genera of cyanobacterial blooms in Taihu Lake, were significantly higher than those in other cyanobacterial strains. The conjugative transfer efficiency for bacterial assimilation of ARGs in cyanobacteria was facilitated by increasing temperature and cyanobacterial cell concentration. Our results demonstrated that cyanobacteria could act as a significant reservoir and source for the acquisition and dissemination of ARGs in aquatic environments, hence the definition of negative ecological effects of cyanobacterial blooms was expanded.

1. Introduction

The extensive application, continuous discharge and incomplete degradation of antibiotics lead to their ubiquitous presence in the aquatic environment (Martínez, 2008). Antibiotics can directly alter microbiota by selecting (multi)drug-resistant bacteria, and promote the horizontal gene transfer that contributes to the transmission of antibiotic resistance genes (ARGs) among bacteria via the genetic mobile elements (plasmids, transposons and integrons) (Povolo and Ackermann, 2019). The environmentally persistent ARGs are now considered as emerging environmental pollutants and the increased morbidity associated with their dissemination has become a great concern of public health on a global scale (Hernando-Amado et al., 2019). Recent studies have focused on the widespread occurrence of ARGs in diverse aquatic environmental matrices under antibiotic pressure (Klein et al., 2018), such as surface water and sediments (Shao et al., 2018), sewages (Xu et al., 2015), hospital wastewaters (Rodriguez-Mozaz et al., 2015), aquaculture discharges (Seyfried et al., 2010), freshwater biofilms (Guo et al., 2018), etc.

Susceptible bacteria may become resistant to antibiotics via mutation during antibiotic exposure (Andersson et al., 2019). ARGs disseminate through two pathways, vertical transfer (bacterial reproduction) and horizontal transfer (conjugation, transduction and transformation) (Perry et al., 2014). ARGs may be located on chromosomes and on some mobile genetic elements, and present as intracellular DNA (iDNA) and extracellular DNA (eDNA) in environments (Guo et al., 2018). eDNA is relatively more abundant and stable than iDNA in organic matters, soils and sediments due to its absorption ability (Mao et al., 2014). iDNA is spread through either conjugation (transfer of mobile genetic elements) or transduction (use of bacteriophages as transporters of genetic information), while eDNA is assimilated by naturally competent bacteria via transformation (uptake of naked DNA) (Mao et al., 2014). Conjugation, mainly mediated by conjugative plasmids, is considered as the principle mode for the dissemination of ARGs among environmental bacteria (Sørensen et al., 2005). Conjugation requires membrane crossing and cell-to-cell contact

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between donor and recipient bacteria (Achtman et al., 1978), while gram-negative bacteria possess a number of outer membrane porin proteins that are necessary for specific and nonspecific pore formation and membrane transport (Achouak et al., 2001). Natural transformation for DNA uptake can occur broadly for some specific microbial species in various environmental media (Overballe-Petersen et al., 2013). Approximately 90 bacterial species, mostly human pathogens, have been identified as naturally transformable (Johnsborg et al., 2007). Both eDNA and iDNA can be used for gene transfer and manipulation, which may play a key role in horizontal gene transfer for cyanobacterial species (Cohen and Gurevitz, 2006). Since ARGs presenting as either eDNA or iDNA may possess significant differences in their mobility and availability to indigenous bacteria, it is particularly meaningful to recognize whether the partitioning, fate and transport of these two forms of DNA are fundamentally different, and thus contribute to ARG propagation in different manners.

Increasing frequency, magnitude and duration of cyanobacterial blooms have been observed as the visible symptom of accelerated eutrophication in freshwater and marine ecosystems worldwide (Huisman et al., 2018). The rapid and excessive proliferation of cyanobacteria increases turbidity and diminishes water quality, and leads to odours, scums, oxygen depletion as well as cyanotoxin production (Paerl et al., 2011). Cyanobacterial blooms are reported to smother submerged aquatic vegetation, destroy habitats for benthic invertebrates and fishes, and threaten drinking water safety (Genuário et al., 2016). As the largest group of gram-negative photosynthetic prokaryotes, cyanobacteria are structurally similar to bacteria and therefore are more susceptible to antibiotics' mode of action than fishes, crustaceans, and algal species (Välitalo et al., 2017). As pointed out by Dias et al. (2015), cyanobacteria may harbour ARGs for the following reasons when they are exposed to antibiotic pollution: (1) they contain mobile genetic units such as transposable elements and plasmids for horizontal gene transfer, the main mechanism of ARG propagation among distinct microbiota (Christiansen et al., 2008; Lin et al., 2011); (2) some cyanobacterial strains exhibit antibacterial activity and evolve self-protection mechanisms for the mutation and evolution of ARGs (Madhumathi et al., 2011; Wright, 2007); (3) plasmids may determine cyanobacterial resistance to antibiotics at environmentally relevant concentrations (Chen et al., 2008). As the main vector carrying multiple ARGs, RP4-like plasmids play a critical role in mediating the horizontal transfer of DNA into a wide range of gram-negative bacteria including cyanobacteria and contributing to the bacterial dissemination of ARGs in the water environment (Wang et al., 2015). We hypothesize that cyanobacteria may act as a persistent carrier and/or refuge of ARGs and perform an important function in the transfer of ARGs between biotic elements of aquatic ecosystems in the context of cyanobacterial blooms. Previous attempts to study the emergence and transfer ability of ARGs associated with cyanobacteria were based on laboratory experiments (Cameron and Pakrasi, 2011; Dias et al., 2015, 2019; Elhai et al., 1997). However, few studies to date have explored the potential spread and persistence of ARGs in natural cyanobacteria samples.

The specific objectives of this study are to: (1) identify and quantify the distribution of ARGs in cyanobacteria; (2) investigate the persistence of ARGs in cyanobacterial eDNA and iDNA; (3) evaluate the horizontal transfer ability of ARGs mediated by plasmids between bacteria and cyanobacteria. The study would provide a promising route to interpret how the aquatic ecosystem reacts against antibiotic pollution and define to what extent cyanobacteria may contribute to the propagation of ARGs in the antibiotic-polluted aquatic environment.

2. Materials and methods

2.1. Study sites and sample collection

Taihu Lake, the third largest freshwater lake in China, has a surface area of 2 338 km^2 with an average depth of 1.9 m and over 100 tributaries. Taihu basin is densely populated and highly urbanized, with only 0.4% of China's land area supporting 40 million residents and 15% of Gross Domestic Production (GDP) in China. The upstream river networks in the northwest part of the Taihu basin are the most heavily polluted area, and account for 70% of the pollution loads entering the lake (Yi et al., 2017a, 2017b). In recent years, excessive anthropogenic nutrient loading from the Taihu basin has caused severe eutrophication and frequent recurrence of harmful cyanobacterial blooms in Taihu Lake. Intensive discharges of antibiotic residues from municipal sewage, livestock and poultry breeding wastewater, aquaculture wastewater, reclamation runoffs, and untreated rural domestic sewage have led to the prevalence and dissemination of multiple ARGs at a basin scale (Zhang et al., 2015). The most common antibiotics and their reported concentrations in Taihu Lake are tetracyclines (tetracycline < LOQ-125 ng/L, chlortetracycline ND-142.5 ng/L, doxycycline ND-947.0 ng/L, and oxytetracycline ND-72.8 ng/L), sulfonamides (sulfamethoxazole ND-114.7 ng/L, sulfathiazole ND-134.5 ng/L, sulfamethazine ND-654.0 ng/L, and sulfadimethoxine < LOQ-210.0 ng/L), and macrolides (erythromycin ND-624.8 ng/L and roxithromycin ND-218.3 ng/L) (Xie et al., 2017; Xu et al., 2014; Zhou et al., 2016). The most common ARGs in Taihu Lake were *tet*A (10^4–10^5 copies/mL) and *tet*C (10^5 copies/mL) (Zhang et al., 2009).

Eight sampling sites were carefully selected in the northwest region of the Taihu basin, including urban areas, rural areas, inflow tributaries and bloom-forming areas of Taihu lake (Fig. 1). The bloom season of Taihu Lake lasted from May to September in the year 2018 (Fig. S1). Two sampling campaigns were conducted in the non-bloom season (March 2018) and bloom season (July 2018), respectively. Water samples were collected in triplicate at 0.5 m depth below the surface and sieved through a 1-mm mesh to remove fine roots, coarse-grained particles (such as cobbles, gravels, sandy pebbles, large soil particles, etc.) and macro debris. Each sample was partitioned into two subsamples, one for DNA extraction and the other for stock culturing (Fig. S2). The pre-filtered water samples were then passed through glass-fiber filters (Whatman GF/C 0.45-μm pore size) to collect cyanobacterial cells before transporting them to laboratory in sterile containers at −20 °C. Cyanobacterial density was determined by microscopic cell counting at 400× ~ 1000× magnification (Axioskop 40 Pol, Carl Zeiss, Göttingen, Germany). Taxonomic composition was identified at least to genus, and to species whenever possible, according to Hu's standard protocol (Hu and Wei, 2006).

2.2. DNA extraction and ARGs analysis

The filtered cyanobacterial samples were gently rinsed in deionized water and centrifuged as suspensions in 50 mL sterile BG11 medium (4000 rpm, 4 °C, 5 min). The composition of BG11 culture medium is shown in Table S1. To eliminate bacterial contamination, the cyanobacteria samples were sequentially subjected to the treatment of lysozyme-sodium dodecyl sulfate (SDS) solution (0.005% Tween-80, 0.1 M ethylene diamine tetraacetic acid, 0.5 mg/mL lysozyme, 0.25% SDS, 20 °C, 10 min) and ice-cold Tris–buffered saline (TBS) solution (50 mM Tris–HCl, 200 mM NaCl pH 7.4, 4 °C, 10 min) before centrifuging and washing three times in deionized water. Epi-fluorescence microscopic examination, polymerase chain reaction (PCR) amplification and denaturing gradient gel electrophoresis (DGGE) of 16S rDNA were conducted to verify that only cyanobacteria but no other eubacteria or archaea survived in these environmental samples (Text S1). The treated cyanobacterial cells were lyophilized for dry weight measurement and stored at −20 °C for genomic DNA extraction.

The methods for extraction of eDNA and iDNA from cyanobacteria samples are described in detail in a previous study (Guo et al., 2018) and used here with little modification. Approximately 100 mg lyophilized cyanobacteria cells were intensively washed with NaH_2PO_4 buffer (0.12 M, pH 8.0) and polyvinyl polypyrrolidone (PVPP) to separate and dissolve the eDNA tightly bound to cyanobacterial cell surfaces. After

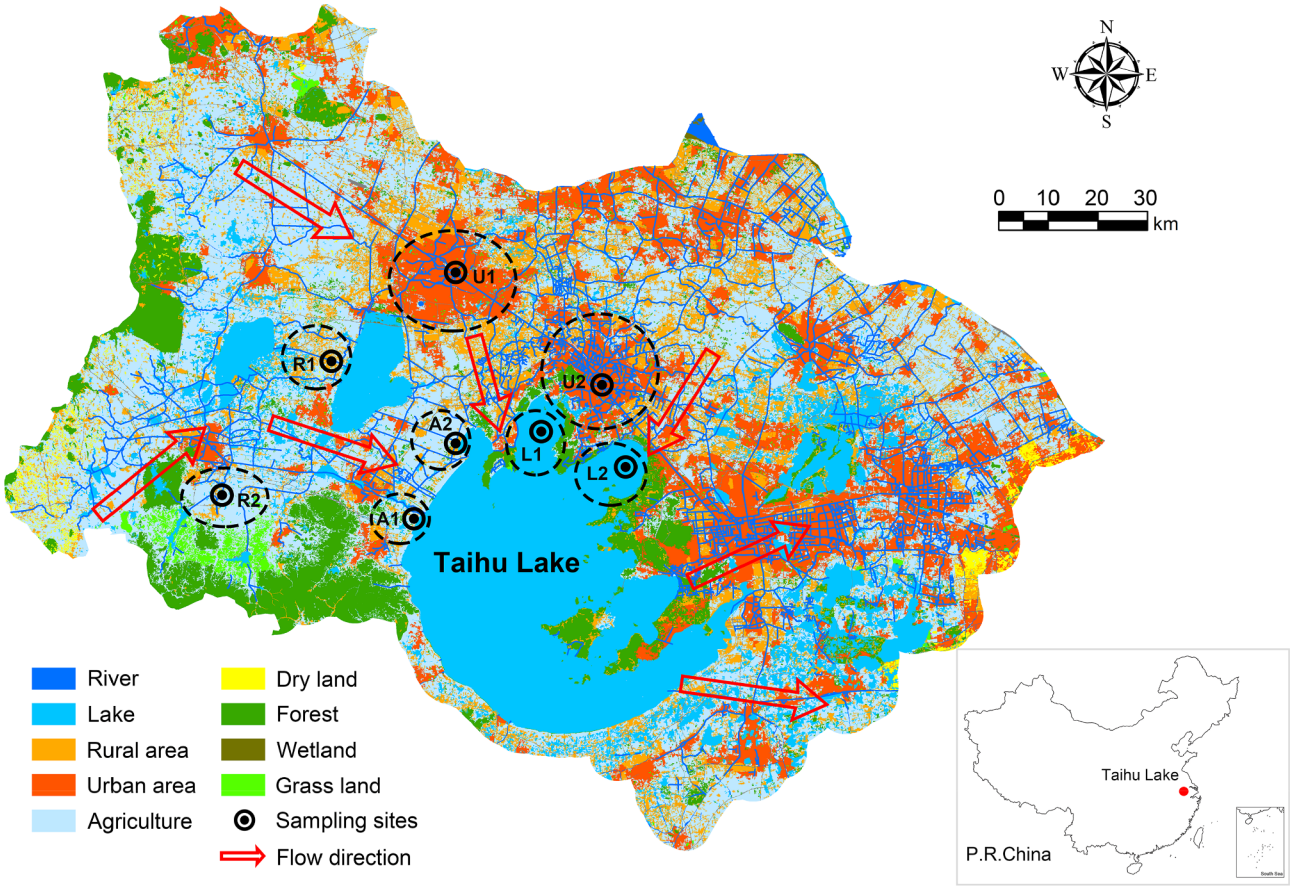

Fig. 1. Sampling sites in the northwest region of the Taihu Lake basin. The study area accounts for the major partition of pollution loads in Taihu Lake. Sites U1 and U2 are located in densely populated urban areas and receive domestic sewage discharges. Sites R1 and R2 are located in agriculturally influenced rural areas with significant loads of livestock and breeding wastewater. Sites A1 and A2 are located in main inflow tributaries, which connect Taihu Lake and the upstream river networks. Sites L1 and L2 are located in Meiliang Bay and Gonghu Bay, respectively, which are representative cyanobacterial bloom-forming areas of Taihu Lake.

shaking (250 rpm, 25 °C, 10 min) and centrifuging (10 000 rpm, 4 °C, 10 min), the supernatants were collected in a new tube on ice and the pellets were extracted twice again with the same procedure. The total supernatants of three successive extractions were combined and filtered through cellulose nitrate membrane filters (Whatman 0.2-μm pore size, Maidstone, United Kingdom). The filtrate was used for eDNA extraction and the final pellets and filters were used for iDNA extraction. The extraction yield and quality of DNA was verified by UV spectrophotometry (Nanodrop ND2000, ThermoFisher Scientific, Waltham, MA, United States) and gel electrophoresis.

Total DNA of cyanobacteria samples were extracted and purified by a combination of physical and chemical cell-lysing techniques using the MO BIO Power Biofilm DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, United States), with an extraction efficiency from 68.6% to 82.4% in this study. The lysing buffer provided with the kit was used to resuspend pellets and samples were then bead-beaten with a vortex plate (Vortex Genie 2 and Vortex Adapter, Bohemia, NY, United States). The crude extracts were further processed following manufacturer's instructions. All forms of DNA were stored at −20 °C until PCR and quantitative real-time PCR (qPCR) testing for ARGs.

Six classes of ARGs, class 1 integrase gene (*intI*1), and 16S rRNA gene were detected in triplicate by qPCR using the SYBR Green approach. Target ARGs included sulfonamide resistance genes (*sul*1, *sul*2, *sul*3, *sul*A), tetracycline resistance genes (*tet*A, *tet*B, *tet*D, *tet*E, *tet*J, *tet*K, *tet*M, *tet*O, *tet*Q, *tet*S, *tet*W), macrolide resistance genes (*erm*B, *erm*C, *ere*A, *ere*B, *mph*A, *mph*B), quinolone resistance genes (*qnr*B, *qnr*S, *aac (6′)*-Ib), β-lactam resistance genes (bla_{TEM}, bla_{OXA}, bla_{OXY}, bla_{SHV}, bla_{CTX-M}), and aminoglycoside resistance genes (*str*A, *str*B). The selected ARGs included, but were not limited to, the most prevalent ARGs and the corresponding ARGs of the most common antibiotics in Taihu Lake. The ARG amplification procedure and specific primers for PCR and qPCR are detailed in Text S2, Tables S2 and S3.

2.3. Assessment of ARGs distribution in cyanobacteria-associated eDNA and iDNA from semi-continuous culturing

The cyanobacterial subsamples for stock culturing were subjected to successive rounds of serial dilution and were streaked across BG11 agar plates. The native cyanobacterial isolates were successfully maintained in the laboratory culture chamber as stock cultures of monostrains. To minimize the possible side effect of intracellular nutrient storage and nutrient transfer from environmental samples on the growth, lipopolysaccharide content, and protein content of cyanobacterial experimental cultures, the cyanobacterial isolates for the ARG persistence test were starved for nutrients in sterile nitrogen- and phosphorus-free BG11 medium with constant shaking at 25.0 ± 0.5 °C under a 12/12 h light/dark photoperiod with light intensity of 50 μE/m/s. All containers used were autoclaved at 120 °C and 1.2 atm for 120 min as aseptic techniques. After a 2-week incubation, cyanobacterial cells were harvested at the exponential phase by centrifugation (4 000 rpm, 4 °C, 5 min) and used as inoculums for ARG persistence tests at an initial density of 750 ± 153 cells/mL.

ARG persistence tests were performed in a temperature- and light-controllable microcosm system based on a simplified OECD 308 test. To simulate a desirable ambient temperature and light intensity for cultivation of cyanobacteria under hydrodynamic conditions, the

microcosm system was installed in a thermostatic chamber and exposed to photosynthetically active radiation provided by fluorescent lights (Fig. S3). Cyanobacterial cultures were inoculated with 1 L of sterile BG11 medium in the microcosm, each in triplicate, and the cyanobacterial suspension was agitated at 50 rpm with the magnetic stirrer at 20 °C and 30 °C, respectively. A certain volume of the test medium with cyanobacterial cells was collected from the settling pond and subcultured with 1 L of fresh BG11 medium in the same microcosm every week. The semi-continuous tests lasted for eight subculture cycles, and eDNA as well as iDNA of cyanobacterial strains were extracted from periodic samples at the end of each intergeneration. All experiments were performed with three biological replicates.

2.4. Determination of plasmid conjugative frequency

To assess the conjugative transfer potentials of plasmids to cyanobacteria under the interactive effects of temperature and cyanobacterial density, liquid mating assays were conducted in the microcosm system using isolated cyanobacterial strains as the recipient strain. The strain of rifampicin resistance (Rif^R) *Escherichia coli* K12 (*E. coli* ATCC47076), harbouring the plasmid RP4 that carried kanamycin resistance (Km^R), was used as the RP4 donor strain. The four recipient isolates, *Synechococcus* sp. PCC 7942, *Synechocystis* sp. PCC 6803, *Anabaena* sp. PCC 7120 and *Microcystis aeruginosa* PCC 7806, were negative in the screening of Rif^R (Text S3) and the qualitative PCR screening of *tra*G gene (RP4 indicator) and *aph*A gene (Km^R gene on RP4) (Text S4). The exponentially growing culture of recipient strains was fragmented to pellets or filaments by cavitation and then resuspended in 1 L of fresh BG11 medium in the microcosm. Fifty-millilitre donor strains were incubated overnight in Luria-Bertani (LB) liquid medium (1% tryptone, 0.5% NaCl, 0.5% yeast extract) on a shaker incubator (200 rpm, 30 °C) and then diluted to reach microbial concentrations (optical density) of $OD_{600} = 0.5$.

For each mating, aliquots of the donor and recipient cultures were mixed in the plasmid RP4 horizontal transfer microcosm and incubated under cyanobacterial growth conditions. The microcosm was supplemented with 1% (V/V) *E. coli* K12 donor strains (plasmid RP4 concentration of approximately 5 μg/mL). Three influencing factors, including concentration of recipient strains, mating time, and temperature, were optimized by response surface methodology (Text S5, Tables S4 and S5). Statistical analysis of the response surface modelling was performed (Text S6, Figs. S4 and S5, Tables S6 and S7). Each treatment was set up in triplicate. A recipient-free microcosm was used as control. The selected ranges of recipient strain concentration (10^5–10^8 cells/mL) and temperature (16–36 °C) covered most reported cyanobacterial densities and water temperatures in natural aquatic environments. The mating time was designed as 48 h because the plasmid RP4 horizontal transfer had reached a plateau by 48 h in pre-experiments. During mating, periodic samples (10 mL) were harvested from the microcosm and serially diluted for plate counting and DNA extraction. Appropriate dilutions were spread with glass beads on BG11 selective plates and the bacterial colonies were counted as colony-forming units per millilitre culture (CFU/mL). The number of donor strains carrying plasmid RP4 (N_2, Rif^R and Km^R) and the number of total cultivable donor strains (N_1, Rif^R) were enumerated on plates of BG11 selective agar plus 5% Difco agar containing 40 mg/L of rifampicin with and without 60 mg/L kanamycin, respectively. The counting plate method was established according to Clinical and Laboratory Standards Institute (CLSI) 2005 guidelines, as detailed in Text S3. The presence of plasmid RP4 and transformation of ARGs in transconjugants were confirmed by PCR and qPCR, as described in Text S4. The donor and recipients sampled from microcosms were plated onto BG11 tri-antibiotic plates as negative controls to rule out the possibility of spontaneous mutation. The conjugative frequency (Y) was calculated as $Y = (N_1 - N_2)$ (CFU/mL)$/N_1$ (CFU/mL) × 100%, where N_1 and N_2 are the number of total cultivable donor strains and donor strains carrying plasmid RP4, respectively.

2.5. Statistical analysis

Heatmaps were generated with qPCR data of ARG absolute abundance expressed in copies per gram dry weight of cyanobacterial cells. The relative abundance of ARGs was calculated by normalizing ARG gene copies to 16S rRNA gene copies (ARGs/16S rRNA). To visualize the co-occurrence patterns between cyanobacterial taxa and ARGs, we constructed the polar contours between cyanobacterial biomass and ARG abundances by calculating all possible pairwise Spearman's rank correlations based on the cell density of different cyanobacterial genera and ARG abundances in both eDNA and iDNA. Correlations between two items were considered statistically significant if the Spearman's p-value was below 0.05. To further reduce false-positives, we restricted our analysis to cyanobacterial genera and ARGs present both in more than 20% of the environmental samples. Study area was plotted using ArcGIS 10.2 (Esri, New York, NY, United States). Heatmap, polar contour plots and bar charts were generated by OriginPro 2019 (OriginLab Corporation, Northampton, MA, USA). Analysis of variance (ANOVA) and *t*-test were conducted using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Response surface modelling and graphical optimization were performed using Design Expert 8.0 (Stat-Ease Inc., Minneapolis, MN, United States).

3. Results

3.1. Occurrence and abundance of ARGs in cyanobacteria

Twenty-eight of 33 target genes were detected in all the sampling sites during bloom and non-bloom periods (Fig. 2). Fourteen genes, including 16S rRNA, *int*I1, *sul*A, *sul*1, *sul*2, *tet*A, *tet*B, *tet*E, *erm*B, *erm*C, *ere*A, *qnr*B, bla_{TEM}, *str*A, were detected with relatively high abundances ($> 10^6$ copies per gram dry weight of cyanobacterial cells). The abundance of class 1 integron (*int*I1), and sulfonamide resistance genes (*sul*1, *sul*2, *sul*3, *sul*A), and major tetracycline resistance genes (*tet*A, *tet*B, *tet*D, *tet*E) in cyanobacteria was significantly higher in the bloom period than in the non-bloom period ($p < 0.05$). The average concentration of most ARGs at different sampling sites followed the order of rural area > tributaries ≈ urban area > lake area during the bloom period, and tributaries > lake area > urban area ≈ rural area during the non-bloom period.

3.2. Contributions of ARGs from eDNA and iDNA in cyanobacteria

The 16S rRNA gene, class 1 integron and four ARGs with relatively high abundances (*sul*1, *tet*A, *qnr*B and bla_{TEM}) were selected to investigate their distribution in eDNA and iDNA in cyanobacteria (Fig. 3). The abundance of most target genes was significantly higher in eDNA than in iDNA ($p < 0.05$). The abundances of tested genes in iDNA contributed a larger proportion to the total abundance in the cyanobacteria samples taken from the lake area than in those from other sites. The contribution of ARGs from eDNA in cyanobacteria was significantly lower in the bloom period than in the non-bloom period ($p < 0.05$). The relative abundances of ARGs in both forms of DNA from different cyanobacterial strains were statistically comparable (Fig. 4). The ARG abundances followed the order of *Anabaena* > *Synechococcus* > *Synechocystis* ≈ *Microcystis* > *Planktothrix* ≈ *Raphidiopsis* ≈ *Oscillatoria* ≈ *Aphanizomenon*. The relative abundances of ARGs in *Microcystis* and *Synechococcus* were significantly higher than those in other strains ($p < 0.05$).

3.3. Persistence of ARGs in eDNA and iDNA of cyanobacteria

The variation of ARGs distribution in both forms of DNA at different temperatures during the semi-continuous cultivation was characterized,

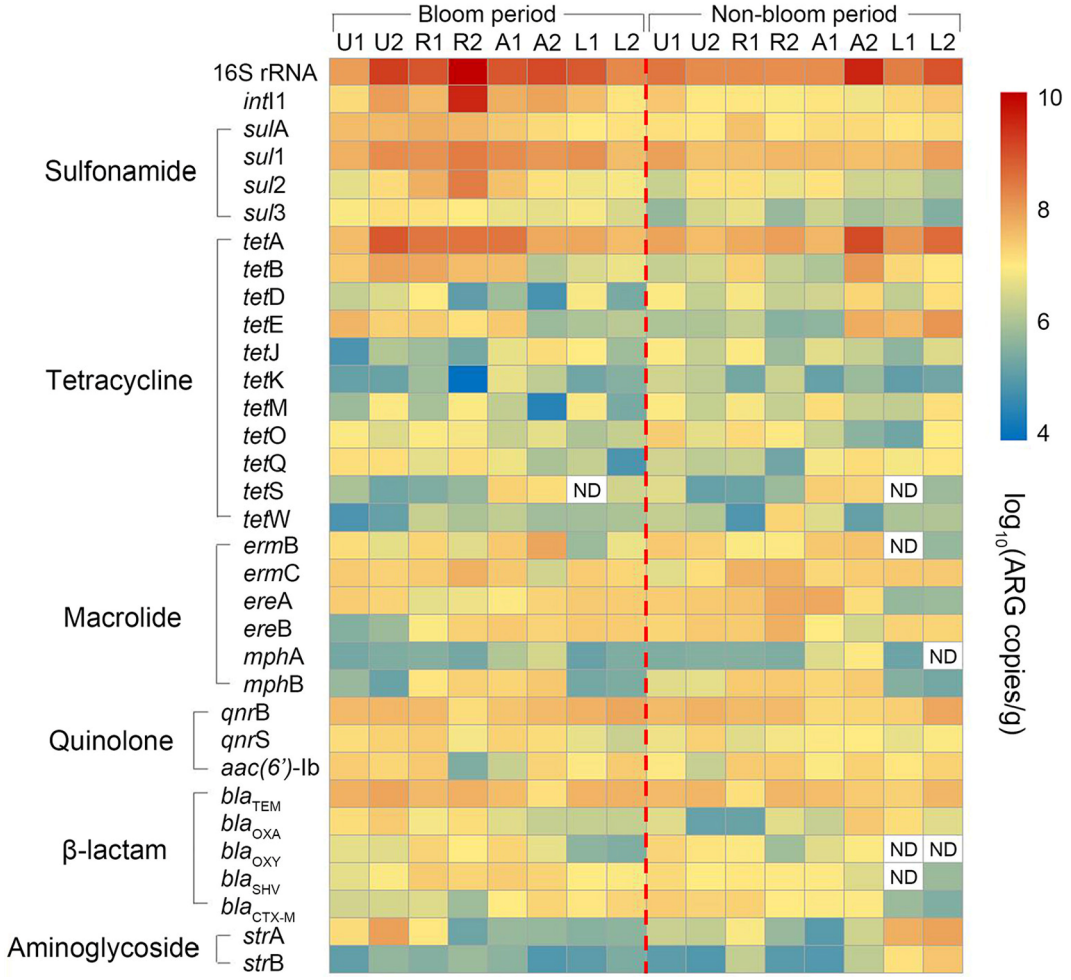

Fig. 2. Heatmap of the absolute abundances of 16S rRNA gene, class 1 integrase gene, and 6 classes of antibiotic resistance genes in cyanobacteria (subsample 1, ND: not detected). The figure shows the spatial–temporal distribution of target genes. The colour intensity in the panel indicates the log values of the gene concentrations expressed as $\log_{10}$(copies per gram dry weight of cyanobacterial cells).

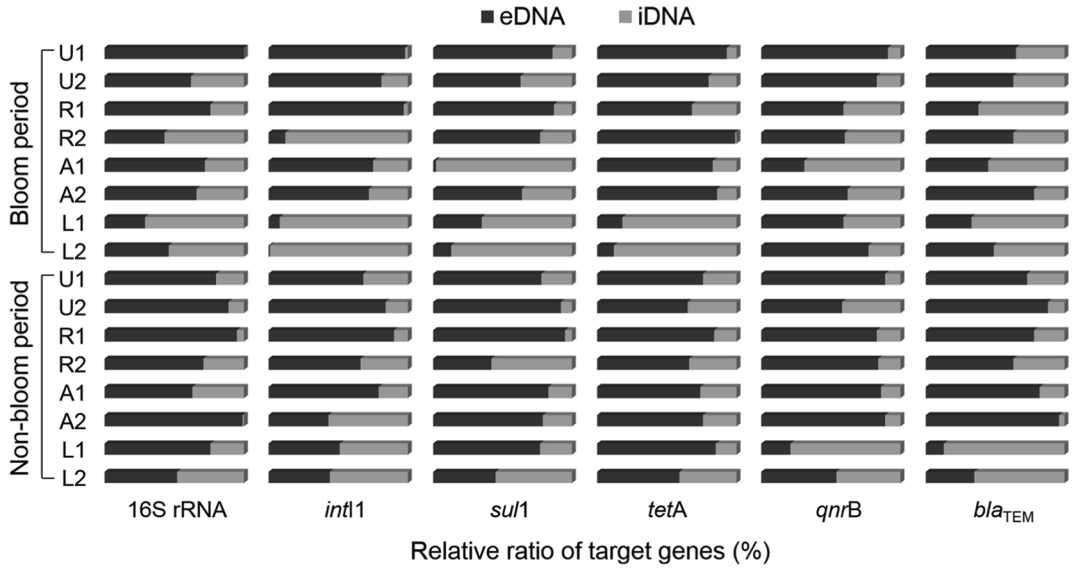

Fig. 3. Distribution of target genes in eDNA and iDNA of cyanobacteria (subsample 1). The figure reveals the contributions of ARGs from eDNA and iDNA in cyanobacteria. Relative ratios of 16S rRNA, *intI*1, *sul*1, *tet*A, *qnr*B and bla_{TEM} are calculated with their absolute abundances.

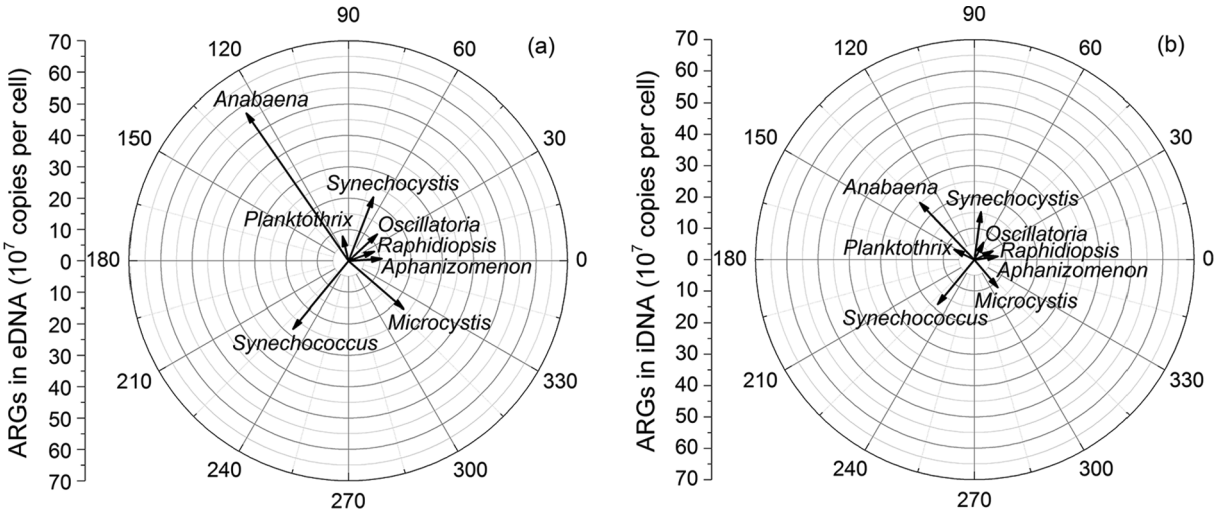

Fig. 4. Relative abundance of ARGs in eDNA (a) and iDNA (b) of different cyanobacterial strains (subsample 2). The length of arrows indicates the absolute abundance of ARGs in cyanobacterial strains. The angle of arrows indicates the relative abundance of ARGs in cyanobacterial strains. The absolute abundances of ARGs are expressed in copies per gram dry weight of cyanobacterial cells. The relative abundances of ARGs are normalized to 16S rRNA gene copies (ARGs/16S rRNA).

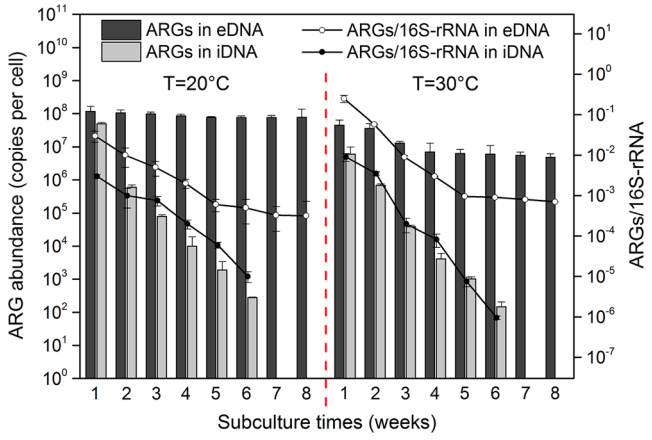

Fig. 5. Persistence of ARGs in eDNA and iDNA of cyanobacteria (subsample 2) at different temperatures. The columns indicate the variation in the absolute abundances of ARGs. The lines indicate the variation in the relative abundances of ARGs. The absolute abundances of ARGs are expressed in copies per gram dry weight of cyanobacterial cells. The relative abundances of ARGs are normalized to 16S rRNA gene copies (ARGs/16S rRNA). Error bars represent the standard deviation of the mean (n = 3).

as shown in Fig. 5. The ARG abundance in iDNA decreased rapidly and became undetectable by the seventh week, while the ARG abundance in eDNA remained stable and fairly high. The relative abundance of ARGs in eDNA was significantly higher than that in iDNA ($p < 0.05$). The attenuation rate of the ARGs/16S rRNA genes ratio in iDNA was higher than that in eDNA. The decrease in the relative abundance of ARGs for eDNA gradually ceased after 8 successive subcultures. A significantly lower absolute abundance but higher relative abundance of ARGs from eDNA was observed at 30 °C over the whole period ($p < 0.05$). The variation of ARG relative abundance in iDNA at 20 °C and 30 °C showed no statistical difference ($p > 0.05$).

3.4. Bacterial assimilation of ARGs in cyanobacteria

The conjugative transfer frequency of plasmid RP4-carrying ARGs from donor strain (*E. coli* K12) to cyanobacterial recipient strains had a significant positive relation with temperature and recipient concentration ($p < 0.05$) (Fig. 6). With temperature increasing from 16 to 36 °C and recipient strain concentration increasing from 10^5 to 10^8 cells/mL, the conjugative transfer frequency of plasmid RP4 increased from 0.00005% to 0.1358%. The transfer efficiency was much higher in *Anabaena* sp. PCC 7120 than in *Synechococcus* sp. PCC 7942 (2–67 fold), *Synechocystis* sp. PCC 6803 (3–246 fold) and *Microcystis aeruginosa* PCC 7806 (26–716 fold). The variation of *tra*G and *aph*A genes followed a similar trend, and a significantly positive correlation was found between their relative abundances ($p < 0.01$) (Fig. S6), suggesting that the proliferation and propagation of Km^R in cyanobacteria could be attributed to the horizontal transfer of plasmid RP4.

4. Discussion

The class 1 integron (*int*I1) encoding type 1 integrase enzyme was found to be ubiquitous in cyanobacteria and was detected with high numbers of gene copies in the study area. Integron gene sequences contribute to the spread of antibiotic resistance by facilitating horizontal gene transfer of ARGs between microbes and incorporation into bacterial chromosomes (Luo et al., 2010). Compared to other mobile genetic elements, *int*I1 exhibits enhanced maintenance and propagation of *sul*- and *tet*-ARGs characteristics (Yang et al., 2017). We found that the dominant sulfonamide resistance genes, *sul*A and *sul*1, were indeed significantly more abundant than other ARGs ($p < 0.05$), with the exception of *tet*A gene. Tetracycline resistance was featured by the absolute dominance of *tet*A gene, followed by *tet*B and *tet*E genes. The abundance of *tet*A was 1–3 orders of magnitude higher than other *tet*-genes in this study. The *tet*A gene is one of the most frequently detected tetracycline resistance genes in the aquatic environment (Dang et al., 2017). Antibiotic efflux pumps, ribosomal protection proteins, inactivating enzymes, and mutations within 16S rRNA are four different strategies used by bacteria for tetracycline resistance (Roberts, 2005). Mainly harboured by gram-negative bacteria (Chopra and Roberts, 2001), *tet*A, *tet*B and *tet*E encoding antibiotic efflux pumps (Nguyen et al., 2014) were the dominant tetracycline resistance genes in the cyanobacteria samples. The *qnr*B and bla_{TEM} genes were the most abundant quinolone and β-lactam ARGs detected in the present study, respectively, which were generally reported to be mediated by the acquisition of conjugative plasmids rather than conferred through chromosomal mutations (Colomer-Lluch et al., 2011). For macrolide and aminoglycoside resistance, the plasmid-borne *ere*A and *str*A genes (Roberts, 2008) were found to be the dominant genes of their corresponding ARG groups, respectively. The *sul*1, *sul*2, *tet*A, *tet*C, *str*A, *str*B,

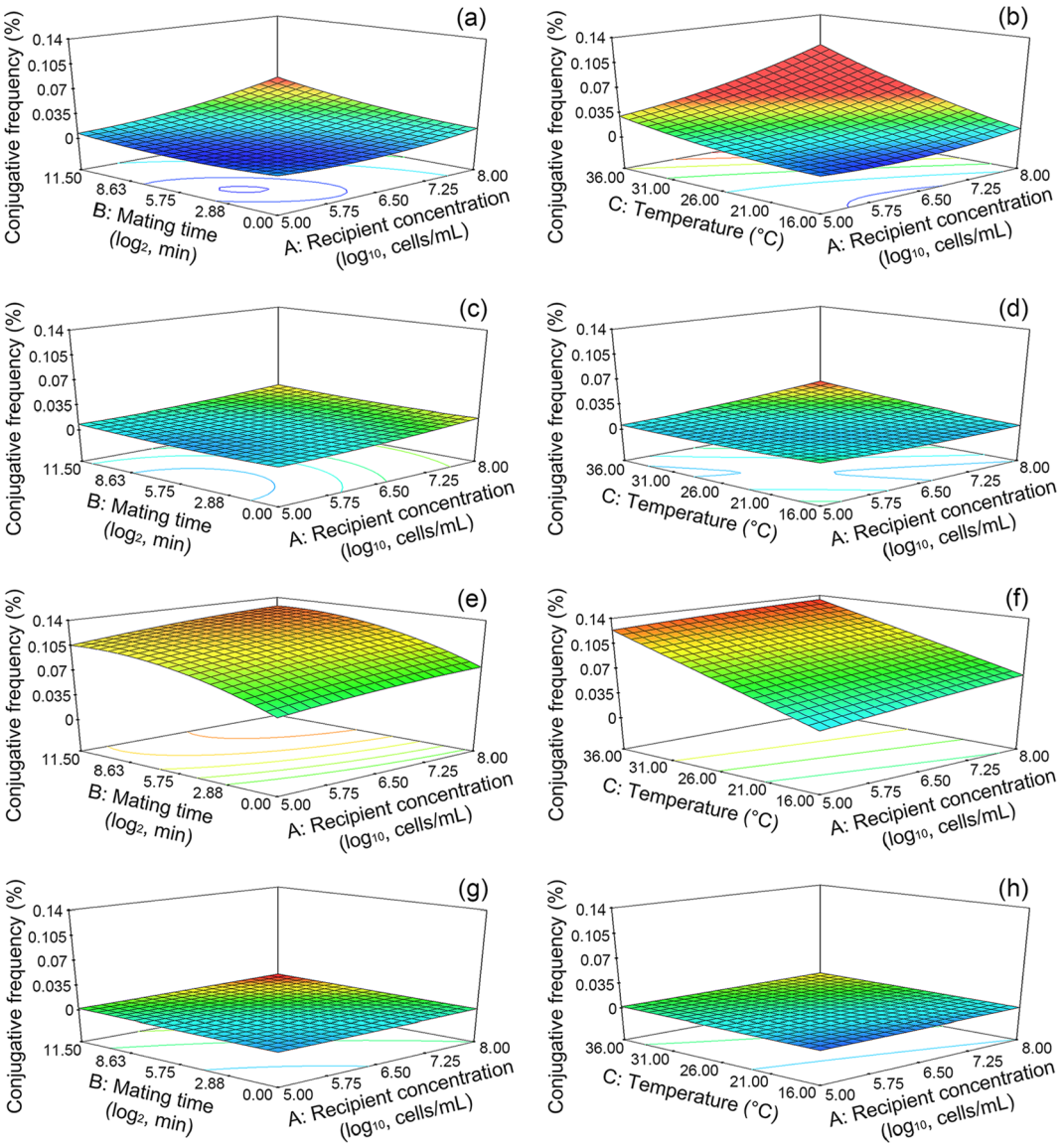

Fig. 6. Response surface model plots for conjugative transfer frequency in different cyanobacterial genera as function of recipient concentration, mating time, and temperature: *Synechococcus* sp. PCC 7942 (a & b), *Synechocystis* sp. PCC 6803 (c & d), *Anabaena* sp. PCC 7120 (e & f), and *Microcystis aeruginosa* PCC 7806 (g & h). This figure shows the effects of environmental factors on the propagation of ARGs mediated by conjugative transfer of plasmids. Each model graph is a combination of three-dimensional surface plot and contour plot.

*qnr*B, and *qnr*S genes detected in the cyanobacteria samples were the common ARGs reported in water and sediments of Taihu Lake (Han et al., 2013; Yang et al., 2017; Zhang et al., 2009). The class 1 integron (*int*I1), *sul*1, *str*A, and *str*B genes were also detected in freshwater cyanobacterial strains *Planktothrix agardhii* and *Planktothrix mougeotii* (Dias et al., 2019).

The higher concentrations of ARGs detected in the cyanobacteria samples during the bloom period than during the non-bloom period could be attributed to the seasonal variation of antibiotic pollution in the overlying water and sediment. When exposed to an environment with relatively high concentrations of antibiotics, antibiotic resistant variants are known to be generated in microbes via gene mutation and/or horizontal gene transfer (Knapp et al., 2008). Both human- and animal-derived drugs significantly contribute to the antibiotic contamination in the highly developed Taihu Lake Basin (Xu et al., 2014). The relatively high antibiotic concentrations reported in bloom season are related to the elevated surface runoffs into receiving rivers and lakes due to strong precipitation (Fig. S7) and excessive use of veterinary antibiotics for promoting growth or preventing diseases (Xu et al., 2018). This also explains the overall trend of higher ARG concentrations in the rural area than at other sites during the bloom period in our study, where the emission of veterinary antibiotics from aquaculture farming, livestock and poultry breeding was notably enhanced (Wang et al., 2019). Sulfonamides and tetracyclines are the main types of veterinary antibiotics used and contribute the majority of the antibiotic load in the study area (Qin et al., 2018; Song et al., 2016), explaining the high levels of *sul*- and *tet*-ARGs under antibiotic selective pressure. Meanwhile, the rapid proliferation of cyanobacterial cells during the bloom period might promote the bacterial reproduction of ARGs (vertical transfer) and potentially increased the density of mobile genetic elements such as plasmids and integrons, which acted as important media for the horizontal transfer of ARGs.

Other chemical pollutants, which were discharged with wastewater and carried by eroded soils that are flushed into receiving rivers and lakes with surface runoffs during rainfall events, might also play one of the vital roles for the seasonal distribution patterns of ARGs. The positive correlation between ARGs and certain metals indicated that the antibiotic resistance of microbes may relate to their metal resistance

ability or co-resistance to metal pressures (Guo et al., 2018). The presence of metals has been shown to stimulate horizontal gene transfer among bacteria (Klümper et al., 2016). The occurrence and dissemination of ARGs can be regulated by organic carbon, as ARGs are found to be associated with organic carbon and be further protected from nuclease degradation (Demanèche et al., 2001). Exposure to the non-antibiotic antimicrobial agents, such as biocides, can induce or select for bacterial adaptations that result in decreased susceptibility to one or more antibiotics, which may occur via cellular mechanisms that are linked to genes for antibiotic resistance or via non-specific mechanisms such as mobilization of genetic elements or mutagenesis (Wales and Davies, 2015). In addition, exogenous microorganisms and eDNA containing ARGs could be washed out from aquaculture farming, livestock and poultry breeding into surface waters by rain-runoff, which might potentially increase the abundance of ARGs during the bloom season.

The significantly higher ARG concentrations and slower degradation rates in eDNA implied higher persistence of ARGs in eDNA. Most target ARGs, including tetracycline, sulfonamide, quinolone and β-lactam resistance genes, are plasmid-mediated or integron-mediated (Dang et al., 2017). Such mobile genetic elements exhibit long-term stability in the aquatic environment, explaining the conjugation-assisted persistence of ARGs in eDNA (Lopatkin et al., 2017). Our results suggested that cyanobacteria-associated eDNA could be a vital reservoir for ARGs, which increase the potential for the proliferation and propagation of ARGs in the aquatic ecosystem through indigenous microbial communities. In this study, the greater contribution of ARGs from iDNA in the lake area may be associated with the relatively higher cyanobacterial density (recipient concentration) at these sampling sites (Fig. S8), promoting the conjugative transfer of ARGs between cyanobacterial cells. Among the cyanobacterial strains, *Anabaena*, *Synechococcus*, *Synechocystis*, and *Microcystis* can adsorb almost all exogenous DNA containing ARGs through natural transformation (Elhai et al., 1997; Zang et al., 2007). *Microcystis* and *Synechococcus* have been identified as the two most dominant cyanobacteria in the hypertrophic water column of Taihu Lake (Ye et al., 2011), explaining the high relative abundance of ARGs in eDNA of these two strains. Although cyanobacteria may share common responses to antibiotics, the resistance of cyanobacteria species is related to specific antibiotic susceptibility patterns (Dias et al., 2015). As indicated by Dias et al. (2015), further studies using a higher number of isolated strains will be necessary to identify putative antibiotic susceptibility and to map the individual resistance phenotypes/genotypes related to cyanobacteria species, genera or orders.

The persistence of ARGs in cyanobacteria samples could be largely dependent on the decay rates of DNA. Accompanied with cyanobacterial proliferation and iDNA degradation, conversion of iDNA to eDNA occurs during cell death (Mao et al., 2014). Since the ARG concentration in iDNA was lower than the detection limit after the seventh week, the conversion of iDNA into eDNA could not be considered to be an artefact of the persistence of ARGs in eDNA for the last two weeks. Although subject to biotic and abiotic degradation, eDNA acts as free DNA from lysed cells and remains accessible to other competent bacterial cells in natural habitats (Zhu, 2006). Previous studies have demonstrated that bacterial eDNA can persist in soils, freshwater and sediments for months, especially when bound to soil clay, minerals, biofilms, and humic substances (Nielsen et al., 2007). The sedimentation of cyanobacterial cells after the decay of blooms may lead to the subsequent redistribution of cyanobacteria-associated eDNA to benthic microorganisms with sediment disturbance and remobilization events. Our results supported the hypothesis that cyanobacteria-associated eDNA carrying ARGs could persist in aquatic environments and act as an important source for the dissemination of ARGs. The slower decrease of the ARGs/16S rRNA genes ratio in eDNA corroborated that chromosomal DNA (16S rRNA) was more rapidly degraded than the plasmid DNA (ARGs) in eDNA.

A lower persistence of ARGs was recorded at higher temperature in cyanobacterial eDNA. Environmental DNA degradation is time-dependent and can be accelerated at higher water temperatures (Tsuji et al., 2017). Exposure to high temperatures leads to single-stranded and fragmented DNA molecules, which become inefficient substrates for natural transformation (Eichmiller et al., 2016). The stability of DNA molecules can benefit from the decreasing nucleolytic activity of enzymes and other reactive chemicals at lower temperatures (Nielsen et al., 2000). However, cyanobacterial biomass productivity can be substantially elevated under high temperature conditions, which are sub-optimal for competence development and gene uptake by natural transformation. The increase in cyanobacterial cells may facilitate plasmid conjugative transfer and compensate the negative effects of high temperature on the persistence of ARGs in cyanobacterial eDNA.

This study assessed the conjugative transfer frequency of ARGs from *E. coli* strains to different cyanobacterial strains under the interactive effect of temperature and recipient concentration by applying response surface models. As gram-negative photoautotrophic prokaryotes, cyanobacteria, including the four tested strains in this study, have been identified as dominant plasmid RP4 recipients that are more likely to acquire ARGs during the conjugative transfer of plasmids (Cohen and Gurevitz, 2006). However, the ability to regulate ARG expression in cyanobacteria related to plasmid conjugation under different environmental conditions has not yet been reported. To the best of our knowledge, the present research is the first insightful exploration of both high temperature and elevated cyanobacterial cell concentration facilitating the conjugative transfer of ARGs in cyanobacteria mediated by widely distributed plasmid RP4. It has been reported that the conjugation frequency of plasmid RP4 between two species of *Pseudomonas* in biofilm reactors increases with elevated temperature (Ehlers and Bouwer, 1999). The tested cyanobacterial strains, such as *Microcystis aeruginosa*, are common toxic bloom-forming cyanobacterium in freshwater ecosystems and their large-scale blooms have been reported as the visible symptom of accelerated eutrophication worldwide (Donald et al., 2013). Global warming is reported to favour cyanobacterial blooms in different manners, and cyanobacteria respond more strongly to rising temperature than eukaryotic algae (Visser et al., 2016). The increased cyanobacterial recipient concentration may allow the donor pilus to attach to and access the recipient cells, thereby assisting cell-to-cell contact to promote the conjugative transfer of plasmid RP4 (Wang et al., 2015). Our results implied that the widespread dissemination of ARGs from resistant bacteria to indigenous cyanobacteria could be enhanced by conjugative transfer processes throughout cyanobacterial populations under the context of global warming and bloom-formation.

Since conjugation occurs more frequently among closely related bacterial strains (within genera) or species but occurs at a relatively low frequency across genera (DeBruyn et al., 2012), the natural transfer frequency of plasmid RP4 from *E. coli* to cyanobacteria was found to be quite low in this study. The transfer frequency might be underestimated because the selective pressure exerted by antibiotics in real water environments could enhance the horizontal transfer of conjugative plasmids (Dang et al., 2017). Meanwhile, vertical transfer of acquired resistance by transconjugant reproduction or replication may occur simultaneously with horizontal transfer during the proliferation of multi-resistant cyanobacteria. Considering their enormous population, sufficient cellular buoyancy, and dominant function in the aquatic food chain, cyanobacteria have demonstrated the ability to act as an important reservoir or source of exogenous ARGs, and to potentially enhance the propagation of ARGs among aquatic organisms from different trophic levels.

5. Conclusions

This study confirmed that cyanobacteria could serve as an important reservoir and source for ARG maintenance and propagation in

aquatic environments. Some frequent ARGs like *tet*A, *sul*A and *sul*1 were detected with relatively high abundances in cyanobacteria samples isolated from Taihu Lake. The contribution and persistence of ARGs were higher in eDNA than in iDNA from cyanobacteria. Although ARGs in cyanobacterial eDNA were more stable at lower temperature, higher concentration and higher bacterial assimilation efficiency for ARGs in cyanobacterial strains were recorded during the warm bloom season. This study provided insights to the mechanisms governing the widespread dissemination of ARGs via cyanobacteria. The potential risks of harmful cyanobacterial blooms may be a greater hazard than previously perceived.

CRediT authorship contribution statement

Zhiyuan Wang: Conceptualization, Data curation, Investigation, Methodology, Writing - original draft, Writing - review & editing. **Qiuwen Chen:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Jianyun Zhang:** Supervision, Project administration. **Tiesheng Guan:** Methodology, Validation, Data curation. **Yuchen Chen:** Investigation, Formal analysis, Data curation. **Wenqing Shi:** Data curation, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2020.106034.

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