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The impacts of different high-throughput profiling approaches on the understanding of bacterial antibiotic resistance genes in a freshwater reservoir

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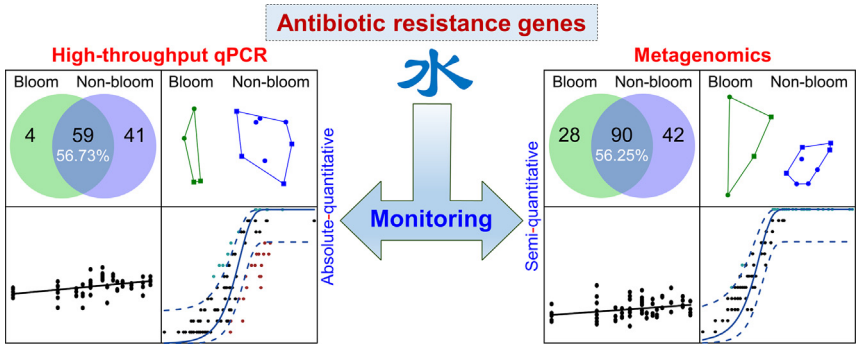
HIGHLIGHTS

- High-throughput qPCR and metagenomic approaches were used to profile bacterial ARGs.
- More ARG subtypes were revealed by metagenomics than high-throughput qPCR.
- Similar ARGs dynamic patterns were revealed by different ARG profiling approaches.
- High-throughput qPCR approach is suitable for routine environmental ARG monitoring.
- Metagenomics is an ideal tool for more comprehensive survey of environmental ARGs

GRAPHICAL ABSTRACT



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ABSTRACT

Bacterial antibiotic resistance genes (ARGs), a kind of emerging environmental pollutants, greatly threat human health through pathogenic bacteria. High-throughput quantitative PCR (HT-qPCR) and metagenomic approaches are two popular tools applied in aquatic environmental ARGs monitoring. However, current poor knowledge of different ARG profiling approaches' impacts on the understanding of the ARGs in aquatic environments greatly limit the further field application of these two approaches. For filling such knowledge gap, this study simultaneously employed these two approaches to examine and compare the ARGs in a freshwater reservoir across space and time. We found metagenomic approach detected more ARG subtypes and much higher bacitracin resistance genes' abundances than HT-qPCR. In general, HT-qPCR and metagenomics analyses both revealed similar ARG dynamic patterns and co-occurrence patterns between ARGs and bacterial taxa as well as the relationships between ARGs and environmental factors. Our results indicated the impacts of different ARG profiling approaches on the understanding of bacterial ARGs might be minor or negligible. HT-qPCR approach has the superiorities of time-saving, absolute quantification, low requirement for bioinformatics skills but also has some drawbacks including higher PCR amplification & primer bias, higher primer dependency and relative lower ARG subtype quantification capability compared to metagenomic approach. We suggest HT-qPCR approach can be employed for routine aquatic environmental monitoring, and metagenomic approach could be applied in comprehensive

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surveys for getting more ARG subtype information. Our data can be a useful reference for choosing right ARG profiling approaches for bacterial ARGs monitoring and risk assessment in aquatic environments.

1. Introduction

Antibiotic resistance genes (ARGs) confer antibiotic resistance to their hosts and lead to the failure of antibiotic therapy, the most effective measure to defeat pathogen infections in the last century (Baker et al., 2017; Qiao et al., 2018). ARGs are natural and ubiquitous in the environment but their emergence, evolution and spread become faster and faster since the first antibiotic, penicillin, was invented and widely used in clinic, agriculture, cultivation industry and other fields (Martínez, 2008; Allen et al., 2010; Knapp et al., 2010; Fortunato et al., 2018). Nowadays, ARGs are regarded as "emerging environmental pollutant" and environmental ARG pollutions have attracted great public concerns for their high potential threat on human's health (WHO, 2018). Natural environments, especially for aquatic environments, are important reservoirs of ARGs and play critical roles in the evolution, spread and cross-species transfer of antibiotic resistance (Baquero et al., 2008; Martínez, 2008; Allen et al., 2010; Marti et al., 2014). ARGs in aquatic environments can enter human bodies through direct pathways by drinking or touching and through indirect pathway by aquatic product consuming, then ARGs are likely transferred to the bacteria hosted in human bodies through horizontal gene transfer. Great efforts were made on environmental ARGs monitoring and risk evaluation in aquatic environments including lakes (Liu et al., 2018; Yang et al., 2018; Bondarczuk and Piotrowska-Seget, 2019; Yang et al., 2019), sea (Yang et al., 2019; Calero-Cáceres and Balcázar, 2019), rivers (Rodriguez-Mozaz et al., 2015; Zheng et al., 2018; Chen et al., 2019), reservoirs (Guo et al., 2018; Chen et al., 2019), drinking water (Bai et al., 2019; Ma et al., 2019) and the influents and effluents of water and sewage treatment plants (Yang et al., 2014; Li et al., 2015; Karkman et al., 2018; An et al., 2018; Ju et al., 2019). Environmental ARGs are extremely diverse and complex in natural waterbodies, and these properties increase the difficulty of precise detection (Liu et al., 2018). Public calls for reliable and standard ARG profiling approaches for monitoring and evaluating the potential risks of environmental ARGs, especially the ARGs in aquatic environments, to fill critical knowledge gaps related to the environmental dimensions of antibiotic resistance (Allen et al., 2010; McArthur and Tsang, 2017; Larsson et al., 2018).

For fulfilling such requirement, various ARGs profiling approaches, including conventional quantitative PCR (qPCR), micro-array, high-throughput quantitative PCR (HT-qPCR) and metagenomic sequencing approaches, have been developed in recent decades (Su et al., 2017; Waseem et al., 2019). Currently, HT-qPCR and metagenomic approaches are two powerful ARGs profiling approaches applied in environmental ARG monitoring (Schmieder and Edwards, 2012; Waseem et al., 2019). Compared with traditional ARGs profiling approaches, both HT-qPCR and metagenomic approaches possess high-capacity of ARG profiling that can provide much more ARG subtype information but spend less time (Su et al., 2017; Waseem et al., 2019). HT-qPCR approach can simultaneously detect thousands of nano-liter qPCR reactions per run with the help of high-throughput analysis platforms (WaferGen SmartChip Real Time PCR System, Applied Biosystem Open Array Platform, Bio-Rad CFX384TM Real-Time PCR Dectection System, Microfluidic Dynamic ArrayTM System, etc.) (Waseem et al., 2019). Metagenomic approach can employ shot-gun metagenomic sequencing to gain base sequences of DNA fragments (known as "reads") from the whole genome of target organisms or environmental samples and then decode ARG profiles through the annotation of unassembled reads or assembled sequences ("contigs" or "open-reading frame") in ARG databases, such as the antibiotic resistance genes database (ARDB) (Liu and Pop, 2009), the comprehensive antibiotic resistance database (CARD) (McArthur et al., 2013), and the structured ARG reference database (SARG) (Yang et al., 2016). Three studies simultaneously used HT-qPCR and metagenomic approaches to profiling ARGs in the microbiomes of swine intestine (Looft et al., 2012), murine gut (Stedtfeld et al., 2017) and sludge (Tian et al., 2019), but they only simply compared the difference on detected types and abundances of ARGs for the purpose of using metagenomics analyses to verify HT-qPCR results. Till now, no study was conducted typically focusing on exploring the exact impacts of different ARG profiling approaches on the understanding of environmental ARG dynamics interpreted by common-used downstream statistics analyses and systematically comparing the applicability of HT-qPCR and metagenomic approaches in environmental ARG studies.

This study simultaneously employed HT-qPCR and metagenomic approaches to profile ARGs of same water samples from a freshwater reservoir under cyanobacteria bloom and non-bloom conditions. The differences in the compositions and spatiotemporal dynamics of ARG profiles as well as the effects of environmental variables on ARGs respectively achieved by HT-qPCR and metagenomics analyses were identified. In this study, we aimed at (1) exploring the impacts of different ARG profiling approaches on ARG profile-based downstream analysis results and (2) providing valuable suggestions on the selection of ARG profiling approaches applied in future environmental ARGs monitoring.

2. Material and methods

2.1. Sample collection, measurements of environmental variables and DNA extraction

Xidong Reservoir is a typical subtropical stratified freshwater reservoir located in Xiamen, China. Water samples were collected twice a month from October to December 2014 at selected time (Guo et al., 2018; Liu et al., 2019). Totally, 12 water samples were respectively collected in 6 times of field-samplings from surface-layer (0.5 m) and bottom-layer (25 m) of the water column of Xidong Reservoir. A cyanobacterial bloom dominated by *Microcystis aeruginosa* was observed in October, and the reservoir ecosystem recovered in November (Xue et al., 2017; Guo et al., 2018). Detailed procedures of environmental variables measurements and DNA extraction were shown in supplementary information (Methods S1 & S2).

2.2. Quantification 16S rRNA gene by real-time quantitative PCR

Real-time quantitative PCR was carried out for quantification of absolute copy number of 16S rRNA genes by LightCycler® 480 System according to a method reported in our previous study (Guo et al., 2018). All samples were quantified in triplicate. For quality control, standard curves as well as negative and positive controls were conducted following the methods described in two previous studies (Schmittgen and Livak, 2008; Ouyang et al., 2015).

2.3. Quantification of ARGs by HT-qPCR analysis

For quantification of ARGs, HT-qPCR was performed by a SmartChip Real-time PCR system (Warfergen Biosystems, Fremont, CA, USA) according to the protocol described by Su et al. (2015) and Liu et al. (2018). One pair of 16S rRNA gene primer and 285 pairs of ARG primers targeting 214 ARG subtypes were used for HT-qPCR analyses. Detailed

primer information, protocol and data of HT-qPCR analysis as well as absolute ARG copy number calculation method were described previously (Liu et al., 2018).

2.4. Quantification of ARGs by metagenomics analysis

In this study, shotgun library was constructed by using NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instruction. Briefly, DNA was fragmented to a size of 300 bp by ultra-sonication, then DNA fragments were, in turn, ligated with index barcode sequences, end-polished, A-tailed, ligated with adaptor for Illumina sequencing and finally amplified by PCR. PCR products were purified with AMPure XP system (Beckman Coulter, Beverly, MA, USA). Libraries were assessed for size distribution by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and then quantified by real-time PCR.

The clustering of the index-barcoded samples was carried out on a cBot Cluster Generation System (Illumina Inc., San Diego, CA, USA) following the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina HiSeq2500 platform (Illumina Inc., San Diego, CA, USA) using a paired-end (2 × 150 bp) sequencing strategy. A total of 129,514.62 Mbp reads (10,792.89 Mbp reads per sample in average) for 12 sequenced samples were achieved. All metagenomic sequence data had been uploaded in the public NCBI sequence read archive (SRA) database under the BioProject number PRJNA416667.

To guarantee the accuracy and reliability of metagenomic sequencing data, data filtration of raw reads was performed to get the clean reads by removing adaptor-contaminated (≥ 15 bp sequence overlap with adapter sequence), ambiguous (≥ 10 ambiguous nucleotides) and low-quality reads (≥ 40 nucleotides with quality score below 38). After data filtration, 129,117.47 Mbp clean reads (about 10 Gb of clean reads data per sample in average) were achieved for further ARG annotation in SARG database following ARGs-OAP (online analysis pipeline) with default settings (Yang et al., 2016). In this study, 7.17×10^7 pairs of reads with 150 bp length per sample were achieved in average. Compared with the sequencing depth in similar study (Li et al., 2015), the sequencing depth of our 12 samples was sufficient to characterize the ARG profile at subtype level.

2.5. Statistical analyses

In order to compare ARGs abundance quantified by HT-qPCR and metagenomic approaches, normalization of ARG absolute abundance data was carried out. In HT-qPCR data-based downstream analysis, the normalized abundance of ARG was defined as the ratio of absolute ARG abundance of all bacteria to absolute 16S rRNA gene abundance of all bacteria (the ARGs copy number /16S rRNA gene copy number). For metagenomics data-based downstream analyses, the normalization of metagenomics data was automatically conducted by SARG database using ARG-OAP online pipeline (Yang et al., 2016). Briefly, the normalized abundance of the ARG type or subtype was calculated based on the ARG read abundance (ppm: reads per one million reads) acquired via metagenomic sequencing following the method built by Li et al. (2015).

The bacterial taxonomic communities were profiled by Illumina sequencing and the detailed information was described in supplementary information (Method S3). The bacterial OTU sequence data of all bacteria were calculated by normalizing to the ratio of the least DNA sequence concentration (copy/L) of all samples to those of selected sample. Finally, the percentages of bacterial OTU sequence numbers were used for network analysis.

ARG abundance and richness data were visualized in column charts using Origin v8.0 software (Origin Lab, Northampton, MA, USA). Box charts and scatter plots were performed in SigmaPlot v12.0 (Systat Software Inc., Chicago, IL, USA). Shannon-Wiener index computation, nonmetric multi-dimensional scaling (NMDS) ordination, Bray-Curtis dissimilarity computation and analysis of similarities (ANOSIM) of ARG profiles were performed in the PRIMER v7.0 (Clarke and Gorley, 2015). For the comparison of more than two independent groups, the Kruskal-Wallis test was performed in SPSS v22.0 (IBM Corp., Armonk, NY, USA).

Redundancy analysis (RDA) was performed in CANOCO program v4.5 (Microcomputer Power, Ithaca, NY, USA) (ter Braak and Šmilauer, 2002). The absolute copy numbers of ARGs measured by HT-qPCR, the ARG read abundance quantified by metagenomic approach and log (x + 1)-transformed data of environmental variables, except pH, were used for RDA analysis. In order to exclude the influence of collinearity among environmental variables on RDA analysis results, we sequentially removed environmental variables with the highest variance inflation factor, until all variance inflation factors were <10. Then, a forward selection procedure with 999 Monte Carlo permutation tests was conducted to identify the environmental variables significantly ($P < 0.05$) correlated with the profile of ARGs.

For exploring temporal pattern of ARG composition dynamics, time-lag analysis was conducted according to our previous study (Guo et al., 2018). The neutral community model (NCM) was employed to evaluate the effects of stochastic processes on ARG profile using the method described previously (Sloan et al., 2006; Guo et al., 2018). All related computations and visualizations were performed in R software v3.4.3 (R Core Team, 2017).

The Spearman's rank correlation-based network analysis was employed to explore co-occurrence pattern between ARGs and bacterial taxa using the "picante" package in R software (Kembel et al., 2010; Li et al., 2015; R Core Team, 2017). The normalized bacterial sequence number by Illumina sequencing, the percentages of the absolute copy numbers of ARGs measured by HT-qPCR and the percentages of the ARG read abundances quantified by metagenomic approach were used for network analyses. Finally, networks were visualized by Gephi software v0.9.2 (available at: https://gephi.org/).

3. Results and discussion

3.1. The impacts of different ARG profiling approaches on ARG compositions

In total, 104 and 160 ARG subtypes conferring resistance to 10 and 18 classes of antibiotics were detected in all samples by HT-qPCR and metagenomics analyses, respectively (Fig. 1A). Higher total normalized abundances of all ARGs were revealed by metagenomics analysis than by HT-qPCR analysis (Fig. 1B). Further, we found great differences in bacitracin resistance genes abundances (HT-qPCR vs. metagenomics: $0–2.23 \times 10^{-5}$ vs. $1.30–3.05 \times 10^{-2}$) which resulted in different dominant ARGs identified by HT-qPCR (multidrug resistance genes) and metagenomics (bacitracin and multidrug resistance genes) analyses (Fig. 1B, C). Lower normalized abundances and richness of ARGs in HT-qPCR results (Fig. 1) demonstrated the metagenomic approach might provide more comprehensive information of aquatic environmental ARGs than HT-qPCR approach, and three reasons may explain the differences observed in such comparison.

The first reason is limited availability of ARG primers for HT-qPCR analysis. Current-used ARG primers were mainly designed based on part of representative ARG sequences archived in ARGs databases (Looft et al., 2012; Ouyang et al., 2015; Zhu et al., 2017), while metagenomic approaches which identify ARGs by aligning all environmental microorganism sequences to all ARGs-like sequences in ARGs databases without the limit on primers could certainly detect more ARGs and report higher abundances and richness of ARGs. In pace with increasing works on ARG primer design, booming primers targeting environmental ARGs will be available for HT-qPCR analysis (i.e. Looft et al., 2012; Zhu et al., 2013; Ouyang et al., 2015; Stedtfeld et al., 2018), so that more comprehensive and reliable information of environmental ARGs can be provided by HT-qPCR analysis as time

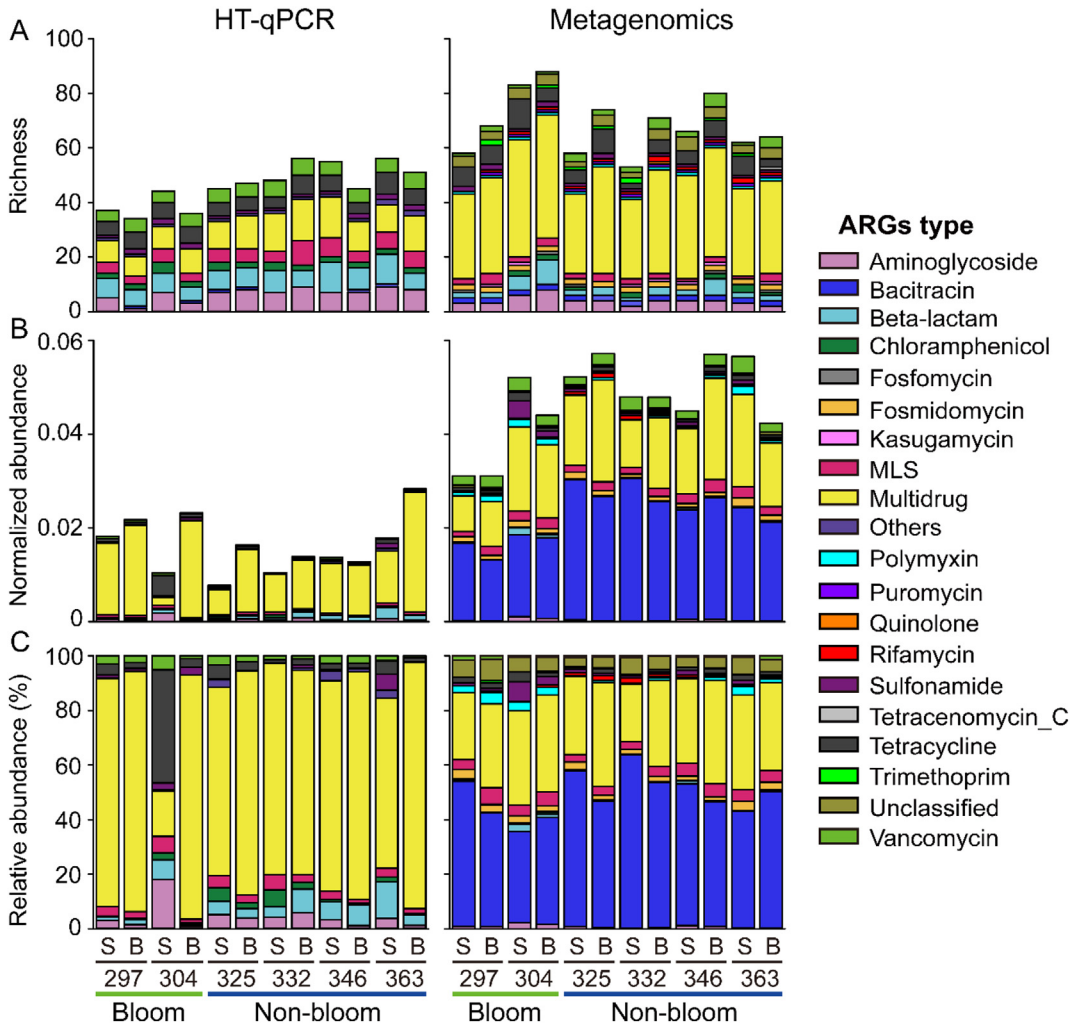

Fig. 1. Comparison of the ARG profiles achieved by HT-qPCR and metagenomics analyses. (A) The richness of ARGs based on detected number of ARG subtypes; (B) Normalized abundances of ARGs; (C) The relative abundances of ARGs calculated based on normalized ARG abundance. ARGs were classified into twenty types according to antibiotics classes they confer resistance to. "S" and "B" represents surface water samples (0.5 m depth) and bottom water samples (25.0 m depth), respectively. "Bloom" and "Non-bloom" means the water samples were collected in cyanobacterial bloom period (days 297 and day 304 of the year 2014) or in non-bloom period (day 325, 332, 346 and 363 of the year), respectively. MLS: macrolide, lincosamide and streptogramin; Others: the ARGs conferring resistance to other classes of antibiotics including nitroimidazole, lantibiotic and pyrazinamide; Unclassified: the ARGs with no clear classification in the structured ARG reference database.

goes on. However, the increase of ARGs-like sequences in ARGs database would be faster than the increase of new-validated ARG primers for HT-qPCR analysis due to current wide application of DNA sequencing technologies in environmental investigation which can provide thousands of new ARGs-like sequences in just one investigation. Hence, metagenomic approaches would be likely to provide more comprehensive ARG information than HT-qPCR for a long time to come.

The second reason is the PCR amplification bias in HT-qPCR analysis. In HT-qPCR analysis, different ARG primers requiring different optimum PCR conditions for amplifications of ARGs were simultaneously reacted in one platform with same PCR condition. The primers of some ARGs might not work in their optimum PCR conditions in this study, thus the detected abundances of those ARGs might be lower than their actual values in environment. Amplification bias might also be existed but could be relative fewer in metagenomics analysis due to the mechanisms of metagenomic sequencing (Schmieder and Edwards, 2012; Li et al., 2015). For such reason, metagenomic approaches were often employed for verifying the ARG results of HT-qPCR analysis in previous studies (Looft et al., 2012; Stedtfeld et al., 2017; Tian et al., 2019).

The third reason is the use of inadequate ARG primers. In this study, the primers for bacitracin resistance genes amplification were designed only for targeting *bacA* genes in *Escherichia coli* whose abundance was very low in studied reservoir (Liu et al., 2019), while bacitracin resistance genes were distributed in a wide range of bacteria taxa (Stedtfeld et al., 2017; Yang et al., 2019).

No significant difference was observed in richness and Shannon-Wiener index of ARGs between HT-qPCR and metagenomic approaches (Fig. S1). The richness and Shannon-Wiener index of ARGs in Xidong Reservoir were comparable to those of ARGs in sediment, ocean and drinking water but were obviously lower than those of ARGs in sewage treatment plant and livestock (Fig. S1). Recently, Yang (2019) also reported Shannon-Wiener indexes of lake and sea water were comparable.

3.2. The impacts of different ARG profiling approaches on interpreting the effects of environmental variables on ARG profiles

As Venn diagram (Guo et al., 2018; Zhao et al., 2018), NMDS (Li et al., 2015; An et al., 2018; Guo et al., 2018) and RDA analysis (Yang et al., 2014; Chen et al., 2016; McCann et al., 2019; Yang et al., 2019) were common-used tools for exploring the relationships between environmental variables and ARG profiles, we compared the results of these

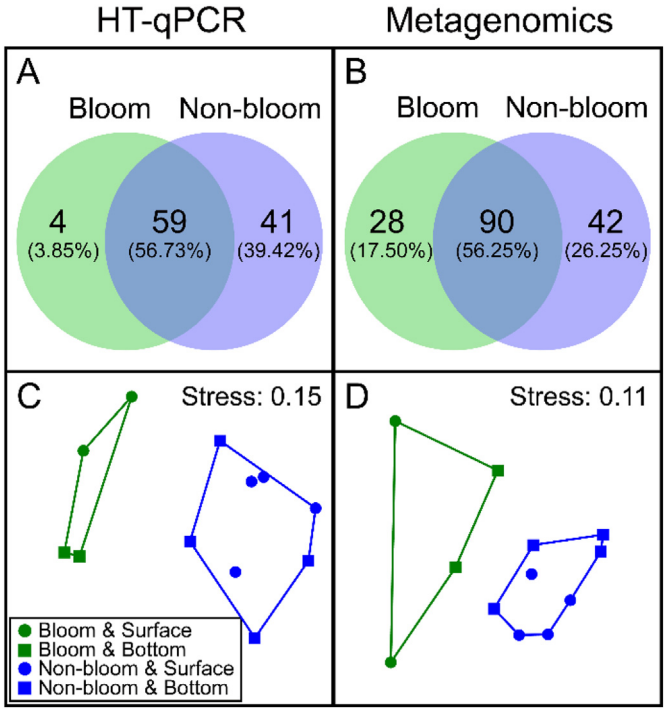

Fig. 2. Similar effects of cyanobacterial bloom on bacterial ARGs in the water of Xidong Reservoir were respectively revealed by HT-qPCR and metagenomics analyses. Venn diagrams show ARG subtype number in cyanobacteria bloom and non-bloom periods achieved by HT-qPCR (A) and metagenomics analyses (B), respectively. The numbers without and in brackets in Venn diagrams represent the amount and the percentages of unique or shared ARG subtypes in cyanobacteria bloom period and in non-bloom period, respectively. Nonmetric multi-dimensional scaling (NMDS) ordinations show the similarity of ARG profiles between different water stratifications (surface-layer and bottom-layer) and between different cyanobacterial bloom periods (bloom period and non-bloom period) achieved by HT-qPCR (C) and metagenomics analyses (D), respectively. In NMDS ordinations, a circle or square symbol in green or blue colour represents the ARGs in a surface-layer or bottom-layer water sample during cyanobacteria bloom period or non-bloom period, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

three analyses based on ARG profiles quantified by HT-qPCR and metagenomic approaches to evaluate the impact of different ARG profiling approaches on understanding the relationships between environmental variables and ARGs (Figs. 2 and 3).

The ARGs of different cyanobacterial bloom periods (cyanobacteria bloom period and non-bloom period) profiled by HT-qPCR and metagenomic approaches were showed in Venn diagrams (Fig. 2A and B). The percentages of shared ARG subtypes measured by HT-qPCR (56.73%) and metagenomics (56.25%) analyses were almost identical (Fig. 2A and B). NMDS analysis results based on ARGs data from different ARG profiling approaches (Fig. 2C and D; Table 1) both showed significant difference ($P < 0.01$) between the ARG profiles in cyanobacterial bloom period and in non-bloom period and no significant difference ($P > 0.05$) between the ARG profiles in surface water and in bottom water, indicating we could achieve similar interpretations on the effects of cyanobacterial bloom on ARG profiles, no matter HT-qPCR or metagenomic approach was used for profiling aquatic bacterial ARGs in the studied reservoir. More importantly, RDA analyses based on HT-qPCR and metagenomics data both identified that pH was the only environmental variable significantly correlated with the ARGs ($P < 0.01$, Fig. 3), thereby revealing that the selection of different ARG profiling approaches might have little impact on interpreting the relationships between ARGs and environmental variables. Slight difference in variance of ARGs explained by RDA axes (Fig. 3) was observed and may be due to the differences in the ARG abundances quantified by different ARG profiling approaches. The similar conclusions drawn by Venn diagram, NMDS and RDA analyses (Figs. 2 and 3) indicated the impacts of different ARG profiling approaches (HT-qPCR and metagenomic approaches) on interpreting environmental variables' effects on ARG profile in our studied reservoir may be negligible.

3.3. The impacts of different ARG profiling approaches on identifying co-occurrence patterns between ARGs and bacterial taxa

Network analysis has been widely used in exploring co-occurrence patterns between ARGs and bacterial taxa, and can successfully reflect the potential ARGs-bacteria relationships and identify possible host of ARGs (Li et al., 2015; Liu et al., 2018) which is important for risk evaluation of antibiotic resistance spread (Berendonk et al., 2015). The co-occurrence networks between bacterial taxa at family level and ARGs quantified by HT-qPCR and metagenomics analyses were compared and were shown in Fig. S2 and Fig. S3. Detailed topological properties of networks are presented in Table S1.

The majority of network nodes representing ARGs or bacterial taxa based on HT-qPCR data (Fig. S2A) were belonged to four ARG types including beta-lactam, MLS (macrolide, lincosamide and streptogramin), multidrug, and tetracycline and four bacterial phyla including *Proteobacteria*, *Actinobacteria*, *Chloroflexi* and *Firmicutes*. Such result is similar to the result based on metagenomics data that most of nodes representing ARGs and bacterial taxa were belonged to four ARG types (animoglycoside, beta-lactam, fosmidomycin and multidrug resistance genes) and four bacterial phyla (*Proteobacteria*, *Actinobacteria*, *Chloroflexi* and *Firmicutes*) (Fig. S2B). No or only one strong correlation between cyanobacteria and bacitracin or multidrug resistance genes was respectively observed in HT-qPCR and metagenomics analyses (Fig. S2). Apart from the number of nodes and edges, other topological properties of ARG-bacterial taxa correlation networks on the basis of HT-qPCR and metagenomics data were also similar to some extent (Table S1).

As shown in Fig. S3 and Table S1, our network analysis results based on HT-qPCR and metagenomics data both showed remarkable differences between the ARGs-bacterial taxa networks in cyanobacterial bloom period with higher modularity (0.873 and 0.907, respectively) and the networks in non-bloom period with slightly lower modularity (0.856 and 0.785, respectively), suggesting cyanobacterial bloom might change the ARGs-bacterial taxa co-occurrence patterns in the studied freshwater reservoir.

The similar complicated ARGs-bacteria taxa co-occurrence patterns observed in the networks based on HT-qPCR and metagenomics data (Fig. S2 and S3) revealed that the influences of different ARG profiling approaches on the general understanding of ARGs-bacterial taxa co-occurrence patterns might be little or negligible despite that some minor difference may be existed in detected ARG subtype numbers and abundances.

3.4. The impacts of different ARG profiling approaches on deciphering the spatiotemporal dynamic patterns of ARGs

Time-lag analysis was firstly used for determining the rates and patterns of variability in ecological communities (Collins et al., 2000) and then was modified for exploring the temporal dynamic pattern of ARG profiles (Guo et al., 2018). According to the linear regression of all data points and *P*-value, the temporal stability of the component of ARGs can be evaluated (Guo et al., 2018). The neutral community model (NCM) is also a powerful tool for evaluating the relative role of stochastic processes in explaining bacterial taxonomic and functional (i.e. ARGs) profiles assembly (Sloan et al., 2006; Guo et al., 2018). In order to evaluate the impacts of different ARG profiling approaches on exploring the spatiotemporal dynamic patterns of ARGs, time-lag analysis and NCM were conducted on the basis of ARG occurrence quantified

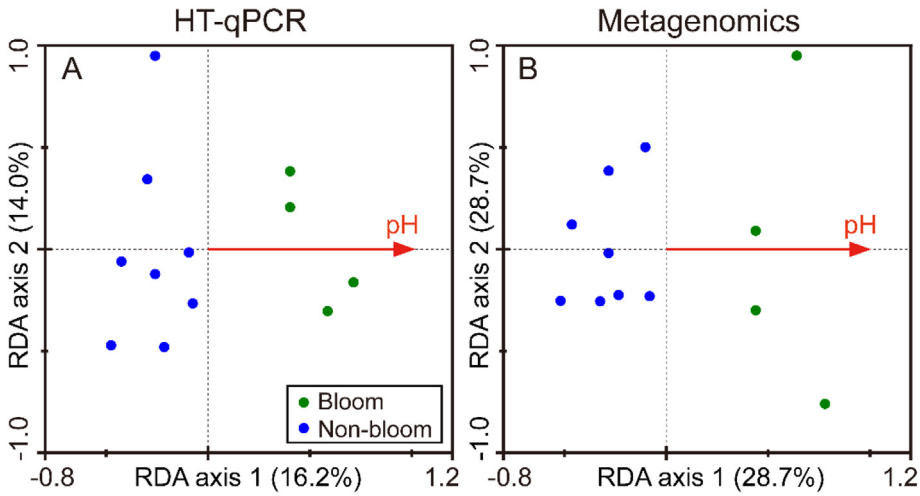

Fig. 3. Same environmental variable significantly correlated with ARGs was respectively identified by the redundancy analyses based on HT-qPCR and metagenomics data. Only the environmental variable significantly ($P < 0.01$) correlated with the ARG profiles was presented in redundancy analysis (RDA) plots. Green or blue circle symbols represent the ARGs in the water collected during cyanobacterial bloom period or non-bloom period, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by HT-qPCR and metagenomic approaches in this study and the results were compared (Fig. 4).

Time-lag analysis results based on ARGs data from HT-qPCR and metagenomics analyses were similar because the dissimilarity of ARGs composition significantly ($P < 0.05$) increased over time, both suggesting that cyanobacteria bloom maybe enhanced the homogeneity of antibiotic resistomes in the reservoir (Fig. 4A and B). The medium and large fraction of the compositional variation in the ARGs which can be explained by the NCM. The fitting results of NCM were similar that the ARG profiles achieved by HT-qPCR and metagenomics analyses were well fit ($R^2 = 0.52$ for HT-qPCR data, $R^2 = 0.91$ for metagenomics data) to the NCM (Fig. 4C and D), revealing the stochastic processes maybe greatly shape the dynamics of ARG profiles in the reservoir. The highly similar conclusion respectively drawn by time-lag analysis and NCM based on HT-qPCR and metagenomics data indicated that the impacts of different ARG profiling approaches on the understanding of the spatiotemporal dynamic patterns of ARGs might be slight or negligible.

3.5. Comparison of the advantages and disadvantages of HT-qPCR and metagenomic approaches

For figuring out which approach will be more suitable for environmental ARGs monitoring, the costs of time and money for ARG profiling by HT-qPCR and metagenomic approaches were compared (Table 2).

Table 1
Analysis of similarity showing same effects of cyanobacterial bloom or water depth (surface and bottom) on ARG profiles based on different ARG profiling approaches.

ARG profiling approach	Group	Global R	*P*
HT-qPCR	Bloom vs. non-bloom periods	0.825	0.002**
	Surface vs. bottom	−0.065	0.639
Metagenomics	Bloom vs. non-bloom periods	0.717	0.002**
	Surface vs. bottom	0.043	0.255

The analysis of similarity statistic compares the mean of ranked dissimilarities between groups to the mean of ranked dissimilarities within groups. Global R value close to "1" suggests dissimilarity between groups while global R value near "0" suggests an even distribution of high and low ranks within and between groups. Global R value below "0" indicates that dissimilarities are greater within groups than between groups.
** $P < 0.01$.

As triplicate should be at least carried out in ARG quantification for proving data reliability, one Wafergen SmartChip containing 5184 nano-wells allows for up to ARGs detection of five environmental samples per run if 296 primer sets (containing 285 ARG primers in this study) are used (Guo et al., 2018). According to our practical experience, current cost of HT-qPCR analysis encompassing the costs of relevant reagents (i.e. LightCycler 480 SYBR Green I Master mix), consumable materials (i.e. Wafergen SmartChip) and instrument rental is approximately $500 for ARG profiling of five samples. So, the cost of HT-qPCR-based ARG profiling was calculated to be about $100 per sample in the condition that the number of sample amount is a multiple of 5 (Table 2). In our research team, experienced researchers who are familiar with HT-qPCR analysis procedures can complete three times of HT-qPCR analysis and profile the ARGs of 15 environmental samples in one day. As people usually work 5 days in one week, we estimated that an experienced researcher has the capability of profiling the ARGs of 75 samples per week (Table 2).

The costs of money and time for metagenomics-based ARGs analysis were estimated based on DNA library construction, metagenomic sequencing and data cleaning in this study. As ARGs are normally in low abundance in most of natural environments compared with other genes (i.e. 16S rRNA genes), >10 Gb high-quality metagenomic sequencing data (about 7×10^7 pairs of sequencing reads with 150 bp length) would be suitable in order to prove sufficient sequencing depth (Li et al., 2015). Currently, the total price of the services including library construction, metagenomic sequencing and data cleaning is about $200 per sample with >10 Gb sequencing data. The cost of ARGs annotation on SARG database is negligible because this ARG database is now free for everyone (Yang et al., 2016). Hence, we finally estimated the total cost of metagenomics-based ARG profiling of one sample is about $200 (Table 2). Ideally, main procedures of metagenomic ARG profiling includes DNA library construction, metagenomic sequencing, data cleaning and ARGs annotation which may respectively cost about 1, 3, 1 and 2 days for one batch of metagenomics analysis of 12 environmental samples for maximizing the utilization rate of lanes in sequencing platform. In the actual situation, more time is likely to be spent on those procedures for many unpredictable conditions.

Obviously, less time and money are required for obtaining ARG profiles of same samples by HT-qPCR than by metagenomics analysis nowadays (Table 2). The cost of time and money of HT-qPCR and metagenomic sequencing will decrease in pace with the advances in related technologies. Actually, data from National Human Genome

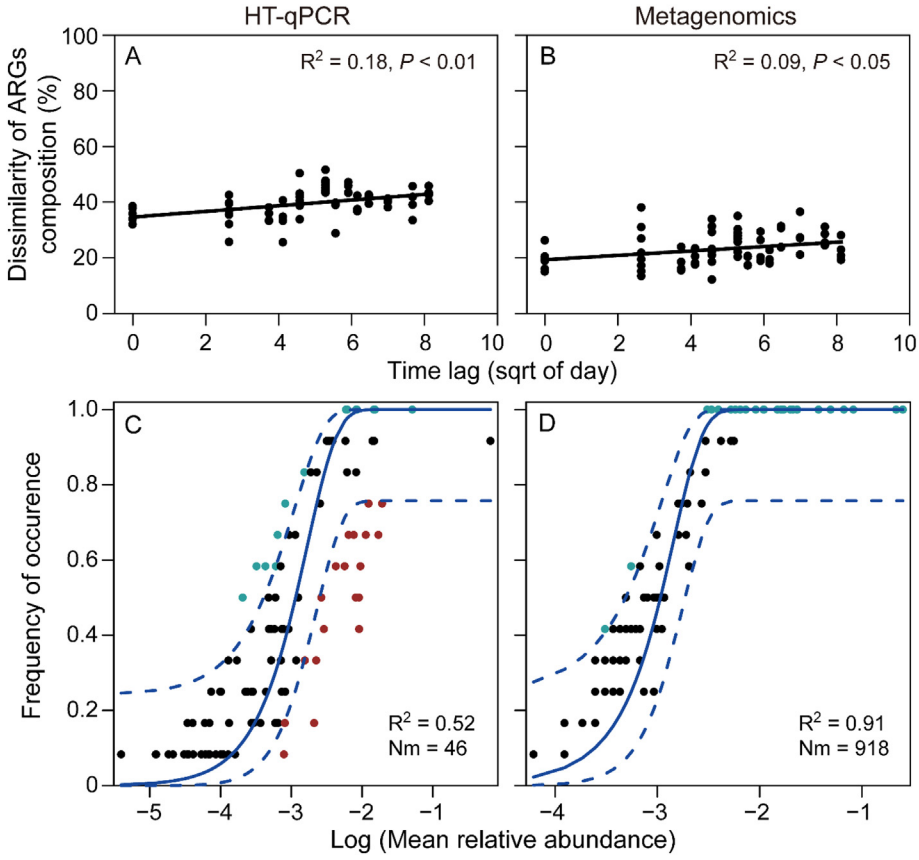

Fig. 4. Comparison of temporal compositional dynamics (A, B) and neutral community model fitness (C, D) of the ARG profiles in all samples revealed by HT-qPCR (A, C) and by metagenomics (B, D) analyses, respectively. Temporal compositional dynamics of all ARGs were analyzed based on time-lag regression analyses using ARG data. Data for testing neutral community model fit was the absolute ARG abundances achieved by HT-qPCR analysis and the normalized ARG read number achieved by metagenomics analyses. Solid blue line represents the predicated occurrence frequency; dashed blue lines represent 95% confidence intervals around the model prediction. ARGs that occur more or less frequently than predicted by neutral community model are shown in different colors. Nm means community size (N) times immigration (m), and R^2 indicates the fit to neutral community model. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Research Institute (NHGRI) Genome Sequencing Program showed the cost of sequencing had continuously sharp decreases in recent decade (Schmieder and Edwards, 2012; Wetterstrand, 2018).

Although HT-qPCR approaches have the advantages including quick (less cost of time per sample) and cheap (less cost of money per sample), they are still highly limited in the capability of providing comprehensive ARG information because of their high dependency on ARG primers. Current available ARG primers are very limited (285 ARG primer pairs for 214 ARG subtypes in this study). Hence, HT-qPCR approaches could only provide the information of hundreds of ARG subtypes, while metagenomic approaches using SARG database could provide information about 1209 ARG subtypes (Yang et al., 2016). However, metagenomic approaches require researchers should have good skills and professional experience in bioinformatics analyses which may limit the application of such approaches in environmental ARG survey. Even more and more bioinformatics analysis pipelines or softwares have been improved or developed (McArthur and Tsang, 2017), it is still a hard work for most of researchers who are not experts in bioinformatics to capture ARG information from environmental metagenomes containing millions of, even billions of, bacterial nucleotide sequences.

Routine environmental monitoring puts forward relative high demands on detection speed, cost of money and operation simplicity but a relative low demand in comprehensiveness of ARG information, because routine environmental monitoring would be made in high frequency and focus on some specific or typical ARGs posing high threats to human health, such as tetracycline and carbapenems resistance genes (Berendonk et al., 2015). HT-qPCR approach well meets such requirements and would be suitable for routine environmental ARG monitoring. More importantly, HT-qPCR approach can achieve absolute quantification of ARGs at subtype level, while metagenomic approach is only a semi-quantitative method for ARGs quantification. More comprehensive and systematic surveys of environmental ARGs require tools which can provide most comprehensive information of ARGs in investigated environments but do not have high demands in detection speed, cost of money and operation simplicity because such surveys would not

Table 2
Comparison of the advantages and disadvantages of the application of HT-qPCR and metagenomic approaches employed in this study on aquatic environmental ARG monitoring.

	HT-qPCR	Metagenomics
ARG abundance quantification	Absolute quantitative	Semi-quantitative
ARG subtype quantification	Less (214 ARG subtypes)	More (1209 ARG subtypes)
Detection cost	Low ($100 per sample)	High ($200 per sample)
Detection speed	Fast (75 samples per week)	Slow (12 sample per week)
PCR amplification & primer bias	High	Low
Primer dependency	High	Low
Bioinformatics skill requirement	Low	High

be high-frequently conducted (i.e. Zhu et al., 2017), just like census. So, metagenomic approach can be one of good choices for such general and comprehensive surveys of environmental ARGs.

4. Conclusion

Similar interpretations on the effects of environmental variables on ARG profiles and the spatiotemporal dynamic patterns of ARGs were achieved by HT-qPCR and metagenomics analyses, although more ARGs subtypes were detected by the metagenomic approach. The impacts of different ARG profiling approaches on the understanding of bacterial ARGs in aquatic environment could be minor or negligible. Our study can guide researchers in selecting an ideal environmental ARG profiling approach for different monitoring purposes. However, with the developments of qPCR, sequencing, bioinformatics and other technologies, HT-qPCR and metagenomic approaches may overcome their current weaknesses and have higher application potentials in monitoring and risk evaluation of environmental ARGs.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Author contributions

JY designed the experiments. YG performed the high-throughput qPCR and real-time qPCR. XL, PX, YG and LL performed the DNA sequencing and bioinformatics. LL and JY collected the samples and determined the environmental parameters. XL, PX and JY wrote the first draft of the manuscript, and all authors contributed to and have approved the final manuscript.

Appendix A. Supplementary data

Supplementary Methods S1–S3, Supplementary Figs. S1-S3 and Supplementary Table S1 showing additional study details. The supplementary data to this article can be found online at doi: https://doi.org/10.1016/j.scitotenv.2019.133585.

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