Environment International 117 (2018) 292–299

Contents lists available at ScienceDirect

Environment International

journal homepage: www.elsevier.com/locate/envint

Large-scale biogeographical patterns of bacterial antibiotic resistome in the waterbodies of China

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ARTICLE INFO

Keywords:
Antibiotic resistance genes
Functional biogeography
Anthropogenic activities
Community ecology
Geographical distance
Multidrug resistance gene
Lake and reservoir

ABSTRACT

Antibiotic resistance genes (ARGs) are widespread in aquatic environments, but we know little about their biogeographical distribution and occurrence at national scales. Here we analyzed the patterns of ARGs from 42 natural waterbodies (natural lakes and reservoirs) across China using high-throughput approaches. The major ARGs were multidrug genes and the main resistance mechanism was the efflux pump. Although the absolute abundance of ARGs (gene copies/L) in the south/central waterbodies was similar to the northern waterbodies, the normalized abundance of ARGs (ARGs/16S rRNA gene copy number) was higher in the south/central waterbodies than in the north (mainly because of the aminoglycoside and multidrug resistance genes). Human activities strongly correlated with the normalized abundance of ARGs. The composition of ARGs in the waterbodies of south/central China was different from that in the north, and ARGs showed a distance-decay relationship. Anthropogenic factors had the most significant effects on this spatial distribution of ARG composition, followed by the spatial, bacterial and physicochemical factors. These indicate that the ARGs exhibited biogeographical patterns and that multiple ecological mechanisms - such as environmental selection (human activities and local physicochemical parameters) and dispersal limitation - influence distribution of ARGs in these waters. In general, our results provide a valuable ecological insight to explain the large-scale dispersal patterns in ARGs, thereby having potential applications for both public health and environmental management.

1. Introduction

Antibiotics have been widely used to treat bacterial infections since the 1940s and have revolutionized global medicine (Allen et al. 2010; Marti et al. 2014). However, the widespread occurrence of microbial resistance to antibiotics has become 'possibly the best example of human-driven evolution in action' at a global scale (Gillings and Paulsen 2014), with the first cases of antibiotic resistance typically being reported only a few years after the first use of a particular antibiotic (Pruden 2014). This has been considered as a serious public health problem by some scientists for several decades, and has led to the frighteningly plausible predictions of the end of effective antibiotics in medicine (Levin and Andreasen 1999). Clearly, the control of antibiotic resistance in medical settings, such as hospitals, is important, but it has become apparent that the wider environment has become a potentially major pool of, and source of, antibiotic resistance genes (ARGs) (Pehrsson et al. 2016). In particular, the extensive use of antibiotics by humans, and their subsequent release into aquatic ecosystems in treated and untreated sewage, hospital waste, aquaculture discharges, and agricultural runoff have made aquatic environments crucial for the occurrence, exchange, evolution and spread of ARGs (Allen et al. 2010; Pruden 2014; Rodriguez-Mozaz et al. 2015; Y.G. Zhu et al. 2017c). However, there is still a lack of fundamental data on the distributions and effects of ARGs in natural waters (Czekalski et al. 2015).

Previous biogeographical studies have largely used 16S rRNA gene (via either sequencing or fingerprinting) as a standard approach for

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https://doi.org/10.1016/j.envint.2018.05.023
Received 12 November 2017; Received in revised form 3 May 2018; Accepted 9 May 2018

determining microbial diversity and differences in composition between communities, but it can be difficult to know how variation in taxonomic composition relates to ecosystem function (Louca et al. 2016). More recently, there has been a growing consensus on the need for a better representation of functional traits in biogeography - an approach which is better developed in macro-organism studies than in microorganisms (Violle et al. 2014). One of the lines of evidence that microorganisms display spatial biogeographical patterns is the distance-decay relationship (i.e. A decline in similarity with increasing geographical distance) (Hanson et al. 2012). This relationship shows that different locations harbor microorganisms that differ in taxonomic and functional composition. In functional biogeography, some studies found no significant correlation between geographical distance and microbial functional differences, implying that the functional traits available to a specific community have no physical constraints on their dispersal (Angermeyer et al. 2016; Raes et al. 2011). However, other studies suggest microbial functional genes have a biogeographical provincialism, as these functional genes correlated to geographical distance (Haggerty and Dinsdale 2017; Kelly et al. 2014). To date, we still know little about the whether the ARGs show a distance-decay relationship, and how the four fundamental processes that drive the biogeography of microbial taxonomic communities - selection, drift, dispersal and mutation - affect the ARG biogeographical patterns (Hanson et al. 2012). Studies on the biogeography of ARGs can enhance our knowledge on the dispersal mechanism of ARG across ecosystems. It can also contribute to strategies for prevention and control of the emergence, spread, enrichment and exchange of ARGs in both natural waters and manmade ecosystems (Berendonk et al. 2015).

In this study, we used high-throughput quantitative PCR (HT-qPCR) to investigate the ARGs along a latitudinal gradient ranging from 24 to 50°N (over 2700 km) in 42 lakes and reservoirs across China. China has a greater area than all of the countries of Europe put together - because of the size of the country, this is effectively a continental scale survey of the distribution of ARGs in waterbodies. We aimed to determine the abundance and diversity patterns of ARGs at a large-scale in the Chinese lakes/reservoirs and explore how the ecological processes and mechanisms that drive these biogeographical patterns of ARGs.

2. Materials and methods

2.1. Study area, sampling and physicochemical analysis

A total of 42 Chinese lakes and reservoirs were sampled during July and August 2012 (Fig. 1 and Table S1). As the emphasis in this study was on maximizing the number of different sites across China, only one collection was taken at each lake/reservoir. In order to guarantee the representation of each sample, first, the sample site was located at the center of each lake/reservoir where the water was mixed well. Second, we collected several water samples every 50 cm in the epilimnion, and then mixed them very well. Only one mixed sample was taken at the center of each waterbody (natural lake or reservoir). These waterbodies spanned a south-north gradient of over 2700 km and ranged from approximately 24 to 50°N. The study covered several major climate types; comprising five regions based on their climate and geographical characteristics: FJ (included 5 reservoirs) - Fujian province, southeast China; CJ (9 lakes) - the lower and middle reaches of Changjiang River, China; ECC (6 lakes) - east central China, IM (13 lakes) - Inner Mongolia, north China; NEC (9 lakes) - northeast China. All of these lakes have been featured in our previous work on large scale patterns in microbial taxonomic community diversity (Ju et al., 2014; Liu et al. 2015). We further classified these five regions into two large regions, that is, south/central China (included FJ, CJ, and ECC) and north China (included IM and NEC) based on the human population, economy and climate. Water samples were collected using a polypropylene bottle. The bottle was sterilized in an autoclave and pre-rinse by lake/reservoir water. All water samples were stored in the dark at 4°C and filtered within two hours.

Water temperature (WT), electrical conductivity (EC), pH, dissolved oxygen (DO) and turbidity (Turb) of the epilimnion layer, water depth of sampling site, water transparency (Trans) and concentrations of chlorophyll *a* (Chl *a*), total nitrogen (TN) and total phosphorus (TP) were measured using the methods described in our previous study (Liu et al. 2015).

2.2. DNA extraction

Water samples of approximately 500 ml were collected for ARG analyses and filtered through a 0.22-μm pore size polycarbonate filter (47 mm diameter, Millipore, Billerica, MA, USA) as described previously (Liu et al. 2015). Total lake/reservoir microbial DNA was extracted directly from the filter using the FastDNA SPIN Kit and the FastPrep Instrument (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. All DNA samples were checked for quality using the agarose gel electrophoresis and quantified using the NanoDrop ND-100 device (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. High-throughput quantitative PCR of ARGs

We analyzed the ARGs in 42 lakes and reservoirs using high-throughput quantitative PCR (HT-qPCR). HT-qPCR of ARGs was performed using the SmartChip Real-time PCR (Wafergen Biosystems, Fremont, CA, USA) as described previously (Wang et al. 2014; Takara Bio USA, 2017). A total of 296 primer pairs were used to target one 16S rRNA gene, 285 ARGs (resistance to major classes of antibiotics), 8 transposase genes, one universal class I integron-integrase gene (*intI*), and one clinical class 1 integron-integrase gene (*cintI*) (Table S2). These 285 ARGs confer resistance to major classes of antibiotics (Table S2). All these primer sets were designed, used, and validated in the previous studies (Looft et al. 2012; Su et al., 2015; Zhu et al. 2013, YG Zhu et al., 2017c). Antibiotic resistance-gene reference sequences were harvested from (1) the Antibiotic Resistance Genes Online database (http://ardb.cbcb.umd.edu/); (2) a National Center for Biotechnology Information (NCBI) search for resistance-gene sequences; and (3) literature searches. All qPCRs were performed in triplicate, and a non-template negative control was included. The results of the HT-qPCR were analyzed with the standard settings: (1) any wells with multiple melting peaks and/or amplification efficiency < 1.8 or > 2.2 were discarded, and (2) a threshold cycle (Ct) 31 was used as the detection limit and only sample with three replicates simultaneously (Ct < 31) were regarded as positive. The relative copy number of ARGs, MGEs and 16S rRNA gene was calculated according to a previous study (Eq. 1) (Looft et al. 2012; Muurinen et al. 2017).

$$\text{Relative gene copy number} = 10^{((31-Ct)/(10/3))} \quad (1)$$

where Ct refers to high-throughput quantitative PCR results, 31 refers to the detection limit, and 10/3 refers to the 10-fold difference in gene copy numbers is 10/3 cycles, if the efficiency is close to 100%.

To obtained the absolute abundance of ARGs, we quantified the absolute copy number of 16S rRNA gene (gene copies/L) using a Lightcycler 480 instrument (Roche, Basel, Switzerland) with a SYBR® Green approach as previously described (Ouyang et al. 2015). A significantly high correlation between the relative copy number of 16S rRNA gene from SmartChip Real Time System and the absolute copy number of 16S rRNA gene from Roche 480 was observed ($r = 0.95$, $P < 0.01$). Therefore, we transformed the relative copy number of ARGs to absolute copy number according to a previous study (Eq. 2) (Ouyang et al. 2015).

$$\text{ARG}_{\text{SmartChip}} \text{ (or MGE}_{\text{SmartChip}})/16\text{S}_{\text{smartChip}} = \text{ARG}_{\text{absolute}} \text{ (or MGE}_{\text{absolute}})/16\text{S}_{\text{Roche480}} \quad (2)$$