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RESEARCH ARTICLE

Bacterial viability and diversity in a landscape lake replenished with reclaimed water: a case study in Xi'an, China

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Abstract

To understand the characteristics of bacterial viability and diversity in landscape waters replenished with reclaimed water, the typical landscape lake using reclaimed water was investigated in this study. Samples were collected from a reclaimed water inlet (P1), a reclaimed water distribution outlet (P2), and a landscape lake replenished by reclaimed water (P3). By means of measuring adenosine triphosphate (ATP), flow cytometry (FCM), and 16S rRNA gene high-throughput sequencing, the bacterial viability and diversity in reclaimed water distribution system and landscape lake were illustrated. The bacterial ATP contents at P1, P2, and P3 were 3.55 ± 1.79 ng/L, 3.31 ± 1.43 ng/L, and 18.97 ± 6.39 μg/L, and the intact bacterial cell concentrations were $5.91 \pm 0.52 \times 10^4$ cells/mL, $7.95 \pm 2.58 \times 10^4$ cells/mL, and $5.65 \pm 2.10 \times 10^6$ cells/mL, respectively. These results indicated a significant increase of bacterial viability in the landscape lake. The Shannon diversity index of 6.535, 7.05, and 6.886 at P1, P2, and P3, respectively, demonstrated no notable change of bacterial diversity from reclaimed water distribution system to landscape lake. However, the relative abundance of *Pseudomonas* sp. at P3 was significantly higher than that at P1. These findings indicated that viable but non-culturable (VBNC) bacteria could be revived in the landscape lake. The bacterial viability during reclaimed water reuse should deserve special attention.

Keywords Bacterial viability and diversity · VBNC bacteria · Reclaimed water distribution system · Landscape lake replenished with reclaimed water

Chongmiao Zhang and Pengcheng Xu contributed equally to this work.

Highlights
- Bacterial viability and diversity were investigated in the landscape reuse system
- Bacterial viability increased greatly in the landscape lake
- VBNC bacteria obviously revived when replenishing the landscape lake
- Bacterial diversity changes slightly except several phyla and genus

Main finding VBNC bacteria revived during replenishing of a landscape lake, while bacterial diversity showed no great change.

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Introduction

Water shortage impacts approximately 1.6 billion people around the world, and this number is predicted to double by 2050 due to the impacts of climate change and population growth (Gosling and Arnell 2016). Continuing urbanization and industrialization has brought about an increasing growth of urban water use, giving rise to a crisis of water scarcity for urban residents, including the need to replenish landscape lakes with surface water (Chen et al. 2017). Reclaimed wastewater widely used as a supplemental water resource, because of its stable effluent quality and constant availability (Michael-Kordatou et al. 2015; Teklehaimanot et al. 2015), can be used to replenish landscape lake (Chen et al. 2017). Therefore, the use of reclaimed wastewater is expected to increase in many areas around the world, especially in inland areas.

Although reclaimed wastewater can alleviate the urban water resource scarcity to some extent, some of its constituents, such as micropollutants and microorganisms, are a cause for concern because of their potential impacts on human health and ecological safety (Cai and Zhang 2013; Jjemba et al. 2010). In general, pathogens can be effectively removed or inactivated during reclaimed water production. However, some can survive the process and reproduce in distribution pipelines and during storage. These bacteria cause water safety issues such as biofilm formation and microbial-mediated corrosion and even spread infectious diseases (Jjemba et al. 2010; Mccarty et al. 2011; Thayanukul et al. 2013). Bacterial survival and reproduction are usually described by measurements of bacterial viability and diversity. Recent studies report on the occurrence of pathogens in reclaimed water distribution system. These include enteroviruses (Zhou et al. 2015), *Escherichia coli* (Zhou et al. 2015) and opportunistic pathogens (Garner et al. 2018) such as *Legionella*, *Mycobacterium*, and *Pseudomonas*. In addition, viable but non-culturable (VBNC) bacteria can survive and reproduce during the reuse of reclaimed water, causing water-borne diseases mainly via aerosol inhalation/ingestion (Jjemba et al. 2015; Jjemba et al. 2010). Nonetheless, other non-ingestion routes favored by VBNC bacteria need careful consideration, for which research is currently lacking. Detailed insight into the bacterial activity and diversity in the landscape lake replenished with reclaimed water, including changes in the bacterial community and presence of VBNC bacteria, is particularly needed to guide the safe use of reclaimed water.

With the introduction of gene sequencing method, the study of microbial community characteristics in water and wastewater treatment process is more in-depth (Hu et al. 2012; Pinto et al. 2012). The 16S rRNA gene high-throughput sequencing technology has been widely used in study of the aquatic environment (Prest et al. 2014; Zhang et al. 2017; Limayem et al. 2019), including changes in bacterial diversity during reclaimed water production and distribution (Lin et al. 2016a, b). Beyond the advantage of its high genetic resolution, the 16S rRNA gene high-throughput sequencing technology reduces costs and runs with short time (Oliveira et al. 2018; Casero et al. 2019). Due to the increasing number of whole-genome sequence in recent years and the emergence of automatized pipelines and algorithms in publically open/free databases (Oliveira et al. 2018; Casero et al. 2019), the cost of 16S rRNA gene high-throughput sequencing technology keeps falling, thus reducing the cost of obtaining bioinformatics. Thus, the 16S rRNA gene high-throughput sequencing technology is conducive for identifying species at low abundance.

Flow cytometry (FCM) was first developed in the 1960s, based on a culture-independent analysis technique, and has been successfully applied to the characterization of bioinformatics (Coggins et al. 2020). FCM is a rapid, accurate, quantitative, and repeatable technique, combined with fluorescent dyes can represent total bacterial cell concentrations, intact bacterial cell concentrations, and bacterial fingerprint information (Zipper et al. 2004; Wang et al. 2010; Prest et al. 2013; Prest et al. 2014; Blatchleyiii et al. 2018; Yang et al. 2019; Coggins et al. 2020). FCM also has been widely used to evaluate and monitor the total and intact bacterial cell concentrations during water treatment and distribution in the past decade (Lautenschlager et al. 2013; Prest et al. 2014). Thus, the combination of 16S rRNA gene high-throughput sequencing technology and real-time FCM can provide enough bacterial information to reveal the changes in bacterial viability and diversity in a reclaimed water reuse system.

In this study, the FCM and 16S rRNA gene high-throughput sequencing technologies were used to characterize bacterial viability and diversity, respectively, in a reclaimed water reuse system, composed of reclaimed water distribution pipeline and a landscape lake. At present, knowledge of bacterial viability and diversity during reclaimed water reuse is lacking. Therefore, this study will provide managers and users with in-depth information about bacterial growth during the use of reclaimed water for replenishing landscape lakes. Also, it will promote the application of reclaimed water for landscape use in order to alleviate the problem of urban water scarcity.

Materials and methods

Description of the reclaimed water landscape use system

Figure 1 illustrates the reclaimed water recycling system investigated in this study. This system has been in operation for over 5 years, including a circulation of used water collection, water reclamation process, and reclaimed water distribution system (Wang et al. 2015; Ma et al. 2016). The water reclamation process consists of anaerobic-anoxic-oxic biological

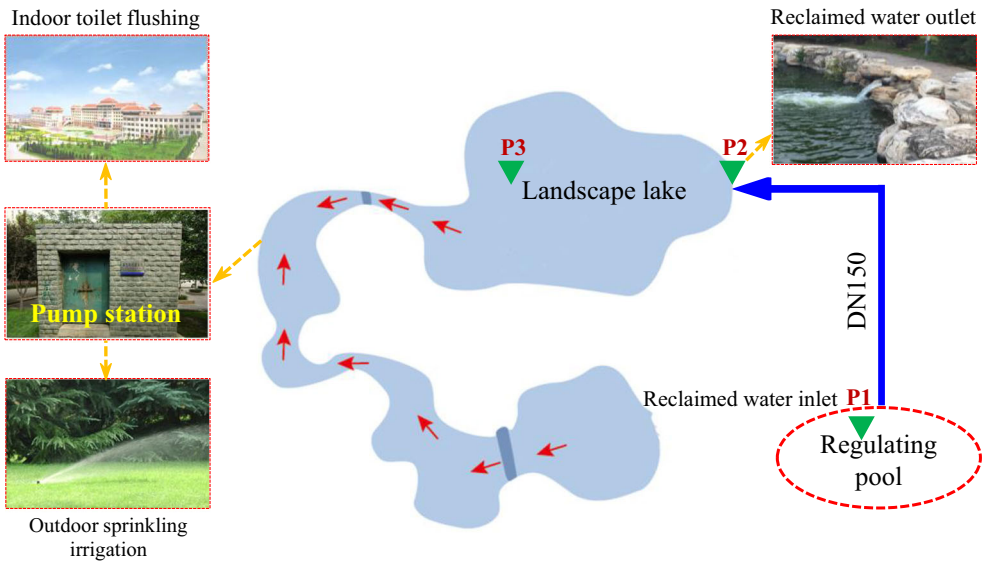

Fig. 1 Schematic diagram of reclaimed water reuse system (Wang et al. 2015)

treatment (AAO), filtration by a membrane bioreactor (MBR), and chlorine disinfection with sodium hypochlorite. Finally, the reclaimed water is used for various non-potable purposes, i.e., landscaping, toilet flushing, and green-belt irrigation. The system can provide approximately 2000 m^3 of water a day. To meet the requirements for landscaping, about half of the reclaimed water is transported directly to a landscape lake located at the center of the campus located in Xi'an, Shaanxi Province, China. The other about half of the reclaimed water is transported directly to buildings for toilet flushing or outdoor for green-belt irrigation, which is independent of the supply of the landscape lake. The reclaimed water is transported through a 0.8-km pipeline made from cast iron, with a diameter of 150 mm. The total storage volume of the landscape lake is about 5000 m^3, with a depth of 0.8–1.0 m and hydraulic retention time (HRT) of circa 5 days.

Sample collection and pre-treatment

Water samples were collected once a month between November 2016 and June 2017 from three sampling sites in the reclaimed water reuse system (Fig. 1), namely (1) the reclaimed water inlet (P1); (2) the reclaimed water outlet (P2); and (3) the landscape lake (P3). Samples were collected on sunny days to avoid the influence of rainfall and surface runoff. On each sampling occasion, 5 L of water were collected from each of P1, P2, and P3 in brown glass bottles, which were sterilized before use (121 °C, 25 min), and then immediately taken to the laboratory for analysis.

Chemical analysis

Chemical indexes, including chemical oxygen demand (COD), soluble chemical oxygen demand (SCOD), total organic carbon (TOC), NH_4^+-N, total phosphorus (TP), residual total chlorine (Cl), and electric conductivity, were measured in this study. NH_4^+-N was measured by Nessler reagent method. COD and SCOD were measured by potassium dichromate digestion method. For SCOD, water samples were firstly filtered through a mixed cellulose ester membrane with pore size of 0.45 μm. TOC was measured by combustion oxidation-non-dispersive infrared absorption method. TP was measured by molybdenum antimony spectrophotometry method. Cl was measured by iodometric method. Electric conductivity was measured by a portable conductivity meter (HQ30d, Hach Corp., USA). The detailed information was according to the standard methodology (SEPA 2006).

Bacteria count for fecal coliforms and heterotrophic plate counts

Fecal coliform (FC) and *Escherichia coli* (*E. coli*) were enumerated by filter membrane method, which was filtration membrane culture and plate count following standard methods (SEPA 2006). Heterotrophic plate counts (HPC) were enumerated by immersion method, which was nutrient agar medium culture following standard methods (SEPA 2006). The details of enumeration and counting for FC, *E. coli*, and HPC are described in the supplementary materials.

Bacterial viability: adenosine triphosphate and flow cytometry

Bacteria adenosine triphosphate (ATP) was analyzed using a BacTiter-Glo™ Microbial Cell Viability Assay kit (G8232, Promega, USA). One hundred microliters of reagent was added to each sample (100 μL), then mixed in a shaking incubator at 30 °C for 5 min. Luminescence in relative light

units (RLU) was measured using a luminometer (LB 962 CentroLIA/PC, Berthold Technologies, Germany). Then, RLU was converted to ATP concentrations according to a calibration curve. The calibration curve was derived from a pure ATP standard (A2383, Sigma, USA). The cellular ATP was calculated as the total ATP minus extracellular ATP (Xu et al. 2017). Extracellular ATP was detected from samples filtered with a syringe filter (0.1 μm). The detection method was described in a previous study (Xu et al. 2017).

Flow cytometric measurements were performed in the BD Accuri C6® flow cytometer (BD Accuri cytometers, USA) equipped with an air-cooled 488-nm argon laser. A threshold of 500 on the green fluorescence channel (FL1) was set to eliminate background noise and ensure the reliability of data. Fifty microliters of each 1 mL sample was measured at a slow flow rate. The CFlow Plus software was used to analyze bacterial membrane permeability. Green fluorescence in cells stained with SGI was collected in the FL1 channel (530 ± 30 nm), whereas red fluorescence in cells labeled with PI was collected in the FL3 channel (> 630 nm) (Xu et al. 2017). A stock solution of SYBR® Green I (SGI, 1:100 dilution in DMSO; S7563, Molecular Probes, USA), a green fluorescent dye which is commonly used to identify both living and dead cells, combined with PI (30 mmol/L; P4170, Sigma, USA), a red fluorescent dye which can only cross permeabilized membranes, was prepared as described previous study (Prest et al. 2013), and then stored at − 20 °C until use. The combination of SGI and PI (SGI + PI) could be used to distinguish cells with permeabilized membranes from the total cells. The details of diluting and staining of water samples for FCM detection are described in the supplementary materials.

Bacterial diversity

Bacterial diversity was profiled using gene amplicon sequencing with barcoded primers (515F/806R) targeting the V4 region of the 16S rRNA gene. DNA was first extracted, and then underwent high-throughput sequencing sequence analysis. A volume of 2-L water sample was concentrated by filtering a mixed cellulose ester membrane with pore size of 0.22 μm; the sample was transited into a sterile 50-mL centrifuge tube. Water samples were taken for DNA analysis using the E.Z.N.A.™ Water DNA Kit (Omega, Inc., USA). Two percent agarose gel electrophoresis was used to check the quantity and purity of the extracted DNA, respectively. The 16S gene high-throughput sequencing method was used to explore the microbial composition of the bacterial communities. The amplification of the V4 region in the bacterial 16S rRNA gene was performed using the 515F (5′-adaptor B-Barcode-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-adaptor B-Barcode-GGACTACHVGGGTWTCTAA-3′). Thermal cycling consisted of initial denaturation at 98 °C for 1 min, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and elongation at 72 °C for 30 s, finally 72 °C for 5 min. Initial data were processed by FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/) (Magoč and Salzberg 2011), Qiime (V1.7.0, http://qiime.org/scripts/split_libraries_fastq.html) (Caporaso et al. 2010), and Mothur software (http://www.mothur.org) to obtain the available sequences. The effective sequences were clustered in operational taxonomic units (OTUs) using Uparse software (Uparse v7.0.1001, http://drive5.com/uparse/) (Edgar 2013) at 97% similarity. The taxonomic classification of sequences was accomplished using Mothur method and SSUrRNA Database (Quast et al. 2013) of SILVA (http://www.arb-silva.de/) (Wang et al. 2007), which passed a threshold of 80%. A heat map was created using the R g-plots package to show the similarities and differences between the three samples and highlight the most abundant groups. The detailed steps are described in the supplementary materials.

Data analysis

Statistical analysis was conducted using Microsoft Excel 2010 and SPSS software (v. 19.0; SPSS IBM, Armonk, NY, USA), and graphs were constructed using Microsoft Excel 2010 and OriginPro 17.0. Relationships between variables were tested for significance using a t test and associated P values, with alpha set at 0.05. Spearman correlation coefficient was calculated in order to evaluate the relationship among FIB, HPC, ATP, FCM data, and water chemical indexes. We calculated Shannon diversity indexes for each site.

Results and discussion

Water quality in the reclaimed water reuse system

As shown in Table 1, the water quality within the distribution pipeline and in the landscape lake meet the standards required for reclaimed water for reuse in landscape features, irrigation, and toilet flushing (MWR 2006). Overall, inorganic indexes (i.e., NH_4^+-N and TDS) and organic indexes (i.e., UV254, COD, SCOD, and TOC) showed a slight difference between P1 and P2. By contrast, organic indexes (i.e., UV254, COD, SCOD, and TOC) were higher in P3 compared with P2. Salt and nitrogen in the reclaimed water may cause pipeline corrosion and eutrophication. Our results show that salt and nitrogen did not exceed the required standards, which indicate that these compounds do not accumulate in the landscape lake. On the other hand, non-point source pollution can be negligible when compared with the environmental capacity of the landscape lake (Ma et al. 2016). COD was twice as high in P3 as it was in P2, while TOC in P3 was just 10% higher than that in P2. These results indicate that the slightly higher

Table 1 Reclaimed water quality in the reclaimed water reuse system

Index	Reclaimed water inlet (P1)	Reclaimed water outlet (P2)	Lake water (P3)
NH_4^+-N (mg/L)	0.43 ± 0.27	0.24 ± 0.09	0.21 ± 0.09
COD (mg/L)	26.34 ± 6.77	17.89 ± 7.37	39.27 ± 7.22
SCOD (mg/L)	20.18 ± 5.11	16.14 ± 7.85	25.84 ± 7.38
TOC (mg/L)	4.22 ± 0.65	4.2 ± 0.63	4.72 ± 0.55
UV254 (1/cm)	0.077 ± 0.029	0.077 ± 0.033	0.086 ± 0.032
TP (mg/L)	2.63 ± 0.25	1.98 ± 1.01	0.75 ± 0.56
Electric conductivity (μs/cm)	842.38 ± 29.76	857.17 ± 2.27	776.75 ± 11.08
Cl (mg/L)	0.96 ± 0.11	0.34 ± 0.19	0.06 ± 0.02

Values are presented as average value ± standard deviation. There are 8 measurements during water sample collection period

concentrations of COD in the landscape lake may not have been caused by organic carbon input from non-point sources (Ma et al. 2016). The residual total chlorine decreased slightly during transportation within the distribution pipeline due to the short residence time and remained at 0.34 ± 0.19 mg/L on exit from the pipeline. However, the residual total chlorine decreased sharply within the landscape lake to 0.06 ± 0.02 mg/L, and at times below the detection limit.

Changes in FIB and HPC concentrations

There was a slight difference in the concentrations of FC and *E. coli* between P1 and P2, but the difference was more significant between P3 and the other two sampling sites (Fig. 2). The detection rates of FC and *E. coli* in P1 and P2 were only 25% (number of positive samples was 4) at the concentrations of less than 1 cfu/100 mL for both. Zhou et al. (2015) reported that almost all pathogens, including FC and *E. coli*, can be removed or inactivated by MBR filtration followed by disinfection with 2.5 mg/L sodium hypochlorite (NaClO). The detection rates of FC and *E. coli* at P3 were 100% at the concentrations of 14 ± 4 cfu/100 mL and 15 ± 6 cfu/100 mL (number of positive samples was 16), respectively. Zhou et al. (2015) demonstrated that FC was occasionally detected in landscape lakes, and the detection rate of *E. coli* in such lakes was only 51.5%. In principle, *E. coli* and FC can be inactivated by NaClO. However, bacteria can be transferred to VBNC status in adverse environments (Oliver 2010), while retaining the ability to reproduce when conditions become suitable again.

The concentrations of HPC at P1, P2, and P3 were 5.6 ± 0.6 × 10^3 cells/mL, 6.6 ± 3.5 × 10^3 cells/mL, and 13.2 ± 3 × 10^3 cells/mL, respectively (Fig. 2). The difference in HPC concentrations between P3 and P2 was significant ($p < 0.001$), indicating a regrowth of bacteria in the landscape lake. No such change was observed within the distribution pipeline ($p = 0.094$). Very few bacteria can be detected using the culture method (Van Nevel et al. 2017), and more than 99% of microorganisms are uncultured bacteria (Achtman and Wagner 2008; D'Onofrio et al. 2010). In fact, it is

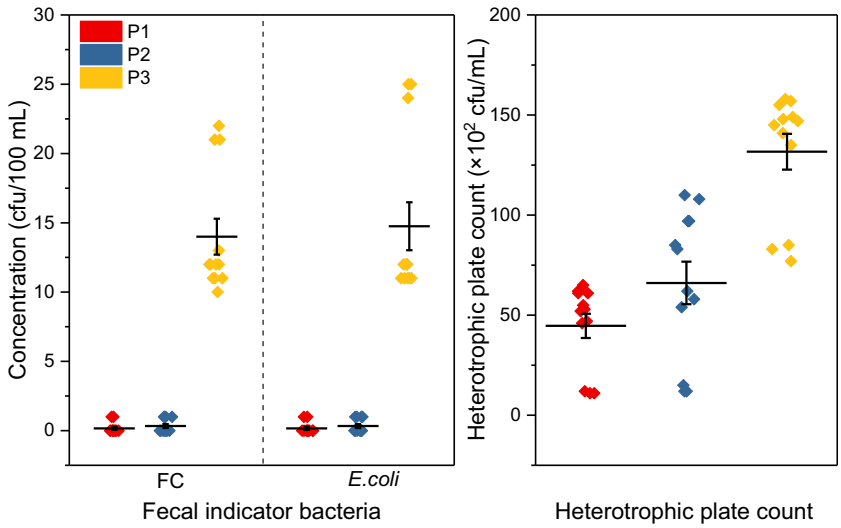

Fig. 2 Changes in fecal indicator bacteria and heterotrophic plate count in the water reuse system

underestimating to detect HPC using the culture method as the bacteria can be transferred into VBNC status because of chlorine disinfection.

Bacterial viability by measuring ATP and FCM

The bacterial ATP contents at P1, P2, and P3 were 3.55 ± 1.79 ng/L, 3.31 ± 1.43 ng/L, and 18.97 ± 6.39 μg/L, respectively (Fig. 3a). The bacterial ATP content changed slightly within the distribution pipeline but increased sharply within the landscape lake. Based on the conversion formula of 8.9×10^{-8} ng ATP/cell (Hammes et al. 2010), the bacteria counts at P1, P2, and P3 were $3.99 \pm 2.01 \times 10^4$ cells/mL, $3.72 \pm 1.61 \times 10^4$ cells/mL, and $2.13 \pm 0.72 \times 10^8$ cells/mL, respectively. As an alternative method for HPC detection, bacterial ATP detection is fast and simple and can be used to detect active bacteria (Hammes et al. 2010; Wielen and van der Kooij 2010). However, conventional bacterial ATP detection is limited by the lack of standardized comparative methods (Hammes et al. 2010). Riemann (1979) reported that more than 76% of bacterial ATP originated from extracellular ATP in eutrophic lake water samples. This may be the reason for the sharp increase in bacterial ATP at P3 compared with P2. By contrast, previous studies have shown that the bacterial ATP content of chlorinated water samples increases with prolonged residence time, implying bacterial resuscitation (Nescerecka et al. 2014). No obvious bacterial resuscitation was found in this study, due to the short HRT of the distribution pipeline.

The total bacterial cell concentrations at P1, P2, and P3 were $2.30 \pm 1.28 \times 10^6$ cells/mL, $2.35 \pm 0.63 \times 10^6$ cells/mL, and $17.98 \pm 12.75 \times 10^6$ cells/mL (Fig. 3b). Furthermore, the proportions of intact bacterial cells at P1, P2, and P3 were 3.9 ± 0.5%, 4.1 ± 0.1%, and 20.6 ± 1.7%, respectively (Fig. 4). Therefore, the intact bacterial cell concentrations in P1, P2, and P3 were $5.91 \pm 0.52 \times 10^4$ cells/mL, $7.95 \pm 2.58 \times 10^4$ cells/mL, and $5.65 \pm 2.10 \times 10^6$ cells/mL, respectively. Thus, the total bacterial cell concentrations and intact bacterial cell concentrations of the reclaimed water increased only slightly from P1 to P2 ($p = 0.056$), but increased significantly from P2 to P3 ($p = 0.006$).

FCM total bacterial cell concentrations, percentage of intact bacterial cell, and intact bacterial cell concentrations revealed changes of VBNC bacteria in the reclaimed water samples within the distribution pipeline and the landscape lake. These changes were not detected with conventional analysis (i.e., HPC and ATP). FCM is a widely used technique. It is fast, accurate, quantitative, and repeatable. When used with the mixed nucleic acid dyes of SGI and PI (SGI + PI), it can be used to characterize total bacterial cells and intact bacterial cells (Prest et al. 2014) with distribution pipelines (Nescerecka et al. 2014; Prest et al. 2016; Van Nevel et al. 2016) and during storage (Wang et al. 2008). In terms of the distribution pipeline, Nescerecka et al. (2014) have demonstrated that bacteria in chlorinated water samples can increase from 1×10^4 cells/mL to 4×10^5 cells/mL, indicating bacterial resuscitation. However, no apparent bacterial resuscitation was found in this study, possibly due to the short HRT of the reclaimed water distribution.

Our results indicate that HPC, ATP, and intact bacterial cell concentrations increase slightly during reclaimed water distribution, which means that bacterial viability also increases slightly. However, these indexes at P3 were considerably higher than P2, which indicate that bacterial viability increased sharply in the landscape lake. We infer from these results of the resurgence of VBNC bacteria in the landscape lake, albeit no notable change occurred within the distribution pipeline.

In this study, quantitative information was obtained by FCM, and not from relative abundance data alone. FCM has clear potential for applications in reclaimed water distribution system and landscape lake monitoring. However, FCM also has inherent deficiencies, such as its inability to

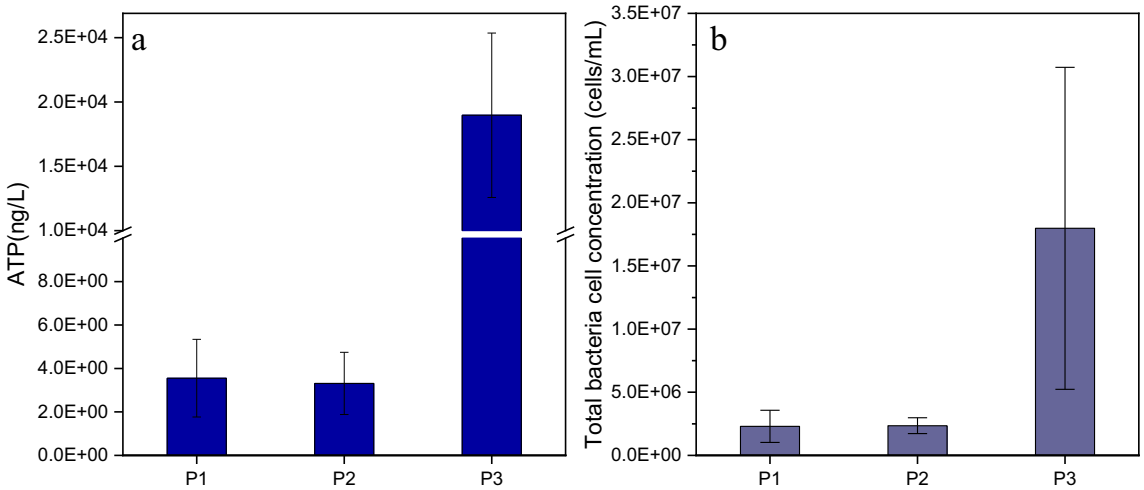

Fig. 3 Changes in ATP and total bacterial cell concentrations in the water reuse system

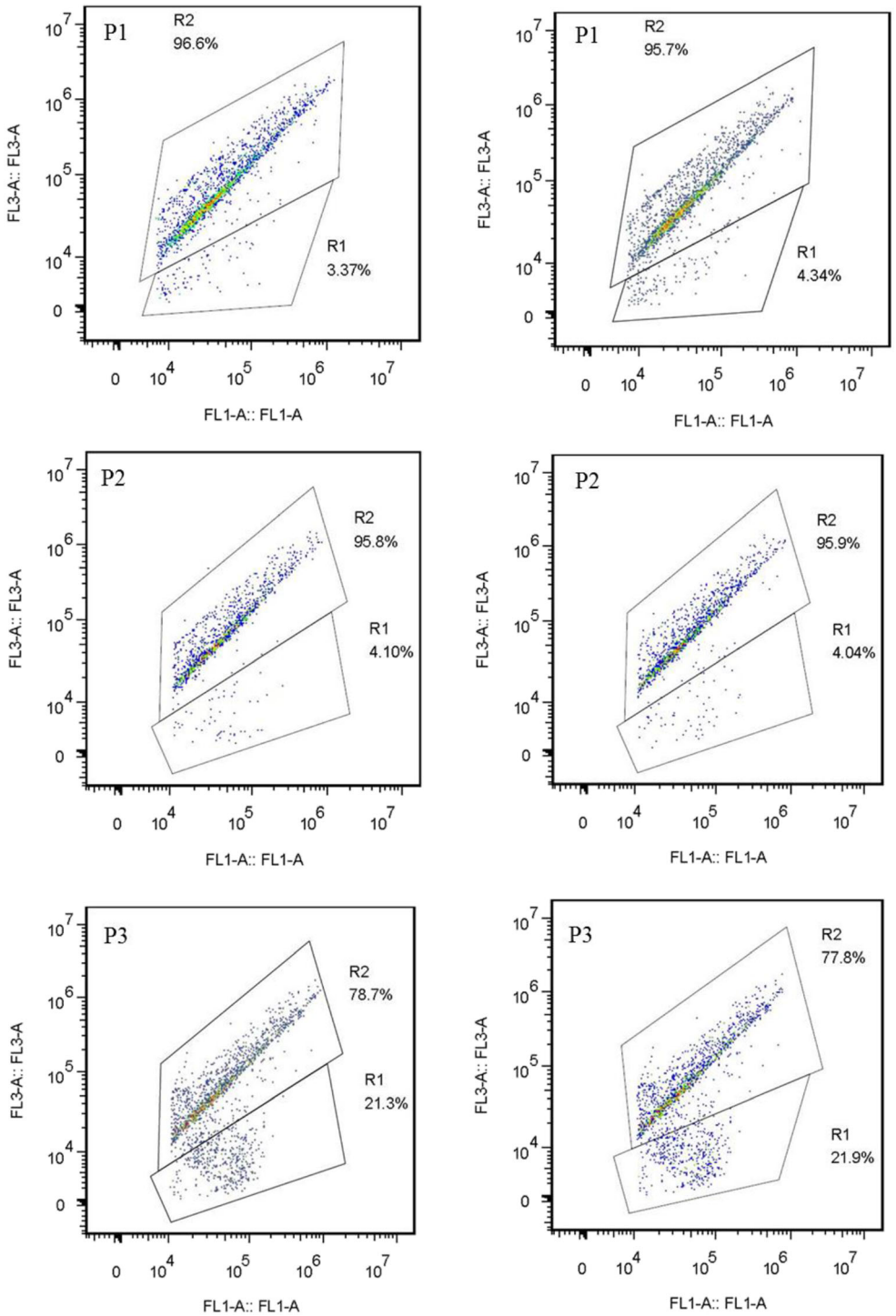

Fig. 4 Changes in intact bacterial cell percentage in the water reuse system

recognize changes in populations of specific bacteria, and such detailed information needs to be obtained by other more cumbersome methods (e.g., qPCR and PMA-qPCR) (Lin et al. 2016a, b). On the other hand, the bacterial viability characterized by the FCM data is based on cell membrane damage, while bacteria with damaged membranes may still have a certain survival and reproduction capabilities.

Bacterial community structure by 16S rRNA gene high-throughput sequencing

The Shannon diversity indexes at P1, P2, and P3 were 6.535, 7.05, and 6.886, respectively. As shown in Fig. 5, *Proteobacteria* was the dominant phylum across all sampling sites, with a relative abundance at P1, P2, and P3 of 88.96%, 84.04%, and 71.20%, respectively. *Bacteroidetes* was the

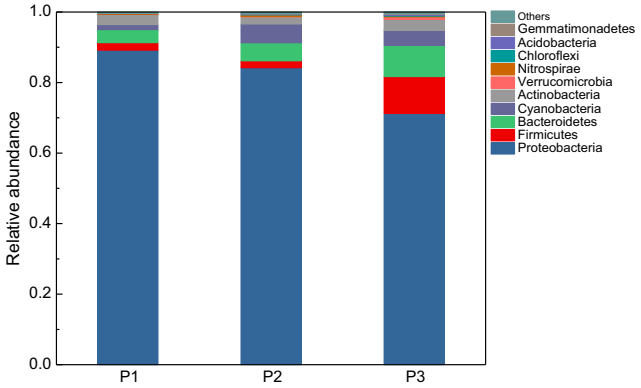

Fig. 5 Changes in the relative abundance of phyla in the water reuse system

second most dominant phylum at P1 and P2, and third dominant at P3. The relative abundance of *Bacteroidetes* at P1, P2, and P3 was 3.77%, 5.18%, and 8.99%. In addition, the relative abundance of *Firmicutes* at P1, P2, and P3 was 2.2%, 2%, and 10.26% and that of *Cyanobacteria* at P1, P2, and P3 was 1.39%, 4.18%, and 5.2%. Furthermore, the relative abundance of *Actinobacteria* at P1, P2, and P3 was 2.9%, 2.1%, and 3.25%. Overall, the relative abundance of phyla changed slightly within the distribution pipeline. However, the relative abundance of *Proteobacteria* decreased by 17.76% in the landscape lake, while those of *Firmicutes* and *Bacteroidetes* increased by 8.02% and 5.22%, respectively.

In addition, as shown in Fig. 6, the proportions of high nucleic acid content (HNA) cells in P1, P2, and P3 were 67.6 ± 0.4%, 71.0 ± 1.0%, and 71.6 ± 0.7%, respectively. Slight differences of HNA cells between P1, P2, and P3 samples were reflected in the FCM fingerprints. The increase in HNA cells can be due to the growth of specific bacterial species (Vila-Costa et al. 2012). The relative abundance analysis of the 16S high-throughput sequencing data allowed for characterizing the change between P1, P2, and P3 samples and revealed that the main bacterial phyla present in both water samples were *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Fig. 5). The percentage of *Proteobacteria* decreased from 88.96 to 71.20%, while the percentage of *Bacteroidetes* increased from 3.77 to 8.99% and *Firmicutes* increased from 2.2 to 10.26%, of the total bacterial population. These bioinformatics also demonstrated that the bacterial community undergoes slight changes in the system.

As shown in Fig. 7, the relative abundances of the top five most abundant genera at P1, namely *Zoogloea*, *Sphingobium*, *Novosphingobium*, *Dechloromonas*, and *Pseudomonas*, were 13.88%, 8.93%, 8.65%, 8.51%, and 5.44%, respectively. The relative abundances of *Permianibacter*, *Acinetobacter*, *Pseudomonas*, *Limnohabitans*, and *Novosphingobium* at P2 were 11.58%, 9.22%, 7.33%, 6.88%, and 5.39%, respectively. The genera, as mentioned above, all belong to *Proteobacteria*. Also, the top five genera at P3 were *Pseudomonas*, *Limnohabitans*, *Bacillus*, *Flavobacterium*, and *Lactococcus*. The relative abundances of *Pseudomonas* and *Limnohabitans* at P3 were 11.94% and 9.26%, which belong to *Proteobacteria*. Conversely, the relative abundances of *Bacillus* and *Lactococcus* in P3 were 5.29% and 1.9%, which belong to *Firmicutes*. *Flavobacterium*, which belongs to *Bacteroidetes*, also showed a relative abundance of 3.75%, at P3.

Pathogenic bacteria in the reclaimed water reuse system

As shown in Fig. 8, the relative abundances of *Pseudomonas*, which was the dominant genus, at P1, P2, and P3 were 5.55%, 7.34%, and 11.84%, respectively. Also, the relative abundances of *Acinetobacter* at P1, P2, and P3 were 3.04%, 4.15%, and 1.90%, respectively, which was the second most dominant genus.

Moreover, the relative abundances of *Mycobacterium* at P1, P2, and P3 were 0.05%, 0.09%, and 0.03%, respectively. *Mycobacterium* is insensitive to chlorine disinfection environment (Kim et al. 2013), and this resistance is further improved by coexistence with *Amoeba* (Thomas et al. 2008). Furthermore, the relative abundances of *Legionella* at P1, P2, and P3 were 0.06%, 0.03%, and 0.04%, respectively. Similar to *Mycobacterium*, *Legionella* can coexist with *Amoeba*, and it is also tolerant of residual chlorine in distribution pipelines (Buse and Ashbolt 2012). *Legionella* is ubiquitous in reclaimed water (Jjemba et al. 2015) and can infect children and the elderly (Collier et al. 2012).

Also, the relative abundances of *Enterococcus* at P1, P2, and P3 were 0.015%, 0.017%, and 0.012%, respectively, whereas that of *Staphylococcus* was 0.199%, 0.007%, and 0.016%, respectively. *Staphylococcus* can cause a variety of diseases (e.g., food poisoning) in humans and animals through osmotic toxins. *Clostridium* also showed a relative abundance of 0.017%, 0.027%, and 0.017%, respectively at P1, P2, and P3, whereas that of *Arcobacter* was 0.09%, 0.1%, and 0.024%, respectively. *Clostridium* can tolerate harsh environments, e.g., high hydrostatic pressure and disinfectant (Omidbakhsh 2010).

Overall, the relative abundance of *Pseudomonas* doubled from 5.55% in the reclaimed water to 11.84% in the landscape lake, while those of *Mycobacterium*, *Legionella*, *Enterococcus*, *Clostridium*, and *Arcobacter* showed only minimal changes. The 16S rRNA gene high-throughput sequencing technology can only provide information on relative abundance (Lin et al. 2014), but the causes of these community changes are not yet clear. Nonetheless, competition between bacterial species can result in the differential growth of specific bacterial populations (Egli 2010; Prest et al. 2014). In addition, other microorganisms (e.g., fungi, viruses, and

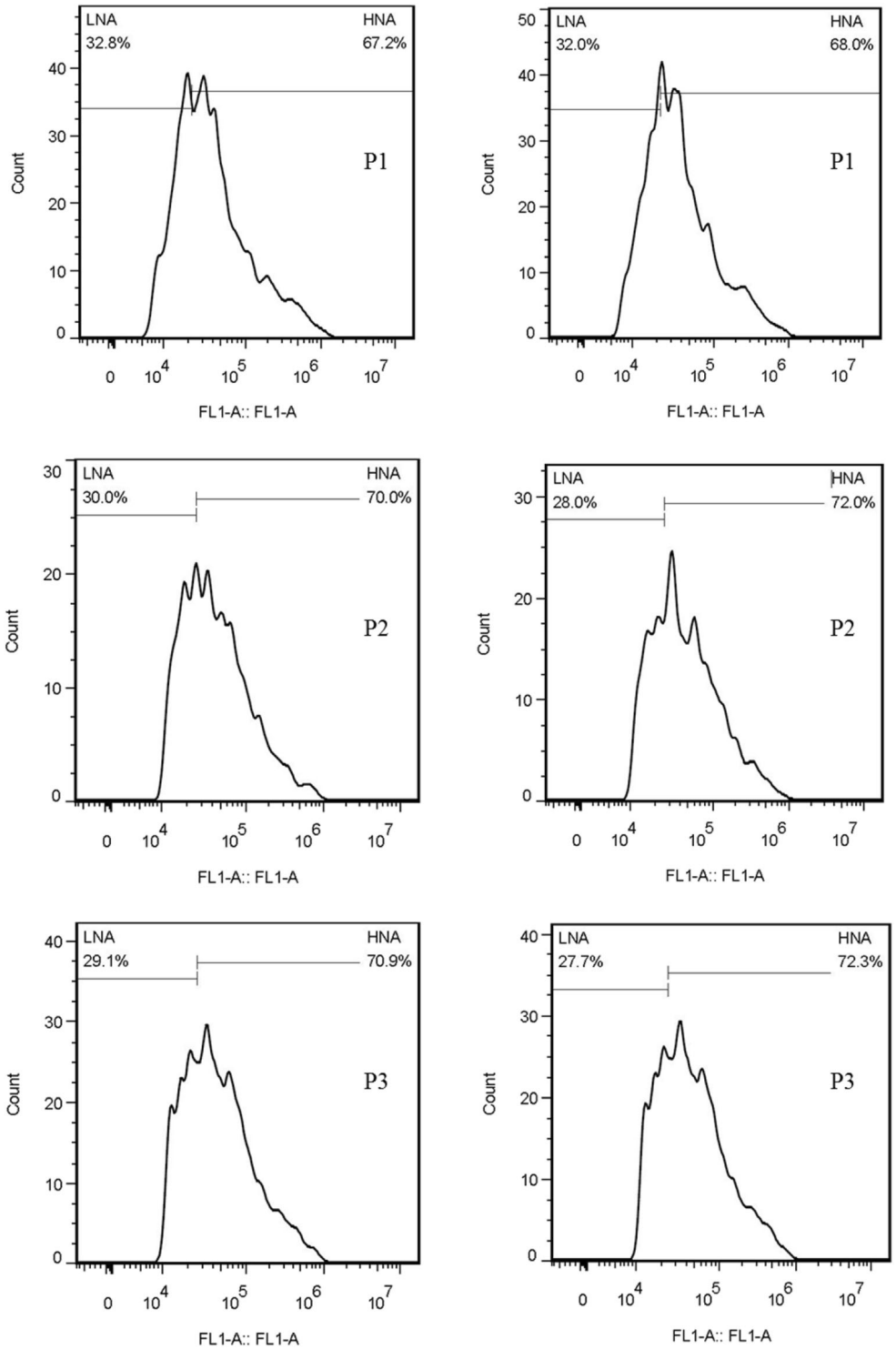

Fig. 6 Changes in high nucleic acid content (HNA) cells in the water reuse system

protozoa) can target specific bacterial species, leading to their decline or even disappearance (Prest et al. 2014). Considerably more knowledge on bacterial community diversity during reclaimed water distribution and replenishment of landscape lakes is needed, and 16S high-throughput sequencing technology could help gain knowledge about microbial dynamics in such a system to some extent.

Relationship between FIB, HPC, ATP, FCM data, and water chemical indexes

FC and *E. coli* showed negative correlations with inorganic nutrient indexes (i.e., NH_4^+-N, TP, residual total chlorine, and electric conductivity), but positively correlated with organic nutrients indexes (i.e., TOC, COD, and SCOD) (Table 2).

Fig. 7 Changes in the relative abundance of bacteria genera in the water reuse system

Similarly, total bacterial cell concentrations (TBCC) and intact bacterial cell concentrations (IBCC) were positively correlated with organic nutrients indexes (i.e., TOC, COD, and SCOD), and negatively correlated with inorganic nutrients indexes (i.e., NH_4^+-N, TP, residual total chlorine, and electric conductivity) (Table 2). Conversely, HPC showed negative correlations with NH_4^+-N, residual total chlorine, and organic nutrients indexes (i.e., TOC, COD, and SCOD), but positively correlated with TP and electric conductivity (Table 2).

These results show that residual total chlorine and organic nutrients indexes (i.e., COD, SCOD, and TOC) have a strong correlation with almost all the bacteriological indexes. Residual total chlorine was the most important factor. Previous research has shown that the concentration of organic carbon should be much lower than 10 ppb in order to constrain bacterial regrowth in distribution systems (Williams et al. 2015). This restrictive condition is unrealistic for reclaimed water reuse in landscape lakes. In addition, both

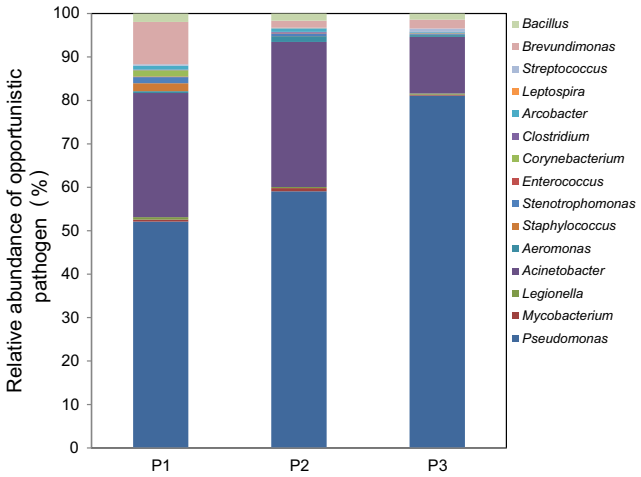

Fig. 8 Changes in pathogenic bacteria in the water reuse system

Mycobacterium spp. and *Legionella* spp. were positively correlated with phosphorus in the reclaimed water distribution system, while *Legionella* spp. was positively correlated with ammonia (Garner et al. 2018). These findings indicate that ammonia and phosphorus, as well as carbon, are worthy of further study. Furthermore, chlorine disinfection is known to destroy or damage the surface structure of bacteria (Cho et al. 2010). The N-terminal amino acids of peptidoglycan located on bacterial walls could be oxidized during chlorine disinfection (Pattison and Davies 2012), resulting in morphological changes. Mild membrane damage has been observed under low doses of chlorine disinfectants, while much more severe damage was observed under high doses (25 mg/L chlorine for 30 min) (Xu et al. 2017). Moreover, *E. coli* can quickly lose culturability under low doses of chlorination (Xu et al. 2017).

The average removal rates of *E. coli* were 0.38-log, 1.08-log, and 2.06-log at the exposure doses to chlorine 0.5 mg/L, 1.0 mg/L, and 2.0 mg/L, respectively, for 30 min (Xu et al. 2017). In this study, residual total chlorine was greatly reduced in the landscape lake (Table 1). Without the inhibitory effect of residual chlorine, bacteria can recover and resuscitate, as was illustrated by the increase of FC, *E. coli*, HPC, total bacterial cell concentrations, and intact bacterial cell concentrations in this study.

Conclusions

Bacterial viability and diversity in the reclaimed water reuse system were investigated, including reclaimed water distribution and a landscape lake. Overall, bacterial viability was shown to increase, while no notable change of bacterial diversity in the reclaimed water reuse system.

- Indexes of HPC and IBCC increased slightly within the reclaimed water distribution system, demonstrating that bacteria are not easily disturbed by external factors in a short-range closed pressurized pipeline.
- HPC, ATP, and IBCC showed significant increases in the landscape lake, indicating that landscape lakes may provide favorable conditions for the survival of bacteria.
- Minimal changes were demonstrated of the bacterial diversity in the reclaimed water reuse system, except for phyla such as *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* and the genus *Pseudomonas*.
- VBNC bacteria were revived in the landscape lake (P3). Thus, the bacterial viability should be specially considered, in particular when used for replenishing landscape lakes (from P2 to P3).

Overall, the findings of this study can serve as a guide for monitoring of microbial water quality in landscape lakes replenished with reclaimed water. Nonetheless, further research on the quantitative detection of opportunistic pathogens (e.g., *Mycobacterium*, *Legionella*) is necessary; as such, a study would provide further in-depth risk assessment of exposure to opportunistic pathogens.

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Table 2 Relationship between FIB, HPC, FCM data, and water chemical indexes

	FC	*E. coli*	HPC	ATP	TBCC	IBCC
NH_4^+-N	− 0.355	− 0.037	− 0.093	0.255	− 0.027	− 0.026
COD	0.632**	0.760**	− 0.213	− 0.165	0.27	0.329
SCOD	0.258	0.473*	− 0.266	0.097	0.183	0.206
TOC	0.261	0.435*	− 0.169	− 0.355	0.227	0.245
UV254	− 0.337	− 0.045	0.063	0.314	− 0.084	− 0.141
TP	− 0.764**	− 0.584**	0.036	− 0.045	− 0.342	− 0.426*
Electric conductivity	− 0.748**	− 0.744**	0.138	− 0.554*	− 0.335	− 0.408*
Cl	− 0.703**	− 0.715**	− 0.161	− 0.164	− 0.263	− 0.354

A double asterisk means that when confidence (double test) is 0.01, the correlation is significant, and a single asterisk means that when confidence (double test) is 0.05, the correlation is significant

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