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Benzyldimethyldodecyl ammonium chloride shifts the proliferation of functional genes and microbial community in natural water from eutrophic lake

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2 Benzyldimethyldodecyl Ammonium Chloride Shifts the Proliferation of Functional

3 Genes and Microbial Community in Natural Water from Eutrophic Lake

4

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6

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10

11

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Abstract

Benzylalkyldimethylethyl ammonium compounds are pervasive in natural environments and toxic at high concentrations. The changes in functional genes and microbial diversity in eutrophic lake samples exposed to benzyldimethyldodecyl ammonium chloride (BAC) were assessed. BAC exerted negative effects on bacteria abundance, particularly at concentrations of 100 $\mu\text{g L}^{-1}$ and higher. A significant increase in the number of the quaternary ammonium compound-resistant gene *qacA/B* was recorded within the 10 $\mu\text{g L}^{-1}$ treatment after the first day of exposure. Not all antibiotic resistance genes increased in abundance as the concentrations of BAC increased; rather, gene abundances were dependent on the gene type, concentrations of BAC, and contact time. The nitrogen fixation-related gene *nifH* and ammonia monooxygenase gene *amoA* were inhibited by high concentrations of BAC after the first day, whereas an increase of the nitrite reductase gene *nirK* was stimulated by exposure. Microbial communities within higher treatment levels (1 000 and 10 000 $\mu\text{g L}^{-1}$) exhibited significantly different community composition compared to other treatment levels and the control. Selective enrichment of *Rheinheimera*, *Pseudomonas*, and *Vogesella* were found in the higher treatment levels, suggesting that these bacteria have some resistance or degradation capacity to BAC. Genes related with RNA processing and modification, transcription, lipid transport and metabolism, amino acid transport and metabolism, and cell motility of microbial community function were involved in the process exposed to the BAC stress.

Keywords: *Cyanobacteria*; *qacEΔ1*; *nirK*; *Rheinheimera*; microbial diversity

34 **Capsule:** Shift pattern in the proliferation of functional genes and microbial
35 community in natural water from eutrophic lake exposed to BAC was assessed.
36

1. Introduction

Quaternary ammonium compounds (QACs) are a major class of cationic surfactants in disinfectants, biocides, detergents, and dispersants used across domestic, agricultural, industrial and clinical products { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Benzylalkyldimethylethyl ammonium compounds are one of the most prevalent QACs in natural environments, commonly occurring as effluents from wastewater treatment plants, hospitals, and laundry wastewater { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Benzylalkyldimethylethyl ammonium compounds in surface water are often detected in the $\mu\text{g L}^{-1}$ level { ADDIN EN.CITE

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59 environment</title><secondary-title>Science of The Total Environment</secondary-
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 63 519</volume><keywords><keyword>Quaternary ammonium compounds
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 65 word><keyword>Toxicity</keyword><keyword>Determination</keyword></keywo
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 70 main.pdf?_tid=dc0dd0e8-1450-11e7-aa6c-
 71 00000aacb35f&acdnat=1490772498_0384763c3f545e833f59411b45eefa4e</url
 72 ></related-urls></urls><electronic-resource-
 73 num>http://dx.doi.org/10.1016/j.scitotenv.2015.03.007</electronic-resource-
 74 num></record></Cite></EndNote>}. The concentrations of benzyldimethyldodecyl
 75 ammonium chloride (BAC), a type of benzylalkyldimethylethyl ammonium
 76 compounds, in the surface water downstream from five wastewater treatment plants in
 77 the US ranged from 2.7 to 5.8 $\mu\text{g L}^{-1}$ { ADDIN EN.CITE
 78 <EndNote><Cite><Author>Ferrer</Author><Year>2001</Year><RecNum>1200</R
 79 ecNum><DisplayText>(Ferrer and Furlong, 2001)</DisplayText><record><rec-
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 num></record></Cite></EndNote>}. The concentrations of benzylalkyldimethylethyl
 ammonium compounds, including BAC, in Taiwanese river water were detected in the
 range 2.5 - 65 $\mu\text{g L}^{-1}$ { ADDIN EN.CITE
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 107 H.</author><author>Liao, Y. H.</author></authors></contributors><auth-
 108 address>Natl Cent Univ, Dept Chem, Chungli 32054, Taiwan</auth-
 109 address><titles><title>Determination of alkylbenzyldimethylammonium chlorides in
 110 river water and sewage effluent by solid phase extraction and gas chromatography
 111 mass spectrometry</title><secondary-title>Analytical Chemistry</secondary-
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 127 num>DOI 10.1021/ac000655i</electronic-resource-
 128 num><language>English</language></record></Cite></EndNote>}. The total
 129 concentration of BAC and other QACs in surface water samples from the Gdańsk
 130 City in Poland were found in the range 72.5 - 342 µg L⁻¹ { ADDIN EN.CITE
 131 <EndNote><Cite><Author>Olkowska</Author><Year>2013</Year><RecNum>120
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 137 Ewa</author><author>Polkowska, Żaneta</author><author>Namieśnik,
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 140 surfactants in surface water samples</title><secondary-title>Talanta</secondary-
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 145 chromatography-conductivity detection</keyword><keyword>Surface water
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 151 num>http://doi.org/10.1016/j.talanta.2013.04.083</electronic-resource-
 152 num></record></Cite></EndNote>}.
 153 Benzylalkyldimethylethyl ammonium compounds could inhibit cell growth via
 154 cytoplasmic membrane disruption { ADDIN EN.CITE { ADDIN EN.CITE.DATA
 155 }}, so, they can be toxic to aquatic life without target organisms, such as fish {
 156 ADDIN EN.CITE <EndNote><Cite><Author>Van de
 157 Voorde</Author><Year>2012</Year><RecNum>1308</RecNum><DisplayText>(Va
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 163 Antoine</author><author>Lorgeoux, Catherine</author><author>Gromaire, Marie-
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 165 Ghassan</author></authors></contributors><titles><title>Analysis of quaternary
 166 ammonium compounds in urban stormwater samples</title><secondary-
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169 2>Environ. Pollut.</abbr></periodical><pages>150-
 170 157</pages><volume>164</volume><number>Supplement
 171 C</number><keywords><keyword>Benzalkonium</keyword><keyword>Liquid
 172 chromatography</keyword><keyword>Mass
 173 spectrometry</keyword><keyword>Water</keyword><keyword>Particles</keyword
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 178 ></related-urls></urls><electronic-resource-
 179 num>https://doi.org/10.1016/j.envpol.2012.01.037</electronic-resource-
 180 num></record></Cite></EndNote>}, algae { ADDIN EN.CITE { ADDIN
 181 EN.CITE.DATA }} and microorganisms { ADDIN EN.CITE { ADDIN
 182 EN.CITE.DATA }}. The green algae *Chlorella vulgaris* and the water flea *Daphnia*
 183 *magna* are two organisms frequently used to assess the toxicity of
 184 benzylalkyldimethylethyl ammonium compounds on aquatic environments { ADDIN
 185 EN.CITE { ADDIN EN.CITE.DATA }}. The 48-h EC₅₀ of *Daphnia magna* after
 186 exposure to BAC was recorded as 0.041 mg L⁻¹ { ADDIN EN.CITE { ADDIN
 187 EN.CITE.DATA }} and the 96-h EC₅₀ of *Chlorella vulgaris* was 0.203 mg L⁻¹ {
 188 ADDIN EN.CITE
 189 <EndNote><Cite><Author>Zhu</Author><Year>2010</Year><RecNum>1144</Rec
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 196 Runliang</author><author>Wang, Xueye</author><author>Zheng,
 197 Xiaoyan</author></authors></contributors><titles><title>A DFT-based QSAR study
 198 of the toxicity of quaternary ammonium compounds on *Chlorella*
 199 *vulgaris*</title><secondary-title>Chemosphere</secondary-
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 210 ></related-urls></urls><electronic-resource-
 211 num>http://dx.doi.org/10.1016/j.chemosphere.2010.03.044</electronic-resource-
 212 num></record></Cite></EndNote>}. Acute effects of BAC could occur at tens to

213 hundreds of $\mu\text{g L}^{-1}$ levels for *Daphnia magna* and *Ceriodaphnia dubia*, while
 214 genotoxic effects at DNA damage level, the lowest adverse effect levels were 0.4
 215 and 4 ng L^{-1} for *D. magna* and *C. dubia*, respectively { ADDIN EN.CITE
 216 <EndNote><Cite><Author>Lavorgna</Author><Year>2016</Year><RecNum>1191
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 223 Brigida</author><author>Parrella, Alfredo</author><author>Isidori,
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 225 the quaternary ammonium compound benzalkonium chloride (BAC) using *Daphnia*
 226 *magna* and *Ceriodaphnia dubia* as model systems</title><secondary-
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 238 ></related-urls></urls><electronic-resource-
 239 num>http://doi.org/10.1016/j.envpol.2015.11.042</electronic-resource-
 240 num></record></Cite></EndNote>}. It should be paid more attention, because these
 241 effective concentrations are much lower than BAC concentrations detected in surface
 242 waters.
 243 Benzylalkyldimethylethyl ammonium compounds not only caused negative
 244 influence on the organisms but also resulted in changes of antibiotic resistance genes
 245 in engineered environment { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The
 246 enhanced selection and spread of antimicrobial genes by these compounds have been
 247 regarded as a threat to human health { ADDIN EN.CITE
 248 <EndNote><Cite><Author>Hegstad</Author><Year>2010</Year><RecNum>1312<
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 254 K.</author><author>Langsrud, S.</author><author>Lunestad, B.
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 256 M.</author><author>Yazdankhah, S. P.</author></authors></contributors><auth-

address>Department of Microbiology and Infection Control, Reference Centre for
 Detection of Antimicrobial Resistance, University Hospital of North Norway, Tromsø,
 Norway. kristin.hegstad@uit.no</auth-address><titles><title>Does the wide use of
 quaternary ammonium compounds enhance the selection and spread of antimicrobial
 resistance and thus threaten our health?</title><secondary-title>Microb Drug
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 num>10.1089/mdr.2009.0120</electronic-resource-num><remote-database-
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279 provider><language>eng</language></record></Cite></EndNote>}. Besides,

280 denitrification process was also inhibited by BAC in engineered environment {

281 ADDIN EN.CITE

282 <EndNote><Cite><Author>Hajaya</Author><Year>2012</Year><RecNum>1321</

283 RecNum><DisplayText>(Hajaya and Pavlostathis,

284 2012)</DisplayText><record><rec-number>1321</rec-number><foreign-keys><key

285 app="EN" db-id="vzededvvherd97ep2db5pwr1fe5trtad95r0"

286 timestamp="1511820333">1321</key></foreign-keys><ref-type name="Journal

287 Article">17</ref-type><contributors><authors><author>Hajaya, Malek

288 G.</author><author>Pavlostathis, Spyros

289 G.</author></authors></contributors><titles><title>Fate and effect of benzalkonium

290 chlorides in a continuous-flow biological nitrogen removal system treating poultry

291 processing wastewater</title><secondary-title>Bioresource Technology</secondary-

292 title></titles><periodical><full-title>Bioresource Technology</full-title><abbr-

293 1>Bioresource Technol</abbr-1><abbr-2>Bioresource. Technol.</abbr-

294 2></periodical><pages>73-

295 81</pages><volume>118</volume><number>Supplement

296 C</number><keywords><keyword>Benzalkonium

297 chlorides</keyword><keyword>Biological nitrogen

298 removal</keyword><keyword>Nitrification</keyword><keyword>Denitrification</k

299 eyword><keyword>Quaternary ammonium

300 compounds</keyword></keywords><dates><year>2012</year><pub-

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></related-urls></urls><electronic-resource-
num>https://doi.org/10.1016/j.biortech.2012.05.050</electronic-resource-
num></record></Cite></EndNote>}. However, few studies have focused on the effect
of benzylalkyldimethylethyl ammonium compounds on microbial diversity and
nitrogen cycling genes in natural aquatic environments.

In this study, microcosm tests were constructed from water samples collected
from Nanhu Lake, a eutrophic lake in the city of Wuhan, Hubei Province in Central
China. In this study, we hypothesized that: (1) BAC influenced the abundance of
bacteria, including *Cyanobacteria*, in the eutrophic lake; (2) BAC affected the spread
of quaternary ammonium compound-resistant genes and antibiotic resistance genes;
(3) BAC may influence abundances of *amoA*, *nifH* and *nirK* and affect the nitrogen
cycle; and (4) microbial diversity and community composition adapted to BAC
depended on the dose of BAC and contact time. The information provided in this
study will be beneficial to understand the effects of BAC on aquatic microbial
ecosystem in the natural lake environments.

2. Materials and Methods

2.1 Materials and setup of freshwater microcosm preparation

To assess the influence of BAC on freshwater microcosms, we followed the
research protocol outlined in previous study about effect of ionic liquid on the

proliferation of antibiotic resistance genes { ADDIN EN.CITE
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 Quanhua</author><author>Mao,
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 the Proliferation of Antibiotic Resistance Genes Mediated by Class I
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 num>10.1021/ez500103v</electronic-resource-num><access-
 date>2014/07/24</access-date></record></Cite></EndNote>}. Briefly, water samples

were collected from different parts of the eutrophic Nanhu Lake in Wuhan in September 2016, and mixed to get homogenous freshwater microcosms. To remove sediment and collect the supernatant, the water samples were placed in a refrigerator set at 4°C and left undisturbed for 3 h. The water quality of the supernatant was recorded as: pH 7.95, dissolved oxygen 10.38 mg L⁻¹, total nitrogen 11.35 mg L⁻¹ and total phosphorus 0.59 mg L⁻¹. The freshwater microcosms were set up in triplicate in 1 L bottles containing 800 mL of freshwater. BAC (CAS No. 139-07-1) with 99% purity was purchased from Sigma-Aldrich Co. LLC. The freshwater microcosms were spiked with BAC at nominal concentrations of 0, 10, 100, 1000 and 10 000 µg L⁻¹. All experiments were carried out outdoors over an interval of seven sequential days without rain (starting on September 5, 2016) to simulate natural exposure. The bottles were open to the environment and the volumes were adjusted every two days with sterilized deionized water.

2.2 DNA extraction

The water samples (800 mL) were pre-filtered through GF/A filters (1.6 µm, Whatman) and collected on polyvinylidene fluoride (PVDF) membrane filters (0.22 µm, Millipore) using a vacuum pump. The total genomic DNA was extracted using E.N.Z.A Water DNA Kit (Omega, USA) and purified by using the GeneClean Spin Kit (QBiogene, Carlsbad, CA) as described by the manufacturer. The quality and concentration of DNA was evaluated by 1% agarose gel electrophoresis and spectrophotometer analysis at 260 nm (NanoDrop ND-2000c, Thermo, USA).

2.3 Quantification of bacteria and functional genes by real-time PCR

Real-time polymerase chain reactions (qPCRs) were performed to determine the total abundance of bacteria (i.e., number of 16S rRNA gene sequences) and functional genes, including quaternary ammonium compound-resistant genes, nitrogen-cycling genes, and antibiotic resistance genes, present in the water samples. Plasmids with targeted genes were constructed as the standards according to the methods of previous literatures { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Twenty microliters of the reaction mixture of qPCR were prepared and carried out in a 96 well plate by the 7500 Fast real-time PCR system (PE Applied Biosystem, USA) according to the manual and protocol of the 7500 Fast real-time PCR system (<https://www.thermofisher.com/order/catalog/product/4351107?SID=srch-srp-4351107>). The primers and cycle conditions of total bacteria and functional genes were shown in Table S1 (See supplementary materials). Melting curve analysis was applied to check the purity of the amplified products and performed for temperatures ranging from 60 to 95°C. The abundances of total bacteria and functional genes were calculated by comparing the threshold cycle (C_t) values of each sample with the standard curve.

2.4 16S rRNA gene sequence analysis

The genomic DNA extracts served as a template for the PCR amplification of the V2-V4 region of 16S rRNA using the primer set 338F/806R (5'-ACTCCTACGGGAGGCAGCAG-3' and 5'-GGACTACHVGGGTWTCTAAT-3') { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. The amplified DNA was subjected to agarose gel electrophoresis and purified using AxyPrep DNA Gel Extraction Kit

389 (Axygen Biosciences, Union City, CA, USA). A mixture of the amplicons was then
 390 used for sequencing on an Illumina MiSeq platform according to the standard
 391 protocols at Majorbio Bioinformatics Technology Co., Ltd. (Shanghai, China). In
 392 brief, raw fastq files were quality-filtered by Trimmomatic and merged by FLASH
 393 with the following criteria: (i) The reads were truncated at any site receiving an
 394 average quality score <20 over a 50 bp sliding window. (ii) Sequences whose overlap
 395 being longer than 10 bp were merged according to their overlap with mismatch no
 396 more than 2 bp. (iii) Sequences of each sample were separated according to barcodes
 397 (exactly matching) and primers (allowing 2 nucleotide mismatching), and reads
 398 containing ambiguous bases were removed. Operational taxonomic units (OTUs)
 399 were clustered with 97% similarity cutoff using UPARSE (version 7.1
 400 <http://drive5.com/uparse/>) with a novel 'greedy' algorithm that performs chimera
 401 filtering and OTU clustering simultaneously { ADDIN EN.CITE
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417 ISI>://WOS:000325073800023</url></related-urls></urls><electronic-resource-
418 num>10.1038/Nmeth.2604</electronic-resource-
419 num><language>English</language></record></Cite></EndNote>}. The taxonomy
420 of each 16S rRNA gene sequence was analyzed by RDP Classifier algorithm
421 (<http://rdp.cme.msu.edu/>) against the Silva (Release128 <http://www.arb-silva.de>) 16S
422 rRNA database using confidence threshold of 70%. RDP classifier work was done
423 within the QIIME environment.

424

425 **2.5 Statistical analysis**

426 The richness and diversity of the bacterial communities within each treatment
427 were calculated with the Chao1 richness index and Shannon diversity index within
428 Mothur software, respectively { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}.
429 Differences in the number of functional genes within treatments were measured with a
430 series of one-way analysis of variance (ANOVA). Heatmap of tested functional genes
431 and microbial composition in surface water and Wilcoxon rank-sum test were
432 performed by ‘gplots’ and ‘clusrank’ packages using R v. 3.2.5 (R Foundation for

Statistical Computing, Vienna, Austria), respectively. The Pearson correlation analysis between different genes and microbial community composition was carried out using SPSS software (IBM Co., USA) and showed by Gephi software v. 0.9.1(Gephi, WebAtlas, France) { ADDIN EN.CITE <EndNote><Cite><Author>Bastian</Author><Year>2009</Year><RecNum>961</RecNum><DisplayText>(Bastian et al., 2009)</DisplayText><record><rec-number>961</rec-number><foreign-keys><key app="EN" db-id="x095edwrr995rve0vtipte09wd9ptz2trr5t" timestamp="1503503495">961</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Bastian, Mathieu</author><author>Heymann, Sebastien</author><author>Jacomy, Mathieu</author></authors></contributors><titles><title>Gephi: an open source software for exploring and manipulating networks</title><secondary-title>Icws</secondary-title></titles><periodical><full-title>Icws</full-title></periodical><pages>361-362</pages><volume>8</volume><dates><year>2009</year></dates><urls></urls></record></Cite></EndNote>}. Functional profiling of microbial communities based on 16S rRNA data was carried out using PICRUSt software { ADDIN EN.CITE { ADDIN EN.CITE.DATA } }.

3. Results

3.1 Effect of benzyldimethyldodecyl ammonium chloride on amount of total bacteria

The number of 16S rRNA copies in natural water decreased with the presence of BAC, even at low amounts (e.g., 10 µg L⁻¹) (Fig. 1). The numbers of 16S rRNA copies for 100, 1000, 10 000 µg L⁻¹ of BAC on the first day were $1.50 \pm 0.04 \times 10^9$, $1.49 \pm 0.13 \times 10^9$, and $1.26 \pm 0.04 \times 10^9$ copies per liter, which were significant lower than that of control with value of $2.06 \pm 0.14 \times 10^9$ copies per liter ($p < 0.05$). The numbers of 16S rRNA copies for all the treatments decreased from first day to the seventh day. There were no significant differences between the numbers of 16S rRNA copies with the 100, 1000, or 10 000 µg L⁻¹ treatments, but the numbers of 16S rRNA copies within these treatments were lower than that of control ($p < 0.05$). Based on these results, the presence of BAC clearly exerted a negative effect on bacteria growth, especially at concentrations of higher than 100 µg L⁻¹.

3.2 Effect of benzyldimethyldodecyl ammonium chloride on abundance of functional genes

The exposure time and concentrations of BAC influenced the abundances of most functional genes at different significant levels ($p < 0.05$, $p < 0.01$, and $p < 0.001$), except the effect of time, and interaction effects of time and concentrations of BAC on the proliferation of *tetM* and *qnrD* (Table 1).

3.2.1 Quaternary ammonium compound-resistant genes and antibiotic resistance genes

Two quaternary ammonium compound-resistant genes (*qacEAI* and *qacA/B*) and six antibiotic resistance genes (*sulI*, *tetA*, *tetM*, *qnrD*, *strA*, and *bla_{CTX-M}*) were assessed in this study (Fig. 1). There was no significant difference between the control

and lowest treatment level ($10 \mu\text{g L}^{-1}$) for the *qacEΔ1*, *sul1*, *tetM*, *qnrD* and *strA* both on the first day and seventh day. The numbers of *sul1*, *tetA* and *tetM* copies increased as the concentration of BAC increased both on the first and seventh day. *qacA/B* exhibited an increase trend from the control to the highest treatment level on the first day, but no significant differences in the numbers of *qacA/B* copies were found between any of the treatments on Day 7 ($p > 0.05$). The effect of BAC on the proliferation of *strA* and *qacEΔ1* showed similar trend. On the first day, a sharp increase in the abundances of *strA* and *qacEΔ1* exposed to BAC was observed at concentration of $1\,000 \mu\text{g L}^{-1}$, but no significant difference was found between the abundances of *strA* and *qacEΔ1* at the treatment levels of 100, 1 000 and $10\,000 \mu\text{g L}^{-1}$ on Day 7 ($p > 0.05$). *qnrD* abundances at highest treatment level of $10\,000 \mu\text{g L}^{-1}$ on the seventh day exhibited significant difference with the control, while no significant differences were found between all the other experimental treatments ($p > 0.05$). The effect of BAC on the abundances of *bla_{CTX-M}* gene increased significantly compared with the control even at the lowest treatment level of $10 \mu\text{g L}^{-1}$ on Day 1 ($p < 0.05$). However, there was no significant difference in the abundances of *bla_{CTX-M}* between the control, 10, 100, or $1\,000 \mu\text{g L}^{-1}$ treatments on Day 7.

3.2.2 Nitrogen-cycling genes

The response of nitrogen-cycling genes exposed to different concentrations of BAC is shown in the Fig. 1. Generally, the abundances of *nifH* and *amoA* showed a decrease trend as BAC concentrations increased on Day 1, but the *nirK* showed an increase trend. However, no significant difference within the abundances of *amoA*

was found between the control and different treatment levels on Day 7. There was no significant difference in the numbers of *nifH* copies between control and the lowest treatment level (10 µg L⁻¹) on Day 1 and Day 7. Yet, on both Day 1 and Day 7, the abundances of *nifH* within the 100, 1000, and 10 000 µg L⁻¹ treatments were significantly lower than that within the control ($p < 0.05$). The abundances of *nirK* on Day 1 exhibited a slow increase from the control to the 1 000 µg L⁻¹ treatment and a sharp increase as BAC concentrations reached 10 000 µg L⁻¹. After seven days, the numbers of *nirK* copies within 1000 and 10 000 µg L⁻¹ treatments were still significant higher than that of the control ($p < 0.05$).

3.3 Effect of benzyldimethyldodecyl ammonium chloride on bacterial community

3.3.1 Bacterial community richness and diversity

The bacterial valid reads obtained from each treatment ranged from 30 963 to 42 619, normalized to 30 900 to compare richness and diversity of bacteria community (Table 2). On Day 1, the control treatment had the highest number of operational taxonomic units (OTUs) with 560, followed by the 10, 100, 1000 and 10 000 µg L⁻¹ treatments. After seven days, the 10 and 100 µg L⁻¹ treatments had the highest number of OTUs (451), followed by the control, 1000 and 10 000 µg L⁻¹ treatments. On Day 1, Shannon diversity indices declined from control to the highest BAC concentration; however, on Day 7, Shannon diversity indices increased as the concentrations increased from the control to 10 000 µg L⁻¹. On Day 7, richness within two high concentration treatments (1 000 and 10 000 µg L⁻¹) remained lower than those of the control, 10 and 100 µg L⁻¹ treatments. There is an obvious difference in the diversity

at Day 1 but not at Day 7, which may be due to the degradation of BAC by the microorganisms as the contact time increased. Evidence showed that the BAC could be mineralized by enriched *Pseudomonas* sp. from returned activated sludge within 300 h (Khan et al., 2015).

3.3.2 Bacterial community structure

The compositions and cluster heatmap of bacterial community exposed to BAC were shown in Fig.2. The bacterial compositions within the 10 and 100 $\mu\text{g L}^{-1}$ treatment between Day 1 and Day 7 as well as the control were similar (group A), higher concentrations (1000 and 10 000 $\mu\text{g L}^{-1}$) treatments were classified in the other group (group B) (Fig. 2b). Bacteria within the class *Cyanobacteria* was the highest in abundance of group A (Fig. 2a). *Sphigobacteriia*, *Betaproteobacteria*, *Phycisphaerae*, *Acidobacteria* and *Alphaproteobacteria* were also important components in group A (Fig. 2a). The group B could be divided into two small cluster. Cluster I including higher concentrations (1000 and 10 000 $\mu\text{g L}^{-1}$) treatments on Day 1, which showed that *Gammaproteobacteria* was important composition of bacterial community in this group. *Flavobacteriia* and *Betaproteobacteria* were also the large proportion of the composition of bacterial community in the 1 000 and 10 000 $\mu\text{g L}^{-1}$ treatment on Day 1, respectively (Fig. 2a). The higher concentrations (1000 and 10 000 $\mu\text{g L}^{-1}$) treatments on Day 7 constituted the Cluster II, in which *Cytophagia*, *Sphigobacteriia*, *Alphaproteobacteria*, and *Betaproteobacteria* were the main composition of bacterial community (Fig. 2a). Genus difference between the group A and group B was analyzed via Wilcoxon rank-sum test (Fig. 3). It is observed that the proportions of

Cyanobacteria, *Microcytis*, *Synechococcus*, unclassified_f_Family, and CL500-3 (*planctomycetes*) in group A were higher than those in group B ($p < 0.05$), indicating these kinds of bacteria were inhibited or killed by high concentrations of BAC. Group B had higher proportion of *Pseudomonas*, *Vogesella* and *Rheinheimera* at $p < 0.1$ level.

3.3.3 Functional profile prediction based on the 16S information

The functional community profiles for each sample based on clusters of orthologous groups (COG) were created (Fig. S1a, Supplementary materials). The heatmap also showed that the functional community profiles were classified also into two groups (Fig. S1b, Supplementary materials), which were the same as those clustered based on the community structure. A significant increase in genes associated with RNA processing and modification, transcription, lipid transport and metabolism, amino acid transport and metabolism, and cell motility was found in group A than group B at $p < 0.05$ via t-test (Fig. 4).

3.4 Correlation analysis between the functional genes and microbial community

The Pearson correlation coefficients between the functional genes and bacterial community composition with p values less than 0.05 were shown in Fig. 5 using Gephi software. *sulI*, *qacEΔI* and *tetM* showed positive significant correlations with most other antibiotic resistance genes. *Cyanobacteria* exhibited significant negative correlations with most antibiotic resistance genes. Bacteria of *Gammaproteobacteria* and *Betaproteobacteria* showed positive correlations with antibiotic resistance genes. *Gammaproteobacteria* and *Betaproteobacteria* showed evidence that they were the

important groups of multi-antibiotic resistance bacteria in surface water of the environment { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Hence, our results also confirmed that shifts in proliferation of antibiotic resistances and microbial community were correlated with each other exposure to BAC.

4. Discussion

4.1 Effect of BAC on functional genes

Selective enrichment of a population, advantageous mutations, and the transfer of ecologically important genes are important mechanisms for the adaptation of microbial communities to toxic pollutants { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. *qacA/B* has been shown to be wide-spread among gram-positive bacteria, such as *Staphylococci*, while *qacEΔ1* has been wide-spread among gram-negative bacteria, especially *Enterobacteriaceae* and *Pseudomonas* { ADDIN EN.CITE

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 592 Gram-negative bacteria were shown to have high insensitivity to antimicrobials
 593 compared with gram-positive bacteria in shallow urban lakes { ADDIN EN.CITE {
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 606 heterotrophic bacterial community in a small eutrophic lake (Priest
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 urls></urls></record></Cite></EndNote>}. In this study, the abundances of *qacEΔ1*
 were 110-1495 times higher than *qacA/B* and proportion of *Pseudomonas* increased at
 high concentrations of BAC compared to low levels of BAC (Fig. 2 and 3). These
 results indicated *qacEΔ1* may play a more direct role in the adaptation of bacteria
 exposed to BAC. Among the antibiotic resistance genes examined, *qnrD* was the most
 insensitive to BAC exposure. Quinolones inhibit the DNA gyrase of bacteria which
 could be protected by the 214-amino-acid pentapeptide repeat protein encoded by
qnrD { ADDIN EN.CITE
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 sterilization mechanisms of BAC and quinolones may account for the insensitivity of
qnrD gene to BAC exposure. An increase of the antibiotic resistance genes encoding
 efflux pump has been found in the long-term exposure of aerobic microbial
 communities within engineered, unnatural systems to BAC { ADDIN EN.CITE
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In this study, not only did the abundance of efflux pump antibiotic resistance genes
increase with increasing concentrations of BAC, but other antibiotic resistance gene
types (e.g., ribosomal protection protein, *tetM*) also increased. The majority of
plasmids carrying both antibiotic resistance genes and biocide (e.g. BAC) /metal
resistance genes (BMRGs) are found to be conjugative (Pal et al., 2015), which may
result in the increase in abundances of different kind of antibiotic resistance genes
exposed to BAC.

The changes in abundances of eleven genes exposure to BAC could be classified
into four groups (Fig. 6). The first group included *tetA*, *qacEΔ1* and *strA*, which
showed higher abundances to BAC exposure compared with the control both on Day
1 and Day 7, indicating the selective pressure of BAC on these genes always existed
during the experimental period. *amoA* and *nifH* constituted the second group, which
exhibited lower abundances to BAC exposure, indicating BAC had negative effect on
the proliferation of these two genes and influenced nitrogen cycle in the aquatic
system. BAC has showed evidence that it initially inhibited the nitrification efficiency

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 antibiotics, which have been proved to have a significant and rapid negative impact on
 the presence of *amoA* in soils { ADDIN EN.CITE
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 798 ></related-urls></urls><electronic-resource-
 799 num>http://dx.doi.org/10.1016/j.aquatox.2013.12.008</electronic-resource-
 800 num></record></Cite></EndNote>}. More research is still needed to investigate the
 801 effect of BAC on the nitrogen cycle based on the changes of nitrogen removal rate
 802 and denitrifer community in the natural water system. The third group composed
 803 *qnrD*, *nirK* and *tetM*. Although *nirK* and *tetM* abundances showed increase at high
 804 concentrations of BAC, a low multiple was observed for *nirK* and *tetM* compared
 805 with *tetA*, *qacEΔ1* and *strA*. *nirK* was not only an important nitrogen cycling gene,
 806 but also played important role in the response to different pollutants, such as

807 wastewater { ADDIN EN.CITE

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 resource-num></record></Cite></EndNote>}. *nirK* may be a multipurpose gene and
 not specificity for pollutants, resulting in slight increase in the abundances exposure

to BAC. Hence, BAC had less effect on the proliferation or spread of these three genes (*qnrD*, *tetM* and *nirK*). *qacA/B*, *sulI* and *bla_{CTX-M}* were clustered as the four group, which showed higher abundances to BAC exposure compared with control on Day 1 but not on Day 7. These results indicated the BAC could exert different selective pressure on the quaternary ammonium compound-resistant genes, antibiotic resistance genes and nitrogen-cycling genes.

4.2 Selective enrichment of specific bacteria exposure to BAC in aquatic system.

Selective enrichment of *Rheinheimera*, *Pseudomonas*, and *Vogesella* was found in the high BAC treatments (Fig. 3), suggesting that these bacteria have resistance or degradation capacity to BAC. In engineered BAC-fed communities, *Pseudomonas* has been identified as the dominant species (over 50%), followed by *Citrobacter* { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. In domestic drain biofilm microcosms, *Falvorbacterium*, *Sphingobacterium*, *Sediminibacterium*, and *Niabella* were also enriched after exposure to BAC { ADDIN EN.CITE <EndNote><Cite><Author>Forbes</Author><Year>2017</Year><RecNum>1183</RecNum><DisplayText>(Forbes et al., 2017)</DisplayText><record><rec-number>1183</rec-number><foreign-keys><key app="EN" db-id="vzededvvherd97ep2db5pwr1fe5trtad95r0" timestamp="1491791240">1183</key></foreign-keys><ref-type name="Journal Article">17</ref-type><contributors><authors><author>Forbes, Sarah</author><author>Cowley, Nicola</author><author>Humphreys, Gavin</author><author>Mistry, Hitesh</author><author>Amézquita,

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 num></record></Cite></EndNote>}. These highlights similarities and differences in
 the response of microbial diversity in natural lake water and engineered systems. The
 occurrence of enriched *Pseudomonas* spp. communities after the introduction of
 quaternary ammonium compounds has previously been observed by U. Tezel et al {
 ADDIN EN.CITE { ADDIN EN.CITE.DATA }}. Additionally, *Rheinheimera*
 species isolated from freshwater culture pond and sea sediment exhibited
 antimicrobial activity { ADDIN EN.CITE { ADDIN EN.CITE.DATA }}.
Flavobacterium and *Pseudomonas* species from natural and clinical environments
 have been proven to contain antiseptic-resistance genes *qacE* and *qacEΔ1* { ADDIN
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 918 urls><url>https://www.ncbi.nlm.nih.gov/pubmed/9503610</url></related-
 919 urls></urls></record></Cite></EndNote>}. *Vogesella* has seldom been reported to be
 920 related with exposure of BAC, but along with *Pseudomonas* and *Flavobacterium*, was
 921 prominent in the toxic organic pollutants (pyrene and benzo[a]pyrene) removed from
 922 lake sediments { ADDIN EN.CITE
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 942 `tary-information</electronic-resource-num></record></Cite></EndNote>}`.

943 **4.3 Effect of BAC on microbial community and function**

944 Microbial abundance and diversity were affected by the varying concentrations
 945 of BAC. Our study showed that within water samples exposed to the highest BAC
 946 concentrations (i.e., 10 000 µg L⁻¹) microorganism abundance decreased by 38.72%
 947 on Day 1 (Fig. 1). The EC₅₀ of BAC for these microbial communities, based on
 948 quantitation of 16S rRNA, in natural water from eutrophic lake was more than 10 000
 949 µg L⁻¹. The acute toxicity on *Photobacterium phosphoreum* obtained an EC₅₀ in the
 950 range of 0.1-1 mg L⁻¹ for both alkyl trimethyl ammonium halides (ATMAC C12-16)
 951 and alkyl benzyl dimethyl ammonium halides (BAC C12-16) { ADDIN EN.CITE
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 973 ></related-urls></urls><electronic-resource-num>http://doi.org/10.1016/S0269-
 974 7491(99)00322-X</electronic-resource-num></record></Cite></EndNote>}. The
 975 difference in the resistance of quaternary ammonium compounds for *Photobacterium*
 976 *phosphoreum* and the total microbial community in the natural environment may be
 977 due to the ability of some microbes to tolerate and degrade quaternary ammonium
 978 compounds. Microbial communities exposed to quaternary ammonium compounds in
 979 engineered system have been characterized by a versatile repertoire of antibiotic
 980 resistance genes and cell envelope modification systems { ADDIN EN.CITE {
 981 ADDIN EN.CITE.DATA }}. The transcriptome analysis of *Listeria monocytogenes*
 982 exposed to quaternary ammonium compound benzethonium chloride revealed cell

983 wall synthesis, sugar uptake, and motility were involved in the response { ADDIN
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 996 involving cell wall synthesis, sugar uptake, and motility</title><secondary-
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1005 urls></urls><electronic-resource-num>10.3389/fmicb.2014.00068</electronic-
 1006 resource-num><remote-database-name>PMC</remote-database-
 1007 name></record></Cite></EndNote>}. Energy production, amino acids, carbohydrates
 1008 and lipids metabolism were involved in the multiple adaptive routes of *Salmonella*
 1009 *enterica* for stress of biocide and antibiotic { ADDIN EN.CITE
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 resource-num></record></Cite></EndNote>}. Our results firstly indicated that the
 functional profiles of aquatic microbial community involved in the adaption process
 to BAC stress, such as genes related with RNA processing and modification,
 transcription, lipid transport and metabolism, amino acid transport and metabolism,
 and cell motility.

4.4 Shift pattern in proliferation of functional genes and microbial community

The functional genes within aquatic microbial community seemed to be more
 sensitive to BAC exposure. Most of the genes examined in this study, such as *qacEΔ1*,
sull, *tetM*, *strA*, *nifH* and *nirK*, exhibited significantly different abundances in the
 treatments with over 100 μg L⁻¹ BAC on Day 1, compared to the abundances of these
 genes in the control. The abundances of *bla_{CTX-M}*, *tetA*, *amoA* and *qacA/B* in the 10 μg
 L⁻¹ treatment on Day 1 were significantly different compared with control. Based on
 the results of functional genes and microbial diversity, the changes in abundances of
 functional genes exposure to BAC at lower concentrations were observed before
 significant changes in microbial community compositions occurred. This may be due
 to the limitation of 16S rRNA gene sequence method. Although 16S rRNA gene
 sequence can exhibit biases by amplifying species unequally and also capture a

broader range of microbiome diversity, a lower sensitivity and resolution existed for this method { ADDIN EN.CITE

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title></titles><pages>165-
176</pages><dates><year>2011</year></dates><urls></urls></record></Cite></En
dNote>}. This work using qPCR and 16S rRNA gene data analyzed the changes in
abundances of specific functional genes and microbial diversity and function exposed
to BAC, which were summarized in the Fig. 7 as following: (1) BAC could influence
the level of specific functional genes even at low level of BAC (10 µg L⁻¹), such as
bla_{CTX-M} and *tetA*; (2) specific bacterial species were enriched due to the stress of
BAC, such as *Rheinheimera*, *Pseudomonas*, and *Vogesella*; (3) changes in microbial
diversity and function were found significantly at high levels of BAC. The

1071 concentrations of BAC and QACs in most studied surface water were less than 20 μg
 1072 L^{-1} and 100 $\mu\text{g L}^{-1}$, respectively { ADDIN EN.CITE
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 num><http://doi.org/10.1016/j.talanta.2013.04.083></electronic-resource-
 num></record></Cite></EndNote>}. Combined with the discovery of this study, it
 could conclude that environmental concentrations of BAC did not obviously influence
 the aquatic microbial composition and function, only affected the proliferation of
 specific functional genes. The specific species enriched gave light for isolating
 bacteria degrading the BAC from natural environment. qPCR and 16SrNA used in
 this study showed that BAC could shifts the proliferation of specific functional genes
 and microbial community at DNA level. Further studies are still needed to identify the
 main pathways of BAC and key players in the nutrient cycling influenced by BAC in
 aquatic ecosystems, using metatranscriptome at RNA level or functional
 metaproteomic approach at protein level.

5. Conclusion

In this study, BAC was applied to discover its effect on abundances of functional
 genes and microbial diversity. Changes within important functional genes in natural
 water exposed to BAC were different dependent on the gene type, concentrations of

1137 BAC and exposure time. High concentrations of BAC more than 1 000 $\mu\text{g L}^{-1}$
1138 significantly influenced the microbial diversity and community composition. Low
1139 concentrations had significant influence on the abundances of specific genes but less
1140 effect on microbial composition. The changes of BAC transformation and nutrients
1141 were not recorded in this study, hence, metaproteomic and metatranscriptomic may be
1142 needed to discover relationship between the key microbial species and pathway of
1143 BAC in the aquatic microbial ecosystem in the further research.

1144 **Acknowledgements**

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1147 Academy of Sciences [grant number 2015282].

1148 **Conflicts of interest**

1149 none

1150 **References**

1151 { ADDIN EN.REFLIST }

1152 Table 1 Statistically significant differences of functional genes based on two-way ANOVA

Variables	<i>qacΔE1</i>	<i>qacA/B</i>	<i>sulI</i>	<i>tetA</i>	<i>tetM</i>	<i>qnrD</i>	<i>strA</i>	<i>blaTXM</i>	<i>amoA</i>	<i>nifH</i>	<i>nirK</i>
Time	**	***	***	*	ns	ns	***	***	***	***	***
Concentrations	***	**	***	***	***	*	***	***	***	***	***
Time × Concentrations	***	*	**	***	ns	ns	***	**	***	**	**

1153 **p* <0.05; ***p* <0.01; ****p* <0.001;

1154

1155 Table 2. Bacterial community diversity in water microcosms exposed to BAC after
 1156 one day (Day 1) and seven days (Day 7).

Concentration of BAC	Day 1			Day 7		
	OTUs	Chao1	Shannon	OTUs	Chao1	Shannon
Control (0)	560	589.5	3.48	376	460.4	2.51
10	520	553.5	2.81	451	509.9	2.82
100	513	586.1	2.83	451	497.5	2.80
1 000	250	325.0	2.59	366	411.9	3.47
10 000	154	201.5	2.14	256	279.6	3.98

1157

Figure captions

Fig. 1 The copies of 16S rRNA, quaternary ammonium compound-resistant genes (*qacEΔ1* and *qacA/B*), antibiotic resistance genes (*sul1*, *tetA*, *tetM*, *qnrD*, *strA*, and *bla_{CTX-M}*) and nitrogen cycling related genes (*amoA*, *nifH* and *nirK*) in water microcosms exposed to BAC after one day (1 d) and seven days (7 d). Different letters over the bars indicate statistically significant differences at $p < 0.05$ level in One-way ANOVA.

Fig. 2 Microbial community composition at the level of the Class in water microcosms exposed to BAC after one day (D 1) and seven days (D 7) (a), and clusters were analyzed using heatmap (b). The number after D1 and D7 means the concentrations of BAC.

Fig. 3 Difference of genus in the two groups was analyzed using Wilcoxon rank-sum test responded to exposure of BAC.

Fig. 4 The difference between the specific functional profiles of microbial community based on 16S sequence at $p < 0.05$ level between group A and Group B.

Fig. 5 The Pearson correlation between the functional genes and microbial composition using Gephi software. The p values showed in the figure were all less than 0.05. Lines with pink color indicated negative correlation, and lines with green color indicated positive correlation.

Fig. 6 The heatmap of quaternary ammonium compound-resistant genes, antibiotic resistance genes and nitrogen-cycling genes exposure to different concentrations of BAC after one day (D 1) and seven days (D 7). (The log values for each gene were normalized to the corresponding log values of D1_control).

1181 Fig. 7 The schematic map of shifts in proliferation of functional genes and microbial
1182 community influenced by BAC stress.