Journal of Water & Health

Journal of Water and Health Vol 19 No 6, 895 doi: 10.2166/wh.2021.171

Short-term exposure to benzalkonium chloride in bacteria from activated sludge alters the community diversity and the antibiotic resistance profile

Luz Chacón [a,*], Maria Arias-Andres [b], Freylan Mena [b], Luis Rivera [a], Lorena Hernández[c], Rosario Achi [a], Fernando Garcia[d] and Keilor Rojas-Jimenez [e]

[a] Instituto de Investigaciones en Salud (INISA), Universidad de Costa Rica, P.O. Box: 11501-20160, San José, Costa Rica
[b] Instituto Regional de Estudios en Sustancias Tóxicas (IRET), Universidad Nacional, Campus Omar Dengo, P.O. Box 86-3000, Heredia, Costa Rica
[c] Centro de Investigación en Productos Naturales (CIPRONA), Universidad de Costa Rica, P.O. Box: 11501-20160, San José, Costa Rica
[d] Centro de Investigación en Enfermedades Tropicales (CIET), Universidad de Costa Rica, P.O. Box: 11501-20160, San José, Costa Rica
[e] Escuela de Biología, Universidad de Costa Rica, P.O. Box: 11501-20160, San José, Costa Rica
*Corresponding author. E-mail: luz.chacon@ucr.ac.c.cr

LC, 0000-0003-2506-0619; MA, 0000-0002-4678-765X; FM, 0000-0002-1215-0378; LR, 0000-0001-8739-7466; RA, 0000-0002-4662-3073; KR, 0000-0003-4261-0010

ABSTRACT

The continuous introduction of cleaning products containing benzalkonium chloride (BAC) from household discharges can mold the microbial communities in wastewater treatment plants (WWTPs) in a way still poorly understood. In this study, we performed an *in vitro* exposure of activated sludge from a WWTP in Costa Rica to BAC, quantified the changes in *intI1*, *sul2*, and *qacE/qacEΔ1* gene profiles, and determined alterations in the bacterial community composition. The analysis of the qPCR data revealed elevated charges of antibiotic resistance genes in the microbial community; after BAC's exposure, a significant increase in *the qacE/qacEΔ1* gene, which is related to ammonium quaternary resistance, was observed. The *16S rRNA* gene sequences' analysis showed pronounced variations in the structure of the bacterial communities, including reduction of the alpha diversity values and an increase of the relative abundance of Alphaproteobacteria, particularly of *Rhodospseudomonas* and *Rhodobacter*. We confirmed that the microbial communities presented high resilience to BAC at the mg/mL concentration, probably due to constant exposure to this pollutant. They also presented antibiotic resistance-related genes with similar mechanisms to tolerate this substance. These mechanisms should be explored more thoroughly, especially in the context of high use of disinfectant.

Key words: activated sludge, antimicrobial resistance, bacterial community, benzalkonium chloride, wastewater treatment

HIGHLIGHTS

- *In vitro* BAC's exposure enhances qacE/qacEΔ1 gene presence in bacterial communities from AS.
- AR related genes in AS's microbial communities from tropical countries are reported.
- The BAC exposure can alter the AS microbial community composition.
- Putative nitrifiers are enhanced by BAC's exposure.
- The presence of intI-1 gene in AS could indicate the anthropogenic spread of microbial resistance into the environment.

INTRODUCTION

The use and release of antimicrobial substances into the environment from urban uses (houses, hospitals, factories, for example) and agricultural activities (horticulture, aquaculture, and livestock production) cause concern and require urgent attention. Large amounts of disinfectants were released into the environment before the COVID-19 pandemic. Over 450,000 kg per year of cleaning products, as quaternary ammonium compounds (QAC), were manufactured or imported alone in the USA (Hora *et al.* 2020). Among the QACs, benzalkonium chloride (BAC) is the most common surfactant, composed of a mix of chlorides of alkyldimethylbenzylammonium. The primary mechanism of action involves a general perturbation of lipid bilayers of membranes leading to a generalized and progressive leakage of cytoplasmic materials to

the environment (Gilbert & Moore 2005). Owing to its action mechanism, BAC is one of the most recommended disinfectants against the SARS-CoV-2 virus (US Environmental Protection Agency 2020).

BAC concentration in domestic wastewater treatment plants (WWTPs) was estimated at 9.9 μg/L, and 2.2–2.8 ml/L in effluents from hospitals (Martínez-Carballo *et al.* 2007), but currently, higher BAC concentrations are expected due to the COVID-19 pandemic (Hora *et al.* 2020). Biodegradation and adsorption are the major removal pathways at WWTPs, but some studies have revealed incomplete degradation and complete compound adsorption in the activated sludge (Clarke & Smith 2011). The activated sludge (AS) constitutes the principal agent used for the biological purification of industrial and domestic effluents; some AS functions include nitrogen fixation, nitrification, ammonification, and other biochemical processes (Gernaey & Sin 2008). Also, the microbial structure of AS influences receiving water bodies (Numberger *et al.* 2019). The microbial composition of each AS is shown to be specific for each geographical area and the organic substrates of each sewage; however, the main phylum found in AS is *Proteobacteria,* followed by the phyla *Firmicutes, Bacteroidetes*, and *Actinobacteria* (Zhang *et al.* 2012).

Some studies in temperate regions have shown that the environmental concentrations of BAC can alter the microbial communities from activated sludge and interfere with the depuration process; concentrations higher than 2.0 mg BAC per gram of solids inhibited enzyme activity, and a long-term exposure reduced the microbial community diversity and selected for BAC-resistant bacteria as *Pseudomonas* (Chen *et al.* 2018). A recent review about disinfectant resistance includes *qac* genes as important efflux pumps that can export harmful molecules as disinfectants and antibiotics outside the bacterial cell. These genes might be either chromosomally encoded or in plasmids and can be eventually transferred to other bacteria by conjugation and transduction process and are involved in the spread of antibiotic resistance genes in the environment. Moreover, disinfectant and antibiotic resistance genes can be integrated by site-specific recombination in an *aatI* site of class I integrons, composed by an integrase gene (*intI*) followed by disinfectant (*qacE*), and antibiotic resistance genes as *sul* family genes (McCarlie *et al.* 2020). The WWTPs receive disinfectants, antibiotics, and heavy metals; they generate a selection pressure for antibiotic-resistant microorganisms. For this reason, they are well recognized as hot spots of antibiotic resistance spread (Karkman *et al.* 2018).

In Costa Rica, the most common system for wastewater treatment is AS, since tertiary or quaternary disinfection steps are not mandatory in national legislation (Ruiz Fallas 2012; Mora-Alvarado & Portuguez-Barquero 2016). This study explores the impact of a higher BAC exposure to a bacterial community from activated sludge from a municipal WWTP in Costa Rica, regularly exposed to low BAC concentrations. Additionally, we analyzed changes in bacterial composition (using *16S rRNA* gene sequencing) and the antibiotic resistance genes load (quantitative PCR detection of *intI1, sul2*, and *qacE/qacEΔ* genes).

MATERIALS AND METHODS

Reagents

Benzalkonium chloride (BAC) (≥95.0% Fluka 12060 Sigma), methanol (MeOH) (>99.8% grade HPLC, Lot 1687318324, Merck 1.06018.4000 (DS228)), hydrochloric acid (HCl) (37% Merck GR 37.2500 133 K16502817), dimethanechloride (DMC) (Merck 1.06054.4000, Lot 1584154 114, G.C. grade, purity >99.8%), sodium sulfate (NaSO4), acetonitrile (ACN), methanol and formic acid, Optima LC-MS grade was purchased from Fisher Chemical, and water was purified by using a Thermo Scientific system (OH, USA). The benzalkonium chloride (BAC) standard was obtained from Sigma Aldrich (≥95.0% Fluka 12060).

Experiment design and sampling

As a representative of the most common type of WWTPs in Costa Rica, we selected a small size residential plant (serving less than 4,000 inhabitants, see operative details in the supplementary material S1) located in the Costa Rican Central Valley (9°55′14″N, 84°14′34″W, 1,400 m above sea level). We collected a sample of the AS (4 L) from an aeration tank (4 m deep) using a metal bucket at approximately 50 cm of depth. The sample was then transferred to a sterile amber glass container, kept at a temperature of 4 °C, and immediately transported to the laboratory. Later, the sample was divided into three portions corresponding to three treatments. The first portion (T0) was frozen immediately and used as a baseline. For replication purposes, the second portion (T1) was homogenized, distributed in 3 aliquots of 500 mL in Erlenmeyers of 1 L, and kept at 20 °C (environmental temperature) with aeration for 12 hr until the enrichment test was performed. T1 sample was enriched with a nominal concentration of 10 mg BAC/L; for the sludge dosing, we used a solution of 100 mg/L of BAC diluted with ultrapure water. For dosed concentration and exposition time selection, previous pilot studies were carried

out using oxidation substrates rates' changes as a parameter for measuring global BAC-induced variations in bacterial community behavior (data are not shown). The three containers were enriched and incubated with aeration at a temperature of 20 °C for 96 hr. The third portion (T2) was used as process control, and followed the same protocol as T2 except that it did not have enrichment with BAC. The general experiment procedure is described in Figure 1.

BAC extraction and quantification

BAC extraction was carried out from all samples enriched (T1) and without dosing (T0 and T2). Twenty mL (triplicates) of each sample were filtered using vacuum equipment, retaining the solids with a 47 mm fiberglass filter (VWR). Further details of the extraction are described in the supplementary material. The last concentration step was carried out under N_2 flow. The residue was re-suspended in 1 mL of Acer (these tubes had been previously weighed and were weighed again after adding the Acer). The extracts were filtered through a PDVF syringe of 0.22 μm and transferred to previously weighed vials. For BAC quantification, all samples were analyzed using an Acquity Ultra Performance Liquid Chromatography system (UPLC), consisting of Waters Acquity binary solvent manager, autosampler, and Photodiode Array Detector (PDA) coupled with a Quadrupole Time of Flight (Q-ToF) (Waters Synapt G1), (Waters Corp., Milford, MA, USA) in series. The details of the chromatographic process are described in the supplementary material. The three BAC homologs were identified according to their retention time and high-resolution molecular mass determination, and comparisons with reference standards were done.

Molecular analyses

Three independent DNA extractions were performed from enriched sludge (T1) and the non-enriched control sludge (T2) samples after 96 hr experiment, and each of the three sub-samples was analyzed (T1 n=9 and T2 n=9). Also, six independent DNA extractions were carried out to the baseline sludge (T0 n=6). All DNA extractions (T0, T1, and T2 samples) were conducted in parallel. We used the NucleoSpin® Tissue Kit (Macherey Nagel®, Germany) to extract DNA, following the manufacturer's protocol using 1 mL of sludge sample previously homogenized. The V1–V3 regions of the *16S rRNA* gene were sequenced using primers 27F and 518R primers with a Roche GS FLX. Sequencing services were provided by Macrogen (Seoul, Republic of Korea).

For antibiotic resistance gene assays, quantitative PCR (qPCR) for *qacE/qacEΔ1, intI1, sul2*, and *16S rRNA* were assessed using StepOnePlus™ Real-Time PCR thermocycler (Thermo Fisher, USA). Standard calibration curves were carried out using

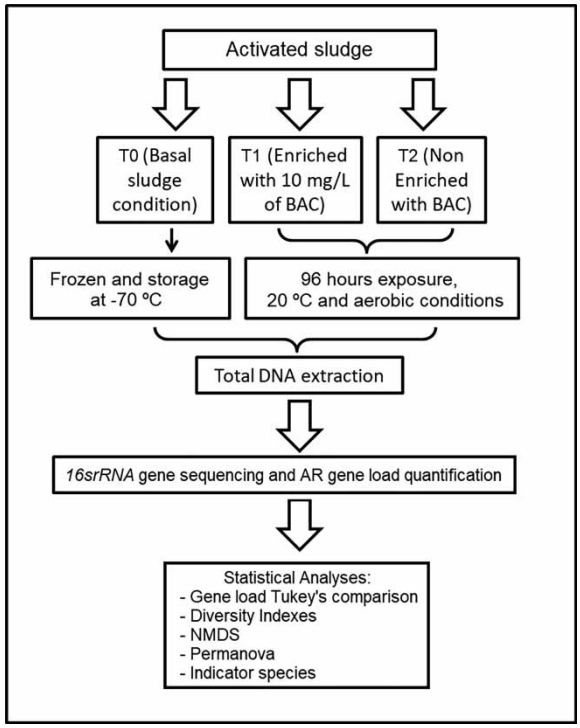

Figure 1 | Flow chart of experimental procedures followed with activated sludge samples.

each gene's purified, quantified amplicon as previously described (Di Cesare *et al.* 2013). Each gene amplicon was visualized by electrophoresis (60 min at 60 V, 1.5% agarose gel), the amplicon extraction was carried out with NucleoSpin Gel PCR Clean-Up (Macherey-Nagel, USA) according to the manufacturer's instructions and quantified by NanoDrop 2000c spectrophotometer (ThermoFisher, USA). With the absolute quantity of DNA for each amplicon, a gene copy number estimation was conducted using the theoretical molecular mass of each amplicon sequence, according to the sequences deposited in the NCBI database. Multi-resistant *Escherichia coli* PTA-A0653-2 (GeneBank WAAM01000041.1) was used as a positive control for all studied genes. For qPCR assays, PowerUp™ SYBR™ Green Master Mix (Applied Biosystems™, USA) was used according to the manufacturer's instructions, 5 μL of DNA of each sample was used. The qPCR program, primers, primers' concentration, and expected amplicon are detailed in Table 1. Melt curve analysis was performed from 60 °C to 95 °C with a continuous increment of 3% °C. Efficiencies and R^2 averages for the tested genes were 90.78% and 0.999 for *qacE/qacEΔ1*, 80.81% and 0.989 for *intI1*, 96.02% and 0.992 for *sul2*, and 103.64% and 0.976 for *16S rRNA*. The limits of quantification for each gene were the minimum concentration detected with a standard linear curve (Di Cesare *et al.* 2015), for all assays were ten copies μL^{-1}. The abundances of the different genes were expressed as gene copies mL^{-1}.

Bioinformatic analyses

The *16S rRNA* gene sequences were paired and quality filtered using Mothur v1.39.5 (Kozich *et al.* 2013). Subsequent processing was performed with the SILVA NGS v1.3 pipeline (Quast *et al.* 2013), including the alignment against the SILVA SSU rRNA SEED using SINA v1.2.10 (Pruesse *et al.* 2012), operational taxonomic unit (OTU) clustering at a 0.03 distance cut-off with Cd-hit v3.1.2 (Li & Godzik 2006), and taxonomic classification by local nucleotide BLAST search against SILVA SSU Ref dataset 132 using blastn (Camacho *et al.* 2009). This process resulted in 274,368 bacterial sequences (sample average=23,833, range=5,522 to 27,966).

Statistical analyses

The statistical analyses and visualizations were performed in R (R Core Team 2019). We used Vegan (Oksanen *et al.* 2019) to calculate alpha diversity estimators (richness and Shanon), the non-metric multidimensional scaling analyses (NMDS), and to perform the permutational analysis of variance (Permanova) on OTU tables normalized to the relative abundance of each sample. Indicator Species package (De Cáceres 2013) was used for indicator species analysis. Additionally, to analyze differences between the abundance of each studied gene and diversity indexes, a Kruskal–Wallis non-parametric test was done. Previously, each antimicrobial resistance gene load was normalized by *16S rRNA* gene load to obtain a relative gene load used in the Kruskal–Wallis test (Thorsten 2021).

Table 1 | Primers and conditions for qPCR reactions

Gene	Primer	Product size (bp)	Primer concentration (nM)	Cycling conditions (40 cycles)	Reference
16S	Bact1369F: 5′-CGGTGAATACGTTCYCGG-3′; Prok1492R: 5′-GGHTACCTTGTTACGACTT-3′	142	500	Initial denaturation: 95 °C, 2 min; Denaturalization: 95 °C, 15 sec; Annealing: 55 °C, 60 sec	Di Cesare *et al.* (2015)
intI1	intI1LC1: 5′-GCCTTGATGTTACCCGAGAG-3′; intI1LC5: 5′-GATCGGTCGAATGCGTGT-3′	196	500	Initial denaturation: 95 °C, 2 min; Denaturalization: 95 °C, 15 sec; Annealing: 60 °C, 60 sec	Barraud *et al.* (2010)
sul(II)	SulIIIF: 5′-TCCGGTGGAGGCCGGTATCTGG-3′; SullIIR: 5′-CGGGAATGCCATCTGCCTTGAG-3′	191	500	Initial denaturation: 95 °C, 2 min; Denaturalization: 95 °C, 15 sec; Annealing: 60 °C, 60 sec	Pei *et al.* (2006)
qacE/qacEΔ1	qacEF: 5′-GGCTTTACTAAGCTTGCCCC-3′; qacER: 5′-CATACCTACAAAGCCCCACG-3′	189	500	Initial denaturation: 95 °C, 2 min; Denaturalization: 95 °C, 15 sec; Annealing: 55 °C, 60 sec	Szczepanowski *et al.* (2009)

RESULTS

We confirmed the presence of BAC in the three types of samples of analyzed AS (treatments T0, T1, and T2). The background concentration of the compound in the sludge before treatment (T0) was 1.46 mg/L, and after 96 hr incubation (T2) the concentration was 3.14 ± 1.5 mg/L. The BAC concentration of the enriched sample (T1) was 14.19 mg/L immediately after exposure, and after 96 hr of exposure, the concentration decreased to 2.27 ± 0.67 mg/L.

Significant changes occurred in antibiotic resistance-associated genes present in the sludge after treatment with BAC (Figure 2, Table S1, supplementary material). *qacE/qacEΔ1* was significantly higher in the treated sludge (T1) compared to the T0 and T2 (ρ=0.0066). Conversely, the load of *intI1* was higher in the original sludge (T0) in comparison with both the samples that were exposed to BAC (T1) and the laboratory experiment control (T2) (ρ=0.0096). No differences in *sul2* gene load were found among samples (ρ=0.8061).

The microbial composition analyses identified 1,127 bacterial OTUs from the 274,368 sequences analyzed. In general, Proteobacteria was the most abundant phylum in the sludge samples analyzed, representing around 53% of the sequences. This group was followed by Bacteroidetes (11%), Chloroflexi (7%), Planctomycetes (7%), and Acidobacteria (3%), while nearly 10% of the bacterial sequences could not be assigned to any phylum (Figure 3). Within Proteobacteria, Alphaproteobacteria represented 26.6% of the total sequences while Gammaproteobacteria represented 23.7% and Deltaproteobacteria 2.7%. Some differences can be observed between each treatment (Table 2). For example, in dosed samples (T1 treatment), the presence of Alphaproteobacteria is higher than other treatments; meanwhile, other phyla such as Bacteroidetes, Patescibacteria, and Planctomycetes decreased.

Richness diversity index revealed a significant diversity decrease between the baseline sludge (T0, 362 ± 53) and the samples from the controlled exposure T1 (259 ± 41) and T2 (263 ± 62) (ρ=0.0129). Shannon diversity presents a tendency to decrease between treatments, T0 (4.64 ± 0.05), T1 (4.59 ± 0.07), and T2 (4.54 ± 0.10); however, non-statistical differences were found (ρ=0.1062). In this context, the application of indicator species analysis corroborated differences between the three sample groups (Table 3). For example, T1 samples only present *Methyloversatilis* sp. as indicator species of the BAC exposure, T2 samples showed *Aminobacter* sp. as an indicator, and T0 samples showed high specie number. Table 3 summarizes the identified indicator species.

Finally, NMDS analysis showed differences between the three analyzed microbial communities. The samples within the experimental treatments clustered together more than the baseline sample, but still T0 (baseline community), T1 (enriched with 10 mg BAC/L), and T2 (non-enriched control) were separated from each other (Figure 4). The clustering was consistent with the Permanova analysis, which determined significant differences (p=0.001, α=0.001) between the three treatments, showing the impact of BAC exposure on the structure of the bacterial communities. Concomitantly, we also observed a reduction in the values of α-diversity indexes in both T1 and T2 compared to the baseline community (Figure 5).

DISCUSSION

The study results showed that BAC concentration in activated sludge samples induced changes in the antibiotic resistance-associated genes studied. It is worth mentioning that BAC was detected in the baseline samples (T0) indicating this compound's frequent and extensive use. A significant increase in the copy load of *qacE/qacEΔ1* was determined in the samples exposed to BAC (T1, Figure 2) concerning the other treatments. This gene copy increase suggests a specific selection process by the presence of BAC. Previously, *qacE/qacEΔ1* was related to resistance to quaternary ammonium compounds as it encodes for an efflux pump of the SMR family (Bay *et al.* 2008). This gene usually is present in mobile genetic elements such as integrons and plasmids (Chuanchuen *et al.* 2007). Accordingly, other studies have shown a similar response in microbial communities exposed to BAC (Kim *et al.* 2018; Yang & Wang 2018).

Concerning the *intI1* gene, its load was higher in T0 samples (baseline), whereas the *sul2* gene did not show differences between treatments; although class 1 integrons carrying *intI1* gene are usually associated with *sul* genes conferring resistance to sulfonamides. There are some differences among the *sul* genes: *sul1* usually is linked to other antibiotic resistance genes in class 1 integrons, while *sul2* is generally located on small nonconjugative or large transmissible multi-resistance plasmids (Sköld 2000; Antunes *et al.* 2005). The prevalence of *sul* genes may explain, at least partially, the results observed in this study. In addition, other factors should be considered for our findings, including: (1) the reduction in the diversity of microbial communities could be related to the reduction of the antibiotic resistance gene loads; (2) the surviving mechanisms related to

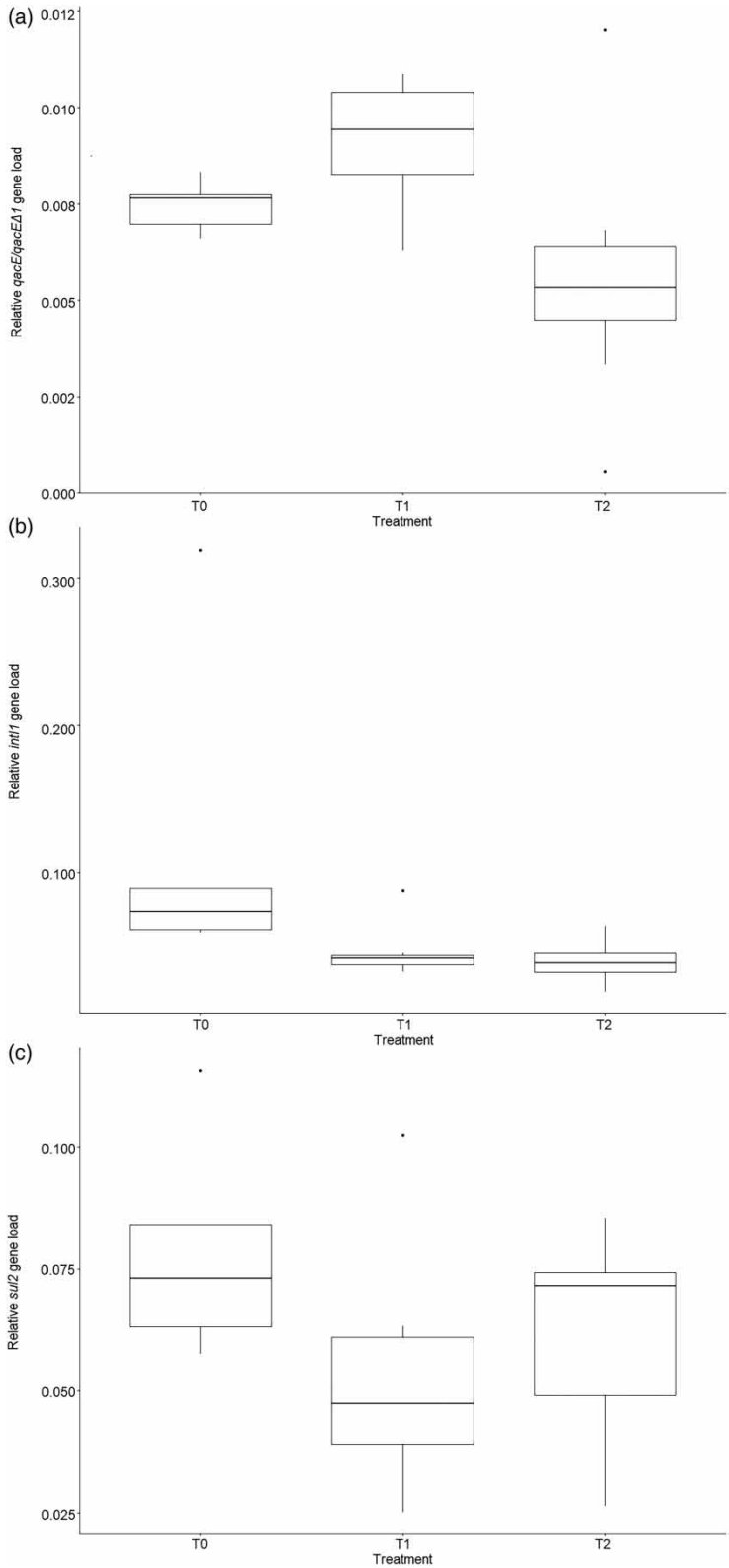

Figure 2 | Gene load boxplot for T0 samples (baseline samples), T1 samples (enriched AS with BAC), and T2 samples (non-enriched control in lab conditions). (a) Corresponds to *qacE/qacEΔ1* gene, (b) to *intI1* gene, and (c) to *sul2* gene.

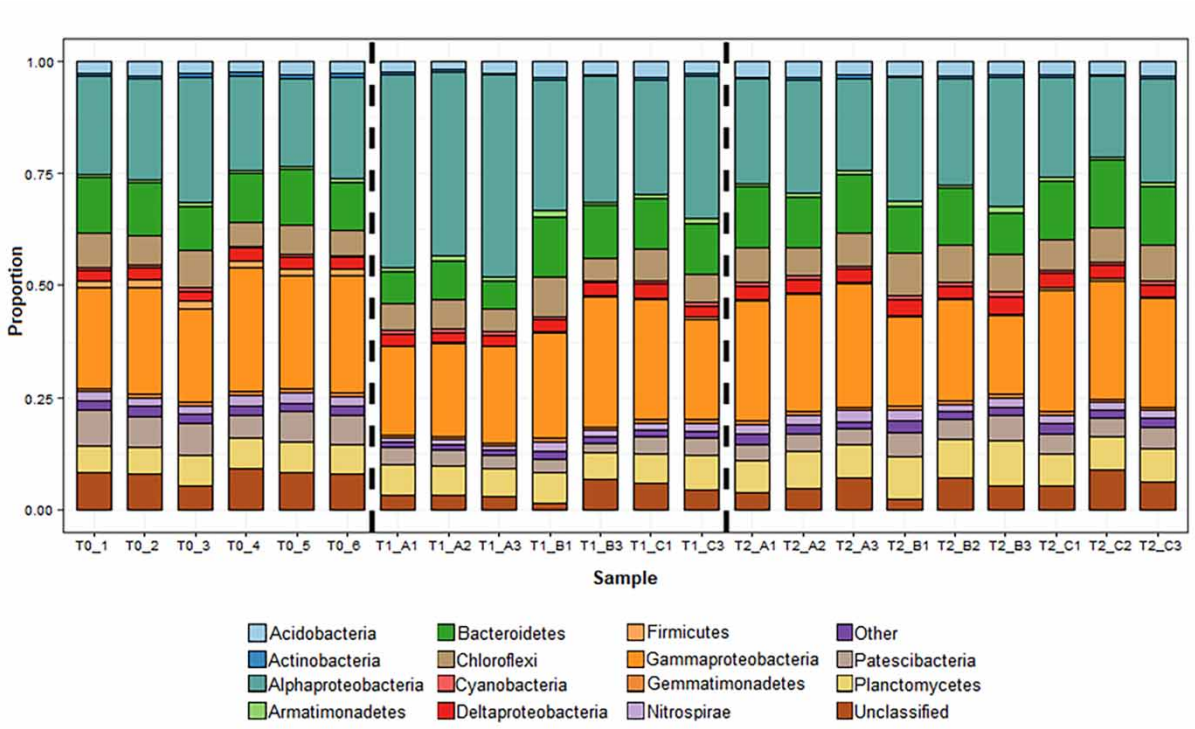

Figure 3 | Major groups' composition from T0 samples (baseline samples), T1 samples (enriched AS with BAC), and T2 samples (non-enriched control in lab conditions). The number indicated after the treatment indication corresponds to an analyzed replica of each treatment.

Table 2 | Most abundant genera in activated sludge samples: before BAC enrichment (T0), after 96 hr exposure to BAC (T1), and non-exposed control at laboratory conditions (T2)

		T0 (n=6)		T1 (n=7)		T2 (n=9)	
Genera	**Group**	**RA**	**SD**	**RA**	**SD**	**RA**	**SD**
JGI 0001001-H03	Acidobacteria	0.69	0.10	1.06	0.34	1.29	0.12
Kouleothrix	Chloroflexi	2.55	0.59	2.20	0.68	2.92	0.69
Nitrospira	Nitrospirae	2.05	0.21	1.44	0.39	2.01	0.33
SM1A02	Planctomycetes	0.91	0.10	1.64	0.70	1.42	0.33
Dongia	Alphaproteobacteria	1.80	0.35	3.14	0.65	2.32	0.51
Methylorosula	Alphaproteobacteria	0.51	0.20	1.13	0.27	0.62	0.13
Hyphomicrobium	Alphaproteobacteria	0.73	0.13	2.42	0.59	2.00	0.43
Bradyrhizobium	Alphaproteobacteria	0.76	0.31	1.61	1.00	0.65	0.16
Rhodopseudomonas	Alphaproteobacteria	0.82	0.14	1.78	1.09	1.19	0.44
Rhodobacter	Alphaproteobacteria	0.85	0.20	1.38	0.26	0.81	0.05
Ideonella	Gammaproteobacteria	0.44	0.10	1.35	0.57	0.74	0.33
Dechloromonas	Gammaproteobacteria	0.64	0.11	2.54	1.62	4.62	1.83
OM60(NOR5) clade	Gammaproteobacteria	1.05	0.24	0.69	0.32	1.08	0.37
Arenimonas	Gammaproteobacteria	3.0	0.80	1.79	0.39	2.28	0.41

Table 3 | Indicator species identified by treatment

Treatment	Indicator OTU	*p* value
T0 (Baseline)	*Roseburia* sp.	0.001***
	Dorea sp.	0.001***
	Bacteroides sp.	0.001***
	Prevotella 9 sp.	0.001***
	Rivicola sp.	0.001***
	Balutia sp.	0.001***
T1 (Enriched with BAC)	*Methyloversatilis* sp.	0.001***
T2 (Non-enriched control)	*Aminobacter* sp.	0.015*

*Significance 0.05.
***Significance <0.0001.

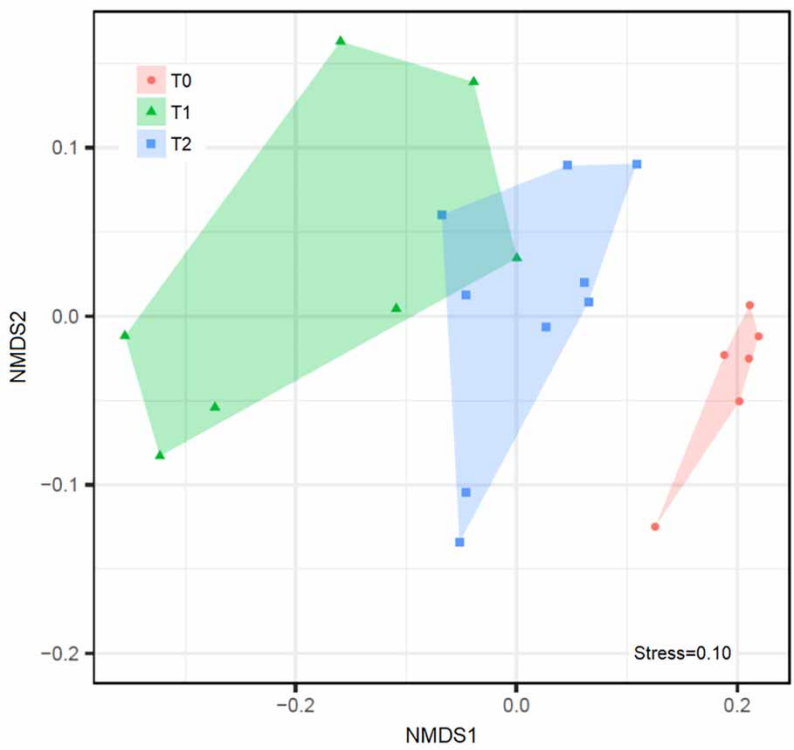

Figure 4 | NMDS from T0 samples (baseline samples), T1 samples (enriched AS with BAC), and T2 samples (non-enriched control in lab conditions).

BAC's response are general and, in some situations, are not related to antibiotic resistance response; and, (3) the microbial communities are highly resilient to antimicrobial substances like BAC.

In the studied Costa Rican municipal WWTPs, we found antimicrobial resistance genes in a similar relative load to that found previously in other latitudes (Di Cesare *et al.* 2016). The *intI1* gene has been used as a marker of anthropogenic pollution since it is commonly linked to disinfectants, antibiotics, and heavy metal resistance genes, it has penetrated pathogenic and commensal bacteria from humans and animals, and its abundance can rapidly change in response to environmental pressures (Gillings *et al.* 2015). Our results indicate the possible spread of the *intI1* gene in AS and the associated risk of resistance genes' horizontal transference in this ecosystem.

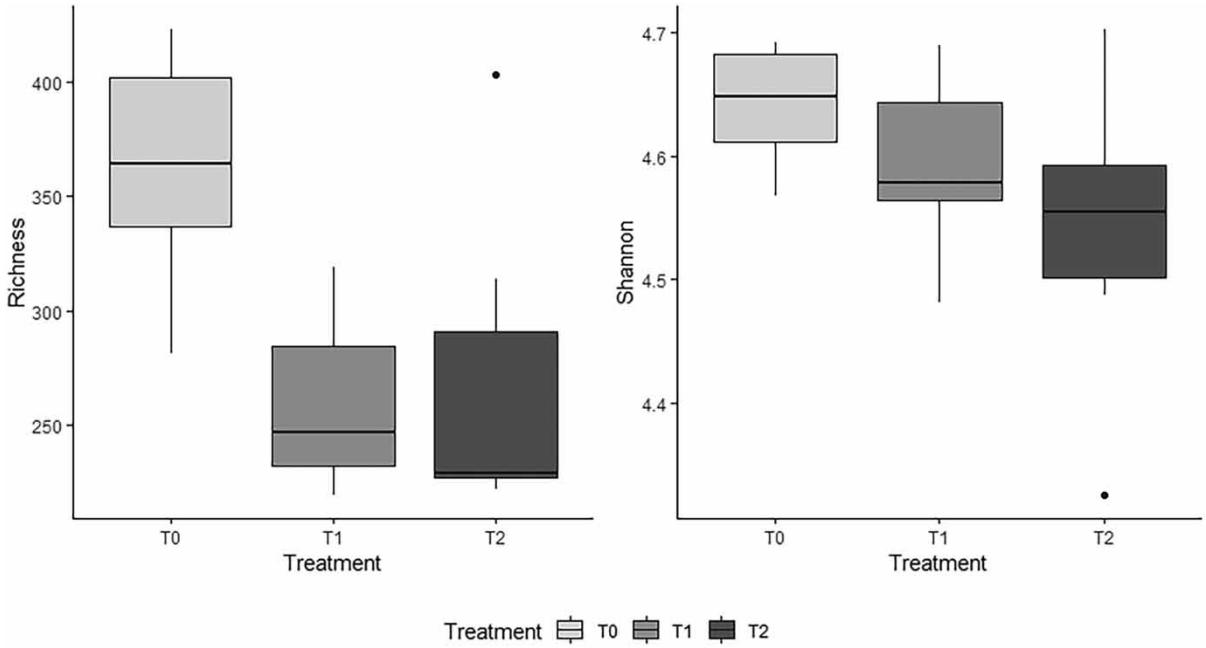

Figure 5 | Diversity α indexes for the sludge samples analyzed: T0 (baseline samples), T1 (enriched AS with BAC), and T2 (non-enriched control in lab conditions). Left panel corresponds to richness and right panel to Shannon diversity.

The sludge community composition obtained in this study is consistent with previous studies where Proteobacteria (and, more specifically, Alphaproteobacteria), followed by Bacteroidetes, Acidobacteria, and Chloroflexi are predominant phyla in AS (Xia *et al.* 2018). In addition, previous studies have shown a lower microbial diversity in BAC-degrading communities than non-exposed communities (Oh *et al.* 2013). Those findings are consistent with our results, showing a reduction of the richness and α-diversity in the samples exposed to BAC (Figure 4).

The most abundant genera in all treatments (Table 2) are associated with nitrogen fixation, nitrifiers, denitrifiers, methylotrophs, and others associated with bulking in WWTP. However, no functional or genetic analyses were performed to assess microbial nitrogen metabolism in the studied samples. For example, *Nitrospira*, a well-known nitrifier (Tian *et al.* 2017), decreased its relative abundance in BAC-enriched sludges. In this context, nitrification has been found to decrease at BAC concentrations of 2 mg/L (Hajaya & Pavlostathis 2013). On the other hand, *Rhodobacter* and *Methylorosura* (Alphaproteobacteria) increased in the BAC-treated sludge. *Rhodobacter* is a genus with photosynthetic capacity and nitrogen fixation and assimilation capabilities (Mackenzie *et al.* 2007), while *Methylorosula* is a methylotrophic bacterium associated with low temperatures (Berestovskaya *et al.* 2012).

Rhodobacter and *Rhodopseudomonas* (also Alphaproteobacteria) are involved in the denitrification process during AS treatment (Lu *et al.* 2014). A previous study showed that the denitrification process efficiency was reduced linearly to 64% in a mixed culture of nitrate-reducing bacteria exposed to 50–100 mg/L of BAC (Hajaya & Pavlostathis 2013). Previous studies have shown that these genera also share BAC resistance features in their annotated genomes: *sugE* gene presence in *Rhodopseudomonas palustris* genome (Larimer *et al.* 2004) and SMR efflux transporters' genes in *Rhodobacter sphaeroides* and *R. capsulatus* genomes (Kontur *et al.* 2012; Ding *et al.* 2014). As previously mentioned, the SMR pumps such as *sugE* and *qacE* are described as transporters that confer resistance to BAC and other quaternary ammonium compounds (Zou *et al.* 2014).

Using indicator species analysis (Table 3), we identified the genus *Methyloversatilis* (Rhodocyclaceae, Proteobacteria) as an indicator in BAC's exposed samples. This genus can be responsible for denitrification, nitrogen fixation, and the assimilation of single carbon compounds (Smalley *et al.* 2015). In the context of our study, the role of *Methyloversatilis* could be associated with processes related to the degradation of quaternary amines (such as benzalkonium chloride in AS, mainly when BAC is the primary source of nitrogen and carbon resources). *Methyloversatilis* sp. as indicator species is consistent with previous studies showing the impact of BAC exposure in the nitrogen cycle (Hajaya & Pavlostathis 2013). Additionally, the whole

genome sequence of *Methyloversatilis universalis* FAM5 shows 20 annotated genes related to efflux pumps and a *sugE* gene (Kittichotirat *et al.* 2011).

We hypothesize that the increase in the relative abundance of putative denitrifiers is part of the adaptability response of the AS microbial communities when they are exposed to BAC in concentrations below 50 mg/L. Further measurements of the nitrification-denitrification potential under these concentrations and BAC resistant phenotype confirmation should be performed in parallel to confirm this hypothesis. Finally, we found some genera previously described as BAC degraders, such as *Pseudomonas* and *Achromobacter* (Ertekin *et al.* 2016); however, these were found in abundances below 1%.

CONCLUSIONS

Our findings confirm that the use of cleaning products containing BAC, at a domestic level, can alter bacterial communities in activated sludges of a WWTP, as that studied in a tropical country such as Costa Rica. Furthermore, it suggests that BAC can alter some antibiotic resistance genes of the bacterial community and select some bacterial groups that can replace traditional microorganisms to maintain the nitrogen cycling in the microbial community. Nevertheless, the ultimate effects of these disinfectants on the ecology and evolution of tropical aquatic communities should be further studied.

ACKNOWLEDGEMENTS

This work was funded by CONARE and Vicerrectoría de Investigación of Universidad de Costa Rica. We thank Dr José Bonilla from CIBCM-UCR for his valuable suggestions.

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

REFERENCES

Antunes, P., Machado, J., Sousa, J. C. & Peixe, L. 2005 Dissemination of sulfonamide resistance genes (sul1, sul2, and sul3) in Portuguese Salmonella enterica strains and relation with integrons. *Antimicrob. Agents Chemother.* **49**, 836–839. https://doi.org/10.1128/AAC.49.2.836-839.2005.

Barraud, O., Baclet, M. C., Denis, F. & Ploy, M. C. 2010 Quantitative multiplex real-time PCR for detecting class 1, 2 and 3 integrons. *J. Antimicrob. Chemother.* **65**, 1642–1645. https://doi.org/10.1093/jac/dkq167.

Bay, D. C., Rommens, K. L. & Turner, R. J. 2008 Small multidrug resistance proteins: a multidrug transporter family that continues to grow. *Biochim. Biophys. Acta – Biomembr.* **1778**, 1814–1838. https://doi.org/10.1016/j.bbamem.2007.08.015.

Berestovskaya, J. J., Kotsyurbenko, O. R., Tourova, T. P., Kolganova, T. V., Doronina, N. V., Golyshin, P. N. & Vasilyeva, L. V. 2012 Methylorosula polaris gen. nov., sp. nov., an aerobic, facultatively methylotrophic psychrotolerant bacterium from tundra wetland soil. *Int. J. Syst. Evol. Microbiol.* **62**, 638–646. https://doi.org/10.1099/ijs.0.007005-0.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K. & Madden, T. L. 2009 BLAST+: architecture and applications. *BMC Bioinformatics* **10**, 421. https://doi.org/10.1186/1471-2105-10-421.

Chen, M., Zhang, X., Wang, Z., Liu, M., Wang, L. & Wu, Z. 2018 Impacts of quaternary ammonium compounds on membrane bioreactor performance: acute and chronic responses of microorganisms. *Water Res.* **134**, 153–161. https://doi.org/10.1016/j.watres.2018.01.073.

Chuanchuen, R., Khemtong, S. & Padungtod, P. 2007 Occurrence of qacE/qacEDelta1 genes and their correlation with class 1 integrons in salmonella enterica isolates from poultry and swine. *Southeast Asian J. Trop. Med. Public Health* **38**, 855–862.

Clarke, B. O. & Smith, S. R. 2011 Review of 'emerging' organic contaminants in biosolids and assessment of international research priorities for the agricultural use of biosolids. *Environ. Int.* **37**, 226–247. https://doi.org/10.1016/j.envint.2010.06.004.

De Cáceres, M. 2013 *How to use the Indicspecies Package (ver. 1.7.1)*. Available from: https://cran.r-project.org/web/packages/indicspecies/vignettes/indicspeciesTutorial.pdf

Di Cesare, A., Luna, G. M., Vignaroli, C., Pasquaroli, S., Tota, S., Paroncini, P. & Biavasco, F. 2013 Aquaculture can promote the presence and spread of antibiotic-resistant Enterococci in marine sediments. *PLoS One* **8**, 1–8. https://doi.org/10.1371/journal.pone.0062838.

Di Cesare, A., Eckert, E. M., Teruggi, A., Fontaneto, D., Bertoni, R., Callieri, C. & Corno, G. 2015 Constitutive presence of antibiotic resistance genes within the bacterial community of a large subalpine lake. *Mol. Ecol.* **24**, 3888–3900. https://doi.org/10.1111/mec.13293.

Di Cesare, A., Fontaneto, D., Doppelbauer, J. & Corno, G. 2016 Fitness and recovery of bacterial communities and antibiotic resistance genes in urban wastewaters exposed to classical disinfection treatments. *Environ. Sci. Technol.* **50**, 10153–10161. https://doi.org/10.1021/acs.est.6b02268.

Ding, H., Moksa, M. M., Hirst, M. & Beatty, J. T. 2014 Draft genome sequences of six Rhodobacter capsulatus strains, YW1, YW2, B6, y262, r121, and DE442. *Genome Announ.* **2**, 1–2. . https://doi.org/10.1128/genomeA.00050-14.Copyright.

Ertekin, E., Hatt, J. K., Konstantinidis, K. T. & Tezel, U. 2016 Similar microbial consortia and genes are involved in the biodegradation of benzalkonium chlorides in different environments. *Environ. Sci. Technol.* **50**, 4304–4313. https://doi.org/10.1021/acs.est.5b05959.

Gernaey, K. V. & Sin, G. 2008 *Wastewater Treatment Models, Encyclopedia of Ecology*. Elsevier Inc. https://doi.org/10.1016/b978-008045405-4.00828-4

Gilbert, P. & Moore, L. E. 2005 Cationic antiseptics: diversity of action under a common epithet. *J. Appl. Microbiol.* **99**, 703–715. https://doi.org/10.1111/j.1365-2672.2005.02664.x.

Gillings, M. R., Gaze, W. H., Pruden, A., Smalla, K., Tiedje, J. M. & Zhu, Y.-G. 2015 Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* **9**, 1269–1279. https://doi.org/10.1038/ismej.2014.226.

Hajaya, M. G. & Pavlostathis, S. G. 2013 Modeling the fate and effect of benzalkonium chlorides in a continuous-flow biological nitrogen removal system treating poultry processing wastewater. *Bioresour. Technol.* **130**, 278–287. https://doi.org/10.1016/j.biortech.2012.11.103.

Hora, P., Pati, S. G., McNamara, P. J. & Arnold, W. A. 2020 Increased use of quaternary ammonium compounds during the SARS-CoV-2 pandemic and beyond consideration of environmental implications. *Environ. Sci. Technol. Lett.* **7**, 622–631. https://doi.org/10.1021/acs.estlett.0c00437.

Karkman, A., Do, T. T., Walsh, F. & Virta, M. P. J. 2018 Antibiotic-resistance genes in waste water. *Trends Microbiol.* **26**, 220–228. https://doi.org/10.1016/j.tim.2017.09.005.

Kim, M., Weigand, M. R., Oh, S., Hatt, J. K., Krishnan, R., Tezel, U., Pavlostathis, S. G. & Konstantinidis, K. T. 2018 Widely used benzalkonium chloride disinfectants can promote antibiotic resistance. *Appl. Environ. Microbiol.* **84**, e01201–18. https://doi.org/10.1128/AEM.01201-18.

Kittichotirat, W., Good, N. M., Hall, R., Bringel, F., Lajus, A., Medigue, C., Smalley, N. E., Beck, D., Bumgarner, R., Vuilleumier, S. & Kalyuzhnaya, M. G. 2011 Genome sequence of Methyloversatilis universalis FAM5T, a methylotrophic representative of the order Rhodocyclales. *J. Bacteriol.* **193**, 4541–4542. https://doi.org/10.1128/JB.05331-11.

Kontur, W. S., Schackwitz, W. S., Ivanova, N., Martin, J., LaButti, K., Deshpande, S., Tice, H. N., Pennacchio, C., Sodergren, E., Weinstock, G. M., Noguera, D. R. & Donohue, T. J. 2012 Revised sequence and annotation of the Rhodobacter sphaeroides 2.4.1 genome. *J. Bacteriol.* **194**, 7016–7017. https://doi.org/10.1128/JB.01214-12

Kozich, J. J., Westcott, S. L., Baxter, N. T., Highlander, S. K. & Schloss, P. D. 2013 Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* **79**, 5112–5120. https://doi.org/10.1128/AEM.01043-13.

Larimer, F. W., Chain, P., Hauser, L., Lamerdin, J., Malfatti, S., Do, L., Land, M. L., Pelletier, D. A., Beatty, J. T., Lang, A. S., Tabita, F. R., Gibson, J. L., Hanson, T. E., Bobst, C., Torres, T. Y., Peres, J. L., Harrison, C., Gibson, F. H., & Harwood, J. & S, C. 2004 Complete genome sequence of the metabolically versatile photosynthetic bacterium Rhodopseudomonas palustris. *Nat. Biotechnol.* **22**, 55–61. https://doi.org/10.1038/nbt923.

Li, W. & Godzik, A. 2006 Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**, 1658–1659. https://doi.org/10.1093/bioinformatics/btl158.

Lu, H., Chandran, K. & Stensel, D. 2014 Microbial ecology of denitrification in biological wastewater treatment. *Water Res.* **64**, 237–254. https://doi.org/10.1016/j.watres.2014.06.042.

Mackenzie, C., Eraso, J. M., Choudhary, M., Roh, J. H., Zeng, X., Bruscella, P., Puskás, Á. & Kaplan, S. 2007 Postgenomic adventures with Rhodobacter sphaeroides. *Annu. Rev. Microbiol.* **61**, 283–307. https://doi.org/10.1146/annurev.micro.61.080706.093402.

Martínez-Carballo, E., Sitka, A., González-Barreiro, C., Kreuzinger, N., Fürhacker, M., Scharf, S. & Gans, O. 2007 Determination of selected quaternary ammonium compounds by liquid chromatography with mass spectrometry. Part I. application to surface, waste and indirect discharge water samples in Austria. *Environ. Pollut.* **145**, 489–496. https://doi.org/10.1016/j.envpol.2006.04.033.

McCarlie, S., Boucher, C. E. & Bragg, R. R. 2020 Molecular basis of bacterial disinfectant resistance. *Drug Resist. Updat.* **48**, 100672. https://doi.org/10.1016/j.drup.2019.100672.

Mora-Alvarado, D. A. & Portuguez-Barquero, C. F. 2016 Cobertura de la disposición de excretas en Costa Rica en el periodo 2000-2014 y expectativas para el 2021. (Excreta disposal coverage in Costa Rica from 2000 to 2014 and outlook for 2021). *Rev. Tecnol. en Marcha* **29**, 43. https://doi.org/10.18845/tm.v29i2.2690.

Numberger, D., Ganzert, L., Zoccarato, L., Mühldorfer, K., Sauer, S., Grossart, H. P. & Greenwood, A. D. 2019 Characterization of bacterial communities in wastewater with enhanced taxonomic resolution by full-length 16S rRNA sequencing. *Sci. Rep.* **9**, 1–14. https://doi.org/10.1038/s41598-019-46015-z.

Oh, S., Tandukar, M., Pavlostathis, S. G., Chain, P. S. G. & Konstantinidis, K. T. 2013 Microbial community adaptation to quaternary ammonium biocides as revealed by metagenomics. *Environ. Microbiol.* **15**. https://doi.org/10.1111/1462-2920.12154

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., Mcglinn, D., Minchin, P. R., Hara, R. B. O., Simpson, G. L., Solymos, P., Stevens, M. H. H., Szoecs, E. & Wagner, H. 2019 *Package 'Vegan'. Community Ecology Package*. Available from: https://cran.r-project.org/web/packages/vegan/vegan.pdf

Pei, R., Kim, S.-C., Carlson, K. H. & Pruden, A. 2006 Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). *Water Res.* **40**, 2427–2435. https://doi.org/10.1016/j.watres.2006.04.017.

Pruesse, E., Peplies, J. & Glöckner, F. O. 2012 SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes. *Bioinformatics* **28**, 1823–1829. https://doi.org/10.1093/bioinformatics/bts252.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yaza, P., Peplies, J. & Glöckner, F. O. 2013 The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* **41**, 590–596. https://doi.org/10.1093/nar/gks1219.

R Core Team. 2019 *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria.

Ruiz Fallas, F. 2012 *'Gestión de las Excretas y Aguas Residuales en Costa Rica" Situación Actual y Perspectiva. (Excreta disposal and wastewater management in Costa Rica: Current situation and perspectives) San José*. Available from: https://www.aya.go.cr/centroDocumetacion/catalogoGeneral/Gesti%C3%B3n%20de%20las%20Excretas%20y%20Aguas%20Residuales%20en%20Costa%20Rica%20%20Situaci%C3%B3n%20Actual%20y%20Perspectiva.pdf

Sköld, O. 2000 Sulfonamide resistance: mechanisms and trends. *Drug Resist. Updat.* **3**, 155–160. https://doi.org/10.1054/drup.2000.0146.

Smalley, N. E., Taipale, S., De Marco, P., Doronina, N. V., Kyrpides, N., Shapiro, N., Woyke, T. & Kalyuzhnaya, M. G. 2015 Functional and genomic diversity of methylotrophic Rhodocyclaceae: description of methyloversatilis discipulorum sp. nov. *Int. J. Syst. Evol. Microbiol.* **65**, 2227–2233. https://doi.org/10.1099/ijs.0.000190.

Szczepanowski, R., Linke, B., Krahn, I., Gartemann, K.-H., Gützkow, T., Eichler, W., Pühler, A. & Schlüter, A. 2009 Detection of 140 clinically relevant antibiotic-resistance genes in the plasmid metagenome of wastewater treatment plant bacteria showing reduced susceptibility to selected antibiotics. *Microbiology* **155**, 2306–2319. https://doi.org/10.1099/mic.0.028233-0.

Thorsten, P. 2021 *Package 'PMCMRplus'. Calculate Pairwise Multiple Comparisons of Mean Rank Sums Extended*. Available from: https://cran.r-project.org/web/packages/PMCMRplus/PMCMRplus.pdf

Tian, S., Tian, Z., Yang, H., Yang, M. & Zhang, Y. 2017 Detection of viable bacteria during sludge ozonation by the combination of ATP assay with PMA-Miseq sequencing. *Water* **9**, 166. https://doi.org/10.3390/w9030166.

US Environmental Protection Agency 2020 *List N: Disinfectants for Use Against SARS-CoV-2 (COVID-19) [WWW Document]*. Available from: https://www.epa.gov/pesticide-registration/list-n-disinfectants-use-against-sars-cov-2-covid-19 (accessed 9.2.2020).

Xia, Y., Wen, X., Zhang, B. & Yang, Y. 2018 Diversity and assembly patterns of activated sludge microbial communities: a review. *Biotechnol. Adv.* **36**, 1038–1047. https://doi.org/10.1016/j.biotechadv.2018.03.005.

Yang, Y. & Wang, W. 2018 Benzyldimethyldodecyl ammonium chloride shifts the proliferation of functional genes and microbial community in natural water from eutrophic lake. *Environ. Pollut.* **236**, 355–365. https://doi.org/10.1016/j.envpol.2018.01.059.

Zhang, T., Shao, M. F. & Ye, L. 2012 454 pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. *ISME J.* **6**, 1137–1147. https://doi.org/10.1038/ismej.2011.188.

Zou, L., Meng, J., McDermott, P. F., Wang, F., Yang, Q., Cao, G., Hoffmann, M. & Zhao, S. 2014 Presence of disinfectant resistance genes in Escherichia coli isolated from retail meats in the USA. *J. Antimicrob. Chemother.* **69**, 2644–2649. https://doi.org/10.1093/jac/dku197.

First received 13 July 2021; accepted in revised form 8 September 2021. Available online 6 October 2021