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Fate and persistence of antibiotic-resistant bacteria and genes through a multi-barrier treatment facility for direct potable reuse

Lina Wallmann, Jörg Krampe, Josef Lahnsteiner, Elena Radu, Pierre van Rensburg, Katarzyna Slipko, Markus Wögerbauer and Norbert Kreuzinger

ABSTRACT

Given the availability of technological solutions and guidelines for safe drinking water, direct potable reuse of reclaimed water has become a promising option to overcome severe lack of potable water in arid regions. However, the growing awareness of the presence of antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARG) in corresponding raw wastes has led to new safety concerns. This study investigated the fate of ARB and intracellular and extracellular ARG after each treatment step of an advanced water treatment facility in Windhoek, Namibia. The New Goreangab Water Reclamation Plant (NGWRP) produces drinking water from domestic secondary wastewater treatment plant effluent and directly provides for roughly a quarter of Windhoek's potable water demand. Procedures to study resistance determinants were based on both molecular biology and culture-based microbiological methods. TaqMan real-time PCR was employed to detect and quantify intracellular resistance genes *sul1, ermB, vanA, nptII* and *nptIII* as well as extracellular resistance gene *sul1*. The NGWRP reduced the amount of both culturable bacterial indicators as well as the resistance genes to levels below the limit of detection in the final product. The main ozonation and the ultrafiltration had the highest removal efficiencies on both resistance determinants.

Key words | antibiotic resistance, ARB, ARG, direct potable reuse

HIGHLIGHTS

- The final product contained none of the antibiotic resistance genes investigated.
- A subsequent series of water treatment steps can decrease antibiotic resistance genes to below LOD.
- All steps in the treatment train decreased the abundance of the *sul1* resistance gene except for the pre-ozonation and the biological activated carbon.
- In the final product, extracellular and intracellular *sul1* resistance genes were below LOD.

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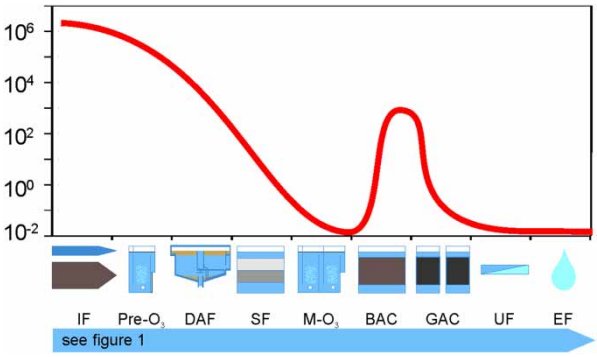
GRAPHICAL ABSTRACT



INTRODUCTION

Population growth in various countries of the world together with the effects of climate change leads to a continuous increase of pressure on the availability of water resources for irrigation of agricultural crops and potable water (USEPA 2012). The use of reclaimed water can help address the resulting challenges by regarding wastewater (WW)/used water (UW) as a valuable key resource that can be recovered rather than considered a waste product. An alternative to relying on traditional raw water sources for water supply therefore is direct potable reuse (DPR), a process in which the reclaimed water is not re-introduced into the environment (groundwater or surface water) but directly reused for drinking water supply and further human consumption (USEPA 2018). DPR is a suitable method to augment water supplies when necessary, and technologies exist that reliably produce safe potable water that continuously meets drinking water regulations (du Pisani 2006; Leverenz *et al.* 2011). Current quality guidelines include an extensive list of microbial indicators, requirements for organic matter and suspended particles and physicochemical parameters (Lahnsteiner Du Pisani & Menge 2013; Water Reuse Research Foundation 2015; Hong *et al.* 2018).

However, beside these conventional parameters, organic trace chemical constituents that have survived treatment are currently under close investigation by scientists and advisory boards regarding their significance for human health. One group of these trace constituents are pharmaceuticals and their metabolites, amongst which belong the antibiotics (Dulio *et al.* 2018). They have been and are extensively used for disease treatment and prophylaxis in humans as well as in animal husbandry (Bouki *et al.* 2013). Their effectiveness is essential for the success of modern medicine (Davies & Davies 2010; White & Hughes 2019). Yet, the increasing use of antibiotics brings along the increased occurrence of antibiotic resistance (AR) which is the ability of bacteria to withstand the antibiotic and compensate its effect (Davison *et al.* 2000). AR has serious implications for our modern medical practice because it threatens the performance of antibacterial treatment and prophylaxis. Health and economic burdens connected with AR are 'an increased risk of worse clinical outcomes and death' and an increase in consumed healthcare resources (World Health Organization 2014). Accordingly, AR is listed amongst the top ten global health threats (World Health Organization 2015).

Numerous studies show that WW is laden with antibiotic-resistant bacteria (ARB) and associated antibiotic resistance genes (ARG). This is because partially metabolized antibiotic remnants as well as ARB themselves are excreted from humans, collected in sewer systems and further concentrated at wastewater treatment plants (WWTP) along with all other biological and chemical UW (WW) constituents (Kreuzinger 2015). Resistant heterotrophic and faecal coliform bacteria are present in raw

and treated WW and may reach the environment and drinking water sources through effluent discharge. If they are not eliminated during drinking water treatment, there is a risk that they may spread into the human microbiome through drinking water consumption (Cooke 1976; Vaz-Moreira *et al.* 2014). A number of previous studies detected ARB and ARG in drinking water (Xi *et al.* 2009; Sanganyado & Gwenzi 2019). Removal efficiencies of conventional WWTP for antibiotic traces, ARBs and ARGs vary and are dependent on the physiology of microorganisms and operating conditions of the treatment system (Michael *et al.* 2013). Therefore, it is necessary to address the concern of AR in the DPR context and expand the scientific knowledge on advanced water treatment technologies to provide a safeguard against AR dissemination during subsequent reuse.

AR can spread through the selection of ARB and the exchange of genetic material (ARG) that conveys the resistance by horizontal gene transfer based on transformation (incorporation of free DNA), transduction (phage mediated) and conjugation (direct exchange by bacteria). Therefore, the investigation of both ARB and ARG is important in order to understand AR proliferation. Both determinants have recently been studied in UW and environmental samples by means of resistance gene detection and culture-dependent estimates of live bacterial indicators.

For this reason, one part of this study is concerned with the detection of resistant phenotypes using sulphamethoxazole (SMX) resistance as a high abundance lead parameter. This allows us to follow the removal of ARB throughout the treatment train without losing the signal in an early stage treatment process. For the same reason, SMX resistance genes are used to follow the abundance of ARG through the technological steps of DPR. Resistance genes are cell-associated within the host's genome or plasmids. However, microbial death, as can occur during water treatment processes, leads to the release of the genetic material. Occurrence and persistence of free DNA in water and soil environments have been reported (Nielsen *et al.* 2007), but the associated risks for the spread of AR coming from free resistance genes are still unknown. The concern is that free DNA facilitates transformation by direct uptake of extracellular DNA (Zhang *et al.* 2018). Therefore, this study includes an assessment of resistance genes on free environmental DNA within the DPR process.

Within that frame, this study investigates the efficiency of the treatment train at New Goreangab Water Reclamation Plant (NGWRP) and its treatment steps on the reduction of ARB and ARG by culture-dependent methods and quantification of ARG by TaqMan real-time PCR. It aims to address the following research questions:

- Does the treatment process at NGWRP as a whole reduce the abundance of (i) resistant bacteria, (ii) intracellular resistance genes and (iii) extracellular resistance genes?
- To what extent do the individual treatment technologies at NGWRP reduce the abundance of (i) resistant bacteria, (ii) intracellular resistance genes and (iii) extracellular resistance genes?

Therefore, the study focuses on life bacterial indicators resistant to SMX, the intracellular indicator resistance genes *sul1* (sulphonamides), *ermB* (macrolides), *vanA* (vancomycin), *npt II* and *npt III* (kanamycin – aminoglycosides) and extracellular resistance gene *sul1*.

METHODS

Study area and site

Namibia's capital, Windhoek, has encountered recurring periods of limited water supply and suffered from great droughts (Lahnsteiner & Lempert 2007). In order to augment the available water supply, the city covers a quarter of its drinking water demand through DPR. The NGWRP produces purified drinking water from pre-treated municipal WW at a maximum capacity of 21,000 m^3/day and includes a series of advanced biological and chemical purification steps. An overview of the treatment train applied at the NGWRP system is shown in Figure 1. Before being distributed to the point of use (POU) (10), it consists of pre-ozonation (2), flocculation and dissolved air flotation (3), rapid sand filtration (4), main ozonation (5), biological activated carbon (6), granular activated carbon (7), ultrafiltration membranes (8), pH adjustment and chlorination (9). A flow chart summarizing average hydraulic flow is provided in the Supplementary Material, and a detailed description of the plant, as well as quality control and operational parameters

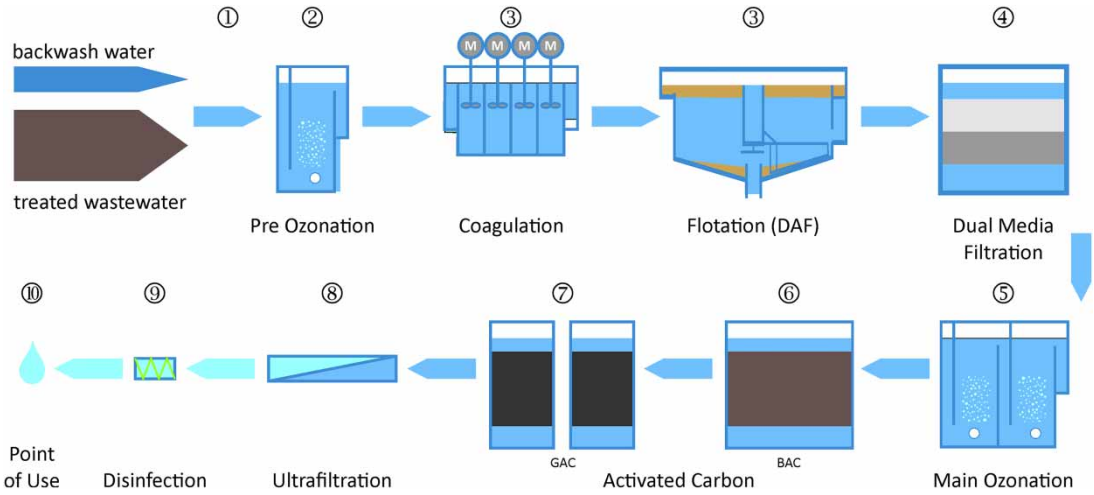


Figure 1 | System overview of the purification steps at the NGWRP.

are provided by du Pisani (2006), Lahnsteiner *et al.* (2013), Onyango *et al.* (2014) and Lahnsteiner *et al.* (2018).

Besides providing safe drinking water, this combination of a broad range of treatment technologies is of interest as these treatment technologies are proposed to be suitable for the removal of all possible forms of microcontaminants (Fatta-Kassinos *et al.* 2015) or contaminants of emerging concern (CEC), respectively.

Sampling

For this study, water samples at nine sampling points representing the effluents of various treatment steps at NGWRP were collected in the third week of September 2018 by the facility's routine sampling team. A reducing agent (sodium thiosulphate) that neutralizes potential free chlorine was added to the sample 9-EF final product as well as 10-POU to maintain the sample's original bacterial state.

Considering typical fluctuations and variation of the microbial load in urban WW depending, e.g., on seasons and other effects, the representativeness of the samples was evaluated by comparing CFU counts obtained in this study with results from routine monitoring of the plant over a longer period. Details on this evaluation can be found in the section 'Representability of Sampling Period' in the Supplementary Material. Since the city of Windhoek distributes a blend of the final product from NGWRP, Von Bach Dam Water Treatment Plant (BD) and borehole water, additional samples for environmental background detection were taken at a hotel tap (POU), BD and Borehole 12/1b (BH). This borehole was chosen due to its relatively recent construction in 2017 and the fact that it has not been subject to groundwater recharge by reclaimed water from NGWRP. Table 1 lists all sampling points and their abbreviations used throughout this work.

Selection of AR determinants

The following antimicrobial resistance genes have been selected for our analyses due to their clinical relevance

Table 1 | Sampling points and abbreviations used numbered according to the system overview in Figure 1

Number	Sample	Abbreviation
1	Influent mix	1-IF
2	After pre-ozonation	2-Pre-O_3
3	After coagulation + dissolved air flotation	3-DAF
4	After dual media filtration	4-SF
5	After main ozonation	5-M-O_3
6	After biological activated carbon filtration	6-BAC
7	After granular activated carbon filtration	7-GAC
8	After ultrafiltration	8-UF
9	Final product	9-EF
10	Point of use	10-POU
11	Borehole 12/1B	11-BH
12	Von Bach Dam Reservoir Treatment Plant	12-BD

and their prevalence in human and animal pathogens and in natural environments. The RNA methyltransferase *ErmB* confers resistance to critically important macrolide–lincosamine–streptogramin (MLS) antibiotics like erythromycin, azithromycin and clarithromycin and is prevalent in Gram-positive enterococci (Portillo *et al.* 2000; WHO 2017). *Sul1* is a resistant dihydropteroate synthase which mediates tolerance to a broad group of sulphonamide antibiotics (sulphadiazine, sulphadimidine, SMX, etc.) (Sköld 2001). This gene is frequently found in Gram-negative enterobacteria but also in environmental pathogens like *Pseudomonas aeruginosa* (Gu *et al.* 2007). SMX is a broad-spectrum antibiotic. Hence, its remnants as well as resistant bacteria and related ARG (*sul1*) have been frequently identified in WW and drinking water (e.g., Xi *et al.* 2009; Vaz-Moreira *et al.* 2014; Sanganyado & Gwenzi 2019). Due to the high abundance in these waters, it is suitable for the evaluation of advanced treatment processes because ARGs with low concentrations may quickly reach their detection limit, which would eliminate the chance of evaluating reduction efficiencies (Hembach *et al.* 2019). *VanA* encodes a D-Ala-D-Ala ligase homologue which confers resistance to the second-line glycopeptide antibiotics vancomycin and teicoplanin. Both substances block bacterial cell wall synthesis (Marshall *et al.* 1997; WHO 2019). *VanA* is predominantly identified as a resistance determinant in vancomycin-resistant enterococci (VRE) and constitutes an important burden in clinical intensive care unit settings and sepsis (De Angelis *et al.* 2014; Papanicolaou *et al.* 2019). The aminoglycoside phosphotransferases *nptII* and *nptIII* inactivate critically important aminoglycosides like amikacin (*nptIII*), kanamycin and neomycin (*nptII* + *nptIII*) (Woegerbauer *et al.* 2014). Both genes are the most frequently applied AR marker genes in plant gene biotechnology (Miki & McHugh 2004). They show a unique prevalence and distribution pattern in agricultural ecosystems (Woegerbauer *et al.* 2015). Enterococcal pathogens are frequent sources of *nptIII* (Woegerbauer *et al.* 2014). With the exception of *nptII* which is characterized by a low prevalence in natural environments and in clinical pathogens (Woegerbauer *et al.* 2014, 2015), all remaining selected resistance genes are frequently present in gut bacteria and, thus, supposed to be prevalent constituents of an average WW microbiome.

Cultivation of bacterial indicators

The number of viable heterotrophs as well as coliform bacteria in the samples was estimated by the filtration-based CFU method. This was done according to the plant's SOPs in order to allow for the comparison of results from the sampling campaigns with the monitoring data of NGWRP to show the representativeness of the conditions during the sampling campaigns. The methods were adapted to access the number of SMX-resistant CFU in the samples.

Heterotrophic plate count

Tryptone Glucose Extract Agar (TGEA) (Sigma-Aldrich) was used for the heterotrophic plate count (HPC) with an incubation time of 48 h at 34 °C. The medium was prepared according to the manufacturer's instructions.

Coliforms

Coliforms and *Escherichia coli* were enumerated on mFC agar (Difco, Rosolic Acid Difco) after incubation for 24 h at 37 °C. The medium was prepared according to the manufacturer's instructions. Both media belong to the established routine CFU monitoring of NGWRP.

Antibiotic selective media

To prepare the antibiotic selective plates, 750 mg of SMX (Sigma-Aldrich) were dissolved in 1.5 L of distilled autoclaved water to reach a final concentration of 0.5 mg/L. This concentration was chosen according to the clinical minimum inhibitory concentration (MIC) of sulphonamides for Enterobacteriaceae, other non-Enterobacteriaceae and *Staphylococcus spp.* (EUCAST 2017). The required amount of agar powder specified by the producer was then added to the water supplemented with antibiotic, and the protocol proceeded by following the manufacturer's instructions. This was possible because SMX can be autoclaved as accessed in an antibiotic integrity previously performed (see Supplementary Material).

Filtration and counting

Samples were filtered through 0.45 μm cellulose nitrate membrane filters (Pall Corporation) and placed on plain agar (−) and agar supplemented with 0.5 mg/L SMX (+). Applied sample volumes and dilutions with sterile saline used for plating are summarized in the Supplementary Material and were derived from routine CFU monitoring at NGWRP and pretests. The manifold was rinsed with autoclaved water and sterilized with a gas burner between filtration steps. Samples were plated in triplicates. As a control for cross-contamination, two no-sample negative controls were taken by filtering 400 mL of the autoclaved saline during the filtration process. The controls were incubated amongst the other samples (see Supplementary Material).

According to the Standard Methods for the Examination of Water and Wastewater Section 9010 (APHA 1999), plates of 30–300 colonies should be counted, which would define 30 colonies as the limit of quantification (LOQ) of the maximum amount of sample volume that could be filtered. Against the standard methods, this study accepted plates of five colonies or more, due to a combination of limited knowledge of the correct dilutions for sampling sites not implemented in the routine monitoring at NGWRP and the strict time constraint for the onsite work. Results of colony counts between 5 and 30 are marked in the tables provided. The limit of detection (LOD) is herein defined as five colonies per plate at the maximum volume that could be filtered.

Log removal value

For the evaluation of reduction efficiencies of the individual treatment units, log removal values (LRV) were calculated as follows:

$$LRV = Log_{10}\left(\frac{CFU\ before\ treatment}{CFU\ after\ treatment}\right)$$

The number does not reflect on the type of removal, which may be physical removal or inactivation of the bacteria. Where the treatment brought the CFU to undetectable levels, LRV were calculated with the respective LOD, listed in the Supplementary Material.

DNA preparation

Sample preparation for intracellular DNA extraction

Due to equipment limitations at NGWRP and no possibility for onsite DNA extraction, samples received a special fixation step prior to transport to the laboratory in Vienna, Austria. Samples were taken and treated in triplicate, but only one technical replicate was used for final analysis by TaqMan real-time PCR, as the other replicates were 'consumed' for optimizing the DNA extraction procedure (see the 'Intracellular DNA extraction' section).

All samples were prepared for transportation and subsequent DNA extraction by filtering between 400 mL and up to 3 L through sterile 0.45 μm cellulose nitrate filters (Pall Corporation) with the aid of a multi-way vacuum filtration system. Sample-specific volumes can be found in the Supplementary Material and reflect the maximum sample volume that could be filtered. After filtration, filters were placed in sterile Petri dishes and dried in a dry oven for 24 h at 40–50 °C in order to inhibit bacteria and nuclease activity. 400 mL of autoclaved saline prepared from distilled water was filtered as a no-sample negative control (NC-F) twice throughout the filtration process after rinsing the manifold with autoclaved distilled water and sterilization with a gas burner as a negative control. An additional negative control of the dry oven condition (NC-DO) was prepared by having an empty sterile filter in a Petri dish undergo the drying process amongst the other samples. All negative controls and samples were treated alike. After drying, the filters were rolled and transferred into individual sterile and empty disruptor tubes of the DNA extraction kit E.Z.N.A. Water DNA Kit (Omega Bio-tek) for safe transportation by aeroplane at ambient temperature. Upon arrival in Vienna, all disruptor tubes were stored in an exicator at room temperature until DNA extraction in the following week.

Intracellular DNA extraction

Intracellular nucleic acid extraction took place at TU Wien in Vienna, Austria. The cellular metagenomic DNA collected in the filters was extracted with the E.Z.N.A. Water DNA Kit (Omega Bio-tek). Procedures were carried out according to the manufacturer's instructions with the

following modifications resulting from two optimization steps: Lysis was performed without 2-Mercaptoethanol on a FastPrep-24 homogenizer (MP Biomedicals) once for 30 s at 4 m/s to chop the filter and three times for 40 s at 6 m/s to lyse released cells. The isopropanol precipitation step was omitted due to initially low DNA yield. The Elution Buffer was heated to 60 °C prior to its use, and the DNA was eluted off the column twice with 50 μL of Elution Buffer. In order to control for cross-contamination, extractions with an empty, sterile filter (NCex-wF) and without a filter (NCex-w/oF) were performed. In order to control the performance of the DNA extraction, 200 mL of saline with 100 μL of a Library Efficiency™ DH5α™ Competent Cells (Invitrogen) culture were treated the same way as the samples and extracted as a positive control (PCex).

The amount of extracted DNA in the individual samples was quantified prior to gene analysis, and results are provided in the Supplementary Material.

Extracellular DNA extraction

Extracellular DNA occurs when eukaryotes, as well as prokaryotes, release their cell-associated DNA into the surroundings by means of active excretion, excrements or cell death (Nielsen *et al.* 2007). As the chain of treatment steps in the investigated WWTPs leads to an increased occurrence of microbial cell death, one section of the ARG analysis is dedicated to extracellular DNA.

We herein define the term 'free DNA' solely to extracellular DNA, which is DNA of any size that is found to be freely dispersed in the water column after a filtration step outside of cells and may or may not be attached to organic molecules found within the WW matrix as, e.g., humic acids. Investigating the presence of extracellular resistance genes is of interest, as the direct uptake of free DNA is, next to conjugation and transduction, one of the means of ARG propagation (Zhang *et al.* 2018).

The amount of extracted DNA in the individual samples was quantified prior to gene analysis, and results are provided in the Supplementary Material.

Protocol of extracellular DNA extraction. Extracellular DNA was extracted from samples one to nine (see Table 1) immediately after sampling on site. Sample 10 was extracted along with the other samples around 2 h after sampling, and samples 11 and 12 were excluded from this method due to logistic complications.

A volume of 10 mL sample was filtered through individual 0.2 μm sterile filtration units (Nalgene). 5 mL of the filtrate and 200 μL of magnetic beads were added to 5 mL of Binding Buffer of the MagNA Pure LC Total Nucleic Acid Isolation Kit (Roche). The 50 mL tubes were mounted onto a Thermo Mixer Compact (Eppendorf) with tape and shaken at 130 rpm for 30 min. For subsequent removal of the Binding Buffer, the magnetic beads were collected by applying a magnetic field to the bottom of the tube. 1 mL of Wash Buffer 1 was added to the beads, mixed well and the solution was transferred to a microcentrifuge tube. The beads were sedimented by magnetic forces, and Wash Buffer 1 was removed. The same procedure was carried out for Wash Buffer 2 and 3. Upon removal of Wash Buffer 3, the tubes were set to air dry for 20 min before adding 50 μL of heated Elution Buffer. For the elution, the microcentrifuge tubes were shaken at 250 rpm for 10 min at 40 °C. Finally, the Elution Buffer containing the extracted free DNA was separated from the beads.

The same procedure was carried out with 5 mL molecular water as a no-sample extraction negative control (NCex). 50 μL of a heterogenous effluent culture grown on agar that was supplemented with 0.5 mg/L SMX added to 5 mL of molecular grade water and served as a positive control (PCex) for extraction. Samples were extracted in duplicate and transported to Vienna by aeroplane in an insulated cooler box. They were frozen at −20 °C immediately upon arrival in Vienna and stored there until further analyzed by PCR and qPCR.

DNA quantitation

Concentrations of the extracted DNA were measured by fluorescence staining of double-stranded DNA (Quant-it PicoGreen dsDNA Assay Kit, Thermo Fisher Scientific). A low-range standard curve from 2 to 1,000 ng/mL was prepared from the kit's lambda DNA standard. The LOQ of this method is 1 ng/mL.

Quantitative gene analysis

Quantitative TaqMan real-time PCR was used to estimate the copy number of intracellular bacterial 16S rRNA gene

and five different ARGs inactivating four major classes of antibiotics: *sul1* (sulphonamides), *vanA* (vancomycin), *ermB* (macrolides), *nptII* and *nptIII* (aminoglycosides). All genes were quantified in samples 1–12 (see Table 1) except for *nptII* and *nptIII*, which were only quantified in samples 1-IF, 7-GAC, 10-POU and 12-BD. Free DNA samples where only analyzed for the *sul1* resistance gene due to its high abundance at all sampling points except 11-BH and 12-BD.

Protocol of qPCR

10 µL qPCR reaction mixture consisted of 5 µL LightCycler® 480 Probes Master Mix, 2.5 µL nuclease-free water, 0.5 µL gene-specific TaqMan Assay (Ingenetix, Austria, kit specification, see Supplementary Material) and 2 µL DNA template. Controls were run with molecular biology grade water (Sigma-Aldrich, Austria) as a template. The qPCR amplifications were performed in the 96-well microtiter plate format on a LightCycler® 480 System (Roche, Life Science, Austria) under the following cycling conditions: initial denaturation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, annealing for 30 s at 60 °C and elongation at 72 °C for 10 s. All qPCR assays were analyzed in three technical replicates. Absolute quantification was performed using the second derivative maximum method of the LC480 software and logarithmic dilutions of the respective plasmid standards for calculating standard curves and amplification efficiencies.

Standards, LOQ and LOD

The validation of the qPCR systems for the ARGs (*sul1, vanA, ermB, nptII, nptIII)* and the 16S rRNA gene was done following Paul Ehrlich Institute (PEI) recommendations regarding tests of blood products for HBV, HCV and HIV (Forootan *et al.* 2017). In order to determine the 95% LOD and the LOQ, a total number of 120 measurement points (five dilution steps in eight replicates in three qPCR runs) were performed. All reference standards were purchased from Ingenetix GmbH, and the calibration curve was obtained from their semi-logarithmic (1:5) dilutions. Due to a ubiquitous 16S rDNA presence in qPCR reagents resulting in a trace background, the definition of 95% detection limit was not possible. The data were analyzed using the generalized linear mixed model (GLMM), and the LOD was determined as the value at which a positive dilution is detected by the estimated model with a probability of at least 95%. The quantification limit was determined using the following formula:

$$\mathrm{LOQ} = 10 \times \sigma / S$$

where S is the slope of the linear regression of the standard curve and σ is the standard deviation of the response variable.

Limits and specifications can be found in the Supplementary Material. The LODs and LOQs are reported in gene copies per assay. This results in an effective sample-specific LOD and LOQ depending on the sample volume used for DNA extraction and the amount of DNA used for qPCR. Sample-specific LOD and LOQ are given in the Supplementary Material.

Log removal values

For the evaluation of removal efficiencies of the individual treatment units, LRV were calculated as follows:

$$LRV = Log_{10}\left(\frac{copies/mL\ before\ treatment}{copies/mL\ after\ treatment}\right)$$

The number does not reflect the type of removal, which may be physical removal or destruction of the gene. Where the treatment reduced the abundance below detectable levels, LRV were calculated with the respective LOD.

Statistics

Results were analyzed with statistic tools available in SigmaPlot 13 (Systat Software Inc.). A student's *t*-test was performed to test if the change that occurred with the treatment steps was greater than expected by chance. Statistical significance was set at $\alpha = 0.05$. A test for outliers was performed in Excel (Microsoft) according to the interquartile range method described by Aguinis *et al.* (2013). The limits of detection and quantification were applied to the means of the triplicate data sets.

RESULTS AND DISCUSSION

As the NGWRP applies a set of state-of-the-art technologies into one multi-barrier treatment train, it is not possible to directly compare the total LRV with single-step technologies. Hence, only the individual treatment technologies that have been assessed for their reduction efficiencies can be discussed and compared with the literature.

Bacterial counts

Addressing research question part (i) in regard to the reduction of the abundance of ARB, the following section will present CFU results and discuss the effects of the sequential treatment units of the NGWRP treatment train on the abundance of SMX-resistant heterotrophic bacteria and coliforms, respectively. Figure 2 gives a summary of the results in CFU/mL. The raw CFU data and individual graphs can be found in the Supplementary Material.

Total HPC and coliforms

The NGWRP reduced the HPC as well as coliforms from 1.6×10^4 and 1.9×10^3 CFU/mL, respectively, to below the LOD in the final product (9-EF) with corresponding total log reductions of 6.5 and 3.8 or higher. The pre-ozonation treatment only decreased the HPC slightly to 3.1×10^4 CFU/mL and had no significant effect on the total coliforms resulting in 1.2×10^3 CFU/mL at 2-Pre-O_3. As the results after 3-DAF are likely to be influenced by permanganate dosing in close proximity to the sampling spot and therefore are not representative of the whole process, they are excluded from the evaluation. There were HPC and coliforms of 2.2×10^3 and 2.3×10^1 CFU/mL detected at 4-SF, which then decreased by 4.7 LRV to 4.1×10^{-2} CFU/mL and 3.2 LRV to 1.4×10^{-2} CFU/mL at 5-M-O_3. The main-ozonation treatment had the highest removal efficiency for HPC and FC. Those LRV only differ by 0.1 to those that Hiller *et al.* (2019) summarized by comparing nine studies on tetracycline-resistant bacteria in the WWT process. The HPC at 6-biologically activated carbon (BAC) came close to that of the influent with 8.4×10^3 CFU/mL. The granular activated carbon treatment at 7-GAC decreased this by an LRV of 1.5 to 2.7×10^2 CFU/mL. A further decrease of 2.8 LRV was achieved by the ultrafiltration process at 8-UF to 4.6×10^{-1} CFU/mL. All reductions of total HPC in the treatment train were statistically significant. The three additional

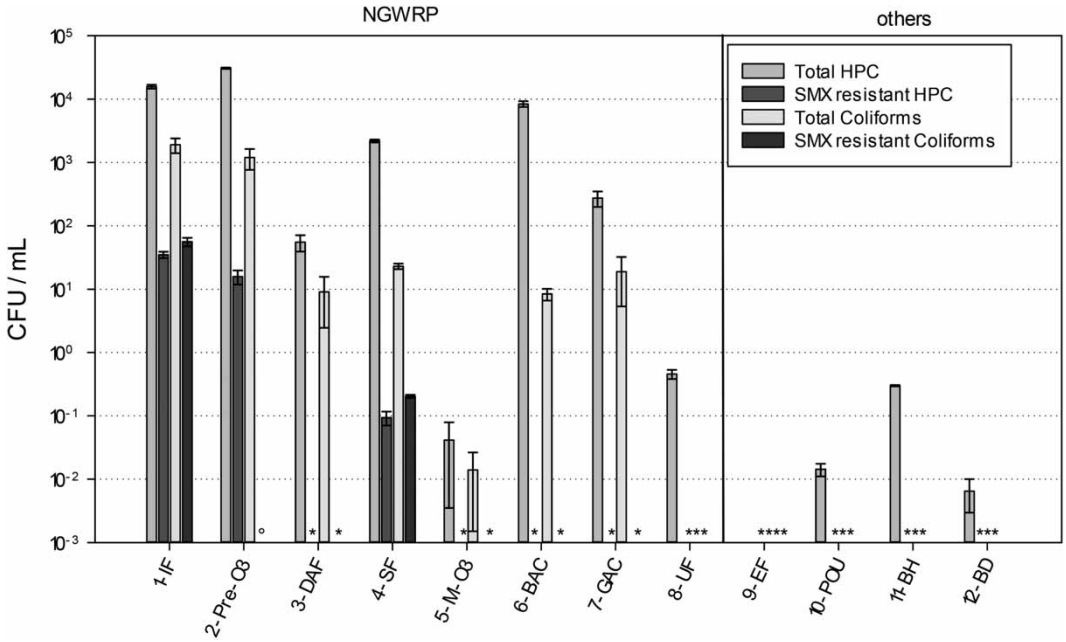


Figure 2 | CFU of total and SMX-resistant heterotrophs as well as total and SMX-resistant coliforms after individual treatment steps at NGWRP and other reference samples, (mean ± SD) <LOQ marked '°'; <LOD marked '*'.

drinking water sources, POU, BH and BD, showed HPC of 1.4×10^{-2}, 3.0×10^{-1} and 6.5×10^{-3}, respectively.

Resistant HPC and coliforms

The NGWRP reduced the SMX-resistant HPC as well as coliforms from 3.5×10^{1} and 5.6×10^{1} CFU/mL at 1-IF, respectively, to below the LOD in the final product (9-EF) with corresponding log reductions of 5.3 and 4 or higher. Of the total heterotrophs in the influent, 0.2% showed SMX resistance. This ratio was considerably higher for the coliforms with 2.9% SMX resistance.

The change in SMX-resistant HPC between 1-IF and 2-Pre-O_3 was not statistically significant. The number of coliforms after 2-Pre-O_3 could not be obtained due to too high dilution steps applied based on pre-tests and results from sensitive HPC and coliforms. As stated above, the results at 3-DAF are likely to have been influenced by permanganate dosing in proximity and are therefore excluded from the evaluation. At 4-SF, the HPC was 9.3×10^{-2} CFU/mL and the coliforms were 2.0×10^{-1} CFU/mL. Up until the sand filtration, the NGWRP achieved a log reduction of 2.5 ± 0.1 on SMX-resistant heterotrophic and coliform bacteria. This would be the LRV to compare with other drinking water treatment plant (DWTP) trains that apply a more conventional technical setup.

Subsequent main ozonation brought the CFU of resistant HPC and FC to levels below the LOD and SMX-resistant bacteria were not detected downstream at any point nor at 10-POU, 11-BH and 12-BD. This is a major finding because the total CFU of sensitive phenotypes significantly increased after BAC to about the same level as in the influent as a consequence of biological activity (BAC). This suggests that in contrast to sensitive phenotypes, there is no major proliferation of resistant HPC and coliforms on the carbon bed.

Obviously, the resistant CFU do not behave similarly to the total CFU. The ratio between total and resistant CFU of both HPC and coliforms is calculated and presented in Figure 3. It shows that 1-IF had the highest percentage of viable culturable SMX-resistant bacteria, followed by the heterotrophs at 2-Pre-O_3. At 4-SF, the proportion of resistant HPC was as little as 0.004% and does not show up in the graph, whereas the one of the coliforms appears at 0.9%

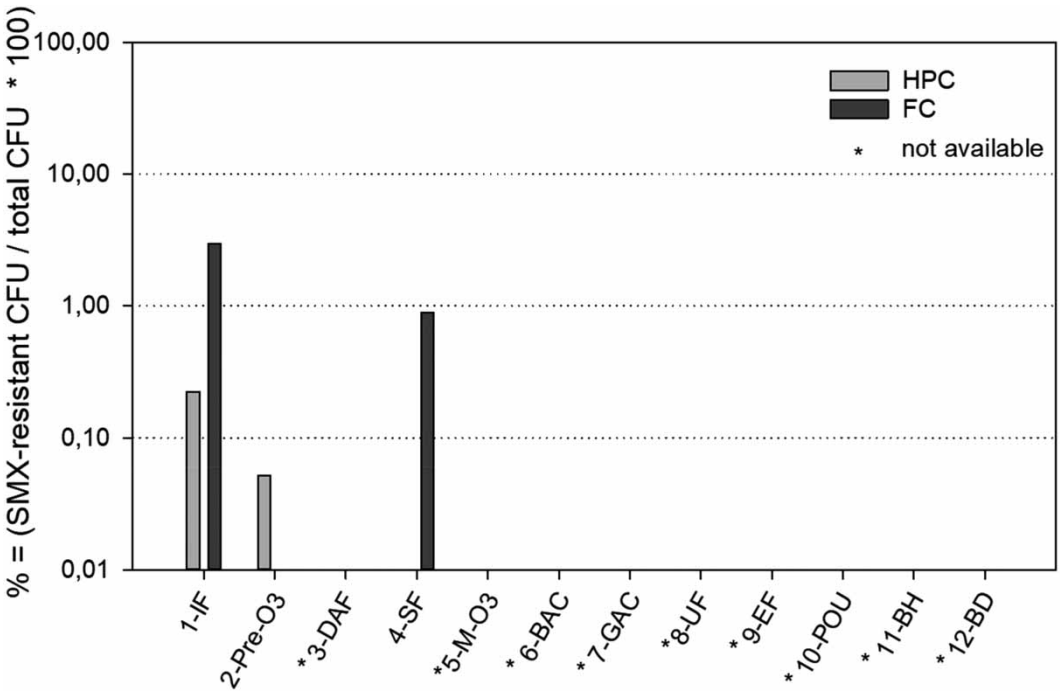


Figure 3 | Number of resistant CFU in relation to the total CFU.

and is considerably higher. Of the ratios that could be obtained, the coliforms have a proportionally higher share and occurrence of resistance to SMX compared with the HPC.

Gene quantification

Quantification of intracellular resistance genes and absolute copy numbers

The ARG *sul1, ermB, vanA, nptII* and *nptIII* were quantified by TaqMan real-time PCR in order to evaluate the effects of the treatment train and individual treatment units on their abundance. The results of the negative controls are listed in the Supplementary Material and suggest that there is a *sul1* background at Cp 37.7 and a 16S background that arises as of Cp 34.3. Consequently, this leads to an inability to detect lower gene abundances for the respective genes in samples with Cp-values of that or higher. LOD and LOQ for all sampling points and genes investigated are summarized in the Supplementary Material.

Treatment train from raw feed to sand filtration. Gene copies per millilitre at all sampling sites are depicted in Figure 4 and summarized in the Supplementary Material. The resistance gene *ermB* was present at an abundance of 1.2×10^2 copies/mL in the influent 1-IF, but already was below LOQ at 2-Pre-O_3 and below LOD at all other sampling sites. *VanA* was below LOD at all sampling sites.

NptII and *nptIII* were detected in the influent 1-IF at abundances below the LOQ and were not detectable at all other respective sampling sites.

As expected, *sul1* was by far the most abundant resistance gene and present at most steps in the treatment train allowing for the intended evaluation of the LRVs in the subsequent treatment steps. *Sul1* was detected at an abundance ranging from 1.55×10^5 copies/mL at 1-IF to levels under the LOD at 9-EF, which is an LRV reduction of at least 5.6 for the whole treatment train. Whereas an insignificant rise in the *sul1* abundance between 1-IF and at 2-Pre-O_3 could be observed, it dropped by about two orders of magnitudes at 3-DAF to 2.73×10^3 copies/mL (LRV 1.8), followed by 4-SF resulting in gene abundance of as little as 1.45×10^2 copies/mL (LRV 1.3). Hiller *et al.* (2019) point out that there is no consistency in LRV of sand filtration in the 17 studies they compared, but the average LRV reported in this study is within the range of our findings.

Against expectations, the change in the abundance of *sul1* behaved differently compared with the reduction of the bacterial 16S rRNA marker gene among the different treatment steps. Whereas the LRV of 16S genes between 2-Pre-O_3 and 4-SF is in the range of 1 LRV per treatment step, the removal of *sul1* copies is steadily increasing from below 1 LRV after 2-Pre-O_3 to close to 2 after sand filtration, indicating a continuous gradual decrease of the relative abundance of *sul1* resistance genes.

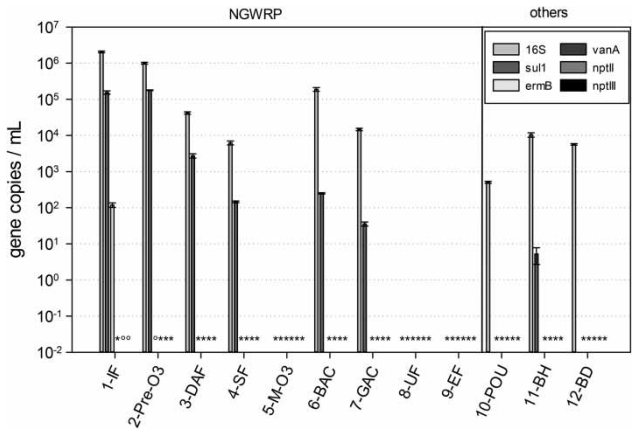


Figure 4 | Absolute gene abundance in copies/mL of the resistance genes *sul1*, *ermB*, *vanA*, *nptII*, *nptIII* and bacterial marker gene 16S rRNA, error bars indicate the mean and standard deviation of three technical replicates in one qPCR run, <LOQ marked '○'; <LOD marked '*'.

Main ozonation

The highest removal for *sul1* of at least 2.5 was achieved by the main-ozonation treatment reducing the *sul1* gene abundance to below LOD. Since ozonation conditions are optimized for the local water matrix and therefore vary in different treatment plants, a direct comparison of LRVs is difficult. Stange *et al.* (2019) report 4.3–4.6 log reduction of ARG in a laboratory-scale test with *E. coli* and an ozone dose of 1 mg/L, which is a higher reduction efficiency with ten times less ozone concentration than that at the main ozonation at NGWRP. Quite the opposite was found, when Zhuang *et al.* (2015) investigated different disinfection procedures on the effluent of a municipal WWTP in

China. The authors report that an ozone concentration of 27 mg/L only resulted in a log reduction of 0.6 on 16S rRNA genes. Increasing LRV were achieved by raising the ozone dose up to 98 mg/L.

Examples from the literature with contradicting results indicate that for assessment of the efficiency of ozonation for both, the removal of organic trace substances and microbial parameters, the ozone concentration alone is not a suitable parameter. It has to be considered that the UW matrix (mainly suspended solids, organic compounds and NO_2) consumes oxygen radicals depending on its constituents' kinetic reaction constant for ozone (k_{O3}), which weakens the disinfective effect. In contrast to drinking water ozonation, where the organic background is usually very low and therefore of no significance, the specific ozone dose has to be considered for the ozonation of UW instead of the ozone concentration in mg/L. The specific ozone dose relates the ozone concentration to the DOC background of the water treated resulting in mg O_3 per mg DOC. 0.4–1 mg O_3/mg DOC are usually applied in advanced WW treatment. If no specific ozone dose is provided – which is the case in most publications – results based on ozone concentrations alone cannot be compared.

At the NGWRP typically a specific ozone dose of 3–3.5 mg O_3/mg DOC is applied (Lahnsteiner *et al.* 2018). This is 3–7.5 times higher than in aforementioned advanced WW treatment. The reason is that the primary aim in DPR is the reliable inactivation of virus and protozoa needing higher specific ozone concentrations. During ozonation disinfection, oxygen radicals interact with the cell surface inactivating its function but rarely oxidize the cell's interior content (in contrast to UV disinfection). This may be the underlying reason for the observation that the LRV for bacterial counts is 3.2 compared with 2.6 for 16S rRNA genes and 2.5 for *sul1* in the same sample.

BAC and GAC. The absolute abundance of the *sul1* gene (2.49×10^2 copies/mL) and the 16S rRNA genes (1.88×10^5 copies/mL) increased significantly after the biological activated carbon step (6-BAC LRV_{16S} −4.1, LRV_{sul1} −2.8) whereas the relative abundance stayed in the same order of magnitude as before the main-ozonation treatment (see Table 14 in the Supplementary Material). This is also in accordance with the CFU results that show resistant HPC and coliforms under the LOD after the BAC even though the total bacterial counts increased (LRV_{HPC} −5.3, LRV_{FC} −2.8).

The opposite was reported by Xu *et al.* (2016) in their study of a DWTP that employs similar technologies to the ones at NGWRP. They suggest that their observed increase in relative abundance may have been due to selection pressure that was acting on the bacteria growing in the activated carbon from adsorbed antibiotic micropollutants. In conclusion, the BAC does not remove ARG but instead gene abundance returned to the value upstream of the main ozonation. This is likely due to the bacterial community proliferating on the carbon particles. Subsequently, the 7-GAC results in a log reduction of 0.8 to 3.56×10^1 copies/mL.

UF. *Sul1* resistance genes were below LOD at 8-UF (LRV 1.9), 9-EF, 10-POU, 12-BD and 5.25×10^0 copies/mL at 11-BH. Ultrafiltration has recently been shown to be the most promising removal technology for microcontaminant separation including ARB and ARG (Fatta-kassinos *et al.* 2015). Experiments on ozonation and ultrafiltration reduction efficiencies for ARB and ARG with real WW by Hembach *et al.* (2019) showed that the ultrafiltration was able to reduce *sul1* abundance to below the LOD (LRV 6-7). This was achieved by a membrane cut-off (20 nm) half of that at NGWRP. The reduction efficiency of this technology strongly depends on the cut-off/nominal pore size of the applied membrane (Hiller *et al.* 2019). A considerable disadvantage of this technology is its non-destructive nature. Hembach *et al.* (2019) found that the retentate water contained 2 log units higher concentrations of ARB and ARG than the influent. Considering ARB and ARB removal in DWT facilities, treating the backwash water separately could be an option to increase the removal efficiency, yet the potential of this needs to be further investigated.

The effects of the final treatment steps – chlorination and pH-stabilization – on the genes cannot be deduced since even for *sul1* results were below LOD after ultrafiltration. Stabilization by chlorination is an important measure to prevent the regrowth of pathogens (including ARB) in storage tanks and the drinking water distribution system. Generally, chlorination has been suggested to have a relatively consistent reduction efficiency on *sul1* resistance genes of below 1.5 LRV (Hiller *et al.* 2019).

All reductions were statistically significant except for the pre-ozonation treatment at 2-Pre-O_3. There were no outliers identified for all gene quantification results.

Relative abundance of the *sul1* resistance gene

Figure 5 shows the number of *sul1* resistance genes per millilitre normalized to the number of bacterial 16S rRNA genes in each sample. This provides a proportional representation of the resistance gene relative to the bacterial load of each sample indicating the potential selection of ARBs by operational conditions. As only *sul1* was detected throughout an extended number of treatment steps (see Figure 4), relative abundance was only calculated for *sul1*.

S*ul1* gene copy numbers related to 16S rRNA gene numbers show a ratio between 0.0001 at 11-BH and 0.008 in the influent 1-IF to NGWRP. The proportion rises to 0.18 after the pre-ozonation treatment at 2-Pre-O_3. It is also visible that the proportion of resistance gene is larger in samples before the main-ozonation treatment than afterwards. The difference in proportion of ARG at 1-IF, 2-Pre-O_3, 3-DAF and 4-SF is less than one order of magnitude but differs by roughly one magnitude between samples before and after 5-M-O_3. As noted earlier, it is suggested that the main-ozonation treatment has a strong effect on the relative abundance of the resistance gene.

Log removal values

LRV for gene copies were calculated as stated in the 'Methods' section for *sul1* and 16S rRNA and results shown in Figure 6 and in the Supplementary Material.

The NGWRP achieved a total log reduction of at least 5.6 for the 16S rRNA gene, 5.1 for the *sul1* and 2.6 for the *ermB* resistance gene. The LRV for the other resistance genes could not be calculated due to concentrations below LOD after the first treatment step. All treatment units decreased both the *sul1* and 16S gene abundance, except for the biological activated carbon which was to be expected

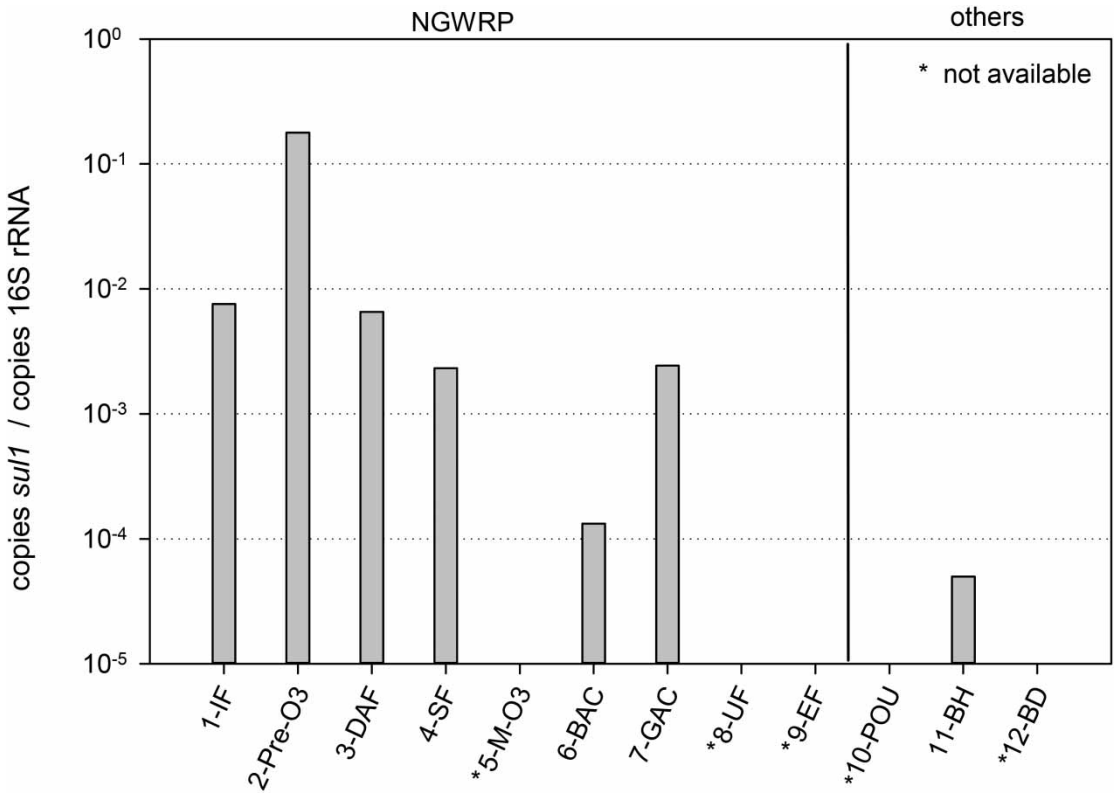


Figure 5 | Copies of resistance genes per mL normalized to the number of bacterial 16S rRNA genes per mL at all sampling sites; '*': no calculation possible due to numbers < LOD.

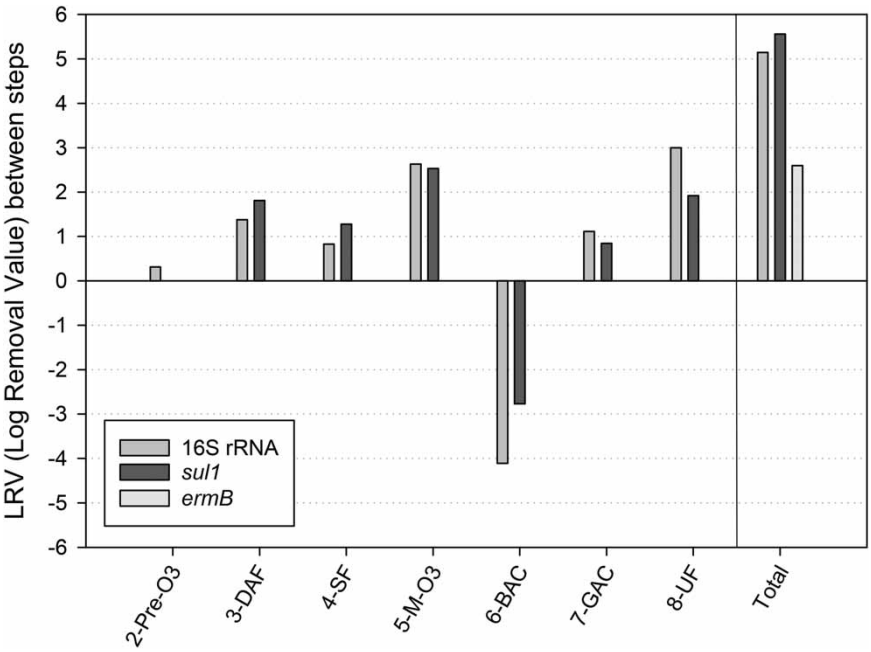


Figure 6 | LRV of the individual treatment units and the total removal of the NGWRP on the intracellular 16S rRNA gene, *sul1* and *ermB* resistance gene.

since its function is based on the microbial community on the carbon particles.

The NGWRP effectively reduces the abundance of intracellular resistance genes below the LOD. Furthermore, the results of the 16S rRNA gene quantification indicate that the NGWRP reduces the bacterial load to undetectable levels confirming HPC results. This is far below levels found in the two other drinking water sources used for the same supply area: the borehole water (11-BH) and Von Bach Dam (12-BD). In terms of water quality regarding AR, the results propose that controlled aquifer recharge with the final product of NGWRP is unlikely to cause a detectable increase in the abundance of ARB and ARG in the aquifer water and thus could be considered safe.

In that regard, it has to be mentioned that based on the intrinsic analytical approach by real-time PCR, quantification of gene numbers technically represents gene fragments between the primer pair and not necessarily functional genes that would be required for AR phenotypic gene expression. This means that real-time PCR tends to overestimate the presence of functional ARGs by amplifying gene fragments too. On the other hand, this potential over estimation represents a worst case scenario for gene numbers and allows us to follow LRV over a wider range of the treatment train. Furthermore, it reinforces the result that at the end of the NGWRP, no functional ARG can be detected any more, supporting the efficiency of the treatment train for the removal of ARGs.

Extracellular *sul1* resistance gene

Free DNA may facilitate transformation through direct uptake of extracellular resistance genes (Zhang *et al.* 2018), and it is assumed that the treatment train causes a release of cellular DNA through bacterial cell death. Therefore, all treatment train samples as well as the reference sample 10-POU were analyzed for the presence of extracellular *sul1* resistance gene by TaqMan real-time PCR. However, this analysis was limited due to the novel free DNA extraction approach applied, missing information of the extraction efficiency and a relatively high *sul1* background signal that was detected in the NCex at a Cp-value of 35.2. Therefore, all samples with a Cp of that or higher were considered to be below detectable levels. Only the first sampling point 1-IF with 8.5×10^3 and 3.9×10^4 copies/mL respectively (Cp 31.6 and 30.8) showed free extracellular *sul1* copies above that threshold. The concentrations of all other samples were

beyond the background signal. Thus, only the influent 1-IF to the NGWRP could be evaluated for the presence of the extracellular *sul1* gene. Raw data can be found in the Supplementary Material.

Although there are not many resources reporting on the quantity of free DNA in treated WW, two sources report similar results. Zhang *et al.* (2018) also identified the presence of free *sul1* resistance genes of roughly 10^4 copies/mL in the secondary effluent of a municipal WWTP. Similarly, Yuan *et al.* (2019) report on the presence of free *sul1* resistance genes in the secondary effluent of a WWTP in China with 10^3 copies/mL. Since the detection of the gene is below background levels in all samples of the treatment train except in the influent, it is assumed that the NGWRP had a decreasing effect on free *sul1* resistance genes as early as in the first pre-ozonation step. Yet, this decrease cannot be quantified, and neither can the reduction efficiency of the individual treatment technologies. Furthermore, the treatment train did not lead to a detectable amount of free *sul1* resistance gene released from SMX-resistant bacteria dying off during the treatment train. Both the removal of about 10^2/mL SMX-resistant heterotrophs (see Figure 2) and intracellular sul1 genes from the inflow during the treatment train (see Figure 6) could potentially have resulted in a release of extracellular *sul1* copies that was not detected. A huge amount of uncertainties lie in the use of the magnetic beads extraction method for free DNA with the MagNa Pure isolation kit in such complex matrices as WW. The method shows potential but needs to be improved and evaluated for its performance and suitability for variable sample quality parameters. The analysis can further be broken down into whether or not the free DNA is attached to other particles. This may influence the effectiveness of treatment methods and its stability in the environment. For example, adsorbed free DNA may be caught on membranes more efficiently along with the particle whereas the flexibility of small linear DNA fragments may result in lower retention (Slipko *et al.* 2019). However, Yuan *et al.* (2019) show that the percentage of particle-associated *sul1* resistance genes in the secondary effluent was roughly 90% of the total *sul1* abundance. According to this, the separation between the two fractions would not have made an essential difference to for this study.

CONCLUSION

In order to gain an understanding of the impact of combined water treatment technologies on the removal of ARB and ARG, this study quantified viable bacteria possessing resistance to SMX and ARG *sul1*, *ermB*, *vanA*, *nptII* and *nptIII* after each treatment step at NGWRP and of three additional drinking water sources in Windhoek, Namibia. The resulting observations aim to provide a suggestion for possible combinatory treatment technologies that can be employed to reduce the discharge of ARB and ARG into potable reuse systems or the environment.

Regarding the research questions, the main findings of this study can be summarized as follows:

- The NGWRP statistically significantly reduced the abundance of total and SMX-resistant heterotrophic bacteria as well as total and SMX-resistant coliforms down to undetectable levels below LOD in the final effluent.
- All treatment technologies had a reducing effect on heterotrophic bacteria except for the pre-ozonation and the biological activated carbon. Furthermore, all treatment technologies had a reducing effect on coliform bacteria except for the biological activated carbon and the granular activated carbon. Both of these observations were not unexpected due to the technical setup of the two technologies and their task in the treatment train.
  The main ozonation and the ultrafiltration had the highest reduction efficiencies.
  There were no SMX-resistant heterotrophs or coliforms detected downstream the main-ozonation treatment nor at POU.
- The NGWRP reduced the abundance of intracellular *sul1* resistance genes to undetectable levels below LOD.
- All treatment technologies decreased the abundance of intracellular *sul1* resistance genes except for the pre-ozonation and the biological activated carbon.
  The main ozonation and the ultrafiltration had the highest reduction efficiencies.
- Despite removal below LOQ being observed for extracellular *sul1*, methodological limitations regarding efficiency and yield of extracellular DNA recovery from samples did not allow us to follow the removal of

ARGs located on free extracellular DNA throughout the whole treatment train.

The results show that the advanced multi-barrier system for potable reclamation at NGWRP reduced SMX-resistant bacteria and the investigated *resistance* genes to undetectable levels below LOD (LODs for individual sampling sites, see Supplementary Material). This shows that an advanced technical treatment train is able to remove ARB as well as ARG to levels below 1 copy per 100 mL and with this it may be possible to reach recommended prospective removal targets in the future.

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DATA AVAILABILITY STATEMENT

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

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