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Elevated levels of antibiotic resistance in groundwater during treated wastewater irrigation associated with infiltration and accumulation of antibiotic residues

Ioannis D. Kampouris [a,*], Nikiforos Alygizakis [b,c], Uli Klümper [a], Shelesh Agrawal [d], Susanne Lackner [d], Damiano Cacace [a], Steffen Kunze [a], Nikolaos S. Thomaidis [c], Jaroslav Slobdonik [b], Thomas U. Berendonk [a,*]

[a] Technische Universität Dresden, Institute of Hydrobiology, Chair of Limnology, Zellescher Weg 40, 01062 Dresden, Germany
[b] Environmental Institute, Okružná 784/42, 97241 Koš, Slovak Republic
[c] Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis Zografou, 15771 Athens, Greece
[d] Technical University of Darmstadt, Institute IWAR, Chair of Wastewater Engineering, Franziska-Braun-Straße 7, 64287 Darmstadt, Germany

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ABSTRACT

Treated wastewater irrigation (TWW) releases antibiotics and antibiotic resistance genes (ARGs) into the environment and might thus promote the dissemination of antibiotic resistance in groundwater (GW). We hypothesized that TWW irrigation increases ARG abundance in GW through two potential mechanisms: the contamination of GW with resistant bacteria and the accumulation of antibiotics in GW. To test this, the GW below a real-scale TWW-irrigated field was sampled for six months. Sampling took place before, during and after high-intensity TWW irrigation. Samples were analysed with 16S rRNA amplicon sequencing, qPCR of six ARGs and the class 1 integron-integrase gene *intI1*, while liquid chromatography tandem mass spectrometry was performed to detect antibiotic and pharmaceutical residues. Absolute abundance of 16S rRNA in GW decreased rather than increased during long-term irrigation. Also, the relative abundance of TWW-related bacteria did not increase in GW during long-term irrigation. In contrast, long-term TWW irrigation increased the relative abundance of *sul1* and *intI1* in the GW microbiome. Furthermore, GW contained elevated concentrations of sulfonamide antibiotics, especially sulfamethoxazole, to which *sul1* confers resistance. Total sulfonamide concentrations in GW correlated with *sul1* relative abundance. Consequently, TWW irrigation promoted *sul1* and *intI1* dissemination in the GW microbiome, most likely due to the accumulation of drug residues.

1. Introduction

Over the past years, several studies reported that pharmaceutical residues, including antibiotics, and antibiotic resistance genes (ARGs) remain in treated wastewater (TWW) in relatively high concentrations (Caucci et al., 2016; Alygizakis et al., 2020). Thus, TWW discharge has the potential to release high loads of antibiotics and ARGs into the environment (Caucci et al., 2016; Berendonk et al., 2015; Cacace et al., 2019; Alygizakis et al., 2020). Therefore, concerns have emerged regarding the spread of antibiotic resistance through agricultural practices involving TWW, such as TWW irrigation and managed aquifer recharge (MAR) (Guo et al., 2017; Smalla et al., 2018). Specifically, several studies have investigated the impact of TWW irrigation on ARG prevalence in soil, crops and subsoil pore water (Wang et al., 2014; Han et al., 2016; Dalkmann et al., 2012; Jechalke et al., 2015; Cerqueira et al., 2019a, 2019b, 2019c; Marano et al., 2019; Kampouris et al., 2021a, 2021b). However, the number of studies investigating the prevalence of ARGs in groundwater (GW) environments below TWW irrigated agricultural fields is limited. Szekeres et al. (2018) found that GW wells close to human settings exhibited elevated levels of ARGs, presumably due to anthropogenic pressures. Further, tetracycline and erythromycin ARGs were identified in the GW of MAR sites (Böckelmann et al., 2009b). On the contrary, the ARGs bla_{TEM} and *qnrS* occurred in TWW used in a MAR site in Israel, but did not appear in the GW (Elkayam et al., 2018).

Furthermore, the presence of ARGs in TWW is regularly connected

* Corresponding authors.
E-mail addresses: ioannis.kampouris@tu-dresden.de (I.D. Kampouris), thomas.berendonk@tu-dresden.de (T.U. Berendonk).

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with that of the integrase gene *intI1* (Marano et al., 2019; Gatica et al., 2016), which is associated with mobile genetic elements (MGEs) and horizontal gene transfer (HGT) (Gillings, 2017). Usually, several, diverse ARGs are part of the mobile class 1 integron gene cassettes alongside *intI1* (Gatica et al., 2016). The increase in *intI1* relative abundance is not only related to ARG dissemination, but also to stress experienced by bacterial communities connected to anthropogenic activities, such as exposure to high loads of antibiotics, pharmaceuticals, disinfectants and metals (Gillings, 2017). Moreover, stress can increase the mobility potential of MGEs (Klümper et al., 2017; Wang et al., 2019). Previous studies reported that carbamazepine, a non-antibiotic drug that induces bacterial stress responses and promotes HGT (Wang et al., 2019), has been detected in high concentrations in GW during TWW irrigation (Ternes et al., 2007; Lesser et al., 2018).

In addition to carbamazepine, antibiotics have regularly been detected in GW environments in Europe, the US and Asia (Szekeres et al., 2018; Barber et al., 2009; Avisar et al., 2009; Chen et al., 2018). Among those, sulfonamides, a class of antibiotics of synthetic origin (Underwood et al., 2011a, 2011b), occurred in high concentrations in TWW (Johnson et al., 2015). Sulfonamide class antibiotics were able to persist in GW environments, especially during TWW irrigation or MAR operations (Barber et al., 2009; Avisar et al., 2009; Underwood et al., 2011a, 2011b; Szekeres et al., 2018). Among those, the antibiotic sulfamethoxazole has occurred in GW samples with concentrations reaching up to 1100 ng/L (Barber et al., 2009). These high concentrations are close to the predicted no effect concentration (PNEC) for positive selection of sulfamethoxazole-resistance (16,000 ng/L) (Bengtsson-Palme and Larsson, 2016). Nevertheless, these PNECs are predicted based on models that depend on data from cultivated bacteria, while GW environments contain a high fraction of yet-to-be-cultivated bacteria (Anantharaman et al., 2016). Thus, whether selection for sulfamethoxazole-resistance in GW bacterial communities could occur in lower concentrations than predicted from the current PNEC models remains to be elucidated.

Previously we found that TWW irrigation increased the ARG abundance in topsoil and subsoil pore-water microbiota at a depth of 1.2 m (Kampouris et al., 2021a, 2021b). However, it remained unclear whether TWW irrigation can similarly affect the much deeper lying GW environment. Thus, we here investigated the impact of TWW irrigation on ARG and *intI1* dissemination and prevalence in GW at ten-meter depth of a real-scale and commercially operated TWW irrigated field. Such an impact would be possible through two main hypothesized mechanisms: (a) the infiltration of ARG carrying bacteria from TWW irrigation into the GW and (b) the accumulation of antibiotics from TWW in the GW. The hypothesized mechanisms were tested by sampling the GW of the commercially operated field subjected to irrigation with secondary TWW. The GW was sampled over a period of six months, prior, during and after high-intensity TWW irrigation. ARG and *intI1* dynamics were determined using quantitative Real Time PCR (qPCR). 16S rRNA high throughput sequencing was performed to examine the bacterial community structure. Further, the infiltration of drug residues from TWW to GW was determined using liquid chromatographic separation with tandem mass spectrometric detection (LC-MS/MS).

2. Materials and methods

2.1. Sampling

2.1.1. Description of the sampling area

Samples were collected from the facilities of Braunschweig Wastewater Association (BWA) (Paranychianakis et al., 2015; Ternes et al., 2007). BWA is one of the few companies that commercially operate TWW irrigation in real-scale agricultural fields in Germany. The BWA is located in Wendeburg (Lower Saxony, Germany) and performs the treatment of municipal wastewater in the local area. The Urban Wastewater Treatment Plant (UWTP) of BWA receives approximately 60,000 m^3 WW/d, a population equivalent of 350,000. TWW is then used for irrigation of the agricultural field in question. The soil of the field (sandy soil, cambisol, N: 52.359500, E: 10.399833; Fig. S1) contains a high percentage of sand (over 90% percent) (Ternes et al., 2007; Kampouris et al., 2021a, 2021b). The soil is deficient in nutrients and hence usually not suitable for systematic agricultural use. The farmers counter the lack of nutrients through TWW irrigation (Paranychianakis et al., 2015). Further information regarding the soil and its physicochemical characteristics can be found in Ternes et al. (2007) and Kampouris et al. (2021a). Irrigation is performed with TWW subjected to conventional secondary biological treatment. Occasionally, the TWW is mixed with digested sludge (TWW & DS), depending on the nutrient demand of crops (rye, maize or rapeseed) and amount of natural precipitation. The crops are grown for the production of biogas.

2.1.2. Sampling

The GW sampling started at the end of June 2018 (28/06/2018). June 2018 was chosen as the starting point for this study, as the potential effect of previous TWW irrigation in the eight month leading up to the study was minimized, considering the commercial operation of the field. The last irrigation event ahead of the start of the study occurred in the middle of March (14/03/2018). Thus, no irrigation occurred in the three months immediately leading up to the study. In addition, the field was not irrigated from October 2017 until February 2018, while during February-March 2018 only low intensity irrigation occurred. During this period, three individual irrigation events took place, with a total volume of irrigation water per irrigation event of 35 mm^3 per m^2.

From the beginning of the study in the end of June 2018 to the middle of August 2018 the field was intensively irrigated with TWW & DS. Irrigation then switched to only TWW until October 2018, in accordance with the farmers' plan for crop-cultivation. The field was, during this period, irrigated 10–14 times per month, resulting in 350–420 mm^3/m^2/month. Samples were taken prior to the start (June 2018), after one (July 2018) and three months (September 2018) of high-intensity irrigation. Irrigation ceased at the start of October 2018. A final sample was taken approximately two months after the irrigation break in December 2018. At each time-point, three samples each were taken from three GW wells (n = 9) located in the field at a depth of 10 m. One additional sample was taken from each well for drug residue profile analysis.

Water samples were stored on ice and transferred to the lab immediately. Bacteria from water samples were captured by filtration (polycarbonate, 0.2 μm pore size, 47 mm diameter, Sartorius, Germany) within 24 h of sampling. The filtration volume was 0.5 L for irrigation water and 2.5 L for GW samples. Additionally, samples from July, September and December 2018 were stored at −20 °C for subsequent chemical analysis. Due to the severe heat wave during June 2018 the volume of sampled GW in June 2018 was insufficient for performing both DNA extraction and preserving samples for chemical analysis. Thus, no additional sample from June 2018 was taken and stored for LC-MS/MS.

2.2. Liquid chromatography tandem mass spectrometry (LC-MS/MS)

2.2.1. Chemicals and reagents

Acetonitrile (ACN) and Methanol (MeOH) LC–MS grade was purchased from Merck (Darmstadt, Germany). Formic acid (FA) with purity 99% was obtained from Sigma–Aldrich, Fluka (Buchs, Switzerland). Distilled water was provided through Milli-Q purification (Millipore Direct-Q UV, Bedford, MA, USA). Atlantic HLB-M disks were purchased from Labicom (Olomouc, Czechia) and RC syringe filters (4 mm diameter, 0.2 μm pore size) from Phenomenex (Torrance, CA, USA).

2.2.2. Sample preparation and instrumental analysis

The sample preparation protocol involved sample clean up and preconcentration by 4000 times using automatic solid phase extraction by

HORIZON SPE-DEX 4790. The conditioning and extraction program for the preparation of the samples can be found in Table S4. Extracts were evaporated using a gentle stream of nitrogen and reconstituted to 250 μL (50:50 methanol:water). Before analysis, extracts were filtered through RC syringe filters of 4 mm diameter and 0.2 μm pore size.

Instrumental analysis was performed with a Thermo UHPLC Accela system connected to a triple quadrupole (TSQ Quantum Access, Thermo Electron Corporation, USA) equipped with an electrospray ionization source (Thermo IonMAX) in positive mode. Chromatographic separation was accomplished on an Atlantis T3 C18 column (100 mm × 2.1 mm, 3 μm) from Waters (Milford, MS, USA) at a constant flow rate of 100 μL min^{-1}. The mobile phase, the gradient elution programs and the ESI parameters are presented in Table S5. Identification and quantification were performed under selected reaction monitoring (SRM) mode. The transitions between the precursor ion and the two most abundant product ions were recorded for all target compounds. This allows achieving four identification points per compound (2002/657/EC). SRM transitions for each substance were optimized by infusion of standard reference solutions at average concentration levels of 1 mg L^{-1}. The optimized ionization mode, fragmentation voltages and collision energies for each antibiotic (41 in total) are summarized in Table S6. Thermo LCquan 2.7 (CA, USA) was used to analyze the LC-MS/MS data. More details about the instrumental method can be found in Thomaidis et al. (2016).

2.3. DNA extraction, qPCR and sequencing

DNA extractions were performed using the DNeasy PowerWater Kit (Qiagen, Germany) according to the manufacturer's instructions. The quantity and quality of DNA was measured by NanoDrop (Thermo Fischer Scientific, Germany). Quantitative real-time PCR (qPCR) analysis was performed for eight genes (*sul1*, *intI1*, *qnrS*, *tet*(M), bla_{OXA-58}, bla_{TEM}, $bla_{CTX-M-32}$ and 16S rRNA). The selection of genes was based on the framework for TWW monitoring established by the NEREUS (www.nereus-cost.eu) and ANSWER-ITN (www.answer-itn.eu) networks (Rocha et al., 2020; Cacace et al., 2019). In short, the genes *sul1*, *qnrS* and *tet*(M) and bla_{OXA-58} were selected due to their clinical importance and high abundance in TWW across European countries (Caucci et al., 2016; Cacace et al., 2019; Alygizakis et al., 2020) and TWW irrigated soil (Kampouris et al., 2021a). The two final β-lactamase genes bla_{TEM} and $bla_{CTX-M-32}$ occur in low abundance in TWW (Cacace et al., 2019), while they have shown natural prevalence in soil microbiota (Gatica et al., 2015; Kampouris et al., 2021a). Apart from the ARGs, the class 1 integron-integrase gene *intI1* is commonly used as a genetic marker for anthropogenic pollution and frequently part of mobile gene cassettes that carry ARGs (Gatica et al., 2016). Reactions were performed on a MasterCycler RealPlex (Eppendorf, Germany) at 20 μL final volume with 10 μL of 2x Luna Universal qPCR Master Mix (New England Biolabs, Germany) and 20 ng sample DNA per reaction. Further details about reagents, primers and annealing temperature for each gene are given in Tables S2 and S3. Standard curves with an efficiency of 0.9–1.1 and $R^2 \geq 0.99$ were accepted (Table S3), and melting curve analysis was performed to assess the amplicons' specificity.

The lowest dilution in the qPCR standard curve that still retained a positive and specific amplification signal was set as the limit of quantification (LOQ). Depending on the gene target, the LOQ varied. For example, 16S rRNA gene had a LOQ of 4000 copies per reaction, while *sul1* had a LOQ of 40 copies per reaction and $bla_{CTX-M-32}$ of 4 copies per reaction. Details for the remaining genes are given in Tables S2 and S3. Screening for potential PCR inhibition was performed by spiking a plasmid containing a gene, which was rarely detected in the samples at very low abundance ($bla_{CTX-M-32}$, spiking concentration $4*10^6$ copies/μL). The absolute abundance for each gene was finally calculated from the filtrated volume, the dilution factor (copies/L) and the relative abundance of each gene was calculated as the ratio of gene copies per copy of the 16S rRNA gene.

The GW/TWW-Group replicates were pooled (in equimolar concentrations) with a final concentration of 5 ng/μL and were analysed with the 16S Ion Metagenomics Kit™ (Thermo Fisher Scientific, Germany) for amplification and sequencing of multiple parallel variable regions. The protocols for 16S rRNA library preparation for parallel variable regions sequencing and processing of the sequences were described previously in Orschler et al. (2019). Briefly, for each sample, two PCR reactions were prepared, one for each of the 2 primer pools included in the 16S Ion Metagenomics Kit™ (Thermo Fisher Scientific, Germany). Each PCR reaction consisted of 3 μL 10X primer mix, 6 μL of sample, 15 μL 2X mastermix, 6 μL nuclease free water. PCR conditions included initial denaturation at 95°C for 10 min, 20 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 20 s; and a final cycle of 72 °C for 7 min 100 nanograms of each amplicon were processed to the amplicon library using the Ion Xpress Plus Fragment Library Kit™, and each sample was tagged using Ion Xpress Barcode Adapters™ (Thermo Fisher Scientific), according to the manufacturer's protocol. Each sample library was adjusted to a 10 picomolar concentration. All samples were pooled in equal volumes and processed using the ION Chef system (Thermo Fisher Scientific) according to the manufacturer's instructions. Sequencing was performed on the Ion Torrent (ION Torrent Ion S5) using a 530 chip. Base calling and run demultiplexing were conducted in Torrent Suite version 4.4.2 (Thermo Fisher Scientific) with default parameters. Raw sequencing data was submitted to the sequencing read archive (SRA) (bioproject accession number: PRJNA713765).

2.4. Data processing and statistical analyses

Every sample below LOQ was placed at 1 copy/L for absolute abundance and 10^{-8} relative abundance (one order of magnitude below the minimum possible relative abundance $\sim 10^{-7}$). The data was $\log_{10}$-transformed prior to any graphical representation or statistical analysis. The program R (R Core Team, 2019; v. 3.5.3) was used for graphical representations and statistical analyses. Specifically, the packages "ggplot" (Wickham, 2016, v.3.3) and "ggpubr" (v. 0.2.2, Kassambara, 2019) were used for the generation of plots. Significant differences were assessed with the Wilcoxon rank sum test or Student's *t*-test and in case of group comparisons with the Kruskal-Wallis test (package "ggpubr"). Correlations were analysed with Kendall rank correlation (package "ggpubr"). Multiple comparisons were performed with Dunn's test and Benjamini-Hochberg correction (package "dunn's test", v1.3.5, Dinno, 2016) to assign significant differences from pairwise comparisons. Comparisons with p-values below 0.05 were considered statistically significant ($\alpha = 0.05$).

Sequencing data was analysed using the DADA2 package (v 1.14.1, Callahan et al., 2016), by filtering the low quality sequences, excluding reads longer than 260 bp, while filtering potential chimeric sequences. Then, operational taxonomical units (OTU) were picked based on de novo clustering at 97% identity, corresponding to species level. Sequences were classified based on the SILVA taxonomy database (97% confidence threshold, version 132, Quast et al., 2013). For the analysis and graphical representation of bacterial community data, the package "phyloseq" (McMurdie and Holmes, 2013) was used.

3. Results

3.1. Bacterial abundance in groundwater did not increase due to TWW irrigation

To evaluate whether groundwater (GW) is polluted through infiltration of TWW associated bacteria during TWW irrigation events, both, the irrigation water and the GW were analyzed with qPCR and 16S sequencing. The TWW & DS irrigation water contained the highest absolute bacterial abundance of 16S rRNA gene (10.0 ± 0.1 $\log_{10}$ copies/L, Fig. 1A). Both, TWW irrigation water (9.7 ± 0.2 $\log_{10}$ copies/L) and GW prior to irrigation (9.5 ± 0.2 $\log_{10}$ copies/L) had significantly lower

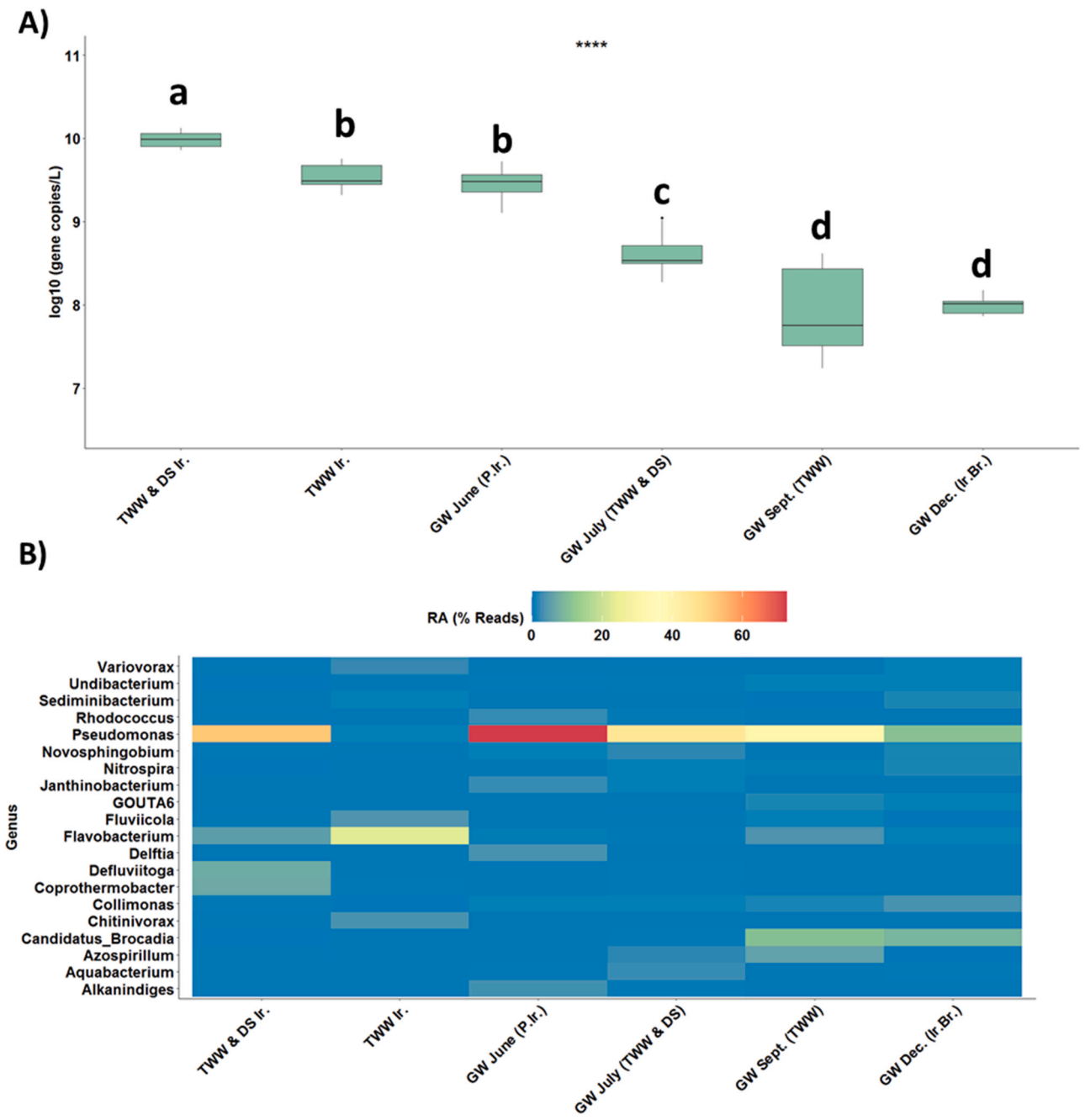

Fig. 1. A) Absolute abundance of 16S rRNA (gene copies/L) in the groundwater and the irrigation waters, based on the qPCR analysis. Kruskal-Wallis test, $p = 1.4*10^{-12}$, n = 9. Letters from "a" to "d" were assigned to non-significantly different groups after multiple pairwise comparison with Dunn's test along with Benjamini-Hochberg correction. The p-value cut-off for significance was set at 0.05. B) Relative abundance (% reads) of the 20 most abundant bacterial genera of irrigation waters and groundwater, based on the 16S amplicon sequencing. Given in parenthesis is the status of irrigation during each groundwater sampling. Ir.= Irrigation, P.Ir.=Prior Irrigation, GW=Groundwater, TWW = Treated Wastewater, DS = Digested sludge, Ir. Br. = Sampling after the two-month irrigation break.

absolute bacterial abundance than TWW & DS irrigation water ($p < 0.05$, Dunn's test, n = 9, Fig. 1A), but were insignificantly different from one another ($p > 0.05$, Dunn's test, n = 9, Fig. 1A).

Rather than an increase, a gradual decrease of GW bacterial abundance occurred during long-term, high-intensity TWW irrigation. Specifically, the absolute bacterial abundance decreased from 9.5 ± 0.2–8.6 ± 0.2 $\log_{10}$ copies/L, during the first month of irrigation (June-July 2018, $p < 0.05$, Dunn's test, n = 9, Fig. 1A). After three months of irrigation (September 2018), only 7.9 ± 0.5 $\log_{10}$ copies/L were detected, significantly less than in July 2018 ($p < 0.05$, Dunn's test, n = 9, Fig. 1A). After the two-month irrigation break (December 2018) the absolute abundance of 16S rRNA gene increased slightly, but not significantly to 8.0 ± 0.1 $\log_{10}$ copies/L compared to September 2018 ($p > 0.05$, Dunn's test, n = 9, Fig. 1A). Despite the high absolute numbers of bacteria introduced into the soil through continuous, intensive TWW/TWW & DS irrigation, no increase in bacterial abundance in the underlying GW environments occurred.

To further verify the limited bacterial contamination of GW due to TWW irrigation, the bacterial community profiles of TWW, TWW & DS and GW samples were analyzed (Fig. 1B). No increase of TWW related genera occurred in the GW during long-term irrigation (Fig. 1B). For example, *Pseudomonas* relative abundance showed a linear but non-significant reduction one month after the start of irrigation (72.6–45.4%), three months after irrigation (45.1–31.3%) and after the

irrigation break (31.3–10.7%) (Mann-Kendall test, $\tau = -1$, $p = 0.089$, Fig. 1B). The genus *Escherichia/Shigella*, commonly used as fecal indicator bacteria was present in TWW (0.1%) and TWW & DS (0.01%), but not detected in GW as a consequence of irrigation. Consequently, direct bacterial contamination of GW with TWW-related bacteria during TWW irrigation was limited.

3.2. Elevated concentrations of sulfamethoxazole and carbamazepine in the groundwater of the TWW irrigated field

Apart from bacteria, TWW contains drug residues that can infiltrate into the GW. Thus, the drug-residue profile of irrigation waters and GW was analyzed. As expected, the irrigation waters contained several antibiotic and non-antibiotic pharmaceutical residues. The most frequent class of detected antibiotics was sulfonamides, especially sulfamethoxazole detected at concentrations of 83.5 ng/L in TWW & DS (July 2018) and 61.85 ng/L in TWW (September 2018, Fig. 2). Besides sulfamethoxazole, doxycycline (tetracycline) was present in high concentrations (194.5 ng/L) in TWW & DS (July 2018) but not detected in TWW (September 2018, Fig. 2). Other antibiotics detected in TWW/TWW & DS included lincomycin (macrolide), metronidazole (nitroimidazole) and ofloxacin (quinolone) (Fig. 2). Further, a multitude of non-antibiotic pharmaceuticals (e.g. carbamazepine, ibuprofen and hydrochlorothiazide) were present in the irrigation water (Fig. 2).

However, only a minor fraction of the drug residues was able to infiltrate and persist in the GW during irrigation (Fig. 2). The compounds detected in GW were lincomycin, metronidazole, ofloxacin, carbamazepine, ibuprofen, hydrochlorothiazide as well as several sulfonamide class antibiotics (Fig. 2). However, the concentrations of the majority of drug residues was low, close to the LOQ of the chemical analysis method. An exception were four compounds: carbamazepine, ibuprofen, hydrochlorothiazide and sulfamethoxazole were abundant in the GW in high concentrations after previously being identified in irrigation water in high concentrations as well (Fig. 2). Among these four, carbamazepine and sulfamethoxazole displayed the highest concentrations in GW (Fig. 2). Specifically, sulfamethoxazole concentrations increased significantly during TWW irrigation from 98.2 ± 39.8 ng/L (July 2018) to 301.9 ± 33.8 ng/L (September 2018, Student's *t*-test, $p = 0.0057$, $n = 3$) (Fig. 2). Surprisingly, even after a two months irrigation break, the concentrations remained significantly elevated but with far higher variation among replicates (406.9 ± 204.0 ng/L, Fig. 2).

While carbamazepine concentrations were equally high, with concentrations of 272.3 ± 185.1 ng/L (July 2018), 168.5 ± 18.9 (September 2018) and 183.8 ± 101.4 ng/L after the irrigation break (Fig. 2), no significant increase with prolonged irrigation was detected. Thus, sulfamethoxazole and carbamazepine co-occurred as the main drug-residue contaminants in the GW, with only sulfamethoxazole increasing during TWW irrigation. Consequently, while TWW-related bacteria did not infiltrate the GW, several drug residues did and even persisted after the irrigation break.

3.3. TWW irrigation promotes sul1 and intI1 dissemination in groundwater

To evaluate whether TWW irrigation promotes the dissemination of ARGs in the GW, irrigation waters and GW samples were analyzed by qPCR. TWW and TWW & DS irrigation water contained the tested ARGs as well as the integrase gene *intI1*, with the exception of the bla_{TEM} gene (Fig. S2). The genes *intI1* and *sul1* showed the highest relative abundance in the irrigation waters (*intI1:* -1.8 ± 0.5 *sul1:* -2.0 ± 0.5 $\log_{10}$ copies/16s rRNA, Fig. S2). The genes *qnrS,* bla_{OXA-58} and *tet*(M) showed one-order of magnitude lower relative abundance than *sul1* and *intI1* (*qnrS*: -3.4 ± 0.8, bla_{OXA-58}:-3.2 ± 0.7 & *tet*(M):$- 3.8 \pm 0.3$ $\log_{10}$ copies/16S rRNA; Fig. S2). The gene $bla_{CTX-M-32}$ had the lowest abundance among detected genes in irrigation water (-4.7 ± 0.9 $\log_{10}$ copies/16S rRNA).

Of the detected genes in the irrigation water, the relative abundances of *sul1* and *intI1* increased significantly and continuously in GW during TWW irrigation (Kruskal Wallis, $p < 0.0001$, $n = 9$, Fig. 3A). Between June and July 2018, the relative abundance of *sul1* slightly but not significantly increased from -6.0 ± 2.1 to -3.9 ± 0.5 $\log_{10}$ copies/16s rRNA (Dunn's test, $p > 0.05$, $n = 9$, Fig. 3A). The observed increase

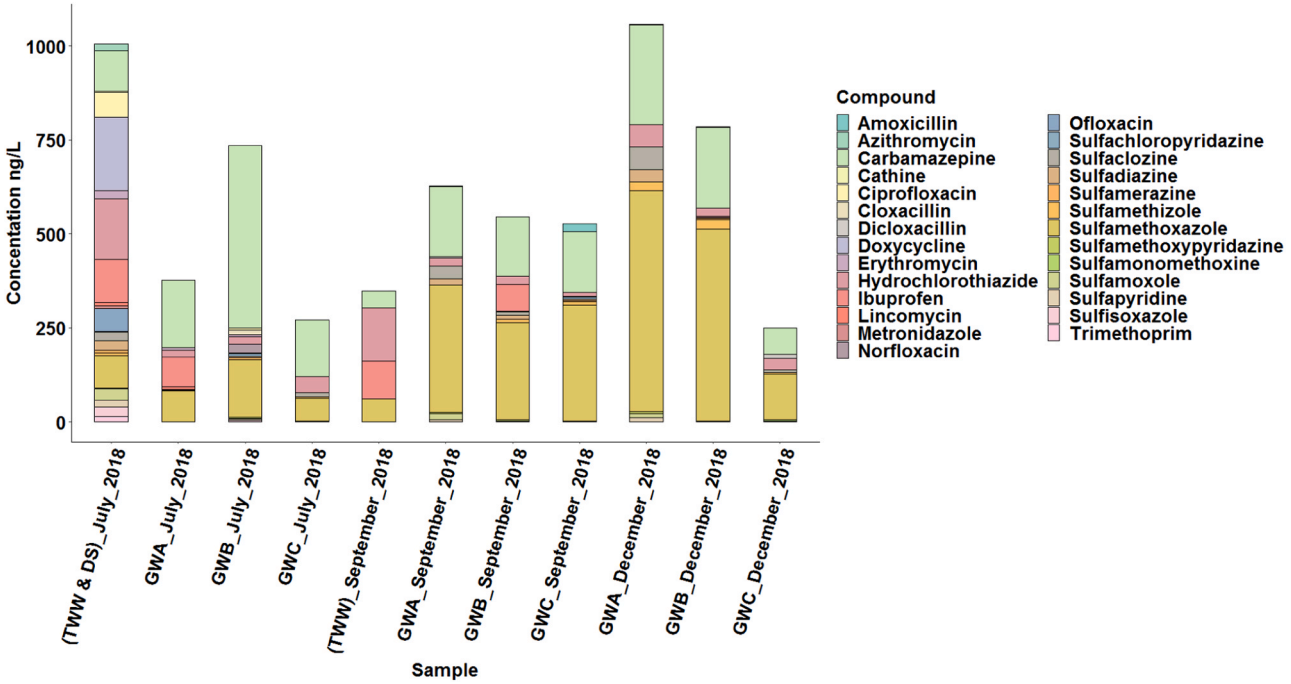

Fig. 2. Concentration of antibiotic and non-antibiotic drug residues detected in the irrigation waters and the respective groundwater wells (GWA, GWB and GWC) from July 2018 (one month of high intensity irrigation), September 2018 (three months of high intensity irrigation) and December (two months after irrigation break). GW = Groundwater, TWW = Treated Wastewater, DS = Digested sludge.

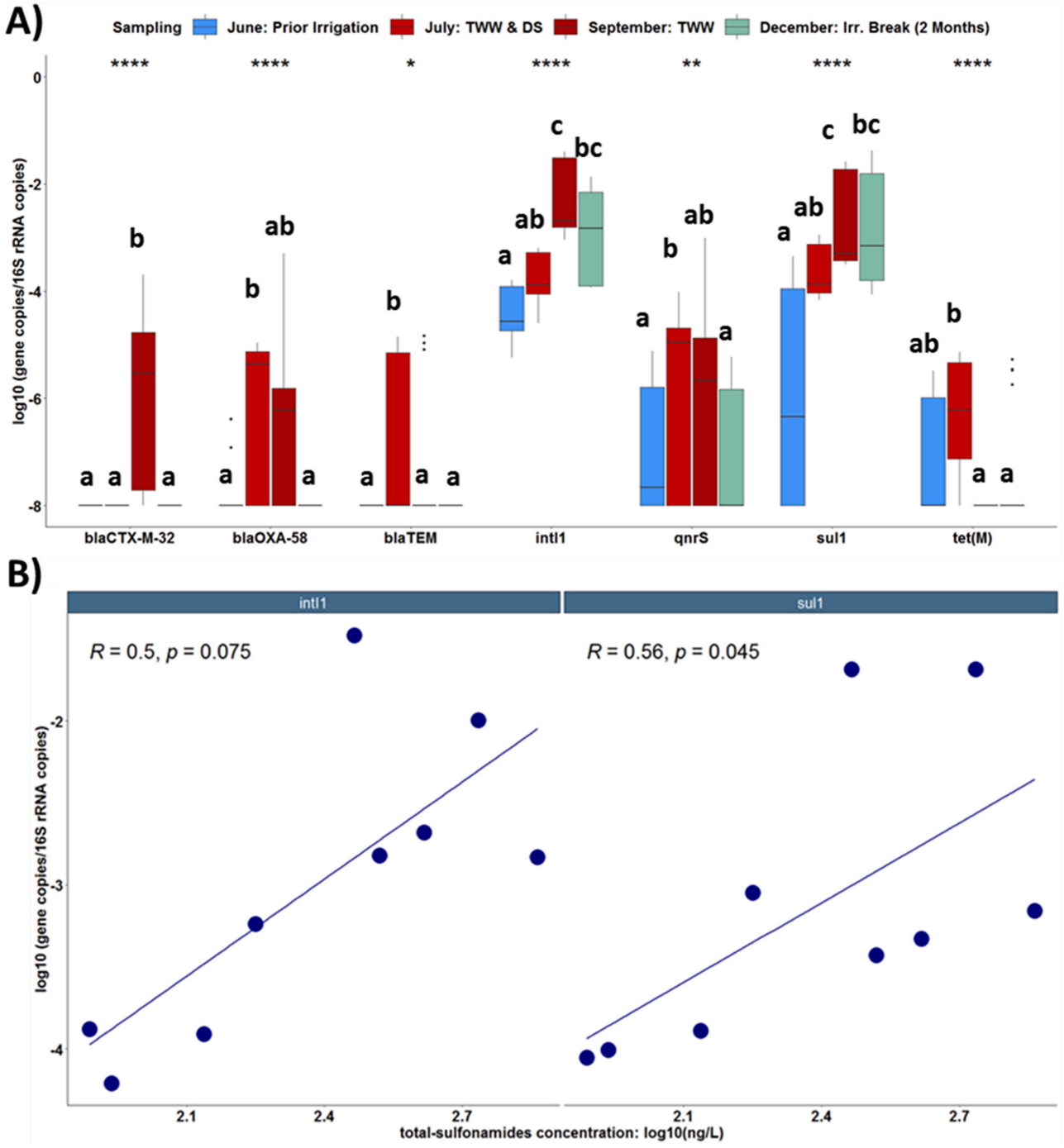

Fig. 3. A) Log_{10} transformed relative abundance of ARGs and *intI1* to 16s rRNA gene copies. The samples were taken from the groundwater of the selected field (depth 10 m). The sampling started from June 2018 (prior irrigation) and lasted until December 2018. We sampled in one (June 2018) and three months (September 2018) of high intensity irrigation. The high intensity irrigation was continued until end of October 2018. A last sampling took place in December 2018, two months after the irrigation operation ceased. TWW=Treated Wastewater, DS= Digested Sludge; Kruskal-Wallis test *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, $n = 9$. Letters from "a" to "c" were assigned to non-significantly different groups after multiple pairwise comparison with Dunn's test along with Benjamini-Hochberg correction. B) Kendall rank correlation of the median *sul1* and *intI1* relative abundance of each well (Fig. 3A) and the total sulfonamide concentration (ng/L) of each sampled well (Fig. 3B) (total $n = 9$).

continued after three months of irrigation to -2.8 ± 0.8 log_{10} copies/16S rRNA, leading to a significant difference when compared to June 2018 (Dunn's test, $p < 0.05$, $n = 9$, Fig. 3A). Despite the irrigation break, *sul1* relative abundance remained stable at 2.9 ± 1.0 log_{10} copies/16S rRNA and was significantly higher than prior to the irrigation-start (Dunn's test, $p < 0.05$, $n = 9$, Fig. 3A). Kendall rank correlation was performed with *sul1* relative abundance (average abundance per well) and total concentration of sulfonamide class antibiotics in each well. The *sul1* relative abundance correlated significantly with total concentration of sulfonamide class antibiotics ($R = 0.56$, $p = 0.045$, $n = 9$, Fig. 3B).

The integrase gene *intI1,* followed similar patterns in terms of relative abundance as *sul1*. The *intI1* relative abundance increased slightly but not significantly between June and July 2018, from -4.4 ± 0.5 to

-3.7 ± 0.4 $\log_{10}$ copies/16S rRNA (Dunn's test, $p > 0.05$, $n = 9$, Fig. 3A). This increase continued after three months of irrigation to -2.3 ± 0.6 $\log_{10}$ copies/16S rRNA, significantly higher than June and July 2018 samplings (Dunn's test, $p < 0.05$, $n = 9$, Fig. 3A). A slight decrease occurred after the two-month irrigation break to -2.9 ± 0.8 (Fig. 3A). Despite this decrease, the *intI1* relative abundance in GW remained significantly higher when compared to prior the start of TWW irrigation (Dunn's test, $p < 0.05$, $n = 9$, Fig. 3A). However, *intI1* relative abundance did not significantly correlate with total concentrations of sulfonamide class antibiotics ($R = 0.5$, $p = 0.057$, $n = 9$, Fig. 3B).

Regarding the remaining ARGs, the relative abundance of *qnrS* displayed a limited increase during TWW irrigation. The *qnrS* relative abundance significantly increased from -7.0 ± 1.2 to -5.8 ± 1.6 copies/16S rRNA (Dunn's test, $p < 0.05$, $n = 9$, Fig. 3A) and remained stable at -5.8 ± 1.7 copies/16S rRNA, after three months of irrigation (September 2018). Other than *sul1* and *intI1*, its relative abundance decreased significantly down to -7.1 ± 1.2 copies/16S rRNA after the two-month irrigation break (Dunn's test, $p < 0.05$, $n = 9$, Fig. 3A). No specific patterns were observed for any of the other tested genes. In addition, the relative abundance of any of the remaining ARGs did not correlate with the total concentration of any of the antibiotic classes ($p > 0.05$, $n = 9$, Fig. S3).

Therefore, during long-term TWW irrigation *qnrS* slightly increased in the GW, while *sul1* and *intI1* relative abundance showed a stronger, consistent and significant increase. In addition, *sul1* abundance correlated with the total concentration of sulfonamide class antibiotics, indicating a contribution of sulfonamide persistence to *sul1* dissemination.

4. Discussion

Despite the importance of groundwater (GW), ARG dynamics within GW environments remain underexplored. Here, we demonstrated that TWW irrigation promoted the spread of ARGs, specifically *sul1* and *intI1*, in GW environments. After irrigation, GW contained elevated concentrations of sulfonamide class antibiotics (especially sulfamethoxazole), which correlated with the relative abundance of *sul1*. Thus, TWW irrigation increased ARG abundance corresponding to the accumulation of antibiotics, supporting the second hypothesized mechanism of TWW irrigation impacts on ARG in GW.

TWW, subjected to conventional biological wastewater treatment only, regularly contains high amounts of human opportunistic pathogens, including fecal indicator bacteria, such as *E. coli* (Petousi et al., 2019). This has raised initial concerns regarding the contamination of GW reservoirs through the bacterial load introduced by TWW irrigation (Ayni et al., 2011). In the present study, no increase in the absolute bacterial abundance of GW due to TWW irrigation was observed. Moreover, the genus *Escherichia/Shigella*, commonly used as fecal indicator bacteria, was identified in TWW but not detected in the GW microbiome after irrigation. Accordingly, Elkayam et al. (2018) reported that TWW-related opportunistic pathogens do often not persist during soil passage. Absolute bacterial abundance decreased in the GW during continued TWW irrigation. To our knowledge, such log-fold fluctuations of absolute bacterial abundance prior and after long-term irrigation in GW have not been reported yet. A knowledge gap remains on whether the herein observed decrease was directly related to TWW irrigation or whether the absolute bacterial abundance in the GW microbiome fluctuates naturally over long-term periods. Follow up long-term studies in agriculturally-impacted and pristine GW environments could tackle this gap.

Bacteria introduced through TWW irrigation were most likely outcompeted by indigenous microbes and did not invade the GW microbiome. This trend was previously shown for the microbiomes of TWW irrigated soil and subsoil pore-water and was here confirmed for GW microbiota (Kampouris et al., 2021a, 2021b). This further supports that soil filtration during TWW irrigation could serve as a suitable low-cost additional barrier option for TWW bacteria. Despite the incapability of TWW bacteria to persist in soil (Marano et al., 2019; Obayomi et al., 2019; Kampouris et al., 2021a), TWW bacteria can still potentially transfer their ARGs to soil bacteria (Kampouris et al., 2021a). These gene transfer events can then result in ARG carrying bacteria infiltrating the subsoil (Kampouris et al., 2021b) and ultimately reaching the GW.

While retention of TWW bacteria was achieved during soil passage, the occurrence of several micro-contaminants in GW suggests that drug-residues introduced through TWW irrigation are not equally well retained. Especially sulfamethoxazole and carbamazepine displayed elevated concentrations, close to TWW irrigation levels. Similar elevated sulfamethoxazole concentrations have been reported in groundwater wells during a monitoring study for baseline pharmaceutical concentrations across the USA (Barnes et al., 2008) and in a phreatic aquifer of TWW irrigation in Israel (Avisar et al., 2009). Sulfonamide class antibiotics, such as sulfamethoxazole, are not fully eliminated during wastewater treatment (Göbel et al., 2005), and have previously been detected in investigations of this specific field (Ternes et al., 2007). Specifically, sulfamethoxazole occurred in elevated concentrations in the GW of the TWW irrigated field, with a sharp increase from one to three months of irrigation. Sulfamethoxazole even persisted at high levels in the GW matrix after a two-month irrigation break. This persistence can be explained by low biodegradation rates compared to other pharmaceuticals due to the synthetic nature of sulfonamide class antibiotics (Underwood et al., 2011a, 2011b). Still, observed sulfamethoxazole concentrations (406.9 ± 204.0 ng/L) remained far below the PNEC values predicting selection of sulfamethoxazole resistance in environmental bacteria (16,000 ng/L) (Bengtsson-Palme and Larsson, 2016). Hence, positive selection for sulfonamide resistance might be possible at concentrations lower than previously suggested. However, in complex environmental habitats it remains difficult to mechanistically disentangle the observed antibiotic resistance dynamics.

The second main contaminant, carbamazepine, has been reported in GW during TWW infiltration at high concentrations as well (Clara et al., 2004), but here did not significantly increase during irrigation. A few other drug residues (lincomycin, metronidazole, ofloxacin, carbamazepine, ibuprofen, hydrochlorothiazide and several sulfonamide class antibiotics) persisted TWW infiltration in the present study, however, in much lower concentrations.

TWW irrigation promoted the dissemination of *sul1* and *intI1* in the GW microbiome, due to the accumulation of drug residues in GW. The *sul1* gene confers resistance to the detected sulfonamide class antibiotics through encoding of a dihydropteroate synthase, which has low affinity for sulfonamides and can hence bypass its inactivation (Reis et al., 2018). The *sul1* gene is frequently present in TWW as one of the most abundant ARGs (Cacace et al., 2019; Kampouris et al., 2021a,b;). Remarkably, *sul1* relative abundance in GW increased so significantly during long-term irrigation that it ultimately reached similar levels as detected in the irrigation waters. The increase of *sul1* abundance was positively correlated with concentrations of sulfamethoxazole and total sulfonamide class antibiotics in the GW. Thus, the introduction and persistence of sulfonamide class antibiotics and especially sulfamethoxazole through TWW irrigation provides a mechanistic explanation behind the successful dissemination of *sul1* in GW.

Apart from *sul1,* the integrase gene *intI1* increased during long-term TWW irrigation. The *sul1* gene is frequently part of mobile *intI1* gene cassettes (Gillings, 2017). Thus, co-selection may occur for *intI1,* due to the positive selection pressure *sul1* is subjected to from sulfonamide class antibiotics. Furthermore, consistent GW bacterial community profiles during irrigation supports that HGT might be the main contributor to *sul1* and *intI1* dissemination, since no significant addition of TWW-related bacteria occurred. Carbamazepine, which occurred in high concentrations in GW is known to function as bacterial stressor and can at elevated levels enhance plasmid transfer (Wang et al., 2019, 2020). Thus, the occurrence of carbamazepine in GW, might additionally accelerate the dissemination of *intI1* and *sul1*. However,

mechanistically-oriented experiments are needed to disentangle the contributions of selection, co-selection and horizontal gene transfer connected to sulfamethoxazole and carbamazepine pollution on the *sul1* and *intI1* dynamics in GW.

TWW remains a necessary countermeasure for depleting freshwater resources in semi-arid and arid areas (Ternes et al., 2007; Maaß and Grundmann, 2016; Paranychianakis et al., 2015). GW environments are important freshwater reservoirs and common freshwater resources. Since drug residues are able to persist during soil filtration and reach the GW in high concentrations, we recommend that TWW should only be used for irrigation after the successful elimination of drug residues. Concentrations of these contaminants need to be reduced to ensure minimal risks of TWW irrigation to GW environments. This can be achieved by low-cost measures (e.g. long hydraulic retention time) (Ejhed et al., 2018), which could further support the aspect of the green and circular economy of TWW reuse.

Here, the use of TWW subjected to secondary (biological) treatment, which usually contains a high bacterial load (Cacace et al., 2019; Manaia et al., 2018), seemed to cause negligible bacterial infiltration into the GW. However, the overlying soil and especially crops that come into direct contact with TWW bacteria could be impacted more significantly (Libutti et al., 2018; Tripathi et al., 2019; Petousi et al., 2019). Opportunistic pathogens and pathogenic bacteria (e.g. *Salmonella* spp. or *E. coli)* may colonize crops (Jechalke et al., 2015; Araújo et al., 2017), including fresh produce (Blau et al., 2018). For example, total coliform abundance has shown to be increased on grapes of vineyards irrigated with TWW subjected to secondary treatment only (Petousi et al., 2019). The occurrence and survival of enterohemorrhagic *E. coli* of fecal origin in crops has caused severe bloody diarrhea outbreaks across the world, leading to increased hospitalization and death rates (Viazis and Diez-Gonzalez, 2011). However, the accumulation of bacteria on plants is less of a problem, when irrigated crops are directly utilized for biogas production, as bacteria do not reenter the human microbiome through the food chain. Thus, in case crops and especially fresh produce are intended for food consumption, TWW irrigation with high bacterial load still might pose several risks for human health (Blau et al., 2018; Petousi et al., 2019).

In the present study, we confirmed that TWW irrigation promotes the dissemination of the sulfonamide ARG *sul1* along with the integrase gene *intI1* to GW microbiota. Additionally, the correlation of *sul1* relative abundance with total sulfonamide concentrations in GW provides a mechanistic explanation behind *sul1*'s successful dissemination. Therefore, further monitoring and reduction of sulfonamides and *sul1* in TWW could minimize the impact on GW environments during TWW irrigation. By overcoming these impacts, the proper use of TWW irrigation as a necessary countermeasure against freshwater and especially GW resources depletion can be ensured.

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CRediT authorship contribution statement

Ioannis D. Kampouris: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Nikiforos Alygizakis**: Investigation, Writing – review & editing. **Uli Klümper:** Formal analysis, Methodology, Writing – review & editing. **Shelesh Agrawal:** Investigation, Writing – review & editing. **Susanne Lackner:** Resources, Writing – review & editing. **Damiano Cacace:** Methodology, Investigation. **Steffen Kunze:** Methodology, Investigation. **Nikolaos S. Thomaidis:** Resources. **Jaroslav Slobdonik:** Resources. **Thomas U. Berendonk:** Funding acquisition, Resources, Conceptualization, Writing – review & editing, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

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