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Occurrence of Antibiotic-Resistant Genes and Bacteria in Household Greywater Treated in Constructed Wetlands

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Abstract: There is a growing body of knowledge on the persistence of antibiotic-resistant genes (ARGs) and antibiotic-resistant bacteria (ARB) in greywater and greywater treatment systems such as constructed wetlands (CWs). Our research quantified ARGs (*sul1*, *qnrS*, and bla_{CTXM32}), class one integron (*intI1*), and bacterial marker (16S) in four recirculating vertical flow CWs in a small community in the Negev desert, Israel, using quantitative polymerase chain reaction (qPCR). The greywater microbial community was characterized using 16S rRNA amplicon sequencing. Results show that CWs can reduce ARG in greywater by 1–3 log, depending on the gene and the quality of the raw greywater. Community sequencing results showed that the bacterial community composition was not significantly altered after treatment and that Proteobacteria, Epsilonbacteraeota, and Bacteroidetes were the most dominant phyla before and after treatment. Pseudomonas, Citrobacter, Enterobacter, and Aeromonas were the most commonly identified genera of the extended spectrum beta lactamase (ESBL) colonies. Some of the ESBL bacteria identified have been linked to clinical infections (*Acinetobacter nosocomialis*, *Pseudomonas fulva*, *Pseudomonas putida*, *Pseudomonas monteilii*, and *Roseomonas cervicalis*). It is important to monitor *intI1* for the potential transfer of ARGs to pathogenic bacteria.

Keywords: antibiotic resistance; greywater; constructed wetlands; greywater treatment systems; extended spectrum beta lactamase

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1. Introduction

Household greywater is commonly reused in remote and water-stressed regions because it has a lower content of fecal matter than sanitary sewage, reducing the risks associated with microbial contaminants. Although greywater has lower risks and is widely used for non-potable purposes, there are potential biological health risk concerns due to bacteria shed from skin, and small amounts of fecal matter and urine released during bathing [1]. Greywater typically consists of effluent from laundries, sinks, baths, and showers. It may also include water from kitchen sinks but does not include water from toilets. Greywater is reused to irrigate edible and non-edible crops, dust suppression, and toilet flushing [1,2]. While raw greywater is typically not suitable for direct reuse, simple onsite greywater treatment technologies can be operated and maintained by homeowners. Thus, the reuse of treated greywater can address the combined challenges of water scarcity and water quality while conserving freshwater resources.

Constructed wetlands (CWs) are a practical and effective technology for onsite greywater treatment and reuse with low maintenance requirements. These systems typically function as extensive biofilters that incorporate wetland vegetation and natural biofilm carrier materials, such as gravel. Without using constructed wetlands or other wastewater treatment systems, irrigation with raw greywater can lead to temporary soil hydrophobicity,

disrupt soil microbiome communities, and persist in groundwater [3–5]. However, a review found that treated greywater does not seem to negatively affect soil microbiomes [6].

Effluents from CWs are typically reused onsite, without the need for extensive reclaim water conveyance systems [1,7–9]. Recirculating vertical flow CWs (RVFCWs) are a type of CW design that have been shown to treat greywater economically [10]. RVFCWs also reduce water losses by evapotranspiration, which is important in arid regions [10].

Although not yet regulated worldwide, there are still unknown health risks associated with greywater reuse due to the potential release of antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic-resistant genes (ARG) [1]. ARG and ARB are worldwide concerns within public health and engineering [11,12]. It is projected that by 2050, the number of deaths from ARB-related infectious diseases will outnumber all current causes of death [13]. The World Health Organization has developed a plan to mitigate the spread of ARG-related infectious diseases by reducing consumption of antibiotics and increasing research related to antimicrobial resistance surveillance. Although only a limited number of household scale RVFCWs were studied, this research adds to the growing body of work related to ARG and ARB surveillance worldwide.

Wastewater generally contains low levels of antibiotics; however, these low levels can trigger a resistance response from bacteria [14–16]. There are a few possibilities for the emergence of ARB and ARG in greywater, from human excretions, food, or the evolution of resistance in greywater due to exposure of microbes to micropollutants, such as biocides and antibiotics present in the untreated greywater [17]. Contaminants such as heavy metals and disinfectants can also promote antibiotic resistance in bacteria [18–20]. For example, exposure of greywater bacteria to the biocide triclosan increased ARB in irrigated soil [21]. Triclosan, among other micropollutants, was detected in greywater at a significant level in Israel [17]. Additionally, antibiotics such as ciprofloxacin and sulfamethoxazole were detected at concentrations ranging from 1.3 to 1593 ng/L in greywater used for irrigation in the West Bank, Palestinian Territories [22].

Although it has previously been demonstrated that CWs effectively remove conventional pollutants (TOC, COD, TSS, among others) and pathogenic bacteria from greywater [23,24], there is limited information on the efficacy of CWs for ARG and ARB removal. We hypothesized that raw greywater contains diverse ARGs that are removed during treatment and that microbial diversity is modified within CWs concurrently with chemical and microbial pollutants. In this study, changes in the raw and treated greywater microbial community were evaluated in parallel with the overall performance of onsite CWs for the treatment of conventional pollutants. Concentrations of specific ARGs (*sul1*, *intI1*, bla_{CTXM32}), corresponding to resistance to sulfonamides, beta-lactams, and fluoroquinolone antibiotics, were enumerated in raw and CW-treated greywater. The presence of extended-spectrum beta-lactam (ESBL) resistant bacteria was examined, and their phylogenetic distribution in raw and treated greywater was analyzed. The results have implications for arid regions where greywater is treated in CWs before reclamation for irrigation and other reuse applications. Thus, the overall goal of this study was to investigate the presence of ARB and ARG in greywater and their fate in CWs.

2. Materials and Methods

2.1. System Description and Sampling

Four RVFCWs were examined in this study (CW1, CW2, CW3, CW4). The systems were located at single-family households inhabited by 4–6 people (parent and adolescents) in Israel's central Negev desert. Greywater from each household was first collected in a 400 L sedimentation basin (raw greywater) from which it was pumped into the RVFCW two times per day to achieve a maximal hydraulic retention time of 12 h. Each RVFCW was composed of two containers with an upper and lower portion composed of two 500 L plastic containers (1.0 m × 1.0 m × 0.5 m) placed atop each other (Figure 1). The upper perforated container contained a planted three-layer bed, a 5 cm top layer of woodchips, followed by a 35 cm middle layer of tuff gravel and a 10 cm lower layer of limestone

pebbles. The lower container served as a reservoir with a hydraulic retention time of 8 h. Greywater trickled from the upper container through the biofilter medium to the lower container and was recirculated back to the upper container. The recirculation rate in the RVFCW was 300 L/h [25]. After 15 or more recirculation cycles, the effluent was discharged for landscape irrigation reuse. Greywater from these households consisted of effluents from laundries, showers, and bathroom sinks. Detailed descriptions of the RVFCWs have been published previously [10,25,26]. Samples were collected monthly over three months between August and October (overall 12 samples of raw greywater and 12 of treated greywater) and brought to the laboratory for analysis within 30 min of collection. A total of 1 L of raw and 1–2 L of treated greywater were collected from each household during each sampling campaign.

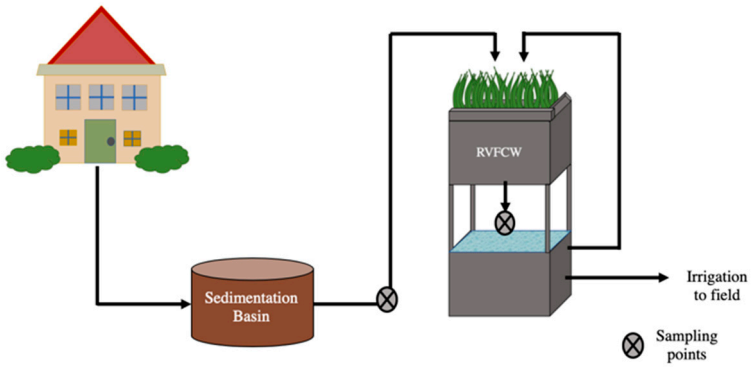

Figure 1. Recirculating vertical flow constructed wetland schematic.

2.2. Water Quality Analysis

Standard methods [27] were used to measure 5-day biochemical oxygen demand (BOD_5) (5210), total suspended solids (TSS) (2540), pH (4500-H^+), and conductivity (2510). Turbidity was measured using a Sper Scientific Turbidity Meter-860040 (Scottsdale, Arizona, AZ, USA). Total organic Carbon (TOC) and total nitrogen (TN) were measured using an Analytik Jena TN/TOC analyzer [28,29].

2.3. Microbial Enumeration

HiCrome agar media (HiMedia, Mumbai, India) was used to enumerate *E. coli* using the spread plate method (9215C) [27]. After spreading decimal dilutions, the plates were first incubated at 30 °C for 4 h, then incubated at 44 °C for 20 h. CHROMagar™ ESBL plates (Hylabs®, Rehovot, Israel) were used to enumerate ESBL producing bacteria. ESBL agar comprised Digalski and MacConkey agar containing cefotaxime and ceftazidime, respectively. The spread plate method was used to spread 100 µL of decimal dilutions of raw and treated greywater on the plates. Plates were incubated for 20–24 h at 37 °C. Colonies were identified based on their color and morphology and were then purified on LB plates and identified based on their 16S rRNA gene sequences [30].

2.4. DNA Extraction and ARG Analysis

DNA was extracted from untreated and treated greywater samples using Qiagen DNeasy PowerWater Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. All extracted DNA quantity and quality were measured using a Nanodrop Spectrophotometer ND-1000 (Wilmington, DE, USA). Polymerase chain reaction (PCR) and quantitative PCR (qPCR) were used to determine the presence and number of genes in the raw and treated greywater. ARGs quantified in this study were *qnrS*, bla_{CTXM32}, and *sul1* (Table 1). These genes were selected because they correspond to commonly prescribed antibiotics and their frequent detection in wastewater samples [31–33]. Other genes used in the analysis were 16S rRNA and class 1 integrase (*intI1*) (Table 1). A list of primers and sequences is shown in Table 1. The Bioline master mix was SYBR No-ROX mix. qPCR was

run on CFX96 Real-Time System C1000 Thermal Cycler from Biorad (Hercules, CA, USA). All efficiency values were between 90 and 110%. All samples and standards were run in duplicate or triplicate. Standards were diluted from plasmid pNORM1 ranging from 2.11 $\times$ 10^2–2.11 $\times$ 10^7 gene copies/µL DNA [31]. Negative controls were an enzyme, primers, and DNA-free water. DNA from PCR and qPCR runs were examined in 2% agarose gel electrophoresis using Bio-Rad PowerPac Basic (Hercules, CA, USA). Gels were viewed and photographed using Bio-Rad Gel Doc XR+ Documentation System/Universal Hood II (Hercules, CA, USA) with software Quantity One Version 4.6.9 (Basic, Hercules, CA, USA).

2.5. Raw and Treated Greywater Microbial Community Analysis

The microbial community in the raw and treated greywater was analyzed using 16S rRNA gene amplicon sequencing [34]. DNA from the raw and treated greywater was amplified by PCR before sequencing. The reaction was performed in a Life ECO PCR System (Bio-Rad Laboratories); each 20 µL PCR reaction contained 10 µL Bio-Ready-Mix (Bio-lab, Jerusalem, Israel) primers (200 nM) CS1-341F ACACTGACGACATGGTTCTACACCTACGGGAGGCAGCAG and CS2-806R TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT [35] and an average of 11 ng templet DNA. PCR thermocycler conditions were 5 min at 95 °C, followed by 26 cycles of 95 °C (30 s) → 55 °C (45 s) → 68 °C (30 s), and finalizing the reaction with 68 °C (7 min).

2.6. Sequencing and Statistical Analysis

Sequencing and primary processing of the raw data were performed by the University of Illinois at Chicago Core for Research Informatics (UICCRI). Sequencing of 16S rRNA was done using the Illumina MiSeq platform, with CS1-341F-CS2-806R primers for bacteria [34,35]; forward and reverse reads were merged using PEAR [36], and chimeric sequences were identified using the USEARCH algorithm as compared with a reference database silva_132_16S.973 [37]. Annotated sequences with taxonomic information were generated using USEARCH2 with silva_132_16S.973 as reference database; the resulting data set was analyzed with MicrobiomeAnalyst using the default settings and normalized by rarefaction to the minimum library size [38]. A Pearson's correlation analysis was conducted for the water quality parameters ($p < 0.05$). Raw reads were deposited in the NCBI sequence read archives (SAR) under Bio Project accession number PRJNA725961.

Table 1. Target genes, primers, amplicon size, and partial sequences used for PCR and qPCR detection of ARG.

Target Gene	Primer	Amplicon Size	Sequence	Associated Antibiotic or Other Conditions	Resistant to	References
16s rDNA	331	195 bp	TCCTACGGGAGGCAGCAGT	N/A	N/A	[31,39,40]
	518		ATTACCGCGGCTGCTGG			
intI1	intILC5_fw	196 bp	GATCGGTCGAATGCGTGT	Wastewater treatment, clinical settings, food, groundwater, and other anthropogenic sources	N/A	[31,41]
	intILC1_rv		GCCTTGATGTTACCCGAGAG			
qnrS	*qnrS*rtF11	118 bp	GACGTGCTAACTTGCGTG	Fluoroquinolones	Fluoroquinolones	[31,42]
	*qnrS*rtR11		TGGCATTGTTGGAAACTT			
sul1	*sul1*-FW	162 bp	CGCACCGGAAACATCGCTGCAC	Sulfonamides	Sulfonamides	[31,33,43]
	sul1-RV		TGAAGTTCCGCCGCAAGGCTCG			
bla_{CTXM32}	CTX-M32-Fw	156 bp	CGTCACGCTGTTGTTAGGAA	Beta-Lactams	Amoxicillin, Cefotaxime, Ceftazidime, Cefepime, Piperacillin, Cephalothin, Cefoxitin, Cefuroxime	[31,33]
	CTX-M32-Rv		CGCTCATCAGCACGATAAAG			

3. Results and Discussion

3.1. Water Quality Results

All tested CWs had excellent performance in removing solids and organic matter (Table 2). Average effluent turbidity and TSS levels in the treated greywater were low, with average concentrations of 14 NTU and 10.8 mg/L, respectively. Similarly, average effluent TOC and BOD_5 concentrations were low, at 5.76 mg/L and 3.9 mg/L, respectively. The results were similar to the previously documented performance of the CWs investigated in this study demonstrating the longevity of the CWs [22,26,30]. Conductivity and pH slightly changed after treatment, while TN was reduced 33% from 9.46 ± 5.31 mg/L to 5.76 ± 3.25 mg/L. Some TN in the effluent is advantageous as the water is reused for irrigation as the remaining nitrogen can reduce the need for fertilizers. Since the greywater in the system is well aerated, nitrogen removal is mainly related to solids removal and plant and microbial assimilation. Removal of *E. coli* was 2 $\log_{10}$ reduction of magnitude, suggesting that disinfection is required for safe reuse. For Israeli reuse standards for unrestricted irrigation, the final effluent concentration is 10 fecal coliform units per 100 mL or less [44]. To reach a concentration of 10 fecal coliform units or less, a further 4 log reduction would be required.

Table 2. Water quality parameters for raw and treated greywater in the CWs with the corresponding percent reduction.

Parameter	Average and Standard Deviation of Raw Greywater and Treated Greywater		Percent Reduction
	Raw Greywater	Treated Greywater	
BOD_5 (mg/L)	89.3 ± 102.8	3.9 ± 3.0	95.7
pH	7.31 ± 0.41	7.54 ± 0.29	N/A
TSS (mg/L)	150.7 ± 194.1	10.8 ± 16.7	92.9
Conductivity (µS/cm)	776.5 ± 286.5	742.9 ± 205.5	4.3
Turbidity (NTU)	228.6 ± 205.1	14.1 ± 16.4	93.8
TOC (mg/L)	31.9 ± 30.8	5.76 ± 3.25	81.9
TN (mg/L)	9.46 ± 5.31	5.76 ± 3.25	35.6
E. coli	1.15×10^7	2.61×10^5	97.7

3.2. Overall ARG Results

All ARGs investigated in this study were detected in both raw and treated greywater (Figure 2). Genes were not completely removed from the final effluent (treated GW), which was expected since wastewater treatment systems typically do not completely remove all ARGs [45]. The abundance of genes in decreasing order was: 16S, *intI1*, *sul1*, *qnrS*, and bla_{CTXM32}, with log removals ranging from approximately 1 to 4 logs, apart from bla_{CTXM32} (1.45 log reduction). Results from this study are similar to those of other studies [31,46], where *intI1* and *sul1* were more abundant in wastewater than *qnrS* and bla_{CTXM32}, which were below the limit of detection. This relatively high reduction of ARG may be attributed to multiple factors, such as the removal of solids or biomass [47], long recirculation time of the treated water (6–12 h), and the combination of aerobic and anaerobic processes within the CW [48]. Even with significant ARG reductions, the ARG concentration in the final effluent should be considered for the end-user. Various studies have shown that ARG or other bacterial genes are not completely removed in centralized or onsite wastewater treatment processes [30,32,46,49]. CW3 had lower reductions than the other CWs for the most abundant gene detected (16S, *intI1*, *sul1*), ranging from 1–1.5.log reduction (Figure 2). However, statistically, this system was not an outlier ($p = 0.05$). Although ARG abundance does not reflect viable ARBs, lower ARG reduction could be attributed to lower influent

ARG concentrations. Even when bacteria or other pathogens are not completely removed, the greywater can be reused for non-potable applications depending on the end-use [50].

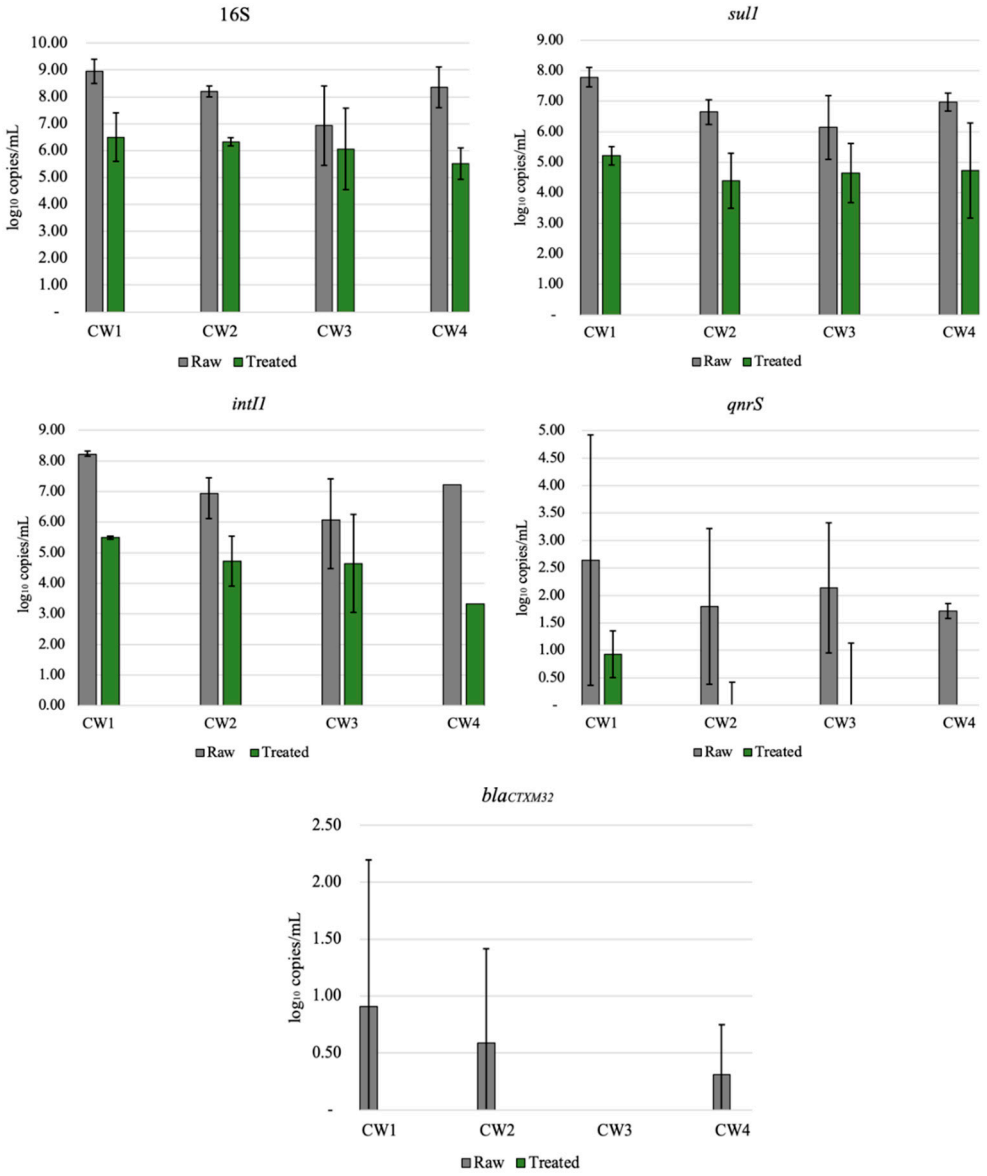

Figure 2. Relative abundance of 16S, *sul1*, *qnrS*, *intI1*, and bla_{CTXM32} before (untreated) and after (treated) treatment in all four recirculating vertical flow constructed wetlands. The results are average of triplicates ± standard deviation.

3.3. Sul1, bla_{CTXM32}, and qnrS

Results from qPCR (Figure 2) showed that ARG removal varied by household and gene type. ARG removal may be dependent on the specific gene studied [32]. The *sul1* gene was abundant in raw greywater for all households, with an average 2.58 log reduction and effluent concentrations ranging from 4.24×10^4 to 4.64×10^5 gene copies mL (Figure 2). The *sul1* gene has been found in relatively high concentrations in effluents from other CWs and wastewater treatment plants [51–54]. The final concentrations of *sul1* genes were relatively high, but the overall reduction in the CWs was comparable to a wastewater treatment plant. In comparison with our results, various studies of wastewater treatment plants with primary sedimentation, biological treatment, filtration, and disinfection have reported similar *sul1* reductions (approximately 1–3 logs) [55]. Gao et al. [55] found a strong positive correlation between sulfonamide resistant genes (*sul1* and *sul2*) and sulfonamide

drug abundance at a wastewater treatment plant, while [56] found no correlation with *sul* genes and sulfonamides. Since the CWs in this study each treated greywater from a single household, it is possible that variations in antibiotic use in the household were related to ARG abundance.

Relatively low counts of bla_{CTXM32} genes were detected in raw and CW-treated greywater, indicating that beta-lactam antibiotics are probably not commonly prescribed in the community studied. Alternatively, we may have underestimated beta-lactam resistance because there are numerous beta-lactamases. In earlier studies on ARB in these systems, some of the ARB isolates that were resistant to tetracycline were also harboring genes such as bla_{TEM}, bla_{SHV}, $bla_{\text{OXA-2}}$, and $bla_{\text{OXA-10}}$ [30]. Therefore, single-gene screening does not provide a broad view of resistance. *QnrS* encoded for resistance to fluoroquinolones and was detected in raw and treated greywater in all CWs, with an average 2.20 log reduction with the initial concentrations varying for all households. In all of the treated GW, abundances of this gene were low and ranging between zero and 3.9×10^1 gene copies per mL. CW4 was able to altogether remove *qnrS* from the final effluent after treatment in the CW; however, the initial concentrations were relatively low (5.3×10^1 gene copies/mL).

3.4. 16S and IntI1

The 16S gene was used as a general bacterial marker gene and was abundant in raw and treated greywater, as shown in Figure 2. The observed 2.11 log reduction is similar to values reported for decentralized wastewater treatment systems for total bacteria removal [56]. The lowest removal of 16S was observed in CW3, with less than 1 log reduction. The presence of *intI1* in wastewater is significant because it represents the potential for horizontal transfer of ARGs among microorganisms [57,58]. *IntI1* was very abundant in both raw and CW-treated greywater, with an average of 2.40 log reduction, similar to *sul1*. Although *intI1* was reduced to 2–3 logs, the final concentrations ranged from 2.1×10^4 to 8.3×10^6 gene copies/mL. *IntI1* is generally associated with anthropogenic environmental pollution; it is persistent in wastewater after treatment and is often present in pathogenic infections [59,60]. A study of three rivers by Makowska et al. [32] observed that *intI1* abundance increased almost 2-fold in all of the rivers after wastewater effluent was mixed with receiving waters, signifying anthropogenic influences. Benami et al. [26] examined gene reduction of fecal indicator and pathogenic bacteria in greywater using qPCR analysis; gene copies were reduced by less than 1 log except for one bacteria type, which was reduced by more than 1 log. The reduction found was less than the log reduction observed in our study (2.2–3 log) [26]. As previously mentioned, all genes do not behave the same way in a single system. Still, a more significant gene reduction of ARG than pathogenic bacteria draws attention to the differences between the types of genes studied in the CWs.

A correlation analysis was performed on the water quality parameters for raw and treated greywater and ARG (Table 3). TOC was positively correlated with TSS, 16S, *intI1*, *sul1*, and *qnrS*, while TSS was positively correlated with bla_{CTXM32}. The positive correlations with TOC could signify the relationship between organic carbon and ARG. The positive relationship between TSS and bla_{CTXM32} may be an artifact, considering the low concentrations of bla_{CTXM32} in both raw and treated greywater. 16S was positively correlated with *intI1*, *sul1*, and *qnrS*, as expected, since 16S is a bacterial marker. The positive correlation between *intI1* and *sul1* has been identified in previous studies [32], and this correlation is significant because *sul1* can possibly be transferred to commensal or pathogenic bacteria via horizontal gene transfer.

Table 3. Pearson's correlation analysis of raw and treated water quality parameters and ARG in the CWs.

	TOC	TN	BOD	TSS	16S	bla_{CTXM32}	*IntI1*	*Sul1*	*qnrS*
TOC	1.000								
TN	0.263	1.000							
BOD	0.190	0.116	1.000						
TSS	0.780	0.338	0.334	1.000					
16S	0.891	0.196	0.238	0.349	1.000				
bla_{CTXM32}	−0.028	0.211	0.335	0.780	0.091	1.000			
IntI1	0.924	0.171	0.322	0.687	0.849	0.498	1.000		
sul1	0.929	0.189	0.223	0.502	0.951	0.219	0.951	1.000	
qnrS	0.904	0.183	0.102	0.247	0.901	−0.105	0.799	0.935	1.000

3.5. Community Structure in Raw and Treated Greywater

DNA was extracted from the microbial community of the raw and treated greywater, and the 16S rRNA genes were sequenced to establish the community structure before and after treatment in the CWs. Using bray distance analysis shows that the community composition did not change after treatment for all households (Figure 3).

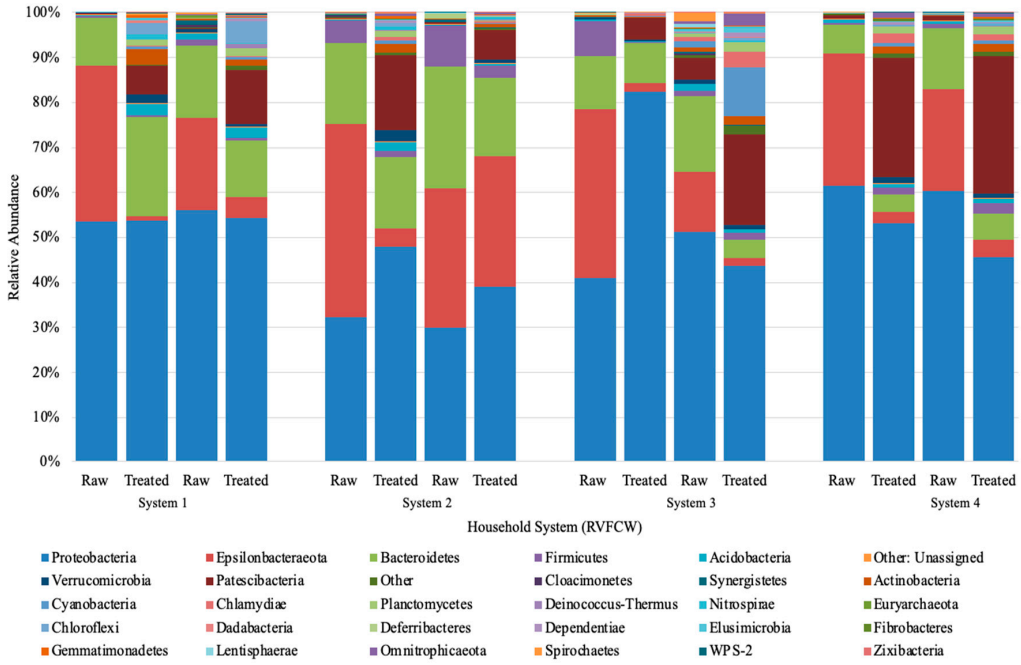

Figure 3. Next-generation Illumina Miseq amplification phylum results for raw and treated greywater. The abundances presented are of duplicates samples sequenced independently from the same system. Phyla with less than 100 OTU were omitted. System 1–4" refers to "CW1–4".

Proteobacteria, Epsilonbacteraeota, and Bacteroidetes phyla dominated the raw and treated greywater (Figure 3). Other phyla with a lower relative abundance that increased in treated greywater were Patescibateria, Acidobacteria, and Verrucomicrobia, while Firmicutes varied slightly. Although treated samples were more biodiverse, the abundance of more minor bacterial phyla (e.g., Firmicutes, Acidobacteria, Cyanobacteria) were generally below 2% (Figure 3). This increase in biodiversity could be due to several factors, including physical, chemical, and biological treatment processes in the CW, such as filtration, aerobic and anaerobic biodegradation, competition, predation, and plant uptake [61].

The relatively high abundance of Proteobacteria was expected and only increased slightly after treatment in CW1 and CW2, while in CW4, Proteobacteria decreased, and CW3 showed varying trends on different sampling days. Of the Proteobacteria classes identified, Gammaproteobacteria was the most abundant (85.3%). In addition, most of the colonies isolated on the ESBL plates belonged to this class (Table 4). There were relatively small changes in the class percentages as Pseudomonodaceae decreased ~1 log and Aeromonadaceae decreased ~0.5 log in the final effluent, and Enterobacteriaceae increased ~1 log (Figure 3). In prior studies, a ~1 log decrease in Pseudomonas and *E. coli*, which come from the Gammaproteobacteria class, was also observed in CW using both qPCR and culturable methods [26]. These combined results indicate that the performance of the CWs is steady for bacterial reduction; however, in our study, we observed a greater log reduction, approximately 2–3 logs. Overall, a greater log reduction is more advantageous as it ultimately reduces the ARGs introduced into the environment and the public.

Table 4. 16S rRNA bacterial identification sequencing results from ESBL isolates for all raw greywater treatment systems with corresponding ascension numbers. The ID is based on the system (CW1–CW4) and the isolate running number.

Isolate ID	Isolate Identification	Ascension Number
CW1_1	*Aeromonas* sp.	MT322960.1
CW1_2	*Aeromonas caviae*	MK301540.1
CW1_3	*Pseudomonas* sp.	MT512028.1
CW1_4	*Pseudomonas* sp.	EF442068.1
CW1_5	*Aeromonas* sp.	MF148425.1
CW1_6	*Pseudomonas nitroreducens*	MT472129.1
CW1_7	*Acinetobacter nosocomialis*	MT540255.1
CW1_8	*Pseudomonas* sp.	MF372961.1
CW2_1	*Pseudomonas fulva*	MT634251.1
CW2_2	*Pseudomonas putida*	MT641244.1
CW2_3	*Citrobacter* sp.	MH341951.1
CW2_4	*Pseudomonas guariconensis*	MT436398.1
CW2_5	*Pseudomonas* sp.	MT376777.1
CW2_6	*Pseudomonas* sp. G	MT256213.1
CW2_7	*Pseudomonas* sp.	MT507070.1
CW2_8	*Pseudomonas guariconensis*	MT436398.1
CW2_9	*Elizabethkingia* sp.	MN540122.1
CW3_1	*Pseudomonas* sp.	LC549486.1
CW3_2	*Pseudomonas nitroreducens*	MT472129.1
CW3_3	*Pseudomonas viridiflava*	MT386110.1
CW3_4	*Aeromonas caviae*	MN582971.1
CW3_5	*Aeromonas caviae*	MN582971.1
CW3_6	*Pseudomonas* sp.	GQ456130.1
CW3_7	*Aeromonas hydrophila*	MT605959.1
CW3_8	*Roseomonas cervicalis*	MF372961.1
CW4_1	*Pseudomonas putida*	MT641244.1
CW4_2	*Pseudomonas putida*	CP045551.1
CW4_3	*Stenotrophomonas* sp.	MT649753.1
CW4_4	*Pseudomonas* sp.	CP045553.1
CW4_5	*Pseudomonas monteilii*	MW245841.1
CW4_6	*Aeromonas* sp.	MK834723.1

The second most identified phylum in our study was Epsilonbacteraeota, which was more abundant in raw greywater (Figure 3). Nitrifying bacteria, such as Nitrosomonas, belong to the phylum Epsilonbacteraeota, accounting for nitrification or denitrification [62]. Epsilonbacteraeota decreased from 25% to 14% in the final effluent of all household systems (Figure 3). Although Epsilonbacteraeota was detected for sequencing, none were isolated on the cultured ESBL plates. Bacteroidetes was the third most identified phylum. Based on the community sequencing results, the relative abundance of Bacteroidetes varied for each CW; however, variations were minor. The relative abundance of Bacteroidetes did not change significantly after treatment in the CWs, decreasing from 13% to 11% (Figure 3). Based on the results from the PCoA plot using bray distance analysis, the community composition from raw to treated greywater did not change considerably after treatment in the CWs (Figure 4). However, the results demonstrate that the overall distribution of bacteria was reduced after treatment.

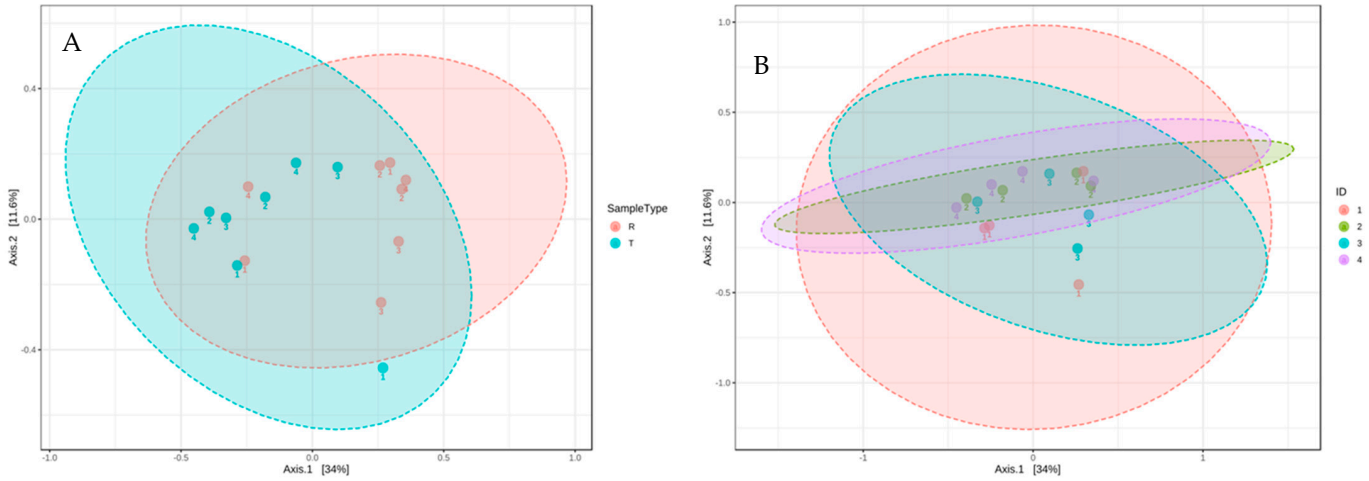

Figure 4. PCoA 2-D plot using bray distance and the explained variances are shown in brackets. R indicates raw greywater and T indicates treated greywater. ID 1,2,3,4 corresponds to CW1, CW2, CW3, CW4, respectively. (**A**) Community analysis of raw and treated samples. (**B**) Community analysis by household.

3.6. ESBL Isolates

Bacteria in raw greywater were isolated from the ESBL plates and were identified using 16S rDNA sequencing (Table 4). *Pseudomonas* was the most prevalent genus and was identified in all households. Other commonly identified bacteria were *Citrobacter* and *Aeromonas*. A few bacteria identified from the ESBL isolates have been identified in hospital environments, have caused clinical infections, and have been detected in immunocompromised individuals (*Acinetobacter nosocomial*, *Pseudomonas fulva*, *Pseudomonas putida*, *Pseudomonas monteilii*, and *Roseomonas cervicalis*) [63–67].

Pseudomonas putida was identified in two households and has been reported to cause clinical resistance to carbapenems and cephems (Table 4) [68]. In the same study, *Pseudomonas putida* transferred resistance genes to *Pseudomonas aeruginosa* via conjugation and transformation, which conferred resistance to amikacin and beta-lactams. Although *Pseudomonas aeruginosa* was not isolated on ESBL agar in this study, it was identified in the same CWs from a previous study [69]. Benami et al. [69] reported that opportunistic pathogens (*Enterococcus faecalis* and *Pseudomonas aeruginosa*) were detected in treated greywater. Although these pathogens were isolated our study, that does not negate their presence in raw or treated greywater as indicated by proteobacteria in the water microbiome. Other ESBL bacteria (*Aeromonas hydrophila*, *Pseudomonas guariconensis*, and *Pseudomonas viridiflava*) have been identified in outdoor, zoonotic, and greywater-impacted environments [22,70–73].

When considering the larger narrative of ARG and pathogens, it is pertinent to consider the transfer of resistant genes from pathogenic to non-pathogenic organisms and the health

risks associated with gene transfer [74]. Combining previous knowledge of pathogenic microorganisms with this current research on *intI1*, *sul1*, and *qnrS*, there is a potential for these ARGs or other ARGs to be transferred to pathogenic or non-pathogenic bacteria. This research was conducted in a relatively well-resourced desert community of primarily single-family households with greywater treatment and reuse systems. However, greywater is often not treated before reuse in many other water-stressed communities in Israel and globally [22].

Figure 5 shows the distribution of the most identified phylum in the constructed wetlands. Results from the heat map show that the abundance of specific bacteria varies from raw to treated greywater and the distribution changes considerably. The synchronicity between the CWs is also relatively low as well with varied bacteria populations. Before treatment in CW4, Lentisphaeria and Fibrobacteria were abundant; however, after treatment, they were scarcely detected and vice versa for Chlamydiae and oxyphotobacteria (cyanobacteria) (Figure 5).

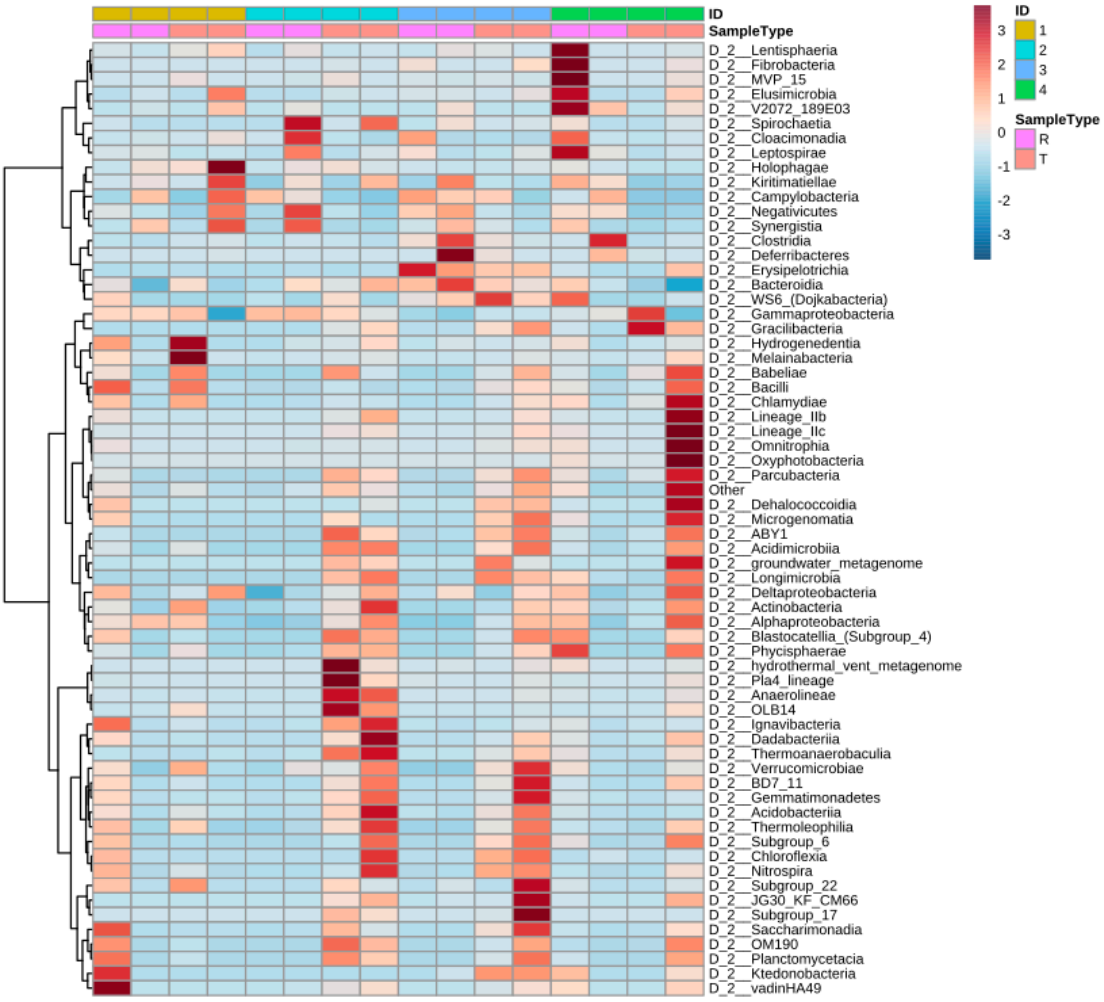

Figure 5. Heat map presenting changes (abundances) of the different classes of bacteria across systems and water type (raw or treated). ID 1,2,3,4 corresponds to CW1, CW2, CW3, CW4, respectively, and R represent raw greywater and T represents treated greywater.

4. Conclusions

This work demonstrates that it is advantageous to treat greywater before reuse and provides insights into the fate of ARB and ARG in household greywater treatment systems. There is a chance that these genes could interact in wastewater treatment systems or other aquatic environments and be transferred to potentially pathogenic bacteria via natural

transformation or conjugation. This is important if homeowners reuse treated effluent for irrigation because ARG may persist in these systems. Although the effluent microbial community was not significantly altered, the CWs studied substantially reduced the amount of ARG, ARB, bacteria, *E. coli*, solids, and organic matter in greywater. A primary concern with abundant ARG in wastewater is the presence of *intI1*. In this research, we found that *intI1* was correlated with ARG *sul1*. When ARGs are in abundance, *intI1* can increase the transfer rate of ARGs to nonresistant bacteria. The presence of *intI1* and other ARGs could confer the transfer of ARG into bacteria and increase the overall amount of ARB present in wastewater and natural waters. This raises concerns when considering public health.

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