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Removal of bacterial contaminants and antibiotic resistance genes by conventional wastewater treatment processes in Saudi Arabia: Is the treated wastewater safe to reuse for agricultural irrigation?

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Abstract

This study aims to assess the removal efficiency of microbial contaminants in a local wastewater treatment plant over the duration of one year, and to assess the microbial risk associated with reusing treated wastewater in agricultural irrigation. The treatment process achieved 3.9 logs removal of heterotrophic bacteria and up to 3.9 logs removal of fecal coliforms. The final chlorinated effluent had 1.8×10^2 MPN/100 mL of fecal coliforms and fulfils the required quality for restricted irrigation. 16S rRNA gene-based high-throughput sequencing showed that several genera associated with opportunistic pathogens (e.g. *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Legionella*, *Mycobacterium*, *Neisseria*, *Pseudomonas* and *Streptococcus*) were detected at relative abundance ranging from 0.010 to 21 % of the total microbial community in the influent. Among them, *Pseudomonas* spp. had the highest approximated cell number in the influent but decreased to less than 30 cells/100 mL in both types of effluent. A culture-based approach further revealed that *Pseudomonas aeruginosa* was mainly found in the influent and non-chlorinated effluent but was replaced by other *Pseudomonas* spp. in the chlorinated effluent. *Aeromonas hydrophila* could still be recovered in the chlorinated effluent. Quantitative microbial risk assessment (QMRA) determined that only chlorinated effluent should be permitted for use in agricultural irrigation as it achieved an acceptable annual microbial risk lower than 10^{-4} arising from both *P. aeruginosa* and *A. hydrophila*. However, the proportion of bacterial isolates resistant to ≥ 6 types of antibiotics increased from 3.8% in the influent to 6.9% in the chlorinated effluent. Examples of these antibiotic-resistant isolates in the chlorinated effluent include *Enterococcus* and *Enterobacter* spp. Besides the presence of antibiotic-resistant bacterial isolates, tetracycline resistance genes tetO, tetQ, tetW, tetH, tetZ were also present at an average 2.5×10^2 , 1.6×10^2 , 4.4×10^2 , 1.6×10^1 and 5.5×10^3 copies per mL of chlorinated effluent. Our

24 study highlighted that potential risks associated with the reuse of treated wastewater arise not
25 only from conventional fecal indicators or known pathogens, but also from antibiotic-resistant
26 bacteria and genes.
27

1. Introduction

Agricultural irrigation accounts for ~ 70% of the freshwater use worldwide (FAO 2005), and imposes a serious burden on water supplies in water-stressed countries like Saudi Arabia. To mitigate the stress on water resources, Saudi authorities have commenced a countrywide initiative to develop the local market for treated wastewater to meet the needs of agricultural irrigation (Al-Jassem 2012). Despite intense efforts to develop treated wastewater as an alternative water resource for agricultural irrigation, reuse of wastewater is still not widely acceptable in Saudi Arabia as exemplified from the low reuse rates (Abu-Rizaiza 1999, KICP 2010).

The reluctance to reuse treated wastewater is, in part, due to a lack of understanding on the efficacy of wastewater treatment plants (WWTPs) in removing contaminants. The general perception among the local public is that the WWTPs in a developing country like Saudi Arabia may have inefficient treatment processes that would lead to incomplete removal of microbial contaminants (e.g. pathogens and fecal indicators). There are at least 30 major sewage treatment facilities in Saudi Arabia (UNEP 1999). The majority of these facilities utilize secondary treatment that involves biological processes like activated sludge trickling filters, aerated lagoons and rotating biological contactors. Agricultural workers can be directly exposed to these microbial contaminants through occupational contact if wastewater were not well-treated and reused for irrigation. Public consumers can also be indirectly exposed through consumption of food produce that is contaminated by insufficiently treated wastewater. In order to promote acceptance of the use of these waters, it would be essential to first implement systematic monitoring efforts on the treatment efficiency of local WWTP, and assess any potential microbial risks associated with reusing the treated water.

Evaluation of treatment efficiency is generally based on monitoring conventional water quality parameters that include heterotrophic bacterial counts and the abundance of coliforms. However, emerging contaminants like antibiotic-resistant bacteria (ARB) and antibiotic resistance genes (ARGs) are also of increasing concern. Surveillance efforts to document the incidence of ARB revealed that *Pseudomonas aeruginosa*, *Acinetobacter* spp., *Staphylococcus aureus* and *Klebsiella pneumoniae* were the most prevalent resistant pathogens reported among nosocomial infections in Saudi Arabia (Aly and Balkhy 2012, Balkhy et al. 2006, Memish et al. 2012). Although there are limited epidemiological studies that detail the death burden arising from antibiotic-resistant pathogens in Saudi Arabia, local practitioners have reported that pediatric patients are most susceptible to infections caused by antibiotic-resistant pathogens (Bukhari and Al-Otaibi 2009, Shibl et al. 2009). To illustrate, 5 out of 80 children with community-onset methicillin-resistant *Staphylococcus aureus* (MRSA) succumbed to the infection, while fatalities arising from invasive pneumococcal meningitis ranged from 0-22% among children in the Arabian Peninsula and Egypt (Shibl et al. 2009).

Infections by antibiotic-resistant pathogens are not limited to the hospital settings. Since 1990s, there is a statistically significant increase in community-acquired infections caused by antibiotic-resistant *Escherichia coli* (Al-Tawfiq 2006b) and *S. aureus* (Al-Tawfiq 2006a, Yezli et al. 2012). The increase in ARB in community settings has been attributed in part to the ready availability of over-the-counter antibiotics, a lack of public knowledge towards responsible use of antibiotics and antibiotic misuse in animal husbandry (Al-Tawfiq et al. 2010). In recent years, there is increasing global recognition that wastewater treatment processes can become hotspots for antimicrobial resistance and the subsequent dissemination of the resistance genes into environment (Michael et al. 2013, Pruden 2014, Rizzo et al. 2013). A WWTP that does not

effectively remove ARB is likely to further compound antimicrobial resistance threats if such partially treated wastewater were to be reused. As such, microbial contaminants related to ARB and ARGs should also be monitored alongside with the conventional indicators.

The overall aims of this study are (i) to assess the removal efficiency of microbial contaminants in wastewater by a local WWTP, and (ii) to evaluate the microbial risk associated with reusing the wastewater. To achieve these aims, wastewater samples were collected at different stages of the treatment process over the duration of one year. The approaches undertaken to evaluate the efficacy of this WWTP were to; firstly, determine the removal efficiency of heterotrophic bacterial counts, total and fecal coliforms. Secondly, high-throughput sequencing was performed to characterize the microbial community in wastewater samples, with emphasis given to determine the occurrence of bacterial genera associated with opportunistic pathogens and those that persist throughout the wastewater treatment process. Thirdly, ARB and tetracycline resistance genes were also evaluated using culture-based approaches and quantitative PCR (qPCR) to provide insight into the occurrence of these emerging contaminants. These approaches provide datasets related to the abundance of opportunistic pathogens determined in the wastewater samples, which can be used in quantitative microbial risk assessment (QMRA). The QMRA approach, in turn, provides estimates on the microbial risk faced by the agricultural workers if the wastewater were to be reused for irrigation.

2. Materials and methods

2.1. Sampling site and samples collection

Wastewater was obtained from a wastewater treatment plant (WWTP) in Jeddah, Saudi Arabia. The exact location of the WWTP cannot be disclosed due to a confidentiality agreement with the plant operators. The treatment process in the WWTP consisted of initial settling of solids in a primary clarifier, followed by conventional aerobic activated sludge treatment. The hydraulic retention time (HRT) and sludge retention time (SRT) in the activated sludge tank was 6 h and 15 d, respectively. Biomass from the activated sludge tank was then channeled for further settling of solids in a secondary clarifier prior to disinfection. The amount of treated wastewater that flowed through this WWTP was 19700 m³/d (KICP 2010). Operators could not provide precise information on the contact time and chlorine dosage. Sampling was performed at three locations along the treatment process, namely after primary settling and before the conventional activated sludge process (i.e., influent), after activated sludge process (i.e., effluent) and after chlorination (i.e., chlorinated effluent). Influent, effluent and chlorinated effluent samples were collected in December 2012, April 2013, September 2013 and October 2013. To prevent cross-contamination, chlorinated effluent was first collected during each sampling trip, followed by effluent and lastly influent samples. Influent samples were aseptically collected in 1 L bottles, while effluent and chlorinated effluent sample were aseptically collected in 20 L bottles. Prior to collection, sampling bottles were rinsed twice with their respective wastewater samples. After collection, samples were immediately transported to the laboratory and stored at 4 °C. Sample processing and analyses were performed within 12 h of collection. Information regarding sample processing and analyses were detailed in following sections 2.2 and 2.4.

2.2. Enumeration of heterotrophic bacteria and coliforms

Enumeration of heterotrophic bacteria and coliforms was performed for the wastewater samples collected at different stages of the WWTP. Abundances were compared to determine the removal efficiency of the treatment processes. Dilutions of influent and effluent were prepared in sterile 0.86% w/v normal saline solution (NSS, 8.6g NaCl/L). Serial dilutions (10^0 , 10^1 , 10^2 and 10^3) of samples were used to obtain colony counts ranging between 30 – 300 colony forming units (CFUs) per plate. Samples, along with the control containing only NSS, were individually inoculated onto the media agar by the spread-plate method and incubated at 37 °C overnight. Chlorinated effluent was anticipated to have low microbial counts and to be inappropriate for dilutions. Therefore, 0 mL (i.e., control), 10 mL, 50 mL and 100 mL of the water samples were filtered through 0.4 µm Whatman Nuclepore™ track-etched polycarbonate membrane filters (GE Healthcare Life Sciences, Little Chalfont, UK), and the filters were placed on media agar for incubation to allow colony growth and enumeration. Heterotrophic plate counting was performed on nutrient agar. Most Probable Number (MPN) determination for coliforms was conducted as described previously (APHA et al. 2012c). The standard EPA method number 9221 was used to estimate total and fecal coliform bacterial densities. Growth media were prepared from commercially available dry mixes, namely, Fluka lauryl sulfate and brilliant green bile lactose broth (Sigma-Aldrich, Buchs, Switzerland) were used for total coliforms; and Fluka EC broth (Sigma-Aldrich, Buchs, Switzerland) was used for fecal coliforms. The growth media were dissolved in deionized water, dispensed into the inoculation tubes, capped and individually autoclaved at 121 °C for 20 min prior to the experiments. Samples were then aseptically dispensed into the sterile growth media at room temperature, and then examined for the coliforms based on protocols detailed in the standard EPA method number 9221. A set of

growth media that were not inoculated with any samples were also incubated as control blanks.

All control blanks were absent of heterotrophic bacteria and coliforms.

2.3. Isolation and phylogenetic identification of bacterial isolates based on 16S rRNA genes

A total of 290 bacterial cultures (n = 133 from influent, n = 85 from effluent, and n = 72 from chlorinated effluent) were isolated and evaluated for their phylogenetic identities by sequencing the 16S rRNA genes. Individual colonies representing heterotrophic bacteria, total coliforms and fecal coliforms were picked and re-streaked twice to obtain pure cultures. Each isolate was subsequently enriched in Fluka nutrient broth No. 1 (Sigma-Aldrich, Buchs, Switzerland) at 37 °C overnight. DNA was first extracted from each bacterial isolate based on manufacturer's protocol as described for DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). The 16S rRNA gene sequences were then PCR-amplified using forward primer 11F (5'-GTTYGATYCTGGCTCAG-3') and reverse primer 1492R (5'-GGYTACCTTGTTACGACTT-3'). Each PCR reaction contained 0.3 µL of 0.025 U/µL Ex Taq polymerase (Takara Bio, Dalian, China), 25 µL of 2X Epicentre Biotechnologies FailSafe™ Premix F (Illumina, Madison, WI, USA), 1 µL each of 10 µM each of forward and reverse primer, 22 µL H₂O and 1 µL of DNA template. Thermal cycling was conducted on C1000 Touch Thermocycler (BioRad Laboratories Inc., Hercules, CA, USA) with the following conditions: an initial denaturation stage at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 50 °C for 45 s and extension at 72 °C for 60 s, and a final extension stage at 72 °C for 10 min. The presence of PCR product was verified using Invitrogen SYBR® green nucleic acid gel stain (Thermo Fisher Scientific, Carlsbad, CA, USA) and gel electrophoresis. Controls for PCR reactions were negative for amplification. 16S rRNA gene amplicons obtained from the bacterial isolates were

of the correct size of ~1500 bp, and were purified using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The purified products were then sent in to the KAUST Genomics core lab for Sanger sequencing on ABI 3730 XL using the primer 338F (5'-ACTCCTACGGGAGGCAGC-3'). Gene sequences were matched against the National Center for Biotechnology Information (NCBI) 16S rRNA sequences database using Blastn to determine the gene identities of isolates.

2.4. 16S rRNA gene-based high-throughput sequencing of wastewater samples to determine microbial communities

The total microbial communities in the different types of wastewater samples were determined by performing 16S rRNA gene-based high-throughput sequencing on Ion PGM Torrent platform. The wastewater samples were prepared for DNA extraction by first concentrating the biomass. To illustrate, 30 mL of influent sample was rinsed with 20 mL 1X PBS buffer in a sterile plastic centrifuge tube, and the contents were centrifuged at 12,000 x g for 15 min to obtain a cell pellet for DNA extraction. Effluent and chlorinated effluent samples were filtered through 0.4 µm Whatman Nuclepore™ track-etched polycarbonate membrane filters (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK) in volumes varying between 200 to 900 mL. The filters with the concentrated biomass were then aseptically cut into 4-5 strips and used for DNA extraction. DNA was extracted using the UltraClean® Soil DNA Isolation Kit (MoBio, Carlsbad, CA, USA) with slight modifications to the manufacturer's protocol. Briefly, 12 µL of 100 mg/mL lysozyme and 12 µL of 1 mg/mL achromopeptidase were each added to the extraction buffer, and the sample mixture was incubated at 37 °C for 1 h before DNA extraction. Barcoded PCR for 16S rRNA gene-based Ion PGM sequencing was carried out with primer pairs 515F: (5'-Barcode-GTGYCAGCMGCCGCGGTA-3') and 909R: 5'-

CCCCGYCAATTCMTTTRAGT-3'). PCR reaction mixtures were prepared as described earlier. The thermal cycling program included an initial denaturation stage at 95 °C for 3 min, followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 45 s and extension at 72 °C for 60 s, and then a final extension stage at 72 °C for 10 min. The presence of PCR product was verified using Invitrogen SYBR® green nucleic acid gel stain (Thermo Fisher Scientific, Carlsbad, CA, USA) and gel electrophoresis. Controls for PCR reactions were negative for amplification. Amplicons from the influent, effluent and chlorinated effluent DNA samples were of the correct anticipated size of ~450 bp, and were gel-purified with the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The concentrations were then measured by Invitrogen Qubit® 2.0 fluorometer (Thermo Fisher Scientific, Carlsbad, CA, USA). Purified amplicons were submitted to KAUST Genomics Core lab for sequencing on an Ion Torrent™ 314 chip.

2.5. 16S rRNA gene-based high-throughput sequencing data analyses

High-throughput sequencing data was analyzed to provide information on the total microbial community, with emphasis given to determine the occurrence of bacterial genera associated with opportunistic pathogens and those that persist throughout the WWTP. All sequences were first sorted by the KAUST Bioinformatics team based on the barcodes and a Phred score of >20. The sorted sequences were then trimmed off for the primers, barcodes and adaptor sequences, and removed of any sequences that were less than 350 nt in length. The sequences were then further checked for chimeras on UCHIME (Edgar et al. 2011) by referencing to a core reference set that was downloaded from Greengenes (i.e., gold strains gg16 – aligned.fasta, last modified on 19 March 2011). A total of 1400,451 sequences were obtained. The chimera-free fasta files were then used for subsequent analysis on the RDP pipeline (Cole et al. 2009). RDP Classifier was

used for taxonomical assignments of the aligned 16S rRNA sequences at 95% confidence level (Cole et al. 2009). Chimera-free sequences were also aligned using the RDP Infernal Aligner, and aligned files were submitted to their individual cluster (Wang et al. 2007). The cluster files were then used to generate rarefaction curves (Figure S1). Microbial richness for each sample was denoted from the rarefaction curves based on a defined sequencing depth of 4,400 sequences. To further perform an OTU-based analysis, all chimera-removed fasta files were combined together with an in-house written Perl Script. The combined sequence file was then identified for the unique OTUs at 97% 16S rRNA gene similarity using CD-Hit (Li and Godzik 2006). The output file denotes the abundance of each unique OTU in each barcoded sample, and the nucleotide sequence of each unique OTU.

2.6. Quantitative microbial risk assessment (QMRA)

The microbial risks arising from reusing the influent, effluent and chlorinated effluent in agricultural irrigation were further evaluated by QMRA. Specifically, QMRA was performed for *Pseudomonas aeruginosa* and *Aeromonas hydrophila* as these two opportunistic pathogenic species were detected during cultivation-based analyses. At a 95% confidence interval, the median numbers of *Pseudomonas* or *Aeromonas* spp. cells in the respective wastewaters were multiplied with their corresponding fraction of *P. aeruginosa* or *A. hydrophila* to determine the concentration of opportunistic pathogenic species in the water sample. An assumed 2.0×10^{-6} probability of transmission of opportunistic pathogenic species to host was used (Gerba and Choi 2006). The main exposure route considered for QMRA was dermal exposure to liquid particulates by the agricultural workers during irrigation events, and exposure assessment data was obtained from USEPA exposure factors handbook 2011 edition (USEPA 2011). Briefly, 33% of the total 15310 cm^2 body surface area was assumed to be exposed during irrigation, with

0.14635 mL/cm² aqueous particulates from water adhering to exposed body surface. The exposure concentration of opportunistic pathogenic species was obtained by multiplying the exposed surface area with concentration of adhered particulates, concentration of opportunistic pathogenic species in water sample and the probability of transmission. The final exposure dose was obtained by first assuming a farm size of 180 m², an irrigation frequency of 4 days per week, with duration of 20 min per irrigation event. In addition, it was assumed that 180 mL/min of water would be used to irrigate per m² of farm. The k constants for the opportunistic pathogenic species were obtained from their LD₅₀ dose using the exponential models, and are 1.05 x 10⁻⁴ and 1.39 x 10⁻⁸ for *P. aeruginosa* (Hazlett et al. 1978) and *A. hydrophila* (Chakraborty et al. 1987), respectively.

Point estimates of risk were determined using formulae below:

Point estimate of risk from *P. aeruginosa*, $P_{P.aeruginosa} = 1 - \exp(-1.05 \times 10^{-4} * \text{exposed dose})$

Point estimate of risk from *A. hydrophila*, $P_{A.hydrophila} = 1 - \exp(-1.39 \times 10^{-8} * \text{exposed dose})$

Annual risk was further determined using the formula below:

Annual risk = $1 - (1 - \text{Point estimate of risk})^{(\text{number of exposure days per annum})}$

Annual risk was evaluated against an acceptable microbial risk of 10⁻⁴ (Smeets et al. 2009).

Further elaboration on the QMRA analyses is detailed in the Supplementary Text.

2.7. Determination of gene copy numbers by quantitative PCR (qPCR)

DNA extracted from influent, effluent and chlorinated effluent was quantified for their copy number of various tetracycline resistance genes and 16S rRNA genes. The copy numbers were determined by qPCR using primers designed in past studies (Amann et al. 1990, Aminov et al. 2002, Aminov et al. 2001, Frank et al. 2007). Primer sequences, amplification efficiencies and annealing temperatures were shown in Table S1. qPCR standards for the respective genes (i.e.,

tetO, tetQ, tetW, tetH, tetZ and 16S rRNA gene) were prepared by first cloning target gene
 amplicons into either pGEM[®]-T easy vector (Promega, Madison, WI, USA) or Invitrogen
 pCR[™]4-TOPO[®] TA vector (Thermo Fisher Scientific, Carlsbad, CA, USA). Plasmids carrying
 the gene inserts were harvested from transformed cells using PureYield[™] Plasmid Miniprep
 System (Promega, Madison, WI, USA). The extracted plasmids were sequenced to verify the
 gene insertions (Table S2). Plasmid copy numbers per μL were calculated based on the
 concentration of extracted plasmid DNA and the known sizes of vector and insert, and were
 individually diluted in series within the range of 10^{10} to 10^2 copies/ μL to produce a 6-points
 qPCR standard curve for each of the tested gene. To determine if there were qPCR inhibitors in
 the samples, a dilution series (i.e., 0, 2, 3, 4, 5, 10 and 50-fold dilution) of the influent, effluent
 and chlorinated effluent collected from December 2012 and April 2013 were individually
 prepared for qPCR to target the 16S rRNA genes (Figure S2). qPCR, following the relative
 standard curve method, was performed using Applied Biosystems[®] 7900HT Fast Real-Time
 PCR system with 384-well block module (Thermo Fisher Scientific, Carlsbad, CA, USA) per
 manufacturer's instructions. In brief, amplifications to obtain standard curves were performed in
 triplicate, while samples and no template controls (NTCs) were run in duplicates. All standard
 dilution series and NTCs were run alongside the samples in 384-well plates. Each reaction
 volume of 20 μL contained 10 μL of Applied Biosystems[®] Power SYBR[®] Green master mix
 (Thermo Fisher Scientific, Carlsbad, CA, USA), 0.4 μL of each primer (10 μM), 1 μL of 5-fold
 diluted DNA template from each sample (i.e., with a dilution amounting to a final 1 to 35 ng of
 DNA template among the different samples) and 8.2 μL H₂O. The reactions for amplification
 were carried out using the standard curve (AQ) assay and standard mode on Applied
 Biosystems[®] SDS version 2.3 software (Thermo Fisher Scientific, Carlsbad, CA, USA). The

thermal cycling protocol in the standard mode comprised of 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min, 40 cycles of 95 °C for 15 min and 1 min at an annealing temperature appropriate for the respective primers. The appropriate annealing temperature for primers targeting tetQ gene was 55 °C, while that for 16S rRNA gene and tetO gene was 57 °C. The annealing temperature for primers targeting tetW, tetH and tetZ genes was 60 °C. Melting curve analysis was performed with a dissociation cycle that included an increment of temperature from 60 °C to 95 °C, at an interval of 0.5 °C for 5s. The quantification cycle (C_q) and dissociation curve were analyzed by the SDS version 2.3 software. The C_q value was automatically defined by the software as the cycle number at which fluorescence passes the detection threshold. Standard curve for each tested gene was obtained by plotting the C_q value for each dilution point against the log-transformed concentration of each dilution. The amplification efficiencies of the standards for 16S rRNA gene, tetO, tetQ, tetW, tetH and tetZ were 90.2%, 88.2%, 99.5%, 92.9%, 104.8% and 97.5%, respectively. The R-squared values of the standards for 16S rRNA gene, tetO, tetQ, tetW, tetH and tetZ ranged from 0.97 to 0.99. Thereafter, the copy numbers of each tested gene in the samples were determined by fitting into the standard curve. All NTCs of tetO, tetQ, tetW, tetH and tetZ were negative with non-determinable C_q values. Samples were deemed to be absent of tetO, tetQ, tetW, tetH or tetZ genes if the C_q for the lowest detectable concentration of target genes in a sample was 35. All NTCs of reference 16S rRNA genes were negative with C_q values > 32. Therefore, samples were deemed to have non-detectable abundance of 16S rRNA genes and ignored if the C_q in a sample was > 29.

2.8. Antibiotic susceptibility of 290 bacterial isolates from wastewater

The 290 viable bacterial colonies that were isolated from the influent, effluent and chlorinated effluent were examined for their resistance/susceptibility profiles to various antibiotics. The

tested antibiotics include 30 µg/mL each of ampicillin, kanamycin, erythromycin and tetracycline, as well as 8 µg/mL each of ceftazidime, ciprofloxacin, chloramphenicol and meropenem. Bacterial isolates were individually grown overnight at 37 °C in the presence of each of the antibiotics and in the absence of any antibiotics to serve as a control. The optical density of the growth medium at wavelength 600 nm (OD₆₀₀) was subsequently measured using the Spectromax 340pc microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The threshold value to determine the status of a bacterial isolate as resistant to the antibiotic was set at > 50% of the control OD₆₀₀ value, which mostly ranged from an OD₆₀₀ value of 0.7 to 1.2.

2.9. Measurement of total suspended solids and chlorine content in wastewaters

The amount of total suspended solids (TSS) and chlorine in the wastewaters were measured to determine the physical and chemical properties of these samples. Total suspended solids (TSS) measurements were performed according to standard method 2540D (APHA et al. 2012b). Briefly, Grade 691 glass microfiber filters that retained particles ≥ 1.5 µm in diameter (VWR International, Wayne, PA, US) were weighed. Thereafter, 100 mL of wastewater sample was each filtered through individual filters, and the filters were then dried at 105 °C. After desiccation, the filters and the retained particulates were weighed again, and the differences in both weight measurements before and after filtration were calculated to determine TSS in each wastewater sample. Turbidity in wastewater samples was also measured using a Hach model 2100AN nephelometric turbidimeter (Hach, Loveland, CO, US) according to standard method 2130B (APHA et al. 2012d). The DPD/KI (N, N-diethyl-p-phenylenediamine/potassium iodide) colorimetric standard method 4500-Cl G was used to determine total chlorine concentration in chlorinated effluent samples (APHA et al. 2012a). Briefly, the DPD indicator solution and KI

crystals react with the free and combined chlorine in the sample to produce red color.

Absorbance at 515 nm was then measured to derive total chlorine concentration.

2.10. Nucleotide sequence accession numbers

All high-throughput sequencing files were deposited in the Short Read Archive (SRA) of the European Nucleotide Archive (ENA) under study accession number PRJEB6929. All Sanger-based sequences of the 16S rRNA genes of multidrug-resistant bacterial isolates mentioned in Section 3.6 were listed in Table S3.

3. Results

3.1. Removal efficiency of heterotrophic bacteria, coliforms and microbial cell numbers

The removal efficiency of heterotrophic bacteria by the conventional activated sludge process was, on average, 2.0 ± 0.031 logs ($n = 4$) while chlorination of effluent achieved a further 1.9 ± 0.13 logs ($n = 4$) removal (Figure 1A). A similar observation was made for the total and fecal coliforms (Figure 1B and 1C). Conventional activated sludge process achieved an average log removal of 1.7 ± 0.25 logs ($n = 4$) and 1.9 ± 0.068 logs ($n = 4$) for total and fecal coliforms, respectively. Removal efficiencies for coliforms by the conventional activated sludge process were lower when compared to the average 2.0 ± 0.0 logs ($n = 4$ for total coliforms, $n = 4$ for fecal coliforms) removal by chlorination of the effluent. Depending on the sampling period, the primary influent contained an abundance of heterotrophic bacteria ranging from $10^{7.5}$ to $10^{8.5}$ CFU/100 mL (Figure 1A) and an abundance of fecal coliforms ranging from $10^{5.2}$ to $10^{6.0}$ MPN/100 mL (Figure 1C). The abundance of heterotrophic bacteria then decreased to an abundance ranging from $10^{2.9}$ to $10^{6.1}$ CFU/100 mL in the final chlorinated effluent (Figure 1A). The abundance of total coliforms ranged from $10^{1.3}$ to $10^{2.5}$ MPN/100 mL (Figure 1B), while that

of fecal coliforms ranged from $10^{1.3}$ to $10^{2.7}$ MPN/100 mL (Figure 1C) in the final chlorinated effluent. A further approximation of the cell numbers based on extracted DNA yield and an assumed 10 fg DNA per cell genome (Navarro et al. 2006) was obtained (Figure 1D). The approximate cell numbers in the non-chlorinated effluent were on average 2.0 ± 0.027 logs ($n = 4$) lower than that in the primary influent, decreasing from an average $10^{9.5} \pm 10^{0.60}$ cells/100 mL ($n = 4$) to $10^{8.3} \pm 10^{0.50}$ cells/100 mL ($n = 4$). With the exception of the December 2012 sampling point which noted a slight increase in the cell numbers from $10^{8.1}$ /100 mL in the non-chlorinated effluent to $10^{8.5}$ /100 mL in the chlorinated effluent, chlorination generally achieved an average 1.8 ± 0.84 logs removal of the approximated cell numbers ($n=3$). However, there still remained an average $10^{7.8}$ cells/100 mL ($n = 4$) in the chlorinated effluent.

3.2. Microbial community along the treatment process

The average microbial richness increased from $1.2 \times 10^3 \pm 3.4 \times 10^2$ OTUs ($n = 4$) in the primary influent to $1.7 \times 10^3 \pm 5.4 \times 10^2$ OTUs ($n = 4$) in the chlorinated effluent during the entire sampling period (Figure 2). The phylum *Proteobacteria* was predominant in all three types of wastewater, with an average relative abundance of 60 ± 27 % and 34 ± 16 % in the influent ($n = 4$) and chlorinated effluent ($n = 4$), respectively. The decrease in the *Proteobacteria* was associated with a corresponding increase in the relative abundance of phylum *Cyanobacteria* and *Actinobacteria*, as well as unclassified *Bacteria* in the chlorinated effluent (Figure 2). OTU-based analysis further identified 45 OTUs that were not effectively removed by the treatment train as they were consistently found in all of the influent, effluent and chlorinated effluent samples. Seven of the 45 OTUs were identified to be *Aeromonas* spp. Genera *Tolumonas* spp., *Bacteroides* spp. and *Cloacibacterium* spp. each accounted for 4 OTUs (Figure 3B).

Furthermore, 16 OTUs were unique to only the chlorinated effluent and included 4 OTUs of *Pseudomonas* spp. (Figure 3C).

3.3. Genera associated with opportunistic pathogens

Several genera associated with opportunistic pathogens (e.g. *Acinetobacter*, *Aeromonas*, *Arcobacter*, *Legionella*, *Mycobacterium*, *Neisseria*, *Pseudomonas* and *Streptococcus*) were detected at relative abundance ranging from 0.010 to 21% of the total microbial community in the influent (Table 1). The relative abundances of these genera, except *Mycobacterium*, decreased from the influent to the effluent and chlorinated effluent, and remained at a range of 0.010 to 0.58% of total microbial community (Table 1). The cell numbers of individual genera per 100 ml of water sample was further approximated by multiplying the relative abundance with the total cell numbers per 100 mL of each sample (Figure 1D). Genus *Pseudomonas* had the highest approximated cell number in the influent but decreased to less than 30 cells/100 mL in both types of effluent. This observation was further supported by the culture-based approach, which recovered 8 *P. aeruginosa* isolates out of a total of 133 isolates from influent and 1 out of 85 isolates from effluent. No *P. aeruginosa* was isolated in the chlorinated effluent throughout the sampling period, indicating that the treatment schematic was effective in the removal of this opportunistic pathogenic species. Similarly, 11, 6 and 4 isolates identified to be *Aeromonas hydrophila* were isolated from influent, effluent and chlorinated effluent, respectively.

3.4. Microbial risk associated with reuse of water for agricultural irrigation

Quantitative microbial risk assessment (QMRA) was performed for genera *Pseudomonas* and *Aeromonas* as they accounted for a higher relative abundance and cell numbers compared to the other genera associated with opportunistic pathogens (Table 1). Phylogenetic identification of isolates cultivated from influent and effluent denoted the presence of *P. aeruginosa* at an

individual fraction of 0.060 and 0.012 relative to the total isolates cultivated from each type of wastewater. No *P. aeruginosa* were detected in the chlorinated effluent. Therefore, the median exposure doses of *P. aeruginosa*, over a 95% confidence interval, decreased from 5.6×10^4 cells per irrigation event using untreated influent to 2.4 cells per event using non-chlorinated effluent to negligible exposure dose of this bacterium if chlorinated effluent were to be reused for irrigation (Supplementary Text). QMRA indicated that the use of non-chlorinated effluent would impose a point estimate of 2.6×10^{-4} or an annual risk of 4.8×10^{-2} by *P. aeruginosa* on farmers who irrigate with the wastewater, while primary influent is definitely not recommended as the point estimate of risk alone would be more than 9.9×10^{-1} over a 95% confidence interval. The fraction of *A. hydrophila* detected in influent, effluent and chlorinated effluent was 0.083, 0.071 and 0.056, respectively. Therefore, the median exposure doses of *A. hydrophila*, over a 95% confidence interval, decreased from 7.0×10^2 cells per irrigation event using primary untreated influent to 9.4×10^1 cells per irrigation event using effluent and finally, to 9.0 cells per irrigation event using chlorinated effluent. Point estimated risk imposed by *A. hydrophila* on the farmers was lower than that from *P. aeruginosa*. At a 95% confidence interval, point estimate risk arising from *A. hydrophila* ranged from 9.6×10^{-6} (i.e., from influent) to 1.2×10^{-7} (i.e., from chlorinated effluent) per event. Consequently, farmers who irrigate with the influent, effluent and chlorinated effluent may face an annual risk of 1.9×10^{-3} , 2.5×10^{-4} and 2.4×10^{-5} , respectively, arising from *A. hydrophila* and over a 95% confidence interval.

3.5. Evaluation for qPCR inhibitors and determination of antibiotic resistance genes abundance in wastewater

To determine if qPCR inhibitors were present in the extracted DNA of wastewater samples, the abundance of 16S rRNA genes in serially diluted samples were quantified by qPCR approach.

With the exception of the influent samples collected from December 2012, the log copies of 16S rRNA genes per ng DNA exhibited less than 0.1 log₁₀ differences in the copy numbers across the different dilution folds (Figure S2). PCR inhibitors in the December 2012 influent sample adversely impacted the amplification efficiency, resulting in 1 log lower copies of 16S rRNA genes per ng DNA when the sample was not diluted (Figure S2). Based on this observation, all DNA samples were diluted 5-fold prior to qPCR. Copy numbers of tetracycline resistance genes (tetO, tetQ, tetW, tetH and tetZ) were quantified by qPCR, and normalized against the volume of samples extracted for their genomic DNA (Figure 4A and 4B) or against the copy numbers of 16S rRNA genes in each sample (Figure 4C and 4D). Influent samples (n = 4) had the highest abundance of all tested tetracycline resistance genes, with average abundances of $9.7 \times 10^4 \pm 1.1 \times 10^5$, $8.7 \times 10^4 \pm 1.0 \times 10^5$, $1.8 \times 10^5 \pm 1.5 \times 10^5$, $5.6 \times 10^4 \pm 8.9 \times 10^4$ and $2.2 \times 10^5 \pm 1.8 \times 10^5$ copies of tetO, tetQ, tetW, tetH and tetZ, respectively, per mL of influent (Figure 4A-4B). The copy numbers of these resistance genes decreased by nearly 3 logs from the influent to effluent. A further 1.9, 1.8 and 0.77 logs removal was observed for the tetQ, tetW and tetH, respectively, from the effluent to chlorinated effluent. This resulted in an average $1.6 \times 10^2 \pm 1.5 \times 10^2$, $4.4 \times 10^2 \pm 4.4 \times 10^2$ and $1.6 \times 10^1 \pm 1.5 \times 10^1$ copies of tetQ, tetW and tetH, respectively, per mL of final chlorinated effluent (n = 4). A slight increase of tetO and tetZ to an average $2.5 \times 10^2 \pm 2.4 \times 10^2$ and $5.5 \times 10^3 \pm 4.7 \times 10^3$ copies/mL, respectively, were observed in the final chlorinated effluent. When the tetracycline resistance genes were normalized to the total copies of 16S rRNA genes, it was observed that all resistance genes encoding ribosomal protection protein decreased by 1.9 to 2.0 logs removal from the influent to the final chlorinated effluent (Figure 4C). The tetracycline resistance genes tetH and tetZ, both encoding for efflux pump mechanisms, increased by 1.5 and 3.5 logs, respectively, in the chlorinated effluent (n = 4) compared to the

influent ($n = 4$). After chlorination, there remained an average relative abundance of $8.2 \times 10^{-4} \pm 1.6 \times 10^{-3}$ copies tetH per copy of 16S rRNA gene and $1.2 \times 10^{-1} \pm 2.1 \times 10^{-1}$ copies tetZ per copy of 16S rRNA gene (Figure 4D).

3.6. Antibiotic Resistance Profiles

Bacterial isolates from influent ($n_{\text{inf}} = 133$), effluent ($n_{\text{eff}} = 85$) and chlorinated effluent ($n_{\text{chl.eff}} = 72$) were tested for their antibiotic resistance profiles. Resistance against 30 $\mu\text{g/mL}$ ampicillin was most prevalent with about 72% of the total isolates being resistant to this antibiotic. This is followed by resistance against 30 $\mu\text{g/mL}$ erythromycin, 8 $\mu\text{g/mL}$ chloramphenicol, 30 $\mu\text{g/mL}$ kanamycin, 8 $\mu\text{g/mL}$ ceftazidime and 30 $\mu\text{g/mL}$ tetracycline, where an average 51%, 46%, 41%, 32% and 21% of the total recovered isolates were resistant to these antibiotics, respectively. Bacterial isolates that were resistant to 8 $\mu\text{g/mL}$ of each meropenem and ciprofloxacin were also detected. However, there was a relatively lower percentage of isolates that were resistant to meropenem (12% of total isolates) and ciprofloxacin (6.6%). The fraction of isolates that was resistant to meropenem increased from the influent to chlorinated effluent (Figure 5A). To illustrate, about 6.8% of the isolates recovered from influent were resistant to meropenem, but the percentage increased to 24% of the isolates recovered from chlorinated effluent (Figure 5A). Only 30 of the total 290 isolates, i.e., 10% of the total isolates, were non-resistant. Isolates resistant to three antibiotics represented the largest percentage in influent (25%), with less than 20% of the influent isolates resistant to ≥ 5 types of antibiotics. However, there was an increase in the percentage of isolates (28%) recovered from the chlorinated effluent that was resistant to ≥ 5 types of antibiotics (Figure 5B). For example, only 3.8% of the influent isolates were resistant to six antibiotics and this percentage increased to 6.9% of the chlorinated effluent isolates. Differences in the resistant bacterial strains were further observed in the three types of samples.

Majority of the isolates recovered from influent and were resistant to more than six types of antibiotics were identified to be Enterobacteriaceae (e.g. *Klebsiella* and *Escherichia*) and *Pseudomonas*. After chlorination of the effluent, partial 16S rRNA gene-based identification of the isolates revealed that majority of these multi-drug resistant isolates recovered from chlorinated effluent were *Enterobacter* and *Enterococcus* spp. (Table S3). The antibiotic resistance profiles of these bacterial isolates further showed that most of these isolates were resistant to almost all of the tested antibiotics except meropenem and ciprofloxacin (Table S4).

4. Discussion

The Draft 2010 Saudi Water Act has laid out specific regulations stating that wastewater has to be subjected to at least secondary treatment prior to reuse (KICP 2010). Generally, a secondary treatment process would be comprised of a primary clarifier where the wastewater is first removed of suspended solids, a biologically active tank where the organic constituents are degraded, and a clarifier which provides physical decantation of the microbial biomass from the supernatant effluent (Tamaki et al. 2012). This effluent is further chlorinated to decrease microbial activity and to achieve a permissible level of fecal coliforms in the final effluent. The Draft 2010 Saudi Water Act further stipulated that weekly monitoring is required for all wastewater treatment plants to ensure that wastewater is sufficiently treated prior to discharge or reuse. This study addresses the stipulated needs of the Draft 2010 Saudi Water Act by (i) assessing the removal efficiency of microbial contaminants in wastewater by a local WWTP, and (ii) to evaluate the microbial risk faced by the agricultural workers if the wastewater were to be reused for irrigation.

Our findings showed that the concentrations of traditional bacterial indicators (i.e., heterotrophic bacteria and coliforms) in the influents were fairly consistent across the sampling

period (Figure 1), and that the local WWTP was able to achieve, in total, 1.7 to 2 logs removals of traditional bacterial indicators along the entire treatment process. These removal efficiencies were comparable to those reported by past studies performed in the eastern region of Saudi Arabia (Saleem et al. 2000, 2003) and in other geographical locations, namely the US, Canada, France and Belgium (George et al. 2002, Harwood et al. 2005, Wery et al. 2008, Zhang and Farahbakhsh 2007). To illustrate, the WWTP in Canada was able to achieve approximately 1.9 and 2 logs removal of total and fecal coliforms, respectively, by a conventional activated sludge process operated at a HRT of 7 h (Zhang and Farahbakhsh 2007). Similarly, WWTPs in US, France and Belgium which were operated without tertiary nutrient removal, and hence a shorter HRT, were able to remove fecal coliforms by 1 to 2 logs (George et al. 2002, Harwood et al. 2005).

The removal efficiency of culturable fecal coliforms was related to higher HRT or to the presence of more efficient tertiary treatment processes (George et al. 2002, Rose et al. 2004), both of which were lacking in the local WWTPs examined in this study and by Saleem et al. As such, the removal efficiency achieved by the local WWTP was lower than the 3-4 logs achieved when activated sludge processes were operated at an extended HRT of 3-5 d (George et al. 2002). In addition, although chlorination accounted for 2 logs reduction in fecal coliforms (Figure 1C), this reduction was lower than the ~5 logs achieved when chloramine was applied to secondary effluent (Harwood et al. 2005). It is likely that disinfection was not properly conducted in this examined WWTP as the local operators were unable to provide precise information on the contact time and combined chlorine dose used during the disinfection step. Nevertheless, we were able to detect 0.20-0.5 mg/L of combined chlorine residual in the chlorinated effluent, suggesting that chlorine disinfection is indeed carried out. Alternatively, an

increase in the effluent particle size and TSS exhibited a negative correlation with the chlorine inactivation efficacy (Winward et al. 2008), and may have adversely affected the disinfection efficacy in the local WWTP. To illustrate, it was observed that the final effluent quality varied during the course of this study. The effluent collected in Oct 2013 sampling trip had a turbidity of 4.4 NTU and TSS of 6.8 mg/L. The turbidity and amount of TSS in this sample were more than 5-fold higher than that measured in the other effluent samples collected from other time points. The fluctuation in the effluent quality coupled with the lack of information on chlorination contact time may have suggested an ineffective disinfection process that explained for the presence of viable traditional bacterial indicators remaining in the chlorinated effluent.

Depending on the type of irrigation, the current permissible level of fecal coliforms in wastewater that is used for restricted irrigation is limited at < 1000 CFU/100 mL, and at < 2.2 CFU/100 mL for unrestricted irrigation (Al-Jasser 2011). Given that there are $\sim 1.8 \times 10^2$ MPN/100 mL of fecal coliforms in the final effluent, the treated wastewater examined in this study was therefore only appropriate for use in restricted irrigation. Our molecular-based approaches also suggest that some bacterial OTUs were able to pass through the entire treatment train as they were ubiquitously detected in all collected samples. Examples of such bacterial OTUs include *Pseudomonas* spp. that were frequently isolated in the untreated wastewater and non-chlorinated effluent. Some of these isolates were further identified at the species level to be mainly *Pseudomonas aeruginosa*, an opportunistic pathogenic species that caused 10-20% of nosocomial infections (Bodey et al. 1983) as well as morbidity and mortality in cystic fibrosis patients (Davies 2002).

There are limited published studies that assess the non-nosocomial infection rates arising from *P. aeruginosa* in reused water. However, past hospital outbreaks of antibiotic resistant *P.*

aeruginosa were traced to contaminated tap water supplies (Muscarella 2004, Rudnick et al. 1996). These studies suggested that immuno-compromised patients can be particularly vulnerable to infections from *P. aeruginosa* upon exposure to contaminated waters. As the Saudi Arabian government intends to encourage reused water as a national water tap, a more thorough assessment of the risks arising from *P. aeruginosa* in reuse water should be undertaken to minimize the increase in community-acquired infections spreading among and from farmers who irrigate with treated wastewaters.

Our QMRA analyses suggested that non-chlorinated effluent, despite having gone through the secondary treatment process, remained unsafe for reuse. Chlorinated effluent was however suitable for restricted irrigation as *P. aeruginosa* did not persist after chlorination and was instead replaced by other *Pseudomonas* spp. of environmental origins, namely *P. syringae*, *P. pohangensis* and *P. stutzeri*. Although microbial agents such as *Aeromonas hydrophila* remained viable in the chlorinated effluent, the annual microbial risk faced by the farmers from *A. hydrophila* in chlorinated effluent remained within the acceptable probability of 10^{-4} . Coupled with the absence of *P. aeruginosa*, it was determined that chlorinated effluent obtained from the local WWTP may be more suitable for agricultural irrigation. Nevertheless, there is a need to consider the chemical risks arising from toxic disinfectant byproducts (DBPs) that may be formed during chlorination (Mitch and Sedlak 2002, 2004).

In addition to the DBPs, antibiotic resistance genes were also detected in the chlorinated effluent, albeit at an abundance that was 2 logs lower than that in the influent. Specifically, the abundance of *tetO* and *tetW* remained at an abundance of 2.5×10^2 copies and 4.4×10^2 copies per mL of chlorinated effluent, respectively. These abundances approximate to the abundance of 5.0×10^1 to 2.0×10^2 *tetO* or *tetW* copies per mL of chlorinated effluent detected in a WWTP

with similar treatment processes (Fahrenfeld et al. 2013, Hong et al. 2013). The abundance of tetZ per copy of 16S rRNA gene increased by more than 17-fold after chlorination of the effluent (Figure 4D), suggesting an enrichment of bacterial populations that possessed tetZ genes. One of the primary inactivation mechanisms of chlorination is achieved through the reaction of nitrogenous compounds in proteins (McDonnell and Russell 1999). A proposed mechanism for bacteria to survive this oxidative stress is through up-regulation of the efflux pumps to maintain an acceptable intracellular concentration of the residual chlorine. This may in turn result in the enrichment of bacterial populations that would possess genes related to efflux pumps (Shi et al. 2013), and hence explain the increase in the tetZ gene per copy of 16S rRNA gene. Despite a positive detection of antibiotic resistance genes in the treated water, the abundance of antibiotic resistance genes required to impose a substantial risk to the public health through horizontal gene transfer remains unknown (Ashbolt et al. 2013, Hong et al. 2013, Pruden 2014). Until more epidemiological studies are initiated to assess the risks, these uncertainties will continue to impede the ability to establish guidance on the permissible concentration of antibiotic resistance genes in treated wastewater.

Besides the slight increase in antibiotic resistance genes, the proportion of bacterial isolates that were resistant to ≥ 5 types of antibiotics also increased after chlorination. These bacterial isolates include *Enterococcus* spp. and *Enterobacter* spp. In particular, *Enterobacter* spp. of Enterobacteriaceae were resistant to a wide range of tested antibiotics including ampicillin, kanamycin, erythromycin, tetracycline, ceftazidime, chloramphenicol and meropenem (Table S4). Our observation was in agreement with previous studies which reported that the collective proportion of *Escherichia coli* resistant to antibiotics, for example cephalothin, amoxicillin and beta-lactams increased significantly in the effluent as compared to the influent (da Silva et al.

2007, Galvin et al. 2010, Mokracka et al. 2012). These studies collectively suggest that ARB including Enterobacteriaceae may be selectively enriched upon chlorination of effluent.

Other ARB, such as *Acinetobacter* spp., may have also increased in proportion after chlorination (Zhang et al. 2009). However, the use of culture media like nutrient agar, lauryl sulfate, brilliant green bile lactose and EC broth may have inadvertently biased the isolation towards certain bacterial species like Enterobacteriaceae. These culture-based biases can also cause inaccuracies in the QMRA analyses. To illustrate, the calculation of microbial risk arising from *P. aeruginosa* was dependent on the concentration of *P. aeruginosa* in the water sample. The inability to grow viable but non-culturable (VBNC) *P. aeruginosa* due to heavy metals (Dwidjosiswojo et al. 2011) or chlorine in wastewater would mean that it is technically challenging to provide a precise concentration of *P. aeruginosa* and hence an accurate determination of the microbial risks. Alternatively, qPCR can be used to determine the copy numbers of *P. aeruginosa* in the water samples. However, PCR inhibitors were present in different concentrations in the influent and effluent samples (Figure S2), which in turn can affect amplification and a precise determination of targeted gene copies. For example, the qPCR-based determination of tetO genes was carried out at a slightly non-optimal amplification efficiency of 88%, and may have led to an underestimation of the actual abundance of this gene (Table S1). Even if a precise determination of the number of opportunistic pathogenic species was possible, accurate quantification of risks would still be affected by various assumptions that the QMRA analyses in this study were built upon. To illustrate, the frequency and duration of irrigation were assumed at 4 days per week and 20 min per irrigation event. Depending on the farm management practices, these assumptions may not be valid across different farms and the determined microbial risks may not apply to each of these farms. Furthermore, this study investigates only

bacterial surrogates and pathogens in wastewater samples grabbed over 4 time-points. Future experiments should increase sampling frequency to collect more samples, and to examine the removal efficiency of pathogenic viruses, protozoan parasites and helminthes that are also major contributors to morbidity and mortality throughout the world.

Despite the limitations of this study, this is one of the first studies that have highlighted the occurrence of ARB and ARGs in the treated effluent from a WWTP in Saudi Arabia. The detection of bacterial isolates that exhibited multi-drug resistance in the influent and non-chlorinated effluent could raise concerns pertaining to reusing these waters as these antibiotic-resistant strains will be clinically harder to treat with a typical antimicrobial therapy. In places that only rely on treatment processes as outlined in this study, existing infrastructure may have to be retrofitted with other technologies such as ultraviolet disinfection and/or membrane filtration to ensure a substantial removal of antibiotic resistant bacteria and their incorporated resistance genes. To illustrate, 10-20 mJ/cm² of UV doses can be used to reduce gram-negative ARB by 4 to 5 logs (McKinney and Pruden 2012). However, a high UV dose is required to reduce antibiotic resistance genes by more than 2 logs (McKinney and Pruden 2012, Zhuang et al. 2014). This is not economically viable for routine operation. Instead, ultrafiltration membranes could be used to reduce plasmid-associated antibiotic resistance genes in wastewaters by more than 3 logs (Breazeal et al. 2013). Alternatively, in cases where physical modification of the treatment system is not possible, the microbial load that is exposed to the agricultural farmers can be decreased through better management practices. Examples of these management practices include (i) using subsurface drip irrigation instead of sprinkler systems to minimize exposure to aerosolized treated wastewater, (ii) discouraging the use of stored treated wastewater to minimize the associated microbial regrowth issues, (iii) harvesting crops after a certain number

of days from the last irrigation event, depending on the die-off rates of the pathogenic bacteria and ARB, and (iii) wearing personal protective gear or clothing when irrigating fields with treated wastewater.

5. Conclusion

As more water-scarce countries explore the option of using treated wastewater as an alternative water source, it remains important to first audit for the associated microbial risks faced by the farmers when they irrigate with these wastewaters. This would allow for the safe use of treated water for irrigation of food crops, and contribute to balancing water and food security issues with minimal detrimental health and environment impacts. A one-year monitoring survey at a WWTP in Saudi Arabia revealed that the current treatment processes achieved the required quality for restricted irrigation but not for unrestricted irrigation. The final chlorinated effluent had 1.8×10^2 MPN/100 mL of fecal coliforms and QMRA suggested that the annual risks arising from *P. aeruginosa* and *A. hydrophila* in the chlorinated effluent were within the acceptable probability of 10^{-4} . However, the proportion of multi-drug resistant bacteria isolated from the chlorinated effluent increased as compared to the influent, and ARGs remained detectable in the chlorinated effluent. Although the associated risks arising from these antibiotic-associated contaminants remain unknown, our study highlighted that the assessment of potential risks pertaining to water reuse should not be restricted to conventional fecal indicators or known pathogens. Instead, antimicrobial resistance threats should also be incorporated into the risk assessment and planning to minimize any potential detrimental outcomes on the general public and agricultural farmers.

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Table 1. The average relative abundance $\pm$ standard deviation of genera associated with opportunistic pathogens. These genera were within the detectable limits by 16S rRNA gene-based high-throughput sequencing. The estimated average number of cells per 100 mL $\pm$ standard deviation was denoted within the brackets. * denoted quantitative microbial risk assessment (QMRA) was performed for these genera.

Genera	Influent (n = 4)	Effluent (n = 4)	Chlorinated effluent (n = 4)
<i>Acinetobacter</i>	1.2 $\pm$ 1.1% (4.6 x 10 ³ $\pm$ 4.5 x 10 ³)	0.38 $\pm$ 0.23% (1.2 x 10 ² $\pm$ 1.3 x 10 ²)	0.28 $\pm$ 0.32% (3.1 x 10 ¹ $\pm$ 3.4 x 10 ¹)
<i>Aeromonas</i> *	0.50 $\pm$ 0.38% (3.5 x 10 ³ $\pm$ 6.0 x 10 ³)	0.56 $\pm$ 0.62% (1.1 x 10 ² $\pm$ 9.1 x 10 ¹)	0.38 $\pm$ 0.43% (2.8 x 10 ¹ $\pm$ 3.8 x 10 ¹)
<i>Arcobacter</i>	4.9 $\pm$ 4.9% (1.4 x 10 ⁴ $\pm$ 1.2 x 10 ⁴)	0.55 $\pm$ 0.20% (1.6 x 10 ² $\pm$ 1.8 x 10 ²)	0.18 $\pm$ 0.18% (3.7 x 10 ¹ $\pm$ 6.0 x 10 ¹)
<i>Legionella</i>	0.014 $\pm$ 0.022% (4.3 x 10 ¹ $\pm$ 7.1 x 10 ¹)	0.50 $\pm$ 0.70% (6.8 x 10 ¹ $\pm$ 9.3 x 10 ¹)	0.25 $\pm$ 0.44% (6.9 x 10 ¹ $\pm$ 1.4 x 10 ²)
<i>Mycobacterium</i>	0.035 $\pm$ 0.047% (1.1 x 10 ² $\pm$ 1.5 x 10 ²)	0.20 $\pm$ 0.13% (3.0 x 10 ¹ $\pm$ 1.3 x 10 ¹)	0.58 $\pm$ 0.66% (7.4 x 10 ¹ $\pm$ 7.9 x 10 ¹)
<i>Neisseria</i>	0.047 $\pm$ 0.043% (1.9 x 10 ² $\pm$ 1.9 x 10 ²)	0.010 $\pm$ 0.00% (4.0 $\pm$ 5.9)	0.0095 $\pm$ 0.013% (1.0 $\pm$ 1.1)
<i>Pseudomonas</i> *	21 $\pm$ 24% (1.9 x 10 ⁵ $\pm$ 2.9 x 10 ⁵)	0.076 $\pm$ 0.073% (3.0 x 10 ¹ $\pm$ 4.1 x 10 ¹)	0.072 $\pm$ 0.061% (1.3 x 10 ¹ $\pm$ 1.9 x 10 ¹)
<i>Streptococcus</i>	0.48 $\pm$ 0.26% (2.1 x 10 ³ $\pm$ 2.1 x 10 ³)	0.11 $\pm$ 0.031% (3.0 x 10 ¹ $\pm$ 3.1 x 10 ¹)	0.091 $\pm$ 0.078% (1.2 x 10 ¹ $\pm$ 1.2 x 10 ¹)

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Figure 1. Comparison of the conventional water quality parameters (A) heterotrophic plate count, (B) total coliforms, (C) fecal coliforms, (D) approximated total cells, in the influent (inf), effluent (eff) and chlorinated effluent (chl. eff) sampled from the wastewater treatment plant at four sampling periods, namely December 2012, April 2013, September 2013 and October 2013. Approximation of total cells is determined by assuming 10 fg of DNA per cell, and dividing the total extracted DNA yield from each sample with this assumed constant. Dark gray circular dot located in between influent and effluent in each of the panels annotates the average log removal achieved by the activated sludge process. Dark gray circular dot located in between effluent and chlorinated effluent in each of the panels annotates the average log removal achieved by chlorination.

Figure 2. The dominant microbial phyla detected in influent (inf), effluent (eff) and chlorinated effluent (chl. eff) sampled from the wastewater treatment plant. The circumference of the circle is proportional to the average relative abundance of the particular phylum with respect to the total microbial community within the particular type of water sample. Microbial richness in each of the water type is represented by the average number of operational taxonomic units (OTUs) identified at a sequencing depth of 4400 reads and based on 97% 16S rRNA gene similarity.

Figure 3. The number of operational taxonomic units (OTUs) ubiquitously identified in all of the influent, effluent and chlorinated effluent samples are arranged in a (A) venn diagram to illustrate the number of OTUs that were uniquely associated with each of the water type, and the number of OTUs that were detected in two or more types of the water. (B) Phylogenetic identities of the 45 OTUs that were present in all influent, effluent and chlorinated effluent samples, and (C) phylogenetic identities of the 16 OTUs that were only present in the chlorinated effluent.

Figure 4. The average abundance of tetracycline resistance genes (A-B) per mL of water or normalized against (C-D) the total copies of 16S rRNA genes. tetO, tetQ and tetW denote genes that encode for tetracycline resistance via the ribosomal protection protein. tetH and tetZ denote genes that encode for tetracycline resistance via the efflux pumps.

Figure 5. The antibiotic resistance profiles of bacteria isolated from the influent, effluent and chlorinated effluent. (A) Percentage of isolates normalized against the total number of isolates obtained from that respective wastewater, and which demonstrates resistance to the tested antibiotics. (B) Percentage of isolates normalized against the total number of isolates obtained from that respective wastewater, and which demonstrates resistance to the stipulated number of antibiotics, R.

A total of 133, 85 and 72 isolates were obtained from the influent, effluent and chlorinated effluent, respectively. Isolates were tested for resistance to 30 µg/mL each of ampicillin (amp), kanamycin (kan), erythromycin (erm) and tetracycline (tet), as well as 8 µg/mL each of ceftazidime (cef), chloramphenicol (chl), meropenem (mer) and ciprofloxacin (cip).

Highlights

- Local wastewater treatment process achieves 3.88 logs removal of heterotrophic bacteria.
- 3.99 logs removal of fecal coliforms was also achieved.
- No *Pseudomonas aeruginosa* were present in chlorinated effluent but *Aeromonas hydrophila* was.
- Tetracycline resistance genes and antibiotic-resistant bacteria remain too.
- QMRA denoted that chlorinated effluent should be permitted for use in agricultural irrigation.

Supplementary Text. Elaboration on the quantitative microbial risk assessment (QMRA) conducted on *Pseudomonas aeruginosa* and *Aeromonas hydrophila*, and the assumptions made to facilitate QMRA.

Parameters	Annotation	Assumed value	Ref.
Size of farm (m ²)	A	250	
Water usage (ml/min/m ²)	B	180	
Irrigation frequency (days per week)	C	4	
Duration of irrigation (min/event)	D	20	
Fraction of total body surface area exposed during farming activities	E	0.33	(USEPA 2011)
Total body surface area (cm ²)	F	15310	
Liquid volume adhered onto exposed skin (ml/cm ²)	G	0.14635	
Transmission probability of bacterium to host	H	2.00×10^{-6}	(Gerba and Choi 2006)

Pseudomonas aeruginosa

Parameters	Annotation	Type of wastewater			Ref.
		Influent	Effluent	Chlorinated effluent	
Median cell numbers of genus <i>Pseudomonas</i> per mL over 95% confidence interval	I	705	0.156	0.0506	
Proportion of <i>Pseudomonas aeruginosa</i> isolated from the respective water	J	0.06015	0.011765	0	
Exposure dose (cells/event), $X = A * B * D * E * F * G * H * I * J$		5.64×10^4	2.44	0	
LD ₅₀ (CFU)		6.61×10^3			(Hazlett et al.)

k (exponential model)	1.05 x 10 ⁻⁴			1978)
Point estimate of risk = 1 – exp (-k * exposed dose)	9.97 x 10 ⁻¹	2.56 x 10 ⁻⁴	0	
Annual risk = 1- (1-point estimate)^(C * number of weeks * number of months)	1	4.80 x 10 ⁻²	0	

Aeromonas hydrophila

Parameters	Annotation	Type of wastewater			Ref.
		Influent	Effluent	Chlorinated effluent	
Median cell numbers of genus <i>Aeromonas</i> per mL over 95% confidence interval	I	6.31	0.995	0.121	
Proportion of <i>Aeromonas hydrophila</i> isolated from the respective water	J	0.082707	0.070588	0.05556	
Exposure dose (cells/event), $X = A * B * D * E * F * G * H * I * J$		6.95×10^2	9.35×10^1	8.98	
LD ₅₀ (CFU)		5.0×10^7			
k (exponential model)		1.39×10^{-8}			(Chakraborty et al. 1987)
Point estimate of risk = $1 - \exp(-k * \text{exposed dose})$		9.63×10^{-6}	1.30×10^{-6}	1.24×10^{-7}	
Annual risk = $1 - (1 - \text{point estimate})^{(C * \text{number of weeks} * \text{number of months})}$		1.85×10^{-3}	2.49×10^{-4}	2.39×10^{-5}	

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Table S1. Sequences of the oligonucleotide primers used in this study.

Primer	Target Gene	Sequence	Standard curve equation Amplification efficiency, %	Annealing Temp. (°C)	Amplicon Size (bp)	Reference
27-F	16S rRNA	AGAGTTTGATCCTGGCTCAG	y = -3.58x + 37.4 R ² = 0.99 90.2%	57	348	(Frank et al. 2007)
338-R		GCTGCCTCCCGTAGGAGT	(Amann et al. 1990)			
Ribosomal protection protein genes						
tetO-F	tetO	ACGGARAGTTTATTGTATACC	y = -3.64x + 43.5 R ² = 0.98 88.2%	57	171	(Aminov et al. 2001)
tetO-R		TGGCGTATCTATAATGTTGAC				
tetQ-F	tetQ	AGAATCTGCTGTTTGCCAGTG	y = -3.33x + 36.2 R ² = 0.97 99.5%	55	169	
tetQ-R		CGGAGTGTCAATGATATTGCA				
tetW-F	tetW	GAGAGCCTGCTATATGCCAGC	y = -3.51x + 39.1 R ² = 0.98 92.9%	60	168	
tetW-R		GGGCGTATCCACAATGTTAAC				
Efflux pump genes						
tetH-F	tetH	CAGTGAAAATTCACTGGCAAC	y = -3.21x + 39.2 R ² = 0.98 105%	60	185	(Aminov et al. 2002)
tetH-R		ATCCAAAGTGTGGTTGAGAAT				
tetZ-F	tetZ	CCT TCT CGA CCA GGT CGG	y = -3.38x + 40.0 R ² = 0.99 97.5%	60	210	
tetZ-R		ACC CAC AGC GTG TCC GTC				

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Table S2. Sequences of qPCR standards. Sequence region that is perfectly matched to the forward primer sequence is denoted in red. Sequence region that is perfectly matched to the reverse-complemented reverse primer sequence is denoted in blue.

Name of qPCR standard	Sequence
pCR-tetO	ACGGAGAGTTTATTGTATACCAGTGGTGCAATTGCAGAACCAGGGAGCGTAGATAAAAGGCACAACA AGGACAGATACAATGAATTTGGAGCGTCAAAGGGGAATCACTATCCAGACAGCAGTGACATCTTTTC AGTGGGAGGTAAAGTCAACATTATAGATACGCCCA
pGEM-tetQ	AGAATCTGCTGTTTGCCAGTGGANCAACGGAAAAGTGGCGCTGTGTGGATAATGGTGACACCATAACGGACTC TATGGATATAGAGAAACGTAGAGGAATTACTGTTCCGGCTTCTACGACATCTATTATCTGGAATGGTGTGAAAT GCAATATCATTGACACTCCG
pGEM-tetW	GAGAGCCTGCTATATGCCAGCGGAGCCATTTTCAGAACCAGGGAGCGTCGAAAAAGGGACAACGAGG ACGGACACCATGTTTTTTGGAGCGGCAGCGTGGGATTACCATTCAAGCGGCAGTCACCTCCTTCCAGT GGCACAGATGTAAAGTTAACATTGTGGATACGCC
pCR-tetH	CAGTGAAAATTCAGTGGCAACCCATTACGGTGTGCTATTAGCGCTCTATGCTACCATGCAGGTTATTT TTGCTCCTATTCTAGGACGACTGTCTGATAAATACGGCAGAAAACCCATCTTGCTGTTTTCCCTTTTA GGCGCGGCACTCGACTATCTTTTAATGGCATTCTCAACCACACTTTGGAT
pGEM-tetZ	CCTTCTCGACCAGGTCGGTGCCCCGACGACATGATCCCACTGCACGTCGGACTACTGACAGCGCTCTATGCGA TCATGCAGTTTCTTTGCGCCCCGATCCTTGGCCGACTCTCTGACCGTTTCGGACGCCGCCGCGTGCTTGTCGCCT CCCTCGCAGGCGCGACGATCGACTACCTCGTGCTCGCACTGACGGACACGCTGTGGGT
pGEM-16S	AGAGTTTGATCCTGGCTCAGATTGAACGCTGGCGGCAGGCCTAACACATGCAAGTCGAACGGTAACAGGAAGA AGCTTGCTTCTTTGCTGACGAGTGGCGGACGGGTGAGTAATGTCTGGGAAACTGCCTGATGGAGGGGGATAACT ACTGGAAACGGTAGCTAATACCGCATAACGTGCGAAGACCAAAGAGGGGGACCTTCGGGCCCTCTTGCCATCGG ATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGA GGATGACCAGCCACACTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGC

Table S3. Phylogenetic identities of bacterial isolates that exhibited resistance to six or more types of antibiotics.

Isolate name	Best matched 16S rRNA gene identity	Partial 16S rRNA gene sequence (5'-3')
Influent		
IC1	<i>Aeromonas caviae</i>	TGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGT AGCTAATATCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAG ATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGT AGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCTTG ACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC TGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTG GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAG TGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTGCTCAGCTCGTGTGCTGAGATGTTGGGTTAAGT CCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAAGTCAAGGGAGACTGCCCGT GATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCT ACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGA
IC2	<i>Klebsiella pneumoniae</i>	TGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGT TGAGGTTAATAACCTTGTGCGATTGACGTTACCCGCGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGC GGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGT CGGATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGG GGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCTT TGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACG CCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACC GCCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCAT GTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTCCAGAGATGGAT TGGTGCCTTCGGGAAGTGTGAGACAGGTGCTGCATGGCTGTGCTCAGCTCGTGTGTTGTGAAATGTTGGGTTA AGTCCCAGCAACGAGCGCAACCCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAAGTCAAAGGAGACTGCCA GTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTG CTAC
JWW2 PI -12	<i>Pseudomonas aeruginosa</i>	CCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAA GTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA ATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTCAGCAAGTTGGA TGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATCCAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTG GAATTTCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGAACACAGTGGCGAAGGCGACCACTGGGA CTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCCGT AAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTAACGCGATAAGTCGACCGCCT GGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGG TTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCAGAGATGGATTGGT

		GCCTTCGGGAACCTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGAGATGTTGGGTAAAGTC CCGTAACGAGCGCAACCTTGTCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGAGACTGCCGGTGAC AAACCGGAGGAAGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGCTACACACGTGCTACAAT G
FI3	<i>Shigella sonnei</i>	GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGGGGAGGAAGGGAGTAA AGTTAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTAAAGTCAG ATGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGT AGAATTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCTGG ACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGTCGACTTGGAGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACGCC TGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTTCAGAGATGAATATG GTGCCTTCGGGAACCTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTGAAATGTTGGGTAA GTCCCGCAACGAGCGCAACC
FI4	<i>Pseudomonas aeruginosa</i>	AGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGT TAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT ACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATG TGAAATCCCCGGGCTCAACCTGGGAAGTGCATCCAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGG AATTTCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGAC TGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA AACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTAACGCGATAAGTCGACCCGCTG GGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT TTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTG CCTTCGGGAACCTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTCC CGTAACGAGCGCAACCCTTGTCTTAGTTACCAGCAC
PI4	<i>Enterobacter hormaechei</i>	TGCCGCGTGTATGAAGAAGGCCTTCGGATTGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTAAATA ACCTCATGAATTGACGTTACCCGCAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAA TCCCCGGGCTCAACCTGGGAAGTGCATTCGAAACTGGTGGGCTAGAGTCTTGTAGAGGGGGGTAGAATTCC AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCTGGACTGAGAC TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAACGAT GTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCCGCTGGGGAGT ACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT CGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGCTTTGGTGCCTTCG GGAACCTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGAAATGTTGGGTAAAGTCCCGCAAC GAGCGCAACCCTTAT
BI1	<i>Enterococcus faecium</i>	AAGTCTGACCGAGCACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAACTCTGTTGTTAGAGAAGAAC AAGGATGAGAGTAACTGTTTCATCCCTTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAG CCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTA

		<p>AGTCTGATGTGAAAGCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGA GGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGC TCTCTGGTCTGTAAGTACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC ACGCCGTAAACGATGAGTGCTAAGTGTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCA CTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGGCCCGCACAAGCGGTGGA GCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGAT AGAGCTTCCCCTTCGGGGGCAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTGTCGTGAGATGTTGG GTTAAGTCCCGCAACGAGCGCAACCCTTATTGTTAGTTGCCATCATTAGTTGGGCACTCTAGCAAGACTGC CGGTGACAAACCGGAGGAGGTGGGGATGACGTCAAATCATCATGCCC</p>
IP7	<i>Aeromonas hydrophila</i>	<p>TGCCCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTTGATGCCTAATA CGTATCAACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA GGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAAA GCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAATTCC AGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGAC TGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT GTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCTGGGGAGT ACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT CGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGTAGAGATACGGGAGTGCCTTCG GGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTCGTGAGATGTTGGGTAAAGTCCCGCAAC GAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGGAGACTGCCGGTGATAAACC GGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCTCTTACGGCCAGGGCTACACACGTGCTACAATGGC GCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCAAAAAGCGCGTCGTAGTCCGGATCGGAGT CTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAATACGTTCCC GGGCCTTGTAACACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGAAGTAGATAGCTTA</p>
PI7	<i>Escherichia coli</i>	<p>GCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTTCAGCGGGGAGGAAGGGAGTAAAGT TAATACCTTTGCTCATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTAAAGTCAGATGT GAAATCCCCGGGCTCAACCTGGGAAGTGCATCTGATACTGGCAAGCTTGAGTCTCGTAGAGGGGGGTAGAA TTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGA AGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA CGATGTCGACTTGAGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGG GAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTT AATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTCCAGAGATGAAATGGTGCC TTCGGGAAGTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTTGTGAAATGTTGGGTAAAGTCCCG CAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAA ACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAAT GGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTGCTAGTCCGGATTGA GTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCC CCGGGCCTTGTAACACACCGCCCGTCACACCATGG</p>

Effluent		
JWW2 BE 11	<i>Staphylococcus saprophyticus</i>	CTGACGGAGCACGCCGCGTGAGTGATGAAGGGTTTCGGCTCGTAAAACTCTGTTATTAGGGAAGAACAAC GTGTAAGTAAGTGTGCACGTCTTGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGC GGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGTAGGCGGTTTCTTAAGTC TGATGTGAAAGCCACGGCTCAACCGTGGAGGGTCATTGGAACTGGGAACTTGAGTGCAGAAGAGGAA AGTGGAAATTCATGTGTAGCGGTGAAATGCGCAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTC TGGTCTGTAAGTACGCTGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATACCCTGGTAGTCCACGC CGTAAACGATGAGTGCTAAGTGTAGGGGGTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCC GCCTGGGGAGTACGACCGCAAGGTTGAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCAT GTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAATCTTGACATCCTTTGAAAACCTCTAGAGATAGAG CCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCATG
PE5	<i>Bacillus pumilus</i>	TGAAGGTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCAGAGTAAGTGCACCTTGA CGGTACCTAACAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTT GTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTAAC CGGGGAGGGGCATTGGAACTGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCACGTGTAGCGGTG AAATGCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAAGTACGCTGAGGAG CGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGT TAGGGGGTTTTCCGCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGA CTGAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCG AAAAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAAAGATAGGGCTTCCCTTCGGAGACAGAGTGA CAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCTT TGATCTTAGTTGCCAGCATTAGTTGGGCACTCTAAGGTGACTGCCGGTGACAACTGGAGGAAGGTGGGG ATGACGTCAAATCATCATGCCCCCTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTG CGAGATCGCGAGGTTTAGCCAATCCTCATAAATCTGTTCTCAGTTCGGATCGCAGTCTGCAACTCGACTGCG TGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTG
PE8	<i>Enterococcus faecalis</i>	TCTGACCGAGCACGCCGCGTGAGTGAAGAAGGTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAG GACGTTAGTAACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCG CGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGT CTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAACCTGGGAGACTTGAGTGCAGAAGAGGA GAGTGGAAATTCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCT CTGGTCTGTAAGTACGCTGAGGCTCGAAAGCGTGGGGAGCAAAACAGGATTAGATACCCTGGTAGTCCACG CCGTAAACGATGAGTGCTAAGTGTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTC CGCCTGGGGAGTACGACCGCAAGGTTGAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCA TGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTTTGACCACTCTAGAGATAGA GCTTTCCTTCGGGGACAAAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTA AGTCCCGCAACGAGCGCAACCTTATTGTTAGTTGCCATCATTTAGTTGGGCACTCTAGCGAGACTGCCGGT GACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTATGACCTGGGCTACACACGTGCTA CAATGGGAAGTACAACGAGTCGCTAGACCGCGAGGTCATGCAATCTCTTAAAGCTTCTCTCAGTTCGGAT TGCAGGCTGCAACTCGCCTGCATGAAGCCGGAATCGCTAGTAATCGCGGATCAGCACGCCGCGTGAATACG TTCCCGG

EHPC7	<i>Klebsiella variicola</i>	TGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGGGGAGGAAGGCGGTG AGGTTAATAACCTTGTTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGG TAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCG GATGTGAAATCCCCGGGCTCAACCTGGGAACCTGCATTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGG GTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCT GGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGC CGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAATCGACCG CCTGGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATG TGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCAGAGATGGATT GGTGCCCTTCGGGAACCTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTGAAATGTTGGGTTAA GTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTTCCGGCCGGAACTCAAAGGAGACTGCCAG TGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCT ACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGG ATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTACGGTGAAT ACGTTCCCGGGCCTTGTACACACCGCTCGTCACACCAT
FCE9	<i>Pseudomonas aeruginosa</i>	CCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAA GTTAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTA ATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGA TGTGAAATCCCCGGGCTCAACCTGGGAACCTGCATCCAAAACCTACTGAGCTAGAGTACGGTAGAGGGTGGTG GAATTTCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAAGTGGCGAAGGCGACCACCTGGA CTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT AAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTAACGCGATAAGTCGACCGCCT GGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGGCCCGCACAAAGCGGTGGAGCATGTGG TTTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCAGAGATGGATTGGT GCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCTGTGAGATGTTGGGTAAAGTC CCGTAACGAGCGCAACCCTTGTCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGA CAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTAC AATGGTCCGTACAAAGGTTGCCAAGCCGCGAGGTGGAGCTAATCCATAAAACCGATCGTAGTCCGGAT CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGATGTCACGGTGAATACG TTCCCGGGCCTTGTACACACCGCCCCGTCACACC
Chlorinated effluent		
CE3	<i>Enterococcus</i> spp.	CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG GCGGTTTCTTAAGTCTGATGTGAAATCCCCCGGCTCAACCGGGGAGGGGCATTGGAACTGGGAGACTTGA GTGCTGAAGAGGAGAGTGGAAATCCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCGGTGG CGAAGGCGGCTCTCTGGACTGTAAGTACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTTGGAGGTTGGAGGGTTCCGCCCTTCTTGTGAACTAA ACGCTTTAATCAATCCCCCGGGGAAGTACGACCCCAAGGTAGAAGTCTCAATGAATTGGACGGGGGGCCCC CCCAACGGGGGGGAGCATGGGGGTTAATTTTCATGCACACCCCGAAAACTTAAGTGGCTTGGACTTCCTA TTGACTTATCCTAAAAAGTATTTGGTTGCCTTCTCGGAAATCTGATAAACGGTGGCGTGCTGGGCTTGGTCG

		TTCAGCTTCCGGGTCGTGAGAATTGTTGGGTAAAGTTCCCGCCAACCGAAGCCCACACCCCTTATCTTGTTA TGTTGCCAATCGTCTAGGTCTGGGCACACTCTCAAACGAGAGACTGTGCCGGTGACTAAACTCGGAGAGGA AGGTTGGGGATTGACCGTCAAATCATCATGGCCCCCTTATC
TPCE2	<i>Enterobacter hormaechei</i>	TGCCGCGTGTATGAAGAAGGCCTTCGGATTGTAAAGTCTTTCGTCGGAGAGGAAGGTGTTGAGGTAAATAA CCTCGTCAATTGACGTTACCCAAAGAAGAAGCCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG GGGGCAAGCGTTGATCGGATTTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCTGATGTGAAATC CCCGGGCTCAACCTGGGAACTGCATTGGAACTGGGAGGCTTGAGTCTTGAAGAGGGGGGTGGAATTCCAT GTGTAGCGGTGAAATGCGTAGATATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACTGTAAGTGT ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGT CGACTTGGAGGTTGTGCCCTTGAGGCCTGGCTTGGCGAGCTAACGCGTTTAGTCAATCGCCTGGGGAATAC CGACGCCAGGGTAAAACTTCAATGAATTGACGGGGGGCCGCACCAGCGGTGGAACATGGGGGTTAATTTTC ATGCAACGCGAAAAACCTTACCTACTCTTGACATCCCTATGACTTCTCAGAGATGCTTTGTTGCCTTCCGGA ACTCTGAGGACGGGGGTGGCTGGGTGTTCTTAGCCCCCTGGTGTGGAAAGGTTGGGTAAATCCCCCACC CGCCAACCTTAATTTGTTGGTGGCAGTCGAGTTCAGGTTTCGGGCACTCTAACGGAGACTGCCAGTGATAAA CTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCTTATGACCTAGGGCTACACACGTGCTACAATG GGCGACATACACGAGTACGCGACCCTCGCGAGATCATGCGAGATCTCATAAAGCTGTCGTCGTCAGTTCGG GATTGCAGTCTGCAACTCGACTGCATGAAGTCGGAATCGCTAGTAATCGTGGATCA
TPCE4	<i>Enterococcus</i> spp.	CACGCCGCGTGAGTGAAGAAGGTTTTTCGGATCGTAAAACTCTGTTGTTAGAGAAGAACAAGGACGTTAGTA ACTGAACGTCCCCTGACGGTATCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACG TAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTCTTAAGTCTGATGTGAA AGCCCCCGGCTCAACCGGGGAGGGTCATTGGAACTGGGAGACTTGAGTGCAGAAGAGGAGAGTGGAATT CCATGTGTAGCGGTGAAATGCGTAGATATATGGAGGAACACCAGTGGCGAAGGCGGCTCTCTGGTCTGTAA CTGACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA TGAGTGCTAAGTGTTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCAAACGCATTAAGCACTCCGCTGGGGA GTACGACCGCAAGGTTGAACTCAAAGGAATTGACGGGGGGCCGCGCCCCAACGCGGTGAGAGCATGGTGG TTTAATCTCGAGAGCACACGGCGAGAGAACCTATACTCAGTCTCTTGCACTCTCCTATTGACCACACTCTAA AAGATATGATGCTTTTCCCTCTTTCGGGGGATCAAAGTTGACCGGGTGGGTGCCATTGGGTGTGCCGTCCCAG CTCTCTGTGTCGTGGAGAGATGGTTGGGGTAAAGTTCCCGCAACCAGAGCGGCACACCCCTTATATTCGTTT AGGTGCGCAATCGCATTTAGAGTTGGGCAACTCTCTAAGCGAGAAACTGGCCCGGTGGAACAAACCGGAG AGGAAGGTGGGGATATGAACGTTCAAATCATCATGGCCCCCTTAAT
TPCE7	<i>Enterococcus faecalis</i>	CGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAG GCGGTTTCTTAAGTCTGATGTGAAATCCCCCGGCTCAACCGGGGAGGGGCATTGGAACTGGGAGACTTGA GTGCTGAAGAGGAGAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGATATCTGGAGGAACACCGGTGG CGAAGGCGGCTCTCTGGACTGTAAGTACGCTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATAC CCTGGTAGTCCACGCCGTAAACGATGAGTGCTTGGAGGTGGAGGGTTTCCGCCCTTCCTTGCTGAGCTAAA CGCTTTAATCAATCGGCCGGGGAAGTACGACCGCAGGGTTGAAATTCAATGGATTTGACGGGGGGCCGCAC AACCGTGGAAGCTGGTGGTTTATTTTCATGCCACGCCAAAAAACCTTACTACGCCTTGACTTCCTTTGACTTC TCTAAAGATATATGGTGCCTTTTCGGAGATCTGATGACAGGTGTGGCAGGGTTTCCGTCACCTCGGGTCGGG AAAGGTTGGGTAAATTCCCGCACCAACCGCAACCTTTTATCGTTAGTTGCAATCGATCTAGTTCGGGCACTC TAACGAGACTGCCGGTGATAAACCTGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCT

		AGGGCTACACACGTGCTACAATGGGCG
TPCE9	<i>Enterobacter</i> spp.	<p>CGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACT CCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGC AGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTGAAACTGGCAGGCTA GAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGT GGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGCGGAGCAAACAGGATTAGAT ACCCTGGTAGTCCACGCCGTAAACGATGTGCACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTA ACGCGTTAAGTCAACCGCCTGGGGGAATACGGACGCAAGGTTAAACTCAAATGAATTGACGGGGGGCCC CGCACCAAGCGGGTGGAGACATGTTGGTTTTTATTTTCGATGCCCACGCCGAAGAGACCTTACCTACGTGCTT GACAATCCCCGATGAGACTTATTCAAAGAAGTGCAGTATGGTTGCCCTCTCGGGAGACTTCTGAGAGACA AGGTGGCTGGCATGGGCTGTGTCGTCTACGCTTCCGGGTGTGCGTGGAATAATGTGTGGGGTTAAGTTCCCC CGCAACCAAGCCGCAAAACCCTTATATCTCTTTGATTGGCCAGTCGGTCTCAGGCTGGGGAACCTCTCAAAAG AGAGACTGTGCCAGTGTGATAAACTGGGAGGAGAGGTGGGGGGTATGACGTCAAGTCCATTCAATTGGGCC CTTAACGAACCTATAGGGCCTACACACCACGGTGCTACCAATGGGCGACGATACAAAGAAGAAGCGAACCT CGCGAAGAGCAAGCGGATCTCATAAAAGTGTGTCGTAGTCCGGATTGGAGGTCTGCAACTCGACTGCATG AAGTCGGAATCGCTAGTAATCG</p>
TPCE10	<i>Enterobacter hormaechei</i>	<p>AGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTTCAGCGGGGAGGAAGGTGTTGAGGT TAATAACCTCAGCAATTGACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAAT ACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATG TGAAATCCCCGGGCTCAACCTGGGAACTGCATTGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAG AATTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGAC AAAGACTGACGCTCAGGTGCGAAAGCGTGCGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT AACGATGTGCACTTGGAGGTTGTGCCCTTGAGGCGTGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTG GGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGGCCGCACAAGCGGTGGAGCATGTGGT TTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGCTTTGGTG CCTTCGGGAACCTCTGAGACAGGTGCTGCATGGCTGTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTAAAGTCCC GCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTGTCAGGCCGGGAACCTCAAAGGAGACTGCCAGTGATA AACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGAGCCCTTACGAGTAGGGCTACACACGTGCTACAA TGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTGCTAGTCCGGATTG CAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGAATCAGAATGCCACGGTGAATACGT TCCC</p>
TPCE11	<i>Enterobacter hormaechei</i>	<p>CGACTTACGCGTTAGCTCCGGAAGCCACGCCTCAAGGGCACAACCTCCAAGTCGACATCGTTTACGGCGTG GACTACCAGGGTATCTAATCCTGTTTGTCTCCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGC CGCCTTCGCCACCGGTATTCCTCCAGATCTCTACGCATTTACCGCTACACCTGGAATTCTACCCCCCTCTAC AAGACTCTAGCCTACCAGTTTCGAATGCAGTTCAGGTTGAGCCCGGGGATTTACATCCGACTTGACAG ACCGCCTGCGTGCGCTTTACGCCAGTAATTCCGATTAACGCTTGACCCCTCCGTATTACCGCGGCTGCTGG CACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGAGGTTATTAACCTCAACACCTTCCTCC CCGCTGAAAGTACTTTACAACCCGAAGGCCCTTCTTCATACACGCGGCATGGCTGCATCAGGCTTGCGCCCA TTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCTGGACCGTGCTCAGTTCCAGTGTGGCTGATCAT CCTCTCAGACCAGCTAGGGATCGTCGCCTAGGTGAGCCGTTACCTCACCTACTAGCTAATCGCATCTGGGTA</p>

		CATCCGATGGCAAGAGGCCCGAAGGTCCCCCTCTTTGGTCTTGCGACGTTATGCGGTATTAGCTACCGTTTC CAGTAGTTATCCCCCTCCATCAGGCAGATTCCAGACATTACTCACCCGTCCGCCACTCGTCAGCAAAGCA GCAAGCTGCTTCCTGTTACCGTTGACTTGTCATGTGTTAGGCCTGCCGCCA
CE3	<i>Pseudomonas pseudoalcaligenes</i>	AGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGT TAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT ACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTTGTAAAGTTGGAGGT GAAATCCCCGGGCTCAACCTGGGAAGTGCCTCCAAAAGTGCATGACTAGAGTACGGTAGAGGGTAGTGGA ATTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACTACCTGGACT GATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA ACGATGTCAACTAGCCGTTGGGTACCTTGAGTACTTAGTGCGCAGCTAACGCATTAAAGTTGACCGCCTGG GGAGTACGGCCGCAAGGTTAAAACCTCAAATGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTGGTT TAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGGTGC CTTCGGGAAGTACAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGTCCC GTAACGAGCGCAACCCTTGTCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGAC AAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTACA ATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATC GCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACG TTCCCGGGCCTTGACACACCGCCCGTCACACCATGGGAGTGGGTGCTCCAGAAGTAGCTAGTCTAACCTT CGGGGGACGGTTACCACGGAGTGATTCATGACTGGGG
CP4	<i>Aeromonas jandaei</i>	GCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAAGT AGCTAATATCTGCTGGCTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGT AATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAG ATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAAGTGTCCAGCTAGAGTCTTGTAAGAGGGGGGT AGAATTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCTTG ACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG TAAACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCC TGGGGAGTACGGCCGCAAGGTTAAAACCTCAAATGAATTGACGGGGGCCCGCACAAAGCGGTGGAGCATGTG GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAG TGCCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAGATGTTGGGTAAAGT CCCGCAACGAGCGCAACCCCTGTCTTTGTTGCCAGCACGTAATGGTGGGAAGTCAAGGGAGACTGCCGGT GATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCT ACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCTAGTCCGG ATTCGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTTGCGGTGAA TACGTTCCCG
FCCE2	<i>Pseudomonas</i> spp.	AGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGT TAATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGCCGCGGTAAT ACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATG TGAAATCCCCGGGCTCAACCTGGGAAGTGCATCCAAAAGTACTGAGCTAGAGTACGGTAGAGGGTGGTGG AATTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGAC TGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA

		<p> AACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGCGCAGCTAACGCGATAAGTCGACCGCCTG GGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT TTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCAGAGATGGATTGGGT GCCTTCGGGAACTCAGACACAGGGTGCTGCATGGCTGTCGTCAGCTCGTGTGCTGAAATGTTGGGTTAAGT CCCGTAACGAGCCGCAACCCCTTGTCCTTAGTTACCAGCAACCTTCGGGGTGGGCACCTTCTAAGAAGACTGC CGGTGGACCAACCGGAGGAAGGTGGGGGATGACGTCAAGTCATCATGGCCCTTAACGGCCAGGGCTACC ACCACGTTGCTACAATGGTCGGTACCAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCAATAAAACC GATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGT </p>
CP9	<i>Citrobacter braakii</i>	<p> GCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCGAGGAGGAAGGCATTGTG GTTAATAACCACAGCGATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGA TGTGAAATCCCCGGGCTCAACCTGGGAAGTGCATCCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGTA GAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGA CAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT AAACGATGTCGACTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCT GGGGAGTACGGCCGCAAGGTTAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG TTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTTGGCAGAGATGCCTTAGTG CCTTCGGGAAGTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTGTTGTGAAATGTTGGGTTAAGTCCC GCAACGAGCGCAACCCCTTATCCTTTGTTGCCAGCGATCCGGTCGGGAAGTCAAAGGAGACTGCCAGTGATA AACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACAGTGCTACAA TGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTATGTCGTAGTCCGGATTG AGTCTGCAACTCGACTCCATGAAGTCGGAATCGTAGTAATCGTGCATCA </p>

Supplementary Table S4. Antibiotic resistance profiles for the bacterial isolates resistant to 6 and more antibiotics. Gray shaded cell denotes that the bacteria was resistant to that particular antibiotic. Clear cell indicate that the bacteria was susceptible to that particular antibiotic. Amp, Kan, Erm, Tet, Cef, Chl, Mer, Cip denote ampicillin, kanamycin, erythromycin, tetracycline, ceftazidime, chloramphenicol, meropenem and ciprofloxacin.

Isolate name	Resistance profiles							
	Amp	Kan	Erm	Tet	Cef	Chl	Mer	Cip
IC1								
IC2								
JWW2 PI-12								
FI3								
FI4								
PI4								
BI1								
IP7								
PI7								
JWW2 BE11								
PE5								
PE8								
EHPC7								
FCE9								
CE3								
TPCE2								
TPCE4								
TPCE7								
TPCE9								
TPCE10								
TPCE11								
CE3								
CP4								
FCCE2

Supplementary Figure S1. Rarefaction curves for influent, effluent and chlorinated effluent samples collected from Dec 2012 through Oct 2013.

Supplementary Figure S2. The average abundance of 16S rRNA genes per ng DNA extracted from influent, effluent and chlorinated effluent samples. Influent, effluent and chlorinated effluent were collected on December 2012 and April 2013. DNA from each sample was diluted over 0, 2, 3, 4, 5, 10 and 50-fold, and individually prepared for qPCR to determine if there were qPCR inhibitors in the samples.