DOES THE DISINFECTION OF PUBLIC WATER SUPPLIES INCREASE ANTIBIOTIC RESISTANCE LEVELS?

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Abstract

Antibiotic resistance is a substantial threat to public health. Recently researchers have suggested that the disinfection of public water supplies increases antibiotic resistance levels. This study investigated the levels of antibiotic resistance genes prior to treatment and disinfection and from within the distribution system of 18 water utilities (14 systems used groundwater as a source; 4 used surface water as a source). Samples were analyzed by real-time qPCR and microfluidic qPCR targeting total bacteria, 19 antibiotic resistance genes, 3 genes encoding the integrase of 3 different classes of integrons, and a gene encoding for quaternary ammonium compound resistance.

This study found that treatment and disinfection significantly ($P < 0.05$) reduced the concentration of 16S rRNA genes, *tet*(A) and *intI1* by approximately 90 percent (1 log-unit reduction) in groundwater systems and approximately 99 percent (2 log-unit reduction) in surface water systems. In groundwater systems, there was no significant change ($P < 0.05$) in the relative abundance of *intI1* or *tet*(A). In surface water systems, there was no significant change ($P < 0.05$) in the relative concentration of *tet*(A), but there was a significant increase in the relative abundance of *intI1* (increase of 0.3 log-units, $P < 0.01$). In conclusion, this study suggests that disinfection of public water supplies decreases the total number of antibiotic resistance genes, thus supporting a century of research demonstrating the public health benefits of the disinfection of drinking water supplies.

Table of Contents

List of Figures

Main Body Figures

Appendices Figures

List of Tables

Main Body Tables

Appendices Tables

1. Introduction

The World Health Organization has identified antibiotic resistance as one of the biggest threats to global health, food security and development because antibiotic resistant bacterial infections are substantially more expensive to treat and are associated with increased mortality rates (Leeb, 2004; Shrestha et al., 2018; Spellberg et al., 2011; WHO, 2018). Of the yearly two million antibiotic resistant infections in the United States, approximately 23,000 are fatal (CDC, 2017). In addition, models predict that the global death rate from antibiotic resistant bacteria will reach ten million people per year by 2050 (De Kraker, et al., 2016).

Since the first clinical use of penicillin in 1941, antibiotics have played a crucial role in protecting human health (American Chemical Society, 1999). Antibiotics are naturally produced antimicrobial agents that selectively kill or inhibit the growth and survival of targeted bacteria (Bender et al., 2018). Antibiotics target specific fundamental processes of bacteria including cell wall synthesis, protein synthesis and DNA replication and repair (Hash, 1972; Walsh, 2000). Selective toxicity allows antibiotics to treat bacterial infections without harming the infected individual (human or animal), and distinguishes antibiotics from disinfectants; disinfectants are chemicals that indiscriminately kill microorganisms (Bender et al., 2018).

An increase in antibiotic use, both appropriate and improper, is believed to contribute to the increased prevalence of antibiotic resistant bacteria. A study that analyzed antibiotic use in 76 countries found that the antibiotic consumption rate increased 39 percent from 2000 to 2016 (Klein et al., 2018). In addition to the increase in the overall use of antibiotics, antibiotics are also taken unnecessarily. The CDC found

that approximately 30 percent of all human antibiotics prescribed in the US are not actually needed to treat the infection for which they were prescribed (CDC, 2018).

The lack of new antibiotic development exacerbates the issues associated with the proliferation of antibiotic resistant bacteria. Developing a new antibiotic takes approximately 15 years; therefore, new antibiotics would optimally be developed before resistance to current antibiotics is detected (Welte, 2016). However, the development of new antibiotics has been steadily decreasing over the past three decades (Ventola, 2015). Without developing new antibiotics, it will become nearly impossible to solve the antibiotic resistance crisis.

Since the discovery of antibiotics, there has been concern about antibiotic resistance; Alexander Fleming warned of misuse of his wonder drug, penicillin, in 1946—only five years after its first clinical use (Bartlett et al., 2013). Antibiotic resistant bacteria can survive clinically relevant exposure to antibiotics that are known to kill other sensitive bacteria of the same strain (Institute of Medicine (US), 2010). Because antibiotics are selectively toxic, most bacteria are intrinsically resistant to some antibiotics. For example, gram negative bacteria are less affected by penicillins because gram negative bacteria lack the thick outer peptidoglycan cell wall that penicillins target (E. L. Smith, 1976).

There are several other mechanisms of antibiotic resistance that bacteria can develop, including enzymatic antibiotic modification, target modification, reduced uptake/ increased efflux, and antibiotic bypass through alternative target production (Hawkey, 1998). Enzymatic antibiotic modification occurs when bacteria produce enzymes that change the structure of antibiotics such that the antibiotic is unable to affect

its intended target, and is therefore no longer harmful to the bacteria (Poole, 2002). For example, the bacterial enzyme β-lactamase inactivates β-lactam antibiotics by hydrolyzing the β-lactam ring (Hawkey, 1998; Poole, 2002). Target modification involves chemically or structurally altering the target within the bacterial cell (Hawkey, 1998; Walsh, 2000). The newly modified bacterial target reduces the bactericidal/ bacteriostatic effects of the antibiotic by interfering with the interaction between the antibiotic and the target (Hawkey, 1998; Walsh, 2000). For example, some erythromycin-resistant bacteria can create slightly chemically different ribosomal structures via methylation in order to prevent erythromycin from binding to the targeted ribosomal subunits (Walsh, 2000).

In addition to antibiotic and target modification, bacteria can also modulate the uptake of an antibiotic by decreasing permeability or increasing excretion (Hawkey, 1998; Poole, 2002; Walsh, 2000). Genes encoding efflux pumps, such as *tet*(A), allow bacteria to pump out the antibiotic faster than the antibiotic can diffuse into the cell, keeping antibiotic concentration below inhibitory concentrations (Walsh, 2000). Bacteria can also protect against antibiotic action by producing alternatives targets. One such case occurs when enterococci alter their cell walls to prevent vancomycin from binding to a specific structural amino acid (D-Ala-D-Ala) (Poole, 2002; Walsh, 2000). Vancomycin-resistant enterococci express several genes (such as *vanA*) in order to produce a different structural amino acid (D-Ala-D-Lac) allowing the bacteria to continue cell wall synthesis while rendering vancomycin ineffective (Poole, 2002; Walsh, 2000).

Bacteria can become resistant to antibiotics through mutation and vertical evolution, or by acquiring genes through horizontal gene transfer. A point mutation could

confer antibiotic resistance to a bacterium, and through vertical evolution and natural selection, subsequently confer resistance to its descendants (Jury et al., 2011). In addition to vertical evolution, bacteria can acquire resistance through horizontal gene transfer. There are three mechanisms of horizontal gene transfer: conjugation (exchange of genetic material between bacteria), transduction (injection of DNA from bacteriophages) and transformation (uptake of naked DNA) (Jury et al., 2011). Horizontal gene transfer allows bacteria to rapidly acquire antibiotic resistance, thus leading to substantial dissemination of antibiotic resistance genes in the environment (Bengtsson-Palme et al., 2016; Goldman, 2004; Martínez, 2008).

Bacteria can also acquire antibiotic resistance via environmental selection pressures from compounds that are not antibiotics, especially heavy metals. Alternative environmental selection pressures can confer resistance in multiple ways, including co-resistance and cross-resistance (Baker-Austin et al., 2006). Co-resistance occurs when multiple genes encoding for resistance exist on the same genetic element (Canton & Ruiz-Garbajosa, 2011). For example, some metal resistance genes, such as mercury resistance genes, and antibiotic resistance genes have been known to occur on the same plasmids (Baker-Austin et al., 2006; Foster, 1983). Cross-resistance occurs when resistance to one toxic agent confers resistance to other chemically similar toxic agents (Canton & Ruiz-Garbajosa, 2011). One such case is the ability of an efflux pump to excrete both metals and antibiotics (Baker-Austin et al., 2006; Mata, Baquero, & Pérez-Díaz, 2000).

There are numerous reservoirs of antibiotic resistance; several studies have found antibiotic resistant bacteria in wastewater treatment plants (LaPara et al., 2011; Pruden et

al., 2006), agricultural soils (Ghosh & Lapara, 2007; Sandberg & LaPara, 2016; Seveno et al., 2002), and even drinking water (Armstrong et al., 1981; Dias et al., 2020; Pruden et al., 2006). The presence of antibiotic resistant bacteria in surface water (Bell et al., 1980), groundwater (McKeon et al., 1995) and in drinking water (Armstrong et al., 1981) has been well documented since the 1980's using cultivation-based methods. As technology advanced, researchers began using metagenomic methods and bioinformatics to quantify antibiotic resistant bacteria and classify resistance types present in samples. Many different types of antibiotic resistance genes have been found in drinking water, including β-lactam resistance, single drug resistances (i.e. vancomycin, tetracycline, sulfonamide, etc.), and multi-drug resistance (Jia et al., 2015). Additionally, genes associated with antibiotic resistance (i.e. efflux pumps, integrons, etc.) and genes encoding for disinfection resistance (i.e. quaternary ammonia compound resistance) have also been detected (Dias et al., 2020; Shi et al., 2013).

The impact of disinfection has been a focus within the investigation of antibiotic resistant bacteria in drinking water (Bergeron et al., 2015; Jia et al., 2020; Xi et al., 2009). Relative abundance, or the ratio of antibiotic resistant bacteria to the total number of bacteria, is the method most often used to determine the effect of disinfection on antibiotic resistant bacteria; researchers compare the ability of disinfection to remove antibiotic resistance genes to its ability to remove total bacteria. Several metagenomic and culture-dependent studies have found that chlorine disinfection of public water supplies increases the relative abundance of antibiotic resistance genes by at least 10 percent (Armstrong et al., 1982; Shi et al., 2013). A conclusion frequently drawn by researchers is that if disinfection is less effective at reducing antibiotic resistance bacteria

(i.e. an increase in relative abundance), than disinfection co-selects for antibiotic resistance (Armstrong et al., 1982; Jia et al., 2015; Shi et al., 2013).

This study was performed to analyze the effect of disinfection of public water supplies on antibiotic resistant bacteria using quantitative polymerase chain reaction (qPCR). I hypothesized that disinfection would decrease the total number of total bacteria and genes associated with antibiotic resistance, but that disinfection would not significantly increase the relative abundance of genes associated with antibiotic resistance. These hypotheses were drawn from a century of evidence describing the benefits of disinfection of public water supplies. A total of 18 drinking water distribution systems were studied: 11 systems that provide disinfected groundwater, 2 systems that use groundwater as their source that provide non-disinfected water, and 4 systems that provide treated and disinfected surface water. Samples were collected prior to treatment and from within the distribution system to determine the effect of treatment (especially disinfection), on antibiotic resistance genes in drinking water. Real-time qPCR was used to quantify total bacteria, *intI1*, and *tet*(A) in source water and water from the distribution system. Microfluidic qPCR was used to quantify total bacteria, 3 genes encoding for the integrase of 3 different classes of integrons (*intI1*, *intI2*, *intI3*), *qacF* and 19 antibiotic resistance genes.

2. Materials and Methods

2.1 Site Description

A total of 96 samples were collected between July 2016 and July 2019. Untreated water samples were collected from water utilities and finished water samples were collected from within their respective distribution systems. Samples were collected from 11 utilities that provide disinfected groundwater (GWD), 2 utilities that provide undisinfected groundwater (GWND) and 4 utilities that provide treated and disinfected surface water (SW). Additional information on these utilities is provided in Table 1.

Table 1. Summary of the approximate population served and disinfection technology of each utility.

Name	Approximate Population Served	Primary Disinfectant	Residual Disinfectant
GWD1	65,000	Chlorine	Chlorine
GWD2	500	Chlorine	Chloramine
GWD3	500	Chlorine	Chloramine
GWD4	67,000	Chlorine	Chlorine
GWD5	28,000	Chlorine	Chloramine
GWD6	165	Chlorine	Chlorine
GWD7	1,500	Chlorine	Chlorine
GWD8	3,400	Chlorine	Chlorine
GWD9	116,000	Chlorine	Chlorine
GWD10	6,000	Chlorine	Chlorine
GWD11	200	Chlorine	Chlorine
GWND1	13,400	None	None
GWND2	115	None	None
SW1	420,000	Chlorine	Chloramine
SW2	38,600	Ozone	Chloramine
SW3	66,000	Ozone, UV	Chloramine
SW4	307,000	Chlorine	Chloramine

2.2 Sample Collection

Samples were collected using REXEED 25S ultrafiltration membranes (Asahi Kasei, Tokyo, Japan), as described by Smith and Hill (C. M. Smith & Hill, 2009). Samples were collected from various types of faucets from within water treatment facilities and from the distribution system. Sample were collected from utility sinks or outdoor hose spigots to limit the effects of premise plumbing. All faucets were flame sterilized, and sterile autoclaved plumbing fittings were used. A flowmeter (GPI: Sparta, NJ; Assured Automation: Roselle, NJ) was used to measure both flow rate and total volume. For both groundwater and finished water, the goal was to filter 600-800 L. Due to high particulate concentrations, the goal was to filter 40 L of untreated surface water. After sample collection, the membranes were stored at 4 °C, transported back to the lab, and processed within 48 hours.

2.3 Sample Processing

After sample collection, membranes were backflushed with 500 mL of sterile backflushing solution (0.50% Tween-80, 0.01% sodium hexametaphosphate, 0.001% Y-30 antifoam emulsion) Unused, sterile membranes were also backflushed to serve as negative controls. After backflushing, polyethylene glycol (final concentration = 8% w/v), beef extract (final concentration = 1% w/v), and sodium chloride (final concentration = 0.2 M) were added and mixed for 1 hour at 4 °C, and then stored at 4 °C incubated at 4 °C for 24 hours. The particulate material in this solution was the concentrated by centrifugation 4 °C for 45 minutes at 12,000 × g resuspended in 10X Tris-EDTA buffer (pH 8.0) and stored at -20 °C.

2.4 DNA Extraction

For each sample, 300 µL of concentrated particulate matter was mixed with 700 µL of lysis buffer (5% m/v sodium dodecyl sulfate, 120 mM sodium phosphate buffer, pH 8.0), subjected to three freeze-thaw cycles, and then incubated for 90-minute at 70 °C. Metagenomic DNA was then extracted using a MP Bio FastDNA (MP Biomedicals, Santa Ana, CA) according to the manufacturer's protocol. Purified DNA extracts were stored at -20 °C.

2.5 Real-time Quantitative Polymerase Chain Reaction (qPCR)

Real-time qPCR was performed using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each reaction had a total volume of 20 µL, comprised of SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, Hercules, CA), 20 mg of bovine serum albumin (BSA), optimized quantities of forward (8 µmol) and reverse (4 µmol) primers, and 2 µL of sample template DNA. Thermal cycles consisted of an initial denaturization step (1 minute, 95°C) followed by 40 cycles of denaturization (15 seconds, 95°C) and a combined annealing/ extension step (1 minute, 60°C). Standard curves were created using serially 10-fold diluted gBlocks® Gene Fragments (Integrated DNA Technologies, Skokie, IL). Each standard curve was comprised of 8 sequential standards, had an R^2 value greater than 0.99, and an amplification efficiency between 85 and 100 percent.

Upon completion of real-time qPCR, melt curves were analyzed for evidence of nonspecific amplification. Amplification curves were visually inspected and compared to

standards to ensure that target genes amplified without inhibition. If inhibition was identified, samples were diluted 10-fold with nuclease-free water to eliminate inhibition.

Three genes were targeted by real-time qPCR: 16S rRNA gene (total bacterial biomass), *tet*(A) (tetracycline resistance), and *intI1* (integrase of class 1 integrons). *tet*(A) encodes for a tetracycline efflux pump (Levy et al., 1999) and has been detected in previous studies (Burch et al., 2014; Sandberg & LaPara., 2016). Integrons are genetic units that modulate the expression of functional genes contained in gene cassettes (Hall & Collis., 2006); *intI1* was analyzed in this study due to the association of integrons with multidrug resistance and horizontal gene transfer (Mazel, 2006). A summary of primer sequences used for real-time qPCR is included in Table 2. A summary of the synthetic double stranded DNA used as standards for real-time qPCR is located in Table A1 in Appendix A.

Table 2. Summary of forward (F) and reverse (R) primer sequences used for real-time qPCR.

Target Gene	Description	Assay Sequence (5'→3')	Assay Reference
16S rRNA	Total Bacterial Quantification	F: CCT ACG GGA GGC AGC AG R: ATT ACC GCG GCT GCT GG	(Muyzer et al., 1993)
intI1	Class 1 Integrase	F: CCT CCC GCA CGA TGA TC R: TCC ACG CAT CGT CAG GC	(Goldstein et al., 2001)
tet(A)	Tetracycline Resistance	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	(Ng et al., 2001)

2.6 Microfluidic Quantitative Polymerase Chain Reaction (MF-qPCR)

Microfluidic quantitative polymerase chain reaction (MF-qPCR) was conducted using a Fluidigm (South San Francisco, CA) Biomark Gene Expression 192.24 Dynamic Array™ integrated fluidic circuit chip. The chip was loaded according to the appropriate

protocol provided by Fluidigm (PN 100-7222 C1). The chip was prepared by an MX IFC Controller (Fluidigm; South San Francisco, CA) and read by a Biomark HD. The thermal protocol consisted of an initial annealing step (1 minute, 95 °C), 40 cycles of 5 seconds at 96 °C and 47 seconds at 60 °C, followed by 3 seconds at 60 °C and slow heating to 95 °C at a rate of 1 °C per 3 seconds.

In order to increase the concentration of template DNA, preamplification (specific target amplification, STA) was performed prior to MF-qPCR. The STA was conducted using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA); reactions consisted of 8 µL, containing SsoFast EvaGreen Supermix with Low ROX, 2 µL of a mixture of 20 µM of each primer, and 2 µL of template DNA. The thermal cycle for the STA included an initial denaturization (10 minutes, 95°C) followed by 17 cycles of denaturization (15 seconds, 95 °C) and annealing/ extension (4 minutes, 60 °C). After the STA was complete, the STA products were diluted 10-fold with nuclease-free water and stored 4 °C for approximately 24 hours. The STA was not conducted using the primers targeting 16S rRNA genes because sufficient quantities were already present in the samples.

Twenty-four genes were targeted using MF-qPCR. These genes included 16S rRNA genes, 3 genes encoding the integrase of 3 different classes of integrons (*intI1*, *intI2*, and *intI3*), qacF (gene encoding for quaternary ammonium compound resistance), and 19 antibiotic resistance genes. A summary of primer sequences used for MF-qPCR is included in Table A2 located in Appendix A. A summary of the synthetic double stranded DNA used as standards for MF-qPCR is included in Table A3 located in Appendix A.

2.7 Data Analysis

For all genes except for 16S rRNA genes, the limit of detection for qPCR was assumed to be equal to one half of the lowest standard that successfully amplified; this was equal to 5 genes. For the 16S rRNA gene, the detection limit was assumed to be 2,000 genes based on an estimation of the quantity of genes in the no-template controls. For statistical purposes, if a gene was not detected in a sample, the quantity was assumed to be one half of the detection limit (i.e., 2.5 for all genes except for the 16S rRNA gene; 1,000 for 16S rRNA genes). The resulting gene concentration was then calculated depending on the volume of sample collected; therefore, the limit of detection for each sample varied depending on the sample volume.

For pairwise comparisons of data sets, a Student's t-test (parametric) and a Wilcoxon rank-sum test (non-parametric) were performed. The threshold of statistical significance *a priori* was assumed as $\alpha = 0.05$. Statistical analysis was conducted using MATLAB R2018b. Statistical comparisons were performed only on data sets that had a minimum of 25 percent of the samples containing values greater than the limit of detection.

3. Results

3.1 Groundwater Source: Real-time qPCR

In the public water supplies that perform disinfection, the bacterial biomass concentration in the untreated groundwater was $10^{5.7 \pm 0.8}$ 16S rRNA gene copies L^{-1}, whereas the bacterial biomass concentration from within these corresponding distribution systems was $10^{4.6 \pm 0.9}$ 16S rRNA gene copies L^{-1} (Fig. 1A). This reduction in bacterial biomass concentration was statistically significant using both a Student's t-test ($P_t < 10^{-4}$) and a Wilcoxon rank-sum test ($P_W < 10^{-4}$). In contrast, in the public water supplies that did not perform disinfection, the concentration of bacterial biomass in the untreated groundwater samples was $10^{6.7 \pm 0.5}$ 16S rRNA gene copies L^{-1}, which was not significantly different from the bacterial biomass levels in the corresponding samples collected from within the distribution system ($10^{6.5 \pm 0.7}$ 16S rRNA gene copies L^{-1}; P_t = 0.4; P_W = 0.5) (Fig. 1B).

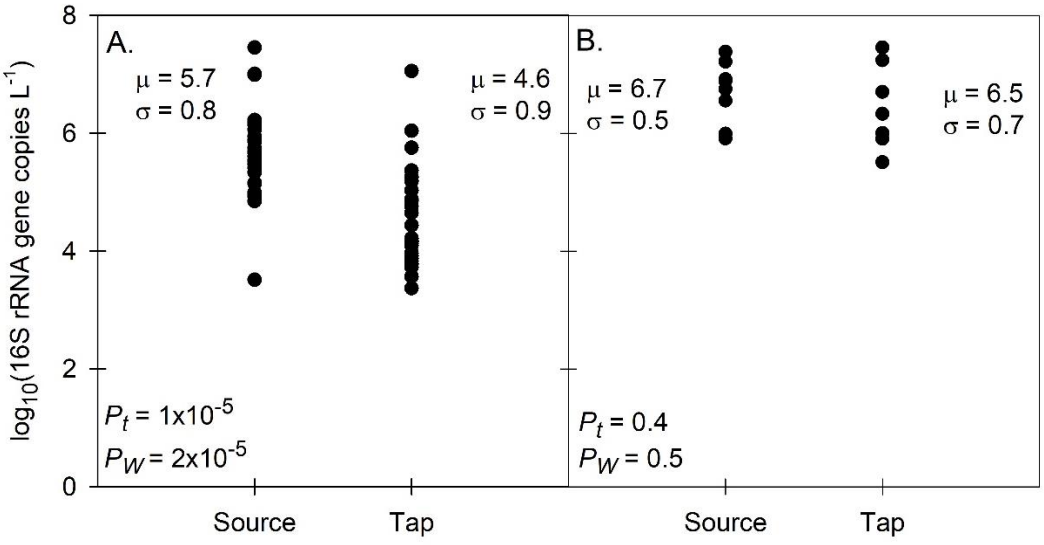

Figure 1. The mean (μ) and standard deviation (σ) of 16S rRNA gene concentrations at the source and from the distribution system (Tap) in (A) systems that provide disinfected groundwater and (B) systems that provide undisinfected groundwater. The mean (μ) and standard deviation (σ) of the source water are shown in the top right corner of each panel. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

Similarly, the untreated water samples from the systems that provide disinfected groundwater ($10^{3.5 \pm 0.4}$ gene copies L^{-1}) had significantly higher concentrations of *intI1* genes than the water from within the distribution system ($10^{2.7 \pm 0.8}$ gene copies L^{-1}; $P_t < 10^{-4}$; $P_W < 10^{-3}$; Fig. 2A). Likewise, in the samples from the public water systems that provide disinfected groundwater, the concentration of *tet*(A) in the untreated water was $10^{2.7 \pm 0.6}$ gene copies L^{-1}, which was significantly higher than the *tet*(A) concentration in the water collected from within the distribution system ($10^{1.8 \pm 0.7}$ gene copies L^{-1}; $P_t < 10^{-4}$; $P_W < 10^{-4}$; Fig. 2C). In contrast, the public water utilities that provide non-disinfected groundwater had statistically similar concentrations of *tet*(A) and *intI1* (*intI1*: $P_t = 0.2$; $P_W = 0.2$; *tet*(A): $P_t = 0.9$; $P_W = 1.0$) at both the source ($10^{4.8 \pm 0.3}$ *intI1* gene copies L^{-1}; $10^{3.8 \pm 0.4}$ *tet*(A) gene copies L^{-1};) and within the distribution system ($10^{4.5 \pm 0.3}$ *intI1* gene copies L^{-1}; $10^{3.5 \pm 0.4}$ *tet*(A) gene copies L^{-1}; *intI1*: Fig. 2B; *tet*(A): Fig. 2D).

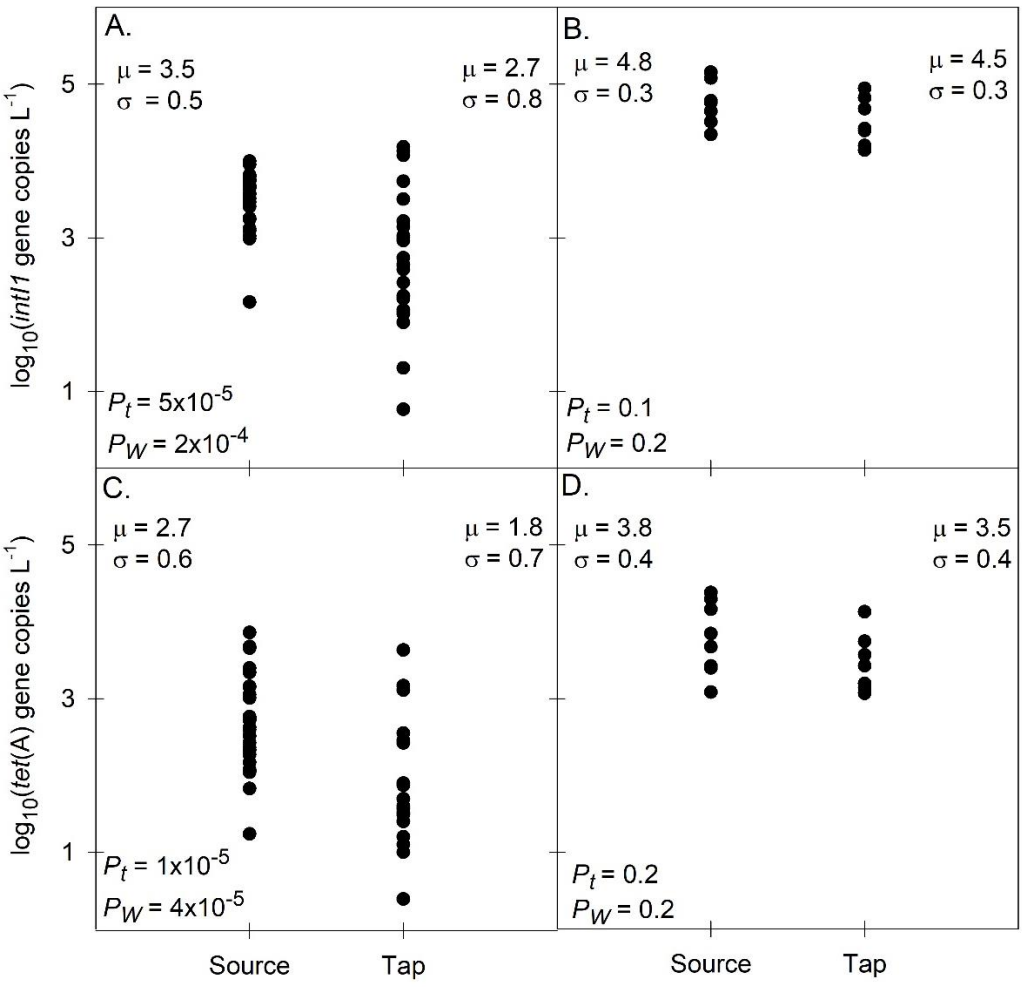

Figure 2. The mean (μ) and standard deviation (σ) of *intI1* and *tet*(A) concentrations at the source and from the distribution system (Tap). (A) *intI1* in systems that provide disinfected groundwater, (B) *intI1* in systems that provide undisinfected groundwater, (C) *tet*(A) in systems that provide disinfected groundwater, and (D) *tet*(A) in systems that provide undisinfected groundwater. of the source water are shown in the top right corner of each panel. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

Because total bacterial levels decreased so substantially from source to the distribution system, it is also pertinent to ask whether the relative quantity of antibiotic resistance genes (*tet*(A):16S rRNA genes; *intI1*:16S rRNA genes) is affected by disinfection. For the disinfected systems, the ratio of *intI1* to the total bacterial biomass in the untreated water ($10^{-2.2 \pm 0.7}$) was not significantly different than the ratio measured in the water from within the distribution system ($10^{-1.8 \pm 0.6}$; $P_t = 0.1$; $P_W = 0.2$; Fig. 3A). A similar result was observed *tet*(A):16S rRNA genes in these systems; the ratio of *tet*(A):16S rRNA genes for untreated water was $10^{-3 \pm 0.6}$, which was not significantly different than *tet*(A):16S rRNA genes in the water collected from within the distribution system ($10^{-2.6 \pm 0.8}$; $P_t = 0.2$; $P_W = 0.3$; Fig. 3C). Similarly, in the non-disinfected groundwater systems, *intI1*:16S rRNA genes (Fig. 3B) and *tet*(A):16S rRNA genes (Fig. 3D) did not significantly change ($P_t > 0.7$, $P_W > 0.5$) from the untreated source water collected (*intI1*:16S rRNA genes = $10^{-1.9 \pm 0.7}$; *tet*(A):16S rRNA genes = $10^{-2.9 \pm 0.4}$) to water collected from within the distribution system (*intI1*:16S rRNA genes = $10^{-2.1 \pm 0.7}$; *tet*(A):16S rRNA genes = $10^{-3.0 \pm 0.8}$).

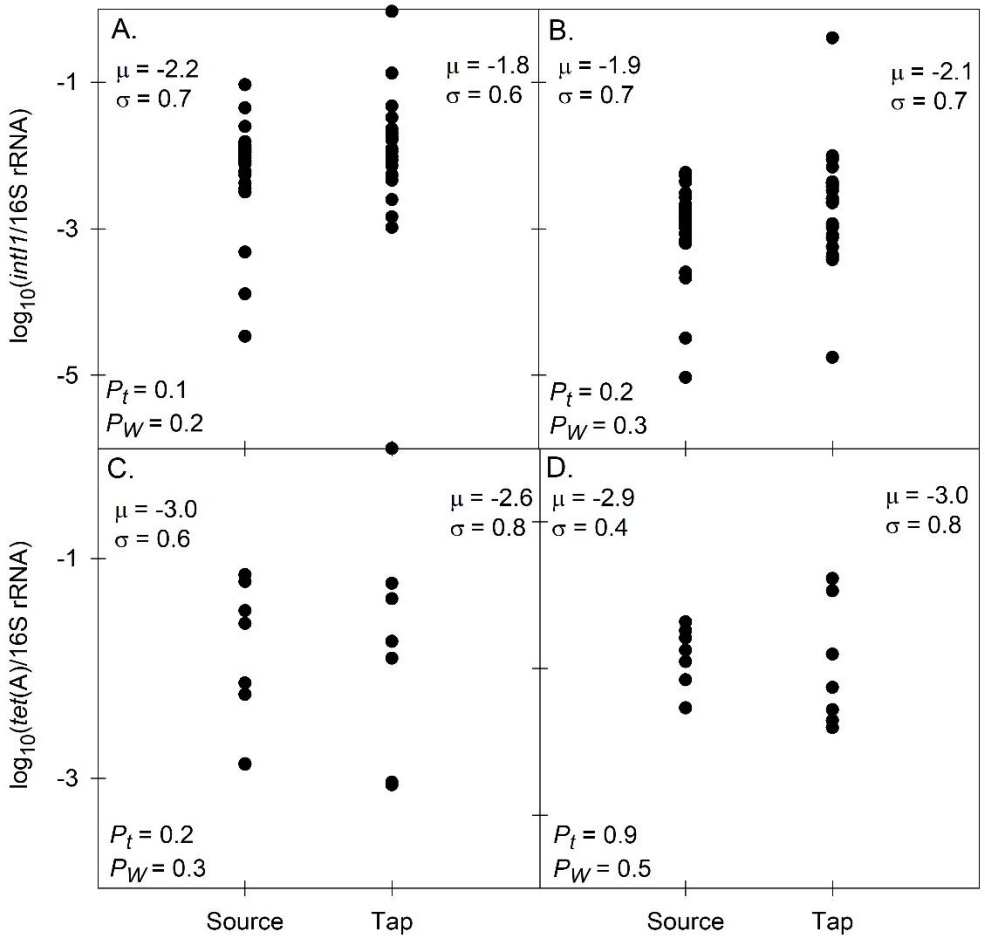

Figure 3. The mean (μ) and standard deviation (σ) of normalized *intI1* and *tet*(A) concentrations at the source and from the distribution system (Tap). (A) normalized *intI1* in systems that provide disinfected groundwater, (B) normalized *intI1* in systems that provide undisinfected groundwater, (C) normalized *tet*(A) in systems that provide disinfected groundwater, and (D) normalized *tet*(A) in systems that provide undisinfected groundwater. of the source water are shown in the top right corner of each panel. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

3.2 Groundwater Source: Microfluidic qPCR

Microfluidic qPCR (MF-qPCR) was also performed targeting the 16S rRNA gene, 3 genes encoding the integrase of 3 different classes of integrons (*intI1*, *intI2*, *intI3*), *qacF*, and 19 antibiotic resistance genes (Table 3 and Table 4). In the systems that practiced disinfection, only 5 genes (16S rRNA genes, *intI1*¸*tet*(A), *mexB*, bla_{SHV}) were detected in at least 25 percent of the samples collected from the groundwater prior to treatment and in samples collected from the distribution system. In the systems that do not practice disinfection, the same 5 genes were frequently detected (>75 percent of samples) in both the raw water as well as water samples collected from the distribution system. In addition, bla_{OXA} was detected in about half of the samples collected (5 out of 8 raw water samples; 2 out of 7 distribution system samples) and was therefore included in statistical analysis. Curiously, *sul1* was not detected in any of the raw water samples but was detected in 5 out of the 7 samples collected from within the distribution system with undisinfected water.

Table 3. Number of detects, mean concentration, and standard deviation of genes detected in disinfected groundwater with MF-qPCR. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

	Prior to Treatment (n = 27)			Distribution System (n = 25)			Statistics	
Gene	Number of Detects	Average $\log_{10}$(gene copies L^{-1})	Standard Deviation	Number of Detects	Average $\log_{10}$(gene copies L^{-1})	Standard Deviation	P_t	P_W
16S rRNA	26	6.4	0.7	24	6.3	0.4	0.3	0.2
ampC	0	-	-	0	-	-	-	-
bla$_{OXA}$	3	-	-	2	-	-	-	-
bla$_{SHV}$	24	3.8	0.7	20	3.4	0.7	0.05	0.03
ereB	0	-	-	0	-	-	-	-
ermB	2	-	-	0	-	-	-	-
ermF	0	-	-	1	-	-	-	-
impl3	0	-	-	0	-	-	-	-
intI1	17	2.7	0.5	6	2.5	0.4	0.08	0.09
intI2	1	-	-	0	-	-	-	-
intI3	0	-	-	0	-	-	-	-
mefE	0	-	-	0	-	-	-	-
mexB	22	3.2	0.7	6	2.7	0.8	9×10^{-3}	7×10^{-4}
qacF	0	-	-	0	-	-	-	-
qnrA	0	-	-	0	-	-	-	-
strB	0	-	-	1	-	-	-	-
sul1	1	-	-	3	-	-	-	-
sul2	1	-	-	0	-	-	-	-
sul3	0	-	-	0	-	-	-	-
tet(A)	9	2.6	0.8	6	2.5	0.4	0.6	0.5
tet(M)	0	-	-	0	-	-	-	-
tet(W)	0	-	-	0	-	-	-	-
tet(X)	1	-	-	0	-	-	-	-
vanA	0	-	-	0	-	-	-	-

Table 4. Number of detects, mean concentration, and standard deviation of genes detected in undisinfected groundwater with MF-qPCR. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

	Prior to Treatment (n = 8)			Distribution System (n = 7)			Statistics	
Gene	Number of Detects	Average $\log_{10}$(gene copies L^{-1})	Standard Deviation	Number of Detects	Average $\log_{10}$(gene copies L^{-1})	Standard Deviation	P_t	P_W
16S rRNA	7	6.9	1.0	7	7	1.0	0.9	0.8
ampC	0	-	-	0	-	-	-	-
blaOXA	5	-	-	2	-	-	-	-
blaSHV	7	4.6	0.9	7	4.4	0.8	0.7	0.5
ereB	0	-	-	0	-	-	-	-
ermB	0	-	-	0	-	-	-	-
ermF	0	-	-	0	-	-	-	-
impl3	0	-	-	0	-	-	-	-
intI1	7	3.7	0.6	6	3.6	0.7	0.7	0.7
intI2	0	-	-	0	-	-	-	-
intI3	0	-	-	0	-	-	-	-
mefE	0	-	-	1	-	-	-	-
mexB	8	4.1	0.4	7	3.7	0.5	0.2	0.2
qacF	0	-	-	0	-	-	-	-
qnrA	0	-	-	0	-	-	-	-
strB	0	-	-	1	-	-	-	-
sul1	0	-	-	5	-	-	-	-
sul2	1	-	-	1	-	-	-	-
sul3	2	-	-	0	-	-	-	-
tet(A)	6	4.1	0.9	6	4.1	0.9	1.0	1.0
tet(M)	0	-	-	0	-	-	-	-
tet(W)	0	-	-	0	-	-	-	-
tet(X)	0	-	-	0	-	-	-	-
vanA	0	-	-	0	-	-	-	-

Using MF-qPCR, the concentrations of total bacterial biomass and *tet*(A) were statistically similar between samples collected from the source and from within in the distribution system in utilities that provide disinfected groundwater ($P_t > 0.3$; $P_W > 0.2$). In contrast, the concentration of *intI1* declined substantially from groundwater ($10^{2.7 \pm 0.5}$ genes L^{-1}) to the distribution system ($10^{2.5 \pm 0.4}$ genes L^{-1}) but did not satisfy the *a priori* threshold for statistical significance ($P_t = 0.08$; $P_W = 0.09$). Similarly, the concentrations of total bacterial biomass, *intI1* and *tet*(A) in the utilities that do not practice disinfection did not significantly change from source to the distribution system ($P_t > 0.7$, $P_W > 0.7$).

The levels of bla_{SHV} and *mexB* significantly decreased in the utilities that provide disinfected groundwater but did not significantly change from source to distribution system in the systems that do not practice disinfection. For the utilities that provide disinfected groundwater, the untreated water had a concentration of $10^{3.8 \pm 0.7}$ bla_{SHV} genes L^{-1}, which was significantly higher than the concentration of bla_{SHV} genes in the treated water from within the distribution system ($10^{3.4 \pm 0.7}$ genes L^{-1}, $P_t = 0.05$, $P_W = 0.03$). The disinfected groundwater systems also had a significant decrease in the levels of *mexB* from source ($10^{3.2 \pm 0.7}$ genes L^{-1}) to distribution system ($10^{2.7 \pm 0.8}$ genes L^{-1}, $P_t = 9 \times 10^{-3}$, $P_W = 7 \times 10^{-4}$). In the samples collected from the utilities that provide undisinfected groundwater, the levels of bla_{SHV} did not significantly change from the untreated water ($10^{4.6 \pm 0.9}$ genes L^{-1}) to the water from within the distribution system ($10^{4.4 \pm 0.8}$ genes L^{-1}, $P_t = 0.7$, $P_W = 0.5$). Similarly, the concentration of *mexB* in the raw water was $10^{4.1 \pm 0.4}$ genes L^{-1}, which was not significantly different than the concentration in the water from within the distribution system ($10^{3.7 \pm 0.5}$ genes L^{-1}, $P_t = 0.2$, $P_W = 0.2$).

3.3 Surface Water Source: Real-time qPCR

The total bacterial biomass in utilities that provide disinfected surface water was significantly ($P_t = 1\times10^{-3}$, $P_W = 3\times10^{-3}$) reduced from the source ($10^{8.5 \pm 1.2}$ 16S rRNA gene copies L^{-1}) to the distribution system ($10^{6.6 \pm 1.0}$ 16S rRNA genes L^{-1}; Fig. 4A). Likewise, treatment and disinfection significantly reduced the levels of *intI1* and *tet*(A) from the source to the distribution system (Fig.4B, 4C). The concentration of *intI1* in the untreated surface water was $10^{5.9 \pm 1.1}$ genes L^{-1}, whereas the concentration of *intI1* in the water from within the distribution system was $10^{4.3 \pm 1.0}$ genes L^{-1} ($P_t = 6\times10^{-3}$, $P_W = 9\times10^{-3}$). Treatment and disinfection also reduced the concentration of *tet*(A) from the source ($10^{5.1 \pm 0.5}$ gene L^{-1}) to the distribution system ($10^{3 \pm 0.3}$ gene copies L^{-1}, $P_t < 10^{-8}$, $P_W < 10^{-3}$).

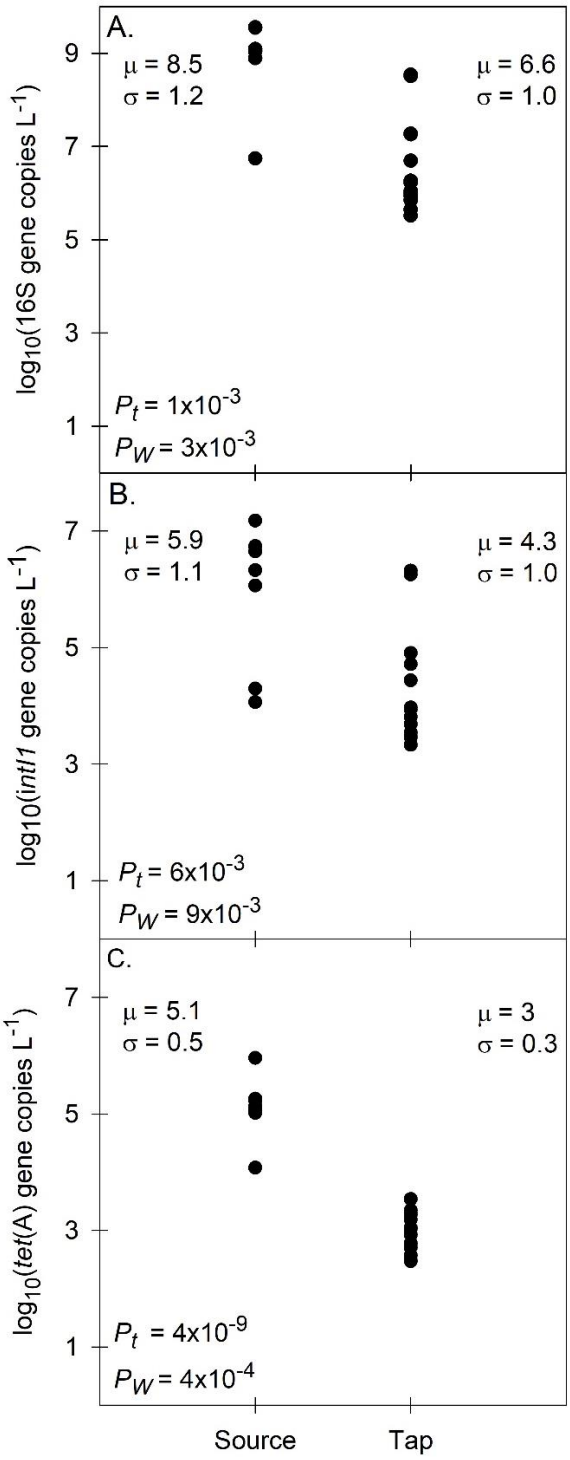

Figure 4. The mean (μ) and standard deviation (σ) of (A) 16S rRNA gene concentration, (B) *intI1* gene concentration and (C) *tet*(A) gene concentration at the source and from the distribution system (Tap) in systems that provide disinfected surface water. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

Similar to the groundwater systems, the relative quantity of antibiotic resistance genes in surface water systems was quantified to determine if it was affected by treatment and disinfection. The ratio of *intI1*:16S rRNA genes significantly increased from the source ($10^{-2.6 \pm 0.4}$) to the distribution system ($10^{-2.3 \pm 0.1}$; $P_t = 9\times10^{-3}$; $P_W = 3\times10^{-3}$; Fig. 5A). In contrast, the ratio of *tet*(A):16S rRNA genes was $10^{-3.4 \pm 1.3}$ in the untreated surface water, which was not significantly higher than the ratio of *tet*(A):16S rRNA genes in the water from within the distribution system ($10^{-3.6 \pm 1.0}$; $P_t = 0.7$; $P_W = 0.9$; Fig. 5B).

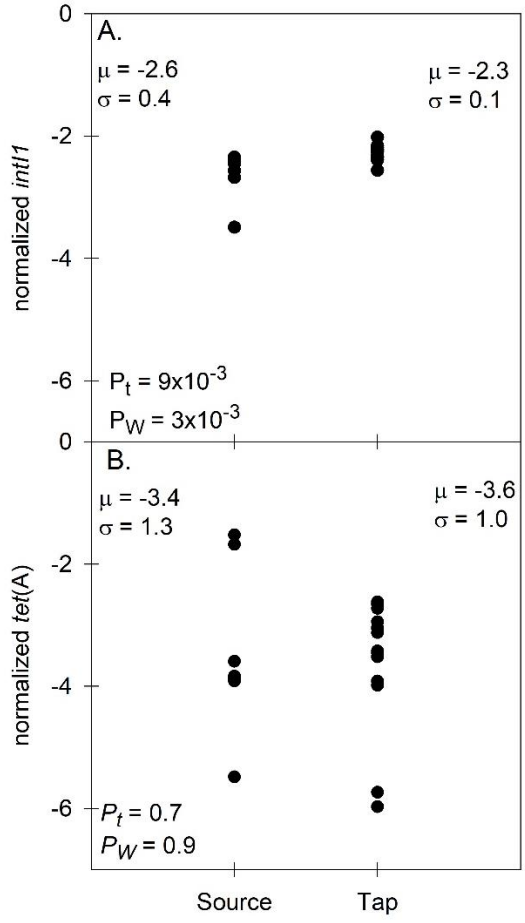

Figure 5. The mean (μ) and standard deviation (σ) of normalized *intI1* and *tet*(A) concentrations at the source and from the distribution system (Tap) in systems that provide disinfected surface water. (A) normalized *intI1* concentration (B) normalized *tet*(A) concentration. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

3.4 Surface Water Source: Microfluidic qPCR

Additional analyses were conducted using MF-qPCR targeting the 16S rRNA gene, 3 genes encoding the integrase of 3 different classes of integrons (*intI1*, *intI2*, *intI3*), *qacF*, and 19 antibiotic resistance genes (Table 5). Only 6 genes (16S rRNA, *intI1*, *tet*(A), *blaSHV*, *mexB*, *sul1*) were detected in at least 25 percent of the untreated source water samples and the samples from water within the distribution system

Table 5. Number of detects, mean concentration, and standard deviation of genes detected in disinfected surface water with MF-qPCR. P_t represents the *P*-value from a Student's t-test and P_W represents the *P*-value from a Wilcoxon rank sum test.

	Prior to Treatment (n = 7)			Distribution System (n = 13)			Statistics	
Gene	Number of Detects	Average $\log_{10}$(gene copies L^{-1})	Standard Deviation	Number of Detects	Average $\log_{10}$(gene copies L^{-1})	Standard Deviation	P_t	P_W
16S rRNA	7	9.7	0.8	13	7.0	0.6	8.9×10^{-8}	4.9×10^{-4}
ampC	0	-	-	0	-	-	-	-
bla$_{OXA}$	0	-	-	0	-	-	-	-
bla$_{SHV}$	6	6.1	1.0	13	4.5	0.4	1.0×10^{-4}	9.0×10^{-3}
ereB	0	-	-	0	-	-	-	-
ermB	0	-	-	1	-	-	-	-
ermF	3	-	-	2	-	-	-	-
impl3	1	-	-	0	-	-	-	-
intI1	6	6.5	1.1	13	4.3	0.5	1.9×10^{-5}	4.3×10^{-3}
intI2	0	-	-	0	-	-	-	-
intI3	2	-	-	1	-	-	-	-
mefE	0	-	-	3	-	-	-	-
mexB	6	5.5	0.8	10	3.3	0.5	1.3×10^{-6}	6.6×10^{-4}
qacF	1	-	-	0	-	-	-	-
qnrA	0	-	-	0	-	-	-	-
strB	3	-	-	0	-	-	-	-
sul1	6	5.8	0.9	8	3.3	0.8	7.9×10^{-6}	8.7×10^{-4}
sul2	5	-	-	1	-	-	-	-
sul3	0	-	-	0	-	-	-	-
tet(A)	6	4.9	0.5	5	2.9	0.5	1.4×10^{-7}	3.6×10^{-4}
tet(M)	0	-	-	0	-	-	-	-
tet(W)	0	-	-	0	-	-	-	-
tet(X)	0	-	-	0	-	-	-	-
vanA	0	-	-	0	-	-	-	-

Similar to the real-time qPCR results, the concentration of total bacterial biomass significantly decreased from $10^{9.7 \pm 0.8}$ 16S rRNA gene copies L^{-1} at the source to $10^{7.0 \pm 0.6}$ 16S gene copies L^{-1} within the distribution system ($P_t < 10^{-7}$; $P_W < 10^{-3}$). Likewise, the levels of *intI1* and *tet*(A) in the untreated surface water (*intI1*: $10^{6.5 \pm 1.1}$ genes L^{-1}; *tet*(A): $10^{4.9 \pm 0.5}$ genes L^{-1}) were significantly higher than the levels in the disinfected water from the distribution system (*intI1*: $10^{4.3 \pm 0.5}$ genes L^{-1}; $P_t < 10^{-4}$; $P_W = 4 \times 10^{-3}$; *tet*(A): $10^{2.9 \pm 0.49}$ genes L^{-1}; $P_t < 10^{-6}$; $P_W < 10^{-3}$).

Treatment and disinfection reduced the concentrations of bla_{SHV}, *mexB* and *sul1* from the untreated surface water to the samples collected from within the distribution system. The concentration of bla_{SHV} in the raw source water was $10^{6.1 \pm 1.0}$ genes L^{-1}, which was significantly higher than the concentration in the water collected from within the distribution system ($10^{4.5 \pm 0.4}$ genes L^{-1}; $P_t < 10^{-3}$; $P_W = 9 \times 10^{-3}$). The levels of *mexB* also significantly decreased from the untreated surface water ($10^{5.5 \pm 0.8}$ genes L^{-1}) to samples collected from the distribution system ($10^{3.3 \pm 0.5}$ genes L^{-1}; $P_t < 10^{-5}$; $P_W < 10^{-3}$). Likewise, the concentration of *sul1* was significantly higher at the source ($10^{5.8 \pm 0.9}$ genes L^{-1}) than in samples collected from within the distribution system ($10^{3.3 \pm 0.8}$ genes L^{-1}; $P_t < 10^{-5}$; $P_W < 10^{-3}$).

4. Discussion

This study clearly demonstrates that the treatment and disinfection of public drinking water supplies significantly reduces the concentrations of total bacteria (16S rRNA genes), antibiotic resistance genes, and *intI1* in public water supplies from both groundwater and surface water sources. Treatment and disinfection resulted in a ~1 log reduction in all three of these genes in groundwater systems and ~2 log reduction in all three of these genes in surface water systems. This conclusion supports more than a century of research and data that the treatment of disinfection of drinking water protects public health from microbial pathogens (American Water Works Association, 2004; Crittenden et al., 2012).

I also conclude that treatment and disinfection have no significant effect on the relative concentrations of *tet*(A) in public water supplies. This contrasts a couple of recent studies that suggested drinking water disinfection results in an increase in antibiotic resistance levels in the finished water. One study used metagenomic sequence analysis to conclude that chlorine disinfection increased the relative abundance of antibiotic resistance genes in drinking water; however, close inspection of their data suggests that the relative abundance of individual antibiotic resistance genes increased by less than 2-fold (Jia et al., 2015). Another study also used metagenomic sequence analysis to conclude that chlorination increased the relative abundance of several antibiotic resistance genes 10- to 100-fold; however, their results were specific to samples collected immediately following chlorine disinfection (Shi et al., 2013).

Disinfection of drinking water had different effects on *intI1* (integrase of class 1 integrons) depending on the source water type. Integrons allow for the expression and

modulation of exogenous gene cases, which has been linked to the proliferation of antibiotic resistance genes via horizontal gene transfer (Mazel, 2006). The most common structure of class 1 integrons includes the *qacE*Δ and *sul1*, which evolved relatively recently in response to substantial use of quaternary amines and sulfonamides, respectively (Gillings, 2014). I hypothesize, therefore, that disinfection has profoundly different impact on the bacteria that harbor integrons depending on the source water; the integrons found in groundwater have been largely unaffected by human antibiotic use (i.e. the water age is older than the antibiotic era), whereas the integrons in surface waters are almost impacted by anthropogenic activities (Gillings et al., 2015).

In addition to source variation (groundwater vs surface water), I collected multiple samples from many utilities to capture temporal and water characteristic differences in order to conduct robust statistical analyses. Characteristics of drinking water utilities are inherently different; source type, water age, and treatment type vary widely. It is well-known that statistical precision and confidence increases with number of independent samples (Prosser, 2010). Replication and comprehensive statistical analyses are incredibly important when using the relative abundance metric; a limited number of samples will not accurately characterize the variance of the data. Furthermore, taking the ratio of two measurements can substantially increase the standard deviation of the data, which means that the change in relative abundance is more likely to be found as statistically insignificant. By analyzing two different source types and numerous utilities, this study provides a broader analysis of the impact of treatment and disinfection on the propagation of antibiotic resistance genes in drinking water.

The investigation of antibiotic resistance genes in drinking water systems poses a unique and difficult challenge. One fundamental challenge is the low concentration of bacterial biomass in drinking water, within which antibiotic genes are relatively uncommon. To help resolve this problem, I used a high-volume, dead-end filtration method (C. M. Smith & Hill, 2009) to collect very high sample volumes (>500 L). Even then, many of the samples analyzed in this study had gene quantities below the detection limit of qPCR (via either real-time or microfluidic qPCR). In addition, there are numerous different antibiotic resistance genes that could be studies; I used real time qPCR to examine genes well-known to be abundant in drinking water (Jia et al., 2015; Shi et al., 2013) and supplemented these analyses with microfluidic qPCR to quantify additional antibiotic resistance genes (Sandberg et al., 2017).

My study could have been improved by examining additional water utilities that use surface water as a source but do not practice disinfection (i.e. as a no-disinfection 'control'). While such facilities exist (Waak et al., 2019), they are typically not found in the United States. Additionally, more surface water utilities that practice disinfection and more groundwater utilities that do not practice disinfection could have been analyzed to increase the sample size for broader statistical analyses.

In conclusion, this study demonstrates that the treatment and disinfection of public water supplies significantly reduces the concentrations of total bacteria, antibiotic resistance genes, and *intI1*. My research also showed that drinking water treatment and disinfection had no significant ($P < 0.05$) on the relative concentration of *tet*(A). The effect of treatment and disinfection on *intI1*, however, differed depending on the source water type. I hypothesize that since groundwater is much older than surface water, the

integrons in surface water have evolved to provide a selective advantage during drinking water disinfection. In contrast, the integrons in groundwater are in organisms that been effectively shielded by the relatively recent (<100 years) wide-scale use of antibiotics. This study further demonstrates that the disinfection of water supplies is effective at protecting public health, in this case by reducing the quantity of antibiotic resistance genes in drinking water. The propagation of antibiotic resistance will continue to challenge society; therefore, further research is necessary to study potential reservoirs, (such as drinking water), mechanisms and solutions.

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6. Appendix A: qPCR Assay and Standard Sequence Information

Table A1: Summary of synthetic double stranded DNA used as standards for real-time qPCR.

Gene	GenBank Accession Number	Standard Sequence (5'→3')
16S rRNA	KR190116	CGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAT TGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGT GTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGC GGGGAGGAAGGGAGTAAAGTTAATACCTTTGCTCATTG ACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCC AGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTA
intI1	KR262557.1	GAGCCCTTGCCCTCCCGCACGATGATCGTGCCGTGATCG AAATCCAGATCCTTGACCCGCAGTTGCAAACCCTCACT GATCCGCATGCCCGTTCCATACAGAAGCTGGGCGAACA AACGATGCTCGCCTTCCAGAAAACCGAGGATGCGAACC ACTTCATCCGGGGTCAGCACCACCGGCAAGCGCCGCGA CGGCCGAGGTCTTCCGATCTCCTGAAGCCAGGGCAGAT CCGTGCACAGCACCTTGCCGTAGAAGAACAGCAAGGCC GCCAATGCCTGACGATGCGTGGAGACCGAAACC
tet(A)	KF483599.2	GACGGCACAGGCTACATCCTGCTTGCCTTCGCGACACG GGGATGGATGGCGTTCCCGATCATGGTCCTGCTTGCTTC GGGTGGCATCGGAATGCCGGCGCTGCAAGCAATGTTGT CCAGGCAGGTGGATGAGGAACGTCAGGGGCAGCTGCA AGGCTCACTGGCGGCGCTCACCAGCCTGACCTCGATCG TCGGACCCCTCCTCTTCACGGCGATCTATGCGGCTTCTA T

Table A2: Summary of primer sequences used for Microfluidic qPCR.

Gene	Description	Assay Sequence (5'→3')	Assay Reference
16S rRNA	Total Bacterial Quantification	F: CCT ACG GGA GGC AGC AG R: ATT ACC GCG GCT GCT GG	(Muyzer et al., 1993)
ampC	Ampicillin Resistance	F: CCT CTT GCT CCA CAT TTG CT R: ACA ACG TTT GCT GTG TGA CG	(Szczepanowski et al., 2009)
bla_{OXA}	ß-lactam Resistance	F: TGA TGA TTG TCG AAG CCA AA R: GCC TGT AGG CCA CTC TAC CC	(Ross & Topp, 2015)
bla_{SHV}	ß-lactam Resistance	F: AAC GGA ACT GAA TGA GGC GCT R: TCC ACC ATC CAC TGC AGC AGC T	(Chia et al., 2005)
ereB	Erythromycin Resistance	F: TCT GCA TTA TGC CAA CGG TA R: TCT GCT CAC TTT GTG GGT TTT	(Szczepanowski et al., 2009)
ermB	Erythromycin Resistance	F: GAT ACC GTT TAC GAA ATT GG R: GAA TCG AGA CTT GAG TGT GC	(Chen et al., 2007)
ermF	Erythromycin Resistance	F: CGA CAC AGC TTT GGT TGA AC R: GGA CCT ACC TCA TAG ACA AG	(Ma et al., 2011)
imp13	ß-lactam Resistance	F: AGG AGC GGC TTT ACC TGA TT R: CGC TCC ACA AAC CAA TTG AC	(Szczepanowski et al., 2009)
intI1	Class 1 Integrase	F: CCT CCC GCA CGA TGA TC R: TCC ACG CAT CGT CAG GC	(Goldstein et al., 2001)
intI2	Class 2 Integrase	F: GAC GGC TAC CCT CTG TTA TCT C R: TGC TTT TCC CAC CCT TAC C	(Barraud et al., 2010)
intI3	Class 3 Integrase	F: GGA TGT CTG TGC CTG CTT G R: GCC ACC ACT TGT TTG AGG A	(Barraud et al., 2010)
mefE	Macrolide Resistance	F: CCT GCA AAT GGC GAT TAT TT R: AAT AGC AAG CAC TGC ACC AG	(Szczepanowski et al., 2009)
mexB	Multi-drug Efflux Pump	F: GTG TTC GGC TCG CAG TAC TC R: AAC CGT CGG GAT TGA CCT TG	(Mcnamara et al., 2014)
qacF	Quaternary Ammonia Disinfectant Resistance	F: TGG CTG TTT CAA TCT TTG GC R: GCC CAT ACA GCG TAA GCA AT	(Szczepanowski et al., 2009)
qnrA	Quinolone Resistance	F: AGG ATT TCT CAC GCC AGG ATT R: CCG CTT TCA ATG AAA CTG CA	(Cummings et al., 2011)
strB	Streptomycin Resistance	F: CGC AGT TCA TCA GCA ATG TC R: GCC TGT TTT TCC TGC TCA TT	(Szczepanowski et al., 2009)
sul1	Sulfonamide Resistance	F: CCG TTG GCC TTC CTG TAA AG R: TTG CCG ATC GCG TGA AGT	(Heuer & Smalla, 2007)
sul2	Sulfonamide Resistance	F: GAC AGT TAT CAA CCC GCG AC R: GTC TTG CAC CGA ATG CAT AA	(Szczepanowski et al., 2009)
sul3	Sulfonamide Resistance	F: TCC GTT CAG CGA ATT GGT GCA G R: TTC GTT CAC GCC TTA CAC CAG C	(Pei et al., 2006)
tet(A)	Tetracycline Resistance	F: GCT ACA TCC TGC TTG CCT TC R: CAT AGA TCG CCG TGA AGA GG	(Ng et al., 2001)

tet(M)	Tetracycline Resistance	F: GTG GAC AAA GGT ACA ACG AG R: CGG TAA AGT TCG TCA CAC AC	(Ng et al., 2001)
tet(W)	Tetracycline Resistance	F: GAG AGC CTG CTA TAT GCC AGC R: GGG CGT ATC CAC AAT GTT AAC	(Aminov et al., 2001)
tet(X)	Tetracycline Resistance	F: AGC CTT ACC AAT GGG TGT AAA R: TTC TTA CCT TGG ACA TCC CG	(Ghosh et al., 2009)
vanA	Vancomycin Resistance	F: GTA GGC TGC GAT ATT CAA AGC R: CGA TTC AAT TGC GTA GTC CAA	(Bell et al., 1998)

Table A3: GenBank Accession Numbers for the synthetic double stranded DNA used as standards for qPCR.

Gene	GenBank Accession Number
16S rRNA	KR190116
ampC	KR010387.1
bla_{OXA}	AY007784.1
bla_{SHV}	KT218681.1
ereB	AF466411.1
ermB	KF864551.1
ermF	KP265720.1
imp13	JN091097.1
intI1	KR262557.1
intI2	KF534916.1
intI3	HE616889.1
mefE	EU908679.1
mexB	L11616.1
qacF	KC441951.1
qnrA	JN103331.1
strB	NG_041759.1
sul1	KR338352.1
sul2	KM194586.1
sul3	AY617070.1
tet(A)	KF483599.2
tet(M)	KJ545575.2
tet(W)	KC790464.1
tet(X)	JQ990987.1
vanA	LC088032.1

Table A4: Summary of synthetic double stranded DNA used as standards for Microfluidic qPCR.

Genes	Sequence (5' → 3')
sul1	CTTGGACTATAACATTCAAGCTATGTCGGGCTTTATTTCAGGAG GCGGAATGCAGGGCGATATGGGTGCAAAAGACAAATACATGGC AGATTCTGTGCTGTGGCATTTAA**AAAACCCACAAAGTGAGCAG** **A**aagtgatagtaaaagaaacc**GATACCGTTTACGAAATTGG**AACAGGTAA AGGGCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTA ACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGA AAAATTAAAACTGAATACTCGTGTCACTTTAATTCACCAAGATA TTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTT GGGAATATTCCTTACCATTTAAGCACACAAATTATTAAAAAAGT
ereB	GGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAG
ermB	AAGGATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGG
ermF	GTTGCTCTT**GCACACTCAAGTCTCGATTC**agcaattgctctattgaaaa**CG** **ACACAGCTTTGGTTGAAC**ATTTACGAAAATTATTTTCTGATGC CCGAAATGTTCAAGTTGTCGGTTGTGATTTTAGGAATTTTGCAG TTCCGAAATTTCCTTTCAAAGTGGTGTCAAATATTCCTTATGGC ATTACTTCCGATATTTTCAAAATCCTGATGTTTGAGAGTCTTGA AAATTTTCTGGGAGGTTCCATTGTCCTTCAGTTAGAACCTACAC AAAAGTTATTTTCGAGGAAGCTTTACAATCCATATACCGTTTTC TATCATACTTTTTTTGATTTGAAA**CTTGTCTATGAGGTAGGTC** **C**tgaaagtttc
qacF *qnrA* *tet*(W) *intI2* *intI3*	tggatatttc**TGGCTGTTTCAATCTTTGGC**GAGGTCATCGCAACTTC CGCACTGAAGTCTAGCCATGGATTCACTAGGTTAGTTCCTTCCG TTGTAGTTGTGGCTGGTTACGGGCTTGCGTTCTATTTCTTGTCTC TCGCGCTCAAGTCCATTCCGGTCGGT**ATTGCTTACGCTGTATG** **GGC**tgggcttggctttcagcaag**AGGATTTCTCACGCCAGGATT**TGAGTG ACAGCCGTTTTCGCCGCTGCCGCTTTTATCAGTGTGACTTCAGC CATTGCCAGCTAAGGGATGCCAGTTTCGAGGAT**TGCAGTTTCA** **TTGAAAGCGG**cgccatcgaagaccttgacg**GAGAGCCTGCTATATGCCA** **GC**GGAGCCATTTCAGAACCGGGGAGCGTCGAAAAAGGGACAA CGAGGACGGACACCATGTTTTTGGAGCGGCAGCGTGGGATTAC CATTCAAGCGGCAGTCACTTCCTTCCAGTGGCACAGATGTAAA**G** **TTAACATTGTGGATACGCCC**ggccacatggagcaagccta**GACGGCTAC** **CCTCTGTTATCTC**TGCAAATGAAGTGCAACGCATTTTGCAGGT TATGGATACTCGCAACCAAGTTATTTTTACGCTGCTGTATGGTG CAGGTTTGCGCATTAATGAATGCTTGCGTTTGCGGGTTAAAGAT TTTGATTTTGATAATGGCTGCATCACTGTGCATGAC**GGTAAGG** **GTGGGAAAAGCA**gaaacagccttgcaccgttc**GGATGTCTGTGCCTGCT** **TG**CAGCAAGTGGGTGGCGAATGAGTGGCGCAGGGTGTGGACAG ATACGTGTTTGGCAATGCCAGCCTGAACTACCGCTTTTTTTAGTT GCCGGTTCAGTCTT**TCCTCAAACAAGTGGTGGC**ggcgctcaac

blaOXA *blaSHV* *strB* *tet*(M)	gttaaagact**TGATGATTGTCGAAGCCAAA**CGTGATTGGATACTACG TGCCAAAACAGGCTGGGATGGTCAAATGGGTTGGTGGGTCGGT TGGGTAGAGTGGCCTACAGGCccagtattttaccgctggga**AACGGAACTG** **AATGAGGCGCT**TCCCGGCGACGCCCGCGACACCACTACCCCGG CCAGCATGGCCGCGACCCTGCGCAAGCTGCTGACCAGCCAGCG TCTGAGCGCCCGTTCGCAACGGC**AGCTGCTGCAGTGGATGGT** **GGA**cgatcgggtcgtcggccccg**CGCAGTTCATCAGCAATGTC**TTCTATA GGTTTCAATCCCTTGACGATTGCAGGAGTCCCGTCTGGCAATGA AACTTTCCAAACGAGGCTGGAAAAGGTGTCCGCAATGAGAACA GGTTGCGAAACGTGCC**AATGAGCAGGAAAAACAGGC**ggcatgaac aattaggaagc**GTGGACAAAGGTACAACGAG**GACGGATAATACGCT TTTAGAACGTCAGAGAGGAATTACAATTCAGACAGGAATAACC TCTTTTCAGTGGGAAAATACGAAGGTGAACATCATAGACACGC CAGGACATATGGATTTCTTAGCAGAAGTATATCGTTCATTATCA GTTTTAGATGGGGCAATTCTACTGATTTCTGCAAAAGATGGCGT ACAAGCACAAACTCGTATATTATTTCATGCACTTAGGAAAATGG GGATTCCCACAATCTTTTTTATCAATAAGATTGACCAAAATGGA ATTGATTTATCAACGGTTTATCAGGATATTAAAGAGAAACTTTC TGCCGAAATTGTAATCAAACAGAAGGTAGAACTGTATCCTAAT AT**GTGTGTGACGAACTTTACCG**aatctgaaca
ampC *impl13* *vanA* *sul2* *sul3*	ttaattaccg**CCTCTTGCTCCACATTTGCT**GCCCCTCAACAAATCAA CGATATTGTGCATCGCACAATTACCCCGCTTATAGAGCAACAAA AGATCCCGGGTATGGCGGTGGCGGTAATTTATCAGGGTAAACC TTATTACTATACCTGGGGCTATGCGGACATCGCCAAAAAGCAGC C**CGTCACACAGCAAACGTTGT**ttgagttaggttactgccgc**AGGAGCGGC** **TTTACCTGATT**TAAAAATCGAGAAGCTTGAAGAAGGTGTTTTT GTTCATACATCGTTCGAAGAGGTTAACGGTTGGGGGGTTGTTAC TAAACACGGTTTAGTGGTGCTTGTAAACACAGACGCCTATCTAA TTGACACTCCATTTACTGCTACAGACACTGAAAAATTA**GTCAAT** **TGGTTTGTGGAGCG**cggctatgaatatccctttt**GTAGGCTGCGATATTC** **AAAGC**TCAGCAATTTGTATGGACAAATCGTTGACATACATCGTT GCGAAAAATGCTGGGATAGCTACTCCCGCCTTTTGGGTTATTAA TAAAGATGATAGGCCGGTGGCAGCTACGTTTACCTATCCTGTTT TTGTTAAGCCGGCGCGTTCAGGCTCATCCTTCGGTGTGAAAAAA GTCAATAGCGCGGACGAA**TTGGACTACGCAATTGAATCG**gcaag acaatcgtctcgctc**GACAGTTATCAACCCGCGA**CGCAAGCCTATGCCT TGTCGCGTGGTGTGGCCTATCTCAATGATATTCGCGGTTTTCCA GACGCTGCGTTCTATCCGCAATTGGCGAAATCATCTGCCAAACT CGTCG**TTATGCATTCGGTGCAAGAC**gggcaggcaggttgatgcac**TCCG** **TTCAGCGAATTGGTGCAG**CTACTAAAGTTGAAACGAATCCGG AAGAGGTTTTTACTTCCATGATGGAATTTTTTAAAGAAAGAATT GCTGCTTTAGTTGAG**GCTGGTGTAAGGCGTGAACGAA**ttattcttga

	attatgactt**AGCCTTACCAATGGGTGTAAA**TATTGCTGATAAAAAA
	GGCAATATTTTATCCACAAAAAATGTAAAGCCCGAAAATCGAT
	TTGACAATCCTGAAATAAACAGAAATGACTTAAGGGCTATCTTG
	TTGAATAGTTTAGAAAACGACACGGTTATTTGGGATAGAAAAC
	TTGTTATGCTTGAACCTGGTAAGAAGAAGTGGACACTAACTTTT
	GAGAATAAACCGAGTGAAACAGCAGATTTGGTTATTCTTGCCA
	ATGG**CGGGATGTCCAAGGTAAGAA**aatttgttacctagtgccat**CCTGCA**
	AATGGCGATTATTTTTTACCTTACAGAAAAAACAGGATCTGCG
	ATGGTCTTGTCTATGGCTTCATTAGTAGGTTTTTTACCCTATGCG
tet(X)	ATTTTGGGACCTGCCATTGGTGTGCTAGTGGATCGTCATGATAG
mefE	GAAGAAGATAATGATTGGTGCCGATTTAATTATCGCAGCAG**CT**
mexB	**GGTGCAGTGCTTGCTATT**gttgcattcttgacttccag**GTGTTCGGCTCG**
tet(W)	**CAGTACTC**GATGCGCATCTGGCTCGACCCGGCCAAGCTGAACA
	GCTACCAGCTGACCCCCGGCGACGTGAGCAGCGCGATCCAGGC
	GCAGAACGTGCAGATTTCCTCCGGCCAGCTCGGCGGCTTGCCGG
	CGGTCAAGGGCCAGCAGCTCAACGCCACCATCATCGGCAAGAC
	CCGCCTGCAGACCGCGGAGCAATTCGAGAACATCCTGCT**CAAG**
	GTCAATCCCGACGGTTcccaggtgcggaccttgacg**GAGAGCCTGCTAT**
	ATGCCAGCGGAGCCATTTCAGAACCGGGGAGCGTCGAAAAAG
	GGACAACGAGGACGGACACCATGTTTTTGGAGCGGCAGCGTGG
	GATTACCATTCAAGCGGCAGTCACTTCCTTCCAGTGGCACAGAT
	GTAAA**GTTAACATTGTGGATACGCCC**ggccacatgg

Appendix B: Method Correlation

Three genes (16S rRNA genes, *tet*(A), and *intI1*) were quantified by both real time qPCR and by MF-qPCR. Results were compared with linear regression and by a Pearson's correlation test (done using MATLAB R2018b). A Pearson's coefficient of 1 indicates a perfect positive linear correlation. Although results varied, the results of the MF-qPCR statistically correlated with each the results of real time PCR for each gene ($P_\rho < 0.05$ Fig. B1).

All genes showed a significant, positive correlation between methods ($\rho > 0.49$; $P_\rho < 0.013$). Linear regression analysis shows that real-time qPCR systematically predicted higher concentrations than MF-qPCR; for values detected by both methods, the $\log_{10}$ values of the MF-qPCR results were typically 70 to 80 percent of the $\log_{10}$ values produced by real-time qPCR.

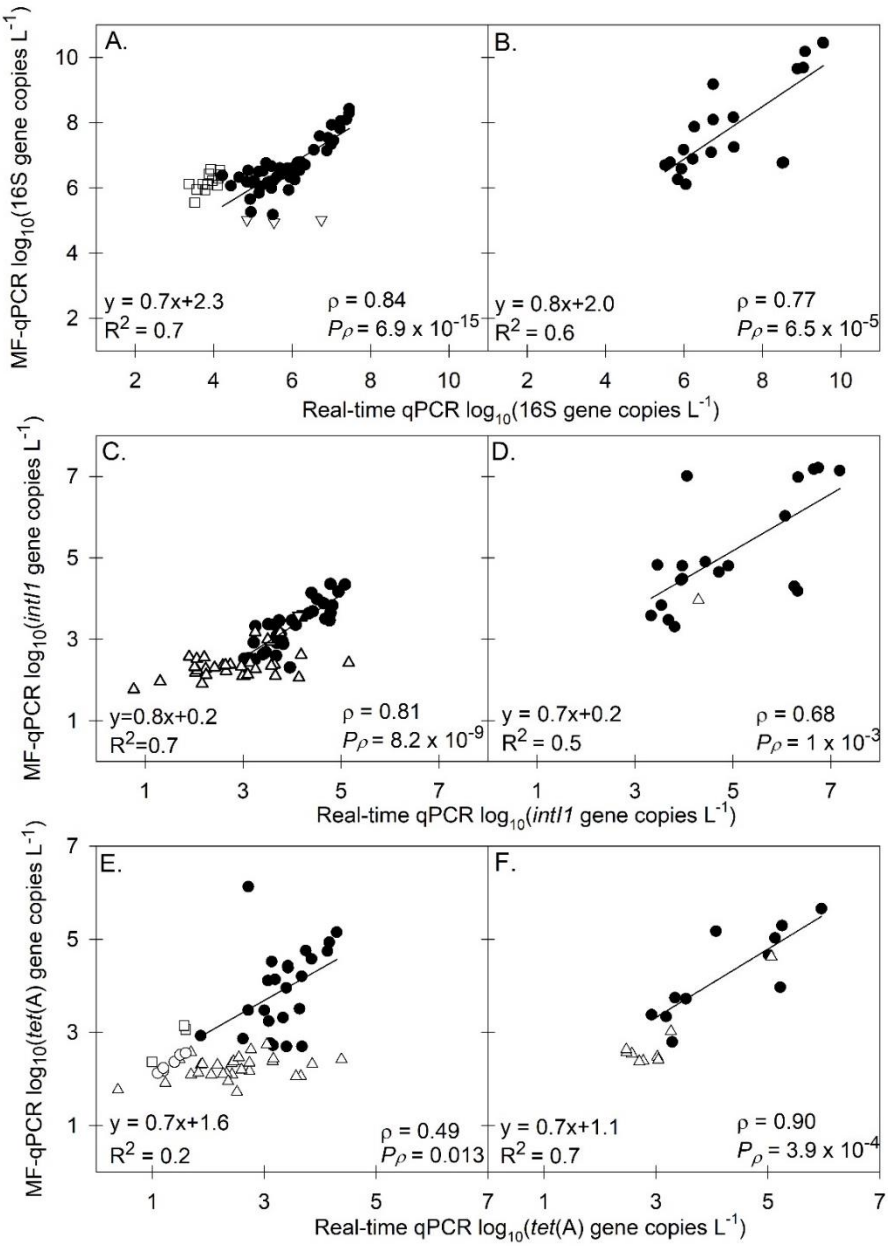

Figure B1. Comparison of real-time qPCR data with microfluidic qPCR data for (A) 16S rRNA genes in groundwater, (B) 16S rRNA genes in surface water, (C) *intI1* in groundwater, (D) *intI1* in surface water, (E) *tet*(A) in groundwater and (F) *tet*(A) in surface water. Closed circles represent data that were above the limit of detection using both methods. Open circles represent data that were below the limit of detection using both methods. Open squares represent data that were below the limit of detection for real-time qPCR but above the limit of detection using MF-qPCR. Open triangles represent data that were below the limit of detection for MF-qPCR but greater than the limit of detection using real-time qPCR. The bottom left corner of each panel shows the linear regression line and fit coefficient (R^2) describing the data detected with both methods. The bottom right corner of each panel shows the Pearson's coefficient (ρ) and associated *P*-value (P_ρ) conducted on the data detected by both methods.