Groundwater Research Report
WR05R006

EVALUATION OF ON-SITE WASTEWATER TREATMENT AS A SOURCE OF ANTIBIOTIC RESISTANCE GENES IN GROUNDWATER

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Funding Year: 2005

Evaluation of On-Site Wastewater Treatment as a Source of Antibiotic Resistance Genes in Groundwater

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A report to

The University of Wisconsin System Groundwater Research Program

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Project Duration: July 2005 – June 2007

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PROJECT SUMMARY

Title:	Evaluation of On-Site Wastewater Treatment as a Source of Antibiotic Resistance Genes in Groundwater
Project ID:	WR05R006
Investigators:	Katherine D. McMahon, Assistant Professor, Department of Civil and Environmental Engineering Erin E. Seyfried, Graduate Student, Department of Civil and Environmental Engineering
Period of Contract:	7/1/2005 – 6/30/2007

Background/Need: Concerns surrounding the presence of pharmaceutically active compounds in our water resources are driving efforts to characterize their occurrence, fate, and transport in the environment. One potential consequence of the release of antibiotics is the proliferation of resistant bacteria. This work addresses the need to identify sources of antibiotic resistant bacteria in the environment. Onsite-wastewater treatment systems are evaluated as a potential source of genes encoding antibiotic resistance.

Objectives:

1. Characterize the genotypic diversity of genes conferring resistance to tetracycline, in effluent from individual onsite wastewater treatment systems.
2. Assess the impact of onsite wastewater treatment systems on the occurrence of tetracycline resistance genes in groundwater.

Methods: The study site was a recently constructed, unsewered subdivision in Dane County, called Savannah Valley. Septic tanks were installed at two residential sites in 2004. Water samples were collected in 2004, 2005, and 2006 from septic tanks, wells installed down-gradient of the septic tanks, and household water supplies. Molecular tools were used to qualitatively screen for the presence of ten tetracycline resistance genes (tet^R). A quantitative technique was used to assess the abundance of three tet^R in the septic tanks to compare their concentrations to those found in other aquatic systems.

Results and Discussion: Generally, septic tank effluent contained the most number of different types of tet^R and private water supplies contained the fewest. This is not surprising given that the water supply wells were deep and unlikely to have been impacted by septic tank effluent. The few types detected in water supplies may be present in naturally occurring bacterial communities in groundwater or could have been introduced as a result of past agricultural activity. The tet^R detected in the monitoring wells may have originated from septic tank effluent, given that bromide tracer experiments predicted the average residence time between the septic tanks and the monitoring wells was on the order of 30 to 60 days. However, the tet^R detection frequency in monitoring wells was comparable across years, suggesting that occupation of the residences contributing to the septic tanks did not cause an increase in the

number tet^R that could be detected over the study period. Longer term monitoring targeting these ten tet^R as well as genes encoding resistance to other antibiotics is necessary to confirm this observation. The concentrations of three tet^R were higher in one septic tank than in another, which might be explained by resident lifestyle differences or the length of septic tank operation.

Conclusions/Implications /Recommendations: Genes expected to encode resistance to tetracycline were detected using a cultivation-independent molecular approach in private deep well water supplies, septic tank effluent, and downgradient monitoring wells at a newly developed subdivision. These genes were detected most frequently in septic tank effluent, presumably because bacteria carrying the genes were present in the septic tanks. The concentrations of resistance genes in the septic tanks were several orders of magnitude higher than those observed in treated municipal wastewater effluent. Some of these genes may have been transported in the subsurface to the monitoring wells, but in some cases genes were detected in the monitoring wells that were not present in the corresponding septic tanks. Therefore, we hypothesize that past agricultural activity may have contributed to the presence of resistance genes in subsurface bacteria. Longer-term sampling over several more years combined with higher spatial resolution is required to adequately test this hypothesis.

Related Publications: None.

Key Words: Antibiotics, septic tanks, onsite wastewater treatment

Funding: UWS

INTRODUCTION

The emergence and spread of antibiotic resistance in bacteria is a major public health issue (Levy, 2002). Significant quantities of antibiotics are released into the natural environment in treated wastewater effluent and through use in confined animal feeding operations (Halling-Sorensen et al., 1998). Many of these compounds can now be readily detected in our water resources (Kolpin et al., 2002, Lindsey et al., 2001, Yang and Carlson, 2003), leading to increasing concerns regarding their contribution to the presence and persistence of resistance in populations of both pathogenic and non-pathogenic microbes. These concerns are fueled partly by a lack of critical information regarding the movement of resistance genes within and between commensal microbes and microbial populations in the environment (Isaacson and Torrence, 2002). Government and regulatory agencies with a potential interest in controlling antibiotic release through wastewater discharge and agricultural use, in the future, need this kind of information in order to develop sound policy.

To understand the ecology of antibiotic resistance, it is necessary to characterize the occurrence, fate, and transport of both the antibiotics and the antibiotic resistance genes. Antibiotics are found in groundwater (Lindsey et al., 2001) and surface waters (Kolpin et al., 2002, Yang and Carlson, 2003) generally at submicrogram per liter concentrations. A few recent studies focusing on antibiotic resistance genes as emerging contaminants measured their prevalence and abundance across human "impact" gradients in their systems. Pei et al. (Pei et al., 2006) surveyed sediments from a river in a mixed-use landscape for antibiotic resistance genes and observed higher resistance gene concentrations with increasing urban and agricultural impact. Auerbach et al. (Auerbach et al., 2007) demonstrated that municipal wastewater treatment plants disseminate many tetracycline resistance genes (tet^R). Other potential sources of antibiotics and their associated resistance determinants include aquaculture (Mellon et al., 2001, Miranda et al., 2003), animal feedlots (Campagnolo et al., 2002), swine waste lagoons (Aminov et al., 2002, Chee-Sanford et al., 2001, Ng et al., 2001), and runoff from manure-amended and effluent irrigated agricultural lands (Pedersen et al., 2005). With so many routes through which antibiotics and antibiotic resistance genes can enter the natural environment, establishment of resistance reservoirs in natural microbial communities appears likely. A better understanding of the relative contributions of these sources to environmental resistance reservoirs is needed to prioritize targets for reducing antibiotic use or release.

Onsite wastewater treatment systems are known to contain antibiotic resistant bacteria (Pillai et al., 1997), and malfunctioning systems have been shown to lead to an increase in the proportion of resistant culturable bacteria present in adjacent surface waters (Harwood et al., 2000). However, these and other previous studies on onsite wastewater treatment systems employed only phenotypic culture-based methods to detect resistance. In this study, modern molecular techniques were used to selectively target different classes of tet^R, thereby avoiding the inherent bias of cultivation-based methods. Furthermore, little is known about the release of antibiotics or resistant organisms from more modern onsite wastewater treatment systems. In this study we sought to monitor the profile of antibiotic resistance in newly installed septic systems and the water impacted by them over the first three years of operation.

Our study site was the Savannah Valley subdivision in Dane County, Wisconsin. The Wisconsin Geological and Natural History Survey installed over twenty water-table monitoring wells

beginning in October 2001, and the first home was completed in May 2003. Deeper bedrock wells were drilled to serve as drinking water supplies for residences. The two residences selected for monitoring were constructed in 2004. These residences were also the subjects of focused study by Wilcox (2007) as part of another project supported through the Groundwater Coordinating Council, DNR Project #178. Wilcox monitored the concentrations of emerging organic wastewater contaminants such as caffeine, 17β-estradiol, and acetaminophen between 2004 and 2006. During the same period, we collected water samples and assessed the tetracycline resistance "fingerprint" of the bacterial communities in the water.

Water samples were collected in collaboration with Jeffery Wilcox in March 2004, October 2005, and August 2006. Total community DNA was extracted from each sample and polymerase chain reaction (PCR) was used to specifically detect the presence of ten genetically distinct types of tet^R. A quantitative PCR technique was also used to measure the concentration of two tet^R in order to compare their abundances in septic tanks to that observed in conventional centralized municipal wastewater treatment plants.

PROCEDURES AND METHODS

Water samples were collected from wells and septic tanks in the Savannah Valley subdivision in Dane County, WI between 2004 and 2006 (Table 1, Figure 1). This site was characterized as part of a related project and detailed site descriptions, hydrology, and groundwater chemistry findings are reported elsewhere (Wilcox, 2007, Wilcox et al., 2005). Monitoring wells MW-20 and MW-22 were located downgradient from the septic tank leachfields associated with SE5 and SE8, respectively. Monitoring well MW-02 was located on a plot that was not developed (and had no septic tank), and was included in the study to serve as an unimpacted reference. The private water supplies WS5 and WS8 were deep bedrock wells at residences with septic tanks corresponding to SE5 and SE8, respectively. The residence with SE5 was occupied from early 2004 onward and the residence with SE8 was occupied from November 2004 ownward.

Water was collected from wells and septic tanks using peristaltic pumps and new Teflon tubing, after which it was transferred to sterile 2-L polycarbonate bottles. Samples were stored on ice during transport back to the laboratory for filtration within 4 hours. Bacteria in each water sample were concentrated by vacuum filtration onto three replicate 0.2-µm filters (Supor-200; Gelman Sciences, Ann Arbor, MI). Filters were stored at -80 ^{o}C until DNA could be extracted.

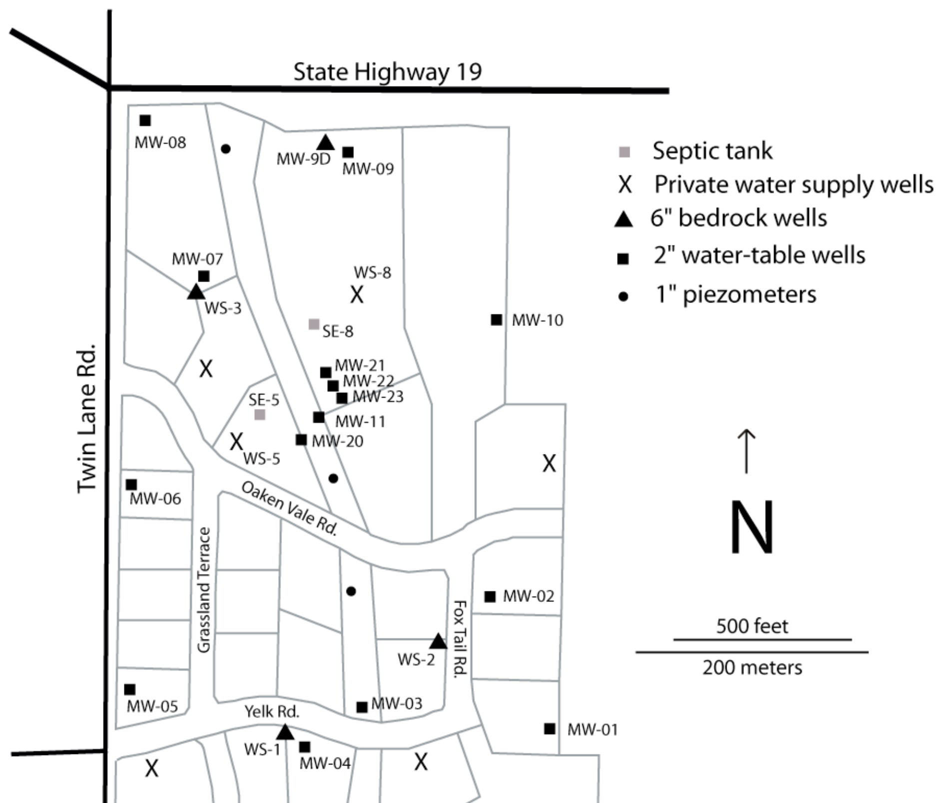
Table 1. Sample sites and the months during which samples were collected.

Sample site name	Description	Samples collected
MW-11	Monitoring well 11	March 2004
MW-02	Monitoring well 02	October 2005, August 2006
MW-20	Monitoring well 20	March 2004, October 2005, August 2006
MW-22	Monitoring well 22	October 2005, August 2006
WS5	Water supply 5	October 2005, August 2006
WS8	Water supply 8	October 2005, August 2006
SE5	Septic effluent 5	March 2004, October 2005, August 2006
SE8	Septic effluent 8	March 2004, October 2005, August 2006

DNA extraction

DNA was extracted from bacteria trapped on filters using the FastDNA kit according to the manufacturer's instructions (Qbiogene, Irvine, CA), with minor modifications. Filters were cut in half and each half was used in parallel extractions. Binding matrix was combined in the spin column prior to elution so that DNA from the entire filter could be eluted in the recommended 50 µl. Concentrations of purified DNA were determined by spectrofluorometry (SPECTRAmax GEMINI XS®, Molecular Devices Corporation, Sunnyvale, CA) using the PicoGreen® dsDNA quantitation assay (Quant-it™ PicoGreen® dsDNA Assay Kit, Invitrogen Corporation, Carlsbad, CA). Extractions were diluted to 2 ng DNA/µL in sterile, TE buffer (10mM Tris-HCl, 1mM EDTA [pH=8]).

Figure 1. Map of Savannah Valley subdivision with sample locations labeled as described in Table 1.



Qualitative PCR.

Presence/absence PCR was conducted to screen for ten distinct tet^R (Table 2). Amplification was conducted in 25-μL reaction volumes containing 1× PCR Buffer (Applied Biosystems, Foster City, CA), 3 mM $MgCl_2$, 400 μM each deoxynucleoside triphosphate (dNTP), 400 nM of each forward and reverse primer, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) using an iCycler thermal cycler (BioRad, Hercules, CA). The thermal cycle was: initial denaturation at 95°C for 7 min; 40 cycles of 95°C for 45 s, annealing (various temperatures, see Table 2) for 45 s, and 72°C for 90 s; and a final extension of 72°C for 7 min. Reactions targeting ten tet^R genes were carried out using previously designed primer sets (Table 2) and 2 ng of template DNA. A DNA template mass of 2 ng per reaction was previously found to be optimal for such reactions (Auerbach et al., 2007). PCR product aliquots (4 μL) were visualized by electrophoresis on 1.5% (wt/vol) agarose gels stained with ethidium bromide and denoted as "+" when a DNA band could be identified on the gel.

Table 2. Primers used to target tet^R in this study.

Targeted tet^R Class		Sequence (5'→ 3')	Annealing Temperature (°C, T_A)	Amplicon Size (bp)	Reference
tet(A)	FW RV	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG	61.3	210	(Ng et al., 2001)
tet(B)	FW RV	TAC GTG AAT TTA TTG CTT CGG ATA CAG CAT CCA AAG CGC AC	58	206	(Aminov et al., 2002)
tet(D)	FW RV	AAA CCA TTA CGG CAT TCT GC GAC CGG ATA CAC CAT CCA TC	63.1	787	(Ng et al., 2001)
tet(E)	FW RV	GGT ATT ACG GGA GTT TGT TGG AAT ACA ACA CCC ACA CTA CGC	61.2	199	(Aminov et al., 2002)
tet(G)	FW RV	GCA GAG CAG GTC GCT GG CCY GCA GAG GAA GCC AGA AG	61.3	134	(Aminov et al., 2002)
tet(M)	FW RV	ACA GAA AGC TTA TTA TAT AAC TGG CGT GTC TAT GAT GTT CAC	61.2	406	(Ng et al., 2001)
tet(O)	FW RV	ACG GAR AGT TTA TTG TAT ACC TGG CGT ATC TAT AAT GTT GAC	60	171	(Aminov et al., 2001)
tet(Q)	FW RV	TTA TAC TTC CTC CGG CAT CG ATC GGT TCG AGA ATG TCC AC	61.2	904	(Ng et al., 2001)
tet(S)	FW RV	CAT AGA CAA GCC GTT GAC C ATG TTT TTG GAA CGC CAG AG	65	667	(Ng et al., 2001)
tet(W)	FW RV	GAG AGC CTG CTA TAT GCC AGC GGG CGT ATC CAC AAT GTT AAC	64	168	(Aminov et al., 2001)

To assess reproducibility, duplicate PCR reactions were performed for each permutation of sample and primer set, with each assay including positive and negative controls. Positive controls were purified PCR products amplified from plasmids carrying the appropriate tet^R gene (Aminov et al., 2002, Aminov et al., 2001). PCR conditions were the same as described above. Products were purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA), and 2 ng of DNA was used per positive control reaction. Negative controls contained an additional 1 μL of sterile water. Detection limits were determined for each primer pair by a serial dilution of the

positive control template. Detection limits for tet^R genes were determined by constructing titration curves of known gene copies per µL of template and running these dilutions on an agarose gel until no product was visualized. Product for each gene typically disappeared below 10 copies/µL.

Quantitative PCR.

We measured the concentration of tetG, tetO, and tetQ in septic tank effluent using SYBR-Green quantitative real time PCR. Three primer sets were designed to amplify target gene regions from plasmids carrying the target tet^R gene, to generate template for standard curves (Table 3). Specificity was verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Amplification was conducted in 50-µL reaction volumes containing 1× PCR Buffer (Applied Biosystems, Foster City, CA), 3 mM $MgCl_2$, 400 µM of each deoxynucleoside triphosphate (dNTP), 400 nM of each forward and reverse primer, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems) using an iCycler thermal cycler (BioRad, Hercules, CA). The thermal cycle was: initial denaturation at 95°C for 7 min; 40 cycles of 95°C for 45 s, annealing at 60°C for 45 s, and 72°C for 90 s; and a final extension of 72°C for 7 min. PCR product aliquots (4 µL) were visualized by electrophoresis on 1.5% (wt/vol) agarose gels stained with ethidium bromide. The remaining PCR product was purified using the QIAquick PCR Purification kit (QIAGEN, Valencia, CA). Concentrations of purified PCR product were quantified using either microspectrophotometry with a Gemini SpectraMax spectrofluorometer (Bucher Biotech, Basel, Switzerland) or fluorometry with the Quant-iT™ PicoGreen® dsDNA Assay kit (Molecular Probes/Invitrogen, Eugene, OR).

Equation 1 was used to determine the copy number of genes per µL of the PCR purified product.

$$\textit{Copy of genes/µL DNA} = \frac{c * N_A}{l * b * 10^6} * \frac{\text{DNA elution volume } (\mu\text{l})}{\text{water sample volume (L)}} \qquad \textbf{Equation 1}$$

Where c is the concentration of template in µg/µL, N_A is Avogadro's number (6.02×10^{23}/mol), l is the length of amplicon containing the target gene, and b is the weight of 1bp DNA ($660\ g \cdot bp^{-1} \cdot mol^{-1}$).

Seven-point calibration curves for qPCR were produced by six tenfold serial dilutions of positive controls in duplicate within each assay, from 10^7 to 10^1 target copies per reaction. Standard curves were accepted only if correlation coefficients were greater than 0.998 and the PCR efficiencies were between 94.3% and 101.2%.

Table 3. Primers used to target tet^R by quantitative real time PCR.

Targeted tet^R Class		Sequence (5'→ 3')	Annealing Temperature (°C, T_A)	Amplicon Size (bp)	Reference
tet(G)	FW RV	GCA GAG CAG GTC GCT GG CCY GCA GAG GAA GCC AGA AG	61.3	134	(Aminov et al., 2002)
tet(O)	FW RV	ACG GAR AGT TTA TTG TAT ACC TGG CGT ATC TAT AAT GTT GAC	60	171	(Aminov et al., 2001)
tet(Q)	FW RV	AGA ATC TGC TGT TTG CCA GTG CGG AGT GTC AAT GAT ATT GCA	63	169	(Aminov et al., 2001)

Positive Control Primers					
tet(G)	FW RV	GTC TCG GCC TCA TCA TGC CCG T GAG TGC TGT GAA GCC AAG CGG TC	60	1,016	*This Study*
tet(O)	FW RV	GGG CTA TTG GAG TTA TTT ACC CTC TAA TAG TTC ATC GTT TCC C	60	651	*This Study*
tet(Q)	FW RV	CTC ATA TGC TAC GAG GAG GTA TGC CGC TTG GGG TAT CTGC	60	1,729	*This Study*

RESULTS AND DISCUSSION

PCR was successfully used to detect tet^R in water collected from private water supply wells, monitoring wells, and septic tank effluent. In March 2004 two monitoring wells and two septic tanks were sampled (Table 4). Notably, all six tet^R were detected in SE5, but only three were detected in SE8. The resistance profile in the monitoring wells was the same as that observed in SE8.

Table 4. Tetracycline resistance genes detected using presence/absence PCR in March 2004 samples

March 2004	**Sample collection site**			
Targeted tet^R Class	MW-11	MW-20	SE5	SE8
tet(A)	-	-	(+)	-
tet(B)	(+)	(+)	(+)	(+)
tet(D)	-	-	(+)	-
tet(E)	-	-	(+)	-
tet(M)	(+)	(+)	(+)	(+)
tet(Q)	(+)	(+)	(+)	(+)
Percent of genes detected	50%	50%	100%	50%

In October 2005 three monitoring wells, two private water supplies, and two septic tanks were sampled (Table 5). Again, the most tet^R were detected in SE5, though SE8 also had a 50% detection frequency. The monitoring wells had between a 10% and 40% detection frequency, with tet(B) being detected in all three. It is notable that MW-02 had a higher detection frequency than MW-20 or MW-22 although it was located farther away on an undeveloped plot. None of the ten tet^R were detected in one of the private water supplies, but three were detected in the other.

In August 2006 the same monitoring wells, private water supplies, and septic tanks were sampled as in the previous year. The monitoring wells had similar detection frequencies and tet^R profiles as in 2004 and 2005. The private water supplies each contained only one of the monitored genes, tet(G). This gene may have been present at levels near the detection limit since it was not detected in October 2005, or it could have entered the groundwater near the deep wells sometime between the two sample dates. Notably, tet(B), tet(D), and tet(Q) were detected in 2005 but not 2006, suggesting they were also present at levels near the detection limit or that the populations harboring the genes were no longer present in the water supply wells in 2006. The septic tanks

still contained the largest number of detected tet^R, but tet(S) and tet(B) were no longer detected in SE5.

Table 5. Tetracycline resistance genes detected using presence/absence PCR in October 2005 samples

October 2005	Sample collection site						
Targeted tet^R Class	MW-02	MW-20	MW-22	WS5	SE5	WS8	SE8
tet(A)	-	-	-	-	-	-	-
tet(B)	(+)	(+)	(+)	(+)	(+)	-	(+)
tet(D)	(+)	(+)	-	(+)	(+)	-	(+)
tet(E)	-	-	-	-	-	-	-
tet(G)	(+)	(+)	-	-	(+)	-	(+)
tet(M)	-	-	-	-	-	-	-
tet(O)	(+)	-	-	-	(+)	-	(+)
tet(Q)	-	-	-	(+)	(+)	-	(+)
tet(S)	-	-	-	-	(+)	-	-
tet(W)	-	-	-	-	(+)	-	-
Percent of genes detected	40%	30%	10%	30%	70%	0%	50%

Table 6. Tetracycline resistance genes detected using presence/absence PCR in August 2006 samples

August 2006	Sample collection site						
Targeted tet^R Class	MW-02	MW-20	MW-22	WS5	SE5	WS8	SE8
tet(A)	-	-	-	-	-	-	-
tet(B)	(+)	-	-	-	-	-	(+)
tet(D)	(+)	(+)	-	-	(+)	-	(+)
tet(E)	-	-	-	-	-	-	-
tet(G)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
tet(M)	-	-	-	-	-	-	-
tet(O)	-	-	-	-	(+)	-	(+)
tet(Q)	(+)	-	-	-	(+)	-	(+)
tet(S)	-	-	-	-	-	-	-
tet(W)	-	-	-	-	(+)	-	(+)
Percent of genes detected	40%	20%	10%	10%	50%	10%	60%

Quantitative real-time PCR (qPCR) was used to measure the concentrations of tet(G), tet(O), and tet(Q) in the two septic tank effluents (SE5 and SE8) (Figure 2). The samples from 2004 and 2005 were pooled before analysis to obtain and average value. The concentrations of these genes were also measured in similar kinds of samples: creek water upstream of a municipal wastewater treatment plant discharge, the treated municipal wastewater effluent, and the confluence of the stream and the discharge. These are also presented in Figure 2 for comparative purposes. Concentrations of tet^R in the septic tank effluents were several orders of magnitude higher than

those in treated municipal effluent. This is not surprising since municipal wastewater treatment plants produce a higher quality effluent with fewer viable bacterial cells. Notably, SE5 had consistently higher concentrations of the three tet^R. This is consistent with the findings based on presence/absence PCR, in which SE5 usually contained more different types of tet^R (Tables 4, 5, and 6). This could be explained by the fact that the SE5 septic tank had been in operation for almost one full year longer than the SE8 septic tank at the time of sampling. However, it is also possible that the residents discharging to the SE5 septic tank had some lifestyle difference that led to more proliferation of tetracycline resistant bacteria in their septic tank.

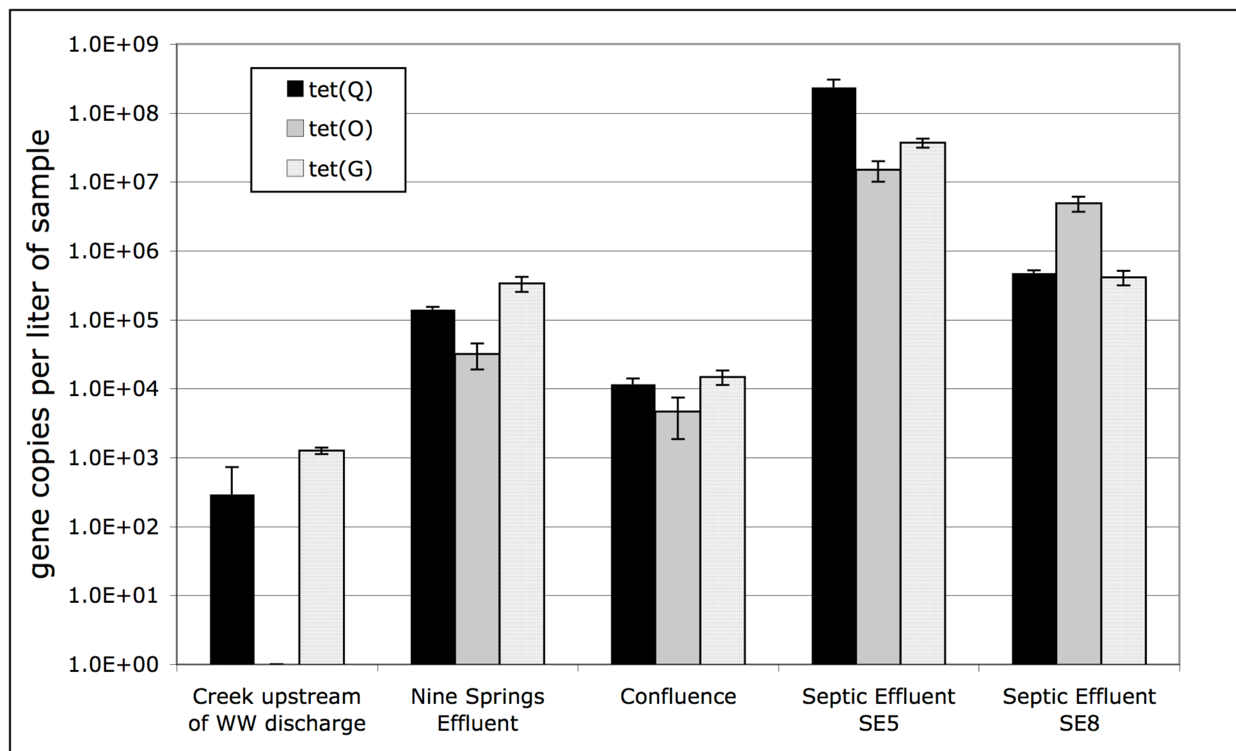


Figure 2. Quantitative PCR analysis of gene copy concentration in two septic tank effluent samples and other reference samples from comparable systems

Summary. Generally, septic tank effluent contained the most number of different types of tet^R and private water supplies contained the fewest. This is not surprising given that the water supply wells were deep and unlikely to have been impacted by septic tank effluent. The few types detected in water supplies may be present in naturally occurring bacterial communities in groundwater or could have been introduced as a result of past agricultural activity. The tet^R detected in the monitoring wells may have originated from septic tank effluent, given that bromide tracer experiments predicted the average residence time between the septic tanks and the monitoring wells was on the order of 30 to 60 days (Wilcox, 2007). However, the tet^R detection frequency in monitoring wells was comparable across years, suggesting that occupation of the residences contributing to SE5 and SE8 did not cause an increase in the number tet^R that could be detected over the study period. Longer term monitoring targeting these ten tet^R as well as genes encoding resistance to other antibiotics is necessary to confirm this observation. The

concentrations of three tet^R were higher in SE5 than in SE8, which might be explained by either that the residents using the SE5 septic tank had a lifestyle difference that caused a higher amount of resistant bacteria to be present in their septic tank or the longer operation of the SE5 septic tank.

CONCLUSIONS AND RECOMMENDATIONS

Genes expected to encode resistance to tetracycline were detected using a cultivation-independent molecular approach in private deep well water supplies, septic tank effluent, and downgradient monitoring wells at a newly developed subdivision. These genes were detected most frequently in septic tank effluent, presumably because bacteria carrying the genes were present in the septic tanks. The concentrations of resistance genes in the septic tanks were several orders of magnitude higher than those observed in treated municipal wastewater effluent. Some of these genes may have been transported in the subsurface to the monitoring wells, but in some cases genes were detected in the monitoring wells that were not present in the corresponding septic tanks. Therefore, we hypothesize that past agricultural activity may have contributed to the presence of resistance genes in subsurface bacteria. Longer-term sampling over several more years combined with higher spatial resolution is required to adequately test this hypothesis.

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