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Tetracycline Residues and Tetracycline Resistance Genes in Groundwater Impacted by Swine Production Facilities

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TETRACYCLINE RESIDUES AND TETRACYCLINE RESISTANCE GENES IN GROUNDWATER IMPACTED BY SWINE PRODUCTION FACILITIES

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Antibiotics are used at therapeutic levels to treat disease; at slightly lower levels as prophylactics; and at low, subtherapeutic levels for growth promotion and improvement of feed efficiency. Over 88% of swine producers in the United States gave antimicrobials to grower/finisher pigs in feed as a growth promoter in 2000. It is estimated that ca. 75% of antibiotics are not absorbed by animals and are excreted in urine and feces. The extensive use of antibiotics in swine production has resulted in antibiotic resistance in many intestinal bacteria, which are also excreted in swine feces, resulting in dissemination of resistance genes into the environment.

To assess the impact of manure management on groundwater quality, groundwater samples have been collected near two swine confinement facilities that use lagoons for manure storage and treatment. Several key contaminant indicators—including inorganic ions, antibiotics, and antibiotic resistance genes—were analyzed in groundwater collected from the monitoring wells. Chloride, ammonium, potassium, and sodium were predominant inorganic

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constituents in the manure samples and served as indicators of groundwater contamination. Based on these analyses, shallow groundwater has been impacted by lagoon seepage at both sites. Liquid chromatography-mass spectroscopy (LC-MS) was used to measure the dissolved concentrations of tetracycline, chlortetracycline, and oxytetracycline in groundwater and manure. Although tetracyclines were regularly used at both facilities, they were infrequently detected in manure samples and then at relatively trace concentrations. Concentrations of all tetracyclines and their breakdown products in the groundwater sampled were generally less than 0.5 µg/L.

Bacterial tetracycline resistance genes served as distinct genotypic markers to indicate the dissemination and mobility of antibiotic resistance genes that originated from the lagoons. Applying PCR to genomic DNA extracted from the lagoon and groundwater samples, four commonly occurring tetracycline (tet) resistance genes—tet(M), tet(O), tet(Q), and tet(W)—were detected. The detection frequency of tet genes was much higher in wells located closer to and down-gradient from the lagoons than in wells more distant from the lagoons. These results suggested that in the groundwater underlying both facilities tetracycline resistance genes exist and are somewhat persistent, but that the distribution and potentially the flux for each tet gene varied throughout the study period.

BACKGROUND

In commercial swine production antibiotics are used therapeutically to treat existing disease conditions, prophylactically at subtherapeutic doses when pathogens are present or animals are in high stress situations, and subtherapeutically to enhance growth. In addition, metaphylaxis, or the timely mass medication of entire groups of animals, is a common practice in the pig and poultry industry. Because there are obvious therapeutic effects of both metaphylaxis and prophylaxis the term nontherapeutic is considered inaccurate. Subtherapeutic concentrations of antimicrobials are commonly added to animal feed and/or drinking water sources as growth promoters, and have been a regular part of swine production since the early 1950's (1). However, when used in this manner, antibiotics can select for resistant bacteria in the gastrointestinal tract of production animals, providing a potential reservoir for dissemination of drug resistant bacteria into other animals, humans, and the environment (2). Bacteria have been shown to readily exchange genetic information in nature, permitting the transfer of different resistance mechanisms already present in the environment from one bacterium to another (3–5). Transfer of resistance genes from fecal organisms to indigenous soil and water bacteria may occur (6–9) and because native populations are generally better adapted for survival in aquatic or terrestrial ecosystems there is also the likelihood of resistance trait persistence in natural environments. This has led to the current scrutiny over antibiotic use in the livestock industry particularly for pig and poultry production.

Many antibiotics used in animal agriculture are poorly absorbed in the gut and consequently substantial amounts of these compounds and their breakdown products are excreted. Elmund and colleagues (10) estimated that as much as 75% of the antibiotics administered to feedlot animals could be excreted into the environment. Manure and waste slurries potentially contain significant amounts of antibiotics and their presence can persist in soil after land application (11,12). Feinman and Matheson (13) suggested that about 25% of the oral dose of tetracycline is excreted in feces and another 50%–60% is excreted unchanged or as an active metabolite in

Table 1 Commonly used antibiotics in the pig and poultry production industry

Pigs	Poultry
^a Chlortetracycline, Oxytetracycline	Bambermycin
^b Bacitracin	Amprolium
Tylosin	Ethopabate
Sulfamethazine	Roxarsone
Carbadox	Virginiamycin
Lincomycin	Salinomycin
Virginiamycin	Bacitracin
Penicillin	Monensin
	Lincomycin

^aApproximately 48% of total antibiotic fed to swine in 1990s.

^bUsed in 52% of swine operations reported in a 1995 USDA survey.

urine. Oral administration of tylosin resulted in a maximum of 67% of the antibiotic excreted, mainly in the feces. Regardless of route of excretion, most of the antibiotics administered to production animals, as well as their resultant metabolites, are eliminated via feces and urine. These animal waste products are generally stored before disposal into the environment. The most common method to dispose of swine effluent in the United States is through land application, where application of liquid manure at agronomic rates can produce crop yields that equal those obtained with chemical fertilizers (14). The most commonly used antibiotics in the pig and poultry industries are tetracyclines and bacitracin (Table 1). In this manuscript we briefly review pathways for entry of antibiotics into the environment, management of animal waste from production agriculture, antibiotic resistant bacteria in manure, and antibiotic occurrence in the surface and groundwater environment. In addition, we describe current research in our laboratories concerning tetracycline residues and tetracycline resistance genes in groundwater impacted by swine production facilities.

Pathways for Entry of Antibiotics into the Environment

As noted previously, many antibiotics are not completely absorbed in the gut resulting in the parent compound and its metabolites being excreted in feces and urine (13,15,16). The land application of livestock manure provides large areal scale for introduction of antibiotics into the environment. The excretion of waste products by grazing animals, atmospheric dispersal of feed and manure dust containing antibiotics, and the incidental release of products from spills or discharge are also potential pathways into the environment. Once released into the environment, antibiotics can be transported either in a dissolved phase or (ad)sorbed to colloids or soil particles into surface- and ground-water (17–20). Studies have shown that under a broad range of environmental conditions tetracyclines (tetracycline, chlortetracycline, and oxytetracycline) can adsorb strongly to clays (21–24), soil (20), and sediments (25). Because of the strong sorption of the tetracycline and macrolide antibiotics, their mobility in the environment may be facilitated by transport with manure and soil colloidal material (26,27).

Manure generated at animal confinement facilities is generally stored in lagoons, surface storage structures, or pits. Boxall and colleagues (15) compiled persistence data for various antibiotic classes in manure. Half-lives for all antibiotic classes were less than the anticipated storage period of manure, thus allowing for significant degradation of the parent compounds prior to land application. However, tetracyclines were among the most persistent with half-lives approaching 100 days. In addition, tetracycline concentrations were generally higher than macrolide, β -lactams, and sulfonamides in manure samples with tetracycline concentrations in some swine lagoons as great as 1 mg/L (17). Although there is little data available in the published literature, it is likely that, although biodegradation and abiotic degradation occurs, the primary mechanism for tetracycline loss was sorption to manure solids. This suggests that application of manure to agricultural fields likely introduces tetracycline breakdown products into the environment along with the parent compound. However, persistence data for tetracycline degradation products is scarce. Gavalchin and Katz (12) concluded that the longer an antibiotic persists in the environment in an active form, the greater the potential for indigenous bacterial populations to be affected. In addition, biologically-active antibiotics (or antibiotic breakdown products) introduced to the environment may confer a selective advantage for indigenous bacteria carrying resistance genes or exert selective pressure for acquisition of resistance genes in indigenous bacteria.

Management of Animal Waste from Production Agriculture

Over the last 25 years swine production has largely shifted from smaller integrated farming systems to concentrated animal feeding operations (CAFOs) that may house thousands of animals. In 1984, there were approximately 690,000 U.S. producers producing 20 billion pounds of pork. By 2000, about 95,000 producers were producing 26 billion pounds of pork (27,28). Due to geographic patterns of feed grain production and other market forces CAFOs have become concentrated in certain geographic regions in the United States, primarily North Carolina and the Midwest. USDA surveys performed in 2000 found that 28.3% of swine facilities were located within 1/2 mile of another swine production site and 53.9% were within one mile of another site (28). Thus, in some regions of the United States CAFOs are concentrated to the point that manure production is likely in excess of what the local land base can absorb without environmental consequences. With the advent of CAFOs large quantities of waste are concentrated in a single location and/or region, and producers may only own sufficient land to site their facilities. Swine typically produce 635 kg (1.4 tons) each of fresh manure in the 5–6 months it takes to grow them to a market weight of 114 kg (250 lbs). On a national scale, quantities of manure generated are massive—the National Agricultural Statistics Service estimated that in 2002 185 million head of swine were sold in the U.S. These animals would have produced some 117,475,000 Mg (1.3×10^8 tons) of fresh manure. This waste, containing nutrients, antibiotic residues, and antibiotic resistant bacteria is collected and stored prior to land application.

Antibiotic Resistant Bacteria in Manure

Antibiotic resistance among commensal bacteria represents a major avenue for the development of resistance in bacterial pathogens because resistance increases first

in commensals and is transferred to pathogens later. First, commensal gut bacteria are likely to be highly efficient contributors to resistance because the numbers of commensal bacteria in the intestinal ecosystem are large, often more than 10^{14} bacteria from several hundred species (2). Anaerobic bacteria dominate this ecosystem and number 10^{11} – 10^{12} per g of intestinal content whereas enterobacteria and enterococci are relatively minor players ranging from 10^6 – 10^8 per g of intestinal content. Second, the commensal genetic pool is so large and encompasses the potential for many different mechanisms of conferring resistance. Third, resistant commensal bacteria may be selected each time an antibiotic is administered, regardless of the health status of the animal. This microbial population is excreted in feces and stored as manure where it undergoes changes in the numbers and proportions of the dominant bacterial species. An analysis of stored swine manure indicated that the predominant culturable microorganisms from these environments were obligately anaerobic, low mol% G + C Gram-positive bacteria (Firmicutes) comprised of members of Clostridial, Eubacterial, and Lactobacillus/Streptococcus phylogenetic groups (29).

Although reports of the percentage of viable, culturable antibiotic-resistant bacteria in swine effluent vary, it is clear that antibiotic resistance is a common phenomenon. Japanese studies in the 1980's of coliforms in swine waste found that 97 percent of *E. coli* were resistant to at least one of the following antibiotics: ampicillin, furatrizine, chloramphenicol, kanamycin, streptomycin, sulfonamides, or tetracycline (30). Haack and Andrews (31) found that 71 percent of *Enterococcus faecalis* isolates from farrowing house effluent were resistant to tetracycline. Cotta and colleagues (29) found that 4%–32% of the bacteria in swine manure were resistant to tylosin, depending on the depth from which the sample was collected in the manure holding pits.

Antibiotic Occurrence in the Surface and Groundwater Environment

Surface water. The USGS has a comprehensive stream monitoring network throughout the United States and have developed state-of-the-art analytical techniques such as Liquid chromatography coupled with tandem Mass spectroscopy (LC-MS-MS) to be able to detect and quantify the contaminants at environmentally relevant concentrations. A recent study by the USGS (18) conducted a reconnaissance of the occurrence of pharmaceuticals, hormones, and other organic wastewater contaminants in water resources. They sampled 139 streams across 30 states during 1999 and 2000. Table 2 lists of the most commonly detected antibiotics found in filtered stream samples. Carbodox, doxycycline, enrofloxacin, sarafloxacin, sulfachlorpyridazine, sulfamerazine, sulfathiazole, and virginiamycin were not detected in any samples. Many of these compounds are commonly used in livestock operations, but were not detected in stream water samples, suggesting limited transport to surface waters in the aqueous phase. When detected, the maximum antibiotic concentrations were generally less than 1.7 µg/L.

Yang and Carlson (19) investigated the occurrence of five tetracycline and six sulfonamides in water collected along the Cache la Poudre River, Colorado. No antibiotics were detected in the pristine mountain stretch of the river. Few sulfonamides were detected along the entire river. However, the frequency of detection and concentration of the tetracyclines increased as the river water quality became impacted

by urban and agricultural sources. Tetracycline concentrations in filtered samples ranged from 0.08 to 0.30 $\mu\text{g}/\text{L}$. Photolysis, biodegradation, and sorption of the tetracyclines could have occurred in various reaches of the stream but they concluded that proximate agricultural activity influenced tetracycline occurrence in the river.

Investigating surface and ground waters, Campagnolo and colleagues (17) detected antibiotics in 31% and 67% of the samples collected near swine and poultry confinement facilities, respectively. Concentrations for all antibiotics in the water were all less than 10 $\mu\text{g}/\text{L}$ even though manure samples contained concentrations up to 1 mg/L (chlortetracycline).

Groundwater. Few studies were found that determined the occurrence of veterinary antibiotics in groundwater. Krapac and colleagues (20) collected shallow (< 8 m) groundwater samples near two swine confinement facilities. Using LC-MS to detect the antibiotics, fewer than five percent of the samples contained any of the tetracyclines at either of the facilities. Parent tetracycline compounds were detected in a small number of groundwater samples collected from wells that had also been significantly impacted by manure seepage as evident by elevated chloride, ammonium, and potassium concentrations. Tetracycline breakdown products were detected in some groundwater samples even when the parent compound was not detected. When detected, antibiotic concentrations were less than 0.5 $\mu\text{g}/\text{L}$.

Hirsch and colleagues (32) collected more than 30 groundwater samples from agricultural areas in Germany containing large numbers of animal confinement facilities. Eighteen antibiotics representing macrolide, sulfonamides, penicillin, and tetracycline classes of compounds were analyzed by LC-MS. Sulfonamide residues were detected in four samples, but none of the other antibiotics were detected in the groundwater. The authors concluded that sulfonamides in two of the samples were the result of sewage irrigation and sulfamethazine detected in the other samples was likely from veterinary use.

LONG-TERM MONITORING OF THE OCCURRENCE OF TETRACYCLINE RESIDUES AND TETRACYCLINE RESISTANCE GENES IN GROUNDWATER NEAR SWINE PRODUCTION FACILITIES

The protection and maintenance of the quality of our water resources has been a particular focus of attention over the past 25 years and remains a high priority in the U.S. Groundwater constitutes about 40% of the water used for public supply and provides drinking water for more than 97% of the rural population (33). Agriculture and, in particular, the increase in confined animal feeding operations (CAFO's) and the resulting need for effective manure management has heightened the concern for safe and sustainable waste handling and treatment practices. Issues of animal waste treatment and water quality control must be addressed in ways that minimize the risk of chemical and (micro)biological contamination in the environment. The challenge to livestock producers, regulatory agencies, and the public is to design and implement environmentally sustainable systems. In order to meet this challenge accurate data on the type, occurrence, and extent of contamination from CAFO's must be determined and made available. One major concern for groundwater is pollution due to leaks from manure holding lagoons and deep pits. Monitoring studies have shown that

seepage from animal waste lagoons has affected groundwater quality at numerous locations (34). Detailed investigations near livestock waste lagoons and deep pit systems have demonstrated chemical and biological contamination and its impact on groundwater quality (34–36). However, few studies have addressed the long-term impact of CAFO's on surface and groundwater quality.

Information on the persistence and dissemination of antibiotic resistance genes in bacteria is of fundamental importance in assessing risks in water quality. The detection of specific genes and their hosts is an important component of disease detection and prevention, food safety, and epidemiological surveillance. At present, the detection of bacteria in water and soil relies heavily on cultivation techniques (37). More precise identification of isolates requires further biochemical and immunological testing. These methods are often time consuming and expensive and can lack specificity, sensitivity, and reliability. The use of molecular techniques is growing rapidly in the environmental microbiology field. The primary advantage of these techniques is that they provide rapid, sensitive, and specific detection and identification without the requirement for growth and isolation. Commonly used molecular microbial techniques are based on unique sequence features of genes to detect and identify microorganisms. PCR amplification of nucleic acids is now widely used to enable detection of low levels of target sequences, and has become a key procedure in the detection and identification of bacteria and genes from a variety of environments including soil, water, and fecal material (38–40).

Because specific classes of antibiotics can be characteristic of the application in which they are used multiple antibiotic resistance analyses of bacteria have been used to identify sources of fecal pollution (e.g., human, poultry, cattle, swine) in environmental samples (41–43). Analysis of antibiotic resistance genes using molecular-based PCR methods can provide a rapid and convenient method for tracking the source of fecal contamination in surface and groundwater. Similar to the strategy used in microbial diversity studies, the starting point in the design of probes and primers for detection of antibiotic resistance genes is a robust phylogenetic analysis. These analyses demonstrate that a great diversity of antibiotic-resistant genes are present in swine lagoon and pit effluent. For example, Aminov and colleagues (41,45) and Chee-Sanford and colleagues (46) found the tetracycline resistance efflux genes (*tet* B, C, E, H, Y, Z) and the ribosomal protection protein (RPP) genes (*tet* W, O, Q, M, S, T, B[P], and *otr* A) were all present in a single swine waste lagoon. Many of these genes are found in large numbers in lagoon effluent. For example, Smith and colleagues (47) detected 10^5 copies per 50 μ L of *tet* genes O, W, and Q combined in a cattle feedlot lagoon. PCR is also helpful to phylogenetically classify antibiotic resistance genes. In the following section we describe current research in our laboratories concerning long-term monitoring of the occurrence of tetracycline residues and tetracycline resistance genes in groundwater near swine confinement facilities.

METHODS

Site Geology and Facility Operations

Groundwater quality at two swine confinement facilities located in Illinois, USA that use lagoons for manure storage or treatment have been monitored for

up to six years. These facilities are identified as sites A and C. The geographic location of the facilities cannot be disclosed because of a confidentiality agreement with the producers.

Site A, which started in February of 1995, is a finishing operation that houses 4,000 animals (Figure 1). The facility incorporates a two-stage waste handling system in which a concrete settling basin collects most of the solids prior to the supernatant liquid passively entering an earthen lagoon. The lagoon is approximately 1.2 ha and unlined. No special construction techniques were used to compact the soil during lagoon construction. The average depth of liquid in the lagoon during our study

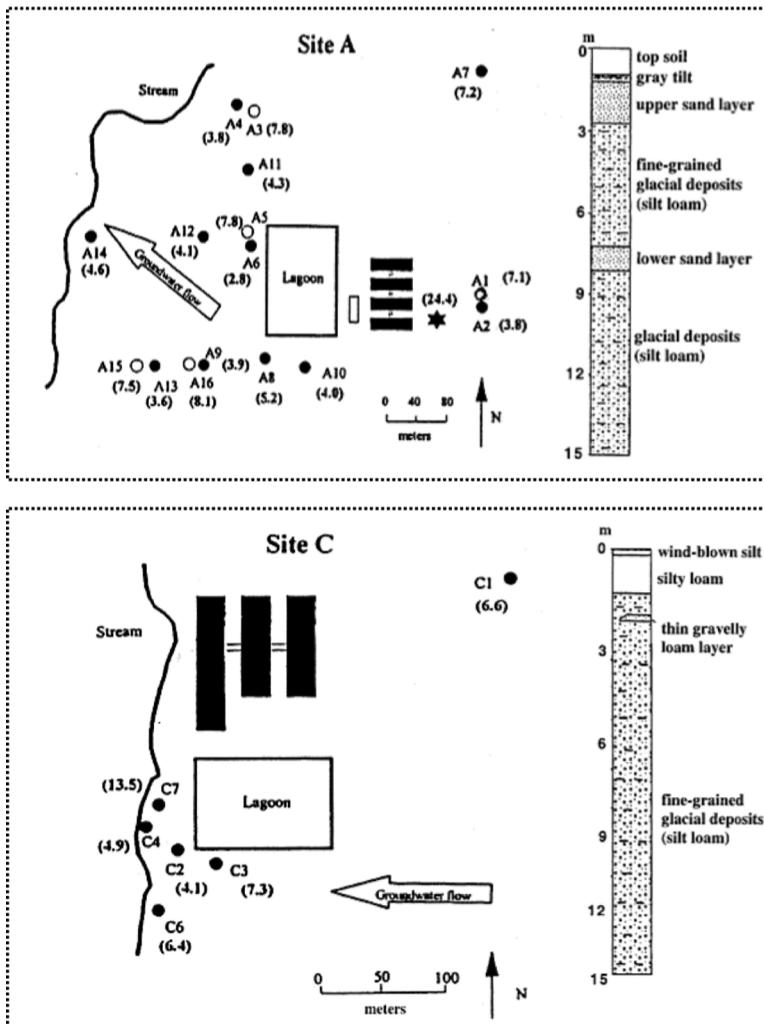


Figure 1 Site A and C well locations and groundwater flow direction. Stratigraphic columns indicate the location of sand layers. The locations of monitoring wells are indicated by circles. Open circles at site A represent wells finished in deeper sand layer. Numbers in parenthesis are well depths (m). The dark rectangles represent confinement buildings.

was about 1.5 m. The concrete settling basin is periodically pumped and the manure is applied to crop fields both on- and off-site. Site A is located on glacial outwash and terrace deposits along a stream valley that is incised into a till plain formed during the Illinois Episode of glaciation. The top soils are silt or silty clay loams developed on alluvial deposits that are 1.3 to 2 m thick. These deposits overlie a 0.6- to 1.3-m thick upper layer of fluvial silty sand and gravel outwash which is continuous across the site. Twelve of the 16 monitoring wells were installed in this upper sand layer. Slug test results indicated that this upper sand has a saturated hydraulic conductivity of approximately 6.8×10^{-4} m/s. Below the silty sand and gravel is 1.6 to 3 m of silt loam diamicton which may be colluvial. Below the silt loam diamicton lies a 1- to 2-m thick lower sand layer composed of sand and gravel outwash that is being used locally as an aquifer. Four monitoring wells were installed in this lower sand layer. The saturated hydraulic conductivity of this deeper sand was estimated to be 8.2×10^{-6} m/s based on slug tests. Below this sand and gravel is more silt loam diamicton. Logs from water wells drilled in the vicinity show the presence of discontinuous sand and gravel outwash units below the diamicton that are used locally as aquifers. The multiple sand layers make this site particularly susceptible to leachate migration from the lagoon.

Site C is a farrowing and nursery operation that began operations in the fall of 1992 (Figure 1). Prior to 1998 the facility housed 1,250 sows and expanded in 1998 to 2,500 sows. The facility uses a single-stage lagoon. Lagoon water is recycled to partially fill and flush the shallow pits below the confinement buildings. The lagoon is approximately 0.8 ha and unlined. The average depth of waste in the lagoon during our study was about 6 m. Waste has never been applied to the crop fields surrounding the lagoon. Site C is located on a glacial till plain formed during the Illinois Episode of glaciation. It is underlain by a silt loam glacial diamicton 3- to 15-m thick that overlies shale bedrock. Thin (<30-cm thick) glacial gravelly loam layers were found in two of the seven borings at the site. Large-diameter wells and ponds are the predominant sources of drinking water in the area. Six monitoring wells were installed at depths less than 11 m at this facility (Figure 1).

The water table is approximately 2 m below the surface at both sites. Groundwater flow direction at the facilities was determined from water level measurements made prior to sampling. Groundwater levels fluctuated throughout the study period less than 2 m at each site. Groundwater flow at site A was in a northerly direction while flow at site C was to the west.

Groundwater Sampling

Water levels in each well were determined using an electronic water level indicator prior to sample collection. Polyethylene bailers dedicated to each well were sterilized using an alcohol wash and a deionized water rinse prior to sample collection. Following well purging recommendations presented by Gibb and colleagues (48) 1.5–3 well volumes of groundwater were removed from each well, depending on well recovery, before collection of the samples. Samples were collected for anion, cation, and antibiotic analyses as well as for DNA extraction. All groundwater and manure samples to be analyzed for cations and anions were filtered through 0.45- μ m filters. Samples for ammonia analyses were not filtered. Antibiotic samples were

stored in amber glass bottles and filtered through 0.70- μm glass fiber filters in the laboratory. Sample preservation techniques, as outlined in American Public Health Association (37), were followed.

Inorganic Analysis

Anion concentrations were determined by ion chromatography (49) and cation concentrations by inductively coupled argon plasma spectrophotometry (ICP) (37). Detection limits for chloride, nitrate-N, phosphate-P, and sulfate were 1, 0.2, 0.3, and 5 mg/L, respectively. Detection limits for the ICP analyses were in the range of 1 $\mu\text{g/L}$ for constituents such as Be, La, and Sc to 10 $\mu\text{g/L}$ for most of the metals. Ammonia-N concentrations were determined by electrode and had a detection limit of 10 $\mu\text{g/L}$ (37,50). Electrical conductivity, pH, oxidation/reduction potential, and temperature were determined in the field using electrodes according to standard methods (37).

Antibiotic Detection

The detection and quantitation of antibiotics in groundwater and manure samples were performed by Liquid Chromatography-Mass Spectroscopy (LC-MS), High Pressure Liquid Chromatography (HPLC), and Enzyme Linked Immunosorbent Assay (ELISA). LC-MS analyses followed methods outlined in Zhu and colleagues (51) and Kolpin and colleagues (18). Briefly, a 125 or 500 mL groundwater sample was prepared by adding 0.5 g of $\text{Na}_2\text{-EDTA}$ adjusted to a pH of 3 with H_2SO_4 and then passed through Waters Oasis HLB solid-phase extraction (SPE) cartridges. The SPE cartridges were then eluted with 5 mL of methanol and 2 mL of methanol with 5% ammonia hydroxide (52), or with 2.5 mL methanol containing 0.5% formic acid (51). The sample eluate was injected without additional treatment, or evaporated to 20 μL using nitrogen evaporation and taken up in 300 μL of water with 20 mmol ammonia acetate adjusted to a pH of 5.7 with ammonia acetate. The sample eluates were frozen until analysis. The eluates were then analyzed using a HPLC with a PDA at 450 nm, by LC-MS or by LC-MS-MS with an electro-spray ionization source.

DNA Extraction

Prior to DNA extraction, one-liter of groundwater or 100 mL of lagoon sample were centrifuged at $17,700 \times g$ for 20 min at 4°C . The supernatants were discarded and the bacterial pellets were washed three times with a phosphate-buffered saline solution (120 mM NaH_2PO_4 [pH 8.0], 0.85% NaCl). Total DNA was extracted from the pellets by the method of Tsai and Olsen (53). Briefly, the pellets were resuspended in 400 μL of lysis solution (0.15 M NaCl, 0.1 M EDTA [pH 8.0]) containing 15 mg of lysozyme/mL, and incubated at 37°C for 2 h, and then 400 μL of 0.1 M NaCl-0.5 M Tris-HCl (pH 8.0)-10% sodium dodecyl sulfate was added. Samples were incubated for 30 min at 37°C . Three cycles of freezing in -80°C and thawing in a 65°C water bath were conducted to release DNA from microbial cells in the pellets. Proteinase K was added to a final concentration of 50 $\mu\text{g/mL}$, and the mixture was incubated for 30 min at 37°C , centrifuged, and supernatant collected. The crude DNA was

purified with polyvinylpolypyrrolidone (PVPP) and Sepharose 2B, as described by Zhou and colleagues (54) and Miller (55).

Polymerase Chain Reaction (PCR) Detection of Tetracycline Resistance Genes

PCR was conducted to monitor the distribution of four tetracycline resistance genes, *tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*, using the class-specific primer sets described in Aminov and colleagues (44,45). A reaction mixture containing 0.5 μ M of each primer, 1.5 mM MgCl₂, 0.2 mM each deoxyribonucleotide triphosphate, PCR Buffer II, 1.25 U of AmpliTaq Gold DNA polymerase (Applied BioSystems, Foster City, CA) and 1 μ L (10 ng) of template DNA in a total volume of 25 μ L was prepared. PCR amplification was carried out with a GeneAmp PCR System 2700 thermocycler (Applied BioSystems, Foster City, CA). The temperature program consisted of denaturation at 94°C for 10 min, followed by 40 cycles consisting of 94°C for 30 s, annealing for 30 s, and extension at 72°C for 30 s and a final extension at 72°C for 10 min. The annealing temperatures used for amplification of different target genes were as follows: *tet(M)*, 55°C; *tet(O)*, 60°C; *tet(Q)*, 63°C; and *tet(W)*, 64°C. The control reactions included PCR amplification with sterile water as the negative control template for all primer sets and the positive control strains for each primer set as described previously (44,45). PCR product aliquots (5 μ L) were analyzed by electrophoresis on 2.0% (wt/vol) agarose gel and were stained with ethidium bromide.

Quantitation of *tet* Genes by Real-Time PCR

PCR amplifications for the quantification of *tet(M)* and *tet(Q)* in total DNA from lagoon samples were performed with a GeneAmp 9600 thermocycler coupled with a GeneAmp 5700 sequence detection system (Applied BioSystems, Foster City, CA). The SYBR Green PCR Core Reagents kit was used for PCR amplification. The reaction mixture in 25 μ L of the final volume consisted of 0.5 μ M of each primer, 1.5 mM MgCl₂, 0.2 mM each deoxyribonucleotide triphosphate, SYBR Green PCR buffer, 1.25 U of AmpliTaq Gold DNA polymerase and 1 μ L (10 ng) of template DNA. The thermal profile for all SYBR Green PCRs was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 30 s and annealing at temperature described above for 30 s, and 72°C for 30 s and a final extension at 72°C for 7 min. The dilution series of the plasmid standard for the respective genes was run along with the unknown samples for the corresponding gene controls and each sample was duplicated. Quantitation was done by using standard curves made from known concentrations of plasmid DNA containing the respective amplicon for each primer set. All reactions were repeated in triplicate to ensure the reproducibility of the results.

RESULTS AND DISCUSSION

Occurrence of Inorganics in Groundwater and Manure

Samples were collected on a quarterly basis and analyzed for 35 inorganic constituents. Chloride, ammonium, potassium, and sodium, the predominant constituents in

Table 2 Average concentrations (mg/L) in groundwater, tile, and manure samples collected from August 1996 to October 2003. Highlighted wells have been significantly impacted by manure seepage from the lagoon. *Background wells

Well	EC ($\mu\text{s}/\text{cm}$)	Cl	NO_3	$\text{NH}_3\text{-N}$	K	Na
A1*	929 \pm 75	29.0 \pm 3	127 \pm 78	<0.1 \pm 0	<1 \pm 0.9	9.7 \pm 1.4
A2*	725 \pm 148	12.8 \pm 7.3	102 \pm 39.1	<0.1 \pm 0	<1 \pm 0.7	23.0 \pm 49.1
A3	657 \pm 42	34.3 \pm 3.9	<1 \pm 0.2	1.1 \pm 0.4	2.0 \pm 2.1	49.1 \pm 4.3
A4	983 \pm 127	47.0 \pm 25.1	12.8 \pm 19.4	<0.1 \pm 0.1	<1 \pm 0.4	10.4 \pm 3.5
A5	822 \pm 300	48.2 \pm 36.3	<1 \pm 0.2	12.3 \pm 14.5	11.4 \pm 9.1	75.6 \pm 22.2
A6	5938 \pm 1500	408 \pm 129	7.9 \pm 30.7	507 \pm 202	432 \pm 156	168 \pm 48.2
A7*	544 \pm 40.7	10.3 \pm 1.1	<1 \pm 0.2	2.2 \pm 0.3	<1 \pm 0.6	14.8 \pm 1.8
A8	5214 \pm 3676	273 \pm 241	7.1 \pm 18.2	496 \pm 529	412 \pm 299	152 \pm 111
A9	4462 \pm 1874	159 \pm 32	<1 \pm 0	326 \pm 222	291 \pm 252	181 \pm 68.6
A10	684 \pm 76.5	14.8 \pm 4.4	130 \pm 40.3	<0.1 \pm 0.1	<1 \pm 0.6	11.5 \pm 2.9
A11	5066 \pm 2048	405 \pm 108	<1 \pm 0	395 \pm 215	372 \pm 297	182 \pm 57.2
A12	2501 \pm 1632	296 \pm 139	<1 \pm 0	112 \pm 128	100 \pm 129	104 \pm 69.9
A13	1397 \pm 753	172 \pm 135	20.7 \pm 28.2	0.2 \pm 1.1	<1 \pm 0.3	35.2 \pm 32.8
A14	943 \pm 187	58.9 \pm 47.7	3.2 \pm 7.5	<0.1 \pm 0.2	<1 \pm 0.3	12.0 \pm 5.5
A15	721 \pm 45.3	20.3 \pm 2.9	<1 \pm 0.2	0.4 \pm 0.2	<1 \pm 0.4	20.8 \pm 1.0
A16	789 \pm 92.1	30.5 \pm 9.3	<1 \pm 0.2	4.2 \pm 3.6	4.2 \pm 3.3	31.9 \pm 3.4
Lagoon	11687 \pm 1183	792 \pm 130	1.9 \pm 4.3	886 \pm 183	1723 \pm 232	365 \pm 53.6
C1*	698 \pm 42.4	24.2 \pm 14.3	4.0 \pm 4.0	0.2 \pm 0.2	2.0 \pm 2.0	117 \pm 13.5
C2	1727 \pm 220	48.3 \pm 39.2	25.7 \pm 12.6	<0.1 \pm 0.1	<1 \pm 1.5	156 \pm 19.6
C3	3957 \pm 2740	4.4 \pm 133	<1 \pm 14	5.5 \pm 562	6.8 \pm 283	227 \pm 86.3
C4	919 \pm 268	38.5 \pm 14.5	12.0 \pm 20.3	<0.1 \pm 0.6	6.3 \pm 4.9	44.0 \pm 20.1
C6	1416 \pm 57.9	142 \pm 8.1	<1 \pm 0.2	3.1 \pm 0.8	1.3 \pm 1.2	155 \pm 11.3
C7	1307 \pm 299	133 \pm 12.4	<1 \pm 0.5	6.6 \pm 4.0	2.6 \pm 1.6	219 \pm 24.8
Lagoon	7085 \pm 690	357 \pm 98.6	<1 \pm 1.0	811 \pm 1043	693 \pm 94.8	214 \pm 45.3

the manure samples, served as indicators of groundwater contamination (Table 2). Shallow groundwater has been impacted by lagoon seepage at both sites. Migration of contaminants as much as 30 m down-gradient of the lagoon at site C and 150 m at site A can be attributed to the difference in the local geologic conditions at the sites. At site A, there is a shallow (3 m below ground surface), continuous sand layer that likely intersects the bottom of the lagoon and provides a pathway for contaminant migration into the surrounding groundwater. Wells located in this shallow that have been significantly impacted include A6, A8, A9, A11, A12, and A13. A deeper (8 m below ground surface) sand layer did not appear to be significantly impacted by lagoon seepage.

Occurrence of Antibiotics in Groundwater and Manure

LC-MS was used to measure the dissolved concentrations of tetracycline, chlortetracycline, and oxytetracycline in groundwater and manure collected between 2000 and 2004. Fewer than five percent of groundwater samples contained any of the tetracyclines at either of the facilities (Table 3). Parent tetracycline compounds were detected in few of the groundwater samples collected from wells that have been significantly impacted by manure seepage as evident by elevated chloride, ammonium, and potassium concentrations. Only two groundwater samples, collected from wells A7 (background) and A11, contained the parent compound, oxytetracycline, at site A.

Table 3 Number of tetracycline detections in groundwater and manure at site A and C based on LC-MS analysis. Samples collected from September 2000 to March 2004. Numbers in parenthesis represent concentration ($\mu\text{g/L}$) range. Tet. = tetracycline, Chlor. = chlortetracycline, Oxy. = oxytetracycline, Antet. = anhydrotetracycline, Btet = Beta-Apooxytetracycline, Anchlor = anhydrochlortetracycline

	Parent compounds				Breakdown products			
	N	Tet.	Chlor.	Oxy.	N	Antet.	Btet.	Anchlor.
SITE A								
All samples	52	4	5	5	27	3	6	4
Groundwater	45	0	0	2 (0.08–0.13)	24	1 (0.1)	3 (0.1–0.3)	3 (0.2–0.3)
Manure	7	4 (0.4–8.2)	5 (0.1–14)	3 (0.35–0.41)	3	2 (0.2)	3 (0.1–0.4)	1 (0.4)
SITE C								
All samples	28	4	2	1	10	2	2	2
Groundwater	21	1 (0.4)	0	0	8	0	0	0
Manure	7	3 (2.6–8.5)	2 (8.9–130)	1 (4.26)	2	2 (0.65–0.77)	2 (0.44–1.9)	2 (0.12–0.28)

Similarly, at site C, only well C4 contained tetracycline. Most of the wells had been sampled multiple times during the project period and in all cases only one of the groundwater samples from each of the wells contained detectable concentrations of antibiotic. Because of the low number of detections there were no apparent spatial or temporal trends regarding antibiotic occurrence.

During the project, tetracycline breakdown products (anhydrotetracycline, beta-apooxytetracycline, and anhydrochlortetracycline) were added to the analytical method and detected in selected groundwater samples at site A even when the parent compound was not detected (Table 3). The tetracyclines and their breakdown product concentrations in groundwater were generally less than $0.5 \mu\text{g/L}$. Although the tetracyclines are used at both facilities, they were not detected in every manure sample and were detected at relatively small concentrations. Chlortetracycline was detected at the largest concentration at site C (Table 3).

Two other analytical techniques, HPLC and ELISA, have also been used to determine the dissolved concentration of the tetracyclines (data not shown). In general, LC-MS detected fewer of the tetracyclines than the other techniques because the combination of chromatographic and mass spectra analysis provided better specificity or the ability to detect and confirm a particular antibiotic. HPLC utilized chromatographic separation that can cause, in these very complex sample matrices, other compounds to be identified as the antibiotic of interest. ELISA is cross-reactive to all the tetracyclines and cannot differentiate between specific tetracyclines. Although these techniques have limitations, they can serve as screening techniques and because of their reduced cost allow more samples to be analyzed. Despite the limitations of these techniques, it is of interest that fewer than 25% of the samples contained tetracycline and less than 10% of the samples contained chlortetracycline or oxytetracycline, suggesting a trend similar to the LC-MS data. Our data suggest that the tetracyclines do not readily migrate from manure seepage into groundwater.

Monitoring Tetracycline Resistance Gene Patterns

We have been monitoring tetracycline resistance genes in lagoon and groundwater samples to detect the dissemination of antibiotic resistance genes as a marker

for the spread of antibiotic resistant bacteria. Among seven of the *tet* genes encoding ribosomal protection proteins, four—*tet*(M), *tet*(O), *tet*(Q) and *tet*(W)—were frequently detected in groundwater in a preliminary study (46). PCR detection for the four *tet* genes—*tet*(M), *tet*(O), *tet*(Q) and *tet*(W)—in the lagoon and groundwater samples collected from 2000 through 2003 indicated that all four tet genes were detected in groundwater samples from site A during the three-year period (Table 4). The detection frequency of *tet* genes fluctuated and no clear pattern was observed

Table 4 Distribution of tetracycline resistance genes in lagoon and groundwater samples from 2000 through 2003

Sample gene	Detection of tetracycline resistance genes																				Frequency. ^b (%)				
	Period 1 ^a				Period 2				Period 3				Period 4				Period 5					Period 6			
	M	O	Q	W	M	O	Q	W	M	O	Q	W	M	O	Q	W	M	O	Q	W		M	O	Q	W
Site A																									
Lagoon	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
A7 bkg. ^c	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	+	+	25
A1 bkg.	N	N	N	N	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	35
A2 bkg.	N	N	N	N	N	N	N	N	N	N	N	N	+	-	+	-	+	+	+	+	+	+	+	+	50
A10	-	-	-	-	-	+	+	-	+	+	+	+	-	+	+	+	+	+	+	+	+	-	-	-	42
A8	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	96
A9	+	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	92
A16	+	+	+	+	N	N	N	N	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	80
A13	+	+	+	+	-	+	+	-	+	+	N	N	N	N	+	+	+	+	-	-	-	-	-	-	60
A15	+	-	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	54
A6	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	88
A5	+	+	+	+	-	-	+	-	-	-	+	-	-	-	+	+	+	+	+	+	-	+	+	+	54
A12	-	-	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	83
A14	-	-	+	-	-	+	+	+	-	-	-	-	-	+	-	+	+	+	+	+	-	+	-	+	38
A11	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	88
A3	+	+	+	+	N	N	N	N	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	+	35
A4	-	-	-	-	-	+	+	-	-	-	+	-	-	+	+	-	+	-	-	-	-	+	-	+	29
Site C																									
Lagoon	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	100
C1 bkg.	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+	25
C3	-	-	-	-	+	+	+	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+	+	46
C2	+	-	+	+	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	+	+	+	46
C4	-	-	-	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	58
C6	+	+	+	+	+	-	+	+	-	-	-	-	-	+	-	-	+	-	+	-	+	-	-	-	46
C7	+	+	+	+	N	N	N	N	N	N	N	N	-	-	-	-	-	-	-	-	-	-	+	+	38

^aSampling period. Period 1-April 18, 2000 for site A and April 13, 2000 for site C; Period 2-May 29, 2001 for site A and May 22, 2001 for site C; Period 3-September 5, 2001 for site A and August 28, 2001 for site C; Period 4-January 8, 2002 for site A and January 16, 2002 for site C; Period 5-April 17, 2002 for site A and May 1, 2002 for site C; Period 6-March 26, 2003 for site A and February 19, 2003 for site C.

^bPercentage of positive signals within each row. N excluded from the calculation.

^cBackground well located up-gradient of the lagoon.

N=No sample.

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between sampling periods. However, detection frequency was much greater in the wells located close and downgradient of the lagoon in the direction of groundwater flow than in wells more distant from the lagoon. On the other hand, large differences in *tet* gene detection were observed between sampling periods at site C. Most of the *tet* genes were at concentrations less than the detection limit in groundwater samples collected during the second quarter of 2001 (period 3, Table 4), whereas most of the *tet* genes were detected in samples collected during the fourth quarter of 2003 (period 6, Table 4). The lowest detection frequency for *tet* genes was observed in samples from the background wells (A7 and C1) located up-gradient from the lagoons. These results suggested that although the distribution of tetracycline resistance genes in the groundwater underlying both pig farms was not stable, they persisted through the three-year study period. Based on the relationship between detection frequency of *tet* genes and well location, geological conditions such as the presence of a sand layer or groundwater flow influenced dissemination of resistance genes in the environment.

To determine the impact of lagoon seepage on *tet* gene distribution, monitoring wells were selected at site A using the value of electrical conductance (EC) as an indicator of contaminants from manure in the lagoon. Then the detection frequency of *tet* genes during the three-year study period was correlated with EC values for selected monitoring wells and the lagoon. This provided a clear correlation between detection frequency of *tet* genes and EC, that is, higher detection frequency of *tet* genes was observed in the wells having higher EC values. For example, groundwater samples from wells A6, A8, A9, A11, and A12 exhibited the largest conductivities (2,500 to 5,938 $\mu\text{S}/\text{cm}$), suggesting a significant impact of lagoon seepage on groundwater quality, and also exhibited the greatest frequency for containing the *tet* genes (83.3 to 95.8%) (Tables 2 and 4). These data indicate that lagoon seepage may not only be a source of inorganic contaminants but can also contain tetracycline resistant determinants.

Quantitation of *tet* Genes by Real-time PCR

The concentration of *tet* genes in groundwater is also critical when monitoring the impact of swine production systems on the environment. For this purpose we developed and validated a real-time PCR assay which allowed us precise and sensitive quantitation of *tet* genes. We first validated quantitation and sensitivity using a serially diluted (equivalent to $29 \sim 2.9 \times 10^7$ copy of target) cloned plasmid containing the *tet(Q)* gene (Figure 2). The assay for *tet(Q)* showed a typical standard amplification profile, and high correlation in the standard curve ($r^2 = 0.9972$). We also observed a similar result in the assay for the *tet(M)* gene. These results validated the quantitation and sensitivity of the assays for both genes. Quantitation of *tet(M)* and *tet(Q)* in lagoon samples collected from both sites was also conducted. Quantitation was carried out for each sample in duplicate on three different microtiter plates ($n = 6$). Standard deviations for each assay value were small, and the coefficient of variation was 12.66% for the assay of *tet(M)* and 16.26% for that of *tet(Q)*. Although a large decrease in concentration was observed during the first two sampling periods, the levels of *tet(M)* and *tet(Q)* genes were relatively stable in the

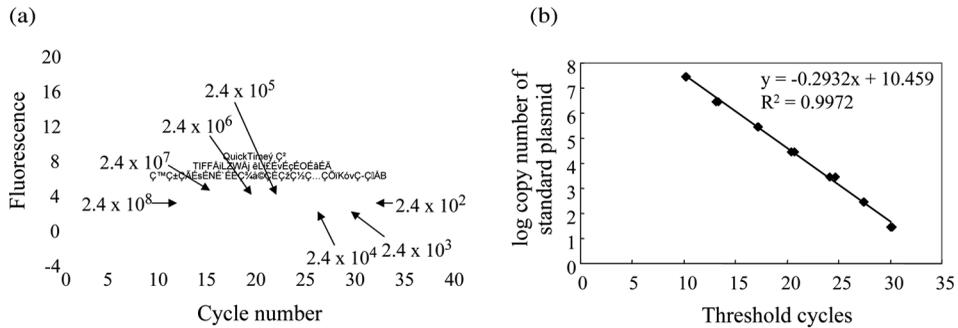


Figure 2 Amplification (a) and standard curve (b) of real-time PCR assay for *tet(Q)*. Serially diluted cloned plasmid (240 to 2.4×10^8 copy) was used as template. Duplicate was made at each amount of plasmid. The horizontal line in the panel A indicates threshold line. The threshold cycles (C_T , cycle number when the fluorescence reached threshold line) were obtained from the amplification curve shown in panel A, and C_T values were plotted against respective *tet(Q)* cloned plasmid copy number for construction of the standard curve shown in panel B.

lagoon through the monitoring period (Table 5). It is unknown what caused the large decrease in *tet* concentrations between those periods. However, the concentration of *tet(Q)* was much greater than that of *tet(M)* in the lagoons at both sites, suggesting that the risk for dissemination of *tet(Q)* from manure is much higher than that for *tet(M)*.

Table 5 Quantitation of *tet(M)* and *tet(Q)* genes in lagoon samples by real-time PCR

Sampling date	Target amount (% of 16S rDNA) ^a	
	<i>tet (M)</i>	<i>tet (Q)</i>
Site A		
May 29, 2001	3.21 ± 0.47	13.01 ± 1.22
Sept. 5, 2002	0.52 ± 0.05	3.14 ± 0.36
Jan. 8, 2002	0.90 ± 0.11	6.58 ± 1.44
Apr. 17, 2002	0.68 ± 0.03	5.45 ± 0.45
Jul. 16, 2002	1.54 ± 0.17	6.91 ± 0.22
Nov. 19, 2002	0.32 ± 0.06	2.43 ± 0.30
Mar. 26, 2003	1.35 ± 0.14	5.87 ± 1.26
Site C		
May 22, 2001	0.96 ± 0.03	21.03 ± 1.36
Aug. 28, 2001	0.32 ± 0.03	10.55 ± 1.34
Jan. 16, 2002	0.33 ± 0.03	1.20 ± 0.39
May 1, 2002	0.76 ± 0.26	6.58 ± 1.11
Jul. 30, 2002	0.26 ± 0.02	5.00 ± 0.77
Oct. 29, 2002	0.66 ± 0.04	5.34 ± 1.34
Feb. 19, 2003	0.99 ± 0.17	3.34 ± 0.97

^aQuantitation values were expressed as the ratio of copy number of target gene to that of the total bacterial 16S rDNA \pm standard deviation. Each sample was analyzed in duplicate on three different microtiter plates ($n = 6$).

CONCLUSIONS

Several key contaminant indicators, including inorganic ions, antibiotics, and antibiotic resistance genes and bacteria were analyzed in groundwater collected from 23 monitoring wells. Chloride, ammonium, potassium, and sodium were predominant constituents in the manure samples and served as indicators of groundwater contamination. Based on analysis of these constituents shallow groundwater at both sites has been impacted by lagoon seepage. The extent of migration of contaminants down gradient from the lagoons and the magnitude of contaminant concentrations in groundwater were significantly greater at site A than at site C. Migration of contaminants as much as 30 m down-gradient of the lagoon at site C and 150 m at site A can be attributed to the difference in the local geologic conditions at the sites.

Parent tetracycline antibiotics were detected in a few groundwater samples collected from wells impacted by manure seepage as evidenced by elevated chloride, ammonium, and potassium concentrations. Breakdown products of the tetracyclines were detected in selected groundwater at site A even when the parent compound was not detected. The tetracyclines and their breakdown product concentrations in groundwater were generally less than 0.5 µg/L. Although tetracyclines are used at both facilities, they were not detected in every manure sample and were detected at relatively small concentrations. It is likely that the affinity and nonreversibility of the sorption of tetracycline antibiotics to soil minerals and organic matter account for the relatively few detections and small concentrations in our manure and groundwater samples.

Of the seven *tet* genes encoding ribosomal protection proteins, only four—*tet(M)*, *tet(O)*, *tet(Q)*, and *tet(W)*—were frequently detected in groundwater and manure samples. The gene detection frequency was much greater in the wells located close to and down-gradient of the lagoon in the direction of groundwater flow than in wells more distant from the lagoon. Large differences in *tet* gene detection was observed between sampling periods at site C. These results suggest that the distribution of tetracycline resistance genes in the groundwater underlying both pig farms was not stable, but did persist through the three-year study period. The members of the complex bacterial community in the groundwater samples fluctuated during the study period and the dominant bacterial species also differed between the lagoon and corresponding groundwater samples. Although the concentrations of *tet(M)* and *tet(Q)* genes were relatively stable in the lagoon samples through the monitoring period, the larger concentrations of *tet(Q)* compared to *tet(M)* in the lagoons at both sites indicates that the risk for dissemination of *tet(Q)* from manure is much greater than that for *tet(M)*.

Sequence analysis of the *tet(M)* gene indicates that the sources of *tet* genes in lagoon and the background groundwater samples differ. The origin or source of genetic contamination in the background well is currently unknown, emphasizing the importance of sampling surface water and soil in the vicinity of these wells to track the potential source of resistance genes.

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