Report for 2002IA10B: Fate and transfer of antibiotic-resistance genes excreted by farm animals

There are no reported publications resulting from this project.

Report Follows:

Problem and Research Objectives

Antibiotic resistance is a public health concern of great urgency due to a growing inefficacy of antimicrobial agents to treat infectious diseases. This is mainly due to the propagation of antibiotic resistance genes among bacteria, which is exacerbated by the potential overuse of antimicrobials in humans and the intensive use of antibiotics in animal agriculture for non-therapeutic purposes such as growth promotion and disease prevention (Mellon et al., 2001). Recent studies have found that antibiotic resistance genes occur in bacteria in the environment as a direct result of animal agriculture (e.g., swine production facilities), and that soil and groundwater in the vicinity of such facilities may be *potential sources of antibiotic resistance in the food chain* (Chee-Sanford et al., 2001). However, such genes have not yet been considered as environmental pollutants, and little is known about the fate and transport of antibiotic resistance genes when released to the environment as a result of direct runoff, groundwater infiltration from lagoons, or manure spreading activities. Critical knowledge gaps include the rate and extent of gene propagation (including bacterial migration and inter-specific gene transfer from enteric to soil bacteria) and the effect of environmental factors such as soil characteristics and water chemistry on the persistence of antibiotic resistance. Learning about these issues is important to assess the impact of antibiotic resistance genes on public and environmental health and to determine the need for regulatory action in states where animal agriculture is common.

This study addresses the effect of antibiotic exposure (e.g. tetracycline (TC)) on indigenous soil microorganisms in simulated runoff infiltration conditions. Emphasis was placed on addressing the following issues: (1) persistence of TC in a flow through column, (2) effect of the presence of an antibiotic on the microbial community (populations) within a flow through column, and (3) genetic characterization of the microorganisms isolated from the columns exposed to the antibiotic.

The main goals of the ongoing research are to:

Short Term

1. Characterize the fate and transport of TC in soil.
2. Determine the effect of sustained TC exposure on the development of TC-resistant strains.

Long Term

1. Monitor the development and relative abundance of TC-resistant strains.
2. Genotypic characterization of resistant strains.
3. Model the resistance gene transfer.

Methodology

Flow-through Columns. Two cylindrical, 30-cm-long, flow-through glass columns (Kontes Glass Company, Vineland, NJ) were modified with six sample ports located at 2, 5, 9, 14, 19, and 24 cm from the bottom inlet of the column. Inlet and outlet three-way valves were placed at the respective locations. The columns were secured in a vertical

position and tightly packed with soil (University of Iowa Softball Field). The columns were wrapped in aluminum foil to minimize algal growth and possible antibiotic photodegradation.

Two-L reservoir bottles were equipped with 3-hole caps (Kontes Glass Company, Vineland, NJ) and wrapped in aluminum foil. Masterflex Neoprene® tubing (Cole-Parmer Co.) and a Masterflex peristaltic pump (Cole-Parmer Co.) were used for the delivery of the feed solution. The pump flow rate was adjusted as to achieve a column flow rate range between 3.0 and 4.0 mL/hr. The flow rate for the control column (TC -) was approximately 3.4 mL/hr and 3.6 mL/hr for the TC-enriched column. Bromide tracer studies were conducted on both columns prior to addition of the feed solutions.

The feed solution for both columns consisted of synthetic ground water (von Gunten and Zorbist, 1993) as nutrient source and sodium acetate as a carbon source (10 mg/L). In addition, one feed solution was amended with tetracycline-hydrochloride (T3383, Sigma Co.) at 10-50 mg/L.

Concentration Profiles. The concentrations of acetate and tetracycline were monitored along the column length (inlet, outlet, and sample ports) approximately every two months since column initiation. Standard curves for both chemicals were prepared monthly to ensure measurement accuracy. Acetate concentrations were measured via an anion chromatograph equipped with an auto-sampler apparatus (Alltech 570), an IonPAc AS14 column (Dionex), and a conductivity detector (Dionex). Tetracycline content was analyzed via a manual injection HPLC pump (Alltech 426) equipped with a HPLC column (Supelco, Discovery C8, 59353-U) and a variable wavelength detector (Dionex). Detection conditions were as follows: 680mL 0.1 Ammonium Oxalate, 270mL Dimethylformamide, 50 mL 0.2M Dibasic Ammonium Phosphate (pH 7.6), at 1 mL/min, 20 µL, Isocratic, Ambient, UV at 280 nm. The elution time for tetracycline was approximately 3 min.

Microbial Enumeration. Initially, agar plate counts for the enumeration of microbial populations were performed. The effluent from both columns was collected and 100 µL were streaked onto the R2A agar plates, with the intent to quantify the total heterotrophic populations. R2A plates enriched with tetracycline (50 mg/L) were also streaked with the column effluent in order to quantify the antibiotic-resistant microorganisms. Several attempts with this method yielded variable and irreproducible results.

A modified MPN 96-well plate technique was adapted for microbial enumeration of the column effluent. Growth media containing succinate and resazurin solution was used for the enumeration of the total heterotrophic population, and tryptic soy broth enriched with tetracycline (50 mg/L) was used for the antibiotic-resistant microorganisms. This quantification was based on visual scoring of the color change (blue to red for resazurin) and growth-induced TSB-turbidity development and subsequent statistical analysis.

Genetic Analysis. Effluent from the tetracycline exposed column was used for the isolation of antibiotic-resistant strains. Tetracycline enriched R2A agar plates were streaked with the effluent and incubated at 30°C for periods of 2-5 days, depending on the growth rates (appearance of colonies). Individual colonies were restreaked onto TC-enriched R2A agar plates, incubated, isolated, and restreaked a second time in order to ensure strain "purity."

Bacterial DNA was extracted with kits according to manufacturers' protocols (Qiagen). A Mastercycler® thermocycler device (Eppendorf) was purchased for the Polymerase Chain Reaction (PCR) gene detection techniques. PCR amplification was performed on the extracted DNA according to the protocols provided in the reaction kits (PanVera). The typical (50 µL) reaction mixture consisted of 0.25 µL DNA polymerase (Ex Taq), 5.0 µL 10X buffer, 4.0 µL dNTP mix (2.5 mM), 5.0 µL DNA template, 4.0 µL primers (forward and reverse), and 27.75 µL H_2O. The amplification was performed as previously described by Aminov, et.al. (2001). Briefly, the cycle steps were: (1) an initial denaturation at 94°C (5 min) followed by 25 cycles of 94°C (30s), (2) annealing at 30s and 30s extension (72°C), and (3) extension at 72°C (7 min). The annealing temperatures and the sequences for each primer are shown in Table 1.

DNA primers were constructed for the following tet-determinants coding for Ribosomal Protection Proteins (RPP): TetB(P), Tet(M), Tet(O), Tet(Q), Tet(S), Tet(T), Tet(W), and OtrA. PCR products were analyzed by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide. The expected sizes of the amplification products were, 168bp for TetW, 169bp for TetB(P), Tet(Q), Tet(S), Tet(T), 171bp for Tet(M), Tet(O), and 212 bp for OtrA. 16S ribosomal DNA (rDNA) analysis of the isolated strains was also conducted.

Table 1: PCR Primers targeting RPP classes (Aminov et al., 2001)

Primer	Class Targeted	Annealing T (°C)	Sequence
TetB/P-FW	Tet B P	46	AAAACTTATTATATTATAGTC
TetB/P-RV			TGGAGTATCAATAATATTCAC
TetM-FW	Tet M	55	ACAGAAAGCTTATTATATAAC
TetM-RV			TGGCGTGTCTATGATGTTCAC
TetO-FW	Tet O	60	ACGGARAGTTTATTGTATACC
TetO-RV			TGGCGTATCTATAATGTTGAC
OTR-FW	Otr A	66	GGCATYCTGGCCCACGT
OTR-RV			CCCGGGGTGTCGTASAGG
TetQ-FW	Tet Q	63	AGAATCTGCTGTTTGCCAGTG
TetQ-FW			CGGAGTGTCAATGATATTGCA
TetS-FW	Tet S	50	GAAAGCTTACTATACAGTAGC
TetS-FW			AGGAGTATCTACAATATTTAC
TetT-FW	Tet T	46	AAGGTTTATTATATAAAAGTG
TetT-FW			AGGTGTATCTATGATATTTAC
TetW-FW	Tet W	64	GAGAGCCTGCTATATGCCAGC
TetW-FW			GGGCGTATCCACAATGTTAAC

FW= forward, RV= reverse

Principal Findings and Significance

Initially, tetracycline concentrations were monitored in the influent and effluent of the exposed column. Approximately 97% of the initial tetracycline was degraded within the column length (Figure 1).

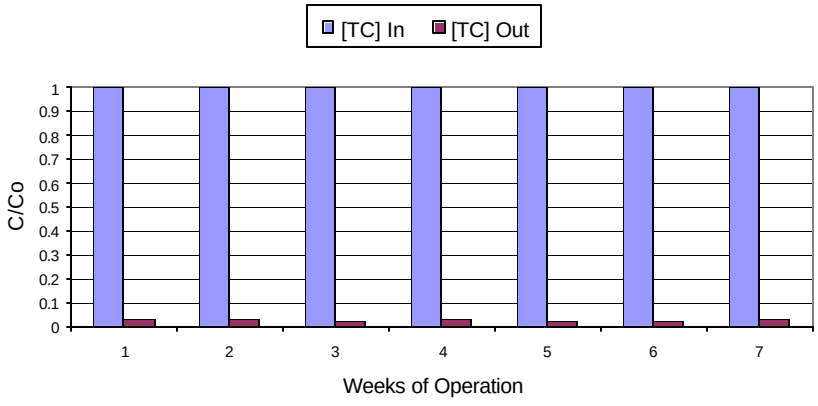

Figure 1: Inlet and outlet standardized antibiotic concentrations for TC-enriched column (operation start date: 8/9/2002)

Tetracycline and acetate concentration profiles along the length of the columns were monitored in order to determine the antibiotic degradation behavior within the column and monitor microbial utilization of the carbon source. Three column-length profiles have been performed since column initiation. A representative profile is shown in Figure 2. All obtained profiles exhibited similar acetate and tetracycline behaviors within the columns.

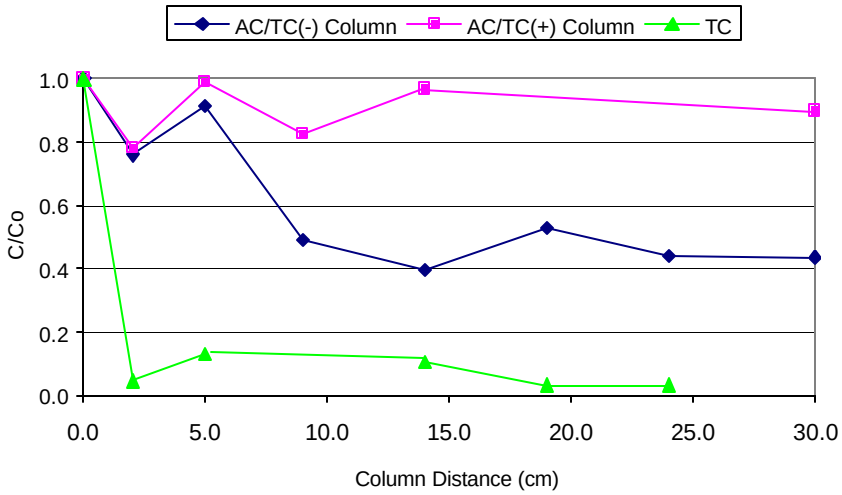

Figure 2: Tetracycline and acetate concentration profiles (12/2002)

The degradation of tetracycline appears to take place within the first 2.5 cm of the column and presumably occurred upon initial contact with the column soil (no sampling port was available at this location). Batch studies conducted to understand the behavior of tetracycline when in contact with soil are described later in the report. Trace amounts of the antibiotic were present throughout the length of the column and at the outlet sampling port.

Assuming that acetate consumption is indicative of microbial activity, acetate profiles for the two columns suggest decreased microbial concentrations within the TC-enriched column. The presence of approximately 80-90% of the original acetate added to the TC-enriched column (Fig.2) suggests a very low level of acetate consumption by the microorganisms present in the column, which, in turn, can be assumed as an overall lower number of microbes than in the non-enriched column, where the acetate concentrations appear to be oscillate between 40-60% of the feed concentration. The removal of acetate in the non-enriched column may also be assumed as a process limited by the dissolved oxygen (DO) concentration.

MPN microbial counts corroborated the acetate profile data with a lower total heterotrophic population in the TC-enriched column. The antibiotic-resistant populations in both columns were also significantly smaller than the respective total heterotrophic populations, but there seemed to be no statistical difference between the resistant counts of the two columns (Figure 3). However, the percentage of resistant microorganisms increased in the TC-enriched column from approximately 4% to 35%.

Again, this was apparently due to the decrease of the total heterotrophic population in the enriched column, rather than to the development of antibiotic resistance in the indigenous strains.

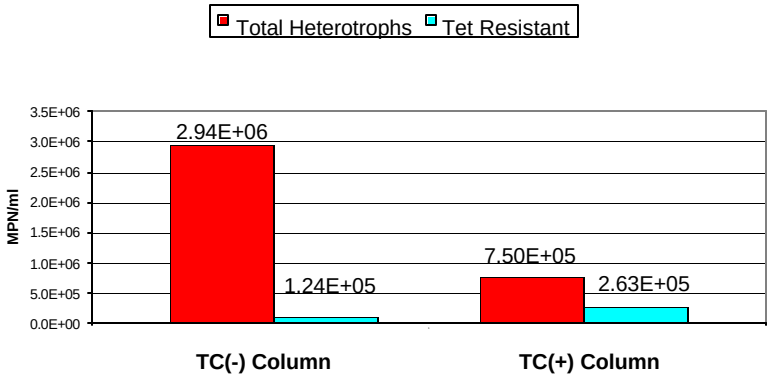

Figure 3: MPN microbial enumeration

Tetracycline has been reported to be highly unstable in light conditions due to photodegradation. Batch experiments were performed to assess the stability of an aqueous tetracycline solution under light and dark conditions. Two 100-mL solutions were prepared with one beaker completely covered in aluminum foil to simulate dark conditions and with the second beaker exposed to environment light. Surprisingly, no statistical differences were observed for the degradation rates of the two solutions, with approximately 85% of the original tetracycline remaining after 400 hours (Figure 4).

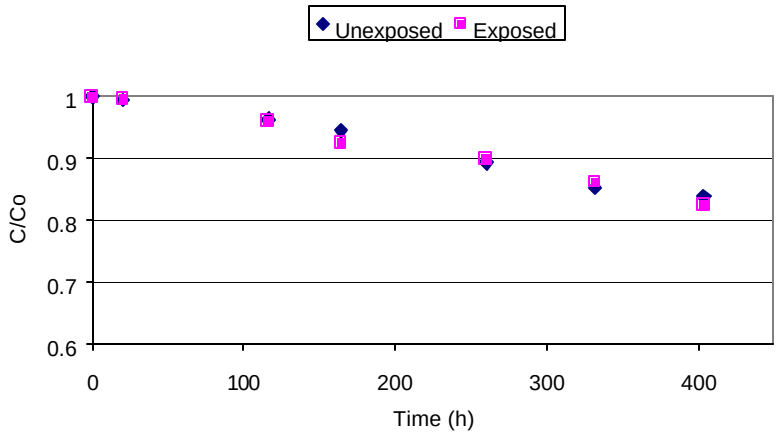

Figure 4: Tetracycline degradation under light (exposed) and dark (unexposed) conditions

To investigate the behavior of tetracycline when in contact with soil environments, batch studies were performed with the same soil source that was used for the column packing. 250-mL amber-glass reaction bottles were filled with 100-mL of distilled water and 10 grams of soil. The solution was mixed thoroughly by vigorous shaking and the initial pH was measured (pH ≈ 7). A 100 mg/L tetracycline solution was prepared, and the pH was

also measured (pH ≈ 4). Fifty mL of the TC solution were added to the soil mixture and shaken immediately. The pH and the tetracycline concentrations of the resulting solution were measured within 1 minute of the TC addition. Approximately 96% of the initial antibiotic was removed upon contact with the soil, which concurs with the tetracycline degradation behavior observed in the tet-enriched column. Along with the disappearance of the antibiotic, a rise in pH of the solution is observed, suggesting some form of *alkaline hydrolysis* as the mechanism of tetracycline degradation (Figure 5).

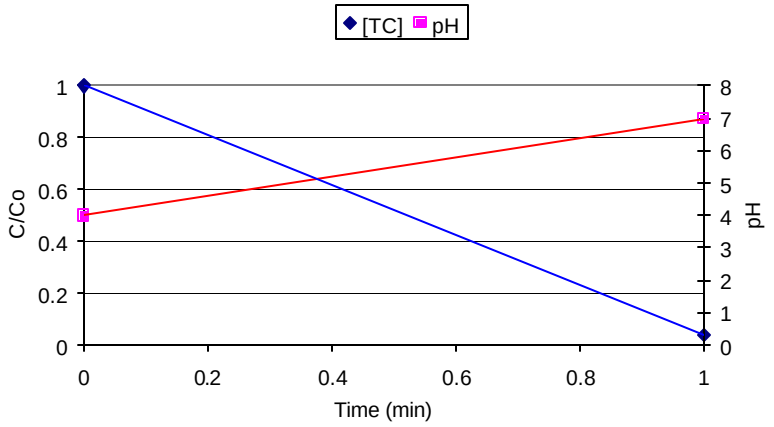

Figure 5: Batch degradation of TC and pH behavior

Microbial strains (courtesy of Dr. Svetlana Kocherginskaya, UIUC) carrying the tet-determinants of interest were grown and the plasmids containing these determinants were extracted. PCR amplification of the template DNA yielded products approximately 160-220 bp., which is in agreement with previously published results (Aminov et al., 2001). The positive PCR controls are shown in Figure 6 with standard DNA ladder samples flanking the amplified products. The remaining determinants (TetB(P), TetW, TetT) have been successfully amplified but are not shown.

Isolation, DNA extraction, and PCR amplification steps were followed with the TC-resistant strains harvested from the TC-enriched column, but, to date, no RPP-tet-determinant has been detected in any of these strains. Since the presence of extracted DNA was confirmed by gel electrophoresis, it may be assumed that the isolated TC-resistant strains do not carry the determinants we had targeted. We are currently screening for the genes coding the Ribosomal Protection Protein (RPP), which represent a portion of the total TC-resistance determinants. Genes responsible for the efflux pump mechanism for antibiotic excretion present another important resistance mechanism available to the microorganisms. Thus, it is very likely that the isolated TC-resistant strains contained one of the efflux pump genes, explaining their ability to grow on antibiotic medium without carrying the RPP determinants we had targeted.

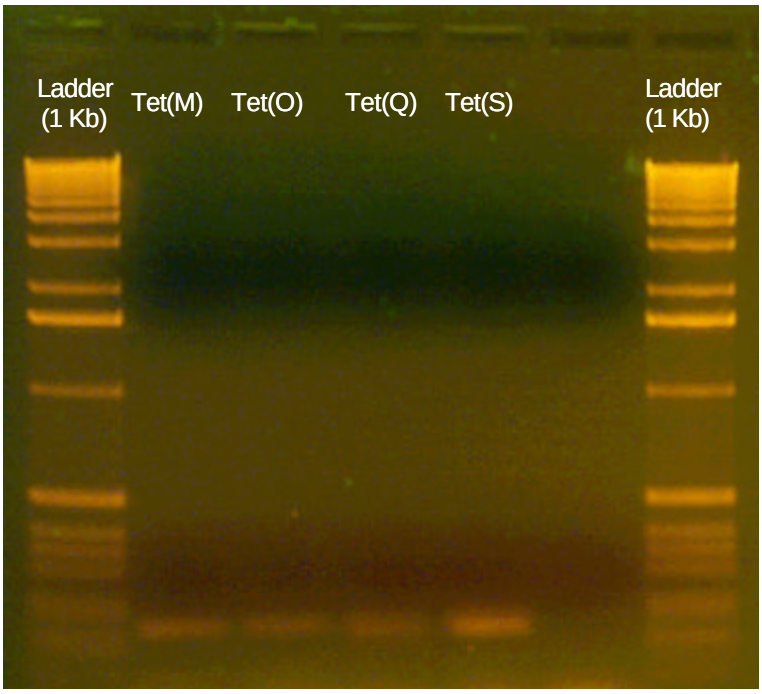

Figure 6: Tet-resistant strains, positive controls
(amplicon sizes 169-171 bp.)

Future Research Directions

Currently, antibiotic resistance could be considered to be an environmental pollution problem with gene vectors as the target contaminants. However, little is known about the fate and transfer of such gene vectors when released to the environment during direct runoff, groundwater infiltration from lagoons, or manure spreading activities (Figure 7). Critical knowledge gaps include the rate and extent of gene propagation (including bacterial migration and inter-specific gene transfer from enteric to soil bacteria) and the effect of environmental factors and sustained antibiotic exposure on the persistence of antibiotic resistance.

Accordingly, one possibility for future research would be the expansion of tet-resistance determinants targeted. This would include the current screening for the RPP genes as well as the efflux pump determinants (TetA-TetE, TetG, TetH, TetK, TetL, TetA(P), OtrB). Upon successful genetic detection and analysis of the tet-resistant strains, the spatial distribution of the resistance genes within the flow-through columns will be addressed in detail. This approach will not only provide a more complete idea of the type of resistance genes present in the columns, but it will also facilitate the mathematical modeling of the dynamics of propagation of these genes, which is an objective of this research project. Determining the fate, transport, transfer, and decay kinetics of gene vectors will thus help formulate more accurate mathematical models to support regulatory and management decisions (e.g., to set total maximum daily loads [TMDLs]).

The effect of antibiotic dosing will also be addressed in the near future. The on/off impact of antibiotic application will be investigated in order to elucidate the effect of such administration on the microbial communities, first, by means of microbial enumeration, and, second, by genetic analysis. The shifts in tet-resistant population will be monitored during and after antibiotic application with a predicted decrease in the amounts of resistant microbes after the withdrawal of the antibiotic from the column feed solutions. Upon this removal, the time necessary for the community to return to the "original" (pre-antibiotic) state will be monitored in order to ascertain the effect of antibiotic dosing as experienced in animal agricultural applications (also periodic).

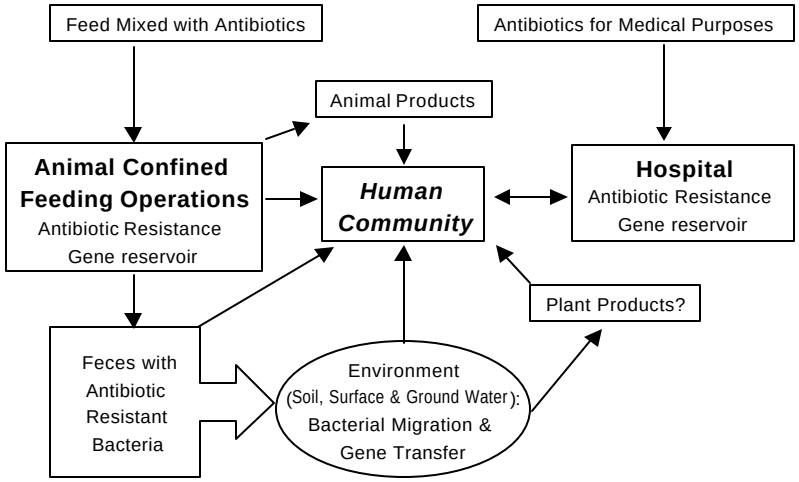

Figure 7. Potential Exposure Pathways for Antibiotic Resistance Genes

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