

Report for 2003IA49B: Amplification and Attenuation of Tetracycline Resistance in Soil Bacteria: Aquifer Column Experiments

There are no reported publications resulting from this project.

Report Follows

Amplification and Attenuation of Tetracycline Resistance in Soil Bacteria: Aquifer Column Experiments

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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, 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) on indigenous soil microorganisms in simulated runoff infiltration conditions. The main goals are to:

Short Term

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

Long Term

1. Monitor development of resistant strains
2. Genotypic characterization of resistant strains
3. Model the resistance gene transfer

Methodology

General Approach. Flow-through columns packed with soil were used to mimic runoff and infiltration of TC-contaminated agricultural drainage and to evaluate changes in the total heterotrophic and Tef^r microbial populations during and after sustained TC exposure. Acetate, which is a common product of animal waste breakdown and is likely to be present in farm runoff, was added as a carbon source. A control column without TC was also run. Emphasis was placed on enumerating and characterizing bacteria in the column effluent to focus on mobile bacteria with a higher potential to reach a human recipient.

Along with population enumerations, TC and acetate concentration profiles were monitored along the length of the columns to investigate TC stability and acetate utilization patterns. Tet^r microorganisms isolated from the effluent of the TC-enriched column were identified by genetic analysis and screened for the tet-determinants responsible for TC resistance. The recovery of the microbial populations after TC exposure ceased was also characterized by monitoring the percentage of Tet^r heterotrophs in the column effluent.

1. *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 24cm. 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 feed solution delivery. The pump flow rate was adjusted to achieve a column flow rate range between 3.0–4.0 mL/hr. The flow rate for the control column (TC -) was approximately 3.4 mL/hr and 3.6 mL/hr for the exposed (TC +) 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.

2. *Concentration Profiles:* The concentrations of acetate and tetracycline were monitored along the column length (inlet, outlet, and sample ports). 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).

3. *Microbial Counts:* 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 irreproducible results.

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

4. *Genetic Analysis*: Effluent from the TC-exposed column was used for the isolation of antibiotic-resistant strains. TC-enriched (50 mg L⁻¹) R2A agar plates (Difco Laboratories) were streaked with column effluent (0.1 mL) and incubated at 30°C 2–5 days, depending on the growth rates (appearance of colonies). Individual colonies were re-streaked onto TC-enriched R2A agar plates, incubated, isolated, and re-streaked a second time in order to ensure strain “purity.”

Bacterial DNA was extracted from selected colonies with kits according to manufacturers’ protocols (Qiagen). A Mastercycler® thermocycler device (Eppendorf) was used 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 final concentrations of the PCR reagents in a 50 µL reaction mixture were: 1.25U DNA polymerase (Ex Taq), 1X reaction buffer, 200 µM deoxynucleoside triphosphate, and 0.2 µM primers (forward and reverse). Primers were constructed (according to Table 1) 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. 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 at 94°C (30s); (2) annealing at 30s and 30s extension (72°C); and (3) extension at 72°C (7 min). The annealing temperatures for each primer are shown in Table 1. Reaction products were analyzed by electrophoresis on a 1.2% (wt/vol) agarose gel containing ethidium bromide.

**Table 1. PCR Primers targeting ribosomal protection protein (RPP) classes
(Source: Aminov *et al.*, 2001)**

Tet-determinant targeted	Primer Sequence	Amplicon size (bp)	Annealing Temperature (°C)
TetBP-F	AAAAC TTATTATATTATAGTC	169	46
TetBP-R	TGGAGTATCAATAATATTCAC		
TetM-F	ACAGAAAGCTTATTATATAAC	171	55
TetM-R	TGGCGTGTCTATGATGTTTAC		
TetO-F	ACGGARA GTTTATTGTATACC	171	60
TetO-R	TGGCGTATCTATAATGTTGAC		
OtrA-F	GGCATYCTGGCCCA CGT	212	66
OtrA-R	CCCGGGGTGTCGTASAGG		
TetQ-F	AGA ATCTGCTGTTTGCCAGTG	169	63
TetQ-R	CGGAGTGTCAATGATATTGCA		
TetS-F	GAAAGCTTACTATACAGTAGC	169	50
TetS-R	AGGAGTATCTACAATATTTAC		
TetT-F	AAGGTTTATTATATAAAAAGTG	169	46
TetT-R	AGGTGTATCTATGATATTTAC		
TetW-F	GAGAGCCTGCTATATGCCAGC	168	64
TetW-R	GGCGTATCCACAATGTTAAC		

Primers targeting efflux-pump tet-determinants were constructed according to Table 2. Amplification conditions were as described by Furushita *et al.*, (2003) and included 30 cycles of 60s at 94°C, 45s at annealing temperatures shown in Table 2, and 90s at 72°C followed by a final extension of 300s at 72°C. PCR reaction products were purified with the QIAquick® PCR Purification Kit (Qiagen) according to manufacturer's protocol. Restriction digests of the PCR products were attempted with the following endonucleases (to identify the following *tet* determinants): *SmaI* (TetA), *SphI* (TetB, TetD, TetY), *Sall* (TetC), *NdeII* (TetE, TetH, TetJ), and *EcoRI* (TetG). The amplicons were analyzed by gel-electrophoresis as described above.

Table 2. PCR Primers targeting efflux-pump determinants
(Source: Furushita *et.al.* 2003)

Tet-determinant targeted	Primer Sequence	Amplicon size (bp)	Annealing Temperature (°C)
TetA-F	CGCYTATATYGCCGA YATCAC	417	55
TetA-R	CCRAAWKCGGCWAGCGA		
TetB-F	GGDATTGGBCTTATYATGCC	967	50
TetB-R	ATMACKCCCTGYAATGCA		
TetC-F	CGCYTATATYGCCGA YATCAC	417	55
TetC-R	CCRAAWKCGGCWAGCGA		
TetD-F	GGDATTGGBCTTATYATGCC	964	50
TetD-R	ATMACKCCCTGYAATGCA		
TetE-F	GGDATTGGBCTTATYATGCC	650	50
TetE-R	AWDGTGGCDGGAATTTG		
TetG-F	TATGCRTTKATGCAGGTC	917	50
TetG-R	GACRAKCCAAACCCAACC		
TetH-F	GGDATTGGBCTTATYATGCC	650	50
TetH-R	AWDGTGGCDGGAATTTG		
TetJ-F	GGDATTGGBCTTATYATGCC	650	50
TetJ-R	AWDGTGGCDGGAATTTG		
TetY-F	TATGCRTTKATGCAGGTC	911	50
TetY-R	GACRAKCCAAACCCAACC		

Principal Findings and Significance

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

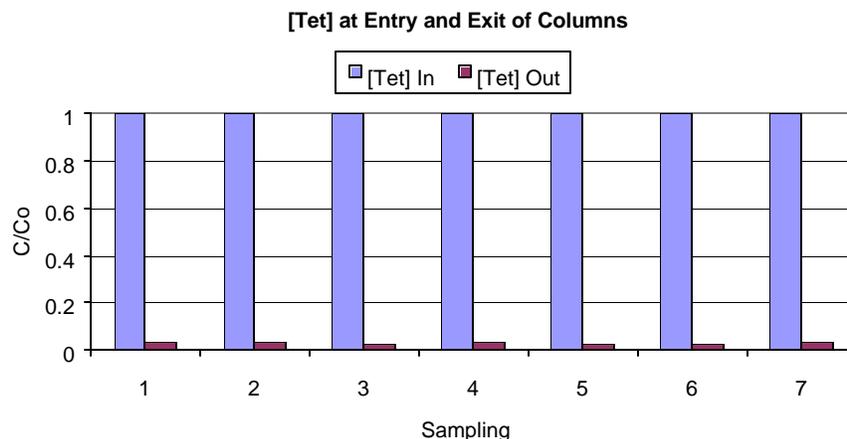


Figure 1: Inlet and outlet standardized tetracycline concentrations

Tetracycline and acetate concentration profiles along the length of the columns (Figure 2) were monitored in order to determine the antibiotic degradation behavior within the column and monitor microbial utilization of the carbon source.

A significant decrease in the aqueous TC concentration occurred near the inlet, and only trace amounts of TC (approximately 4 percent of influent concentration) were detectable throughout the length of the column. TC removal was presumably due to abiotic degradation upon contact with soil under neutral or mild alkaline conditions (pH=6.5–9.0). Furthermore, although TC is very soluble in water ($S=1,700$ mg/L, $\log k_{ow}=-1.19$), sorption by other mechanisms than hydrophobic partitioning (e.g., cation bridging at clay surfaces and surface complexation) probably contributed to TC removal from the aqueous phase. It is unlikely that TC removal was due to microbial degradation because very fast TC removal (96% within one minute) was observed in batch studies where TC (100 mg/L, pH=4) was added to soil slurries (pH=7).

Assuming that acetate consumption is indicative of microbial activity, acetate profiles for the two columns suggest decreased microbial presence within the TC-enriched column. The presence of fewer microorganisms within the column could be attributed to the antibiotic dosages and the selective pressure exerted on the indigenous strains.

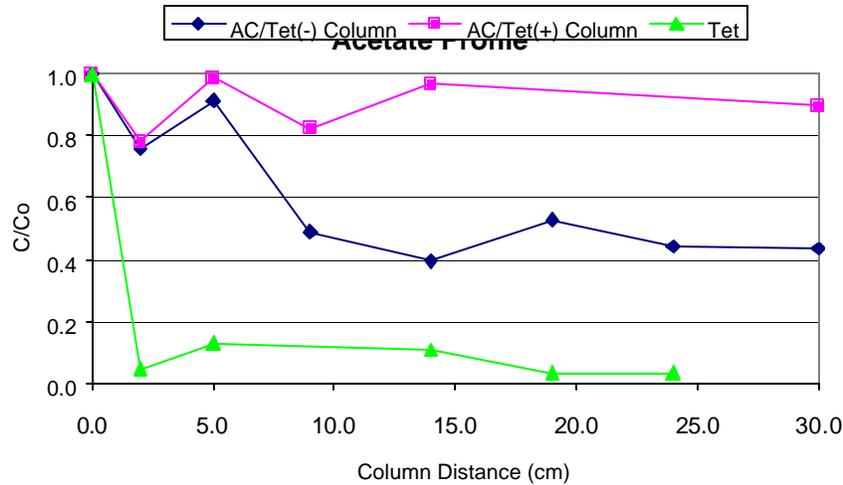


Figure 2: Tetracycline and acetate concentration profiles (12/2002)

TC exerted a significant bacteriostatic effect, decreasing the MPN concentration of total heterotrophs eluting from the TC-amended column by one order of magnitude compared to the control (Figure 3A). Nevertheless, the effluent concentration of Tet^r bacteria was significantly higher for the TC-amended column than for the control ($p < 0.05$) (Figure 3B). Thus, TC exerted selective pressure for the development and maintenance of antibiotic resistance in soil bacteria, even though, to the best of our knowledge, potential Tet^r gene donors such as enteric Tet^r bacteria that could be excreted by farm animals were not initially present in this soil. Whereas TC concentrations decreased rapidly near the inlet, some TC degradation products such as 5a-6-anhydrotetracycline and 5a,6-anhydrochlorotetracycline (none of which were examined) are known to retain antimicrobial properties (Halling-Sorensen et al., 2002). Thus, it is plausible that TC degradation products, which may result from abiotic reactions, also contributed to the selective pressure for Tet^r bacteria.

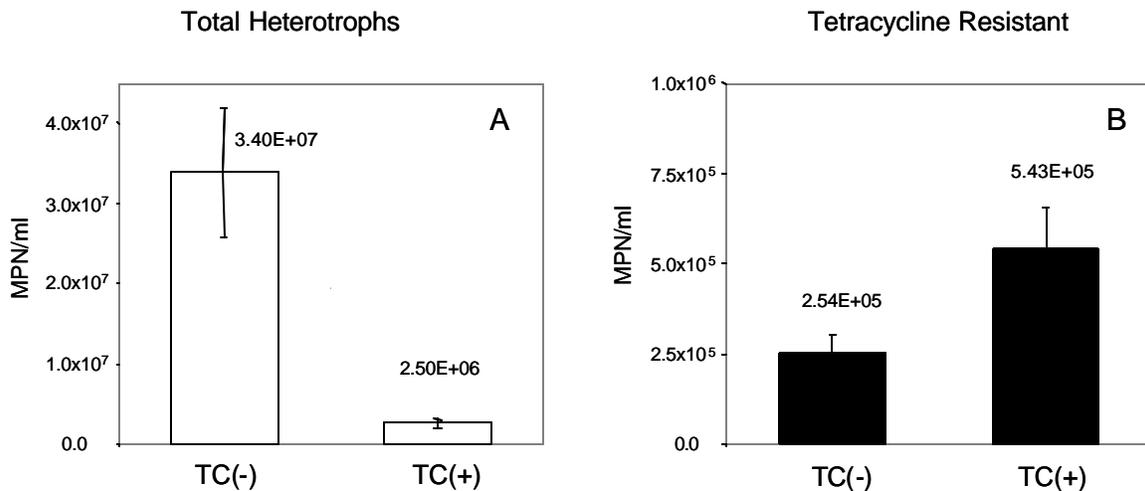


Figure 3: Effluent microbial concentrations from two columns. The TC(+) column was exposed to tetracycline during 300 days, whereas the TC(-) column served as an unexposed control. The concentration of total heterotrophs was significantly lower in the TC(+) column (panel A), while Tet^r bacteria concentrations were significantly higher ($p < 0.05$) (B).

Tetracycline has been reported highly unstable in light conditions due to photodegradation characteristics. Batch experiments were performed to assess the stability of an aqueous tetracycline solution under light and dark conditions. Two 100mL solutions were prepared with one beaker completely covered in aluminum foil, simulating dark conditions, and 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 both solutions remaining after 400 hours (Figure 4).

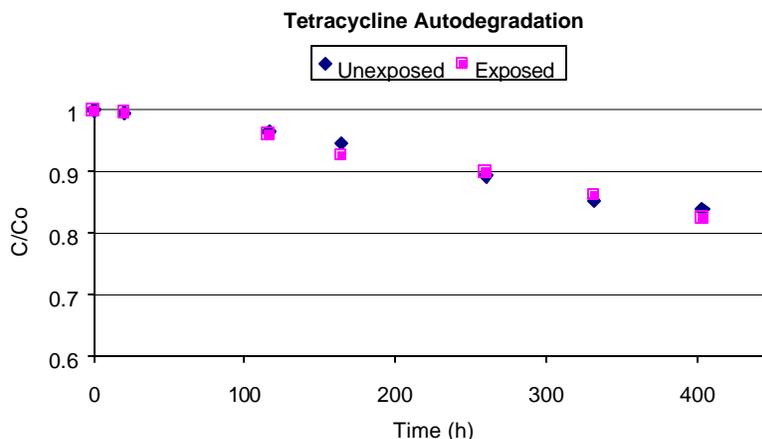


Figure 4: Tetracycline degradation under light (exposed) and dark (unexposed) conditions

To further 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 di-water and 10 grams of soil. The solution was mixed thoroughly by vigorous shaking, and the initial pH was measured (pH \approx 7). A 100 mg/L tetracycline solution was prepared, and the pH was also measured (pH \approx 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—degraded upon contact with the soil—which concurs with the tetracycline degradation behavior observed in the tet-enriched column (Figure 2). 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).

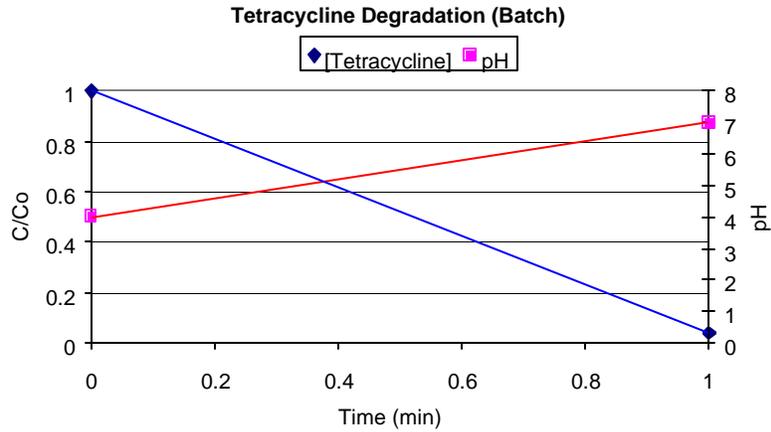


Figure 5: Batch degradation of TC and pH behavior

TC addition to the treated column was stopped after 300 days. Following a two-week lag, this resulted in a significant decrease in the percentage of Tet^r heterotrophs, from about 25% after sustained TC exposure to the pre-exposure and control levels (1 to 2%) within 30 days (Figure 6). This trend was due both to a rebound of total heterotrophs (with a related increase in acetate consumption) as well as to a significant decrease in Tet^r bacteria concentration. It should be pointed out that the concentration of total heterotrophs eluting from the column previously enriched with TC did not reach the same levels eluting from the control column within the 30-day monitoring period, possibly due to some residual antibiotic activity. Nevertheless, Figure 6 suggests that discontinuing TC exposure or curtailing its use should enhance natural attenuation mechanisms that mitigate the spread of resistance vectors.

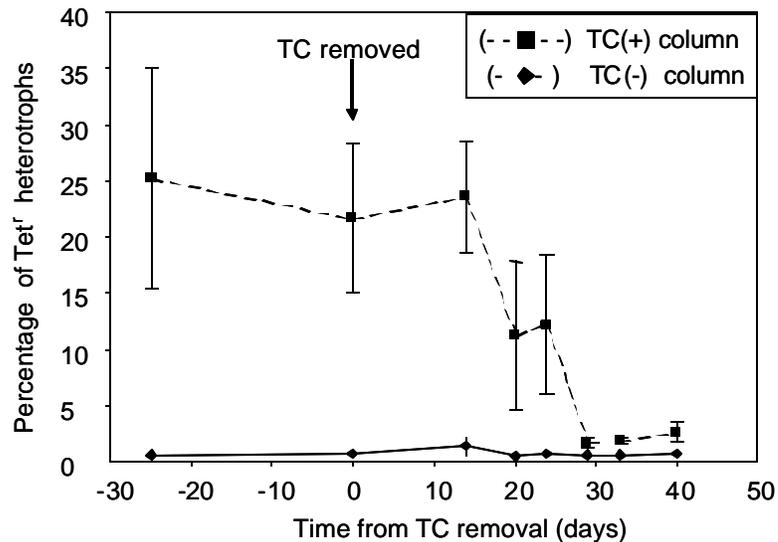


Figure 6: Decrease in the percentage of Tet^r heterotrophs after TC removal from column feed solution.

Tet^r bacteria were isolated from the TC-enriched column effluent by plating on Tet-R2A agar. Such isolates represent mobile bacteria that could reach human recipients. Two types of Tet^r microbial colonies were consistently detected and isolated. The first isolate was identified on the basis of its 16S rRNA sequence (using the BLAST database) as *Burkholderia cepacia*, which is a common soil bacterium. The second isolate was identified on the basis of its 26S rRNA sequence (using the NTBI database) as *Rhodotorula mucilaginosa*, which is a fungus. The identity of this yeast was confirmed by analysis with an API 20 C AUX yeast system kit. Both identifications were performed by Microbial Insights Inc. (Rockford, TN).

Burkholderia cepacia was screened for 17 tet-determinants, coding for both ribosomal protection proteins and efflux pumps, and was found to carry an efflux pump gene (TetA or TetC) (Figure 7). Discerning whether the determinant was TetA or Tet C was not possible because the same primer was used for both genes, and restriction digest attempts of the amplicon did not result in detectable fragments.

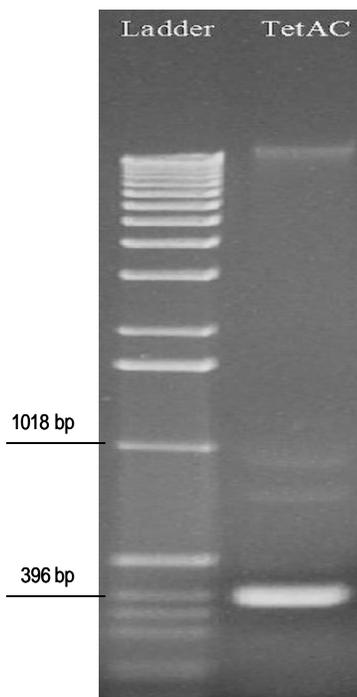


Figure 7: PCR amplification of the TetA/C gene determinant of *B. cepacia*, coding for an efflux pump. Amplicon size of approximately 417 bp along with 1 Kb ladder are presented.

The development of TC resistance in soil bacteria exposed to TC suggests that indigenous soil microorganisms may serve as reservoirs for the propagation (and possibly the amplification) of antibiotic resistance and potentially pose a direct hazard to public health. *B. cepacia* has been a focus of attention due to its (opportunistic) pathogenic characteristics (Kiska et al., 1996, Govan et al., 1996). Yet, *B. cepacia* has also received considerable attention due to its broad substrate specificity for application in

bioremediation processes (Bourquin et al., 1997, Steffan et al., 1999). Thus, the correlation between such a widely employed bacterium and its potential for disease propagation due to acquired antibiotic resistance may, in the future, influence the selection process and feasibility of some bioaugmentation practices.

Interestingly, the fungal microorganism (*R. mucilaginosa*) was only detected on R2A plates during the TC-enrichment period. This yeast was never detected in the effluent from the control column without TC or in the treated column effluent after TC exposure ceased. Since yeasts are relatively insensitive to TC (e.g., TC is produced by a yeast, *Saccharomyces*), we speculate that *R. mucilaginosa* proliferation during TC exposure was due to the inhibition (or death) of bacteria that were antagonistic to this yeast. This implies that TC exposure might affect microbial community structure, not only through its direct bacteriostatic effect, but also indirectly by influencing microbial interactions among different populations.

This study investigated the phenotypic response of soil microbial communities exposed to selective pressure by tetracycline. Sustained exposure to TC resulted in a significant increase in the concentration of tetracycline-resistant soil bacteria, as well as a large decrease in the concentration of total heterotrophs. This suggests that TC release to the environment by animal agriculture is conducive to the development and amplification of antibiotic resistance, with soil bacteria serving as resistance reservoirs for Tet^r continuance. Nevertheless, removing the selective pressure by TC resulted in phenotypic shifts that returned the microbial community to initial conditions within one month, which implies that discontinuing TC exposure or curtailing its use should enhance natural attenuation mechanisms that mitigate the spread of resistance vectors.

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