

**ARDI Grant 04-633, Final Report**

Title: Environmental impacts of spreading hog manure on pasture:  
pathogens and antibiotic resistance in water

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## **Executive non-technical summary**

### Background

1. Over the course of the past 3 years research has been occurring at a cattle pasture located in the RM of La Broquerie, Manitoba.
2. The aim of the research was to address concerns expressed by the public and regulatory authorities regarding the use of hog manure as an alternative to commercial fertilizer. Although livestock manure is a more economically viable method to increase pasture productivity questions arise regarding the microbial effect of the manure on other close knit ecosystems such as grazing cattle, soil, and groundwater.

### Objectives

1. Determine whether *E. coli* found in hog manure were being transferred and surviving in the surrounding environment.
2. Determine whether the application of manure to land causes an increase in the survival of more virulent bacteria in the soil or grazing cattle
3. Develop a more robust method using entire microbial community analysis and antibiotic resistance characteristics of the bacteria to source track and more thoroughly identify microbial contamination originating from manure.

### Results

1. Antibiotic resistance (AR) profiles of *E. coli* found in hog manure, soil, and cattle feces were developed. Ideally, each *E. coli* isolate should have a unique AR profile, which is similar to

a fingerprint for bacteria. This was done to determine whether the *E. coli* in the manure were transferring to the cattle or the soil.

2. *E. coli* was not observed to transfer or survive in the soil or the grazing cattle. However, virulent bacteria, which are potentially pathogenic in nature, were seen to have increased survival in the soil and grazing cattle from manure treated plots.
3. Microbial community analysis was performed using molecular techniques, which allow for the identification of predominant bacteria in groundwater. The molecular technique used, T-RFLP, produces a bacterial fingerprint of the sample and ideally, if the groundwater fingerprint overtime begins to appear increasingly similar to the hog manure profile, groundwater contamination with hog manure organisms is likely occurring.
4. Over time, the groundwater fingerprint did not appear to shift to mimic that of hog manure, indicating that microbial contamination was not likely occurring but the fingerprint of the groundwater did none the less change over time.
5. Nutrient concentrations in groundwater were analyzed over time and compared against the changing groundwater profile to determine whether the nutrients in groundwater were having and effect on the changing groundwater community.
6. Results showed that nitrogen and chloride, which are known to be the most mobile nutrients in the soil, had the largest effect on the groundwater microbial community, and are potentially causing a shift in the microbial population.
7. Groundwater and hog manure microbes were then tested for resistance to 5 different antibiotics. The overlap region of antibiotic resistant microbes found to occur in both hog manure and groundwater was fairly low, ranging from 0 – 9% of the total communities.

8. In order to source track the bacteria from manure to groundwater, resistance profiles were also determined for groundwater and hog manure, and of the 91 antibiotic resistance profiles obtained, 60 profiles were found to be unique to groundwater.
9. This means that there is already naturally occurring resistance in the environment that may not be related to the use of antibiotics in swine production. Also, when comparing the resistance patterns obtained from hog manure and those obtained from groundwater, results showed that hog manure was not very similar to groundwater, indicating that very few organisms are being transferred from manure to groundwater.

### Conclusion

1. Application of manure to land does not appear to pose a serious risk with regards to the microbial contamination of underlying groundwater.
2. Significant transfer and survival of manure organisms in groundwater was not observed following application of hog manure, however there is still bacterial overlap region between the two communities.
3. In order to determine whether the microbes that are shown to occur in both groundwater and hog manure is of concern future studies will focus on species specific identification and quantification of the bacteria found within the overlap.

## Introduction

Application of manure to land as an alternative to commercial fertilizer has created concern among the public and regulatory authorities in recent decades which have led to increased scrutiny of livestock production systems. Concern primarily arises following major outbreaks that link animals and agricultural practices to the cause of human illness. The risks associated with spreading manure and the potential to contaminate raw produce have been clearly identified, however risks associated with the contamination of groundwater water in Canada as a result of manure application to land requires further study. Often, much attention is paid to the increased concentration of nutrients (nitrates and phosphates) reaching nearby waterways, however, more often than not the majority of human illness that has been linked to agriculture and contamination of waterways, is of microbial origin.

Over a three year period commencing in the spring of 2004 and ending September of 2006 extensive work has been done to determine the risks associated with spreading manure on agricultural land. Transfer of *Escherichia coli* from manure to cattle, from cattle through soil, and from manure to soil has been studied, and these *E. coli* strains have been analyzed for virulence. Culture independent techniques were used to monitor microbial community structure changes in groundwater, soil, and cattle feces as a result of manure application; this was sought to be a more comprehensive method to identify manure contamination of environmental samples. In addition we investigated the ability of antibiotic resistance determinants to be transferred from hog manure to the groundwater.

## **Materials and Methods**

### *Experimental site*

Establishment of the experimental site took place in the fall of 2003, and is located 13 km south of La Broquerie, Manitoba. The site was divided into 12 separate plots and consisted of six separate treatments that were arranged in duplicate. Treatments consisted of three manure application rates: full rate (110 lb available N ac<sup>-1</sup>), split rate (55 lbs available N ac<sup>-1</sup>), and control (no manure); and two forage management practices: cattle grazed and mechanically harvested. A total of 15 wells were drilled to gain access to underlying groundwater, and direction of groundwater flow was determined by Manitoba Water Stewardship to be from south to east/north-east. Wells were situated down-gradient from each plot, and an additional three wells were located on the outer west side of the experimental site to monitor the water quality coming into the experimental site from surrounding properties. Soil type was classified as 3M, which is characteristic of coarse soil texture and quick drainage. Replicate 1 is situated on the west side of the site and has an underlying clay soil fraction where as replicate 2, located on the east side does not contain a clay fraction.

### *Manure application*

Manure application began in the fall of 2003 where all split rate plots received 55 lbs available N ac<sup>-1</sup>. The second manure application followed in the spring of 2004 with full rate plots receiving 110 lbs available N ac<sup>-1</sup> and split rate plots again being treated as described above. Application

occurred as described above for the following three years (2004, 2005, and 2006). Swine manure samples were collected for each of the following dates and microbial analysis was performed.

Manure application schedule:

1. October 23, 2003 - Only split rate plot application.
2. May 10, 2004 (rep 1) – Split and full rate application completed.
3. May 25, 2004 (rep 2) – Split and full rate application completed.
4. October 24, 2004 – Only split rate application.
5. April 26, 2005- April 27, 2005 - Split and full rate application completed.
6. October 13, 2005 - Only split rate application.
7. April 25, 2006-April 27, 2006 - Split and full rate application completed.
8. October 3, 2006 - Only split rate application.

### *Sample collection*

Soil samples were collected and composited based by plot first in 2004 following manure application in the spring; in 2005 prior to manure application in the spring, one week following manure application and again one month following manure application; and in 2006 soil samples were collected on a monthly basis during the months of April-September. Samples were taken from depths of 30cm to 120cm.

Cattle fecal samples were collected once every 28 day sample period in June, July, and August of 2004; in May, June, and July of 2005; and again in May, June, and July of 2006 and samples were composited based on plot. Cattle fecal samples were taken prior to introducing the cattle onto the field and this corresponds to the first sample period listed above.

Groundwater samples were taken from all 15 samples wells in September of 2005 and again following fall manure application in November of 2005. Samples for 2006 were collected on a monthly basis beginning in April, prior to spring manure application, and ending in September. Well depth ranged from 2 to 4 meters and sample collection was done from 10 cm below the top of the screen when water levels were above the screen height and when the water table was below screen height, samples were taken from 10 cm below the water table.

#### *Antibiotic resistance patterns among Escherichia coli*

Fresh soil, cattle feces, and hog manure samples were collected during the 2004 and 2005 sample periods and stored at 4 °C prior to processing. Samples were enriched in 100 ml of buffered peptone water and streaked on Eosin methylene blue agar. *Escherichia coli* isolates were identified using standard methods. Antibiotic susceptibility testing was done using the standard Kirby-Bauer disk diffusion method, in which the zone of inhibition around the antibiotic disk indicates the extent to which a single *E.coli* isolate is resistant to the antibiotic in question. Six antibiotics were tested against multiple *E. coli* isolates originating from hog, soil and cattle samples. Antibiotics used include: Ampicillin, Ciprofloxacin, Streptomycin, Tetracyclin, Imipenem, and Erythromycin. The mean disk diffusion zone for *E.coli* isolates originating from

each sample type (hog manure, soil and cattle feces) was determined for each antibiotic used. Antibiotic resistance (AR) patterns were then created and fingerprints were assigned to each isolate based on their antibiotic resistance patterns. AR patterns are used as a source tracking method to identify whether the *E. coli* isolates from the manure are the same isolates that are transferred to, or surviving in the cattle or soil. Soil and cattle AR patterns were studied over time and by treatment.

#### *Genotypic diversity of Escherichia coli isolates*

*E. coli* is known to be the most common commensal bacteria in the digestive tract as well as being the most common human-acquired pathogen (Cermont et al. 2001). In order to determine the genotype of the strains of *E. coli*, along with the diversity of the *E. coli* strains that were isolated from soil, cattle feces and hog manure during the 2004/2005 sample periods, isolates were classified based on four phylogenetic groups (A, B1, B2 and D). Isolates that fall into groups A or B1 are typically classified as non-pathogenic, where as isolates that fall into groups B2 and D tend to be more virulent type. Classification of the isolates into the four groups was done using the polymerase chain reaction, a molecular technique, that allow for the identification of certain genes. Isolates possessing the *chuA* gene were designated as B2 or D and those without the gene were designated as A or B1. The *yiaA* gene was then used to differentiate between B2 and D genotype and the TSPE4.C2 DNA fragment was used to differentiate between the A and B1 genotype. Isolates designated to each phylogenetic group were then matched up with the sample type and treatment that they were originally sampled from to determine whether there was a difference in the quantity of virulent *E. coli* found in the split and full treatment plots.

### *Microbial ecology analysis*

Hog manure, soil, groundwater and cattle feces were processed from 2005 and 2006 samples periods. DNA was extracted from all samples and T-RFLP, a molecular technique, was used to determine the microbial fingerprint of each sample. A microbial fingerprint was obtained from each sample. This allowed for the identification of total changes in the microbial community structure of each sample type over time with the application of hog manure as well as over treatment. Clustan, a statistical software package was used to show how closely related each sample was with regards to their microbial profiles and samples were grouped based on their similarity.

Groundwater nutrient concentrations were obtained by Manitoba Water Stewardship and microbial fingerprints of the groundwater samples were analyzed in relation to nitrogen, phosphorus, and chloride concentrations found in the groundwater along with the time of the year that the samples were collected and the water levels at the time of sampling. This multivariate statistical analysis was used to evaluate any effect that nutrient concentrations in groundwater may be having on the microbial populations of groundwater samples. The same analysis will be performed on soil samples in the future but was not part of the current project.

### *Multiple antibiotic resistance profiling*

The basis of multiple antibiotic resistance profiling of microbial community samples is that microorganisms originating from different animal host sources will become exposed to the antibiotics used on their hosts. Once these bacteria become adapted to their environment they develop an antibiotic resistance fingerprint characteristic of their host environment. Hog manure and groundwater samples were resuscitated in buffered peptone water from the 2006 samples periods. Antibiotic resistant organisms were grown in three different media types: buffered peptone water (BPW); brain heart infusion (BHI) broth; or Luria-Bertani (LB) broth containing different combinations of antibiotics. Antibiotics and antibiotic combinations used in this study include: erythromycin, tetracycline, chlorotetracyclin, penicillin-G, a combination of erythromycin and tetracycline, and a combination of chlorotetracyclin, sulfamethazine, and penicillin-G. DNA was extracted from all samples (this includes all three media types containing all the varied combinations of antibiotics described above) and T-RFLP was again performed, to determine the antibiotic resistance fingerprint of the groundwater and hog manure bacterial communities. Clustan was used to determine how closely related each sample was with regards to their microbial profiles and samples were grouped based on their similarity.

## Results and Discussion

### *Antibiotic resistance patterns among Escherichia coli*

Mean disk diffusion zones of *E. coli* isolated from cattle feces remained relatively constant by treatment and over time for the majority of antibiotics. There was a significant increase in resistance to streptomycin over the three periods (Table 1). Mean disk diffusion zones of *E. coli* isolated from soil also remained fairly constant by treatment and over time (Table 2). The zone of inhibition around tetracycline was significantly reduced in the split and full rate plots as compared to the control, however according to the resistance breakpoint, isolates were still not considered resistant to tetracycline. Isolates retrieved from soil were consistently resistant to erythromycin over time and by treatment, even those taken from control plots. This indicates a natural resistance that is resident in soil. Resistance profiles indicate that *E. coli* from hog manure are not being transferred very often or surviving in the soil for more than 6 weeks to 3 months (Table 3).

Reliance on *E. coli* as a sole indicator for fecal contamination may lead to false negatives, and precludes the chances of detecting other pathogens. It is also important to note that microorganisms are capable of entering into a viable but non-culturable state and it is therefore important to utilize molecular techniques which allow for the identification of a wide range of bacteria without the need for culturing. By focusing on microbial community structure changes in groundwater, soil and cattle feces, a more thorough and possibly more reliable method to identify fecal contamination will be obtained.

### *Genotypic diversity of Escherichia coli isolates*

Although *E. coli* does not survive as a whole or transfer from swine manure to the soil or cattle, results suggest that B2 and D genotypes do increase as a percentage of the total (Table 4).

Control plots had a lower percentage of pathogenic genotypes than did the split and full treatment plots, suggesting the accumulation of pathogenic genotypes in soil and cattle following the application of swine manure. This makes sense because pathogenic bacteria tend to have a functional advantage under stressful conditions in comparison to commensal bacteria (Hacker & Carniel, 2001).

### *Microbial ecology analysis*

Microbial community structure changes in soil, groundwater, and cattle feces with the use of T-RFLP does allow for a more robust analysis of the overall microbial impact of applying hog manure to pasture. When microbial communities from all four ecosystems (hog manure, cattle feces, soil and groundwater) were analyzed for similarity between communities, hog manure consistently clustered separately from groundwater, soil and cattle feces based on a 95% difference in microbial community profiles (Figure 2).

Groundwater, soil and cattle feces clustered somewhat separately but were much more similar with in comparison to their microbial fingerprints than that of hog manure. In other words, the difference between groundwater, soil, and cattle was much less than hog manure. The

ecosystems of groundwater, soil and cattle are much more closely knit than hog manure, which could be the reason for the similarity in microbial profiles. No time or treatment effect was observed in the soil or cattle feces indicating stability of these ecosystems. There was however a time effect with the groundwater, which appears to result from the application of hog manure. The microbial profiles of groundwater change over time, however the groundwater community structure did not become more similar to to hog manure (Figure 4). This means that very few microorganisms from hog manure get into the hog manure, and the changes in groundwater microbial community structure must be because of some other reason.

Multivariate analysis of nutrient concentrations (nitrate, phosphorus, and chloride) in groundwater, along with the time of the year that the sample was taken, and the groundwater level at the time of sampling was done to determine the effects of these various parameters on the changing microbial profiles of groundwater. Results indicate that nitrate had the largest effect on the microbial community structure of groundwater, followed by chloride (Figure 5). Nitrate and chloride were the most influential mainly due to their increased mobility through the soil. All other parameters measured were not as influential. The same nutrient multivariate analysis will be completed on the microbial profiles of soil in the future. Soil analysis was not part of the current funded project.

Phylogenetic analysis was done in order to identify the bacteria found within the soil, hog manure, cattle feces and groundwater. This was done to identify specific bacterial similarities between all four communities, and to more closely monitor the bacterial changes that occur in the groundwater. Figure 6 shows the microbial profiles of soil, cattle feces, and hog manure.

Figure 7 shows the changing microbial fingerprint of groundwater over time. Results indicate that Cyanobacteria, which are responsible for assimilatory nitrate reduction, are increasing with each manure application. Firmicutes and Proteobacteria, are the most predominant phyla found in groundwater, and Proteobacteria seem to increase slightly in groundwater with each manure application. Within the phylum Proteobacteria, bacteria such as *Salmonella* and *Escherichia coli* are found, however it is yet to be determined whether hog manure application promotes survival of these specific bacteria in groundwater.

#### *Multiple antibiotic resistance profiling*

Resistance was observed against all five antibiotics tested in both hog manure and groundwater. In total, 303 different terminal restriction fragments (T-RF), which are indicative of unique bacterial species, were found in groundwater and hog manure and the identity of the T-RFs that were found in both hog manure and groundwater are indicated (Table 6). The overlap region of antibiotic resistant microbes found to occur in both hog manure and groundwater was fairly low, ranging from 0 – 9% depending on the antibiotic enrichment pattern and the type of media used (Figure 7).

A total of 91 multiple antibiotic resistance (MAR) profiles were found (Table 7). Of the 91 MAR profiles observed, 60 were found to be unique to groundwater, indicating that there is already naturally occurring resistance in the environment that probable are not be related to the use of antibiotics in swine production. As the number of antibiotic enrichments increased, the number of unique T-RFs per sample decreased (Table 5). A higher number of species found in

groundwater were resistant to sulfamethazine alone in comparison to species found in hog manure, and the number of unique species found to be resistant to only erythromycin was higher in hog manure than in groundwater. Multivariate analysis was used to compare the similarity of hog manure MAR profiles to groundwater MAR profiles (Figure 8). Hog manure profiles did not cluster closely with the groundwater profiles. This indicates that few organisms are being transferred from hog manure to groundwater.

### **Conclusions and Implications**

At this point, application of manure to land does not appear to pose a serious risk with regards to the microbial contamination of underlying groundwater. This includes transfer and survival of the general microbial community of hog manure, as well as pathogenic and antibiotic resistant bacteria. However, further research is warranted in this area due to the possible shortcomings of the methods used to monitor the transfer of microorganisms from manure to soil and to underlying groundwater. Although significant microbial community shifts in groundwater were not observed following application of hog manure, there are still bacterial overlap region between the two communities. In order to determine whether this overlap region is of concern future studies will focus on species specific identification and quantification of the bacteria found within the overlap.

## **Financial reporting**

Final financial reports will be provided by Budgets and Grants. Contact is:

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## **Training of students, technicians, and post-doctoral scientists**

This project has been successful in training a number of individuals who will be able to apply their knowledge to problems in sustainable agriculture in Canada and other places in the world.

1. Dr. Bu Dengpan – He did a lot of the initial work on the antibiotic resistance of the *E. coli* isolates obtained from the site. Dr. Dengpan returned to China after his work at the U of M and heads a program in gut and environmental microbiology at the Chinese Academy of Agricultural Sciences. There are ongoing collaborations with Dr. Dengpan.
2. Ms. Stephanie Cheng – She was a technician who developed the antibiotic profiling molecular profiling technology to assess the overlap between the hog manure and groundwater. After one year on the project Stephanie elected to continue her studies in dentistry at the University of British Columbia.

3. Ms. Ainsley Little – Ainsley initially started working on this project as a summer student and then as a masters student. She has recently transferred to a PhD program and will continue working on issues related to the above project but funded from other grants.
4. Summer students – There have been a large number of summer student who have worked on various aspects of this project during the last three years.

## **Publications**

### *Conference proceedings*

1. Cheng, Stephanie, Rick Holley, Kim Ominski, Ainsley Little, and Denis O. Krause. 2007. "Community analysis of antibiotic resistant microbes in hog manure and groundwater." Canadian Nutrition Congress. Winnipeg, June 18 – 21.
2. Little, A.C., K.H. Ominski, K.M. Wittenberg, and D.O. Krause. 2006. "Phylogenetic analysis of environmental samples in a hog manure amended site." Proceeding of the 27th Western Nutrition Conference. Winnipeg, MB, September 19-20:251.

### *Peer reviewed publications*

There are a number of peer reviewed publication been prepared as part of Ainsley Little's PhD thesis.

### *Websites*

The information on this project is publically accessible at the following websites:

1. The National Centre for Livestock and the Environment.  
<http://www.umanitoba.ca/afs/ncle/index.html>
2. La Broquerie Research Project. <http://www.umanitoba.ca/afs/labroquerie/>
3. Gut Microbiology website. [www.gutmicro.com](http://www.gutmicro.com)

## **Extension**

A large number of extension activities have been carried out in relation to this project:

### *Radio Interviews*

1. 2007. *Swine Manure Unlikely as Major Contributor to Antibiotic Resistance*, Farmscape, Bruce Cochran (July 26) Episode 2548
2. 2007. *Research Identifies Environmentally Sustainable Management Practices to Improve Pasture Productivity*, Farmscape, Bruce Cochran (July 28) Episode 2550
3. 2004 *Public perception expected to force antibiotics out of swine diets*, Farmscape, Bruce Cochran (April 22) Episode 1499
4. 2004 *Several Compounds Show Potential for Replacing Subtherapeutic Antibiotics*, Farmscape, Bruce Cochran (April 23) Episode 1500

*Government, producers, visitor talks and presentations*

1. Government talk (2005) Best-management practices in livestock production to reduce pathogen and antibiotic resistance. Talk to Manitoba Rural Adaptation Council. Hytek field site, La Broquerie, MB (26<sup>th</sup> July 2005). ~ 50 participants
  
2. Producer talk (2005) Reduction of pathogens and antibiotic resistance in manure amended pastures. Talk to Manitoba Pork Council and producers. Hytek field site, La Broquerie, MB (11<sup>th</sup> August 2005). ~ 50 participants
  
3. Eastman Provincial Pasture Tour (2007). Speaker: July 25 and 26, Environmental impact of spreading hog manure on pasture,
  
4. Manitoba Forage Council La Broquerie Workshop (2007). Organizer and presenter, Presentation and organizing material on the role of zoonotic pathogens in soil and groundwater. This was a wrap up for industry and all other interested parties on the La Broquerie I project.
  
5. Living with Livestock Conference (2007). Organizing committee, Organized program and site visits, Living with Livestock Conference, 25-26 June 2007. The La Broquerie site was visited and information on the current project was presented

## **Public policy**

The research conducted in this project was used as input to the report to the Manitoba Clean Environment Commission on the Hog Industry.

Weblink: [http://www.umanitoba.ca/afs/pdf/CEC\\_Final\\_Report\\_UM\\_October\\_23\\_2007.pdf](http://www.umanitoba.ca/afs/pdf/CEC_Final_Report_UM_October_23_2007.pdf)

Table 1. Mean disk diffusion zones and resistance breakpoints for *E. coli* isolated from cattle fecal samples

Antibiotic agents	Breakpoint <sup>1</sup> (mm)	Disk diffusion zones for treatment (mm)			Disk difusion zones for period (mm)		
		Control	Full	Split	Period 1	Period 2	Period 3
Ampicillin AM-10	≤13	16.5	16.5	16.6	15.99	16.59	17.03*
Ciprofloxacin CIP-5	≤15	29.4	29.0	28.5	27.72	30.90	28.34*
Streptomycin S-10	≤11	12.1	10.1	11.7	12.27	11.16	10.45*
Tetracycline TE-30	≤14	16.4	15.6	16.8	16.06	16.07	16.65
Imipenem IPM-10	≤13	24.9	29.3	24.3	23.79	25.08	24.14
Erythromycin E-15	≤13	11.7	11.6	11.5	10.19	12.57	11.95*

<sup>1</sup>Point at which isolates are considered to be resistant to specified antibiotic

\*Treatment/period means within specific rows show significant difference ( $p < 0.05$ )

Antibiotic agents	Breakpoint <sup>1</sup> (mm)	Disk diffusion zones for treatment (mm)			Disk diffusion zones for period (mm)		
		Control	Full	Split	Period 1	Period 2	Period 3
Ampicillin AM-10	≤13	14.7	13.6	12.9	12.74	15.38	13.00*
Ciprofloxacin CIP-5	≤15	28.7	28.6	28.7	28.20	29.16	28.69
Streptomycin S-10	≤11	12.9	11.9	12.5	12.64	12.14	12.49
Tetracycline TE-30	≤14	19.0	16.5	16.6*	17.81	17.48	16.85
Imipenem IPM-10	≤13	21.9	19.5	20.9*	20.28	21.07	21.04
Erythromycin E-15	≤13	10.1	10.2	9.6	10.09	9.90	9.89

<sup>1</sup>Point at which isolates are considered to be resistant to specified antibiotic

\*Treatment/period means within specific rows show significant difference ( $p < 0.05$ )

Table 3. Mean disk diffusion zones and resistance breakpoints for <i>E. coli</i> isolates				
Antibiotic agents	Breakpoint <sup>1</sup> (mm)	Disk diffusion zones (mm)		
		Cattle fecal (n=186)	Hog manure (n=44)	Soil (n=84)
Ampicillin AM-10	≤13	16.2 <sup>a</sup>	9.1 <sup>b</sup>	13.5 <sup>c</sup>
Ciprofloxacin CIP-5	≤15	28.7 <sup>a</sup>	32.2 <sup>b</sup>	28.6 <sup>a</sup>
Streptomycin S-10	≤11	11.5 <sup>a</sup>	12 <sup>a</sup>	12.5 <sup>a</sup>
Tetracycline TE-30	≤14	16.5 <sup>a</sup>	6.1 <sup>b</sup>	17.4 <sup>a</sup>
Imipenem IPM-10	≤13	24.2 <sup>a</sup>	26.4 <sup>b</sup>	20.9 <sup>c</sup>
Erythromycin E-15	≤13	11.4 <sup>a</sup>	12.2 <sup>ab</sup>	9.9 <sup>c</sup>

<sup>1</sup>Point at which isolates are considered to be resistant to specified antibiotic

<sup>a-c</sup>Different subscripts represent significantly different values (p<0.05)

Source (Total No. of isolates)	Phylogenetic group	No. of isolates per group <sup>a</sup>	% of total isolates per group <sup>b</sup>	% of isolates per group and treatment <sup>c</sup>		
				Control	Full	Split
Fecal (243)	A	11	4.5	27.3	63.6	9.1
	B1	64	26.3	35.9	32.8	31.3
	B2	116	47.7	24.1	39.7	36.2
	D	52	21.5	26.9	46.2	26.9
Hog Manure (71)	A	13	18.3	n/a	n/a	n/a
	B1	14	19.7	n/a	n/a	n/a
	B2	35	49.9	n/a	n/a	n/a
	D	9	12.7	n/a	n/a	n/a
Soil (164)	A	36	22	63.9	8.3	27.8
	B1	43	26.2	27.9	20.9	51.2
	B2	18	11	27.8	38.9	33.3
	D	67	40.8	31.3	44.8	23.9

<sup>a</sup> Number of isolates belonging to each phylogenetic group within a source  
<sup>b</sup> Percentage of the total number of isolates belonging to each phylogenetic group within a source  
<sup>c</sup> Percentage of isolates within each phylogenetic group detected in each treatment application  
 n/a Not applicable

Table 5. Richness and diversity indices for T-RFs detected in swine manure, ground water and both in ground water and swine manure in eight antibiotic enrichments.

Antibiotic enrichment <sup>a</sup>	No AB	E	T	ET	C	S	P	CSP
Index	Swine manure							
Richness								
ICE Mean	432	330	128	30	438	274	216	77
Chao 2 Mean	151 <sup>b</sup>	126	71	15 <sup>b</sup>	148	102	81	41
MM Mean	142	108	91	25	33	20	89	35
Diversity								
Shannon Mean	3	3	3	2	3	3	3	2
Simpson Mean	431 <sup>c</sup>	352 <sup>c</sup>	176 <sup>c</sup>	30 <sup>c</sup>	595 <sup>c</sup>	378 <sup>c</sup>	325 <sup>c</sup>	19 <sup>c</sup>
	Ground water							
Richness								
ICE Mean	169.6 <sup>d, e</sup>	181.1	126.6	89.5 <sup>d</sup>	141.7	166.9	126.8	102.1 <sup>e</sup>
Chao 2 Mean	139.1 <sup>f, g</sup>	138.4	105.5	77.3 <sup>g</sup>	117.7	143.3	109.3	83.3 <sup>f</sup>
MM Mean	243.6	278.1	246.2	107.4	373.6	144.9	161.9	116.4
Diversity								
Shannon Mean	4	4	3.5	3.5	3.5	4	3.8	3.6
Simpson Mean	99.9	107.7	66.5	53.4	59.2	80.4	72.5	57.1

**a.** E, erythromycin; T, Tetracycline; ET, combination of erythromycin and tetracycline; C, chlortetracycline; G; No AB, no antibiotic added. S, sulfamethazine; P, penicillin-G; CSP, combination of chlortetracycline and sulfamethazine and penicillin-

**b-g.** Values with a common superscript indicate antibiotic enrichments that are significantly different than the no antibiotic enrichment ( $P > 0.05$ ).

Table 6. Phylogenetic analysis of terminal restriction fragments (T-RFs) found both in swine manure and ground water plot samples from chlortetracycline, sulfamethazine and penicillin-G (CSP) antibiotic enrichments and erythromycin and tetracycline (ET) antibiotic enrichments.

T-RF(bp) <sup>a</sup>	Medium <sup>b</sup>	Plot <sup>c</sup>	Total accession numbers <sup>d</sup>	Identity <sup>e</sup>
CSP Enrichment				
53	LB	6	0	Unmatched
	BPW	7, 8		
84	BHI	8	58	phylum <a href="#">Proteobacteria</a> (83%) class <a href="#">Alphaproteobacteria</a> (78%)
212	BPW	7	480	phylum <a href="#">Proteobacteria</a> (81%) class <a href="#">Gammaproteobacteria</a> (66%)
217	BHI	10	258	phylum <a href="#">Proteobacteria</a> (57%) class <a href="#">Gammaproteobacteria</a> (53%)
574	LB	1, 3, 6, 7, 8, 9, 11, 12	1011	phylum <a href="#">Firmicutes</a> (46%) class <a href="#">Bacilli</a> (34%)
ET Enrichment				
576	BHI	1, 3, 4, 8, 9, 12	1047	phylum <a href="#">Firmicutes</a> (66%) class <a href="#">Bacilli</a> (55%)
	LB	1, 3, 5, 6, 7, 9		
577	BPW	3, 4, 6, 8, 9, 11,12	943	phylum <a href="#">Firmicutes</a> (73%) class <a href="#">Bacilli</a> (61%)
	LB	1, 7, 9, 11, 12		
579	BPW	1, 4, 8, 9, 12	776	phylum <a href="#">Firmicutes</a> (88%) class <a href="#">Bacilli</a> (75%)
580	BHI	3, 6, 8	789	phylum <a href="#">Firmicutes</a> (88%) class <a href="#">Bacilli</a> (74%)

a. Size of detected T-RF in base pairs (bp).

b. Medium from which T-RF was detected. BPW, buffered peptone water; BHI, brain heart infusion broth; and LB, Luria-Bertani broth.

c. Groundwater plot from which T-RF was detected. See Fig. 1 for plot description.

d. Total number of accession numbers that matched to experimental T-RF size within constructed MiCA database.

e. Most probable identity of T-RF. Numbers in parenthesis represent the percentage of accession numbers that fell within that phylum or class of bacteria.



Table 7. continued												
E-T-C-CSP	0.9	0	0	0	0	0	0	0	0	0	0	0
E-T-ET	0.9	0	0	0	0	0	0	0	0	2.4	0	0
E-P-CSP	0.9	0	0	0	0	0	0	0	0	0	0	0
T-C-S	0.9	1.3	0	0	0	0	0	2.3	0	0	0	0
ET-C-CSP	0.9	0	0	1.4	0	0	0	0	1.5	0	0	0
E-ET	0.9	0	0	1.4	0	0	0	0	0	0	2.5	0
T-C	0.9	0	0	0	0	0	0	0	4.5 <sup>b</sup>	2.4	0	0
T-S	0.9	0	0	0	1.4	0	0	0	0	0	0	0
ET-CSP	0.9	0	1.6	1.4	0	2.6	0	0	0	0	0	0
S-CSP	0.9	3.8 <sup>b</sup>	0	4.1 <sup>b</sup>	0	0	0	0	0	2.4	2.5	0
P-CSP	0.9	2.6	1.6	1.4	0	2.6	0	0	3.0 <sup>b</sup>	0	0	10.0 <sup>b</sup>
ET-C-S-P	0.9	1.3	0	1.4	0	2.6	0	0	0	0	2.5	0
C-CSP	0.9	0	0	1.4	0	2.6	2.3	0	1.5	0	0	0
E-T-P-CSP	0.9	0	0	0	1.4	0	0	0	0	0	0	0
Other	0	15.4 <sup>b</sup>	15.4 <sup>b</sup>	25.6 <sup>b</sup>	22.8 <sup>b</sup>	32.4 <sup>b</sup>	22.8 <sup>b</sup>	14.7 <sup>b</sup>	18.9 <sup>b</sup>	18.1 <sup>b</sup>	12.9 <sup>b</sup>	14.3 <sup>b</sup>

**a.** E, erythromycin; T, tetracycline; ET, combination of erythromycin and tetracycline; C, chlortetracycline; S, sulfamethazine; P, penicillin-G; CSP, combination of chlortetracycline and sulfamethazine and penicillin-G. The resistance patterns under the heading of other, consisted of 60 patterns not detected.

**b.** Ground water plot MAR pattern percentages that are significantly different from swine manure MAR pattern percentages ( $p < 0.05$ ).

**Figure 1.** Hierarchical cluster of 2005 and 2006 soil, groundwater, cattle feces and hog manure. First letter in sample label denotes sample type (S = soil, W = groundwater, C = cattle feces, and H = hog manure). T1 – T9 denotes chronological order of sample times throughout the two year sample period. Plot treatment follows: C = control plot, F = full rate plot, H = split rate plot, BG = background plot. Hay and Grazed denote the forage management practice used.

**Figure 2.** Hierarchical clusters of 2005 and 2006 groundwater and hog manure samples clustered based on average linkage, squared Euclidean distance method.

**Figure 3.** Hierarchical clusters of 2005 and 2006 groundwater samples clustered based on average linkage, squared Euclidean distance method.

**Figure 4.** Ordination biplot of groundwater T-RF microbial profiles and geochemical characteristics of groundwater obtained by detrended canonical correspondence analysis (DCCA). Samples taken at different time periods are represented by different symbols. Manure treated plots (split, and full rate) are represented by shaded symbols and non-manured plots are represented by non-shaded symbols. Increased distance between samples indicates an increased dissimilarity between microbial profiles and arrow length indicates the relative impact of that parameter on microbial profiles as a whole. Distance between the arrow head and groundwater sample indicates the influence of that parameter on the microbial profile of the sample. Arrows in opposite directions indicate a negative correlation, and arrows at 90° to one another indicates no correlation.

**Figure 5.** Microbial composition of soil per treatment at five separate time periods during 2005 and 2006 at the phylum level.

**Figure 6.** Microbial composition of groundwater at five separate time periods during 2005 and 2006 at the phylum level.

**Figure 7.** Distribution of terminal restriction fragments (T-RFs) from swine manure and ground water samples cultured in three different media: buffered peptone water (A), brain heart infusion (B) broth and Luria-Burtani (C). Samples were cultured under eight antibiotic treatments, denoted as E, erythromycin; T, tetracycline; ET, combination of erythromycin and tetracycline; C, chlortetracycline; S, sulfamethazine; P, penicillin-G; CSP, combination of chlortetracycline and sulfamethazine and penicillin-G; No AB, no antibiotic added. Similar symbols (▲, • or +) indicate where one common T-RF is detected among that region of T-RFs found in both Ground Water and Swine Manure.

**Figure 8.** Multivariate analysis of MAR incidence profiles to assess the degree of similarity between swine manure and ground water samples.

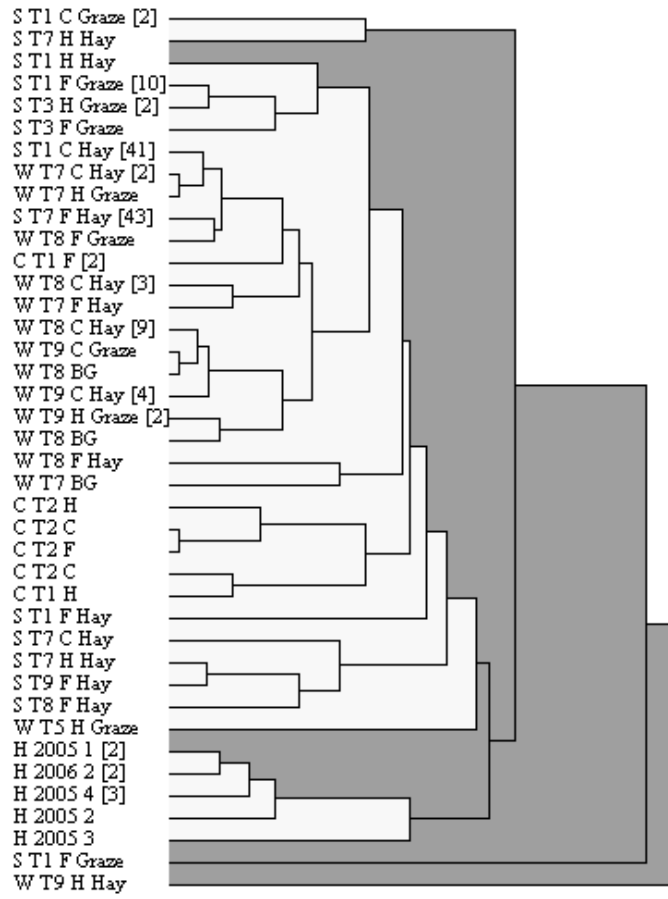


Figure 1

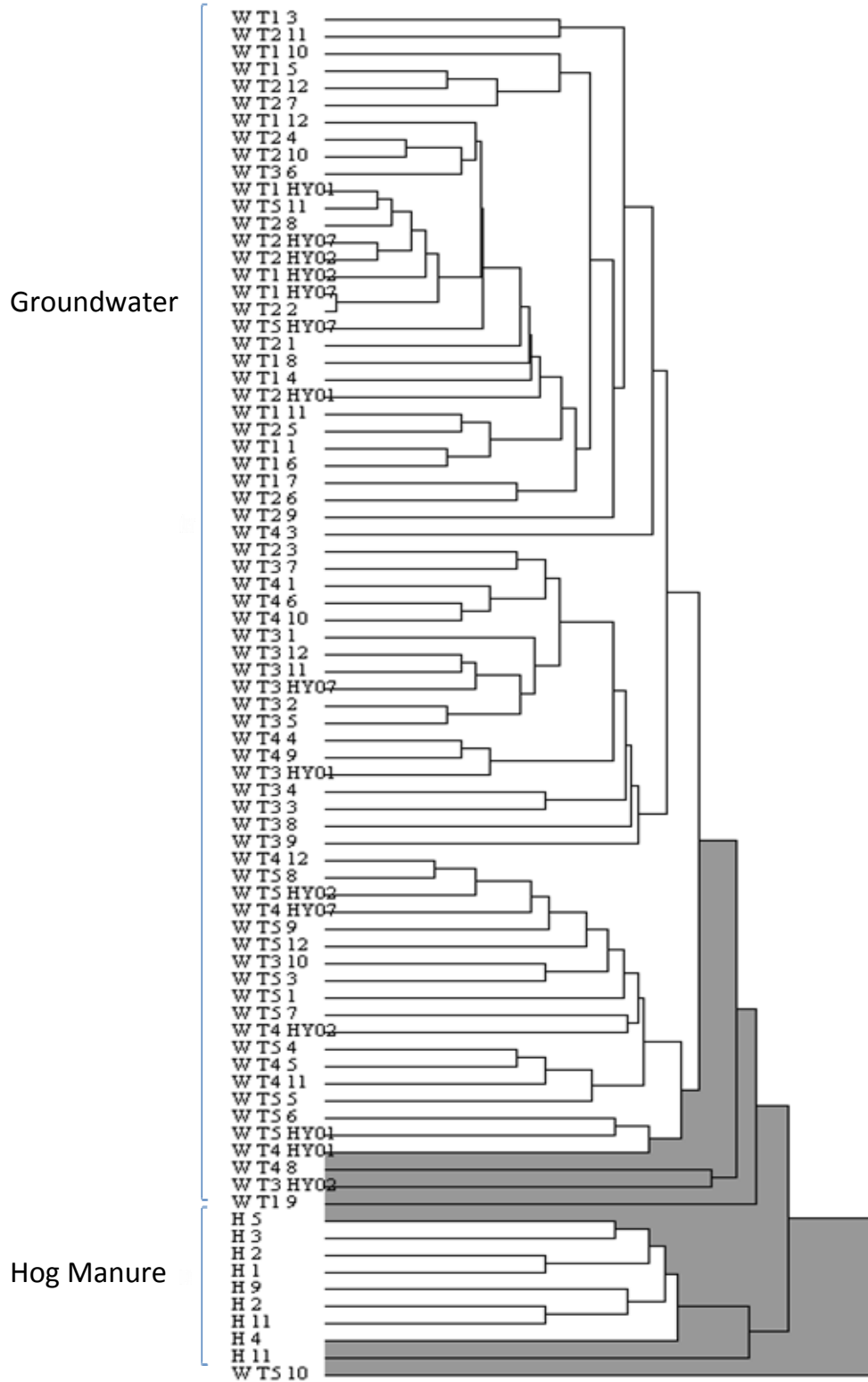


Figure 2

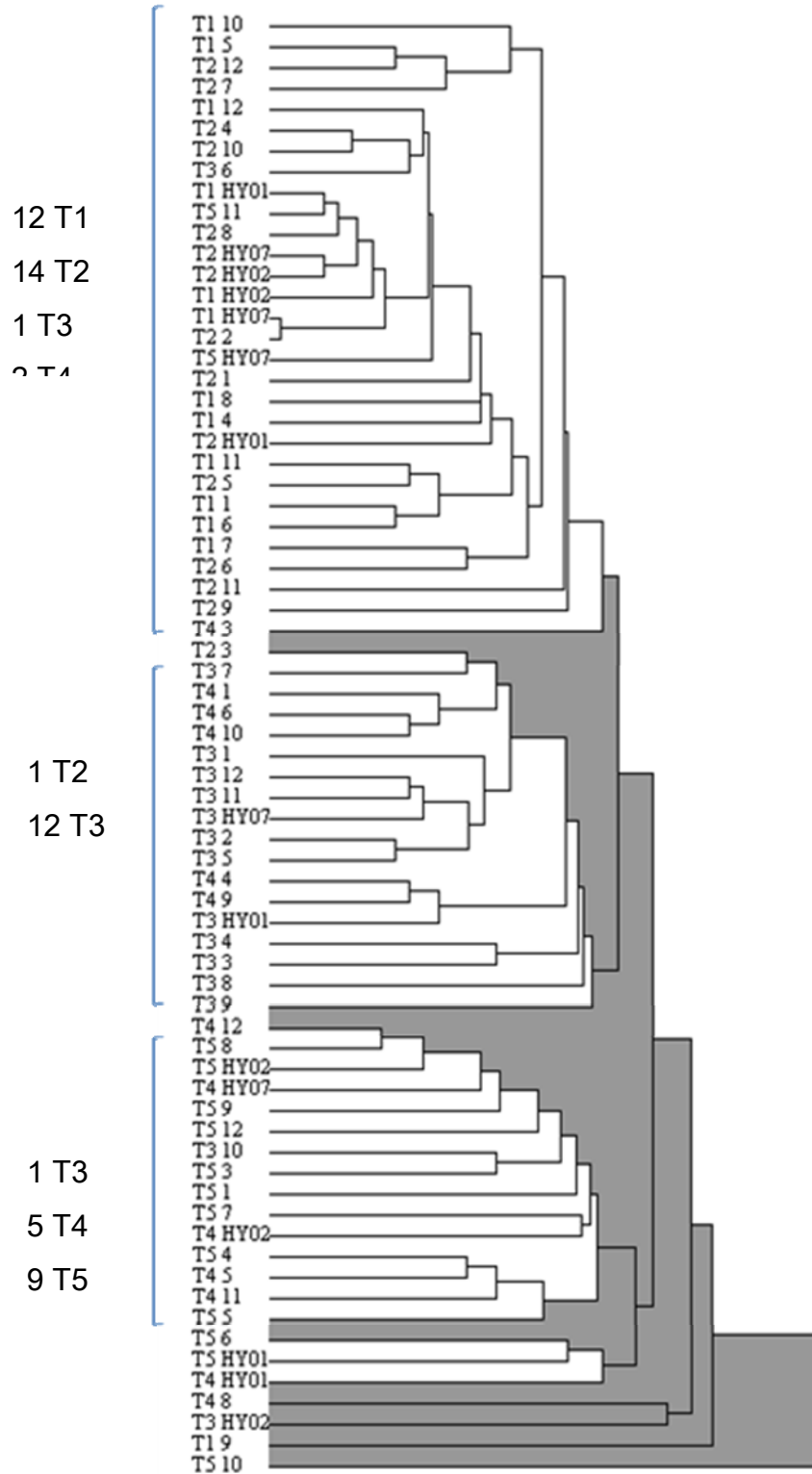


Figure 3

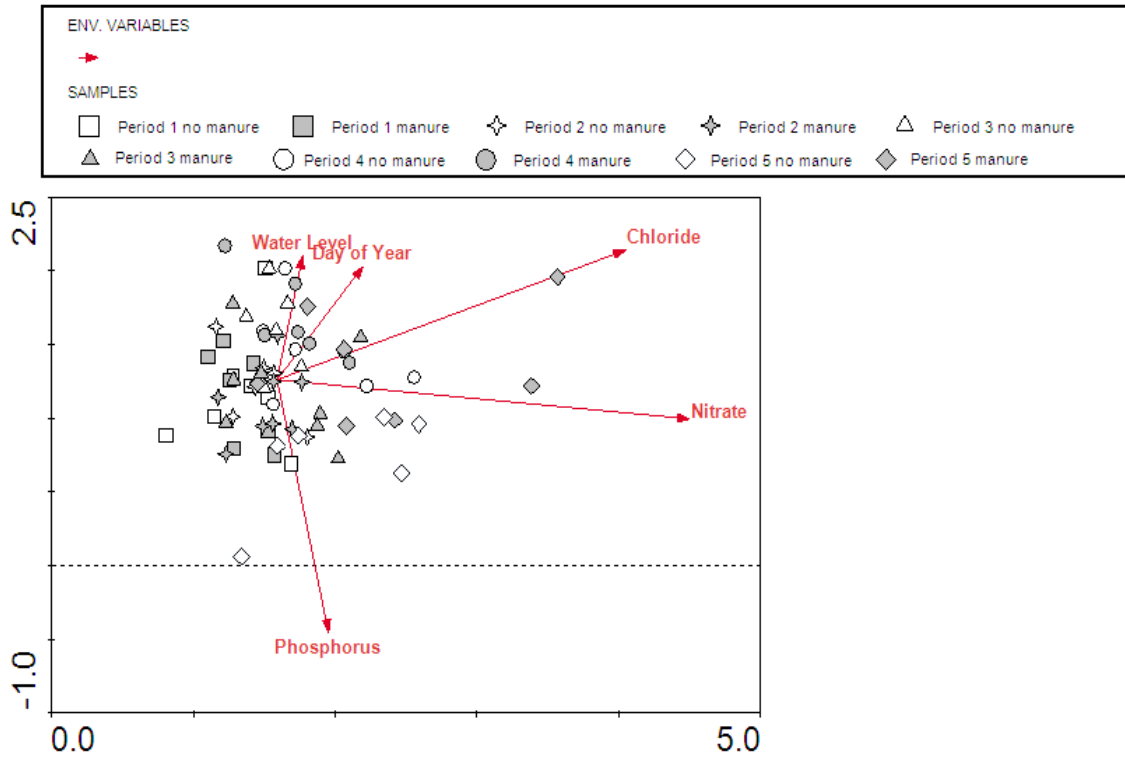


Figure 4

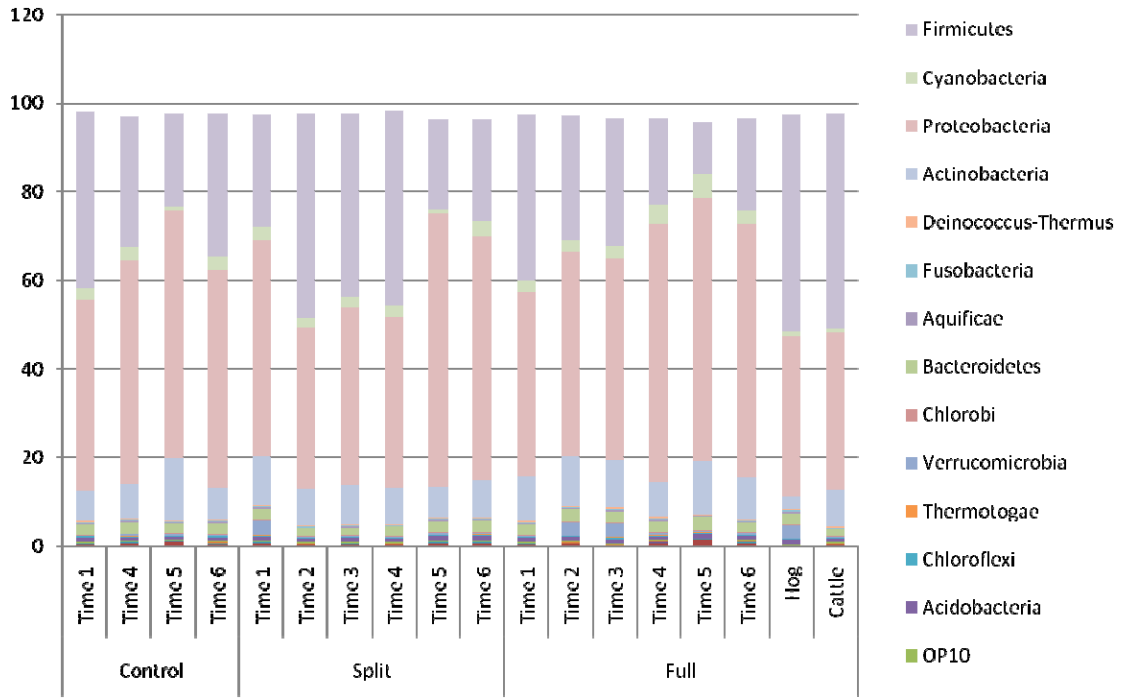


Figure 5

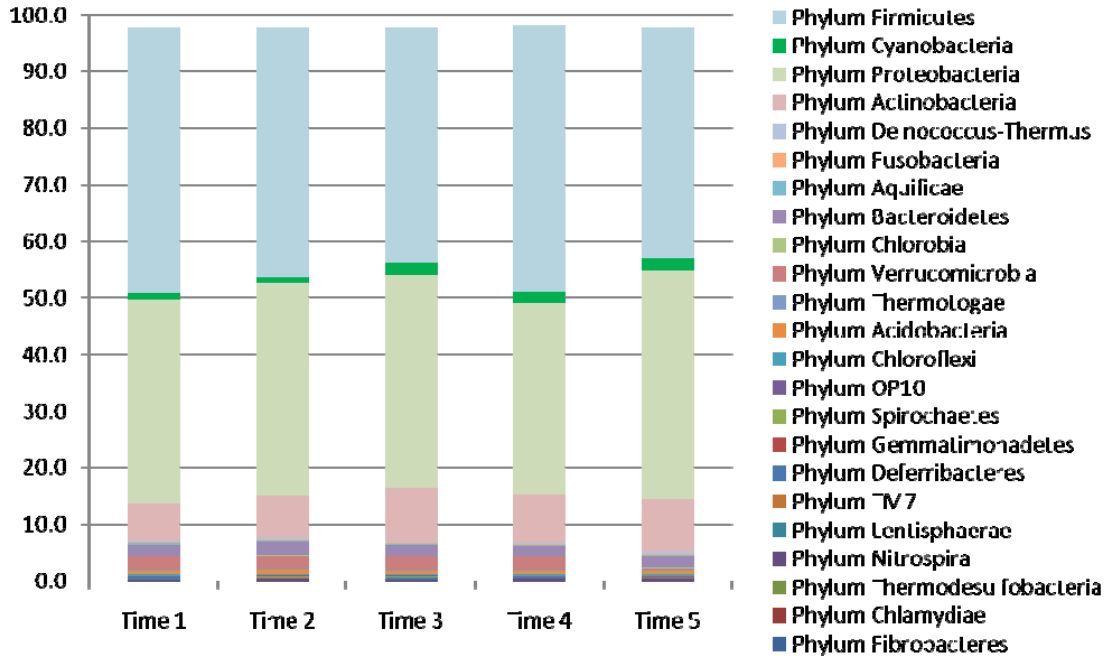


Figure 6

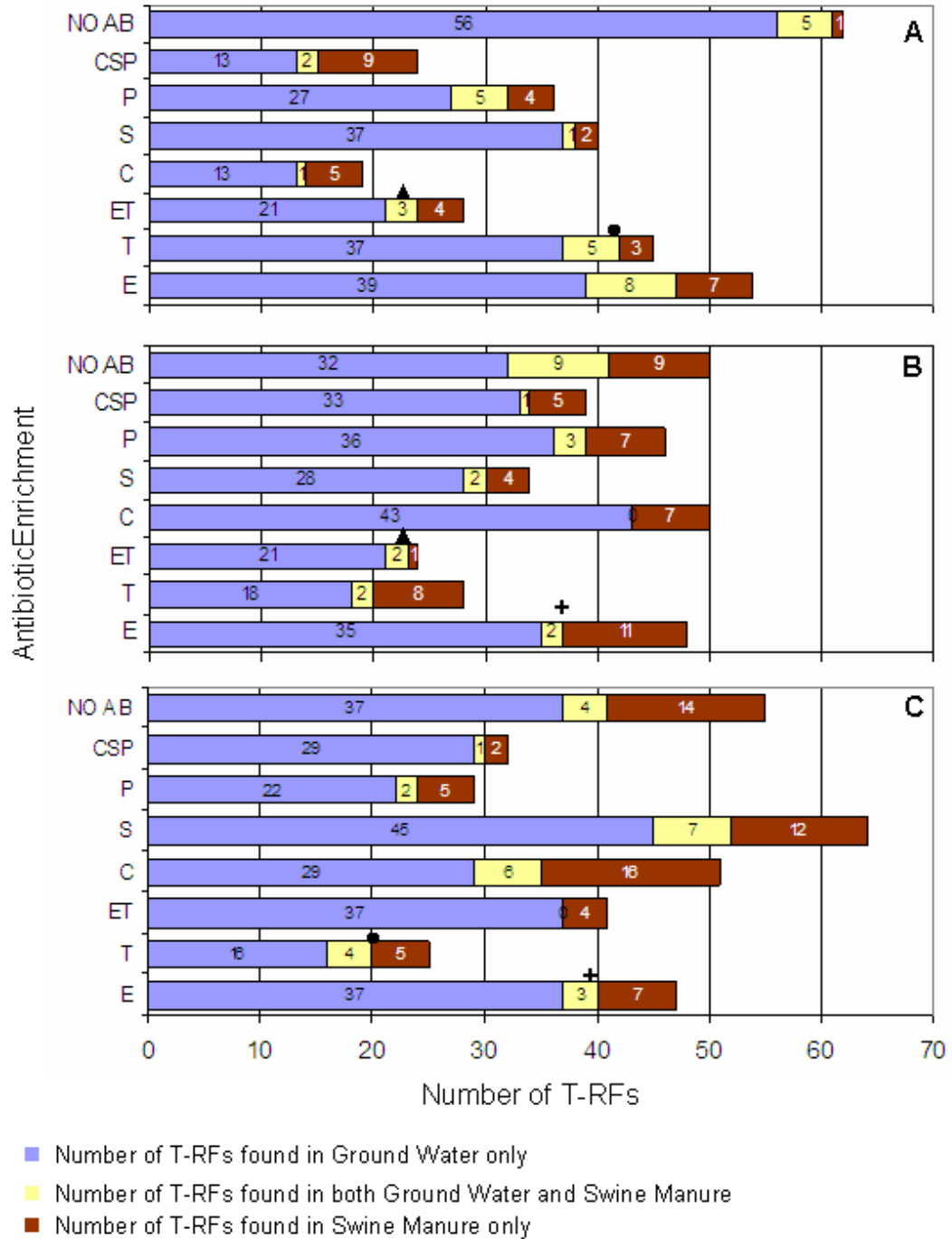


Figure 7