RESEARCHREPORT
ENVIRONMENT

Title: Special Call for Review of the Critical Literature: Fate and Transport of Antibiotic Residues and Antibiotic Resistance Genetic Determinants during Manure Storage, Treatment, and Land Application with emphasis on the Environmental Persistence and Transferability of these Determinants - **NPB# 04-161**

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GLOSSARY

Agricultural wastes are by-products of production systems that are not fully utilized within the confines of the production unit and burden the natural environment. However, wastes generated by agricultural production are generally natural by-products which can be recycled in the natural environment. Wastes are resources out of place and inherent by-products of agricultural production.

The study of **agricultural waste management** includes: waste generation rates and properties, their collection, storage, treatment and processing, and the utilization and final disposition of all wastes associated with agricultural production.

Livestock waste is a term sometimes applied to manure that may also contain bedding, spilled feed, water, or soil. It also includes wastes not particularly associated with manure, such as milking center or washing wastes, and milk, hair, feathers, chemicals or other debris.

Solid manure is a combination of feces, urine, and bedding with little or no extra water added. It is usually found in loafing barns, swine hoop houses, calving pens, and open lots with good drainage.

Semi-solid manure has little bedding and usually no extra water added. In most cases, little drying occurs before handling. During wet weather the manure scraped from open lots can also be semi-solid in nature.

Liquid manure is usually less than 8.0% solid material. Wash water, runoff, precipitation, and so forth dilute the manure and lower the solids content. Often sufficient water is added to semisolid manure to facilitate its agitation and removal. If placed in an anaerobic treatment lagoon, a significant amount of dilution water is added to raw manure, in order to control odors and enhance microbial breakdown of manure components.

Effluent is the liquid discharge from a waste treatment process.

Antibiotics are low molecular weight antimicrobial compounds that are effective at low concentrations in inhibiting a cellular target essential for bacterial growth and survival.

Antibiotic resistance is resistance to a given antibiotic. It is said to be intrinsic (*constitutive*) if cells which lack the target site and/or which are impermeable to the antibiotic. Cells may *acquire* resistance by mutation or by gene transfer mechanisms such as conjugation, transduction or transformation.

Broad-spectrum antibiotics are antibiotics active against many different species of bacteria.

Cell wall (bacterial) is the peptidoglycan layer covering the cytoplasmic membrane that gives the bacterium its shape and maintains its structural integrity.

Conjugation is the transfer of DNA mediated by conjugal plasmids or conjugal transposons; requires cell to cell contact but can occur between distantly related bacteria or even bacteria and eukaryotic cells; can transfer long fragments of DNA.

Fitness is the ability of a bacterium to grow under conditions it is experiencing.

Gene is a DNA sequence that encodes a single protein; promoter plus open reading frame.

Gene transfer element is a DNA segment (e.g. plasmid, conjugative transposon) that can transfer from one bacterium to another.

Genome is the entire complement of DNA possessed by a cell. In the case of bacteria it includes both the chromosome and any plasmids present in the strain.

Genus (bacterial) is a group of species that are closely related to each other. The genus name is the first name e.g. *Escherichia coli – Escherichia* is the genus name.

Gram-negative bacteria stain red with the Gram stain procedure and have a thin peptidoglycan layer and an outer membrane.

Gram-positive bacteria stain blue with the Gram stain procedure and have a thick peptidoglycan layer and no outer membrane.

Horizontal gene transfer (also **Lateral gene transfer)** is defined to be the movement of genetic material (i.e. DNA) between bacteria other than by *vertical transfer* or descent in which information travels through the generations as the cell divides. Horizontal gene transfer is common among bacteria, even very distantly related ones. This process is thought to be a significant cause of increased drug resistance; when one bacterial cell acquires resistance, it can quickly transfer the resistance genes to many species. There are three common mechanisms for horizontal gene transfer: conjugation, transduction and transformation.

Host range is a term used to denote the range of bacteria to which a segment of DNA (e.g. plasmid) can be transferred.

Integron is a segment of DNA that contains an antibiotic resistance gene and integrates in a specific site on a plasmid; more than one integron can integrate end-to-end to create a plasmid with multiple antibiotic resistance genes.

Minimum Inhibitory Concentration (MIC) is the lowest concentration of a given antibiotic that inhibits a given type of bacteria under given conditions.
Mutation is a change in DNA sequence that can create or destroy a promoter or when it occurs within an open reading frame can change the amino acid sequence of the protein encoded by the open reading frame or prematurely terminate the protein.

Open Reading Frame (ORF) is a DNA sequence that encodes a single protein.

Plasmid is a covalently closed, double-stranded DNA circle that can replicate autonomously.

Promoter is a DNA sequence upstream of an open reading frame that binds RNA polymerase and signals it to transcribe the gene.

R Plasmid is a plasmid containing one or several transposons with resistance genes.

Phylogeny is the study of evolutionary relatedness among organisms as they change through time. It includes the discovery of these relationships, and the study of the causes behind this pattern.

Phylogenetic tree (also **Dendrogram** or **Cladogram**) is a diagram, resulting from a phylogenetic analysis, which depicts a hypothetical branching sequence of lineages leading to the taxa under consideration. The points of branching within a dendrogram are called nodes. All taxa occur at the endpoints of the dendrogram.

Selection is the condition that enables some bacteria to grow more rapidly than others e.g. an antibiotic selects for bacteria that are resistant to it and against bacteria that are sensitive or susceptible to it. This occurs through differential reproduction—those with the favored feature produce more offspring than those with the other feature, such that they become a greater percentage of the population in the next generation.

Species (bacterial) is a group of bacterial strains that are closely related to each other (>70% DNA-DNA similarity). The species name is the second name e.g. *Escherichia coli – coli* is the species name.

Transduction is the transfer of DNA by (bacterio)phage requires that the donor and recipient share cell surface receptors for phage binding and thus is usually limited to closely related bacteria; the length of DNA transferred is limited by the size of the phage head.

Transformation is the uptake of naked DNA; common mode of horizontal gene transfer that can mediate the exchange of any part of a chromosome; this process is most common in bacteria that are naturally transformable; typically only short DNA fragments are exchanged.

Transposon is a DNA segment that is capable of integrating into a genome.

EXECUTIVE SUMMARY

Antibiotics are used at therapeutic levels to treat disease, at slightly lower levels as prophylactics, and at low, sub-therapeutic 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 waste holding environments. 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.

Antibiotics used in animal agriculture can enter the environment via a number of routes including the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drug. 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. Laboratory investigations have documented the physico-chemical interactions of most veterinary antibiotics with soil and soil components. Determining what concentrations of antibiotics are environmentally relevant is important in making inferences about the actual impacts of antibiotics detected in the environment but there is limited field information on the biological activity of low concentrations of antibiotics and antibiotic residues, and the fate and transport of antibiotics. Much of the information is simply occurrence or frequency data. In general, the available field data suggests that antibiotics are relatively immobile in the environment and when detected are at the lower µg/L range. Antibiotics are generally not very persistent in manure with half-lives generally less than 100 days. Thus, the application of manure to agricultural fields will likely introduce antibiotic breakdown products to the environment. However, data are very scarce concerning the occurrence, fate and transport of antibiotic breakdown products in the environment. In addition, little information is available concerning the biological activity of breakdown products or for parent antibiotics that are sorbed to soil components. More sensitive techniques are required to assess the persistence of antibiotics and their residues in the environment.

The differences of manure storage systems and land application methods can affect the dissemination of antibiotic resistance genes into the environment. Intestinal bacteria in manure that are introduced to the environment, can survive in soil for as long as 8 weeks to 6 months, but vary depending on species and temperature. DNA from viable, as well as dead, bacteria could be a source of antibiotic resistance genes. More research is needed on how bacterial populations are partitioned in pit/lagoon environments; what proportion and types are associated with the solid and liquid phases This has practical implications for waste management from both an antibiotic resistance perspective as well as pathogen reduction, and will become of much greater importance as the industry inevitably moves toward separation of liquids and solids in order to comply with phosphorus-based limits on land application of manure. In addition, more research is needed to obtain a thorough understanding of the effect of soil heterogeneity on spatial and temporal patterns of microbial communities and the potential for gene transfer. Quantitative measurements of antibiotic resistance gene levels are needed, in addition to measurements of diversity and frequency, in order to address questions of accumulation and persistence of these determinants. Bacteria and

viruses have great potential to move deep into the subsurface environment. Several studies concluded that application of animal manure to soil can readily lead to groundwater contamination with fecal bacteria, especially under moist soil conditions, and that macropores are important in the transport of bacteria through soil.

Phylogenetic analysis indicates that antibiotic resistance genes have evolved and been maintained in bacteria prior to the modern antibiotic era, even though the origin and purpose of these genes is not yet clear. These resistance genes are exchanged among a broad range of bacteria. There is evidence that increased occurrence of lateral gene transfer has occurred recently, most likely accelerated by indiscriminate use of antibiotics. Trace amounts of antibiotics, chemicals or other substances such as heavy metals could act as a selective pressure for the maintenance and transfer of antibiotic resistance genes. Very little is known about gene transfer between bacteria in the environment and the maintenance or reduction of antibiotic resistance in manure storage environments. In order to obtain key data on gene flow, higher throughput and more sequencing of antibiotic resistance genes both from known organisms and bulk genomic DNA from different environments is essential.

Introduction

Antibiotics are routinely used in the livestock industry to treat and prevent disease. In addition, 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 (Cromwell 2001). 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 (Andremont 2003). 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 (Stewart 1989; Amabile-Cuevas and Chicurel 1992; Salyers and Amabile-Cuevas 1997). Transfer of resistance genes from fecal organisms to indigenous soil and water bacteria may occur (Lorenz and Wackernagel 1994; Daane et al. 1996; DiGiovanni et al. 1996; Nielson et al. 2000), 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.

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 et al. (1971) 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 (Donohoe 1984; Gavalchin and Katz 1994). Feinman and Matheson (1978) 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 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 (Schmitt 1995). In the following review, we describe current knowledge concerning antibiotics and their use in swine production, the fate and transfer of antibiotic residues and antibiotic resistance determinants during manure storage, treatment and after land application with emphasis on the environmental persistence and transferability of these determinants. We conclude with a summary of findings and make recommendations for future research.

Antibiotic use in animal agriculture

In commercial livestock 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. A survey carried out by the Animal Health Institute of its members reported that ionophores/arsenicals were the most commonly used antimicrobials in animal production overall (Table1; AHI 1999). The most commonly used antibiotics in pig and poultry production are listed in Table 2, with predominant uses of tetracyclines and bacitracin in the swine industry. There are significant discrepancies in the amount and rationale of antibiotic usage in agriculture. Two separate estimates of antibiotic usage in agriculture were recently reported by AHI (AHI 2001) and Union of Concerned Scientists (UCS 2001). The AHI reported a total of 20.5 million pounds of antibiotics sold for all animal use in 1999. Of the 20.5 million pounds, 17.7 million pounds were

used for treatment and prevention of disease and only 2.8 million pounds were used for improving feed efficiency and enhancing growth. In contrast, the UCS (2001) reported 24.6 million pounds of antibiotic were used for non-therapeutic purposes in the swine, poultry and cattle industries. If antimicrobial use for therapeutic purposes in animals is included the tonnage would be even higher. According to UCS (2001), livestock use accounts for the major share of total antimicrobials used in the United States estimated at 50 million pounds annually, based on extrapolation from a 1989 Institute of Medicine report (IOM 1989). While there appears to be a lack of accurate, publicly available data to determine the animal species, dosage, and usage of antimicrobial products in the United States, it is clear that antibiotics are in heavy use in animal agriculture.

Pathways for entry of antibiotics into the environment

Antibiotics used in animal agriculture can enter the environment via a number of routes including the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drugs. 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 (Boxall et al. 2004; Halling-Sorensen et al. 1998; Feinman and Matheson 1978). 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 (Campagnolo et al. 2002; Kolpin et al. 2002; Yang and Carlson 2003; Krapac et al. 2004).

Chemical characteristics of antibiotics that effect their mobility in the environment

Veterinary antibiotics comprise a group of organic compounds that have a wide variety of functional groups that affect their chemical properties. The octanol-water partition coefficient (K_{ow}) is used as a general measure of hydrophobicity, and most antibiotics have log K_{ow} values less than 5 indicating that they are relatively non-hydrophobic (Tolls 2001). Additionally, water solubility for the antibiotics generally exceed 1 g/L suggesting that they are relatively hydrophilic compounds (Table 3). Tolls (2001) and Boxall et al. (2004) compiled data of sorption coefficients (K_d) for a variety of antibiotics, soils, and soil components measured over the course of many studies. Based on the K_d values, antibiotics exhibit a range of affinities for the solid phase (K_d 0.2 -6,000 L/kg) with consequent effects on their mobility in the environment. Estimations of antibiotic organic carbon-normalized sorption coefficients (K_{oc}) by using a compound's K_{ow} generally results in underestimation of K_{oc} values, suggesting that mechanisms other than hydrophobic partitioning such as cationic exchange, surface complexation, and hydrogen bonding are the likely mechanisms for antibiotic sorption to soils. Many of the acid disassociation constants (pK_a) for antibiotics are in the range of soil pH values suggesting these compounds can protonate or deprotonate in soil solutions such that their speciation depends on the soil solution pH (Tolls 2001).

Studies have shown that under a broad range of environmental conditions, tetracyclines (tetracycline, chlortetracycline, and oxytetracycline) can adsorb strongly to clays (Pinck et al. 1961 a,b ; Sithole and Guy 1987), soil (Krapac et al. 2004) and sediments (Rabolle and Spliid 2000). Macrolides such as tylosin have a weaker tendency to sorb to soil materials (Rabolle and Spliid 2000), whereas sulfonamides exhibit weak sorption to soil, and likely are the most mobile of the antibiotics (Tolls

2001). Pinck et al. (1962) determined that two macrolide antibiotics (carbomycin and erythromycin) sorbed significantly (231-263 mg/g) to montmorillonite and to a much lesser extent (0 to 39 mg/g) to vermiculite, illite and kaolinite. Haung et al. (2001) conducted a literature review on the fate of antibiotics in the environment and concluded that there was little information on the sorption of aminoglycoside and β-lactam antibiotics. Because aminoglycosides can be protonated under acidic conditions, they could be sorbed to clay minerals under certain conditions while β-lactams are highly polar compounds and would not be expected to sorb readily to soil components (Haung et al. 2001). 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 (Kolz et al. 2005a).

Antibiotics are generally introduced from livestock operations via water into the environment, and thus hydrolysis can be an important degradation pathway. β-Lactams, macrolides, and sulfonamides appear to be the most susceptible classes of antibiotics to hydrolysis (Haung et al. 2001). At near neutral pH's, tylosin A was found to have a hydrolysis half-life of 300 to 500 hours at 60° C (Paesen 1995). At more environmentally relevant temperatures, these half-lives are expected to be longer. Doi and Stoskopf (2000) determined that under relatively high temperatures (43° C) the half-life of oxytetracycline in deionized water was 0.26 days, but was relatively stable at 4° C. β-Lactams are rapidly hydrolyzed under mild acidic and basic conditions (Hou and Poole 1969; Haung et al. 2001).

Photolysis can be another abiotic transformation process affecting antibiotics introduced into the environment. The photodegradation of antibiotics in soil can occur at the soil-atmosphere interface and at the surface of liquid manure. Soils can provide a much different photodegradation environment than aqueous solution and transformation rates can vary significantly in soils compared to those in waters (Balmer et al. 2000). Photodecomposition of oxytetracycline was three times more rapid under light than dark conditions (Doi and Stoskopf 2000). Haung et al. (2001) concluded that the quinolones and tetracylines are susceptible to photodegradation. Halling-Sorenson (2000) suggested that tylosin may be resistant to photolysis because it has only limited light absorbance in the visible spectrum. Boxall et al. (2004) reviewed the literature and determined that sulfonamides would not be readily photodegraded. Beausse (2004) concluded that photodecomposition of antibiotics under field conditions were negligible when compared with other abiotic processes.

Persistence of antibiotics in manure

As discussed earlier, manure generated at animal confinement facilities is generally stored in lagoons, surface storage structures, or pits. Boxall et al. (2004) compiled persistence data for various antibiotic classes in manure (Table 4). 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. Quinolones and tetracyclines were the most persistent with half-lives approaching 100 days. Kolz et al. (2005b) determined that 90% of tylosin, tylosin B, and D was lost within 30 to 130 hours in anaerobic manure slurries at 22 °C. In aerobic manure slurries 90% of tylosin was lost in 12 to 26 hours. Kolz et al. (2005a,b) concluded that although biodegradation and abiotic degradation occurred, the primary mechanism for tylosin loss was sorption to manure solids. They also detected residual tylosin and its breakdown product (dihydrodesmycosin) in the slurries after eight months. Tetracycline concentrations were generally higher than macrolide, β-lactams, and sulfonamides in manure samples (Table 5). Tetracycline concentrations in some swine lagoons were as

great as 1 mg/L (Camagnolo et al. 2002). These data suggest that application of manure to agricultural fields likely introduces breakdown products into the environment along with the parent compound. However, persistence data for degradation products was not found in the reviewed literature.

Management of animal waste from production agriculture

Historically, until the mid- to late 1970s, hog production was usually part of larger integrated farming operations that produced small grains, corn and soybeans. Over the last 25 years swine production has largely shifted from such 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 (NASS 2002). 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 (USDA 2001). 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.

Under the earlier integrated system of production, producers typically owned large tracts of land necessary for agronomic activity. Waste and effluent from a modest number of animals was applied rotationally over different fields, effectively diluting nutrients, antibiotic residues, and antibiotic resistance genes in the environment. 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.

Methods of waste storage vary among operations, but usually follow one of three primary types: a slatted floor over a deep concrete pit; a slatted floor over a shallow pit with outdoor areas for slurry storage, and a slatted floor over a shallow pit with outdoor anaerobic lagoon treatment. Deep pit storage requires the least management; waste accumulates in the pit and is only moved once when it is removed for disposal. No mechanical parts like piping or valves are required and the land area required to store waste is minimized. Deep pits are expensive to build and manure gas buildup in the buildings above the pits can be a problem. Shallow pits with outdoor slurry storage in tanks or concrete or earthen basins are less expensive to build and reduce the amount of odor and gas inside the buildings, but require more intensive management and a mechanical means of transferring the waste from the pit into storage. These systems also require more land area for waste storage than deep pits and manure is more visible to the public. Shallow pits with outdoor lagoon treatment require both a means of transferring waste from the pit to the lagoon and enough land on which to site the lagoon. In lagoon systems, manure solids are partially degraded and organic nitrogen is converted to inorganic forms and released from the lagoon through ammonia volatilization. The loss of nitrogen and the sequestering of much of the phosphorus in wastes in lagoon sludge can reduce the amount of land required for waste disposal at

agronomic rates. Lagoons can significantly reduce the odor associated with effluent, but require handling a large volume of liquid material.

In order to utilize and dispose of the manure effluent, CAFO operators often contract with neighboring growers to apply effluent to their land or apply it to land surrounding the facility. In the United States the crop cycles coincide with seasonal cycles with the application of manure occurring between crop cycles. For many locations, manure is stored for six months to one year before being applied to crop fields as fertilizer. For example, livestock management facilities regulations in Illinois require that manure lagoons be designed for a minimum of 270 days storage (IDOA 2002). Effluent differs from fresh manure in that it has a much greater volume due to the addition of water. Fresh swine waste contains approximately ten percent solids, while the solids content of deep pit effluent range from 4 to 8 percent and the solids content of a lagoon range from less than 0.5 to 1 percent (Fulhage and Post 2005). Thus in a lagoon system there is a tenfold increase in waste volume over fresh manure, although the effluent has a much lower nutrient content. Because it is costly to transport liquid effluent any great distance, there is an incentive to apply effluent as close to the source as possible.

This practice had become so widespread that by the 1990s the Environmental Protection Agency (EPA) began an effort to require nutrient management plans for CAFOs. Initially, nutrient management plans were nitrogen based, requiring manure to be applied at a rate that would not exceed crop nitrogen requirements. Swine manure, however, has a high phosphorus content relative to nitrogen content; as excreted, swine manure contains a ratio of approximately 0.86:1 P_2O_5: N (LPES 2005). Applying effluent to meet the nitrogen requirements of a crop often leads to a buildup of phosphorus in the soil, in some instances to values in excess of 2,000 mg/kg of total soil phosphorus (Lehmann 2005). Regulations are being implemented that require phosphorus-based nutrient management plans with soil phosphorus test values limited to state-set thresholds. Attempts by the EPA to use the Clean Water Act to mandate nutrient management standards for effluent applied systems have become bogged down in lawsuits as recently as mid 2005, although many states have regulations currently in effect. Nowhere in this discussion of how best to establish nutrient management plans is the issue of antibiotic resistance addressed. Although not an issue of “nutrient management” per se, the occurrence of antibiotic resistance (antibiotic resistant bacteria and resistance genes) in a soil is affected by many of the same factors affecting nutrient management: holding system type, pretreatment of effluent, soil characteristics, method and timing of application, and application site topography.

Effluent application to soil

The method of effluent application to soil can greatly affect the movement and persistence of both susceptible and antibiotic-resistant bacteria, thus having a substantial influence on the overall degree of antibiotic resistant phenotypes associated with a particular soil. The three primary methods used to apply effluent include surface application, surface application followed by incorporation, and direct soil injection. Typically, a particular method is chosen for reasons unrelated to management of antibiotic resistance; it is usually focused on cost, odor control, prevention of nitrogen loss, and minimization of off-site effects.

Because surface application has been associated with nitrogen loss, it is often considered “environmentally unfriendly,” yet it has merits as a method of managing pathogen loads and possibly, antibiotic resistance. Hutchison (2004) reported that the mean D-value, or time needed to reduce the item being measured by one order of magnitude, for four zoonotic pathogens, *Salmonella* sp., *E. coli* 0157, *Listeria* sp., and

Campylobacter sp., was 1.42 days for unincorporated pig slurry and 2.48 days for slurry incorporated immediately after application. These pathogens also declined at similar rates regardless of season (summer versus winter). This suggests that desiccation may be an important factor in the population decline because more intense UV radiation in the summer would be expected to accelerate cell mortality (Booth 2001; Hoerter 2005). Again, these studies do not address antibiotic resistance specifically; however, a tenfold reduction in pathogenic bacteria in a day and a half suggests that the volume of antibiotic resistance genes entering the soil would be substantially reduced using this application method if effluent remained on the soil surface for more than a few days. It should be noted that a significant rainfall event following surface application of effluent would move resistant bacteria and resistance genes into the soil profile and off the field to other locations via surface flow (Saini 2003). Additionally, surface applications to frozen soil are usually avoided because of the likelihood of significant runoff.

The application of effluent followed by delayed incorporation may be a viable way to reduce the quantity of antibiotic-resistant bacteria and resistance genes entering the soil, given the findings discussed above, but is unlikely to be widely used by producers. The primary reason to incorporate surface-applied effluent is to limit the loss of nitrogen as ammonia and nitrous oxides by as much as 50 percent compared to surface application alone (Rotz 2004), lessen odor, and minimize the likelihood of runoff. To achieve these goals, effluent should be incorporated immediately following application; however, this practice transfers bacteria and resistance genes directly into the soil environment, where an entirely different set of dynamics exist. The preferred method of application from a nutrient management standpoint is deep injection into the soil, which eliminates the nitrogen loss associated with other methods, vastly reduces odor, and virtually eliminates the possibility of runoff. However, this method moves bacteria and resistance genes directly into the soil.

Manure volume and frequency of application

Another factor affecting levels of antibiotic resistance bacteria and genes in soil is the amount of effluent applied and the frequency of application. Effluent application rates are highly variable from farm to farm and field to field. Fields receiving multiple, high-volume (20,000 - 30,000 gallons/acre) applications of effluent each year show consistently greater diversity and occurrence of tetracycline resistance genes than do fields receiving moderate volume (10,000 -15,000 gallons/acre) applications of effluent on a two- or three-year rotation (Maxwell et al. 2006). In addition, the spatial heterogeneity of antibiotic resistance bacteria or genes within any a field likely occurs due to the variability in the solids content of swine effluent being applied. O'Dell (1995) found the solids content in 18 tank loads of swine effluent that had been agitated for 24 hours prior to application ranged from 4 to 10 grams per liter. Excreted antibiotics and antibiotic residues from many commonly used antibiotics are often concentrated in the solid phase because of sorption dynamics (Tolls 2001; Loke 2002; Kolz 2005a,b), and bacterial populations may be greater in the solid phase, although this is an issue that requires further investigation. Sampling multiple locations in a field following swine effluent application, Maxwell et al. (2006) determined that the frequency and occurrence and level of tetracycline resistance genes were not evenly distributed. Because of the heterogeneous nature of soil and tankloads of swine effluent, it is expected that there will be “hotspots” of antibiotic resistance in a field. More research is needed to determine how much antibiotic resistance variability exists in effluent-applied fields and how significant this variability is with regard to maintaining antibiotic resistance in agricultural environments.

Antibiotic occurrence in the environment

Soil and surface runoff

The practice of spreading manure onto agricultural fields as a source of fertilizer can introduce antibiotics into the soil ecosystem. As noted above, the three primary methods used to apply effluent include surface application, surface application followed by incorporation, and direct soil injection, all of which bring soil and effluent containing antibiotics together. There have been several laboratory studies evaluating the chemical behavior of antibiotics sorbing to soil components (see antibiotic chemistry section). Gavalchin and Katz (1994) for example determined the persistence of seven antibiotics in a soil-feces matrix under laboratory conditions and found that the order of persistence was chlortetracycline > bacitracin > erythromycin > streptomcycin $\geq$ bambermycin $\geq$ tylosin $\geq$ penicillin with regard to their detection in the soil.

Until recently information regarding the occurrence, fate and transport of antibiotics under field conditions has been limited. Hamscher et al. (2002, 2005) investigated a sandy soil that had repeated manure applications and detected tetracycline and chlortetracycline down to a depth of 30 cm. The largest tetracycline and chlortetracyline concentrations (198 and 7.3 μg/kg, respectively) were detected at a depth of 10-20 cm and 20-30 cm respectively. Sulfamethazine was generally not detected or was less than quantification limits in the soil samples, but was detected in groundwater collected at a depth of 1.4 m. Oxytetracycline, sulfadiazine, sulfathiazole, sulfamerazine, sulfamethoxypyridazine, sulfamethoxazole, sulfadimethoxine and tylosin were not detected in any soil or groundwater samples. They concluded that tetracyclines can enter the environment in significant concentrations after repeated manure application that can then accumulate in soil. However, none of the antibiotics were detected at soil depths greater than 30 cm and only sulfamethazine was detected in groundwater suggesting limited transport, even in sandy soils.

Boxall et al. (2002) investigated the movement of sulfachlorpyridazine (SCP) in clay loam and sandy loam fields that received swine manure spiked with SCP. They determined that SCP was mobile and readily entered the field drain at the clay site with a maximum concentration of 590 μg/L detected seven days after manure application. SCP concentrations in soil pore water at the sandy site were significantly lower (max. concentration 0.78 μg/L) than the clay site, and did not relate to the laboratory sorption studies that predicted larger soil water concentrations. These smaller concentrations were hypothesized to be the result of SCP degradation. The study also showed that the reported SCP concentrations would not significantly affect aquatic organisms, and further, illustrates the need for field-scale validation.

The transport of sulfachlorpyridazine, (SCP) oxytetracycline (OTC), and tylosin in a clay soil was investigated by Kay et al. (2004). They detected SCP and OTC in soil at concentrations up to 365 and 1691 μg/kg, respectively. Similar to other investigations, these compounds were not detected below a depth of about 37 cm. They also detected these compounds in tile drainage at peak concentrations of 613 and 36 μg/L, respectively. Tylosin was not detected in any samples suggesting its rapid degradation in the manure. Of interest, they determined that sediment-associated transport of antibiotic was less important than transport in the aqueous phase. Only 0.004% of the OTC that was applied was in the particulate phase, representing 23% of the OTC lost to tile drainage. They concluded that the antibiotics behaved similarly to pesticides under these field conditions, and that tile drainage may be a significant route for these compounds to migrate to surface waters. However, the manure in this study was surface-applied without incorporation into the soil and the authors suggest that tillage prior to or during manure application may limit transport of antibiotics.

Swine manure spiked with sulfadiazine and sulfathiazone was irrigated on to grassland (Burkhardt et al. 2005). Less than 5% of sulfonamide applied was lost to runoff. Sulfonamide losses were 10 to 40 times greater on the manured plots when compared to control plots likely because the manure caused sealing of the soil surface resulting in more runoff and also because the high manure pH may have caused deprotonation of the sulfonamides resulting in decreased sorption to the soil. This study again illustrates the need for field studies to validate laboratory fate data. Kay et al. (2005) investigated the surface transport of sulphachloropyridazine, oxytetracycline, and tylosin spiked in swine manure that was applied to wheat stubble in a clay loam soil. The manure was not incorporated into the soil. Tylosin was not detected in any samples. The mass of SCP and OTC lost in surface runoff ranged from 0.42 to 0.07% respectively with the largest concentrations being 703 and 72 μg/L. Although the authors concluded that surface runoff may be a route in which antibiotics could reach surface waters, incorporation of the manure into the soil during application would likely reduce surface transport of the antibiotics.

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 LC-MS-MS to be able to detect and quantify the contaminants at environmentally relevant concentrations. A recent study by the USGS (Kolpin et al. 2002) 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 6 lists of the most commonly detected antibiotics found in filtered stream samples. Carbodox, doxycycline, enrofloxacin, sarafloxacin, sulfachlorpyridazine, sulfamerazine, sulfathiazole, 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 (2003) 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 μg/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 et al. (2002) 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 μg/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 et al. (2004) collected shallow (<8 m) groundwater samples near two swine confinement facilities. Using LC-MS to detect the antitibotics, 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 μg/L.

Hirsch et al. (1999) 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.

Antibiotic resistant bacteria in manure

Antibiotic resistance among commensal bacteria represents a major avenue for the development of resistance in bacterial pathogens, since 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 (Andremont 2003). 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} to 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 (Cotta et al. 2003).

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 (Hanzawa 1984). Haack and Andrews (2000) found that 71 percent of *Enterococcus faecalis* isolates from farrowing house effluent were resistant to tetracycline. Cotta et al. (2003) found between 4 to 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.

<u>Persistence of resistant bacteria during manure storage</u>

As noted above, deep waste holding pits located beneath the slatted floors of hog barns have 4-8 times the amount of solid material suspended in liquid relative to a lagoon holding system, where most of the solid fraction settles out as sediment. Little research has been conducted to determine the affects of the various manure storage techniques on overall bacterial populations and the corresponding genetic pool that

includes antibiotic resistance genes. Investigations that have been done in this area generally involve human or livestock pathogens. Hutchison (2004) studied zoonotic agents in fresh wastes, akin to a below barn holding pit, and stored wastes, analogous to the second stage of a two stage lagoon. A general trend was identified that suggests that storage of waste without addition of fresh waste reduces pathogen numbers. Total bacterial populations may also decrease in stored effluent; however, this hypothesis has yet to be adequately tested. Likewise, a comparison is warranted between the number and diversity of particular antibiotic resistance determinants in lagoon and pit systems. Recent findings indicate that pit systems are similar to lagoons in the diversity of tetracycline determinants (Maxwell et al. 2006). However, relative quantities of these genes in different systems have not been determined. There is strong evidence that thermophilic aerobic or anaerobic digestion of swine manure in a reactor can reduce pathogen loads by more than four orders of magnitude (Sobsey et al. 2001), although the effects of the digestion on other pathogenic or nonpathogenic antibiotic-resistant bacteria and resistance genes is generally unknown. Mesophilic anaerobic digestion of swine slurry in bench scale sequencing batch reactors at a temperature of 20° C for 20 days reduced total coliform populations by 98 to 100 percent and *E. coli* populations by 99 to 100 percent (Cote 2005). Although coliform bacteria from livestock typically exhibit some degree of antibiotic resistance, it is unknown what effect mesophilic digestion would have on the overall antibiotic resistance of swine waste bacteria.

Survival and transport of bacteria in the environment

Land application of animal manure, with its high concentration of microbial biomass, is a significant route for the introduction of new bacteria into the terrestrial environment, including potential pathogens (e.g., *E. coli* O157:H7) and some human enteric viruses (e.g. rotavirus). The persistence and transport of these organisms in the environment continues to be a concern for environmental quality, food safety, as well as human and animal health.

Persistence of viable bacteria and genes

It has been well documented that many microorganisms survive the transition from effluent pit or lagoon into soil (Kibbey et al. 1978; Chandler et al. 1981; Stoddard 1998; Bolton, Byrne et al. 1999; Lee and Stotzky 1999; Jiang et al. 2002; Guan and Holley 2003; Boes et al. 2005). Most investigations have however, focused on pathogens of clinical interest. The length of time that introduced organisms can persist in the soil varies with temperature, moisture, pH and the indigenous community present. Table 7 lists the persistence times of four well-studied pathogens in different environments and at different temperatures. A recent study examining the survival of *E. coli* and *Salmonella typhimurium* applied to a clay soil with swine effluent, however, found a considerably shorter persistence time than that listed in Table 7 (21 days for *E. coli* and 7 days for *Salmonella typhimurium*; Boes et al. 2005) highlighting the great amount of variation in survival times under varying environmental conditions. Sengelov et al. (2003) studied the persistence of culturable, heterotrophic, aerobic, tetracycline resistant bacteria on four Danish farm soils following pig slurry application at variable application rates. An increase in numbers of resistant bacteria was seen following application, with greater increases occurring in the more heavily manured soils. However, five months following application the proportion of tetracycline resistant bacteria in all of the treated soils had returned to levels within the range of the non-manured control samples. Andrews et al. (2004) examined the persistence of enterococci bacteria in autoclaved and native soil microcosms. In native microcosms, enterococci declined from 4.8 X 10^5 colony forming units (CFU) per gram soil to less

than 10 CFU per gram over a five week period. It should be noted that only a fraction of all bacteria present in pit or lagoon slurry can be cultured, so current understanding of the persistence of introduced organisms is inherently limited. Gavalchin and Katz (1994) concluded that the longer an antibiotic persists in the soil in an active form, the greater the potential for native soil bacterial populations to be affected. In addition, biologically active antibiotics (or antibiotic breakdown products) introduced to the soil may confer a selective advantage for indigenous bacteria carrying resistance genes, or exert selective pressure for acquisition of resistance genes in indigenous soil bacteria.

Transport of bacteria and viruses into groundwater

Bacteria and viruses have great potential to move deep into the subsurface environment, and can even penetrate an aquitard and reach a confined aquifer. The extremely small size of viruses allows them passage through sediment pores that would trap much larger bacteria and protozoa. Many studies have shown that bacterial indicator system does not accurately reflect the occurrence of viruses in aquatic system (Goyal 1983; Payment and Armon 1989; Nasser et al. 1993; Scandura and Sobsey 1997; Nasser and Oman 1999). Depending on sediment chemistry, they may not be completely attenuated by adsorption onto sediment grains. Viruses have been found in groundwater at the depth of 67 m and are reported to move horizontally as far as 408 m in glacial till and 1600 m in fractured limestone (Keswick and Gerba 1980; Robertson and Edberg 1997). Wellings et al. (1975) reported that viruses are capable of surviving at least 28 days in groundwater. Wellings et al. (1974) found that viruses may survive chlorination, sunlight, spraying and percolation through sandy soil. Bacteria and viruses are widely available for transport within groundwater systems due their common occurrence in drinking water wells (Gerba and Rose 1990). Keswick and Gerba (1980) completed a pioneering literature review and developed recommendations for research to better understand the occurrence and transport of viruses in groundwater.

Macler (1995) estimated that 20-25% of groundwater sources in the United States are contaminated with microbial pathogens which include more than 100 types of viruses. During 1999-2000, 39 outbreaks of waterborne disease were reported in the United States, including 22 microbe-related incidents and 28 groundwater-linked incidents (Lee et al. 2002). Analysis of groundwater sites, located in 35 states, showed that 141 of 448 sites (31.5%) were found to contain at least one type of virus (Abbaszadegan et al. 2003). Other research has shown that more than half of waterborne disease outbreaks in the USA are attributable to drinking groundwater (Craun et al. 1997; Lee et al. 2002; U.S. EPA 2000). The processes of viral transport are extremely complex. There is much literature discussing the major factors affecting subsurface viral transport, including temperature, moisture content, pH, hydraulic conditions, organic matter, adsorption and desorption, salt content, type of virus, virus inactivation (i.e., virus decay), soil properties, rainfall, source of virus and water table depth. Among the complex combination of those factors, the numerical models often consider advection, dispersion, sorption, and inactivation to be the major processes affecting virus transport. Comprehensive, detailed reviews of virus transport and survival are contained in the following publications (Gerba and Bitton 1984; Yates and Yates 1988; Bitton and Harvey 1992; Schijven and Hassanizadeh 2000; Azadpour-Keeley et al. 2003; Cherry et al. 2006).

Colloids, such as viruses, may move substantially faster than dissolved solutes, like bromide, in subsurface environments as a result of preferential flow of the colloids through the large apertures offered by fractures and root holes. The solutes have a higher probability than the colloids of entering smaller pores, resulting in more tortuous, longer, and ultimately slower flow paths. Colloids, including viruses, cannot enter the

much smaller pores between clay grains. In clay formations, fractures and root holes can be as large as 100 µm, 5000 times larger than the smallest viruses (Hinsby et al. 1996), but viruses can pass through fractured clay till in apertures as small as 3 to 5 µm (Sims 1993). McKay et al. (1993) showed that approximate 50% of clay pore throats are smaller than 65 nm, which is about the same size as enteric viruses. The same study demonstrated that the viruses PRD-1 and MS-2 move through fractured clay at velocities of 2 - 5 m/day, which is 100 to 200 times faster than bromide. Hinsby et al. (1996) measured PRD-1 and MS-2 virus velocities of 4 - 360 m/day through clay with fractures and root holes.

Many numerical models representing bacteria and virus transport are available publicly. Azadpour-Keeley et al. (2003) compared various subsurface virus transport models. These models are based on the primary processes of viral transport: advection, dispersion, sorption, and inactivation (Loveland et al., 2003) and are beyond the scope of the present review. Since the processes simulated by computer models are highly heterogeneous on a field-scale and differ widely by virus type, model outputs are associated with a great deal of uncertainty (Yates and Jury 1995). Yates et al. (2000) compared predicted virus concentrations from CANVAS (Park et al. 1994) and HYDRUS-2D (Simunek et al. 1999) in a septic system leach field. CANVAS predictions either overestimated or underestimated the field data. Although HYDRUS-2D accurately predicted the virus breakthrough curves, the model required extensive input data and advanced expertise that are not generally available and very expensive to obtain. Moreover, a separate model would then be needed for predicting virus movement through an aquitard into a confined aquifer, but to our knowledge this type of model does not exist. According to Yates, virus transport models are either too complex or the model predictions too uncertain for routine use by drinking water utilities for determining whether wells are in regulatory compliance for acceptable virus concentrations (Cherry et al. 2006). If it is reasonable to assume that the longest survival time for enteric viruses is one to two years, water taking 10 to 100 years to reach a confined aquifer is unlikely to transport infectious viruses. It is not entirely clear, however, that water would take such a long time to reach a confined aquifer. The estimation of groundwater travel times is not straightforward in highly heterogeneous settings, and measurements of groundwater age of the bulk fluid may not reflect microscopic contributions via preferential flow that, albeit small, contain measurable infectious viruses (Powell et al. 2003).

Several studies have focused on bacterial occurrence and movement in soil caused by manure application. For example, Unc and Goss's (2003) study of the vadose zone at sites of manure application established that the potential for fecal bacteria to be transported to depth in soil is correlated with the water content of the manure. They concluded that application of animal manure to soil can readily lead to groundwater contamination with fecal bacteria, especially under moist soil conditions, and that macropores, but not total porosity, are important in the transport of bacteria through soil. The study of McMurry et al. (1998) using poultry manure showed that preferential fecal coliform movement corresponded to preferential water movement in each soil block sampled, whether sod-covered or tilled. Their findings were consistent with those of Howell et al. (1995) that found that groundwater contamination by fecal coliform infiltration through well-structured soil may be significant during even modest rainfall.

Mechanisms of antibiotic resistance gene transfer in the environment

Once antibiotic resistant bacteria and antibiotic resistance genes enter the soil, primary factors that determine the persistence and fate of the introduced determinant in

the soil environment include: the presence of the gene in a viable bacterium or bacteriophage; the presence of the gene in a dead organism; and the behavior of free genetic material including uptake by cells or sorption to mineral or organic colloids. As long as a resistance gene is present in the soil the possibility of lateral gene transfer exists. Genetic mechanisms involved in lateral exchange of antibiotic resistance genes may include: (i) conjugative transfer (plasmids, transposons, integrons on plasmids or transposons, mobilization of nonconjugative and NBU elements, integrative and conjugative elements, etc.); (ii) transformation by naked DNA, which includes the naturally competent state of some bacteria as well as environmentally induced competence (the presence of calcium, lightning, etc.); and (iii) transduction by bacterial viruses called phage. The potential for lateral transfer of antibiotic resistance genes in soil by these three mechanisms, as well as the persistence of resistance genes outside of living organisms, is discussed in the following section. In general, it is thought that conjugative mechanisms for gene transfer in the environment are more frequent and significant because they are broad host range and can thus cross genus and species boundaries resulting in gene swapping in the environment.

Conjugation

When introduced antibiotic resistant bacteria persist as viable cells in the soil, the potential exists for the transfer (or mobilization) of a resistance gene from the introduced organism to an indigenous soil bacterium through the conjugation process. Conjugation requires physical cell-to-cell contact, so the environment in which the bacteria are growing will have a major effect on the frequency of conjugation events. For example, a high frequency of conjugation events occurs in animal enteric systems that have high bacterial populations in close physical proximity to one another and are in a hydrated environment (Salyers et al. 1995). Aquatic environments where bacteria have greater freedom of movement and bacterial consortia can form biofilms also exhibit increased chances of bacterial contact that could lead to conjugation events. Soil however, is a very heterogeneous environment both at the micro- and macro- scales. Differences in soil type and structure can vary throughout a field, and soil micro-aggregate size and structure can vary over a distance of millimeters. Soils also vary widely in their degree of hydration, based both on water input through precipitation or irrigation and physical properties that influence water holding capacity. The numbers and proximity of bacteria to one another, and the potential for conjugation, will likewise vary with differences in the soil environment at any given time and location. While it is thought that conjugation occurs much less frequently in soils than the other environments mentioned above, it certainly does occur and could be a primary mechanism for antibiotic resistance gene transfer and maintenance in soil.

Many resistance genes are harbored on mobile genetic elements such as transposons, integrons, or plasmids and can be readily transferred from among both members of the same species, and bacteria of diverse genera under the right conditions. Many microcosm studies have documented plasmid transfer in soil environments, and plasmid transfer from introduced organisms into indigenous bacteria (Wellington 1992; DiGiovanni et al. 1996; Lee and Stotzky 1999; Heuer et al. 2002; Andrews et al. 2004). In particular, Andrews et al. (2004) examined the persistence of the conjugative transposon Tn916 associated with antibiotic resistance in autoclaved and native soil microcosms treated with swine effluent containing large populations of enterococci carrying Tn916. In autoclaved microcosms, persistence of the transposon closely correlated with the persistence of its enterococci host. In native microcosms however, Tn916 was still detected at substantial levels 6 weeks after treatment with effluent despite the fact that the introduced host enterococci were undetectable. This

finding suggests that gene transfer of elements associated with antibiotic resistance is indeed occurring or the transposon is somehow being maintained in an extracellular manner.

Plasmid transfer through conjugation among diverse genera has occured in the soil rhizosphere under field and simulated microcosm conditions (van Elsas et al. 1998). Higher rates of gene transfer were found in rhizosphere soil relative to bulk soil (Lilley and Bailey 1997). Daane et al. (1996) found that earthworm activity increased the mobilization of a mercury resistance plasmid from introduced organisms to indigenous bacteria because they helped to disperse bacteria in the soil. It also appears that the presence of effluent itself may increase the likelihood of conjugal transfer in the soil. Gotz and Smalla (1997) found a tenfold increase in plasmid transfer in soils receiving manure application relative to those that had not. Several studies have documented sub clinical amounts of antibiotics in soils following effluent application (see antibiotic occurrence in the environment section) The presence of antibiotics, even at very small concentrations, can stimulate conjugation and the transfer of resistance genes by as much as 10,000 fold in enteric systems (for a comprehensive review see Salyers et al. 1995). Ohlsen et al. (2003) found that 0.1 µg/mL of the antibiotic, gentamicin, increased plasmid transfer in *Staphylococcus aureus.* This concentration is less than clinical concentrations but greater than what has been detected in soil environments. It is not known if environmentally relevant concentrations can stimulate similar transfer, but the conditions and potential do exist.

Transduction

DNA and resistance genes can also be transferred between introduced and indigenous bacteria by viruses called transducing bacteriophages. Gene transfer can be by one of two methods, generalized or specialized transduction. In the case of generalized transduction, random genes are transferred by bacteriophage following host cell lysis into new bacterial hosts. During specialized transduction, only host DNA adjacent to the site where the phage inserted itself is transferred. Although little is known about the potential for antibiotic resistance gene transfer by transduction, this gene mobility mechanism could be viable in soil systems. Jiang and Paul (1998) determined that in marine environments containing relatively large phage populations, transduction could be a significant mechanism of horizontal gene transfer. It was thought for many years that phage densities in soil were too small to account for much, if any, gene transfer. Recent work by Ashelford et al. (2003) found a greatly elevated abundance of bacteriophage in soil systems, leading them to conclude that numbers of phage particles in the soil average around 1.5×10^8, equivalent to between one and four percent of total bacterial populations. Earlier work by Ogunseitan et al. (1992) determined the distribution of bacteriophage infecting *Pseudomonas aeruginosa* in water, sediment, sewage, and soil. While sewage had the greatest diversity and numbers of bacteriophage, between 2 and 37% of *P. aeruginosa* in soil samples also harbored bacteriophage. These findings indicate more research is needed to determine the overall importance of transduction in soil systems and also focus on the efficiency of this mechanism for transfer of antibiotic resistance genes in the soil environment.

Transformation

Naked DNA can persist in the soil without residing in a living host and can be acquired by a new living host through the process of transformation (for a review see Lorenz and Wackernagel 1994). Given the large numbers (10^9 to 10^{11}) of total bacteria per gram of soil, there is likely a large pool of dead, and viable but not culturable, organisms present at any given time that contribute DNA to the pool available for

transformation. Although not well understood, the length of time antibiotic resistance genes can persist in dead cells in the environment involves factors such as the presence of a cell wall and enzyme activity. A microcosm study using a soil environment documented the transformation of kanamycin sensitive *Acinetobacter* sp. strain BD413 with cell lysates of other *Acinetobacter* strains, *Pseudomonas fluorescens*, and *Burkholderia cepacia*, which conferred kanamycin resistance to the transformed bacteria (Nielsen et al. 2000). This study also found that the ability of the lysates to transform the recipient declined by 31% in transforming activity after an hour in non-sterile soil. A related study found that plant exudates in the soil rhizosphere had a stimulatory effect on the transformation of *Acinetobacter* sp. strain BD413 (Nielsen and van Elsas 2001). While these studies document the ability of free DNA in soil to transform bacteria, DNA sorbed to soil colloids also has the potential to effect transformation. Gallori et al. (1994) documented that both chromosomal and plasmid DNA bound to clay particles were capable of transforming bacteria in non-sterile soil. Crecchio and Stotzky (1998) observed the transformation of *Bacillus subtilis* by DNA bound to humic acids, and Lee and Stotzky (1999) noted that in non-sterile soils, the addition of montmorillonite clay, known to bind both DNA and endonucleases, increased the transformation rate of *B. subtilis* by a plasmid coding for chloramphenicol resistance.

Sorption

Another mechanism that allows DNA and antibiotic resistance genes to persist in soil, outside of living organisms, is through sorption to organic or mineral colloids. Blum et al. (1997) found that of three agricultural soils sampled, all were capable of adsorbing the total DNA of the microbial community (>13ug/g soil) within them. While DNA has been shown to sorb to sand (Lorenz and Wackernagel 1987), it is thought that the clay size fraction is the primary constituent sorbing DNA in soil systems. It is well documented that both chromosomal and plasmid DNA sorb to clay particles in the soil, particularly at neutral or lower pH values (Ogram et al. 1988; Demaneche et al. 2001) and at large concentrations of multivalent cations (Paget et al. 1992). A recent study by Cai et al. (2006) offers new insights into DNA/clay interactions. The fine textured 2:1 swelling clays, e.g. montmorillonite, bind DNA primarily though electrostatic forces which are relatively weak and allow bound DNA to desorb fairly easily. The coarser textured 1:1 non-swelling clays, e.g. kaolinite, bind DNA primarily through ligand exchange and possibly hydrogen bonding, resulting in a much stronger bond with less likelihood of DNA desorption. The fine clays, to which DNA is weakly bound, greatly influence how much DNA is sorbed in a soil, whereas the coarse clays are more responsible for the overall affinity for a soil to strongly bind DNA. Given this information, the hypothesis that DNA (and antibiotic resistance genes) would persist longer in soils with greater coarse clay contents warrants investigation. In addition, humic acids in soils have the ability to bind substantial amounts of DNA. Crecchio and Stotzky (1998) reported DNA binding to humic acids in vitro at rates of 60-80 μg/mg of humic acid.

DNA adsorbed to soil colloids is protected to varying degrees from enzyme (DNAse) activity and persists much longer than free DNA in the soil environment: from months (Recorbet et al. 1993; Romanowski et al. 1993) to as much as two years (Gebhard and Smalla 1999). Two primary mechanisms are thought to account for the retardation of sorbed DNA degradation. The first mechanism suggests that bound DNA is physically protected from contact by endonucleases. This mechanism has not been thoroughly investigated as confirmed by the lack of literature addressing this subject. The second mechanism involves sorption of endonucleases to clay, which physically separates the enzyme from DNA and causes conformational changes in the enzyme

resulting in decreased activity. Several studies document the occurrence of this phenomenon (Khanna and Stotzky 1992; Demaneche et al. 2001).

Detection of antibiotic resistance genes

Information on the persistence and dissemination of antibiotic resistance genes in bacteria is of fundamental importance in assessing environmental impacts and risks related to 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 (APHA 1992). 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 advantages of these techniques are that they provide rapid, sensitive, and specific detection and identification of bacteria 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. Polymerase chain reaction (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 (Josephson et al. 1993; Karch et al. 1995; Wang et al. 1996). Gene probes and PCR-based methods are useful for detection and quantification of specific genes and their expression, as well as identification of microbial populations. Molecular fingerprinting tools and robotic technology has facilitated more accurate and sensitive microbial characterization of complex environmental samples and has proven to be essential in providing more informative data in environmental monitoring studies.

There are several platforms currently used to analyze nucleic acid (DNA and RNA) sequences but these approaches are largely serial and, in the case of cloning amplicons and then sequencing, is laborious and time consuming. Microarray technology is a powerful tool that can be used for simultaneous detection of thousands of genes or target sequences on a single glass slide. However, the technology is versatile enough to be used for bacterial detection and DNA typing of specific bacterial strains including pathogens. This technology opens up the potential for high throughput because of its high degree of parallelism and the capacity to assay a large number of samples in a short time. The development of novel microchip gene arrays for rapid detection and quantitation of specific resistance genes would provide a major advance over culture-dependent methods for assessment of antibiotic resistance and microbial pathogens. Recent developments, referred to as real-time nucleic acid amplification technologies can further reduce overall test times by replacing time consuming post-amplification electrophoresis or hybridization with sensitive, quantitative, solution based detection based on fluorescence resonance energy transfer. Efforts to harness real-time detection strategies and couple them with microarray technologies will help to meet challenges of detecting, identifying and quantifying natural microbial populations in environmental samples. Microarray technology is already being developed specifically to detect and identify antimicrobial resistance in clinical and environmental bacteria (Call et al. 2003; Volokhov et al. 2003).

Since 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 (Kaspar et al. 1990; Pillai et al. 1997; Wiggins et al. 1999). 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 et al. (2001, 2002) and Chee-Sanford et al. (2001) 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 (2004) detected 10^5 copies per 50 µL of *tet* genes O, W, and Q combined in a cattle feedlot lagoon. PCR is also helpful to phylogenetically classify antibiotic resistance genes. Thus in the next section a brief overview of the current status of evolution and molecular ecology of antibiotic resistance genes will be presented.

Evolution and ecology of antibiotic resistance genes

The final section of this review attempts to answer a number of vexing questions that arise when considering antibiotic resistance in an evolutionary and ecological context rather than from a clinical and modern medical perspective as is most often the case. Some of these questions are:

1.) Is animal agriculture the source of antibiotic resistance genes that are detected in the environment and what is their evolutionary origin?
2.) How can we distinguish between natural evolution and descent versus recently acquired or horizontal transfer of antibiotic resistance genes?
3.) What is the pool or reservoir of antibiotic resistance genes in the intestinal tract of production animals and in the environment?
4.) Why does antibiotic resistance persist even when antibiotic use is terminated.

Antibiotic resistance has received considerable attention due to the problem of emergence and rapid expansion of antibiotic resistant pathogenic bacteria. The common perception, however, is that the antibiotic resistance problem is exclusively associated with the overuse and misuse of antibiotics in humans and animals. While this perception is accurate regarding the dissemination of clones of pathogenic bacteria with resistance mechanisms due to mutational events and strong positive selection of mutants, the majority of antibiotic resistances are thought to be cases of acquired resistance through the transfer of antibiotic resistance genes from other bacteria. If this is correct, then there must be a reservoir where these resistance genes originated and from which they were subsequently transferred.

<u>Origin of antibiotic resistance genes</u>

It was reported over two decades ago that aminoglycoside-inactivating enzymes in actinomycetes were similar to those present in clinical isolates of antibiotic-resistant bacteria (Benveniste and Davies 1973). Subsequently, it was reported that many antibiotic preparations contained enough DNA to be visible on agarose gels and that this DNA could be transformed into enterobacteria and vibrios resulting in antibiotic resistant clones (Chakrabarty et al. 1990). Webb and Davies (1993) confirmed the presence of antibiotic resistance gene sequences in a number of antibiotic preparations employed for human and animal use. They hypothesized that residual DNA encoding drug resistance in antibiotic preparations has been a factor in the rapid development of multiple antibiotic resistance due to the acquisition of this DNA by bacteria. Recently,

evaluation of this residual DNA with a more sensitive fluorescence detection technique confirmed that many antibiotic preparations, both of research and clinical grade, contain detectable amounts of DNA (Woegerbauer et al. 2005). Moreover, in most cases this DNA is specific to the antibiotic producing strain and contains the corresponding resistance genes. However, attempts to demonstrate *in vivo* transformation by these antibiotic preparations were unsuccessful (Woegerbauer et al. 2005). Horizontal gene transfer events are difficult to reproduce in a laboratory because transfer frequencies may be too low but, fortunately, there is the possibility of retrospective analysis using phylogenetic tools to demonstrate past lateral gene transfer events.

Use of phylogeny to infer evolutionary origins of antibiotic resistance genes

Molecular phylogeny is the comparative analysis of gene sequences, at the nucleotide or amino acid level, to gain information on an organism's evolutionary relationships. The result of a molecular phylogenetic analysis can be displayed in a phylogenetic tree to show these relationships. This approach was used to analyze the evolutionary history of antibiotic resistance genes encoding ribosomal protection proteins (RPP's) that function as alternative elongation factors and confer resistance to tetracyclines (Connell et al. 2002, 2003). This phylogenetic analysis suggested early branching and long independent diversification of at least eight clusters of RPP's that had diverged well before the modern "antibiotic era" with no indication of transfer of antibiotic resistance genes from antibiotic producing strains to pathogenic or commensal bacteria (Aminov et al. 2001). The phylogenetic analysis was repeated on a much more comprehensive dataset and it confirmed the monophyletic origin (i.e. descended from a single common ancestor) of the RPP genes, with an early branching event separating them from the other group of elongation factors, EF-G, encoded by the *fusA* genes (Fig. 1). The next branching event, supported by a 100% bootstrap confidence value, separated the genes from the streptomycetes/*Agrobacterium tumefaciens* clade (cluster 2) and the rest of commensal/pathogenic bacterial clade (cluster 1). The RPP gene sequence of *A. tumefaciens,* a soil/plant pathogenic bacterium, although clustered with the sequences of streptomycetes, is very divergent and does not reflect a horizontal gene transfer event. A community genomic or metagenomic approach to recover additional diversity of the *tet* genes from uncultivated bacteria in the environment has resulted in generation of additional sequences shown in Fig. 1 that are interspersed among genes from commensal and pathogenic bacteria (Yu et al. 2005).

This phylogenetic analysis can be extended to other gene families to determine if phylogenetic evidence of gene migration from antibiotic-producing bacteria to human and animal commensal and pathogenic bacteria can be traced. The clinically relevant *erm* gene family encode enzymes that catalyze S-adenosyl-L-methionine-dependent methylation of a specific adenine residue in the 23S rRNA molecule that sterically protects the ribosomes from binding the macrolide, lincosamide and streptogramin B antibiotics and subsequent inhibition of protein biosynthesis. Acquisition of these methylase genes confers bacterial resistance to erythromycin. The phylogeny of this *erm* gene family is presented in Figure 2. The phylogenetic tree shows that the origin of this gene family is polyphyletic (i.e. having more than one line of evolution) and, as with the RPP genes, there is no indication of gene exchange between the commensal/pathogenic bacteria clade (cluster 1, Fig. 2) and the group clustered with antibiotic producers (cluster 2, Fig. 2). Antibiotic producers in cluster 2 are exemplified by sequences from *S. fradiae, S. venezualae, Micromonospora griseorubida, S. lincolnensis, S. ambofaciens, S. thermotolerans, Aeromicrobium erythreum,* and *Saccharopolyspora erythrea* and in no case are these sequences clustered with human

and animal-associated bacteria. Cluster 2, however, does contain a group of bacteria of medical and veterinary importance, which carry the *erm*(X) gene but again, this subcluster branched early in the evolution of the genes from antibiotic producing bacteria (Fig. 2).

Another independent phylogenetic analysis of these two and other antibiotic resistance gene families supports the conclusion that there is no evidence for transfer of antibiotic resistance genes from antibiotic producing bacteria to human- and animal-associated bacteria (Lau et al. 2004). A recent study also demonstrated that the residual DNA found in antibiotics is unlikely to be a major factor influencing lateral transfer of antibiotic resistance genes in clinical settings (Woegerbauer et al. 2005). The question, therefore, remains, what is the origin of antibiotic resistance genes in human- and animal-associated bacteria?

Recent work on evolutionary history of ß-lactamase genes in *Klebsiella oxytoca* has suggested that these genes evolved over the past 100 million years in this host, without concomitant evolution of the antimicrobial resistance phenotype and the phylogenies of ß-lactamase and **house-keeping genes** are highly congruent in this organism (Fevre et al. 2005). While the functional role of antibiotic resistance genes in antibiotic-producing bacteria is evident (protection against the antibiotics synthesized), their presence and function in bacteria from other ecological niches with no or limited exposure to soil bacteria (e.g., the gastrointestinal tract) is more challenging to explain. The most plausible explanation for this phenomenon is that these genes might have served some other metabolic function(s) rather than providing antibiotic resistance in the modern antibiotic era. Antibiotics target vital cellular processes such as cell wall integrity and various biosynthetic processes that are essential for cell division, growth and metabolism and it is logical that mechanisms exist to support and protect these fundamental cellular needs. For example, methylation by Dim1p (the eukaryotic ortholog or functional equivalent of bacterial methylases), is essential to cellular integrity, probably due to its function as a quality control mechanism in ribosome synthesis (Lafontaine et al. 1998). Likewise, the bacterial ortholog may have been involved in a similar function in the bacterial cell before assuming the role of protecting ribosomes against the binding of macrolide antibiotics.

On the other hand, phylogenetic analysis also suggests that rapid movement of antibiotic resistant genes to taxonomically divergent commensal and pathogenic bacteria has occurred in the recent past and can most probably be attributed to accelerated horizontal transfer within bacteria in the “antibiotic era”. Tetracycline resistance genes like *tet*(M) gene can be found in both gram-positive and gram-negative bacteria (Fig. 1) and is known to be transferred by transposon *Tn*916. The *tet*(W) gene is virtually identical in a range of bacterial species (*Megaspaera elsdenii*, *Bifidobacterium longum, Roseburia hominis, Mitsuokella multacida,* and *Butyrivibrio fibrisolvens*) suggesting very recent lateral gene exchange events between human, pig and cattle digestive tracts as well as a probiotic bacterium. The precise genetic mechanisms responsible for the movement of *tet*(W) between the gut ecosystems of different species are presently unknown but presumably involves an unidentified mobile element. The vast majority of bacteria carrying *tet* genes are of intestinal or genital origin, and horizontal gene exchange between these, as well as transient bacteria, is considerable. The case of the *erm* genes is very similar with lateral gene transfer of the *erm*(B) and *erm*(C) genes between gram-positive and gram-negative bacteria (Fig. 2). Retrospective analysis of the *erm* and *tet* genes in *Bacteroides* and other predominant intestinal bacteria suggested that these genes have been disseminated rapidly among human populations in hospitals and in the community over the past three decades (Shoemaker et al. 2001), which coincides with the “antibiotic era”. Molecular analysis of

human, pig, and poultry *Enterococcus faecium* isolates and their *erm*(B) genes also suggests that horizontal exchange of antibiotic resistance genes is more important in dissemination of antibiotic resistance than direct transmission of resistant strains (De Leener et al. 2005). Factors contributing to the accelerated lateral transfer of antibiotic resistance genes and their rapid evolution will be discussed in the following section.

Factors contributing to dissemination of antibiotic resistance genes

One obvious evolutionary factor contributing to the dissemination of antibiotic resistance genes is the ever-increasing production and consumption of antibiotics for various purposes, ranging from treatment of disease to the practice of feeding antibiotics to domestic animals to promote health and growth. The pressure imposed by antibiotic use obviously selects for antibiotic resistant bacteria but the resistance problem does not disappear when the selection pressure is removed, an “easy-to-get, hard-to-lose phenomenon” (Salyers and Amabile-Cuevas 1997). Although it is generally thought that the maintenance of antibiotic resistance determinants imposes an additional metabolic cost on the bacterial cell and that resistance genes will be eliminated from the population once the selective pressure is removed, there are many examples demonstrating how adaptable bacteria are and how this plasticity may allow them to ameliorate the metabolic cost to maintain resistance (Lenski 1997; Enne et al. 2005; Ramadhan and Hegedus 2005). Moreover, the acquisition of an antibiotic resistance genotype may actually increase the fitness of certain bacteria in the absence of antibiotic selective pressure allowing rapid emergence and dissemination of antibiotic resistance on a worldwide scale (Enne et al. 2004; Luo et al. 2005). The amelioration of the fitness cost required to maintain antibiotic resistance may be one of the reasons why for example, in wild animals, antibiotic resistance genes persist in the absence of selection imposed by the presence of antibiotics (Gilliver et al. 1999). If this is the case, the dissemination of antibiotic resistance becomes a self-perpetuating process, replacing the antibiotic susceptible genotype in the absence of any antibiotic selective pressure. Therefore, the release of antibiotic resistance genes into the environment becomes a critical control point as evidenced by the fact that in areas with historically low level of agricultural antibiotic use the frequency of antibiotic resistance gene carriage is also very low (Osterblad et al. 2001).

Another consequence of prolonged antibiotic usage could be selection of a novel gene variant/recombinant that may confer higher minimum inhibitory concentrations (MICs). Mosaic recombinant *tet*(O/W/O) variants (Fig. 1) were first isolated from *Megasphaera elsdenii* from swine (Stanton and Humphrey 2003). The previously characterized *tet*(32) gene from the human commensal bacterium *Clostridium* sp. was also shown to be a mosaic recombinant, *tet*(O/32/O) (Stanton et al. 2005). Analysis of the published *tet*(O) gene sequence from *Campylobacter coli* (Batchelor et al. 2004) suggests that this is a double-crossover recombinant and should be described as a *tet*(O/M/O). Characteristically in recombinants this gene forms a separate entity in the phylogenetic tree (Fig. 1). The recombinant variants of the gene appeared to have higher MICs for tetracycline which might explain the selection and persistence of these mosaic recombinants (Stanton et al. 2004). Thus, commensal bacteria serve both as reservoirs of antibiotic resistance genes and as a dynamic breeding ground for evolution and generation of novel, diverse antibiotic resistance genes.

Selection for antibiotic resistance variants

The use of antibiotics at low sub-inhibitory concentrations represents another major route for accelerated horizontal transfer and dissemination of antibiotic resistance genes. It was noted more than two decades ago, that sub-inhibitory concentrations of ß-

lactams enhanced the transfer of tetracycline resistance plasmids in *Staphylococcus aureus* by up to 1000-fold (Barr et al. 1986). Previous growth of a donor *Bacteroides* strain on low concentration of tetracycline also accelerated the mobilization of a resident non-conjugative plasmid by chromosomally encoded tetracycline conjugal elements (Valentine et al. 1988). The exposure of donor *Bacteroides* cells to low concentration of tetracycline appears to be a pre-requisite for the excision of the CTnDOT family of conjugative transposons from the chromosome and conjugal transfer of the excised elements since virtually no transfer occurs without tetracycline induction of donor cells (Stevens et al. 1993; Whittle et al. 2002). Incorporation of tetracycline at sub-inhibitory concentrations in the mating medium also substantially enhances *Tn*916-mediated conjugal transfer (Showsh and Andrews 1992). The similar stimulatory effect of tetracycline on conjugation transfer was also demonstrated for the conjugative transposon *Tn*925 (Torres et al. 1991). These *in vitro* results have been reproduced in *in vivo* models as well. In gnotobiotic mice, for example, the presence of a low concentration of tetracycline in the drinking water resulted in a 10-fold increase in the frequency of transfer of conjugative transposon *Tn*1545 from *Enterococcus faecalis* to *Listeria monocytogenes* in the digestive tract (Doucet-Populaire et al. 1991). In gnotobiotic rats, the presence of tetracycline was the major factor causing higher numbers of *Tn*916 transconjugants (Bahl et al. 2004) suggesting that the enhancement of conjugal transfer of antibiotic resistance-carrying transposons in the presence of sub-inhibitory concentration of antibiotics is not only an *in vitro* phenomenon, but can also take place in the gut ecosystem. At present, however, the transfer process has been studied in gnotobiotic animals, which may not be representative of conditions occurring in the normal gut containing a dense and diverse bacterial population. Further development of sensitive and accurate molecular tracking techniques will allow study of this process in conventional animals.

Co-selection of antibiotic resistance genes

Many antibiotic resistance genes reside on large self-transmissible genetic elements such as conjugative plasmids and transposons, which have sufficient capacity to carry multiple genes, including those encoding antibiotic, heavy metal and biocide resistances. The physical linkage between the R plasmids and resistances to heavy metals was reported almost 40 years ago (Smith 1967). Subsequent research demonstrated that the genetic linkage between antibiotic resistance and mercury resistance in enterobacteria had occurred prior to the late 1950s in Japan (reviewed in Liebert et al. 1999). This genetic element, *Tn*21 encodes a mercury-resistance operon, transposition functions, and resistances to streptomycin/spectinomycin and sulphonamides. The resistances genes and other Open Reading Frames (ORF's) are carried on an integron, thus allowing capture of antibiotic-resistance cassettes that can be thought of as portable, transferable multi-gene transfer casettes carried by bacteria. Interest in possible co-selection of antibiotic resistance genes by exposure to mercury was sparked by a publication suggesting that exposure to mercury from dental amalgams increased the incidence of multiple antibiotic resistance plasmids in the normal microbiota of non-medicated subjects (Summers et al. 1993). Despite intensive follow up studies on the possible link between exposure to mercury from dental fillings and antibiotic resistance, the results remain controversial (Osterblad et al. 1995; Lorscheider et al. 1995; Edlund et al. 1996). The contribution of co-selection by mercury was difficult to ascertain because of very high levels of antibiotic resistance already present in the gut of control groups.

In order to survive under various environmental conditions, many of which are stressful, cells have a repertoire of genes that they can choose to express or silence

according to their needs. Among this vast collection of genetically controlled networks widely present in bacteria, the SOS response is an inducible DNA repair system that allows bacteria to survive sudden increases in DNA damage (Michel 2005). The name is derived from the group of SOS genes that are regulated by repressor binding. Stress has been shown to induce the mobility of transposons and insertion sequences (Levy et al. 1993; Ilves et al. 2001). In particular, the SOS response, which is induced by DNA damaging agents such as mitomycin C and antibiotics such as fluoroquinolones and dihydrofolate reductase inhibitors, increases the rate of horizontal transfer of antibiotic resistance genes more than 300-fold (Beaber et al. 2004). More importantly, the use of the SOS response-inducing antibiotics may co-select other antibiotic resistance genes that are physically linked on a mobile genetic element (Hastings et al. 2004). Molecular mechanisms of stress-induced mutagenesis and lateral gene transfer are still under investigation but the reduced use of SOS-response inducing antibiotics at least in food animals and as growth promoters, seems a reasonable method to limit the dissemination of antibiotic resistance genes to other bacteria and the environment.

Summary and research shortfalls

Fate and transport of antibiotic residues in the environment

1. Antibiotics used in animal agriculture can enter the environment via a number of routes including the drug manufacturing process, disposal of unused drugs and containers, and through the use and application of waste material containing the drug. Many antibiotics are not completely absorbed in the gut resulting in the parent compound and its metabolites being excreted in feces and urine. The land application of livestock effluent 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.
2. Antibiotics are generally very water soluble and have several functional groups, that depending on the pH, affects their behavior in the environment. The octanol-water partition coefficient (K_{ow}) is used as a general measure of hydrophobicity, and most antibiotics have log K_{ow} values less than 5 indicating that they are relatively non-hydrophobic. Mechanisms other than hydrophobic partitioning such as cationic exchange, surface complexation, and hydrogen bonding are the likely mechanisms for antibiotic sorption to soils.
3. Laboratory investigations have documented the physico-chemical interactions of most veterinary antibiotics with soil and soil components. These investigations began in the early 1960's and continue today. Some of the earlier field studies used High Pressure Liquid Chromatography (HPLC) analysis which does not provide the selectivity and sensitivity required to detect antibiotics at concentrations commonly found in the environment. There is limited field information on the fate and transport of antibiotics and much of the information is simply occurrence or frequency data. The development of new sample concentration and analytical techniques, such as solid phase extraction (SPE) and Liquid Chromatography-Mass Spectrometry (LC-MS), that can detect antibiotics at environmentally relevant concentrations, has lead to determining the occurrence of many veterinary antibiotics in various matrices (soil, sediments, water, sludge).
4. Antibiotics are generally not very persistent in manure with half-lives generally less than 100 days. Thus, the application of manure to agricultural fields will likely introduce antibiotic breakdown products to the environment.
5. In many of the runoff investigations, manure was surface applied without

incorporation into the soil. Current practices in many areas of the United States incorporate manure into the soil during application. In general, the available field data suggests that antibiotics are relatively immobile in the environment and when detected are at the lower µg/L range. Pharmaceutical companies in many cases have significant data on the environmental fate of many antibiotics that are not publically available.

Fate and transport of antibiotic resistant bacteria and genes in the environment

1. The differences of manure storage systems and land application methods can affect the dissemination of antibiotic resistance genes into the environment. Enteric bacteria in the manure that are introduced to the environment, can survive in soil for as long as 8 weeks to a year but vary depending on species and temperature. DNA from viable, as well as dead bacteria, could be a source of antibiotic resistance genes.
2. Phylogenetic analysis indicated that antibiotic resistance genes have evolved and have been maintained in bacteria prior to the modern antibiotic era, even though the origin and purpose of these genes is not yet clear. These resistance genes are exchanged among a broad range of bacteria. There is evidence that increased occurrence of lateral gene transfer has occurred recently, most likely accelerated by indiscriminate use of antibiotics. Trace amounts of antibiotics or other chemicals such as heavy metals could act as a selection pressure for maintenance and transfer of antibiotic resistance genes.

Transport of bacteria and viruses into groundwater

1. Bacteria and viruses have great potential to move deep into the subsurface environment. Approximate 20-25% of groundwater sources in the United States are contaminated with microbial pathogens which include more than 100 types of viruses. The processes of viral transport are extremely complex. Several studies concluded that application of animal manure to soil can readily lead to groundwater contamination with fecal bacteria, especially under moist soil conditions, and that macropores are important in the transport of bacteria through soil. However, many studies have shown that bacterial indicators do not accurately reflect the occurrence of viruses in aquatic systems.
2. Mathematical models representing bacteria and virus transport are publicly available. These models might not adequately predict whether an aquifer will be contaminated by bacteria. Since the processes simulated by computer models are highly heterogeneous on a field-scale and differ widely by virus type, model outputs are associated with a great deal of uncertainty. Virus transport models are either too complex or the model predictions too uncertain for routine use by drinking water utilities for determining whether wells are in regulatory compliance for acceptable virus concentrations. It is advised that these models should be used for research purposes only.

Future Research

Antibiotic residues in the environment

1. Data are very scarce concerning the occurrence, fate and transport of antibiotic breakdown products in the environment. In addition, little information is available concerning the biological activity of the breakdown products or for parent antibiotics that are sorbed to soil components. More sensitive techniques are required to assess the persistence of antibiotics and their residues in the environment.

2. Determining what concentrations of antibiotics are environmentally relevant is important in making inferences about the actual impacts of antibiotics detected in the environment. More laboratory and field research is needed to determine the biological activity of antibiotic degradation products.

Antibiotic resistant bacteria and genes in the environment

1. More research is needed on how bacterial populations are partitioned in pit/lagoon environments. The proportion and types of bacteria associated with the solid phase and liquid phases are not well understood. This has practical implications for waste management from both an antibiotic resistance perspective as well as pathogen reduction, and will become of much greater importance as the industry inevitably moves toward separation of liquids and solids in order to comply with phosphorus-based limits on land application of manure.
2. In addition, more research is needed to obtain a thorough understanding of the effect of soil heterogeneity on spatial and temporal patterns of microbial communities and the potential for gene transfer.
3. Very little is known about gene transfer between bacteria in the environment and the maintenance or reduction of antibiotic resistance in manure storage environments. Does the type of waste and management of waste holding facility enhance or retard gene transfer? Are pit/lagoon solids a more concentrated environment of residues and antibiotic resistance genes and could this be a good model system to address gene transfer and persistence issues?
4. Many studies used DNA extracted from environmental samples to infer conditions present in soils at the time of sampling. This DNA likely originated from both live and dead organisms. That proportion of DNA originating from dead organisms could represent historical DNA that is not representative of present practices. Thus an RNA or protein based approach would provide a more accurate picture of current conditions and practices. To accurately represent viable organisms and their genomes in bulk environmental samples requires validation of RNA and protein extraction protocols. Quantitative measurements of antibiotic resistance gene levels are needed, in addition to measurements of diversity and frequency, in order to address questions of accumulation and persistence of these determinants.
5. In order to obtain key data on gene flow, higher throughput and more sequencing of antibiotic resistance genes both from known organisms and bulk genomic DNA from different environments is essential. Among the determinants detected in environmental samples, what are considered background versus introduced genes?
6. Preliminary results show a wide taxonomic range of tet-resistant environmental isolates, and we should take a closer look at the sequences of the determinants carried by these isolates. Multiple genes conferring resistance to the same drug are found in some environmental isolates; is this a more common occurrence among certain taxa? Are these functionally active genes in these cells? The effect of antibiotic residues on bacterial growth or on the selection of resistance should be examined. Identify natural selection mechanisms including co-selection in the environment. The cultivation effort for antibiotic resistance has mainly focused on pathogenic bacteria. Carriers of antibiotic resistance genes in the environment should be identified to evaluate the gene transfer between bacteria in the gut and manure and into soil bacteria.

Viral and bacterial transport

1. To estimate the transit time and dilution/concentration of contaminants in groundwater a modeling approach should be employed.
2. However, no single virus or bacterium type has transport characteristics that can be adequately modeled to accurately describe the groundwater transport of the whole population. The most confident and feasible future assessment is to delineate a conceptual transport model based on more accurate and efficient data from the simpler, standardized, and more affordable testing methods.

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Table 1. Survey of most commonly used antibiotics in animal production (millions of pounds)
(AHI 1991)

Antibiotic class	Amount	
Ionophores/Arsenicals		9.70
Other antibiotics -includes macrolides, lincosamides, polypeptides, streptogramins, cephalosporins	5.90	
Tetracyclines	3.23	
Penicillins	0.871	
Sulfonamides		0.471
Aminoglycosides	0.240	
Fluoroquinolones	0.038	

Table 2. 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

[a]approximately 48% of total antibiotic fed to swine in 1990's
[b]used in 52% of swine operations reported in a 1995 USDA survey

Table 3. Chemical and fate properties of selected veterinary antibiotics (modified from Tolls 2001; Baxall et al. 2004; Beausse, 2004).

Antibiotic	Solubility (water)	log KOW	Log K_{OC}	Kd (L/kg)	Chemical/degradation information	Mobility
Lincomycin (hydrochloride salt)	freely	ND	ND	ND	pKa 7.6 In spiked soil 10 ppm undetectable after 11 weeks and 80% lost after 7 weeks	Immobile especially in high organic matter/clay soil based on manufacturer column tests.
Sulfathiazole	0.6 g/L	0.05	2.30	4.9	pKa_1 2, pKa_2 7.24	Medium mobility based on Kd
Sulfamethazine	1.5 g/L	0.89	1.78 - 2.32	0.6-3.1	pKa_1 2.65, pKa_2 7.65 Is biodegradable but persistent in water phase	High to medium based on Kd
Tylosin	5 g/L	3.5	2.74 - 3.90	8.3-240	pKa 7.1 Stable at pH 4 to 9, <pH 4 desmycosin is formed.	Low to immobile based on Kd
Virginiamycin	54-80 mg/L	1.5-1.7	2.7-2.8	ND	$T_{1/2}$: 87-173 days 89% inactivated within 18 days and ND after 84 days. Activity decreases rapidly in water and increasing temperature. In alkaline pH degrades.	Immobile due to low water solubility, high lipophilicity and rapid inactivation in soil.
Tetracycline	1.7 g/L	-1.19	ND	>400-1620	pKa_1- 3.30, pKa_2-7.68, pKa_3-9.69	Immobile based on Kd
Chlortetracycline	0.6 g/L	-0.62	ND	282-2608	$T_{1/2}$ in manure 1 week at 37 C& >20 days at 4 or 28 C 85% of CTC added to soil was recovered.	Immobile based on Kd
Oxytetracycline	1 g/L	-1.22	1.2-5.0	0.3-1030	pKa_1 3.27, pKa 7.32, pKa_3 9.11 Stable compared to CTC	Immobile based on Kd

Ciprofloxacin	30 g/L	0.4	4.78	430	pKa_1 5.9, pKa_2 8.89	Immobile based on Kd
Enrofloxacin	130 g/L	1.1	4.22 - 5.89	260-6310	pKa_1 6.27, pKa_2 8.3	Immobile based on Kd
Penicillin	4 g/L	1.87	ND	ND	pKa 2.79 Unstable rapidly degrades to penicilloic acid. $T_{1/2}$ <7 days	Weakly sorbed to soils

ND - not determined or not found in the literature reviewed

Table 4. Antibiotic persistence in manure (modified from Boxall et al. 2004)

Antibiotic class	Half-life (days)
Aminoglycosides	30
β-lactams	5
Macrolides	<2-21
Quinolones	100
Sulfonamides	<8-30
Tetracyclines	100

Table 5. Antibiotic concentrations detected in samples of swine and poultry lagoon manure

Antibiotic	Concentration	Reference
Lincomycin	2.5-240 (µg/L)	Camagnolo et al. 2002
Chlortetracycline	68-1000 (µg/L)	Camagnolo et al. 2002
	0.1 (mg/kg)	Hamscher et al. 2002
	<0.5-1.0 (mg/kg)	Hamscher et al. 2005
Tetracycline/Oxytetracycline	25-410 (µg/L)	Camagnolo et al. 2002
	4.0 (mg/kg)	Hamscher et al. 2002
	14.1-41.2 (mg/kg)	Hamscher et al. 2005
Sulfamethazine	2.5-380 (µg/L)	Camagnolo et al. 2002
	0.13-8.7 (mg/kg)	Haller et al. 2002
	0.2-7.2 (mg/kg)	Hamscher et al. 2005
Sulfadimethoxine	2.5 (µg/L)	Camagnolo et al. 2002
Erythromycin	2.5 (µg/L)	Camagnolo et al. 2002
Penicillin G	2.1-3.5 (µg/L)	Camagnolo et al. 2002

Table 6. Detection frequency and maximum concentrations of selected antibiotics detected in filtered stream samples

Antibiotic	Frequency of Detection (%)	Maximum Conc. (µg/L)
Trimethoprim	27.4	0.30
Erythromycin-H	20.2	1.5-1.7
Lincomycin	21.5	1.7
Sulfamethoxazole	19.0	0.52
Tylosin	13.5	0.28
Roxithromycin	4.8	0.18
Ciprofloxacin	2.6	0.03
Chlortetracycline	2.4	0.69
Oxytetracycline	1.2	0.34

Table 7. Persistence times of pathogenic bacteria in different environments

Environment	Temperature	Current understanding of survival time			
		Salmonella	*Campylobacter*	*Yersinia enterocolitica*	*E. coli* 0157:H7
Water	≤0º C	~ 6 months	≤ 8 weeks	> 1 year	> 300 days
	~5º C	~ 6 months	1 week - 4 months	> 1year	> 300 days
	~30º	~ 6 months	~4 days	~10 days	~84 days
Soil	≤0º C	> 6 months	≤ 28 weeks	> 1 year	> 300 days
	~5º C	≤ 28 weeks	~ 2 weeks	> 1 year	~ 100 days
	~30º	~ 4 weeks	~ 1 week	~ 10 days	~ 2 days
Slurry		≤ 75 days	> 112 days	≥ 28 days	≤ 100 days
Dry surfaces		≤ 7days	~ 1 day	~ 1 day	~ 1 day
References		Santo Domingo et al., 2000 Mitscherlich and Marth, 1984 Zibilske and Weaver, 1978 Guo et al., 2002	Buswell et al., 1998 Rollins and Colwell, 1986 Mitscherlich and Marth, 1984 Blaser et al., 1980	Karapinar and Gonul, 1991 Chao et al., 1988	Zhao et al., 1995 Tauxe, 1997 Cieslak et al., 1993 Wang and Doyle, 1998

Figure Legends

FIG. 1. Phylogenetic analysis of tetracycline resistance genes encoding ribosomal protection proteins performed using the neighbor-joining or distance method to construct the phylogeny representing the relationship between the gene sequences. The sequence of the *A. aeolicus* fusA gene for translation elongation factor EF-G is used as the outgroup for rooting the tree and help to define the pathways for the evolution of genes encoding ribosomal protection proteins. Numbers above each node show the percentage of tree configurations that occurred during 1000 bootstrap trials. Bootstrap values provide a statistical estimate of how reliable the branch points and subsequent groupings are. The scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of sequences used in this analysis are given in parenthesis.

FIG. 2. Unrooted phylogenetic tree of macrolide resistance genes encoding methylases produced using the neighbor-joining method. Unrooted trees specify the relationships between the gene sequences included in the analysis. Numbers above each node show the percentage of tree configurations that occurred during 1000 bootstrap trials. The scale bar is in fixed nucleotide substitutions per sequence position. GenBank accession numbers of sequences used in this analysis are given in parenthesis.