DISSERTATION

ANTIBIOTIC RESISTANCE GENES (ARG) AS ENVIRONMENTAL CONTAMINANTS: OCCURRENCE IN CACHE LA-POUDRE RIVER WATERSHED AND RESPONSE TO BIOLOGICAL TREATMENT

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY RUOTING PEI ENTITLED ANTIBIOTIC RESISTANCE GENES (ARG) AS ENVIRONMENTAL CONTAMINANTS: OCCURRENCE IN CACHE LA-POUDRE RIVER WATERSHED AND RESPONSE TO BIOLOGICAL TREATMENT BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

ANTIBIOTIC RESISTANCE GENES (ARG) AS ENVIRONMENTAL CONTAMINANTS: OCCURRENCE IN CACHE LA-POUDRE RIVER WATERSHED AND RESPONSE TO BIOLOGICAL TREATMENT

In this study antibiotic resistance genes (ARG) were investigated as emerging environmental contaminants. The spread of ARG is of major concern because they are the agents that allow pathogenic microorganisms to become resistant to antibiotics, thus rendering antibiotics ineffective for fighting disease. ARG were quantified in various environmental compartments, which provided a means to assay the environmental impact of corresponding antibiotics in the environment, which originate from both human and agricultural sources. An initial baseline study was conducted and demonstrated a relationship between the number of ARG present in the sediments of the Cache La Poudre (Poudre) River and the relative levels of adjacent urban and agricultural activities. Five Poudre River sites of various urban and agricultural impacts were monitored for quantities of four ARG [*tet*(W), *tet*(O), *sul*(I), and *sul*(II)] on five sampling dates for over a one year period using quantitative real-time polymerase chain reaction. It was found that while a consistent spatial pattern was observed with the levels of ARG relating to the level of human and agricultural activity, there was no clear temporal pattern in the levels of ARG. It was also observed that dairy lagoon water had significantly higher concentrationsof ARG than adjacent irrigation ditch water, which had greater

concentrations than the neighboring Poudre River sediments, suggesting a potential pathway for the spread of ARG from farms to the river. To take a step toward minimizing the spread of the ARG from agricultural sources, the response of six ARG corresponding to three antibiotic classes (tetracycline, sulfonamide, and macrolide) to biological treatment was studied in anaerobic and aerobic lagoon water at ambient and reduced temperatures. It was found that *tet*(W), *tet*(O), *sul*(I), and *sul*(II) ARG responded differently to the different aerobic/anaerobic and temperature treatments. The two macrolide ARG, *ere*A and *msr*A, were present at very low levels and showed no response to any of the treatments. Overall, anaerobic treatment was the most effective for achieving final ARG levels that were equal to initial levels. No treatments reduced ARG below the initial levels, while some treatments resulted in higher ARG concentrations. This study is the first to quantitatively explore the levels of various ARG in environmental compartments and to investigate the effect of biological treatment. Future work is needed to further characterize ARG pathways and behavior in the environment and to explore alternative approaches for reducing ARG present in animal waste prior to land-application.

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LIST OF ABBREVIATIONS

ARG: Antibiotic Resistance Genes

Ab: Antibiotic

CAFOs: Concentrated Animal Feeding Operations

CDC: Center for Disease Control and Prevention

COD: Chemcial Oxygen Demend

DO: Dissolved Oxygen

VRE: Vancomycin Resistance

MRSA: Methicillin Resistant *S. aureus*

MON: Monensin

OTC: Oxytetracycline

OECD: Organization for Economic Cooperation and Development

PBS: Phosphate Buffered Saline

Poudre: Cache La Poudre

PCR: Polymerous Chain Reaction

Q-PCR: Quantitative Real-time Ploymerous Chain Reaction

SMX: Sulfamethoxazole

TYL: Tylosin

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CHAPTER1: INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 THE PROBLEM OF ANTIBIOTIC RESISTANCE

Ever since antibiotics became widely available in the 1940s, they have revolutionized human health and modern medical history. After more than 50 years of widespread use, however, many antibiotics do not play the same role they once did. For example, not long after the introduction of penicillin, a bacterium known as *Staphylococcus aureus* began developing penicillin-resistant strains. By the end of the 1980s, 70 to 80 % of *S. aureus* isolates became resistant to penicillin (Atkinson *et al.*, 1984). Today, antibiotic-resistant strains of *S. aureus* bacteria, as well as various enterococci-bacteria that colonize the intestines, are common and pose a global health problem. Bacteria have a remarkable array of tools at their disposal to overcome antibiotics. Through natural selection, bacteria will develop resistance to virtually every antibiotic if given enough time and enough antibiotic use, so eventually resistance may occur to every antibiotic (Levy, 1998a). After bacteria have become carriers of antibiotic resistance genes (ARG), they can be spread rapidly through horizontal genetic exchange. This may take place through conjugation (transfer of DNA from one cell to another by direct cell to cell contact), transduction (DNA is transferred from one cell to another by a bacteriophage), or transformation (DNA is taken up from the external environment and incorporated into the genome; Hartl and Jones, 1998). The three processes yield DNA that can be replicated and passed on to progeny. Transposons are another mechanism

contributing to the spread of ARG; they are transposable elements that are small regions of DNA that can move from one place to another in the genome. Genes in transposons can be transferred between bacterial hosts by transposition into bacterial plasmids, which can then undergo conjugation (Hartl and Jones, 1998).

Each year in the United States, 160 million prescriptions are written for antibiotics and 22.7 million kg (25,000 tons) of antibiotics are prescribed, approximately 50 % for use by patients and 50 % for use in animals, agriculture, and aquaculture (Levy, 1997). Since the more an antibiotic is used, the greater the selective pressure on resistant microbes and the faster resistance spreads, the spread of ARG and the resulting appearance of drug-resistant pathogens is a growing threat to all people, especially in healthcare settings. Each year nearly 2 million patients in the United States acquire an infection in a hospital. Of those patients, about 90,000 die as a result of the infection (CDC, 2001). More than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the drugs most commonly used to treat them. Persons infected with drug-resistant organisms are more likely to have longer hospital stays and require treatment with second- or third-choice drugs that may be less effective, more toxic, and/or more expensive (CDC, 2001).

1.1.2 ANTIBIOTIC INPUTS TO THE ENVIRONMENT

Antibiotics are used in animals to prevent and/or treat diseases and to promote growth in livestock production facilities, such as concentrated animal feeding operations (CAFOs). A significant amount of these antibiotics (up to 75%; Elmund *et al.*, 1971; Feinman and Matheson, 1978) are excreted as active metabolites. Thus animal waste presents a major potential source of antibiotic input to the environment (Haapapuro *et al.*,

1997; Sweeten, 1992). Discharge of treated municipal, hospital, and veterinary wastewaters present additional sources.

The appearance of antibiotic contamination in the environment has recently been confirmed. A study conducted by the United States Geological Survey in 1999 and 2000 indicated that, out of a network of 139 streams across 30 states, 95 contained antibiotics, which was 80% of the streams sampled (Kolpin, 2002). These released antibiotics may contribute to the emergence of strains of disease-causing bacteria that are resistant to even high doses of these drugs (ASM, 2002; Chee-Sanford *et al.*, 2001; Goni-Urriza *et al.*, 2000; Guardabassi *et al.*, 1998), and thus may compromise the effectiveness of antibiotics in humans.

1.1.3 STUDIES IN COLORADO

In recent studies conducted at Colorado State University, the occurrence of four classes of antibiotics (tetracyclines, sulfonamides, macrolides and ionophores) has been investigated in environmental samples. Analysis to date has included natural water, animal and human wastewaters, irrigation ditches, and river sediments (Kim and Carlson, 2005; Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b). In particular, within the mixed-landscape of the Cache La Poudre (Poudre) River watershed, the presence of the above four classes of antibiotics in water and sediments were found to be related with urban and agricultural activity. Generally, the number of antibiotics present and their concentrations increased as agricultural and urban activities increased along the Poudre River from its pristine origins in the Rocky Mountains, where no antibiotic contamination has yet been found. Relationships could also be identified between antibiotics known to be specific to human or agricultural use,

and their most likely inputs (Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b).

Based on these results it is clear that studies are needed to assess the impact of antibiotics released into the environment and their potential ultimate effect on human health and the environment. First, there is a need for the development and application of methods to quantify the actual number of ARG in the environment. The quantity of ARG may serve as a good indicator of the bioavailability of antibiotics in the environment. Also, as suggested recently by Rysz and Alvarez (2004), the genes themselves could be considered to be "pollutants," as their wide-spread dissemination is clearly undesirable. In addition, to assess the environmental impact of the land application of animal wastes, the fate of these compounds in on-farm waste management facilities (such as lagoons) must be determined. Understanding the fate of antibiotics and ARG during treatment may help to guide the design of waste management facilities and reduce the environmental impact of land application of animal wastes.

1.2 LITERATURE REVIEW

1.2.1 MECHANISMS OF ANTIBIOTICS

1.2.1.1 Molecular Mechanisms of Antibiotic Resistance

Antibiotics that are available have three proven targets to effectively inhibit bacteria: cell wall synthesis, protein synthesis, and DNA replication (Walsh, 2000). Penicillins, cephalosporins, and the vancomycin family of glycopeptide antibiotics target the peptidoglycan layer in the cell-wall assembly. The first two target the enzymes involved in peptide crosslinking, while vancomycin ties up the peptide substrate and thereby prevents it from reacting with either the transpeptidases or the transglycosylases

(Williams, 1996). Both mechanisms cause the failure to make peptidoglycan crosslinks, which leads to a weaker wall that predisposes the treated bacteria to a lethal lysis of the cell wall layer. Given the large number of molecular steps involved in initiation, elongation and termination of protein assembly by the ribosome, it is not surprising that protein synthesis provides a multifaceted target for antibiotics. Antibiotics such as the erythromycin of the macrolide class, the tetracyclines and the aminoglycosides are in this category (Brisson, *et al.*, 1988; Chopra, 1985; Forumy *et al.*, 1996). The last target, DNA replication and repair is represented by a synthetic antibiotic: the fluoroquinolones, such as ciprofloxacin, which kill bacteria by targeting the enzyme DNA gyrase (an enzyme responsible for uncoiling the intertwined circles of double-stranded DNA that arise after each round of DNA replication) (Shen, 1993).

Bacteria can resist antibiotics through three strategies: 1) pump out the antibiotic, 2) alter the antibiotic structure, and 3) modify the target structure (Walsh, 2000). The efflux pumps are variants of membrane pumps used by all bacteria to move lipophilic or amphipathic molecules in and out of the cells. The drug is pumped out faster than it can diffuse in, so intrabacterial concentrations are kept low and ineffectual to make sure bacterial protein synthesis proceeds without impact. Bacteria that become resistant to tetracyclines commonly overproduce related membrane proteins that act as an export or efflux pump for the drug (Levy, 1992; Paulsen *et al.*, 1996). Equivalent drug efflux pumps have been observed in several bacteria, including *Staphylococcus*, which have the potential to become resistant to the erythromycin in the macrolide class of antibiotics (Paulsen *et al.*, 1996; Ross *et al.*, 1990). The second strategy of resistance is destruction of the structure of the antibiotic. The classic case is the hydrolytic deactivation of the β-

lactam ring in the penicillins and cephalosporins by modification of the hydrolytic enzyme β –lactamase by resistant bacteria (Philippon *et al.*, 1985). The third resistance strategy is alteration of the target in the resistant bacteria. For example, erythromycin-resistant bacteria can mono- or dimethylate a specific adenine residue, A2058, in the peptidyl transferase loop of the 23S RNA component of the ribosome. The modification is carried out by the methyl transferase enzyme Erm, which does not impair protein biosynthesis but does lower the affinity of all the members of the erythromycin drugs for the RNA. The Erm mechanism is the main resistance route in drug-resistant clinical isolates of *S. aureus* and is present in erythromycin-producing organisms as a self-immunity mechanism (Bussiere, *et al.*, 1998).

1.2.1.2 Mechanism of the Spread of ARG

Horizontal genetic exchange is one mechanism by which antibiotic resistance is spread. Horizontal genetic exchange is the "movement of genetic information between bacteria other than by descent" (Maiden, 1998). Horizontal genetic exchange that takes place through conjugation is a sexual process. Transduction (DNA is transferred from one cell to another by a bacteriophage) and transformation (DNA molecule is taken up from the external environment and incorporated into the genome) are two non-sexual means by which genes may be exchanged (Hartl and Jones, 1998). All three processes yield DNA that can be replicated and passed on to progeny.

Conjugation plays a large role in the spread of antibiotic resistance in bacteria. This process involves direct cell-to-cell contact of two bacterial cells, and the subsequent transfer of DNA. Although conjugation is usually considered to be a species-specific phenomenon, it has also been observed to occur between species that are unrelated. For

this reason, a large gene pool is available from which bacteria can exchange and acquire new genetic material (Guiney, 1984). Sex pili make contact between the donor and the recipient cell. Once the two cell walls are in contact, this allows a mating bridge to form. The plasmid DNA in the donor, possibly containing ARG, is nicked in one strand; this strand proceeds into the recipient cell by undergoing rolling-circle replication (Hartl and Jones, 1998). Complementary copies of plasmid DNA are produced in both the donor and the recipient cells. Finally, the linear plasmid in the recipient becomes circular and is ligated, and then both of the cells have a copy of the plasmid.

Transduction occurs when a bacteriophage carries DNA from one species to another. When a bacteriophage destroys its current host and invades a new one, it may carry pieces of chromosomal DNA or plasmids from the previous host. An occasional phage may carry some bacterial DNA. Recombination can then occur between the phage (carrying bacterial DNA) and the new host's bacterial DNA. Transfer of DNA thus occurs from one bacterial cell to another, carried by a bacteriophage (Lacey, 1984; Hartl and Jones, 1998). This provides another method for the spread of resistance among bacteria.

Transformation is yet another method of acquiring resistance. During transformation, bacterial cells take up DNA from the surrounding environment. Certain requirements exist in order for transformation to take place. First, exogenous DNA must be present in the immediate environment. Bacteria must have mechanisms that allow the DNA to be taken up through the bacterial cell walls. Also, the DNA must be incorporated into the chromosome of the host, often by homologous recombination. During homologous recombination, parts of the chromosome are replaced with related

DNA (Maiden, 1998). Restriction modification systems play a role in transformation as well as in conjugation. However, it is thought that since these modification systems generate both DNA ends and smaller fragments, restriction modification may actually increase the chance of recombination with incorporated fragments. This could occur because recombination occurs more frequently if the ends are homologous.

The basic mechanisms of transformation, transduction, and conjugation provide a starting point for more complex mechanisms of the spread of antibiotic resistance. For example, transposons are another mechanism contributing to the spread of ARG. Transposable elements are small regions of DNA that can move from one place to another in the genome. Therefore, these play a role in evolution of antibiotic resistance as well, by providing yet another method of genetic exchange. Transposable elements that contain genes in the central region are called transposons. This central sequence may contain ARG corresponding to one or more antibiotics, which makes multiple antibiotic resistance possible. Genes in transposons can be transferred between bacterial hosts by transposition into bacterial plasmids, which can then undergo conjugation (Hartl and Jones, 1998). Transposons make multiple antibiotic resistance not only possible, but very likely; this mechanism provides an easy and efficient way for the transfer of resistance to several antibiotics to be spread at one time.

1.2.2 THE POTENTIAL CRISIS OF ANTIBIOTIC RESISTANCE

About fifty years ago, antibiotics became widely available, and they revolutionized human health by eliminating many previously untreatable disease. But with each passing decade, bacteria continue to defy not only single but multiple antibiotics. In 1941, virtually all strains of *Staphylococcus aureus* worldwide were

susceptible to penicillin G, but resistance to this drug began to emerge very soon. By the end of 1980s, 70 to 80 % of *S. aureus* isolates were resistant to penicillin (Atkinson *et al.*, 1984). Methicillin and other semisynthetic penicillins were successful in treating penicillin-resistant *S. aureus* infections until the 1980s, when methicillin resistant *S. aureus* (MRSA) became endemic in many hospitals (Panlilio *et al.*, 1992). Since the emergence of MRSA, the glycopeptide vancomycin has been the only uniformly effective treatment for staphylococcal infections. Then in May 1996, the world's first documented clinical infection due to *S. aureus* with intermediate resistance to glycopeptides (glycopeptide-intermediate S. aureus) was diagnosed in a patient in Japan (Hiramatsu, 1997). Both the frequency of resistance in bacteria and the numbers of drugs to which they are resistant are increasing. Multidrug resistance marked the 1990s, as evidenced by the report by Glynn *et al.* in 1998. A strain of *Salmonella enterica* serotype typhimurium, known as definitive type 104 (DT104), in the United States was found to be resistant to five drugs (Glynn *et al.*, 1998).

As we have learned about the mechanisms and epidemiology of resistance to antimicrobial drugs, it has become clear that bacteria have a remarkable array of tools at their disposal to overcome antibiotics. Through natural selection, bacteria will develop resistance to virtually every antibiotic if given enough time and enough antibiotic use, so eventually resistance may occur to every antibiotic (Levy, 1998a). The time to resistance, however, may vary considerably. For example, penicillin-resistant *Streptococcus pneumoniae* took 25 years to emerge as a clinical problem, but fluoroquinolone-resistant Enterobacteriaceae became problematic after 10 years (Levy, 1998b). With some bacteria, resistance to new drugs has emerged much more rapidly.

The spread of antibiotic resistant pathogens is a growing problem in the U.S. and around the world. A recently report from World Health Organization (WHO) (WHO 2000) discussed on antibiotic resistance as one of the most critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance." According to the report, more than two million Americans are infected each year with resistant pathogens and 14,000 die as a result. Another report compiled by the U.S. Centers for Disease Control and Prevention (CDC) indicated that over 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the drugs most commonly used to treat them. Subsequently, people infected by drug-resistant organisms tend to stay in hospital longer and require treatment with other drugs that may be less effective, more toxic, and/or more expensive (CDC, 2001). The problem of the rapid spread of antibiotic resistance has reached an alarming extent. For example, vancomycin is currently considered to be the most powerful antibiotic of "last resort," yet within 10 years the incidence of vancomycin-resistant *Enterococci* (VRE) increased in the United States from 0% to 25% (CDC 2001b, Willems *et al.*, 2005).

The more an antibiotic is used, the greater the selective pressure on resistant microbes and the faster resistance spreads. Each year in the United States, 160 million prescriptions are written for antibiotics and 22.7 million kg (25,000 tons) of antibiotics are prescribed, approximately 50 percent for use by patients and 50 percent for use in animals, agriculture, and aquaculture (Levy, 1997). In a population of 275 million, this degree of use in terms of exposure is equivalent to nearly 30 prescriptions per 100 persons per year and to 4.1 kg (9 lb) of antibiotics per 100 persons per year. This enormous level of use of antibiotics has great potential for selecting for or enhancing the growth of

resistant strains. Even worse, perhaps only half of the use is appropriate, meant to cure bacterial infections and administered correctly— in ways that do not strongly encourage resistance. Researchers at the CDC have estimated that some 50 million of the 150 million outpatient prescriptions for antibiotics every year are unneeded (Levy, 1998a). For example, more than 80 % of the physicians present admitted to having written antibiotic prescriptions on demand to treat colds and other viral infections that cannot be cured by the drugs (Levy, 1998a). In addition, people often fail to finish the full course of treatment, or share leftovers with their family and friends in less than therapeutic amounts. The improper dosing will fail to eliminate the disease agent completely and will, furthermore, encourage growth of the most resistant strains, which may later produce hard-to treat disorders.

As mentioned above, over 50% of the 22.7 million kg (50 million lb) of antibiotics produced in this country is used in animals. Of this, more than 80 % by weight is used subtherapeutically for growth promotion; the rest is for therapy (Levy, 1998). Clearly, this long-term exposure to low doses for growth promotion is the perfect formula for selecting increasing numbers of resistant bacteria in treated animal, which may then pass the microbes to workers at the farm and, more broadly, to people who prepare and consume undercooked meat. A direct consequence for this is the ineffectiveness for the treatment of disease in humans. For instance, the use of avoparcin, a glycopeptide growth promoter, has selected for vancomycin-resistant *Enterococci* among animals (Klare *et al.*, 1995). The same vancomycin-resistant clone of *Enterococci* has been found in animals and people (Van den Bogaard *et al.*, 1997). Likewise, the use of the growth promoter virginiamycin has selected for *Enterococci* with resistance to it,

but more importantly, to quinupristin–dalfopristin (Synercid), an antibiotic that was recently approved for use in humans (Welton *et al.*, 1998). Therapeutic use of antibiotics in animals also leads to the selection of resistant strains of consequence to human health. For example, the resistance strains to fluoroquinolones spreads. Soon after fluoroquinolones were given to animals in the Netherlands, fluoroquinolone resistance appeared in another zoonotic organism, *campylobacter* (Endtz *et al.*, 1991). With the introduction of quinolones for use in poultry, resistant strains of campylobacter are now appearing in the United States (Smith *et al.*, 1998).

In agriculture, antibiotics are also applied to fruit trees for controlling or preventing bacterial infections. While the high concentrations may kill all the bacteria on the trees, the lingering antibiotic residues can encourage the growth of resistant bacteria that later colonize the fruit during processing and shipping. Besides the residues, the aerosols can also land on nearby trees and food plants, where they might be too dilute to eliminate full-blown infections but are still capable of killing off sensitive bacteria and thus giving the edge to resistant versions. The resistant bacteria can reach and survive in the intestinal tract of people through the food chain. This has been demonstrated by the research conducted by Denis E. Corpet of the National Institute for Agricultural Research in Toulouse, France. This research showed that when human volunteers went on a diet consisting only of antibiotic-free foods, the number of resistant bacteria in their feces decreased 1,000-fold (Levy, 1998).

In summary, the extensive worldwide exploitation of antibiotics in medicine, animal care and agriculture constantly selects for strains of bacteria that are resistant to the drugs. The fact that the spread of ARG is undesirable and has implications for human

health defines them as "contaminants". Therefore, we need to minimize the environmental impact of these contaminants. The main strategy for minimizing the spread of these contaminants is based on reducing the extent of exposure to antibiotics. The smaller exposure to antibiotics, the less likely the selection and transfer of resistance traits among environmental bacteria and the lower the probability that a resistant pathogen will arise (Levy, 1998b). Consumers and health care professionals should be educated to reduce the misuse of antibiotics for viral diseases. The use of antibiotics in animals should be limited to prophylaxis and therapy in a way that does not perpetuate the selection of resistance. Antibiotics used for controlling or preventing bacterial infections in crops and fruit trees should also be appropriate to prevent the spread of antibiotic resistant. The above strategies are our chief allies in preventing a potential crisis of antibiotic resistance (Levy, 1998b).

1.2.3 THE PATHWAYS OF ANTIBIOTICS AND ARG IN THE ENVIRONMENT

The pathways of different types of antibiotics are summarized in Figure 1 and explained as follows (information was collected and summarized from USDA website: http://www.usda.gov/wps/portal/usdahome): Antibiotics may roughly be divided into those used primarily in humans versus those used in animals. The animal antibiotics may further be subdivided into substances used as growth promoters for livestock production, therapeutics in livestock production, or as feed additives in fish farms. The antibiotics used by humans will enter the sewer system with the urine and feces and reach the wastewater treatment plant. Antibiotics entering into wastewater treatment plants may be divided among three possible fates: they could be mineralized to carbon dioxide and water; they could be retained in the sludge; or they may pass through the wastewater

treatment plant unchanged (Kim *et al.*, 2005; Ingerslev and Halling-sørensen, 2000; Pérez *et al.*, 2005). Antibiotics that have been retained in the sludge will be spread in the environment if the sludge is dispersed on fields. Run-off from soil could bring the antibiotics to aquatic environments. Antibiotics that pass through the treatment plant will end up as direct inputs to the aquatic environment. The antibiotics used for animals in CAFOs will mostly end up in manure, compost or lagoons used to treat animal waste. These antibiotics will affect soil environment after the manure is dispersed on the fields. Run-off of substances from the fields could also bring the antibiotics to an aquatic environment. It is possible that antibiotics dispersed on fields may be mineralized in the soil column or will reach the groundwater as parent compounds or metabolites. Antibiotics used in fishfarms will be exposed directly to the receiving waters, and as a result, affect the aquatic environment.

1.2.4 BIODEGRADATION OF ANTIBIOTICS

Significant amounts of antibiotics used for human and veterinary purposes (up to 75%; Elmund *et al.*, 1971; Feinman and Matheson, 1978) are excreted unchanged or as metabolites in feces or urine. Thus animal waste presents a major potential source of antibiotic input to the environment (Haapapuro *et al.*, 1997; Sweeten, 1992). A major route for entry of veterinary pharmaceuticals into watersheds is through land application of animal manure or effluent and spills of animal waste at facilities using these drugs (Boxall *et al.*, 2001; Daughton and Ternes, 1999). To assess the environmental impact of the land application of animal wastes, the fate of these compounds in the waste management facilities in the farm must be known. The most common practice for collection and storage of animal waste is some form of lagoon, pond or tank. Animal

wastes are commonly stored in lagoons for several months before land application as a fertilizer. Typical lagoons may have a reduction in solids, nutrients, odor and sludge volume (Zhang, 2001); they also act as aerobic or anaerobic bioreactors utilizing bacteria to decompose organic materials that make up the bulk of the waste, including the pharmaceuticals. During the biodegradation process, a chemical maybe either mineralized or transformed to other degradation products. There is a need for environmental engineers to understand the process of biodegradation of pharmaceuticals in order to design more effective animal wastewater lagoons, and to reduce their environmental effects.

Studies have investigated the degradation of certain antibiotics within different environmental contexts, such as manure lagoons, soil manure slurries, surface waters, animal excreta, soils, activated sludge and at different stages of sewage treatment. Kolz et al. (2005) investigated the disappearance of tylosin in lagoon slurry. The results of their study indicated that the majority of tylosin was rapidly sorbed and degraded, with 90% disappearance occurring in less than five days. Tylosin disappearance started with a rapid initial loss followed by a slow removal phase. Both biodegradation and abiotic dissipation occur, and most likely, the abiotic dissipation is due to the strong sorption during the rapid initial phase. Residual tylosin remained detectable after 8 months of incubation, indicating that degradation in lagoons is incomplete and residues will enter agricultural field during land application.

In the scenario of antibiotics in soil, the biodegradation process is affected by soil properties. Anaerobic conditions are known to prevail in soil that is more than 0.7 m in depth while aerobic conditions are believed to be present in large parts of the topsoil that

is less than 0.25 m in depth. Aerobic biodegradation in the topsoil is therefore believed to be the dominating process (Ingerslev and Halling-Sørensen, 2001). Sorption of the antibiotics to the soil particles may reduce the bioavailability and thus the biodegradability, as is observed with other chemicals (Hatzinger and Alexander, 1997). Organic materials in the soil can either enhance (Shimp and Pfaender, 1985) or reduce (Zaidi and Mehta, 1995) biodegradation. The soil biomass consists of a large number of many bacterial species and may therefore have a high capacity for biodegradation of antibiotics. This population can also be strongly affected by bacteria that are transferred from the manure to the soil as these bacteria previously might have been exposed to antibiotics and therefore may hold a capacity for biodegradation of antibiotics (Ingerslev and Halling-Sørensen, 2001). Studies designed to measure the biodegradability of tylosin in soil–manure slurries concluded that tylosin and its degradation products disappeared rapidly (Ingerslev and Halling-Sørensen, 2001). Degradation half-lives for tylosin in soil–manure slurries ranged between 4.1 and 8.1 days. It was also concluded in this study that mineralization of tylosin and its degradation products is likely to occur rapidly since their study methodologies were designed to cover and detect a wide range of possible metabolites.

In the scenario of antibiotics in water, their biodegradation behavior in surface water is determined by numerous factors such as temperature, availability of organic and inorganic nutrients (Alexander, 1994), and the presence of oxygen. The consumption and size of the biomass and the presence of suspended sediments in water are factors of particular importance (Ingerslve and Nyholm, 2000). Furthermore, it is well known that biodegradation processes are inhibited by the absence of oxygen and finally, as is well-

established, biodegradation rates depend on the concentration of the chemical being degraded (Alexander, 1985; Nyholm and Ingerslev, 1997). In the study conducted by Ingerslev *et al.* (2000) for a simulated surface water system, biodegradation of oxytetracycline (OTC) under aerobic conditions was initiated without a lag phase but had a very long half-life (37-175 days), while tylosin was biodegraded after a considerable lag phase and had a slower biodegradation rate. Under anaerobic conditions, OTC and TYL were degraded at lower biodegradation rates with longer lag periods in comparison to aerobic conditions. Adding sediment and activated sludge to water systems stimulated the removal of OTC and TYL, which was considered to be due to increased biomass concentration and sorption to the particles (Ingerslev *et al.*, 2000).

It is also important to evaluate the degradation rate of antibiotics in excreted feces. Studies conducted by Teeter and Meyerhoff (2002) indicated that TYL residues degraded rapidly in cattle, chicken, and swine excreta. TYL declined to less than 6.5% of the initial level after 30 days in all animal excreta tested and fell below the detection limits (2.23 ug/g) in chicken feces after a 30 day period. These rapid half-lives indicate that TYL residues will not be persistent in the environment. This result might be consistent with the low percentage of surface water containing TYL. The survey conducted by Kolpin *et al.* (2002) indicated the transient nature of TYL in the environment by analyzing 104 surface water samples from sites where TYL might be found. Only 13.5% of the samples assayed contained TYL, and those only had concentrations ranging from 0.04 to 0.28 mg/L.

The fate of antibiotics in the waste water treatment process is obviously very important since the effluent will be discharged directly to the environment. Studies have

been conducted to investigate the removal of antibiotics in wastewater treatment plants. Kim *et al.* (2005) concluded that sorption was found to be the principle removal mechanism for tetraycyline in active sludge, and no evidence of biodegradation for tetracycline was observed. Two lines of evidence were provided for this conclusion. First, the inhibited and noninhibited biomass showed strong similarities in the extended period time of degradation in the batch reactor that contained elevated tetracycline concentration and a tetracycline adapted sludge. Second, no tetracycline metabolites were observed during the extended biodegradation process. In contrast, biodegradation of sulfonamides in activated sludge occurred after lag phases of 7 to 10 days at 20 °C in the nonadapted sludge (Ingerslev and Halling-Sørensen, 2000). Compounds were degraded within a few days after the lag phase. Both lag phases and degradation rates were three to four times longer at 6 °C. The author also concluded that if bacteria are capable of degrading one sulfonamide substance, they may also degrade many other sulfonamides, since the adapted bacterial culture was able to degrade other sulfonamides in a rapid and uniform way. Thus it was concluded that there is no need to make detailed investigations of single sulfonamide drugs. There are very limited studies investigating the biodegradation of monensin (MON) to date.

In summary, OTC, SMX, MON and TYL are all not readily biodegradable based on the European system of standardized legal tests for assessing the biodegradability of chemicals (Alexy *et al.*, 2004). The European system tests are standardized methods for assessing the biodegradability of organic compounds using closed bottle tests and utilized by many European countries such as Germany and Denmark. However, the closed bottle test as Organization for Economic Cooperation and Development (OECD) 301D

described may not be used to conclude that drugs are persistent in the environment since they are only operated for 28 days without application of adapted biomass. Given a long enough time and presence of degraders, the biodegradation of these compounds could occur. However, compared to other known recalcitrant compounds (e.g., pentachlorophenol), the biodegradation of those compounds is slow. It is crucial for an assessment of their removal in a WWTP to take into consideration the hydraulic retention times in the various treatment stages. With typical values of between 8 and 12 h (Halling-Sørensen *et al.* 2000), this time period is most likely too short to provide a sufficient reaction time for complete elimination of these compounds. For this reason, despite potential biodegradation during sewage treatment, residual fractions of the compounds are still detectable in the final effluent.

1.2.5 RESPONSE OF ARG TO BIOLOGICAL TREATMENT

There has been very limited research conducted on the response of ARG to biological treatment processes. In a recent study conducted by Storteboom (2006), the response of ARG such as *tet*(W) and *tet*(O) to composting was investigated. It was concluded that there was no significant differences between high level and low level manure management with respect to the response of either *tet*(W) or *tet*(O), except for *tet*(W) in high level managed horse manure spiked with antibiotics. High level management was defined as amending, watering, and turning, while low level managements was defined as no amending, watering, or turning. Different patterns for the response of *tet*(W) and *tet*(O) were also observed. ARG *tet*(O) followed a first order degradation curve while ARG tet(W) actually increased in all treatments at first before finally decreasing. However, in dairy and beef manure, both *tet*(W) and *tet*(O) attenuated

continually in most treatments with time, again with no major difference between high level or low level manure management.

1.2.6 ARG STUDIED IN THIS RESEARCH

1.2.6.1 Tetracycline ARG *tet*(W) and *tet*(O)

Tet(W) and *tet*(O) are two of the eleven ribosomal protection genes that have been identified. They have been found in a variety of genera including Gram-positive, Gram-negative bacteria (Fletcher and Macrina, 1991; Depala and Roberts, 1995; Barbosa, *et al.*, 1999; Billington, *et al.*, 2002). They have been detected among a diverse range of environmental samples like swine feces, dairy lagoons, river sediments, irrigation ditch waters and even drinking waters (Aminov *et al.*, 2001, Pei *et al.*, 2006, Pruden *et al.*, 2006). Both *tet*(W) and *tet*(O) have been found to be associated with a conjugative transposon although tet(O) was originally discovered on plasmids only (Giovanetti *et al.*, 2003; Melville *et al.*, 2004; Brenciani *et al.*, 2004). It is interesting that many of these conjugative elements often code for rRNA methylase or macrolide efflux genes (Giovanetti *et al.*, 2003; Brenciani *et al.*, 2004). These conjugative element may allow antibiotic resistance genes to be maintained in the bacterial populations without selective pressure, while allowing flexibility and the ability to form new linkages between genes in the host bacteria (Luna and Roberts 1998).

1.2.6.2 Sulfonamide ARG *sul*(I) and *sul*(II)

sul(I) and *sul*(II) are ribosomal protection genes. The chromosomal mutations in the *dhps* genes can stop the selectivity of the sulfonamide to the enzyme dihydropteroate synthase (DHPS), which catalyzes the formation of dihydropteroic acid (folic acid) in

bacteria (Huovinen *et al.*, 1995). Both are plasmid-borne genes conferencing resistance to sulfonamide (Nakaya *et al.*, 1960; Lawn *et al.*, 1967; Swedberg and Sköld, 1983). To date, *sul*(I) and *sul*(II) are present in many different species, but mainly in Gram-negative bacteria in animal and human faeces, and have been shown to be distributed equally among resistant isolates (Huovinen *et al.*, 1995; Sköld 2000; Grap *et al.*, 2003, Guerra et al., 2003; Perreten and Boerlin, 2003; Infante *et al.*, 2005).

1.2.6.3 Macrolide ARG *ere*(A) and *msr*(A)

Ere(A) is efflux genes that allow the cell to pump out the macrolide while the *msr*(A) codes for an inactivating enzyme. *Ere*(A) has been found in plasmids or in the chromosome while *msr*(A) is often associated with plasmids (Matsuoka, et al., 1997; Lina, et al., 1999; Reynolds, et al., 2003; Sutcliffe and Leclercq, 2003). They have been identified in both Gram-positive and Gram-negative bacteria (Ojo *et al.*, 2006; Sutcliffe and Leclercq, 2003), and in both aeroboes and anaerobes (Reig *et al.*, 2001, Jalava and Marttila, 2004)

1.2.6.4 Detection of ARG in the environment

Various studies have confirmed the presense of ARG in surface waters, river sediments, municipal wastewater treatment facilities, irrigation ditch water, drinking water, animal waste lagoon systems and ground water underlying lagoon systems (Chee-sanford *et al.*, 2001, Pruden *et al*, in press). Studies have also demonstrated the seepage of ARG from waste lagoons to groundwater (Krapac, *et al.*, 1998), therefore, it is necessary to improve treatment technologies such as lagoons to reduce the spread of ARG, given that groundwater constitutes about 40% of the water used for public supplies

and provides drinking water for more than 97% of rural populations in the US (http://water.usgs.gov/wid/html/GW.html).

1.3 OBJECTIVES

The purpose of this study was four-fold: *(1)* to develop methods for quantifying the number of ARG in environmental samples; and *(2)* to apply these methods in assessing and quantifying ARG present in the sediments of the Poudre River, a model river system that is zonated with respect to pristine, urban, and agricultural impacts; *(3)* to apply these methods in assessing the spatial and temporal distribution of ARG within Poudre River watershed; *(4)* to apply these methods in determining the effect of biological treatment on three families of ARG. In particular, quantitative real-time polymerase chain reaction (Q-PCR) methods targeting tetracycline, sulfonamide, and macrolide resistance genes were developed and applied to investigate the fate of ARG. All of these families of ARG correspond to antibiotics that are widely used both in animals and in people (Aminov *et al.*, 2001, Huber, 1971; Perreten and Boerlin, 2003). A culture-based approach was also implemented as a broad method of quantifying resistant microbes, including resistance incurred by genes that may not have been previously described. Different environmental samples were collected within the watershed at different times and locations, and the ARG were quantified to ascertain their temporal and spatial distributions. Finally, quantification of ARG during biological treatment was also conducted to investigate the potential of "bioremediating" ARG.

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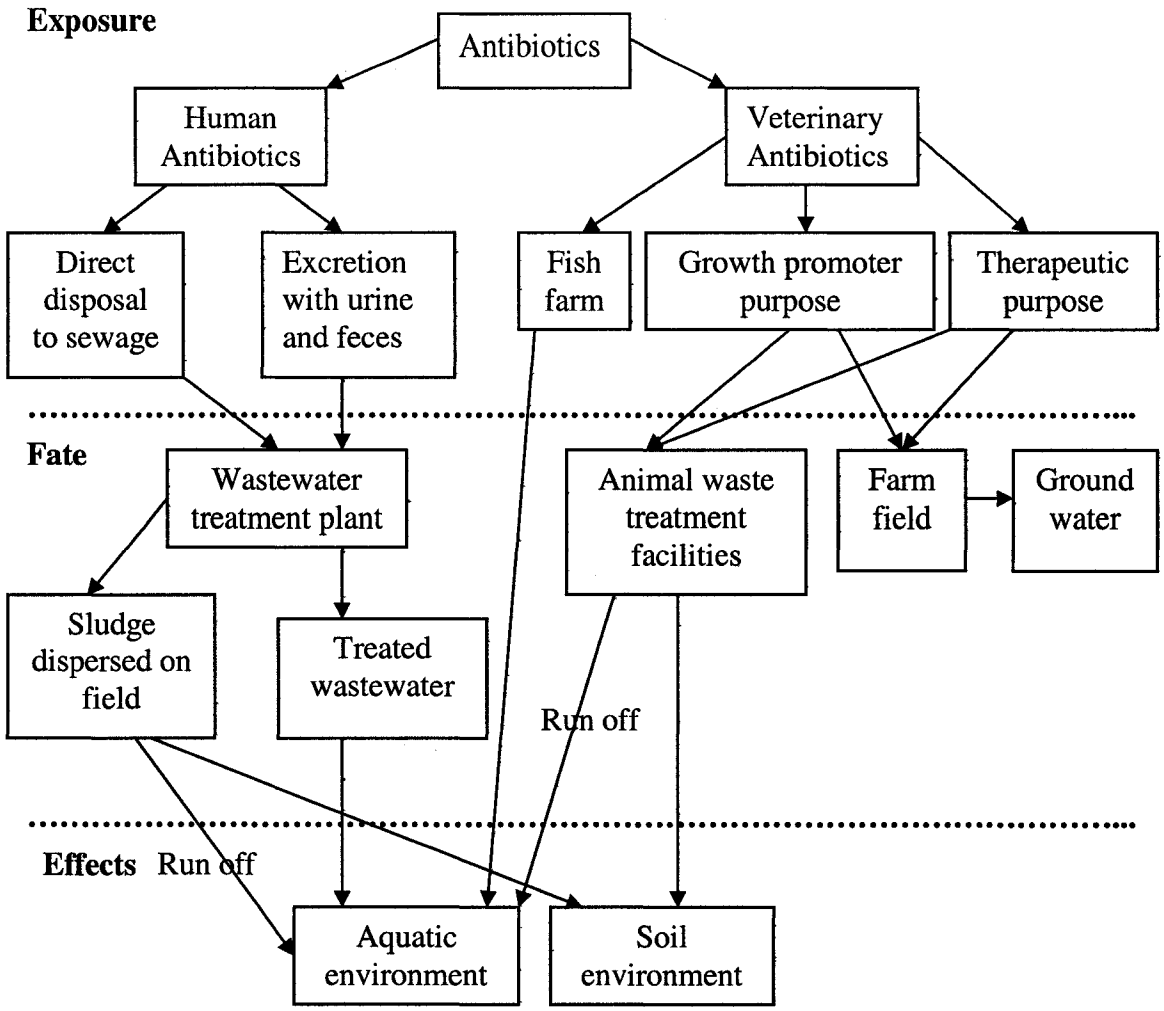

Figure 1. Pathways of antibiotics in the environment.

CHAPTER2: DEVELOPMENT OF METHODS TO QUANTIFY ANTIBIOTIC RESISTANCE GENES (ARG) IN THE ENVIRONMENT

2.1 REAL-TIME QUANTITATIVE POLYMERASE CHAIN REACTION (Q-PCR)

Real-time quantitative polymerase chain reaction (Q-PCR) is a technique used to quantify a relatively small amount of a target PCR product (DNA, cDNA or RNA). Q-PCR is based on the detection of the fluorescence produced by a reporter molecule which increases as the amplification reaction proceeds (Lee, *et al.*, 1993; Livak *et al.*, 1995). This occurs due to the accumulation of the PCR product with each cycle of amplification. These fluorescent reporter molecules include dyes that bind to the double-stranded DNA (i.e. SYBR® Green) or sequence specific probes (i.e. Molecular Beacons or TaqMan® Probes) (Morrison *et al.*, 1998; Heid *et al.* , 1996; Mhlanga and Malmberg 2001). The fluorescence of the reporter dye increases as the product accumulates with each successive cycle of amplification. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during the exponential phase where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. If a graph is drawn between the log of the starting amount of template and the corresponding increase in the fluorescence of the reporter dye during real-time PCR, a linear relationship is observed. By constructing a standard curve between the known number of initial copies of targeting templates and a fluorescence threshold value C_T, which is defined as the cycle number at

which the fluorescence emission exceeds the fixed threshold, the starting amount of templates can be quantified.

SYBR® Green (Molecular Probes, Inc, Eugene, OR) is the most widely used double-strand DNA-specific dye reported for Q-PCR. SYBR® Green binds to the minor groove of the DNA double helix. In the solution, the unbound dye exhibits very little fluorescence. This fluorescence is substantially enhanced when the dye is bound to double strand DNA. SYBR® Green remains stable under PCR conditions, and the optical filter of the thermocycler can be affixed to harmonize the excitation and emission wavelengths. Although these double-stranded DNA-binding dyes provide the simplest and cheapest option for real-time PCR, the principle drawback to intercalation based detection of PCR product accumulation is that both specific and nonspecific products generate a signal (Morrison *et al.*, 1998; Ririe *et al.*, 1997).

Specific detection of real-time PCR is done with oligonucleotide probes labeled with both a reporter fluorescent dye and a quencher dye. Various probes based on different chemistries are available for real-time detection. These include Molecular Beacons and TaqMan® Probes. TaqMan probes are oligonucleotides longer than the primers (20-30 bases long with a Tm value of 10 °C higher) that contain a fluorescent dye usually on the 5' end, and a quenching dye typically on the 3' end. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing (this is called FRET = fluorescence resonance energy transfer) (Hiyoshi, and hosoi, 1994; Chen *et al.*, 1997). Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. TaqMan probes are designed to anneal to an internal region of a PCR product. When the polymerase

replicates a template on which a TaqMan probe is bound, its 5' end exonuclease activity cleaves the probe (Holland *et al.*, 1991). This ends the activity of quencher (no FRET), and the reporter dye starts to emit fluorescence which increases in each cycle proportional to the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye.

Molecular beacons also contain fluorescent (FAM, TAMRA, TET, ROX) and quenching dyes (typically DABCYL) at either end but they are designed to adopt a hairpin structure while free in solution to bring the fluorescent dye and the quencher in close proximity for FRET to occur. They have two arms with complementary sequences that form a very stable hybrid or stem. The close proximity of the reporter and the quencher in this hairpin configuration suppresses reporter fluorescence. When the beacon hybridizes to the target during the annealing step, the reporter dye is separated from the quencher and the reporter fluoresces (FRET does not occur). Molecular beacons remain intact during PCR and must rebind to target every cycle for fluorescence emission. This will correlate to the amount of PCR product available (Vet *et al.*, 2002).

2.2 PRIMER DESIGN

At the time of this study, no PCR primers suitable for Q-PCR quantification of sulfonamide or macrolide ARG had previously been published. Therefore, it was necessary to design new primers and to develop new protocols for quantitative detection of these two ARG families in environmental samples.

2.2.1 SULFONAMIDE ARG PRIMER DESIGN

All currently available nucleotide sequences encoding sulfonamide ARG were downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/). Sequences were aligned with the multiple-sequence alignment program CLUSTALX 1.81 (Thompson *et al.*, 1997). Sequences within clusters were separately aligned and compared with each other in order to create consensus sequences for the primer design templates using FastPCR (Kalendar, 2004). The size of the PCR product was specified in the range of 100 bp to 350 bp for Q-PCR suitability. Specificity was verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Purified PCR products obtained from sediment DNA extract were cloned and sequenced in order to further confirm specificity. The four sets of *sul* primers from which verifiable target products were obtained are shown in Table 1. The distance tree of different clusters of sulfonamide ARG is shown in Appendix A, Figure 1.

2.2.2 MACROLIDE ARG PRIMER DESIGN

Macrolide ARG primers were designed as described above for sulfonamide ARG. All known gene sequences were downloaded from NCBI, a preliminary alignment was created using ClustalX, and a consensus sequence was built for each cluster. The consensus sequence was used for subsequent primer design using FastPCR Software (Kalendar, 2004). One set of primers was designed for each of following resistance mechanisms: alternate the antibiotic structure and efflux pump, and they are listed in Table 2. The clusters formed on the distance tree for macrolide ARG indicated that the nomenclature of macrolide ARG in NCBI was misleading and/or inaccurate, since the overlap among the three mechanisms of resistance within various clusters was revealed.

A table that summarizes the overlaps within different clusters was generated from the tree and included in Appendix A (Table 1).

2.3 DEVELOPMENT OF Q-PCR PROTOCOLS

Q-PCR protocols were developed using the intercalating dye SYBR Green I (Molecular Probes, Inc., Eugene, OR) to transition from traditional PCR to real-time Q-PCR. Because SYBR Green I binds non-specifically to all double-stranded DNA, it was important to ensure that the signals obtained were from specific products rather than primer-dimer or other non-specific products. This was accomplished by: ***(1)*** optimizing the Q-PCR conditions, including annealing temperature, amount of time for each step in the thermal cycling protocol, and primer concentration; ***(2)*** routinely implementing a melt curve analyses at the end of each program to verify the purity of the amplification product based on the melting temperature (T_m) and to discriminate against primer-dimers, which generally melt at a lower temperature than specific products; ***(3)*** verifying the expected product size by gel electrophoresis; and ***(4)*** cloning and sequencing of products formed. DNA sequence analysis of clone inserts was performed by SeqWright DNA Technology Service (Houston, TX). Specificity to target genes was confirmed using the BLAST alignment tool. All clones sequenced were verified to match the target genes. According to the above criteria, Q-PCR protocols were optimized to quantify the number of sulfonamide resistance genes *sul*(I), *sul*(II), *ere*(A) and *msr*(A), using the designed primers. Two tetracycline ARG encoding ribosomal protection factors, *tet*(W) and *tet*(O), were also transitioned to Q-PCR using previously described primers (Aminov et al., 2001; Chee-Sanford et al., 2001). Q-PCR reactions were performed with a SmartCycler (Cepheid, Sunnyvale, CA) in a 25 μL reaction mixture [1× SYBR Green PCR Master

Mix (Qiagen, Valencia, CA), 0.2 μM of each primer, and 1 μL of template] with a temperature program of 15 min at 95 °C (initial denaturing and Hot Start Taq activation), followed by 50 cycles of: 15 s at 95 °C; 30 sec at the annealing temperature [65 °C for *sul*(I), 57.7 °C for *sul*(II), 60 °C for *ere*(A), 60 °C for *msr*(A), 60 °C for *tet*(W), and 50.3 °C for *tet*(O)]; and 30 s at 72 °C (optical window on) followed by a final melt curve stage with temperature ramping from 60 °C to 95 °C.

2.4 Q-PCR QUANTIFICATION OF TOTAL EUBACTERIA

In order to account for potential differences in the overall size of the microbial population at the sites, a standard curve was generated using "universal" primers 1396F and 1492R and a TaqMan probe targeting Bacterial 16S rDNA as described in Suzuki *et al.* (2000).

2.5 Q-PCR STANDARD CURVES AND QUANTIFICATION

To generate positive controls for calibration, genes from the site sediment DNA extractions were PCR-amplified using traditional PCR and cloned using the TOPA TA Cloning Kit (Invitrogen, Carlsbad, CA). Clones containing inserts of the correct size were re-amplified using vector-specific primers and quantified by agarose gel electrohporesis. The log copy number of genes per μL DNA template solution for y axis of the calibration curve was calculated by equation (1).

$$\text{Log copy of genes / μL DNA} = \log\frac{b\times c}{L\times a\times 10^{12}} \qquad (1)$$

In which:

a = weight of kb DNA per pmol (1 kb DNA = 0.66 μg/pmol);

b = Avogadro's constant (6.022×10^{23}/mol)

L = length of template containing the target gene

c = concentration of template in μg/μL

Three approaches were taken to investigate the effect of the DNA extraction matrix and the potential for threshold cycle (C_T) value suppression. *(1)* To determine if the concentration of background DNA affects the amplification of the desired genes, two concentrations (140 ng/ul and 50 ng/μL) of additional non-target calf thymus DNA (BioRad, Hercules, CA) was spiked into the calibration control templates. Ten fold serial dilutions of both solutions (140 ng/μL *Calf thymus* + 3 ng/μL desired gene and 50 ng/μL *Calf thymus* + 3 ng/μL desired gene) were made up to 10^{-10} to determine the detection limit. For each type of gene, at least 6 replicates of ten different concentrations at two carrier DNA levels were used to create the standard curves. The detection limit for each gene was determined by the maximum dilution that produced a consistent C_T value (within 5%). The detection limits for *sul*(I), *sul*(II), *ere*(A), *msr*(A), *tet*(W), and *tet*(O) were observed to be 8, 7, 10, 8, 1, and 1 copies of genes per 25 μL assay, respectively. *(2)* The impact of PCR inhibitors potentially present in the DNA extract from the sediments was also investigated. DNA extract samples were spiked with known amounts of template and the difference in C_T value between the matrix and the control was determined. The matrix test was performed for each of the four resistance genes at two different concentrations of spiked template (10× and 100× of the concentration of the sediment DNA) and a consistent value was obtained for each DNA with respect to the percent suppression observed. *(3)* The impact of PCR inhibitors potentially present in the DNA extract from the diary lagoon water was investigated using dilution method. Serial dilutions 1:5, 1:10, 1:20, 1:40 and 1:60 were made from DNA extractions, and tested to

see if an appropriate dilution range could be found in which PCR inhibition could be eliminated. The results indicated that a dilution factor of 1:30 or 1:40 eliminated inhibition without diluting the DNA extracts lower than the detection limits for diary lagoon samples. The suppression for 16S rRNA genes was determined using the spiked method mentioned above for dairy lagoon water, because dilution did not effectively overcome inhibition.

Correlation coefficients were more than 99% ($R^2 > 0.99$) for all calibration curves, indicating that the relationship between the C_T value and the log copy number of genes per Q-PCR reaction was linear over 8 orders of magnitude. The levels of detection (LOD) for *sul*(I), *sul*(II), *ere*(A), *msr*(A), *tet*(W), and *tet*(O) were observed to be 8, 7, 10, 8, 1, and 1 copies of genes per 25 μL assay, respectively, which were then implemented as the level of quantification (LOQ) in the calibration curves. It was determined that the background DNA concentration had no significant effect on the amplification of target genes, as all C_T values fell within 5% of each other, regardless of the concentration of calf thymus DNA. However, the matrix study revealed that suppression of the C_T value by the DNA extract matrix was apparent and varied between sites. Little to no suppression was observed in DNA extract from the pristine site, while the matrix from the impacted sites lowered the gene copy number estimate by as much as 20× and an average of 4×. A suppression factor was determined for each DNA matrix and primer combination as noted in equation 2, where *c* is the number of templates enumerated in the control, *t* is the number of templates enumerated in DNA extraction matrix, and *m* is the number of templates enumerated when the same control is spiked into the DNA extraction matrix:

$$S = (c + t) \div m \quad (2)$$

Quantification of ARG at the sites thus took into account the mass of sediment extracted (0.5 g), any losses observed during DNA cleaning steps (as a fraction), and the PCR suppression factor. Therefore the copy of genes / gram of sample was determined according to equation 3:

$$\frac{gene\ copies}{g\ \text{sample}} = d \times \left(\frac{50\mu l\ DNA\ extract}{0.5\ g\ \text{sample}} \right) \times e \times S \quad (3)$$

where d is the copy of genes / μL DNA extract determined from calibration curve, e is the correction factor for losses during cleaning the DNA extract (1 / loss fraction), and S is the matrix suppression factor (eq. 2). Although several kits and conditions were tested for Q-PCR, in general it was found that Q-PCR was more susceptible to DNA extraction matrix inhibition and thus all other genes in this study were detected by traditional PCR, but could not be quantified by Q-PCR. For dairy lagoon samples, each sample was analyzed in triplicate for all six genes. Samples along with standards over 7 orders of magnitude were run in three replicates as well as negative controls in every run. The relative levels of ARG were determined as copies per milliters of lagoon water and normalized to 16S rRNA genes.

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TABLE 1. PCR primers designed in this study to target sulfonamide resistance gene families

Primer	Class trageted	Sequences	Regular PCR annealing temp (°C)	Q-PCR annealing temp (°C)	Amplicon Size (bp)	Resistance Mechanisms
Sul I-FW	*Sul I*	cgcaccggaaacatcgctgcac	55.9	65	163	modify the target structure
SulI-RV		tgaagttccgccgcaaggctcg				
Sul II-FW	*Sul II*	tccggtggaggccggtatctgg	60.8	57.5	191	modify the target structure
Sul II-RV		cgggaatgccatctgccttgag				
Sul III-FW	*Sul III*	tccgttcagcgaattggtgcag	60	61	128	modify the target structure
Sul III-RV		ttcgttcacgccttacaccagc				
Sul A-FW	*Sul A*	tcttgagcaagcactccagcag	60	60	299	modify the target structure
Sul A-RV		tccagccttagcaaccacatgg				

[a]FW, forward; RV, reverse

TABLE 2. PCR primers designed in this study to target macrolide resistance gene families

Primer	Class targeted	Sequences	Traditional PCR annealing temp (°C)	Q-PCR annealing temp (°C)	Amplicon Size (bp)	Resistance Mechanisms
ereA-FW	*ere*(A)	atgacgtggagaacgaccag	60	60	101	alter the antibiotic structure
*ereA*RV		ccgacaattcgggcgccctcaat				
msrA/B-FW	*msr*(A/B)	ctggaacggttgaaacggatggc	60	60	143	pump out the antibiotic
msrA/B-RV		accaccactcatactgtcggttg				

[a]FW, forward; RV, reverse

CHAPTER3: EFFECT OF RIVER LANDSCAPE ON THE SEDIMENT CONCENTRATION OF ANTIBIOTICS AND CORREPONDING ANTIBIOTICS RESISTANCE GENES (ARG)

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* Note: Antibiotic measurements were conducted by Sung-Chul Kim, all other analyses were conducted by Ruoting Pei

3.1 ABSTRACT

The purpose of this study was to quantify antibiotic resistance genes (ARG) in the sediments of the mixed-landscape Cache La Poudre River, which has previously been studied and shown to have high concentrations of antibiotics related to urban and agricultural activities. River sediments were sampled during two events (high-flow and low-flow) from five sites with varying urban and agricultural impact levels. Polymerase-chain-reaction (PCR) detection assays were conducted for four sulfonamide resistance gene families, using newly designed primers, and five tetracycline resistance gene families, using previously published primers. S*ul*(I), *sul*(II), *tet*(W), and *tet*(O) gene families were further quantified by real-time quantitative polymerase chain reaction (Q-PCR). Resistance to four classes of antibiotics (tetracyclines, sulfonamides, ionophores, and macrolides) was also investigated using a culture-based approach. The quantities of resistance genes normalized to the 16S gene copy number were significantly different between the sites, with higher resistance gene concentrations at the impacted sites than at the pristine site. Total resistant CFUs were over an order of magnitude lower at the pristine site, but differences were less apparent when normalized to the total CFUs. Six tetracyclines and six sulfonamides were also quantified in the sediments and were found to be highest at sites impacted by urban and agricultural activity, with no antibiotics detected at the pristine sit. To the knowledge of the authors, this study is the first to demonstrate a relationship between urban and agricultural activity and microbial resistance in river sediments using quantitative molecular tools.

3.2 KEYWORDS:

Antibiotic resistance; quantification; sediments; tetracyclines; sulfonamides

3.3 INTRODUCTION

Pharmaceutical compounds including antibiotics, hormones, and steroids are widely used to prevent and/or treat diseases and to promote animal growth in livestock production facilities, such as concentrated animal feeding operations (CAFOs). In particular, one half of the fifty million pounds of antibiotics produced each year in the U.S. is used for agriculture and 90% of these are used for growth promotion (Levy, 1998). A significant amount of these antibiotics (up to 75%; Elmund *et al.*, 1971; Feinman and Matheson, 1978) are excreted as active metabolites. Thus animal waste presents a major potential source of antibiotic input to the environment (Haapapuro *et al.*, 1997; Sweeten, 1992). Discharge of treated municipal, hospital, and veterinary wastewaters present additional sources. A recent study conducted by the United States Geological Survey in 1999 and 2000 indicated that, out of a network of 139 streams across 30 states, 95 contained antibiotics (Kolpin, 2002). Now that antibiotic contamination in the environment has been confirmed, a growing concern is that the release of antibiotics into the environment may contribute to the emergence of strains of disease-causing bacteria that are resistant to even high doses of these drugs (ASM, 2002; Chee-Sanford *et al.*, 2001; Goni-Urriza *et al.*, 2000; Guardabassi *et al.*, 1998). Therefore, there is a need to better understand the relationship between antibiotics in the environment and their ultimate impact on the emergence of microbial resistance.

Recently the occurrence of four classes of antibiotics (tetracyclines, sulfonamides, macrolides and ionophores) has been investigated in environmental samples such as

natural water, animal and human wastewaters, irrigation ditches, and river sediments (Kim and Carlson, 2005; Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b). In particular, within the mixed-landscape of the Cache La Poudre (Poudre) River watershed, the presence of these four classes of antibiotics in water and sediments were found to relate to urban and agricultural activity. Generally, the number of antibiotics present and their concentrations increased as agricultural and urban activates increased along the Poudre River from its pristine origins in the Rocky Mountains, where no antibiotic contamination has yet been found. Relationships could also be identified between antibiotics known to be specific to human or agricultural use, and their most likely inputs (Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b). Based on these results it is clear that studies are needed to assess the impact of antibiotics released into the environment and their potential ultimate effect on human health and the environment. In particular, there is a need for the development and application of methods to quantify actual resistance genes in the environment. The quantity of antibiotic resistance genes (ARG) may serve as a good indicator of the bioavailability of antibiotics in the environment. Also, as suggested recently by Rysz and Alvarez (Rysz and Alvarez, 2004), the genes themselves could be considered to be "pollutants," as their wide-spread dissemination is clearly undesirable.

The purpose of this study was two-fold: ***(1)*** to develop methods for quantifying ARG in environmental samples; and ***(2)*** to apply these methods in assessing and quantifying ARG present in the sediments of the Poudre River, a model river system that is zonated with respect to pristine, urban, and agricultural impacts. In particular,

quantitative real-time polymerase chain reaction (Q-PCR) methods targeting tetracycline and sulfonamide resistance genes were developed and applied. Both of these classes of antibiotics are widely used in both animals and in humans (Aminov *et al.*, 2001, Huber, 1971; Perreten and Boerlin, 2003). A culture-based approach was also implemented as a broad method of quantifying microbial resistance, including resistance incurred by genes that may not have been previously described. Finally, various tetracyclines and sulfonamides were also quantified in the sediment samples in order to compare the concentrations of antibiotics observed with those of ARG.

3.4 MATERIALS AND METHODS

3.4.1 STUDY AREA AND SAMPLING SITES

The Poudre River in northern Colorado has many unique attributes that make it an excellent model watershed to compare pristine, urban, and agricultural impacts. These attributes include: ***(1)*** the semi-arid nature of the front range of Colorado which results in only a small number of tributaries to the Poudre River; ***(2)*** the predominance of point sources in the urban landscape of Fort Collins versus non-point sources in the agricultural areas outside of the city; and ***(3)*** the source of the river being primarily snowmelt with minimal anthropogenic influences (Yang and Carlson, 2003; Yang and Carlson, 2004b). Five sites along the Poudre River were monitored and are summarized in Table 1. A map of this study site has been previously published (Yang and Carlson, 2003). Sample site 1 was chosen to represent the pristine area without anthropogenic influence and is located at the river origin. The river originates near the continental divide in the Rocky Mountain National Park and flows through steep mountainous terrain for approximately 43 miles before entering the front range city of Fort Collins. To date, no antibiotic compounds

have been detected at site 1 (Kim and Carlson, 2005; Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b). Site 2 is located just prior to where the river enters Fort Collins and represents an area influenced by light agricultural activities (a few small-scale dairies and cattle feed operations). Sample site 3 is located at the point of discharge for the Drake Wastewater Reclamation Facility after the river travels through the city and represents an urban area influenced by wastewater discharge. Sample site 4 is downstream of Fort Collins but upstream of the next urban area, Greeley, CO. Between Fort Collins and Greeley there are at least six CAFOs and multiple smaller animal feed facilities, therefore, site 4 represents an area heavily influenced by agriculture activities. Site 5 is downstream of Greeley and represents both urban and agriculture influences since Greeley is home to several CAFOs near the town and a beef processing plant that processes up to 3000 animals per day. Previous work has documented the occurrence of several different classes of antibiotic compounds, including: tetracyclines; sulfonamides; macrolides; β-lactams; and ionophores, some are as high as the microgram per kilogram range in the most heavily impacted sites 3-5 (Kim and Carlson, 2005; Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b).

3.4.2 SAMPLING

Sediment samples were collected in April 2004 (high-flow, 6.8 cfs) and February 2005 (low-flow, 0.8 cfs) along the Poudre River at the five sites. The upper sediments (about 5 cm) from the middle and two sides of a cross-section at each site were sampled and composited. Samples were collected using a shovel and mixed well in sterilized centrifuge tubes. Fifty-five grams of mixed sample at each site were stored at -80 °C for

subsequent molecular analysis. The remainder of the samples were processed within 8 hours for viable culturing.

3.4.3 HETEROTROPHIC PLATE COUNTS ON ANTIBIOTIC-SELECTIVE MEDIA

10× fold serial dilutions were prepared with a 10^{-1} dilution defined as one gram of sediment sample diluted in 9 mL of sterilized phosphate buffered saline (PBS, pH = 7.4). Diluted sediment samples were directly plated onto R2A agar medium (Difco, Sparks, MD) dosed with and without antibiotics. The concentrations of antibiotics were: chlortetracycline (CTC) 70.55 mg/L; oxytetracycline (OTC) 45.55 mg/L; meclocycline (MCC) 560.4 mg/L; sulfamethazine (SMT) 281.8 mg/L; sulfamethoxazole (SMX) 50.4 mg/L; erythromycin (Ery) 18.1 mg/L; Tylosin (TYL) 547.45 mg/L and monensin (MON) 11 mg/L. For water soluble antibiotics, the concentrations were chosen as five times the average reported LD_{50} values, whereas for insoluble antibiotics (SMX, SMT, MON, MCC and OTC) the concentrations were chosen as the maximum amount that dissolved readily in water and when subsequently added to the melted agar did not affect solidification. Plates were incubated at 37 °C for 48 hours, and thereafter at room temperature (shielded from light to prevent antibiotic degradation) for another week before determining the colony forming units (CFUs). This was in order to ensure that slow-glowing organisms were included.

3.4.4 DNA EXTRACTION AND PURIFICATION

DNA was extracted from sediment samples using the FastDNA Spin Kit for Soil (QBiogene, Carlsbad, CA). To aid in downstream quantification, exactly 0.5 gram of sample from each site was used for DNA extraction. The steps were followed according

to the protocol provided by the manufacturer. DNA was further purified using the Geneclean Spin Kit (QBiogene, Carlsbad, CA) to minimize PCR inhibition. The concentration of DNA before and after purification was determined and the percent recovery was recorded.

3.4.5 PRIMER DESIGN

All currently available nucleotide sequences encoding sulfonamide resistant genes were downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/). Proteins, antibiotic resistance islands, and plasmids encoding sulfonamide resistance genes were included mainly from pathogenic carriers, including: *Typhimurium* sp., *Salmonella typhimurium, Salmonella enterica, Streptomyces coelicolor, Streptococcus pneumoniae, Corynebacterium diphtheriae, Escherichia coli, Photorhabdus luminescens, Enterica serovar, Actinobacillus pleuropneumoniae,* and *Pasteurella multocida.*

Sequences were aligned with the multiple-sequence alignment program CLUSTALX 1.81 (Thompson *et al.*, 1997). Sequences within clusters were separately aligned and compared with each other in order to create consensus sequences for the primer design templates using FastPCR (Kalendar, 2004). The size of the PCR product was specified in the range of 100 bp to 350 bp for Q-PCR suitability. Specificity was verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Purified PCR products obtained from sediment DNA extract were cloned and sequenced in order to further confirm specificity. The four sets of *sul* primers from which verifiable target products were obtained are shown in Table 2.

3.4.6 PCR ASSAYS FOR DETECTION OF RESISTANCE GENES

Qualitative PCR assays were performed in order to determine which of the four sulfonamide ARG and the five tetracycline ARG encoding ribosomal protection (Aminov *et al.*, 2001) were detectable at the sites. These assays were carried out using the Eppendorf MasterTaq kit (Eppendorf, Westbury, NY) in a 25 μL volume reaction. The PCR master mixture consisted of 5 μL 5× buffer; 2.5 μL 10× buffer; 0.2 mM dNTPs; 0.2 μM primers; 1.75 units of Taq DNA polymerase, and 1 μL of template. The temperature program consisted of initial denaturing at 95 °C, followed by 40 cycles of: 15 s at 95 °C; 30 s at the annealing temperature [Table 2 for *sul* genes, 60 °C for *tet*(W), 50.3 °C for *tet*(O), 56 °C for *tet*(S), 43.9 °C for *tetB*(P), 43.9 °C for *tet*(T) - *note these vary from Aminov et al.* (2001)]; 30 s at 72 °C, and a final extension step for 7 min. at 72 °C.

3.4.7 REAL-TIME QUANTITATIVE PCR (Q-PCR)

Q-PCR protocols were developed using the intercalating dye SYBR Green I (Molecular Probes, Inc., Eugene, OR) to transition from traditional PCR to real-time Q-PCR. Because SYBR Green I binds non-specifically to all double-stranded DNA, appropriate measures were taken to ensure that the signals obtained were from specific products rather than primer-dimer or other non-specific products. Q-PCR protocols were optimized to quantify sulfonamide resistance genes *sul*(I) and *sul*(II), using the designed primers, and *tet*(W) and *tet*(O), using previously described primers (Aminov et al., 2001; Chee-Sanford et al., 2001). Q-PCR reactions were performed with a SmartCycler (Cepheid, Sunnyvale, CA) in a 25 μL reaction mixture [1× SYBR Green PCR Master Mix (Qiagen, Valencia, CA), 0.2 μM of each primer, and 1 μL of template] with a temperature program of 15 min at 95 °C (initial denaturing and Hot Start Taq activation),

followed by 50 cycles of: 15 s at 95 °C; 30 sec at the annealing temperature [65 °C for *sul*(I), 57.7 °C for *sul*(II), 60 °C for *tet*(W), and 50.3 °C for *tet*(O)]; and 30 s at 72 °C (optical window on) followed by a final melt curve stage with temperature ramping from 60 °C to 95 °C.

3.4.8 Q-PCR STANDARD CURVES AND QUANTIFICATION

To generate positive controls for calibration, genes from the site sediment DNA extractions were PCR-amplified using traditional PCR and cloned using the TOPA TA Cloning Kit (Invitrogen, Carlsbad, CA). The log copy number of genes per μL DNA template solution for y axis of the calibration curve was calculated by equation (1).

$$\text{Log copy of genes / } \mu\text{L DNA} = \log\frac{b \times c}{L \times a \times 10^{12}} \qquad (1)$$

In which:

a = weight of kb DNA per pmol (1 kb DNA = 0.66 μg/pmol);

b = Avogadro's constant (6.022×10^{23}/mol)

L = length of template containing the target gene

c = concentration of template in μg/μL

Two approaches were taken to investigate the effect of the DNA extraction matrix and the potential for threshold cycle (C_T) value suppression. ***(1)*** To determine if the concentration of a background DNA affects the amplification of the desired genes, two concentrations (140 ng/ul and 50 ng/μL) of additional non-target calf thymus DNA (BioRad, Hercules, CA) was spiked into the calibration control templates. Ten fold serial dilutions of both solutions (140 ng/μL *Calf thymus* + 3 ng/μL desired gene and 50 ng/μL *Calf thymus* + 3 ng/μL desired gene) were made up to 10^{-10} to determine the detection limit. For each type of gene, at least 6 replicates of ten different concentrations at two

carrier DNA levels were used to create the standard curves. The detection limit for each gene was determined by the maximum dilution that produced a consistent C_T value (within 5%). *(2)* The impact of PCR inhibitors potentially present in the DNA extract from the sediments was also investigated. DNA extract from each of the five sites was spiked with known amounts of template and the difference in C_T value between the matrix and the control was determined. The matrix test was performed for each of the four resistance genes at two different concentrations of spiked template (10× and 100× of the concentration of the sediment DNA) and a consistent value was obtained for each DNA with respect to the percent suppression observed.

Correlation coefficients were more than 99% ($R^2 > 0.99$) for all calibration curves, indicating that the relationship between the C_T value and the log copy number of genes per Q-PCR reaction was linear over 8 orders of magnitude. The levels of detection (LOD) for *sul*(I), *sul*(II), *tet*(W), and *tet*(O) were observed to be 8, 7, 1, and 1 copies of genes per 25 μL assay, respectively, which were then implemented as the level of quantification (LOQ) in the calibration curves. It was determined that the background DNA concentration had no significant effect on the amplification of target genes, as all C_T values fell within 5% of each other, regardless of the concentration of calf thymus DNA. However, the matrix study revealed that suppression of the C_T value by the DNA extract matrix was apparent and varied between sites. Little to no suppression was observed in DNA extract from the pristine site, while the matrix from the impacted sites lowered the gene copy number estimate by as much as 20× and an average of 4×. A suppression factor was determined for each DNA matrix and primer combination as noted in equation 2, where c is the number of templates enumerated in the control, t is the number of

templates enumerated in DNA extraction matrix, and m is the number of templates enumerated when the same control is spiked into the DNA extraction matrix:

$$S = (c + t) \div m \qquad (2)$$

Quantification of ARG at the sites thus took into account the mass of sediment extracted (0.5 g), any losses observed during DNA cleaning steps (as a fraction), and the PCR suppression factor. Therefore the copy of genes / gram of sediment was determined according to equation 3:

$$\frac{gene\ copies}{g\ \text{sediment}} = d \times \left(\frac{50\mu l\ DNA\ extract}{0.5\ g\ \text{sediment}} \right) \times e \times S \qquad (3)$$

where d is the copy of genes / μL DNA extract determined from calibration curve, e is the correction factor for losses during cleaning the DNA extract (1 / loss fraction), and S is the matrix suppression factor (eq. 2). Although several kits and conditions were tested for Q-PCR, in general it was found that Q-PCR was more susceptible to DNA extraction matrix inhibition and thus the remaining genes were detected by traditional PCR, but could not be quantified by Q-PCR.

In order to account for potential changes in the overall size of the microbial population at the sites, a standard curve was generated using "universal" primers and a TaqMan probe targeting Bacterial 16S rDNA as described in Suzuki *et al.* (2000).

3.4.9 QUANTIFICATION OF ANTIBIOTICS

For quantification of antibiotics in sediments, McIlvaine Buffer solution (pH 4.0) was used to pre-extract tetracyclines and sulfonamides from the solid phase to the liquid phase, followed by solid phase extraction (SPE) to clean-up and concentrate the sample. High performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS)

operated in positive mode with electrospray ionization (ESI) was used to separate and detect the tetracyclines and sulfonamides. Detailed information on the quantification of tetracyclines and sulfonamides in sediments is described elsewhere (Kim and Carlson, 2005).

3.5 RESULTS

3.5.1 HETEROTROPHIC PLATE COUNTS ON ANTIBIOTIC-SELECTIVE MEDIA

In terms of absolute numbers of resistant CFU per gram of sediment summed for all 8 antibiotic R2A media, the pristine site was found to be the lowest at 1.9×10^3 CFU/gram sediment for the high-flow sampling event and 5.7×10^4 CFU/gram sediment for the low-flow event. Site 2 and Site 4 were the next lowest, with site 2 at 2.0×10^5 and 4.4×10^6 CFU/gram sediment for the high-flow and low-flow events, respectively, and site 4 at 1.0×10^5 and 1.6×10^7 CFU/gram sediment, respectively. Sites 3 and 5 were the highest for both the high-flow and low-flow events, at 1.1×10^6 and 2.4×10^7 CFU/gram sediment respectively for site 3, and 2.5×10^6 and 5.6×10^7 CFU/gram sediment respectively for site 5. For all sites the number of resistant CFUs was higher during the low-flow sampling event.

Because the total CFU growing on control plates varied significantly among the sites (3 orders of magnitude difference between site 1 and site 5 for both events), there was no strong contrast between the five sites when the resistant CFU data were normalized to the control CFU. The normalized heterotrophic CFU data are plotted in Fig. 1 for the high-flow sampling event and Fig. 2 for the low-flow sampling event. A high level of resistance to monensin, an ionophore antimicrobial exclusively used in agriculture, was noted at site 3, site 4, and site 5, especially during the high-flow event.

3.5.2 PCR ASSAYS FOR DETECTION OF RESISTANCE GENES

The qualitative occurrence data for tetracycline and sulfonamide resistance gene families at the sites obtained by PCR assays are summarized in Table 3. During the high-flow event it was readily apparent that the number of different sulfonamide and tetracycline ARG detected increased as activity at the sites increased. Statistical analysis of the binomial data fit to a generalized linear model indicated that site 1 and site 5 ($p=0.01$) and site 2 and site 5 ($p=0.01$) were significantly different for the high-flow sampling event, and that there was no statistical difference between the most heavily impacted sites. In contrast, there was no significant difference between any of the sites during the low-flow event, and only *sul*(II) was detected exclusively at the three most heavily impacted sites.

3.5.3 QUANTIFICATION OF RESISTANCE GENES AT THE SITES

The numbers of copies of the four resistance genes quantified at each site are plotted normalized to the number of copies of Bacterial 16S rRNA genes in Fig. 3 for the high-flow and Fig. 4 for the low-flow sampling events. Normalizing to the number of copies of 16S rRNA genes provided a means to assess the level of resistance proportional to the size of the overall population. The total number of copies of Bacterial 16S rRNA genes was found to be relatively consistent between sites (within 1 order of magnitude, except site 2, which was 1-2 orders of magnitude lower for both events).

The total concentrations of *sul*(I) and *sul*(II) together were the highest at site 3 and site 5, where there was heavy influence from both urban and agricultural activity. *Sul*(I) and *sul*(II) were present at site 4, but significantly lower in concentration than they were at site 3 and site 5. Site 2 showed concentrations of *sul*(I) equal to or greater than those

observed at sites 3 and 5, however *sul(II)* was consistently absent. *Sul*(II) was also absent from site 1 in both sample events, while *sul*(I) was 2 to 3 orders of magnitude lower during the high-flow event, and below Q-PCR detection in the low-flow event.

In the high-flow sampling event, the total *tet* genes followed the same trend as the total *sul* genes, with sites 3 and 5 the highest, followed by site 4, and detection of only one of the pair of genes at site 2 and site 1. In the low-flow event, however, there was no significant difference between site 1 and any of the other sites for *tet*(O), and *tet*(W) was significantly different (lower) only at site 1. *Tet*(W) and *tet*(O) genes were lower in concentration than *sul* genes at all sites.

3.5.4 QUANTIFICATION OF ANTIBIOTICS

The concentrations of tetracycline and sulfonamide antibiotics found in the sediments are presented in Table 4. As has been observed in previous studies, the concentrations of tetracyclines and sulfonamides were highest at sites 3, 4, and 5. It was also observed that the antibiotic concentrations were higher during the low flow sampling event. Correlation analyses conducted comparing the concentration of the antibiotics in the sediments and concentrations of genes did not reveal statistically significant relationships for either the high-flow or the low-flow sampling events (R^2 ranged from 0.12 to 0.84, p-value ranged 0.92 to 0.47).

3.6 DISCUSSION

3.6.1 APPLICABILITY OF THE METHODS

The Q-PCR methods developed in this study provide a useful means to quantify ARG in the environment and thus bioassay and monitor the relative impact of antibiotic

use. Q-PCR also allows for direct quantification of ARG as a new class of "pollutants" in and of themselves. While all three levels of data gathered in this study (CFU, PCR presence / absence assay, Q-PCR) revealed similar overall trends, there were some differences observed. For example, the CFUs on control plates without antibiotics varied over three orders of magnitude between sites, while the quantities of 16S rRNA genes were relatively consistent between sites. Because PCR of 16S rRNA genes would quantify the DNA of both live and dead cells, it cannot be determined whether the culturing approach was biased, or if there were varying proportions of 16S rRNA genes corresponding to dead cells at the five sites. In particular, this affected interpretation of the data at site 1, where the CFU growing on control plates were lowest and thus provided a high estimate of the proportion of resistant CFU. However, site 1 is also the most pristine ecologically and therefore would be expected to be the least amenable to growth on a Petri dish. On the other hand, 16S rRNA genes are well-studied and increasingly being used as biomarkers for normalizing quantitative data in the environment. The Q-PCR method also is less cumbersome and provides results within 3 hours, including DNA extraction.

In applying Q-PCR to environmental samples, however, one needs to be aware of the potential impacts of PCR inhibitors. In this study there were some genes detected by the PCR assay that could not be quantified by Q-PCR because Q-PCR was apparently more sensitive to inhibitors. Inhibitors present in the DNA extraction matrix were also a factor in Q-PCR in that they lowered the estimated gene concentration by an average of 4× and as high as 20× in one case. However, this was easily corrected for by determining the suppression factors in spiked matrix control tests.

3.6.2 LAND-USE AND OCCURRENCE OF RESISTANCE

To the knowledge of the authors, this study is the first report of an increase in ARG in river sediments corresponding to increases in human and agricultural activity. This is consistent with the findings of previous studies that have shown that as the Poudre River runs through pristine, urban, and agriculturally influenced areas, the concentrations of various antibiotic compounds increase (Kim and Carlson, 2005; Yang and Carlson, 2003). To date, no antibiotics have been detected at site 1 which is considered not to have any anthropogenic influences, and the highest concentrations of antibiotics have been found at sites 3, 4, and 5, which are the most heavily impacted. Similarly, the kinds and quantities of resistance genes detected at site 1 were consistently lower at site 1, in most cases by orders of magnitude. It should be noted that detection of resistance genes at site 1 was not unexpected considering that resistance genes are naturally-occurring. This was also consistent with the observed growth of some heterotrophic bacteria on plates with sulfonamides and tetracyclines. The characteristics of site 1 thus provided a good means of characterizing the background level of resistance for comparison to impacted sites.

Other researchers have recently investigated the presence of resistance genes in animal feedlot lagoons and areas immediately adjacent. For example, Chee-Sanford *et al.* (2001) demonstrated a high occurrence of tetracycline resistance genes in the groundwater underlying swine production facilities using traditional PCR assays. More recently, Smith *et al.* (2004) developed a TaqMan Q-PCR assay for *tet*(W), *tet*(O), and *tet*(Q) and observed a correlation with the concentrations of these genes and the concentration of tetracyclines in 18 different lagoons. The present study further supports

such findings and takes the ARG dissemination pathway a step further by documenting their occurrence in impacted river sediments.

The lack of a statistically-significant correlation in this study between the concentration of antibiotics in the sediments and the concentration of resistance genes could be the result of various factors. For example, other types of resistance genes may have been present, but not analyzed. In this case, while numerous tetracycline and sulfonamide ARG have been reported in the literature, PCR primers were developed or applied for only 15 *sul* and *tet* genes total, 9 of which tested positive in the PCR assay, and only 4 were quantified. To fully characterize the correlation, full discovery and quantification of all resistance genes would be required. It should also be pointed out that microbial populations may retain resistance genes long after their initial exposure, which could additionally contribute to a lack of direct correlation. Also, lower concentrations of antibiotics may actually enhance the development of resistance, considering that very high concentrations are often lethal even to resistant cells. Such phenomena would be of significant interest in developing a model of understanding of the spread of ARG and strategies for minimizing their impact. Recent work investigating the fate of antibiotic resistant organisms in column studies suggests that about one month was required for resistance levels to return to baseline after tetracycline was removed from the feed (Rysz and Alvarez, 2004).

3.6.3 SULFONAMIDE RESISTANCE GENES

Sulfonamides are used both in animals and in humans, though generally more-so in humans. This is because sulfonamides are used primarily as therapeutic agents, rather than as routine growth-promoters. The target of sulfonamide antibiotics is the enzyme

dihydropteroate synthase (DHPS) in the folic acid pathway. *Sul*(I) and *sul*(II) are two alternative sulfonamide resistant DHPS genes found in Gram-negative bacteria (Sköld, 2000). In a case study of pathogenic *E. coli* from various livestock in Switzerland by Lanz *et al.* (2003), about 70% of the sulfonamide resistant isolates from pigs could be explained by the presence of *sul*(I) and *sul*(II). Both of these genes have also been detected in human pathogens such as *Salmonella typhimurium*, *E. coli*, and *Streptococcus pneumoniae*. The most commonly detected sulfonamide resistance genes in this study were also mainly *sul*(I) and *sul*(II), and based on the research of Lanz *et al.*, their likely inputs may be from the CAFOs that are prolific in northern Colorado.

3.6.4 TETRACYCLINE RESISTANCE GENES

Tet(W) and *tet*(O) are both common in anaerobic intestinal and rumen environments and both incur resistance by ribosomal protection mechanisms (Barbosa *et al.*, 1999; Taylor *et al.*, 1987). *Tet*(O) is found in both Gram-positive and Gram-negative bacteria (Roberts, 1997), and approximate estimates suggest that up to 5% of the bacteria in bovine rumen and swine intestines may carry the *tet*(O) gene (Aminov *et al.*, 2001). *Tet*(W) is also found in bovine and sheep rumen as well as human intestinal isolates (Melville *et al.*, 2004; Scott *et al.*, 2000). In a study by Smith *et al.* (25) *tet*(O) and *tet*(W) along with *tet*(Q) were the most commonly found tetracycline resistance genes in 18 different feedlot lagoons. Thus, the presence of the *tet*(W) and *tet*(O) resistance genes may be a good indicator of fecal contamination from humans and/or animals. If *tet*(O) and *tet*(W) are truly indicators of fecal contamination, then the present study suggests that such contamination is wide-spread at the impacted sites, especially during high-flow events, which may be the result of transport of fecal bacteria in runoff. *Tet* genes may

have been lower at site 5 during the low-flow event, even though the concentration of tetracyclines was double, because there was less runoff. On the other hand, both *tet*(O) and *tet*(W) have been cited as being promiscuous in their ability to spread among and across populations (Billington *et al.*, 2002; Smith, *et al.*, 2004), and *tet*(W) in particular has recently been identified as being associated with a conjugative transposon (Melville *et al.*, 2004). Examples of *tet*(W) carrying strains include *Mitsuokella multiacidus*, *Selenomonas ruminantium,* and *Butyrivibrio fibrisolvens* (Barbosa *et al.*, 1999). Further investigation is needed in order to determine whether horizontal gene transfer interferes with the use of these genes as indicators of fecal sources of ARG.

3.7 CONCLUSIONS AND FUTURE WORK

This study demonstrates a relationship between human and agricultural activity and elevated levels of ARG in river sediments using quantitative molecular tools. This is an important step in developing a paradigm for ARG as environmental contaminants. Further study is needed in order to better understand the relationship between the use of antibiotics in humans and animals and the spread of ARG. In particular, the relationship between the transport of antibiotics and the transport of ARG in the environment has not been defined. For example, in this study it is not clear whether the elevated levels of resistance quantified in the river sediments were a result of antibiotic selection taking place in the sediments, or if selection occurred upstream and the resistant microbes were later transported there. The fact that the correlation analysis of the antibiotic concentrations and the concentration of corresponding resistance genes was not significant suggests that selection may have taken place upstream.

Another important area of future work will be to build a better understanding and separation of human and animal sources. Certain antibiotics, such as tylosin and monensin, are only used in agriculture and thus provide useful markers for defining agricultural sources (Kim and Carlson, 2005). Similar source tracking methods for resistance genes need to be further developed, such as the denaturing gradient gel electrophoresis (DGGE) profiling approach developed by Aminov *et al.* (2001). Even if it becomes possible to clearly distinguish human and agricultural sources of ARG, it will still be important to consider that even antibiotics used exclusively for agriculture have been demonstrated to co-select for microbial resistance to a variety of antibiotics, including antibiotics used in humans (Aarestrup, 1995). A better understanding of the sources and fates of antibiotics and ARG will facilitate improved modeling of the ultimate impact of antibiotic use on human, animal, and environmental health and may ultimately be applied in developing strategies to mitigate potentially adverse impacts.

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TABLE 1. Poudre River Sampling Site Locations and Characteristics

Site ID	Actual location	Site Characteristics
Site 1	Greyrock National Recreation Trail, Ft Collins, CO	Pristine area without anthropogenic influence
Site 2	Shields street bridge, Ft Collins, CO	Area influenced by light agriculture activities
Site 3	Drake waste water treatment plant, Ft Collins, CO	Urban area influenced by wastewater discharges
Site 4	Hwy 329 bridge, Windsor, CO	Area influenced significantly by agriculture activities
Site 5	Weld country municipal airport, Greeley, CO	Area influenced by both urban and agriculture activities

TABLE 2. PCR Primers Targeting Sulfonamide Resistance Gene Families

Primer	Class targeted	Sequences	Traditional PCR annealing temp (°C)	Q-PCR annealing temp (°C)	Amplicon Size (bp)
sulI-FW[a]	*sul*(I)	cgcaccggaaacatcgctgcac	55.9	65.0	163
sulI-RV		tgaagttccgccgcaaggctcg			
sulII-FW	*sul*(II)	tccggtggaggccggtatctgg	60.8	57.5	191
sulII-RV		cgggaatgccatctgccttgag			
sulIII-FW	*sul*(III)	tccgttcagcgaattggtgcag	60.0	61.0	128
sulIII-RV		ttcgttcacgccttacaccagc			
sulA-FW	*sul*(A)	tcttgagcaagcactccagcag	60.0	60.0	299
sulA-RV		tccagccttagcaaccacatgg			

[a]FW, forward; RV, reverse

TABLE 3. Detection of Resistance Gene Families at Sampling Sites

Gene ID	April 2004 high-flow sampling event					February 2005 low-flow sampling event					+ control
	Site1	Site2	Site3	Site4	Site5	Site1	Site2	Site3	Site4	Site5	
tetB(P)	-	-	-	-	+	-	-	-	-	-	+
tet(O)	+	-	+	+	+	+	+	+	+	+	+
tet(S)	-	-	-	-	+	-	-	-	-	-	+
tet(T)	-	-	+	+	+	-	-	-	-	-	+
tet(W)	-	+	+	+	+	+	+	+	+	+	+
sul(I)	+	+	+	+	+	+	+	+	+	+	+
sul(II)	-	-	+	+	+	-	-	+	+	+	+
sul(III)	-	-	+	+	+	-	-	-	-	-	+
sul(A)	-	-	+	-	+	-	-	-	-	-	+

+:present; -:absent

TABLE 4. Concentrations of Tetracyclines and Sulfonamides at Sampling Sites

	April, 2004 high-flow sampling event (μg/L)					February, 2005 low-flow sampling event (μg/L)				
	site1	site2	site3	site4	site5	site1	site2	site3	site4	site5
Tetracycline (TC)	ND	3.6	8.7	8.4	10.2	ND	11.0	102.7	3.9	24.9
Chlortetracycline (CTC)	ND	3.0	3.1	4.6	3.8	ND	9.6	19.3	15.8	22.0
Oxytetracycline (OTC)	ND	2.4	7.3	7.4	23.6	ND	7.8	56.1	19.0	35.5
Demeclocycline (DMC)	ND	2.1	6.8	2.1	6.9	ND	6.5	14.7	9.5	23.5
Meclocycline (MCC)	ND	29.5	21.6	28.4	41.6	ND	38.3	167.5	26.3	72.0
Doxycycline (DXC)	ND	5.1	10.2	6.3	14.8	ND	13.3	38.9	12.0	25.6
Sum of tetracyclines	ND	***45.7***	***57.7***	***57.2***	***100.9***	ND	***86.5***	***399.1***	***86.4***	***203.5***
Sulfathiazole (STZ)	ND	2.7	4.4	3.5	4.7	ND	2.7	4.4	3.5	4.7
Sulfamerazine (SMR)	ND	ND	ND	ND	ND	ND	1.7	15.0	ND	ND
Sulfamethazine (SMT)	ND	1.7	1	ND	ND	ND	ND	ND	ND	ND
Sulfachloropyridazine(SCP)	ND	ND	3	ND	ND	ND	ND	3.0	ND	ND
Sulfamethoxazloe (SMX)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sulfadimethoxine (SDM)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sum of sulfonamides	ND	***4.4***	***8.4***	***3.5***	***4.7***	ND	***4.4***	***22.4***	***3.5***	***4.7***

ND: Not detected

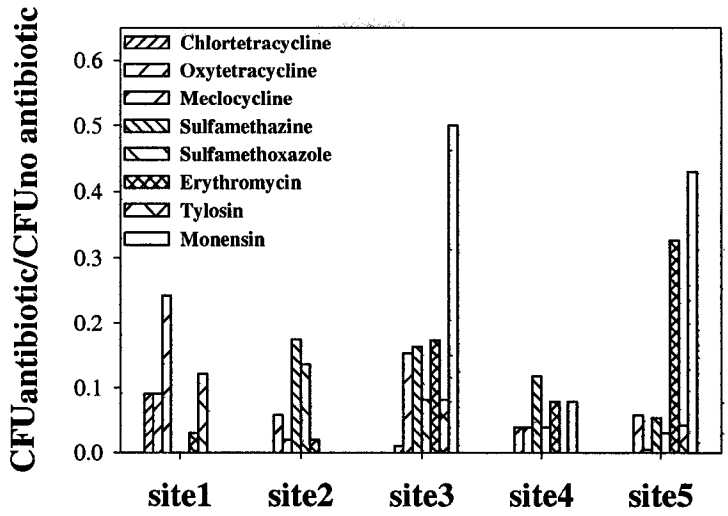

FIG. 1. CFUs on antibiotic media normalized to CFUs on media with no antibiotic added for the high-flow sampling event.

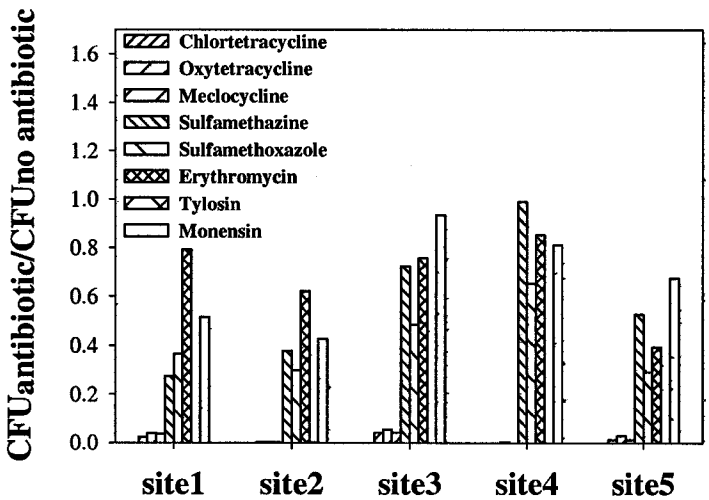

FIG. 2. CFUs on antibiotic media normalized to CFUs on media with no antibiotic added for the low-flow sampling event.

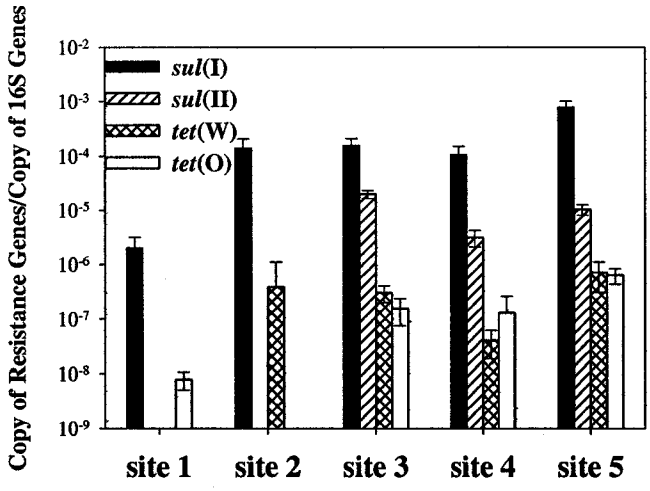

FIG. 3. Copies of resistance genes normalized to the number of Bacterial 16S rRNA genes at different sites for the high-flow sampling event. Error bars indicate the standard deviation of 6 replicates in three independent Q-PCR runs.

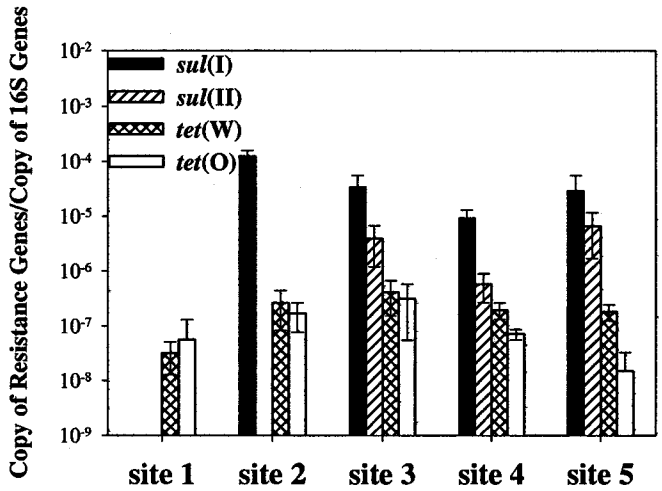

FIG. 4. Copies of resistance genes normalized to the number of Bacterial 16S rRNA genes at different sites for the low-flow sampling event. Error bars indicate the standard deviation of 6 replicates in three independent Q-PCR runs.

CHAPTER4: ANTIBIOTIC RESISTANCE GENES (ARG) AS EMERGING CONTAMINANTS: STUDIES IN NORTHERN COLORADO

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4.1 ABSTRACT

This study explores antibiotic resistance genes (ARG) as emerging environmental contaminants. The purpose of this study was to investigate the occurrence of ARG in various environmental compartments in northern Colorado, including the Cache La Poudre (Poudre) River sediments, irrigation ditches, dairy lagoons, and the effluents of wastewater recycle and drinking water treatment plants. Additionally, ARG concentrations in the Poudre River sediments were analyzed at three time points at five sites with varying levels of urban/agricultural impact and compared with two previously published time points. It was expected that ARG concentrations would be significantly higher in environments directly impacted by urban/agricultural activity than in pristine and lesser impacted environments. Polymerase chain reaction (PCR) detection assays were applied to detect the presence/absence of several tetracycline and sulfonamide ARG. Quantitative real-time PCR (Q-PCR) was used to further quantify two tetracycline ARG (*tet*(W) and *tet*(O)) and two sulfonamide ARG (*sul*(I) and *sul*(II)). The following trend was observed with respect to ARG concentrations (normalized to Eubacterial 16S rRNA genes): dairy lagoon water > irrigation ditch water > urban/agriculturally impacted river sediments ($p<0.0001$), except for *sul*(II), which was absent in ditch water. It was noted that *tet*(W) and *tet*(O) were also present in treated drinking water and recycled wastewater, suggesting that these are potential pathways for the spread of ARG to and from humans. Based on this study, there is a need for environmental scientists and engineers to help address the issue of the spread of ARG in the environment.

4.2 KEYWORDS

Antibiotic resistance genes, tetracyclines, sulfonamides, antibiotics, Q-PCR

4.3 INTRODUCTION

The spread of antibiotic resistant pathogens is a growing problem in the U.S. and around the world. Recently a 2000 World Health Organization report (WHO) (*1*) focused on antibiotic resistance as one of the most critical human health challenges of the next century and heralded the need for "a global strategy to contain resistance." According to the report, more than two million Americans are infected each year with resistant pathogens and 14,000 die as a result. The rapid growth of the problem emphasizes the need for intervention. For example, vancomycin is currently considered to be the most powerful antibiotic of "last resort," yet within 10 years the incidence of vancomycin-resistant *Enterococci* (VRE) increased in the United States from 0% to 25% (*2, 3*). Resistance to penicillin, the antibiotic that originally revolutionized human health 50 years ago, is now as high as 79% in *Staphylococcus pneumoniae* isolates in South Africa (*4, 5*). Alarmingly, diseases that were once considered to be eradicated, such as tuberculosis, are now beginning to make a comeback because of antimicrobial resistance (*1, 6, 7*). As with other dangerous pollutants that spread in the environment and threaten human health, there is a need for environmental scientists and engineers to help address the critical problem of microbial resistance to antibiotics.

The rise of antibiotic resistance is considered to be closely linked with the widespread use of antibiotic pharmaceuticals in humans and animals. In particular, more than one-half of the antibiotics used in the U.S. are administered to livestock for purposes of growth-promotion or to treat infections (*8, 9*). In both animals and humans, up to 95%

of antibiotics can be excreted in an unaltered state (*10, 11*). Some removal has been observed in wastewater treatment plants (WWTPs), however as is true with the larger problem of pharmaceutical compounds, WWTPs are not designed for removal of micropollutants (*12-14*). Residual antibiotics thus are released into the environment where they may exert selection pressure on microorganisms. While over-prescribing or other improper use/disposal of antibiotics in humans is generally considered to contribute to the problem, several studies have also linked agricultural antibiotic use with antibiotic-resistant infections in humans (*15-23*). For example, avoparcin, an antibiotic growth-promoter used in poultry, was recently banned in Europe because of its association with the development of vancomycin-resistant *Enterococci* (*24*).

Because of the direct selection pressure that antibiotics exert on organisms carrying antibiotic resistance genes (ARG), the transport pathways of antibiotic resistant microorganisms and the ARG that they carry are expected to be similar to the pathways of antibiotic pharmaceuticals. In fact, it is likely that ARG persist further in the pathway, considering that in many cases they are maintained in the microbial populations even after the antibiotic selection pressure has been removed (*25-28*). Also, horizontal gene transfer (HGT) is a major mechanism for sharing ARG between microbes and has been documented to occur between non-pathogens, pathogens, and even distantly related organisms, such as gram positive and gram negative bacteria (*25, 29-31*). In many cases, ARG have been discovered to occur as part of multiple antibiotic resistant (MAR) super integrons, which may contain over one hundred ARG cassettes (*32*). These MAR super integrons cause multi-resistance in organisms, meaning that even when very different antibiotics are used, one antibiotic may co-select for resistance to other antibiotics (*5, 33*).

MAR gene cassettes and ARG are notorious for being associated with plasmids and/or transposons that facilitate HGT. Finally, even if cells carrying ARG have been killed, DNA released to the environment has been observed to persist, to be protected from DNAse, especially by certain soil/clay compositions, and eventually be transformed into other cells (*34-36*). For all of these reasons, ARG in and of themselves can be considered to be emerging "contaminants" for which mitigation strategies are needed to prevent their widespread dissemination.

The purpose of this study was to document the occurrence of tetracycline and sulfonamide ARG in various environmental compartments in northern Colorado. These two ARG groups were chosen because sulfonamide and tetracycline antibiotics have been previously characterized in Poudre River sediments and shown to relate to urban/agricultural activity (*37*). The breadth of the study included: Cache La Poudre (Poudre) River sediments, dairy lagoon water, irrigation ditch water, and a wastewater recycle plant (WRP) and two drinking water treatment plants (DWTP). The hypothesis was that environmental compartments most directly impacted by urban/agricultural activity would have significantly higher concentrations of ARG than less impacted and pristine environments. Irrigation ditch waters, which were directly adjacent to farms, were investigated as a potential pathway of ARG from farms to the Poudre River, while the WRP and the DWTPs were explored as potential routes of human environmental input and consumption. The presence/absence of several ribosomal protection factor tetracycline ARG and folic acid pathway sulfonamide ARG was determined using a polymerase chain reaction (PCR) detection assay and four commonly occurring ARG were further quantified by quantitative real-time PCR (Q-PCR). Documenting the

baseline occurrence of ARG in a cross-section of environmental compartments will take a step toward understanding and modeling the fate and transport phenomena associated with these emerging contaminants.

4.4 EXPERIMENTAL SECTION

4.4.1 POUDRE RIVER SEDIMENT SAMPLING

Because of its pristine origins and zonation corresponding to land-use, the Poudre River has served as a good model for relating human and agricultural activities with the occurrence of antibiotic pharmaceuticals (*37*) and ARG (*38*). Five sampling sites were the focus of this study, numbered sequentially in the direction of flow from west to east, with the following characteristics: site 1, pristine location at the river origin in The Rocky Mountains; site 2, light agriculture influenced area; site 3, urban-influenced area at the outlet of the Fort Collins Drake WWTP; site 4, heavy agriculture influenced area between Fort Collins and Greeley; and site 5, heavy agriculture and urban influenced area just east of Greeley, which is a major center for the meat-packing industry. Over 90 confined animal feeding operations (CAFOs), dairies, and ranches are located between sites 3 and 5. Further attributes of the Poudre River watershed that contribute to its suitability for investigating the impacts of urban and agricultural activity on antibiotics and ARG have been described previously (*37, 38*).

Sediment samples were collected along the Poudre River at the five sites on August 18, 2005, October 27, 2005, and February 17, 2006. The flow rates on these three dates were 1.04 cms, 14.19 cms, and 0.14 cms, respectively (USGS station number 06752260, Fort Collins, CO). Sampling at three points in time provided insight into potential temporal variations in ARG concentrations, and the February 17th date is exactly

one year later than a previously published sampling date (*38*). The upper sediments (about 5 cm) from the middle and two sides of a cross-section at each site were sampled and composited. Samples were collected using a shovel and mixed well in sterilized centrifuge tubes. Fifty-five grams of mixed sample at each site were stored at -80 °C for subsequent molecular analysis.

4.4.2 BULK WATER SAMPLING

Irrigation ditch waters were investigated as a potential pathway of ARG from farms to the Poudre River. Grab samples of bulk water were collected in sterile containers from irrigation ditches on August 18, 2005, corresponding to the August sampling date of the Poudre River sediments. All irrigation ditches were located between site 4 and site 5 on the Poudre River within a 3.5 km x 2 km zone north of the river and a total of ten locations were sampled. In order to investigate a potential source of ARG within this zone, a microaerophillic dairy lagoon (~ 1mg/l dissolved oxygen in upper 1 m) and an anaerobic dairy lagoon (0 mg/l dissolved oxygen) from an anonymous farm located 8 km from site 5 was sampled October 20, 2005. Finally, source water, and pre-chlorinated and post-chlorinated bulk water were collected from two anonymous DWTPs and an anonymous WRP in northern Colorado in February, 2005. The DWTP was studied as a potential direct route of ARG to consumers, and the WRP was considered a potential human input into the environment. In order to collect fine particulates from the dilute aqueous ditch water, DWTP, and WRP samples for subsequent analysis, 500 ml of well-mixed sample was filtered using a 0.45 micron glass fiber filter (Whatman). This concentration step was not required for dairy lagoon samples.

4.4.3 DNA EXTRACTION

DNA was extracted from 0.5 g of composited sediment using the FastDNA Spin Kit for Soil (MP Biomedicals) and from 1.8 mL of dairy lagoon water using the Ultraclean Microbial DNA Kit (MoBio Laboratories, Inc.) according to manufacturer protocol. Both approaches employ a bead-beating procedure. For fine particulates collected on filters from bulk water, the filters were cut into small pieces and added directly to the extraction tubes. Extraction yield and the quality of the DNA were verified by agarose gel electrophoresis and spectrophotometry.

4.4.4 DETECTION AND QUANTIFICATION OF ARG

Polymerase chain reaction (PCR) detection assays were used for broad-scale screening of the presence/absence of five ribosomal protection factor tetracycline ARG (*tet*(BP), *tet*(O), *tet*(S), *tet*(T), and *tet*(W)) (*39*) and four folic acid pathway sulfonamide ARG (*sul*(I), *sul*(II), *sul*(III), and *sul*(A)). Development and validation of *sul* primers was described in Pei et al (*38*). Positive controls consisted of cloned and sequenced PCR amplicons obtained from Poudre River sediments. Both positive and negative controls were included in every run and negative signals were confirmed by spiking positive control template into the sample in order to verify a signal. Forty cycles were used in order to improve chances of product formation from low initial template concentrations. Further details on reaction mixes and temperature programs are available in Pei et al. (*38*), note that annealing temperatures for *tet* primers vary from Aminov et al. (*39*). Two tetracycline ARG (*tet*(W) and *tet*(O)) and two sulfonamide ARG (*sul*(I) and *sul*(II)) that were commonly occurring according to the PCR presence/absence assays were further quantified by real-time quantitative PCR (Q-PCR) using a SybrGreen approach. For

further details on Q-PCR methods, see Pei et al. (*38*). Eubacterial 16S rRNA genes were quantified according to the TaqMan Q-PCR method described by Suzuki et al. (*40*) so that ARG could be normalized to the total bacterial community. This provided a means to correct for potential variations in extraction efficiencies. By quantifying 16S rRNA genes, it was also possible to compare ARG proportionally between samples of different overall population size. Matrix effects associated with extraction of DNA from environmental samples were corrected for by performing spiked matrix control tests and determining template suppression factors as described in Pei et al. (*38*). All Q-PCR analyses were performed using a Cepheid SmartCycler (Sunnyvale, CA).

4.4.5 STATISTICS

The influences of the environment (sites, ditch water, and dairy lagoons) on the normalized and non-normalized copies of ARG were analyzed using the Mixed Procedure, which fits a variety of mixed linear models to data. This provides the flexibility of simultaneously modeling means, variances and covariances (*41-44*). Using this test, it was thus possible to comprehensively compare overall differences between different environmental compartments with respect to ARG concentrations. For comparison of the five Poudre River sites, multiple sampling time points were treated as replicates. Mixed Procedures were conducted using SAS 9.0 (SAS Institute Inc, Cary, NC). A p-value < 0.05 was considered to indicate significance. Averages and standard deviations of all data were determined using Microsoft Excel, 2003.

4.5 RESULTS AND DISCUSSION

4.5.1 OCCURRENCE OF ARG IN NORTHERN COLORADO

Fig. 1 summarizes the Q-PCR data obtained for the four ARG at the five Poudre River sites, while Fig. 2 summarizes the same analyses for the ditch waters and dairy lagoon water. In comparing August, 2005 data for the Poudre River sediments with the dairy lagoon and ditch water, the following trend is observed with respect to ARG concentrations: dairy lagoon water > ditch water > river sediments ($p< 0.0001$), for all ARG except *sul*(II), which was absent from the ditch waters. This is based on pooling of all 10 ditch water sites, the two dairy lagoons, and sites 4 and 5, which were directly adjacent to the ditch water sampling locations. Within each of these three pools, there was no statistical difference observed among the samples. Therefore, it was observed as expected that environmental compartments most directly impacted by human/agricultural activity showed higher concentrations of ARG. This trend is even stronger in considering absolute quantities of ARG (not normalized to 16S rRNA genes), because the concentration of cells in the dairy lagoon water was orders of magnitude higher than that of the ditch water or the sediments.

In developing a hypothetical pathway for ARG, a trend is not as clear. The overall trend in terms of ARG concentrations of dairy lagoon water > ditch water > river sediments suggests that on-farm compartments, such as lagoons may be the source of ARG, which are subsequently attenuated in ditch water before reaching Poudre River sediments. However, this trend is not supported in terms of *sul*(II), which is entirely absent from the ditch water and therefore cannot be the source of what is observed in the Poudre River sediments. An alternative source of the *sul*(II) that appears at site 4 and site

5 could instead be human inputs. This is supported by the data presented in Fig. 1, in which it is observed that *sul*(II) is consistently present at high levels on average at site 3, which is at the point of discharge of the Drake WWTP, while consistently lower (comparing each date sampled) at site 4 (entirely absent for the October event), and equivalent or lower at site 5, which has mixed human/agricultural inputs. Because *sul*(II) is present in the dairy lagoon waters, it must also have agricultural sources, but it may attenuate too quickly to be transported to the ditches and subsequently to the river sediments. Based on this study and a previous study (*38*), it is appears that of the four ARG quantified, *sul*(II) is the most sensitive indicator of human/agricultural impact, and thus it is suggested that it attenuates quickly in the absence of direct inputs. The other ARG in the Poudre River sediments at site 4 and site 5 may be of either/both human and agricultural origin, since they followed a decreasing trend from the dairy lagoon through the ditch water, but were also present at site 3.

In addition to having higher concentrations of three out of four of the ARG, the dairy lagoon water was also observed to have more different kinds of ARG present than the irrigation ditch water according to the PCR assay (Table 1). Together with the Q-PCR results, these data further support the concept that there is some attenuation of ARG between any linkages that may connect dairy lagoon water and irrigation ditch water. Future work should implement ARG fingerprinting/source tracking to fully characterize the potential pathways.

4.5.2 TEMPORAL VARIATION OF ARG IN POUDRE RIVER SEDIMENTS

As observed in a previous study that compared a high-flow sampling point (6.8 cms, April 2004) with a low-flow sampling point (0.6 cms, February 2005), the ARG

concentrations in the Poudre River sediments are variable with time (*38*). In order to better understand temporal variations in ARG concentrations, the Poudre River sediments were sampled at three additional time points and compared with the two previously published time points. The February sampling point in this study took place exactly one year after the previous February event. In support of the relationship between ARG concentration and relative environment impact observed above, the pristine site (site 1) consistently had the lowest average concentrations of ARG with time, with *sul*(II) completely absent, and no individual ARG consistently present at all five sampling times (Fig. 1). In comparing presence/absence of ARG, site 2 appears to be the next lowest in terms of overall impacts. For example, *sul*(II) is consistently absent at site 2, and *tet*(O) was absent in one of the five sampling events, whereas these genes were consistently present at sites 3, 4, and 5. In terms of ARG concentrations, *tet*(W) and *tet*(O) at site 2 were equal or less than site 3, however, these two genes were sometimes higher and sometimes lower than at sites 4 and 5. Based on ARG averages and presence/absence of ARG, sites 1 and 2 were the least impacted, as expected.

In applying the Mixed Procedure to the data, in which the time points were pooled as replicates, it was found that there was no statistical difference between the five sites for the 16S normalized data, except in the case of *sul*(II) (p=0.0117). However, in performing the same test with non-normalized data, it was found that site 1 and site 2 were statistically lower than sites 3, 4, and 5 in terms of *sul*(I) (p=0.00296), *sul*(II) (p=0.0199), and *tet*(O) (p=0.0102). Though normalizing to 16S genes provides a comparison of ARG as a proportion of the total population, arguably it may be the absolute quantities of ARG that are more critical.

While spatial variations in ARG could be fairly well-characterized, it is difficult to identify clear temporal patterns. Comparison of the two February sampling dates that were exactly a year apart provides some insight. All four genes were either the same on average for both events (*tet*(O) for sites 1 and 4, and *sul*(II) for site 4 and 5) or higher in the 2006 event (all other genes, except *sul*(II) at sites 1 and 2, where it was not present) (Fig. 1). This suggests the possibility that all ARG are increasing in concentration with time. However, the trends in between these two dates do not support this. Only *tet*(W) and *tet*(O) at site 3 increase consistently with time. All remaining ARG at the five sites either decrease before increasing (e.g., *tet*(W) at site 2 and *sul*(II) at site 3), are constant and then increase (e.g., *tet*(O) at site 2 and *tet*(W) at site 1), or increase and then decrease (e.g., *tet*(W) at site 4 and site 5) (Fig. 1). Therefore, no clear trend was identified with time.

It was also attempted to analyze trends in the data with respect to river flow rate. This was of interest because flow rate directly relates to run-off and non-point source inputs, which were hypothesized in the previous study to play a role in the observed increase in the number of kinds of ARG detected in Poudre River sediments (*38*). The October, 2005, sampling date provided a second sampling date at high-flow (14.9 cms), compared to the previously published April, 2004, high-flow sampling date (6.8 cms) (all other dates were at or below 1.0 cms). Interestingly, all four ARG increased on average at site 5 in comparing the high-flow October event with the immediately previous low-flow event in August (Fig. 1). At site 4, *tet*(W) and *tet*(O) increased, but *sul*(II) stayed the same, and *sul*(I) went down. There was no effect at all at site 3, which is affected primarily by point-discharge rather than run-off, site 2 or site 1. However, attempts to

plot ARG concentrations versus flow rate did not reveal any clear trend. Thus, it is still not possible to make a conclusive judgment on the effect of flow rate on ARG concentrations, though the role of non-point source inputs merits further investigation. To accomplish this, it would be necessary to gather more data with time/flow, or else monitor a much more controlled and smaller-scale system.

4.5.3 WASTERWATER RECYCLE PLANT (WRP) AND DRINKING WATER TREATMENT PLANTS (DWTPS)

A PCR presence/absence assay was conducted on the influent, intermediate effluent, and final effluent of two drinking water treatment plants (DWTP "a" and DWTP "b"), and the pre-chlorinated and chlorinated effluent of a wastewater recycle plant (WRP). It was observed that both *tet*(W) and *tet*(O) were present at detectable levels in all samples except the source water for DWTP "a" (Fig. 3). This indicates that the same two genes that were common in various environmental compartments in northern Colorado, are also present in treated recycled wastewater and bulk drinking water. These two genes also showed a response to the level of impact, e.g. they were highest in dairy lagoon water and ditch water and lowest on average at the pristine site. Based on the intensity of the signal, they were also higher in the recycled wastewater than in the drinking water, as would be expected. Though these two ARG are not directly associated with any known human pathogens, they may be indicators of links between human/agricultural activity and ARG in drinking water. Considering that drinking water is a direct route to human consumers, this emphasizes the need to better understand the pathways by which ARG are spread in the environment and potential ways that the spread of ARG may be reduced. For example, vancomycin resistance genes were found

in drinking water biofilms in a recent study (*45*). Considering that vancomycin is typically the antibiotic of last resort when all else fails, this underscores the need to address this issue before it is too late. One possibility may be to make simple modifications to wastewater and drinking water treatment plants to reduce the spread of ARG.

4.5.4 ARG AS EMERGING CONTAMINANTS

Based on this study it is clear that ARG are present in various environmental compartments, including river sediments, irrigation ditch water, dairy lagoon water, DWTPs, and a WRP. Furthermore, quantitative techniques incorporating Q-PCR provide a means to compare concentration of ARG associated with the known urban and agricultural impacts, which provides a more direct measure than previous culture-based methods. Based on this occurrence survey, it is argued that ARG are emerging contaminants that need to be further studied in the paradigm of environmental science and engineering. The concept of ARG as "pollutants" has also been suggested by Rysz and Alvarez (*46*).

It should be noted that besides the tetracycline and sulfonamide ARG that were the focus of this study, there are numerous other ARG that have been described in the literature, and likely even more that have not yet been discovered, each potentially with its own unique properties. Thus, each ARG may have different behaviors with respect to fate and transport and response to physical, chemical, and/or biological treatment. In terms of defining fate and transport characteristics of ARG in general, it is expected that their behavior will be distinct in comparison to "typical" contaminants. For example, ARG may be sequestered with bacteria which are themselves transported, or they may be

present as naked DNA bound to clay particles (*47*). Furthermore, ARG may actually amplify in the environment under some conditions. This is indeed a unique contaminant property. Considering the significance of the problem of the spread of antibiotic resistance, further effort by environmental researchers to better understand these emerging contaminants is well-warranted. This is especially true as the rate of discovery and development of new antibiotics is continually declining (*48*), while the corresponding development and spread of resistance is occurring at a rapid pace. Based on this study, understanding ARG as emerging contaminants can add a new and important angle to helping to approach this important problem.

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Brief: This study explores the distribution of sulfonamide and tetracycline antibiotic resistance genes (ARG) in various environmental compartments in northern Colorado with respect to urban/agricultural impacts.

FIGURES

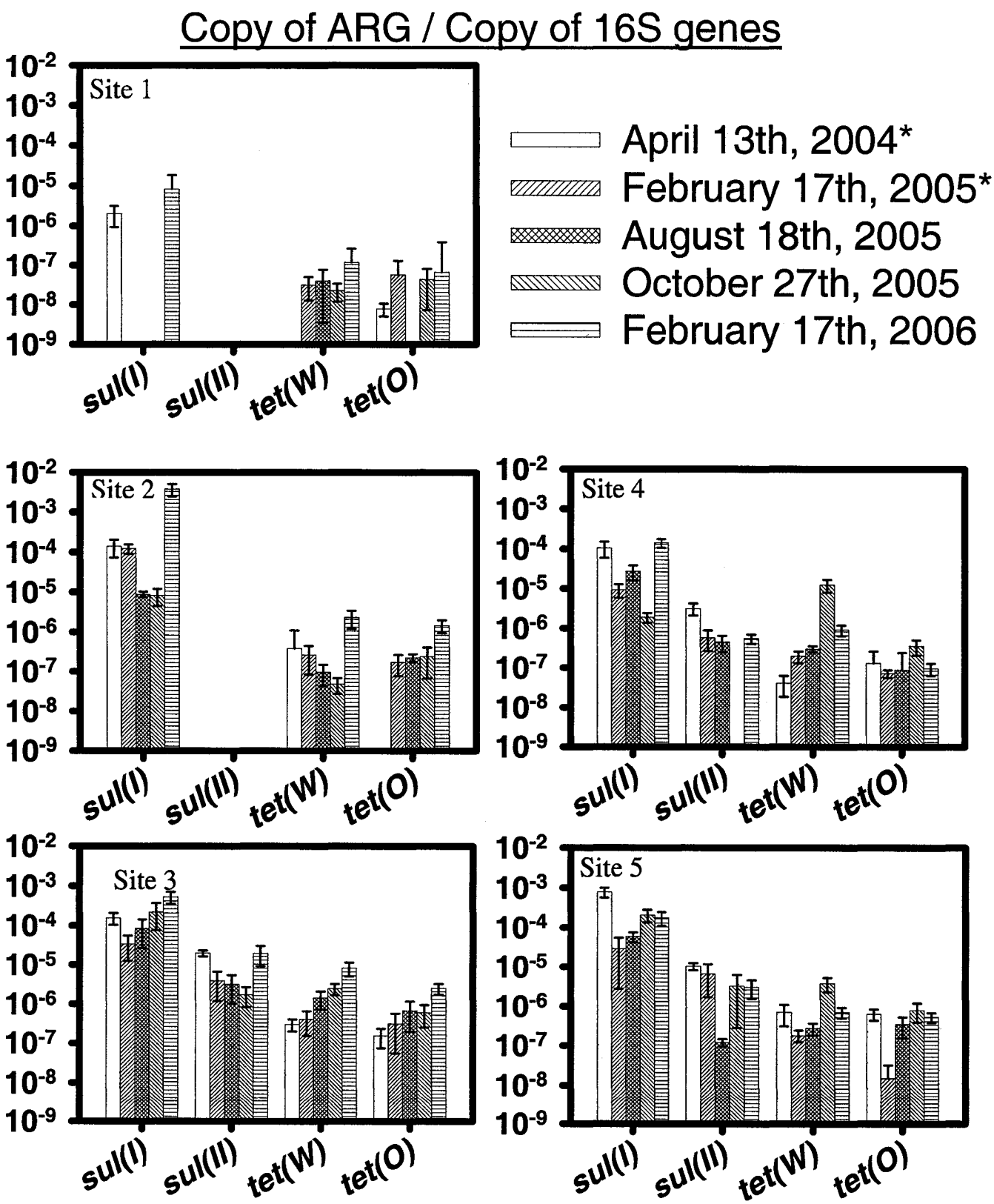

Figure 1. Distribution of four ARG [*sul*(I), *sul*(II), *tet*(O), and *tet*(W)] in Poudre River sediments on three sampling dates, compared to *two previously published sampling dates (April 13th, 2004 and February 17th, 2005 (*38*)), as determined by Q-PCR. site1: pristine site; site 2: light agricultural activity; site 3: heavy urban activity; site 4: heavy agricultural activity; and site 5: heavy urban and agricultural activity. Error bars represent standard deviation of six measurements from three independent Q-PCR runs analyzing DNA extract from composite samples.

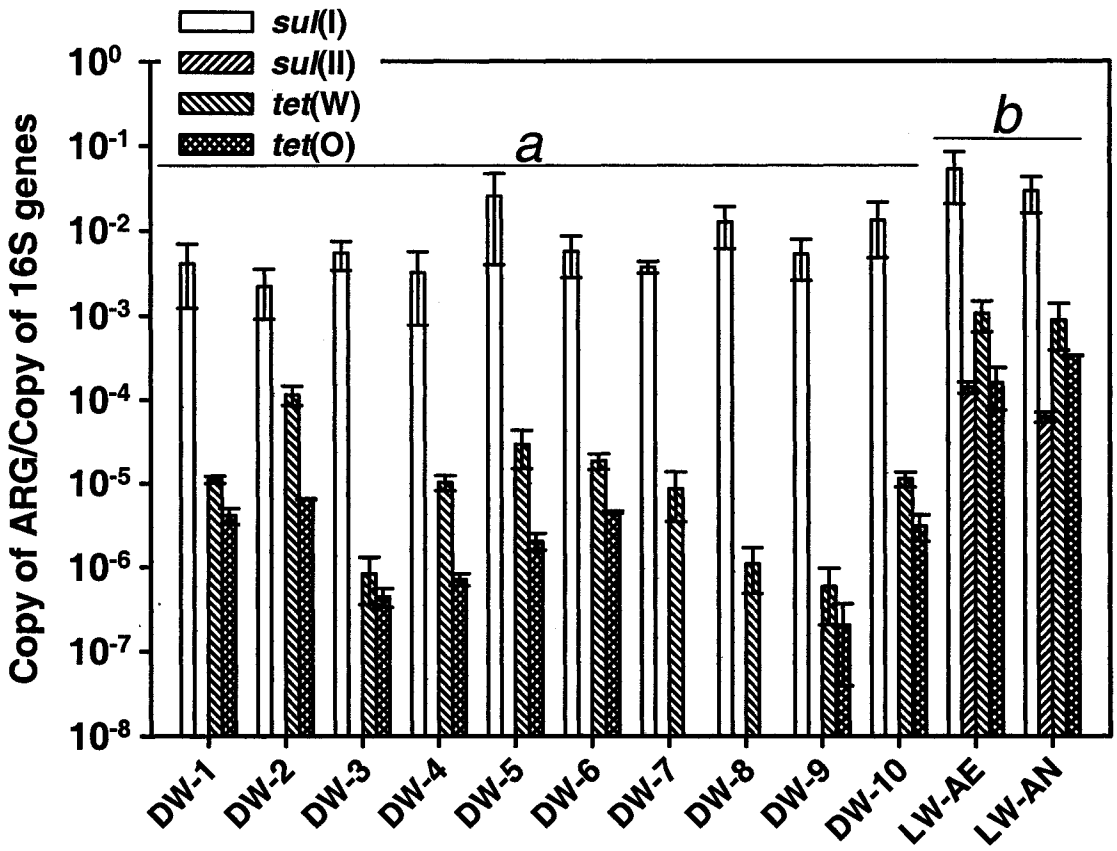

Figure 2. Distribution of four ARG [*sul*(I), *sul*(II), *tet*(O), and *tet*(W)] at ten sampling points of irrigation ditch water (DW-1 – DW-10) located between site 4 and site 5 compared with that of a microaerophillic dairy lagoon (LW-AE) and an anaerobic dairy lagoon (LW-AN). DW samples were concentrated from 500 ml, and LW samples were extracted directly from 1.8 ml. All samples were normalized to the total 16S rRNA genes. Error bars represent three independent Q-PCR runs in duplicate. *a* and *b* indicate that the data sets fell into two statistically different groups, according to the Mixed Procedure.

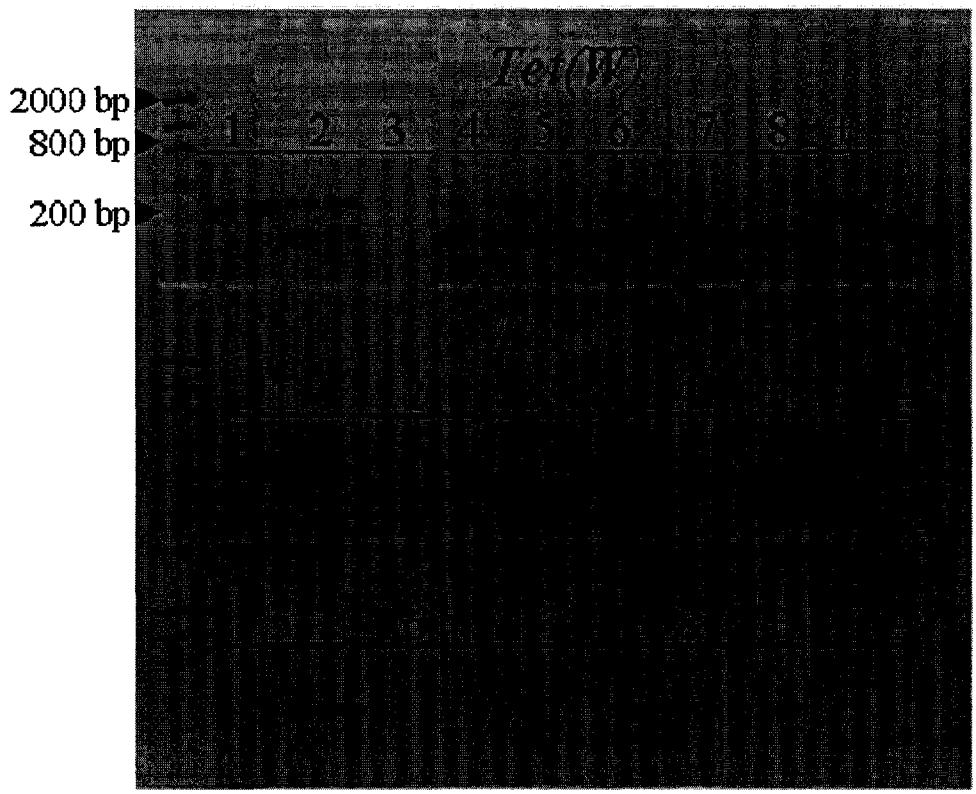

Figure 3. Agarose gel analysis of PCR presence/absence (in duplicate) of two ARG families: *tet*(W) and *tet*(O). + = positive control, - = negative control. Presence of band at same molecular weight as + indicates presence of ARG. 1=wastewater recycle plant (WRP) effluent; 2=WRP chlorinated effluent; 3= drinking water treatment plant (DWTP) "a" influent; 4= DWTP "a" treated water pre-chlorination; 5= DWTP "a" treated water post-chlorination; 6= DWTP "b" influent water; 7= DWTP "b" treated water pre-chlorination; 8= DWTP "b" treated water post-chlorination. Band appearing below 200bp is consistent with primer dimer.

TABLES

Table 1. PCR Presence/Absence Assay of Various ARG in Ditch (DW)[a] and Dairy Lagoon (LW) Water[b]

ARG	DW-1	DW-2	DW-3	DW-4	DW-5	DW-6	DW-7	DW-8	DW-9	DW-10	LW-AE	LW-AN	+ Control
tet(BP)	-	-	-	-	-	-	-	-	-	-	-	-	+
tet(O)	+	+	+	+	+	+	-	-	+	+	+	+	+
tet(S)	-	-	-	-	-	-	-	-	-	-	-	-	+
tet(T)	-	-	-	-	-	-	-	-	-	-	+	+	+
tet(W)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(I)	+	+	+	+	+	+	+	+	+	+	+	+	+
sul(II)	-	-	-	-	-	-	-	-	-	-	+	+	+
sul(III)	-	-	+	+	+	-	-	-	-	-	+	+	+
sul(A)	-	-	-	-	-	-	-	-	-	-	-	-	+

[a]Collected August 18, 2005
[b]Collected October 20, 2005

CHAPTER5: RESPONSE OF TETRACYCLINE, SULFONAMIDE, AND MACROLIDE ANTIBIOTIC RESISTANCE GENES (ARG) TO BIOLOGICAL TREATMENT IN DAIRY LAGOON WATER[†]

(This Chapter will be submitted for publication to *Environmental Science and Technology*)

5.1 ABSTRACT

Lagoons are a commonly used management strategy for treatment of animal wastes. Antibiotics present in lagoons can impact the levels of antibiotic resistance gene (ARG), which may then enter into watersheds through land application of the residue. To understand the fate of antibiotic resistance genes (ARG) in lagoons before the treated residuals are land applied, dairy lagoon water was subjected to anaerobic or aerobic treatment at 20 °C or 4 °C. Three different conditions were compared: Antibiotic (Ab) spiked, Ab spiked and killed, and Background (unamended). For the Ab spiked conditions, four antibiotics from four different classes were added: oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TYL) and monensin (MON), each at 20 mg/L. The response of ARG was monitored using quantitative real-time polymerase chain reaction (Q-PCR). The results indicated various responses of different ARG to biological treatment. There was no difference in response of *tet*(W) to aerobic versus anaerobic treatment, with an overall pattern of increase followed by decrease to initial levels in most Ab spiked conditions. The level of *tet*(O) responded differently under aerobic

[†] The effect of lagoon treatment on antibiotics will be included in the dissertation of Jongmun Cha. Co-authors: A. Pruden, J. Cha, K.H. Carlson.

versus anaerobic treatments but increased to highest levels at 20 °C under anaerobic treatment and at 4 °C under aerobic treatment before returning to initial levels. The two sulfonamide ARG (*sul*(I) and *sul* (II)) showed more similar patterns and increased in all biological treatments where antibiotics were spiked, and failed to return to initial levels at 4 °C and some 20 °C conditions. The level of two macrolide ARG (*ere*(A) and *msr*(A)) were lower than the other ARG classes and remained constant in all treatments and conditions. Addition of NaN_3 and/or $HgCl_2$ did not appear to effectively kill biological activity in the controls, and *tet*(W) and *tet*(O) actually showed the greatest increase in these treatments. This study is the first to quantitatively explore the effect of lagoon treatment on the levels of various classes of ARG.

5.2 INTRODUCTION

Substantial quantities of antibiotics are used in human and veterinary medicine. Antibiotics are widely used to prevent and/or treat diseases and to promote animal growth in livestock production facilities, such as concentrated animal feeding operations (CAFOs). In particular, one half of the fifty million pounds of antibiotics produced each year in the U.S. is used for agriculture and 90% of these are used for growth promotion (Levy, 1998). Significant amounts of antibiotics used for human and veterinary medicine (up to 75%; Elmund *et al.*, 1971; Feinman and Matheson, 1978) are excreted unchanged or as metabolites in faeces or urine. Thus animal waste presents a major potential source of antibiotic input to the environment (Haapapuro *et al.*, 1997; Sweeten, 1992). These antibiotics have the potential to contribute to the spread of antibiotic resistance genes (ARG), which arguably are contaminants in and of themselves (Pruden *et al.*, 2006).

The spread of ARG are of major concern considering that they impart the ability of disease-causing microorganisms to resist medical treatment. The spread of antibiotic resistant pathogens is of major concern. In 1995, the U.S. Centers for Disease Control and Prevention (CDC) launched a national campaign to reduce antimicrobial resistance through promotion of more appropriate antibiotic use, since drug-resistant pathogens are a growing threat to all people, especially in healthcare settings (CDC, 2001). Each year nearly 2 million patients in the United States acquire an infection in a hospital. Of those patients, about 90,000 die as a result of their infection. More than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the drugs most commonly used to treat them (CDC, 2001). The rapid growth of antibiotic resistance has been a problem since the discovery of antibiotics. Virtually all strains of *Staphylococcus aureus* worldwide were susceptible to penicillin in 1941, but by the end of 1980s, 70 to 80 percent of *S. aureus* isolates were resistant to penicillin (Atkinson *et al.*, 1984). Other antibiotics like methicillin and other semisynthetic penicillins were successful in treating penicillin-resistant *S. aureus* infections until the 1980s, when methicillin resistant *S. aureus* (MRSA) became endemic in many hospitals (Panlilio *et al.*, 1992). Since the emergence of MRSA, the glycopeptide vancomycin has been the only uniformly effective treatment for staphylococcal infections. Thus, a better understanding of the behavior of ARG and the various pathways by which they are spread is needed. This will help to develop a comprehensive strategy to contain resistance and protect human health. The behavior of ARG in the greater environment is of particular interest, considering that relatively little is known about the routes by which ARG may be spread.

Animal waste has been demonstrated to be a major source of ARG (Levy, 1998; Halling-Sørensen *et al*, 1998). A common practice for collection, storage and treatment of animal waste is some form of lagoon, pond, or tank. Animal wastes are usually stored in lagoons with a retention time of several months (six month is typical, Barker, 1996) before land application of the residual such as a fertilizer or soil conditioner. Well-functioning lagoons have a reduction in solids, nutrients, odor and sludge volume (Zhang, 2001), and they also act as aerobic or anaerobic bioreactors utilizing bacteria to decompose organic materials that make up the bulk of the waste. During the biodegradation process, organic materials may be either mineralized or transformed to other degradation products. However, in the case of ARG, the effect of treatment is unknown. Because ARG are harbored within microbes and are selected for in the presence of antibiotics, they have the potential to actually amplify in response to treatment. Conversely, considering that ARG are organic molecules, they may also be biologically degraded. Therefore, there is a need to develop an understanding of the effect of biological treatment on ARG and to identify effective strategies for minimizing the spread of ARG via environmental pathways.

The purpose of this study was to determine the effect of dairy lagoon treatment on the relative levels of three families of ARG. A 2x2 study design was implemented, aerobic versus anaerobic and 20 °C verses 4 °C, under the following conditions: Antibiotic spiked, antibiotic spiked and killed with NaN_3 and/or $HgCl_2$ (Ab spiked and killed), and no amendment (Background). Oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TYL) and monensin (MON) were all added for antibiotic spiked conditions. These were chosen to represent four classes of antibiotics: tetracyclines,

sulfonamides, macrolides, and inophores, whose occurrence has been confirmed in environmental samples such as natural water, animal and human wastewaters, irrigation ditches, and river sediments within the Cache La Poudre (Poudre) river watershed (Kim and Carlson, 2005; Yang and Carlson, 2003; Yang *et al.*, 2004; Yang and Carlson, 2004a; Yang and Carlson, 2004b). In particular, within the mixed-landscape of Poudre river watershed, certain antibiotics within each class were found to be indicators of human versus agricultural sources. Quantitative real-time polymerase chain reaction (Q-PCR) assays targeting tetracycline (*tet*(W) and *tet*(O)), sulfonamide (*sul*(I) and *sul*(II)), and macrolide (*ere*(A) and *msr*(A)) ARG were used to monitor ARG levels with time. This study is the first to quantitatively explore the effect of lagoon treatment on the levels of various classes of ARG.

5.3 MATERIALS AND METHODS

5.3.1 SAMPLING AND SYSTEM SET UP

Lagoon water was collected from a dairy farm in northern Colorado on October 20th, 2005 for the ambient temperature (20 °C) study to represent end of summer conditions and on March 16th, 2006 for the reduced temperature (4 °C) study to represent winter conditions prior to turnover. Both aerobic and anaerobic lagoon water were collected at the dairy farm from an aerobic (~1 mg/L DO) and an anaerobic (0 mg/L DO) lagoons and used in the corresponding experiments. The purpose of the reduced temperature experiment was to simulate winter conditions, and thus identify any potential temperature effects on treatment, such as reduced kinetic rates.

Oxytetracycline (OTC), sulfamethoxazole (SMX), tylosin (TYL) and monensin (MON), were chosen to represent the tetracycline, sulfonamide, macrolide and ionophore

classes of antibiotics that are commonly used in CAFOs. They were added to the antibiotic spiked conditions. All compounds were obtained from Sigma Chemical Co. (St. Louis, MO). For aerobic reactors, three sets of bottle reactors (in duplicate), were prepared in the following way: to each 9L glass bottle, 8L aerobic lagoon water was added; for the spiked experiment set (Ab spiked), 20 mg/L of OTC, SMX, TYL and MON were added; for the killed control set (Ab spiked and killed), besides the above four antibiotics, 50 g/L sodium azide was added to eliminate biological activity; for background control set (Background), only the unamended aerobic lagoon water was added. Fish tank aeration pumps (Profile Aquarium air pump 4000, Taiwan) were used for aerating and mixing the reactors. The experimental conditions are summarized in Table 1.

For anaerobic bioreactors, all compounds added were the same as the aerobic reactors, except for the Ab spiked and killed condition. In addition to 50 g/L sodium azide, 0.1 g/L mercuric chloride were also added to eliminate anaerobic microbial activity, considering that sodium azide specifically targets aerobic microorganisms. To make sure the anaerobic reactors were maintained oxygen-free, the bottles were sealed with rubber stoppers modified with three different ports, which were the nitrogen gas purge inlet, the sampling outlet, and the gas production monitoring outlet. A schematic drawing for one anaerobic bottle reactor is shown in Figure 1. The headspace of each anaerobic bottle reactor was purged with nitrogen gas for ten minutes after filling. Gas production was read from the volume reading on the syringe before the nitrogen gas was purged. To sample the anaerobic reactor, the gas production measurement line was closed with the one way stopcock, and nitrogen gas was purged into the bottle. The positive pressure

formed within the bottle will purge the samples out. About 150 mL sample was taken for testing of chemical properties, including concentration of antibiotics, chemical oxygen demand (COD), pH, dissolved oxygen (DO), and temperature. Another 45 mL sample were taken and stored at -20 °C for molecular analyses.

The lagoon experiment was repeated at a smaller scale at a reduced temperature of 4 °C to identify any potential temperature effects on treatment. Only one reactor was set up for each Ab spiked set and Ab spiked and killed set under both anaerobic and aerobic treatments. For the anaerobic background set, sixty 50 mL amber serum bottles were filled with anaerobic lagoon water. After purging with nitrogen gas, they were sealed with butyl rubber stoppers without any additional spike of antibiotic compounds. Ten 300 mL serum bottles filled with aerobic lagoon water and aerated by the fish tank pump were used as the aerobic background set, and also were sampled randomly with time.

For Q-PCR analysis, DNA extracts from 6 sample dates for each class of ARG (tetracycline, sulfonamide and macrolide) were chosen based on the antibiotic degradation process (data not shown, see Jongmun Cha's dissertation). Sampling dates for tetracycline ARG were days 0, 19, 26, 39, 74 and 144; for sulfonamide ARG days 0, 10, 19, 39, 60 and 144; and for macrolide ARG days 0, 8, 19, 26, 39 and 144. Since the 4 °C experiments were repeated for general comparative purpose only, sample dates were the same for all classes: day 0, 12, 26, 54, 89 and 107.

5.3.2 DNA EXTRACTION

DNA was extracted from lagoon water samples using the Mo Bio Ultra Clean Microbial DNA isolation kit (MioBio Laboratories Inc, Carlsbad, CA). To aid in

downstream quantification, exactly 1.8 mL of sample was used for DNA extraction. The steps were followed according to the protocol provided by the manufacturer.

5.3.3 PRIMER DESIGN

Primers were designed in order to detect and quantify macrolide ARG. All currently available nucleotide sequences encoding macrolide resistant genes were downloaded from the GenBank Database (http://www.ncbi.nlm.nih.gov/). Sequences were aligned with the multiple-sequence alignment program CLUSTALX 1.81 (Thompson *et al.*, 1997). Sequences within clusters were separately aligned and compared with each other in order to create consensus sequences for the primer design templates using FastPCR (Kalendar, 2004). The size of the PCR product was specified in the range of 100 bp to 350 bp for Q-PCR suitability. One set of primers for each mechanism of bacterial resistance to macrolides, *msr*(A)-alter the antibiotic structure and *ere*(A)-efflux pump was designed (Table 2). Specificity was verified using the BLAST alignment tool (http://www.ncbi.nlm.nih.gov/blast/). Purified PCR products obtained from bioreactor DNA extract were cloned and sequenced in order to further confirm specificity. Because no known monensin ARG are currently available in GenBank, monensin ARG primers could not be designed for this study.

5.3.4 REAL-TIME QUANTITATIVE PCR (Q-PCR)

Q-PCR protocols were optimized to quantify the number of macrolide ARG *ere*(A) and *msr*(A) using the designed primers, and sulfonamide ARG *sul*(I) *sul*(II), and tetracycline ARG *tet*(W) and *tet*(O), using previously described primers (Pei et al., 2006; Aminov et al., 2001; Chee-Sanford et al., 2001). Q-PCR was performed using a

ABI7300 Real-Time PCR system (Applied Biosystems, Foster City, CA) and a 25 μL reaction mixture [1× Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA), 0.2 μM of each primer, and 1 μL of template] with a temperature program of 15 min at 95 °C (initial denaturing and Hot Start Taq activation), followed by 50 cycles of: 15 s at 95 °C; 30 sec at the annealing temperature [65 °C for *sul*(I), 57.7 °C for *sul*(II), 60 °C for ere(A), 60 °C for msr(A), 60 °C for *tet*(W), and 50.3 °C for *tet*(O)]; and 30 s at 72 °C (optical window on) followed by a final dissociation stage on manufacture's recommendations. In order to account for the variations in overall extraction efficiency and the total bacterial community, the 16S rRNA gene was quantified using the TaqMan Q-PCR assay using 1369F and 1492R as described in Suzuki *et al.* (2000). Reactions were performed in a 25 μL reaction mixture [1× TaqMan® PCR Master Mix (Applied Biosystems, Foster City, CA), 0.1 μM of each primer, 0.15 μM 16S rRNA gene TaqMan Probe and 1 μL of template] with a temperature program of 15 min at 95 °C (initial denaturing and Hot Start Taq activation), followed by 50 cycles of: 15 s at 95 °C, 30 s at 53 °C and 27 s at 72 °C (optical window on).

Dilution tests were conducted to determine if an appropriate dilution range could be found in which PCR inhibition was eliminated. The results indicated that a dilution factor of 1:30 (for 20 °C experiment) and 1:40 (for 4 °C experiment) eliminated inhibition in ARG assays without diluting the DNA extracts below the detection limits. The suppression for 16S rRNA genes was determined using a spiked matrix method to determine a suppression factor to correct for inhibition published in Pei *et al.* (2006). Each ARG was quantified in each sample along with standards over 7 orders of magnitude in three replicates with negative controls in every run. The relative levels of

ARG were determined as copies per milliliter of lagoon water and normalized to copies of 16S rRNA genes per milliliter. The detection limit for each gene was determined by the maximum dilution that produced a consistent C_T value (within 5%), and they are all less than 10 copies per μL DNA extracts.

5.3.5 STATISTICS

Statistical analyses were conducted using SAS 9.0 (SAS Institute Inc, Cary, NC). The response of each ARG to different treatments and conditions in dairy lagoon water was analyzed using the Mixed Procedure, which fits a variety of mixed linear models to data. This provides the flexibility of simultaneously modeling means, variances and covariances (Searle 1988; Littell *et al.*, 1996; Verbeke and Molenberghs, 1997; Singer 1998). Therefore, using this test, the response of each ARG and the overall trend among different treatments could be comprehensively compared. Dixon's Extreme Value Test was used to test for statistical outliers rejected. For each ARG, aerobic and anaerobic data were pooled together, respectively, to determine if the responses of ARG to the two treatments were significantly different. Data from the 20 °C and 4 °C experiments were also respectively pooled to determine the effect of temperature on ARG response. To compare the three conditions (Ab spiked, Ab spiked and killed and Background), data from duplicate treatments were pooled together. A p-value < 0.05 was considered to indicate significance. Averages and standard deviations of all data were determined using Microsoft Excel, 2003.

5.4 RESULTS AND DISCUSSION

5.4.1 RESPONSE OF ANTIBIOTICS TO TREATMENT

The response of antibiotics to lagoon treatment is the subject of a separate study (Cha, in progress). It was observed that the concentrations of OTC, SMX, TYL and MON all decreased with time in the Ab spiked condition. Also, all antibiotics showed a higher rate of degradation in the Ab spiked than in the Ab spiked and killed condition, demonstrating that biological activity played a significant role in degrading the antibiotics. However, after 144 days of treatment at 20 °C, OTC, SMX, TYL and MON had not dissipated completely in any of the treatments except the following: OTC in the Ab spiked condition under aerobic treatment and Ab spiked condition under anaerobic treatment; SMX in Ab spiked condition under aerobic treatment; and MON in the Ab spiked conditions under aerobic treatment. At 4 °C, after 107 days, all of the antibiotics had decreased in concentration in the Ab spiked condition, at a slower rate than at 20 °C, but none were completely dissipated. Analysis of antibiotics in the Backround treatment on day 0 revealed that all antibiotics were below the detection limit, except TYL, which was initially present at 6 μg/L

5.4.2 RESPONSE OF TETRACYCLINE ARG TO TREATMENT

The copies of ARG normalized to copies of Bacterial 16S rRNA genes are shown in Figure 2 and Figure 3 for *tet*(W) and *tet*(O), respectively. *Tet*(W) increased in all Ab spiked and killed conditions, reaching the highest level on day 26 at both 20 °C and 4 °C before they dissipated back to the initial level. The peak level of *tet*(W) under anaerobic treatment was not significantly different than that under aerobic treatment ($p=0.0867$). *Tet*(W) has been found in both aerobes and anaerobes although more frequently in oral

and intestinal anaerobes (Roberts, 2005, Roberts 2005b). The results in this study indicate that *tet*(W) was not significantly different between either aerobic and anaerobic treatment, which supports a distribution of *tet*(W) within both aerobic and anaerobic microorganisms. At 20 °C, the levels of *tet*(W) were significantly different among the three conditions (Ab spiked, Ab spiked and killed, and Background), with the levels of *tet*(W) in Ab spiked and Ab spiked and killed significantly different from that of the Background condition ($p= 0.0004$ and $p<0.0001$ for Ab spiked vs. Background and Ab spiked and killed vs. Background, respectively for aerobic treatment; $p= 0.0019$ and $p<0.0001$ for Ab spiked vs. Background and Ab spiked and killed vs. Background, respectively for anaerobic treatment). The level of *tet*(W) in Ab spiked and killed were significantly higher than that in Ab spiked for aerobic treatment ($p=0.0003$), but not for anaerobic treatment ($p=0.0537$). Studies have shown that some genetic elements encode both antibiotic resistance and heavy-metal resistance to mercuric compounds (Nakahara *et al.*, 1977; Tanaka *et al.*, 1983), and a self-transferable plasmid containing *tet*(B) (tetracycline resistance gene) and *merA* (mercuric resistance gene) has also been found in microbes (Guerra et al., 2002), therefore, it is possible that *tet*(W) and *tet*(O) could be linked with mercuric resistance genes, which may have co-selected for tetracycline ARG in the Ab spiked and killed condition. The levels of *tet*(W) were statistically the same as the initial levels in all treatments and conditions and were attained at approximately Day 74 at 20 °C, Day 89 at 4 °C under aerobic treatment and Day 74 at 20 °C, Day 55 at 4 °C under anaerobic treatment. The levels of *tet*(W) were significantly higher at 4 °C than at 20 °C ($p<0.0001$) for both aerobic and anaerobic treatments, but was likely due to the higher initial levels of *tet*(W) at 4 °C ($p<0.0001$).

In contrast to *tet*(W), the levels of *tet*(O) ARG were significantly different between aerobic and anaerobic treatment at 20 °C, with levels of *tet*(O) under aerobic treatment being significantly lower than under anaerobic treatment ($p<0.0001$). Similarly, at 4 °C, the level of *tet*(O) responded to aerobic and anaerobic treatments differently (Figure 3), but aerobic treatment showed a higher number of copies than anaerobic. The levels of *tet*(O) in Ab spiked and killed conditions rose to the highest levels under both aerobic treatments. Under anaerobic treatment, both the Ab spiked and Ab spiked and killed rose equivalently above the Background at 20 °C; however, there was no response above the background at 4 °C. Ab spiked conditions were associated with a significant increase in *tet*(O) in all experiments except for the anaerobic treatment at 4 °C. The levels of *tet*(O) were statistically the same as the initial levels in all treatments and were attained around Day 74 under aerobic treatment at 20 °C, Day 55 under aerobic treatment at 4 °C, Day 144 under aerobic treatment at 20 °C and Day 0 for anaerobic treatment at 4 °C. Since the host range of *tet*(O) is narrower than that of *tet*(W) (Roberts 2005), it is possible that the microbes that carry *tet*(O) are only active under certain combinations of oxygen and temperature conditions. The levels of *tet*(O) were significantly higher at 4 °C than at 20 C ($p<0.0001$) under aerobic treatment, this is likely due to the higher initial levels of *tet*(O) at 4 °C ($p<0.0001$).

5.4.3 RESPONSE OF SULFONAMIDE ARG TO TREATMENT

The copies of ARG normalized to copies of Bacterial 16S rRNA genes are shown in Figure 4 and Figure 5 for *sul*(I) and *sul*(II), respectively. In contrast to tetracycline ARG, the two sulfonamide ARG showed relatively similar response to treatments.

The level of *sul*(I) increased under Ab spiked conditions, reaching the highest level on Day 12 under aerobic treatment at 20 °C and on Day 60 under anaerobic treatment at 20 °C before they dissipated back to the initial level. Although the trend was the same for aerobic and anaerobic treatment, *sul*(I) under Ab spiked condition for aerobic treatment was significantly higher than under anaerobic treatment ($p=0.0137$), but attenuated after 19 days at 20 °C. All Ab spiked conditions, showed levels of *sul*(I) that were significantly higher than Ab spiked and killed conditions (p value ranged from 0.0113 to 0.017). The Ab spiked condition was also significantly higher than the Background condition ($p=0.0247$), with the Ab spiked and killed conditions statistically equal to the Background conditions ($p=0.5448$). The results indicated that under anaerobic treatment, the microbial activities of *sul*(I) AGR carriers were better eliminated by the NaN_3 and $HgCl_2$ than the tetracycline ARG classes. The main difference between treatment at 20 °C and 4 °C was that *sul*(I) levels still did not return to initial levels after more than 100 days of treatment under the Ab spiked condition. The final levels of the *sul*(I) ARG were notably higher than the initial levels in all Ab spiked conditions except at 4 °C. The fact that the final *sul*(I) level in the anaerobic 20 °C treatment was also slightly higher than the initial indicates that *sul*(I) attenuation is slower under anaerobic and low temperature treatment.

The level of *sul*(II) increased in all Ab spiked conditions, reaching the highest level on Day 60 under both aerobic and anaerobic treatment at 20 °C before dissipating back to the initial level. Again, although the trend was the same for both aerobic and anaerobic treatments, the peak levels of *sul*(II) under aerobic treatments were significantly higher than under anaerobic treatment ($p<0.0001$). Similar to *sul*(I) under

aerobic treatment at 20 °C, the level of *sul*(II) in the Ab spiked conditions at 20 °C was significantly higher than the Background condition ($p < 0.0001$). In all treatments, the levels of *sul*(II) were significantly higher in Ab spiked than the Ab spiked and killed conditions (p value ranged from less than 0.0001 to 0.0007). As observed with *sul*(I), *sul*(II) in the Ab spiked and killed conditions were all statistically equal to or lower than the Background. Specifically, the Ab spiked and killed condition was lower than the Background after 60 days at 20 °C, but consistently equivalent to background at 4 °C. The final levels of *sul*(II) were higher than initial in all Ab spiked conditions, and in the Background when incubated at 20 °C under aerobic treatment ($p=0.0173$) but not for anaerobic treatment at 4 °C ($p=0.0936$). Overall, *sul*(II) ARG were best attenuated under anaerobic treatment at 4 °C.

5.4.4 RESPONSE OF MACROLIDE ARG TO TREATMENT

The copies of ARG normalized to copies of Bacterial 16S rRNA genes are shown in Figure 6 and Figure 7 for *ere*(A) and *msr*(A), respectively. The macrolide ARG were present at low levels and reamained consistent in all treatments and conditions. It is interesting that macrolide ARG were found at the lowest levels among the three groups of ARG investigated, considering that tylosin was the only antibiotic found to be present initially in the Background condition. Of all of the antibiotics, TYL also degraded the slowest. Perhaps because TYL persisted at high levels, it was actually lethal to bacteria carrying macrolide ARG. Considering that TYL is the most water-soluble of the antibiotics investigated, it may also have been more bioavailable. A summary of the treatment effects on ARG is provided in Table 3.

5.4.5 COMPARISION TO OTHER TREATMENT APPLICATIONS

In a recent study conducted by Storteboom (2006), the response of ARG such as *tet*(W) and *tet*(O) to low level and high level manure management for both pilot and large-scale field study was investigated. It was concluded that the type of treatment application had little effect on the final levels of ARG in the manures. However, the response of *tet*(W) differed between the pilot field study and the large-scale field study. *Tet*(W) increased before dissipating in pilot field study while it decreased without an increase during large-scale field study. The dissipation patterns of *tet*(O) were consistent decreased without any initial increase in both studies. The present study investigated the treatment of animal waste in lagoons (aerobic vs. anaerobic, 20 °C vs. 4 °C), which is much more dilute than manure. The level of *tet*(W) was observed to be significantly higher than *tet*(O) within the same treatment or conditions. The dissipation pattern of *tet*(W) in this study was similar to the pilot field study conducted by Storteboom, while the dissipation pattern of *tet*(O) was distinctly different than either the pilot or large-scale study, considering that tet(O) increased before dissipating back to initial levels. It was found that the ratios of the level of ARG in the lagoon water to the dairy manue from the large-scale field study:

$$Ratio = \frac{ARG_{lagoon}}{16S_{lagoon}} \div \frac{ARG_{dairy\ manue}}{16S_{dairy\ manue}}$$

was found to be 0.029 for *tet*(W) and 0.024 for *tet*(O).

5.5 OVERALL RECOMMENDATIONS

Based on this study, the responses of tetracycline, sulfonamide and macrolide ARG can be compared. All three ARG groups showed distinctly different trends.

Generally, the level of tetracycline ARG increased significantly under the Ab spiked and killed conditions while the level of sulfonamide ARG increased significantly only in the Ab spiked conditions. Tetracycline ARG are associated with conjugative transposons which allow for horizontal gene transfers to unrelated genera (Luna and Roberts 1998; Giovanetti *et al.*, 2003; Brenciani *et al.*, 2004; Melville *et al.*, 2004). In comparison, the sulfonamide ARG *sul*(I) and *sul*(II) are plasmid-borne (Nakaya *et al.*, 1960; Lawn *et al.*, 1967; Swedberg and Sköld, 1983) genes. As a result, the host range of tetracycline ARG might be wider than the plasmid-borne sulfonamide ARG. This might explain why NaN_3 and $HgCl_2$ did not eliminate the biological activities of the tetracycline ARG carriers since they have a wider host range that might be able to survive even under high dosage of biocids used to eliminate the biological activities. The fact that the concentration of SMX in Ab spiked and killed conditions remained constant while OTC in the Ab spiked and killed condition degraded with time at 20 °C (Cha, in prgress) demonstrates that there was biological activity among tetracycline degraders in the Ab spiked and killed conditions, which supports the above hypothesis. Selection pressure drives the evolution, maintenance, amplification, and horizontal transfer of ARG that confer resistance (Aarestrup *et al.*, 2001; Berge *et al.*, 2005; Poppe *et al.*, 2005). Since macrolides are less commonly used in dairy farms (Raymond *et al.*, 2006), there would likely be less selective pressure for tylosin resistance, and therefore the host range of tylosin ARG might be less then the tetracycline and sulfonamide ARG. In this case, the concentration of tylosin at 20 mg/L may have eliminated all of the tylosin resistant bacteria, which would explain why macrolide ARG showed no responses to any of the treatments.

On the basis of this study, some practical guidelines for lagoon management and reduction of ARG inputs into the environment are provided. To reduce the level of *tet*(W) ARG prior to land application, both aerobic and anaerobic treatment at 20 °C were effective, corresponding in practice to aeration in summer and no aeration in winter. In the case of *tet*(O) ARG, aerobic treatment at 20 °C and anaerobic treatment at 4 °C were effective. For *sul*(I) ARG, anaerobic treatment at 20 °C and 4 °C, and for *sul*(II) ARG, anaerobic treatment at 20 °C were most effective, though no treatment of *sul*(II) was observed to effectively return this ARG to initial levels. The macrolide ARG *msr*(A) and *ere*(A) showed no response to treatment. Therefore, overall anaerobic treatment may be the most universally effective biological approach for managing ARG while also attractive for minimizing operation and maintenance costs. It is interesting that it was not possible to reduce any ARG below initial levels in any treatments, but land applicaton after treatment is still recommended since the concentration of antibiotics decreased during treatment in addition to other benefits, even if the level of ARG did not dissipate below background. However, the batch reactors in this study were not continuous flow reactors, supplying organic carbon and nutrients during treatment may have led to the further reduction of ARG below the initial levels. Therefore, future research using continuous flow reactors to simulate field conditions is suggested. In this study, time appeared to be a significant factor in treatments. If lagoon treatment is terminated too early prior to land application, then it may be possible to actually increase the spread of ARG in the environment. It would be worthwhile for future studies to further explore the effect of longer treatment times as well as combinations with on farm physical and chemical approaches to treatment.

Endnote:

Following this experiment, a study was conducted to determine the levels of ARG in the solid separators prior to lagoon treatment. This was to provide insight into the levels of ARG prior to reaching the lagoons. It was found that the ratios of the level of ARG in the lagoon water to the solids from the solid seperators:

$$Ratio = \frac{ARG_{lagoon}}{16S_{lagoon}} \div \frac{ARG_{solid\ separator}}{16S_{solid\ separator}}$$

This ratio was found to be 289 for *sul*(I), 2.51 for *sul*(II) and 4 for *tet*(W). *Tet*(O) ARG were absent from the solid separator but present in lagoon water. Therefore, all ARG were higher in the lagoon water than in the solid separators when normalized to 16S genes, suggesting that ARG actually increase proportionally within the bacterial populations between the two treatment steps. However, absolute values of ARG were higher in the solids separator for *sul*(II) and *tet*(W).

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TABLE 1. Summary of experimental conditions

Matrix Components	Aerobic Reactors			Anaerobic Reactors		
	Ab Spiked	Ab spiked and killed	Background	Ab Spiked	Ab spiked and killed	Background
Oxytetracycline (OTC) 20 mg/L	+	+	-	+	+	-
Sulfamethoxazole (SMX) 20 mg/L	+	+	-	+	+	-
Tylosin (TYL) 20 mg/L	+	+	-	+	+	-
Monensin (MON) 20 mg/L	+	+	-	+	+	-
Sodium Azide (SZ) 50 g/L	-	+	-	-	+	-
Mercuric chloride (ML) 0.1 g/L	-	-	-	-	+	-
Dairy Lagoon Water (DLW) 8 L	+	+	+	+	+	+

TABLE 2. PCR Primers Targeting the Macrolide Antibiotic Resistance Genes

Primer	Family targeted	Sequences	Q-PCR annealing temp (°C)	Amplicon Size (bp)
ereA-FW	*ere*(A)	Atgacgtggagaacgaccag	60	101
ereA-RV		ccgacaattcgggcgccctcaat		
msrA/B-FW	*msr*(A)	ctggaacggttgaaacggatggc	60	143
msrA/B-RV		accaccactcatactgtcggttg		

[a]FW, forward; RV, reverse

TABLE 3. Summary of Conditions That Initially Increased ARG Levels

	Aerobic		Anaerobic	
	20 °C	4 °C	20 °C	4 °C
tet(W)	Ab spiked and killed* Ab spiked	Ab spiked and killed* Ab spiked	Ab spiked and killed* Ab spiked	Ab spiked and killed* Background
tet(O)	Ab spiked and killed*	Ab spiked and killed* Ab spiked	Ab spiked and killed* Ab spiked	-
sul(I)	Ab spiked*	Ab spiked*+ Background	Ab spiked*	Ab spiked*+
sul(II)	Ab spiked* Background	Ab spiked*+	Ab spiked* Background	Ab spiked*+
ere(A)	-	-	-	-
msr(A)	-	-	-	-

*highest increase; +Did not return to initial level; - No response

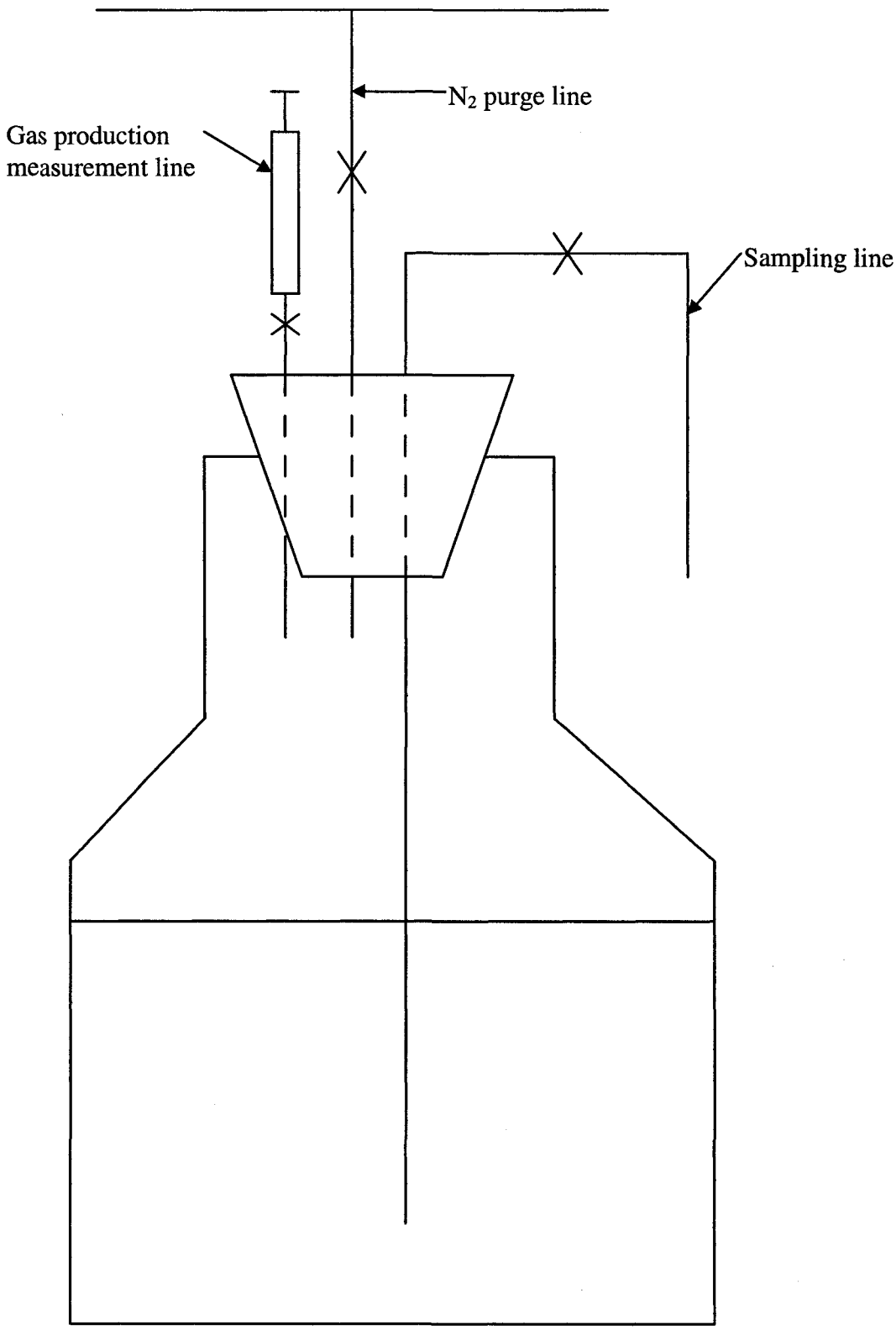

Figure 1. Anaerobic bottle reactor schematic.

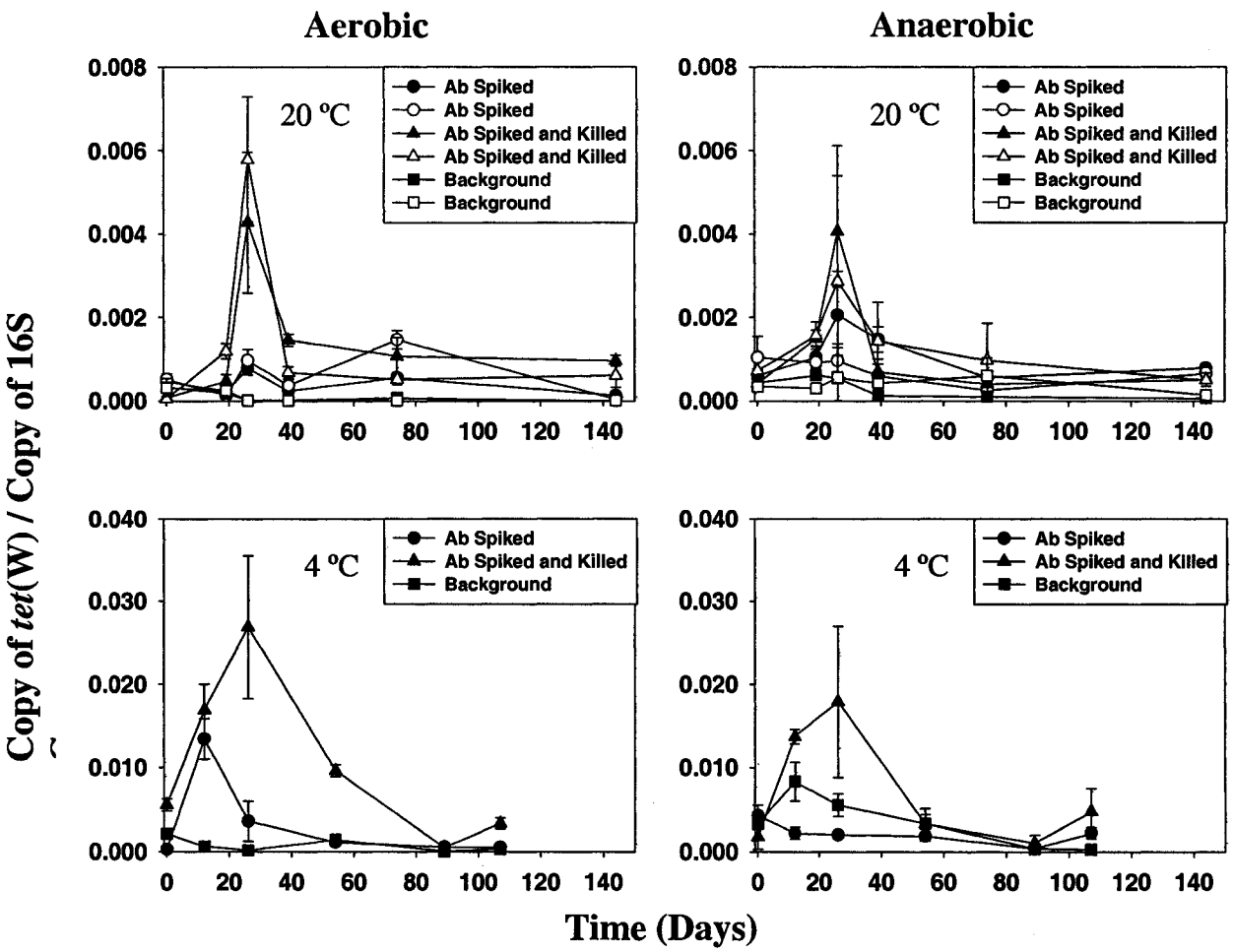

Figure 2. Copies of *tet*(W) ARG normalized to the number of Bacterial 16S rRNA genes in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment conducted in duplicate. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions for both temperatures were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of replicate Q-PCR measurements.

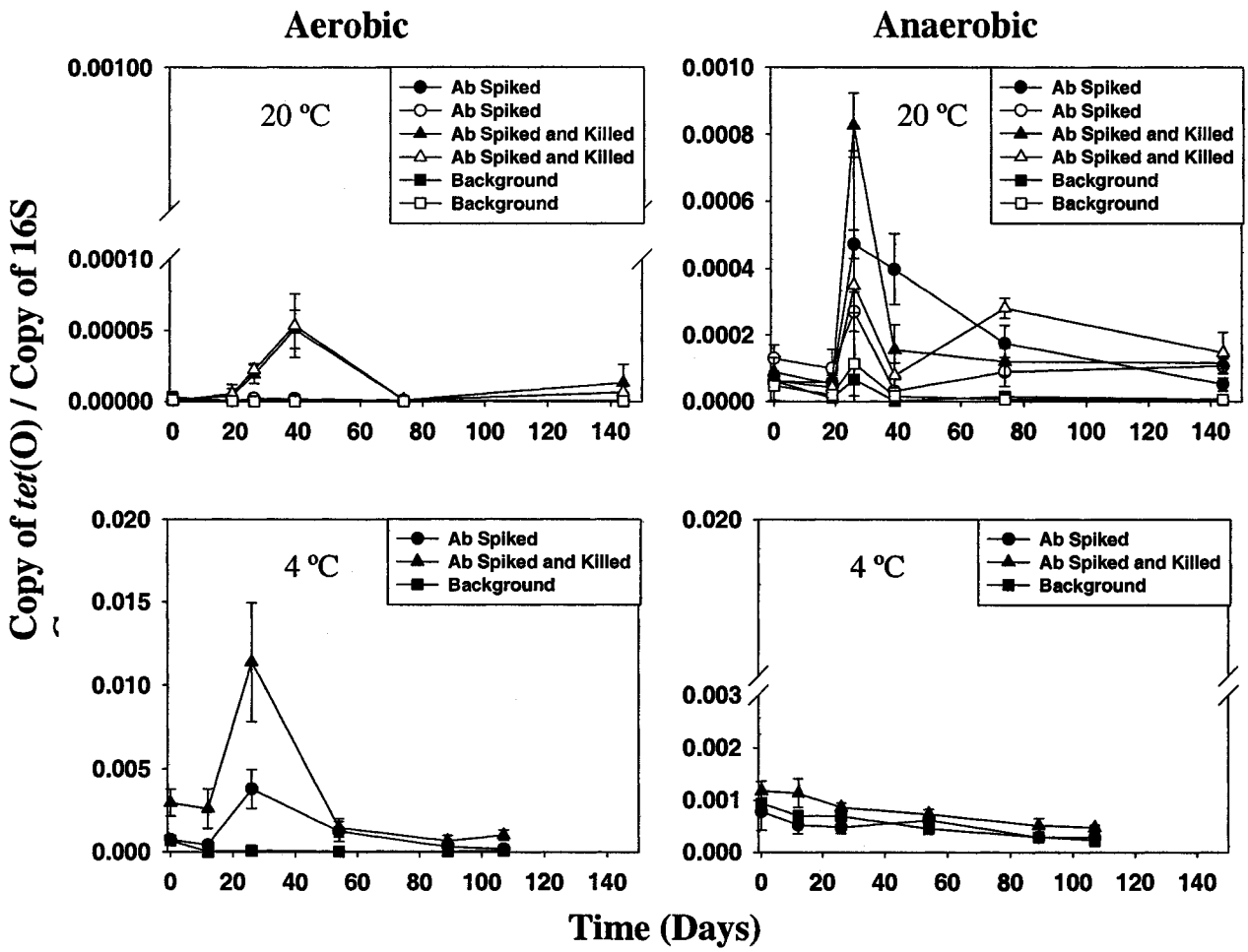

Figure 3. Copies of *tet*(O) ARG normalized to the number of Bacterial 16S rRNA genes in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment conducted in duplicate. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions for both temperatures were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of replicate Q-PCR measurements. The Y scale breaks were 0.0001 to 0.0095 for aerobic treatment at

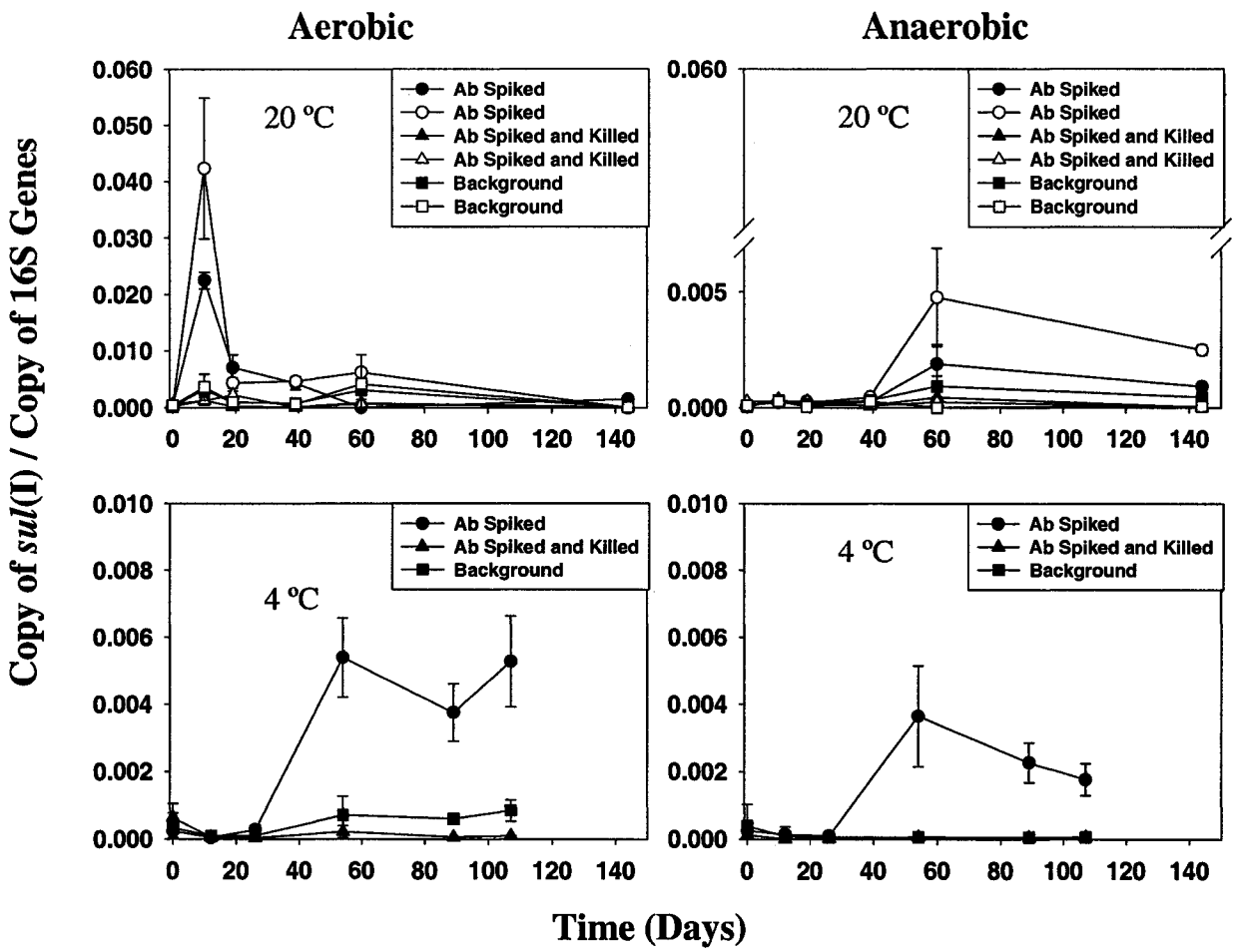

Figure 4. Copies of *sul*(I) ARG normalized to the number of Bacterial 16S rRNA genes in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment conducted in duplicate. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions for both temperatures were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of replicate Q-PCR measurements. The Y scale breaks for anaerobic treatment at 20 °C was from 0.0009

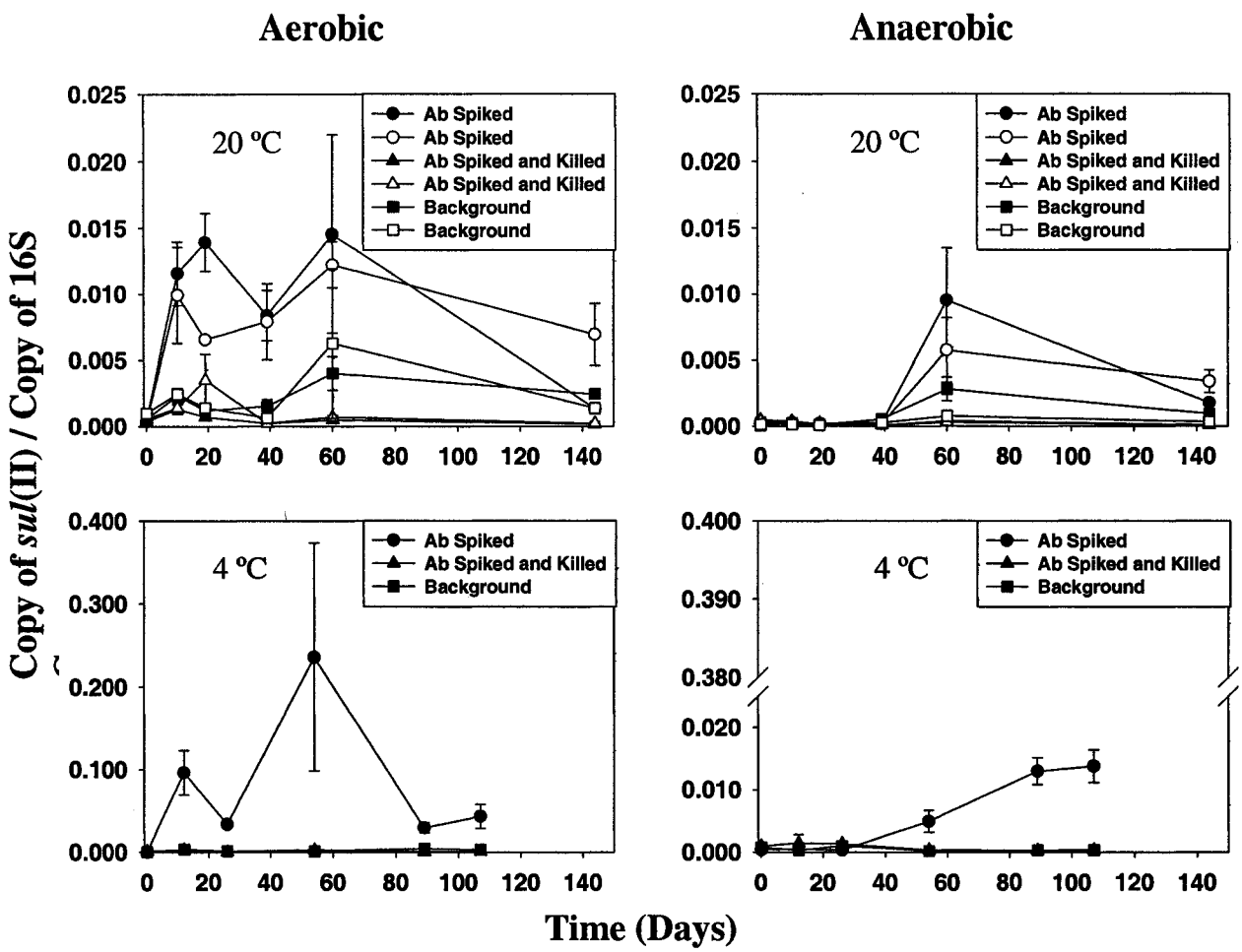

Figure 5. Copies of *sul*(II) ARG normalized to the number of Bacterial 16S rRNA genes in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment conducted in duplicate. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions for both temperatures were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of replicate Q-PCR measurements.The Y scale breaks for anaerobic treatment at 4 °C was from 0.0025 to 0.038.

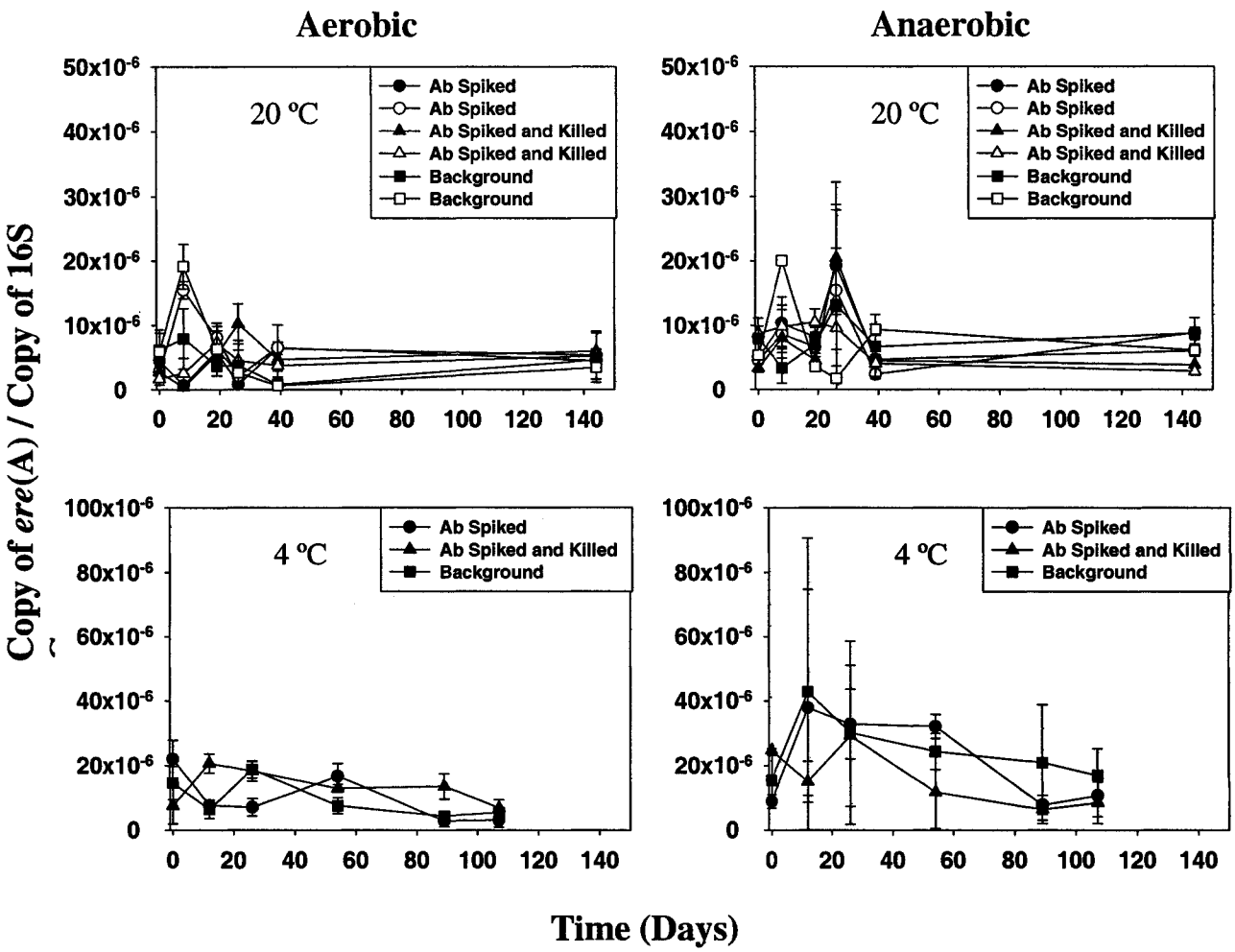

Figure 6. Copies of *ere*(A) ARG normalized to the number of Bacterial 16S rRNA genes in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment conducted in duplicate. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions for both temperatures were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of replicate Q-PCR measurements.

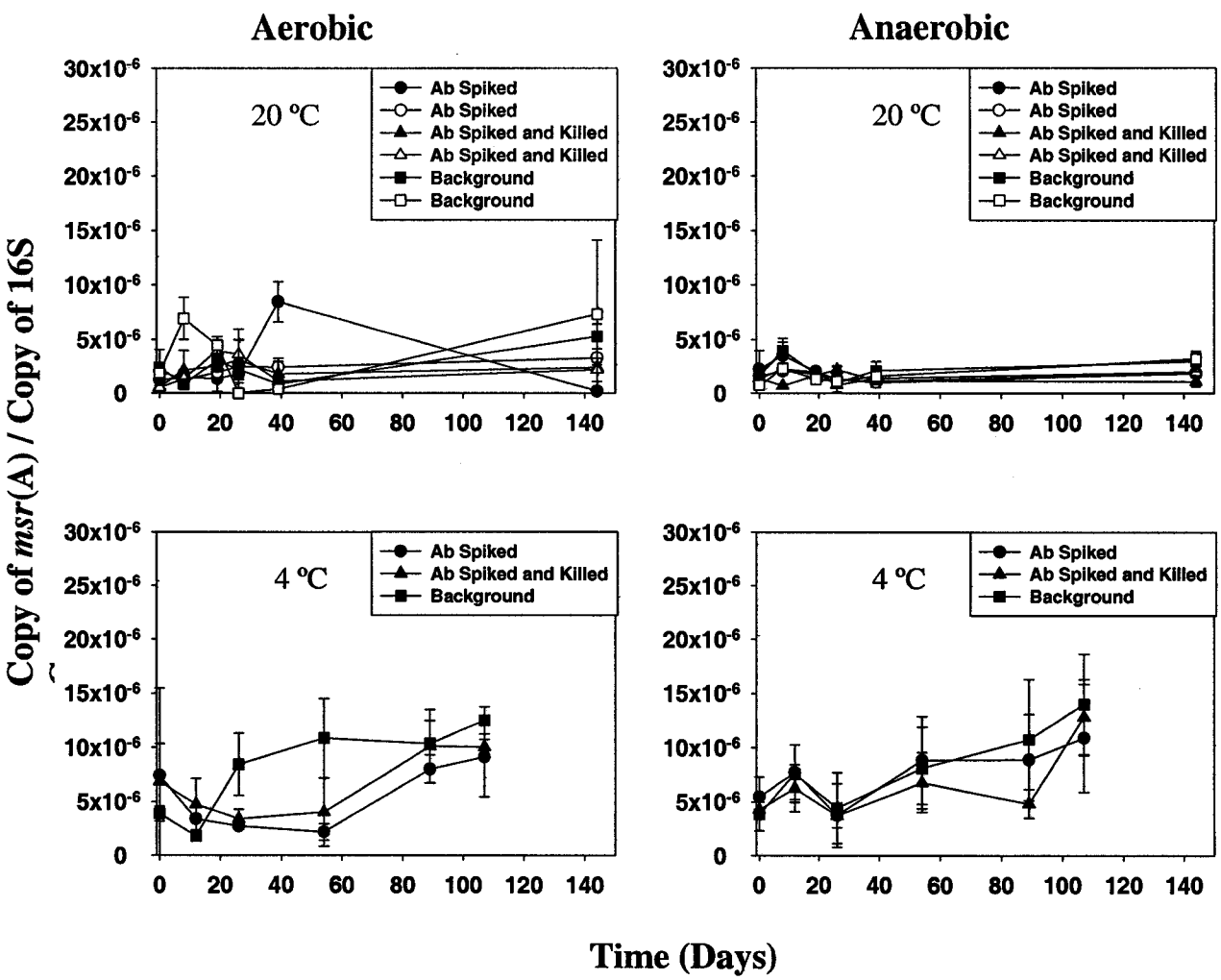

Figure 7. Copies of *msr*(A) ARG normalized to the number of Bacterial 16S rRNA genes in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment conducted in duplicate. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions for both temperatures were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of replicate Q-PCR measurements.

CHAPTER 6: CONCLUSIONS AND FUTURE WORK

In this study, a molecular biological tool, Q-PCR, was developed for quantifying ARG in different environmental samples. This provided a way to quantitatively assess the occurrence of ARG in the environment as they relate to human and agricultural activities, as well as the presence of antibiotic contaminants. Using the Poudre River watershed as a model system, it was demonstrated that there is a relationship between land-use and levels of ARG in the environment. This may be an indicator of corresponding antibiotic contamination in the environment, and highlighted the need to better characterize potential sources of antibiotics and ARG and to develop strategies for their containment.

Considering that dairy lagoons were identified as a potential source of ARG in a spatial survey of the Poudre watershed, the effect of lagoon treatment on ARG was investigated. Lagoons are commonly employed in on-farm animal waste management, and optimizing their performance prior to the application of the residual could help reduce the spread of ARG in the environment. The responses of the different ARG studied [*tet*(W), *tet*(O), *sul*(I), *sul*(II), *msr*(A), and *ere*(A)] varied considerably with respect to their response to the different treatments (aerobic at 20 °C and 4 °C and anaerobic at 20 °C and 4 °C). While most of the ARG returned to initial levels after any observed increases in response to treatment, no ARG were reduced below the initial level. This indicates that biological treatment may not be ideal for reducing ARG, but that if it is employed, it is important to wait a sufficient period of time before land-

applying the residuals, or the result may be further spreading the ARG. Physical or chemical treatment approaches may destroy antibiotics while also destroying ARG and the bacteria that harbor them. It is also possible that biological treatment would be more effective if continuously amended with a carbon source, such as is typical in on-farm lagoons.

In summary, this study was an important step in viewing ARG as emerging contaminants, and provided methods to track the occurrence of ARG within various environmental compartments and the response to biological treatment processes like dairy lagoons. Future studies should further investigate the fate and transport properties of ARG in the environment. For example, are ARG transported farther than antibiotics, or rather, is a direct correlation between ARG and antibiotics maintained? Future studies are also recommended that further explore potential treatment options for ARG. To be successful, such approaches should work with existing on-farm approaches and infrastructure. In considering ARG as contaminants, they have unique properties: they can be transported within living bacterial hosts or present as naked DNA bound to clay particles which may then be transported. As observed in this study, they may also amplify in the environment under certain conditions. Further studies to explore the methods to track and distinguish different transport and amplification properties of ARG are recommended, so that models may be developed to describe the spread of ARG in the environment. Development source-tracking approaches for ARG will be instrumental in developing fate and transport models, allowing watershed managers to take action and help reduce the spread of ARG. Future research should also evaluate additional ARG groups, especially those that most directly affect human health, such as ARG related to

"last resort" antibiotics like vancomycin. Then, strategies targeting the spread of such critical ARG, which are arguably life threatening, should be developed.

Appendix A: Supporting Information for PCR Primer Design

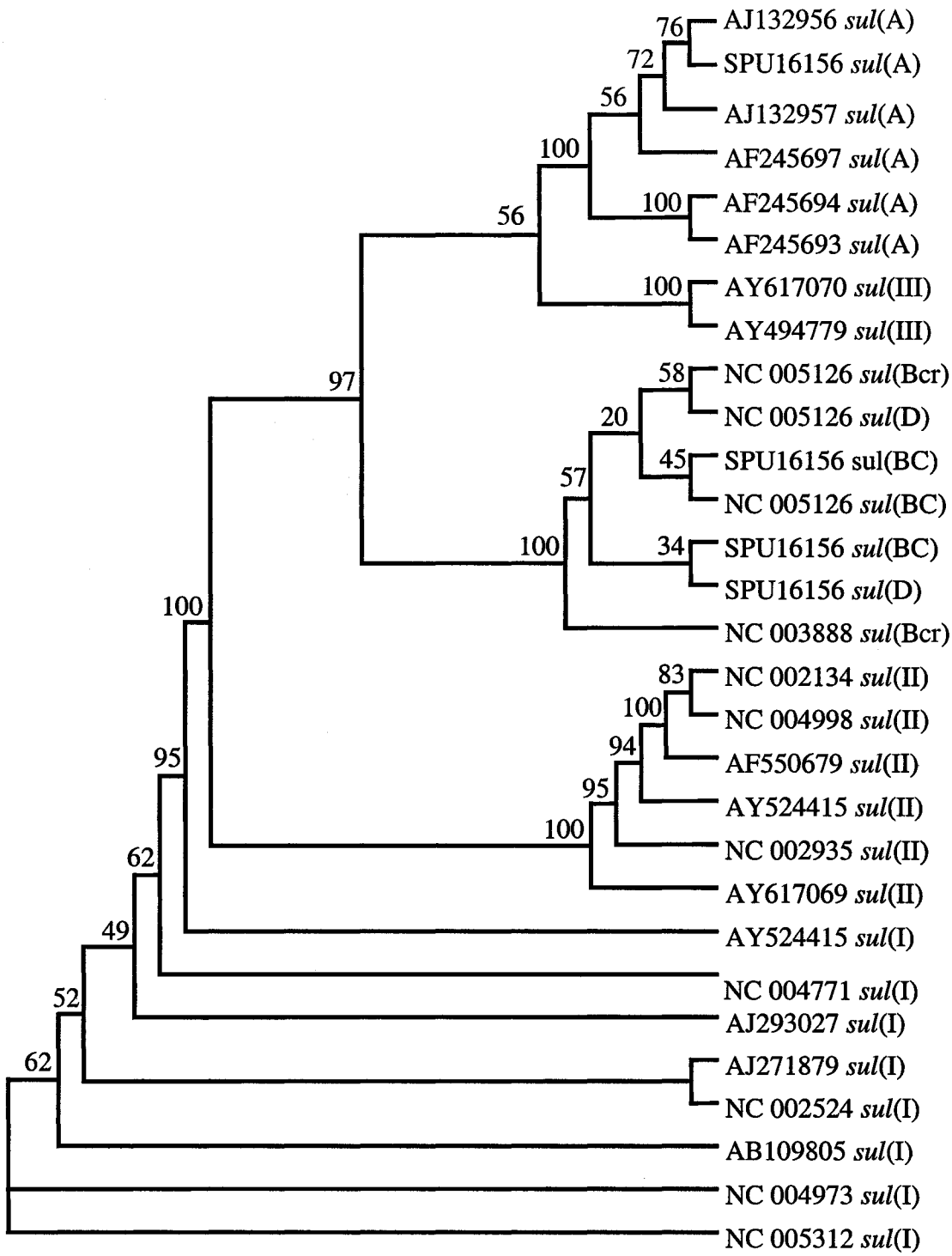

FIG. 1. Distance tree of different clusters of sulfonamide resistance genes constructed using a distance matrix with 1000 bootstrap replicates. Bootstrap values > 50% are indicated at the corresponding nodes. GenBank accession numbers are indicated for individual sequences.

Table 1 Distance tree summary for macrolide resistance genes

SN	Protein ID - Cluster #1		
M201	antibiotic resistance rRNA adenine methyltransferase		783
M466	rRNA (adenine-N6-)-methyltransferase	ermSV	793
M198	putative membrane transport protein		1335
M200	putative transferase		1005
M7	putative macrolide O-acyltransferase	mdmB	1179
M248	putative macrolide O-acyltransferase	mdmB	1179
m122	putative macrolide resistance protein		936
M62	macrolide-specific ABC-type efflux carrier	macB	1962
M64	macrolide-specific ABC-type efflux carrier	macB	1962
M160	macrolide-specific ABC-type efflux carrier	macB	1962
M283	macrolide-specific ABC-type efflux carrier	macB	1962
M29	macrolide 2'-phosphotransferase	mphA	1050
M361	macrolide 2'-phosphotransferase	mphA	1050
M453		ermE2	1220
M203	putative O-methyltransferase		672
M307	putative macrolide C12,C13-epoxidase	chmPII	1206
M308	putative macrolide C8-hydroxylase	chmPI	1224
M16	macrolide-transport ATP-binding protein ABC transporter		1629
M23	macrolide-transport ATP-binding protein ABC transporter		1629
M178	macrolide-transport ATP-binding protein ABC transporter		1629
M51	macrolide-transport ATP-binding protein ABC transporter		1629
M193	macrolide-transport ATP-binding protein ABC transporter		1629
M401	adenine rRNA methylase confers resistance to macrolide-lincosamide (ML) agents	erm(38)	1161
M402	adenine rRNA methylase confers resistance to macrolide-lincosamide-streptogramin B (MLSb) agents	erm	300
M381	adenine rRNA methylase similar to Mycobacterium fortuitum Erm(39) encoded by GenBank Accession Number AY487229; similar to Mycobacterium smegmatis Erm(38) encoded by GenBank Accession Number AY154657	erm(40)	756
M22	Probable conserved integral membrane transport (efflux) protein, possibly member of major facilitator superfamily (MFS), highly similar to O32859|TAP PROTEIN multidrug-resistance efflux pump from Mycobacterium fortuitum		1260
M177	member of major facilitator superfamily (MFS), highly integral membrane transport (efflux) protein, possibly similar to O32859|TAP PROTEIN multidrug-resistance efflux pump from Mycobacterium fortuitum Probable conserved		1260
M15	Probable conserved pump from Mycobacterium fortuitum member of major facilitator superfamily (MFS), highly similar to O32859|TAP PROTEIN multidrug-resistance efflux integral membrane transport (efflux) protein, possibly		1260
M429	putative adenine rRNA methylase Erm; similar to Mycobacterium tuberculosis ErmMT	erm	440
M382	putative macrolide 2'-phosphotransferase		984
M28	putative macrolide-efflux protein		1032
M314	putative macrolide-efflux protein	TTHA0028	1032
M288	probable macrolide-efflux transmembrane protein		1080
M379	probable macrolide-efflux transmembrane protein		1080
M114	macrolide efflux ABC transporter, ATP-binding/permease protein	macB	1959

M118	macrolide efflux ABC transporter, ATP-binding/permease protein	macB	1959
M202	putative antibiotic resistance macrolide glycosyltransferase	SCO6090	1257
M473	macrolide glycosyl transferase	mgt	1257
M457	adenine methylase	ermB	738
M463	macrolide-resistance determinant	carA	192
M204	putative glycosyl transferase	SCO0040	1224
M388	Macrolide glycosyltransferase		1215
M52	MACROLIDE-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER		1776
M194	MACROLIDE-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER		1776
M18	Probable first part of macrolide-transport ATP-binding protein ABC transporter (see citation below), similar to many ATP-binding proteins ABC transporter		1119
M25	Probable first part of macrolide-transport ATP-binding protein ABC transporter (see citation below), similar to many ATP-binding proteins ABC transporter		1119
M176	Probable first part of macrolide-transport ATP-binding protein ABC transporter (see citation below), similar to many ATP-binding proteins ABC transporter		1119
M24	PROBABLE SECOND PART OF MACROLIDE-TRANSPORT -BINDING PROTEIN ABC TRANSPORTER		654
M175	PROBABLE SECOND PART OF MACROLIDE-TRANSPORT -BINDING PROTEIN ABC TRANSPORTER		654
M17	Probable second part of macrolide-transport ATP-binding protein ABC transporter (see citation below), with similarity to C-terminal end of putative ABC transporters/ATP binding proteins		654
M309	putative macrolide C20-hydroxylase	chmHI	1263
M14	Also similar to transferases e.g. P77026 MACROLIDE 2'-PHOSPHOTRANSFERASE II from Escherichia coli (279 aa)	lipG	906
M50	Also similar to transferases e.g. P77026 MACROLIDE 2'-PHOSPHOTRANSFERASE II from Escherichia coli (279 aa)	lipG	906
M21	Also similar to transferases e.g. P77026 MACROLIDE 2'-PHOSPHOTRANSFERASE II from Escherichia coli (279 aa)	lipG	906
M19	Also similar to transferases e.g. P77026 MACROLIDE 2'-PHOSPHOTRANSFERASE II from Escherichia coli (279 aa)	lipG	906
M179	Also similar to transferases e.g. P77026 MACROLIDE 2'-PHOSPHOTRANSFERASE II from Escherichia coli (279 aa)	lipG	906
M195	PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN Also similarity with several MACROLIDE-EFFLUX PROTEINS		1326
M207	probable hydrolase Similar to M. tuberculosis putative hydrolase Rv0646c TR:P96935 (EMBL:Z92772) (301 aa); Fasta score E(): 0, 71.3% identity in 303 aa overlap, and to others e.g. Escherichia coli macrolide 2'-phosphotransferase II	lipG	915
M206	PROBABLE MACROLIDE-EFFLUX TRANSMEMBRANE PROTEIN	RSp0310	1353
M225	PROBABLE MACROLIDE-EFFLUX TRANSMEMBRANE PROTEIN	RSp0310	1353
M125	probable macrolide-efflux transmembrane protein		1236
M13	UNKNOWN; POSSIBLY INVOLVED IN TRANSPORT OF MACROLIDE ACROSS THE MEMBRANE.		1326
M20	UNKNOWN; POSSIBLY INVOLVED IN TRANSPORT OF MACROLIDE ACROSS THE MEMBRANE.		1326
M49	PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN Also similarity with several MACROLIDE-EFFLUX PROTEINS		1326
M172	PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN Also similarity with several MACROLIDE-EFFLUX PROTEINS		1326
M196	PROBABLE CONSERVED INTEGRAL MEMBRANE PROTEIN Also similarity with several MACROLIDE-EFFLUX PROTEINS		1326
M455	putative ABC transporter putative macrolide antibiotic resistance		402
M464	acyB1 or carE 4'-O-acylation of macrolide antibiotics		1167

M209	possible acyltransferase Similar to several acyltransferases including: Streptomyces thermotolerans macrolide antibiotics 3-O-acyltransferase TR:Q56074 (EMBLD30759:) (389 aa);	ML1101	1140
M161	putative drug-efflux protein Similar to Escherichia coli macrolide-specific efflux protein MacA		1203
M284	putative drug-efflux protein Similar to Escherichia coli macrolide-specific efflux protein MacA		1203
M63	macrolide-specific ABC-type efflux carrier	macB	1962
M65	macrolide efflux protein MacA	macA	1287
M113	macrolide efflux ABC transporter ATP-binding/permease protein	macB	1959
M117	macrolide efflux protein MacA		1152
M26	PROBABLE MACROLIDE-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER		1677
M170	PROBABLE MACROLIDE-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER		1677
M53	PROBABLE MACROLIDE-TRANSPORT ATP-BINDING PROTEIN		1677
M192	PROBABLE MACROLIDE-TRANSPORT ATP-BINDING PROTEIN ABC TRANSPORTER		1677
M208	probable O-methyltransferase involved in the synthesis of the macrolide antibiotic midecamycin	ML1075	675
M27	POSSIBLE ACYL-CoA DEHYDROGENASE FADE19 (MMGC) UNKNOWN, BUT SEEMS INVOLVED IN METABOLISM OF SMALL BRANCHED-CHAIN FATTY ACIDS AND MACROLIDE ANTIBIOTIC PRODUCTION	fadE19	1185
M171	POSSIBLE ACYL-CoA DEHYDROGENASE FADE19 (MMGC) UNKNOWN, BUT SEEMS INVOLVED IN METABOLISM OF SMALL BRANCHED-CHAIN FATTY ACIDS AND MACROLIDE ANTIBIOTIC PRODUCTION	fadE20	1185
M454	putative streptomycin 6-kinase homolog putative macrolide antibiotic resistance		517
M10	macrolide 2'-phosphotransferase I similar to BAB12239.1 macrolide 2'-phosphotransferase I Mph(A) of Escherichia coli	mph(A)	906
M220	macrolide 2'-phosphotransferase confers erythromycin and roxithromycin resistance	mph(A)	920
M213	macrolide 2'-phosphotransferase confers resistance against erythromycin and roxithromycin resistance	mph(A)	906
M449	macrolide 2'-phosphotransferase I Mph(A)	mph(A)	906
M321	macrolide 2'-phosphotransferase I	mph(A)	906
M384	macrolide 2'-phosphotransferase I	mphA	906
M139	macrolide 2'-phosphotransferase I	mph(A)	906
M436	macrolide 2'-phosphotransferase I	mphA	906
M138	macrolide 2'-phosphotransferase	mphA	906
M210	putative glycosyl transferase Similar to Streptomyces venezuelae desVII, glycosyl transferase from a macrolide antibiotic biosynthesis gene cluster	ML0125	1317
M211	putative glycosyl transferase Similar to Streptomyces venezuelae desVII, glycosyl transferase from a macrolide antibiotic biosynthesis gene cluster	ML0128	1308
M1	Putative macrolide resistance protein		870
M181	Putative macrolide resistance protein Similar to Micromonospora griseorubida mycinamicin-resistance protein MyrA SW:MYRA_MICGR (P37000)		870
M12	macrolide efflux ABC transporter, ATP-binding/permease protein	macB	1971
M89	macrolide efflux ABC transporter, ATP-binding/permease protein	macB	1971
M115	macrolide efflux ABC transporter, ATP-binding/permease protein	macB	1974
M119	macrolide efflux ABC transporter, ATP-binding/permease protein	macB	1974
M430	23S ribosomal RNA methyltransferase ErmML confers resistance to erythromycin and other macrolide-lincosamide-streptogramin B (MLS) antibiotics	ermML	846
M431	23S ribosomal RNA methyltransferase ErmML confers resistance to erythromycin and other macrolide-lincosamide-streptogramin B (MLS) antibiotics	ermML	846
M174	Macrolide specific ABC-type transporter, ATP-binding protein	macB	1953
M311	Macrolide specific ABC-type transporter, ATP-binding protein	macB	1953

M180	Putative integral membrane acyltransferase Similar to Mycobacterium tuberculosis hypothetical 43.3 kDa protein Rv0228 or MT0238 or MTCY08D5.23 TR:P96410 (EMBL:Z92669) (407 aa) fasta scores: E(): 1.2e-31, 38.84% id in 381 aa, and to Streptomyces thermotolerans macrolide antibiotics 3-O-acyltransferase AcyA		1140
M336	probable macrolide efflux protein		1317
M400	probable macrolide efflux protein		1317
M383	macrolide 2'-phosphotransferase I repressor A	mphR	585
M450	repressor protein MphR(A); Mrx; macrolide 2'-phosphotransferase I Mph(A)		585
M232	MACROLIDE-EFFLUX PROTEIN		1239
M73	moderate macrolide resistance-conferring membrane protein		1074
M399	23S rRNA N-6-methyltransferase	ermX	762
M446	ErmX	ermX	855
M445	ErmX	ermX	855
M140	macrolide-specific ABC-type efflux carrier	macB	1953
M162	macrolide-specific ABC-type efflux carrier	macB	1953
M129	putative permease component of ABC transporter Similar to C-terminus of Escherichia coli macrolide-specific ABC-type efflux carrier MacB or B0879 SWALL:MACB		1221
M157	putative permease component of ABC transporter Similar to C-terminus of Escherichia coli macrolide-specific ABC-type efflux carrier MacB or B0879 SWALL:MACB		1221
M9	macrolide transport protein (ABC superfamily, atp_bind (N-terminal), membrane (C-terminal))	macB	1947
M91	macrolide-specific ABC-type efflux carrier	macB	1947
M173	probable macrolide-specific efflux protein		966
M310	probable macrolide-specific efflux protein		966
M3	macrolide-binding protein FKBP12		851
M112	macrolide-binding protein FKBP12		851
M4	macrolide-binding protein FKBP12		405
M141	macrolide-specific efflux protein maca precursor	macA	1197
M163	macrolide-specific efflux protein maca precursor	macA	1197
M126	putative transport/efflux component protein Similar to Yersinia pestis probable macrolide-specific efflux protein MacA precursor or YPO1364 or Y2814 SWALL:MACA		1101
M154	putative transport/efflux component protein Similar to Yersinia pestis probable macrolide-specific efflux protein MacA precursor or YPO1364 or Y2814 SWALL:MACA		1101
M254	merB	merB	651
M317	mlr4194 similar to macrolide glycosyltransferase	mlr4194	1308
M66	ABC macrolide efflux transporter MacB, fused ATPase and inner membrane domains	macB	1998
M68	ABC macrolide efflux transporter MacB, fused ATPase and inner membrane domains	macB	1998
M70	ABC macrolide efflux transporter MacB, fused ATPase and inner membrane domains	macB	1998
M238	macrolide 2'-phosphotransferase	mphB	903
M291	macrolide 3'-phosphotransferase	mphB	903
M36	similar to macrolide-efflux transporter	yjbB	1218
M186	similar to macrolide-efflux transporter	yjbB	1218
M304	similar to macrolide-efflux transporter; RBL04922	yjbB	1281
M349	similar to macrolide-efflux transporter; RBL04922	yjbB	1281
M8	EcoGene suggests alternate start codon; macrolide-specific efflux protein; putative membrane; Not classified	macA	1143
M90	macrolide-specific efflux protein	macA	1143

M55	macrolide-specific efflux protein maca precursor	macA	1143
M428	macrolide-specific efflux protein maca precursor	macA	1143
M42	Macrolide-specific efflux protein MacA	macA	1113
M182	macrolide-specific efflux protein maca precursor	macA	1113
M33	similar to macrolide 2'-phosphotransferase	ycbJ	921
M191	similar to macrolide 3'-phosphotransferase	ycbJ	921
M432	homologue of Orf4 of macrolide 2'-phosphotransferase of E. coli	ycbJ	630
M169	Fusaric acid resistance protein probable macrolide-specific efflux protein macA precursor, HlyD family secretion protein hypothetical protein		1749
M312	Fusaric acid resistance protein probable macrolide-specific efflux protein macA precursor, HlyD family secretion protein hypothetical protein		1749
M128	putative permease component of ABC transporter C-terminus is similar to Escherichia coli macrolide-specific ABC-type efflux carrier MacB or B0879 SWALL:MACB		1266
M156	putative permease component of ABC transporter C-terminus is similar to Escherichia coli macrolide-specific ABC-type efflux carrier MacB or B0880 SWALL:MACB		1266
M76	glycosyltransferase; possible macrolide glycosyltransferase		1209
M101	glycosyltransferase; possible macrolide glycosyltransferase		1209
M325	glycosyltransferase; possible macrolide glycosyltransferase		1209
M353	glycosyltransferase; possible macrolide glycosyltransferase		1209
M263	Macrolide-efflux protein		1230
M421	Macrolide glycosyltransferase		1209
M300	similar to macrolide glycosyltransferase; RBL03304	yojK	1188
M345	similar to macrolide glycosyltransferase; RBL03305	yojK	1188
M468	rRNA methylase	ermC	224
M39	hypothetical protein similar to macrolide glycosyltransferase	yojK	1218
M34	similar to macrolide glycosyltransferase	ydhE	1146
M190	similar to macrolide glycosyltransferase	ydhE	1146
M301	similar to macrolide glycosyltransferase; RBL02291	ydhE	1221
M346	similar to macrolide glycosyltransferase; RBL02291	ydhE	1221
M270	Macrolide glycosyltransferase		1002
M415	Macrolide-efflux protein		1230
M271	Macrolide glycosyltransferase		1194
M410	Macrolide-efflux protein		1203
M111	adenine methylase macrolide resistance	erm(A)	152
M338	putative glycosyltransferase similar to macrolide glycosyltransferase		1170
M224	macrolide efflux pump, putative		1635
M281	macrolide efflux pump, putative		1635
M334	macrolide efflux pump, putative		1635
M374	macrolide efflux pump, putative		1635
M368	macrolide efflux pump, putative		1635
M255	macrolide glycosyltransferase		1173
M276	macrolide glycosyltransferase		1173
M164	macrolide transport protein (ABC superfamily, atp_bind (N-terminal), membrane (C-terminal))	macB	1995
M285	macrolide transport protein (ABC superfamily, atp_bind (N-terminal), membrane (C-terminal))	macB	1995
M56	Probable macrolide acetyltransferase		657
M258	macrolide-efflux protein, major facilitator (MFS) superfamily		1248
M279	macrolide-efflux protein, major facilitator (MFS) superfamily		1248
M274	Macrolide-efflux protein		1248
M407	Macrolide-efflux protein		1248
M303	similar to macrolide glycosyltransferase	yjiC	1191
M221	putative Macrolide 2'-phosphotransferase	ycbJ	912

M348	similar to macrolide glycosyltransferase	yjiC	1191
M297	Glycosyl transferase Family 1 similar to macrolide glycosyltransferase	yjiC	1191
M187	similar to macrolide glycosyltransferase	yjiC	1179
M188			1221
M37	hypothetical protein similar to macrolide glycosyltransferase	yjiC	1179
M296	putative Macrolide 2'-phosphotransferase	ycbJ	912
M299	similar to macrolide 2'-phosphotransferase	ycbJ	912
M344	similar to macrolide 2'-phosphotransferase; RBL00987	ycbJ	912
M222	putative macrolide-efflux protein	yvqJ	1263
M350	similar to macrolide-efflux protein	yvqJ	1263
M305	similar to macrolide-efflux protein	yvqJ	1263
M298	putative macrolide-efflux protein	yvqJ	1263
M40	similar to macrolide-efflux protein	yvqJ	1269
M185	similar to macrolide-efflux protein	yvqJ	1269
M86	Macrolide-efflux protein		1110
M87	Macrolide-efflux protein		1110
M257	macrolide-efflux protein, major facilitator (MFS) superfamily		1263
M278	macrolide-efflux protein, major facilitator (MFS) superfamily		1263
M302	similar to macrolide-efflux protein	ykuC	1302
M347	similar to macrolide-efflux protein	ykuC	1302
M38	similar to macrolide-efflux protein	ykuC	1293
M252	hypothetical protein similar to Bacillus halodurans macrolide 2'-phosphotransferase in GenBank Accession Number AP001508	mph1	250
M135	macrolide efflux pump, putative		360
M467		ermC	30
M226	macrolide-efflux determinant	mefE	240
M80	macrolide efflux protein		1284
M105	macrolide efflux protein		1284
M329	macrolide efflux protein		1284
M357	macrolide efflux protein		1284
M269	macrolide efflux protein		1284
M414	macrolide efflux protein		1284
M260	macrolide efflux protein		1239
M423	macrolide efflux protein		1239
M143	macrolide-efflux protein, putative		1179
M282	macrolide-efflux protein, putative		1179
M341	putative protein similar to Bacillus halodurans macrolide 2'-phosphotransferase in GenBank Accession Number AP001508	mph1	250
M41	similarities with macrolide-efflux pump		1206
M184	unnamed protein product; Some similarities with macrolide-efflux pump. Putative transmembrane protein		1206
M137	putative macrolide-efflux protein	mreA	933
M318	putative macrolide-efflux protein	SPs1086	933
M54	putative macrolide-efflux protein	SPs1086	933
M153	putative macrolide-efflux protein	mreA	933
M230	putative macrolide-efflux protein		933
M322	macrolide-efflux protein		946
M452	macrolide-efflux protein	mreA	936
M205	macrolide-efflux protein	gbs1032	940
M339	macrolide-efflux protein mreA/riboflavin biosynthesis protein RibF		933
M337	putative macrolide-efflux protein		921
M236	1-acyl-sn-glycerol-3-phosphate acyltransferase macrolide-efflux protein		1869
M237	macrolide-efflux protein		1869
M275	Macrolide-efflux protein		1203
M408	Macrolide-efflux protein		1203

M44	hypothetical protein Similar to macrolide-efflux protein		1233
M123	macrolide-efflux protein		1248
M239	macrolide-efflux transporter		1218
M292	macrolide-efflux transporter		1218
M217	adenine methylase macrolide resistance	erm(A)	145
M84	macrolide-efflux protein		1260
M109	macrolide-efflux protein		1260
M273	macrolide-efflux protein		1260
M409	macrolide-efflux protein		1260
M332	macrolide-efflux protein		1260
M360	macrolide-efflux protein		1260
M262	macrolide-efflux protein		1218
M420	macrolide-efflux protein		1218
M375	macrolide-efflux protein		1227
M378	macrolide-efflux protein		1227
M35	similar to macrolide-efflux transporter	yfmI	1221
M189	similar to macrolide-efflux transporter	yfmI	1221
M59	hypothetical protein similarity to unknown protein: macrolide-efflux determinant related PAB0754 - Pyrococcus abyssi (strain Orsay); PIR:D75093		1107
M212	conserved hypothetical protein similarity to unknown protein: macrolide-efflux determinant related PAB0754 - Pyrococcus abyssi (strain Orsay); PIR:D75093		1107
M67	macrolide secretion protein MacA, HlyD family	macA	1368
M69	macrolide secretion protein MacA, HlyD family	macA	1368
M71	macrolide secretion protein MacA, HlyD family	macA	1368
M48	O-methyltransferase-like protein macrolide antibiotics synthesis		618
M47	macrolide 2'-phosphotransferase-like protein		1263
M313	putative transferase Weakly similar to Escherichia coli macrolide 2'-phosphotransferase I MphA SWALL:Q47396 (EMBL:D16251)		1176
M385			845
M470	macrolide-lincosamide-streptogramin B-resistance protein	ermM	735
M471	macrolide-lincosamide-streptogramin B-resistance protein	mls	735
M229	rRNA methylase mediates resistance to macrolides, lincosamides and streptogramin B antibiotics	ermC	735
M425	Bacillus subtilis plasmid pIM13 macrolide-lincosamide-streptogramin B resistance protein	mlsR	735
M426	Bacillus subtilis plasmid pIM13 macrolide-lincosamide-streptogramin B resistance protein	mlsR	735
M427	Bacillus subtilis plasmid pIM13 macrolide-lincosamide-streptogramin B resistance protein	mlsR	735
M386	macrolide-lincosamide-streptogramin B-resistance protein	mls	735
M405	Bacillus subtilis plasmid pIM13 macrolide-lincosamide-streptogramin B resistance protein	mlsR	735
M5	rRNA adenine N-6-methyltransferase (Macrolide-lincosamide-streptogramin B resistance protein) (Erythromycin resistance protein)	ermC	735
M120	hypothetical protein rRNA adenine N-6-methyltransferase (Macrolide-lincosamide-streptogramin B resistance protein) (Erythromycin resistance protein)	ermC	735
M110	inducible macrolide, lincosamide and streptogramin B resistance	ErmC	735
M121	inducible macrolide, lincosamide and streptogramin B resistance	ErmC	735
M245	23S RNA methylase	ErmC	735
M477	23S RNA methylase	ErmC	735
M214	rRNA adenine N-6-methyltransferase (Macrolide-lincosamide-streptogramin B resistance protein)	ErmC	735

M391	adenine methylase confers upon Staphylococcus aureus resistance to macrolide, lincosamide, and streptogramin type B (MLS) antibiotics mediated by a specific N6-dimethylation of adenine in 23 S rRNA; confers erythromycin-resistance	erm	735
M465	adenine methylase	ermC	735
M469	rRNA methylase	ermC	224
M443	Erm(B) truncated leader peptide	erm(B)-L	84
M31	Macrolide-specific ABC-type efflux carrier		1464
M363	Macrolide-specific ABC-type efflux carrier		1464
M366	involved in macrolide efflux	mat(A)	1464
M377	Mel protein to staphylococcal macrolide resistance protein MsrSA; putative ATP-binding protein	mel	1464
M93	ABC transporter involved in macrolide resistance	msr(D)	1464
M286	rRNA adenine N-6-methyltransferase 1	ermA1	732
M287	rRNA adenine N-6-methyltransferase 2	ermA2	732
M167	rRNA adenine N-6-methyltransferase 1	ermA1	732
M168	rRNA adenine N-6-methyltransferase 2	ermA2	732
M216	adenine methylase resistance to macrolide-lincosamide-streptogramin B antibiotics	erm(33)	744
M246	macrolide-specific efflux protein macA		1173
M249	macrolide-specific efflux protein macA		1173
M451	erythromycin resistance protein contains two putative macrolide resistant genes	msrA	1467
M456	silence of this gene makes susceptibility to macrolide antibiotics	msrA	1467
M459	macrolide antibiotics resistance gene		1467
M142	rRNA methyltransferase macrolide-lincosamide-streptogramin B resistance protein from Bacteroides thetaiotaomicron Tcr Emr 7853 conjugative transposon		735
M150	erythromycin ribosome methylase macrolide-lincosamide resistance	ermB	739
M149	erythromycin ribosome methylase macrolide-lincosamide resistance	ermB	732
M151	erythromycin ribosome methylase macrolide-lincosamide resistance	ermB	732
M148	erythromycin ribosome methylase macrolide-lincosamide resistance	ermB	732
M152	erythromycin ribosome methylase macrolide-lincosamide resistance	ermB	739
M440	erythromycin resistance methylase ErmB; resistance determinant in clinical isolate	ermB	738
M444	erythromycin resistance methylase	ermB	738
M406	MLS resistance protein macrolide-lincosamide-streptogramin B resistance		738
M72	MLS methylase ORF7; similar to MLS methylase, macrolide resistance genes ermB, GenBank Accession Number CAC29169		738
M372	macrolide resistance determining protein	ermB	738
M447	erythromycin resistance methylase	ermB	747
M434	rRNA methylase	erm(B)	738
M460	adenine methylase rRNA methylase; inducible resistance to macrolide-lincosamide-streptogramin B antibiotics	ermB	738
M439	erythromycin resistance methylase	erm(B)	738
M441	erythromycin resistance methylase ErmB; resistance determinant in clinical isolate	ermB	738
M458	adenine methylase resistance to macrolide/lincosamide antibiotics	ermB	738
M147	erythromycin ribosome methylase macrolide-lincosamide resistance	ermB	732
M438	rRNA methyltransferase Erm2B	erm2B	738
M251	probable macrolide efflux protein similar to Streptococcus pneumoniae ABC transporter in GenBank Accession Number AF376746	mel	1491
M340	probable macrolide efflux protein similar to Streptococcus pneumoniae ABC transporter in GenBank Accession Number AF376747	mel	1491
M77	macrolide efflux protein		1230
M102	macrolide efflux protein		1230
M326	macrolide efflux protein		1230
M354	macrolide efflux protein		1230
M264	macrolide efflux protein		1230
M416	macrolide efflux protein		1230
M75	macrolide efflux protein		1215

M100	macrolide efflux protein		1215
M324	macrolide efflux protein		1215
M352	macrolide efflux protein		1215
M261	macrolide efflux protein		1200
M422	macrolide efflux protein		1200
M166	putative membrane protein Weakly similar to Enterococcus sp. 130, Staphylococcus aureus, Streptococcus intermedius, Streptococcus salivarius, Streptococcus sp. 6, and Streptococcus pneumoniae macrolide efflux protein E MefA or MefE SWALL		1194
M241	putative membrane protein Weakly similar to Enterococcus sp. 130, Staphylococcus aureus, Streptococcus intermedius, Streptococcus salivarius, Streptococcus sp. 6, and Streptococcus pneumoniae macrolide efflux protein E MefA or MefE SWALL		1194
M219	putative ABC transporter putative efflux pump involved in antibiotic resistance	msrC	405
M448	acquired macrolide resistance-like protein		1479
M46	macrolide-specific ABC-type efflux carrier	macB	2091
M404	macrolide-specific ABC-type efflux carrier	macB	2091
M165	putative permease protein Similar to Clostridium perfringens probable ABC transporter cpe1540 SWALL:Q8XK63 (EMBL:AP003190) (394 aa) fasta scores: E(): 1.1e-34, 34.42% id in 398 aa, and to the C-terminal region of Escherichia coli macrolide-specific ABC-type efflux carrier MacB or b0879 SWALL		1179
M240	putative permease protein Similar to Clostridium perfringens probable ABC transporter cpe1540 SWALL:Q8XK63 (EMBL:AP003190) (394 aa) fasta scores: E(): 1.1e-34, 34.42% id in 398 aa, and to the C-terminal region of Escherichia coli macrolide-specific ABC-type efflux carrier MacB or b0879 SWALL		1179
M6	macrolide 2'-phosphotransferase II	mphBM	900
M116	macrolide 2'-phosphotransferase II	mphBM	900
M218	Macrolide 2'-phosphotransferase II	mpbBM	900
M247	macrolide-specific efflux protein macB		1926
M250	macrolide-specific efflux protein macB		1926
M462	23S rRNA methlyase erythromycin resistance determinant	ermQ	787
M227	putative macrolide efflux protein		1218
M228	putative macrolide efflux protein		1218
M234	Macrolide-efflux protein	mreA	918
M316	macrolide-efflux protein		918
M256	macrolide-efflux protein, major facilitator (MFS) superfamily		1326
M277	macrolide-efflux protein, major facilitator (MFS) superfamily		1326
M97	macrolide-efflux protein	mef	1326
M133	macrolide-efflux protein	mef	1326
M145	macrolide-efflux protein	mef	1332
M60	macrolide-efflux determinant		1212
M290	403aa long hypothetical macrolide-efflux determinant	PH1098	1212
M61	macrolide-efflux determinant related		1197
M45	probable macrolide-specific efflux protein	macA	1173
M403	probable macrolide-specific efflux protein	macA	1173
M333	macrolide 2-phosphotransferase, putative		897
M424	macrolide 2-phosphotransferase, putative		897
M367	macrolide 2-phosphotransferase, putative		897
M373	macrolide 2-phosphotransferase, putative		897
M280	macrolide 2-phosphotransferase, putative		897
M223	macrolide 2-phosphotransferase, putative		897
M74	macrolide 2-phosphotransferase		897
M99	macrolide 2-phosphotransferase		897
M323	possible macrolide 2-phosphotransferase		897
M351	possible macrolide 2-phosphotransferase		897

M58	macrolide 2'-phosphotransferase		897
M294	macrolide 2'-phosphotransferase		897
M57	macrolide efflux protein		1275
M293	macrolide efflux protein	OB0402	1275
M267	Macrolide-efflux protein		1254
M419	Macrolide-efflux protein		1254
M474	macrolide-lincosamide-streptogramin B resistance methylase	ermD	1252
M475	rRNA methyltransferase	ermJ	1287
M476	erythromycin-inducible 23S ribosomal RNA methylase	ermK	864
M132	macrolide-efflux determinant	mefE	240
M387	ribosomal methylase Erm34 confers resistance to macrolides, lincosamides and streptogramins B		1200
[illegible]	[illegible]	[illegible]	[illegible]
M369	macrolide-efflux protein involved in macrolide resistance	mef	1218
M370	macrolide-efflux protein involved in macrolide resistance	mef	1218
M371	macrolide-efflux protein involved in macrolide resistance	mef	1218
M376	macrolide efflux protein E effluxes erythromycin, 14- and 15-member macrolides	mefE	1218
M390	macrolide-efflux protein A effluxes erythromycin; 14 and 15-member; ABC transporter	mefA	1218
M393	macrolide-efflux protein Mef; involved in macrolide resistance	mef	1218
M395	macrolide-efflux protein Mef; involved in macrolide resistance	mef	1218
M364	Macrolide-efflux protein		1218
M461	macrolide-efflux protein effluxes erythromycin and other 14 and 15-member macrolides	mef	1277
M365	involved in macrolide efflux	mef(A)	1218
M32	Macrolide-efflux protein		1218
M289	macrolide-efflux protein confers resistance to macrolide antibiotics	mefA	1218
M396	macrolide-efflux protein Mef; involved in macrolide resistance	mef	1218
M215	macrolide-efflux protein	mefA	933
M92	macrolide efflux protein efflux protein involved in macrolide resistance	mef	1218
M389	macrolide-efflux protein	mef	1218
M397	macrolide-efflux protein	mef	1218
M398	macrolide-efflux protein	mef	1218
M394	macrolide-efflux protein	mef	1218
M2	macrolide-efflux protein	mef	1218
M81	permease; possible macrolide-efflux protein		1239
M106	permease; possible macrolide-efflux protein		1239
M130	putative membrane protein		1212
M158	putative membrane protein		1212
M144	putative macrolide efflux pump		1212
M343	putative macrolide efflux pump		1212
M197	multidrug transporter previous name:mef214	mdt(A)	1268
M83	macrolide efflux protein		1221
M108	macrolide efflux protein		1221
M331	macrolide efflux protein		1221
M359	macrolide efflux protein		1221
M85	Macrolide-efflux protein		1263
M88	Macrolide-efflux protein		1263
M265	Macrolide-efflux protein		513
M417	Macrolide-efflux protein		513
M79	macrolide-efflux protein		1227
M104	macrolide-efflux protein		1227
M328	Macrolide-efflux protein		1227
M356	Macrolide-efflux protein		1227
M412	Macrolide-efflux protein		1227

M413	Macrolide-efflux protein		1227
M268	Macrolide-efflux protein		1227
M82	macrolide efflux protein		1203
M107	macrolide efflux protein		1203
M330	macrolide efflux protein		1203
M3558	macrolide efflux protein		1203
M272	Macrolide-efflux protein		1203
M411	Macrolide-efflux protein		1203
M43	Macrolide-specific ABC-type efflux carrier protein MacB	macB	1944
M183	Macrolide-specific ABC-type efflux carrier protein MacB	macB	1944
M253	,similar to Escherichia coli macrolide 2'-phosphotransferase I in GenBank Accession Number D16251	mph2	630
M342	putative protein similar to Escherichia coli macrolide 2'-phosphotransferase I in GenBank Accession Number D16251	mph2	630
M30	Macrolide-efflux protein		1209
M362	Macrolide-efflux protein		1209
M95	macrolide-efflux protein	mefE	1176
M96	macrolide-efflux protein	mefE	1176
M11	UNKNOWN; LIPOLYTIC ENZYME INVOLVED IN CELLULAR METABOLISM similar to transferases e.g. P77026 MACROLIDE 2'-PHOSPHOTRANSFERASE II from Escherichia coli (279 aa)	lipG	1209
M94	macrolide-efflux protein	mefE	1209
M136	putative efflux protein,best non-GAS blastp hit: gb|AAK99775.1| (AE008470),ABC transporter membrane-spanning permease - macrolide,efflux [Streptococcus pneumoniae R6]		1176
M433	putative efflux protein best non-GAS blastp hit: gb|AAK99775.1| (AE008470)ABC transporter membrane-spanning permease - macrolide efflux [Streptococcus pneumoniae R6]	SpyM3_0386	
M233	ABC transporter membrane-spanning permease -macrolide efflux	mefE	1212
M235	ABC transporter membrane-spanning permease -macrolide efflux	ABC-MSP	1179
M98	probable macrolide-efflux protein	mef	1224
M134	probable macrolide-efflux protein	mef	1225
M131	putative O-acetyl transferase (capsular polysaccharide synthesis enzyme o-acetyl transferase)		633
M159	putative O-acetyl transferase (capsular polysaccharide synthesis enzyme o-acetyl transferase)		634
M259	macrolide-efflux transporter		1203
M295	macrolide-efflux transporter		1203
M124	Macrolide-efflux protein	FN1168	909
M231	Macrolide-efflux protein		909
M442	putative ABC transporter, ATP-binding protein; putative macrolide efflux genetic assembly		1485
M315	macrolide efflux protein, putative		1179
M242	hypothetical protein, similar to macrolide-efflux determinant	MW0062	1221
M306	hypothetical protein, similar to macrolide-efflux determinant	MW0062	1221
M78	permease; possible macrolide-efflux protein		1254
M103	permease; possible macrolide-efflux protein		1254
M327	permease; possible macrolide-efflux protein		1254
M355	permease; possible macrolide-efflux protein		1255
M266	Macrolide-efflux protein		1263
M418	Macrolide-efflux protein		1263
M146	macrolide-lincosamide-streptogramin B-resistance protein	ermF	801
M435	macrolide-lincosamide-streptogramin-resistance	ermF	801
M392	rRNA methyltransferase,macrolide-lincosamide resistance	ermF	801
M472	methyltransferase	ermFU	813

M437	macrolide-lincosamide-streptogramin B resistance protein	erm(35)	801
M243	hypothetical protein similar to macrolide efflux protein	alr2188	1308
M319	similar to macrolide-efflux pump	alr2189	1308
M244	hypothetical protein similar to macrolide efflux protein		1308
M320	similar to macrolide efflux protein	alr2215	1308
M335	macrolide efflux protein, putative		1245
M380	macrolide efflux protein, putative		1245
M127	putative transmembrane acyltransferase,Similar to Streptomyces avermitilis putative macrolide O-acyltransferase MdmB or sav3790 SWALL:Q82GV6	MdmB	1041
M155	putative transmembrane acyltransferase,Similar to Streptomyces avermitilis putative macrolide O-acyltransferase MdmB or sav3790 SWALL:Q82GV6	MdmB	1041

Appendix B: Copy of ARG normalized to initial levels of ARG

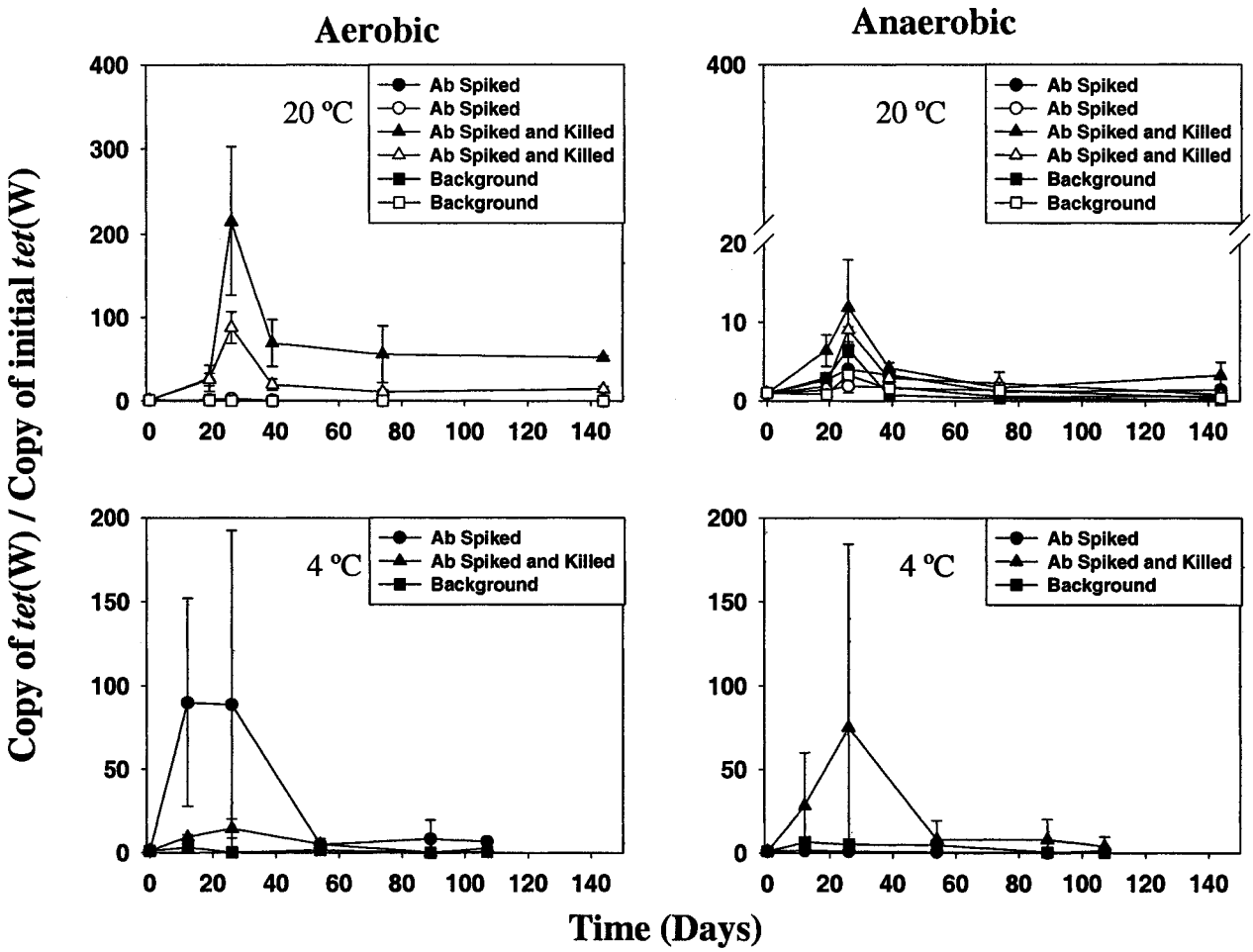

Figure 1. Copies of *tet*(W) ARG normalized to the initial level of *tet*(W) ARG in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions, in duplicate for 20 °C were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviations of Q-PCR replicates. The Y scale breaks was 20 to 390 for anaerobic treatment at 20 °C.

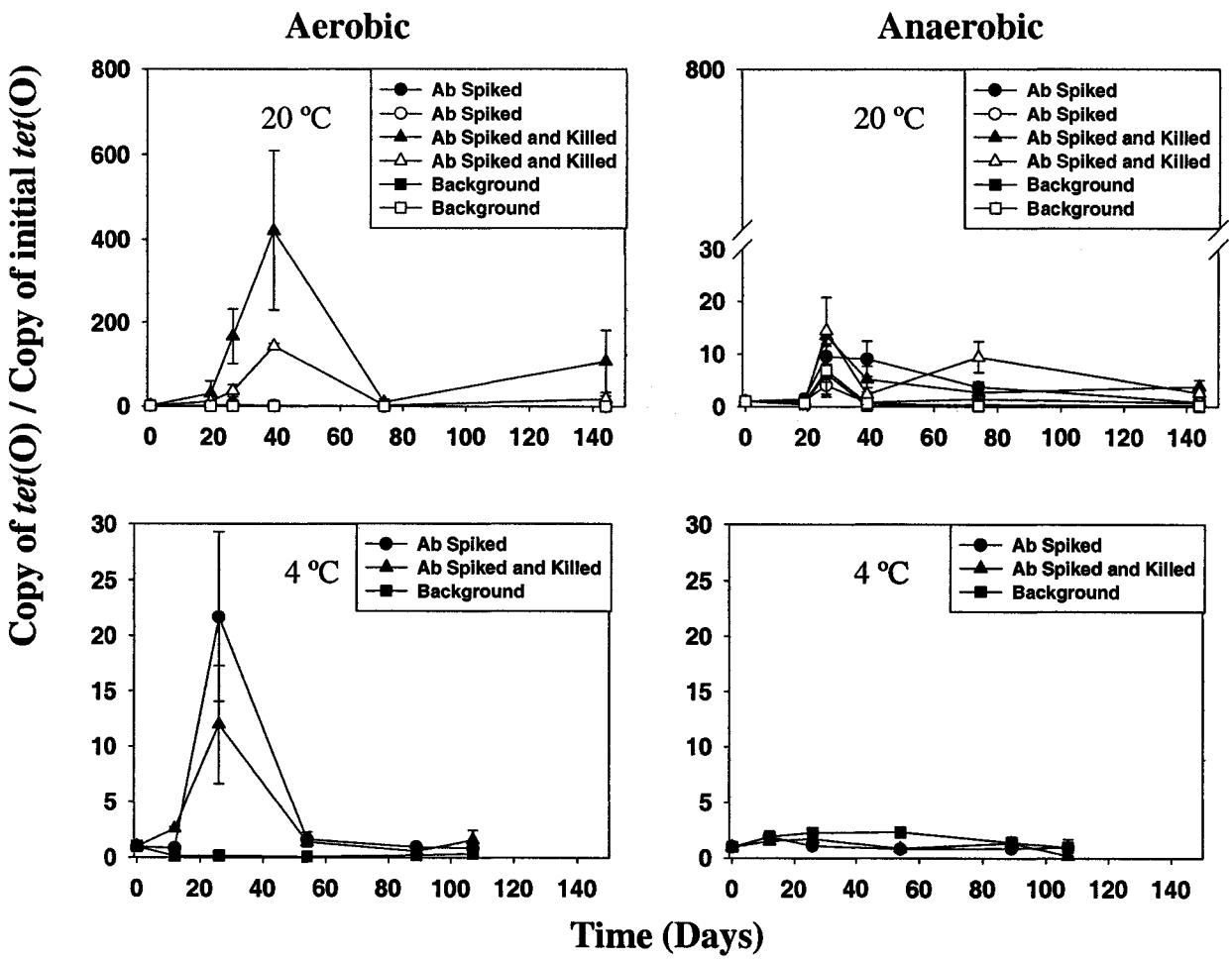

Figure 2. Copies of *tet*(O) ARG normalized to the initial level of *tet*(O) ARG in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions, in duplicate at 20 °C, were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of Q-PCR replicates. The Y scale breaks was 30 to 790 for aerobic treatment at 20 °C.

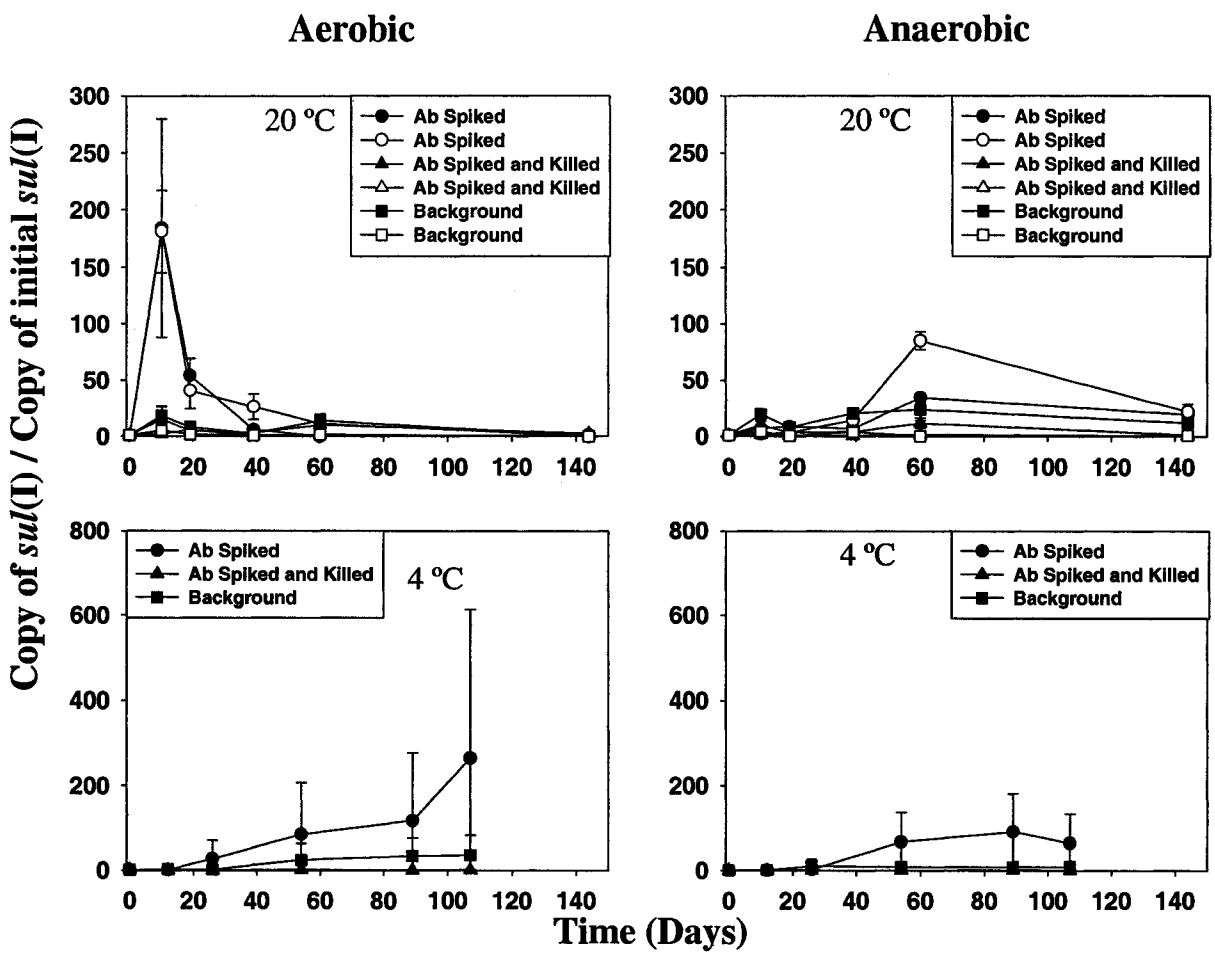

Figure 3. Copies of *sul*(I) ARG normalized to the initial level of *sul*(I) ARG in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions, in duplicate at 20 °C, were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of Q-PCR replicates.

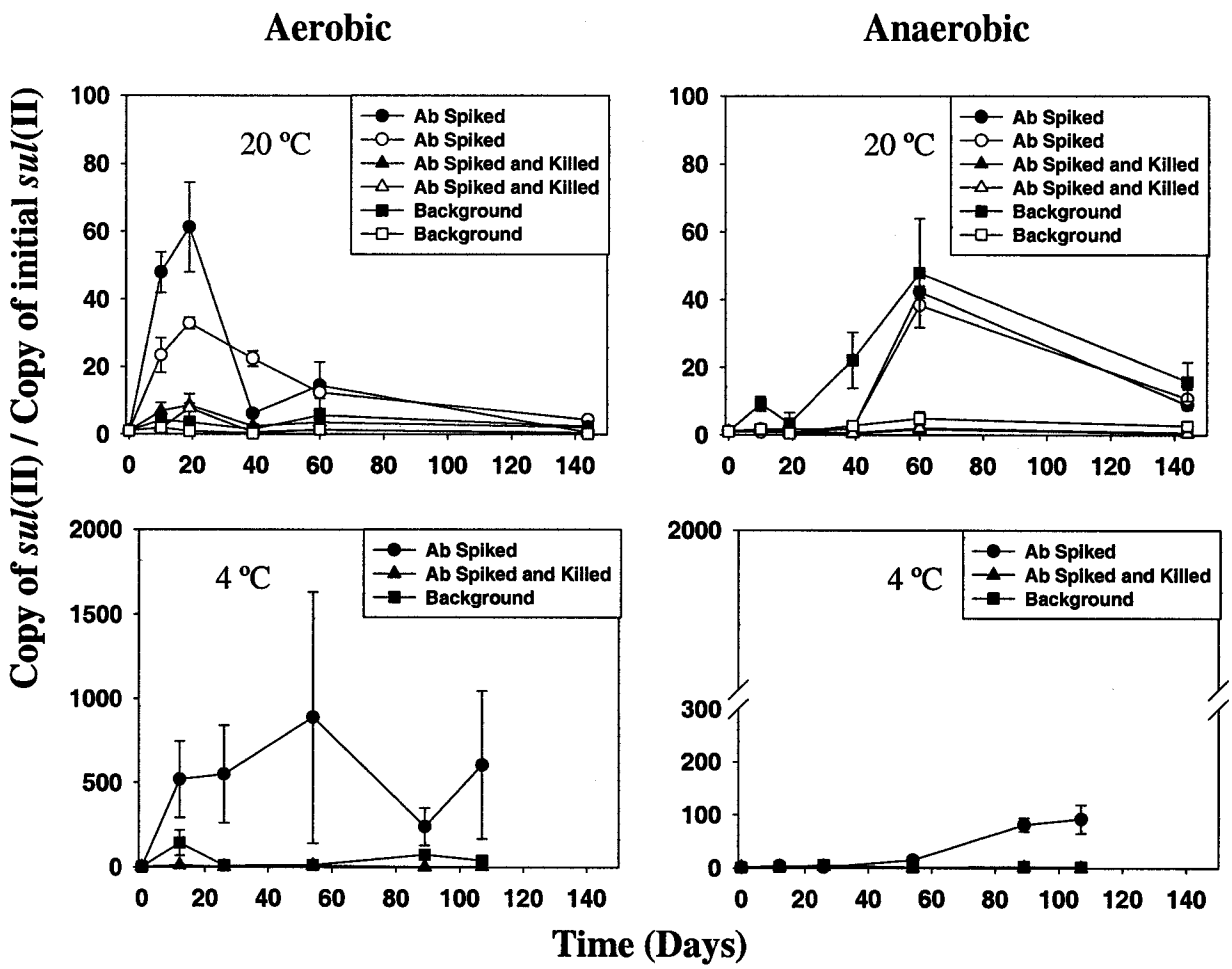

Figure 4. Copies of *sul*(II) ARG normalized to the initial level of *sul*(II) ARG in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions , in duplicate at 20 °C, were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of Q-PCR replicates. The Y scale breaks for anaerobic treatment at 4 °C was from 300 to 1900.

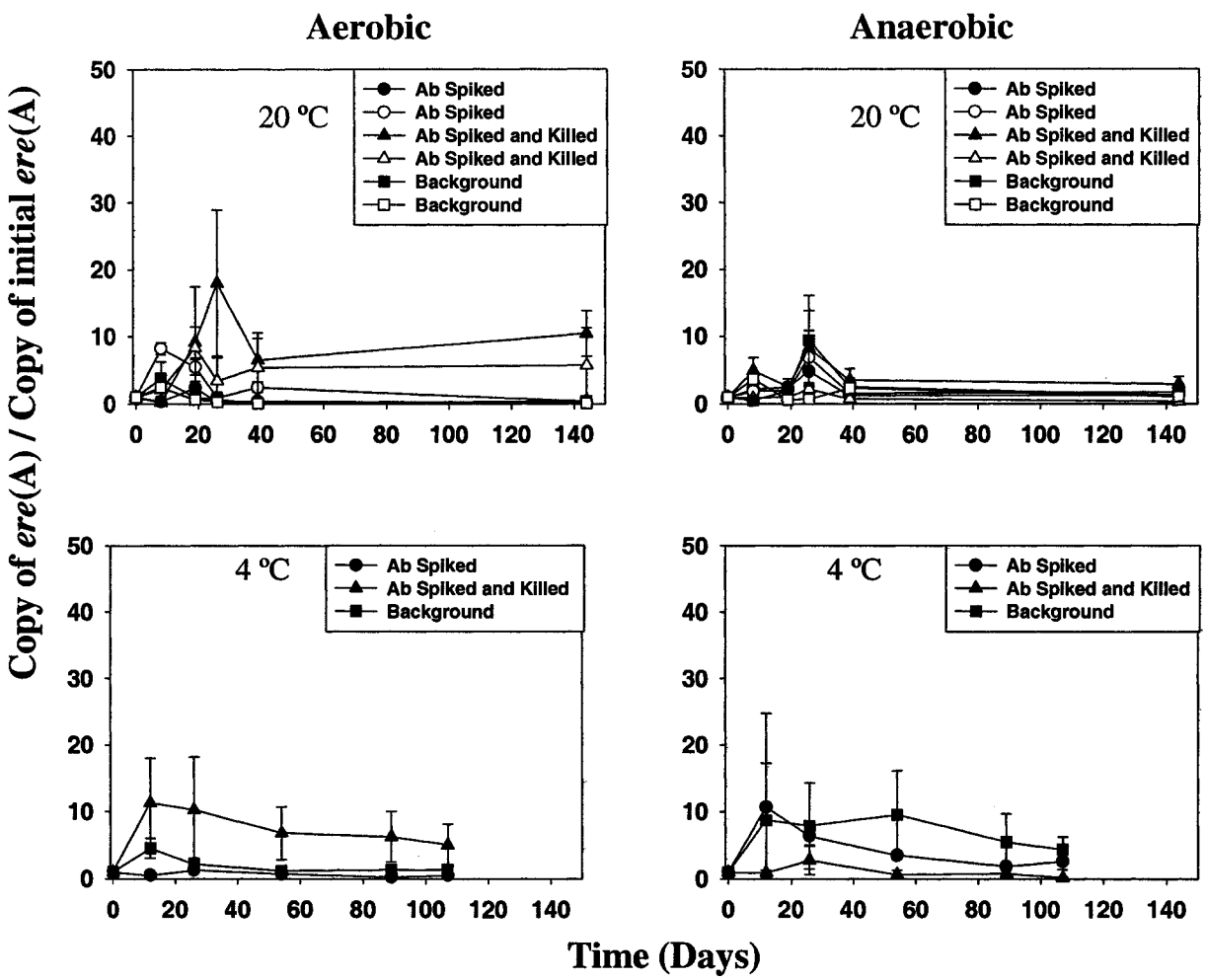

Figure 5. Copies of *ere*(A) ARG normalized to the initial level of *ere*(A) ARG in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions, in duplicate at 20 °C, were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment], **3)** Background = unamended lagoon water. Error bars are standard deviation of Q-PCR replicates.

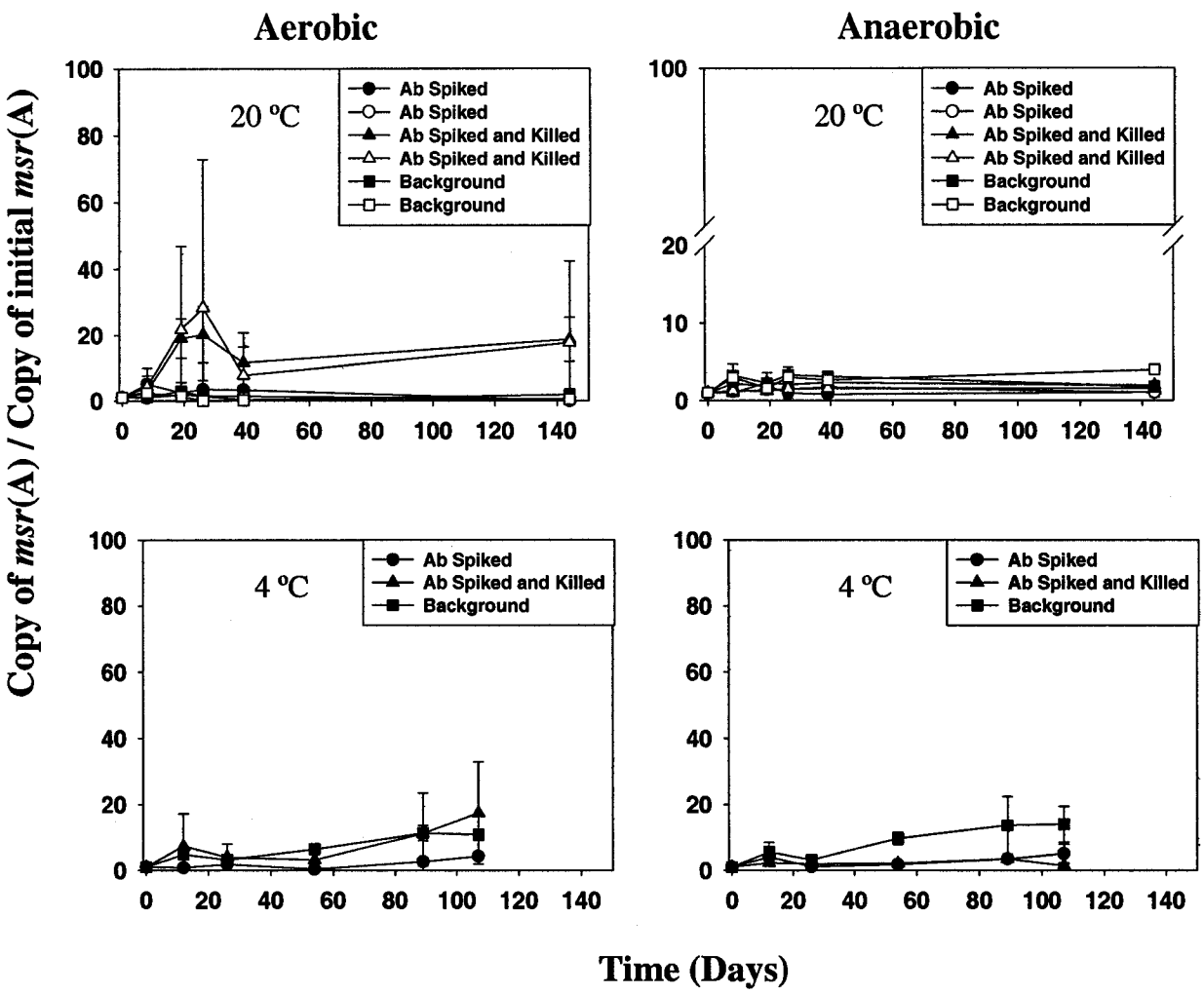

Figure 6. Copies of *msr*(A) ARG normalized to the initial level of *msr*(A) ARG in incubated dairy lagoon water. Top two figures: 20 °C under aerobic (left) and anaerobic (right) treatment. Bottom two figures: 4 °C under aerobic (left) and anaerobic (right) treatment. The three conditions, in duplicate at 20 °C, were: **1)** Ab spiked = OTC+SMX+Tyl+Mon each added at 20 mg/L, **2)** Ab spiked and killed = OTC+SMX+Tyl+Mon+NaN_3 (50 mg/L) [+$HgCl_2$ (0.1 mg/L) for anaerobic treatment] **3)** Background = unamended lagoon water. Error bars are standard deviation of Q-PCR replicates. The Y scale breaks for anaerobic treatment at 20 °C was from 20 to 90.

Appendix C: ARG distribution among irrigation ditches

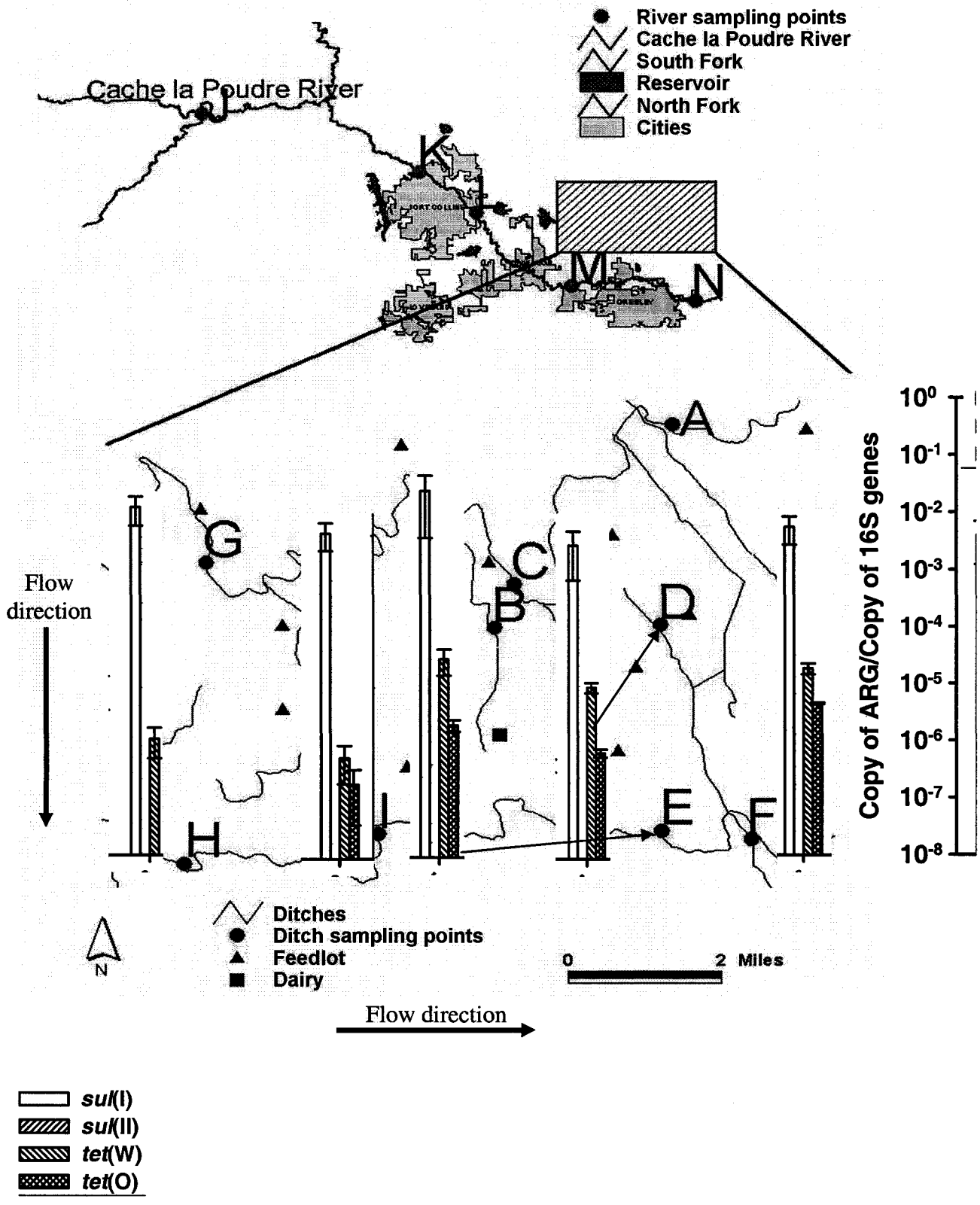

Figure C-1 ARG distrubutions among irrigation ditchs

In order to gain an idea of the spatial distribution of ARG within the irrigation ditch study area with respect to flow, ARG data were superimposed over a map of the site (Fig. C-1). Irrigation ditch water flows from the north to the south and east to west before flowing into the Poudre River. The level of *tet*(W) and *tet*(O) increased at site F significantly when ditch water flowed from site D to site F, while the level of *sul*(I) was statistically the same. A similar trend was observed when ditch water flowed from H to E, the level of *tet*(W) and *tet*(O) increased significantly at site E, while the level of *sul*(I) was statistically the same among the three sites. Tet(O) was absent at site H, but was present at both site I and site E, where the ditch water passed a feedlot near site I. Therefore, the feedlot near site I might be the source of tet(O) ARG down stream of site I. Overall it appears that ARG levels may increase as they flow through the irrigation ditches, prior to entering the Poudre River.