Antibiotics as Environmental Pollutants:

Associated Public Health Threats and Residues in Animal Protein and Biosolids

by

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ABSTRACT

This dissertation studies the larger issue of antibiotic resistance with respect to how antibiotics are being introduced into the environment, focusing on two major anthropogenic pathways: animal husbandry for human consumption, and the recycling of wastewater and municipal sludge generated during conventional biological sewage treatment.

For animal production on land (*agriculture*) antibiotics are often used for growth enhancement and increased feed efficiency. For animal production in water (*aquaculture*) antibiotics are often used as a prophylactic. I found that the same antibiotics are being used in both industries and that the same strains of human pathogens have also been isolated from both sources, expressing identical resistance mechanisms. In U.S. seafood, five out of 47 antibiotics screened for were detected at levels of 0.3 to 7.7 ng/g fresh weight. Although compliant with FDA regulations, the risk for resistance still exists, as even low antibiotic concentrations have been shown to exert selective pressure on bacteria.

Similarly low concentrations of antibiotics were found in U.S. biosolids at levels of 0.6 to 19.1 ng/g dry weight. Of the five antibiotics detected, two have never been reported before in biosolids. Three have never been reported before in U.S. biosolids. Using the raw numbers obtained from antibiotic screenings in biosolids, I assessed the impact of employing four different LC-MS/MS methods, concluding that analysts should experimentally determine the most appropriate quantitation method based on the analyte targeted, matrix investigated, and research goals pursued. Preferred quantitation

approaches included the isotope dilution method with use of an analogous standard and, although time and resource demanding, the method of standard addition.

In conclusion, antibiotics introduced into the environment via agriculture, aquaculture, and wastewater recycling pose a combination of chemical and biological threats. Aside from exerting outright chemical toxicity to non-target organisms, antibiotic residues can promote the development of multi-drug resistance in human pathogens. Public health protection approaches to stem the risks posed by animal husbandry may include reserving drugs for exclusive, human use, decreasing their usage altogether, improving reporting efforts, reevaluating existing regulations on agricultural and aquacultural antibiotic usage, and improved risk assessment for biosolids application on land.

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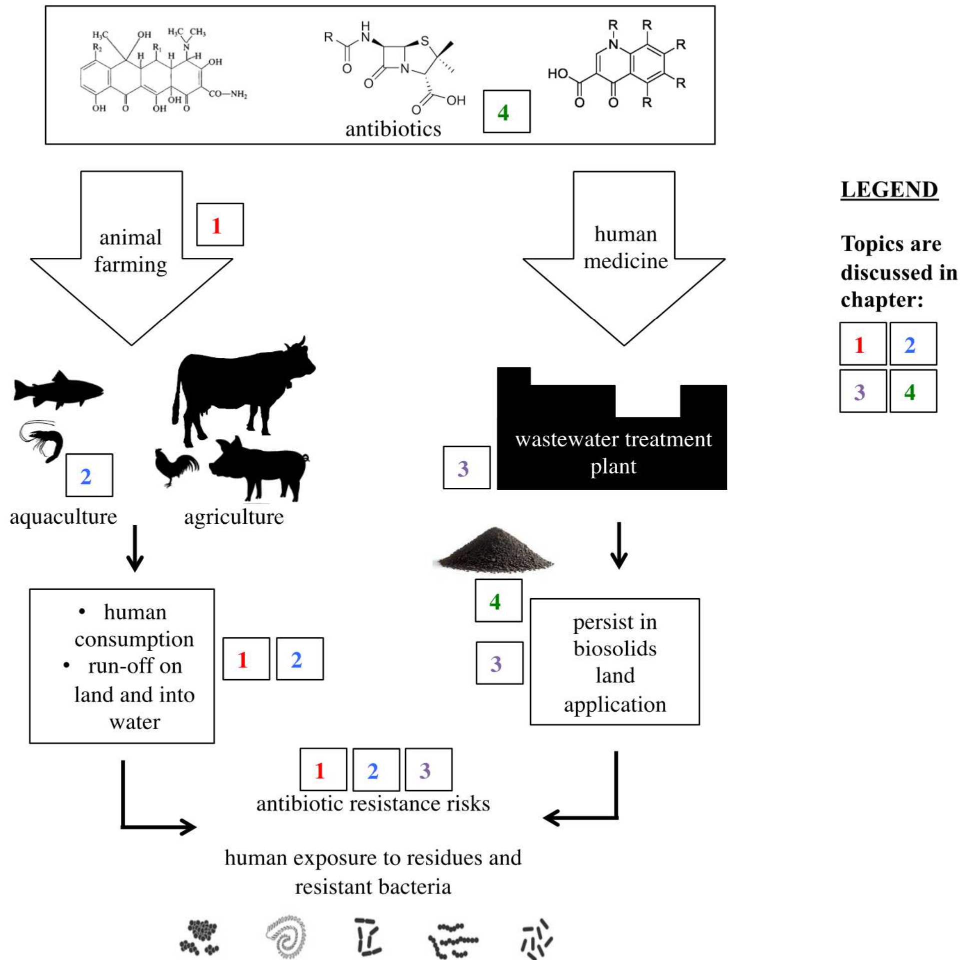
PREFACE

Antibiotics are life-saving compounds that are now seeing resistance from many important human pathogens. This issue is exacerbated by the fact that antibiotics are not only used in human medicine, but also in animal farming. Wastewater treatment plants, the gateway between chemicals used by metropolitan human societies and the environment, are also not optimized to filter out antibiotics, but rather, many chemical groups as a whole, and thus result in antibiotics being introduced into the environment. This dissertation explores these two issues and the mass spectrometry quantitation methods typically used to obtain environmental and food safety data.

Hypothesis: The current human antibiotic usage practices lead to detectable levels of residues in farmed animal flesh and wastewater treatment by-product biosolids, and these levels pose antibiotic resistance risks.

Objectives: 1) Compare and contrast antibiotic usage in land-based and water-based animal farming and assess resistance risks based on published data; 2) Analyze representative seafood samples from the southwest U.S. for commonly used aquaculture antibiotics and assess resistance risks; 3) Develop and apply a liquid chromatography tandem mass spectrometry antibiotics method to analyze nationwide U.S. biosolids samples from the 2006/2007 EPA Targeted National Sewage Sludge Survey; 4) Evaluate how four different quantitation methods applied to identical mass spectrometry raw data affect the results obtained; calculate the magnitude of matrix effects on concentration

results for antibiotics in biosolids, and to also analyze published literature for trends in quantitation method usage.



Overview figure. Flow of antibiotics into the environment and associated risks. Antibiotics used in animal farming and human medicine may eventually reach the environment and promote resistance development. Boxes with numbers indicate the chapter that addresses this part of the antibiotic flow cycle.

Methods: All meta-analyses of data were performed using references published in peer-reviewed journals as well as in non-academic literature from organizations such as the World Health Organization (WHO) and Food and Drug Administration (FDA). Using

liquid chromatography tandem mass spectrometry, seafood and biosolids samples were processed and analyzed for antibiotic content. Raw results from biosolids analyses were used for quantitation of drug residues using four different analytical methods: isotope dilution with stable isotope-labeled analogs of the analytical target, isotope dilution with heavy-labeled standards non-analogous to the analytical target, method of standard addition, and external calibration.

CHAPTER ONE. DOES THE RECENT EMERGENCE OF AQUACULTURE CREATE ANTIBIOTIC RESISTANCE THREATS DIFFERENT FROM THOSE ASSOCIATED WITH LAND ANIMAL PRODUCTION IN AGRICULTURE?

ABSTRACT

Important antibiotics in human medicine have been used for many decades in animal agriculture for growth promotion and disease treatment. Several publications have linked antibiotic resistance development and spread with animal production. Aquaculture, the newest and fastest growing food production sector, may promote similar or new resistance mechanisms. This review of 650+ papers from diverse sources examines parallels and differences between land-based agriculture of swine, beef, and poultry and aquaculture. Among three key findings was, first, that of 51 antibiotics commonly used in aquaculture and agriculture, 39 (or 76%) are also of importance in human medicine; furthermore, six classes of antibiotics commonly used in both agriculture and aquaculture are also included on the World Health Organization's (WHO) list of critically important/highly important/important antimicrobials. Second, various zoonotic pathogens isolated from meat and seafood were observed to feature resistance to multiple antibiotics on the WHO list, irrespective of their origin in either agriculture or aquaculture. Third, the data show that resistant bacteria isolated from both aquaculture and agriculture share the same resistance mechanisms, indicating that aquaculture is contributing to the same resistance issues established by terrestrial agriculture. More transparency in data collection and reporting is needed so the risks and benefits of antibiotic usage can be adequately assessed.

INTRODUCTION

Antibiotics are arguably the most successful and important family of drugs developed for the protection of human health. Since the discovery of penicillin in 1928, over 100 antibiotics have been discovered and used, with the majority of these being introduced before 1970 (Davies, 2006). With the unveiling of each new antibiotic class, resistant bacterial strains were soon identified thereafter, and treatment of some are now a major medical challenge. Today, approximately 70% of characterized nosocomial infections are resistant to at least one clinically relevant antibiotic (Zhang et al., 2011a). Moreover, many strains have been discovered that exhibit multi-drug resistance (MDR) to nearly all commonly available classes of antibiotics (Nikaido, 2009). Coded by antibiotic resistance genes (ARGs), resistance mechanisms such as efflux pumps have made many zoonotic pathogens extremely difficult to treat, forcing doctors to use antibiotics of last resort, example, the fluoroquinolone ciprofloxacin, to treat pathogenic *Escherichia coli* strains (WHO, 2014).

Usage of antibiotics in the production of food animals to sustain and nurture the world's continually increasing human population has contributed to the development of antibiotic resistance (Mathew, 2007). In agriculture – referred to in this review as the farming of swine, poultry, and cattle – uses of antibiotics include disease prevention, treatment, control, and application as growth-promoting antibiotics (GPA) in order to improve feed utilization and production (EU, 2005). The jurisdictions for specific antibiotics allowed and their usage in agriculture vary depending on the location; for example in the

European Union (EU), use of antibiotics for growth promotion is not allowed (EU, 2005). In aquaculture – referred to in this review as the production of aquatic seafood in captivity but excluding plants – application of antibiotics is regulated sparingly, differing greatly from country to country with little to no enforcement in many of the countries that produce the majority of the world's aquaculture products (Pruden et al., 2013). Usage purposes are the same as those in agriculture, with the exception that in aquaculture, prophylactic treatment is more common (Cabello, 2006). Previous research has linked agricultural antibiotic usage practices with antibiotic resistance development, resulting in calls for more judicious usage of antibiotics (Mathew et al., 2014; Silbergeld et al., 2008). Many studies have found drug resistant bacterial strains in agricultural facilities, whether originating in the meat itself (Rasheed et al., 2014; Ta et al., 2014; Asadpour, 2012) or in the surrounding environment (Hsu et al., 2014; Li et al., 2013a; Knapp et al., 2010). The same has been shown for aquaculture (Sapkota et al., 2008; Shah et al., 2014; Ryu et al., 2012), triggering repeated calls for improved regulation and enforcement (Pruden et al., 2013). Efforts to document resistance have increased in recent years, a notable one being the National Antimicrobial Resistance Monitoring System (NARMS) that was established in 1996 as a collaboration between the U.S. Food and Drug Administration (FDA) Center for Veterinary Medicine (CVM, 2011), the U.S. Department of Agriculture (USDA), and the Centers for Disease Control and Prevention (CDC). However, the role of antibiotic usage in agriculture and aquaculture in the development of resistance and dissemination of ARGs is still poorly understood.

Acknowledging the recent growth of aquaculture as a major agricultural sector, this review explores similarities and differences between antibiotic resistance risks associated with agriculture and aquaculture. Specifically, I address whether the recent rise of aquaculture is creating new resistance issues or whether it is simply exacerbating the same ones already established for agriculture. To answer this question, I first discuss how antibiotics have been traditionally used in these industries around the world. I then focus on peer-reviewed academic literature contributions containing data on resistance development in foodborne pathogens. And finally, I use the United States as a case study to discuss in more detail specific issues identified in the global analysis.

METHODOLOGY

A systematic review was conducted of over 650 reports (see Appendix B for full list) extracted from the peer-reviewed academic literature, non-government organizations (NGOs), industry, and government (see Supplemental Information for full list of documents reviewed). Initial searches started with Web of Science and Google Scholar using key terms "antibiotics", "livestock", "agriculture", "aquaculture", and "food production". Additional articles were identified using each article's reference section and further searches were conducted depending on the topic section. Information was also obtained through conversations with food production experts. When possible, the most recent peer-reviewed academic literature was used as the cited reference. A total of 98 key sources are cited in-text to illustrate key issues, show novel data or ways of analysis,

and highlight key research gaps still awaiting attention in future studies. A full list of references is available as supplemental information.

Animal Farming and Antibiotic Usage

In addition to the search terms above, various country/region names were searched alongside (European Union, Brazil, China, etc.). Each jurisdiction's official government website was further surveyed to collect relevant data. Non-government documents such as ones from the Food and Agricultural Organization (FAO) were also extensively reviewed in this section.

Foodborne Pathogens and Antibiotic Resistance Mechanisms

A separate search was conducted to analyze the link between antibiotic resistance and animal production. The initial search of literature on Web of Science started with the search terms "antibiotics, resistance, and agriculture" and "antibiotics, resistance, and aquaculture/seafood" (see supplemental information). These results were then filtered based on title to exclude topics that are not covered in this review (see exclusion criteria in supplemental information). Further literature searches were conducted as needed using terms such as "drug resistance, seafood, and antimicrobials" in order to find articles not captured in the primary search.

United States Agriculture and Aquaculture

Much of these data were collected from governmental websites and through personal communications with personnel from various organizations such as the National Oceanic

and Atmospheric Administration (NOAA) and the National Resources Defense Council (NRDC).

The cutoff date for the literature search was September 1, 2014. Information from the 2007 U.S. Agriculture Census, kindly provided by the Food and Water Watch in raw and processed data formats, served to create the composite Geographic Information Systems (GIS) illustrations in Figure 5. Whenever possible, an update to currently reported data is provided.

The use of terminology in the field of drug resistance is not always consistent. In this dissertation, I define prophylaxis as the precautionary administration of antibiotics at levels predetermined to be therapeutic in the absence of disease (sometimes also termed "disease prevention"). "Sub/non-therapeutic" usage of antibiotics refers to the usage of these compounds for growth promotion at concentrations lower than the dosages required to effectively inhibit the growth of harmful bacteria.

AGRICULTURE VS. AQUACULTURE

Animal Farming and Antibiotic Usage

Over the last sixty years, worldwide production of swine, poultry, and cattle has grown continuously, with poultry outpacing the others (**Figure 1-1A**). World aquaculture production only became a major animal production industry around 1985 (**Figure 1-1B**). Before then, it was a largely non-commercial affair, representing a traditional way of life

for centuries and often providing the sole reliable source of nourishment for its producers (Cole et al., 2009). Reasons for the recent growth of aquaculture include an increased demand for what is now recognized as a healthy protein choice, advances in seafood feed production, depleted wild fish stocks, and improvements in farming facilities enabling high-density farming (Sapkota et al., 2008; Cole et al., 2009). Total seafood production is now almost evenly split between wild-caught and farmed with the former steadily becoming stagnant in volume for the past two decades.

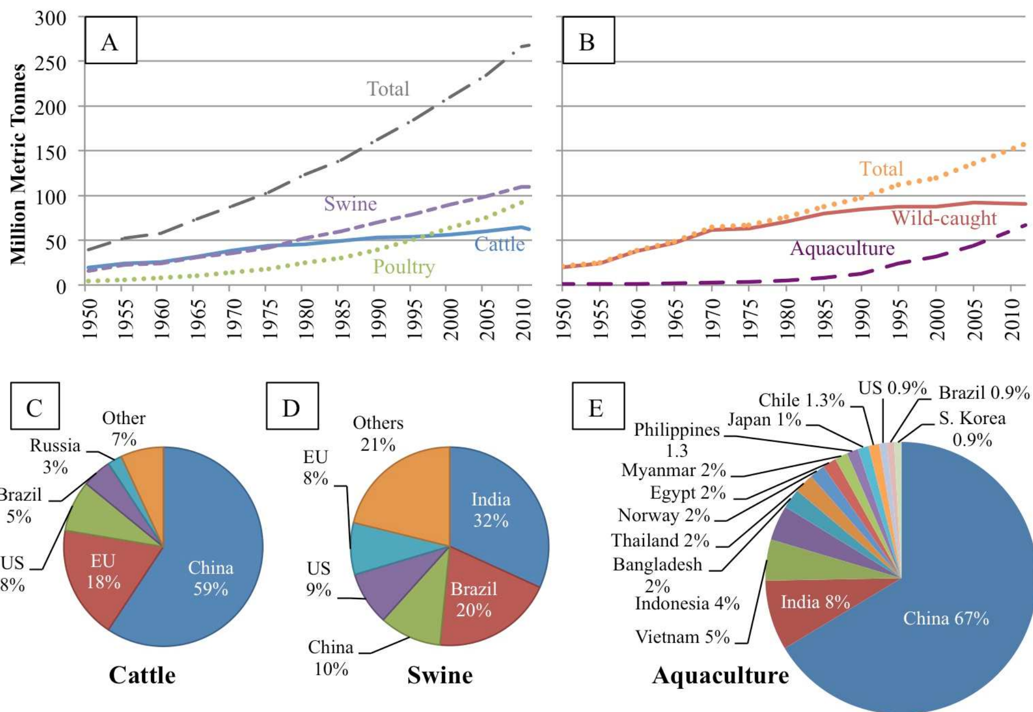


Figure 1-1. Animal production values 1950-2011 and top producing countries of cattle, swine, and aquaculture. **A)** 1950-2011 world production of swine (purple), cattle (blue), poultry (green), and total for all three (gray). **B)** 1950-2011world production of total seafood (orange), wild-caught seafood (red), and aquacultured seafood (purple). **C)** Top 5 cattle producing countries in 2013, counting only beginning stocks by head. **D)** Top 5 swine producing countries in 2013, counting only beginning stocks by head. **E)** Top 15 aquaculture producing countries in 2010 by percentage of total world production. (USDA Production, Supply, and Distribution, 2014; FAOSTAT, 2014; FAO The State of World Fisheries and Aquaculture, 2012; FAO FishStat, 2010).

Figure 1-1 panels C-E show the top countries that produce cattle, swine, and aquacultured seafood. Perhaps the most important detail here is that the majority (>90%) of aquaculture occurs in Asia whereas agriculture's concentrated animal feeding operations (CAFOs) that confine large populations of animals in buildings or feedlots (Silbergeld et al., 2008) can be found distributed across several regions. Aquaculture facilities vary in design, with some keeping animals contained in ocean nets and others in secluded ponds or reservoirs. In Asia, aquaculture often links to the natural water environment (Rico et al., 2012). Many of these freshwater farms irrigate or flow through ponds that often tie with water reservoirs, lakes, and rivers (Rico et al., 2012). Brackish water aquaculture has more than doubled over the past decade and is primarily producing shrimp in coastal ponds and tanks (Rico et al., 2012).

Data regarding the classes and amounts of antibiotics used for agriculture and aquaculture depends on the region. For example, in 2003, salmon aquaculture in Chile used about 0.5 kg of antibiotic for each kg of salmon produced, whereas the amount in Norway was 0.002 kg (Buschmann et al., 2009). **Figure 1-2** shows the most recent data available regarding antibiotic sales in the U.S. and the EU (25 countries). It is important to keep in mind that antibiotic sales do not equate to antibiotic usage, and usage information is not readily available or even reported in most cases. In both regions, the tetracycline class is the largest class of antibiotics sold, comprising about 40% of total sales. Similar reliable data from other regions of the world proved to be unavailable. Antibiotic sales and usage in India are not regulated (Ganguly et al., 2011; NICD, 2011). In China, two different reports of antibiotic usage were found, one stating the annual usage in animal feeds as

6000 tons (Zhao et al., 2010) and the other stating over 8000 tons were used annually in animal husbandry (Chen et al., 2012). In Brazil, it has been reported that the most commonly used antibiotic classes are fluoroquinolones (34% of total antibiotics), ionophores (20%), and macrolides (10%) (Regitano and Leal., 2010). Overall, worldwide usage of antibiotics in both animal production and human medicine has increased in recent decades; agriculture accounts for the majority of drugs used, and the mass of antibiotics used for the production of terrestrial food animals is estimated to exceed the amount of drugs used in aquaculture (Marshall and Levy, 2011).

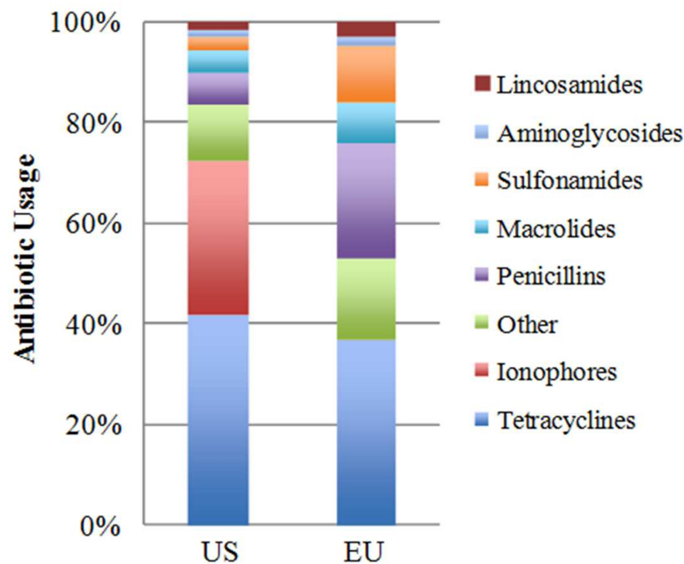


Figure 1-2. Antibiotic classes sold annually for use by animal production industries in U.S. and EU (25 countries) in 2011. Total volume sold in the U.S. is approximately 13.5 million kg. Total sold in EU is approximately 8.4 million kg. (FDA, 2011; EMA, 2011).

How the antibiotics are used depends on the location and is not typically reported. Global trends in agriculture, aquaculture, and human medicine point to a steady increase in the usage of antibiotics. The most important delineation in usage is whether antibiotics are used for growth promotion. Among the top five cattle- and swine-producing countries (see **Figure 1C-D**), only the EU has a confirmed ban on use of GPAs (EU, 2005). In the US, ionophores are used only in animals for growth promotion; a usage which is probably true in Brazil as well where ionophores are also reported to be commonly used (Regitano and Leal, 2010). It should be noted that ionophores are typically reserved for animal usage and not for human usage, unlike the other antibiotic

classes (Chapman et al., 2010). These drugs can alter the stomach microorganisms in livestock to increase feed efficiency and energy extraction in the conversion of feeds (Coffman, 1999). As **Figure 1-2** shows, ionophores are absent from EU antibiotic sales because of the 2006 ban on usage of GPAs in food animals (Maron et al., 2013; EU, 2005). Although there is no law against GPA usage in the US, the FDA has recently issued formal guidance to industry strongly urging drug companies to withdraw their GPAs and/or convert their usage guidelines to "therapeutic only" (FDA #213, 2013). In China and Russia, antibiotic usage in animals is restricted to using only non-human medicine drugs (Sarmah et al., 2006) and since 2003, several reforms have been attempted in China to improve food safety (Broughton and Walker, 2010). However, reports of medically important antibiotics such as tetracyclines being used (Jin, 1997) and detections of illegal veterinary antibiotics like chloramphenicol in Chinese waters suggest that enforcement of the regulation is lax (Hu et al., 2010; Chen et al., 2012). Today, unlike in the EU (Maron et al., 2013), no veterinary prescriptions are required in China for use of antibiotics in animals (Maron et al., 2013). One of the first steps that can be taken to ensure better monitoring of antibiotic usage is to require veterinary prescriptions when antibiotics are used in animals (Mathew et al., 2007; Cabello, 2006; Maron et al., 2013). This approach is being favored in India, as reported in 2011 in a national policy document outlining details to contain antibiotic resistance (NICD, 2011). Whereas data on actual implementation of such measures are scarce, the current trend in published papers indicates that many countries are taking steps to better regulated and report antibiotic usage.

The data presented above is for all antibiotics used in animal production, which includes aquaculture. Specific data for antibiotic usage patterns in aquaculture is available mostly in non-academic literature from the FAO and reports based on surveys as to what antibiotics are commonly used. In 2008, a review article identified three antibiotics to be in common use in aquaculture: oxytetracycline, oxolinic acid, and chloramphenicol (Sapkota et al., 2008). A more recent survey conducted by the FAO of 21 countries engaging in aquaculture confirmed continued use of oxytetracycline as the top antibiotic applied in the treatment of disease in all major seafood species (Alday-Sanz et al., 2012). Florfenicol and trimethoprim/sulfadiazine were next in line with respect to usage frequency. Oxytetracycline was also reported as the most widely used antibiotic for prophylactic treatment. A total of 24 countries were surveyed, including 11 of the top 15 aquaculture producers; the four countries missing from the survey were Egypt, Japan, South Korea, and Myanmar.

To assess the similarities and differences in antibiotics used for agriculture, aquaculture, and human health, the 2011 World Health Organization (WHO) list of important antimicrobials was compared to the above data (WHO, 2012). The WHO list is a categorization system of 260 antimicrobials created in an effort to contain antimicrobial resistance development and spread and to reserve key drugs for human medicine (WHO, 2007). This list was intended for public health and animal health authorities as a reference for prioritizing risk assessment with respect to antibiotic resistance development. Two criteria are considered for inclusion on this list: first, the antibiotic must be the sole or one of a few limited available therapies to treat serious human

diseases; and second, it must be used to treat diseases caused either by a) organisms that may be transmitted to humans from non-human sources or b) human diseases caused by organisms that may acquire resistance genes from non-human sources. “Critically important” antimicrobials (n=162) meet both criteria. “Highly important” antimicrobials (n=88) meet one of the two criteria, and “important” antimicrobials (n=10) meet neither criterion but are still recognized as drugs of importance in human medicine. In this paper, antibiotics from all three classes were screened for usage similarity with results shown in **Figure 1-3** (excluding antibiotics listed for veterinary use only). Six common classes of antibiotics (aminoglycosides, macrolides, penicillins, quinolones, sulfonamides, tetracyclines) on the WHO list are regularly used in agriculture and aquaculture. Of the 51 antibiotics reported to be used by the top agriculture and aquaculture countries, 39 are on the WHO list. Of these 39 antibiotics, only 2 are listed as “important”; the other 37 are either “critically important” or “highly important”. These numbers indicate that there is extreme crossover of antibiotic usage in human medicine and animal food production. It is important to note that data provided in **Figure 1-3** most likely underestimate the antibiotics actually used as this information is not reported and recorded systematically. The most important message from these data is that several of the same classes of antibiotics are used for both human medicine and animal production. This parallel antibiotic usage may be promoting similar resistance issues in both aquaculture and agriculture.

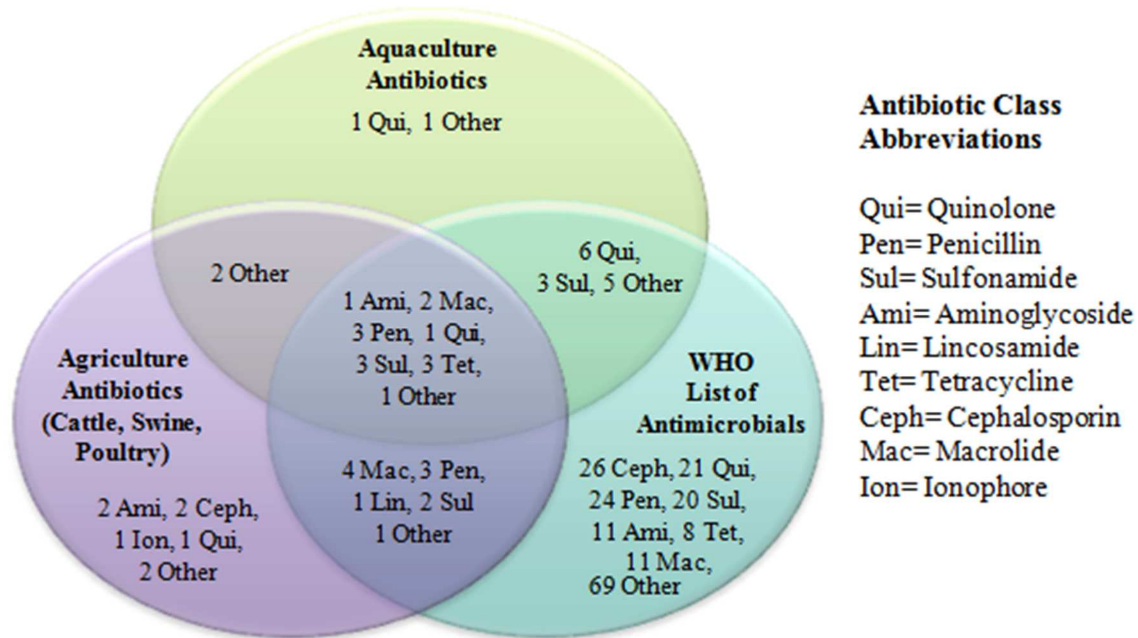


Figure 1-3. Common antibiotics used in aquaculture, agriculture, and included in the 2011 WHO antimicrobials list. Displayed as number of antibiotics followed by antibiotic class. Aquaculture antibiotics include the ones reported to be used by top 15 aquaculture-producing countries. Agricultural antibiotics include the ones used in cattle, swine, and poultry farming. WHO antibiotics are ones on the antimicrobial list in all three labels: "critically important", "highly important", and "important".
(Yuan and Chen, 2012; Kemper, 2008; Hao et al., 2007; WHO, 2012; Sarmah et al., 2006; Sapkota et al., 2008).

Aquaculture: qui-sarafloxacin; other- miloxacin.
WHO: excludes antibiotics used solely for veterinary use. See reference WHO, 2012 for full list.
Agriculture: ami- apramycin*, neomycin; ceph- cefquinome*, ceftiofur*; ion- monensin; qui-marbofloxacin*; other- virginiamycin*, narasin.
Agriculture and Aquaculture: other- tiamulin, ormetoprim.
Agriculture and WHO: mac- kanamycin, oleandomycin, spectinomycin, streptomycin; pen- cloxacillin, dicloxacillin, oxacillin; lin- lincomycin; sul- sulfamethazine, sulfathiazole; other- tylosin
Aquaculture and WHO: qui- norfloxacin, ciprofloxacin, pefloxacin, oxolinic acid, nalidixic acid, flumequine; sul- sulfadiazine, sulfamerazine, sulfamethoxazole; other- chloramphenicol, colistin, florfenicol, furazolidone, thiamphenicol.
Aquaculture, Agriculture, and WHO: ami- gentamicin; mac- spiramycin, erythromycin; pen- amoxicillin, ampicillin, penicillin G; qui- enrofloxacin; sul- sulfadimethoxine, sulfadimidine, sulfapyridine; tet-chlortetracycline, oxytetracycline, tetracycline; other- trimethoprim.
* These agriculture antibiotics are included in the WHO list but are reserved for veterinary use only.

Foodborne Pathogens and Antibiotic Resistance Mechanisms

As shown in the previous section, the antibiotics used in agriculture and aquaculture span many of the same antibiotic classes. Thus, as agriculture has been using antibiotics for much longer than aquaculture has, I ask whether the same resistance mechanisms exist in both or if the latter is promoting the development of new ones. In this section, I identified reported bacterial pathogens from meat and seafood, characterized how resistance may develop, and looked for resistance development pathways in agriculture and aquaculture. To relate the isolated strains to human health risks, I focused our identified strains on zoonotic foodborne pathogens.

The most prevalent and serious emerging pathogens in agricultural meat products are *Campylobacter jejuni*, *Salmonella enterica* serovar Typhimurium DT104, and *E. coli* O157:H7 (Mor-Mur and Yuste, 2010). Often, these products are contaminated during handling and processing in the CAFOs where the animals are slaughtered. Pathogens present in feces and/or animal hides often are transferred to edible fractions, or spread as aerosols produced during dehiding, evisceration, and carcass splitting (Mor-Mur and Yuste, 2010). In aquaculture, foodborne diseases are not as well documented, but the literature shows that *Salmonella* and *Vibrio* spp. are likely to be the most common pathogens detected in seafood, with *Listeria monocytogenes*, *Aeromonas*, and *Clostridium* spp. becoming emerging threats (Feldhusen, 2000; Herrera et al., 2006; Normanno et al., 2006). Cases of human infections from seafood most often arise from handling, such as contact with the wash water or through processing in the food industry, and by oral consumption of infected fish or related products (Novotny et al., 2004).

Aside from the potential to cause infections in the people that are exposed, these bacteria, along with others that are less often found, are capable of developing and spreading antibiotic resistance. In both agriculture and aquaculture, development/persistence of resistance can occur when these bacteria are exposed to sub-therapeutic concentrations of antibiotics (Sapkota et al., 2007). In terrestrial agriculture, this exposure occurs when antibiotics used for growth promotion are added as a CAFO feed additive over a period of time for fattening and for increased feed efficiency (Phillips et al., 2004). In the US, about 55% of all antibiotic usage in cattle is during the feedlot stage of cattle production (Mellon et al., 2001). The feedlot stage is when the animals weigh in between 700 and 1200 lbs, with average antibiotic dosages estimated at 80 mg/animal/day for about 120 days (Mellon et al., 2001). This means that these cattle are subject to sub-therapeutic antibiotic concentrations for almost one third of a year.

The commonly cited rationale behind using GPAs is an economic benefit, with average increases in animal mass reported in the range of four to eight percent (Butaye et al., 2003). Other advantages reported in the literature include an improvement of animal health, decreases of bacterial contamination in animal products, a reduction of adverse environmental impacts such as greenhouse gas emissions, and prevention of water eutrophication (Hao et al., 2007). However, an economic analysis of using antibiotics in commercial broiler chickens for growth promotion showed that the net economic effect of using GPAs is negative, with an estimated lost value of $0.0093 per chicken or about 0.45% of the total cost; the positive production changes associated with antibiotic use reportedly were insufficient to offset the cost of more expensive feed (Graham et al.,

2007). The latter study did not consider the potential benefits of GPA removal in terms of preventing external costs from medical and public health burdens resulting from antibiotic-resistance infections. Considering such would further increase the cost incurred by the use of antibiotics. No other such analysis is available in the literature, and more are needed to assess the economic impact of using GPAs.

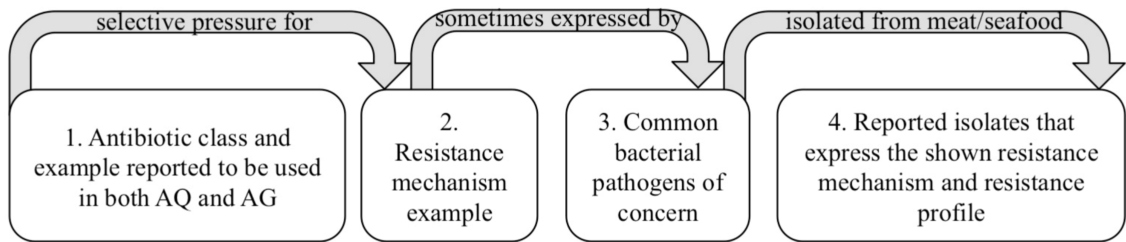
In aquaculture, sub-therapeutic exposure concentrations are mostly encountered after the prophylactic use of antibiotics. Unconsumed fish feed and feces can contain residues that persist in the surrounding environment (Cabello, 2006), allowing for bacteria to be exposed to low concentrations that can select for resistance. The exposed bacteria then can spread ARGs to the natural microbiota in nearby ecosystems, which may pose a greater threat than low levels of residues, as resistance genes may persist for decades due to the marginal impact of gene maintenance on fitness (Pruden et al., 2013). As previous studies suggest that the environment already harbors ARGs (Marti et al., 2014), the mixing of residues that is made easier via the water pathway make aquaculture more likely to spread contaminants compared to agriculture. In many cases, these compounds are only slightly transformed, or even unchanged and conjugated to polar molecules, allowing for easier dispersion in water (Kemper, 2008) The added potential impacts on the environment include direct antibiotic toxicity in natural microbiota, flora, and fauna, have been voiced in literature (Rico et al., 2012; Baquero et al., 2008). However, not all detected antibiotic concentrations are environmentally relevant enough to negatively impact invertebrates and fish (Zounkova et al., 2011; Park and Choi, 2008). These reports in literature indicate that the risks associated with antibiotic residues in aquaculture may

vary depending on the situation and that there is a gap in knowledge regarding residues and their effects on resistance development. It must be noted that the usage of antibiotics in animal production has provided many benefits as well. Antibiotics have allowed for animal health to be improved, increasing economic gain for the farmers, as pathogens are significantly reduced when antibiotics are utilized (Phillips et al., 2004; Hao et al., 2007). However, despite these benefits, I cannot ignore the risks and potential negative human health and environmental impacts.

To compare the potential for agriculture and aquaculture to be developing the same mechanisms of antibiotic resistance, I reviewed reports in literature of bacterial isolates resistant to commonly used antibiotics in these food production industries. In agriculture, four common resistance mechanisms have been identified (**Figure 1-4**). These categories are presented very broadly to be more inclusive; “altered intracellular target” can mean any mutation that allows for ribosomal active site changes or an RNA polymerase mutation that leads to reduced binding of the antibiotic (Giedraitiene et al., 2011). Antibiotics in many classes can be ineffective against these mechanisms; both macrolides and penicillins can be pumped out of the bacterial cell by efflux pumps, for example. In other words, co-resistance can occur with any of these mechanisms. The zoonotic pathogens of concern listed in **Figure 1-4** are typical examples of bacteria exhibiting the common resistance mechanisms. For example, *P. aeruginosa* is well known for expressing MDR efflux pumps (Nikaido and Pages, 2012). Examples of these pathogens isolated from agriculture that have been molecularly shown to harbor each resistance

mechanism's ARGs are also shown in **Figure 1-4**. Many are resistant to several antibiotics, but ones commonly used in agriculture are noted.

The same four mechanisms were also found to be associated with aquaculture. Zoonotic pathogens resistant to aquaculture antibiotics have been isolated from seafood containing all of the four resistance mechanisms (Ryu et al., 2012; Uddin et al., 2013; Meng et al., 2011; Nawaz et al., 2012). Some of these microbes are relevant pathogens in agricultural products as well (i.e., *Salmonella*). Tetracycline resistance is the most commonly seen resistance among bacterial isolates from aquaculture; a recent study showed that as the number of resistance reports increased, so did the incidence of tetracycline resistance (Done and Halden, 2015). Among 23 publications on drug resistant bacteria isolated from seafood for human consumption, 21 reported resistance to at least one antibiotic belonging to the class of tetracyclines. This previous study only reported publications from 2003-2013 and limited the search to bacterial strains from seafood products only (excluding aquaculture facilities, the surrounding water, etc.). If the exclusions were not applied, the number of resistant strains isolated would most likely increase. The major issue with detections of specific resistance determinants such as efflux pumps is the ability of these genes to be spread via horizontal gene transfer, possibly to bacteria that are even more pathogenic to humans. In both aquaculture and agriculture, native environmental bacteria are mixed with zoonotic bacteria, providing a situation where resistance can develop, spread, and linger amongst them. The biggest human health risk is coming into contact with pathogenic bacteria that are also resistant to multiple antibiotics, especially ones from different classes.



1. Antibiotic Class (Example in AQ & AG)	2. Resistance Mechanism	3. Pathogens of Concern	4. Pathogen Isolates Detected Resistances
Tetracyclines (Oxytetracycline) Macrolides (Erythromycin) Penicillins (Ampicillin) Quinolones (Enrofloxacin)	Efflux pumps	*P. aeruginosa* *E. coli* *S. pneumoniae* *Salmonella spp.*	AG: *Salmonella* (11) -Ampicillin, Tetracycline AQ: *P. aeruginosa* (65) -Ampicillin
β-Lactams (Penicillin)	Cell wall changes (*e.g.* permeability)	*S. aureus* *N. gonorrhoeae* *E. faecium* *E. coli* *H. influenzae* *S. pneumoniae*	AG: *Salmonella* (66) -Tetracycline, Ampicillin, Sulfamethoxazole AQ: *S. aureus* (67) -Tetracycline, Ampicillin, Sulfamethoxazole
β-lactams (Ampicillin) Aminoglycosides (Gentamicin)	Alter/ inactivate antibiotic (*e.g. β*-lactamase)	*K. pneumoniae* *E. coli* *M. catarrhalis* *B. fragilis*	AG: *Salmonella* (68) - Ampicillin, Tetracycline, Nalidixic Acid AQ: *E. coli* (18) - Ampicillin, Tetracycline, Chloramphenicol, Nalidixic Acid
Macrolides (Erythromycin) Tetracyclines (Oxytetracycline)	Altered intracellular target (*e.g.* ribosome)	*S. aureus* *S. pneumoniae* *S. pyogenes*	AG: *Enterobacter aerogenes* (69) - Tetracycline, Trimethoprim & Sulfamethoxazole AQ: *E. coli* (70) - Tetracycline, Streptomycin

Figure 1-4. Resistance mechanism development in agriculture and aquaculture. Top panel explains how each row exhibits a resistance mechanism. Each row in chart is an example via a different resistance mechanism. Each resistance mechanism can allow bacteria to be resistant to many classes of antibiotics (leftmost column). Antibiotics reported to be used in agriculture and aquaculture (column 1) can select for resistance mechanisms (column 2) that are sometimes expressed by common pathogens listed here are examples (column 3). Column 4 shows bacterial isolates reported in literature that are resistant to the stated antibiotics *and* have been genetically shown to express the resistance mechanism in that row. AG= isolate from agriculture; AQ= isolate from aquaculture. Reference numbers for the publications are noted with the bacterial strain. Strain genera are as follows: P = *Pseudomonas*, E = *Escherichia*, S = *Streptococcus pneumoniae*/*pyogenes* or *Staphylococcus aureus*, N = *Neisseria*, E = *Enterococcus*, H = *Haemophilus*, K = *Klebsiella*, M = *Moraxella*, and B = *Bacillus*. Resistance mechanisms from Giedraitiene et al., 2011. References: **11**=Ta et al., 2014 **18**= Ryu et al., 2012 **65**= Uddin et al., 2013 **66**= Chen et al., 2004 **67**= Meng et al., 2011 **68**= Van et al., 2007 **69**= Jiang and Shi, 2013 **70**= Nawaz et al., 2012

As noted above, several such cross-resistant isolates have already been found in terrestrial agriculture and aquaculture. These data suggest that identical resistance mechanisms are being promoted and developed in both agriculture and aquaculture. Alarmingly, some of the same pathogens have been isolated from both seafood and meat. Different strains of MDR *Salmonella* were isolated containing the same resistance genes from both shellfish and pork (Van et al., 2007). Similarly, *E. coli* strains isolated from pork, beef, poultry, and fish were resistant to several tetracyclines (Koo and Woo, 2011). This review only focuses on human health risks posed by edible animal products themselves, but it should be noted that additional risks result from the processing and handling of all materials involved, such as the disposal of animal feces containing resistant bacteria (Tadesse et al., 2013). The studies available and examined for this work show that the same resistance mechanisms are being promoted in agricultural and aquacultural environments (including processing and handling), thereby allowing for resistance to develop and spread via food and the environment, resulting in significant human health threats.

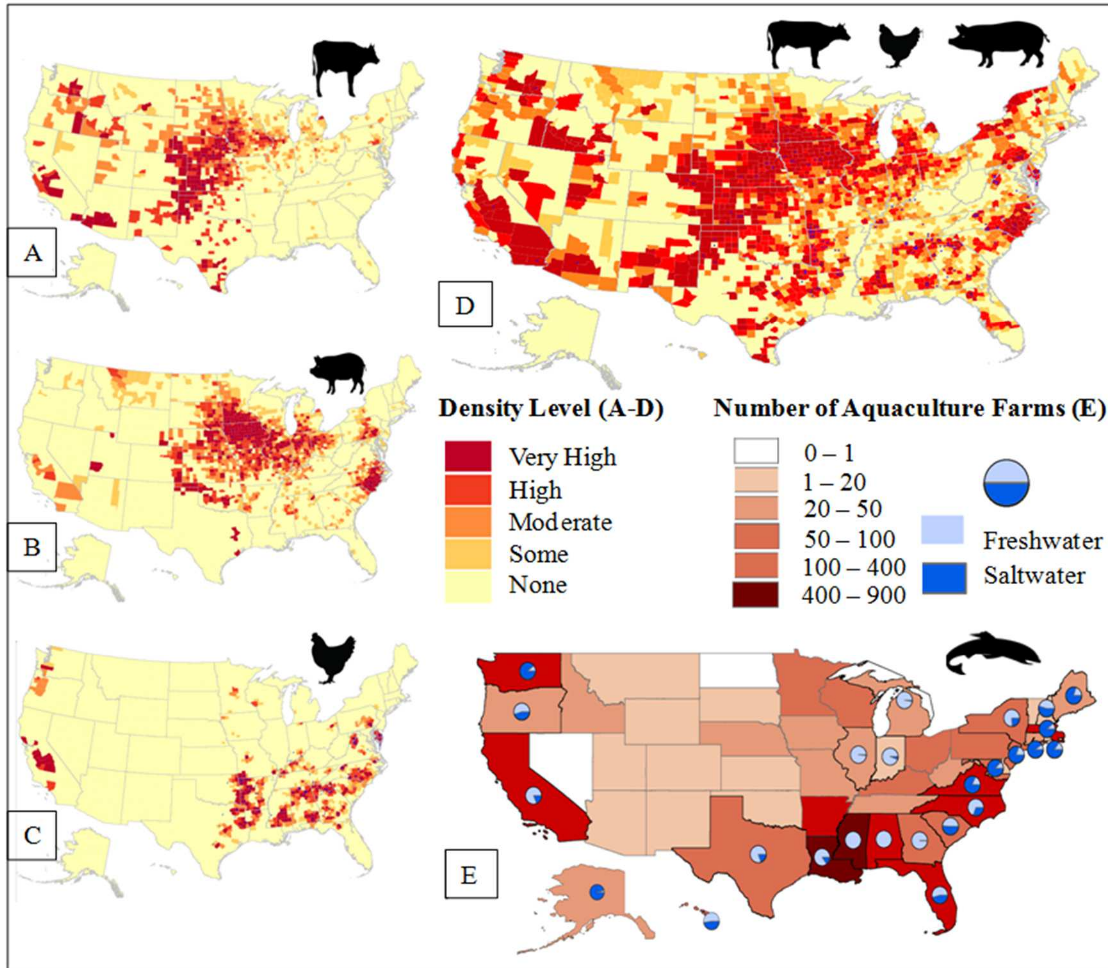


Figure 1-5. 2007 density maps of cattle, swine, poultry, and combined values of production and 2005 number of aquaculture farms in US. 2007 U.S. density of **A)** cattle, **B)** swine, **C)** poultry, and **D)** combined production. Maps A-C show animal density by county. For map A cattle density level: very high = > 17,400; high = 7,300-17,400; moderate = 2,175-7,299; some = < 2,175; none = 0. For map B swine density level: very high = > 48,500; high = 19,000-48,500; moderate = 9,500-18,999; some = < 9,500; none = 0. For map C poultry density level: very high = > 2.75 million; high = 1-2.75 million; moderate = 350-999 thousand; some = < 350 thousand; none = 0. For map D combined production, the total number of livestock across different animals types was calculated using the U.S. Department of Agriculture definition of a livestock unit, which is 1000 pounds (454 kg) of live weight. Map D county density level (in livestock units): very high = > 13,200; high = 5,200-13,200, moderate = 2,000-5,199; some = < 2,000; none = 0. **E)** 2005 U.S. density of aquaculture production by number of reported farms, with percentage of farm being freshwater or saltwater indicated in blue pie charts. States without a pie chart contain fully freshwater operations. (Food and Water Watch, 2007; Department of Agriculture, 2005).

CASE STUDY: UNITED STATES AGRICULTURE AND AQUACULTURE

Animal Farming and Antibiotic Usage

The U.S. is one of the largest producers of agriculture in the world, ranking (counting beginning year stock numbers) 4th in 2013 cattle production at approximately 89 million head and 3rd in swine production at approximately 66 million head (USDA Production, Supply, and Distribution, 2014). As seen in **Figure 1-5**, the cattle and swine industries dominate over the poultry industry, with much higher densities reported for many of the U.S. counties and states shown. These data (**Figure 1-5A-D**) are from the 2007 USDA Agricultural Census, which conducts a new survey every five years (the 2012 report is expected to be released within the next year). Shown at the county level, the majority of the U.S. cattle, swine, and poultry farming is done in the Great Plains states and along the west border of the Mississippi river. These geographic locations differ, as one would expect, from the locales of aquaculture, which are largely situated near the ocean and along the Gulf of Mexico (**Figure 1-5E**)**.**

Aquaculture can be divided into freshwater and saltwater culture (**Figure 1-5E**). By value of production, saltwater and freshwater aquaculture in the U.S. contributed approximately $800 and $550 million dollars, respectively, in 2011 (NOAA, 2012). About two-third by value of saltwater (or marine) aquaculture consists of mollusks such as oysters, clams, and mussels (NOAA, 2014A). This type of aquaculture takes place in cages that are located on the ocean floor or suspended in water column (NOAA, 2014B). The majority of this farming is done in the northwest region of the U.S. (see **Figure 1-5E** for blue pie

chart inserts) and in Washington and Oregon. Freshwater aquaculture is predominated by trout, catfish, and tilapia (NOAA, 2012A). **Figure 1-5** only shows the density of aquaculture farms contained in each state based on the 2005 Agricultural Census, but these numbers don't necessarily reflect the amount of production. The top 5 aquaculture states by value in 2005 were as follows: Mississippi, Arkansas, Alabama, Louisiana, and Washington, together producing about a half a billion dollars worth of products, which is about half of the total U.S. value produced (USDA, 2005).

Table 1-1. Total reported U.S. antibiotic usage (in million kg) by animal industry and for human health.

Reporting Source	Year Reported[a]	Total Amt. Sold for Food Production Animals (Million kg)	Reported Sub-Therapeutic Usage[b] Million kg (% of Total Animal Amount)	Total Human Usage (Million kg)	% of Total AB Sold is for Animals	Reference
AHI	2001	8.1	1.4 (18%)	14.6	35%	Mellon et al., 2001
UCS	2001	12.5	11 (88%)	3	70%	Mellon et al., 2001
USFRA	2007	NR	(13%)	NR	NR	USFRA, 2007
FDA; Rep. Slaughter	2009	13.1	NR	3.3	80%	FDA, 2010; Slaughter, 2011
CSPI, NRDC, This Review	2011	13.5	NR	3.3	80%	FDA Drug Use Review, 2012; FDA, 2011; NRDC, 2014; DeWaal and Grooters, 2013

[a]Year reported does not always correspond to year data was collected/formulated. NR= not reported in publication.
[b]Reported sub-therapeutic usage, does not differentiate between amounts of antibiotics used for prophylaxis, metaphylaxis, growth promotion, or feed efficiency.

As production of cattle, poultry, and swine expanded to large-scale productions over the last half-century, the usage of antibiotics in agriculture has also become the norm and has

greatly increased. Based off of FDA reports, I calculated that in 2011, 80% of the antibiotics sold by weight were designated for animal usage (FDA, 2012; FDA, 2011). This percentage was calculated from the annual FDA released summary report on antimicrobials sold/distributed for food-producing animals (13.5 million kg) and from the FDA drug use review, where sales numbers for human medicine usage (3.29 million kg) were obtained (FDA, 2011). Similar numbers have previously been reported by several other NGOs, including the Natural Resources Defense Council (NRDC, 2014; DeWaal and Grooters, 2013), the UCS, and the Center for Science in the Public Interest, among others (**Table 1-1**). These organizations primarily based their estimates on annual FDA summary reports for antimicrobials. However, the numbers reported by the Animal Health Institute (AHI) are much different, resembling those reported by the U.S. Farmers and Ranchers Alliance, another entity representing the industry. The AHI estimates that only about 35% of antibiotics in the U.S. is used in animals for food production (Mellon et al., 2001).

A second data discrepancy requiring more transparency is what antibiotics are annually used in animal production as well as their frequency of usage. This reporting is difficult in part because animal producers are not required to report this information, but also because “non-therapeutic” or “sub-therapeutic” usage of antibiotics can mean different things. As the FDA allows antibiotics to be used for growth promotion, feed efficiency, disease and metaphylaxis, it is hard to specifically enumerate the amount of antibiotics used in each of these categories (MacDonald and Wang, 2011). Thus, it must be noted that the numbers reported in **Table 1-1** column “Reported Sub-Therapeutic Usage” are

only estimates by a few organizations and that these numbers may not reflect the situation accurately. As the FDA is now required to report antimicrobial usage numbers, the next step would be to report what the antibiotics are used for. Recent FDA/CVM guidance now provides recommendations for industry to voluntarily align their products with FDA #209 (FDA #209, 2012). This guidance includes two principles: 1) limiting medically important antimicrobials to uses in food-producing animals that are considered necessary for assuring animal health and 2) limiting these usages to only those with veterinary oversight or consultation (FDA #209, 2012). These guidelines encourage better documentation and usage practices.

With regards to aquaculture production, the U.S. produces a relatively low amount compared to other countries. This is partly due to the fact that China provides close to 70% of total aquaculture products, as well as the fact that the U.S. imports about 90% of its seafood. There is a major effort in place to expand the aquaculture industry in the US, so that the reliance on imported fish is reduced. The U.S. is a leading global consumer of fish and fishery products, and yet only about 5-7% of the national supply comes from its aquaculture industry (NOAA, 2014B). It has been estimated that up to 433,000 lbs (approximately 196,000 kg) of antibiotics were used in 2002 in U.S. aquaculture (Benbrook, 2002). These data indicates that the vast majority (approximately 80%) of animal antibiotics used in the U.S. are used in agricultural animal production (see **Table 1-1**). Antibiotics do not improve growth or feed efficiency in fish like they have been reported to do in certain livestock (NOAA, 2014C). The usage of vaccines has also greatly limited antibiotic usage in the US, and at present, only three antibiotics are

registered and sold for disease control in fish: oxytetracycline, florfenicol, and sulfadimethoxine/ormetoprim (FDA, 2014). Thus, it can be assumed that the majority of the antibiotics used for food-producing animals in the U.S. are in livestock, which is most likely the case in other countries as well (Marshall and Levy, 2011).

Foodborne Pathogens and Detected Resistance

In the US, foodborne pathogens of concern in agricultural meats are *E. coli, Salmonella,* and *Campylobacter*. The NARMS Retail Meat Annual Report of 2011 identifies *E. coli* as the most commonly detected bacterium in all retail meat products (CVM, 2011). Out of 1,920 retail meats tested in 2011, 55.7% were found to culture positive for *E. coli.* Although no isolates were resistant to ciprofloxacin, some isolates were shown to be resistant to third-generation cephalosporins, and co-resistances to other *β*-lactam compounds were reported. For *Salmonella*, the three serotypes most commonly detected were Typhimurium, Kentucky, and Heidelberg. Resistance to ampicillin rose from 17% of isolates in 2002 to 41% in 2011. A similar trend was seen for third-generation cephalosporins (from 10% to 34%). Most concerning is the fact that 45% of retail chicken harbored isolates featuring resistance to three or more classes of antimicrobials. Approximately 27% showed resistance to at least 5 classes. With regards to *Campylobacter*, the species *jejuni* and *coli* were most commonly detected. The majority of the isolates (90%) were from retail chicken. Although macrolide resistance has remained low, tetracycline resistance increased by about 10% of isolates for both species from 2010 to 2011. MDR was low in *Campylobacter*, as only 9 out of 634 isolates were resistant to at least three antimicrobial classes. *Enterococcus* (*faecalis* and *faecium*) is

used as a sentinel for antibiotic selection pressures by anti-gram-positive antibiotics. Vancomycin resistance was not detected, and streptogramin resistance has significantly decreased in retail chicken from 56% of isolates in 2002 to 27% in 2011. Overall, it seems that most of the risk is from gram-negative bacteria and gram-positive bacteria pose a lesser risk to humans in retail meats. In reference to **Figure 1-4**'s resistance pipelines, these data support the notion that feeding food production animals with antibiotics like ampicillin and tetracycline may contribute to the increased drug resistances observed in the U.S. as shown in NARMS data (CVM, 2011).

In U.S. aquaculture, as most of the seafood is imported, foodborne pathogens of concern are often ones that are considered food safety risks overseas as well. In 2004, it was reported that eating contaminated seafood resulted in about 15% of the reported foodborne outbreaks in the U.S. This is a greater percentage than was found for either meat or poultry, which are consumed at volumes eight and six times higher than those of seafood (Rakowski, 2012). Our literature search shows that *Vibrio* spp. and *Salmonella* are the most commonly isolated zoonotic pathogens from seafood. Specifically, *V. vulnificus,* followed by *parahaemolyticus,* are the two most important *Vibrio* spp. noted, causing gastroenteritis that may lead to septicemia (Powell, 1999). *Vibrio* spp. are a natural inhabitant of many aquatic organisms and are the leading cause of seafood-related deaths in the U.S. (William et al., 2014). Mostly a concern in oysters, *Vibrio* spp. have been isolated and characterized in several studies (Reynaud et al., 2013; Turner et al., 2013; Givens et al., 2014). Antibiotic residue in bivalves is not a significant concern because they are not fed feed as they are filter feeders that survive on particles in the

water (NOAA, 2014C). *Salmonella* are an issue in almost all types of seafood, and species distribution is broad, with frequently reported serotypes including Weltevreden, Senftenberg, Lexington, and Paratyphi-B (Heinitz et al., 2000). Mostly of human origin, *Salmonella* also causes gastroenteritis, and primarily contaminates seafood during processing (Amagliani et al., 2012). This is similar to agricultural meat products, where *Salmonella* is also an important foodborne pathogen. Recent seafood outbreaks include three in 2011 where a total of 168 cases resulted in 48 hospitalizations and 1 death (DeWaal and Grooters, 2013). The *Salmonella* isolated in the latter study were all resistant to ampicillin, tetracycline, and amoxicillin/clavulanic acid, all of which are on the WHO list. These data suggest that resistance in zoonotic pathogens isolated from commonly eaten meats and seafoods is prevalent and a growing concern for the food industry.

CONCLUSIONS

Swine, cattle, and poultry agriculture all have relied on antibiotic usage for over half a century, promoting the development and spread of antibiotic resistance. As aquaculture continues to grow, the knowledge gap regarding how antibiotic usage, development of resistance mechanisms, and human health risks connect with each other must be filled with scientific research and results. Here, I present data showing that agriculture and aquaculture share many similarities, from the antibiotics used to the resistance mechanisms shared by the zoonotic pathogens corresponding to these two important food production sectors. The bacteria isolated from both meat and seafood have been reported

to display resistance to antibiotics commonly applied in animal production. From the data gathered here, it is concluded that the recent growth of aquaculture is contributing to the development of the same resistance mechanisms also seen in agricultural production. The usage of antibiotics provides selective pressure that can accelerate ARG development and spread. As zoonotic pathogens have been isolated exhibiting resistance mechanisms known to be effective against multiple antibiotics, co-resistance is increasingly becoming a major concern. The lack of data and discrepancies in existing data regarding antibiotic usage contribute to the fact that it is challenging at present to accurately determine the magnitude of influence both aquaculture and agriculture has on resistance development. However, as water provides a constant and facile mechanism for dispersal of drug residues, microbial pathogens, and resistance genes, aquaculture will continue to pose a threat that may increase as the demand for seafood increases.

TRANSITION ONE

Antibiotics are commonly used in agriculture to prevent and treat bacterial infections, but also to promote growth in cattle, swine, and poultry. As these antibiotics leach into the environment, several human and environmental health issues arise, the most prominent of which being antibiotic resistance. As Chapter 1 discusses, opportunities and likelihood of migration (movement) of antibiotics is greater in aquatic than in terrestrial environments. Thus, it is (even more) important to monitor antibiotic usage in aquaculture. The U.S. imports over 90% of its seafood from other countries, ones where antibiotic regulation may be more lax or absent all together. Chapter 2 conducts a wide reconnaissance of 47 antibiotics in 27 seafood samples from 11 countries. The next three chapters use liquid chromatography mass spectrometry as a valuable tool for detecting key human health antibiotics in seafood (Chapter 2) and biosolids (Chapter 3).

CHAPTER TWO. RECONNAISSANCE OF 47 ANTIBIOTICS AND ASSOCIATED MICROBIAL RISKS IN SEAFOOD SOLD IN THE UNITED STATES

ABSTRACT

Aquaculture production has nearly tripled in the last two decades, bringing with it a significant increase in the use of antibiotics. Using liquid chromatography/tandem mass spectrometry (LC-MS/MS), the presence of 47 antibiotics was investigated in U.S. purchased shrimp, salmon, catfish, trout, tilapia, and swai originating from 11 different countries. All samples (n= 27) complied with U.S. FDA regulations and five antibiotics were detected above the limits of detection: oxytetracycline (in wild shrimp, 7.7 ng/g of fresh weight; farmed tilapia, 2.7; farmed salmon, 8.6; farmed trout with spinal deformities, 3.9), 4-epioxytetracycline (farmed salmon, 4.1), sulfadimethoxine (farmed shrimp, 0.3), ormetoprim (farmed salmon, 0.5), and virginiamycin (farmed salmon marketed as antibiotic-free, 5.2). A literature review showed that sub-regulatory antibiotic levels, as found here, can promote resistance development; publications linking aquaculture to this have increased more than 8-fold from 1991-2013. Although this study was limited in size and employed sample pooling, it represents the largest reconnaissance of antibiotics in U.S. seafood to date, providing data on previously unmonitored antibiotics and on farmed trout with spinal deformities. Results indicate low levels of antibiotic residues and general compliance with U.S. regulations. The potential for development of microbial drug resistance was identified as a key concern and research priority.

INTRODUCTION

It is estimated that within the next few years, aquaculture will account for almost 40% of total global seafood production by weight, up from 4% in 1970 (FAO, 2013; Cole et al., 2009). This increase to a projected worldwide production of 83 million metric tons in 2013 has been due to a heightened demand for seafood, improved aquaculture techniques, emergence as a key cash crop in certain regions of the world, and recognition as a cheaper way to obtain high-quality protein (Cole et al., 2009; Sapkota et al., 2008). However, as production surges, many aquaculture facilities resort to antibiotics to combat diseases in an environment that creates ample opportunities for bacterial pathogens to thrive (Cabello, 2006). Antibiotics are also commonly used as a prophylactic, sometimes on a daily basis (Defoirdt et al., 2011). Although some promising alternatives such as short-chain fatty acids and bacteriophage therapy have been proposed, many are not ready for mass usage (Defoirdt et al., 2011). Developed vaccines show promise in reducing antibiotic usage (Cabello, 2006), but are only available to treat certain diseases and are not as cost-effective as antibiotics. Thus, the usage of antibiotics in aquaculture remains high.

Consequences associated with the use of antibiotics in aquaculture include the spread of antibiotics into the environment (Christensen et al., 2006; Baker-Austin et al., 2008), residual concentrations left in seafood, high exposure by aquaculture facility personnel, and antibiotic resistance development (Sapkota et al., 2008; Cabello, 2006). Another issue is the impact of antibiotics on the animals themselves, such as potential changes in

genetic expression (Barros-Becker et al., 2012; Lunden et al., 1998) and physiological anomalies. These physiological anomalies include malformation of the spine reported in fish exposed to oxytetracycline (Lunden et al., 1998; Toften and Jobling, 1996).

Many of the antibiotics used in aquaculture are also used in human medicine (Heuer et al., 2009). Amoxicillin and ampicillin are commonly prescribed for treating bacterial infections such as pneumonia and gastroenteritis (Struthers and Westran, 2003). As fish are a potential source of bacterial pathogens for humans, it is important to monitor the spread of antibiotic resistance amongst seafood (Novotny et al., 2004). Resistance to the most commonly applied antibiotics has been found in previous studies (Sapkota et al., 2008; Ryu et al., 2012; Nawaz et al., 2009; Ponce et al., 2008), including several that are multi-drug resistant (MDR) to many classes of antibiotics important in treating human infections (Ponce et al., 2008; Zhao et al., 2003; Labella et al., 2013; Chiu et al., 2013) Thus, detecting and monitoring antibiotic residues in seafood is critically important to reduce potential environmental and human health risks.

A large portion of aquaculture takes place in countries with few regulations and limited enforcement (Pruden et al., 2013), creating the need to monitor imported seafood strictly for antibiotic residues and presence of pathogens. In this study, twenty-seven seafood samples were collected by the National Oceanic and Atmospheric Administration (NOAA) from stores in Arizona and California for analysis. Samples included five of the top ten most consumed seafood varieties in the US: shrimp, tilapia, catfish, swai, and Atlantic salmon. Trout with visible deformed spines were also analyzed. Using liquid

chromatography tandem mass spectrometry (LC-MS/MS), 47 antibiotics identified from literature as drugs of concern were analyzed for using two methods. I also conducted a meta-analysis of published data on antibiotics and resistance development to note trends in aquaculture over the last few decades.

MATERIALS AND METHODS

Samples and Preparation

A collaborating NOAA consumer safety officer obtained samples (*n*= 27) from retail grocery stores in Arizona and California (in southwest U.S.) over a period of three months from June to August in 2012 (**Table 2-1**). Samples originated from 11 different countries. Each sample was sold as a pre-packed unit or bought from store counter displays, meaning that each sample sometimes included multiple fish. Negative controls consisted of catfish donated from Louisiana State University that were never exposed to antibiotics. Normal and deformed rainbow trout (*n*=3 for each) were obtained to survey the potential link between antibiotic exposure and spinal deformities. Atlantic salmon marketed as "antibiotic-free" was also obtained from a local health food store.

Whole fish were filleted and only edible parts were used for analysis. Shrimp (*n*=6), tilapia (*n*=3), catfish (*n*=5), rainbow trout (*n*=6), Atlantic salmon (*n*=5), and swai (*n*=2) were stored at minus 20°C prior to processing by homogenization, using a commercial meat grinder (STX Turbo Force 3000 Series Electric Meat Grinder, Lincoln, Nebraska).

Table 2-1. Aquaculture information and demographics on samples used in this study.

General Information for the U.S.				**This Study**			
Seafood Type	**2011 Rank**[a]	**2012 Imports & Value**[b]	**2011 Production & Value**[c]	**Composite Sample #**[d]	**Origin # of Samples**[e]	**Fillet (F) or Whole (W)**	**Pack-aged**[f]
Shrimp	1	531,840 $4,440M	148,000 $6M	1. Farmed Shrimp	Ind-2; Tha-1; Ban-1; Vie-1	W	Y
				2. Wild-caught Shrimp	Mex-1	W	N
Tilapia	5	227,440 $970M	10,000 $54M	3. Farmed Tilapia	Pan-1; Chi-2	F	Y
Catfish	7	107,690 $370M	163,000 $395M	4. Farmed Catfish	U.S.-2	W	N
				5. AB-Free Farmed Catfish[g]	U.S. LSU-3	W	N
Trout	N/A	9310 $70M	15,300 $53M	6. Farmed Trout w/ D Spine	U.S.-3	W	N
				7. Farmed Trout w/ Normal Spine	U.S.-3	W	N
Salmon	3	120,640 $720M	373,000 $720M	8. Farmed International Atlantic Salmon	Can-2 Chl-1	F	Y
				9. Farmed AB-Free Atlantic Salmon[h]	Sco-1		
				10. Farmed U.S. Atlantic Salmon	U.S.-1		
Swai	6	N/A[i]	N/A[i]	11. Farmed Swai	Vie-2	F	Y

[a]Rank in most consumed seafood. Data from National Fisheries Institute (National Fisheries Institute, 2013).
[b]Units: metric tons and millions of U.S. dollars. Fresh and frozen seafood imported for human consumption. Data from National Oceanic and Atmospheric Administration (NOAA) for the 50 U.S. states, District of Columbia, Puerto Rico, and the U.S. Virgin Islands (NOAA, 2012). Numbers have been rounded.
[c]Units: metric tons and millions U.S. dollars. Commercial U.S. landings and aquaculture. Data from NOAA (NOAA, 2012). Numbers have been rounded. 2012 U.S. aquaculture data were unavailable, thus limiting reported values to 2011 data. [d]11 total composites were made.
[e]Ind= Indonesia, Tha= Thailand, Ban= Bangladesh, Vie= Vietnam, Mex= Mexico, Pan= Panama, Chi= China, U.S.= United States, LSU= Louisiana State University, Can= Canada, Chl= Chile, Sco= Scotland.
[f]Pre-packaged seafood was provided in factory-sealed plastic packages.
[g]Catfish bred from eggs for research purposes never exposed to antibiotics were provided by Dr. Javier Santander of Arizona State University and from Louisiana State University.
[h]Salmon sold as “antibiotic-free” salmon.
[i]Swai is also marketed as pangasius, channel catfish, catfish, basa, and tra, among other names. Thus, import data were not available, due to this inconsistency in labeling.

Between processing of individual samples, the grinder was cleaned with water and soap, and then rinsed with acetone, ethanol, and distilled water three times each. Composite samples were prepared by pooling equal amounts of individual samples to result in 11

composite samples: farmed shrimp, wild-caught shrimp, farmed tilapia, farmed catfish, antibiotic-free catfish, farmed rainbow trout with normal spine, farmed rainbow trout with deformed spine, farmed international Atlantic salmon, farmed antibiotic-free Atlantic salmon, farmed U.S. Atlantic salmon, and farmed swai (**Table 2-1**).

Sample Analysis

Samples pre-processed as described above were frozen and shipped to a commercial laboratory (AXYS Analytical Services Ltd., Sydney, British Columbia, Canada). Approximately 2.5 grams fresh weight (wet weight) of homogenized seafood was subsampled and spiked with isotope-labeled surrogates. Samples were then extracted by bath sonication with 15 mL acetonitrile that was acidified to pH 2 using 0.14 M NaH_2PO_4/ 85% H_3PO (1.93 g $NaH_2PO_4 \cdot H_2O$, 99 mL reagent water, 1 mL 85% H_3PO_4). The extract was then treated with 500 mg of solid ethylenediaminetetraacetic acid (EDTA). Resultant extracts were then filtered and cleaned using solid phase extraction (Waters Oasis HLB SPE cartridges 20 cm^3/1g LP; Hartford, CT). For each sample, 30 mL of extract was diluted to 200 mL total with ultra pure water. Prior to sample loading, the cartridges were conditioned using 20 mL of methanol, 6 mL ultra pure water, and 6 mL pH 2 water. The cartridges were then washed with 10 mL of ultra pure water and subsequently dried under a vacuum. Analytes were eluted using 12 mL methanol, and the eluate concentrated under vacuum to a volume of 4 mL prior to analysis. The full 2.5 g of sample was extracted and contained in the final 4 mL extract.

Samples were analyzed by positive electrospray ionization on a triple quadrupole LC-MS/MS in multiple reaction monitoring (MRM) mode using a Waters Micromass Quattro

Ultima LC-MS/MS system paired with a Waters LC 2795. Chromatography was conducted using reverse-phased C_{18} column (Waters, Milford, MA). A total of 60 pharmaceuticals were analyzed according to the AXYS Method MLA-075, a modification of the USEPA Method 1694 as described previously (Chari and Halden, 2012). Out of the 60 analytes screened for, 47 were antibiotics, and are the focus of this paper. All analytes and instrument parameters are listed in **Appendix A Table A1 and A2**. Two methods were used on the same extract (injection volume: 10 uL) to analyze for tetracyclines and non-tetracyclines, respectively. The tetracyclines method, totaling 30 minutes in duration, had solvent A consisting of an equal mixture of acetonitrile and methanol with 0.5 mM oxalic acid and 0.5% (v/v) formic acid; solvent B consisted of HPLC-grade water containing 0.5 mM oxalic acid and 0.5% (v/v) formic acid. The starting mixture was 10% solvent A (flow rate 0.2 mL/min), increased to 90% A by minute 20 at a flow rate of 0.23 mL/min. The non-tetracyclines method had a run time of 33 min, using as solvent A HPLC-grade water with 0.1% formic acid and 0.1% ammonium formate, and as solvent B a mixture of equal amounts of acetonitrile and methanol. The starting mixture was 95% solvent A (flow rate 0.15 mL/min), increased to 100% solvent B by minute 23 at a flow rate 0.3 mL/min. For the 10 of the 60 total compounds for which a respective stable-isotope labeled analog was available, the concentration was determined using the isotope dilution technique (Halden and Paull, 2005). For the remaining 50 compounds where a labeled analog was not available, the concentration was determined using an alternate isotope-labeled internal standard (see supplemental information).

Precision between intraday samples and duplicates was expressed as relative percent difference (RPD), which was calculated using the following expression as reported previously (McClellan and Halden, 2010):

$$RPD\ [\%] = \frac{|C_{sample} - C_{duplicate}| \times 100}{(C_{sample} + C_{duplicate})/2} \qquad \text{(Eq. 1)}$$

where C_{sample} and $C_{duplicate}$ are the concentrations detected in the original sample and in its duplicate, respectively.

Quality Assurance and Control

Several tests were performed before and during sample analysis to ensure system and laboratory performance. Initial calibration was performed using labeled surrogates, recovery standards and authentic targets to encompass the working concentration range. Retention times of native and labeled compounds had to be within 0.4 minutes of the respective retention time established during the previous calibration. A mid-level solution was analyzed every 12 hours or every 20 samples, whichever occurred first. All calibration curves consisted of at least 5 consecutive calibration levels. Native compounds with labeled surrogate standards had to elute within 0.1 minutes of the associated labeled surrogates in order to be authenticated. Method blanks and matrix spikes to evaluate recovery rates were also conducted, and duplicates were also analyzed for 5% of test samples within each batch on the same day (containing 7 or more test

samples). Method detection limits (MDLs) were determined as specified by EPA Federal Regulation 40 CFR Part 136, Appendix B.

Meta-Analysis of the Peer-reviewed Literature for Antibiotic Resistance Articles

A literature search of the Web of Knowledge was performed for studies published between 2003 and November 2013 using the search terms "antibiotic resistance AND aquaculture" and "antibiotic resistance AND seafood" to identify relevant strains of bacteria isolated from seafood shown to contain antibiotic resistant microorganisms. Only microbial strains isolated from finned fish or shrimp were included to make it relevant to this study and only seafood for human consumption was included; strains further had to show resistance to one or more specific antibiotics (as opposed to mere classes of antibiotics). Resistance to only four antibiotic classes, tetracyclines, sulfonamides, penicillins, and quinolones, was examined because these are the top drug classes customarily screened for in our study.

The same search words were used to identify connections between antibiotic resistance and aquacultural practices (i.e., sediment, water pollution, resistant strains found on aquaculture facilities or seafood). Articles focusing on non-antibiotic pathogen reduction methods and/or ornamental fish were excluded. No publication-year limit was employed.

Calculation of Theoretical Maximum Concentrations in Individual Samples Used in Composites

This study employed a composite sampling approach. Samples were pooled to create 11 composites from 27 individual samples. Theoretical maximum concentrations in individual samples processed were calculated using the conservative formula:

$$C_{composite} \text{ x } n \text{ samples in pool} = C_{individual\ sample} \qquad \text{(Eq. 2)}$$

where $C_{composite}$ is the concentration determined experimentally in the pool of samples, n is the number of samples contributing to the pool, and $C_{individual\ sample}$ is the calculated theoretical maximum concentration of the analyte in individual samples contributing to the pool. Each composite sample was constructed from a different number of individual samples, depending on the species. See **Table 2-1** for a complete list.

RESULTS AND DISCUSSION

Method Performance

As this paper focuses on antibiotics, further discussion will only pertain to the 47 antibiotic analytes that were screened for. Method detection limits for the various antibiotics ranged from 0.1 ng/g (roxithromycin/sulfadimethoxine) to 25.5 ng/g (minocycline) fw of seafood (**Table 2-2**; **Appendix A Table A2**). Recoveries of the 47 antibiotics ranged from 15.9% (4-epianhydrochlortetracycline) to 138% (sulfathiazole), with the majority (35 out of 47) placing in the preferred range of 70 to 130% (**Table 2-2**).

No laboratory contamination was observed in method blanks. Method performance in this study was favorable and comparable to previously reported results (McClellan and Halden, 2010; Love et al., 2012).

Occurrence of Antibiotics in Seafood

Seven out of eleven composite samples were found to have detectible quantities of antibiotics, including oxytetracycline, 4-epioxytetracycline, sulfadimethoxine, ormetoprim, and virginiamycin (**Table 2-2**). The most commonly detected antibiotic was oxytetracycline, which is the number one used antibiotic in aquaculture, with 12 of the top 15 aquaculture-producing countries reporting usage (Sapkota et al., 2008). It was detected at a concentration of 8.6 ng/g fw, along with its 4-epimer at 4.1 ng/g fw, in farmed international Atlantic salmon comprised of samples from Chile and Canada (**Figure 2-1**), which are among the top four salmon-producing countries (FAO, 2013). As the 4-epimer is a known degradation product of oxytetracycline (Loke et al., 2003) it is likely that a higher oxytetracycline concentration was originally in these samples. Tetracyclines are regulated in the U.S. as a sum of all parent antibiotics and their 4-epimers (FDA, 2013). The resultant combined concentration in farmed international Atlantic salmon of 12.6 ng/g was still under the maximum permitted concentration of 2 µg/g in finfish (**Table 2-3**).

The unexpected detection of oxytetracycline at a concentration of 7.7 ng/g fw in wild-caught shrimp imported from Mexico may be due to several reasons. Unintentional or intentional mislabeling of the product and cross-contamination of seafood during

handling, processing and packaging are possible. Uptake of the drug from coastal waters and sediments impacted by inputs of raw and treated wastewater (Kim and Carlson, 2007A) also could explain the observed detection but ultimately the origin of contamination remains unknown.

Table 2-2. Antibiotics analyzed, recovery percentages, method detection limits, and concentrations detected in seafood samples in units of ng/g fresh weight.

Antibiotic Class	Compound, Recovery %, (MDL[a]), Concentration If Detected	
	DETECTED	NOT DETECTED
Tetracyclines	Oxytetracycline, 100, (2.4), 7.7[2], 2.7[3] 3.9[6], 8.6[8] 4-Epioxytetracycline, 112.5, (3.9), 4.1[8]	Anhydrochlortetracycline, 46.8, (7.4); Anhydrotetracycline, 137.5, (6.0); Chlortetracycline, 130.5, (9.2); Demeclocycline, 97.7, (6.0); Doxycycline, 117, (2.4); 4-Epianhydrochlortetracycline, 15.9, (24.1); 4-Epianhydrotetracycline, 104.1, (6.2); 4-Epichlortetracycline, 104, (9.1); 4-Epitetracycline, 130.5, (4.2); Isochlortetracycline, 87.2, (2.4); Minocycline, 109.5, (25.5); Tetracycline, 135, (3.5)
Sulfonamides	Sulfadimethoxine, 79.5, (0.2), 0.3[1]	Sulfachloropyridazine, 83, (0.6); Sulfadiazine, 102.3, (0.6); Sulfamerazine, 111, (0.2); Sulfamethazine, 109, (0.4); Sulfamethizole, 85.5, (0.9); Sulfamethoxazole, 112.4, (0.2); Sulfanilamide, 56.5, (6.0); Sulfathiazole, 138, (0.6)
Macrolides	Virginiamycin, 89.5, (4.2), 5.2[9]	Azithromycin, 97.7, (0.7); Clarithromycin, 96.4, (0.6); Erythromycin-H_2O, 117, (0.9); Lincomycin, 129.5, (1.2); Roxithromycin, 75.1, (0.1); Tylosin, 72.1, (2.4);
Quinolones	-	Ciprofloxacin, 99.6, (2.); Clinafloxacin, 119, (2.6); Enrofloxacin, 119, (1.2); Flumequine, 104.7, (0.6); Lomefloxacin, 72.7, (1.2); Norfloxacin, 114, (6.); Ofloxacin, 81.8, (0.6); Oxolinic Acid, 54.8, (0.3); Sarafloxacin, 65.7, (0.6)
Penicillins	-	Cloxacillin, 86, (1.2); Oxacillin, 87.7, (1.2); Penicillin G, 28.3, (1.2); Penicillin V, 120.5, (1.2)
Cephalosporin	-	Cefotaxime, 65.1, (9.9)
Other	Ormetoprim, 93.1, (0.4), 0.5[10]	Carbadox, 24.7, (0.6); Trimethoprim, 91.5, (0.6)

Superscripts of detected concentrations indicate sample number; see Table 1 for additional sample information.
[a]Highest method detection limit (MDL) for each analyte is reported. See Table A2 in the Appendix A for all MDLs.

Oxytetracycline was also detected at concentrations of 2.7 and 3.9 ng/g fw, respectively, in farmed tilapia and in farmed rainbow trout with visibly deformed spines (**Figure 2-**

2A). Oxytetracycline was not detected above the detection limit of 2.4 ng/g in trout without visible spinal deformities (supplemental information T2). Detection of the latter corroborates earlier reports that this antibiotic may cause spinal deformities in certain species (Toften and Jobling, 1996); however, due to the limited number of individual samples available (*n* =3), the present study was underpowered and cannot ascertain causation. As trout is a major market in the U.S., with over 700 trout-rearing farms (Agricultural Marketing Research Center, 2013), further work with a larger sample size is needed to elucidate the connection between oxytetracycline dosing and spinal deformities in trout and other fish species. Among the large group of sulfonamides, only sulfadimethoxine was detected and only in a single seafood variety, in farmed shrimp at 0.3 ng/g fw. Sulfadimethoxine reportedly is used by 4 of the top 15 aquaculture-producing countries (Sapkota et al., 2008). Yet, although screened for previously (Won et al., 2011; Tittlemier et al., 2007) and several detection methods have been developed (Gehring et al., 2006; Villar-Pulido et al., 2011), the result reported here constitutes the first detection of this drug in shrimp. There is no U.S. MRL set for this drug in shrimp, although it is regulated in salmonids and catfish at a level of 0.1 µg/g fw (**Table 2-3**).

Figure 2-1. Map showing countries from which seafood samples originated (*n*, number of samples).

Ormetoprim, an antibiotic commonly used with sulfonamides, was detected at a concentration of 0.5 ng/g fw in farmed Atlantic salmon from the U.S. This concentration is about 200 times less than the regulatory limit of 0.1 μg/g.

Contrary to the label stating culturing without antibiotics, virginiamycin was found at a concentration of 5.2 ng/g fw in farmed Atlantic salmon. The apparent presence of virginiamycin indicates that either the labeling was inaccurate or contamination of the seafood occurred. Although the detected concentration was much lower than the regulatory limit of 0.1 μg/g (**Table 2-3**), this finding is still important, as it indicates that the "antibiotic-free" label does not always accurately represent whether antibiotics are absent or present.

The occurrence of antibiotics in seafood above method detection limits in the low ng/g range attained here appears to be the exception rather than the norm. Five antibiotics were detected at low ng/g concentrations in this survey. The present study is the first to consider the top consumed seafoods in the U.S. as well as the first to survey a large

Table 2-3. Maximum Residue Limits (MRLs) of antibiotics allowed for the USA, EU, Chile, and CODEX (µg/g fresh weight). For antibiotics lacking regulatory guidelines in seafood, values are given for other food animal varieties when available.

Antibiotic	US[a]	EU[b]	Chile[c]	CODEX[d]
Carbadox	0.03[e]	-	-	-
Cloxacillin	0.01[g]	0.3[m]	-	-
Doxycycline	2[f]	0.1[i]	-	-
Enrofloxacin	0.1[h]	0.1[n]	0	-
Tetracyclines[r]	2[f]	0.1[o]	-	-
Erythromycin-H_2O	0.1[g]	0.2[m]	0.2[m]	0.1[q]
Lincomycin	0.1[i]	0.1[m]	-	0.2[q]
Ormetoprim	0.1[j]	-	-	-
Oxytetracycline	2[f]	0.1[o]	0.12[m]	0.2[m]
Penicillin G	0[k]	0.05[m]	-	0.05[i]
Penicillin V	0[k]	-	-	-
Sulfadimethoxine	0.1[j]	0.1 (sum of sulfonamides) 0.1[o]	-	-
Sulfamerazine	0[l]		-	-
Sulfathiazole	0.1[i]		-	-
Tetracycline	2[f]		-	0.2[p]
Tylosin	0.2[g]	0.1[m]	-	0.1[g]
Virginiamycin	0.1[i]	-	-	-

[a]FDA USDA CFR 21 (FDA, 2013).
[b]EU commission regulation no. 37/2010, Dec. 2009 (EU, 2013).
[c]FAO 2012 Report (Bravo, 2012).
[d]Codex Alimentarius Commenssion (CAC, 2009).
[e]Swine liver.
[f]Sum of tetracyclines in finfish.
[g]Cattle.
[h]Cattle liver.
[i]Swine.
[j]Salmonids and catfish.
[k]Different forms of penicillin are not differentiated. Chicken.
[l]Trout.
[m]All fish.
[n]Sum of ciprofloxacin and enrofloxacin.
[o]Sum of 4-epimer plus parent drug.
[p]Sum of parent drugs.
[q]Poultry.
[r]Includes 4-epianhydrotetracycline, 4-epianhydrotetracycline, 4-epichlortetracycline, 4-epioxytetracycline 4-epitetracycline, demeclocycline, isochlortetracycline, minocycline. Currently unregulated/information not available for: anhydrochlortetracycline, anhydrotetracycline, azithromycin, cefotaxime, clarithromycin, clinafloxacin, omefloxacin, norfloxacin ofloxacin, and roxithromycin. Currently, no MRLs have been set in U.S. for ciprofloxacin, flumequine, oxacillin, oxolinic acid, sarafloxacin, and trimethoprim.

number of antibiotics. The majority of these antibiotics have never been screened for in our food supply. This study also represents samples from 11 countries (**Figure 2-1**), 8 of which are among the top 15 aquaculture-producing countries (Sapkota et al., 2008). Results of this study of modest sample size suggest that seafood, regardless of whether wild-caught, farmed, imported, or domestically produced, is typically compliant with U.S. chemical regulations. However, the results need further confirmation, ideally by studies featuring a large sample size.

Antibiotic Resistance Development in Seafood

Although the concentrations reported here are less than the FDA allowed maxima, these sub-therapeutic drug concentrations can often select for and enrich resistant bacteria (Andersson and Hughes, 2012). There has been a notable increase in resistant microbial strains associated with the antibiotics and seafoods examined in this study. Out of 179 *Escherichia coli* strains isolated from commercial seafood in a study by Ryu et al., 55 strains were found to be resistant to tetracycline (Ryu et al., 2012). Another 34 strains were found to hold intermediate resistance to tetracycline, which can be affected and selected for by sub-therapeutic antibiotic concentrations. Nawaz et al. also reported isolation of MDR *Klebsiella* spp. bacteria from imported shrimp obtained from grocery stores (Nawaz et al., 2012). The identification of these strains may be interpreted as being the result of extensive human use and misuse of antibiotics in the clinic, community, agriculture, and in animal husbandry such as aquaculture (Andersson and Hughes, 2012). The top antibiotics used by heavy aquaculture producers include the following: oxytetracycline, oxolinic acid, chloramphenicol, erythromycin, furazolidone,

trimethoprim, sulfadiazine, ampicillin, florfenicol, flumequine, and sulfadimethoxine (Sapkota et al., 2008). All of these antibiotics are included on the WHO list of critically/highly important antibiotics for human health (Heuer et al., 2009, Nawaz et al., 2012; WHO, 2007). Multiple studies in the last three decades have revealed resistance to many of these antibiotics, the majority of which were screened for in this study (**Figure 2-3A).** The fact that seafood examined for bacteria has resulted in isolates belonging to pathogenic genera causing infections in humans (e.g., *Salmonella*, *Vibrio*, *Escherichia*) (Baker-Austin et al., 2008; Ryu et al., 2012;Ponce et al., 2008) increases the likelihood of resistance spread from aquaculture to people. This poses a risk to consumers as well as employees coming into contact with the seafood from production to store delivery.

Indeed, literature volume statistics summarized in **Figure 2-3** show that the topic of resistance to many antibiotics screened here is a major area of concern for the aquaculture community. The number of publications linking resistance to seafood has increased by 800% between the 1990s and today (**Figure 2-3B**). The majority of papers report the ineffectiveness of tetracycline and oxytetracycline as one of the most commonly seen resistances. The observed publication trend also acknowledges an increased awareness of the fact that exponential growth has taken place in the aquaculture industry in the past few decades. This trend also suggests an association between the heavy usage of oxytetracycline (the number one used antibiotic in aquaculture) and resistance development.

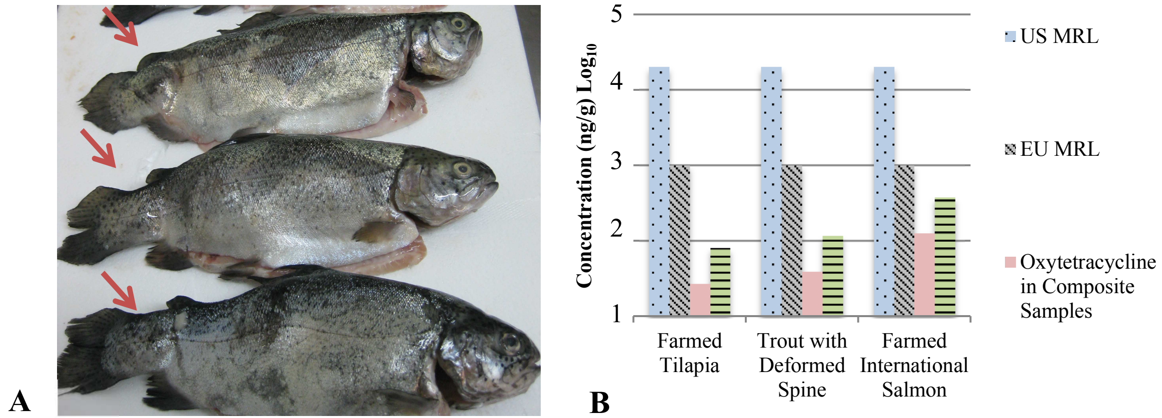


Figure 2-2. Farmed trout with visible spinal deformities and applicable U.S. and EU MRLs in composite and individual samples. Panel A shows an image of spinal deformities in trout analyzed in this work. Arrows indicate abnormal spinal curvatures (Photo credit: Don McBride, NOAA, 2012). Panel B shows a comparison of oxytetracycline concentrations determined in this study to maximum residue limits (MRLs) allowed in the United States (US) and the European Union (EU) (FDA, 2013; EU, 2013). Concentrations of oxytetracycline and 4-epioxytetracycline in farmed international salmon were added, as regulation is for maximum total tetracyclines.

Some bacterial strains identified in our literature review were found to be completely or intermediately resistant to certain antibiotics (Ryu et al., 2012; WHO, 2007). Furthermore, the transfer of plasmids among bacteria on seafood has been reported (Ferrini et al., 2008). Strains were found to have minimal inhibitory concentrations (MIC) far lower than the MIC requirement for the "resistant" classification, indicating that very low concentrations of antibiotics can select for resistance. One study found that only about half of the isolates from their aquaculture samples had MICs above the "resistant" concentration of 128 μg/mL; some isolates exhibited MICs as low as 0.25 μg/mL, over 500 times less than the classification of resistance-promoting concentration (Guglielmetti et al., 2009). In Chile, the reported dose of oxytetracycline through feed is 100-120 μg per g fish per day, administered for 14-21 days, depending on the disease (Akinbowale et al., 2006). In China, the preventative dose for the fluoroquinolone compound oxolinic acid is 10-20 μg per g fish per day for 4-7 days (Bravo, 2012). These concentrations

currently in use are known to exert selective pressure. Since many of these antibiotics also are used in human medicine, selective pressure may promote the occurrence of resistant strains of potential human health concern. Overall, the information compiled in **Figure 2-3** shows that the development and occurrence of drug resistant bacteria in seafood is an issue that is both timely and of notable importance. Thus, to ensure the safety of the food supply in the U.S. and abroad, the monitoring of seafood has to focus on both the residues of aquacultural drugs themselves and the drug resistance in pathogens these antibiotics can trigger.

Study Limitations

This study employed composite sampling. This approach is well suited for the economical screening of a large number of analytes and for accurately determining average concentrations therein (Yuan and Chen, 2012; Baron et al., 2014). This method of sampling was chosen here because the purpose of this study was to conduct a large-scale screening of many analytes. However, this methodology is inappropriate for determining the full range of concentrations (i.e., minima and maxima) as well as detection frequencies. Accordingly, theoretical maximum concentrations of oxytetracycline and sulfadimethoxine were calculated for individual samples and the resultant values represent conservative estimates that are likely higher than the true concentration. The oxytetracycline values of 8.1, 11.7, and 37.8 ng/g calculated, respectively, in farmed tilapia, farmed trout with spinal deformities, and farmed international salmon are well below the U.S. limit of 2,000 ng/g (**Figure 2-2B**). Note that the concentration of 37.8 ng/g calculated for salmon includes both oxytetracycline and 4-

epioxytetracycline; it is provided in this form because tetracyclines are regulated as a sum of drugs of this class. Values calculated for sulfadimethoxine (1.7 ng/g for each country's sample) is also significantly under U.S. regulatory limits.

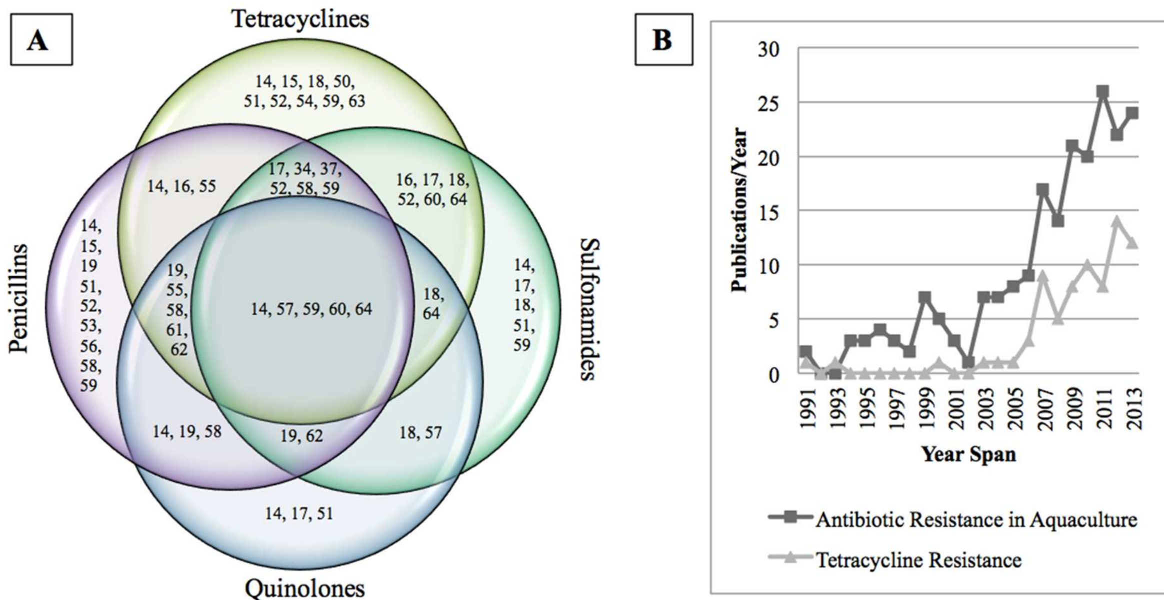


Figure 2-3. Published studies reporting resistant bacteria isolated from aquaculture and seafood. Panel A shows select studies from 2003-2013 reporting the presence of bacteria resistant to 4 groups of antibiotics found on seafood available for human consumption. Numbers correspond to references. Panel B shows the number of publications featuring antibiotic resistance development in aquaculture and seafood (dark gray) and number of publications featuring resistance to the antibiotic class of tetracyclines (light gray).

References: 14= Ryu et al., 2012 **15**= Nawaz et al., 2009 **16**= Ponce et al., 2008 **17**= Zhao et al., 2003 **18** = Labella et al., 2013 **19**= Chiu et al., 2013 **50**= Fallah et al., 2013 **51**= Ansari et al., 2011 **52**=Khan et al., 2009 **53**= Kumar et al., 2013 **54**= Budiati et al., 2013 **55**= Raissy et al., 2012 **56**= Deekshit et al., 2012 **57**= Yan et al., 2010 **58**= Kakatkar et al., 2011 **59**= Liu et al., 2009 **60**= Kumar et al., 2009 **61**= Adeyemi et al., 2008 **62**= Thayumanavan et al., 2003 **63**= Kim et al., 2004 **64**= Sarter et al., 2006

Another limitation is that sampling was done only in Arizona and California. The obtained results may not necessarily apply to other states and alternate sources (i.e., countries) of commercial seafood. Many wild-caught seafood varieties were not available for this survey because the vast majority of seafood for consumption in the U.S. is only readily available from aquaculture operations. Also, as I obtained fresh seafood in the

form most consumers choose, samples were either whole animals or fillets and either pre-packaged or loose, which means that variation in handling and processing by the producer may affect antibiotic preservation and degradation in the tissue. This variation, as well as antibiotic sources that do not originate from aquaculture, could also have contaminated the seafood and affected our data.

Samples were collected in June-August, 2012 and analyzed in November 2012, following storage for 3-5 months at -20°C. A previous study, examining the effect of sample storage at -18°C, showed that tetracyclines, sulfonamides, quinolones, macrolides, and aminoglycosides are stable and remain intact structurally and quantitatively, as demonstrated using a porcine muscle matrix (Berendsen et al., 2011). However, penicillins were observed to attenuate, by about 30% and 20%, respectively, for ampicillin and cloxacillin over the course of 3-6 months (Berendsen et al., 2011). Hence, the concentrations of penicillins at the time of purchase in samples of seafood analyzed here may have been higher than the values of less than <1.2 to <1.6 ng/g fw reported here.

Our sample size of 27 is of a magnitude similar to other studies that utilized composite sampling to investigate poorly characterized potential human exposure sources (Kim et al., 2007B; Kim et al., 2008). The goal of the present work was not necessarily to identify specific antibiotics in individual samples, but rather to conduct a large-scale screening of U.S. seafood to assess whether there is a need for more aggressive monitoring. Whereas the present dataset cannot prove the safety or danger of imported seafoods, it provides an

incremental, yet significant step forward in assessing the safety of the U.S. seafood supply. Data made available here suggest that there is no immediate threat to human health from trace levels of the analytes surveyed in this work. However, additional studies using a larger sample size would be beneficial to confirm the findings and conclusions of the results obtained here.

Our literature review considered only a subset of papers based on the inclusion criteria stated. A less stringent search would have resulted in an even larger body of literature supporting the conclusion reached here that the promotion of antibiotic resistance constitutes a major health concern in aquaculture.

CONCLUSIONS

This study surveyed the concentrations of 47 antibiotics in 6 different seafood varieties originating in 11 countries purchased exclusively from the southwestern U.S. All samples studied demonstrated compliance under current federal regulations, suggesting that they are chemically safe to consume. This conclusion could be drawn from the analysis of pooled samples, an approach that did not permit to determine the actual concentration in each individual sample entering the survey, however. Five antibiotics were found at detectable levels and estimated concentrations were relatively low (0.3-8.6 ng/g fw). However, the development and spread of antibiotic resistance is a public health priority that is divorced from the regulatory limits designed to prevent adverse outcomes from human ingestion of drugs. Antibiotics present at levels well below regulatory limits still

can promote the emergence of (multi-) drug resistant microorganisms. Future studies are warranted to fully understand the connection between aquacultural use of antibiotics, development of drug resistance, human exposure to resistant pathogens, and ensuing morbidity and mortality in seafood consumers. The trend in the last 3 decades of notable increases in the number of resistant and multi-drug resistant strains identified in seafood is of concern. Monitoring studies such as the present work are one of multiple steps required to understand and manage potential risks posed by use of antibiotics in aquaculture and in society at large. The present study was limited in sample size and employed sample pooling. It is desirable to perform additional surveys to confirm the findings and preliminary conclusions reported here.

TRANSITION TWO

Antibiotics reach the environment in two primary pathways, via animal husbandry and through wastewater treatment plants (WWTPs). WWTPs may serve as urban public health observatories; an entire community reaches these plants for decontamination of biological and chemical contaminants. Often, contaminants of concern include important microbes such as *Escherichia coli* and hepatitis viruses. However, chemical contaminants must also be monitored as many of the compounds entering the plant may act as carcinogens, endocrine disruptors, antibiotic resistance promoters, and/or ecological toxicants upon incomplete removal and discharged into the natural ecosystems. Using the largest and most current repository of U.S. biosolids, I selected samples to screen for 9 antibiotics on the World Health Organization list of important antimicrobials and commonly used in human health and aquaculture. Biosolids, the semi-solid byproduct of municipal sewage treatment, are often applied on agricultural land, making them a very important product to monitor for chemical contaminants, especially ones that will affect agricultural settings. In the case of antibiotics, increasing opportunities for unwanted microbial drug resistance in these agricultural fields will not only endanger the workers on these fields, but also potentially the downstream consumer that these crops may reach. In Chapter 3, I examined whether biosolids contain detectable levels of key antibiotics used in human medicine.

CHAPTER THREE. OCCURRENCE OF NINE ANTIBIOTICS IN ARCHIVED BIOSOLIDS FROM THE U.S. EPA TARGETED NATIONAL SEWAGE SLUDGE SURVEY

ABSTRACT

The occurrence of nine antibiotics was investigated in archived biosolids from wastewater treatment plants in 12 states sampled as part of the 2006/2007 U.S. Environmental Protection Agency (EPA) Targeted National Sewage Sludge Survey. Using liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis, five antibiotics were detected at the following average concentrations (ng/g dry weight): nalidixic acid (19.1), oxolinic acid (2.7), erythromycin (0.6), oxytetracycline (4.5), and ampicillin (14.8). Four were not detected in any samples (< MDL): sulfadimidine (<1.0), sulfadimethoxine (<0.5), NP-AOZ ((3-(2-nitrobenzylidenamino)-2-oxazolidinone), furazolidone metabolite; <20.0), and spiramycin (<2.0). At least one targeted antibiotic was found in 83% of samples analyzed. Oxytetracycline and erythromycin concentrations were lower than those previously reported for these samples by the EPA, suggesting that degradation of antibiotics had occurred during storage. This is the first report of oxolinic acid and ampicillin in biosolids worldwide and, along with nalidixic acid, the first report of these three antibiotics in U.S. biosolids. Occurrence data for key antibiotics used in human medicine may help to inform risk assessments for biosolids application on croplands.

INTRODUCTION

The efficacy of antibiotics, arguably the most important class of life-saving compounds in human medicine, is now threatened by widespread microbial resistance due in part to overuse in human medicine and agricultural and aquacultural food animal production. Recent research has shown that wastewater treatment plants (WWTPs) are a dispersal route of antibiotic residues, resistant bacteria, and resistance genes into the water environment (Pruden, 2013). Among the two process streams exiting WWTPs, biosolids may be an important route of contaminant releases in addition to treated effluent. The U.S. Environmental Protection Agency (EPA) defines biosolids as treated solids produced from wastewater treatment that are nutrient-rich and can be safely recycled and applied as fertilizer (EPA, 2012). It is estimated that over eight million dry tons were generated in 2006 in the United States (EPA, 2006). Half of this mass is applied on land, and the remainder is either being incinerated or placed in landfills (Kinney et al., 2008; EPA, 2012).

Several research gaps exist regarding the occurrence of antibiotics in biosolids applied on farmland. The identity and concentration in biosolids of many antibiotics is still not fully understood. This is a concern because antibiotics are biologically active compounds and may potentially retain their activity in biosolids for a long time (Jjemba, 2002). To the best of my knowledge, ampicillin, spiramycin, furazolidone, and nalidixic acid are four antibiotics that have never before been monitored in U.S. biosolids. Ampicillin and nalidixic acid are commonly used in human medicine. Screening of East Asian sewage

sludges (Matsuo et al., 2011; Gao et al., 2012b; Li et al., 2013b; Chen et al., 2013; Jia et al., 2011) resulted in only a single report on the occurrence of nalidixic acid at approximately 10 ng/g dry weight (Chen et al., 2013). No publications screening for furazolidone have been published to date.

Oxytetracycline, sulfadimethoxine, sulfadimidine (aka sulfamethazine), erythromycin, and oxolinic acid have been previously screened for in multiple studies. Among the most notable is the publication by the U.S. Environmental Protection Agency (EPA) in 2009 that surveyed the occurrence of 44 antibiotics in a Targeted National Sewage Sludge Survey (TNSSS) conducted in 2006/2007 (EPA, 2009). In this survey, oxytetracycline, sulfadimethoxine, sulfadimidine, and erythromycin were detected in approximately 38, 7, 3, and 93% of 84 samples, respectively. Detected concentrations resided in the ng/g to μg/g range. Other publications produced similar results, with some papers reporting detections in the same range (Garcia-Galan et al., 2013; Gao et al., 2012a; Ding et al., 2012; Chen et al., 2013) and some reporting non-detects (Tang et al., 2009; Gago-Ferrero et al., 2015). Overall, oxytetracycline and erythromycin are some of the most commonly detected antibiotics reported in the published literature.

The above mentioned drugs are among the most medically important antibiotics, as defined by the World Health Organization (WHO, 2012). Together, these antibiotics span six medically important classes: penicillins, sulfonamides, quinolones, nitrofurans, macrolides, and tetracyclines. These antibiotics, such as the quinolones nalidixic acid and oxolinic acid, are often used to treat a variety of Gram positive and Gram negative bacterial infections (Jia et al., 2012). Presence of antibiotics in biosolids signals

widespread use as well as their persistence during wastewater treatment. Drug residues in land-applied sludge are a potential human health concern, directly due to their inherent toxicity and indirectly through their ability to promote antibiotic resistance, a medical issue that is on the rise globally (CDC, 2015). Aside from their importance in human medicine, these antibiotics are also increasingly important in the farming of food animals for human consumption, especially in aquaculture, the fastest growing agricultural sector in the world today (Sapkota et al., 2008; Heuer et al., 2009). Thus, the monitoring of antibiotics in biosolids destined for agricultural fields is important for understanding their fate during wastewater treatment and mass loadings to agricultural soils.

The purpose of the present study was to determine the concentration of nine medically important antibiotics in archived biosolids from the 2006/2007 U.S. EPA TNSSS. Four of the targeted drugs have never been screened for previously in U.S. biosolids. Using liquid chromatography tandem mass spectrometry (LC-MS/MS), I screened for ampicillin, erythromycin, nalidixic acid, furazolidone, oxolinic acid, oxytetracycline, spiramycin, sulfadimethoxine, and sulfadimidine in biosolids samples from a dozen samples across the continental U.S.

MATERIALS AND METHODS

Samples

Biosolids grab samples were collected by the EPA as described previously (EPA, 2009; Venkatesan et al., 2014; see **Appendix A Table A3** for full EPA sampling locations).

Out of these, 12 samples were randomly chosen, four from each of the four U.S. regions (Northeast n=9 states, South n=16, Midwest n=12, West n=11) (**Figure 3-1**). The regions were previously determined by the EPA during their sampling in 2006/2007. Composites of all samples in the each of the four regions were used for method development, analyte recovery, and method detection limit (MDL) determination.

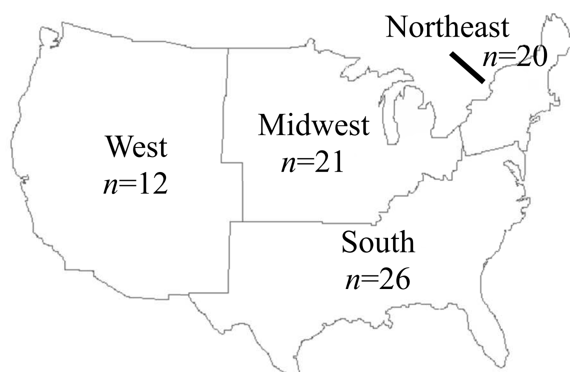


Figure 3-1. EPA organization of sampling geography. Number of states are noted after n =.

Materials

Analytical standards of antibiotics AMP (ampicillin), ERY (erythromycin), NDA (nalidixic acid), OXA (oxolinic acid), OXY (oxytetracycline), SDD (sulfadimidine), SPI (spiramycin), SUL (sulfadimethoxine), NP-AOZ (3-(2-nitrobenzylidenamino)-2-oxazolidinone) and LC-MS grade acetonitrile (ACN), water, acetic acid, and methanol (MeOH) were purchased from Sigma-Aldrich (St. Louis, MO). NP-AOZ is a metabolite of furazolidone and was used as the analytical target in this study (Vass et al., 2005). Ortho-phosphoric acid (85%) was purchased from Fisher Scientific (Waltham, MA, USA). Ultra pure water (18.3 Ohm) was provided by a NANOpure water system (Elga; Woodridge, IL, USA). Three isotopically-labeled analogs were also purchased from Sigma-Aldrich (St. Louis, MO): erythromycin-(*N,N-dimethyl-*$^{13}C_2$), 3-(2-nitrobenzylidenamino-)-2-oxazolidinone-d_4, and sulfadimethoxine-(*phenyl-*$^{13}C_6$).

Individual stock solutions of 1.0 g/L were created for each analyte in MeOH. The exceptions were ERY, which was purchased at a concentration of 1.0 g/L in water, and NDA, which was dissolved in 1% 0.1 M NaOH to increase solubility (Dinh et al., 2011). Combined standards were created of all antibiotics ranging from concentrations 0.5 μg/L to 100 mg/L and kept at -20 °C. All glassware used was baked at 550°C overnight (Thermolyne; Thermo Scientific; Waltham, MA, USA); caps were acid-washed using 10% HCl and thoroughly rinsed three times with ultrapure water and allowed to air dry prior to use.

Extraction

Approximately 0.5 g of biosolids dry weight (dw) was weighed into 4 mL ashed glass vials and 100 ng of each isotopically labeled analog standards (NP-AOZ-d_4, ERY-$^{13}C_2$, and SUL-$^{13}C_6$) were spiked in. Three times the biosolids mass (approximately 1.5 mL) of acetonitrile (pH 2 with 85% ortho-phosphoric acid) was added to each vial and the samples were shaken on a MaxQ 2000 horizontal shaker at 200 rpm (Thermo Scientific) for 6 h while wrapped in aluminum foil to exclude light. The vials were then centrifuged at 1800 rpm for 15 min (Eppendorf 5810R) and the entire supernatant was transferred to a new 4 mL glass vial. 1.5 mL of ACN was added again to each vial and the sample was vortexed until homogenized and re-centrifuged as above. The supernatants were combined and evaporated under N_2 stream (ReactiVap Evaporator- Thermo Scientific) until volume was approximately 2 mL. The entire extract was contained in the 2 mL. Extracts were stored at -20 °C and centrifuged immediately before analysis.

LC-MS/MS

Mass spectrometric analyses were carried out on an API 4000 instrument (Applied Biosystems, Framingham, MA, USA), coupled to a Shimadzu Prominence HPLC (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA) and controlled by Analyst 1.5 software (Applied Biosystems, Framingham, MA, USA). Separation was carried out using XBridge BEH C_8 Column, (130 Å, 3.5 μm particle size, 4.6   150 mm; Waters, Milford, MA, USA). The mobile phase consisted of solvent A (30 mM acetic acid water) and solvent B (MeOH) flowing at a rate of 600 μL/min with a total runtime of 10 min. The solvent gradient program consisted of a hold at 30% solvent B for 1.5 min, a ramp to 60% solvent B over 30 seconds, then a ramp up to 80% solvent B over 1.5 min. Solvent B was then held at 80% for 2 min followed by a decrease to 30% over 30 seconds. The column was then equilibrated at 30% for 3 min before the next injection of 50 μL. Analytes were introduced to the mass spectrometer using an electrospray ionization probe in positive mode. Optimized conditions for the ionization and fragmentation of the analytes are specified in **Appendix A Table A4**. Two transition ions were used for each analyte. The one giving the highest signal was used as the quantitation ion and the one giving the second highest signal was used as the confirmation ion.

Using Analyst 1.5 software (Applied Biosystems, Framingham, MA, USA), peak areas were smoothed and integrated automatically and then individually inspected and adjusted as needed to create robust calibration curves. For compounds with an isotopically labeled analog (ERY, SUL, NP-AOZ), quantitation was conducted using the isotope-dilution method. For all other compounds, quantitation was conducted using the method of

standard addition. Standard addition was conducted as follows: five identical aliquots of final extract were spiked with increasing known masses of analyte prior to analysis. A six-point calibration curve was created using these five concentrations plus the unspiked extract and the slope and abscissa were used to find the unknown concentration of the unspiked extract. Duplicate extractions were performed for 90% of the samples and the concentration of the duplicate sample was quantified using the standard addition curve of the primary sample (i.e., using the extract of the primary sample). Standard addition was used for quantitation instead of external calibration to account for matrix effects (Koester et al., 1990; Garcia-Rodriguez et al., 2014; Tusiimire et al., 2015). Absolute recoveries were determined by spiking 100 ng of analyte into composite samples (where background levels were determined to be non-detects of target analytes) prior to extraction and calculating the mass recovered in units of percent. Absolute areas under the curve were used with the $y=mx+b$ equation obtained via standard addition for each of the six analytes for which isotopically-labeled analogs were lacking.

Quality Assurance

Calibration accuracy was verified for each batch using a calibration standard with native and isotopically-labeled analogs of the target analytes. Blanks were run before and after each batch, as well as in between every seven samples at a minimum. Retention times had to be within ±15 s of the value established during initial calibration. Lab blanks were analyzed to confirm absence of laboratory contamination. Precision between samples and duplicates was expressed as relative percentage difference (RPD), which was calculated using the following expression:

$$RPD\ [\%] = \frac{|C_{sample} - C_{duplicate}| \times 100}{\frac{C_{sample} + C_{duplicate}}{2}}$$

where C_{sample} and $C_{duplicate}$ are the concentration detected in the original sample and in its duplicate, respectively. Matrix spikes were performed for composited samples to evaluate recovery rates. Spikes of analyte into the sample extracts were conducted to confirm all detections via the increases of peak areas at the anticipated retention times.

RESULTS AND DISCUSSION

Data Quality Assurance

Laboratory blanks showed no detections for any of the analytes. Relative recoveries for ERY, NP-AOZ and SUL were 103.4, 77.2, and 68.2%, respectively (**Table 3-1**). Absolute recoveries of the analytes ranged between 12.5 and 40.4% with an average of 29.3% and standard deviations between 0.9 and 6.9% (**Table 3-1**). These recoveries are consistent with the range of absolute recoveries reported in literature for the detection of antibiotics in sewage sludge. Recovery percentages of 21% and 31% have been observed for SUL and OXY, respectively (Shafrir and Avisar, 2012). The 2009 EPA study conducted by AXYS Analytical (Sidney, Canada) reported an acceptable recovery range of 5-200% for some antibiotics in biosolids (EPA, 2009). The EPA acceptable recovery ranges for the five compounds in this study that were also monitored in their study are: ERY 50-158%, oxolinic acid 42-124%, SUL 50-120%, SDD 50-142%, and OXY 50-183%. The lower than ideal (70-130%) recoveries may be explained by any one or a combination of the following reasons: 1) complexity of biosolids matrix, 2) inefficiency

of extraction method, or 3) diversity in analyte structure (**Figure 3-2**). Sample duplicates revealed relative percentage differences (RPD) between 5 and 32%, with seven out of nine analytes having a RPD below 20%. The average RPD for the five detected analytes was 12.4%. These RPDs are similar to reported values of precision for antibiotics previously reported as relative standard deviation in the range of 9 (OXY) to 14% (SDD) (Gago-Ferrero et al., 2015) and under 23% (Garcia-Galan et al., 2013).

Method detections limits (MDLs) ranged from 0.1 ng/g (OXA) to 20.0 ng/g (NP-AOZ) (**Table 3-1**). Published studies report MDLs ranging from low concentrations of 0.02 ng/g (for SUL, Gao et al., 2012b) to high concentrations of 500 ng/g (for OXY, Tang et al., 2012). Our limits are consistent with the ones reported in literature for the detection of antibiotics in sewage sludge.

Occurrence of Antibiotics in Biosolids

Out of the nine antibiotics screened for in this study, five were detected in at least one sample. The majority of samples (83.3%) showed the presence of at least one antibiotic, with 33.3% showing the presence of at least two.

Oxytetracycline, the most frequently detected antibiotic, was found in five samples at concentrations 1.0, 2.7, 3.7, 5.2, and 9.7 ng/g. All concentrations were lower than those reported by the EPA (**Table 3-2**). In fact, two of the five detections were labeled as "non-detects" by the EPA. This study achieved a lower MDL (0.5 ng/g) for oxytetracycline than the ones reported by the EPA of 38.8 and 37.2 ng/g (one for each of the two

samples), suggesting that they may have been non-detects because the concentrations present were lower than the MDLs of the EPA study.

Table 3-1. Method performance and concentrations (ng/g dry weight) of antibiotics in U.S. biosolids.

Targeted Compound	CAS #	Recovery (%)[b]		Method Detection Limit (ng/g)	Mean Biosolids Concentration (ng/g) (min, max)	RPD (%)[c]	Detection Frequency (%)
		Absolute	Relative				
AMP[a]	69-53-4	39.9±2.8		10.0	14.8	5	8.3
ERY	114-07-8	35.9±5.8	103.4±16.9	0.3	0.6 (0.4, 1)	18±19	33.3
NDA[a]	389-08-2	30.4±5.4		9.0	19.1 (9.4, 33.2)	16±3	33.3
NP-AOZ	19687-73-1	26.7±0.9	77.2±2.4	20.0	ND	19±5	-
OXA	14698-29-4	30.3±4.3		0.1	2.7 (0.1, 5.2)	10±10	16.7
OXY	2058-46-0	40.4±6.9		0.5	4.5 (1, 9.7)	13±19	41.7
SPI	8025-81-8	12.5±6.7		2.0	ND	32±19	-
SUL	122-11-2	23.5±2.2	68.2±6.5	0.5	ND	16±9	-
SDD	57-68-1	24.0±4.6		1.0	ND	27±8	-

[a]Concentrations of analytes lacking isotopically-labeled analogs are not recovery-corrected. [b]Relative recoveries were determined using area ratios of analyte to isotopically-labeled analog standards. Absolute recoveries were determined using absolute areas instead of area ratios. [c]RPD: relative percentage difference; was determined as an average of RPDs for each duplicate sample set. RPDs for non-detects were calculated using duplicate matrix spikes. ND= non-detect.

The higher MDLs reported by the EPA may be due to the fact that the present analytical method screened for nine compounds while the EPA method screened for 97 compounds in two ranging from pharmaceuticals to hormones in two analytical methods (EPA, 2009). The EPA did report in two samples at concentrations 57.9 and 64.2 ng/g for which I found non-detect values (< 0.5 ng/g). It is likely that degradation of oxytetracycline occurred during storage, which may explain the low concentrations found and the absence of detections in two of the archived samples. Another explanation for the

different results may be the method of quantitation used. Standard addition was used here in order to account for matrix effects and recovery percentages as well as to positively confirm detections (**Figure 3-3**); however, the EPA study used the isotope dilution method with isotope labeled proxy standards rather than isotope labeled analogs of the target analyte. Oxytetracycline was quantified against thiabendazole-d_6. The effect of using different quantitation methods is discussed in the next chapter.

Just like with oxytetracycline, the four detections of erythromycin in this study, 0.5, 0.4, 1.0, and 0.6 ng/g, were all significantly lower than the concentrations reported by the EPA (39.1, 44.8, 50.2, and 15.9 ng/g, respectively). Their detected concentrations range from 3.1 to 28.3 ng/g. Of the eight samples in this study that did not result in ERY detections, the EPA study reported detections in all but two of them. The four samples that had detections in this study and in the EPA study were among the highest ERY detections, suggesting that non-detects here were most likely due to degradation of target analyte during the prolonged, multi-year storage.

Together, oxytetracycline and erythromycin are among the most frequently screened for and most often detected antibiotics reported in the literature (**Figure 4**), likely because these are popular antibiotics used in human medicine. Erythromycin is often used in common respiratory and skin infections among other diseases (Bpac, 2013; Amsden, 2005). Both are broad spectrum antibiotics for which increasing antibiotic resistance has been reported in the past decades (Alvarez-Elcoro and Enzler, 1999). These data imply that erythromycin and oxytetracyline either do not degrade effectively during wastewater

treatment and instead stay in biosolids, or are used at such high concentrations that WWTPs cannot efficiently remove them, or a combination of both factors.

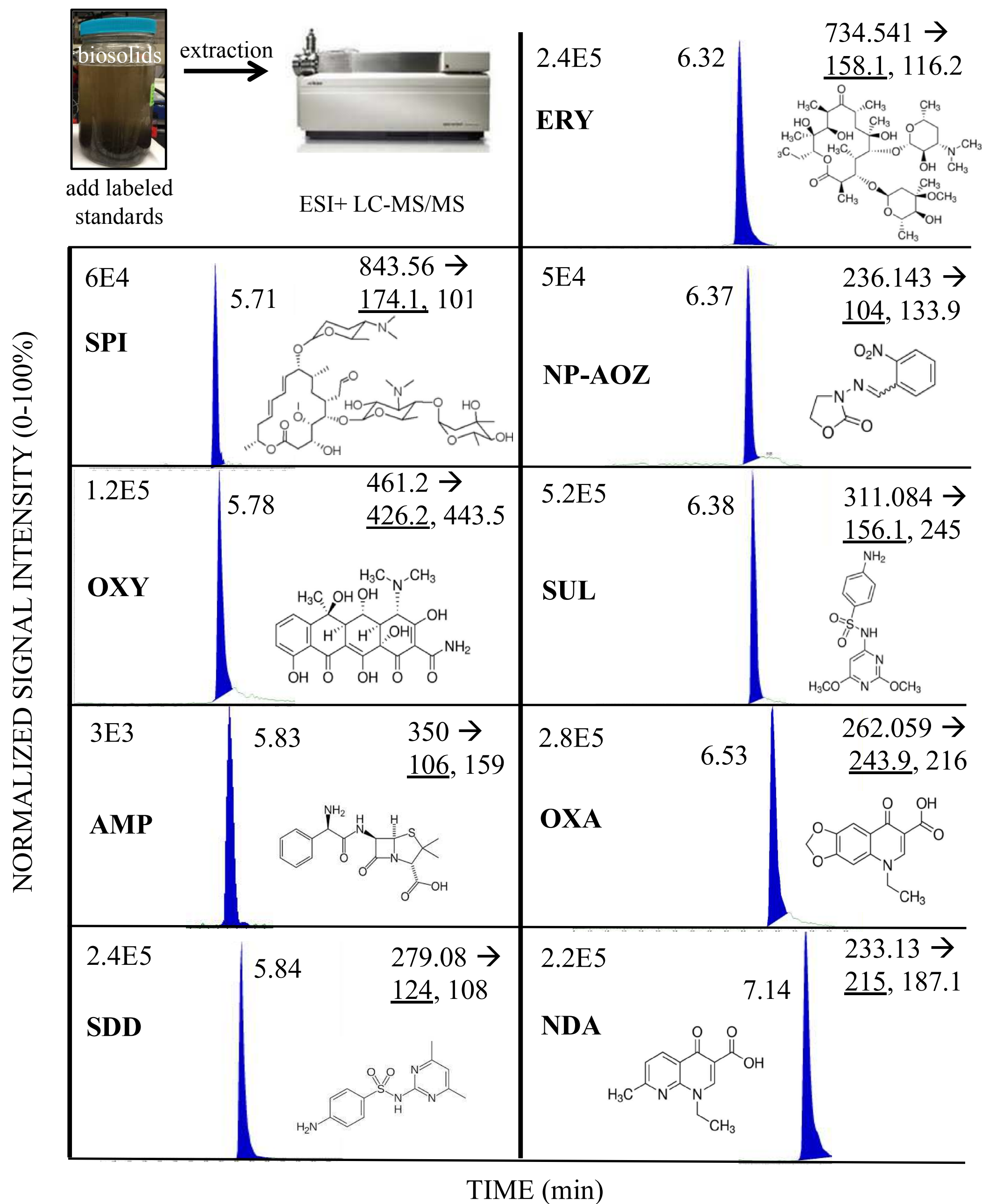


Figure 3-2. Structures, transitions (parent ion *m/z* → <u>quantitation product ion *m/z*</u>, confirmation ion *m/z*), and LC-MS/MS chromatograms. All standards are 10 µg/L standards except for AMP which is 3 µg/L. Number next to y-axis is the intensity. Number next to analyte peak is the retention time.

Table 3-2. Antibiotic detections (ng/g dry weight) in this study and in the previous 2009 EPA screening of erythromycin, oxytetracycline, and oxolinic acid.

<table>
<tr><th></th><th colspan="2">ERY</th><th colspan="2">OXY</th><th colspan="2">OXA</th></tr>
<tr><th>US Region</th><th>This Study</th><th>EPA</th><th>This Study</th><th>EPA</th><th>This Study</th><th>EPA</th></tr>
<tr><td rowspan="4">Northeast</td><td rowspan="2"><0.3</td><td>6.4</td><td rowspan="3"><0.5</td><td><39.1</td><td rowspan="9"><0.1</td><td><3.9</td></tr>
<tr><td>17.9</td><td><40.8</td><td><4.0</td></tr>
<tr><td>1.8</td><td>39.1</td><td>57.9</td><td><3.9</td></tr>
<tr><td>0.8±0.2</td><td>44.8</td><td>9.7±1.8</td><td><38.8</td><td><3.8</td></tr>
<tr><td rowspan="4">West</td><td><0.3</td><td>13</td><td><0.5</td><td>64.2</td><td><4.3</td></tr>
<tr><td>1.2</td><td>50.2</td><td>1±0.1</td><td>87</td><td><5.6</td></tr>
<tr><td rowspan="5"><0.3</td><td>28.3</td><td><0.5</td><td><40.7</td><td><5.6</td></tr>
<tr><td>3.1</td><td>3.7±0.8</td><td>75.5</td><td><3.6</td></tr>
<tr><td rowspan="4">Midwest</td><td>16.4</td><td rowspan="6"><0.5</td><td><41.2</td><td><4.1</td></tr>
<tr><td><1.9</td><td><39.4</td><td>5.2±0.5</td><td><3.9</td></tr>
<tr><td><1.9</td><td><38.5</td><td rowspan="4"><0.1</td><td><3.8</td></tr>
<tr><td>0.9±0.1</td><td>15.9</td><td><40.4</td><td><4.0</td></tr>
<tr><td rowspan="4">South</td><td rowspan="4"><0.3</td><td>3.9</td><td><39.8</td><td><3.6</td></tr>
<tr><td>24</td><td><41.5</td><td><5.4</td></tr>
<tr><td>3.7</td><td>2.7±0.1</td><td><37.2</td><td><3.7</td></tr>
<tr><td>16.2</td><td>5.2</td><td>98.9</td><td>0.1</td><td><3.1</td></tr>
</table>

Concentrations in bold are discussed in the text. Each row presents data for one sample. MDLs are shown as < MDL ng/g if the result is a non-detect. Detections in this study and in the EPA study are matched up by row.

Oxolinic acid was detected in this study in two samples at concentrations of 5.2 and 0.1 ng/g (**Table 2**). The EPA did not report any detections and had MDLs of 3.94 and 3.18 ng/g, respectively. Oxolinic acid could very well have been present in the EPA sample but may have gone undetected due to differences in analytical method detection limits and losses during extraction. I report the first detection of oxolinic acid in biosolids. Oxolinic acid is a quinolone antibiotic that was previously screened for in three other studies (Okuda et al., 2009; McClellan and Halden, 2010; Jia et al., 2011). These had MDLs of 2.9 ±0.5, 0.03, and 5.8 ng/g. The fact that our low detections of 0.1 and 5.2 ng/g

are within the range of these MDLs suggest that these studies may also have had oxolinic acid present in their samples but were unable to detect them.

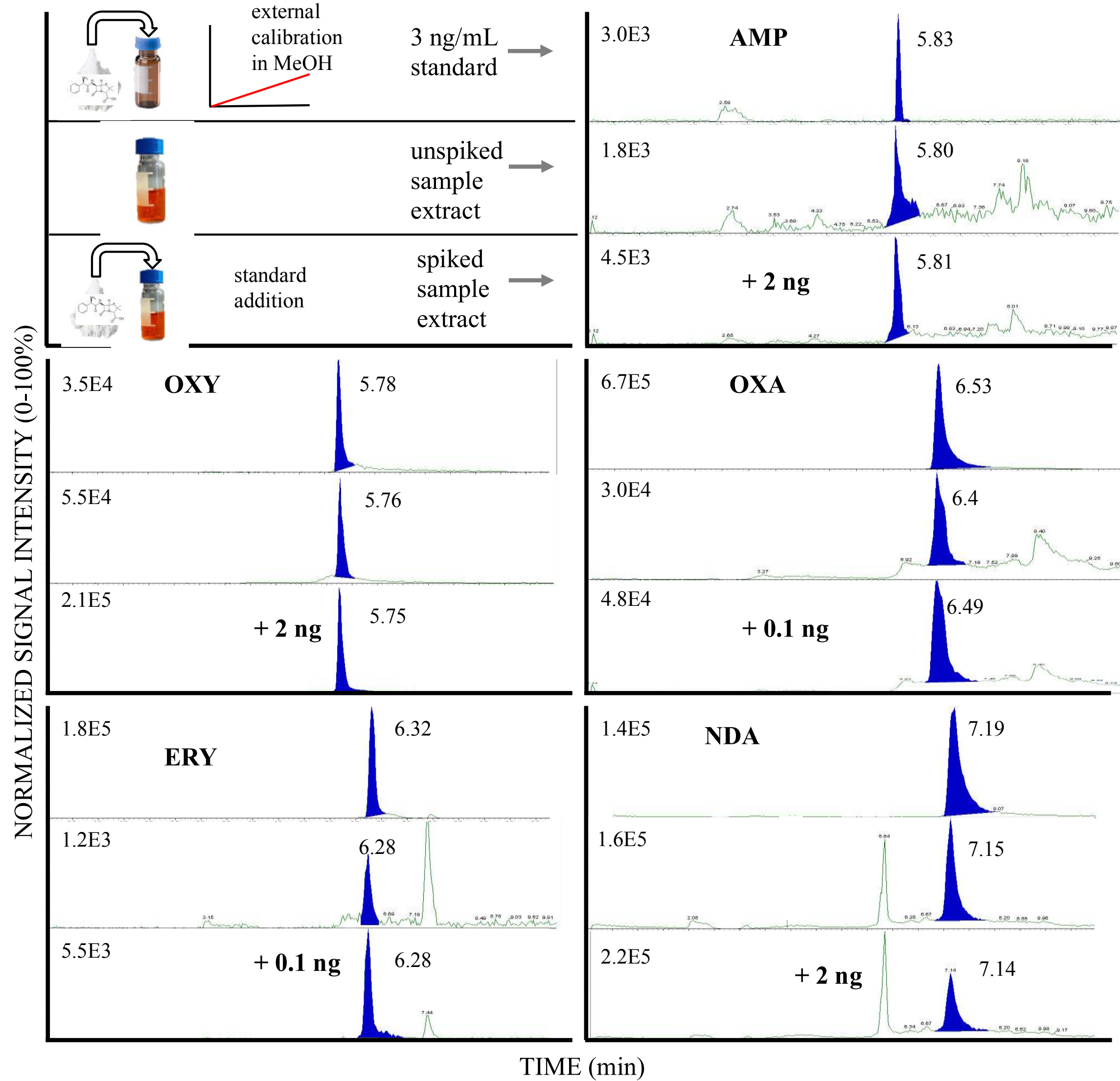
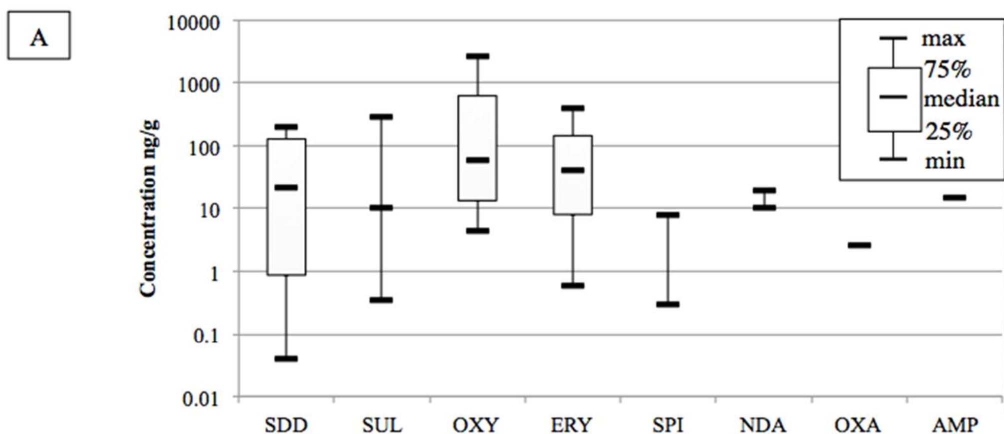


Figure 3-3. LC-MS/MS chromatograms of 3 ng/mL standards, sample extracts, and standard addition spikes to the extract of five detected antibiotics. Number next to peak is the retention time and number next to y-axis is the intensity.



B

	SDD	SUL	OXY	ERY	SPI	NDA	OXA	AMP
Min	0.04	0.34	4.5	0.97	0.3	10	2.7	14.8
Median *(detects only)*	21.2	10	60.0	41	-	-	-	-
Median *(all data*)*	4	1.3	84.0	39	8.46	10	2.9	14.8
Max	200	280	2645	390	7.8	19.1	2.7	14.8
References (*detects*)	*1-4, 7-10, 12, 17*	*3, 11, 15*	*1, 6, 9, 10, 12, 14, 17, 20, 21, 23*	*5-9, 11-13, 17, 23*	*8, 11*	*17, 23*	*23*	*23*
References (*non-detects*)	*5, 6, 20, 21, 23*	*6, 8, 14, 16, 20-23*	*18*	*10*	*23*	*19*	*6, 19, 20*	*20, 22*
	n_d=10 n_n=5	n_d=3 n_n=7	n_d=10 n_n=1	n_d=10 n_n=1	n_d=2 n_n=1	n_d=2 n_n=1	n_d=1 n_n=3	n_d=1 n_n=2

n_d= number of publications with at least 1 detect n_n= number of publications with all non-detects

Figure 3-4. Range of reported concentrations and respective references in published studies. **A)** Reported concentrations of antibiotics detected in biosolids (including this study) presented in log-scale. For studies that reported multiple concentrations, averages were taken. **B)** References for reports that found at least one detection (**References** *detects*) and references that found non-detects in all samples (**References** *non-detects*), as well as minimums, medians, and maximum values for the former in ng/g. Some studies found detections in some samples and non-detects in others. These are listed as "detects". *Median for all reports, including MDL concentrations of non-detects. Furazolidone (NP-AOZ) was also not detected in this study, the first to screen for this analyte in biosolids. This figure excludes concentrations reported by the EPA on the TNSSS.

References: 1. Pamreddy et al., 2013 **2.** Garcia-Rodriguez et al., 2014 **3.** Garcia-Galan et al., 2013 **4.** Nieto et al., 2010 **5.** Tang et al., 2009 **6.** McClellan and Halden, 2010 **7.** Yan et al., 2014 **8.** Li et al., 2013 **9.** Zhou et al., 2013 **10.** Gao et al., 2012a **11.** Gao et al., 2012b **12.** Ding et al., 2012 **13.** Xu et al., 2007 **14.** Shafrir and Avisar, 2012 **15.** Lillenberg et al., 2010 **16.** Lillenberg et al., 2009 **17.** Chen et al., 2013 **18.** Tang et al., 2012 **19.** Jia et al., 2011 **20.** Gago-Ferrero et al., 2015 **21.** Okuda et al., 2009 **22.** Matsuo et al., 2011 **23.** this study.

Nalidixic acid was detected here in four samples ranging from 9.4 to 33.2 ng/g. This is the second detection of nalidixic acid in biosolids, and the first in U.S. biosolids. Only two other papers have screened for the presence of this quinolone in biosolids (Jia et al., 2011; Chen et al., 2013). Only one of them detected nalidixic acid, reporting an average concentration of 10 ng/g in sewage sludge samples from 20 cities in China (Chen et al., 2013) and a detection frequency of 16.7%. Our detection frequency of 33.3% suggests that nalidixic acid is present in U.S. biosolids as well.

The penicillin class of antibiotics, which contains ampicillin screened for here, was among the top prescribed antibiotic classes in 2010 (Hicks et al, 2013). I found one study that previously looked for ampicillin in biosolids (Matsuo et al., 2011). This study reported non-detects for their sludge samples (n=3) that were obtained from one Japanese municipal WWTP. The detection of ampicillin in this study is the first report of its presence in U.S. biosolids at (14.8 ng/g) dw.

Sulfadimidine, spiramycin, NP-AOZ, and sulfadimethoxine were not detected in this study. The EPA study also did not detect any sulfadimidine or sulfadimethoxine residues in these samples. It is surprising that these two sulfonamide drugs were not detected, as several reports in the published literature reported detections ranging from a 0.04-200 ng/g for sulfadimidine and 0.34-280 ng/g for sulfadimethoxine (see **Figure 3-4** for references).

Many of the antibiotics most likely degraded during storage and their levels dropped below MDLs. As most biosolids are stored in storage tanks for days to months before land applications (Wu et al., 2008) the chemical interactions between pharmaceuticals like antibiotics with other biosolids components and external factors such as temperature and oxygen content can greatly affect antibiotic stability. Few other studies in literature show experimentally-derived data regarding antibiotics and the factors affecting their degradation patterns in biosolids (and soils); however, reported experiments suggest that several factors contribute to the degradation rate of antibiotics, some relevant to this study (temperature, storage time), and some more relevant to the land application of biosolids (mixture ratio with soil, soil type, biosolids type). Half-lives of antibiotics can vary from days to years (Monteiro et al., 2009; Walters et al., 2010) even for pharmaceuticals within the same therapeutic class (Schlusener and Bester, 2006). A recent study published experimentally-determined half-lives of select antibiotics in outdoor biosolids-amended soil mesocosms (Walters et al., 2010). Although our detected analytes were not included in the half-life calculations, other antibiotics in the same classes can be noted here. For tetracyclines, quinolones, and macrolides, the ranges were, respectively, 55-630, 866-3466, and 360-770 days. These data in literature indicate that degradation patterns vary greatly and the non-detects as well as detected concentrations are a result of many different factors. Thus, it must be a research priority to determine what factors lead to quicker degradation of biologically active pharmaceuticals so land application of biosolids can be made safer.

This study also employed raw extracts for direct injection into the LC-MS/MS, which is not a common technique. This was done because efforts to treat the extract prior to injection (SPE, filtration) did not yield better results and so were forsaken to save time. Previous studies have reported that SPE may not always be necessary. Large volume injection (LVI) constitutes the direct injection of a large sample volume into a high-performance LC column with only minimal sample pre-treatment, such as centrifugation (Chiala et al., 2008; Backe and Field, 2012). Although this technique injects more volume (between 100-5000 µL) than the amount injected in this study (50 µL), the same concepts can still be applied. Past studies have reported that LVI involves minimal sample handling, an increase in sensitivity and accuracy (sometimes; due to negligible loss of target analyte). LVI has not been very commonly used but may prove to be an alternative to SPE-based methods. Here we show that analysis of extracts that have only been centrifuged and frozen prior to injection may also be an alternative to SPE-based methods. Depending on the analyte and matrix, direct injection of the extract may be better than or produce similar results as SPE-based preparation methods.

Human health risks associated with the detection of antibiotics in biosolids largely revolve around antibiotic resistance development. Studies looking at the risk of coming into contact with bacteria containing resistance genes suggest that the land application of biosolids is a potential route of exposure to pathogenic bacteria that are under selective pressure to become resistant (Rahube et al., 2014; Burch et al., 2014). The mix of many different kinds of bacteria, antibiotics, metals, and other antimicrobials such as triclosan increase the risk for co- and cross-resistance to develop in biosolids (Flores and Jay,

2014; Carey and McNamara, 2015). As many antibiotics and antibiotic-resistant bacteria can survive WWTP processes (Uyaguari et al., 2011), it is important to monitor the presence of antibiotics destined for land application to reduce the potential contact of resistant genes with human pathogens.

Biosolids for land application are not regulated for the presence of antibiotics in the U.S. In fact, the only two things that are regulated are microbes (pathogen load) and ten heavy metals (EPA Part 503). In addition to these set maximum concentrations, biosolids must also meet site restrictions depending on the purpose of the land amendment (e.g., parks, agricultural, home gardens). The data that this study and other published papers contribute indicate that other non-biological and non-metal pollutants are extant in biosolids that also merit consideration for better monitoring and potential regulation. The biological activity that is retained in many of the antibiotics in biosolids poses potential dangers to ecosystems that may be affected by small concentrations (e.g., sub-lethal/non-lethal) of these compounds (Andersson and Hughes, 2012). As mentioned above, antibiotic resistance is a key issue, with recent data showing that antibiotics can shape the multi-level population biology of bacteria as well (Baquero et al., 2013). In view of such emerging information, a more detailed assessment of risks posed by antibiotic residues in biosolids is warranted.

Study Limitations

The prolonged storage of samples (8-9 years) between sampling event and analysis most likely affected the chemical structures and thus allowed for transformation of certain

analytes to occur; however, previous works have been published that took advantage of available archived biosolids (Hale et al., 2012; Xue et al., 2015). The results of this study should be viewed as conservative estimates of actual concentrations. Low recoveries were also seen for all analytes, meaning that detected concentrations are most likely underestimates of the true values. As only 12 samples were analyzed, four from each of the four regions, samples should not be seen as representative of the entire repository nor the region.

CONCLUSIONS

In this study, I screened for nine medically important antibiotics in 12 samples, four from each of the four U.S. regions geographically delineated by the EPA 2009 Targeted National Sewage Sludge Survey. Four of these analytes have never been screened for in U.S. biosolids. This study reports the first detections of oxolinic acid and ampicillin in biosolids, and the first for nalidixic acid in biosolids from the U.S. Out of the five compounds that were screened for previously by the EPA, three were found at much lower concentrations, suggesting that degradation of antibiotics occurred during storage. Different quantitation methods were also used in this study, which may also have led to different concentrations reported for the same analytes in the same samples. Compared to the EPA study, the present study had superior (i.e., lower) MDLs. Regardless, the presence of two newly-detected antibiotics and the detection of three others in archived U.S. biosolids shows that antibiotics are present and may negatively impact human and environmental health. The extent of this problem and the magnitude of risk ought to be

subject of additional research and potentially may lead to the conclusion that current regulations are inadequate to properly protect ecosystems and human populations.

TRANSITION THREE

The detection of antibiotics in seafood and biosolids required the usage of liquid chromatography tandem mass spectrometry (LC-MS/MS), a methodology currently representing the gold standard of analytical tools for the identification and quantitation of small amounts of organic contaminants in complex sample matrices; however several factors can affect the accurate analysis of many chemicals such as antibiotics. One major factor is the quantitation method used. In Chapter 4, four different quantitation methods are used to explore the impact of the quantitation method used and a literature analysis is conducted to determine choice of analytical method trends. As some methods are more susceptible to interferences such as matrix effects, this chapter aims to see what differences, if any, can be seen in using four popular analytical methods: isotope dilution with heavy-labeled analogs, isotope dilution with heavy-labeled nonanalogs, external dilution, and standard addition.

CHAPTER FOUR. LITERATURE META-ANALYSIS AND EXPERIMENTAL COMPARISON OF FOUR DIFFERENT ANALYSIS STRATEGIES FOR LC-MS/MS QUANTIFICATION OF ANTIBIOTIC RESIDUES IN BIOSOLIDS

ABSTRACT

This study explored the impact of using four different calibration methods on the quantitation of antibiotics in nationwide biosolids by liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Ten previously analyzed samples showing detections of antibiotics (Chapter 3) were scrutinized as to the impact of using the following quantitation approaches: (i) external calibration; (ii) isotope dilution method with proxy compounds rather than true structural analogs; (iii) isotope dilution with structurally identically analog standards; and (iv) the method of standard addition. Results showed that the use of different calibration and quantitation techniques impacted the studied analytes in different ways. Concentrations obtained from quantitation of erythromycin using an isotopically-labeled analog were statistically different from those obtained using external calibration or standard addition (p<0.05). However, concentrations obtained for oxytetracycline using the method of standard addition were statistically indistinguishable from those obtained using external calibration (p=0.13) although using three non-analogous isotopically-labeled standards, ERY-$^{13}C_2$, NP-AOZ-d_4, and SUL-$^{13}C_6$ did produce differing results (p<0.05). Matrix effects were also quantified for spiramycin, NP-AOZ, and sulfadimethoxine using composite samples from four U.S. regions. Ion enhancement was as high as 734% (spiramycin) and ion suppression reduced signal intensity in organic extracts of biosolids by as much as 88%

(NP-AOZ). MDLs obtained for the analytes also showed great variation depending on the quantitation method used, with the presumed accurate method utilized in Chapter 3 generally being lower than the rest. This study shows that biosolids are a very complex matrix that can enhance or suppress ion signal (range of 12-734% of signal) and that in the absence of isotopically-labeled analogs the most accurate alternate quantitation method may need to be experimentally determined depending on the analyte. Analysis of published literature (*n*=61) indicated that isotope dilution (with non-analogous and analogous standards) is more commonly used than standard addition and external calibration, although standard addition usage has increased in recent years. Future studies should report with more detail their exact quantitation method and justify their choice of quantitation method.

INTRODUCTION

Liquid chromatography mass spectrometry (LC-MS) and tandem mass spectrometry (LC-MS/MS) are being applied extensively in environmental monitoring for their applicable analyte spectrum, speed, sensitivity, accuracy, precision, and suitability for high-throughput analysis of emerging pollutants in complex environmental matrices (Richardson, 2011). Many organic emerging contaminants, such as pharmaceuticals and personal care products (PPCPs), have been identified and quantified using LC-MS and more recently LC-MS/MS (quadrupole or time-of-flight) technology. Although tandem mass spectrometry allows for the exclusion of many unwanted interferences in quadrupole one and observation of characteristic transformation products as identifiers,

matrix components present in environmental samples are known to interfere with both the identification and quantitation of analytes, especially when electrospray ionization (ESI) is used (Raji and Schug, 2008; Garcia-Rodriguez, 2014; Hao et al., 2007). Co-eluting compounds and mobile phase additives can also introduce interferences that suppress or enhance the analyte signal (Gomes et al., 2004). Whereas the exact mechanisms of ion suppression and enhancement are still under investigation, studies have shown that these matrix-induced phenomena can affect the performance of LC-MS/MS (Zhang et al., 2011).

Methods have been created in recent years to increase the sensitivity of LC-MS/MS and to decrease the potential impact of interferences. Ways to reduce interferences include extraction of target analyte and cleanup procedures as well as eluent additives (i.e., formic acid) to increase ionization of wanted analytes (Gomes et al., 2004). Extraction procedures vary greatly, with examples in the literature including ultrasound-assisted extraction (Yu and Wu, 2012), microwave-assisted extraction (Azzouz and Ballesteros, 2012), pressurized liquid extraction (Pamreddy et al., 2013), and solid-phase (micro)extraction (Gao et al., 2012; Zhang et al., 2011b) to name just a few.

To compensate for analyte loss during extraction and MS analysis, isotopically-labeled analogs of the native compounds are often used as internal standards using the so-called isotope dilution method (Pedrouzo et al., 2011; Cappiello et al., 2008). These surrogates are chemically the same as the target analyte with the exception that certain atoms featured an increased mass (i.e., ^{2}H (deuterium) vs. ^{1}H or ^{13}C vs. ^{12}C), implying that they

will behave the same as the native analytes of interest during pre-MS treatment but will be differentiated during MS by their specific mass differences (Hernandez et al., 2005). The ratio of native-to-surrogate concentrations is preserved throughout extraction and analysis, thus the original native analyte concentration can be calculated if a known surrogate mass is spiked initially and recovered only partially (<100% absolute recovery) (Halden and Paull, 2004). Adjusting analytical results for incomplete surrogate recovery enables reporting of relative recoveries that are normalized for losses occurring during sample workup and analysis; however, isotopically-labeled surrogates are not available for every analyte of interest (Hernandez et al., 2005). Even when available they can be very expensive, from a few hundred to several thousands of dollar for a few milligrams, depending on whether they are off-the-shelf products or custom synthesized in a small batch. When isotopically-labeled surrogates are unavailable, the analyst often selects from the following choices: (i) use an isotopically-labeled analog of a compound that is non-identical but similar to the target analyte of interest (Tang et al., 2009); (ii) use no surrogate standard but perform the method of standard addition to account for non-ideal chemical behavior during analysis (Lillenberg et al., 2009); or (iii) use external calibration and forego calculation of relative recoveries, arriving at quantitative estimates that frequently are considered as "lower bounds" of the true concentration (Pamreddy et al., 2013). The first method is, but not identical to, isotope dilution using analog standards; both require surrogate standard addition prior to analyte extraction so any losses taking place over the entire extraction process can be taken into account at the end. The second requires the spiking of chemically known increasing amounts of identical native analyte into the final extract (or spiked in at the beginning) just prior to injection

into the LC-MS/MS, so a calibration curve can be created for each individual sample using the known, spiked amounts. The third dilutes the chemically identical target analyte in a solvent (i.e., MeOH) to create an external calibration curve.

Few studies have explored systematically how different calibration and quantitation approaches impact the quality and range of analytical results. To our knowledge, only one study analyzed how standard addition, external calibration, and internal isotopically-labeled standards can affect quantitation results (vom Eyser et al., 2015). This study quantified 12 pharmaceuticals in biochar and biosolids using these quantitation methods and found that using standard addition prior to the entire extraction procedure yielded the best recovery rates by compensating all losses and matrix effects. Another recent study compared 52 analytical methods used to measure contaminants of concern in water (Vanderford et al., 2014). Results from 25 research and commercial laboratories using various MS instruments (GC and LC-MS) showed that LC-MS/MS coupled with isotope dilution most accurately quantified the majority of the compounds, including an antibiotic also quantified here, erythromycin. However, the purpose of this study was not to look at analysis methods, but rather, instrument analytical methods. A third study examined how five different calibration approaches affected results for quantifying proteins (Nouri-Nigjeh et al., 2014). Although the calibration approaches are not comparable to ones here, as different methods are required for the analysis of proteolytic peptides, the goal of the study was the same. The study reported that different results were obtained from the different methods even though the same plasma samples were used.

In this paper, we employed four quantitation methods for the analysis of nine antibiotics in 12 samples from the U.S. Environmental Protection Agency's (EPA) 2006/2007 National Targeted Sewage Sludge Survey. The four quantitation methods examined included: standard addition (immediately prior to LC-MS/MS), external calibration, isotope dilution with a heavy-labeled analog of the native analyte, and isotope dilution with a non-analog of the native analyte. To our knowledge, this study is the first to compare the isotope dilution method using a surrogate non-analog standard with the three other quantitation methods. This method was included because it has been commonly used in literature (Evans et al., 2015; Dorival-Garcia et al., 2015). The goal of the work was to look for trends in quantitation method accuracy and analyze whether certain methods were better performing than others.

MATERIALS AND METHODS

Materials

All materials, extraction methods, and LC-MS/MS procedures were previously described in Chapter 3. The results of detecting five antibiotics in ten biosolids samples were used for the analyses here.

Isotope Dilution Method Quantitation

The isotope dilution method was conducted as follows: 100 ng of heavy-labeled standards (SUL-$^{13}C_6$, ERY-$^{13}C_2$, NP-AOZ-d_4) were spiked prior to extraction in each sample. The equivalent amount was added to the external calibration standards. The ratio

of area under the curve (signal responses) of the native analyte: isotopically-labeled analog was used to create a calibration curve from which the native analyte concentration was estimated according to the equation below:

$$area\ ratio = \frac{area\ under\ signal\ curve\ of\ native\ analyte}{area\ under\ signal\ curve\ of\ isotopically{-}labeled\ analog} \qquad \text{(Eq. 1)}$$

Isotope dilution method using non-analog standards was conducted as noted above, except that the ratio used was the native analyte: non-analog isotopically-labeled standard signal areas.

$$area\ ratio = \frac{area\ under\ signal\ curve\ of\ native\ analyte}{area\ under\ signal\ curve\ of\ isotopically{-}labeled\ non{-}analog} \qquad \text{(Eq. 2)}$$

Standard Addition Quantitation

Standard addition quantitation was conducted as follows: with obtained extracts of the samples, increasing amounts of native analyte were added immediately prior to injection into the mass spectrometer. Five additional concentrations using the same extract were created (concentrations 0.2, 0.4 1, 2, and 4 µg/L if in clean matrix). The unspiked extract and these five additional concentrations were run through the mass spectrometer and the obtained six signal responses together created a sample-specific curve (Tusiimire et al., 2015). Detections resulted in the following equation where a positive signal response (or area count) was yielded for x=0:

$$y = mx + b \qquad \text{(Eq. 3)}$$

where *y* is the signal of area under response curve, *m* is the slope, *x* is the concentration, and *b* is the y-intercept (i.e., the signal when the spiked mass of analyte is zero).

This area count is corresponding to the signal response of the unknown mass of antibiotic present in the extract prior to spiking. To calculate the corresponding concentration, the absolute value of the x-intercept was used as the corrected, estimated concentration of the analyte present in the sample prior to spiking. Thus, obtained slopes and abscissa of each standard addition equation were used to find the unknown concentration. This process was repeated for each sample and each analyte investigated.

External Calibration Quantitation

External calibration was conducted as follows: analytes dissolved in MeOH of increasing amounts were used to create a linear curve that was then used to estimate analyte concentrations in samples. No further corrections were made for sample matrix effects on ionization or extraction losses during sample processing.

Signal Response Quantitation

Matrix effects were also calculated by obtaining the signal response (SR) using the following equation (Rodriguez-Alvarez, et al., 2014):

$$signal\ response\ (\%) = \frac{response\ of\ spiked\ sample}{response\ of\ standard} \times 100 \qquad \text{(Eq. 4)}$$

Where the response of spiked sample equaled the area under the signal curve of analytes in samples that were spiked with 100 ng of native compound prior to extraction, and response of unspiked samples equaled the area under the signal curve of analytes in samples that did not have spiked antibiotics prior to extraction. The response of standard equaled the area under the signal curve of analytes in MeOH (standards). A SR of 100% indicates a lack of matrix effects; an SR% <100% indicates signal suppression, whereas an SR% > 100% indicates signal enhancement.

Statistical Analysis of Data Sets (t-Test)

The Student's t-test was used to analyze whether there was a difference between reported values in Chapter 3 and the values obtained in this study using different quantitation methods. The data was assumed to be normally distributed. The α was set at 0.05 and a two-tailed, paired t-test was run between reported erythromycin, oxytetracycline, and nalidixic acid results. Duplicates of each sample were factored into the analysis except for two samples with detected nalidixic acid concentrations that did not have a duplicate. T-test calculations comparing erythromycin concentrations obtained using external calibration were calculated in two ways. The first way used only four values (from two samples) as two values resulted in non-detects (see Chapter 3). The second used all eight values and inputted the non-detects as the $MDL/\sqrt{2}$. Thus, for these t-tests with external calibration of erythromycin, two p-values are reported.

Method Detection Limits Calculations

Method detection limits (MDL) for all detected antibiotics were calculated using results from composite samples where composites of each of the four U.S. regions were used. This was done because the original MDL calculations were conducted using composite samples, so the same values were used here in order to compare them. Each of the quantitation methods were applied to analyte peak areas used for MDL quantitation in Chapter 3 and reported here.

Quality Assurance

See Chapter 3 for full details on quality assurance regarding obtained signals for areas under the curves. For all new calculations reported in this study, duplicate sample results were used and the average was reported with the distance between the average and the min/max also being reported.

Meta-Analysis of the Published Literature

A literature search was conducted using Web of Science for years 2000-2015 to analyze quantitation methods used in LC-MS/MS publications. The search terms "liquid chromatography mass spectrometry pharmaceuticals" paired with "biosolids" and then paired with "sewage sludge". The resulting abstracts were individually screened. Experiments using soil as biosolids, involving spiking in analytes just for method development, and drugs of abuse analyte papers were excluded. Papers using diode array detectors were also excluded. Educated guesses were made when possible (e.g., if the paper said "internal standards were used" and listed labeled analogs and non-analogs in

the materials section, it was presumed that isotope dilution with both kinds of standards was used). In cases of extreme uncertainty regarding the method utilized, the author was contacted and if no response was received, the paper was excluded. A total of 61 papers were analyzed for standard addition (see Appendix C for complete list of analyzed references), external calibration, isotope dilution with analogous standards, and isotope dilution with non-analogous standards. Papers that used multiple methods were included in the total count of each of those methods.

RESULTS AND DISCUSSION

Quantitation Of Antibiotics In Biosolids Using Different Methods

A Student's *t*-test was conducted for three antibiotics, erythromycin, oxytetracycline, and nalidixic acid, to compare four quantitation methods (**Table 4-1**). For discussion purposes, it is being assumed that for erythromycin, the accurate concentrations are the ones reported using isotope dilution with ERY-$^{13}C_2$. For all others, it is being assumed that standard addition concentrations are the most accurate.

When excluding the non-detects that resulted in external calibration of erythromycin, the concentrations quantitated using ERY-$^{13}C_2$ differed from the concentrations quantitated using external calibration (p=0.04), NP-AOZ-d_4 (p=0.04), and standard addition (p=0.02) but did not differ from values obtained using SUL-$^{13}C_6$ (p=0.13) (**Table 4-2**). T-tests were also run amongst the newly calculated concentrations using isotope dilution and the results indicate that they were not different from each other. Interestingly, external

calibration results were different than standard addition results (p=0.04). However, when using the p-values obtained by using the concentration of MDL/√2 for the non-detected concentrations, results indicated that external calibration concentrations were different than concentrations obtained from all other quantitation methods (p=<0.00). This second method of calculation also concluded that external calibration produced different results than using erythromycin's analogous standard of ERY-$^{13}C_2$.

The p-values calculated from these results demonstrate that isotope dilution using ERY-$^{13}C_2$, standard addition, and external calibration are all different from each other. It cannot be concluded whether one quantitation method is better than another one; that is not the purpose of a t-test. However, if isotope dilution using ERY-$^{13}C_2$ is considered the accurate method for comparison purposes here, based on the obtained p-values in Table 4-2, it appears that using external calibration and standard addition produced different, and perhaps less accurate, results. Using isotope dilution with NP-AOZ-d_4 did not change the results for this compound but using isotope dilution with SUL-$^{13}C_6$ did.

For oxytetracycline, it appears that all quantitation methods yielded statistically different results with the exception of two pairings. External calibration and standard addition results did not differ from each other (p=0.13) and ERY-$^{13}C_2$ and NP-AOZ-d_4 isotope dilution methods also did not differ from each other (p=0.21). These results show the differences that can be achieved from using different quantitation methods. If presuming standard addition concentrations as the accurate concentrations, results show that external

calibration is the only one that did not give statistically different concentrations, and, thus, is the most similar.

Table 4-1. Concentrations (ng/g dw) of antibiotics detected in biosolids samples quantified using different quantitation methods.

	Sample	External Calibration	[a]ERY-$^{13}C_2$	[a]NP-AOZ-d_4	[a]SUL-$^{13}C_6$	Standard Addition
ERY	NE 3	0.3±0.0	**0.5±0.1**	1.0±0.3	1.2±0.3	0.2±0.0
	NE 4	ND/0.07[#]	**0.4±0.1**	0.4±0.1	0.5±0.1	1.8±0.3
	W 2	0.1±0.0	**1.0±0.0**	1.1±0.1	0.4±0.0	2.5±0.1
	MW 4	ND/0.07[#]	**0.6±0.1**	1.4±0.2	1.3±0.2	1.2±0.1
OXY	NE 4	6.7±1.6	24.1±3.0	25±3.0	30.7±0.8	**9.7±1.8**
	W 4	11.8±3.1	110.4±8.0	28.9±4.6	545.6±110.2	**3.7±0.8**
	W 2	1.5±0.1	10.4±0.8	10.3±0.6	10.3±0.5	**1.0±0.1**
	S 3	5.7±0.5	13.7±0.6	16.2±0.3	358.4±16.9	**2.7±0.1**
	S 4	7.1±0.2	27.6±0.1	30.9±0.5	402.1±5.9	**5.2±0.0**
NDA	MW 1	1.4±0.1	9.5±0.3	11.3±0.0	7.4±0.1	**9.4±0.6**
	W 4*	13.6	1390.7	58.3	102.4	**33.2**
	NE 4	2.3±0.4	106.1±42.5	22.5±3.5	11.3±2.1	**18.8±2.6**
	NE 2*	1.9	8.7	18.9	10.6	**15**
AMP	S 4	16.4±0.3	46.1±1.8	14.8±4.4	667±45	**14.8±0.4**
OXA	MW 3	1.4±0.1	74.1±4.6	3.6±0.8	5.1±0.9	**5.2±0.5**
	S 4	0.05±0.0	12.6±0.8	0.8±0.1	0.5±0.0	**0.1±0.0**

Names indicate sample region origin. In bold are the concentrations reported in Ch 3. Averages of duplicate extractions are shown with ± as the distance between it and the min/max. Values of ±0.0 resulted after rounding. [a]Standards for isotope dilution method. *Only a single sample was extracted. [#]Second value indicates result of MDL/√2.

Table 4-2. P-values for comparing erythromycin, oxytetracycline, and nalidixic detections using the different quantitation methods.

ERYTHRO-MYCIN*		External Calibration	Isotope Dilution			Standard Addition
			ERY-$^{13}C_2$	NP-AOZ-d_4	SUL-$^{13}C_6$	
External Calibration			0.04/0.00	0.11/0.00	0.11/0.00	0.04/0.00
Isotope Dilution	ERY-$^{13}C_2$			0.04	0.13	0.02
	NP-AOZ-d_4				0.58	0.27
	SUL-$^{13}C_6$					0.15

OXYTETRA-CYCLINE		External Calibration	Isotope Dilution			Standard Addition
			ERY-$^{13}C_2$	NP-AOZ-d_4	SUL-$^{13}C_6$	
External Calibration			0.03	0.00	0.01	0.13
Isotope Dilution	ERY-$^{13}C_2$			0.21	0.01	0.03
	NP-AOZ-d_4				0.01	0.00
	SUL-$^{13}C_6$					0.01

NALIDIXIC ACID		External Calibration	Isotope Dilution			Standard Addition
			ERY-$^{13}C_2$	NP-AOZ-d_4	SUL-$^{13}C_6$	
External Calibration			0.28	0.01	0.18	0.00
Isotope Dilution	ERY-$^{13}C_2$			0.31	0.29	0.30
	NP-AOZ-d_4				0.92	0.13
	SUL-$^{13}C_6$					0.56

Red highlights indicate quantitation methods used in Chapter 3 that are assumed to yield the most reliable estimate of the true value. *P-values calculated for comparing external calibration with the other methods were calculated in two different ways (see Methods). The first p-value represents the answer calculated when four of the eight concentrations (two for each sample, as there were duplicates) calculated using external calibration for erythromycin were not included in the t-test because these concentrations resulted in non-detects. The second p-value represents the answer calculated when these four concentrations were calculated as the MDL/$\sqrt{2}$.

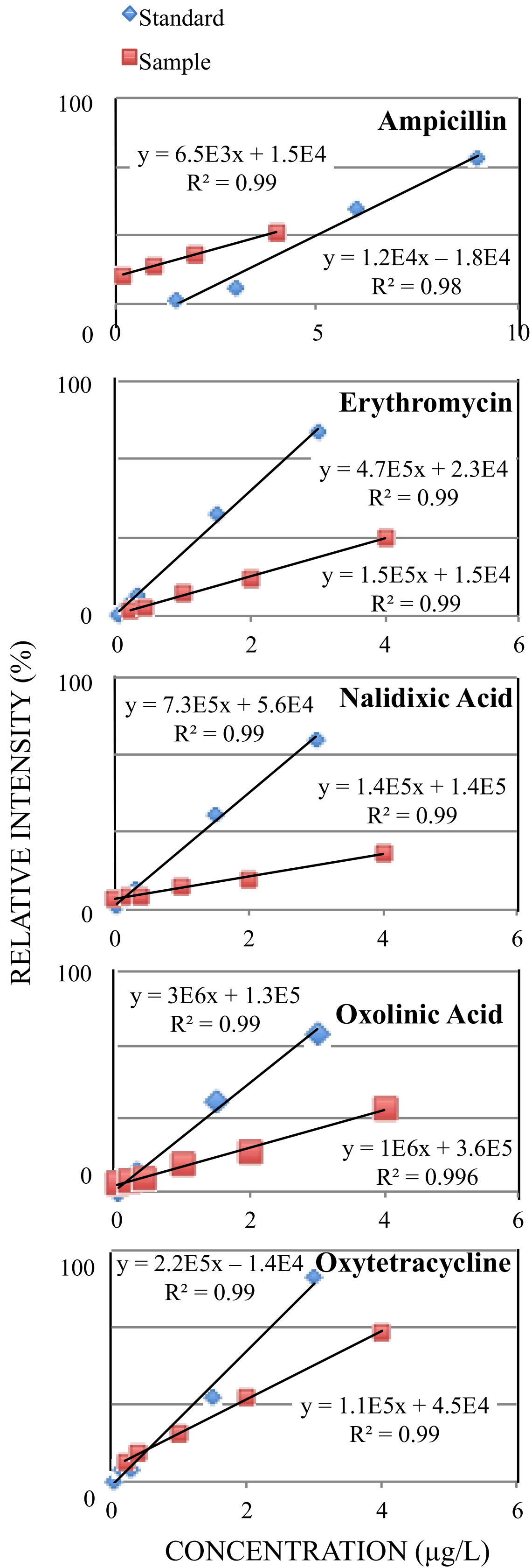
Nalidixic acid comparisons showed that most pairings were statistically similar, with the exception of two pairings. Standard addition and external calibration yielded differing results ($p<0.01$) and external calibration and isotope dilution using NP-AOZ-d_4 yielded differing results ($p=0.01$). For this compound, it appears that using a non-analog isotope as a standard universally did not statistically change the concentrations calculated, but using standard addition did. Based on these numbers alone, it may be concluded that using non-analog isotopes resulted in the most accurate data for nalidixic acid quantitation in biosolids.

The MDLs for each of the five detected antibiotics in Chapter 3 were also re-determined by applying the different quantitation methods to the peak areas obtained for the five detected analytes. The numbers reported in **Table 4-3** show the large range of MDLs obtained for each compound. It is important to note that these values should only be compared to each other as they were determined using composite samples and thus should not be applied to Table 4-1, which lists concentrations of individual values. As expected, using different quantitation methods with the same signal of the area under the curve will yield different results for each compound. Results for the four compounds that originally used standard addition for quantitation showed a very high range of MDLs. For example, nalidixic acid, with a reported MDL of 9.0 ng/g in Chapter 3, now has a range

of 1.0-321.1 ng/g. Ampicillin, with a reported MDL of 10.0 ng/g, now has a range of 10-90.2 ng/g. In general, the MDL obtained using the assumed accurate method is either in the middle (i.e., erythromycin and nalidixic and oxolinic acids) or on the lower end of the range of concentrations (e.g., oxytetracycline, ampicillin). This suggests that the "accurate" methods may also have the lower MDLs.

Table 4-3. MDLs for composite samples in ng/g dw determined from different quantitation methods. MDLs in red indicate the presumed accurate value determined in Chapter 3.

Analyte	External Calibration	Isotope Dilution with Standard as:			Standard Addition
		ERY-$^{13}C_2$	NP-AOZ-d_4	SUL-$^{13}C_6$	
Erythromycin	0.1	**0.3**	0.6	0.3	0.2
Oxytetracycline	3.4	181.1	13.5	12.6	**0.5**
Nalidixic Acid	1.0	321.1	12.8	6.4	**9.0**
Oxolinic Acid	0.1	13.6	0.5	0.04	**0.1**
Ampicillin	11.5	90.2	39.7	36.9	**10.0**

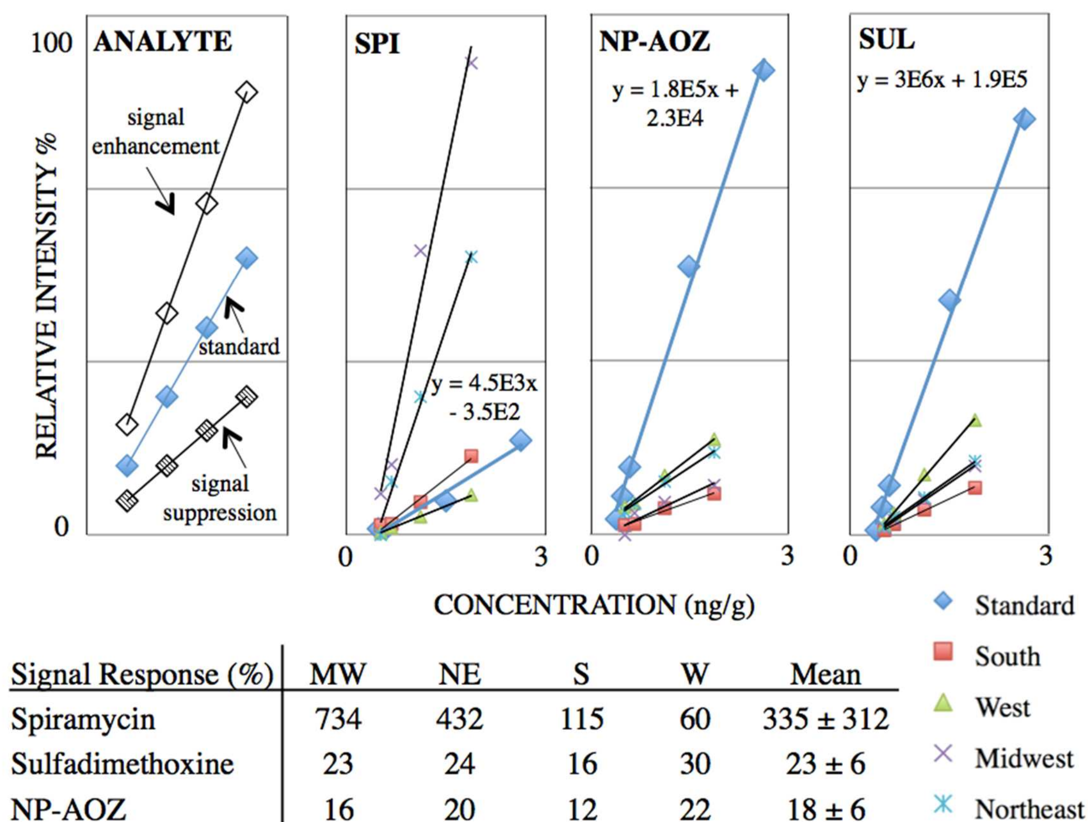


Matrix Effects: Ion Enhancement and Suppression

For all five detected antibiotics (ampicillin, erythromycin, nalidixic acid, oxolinic acid, and oxytetracycline) the calibration curves obtained with standard addition and external calibration were different (**Figure 4-1**). This likely is due to ion suppression/enhancement from interferences present in the extracted matrix. It must be noted here that the raw extract was used in these analyses without the aid of a clean-up method. This was done because undesirably low recoveries resulted (see Chapter 3) when solid-phase

Figure 4-1. Calibration curves resulting from the use of standard addition method (sample) and external calibration (standard) for individual samples. *Ampicillin has a different x-axis because the standard curve had a higher linear range.

Extraction (SPE) was utilized as a cleanup step. The use of raw extract still resulted in low recoveries, but these were much higher (20-40%) and produced more repeatable results than when SPE was utilized. As reliable recoveries and precision were still achieved, the raw extract was used for further analyses.



Signal Response (%)	MW	NE	S	W	Mean
Spiramycin	734	432	115	60	335 ± 312
Sulfadimethoxine	23	24	16	30	23 ± 6
NP-AOZ	16	20	12	22	18 ± 6

Figure 4-2. Matrix effects and standard addition curves compared to standard curves. (Top) Spiramycin, NP-AOZ, and sulfadimethoxine standard addition curves of composited samples from the four U.S. regions compared to the external calibration standard (blue diamonds). Equations belong to the standard curve. First panel on left shows that analyte curves with steeper slopes than the standard indicate signal enhancement. Analyte curves with less steep slopes indicate signal suppression. (Bottom) Signal response was calculated (see Materials and Methods) for each composite sample and analyte. Responses above 100% indicate signal enhancement. Responses below 100% indicate signal suppression.

But because raw extracts were used, there are presumably a large number of interfering compounds in our extract that could have caused ion suppression and enhancement. ESI is the preferred ionization mode as it is universally applicable for polar compounds and

can be applied to the ionization of many different kinds of analyte (Hernandez et al., 2005). The major drawback of ESI is its susceptibility to unwanted influences from the sample matrix (Stahnke et al., 2012) which likely were abundant in our raw extracts. This probably is a factor contributing to the differences in calibration curves.

A common discrepancy is that the slopes feature 2-5 fold differences in magnitude. Slopes steeper than the external calibration curve, which can be assumed as the "ideal" (with no interferences), are ones showing signal or ion enhancement (**Figure 4-2**). Slopes less steep than the "ideal" curve are ones showing signal or ion suppression. Both situations may lead to severe errors in quantitation (Hernandez et al., 2005).

As calculated according to the equation in Eq. 4, signal response (SR) percentages are given in Figure 4-2. Matrix effects could manifest as signal enhancement (SR>100%) or signal suppression (SR<100%). Signal enhancement, exhibited by three samples in the graph of the spiramycin calibration curves, all have SRs greater than 100%. These values of 734, 432, and 115%, respectively, show that these matrices increased the signal 7.34, 4.32, and 1.15-fold above the response obtained in pure MeOH. Here, pure MeOH is the "ideal" signal as it has no matrix interferences and is the eluent the LC-MS/MS method is based on. All other samples showed an SR of less than 100% indicating that ion suppression occurred, with a range of 12-60% of the response obtained using MeOH. The sample curves presented are calibration curves obtained from standard addition using composite samples of the four different U.S. regions. It should be noted that the individual samples in each composite could have different SR percentages for each of the

analytes. Matrix effects on spiramycin showed the greatest signal enhancement (up to 734%) to the greatest signal suppression (up to 60%) in the following order of samples: Midwest (734%), Northeast (432%), South (115%), and West (60%). For NP-AOZ and SUL, the order for least suppressing to most suppressing is: West (NP-AOZ 30%/ SUL 22%), Northeast (24%/20%), Midwest (23%/16%), and South (16%/12%). Very little differences were seen for NP-AOZ and SUL from sample to sample (7% difference in Midwest sample; 4% in Northeast and South, 8% in West), suggesting that depending on the analyte, general trends may perhaps be seen in certain types of matrices. A smaller standard deviation indicates that a general assumption may be true for these two compounds and how they are suppressed in U.S. biosolids, but must be validated with more samples.

Data for SPI showcase that it is impossible to make a generic statement about matrix effects, not even for a single compound and a single type of sample matrix. It is clear that a general statement regarding matrix effects cannot be made with confidence, although it appears that signal enhancement is more common than is signal suppression. The causes for signal enhancement are not well understood (Stahnke et al., 2012); however, with LC-MS/MS by ESI, ionization suppression is a well-known phenomenon (Mei et al., 2003). For suppression, it is assumed that matrix components may outcompete the target analytes during ionization. In other words, the target analyte is suppressed due to loss of charge (Gosetti et al., 2010). These components can range from inorganic electrolytes to organic molecules such as carbohydrates. Other reasons for suppression include co-eluting compounds, mobile phase additives, and equipment design (Gomes et al., 2014).

The extraction process may also introduce interfering compounds such as plastic polymer residues and phthalates (Mei et al., 2003). Future studies should focus on signal suppression/enhancement with more analytes in biosolids matrices to look for patterns and key influencing factors.

Table 4-4. Potential limitations each quantitation method may be subject to.

Results May Be Limited By:	External Calibration	Isotope Dilution (non-analog)	Isotope Dilution (analog)	Standard Addition
Extraction Losses	V	S[1]	R	R
Matrix Effects	V	S[1]	R	R
Costly Labeled Standards	R	S	V	R
Availability of Labeled Standard	R	S	V	R
Increased Preparation Time	R	S[2]	S[3]	V
Extra Lab Materials Needed	R	S[2]	S[3]	V

A value of "R" = robust; this method is not affected by this limitation. A value of "S"= susceptible; this method may be affected by this limitation. A value of "V"= vulnerable; this method is most likely affected by this limitation. [1]As these analog standards are not chemically identical to the target analyte, extraction losses and matrix effects may affect the obtained signal. [2]May be susceptible if multiple non-analog standards are tested to experimentally determine best fitting standard. [3]May be susceptible if optimization of analog standard on mass spectrometer proves to be difficult.

Strengths and Weaknesses of the Quantitation Methods Evaluated

Many potential issues exist in LC-MS/MS analysis of analytes (**Table 4-4**). The predominantly accepted method for quantitation is using isotope dilution with a stable isotope-labeled analog of the target analyte. The labeled analog is introduced at the beginning of extraction and therefore accounts for recovery losses during sample preparation procedures, whether it be due to inefficient extraction, analyte interactions with the matrix, or speciation differences due to pH, among all possible reasons. The labeled analog also chemically acts the same way as the native compound, thus it is

subject to the same matrix effects and ionization pattern regardless of what mass spectrometer is used; however, these compounds are costly, not always commercially available, and may prove to be time-consuming to obtain and optimize on the mass spectrometer so other heavy-labeled standards may sometimes be used (Tang et al., 2009). These standards are not the same as the target analyte. For example, a heavy labeled thiabendazole-d_6 was used to quantitate oxytetracycline (EPA, 2009). The approach to using these surrogate labeled standards is to ensure that they have the same response pattern as the target analyte. This means that they must be extracted the same way, yield the same recovery percentage, and are subject to the same matrix effects and ionization patterns. This may prove to be more time-consuming and costly in the long run, as the selection of this non-analog standard requires experimentation since ionization behaviors can be so different from compound to compound (Sancho et al., 2002) and from samples to sample; however, if a surrogate labeled proxy is already available, it may be easier and cheaper to use it as a standard in the isotope dilution method instead of purchasing the actual target compound's isotopically-labeled counterpart. As sample preparation counts for 70-90% of time and significantly affects reliability and quality of data (Garcia-Rodriguez et al., 2014), it is important to take all factors in Table 4-2 into account.

Standard addition and external calibration do not require the usage of isotopically-labeled chemicals (**Figure 4-3**). Standard addition sees the addition of increasing amounts of native analyte to the extract to form a calibration curve that then can be used to back-calculate the actual concentration, if there is a detection. Ionization patterns and matrix

effects of the extract are factored into this analysis, but this method does not take into account extraction recoveries as the standards are spiked after extraction; however, concentrations can be recovery-corrected. It is important to keep in mind that standard addition is time-consuming as multiple concentration vials need to be created for every individual sample and thus may not be a viable method for commercial labs and/or high-throughput analyses where using one vial per sample and use of an auto-injector is commonly established. External calibration is the usage of native analytes dissolved in a clean matrix such as MeOH to create a standard curve. This curve does not take into consideration extraction recoveries or matrix effects.

Thus, the quantitation method used will vary depending on matrix, analyte, and lab resources. Analytically, looking at the four methods in Figure 4-3 and taking into account the issues in Table 4-2, it could be argued that the "best" quantitation method is the isotope dilution method with analog standards (panel C). It must still be kept in mind that severe matrix effects can lead to poor sensitivities regardless (Hernandez et al., 2005). A simple dilution of the extract can be used to minimize matrix effects, but this will also minimize differences between samples and target analyte levels (Hernandez, et al. 2005).; however, if the matrix effect can be decreased, satisfactory results can be obtained without the use of an analog isotope standard (Sancho et al., 2002). Thus, proper clean-up and analyte ionization (i.e., chromatography optimization) must be top concerns even when using isotopically-labeled analogs. Arguably, the second "best" method is standard addition. Using the same compound for a calibration curve in the same sample matrix is ideal but the time-consuming nature of this method makes it less appealing. External

calibration and isotope dilution with non-analog standards arguably is the least accurate methods, although they are the easiest and most efficient methods. In a situation where concentration isn't as important than the confirmation of the analyte presence, external calibration may suffice analysis goals; however, if the exact concentration is needed, using isotope dilution with an analog standard and standard addition methods should be employed.

Meta-Analysis of the Published Literature

The analysis of the published LC-MS/MS studies indicates that from 2000 to 2015, internal standards were used most frequently. Standards that were analogous to the analytes of interest were used most frequently (in 37 studies; ~59%) followed by the usage of standards that were not analogous to the analyte of interest (in 28 studies; 44%). The usage of standard addition and external calibration were less frequent (in 29% and 25% of the studies, respectively). It is important to keep in mind that some studies employed multiple methods and so were counted twice. These numbers show that using surrogate standards, whether analogous or non-analogous to the target analyte, are far more common than standard addition and external calibration. As using surrogate standards are generally considered more accurate since they are internal, meaning that they are added into the sample prior to extraction, these results are not surprising. It is interesting to note that as mass spectrometry instrumentation improved over the years, and analytical chemists developed more and more isotopically-labeled standards, the use of external calibration seems to be decreasing. It is also interesting to note that standard addition usage is increasing. One paper noted that standard addition was used because

isotopically-labeled standards were not available and the non-analog standards that they tested didn't correct for ion suppression (Echeverria et al., 2014). Another paper actually tested multiple methods before settling on standard addition (vom Eyser et al., 2015). The data presented in this chapter suggest that different quantitation methods can produce different results, and that it is important to experimentally determine what method is best suited for each analyte and sample matrix. This means that the data in many of these papers could have been negatively affected by the choice of their quantitation method. The present study shows how the usage of isotopically-labeled non-analogous standards in biosolids greatly varies depending on the standard of choice and analyte of interest. The majority of papers that reported using these standards did not specify what standard was used for what analyte. The usage of terminology also differed greatly; standard addition and matrix-matched calibration are presumed to be the same technique in this analysis as the descriptions of both appeared to be identical methods. The differentiation between internal and external standards is important, as the former means that the standard is present during the entire extraction process. However, merely stating that an internal standard was used does not specify exactly how it was used as the reason could be for recovery, quantitation, matrix effects determination or any other reason. Similarly, educated guesses had to also be made for papers mentioning that a calibration curve was used for quantitation but did not state what was used to make the calibration curve. These were presumed to be external calibration if no isotopically-labeled standards were included in the "materials" section of the paper.

The trend in LC-MS/MS papers over the years did show an increase in efforts to reduce and quantitate matrix effects (Arbelaez et al., 2014; Chu and Metcalfe, 2007). A few even

used a different method for quantitation but then switched to standard addition for analyzing matrix effects (Chu and Metcalfe, 2007; Lajeunesse et al., 2012; Ding et al., 2011). Future studies need to include in their quantitation how matrix effects were circumvented.

A few key points can be taken away from this literature analysis. The first is that using isotopically-labeled standards is more common than using standard addition and external calibration (although it appears that the use of standard addition is increasing and the use of external calibration is decreasing). The second is that the data obtained in this study indicates that many of these published papers where a quantitation method was chosen without prior experimental evidence indicating that the chosen method is the most accurate may have reported concentrations that were not the best estimate of the true value. The third is that although many papers did indeed take into account (or at least note) potential matrix effects on obtained data, few specified reasons as to why their method of quantitation was chosen and fewer still experimentally determined the best standards for usage. This leads to the final point that the rigor in LC-MS/MS quantitation needs to be strengthened and better reporting of quantitation methods needs to occur. Many of the "methods" sections in the analyzed papers were vague regarding quantitation. Certain papers were excluded if there was no response from the author. This highlights the need for authors to be more specific in their reporting of how quantitation was conducted (perhaps in the supplemental information section) and the need for uniformity of language to be used.

Study Limitations

This study had two main limitations. The first is that few samples were used. In Chapter 3, only 12 samples were analyzed and ten were found to contain detectable amounts of antibiotics. Thus, general conclusions must be taken with caution as the trend may change with the analysis of more samples. The second limitation is that few analytes were screened for. Out of the nine antibiotics in Chapter 3, five were detected and thus analyzed in this chapter; however, this is the first study where the same LC-MS/MS data was analyzed using different quantitation methods. Thus, this study establishes the need for future studies where a larger sample size is used with more analytes of interest, not just antibiotics.

The literature search was only limited to pharmaceuticals reported in biosolids. Different analytes in different matrices may produce different patterns in quantitation method usage. However, the conclusions reached here regarding how methods should be chosen and improvements in the reporting of method details can be applied to all LC-MS/MS analyses.

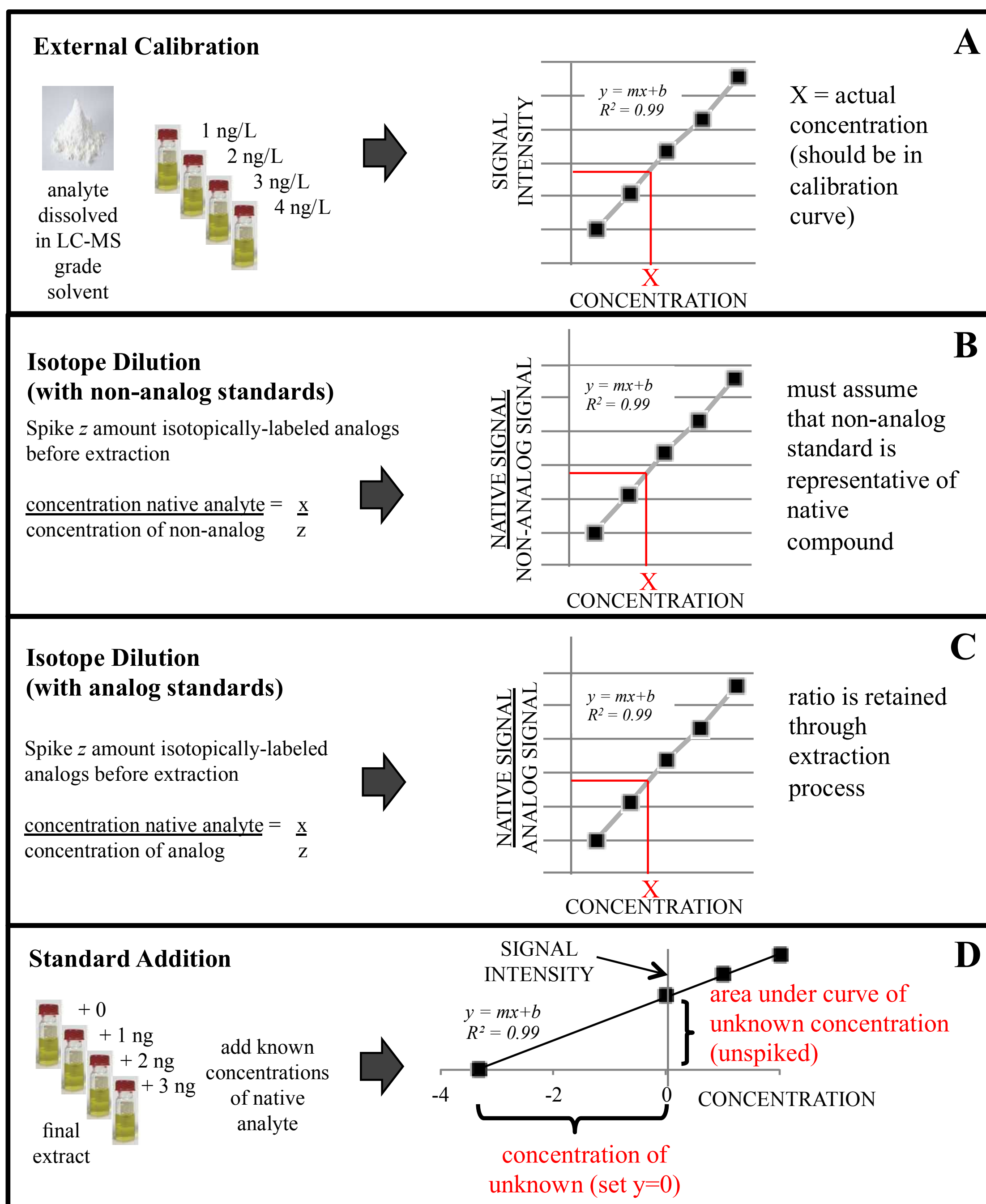


Figure 4-3. Methods for quantitating analytes on LC-MS. **A)** External calibration curve is analyte standard in clean solvent at increasing concentrations. Calibration curve is used to find concentration of unknown. **B)** Isotopically-labeled analogs are spiked into the sample prior to extraction. The ratio is conserved through extraction and the end calibration curve is based on the ratio of the signals. **C)** Instead of the denominator being the isotopically-labeled analog signal, the non-analog isotopically-labeled standard signal is used. **D)** Standard addition method utilizes spikes of known analyte amounts into the final extract. Curve obtained is used to back-calculate for unknown concentration.

CONCLUSIONS

This chapter is the first study to systematically explore the impact of these four different, yet common quantitation approaches in the use of LC-MS/MS for antibiotics analysis in biosolids. Using the same LC-MS/MS data results for five detected analytes, different concentration results were obtained using four different quantitation methods. As these methods are commonly used in literature, it is important to evaluate their accuracy as well as their strengths and weaknesses. Based on the results and theoretical considerations raised in this chapter, it is concluded that isotope dilution with a structurally identical analog standard is the preferred quantitation method. In situations where it cannot be applied, the next best choice is standard addition due to this method's ability to account for of matrix effects in its results. Using external calibration and isotope dilution with non-analog standards run the risk of the results being influenced in an unpredictable fashion by matrix effects, recovery losses, and different signal patterns through ionization. Finally, in the case of antibiotics in biosolids, it appears that although signal suppression is more common than signal enhancement, both can still be observed, even for the same analyte in different biosolids matrices (spiramycin). Though some quantitation methods presented here are better than others, it is still important to evaluate which may be best suited for each study as different variables exist (i.e., availability of standard). The literature analysis indicated that isotope dilution (with analogous and non-analogous standards) was reported to be used more often than standard addition and external calibration were. The trend in recent years is to consider matrix effects in data analysis, and thus standard addition has become more common. More detailed reporting

of quantitation methods and uniformity of terminology should be used in future reports. As the information presented here is relevant to antibiotics in biosolids, more comparative studies should be conducted in the future with more analytes and matrices to better judge the strengths and weaknesses of each LC-MS/MS quantitation method.

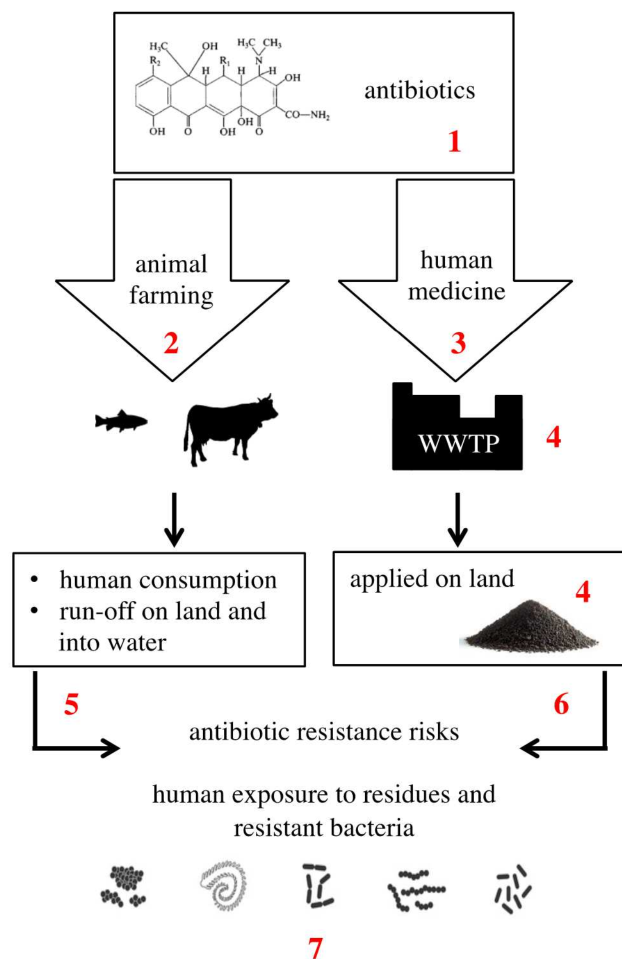
CHAPTER FIVE. RESEARCH IMPLICATIONS AND FUTURE DIRECTIONS

Antibiotics are arguably the most important human chemicals to have ever been discovered. They have saved countless lives and have the potential to save countless more. In order for their efficacy to continue, we as a society must be more prudent with their usage. This dissertation explored the two main ways antibiotics are anthropogenically introduced into the environment. The first way is through animal farming, where antibiotics are often used for growth promotion, disease prevention, and treatment. The second way is through persistence in wastewater treatment plants, where antibiotics sometimes accumulate in biosolids, a solid by-product frequently applied on land. This dissertation contributes knowledge to both these areas, as well as technical data regarding how antibiotics can be quantified for these purposes.

The farming of animals on land (agriculture) and in water (aquaculture) strives to meet the many growing demands of the human population. Aside from providing critical food protein, other essential products include dairy, wool for clothing, fish oil, as well as food for other animals. These industries support the livelihoods for billions of people around the planet. It is important to find more sustainable and economical practices to continue the development of these industries without causing irreparable harm and threatening future generations. Chapters one and two discuss how antibiotics are used in agriculture and aquaculture. Predominantly, there are two main issues. The first is that not enough reporting of key antibiotic statistics is occurring (Figure 5-1, questions 1-2). We as a society don't know what antibiotics are used, in what concentrations, at what frequencies,

and for what reasons. This is especially true in Asian countries where the majority of aquaculture is taking place. The second is that many negative consequences are being reported in literature regarding animal farming and its role in promoting the development of antibiotic, which is indeed occurring. Specific genetically-identified resistances (i.e., efflux pumps) to individual or classes of antibiotics have been reported. Of greatest concern is the occurrence of cross- and co-resistances in many different environmental and animal farming matrices. As aquaculture continues to grow, the potential for resistance spread through water increases and the urgency to improve animal farming practices becomes more and more apparent.

In this dissertation, a comprehensive study was conducted by screening for 47 antibiotics in 27 seafood samples from 11 different countries. This was the first U.S. screening of top consumer choice fresh seafood for a large number of medically important antibiotics. Low concentrations of five antibiotics were found to be in compliance with U.S. (and EU) regulations. Although these seafoods are deemed chemically safe to consume, the detection of antibiotics still points to a problem as they should have cleared out of the fish by the time they reached the market. The detection of antibiotics in wild-caught shrimp and farmed salmon marketed as antibiotic-free also brings up issues of possible contamination and mislabeling. Low concentrations of antibiotics like the ones detected here have been shown to exert selective pressure on bacteria to develop resistance. Literature publications report that more and more resistant strains of bacteria have been identified in recent years, some of which are pathogenic to humans.



Research Gaps/Needs

1. What antibiotics are used? At what concentrations and in what ways?
2. How does resistance develop in animal farms? What concentrations/conditions are necessary? Uniformity of usage need to be established.
3. Public health measures need to improve how antibiotics are 1) available and 2) used/prescribed.
4. Degradation patterns of antibiotics need to be researched.
5. Where do antibiotic residues end up after going through an animal farm? How long do they stay in a biologically active structure?
6. What human exposure pathways are associated with biosolids application?
7. How do sub-lethal antibiotic concentrations affect bacterial communities?

Figure 5-1. Research gaps, needs, and questions that future research should focus on.

Antibiotics are introduced into the environment via wastewater treatment as well. Wastewater treatment plants serve to recycle human wastes as well as take out harmful biological and chemical contaminants that may negatively impact the environment and human health. With the large range of contaminants, it is unrealistic to have products from this process that are completely void of health hazards. Thus, this dissertation looks at biosolids, which are known to concentrate just the organic and inorganic contaminants and are regularly applied on land. Using samples from the EPA's 2006/2007 Targeted National Sewage Sludge Survey, five antibiotics (out of nine screened for) were detected

at concentrations between 0.1 and 33.2 ng/g dry weight. This study reports the first detection of ampicillin and oxolinic acid in biosolids, and the first detection of these two antibiotics along with nalidixic acid in U.S. biosolids. Oxytetracyline was the most often detected antibiotic, found in five out of 12 samples (41.7%). Interestingly, this was also the most often detected antibiotic in the seafood study (detected in four out of 11 composite samples~ 36.6%). Oxytetracycline is the most popular antibiotic to be used in aquaculture and is also popular in human medicine. These results confirm that medically important antibiotics are being introduced into the environment via animal farming and biosolids land application pathways.

The selection of quantitation methods to analyze data using LC-MS/MS, as discussed in Chapter 4, affects the results obtained. It is necessary to define the quality of data before considering their implications. This dissertation conducts an initial study to analyze how four different commonly reported quantitation methods may affect resulting concentrations of antibiotics in biosolids. A meta-analysis of studies reporting detections of pharmaceuticals in biosolids was also completed to determine the frequency of usage for each of these methods. It is concluded that using isotopically-labeled analogs in the isotope dilution method is the most accurate, followed by standard addition. External calibration and isotope dilution with non-analog standards should be used with discretion and experimentation in the lab should occur in order to determine which is better suited for each specific analyte. The literature analysis indicated that although the use of isotopically-labeled standards is more common than standard addition and external calibration, standard addition usage has been increasingly, perhaps due to the realization

that matrix effects play a large role in data quality. Biosolids as a complex matrix also was shown to enhance and suppress ion signals depending on the analyte considered. Ideally, corrections can be made to already published data if patterns can be identified based on quantitation method, analyte, and matrix. The results in this dissertation were based on too few samples for this analysis to be conducted here, but this research does set the basis for more detailed studies in the future.

Just as with the usage of antibiotics in animal farming, the application of biosolids containing antibiotics on land can also promote antibiotic resistance. Studies have been published that look at the risk in consuming crops grown under exposure of bacteria exhibiting resistance genes, how WWTPs influence the concentration and dissemination of antibiotic-resistant genes into the environment, the presence of resistant bacteria and resistance genes in soils and biosolids, as well as the development of multidrug resistance in the environment. These issues are congruent with resistance issues posed by the usage of antibiotics in animal farming. Both must be evaluated and addressed in order to effectively reduce the current promotion of antibiotic resistance in the environment.

Many research gaps and needs (some of which are mentioned above) are necessary to fully understand how antibiotic resistance is developing and how to protect the current efficacy of these drugs. Above all, eliminating the unnecessary usage of antibiotics in the beginning is key (Figure 5, question 3). Not only will this reduce the amount of antibiotics to be accounted for later, it will also allow for easier regulation and uniformity of usage practices to be adopted across the globe. This is a goal that is, realistically,

difficult to achieve and enforce as antibiotics are available from many sources without a prescription. However, much progress has been made, especially in animal farming. Examples have already been set in Europe where the usage of antibiotics is limited to only disease treatment in many countries. In the case of swine, no negative effects were observed on productivity, the number of Danish pigs produced per sow, average daily weight gain achieved, or the amount of feed used to produce a kilogram of meat. This example can be followed in all countries with the help of farmers and government support. Initiatives such as the one in the U.S. published earlier this year in March, 2015 by President Barack Obama's administration aim to guide action by public health and veterinary officials in an effort to slow the development of resistance. One result this plan strives for is to eliminate the use of medically-important antibiotics for growth promotion in food animals. Initiatives such as this one need to be adopted and actively pursued by all countries that produce animals for human consumption. As antibiotic usage statistical information is lacking, this initiative will hopefully strengthen surveillance and reporting in the U.S. as well.

In terms of eliminating antibiotic resistance promotion through WWTPs, the most economical thing is probably not to re-engineer existing WWTPs to be more efficient at transforming pharmaceuticals, but to instead treat biosolids prior to land application. However, before this happens, more information is needed regarding exactly how resistance is being promoted in biosolids and in the soil. DNA is easily damaged with UV light, and the water treatment process is typically very rigorous with regard to eliminating biological contaminants. Thus, more needs to be understood about how genes and

microbes survive to spread resistance and where there are some that are more likely to survive certain kinds of water treatment, biosolids storage, and application (Figure 5-1, question 4). After this information is obtained, specific treatment of biosolids can be implemented depending on the final destination and usage on land (i.e., as fertilizer, as ground cover, etc.).

The end fate of biologically active antibiotics is also largely unknown. This partly has to do with the uncertainty in degradation patterns and half-lives in the environment, but also because run-off of chemicals from soils/agricultural fields/animal farms into surrounding ecosystems occurs and therefore makes it hard to track where antibiotics end up (Figure 5-1, question 5). Human exposure pathways also need be studied; risk-assessment analyses need to be done with the published concentrations of antibiotics that have been found in the environment (Figure 5-1, question 6).

Above all, there is a major research need to understand when and where antibiotic resistance develops and under what conditions. We already understand that bacteria can survive in the presence of toxic chemicals (antibiotics) to live and procreate. But we don’t know the importance of sub-lethal concentrations of drugs in the promotion and maintenance of heritable drug- and multidrug resistance. Basic research on this topic has recently been started (Nair et al., 2012; Mirani and Jamil, 2011) and now needs to be continued and applied to animal farms and WWTPs (Figure 5-1, question 7).

The efficacy of antibiotics can be preserved if judicious usage is agreed upon by both the animal production and human health sectors. Eliminating the usage of antibiotics as a growth promoting compound in animal husbandry will drastically reduce the amount of antibiotics used and decrease opportunities for antibiotic resistance to develop. Agreements to reserve certain antibiotics (or classes of antibiotics) for just human medicine will also eliminate the intersection of drugs used for humans and animals. This dissertation contributes new data regarding antibiotic concentrations in U.S. seafoods and biosolids, as well as a new LC-MS method for the multiclass detection of key human and animal health antibiotics. This dissertation also contributes public health data regarding antibiotic resistance in animal husbandry practices and technical information regarding LC-MS quantitation methods. Together with lawmakers and public health officials, scientists can help prevent antibiotics from becoming obsolete and create consensus to reduce the unnecessary usage of antibiotics and preserve their efficacy.

CHAPTER SIX. REFERENCES

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APPENDIX A

SUPPORTING INFORMATION

Table A1. All pharmaceuticals analyzed and their respective detection and quantification parameters.

Compound	CAS	Function/Use	RT[b]	Precursor Ion	Product Ion	Quantified Against	RPD[c]	% Recovery
Acetaminophen	103-90-2	pain-reliever	4.68	152.2	110	$^{13}C_2$, ^{15}N-Acetaminophen	0.638	107
Anhydrochlortetracycline[a]	4497-08-9	antibiotic	20.63	461.2	444	d_6-Thiabendazole	4.96	46.75
Anhydrotetracycline[a]	4496-85-9	antibiotic	16.45	427.2	409.8	d_6-Thiabendazole	7.49	137.5
Azithromycin	83905-01-5	antibiotic	13.55	749.9	591.6	$^{13}C_3$-Trimethoprim	2.74	97.9
Caffeine	58-08-2	stimulant	9.32	195	138	$^{13}C_3$-Caffeine	8.92	92.65
Carbadox	6804-07-5	antibiotic	10.53	263.2	231.2	$^{13}C_3$-Trimethoprim	2.79	24.65
Carbamazepine	298-46-4	anticonvulsant	15.38	237.4	194.2	d_{10}-Carbamazepine	4.78	97.85
Cefotaxime	63527-52-6	antibiotic	10.09	456.4	396.1	$^{13}C_3$-Trimethoprim	12.5	65.1
Chlortetracycline[a]	57-62-5	antibiotic	11.9	479	444	d_6-Thiabendazole	3.94	130.5
Ciprofloxacin	85721-33-1	antibiotic	11.17	332.2	314.2	$^{13}C_3$, ^{15}N-Ciprofloxacin	9.31	99.6
Clarithromycin	81103-11-9	antibiotic	17.61	748.9	158.2	$^{13}C_6$-Sulfamethazine	9.46	96.35
Clinafloxacin	105956-97-6	antibiotic	12.56	366.3	348.1	$^{13}C_3$, ^{15}N-Ciprofloxacin	21.3	119
Cloxacillin	61-72-3	antibiotic	16.82	468.1	160.1	$^{13}C_3$-Trimethoprim	3.08	85.95
Dehydronifedipine	67035-22-7	hypertension	16.65	345.1	284.1	$^{13}C_3$-Trimethoprim	5.05	108
Demeclocycline[a]	127-33-3	antibiotic	9.63	465	430	d_6-Thiabendazole	3.59	97.65
Diltiazem	42399-41-7	heart conditions	15.34	415.5	178	$^{13}C_3$-Trimethoprim	3.94	96.8
Digoxigenin	1672-46-4	aglycon of digoxin	12.68	391.2	355.2	$^{13}C_3$-Trimethoprim	2.93	55.6
Digoxin	20830-75-5	heart conditions	16.58	798.5	651.3	$^{13}C_3$-Trimethoprim	1.92	103
Diphenhydramine	58-73-1	antihistamine	14.57	256.2	167	$^{13}C_3$-Trimethoprim	5.69	66.4
Doxycycline[a]	564-25-0	antibiotic	14.4	445.2	428.2	d_6-Thiabendazole	1.83	117
Enrofloxacin	93106-60-6	antibiotic	11.22	360.2	316	$^{13}C_3$, ^{15}N-Ciprofloxacin	23.3	91.45
4-Epianhydrochlortetracycline[a]	158018-53-2	antibiotic	18.9	461.2	444	d_6-Thiabendazole	17.9	15.85
4-Epianhydrotetracycline[a]	4465-65-0	antibiotic	16.45	427.2	409.8	d_6-Thiabendazole	20.5	104.1
4-Epichlortetracycline[a]	14297-93-9	antibiotic	9.92	479	444	d_6-Thiabendazole	7.17	104
4-Epioxytetracycline[a]	14206-58-7	antibiotic	6.51	461.2	426.2	d_6-Thiabendazole	2.18	112.5
4-Epitetracycline[a]	23313-80-6	antibiotic	5.71	445.2	410.2	d_6-Thiabendazole	3.86	130.5
Erythromycin-H_2O	114-07-8	antibiotic	16.9	716.2	158	$^{13}C_2$-Erythromycin anhydrate	2.01	117
Flumequine	42835-25-6	antibiotic	15.25	262	173.7	$^{13}C_3$-Trimethoprim	11.8	104.7
Fluoxetine	54910-89-3	serotonin inhibitor	16.967	310.1	148	d_5-Fluoxetine	2.13	115.5
Isochlortetracycline[a]	514-53-4	antibiotic	9.95	479	462	d_6-Thiabendazole	0.597	87.15
Lincomycin	154-21-2	antibiotic	9.47	407.2	126	$^{13}C_3$-Trimethoprim	0.703	129.5

Lomefloxacin	98079-51-7	antibiotic	11.14	352.2	308.1	$^{13}C_3$, ^{15}N-Ciprofloxacin	19.9	72.65
Miconazole	22916-47-8	antifungal	20.93	417	161	$^{13}C_3$-Trimethoprim	4.13	42.25
Minocycline[a]	10118-90-8	antibiotic	3.43	458	441	d_6-Thiabendazole	11.7	109.5
Norfloxacin	70458-96-7	antibiotic	10.59	320	302	$^{13}C_3$, ^{15}N-Ciprofloxacin	13.7	114
Norgestimate	35189-28-7	progestogen	21.8	370.5	124	$^{13}C_3$-Trimethoprim	10.0	44.75
Ofloxacin	82419-36-1	antibiotic	10.53	362.2	318	$^{13}C_3$, ^{15}N-Ciprofloxacin	17.2	81.8
Ormetoprim	6981-18-6	antibiotic	10.53	275.3	259.1	$^{13}C_3$-Trimethoprim	9.08	93.05
Oxacillin	66-79-5	antibiotic	16.3	434.1	160.2	$^{13}C_3$-Trimethoprim	6.57	87.7
Oxolinic Acid	14698-29-4	antibiotic	13.11	262.1	244	$^{13}C_3$-Trimethoprim	0.458	54.8
Oxytetracycline [a]	79-57-2	antibiotic	7.29	461.2	426.2	d_6-Thiabendazole	0.221	100
Penicillin G	61-33-6	antibiotic	14.46	367.1	159.9	$^{13}C_3$-Trimethoprim	2.93	28.3
Penicillin V	87-08-1	antibiotic	15.29	383.2	159.9	$^{13}C_3$-Trimethoprim	2.40	120.5
Roxithromycin	80214-83-1	antibiotic	17.83	837.6	679	$^{13}C_6$-Sulfamethazine	1.44	75.05
Sarafloxacin	98105-99-8	antibiotic	12.29	386.1	299	$^{13}C_3$, ^{15}N-Ciprofloxacin	11.2	65.7
Sulfachloropyridazine	80-32-0	antibiotic	10.97	285	156	$^{13}C_6$-Sulfamethazine	13.6	82.95
Sulfadiazine	68-35-9	antibiotic	5.32	251.2	156.1	$^{13}C_6$-Sulfamethazine	8.97	102.3
Sulfadimethoxine	122-11-2	antibiotic	13.33	311	156	$^{13}C_6$-Sulfamethoxazole	0.148	79.5
Sulfamerazine	127-79-7	antibiotic	8.78	265	156	$^{13}C_6$-Sulfamethazine	18.1	111
Sulfamethazine	57-68-1	antibiotic	10.31	279	156	$^{13}C_6$-Sulfamethazine	5.54	109
Sulfamethizole	144-82-1	antibiotic	10.09	271	156	$^{13}C_6$-Sulfamethoxazole	17.6	85.5
Sulfamethoxazole	723-46-6	antibiotic	11.33	254	156	$^{13}C_6$-Sulfamethoxazole	31.4	112.4
Sulfanilamide	63-74-1	antibiotic	2.02	190	155.8	$^{13}C_6$-Sulfamethazine	10.0	56.5
Sulfathiazole	72-14-0	antibiotic	8	256.3	156	$^{13}C_6$-Sulfamethoxazole	4.67	138
Tetracycline[a]	60-54-8	antibiotic	7.74	445.2	410.2	d_6-Thiabendazole	5.57	135
Thiabendazole	148-79-8	fungicide	10.59	202.1	175.1	d_6-Thiabendazole	5.37	92.9
Trimethoprim	738-70-5	antibiotic	9.94	291.2	230	$^{13}C_3$-Trimethoprim	1.84	91.45
Tylosin	1401-69-0	antibiotic	16.37	916.6	772.5	$^{13}C_6$-Sulfamethazine	16.9	72.1
Virginiamycin	11006-76-1	antibiotic	17.4	526.3	508.3	$^{13}C_3$-Trimethoprim	4.98	89.45
1,7-Dimethylxanthine[d]	611-59-6	stimulant	7.02	181.2	124	$^{13}C_3$-Caffeine	3.55	299.5[d]

[a]Analytes were determined by one LC-MS/MS method; all others were determined using a second method.
[b]Retention Time.
[c]Relative percent difference from matrix spike and matrix spike duplicate.
[d]Co-elutes with its isomer theophylline, so % recovery is calculated from the reported maximum possible concentration.

Table A2. Concentrations/detection limits of pharmaceuticals determined in composite samples reported on a ng/g fresh weight basis.

Composite Sample Number Analytes	1. Farmed Shrimp	2. Wild Shrimp	3. Farmed Tilapia	4. Farmed Catfish	5. Antibiotic-Free Farmed Catfish	6. Trout with Deformed Spine	7. Trout with Normal Spine	8. Farmed International Atlantic Salmon	9. Farmed Antibiotic-Free Atlantic Salmon	10. Farmed US Atlantic Salmon	11. Farmed Swai
Acetaminophen	<6	<5.9	<6	<5.9	<5.9	<6	<5.9	<6	<6.0	<6.0	<6.0
Anhydrochlortetracycline	<6.5	<5.9	<6	<5.9	<7.8	<6	<5.9	<6	<6.0	<6.0	<6.0
Anhydrotetracycline	<6	<5.9	<6	<5.9	<5.9	<6	<5.9	<6	<6.0	<6.0	<6.0
Azithromycin	<0.6	<0.7	<0.6	<0.6	<0.6	<0.6	<0.6	<0.7	<0.1	<0.7	<0.6
Caffeine	<6	<5.9	<6	<5.9	<5.9	<6	<5.9	<6.7	<6.0	<6.0	<6.0
Carbadox	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<06	<0.6	<0.6	<0.6
Carbamazepine	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
Cefotaxime	<9.1	<7.9	<8	<7.8	<7.9	<8	<7.9	<8	<9.9	<8.0	<8.0
Chlortetracycline	<9.2	<9.2	<8.4	<8.5	<8.4	<8.6	<8.4	<8.6	<8.5	<8.4	<8.9
Ciprofloxacin	<2.4	<2.4	<2.4	<2.3	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4
Clarithromycin	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6
Clinafloxacin	<2.4	<2.4	<2.34	<2.3	<2.4	<2.6	<2.4	<2.4	<2.4	<2.4	<2.4
Cloxacillin	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2
Dehydronifedipine	<0.2	<0.3	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Demeclocycline	<6	<5.9	<6	<5.9	<5.9	6	<5.9	<6	<6.0	<6.0	<6.0
Diltiazem	<0.1	<0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1\	<0.1	<0.1
Digoxin	<2.4	<2.4	<2.4	<2.3	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4
Digoxigenin	<9.7	<20.5	<6	<12.6	<19.0	<30.6	<10.5	<22	<36.9	<24.7	<12.9
Diphenhydramine	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2	<0.2
Doxycycline	<2.4	<2.4	<2.4	<2.3	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4	<2.4
Enrofloxacin	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<1.2	<0.7	<1.2	<1.2	<1.2
4-Epianhydrochlortetracycline	<23.8	<23.6	<23.9	<23.4	<23.6	<23.9	<23.7	<23.9	<24.0	<24.1	<24.1
4-Epianhydrotetracycline	<6	<5.9	<6	<5.9	<5.9	<6	<5.9	<6.2	<6.0	<6.0	<6.0
4-Epichlortetracycline	<8.8	<9.1	<6	<6.5	<5.9	<6.4	<5.9	<6.7	<6.0	<6.0	<7.3
4-Epioxytetracycline	<2.4	<3.9	<2.4	<2.5	<2.6	<2.4	<2.7	4.1	<2.4	<2.4	<2.5
4-Epitetracycline	<3.4	<4.2	<3.1	<3.0	<2.9	<3.2	<3	<2.9	<2.9	<2.9	<3.3
Erythromycin-H2O	<0.9	<0.9	<0.9	<0.9	<0.9	<0.9	<0.9	<0.9	<0.9	<0.9	<0.9
Flumequine	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6	<0.6

Table A3. Facilities samples in the 2006/2007 National Sewage Sludge Survey.

Facility Name and Flow Group	Flow Stratum	City	State
Sugar Creek WWTP	1<MGD<10	Alexander City	AL
Aldridge Creek WWTP	1<MGD<10	Huntsville	AL
Phoenix WWTP	10<MGD<100	Phoenix	AZ
Valley Sanitary District STP	1<MGD<10	Indio	CA
San Francisco	>100 MGD	San Francisco	CA
El Estero WWTP	1<MGD<10	Santa Barbara	CA
Santa Rosa	1<MGD<10	Santa Rosa	CA
Stockton Water Quality Plant	>100 MGD	Stockton	CA
Los Angeles County Sanitation District	10<MGD<100	Whittier	CA
Boulder WWTP	1<MGD<10	Boulder	CO
South Windsor	1<MGD<10	South Windsor	CT
Three Oaks WWTP	1<MGD<10	Estero	FL
Orange County Northwest WRF	1<MGD<10	Orlando	FL
Tampa	1<MGD<10	Tampa	FL
Albany	10<MGD<100	Albany	GA
Americus-Mill Creek	1<MGD<10	Americus	GA
Boone STP	1<MGD<10	Boone	IA
Calumet Water Reclamation Plant	>100 MGD	Chicago	IL
Plainfield WWTP	1<MGD<10	Plainfield	IL
Lake County DPW, New Century STP	1<MGD<10	Vernon Hills	IL
Dupage County-Knollwood STP	1<MGD<10	Wheaton	IL
Blucher Poole WWTP	1<MGD<10	Bloomington	IN
William Ross Edwin WWTP	10<MGD<100	Richmond	IN
Parsons	1<MGD<10	Parsons	KS
Topeka	10<MGD<100	Topeka	KS
Mayfield WWTP	1<MGD<10	Mayfield	KY
Eunice	1<MGD<10	Eunice	LA
Jefferson Parish East Bank WWTP	1<MGD<10	Marrero	LA

Nantucket	1<MGD<10	Nantucket	MA
Salisbury	1<MGD<10	Salisbury	MD
Mechanic Falls Treatment Plant	1<MGD<10	Mechanic Falls	ME
Benton Harbor-St. Joseph WWTP	1<MGD<10	St. Joseph	MI
Wixom WTP	1<MGD<10	Wixom	MI
Festus Crystal City STP	1<MGD<10	Crystal City	MO
Elizabeth City WWTP	1<MGD<10	Elizabeth City	NC
Hillsborough WWTP	1<MGD<10	Hillsborough	NC
Beatrice	1<MGD<10	Beatrice	NE
Wildwood Lower WTF	10<MGD<100	Cape May Court House	NJ
Middlesex County Utility Authority WRC	>100 MGD	Sayreville	NJ
Verona TWP DPW	1<MGD<10	Verona	NJ
Buffalo	>100 MGD	Buffalo	NY
Canajoharie WWTP	1<MGD<10	Canajoharie	NY
Geneva A-C Marsh Creek STP	1<MGD<10	Geneva	NY
NYC DEP- Jamaica WPCP	10<MGD<100	New York City	NY
North Tonawanda STP	1<MGD<10	North Tonawanda	NY
Clermont County Commissioners	1<MGD<10	Batavia	OH
Bedford	1<MGD<10	Bedford	OH
Metropolitan Sewer District Little Miami	10<MGD<100	Cincinnati	OH
Northeast Ohio Regional Sewerage District Southerly WWTP	>100 MGD	Cleveland	OH
Delaware County Alum Creek WWTP	1<MGD<10	Delaware	OJ
Mingo Junction STP	1<MGD<10	Mingo Junction	OH
Duncan public Utilities Authority	1<MGD<10	Duncan	OK
City of Klamath Falls WWTF	1<MGD<10	Klamath Falls	OR

Western Westmoreland Municipal Authority	1<MGD<10	Irwin	PA
Allegheny County Sanitary Authority	1<MGD<10	Pittsburgh	PA
Greater Pottsville Area Sewer Authority	1<MGD<10	Pottsville	PA
Punxsutawney	1<MGD<10	Punxsutawney	PA
South Kingstown WWTF	1<MGD<10	Narragansett	RI
Plum Island WWTP	10<MGD<100	Charleston	SC
Lawson Fork WTP	1<MGD<10	Spartanburg	SC
Elizabethton	1<MGD<10	Elizabethton	TN
Amarillo	10<MGD<100	Amarillo	TX
Dallas Southside WWTP	>100MGD	Dallas	TX
Trinity River Authority of Texas	1<MGD<10	Ellis County	TX
Fredericksburg	1<MGD<10	Fredericksburg	TX
Odo J. Riedel Regional WWTP	1<MGD<10	Schertz	TX
Wagner Creek WWTP	1<MGD<10	Texarkana	TC
Tyler Southside WTP	1<MGD<10	Tyler	TX
Spanish Fork City Corporation	1<MGD<10	Spanish Fork	UT
Buena Vista	1<MGD<10	Buena Vista	VA
Everett City SVC Center MVD	10<MGD<100	Everett	WA
Beaver Dam	1<MGD<10	Beaver Dam	WI
Elkins WWTP	1<MGD<10	Elkins	WV
Huntington	10<MGD<100	Huntington	WV

Table A4. LC-ESI-MS/MS parameters for analysis of antibiotics. The source parameters were set as follows: curtain gas = 30 psi, ion source gas 1 = 80 psi, ion source gas 2 = 80 psi, ion spray voltage= 4000 V, temperature = 700ºC, and collision activated dissociation gas = 10 psi.

Analyte (*m/z*)	Primary (top) & Secondary (bottom) Transitions (*m/z*)	Declustering Potential (V)	Collision Energy (V)	Collision Cell Exit Potential (V)	Retention Time (min)	Dwell Time (ms)
AMP (350.2)	106	56	37	4	5.83	150
	159.9		21	8		20
ERY (734.5)	158.1	81	71	10	6.32	150
	116.2		41	14		20
NDA (233.1)	215	61	23	12	7.14	50
	187.1		37	10		20
NP-AOZ (236.1)	104	41	35	18	6.37	50
	133.9		19	6		20
OXA (262.1)	216	46	29	12	6.53	150
	243.9		41	10		20
OXY (461.2)	426.2	60	29	8	5.78	50
	443.5		21	8		20
SPI (843.6)	174.1	136	55	8	5.71	50
	101		71	8		20
SUL (311.1)	156.1	76	31	8	6.38	50
	245		29	12		20
SDD (279.1)	124.2	71	35	10	5.84	50
	108		41	10		20
Isotopically-labeled Standards						
ERY-$^{13}C_2$ (736.4)	159.9	81	43	8	6.31	150
	83.1		79	14		20
NP-AOZ-d_4 (240.1)	134	41	19	6	6.33	150
	104		33	4		20
SUL-$^{13}C_6$ (285.1)	70.2	71	77	12	6.38	150
	124.1		37	6		20

APPENDIX B

CHAPTER ONE LITERATURE ANALYSIS REFERENCES

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APPENDIX C

CHAPTER FOUR LITERATURE ANALYSIS REFERENCES

1. Arbelaez P, Granados J, Borrull F, Marce RM, Pocurull E. Determination of sedative hypnotics in sewage sludge by pressurized liquid extraction with high-performance liquid chromatography and tandem mass spectrometry. Journal of Separation Science. 2014;37(23):3481-8.
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APPENDIX D

LIST OF GRADUATE PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

1. **Hansa Y. Done**, Benny F.G. Pycke, Arjun K. Venkatesan, April J. Cobos, Rolf U. Halden. (2015). Occurrence of Nine Antibiotics in Archived Biosolids from the U.S. EPA Targeted National Sewage Sludge Survey. *In preparation.*

2. Hansa Y. Done, Arjun K. Venkatesan, Rolf U. Halden. (2015). Literature Meta-Analysis and Experimental Comparison of Four Different Analysis Strategies for LC-MS/MS Quantification of Antibiotic Residues in Biosolids. *In preparation.*

3. **Hansa Y. Done,** Arjun K. Venkatesan, Rolf U. Halden. (2015). Does the Recent Emergence of Aquaculture Create Antibiotic Resistance Threats Different from those Associate with Land Animal Production in Agriculture? *American Association of Pharmaceutical Scientists.* **17**(3):513-24. doi: 10.1208/s12248-015-9722-z

4. **Hansa Y. Done,** Rolf U. Halden. (2015). Reconnaissance of 47 Antibiotics and Associated Microbial Risks in Seafood Sold in the United States. *Journal of Hazardous Materials*. **282**:10-17.

5. Arjun K. Venkatesan, **Hansa Y. Done**, Rolf U. Halden. (2014). United States National Sewage Sludge Repository at Arizona State University – A New Resource and Research Tool for Environmental Scientists, Engineers, and Epidemiologists. PMID: 24824503. *Environmental Science and Pollution Research.*

PRESENTATIONS

Hansa Y. Done, Rolf U. Halden
Occurrence of 9 Antibiotics in Archived Biosolids from the 2006/2007 U.S. EPA Targeted National Sewage Sludge Survey
American Chemical Society 250th Meeting
August, 2015. Boston, MA
Poster Presentation

Hansa Y. Done, Rolf U. Halden
Antibiotics in Aquaculture: Usage, Resistance Issues, and Sustainability
Arizona Board of Regents Meeting
June 2015. Tucson, AZ
Poster Presentation

Hansa Y. Done, Arjun K. Venkatesan, Rolf U. Halden
Aquaculture vs. Agriculture: Antibiotic Usage and Resistance Threats
SETAC Europe 25th Conference
May, 2015. Barcelona, Spain
Poster Presentation

Hansa Y. Done, Rolf U. Halden
Antibiotics and Aquaculture: Detected Residues and Microbial Resistance Risks
American Chemical Society 248th Meeting
August, 2014. San Francisco, CA
Platform Presentation; Poster Presentation

Hansa Y. Done, Rolf U. Halden
Antibiotic Residue Screening in United States Seafood
SETAC Europe 24th Conference
May, 2014. Basel, Switzerland
Platform Presentation

Hansa Y. Done, Rolf U. Halden
Engineering Sustainable Aquaculture: Understanding and Managing the Necessity for Use of Antibiotics.
Biological Design Graduate Program Symposium, Arizona State University, Tempe, AZ
February, 2013. Tempe, AZ
Poster Presentation