Spring 2014

Antimicrobial Resistance of Channel Catfish Intestinal Microflora in the Arkansas and Ninnescah Rivers in Kansas

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ANTIMICROBIAL RESISTANCE OF CHANNEL CATFISH INTESTINAL MICROFLORA IN THE ARKANSAS AND NINNESCAH RIVERS IN KANSAS

being

A Thesis Presented to the Graduate Faculty

of the Fort Hays State University in

Partial Fulfillment of the Requirements for

the Degree of Master of Science

by

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ABSTRACT

Antimicrobial compounds have been used by humans to counteract bacterial infections since 1910. Overuse of these compounds in clinical and agricultural applications has led to rapid evolution and global spread of antimicrobial resistance and rivers are the main receiving body for antimicrobials and resistant bacteria from urban effluents and agricultural runoff. When antimicrobial-resistant bacteria enter the aquatic environment, water acts as a physical pathway for their distribution. Subsequently, resistance genes become established in natural systems and pose threats to human health and ecological processes. Due to these potential threats, antimicrobial resistance in the aquatic environment should be closely monitored.

To improve the understanding of antimicrobial resistance in two river systems in Kansas, intestinal contents from 20 Channel Catfish (*Ictalurus punctatus*) and water samples were taken at eight sites on the Arkansas and South Fork Ninnescah rivers during the spring of 2012. These samples were examined for resistance to six compounds representing major classes of antimicrobials and resistance was observed in 94 isolates. From these isolates, 39 bacteria species were identified by partial sequencing of the 16S ribosomal RNA gene. Resistant species included common isolates from the environment and pathogens of humans and fish. Minimum inhibitory concentrations were determined for bacteria resistant to azithromycin, ciprofloxacin, and tetracycline. Several isolates exhibited no zone of inhibition, indicating they were resistant to the maximum concentration of the assay. Multi-drug resistance was also observed in eight species.

ACKNOWLEDGMENTS

Many people deserve thanks and recognition for their assistance in the completion of this project. I thank my major advisor, Dr. William Stark, for his insight and dedication throughout this project. I greatly appreciate his encouragement to step outside of the box that led me to a new suite of skills and knowledge, making me a well-rounded biologist. I thank my graduate committee, Dr. Yass Kobayashi, Dr. Eric Gillock, and Linda Smith. Their advice during my graduate career and this project was extremely helpful. I thank Dr. Jeff Briggs, Dr. Elmer Finck, and Kacie Vogt for all of the administrative support they provide to the Department of Biological Sciences. Dr. Terry Balthazor and his Balthazor fellowship provided me vital support during the completion of this project. I also thank the Kansas Idea Network of Biomedical Research Excellence (K-INBRE) and Dr. Michael Madden for administration of funding for this research.

I cannot thank my field assistants enough. They endured miserably long and hot days when they did not have to. I thank Ryan Waters, Aaron Austin, Beth Kerr, Abram Lollar, Wes Fleming, Ryan Pinkall, Jeff Seim, Kyle Broadfoot, Casey Pennock, Christian Lollar, Ryan Cox, Jordan Voss and Mark VanScoyoc for their hard work. I also thank the Golden family for access to the Ninnescah River on their property.

The help that I received in the lab was also invaluable. I thank Joanna Fay and Jeff Sekavec for their guidance and dealing with an ecologist in the lab. Trisha Penning deserves my gratitude for the many hours she spent to help isolate bacteria from my samples. I thank Dr. Sam Zwenger and Brad Bott for assistance and training in

bioinformatics software. I also thank Jennifer Pfannenstiel-Klaus for initial editing of this document.

Finally, I greatly appreciate my family and friends for their support throughout my academic career. I could not have accomplished this goal without them. My parents, Risa Stegman and Rick and Cindy Hofmeier, deserve my utmost gratitude for their love and encouragement. I thank my sister Sara, grandparents Lee and Lois Hofmeier, Peggy Lewis, and Jim Piatt, and many aunts, uncles, and cousins for their support. I also thank Chelsea Hawk for her love and support.

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PREFACE

This thesis follows the style of The Journal of Freshwater Ecology.

INTRODUCTION

Bacteria, a common constituent in all natural systems, are ubiquitous in the water, soil, and air. Many species endure environmental extremes, from the freezing and thawing of Arctic permafrost (Rivkina et al. 2000) to the near boiling waters and high acidity levels of hot springs (Roeselers et al. 2007). Bacteria also live within most organisms and are often beneficial to nitrogen fixation in plants (Franche et al. 2009) and digestive function in animals (Cummings & MacFarlane 1997). For example, a species of *Carnobacterium* is a common intestinal microbe in Atlantic Salmon (*Salmo salar*) and is known to inhibit pathogen growth in fish, allowing it to be used in some aquaculture operations as a probiotic (Robertson et al. 2000). In contrast, some species of bacteria can cause diseases that are harmful or deadly to the organisms they infect. *Edwardsiella ictaluri* and *Flavobacterium columnare* cause enteric septicemia and columnaris, respectively, and are the most common diseases in Channel Catfish (*Ictalurus punctatus*), accounting for the greatest economic losses in aquaculture (Schrader 2008). Another widespread bacterium, *Aeromonas salmonicida*, causes ulcers in salmonid and non-salmonid fish species (Wiklund & Dalsgaard 1998). However, the main focus on bacteria is directed to the many species that cause life-threatening illnesses in humans. Bacteria species such as *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* can cause deadly infections in humans (Levy 1998) and *Yersinia pestis*, the bacterium responsible for the Black Plague in the 14th century, killed 17 million to 28 million people in Europe over the course of four years (Perry & Fetherston 1997).

Humans have a long history of using chemicals to counteract bacterial infections. Centuries before antimicrobial drugs, heavy metals were used to treat infectious diseases (Baker-Austin et al. 2006). In 1910, the first contemporary antimicrobial, arsphenamine, was released to counteract bacterial pathogens (Zaffiri et al. 2012). Antimicrobial compounds inhibit the growth and proliferation of bacterial cells by interfering with the production of materials required for growth or cell division (Levy 1998). Several classes of antimicrobials target different products or mechanisms of bacterial reproduction and, in general, these compounds act on cell wall production, protein synthesis, and DNA replication or repair (Walsh 2000).

Glycopeptide and penicillin classes of antimicrobials inhibit cell wall formation by interfering with the production of peptidoglycan, the component that gives strength to bacterial cell walls (Walsh 2000). Vancomycin, a glycopeptide, inhibits cell wall biosynthesis by interacting with the peptide substrate required for peptidoglycan production (Williams 1996). The spectrum of antimicrobial activity of vancomycin is restricted to *Streptococcus* and *Staphylococcus* species and other Gram-positive bacteria (Wilhelm 1991). Penicillins, such as ampicillin, use beta-lactam rings to inactivate binding proteins that are responsible for the final stages of peptidoglycan layer production (Spratt & Cromie 1988). Ampicillin is effective against both Gram-positive and Gram-negative organisms (Acred et al. 1964).

Antimicrobial classes that inhibit protein synthesis include aminoglycosides, macrolides, and tetracyclines (Walsh 2000). Aminoglycosides, such as gentamicin, negatively affect protein synthesis by binding to the 30S ribosome, which causes codon

misreading (Edelmann & Gallant 1977). Gentamicin, a commonly used aminoglycoside, has antimicrobial effects on many Gram-negative bacteria (Edelmann & Gallant 1977). Tetracyclines also bind to the 30S ribosome but interfere with the binding of tRNA to the ribosome complex (Schnappinger & Hillen 1996). Oxytetracycline, a type of tetracycline, is a broad-spectrum antimicrobial with substantial effects against Gram-negative bacteria (Jacobsen & Berglind 1988). Macrolides, such as azithromycin and erythromycin, act similar to oxytetracycline but bind to 50S ribosomes rather than 30S ribosomes (Brisson-Noel et al. 1988). Azithromycin exhibits activity against Gram-positive and Gram-negative bacteria (Peters et al. 1992).

Ciprofloxacin belongs to the quinolone class of antimicrobial compounds. This class of drugs affects bacteria by targeting DNA gyrase, the enzyme responsible for uncoiling double-stranded DNA, thus inhibiting cell division of bacteria (Shen et al. 1989). Ciprofloxacin is a synthetic antimicrobial that has a broad range of activity and is effective against Gram-positive and Gram-negative bacteria (Chin & Neu 1984; Oliphant & Green 2002).

Several mechanisms influence bacterial resistance to the effects of antimicrobials such as efflux pumps, which are present in a wide variety of bacteria to move molecules out of the cells (Walsh 2000). Some species that produce antibiotics use the pumps to export compounds that allow them to better compete with other microbes (Walsh 2000). There also is growing evidence that bacteria use efflux pumps to export antibiotics and other compounds at sub-inhibitory concentrations as a means

of communication (Waters & Bassler 2005; Davies et al. 2006). As a result, many bacteria have the intrinsic ability to remove antimicrobials before they reach an effective concentration within the cell (Walsh 2000). Another mechanism is the use of enzymes to deactivate or destroy the functionality of the antimicrobial (Walsh 2000). Some bacteria, such as *Staphylococcus aureus*, can use the enzyme beta-lactamase to hydrolyze the beta-lactam ring of penicillins, opening the ring and making it ineffective (Philippon et al. 1985). Other bacteria use enzymes to alter the targets of antimicrobials (Walsh 2000). These enzymes can alter the structure of ribosomal components to reduce antimicrobial affinity without compromising protein synthesis, an effective countermeasure against erythromycin class drugs (Bussiere et al. 1998). Some bacteria not only survive but use "antimicrobial" molecules as their only source of carbon (Dantas et al. 2008). These species can subsist on natural and synthetic antimicrobials and represent a phylogenetically diverse group that includes organisms closely related to human pathogens. The presence of these bacteria in the environment suggests that these species already have the metabolic mechanisms to resist clinical antimicrobial agents and could readily share or receive resistance genes from other organisms (Dantas et al. 2008).

While many bacteria naturally possess these genes for self-protection (Alonso et al. 2001; Piddock 2006) and communication (Waters & Bassler 2006), bacteria can receive new resistance genes via mutation and horizontal gene transfer (Walsh 2000; Davies & Davies 2010). The short generation time of bacteria allows for a relatively high frequency of mutation (Martinez & Baquero 2000). In the presence of

antimicrobials, bacteria with mutations that confer resistance develop a competitive advantage over non-resistant forms and are more likely to pass on these resistance genes (Martinez & Baquero 2000; Walsh 2000). However, the dispersal of these genes is not restricted to vertical transfer from parental cells to offspring. Horizontal gene transfer represents a significant mechanism for the dispersal of antimicrobial resistance genes (Pruden et al. 2006) and includes a number of pathways through which genes can be transferred on plasmids or transposons from one bacterium to another. Transfer elements can be transported between bacteria via viral transduction, bacterial conjugation, and transformation from free DNA (Thomas & Nielsen 2005). Additionally, these transfer pathways have been observed between diverse groups of bacteria (Courvalin 1994; Kruse & Sorum 1994). Accordingly, the increase in the prevalence of resistance genes and the diversity of mechanisms for resistance causes the therapeutic efficacy for any antimicrobial to decline shortly after its introduction. Resistance has been observed within months or only a few years after the release of some clinical antimicrobial drugs (Davies 1996). Because antimicrobials act as the primary form of treatment for many infectious diseases, it is critical that their effectiveness is preserved (Walsh 2000).

Antimicrobial resistant (AMR) bacteria are difficult to treat and are a crucial threat to human health. The World Health Organization (2013) reported that resistant pathogens infect over two million Americans each year, causing 23,000 deaths. The incidence of bacteria resistant to one or multiple antimicrobials becomes more common every year (Arias & Murray 2009), with occurrence of vancomycin-resistant

Enterococcus spp. increasing from 0% to 25% within 10 years in the United States (Willems et al. 2005). Antimicrobial-resistant bacteria also have been collected from isolated human populations in Nepal (Walson et al. 2001). This rapid evolution and global spread of resistance can be largely attributed to overuse of antimicrobials in clinical and agricultural applications (Andersson & Levin 1999).

Compounding the threat is the use of antimicrobials for non-therapeutic purposes. Many antimicrobial drugs are used in agriculture as growth promoters to increase animal production (Gaskins et al. 2002). In addition, Kummerer (2010) reported up to 95% of antimicrobial drugs might be unaltered when excreted by humans and other animals. Unfortunately, some unused antimicrobials are discarded directly into sewage systems (Kümmerer 2003), after which they are released directly into the environment (Kümmerer 2010).

Rivers are the main receiving bodies for antimicrobials and resistant bacteria from urban effluents and agricultural runoff (Goñi-Urriza et al. 2000). Resistant organisms from these sources could contaminate surface and ground waters that are used as sources of human drinking water (Kümmerer 2004). The increased input of drugs has dramatically shaped the resistance determinants in the environment, termed „the resistome“ (D'Costa et al. 2006). Once in the aquatic environment, water provides a means of distribution for antimicrobial resistant bacteria to animal and human populations (Baquero et al. 2008; Allen et al. 2010). In addition to physical forces such as water and wind, animal movements provide a biological mechanism for dispersal of resistance genes (Allen et al. 2010). These dispersal mechanisms allow for resistance

genes to become established in natural bacterial ecosystems (Baquero et al. 2008), causing natural environments to serve as reservoirs of antimicrobial resistance genes (Martinez 2008). Most wastewater treatment plants are not designed to remove small chemicals such as antimicrobials, allowing many drugs to enter riverine systems at high concentrations (Batt et al. 2006). Subsequently, selection occurs for resistant organisms in the environment (Goñi-Urriza et al. 2000). The general lack of efficient wastewater treatment threatens to add to resistance as the human population continues to grow. This increased contact between human pathogenic bacteria and resistant bacteria in the environment is likely to encourage gene transfer among these organisms (Martinez 2008).

The growing human population also demands a larger food supply, which has led to an increase in aquaculture (Goldburg & Naylor 2005). Over 200,000 metric tons of Channel Catfish are produced annually in North America (Garibaldi 1996). Up to 114,000 kg of antimicrobials are used annually to treat catfish, with an industry-wide estimate of 200,000 kg annual rate of use in aquaculture (Benbrook 2002). Antimicrobial compounds, with oxytetracycline being the most common, are frequently used as growth promoters and therapeutic treatments for fish diseases (Martinez 2008). These compounds are frequently integrated into food pellets for the fish (Ervik et al. 1994, DePaola et al. 1995). Diseased fish often exhibit a reduced food-intake, which might result in over-feeding. Excess food pellets containing antimicrobial agents could then enter surrounding systems (Ervik et al. 1994). Additionally, oxytetracycline is readily incorporated into calcified structures; thus it is used to mark hatchery-reared fish

(Brooks et al. 1994) and for age validation studies (MacFarlane & Beamish 1987). As a result of this antimicrobial regime, drug residues and resistant bacteria are often transferred from aquaculture ponds to surrounding aquatic environments (Huys et al. 2001). Ervik et al. (1994) documented resistant bacteria in Blue Mussels (*Mytilus edulis*) and antimicrobial agents in muscles of wild fish near an aquaculture facility. Horizontal gene transfer has been observed from fish pathogens to *Aeromonas* spp. and *Escherichia coli*, common human pathogens (Rhodes et al. 2000; Cabello 2006). Aquaculture workers are particularly susceptible because they might be in direct contact with these resistant organisms (McPhearson et al. 1991). Furthermore, multi-drug resistant bacteria have been isolated from ornamental fish, providing an international mechanism for dispersal of resistance genes (Verner-Jeffreys et al. 2009).

Riverine systems have received relatively little attention compared to aquaculture environments in regard to the presence of antimicrobial resistance (McPhearson et al. 1991). Even though non-clinical environments represent the main source of antimicrobials, there is a paucity of information about the effects of resistant bacteria in natural ecosystems (Martinez 2008). Resistant bacteria could have a competitive advantage over non-resistant bacteria, altering natural microbial communities and thus ecological processes (Costanzo et al. 2005; Martinez 2008). Directing research towards the ecology of antimicrobials and resistance in non-clinical environments could provide insight into the evolution of resistance (Pruden et al. 2006). Paradigms of environmental science will soon need to include antimicrobial resistance genes as potential environmental contaminants. Thus, environmental scientists and

researchers are needed to document, monitor, and address the challenge of antimicrobial resistance in bacteria (Pruden et al. 2006). Particular emphasis should be placed on riverine systems and their biota, as these ecosystems receive the majority of antimicrobials and resistant organisms from agriculture and clinical applications (Goñi-Urriza et al. 2000).

Channel Catfish occur throughout Kansas and live in a variety of habitats, ranging from large streams to small impoundments (Cross & Collins 1995). They are primarily carnivores, eating invertebrates and other fish; however, they also consume parts of plants (Cross & Collins 1995). They also are the most-sought fish species by licensed anglers in Kansas (Burlingame 1998). Additionally, Channel Catfish are one of the most commonly raised fish in aquaculture (Chapman 1992), with several hundred ponds in Kansas dedicated to commercial production (Cross & Collins 1995). The large geographic range, common occurrence, generalized habitat and diet preferences, and human importance make the Channel Catfish a good model organism for environmental studies in Kansas.

The goal of this study was to address the following objectives to improve the understanding of AMR bacteria in two large, prairie streams in Kansas: 1) Screen, isolate, and identify bacteria resistant to six compounds representing major classes of antimicrobial drugs; 2) Determine the prevalence of AMR bacteria in Channel Catfish and associated water samples relative to perceived sources in a large urban area and a large fish hatchery; and 3) Quantify the level of resistance of those bacteria.

METHODS

Study area

The Arkansas River is a sandy, prairie stream that runs through Wichita, the most populous city in Kansas. The domestic effluent from Wichita is released into this river. The South Fork (SF) Ninnescah River is morphologically similar to the Arkansas River, making it a hydrologically comparable stream. However, the anthropogenic effects on the SF Ninnescah River primarily are restricted to agricultural runoff from cropland and a state fish hatchery that contains Channel Catfish. These differences in the prevalence of antimicrobial-resistant (AMR) bacteria allowed comparison between domestic and aquaculture effluents.

Sites were selected based on accessibility, water availability, and probability of antimicrobial exposure (Figure 1, Appendix 1). The Lower Arkansas River Water Quality Reclamation Facility is Wichita‟s main waste water treatment plant. Two study sites were selected downstream of Wichita to determine the effects of domestic effluent on AMR bacteria presence. Site AR1 was located 29.5 river km (rkm) downstream of Wichita‟s effluent. Site AR2 was 3.25 rkm downstream of the effluent. Two additional sites were sampled upstream of the Wichita effluent to provide control treatments on the Arkansas River. Site AR3 was 30 rkm upstream and AR4 was 61.5 rkm upstream of the effluent. Sites AR2 and AR3 were sampled twice to increase sample size. On the SF Ninnescah River, two sites were selected downstream of Pratt, KS to determine the effects of hatchery effluent on presence of AMR bacteria. Sites

NR4 and NR3 were 55.75 and 2 rkm downstream of the fish hatchery, respectively. Two sites were sampled upstream of Pratt to act as a control for these perceived effects. Site NR2 was 4.25 rkm upstream of the fish hatchery and site NR1, 7.25 rkm upstream.

Sample collection

Channel Catfish were collected from March to May 2012 in the Arkansas and SF Ninnescah rivers (Figure 1). A barge electrofishing unit was used to capture Channel Catfish. Fish were placed in a cooler with water and transported to an area in the riparian zone for processing. Intestinal samples were obtained by extracting a length of lower intestine and releasing 10 ml of its contents into a 50-ml centrifuge tube partially filled with a sterile phosphate buffered saline (PBS) solution (Liau & Shollenberger 2003). Water samples were collected from the middle of the water column, at the center of each site. Each sample was assigned a unique code for identification. All samples were stored on ice during transportation to Fort Hays State University. Samples were then stored at 4°C until screening.

Sample screening, isolation, and identification

Antimicrobial agar dilution was used to screen intestinal content and water samples against six antimicrobial compounds. Ampicillin (Fisher BioReagents), azithromycin (TCI America), ciprofloxacin (TCI America), gentamicin (Fisher BioReagents), oxytetracycline (EMD Chemicals), and vancomycin (Fisher BioReagents) were diluted individually in Mueller-Hinton (MH) agar (Thermo Scientific) to concentrations (Table 1) considered to be resistance breakpoints (Kerry et al. 1997; Clinical and Laboratory Standards Institute 2012). DePaola et al. (1995),

Miranda and Zemelman (2002), and Taylor (2003) successfully incubated microbes isolated from fish at temperatures ranging from 20–35°C. Samples in this study were lawn-streaked on antimicrobial plates and incubated at 30°C for 24-96 hours. *Escherichia coli* and *Staphylococcus aureus* were used as positive controls throughout the isolation process to ensure the effectiveness of the antimicrobials. After incubation, unique colonies were differentiated by morphology, growth type, and color. The isolation streaking process was completed three times for each selected colony to ensure a pure culture was isolated. Subsequently, Gram staining of isolated colonies was used to determine Gram reaction, cell morphology and grouping, and to confirm isolate purity prior to gene sequencing. After visual characterization, isolates were assigned a unique code. Additionally, colonies from each isolate were grown in Tryptic Soy Broth (TSB) and then frozen for preservation at -80°C in a solution of 60:40 ratio PBS and glycerol.

Morphologically unique isolates were sent to GeneWiz, Inc. (South Plainfield, NJ) for partial sequencing of the 16S ribosomal RNA gene. CodonCode Aligner software was used to correct misreads in the gene sequences. Consensus sequences were then assembled with the software and compared to the GenBank database via Basic Local Alignment Search Tool (BLAST) for putative bacterial identification. The BLAST software is used to locate similar regions in nucleotide and protein sequences from unicellular and multicellular organisms. The first entry provided by BLAST represents the sequence with the highest identity percentage to the gene sequence submitted, and was thus used as the putative species identification. Isolates with an

identity percentage of ≥99% are confident to species-level identification, whereas isolates with a percentage of 95-98% are confident to genus (Barghouthi 2011). Bacteria of the same species and from the same environmental sample, isolated on different antimicrobial agars, were examined for multi-drug resistance.

Minimum inhibitory concentrations

The minimum inhibitory concentration (MIC) is classified as the lowest concentration of an antimicrobial compound that inhibits bacterial growth (Andrews 2001). Determination of this concentration is important for assessing the antimicrobial activity of new drugs and for measuring resistance in bacteria (Andrews 2001). One method of obtaining this information is to complete E-test assays. E-tests are conducted using a plastic strip that contains a pre-defined gradient of antimicrobial compound on one side and a concentration scale on the other side (Citron et al. 1991). The point at which the zone of inhibition intersects the concentration scale is considered the MIC (Citron et al. 1991).

According to manufacturer‟s protocols, minimum inhibitory concentrations (MIC) were determined from E-test strips (bioMérieux, Inc., Durham, NC). E-strips contained one of the following antimicrobials: azithromycin, ciprofloxacin, or tetracycline. Tetracycline was used in place of oxytetracycline to represent the tetracycline class of antimicrobials for the MIC assays because bioMérieux, Inc. did not manufacture oxytetracycline E-strips. Isolates were revived from frozen storage by incubation in TSB at 30°C for 48 hours. The bacteria and media were then transferred to a conical-bottom centrifuge tube and placed in a centrifuge at 4000 rpm for 15

minutes. The media was decanted and discarded, and the pellet of bacteria cells was retained. The pellet of cells was re-suspended and diluted in 0.85% saline solution. Using a spectrophotometer, the bacterial concentration was standardized to 0.5 McFarland standard. The standardized solution of cells was plated by three-way streak onto MH agar plates. The plate was allowed to dry for one minute before an E-test strip was placed on the agar surface. For isolates suspected of multi-drug resistance, two strips were placed, in opposite directions, on each plate. The plate was incubated for 20 hours and the zone of inhibition was examined to determine the MIC (Figure 2).

Prevalence of resistant species

G-tests of goodness-of-fit were completed for the Arkansas and SF Ninnescah rivers to determine if there was a difference in prevalence of resistant bacteria between sites near effluent sources compared to sites farthest from the sources. For these tests, sites AR2 and AR3 were considered near-source sites and the numbers of species at each site were combined, whereas AR1 and AR4 were farther from the domestic effluent on the Arkansas River. Sites NR2 and NR3 were nearest the hatchery source on the SF Ninnescah River, whereas NR1 and NR4 were farthest from the source.

Rarefaction curve and detection effectiveness

The vegan package (version 2.0-3) in R Statistical Program (version 2.15.2) was used to construct a bacterial species rarefaction curve (Figure 3). The “specaccum” function was used to complete 500 permutations of the rarefaction curve. This curve was used to interpret the effectiveness at detecting AMR bacteria species in the study area, given the detection and isolation methods outlined above. Additionally, the

"specpool" function was used to extrapolate the total number of resistant bacteria in the species pool by estimating the number of unobserved species. The Chao model (Chao 1987) within this function assumes that the number of unobserved species is related to the number of rare species within the sample.

RESULTS

Sample screening, isolation, and identification

During spring 2012, intestinal contents were collected from 20 Channel Catfish at eight sites in the Arkansas and South Fork Ninnescah rivers. Water samples were also collected, one from each site. An additional water sample was collected during a resampling effort at site AR2 during a sewage leak from the Wichita treatment plant. The samples yielded 94 resistant isolates after screening and characterization on antimicrobial agar plates; 71 from fish samples and 23 from water samples. The water sample from site AR4 did not yield any isolates that were resistant to the six antimicrobials examined. One catfish from site NR1 also did not yield resistant isolates.

After partial sequencing of the 16S rRNA gene and subsequent BLAST query, 39 bacterial species (Table 2) were identified from the 94 isolates. Twenty-nine resistant species were isolated from fish samples and 13 were isolated from water, with three species occurring in both sample types. The most bacterial species isolated from one fish was six at site AR1. In the SF Ninnescah River, the highest number was five species from one fish at site NR3. The most species isolated from a water sample was four, at sites AR2 and NR1. Site AR2 yielded a total of 12 resistant species isolated from fish, the highest number for a single site (Table 3). Three species were isolated from fish at site NR1, the lowest number from a single site (Table 3).

Pseudomonas was the most common genus isolated and was represented by 14 species in 35 isolates. However, *Pseudomonas gessardii, P. protogens*, and *P. pseudoalcaligenes* comprised 19 of those 35 isolates. The most common species in the study, *Sediminibacterium salmoneum*, comprised 11 of the isolates. Other common species were *Aeromonas bestiarum*, *Providencia heimbachae*, *Serratia fonticola*, and *Shewanella putrefaciens*. Four species were widespread among fish, occurring at five of the eight sample sites and in both rivers (Table 3). Of the 39 species isolated, 15 were resistant to ampicillin, 11 to azithromycin, 12 to ciprofloxacin, 5 to gentamicin, and 11 to oxytetracycline. No Gram-positive isolates were observed with resistance to vancomycin. The eight species that exhibited multi-drug resistance were *Aeromonas bestiarum*, *Oerskovia turbata*, *Pseudomonas mandelii*, *Pseudomonas pseudoalcaligenes*, *Sediminibacterium salmoneum*, *Serratia fonticola*, *Shewanella putrefaciens*, and *Stenotrophomonas maltophilia* (Appendix 2).

Minimum inhibitory concentrations

E-strips were used to determine MIC values for organisms resistant to azithromycin, ciprofloxacin, and tetracycline. The MIC values for azithromycin ranged from 8 to ≥256 µg/ml (Appendix 4). *Pseudomonas pseudoalcaligenes*, *Shewanella putrefaciens*, and *Yersinia intermedia* showed no zone of inhibition indicating they were resistant to at least 256 µg/ml of azithromycin, the maximum concentration on the E-strip. Minimum inhibitory concentrations were not determined for 13 of the 24 azithromycin-resistant isolates because viability was lost between the initial screening and the MIC testing. The MIC values for ciprofloxacin ranged from 4 to ≥32 µg/ml

(Appendix 5). Three isolates from site AR3 were resistant to at least the maximum concentration of 32 µg/ml ciprofloxacin. Two of these three isolates were *Enterococcus faecium*, and the other was *Pseudomonas pseudoalcaligenes*. Tetracycline MIC values ranged from 6 to ≥256 µg/ml (Appendix 7). Six species of bacteria were resistant to at least the maximum concentration of 256 µg/ml tetracycline. Four isolates exhibited lower MIC values (6.0, 16.0, 16.0 and 24.0 µg/ml) than the concentrations in the oxytetracycline-infused MH plates used in the initial screening (Appendix 7).

Prevalence of resistant species

The results of the G-tests suggested that there was no significant difference in the prevalence of resistant bacteria between sites near the sources compared to sites farthest from the sources on the Arkansas River (G=0.445, df=1, P=0.505) or on the SF Ninnescah River (G=1.657, df=1, P=0.198).

Rarefaction curve and detection effectiveness

A species rarefaction curve (Figure 3) was constructed to determine the sampling effectiveness of all AMR bacteria species. The curve was steep on the left after only a few fish were sampled, indicating that a large proportion of the bacterial species diversity has yet to be sampled. The slope was reduced as sample size increases, damping the curve; however, at about 20 fish sampled, the curve maintained a relatively steep slope suggesting there were likely many bacteria species to be isolated. The species pool model estimated a total of 54.6 resistant species (S.E.=17.90) could have been isolated from fish in the study area. These estimates suggested that, on

average, 26 resistant species were missed that could have been detected by this screening process.

DISCUSSION

The results of screening the intestinal flora of 20 Channel Catfish from the Arkansas and South Fork Ninnescah rivers indicated that these fish acted as reservoirs of antimicrobial-resistant bacteria. Resistance was observed to five antimicrobial compounds, all of which are commonly used in clinical settings. Additionally, oxytetracycline is commonly used in aquaculture operations. The antimicrobial activity of vancomycin is restricted primarily to Gram-positive bacteria (Jones 2006). Only Gram-negative species were isolated on vancomycin-infused plates, though it is possible that some Gram-positives were missed during the screening process. Twenty-nine bacteria species isolated from fish exhibited resistance to at least one antimicrobial compound. Thirteen resistant species also were isolated from water samples in both rivers. However, the intestinal contents and water samples only shared three resistant bacteria species. This suggests that the microbial communities were different between the fish and the aquatic environment. The variable diet of Channel Catfish might also provide sources of antimicrobials and resistant bacteria. Intestinal samples in this study contained a variety of food items including algae, crayfish, and other fish species. In addition, Channel Catfish have been documented to move 160 river km during the summer (Wendel & Kelsch 1999) and might be acting as biological mechanisms for the dispersal of resistance genes (Allen et al. 2010).

The Arkansas River has many potential sources of antimicrobials, resistant bacteria, and resistance genes. Wichita, Kansas and several smaller communities discharge domestic effluent into this river and its tributaries. Regardless of the dosage,

it is estimated that up to 95% of antimicrobials are unaltered when excreted by humans and other animals (Kummerer 2010). In general, treatment plants are not designed to remove micro-pollutants such as antimicrobials, allowing many of these compounds to be released into rivers (Hirsch et al. 1999; Kolpin et al. 2002). Once antimicrobials enter the aquatic system, selection for resistant bacteria occurs (Goñi-Urriza et al. 2000). Resistant organisms also have been isolated directly from wastewater effluents (Schwartz et al. 2003). Furthermore, biosolids are often recycled from wastewater treatment plants and applied to agricultural fields. These biosolids can contain antimicrobials and resistant bacteria (Smith 2009), which then enter the river system through runoff. Although domestic effluent is more limited on the SF Ninnescah River, agriculture and aquaculture are prevalent. One of the largest feedlots in south-central Kansas, serving up to 40,000 cattle, is located approximately 12 km north of the SF Ninnescah River. Runoff from this operation could enter the river or its tributaries, providing a potential source of antimicrobial compounds and resistant bacteria. The Pratt Fish Hatchery discontinued use of oxytetracycline in 2011 (2014 email comm. from Mike Hassler, Hatchery Biologist, Kansas Department of Wildlife, Parks, and Tourism; unreferenced). However, the results of this study suggested that resistance genes have become established in the microbial communities within the SF Ninnescah River. The large number of sources throughout the study area, such as those above, might have caused the lack of a statistical pattern in prevalence of resistant bacteria among sites.

Although bacterial studies have been common in aquaculture facilities where antimicrobials are frequently used, few studies have identified bacteria from fish in riverine systems. This lack of information made it difficult to determine if the AMR bacteria isolated in this study were normal flora or pathogens in Channel Catfish. Sarter et al. (2007) documented that *Pseudomonas* spp. composed 35% of the microflora in farmed Shark Catfish (*Pangasius hypophtalmus*) in Viet Nam. Other studies reported *Pseudomonas*, *Aeromonas*, and *Vibrio* to be common genera in fish intestinal contents (Grisez et al. 1997; Spangaard et al. 2000; Huber et al. 2004). The prevalence of *Pseudomonas* and *Aeromonas* species in these hatchery studies was comparable to the results from the present project, but no *Vibrio* species were isolated from fish in the Arkansas and SF Ninnescah rivers.

Many of the bacteria, such as *Sediminibacterium salmoneum*, detected in this study are commonly isolated from aquatic environments. However, several species of bacteria were isolated that are considered potential pathogens of humans. Although observed more commonly in soil, *Achromobacter spanius* has been isolated from blood samples of humans and is considered an opportunistic pathogen for individuals with cystic fibrosis (Coenye et al. 2003; Spilker et a. 2013). Maningo and Watanakunakorna (1995) reported a fatality rate of 44% in humans with lower respiratory tract infections caused by *Stenotrophomonas maltophilia*. Other opportunistic pathogens of humans isolated in this study were *Acinetobacter haemolyticus*, *Brevundimonas diminuta*, *Enterococcus faecium*, *Morganella morganii*, and *Serratia fonticola* (McDermott &

Mylottte 1984; Pfyffer 1992; Edmond et al. 1995; Bergogne-Berezin & Towner 1996; Han & Andrade 2005).

Resistant pathogens of fish also were isolated during this study. *Aeromonas salmonicida* and *A. bestiarum* are responsible for furunculosis in fish, a disease that causes inflammation and lesions in the skin and can cause hemorrhaging of internal organs (Martinez-Murcia et al. 2005). *Psuedomonas plecoglossicida* is responsible for hemorrhagic ascites in some fish, causing the peritoneal cavity to fill with fluid (Nishimori et al. 2000). *Carnobacterium maltaromaticum* can cause kidney disease in salmonid fish species (Loch et al. 2008), but is occasionally used as an aquaculture probiotic and food protectant for its antimicrobial activity against other bacteria (Robertson et al. 2000; Leisner et al. 2007).

Minimum inhibitory concentrations were determined for organisms resistant to azithromycin, ciprofloxacin, and tetracycline. Several isolates did not exhibit a zone of inhibition, indicating they were resistant to at least the maximum concentrations contained on the E-strips. In many cases, these organisms were resistant to antimicrobial concentrations that are not safely achievable in humans. When azithromycin was administered intravenously to humans, a maximum serum concentration of 9.91 μg/ml was documented (Luke et al. 1996). Isolates from fish and water samples in the Arkansas and SF Ninnescah exhibited MIC values ranging from 8 to ≥256 μg/ml azithromycin (Appendix 4). Davis et al. (1996) reported a maximum serum concentration of 6.7 μg/ml ciprofloxacin when the antimicrobial was administered intravenously to patients. Bacteria isolated from fish and water samples in

this study exhibited MIC values ranging from 4 to ≥32 µg/ml ciprofloxacin (Appendix 5). When intramuscularly administered to Common Carp (*Cyprinus carpio*), oxytetracycline was observed at a maximum serum concentration of 56.8 µg/ml (Grondel et al. 1987). This concentration was not achievable through oral administration (Grondel et al. 1987), the most common route in aquaculture. Minimum inhibitory concentrations for oxytetracycline in the present study ranged from 6 to ≥256 µg/ml (Appendix 7). Four isolates exhibited lower MIC values than the concentrations contained in the oxytetracycline-infused plates (6.0, 16.0, 16.0 and 24.0 µg/ml) used during screening. However, these bacteria possessed an intermediate level of resistance and would not be susceptible to antimicrobial inhibition by clinical standards (Clinical and Laboratory Standards Institute 2012). These results suggested that antimicrobial treatment would be limited, if possible at all, for infections caused by these resistant bacteria.

Multidrug resistance presents a major challenge to the treatment of bacterial infections in humans, agriculture, and aquaculture (Kruse & Sorum 1994). Multiple resistance genes often occur on the same plasmid (Levy & Marshall 2004) and dispersal of these mobile genetic elements has been documented among diverse groups of bacteria (Kruse & Sorum 1994). Eight bacteria species exhibited multidrug resistance. *Serratia fonticola* was resistant to ampicillin and oxytetracycline. *Stenotrophomonas maltophilia* was resistant to ciprofloxacin and oxytetracycline. Both species are considered to be potential pathogens of humans and were resistant to antimicrobials frequently used in clinical settings. *Aeromonas bestiarum*, a documented pathogen of

fish, was resistant to gentamicin and oxytetracycline. Other multidrug resistant bacteria from this study are commonly isolated from aquatic systems. The presence of multidrug resistance genes in these rivers and the ability of bacteria to transfer these genes, represent a concern for public health because both rivers are used as sources of drinking water, crop irrigation, and recreation. Furthermore, in the presence of antimicrobials, these highly resistant bacteria might out-compete non-resistant species that provide important ecological services. Current monitoring protocols of aquatic systems are restricted primarily to sediment loads, heavy metals, pesticides, and polychlorinated biphenyls (PCBs). Based on public health concerns and potential ecological effects, it is critical that antimicrobials and resistance genes are added to this list of environmental contaminants.

Future research

The rarefaction curve (Figure 3) constructed from these data maintained a relatively steep slope, suggesting that more resistant species could be detected without exhaustive sampling. The species pool model indicated that a total number of 55 species of resistant bacteria could be collected from fish within the study area. Twenty-nine resistant species were isolated during this study, which suggested perhaps as many as 26 resistant species were not detected. However, this number is probably quite conservative given the coarse nature of morphological screening and the general observation that most bacteria collected in environmental samples cannot be cultured by standard methods like the ones employed here (Dykhuizen 1998).

Additional research is necessary to isolate and identify the normal flora of fish in riverine systems. MacMillan and Santucci (1990) reported that seasonal temperature changes in aquaculture ponds caused changes in the microflora of Channel Catfish. These types of data would allow researchers to determine when and where certain bacteria occur within fish. Such information also would provide more accurate inferences to potential sources of resistance and more specific antimicrobial assays. Isolating resistance genes carried by these bacteria also would allow identification of potential sources of resistance.

Remediation and monitoring of antimicrobial resistance

While the discovery rate of new antimicrobial drugs is declining (Projan & Shlaes 2004), the dispersal and development of resistance is occurring at rapid rates (Pruden et al. 2006). Responsible use of antimicrobials in clinical, agricultural, and aquacultural settings is necessary to curb the spread of resistance. Reduced and improved use of antimicrobials can diminish resistance and potentially allow the drugs to reemerge as effective agents against bacterial infections (Barbosa & Levy 2000). Antimicrobial use could be reduced in aquaculture by integrating management practices that take a holistic approach to disease prevention and treatment. Ensuring the health of the fish by using quality feed, reducing stress, and selective breeding might improve disease resistance (Defoirdt et al. 2011). Improving the aquaculture environment by maintaining good water quality and quarantine procedures also would reduce disease (Defoirdt et al. 2011). Additionally, new methods, such as bacteriophage therapy and quorum-sensing inhibition, have shown potential for disease treatment in aquaculture

(Defoirdt et al. 2011). Probiotic use also has increased in aquaculture (Balcazar et al. 2006). Probiotics can reduce disease by competitive exclusion of pathogens and improved immune response and nutrient uptake in fish hosts (Balcazar et al. 2006). It would be naïve to expect prevention of infectious diseases in all situations without use of antimicrobials. However, new techniques coupled with the rational use of antimicrobials could help to reduce the prevalence and dispersal of resistance.

Currently, most wastewater treatment practices are ineffective at removing antimicrobials (Batt et al. 2006). Improving treatment plants to decrease antimicrobial concentrations in discharged effluent would further reduce the spread of antimicrobial resistance. Nakada et al. (2007) reported removal rates of 88% and 93% for erythromycin and azithromycin, respectively, following ozonation of wastewater. Nanofiltration has been an effective method for removing tetracycline class antimicrobials with removal rates up to 80% (Koyuncu et al. 2008). Ultraviolet radiation is ineffective at removing macrolide antimicrobials (Kim et al. 2009), but this method is effective against antimicrobials that are susceptible to photodegradation such as tetracyclines (Shaojun et al. 2008). Although it is unlikely that a single removal method would be effective at removing all antimicrobials due to the differences in the chemical nature of these compounds, a combination of processes would greatly increase the removal effectiveness of wastewater treatment plants.

Antimicrobial use in the clinical sector is strongly monitored and regulated in the United States, but the same cannot be said for agriculture or aquaculture where there are no central reporting or monitoring entities. The Food and Drug Administration is

responsible for regulating what antimicrobials are approved (McEwen & Fedorka-Cray 2002), but most estimates of antimicrobial use come from industry sources rather than actual usage rates at the production level (Benbrook 2002). Monitoring resistance in the environment is critical to maintain the efficacy of antimicrobial compounds. When a new antimicrobial compound is released, it is necessary that resistance monitoring in the environment begins immediately to determine the rate at which resistance is established. Rivers are areas of particular concern, given the numerous sources of resistance. The Kansas Department of Health and Environment has protocols to monitor heavy metals and PCBs in rivers and tissues of food fish. However, there are no monitoring protocols in place for antimicrobials or resistant bacteria. Mass spectrometry has been an effective method for screening water samples for antimicrobial compounds (Kolpin et al. 2002). Antimicrobial agar dilution, as used in the current study, could be used to screen for the presence of resistant organisms. Although such screening methods would require more labor and finance for laboratory analysis, they could be applied to current protocols without additional field sampling. Given the risks associated with exposure to antimicrobials and resistant bacteria, these compounds and resistant organisms should be included in environmental regulations, monitoring protocols, and warning systems.

LITERATURE CITED

Acred P, Brown DM, Turner DH, Wilson MJ. 1964. Pharmacology and chemotherapy of ampicillin – a new broad-spectrum penicillin. Brit. J. Pharmacol. 18:356-369.

Allen HK, Donato J, Wang HH, Cloud-Hansen KA, Davies J, Handelsman J. 2010. Call of the wild: antibiotic resistance genes in natural environments. Nature Rev. Microbiol. 8:251-259.

Alonso A, Sanchez P, Martinez JL. 2001. Environmental selection of antibiotic resistance genes. Envrion. Microbiol. 3:1-9.

Andersson DI, Levin BR. 1999. The biological cost of antibiotic resistance. Curr. Opin. Microbiol. 2:489-493.

Andrews JM. 2001. Determination of minimum inhibitory concentrations. J. Antimicrob. Chemother. 48:5-16.

Arias CA, Murray BE. 2009. Antibiotic-resistant bugs in the 21st century – a clinical super-challenge. N. Engl. J. Med. 360:439-443.

Balcazar JL, de Blas I, Ruiz-Zarzuela I, Cunningham D, Vendrell D, Muzquiz JL. 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114:173-186.

Baker-Austin C, Wright MS, Stepanauskas R, McArther JV. 2006. Co-selection of antibiotic and metal resistance. Trends Microbiol. 14:176-182.

Baquero F, Martinez JL, Canton R. 2008. Antibiotics and antibiotic resistance in water environments. Curr. Opin. Biotechnol. 19:260-265.

Barbosa TM, Levy SB. 2000. The impact of antibiotic use on resistance development and persistence. Drug Resistance Updates. 3:303-311.

Barghouthi SA. 2011. A universal method for the identification of bacteria based on general PCR primers. J. Indian Microbiol. 51:430-444.

Batt AL, Bruce IB, Aga DS. 2006. Evaluating the vulnerability of surface waters to antibiotic contamination from varying wastewater treatment plant discharges. Env. Poll. 142:295-302.

Benbrook CM. 2002. Antibiotic drug use in U.S. aquaculture. Institute for Agriculture and Trade Policy Report.

Bergogne-Berezin E, Towner KJ. 1996. *Acinetobacter* spp. as nosocomial pathogens: microbiological, clinical, and epidemiological features. Clin. Microbiol. Rev. 2:148-165.

Brisson-Noel A, Trieu-Cuot P, Courvalin P. 1988. Mechanism of action of spiramycin and other macrolides. J. Antimicrob. Chemother. 22:13-23.

Brooks RC, Heidinger RC, Kohler CC. 1994. Mass-marking otoliths of larval and juvenile Walleyes by immersion in oxytetracycline, Calcein, or Calcein blue. N. Am. J. Fish. Man. 14:143-150.

Burlingame MN. 1998. 1995 licensed angler use and preference survey and attitudes toward angling by secondary education students [Master‟s thesis]. Manhattan, KS: Kansas State University.

Bussiere DE, Muchmore SW, Dealwis CG, Schluckebier G, Nienaber VL, Edalji RP, Walter KA, Ladror US, Holzman TF, Abad-Zapatero C. 1998. Crystal structure of ErmC", an rRNA methyltransferase which mediates antibiotic resistance in bacteria. Biochemistry. 37:7103-7112.

Cabello FC. 2006. Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. Environ. Microbiol. 8:1137-1144.

Chao A. 1987. Estimating the population size for capture-recapture data with unequal catchability. Biometrics. 35:523-531.

Chapman FA. 1992. Farm-raised channel catfish. University of Florida: Institute of Food and Agricultural Sciences Extension Circular 1052.

Chin N, Neu HC. 1984. Ciprofloxacin, a quinolone carbolic acid compound active against aerobic and anaerobic bacteria. Antimicrob. Agents. Chemother. 25:319-326.

Citron DM, Ostovari MI, Karlsson A, Goldstein EJ. 1991. Evaluation of the E test for susceptibility testing of anaerobic bacteria. J. Clin. Microbiol. 29:2197-2203.

Clinical and Laboratory Standards Institute. 2012. Performance standards for antimicrobial susceptibility testing. Jan. Suppl:22, Vol. 31.

Coenye T, Vancanneyt M, Falsen E, Swings J, Vandamme P. 2003. *Achromobacter insolitus* sp. nov. and *Achromobacter spanius* sp. nov., from human clinical samples. Int. J. Sys. Evol. Microbiol. 53:1819-1824.

Costanzo SD, Murby J, Bates J. 2005. Ecosystem response to antibiotics entering the aquatic environment. Mar. Poll. Bull. 51:218-223.

Courvalin P. 1994. Transfer of antibiotic resistance genes between Gram-positive and Gram-negative bacteria. Antimicrob. Agents Chemother. 38:1447-1451.

Cross F, Collins J. 1995. Fishes in Kansas. University of Kansas Natural History Museum. Public Education Series No. 14:149-151.

Cummings JH, MacFarlane GT. 1997. Role of intestinal bacteria in nutrient metabolism. Clin. Nutri. 16:3-11.

D'Costa VM, McGrann KM, Hughes DW, Wright GD. 2006. Sampling the antibiotic resistome. Science. 311:374-377.

Dantas G, Sommer MOA, Oluwasegun RD, Church GM. 2008. Bacteria subsisting on antibiotics. Science. 320:100-102.

Davies J, Davies D. 2010. Origins and evolution of antibiotic resistance. Microbiol. Mol. Biol. Rev. 74:417-433.

Davies J, Spiegelman GB, Yim G. 2006. The world of subinhibitory antibiotic concentrations. Curr. Opin. Microbiol. 9:445-453.

Davies J. 1996. Bacteria on the rampage. Nature. 383:219-220.

Davis R, Markham A, Balfour JA. 1996. Ciprofloxacin: an updated review of its pharmacology, therapeutic efficacy, and tolerability. Drugs. 6:1019-1074.

Defoirdt T, Sorgeloos P, Bossier P. 2011. Alternatives to antibiotics for the control of bacterial disease in aquaculture. Curr. Opin. Microbiol. 14:251-258.

DePaola A, Peeler JT, Rodrick GE. 1995. Tetracycline resistance by bacteria in response to oxytetracycline-contaminated catfish feed. J. Aquat. Anim. Health. 7:155-160.

Dykhuizen DE. 1998. Santa Rosalia revisited: Why are there so many species of bacteria? Antonie van Leeuwenhoek. 73:25-33.

Edelmann P, Gallant J. 1977. Mistranslation in *E. coli*. Cell. 10:131-137.

Edmond MB, Ober JF, Weinbaum DL, Pfaller MA, Hwang T, Sanford MD, Wenzel RP. 1995. Vancomycin-resistant *Enterococcus faecium* bacteremia: Risk factors for infection. Clin. Infect. Dis. 20:1126-1133.

Ervik A, Thorsen B, Eriksen V, Lunestad BT, Samuelsen OB. 1994. Impact of administering antibacterial agents on wild fish and blue mussels *Mytilus edulis* in the vicinity of fish farms. Dis. Aquatic Org. 18:45-51.

Franche C, Lindstrom K, Elmerich C. 2009. Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant and Soil. 321:35-39.

Garibaldi L. 1996. List of animal species used in aquaculture. Food and Agriculture Organization of the United Nations. Fisheries. Circular No. 914.

Gaskins HR, Collier CT, Anderson DB. 2002. Antibiotics as growth promotants: mode of action. Anim. Biotech. 13:29-42.

Goldburg R, Naylor R. 2005. Future seascapes, fishing, and fish farming. Front. Ecol. Environ. 3:21-28.

Goñi-Urriza M, Capdepuy M, Arpin C, Raymond N, Caumette P, Quentin C. 2000. Impact of an urban effluent on antibiotic resistance of riverine Enterobacteriaceae and *Aeromonas* spp. Appl. Environ. Microbiol. 66:125-132.

Grisez L, Reyniers J, Verdonck L, Swings J, Ollevier F. 1997. Dominant intestinal microflora of sea bream and sea bass larvae, from two hatcheries, during larval development. Aquaculture. 155:387-399.

Grondel JL, Nouws JFM, De Jong M, Schutte AR, Driessens F. 1987. Pharmacokinetics and tissue distribution of oxytetracycline in carp, *Cyprinus carpio* L., following different routes of administration. J. Fish Diseases. 10:153-163.

Han XY, Andrade RA. 2005. *Brevundimonas diminuta* infections and its resistance to fluoroquinolones. J. Antimicrob. Chemother. 55:853-859.

Hirsch R, Ternes T, Haberer K, Kratz KL. 1999. Occurrence of antibiotics in the aquatic environment. Sci. Total Environ. 225:109-118.

Huber I, Spanggaard B, Appel KF, Rossen L, Nielsen T, Gram L. 2004. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (*Oncorhynchus mykiss*, Walbaum). J. Appl. Microbiol. 96, 117–132.

Huys G, Gevers D, Temmerman R, Cnockaert M, Denys R, Rhodes G, Pickup R, McGann P, Hiney M, Smith P, Swings J. 2001. Comparison of the antimicrobial tolerance of oxytetracycline-resistant heterotrophic bacteria isolated from hospital sewage and freshwater fishfarm water in Belgium. Syst. Appl. Microbiol. 24:122-140.

Jacobsen P, Berglind L. 1988. Persistence of oxytetracycline in sediments from fish farms. Aquaculture. 70:365-370.

Jones RN. 2006. Microbiological features of vancomycin in the 21st century: minimum inhibitory concentration creep, bactericidal/static activity, and applied breakpoints to predict clinical outcomes or detect resistant strains. Clin. Infect. Dis. 42:S13-24.

Kerry J, NicGabhainn S, Smith P. 1997. Changes in oxytetracycline resistance of intestinal microflora following administration of the agent to Atlantic salmon (*Salmo salar* L.) smolts in a marine environment. Aquaculture. 157:187-195.

Kim I, Yamashita N, Tanaka H. 2009. Performance of UV and UV/H_2O_2 processes for the removal of pharmaceuticals detected in secondary effluent of a sewage treatment plant in Japan. J. Haz. Mat. 166:1134-1140.

Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36:1202-1211.

Koyuncu I, Arikan OA, Wiesner MR, Rice C. 2008. Removal of hormones and antibiotics by nanofiltration membranes. J. Membr. Sci. 309:94-101.

Kruse H, Sorum H. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. Appl. Environ. Microbiol. 60:4015-4021.

Kümmerer K. 2003. Significance of antibiotics in the environment. J. Antimicrob. Chemother. 52:5-7.

Kümmerer K. 2004. Resistance in the environment. J. Antimicrob. Chemother. 54:311-320.

Kümmerer K. 2010. Pharmaceuticals in the environment. Annu. Rev. Environ. Resources. 35:57-75.

Leisner JJ, Laursen BG, Prevost H, Drider D, Dalgaard P. 2007. *Carnobacterium*: positive and negative effects in the environment and in foods. FEMS Microbiol. Rev. 31:592-613.

Levy SB, Marshall B. 2004. Antibiotic resistance worldwide: causes, challenges, and responses. Nature Medicine Supp. 10:122-129.

Levy SB. 1998. The challenge of antibiotic resistance. Sci. Am. 46-53.

Liau CH, Shollenberger LM. 2003. Survivability and long-term preservation of bacteria in water and phosphate-buffered saline. Lett. Appl. Microbiol. 37:45-50

Loch TP, Xu W, Fitzgerald SM, Faisal M. 2008. Isolation of a *Carnobacterium maltaromaticum*-like bacterium from systemically infected Lake Whitefish (*Coregonus clupeaformis*). FEMS Microbiol. Lett. 288:76-84.

Luke DR, Foulds G, Cohen SF, Levy B. 1996. Safety, toleration, and pharmacokinetics of intravenous azithromycin. Antimicrob. Agents. Chemother. 40:2577-2581.

MacFarlane GA, Beamish RJ. 1987. Selection of dosages of oxytetracycline for age validation studies. Can. J. Fish. Aquat. Sci. 44:905-909.

MacMillan JR, Santucci T. 1990. Seasonal trends in intestinal bacterial flora of farm-raised Channel Catfish. J. Aquat. Anim. Health. 2:217-222.

Maningo E, Watanakunakorn C. 1995. *Xanthomonas maltophilia* and *Pseudomonas cepacia* in lower respiratory tracts of patients in critical care units. J. Infect. 31:89-92.

Martinez JL, Baquero F. 2000. Mutation frequencies and antibiotic resistance. Antimicrob. Agents Chemother. 44:1771-1777.

Martinez JL. 2008. Antibiotics and antibiotic resistance genes in natural environments. Science. 321:365-367.

Martinez-Murcia AJ, Soler L, Jose Saavedra M, Chacon MR, Guarro J, Stackebrandt E, Jose Figueras M. 2005. Phenotypic, genotypic, and phylogenetic discrepancies to differentiate *Aeromonas salmonicida* from *Aeromonas bestiarum*. Int. Microbiol. 8:259-269.

McDermott C, Mylotte JM. 1984. *Morganella morganii*: epidemiology of bacterermic disease. Infect. Control. 5:131-137.

McEwen SA, Fedorka-Cray PJ. 2002. Antimicrobial use and resistance in animals. Clin. Infect. Dis. Supp. 3:93-106.

McPhearson RM, DePaola A, Zwyno SR, Motes ML, Jr., Guarino AM. 1991. Antibiotic resistance in Gram-negative bacteria from cultured catfish and aquaculture ponds. Aquaculture. 99:203-211.

Miranda CD, Zemelman R. 2002. Bacterial resistance to oxytetraycline in Chilean salmon farming. Aquaculture. 212:31-47.

Nakada N, Shinohara H, Murata A, Kiri K, Managaki S, Sato N, Takada H. 2007. Removal of selected pharmaceuticals and personal care products (PPCPs) and endocrine-disrupting chemicals (EDCs) during sand filtration and ozonation at a municipal sewage treatment plant. Water Research. 41:4373-4382.

Nishimori E, Kita-Tsukamoto K, Wakabayashi H. 2000. *Pseudomonas plecoglossicida* sp. nov., the causative agent of bacterial haemorrhagic ascites of Ayu, *Plecoglossus altivelis*. Int. J. Sys. Evol. Microbiol. 50:83-89.

Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara OB, Simpson GL, Solymos P, Stevens MHH, Wagner H. 2012. vegan: Community Ecology Package. R-package version 2.0-3. http://CRAN.R-project.org/package=vegan.

Oliphant CM, Green GM. 2002. Quinolones: a comprehensive review. Am. Fam. Physician. 65:455-464.

Perry RD, Fetherston JD. 1997. *Yersinia pestis* – etiologic agent of plague. Clin. Microbiol. Rev. 10:35-66.

Peters DH, Friedel HA, McTavis D. 1992. Azithromycin: a review of its antimicrobial activity, pharmacokinetic properties, and clinical efficacy. Drugs. 44:750-799.

Pfyffer GE. 1992. *Serratia fonticola* as an infectious agent. Eur. J. Clin. Microbiol. Infect. Dis. 11:199-200.

Philippon A, Labian R, Jacoby G. 1985. Extended spectrum beta-lactamases. Antimicrob. Agents Chemother. 28:302-307.

Piddock LJV. 2006. Multidrug-resistance efflux pumps – not just for resistance. Nature Rev. Microbiol. 4:629-636.

Projan SJ, Shlaes DM. 2004. Antibacterial drug discovery: is it all downhill from here? Clin. Microbiol. Infect. 10:18-22.

Pruden A, Pei R, Storteboom H, Carlson KH. 2006. Antibiotic resistance genes as emerging contaminants: Studies in northern Colorado. Environ. Sci. Technol. 40:7445-7450.

R Core Team. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, URL: http://www.R-project.org/.

Rhodes G, Huys G, Swings J, McGann P, Hiney M, Smith P, Pickup RW. 2000. Distribution of oxytetracycline resistance plasmids between aeromonads in hospital and aquaculture environments. Appl. Environ. Microbiol. 66:3883-3890.

Rivkina EM, Friedmann EI, McKay CP, Gilichinsky DA. 2000. Metabolic activity of permafrost bacteria below the freezing point. Appl. Environ. Microbiol. 66:3230-3233

Robertson PAW, O'Dowd C, Burrells C, Williams P, Austin B. 2000. Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhyncus mykiss*, Walbaum). Aquaculture. 185:235-243.

Roeselers G, Norris TB, Castenholz RW, Rysgaard S, Glud RN, Kuhl M, Muyzer G. 2007. Diversity of phototrophic bacteria in microbial mats from Arctic hot springs (Greenland). Environ. Microbiol. 9:26-38.

Sarter S, Nguyen HNK, Hung LT, Lazard J, Montet D. 2007. Antibiotic resistance in Gram-negative bacteria from farmed catfish. Food Control. 18:1391-1396.

Schnappinger D, Hillen W. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch. Micobiol. 165:359-369.

Schrader KK. 2008. Compounds with inhibitory activity against the Channel Catfish pathogens *Edwardsiella ictaluri* and *Flavobacterium columnare*. N. Am. J. Aquaculture. 70:147-153.

Schwartz T, Kohnen W, Jansen B, Obst U. 2003. Detection of antibiotic-resistant bacteria and their resistance genes in wastewater, surface water, and drinking water biofilms. FEMS Microbiol. Ecol. 43:325-335.

Shaojun J, Shourong Z, Daqlang Y, Lianhong W, Liangyan C. 2008. Aqueous oxytetracycline degradation and the toxicity change of degradation compounds in photoirradiation process. J. Environ. Sci. 20:806-813.

Shen LL, Baranowski J, Pernet AG. 1989. Mechanism of inhibition of DNA gyrase by quinolone antibacterials: specificity and cooperativity of drug binding to DNA. Biochemistry. 28:3879-3885.

Smith SR. 2009. Organic contaminants in sewage sludge (biosolids) and their significance for agricultural recycling. Phil. Trans. R. Soc. A. 367:4005-4041.

Spangaard B, Huber I, Nielsen J, Nielsen T, Appel KF, Gram L. 2000. The microflora of rainbow trout intestine: a comparison of traditional and molecular identification. Aquaculture. 182:1-15.

Spilker T, Vandamme P, LiPuma JJ. 2013. Identification and distribution of *Achromobacter* species in cystic fibrosis. J. Cystic Fibrosis. 12:298-301.

Spratt BG, Cromie KD. 1988. Penicillin-binding proteins of Gram-negative bacteria. Clin. Infect. Dis. 10:699-711.

Taylor PW. 2003. Multiple antimicrobial resistance in a chronic bacterial infection of koi carp. N. Am. J. Aquaculture. 65:120-125.

Thomas CM, Nielsen KM. 2005. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nature Rev. Microbiol. 3:711-721.

Verner-Jeffreys DW, Welch TJ, Schwarz T, Pond MJ, Woodward MJ, Haig SJ, Rimmer GSE, Roberts E, Morrison V, Baker-Austin C. 2009. High prevalence of multi-drug tolerant bacteria and associated antimicrobial resistance genes isolated from ornamental fish and their carriage water. PLoS ONE. 4.

Walsh C. 2000. Molecular mechanisms that confer antibacterial drug resistance. Nature. 406:775-781.

Walson JL, Marshall B, Pokhrel BM, Kafle KK, Levy SB. 2001. Carriage of antibiotic-resistant fecal bacteria in Nepal reflects proximity to Kathmandu. J. Infect. Dis. 184:1163-1169.

Waters CM, Bassler BL. 2005. Quorum sensing: cell-to-cell communication in bacteria. Annu. Rev. Cell. Dev. Biol. 21:319-346.

Wendel JL, Kelsch SW. 1999. Summer range and movement of Channel Catfish in the Red River of the North. Am. Fish. Soc. Symp. 24:203-214.

Wiklund T, Dalsgaard I. 1998. Occurrence and significance of atypical *Aeromonas salmonicida* in non-salmonid and salmonid fish species: a review. Dis. Aquatic Org. 32:49-69.

Wilhelm, MP. 1991. Vancomycin. Mayo Clin. Proc. 66:1165-1170.

Willems RJL, Top J, van Santen M, Robinson DA, Coque TM, Baquero F, Grundmann H, Bonten MJM. 2005. Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. Emerg. Infect. Diseases. 11:821-828.

Williams DH. 1996. The glycopeptide story – how to kill the deadly „superbugs". Nat. Prod. Rep. 13:469-477.

World Health Organization (WHO). 2013. World Health Organization annual report on antimicrobial drug resistance.

Zaffiri L, Gardner J, Toledo-Pereyra LH. 2012. History of antibiotics. From Salvarsan to Cephalosporins. J. Invest. Surg. 25:67-77.

TABLE 1. Antimicrobials, agar concentrations, and references for resistance breakpoints used during antimicrobial agar screening.

Antimicrobial Compound	Mueller-Hinton Agar Concentration (μg/ml)	Reference for Resistance Breakpoint
Ampicillin Sodium Salt	32	CLSI 2012
Azithromycin Dihydrate	8	CLSI 2012
Ciprofloxacin Hydrochloride Monohydrate	4	CLSI 2012
Gentamicin Sulfate	16	CLSI 2012
Oxytetracycline Hydrochloride	25	Kerry et al. 1997
Vancomycin Hydrochloride	32	CLSI 2012

TABLE 2. Summary table of resistant bacteria species identified from Channel Catfish intestinal contents and water samples from the Arkansas and South Fork Ninnescah rivers in Kansas, the antimicrobials compounds they were resistant to, and presence of multi-drug resistance. Abbreviations representing the compounds are as follows: AM is ampicillin, AZ is azithromycin, CI is ciprofloxacin, GE is gentamicin, and OT is oxytetracycline.

Bacteria Species	Compound(s)	MDR
Achromobacter spanius	CI	
Acinetobacter haemolyticus	OT	
Aeromonas bestiarum	GE, OT	*
Aeromonas salmonicida	AM, OT	
Brevundimonas diminuta	AM	
Carnobacterium maltaromaticum	AM	
Citrobacter freundii	AZ	
Comamonas jiangudensis	AM	
Enterococcus faecium	CI	
Escherichia fergusonii	GE	
Microbacterium flavescens	CI	
Microbacterium hatanonis	CI	
Microbacterium lacus	CI	
Morganella morganii	OT	
Oerskovia paurometabola	CI	
Oerskovia turbata	CI, GE	*
Providencia heimbachae	OT	
Pseudomonas fluorescens	AM	
Pseudomonas fragi	AM	
Pseudomonas gessardii	AM, AZ	

Bacteria Species	Compound(s)	MDR
Pseudomonas lundensis	AM, OT	
Pseudomonas mandelii	AM, AZ	*
Pseudomonas meridiana	AM	
Pseudomonas migulae	AZ	
Pseudomonas plecoglossicida	AM	
Pseudomonas poae	AZ	
Pseudomonas protegens	AM, AZ	
Pseudomonas pseudoalcaligenes	AZ, CI	*
Pseudomonas umsongensis	AM	
Pseudomonas vancouverensis	AM	
Pseudomonas veronii	AZ	
Sediminibacterium salmoneum	AZ, CI, OT	*
Serratia fonticola	AM, OT	*
Shewanella putrefaciens	AZ, CI, OT	*
Sphingobacterium faecium	GE	
Sphingomonas melonis	CI	
Stenotrophomonas maltophilia	CI, OT	*
Vitreoscilla stercoraria	OT	
Yersinia intermedia	AZ	

TABLE 3. Site occurrence table for bacteria species isolated from Channel Catfish in the Arkansas and South Fork Ninnescah rivers with distance (rkm) and direction from the primary effluent. Domestic effluent from Wichita, KS was the presumed source on the Arkansas River while hatchery effluent from the Pratt Fish Hatchery was the presumed source on the SF Ninnescah River.

Site Distance from Primary Effluent:	29.5 rkm Downstrm	3.25 rkm Downstrm	30.0 rkm Upstream	61.5 rkm Upstream	7.25 rkm Upstream	3.25 rkm Upstream	2.0 rkm Downstrm	55.75 rkm Downstrm
Bacterial Species	AR1	AR2	AR3	AR4	NR1	NR2	NR3	NR4
Achromobacter spanius				X				
Aeromonas bestiarum	X	X						
Aeromonas salmonicida salmonicida	X	X						
Brevundimonas diminuta		X						
Carnobacterium maltaromaticum						X		
Citrobacter freundii				X				
Comamonas testosteroni	X							
Enterococcus faecium			X					
Escherichia fergusonii		X						
Microbacterium flavescens							X	
Microbacterium lacus							X	
Morganella morganii morganii			X					
Oerskovia paurometabola				X				
Oerskovia turbata							X	
Providencia heimbachae		X	X	X	X	X		
Pseudomonas fragi		X						X
Pseudomonas gessardii					X	X		X
Pseudomonas lundensis	X	X						
Pseudomonas migulae		X						
Pseudomonas protegens			X	X	X			
Pseudomonas pseudoalcaligenes	X	X	X			X	X	

TABLE 3. (continued)

Bacterial Species	AR1	AR2	AR3	AR4	NR1	NR2	NR3	NR4
Pseudomonas veronii			X			X		
Sediminibacterium salmoneum	X	X		X			X	X
Serratia fonticola	X		X	X			X	X
Shewanella putrefaciens		X	X	X			X	
Sphingobacterium faecium		X						
Sphingomonas melonis						X		
Vitreoscilla stercoraria								X
Yersinia intermedia	X							
Number of bacteria species per site:	8	12	8	8	3	7	7	5

FIGURE 1. Map of sample collection sites for antimicrobial-resistant bacteria on the Arkansas and South Fork Ninnescah rivers.

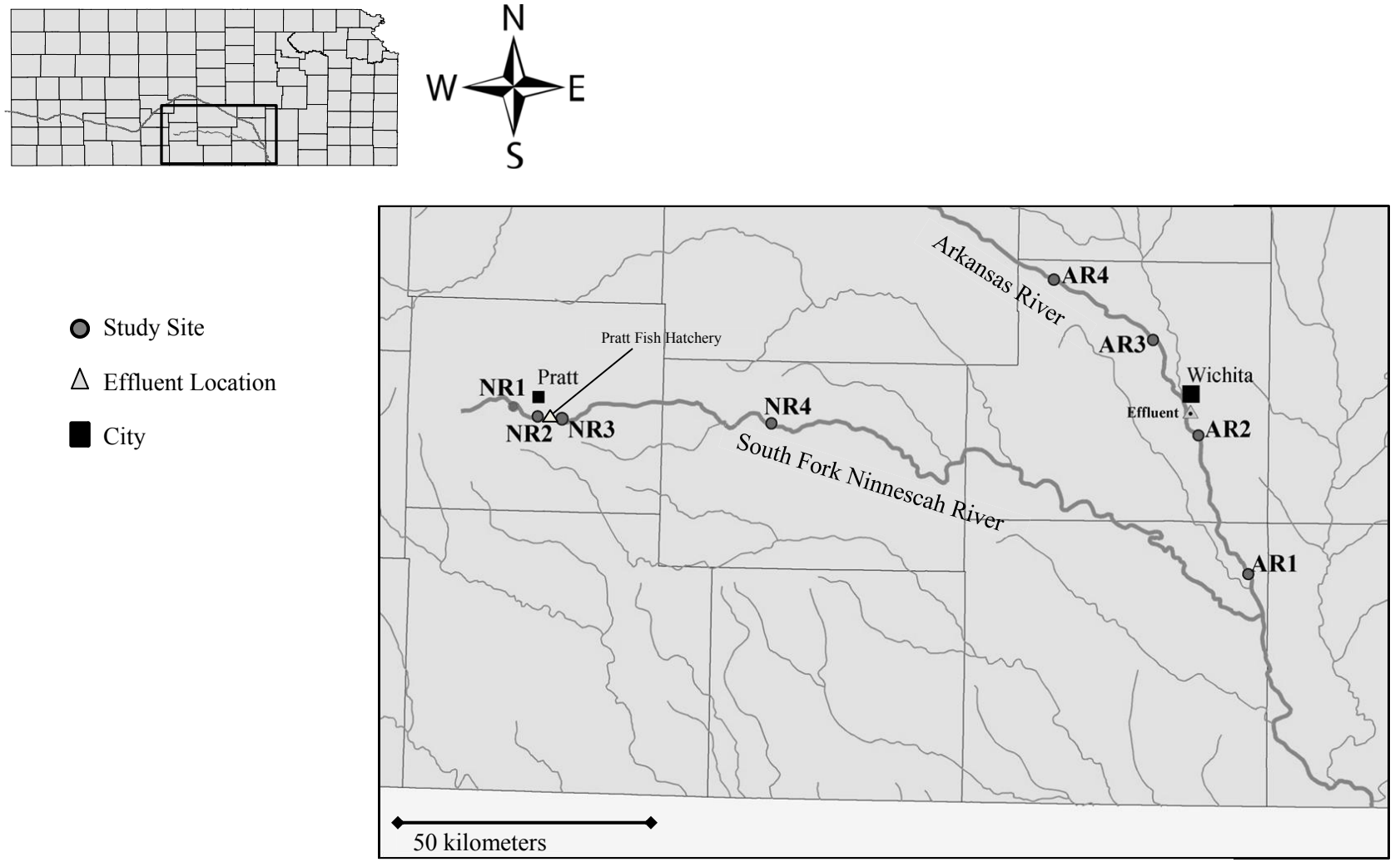


FIGURE 2. Tetracycline E-test results indicating an MIC value of 96.0 µg/ml for *Providencia heimbachae* DSM 3591 isolated from a Channel Catfish at site AR2.

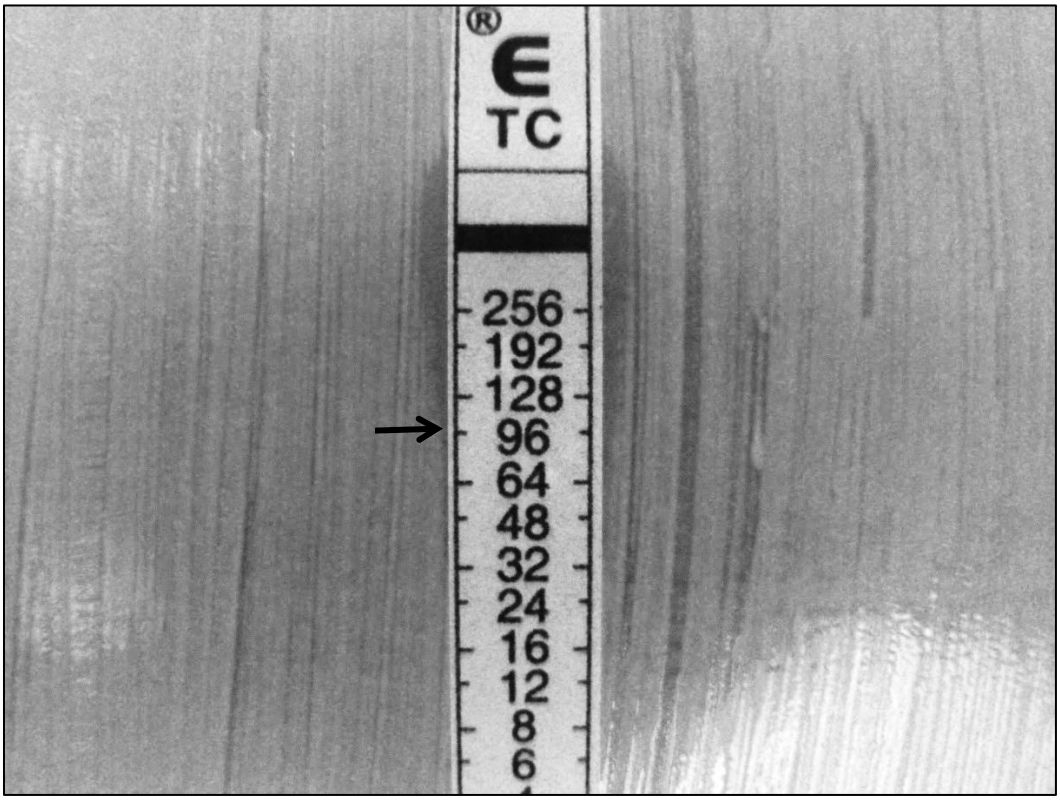
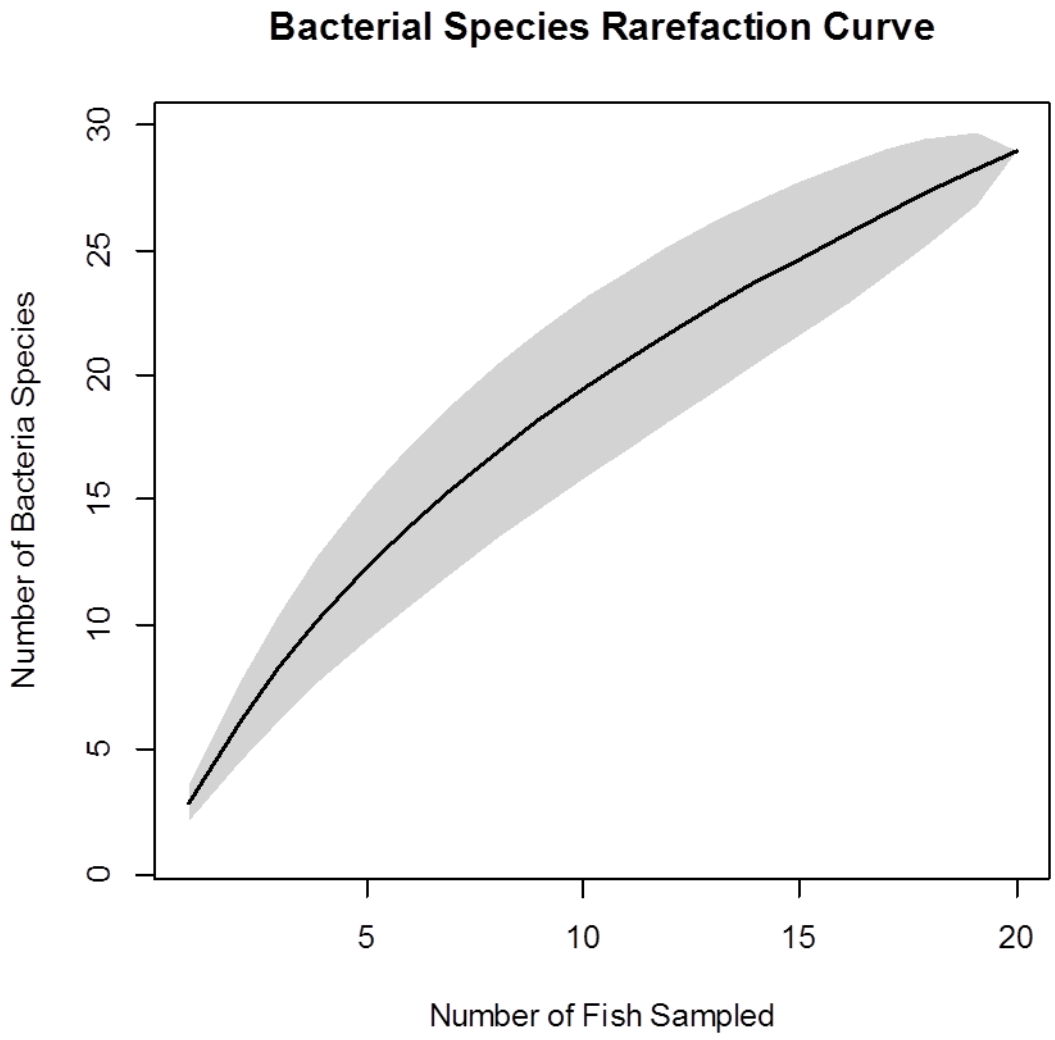


FIGURE 3. Rarefaction curve of bacteria community data of fish from the Arkansas and South Fork Ninnescah rivers with the black line representing the number of bacteria species and the shaded gray area representing the confidence intervals of 500 permutations.



APPENDIX 1. Survey locations for antimicrobial resistant bacteria in the Arkansas and South Fork Ninnescah rivers during the spring of 2012. The main effluent source on the Arkansas River was domestic effluent from Wichita, KS. The main source on the SF Ninnescah River was hatchery effluent from the Pratt Fish Hatchery.

Site	River	River km from Main Effluent Source		County	Date	Latitude	Longitude
AR1	Arkansas River	29.50	Downstream	Sumner	5 May 2012	37.391636	-97.194819
AR2	Arkansas River	3.25	Downstream	Sedgwick	5 & 24 May 2012	37.565953	-97.287307
AR3	Arkansas River	30	Upstream	Sedgwick	6 & 24 May 2012	37.781583	-97.390318
AR4	Arkansas River	61.5	Upstream	Sedgwick	25 May 2012	37.896050	-97.665230
NR1	South Fork Ninnescah River	7.25	Upstream	Pratt	31 March 2012	37.639879	-98.766704
NR2	South Fork Ninnescah River	4.25	Upstream	Pratt	14 April 2012	37.633364	-98.734756
NR3	South Fork Ninnescah River	2	Downstream	Pratt	14 April 2012	37.629086	-98.676670
NR4	South Fork Ninnescah River	55.75	Downstream	Kingman	15 April 2012	37.645499	-98.255655

APPENDIX 2. Multi-drug resistant bacteria species with site, sample, and minimum inhibitory concentration data. Column MIC 1 contains minimum inhibitory concentrations to the associated compound in column AMR 1, whereas column MIC 2 contains the same data for compounds in column AMR 2.

Bacterial Species	Sample Origin	Site	AMR 1	MIC 1 (μg/ml)	AMR 2	MIC 2 (μg/ml)
Aeromonas bestiarum strain CIP 7430	Fish	AR2	OTC	16	Gen	ND
Oerskovia turbata strain 27	Fish	NR3	Cip	6	Gen	ND
Pseudomonas pseudoalcaligenes strain Stanier 63	Fish	AR2	Azi	8	Cip	4
Serratia fonticola strain DSM 4576	Fish	AR4	OTC	≥256	Amp	ND
Shewanella putrefaciens strain Hammer 95	Fish	NR3	Azi	64	OTC	24
Pseudomonas mandelii strain CIP 105273	Water	AR1	Azi	ND	Amp	ND
Sediminibacterium salmoneum strain NJ-44	Water	AR1	Azi	8	Cip	4
Sediminibacterium salmoneum strain NJ-44	Water	NR1	Cip	6	OTC	≥256
Stenotrophomonas maltophilia strain R551-3	Water	AR3	Cip	6	OTC	≥256

APPENDIX 3. Ampicillin-resistant bacteria species with site, sample, and BLAST identity percentage data. Environmental sample ID is the unique code given to fish and water samples. Isolate ID is the unique code given to pure isolates for frozen storage. Symbol * indicates the bacteria species exhibited multi-drug resistance.

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	BLAST Identity %
AR1	AR1-1	Fish	Amp1	*Serratia fonticola* strain DSM 4576	99
AR1	AR1-2	Fish	Amp3	*Comamonas jiangduensis* strain YW1	99
AR2	AR2-1	Fish	Amp5	*Brevundimonas diminuta* ATCC 11568 strain IAM 12691	98
AR2	AR2-2	Fish	Amp6	*Aeromonas salmonicida* subsp. *salmonicida* strain CECT 894	99
AR2	AR2-12	Fish	Amp9	*Pseudomonas lundensis* strain ATCC 49968	99
AR2	AR2-14	Fish	Amp10	*Pseudomonas fragi* strain ATCC 4973	99
AR3	AR3-1	Fish	Amp12	*Pseudomonas protegens* Pf-5 strain Pf-5	99
AR3	AR3-2	Fish	Amp13	*Pseudomonas protegens* Pf-5 strain Pf-5	99
AR3	AR3-4	Fish	Amp16	*Serratia fonticola* strain DSM 4576	99
AR4	AR4-1	Fish	Amp17	*Pseudomonas protegens* Pf-5 strain Pf-5	99
AR4	AR4-1	Fish	Amp18	*Serratia fonticola* strain DSM 4576*	99
NR1	NR1-1	Fish	Amp19	*Pseudomonas protegens* Pf-5 strain Pf-5	99
NR2	NR2-F	Fish	Amp26	*Carnobacterium maltaromaticum* LMA28	99
NR3	NR3-2	Fish	Amp29	*Serratia fonticola* strain DSM 4576	99
NR4	NR4-1	Fish	Amp31	*Serratia fonticola* strain DSM 4576	99
NR4	NR4-2	Fish	Amp32	*Pseudomonas fragi* strain ATCC 4973	99
AR1	AR1-W	Water	Amp35	*Pseudomonas mandelii* strain CIP 105273*	99

APPENDIX 3. (continued)

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	BLAST Identity %
AR2	AR2-W	Water	Amp38	*Pseudomonas plecoglossicida* strain FPC951	99
AR3	AR3-W	Water	Amp40	*Pseudomonas protegens* Pf-5 strain Pf-5	99
NR1	NR1-W	Water	Amp43	*Pseudomonas gessardii* strain CIP 105469	99
NR1	NR1-W	Water	Amp44	*Pseudomonas umsongensis* strain Ps 3-10	99
NR2	NR2-W	Water	Amp45	*Pseudomonas fluorescens* Pf0-1 strain Pf0-1	99
NR2	NR2-W	Water	Amp46	*Pseudomonas vancouverensis* strain ATCC 700688	99
NR3	NR3-W	Water	Amp48	*Pseudomonas meridiana* strain CMS 38	99
NR4	NR4-W	Water	Amp49	*Pseudomonas gessardii* strain CIP 105469	99
NR4	NR4-W	Water	Amp50	*Pseudomonas mandelii* strain CIP 105273	99

APPENDIX 4. Azithromycin-resistant bacteria species with site, sample, minimum inhibitory concentration, and BLAST identity percentage data. Environmental sample ID is the unique code given to fish and water samples. Isolate ID is the unique code given to pure isolates for frozen storage. Symbol * indicates the bacteria species exhibited multi-drug resistance. Abbreviation ND indicates the MIC was not tested due to loss of viability.

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	MIC (µg/ml)	BLAST Identity %
AR1	AR1-2	Fish	Azi2	*Yersinia intermedia* ATCC 29909	≥256	99
AR2	AR2-2	Fish	Azi3	*Pseudomonas migulae* strain CIP 105470	ND	99
AR2	AR2-1	Fish	Azi37	*Pseudomonas pseudoalcaligenes* strain Stanier 63	64	96
AR2	AR2-14	Fish	Azi6	*Shewanella putrefaciens* strain LMG 26268	128	99
AR2	AR2-14	Fish	Azi7	*Pseudomonas pseudoalcaligenes* strain Stanier 63*	8	97
AR3	AR3-1	Fish	Azi8	*Shewanella putrefaciens* strain LMG 26268	192	99
AR3	AR3-2	Fish	Azi9	*Shewanella putrefaciens* strain LMG 26268	≥256	95
AR3	AR3-3	Fish	Azi10	*Shewanella putrefaciens* strain Hammer 95	ND	97
AR3	AR3-3	Fish	Azi11	*Pseudomonas pseudoalcaligenes* strain Stanier 63	ND	97
AR3	AR3-4	Fish	Azi12	*Pseudomonas veronii* strain CIP 104663	ND	99
AR4	AR4-1	Fish	Azi13	*Citrobacter freundii* strain DSM 30039	ND	99
AR4	AR4-2	Fish	Azi14	*Shewanella putrefaciens* strain LMG 26268	ND	96
AR4	AR4-2	Fish	Azi15	*Pseudomonas protegens* Pf-5 strain Pf-5	ND	100
NR1	NR1-1	Fish	Azi16	*Pseudomonas gessardii* strain CIP 105469	192	99
NR2	NR2-F	Fish	Azi19	*Pseudomonas gessardii* strain CIP 105469	ND	99
NR2	NR3-1	Fish	Azi20	*Pseudomonas veronii* strain CIP 104663	ND	99
NR3	NR3-2	Fish	Azi21	*Shewanella putrefaciens* strain Hammer 95*	64	99

APPENDIX 4. (continued)

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	MIC (μg/ml)	BLAST Identity %
NR3	NR3-2	Fish	Azi22	*Pseudomonas pseudoalcaligenes* strain Stanier 63	≥256	97
NR4	NR4-2	Fish	Azi24	*Pseudomonas gessardii* strain CIP 105469	ND	99
AR1	AR1-W	Water	Azi25	*Sediminibacterium salmoneum* strain NJ-44*	8	96
AR1	AR1-W	Water	Azi26	*Pseudomonas mandelii* strain CIP 105273*	ND	99
AR2	AR2-W	Water	Azi27	*Sediminibacterium salmoneum* strain NJ-44	ND	96
AR2	AR2-W2	Water	Azi28	*Pseudomonas gessardii* strain CIP 105469	ND	99
NR4	NR4-W	Water	Azi36	*Pseudomonas poae* strain P 527/13	192	99

APPENDIX 5. Ciprofloxacin-resistant bacteria species with site, sample, minimum inhibitory concentration, and BLAST identity percentage data. Environmental sample ID is the unique code given to fish and water samples. Isolate ID is the unique code given to pure isolates for frozen storage. Symbol * indicates the bacteria species exhibited multi-drug resistance.

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	MIC (μg/ml)	BLAST Identity %
AR1	AR1-1	Fish	Cip39	*Pseudomonas pseudoalcaligenes* strain Stanier 63	6	96
AR2	AR2-2	Fish	Cip7	*Shewanella putrefaciens* strain LMG 26268	8	99
AR2	AR2-14	Fish	Cip9	*Pseudomonas pseudoalcaligenes* strain Stanier 63*	4	97
AR3	AR3-1	Fish	Cip10	*Enterococcus faecium* Aus0004 strain Aus0004	≥32	99
AR3	AR3-1	Fish	Cip11	*Pseudomonas pseudoalcaligenes* strain Stanier 63	≥32	96
AR3	AR3-4	Fish	Cip14	*Enterococcus faecium* Aus0004 strain Aus0004	≥32	97
AR4	AR4-1	Fish	Cip15	*Achromobacter spanius* strain LMG 5911	6	99
AR4	AR4-2	Fish	Cip18	*Oerskovia paurometabola* strain DSM 14281	6	99
NR2	NR2-F	Fish	Cip21	*Sphingomonas melonis* strain DAPP-PG 224	6	93
NR3	NR3-1	Fish	Cip22	*Microbacterium flavescens* strain 401	4	99
NR3	NR3-1	Fish	Cip23	*Microbacterium lacus* strain A5E-52	4	99
NR3	NR3-2	Fish	Cip24	*Oerskovia turbata* strain 27*	6	100
AR1	AR1-W	Water	Cip28	*Sediminibacterium salmoneum* strain NJ-44*	4	96
AR3	AR3-W	Water	Cip33	*Stenotrophomonas maltophilia* strain R551-3*	6	99
NR1	NR1-W	Water	Cip34	*Sediminibacterium salmoneum* strain NJ-44*	6	96
NR1	NR1-W	Water	Cip35	*Microbacterium hatanonis* strain JCM 14558	6	99

APPENDIX 6. Gentamicin-resistant bacteria species with site, sample, and BLAST identity percentage data. Environmental sample ID is the unique code given to fish and water samples. Isolate ID is the unique code given to pure isolates for frozen storage. Symbol * indicates the bacteria species exhibited multi-drug resistance.

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	BLAST Identity %
AR1	AR1-1	Fish	Gen1	*Aeromonas bestiarum* strain CIP 7430	99
AR2	AR2-1	Fish	Gen5	*Sphingobacterium faecium* strain DSM 11690	99
AR2	AR2-2	Fish	Gen7	*Aeromonas bestiarum* strain CIP 7430*	99
AR2	AR2-12	Fish	Gen8	*Escherichia fergusonii* ATCC 35469	99
NR3	NR3-2	Fish	Gen27	*Oerskovia turbata* strain 27*	99

APPENDIX 7. Oxytetracycline-resistant bacteria species with site, sample, minimum inhibitory concentration, and multidrug resistance data. Environmental sample ID is the unique code given to fish and water samples. Isolate ID is the unique code given to pure isolates for frozen storage. Symbol * indicates the bacteria species exhibited multi-drug resistance.

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	MIC (µg/ml)	BLAST Identity %
AR1	AR1-1	Fish	OTC1	*Pseudomonas lundensis* strain ATCC 49968	≥256	99
AR1	AR1-1	Fish	OTC2	*Aeromonas salmonicida* subsp. *salmonicida* CECT 894	6	100
AR1	AR1-1	Fish	OTC3	*Sediminibacterium salmoneum* strain NJ-44	≥256	96
AR1	AR1-2	Fish	OTC4	*Aeromonas bestiarum* strain CIP 7430	64	96
AR2	AR2-1	Fish	OTC5a	*Sediminibacterium salmoneum* strain NJ-44	64	96
AR2	AR2-2	Fish	OTC5b	*Aeromonas bestiarum* strain CIP 7430*	16	100
AR2	AR2-12	Fish	OTC7	*Providencia heimbachae* strain : DSM 3591	96	99
AR2	AR2-14	Fish	OTC8	*Providencia heimbachae* strain : DSM 3591	≥256	99
AR3	AR3-1	Fish	OTC9	*Providencia heimbachae* strain : DSM 3591	192	99
AR3	AR3-4	Fish	OTC12	*Morganella morganii* subsp. *morganii* KT	16	99
AR4	AR4-1	Fish	OTC13	*Providencia heimbachae* strain : DSM 3591	128	99
AR4	AR4-1	Fish	OTC14	*Serratia fonticola* strain DSM 4576*	≥256	99
AR4	AR4-2	Fish	OTC15	*Sediminibacterium salmoneum* strain NJ-44	32	96
NR1	NR1-1	Fish	OTC16	*Providencia heimbachae* strain : DSM 3591	96	99
NR2	NR2-1	Fish	OTC18	*Providencia heimbachae* strain : DSM 3591	128	99
NR3	NR3-2	Fish	OTC20	*Shewanella putrefaciens* strain LMG 26268*	24	99
NR3	NR3-2	Fish	OTC21	*Sediminibacterium salmoneum* strain NJ-44	128	96

APPENDIX 7. (continued)

Site	Environmental Sample ID	Sample Origin	Isolate ID	Bacterial Species	MIC (μg/ml)	BLAST Identity %
NR4	NR4-2	Fish	OTC22	*Vitreoscilla stercoraria* strain Gottingen 1488-6	48	94
NR4	NR4-1	Fish	OTC30	*Sediminibacterium salmoneum* strain NJ-44	64	96
AR2	AR2-W	Water	OTC25	*Acinetobacter haemolyticus* strain DSM 6962	48	97
AR2	AR2-W2	Water	OTC26	*Sediminibacterium salmoneum* strain NJ-44	64	96
AR3	AR3-W	Water	OTC27	*Stenotrophomonas maltophilia* R551-3*	≥256	99
NR1	NR1-W	Water	OTC28	*Sediminibacterium salmoneum* strain NJ-44*	≥256	96

APPENDIX 8. Site occurrence table of resistance to five examined antimicrobial compounds from Channel Catfish intestinal bacteria from the Arkansas and South Fork Ninnescah rivers.

	Antimicrobial Resistance in Fish				
Site	Amp	Azi	Cip	Gen	OTC
AR1	X	X	X	X	X
AR2	X	X	X	X	X
AR3	X	X	X		X
AR4	X	X	X		X
NR1	X	X			X
NR2	X	X	X		X
NR3	X	X	X	X	X
NR4	X	X			X

APPENDIX 9. Table of resistant bacteria and their associated GenBank accession numbers for access to 16S ribosomal RNA gene sequences. Abbreviation N/A indicates sequences were not submitted to GenBank for those isolates.

Isolate ID	Bacteria Species	GenBank Accession
Amp1	*Serratia fonticola* strain DSM 4576	KJ726543
Amp10	*Pseudomonas fragi* strain ATCC 4973	KJ726544
Amp12	*Pseudomonas protegens* Pf-5 strain Pf-5	KJ726545
Amp13	*Pseudomonas protegens* Pf-5 strain Pf-5	KJ726546
Amp16	*Serratia fonticola* strain DSM 4576	KJ726547
Amp17	*Pseudomonas protegens* Pf-5 strain Pf-5	KJ726548
Amp18	*Serratia fonticola* strain DSM 4576	KJ726549
Amp19	*Pseudomonas protegens* Pf-5 strain Pf-5	KJ726550
Amp26	*Carnobacterium maltaromaticum* LMA28	KJ726551
Amp29	*Serratia fonticola* strain DSM 4576	KJ726552
Amp3	*Comamonas testosteroni* CNB-2 strain CNB-1	KJ726553
Amp31	*Serratia fonticola* strain DSM 4576	KJ726554
Amp32	*Pseudomonas fragi* strain ATCC 4973	KJ726555
Amp35	*Pseudomonas mandelii* strain CIP 105273	KJ726556
Amp38	*Pseudomonas plecoglossicida* strain FPC951	KJ726557
Amp40	*Pseudomonas protegens* Pf-5 strain Pf-5	KJ726558
Amp43	*Pseudomonas gessardii* strain CIP 105469	KJ726559
Amp44	*Pseudomonas umsongensis* strain Ps 3-10	KJ726560
Amp45	*Pseudomonas fluorescens* Pf0-1 strain Pf0-1	KJ726561
Amp46	*Pseudomonas vancouverensis* strain ATCC 700688	KJ726562
Amp48	*Pseudomonas meridiana* strain CMS 38	KJ726563
Amp49	*Pseudomonas gessardii* strain CIP 105469	KJ726564
Amp5	*Brevundimonas diminuta* ATCC 11568 strain IAM 12691	KJ726565
Amp50	*Pseudomonas mandelii* strain CIP 105273	KJ726566
Amp6	*Aeromonas salmonicida* subsp. *salmonicida* strain CECT 894	KJ726567
Amp9	*Pseudomonas lundensis* strain ATCC 49968	KJ726568
Azi10	*Shewanella putrefaciens* strain Hammer 95	KJ726597
Azi11	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726598
Azi12	*Pseudomonas veronii* strain CIP 104663	KJ726599
Azi13	*Citrobacter freundii* strain DSM 30039	KJ726569
Azi15	*Pseudomonas protegens* Pf-5 strain Pf-5	KJ726600

APPENDIX 9. (continued)

Isolate ID	Bacteria Species	GenBank Accession
Azi16	*Pseudomonas gessardii* strain CIP 105469	KJ726601
Azi19	*Pseudomonas gessardii* strain CIP 105469	KJ726570
Azi2	*Yersinia intermedia* ATCC 29909	KJ726602
Azi20	*Pseudomonas veronii* strain CIP 104663	KJ726603
Azi21	*Shewanella putrefaciens* strain Hammer 95	KJ726604
Azi22	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726571
Azi24	*Pseudomonas gessardii* strain CIP 105469	KJ726605
Azi25	*Sediminibacterium salmoneum* strain NJ-44	KJ726572
Azi26	*Pseudomonas mandelii* strain CIP 105273	KJ726606
Azi27	*Sediminibacterium salmoneum* strain NJ-44	KJ726607
Azi28	*Pseudomonas gessardii* strain CIP 105469	KJ726608
Azi3	*Pseudomonas migulae* strain CIP 105470	KJ726609
Azi36	*Pseudomonas poae* strain P 527/13	KJ726610
Azi37	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726611
Azi6	*Shewanella putrefaciens* strain LMG 26268	KJ726573
Azi7	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726612
Azi8	*Shewanella putrefaciens* strain LMG 26268	KJ726574
Cip10	*Enterococcus faecium* Aus0004 strain Aus0004	KJ726575
Cip11	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726576
Cip14	*Enterococcus faecium* Aus0004 strain Aus0004	KJ726577
Cip15	*Achromobacter spanius* strain LMG 5911	KJ726578
Cip18	*Oerskovia paurometabola* strain DSM 14281	KJ726579
Cip21	*Sphingomonas melonis* strain DAPP-PG 224	KJ726580
Cip22	*Microbacterium flavescens* strain 401	KJ726613
Cip23	*Microbacterium lacus* strain A5E-52	KJ726614
Cip24	*Oerskovia turbata* strain 27	KJ726615
Cip28	*Sediminibacterium salmoneum* strain NJ-44	KJ726581
Cip33	*Stenotrophomonas maltophilia* strain R551-3	KJ726616
Cip34	*Microbacterium hatanonis* strain JCM 14558	KJ726582
Cip35	*Sediminibacterium salmoneum* strain NJ-44	KJ726617
Cip39	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726583
Cip7	*Shewanella putrefaciens* strain LMG 26268	KJ726584
Cip9	*Pseudomonas pseudoalcaligenes* strain Stanier 63	KJ726585
Gen1	*Aeromonas bestiarum* strain CIP 7430	KJ726586

APPENDIX 9. (continued)

Isolate ID	Bacteria Species	GenBank Accession
Gen27	*Oerskovia turbata* strain 27	KJ726587
Gen5	*Sphingobacterium faecium* strain DSM 11690	KJ726588
Gen7	*Sphingobacterium faecium* strain DSM 11690	KJ726589
Gen8	*Escherichia fergusonii* ATCC 35469	KJ726590
OTC1	*Pseudomonas lundensis* strain ATCC 49968	KJ726618
OTC12	*Morganella morganii* subsp. *morganii* KT	KJ726619
OTC13	*Providencia heimbachae* strain : DSM 3591	KJ726620
OTC14	*Serratia fonticola* strain DSM 4576	KJ726621
OTC15	*Sediminibacterium salmoneum* strain NJ-44	KJ726591
OTC16	*Providencia heimbachae* strain : DSM 3591	KJ726622
OTC18	*Providencia heimbachae* strain : DSM 3591	KJ726623
OTC2	*Aeromonas salmonicida* subsp. *salmonicida* CECT 894	KJ726624
OTC20	*Shewanella putrefaciens* strain LMG 26268	KJ726592
OTC21	*Sediminibacterium salmoneum* strain NJ-44	KJ726625
OTC22	*Vitreoscilla stercoraria* strain Gottingen 1488-6	KJ726593
OTC25	*Acinetobacter haemolyticus* strain DSM 6962	KJ726594
OTC26	*Sediminibacterium salmoneum* strain NJ-44	KJ726595
OTC27	*Stenotrophomonas maltophilia* R551-3	KJ726626
OTC28	*Sediminibacterium salmoneum* strain NJ-44	KJ726627
OTC3	*Sediminibacterium salmoneum* strain NJ-44	KJ726628
OTC30	*Sediminibacterium salmoneum* strain NJ-44	KJ726629
OTC4	*Aeromonas bestiarum* strain CIP 7430	KJ726596
OTC5a	*Sediminibacterium salmoneum* strain NJ-44	KJ726630
OTC5b	*Aeromonas bestiarum* strain CIP 7430	KJ726631
OTC7	*Providencia heimbachae* strain : DSM 3591	KJ726632
OTC8	*Providencia heimbachae* strain : DSM 3591	KJ726633
OTC9	*Providencia heimbachae* strain : DSM 3591	KJ726634
Azi9	*Shewanella putrefaciens* strain LMG 26268	N/A
Azi14	*Shewanella putrefaciens* strain LMG 26268	N/A