Validation of a UPLC-MS/MS method for measuring the extent of antibiotic contamination in seafood and assessing the role of thermal treatment

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SHIVA EMAMI

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Approved:

Ameer Y. Taha, Chair

Daniela Barile

Nitin Nitin

Committee in Charge

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Abstract

Chronic antibiotic exposure in humans can promote the evolution of antibiotic resistant microbes that can directly transfer to humans or host antibiotic resistant genes that can transmit to other infectious human pathogens. Sources of human antibiotic exposure vary, but farmed seafood is of great concern because the use of medicinal antibiotics in aquaculture for prophylactic purposes may be associated with residual levels of antibiotics in seafood products, and ultimately, exposure to humans. Recent studies have also documented the presence of antibiotics in wild seafood, suggesting environmental contamination, but a direct and comprehensive comparison of antibiotic profiles and concentrations in wild versus farmed seafood has not been systematically assessed with validated methods. Additionally, most seafood is cooked prior to human consumption, but detailed analysis of the thermal stability of antibiotics found in seafood remains unknown. The overall objective of this thesis was to validate common methods used for antibiotic extraction from seafood, and apply optimized procedures to test the hypothesis that farmed seafood will contain more antibiotics than wild seafood, and that thermal treatment will degrade antibiotics present in seafood. Method validation involved testing the stability of antibiotic standards stored as mixtures, and checking the extent of seafood matrix effects (i.e. ion suppression or enhancement) on extracted antibiotics measured with ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). Thus, in my first experiment, I investigated the stability of antibiotics stored as mixture in water: methanol for one week at different temperatures, pHs, water: methanol ratios and storage container types (i.e. glass vs. silanized glass), because prior studies had inconclusively suggested that these conditions might affect the stability of antibiotics (Experiment 1). I then explored whether the extraction of antibiotics from salmon, as a representative seafood matrix, is associated with matrix effects that can potentially be minimized with clean-up methods involving column or dispersive solid phase

extraction (Experiment 2). For final experiment (Experiment 3), I used the information gained from my method validation efforts to measure antibiotic residues in both wild-caught and farm-raised fish and shrimp samples produced locally in U.S. and imported from other countries. In addition, I assessed the effect of thermal processing on the degradation of antibiotics in seafood matrices with varying fat levels, as lipids may protect antibiotics from thermal degradation.

I found that antibiotics prepared as a mixture were not stable during one week storage in water: methanol irrespective of temperature and pH and that silanization of glass vials improved the storage stability of some quinolones and macrolides but deteriorated the stability of other antibiotic classes including some amphenicols, B-lactams, macrolides, sulfonamides and dihydrofolate reductase inhibitors (Experiment 1). This led me to conclude that antibiotics should be freshly mixed before use on UPLC-MS/MS. In Experiment 2, I found that salmon matrix components are associated with significant matrix effects, which were not improved with column or dispersive solid phase extraction clean-up. However, using appropriate internal standards that match the polarity of the antibiotics resulted in accurate quantitation of antibiotics despite losses in sensitivity. I therefore used appropriate internal standards for antibiotics quantitation in the seafood survey study in Experiment 3 (n=125), and found that both wild-caught and farm-raised seafood locally produced in U.S. or imported from other countries contained antibiotic residues. I found higher detection frequencies of antibiotics in farmed than wild-caught seafood and in imported than locally produced seafood. Surprisingly, antibiotic concentrations were higher in wild-caught than farm-raised seafood. Finally, I discovered that several antibiotics (quinolones, amphenicols, some macrolides, dihydrofolate reductase inhibitors, lincosamides and sulfonamides) were relatively stable in various fish matrices, irrespective of lipid content, under heat treatment. B-lactams, tetracyclines and a few macrolides were unstable under thermal

treatment. Overall, using validated methods, this study provides new unexpected evidence of widespread contamination of antibiotics in both farmed and wild seafood and that thermal treatment does not degrade several antibiotic classes. The impact of chronic human exposures from seafood on the development of antibiotic resistance warrants immediate investigation.

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1. Introduction

1.1.Antibiotics

Antibiotics are medicines used to treat infections by killing bacteria or preventing their growth. Earlier definitions describe antibiotics as substances naturally produced by microorganisms that are harmful to other microorganisms [1]. Nowadays, " antibiotics", also interchangeably used with "antibacterials", refer to a variety of naturally produced and/ or synthetic substances that kill or inhibit bacteria [2]. The term "antimicrobial" is also sometimes used to refer to compounds that kill or stop bacterial growth. Antimicrobials, however, also act against other microorganisms including fungi, viruses and protozoa [3].

The earliest use of antibiotic-producing microbes goes back to more than 2000 years ago when remedies based on moldy bread were used against open wounds to prevent infection. The use of moldy bread and medicinal earth as remedies for healing infections caused by disease or injury have been mentioned in the Papyrus of Ebers, the oldest medical document written at about 1550 BC [4-5].

The first modern antibiotic drug was introduced by Paul Ehrlich at the beginning of the 1910s. Ehrlich and his colleagues, Sahashiro Hata and Alfred Bertheim, synthesized multiple organoarsenical derivatives of a drug called Atoxyl [6]. Atoxyl was a toxic drug prepared by heating aniline and arsenic acid [7]. Ehrlich and his colleagues then discovered a derivative of Atoxyl (trade name of Salvarsan), that cured syphilis in rabbits [6]. The compound also showed promising results in treating syphilis in an initial cohort of 80 humans subjects [8-9]. Inspired by Ehrlich's work and using similar drug searching techniques, Gerhard Domagk synthesized a sulfonamide prodrug, Prontosil, in 1932 [6].

Penicillin is the first natural (i.e. non-synthetic) antibiotic that was discovered by Alexander Fleming in 1928. Fleming noticed that staphylococcus culture plates exposed to air inhibited the growth of staphylococcus colonies due to mold contamination. His experiments of multiple molds revealed only one strain of Penicillium that reproduced the original observation. The filtrate of the mold broth culture was named "penicillin" [10]. A purification technique was later introduced by group of Oxford researchers which enabled producing sufficient concentrations of penicillin for clinical studies and large-scale production of the drug [11]. The chemical structure of the drug was later discovered by Dorothy Hodgkin in 1945 using X-ray technique [12].

The discovery of penicillin led to further studies on antimicrobial-producing microbes. In the late 1930s, Selman Waksman started investigating the antimicrobial-producing capability of soil actinomycetes and discovered multiple antibiotics including streptomycin [13]. Streptomycin was the first clinical treatment for tuberculosis [14].

The discovery of new antibiotic drug classes surged during the 1940s to 1960s, which is considered the golden age of antibiotic discovery. These drugs were mainly natural products produced by soil actinomycetes and fungi. Many of the important antibiotic classes such as quinolones, macrolides, tetracyclines, polymyxins and lincosamides were discovered during this time [15-19]. Very few antibiotics have been discovered after the 1970s [4]. Although advances in synthetic chemistry have enabled the modification of existing antibiotic structures to produce more effective compounds (e.g. ampicillin from penicillin), no major breakthroughs have been made in discovering new lead compounds [20]. At present, the quest for new lead compounds remains an area of active and much needed research[20].

1.2.Antibiotic classes

The most common antibiotic classes that are used to treat infectious diseases in human and animals include B-lactams, tetracyclines, quinolones and fluoroquinolones, sulfonamides, macrolides, aminoglycosides, amphenicols, lincosamides and dihydrofolate reductase inhibitors including trimethoprim and ormetoprim. The structure and mechanism of action of these antibiotic classes are addressed in this section.

1.2.1. B-lactams

B-lactams are naturally occurring antibiotics and are made of a cyclic amide ring of four members (i.e. B-lactam ring) as their building block. B-lactams include different subclasses, specifically penams, clavams, carbapenems, cephems, oxacephems and monobactams (**Figure 1a**) which differ from each other based on the presence of another ring structure and presence/ absence of heteroatoms including sulfur or oxygen. All of these subclasses are bicyclic except for monobactams which are monocyclic. For instance, penams, clavams and carbapenems contain a five-membered thiazolidine ring fused to the lactam ring, but they differ from each other based on their heteroatoms and double bonds as shown in **Figure 1a**. Cephems contain a six-membered dihydrothiazine ring fused to the B-lactam ring with a sulfur atom. Oxacephems are similar to cephems except that the sulfur atom on the B-lactam ring is replaced by an oxygen atom [21]. Structures of some of the B-lactam antibiotics commonly used for human and or animal applications are shown in **Figure 1b**.

B-lactams kill bacteria by inhibiting cell wall synthesis. They block the synthesis of peptidoglycans present in the cell wall by inhibiting acyl serine transferase enzymes [21]. Acyl serine transferase is required for making peptide linkages between glycan chains of peptidoglycans [22].

1.2.2. Tetracyclines

Tetracyclines are group of antibiotics that are produced naturally from *Streptomyces* spp. The two main antibiotics that these species of yeast produce are tetracycline and oxytetracycline. Doxycycline and minocycline are semi-synthetic derivatives of tetracycline [21]. Tetracyclines comprise four hydrophobic fused rings, A, B, C and D, as the structural backbone. Individual tetracyclines differ from each other based on the functional groups attached to the tetracyclic backbone. **Figure 2a** shows the simplest structure of tetracyclines, i.e. 6-deoxy-6-demethyltetracycline, which shows antibacterial activity [23]. The structure of tetracyclines commonly used for human and animal applications are shown in **Figure 2b**.

Tetracyclines interfere with protein synthesis within bacterial cells. This is because they can bind to the 30S ribosomal subunit, likely via protein 7S and 16S RNA binding sites, thereby preventing the binding of aminoacyl transfer ribonucleic acid (t-RNA) to the ribosome [21, 23]. This prevents t-RNA-mediated transfer of amino acids to a polypeptide that is being assembled, thus inhibiting protein translation.

1.2.3. Quinolones and fluoroquinolones

Quinolones are synthetic antibacterial drugs containing a bicyclic core structure derived from 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid (**Figure 3a**) [15]. Modifications in multiple positions of the core structure has led to the formation of novel quinolones with enhanced antibacterial activity. For example, adding a fluorine atom at position R6 of the core structure has led to the synthesis of fluoroquinolones, which have significantly improved antibacterial activity compared to quinolones. The addition of piperazine and cyclopropyl groups at positions R7 and R1, respectively, have further improved the antibacterial activity of fluoroquinolones (**Figure 3b**) [24]. The structure of some quinolones and fluoroquinolones commonly used for human and animal applications are shown in **Figure 3c**.

Quinolones interfere with bacterial DNA synthesis by inhibiting the activity of DNA gyrase and topoisomerase IV enzymes in bacteria. DNA gyrase and topoisomerase IV are involved in supercoiling of the bacterial DNA and relaxing over-twisted DNA molecules. Generally, DNA gyrase is the main target of quinolones in gram-negative bacteria, whereas topoisomerase IV is the primary target in gram-positive bacteria [25].

1.2.4. Sulfonamides

Sulfonamides are a group of synthetic antibiotics derived from *p*-amino-benzene-sulfonamide (sulfanilamide) (**Figure 4a**). Individual members of sulfonamides differ from each other depending on the substitutions in amine moieties [26]. The structure of some of the sulfonamides commonly used in humans and/or animals are shown in **Figure 4b**.

Sulfonamides are structural analogs of *p*-aminobenzoic acid (PABA), a naturally occurring compound in bacterial cells. Bacteria convert PABA to dihydrofolic acid and tetrahydrofolic acid. Due to the structural similarity to PABA, sulfonamides inhibit PABA utilization by bacterial cells and prevent folic acid synthesis [27]. Interfering with folic acid synthesis prevents bacterial replication because folic acid is needed for cell division.

1.2.5. Macrolides

Macrolides are naturally occurring antibiotics comprising a 12- to 16-member macrolactone ring serving as the structural core; e.g. erythromycin contains a 14-member lactone ring and azithromycin contains a 15-member lactone ring. Sugar moieties are usually attached to the lactone ring at carbon 3 and carbon 5 [28] (**Figure 5**). Azalides are a subclass of macrolides that contain an additional amine-bearing functional group attached to the lactone ring. Ketolides,

another subclass of macrolides, contain a keto group in carbon 3 [29]. The structure of some of the macrolides commonly used for human and/or animal applications are shown in **Figure 5**.

Macrolides interfere with bacterial protein synthesis by binding to the peptidyl transferase center at the 50S ribosomal subunit, which is involved in catalyzing peptide bond formation, thereby preventing the elongation of peptide chains [21].

1.2.6. Aminoglycosides

Aminoglycosides are natural or semisynthetic antibiotics made of an aminocyclitol group linked to amino sugars. Streptomycin, gentamicin and neomycin are examples of naturally isolated aminoglycosides. Netilmicin and amikacin are examples of semisynthetic aminoglycosides [30]. For the majority of aminoglycosides, the aminocyclitol group is 2-deoxystreptamine (4,6-diamino-1,2,3-cyclohexanetriol) (**Figure 6a)**. Streptomycin contains a sterptidine ring instead of a deoxystreptamine. Individual members of aminoglycosides show mono- (in position 4) or di-substitutions (in positions 4,5 and/ or 4,6) on the deoxystreptamine ring [30-31]. The structure of some aminoglycosides used in humans and/or animals are shown in **Figure 6b**.

Aminoglycosides disrupt bacterial protein synthesis by binding to the 30S subunits of bacterial ribosomes. Binding occurs at the 16S rRNA component of the A-site of the 30S ribosomal subunit [31]. The A-site (acceptor site) is one of 3 t-RNA binding sites on the ribosome (A-site, P-site and E-site) that binds to aminoacyl t-RNA, which is responsible for holding the new amino acid to be added to a peptide chain [32]. Binding of aminoglycosides to the A-site of 30S ribosomal subunit results in conformational changes at the A-site that consequently prevents the transfer of peptidyl t-RNA from the A- to P-site on the ribosome; The P-site (peptidyl site) binds to peptidyl t-RNA which is responsible for holding the growing peptide chain. Interfering with this step inhibits protein translation [21, 30-32].

1.2.7. Lincosamides

Lincosamides are antibiotic classes related to lincomycin. Lincomycin is a natural antibiotic, first isolated from *Streptomyces lincolnensis* in a soil sample, made of propylhygrinic acid linked to an amino sugar via a peptide bond. Clindamycin, another commonly used lincosamide, is a chlorinated derivative of lincomycin [33]. The structures of lincomycin and clindamycin are shown in **Figure 7**.

Lincosamides interrupt with protein synthesis in bacterial cells in a similar manner to macrolides. Similar to macrolides, the mode of action involves binding to the 50S subunit of bacterial ribosomes, thus preventing the peptidyl transferase reaction and terminating polypeptide elongation [34-35].

1.2.8. Amphenicols

Amphenicols are a group of natural and semisynthetic antibiotics containing a phenylpropanoid structure with an aromatic ring and a three carbon propene tail[36]. Chloramphenicol and florfenicol are examples of natural and semi-synthetic amphenicols, respectively[37]. Among amphenicols, chloramphenicol is the only antibiotic that has been used in human medicine[36]. However, its use to treat human infections is restricted due to potential toxic effects including aplastic anemia and suspected carcinogenicity [38].

Other amphenicols such as florfenicol and thiamphenicol are used in veterinary medicine and in aquaculture [39]. Chloramphenicol is banned from use in veterinary medicine and aquaculture due to its potential toxic effects. The structure of common amphenicols is shown in **Figure 8**.

Amphenicols kill or stop bacteria by blocking protein synthesis during the translation step. The mode of action involves binding to 50S subunits of bacterial ribosomses and inhibiting peptidyl transferase reaction, thereby preventing elongation of peptide chains [36].

1.2.9. Dihydrofolate reductase inhibitors

Trimetoprim and ormetoprim are synthetic antibiotics (**Figure 9**) that interfere with the synthesis of folic acid by inhibiting dihydrofolate reductase and thereby preventing conversion of dihydrofolic acid to tetrahydrofolic acid which is the active form of folic acid [40]. Trimethoprim and ormetoprim are often used together with sulfonamides such as sulfamethoxazole, which is also involved in blocking folic acid synthesis by inhibiting dihydrofolic acid synthesis. In spite of similar mechanism of action as sulfonamides, they are classed differently due to their distinct chemical structure. Blocking two steps in the folic acid synthesis pathway (i.e. inhibiting dihydrofolic acid synthesis by sulfonamides and preventing conversion of dihydrofolic acid to tetrahydrofolic acid by trimethoprim/ ormetoprim) enables synergistic effect of these antibiotics against the bacteria and stops bacterial replication [41].

1.3.Antibiotics use in agriculture and aquaculture

Antibiotics are commonly used in agriculture and aquaculture farming for both therapeutic and non-therapeutic purposes [42]. Therapeutic uses involve the use of antibiotics to treat an active infection in animal farms such as cattle, swine and poultry. Non-therapeutic uses involve the prophylactic use of antibiotics to prevent opportunistic infections. This is mainly seen in aquaculture farms, where antibiotics are typically mixed with the feed and less commonly via bath treatment and/or injection [43]. The dose, frequency and duration of antibiotic administration are determined based on their pharmacokinetic profile, which is why they vary for different antibiotics and different aquatic animal species. For instance, in the U.S., oxytetracycline is administered to

salmonids at doses of 2.5-3.75 g/100 pound of fish/day for 10 days, whereas sulfadimethozine/ormetoprim mixture is administered at a dose of 50 mg/Kg/day for 5 days [44].

In 2013, the global use of antimicrobial agents in food producing animals was approximately 131,109 tons. This value is estimated to reach 200,235 tons by 2030 [45]. Currently, the ~ 131,109 ton value constitutes approximately 79.5% of the global use of antimicrobials, which means that the human share of antimicrobials use only amounts to 20.5 % [46]. In other words, the majority of antimicrobials (including antibiotics) are used to produce the food that keeps humans alive, versus to treat infections in humans.

Terrestrial animals consume the majority of antibiotics, representing 73.7% of total consumption [46], with the largest proportion consumed in pig farming (~ 50%) followed by chicken (~ 25%), cattle (~ 20%) and sheep (~ 5%). This amount also varies across different countries. For example, in 2013, China used 318 mg antibiotics per Kg of domestically produced animals, whereas, in Norway this amount was 8 mg per Kg of animal [45].

The use of antibiotics in aquaculture constitutes only 5.7% of global consumption as of 2017 [46]. This amounts to 10,259 tons[46]. In spite of this low share, the amount used per biomass is higher in aquatic animals (164.8 mg/Kg) than in terrestrial animals (140 mg/Kg) and in humans (92.2 mg/Kg) and [46]. This means that aquatic animals are likely to accumulate more antibiotics per Kg, over time, compared to terrestrial animals.

Similar to agriculture, antibiotic use in aquaculture also varies among countries and the amount used is influenced by the country's seafood production output. For example, China produced 51.2% of total aquatic animals in 2017 and was the major consumer of antibiotics in aquaculture (57.9% of total global use). India, Indonesia, and Vietnam used the largest amounts of

antibiotics after China, representing 11.3%, 8.6% and 5% of global use, respectively, which is in line with their aquaculture production share of 9.9%, 9.8% and 5.7%, respectively [46].

1.4. Antibiotic drug resistance

A primary concern about the increasing use of antibiotics in agriculture and aquaculture is the emergence of antibiotic resistance microbes in food producing animals and the environment [47-49]. These resistant microbes can either transfer to humans directly or act as reservoirs of antibiotic resistance genes that can transmit to humans indirectly via other pathogens[50-52]. This is particularly concerning as the majority of antibiotics commonly used in agriculture and aquaculture, i.e. penicillins, quinolones, tetracyclines, sulfonamides, macrolides and aminoglycosides [45-46], are classified as "critically important" or "highly important" antibiotics for human use by the World Health Organization (WHO) [53]. Many of these antibiotics are also used in agriculture and aquaculture farming, and this can promote the development of antibiotic resistant infections[52]. Currently, antibiotic resistant infections are responsible for approximately 35,000 deaths per year in U.S. [54] and for 4.95 million deaths worldwide as of 2019 [55].

1.5. Antibiotic resistance mechanisms

Resistance to antibiotics occurs through multiple mechanisms. As discussed in this sub-section, these include modifications to the drug target-site, preventing antibiotics from reaching the target site by increasing efflux and reducing permeability into bacterial cells, direct modification of drugs, and acquisition of resistant genes through horizontal gene transfer [56-57].

1.5.1. Target-site modification

Target site modification is a common mechanism of antibiotic drug resistance. It involves chromosomal mutations of enzymes targeted by antibiotics, or abnormal methylation of ribosomes that bind antibiotics.

An example of target-site modification is the occurrence of random mutations in the genes that encode DNA gyrase (*gyrA*, *gyrB*) and/or topoisomerase IV (*parC*, and *pare*), the primary targets for quinolones. Mutations in these genes will result in amino acid substitutions that change the structure of the target proteins leading to reduced binding affinity of quinolones [25]. Resistance to quinolones in gram-negative and gram-positive bacteria often occurs via mutations in *gyrA* and *parC* genes, respectively [58].

Target site methylation can also lead to antibiotic drug resistance. Macrolides, lincosamides and streptogramin B which interrupt bacterial protein synthesis by binding to the 23S rRNA portion of the 50S ribosomal subunit, can promote resistance via targeted methylation or dimethylation at specific adenine bases within 23S rRNA [58].

1.5.2. Reduced membrane permeability

Cell membrane permeability refers to the ability of an antibiotic to enter the cell and access a target site. In gram-negative bacteria, the outer membrane acts as a barrier against the hydrophilic drugs, and therefore outer membrane proteins are needed to facilitate drug access to cell interior. Random mutations resulting in the inactivation of these proteins can reduce antibiotic entry into the cell, resulting in drug resistance [25]. For example, inactivation of OMPK35 and OMPK36, which are outer membrane porins in *Klebsiella pneumonia*, has been associated with increased resistance to quinolones, cephalosporins and chloramphenicol [59-60].

1.5.3. Increased antibiotic efflux

Efflux pumps are involved in drug export out of bacterial cells. Overexpression of efflux pumps can enhance drugs efflux out of the cell and confer antibiotic resistance [56]. The overexpression of the pumps can occur via multiple mechanisms. These include mutations in regulatory genes controlling efflux pump expression, specifically local repressor genes and global regulatory genes that control transcription of small and large number of genes, respectively. Mutations in the promoter region of the efflux pump gene can also enhance expression of the efflux pumps. Another mechanism of enhanced expression of efflux pumps involves integrating insertion sequences upstream of the efflux pump gene. The insertion sequences might have promoters that can enhance expression of efflux pump genes [61].

1.5.4. Drug inactivation

Bacteria can inactivate antibiotics directly by converting them into inactive metabolites. As described in the next paragraphs, the most common mechanism of antibiotic inactivation by bacteria involves hydrolytic degradation and chemical group transfer[62]. Bacteria can also inactivate antibiotics via redox reactions [62].

Hydrolytic degradation of antibiotics is an important mechanism of antibiotic inactivation. Many antibiotics contain amide and ester linkages and can therefore undergo hydrolysis reactions [63]. An example of antibiotic hydrolytic breakdown is the degradation of B-lactams by β-lactamase enzymes [62]. B-lactams need the lactam ring for their antimicrobial activity, and β-lactamase enzymes expressed by some bacteria lead to the opening of the lactam ring, thus inactivating the antibiotic [64]. Also, macrolides can be hydrolyzed by esterase enzymes in bacteria, resulting in the opening of the lactone ring [62].

Bacteria can also modify antibiotics by adding chemical groups such as acyl, phosphate, glycoside, nucleotidyl, ADP-ribosyl and thiol groups on the antibiotic molecule via transferase enzymes. This biotransformation prevents the antibiotic from binding to its target site thus losing its activity. For example, aminoglycosides could be inactivated by enzymes that transfer acetyl, phosphate and nucleotidyl groups [62].

Redox reactions are less common than hydrolysis or chemical group transfer mechanisms. These reactions involve enzymatic oxidation or reduction of antibiotics. An example is the hydroxylation of tetracyclines which blocks the Mg^{+2}-binding sites on the tetracyclines. Tetracyclines binding to Mg^{+2} cations is required for their antibiotic activity [62].

1.5.5. Acquisition of resistance genes through horizontal gene transfer

Resistance to antibiotics can also occur via the acquisition of antibiotic resistance genes through horizontal gene transfer. Horizontal gene transfer refers to transfer of genes between micro-organisms. Bacteria can acquire external resistance genes via three mechanisms including transformation, transduction and conjugation. Transformation involves the incorporation of a piece of DNA from the surrounding environment into the genetic material of the bacteria by direct uptake. Transduction involves the incorporation of DNA material via a bacteriophage, which then incorporates the DNA into the bacterial cell. Conjugation involves the transfer of DNA material between bacterial cells that are in direct contact. This often occurs via mobile genetic elements including plasmids, conjugative transposons and integrons, acting as gene transferring vehicles [57, 65].

1.6.Antibiotic residues in seafood

Seafood is a source of high value protein that also provides key nutrients including omega-3 polyunsaturated fatty acids, vitamin D and vitamin B12 [66]. Currently, seafood is considered an

essential component of a healthy diet and dietary guidelines advocate the consumption of 8 ounces of seafood per week [66]. This amount provides approximately 250 mg per day of two important omega-3 fatty acids - eicosapentaenoic acid and docosahexaenoic acid [66]. The premise for promoting seafood consumption stems from epidemiological studies showing inverse associations between seafood intake and protection against several morbidities including metabolic disorders, cardiovascular disease and neurological disorders [67-69].

Seafood consumption has substantially grown during the past decades; in 2015, 20.5 kg of fish was consumed per capita compared to 9.0 kg in 1961 [70]. This has been influenced by many factors including rapid population growth, improved living standards, incorporation of seafood consumption in the dietary guidelines and growing awareness of seafood as a healthy food category[71-73]. Increased demand for seafood has been paralleled by minimal growth of capture fisheries since 1990 [70] and the endangerment of several fish species (e.g. Atlantic salmon) [74]. As a result, aquaculture production has continuously increased in order to meet the increased demand for seafood amid declining seafood populations in the environment. In 1970, the aquaculture share of seafood production accounted for approximately 4% of global seafood production[75]. This value surged from 9% in 1980 to 48% in 2011. The aquaculture share of seafood production is projected to reach 60% or more by 2030 [76].

The substantial growth in aquaculture has been accompanied by the increased use of antibiotics in fish farms. Under intense farming practices, the health and performance of aquatic animals are negatively impacted due to the increased stress, resulting in increased possibility of infections[77]. To combat this, antibiotics are used both therapeutically and prophylactically in order to treat and prevent infectious disease [77]. A consequence of increased use of antibiotics in aquatic farms is that the residual concentrations of antibiotics may remain in seafood products, resulting

in increased exposure to consumers. This is consistent with studies showing the presence of antibiotic residues from multiple classes including tetracyclines, quinolones, sulfonamides, macrolides, B-lactams and amphenicols in farm-raised seafood [75, 78-80].

Aquatic animals of natural waters are also exposed to antibiotic residues present in their natural environment. Multiple studies have shown the presence of antibiotic contaminants in water and sediments from coastal and offshore regions [78, 81-82]. Many of these antibiotics can be taken up by wild aquatic animals, as evidenced by studies showing similarities in the antibiotic profile of wild-caught aquatic animals and the surrounding water and sediment samples [78, 83-86]. This is why antibiotics have been widely detected in wild-caught aquatic animals [78-79, 83, 87-88].

Antibiotics can enter the natural waters from various sources, including wastewater, hospital and industrial effluents and animal manure [89-92]. Although these sources undergo decontamination processes, antibiotics are poorly removed by these treatment processes [93-97]. This is why antibiotics are often detected at sites far from the effluent discharge points [97].

Antibiotic contamination in seafood may pose significant risks to human health. Aquatic animals exposed to antibiotics may be a source of antibiotic resistant bacteria [47] that can directly transmit to humans once ingested. Additionally, resistant microbes in seafood could act as a pool of antibiotic resistance genes for human pathogens [77]. In other words, human pathogens can develop the capability to resist antibiotic treatment by acquiring antibiotic resistance genes from resistant microbes [50].

Residual concentrations of antibiotics in seafood may be associated with other detrimental health outcomes in humans, although the evidence is limited. For example, exposure to some antibiotics such as penicillins may induce allergic reactions in sensitive subjects [98-99].

Chloramphenicol is known to cause aplastic anemia and is also a suspected carcinogen [38, 100]. Nitrofurans and their metabolites are generally considered genotoxic and carcinogenic [101]. While, these antibiotics are prohibited from using in food producing animals due to their toxicity [102-103], they are often detected in seafood [79, 104].

1.7.Probing antibiotics in seafood

In view of the habitual use of antibiotics in aquaculture farms, it seems reasonable to routinely monitor their levels in order to better understand the extent of exposure. However, one of prohibitive factors preventing regulatory bodies and laboratories from doing this is that the methods used to quantify antibiotics are not simple or streamlined. They require large solvent volumes and multiple steps. Below is a description of the most common methods used to measure antibiotics in seafood samples.

1.7.1.Antibiotics extraction and detection

In order to measure antibiotic residues in seafood samples, antibiotics need to be extracted from the matrix and separated with liquid chromatography prior to detection with mass-spectrometry. Methods typically used to extract antibiotics from seafood involve extraction with 1) acetonitrile solvent [105-106], 2) acetonitrile containing acid -which is shown to favor extraction of a wide range of acidic and basic antibiotics by influencing their ionization state- [107-110] or 3) the Quick, Easy, Cheap, Effective, Rugged, Safe (QuEChERS) method which uses a combination of acetonitrile, water and salts [111-113]. The QuEChERS method was originally developed for pesticides [114] and then used for antibiotics extraction [111-113]. Recent methods published by FDA research groups have used acetonitrile containing an acid modifier for multi-residue antibiotics extraction from seafood [107, 115] and have sometimes added other additives such as salt and Ethylenediaminetetraacetic acid (EDTA) to the extraction solvent[110].

In the QuEChERS method, antibiotics are extracted using acetonitrile and water usually at a ratio of 4 to 1, and a mixture of salts is added to increase the ionic strength of water and drive the partitioning of antibiotics into the upper acetonitrile phase (increasing the ionic strength of water decreases the solubility of polar antibiotics in water) [116].

pH modifiers have also be used with the QUEChERS method. For instance, acidifying the extraction solvent was reported to improve the extraction recovery of some antibiotics including fluoroquinolones, B-lactams, avermectines and tetracyclines [107, 112]. In the original QUEChERS method developed by Anastadssiades [114], $MgSO_4$ and NaCl are used to facilitate solvent partitioning. However, in the QUEChERS method, $MgSO_4$ is replaced by Na_2SO_4 because quinolones have been found to bind to Mg^{+2} ions, resulting in reduced recoveries [111]. In some cases, citrate or acetate salts are also added to this mixture as buffering agents [111, 113].

Following antibiotic extraction, the samples can be submitted to liquid chromatography systems coupled to a mass-spectrometry detector in order to separate and detect them. Liquid chromatography (LC) is a separation technique that is commonly used for analysis of non-volatile analytes such as antibiotics. In LC, analytes are carried through a column using a mobile phase consisting of various buffers, and separated based on their affinity to the column (i.e. the stationary phase). The separated analytes can then be detected in a mass spectrometry (MS) detector which provides structural confirmatory information of the analyte present in sample.

Mass spectrometers constitute of three components - the ion source, mass analyzer and detector. Analytes separated on a LC column are first ionized in the ion source. Select ions are then separated in the mass analyzer based on their mass to charge ratio (m/z). The separated ions are then detected in the detector. There are many different types of ion sources and mass analyzers. The most common ion sources used with LC are electrospray ionization (ESI) and atmospheric

pressure chemical ionization (APCI). Mass analyzers that have used for antibiotic analysis include triple quadrupole mass analyzer (QqQ)[75, 111, 117] and high resolution mass analyzers including orbital ion trap (Orbitrap) and time of flight (TOF)[107, 115]. LC coupled to QqQ mass spectrometer has been used for targeted multi-residue antibiotic analysis in seafood [75, 111-113, 117]. Ion trap and TOF mass analyzers can provide higher resolution compared to low resolution mass analyzers such as QqQ and can therefore provide high selectivity. As such, they have been used for non-targeted analysis of antibiotics, allowing for the detection of a large number of analytes with high selectivity [107, 115]. However, sensitivity is lower with ion trap/TOF compared to QqQ.

Antibiotics detected by LC coupled to mass spectrometry are quantified using external calibration standards prepared by serial dilution from the stock solutions. In addition, surrogates are sometimes added in order to account for recovery losses.

1.7.2.Other less common methods of antibiotics detection

Immunoassays are alternative methods that have been used for antibiotics detection in seafood [118-120]. Immunoassays rely on antibiotics binding to specific antibodies. These assays are generally sensitive but lack specificity, particularly to structurally similar antibiotics [121]. Sensitivity of immunoassay based methods has been greatly enhanced in recent years by the use of nanofibrous membranes with large surface areas to maximize the number of antibodies available for binding to an antibiotic contaminant. One study reported a more than 10-fold increase in sensitivity using nanofibrous membrane-based ELISA compared to conventional ELISA [122].

There are several setbacks to immunoassays. First, the technique does not allow for simultaneous multi-residue detection as can be done with mass-spectrometry systems. This is because the method requires specific antibodies for many antibiotics, and this could be a challenging process. Furthermore, typically these assays come in the form of an ELISA kit, which

typically allow for the measurement of one antibiotic at a time. This can be both time- consuming and expensive if a sample was to be screened for more than one residue. Another limitation is that unlike mass-spectrometers, immunoassay tests do not provide confirmatory structural information [123-124].

Microbial growth inhibition test is one of the oldest methods for antibiotics detection [125]. The technique uses the inhibitory effects of antibiotics on bacterial growth to infer whether an antibiotic is present or absent in the sample. Although the method is sensitive, it does not inform on the identity of antibiotics in a matrix. Additionally, this method is less sensitive compared to mass spectrometry methods [112].

1.7.3.Limitations of the QUEChERS extraction method

A problem with the acetonitrile and QUEChERS extraction methods is that they do not completely remove components in seafood that might cause "matrix effects". Matrix effects refer to the suppression or enhancement of antibiotic ionization in a mass-spectrometer at the ion source due to the co-eluting matrix components [126]. This could impair the accuracy and sensitivity of analysis if not properly addressed. For example suppression or enhancement of an antibiotic but not the internal standard used to quantify it could underestimate or overestimate antibiotic concentration, respectively, thus making measured values less accurate. This is because suppression of the antibiotic would lead to a lower signal on the mass-spectrometer (because less of it is ionized), resulting in reduced 'apparent' concentration. Conversely, signal enhancement would yield an elevated concentration. Ion suppression can also reduce sensitivity by reducing the signal tied to the compound to levels close to the detection limits. This is why it is important to remove co-eluting matrix components when performing antibiotic analysis.

Antibiotic extraction methods have been tested with clean-up steps in order to reduce the matrix effects. The common clean-up methods used for seafood matrix include solid phase extraction (SPE) [75, 85, 107-108] and dispersive SPE [107, 111], where cartridge sorbent or bulk powder sorbents are used, respectively, to remove lipids, pigments, complex sugars and other compounds from the matrix [111-112, 127-128].

The most common SPE cartridge used for SPE clean-up is a hydrophilic-lipophilic balance (HLB) cartridge. The presence of both hydrophilic and lipophilic polymers in HLB cartridges enables the retention of both polar and non-polar antibiotics [85]. Using HLB columns, polar molecules coming from the matrix are eluted through the column. Non-polar molecules such as triacylglycerols, fatty acids and pigments such as carotenoids are initially retained by the column and be eliminated from the final extract by choosing appropriate elution solvent. This way they could reduce the matrix effects. Although, the use of HLB columns can reduce the matrix effects, but it does not eliminate it.

The other clean-up method, dispersive SPE, relies on using bulk sorbents that could selectively retain interfering molecules from the matrix. In seafood matrices, polar and non-polar lipids are a known source of matrix effects [107]. Primary secondary amines (PSA) and C18 sorbents are typically used to remove polar and non-polar lipids, respectively [129]. Sometimes salts such as $MgSO_4$ or Na_2SO_4 are used along with other sorbents in order to trap the residual amount of water in the final extract to reduce polar interferences in the extract. However, $MgSO_4$ may impair the recovery of tetracyclines and quinolones due to their high affinity to form complexes with divalent cations such as Mg^{+2} [107, 111].

In addition to the clean-up methods, matrix effects could be eliminated by improving the chromatographic separation of the antibiotics on LC. However, this approach could be challenging

in multi-residue analysis because it is difficult to achieve complete separation of matrix components and antibiotics [130].

One alternative to using clean-up methods or LC separation to account for matrix effects is to use matrix-matched calibration standard curves. This involves spiking a matrix extract with different concentrations of antibiotic standards, and generating a standard curve peak area response on the mass-spectrometer. Here, the standard curve used for antibiotic quantification (as described above), accounts for matrix effects because the antibiotics have been spiked into the matrix extract.

This approach has drawbacks because it is difficult to obtain a representative seafood matrix for the calibration curve. For instance, salmon may have different matrix effects than tuna or cod, because of their differing composition despite belonging to the same seafood family. Therefore, it is not possible to use salmon to correct for matrix effects that may be present in cod, or vice versa. Additionally, although accounting for matrix effects with matrix-spiked standards may improve measurement accuracy, sensitivity would still be diminished due to ion suppression [126, 130-131].

There is limited information on the extent of matrix effects from seafood on antibiotics measured by UPLC-MS/MS. One study showed ion suppression and enhancement when antibiotics were extracted from seafood matrices including clam, mussel and fish using the QUEChERS method [112]. In the same study, matrix effects were compensated using matrix-matched calibration curve and isotopically labeled internal standards [112]. One study reported that dispersive SPE clean up using Na_2SO_4, PSA and C18 sorbents (900:50:150 ratio) after QUEChERS extraction reduced matrix effects from several food and seafood matrices [111]. However, the study did not report the seafood matrix used [111]. Other studies that used clean-up methods following antibiotics extraction from seafood did not report on whether matrix effects

were reduced following clean-up. Thus, overall, there is a clear knowledge gap on whether clean-up methods used post QUEChERS extraction can significantly reduce matrix effects associated with seafood.

1.7.4.Antibiotic standard stability

External standard calibration curves are used to quantify multiple antibiotic residues when measured by UPLC-MS/MS. To generate the calibration curve, antibiotics are individually dissolved in a solvent ("stock solution"), mixed and serially diluted to make a range of high to low concentrations of antibiotic standard mix (“calibration standards”). The stock solutions and calibration standards are typically stored at low temperatures for multiple use. Despite this common practice, studies have shown that the stability of individual antibiotic standards is impacted by temperature, pH, solvent type and storage container. Instability of antibiotic standards can negatively impact the reproducibility of the calibration curve and therefore result in inaccurate quantitation.

Low storage temperatures were shown to improve the stability of B-lactam and tetracycline standards stored individually (i.e. as stock solutions and not within a standard mix). B-lactams were stable for approximately one week when stored individually at 4 ˚C [132]. However, at -18 ˚C, they were stable for 2 to >3 months [132]. Similarly, tetracyclines (oxytetracyclin and tetracycline) were stable for 1-2 weeks when stored at 4 ˚C, and for 2 to >3 months when stored at -18 ˚C [132]. In another study, B-lactams stored individually were stable for a shorter duration at -20 ˚C (9 months) than -80 ˚C (1 year) [133]. Additionally this study reported longer stability of B-lactams at -20 ˚C compared to a study by Berendsen et al. [132] which reported only 2 to > 3 months stability of B-lactams at -18 ˚C. Differences between study outcomes remain unresolved.

pH has also been shown to affect antibiotic standard stability. Several antibiotics were shown to degrade when stored in acidic or basic pH compared to neutral pH. B-lactams including ampicillin, cefalotin and cefoxitin degraded at pH 9 compared to acidic and neutral pHs, when stored at 25 ˚C [134]. Conversely, tylosin (a macrolide) degraded faster at pH 2 compared to pH 11 at temperatures of 7 and 22 ˚C [135]. No degradation occurred at pH 5, 7 and 9, suggesting that both low and high pH conditions degrade this antibiotic. This is in general in agreement with another study which showed that tylosin and spiramycin (another macrolide) degraded more at pH 4 and pH 9 compared to neutral pH at 25 ˚C [63]. At low pH, the macrolide erythromycin A was shown to degrade into erythromycin A enol ether and anhydroerythromycin A [136], indicating that new compounds with potential bioactivity are produced upon acid-induced degradation. Amphenicols including chloramphenicol and florfenicol degraded more at pH 4 and pH 9 compared to neutral pH at 25 ˚C [63]. Tetracyclines were shown to be stable at acidic pH and to degrade at high pH [135].

The solvent used to dissolve antibiotics can also affect their stability. For example, B-lactams were shown to degrade in methanol and water: methanol, whereas no degradation occurred when they were stored in water, acetonitrile and water: acetonitrile [137].This is likely due to the formation of B-lactam methyl esters in the presence of methanol [137]. Additionally, antibiotics containing hydrolysable functional groups can undergo hydrolysis when stored in water [63, 134]. The degradation of these antibiotics can potentially be prevented if solvents other than water is used.

The storage stability of antibiotic standards can also be affected by the type of container used to store them. Typically, plain glass, silanized glass and high density polyethylene (HDPE) container vials are used. It was reported that macrolides become unstable (% change in concentration > 20%) in all three container types when stored as family mixture in water at 4 ˚C for one week. Quinolones become unstable when stored in plain glass and HDPE containers. Under

similar storage conditions, B-lactams and tetracyclines were shown to be unstable in HDPE containers and silanized glass, respectively [138].

From the above-mentioned studies, it can be seen that the stability of antibiotic standards is mainly assessed as individual stock solution and that it may be dependent on storage conditions such as temperature, solvent type, pH and storage container. However, no study has assessed the stability of antibiotics as a mixture. This is important to know because practically speaking, the calibration standards used for multi-residue antibiotic analysis are often mixed and stored for multiple uses. Additionally, from the information provided above we could see that different antibiotic classes need a specific solvent, pH, temperature and container type when stored, and a condition appropriate for one class might not be appropriate for another. It is therefore critical to assess the stability of antibiotics as a mixture to be able to determine the optimal storage conditions.

1.8.Thermal degradation of antibiotics

Although a lot of research has investigated the stability of antibiotic standards used for calibration curves, little has been done to assess the stability of food containing antibiotics after thermal treatment. This is important to know because if indeed farmed seafood is a source of antibiotic contamination, then it is imperative to assess whether cooking degrades antibiotics within the matrix or not. Most of the literature to date has tested the effects of thermal treatment on antibiotic degradation in water or other liquid mediums. Data on real food matrices including seafood are limited. Below, I will provide a literature summary on what has been done to date in this topic.

Antibiotic degradation is typically assessed by measuring the difference in concentration before and after heating. Also, antibiotic concentrations measured over multiple timepoints can be

used to obtain degradation kinetic constants which allows comparing antibiotic degradation across different studies and different matrices[139]. Antibiotic degradation is generally hypothesized to follow first order kinetics and the degradation rate constant (k) is calculated by plotting the natural logarithm of antibiotic concentrations as a function of time from which the k is derived from the slope of the linear regression line. For most antibiotics, the degradation rate constant is dependent on the temperature and Arrhenius equation parameters including activation energy (Ea) and collision frequency (A) [139].

Microbial activity can also be used to measure antibiotic degradation during thermal treatment. This is achieved by measuring microbial inhibitory concentration and/or inhibition zone diameter. However, microbial tests may be confounded by the presence of bioactive antibiotic degradation metabolites [139].

B-lactams are thermally unstable. In water, amoxicillin, ampicillin, penicillin G, cloxacillin, dicloxacillin, and oxacillin were shown to degrade by approximately 10% to 60% after heating for 15 min at 100 ˚C [140]. In another study, cloxacillin, dicloxacillin, oxacillin and nafcillin in water degraded by 13-70% when heated at 90 ˚C for 15 min in water [141]. The degradation of B-lactams in seafood samples has not been studied, but in bovine meat, ampicillin was shown to degrade by 25 to 100% at 70 to 98 ˚C applied for 20 to 210 min [142].

In water, tetracycline, oxytetracycline, chlortetracycline and doxycycline were shown to degrade by approximately 10% to 80% relative to baseline values when heated for 15 min at 100 ˚C [140]. Tetracycline and oxytetracycline were more heat-labile than chlortetracycline and doxycycline [140]. The extent of degradation was also temperature dependent as more degradation of tetracycline, chlortetracycline and doxycycline occurred at 121 ˚C compared to 100 ˚C [140]. In seafood, 30 to 100% degradation of oxytetracycline occurred following cooking. In shrimp

samples boiled (100 ˚C, 4 min), fried (180, 1 min) or baked (200 ˚C, 4 min), oxytetracycline degraded by 30 to 60% [143]. Similar changes in oxytetracycline were observed in salmon fried at 100 ˚C for 15 min (60% reduction relative to baseline) [144] and catfish fried at 190 ˚C for 7 to 10 min or baked at 190 ˚C for 45 min a (33-93% reduction relative to baseline) [145-146].

Macrolides have been shown to be stable during heat treatment. In water, the half-lives of spiramycin and tylosin heated at 60 ˚C at pH 7 were 33.1 and 41.4 days, respectively [63]. By comparison, they were stable at ambient temperature (25 ˚C) [63]. Notably, the half-life of these two antibiotics was reduced to 0.73-3.5 days at acidic (pH 4) and basic (pH 9) conditions, suggesting that stability is more dependent on pH than temperature [63]. The antibacterial activity of clarithromycin was not altered following heat treatment at 50 ˚C for 30 min or 121˚C for 15 min in Mueller-Hinton broth (a microbial growth medium). In contrast, josamycin and erythromycin exhibited a 2-16 fold increase in the minimum inhibitory concentrations following heat treatment at 121˚C for 15 min in Mueller-Hinton broth [147]. In meat matrix, a 45% and 47-50% reduction in ivermectin concentration was observed after beef muscle was boiled at 78 ˚C for 9 min or fried at 177 - 192 ˚C for 10 -17 min, respectively [148].

Amphenicols were also shown to be stable to heat treatment but vulnerable to pH. Chloramphenicol and florfenicol degraded with an approximate half-life of 20-38 days at 60 ˚C in pH 7 buffer; under the same conditions at room temperature, no degradation was observed [63]. Similar to macrolides, low and high pH conditions were shown to reduce this half-life to 2.3 to 22.6 days [63]. Boiling amphenicols in water resulted in only a 5-20% reduction in chloramphenicol, florfenicol and thiampheicol concentrations after 2 hours [149]. In shrimp, a 6-29% reduction in chloramphenicol concentration was reported following thermal treatment at 100 and 121 ˚C for 10

to 30 min [150]. Chloramphenicol degraded by 19 and 28% in mussel treated with antibiotics or spiked after sample homogenization when heated for 1 hour at 100 ˚C, respectively [151].

Quinolones were shown to be stable during heating in both water and meat. The antibacterial activity of ciprofloxacin, norfloxiacin, nalidixic acid and ofloxacin, tested by minimum inhibitory concentrations and inhibition zone diameter tests, did not change when these compounds were heated at 50 ˚C for 30 min or 121˚C for 15 min in Mueller-Hinton broth [147]. In shrimp samples, oxolinic acid concentration decreased by 20-30% after boiling (4 min), frying (180 ˚C, 1 min) and baking (200 ˚C, 4 min) [152].

Sulfonamides were shown to be stable during heat treatment in water. Sulfamethoxazole and sulfamethazine degraded by less than 10% when heated in water at 100 and 121 ˚C for 15 min [140]. In Channel Catfish, the degradation of sulfadimethoxine was dependent on the initial concentration and cooking method. At 190 ˚C, a 31, 47 and 62% degradation of sulfadimethoxine was observed relative to baseline after baking for 45 min when initial concentrations were 25, 50 and 100 mg/kg, respectively. Frying at 190 ˚C for 7-10 min resulted in a 7.5, 63.5 and 42.3% reduction compared to baseline when initial concentrations of 25, 50 and 100 mg/kg were used, respectively [153].

Other antibiotics including lincosmaides, such as lincomycin, and dihydrofolate reductase inhibitors including trimethoprim and ormetoprim were shown to be stable under thermal processing. Lincomycin degraded by < 15% when heated in water at 100 and 121 ˚C for 15 min [140]. The antibacterial activity of trimethoprim did not change when heated at 50 ˚C for 30 min or 121˚C for 15 min in Mueller-Hinton broth [147]. Ormetoprim showed a 17.3%, 77.5% and 60.4% reduction after baking Channel Catfish at 190 ˚C for 45 min when initial concentrations were 25, 50 and 100 mg/kg, respectively. Frying Channel Catfish at 190 ˚C for 7-10 min resulted in a 56.8,

83.9 and 44.8% reduction of ormetoprim when initial concentrations were 25, 50 and 100 mg/kg, respectively [153].

From the abovementioned studies, it could be seen that the thermal degradation of antibiotics was mostly studied in water. A limited number of antibiotics were tested in seafood. Thermal degradation in water might not truly reflect degradation in actual food matrix. Indeed, from the studies discussed above, it appears that antibiotics are more resistant to thermal degradation when present in a food matrix compared to water [144]. However, there is a lack of comprehensive assessment of the extent of antibiotic degradation in seafood matrix versus water.

Antibiotics might also degrade to a different extent depending on the seafood matrix composition. Given that hydrolysis is a significant mechanism of antibiotics degradation [63, 134] and that studies have shown less degradation of antibiotics such as oxytetracycline in oil than water under heating [154], antibiotics partitioning into the lipid portion of seafood are likely to be protected from hydrolytic degradation. This is a topic that I will explore in detail in the present thesis (Chapter 4).

1.9.Scientific gaps in the knowledge

There are several scientific knowledge gaps that my thesis aims to address.

First, although there are published data on the stability of individual and class-specific antibiotic standards, the stability of antibiotics stored as a mixture of different classes has not been tested. The stability of antibiotics stored as a mixture is important for multi-residue antibiotic analysis, because this would lead to reproducible calibration curves that could be used to quantify antibiotics within multiple classes. In **Chapter 2**, I explore in detail the effects of temperature, pH, solvents and container type on the stability of antibiotic standards stored as a mixture, in view of

prior studies showing that these parameters affect the stability of antibiotics stored individually or as a class (but not as a multi-class mixture)

The second unknown is that there is no information on the extent of matrix effects on antibiotics extracted from seafood when measured by UPLC-MS/MS. Matrix effects are caused by co-eluting matrix components that can suppress and/ or enhance antibiotic ionizations in the ion source. If present, matrix effects can reduce sensitivity and accuracy of antibiotics analysis. Among seafood samples, salmon remains a challenging matrix to work with because of its high lipid and carotenoid content, which have been shown to cause matrix effects on antibiotics or other contaminants extracted from food matrices [107, 127]. One study reported that approximately 0.5% of salmon matrix components could be extracted into the final extract following QUEChERS extraction [128]. Therefore, it is likely that using the common QUEChERS method to extract antibiotics from salmon will introduce matrix effects on antibiotics. Understanding the matrix contributions of salmon on antibiotic analysis will enable further optimization of the QUEChERS method for other less pigmented and less lipid-rich seafood matrices such as cod, while providing crucial information on whether antibiotic extracts derived from the QUEChERS method yield sensitive and accurate values on UPLC-MS/MS. I address this controversy in **Chapter 3**.

The third unknown is that in spite of information on the prevalence of select antibiotics in farm-raised and wild-caught seafood from different regions in the world [78-80, 155-157], a direct comparison and comprehensive survey of antibiotics in farmed and wild seafood samples with statistically large enough sample size has not been done before. In the past, studies performed in U.S. have typically assessed a few number of samples (< 30) [75] and/ or few number of antibiotic residues (< 10) [158] from aquaculture products.

Lastly, since seafood is commonly consumed after cooking, assessing true exposure to antibiotic residues through seafood consumption requires information on the effect of heating on antibiotic residues. In spite of the available information on antibiotic degradation in model systems, i.e. water, and the few studies that explored a select number of antibiotic degradation in seafood, the effects of thermal treatment on the degradation of multiple antibiotics commonly found in seafood remains unknown. Assessing the thermal degradation of antibiotics in seafood is important in view of studies showing that the kinetics of degradation differ in water compared to food matrices [144, 149, 151]. Additionally, it is not known if the fat composition of seafood can affect the antibiotic degradation. There is reason to expect that fat composition matters because most antibiotics are lipophilic, and therefore the fat content of the matrix might contribute to their thermal stability. In **Chapter 4**, I provide a comprehensive analysis of antibiotic contamination in farmed versus wild fish, and test whether heating affects the kinetics of antibiotic degradation in low- and high-fat fish compared to water and oil. Addressing these unknowns will enable better estimation of exposure and related toxicity, which are also covered in **Chapter 4**.

1.10. Thesis hypothesis and objectives

The overall hypothesis of my thesis is that detailed assessment of calibration curve standard mix stability and matrix effects in fish samples will enable accurate and sensitive quantitation of antibiotic residues in heated and non-heated seafood. My secondary hypothesis is that considerably more antibiotics will be found in farmed compared to wild seafood (because they are purposely applied in aquaculture farming) and that thermal treatment will degrade antibiotics more rapidly in water than in seafood matrix. Another secondary hypothesis is that antibiotics will be more stable to thermal degradation in high-fat fish, compared to low-fat (high protein) fish. The overall objective is to identify and apply stable standards, and sensitive and accurate methods free of

matrix interferences to measure antibiotic residues in seafood, and to test whether antibiotics degrade with heat.

The overall hypothesis will be tested through the following aims:

Aim 1 (chapter 2): assess the effects of temperature, pH, solvent mixture ratios and container type on the stability of antibiotics stored as a mixture

Aim 2 (chapter 3): test whether matrix effects from salmon as a representative matrix reduce the accuracy and sensitivity of antibiotic measurements, and determine whether clean-up methods eliminate matrix effects.

Aim 3 (chapter 4): a) assess the extent of antibiotic contamination in farm-raised and wild-caught seafood from both local (U.S. production) and imported origins; and b) determine the effect of thermal processing on the degradation of antibiotic residues in low- and high-fat seafood matrix relative to water or oil (i.e. pure fat).

The specific hypothesis for each aim are as follows – a) similar to individual antibiotic standards, the stability of antibiotics in mixture will be affected by temperature, pH, solvent type and container type (Aim 1; Chapter 2); b) salmon matrix will result in notable matrix effects on antibiotics during UPLC-MS/MS analysis and that the matrix effects will be eliminated by clean-up methods i.e. SPE and dispersive SPE (Aim 2; Chapter 3); c) farm-raised seafood will contain more and higher concentrations of antibiotic residues than wild-caught seafood, and that thermal treatment will degrade antibiotics less rapidly in high-fat fish matrix compared to low-fat fish, oil and water (Aim 3; Chapter 4).

Chapter 2 is published in journal of “Food Additives and Contaminants: Part A”. Chapter 3 is submitted to “Journal of Chromatography B” and is under review. Chapter 4 is drafted as a manuscript and will be submitted for publication.

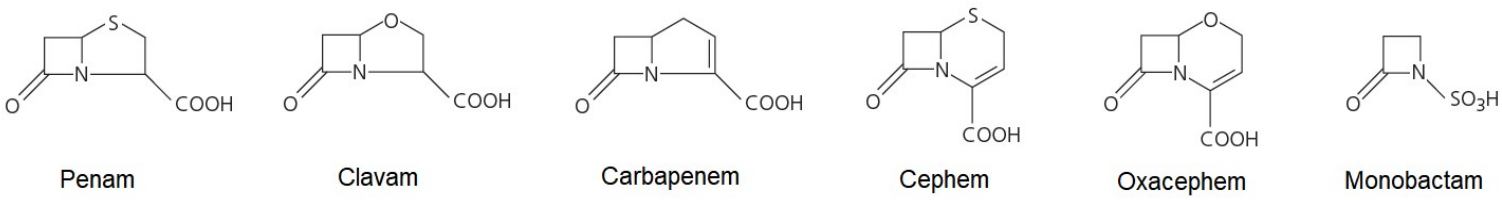

Figure 1a. The main ring structure of B-lactams [21].

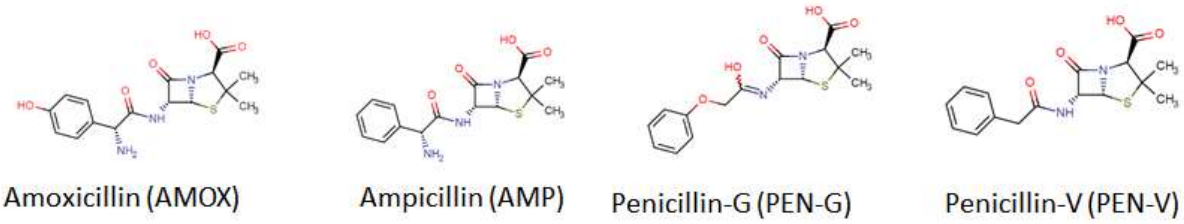

Figure 1b. Structure of B-lactams commonly used in human and animal medicine[159].

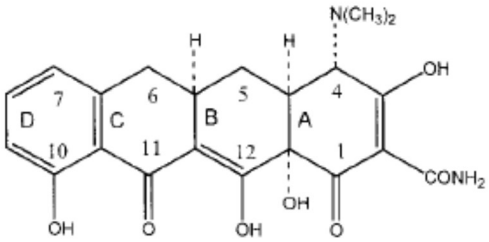

Figure 2a. The structure of 6-deoxy-6-demethyltetracycline, the simplest structure of tetraycline showing antibacterial activity [23].

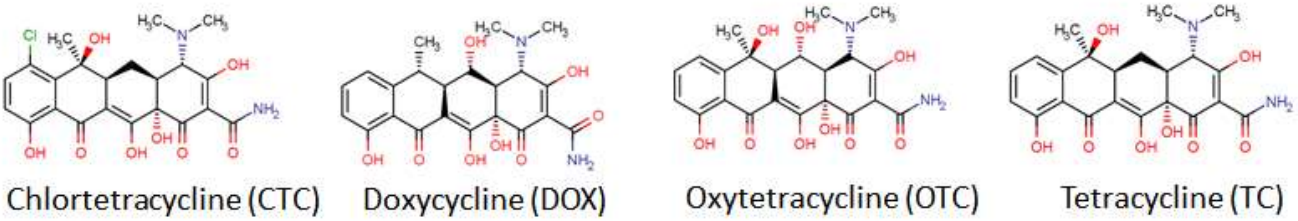

Figure 2b. Structure of some of tetracyclines commonly used for human and animal applications[160].

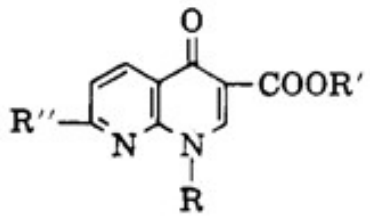

Figure 3a. 1-alkyl-1,8-naphthyridin-4-one-3-carboxylic acid [15].

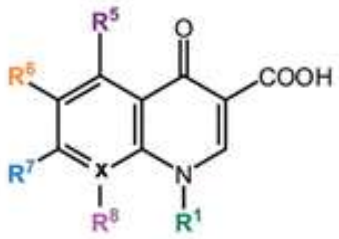

Figure 3b. Modifications in positions R1, R5, R6, R7, R8 and X have resulted in different type of quinolones [24].

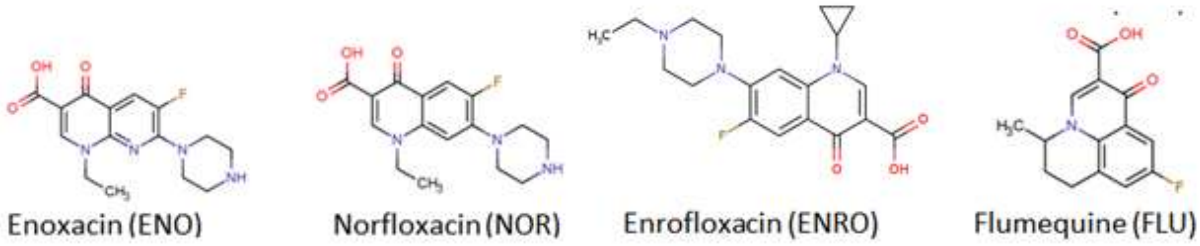

Figure 3c. Structure of some of quinolones and fluoroquinolones commonly used for human and animal applications[161-164].

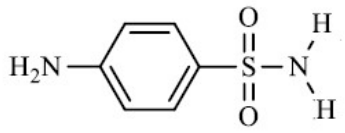

Figure 4a. Structure of sulfanilamide [26].

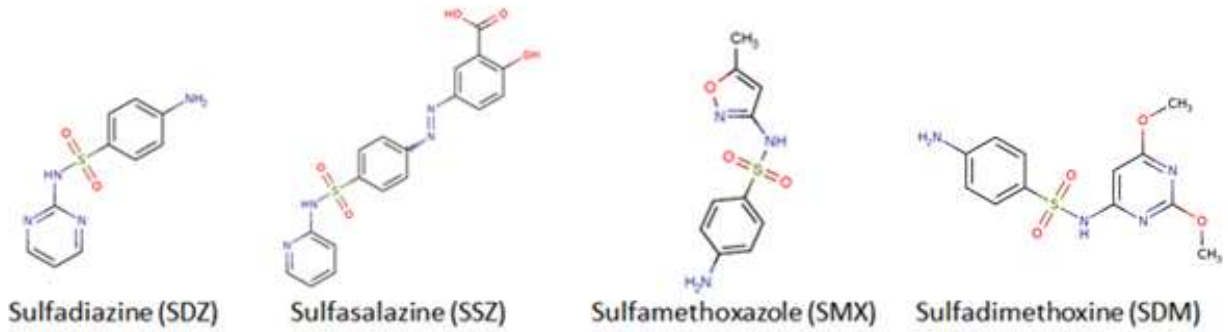

Figure 4b. Structure of some of sulfonamides commonly used for human and animal applications[165-168].

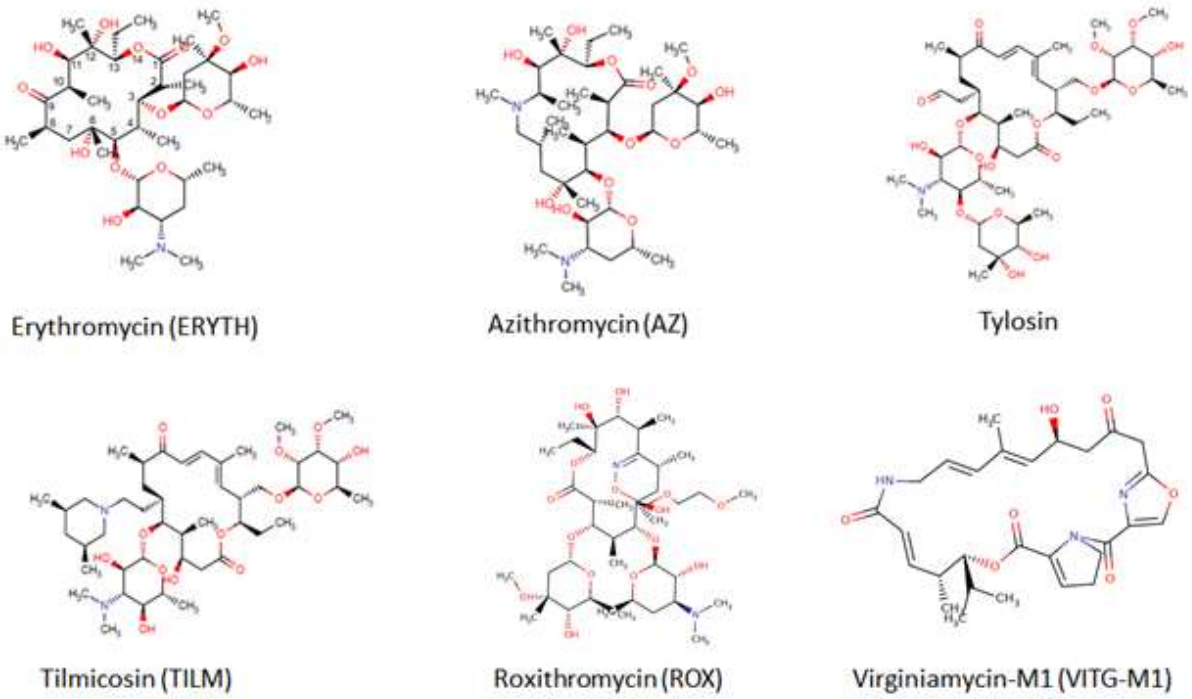

Figure 5. Structure of some of macrolides commonly used for human and animal applications[169-172].

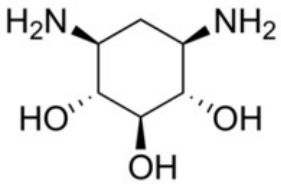

Figure 6a. Structure of 2-deoxystreptamine[173].

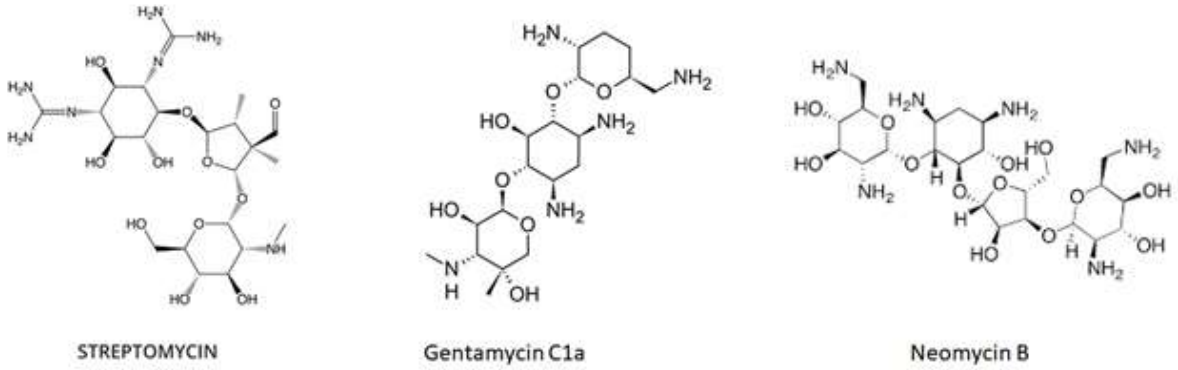

Figure 6b. Structure of common aminoglycosides[174-176].

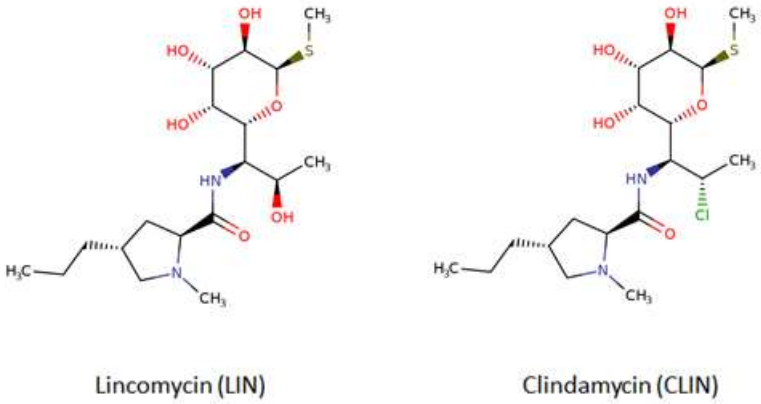

Figure 7. Structure of lincomycin and clindamycin from lincosamides class[177-178].

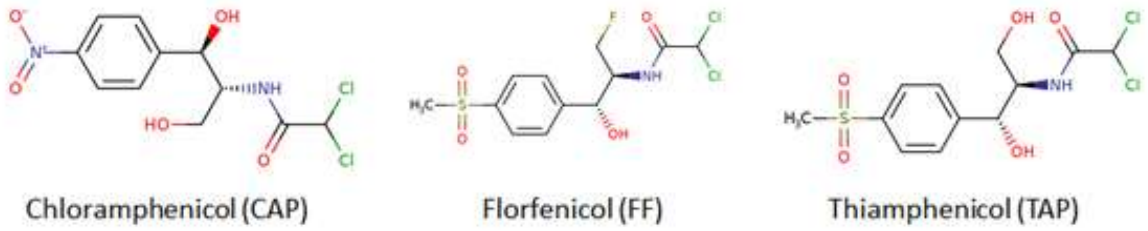

Figure 8. Structure of amphenicols class[179-181].

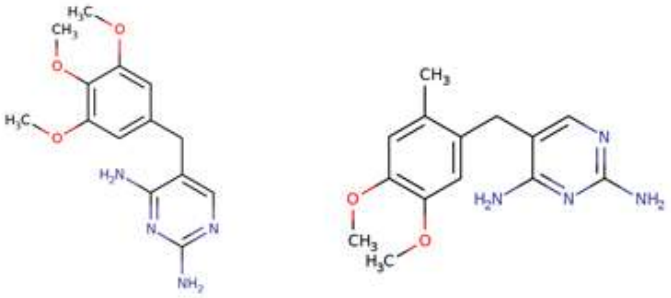

Figure 9. Structure of trimethoprim and ormetoprim[182-183].

Chapter 2: Antibiotic standards stored as a mixture in water: methanol are unstable at various temperatures irrespective of pH and glass container silanization

Shiva Emami [1], Larry A. Lerno [2], Ameer Y. Taha [1*]

Author affiliation:

[1] Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California, Davis, CA, USA, 95616

[2] Food Safety and Measurement Facility, University of California, Davis, CA, USA, 95616

*Corresponding author:
Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California Davis, One Shields Avenue, Davis, CA, USA 95616; Email: ataha@ucdavis.edu

Running title: stability of antibiotic standards in water: methanol

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Abbreviations used

Amoxicillin (AMOX), ampicillin (AMP), azithromycin (AZ), chloramphenicol (CAP), collision energy (CE), chlortetracycline (CTC), ciprofloxacin (CIP), doxycycline (DOX), enoxacin (ENO), enrofloxacin (ENRO), erythromycin (ERYTH), florfenicol (FF), florfenicol amine (FFA), flumequine (FLU), high density polyethylene (HDPE), liquid chromatography-tandem mass spectrometry (LC-MS/MS), lincomycin (LIN), multiple reaction monitoring (MRM), norfloxacin (NOR), ofloxacin-D3 (OFL-D3), Oxolinic acid (OXO), oxytetracycline (OTC), penicillin G (PEN-G), penicillin V (PEN-V), roxithromycin (ROX), sulfadimethoxine (SDM), sulfadiazine (SDZ), sulfamethoxazole (SMX), sulfamethazine-D4 (SMZ-D4), sulfasalazine (SSZ), tetracycline (TC), thiamphenicol (TAP), tilmicosin (TILM), and trimethoprim (TRIM), virginiamycin (VIRG), ultra-high pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Abstract

It is well-established that antibiotics stored individually at their optimal pH and in appropriate solvents, are stable over time. However, limited information exits on the stability of antibiotics from multiple classes when prepared and stored as a mixture prior to multi-residue analysis by mass-spectrometry. This study tested the stability of antibiotic mixtures from eight classes (amphenicols, tetracyclines, sulfonamides, quinolones, macrolides, B-lactams, lincosamides and miscellaneous (i.e. trimethoprim)) in relation to the water: methanol ratio, presence of sodium hydroxide base (to solubilize quinolones), storage temperature and container type including plain and silanized glass vials. Antibiotics were analyzed using ultra-high performance liquid chromatography coupled to tandem mass spectrometry. Several antibiotics, mainly quinolones, tetracyclines and macrolides were unstable when stored as mixture for one week regardless of the water: methanol ratio, storage temperature and presence/ absence of sodium hydroxide. Silanization of the glassware improved the storage stability of quinolones and macrolides, but reduced the storage stability of the tetracyclines and other antibiotics including florfenicol amine, penicillin G, erythromycin and sulfadiazine. Our results show that several antibiotics in water: methanol are unstable when stored as a mixture, and suggest a limited advantage of using base or silanized glass vials for the preparation and storage of antibiotic standard mixtures. Freshly prepared antibiotic standard mixtures are recommended for multi-residue quantitation of antibiotics.

Keywords: Antibiotics, Stability, UPLC-MS/MS, Silanization

Introduction

Antibiotics are frequently used in aquaculture and agriculture farms to prophylactically prevent or treat infections. However, their routine use has been linked to the development of antibiotic resistant genes that can laterally spread to humans [47, 50]. In the U.S., antibiotic resistant infections are responsible for 35,000 deaths per year [184]. In addition to genetic tools which monitor antibiotic drug resistant genes in the food supply [185-186] or in human excrements [187], direct measurement of residues remains an important way to probe antibiotic contamination in the food supply and to estimate exposure risks.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is typically used for the detection and quantitation of antibiotic residues in food and environmental samples [75, 110-111, 138, 188-189]. The use of tandem MS in multiple reaction monitoring (MRM) mode allows the simultaneous detection and monitoring of multiple precursor and product ion transitions [190]. This approach has been used to screen for antibiotics in food, environmental and human samples [85, 110, 191].

Antibiotic quantitation is based on a standard curve as well as spiked labeled surrogates that correct for losses during the extraction and act as internal standards to compensate for matrix effects and instrument variation. Thus, a known amount of antibiotic standard mix is typically prepared at various concentrations and serially diluted to obtain a calibration curve on LC-MS/MS. Quite often, these standard mixtures are prepared individually in stock and stored at cold temperatures until use [132-133, 192].

The stability of antibiotics during storage could be affected by several factors such as temperature [111, 132], pH [134, 136, 193], solvent composition [137] and container type [138]. Quinolones for instance yield irreproducible ionization patterns on positive electrospray mass-spectrometry when

dissolved at high pH [133], despite being more soluble in water when sodium hydroxide is used to raise the pH [194]. Studies have shown that individual antibiotics or antibiotic classes are stable for 6-12 months when dissolved when dissolved individually or by class, in the appropriate solvent (e.g. methanol, water or water/acetonitrile) and stored at -20 or -80 ºC [132-133, 192, 195]. However, when class-specific mixes were stored in water at 4 ºC, some classes (macrolides and quinolones in particular) were found to be unstable (% change in concentration > 20%) after one week [138]. Additionally, stability was dependent on the container type, i.e. plain glass, silanized glass or high density polyethylene (HDPE) containers. Macrolides were found to be unstable in all three types of containers , quinolones were unstable in plain glass and HDPE containers, tetracyclines were unstable in silanized glass and B-lactams were unstable in and HDPE containers [138].

Despite the availability of information on the stability of antibiotics as individual compounds or class-specific mixtures, there is limited information on the stability of multi-class antibiotics mixtures. Thus, this study tested the stability (up to 7 days) of multi-class antibiotic (amphenicols, tetracyclines, sulfonamides, quinolones, macrolides, B-lactams, lincosamides and others) dissolved in various ratios of water: methanol with or without stabilizing additive (sodium hydroxide) at different temperatures (4, -20 and -80 ºC). Additionally, the effect of container type (plain glass vs. silanized glass) on storage stability was tested.

Materials and methods

Materials

LC/MS grade methanol, acetonitrile, toluene and dimethyldichlorsilane were obtained from Fisher Scientific (Hampton, NH, USA). Formic acid and sodium hydroxide (NaOH) were

purchased from Sigma-Aldrich (St. Louis, MO). Antibiotic standards used in this study were from the following classes:

Amphenicols: chloramphenicol (CAP), thiamphenicol (TAP), florfenicol (FF), florfenicol amine (FFA); **Tetracyclines:** tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline HCl (DOX); **Sulfonamides:** sulfadimethoxine (SDM), sulfasalazine (SSZ), sulfamethoxazole (SMX), sulfadiazine (SDZ); **Quinolones:** enrofloxacin (ENRO), oxolinic acid (OXO), flumequine (FLU), ciprofloxacin (CIP), norfloxacin (NOR) and enoxacin (ENO); **Macrolides:** erythromycin (ERYTH), azithromycin (AZ), tylosin A (Tylosin), virginiamycin (VIRG) complex (mixture of VIRG-M1 and VIRG-S1), roxithromycin (ROX), tilmicosin phosphate (TILM); **B-lactams:** ampicillin anhydrous (AMP), penicillin G potassium salt (PEN-G), penicillin V (PEN-V) and amoxicillin (AMOX); **Lincosamides:** lincomycin (LIN), **Others:** trimethoprim (TRIM).

CIP (98%), AMOX (98%), ROX (97%), SDZ (99%), TAP (99.3%), OXO (98%) and FF (98%) were purchased from Fisher Scientific (Hampton, NH, USA). ERYTH (94.8%), DOX (98.8%), NOR (98%), AMP (99.6%), SDM (98.5%), ENRO (99.8%), TC (≥ 98%) and FFA (99.3%) were purchased from Sigma Aldrich (St. Louis, MO). CTC (98.0%), OTC (≥ 95%), FLU (100.0%), ENO (100%), AZ (99.5%), Tylosin (99.8%), VIRG (99.0%), PEN-G (99.5%), PEN-V (98.8%), SSZ (100%), SMX (100%), LIN (98%), TRIM (100%) and TILM (100%) were purchased from Cayman Chemicals (Ann Arbor, MI). CAP (98.5%) was purchased from Crescent Chemical (Islandia, NY). Isotopically labeled standards including CAP-D5 (chemical purity: 98%; isotopic purity: 98.3%), SMX-D4 (chemical purity: 98%; isotopic purity: 99.2%), sulfamethazine-D4 (SMZ-D4; chemical purity: 98%; isotopic purity: 95.9%), AZ-D3 (HPLC purity: 99.86%; isotopic purity: 93.9%), ERYTH-D6 (chemical purity: 95%; isotopic purity: 98.1%), TRIM-D3

(chemical purity: 99.49%; isotopic purity: 99.9%), LIN-D3 (chemical purity: 95%; isotopic purity: 99.6%), CIP-D8 (HPLC purity: 98.91%; isotopic purity: 98.4%) and (R)-Ofloxacin-D3 (OFL-D3, HPLC purity: 99.91%; isotopic purity: 99.7%) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

Study design

Experiment 1: Effect of storage temperature on the stability of antibiotic standards in water: methanol

The goal of this experiment was to assess the stability of antibiotic standard mixture dissolved in water: methanol (90:10 or 50:50 ratios; n=1 per solvent ratio) at different storage temperatures (4, -20 and -80 ºC) for one week. Individual stock solutions of CAP, TAP, FF, FFA, TC, OTC, CTC, DOX, SDM, SMX, ENRO, ERYTH, AZ, VIRG, ROX, TILM, TRIM, LIN, CAP-D5, SMX-D4, SMZ-D4, AZ-D3, ERYTH-D6, TRIM-D3 were prepared in methanol at 1 mg/mL concentration. SSZ, SDZ, Tylosin, LIN-D3 were prepared in methanol at a concentration of 0.5 mg/mL. B-lactams were prepared in Milli-Q water at 1 mg/mL. OXO, FLU, CIP, NOR and ENO were prepared at concentration of 0.5 mg/mL in 0.01 mol/L NaOH in water. CIP-D8 and OFL-D3 were prepared in 0.01 mol/L NaOH in water at concentration of 1 mg/mL. The stock solutions were diluted from 0.5 or 1 mg/mL to individual 'intermediate' solutions of 10 µg/mL using the same solvent as the stock solution.

The individual intermediate solutions were used to prepare antibiotic mixture solutions (working mix) of all antibiotic standards including both unlabeled and labeled standards. Working mixes were prepared at 90:10 and 50:50 water: methanol (by volume) to test whether solvent composition affects storage stability. As described in the next two paragraphs, methanol-soluble

antibiotics were mixed first, followed by water-soluble antibiotics, which include β-lactams and quinolones (except for ENRO because it is methanol soluble).

To make a working mix in 90:10 water: methanol, 10 µL of individual intermediate antibiotics (10 µg/mL) dissolved in methanol were added to 2 mL amber glass vials (Phenomenex, Torrance, CA) and dried under nitrogen. Then, 100 µL methanol, 790 µL of Milli-Q water and 10 µL of individual intermediate solution of antibiotics dissolved in water (four B-lactams) or water with 0.01 mol/L NaOH (containing all 5 quinolones except ENRO, plus their CIP-D8 and OFL-D3 surrogates) were added to the vial to make an antibiotics mixture solution of 100 ng/mL in 90:10 water:methanol.

To make a working mix in 50:50 water: methanol, 10 µL of individual intermediate solution of methanol soluble antibiotics (10 µg/mL) was dried under nitrogen. Then, 500 µL of methanol, 390 µL of water and 10 µL of intermediate solution of individual antibiotics dissolved in water (four β-lactams) or basic water (for all 5 quinolones plus their CIP-D8 and OFL-D3 surrogates, except ENRO) were added to the vial to make a 100 ng/mL mixture.

Both working mixes were diluted to 10 ng/ mL (using the same solvents they were dissolved in), and aliquots of each were separately stored at 4, -20 and -80 ºC for one week. The antibiotic standard mixtures were analyzed on day 1 (preparation day) and day 7 using ultra-high pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Thus, samples were run on UPLC-MS/MS on separate days, but immediately after their storage periods.

Experiment 2: Effect of sodium hydroxide removal from solution on storage stability of antibiotics mixture in water: methanol

In Experiment 1, we observed that several antibiotics were unstable at the three different temperatures after 7 days of storage. We hypothesized that the presence of sodium hydroxide in the antibiotic mixture solution might destabilize antibiotics. This hypothesis was based on a study showing that sodium hydroxide reduced the stability of quinolones measured with UPLC-MS/MS [133]. Therefore, in Experiment 2 sodium hydroxide was not added to the working mix solutions.

Notably, we also observed that solubility was greatly reduced when the 5 water-soluble quinolones (OXO, FLU, CIP, NOR and ENO) and their surrogate standards (CIP-D8 and OFL-D3) were dissolved in water lacking NaOH (all are soluble in water containing NaOH as pointed out above). We therefore tested whether they are soluble in methanol. Only NOR, FLU and ENO were soluble in methanol at 0.2 mg/mL. Therefore, for experiment 2, only NOR, FLU and ENO, as well as ENRO (which is methanol-soluble as pointed above), were tested.

Antibiotics were dissolved in methanol or water (for β-lactams) at a concentration of 0.2-1.0 mg/mL and diluted to 10 µg/mL to make an intermediate solution of each. Working mixes (100 ng/mL) were prepared in two compositions of water:methanol (90:10 and 50:50) by adding 10 µL of the intermediate solution of methanol-soluble antibiotics (10 µg/mL) to 2 mL glass amber vials and drying them under nitrogen. Then, appropriate volumes of methanol and water were added followed by the addition of water-soluble antibiotics (β-lactams; note that these do not require NaOH in order to dissolve in water). Both mixtures were diluted to 10 ng/mL and analysed by UPLC-MS/MS on day 1 and after seven days of storage at −80 °C (n=2 per solvent mixture per day).

Experiment 3: Effect of container type (plain vs. silanized glass) on storage stability of antibiotic standards mixture

The results of Experiments 1 and 2 showed that several antibiotics, particularly tetracyclines, quinolones and macrolides (AZ and TILM), were unstable during one week of storage at 90: 10 and 50: 50 water: methanol, in the presence or absence of NaOH and at different temperatures. We hypothesized that this could be due to the sorption of antibiotics to the container surface as previously pointed out by the Environmental Protection Agency [138]. Thus, in Experiment 3 we tested whether antibiotic sorption to the glass container improves their stability.

The inner surface of glassware (vials (Cat #: AR0-3911-13) and inserts (Cat #: WAT094171) used for LC-MS analysis was deactivated using the silanizing reagent, dimethyldichlorsilane, as described by Ye and Weinberg [196]. Briefly, vials and inserts were first treated with 5% dimethyldichlorsilane in toluene for a few seconds, and then rinsed 3 times with toluene and methanol to remove excess silanizing reagent (1 mL x 3 for vials and 100 uL x 3 for inserts for each solvent). Silanized vials and inserts were then rinsed with similar volumes of Milli-Q water and dried before use.

Antibiotic standards (100 ng/mL) were prepared in both silanized and plain (non-silanized) glassware and in solutions of 90:10 and 50:50 of water:methanol (no base was added). The final working mixture was prepared by diluting the 100 ng/mL mixture in 90:10 or 50:50 of water:methanol to a concentration of 10 ng/mL, as described above. Similar to experiment 2, only quinolones soluble in methanol (i.e., ENO, NOR, ENRO, and FLU) were used ~~for~~in this experiment. Antibiotic mixtures were stored at −80 °C for one week (n=3 per condition). A workflow diagram for experiment 3 is shown in **Figure 1**.

A limitation of experiments 1 and 2 is that the fresh (day 1) and stored (day 7) samples were not captured on the same UPLC-MS/MS run. This means that the observed instability in antibiotic standards during storage may be due to day-to-day variability in UPLC-MS/MS

response. To account for this possibility, in experiment 3, the antibiotic mixtures prepared and stored at −80 °C for one week were analysed alongside freshly prepared mixtures on the same run.

Storage stability of antibiotic standards

Storage stability of each antibiotic during 7-day storage at -80 ˚C was assessed as the % change in peak area from day 1 to day 7 according to equation 1:

$$\% \text{ change in peak area} = \frac{A_7 - A_1}{A_1} \qquad \text{equation 1}$$

Where A_1 is the peak area of antibiotic in freshly prepared solution (day 1) and A_7 is the peak area after 7 days storage at -80 ˚C.

The storage stability criteria for the antibiotics was determined by identifying a tolerance limit (TL) calculated from the variability of MS measurements from two data sets, according to a method described by Desmarchelier et al. [133] with some modifications. The two data sets were from two fresh preparations of the antibiotics mixture ("a" and "b") and were considered as duplicates of each other. Each data set contained 4 groups including antibiotics mixtures dissolved in 90:10 water:methanol and stored in plain glass (P-90-10) or silanized glass (S-90-10), and mixtures dissolved in 50:50 water:methanol and stored in plain glass (P-50-50) or silanized glass (S-50-50). Therefore, in total, four pairs of data were used to calculate the %TL following the steps below:

i. Raw peak areas for each antibiotic were standardized by dividing them by the average peak area from 3 injections.

ii. Standard deviation (SD) of the difference between standardized peak areas of two data sets, SD_i (A_a – A_b) was calculated for each antibiotic (i) and in each pair, i.e. P-90-10, S-90-10, P-50-50 and S-50-50, according to equation 2:

$$SD_i(A_a - A_b) = \sqrt{SD^2_{pooled}\,(\frac{1}{n_a} + \frac{1}{n_b})}$$ equation 2

Where n_a = number of replicates in data set "a" (n_a = 3) and n_b = number of replicates in data set "b" (n_b = 3). SD^2_{pooled} was calculated from equation 3:

$$SD^2_{pooled} = \frac{SD^2_{A_a} + SD^2_{A_b}}{2}$$ equation 3

iii. Then, the median value of SD_i ($A_a - A_b$) for the four pairs was calculated for each antibiotic from equation 4:

$$SD_{i,Median} = Median\,[\,SD_i(A_a - A_b)_{P-90-10}, SD_i(A_a - A_b)_{S-90-} \quad , SD_i(A_a - A_b)_{P-50-50}, SD_i(A_a - A_b)_{S-50-50}]$$ equation 4

iv. Then, a universal SD i.e. $SD_{Universal}$ was calculated based on the $SD^2_{i,\ Median}$ of all antibiotics according to equation 5:

$$SD_{Universal} = \sqrt{\frac{1}{n_i}\sum_{i=1}^{ni} SD^2_{i,\ Median}}$$ equation 5

Where n_i is the total number of antibiotics, i.e. 36.

v. % TL, at 95% confidence level, was calculated according to equation 6:

$$\%\,TL = 1.96 \times SD_{Universal}$$ equation 6

Where 1.96 is the z-score for the 95% probability level. Based on the two data sets of freshly prepared antibiotics mixtures, we calculated $SD_{Universal}$ = 15% and %TL = 29%. Antibiotics were considered to be stable if the % change between peak areas of stored (day 7) and fresh (day 1) samples was in the range of ± 29%.

Instrumentation

Antibiotic analysis was performed using an Agilent 1290 UPLC coupled to a 6460 Agilent triple quadrupole. Chromatographic separation of the antibiotics mixture was performed on

AQUITY BEH C18 column (100 × 2.1 mm, 1.8 µm), using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) running at flow rate of 0.3 mL/min and column temperature of 30 ºC. The mobile phase gradient condition was as follows: initial time: 10% B, 8 min: 20% B, 11 min: 60% B, 13 min: 100% B, 15 min: 100% B, 17 min: 10% B and 20 min: 10% B.

MS/MS analysis was performed using Agilent Jetstream electrospray ionization (ESI) operating in both positive and negative mode and using dynamic MRM scan type. MS source parameters were optimized using a solution of antibiotics mixture (each compound at concentration of 100 ng/mL) dissolved in water: methanol (90: 10) leading to the highest intensity of the precursor ions. Optimized source parameters were as follows: sheath gas temperature of 375 ºC, sheath gas flow of 11 L/min, drying gas temperature of 250 ºC, nozzle voltage of 0V, nebulizer gas pressure of 40 psi and capillary voltage of 3500 V. Product ions were chosen by running the product ion scan of each antibiotic standard. Using optimized source parameters and selected quantifier product ions, collision energy (CE) was then optimized to obtain the highest intensity for product ions. Also, qualifier ions along with their proper CEs were established for confirmation of analyte identity. **Table 1** shows the precursor ion, quantifier and qualifier product ions, fragmentor voltage, and CE for antibiotic standards.

Results

Experiment 1. Effect of storage temperature on storage stability of antibiotics mixture in water: methanol

a) Storage stability of antibiotics mixture in water: methanol (90: 10)

Storing antibiotic standard mixture (each at a concentration of 10 ng/mL) in water: methanol (90: 10) for one week affected their stability, calculated as % change in peak area from

day 1 (baseline) to day 7, regardless of the storage temperature. The percent change in peak area for each compound is presented in **Figure 2**. The same percent change values are reported in table format in **Table S1**. Compared to baseline, we observed a decrease in peak area of 85–89%, 80–84%, and 75–83% for quinolones (ENO, NOR, ENRO, CIP, CIP-D8, OFL-D3) and 92–93%, 80–85%, and 44–53% for tetracyclines in samples stored at 4, −20, and −80 °C, respectively, for one week (**Figure 2**, **Table S1**). Three macrolides - AZ, AZ-D3, and TILM - showed a marked 5–8 fold increase in peak area on day 7 compared to day 1, and this was evident at all storage temperatures (**Figure 2**, **Table S1**). Other antibiotics exhibited increased peak areas at all three temperatures. At 4 °C, increased peak area was observed for other macrolides including ERYTH, ERYTH-D6, Tylosin, ROX, VIRG-M1, and VIRG-S1 (32–70%); sulfonamides including SMX-D4, SMX, SDZ, and SDM (34–39%); and the 'other' antibiotics consisting of TRIM and TRIM-D3 (30–31%). At −20 °C, increased peak areas were observed for macrolides including ERYTH, ERYTH-D6, Tylosin, ROX, VIRG-M1, and VIRG-S1 (29–115%); all sulfonamides (35–102%); TRIM and TRIM-D3 (58%); lincosamides (LIN and LIN-D3; 33–34%); B-Lactams (PEN-G and PEN-V; 36–42%); and the amphenicol, FFA (34%). Only SDZ (29%) and ROX (31%) increased after 1 week storage at −80 °C (**Figure 2**, **Table S1**).

b) Storage stability of antibiotics mixture in water: methanol (50: 50)

Similar to what we observed for antibiotics stored in water:methanol at a 90:10 ratio, storage of antibiotics mixture (10 ng/mL) in water:methanol (50:50) for one week decreased peak areas for quinolones (ENO, NOR, ENRO, CIP, CIP-D8, and OFL-D3) and tetracyclines (TC, OTC, CTC, and DOX) and increased peak areas for AZ, AZ-D3, and TILM at all studied temperatures (**Figure 2**, **Table S1**). The percent decrease in peak area was 90–94%, 88–92%, and 77–82% for quinolones and 99–100%, 94–97%, and 85–88% for tetracyclines in solutions

stored at 4, −20, and −80 °C, respectively. Peak areas for AZ, AZ-D3, and TILM were 7–8.6, 2.4–3.7, and 2–2.7 fold higher on day 7 compared to day 1 in samples stored at 4, −20, and −80 °C, respectively. Other antibiotics such as sulfonamides, macrolides, and β-lactams also showed instabilities (% changes in peak area out of the ± 29% range) during one week storage. A 35-51% increased response was observed for sulfonamides (SMX, SMX-D4, SDZ, and SDM) at −80 °C. An increased response for several antibiotics was also observed at at −20 °C; specifically among all sulfonamides (42–100%), β-lactams (AMOX, PEN-G, and PEN-V; 29–41%), macrolides (ERYTH, ERYTH-D6, Tylosin, ROX, VIRG-M1, and VIRG-S1; 32–60%), lincosamides (LIN and LIN-D3; 37–38%), one amphenicol (FFA; 38%), and other (TRIM and TRIM-D3; 34–35%). At 4 °C only VIRG-S1 increased (34% change from baseline). Amphenicols (FF, TAP-CAP-D5) at −80 °C and β-lactams at 4 °C showed a decreased peak area of 29–39% and 29–41%, respectively, after 7 days in 50:50 water:methanol (**Figure 2**, **Table S1**).

Experiment 2: Effect of sodium hydroxide removal from solution on storage stability of antibiotics mixture in water: methanol

In Experiment 1 we observed that antibiotic mixtures dissolved in various ratios of water: methanol containing sodium hydroxide were not stable (% change in peak area > ± 29% and therefore out of tolerance) during one week storage, at all temperatures tested (4, -20 and -80 ºC). In Experiment 2 we tested whether the added base contributesd to the observed instability of antibiotics, in view of a study showing that quinolones ionize unpredictably on LC-MS/MS at basic pH [133]. Antibiotics were dissolved in water:methanol at 90:10 and 50:50 ratios (concentration = 10 ng/mL) without added sodium hydroxide. Notably, CIP, CIP-D8, OFL-D3, and OXO were not included in the mixture because they did not dissolve in pure methanol used to prepare the stock solutions. Storage stability of the antibiotic mixture solution was tested at −80 °C only.

Similar to experiment 1, decreased peak areas for quinolones (ENO, NOR, and ENRO) and tetracyclines (TC, CTC, OTC, and DOX) and increased peak areas for macrolides (AZ, AZ-D3, and TILM) were observed after one week of storage at −80 °C (**Figure 3**, **Table S3**). The percent decreases in peak area relative to day 1 was 52–71% and 51–61% for quinolones and 71–84% and 78–82% for tetracyclines stored in water:methanol at 90:10 and 50: 50 ratios, respectively. AZ and AZ-D3 increased by 42–60% in water:methanol (90: 10) and by 8.8-fold in water:methanol (50: 50) after one week. The TILM peak area increased by 8.5-fold in water:methanol (50:50) and decreased by 61% in water:methanol (90: 10) relative to baseline (day 1). TRIM and other macrolides including ERYTH and its deuterated form (ERYTH-D6), Tylosin, and ROX showed a 32–134% increase in peak area after one week storage in 90:10 water: methanol, but were stable in 50:50 water:methanol. SMZ-D4 showed an increased peak area of 32–40% in both 90:10 and 50:50 water:methanol during the one-week storage period (**Figure 3**, **Table S3**).

Experiment 3: Effect of container type (plain vs. silanized glass) on storage stability of antibiotics mixture in water: methanol

In experiments 1 and 2, we observed that antibiotic standards were not stable in water:methanol solution during one week storage, irrespective of the storage temperature (4, −20, and −80 °C), water:methanol composition (90:10 and 50:50), and the presence or absence of sodium hydroxide. We hypothesised that the observed instability could be due to the adsorption of antibiotics onto the glass surface during the one-week storage period. In experiment 3, we tested this possibility by storing antibiotic mixtures in silanized and non-silanized glass vials at −80 °C, in both 90:10 and 50:50 water:methanol. The results for this experiment are presented in **Figure 4**, and the values for the percent changes are given in **Table S3**.

a) Storage stability of antibiotic mixture in water: methanol (90: 10)

As shown in **Figure 4** (also **Table S3**), the peak areas of several antibiotics increased by day 7, when stored in 90:10 water:methanol using plain (non-silanized) glass vials. Compared to day 1, quinolones (ENO, NOR, and ENRO) increased by 3.5–5 fold, the macrolides AZ, AZ-D3 and TILM by 6–23 fold, and ROX, Tylosin, VIRG-M1, and VIRG-S1 by 34–52%), the amphenicol TAP by 42%, and other non-class antibiotics (TRIM and TRIM-D3) by 43–45%. The peak areas of tetracyclines decreased during storage by 35–68% (day 7 vs. day 1) (**Figure 4**, **Table S3**). These results are generally consistent with the findings of experiments 1 and 2 except that quinolones showed decreased peak areas in experiments 1 and 2 but increased peak areas in experiment 3 during the one week storage period (**Figures 2**, **3**, and **4**).

In silanized glassware, quinolones were stable during the one week storage period (% change in peak area within ±29%) except for ENRO, which showed a 41% increase in peak area. The peak areas for other antibiotics were not as stable. For instance, we observed a 79% increase in the peak area for TRIM, 152–207% increase ~~for~~in peak areas for AZ, AZ-D3, TILM, ROX, and Tylosin, and 56% increase in peak area for VIRG-S1 on day 7 compared to day 1. Tetracyclines showed an 86–97% reduction in peak area when stored in water:methanol (90: 10) using silanized glass vials (**Figure 4**, **Table S3**).

b) Storage stability of antibiotic mixture in water: methanol (50: 50)

Several antibiotics dissolved in 50:50 water:methanol were unstable one week after storage in plain (non-silanized) glass vials. Peak areas for macrolides (AZ, AZ-D3, and TILM) and quinolones (NOR and ENRO) increased by 3.8–6 fold and 29–40%, respectively, on day 7 compared to baseline (day 1). The peak areas for tetracyclines decreased by 41–55%.

The peak area was higher on day 7 (vs. day 1) for several antibiotics stored in silanized glass vials. These were AZ, AZ-D3, and TILM (32–51%); FFA (213%); PEN-G (69%); TRIM (30%); ERYTH and ERYTH-D6 (27-fold); and ROX and VIRG-M1 (29–37%). Peak areas for tetracyclines and SDZ were lower by 51–69% and ~34%, respectively (**Figure 4**, **Table S3**).

Discussion

This study assessed the stability of 36 labelled and unlabelled antibiotic standards from eight classes dissolved as a mixture in 90:10 or 50:50 water:methanol, with or without sodium hydroxide, over a period of one week. Several antibiotics were unstable (% change in peak area > 29%) during storage, and this was independent of storage temperatures (4, −20, and −80 °C), solvent composition (water:methanol 90:10 and 50:50) (experiment 1, **Figure 2**, **Table S1**), and removal of NaOH from the solution (experiment 2, **Figure 3**, **Table S2**). Quinolones, tetracyclines, and macrolides (AZ, AZ-D3, and TILM) were the most unstable antibiotics during one week storage in water:methanol (experiments 1 and 2). Silanizing the vials used to store antibiotics improved the stability of some antibiotics (quinolones and macrolides) but did not resolve the issue completely (experiment 3).

Consistent with experiments 1 and 2, quinolones, tetracyclines, and macrolides (AZ, AZ-D3 and TILM) were unstable in experiment 3 (% change > 29%) when stored in 90:10 or 50:50 water: methanol for one week (**Figure 4**, **Table S3**). The response was consistent between experiments, except for quinolones, which decreased in water:methanol at 90:10 and 50:50 ratios in experiments 1 and 2 but increased in experiment 3, more so in the water:methanol 90:10 solvent (in non-silanized glass). This discrepancy could be due to the instability of quinolones in water:methanol or their interaction with the glass container as previously reported [138]. It is possible, also, that instrument response changed between sample measurements since fresh (day 1) and

stored (day 7) samples were not analysed on the same UPLC-MS/MS run in experiments 1 and 2 because samples were assayed immediately after preparation on days 1 and 7.

Silanization of the glassware improved the stability of quinolones, particularly at 90:10 water:methanol, and macrolides (AZ, AZ-D3 and TILM) at both water:methanol solvent ratios. However, macrolides were still considered to be unstable in both 90:10 and 50:50 water:methanol because the percent changes in peak areas were out of the tolerance limit (29%). The improved stability of compounds following silanization is in agreement with previous studies assessing the storage stability of quinolones and macrolides during seven-day storage in aqueous solution at 4 °C [138]. Without silanization, quinolones and macrolides are likely to adsorb onto silanol groups on the surface of glass vials and inserts, which act as ion exchange sites for basic functional groups o~~f~~n these antibiotics [197]. This interaction appears to have been minimi~~s~~zed when reactive sites on the glass surface were blocked with the silanizing reagent.

Tetracyclines showed reduced stability during one week storage in either plain or silanized glass vials~~,~~. They were more unstable in silanized vials, particularly when dissolved in 90:10 water:methanol (Figure 4). This is consistent with another study, which showed that glassware silanization reduced the stability of tetracyclines compared to plain non-silanized glass vials [138]. Although the half-life of tetracyclines stored in aqueous solution~~s at~~ (pH =7) at 7 °C has been reported to be long, i.e., ~26, 18, and 46 days for OTC, CTC, and TC, respectively [135], one study reported the degradation of CTC to iso-CTC in tissue samples spiked with CTC and quinolones [198]. However, no degradation was observed when CTC was stored by itself or with other tetracyclines or aminoglycosides[198]. This suggests that the presence of other antibiotic~~s~~ classes in the mixture may contribute to the instability of tetracyclines.

Tetracyclines can also adsorb onto the glass surface via both electrostatic and non-electrostatic interactions involving hydrogen bonding of the amide groups of tetracyclines and the silanol groups of the glass surface [199]. Reduced peak areas in silanized vials compared to non-silanized vials, particularly in 90:10 water:methanol, suggests that hydrophobic methyl-modified surfaces in silanized glass likely enhanced the affinity of tetracyclines to the surface, resulting in decreased storage stability.

Glassware silanization also reduced the storage stability of TRIM and ROX in both 90:10 and 50:50 water:methanol, macrolides (Tylosin and VIRG-S1) in 90:10 water:methanol, and amphenicols (FFA), β-lactams (PEN-G), macrolides (ERYTH and VIRG-M1), and sul~~ph~~fonamides (SDZ) in 50:50 water:methanol (**Figure 4**, **Table S3**). Reduced stabilities were inferred from the increased peak areas during storage for all of these antibiotics except for SDZ, which showed decreased peak area. This indicates that these antibiotics interact with the silanized surface to varying degree (**Table S4**).

Conclusion

Storing antibiotic mixtures in water:methanol for one week reduced the stability of quinolones, tetracyclines, and macrolides (AZ and TILM), irrespective of-storage temperature, water:methanol composition, and the presence or absence of sodium hydroxide. Silanization improved the stability of quinolones and some macrolides (AZ and TILM) but worsened the stability of tetracyclines, TRIM, amphenicols (FFA), β-lactams (PEN-G), other macrolides (ERYTH), and sulfonamides (SDZ). This indicates the limited benefits of glassware silanization towards antibiotics stored as a mixture. Based on these observations, preparing fresh mixtures or individual preparation of antibiotic standards in appropriate solvents is recommended for the reliable analysis of antibiotics in multiresidue methods. In addition, using internal standards that

behave similarly to target antibiotics in terms of interaction with the glass surface could improve the accuracy of quantitation.

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Declaration of interest statement

The authors declare that there is no conflict of interest.

Author contributions

S.E. and A.Y.T. designed the experiments. S.E. performed the experiments, analyzed the data and wrote the manuscript. A.Y.T. reviewed and edited the manuscript. L.A.L. assisted with the UPLC-MS/MS method development.

Supplementary information

The supporting information include the tables presenting the values of the data shown in Figures 2-4.

Table 1. Precursor ion, quantifier and qualifier product ions, fragmentor voltage, and CE for antibiotic standards.

Class	Antibiotics	Abbreviations	Precursor Ion	Fragmentor voltage	Quantifier ion (CE)	Qualifier ion (CE)	Polarity
Amphenicols	Florfenicol amine	FFA	248	75	230.1 (10)	130.1 (20)	Positive
	Thiamphenicol	TAP	353.9	125	184.9 (10)	290.1 (10)	Negative
	Florfenicol	FF	355.9	125	185.1 (10)	118.7 (30)	Negative
	Chloramphenicol	CAP	321	115	152 (10)	193.9 (10)	Negative
	Chloramphenicol-D5	CAP-D5	326.1	90	155.6 (10)	261.9 (10)	Negative
Tetracyclines	Tetracycline	TC	445.1	100	410.1 (15)	427.1 (10)	Positive
	Oxytetracycline	OTC	461.2	90	426 (15)	443 (10)	Positive
	Doxycycline	DOX	445.3	110	428.1 (15)	410.2 (20)	Positive
	Chlortetracycline	CTC	478.7	50	443.9 (20)	462 (15)	Positive
B-Lactams	Ampicillin	AMP	350.1	125	106.1 (20)	160.1 (10)	Positive
	Penicillin G	PEN G	335	110	176 (10)	160.1 (10)	Positive
	Penicillin V	PEN-V	351.2	90	160 (5)	192.2 (5)	Positive
	Amoxicillin	AMOX	365.9	90	114 (20)	207.9(10)	Positive
Quinolones	Norfloxacin	NOR	320.1	130	302.1 (15)	276.1 (15)	Positive
	Ciprofloxacin	CIP	332.1	125	314.1 (20)	288.1 (15)	Positive
	Enoxacin	ENO	321.1	100	303.1 (20)	277.2 (10)	Positive
	Enrofloxacin	ENRO	360.1	125	316.3 (15)	342 (20)	Positive
	Flumequine	FLU	262.1	90	244.1 (20)	202 (30)	Positive
	Ofloxacin-D3	OFL-D3	365.2	110	321.1 (25)	347.1 (20)	Positive
	Ciprofloxacin-D8	CIP-D8	340.2	130	322.2 (20)	296.2 (15)	Positive
	Oxolinic acid	OXO	262.1	90	244.1 (15)	216.1 (30)	Positive
Lincosamides	Lincomycin-D3	LIN-D3	410.2	90	129.1 (30)	362.1 (15)	Positive
	Lincomycin	LIN	407.1	130	126.1 (30)	359.1 (15)	Positive
Sulfonamides	Sulfasalazine	SSZ	399	130	381 (15)	317 (20)	Positive
	Sulfadimethoxine	SDM	311	110	156.1 (15)	245.1 (15)	Positive
	Sulfadiazine	SDZ	251.1	125	156.1 (10)	108.1(20)	Positive
	Sulfamethoxazole	SMX	254.1	90	156.1 (10)	108.1 (20)	Positive
	Sulfamethoxazole-D4	SMX-D4	258	90	112 (20)	160 (10)	Positive
	Sulfamethazine-D4	SMZ-D4	283	125	186 (15)	-	Positive
Macrolides	Azithromycin-D3	AZ-D3	376.7	125	594.4 (10)	82.8 (20)	Positive
	Azithromycin	AZ	375.1	75	591.2 (10)	83 (20)	Positive
	Erythromycin-D6	ERYT-D6	740.3	130	582.2 (15)	164.2 (30)	Positive
	Erythromycin	ERYT	734.3	125	576.3 (15)	158.1 (30)	Positive
	Roxithromycin	ROX	419.3	125	158 (15)	83 (20)	Positive
	Tylosin A	TYLOSIN	916.3	125	174.1 (40)	771.8 (30)	Positive
	Virginiamycin M1	VIRG-M1	526.1	130	508.3 (10)	355.1 (15)	Positive
	Virginiamycin S1	VIRG-S1	824.2	130	205.1 (25)	177.2 (30)	Positive
	Tilmicosin	TILM	869.4	90	696 (25)	174 (30)	Positive
Miscellaneous	Trimethoprim-D3	TRIM-D3	294.2	130	230.1 (25)	123.1 (25)	Positive
	Trimethoprim	TRIM	291.1	130	230.1 (20)	123 (20)	Positive

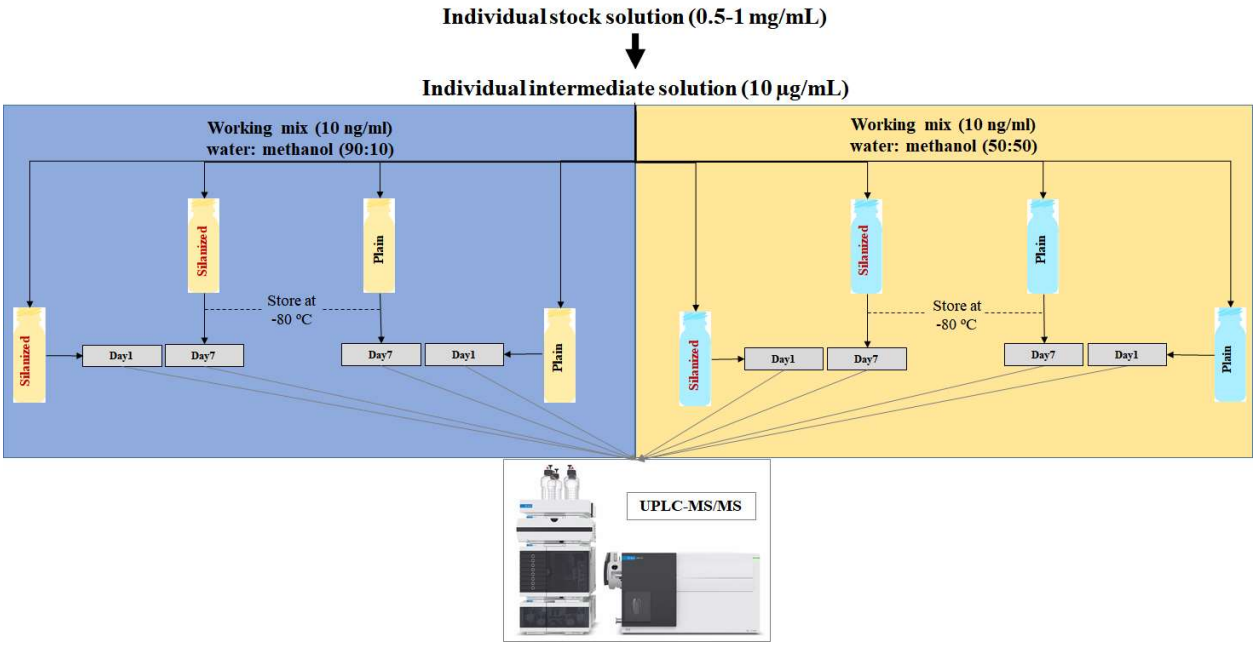

Figure 1. Flow diagram of Experiment 3 which tested the effect of container type (plain vs. silanized glass) on storage stability of antibiotic standards in water: methanol (90: 10) and (50: 50) during one week storage at -80 ˚C.

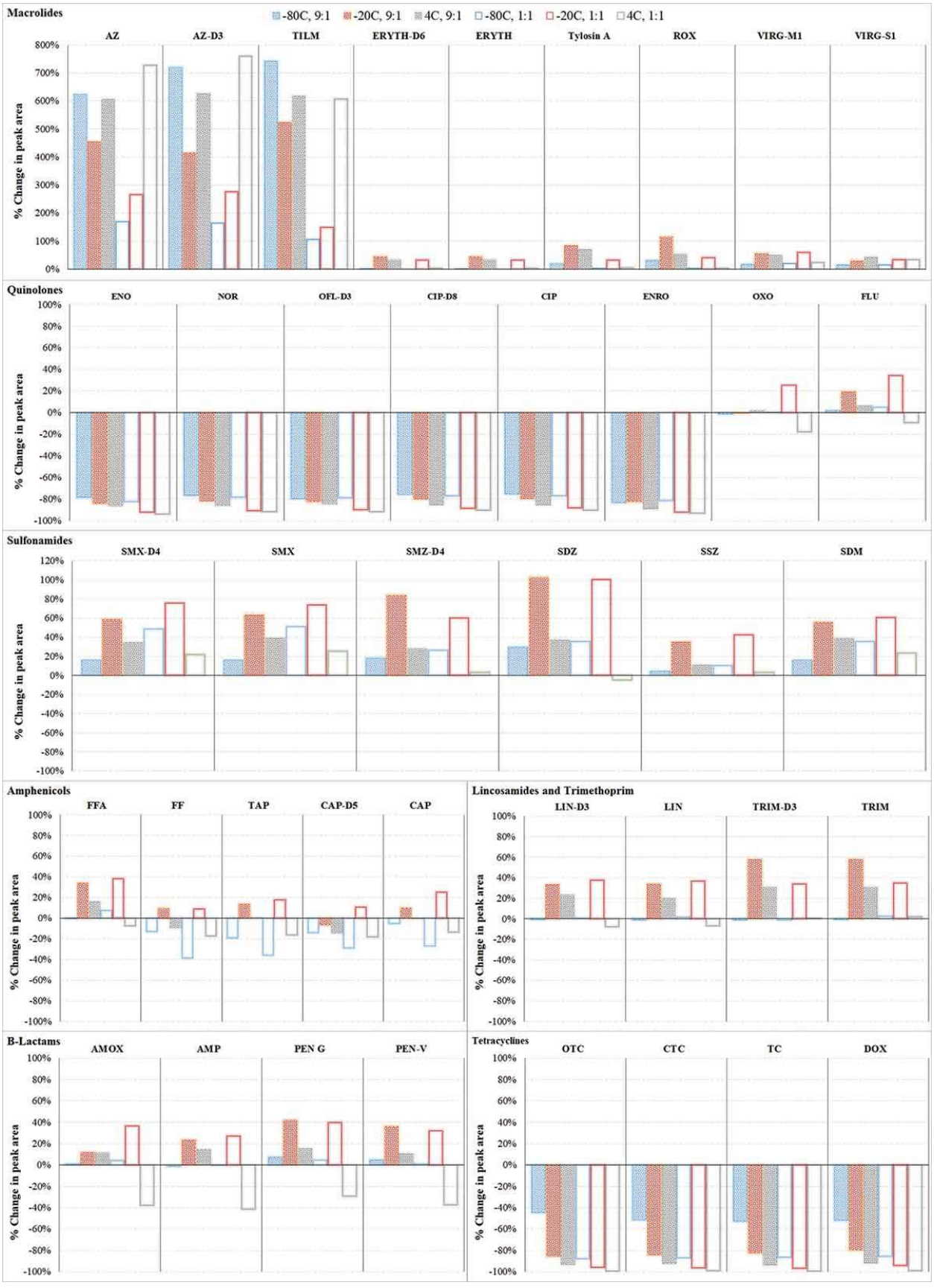
Macrolides
-80C, 9:1
-20C, 9:1
4C, 9:1
-80C, 1:1
-20C, 1:1
4C, 1:1
AZ
AZ-D3
TILM
ERYTH-D6
ERYTH
Tylosin A
ROX
VIRG-M1
VIRG-S1
% Change in peak area
Quinolones
ENO
NOR
OFL-D3
CIP-D8
CIP
ENRO
OXO
FLU
Sulfonamides
SMX-D4
SMX
SMZ-D4
SDZ
SSZ
SDM
Amphenicols
FFA
FF
TAP
CAP-D5
CAP
Lincosamides and Trimethoprim
LIN-D3
LIN
TRIM-D3
TRIM
B-Lactams
AMOX
AMP
PEN G
PEN-V
Tetracyclines
OTC
CTC
TC
DOX

Figure 2.Effect of storage temperature on the stability of antibiotic standard mixture. Antibiotics were dissolved as a mixture in water:methanol at 90:10 and 50:50 ratios and stored at 4, −20 and −80 °C. Samples were analysed using UPLC-MS/MS on day 1 and day 7. “9:1” represents water: methanol solution of (90:10) and “1:1” represents water:methanol solution of 50:50 ratio. Data are shown as % change in peak area between day 7 and day 1 (n = 1).

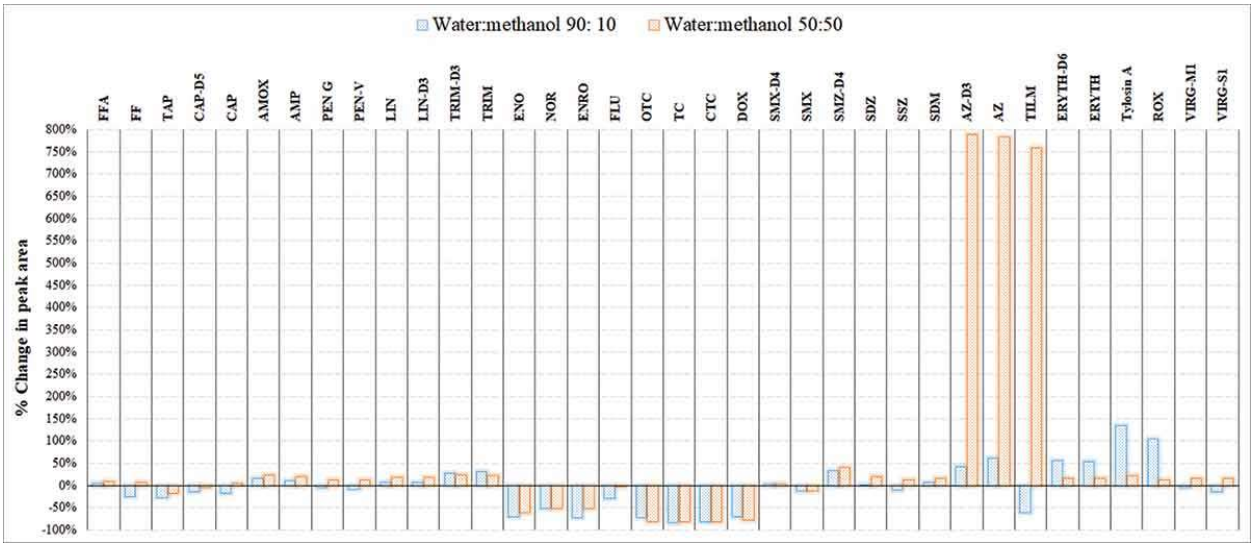

Figure 3.Stability of antibiotics in water: methanol (90:10 and 50:50) during one week storage at −80 °C (experiment 2). In this experiment, sodium hydroxide was not included in the mixture. Data are shown as % change in peak area between day 7 and day 1 (n = 2).

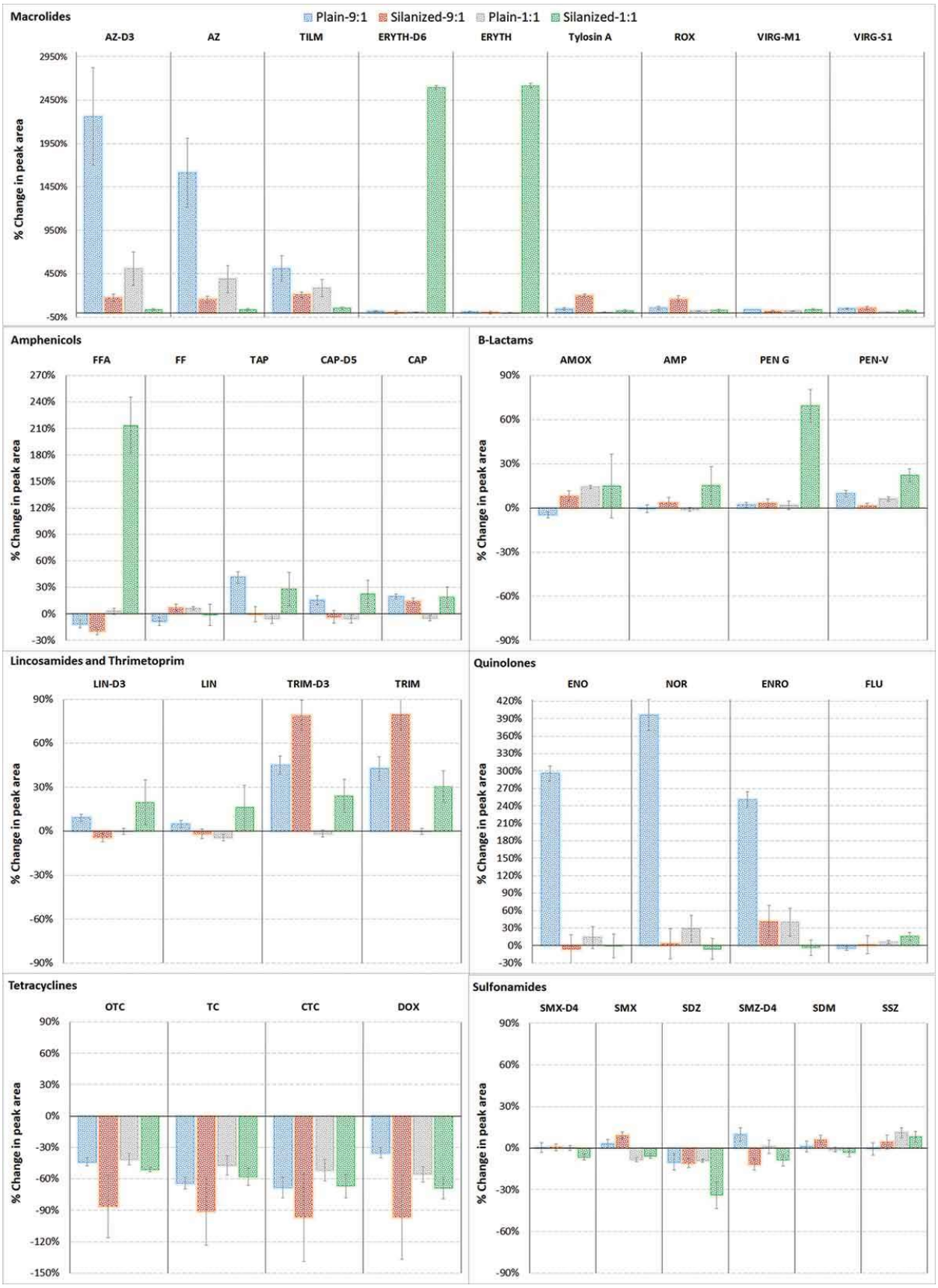
Macrolides
Plain-9:1
Silanized-9:1
Plain-1:1
Silanized-1:1
AZ-D3
AZ
TILM
ERYTH-D6
ERYTH
Tylosin A
ROX
VIRG-M1
VIRG-S1
% Change in peak area
Amphenicols
FFA
FF
TAP
CAP-D5
CAP
B-Lactams
AMOX
AMP
PEN G
PEN-V
Lincosamides and Thrimetoprim
LIN-D3
LIN
TRIM-D3
TRIM
Quinolones
ENO
NOR
ENRO
FLU
Tetracyclines
OTC
TC
CTC
DOX
Sulfonamides
SMX-D4
SMX
SDZ
SMZ-D4
SDM
SSZ

Figure 4. Effect of container type on the storage stability of antibiotic standards in water: methanol (90:10 and 50:50) during one week storage at −80 °C. Data are shown as % change in peak area from day 1 to day 7 (n = 3). “Plain-1:1” represents non-silanized vials and water:methanol solution of 50:50 ratio, “Plain-9:1” represents non-silanized vials and water:methanol solution of 90:10 ratio, “Silanized-1:1” represents silanized vials and water:methanol solution of 50:50, and “Silanized-9:1” represents silanized vials and water:methanol solution of (90:10).

Table S1. Storage stability of antibiotics as a function of storage temperature (-80, -20 and 4 ˚C) and water: methanol ratio (90: 10 and 50: 50) in Experiment 1. Data are shown as % change in peak area on day 7 (n=1) vs. baseline (i.e. day 1, n=1). Antibiotics were considered stable if % change in peak area was in the range of ± 29%.

Class	Antibiotics	Storage T: -80 ˚C				Storage T: -20 ˚C				Storage T: 4 ˚C			
		Water: methanol 90:10		Water: methanol 50-50		Water: methanol 90:10		Water: methanol 50-50		Water: methanol 90:10		Water: methanol 50-50	
		% Change	% of cases between ± 29%	% Change	% of cases between ± 29%	% Change	% of cases between ± 29%	% Change	% of cases between ± 29%	% Change	% of cases between ± 29%	% Change	% of cases between ± 29%
Amphenicols	FFA	-0.1%	100%	7%	40%	34%	80%	38%	80%	16%	100%	-8%	100%
	FF	-13%		-39%		9%		9%		-9%		-17%	
	TAP	-19%		-36%		14%		18%		0%		-16%	
	CAP-D5	-14%		-29%		-7%		11%		-14%		-18%	
	CAP	-5%		-27%		10%		25%		0%		-13%	
B-Lactams	AMOX	1%	100%	4%	100%	12%	50%	37%	25%	11%	100%	-38%	0%
	AMP	-1%		-0.2%		23%		27%		15%		-41%	
	PEN G	7%		5%		42%		40%		15%		-29%	
	PEN-V	5%		1%		36%		32%		11%		-37%	
Lincosamides	LIN-D3	-1%	100%	1%	100%	33%	0%	38%	0%	23%	100%	-8%	100%
	LIN	-1%		2%		34%		37%		20%		-7%	
Trimethorim	TRIM-D3	-1%	100%	-1%	100%	58%	0%	34%	0%	31%	0%	1%	100%
	TRIM	-1%		3%		58%		35%		30%		2%	
Quinolones	ENO	-78%	25%	-82%	25%	-84%	25%	-92%	13%	-86%	25%	-94%	25%
	NOR	-76%		-78%		-82%		-91%		-86%		-92%	
	OFL-D3	-80%		-79%		-82%		-90%		-85%		-92%	
	CIP-D8	-76%		-77%		-80%		-89%		-86%		-90%	
	CIP	-75%		-77%		-80%		-88%		-85%		-90%	
	ENRO	-83%		-82%		-82%		-92%		-89%		-93%	
	OXO	-2%		0%		-1%		25%		2%		-18%	
	FLU	2%		5%		19%		34%		6%		-9%	
Tetracyclines	OTC	-44%	0%	-88%	0%	-85%	0%	-96%	0%	-93%	0%	-99%	0%
	CTC	-51%		-87%		-84%		-96%		-92%		-99%	
	TC	-53%		-87%		-82%		-97%		-93%		-100%	
	DOX	-52%		-85%		-80%		-94%		-92%		-99%	
Sulfonamides	SMX-D4	16%	83%	49%	67%	58%	0%	76%	0%	34%	33%	22%	100%

	SMX	16%		51%		63%		74%		39%		25%	
	SMZ-D4	18%		26%		84%		60%		28%		3%	
	SDZ	29%		36%		102%		100%		37%		-5%	
	SSZ	4%		10%		35%		42%		11%		3%	
	SDM	16%		35%		55%		61%		39%		24%	
Macrolides	AZ	623%	56%	170%	67%	455%	0%	266%	0%	606%	0%	727%	56%
	AZ-D3	718%		165%		415%		276%		626%		760%	
	TILM	742%		107%		523%		150%		617%		608%	
	ERYTH-D6	1%		-4%		44%		32%		32%		3%	
	ERYTH	1%		-4%		44%		33%		33%		3%	
	Tylosin A	19%		3%		84%		33%		70%		5%	
	ROX	31%		4%		115%		41%		53%		3%	
	VIRG-M1	17%		20%		54%		60%		49%		23%	
	VIRG-S1	15%		15%		29%		35%		43%		34%	

Table S2. Stability of antibiotics stored at -80 ºC in water: methanol ratio (90: 10 and 50: 50) without sodium hydroxide (Experiment 2). Data are shown as % change in peak area on day 7 (n=2) vs. day 1 (baseline; n=2). Antibiotics were considered stable if the % change in peak area was in the range of ± 29%.

Class	Antibiotics	Water: methanol (90:10)		Water: methanol (50:50)	
		% change	% of cases between ± 29%	% change	% of cases between ± 29%
Amphenicols	FFA	4%	100%	8%	100%
	FF	-24%		6%	
	TAP	-26%		-17%	
	CAP-D5	-14%		-6%	
	CAP	-17%		5%	
B-Lactams	AMOX	15%	100%	23%	100%
	AMP	10%		19%	
	PEN G	-4%		13%	
	PEN-V	-8%		12%	
Lincosamides	LIN	6%	100%	18%	100%
	LIN-D3	6%		18%	
Trimethoprim	TRIM-D3	28%	50%	24%	100%
	TRIM	32%		22%	
Quinolones	ENO	-71%	0%	-61%	25%
	NOR	-52%		-52%	
	ENRO	-71%		-51%	
	FLU	-29%		-3%	
Tetracyclines	OTC	-72%	0%	-81%	0%
	TC	-84%		-82%	
	CTC	-82%		-82%	
	DOX	-71%		-78%	
Sulfonamides	SMX-D4	3%	83%	3%	83%
	SMX	-12%		-12%	
	SMZ-D4	32%		40%	
	SDZ	0%		20%	
	SSZ	-11%		13%	
	SDM	7%		17%	
Macrolides	AZ-D3	42%	22%	789%	67%
	AZ	60%		783%	
	TILM	-61%		758%	
	ERYTH-D6	55%		16%	
	ERYTH	53%		16%	
	Tylosin A	134%		22%	
	ROX	103%		12%	
	VIRG-M1	-4%		16%	
	VIRG-S1	-14%		15%	

Table S3. Stability of antibiotics stored at -80 ºC in water: methanol (90: 10 and 50: 50 ratio) using silanized or non-silanized glass vials (Experiment 3). Data are shown as % change in peak area on day 7 (n=3) vs. day 1 (baseline; n=3). Antibiotics are considered stable if the % change in peak area was in the range of ± 29%.

Class	Antibiotics	Plain (non-silanized) glass vials				Silanized glass vials			
		Water: methanol (90: 10)		Water: methanol (50: 50)		Water: methanol (90: 10)		Water: methanol (50: 50)	
		% Change in peak area	% cases within ± 29%	% Change in peak area	% cases within ± 29%	% Change in peak area	% cases within ± 29%	% Change in peak area	% cases within ± 29%
Amphenicols	FFA	-11%	80%	3%	100%	-19%	100%	213%	80%
	FF	-8%		6%		7%		-1%	
	TAP	42%		-5%		-1%		28%	
	CAP-D5	16%		-6%		-3%		23%	
	CAP	20%		-5%		14%		19%	
B-Lactams	AMOX	-5%	100%	14%	100%	8%	100%	15%	75%
	AMP	0%		-1%		3%		15%	
	PEN G	2%		2%		3%		69%	
	PEN-V	10%		6%		1%		22%	
Lincosamides	LIN-D3	9%	100%	0%	100%	-4%	100%	20%	100%
	LIN	5%		-4%		-2%		16%	
Trimethoprim	TRIM-D3	45%	0%	-2%	100%	79%	0%	24%	50%
	TRIM	43%		0%		79%		30%	
Quinolones	ENO	296%	25%	14%	50%	-6%	75%	-1%	100%
	NOR	396%		29%		3%		-6%	
	ENRO	251%		40%		41%		-4%	
	FLU	-5%		6%		2%		16%	
Tetracyclines	OTC	-44%	0%	-41%	0%	-86%	0%	-51%	0%
	TC	-64%		-47%		-91%		-58%	
	CTC	-68%		-52%		-96%		-67%	
	DOX	-35%		-55%		-97%		-69%	
Sulfonamides	SMX-D4	0%	100%	0%	100%	1%	100%	-6%	83%
	SMX	3%		-8%		9%		-6%	
	SDZ	-10%		-9%		-11%		-34%	
	SMZ-D4	10%		1%		-12%		-9%	
	SDM	1%		-1%		6%		-3%	

	SSZ	0%		11%		4%		8%	
Macrolides	AZ-D3	2259%	22%	505%	67%	172%	33%	32%	22%
	AZ	1612%		386%		152%		32%	
	TILM	506%		282%		207%		51%	
	ERYTH-D6	16%		7%		2%		2592%	
	ERYTH	8%		-1%		3%		2613%	
	Tylosin A	44%		5%		194%		24%	
	ROX	52%		20%		152%		29%	
	VIRG-M1	34%		20%		19%		37%	
	VIRG-S1	45%		11%		56%		25%	

Table S4. % change in peak area of antibiotics in silanized vials vs. plain vials in freshly prepared antibiotic mixture solution (day 1 samples; i.e. baseline) and as a function of water: methanol ratio (90: 10 and 50: 50) (Experiment 3). Data are shown as % change in peak area in silanized vials (n=3) vs. plain non-silanized vials (n=3).

Antibiotics	% change in peak area in silanized vial compared to plain vial	
	Water: methanol (90:10)	Water: methanol (50:50)
FFA	27%	-75%
FF	9%	-4%
TAP	40%	-12%
CAP-D5	15%	-5%
CAP	7%	-7%
AMOX	-4%	3%
AMP	-4%	-11%
PEN G	-3%	-45%
PEN-V	1%	-11%
LIN-D3	14%	-12%
LIN	9%	-12%
TRIM-D3	-40%	-12%
TRIM	-41%	-13%
ENO	5259%	6201%
NOR	5704%	6543%
ENRO	2608%	3056%
FLU	-24%	-2%
OTC	-19%	106%
TC	5%	159%
CTC	-26%	125%
DOX	-25%	80%
SMX-D4	5%	18%
SMX	4%	3%
SDZ	9%	52%
SMZ-D4	27%	16%
SDM	8%	19%
SSZ	-8%	10%
AZ-D3	610%	2719%
AZ	526%	2449%
TILM	-36%	1530%
ERYTH-D6	-51%	-97%
ERYTH	-55%	-97%
Tylosin A	-89%	-6%
ROX	-94%	-4%
VIRG-M1	-16%	0%
VIRG-S1	-45%	1%

Chapter 3: Antibiotics extracted from pigmented and non-pigmented salmon by the QUEChERS method yield significant matrix effects that reduce the accuracy and sensitivity of analysis by Ultrahigh Performance Liquid Chromatography Coupled to Tandem Mass Spectrometry

Shiva Emami [1], Ameer Y. Taha [1,2*]

Author affiliation:

[1] Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California Davis, One Shields Avenue, Davis, CA, USA 95616

[2] West Coast Metabolomics Center, Genome Center, University of California Davis, One Shields Avenue, Davis, CA, USA 95616

*Corresponding author:

Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California Davis, One Shields Avenue, Davis, CA, USA 95616; Email: ataha@ucdavis.edu

Abstract

Several validated methods exist for the quantitation of antibiotics in seafood with ultra-high pressure liquid chromatography coupled to tandem spectrometry (UPLC-MS/MS). To our knowledge, none have systematically explored the effects of co-eluting matrix components on the accuracy and sensitivity of quantitation. Such "matrix effects" could disproportionally change the ionization of analytes and their respective surrogate/internal standards during UPLC-MS/MS analysis, resulting in over- or under-estimation of antibiotic concentrations. In this study, we measured matrix effects, alongside extraction recoveries for 30 antibiotics and their respective class-specific surrogate standards in Sockeye (pigmented), King (pigmented) and Ivory King (non-pigmented) salmon extracted using the QUEChERS method. A modified QUEChERS method involving dispersive solid phase extraction (SPE) or hydrophilic-lipophilic balance (HLB) SPE clean-up was also used on Sockeye salmon to test whether further clean-up of the sample extract reduces matrix effects. Despite acceptable extraction recoveries for most antibiotics extracted using the QUEChERS method, significant matrix effects were observed in the form of ion suppression (0.1-49%) or enhancement (143-1285%). Only amphenicols were within the optimal range for matrix effects (105-118%) following QUEChERS extraction. Dispersive SPE clean-up did not improve extraction recoveries or matrix effects. HLB SPE, however, improved matrix effects for several antibiotics but reduced percent recovery to <30%. Matrix effects were lower in non-pigmented salmon versus pigmented salmon extracted with the QUEChERS method. Across all types of salmon analyzed, sensitivity of spiked standards were generally lower when matrix effects were high. Accuracy improved when matrix effects were reduced. Our results demonstrate that salmon matrix components, including carotenoid pigmentation, cause matrix effects during antibiotic UPLC-MS/MS analysis that impact sensitivity and accuracy, independent of extraction method.

Running title: Effect of salmon matrix on antibiotic analysis

Keywords: Antibiotics, Salmon, Matrix effects, QUEChERS, UPLC-MS/MS

1. Introduction

The use of antibiotics in aquaculture for therapeutic and/ or prophylactic purposes [77] has led to antibiotic contamination in seafood [75, 158]. Antibiotic residues in seafood pose a public health problem because they can promote the development of antibiotic resistance genes that can laterally transfer to humans [47, 50]. In the US, antibiotic resistance is responsible for 35,000 premature deaths per year [200], which is why these residues are routinely measured in seafood by regulatory agencies.

The analysis of antibiotic residues in seafood is currently performed with ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) [75, 111-113]. Using tandem MS in multiple reaction monitoring (MRM) mode filters unwanted masses and enables simultaneous analysis of multiple antibiotic residues with high selectivity. A limitation of this approach, however, is that co-eluting components from the matrix can interfere with the ionization of analytes, thus causing suppression or enhancement of the MS response. This phenomenon, known as the 'matrix effect' [126], may impact the sensitivity, accuracy and reproducibility of analysis, particularly when ion suppression or enhancement are not uniform across both the analyte and the surrogate (and/or internal standard) used to quantify the analyte [126, 131].

Salmon is one of the most popular seafood consumed in U.S. and constitutes 14% of the total seafood consumption [201]. The majority of the salmon consumed in the U.S. is farm-raised [202] and antibiotic residues have been detected in farmed salmon samples collected from U.S. retail stores [75]. In salmon, antibiotics are commonly extracted using non-polar solvents such as acetonitrile, with or without acid modifiers [75, 107, 110]. Other methods include the QUEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) method [111-112] which was originally developed for pesticides [114] and later extended to antibiotic quantitation in various food matrices including

seafood [111-113]. QUEChERS also involves the use of acetonitrile and water in addition to a mixture of salts to drive the partitioning of antibiotics into the acetonitrile phase [116]. These methods have sometimes been used in conjunction with clean-up methods such as dispersive solid phase extraction (SPE) involving bulk sorbents [107, 111] or column SPE involving hydrophilic-lipophilic balance (HLB) columns [75, 107] in order to reduce matrix effects. While, these methods have often shown good extraction recoveries for several antibiotic classes from salmon [107, 110], there is no information about how matrix effects from salmon may impact the accuracy and sensitivity of antibiotic measurements with UPLC-MS/MS.

Salmon is particularly challenging compared to other types of fish because of its high carotenoid content. In other food matrices (e.g. fruit and vegetables), carotenoids are known to co-extract with other analytes of interest (e.g. pesticides) when using the QUEChERS method [203]. One study showed that removing carotenoids from banana extracts using graphitized carbon black reduced matrix effects when extracting pesticides [127]. Carotenoids such as astaxanthin are present in high amounts in salmonid muscle (3 to 38 mg/ Kg) [204], and they or other lipophilic matrix components can potentially cause ion suppression or enhancement of co-extracted antibiotics as has been documented for pesticides extracted from salmon [128], and for antibiotics extracted from other seafood matrices such as clam (*C. gallina*), mussel (*M. galloprovincialis*) and fish (*P. flesus*) with the QUEChERS method [112].

In the present study, we tested the extent of matrix effects on antibiotics extracted from pigmented and non-pigmented salmon using the QUEChERS method alone or the QUEChERS with dispersive SPE or column SPE (HLB). We also measured extraction percent recovery of spiked antibiotics to determine whether any potential losses in signal intensity are due to matrix effect or simply losses during the extraction, as well as accuracy and method detection limits (i.e.

sensitivity) to determine whether ion suppression or enhancement associated with matrix effects changes these important analytical parameters. Matrix effects can lead to inaccuracies in antibiotic measurements due to disproportional suppression or enhancement of the analyte relative to its surrogate standard, or can cause significant signal suppression leading to reduced sensitivity. Thirty antibiotics belonging to eight classes commonly used in aquaculture farming in several countries [205], banned for use in aquaculture in the U.S. [206], and previously detected in seafood products in the U.S [75] were measured in this study.

We hypothesized that antibiotic extraction from salmon with the QUEChERS method will cause significant matrix effects, resulting in reduced recoveries, decreased accuracy and lower UPLC-MS/MS sensitivity. We also hypothesized, based on prior studies involving other fish matrices, that dispersive or column SPE will improve recovery, accuracy and sensitivity by minimizing matrix effects [107, 111].

2. Materials and methods

2.1. Materials

Sockeye salmon (Open Nature Salmon Sockeye Alaskan Fillet, Wild caught) was purchased from a local supermarket in Davis, CA (USA). King salmon and Ivory King salmon were purchased from Savory Alaska (Leander, TX). Both Sockeye salmon and King salmon are pigmented; Ivory King salmon is not pigmented. LC/MS grade methanol and acetonitrile were obtained from Fisher Scientific (Hampton, NH, USA). Formic acid, sodium sulfate (NA_2SO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO). Trisodium citrate dihydrate (Alfa Aesar) was purchased from Fisher Scientific (Pittsburg, PA). Primary secondary amines (PSA) and C18 endcapped SPE bulk sorbents were purchased from Agilent technologies

(Santa Clara, CA). SPE columns (1g, 20 cc cartridge, OASIS HLB) were purchased from Waters Corp. (Milford, MA). Antibiotic standards used in this study were from the following classes: **Amphenicols:** chloramphenicol (CAP), thiamphenicol (TAP), florfenicol (FF), florfenicol amine (FFA); **Tetracyclines:** tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline HCl (DOX); **Sulfonamides:** sulfadimethoxine (SDM), sulfasalazine (SSZ), sufamethoxazole (SMX), sulfadiazine (SDZ); **Quinolones:** enrofloxacin (ENRO), flumequine (FLU), norfloxacin (NOR) and enoxacin (ENO); **Macrolides:** erythromycin (ERYTH), azithromycin (AZ), tylosin A (TYLOSIN), virginiamycin complex (VIRG-M1 and VIRG-S1), roxithromycin (ROX), tilmicosin phosphate (TILM); **B-lactams:** ampicillin anhydrous (AMP), penicillin G potassium salt (PEN-G), penicillin V (PEN-V) and amoxicillin (AMOX); **Lincosamides:** lincomycin (LIN), **Dihydrofolate reductase inhibitors:** trimethoprim (TRIM), ormetoprim (ORM).

AMOX (98%), ROX (97%), SDZ (99%), TAP (99.3%) and FF (98%) were purchased from Fisher Scientific (Ward Hills, MA). ERYTH (94.8%) DOX HCl (98.8%), NOR (98%), AMP (99.6%), SDM (98.5%), ENRO (99.8%), TC (≥ 98%) and FFA (99.3%) were purchased from Sigma Aldrich (St. Louis, MO). CTC (98.0%), OTC (≥ 95%), FLU (100.0%), ENO (100%), AZ (99.5%), TYLOSIN (99.8%), VIRG (99.0%), PEN-G (99.5%), PEN-V (98.8%), SSZ (100%), SMX (100%), LIN (98%), TRIM (100%) and TILM (100%) were purchased from Cayman Chemicals (Ann Arbor, MI). CAP (98.5%) was purchased from Crescent Chemical (Islandia, NY). Isotopically labeled surrogates including CAP-D5 (chemical purity: 98%; isotopic purity: 98.3%), SMX-D4 (chemical purity: 98%; isotopic purity: 99.2%), sulfamethazine-D4 (SMZ-D4; chemical purity: 98%; isotopic purity: 95.9%), AZ-D3 (HPLC purity: 99.86%; isotopic purity: 93.9%), ERYTH-D6 (chemical purity: 95%; isotopic purity: 98.1%), TRIM-D3 (chemical purity: 99.49%;

isotopic purity: 99.9%), LIN-D3 (chemical purity: 95%; isotopic purity: 99.6%), ENRO-D5 (HPLC purity: 99.61%; isotopic purity: 99.40%), ROX-D7 (HPLC purity: 96.04%; isotopic purity: 99.00%), L-(+)-AMP-D5 (chemical purity: 95%; isotopic purity: 99.00%), and ent-FFA-D3 (chemical purity: 98%; isotopic purity: 98.7%) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada). PEN-V-D5 (chemical purity: ≥98%; isotopic purity: ≥99%) was purchased from Cayman Chemicals (Ann Arbor, MI).

2.2. Antibiotic stock solutions

Individual stock solutions of CAP, TAP, FF, FFA, TC, OTC, CTC, DOX, SDM, SMX, ENRO, ERYTH, AZ, VIRG, ROX, TILM, TRIM, ORM, LIN, CAP-D5, FFA-D3, SMX-D4, SMZ-D4, AZ-D3, ERYTH-D6, TRIM-D3, ROX-D7, ENRO-D5 were prepared in methanol at a concentration of 1 mg/mL. SSZ, SDZ, TYLOSIN, LIN-D3 were prepared at a concentration of 0.5 mg/mL in methanol and FLU, NOR and ENO were prepared at a concentration of 0.1 mg/mL in methanol to ensure their solubility. B-lactams along with their deuterated surrogate PEN-V-D5 were prepared in Milli-Q water (1 mg/mL). AMP-D5, another surrogate used for B-lactams class, was prepared in Milli-Q water at concentration of 0.5 mg/mL.

Individual intermediate solutions of 10 µg/mL of each antibiotic were made in the same solvent as the stock solution and were used to prepare the antibiotics mixture solution (working mix). Working mixes were prepared in water: methanol 1:1 (v/v) prior to the experiment.

2.3. Sample preparation

Salmon fillets were homogenized in dry ice using Sears solid state 10-speed blender at speed 7. The homogenates were stored in loose ziplock bags in a -20 ˚C freezer overnight (~12 hours) to allow the dry ice to sublime. Samples were extracted with the QUEChERS method, or a modified version of the QUEChERS involving dispersive (QUEChERS-dSPE) or column SPE

(QUEChERS-SPE) as described below. Dispersive SPE and column SPE were performed following antibiotic extraction from Sockeye salmon with the QUEChERS method, to test whether extraction recoveries and matrix effects could be improved compared to the original QUEChERS method.

2.4. Experiment 1. Effect of QUEChERS extraction followed by clean-up methods on antibiotic extraction recovery and matrix effects from Sockeye salmon

2.4.1. Extraction recovery

Extraction recovery was determined in Sockeye salmon extracted with the QUEChERS method with or without dSPE and column SPE. Extraction percent recovery was calculated by dividing the peak area in the sample spiked before extraction, to the peak area in sample spiked after extraction. This way of measuring extraction recovery measures true losses of analyte during the extraction.

2.4.1.1. QUEChERS method

Antibiotics from Sockeye salmon matrix were extracted using the QUEChERS method [111]. Approximately, 1 g of salmon (fillet) homogenate was weighed and placed in 50 mL Falcon tubes (Fisher Scientific, cat # LS4541). In order to assess the recovery, samples were spiked with antibiotics working mix at a final concentrations of 20 ng/g per sample, by adding 100 µL of 200 ng/mL antibiotics working mix dissolved in water: methanol (1:1; v/v) (n=5). Control samples consisted of a salmon matrix (n=1) and a method blank (n=1) spiked only with deuterated surrogate standards mix (20 ng/g). The method blank did not contain sample but was extracted in a similar manner as the salmon samples using the same tubes.

To each tube, 8 mL of Milli-Q water, five ceramic beads and 30 mL of acetonitrile were added. The samples were hand-shaken for about 10 seconds and 30 µL formic acid was added to

each sample. The samples were then shaken for 30 min at 200 rpm using the incubator shaker (New Brunswick Scientific, Excella E24 Incubator Shaker series). A salt mixture consisting of 4g Na_2SO_4, 1g NaCl and 1.5 g of trisodium citrate dihydrate was added to the samples and they were hand-shaken for about 10 seconds followed by mechanical shaking for 30 min at 200 rpm. The samples were then centrifuged at 3000 rpm for 10 min at 10 ˚C (SORVALL RT 6000D, rotor H1000B) and the supernatant layer (~ 30 mL of acetonitrile) was transferred to new sets of 50 mL falcon tubes. The supernatant extract was dried under nitrogen and reconstituted in 1 mL of water: methanol (1:1; v/v).

Samples were vortexed for 3 min and sonicated (Branson 1210, Danbury, CT) for 3 min for complete resuspension of the extracts. Samples were transferred to 2 mL centrifuge tubes (Sealrite, USA Scientific, FL), centrifuged at 12000 rpm (13523 ×g) for 2 min (Eppendorf, 5424 R), transferred to filter-containing centrifuge tubes (Ultrafree-MC-VV; PVDF 0.1 µm; Millipore Sigma, MA) and centrifuged for 10 min at 12000 rpm (13523 ×g). The last step was repeated if any visible residues were seen in the tubes. The extracts were transferred to LC vials (Phenomenex, CA) prior to UPLC-MS/MS analysis. Samples were analyzed on the same day of extraction.

2.4.1.2. QUEChERS-dSPE

For the QUEChERS method followed by dSPE, 6 mL of the 30 mL supernatant from the QUEChERS extract was transferred to 15 mL Falcon tubes containing Na_2SO_4/PSA/C18 (900/50/150 mg). The tubes were mechanically shaken for 30 min at 200 rpm using the incubator shaker (New Brunswick Scientific, Excella E24 Incubator Shaker series), and centrifuged at 3000 rpm for 10 min at 10 ˚C (SORVALL RT 6000D, rotor H1000B). Then, 3 mL of the supernatant

layer was taken and dried under nitrogen. Extracts were reconstituted in 100 µL of water: methanol (1:1; v/v) and analyzed by UPLC-MS/MS.

2.4.1.3. QUEChERS-SPE

For the QUEChERS method followed by SPE clean-up, the supernatant (~30 mL) was diluted by bringing the total volume to 200 mL using Milli-Q water. The pH was adjusted to 2.5 using 800 µL formic acid. Samples were then loaded onto OASIS HLB SPE columns (Waters, 20 cc; 1g) pre-conditioned with methanol (20 mL), pure water (6 mL) and pH= 2.5 water (6 mL). The cartridges were washed with Milli-Q water (10 mL) and dried under vacuum for 5 min. Antibiotics were eluted using 12 mL of methanol. The eluent was evaporated under nitrogen to dryness and reconstituted in 1 mL of water: methanol (1:1; v/v).

2.4.2. Matrix effects

To assess matrix effects, a non-spiked Sockeye salmon sample was extracted in the same manner as the other samples as explained in section 2.4.1.1. and the dried extract was reconstituted in 900 µL of water: methanol (1:1; v/v) and then spiked with 100 µL of 200 ng/mL unlabeled and labeled antibiotic standard mix dissolved in water: methanol (1:1; v/v). The resulting concentration of each antibiotic (unlabeled and labeled) was 20 ng/mL of sample extract, at a final volume of 1 mL (n=1). Matrix effects were determined by dividing the peak areas in the spiked matrix extract, by the peak area of each analyte in a separate standard mix vial containing 1 mL of 20 ng/mL antibiotics in water: methanol (1:1; v/v), but no matrix (n=1).

To confirm that the salmon sample itself lacked any antibiotics that might contribute to the matrix effect calculations, a salmon sample spiked with 20 ng (per sample) of deuterated surrogate standards, and extracted with the QUEChERS method as described above (Section 2.4.1.1). Samples were analyzed by UPLC-MS/MS.

For matrix effects assessment using QUEChERS-dSPE, a sample was extracted as described in section 2.4.1.2. and reconstituted (n=1) in 90 µL of water: methanol (1:1; v/v) and 10 µL of 200 ng/mL antibiotic working mix dissolved in water: methanol (1:1; v/v) in LC vials. A parallel vial contained 90 µL of water: methanol (1:1; v/v) and 10 µL of 200 ng/mL antibiotic working mix dissolved in water: methanol (1:1; v/v); i.e. only standard mix but no sample (n=1). Extracts were analyzed on the same day of extraction using UPLC-MS/MS. The water and antibiotic standard volumes were 10 times lower with dSPE method, because 10 times dilution factor was applied for this method, i.e. final reconstitution volume for dSPE was 100 µL and for QUEChERS only and column SPE it was 1 mL.

For matrix effects assessment in the QUEChERS-SPE method, a sample was extracted and reconstituted in 900 µL of water: methanol (1:1; v/v) and 100 µL of 200 ng/mL antibiotic working mix dissolved in water: methanol (1:1; v/v) and run alongside the same volumes of standard mix lacking fish matrix. The samples were vortexed, sonicated, centrifuged and filtered as described above and analyzed using UPLC-MS/MS.

2.5. Experiment 2 - Effect of matrix pigments (carotenoids) on antibiotic extraction recovery and matrix effects

The results from Experiment 1 revealed relatively high matrix effects from Sockeye salmon for most of antibiotics when they were extracted using the QUEChERS method. Matrix effects were not improved by dSPE or column SPE clean-up. Given that the Sockeye salmon used for method development in Experiment 1 has high amounts of carotenoids (astaxanthin; ~ 38 mg/ Kg) [204], we hypothesized that carotenoids might be responsible for the observed matrix effects on antibiotics. To test this hypothesis, wild caught King salmon (orange color; representing salmon matrix containing carotenoids) and Ivory King salmon (ivory white color; representing salmon

matrix without carotenoids) were spiked with 20 ng/g of antibiotics including both unlabeled and surrogate standards and extracted using the QUEChERS method as described in Experiment 1 (n= 5 per fish). Extracts were analyzed with UPLC-MS/MS and recovery and matrix effects were compared. We chose King salmon over Sockeye salmon for this experiment because the ivory white counterpart was available for this type of salmon but not for Sockeye salmon, allowing us to compare the effect of matrix carotenoids on antibiotics extraction.

2.6. Instrumentation

Antibiotic analysis was performed using an Agilent 1290 UPLC coupled to a 6460 Agilent triple quad (UPLC-MS/MS). Chromatographic separation of the antibiotic mixture was performed on AQUITY BEH C18 column (100 × 2.1 mm, 1.8 μm), using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) running at a flow rate of 0.3 mL/min and column temperature of 30 ºC. The mobile phase gradient condition was as follows: initial time: 10% B, 8 min: 20% B, 11 min 60% B, 13 min 100% B, 15 min 100% B, 17 min: 10% B and 20 min: 10% B.

MS/MS analysis was performed using Agilent jet stream electrospray ionization (ESI) operating in both positive and negative mode as shown in **Table 1**. The acquisition method was dynamic multiple reaction monitoring (dMRM) scan type. The MS source parameters were as follows: sheath gas (nitrogen) temperature of 375 ºC, sheath gas flow of 11 L/min, drying gas (nitrogen) temperature of 250 ºC, nozzle voltage of 0V, nebulizer gas pressure of 40 psi and capillary voltage of 3500 V. Collision induced dissociation was carried out using nitrogen in the collision cell. Specific MS/MS parameters including precursor ions, fragmentor voltages, and product ions along with their specific collision energies for each compound are shown in **Table 1**.

2.7. Calculations

Absolute recovery was calculated as follows:

Absolute recovery = [Antibiotic peak area in sample spiked before extraction/ Antibiotic peak area in sample spiked after extraction] × 100

Matrix effects were calculated as follows:

Matrix effects = [Antibiotic peak area in sample spiked with standard mix after extraction/ Antibiotic peak area in standard mix] × 100.

Extraction recovery and matrix effect results were used to link antibiotics to proper surrogates, preferably class specific surrogates, for quantitation purpose and accuracy of quantitation was calculated according to equation below:

Accuracy = [1- (Absolute difference between true concentration and measured concentration / true concentration)] × 100

True concentration in the spiked sample extracts was 20 ng/mL for all antibiotics except for VIRG-M1 and VIRG-S1 which were spiked at 15 and 5 ng/mL, respectively. This is because these two standards were purchased as a single mixture at 75:25 ratio of VIRG-M1: VIRG-S1.

Antibiotic concentrations in spiked samples were calculated by the internal standard calibration method where surrogates were used to correct for both recoveries and matrix effects. A 9-point standard calibration curve (0.5-100 ng/ mL) containing a fixed amount of surrogate standard was made to derive the response factor. Calibration curves were generated by quadratic regression and $\frac{1}{x^2}$ weighting factor was applied [207].

Method detection limits (MDL) were estimated following the procedure suggested by Environmental Protection Agency (EPA; 40 CFR, Appendix B to Part 136 revision 1.11, U.S.) by using the samples spiked with antibiotics:

MDL = $t_{(n-1,1-\alpha=0.99)} \times SD$

Where $t_{(n-1,1-\alpha=0.99)}$ is the student's t value for 99% confidence level and degree of freedom of n-1, and SD represents standard deviation of the concentrations measured in the spiked salmon samples.

2.8. Statistical analysis

Statistical analysis was performed using GRAPHPAD Prism 9.1.0 (La Jolla, CA, USA). In Experiment 1 (effect of clean-up methods on antibiotic extraction recovery and matrix effects), analysis of variance (ANOVA) followed by Dunnett's test was used to compare the results of each group with the control group i.e. QUEChERS extraction without clean-up. In Experiment 2 (effect of matrix pigments on antibiotic extraction recovery and matrix effects), an unpaired t-test was used to compare the recoveries and matrix effects between the two fish matrices.

3. Results

Thirty antibiotics from eight classes were selected for this study and are listed in **Table 1**. A representative MRM chromatogram of the 30 antibiotics spiked into the King salmon at 20 ng/g and extracted using the QUEChERS method alongside 13 antibiotic surrogate standards is shown in **Figure 1**.

3.1. Experiment 1. Effect of QUEChERS extraction followed by clean-up methods on antibiotic extraction recovery and matrix effects from Sockeye salmon

The goal of this experiment was to determine antibiotic extraction recoveries and matrix effects following extraction of Sockeye salmon with the QUEChERS method, and test whether clean-up of the QUEChERS extract with dispersive SPE (QUEChERS-dSPE) or column SPE (QUEChERS-SPE) further reduces matrix effects and yields comparable recoveries. A second goal

was to determine whether the extraction recovery and matrix effects of the surrogate standards behave similar to their class-related antibiotics and use this information to link antibiotics to the proper surrogates for quantitation purposes. Thus, extraction recoveries and matrix effects of 30 unlabeled antibiotic standards and 12 isotopically labeled surrogate standards were determined in Sockeye salmon spiked with 20 ng/g of each antibiotic and extracted with the QUEChERS method, QUEChERS-dSPE and QUEChERS-SPE. A spike level of 20 ng per sample was chosen because it represents less than half the maximum residue levels (MRLs) for most antibiotics (**Table S1**) [200].

3.1.1. Antibiotic extraction recovery

As shown in **Table 2**, the percent recovery of antibiotics extracted with the QUEChERS method was above the acceptable limit (i.e. > 40%) for most compounds - ~56% for lincosamides, ~84% for dihydrofolate reductase inhibitors, 84-187% for quinolones, 70-96% for amphenicols, 42-61% for tetracyclines, 43-83% for sulfonamides, 19-97% for macrolides and 15-77% for B-lactams (except AMOX which was not recovered likely due to degradation). Compared to QUEChERS, the QUEChERS-dSPE method significantly decreased the recovery of CAP-D5 ($p < 0.05$), FFA-D3 ($p < 0.01$), OTC ($p < 0.05$), AMP-D5 ($p < 0.05$), TYLOSIN ($p < 0.05$) and VIRG-M1 ($p < 0.0001$), and increased the recovery of several sulfonamides (SMZ-D4 ($p < 0.01$), SDZ ($p < 0.05$), SMX-D4 ($p < 0.01$), SMX ($p < 0.01$), SDM ($p < 0.01$)) and macrolides (ERYTH-D6 ($p < 0.0001$)). Extraction recoveries significantly decreased for most antibiotics (36 out of 42 compounds) with the QUEChERS-SPE method compared to the QUEChERS method (**Table 2**). This includes 3 compounds (FFA, SSZ and VIRG-S1) which were not recovered with the QUEChERS-SPE method.

3.1.2. Matrix effects

A few background antibiotic peaks were observed in Sockeye salmon, but these constituted less than 3% of the spike peak area (**Table S2**). A few exceptions, however, were TAP, FFA, FLU, VIRG-M1 and SSZ which contributed 8 to 28% of the spike peak areas, likely due to contamination from the salmon (**Table S2**). Background peaks were ignored for the matrix effect calculations as they are constant and do not change the outcome of the calculations.

As shown in **Table 2,** the QUEChERS method resulted in notable matrix effects in the form of ion suppression or enhancement for most antibiotics, where 100% indicates no matrix effects, < 80% indicates ion suppression and > 120% indicates ion enhancement [208]. Ion suppression was observed after QUEChERS extraction for 28 out of 42 antibiotics, and ion enhancement was observed for 11 out of 42 antibiotics. Antibiotics that exhibited ion suppression included lincosamides (LIN and LIN-D3; 32-34%), dihydrofolate reductase inhibitors (TRIM, TRIM-D3 and ORM; 41-49%), quinolones (FLU; 15%), amphenicols (CAP-D5 (35%), FFA and FFA-D3 (0.1%)), sulfonamides (SDZ, SMX, SDM, SSZ, SMX-D4, SMZ-D4; 11-38%), B-lactams (AMP, AMOX, PEN-G, PEN-V, AMP-D5, PEN-V-D5; 1-42%) and macrolides (ERYTH, ERYTH-D6, ROX, ROX-D7, VIRG-M1, VIRG-S1 and TYLOSIN; 3-41%). On the other hand, quinolones including ENO, NOR, ENRO and ENRO-D5 (143-192%), tetracyclines (OTC, CTC, TC, DOX; 526-907%) and macrolides including AZ, AZ-D3 and TILM (171-1285%) showed ion enhancement.

Compared to the QUEChERS method, matrix effects were mostly similar following QUEChERS-dSPE extraction, but were improved following QUEChERS-SPE extraction (i.e. neared 100%) for some antibiotics. For example, lincosamides which exhibited ion suppression with both the QUEChERS and QUEChERS-dSPE methods (matrix effect value 32-37%), had a matrix effect value of 90-03% with the QUEChERS-SPE method. Additionally, improved matrix

effects for dihydrofolate reductase inhibitors (TRIM, TRIM-D3 and ORM; 91-105%), amphenicols (CAP-D5; 89%), sulfonamides (SDZ, SMX, SMX-D4, 87-115%) and B-lactams (AMP and AMOX; 117-121%) were observed following QUEChERS-SPE extraction (**Table 2**). On the other hand, amphenicols including CAP, FF and TAP which showed no matrix effects (105-118%) following conventional QUEChERS extraction, but exhibited ion enhancement following QUEChERS-SPE extraction (CAP, 137%; TAP, 125%; and FF, 163%).

3.2. Effects of matrix pigments (carotenoids) on antibiotic extraction recovery and matrix effects (Experiment 2)

3.2.1. Antibiotic extraction recovery

This experiment examined antibiotic recoveries and matrix effects in King salmon (with carotenoids) and Ivory salmon (without carotenoids) extracted with the conventional QUEChERS method. As shown in **Table 3**, significant differences ($p < 0.05$) in the percent recovery of antibiotics were observed between the two types of salmon for FFA-D3, LIN, LIN-D3, ENO, NOR, ENRO, ENRO-D5, SMX, SMX-D4, SDZ and SMZ-D4, which were lower by 6-14% in King salmon compared to non-pigmented Ivory salmon. On the other hand, significantly greater recoveries (by 6-9%) were observed for AMP-D5, PEN-V and ROX in King salmon compared to non-pigmented Ivory salmon ($p < 0.05$).

3.2.2. Matrix effects

Significant differences in ion suppression and enhancement were observed between King and Ivory salmon for 19 antibiotic standards, as shown in **Table 3**. ERYTH ($p < 0.05$), ERYTH-D6 ($p < 0.05$), ROX ($p < 0.01$), ROX-D7 ($p < 0.05$), VIRG-M1($p < 0.05$) and FLU ($p < 0.05$) were more ion-suppressed (by 2-22%) in King salmon compared to non-pigmented Ivory salmon. TAP was suppressed less in King salmon (83 vs. 71%; $p < 0.05$) compared to non-pigmented Ivory

salmon (**Table 3**). Matrix effects in the form of ion enhancement were observed for amphenicols (FF; $p < 0.05$), quinolones (ENO ($p < 0.001$), NOR ($p < 0.0001$), ENRO ($p < 0.01$), ENRO-D5 ($p < 0.01$)) and macrolides (AZ ($p < 0.0001$), TILM ($p < 0.001$), AZ-D3 ($p < 0.0001$), which were significantly higher in King salmon compared to non-pigmented Ivory salmon by 18-333% (**Table 3**). In addition, ion enhancement for tetracyclines was significantly lower in King salmon than Ivory salmon by 50-179% for OTC, TC DOX and DEM ($p < 0.05$). Overall these data suggest that pigmentation is a potential cause of matrix effects.

Matrix effects were out of the optimal range (80-120%) for 36 antibiotics in non-pigmented Ivory salmon and 35 antibiotics in pigmented King salmon (out of 43 antibiotic and surrogate standards). Optimal matrix effects within 80 to 120% were observed for ORM, FLU, CAP-D5, FF, SMZ-D4, SMX-D4 and SMX in both King and Ivory salmon. Additionally, TAP showed an optimal matrix effect value of 83% in King salmon.

3.3. Method accuracy

Table 4 shows the accuracy of antibiotics following extraction of spiked salmon (at 20 ng/g) with the QUEChERS, QUEChERS-dSPE and QUEChERS-SPE method. Accuracies above 70% were considered acceptable. Quantitation was performed by linking antibiotic standards to proper surrogates, preferably from the same class, based on the extraction recovery and matrix effect results from Experiments 1 and 2 (**Tables 2** and **3**). An antibiotic-to-surrogate peak area ratio of 0.7 to 1.3 was considered as the acceptable criteria for linking the compound to its surrogate (**Table S3**). This criteria was mostly met for the recovery but not for matrix effects. If the criteria was not met for both recovery and matrix effects using a class specific surrogate, a surrogate from another class that behaved similar to the target antibiotic was selected. Surrogates corresponding to each antibiotic is shown in **Table 4**.

As shown in **Table 4**, accuracy was acceptable (>70%) in 11, 13 and 11 out of 26 antibiotics extracted from Sockeye salmon with the QUEChERS, QUEChERS-dSPE and QUEChERS-SPE method, respectively. Accuracy was not calculated for tetracyclines in Sockeye salmon because we did not spike with the proper surrogate standard (DEM) at the time of the experiment. QUEChERS-dSPE and QUEChERS-SPE did not improve accuracy compared to QUEChERS, except for FFA, VIRG-M1 and TYLOSIN where dispersive SPE improved the accuracy from 21-66% to 81-87%. On the other hand, lower accuracy was obtained for AZ extracted with QUEChERS-dSPE (69%) compared to QUEChERS without clean-up (83%).

The QUEChERS method enabled accurate (accuracy > 70%) quantitation of 23 out of 30 antibiotics at 20 ng/g fish spike level in King Salmon and 22 out of 30 antibiotics in Ivory salmon. The antibiotics with low accuracies (<70%) included ENO, NOR, CAP, AMOX, TILM, and SDZ for both King and Ivory salmon. ORM in King salmon, and AMP and VIRG-S1 in Ivory salmon also showed less than 70% accuracy.

3.4. Method detection limit (MDL)

The MDL data are presented in **Table 5**. MDLs ranged from 0.56 ng/g for LIN to 55.44 ng/g for TAP in Sockeye salmon extracted using the QUEChERS method. In King salmon MDLs ranged from 0.35 ng/g for LIN to 17.97 ng/g for TAP. In Ivory salmon MDLs ranged from 0.20 ng/g for TRIM to 10.21ng/g for TAP. MDLs were generally lower in Ivory salmon than King and Sockeye salmon, which are pigmented. Using clean-up methods after QUEChERS did not affect the MDLs in a consistent manner. In some cases, SPE clean-up increased the MDLs, suggesting reduced sensitivity due to analyte losses during clean-up (i.e. reduced percent recovery as shown in **Table 2**).

4. Discussion

This study demonstrates that the standard QUEChERS extraction method has acceptable antibiotic recoveries (>30% [112]) from salmon but is associated with significant matrix effects leading to reduced accuracy and sensitivity. In general, surrogate standards behaved in a similar manner to the antibiotics they quantify in terms of extraction recovery and matrix effects. Matrix effects were improved when column SPE clean-up (but not dSPE) was used post-QUEChERS extraction, but antibiotic percent recoveries were reduced, leading to reduced sensitivity. Salmon pigmentation due to carotenoids resulted in matrix effects, and thus reduced sensitivity for some antibiotics.

The QUEChERS method resulted in acceptable extraction recoveries for most antibiotics (33 out of 42) at the 20 ng/g spike level in Sockeye salmon, but with notable exceptions (**Table 2**). AMOX was not detected at 20 ng/g spike level and AMP and ERYTH were the only antibiotics showing extraction recoveries below 30% (7-27%). This is likely due to degradation during formic acid acidification at the beginning of the extraction as previously reported [107] or transformation into other metabolites at low pH. For instance, ERYTH could transform into other metabolites such as anhydro-ERYTH and ERYTH-enol ether at low pH [209]. Quinolones (ENO, NOR, ENRO and ENRO-D5) showed extraction recoveries above 100% (142-187%) following QUEChERS extraction, likely due to adsorption to the glass vial containing the working mix solution, i.e. spiking mix [138]. Adsorption could reduce antibiotic levels in the samples spiked after extraction compared to samples spiked before extraction, resulting in a calculated extraction recovery value above 100%.

Matrix effects in the form of ion suppression and enhancement were observed for most antibiotics extracted from Sockeye salmon with the standard QUEChERS method. The majority of compounds showed ion suppression in salmon except for tetracyclines, quinolones (ENO, NOR,

ENRO, ENRO-D5) and macrolides (AZ, AZ-D3 and TILM) which showed ion enhancement (**Table 2**). Ion enhancement for AZ, TILM and TC has been reported in other seafood matrices [112], and could be attributed to adsorption of antibiotics to the glass containers (LC vials) used to store them [138, 199] or organic/ inorganic matter from the food matrix itself [210-212]. It is possible that antibiotic sorption onto the glass is reduced in salmon extracts due to preferential sorption to matrix components, resulting in enhanced response in the salmon extract compared to the pure solvent.

Application of dispersive SPE (dSPE) clean-up using the Na_2SO_4/PSA/ C18 (900/50/150) sorbents did not improve matrix effects (**Table 2**). Na_2SO_4 is used to absorb trace amounts of water left in acetonitrile phase, and PSA and C18 are expected to remove polar and non-polar lipids, respectively, from the matrix [129]. The inefficiency of the dispersive SPE in improving matrix effects suggests that other interferences from Sockeye salmon matrix might have caused ion suppression or enhancement.

In contrast, SPE clean-up with HLB columns improved matrix effects for many antibiotics (LIN, LIN-D3, TRIM, TRIM-D3, ORM, SMX, SMX-D4, SDZ, AMP). However, gains in matrix reduction were accompanied with reductions in antibiotic recovery (**Table 2**), leading to reduced sensitivity due to signal loss on the mass-spectrometer (**Table 5**). Antibiotic loss during SPE might be due to column overloading with other matrix components [213] such as lipids and carotenoids, which can reduce the accessibility of active sites available for antibiotic binding. The retention of carotenoids in the column was in fact visible to the experimenter, in the form of an orange color on the column during extraction. Other lipid components co-eluting with antibiotics could also be a factor.

It appears that carotenoids in salmon contributed to the observed matrix effects. This is because pigmented salmon resulted in greater matrix effects for 14 antibiotics compared to non-pigmented salmon (**Table 3**). However, for these antibiotics the matrix effects were still out of the optimal range (70-130%) in non-pigmented samples. This suggests that other matrix components are likely contributing to the matrix effects as well. King salmon has a lower content of carotenoids compared to other types of salmon such as Sockeye salmon (5.4 mg of astaxanthin per kg of flesh in King salmon vs. 28-36 mg/kg in Sockeye salmon)[204]. Therefore, a more pronounced matrix effects could be related to carotenoids if present at high levels such as in Sockeye salmon.

Accuracy was impacted by the observed matrix effects. In Sockeye salmon, high matrix effects were observed for most antibiotic standards (**Table 2**) which is why only 11 out of 26 antibiotics were accurately quantifiable (accuracy > 70%; **Table 4**). However, this value increased to 24 out of 30 antibiotics in King salmon, and to 22 out of 30 antibiotics in Ivory salmon (**Table 4**), which both showed improved matrix effects compared to the Sockeye salmon (**Table 3**). For instance, FLU, SDM, SSZ, PEN-G and VIRG-M1, which showed high matrix effects following extraction from Sockeye salmon using the QUEChERS method (8-17%), were not accurately quantified (accuracy < 70%) (**Table 2 and Table 4**). On the other hand, these antibiotics showed improved matrix effects of 38-131% in King and Ivory salmon and were quantifiable with an accuracy level above 70% (**Table 3 and Table 4**). Due to improved matrix effects, the surrogates and target antibiotics are more likely to behave similarly, which is key for accurate quantitation. It is not yet clear which matrix components are causing these differences in matrix effects and hence accuracy.

MDLs were variable between different salmon matrices and clean-up methods. Generally, MDLs were higher in Sockeye salmon compared to the King and Ivory salmon and clean-up

methods did not have a consistent impact on the MDLs. In a few cases, higher MDLs were observed with the QUEChERS-SPE method compared to the QUECHERS-dSPE and QUECHERS methods. This could be explained by reduced extraction recoveries following SPE clean-up. The higher MDLs for Sockeye salmon compared to the King and Ivory salmon are likely due to greater matrix effects affecting sensitivity.

A limitation of this study is that causes of the observed matrix effects from salmon on antibiotics were not resolved. While we found that salmon carotenoids might partially contribute to the observed matrix effects, other contributing matrix components (e.g. lipids) were not fully characterized. It is possible that other matrix components from salmon such as fatty acids, phospholipids and triacylglycerols contribute to the observed matrix effects on antibiotics. Although PSA and C18 were used in dispersive SPE clean-up to remove polar and non-polar lipids, it is possible that these sorbents in the amounts used were not effective in removing all the lipids. The partial efficiency of column SPE in improving the matrix effects could be attributed to lipids retained by SPE columns. Another possibility is that excess salt used in the QUEChERS might partially remain in the acetonitrile phase, as reported in previous studies [128], resulting in ion suppression or enhancement during UPLC-MS/MS analysis. With these limitations, future studies are required to better understand the specific components in pigmented and non-pigmented seafood that contribute to matrix effects, as well as how salts interact with the ion source to blunt or enhance the signal. This will enable designing more effective extraction and clean-up methods for targeted removal of interfering compounds.

5. Conclusion

This work investigated a) the matrix effects from salmon on antibiotics analysis using UPLC-MS/MS, b) the effectiveness of common clean-up methods in minimizing the matrix

effects, and c) potential contribution of carotenoids from salmon on the matrix effects. The QUEChERS method showed acceptable extraction recoveries but significant matrix effects which were not improved by dSPE clean-up using Na_2SO_4/ PSA/ C18 (900/50/150) sorbents. Column SPE using OASIS HLB column improved matrix effects for some antibiotics but resulted in low extraction recoveries (< 30%) for most antibiotics. Carotenoids at the levels found in King salmon partially contributed to the observed matrix effects. This suggests that other co-extracts from the salmon matrix might be involved in analyte signal suppression or enhancement. Matrix effects compromised the accuracy and sensitivity of the analysis. Therefore, it is critical to characterize the nature of interfering compounds to enable better separation and accurate quantitation of antibiotics in salmon.

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Declaration of interest statement

The authors declare that there is no conflict of interest

Author contributions

S.E. and A.Y.T. designed the experiments. S.E. performed the experiments, analyzed the data and wrote the original draft. A.Y.T. reviewed and edited the manuscript.

Supplementary information

The supporting information include the table presenting the MRLs for antibiotics and table presenting salmon samples background peak areas.

Table 1. Retention time, precursor ion, quantifier and qualifier product ions, fragmentor voltage, and collision energies (CE) for antibiotic and surrogate standards.

Antibiotics	Abbreviations	Retention time	Precursor Ion	Fragmentor voltage	MRM 1 (CE)	MRM 2 (CE)	Polarity
Florfenicol amine-D3	FFA-D3	1.00	251.0	75	233.0 (10)	132.1 (20)	Positive
Florfenicol amine	FFA	1.18	248.0	75	230.1 (10)	130.1 (20)	Positive
Amoxicillin	AMOX	1.46	365.9	90	114.0 (20)	207.9(10)	Positive
Florfenicol	FF	2.10	355.9	125	185.1 (10)	118.7 (30)	Negative
Sulfadiazine	SDZ	2.50	251.1	125	156.1 (10)	108.1(20)	Positive
Ampicillin-d5	AMP-D5	2.72	355.0	75	111.0 (20)	160.0 (10)	Positive
Lincomycin-D3	LIN-D3	2.84	410.2	90	129.1 (30)	362.1 (15)	Positive
Lincomycin	LIN	2.86	407.1	130	126.1 (30)	359.1 (15)	Positive
Ampicillin	AMP	3.78	350.1	125	106.1 (20)	160.1 (10)	Positive
Trimethoprim-D3	TRIM-D3	3.84	294.2	130	123.1 (25)	230.1 (25)	Positive
Trimethoprim	TRIM	3.90	291.1	130	123.0 (20)	230.1 (20)	Positive
Enoxacin	ENO	4.00	321.1	100	303.1 (20)	277.2 (10)	Positive
Sulfamethazine-D4	SMZ-D4	4.30	283.0	125	186.0 (15)	-	Positive
Norfloxacin	NOR	4.44	320.1	130	302.1 (15)	276.1 (15)	Positive
Thiamphenicol	TAP	4.44	353.9	125	184.9 (10)	290.1 (10)	Negative
Oxytetracycline	OTC	4.45	461.2	90	426.0 (15)	443.0 (10)	Positive
Ormetoprim	ORM	4.78	275.0	125	259..2 (20)	123.1 (20)	Positive
Tetracycline	TC	5.33	445.1	100	410.1 (15)	427.1 (10)	Positive
Enrofloxacin	ENRO	5.90	360.1	125	316.3 (15)	342.0 (20)	Positive
Enrofloxacin-D5	ENRO-D5	5.94	365.1	125	321.1 (15)	347.1 (20)	Positive
Demeclocycline	DEM	7.07	465.2	100	447.9 (10)	430.0 (10)	Positive
Sulfamethoxazole-D4	SMX-D4	7.96	258.0	90	112.0 (20)	160.0 (10)	Positive
Sulfamethoxazole	SMX	8.00	254.1	90	156.1 (10)	108.1 (20)	Positive
Chlortetracycline	CTC	9.35	478.7	50	443.9 (20)	462.0 (15)	Positive
Azithromycin	AZ	10.10	375.1	75	591.2 (10)	83.0 (20)	Positive
Azithromycin-D3	AZ-D3	10.10	376.7	125	594.4 (10)	82.8 (20)	Positive
Chloramphenicol-D5*	CAP-D5	10.10	326.1	90	156.0 (10)	261.0 (10)	Negative
Chloramphenicol	CAP	10.10	321.0	115	152.0 (10)	193.9 (10)	Negative
Doxycycline	DOX	10.37	445.3	110	428.1 (15)	410.2 (20)	Positive
Sulfadimethoxine	SDM	10.86	311.0	110	156.1 (15)	245.1 (15)	Positive
Tilmicosin	TILM	11.10	869.4	90	696.0 (25)	174.0 (30)	Positive
Erythromycin-d6	ERYTH-D6	11.72	740.3	130	582.2 (15)	164.2 (30)	Positive
Erythromycin	ERYTH	11.72	734.3	125	576.3 (15)	158.1 (30)	Positive
Penicillin-G	PEN-G	11.90	335.0	110	176.0 (10)	160.1 (10)	Positive
Tylosin A	TYLOSIN	12.03	916.3	125	174.1 (40)	771.8 (30)	Positive
Flumequine	FLU	12.26	262.1	90	244.1 (20)	202.0 (30)	Positive
Sulfasalazine	SSZ	12.39	399.0	130	381.0 (15)	317.0 (20)	Positive
Penicillin-V-D5	PEN-V-D5	12.49	356.0	50	114.0 (10)	160.0 (5)	Positive
Penicillin-V	PEN-V	12.52	351.2	90	160.0 (5)	192.2 (5)	Positive
Roxithromycin	ROX	12.63	419.3	125	158.0 (15)	83.0 (20)	Positive
Roxithromycin-D7	ROX-D7	12.65	422.7	75	158.0 (20)	83.0 (20)	Positive
Virginiamycin-M1	VIRG-M1	12.78	526.1	130	508.3 (10)	355.1 (15)	Positive
Virginiamycin-S1	VIRG-S1	13.21	824.2	130	205.1 (25)	177.2 (30)	Positive

*In Experiment 1, transitions of 155.6 and 261.9 were chosen as MRM1 and MRM2, respectively. After a product ion scan, in Experiment 2 these were changed to 156 and 261, respectively.

Table 2. Percent recovery (mean ± SD; n=5) and percent matrix effects (n=1) of antibiotics extracted from Sockeye salmon spiked with 20 ng/g antibiotics using QUEChERS method or QUEChERS method followed by dispersive SPE or SPE using Oasis HLB columns. Ordinary one-way ANOVA followed by Dunnett's post-hoc test was used to compare differences in recoveries of clean-up methods in comparison to QUEChERS only (as control group). When one group had non-detected values due to negligible recoveries, an unpaired t-test was used to compare the remaining two groups.

Antibiotics class	Antibiotics	Abbreviations	QUEChERS		QUEChERS-dSPE		QUEChERS-SPE	
			Recovery	Matrix effects	Recovery	Matrix effects	Recovery	Matrix effects
Lincosamides	Lincomycin -D3	LIN-D3	56 ± 6	34	54 ± 5	37	0.1 ± 0.1****	93
	Lincomycin	LIN	57 ± 7	32	56 ± 5	34	0.2 ± 0.1****	90
Dihydrofolate reductase inhibitors	Trimethoprim-D3	TRIM-D3	84 ± 11	49	91 ± 9	50	4 ± 1****	105
	Trimethoprim	TRIM	83 ± 10	41	91 ± 9	43	4 ± 1****	90
	Ormetoprim	ORM	84 ± 10	47	92 ± 8	46	5 ± 2****	91
Quinolones	Enrofloxacin-D5	ENRO-D5	142 ± 21	172	138 ± 14	181	16 ± 5****	357
	Enrofloxacin	ENRO	143 ± 20	143	136 ± 12	150	16 ± 5****	295
	Enoxacin	ENO	187 ± 42	187	155 ± 23	211	13 ± 5****	397
	Norfloxacin	NOR	180 ± 33	192	167 ± 18	166	17 ± 6****	432
	Flumequine	FLU	84 ± 9	15	91 ± 13	15	49 ± 4***	20
Amphenicols	Chloramphenicol -D5	CAP-D5	96 ± 24	35	67 ± 15*	46	51 ± 10**	89
	Chloramphenicol	CAP	92 ± 7	105	95 ± 6	102	63 ± 10***	137
	Florfenicol	FF	93 ± 8	114	91 ± 6	117	54 ± 14****	163
	Thiamphenicol	TAP	86 ± 10	118	93 ± 7	98	16 ± 6****	125
	Florfenicol amine -D3	FFA-D3	74 ± 15	0.1	46 ± 10**	0.3	1.3 ± 0.1****	38
	Florfenicol amine	FFA	70 ± 23	0.1	57 ± 18	0.2	ND	39
Tetracyclines	Oxytetracycline	OTC	47 ± 5	907	37 ± 6*	576	3 ± 2****	1521
	Tetracycline	TC	50 ± 6	860	42 ± 5	589	5 ± 3****	1289
	Chlortetracycline	CTC	42 ± 4	526	32 ± 7	422	14 ± 8****	722
	Doxycycline	DOX	61 ± 8	534	57 ± 6	370	31 ± 18**	515
Sulfonamides	Sulfamethazine -D4	SMZ-D4	47 ± 6	26	71 ± 16**	36	10 ± 3***	115
	Sulfadiazine	SDZ	55 ± 6	13	73 ± 13*	17	11 ± 8****	109
	Sulfamethoxazole -D4	SMX-D4	48 ± 4	38	69 ± 11**	50	32 ± 5**	132
	Sulfamethoxazole	SMX	49 ± 4	26	69 ± 11**	33	33 ± 5**	87
	Sulfadimethoxine	SDM	43 ± 5	11	68 ± 17**	15	28 ± 3	23
	Sulfasalazine	SSZ	83 ± 14	11	82 ± 9	14	ND	17
B-Lactams	Ampicillin-D5	AMP-D5	17 ± 2	25	14 ± 2*	36	0.3 ± 0.1****	117
	Ampicillin	AMP	15 ± 2	42	12 ± 3	56	0.4 ± 0.2****	125
	Amoxicillin	AMOX	ND	1	ND	2	0.3 ± 0.1	121
	Penicillin-V-D5	PEN-V-D5	77 ± 19	5	75 ± 9	6	24 ± 5****	10
	Penicillin-V	PEN-V	75 ± 16	5	78 ± 11	6	23 ± 4****	10
	Penicillin-G	PEN-G	60 ± 17	11	77 ± 9	13	4 ± 1****	21
Macrolides	Azithromycin-D3	AZ-D3	81 ± 11	212	84 ± 9	303	9 ± 2****	245
	Azithromycin	AZ	79 ± 11	171	121 ± 61	232	9 ± 2*	190
	Tilmicosin	TILM	82 ± 6	1285	86 ± 5	1006	50 ± 12****	871
	Erythromycin-D6	ERYTH-D6	19 ± 18	23	87 ± 15****	31	2 ± 0.4	32
	Erythromycin	ERYTH	19 ± 18	21	12 ± 2	202	2 ± 0.3*	29
	Roxithromycin-D7	ROX-D7	87 ± 18	4	96 ± 14	3	44 ± 4***	6
	Roxithromycin	ROX	86 ± 17	3	93 ± 14	3	43 ± 5***	6
	Virginiamycin-M1	VIRG-M1	89 ± 3	8	66 ± 7****	7	58 ± 3****	8
	Virginiamycin-S1	VIRG-S1	97 ± 17	17	87 ± 20	11	ND	ND
	Tylosin A	TYLOSIN	76 ± 5	41	60 ± 7*	42	47 ± 10***	47

ND: Not detected, *p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared to QUEChERS

Table 3. Percent recovery (mean ± SD; n=5) and matrix effects (%, mean ± SD, n=5) of antibiotics extracted from Ivory King salmon (white) and King salmon (pink) spiked with 20 ng/g of antibiotics using the QUEChERS method. Unpaired t-test was used to compare differences in recoveries and matrix effects between two salmon matrices.

Antibiotics class	Antibiotics	Abbreviations	Recovery		Matrix effects	
			Ivory king Salmon (white)	King Salmon (pink)	Ivory king Salmon (white)	King Salmon (pink)
Lincosamides	Lincomycin -D3	LIN-D3	59 ± 4	53 ± 3 *	68 ± 6	69 ± 3
	Lincomycin	LIN	60 ± 4	54 ± 3 *	68 ± 6	70 ± 4
Dihydrofolate	Trimethoprim-D3	TRIM-D3	83 ± 6	77 ± 5	77 ± 6	74 ± 2
reductase	Trimethoprim	TRIM	83 ± 6	77 ± 4	76 ± 6	75 ± 2
inhibitors	Ormetoprim	ORM	86 ± 6	82 ± 3	92 ± 8	93 ± 3
Quinolones	Enrofloxacin-D5	ENRO-D5	95 ± 5	84 ± 5 *	491 ± 65	639 ± 24 **
	Enrofloxacin	ENRO	94 ± 4	84 ± 5 *	451 ± 57	552 ± 26 **
	Enoxacin	ENO	78 ± 3	71 ± 5 *	771 ± 77	972 ± 31 ***
	Norfloxacin	NOR	83 ± 4	74 ± 5 *	750 ± 72	1083 ± 37 ****
	Flumequine	FLU	77 ± 7	81 ± 5	95 ± 3	88 ± 6 *
Amphenicols	Chloramphenicol -D5	CAP-D5	97 ± 4	92 ± 8	102 ± 14	103 ± 2
	Chloramphenicol	CAP	112 ± 13	102 ± 11	138 ± 24	159 ± 7
	Florfenicol	FF	107 ± 10	110 ± 12	100 ± 14	118 ± 7 *
	Thiamphenicol	TAP	121 ± 22	133 ± 40	71 ± 8	83 ± 5 *
	Florfenicol amine -D3	FFA-D3	53 ± 9	41 ± 6 *	1 ± 0	1 ± 0
	Florfenicol amine	FFA	47 ± 10	44 ± 4	1 ± 0	1 ± 0
Tetracyclines	Demeclocycline	DEM	40 ± 3	42 ± 4	606 ± 59	525 ± 29 *
	Oxytetracycline	OTC	50 ± 5	54 ± 2	464 ± 47	328 ± 20 ***
	Tetracycline	TC	48 ± 4	49 ± 3	404 ± 40	354 ± 24 *
	Chlortetracycline	CTC	48 ± 4	44 ± 7	408± 31	396 ± 24
	Doxycycline	DOX	59 ± 4	64 ± 4	679 ± 49	500 ± 39 ***
Sulfonamides	Sulfamethazine-D4	SMZ-D4	71 ± 5	59 ± 7 *	90 ± 13	102 ± 4
	Sulfadiazine	SDZ	62 ± 7	48 ± 7 *	53 ± 4	53 ± 3
	Sulfamethoxazole-D4	SMX-D4	69 ± 4	57 ± 7 *	86 ± 10	92 ± 2
	Sulfamethoxazole	SMX	70 ± 4	58 ± 6 *	87 ± 11	92 ± 1
	Sulfadimethoxine	SDM	60 ± 7	53 ± 8	124 ± 12	131 ± 5
	Sulfasalazine	SSZ	72 ± 7	81 ± 5	50 ± 2	49 ± 3
B-Lactams	Ampicillin-D5	AMP-D5	18 ± 1	24 ± 4 *	46 ± 4	46 ± 1
	Ampicillin	AMP	22 ± 3	27 ± 6	55 ± 4	52 ± 2
	Amoxicillin	AMOX	10 ± 2	14 ± 5	14 ± 2	15 ± 1
	Penicillin-V-D5	PEN-V-D5	70 ± 7	77 ± 3	56 ± 3	53 ± 3
	Penicillin-V	PEN-V	70 ± 6	79 ± 5 *	56 ± 3	53 ± 4
	Penicillin-G	PEN-G	53 ± 8	46 ± 3	73 ± 5	67 ± 5
Macrolides	Azithromycin-D3	AZ-D3	80 ± 7	79 ± 2	235 ± 16	333 ± 19 ****
	Azithromycin	AZ	80 ± 7	80 ± 2	236 ± 16	332 ± 20 ****
	Tilmicosin	TILM	90 ± 6	88 ± 2	369 ± 53	530 ± 35 ***
	Erythromycin-D6	ERYTH-D6	9 ± 4	7 ± 2	66 ± 8	48 ± 15 *
	Erythromycin	ERYTH	9 ± 4	7 ± 2	66 ± 8	48 ± 15 *
	Roxithromycin-D7	ROX-D7	71 ± 7	77 ± 4	16 ± 1	14 ± 1 *
	Roxithromycin	ROX	70 ± 7	78 ± 4 *	15 ± 1	13 ± 1 **
	Virginiamycin-M1	VIRG-M1	61 ± 10	66 ± 8	53 ± 4	47 ± 4 *
	Virginiamycin-S1	VIRG-S1	70 ± 13	93 ± 22	43 ± 6	38 ± 4
	Tylosin A	TYLOSIN	74 ± 7	72 ± 4	58 ± 4	57 ± 4

*p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001; N/A: Not applicable.

Table 4. Accuracy (%) of target antibiotics in salmon matrices (Sockeye, King and Ivory King). For Sockeye salmon three extraction methods i.e. QUEChERS, QUEChERS-dSPE and QUEChERS-SPE were tested. King and Ivory King salmon were extracted using QUEChERS only. Data are represented as mean ± SD (n=5). Accuracy values below 70% were considered poor and marked with ǂ. Accuracies for Oxytetracycline, Tetracycline, Chlortetracycline and Doxycycline were not calculated in Sockeye salmon matrix due to the lack of proper surrogate at the time of the experiment.

Class	Antibiotic	Tagged surrogate	Sockeye Salmon			King Salmon	Ivory King Salmon
			QUEChERS	QUEChERS -dSPE	QUEChERS -SPE	QUEChERS	QUEChERS
Lincosamides	Lincomycin	Lincomycin-D3	99 ± 0	99 ± 1	96 ± 3	98 ± 0	98 ± 0
Dihydrofolate reductase inhibitors	Trimethoprim	Trimethoprim-D3	91 ± 1	92 ± 1	97 ± 1	96 ± 1	98 ± 0
	Ormetoprim	Trimethoprim-D3	94 ± 1	97 ± 1	63 ± 4ǂ	66 ± 4ǂ	79 ± 3
Quinolones	Flumequine	Trimethoprim-D3	30 ± 1ǂ	31 ± 3ǂ	-32 ± 59ǂ	70 ± 14	86 ± 7
	Enoxacin	Enrofloxacin-D5	88 ± 9	91 ± 4	72 ± 6	68 ± 5ǂ	63 ± 3ǂ
	Norfloxacin	Enrofloxacin-D5	86 ± 6	88 ± 3	96 ± 3	29 ± 8ǂ	31 ± 2ǂ
	Enrofloxacin	Enrofloxacin-D5	90 ± 1	86 ± 1	95 ± 1	85 ± 1	86 ± 1
Amphenicols	Chloramphenicol	Chloramphenicol-D5	-165 ± 64ǂ	-121 ± 43ǂ	2 ± 57ǂ	37 ± 7ǂ	39 ± 12ǂ
	Florfenicol	Chloramphenicol-D5	-163 ± 57ǂ	-126 ± 44ǂ	-9 ± 74ǂ	75 ± 7	81 ± 7
	Thiamphenicol	Chloramphenicol-D5	-85 ± 74ǂ	-78 ± 36ǂ	51 ± 26ǂ	81 ± 15	91 ± 9
	Florfenicol amine	Florfenicol amine-D3	66 ± 17ǂ	81 ± 22	ND	88 ± 2	83 ± 13
Tetracycline	Oxytetracycline	Demeclocycline	N/A	N/A	N/A	86 ± 5	81 ± 6
	Tetracycline	Demeclocycline	N/A	N/A	N/A	84 ± 6	84 ± 1
	Chlortetracycline	Demeclocycline	N/A	N/A	N/A	85 ± 6	89 ± 3
	Doxycycline	Demeclocycline	N/A	N/A	N/A	82 ± 6	85 ± 4
B-lactams	Ampicillin	Ampicillin-D5	64 ± 8ǂ	64 ± 14ǂ	64 ± 4ǂ	72 ± 5	56 ± 10ǂ
	Amoxicillin	Ampicillin-D5	ND	ND	67 ± 33ǂ	21 ± 4ǂ	17 ± 3ǂ
	Penicillin -V	Penicillin-V-D5	90 ± 8	96 ± 3	96 ± 4	98 ± 1	99 ± 1
	Penicillin -G	Penicillin-V-D5	35 ± 11ǂ	-13 ± 7ǂ	46 ± 8ǂ	75 ± 7	94 ± 5
Macrolides	Virginiamycin-M1	Penicillin-V-D5	39 ± 32ǂ	82 ± 6	-20 ± 43ǂ	72 ± 6	78 ± 5
	Virginiamycin-S1	Penicillin-V-D5	63 ± 39ǂ	61 ± 7ǂ	ND	72 ± 13	64 ± 13ǂ
	Azithromycin	Azithromycin-D3	83 ± 3	69 ± 46ǂ	85 ± 4	95 ± 1	94 ± 1
	Tilmicosin	Azithromycin-D3	-300 ± 35ǂ	-152 ± 21ǂ	-1016 ± 77ǂ	34 ± 3ǂ	56 ± 8ǂ
	Erythromycin	Erythromycin-D6	96 ± 3	98 ± 1	96 ± 1	98 ± 1	98 ± 2
	Roxithromycin	Roxithromycin-D7	93 ± 2	97 ± 1	96 ± 2	90 ± 1	91 ± 2
	Tylosin A	Sulfamethoxazole-D4	21 ± 8ǂ	87 ± 3	61 ± 7ǂ	80 ± 6	72 ± 6
Sulfonamides	Sulfamethoxazole	Sulfamethoxazole-D4	76 ± 1	75 ± 2	75 ± 1	98 ± 2	98 ± 1
	Sulfadimethoxine	Sulfamethoxazole-D4	31 ± 2ǂ	33 ± 3ǂ	16 ± 1ǂ	72 ± 8	74 ± 10
	Sulfasalazine	Penicillin-V-D5	-27 ± 37ǂ	-36 ± 12ǂ	ND	96 ± 3	95 ± 2
	Sulfadiazine	Sulfamethazine-D4	65 ± 3ǂ	55 ± 2ǂ	77 ± 18	45 ± 4ǂ	53 ± 3ǂ

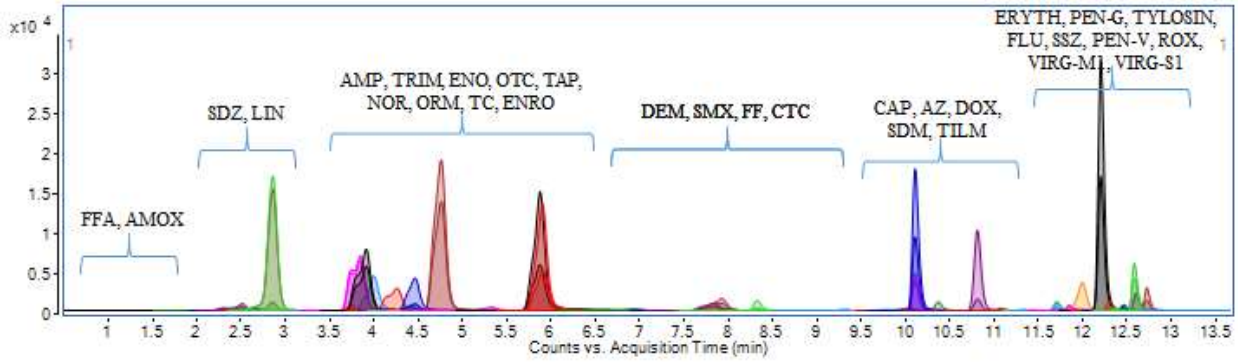
ND: Not detected; N/A: Not applicable

Table 5. Method detection limits (MDLs, ng/g fish) of the target antibiotics in Sockeye, King and Ivory King salmon. In Sockeye salmon MDLs were calculated for the three extraction methods including QUEChERS, QUEChERS-dSPE and QUEChERS-SPE.

Class	Antibiotics	Abbreviations	Sockeye Salmon			King Salmon	Ivory King Salmon
			QUEChERS	QUEChERS-dSPE	QUEChERS-SPE	QUEChERS	QUEChERS
Lincosamides	Lincomycin	LIN	0.56	0.63	2.02	0.35	0.34
Dihydrofolate reductase inhibitors	Trimethoprim	TRIM	1.00	0.47	1.02	0.84	0.20
	Ormetoprim	ORM	0.88	0.38	2.98	3.19	1.96
Quinolones	Flumequine	FLU	0.98	2.09	43.85	10.49	5.44
	Enoxacin	ENO	7.09	3.23	4.39	3.81	2.53
	Norfloxacin	NOR	4.50	2.39	2.13	6.29	1.37
	Enrofloxacin	ENRO	1.04	0.95	0.86	0.55	0.42
Amphenicols	Chloramphenicol	CAP	48.24	32.20	42.90	5.28	8.89
	Florfenicol	FF	42.75	33.19	55.47	5.59	4.88
	Thiamphenicol	TAP	55.44	27.04	19.77	17.97	10.21
	Florfenicol amine	FFA	13.07	17.31	N/A	8.52	10.02
Tetracyclines	Oxytetracycline	OTC	N/A	N/A	N/A	3.65	4.12
	Tetracycline	TC	N/A	N/A	N/A	4.25	0.98
	Chlortetracycline	CTC	N/A	N/A	N/A	4.77	2.37
	Doxycycline	DOX	N/A	N/A	N/A	4.76	2.77
B-lactams	Ampicillin	AMP	5.72	10.82	3.22	4.08	7.84
	Amoxicillin	AMOX	N/A	N/A	36.60	2.74	2.12
	Penicillin -V	PEN-V	9.02	1.91	4.06	1.67	1.22
	Penicillin -G	PEN-G	7.91	5.59	6.14	5.35	3.90
Macrolides	Virginiamycin-M1	VIRG-M1	18.02	3.62	23.95	3.27	3.05
	Virginiamycin-S1	VIRG-S1	8.45	1.33	31.85	2.48	2.38
	Azithromycin	AZ	2.40	41.48	9.63	0.85	0.44
	Tilmicosin	TILM	26.05	15.56	57.63	2.35	5.83
	Erythromycin	ERYTH	2.10	1.14	2.61	1.37	2.23
	Roxithromycin	ROX	1.52	2.45	1.57	1.00	1.68
	Tylosin A	TYLOSIN	6.01	2.29	5.35	4.46	4.72
Sulfonamides	Sulfamethoxazole	SMX	0.50	1.19	0.40	1.57	0.76
	Sulfadimethoxine	SDM	1.29	2.24	0.86	6.32	7.72
	Sulfasalazine	SSZ	27.85	9.01	N/A	3.37	2.65
	Sulfadiazine	SDZ	2.30	1.33	23.55	2.89	2.35

N/A: Not applicable

Figure 1. MRM chromatogram of target antibiotics spiked into King salmon matrix, extracted using QUEChERS (Experiment 2) and reconstituted in methanol: water (1:1; v/v).



Supplementary information:

Table S1. Maximum residue limit (MRL; ng/g) for antibiotics according to U.S. regulations[200].

Class	Antibiotics	MRL (ng/g)	Fish	Other matrices
B-Lactams	AMOX	10	NA	Cattle edible tissue
	AMP	10	NA	Cattle/ swine edible tissue
	PEN-G	50*	NA	Edible tissues of cattle
	PEN-V	50*	NA	Edible tissues of cattle
Amphenicols	CAP	Banned	-	-
	FF**	1000	Fish	-
	TAP	NA	NA	-
Tetracyclines	Sum of tetracycline residues	2000	Finfish muscle	
Quinolones	FLU	NA	NA	NA
	ENO	NA	NA	NA
	ENRO	100***	NA	Cattle liver
	NOR	NA	NA	NA
Sulfonamides	SDZ	NA	NA	NA
	SDM	100	Edible tissues of catfish	-
	SMX	NA	NA	NA
	SSZ	NA	NA	NA
	ROX	NA	NA	NA
Macrolides	TILM	100	NA	Muscle of cattle
	AZ	NA	NA	NA
	Tylosin	200	NA	Muscle of cattle
	VIRG	Exempt	NA	Cattle/ chicken edible tissues****
	ERYTH	100	NA	Cattle edible tissues
Lincosamides	LIN‡	100	NA	Swine muscle
Dihydrofolate reductase inhibitors	TRIM	NA	NA	NA
	ORM	100	Salmonids and catfish	-

NA: Not available

* Penicillin, MRL = 10 ng/g in turkey, 0 ng/g in chicken, milk, swine, egg, milk

**Tolerance for marker residue: FFA. Fish includes catfish muscle, freshwater-reared warmwater finfish (other than catfish) and salmonids muscle/skin

***Tolerance for desethylene ciprofloxacin (marker residue)

****Excluding cattle milk and chicken eggs; swine muscle: 100 ng/g

‡ Exempt in chicken edible tissues

Table S2. Percent contribution of background antibiotics to peak areas. This was calculated by dividing antibiotic peak area in salmon spiked with labeled surrogate standards only before extraction to antibiotic peak area in salmon sample spiked with both unlabeled antibiotics and labeled surrogates after extraction.

Antibiotics	King salmon	Ivory King salmon	Sockeye salmon
AMOX	0%	0%	0%
AMP	0%	0%	0%
PEN-G	0%	0%	0%
PEN-V	0%	0%	0%
ORM	0.02%	0.03%	0%
TRIM	0.03%	0.03%	0%
LIN	0.01%	0.01%	0%
CAP	0%	0%	1%
FF	0%	0%	0%
TAP	0%	0%	11%
FFA	9.1%	5.6%	28%
CTC	0%	0%	0%
OTC	0%	0%	0%
TC	0%	0%	0%
DOX	0.5%	0%	0%
ENO	0%	0%	0%
ENRO	0.4%	0.5%	0%
NOR	0%	0%	0%
FLU	1.5%	0.5%	8%
ERYTH	0.1%	0.1%	0.1%
AZ	0.4%	0.5%	2%
TILM	0%	0%	3%
ROX	0.6%	0.4%	0%
Tylosin	0%	0.01%	0%
VIRG-M1	0.9%	0%	38%
VIRG-S1	0%	0%	0%
SDM	0%	0%	0%
SDZ	0.01%	0%	1%
SMX	0%	0%	0%
SSZ	0%	0%	8%

Table S3. Antibiotic/surrogate recovery and matrix effects ratio in salmon matrices (Sockeye, King and Ivory King). The antibiotic/surrogate recovery ratio was calculated in samples spiked with both antibiotic standard and labeled surrogate before extraction. The antibiotic/surrogate matrix effect ratio was calculated in samples spiked with antibiotic standard and labeled surrogates after extraction. The ratios were calculated for three extraction methods i.e. QUEChERS, QUEChERS-dSPE and QUEChERS-SPE in Sockeye salmon and for QUEChERS extraction in King and Ivory salmon. Antibiotic/surrogate recovery and matrix effects ratio below 0.7 and above 1.3 were considered unacceptable and marked with ǂ. The ratios for oxytetracycline, tetracycline, chlortetracycline and doxycycline were not calculated in Sockeye salmon matrix (Experiment 1) due to the lack of proper surrogate at the time of the experiment.

Antibiotic	Tagged surrogate	Sockeye salmon						Ivory salmon		King salmon	
		QUEChERS		QUEChERS-dSPE		QUEChERS-SPE		QUEChERS		QUEChERS	
		Recovery ratio	Matrix effects ratio	Recovery ratio	Matrix effects ratio	Recovery ratio	Matrix effects ratio	Recovery ratio	Matrix effects ratio	Recovery ratio	Matrix effects ratio
LIN	LIN-D3	1.00	1.05	0.97	1.07	0.97	1.04	1.01	1.01	1.00	1.01
TRIM	TRIM-D3	0.99	0.85	1.00	0.86	1.05	0.86	1.00	1.00	1.00	1.02
ORM	TRIM-D3	1.00	0.96	1.01	0.93	1.49ǂ	0.87	1.03	1.19	1.07	1.26
FLU	TRIM-D3	1.01	0.31ǂ	1.00	0.30ǂ	13.60ǂ	0.19ǂ	0.92	1.24	1.05	1.20
ENRO	ENRO-D5	1.01	0.83	0.98	0.83	0.99	0.83	0.99	0.93	1.00	0.87
ENO	ENRO-D5	1.32ǂ	1.09	1.12	1.17	0.82	1.11	0.82	1.47ǂ	0.85	1.40ǂ
NOR	ENRO-D5	1.27	1.11	1.21	0.92	1.08	1.21	0.87	1.56ǂ	0.88	1.71ǂ
CAP	CAP-D5	0.95	3.01ǂ	1.43ǂ	2.19ǂ	1.24	1.53ǂ	1.16	1.35ǂ	1.11	1.55ǂ
FF	CAP-D5	0.97	3.29ǂ	1.36ǂ	2.51ǂ	1.07	1.82ǂ	1.11	0.98	1.19	1.15
TAP	CAP-D5	0.90	3.39ǂ	1.39ǂ	2.11ǂ	0.31ǂ	1.41ǂ	1.25	0.69ǂ	1.45ǂ	0.80
FFA	FFA-D3	0.94	0.62ǂ	1.24	0.90	ND	1.03	0.91	0.90	1.02	0.91
OTC	DEM	N/A	N/A	N/A	N/A	N/A	N/A	1.23	0.77	1.27	0.63ǂ
TC	DEM	N/A	N/A	N/A	N/A	N/A	N/A	1.19	0.67ǂ	1.15	0.67ǂ
CTC	DEM	N/A	N/A	N/A	N/A	N/A	N/A	1.18	0.67ǂ	1.04	0.75
DOX	DEM	N/A	N/A	N/A	N/A	N/A	N/A	1.46ǂ	1.12	1.52ǂ	0.95
AMP	AMP-D5	0.84	1.66ǂ	0.88	1.56ǂ	1.32ǂ	1.07	1.24	1.19	1.10	1.15
AMOX	AMP-D5	ND	0.04	ND	0.06	0.85	1.03	0.56ǂ	0.30ǂ	0.59ǂ	0.33ǂ
PEN-V	PEN-V-D5	0.97	1.08	1.04	0.97	0.93	1.03	1.00	1.00	1.03	1.00
PEN-G	PEN-V-D5	0.79	2.21ǂ	1.02	2.34ǂ	0.18ǂ	2.10ǂ	0.76	1.31ǂ	0.60ǂ	1.26
VIRG-M1	PEN-V-D5	1.16	1.60ǂ	0.88	1.28	2.38ǂ	0.81	0.87	0.94	0.86	0.89
VIRG-S1	PEN-V-D5	1.26	3.48ǂ	1.16	1.94ǂ	ND	0.00	1.00	0.77	1.23	0.71
AZ	AZ-D3	0.99	0.80	1.43ǂ	0.77	1.11	0.78	1.00	1.00	1.01	1.00
TILM	AZ-D3	1.02	6.05ǂ	1.01	3.32ǂ	5.90ǂ	3.55ǂ	1.13	1.53ǂ	1.11	1.59ǂ
ERYTH	ERYTH-D6	1.00	0.91	0.14ǂ	6.57ǂ	1.00	0.90	0.99	1.00	0.99	1.01
ROX	ROX-D7	0.99	0.88	0.97	0.99	0.99	0.92	0.99	0.91	1.01	0.88
TYLOSIN	SMX-D4	1.58ǂ	1.09	0.87	0.84	1.48ǂ	0.36ǂ	1.07	0.67ǂ	1.27	0.62ǂ
SMX	SMX-D4	1.01	0.69ǂ	1.01	0.67ǂ	1.02	0.66ǂ	1.01	1.01	1.01	1.00
SDM	SMX-D4	0.91	0.28ǂ	0.98	0.30ǂ	0.88	0.17ǂ	0.87	1.44ǂ	0.92	1.42ǂ
SSZ	PEN-V-D5	1.72ǂ	0.29ǂ	1.19	0.27ǂ	ND	0.13ǂ	1.03	0.90	1.05	0.92
SDZ	SMZ-D4	1.17	0.51ǂ	1.02	0.48ǂ	1.11	0.95	0.87	0.59ǂ	0.82	0.52ǂ

ND: Not detected; N/A: Not applicable

Chapter 4: Antibiotics are more concentrated in wild fish compared to farmed fish, and are not readily degraded by thermal treatment

Shiva Emami[1], Zhichao Zhang[1], Nuanyi Liang[1], Nitin Nitin[1,2], Gang Sun[2], Rosalee S. Hellberg[3], Ameer, Y. Taha[1,4*]

Author affiliation:

[1] Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California, Davis, CA, USA, 95616

[2] Department of Biological & Agricultural Engineering, University of California, Davis, CA, 95616, USA

[3] Chapman University, Schmid College of Science and Technology, Food Science Program, One University Drive, Orange, CA, 92866, USA

[4] West Coast Metabolomics Center, Genome Center, University of California Davis, One Shields Avenue, Davis, CA, USA 95616

*Corresponding author:

Department of Food Science and Technology, College of Agriculture and Environmental Sciences, University of California Davis, One Shields Avenue, Davis, CA, USA 95616; Email: ataha@ucdavis.edu

Running title: Antibiotic contamination in seafood and the effects of cooking

Abbreviations used

Amoxicillin (AMOX), ampicillin (AMP), analysis of variance (ANOVA), azithromycin (AZ), chloramphenicol (CAP), chlortetracycline (CTC), demeclocycline (DEM), doxycycline (DOX), enoxacin (ENO), enrofloxacin (ENRO), erythromycin (ERYTH), florfenicol (FF), florfenicol amine (FFA), flumequine (FLU), lincomycin (LIN), norfloxacin (NOR), oxytetracycline (OTC), penicillin G (PEN-G), penicillin V (PEN-V), principal component analysis (PCA), roxithromycin (ROX), sulfadiazine (SDZ), sulfamethazine-D4 (SMZ-D4), sulfadimethoxine (SDM), sufamethoxazole (SMX), sulfasalazine (SSZ), tetracycline (TC), thiamphenicol (TAP), tilmicosin (TILM), trimethoprim (TRIM), virginiamycin (VIRG), ultra-high pressure liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

Abstract

Antibiotic residues have been detected in both farmed and wild-caught seafood, but comparative data on the extent of contamination in domestic (US) and imported farmed and wild seafood are lacking. It is also not known whether antibiotics in contaminated seafood are stable to thermal treatment mimicing cooking conditions. In the present study, we addressed these unknowns by measuring the concentrations of 30 antibiotics (from 8 different classes) routinely used in aquaculture in wild-caught and farm-raised seafood produced in the U.S. or imported from other countries (n=125 samples in total). The effects of thermal treatment were also tested. Several antibiotics were detected more frequently in farm-raised than wild-caught seafood, however, concentrations were significantly higher in wild-caught than farm-raised samples for ampicillin, chlortetracycline, sulfamethoxazole, lincomycin, azithromycin and virginiamycin-S1. The occurrence of antibiotics in imported seafood was statistically more frequent compared to domestically produced seafood ($p < 0.05$ to $p < 0.0001$), although concentrations were mostly comparable. Thermal processing of fish samples spiked with antibiotics degraded B-lactams, tetracyclines and some macrolides. Our findings show new evidence of widespread antibiotic contamination in wild fish irrespective of the source (domestic or imported), reflecting wide-spread environmental contamination. Thermal treatment did not degrade most antibiotics, highlighting the potential for exposure to non-acutely toxic doses of antibiotics with chronic fish intake. Potential implications of these findings to the global spread of antibiotic drug resistance need to be assessed.

Keywords: Antibiotics, Seafood, Wild-caught, Farm-raised, Thermal processing, Cooking

Introduction

Seafood consumption has increased during the past few decades. The amount of fish consumed per person was 9.0 Kg in 1961. This amount has approximately doubled in 2015, reaching 20.5 Kg fish per person [70]. The increase in seafood intake has mainly been attributed to the rapid increase of the world population, improved living conditions and growing public awareness of seafood as a healthy food choice [71]. With capture fisheries reaching their production limit at 90 million metric tons per year since 1990 [70], the production of farm-raised seafood has grown to meet the high demand for seafood. The aquaculture share of global production has increased from 9% in 1980 to 48% in 2011, and it is estimated to further increase to above 60% by 2030 [76].

The substantial growth in production of farm-raised seafood has been accompanied with increased use of antibiotics in aquatic ponds in order to prevent or treat infectious diseases that are more likely to occur under intense farming practices [77]. In 2017, approximately 10,259 tons of antibiotics have been used in aquaculture[46]. This amount is estimated reach 13,600 tons by 2030 (33% increase) [46]. Tetracyclines (oxytetracycline), amphenicols (florfenicol), quinolones (oxolinic acid, flumequine and enrofloxacin), sulfonamides (sulfadiazine) in combination with trimethoprim and B-lactams (amoxicillin) are the most frequently used antibiotics in aquatic ponds[46].

The widespread use of antibiotics in aquaculture has resulted in widespread contamination of farmed seafood with multiple antibiotic classes including tetracycline, quinolones, sulfonamides, macrolides, B-lactams and amphenicols [75, 78-80]. Antibiotic contamination in seafood is concerning because of the emergence of antibiotic resistant bacteria [47] that can directly transmit

to humans or act as hosts for resistance genes that can cross to infectious human pathogens [50-52, 77]. This is concerning because the majority of antibiotics used in aquaculture i.e. penicillins, quinolones, tetracyclines, sulfonamides, macrolides and aminoglycosides [45-46], are categorized as critically or highly important antibiotics for human use by the World Health Organization (WHO) [53]. Thus, developing resistance to these drugs will add to the current epidemic of antimicrobial drug resistance which has resulted in 4.95 million premature deaths worldwide in 2019 [55] and continues to cause approximately 35,000 deaths in the U.S. every year [200].

There is growing concern that wild fish may be exposed to antibiotics present in natural waters. Contamination in natural waters has been attributed to water effluent coming from medical, domestic and industrial wastewater, animal manure, and aquaculture/agriculture wastewater runoffs [89-91]. Several studies have shown that water and sediments from coastal and offshore regions contain antibiotic residues[78, 81-82] which can also accumulate in aquatic animals [78, 83].

Although the presence of antibiotic residues in farm-raised and wild-caught seafood is well-documented [78-80, 155-157], there is limited comparative information on the extent of contamination in wild versus farmed seafood. Wild seafood may be exposed to multiple sources of antibiotics (wastewater, farm and aquaculture effluents), whereas exposure in farmed seafood is often controlled and regulated. To date, studies have measured contamination in a small number of wild or farmed seafood samples [75] or probed for a limited number of antibiotic residues (< 10) [158] used in aquaculture. A large and comprehensive survey of the extent of antibiotic contamination in wild versus farmed seafood from both domestic and imported sources is yet to be done.

One additional factor that has not been considered in seafood antibiotic surveys is whether antibiotics are thermally stable or not. This is important to take into consideration because most seafood is consumed cooked, and assessments of health risks associated with exposure should

factor in the effects of potential thermal degradation. There is data pointing to possible degradation of some antibiotics after thermal treatment [63, 134-135, 140, 147]. Oxolinic acid and oxytetracycline (OTC) were shown to degrade by 20-30% and 30-60%, respectively, in shrimp following boiling (2 to 12 min), frying (180 ˚C, 1 min) and baking (200 ˚C, 4 min) [143, 152]. OTC was reported to degrade by 60% in salmon after frying at 100 ˚C for 15 min[144]. Chloramphenicol (CAP) was shown to degrade by 6-29% in shrimp heated at 100 ˚C and 121 ˚C for 10 to 30 min [150] and by 35-65% in mussels heated at 100 ˚C for 1 hour [214]. Ormetoprim (ORM) and sulfadimethoxine (SDM) were reduced by 54% and 41% after cooking Channel Catfish with smoking at 160-200 ˚C, baking at 190 ˚C and frying at 190 ˚C[153].

At present, there is no information on the effects of thermal treatment on the degradation of several other antibiotic classes, commonly found in wild-caught and farm-raised seafood (e.g. quinolones, B-lactams and macrolides). Also, it is not known whether the lipid content of the matrix impacts thermal degradation. Many antibiotics are lipid-soluble, and may therefore be more protected from thermal degradation in high-fat fish compared to low-fat (mostly protein) fish.

With the abovementioned unknowns, this study was designed to a) survey antibiotic contamination in wild-caught and farm-raised seafood from both domestic and imported sources (fish and shrimp; n=125); b) assess the effects of thermal processing on antibiotic degradation in low- and high-fat fish; c) assess the potential health risks associated with antibiotic exposure from seafood. Thirty antibiotics from eight classes commonly found in seafood were tested in domestic and imported wild-caught and farm-raised seafood collected from local stores in California, U.S. (**Table 1)**. Our coverage included antibiotics routinely used in aquaculture farming in several countries [205], antibiotics banned for use in aquaculture in the U.S. (e.g. CAP) [206], and antibiotics that have been previously detected in seafood products in the U.S [75]. Thermal degradation of these

antibiotics was assessed by spiking wild-caught salmon (high-fat fish), cod (low-fat fish), pure fish oil, pure water and cod fish mixed with fish oil to match the lipid content of salmon, with antibiotic standards and heating them at temperatures comparable to conventional cooking methods. Maximum antibiotic concentrations in raw and cooked seafood were used to calculate the estimated daily intake of antibiotic residues through seafood consumption from which associated health risks were derived.

Materials and methods

Materials

LC/MS grade methanol and acetonitrile were obtained from Fisher Scientific (Hampton, NH). Formic acid, sodium sulfate (NA_2SO_4) and sodium chloride (NaCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trisodium citrate dihydrate (Alfa Aesar) was purchased from Fisher Scientific (Pittsburg, PA, USA). TDT3 aluminum cells were purchased from Washington State University; Engineering shops (Pullman, WA). Thermocouple temperature data logger (OM-EL-USB-TC) was purchased from Omega engineering Inc. (Norwalk, CT).

Antibiotic standards used in this study belonged to the following classes: **Amphenicols:** chloramphenicol (CAP), thiamphenicol (TAP), florfenicol (FF), florfenicol amine (FFA); **Tetracyclines:** tetracycline (TC), oxytetracycline (OTC), chlortetracycline (CTC), doxycycline HCl (DOX); **Sulfonamides:** sulfadimethoxine (SDM), sulfasalazine (SSZ), sufamethoxazole (SMX), sulfadiazine (SDZ); **Quinolones:** enrofloxacin (ENRO), flumequine (FLU), norfloxacin (NOR) and enoxacin (ENO); **Macrolides:** erythromycin (ERYTH), azithromycin (AZ), tylosin A (Tylosin), virginiamycin (VIRG) complex (mixture of VIRG-M1 and VIRG-S1), roxithromycin (ROX), tilmicosin phosphate (TILM); **B-lactams:** ampicillin anhydrous (AMP), penicillin G potassium salt (PEN-G), penicillin V (PEN-V) and amoxicillin (AMOX); **Lincosamides:**

lincomycin (LIN), **Dihydrofolate reductase inhibitors:** trimethoprim (TRIM) and ormetoprim (ORM).

AMOX (98%), ROX (97%), SDZ (99%), TAP (99.3%) and FF (98%) were purchased from Fisher Scientific (Hampton, NH, USA). ERYTH (94.8%), DOX (98.8%), NOR (98%), AMP (99.6%), SDM (98.5%), ENRO (99.8%), TC (≥ 98%) and FFA (99.3%) were purchased from Sigma Aldrich (St. Louis, MO). CTC (98.0%), OTC (≥ 95%), FLU (100.0%), ENO (100%), AZ (99.5%), TYLOSIN (99.8%), VIRG (99.0%), PEN-G (99.5%), PEN-V (98.8%), SSZ (100%), SMX (100%), LIN (98%), TRIM (100%), TILM (100%) and DEM (96%) were purchased from Cayman Chemicals (Ann Arbor, MI). CAP (98.5%) was purchased from Crescent Chemical (Islandia, NY). Isotopically labeled standards including CAP-D5 (chemical purity: 98%; isotopic purity: 98.3%), SMX-D4 (chemical purity: 98%; isotopic purity: 99.2%), sulfamethazine-D4 (SMZ-D4; chemical purity: 98%; isotopic purity: 95.9%), AZ-D3 (HPLC purity: 99.86%; isotopic purity: 93.9%), ERYTH-D6 (chemical purity: 95%; isotopic purity: 98.1%), TRIM-D3 (chemical purity: 99.49%; isotopic purity: 99.9%), LIN-D3 (chemical purity: 95%; isotopic purity: 99.6%), ENRO-D5 (HPLC purity: 99.61%; isotopic purity: 99.40%) and ROX-D7 (HPLC purity: 96.04%; isotopic purity: 99.00%), L-(+)-AMP-D5 (chemical purity: 95%; isotopic purity: 99.00%), and ent-FFA-D3 (chemical purity: 98%; isotopic purity: 98.7%) were purchased from Toronto Research Chemicals (Toronto, Ontario, Canada).

Antibiotic standard preparation

Individual stock solutions of CAP, TAP, FF, FFA, TC, OTC, CTC, DOX, SDM, SMX, ENRO, ERYTH, AZ, VIRG, ROX, TILM, TRIM, ORM, LIN, CAP-D5, FFA-D3, SMX-D4, SMZ-D4, AZ-D3, ERYTH-D6, TRIM-D3, ROX-D7, ENRO-D5 were prepared in methanol at a concentration of 1 mg/mL. SSZ, SDZ, TYLOSIN, LIN-D3 were prepared at a concentration of 0.5

mg/mL in methanol and FLU, NOR and ENO were prepared at a concentration of 0.2 mg/mL in methanol. B-lactams and their deuterated surrogate PEN-V-D5 standard were prepared in Milli-Q water (1 mg/mL) because they are more polar. AMP-D5, another surrogate used for B-lactams quantitation, was prepared in Milli-Q water at a concentration of 0.5 mg/mL.

Individual intermediate solutions of 10 µg/mL of each antibiotic were made in the same solvent as the stock solution and were used to prepare the calibration standards and antibiotic working mixes required for spiking matrices in antibiotics thermal treatment experiment (Experiment B).

The antibiotic working mix (at concentration 500 ng/mL) required for Experiment B was prepared by mixing unlabeled antibiotic standards. First, sub-groups of methanol soluble (tetracyclines, quinolones, macrolides, amphenicols, sulfonamides and dihydrofolate reductase inhibitors) and water soluble antibiotic standards (B-lactams), each at concentration of 1000 ng/mL, were prepared. For methanol-soluble antibiotics subgroup, 180 µL of methanol soluble unlabeled standards from their individual intermediate solution were added to LC vials, followed by evaporating under nitrogen and reconstituting in 1800 µL LC/MS methanol. In order to prepare water-soluble antibiotics, 1080 µL Milli-Q water and 10 µL of four water soluble unlabeled antibiotic standards (B-lactams) from their individual intermediate solution were added to another LC vial. Working mixes were prepared by mixing methanol-soluble and water-soluble subgroups at 1:1 ratio prior to the experiment.

Experiment A. Assessing the prevalence of antibiotic residues in wild-caught and farm-raised seafood

A.1. Seafood samples information and preparation method

A total of 125 seafood samples including 120 fish and 5 shrimp samples were analyzed in this study. Fish samples (fillets, n=120) were obtained from 30 grocery stores in Orange County, California. The 5 shrimp samples were purchased from local stores in Davis, CA.

The fish samples were from sixteen categories including bass, catfish, cod, halibut, mahi-mahi, pangasius, rockfish, rockfish/ snapper, salmon, snapper, sole, swordfish, tilapia, trout, tuna and yellowtail. The samples originated from nineteen countries as shown in **Figure 1**, of which 38 were domestically produced in the U.S., 68 were imported and 14 were from unknown origin. Of the 120 fish samples, 68 were wild-caught, 41 were farm-raised and 11 did not have a known production method based on their label (Unknown). The fish samples were genetically verified using the DNA barcoding as previously reported [215]. The 5 shrimp samples consisted of 3 wild-caught (2 from the U.S. and 1 from Argentina) and 2 farm-raised samples (1 from India and 1 from Indonesia). Detailed information of the fish samples including the seafood type on the label, DNA-identified species, production method and production origin are provided in supplementary **Table S1**.

A.2. Antibiotic extraction from seafood using QUEChERS method

Fish and shrimp samples were thawed at 4 ˚C for approximately 2 hours and homogenized in dry ice using Sears solid state 10-speed blender at speed 4. The homogenates were stored in loose ziplock bags at 4 ˚C overnight (approximately 12 hours) to allow the dry ice to sublime.

Antibiotics from fish and shrimp samples were extracted using the QUEChERS (Quick, Easy, Cheap, Rugged and Safe) method described by Desmarchelier et al. [111]. Approximately, 1 g of seafood homogenate was weighted and placed in 50 mL Falcon tubes (Fisher Scientific, cat # LS4541). Samples were spiked with isotopically labelled surrogate standard mix at a final concentration of 20 ng/g per sample (using the average weight), by adding 40 µL of 500 ng/mL

surrogate mix dissolved in water: methanol (1:1). To each tube, 8 mL Milli-Q water was added. Then, five ceramic beads were added to facilitate homogenization. Beads were pre-soaked 3 times with acetone and 3 times with methanol for a period of 30 min each time and allowed to dry overnight. Acetonitrile (30 mL) was added to each of the samples, and they were hand-shaken for approximately 10 seconds. 30 µL formic acid was added to each sample and tubes were mechanically shaken for 30 min at 200 rpm (New Brunswick Scientific, Excella E24 Incubator Shaker series). A pre-weighed salt mixture consisting of 4g Na_2SO_4, 1g NaCl and 1.5 g of trisodium citrate dihydrate was added to the samples and they were hand-shaken for about 10 seconds followed by mechanical shaking for 30 min at 200 rpm using the incubator shaker. The samples were then centrifuged at 3000 rpm for 10 min at 10 ˚C (SORVALL RT 6000D, rotor H1000B) and the supernatant layer (~ 30 mL of acetonitrile) was transferred to new sets of 50 mL falcon tubes. The supernatant was dried under nitrogen and reconstituted in 1 mL of water: methanol (1:1). The samples were vortexed (3 min), sonicated (Branson 1210, Danbury, CT) for 3 min and transferred to 2 mL centrifuge tubes (Sealrite, USA Scientific, FL). The tubes were centrifuged at 12000 rpm (13523 ×g) for 2 min (Eppendorf, 5424 R) and the samples were transferred to filter-containing centrifuge tubes (Ultrafree-MC-VV; PVDF 0.1 µm; Milipore Sigma, MA) which were also centrifuged for 10 min at 12000 rpm (13523 ×g). The last step was repeated if any visible residues were seen in the tubes. The extracts were transferred to LC vials (Phenomenex, CA) prior to analysis using ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS) as described below. Samples were analyzed on the same day of extraction. Approximately 10-15 samples were extracted and run per day. One modification compared to method described by Desmarchelier et al. [111] was that here we did not perform dispersive solid phase extraction clean-up following QUEChERS extraction due to our

previous results from Chapter 3 showing inefficiency of this clean-up method in improving matrix effects from fish.

Quality control samples consisted of 1) a non-spiked method blank (1 mL; acetonitrile: water (30:8 v/v)) extracted with each batch of 10 to 15 samples alongside the seafood samples to check for possible contamination during the extraction process, and 2) a wild-caught King salmon spiked with all 30 antibiotics at 20 ng/g for each antibiotic was extracted in each batch to monitor the consistency of analysis and accuracy of the quantitation.

A.3. Antibiotics detection and quantification criteria in fish and shrimp samples

Antibiotic concentrations in fish, shrimp, method blanks and quality control samples were calculated by the internal standard calibration method where surrogates were used to correct for both recoveries and matrix effects. A 12-point standard calibration curve (0.001-100 ng/ mL) containing a fixed amount of surrogate standard (20 ng/mL) was made to derive the response factor. The regression equation for the calibration curves was generated by quadratic regression and $\frac{1}{x^2}$ weighting factor was applied to the least-squares regression algorithm [207]. If the concentration in the sample was lower than the concentration in method blank (acetonitrile: water (30:8 v/v)) in the same batch, the sample concentration was reported as below the method blank (< MB). Whenever antibiotics were detected in MB, they were deducted from the sample concentration. Samples without detectable peaks were reported as "Not detected" (ND). The concentrations in samples were compared to the Limits of Detection (LOD) and Limits of Quantitation (LOQ) which were determined as described below (Section A.4). Concentrations lower than LOD and between LOD and LOQ were reported as < LOD and < LOQ, respectively.

Two MRM transitions were monitored for each antibiotic, one ion pair for quantitation purposes (quantifier ions) and one ion pair for qualification purposes (qualifier ions). However,

quantifier ion/ qualifier ion ratios were not applied as the confirmatory criteria of antibiotics detection in seafood samples. This is because the quantifier ion/ qualifier ion ratios were set based on the highest concentration calibration standard at an uncertainty threshold of 20%; in many cases, this threshold was not met for any antibiotic detected at low concentration calibration standards. Therefore, the ratios were not robust enough for low concentrations. Thus, only quantifier MRM transitions were used for quantitation and compound identification.

A.4. Determination of limit of detection (LOD) and limit of quantification (LOQ)

The LOD was calculated according to the Environmental protection agency (EPA) method (EPA; Code of Federal Regulations (CFR) 40, Part 136, Appendix B). A paired t-test was run between the pairs of the calibration points. The LOD was calculated by multiplying the standard deviation (SD) of three replicates at the calibration point that differed significantly ($p < 0.05$) from the ones below it, by the t-value associated with 95% confidence level and a degree of freedom of n-1, as shown in the equation below:

$$LOD = SD \times t_{n-1,\ 1-\alpha = 0.95}$$

The Limit of quantitation (LOQ) was calculated by multiplying the SD by 10 [216]:

$$LOQ = SD \times 10$$

Since seafood samples were analyzed in 3 different UPLC-MS/MS runs, each run included a calibration curve generated from 3 replicates of calibrations standards to calculate the LOD and LOQ per run (**Table S2**).

Experiment B. Effect of thermal treatment of fish on antibiotics degradation

To assess the effect of thermal treatment on antibiotic degradation, 1 g of homogenized cod (low-fat), salmon (high-fat) and cod supplemented with 10% salmon oil were spiked with antibiotics mixture at 20 ng/ per g fish to test whether the fish fat content affects antibiotics

degradation. Matrix controls consisted of Milli-Q water and salmon oil, also spiked with the same level of antibiotic standard mix.

All samples were spiked with 30 antibiotics (no surrogates) and heated at 90 ˚C for 2 hours (n=3 per condition) in custom designed TDT3 aluminum cells (Machine shop, Washington State University) were used to heat the samples. These heating cells were selected to improve the uniformity of temperature distribution in the heating unit [217] (**Figure S1**). The select temperature of 90 ˚C approximates the internal temperature of seafood during conventional oven cooking[218]. The time-temperature profile of all fish types and control matrices during heating process is shown in **Figure S1**.

Homogenized fish (1 g; n=3), fish oil (1 mL, n=3) and water (1 mL; n=3) were put into TDT3 aluminum cells, spiked with 20 ng/g of antibiotic mix containing 30 antibiotics (for fish matrix) or 20 ng/mL of antibiotic mix for oil and water samples. The cells were capped and submerged in a water bath pre-heated at 90 ˚C. A parallel TDT3 cell connected to T-type thermometer containing test sample was included in each batch to monitor the interior temperature of each matrix during thermal processing; temperature was captured with a data logger (OM-EL-USB-TC; Omega engineering Inc., Norwalk, CT). Each cell was heated for 15, 30, 45, 60 and 120 minutes (n=3 per sample type per time-point). At the end of heating, the cells were submerged in an ice bath for 30 seconds to bring the interior temperature to room temperature. The cooled samples were transferred to a -20 ˚C freezer until the heating process for all samples was completed (15-120 min). Samples were put at room temperature for 15 min prior to QUEChERS extraction. Samples were extracted at the same time in order to account for antibiotic contact time with the matrix and heating cell using the same method described above.

Samples were transferred to 50 mL Falcon tubes and the TDT cells were rinsed with 1 mL ACN: water which was transferred to the Falcon tube. Samples were extracted using the QUEChERS method described above. In addition, five contaminated samples from Experiment A were heated in a similar manner to determine whether endogenous antibiotics in the matrix degrade during the thermal treatment.

To evaluate the effects of thermal treatment on 'real samples', we chose 5 contaminated samples from Experiment A and heated them for 20 min in TDT3 cells. Approximately 1 g of raw farmed rainbow trout (n=1), farmed tilapia (n=2), farmed Madai (n=1), and wild Pacific/Atlantic Halibut fish were analyzed at baseline and after 20 min incubation in TDT3 cells maintained at 90 ˚C using a water bath. The antibiotic residue profile and their concentrations are shown in **Table S3**. Sample IDs 014 and 058 refer to two farmed tilapias and sample IDs 070, 088 and 177 refer to farmed rainbow trout, wild Pacific/Atlantic Halibut and farmed Madai, respectively. Their raw counterparts (i.e. baseline) were placed in TDT3 cells and kept at -20 ˚C freezer until the time of extraction. The cooked samples were brought to room temperature after heating by immersing the cells in an ice bath for 30 seconds. The raw and cooked samples were then extracted using the QUEChERS method as explained above.

All samples were run on UPLC-MS/MS along with calibration standards (12 points 0.001 to 100 ng/mL; 3 replicates) and antibiotic concentrations were quantified. Thermal degradation of spiked antibiotics in fish, fish oil and water matrices was assessed by calculating the degradation rate constant (k; min $^{-1}$) based on the following equation:

$$Ln\ \frac{C}{C_0} = -kt$$

The first-order kinetic model has been previously used for studying thermal degradation of antibiotics in food matrices [151].

Instrumentation

Antibiotic analysis was performed using an Agilent 1290 UPLC coupled to a 6460 Agilent triple quadrupole. Chromatographic separation of the antibiotics mixture was performed on AQUITY BEH C18 column (100 × 2.1 mm, 1.8 µm), using 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B) at flow rate of 0.3 mL/min and column temperature of 30 ºC. The mobile phase gradient conditions were as follows: initial time: 10% B, 8 min: 20% B, 11 min: 60% B, 13 min: 100% B, 15 min: 100% B, 17 min: 10% B and 20 min: 10% B.

MS/MS analysis was performed using Agilent Jetstream electrospray ionization (ESI) operating in both positive and negative ionization mode and using dynamic multiple reaction monitoring conditions to scan for quantifier and qualifier ion pair transitions within a specified time window for each analyte. **Table 1** shows the precursor ion, quantifier and qualifier product ions, fragmentor voltage, collision energies (CE), retention time and window and polarity for antibiotic standards. MS source parameters were as follows: sheath gas (nitrogen) temperature of 375 ºC, sheath gas flow of 11 L/min, drying gas temperature of 250 ºC, nozzle voltage of 0V, nebulizer gas pressure of 40 psi and capillary voltage of 3500 V.

Human health risk assessment due to chronic exposure

Measured concentrations in seafood samples were combined with intake data to estimate exposure, and determine whether it exceeds the acceptable daily intake (ADI). This is a measure of toxicity risk assessment based on carcinogenic and non-carcinogenic risk factors. The estimated daily intake (EDI) of antibiotics was calculated for each antibiotic according to the equation below:

$$EDI = \frac{C_{max-raw} \times Daily\ fish\ intake}{Body\ weight}$$

Where: $C_{max\text{-}raw}$ is the maximum concentration of each antibiotic found in raw fish. In order to account for the effect of thermal processing on antibiotic concentrations, a thermal factor was applied to $C_{max\text{-}raw}$ as below:

$$C_{max\text{-}thermal} = C_{max\text{-}raw} \pm (C_{max\text{-}raw} \times C_f)$$

Where C_f is the expected % change in antibiotic concentration during heat treatment for 30 min, derived from the thermal processing experiment (Experiment B). The percent changes were added or deducted from $C_{max\text{-}raw}$ depending on whether concentration increased or decreased during thermal processing.

An estimated daily fish intake of 11.33 g/day for adults (average intake in males and females above 21 years old) [219] and an average body weight of 70 kg[220] was used for the EDI calculation. EDIs were compared to the ADI issued by Ministry of Agriculture and Rural Affairs of the People's Republic of China [221] to assess health risks.

Statistical analysis

Statistical analysis was performed using Graphpad prism Version 9.2.0 or RStudio Version 1.4.1106. A Chi-squared test was used to compare detection frequencies between wild-caught and farm-raised seafood samples, and between imported and domestic samples within the wild-caught and farm-raised groups. “Detection” referred to clear antibiotic peaks that were detected at concentrations above the LOQ, between LOQ and LOD or below the LOD.

A D’Agostino and Pearson test was used to check for the normality of antibiotic concentrations in each of the wild-caught and farm-raised groups. The test showed that concentrations were not normally distributed for most antibiotics. The data were therefore log-transformed and compared using an unpaired t-test (wild-caught versus farmed). Samples with “Unknown” production method were not included in statistical comparison.

A one-was analysis of variance (ANOVA) followed by Tukey's post-hoc test was used to explore differences in concentrations among the wild-domestic, wild-imported, farmed-domestic and farmed-imported groups. A follow-up Principal component analysis (PCA) was used to visualize the distribution of antibiotics among the wild-domestic, wild-imported, farmed-domestic and farmed-imported groups using RStudio Version 1.4.1106. PCA was applied to antibiotic concentrations above the LOQ, between the LOD and LOQ, and below the LOD if a visible peak greater than the blank was detected. K-means clustering was applied to visualize clusters segmentation.

Comparison of the degradation rate constant (k) between different matrices (water, salmon, cod, cod+10% oil and oil) was performed by one-way ANOVA followed by Tuckey's post-hoc test.

Results

Occurrence of antibiotic residues in wild-caught and farm-raised seafood

We first explored antibiotic detectability in the overall cohort at levels of above the LOQ, between LOQ and LOD or below the LOD (n=125 samples). Twenty-nine out of 30 target antibiotics were detected in fish and shrimp samples (**Figure 2**). Twenty-two antibiotics were detected at levels above the LOQ, 3 antibiotics (PEN-G, TAP, CTC) at levels above the LOD but below the LOQ, and 4 antibiotics (NOR, AZ, TILM and TYLSOIN) were at levels below the LOD. DOX was the only antibiotic not detected in any of the samples. Amphenicols (FFA), macrolides (VIRG-M1, ROX) and quinolones (FLU, ENO) were the most frequently detected antibiotics in seafood with detection frequencies of 70% (FFA), 55% (VIRG-M1), 33% (ROX), 38% (FLU) and 36% (ENO) at all detection levels.

We then explored contamination in wild versus farmed seafood. As shown in **Figure 3**, significant differences in antibiotic detection frequencies were observed for 8 antibiotics between wild-caught and farm-raised seafood. TAP ($p < 0.05$), NOR ($p < 0.05$) and VIRG-M1 ($p < 0.0001$) were detected more frequently in wild-caught (15-77%) than farm-raised samples (5-16%). FF ($p < 0.05$), ENRO ($p < 0.0001$), SDZ ($p < 0.05$), LIN ($p < 0.01$) and ERYTH ($p < 0.0001$) were detected less frequently in wild (0-21%) versus farm-raised samples (5-35%).

Antibiotics were detected more frequently in imported than domestic seafood. We found significantly higher detection frequencies for 8 antibiotics in imported (7-37%) than domestic (0-20%) seafood including AMOX ($p < 0.01$), NOR ($p < 0.05$), ENRO ($p < 0.001$), CTC ($p < 0.05$), LIN ($p < 0.0001$), TRIM ($p < 0.01$), ERYTH ($p < 0.001$) and TYLOSIN ($p < 0.01$). Two antibiotics, i.e. PEN-V ($p < 0.05$) and ENO ($p < 0.01$), showed significantly higher detection frequency in domestic than imported seafood (**Figure 4**).

Comparing the antibiotic detection frequencies between domestic and imported origins within farmed-raised seafood, 14 antibiotics were detected at significantly higher frequencies in imported than domestic seafood including B-lactams (AMP, PEN-V, AMOX), amphenicols (FFA), quinolones (NOR, ENRO), sulfonamides (SMX), lincosamides (LIN), dihydrofolate reductase inhibitors (TRIM) and macrolides (AZ, ERYTH, ROX, VIRG-M1, VIRG-S1). On the other hand, 6 antibiotics were detected more frequently in farmed-domestic seafood compared to farmed-imported group and these included TAP, ENO, CTC, SDM, ORM and TILM (**Figure S2**). In wild-caught seafood, 9 antibiotics showed higher detection frequencies in wild-imported than wild-domestic group including B-lactams (PEN-G, AMOX), quinolones (NOR), tetracyclines (CTC), sulfonamides (SDZ), lincosamides (LIN), dihydrofolate reductase inhibitors (TRIM) and

macrolides (TILM, ERYTH and TYLOSIN). Significantly higher detection frequencies in wild-domestic than wild-imported group was only observed for PEN-V and FLU (**Figure S2**).

Overall, the detection frequency of antibiotics was least for farmed-domestic seafood (12 out of 30 antibiotics detected) compared to 28, 24 and 27 antibiotics detected in farmed-imported, wild-domestic and wild-imported seafood, respectively (**Figure S2**).

Concentration of antibiotic residues in wild-caught and farm-raised seafood

Antibiotic concentrations ranged from levels < LOD to 173.15 ng/g in seafood samples (**Figure 5**). Comparing wild- caught vs. farm-raised seafood, significantly higher concentrations were observed in wild-caught than farm-raised seafood for AMP ($p < 0.01$), CTC ($p < 0.05$), SMX ($p < 0.05$), LIN ($p < 0.01$), AZ ($p < 0.01$), and VIRG-S1 ($p < 0.01$). Only OTC was found at significantly higher concentrations in farm-raised seafood than wild-caught seafood ($p < 0.01$) (**Figure 5**).

Within wild-caught seafood, FFA ($p < 0.0001$) and ENRO ($p < 0.05$) were significantly higher in wild-domestic than wild-imported seafood. Within farm-raised seafood, no significant difference was observed between antibiotic concentrations of farmed-domestic versus farmed-imported seafood ($p > 0.05$) (**Figure S3**).

Principal component analysis (PCA)

A PCA plot was used to observe whether there was any meaningful separation of antibiotics among the different production methods (**Figure 6**). Scree plot showed that the first four principal components (PC1, PC2, PC3 and PC4) were the main components contributing to the PCA variance (36.2% of total variance) (**Figure S4**). PC1, PC2, PC3 and PC4 explained 9.6%, 7.8%, 7.1% and 6.2% of the total variance (**Table S4**). The PCA showed separation of farmed-domestic group from other groups likely due to OTC (**Figure 6**). The plot also showed groups

clustering of OTC, ERYTH, ORM and ENO (Cluster I), SDM and VIRG-M1 (Cluster II), NOR and AMP (Cluster III), CAP, TRIM and AZ (Cluster IV), and FLU and ROX (Cluster V) (**Figure 6**), suggesting that these antibiotics might originate from similar sources or have similar bioaccumulation potential.

Effect of thermal treatment on antibiotics degradation

The first order degradation rate constant (k; min^{-1}) for each antibiotic in water, fish oil, cod, cod spiked with fish oil and salmon is shown in **Figure 7**. The first order degradation rate constant (k) values were obtained from plotting the natural logarithm of antibiotic concentrations as a function of time and determined as the slope of the linear regression line (**Figure S5**). Negative values for k indicate thermal degradation of antibiotics. Slope values close to zero (i.e. a horizontal line) indicate minimal degradation.

In fish matrix, significantly negative k values were observed for B-lactams (AMP, PEN-G and PEN-V), tetracyclines (OTC, CTC, TC and DOX), amphenicols (FFA, CAP, TAP, FF), macrolides (VIRG-S1, VIRG-M1, TYLOSIN, ERYTH), sulfonamides (SDM and SSZ) and lincosamides (LIN). Particularly, more negative k values ($k \leq -0.019$; half-life ≤ 36 min), indicating higher degradation, were observed for B-lactams (AMP, PEN-G, PEN-V), tetracyclines (OTC, CTC and DOX), and macrolides (VIRG-M1, VIRG-S1 and TYLOSIN) than other antibiotics (**Figure 7**).

As shown in **Figure 7**, quinolones, macrolides (TILM and ROX), sulfonamides (SMX and SDZ) and dihydrofolate reductase inhibitors (TRIM and ORM) remained unchanged in all matrices during the 2 hours heating period at 90 ˚C.

Amphenicols (FFA) ($p < 0.01$), tetracyclines (DOX) ($p < 0.05$) and macrolides (TYLOSIN ($p < 0.01$) and VIRG-S1 ($p < 0.0001$)) degraded significantly less in high-fat fish (salmon) than

low-fat fish (cod). However, for these antibiotics, no significant differences in the k values were observed between cod and cod spiked with fish oil ($p > 0.05$) (**Figure 7**).

Fish matrix significantly slowed down the degradation of tetracyclines (TC and DOX), amphenicols (FFA and CAP) compared to control matrices (water and fish oil) (**Figure 7**). On the other hand, TYLOSIN ($p < 0.05 – p < 0.0001$), SDM ($p < 0.05$), VIRG-S1 ($p < 0.01 – p < 0.0001$) and ERYTH ($p < 0.02 – p < 0.001$) degraded more significantly in fish matrix than other matrices. Also, B-lactams (PEN-G and PEN-V) degraded more in salmon and fish oil than water ($p < 0.05 – p < 0.01$) (**Figure 7**).

Figure 8 plots the correlation between antibiotics polarity (i.e. Log D) and degradation rate constant (k) in fish (salmon, cod and cod supplemented with fish oil) and control matrices (water and oil). Polar antibiotics degraded more than non-polar antibiotics in fish and control matrices. A positive correlation, significantly different than zero, was observed between antibiotics k and Log distribution (Log D) values, irrespective of the matrix. Additionally, the slope was significantly higher in fish oil than other matrices (**Figure 8**).

AMP, FFA, ENO, ENRO, NOR, ENRO, FLU, LIN, ORM, TRIM, TC, OTC, DOX, SDM, SDZ, SMX, ROX, TYLOSIN and VIRG-M1 were present in the 5 contaminated samples selected for heat treatment for 20 minutes (**Table 2**). There was a 60% reduction in OTC concentration in farm-raised rainbow trout following heat treatment. Other antibiotics did not change during heating (**Table 2**), consistent with their predicted stability (**Figure 7**).

Human health risk assessment

Health risks associated with chronic antibiotic exposure through fish consumption was assessed for adults by calculating EDI for each antibiotic using the highest measured concentration in the samples. A thermal factor was applied to account for the effect of heat on antibiotic

concentrations. The EDIs were compared to published ADI values. Among all antibiotics, FFA followed by OTC showed the highest EDIs in raw seafood, 28.0 and 2.2 ng/Kg body weight per day, respectively. EDI values decreased or remained unchanged by applying the thermal factor. EDI values in both raw and cooked seafood were several order of magnitude lower than the ADI (**Table 3**).

Discussion

This study provides new evidence of widespread contamination of antibiotics in both farmed and wild-type seafood, at concentrations below the published ADI and MRL values, and shows that antibiotics are stable to thermal degradation. Consistent with prior studies, antibiotics were detected more frequently in farm-raised than wild-caught seafood[155] (**Figure 3**), and in imported samples compared to domestic samples (**Figure 4, Figure S2**). Surprisingly, however, measured concentrations were higher for several antibiotics in wild-caught than farm-raised seafood (**Figure 5**).

Prior studies have shown the occurrence (frequency of detection) of antibiotics in both wild-caught and farm-raised seafood samples [75, 78-80], although a direct comparison of the frequency of occurrence and concentrations has not been well documented. Here, we demonstrate that occurrence is higher in farmed compared to wild seafood, whereas concentrations are higher in wild fish compared to farmed fish. The routine use of antibiotics in fish farms for therapeutic and prophylactic purposes[77] explains the high occurrence of antibiotic residues in farmed compared to wild seafood. The high concentrations of antibiotics in wild-caught seafood is indicative of widespread antibiotic contamination in marine environments, and reflect potential bioaccumulation of antibiotics in wild fish. Contamination likely originates from a number of sources including wastewater effluents and agricultural and aquaculture runoffs[157, 222]. Field

studies have shown the occurrence of similar antibiotics in water and aquatic organisms within the same sampling area[83-86]. Several studies have also shown that antibiotics bioaccumulate in aquatic animals from their surrounding medium[223-224]. Also, unlike farmed seafood where exposure to antibiotics is relatively acute and timed, wild fish are likely chronically exposed to antibiotics present in the environment (water and sediment), potentially leading to greater accumulation over time.

Several antibiotics that are not typically used in fish farming were detected in both wild and farmed samples. These include ROX, FLU and ENO, which were previously shown to be detected at high frequencies in culture ponds [157], fish feed[157], farm-raised aquatic animals [79, 112, 157, 225-226], and wild-caught fish[78, 88, 227]. The high abundance of these antibiotics in both wild and farm-raised samples suggests that sources other than direct feeding (e.g. contaminated water or fertilizers) may explain their frequent occurrence [228-229]. Animal manure is often used as a fertilizer in fish farms to enhance fish growth and performance[228, 230], but studies have shown that it contains antibiotic residues[231-232]. Pond waters might also contain antibiotic residues that can contaminate the aquatic animals. It is also possible that these antibiotics were illegally used in aquaculture (or agriculture) and their use was not reported.

Antibiotics detected at higher frequencies (TAP, NOR and VIRG-M1) (**Figure 3**) or higher mean concentrations (AMP, CTC, SMX, LIN, AZ, and VIRG-S1) (**Figure 5**) in wild-caught relative to farm-raised samples are antibiotics commonly used in human and/or veterinary medicine[222] and industry[233]. VIRG is an example of an antibiotic used in corn-based ethanol production in order to prevent bacterial growth during fermentation, and it often remains in corn fermentation byproducts that are used as animal feed [233]. Many of these antibiotics have often been

found in wastewater and animal waste [90-91, 93, 95, 231], resulting in their prolonged presence and accumulation in natural waters.

Environmental contamination is further exacerbated by the fact that many antibiotics are not efficiently removed by wastewater treatment. For instance, SMX has been detected in the effluent of wastewater treatment plants with only 53% removal efficiency[91], and it has been found in water and aquatic animals collected from sampling areas close to water treatment plants, supporting the likelihood of transfer to aquatic animals[78]. Similarly, AZ, a commonly used antibiotic in human medicine [234], as well as LIN and CTC which are used in veterinary medicine[94, 222] have been found in wastewater effluents of wastewater treatment plants [96] and cattle, swine and poultry manure[90, 231].

Antibiotics found in higher frequencies (FF, ENRO, SDZ, LIN and ERYTH) (**Figure 3**) or higher concentration (OTC) (**Figure 5**) in farm-raised than wild-caught seafood have been linked to aquaculture practices. Among these, OTC, SDZ and FF are the most used antibiotics in aquaculture, with 73% of top aquaculture producing countries reporting the use of these antibiotics in fish farming[235]. Approximately 64 and 55% of aquaculture producers use ERYTH and ENRO, respectively [235]. Our findings are also consistent with other studies reporting the detection of OTC, FF, ENRO, ERYTH and SDZ in farm-raised seafood [75, 80, 107, 157, 225, 236]. ERYTH is commonly detected as a dehydrated metabolite not measured in this study, in farm-raised seafood[157, 229] that is shown to be slightly bioactive[237]. Therefore, it is likely that the seafood analyzed in our study originally contained higher concentrations of ERYTH. In our study, OTC was also associated with farmed-domestic samples (**Figure 6**) which is in line with its authorized use in aquaculture in U.S. [238].

Thermal treatment of fish degraded B-lactams (AMP, PEN-G, PEN-V), tetracyclines (OTC, CTC and DOX), and macrolides (VIRG-M1, VIRG-S1 and TYLOSIN) (half-life ≤ 36 min) (**Figure 7**), and this is likely due to hydrolysis, epimerization, dehydration and isomerization. B-lactams and macrolides contain amide, ester and ether linkages which make them susceptible to hydrolysis [63]. The observed degradation of tetracyclines could be due to multiple transformation pathways including epimerization, dehydration and isomerization, which are accelerated by heat [239].

Fish fat content did not affect the degradation of antibiotics during thermal processing. While, amphenicols (FFA), tetracyclines (DOX) and macrolides (TYLOSIN and VIRG-S1) degraded significantly less in high-fat fish (salmon) than low-fat fish (cod), they degraded to the same extent in cod spiked with oil (mimicking high fat condition) and cod (mimicking low fat condition) (**Figure 7**), supporting the notion that fat content does not affect antibiotic degradation. This is in agreement with one study showing that pork fat content did not affect the residual TC concentration after boiling or microwave processing[240].

Fish matrix stabilized some antibiotics against thermal degradation compared to control matrices. In this regard, tetracyclines (TC and DOX) and amphenicols (FFA and CAP) degraded slower in fish than water. Antibiotics within a matrix might bind to metal ions and proteins which could protect them from thermal degradation. Notably, tetracyclines have been shown to form heat stable complexes with metal ions [241] which is why OTC (a tetracycline) was shown to degrade less in salmon, pork and cattle muscle than in water during thermal processing [144, 154, 241].

Antibiotics at levels found in raw and cooked fish do not appear to pose acute risks to human health, based on published ADI values (**Table 2**). In addition, antibiotic concentrations in fish and shrimp samples were below the maximum residue levels (MRLs) set for veterinary drugs

in food producing animals for human consumption (**Table 2**)[200], indicating that the residual amounts of antibiotics in fish samples meet the safety guidelines. However, this risk does not capture the potential for developing antibiotic drug resistance with chronic antibiotic exposure. Additional studies are needed to better estimate risks associated with chronic antibiotic exposures through seafood.

Notably, MRL or ADI values for several antibiotics frequently detected in this study, i.e. FLU, ENO and ROX have not been established (**Table 2**), thus limiting our ability to estimate health risks. In addition, 19 out of 125 samples (15%) contained CAP at levels > LOQ (n=2; 0.28 and 1.40 ng/g), < LOQ (n=5) and < LOD (n=12). CAP is prohibited from food producing animals including seafood in the U.S. and many countries due to its link to aplastic anemia and suspected carcinogenicity[38]. Our study indicates that it may have been illegally used in fish farming, consistent with other reports [226, 242, 226].

Study strengths and limitations

To our knowledge, this is the first study to comprehensively measure antibiotic contamination in large sample set of seafood samples derived from both wild-caught and farm-raised sources. Previous studies have tested small sample sizes (< 30)[75] or a small number of antibiotics (< 10)[158] and have only tested farm-raised seafood. In addition, this study covered a wide range of genetically confirmed fish types (16 categories), enabling broad assessment of true exposure to antibiotics from multiple fish species. This study also quantified losses due to thermal treatment, as most individuals consume seafood cooked.

Several limitations are worth addressing. First, the LOD and LOQ calculated from the calibration curve are likely to be lower than the method detection and method quantitation limits (MDL and MQL) where effects of the fish matrix and extractions process are applied in the

calculating the detection and quantitation limits. Therefore, it is possible that some of the antibiotic concentrations in our study are overestimated. Another limitation is that a few shrimp samples were tested for contamination. Therefore, our study is underpowered in assessing true antibiotics exposure from shrimp. Another limitation is that we did not identify the source of contamination.

Conclusion

Widespread contamination of antibiotics was seen in farmed and wild seafood of domestic and imported origins, suggesting environmental contamination. Antibiotic concentrations were highest in wild seafood, reflecting likely environmental exposure and bioaccumulation in these samples. Thermal processing degraded some antibiotics but many were stable. Future studies are urgently needed to better understand contamination sources and risks associated with chronic exposures through seafood.

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Declaration of interest statement

The authors declare that there is no conflict of interest.

Author contributions

S.E., R.S.H., N.N., G.S. and A.Y.T. designed the experiments. S.E. performed the experiments, analyzed the data and wrote the original draft. Z.Z. and N.L. assisted with the experiments. R.S.H. provided the fish samples, A.Y.T. reviewed and edited the manuscript.

Supplementary information

The supporting information include the information of seafood samples, graphs of antibiotic detection frequencies for domestic and imported samples, table of antibiotic concentrations, time-temperature profile of different matrices during thermal processing.

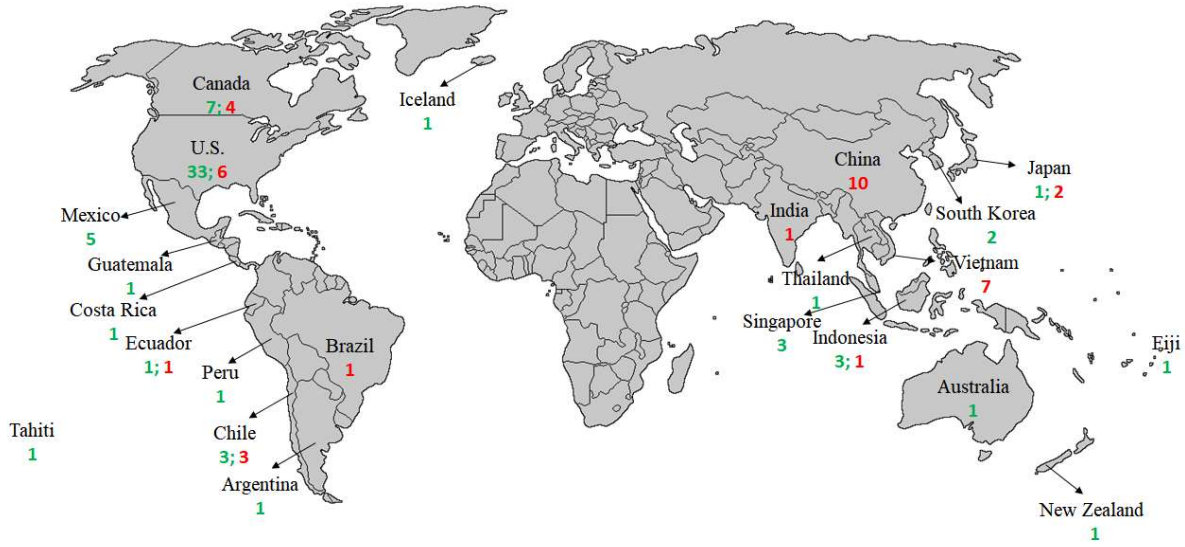


Figure 1. Production origin map of wild-caught (n= 68; green color) and farm-raised (n=36; red color) fish and shrimp samples with known production origin.

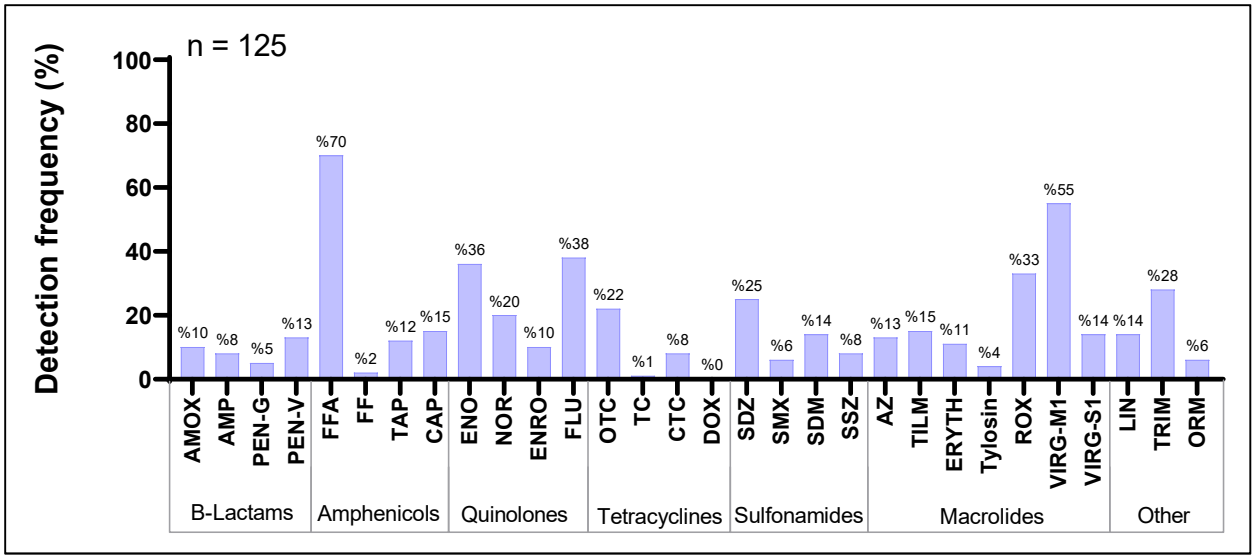
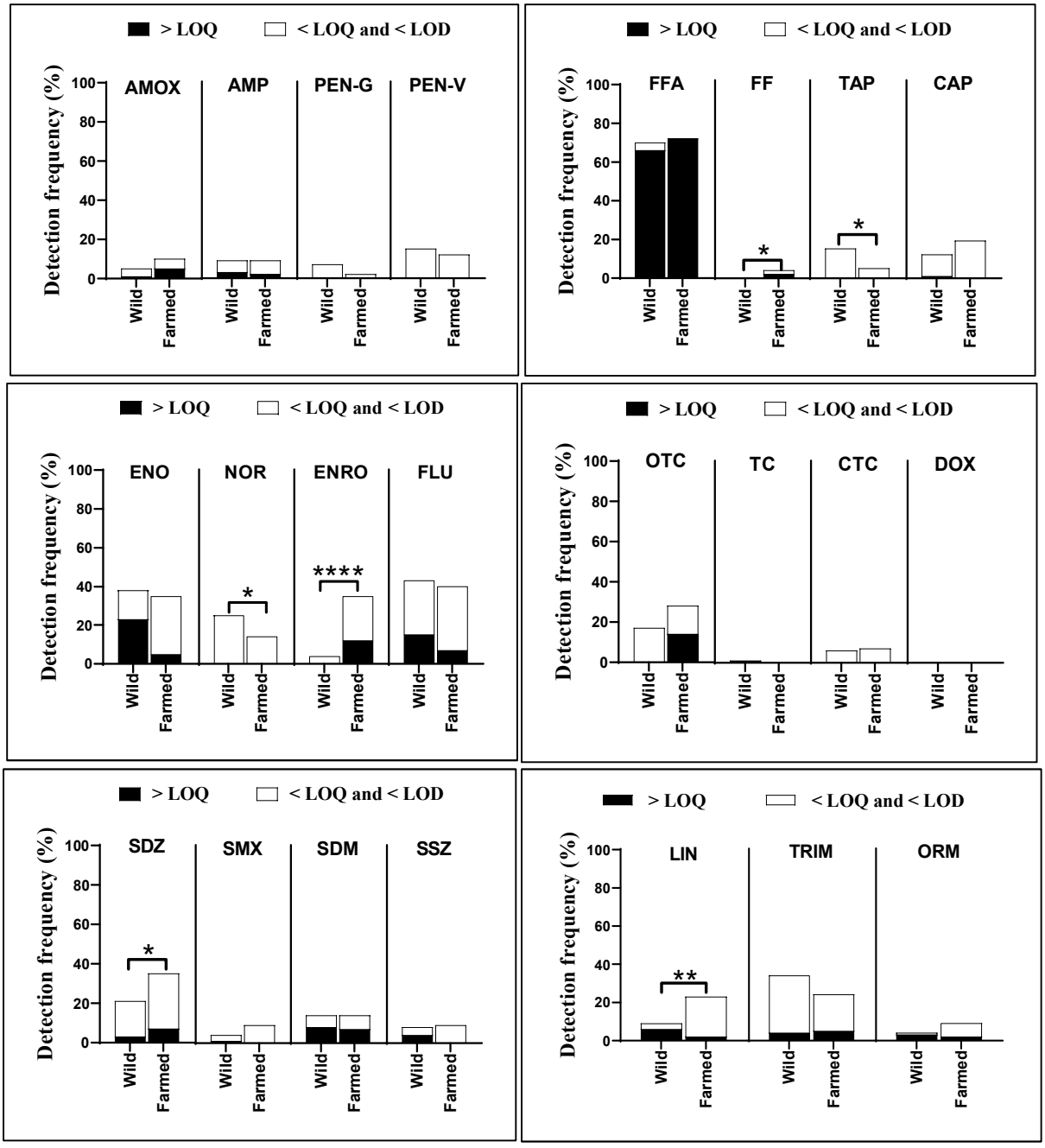


Figure 2. Frequency of detection of antibiotic residues in fish and shrimp samples presented as the sum of all detection levels (above LOQ, between LOQ and LOD and below LOD) in all samples (n=125).


> LOQ
< LOQ and < LOD
Detection frequency (%)
AMOX
AMP
PEN-G
PEN-V
FFA
FF
TAP
CAP
ENO
NOR
ENRO
FLU
OTC
TC
CTC
DOX
SDZ
SMX
SDM
SSZ
LIN
TRIM
ORM
Wild
Farmed
*
**
****

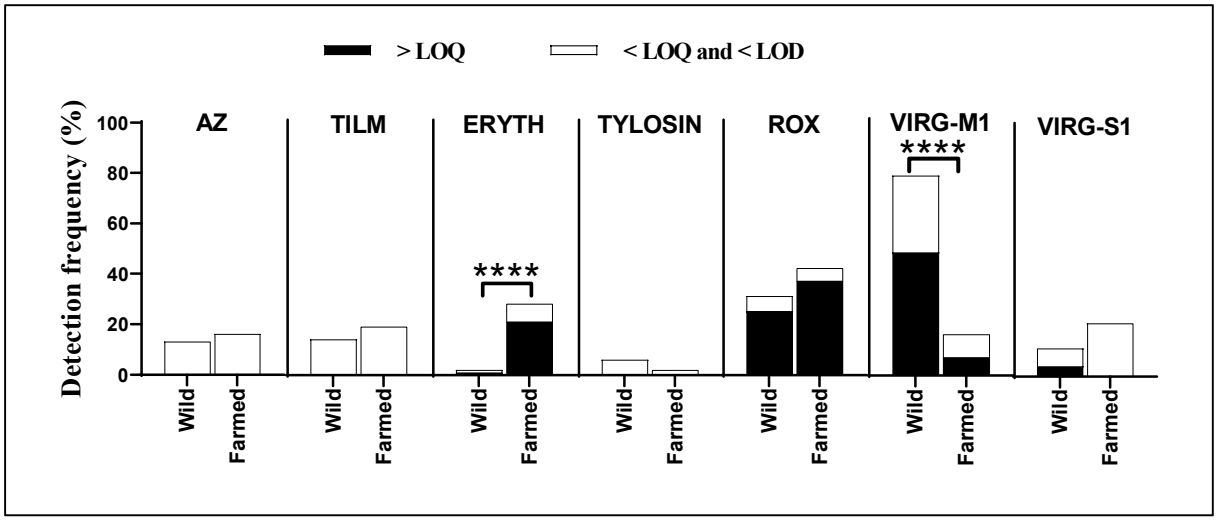
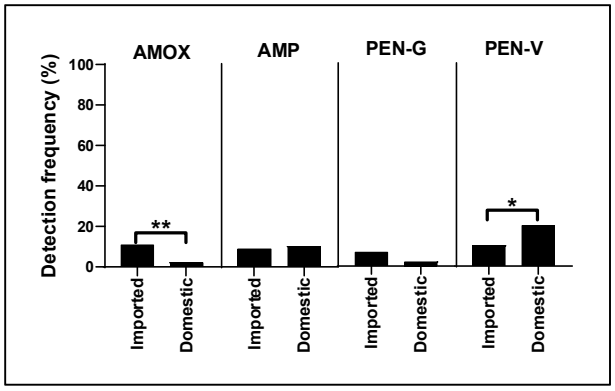
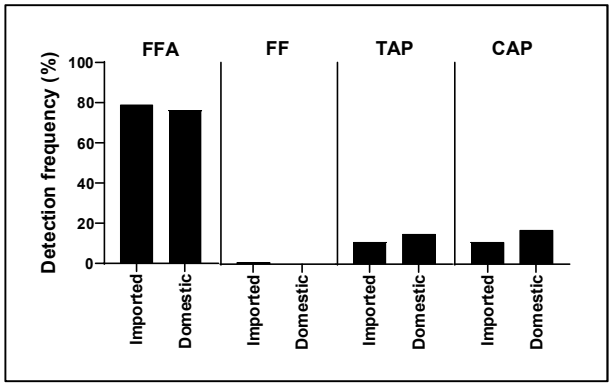
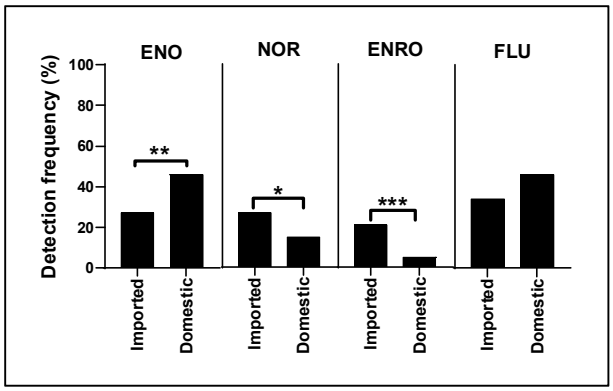
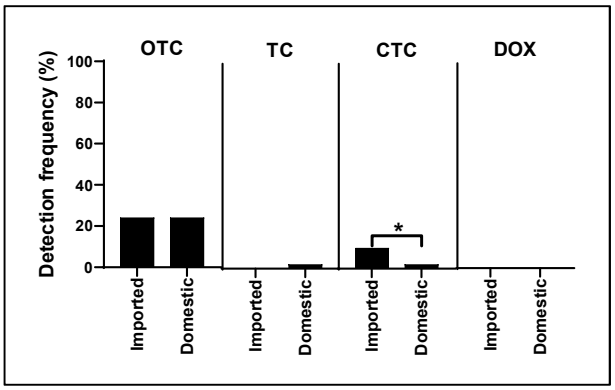


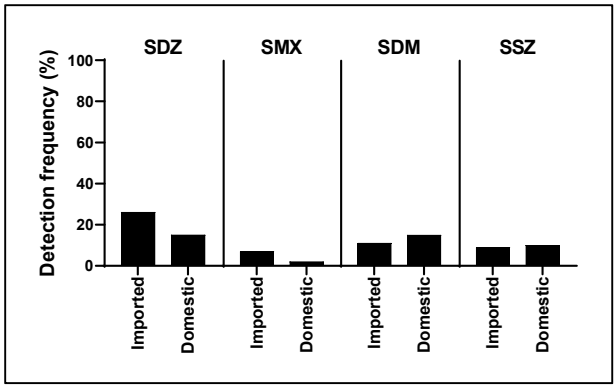
Figure 3. Frequency of detection of antibiotic residues in fish and shrimp samples of wild-caught (n=71) and farm-raised (n=43) production method presented at different levels of above LOQ and between LOQ and LOD + below LOD. Chi-square test was used to assess whether there is difference between wild-caught and farm-raised seafood in terms of antibiotics detection. "****" indicates $p < 0.0001$, "***" indicates $p < 0.001$, "**" indicates $p < 0.01$ and "*" indicates $p < 0.05$.

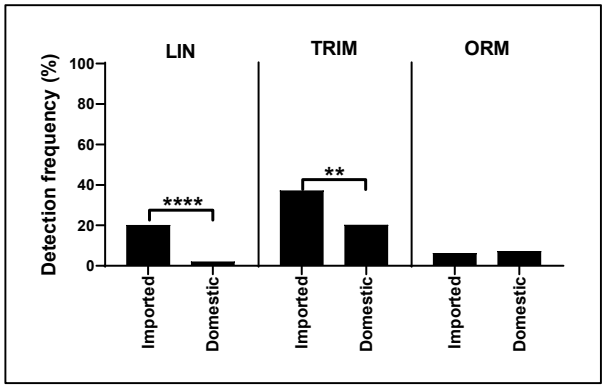

AMOX
AMP
PEN-G
PEN-V
Detection frequency (%)
100
80
60
40
20
0
**
*
Imported
Domestic


FFA
FF
TAP
CAP
Detection frequency (%)
100
80
60
40
20
0
Imported
Domestic


ENO
NOR
ENRO
FLU
Detection frequency (%)
100
80
60
40
20
0
**
*
***
Imported
Domestic


OTC
TC
CTC
DOX
Detection frequency (%)
100
80
60
40
20
0
*
Imported
Domestic


SDZ
SMX
SDM
SSZ
Detection frequency (%)
100
80
60
40
20
0
Imported
Domestic


LIN
TRIM
ORM
Detection frequency (%)
100
80
60
40
20
0
****
**
Imported
Domestic

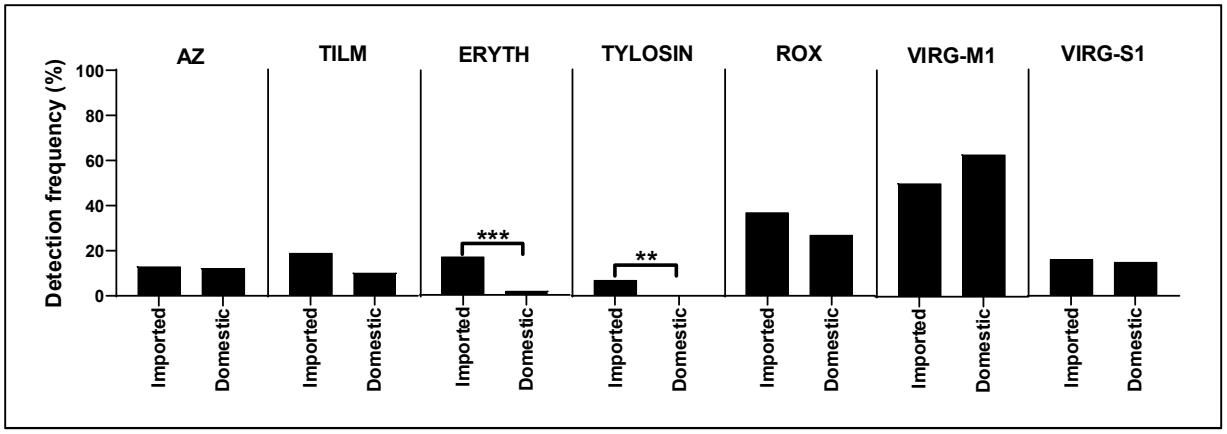

AZ
TILM
ERYTH
TYLOSIN
ROX
VIRG-M1
VIRG-S1
Detection frequency (%)
100
80
60
40
20
0
***
**
Imported
Domestic

Figure 4. Frequency of detection of antibiotic residues in fish and shrimp samples of domestic (n=41) and imported (n=70) presented at all levels of above LOQ, between LOQ and LOD and below LOD. Chi-square test was used to assess whether there is difference between domestic and imported seafood in terms of antibiotics detection frequency. "****" indicates $p < 0.0001$, "***" indicates $p < 0.001$, "**" indicates $p < 0.01$ and "*" indicates $p < 0.05$.

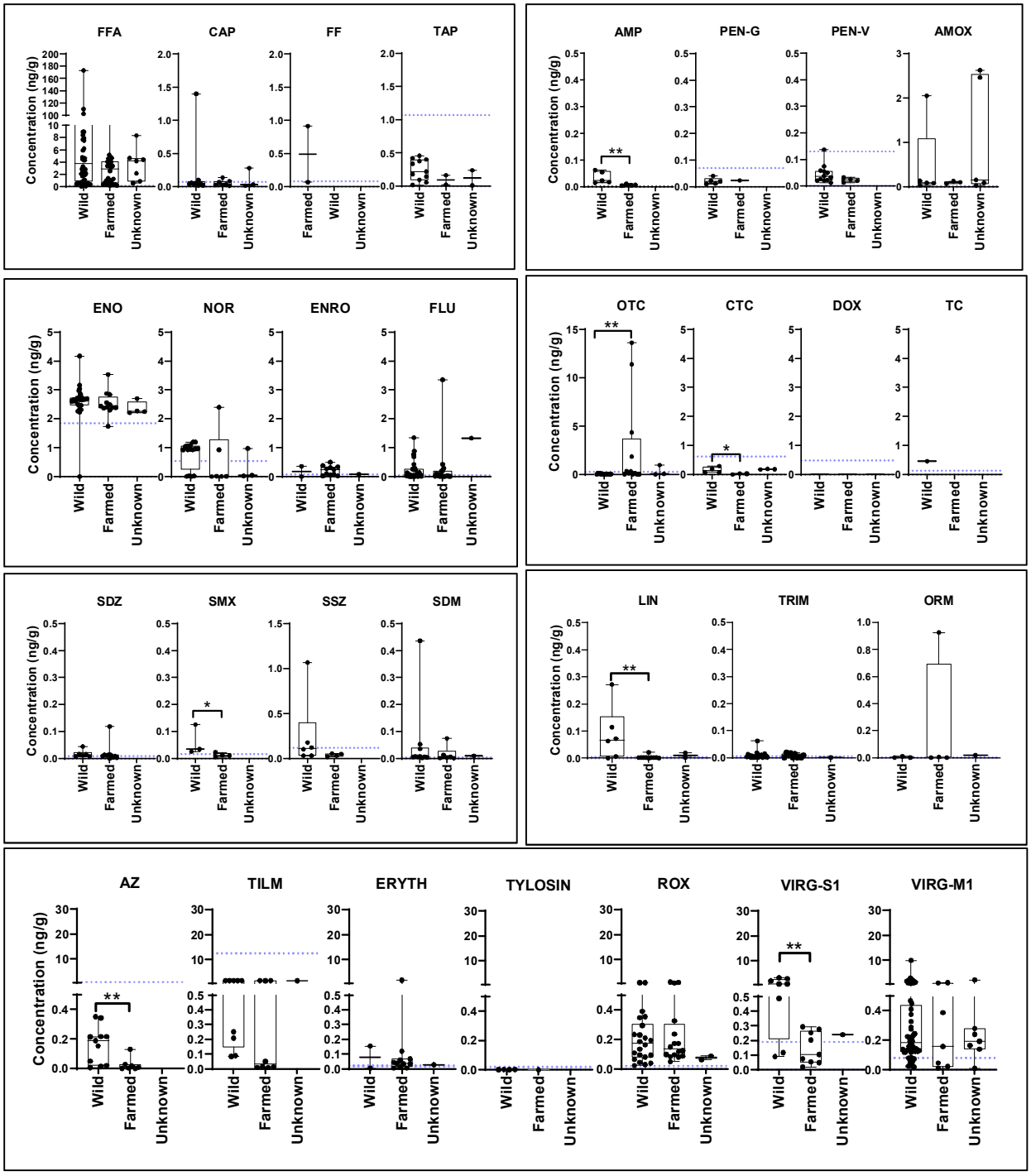


Figure 5. Boxplots of concentrations (ng/g) of antibiotic residues in fish and shrimp samples. Concentrations at levels above LOQ, between LOQ and LOD, and below LOD were included. The dotted line shows the minimum LOQ calculated over 3 runs. Unpaired t-test was performed on log transformed concentrations to test for significant difference between wild-caught and

farm-raised groups. “****” indicates $p < 0.0001$, “***” indicates $p < 0.001$, “**” indicates $p < 0.01$ and “*” indicates $p < 0.05$.

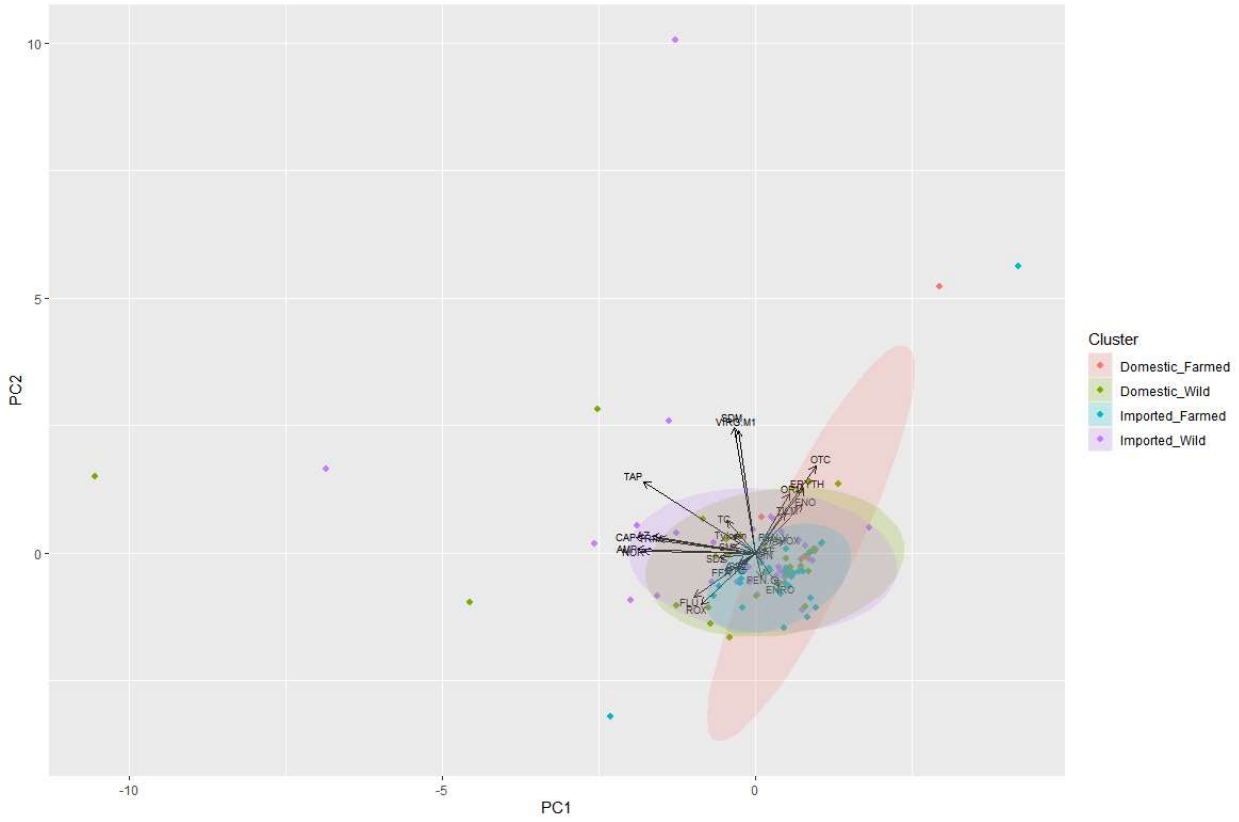
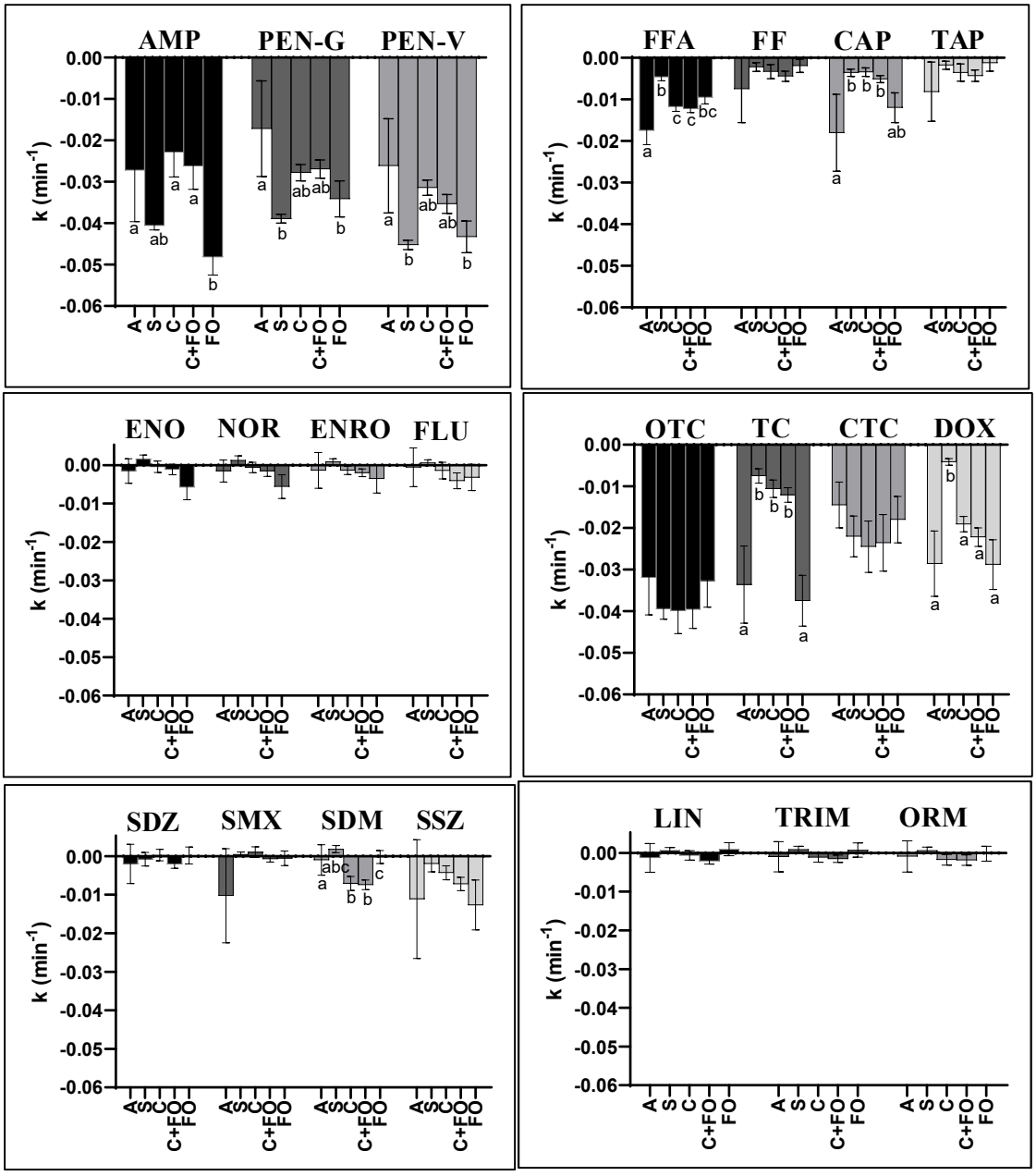


Figure 6. Principal component analysis (PCA) biplot of antibiotics detected in fish and shrimp samples of different production method (wild and farmed) and production origin. Antibiotic concentrations at levels above LOQ, between LOQ and LOD and below LOD were included and concentrations were standardized. Vectors indicate the direction and strength of each variable (antibiotic) to the overall distribution. Positive correlated values point to the same side. Negative correlated values point to opposite sides of the graph.


AMP
PEN-G
PEN-V
FFA
FF
CAP
TAP
ENO
NOR
ENRO
FLU
OTC
TC
CTC
DOX
SDZ
SMX
SDM
SSZ
LIN
TRIM
ORM
k (min-1)
0.00
-0.01
-0.02
-0.03
-0.04
-0.05
-0.06
A
S
C
C+FO
FO

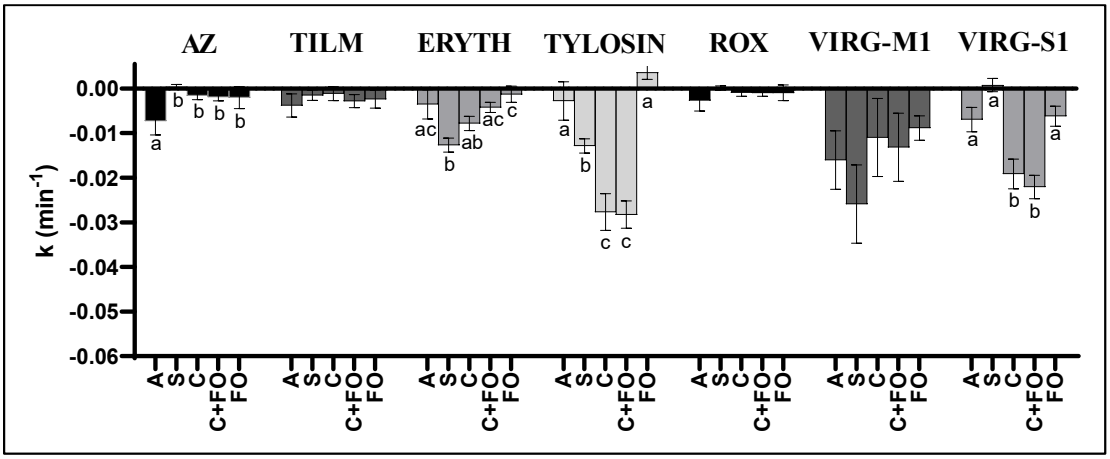
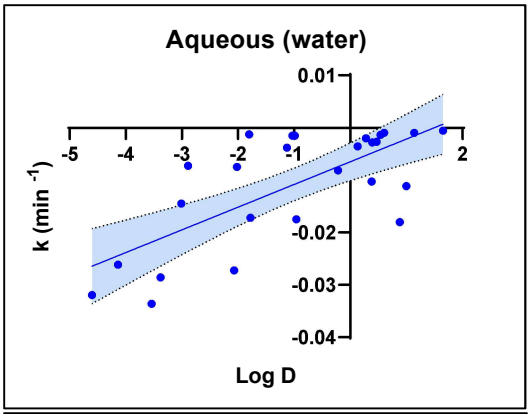
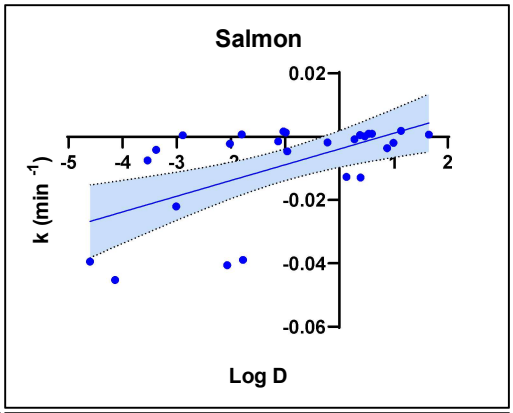
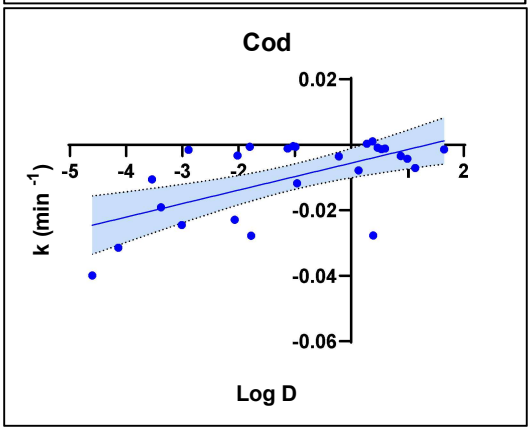
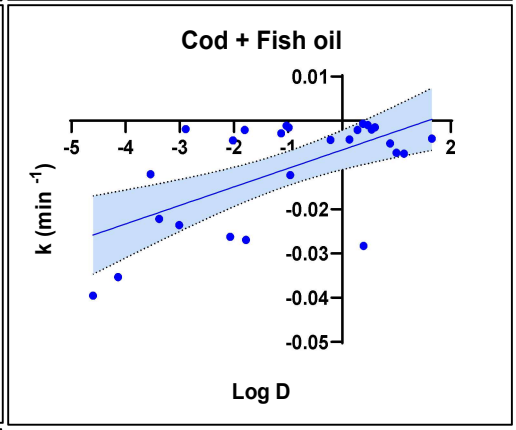


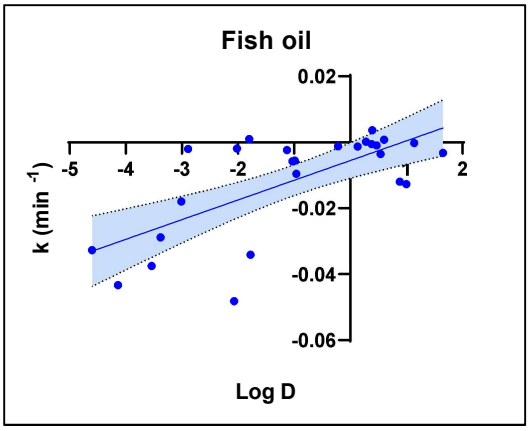
Figure 7. Effect of thermal processing on degradation of antibiotics in fish (cod and salmon), fish oil and water. Antibiotics were spiked into cod (C), salmon (S), cod + 10% fish (salmon) oil (C+FO), fish (salmon) oil (FO) and water (A) at 20 ng/g or 20 ng/mL and were heated at 90 ˚C for 15, 30, 60 and 120 min (n=3 for each matrix and each timepoint). Antibiotic concentrations were transformed by natural log transformation and were fitted into simple linear regression and first order degradation rate constant (k; min^{-1}) were calculated. Negative k indicates degradation of antibiotics during thermal treatment. Different letters indicate statistically significant difference.











	Slope	R^2	P-Value
Aqueous (Water)	0.0043	0.509	<0.0001
Salmon	0.0050	0.346	0.0016
Cod	0.0041	0.378	0.0008
Cod + Fish oil	0.0042	0.388	0.0007
Fish oil	0.0060	0.469	0.0001

Figure 8. The correlation of antibiotics polarity, represented as the log of distribution coefficient (log D) and degradation rate constant (k, min -1) in different matrices of aqueous (water), salmon, cod, cod + fish oil and fish oil (n= 26 antibiotics).

Table 1. Precursor ion (m/z), quantifier and qualifier product ions (ma/z), fragmentor voltage (V), CE (V) retention time (min) and retention time window (min) for antibiotic standards.

Antibiotics		Precursor Ion	Fragmentor voltage	MRM 1 (CE)	MRM 2 (CE)	Retention time	Retention time window	Polarity
Full name	Abbreviation							
Florfenicol amine	FFA	248	75	230.1 (10)	130.1 (20)	1.18	1	Positive
Florfenicol amine-D3	FFA-D3	251	75	233 (10)	132.1 (20)	1	1	Positive
Chloramphenicol	CAP	321	115	152 (10)	193.9 (10)	10.1	1.2	Negative
Florfenicol	FF	355.9	125	185.1 (10)	118.7 (30)	7.96	2.1	Negative
Thiamphenicol	TAP	353.9	125	184.9 (10)	290.1 (10)	4.44	1	Negative
Chloramphenicol-D5	CAP-D5	326.1	90	156 (10)	261 (10)	10.1	1.2	Negative
Oxytetracycline	OTC	461.2	90	426 (15)	443 (10)	4.45	1.2	Positive
Tetracycline	TC	445.1	100	410.1 (15)	427.1 (10)	5.33	2	Positive
Chlortetracycine	CTC	478.7	50	443.9 (20)	462 (15)	9.35	1.6	Positive
Doxycycline	DOX	445.3	110	428.1 (15)	410.2 (20)	10.37	1.6	Positive
Demeclocycine	DEM	465.2	100	447.9 (10)	430 (10)	7.07	2	Positive
Amoxicilin	AMOX	365.9	90	114 (20)	207.9(10)	1.46	1	Positive
Ampicillin	AMP	350.1	125	106.1 (20)	160.1 (10)	3.78	1.2	Positive
Ampicillin-d5	AMP-D5	355	75	111 (20)	160 (10)	2.72	1.6	Positive
Penicillin-V	PEN-V	351.2	90	160 (5)	192.2 (5)	12.52	1	Positive
Penicillin-G	PEN G	335	110	176 (10)	160.1 (10)	11.9	1	Positive
Penicillin-V-d5	PEN-V-D5	356	50	114 (10)	160 (5)	12.49	1	Positive
Trimethoprim	TRIM	291.1	130	230.1 (20)	123 (20)	3.9	1	Positive
Trimethoprim-D3	TRIM-D3	294.2	130	230.1 (25)	123.1 (25)	3.84	1.3	Positive
Ormetoprim	ORM	275	125	259.2 (20)	123.1 (20)	4.78	1.8	Positive
Lincomycin	LIN	407.1	130	126.1 (30)	359.1 (15)	2.86	1	Positive
Lincomycin-D3	LIN-D3	410.2	90	129.1 (30)	362.1 (15)	2.84	1.2	Positive
Enoxacin	ENO	321.1	100	303.1 (20)	277.2 (10)	4	1.8	Positive
Enrofloxacin	ENRO	360.1	125	316.3 (15)	342 (20)	5.9	1	Positive
Norfloxacin	NOR	320.1	130	302.1 (15)	276.1 (15)	4.44	1.2	Positive
Enrofloxacin-D5	ENRO-D5	365.1	125	321.1 (15)	347.1 (20)	5.94	2	Positive
Flumequine	FLU	262.1	90	244.1 (20)	202 (30)	12.26	1	Positive
Sulfamethoxazole	SMX	254.1	90	156.1 (10)	108.1 (20)	8	1.2	Positive
Sulfasalazine	SSZ	399	130	381 (15)	317 (20)	12.39	1	Positive
Sulfadimethoxine	SDM	311	110	156.1 (15)	245.1 (15)	10.86	1.8	Positive
Sulfadiazine	SDZ	251.1	125	156.1 (10)	108.1(20)	2.5	1	Positive
Sulfamethoxazole-D4	SMX-D4	258	90	112 (20)	160 (10)	7.96	1.8	Positive
Sulfamethazine-d4	SMZ-D4	283	125	186 (15)	-	4.3	1.2	Positive
Tylosin A	TYLOSIN	916.3	125	174.1 (40)	771.8 (30)	12.03	1.2	Positive
Tilmicosin	TILM	869.4	90	696 (25)	174 (30)	11.1	1.2	Positive
Azithromycin	AZ	375.1	75	591.2 (10)	83 (20)	10.1	1	Positive
Azithromycin-D3	AZ-D3	376.7	125	594.4 (10)	82.8 (20)	10.1	1.2	Positive
Roxithromycin	ROX	419.3	125	158 (15)	83 (20)	12.63	1	Positive
Roxithromycin-D7	ROX-D7	422.7	75	158 (20)	83 (20)	12.65	1	Positive
Virginiamycin-M1	VIRG-M1	526.1	130	508.3 (10)	355.1 (15)	12.78	1.2	Positive
Virginiamycin-S1	VIRG-S1	824.2	130	205.1 (25)	177.2 (30)	13.21	1	Positive
Erythromycin	ERYTH	734.3	125	576.3 (15)	158.1 (30)	11.72	1	Positive
Erythromycin-d6	ERYTH-D6	740.3	130	582.2 (15)	164.2 (30)	11.72	1.2	Positive

Table 2. Concentrations (ng/g) of antibiotic residues in raw and cooked fish samples. Cooked samples were intact fish pieces heated in TDT3 cells immersed in 90 ºC water batch for 20 min.

Antibiotic	Farmed Rainbow Trout		Farmed Tilapia		Farmed Tilapia		Wild Pacific/ Atlantic Halibut		Farmed Madai	
	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked	Raw	Cooked
AMP	ND	ND	ND	ND	ND	0.003	ND	ND	0.004	ND
PEN-G	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
PEN-V	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TAP	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
CAP	ND	ND	ND	ND	ND	0.003	ND	ND	ND	ND
FFA	1.15	5.05	0.66	2.2	3.8	ND	16.86	17.71	0.36	ND
FF	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ENO	ND	ND	ND	2.44	2.34	2.51	ND	2.57	ND	2.44
ENRO	ND	0.00001	0.33	0.332	0.029	0.343	ND	0.13	0.006	0.085
NOR	3.08	3.07	ND	3.26	3.11	3.46	3.05	3.45	3.18	3.25
FLU	ND	ND	ND	0.006	0.003	0.459	ND	0.214	0.029	0.066
LIN	0.001	<MB	0.0009	<MB	0.005	0.002	ND	0.003	=MB	<MB
ORM	0.462	0.599	0.004	0.011	0.006	0.011	0.003	0.016	<MB	0.006
TRIM	ND	0.004	0.004	0.009	0.006	0.008	ND	0.017	0.002	0.006
TC	ND	ND	ND	0.063	ND	0.052	0.017	0.065	ND	0.046
OTC	4.53	1.7	ND	0.047	ND	ND	ND	0.035	0.017	0.029
CTC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
DOX	ND	ND	ND	ND	ND	0.167	0.052	0.146	0.069	0.094
SDM	0.052	0.067	ND	0.008	0.006	0.016	0.002	0.012	0.003	0.004
SDZ	ND	ND	0.037	0.021	0.031	0.018	0.01	0.011	ND	ND
SMX	0.005	ND	ND	0.023	0.004	0.022	0.006	0.022	ND	0.007
SSZ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
AZ	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
TILM	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ROX	0.416	0.616	0.256	<MB	0.017	0.477	0.039	0.063	0.312	0.491
TYLOSIN	ND	0.003	ND	ND	0.005	0.004	0.001	0.002	0.002	0.004
ERYTH	<MB	<MB	<MB	<MB	<MB	<MB	<MB	<MB	<MB	<MB
VIRG-M1	ND	ND	ND	ND	ND	ND	0.001	0.084	ND	ND
VIRG-S1	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

ND: Not detected; MB: Method blank

Table 3. Estimated daily intake (EDI) of antibiotics from raw and cooked fish evaluated for adults relative to acceptable daily intake (ADI). For cooked fish, EDI was calculated by applying cooking factor (% change during thermal processing for 30 min) obtained from heat treatment of spiked cod and salmon (Experiment B).

Antibiotics class	Antibiotics	EDI-raw (µg/Kg/Day)	EDI-cooked-Cod (µg/Kg/Day)	EDI-cooked-Salmon (µg/Kg/Day)	ADI [a] (µg/kg body weight)	MRL
B-Lactams	AMP	0.00001	0.0000005	0.0000031	0-3	10 [d]
	PEN-G	0.00001	0.0000030	0.0000023	0-30 [b]	50 [e]
	PEN-V	0.00002	0.0000085	0.0000059	NA	50 [e]
	AMOX*	0.00040	NA	NA	0-2	10 [f]
Lincosamides	LIN	0.00004	0.0000358	0.0000441	0-30	100 [g]
Dihydrofolate reductase inhibitors	TRIM	0.00001	0.0000073	0.0000103	0-4.2	NA
	ORM	0.00015	0.0000932	0.0001506	NA	100 [h]
Quinolones	ENO	0.00068	0.0005458	0.0007733	NA	NA
	NOR	0.00039	0.0003063	0.0004388	NA	NA
	ENRO	0.00006	0.0000461	0.0000648	0-6.2	100 [i]
	FLU	0.00054	0.0002772	0.0006529	0-30	NA
Tetracyclines	OTC	0.00220	0.0001550	0.0005882	0-30	2000 [j]
	TC	0.00007	0.0000302	0.0000506	0-30	
	CTC	0.00005	0.0000022	0.0000034	0-30	
	DOX	0.00000	0.0000000	0.0000000	0-3	
Amphenicols	FF	0.00015	0.0001283	0.0001429	0-3 [c]	1000 [k]
	TAP	0.00007	0.0000705	0.0000802	0-5	NA
	FFA	0.02803	0.0167402	0.0265363	NA	NA
	CAP	0.00023	0.0001868	0.0002194	NA	Banned
Sulfonamides	SDZ	0.00002	0.0000196	0.0000203	NA	NA
	SMX	0.00002	0.0000167	0.0000210	NA	NA
	SDM	0.00007	0.0000332	0.0000890	NA	100 [l]
	SSZ	0.00017	0.0001074	0.0001831	NA	NA
Macrolides	AZ	0.00006	0.0000424	0.0000597	NA	NA
	TILM	0.00129	0.0007181	0.0008009	0-40	100 [m]
	ERYTH	0.00028	0.0001895	0.0002015	0-0.7	100 [f]
	TYLOSIN	0.00000	0.0000001	0.0000002	0-30	200 [m]
	ROX	0.00016	0.0001300	0.0001701	NA	NA
	VIRG-M1	0.00161	0.0000272	0.0000179	0-250	Exempt [n]
	VIRG-S1	0.00051	0.0001423	0.0009313	NA	

[a] Acceptable daily intake; [b] µg per person per day; [c] Sum of FF and FFA; [d] Cattle/ swine edible tissue; [e] Penicillin, edible tissues of cattle, MRL = 10 ng/g in turkey, 0 ng/g in chicken, milk, swine, egg, milk; [f] Cattle edible tissue; [g] swine muscle, Exempt in chicken edible tissues; [h] Salmonids and catfish; [i] Cattle liver, Tolerance for desethylene ciprofloxacin (marker residue); [j] Sum of tetracycline residues, finfish muscle; [k] Fish, Tolerance for marker residue: FFA; [l] Edible tissues of catfish; [m] Muscle of cattle; [n] Cattle/ chicken edible tissues excluding cattle milk and chicken eggs; swine muscle: 100 ng/g; NA: Not applicable.
*At spiked level of 20 ng/g fish, AMOX peak was not observed.

Supplementary information

Table S1. Information of fish and shrimp samples analyzed.

Sample Number	Product Name as Advertised	Product Description on Label	Identified Species (DNA barcoding)	Production Method	Country of Origin (use exact wording from point of sale)
P029	Seabass (Patagonian toothfish)	Seabass (Patagonian Tooth Fish)	Antarctic Toothfish (Dissostichus mawsoni)	Wild caught	Product of Korea
P106	Toothfish Chilian Seabass	Toothfish (Chilian Seabass)	Antarctic toothfish	Wild Caught	Product of New Zealand
P120	Chilean Seabass	Chilean Sea Bass Bone-In Skin-On	Antarctic toothfish	N/A	N/A
P068	Chilean Sea Bass Steak	Chilean Sea Bass Steak	Patagonian toothfish (Dissostichus eleginoides)	Wild / Wild Caught	Product of Chile
P115	Previously Frozen & Wild MSC Chilean Sea Bass Fillet Skin On	MSC Chilean Sea Bass Fillet Previously Frozen/Wild Certi fied by: Marine Stewardship Council	Patagonian toothfish	Wild	Product of Australia
P105	Chilean Seabass Kirimi	Chilean Seabass Kirimi	Patagonian toothfish	Wild	Korean
P069	Halibut Steak	Halibut Steak	California flounder	Wild / Wild Caught	Product of CANADA (placard), United States (label)
P065	Halibut Steak	Halibut Steak	California flounder	Wild	Mexico
P061	Halibut Steak Fresh	Halibut Steak	California flounder	Wild	Mexico
P099	Fresh Central Pacific Halibut Fillet	Fresh Central Pacific Halibut Fillet	California flounder	Wild	Product of USA/Product of Mexico
P082	Mahi Mahi Frozen	Mahi Mahi Frozen	Dolphinfish	Farm Raised	N/A
P025	Mahi-mahi fillets	Mahi mahi fillet	Dolphinfish	N/A	N/A
P077	Mahi Mahi Fish Fillet	Mahi Mahi Fillet Fresh	dolphinfish/pomp ano dolphin	N/A	Born, Raised, Harvested China
P091	Previously Frozen Wild Mahi Mahi Fillet	Mahi Mahi Fillet Wild-Prev Frzn	Dolphinfish	Wild Caught	Product of Peru
P026	Mahi-mahi fillets	Mahi mahi	Dolphinfish	Wild caught	Product of Guatemala

P116	Fresh & Wild Swordfish Steak	Swordfish Steak Fresh/Wild	swordfish	Wild	Product of USA
P066	Sword Fish	Fish Department	Swordfish	Wild	Ecuador
P055	Swordfish Portion Frozen 5 oz	Swordfish Portion Frozen 5 oz	Swordfish	Wild	Product of Singapore
P090	Wild Swordfish Steak	Swordfish Steak Wild-Prev Frzn	Swordfish	Wild Caught	Product of Singapore
P006	Swordfish Fresh (T/W)	Swordfish Fresh (T/W)	Swordfish	Wild caught	Chile
P081	Defrosted Swordfish Steak	Swordfish Steak Defrosted	Swordfish	Wild	Product of Singapore
P119	Sword Fish Steak	Wild Sword Fish Fillet	swordfish	Wild	product of Indonesia
P101	Seabass Chilean Portions Minimum 5 oz Previously Frozen	Seabass Chilean Portions Minimum 5 oz Previously Frozen	swordfish	Wild	Product of Chile
P098	Fresh Swordfish Steak	Fresh Swordfish Steak	Swordfish	Wild	Product of Costa Rica/Product of Mexico
P110	Fresh Swordfish	Swordfish	swordfish	Wild Caught	Product of U.S.A.
P072	Swordfish Steak Tp	Swordfish Steak Tp	Swordfish	N/A	Product of USA, Canada, Mexico
P032	Fresh Ahi Yellowfin Tuna	Tuna-ahi steak	Yellowfin Tuna	Wild caught	Product of Tahiti
P060	Tuna Loin Defrosted	Tuna Loin CA Tuna - Frozen Yellow Fin Tuna	Yellowfin Tuna	Wild	Thailand
P087	Wild Ahi Tuna	Ahi Tuna Wild-Prev Frzn	Yellowfin tuna	Wild Caught	Product of Indonesia
P011	Tuna saku	Tuna saku wild	Yellowfin Tuna (CR mini-barcoding); Tuna (Yellowfin Tuna, Blackfin Tuna, Bigeye Tuna) with COI full barcoding	Wild caught	Indonesia
P034	Sushi Tuna	Sushi Tuna	Yellowfin Tuna	N/A	Philippines
P036	Tuna steaks prev frozen	Tuna steaks prev frozen	Bigeye or Yellowfin Tuna	N/A	Product of Indonesia
P062	Tuna	Tuna	Pacific bluefin tuna	Wild	USA
P086	Tuna Steak Ca	Tuna Steak Ca Thu Cat Lat	Pacific bluefin tuna	Wild Caught	Product of USA

	Tuna Cat Khue				
P074	Yellowfin Ahi Tuna Steak Previously Frozen	Tuna Yellow Fin/Ahi Steak Skin-Off Previously Frozen - CO	Southern bluefin tuna	Wild	Product of Indonesia (placard) Fiji (label)
P073	Albacore Tuna Slice Bone In Tp	Albacore Tuna Slice Bone In Tp	Albacore Tuna	N/A	Product of USA, Canada, Mexico
P067	Rex Sole	Rex Sole	Rex Sole	Wild caught	Product of USA
P009	Fresh Rex Sole Skinned	Skinned Rex Sole Fresh	Rex Sole	Wild caught	N/A
P002	Dover Sole Fillet	Sole Fillet Pacific Fresh Wild Caught	Dover Sole	Wild caught	Product of USA
P038	Dover Sole Fillets	Dover Sole Fillets	Dover Sole	Wild caught	Product of USA
P053	Fresh Dover Sole Fillet	Fresh Dover Sole Fillet	Dover Sole	Wild	Product of USA
P097	Fresh Dover Sole Fillet	Fresh Dover Sole Fillet	Dover sole	Wild	Product of USA
P089	Fresh Dover Sole Filet	Dover Sole Fillet Wild-Fresh	Dover sole	Wild Caught	Product of USA
P108	Sole Fillets	Fillets of Sole	Pacific dover sole	Wild Caught	Product of U.S.A.
P113	Fresh & Wild Petrale Sole	Petrale Sole Fresh/Wild	Petrale sole	Wild	Product of USA
P030	Petrale Sole Fillet	Petrale sole fillet	Petrale Sole	Wild caught	Product of U.S.A.
P027	Northern pacific halibut	Northern pacific halibut filet	Halibut (Hippoglossus spp.)	Wild caught	Product of U.S.A.
P088	Northern Halibut Portions	6 oz Halibut Portions Wild-Fresh	Pacific halibut/Atlantic halibut	Wild Caught	Product of USA
P051	Fresh Halibut Portion 5 oz	Halibut Steak Previously Frozen	Pacific halibut	Wild	Product of USA
P109	Alaskan Halibut Fillets	Halibut Fillets	Pacific halibut	Wild Caught	Product of U.S.A.
P112	Fresh & Wild MSC Pacific Halibut Fillet	MSC Pacific Halibut Fillet Fresh/Wild Certified by: Marine Stewardship Council	Pacific halibut	Wild	Product of USA
P007	Halibut Fillet Prev. Frozen (T/W)	Halibut Fillet Prev. Frozen (T/W)	Pacific Halibut	Wild caught	U.S.A.
P001	Fresh Wild Caught Pacific Cod Fillets	True Cod Fillet Fresh	Atlantic Cod	Wild caught	Product of Iceland

P031	Pacific cod	Pacific cod fillet	Atlantic Cod	Wild caught	Product of U.S.A.
P056	Alaska Cod Fillet Previously Frozen	Alaska Cod Fillet Previously Frozen	Pacific cod/Artic cod/Greenland cod	Wild	Product of USA
P078	Defrosted Alaskan Cod Fillet	Cod Fillet Defrosted	Pacific cod/Artic cod/Greenland cod	Wild	Product of USA
P075	Cod Alaskan Fillet Prev Fz	Cod Alaskan Fillet Frozen	Pacific cod/Artic cod/Greenland cod	Wild	Product of USA
P096	Cod Alaskan Fillet Prev Fz	N/A	Pacific cod/Artic cod/Greenland cod	Wild	Product of USA
P085	N/A	Fresh Lind Cod Ca Mu Bong	Pacific cod/Arctic cod/Greenland cod	Wild Caught	Product of Canada
P094	Previously Frozen Wild Cod Fillets	Alaskan Cod Fillet Wild-Prev Frzn	Pacific cod	Wild Caught	Product of USA
P084	Yellow Tail Steak Ca Thu Be Cat Khue	Yellow Tail Steak Ca Thu Be Cat Khue	Yellowtail (Seriola lalandi)	Wild Caught	Product of Mexico
P064	Yellow Tail Fish	Yellow Tail Steak	Yellowtail	Wild	Mexico
P037	Fresh pacfc rockfish flt	Fresh pacfc rockfish flt	Yellowtail rockfish	Wild caught	Product of Canada
P052	Fresh Rockfish Fillet	Fresh Rockfish Fillet	widow rockfish	Wild	Product of Canada
P092	Fresh Pacific Snapper Filet	Pacific Rockfish Fillet Wild-Fresh	widow rockfish	Wild Caught	Product of Canada
P028	Pacific rockfish fillet	Pacific rock fish fillets	Silvergray Rockfish	Wild caught	Product of U.S.A.
P063	Rock Cod Fillet	Fillet of Rock Cod	redbanded rockfish	Wild	Mexico
P107	Fresh Rockfish Red Snapper	Rock Fish Fillets	Rockfish (Sebastes spp.)	Wild Caught	Product of U.S.A.
P114	Fresh & Wild Fresh Pacific Rock Fish Fillets	Fresh Pacific Rock Fish Fillets Fresh Wild	Rockfish (Sebastes spp.)	Wild	Product of USA
P019	Red Snapper Fillet Farm Raised	Whole Clean Red Snapper Fresh/Wild	Blackspotted Rockfish	Farm Raised (placard) Wild (label)	Product of Canada (placard) Product of Brazil (label)
P017	Swai Fish Fillet (Pre Frz) Filete de Pescado Swai	Swai Fish Fillet (Pre Frz) Filete de Pescado Swai	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in	Farm Raised	Product of Vietnam

			seafood list), Basa		
P024	Swai Fillet	Swai fillet farm raised	Sutchi catfish and Basa	Farm raised	Product of Vietnam
P039	Basa Fish Fillet	Red Basa Fish Fillet S/C	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	Farmed	Product of Vietnam
P048	Filete De Pescado Swai Swai Fish Fillet	Filete De Pescado Swai	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	FARM RAISED	Vietnam
P042	Previous Frozen Swai Fillets	Swai Fillet Previously Frozen	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	Farm Raise	Vietnam
P046	Filete De Swai Frozen Swai Fillet	Filete De Swai Frozen Swai Fillet	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	Farm Raised	Product of: Other
P059	Swai Fillet Defrosted	Frozen Swai Fillet	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	Farm	Vietnam
P013	Swai Basa Fillet	Swai Basa Fillet Farm	Sutchi catfish (aka swai), Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	Farm	Vietnam

P005	Swai Fillet Prev Froz Farm Raised	Swai Fillet Prev Froz Farm Raised	Sutchi catfish, Pangasius krempfi (not in seafood list), Pangasius djambal (not in seafood list), Basa	Both Stated (Farm Raised and Wild Caught)	U.S.A.
P045	Filete De Salmon Fresco Fresh Salmon Fish Fillet	Filete De Salmon Fresco Fresh Salmon Fish Fillet	Atlantic salmon	Farm Raised	Product of: Chile
P050	Salmon Fillet Skin On Filete De Salmon Con Piel	Salmon Fillet Skin On Filete De Salmon Con Piel	Atlantic salmon	Farm Raised	Canada
P040	Salmon Fillet	Salmon Fish Fillet S/C	Atlantic Salmon	Farmed	Product of Canada
P020	Salmon steak farmed	Fresh salmon fillet fresh/farmed	Atlantic Salmon	Farmed	Product of Chile
P016	Salmon fillet - Filete De Salmon	Salmon fillet - Filete De Salmon	Atlantic Salmon	Farm Raised	Product of Canada
P004	Atlantic Salmon Fillet	Salmon Fillet Atlantic Fresh Skin on Farm RSD	Atlantic salmon	Farm RSD	Product of Chile
P012	Atlantic salmon fillet	Fresh Atlantic Salmon Fillet Farm	Atlantic Salmon	Farm	Canada
P033	Salmon	Salmon	Atlantic salmon	N/A	Canada
P003	Sockeye Salmon Fillet	Fresh Sockeye Salmon Fresh Wild Caught	Sockeye Salmon	Wild caught	N/A (It appeared that the country of origin information was blocked by a banner on the display case. The information was not visible to the consumer.)
P022	Fresh King Salmon Fillet	King Salmon Fillet	Chinook Salmon	N/A	Product of Canada
P049	Red Tilapia Fillet Filete De Tilapia	Red Tilapia Fillet Filete De Mojarra Roja	Tilapia (Oreochromis sp.) or Pseudocrenilabrus multicolor	Farm Raised	China
P010	Tilapia Fillet	Tilapia Fillet Fresh	Tilapia (Oreochromis spp.)	Farm Raised	N/A
P023	Tilapia Fish Fillet	Tilapia Fillet	Tilapia	BRN,RAISD&HARVST	BRN,RAISD&HARVST China (placard)

					Product of China (label)
P015	Tilapia Fillet (Prev froz) Filete de Mojarra	Tilapia Fillet (Prev Froz) Filete de Mojarra	Tilapia	Farm Raised	Product of China
P021	Whole Clean Tilapia Farmed/Froz en	3-5 Tilapia Fillet Previously Frozen Farmed	Tilapia	Farmed	Product of China
P054	Tilapia Fillet Previously Frozen	Tilapia Fillet Previously Frozen	Tilapia	Farmed	Product of China
P058	Tilapia Fillet Defrosted	Tilapia Fillet	Tilapia	Farm	China
P041	Tilapia Fillet	Tilapia Fish Fillet FRZN/DFRST	Tilapia	Farmed	Product of China
P043	Previous Frozen Tilapia Fillet	Tilapia Fillet Previously Frozen	Tilapia (Blue Tilapia or Nile Tilapia)	Farm Raised	China
P014	Tilapia Fillet	Tilapia Fillet Farm	Mozambique tilapia, tilapia	Farm	China
P071	Catfish Slice	Catfish Slice	channel catfish	Wild	Product of USA
P083	Catfish Nuggets	Catfish Nuggets	channel catfish	N/A	Product of: United States
P018	Fresh Catfish Fillet Previously Frozen/Farm ed	26-30 Raw Headless Shri Previously Frozen Farmed	Channel Catfish	Farmed	Product of China (placard) Product of Ecuador (label)
P008	Catfish Fillet	Catfish Fillet Fresh - Farm Raised	Channel Catfish	Farm Raised	N/A
P093	Fresh Catfish Filet	Catfish Fillet Farm-Fresh	channel catfish	Farm Raised	Product of USA
P100	Catfish Fillet Previously Frozen	Catfish Fillet Previously Frozen	channel catfish	Farmed	Product of USA
P076	Catfish Steak	Fresh Catfish Steaks	channel catfish	Farm	Farm Raised in USA, Born, Raised, Harvested USA
P079	Defrosted Catfish Fillet	Catfish Fillet Defrosted	channel catfish	Farm	Product of USA, Canada, Mexico
P044	Previous Frozen Catfish Fillet	Catfish Fillets Previously Frozen	channel catfish	Farm Raised	China
P057	Catfish Steak Cln	Catfish Steak Cln	channel catfish	Farm Raised	Product of USA
P102	Fresh Rainbow Trout Portions 5 oz	Steelhead Trout Portion 5 oz Skin On	rainbow trout	Farmed/Farm Raised	Product of USA
P070	Steelhead Trout	Fresh Farmed Steelhead Trout	Rainbow trout	Farmed	Product of: USA

P104	Yellowtail Kirimi	Yellowtail Kirimi	Buri (Seriola quinqueradiata)	Farmed	Japan
P035	Sushi yellowtail	Sushi yellowtail	Buri (Seriola quinqueradiata)	N/A	N/A
P117	Fresh Red Snapper Sashimi	Fresh Red Snapper Sashimi	Madai	Farmed	Japan
P118	Premium Red Snapper	Premium Red Snapper	Madai	Wild	Japan
P095	Fresh Rockfish Fillet	Fresh Rockfish Fillet	Pacific Ocean Perch	Wild	Product of Canada
P080	Fresh Pacific Rockfish Fillet	Fresh Pacific Rockfish Fillet	Pacific Ocean Perch	Wild	Product of Canada
P103	Black Cod Kirimi	Black Cod Kirimi	sablefish	Wild	Canada
P111	Wild Mahi Mahi	Mahi Mahi (Dorado)	mahi mahi	Wild Caught	Product of Mexico
P047	Filete De Swai Frozen Red Swai Fillet	Filete De Swai Frozen Red Swai Fillet	Bluespotted Stingray	Farm Raised	Product of: Other
DM	-	Wild caught Salmon	NA	Wild	Product of USA
WFB	-	Wild caught gulf shrimp	NA	Wild caught	Product of USA
W-DOM	-	Shrimp, Wild caught		Wild caught	Product of USA
W-Arg	-	Wild caught Argentine Red Shrimp	NA	Wild caught	Argentina
F-India	-	Shrimp, Farm raised	NA	Farm raised	India
F-Indonesia	-	Raw shrimp	NA	Farm raised	Indonesia

Not applicable

Table S2. Limit of detection (LOD) and limit of quantitation (LOQ) of antibiotics from three separate runs

Antibiotics	Run 1		Run 2		Run 3	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
AMP	0.009	0.03	0.0002	0.0008	0.012	0.04
PEN-G	0.05	0.16	0.030	0.10	0.02	0.07
PEN-V	0.08	0.29	0.04	0.13	0.04	0.13
AMOX	0.03	0.12	0.01	0.02	0.040	0.14
LIN	0.0027	0.01	0.0013	0.0043	0.04	0.15
TRIM	0.0047	0.0162	0.002	0.0062	0.06	0.19
ORM	0.003	0.012	0.0001	0.0004	0.05	0.16
ENO	6.28	21.51	1.42	4.86	0.54	1.85
NOR	3.69	12.62	2.67	9.14	0.16	0.54
ENRO	0.11	0.37	0.021	0.073	0.19	0.66
FLU	0.063	0.214	0.014	0.048	0.03	0.11
OTC	0.09	0.31	0.08	0.27	0.11	0.37
TC	0.04	0.13	0.06	0.22	0.43	1.46
CTC	0.25	0.86	0.182	0.622	3.64	12.47
DOX	0.15	0.52	0.14	0.48	0.27	0.93
FF	0.33	1.12	0.058	0.20	0.02	0.08
TAP	0.31	1.07	0.72	2.46	0.45	1.55
CAP	0.049	0.17	0.021	0.072	0.14	0.48
FFA	0.03	0.11	0.05	0.16	0.06	0.21
SDZ	0.011	0.037	0.003	0.009	0.04	0.13
SMX	0.020	0.07	0.008	0.027	0.005	0.017
SDM	0.0024	0.008	0.001	0.0041	0.02	0.05
SSZ	0.035	0.119	0.03	0.12	0.04	0.12
AZ	4.17	14.28	0.30	1.02	0.22	0.75
TILM	8.14	27.88	3.65	12.49	6.68	22.87
ERYTH	0.0048	0.016	0.007	0.024	0.01	0.02
Tylosin	0.10	0.33	0.01	0.021	0.007	0.02
ROX	0.027	0.092	0.009	0.0293	0.01	0.02
VIRG-M1	0.085	0.29	0.031	0.105	0.02	0.08
VIRG-S1	1.11	3.82	0.43	1.46	0.06	0.19

Table S3. Concentration of antibiotic residues (ng/g) in seafood samples in run 1.

	B-Lactams				Amphenicols				Quinolones				Tetracyclines			
ID	AMOX	AMP	PEN-G	PEN-V	FFA	FF	TAP	CAP	ENO	NOR	ENRO	FLU	OTC	TC	CTC	DOX
029	ND	< MB	ND	ND	ND	ND	ND	ND	ND	<LOD	<LOD	<LOQ	ND	ND	ND	<MB
106	ND	< MB	ND	ND	0.54	ND	ND	ND	ND	<LOD	ND	< MB	ND	ND	<LOD	ND
105	ND	ND	ND	ND	0.36	ND	ND	ND	ND	<LOD	ND	<LOQ	<LOD	ND	<LOQ	ND
068	ND	ND	ND	ND	2.88	ND	ND	<LOQ	ND	ND	ND	<LOQ	ND	ND	<LOD	ND
115	ND	ND	<LOD	ND	0.68	ND	<LOQ	ND	ND	ND	ND	< MB	ND	ND	<LOD	ND
120	2.46	ND	ND	ND	2.19	ND	ND	ND	ND	ND	ND	< MB	ND	ND	<LOD	ND
091	ND	<LOQ	ND	ND	1.88	ND	ND	<LOQ	ND	<LOD	ND	0.23	ND	ND	ND	ND
026	ND	ND	ND	ND	2.03	ND	<LOD	ND	ND	<LOD	ND	0.25	ND	ND	ND	ND
077	ND	ND	ND	ND	4.18	ND	ND	0.28	ND	<LOD	ND	< MB	<LOD	ND	<LOD	ND
025	ND	ND	ND	ND	0.90	ND	ND	ND	ND	ND	ND	< MB	ND	ND	<LOD	ND
082	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	<LOD	ND	< MB	<LOD	ND	ND	ND
069	ND	ND	ND	ND	2.35	ND	ND	<LOD	<LOD	ND	ND	0.29	ND	ND	ND	ND
065	ND	ND	ND	ND	4.65	ND	ND	ND	<LOD	<LOD	ND	<LOD	ND	ND	ND	ND
061	ND	ND	<LOD	ND	7.86	ND	ND	<LOD	<LOD	<LOD	ND	< MB	ND	ND	ND	ND
099	ND	ND	ND	<LOD	2.12	ND	<LOQ	=LOD	<LOD	ND	ND	<LOQ	ND	ND	ND	ND
027	ND	ND	ND	ND	15.14	ND	<LOD	ND	ND	ND	ND	1.35	ND	ND	ND	ND
088	ND	0.05	ND	ND	8.93	ND	<LOQ	1.40	ND	<LOD	ND	0.69	ND	ND	ND	ND
051	ND	0.06	ND	ND	99.11	ND	ND	ND	ND	<LOD	ND	<LOD	ND	ND	ND	ND
109	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	ND	ND	0.28	ND	ND	ND	ND
112	ND	ND	<LOD	ND	7.50	ND	ND	<LOQ	ND	ND	ND	0.77	ND	ND	ND	ND
007	ND	ND	ND	ND	173.15	ND	ND	ND	ND	ND	ND	0.89	ND	ND	ND	ND
118	ND	ND	ND	ND	< LOQ	ND	ND	ND	ND	<LOD	ND	<LOD	ND	ND	ND	ND
117	ND	ND	ND	ND	0.39	ND	ND	ND	ND	ND	ND	3.36	ND	ND	ND	ND
032	ND	ND	ND	<LOD	0.44	ND	ND	ND	<LOD	<LOD	ND	< MB	<LOD	ND	ND	ND
060	ND	ND	ND	ND	12.38	ND	ND	ND	ND	ND	ND	< MB	<LOD	ND	ND	ND
087	ND	ND	ND	ND	3.28	ND	<LOQ	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
011	ND	ND	ND	ND	2.78	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
062	ND	ND	ND	<LOD	ND	ND	ND	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
086	ND	ND	ND	ND	4.36	ND	<LOQ	ND	ND	ND	ND	< MB	<LOD	0.46	ND	ND
074	ND	ND	ND	ND	1.85	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
073	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
034	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	1.33	ND	ND	ND	ND
036	0.16	ND	ND	ND	4.41	ND	<LOD	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
102	ND	ND	ND	ND	1.37	ND	<LOD	ND	ND	ND	ND	< MB	1.86	ND	ND	ND
070	ND	ND	ND	ND	5.20	ND	ND	ND	ND	ND	ND	< MB	11.39	ND	ND	ND
001	ND	ND	ND	ND	1.13	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
031	ND	ND	ND	ND	2.14	ND	ND	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
056	<MB	ND	ND	ND	2.63	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
078	ND	ND	ND	ND	0.81	ND	ND	ND	<LOD	<LOD	ND	< MB	ND	ND	ND	ND
075	ND	ND	ND	ND	0.62	ND	ND	ND	ND	ND	ND	< MB	<LOD	ND	ND	ND
096	ND	ND	ND	<LOD	2.83	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
085	ND	ND	ND	ND	1.74	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
094	ND	< MB	ND	<LOD	ND	ND	ND	ND	ND	ND	ND	0.42	ND	ND	ND	ND
103	ND	ND	ND	ND	0.57	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	ND
064	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
084	ND	ND	ND	<LOD	ND	ND	<LOD	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
035	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	< MB	0.98	ND	ND	ND
104	0.13	ND	ND	ND	1.00	ND	ND	ND	<LOD	ND	ND	< MB	13.61	ND	ND	ND
049	ND	ND	ND	ND	0.42	ND	ND	<LOD	ND	ND	ND	<LOD	<LOQ	ND	ND	ND
010	ND	ND	ND	ND	0.27	ND	ND	<LOQ	<LOD	ND	ND	<LOQ	ND	ND	ND	ND
023	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	< MB	<LOQ	ND	ND	ND
015	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	<LOQ	ND	ND	ND	ND
021	ND	ND	ND	ND	< MB	ND	ND	ND	ND	ND	ND	<LOQ	ND	ND	ND	ND
054	ND	ND	ND	ND	0.38	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	ND	ND
058	<LOQ	ND	ND	ND	0.95	ND	ND	ND	ND	ND	ND	0.29	ND	ND	ND	ND
041	ND	ND	ND	ND	0.20	ND	ND	<LOQ	ND	ND	ND	<LOD	0.32	ND	ND	ND
043	ND	ND	ND	ND	< MB	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	ND	ND
014	ND	ND	ND	ND	1.03	ND	ND	ND	ND	ND	<LOQ	<LOQ	<LOD	ND	<LOD	ND
071	ND	ND	ND	ND	0.51	ND	ND	<LOD	ND	ND	ND	< MB	ND	ND	ND	ND
083	ND	ND	ND	ND	0.59	ND	<LOD	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
018	ND	ND	ND	<LOD	2.57	ND	ND	ND	ND	ND	<LOQ	< MB	ND	ND	ND	ND
008	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	<LOD	ND	ND	ND	ND
093	ND	ND	ND	ND	0.58	ND	ND	<LOD	<LOD	ND	ND	<LOD	ND	ND	ND	ND
100	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	< MB	ND	ND	ND	ND
076	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	<LOD	ND	ND	ND	ND
079	ND	ND	ND	ND	1.28	ND	ND	ND	<LOD	<LOD	ND	<LOD	ND	ND	ND	ND
044	ND	<LOD	ND	ND	0.17	ND	ND	ND	<LOD	ND	~LOD	<LOD	ND	ND	<LOD	ND

057	ND	ND	ND	ND	ND	ND	ND	ND	<LOD	ND	ND	< MB	ND	ND	<LOD	ND

Cont. Table S3. Concentration of antibiotic residues (ng/g) in seafood samples in run 1.

	Sulfonamides				Macrolides							Lincosamides	Dihydrofolate reductase inhibitors	
ID	SDZ	SMX	SDM	SSZ	AZ	TILM	ERYTH	TYLOSIN	ROX	VIRG-M1	VIRG-S1	LIN	TRIM	ORM
029	<LOQ	<LOQ	0.04	0.18	<LOD	<LOD	< MB	< MB	<MB	ND	ND	< MB	<LOD	
106	ND	ND	ND	ND	< MB	ND	< MB	< MB	<MB	ND	ND	< MB	< MB	< MB
105	ND	ND	ND	ND	<LOD	<LOD	< MB	< MB	<MB	ND	ND	< MB	<LOD	< MB
068	ND	ND	ND	ND	< MB	ND	< MB	< MB	<MB	ND	ND	< MB	< MB	< MB
115	ND	ND	ND	ND	< MB	ND	< MB	< MB	0.09	ND	ND	< MB	< MB	< MB
120	ND	ND	ND	ND	< MB	ND	< MB	< MB	<MB	ND	ND	< MB	ND	ND
091	ND	ND	ND	ND	< MB	< MB	< MB	ND	<MB	2.28	ND	< MB	ND	< MB
026	0.04	<LOQ	0.05	0.12	<LOD	<LOD	< MB	< MB	<MB	<LOQ	ND	< MB	0.06	< MB
077	ND	ND	ND	ND	< MB	ND	< MB	ND	<MB	2.10	ND	< MB	< MB	< MB
025	ND	ND	ND	< MB	< MB	< MB	< MB	ND	<MB	ND	ND	< MB	< MB	< MB
082	<LOQ	ND	<LOQ	< MB	<LOD	< MB	< MB	< MB	<MB	<LOQ	ND	< MB	< MB	< MB
069	< IDL	ND	ND	ND	< MB	ND	< MB	ND	<LOQ	<LOD	ND	0.27	ND	< MB
065	< IDL	ND	ND	ND	< MB	< MB	< MB	ND	0.21	1.24	ND	0.06	<LOQ	ND
061	< IDL	ND	ND	ND	< MB	ND	< MB	ND	0.13	<LOQ	ND	0.11	<LOD	ND
099	< IDL	ND	ND	ND	<LOD	ND	< MB	ND	0.23	0.45	ND	0.07	<LOQ	ND
027	ND	ND	<LOQ	ND	< MB	ND	< MB	ND	0.35	ND	ND	< MB	<LOD	ND
088	< IDL	ND	<LOQ	ND	<LOD	ND	< MB	ND	0.18	ND	ND	< MB	<LOQ	< MB
051	ND	ND	ND	ND	<LOD	ND	< MB	ND	0.35	<LOQ	ND	< MB	<LOQ	ND
109	ND	ND	ND	<LOD	< MB	ND	< MB	ND	0.70	<LOQ	<LOD	< MB	ND	ND
112	ND	ND	ND	<LOQ	< MB	ND	< MB	ND	0.39	<LOQ	<LOQ	< MB	ND	ND
007	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOQ	<LOQ	< MB	ND	ND
118	ND	ND	ND	<LOD	< MB	ND	< MB	<LOD	0.75	ND	ND	< MB	<LOQ	ND
117	ND	ND	ND	<LOQ	<LOD	ND	< MB	ND	0.97	ND	ND	< MB	<LOD	ND
032	< IDL	ND	ND	ND	<LOD	<LOD	< MB	ND	< MB	=LOQ	ND	=MB	=MB	< MB
060	ND	ND	ND	ND	< MB	<LOD	< MB	<LOD	<LOQ	1.72	ND	< MB	ND	ND
087	<LOD	ND	ND	ND	< MB	ND	0.15	<LOD	< MB	1.17	ND	< MB	ND	ND
011	ND	ND	ND	ND	< MB	ND	< MB	<LOD	< MB	2.23	ND	< MB	ND	ND
062	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	2.94	<LOD	< MB	ND	ND
086	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	1.61	ND	< MB	<LOQ	ND
074	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	0.38	ND	< MB	<LOD	< MB
073	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOQ	ND	< MB	< MB	< MB
034	< IDL	ND	ND	ND	< MB	<LOD	< MB	ND	< MB	<LOQ	ND	< MB	< MB	ND
036	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOD	ND	< MB	<LOD	< MB
102	<LOD	ND	ND	<LOQ	< MB	<LOD	< MB	ND	= MB	ND	ND	< MB	ND	ND
070	ND	ND	0.07	ND	< MB	<LOD	< MB	ND	< MB	ND	ND	< MB	< MB	ND
001	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOQ	ND	< MB	ND	0.92
031	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOD	ND	< MB	0.018	ND
056	ND	0.13	ND	ND	< MB	ND	< MB	ND	< MB	1.25	ND	< MB	ND	<LOD
078	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	ND	ND	< MB	ND	ND
075	ND	ND	ND	ND	< MB	<LOD	< MB	ND	< MB	<LOQ	ND	< MB	ND	ND
096	< IDL	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOD	ND	< MB	ND	ND
085	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOQ	ND	< MB	0.015	ND
094	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	<LOD	ND	< MB	ND	ND
103	ND	ND	ND	ND	< MB	<LOD	<LOD	ND	< MB	0.96	ND	< MB	ND	ND
064	ND	ND	ND	ND	< MB	<LOD	< MB	ND	< MB	<LOQ	ND	<LOQ	<LOQ	ND
084	ND	ND	0.44	ND	< MB	ND	< MB	ND	< MB	9.92	ND	<LOD	ND	ND
035	ND	ND	ND	ND	< MB	ND	< MB	ND	<LOQ	<LOQ	ND	0.02	ND	ND
104	ND	ND	ND	ND	< MB	<LOD	1.72	ND	< MB	0.39	ND	0.02	ND	ND
049	ND	<LOD	< OD	ND	<LOD	<LOD	< MB	<LOD	<LOQ	ND	ND	<LOD	< MB	ND
010	< IDL	ND	0.013	ND	<LOD	ND	< MB	ND	0.79	ND	ND	< MB	0.019	<LOQ
023	ND	ND	ND	ND	<LOD	<LOD	< MB	ND	0.33	ND	ND	< MB	< LOD	<LOD
015	<LOD	ND	ND	ND	< MB	ND	0.04	ND	1.17	ND	ND	< MB	0.017	< MB
021	<LOD	ND	ND	ND	< MB	ND	< MB	ND	0.15	ND	ND	< MB	< LOQ	< MB
054	ND	ND	ND	ND	< MB	ND	< MB	ND	0.10	ND	<LOD	< MB	< MB	< MB
058	0.12	ND	ND	ND	< MB	ND	< MB	ND	0.12	<LOD	<LOD	< MB	< MB	ND
041	< IDL	ND	ND	ND	<LOD	<LOD	< MB	ND	0.57	ND	ND	< MB	< MB	ND
043	<LOD	ND	ND	ND	< MB	ND	<LOQ	ND	0.12	ND	ND	< MB	< LOQ	ND
014	<LOD	ND	ND	ND	<LOD	<LOD	< MB	ND	0.24	ND	ND	< MB	< MB	< MB
071	ND	ND	ND	ND	< MB	<LOD	< MB	ND	< MB	ND	ND	< MB	< MB	< MB
083	ND	ND	0.010	ND	< MB	ND	< MB	ND	< MB	ND	ND	< MB	ND	< MB
018	ND	ND	ND	ND	< MB	ND	< MB	ND	<LOQ	ND	ND	< MB	< LOQ	0.018
008	< IDL	ND	ND	ND	< MB	ND	< MB	ND	< MB	ND	ND	< MB	ND	<LOD
093	< IDL	ND	ND	ND	< MB	< MB	< MB	ND	< MB	ND	ND	< MB	< MB	ND
100	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	ND	ND	< MB	< MB	< MB
076	ND	ND	ND	ND	< MB	< MB	< MB	ND	< MB	ND	ND	< MB	ND	ND

079	< IDL	ND	ND	ND	< MB	<LOD	< MB	ND	< MB	ND	ND	< MB	ND	< MB
044	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	ND	ND	< MB	< LOD	ND
057	ND	ND	ND	ND	< MB	ND	< MB	ND	< MB	ND	ND	< MB	< MB	ND

Cont. Table S3. Concentration of antibiotic residues (ng/g) in seafood samples in run 2.

	B-Lactams				Amphenicols				Quinolones				Tetracyclines			
ID	AMOX	AMP	PEN-G	PEN-V	FFA	FF	TAP	CAP	ENO	NOR	ENRO	FLU	OTC	TC	CTC	DOX
037	ND	ND	ND	ND	4.31	ND	<LOD	ND	ND	ND	< MB	0.27	<LOD	ND	ND	<MB
052	ND	< MB	ND	ND	0.30	ND	ND	ND	ND	ND	< MB	<LOQ	ND	ND	ND	ND
092	ND	ND	ND	ND	<LOQ	ND	<LOD	ND	ND	ND	< MB	<LOD	ND	ND	ND	ND
028	ND	ND	ND	ND	4.52	ND	ND	ND	ND	ND	< MB	=LOQ	ND	ND	ND	ND
063	ND	ND	ND	ND	0.49	ND	ND	ND	ND	ND	< MB	< MB	< MB	ND	ND	ND
107	ND	ND	ND	ND	8.92	ND	ND	ND	ND	< IDL	< MB	<LOD	ND	ND	ND	ND
114	ND	ND	ND	ND	7.68	ND	ND	ND	ND	< IDL	< MB	0.06	ND	ND	ND	ND
095	ND	< MB	<LOQ	<LOQ	0.63	ND	ND	ND	ND	ND	< MB	<LOQ	< MB	ND	ND	ND
080	ND	ND	ND	ND	4.79	ND	ND	ND	ND	ND	< MB	< MB	< MB	ND	ND	ND
019	ND	ND	ND	ND	3.40	ND	<LOD	ND	ND	ND	< MB	< MB	ND	ND	ND	ND
017	ND	0.006	ND	ND	3.50	ND	ND	ND	<IDL	ND	<LOQ	< MB	ND	ND	ND	<MB
024	ND	ND	ND	ND	4.81	ND	< MB	ND	ND	ND	0.38	< MB	< MB	ND	ND	<MB
039	ND	ND	<LOD	ND	4.59	ND	ND	ND	ND	=MB	0.27	< MB	ND	ND	ND	ND
048	ND	ND	ND	ND	3.41	ND	ND	ND	<LOQ	< MB	<LOQ	< MB	ND	ND	ND	ND
042	< MB	ND	ND	ND	11.69	ND	ND	ND	ND	ND	<LOQ	< MB	ND	ND	ND	<MB
046	< MB	ND	ND	ND	4.04	<LOQ	ND	ND	< IDL	ND	0.50	0.42	ND	ND	ND	ND
059	ND	ND	ND	ND	4.02	ND	< MB	ND	< IDL	ND	0.09	< MB	< MB	ND	ND	ND
013	ND	ND	ND	<LOD	4.44	ND	ND	<LOD	ND	ND	0.31	< MB	ND	ND	ND	ND
005	0.09	ND	ND	ND	4.67	ND	ND	<LOD	ND	ND	0.08	< MB	ND	ND	ND	ND

Cont. Table S3. Concentration of antibiotic residues (ng/g) in seafood samples in run 2.

	Sulfonamides				Macrolides							Lincosamides	Dihydrofolate reductase inhibitors	
ID	SMX	SDZ	SDM	SSZ	AZ	TILM	ERYTH	Tylosin	ROX	VIRG-M1	VIRG-S1	LIN	TRIM	ORM
037	ND	ND	0.007	ND	< MB	ND	< MB	< MB	<LOQ	0.67	ND	< MB	ND	0.002
052	ND	ND	ND	ND	< MB	ND	< MB	ND	<LOQ	<LOQ	ND	< MB	<LOQ	ND
092	ND	ND	0.008	ND	< MB	ND	< MB	< MB	0.23	ND	ND	< MB	<LOQ	ND
028	ND	ND	ND	1.07	< MB	ND	< MB	< MB	< MB	0.21	ND	< MB	ND	ND
063	ND	0.017	ND	<MB	< MB	ND	< MB	ND	< MB	ND	ND	< MB	ND	ND
107	ND	ND	ND	ND	< MB	ND	< MB	< MB	< MB	0.88	ND	< MB	ND	ND
114	ND	ND	0.004	ND	< MB	ND	< MB	ND	0.04	0.12	ND	< MB	0.007	ND
095	ND	ND	ND	ND	< MB	ND	< MB	< MB	0.11	ND	ND	< MB	ND	0.011
080	ND	ND	ND	ND	< MB	ND	< MB	< MB	< MB	0.23	1.93	< MB	<LOQ	ND
019	ND	ND	ND	ND	< MB	ND	< MB	ND	0.17	0.95	ND	< MB	< MB	ND
017	<LOQ	ND	ND	ND	< MB	ND	0.059	< MB	< MB	ND	ND	< LOD	<LOD	< MB
024	ND	ND	ND	ND	< MB	ND	0.043	< MB	< MB	ND	ND	< MB	< MB	< MB
039	ND	0.014	ND	ND	< MB	ND	0.068	< MB	< MB	ND	ND	< LOD	<LOD	ND
048	ND	ND	ND	<MB	< MB	ND	0.033	< MB	0.09	ND	<LOD	< MB	< MB	ND
042	ND	ND	ND	ND	< MB	ND	< LOD	< MB	0.17	ND	<LOD	< MB	< MB	ND
046	ND	0.015	0.004	ND	< MB	ND	0.034	< MB	0.09	ND	ND	< MB	< MB	ND
059	ND	ND	ND	ND	< MB	ND	< LOD	< MB	< MB	ND	ND	< MB	< MB	< MB
013	ND	ND	ND	ND	< MB	ND	0.119	< MB	0.08	ND	ND	< MB	< MB	ND
005	ND	ND	ND	ND	< MB	ND	0.027	ND	0.09	ND	<LOD	< MB	< MB	ND

Cont. Table S3. Concentration of antibiotic residues (ng/g) in seafood samples in run 3.

	B-Lactams				Amphenicols				Quinolones				Tetracyclines			
ID	AMOX	AMP	PEN-G	PEN-V	FFA	FF	TAP	CAP	ENO	NOR	ENRO	FLU	OTC	TC	CTC	DOX
116	ND	ND	ND	=LOD	0.51	ND	< MB	< LOD	2.67	< MB	<MB	=LOD	ND	ND	ND	ND
066	2.05	ND	ND	ND	ND	ND	< MB	ND	2.74	< MB	<MB	<LOD	<LOQ	ND	ND	ND
055	ND	<LOQ	ND	ND	1.12	ND	< MB	ND	ND	= MB	<MB	< MB	<LOD	ND	ND	ND
090	ND	ND	ND	ND	ND	ND	< MB	ND	ND	< MB	<MB	< MB	ND	ND	ND	ND
006	< LOQ	ND	ND	ND	12.46	ND	< MB	ND	2.68	< MB	<MB	< MB	ND	ND	ND	ND
081	ND	ND	ND	ND	ND	ND	< MB	ND	ND	< LOD	<MB	< MB	ND	ND	ND	ND
119	ND	ND	ND	ND	6.23	ND	< MB	ND	2.70	< MB	<MB	< MB	<LOQ	ND	ND	ND
101	ND	ND	ND	ND	ND	ND	< MB	ND	ND	< LOD	<MB	< MB	ND	ND	ND	ND
098	< LOQ	ND	ND	ND	4.60	ND	< MB	ND	2.68	< LOD	<MB	< MB	ND	ND	ND	ND
110	ND	ND	ND	ND	5.92	ND	< MB	ND	2.67	< MB	<MB	< MB	<LOD	ND	ND	ND
072	2.62	ND	ND	ND	ND	ND	< MB	< LOD	2.70	ND	<MB	< MB	ND	ND	ND	ND
067	ND	ND	ND	ND	19.18	ND	< MB	ND	2.67	ND	<MB	ND	<LOD	ND	ND	ND
009	< LOQ	ND	ND	ND	44.85	ND	< MB	ND	ND	ND	<MB	ND	ND	ND	ND	ND

002	ND	ND	ND	ND	110.21	ND	< MB	ND	2.98	< LOD	<MB	<LOD	<LOD	ND	ND	ND
038	ND	ND	ND	< LOD	ND	ND	< MB	ND	2.68	ND	<MB	<LOD	ND	ND	ND	ND
053	ND	ND	ND	ND	36.90	ND	< MB	ND	ND	ND	<MB	< MB	ND	ND	ND	ND
097	ND	ND	ND	ND	20.30	ND	< MB	ND	2.66	ND	<MB	< MB	ND	ND	ND	ND
089	ND	<LOQ	ND	< LOQ	11.03	ND	< MB	ND	2.80	ND	<MB	< MB	<LOD	ND	ND	ND
108	ND	ND	ND	0.14	83.29	ND	< MB	ND	ND	ND	<MB	ND	<LOD	ND	ND	ND
113	ND	ND	ND	ND	102.39	ND	< MB	ND	2.72	ND	<MB	=LOD	<LOD	ND	ND	ND
030	ND	ND	ND	=LOD	14.71	ND	< MB	ND	ND	ND	<MB	<LOQ	ND	ND	ND	ND
DM	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	< LOQ	ND	< MB	ND	ND	ND
003	ND	ND	ND	ND	8.39	ND	< MB	ND	ND	ND	< IDL	ND	ND	ND	ND	ND
022	ND	ND	ND	ND	8.34	ND	< MB	ND	ND	< LOD	=MB	ND	<LOD	ND	ND	ND
033	< LOD	ND	ND	ND	ND	ND	< MB	ND	ND	< LOD	=MB	ND	<MB	ND	ND	ND
045	ND	<LOD	ND	< LOD	3.38	ND	< MB	ND	ND	< LOD	< IDL	ND	ND	ND	ND	ND
050	< LOQ	ND	ND	ND	ND	ND	< MB	ND	ND	< LOD	=MB	ND	<LOD	ND	ND	ND
040	ND	ND	ND	< LOD	3.18	ND	< MB	ND	ND	< LOD	=MB	ND	ND	ND	ND	ND
020	ND	ND	ND	ND	90.18	0.91	< MB	ND	ND	ND	< IDL	ND	4.36	ND	ND	ND
016	ND	ND	ND	ND	4.64	ND	< MB	ND	ND	< LOD	=MB	ND	ND	ND	ND	ND
004	ND	ND	ND	ND	ND	ND	< MB	= LOD	ND	ND	< IDL	ND	0.36	ND	ND	ND
012	ND	ND	ND	ND	3.34	ND	< MB	ND	ND	ND	=MB	ND	< MB	ND	ND	ND
047	ND	ND	ND	ND	ND	ND	< MB	ND	ND	ND	=MB	<LOD	<LOD	ND	ND	ND
WFB	ND	ND	ND	ND	ND	ND	< MB	ND	4.17	= MB	=MB	<LOD	ND	ND	ND	ND
W-DOM	ND	ND	ND	ND	ND	ND	< MB	ND	3.17	ND	=MB	ND	=MB	ND	ND	ND
W-Arg	ND	ND	<LOD	ND	0.33	ND	< MB	ND	2.72	< MB	=MB	<LOD	< MB	ND	ND	ND
F-India	ND	<LOD	ND	ND	0.58	ND	< MB	ND	3.54	< MB	< IDL	ND	ND	ND	ND	ND
F-Indonasia	ND	ND	ND	ND	0.66	ND	< MB	ND	2.84	ND	=MB	ND	ND	ND	ND	ND

Cont. Table S3. Concentration of antibiotic residues (ng/g) in seafood samples in run 3.

	Sulfonamides				Macrolides							Lincosamides	Dihydrofolate reductase inhibitors	
ID	SDZ	SMX	SDM	SSZ	AZ	TILM	ERYTH	TYLOSIN	ROX	VIRG-M1	VIRG-S1	LIN	TRIM	ORM
116	ND	ND	ND	ND	= MB	ND	< MB	< MB	0.19	0.18	< MB	<MB	< MB	ND
066	< IDL	ND	ND	ND	= MB	ND	< MB	< MB	<MB	<LOQ	0.49	<MB	< MB	ND
055	ND	ND	ND	ND	= MB	ND	< MB	= MB	<MB	0.14	=MB	<MB	< MB	ND
090	ND	ND	ND	ND	< MB	ND	< MB	ND	<MB	0.12	ND	<MB	<LOD	ND
006	ND	ND	ND	ND	= MB	ND	< MB	ND	=MB	0.19	ND	<MB	< MB	ND
081	ND	ND	ND	ND	= MB	ND	< MB	ND	=MB	0.24	< LOQ	<MB	<LOD	ND
119	< LOD	ND	ND	ND	= MB	ND	< MB	ND	<MB	0.41	ND	<MB	<LOD	ND
101	ND	ND	ND	ND	= MB	ND	< MB	<MB	0.04	0.13	ND	<MB	< MB	ND
098	ND	ND	ND	ND	= MB	ND	< MB	<MB	0.16	0.14	ND	<MB	<LOD	ND
110	ND	ND	ND	ND	= MB	ND	< MB	ND	0.08	0.16	ND	<MB	< MB	ND
072	ND	ND	ND	ND	< MB	ND	< MB	ND	<MB	0.28	ND	<MB	< MB	ND
067	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	0.20	ND	<MB	=MB	ND
009	ND	ND	ND	ND	= MB	ND	< MB	ND	=MB	0.22	ND	<MB	=MB	ND
002	ND	ND	ND	ND	=LOD	ND	< MB	ND	<MB	<LOQ	ND	<MB	<LOD	ND
038	ND	ND	ND	ND	< MB	ND	< MB	ND	<MB	<LOQ	ND	<MB	<LOD	ND
053	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	0.21	ND	<MB	=MB	ND
097	ND	ND	ND	ND	< MB	ND	< MB	ND	0.29	0.33	ND	=MB	ND	ND
089	< IDL	ND	ND	ND	=LOD	ND	< MB	ND	<MB	0.47	ND	<MB	ND	ND
108	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	0.13	ND	=MB	ND	ND
113	ND	ND	ND	ND	= MB	ND	=MB	ND	<MB	0.12	ND	<MB	ND	ND
030	ND	ND	ND	ND	=LOD	ND	< MB	ND	<MB	=LOQ	0.71	=MB	ND	ND
DM	ND	ND	ND	ND	<LOD	ND	< MB	ND	<MB	ND	ND	<MB	ND	ND
003	< IDL	ND	ND	ND	= MB	ND	< MB	ND	<MB	0.23	ND	<MB	=MB	ND
022	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	0.24	< MB	=MB	=MB	ND
033	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	ND	ND	<LOD	=MB	ND
045	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	ND	< LOQ	<LOD	ND	ND
050	< IDL	ND	ND	<LOD	= MB	ND	< MB	ND	<MB	ND	< LOD	<LOD	=MB	ND
040	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	=LOD	ND	<LOD	=MB	ND
020	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	ND	ND	<LOD	=MB	ND
016	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	<LOQ	< LOQ	= MB	=MB	ND
004	ND	ND	ND	ND	< MB	ND	< MB	ND	<MB	ND	< LOD	< MB	=MB	ND
012	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	ND	ND	<LOD	=MB	ND
047	ND	<LOQ	ND	ND	< MB	ND	< MB	ND	<MB	1.03	< LOQ	<LOD	=MB	ND
WFB	ND	ND	ND	ND	= MB	ND	< MB	ND	<MB	=MB	ND	< MB	=MB	=MB
W-DOM	ND	ND	<LOD	ND	< MB	ND	< MB	ND	<MB	ND	< MB	< MB	=MB	ND
W-Arg	ND	ND	<LOD	ND	< MB	ND	< MB	ND	<MB	<LOQ	< MB	< MB	=MB	ND
F-India	ND	ND	ND	<LOD	< MB	ND	< MB	ND	<MB	ND	ND	< MB	=MB	ND
F-Indonasia	ND	<LOQ	<LOD	ND	< MB	ND	< MB	ND	=MB	ND	ND	< MB	=MB	ND

Table S4. Proportion of the variance explained by each principal componenet

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8	PC9	PC10	PC11
Standard deviation	1.64009	1.48127	1.41547	1.31668	1.23238	1.20722	1.15049	1.13457	1.09545	1.06935	1.0487
Proportion of Variance	0.09607	0.07836	0.07156	0.06192	0.05424	0.05205	0.04727	0.04597	0.04286	0.04084	0.03928
Cumulative proportion	0.09607	0.17443	0.24599	0.3079	0.36214	0.41419	0.46146	0.50744	0.5503	0.59113	0.63041
	PC12	PC13	PC14	PC15	PC16	PC17	PC18	PC19	PC20	PC21	PC22
Standard deviation	1.0217	1.00573	0.99167	0.97497	0.95377	0.90776	0.88312	0.86978	0.84848	0.75849	0.74149
Proportion of Variance	0.03728	0.03612	0.03512	0.03395	0.03249	0.02943	0.02785	0.02702	0.02571	0.02055	0.01964
Cumulative proportion	0.66769	0.70382	0.73894	0.77289	0.80538	0.83481	0.86266	0.88968	0.91539	0.93594	0.95557
	PC23	PC24	PC25	PC26	PC27	PC28					
Standard deviation	0.61012	0.57093	0.48833	0.4643	0.2898	0.08794					
Proportion of Variance	0.01329	0.01164	0.00852	0.0077	0.003	0.00028					
Cumulative proportion	0.96887	0.98051	0.98903	0.9967	0.9997	1					

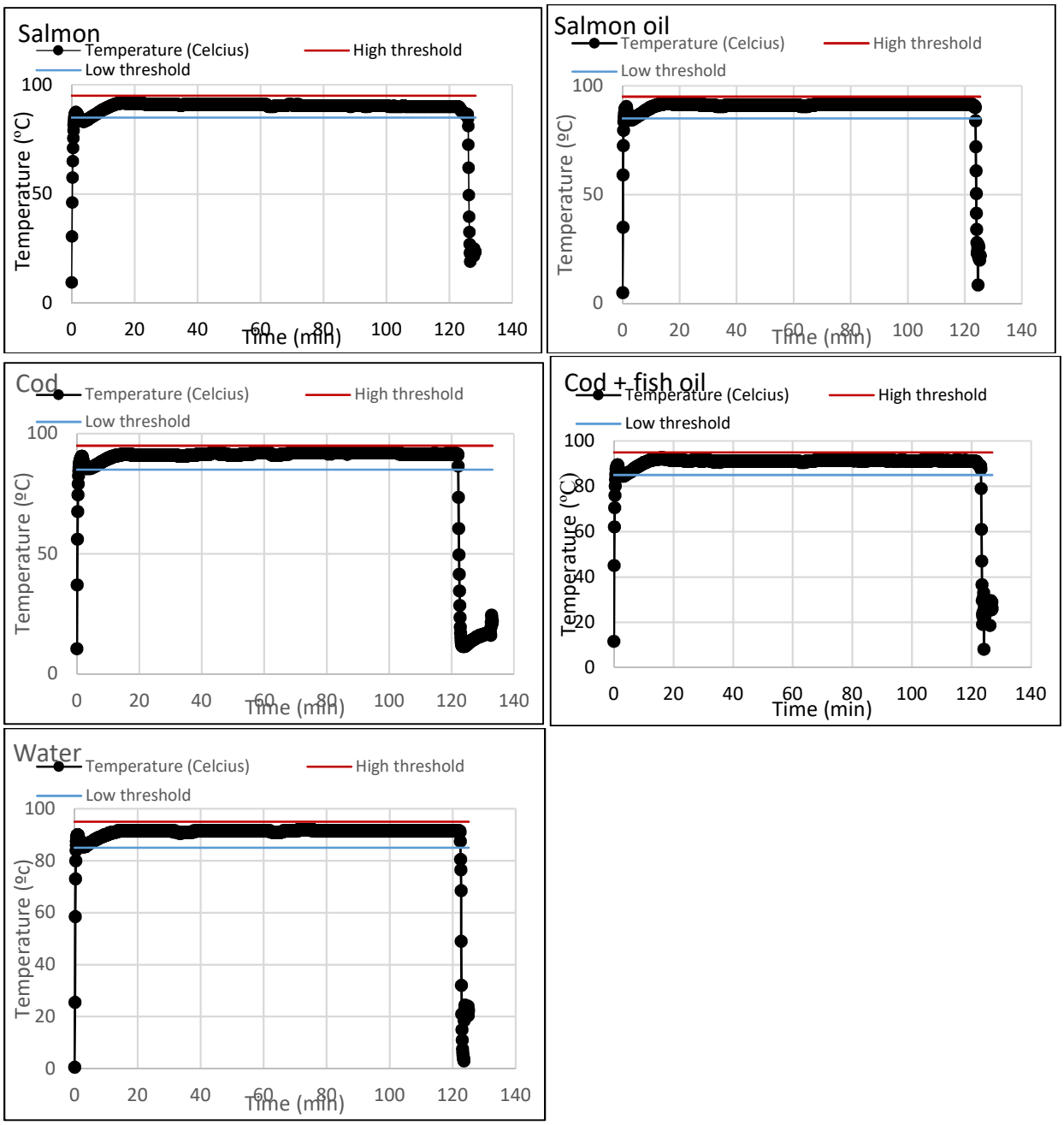
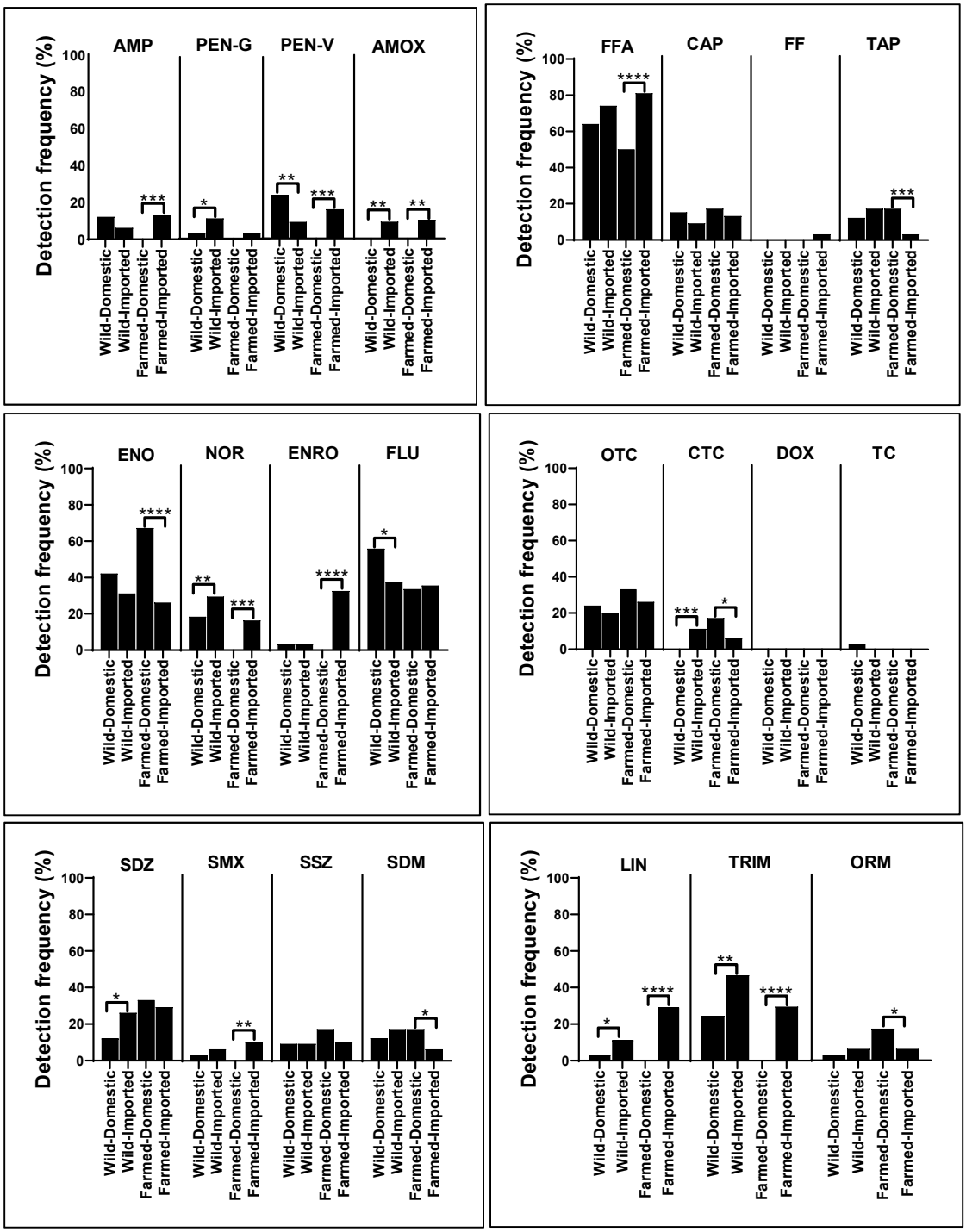


Figure S1. Temperature-time profile of fish (salmon and cod), salmon oil and water heated at 90 C. In order to monitor the temperature fluctuation, highest threshold for temperature set at 95 ºC (red line) and lowest temperature threshold was set at 85 ºC (blue line).


AMP
PEN-G
PEN-V
AMOX
FFA
CAP
FF
TAP
ENO
NOR
ENRO
FLU
OTC
CTC
DOX
TC
SDZ
SMX
SSZ
SDM
LIN
TRIM
ORM
Detection frequency (%)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported

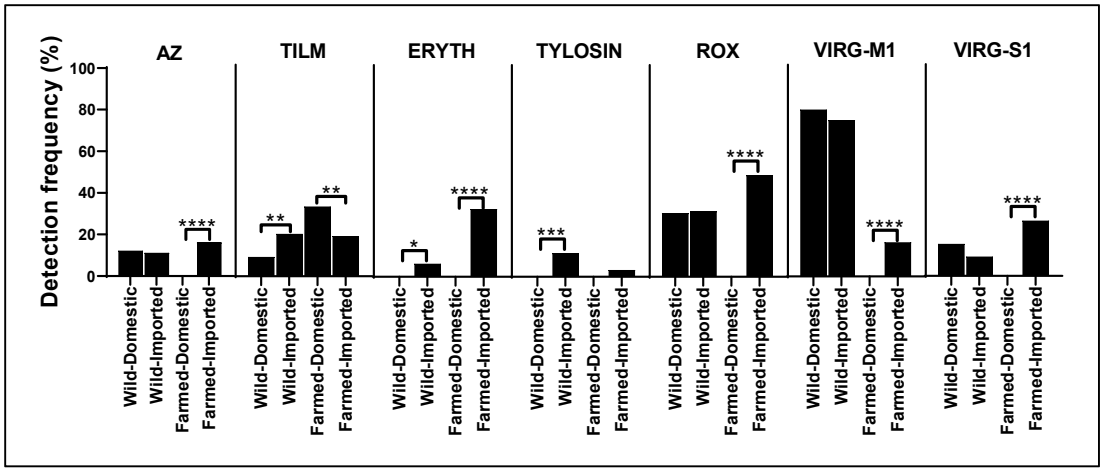
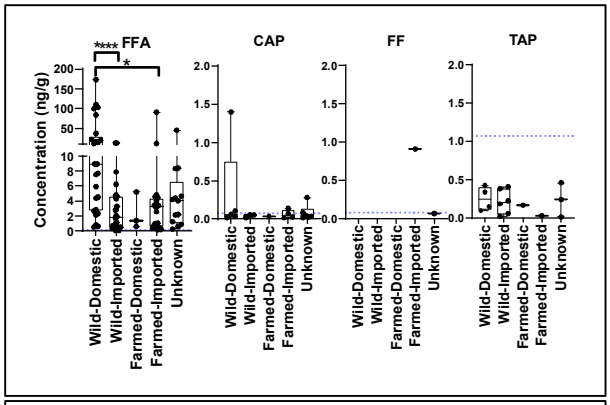
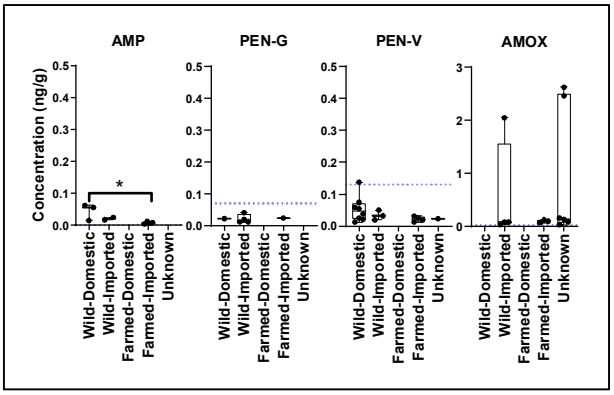
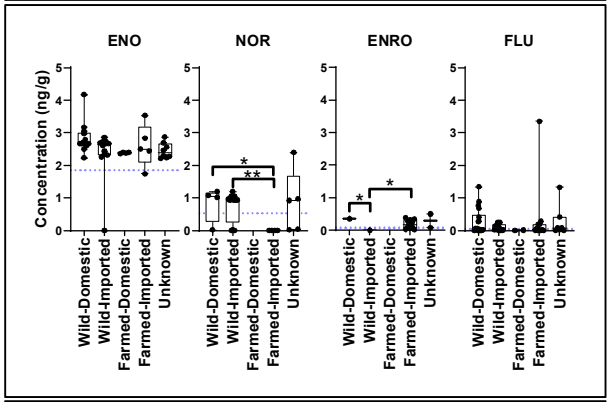
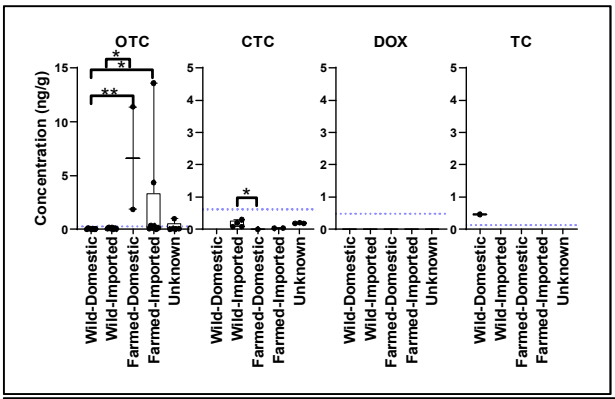


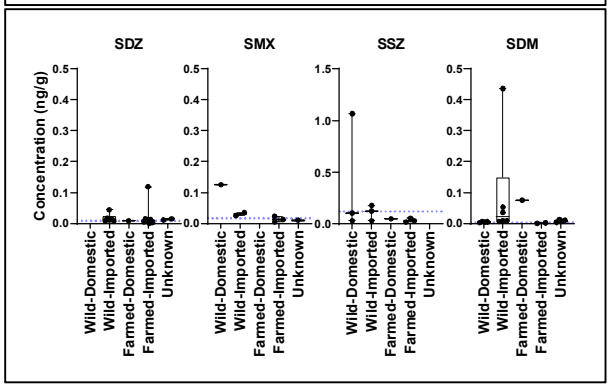
Figure S2. Frequency of detection of antibiotic residues in fish and shrimp samples of wild-imported (n=35), wild-domestic (n=33), farmed-imported (n=31) and farmed-domestic (n=6) production method presented at all levels of above LOQ, between LOQ and LOD and below LOD. Chi-square test was performed between domestic and imported samples within wild-caught and farm-raised groups to test the presence of significant difference. "****" indicates $p < 0.0001$, "***" indicates $p < 0.001$, "**" indicates $p < 0.01$ and "*" indicates $p < 0.05$.

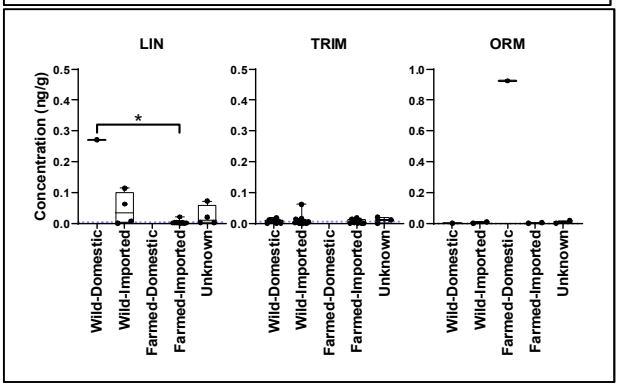

FFA
CAP
FF
TAP
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown


AMP
PEN-G
PEN-V
AMOX
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown


ENO
NOR
ENRO
FLU
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown


OTC
CTC
DOX
TC
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown


SDZ
SMX
SSZ
SDM
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown


LIN
TRIM
ORM
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown

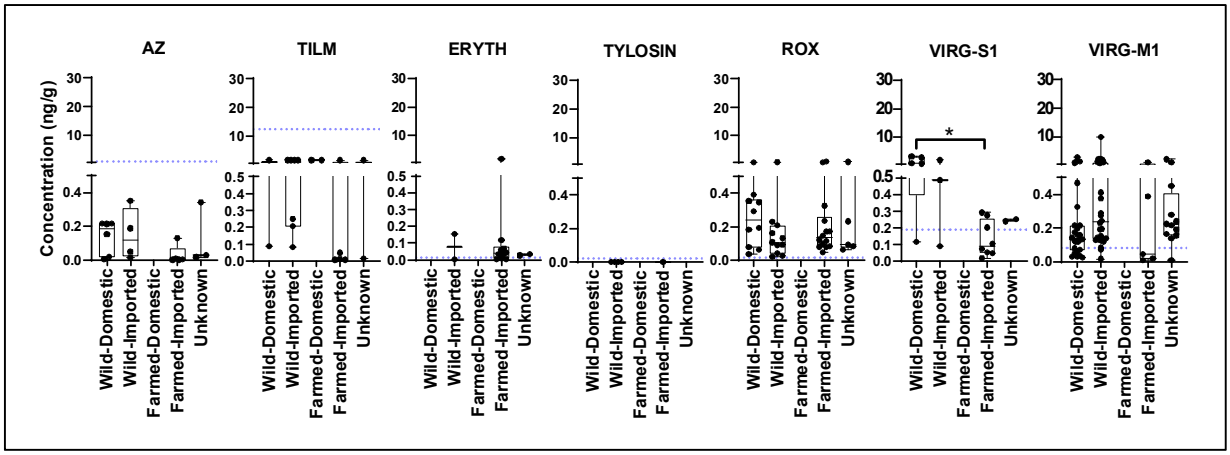

AZ
TILM
ERYTH
TYLOSIN
ROX
VIRG-S1
VIRG-M1
Concentration (ng/g)
Wild-Domestic
Wild-Imported
Farmed-Domestic
Farmed-Imported
Unknown

Figure S3. Boxplots of concentrations (ng/g) of antibiotic residues in fish and shrimp samples. Concentrations at levels above LOQ, between LOQ and LOD, and below LOD were included. The dotted line shows the minimum LOQ obtained from 3 runs. One-way ANOVA followed by tukey's post-hoc test or unpaired t-test was performed on log-transformed concentrations to test for significant difference between groups. "****" indicates $p < 0.0001$, "***" indicates $p < 0.001$, "**" indicates $p < 0.01$ and "*" indicates $p < 0.05$.

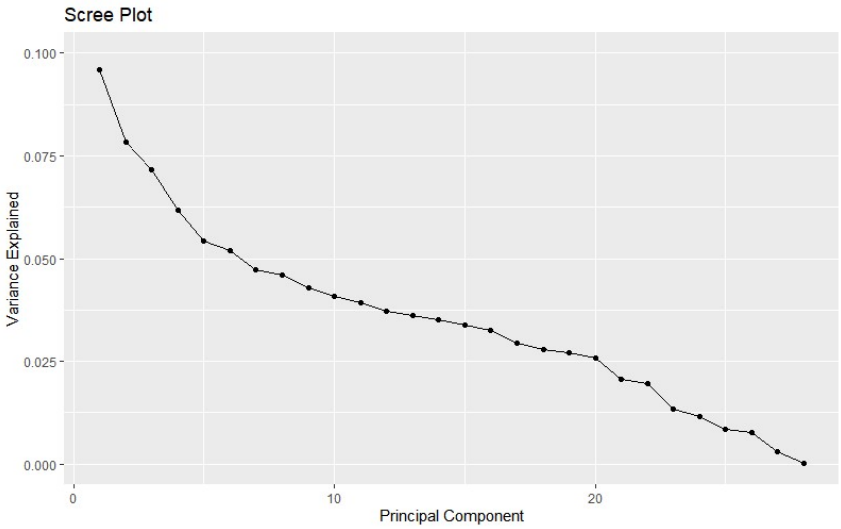
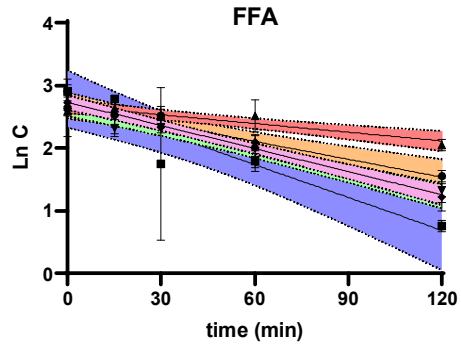
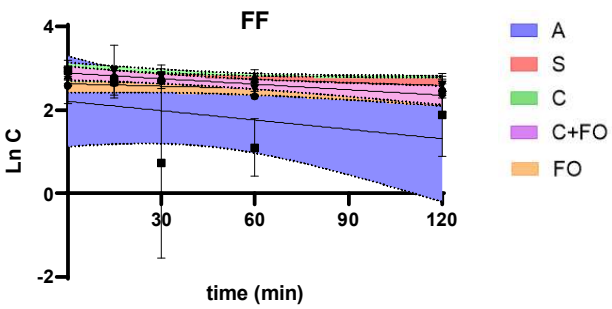
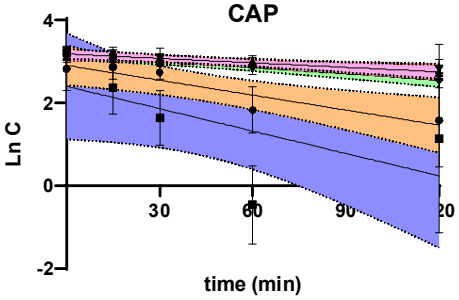


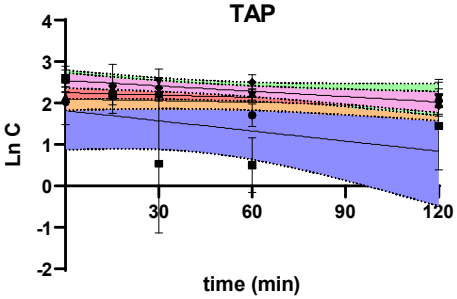
Figure S4. Scree plot showing the variance explained by each principal component in PCA analysis.

Amphenicols

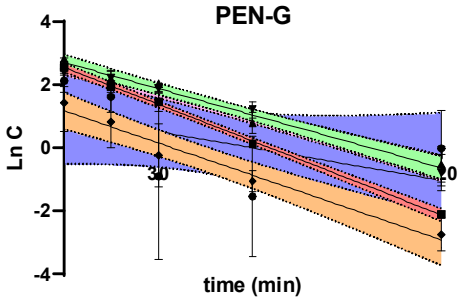


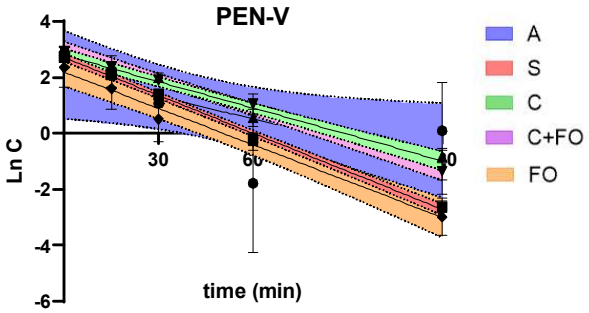


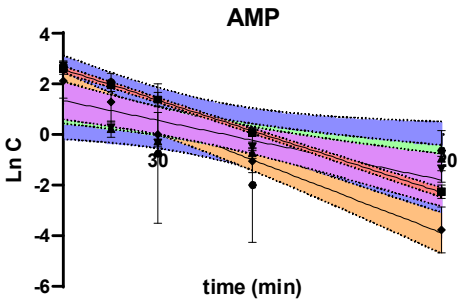




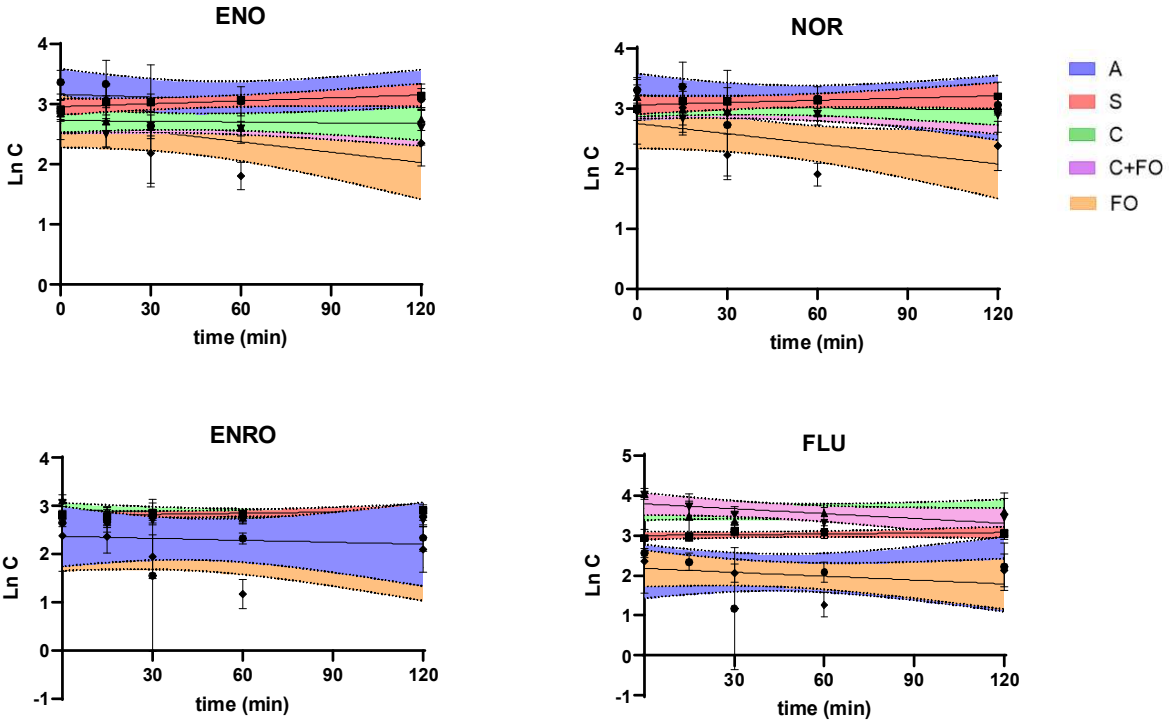
B-Lactams



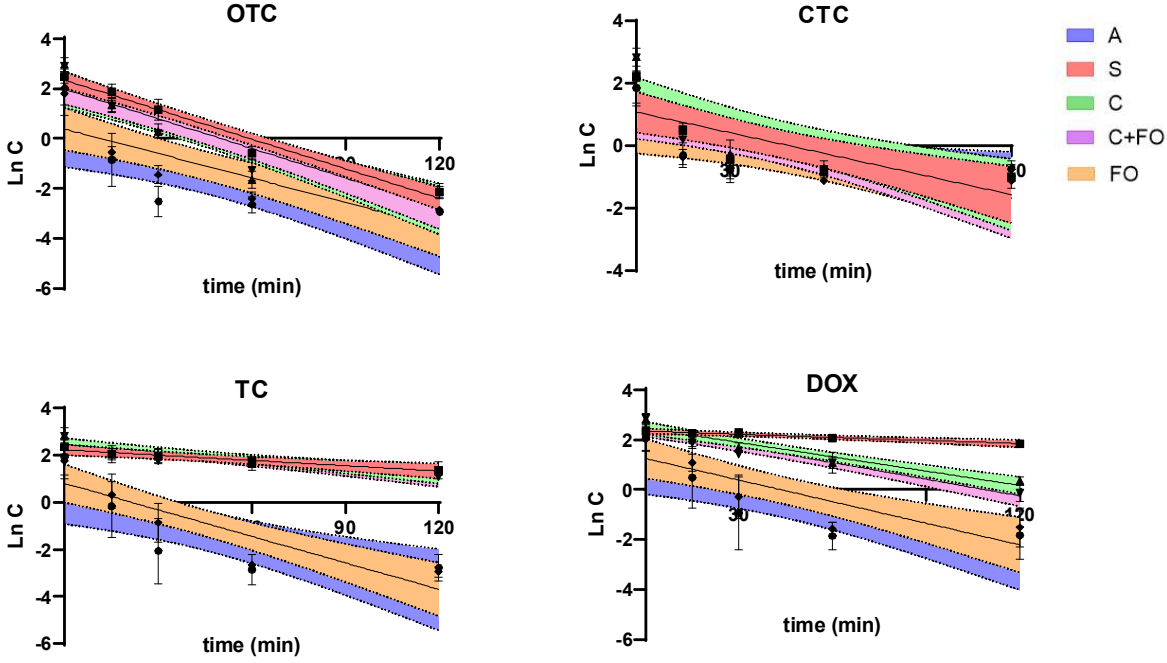




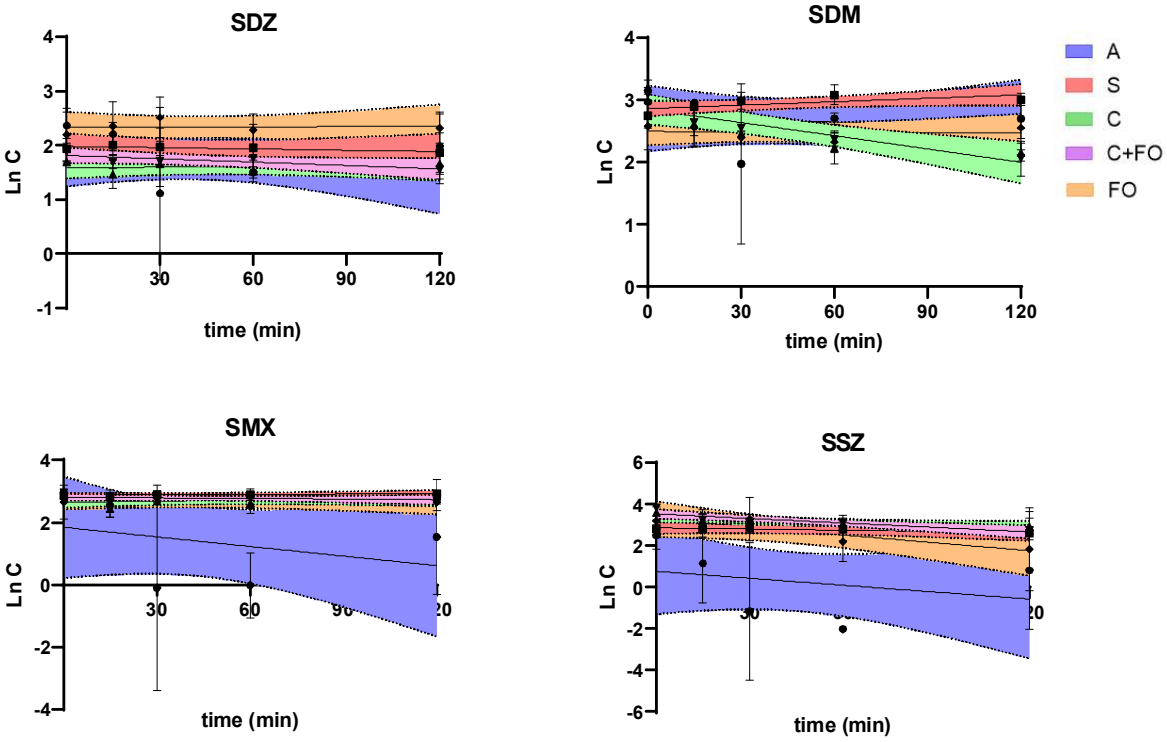
Quinolones



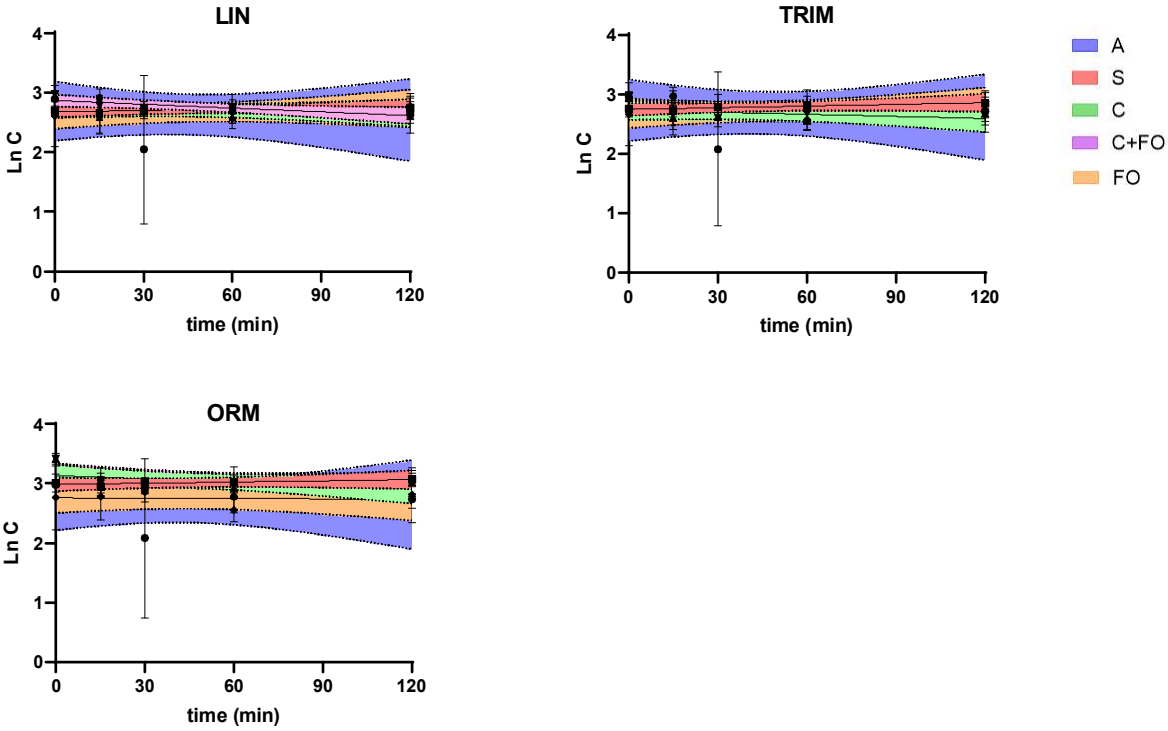
Tetracyclines



Sulfonamides



Lincosamides and dihydrofolate reductase inhibitors



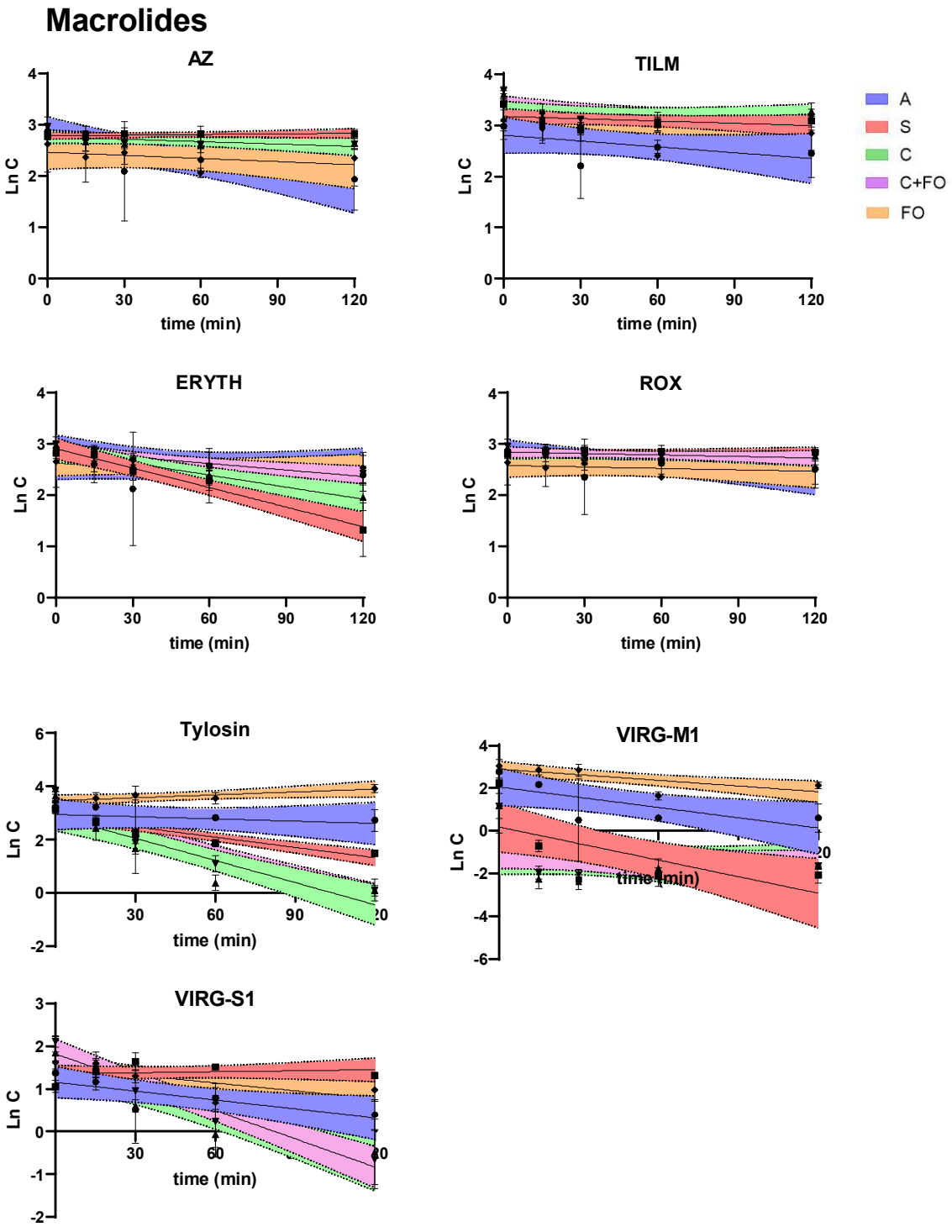


Figure S5. Effect of thermal processing on degradation of antibiotics in fish (cod and salmon), fish oil and water. Antibiotics were spiked into cod (C), salmon (S), cod + 10% fish (salmon) oil (C+FO), fish (salmon) oil (FO) and water (A) at 20 ng/g or 20 ng/mL and were heated at 90 ˚C

for 15, 30, 60 and 120 min. Antibiotic concentrations were transformed by natural log transformation and were fitted into simple linear regression. The slope of the linear fit corresponds to the first order degradation rate constant (k; min^{-1}). The colored region corresponds to 95% confidence interval constructed for the slope.

Chapter 5: Conclusion

My thesis provided new methodological insights in the measurement of antibiotics, and showed that antibiotics are widely prevalent in both wild-caught and farm-raised seafood, and that most are resilient to thermal degradation. For multi-residue antibiotics analysis, I found that antibiotics should be mixed close to the time of analysis (i.e. same day). I made the unexpected discovery that the QUEChERS method commonly used to extract antibiotics from seafood matrixes, reduces the sensitivity and accuracy of antibiotic measurements by causing ion suppression or enhancement on Ultra-high pressure liquid chromatography coupled to tandem mass spectrometry (UPLC-MS/MS). The phenomenon, also known as matrix effects, could not be eliminated by common clean-up methods that use solid phase extraction (SPE), but was compensated using internal standards.

In Chapter 2, I found that antibiotic standards were not stable when stored as a mixture in water: methanol irrespective of the storage temperature (4, -20 and -80 ºC) and presence or absence of sodium hydroxide. Some antibiotics, particularly quinolones, tetracyclines and some macrolides (Azithromycin and Tilmicosin) adsorbed onto the glass surface when stored in methanol: water and at -80 ºC for one week. Silanization of the glass surface improved the storage stability of quinolones and macrolides, indicating that these antibiotics are likely adsorbing onto the glass surface. On the other hand, silanization reduced the storage stability of tetracyclines and some other antibiotics including some of amphenicols, B-lactams, macrolides, sulfonamides and dihydrofolate reductases, implying that these antibiotics interact with the silanized glass surface likely via hydrophobic interactions. The data suggest that silanizing glass surface offers limited benefits towards improving the storage stability of all antibiotic standards. Thus, for analyses

involving multi-residue methods, antibiotic standards should be freshly mixed before UPLC-MS/MS analysis.

In chapter 3, I showed that QUEChERS extraction of antibiotics from salmon, as a representative seafood matrix, introduces matrix effects, in the form of ion suppressions and ion enhancements, on antibiotics analyzed by UPLC-MS/MS. Matrix effects are a problem in UPLC-MS/MS analysis because they can reduce the accuracy and sensitivity of measurements. The use of dispersive SPE clean-up, using bulk sorbents including C18, PSA and Na_2SO_4, following QUEChERS extraction did not improve matrix effects. Clean-up with hydrophobic lipophilic balance SPE columns improved matrix effects for some antibiotics, particularly early eluting analytes such as lincosamides, dihydrofolate reductase inhibitors, amphenicols, sulfonamides and B-lactams, but significantly reduced the antibiotics extraction recoveries. Carotenoids from salmon partially contributed to the observed matrix effects but were not major contributors.

A key finding in Chapter 2 is that the observed matrix effects were compensated by choosing proper internal standards that were linked to their antibiotic analytes (i.e. matched to each antibiotic based on structural similarity and chemical polarity). A limitation of this approach is that while it led to accurate quantitation of antibiotics, sensitivity was still low. Future studies should investigate the components of seafood extracts affecting the ionization of antibiotics in the mass spectrometry ion source, to resolve the matrix effects problem. This will enable the design of effective clean-up methods that remove matrix interferences.

In chapter 4, I used methodological take-aways from Chapters 2 and 3 to measure the extent of antibiotic contamination in seafood samples, and to test whether thermal processing affects the stability of antibiotics in matrix. Additionally, a health risk exposure assessment was performed. Antibiotic residues were found more frequently in farm-raised than in wild-caught seafood.

Additionally, imported seafood contained antibiotics more frequently than locally produced seafood in the U.S. Surprisingly, however, antibiotic concentrations were higher in wild-caught than in farm-raised seafood. This finding provides evidence of widespread environmental contamination of antibiotics in natural waters, and point to the potential bioaccumulation of antibiotics in wild-caught samples. Using this knowledge, I then addressed the question of whether cooking seafood degrades antibiotics, and whether such an effect is dependent on the lipid composition of the sample. This is because many antibiotics are lipid-soluble, leading to the hypothesis that antibiotics may be more stable in fish with high fat content compared to fish with low-fat (high protein) content. Water and oil were used as controls for these experiments.

By cooking fish, most antibiotics remained stable except for B-lactams, tetracyclines and some macrolides. Contrary to the hypothesis, fish fat content did not affect antibiotic degradation. Several antibiotic degraded faster in water than in fish matrix, suggesting that the fish matrix itself stabilizes these compounds possibly by binding to matrix components such as proteins and metal ions. Additionally, polar antibiotics such as B-lactams degraded more than non-polar antibiotics, suggesting hydrolysis is likely the main mechanism of antibiotics degradation.

In Chapter 4, I used the previously obtained exposure values to perform a health risk assessment. Antibiotic residues at the levels present in seafood do not pose toxic risks based on both carcinogenic and non-carcinogenic risk factors. However, this does not preclude the risk of developing antibiotic resistance which could occur at sub-inhibitory antibiotic concentrations [243] such as the levels found in seafood. Also the risks of exposure to vulnerable populations such young children need to be further examined in view of rodent data showing that prenatal exposure to antibiotics increases the risk of developmental abnormalities in the offspring [244-246].

Some limitations should be acknowledged regarding the work presented in this thesis. First, in chapter 2, the stability of the antibiotic standard mixture was only tested in the water: methanol. However, the storage stability of antibiotics in seafood matrix was not addressed. This is relevant because the seafood samples used in Chapter 4 were stored for approximately 2 years at -80 ºC before analysis. Previous studies have shown that tetracyclines, sulfonamides, quinolones, macrolides and aminoglycosides remained unchanged in porcine muscle for at least 3 months when stored at -18 ºC. However, some B-lactams including ampicillin and cloxacillin were reduced by 17-30% in less than 3 months in porcine muscles stored at -18 ºC compared to muscles stored at -70 ºC [132]. These studies point to the possibility that antibiotics within their matrix are likely more stable when stored at -70 ºC or below. However, this needs to be confirmed in future long-term stability studies.

In chapter 3, the matrix effects were compensated by using internal standards. While this approach ensures accuracy of analysis, the sensitivity of analysis still remains impacted. Therefore, it is likely that the antibiotic levels in seafood tested in chapter 4 are underestimated due to the lack of optimization of method sensitivity.

In chapter 4, sampling and testing was only performed in California, since the fish samples were collected from Orange County and the shrimp samples were purchased from grocery stores in Davis. Although these samples came from multiple sources, the results may not be entirely generalizable to other states in the U.S. or other countries. This is because the regional distribution of seafood can vary depending on many factors including consumers preference, regional economies, importing sources, etc. Future studies examining antibiotic contamination in multiple ports of entry and other countries are needed.

One of the knowledge gaps that this thesis did not address is whether bioaccumulation in wild-caught fish explains the higher concentration of antibiotics. Other explanations include the accumulation and slow release of antibiotics in sediments. Understanding why wild-caught seafood is more contaminated than farm-raised seafood is critical for mitigation efforts.

Overall, this thesis provided methodological insights in antibiotic measurements, as well as new information on the extent, source and type of antibiotic exposures from thermally treated and untreated seafood. The findings are important for guiding public health efforts towards reducing the burden of antibiotics in the environment and human exposures. More work is needed to understand whether antibiotics, at the levels seen in this study, may lead to antibiotic drug resistance or adverse health effects in vulnerable populations.

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