Reconnaissance of 47 Antibiotics and Associated Microbial Risks in Seafood Sold in the United States

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Keywords: Aquaculture; Oxytetracycline; Antibiotics; Antibiotic Resistance; Seafood.

Abstract

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

Highlights

- 5 out of 47 antibiotics were detected in shrimp, salmon, tilapia, and trout.
- Oxytetracycline is the most commonly detected antibiotic compound.
- Publications reporting antibiotic resistance in aquaculture have increased 8-fold over 3 decades.
- We report a low risk of drug exposure from consumption of U.S. seafoods.
- We recommend vigilance toward stemming microbial risks.

1. Introduction

It is estimated that within the next few years, aquaculture will account for almost 40% of total global seafood production by weight, up from 4% in 1970 [1, 2]. This increase to a projected worldwide production of 83 million metric tons in 2013 has been due to a heightened demand for seafood, improved aquaculture techniques, emergence as a key cash crop in certain regions of the world, and recognition as a cheaper way to obtain high-quality protein [2, 3]. However, as production surges, many aquaculture facilities resort to antibiotics to combat diseases in an environment that creates ample opportunities for bacterial pathogens to thrive [4]. Antibiotics are also commonly used as a prophylactic, sometimes on a daily basis [5]. Although some promising alternatives such as short-chain fatty acids and bacteriophage therapy have been proposed, many are not ready for mass usage [5]. Developed vaccines show promise in reducing antibiotic usage [4], but are only available to treat certain diseases and are not as cost-effective as antibiotics. Thus, the usage of antibiotics in aquaculture remains high.

Consequences associated with the use of antibiotics in aquaculture include the spread of antibiotics into the environment [6, 7], residual concentrations left in seafood, high exposure by aquaculture facility personnel, and antibiotic resistance development [3, 4]. Another issue is the impact of antibiotics on the animals themselves, such as potential changes in genetic expression [8, 9] and physiological anomalies. These physiological anomalies include malformation of the spine reported in fish exposed to oxytetracycline [9, 10].

Many of the antibiotics used in aquaculture are also used in human medicine [11]. Amoxicillin and ampicillin are commonly prescribed for treating bacterial infections such as pneumonia and

gastroenteritis [12]. As fish are a potential source of bacterial pathogens for humans, it is important to monitor the spread of antibiotic resistance amongst seafood [13]. Resistance to the most commonly applied antibiotics has been found in previous studies [3, 14, 15, 16], including several that are multi-drug resistant (MDR) to many classes of antibiotics important in treating human infections [16, 17, 18, 19]. Thus, detecting and monitoring antibiotic residues in seafood is critically important to reduce potential environmental and human health risks.

A large portion of aquaculture takes place in countries with few regulations and limited enforcement [20], creating the need to monitor imported seafood strictly for antibiotic residues and presence of pathogens. In this study, twenty-seven seafood samples were collected by the National Oceanic and Atmospheric Administration (NOAA) from stores in Arizona and California for analysis. Samples included five of the top ten most consumed seafood varieties in the U.S.: shrimp, tilapia, catfish, swai, and Atlantic salmon. Trout with visible deformed spines were also analyzed. Using liquid chromatography tandem mass spectrometry (LC-MS/MS), 47 antibiotics identified from literature as drugs of concern were analyzed for using two methods. We also conducted a meta-analysis of published data on antibiotics and resistance development to note trends in aquaculture over the last few decades.

2. Materials and Methods

2.1 Samples and Preparation. A collaborating NOAA consumer safety officer obtained samples (n= 27) from retail grocery stores in Arizona and California (in southwest U.S.) over a period of three months from June to August in 2012 (Table 1). Samples originated from 11 different countries. Each sample was sold as a pre-packed unit or bought from store counter displays,

meaning that each sample sometimes included multiple fish. Negative controls consisted of catfish donated from Louisiana State University that were never exposed to antibiotics. Normal and deformed rainbow trout (n=3 for each) were obtained to survey the potential link between antibiotic exposure and spinal deformities. Atlantic salmon marketed as "antibiotic-free" was also obtained from a local health food store.

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

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

[a]Rank in most consumed seafood. Data from National Fisheries Institute [46].
[b]Units: metric tons and millions of U.S. dollars. Fresh and frozen seafood imported for human consumption. Data from National Oceanic and Atmospheric Administration (NOAA) for the 50 U.S. states, District of Columbia, Puerto Rico, and the U.S. Virgin Islands [47]. Numbers have been rounded.
[c]Units: metric tons and millions U.S. dollars. Commercial U.S. landings and aquaculture. Data from NOAA [47]. Numbers have been rounded. 2012 US aquaculture data were unavailable, thus limiting reported values to 2011 data.
[d]11 total composites were made.
[e]Ind= Indonesia, Tha= Thailand, Ban= Bangladesh, Vie= Vietnam, Mex= Mexico, Pan= Panama, Chi= China, U.S.= United States, LSU= Louisiana State University, Can= Canada, Chl= Chile, Sco= Scotland.
[f]Pre-packaged seafood was provided in factory-sealed plastic packages.
[g]Catfish bred from eggs for research purposes never exposed to antibiotics were provided by Dr. Javier Santander of Arizona State University and from Louisiana State University.
[h]Salmon sold as "antibiotic-free" salmon.
[i]Swai is also marketed as pangasius, channel catfish, catfish, basa, and tra, among other names. Thus, import data were not available, due to this inconsistency in labeling.

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

Turbo Force 3000 Series Electric Meat Grinder, Lincoln, Nebraska). Between processing of individual samples, the grinder was cleaned with warm water and soap, and then rinsed separately with acetone, ethanol, and distilled water three times each. Composite samples were prepared by pooling equal amounts of individual samples to result in 11 composite samples: farmed shrimp, wild-caught shrimp, farmed tilapia, farmed catfish, antibiotic-free catfish, farmed rainbow trout of normal habitus, farmed rainbow trout with deformed spine, farmed international Atlantic salmon, farmed antibiotic-free Atlantic salmon, farmed U.S. Atlantic salmon, and farmed swai (Table 1).

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

Samples were analyzed by positive electrospray ionization on a triple quadrupole LC-MS/MS in multiple reaction monitoring (MRM) mode using a Waters Micromass Quattro Ultima LC-MS/MS system paired with a Waters LC 2795. Chromatography was conducted using reverse-phased C_{18} column (Waters, Milford, MA). A total of 60 pharmaceuticals were analyzed according to the AXYS Method MLA-075, a modification of the USEPA Method 1694 as described previously [21]. Out of the 60 analytes screened for, 47 were antibiotics, and are the focus of this paper (Table 2 and SI Table S1). Two methods were used on the same extract (injection volume: 10 uL) to analyze for tetracyclines and non-tetracyclines, respectively. The tetracyclines method, totaling 30 minutes in duration, had solvent A consisting of an equal mixture of acetonitrile and methanol with 0.5 mM oxalic acid and 0.5% (v/v) formic acid; solvent B consisted of HPLC-grade water containing 0.5 mM oxalic acid and 0.5% (v/v) formic acid. The starting mixture was 10% solvent A (flow rate 0.2 mL/min), increased to 90% A by minute 20 at a flow rate of 0.23 mL/min. The non-tetracyclines method had a run time of 33 min, using as solvent A HPLC-grade water with 0.1% formic acid and 0.1% ammonium formate, and as solvent B a mixture of equal amounts of acetonitrile and methanol. The starting mixture was 95% solvent A (flow rate 0.15 mL/min), increased to 100% solvent B by minute 23 at a flow rate 0.3 mL/min. For the 10 of the 60 total compounds for which a respective stable-isotope labeled analog was available, the concentration was determined using the isotope dilution technique [22]. For the remaining 50 compounds where a labeled analog was not available, the concentration was determined using an alternate isotope-labeled internal standard (see supplemental information).

Precision between intraday samples and duplicates was expressed as relative percent difference (RPD), which was calculated using the following expression as reported previously [23]:

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

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

2.3 Quality Assurance and Control. Several tests were performed before and during sample analysis to ensure system and laboratory performance. Initial calibration was performed using labeled surrogates, recovery standards and authentic targets to encompass the working concentration range. Retention times of native and labeled compounds had to be within 0.4 minutes of the respective retention time established during the previous calibration. A mid-level solution was analyzed every 12 hours or every 20 samples, whichever occurred first. All calibration curves consisted of at least 5 consecutive calibration levels. Native compounds with labeled surrogate standards had to elute within 0.1 minutes of the associated labeled surrogates in order to be authenticated. Method blanks and matrix spikes to evaluate recovery rates were also conducted, and duplicates were also analyzed for 5% of test samples within each batch on the same day (containing 7 or more test samples). Method detection limits (MDLs) were determined as specified by EPA Federal Regulation 40 CFR Part 136, Appendix B.

2.4 Meta-Analysis of the Peer-reviewed Literature for Antibiotic Resistance Articles. A literature search of the Web of Knowledge was performed for studies published between 2003 and November 2013 using the search terms “antibiotic resistance AND aquaculture” and “antibiotic resistance AND seafood” to identify relevant strains of bacteria isolated from seafood shown to

contain antibiotic resistant microorganisms. Only microbial strains isolated from finned fish or shrimp were included to make it relevant to this study and only seafood for human consumption was included; strains further had to show resistance to one or more specific antibiotics (as opposed to mere classes of antibiotics). Resistance to only four antibiotic classes, tetracyclines, sulfonamides, penicillins, and quinolones, was examined because these are the top drug classes customarily screened for in our study.

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

2.5 Calculation of Theoretical Maximum Concentrations in Individual Samples Used in Composites. This study employed a composite sampling approach. Samples were pooled to create 11 composites from 27 individual samples. Theoretical maximum concentrations in individual samples processed were calculated using the conservative formula:

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

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

3. Results and Discussion

3.1 Method Performance. As this paper focuses on antibiotics, further discussion will only pertain to the 47 antibiotic analytes that were screened for. Method detection limits for the various antibiotics ranged from 0.1 ng/g (roxithromycin/sulfadimethoxine) to 25.5 ng/g (minocycline) fw of seafood (Table 2; Supplementary Information: Table S2). Recoveries of the 47 antibiotics ranged from 15.9% (4-epianhydrochlortetracycline) to 138% (sulfathiazole), with the majority (35 out of 47) placing in the preferred range of 70 to 130% (Table 2). No laboratory contamination was observed in method blanks. Method performance in this study was favorable and comparable to previously reported results [23, 24].

3.2 Occurrence of Antibiotics in Seafood. Seven out of eleven composite samples were found to have detectible quantities of antibiotics, including oxytetracycline, 4-epioxytetracycline, sulfadimethoxine, ormetoprim, and virginiamycin (Table 2). The most commonly detected antibiotic was oxytetracycline, which is the number one used antibiotic in aquaculture, with 12 of the top 15 aquaculture-producing countries reporting usage [3]. It was detected at a concentration of 8.6 ng/g fw, along with its 4-epimer at 4.1 ng/g fw, in farmed international Atlantic salmon comprised of samples from Chile and Canada (Figure 1), which are among the top four salmon-producing countries [1]. As the 4-epimer is a known degradation product of oxytetracycline [25] it is likely that a higher oxytetracycline concentration was originally in these samples. Tetracyclines are regulated in the U.S. as a sum of all parent antibiotics and their 4-epimers [26]. The resultant combined concentration in farmed international Atlantic salmon of 12.6 ng/g was still under the maximum permitted concentration of 2 µg/g in finfish (Table 3).

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

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

Superscripts of detected concentrations indicate sample number; see Table 1 for additional sample information.

[a]Highest method detection limit (MDL) for each analyte is reported. See Table S2 in the Supplemental Information for all MDLs.

The unexpected detection of oxytetracycline at a concentration of 7.7 ng/g fw in wild-caught shrimp imported from Mexico may be due to several reasons. Unintentional or intentional mislabeling of the product and cross-contamination of seafood during handling, processing and packaging are possible. Uptake of the drug from coastal waters and sediments impacted by inputs of raw and treated wastewater [27] also could explain the observed detection but ultimately the origin of contamination remains unknown.

Oxytetracycline was also detected at concentrations of 2.7 and 3.9 ng/g fw, respectively, in farmed tilapia and in farmed rainbow trout with visibly deformed spines (Figure 2A). Oxytetracycline was not detected above the detection limit of 2.4 ng/g in trout without visible spinal deformities (supplemental information T2). Detection of the latter corroborates earlier reports that this antibiotic may cause spinal deformities in certain species [10]; however, due to the limited number of individual samples available (n =3), the present study was underpowered and cannot ascertain causation. As trout is a major market in the U.S., with over 700 trout-rearing farms [28], further work with a larger sample size is needed to elucidate the connection between oxytetracycline dosing and spinal deformities in trout and other fish species. Among the large group of sulfonamides, only sulfadimethoxine was detected and only in a single seafood variety, in farmed shrimp at 0.3 ng/g fw. Sulfadimethoxine reportedly is used by 4 of the top 15 aquaculture-producing countries [3]. Yet, although screened for previously [29, 30] and several detection methods have been developed [31, 32], the result reported here constitutes the first detection of this drug in shrimp. There is no U.S. MRL set for this drug in shrimp, although it is regulated in salmonids and catfish at a level of 0.1 µg/g fw (Table 3).

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

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

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

The occurrence of antibiotics in seafood above method detection limits in the low ng/g range attained here appears to be the exception rather than the norm. Five antibiotics were detected at low ng/g concentrations in this survey. The present study is the first to consider the top consumed seafoods in the U.S. as well as the first to survey a large number of antibiotics. The majority of these antibiotics have never been screened for in our food supply. This study also represents samples from 11 countries (Figure 1), 8 of which are among the top 15 aquaculture-producing countries [3]. Results of this study of modest sample size suggest that seafood, regardless of whether wild-caught, farmed, imported, or domestically produced, is typically compliant with U.S. chemical regulations. However, the results need further confirmation, ideally by studies featuring a large sample size.

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

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

[a]FDA USDA CFR 21 [26].
[b]EU commission regulation no. 37/2010, Dec. 2009 [48].
[c]FAO 2012 Report [40].
[d]Codex Alimentarius Commenssion (CAC), 2009 [49].
[e]Swine liver.
[f]Sum of tetracyclines in finfish.
[g]Cattle.
[h]Cattle liver.
[i]Swine.
[j]Salmonids and catfish.
[k]Different forms of penicillin are not differentiated. Chicken.
[l]Trout.
[m]All fish.
[n]Sum of ciprofloxacin and enrofloxacin.
[o]Sum of 4-epimer plus parent drug.
[p]Sum of parent drugs.
[q]Poultry.
[r]Includes 4-epianhydrotetracycline, 4-epianhydrotetracycline, 4-epichlortetracycline, 4-epioxytetracycline 4-epitetracycline, demeclocycline, isochlortetracycline, minocycline. Currently unregulated/information not available for: anhydrochlortetracycline, anhydrotetracycline, azithromycin, cefotaxime, clarithromycin, clinafloxacin, omefloxacin, norfloxacin ofloxacin, and roxithromycin. Currently, no MRLs have been set in U.S. for ciprofloxacin, flumequine, oxacillin, oxolinic acid, sarafloxacin, and trimethoprim.

3.3 Antibiotic Resistance Development in Seafood. Although the concentrations reported here are less than the FDA allowed maxima, these sub-therapeutic drug concentrations can often select

for and enrich resistant bacteria [33]. There has been a notable increase in resistant microbial strains associated with the antibiotics and seafoods examined in this study. Out of 179 *Escherichia coli* strains isolated from commercial seafood in a study by Ryu et al., 55 strains were found to be resistant to tetracycline [14]. Another 34 strains were found to hold intermediate resistance to tetracycline, which can be affected and selected for by sub-therapeutic antibiotic concentrations. Nawaz et al. also reported isolation of MDR *Klebsiella* spp. bacteria from imported shrimp obtained from grocery stores [34]. The identification of these strains may be interpreted as being the result of extensive human use and misuse of antibiotics in the clinic, community, agriculture, and in animal husbandry such as aquaculture [33]. The top antibiotics used by heavy aquaculture producers include the following: oxytetracycline, oxolinic acid, chloramphenicol, erythromycin, furazolidone, trimethoprim, sulfadiazine, ampicillin, florfenicol, flumequine, and sulfadimethoxine [3]. All of these antibiotics are included on the WHO list of critically/highly important antibiotics for human health [11, 34, 35]. Multiple studies in the last three decades have revealed resistance to many of these antibiotics, the majority of which were screened for in this study (Figure 3A). The fact that seafood examined for bacteria has resulted in isolates belonging to pathogenic genera causing infections in humans (*e.g.*, *Salmonella*, *Vibrio*, *Escherichia*) [7, 14, 16] increases the likelihood of resistance spread from aquaculture to people. This poses a risk to consumers as well as employees coming into contact with the seafood from production to store delivery.

Indeed, literature volume statistics summarized in Figure 3 show that the topic of resistance to many antibiotics screened here is a major area of concern for the aquaculture community. The number of publications linking resistance to seafood has increased by 800% between the 1990s

and today (Figure 3B). The majority of papers report the ineffectiveness of tetracycline and oxytetracycline as one of the most commonly seen resistances. The observed publication trend also acknowledges an increased awareness of the fact that exponential growth has taken place in the aquaculture industry in the past few decades. This trend also suggests an association between the heavy usage of oxytetracycline (the number one used antibiotic in aquaculture) and resistance development.

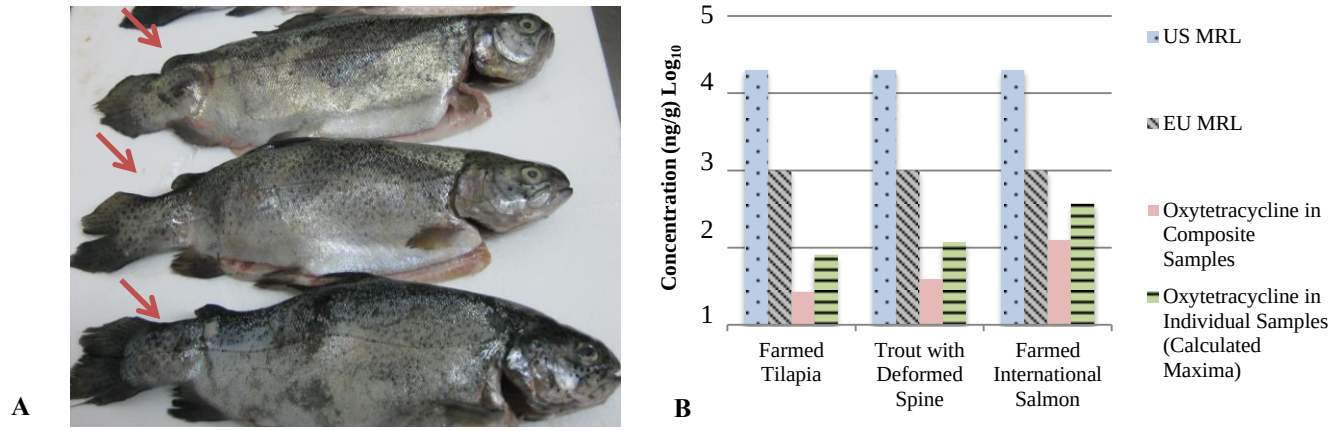

Figure 2. Farmed trout with visible spinal deformities and applicable U.S. and EU MRLs in composite and individual samples. Panel A shows an image of spinal deformities in trout analyzed in this work. Arrows indicate abnormal spinal curvatures (Photo credit: Don McBride, NOAA, 2012). Panel B shows a comparison of oxytetracycline concentrations determined in this study to maximum residue limits (MRLs) allowed in the United States (U.S.) and the European Union (EU) [26, 48]. Concentrations of oxytetracycline and 4-epioxytetracycline in farmed international salmon were added, as regulation is for maximum total tetracyclines.

Some bacterial strains identified in our literature review were found to be completely or intermediately resistant to certain antibiotics [14, 36]. Furthermore, the transfer of plasmids among bacteria on seafood has been reported [37]. Strains were found to have minimal inhibitory concentrations (MIC) far lower than the MIC requirement for the "resistant" classification, indicating that very low concentrations of antibiotics can select for resistance. One study found that only about half of the isolates from their aquaculture samples had MICs above the

"resistant" concentration of 128 µg/mL; some isolates exhibited MICs as low as 0.25 µg/mL, over 500 times less than the classification of resistance-promoting concentration [38]. In Chile, the reported dose of oxytetracycline through feed is 100-120 µg per g fish per day, administered for 14-21 days, depending on the disease [39]. In China, the preventative dose for the fluoroquinolone compound oxolinic acid is 10-20 µg per g fish per day for 4-7 days [40]. These concentrations currently in use are known to exert selective pressure. Since many of these antibiotics also are used in human medicine, selective pressure may promote the occurrence of resistant strains of potential human health concern. Overall, the information compiled in Figure 3 shows that the development and occurrence of drug resistant bacteria in seafood is an issue that is both timely and of notable importance. Thus, to ensure the safety of the food supply in the U.S. and abroad, the monitoring of seafood has to focus on both the residues of aquacultural drugs themselves and the drug resistance in pathogens these antibiotics can trigger.

3.4 Study Limitations. This study employed composite sampling. This approach is well suited for the economical screening of a large number of analytes and for accurately determining average concentrations therein [41, 42]. This method of sampling was chosen here because the purpose of this study was to conduct a large-scale screening of many analytes. However, this methodology is inappropriate for determining the full range of concentrations (*i.e.*, minima and maxima) as well as detection frequencies. Accordingly, theoretical maximum concentrations of oxytetracycline and sulfadimethoxine were calculated for individual samples and the resultant values represent conservative estimates that are likely higher than the true concentration. The oxytetracycline values of 8.1, 11.7, and 37.8 ng/g calculated, respectively, in farmed tilapia, farmed trout with spinal deformities, and farmed international salmon are well below the U.S.

limit of 2,000 ng/g (Figure 2B). Note that the concentration of 37.8 ng/g calculated for salmon includes both oxytetracycline and 4-epioxytetracycline; it is provided in this form because tetracyclines are regulated as a sum of drugs of this class. Values calculated for sulfadimethoxine (1.7 ng/g for each country's sample) is also significantly under U.S. regulatory limits.

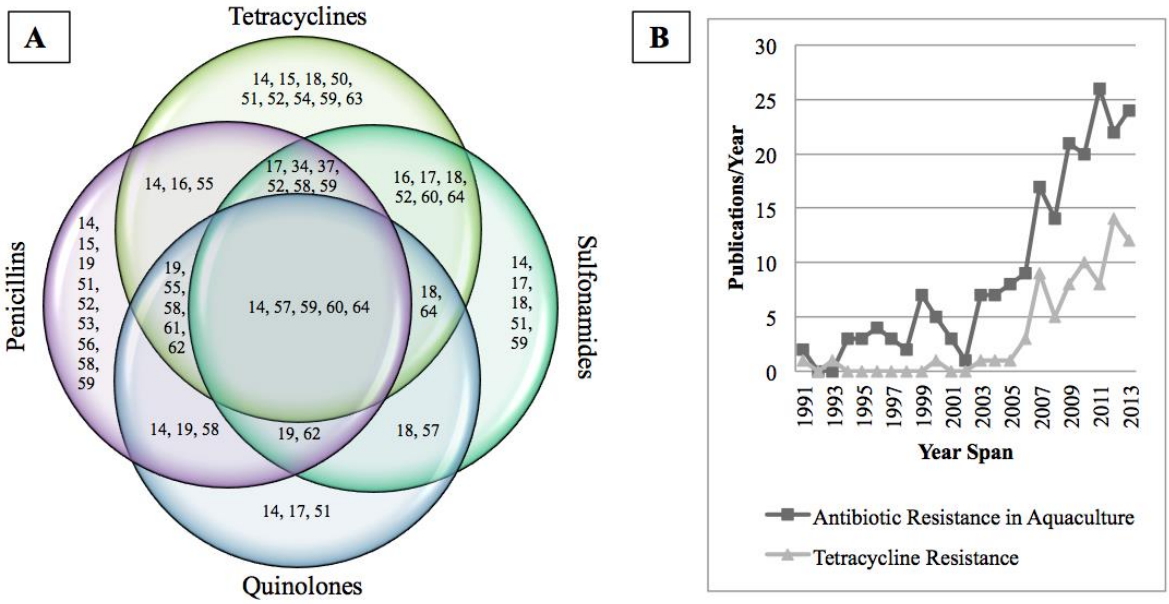

Figure 3. Published studies reporting resistant bacteria isolated from aquaculture and seafood. Panel A shows select studies from 2003-2013 reporting the presence of bacteria resistant to 4 groups of antibiotics found on seafood available for human consumption. Numbers correspond to references. Panel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

Another limitation is that sampling was done only in Arizona and California. The obtained results may not necessarily apply to other states and alternate sources (*i.e.*, countries) of commercial seafood. Many wild-caught seafood varieties were not available for this survey because the vast majority of seafood for consumption in the U.S. is only readily available from aquaculture operations. Also, as we obtained fresh seafood in the form most consumers choose, samples were either whole animals or fillets and either pre-packaged or loose, which means that

variation in handling and processing by the producer may affect antibiotic preservation and degradation in the tissue. This variation, as well as antibiotic sources that do not originate from aquaculture, could also have contaminated the seafood and affected our data.

Samples were collected in June-August, 2012 and analyzed in November 2012, following storage for 3-5 months at -20°C. A previous study, examining the effect of sample storage at -18°C, showed that tetracyclines, sulfonamides, quinolones, macrolides, and aminoglycosides are stable and remain intact structurally and quantitatively, as demonstrated using a porcine muscle matrix [43]. However, penicillins were observed to attenuate, by about 30% and 20%, respectively, for ampicillin and cloxacillin over the course of 3-6 months [43]. Hence, the concentrations of penicillins at the time of purchase in samples of seafood analyzed here may have been higher than the values of less than <1.2 to <1.6 ng/g fw reported here.

Our sample size of 27 is of a magnitude similar to other studies that utilized composite sampling to investigate poorly characterized potential human exposure sources [44, 45] The goal of the present work was not necessarily to identify specific antibiotics in individual samples, but rather to conduct a large-scale screening of U.S. seafood to assess whether there is a need for more aggressive monitoring. Whereas the present dataset cannot prove the safety or danger of imported seafoods, it provides an incremental, yet significant step forward in assessing the safety of the U.S. seafood supply. Data made available here suggest that there is no immediate threat to human health from trace levels of the analytes surveyed in this work. However, additional studies using a larger sample size would be beneficial to confirm the findings and conclusions of the results obtained here.

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

5. Conclusions

This study surveyed the concentrations of 47 antibiotics in 6 different seafood varieties originating in 11 countries purchased exclusively from the southwestern U.S. All samples studied demonstrated compliance under current federal regulations, suggesting that they are chemically safe to consume. This conclusion could be drawn from the analysis of pooled samples, an approach that did not permit to determine the actual concentration in each individual sample entering the survey, however. Five antibiotics were found at detectable levels and estimated concentrations were relatively low (0.3-8.6 ng/g fw). However, the development and spread of antibiotic resistance is a public health priority that is divorced from the regulatory limits designed to prevent adverse outcomes from human ingestion of drugs. Antibiotics present at levels well below regulatory limits still can promote the emergence of (multi-) drug resistant microorganisms. Future studies are warranted to fully understand the connection between aquacultural use of antibiotics, development of drug resistance, human exposure to resistant pathogens, and ensuing morbidity and mortality in seafood consumers. The trend in the last 3 decades of notable increases in the number of resistant and multi-drug resistant strains identified in seafood is of concern. Monitoring studies such as the present work are one of multiple steps required to understand and manage potential risks posed by use of antibiotics in aquaculture and

in society at large. The present study was limited in sample size and employed sample pooling. It is desirable to perform additional surveys to confirm the findings and preliminary conclusions reported here.

Acknowledgements

The authors thank Don McBride of NOAA for acquiring the seafood samples and Dr. Javier Santander of the Biodesign Institute's Center for Infectious Diseases and Vaccinology for providing antibiotic-free catfish. We also thank Isaac Roll and Dr. Arjun Venkatesan of the Center for Environmental Security for editing and for providing the GIS map, respectively. We acknowledge the services provided by AXYS Analytical Services Ltd, British Columbia, Canada. This project was supported in part by the Johns Hopkins Center for a Livable Future and by Award Numbers R01ES015445 and 1R01ES020889 of the National Institute of Environmental Health Sciences (NIEHS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIEHS or the National Institutes of Health (NIH).

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhazmat.2014.08.075.

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