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Development and Strengthening of Radioanalytical and Complementary Techniques to Control Residues of Veterinary Drugs and Related Chemicals in Aquaculture Products

Joint FAO/IAEA Programme
Nuclear Techniques in Food and Agriculture

IAEA
International Atomic Energy Agency

DEVELOPMENT AND STRENGTHENING OF RADIOANALYTICAL AND COMPLEMENTARY TECHNIQUES TO CONTROL RESIDUES OF VETERINARY DRUGS AND RELATED CHEMICALS IN AQUACULTURE PRODUCTS

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DEVELOPMENT AND STRENGTHENING OF RADIOANALYTICAL AND COMPLEMENTARY TECHNIQUES TO CONTROL RESIDUES OF VETERINARY DRUGS AND RELATED CHEMICALS IN AQUACULTURE PRODUCTS

FINAL REPORT OF A COORDINATED RESEARCH PROJECT

PREPARED BY THE
JOINT FAO/IAEA CENTRE OF NUCLEAR TECHNIQUES IN FOOD AND AGRICULTURE

INTERNATIONAL ATOMIC ENERGY AGENCY
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FOREWORD

Fish and seafood farming is becoming more widespread for the inexpensive and intensive production of protein rich foods. As global aquaculture production continues to grow, chemical inputs such as veterinary pharmaceuticals and related substances are increasingly used to control aquaculture related diseases and improve yields. Residues of such inputs, as well as natural toxins in aquaculture products and feeds and contaminants at production sites or in effluents, pose public and environmental health risks that need to be addressed. This calls for robust national regulatory frameworks underpinned by competent analytical laboratories to safeguard both consumers and aquaculture production and to enable international trade in aquaculture products. It was recognized that there was a need for research and development on analytical methods to strengthen laboratory performance in this field, with nuclear and isotopic techniques playing an important role, and for research to better understand the contamination of aquaculture production sites, which has potential public and environmental health implications.

To help address these needs, in 2015 the IAEA initiated the coordinated research project entitled Development and Strengthening of Radioanalytical and Complementary Techniques to Control Residues of Veterinary Drugs and Related Chemicals in Aquaculture Products. The aim of this project was to strengthen the capabilities of Member State analytical laboratories and national chemical residue monitoring programmes, thereby contributing to the improvement of food safety and better aquaculture production and management practices, and in turn contributing to the enhancement of trade in aquaculture products. Within the project, new analytical methods — including improved, environmentally friendly sample preparation techniques — were developed, validated and transferred between Member State laboratories, addressing significant constraints in their capabilities. The project also contributed to knowledge on the contamination of aquaculture production systems. Several standard operating procedures for targeted chemical and microbial contaminants in aquaculture products and production are included as an annex and are available online as a separate supplementary file.

The project was implemented by the IAEA between 2015 and 2021 and involved 11 research contracts, 4 research agreements and 1 observer institution. The IAEA officer responsible for this publication was J.J. Sasanya of the Joint FAO/IAEA Centre of Nuclear Techniques in Food and Agriculture.

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CONTENTS

1. INTRODUCTION

1.1. BACKGROUND

This publication is an output of a Joint FAO/IAEA CRP, D52039 'development and strengthening of radioanalytical and complementary techniques to control residues of veterinary drugs and related chemicals in aquaculture products', which was conducted between 2015 and 2021 to support the monitoring and control of selected antimicrobial residues and contaminants in aquaculture products and production, thus contributing to the improvement of food safety, better aquaculture production and management practices and enhancement of trade in aquaculture products.

Intensive aquaculture is one of the fastest growing areas in food production, contributing almost 50% of the global fish supply [1]. Intensive production practices and the various stress factors involved in aquacultural production, including the prevalence or incidence of fish diseases, demand the use of pharmacologically active substances to enhance production [2]. These agrochemicals, as well as natural and environmental contaminants, present public health risks and trade challenges and need to be controlled. However, a significant constraint in many countries is the lack of laboratory services, especially laboratories using analytical methods validated to internationally acceptable standards, to implement surveillance programmes and support appropriate risk assessment [3].

The overall objective of the CRP was to enhance national control programs for residues of veterinary pharmaceuticals and related chemicals in aquaculture products, feeds and water, as well as the environment at aquaculture production sites. The specific objectives included: 1) to improve laboratory capabilities to collect reliable data on the safety of aquaculture products and aquaculture itself; 2) to assess the cost–effectiveness of new sample preparation techniques and the optimum use of radioanalytical tools to ensure aquaculture product and environmental safety; 3) to assess ways to strengthen laboratory quality assurance and quality control measures; and 4) to enhance understanding of the potential effects of aquaculture inputs and contamination/pollution of aquaculture production systems.

1.2. OBJECTIVE

The objective of this publication is to provide information to help Member States to improve aquaculture practices. This includes information on analytical methodology that can be applied by Member State laboratories to facilitate the detection and quantification of veterinary antimicrobials and associated chemical contaminants in aquaculture products and production systems, including inputs and samples from production sites, for research or regulatory purposes. The publication also provides information, obtained through the application of the analytical methodology developed, on sources of contamination, the depletion of residues in aquaculture products, the effects of current aquaculture production practices on food safety and the environment, and linkages between the use of antimicrobials in aquaculture and the development of antimicrobial resistance. This information will assist Member States in improving testing programmes, risk assessment and aquaculture practices.

1.3. SCOPE

The scope of this publication includes the development and application of analytical methodology using radiometric, isotopic, and complementary techniques for the analysis of

aquaculture products and environmental samples to detect and quantify selected chemical residues and contaminants that are important with respect to food safety. The target analytes include residues of veterinary pharmaceuticals used in aquaculture practices and pharmaceuticals as well as related chemicals from other anthropogenic waste sources, natural toxins, metals, and perfluoroalkyl and polyfluoroalkyl substances. The applications of the methodology include studies to provide information on sources of contamination, the distribution and depletion of certain pharmaceuticals in fish, and links between antimicrobial residues and the development of antimicrobial resistance.

1.4. STRUCTURE

The first part of this publication is organized as follows:

i. Section 1 gives a general background and describes the objective, scope and structure of this publication;
ii. Section 2 provides a summary of the research performed by each of the project participants;
iii. Section 3 summarizes the conclusions arising from the research.

The second part of the publication contains twenty–seven reports from the research participants, with details of the studies performed and their respective results and conclusions. The report ends with an annex consisting of several standard operating procedures presented as supplementary electronic files.

2. SUMMARY OF THE COORDINATED RESEARCH PROJECT RESULTS

The results of the research in this project are presented in twenty–seven papers arranged under four main topics:

— Analytical methods;
— Sources and distribution of contamination;
— Residue depletion;
— Antimicrobial residues and antimicrobial resistance.

The summaries of the papers are presented under what is considered the main topic of each study, with possible crossover. Some of the studies on sources of contamination, for example, required the development of analytical methods for the detection of the compounds being investigated.

2.1. SUMMARY OF RESEARCH RESULTS

2.1.1. Analytical methods

2.1.1.1. Radioreceptor assay screening methods

Screening tests typically employ high throughput, rapid methods, and they are designed to detect, with an acceptable rate of false positive (noncompliant) results and a negligible rate of false negative (compliant) results samples potentially containing unacceptable concentrations of analytes, higher than Codex or other regulatory maximum residue levels or tolerance levels [4]. The radioreceptor assay technique involves ^{3}H or ^{14}C–labeled tracers that compete with

unlabeled residues in the test matrix for specific binding sites in a binding reagent. The radioactivity measured by a liquid scintillation counter in the binding reagent is used to determine concentration of the residues. There are limited applications of the methodology in the testing of aquaculture products and monitoring of contamination of aquaculture systems. Validation exercises for the radioreceptor assay method focusing on selected residues of veterinary pharmaceuticals of importance in aquaculture production in various species, were completed in the CRP. The successful validation of the methodology demonstrated its fitness for purpose for testing in aquaculture.

A collaborative study involving research contract holders in Cameroon, Uganda and a research agreement holder in Belgium (page 13) validated radioreceptor assay tests at concentrations relevant to Codex control levels for a range of antimicrobial residues in different fish species under aquaculture production. The species targeted included catfish, trout, salmon, sea bass, tilapia, lingue, dorade and pangasius. The method was shown to be suitable for the detection of sulfonamides in some of the species at 25 µg/kg, the β–lactams penicillin G, ampicillin, amoxicillin, oxacillin, dicloxacillin and cloxacillin at 25 µg/kg–300 µg/kg, the macrolides erythromycin A, tilmicosin, and tylosin A at 100 µg/kg, the tetracyclines tetracycline and chlortetracycline at 25 µg/kg and oxytetracycline at 100 µg/kg and the aminoglycoside, erythromycin at 25 µg/kg. The method is specific, sensitive, robust and precise.

Research groups in Belgium and Cameroon collaborated in the transfer to Cameroon of the radioreceptor technique for the detection of antimicrobial residues in aquaculture fish (page 19). The method had been validated in Belgium, and an abridged validation, based on the performance criteria recommended in European Commission Decision 2002/657/EC [5], was performed to demonstrate equivalent method–performance in Cameroon. The analytes included β–lactams, tetracycline, sulfonamides, macrolides and chloramphenicol, while the species were tilapia, catfish, carp and kanga. The method met regulatory requirements and is available for use in the aquaculture fish safety monitoring programmes in Cameroon.

A study was carried out in Nigeria to validate radioreceptor assays for the detection of tetracyclines, β–lactams and sulfonamides in a range of seafood and aquaculture products (page 23). Samples used for the validation process included tilapia, catfish, carp, and shrimps. The study followed guidelines of the European Commission Decision 2002/657/EC [5]. The method precision was good and detection capabilities were 50 µg/kg for tetracyclines 25 µg/kg for β–lactams and sulphonamides.

2.1.1.2. Confirmatory methods

Confirmatory methods offer a higher level of certainty than screening tests in the detection and quantification of the target analytes and are often used in regulatory control system as a second tier in combination with high–throughput first tier screening tests. Confirmatory tests are designed to provide results with a false positive (noncompliant) rate as low as possible, and an acceptable false negative (compliant) rate [6]. In typical food control systems, positive results from a screening test are verified using a confirmatory method. Confirmatory tests employ methodology that provides sufficient information to confirm the identity of an analyte and to determine its concentration in the sample. In comparison to screening tests, the techniques used are generally more costly, more involved to apply, need extensive sample extraction and cleanup procedures before analysis, and require expertise to perform the analyses and interpret the results. An example of confirmatory tests is the liquid chromatography tandem mass

spectrometry (LC–MS/MS) [7]. The technique is often used in an isotope–dilution format, with labelled forms of the target analytes, typically labelled with one or more deuterium atoms at non–exchangeable sites in the molecule of ^{13}C as internal standards to improve the precision and quantification capability of the method. Several examples of the development of isotope–dilution confirmatory methods and their application in aquaculture were reported by CRP groups.

Multiclass, multiresidue LC–MS/MS methods were developed by two research groups, in Brazil and in South Africa. In Brazil, a method was developed for the confirmatory analysis of residues of twelve analytes representing four classes of antimicrobials, the tetracyclines, sulfonamides, quinolones and amphenicols, in Nile tilapia from caged aquaculture (page 26). A stable isotope labelled sulphonamide drug (sulfadimethoxine–d6) was used as internal standard. The method developed in South Africa (page 33) could analyse twenty-two representatives of three classes of antimicrobials, the polyether ionophores, tetracyclines and sulfonamides. Good recoveries (80%–113%) and repeatability (<15%) were demonstrated. Following satisfactory performance in proficiency testing, the method is now being used to analyze field samples and is included in the South African National Residue Monitoring Programme.

Several LC–MS/MS analytical methods were also developed for residues of classes of individual classes of veterinary pharmaceuticals or individual compounds, and for shellfish toxins. Some of these methods targeted drugs that are banned for use in food producing animals in many regions of the world because of the potential harmful effects of their residues in humans. These include chloramphenicol and nitrofurans. Although banned, these drugs are frequently used illegally because of their efficacy and low cost. A sensitive LC–MS/MS method was developed and validated according to EU criteria for the analysis of residues of chloramphenicol in fish and shrimp in Brazil (page 41). Validation parameters included detection capability, specificity, stability of standard solutions and stability of the analyte in matrix.

Residues of the nitrofuran class of pharmaceuticals can be difficult to detect because most are metabolised rapidly following administration and detection of the parent compound is not feasible or useful. Analytical methods to detect abuse of these compounds usually target the protein bound metabolites of the drugs, which retain the toxigenicity of the parent compounds. Two methods were reported for various nitrofuran drugs or their metabolites by a research group in China. A new isotopic LC–MS/MS method was developed and validated to determine metabolites of furazolidone, furaltadone, nitrofurazone, nitrofurantoin and nifursol in shellfish (page 46). The limits of quantification ranged from 0.1 μg/kg to 0.2 μg/kg and recovery of the analytes was good (~89% to 112%). The method was used in analysis of field samples including 280 shellfish products. An analytical method was also produced for measuring levels of eight nitrofurans; nitrofurantoin, furazolidone, nitrofurazone, and furaltadone as well as nifursol, nifuroxazide, nifurpirinol, and sodium nifurstyrenate, and six metabolites in shellfish and aquaculture fish (page 51). The method was used to analyze 537 shellfish and fish samples from South China, showing 4.3% of noncompliance in shellfish and 5.0% in fish, semicarbazide (the primary metabolite of nitrofurazone) being the most frequently detected.

The research group in Ecuador completed the development and validation of an LC–MS/MS method for the analysis of sulfonamides in shrimp (page 58). The method scope included sulfadiazine, sulfamethazine, sulfamerazine, sulfanilamide and sulfathiazole and achieved very

good performance, with analyte recovery of 90%–100% from fortified samples at and around the MRLs for the drugs.

A confirmatory analytical method was developed, validated and applied in China for the simultaneous determination of 10 lipophilic shellfish toxins, with detection limits in the range 0.3 μg/kg to 1 μg/kg and quantification in the range 0.7 μg/kg to 3.0 μg/kg (page 63). The method was demonstrated to be fit–for–purpose and can be used for ensuring the safety of mussels, oysters and scallops.

2.1.2. Sources and distribution of contamination

Research groups in Argentina performed a number of studies on the distribution of residues and contaminants and their sources. An isotopic LC–MS/MS analytical method was validated and applied in a survey for 46 antimicrobial residues in aquaculture trout, shad, pacú and salmon in Argentina (page 72). The target analytes included amphenicols, cephalosporins, dihydrofolate reductase inhibitors, fluoroquinolones/quinolones, macrolides, nitrofurans, penicillins, sulfonamides and tetracyclines. The aquaculture fish samples contained residues of 42 of the 46 antimicrobials. Levels of doxycycline, oxytetracycline and sulfamethazine were above MRLs while forbidden drugs such as chloramphenicol, furazolidone and nitrofurantoin were also detected. The results suggested that the residues were from environmental pollution and/or aquaculture production practices.

The distribution of 43 antimicrobials and four of their metabolites in water, sediment and biofilms associated with the Suquía River in Córdoba, Argentina, were investigated (page 80). A number of sample preparation techniques, including solid phase extraction, bead–beating disruption and pressurized liquid extraction, were studied followed by LC–MS/MS analysis. Samples from sites downstream of a wastewater treatment plant were analysed. A range of antimicrobials were found in water, biofilms and sediment samples regardless of the season. The sources were identified as being mainly wastewater treatment plant discharges and urban runoff. Accumulation was more in biofilms than sediments and, as such, biofilms are regarded as excellent bioindicators of environmental contamination with antimicrobials. Another study (page 90) indicated the presence of up to 20 pharmaceuticals, including carbamazepine and two of its metabolites, carbamazepine–10,11–epoxide (CBZ–EP) and 2– hydroxycarbamazepine (2–OH–CBZ), in *Gambusia affinis* and *Jenynsia multidentate* from polluted areas of the Suquía River. *G. affinis* contained all 20 analytes and *J. multidentate* only 15. Carbamazepine and 2–OH–CBZ were found in gills, intestine, liver, brain and muscle of fish, while carbamazepine–10,11–epoxide (CBZ–EP) was detected in gills and muscle only.

A study was undertaken to evaluate anthropogenic sources of pollution and causes of eutrophication in Córdoba, Argentina (page 101). Three reservoirs: San Roque Lake (SRL), Los Molinos Lake and Río Tercero Reservoir, were included in the study. Stable nitrogen isotope ratios ($\delta^{15}N$) were measured in water, plankton, shrimp and fish muscle. The SRL samples showed the highest $\delta^{15}N$ values, suggesting sewage discharge and anthropogenic effects and therefore urgent need for corrective action. SRL is not suitable for fish production for human consumption in its present state. The presence and distribution of 17 metals was also investigated in water, sediments, and aquatic organisms sampled from the San Roque Reservoir in Argentina in the rainy and dry seasons (page 106). Three trophic groups; plankton, shrimp (*Palaemonetes argentinus*) and fish (Silverside, *Odontesthes bonariensis*) were considered, and stable nitrogen isotopes ($\delta^{15}N$) used to investigate trophic interactions. Trophic magnification

factors (TMFs) were calculated. High levels of metals and metalloids mainly Al, Cu, Cr, Fe, Ni and Zn were found in water, but not in sediment. Mercury showed biomagnification in the food web, while most elements were bioconcentrated from water to plankton and then biodiluted from plankton to shrimp and fish. Overall, there was no significant relationship between levels of metals and trophic levels.

The spatial and temporal distribution of antimicrobials was investigated by the research group in Brazil, using a new LC–MS/MS technique (page 118). The method is suitable for analysis of 12 antimicrobials (including oxytetracycline, florfenicol, tetracycline and chlortetracycline) in sediment and surface water. Four fish farms in Brazil were investigated over four seasons. Several antimicrobials were detected in water and sediment and showed seasonal distribution. Fish farming was demonstrated as the source of antimicrobial contamination.

A paper from the research group in Cameroon reports the application of a radioreceptor assay to conduct a pilot study for residues of antimicrobials including macrolides, β–lactams and/or sulfonamides in water and mud from the farming ponds of different fish species (page 126). The objective was to understand possible avenues through which aquaculture products can be exposed to drug residues and therefore provide a basis for regulation and monitoring. The results of this pilot study suggested that water, as well as mud in ponds where fish are farmed accumulates β–lactams and sulfonamides in concentrations that could be harmful for fish and consumers. The presence of aflatoxins in fish farmed in two Cameroonian localities and the potential source of the contaminants was investigated through analysis of feeds, water and mud (page 130). Samples of kanga, tilapia, catfish, and carp as well as water and mud were collected from Mfou and Batié farming sites and analyzed. The targets included total aflatoxins and aflatoxin B1 using 20 µg/kg as a reference value.

The seasonal occurrence and variation of 46 antimicrobial residues used in humans and veterinary production was reported in Chilean wild trout and farmed salmon (page 135). Salmonids contained a range of residues including forbidden substances, with almost the same residue types found in the cold and warm seasons. A comparison was made between levels in wild and farmed salmonids in proximity. The highest frequency and concentrations of the residues were in wild fish, confirming that environmental contamination can be regarded a major concern for South America.

In a study by Lebanese researchers (page 143), levels of 17 active PAHs were investigated in marine and continental (fresh water) Lebanese aquatic systems. The Lebanese seawater was found to be more contaminated than several other study sites on the Mediterranean Sea. The total concentration of PAHs ranged from 55.7 ng/l to 2683.8 ng/l in marine water and from 19.09 ng/l to 2025.03 ng/l in sediments. In freshwater coastal and inland rivers, the total concentration ranges from 465.7 ng/l to 1399.9 ng/l in water and from 72.6 ng/l to 1074.7 ng/l in sediments, presenting higher contamination and detection frequency than the marine sites. The PAHs in Lebanese sediments were found to be of combustion–origin and from industrial sites next to aquatic systems, and heavy traffic, especially along the Lebanese coastline. A few sites had individual PAH levels that may occasionally cause biological adverse effects to benthic organisms, but the ecosystem risk of PAHs in Lebanese sediments is low.

A validated isotopic LC–MS/MS method was established in Peru and used to determine residues of antimicrobials in surface and drinking water and trout tissues from aquacultural production (page 147). Ciprofloxacin, chlortetracycline, and sarafloxacin were detected in

surface water obtained from fish farms and drinking water in Puno city. Oxytetracycline, sulfathiazole, ciprofloxacin and sarafloxacin were detected in trout muscle. The findings indicate that trout–fish farming is contaminated with antimicrobials from agricultural runoff and wastewater.

In South Africa, an LC–MS/MS method was validated and used to study the concentration of 15 perfluoroalkyl substances (PFAS) in four species of farmed marine shellfish and to determine the human daily intake of PFAS, including perfluoropentanoic acid (PFPeA), perfluorooctanesulfonic acid (PFOS), perfluorohexanoic acid (PFHxA) and perfluorotetradecanoic acid (PFTeDA) (page 157). The samples studied were farmed abalone, mussel, oyster and lobster with concentrations in the range 0.12–0.49 ng/g, 4.83–6.43 ng/g, 0.64–0.66 ng/g and 0.22 ng/g, respectively. The estimated daily intakes of PFAS through the consumption of marine shellfish were in the range 0.05 ng/kg bw/d – 1.58 ng/kg bw/d, suggesting that the overall risk of exposure is low.

A cross–sectional study was conducted to better understand aquaculture production practices, including the use of agrochemicals, in parts of Cameroon and how this impacts the safety of aquaculture products (page 166). Farmers (107) who practiced earthen fishpond farming (83.3%) or integrated fish farming, from the central, southern, littoral, and western regions of Cameroon, were included. The results showed that more than half of the farmers involved used agrochemical products, including veterinary drugs, but less than a quarter used these feed additives under proper control, with a veterinary prescription. This constituted a risk of exposure to residues of the drugs in the fish consumed.

2.1.3. Residue depletion

Research was undertaken in Brazil to investigate the effects on a nontarget organism, of a sulfonamide, sulfadiazine, which is used in aquaculture (page 173). The use and improper disposal of sulfadiazine can result in bioaccumulation, bioconcentration and biomagnification in the aquatic environments. Zebra fish (*Danio rerio*) were used as the nontarget organism for the study. ^{14}C–sulfadiazine was administered to evaluate the drug's bioconcentration and depuration after 20 days. A small amount of the drug was found, and no depuration observed. While the bioaccumulation was low, further studies are needed to gain an understanding of the long–term effects of pharmaceuticals such as sulfadiazine on the environment and nontarget organisms.

A study was undertaken in Peru, in collaboration with the research group in Brazil, to investigate the depletion of sulfadiazine in rainbow trout (*Oncorhynchus mykkis*) (page 179). A labelled (^{14}C) molecule was used at a specific activity of 3.5171 MBq/mg, mixed with sulfadiazine in feed added to the water over a period of 7 days, followed by 7 days depuration. The drug accumulated in rainbow trout on day four after the exposure phase and decreased during the purification/depuration phase with an estimated half–life of 4.33 days. Tissue concentrations reached levels greater than the MRL for sulfadiazine, indicating a possible risk for consumers if appropriate withdrawal periods are not adhered to for sufficient depuration.

2.1.4. Antimicrobial residues and antimicrobial resistance

Correlations were observed in two studies in the CRP between the use of antimicrobials in fish farming and the development of antimicrobial resistant bacteria.

The relationship between antimicrobial residues in farmed *Oreochromis niloticus* (Nile tilapia), resistant bacteria, and the sanitary practices of farmers in a reservoir in Brazil was investigated (page 186). Fish from four cage farms sampled at intervals of 3 months over one year. Residues were analyzed by LC–MS/MS and isolated bacteria tested for antimicrobial resistance. Oxytetracycline, tetracycline and florfenicol were detected in fish muscle. Indices for multiple drug resistance were calculated and correlation between the resistance indices and antimicrobial concentrations in the fish was established. Farm management practices that influence hygiene at the cages was also evaluated.

Research was conducted to assess the microbial contamination of Mfou aquaculture production site in Cameroon, and to evaluate the antimicrobial resistance profiles (page 192). Samples included in the study were water, sediment, fishmeal and fish, including African catfish, kanga and Nile tilapia. These were analyzed for at least eleven types of bacterial isolates and fungi. Microbial loads were high and multiresistance was observed in all isolates. Further investigation into the possible causes of the high antimicrobial resistance is recommended. The high microbial loads observed suggest that the fishponds constitute a potential reservoir of zoonotic pathogens, already with resistance to a range of antimicrobial drugs.

2.2. CONCLUSIONS

— Overall, the expected outputs and outcome were realized, and the CRP has been of significant benefit to Member States. In total, thirty–six methods were developed/validated, thirty–six standard operating procedures (SOPs) prepared, and nineteen papers or scientific reports presented. This has contributed to improved analytical methodology, which is transferable and supports the monitoring of residues and contaminants in aquaculture products and production sites. In eight countries, methods have been applied to national residue monitoring programmes both for products consumed locally, including imports, as well as exports. The outcome of one research contract has now resulted in development of a national technical cooperation project (TCP), and additional TCPs are expected to arise to transfer and implement the methodology developed and make use of the knowledge generated in this CRP.

— New radioreceptor assays established have been used for the cost–effective determination of varying levels of residues in aquaculture products. This required primary validation for several veterinary drugs in different fish and involved three countries, followed by transfer to two other laboratories, which have in turn performed secondary validation. This knowledge is being transferred to more countries. In a related development, work on a radioimmunoassay for residues was also initiated.

— Research was done on the uptake and depletion of a ^{14}C–labelled sulfonamide drug in certain fish. This information is important because it helps in understanding the metabolism of the drug, provides a foundation for additional research on the depletion of drugs in food animals, and contributes to the setting of maximum residue limits for drug residues in food.

— Research generated information to help confirm that the presence of certain forbidden drugs/substances in food production is not necessarily due to the intentional use of the drugs in production but may be due to natural occurrence of the substance. For example, a research contract confirmed that semicarbazide (SEM), a metabolite of the antimicrobial drug nitrofuran, which is prohibited for use in food–producing species, is not a conclusive marker for the illegal use of the drug, because SEM was found to be naturally existing in shellfish. Establishing an occurrence–species profile of SEM in shellfish may help in food safety regulation decisions.

— Analytical methods were developed and used to test mycotoxins in aquaculture inputs and associated matrices.

— Research findings contributed knowledge to support understanding of contamination and residue levels in water and related matrices such as sediments, and the potential implications for aquaculture production in general.

— Seventeen PhD, MSc and Post–Doctoral fellows in four Member States benefitted from performing research related to the CRP.

— Isotopic research has demonstrated the presence of a wide range of human and veterinary pharmaceuticals in fish and water, as an indication of anthropogenic exposure. Further, $\delta^{15}N$ was used to demonstrate exposure of phytoplankton, shrimp and fish samples collected in three lakes of Córdoba province in Argentina, to anthropogenic sewage. Thus, research work has helped demonstrate the usefulness of $\delta^{15}N$ in identifying pollution of water bodies whose water is used in aquaculture production.

— Bioaccumulation and bioconcentration of certain drugs used in human medicine, for example carbamazepine, in fish for human consumption, under field and controlled laboratory experiments, has been demonstrated.

— Research contributed to enhanced laboratory competences according to international standards, such as ISO 17025 accreditation of the participating laboratories in Nigeria, South Africa and Uganda. This has significant implications as these institutions have an important mandate in ensuring consumer safety and for facilitation and competitiveness of exports.

— Results indicated that the use of antimicrobials in aquaculture production contributes to the burden of antimicrobial resistant organisms in the environment and in food, with the potential for transfer of these resistant organisms to humans.

— The project resulted in some offshoots that may require follow up CRPs or some other avenue of research. Examples of these areas are: the occurrence and effects of using water contaminated with antimicrobials in alfalfa plant cultures, soil and then feed; determination of the concentrations and assessing human exposure to per– and poly–fluoroalkyl substances (PFAS) in farmed marine shellfish; and the potential of transfer of zoonotic pathogens, already with resistance to a range of antimicrobial drugs, from fishponds into the food chain.

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3. PAPERS

PRIMARY VALIDATION OF RADIO RECEPTOR ASSAY TESTS FOR ANTIMICROBIAL RESIDUES IN AQUACULTURE FISH

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Abstract

Radioreceptor assay tests were validated for a range of antimicrobial residues in different species of aquaculture catfish, trout, salmon, sea bass, tilapia, lingue and pangasius. The analytes studied included sulfonamides, β–lactams, macrolides, tetracyclines and aminoglycosides with the detection capabilities for tetracyclines in the range 25 µg/kg –100 µg/kg; 25 µg/kg to 300 µg/kg for β–lactams; 25 µg/kg for sulfonamides and aminoglycosides and 100 µg/kg for macrolides. Good repeatability was observed with the relative standard deviation for both repeatability and reproducibility in the range 1.2%–15.1%. The tests are group specific and robust and are applicable for the rapid testing of aquaculture fish.

1. INTRODUCTION

Fish farming including aquaculture contributes to food security and well–being [1–3]. Due to stress factors and diseases, the use of antimicrobials such as tetracyclines, macrolides, β–lactams, sulfonamides, and streptomycin is imperative [4–9]. Several tetracyclines are some of the commonest drugs used in aquaculture [10, 11] and for which maximum residue limits (MRLs) have been established [12]. These and other drugs are administered through different means [13, 14] and result in residues of consumer and trade concern [7, 15–20]. Control of these hazards requires reliable techniques and rapid, cost–effective methods such as Charm II radio receptor assay technique suitable for a wide range of antimicrobials such as β–lactams, sulfonamides, tetracyclines, chloramphenicol, quinolones, macrolides and aminoglycosides in fish and other animal products as well as related matrices. The techniques involve ^{3}H– or ^{14}C–labelled antimicrobial tracers that compete for binding sites with residues in matrix with differences in reading used to determine concentration of the residues [21, 22]. Due to limited validation data for detection of antimicrobials in different fish species, a primary validation study was conducted for different aquaculture fish species [23].

2. MATERIALS AND METHODS

2.1.Reagents, materials and equipment

The following were used [23]: antimicrobial test assay kit (Charm Sciences Inc., Lawrence, MA): β–lactams (PMSU–050A); sulfonamides (SMMSU–022C), macrolides (EMSU–023A); tetracyclines (TMSU–025); and streptomycin (STMSU–023A); M2 Buffer, zero and positive control standards, MSU extraction buffer, radioactive labelled tablets; scintillation fluid (OptiFluor O, PerkinElmer), Intronic incubator (Charm Sciences Inc.), liquid scintillator counter (Wallac 1409), centrifuges (Sigma 4K15c and IEC Centra CL–3 centrifuge), blender (R2, Robot Coupe), water bath (Julabo MB13), scintillation vials and a stomacher (AES).

2.2.Preparation of standard reference material and stock solutions

A concentrate containing multiple standards (MSU, Charm Sciences Inc.) was reconstituted in 10 ml of deionized water [23]. The standards included 1000 µg/kg of penicillin G and sulfamethazine; 4000 µg/kg of chlortetracycline and 10000 µg/kg of streptomycin and erythromycin A. These were complemented with other analytical standards from various sources (Sigma Aldrich, Pfizer Inc., US Pharmacopeia Convention and Acros Organics) and used to spike fish in the concentration range 25 µg/kg to 300 µg/kg. Known guidelines [24] were used to validate the method with a protocol applied elsewhere [21].

2.3.Preparation of sample and controls

Fish samples collected from markets in Belgium and tested to confirm absence of the antimicrobials in study were blended and portions (10 g) placed in a polypropylene centrifuge tube, 30 ml of the MSU extraction buffer added, the mixture homogenized in a stomacher for 2 min, incubated in water bath (80°C, 30 min) for determination of streptomycin, macrolides, and beta-lactams; and for tetracyclines or sulfa drugs (80°C, 45 min); cooled content then centrifuged at 3300 rpm for 10 min (4K15C, Sigma) before the supernatant solution adjusted to pH 7.5 analyzed prior to analysis on the liquid scintillation counter.

2.4.Analysis of the residues in the fish samples

For tetracycline, a white tablet containing the binding reagent dissolved in a test tube with 300 µl of water, sample extract or control sample (4 ml) and an orange tablet containing the tracer added, the solution mixed and incubated at 35°C for 5 min before centrifugation for another 5 min. Deionized water (300 µl) was used to dissolve the pellet after removal of the supernatant, 3.0 ml scintillation liquid added and the content analyzed on the Wallac liquid scintillation counter for 60 s on the ^{3}H channel, after the mixture was thoroughly agitated to obtain a cloudy appearance. Determination of the sulfonamides followed the sam procedure and conditions as the tetracyclines. Macrolides was handled similarly with minor difference namely, incubation at 55°C for 2 min; second tablet was green, and reading was done on the ^{14}C channel. The aminoglycosides–streptomycin were determined in the same way as the macrolides although 2 ml of the sample extract or control samples was used, and analysis performed on the ^{3}H channel following incubation of the mixture at 35°C for 2 min. For the β–lactams, a green (binder) and yellow (tracer) tablets were used, 2 ml of matrix/control used and incubation at 55°C for 2 min before analysis on the ^{14}C channel [23].

2.5.Method validation

For the CCβ, blank fish samples (n=20–30) were spiked with the drugs under study, at concentration corresponding to 0.05 MRL, 0.25 MRL, 0.5 MRL, 0.75 MRL and MRL with CCβ determined as the lowest residues concentration in the sample with a minimum of 95% positive results. Repeatability was determined by measuring variations in the levels of residues in the 20–30 samples spiked at the levels. This involved analysis by one analyst over a short interval, using the same method and instrument. Reproducibility was determined when analysis was performed on different days by two different analysts although using the same analytical method and instrument. Minor deliberate changes such as in reading time (for an analyte such as 50 µg/kg amoxicillin) and whether store over night or read immediately, to determine robustness were applied to the method. Potential cross reactivity that could result in false positive results was determined by fortifying residue–free fish with high concentrations of the different drugs.

3. RESULTS AND DISCUSSION

3.1. Counts per minute for blank samples

The blank samples of tilapia, trout, salmon, pangasius, seabass, dorate, catfish, and lingue fish species spiked with binder and tracer on were prepared following known procedure and the counts per minute (cpm) measured with no significant difference in the levels obtained using kits for β–lactams, tetracyclines, macrolides and streptomycin analysis. The levels were much higher, however, for sulfonamides especially for catfish, lingue and pangasius.

3.2. Evaluation of the control points for the different drug residues

The cutoff point between a negative or positive test results result (control point) was determined by fortifying the bank fish samples with the different drugs at the concentrations corresponding to the respective MRLs, except for tetracyclines. Differences in fish matrices are addressed by consideration adding to or subtracting from the average cpm a certain percentage tolerance to minimise false readings [21, 23, 25, 26]. A 20% value was added to the counts from matrices (n=6) containing 25 µg/kg penicillin G (representing beta lactams) to obtain the CP; for sulfonamides 30% was subtracted from value obtained from a matrix spiked with 50 µg/kg with sulfamethazine (for sulfonamides). For tetracycline, 40% was subtracted from the average cpm value; macrolides represented by erythromycin A (100 µg/kg) 20% was added and adding 30% added in the case of streptomycin. The false positive rate was 0% for tetracyclines, β–lactams and sulfonamides, 3.6% for macrolides, and 5% for streptomycin [23]. The CPs in this study were comparable to corresponding cut–off points (Fm) and technical threshold (T) values established elsewhere for validation of screening methods [27] including guidance on acceptable false positive/negative results [27, 28].

3.3. Detection capability for the antimicrobials in selected fish species

The CCβ (lowest analyte concentration detected in the sample with at least 95% positives results) value was 25 µg/kg for tetracycline, chlortetracycline, streptomycin and the sulfonamides (sulfamethazine, sulfadimethoxine, sulfamerazine, sulfadiazine and sulfathiazole; 50 µg/kg for Penicillin G, ampicillin and amoxicillin; 100 µg/kg for oxytetracycline, erythromycin A and tilmicosin, and the highest (300 µg/kg) was for oxacillin, dicloxacillin and cloxacillin [23]. These values were generally below the MRLs except oxytetracycline, tyrosine, oxacillin, dicloxacillin and cloxacillin where the levels were the same and comparable to previous findings [29, 30]. The LODs and LOQs were acceptable and comparable to previous findings following confirmatory analysis of similar residues [31, 32].

3.4. Method repeatability/reproducibility and robustness

The method relative standard deviation (%RSD) was below 12% for tetracyclines, macrolides, β–lactams, aminoglycosides, and sulfonamides [23]. Specifically, the values were 7.8%–9.8% for chlortetracycline and oxytetracycline, 2.8%–6.3% for macrolides, 6.9%–9.7% for β–lactams, 10.01%–11.5% for aminoglycosides; and 1.2%–8.7% for sulfonamides. For reproducibility level was below 15.3% for tetracyclines, macrolides, β–lactams, aminoglycosides, and sulfonamides, specifically 7.2%–11.4% for chlortetracycline and oxytetracycline; 5.8%–8.9% for macrolides; 10.4%–11.2% for β–lactams; 8.9%–15.1% for aminoglycosides and 2.8%–8.3% sulfonamides. There cpm values for pangasius and dorade fortified with β–lactams at 50 µg/kg, were not significantly different [23]. The average cpm for

pagasus was 1089.5 with very low variability/RSD (0.07%) in the 12 test runs conducted while the corresponding values for dorade were 1075.4 and 0.05% [23].

3.5. Specificity and cross reactivity of the technique

While analysis of target drug residues such as macrolides on their respective channels following spiking of known blank samples with 200 µg/k of a representative standards yielded positive results (1478 cpm), a search of residues the nontarget molecules such as aminoglycosides intentionally analyzed on the same macrolide channel on the liquid scintillation counter didn't yield the same positive test results (the reading where above the control point: 2118) an indication that there was no interference or cross reactivity [23]. The findings were observed in the case of sulfonamides, β–lactams, macrolides, and tetracyclines visa vis aminoglycosides channel etc. Similar patterns were reported elsewhere [29].

4. CONCLUSIONS

A radio receptor assay technique has been validated and is suitable for screening residues of tetracyclines, sulfonamides, β–lactams, aminoglycosides and macrolides in catfish, trout, salmon, seabass, tilapia, lingue, dorade and pangasius. Tetracycline and chlortetracycline can be detected at 25 µg/kg and oxytetracycline at 100 µg/kg. Sulfadimethoxine, sulfamerazine, sulfadiazine, sulfathiazole can also be detected at 25 µg/kg in all fish other than catfish, pangasius, and lingue, possibly due to high fat content that could contribute to matrix interference. Erythromycin A, tilmicosin, and tylosin A can be detected in all the fish under study at 100 µg/kg while the β–lactams penicillin G, ampicillin, amoxicillin, oxacillin, dicloxacillin and cloxacillin were detected in the range 25 µg/kg–300 µg/kg and the aminoglycoside at 25 µg/kg. The method is specific, sensitive, robust and performs at a high degree of precision.

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SHORTENED VALIDATION OF A RADIORECEPTOR ASSAY TECHNIQUE FOR VETERINARY DRUG RESIDUES IN FISH

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Abstract

A shortened validation of a radio receptor assay methodology was conducted following the European Commission Decision 2002/657/EC was implemented for β–lactams, tetracycline, sulfonamides, macrolides and chloramphenicol in tilapia, catfish, carp and kanga. Chloramphenicol was detected below 0.3 µg/kg and penicillin G, tetracycline, chlortetracycline and oxytetracycline as well as erythromycin A were detected at 25 µg/kg, 50 µg/kg and 100 µg/kg, respectively. Sulfamethazine) was detected at 25µg/kg. Stability and cross reactivity tests confirmed suitability of the method for application in routine testing and monitoring.

1. INTRODUCTION

Fish contains vital nutrients for human consumption (not excluding use in animal husbandry) [3, 2] including over 20% of protein intake for a large portion of the global population. This has attracted a corresponding exponential increase in fish farming reaching 46% of the total fish production as far back as 2018 but expected to rise to 60% by 2030 [3]. Production in Cameroon like in many parts of the sub–Saharan Africa is also on the rise including aquaculture [4].

The contribution of Africa to the world aquaculture fish production has constantly increased from 0.09% in 2000 to 0.37 % in 2018 [3]. Production in Cameroon like in many parts of the sub–Saharan Africa is also on the rise including aquaculture [4]. In Cameroon more fish is consumed exceeding 400, 000 tons in 2015 with aquaculture's contribution projected to be over 20000 tons per annum [4, 5]. However, more of this fish is still imported at a cost of over US $ 200 million each year [5]. Intensive aquaculture is developing but this requires the of antimicrobials to prevent or treat diseases and improve yield [6]. These chemicals should be used appropriated and be regulated against standards such as the maximum residues levels [7] to safeguard consumers [8] and this involves safety and quality testing using a range of high end and often costly tools such as high performance liquid chromatography (HPLC), liquid chromatography coupled with mass spectrometry (LC–MS), gas chromatography (GC). Rapid and cost–effective screening methods are therefore needed. A good example is the Charm II radioreceptor assay test suitable for a range of analytes in different foods or related matrices [9, 10].

Ensuring that the methods are fit for the job requires validation. Primary validation of Charm II radioreceptor assays was previously conducted at the Institute for Agriculture, Fisheries and Food (ILVO) in Belgium under a collaborative study [11]. To apply this screening elsewhere, a shortened validation was conducted [12]. Validation in one laboratory facilitates quality control and reliable transfer to a recipient laboratory [13] that may conduct A shortened

validation of its own [7]. The shortened validation study for tetracyclines, chloramphenicol, β–lactams, sulfonamides and macrolides in four fish species in Cameroon covered detection capability, repeatability, reproducibility, specificity and robustness [12].

2. MATERIALS AND METHODS

2.1.Chemicals, reagents and equipment

The following were used: multiantimicrobial concentrate standard consisting of chlortetracycline at 4000 µg/kg, erythromycin A at 10000 µg/kg, penicillin G at 1000 µg/kg, streptomycin 10000 µg/kg and sulfamethazine at 1000 µg/kg (Charm Sciences Inc.). Other standards were tetracycline, chloramphenicol, chlortetracycline, enrofloxacin, oxytetracycline, sulfamethazine, sulfamethoxazole, and (Sigma Aldrich, MO, USA). Radioreceptor assay kits β–lactams (PMSU 050E), chloramphenicol (ATBL 012B), macrolides (EMSU 024A), sulfonamides (SMMSU 022H), tetracyclines (TMSU 027). MSU extraction buffer and M2 buffers, controls, radiotracers tablets (Charm Science Inc.); optifluor (PerkinElmer), Intronic incubator, large Bore 80 °C incubator (Charm Sciences Inc.), Charm II Analyser (Sciences Inc LSC 7600), refrigerated centrifuge (Hettich Rotofix 32A D 78532, Germany), food processor (Black & Decker, England), among others [12].

2.2.Study site, sampling and sample handling

The samples were collected from aquaculture farms, located in the localities of Mfou and Batié between September 2018 and October 2019 and handled as detailed elsewhere [12]. Six fish samples weighing 500 g were randomly collected for each species. Blank samples were used for the validation process following testing [10]. The samples (10 g) were extracted using the MSU buffer and further processed prior to detected [12].

2.3.Detection of the antimicrobials in fish

The Charm II 7600 Analyzer was used to detect β–lactams (with PMSU 050E test kit); tetracycline (with TMSU 027 kit); the sulfa drugs (with SMMSU 022H kit); the macrolides (with EMSU 024A kits); chloramphenicol (with ATBL 012B kits), details including the establishment of control points are detailed elsewhere [12].

2.4. Method validation parameters

The parameters validated included detection capability (CCβ), repeatability, reproducibility, robustness and specificity following established guidelines [7, 14]. As detailed elsewhere [12] twenty blank samples were spiked with β–lactams, tetracyclines, macrolides, sulfonamides and chloramphenicol at varying concentrations. The repeatability also involved 20 samples analyzed by the same investigator with at least 5 replicates. The reproducibility involved analyses on different days by two different investigators handling the same equipment. Method robustness involved deliberate varying of analytical conditions such as reading time for different samples following storage at 4°C. Potential cross reactivity and therefore false positive was investigated by analyzing for target drugs when the blank samples are spiked with nontarget drugs.

3. RESULTS AND DISCUSSION

3.1.Bank samples and control points

As detailed elsewhere [12] tilapia, catfish, carp and kanga samples did not contain β–lactams, tetracyclines and macrolides. Chloramphenicol was not detected in tilapia, carp and catfish while kanga and catfish did not contain sulfonamides. Statistical analysis of findings from the blank samples tested using the Charm II radioreceptor assay systems showed no significant difference between results for tilapia, catfish, carp and kanga fish tested with β–lactams and tetracyclines kits. The same was observed for sulfonamide kits in tilapia and carp. Zero control samples were used to determine the control points for tetracycline, while for β–lactams, sulfonamides, macrolides and chloramphenicol, blank fish samples were spiked. The cpm values were 1097, 1354, 1533, 1215 and 1230 for β–lactams, tetracyclines, sulfonamides, macrolides and chloramphenicol, respectively. The findings agreed with established guidelines and previous work [7, 10, 11, 15].

3.2.Validation parameters

The CCβ was established at regulatory limit for chloramphenicol and below the regulatory limit in a range of 0.25–0.5×MRLwith no false negatives for all the drugs. Residues of penicillin G Erythromycin A in tilapia, catfish, carp and kanga at 25 µg/kg and 100 µg/kg, respectively while sulfamethazine was detected at 25 µg/kg in catfish and kanga [12]. Tetracyclines were also detected as reported elsewhere [11, 16]. The tests demonstrated good repeatability with precision values for β–lactams tetracyclines, sulfonamides, macrolides and chloramphenicol) below 15%. The methods also demonstrated reproducibility below 15%. The method was robust as there was no significant change in cpm after varying the measurement time from 0 to 24 h. Furthermore, the methods demonstrated specificity and lack of cross reactivity.

4. CONCLUSIONS

A specific, repeatable and robust radioreceptor assay method for a range of veterinary drug residues in fish in Cameroon has been successfully transferred and used following a shortened validation approach. The method is fit for the testing of penicillin G, tetracycline, oxytetracycline and chlortetracycline as well as erythromycin A in tilapia, catfish, carp and Kanga fortified at 0.5 MRL (25 µg/kg, 50 µg/kg and 100 µg/kg, respectively. Others are sulfamethazine at 25 µg/kg and chloramphenicol at 0.3 µg/kg.

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VALIDATED RADIO RECEPTOR ASSAY METHOD FOR TETRACYCLINES, BETA–LACTAMS AND SULFONAMIDES IN SEAFOOD

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Abstract

A Charm II radioreceptor assay tests for certain tetracyclines, β–lactams and sulfonamides in a range of seafood and aquaculture products including tilapia, catfish, African Threadfin, common Carp and shrimps. Detection capabilities, precision (repeatability and reproducibility) as well as robustness. The latter included analysis of samples at varying periods up to 24 h after preparation and refrigeration. Sample weight and incubation time were also varied with no appreciable differences noted. Samples were detected at as low as 25 µg/kg.

1. INTRODUCTION

Aquaculture practices including a range of species [1] around the world such as periwinkle shrimps, prawns, swimming crabs and lobsters, in Nigeria, most of which are of greater commercial value [2]. Intensive production practices and diseases constrain aquaculture productivity [3] and requires control with chemical such as antimicrobials whose exposure to is associated with negative effects including alteration of the microbial flora [4]. Residues are attributed to failure to observe withdrawal period [5] and application not in accordance with set does or recommendations [6] including maximum residues levels [7]. Cost effective, sensitive screening techniques with short turnaround times are required hence this study [8].

2. MATERIALS AND METHODS

2.1.Materials, reagents and equipment

The following items were used TMSU Tablet Reagents TMSU026B (for Tetracyclines), PMSU Tablet Reagents PMSU050D (for β–lactams), SMMSU Tablet Reagents SMMSU 022G (for sulfonamides), MSU–Multiantimicrobial Concentrate Standard (MSUMA), Tissue Performance Negative Concentrate (TPNC), MSU–Extraction Buffer (MSUEB), M2 Buffer and pH strips (Charm Sciences Inc., Lawrence MA, USA). Others were amoxicillin, ciprofloxacin, tetracycline, chlortetracycline, erythromycin, gentamycin sulphate, lincomycin hydrochloride, oxytetracycline, sulfachloropyridazine and sulfadimethoxine (US Pharmacopoeia); ampicillin and cloxacillin (European Pharmacopoeia); sulfadiazine (Sigma Aldrich); glass test tubes, corks for test tubes, plastic centrifuge tubes, scintillation fluid, food processor, 80^{o}C large bore incubator, vortex mixer; centrifuge (Hettich Rotofix 32) and liquid scintillation counter (Charm Sciences Inc., Lawrence MA, USA).

2.2.Samples, preparation and extraction

The samples including tilapia, catfish, African threadfin, common carp and shrimps were collected from a local market in Lagos, Nigeria and the muscle tissues stored in sample bottles at -18^{o}C [8]. After thawing, 10 g each was extracted following a known protocol [9] and analyzed thereafter as detailed elsewhere [8].

2.3.Tetracycline, β–lactam and sulfonamide assay procedure

As detailed elsewhere [8] tablets containing the binders were dissolved in water (300 µl), the extract or control was added followed by incubation at varying temperatures between 35 (± 2)^{o}C to 65 (± 2)^{o}C for 2 min to 5 min and then centrifuged for 2 min – 5 min at ~33 × 100 rpm (Hettich Rotofix 32 centrifuge). Subsequently the pellet was dissolved in water (300 µl), 3ml scintillation fluid added and analyses conducted on the liquid scintillation counter using ^{3}H channel for sulfonamides and tetracyclines while the β–lactams on the ^{14}C channel [9].

2.4.Method validation

The method was validated for detection capability (CCβ), specificity (selectivity, cross reactivity), ruggedness, robustness, repeatability and reproducibility [8, 9].

3. RESULTS AND DISCUSSION

3.1.Selection of blank samples and control points setup

Fish samples (n = 20) were analysed to rule out the presence of inherent tetracyclines, β–lactams and sulfonamide residues. All the samples were confirmed to be blank and then used for determination of control points [8]. For each control point, six tests were conducted, and relevant correction factors included such as subtracting 40% from the calculated value for tetracyclines, adding 20% for penicillin G and 30% for sulfonamide. The respective levels were 1668, 1405 and 1130 for tetracycline, β–lactams and sulfonamide, respectively [8].

3.2.Validation parameters

The CCβ for oxytetracycline and tetracycline was 50 µg/kg; 100 µg/kg for chlortetracycline was only detected at 100 µg/kg (MRL). For ampicillin, amoxicillin sulfadiazine, sulfadimethoxine and sulfachloropyridazine the value was 25 µg/kg and 100 µg/kg for cloxacillin [8] The method demonstrated specificity as there no observed interference from other non-targeted analytes. Further, minor intentional changes in the experimental conditions such as delayed reading of test results didn't result in significant changes in readings implying that the method is robust. Good precision was also demonstrated as the repeatability and reproducibility were below 10% and acceptable range [8].

4. CONCLUSIONS

A radioreceptor assay method has been demonstrated to be fit for the purpose of analyzing residue of veterinary antimicrobials β–lactams, sulfonamides and tetracyclines in African threadfin, catfish, common carp, shrimps and tilapia collected in Nigeria. The detection ranges from 25 µg/kg to 150 µg/kg for various analytes. The method didn't demonstrate cross reactivity, was robust and showed good precision. The method is ready for use in routine testing and monitoring with confidence that good results would be attained.

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ISOTOPE–BASED LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY FOR MULTIRESIDUES IN CAGE FARMED *OREOCHROMIS NILOTICUS*

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Abstract

A new analytical method has been developed and validated for simultaneous analysis of 12 antimicrobials belonging to amphenicols, tetracyclines, sulfonamides and quinolones in *Oreochromis niloticus*. Samples were treated with Na_2EDTA, acetonitrile, water (with formic acid) and cleaned up using solid phase extraction material before analysis on an LC–MS/MS along with sulfadimethoxine–d6 was used as an internal standard to improve method performance. Good linearity was observed using matrix matched standards. Good limits of quantification (below 4.3 µg/kg) and recoveries (84% to 110%) were determined, and the method used for analysis of field samples in São Paulo State, Brazil.

1. INTRODUCTION

Use of antimicrobials in food production is associated with residues in food chain [1] and these may be parents or their metabolites [2] resulting in health consequences including antimicrobial resistance [3]. One of the common uses is in the aquaculture sector that has grown reaching 60.2% in certain countries such as Brazil [4] with *Oreochromis niloticus* one of the fastest [5] given the country's natural resource–potential [6]. Cage farming of this fish in Brazil involves overcrowding resulting in high mortalities and stresses [7, 8] although other countries do also encounter related challenges [9–14] warranting use of antimicrobials [15] such as florfenicol and oxytetracycline [16].

Testing and surveillance of these drugs is necessary but sample preparation may present challenges to analysts due to complex matrices requiring range of approaches such as solid–liquid extraction [17] or solid phase extraction [18, 19] or immunoaffinity [20] or metal chelate affinity chromatography [21]; Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS) albeit with some challenges [3]; involving subcritical water extraction [22] use of magnetic molecularly imprinted polymer [23], or pressurized liquid extraction [24] and a solid–phase microextraction [25] among others.

These procedures have varying limitations hence the need for improved sample preparation followed by isotopic chromatographic and spectrometric analysis. A study was therefore undertaken to develop and validate a rapid method LC–MS/MS using captiva cartridges, followed by application in field samples.

2. MATERIALS AND METHODS

2.1.Chemicals, reagents and equipment

The following were used: methanol and acetonitrile (HPLC grade, Tedia Company Inc., Fairfield, OH, USA), 99.5% formic acid (JT Baker, Phillipsburg, USA), Na_2EDTA (Sigma Aldrich, Dorset, UK); MilliQ system (Millipore, Bedford, USA); Sigma Aldrich (St. Louis,

USA) standards: oxytetracycline (OTC, 97%), tetracycline (TC, 97.5%), chlortetracycline (CTC, 93%), ciprofloxacin (CFX, 99.5%), enrofloxacin (EFX, 99.0%), sarafloxacin (SAR, 97.2%), norfloxacin (NFX, 99%), sulfathiazole (STZ, 98.0%), sulfadimethoxine–d6 (SDM–d6, 99.4%), florfenicol (FF, 98.0%); ChemService (West Chester, USA) standards sulfadimethoxine (SDM, 99.5%) and sulfamethazine (SMZ, 99.5%) as well as chloramphenicol (CAP, 98.5%, Dr. Ehrenstorfer GmbH, Augsburg, Germany). The antimicrobials were selected based on the Brazilian National plan of residues and contaminants [26]. Other items were ultraturrax Marconi model MA102 (Piracicaba, Brazil); Hitachi CF16RXII centrifuge (Hitachinaka, Japan); polyvinyldifluoride and polypropylene tubes (Agilent Technologies, Wilmington, USA); Manifold Supelco Visiprep.

2.2.Standards, sampling and sample preparation

Individual stock standard solutions (100 µg/ml) were prepared in methanol and stored at -20°C in amber coloured bottles and from this, a working standard solution (1000 µg/l) was prepared using appropriate dilution of the stock solutions in water [27]. Study samples were collected from four nile tilapia cage farms at the Ilha Solteira hydroelectric reservoir, São Paulo. Three cages were selected per farm and fish obtained in triplicate (n=36), shipped in cold condition and kept at -18°C if not analyzed immediately. Samples (5 g minced) were placed in 50 ml test tubes and 50 µl of 1.0 µg/ml sulfadimethoxine–d6, 1 ml of 0.1 M Na_2EDTA solution, 24 ml of acetonitrile:water (70:30, v/v) both with 0.1% formic acid solution were added. Samples were homogenized for 5 min (ultraturrax Marconi model MA102) spined on a centrifuge (Hitachi CF16RXII centrifuge) at 1370 g for 5 min and the supernatant (500 µl) eluted through captiva ND cartridges (3 ml, 0.2 µm). Finally, 2 ml of the resultant solution was analyzed by LC–MS/MS [27].

2.3.LC–MS/MS analyses

Analytes were separated on an Agilent Zorbax Eclipse Plus C18 (3 × 100 mm; 3.5 µm) under the following conditions: mobile phases consisting of Milli Q® water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B); 5% B at linear gradient until 95% of B in 13 min and maintained for 3 min; run for 15 min; re–equilibrated to 5% B for 10 min; 0.4 m/min flow rate; 30 °C;10 µl injection volume. The Agilent 6430 LC–MS/MS (Wilmington, USA) was generally operated in the positive electrospray ionization (ESI) mode for all analytes except FF which was analyzed in negative mode with a slight tweak in the gradient mobile phase where the mobile phase started with 30% B for 2 min, 95% B for 7 min and re–equilibrated to 30% B for the rest of the run. Other instrument parameters were gas flow (9.0 L/min to 11.0 L/min), nebulizer pressure (35 psi to 50 psi), gas temperature (300°C to 400°C); capillary voltage of 4000 V; Nitrogen gas purity of nitrogen 99.99%. Ion fragments were determined by direct infusion of 10 µl of each standard (1.0 µg/ml in acetonitrile) in the mass spectrometer.

2.4.Validation, quality control and quantification procedure

The method was validated for parameters such as linearity, specificity, limits of detection (LOD) and quantification (LOQ), precision and recovery following a known guide [28]. Matrix matched calibrators were prepared using blank fish of known concentration, in the range 5 µg/kg to 400 µg/kg, in triplicate. Blank samples were spiked at 50 µg/kg, 100 µg/kg, and 200 µg/kg in a day and between days (by different analysts). The same concentration values were used to determine recovery with sulfadimethoxine–d6 used as an internal standard.

3. RESULTS AND DISCUSSION

The mass spectrometric parameters are summarized in the Table 1 below.

TABLE 1. MASS SPECTROMETRIC PARAMETERS.

Compounds	Precursor ion	Product ion	Fragmentation energy (V)	Collision energy (V)
Chlortetracycline	479.1	462.2	125	12
	479.1	444.1	125	17
Oxytetracycline	461.2	426	115	16
	461.2	201.1	115	41
Tetracycline	445.2	410.2	115	17
	445.2	154.2	115	30
Sulfadimethoxine	311.1	156	120	16
	311.1	108	120	28
Sulfamethazine	279.1	186	115	12
	279.1	156	115	16
Sulfathiazole	256	156	90	8
	256	108	90	20
Ciprofloxacin	332.1	288.1	125	13
	332.1	245.1	125	22
Enrofloxacin	360.2	342.2	132	17
	360.2	316.2	132	16
Norfloxacin	320.1	302.1	125	20
	320.1	231.0	125	44
Sarafloxacin	386.1	342.1	119	15
	386.1	299.1	119	26
Chloramphenicol	323	305	70	0
	323	275	70	8
Florfenicol	355.9	335.9	139	5
	355.9	185.1	139	13
Sulfadimethoxine–d6	317.1	162.2	65	20

Compounds	Precursor ion	Product ion	Fragmentation energy (V)	Collision energy (V)
	317.1	108.1	65	28

Analytes were identified using a combination of factors including retention time and the ion transitions as shown in table one above and following recommended guidelines [29]. Better peak separation was observed with acetonitrile than with methanol and the optimum dwell time was 50 ms.

3.1.Validation method and quality control

There were no interfering peaks at the retention times for the antimicrobials of interest following analysis of blank fish samples spiked with a mixture of the drugs, followed by clean up with the captiva SPE cartridges. The LOQ was 5.0 µg/kg and very good linearity ($r^2 > 0.99$) was observed following analysis of calibrants in the range 5 µg/kg–400 µg/kg with each level in triplicate. The reference MRLs were 100 µg/kg for most analytes except sarafloxacin (30 µg/kg) and chloramphenicol where there is no MRL [30, 31]. The LODs/LOQs are summarized as follows: CTC = 0.91/3; OTC=1.2/4; TC=1/3.3; SDM=0.3/0.9; SMZ=0.8/2.56; STZ=1.3/4; CFX=0.4/1.2, EFX=0.5/1.5; SAR=1/3.5; FF=1.1/3.6 in µg/kg. The LOQs were comparable to those reported elsewhere [32–34] with the limits detected below MRLs [30, 31].

The interday and intraday day precisions determined by analyzing samples spiked at 50 µg/kg, 100 µg/kg and 200 µg/kg, ranged from 2.8% to 10.3% 2.5% to 10.6%, respectively. The recoveries ranged from 82.8% to 108% with lower values observed among the quinolones. The results are comparable to other findings [35].

3.2.Application to field samples

The method was used to analyze nile tilapia muscle samples (n=36) collected from four cage farms in a high–volume fish producing region of São Paulo State, Brazil Residues of OTC, TC and FF detected as follows. OTC, farm 1, cage 1: 61.6 (± 18.6) µg/kg; cage 2: 20.2 (± 7.5) µg/kg; farm 3, cage 1: 1162.1 (±111.5) µg/kg; TC cage 1: 7.7 (± 0.8) µg/kg; and FF farm 4, cage 1: 524.8 (± 6.3) µg/kg. The presence of OTC and FF was not a surprise since the to two drugs are Oxytetracycline and florfenicol are the only two available molecules licensed for aquaculture production in Brazil [15]. The OTC levels ranged from 15.6 to 1231.8 µg/kg with several cases above MRLs of 100 µg/kg [31] and 200 µg/kg [30]. TC is reported as a by–product in the manufacture of oxytetracycline [36]. The presence of TC is not a surprise therefore given the high levels of OTC.

4. CONCLUSIONS

An analytical method for confirmatory analysis of a range of antimicrobials in targeted caged aquaculture farms in Brazil was developed and validated. The method demonstrated suitability for testing 12 antimicrobials namely, chlortetracycline, oxytetracycline, tetracycline, sulfadimethoxine, sulfamethazine, sulfathiazole, ciprofloxacin, enrofloxacin, norfloxacin, sarafloxacin, chloramphenicol and florfenicol in nile tilapia edible muscles. The fish was collected from caged fish farms located in Ilha Solteira hydroelectric reservoir, São Paulo, formed by Paraná and Grande rivers in Brazil. The method showed good recovery capabilities, selectively and specificity as well as precision with the help of stable isotope labelled standards

such as sulfadimethoxine–d6. Further studies involving more labelled standards can further improve such methods**.**

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A UHPLC–MS/MS METHOD FOR POLYETHER IONOPHORES, TETRACYCLINES AND SULFONAMIDES IN AQUACULTURE FISH AND OTHER TISSUES

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Abstract

A new ultrahigh performance liquid chromatography tandem mass spectrometry method for residues of polyether ionophores, tetracyclines and sulfonamides in animal and aquaculture fish tissues was developed, validated and now available for application. A cost–effective sample preparation method was used, and validation followed EU guidelines with very good rates of recovery (80 %–113%) and repeatability (<15%) were obtained, while the decision limit in the range 50.8 µg/kg – 125.8 µg/kg and detection capability in the range 51.5 µg/kg – 151.7 µg/kg were reported. The method was satisfactorily used for proficiency testing and used to analyze field South African samples.

1. INTRODUCTION

Pharmacologically active veterinary drugs are used widely used in livestock production [1] with applications including prophylaxis, therapy and feed efficiency. Ionophores are an example of the veterinary drugs that disrupt Ca^{2+}, K^{+}, H^{+}, Na^{+} concentration gradients [2]. Monensin one of the ionophores can cause 16 % increase in daily body weight gain in cattle, when used as supplements [3]. Another group, the tetracyclines are widely used to treat a range of infections [4]. Aquaculture production is one of the sectors that has benefited greatly from the use of antimicrobials such as tetracyclines and sulfonamides [5]. Use of these drugs can result in residues that pose health risks [1, 6] including allergic reactions for sulfonamides [7]; muscles and neurologic effects for polyether ionophores [8] as well as effects on the blood cells and teeth in the case of tetracyclines [9]. Proper regulation or outright banning is therefore required to safeguard consumers and promote fair trade [10–12].

Reliable analytical methods are required to determine residues of the drugs in consumer products [1, 5, 11, 13]. The techniques include enzyme linked immunosorbent assay [14–16] that is relatively cheaper although associated with false positive results [11]. Others are chromatographic–mass spectrometric techniques including those with high resolution and applicable for multiple analytes [1, 5, 6, 11, 17–19, 21–24]. Appropriate sample preparation techniques are also required to address challenges associated with complex matrices [1]. Many multiresidue and multiclass analytical methods are laborious and require relatively high volume of solvent [25–29]. Abafe et al., [30] have recently developed and validated a UHPLC–MS/MS inhouse for detection of polyether ionophores, sulfonamide and tetracyclines in bovine, chicken, porcine and aquaculture fish tissues following preparation of the matrices with a modified QuEChERS extraction procedure.

2. MATERIAL AND METHODS

2.1.Chemicals, reagents and equipment/other Material

The following items were used: Tetracycline hydrochloride, chlortetracycline hydrochloride, doxycycline hyclate, oxytetracycline hydrochloride, salinomycin monosodium salt hydrate, narasin, monensin sodium salt hydrate, maduramycin ammonium, lasalocid A sodium salt

solution, sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfadimidine, sulfadoxine, sulfamerazine, sulfamethoxazole, sulfamonomethoxine, sulfapyridine, sulfathiazole and sulfaquinoxaline all above 95% of purity (Sigma Aldrich, South Africa). Others were acetonitrile**,** oxalic acid and sodium sulphate (99.9%, Merck, South Africa); HPLC grade water (Elgastat UHQ water purifier system 18.2 Ωm) and Bondesil C18 powder (Chemetrix, South Africa). The UHPLC–MS/MS (Shimadzu Nexera X2 and ABSciex 4500 series QTRAP triple; a Kinetex® 2.6 µm Biphenyl 100 Å LC Column 50 x 2.1 mm; A Zymark Turbo Vap system.

2.2.Sample preparation

The procedure according to Abafe et al., [30] included use of blank samples: edible aquaculture fish; bovine/porcine liver, kidney and muscle; chicken liver and muscle each weighing 5 (± 0.1) g. These were placed in 50 ml centrifuge tubes and fortified with 1.0 mg/l of the drugs, 15 ml 1% oxalic acid in acetonitrile with 1 % oxalic acid added after mixing on a vortexer for 30 s. The content was shaken for 5 min and spined on a centrifuge (5000 rpm, 10 min, 4°C) before the supernatant was transferred to propylene tubes containing 0.5 (± 0.1) g of Bondesil C18 clean up material. This mixture was also shaken for 15 min, spined on a centrifuge at 5000 rpm for 10 min and 4°C, and the next supernatant transferred into a clean 15 ml propylene tube before evaporating the mixture to dryness using nitrogen. Lastly 0.5 ml of acetonitrile with 0.1 % formic acid was used to dissolve the residue, the content mixed for 30 s on a vortex mixer and pressed through a 0.22 µm syringe filter prior to analysis.

2.3.Instrument conditions

The analytes were separated using a C18 chromatographic column (50 × 2.1 mm, 2.6 µm) maintained at 40°C, and using a mobile phase consisting of ultrapurified water with 0.1 % formic acid (Solvent A) and acetonitrile with 0.1 % formic acid (Solvent B) operated as follows: 5 % solvent B run for 1min, a linear gradient of 40 % B (7 min), linear increase of solvent to 80 % B (3 mins) and then 100 % B (3 min) before equilibration with 5 % B (3 min). The flow rate and sample injection were 0.35 ml/min and 10 µl, respectively. The analytical instrument was operated in the positive ESI and multiple reaction monitoring modes with the ion spray voltage set at 5500 V, using nitrogen as the nebulizer gas, collision gas, curtain gas and turbo gas.

2.4.Method validation

The method was validated following generally acceptable guidelines [31] and the parameters included selectivity, linearity, trueness, repeatability, decision limits and detection capability, limit of quantitation, interday precision and reproducibility. Blank samples (n=7 per matrix) were analyzed each day during the validation period and monitored for potential interfering peaks at a signal–to–noise ratio of three at the retention time of a target analyte in order to determine specificity and selectivity of the method. Matrix matched standard curves in the range 1 µg/kg–200 µg/kg with eight points of concentration although extrapolation was considered. Recovery studies were conducted using blank samples (n=63) at the 0.5×MRL, 1×MRL and 2×MRL levels on three different days.

Trueness was further confirmed through participation in proficiency testing. Additional samples (n=21) also at the three MRL levels were used to determine intraday (repeatability) and between day (reproducibility) precision expressed as % RSD. The experiments were repeated for three different days in order to evaluate the within laboratory reproducibility. Further, 63 fortified samples were used to determine the decision limit (CCα) and detection capability (CCβ). Each

matrix (n=7) was run over a period of three days and the CCα was determined as the sum of the standard deviation (1.64 times) and the specific MRL. The CCβ was the sum of the standard deviation (1.64 times) to the CCα.

A study was also undertaken to investigate signal interference which has effects on analytical results [32]. A procedure previously reported [33] was followed with minor modification. Percent signals of the ratios of spiked matrix to those of the standard were used to determine if signal enhancement or suppression occurred.

3. RESULTS AND DISCUSSION

3.1.UPLC–ESI–MS/MS determination

The standards (1 µg/ml in 0.1% formic acid in methanol) were infused in the positive ESI mode to establish appropriate instrument parameters summarized in Table 1 below.

TABLE 1 SUMMARIZING MS PARAMETERS

Name of analyte	Precursor ion	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision cell exit (CXP)
SGD	215.140>156.1/108.1	30	10/8	20/30	6/4
SDX	311.080>156.2/108.2	70	8/10	30/40	6/8
SMP	281.080>156.2/108.1	70	8	25/30	6/2
SMN	281.100>156.2/92.2	60/70	10	25/40	6
STZ	256.120>156.2/92.1	50	2	25/40	10/15
SMR	265.080>92.0/156	50	14/2	40	15
SPD	250.060>92.1/156.1	60/50	2/14	40/25	15
SDM	279.070>92.1/186.2	60	14	40/25	15
SCP	285.050>156.0/92.1	40/50	14	25/40	15/10

Name of analyte	Precursor ion	Declustering Potential (DP)	Entrance Potential (EP)	Collision Energy (CE)	Collision cell exit (CXP)
SDT	311.100>156.3/92.2	50/60	14/12	25/40	15
SMTX	253.922>156.0/92.0	61	10	21/31	8
SDZ	251.054>155.9/92.0	51	10	21/31	10/8
SQX	301.028>156.0/108.0	6	10	23/37	12/8
Maduramycin	934.370>629.4/393	121	10	35/45	10/12
Narasin	782.369>747.5/729.4	31	10	27/39	10/32
Salinomycin	768.359>733.4/715.4	76	10	27/37	10/32
Monensin	693.279>675.4/461.3	156	10	49/69	28/20
Lasalocid A	613.224>377.2/577.2	146	10	49/45/49	16/13/18
Tetracycline	445.095>154.1/427	70/10/70	2/4/3	40/20/40	6/8/6
Doxycycline	445.106>410.1/321.1/428.1	60/40/40	2/8/8	40/20/20	10
Chlortetracycline	479.000>444.0/462.0/154.0	40/10/40	4/2/4	30/20/30	10/8/8
Oxytetracycline	460.981>425.9/442.9/381.1	70/50/50	8/4/4	30/20/20	8/4/10

Good chromatographic separations were obtained over a run time of 12 min when using the mobile phase containing 0.1 % formic acid in water and 0.1 % formic acid in acetonitrile.

3.2.Optimization of sample preparation

The study developed a simple and generic extraction procedure for simultaneous determination of a range of drug residues including aquaculture fish such as trout, finfish, tilapia and catfish as well other livestock products. Samples (5.0 g) spiked with a mixture of the analytes and treated with organic solvents such as methanol/acetonitrile including buffers such as McIlvaine buffer and succinate buffer, yielding good recoveries [1]. In this study better recoveries were obtained using acetonitrile with formic acid. Oxalic acid was established as a good modifier and suitable alternative in the absence of complexing agents such as ethylenediamine tetra acetic acid (EDTA) which is almost mandatory when analyzing tetracyclines due to a propensity to form chelates resulting in poor recoveries [34]. The appropriate amount of the C18 cleanup material for which good recoveries and excellent reproducibility were obtained with very little (<15%) matrix effects was 0.5 g [30].

3.3.Method validation

The ion intensity ratio of the quantifier and qualifier transitions to corresponding matrix matched standard calibration curves was within ±20% of acceptable range. The analyte retention time in the matrix closely matched (within ±2.5%) that in the standard solution. Method selectivity was confirmed by analyzing 28 control blank samples from all the four species, while considering the absence of interfering peak at the same retention time. Very good linearity ($R^2 > 0.990$) was obtained for all standards following analysis of matrix matched calibration curves (6 to 8 points) in the range of 1 µg/kg–200 µg/kg. The within day repeatability (n=7) was below 5 % for all analytes in the different matrices, while the reproducibility (n =21) ≤15% with the between day (n=21) reproducibility was 3.2% –15% for fish. Method trueness was determined through recovery studies involving 21 replicates for each matrix. The levels for fish were 85% to 110%, while for bovine, chicken and porcine tissues, the ranges were 86%–112%, 85%–110%, 92%–112% and 88%–108%, respectively. The method was successfully subjected to two proficiency testing schemes.

3.4.Decision limit and detection capability

The CCα and CCβ were 111 µg/kg – 126 µg/kg and 122 µg/kg – 151.7 µg/kg for tetracyclines, 113 µg/kg–118 µg/kg, 117 µg/kg – 127 µg/kg for sulphonamides, 51 µg/kg – 52.4 µg/kg and 52 µg/kg–56 µg/kg for polyether ionophores, respectively [30]. The findings agree with previous reports [5, 23, 35]. The LOD was the lowest level in the calibration curve while the LOQs ranged from 1 µg/kg for polyether ionophores to 25 µg/kg for tetracycline and sulphonamides.

The method was used to analyze residues of polyether ionophores, sulfonamides and tetracyclines in field trout, finfish and tilapia liver, muscle and kidney of chicken, pig and bovine tissues obtained locally in South Africa. No residues were detected in the aquaculture finfish, trout and tilapia samples although the other matrices contained monensin, narasin, salinomycin, lasalocid, maduramycin and oxytetracycline [30]. Polyether ionophores are registered for use in various animal species in South Africa [36] and therefore their presence was not a surprise.

4. CONCLUSIONS

A new cost–effective analytical method that is fit for confirmatory testing of residues of polyether ionophores, tetracyclines and sulfonamides in animal and aquaculture fish tissues has been established and is being used to implement South Africa's national residue monitoring programme. Very good recovery rates (80%–113%) and repeatability (<15%) were demonstrated and the method meets established guidelines such as the EU's on decision limit (50.8 µg/kg–125.8 µg/kg) and detection capability (51.5 µg/kg–151.7 µg/kg).

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LC–MS/MS TECHNIQUE FOR CONFIRMATORY ANALYSIS AND CONTROL OF CHLORAMPHENICOL RESIDUES IN FISH AND SHRIMP

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Abstract

A sensitive LC–MS/MS method was developed and validated for confirmatory analysis of chloramphenicol in fish and shrimp. The validation was performed according to the European Union Commission Decision 2002/657/EC and included parameters such as the detection capability (CCβ), specificity, stability of standard solutions and stability of the analyte in matrix. Statistical analysis was performed, and the results indicate that the method is robust when subjected to day–to–day analytical variations. The calculated CCα and CCβ were 0.039 μg/kg and 0.096 μg/kg, respectively.

1. INTRODUCTION

There has been a growing need for analytical methods to support the detection of veterinary drug residues in fish and shrimp as this industry grows [1–3]. One of the major needs is detection of forbidden substances such as chloramphenicol among others. There are new developments in Brazil's fish/shrimp aquaculture industry where indigenous species including tilapia are being introduced. These have hitherto been produced for the local market or for household consumption but could soon reach export marker scales and for which proper control of residues is required.

Different methods have been published for the determination of chloramphenicol residues in aquaculture and other biological matrices, such as enzyme linked immunosorbent assay [4, 5] although this may be associated with false positives. A gas chromatography–electron capture detector method has also been reported [6] although it is rather laborious. Mass spectrometry is increasingly important for confirmatory residue analyses. To ensure detection of chloramphenicol with higher sensitivity and specificity, a simple, rapid and reliable LC–MS/MS method was developed in this study.

2. MATERIALS AND METHODS

2.1.Chemicals and reagents

The chemicals and solvents used were of analytical grade and provided by Merck (Darmstadt, Germany and Synth (São Paulo, Brazil). Chloramphenicol and chloramphenicol–d5 were procured from Sigma Aldrich (Germany). Chloramphenicol–d5 was an internal standard. The apparatus included an UPLC Acquity coupled to a Waters TQD tandem mass spectrometer (Milford, MA, USA) using ESI ionization and MassLynx 4.1 software. The LC–MS/MS operating conditions are summarized in Table 1.

TABLE 1. OPERATING LC–MS/MS CONDITIONS FOR CHLORAMPHENICOL ANALYSIS IN SHRIMP AND FISH SAMPLES.

Parameters	UPLC Conditions			Parameters	MS Conditions
Column	Kinetex C18 (2.1 × 50 mm, 2.6 µm)			Ionization	ESI, negative
Eluent	Water: methanol			Capillary	2.0 kV
Flow rate	0.4 ml/min			Desolvation Temp.	450ºC
Oven temperature	45ºC			Desolvation flow	800 L/hr
Injection volume	10 µl			SIM ion	Chloramphenicol: 321.1 > 257.1, 321.1 > 152 Chloramphenicol–D5: 326.1 > 157.1
Gradient	Time (min)	A (%)	B (%)		
	0.3	90.0	10.0		
	1.7	10.0	90.0		
	1.9	10.0	90.0		
	1.95	90.0	10.0		

2.2.Standard solutions

The chloramphenicol and chloramphenicol–d5 stock solutions (100 µg/ml) were prepared by dissolving the powder in methanol and kept in a freezer at -20°C. Chloramphenicol working standard solutions and respective standard solutions were also prepared by diluting stock with methanol (1 µg/ml). A calibration curve in the range of 0.15 µg/kg–0.90 µg/kg was prepared daily from the 1 µg/ml working standard solutions. Also, 100 µl of each internal standard was added to all calibration preparation tubes. Prepared stock solutions were stored for 6 months while fortification standards were prepared on the same day of analysis.

2.3. Sample extraction and cleanup

Fish/shrimp samples in 20 g portions were sent to the Microbioticos laboratory, transported in isothermal vessels and keep frozen. These were later minced and homogenized. Subsample of 2 g were weighed into 15 ml falcon tube, 5 ml of ethyl acetate added, and the mixer shaken by hand for 1 min. The sample mix was then centrifuged at 3500 rpm for 10 min at 20°C. The supernatant was transferred to a glass tube and dried in a water bath at 50°C under a gentle stream of nitrogen. The residue was then resuspended in 0.35 ml of methanol: water (1:1, v/v). The samples were shaken for 1 min, 0.5 ml of hexane was added, and the content shaken gently before centrifugation at 3500 rpm for 5 min at 20°C. The organic phase was discarded, and the rest of the extract placed in a 1.5 ml vial before injection into the LC–MS/MS for analysis.

2.4. Method validation

Validation parameters such as a linearity using matrix calibration curves, recovery, decision limit (CCα), detection capability (CCβ), precision and accuracy were determined following known guidelines [7]. The calibration curve was determined to check the linear range of the procedure; a linear detector response was obtained in the range 0.15 µg/kg–0.90 µg/kg for the analyte. Three concentration levels were used for validation, including six replicates of shrimp and fish samples (n = 6/matrices) fortified with chloramphenicol for a final concentration of 0.3 µg/kg, 0.45 µg/kg and 0.6 µg/kg. The CCα was determined as the intercept plus 2.33 times the standard error of the within laboratory reproducibility. The CCβ was determined using the signal at CCα plus 1.64 times the standard deviation of the spiked samples of the within laboratory reproducibility. Recoveries were determined by comparing the peak areas from spiked samples with those obtained from standards at similar concentrations.

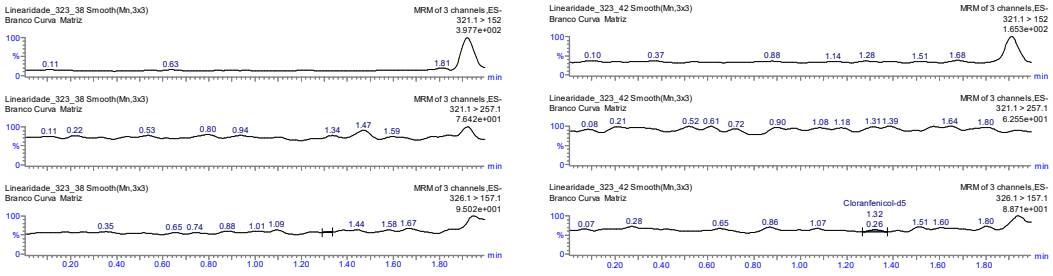
3. RESULTS AND DISCUSSION

Very good linearity with correlation coefficient (R) of 0.9931 was demonstrated in the range 0.15 µg/kg–0.90 µg/kg. The lower LODs were calculated based on a signal to noise ratio of 3 and varied from 0.15 µg/kg to 0.9 µg/kg. The results are shown in Table 2.

TABLE 2. PRECISION AND ACCURACY FOR CHLORAMPHENICOL IN SHRIMP AND FISH.

	Spike level (µg/kg)	Precision (%)	Accuracy (%)
Chloramphenicol	0.30	5.32	103.00
	0.45	6.24	102.08
	0.60	5.22	103.52

The CCα (0.039 µg/kg) and CCβ (0.096 µg/kg) were determined according to the established requirements [7]. Sample chromatograms are shown in Figs. 1 to 3 below.



(a) (b)

FIG. 1. *Chromatogram of a blank shrimp (a) and blank fish (b).*

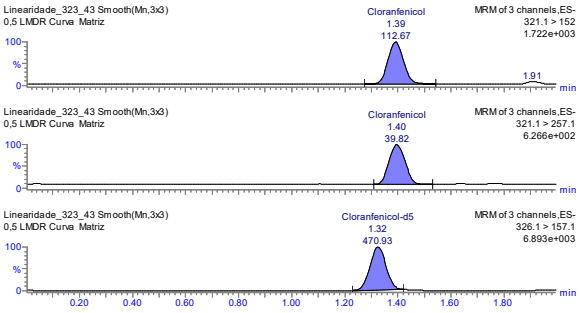


FIG. 2. *Chromatogram of chloramphenicol at 0.15 µg/kg*

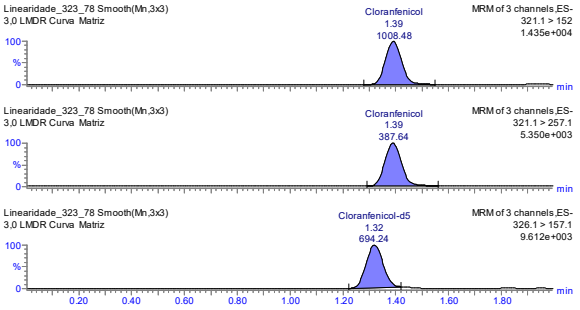


FIG. 3. *Chromatogram of chloramphenicol at 0.90 µg/kg*

4. CONCLUSIONS

A method for the confirmatory analysis of the forbidden substance chloramphenicol in fish and shrimp was developed and validated against the parameters including precision, decision limit, detection capability and accuracy. The method is fast, simple and accurate and can be applied to the control of shrimp and fish consignments to destinations where there is no tolerance of chloramphenicol residues in foods.

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DETERMINATION OF FIVE NITROFURAN METABOLITES IN SHELLFISH BY ISOTOPE DILUTION LC–MS/MS

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Abstract

A new isotopic LC–MS/MS method has been developed and validated to determine metabolites of furazolidone, furaltadone, nitrofurazone, nitrofurantoin and nifursol in shellfish. Sample preparation included acid hydrolysis and derivatization with 2–nitrobenzaldehyde, adjustment of the solution to pH 7.2 (±0.2), extraction with ethyl acetate, evaporation to dryness and addition of the mobile phase as well as hexane before separation of solvents, filtration and injection into the LC–MS/MS. The limits of quantification ranged from 0.1 µg/kg to 0.2 µg/kg using appropriate standards that demonstrated excellent linearity with correlation coefficients and >0.999. Good percentage recoveries (~89% to 112%) were also obtained. The new method has been used to analyze 280 shellfish products.

1. INTRODUCTION

Nitrofurans are synthetic antimicrobial agents widely used in aquatic food production. These include furazolidone, furazolidone, nitrofurazone and nitrofurantoin [1–3]. Nitrofurans and their metabolites are potentially carcinogenic/mutagenic [4, 5]. Although, they are common in aquaculture production due to low costs, nitrofurans are generally forbidden [6] and under strict control [7–9] with minimum required performance limit for relevant analytical method set at 1 µg/kg. Furans, a group of drugs related to these metabolites are also of concern to consumers of animal products [10]. Nitrofurans are rapidly metabolized to 3–amino–2–oxazolidinone (3–amino–2–oxazolidinone, AOZ); 5–methylmorpholine–3–amino-2 oxa 3–amino–5–morpholinomethyl–2–oxazolidinone (3–amino–5–morpholinomethyl–2–oxazolidinone, AMOZ); semicarbazide hydrochloride (SEM), 1–aminohydantoin hydrochloride (AHD) and 3,5– Dinitrosalicyl hydrazide (3,5–dinitrosalicylhydrazide, DNSAH). Nitrofurans also bind to body proteins.

Analysis of nitrofuran metabolites involves a range of chromatographic and biochemical techniques [11–13] although many have poor sensitivity and are prone to false positives. A method for four metabolites has been reported with recoveries in the range 80%–95% at detection limit of 0.5 mg/kg and limit of quantification of 0.3 mg/kg for meat [14]. An LC–MS/MS method with detection in the rage of 0.2 mg/kg–0.5 mg/kg has also been reported [15]. While such confirmatory methods are very important [16–18] they can be improved using stable isotope internal standards. Establishment of an isotope dilution LC–MS/MS method was undertaken to support determination of residues of five nitrofuran metabolites in shellfish.

2. MATERIALS AND METHODS

2.1.Chemicals, reagents and equipment

The following tools and material were used: API QTRAP 4500 Triple Quadrupole Tandem Mass Spectrometer (USA AB SCIEX company); Shimadzu LC 20A high performance liquid chromatograph (Shimadzu, Japan Company); Beckman Coulter AllegraTM X22R high speed centrifuge (US Beckman Company); XS205 Analytical Balance (Mettler Toledo, Switzerland); SW22 oscillating water bath (JULABO, German); NEVAPTM112 Nitrogen blower

(Organomation, USA); MilliQ IQ 7000 ultrapure (Millipore, USA); Centrifuge 5424R desktop, High speed centrifuge (Eppendorf, Germany); Multi reax oscillator (Heidolph Company); AHD, SEM, AOZ, AMOZ, AOZ–d4, AMOZ–d5, AHD–13C3, SEM–^{13}C–$^{15}N_2$ (>98%, Dr. Ehrenstorfer GmbH, Germany); 3,5–dinitrosalicylic acid hydrazine (DNSAH) and similar Internal standard DNSAH–$^{15}N_2$ (purity greater than 98%, Toronto, Canada, Research Chemicals Inc). Acetonitrile, methanol, ethyl acetate, n–hexane (Merck, Germany); ammonium acetate, o–nitrobenzaldehyde, > 99.0% (Fluka, Switzerland); Sodium phosphate (Na_3PO_4), sodium hydroxide (NaOH), hydrochloric acid (HCl) (Analytical grade, Guangzhou Chemical, China); MilliQ® Ultrapure water (18.2 MΩ·cm), Advantage A10 water purification system (Millipore, France).

2.2.Standard solutions

Stock standard and internal standard solutions (1.0 mg/ml) were prepared and stored at -20°C — away from light — from which mixed standard intermediate standards (1.0 µg/ml) and working (50 ng/ml) solutions were also prepared, the latter 2 stored at 4°C. A mixed internal standard solution (100 ng/ml), hydrochloric acid (0.2 mol/l) and 0.1 mol/l o–nitrobenzaldehyde were also prepared and used.

2.3.Sample pre–treatment

Homogeneous shellfish samples (2.0 g) were weighed into appropriate glassware, 5 mixed internal standard solutions 10 g/ml, 10 mL 0.2 mol/L HCl, 200 µl 0.1 mol/l added, the content mixed and shaken overnight at 37°C before addition of 0.3 mol/l Na_3PO_4. The pH was adjusted (2.5 mol/l NaOH to 7.2±0.2), 10 ml ethyl acetate added, and the content centrifuged at 8000 r/min for 5 min at 10°C. The ethyl acetate layer was aspirated (step repeated once) before evaporation of the combined solvents to dryness on water bath at 37°C, using nitrogen. This was reconstituted in a ml of acetonitrile/water (1:9 v/v), 1 ml of acetonitrile saturated n–hexane (1:1, v/v) added, the mixture again centrifuge at 15000 r/min for 5 min, the hexane layer removed (repeated once) and the remaining solution used on the LC–MS/MS.

2.4.Instrumentation

The LC conditions included injection of 10 µl of the solution for separation on a Waters Atlantis® dC18 column (150 mm × 2.1 mm, 5 µm), maintained at 35°C at a flow rate of 0.4 ml/min. Two mobile phases were used, namely A: 200 ml acetonitrile + 800 ml 10 mmol/l ammonium acetate + 0.37 ml ammonia (25 vol%), and phase B: acetonitrile. A gradient elution program was operated as follows: 0 to 0.5 min, 0%B, 0.5 to 5.5 min, 0%B to 80% B, 5.5~6.5 min, 80% B, 6.5 to 6.6 min, 80%B to 0%B; 6.6 to 9.0 min, 0%B.

The MS conditions/parameters were as follows: ESI, multiple reaction monitoring (MRM); ion spray voltage of +5500 V and -4500 V; ion source temperature of 550°C; 20 ms dwell time; curtain gas (CUR) pressure of 40 psi; the atomizing gas (GS1) pressure was 55 psi and the heating auxiliary gas (GS2) pressure was 55 psi.

The precursor (m/z) and product ion (m/z) ions in the ESI and negative modes were: AOZ (236>134/104) at declustering voltage, inlet voltage, collision cell outlet voltage and collision voltage of 68 V, 10 V, 9 V and 17/27 V; AOZ–d4 (240>134/104); 84 V, 7 V, 11/10 V, 20/30 V; AMOZ (335>291/262); 75 V, 10 V, 13 V, 17/22 V; AMOZ–d5 (340>296/265); 60 V, 12 V, 10 V, 17 V; AHD (249>134/104); 72 V, 10 V, 9V, 16/26 V; AHD–$^{13}C3$ (252>134/104); 78 V, 6 V, 7 V, 19/35 V; SEM (209>166/192); 60 V, 9 V, 8 V, 13/15 V; SEM–^{13}C–$^{15}N2$

(212>168/195); 60 V, 8 V, 10 V, 13/15 V; DNSAH (374>181.9/182.9); 60 V, 10 V, 9 v, 30/35 V; DNSAH–^{15}N2 (376>181.9/182.9); 60 V, 10 V, 9 V, 30/35 V.

3. RESULTS AND DISCUSSION

Matrix effect was investigated and calculated as the percent ratio of the analyte peak response in matrix relative to the same peak in the standard in nonmatrix solution. This followed addition of 5 µg/ml of the standards to blank scallop, clam, oyster, and abalone matrices and processing. Suppression of the signals was observed as follows: scallop, 65%–85%, flower beetle/mussel, 45%–85%, oysters, 25%–60%, and abalone, 20%–50%. To correct this problem, stable isotope internal standards are needed, and these made a significant difference.

3.1.Mass spectrometry and chromatographic conditions

The nitrofuran metabolite standards were infused in the instrument to assess suitability for ionization in the positive or negative electrospray modes. All the 5 analytes except DNSAH presented better analytical signals in the positive mode and as such simultaneous positive and negative acquisition were conducted. The effect of the mobile phase on the ionization and detection of the analytes was investigated. This included methanol/water, acetonitrile–acetonitrile/water and inclusion of 0.1% formic acid, 0.1% acetic acid, ammonia, ammonium acetate, and ammonium formate. The mobile phase with 0.1 % formic acid or acetic acid suppressed negative ions; ammonium formate showed tailing while ammonium acetate resulted in better peak shapes and ammonia solution improved ionization sensitivity. It was thus determined that acetonitrile:ammonium acetate solution reduces the baseline noise and improves separation of analytes, especially AMOZ at an optimum flow rate of 0.4 ml/min.

Sample preparation was also optimized. The effect of methanol and dimethyl sulfoxide as suitable solvent for dissolving o–nitrobenzaldehyde as the derivatization agent was studied and it was observed that while the two didn't have a significant difference, DMSO increased the efficiency of AMOZ extraction sixfold. The optimum solvent for reconstitution of the residue following extraction and evaporation was acetonitrile/water (1:9, v/v) as this provided good peak shapes.

3.2.Sample purification conditions

The effect of the defatting solvent was also studied. Acetonitrile was added and saturated with n–hexane followed by pressing the content through a 0.45 µm nylon filter. This was compared to a process where no such treatment was applied. The recovery levels for DNSAH and DNSAH–$^{15}N_2$ reduced due to filtration unlike defatting which generally improved recovery.

3.3.Linearity range, detection limit and quantification limit

Matrix matched standard curves were prepared in the range 0 µg/kg–20 µg/kg (0 µg/kg, 0.2 µg/kg, 0.5 µg/kg, 1.0 µg/kg, 2.0 µg/kg, 5.0 µg/kg, 10 µg/kg, 20 µg/kg) for the scallops, oysters, clams, and abalones and to each of these, the stable isotope labelled internal standard at a single concentration of 5 µg/kg was added. Good linearity (correlation coefficient, $r > 0.999$) was observed in the range of 0.2~20 µg/kg. The LODs/LOQs for AHD, AOZ, AMOZ, SEM and DNSAH in µg/kg were, respectively, 0.1/0/2, 0.05/0.1, 0.1/0.2, 0.1/0.2, 0.05/0.1. The LOD was estimated as 3 times the signal to noise ratio while the LOQ was 10 times.

The recovery studies were performed at 0.5 µg/kg, 1.0 µg/kg, 2.0 µg/kg and 20.0 µg/kg (n=6) and the levels ranged from 88.7% to 112%, at relative standard deviations of 3.9%–13.7%. The

method was then applied to analyze field samples. Shellfish samples (n=280) including scallop, clams, and abalone, and oysters etc were collected and analyzed for the metabolites and 45 samples were found to contain SEM at varying levels from 0.21 μg/kg to 1.86 μg/kg although only 4 samples contained SEM above the national minimum required performance limit (1 μg/kg). Other studies have reported SEM of up to 0.75 μg/kg in seawater and shellfish [19] following analysis of 90 shellfish samples collected from Jincheng and Sishili Bay.

4. CONCLUSIONS

A new isotope dilution LC–MS/MS method was developed and validated for the simultaneous determination of five nitrofuran metabolites, AOZ, SEM, AMOZ, AHD and DNSAH, and applied for analysis of field shellfish samples. The method involved simultaneous positive and negative ESI and MRM analysis. The LOD and LOQ were 0.05 μg/kg–0.1 μg/kg and 0.1 μg/kg–0.2 μg/kg, respectively. The method is applicable to routine laboratory work following detection of 16% of the 280 shellfish products collected.

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DETERMINATION OF EIGHT NITROFURANS IN SHELLFISH AND OTHER FISH

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Abstract

The use of nitrofurans (NFs) in aquaculture is prohibited in many countries and therefore requires proper regulation supported confirmatory analytical methods. A new liquid chromatography–based method coupled to tandem mass spectrometry was developed and validated for measuring levels of nitrofurantoin, furazolidone, nitrofurazone, and furaltadone as well as nifursol, nifuroxazide, nifurpirinol, and sodium nifurstyrenate in shellfish/fish. The limits of detection and quantitation were 0.01 µg/kg–0.2 µg/kg and 0.04 µg/kg–0.5 µg/kg for most of the NFs. Very good recoveries (92%–107%) were recorded and the method applied to analyze 537 field shellfish and fish samples from South China with 4.3 % of noncompliance in shellfish and 5.0% in fish, semicarbazide being the most frequently detected.

1. INTRODUCTION

Nitrofurans (NFs) are synthetic broad–spectrum antimicrobials used for most gram–positive bacteria and gram–negative bacteria, fungi, protozoans [1]. However, due to a range of health concerns, its use in food production is prohibited in several countries [2–4] although illegal use of NFs in breeding of poultry, livestock and aquatic products is still common [4, 5]. Use of the drugs has negative effect on the environment including wildlife and fish [6, 7] and calls for sensitive, reliable methods.

These drugs are rapidly metabolized in 7 min–63 min [8] and metabolites bind to tissue proteins [1]. Several detection methods have been reported [9–12] but LC–MS/MS has high specificity and sensitivity and is useful for analysis of multiple NF metabolites. These are highly polar, and their ionization is poor and one of the solutions is detection of corresponding 2–nitrobenzaldehyde (2–NBA) imine derivatives as reported elsewhere and validated in various aquatic, poultry, and livestock–based products [4, 13–16] although the scope of animal products is still limited.

One of the nitrofurans of consumer and animal production interest is nifursol, a feed additive for preventing histomoniasis in poultry with its metabolite 3,5–dinitrosalicylhydrazide (DNSAH) detected in certain foodstuffs of animal origin [17, 18]. Another feed additive nitrofuran is nifuroxazide, which is commonly used for egg laying chickens in America and treatment of leishmania–induced disease, colitis, and diarrhoea in humans and animals [19, 20]. Very limited work has been reported on detect the nifuroxazide metabolite, 4-hydroxybenzohydrazide (PSH) [19, 21]. Nifurpirinol (NPIR) and sodium nifurstyrenate (NSTY), are effective antibacterial agents used in aquaculture as reported elsewhere [22, 23]). The two metabolites NPIR and NSTY differ slightly from other NFs (–NH_2) [24]. Thus, detection of these two NFs is usually based on their original structure, but reports describing such methods are very limited [21].

To the best knowledge of the investigators in this study, only Kaufmann et al., [21] reported the detection of the seven nitrofuran AOZ, AMOZ, SEM, AHD, DNSAH, PSH and NPIR in a range of animal products. A method for analysis of multiple NFs in shellfish has been lacking,

although illegal use in farming of shrimps and crabs is possible [25]. To protect consumers, it was important that an analytical method was developed/validated to control such forbidden substances as shellfish production in China reached 22.2 million tons in 2017. This study aimed at developing and validating a new method for eight nitrofurans (NPIR and NSTY) and six metabolites (AOZ, AMOZ, SEM, AHD, DNSAH, and PSH) in shellfish. A method for detection of the nitrofurans in freshwater and marine fish was also developed and validated and applied to field aquaculture samples from local markets and water bodies in South China.

2. MATERIALS AND METHODS

2.1.Chemicals and reagents

The following were used: AMOZ, AHD, AOZ, SEM, and NPIR (Dr. Ehrenstorfer GmbH, Augsburg, Germany); PSH and DNSAH (Toronto Research Chemicals Inc., Canada); NSTY (Beijing Manhage BioTech Co. Ltd, China); AOZ–d4, AMOZ–d5, SEM–^{13}C–$^{15}N_2$, and AHD-$^{13}C_3$ (Fluka, Buchs, Switzerland), DNSAH–$^{15}N_2$ (Toronto Research Chemicals Inc). Standards were ≥94% of purity. Other material included acetonitrile, methanol, ethyl acetate, dimethyl sulfoxide, and n-hexane (HPLC grade, Merck, Darmstadt, Germany); ammonium acetate, ≥ 99.0% and 2–NBA ≥99.0% (Fluka, Switzerland). Analytical grade trisodium phosphate (Na_3PO_4 ≥98.0%), neutral alumina oxide (particle size 100 mesh), sodium hydroxide (NaOH, ≥98.0%), ammonium hydroxide (NH_4OH ≥25.0%), and hydrochloric acid (HCl 37.0%) from Guangzhou Chemical Company (Guangzhou, China). Deionized water (18.2 MΩ cm^{-1}) was generated using a MilliQ® Advantage A10 water purification system (Millipore, France).

2.2.Standard solutions

Stock standards solutions for all the analytes were first prepared individually using methanol from which mixed standard solutions for AOZ, AMOZ, DNSAH and PSH (50 µg/l) and SEM, AHD and NPIR (100 µg/l) as well as NSTY (500 µg/l) were prepared. The of the labelled standards were used at 100 µg/l.

2.3.Sample collection

Shellfish samples (n=172) were collected monthly from four sea areas (XXW, ZZD, YMK, and DC) around Dapeng Peninsula (2015–2016) and 225 samples quarterly from local aquaculture markets in Shenzhen, China (2017). The samples included *Chlamys farreri*, *Ruditapes variegatus*, *Perna viridis*, *Haliotis diversicolor supertexta*, *Crassostrea hongkongensis* and *Crassostrea ariakensis*. The freshwater fish (n=140) included as the grass carp and the tilapia as well as marine fish (*Lateolabrax japonicus*). At least 500 g of the sample edible portion was homogenized and kept at -20°C for 3 months as necessary.

2.4.Sample preparation

A portion of the homogeneous sample (2 g) was weighed into a 50 ml polypropylene centrifuge tube and 10 ml of 0.2 mol/l HCl added before vortexing for 1 min followed by addition of 100 µl of mixed internal standard solution (100 µg/l) and 200 µl of 2–NBA solution. The content was further mixed on a vortex and incubated for 16 h in a water bath at 37°C to hydrolyze and thereafter cooled to room temperature. This was followed up with addition of 2 ml of Na_3PO_4 solution (0.3 mol/l) and NaOH 2.5 mol/l to maintain the pH at 7.2 ±0.2. Ethyl acetate (10 ml) was added, and the content mixed at 8000 rpm for 10 min before centrifugation at 10000 rpm for 10 min. Further extraction was performed, the relevant solutions combined and evaporated

to dryness under nitrogen before reconstitution of the residue with 1 ml of acetonitrile/water (10:90, v/v) and removal of the fat by further centrifugation (10000 rpm, 4°C, 5 min) of the mixture with 2 ml of acetonitrile/ n–hexane (1:1, v/v). The hexane layer was discarded, and the lower layer analyzed using the UHPLC–MS/MS.

2.5.UHPLC–MS/MS analysis

The UHPLC conditions involved a Shimadzu LC 20A HPLC system and separation of the analytes (injection volume of 10 µl) on a C18 column: 150 mm × 2.1 mm, particle size 5 µm (Waters Atlantis®) kept at 35°C and using gradient mobile phase: A (acetonitrile: ammonium acetate solution,10 mmol/L, ~80:20 v/v, with 0.37 mL of NH_4OH), and mobile phase B (100% acetonitrile). The programme was as follows: 0% B for 0–0.5 min; 80% B for 0.5–5.5 min; 80% B for 5.5 min – 6.5 min; 0% B for 6.5–6.6 min; 0% B for 6.6–9.0 min all at a flow rate of 0.4 ml/min.

The API 4500 QTRAP MS was operated in positive electrospray ionization, multiple reaction monitoring for AOZ, AMOZ, SEM, AHD, PSH, and NPIR as well as negative mode for DNSAH and NSTY. The curtain gas, ion source gas 1, ion source gas 2, collision gas, and ion source temperature were 40 psi, 55 psi, 55 psi, medium, and 550°C, respectively. Other parameters were: ionspray voltage of +5500 V and -4500 V.

2.6.Method performance

Scallop (*C. farreri*) was used for method optimization although validation involved all species except oyster (*C. ariakensis*). Matrix calibration curves were generated using fortified blank matrices at 0 µg/kg, 0.2 µg/kg, 0.5 µg/kg, 1.0 µg/kg, 2.0 µg/kg, 5.0 µg/kg, 10 µg/kg, 20 µg/kg, and 50 µg/kg for AOZ, AMOZ, PSH, DNSAH; and 0 µg/kg, 0.4 µg/kg, 1.0 µg/kg, 2.0 µg/kg, 4.0 µg/kg, 10 µg/kg, 20 µg/kg, 40 µg/kg, and 100 µg/kg for AHD, SEM, NPIR; and 0 µg/kg, 2 µg/kg, 5 µg/kg, 10 µg/kg, 20 µg/kg, 50 µg/kg, 100 µg/kg, 200 µg/kg, and 500 µg/kg for NSTY. Labelled standards were maintained at 5 µg/kg. Samples fortified at 2 µg/kg were used to determine LODs and LOQs. Samples (n=6 each) were fortified at 0.2 µg/kg, 0.5 µg/kg, 2.0 µg/kg, and 5.0 µg/kg of AOZ, AMOZ, PSH, DNSAH; 0.4 µg/kg, 1.0 µg/kg, 4.0 µg/kg, and 10 µg/kg for AHD, SEM, NPIR; 2 µg/kg, 5 µg/kg, 20 µg/kg, and 50 µg/kg for NSTY. Intraday and interday precision was performed three times in a month. The method was then subjected to proficiency testing.

3. RESULTS AND DISCUSSION

3.1.UHPLC–MS/MS parameters

The NPIR generated the molecule $[M+H]^+$ and NSTY $[M-Na]^-$ as the most intense ions in the ESI negative mode. Addition of 0.1% formic acid, 0.1% acetic acid to the mobile phases suppressed the response from DNASH and NSTY while ammonium formate caused peak tailing in these two analytes. Ammonium acetate provided better peak shapes while further addition of NH_4OH resulted in ionization efficiency and better sensitivity [21]. Acetonitrile was a better solvent with limited matrix interferences compared to methanol. The most suitable mobile phase consisted of acetonitrile, ammonium acetate and NH_4OH.

3.2.Sample preparation

Samples were derivatized using 2–NBA either in methanol or DMSO at 25°C, 37°C, 45°C, and 60°C for 5 h, 10 h, 16 h, and 20 h. Overall DMSO at 37°C for 16 h were the most suitable conditions.

Extraction was conducted using ethyl acetate (liquid–liquid) and solid phase extraction with hydrophilic–lipophilic–balanced (HLB) cartridges as reported elsewhere [25]. Better responses for DNSAH and NSTY were observed when using SPE instead of liquid–liquid extraction, the latter being associated with retention of lipids. Defatting — compared to addition of another clean up step or filtration through 0.45 µm — helped improve the signal. Meanwhile, the most suitable solvent for reconstitution of the extract residues prior to analysis was acetonitrile:water (1:9, v/v).

3.3.Method validation

Using samples spiked at 5 µg/L of the analytes, the recoveries for AHD, NPIR, DNSAH, and NSTY in freshwater fish was 51.0 %–76%. The levels (13.0%–47.3% and 28.6–68.8%, respectively) for marine fish and shellfish were lower. These were, however, improved to 94 %–105% upon addition of stable isotope labelled standards. Where specific labelled standards were missing as for PSH, NPIR, and NSTY, the surrogates AOZ–d4, AMOZ–d5, and DNSAH–^{15}N2, respectively, were used. Thus, final recoveries were improved to 96.0%~104% for freshwater fish, 95%–104%, marine fish, and 94%–105% in shellfish, respectively. Excellent coefficients of regression ($r^2 > 0.999$) were obtained for all the calibration curves for AOZ, AMOZ, PSH (0.2 µg/kg–50 µg/kg), DNSAH (0.4 µg/kg –100 µg/kg), AHD, SEM, and NPIR (2 µg/kg–500 µg/kg) and NSTY. Except for NSTY whose LOD (2 µg/kg) and LOQ (5 µg/kg) values were high, most were in the range 0.01 µg/kg –0.2 µg/kg all below the EU's MRPL of 1 µg/kg. During the determination of recoveries, the interday and intraday variations for shellfish, freshwater fish, and marine fish were 2.4%–9.0% and 0.7%–8.6%, 1.7%–9.6% and 3.3%–10.2%, and 0.8%–9.6% and 1.05–10.7%, respectively. Competence of the method was determined by subjecting it to proficiency testing (FAPAS prawn test material) where AHD and SEM were determined at 2.54 µg/kg and 2.18 µg/kg, against assigned range levels of 2.18 ± 0.48 µg/kg to2.15± 0.47 µg/kg [21].

The method compares favourably with previous findings reporting fewer analytes ranging from four [11, 13, 16, 21, 26] to seven NFs in fish [21]. The validation results in the current study were also in agreement with previous reports on detection of AOZ, AMOZ, SEM, AHD as well as DNASH, PSH, NPIR in fish [21].

3.4.Method application

The new method was used to analyze filed shellfish and freshwater fish. Shellfish (n=397) from Guangdong Province, China were analyzed with AMOZ, SEM and AHD detected. AHD and AOZ were detected in *Hediste diversicolor supertexta* samples in the range 0.12 µg/kg–7.8 µg/kg SEM was detected in *Crassostrea ariakensis H. diversicolor supertexta*, *P. viridis*, *C. farreri*, and *R. variegatus* in decreasing frequency. Several samples were noncompliant at varying frequencies such as SEM, 3.0%; AOZ, 0.8%; and AHD, 0.5%. Forty–seven shellfish samples were analyzed for tissue–bound SEM in shellfish samples collected in 2016 to explore potential sources of SEM using a modified method [25]. *C. ariakensis* (8 of 11), *R. variegates* (5 of 8), *H. diversicolor supertexta* (3 of 8), and *C. farreri* (5 of 14) contained tissue–bound SEM at 0.1 µg/kg–1.7 µg/kg. However, SEM may be of endogenous nature as reported in prawns and crabs [15, 27, 29].

Commercial freshwater and marine fish samples (n=140) were collected between 2014 to 2017 and analyzed. Most of the samples (n=92) contained AOZ while 48 *L. Japonicus* samples contained SEM, AHD, and AMOZ. For the samples that contained nitrofurans, concentrations were in the range 1.1 µg/kg–6.8 µg/kg, all above the MRPL.

4. CONCLUSIONS

A new sensitive and high throughput isotope dilution UHPLC–MS/MS was developed and validated for simultaneous detection of eight nitrofurans in fish and shellfish other than shrimps and crabs. The method was used to analyze field samples (n= 537) and it is therefore applicable to routine monitoring of eight nitrofurans.

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VALIDATION OF AN LC–MS/MS METHOD FOR SULFONAMIDE RESIDUES IN SHRIMPS

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Abstract

Sulfonamides are antimicrobials used in aquaculture practice in Ecuador. Shrimp are now a very important export product and should therefore meet safety standards. Appropriate testing is required. An analytical method for the determination of sulfadiazine, sulfamerazine, sulfamethazine, sulfanilamide and sulfathiazole in shrimp was developed and validated. This included optimization of a liquid–liquid extraction procedure before analysis. Good recoveries (83% – 109%), linearity (> 0.99), robustness, repeatability (<5% CV), reproducibility (<8% CV), critical limits (CCα=11.3 µg/kg; CCβ=19.2 µg/kg) were attained. The method is applicable to the country's residue control programme.

1. INTRODUCTION

In Ecuador, the aquaculture industry has had a great commercial impact. Shrimp represents the country's first non–oil product export with the country being the third largest worldwide exporter of shrimp. According to the Central Bank of Ecuador in 2018, USD 3.23 million was generated from this product sold mainly to countries such as the European Union and the United States of America, markets that are increasingly demanding for quality and safety. Sulfonamides are used to combat infectious diseases in aquaculture practice, but improper use has consequences such as development of antimicrobial resistance, long term toxicity and other adverse effects [1]. Maximum residue limits of 100 µg/kg have thus been established such as in the European Union [2].

The Ecuador's Secretariat of Quality and Safety is responsible for the monitoring of these substances through the monitoring plan for residues in aquaculture products and includes drugs such as sulfonamides for which suitable methods are required. A study was therefore undertaken to develop and validate an LC–MS/MS method for determination of the sulfonamides: sulfadiazine, sulfamerazine, sulfamethazine, sulfanilamide and sulfathiazole in shrimp.

2. MATERIALS AND METHODS

2.1.Reagents and solutions

The material used included Type I grade water obtained from a Milli Q purifier with 0.22 µm pore filter; acetonitrile (HPLC grade), formic acid (98%–100%), C18 (Octadecyl Silane); buffer solution of 10 mM potassium phosphate; mobile phases consisted of water, formic acid and acetonitrile. The solvents were used to prepared matrix matched standards curves at: 25 µg/kg, 50 µg/kg, 100 µg/kg, 150 µg/kg, 200 µg/kg. Others were: Sulfadiazine (SDZ), sulfamerazine (SMZ), sulfamethazine (STZ), sulfathiazole (STZe) sulfafenazole (SFZ) and sulfanilamide (SND).

2.2.Equipment

An Alliance 2695 XE Water Liquid Chromatograph mass spectrometer (Micro Mass, Altrincham, Cheshire, UK) was used, with an inline degassing device, autosampler set at 10°C,

column oven at 30°C, injection volume of 50 µl and flow rate of 300 µl/min and runtime of 12 min (Table 1). The column used for the tests was a YMC Meteoric CORE C18, 2.7 µm, 2.1 × 100 mm, equipped with a XTERRA C18, 5 µm, Vanguard 2.1 mm × 5 mm precolumn. The mobile phase was water: 0.1% formic acid (a) and acetonitrile: 0.1% formic acid (b) was used. Sulfonamides eluted at 2 min to 5.5 min.

TABLE 1 MOBILE PHASE GRADIENT

Time (min)	A %	B %	Flow ml/min
0	100	0	0.35
3	90	10	0.4
4	60	40	0.4
5	20	80	0.35
5.5	0	100	0.35
6	10	90	0.3
6.5	70	30	0.3
7	80	20	0.35
8.5	80	20	0.35
10	100	0	0.35
12	100	0	0.35

The LC–MS/MS was controlled with a Masslynx Software version 4.1. and Quanlynx and parameters are shown in Table 2.

TABLE 2. PRECURSOR AND PRODUCT IONS FOR THE SULFONAMIDES

Sulfas	Precursor Ion (m/z)	Product Ion (m/z)		Cone (V)	Collision (V)
SDZ	251.08	Q	92.25	30	30
		c	156.11	30	15
SMZ	264.93	c	92.00	30	25
		Q	155.94	30	15
STZ	279.18	c	108.25	30	32
		Q	92.29	30	32
SND	172.9	Q	93.15	30	20
		c	66.25	30	30
STZe	255.88	Q	92.10	30	25
		c	155.98	30	20
SFZ	315..1	Q	158.12	30	30
		c	92.20	30	30

Q: Quantification ion c: Confirmation ion

The method was optimized as reported elsewhere [3, 4] while validation followed set guideline [5, 6]. The parameters studied included linearity, matrix effect, selectivity and specificity, accuracy, precision, detection capability and decision limit and ensure application as reported elsewhere [7–9].

2.3.Sample preparation.

Liquid–liquid extraction was investigated for suitability to isolate the sulfonamides from sample matrix. The homogenized shrimp sample (1 ± 0.005 g) was weighed into a test tube, internal standard and 5 ml of acetonitrile added, the mixture homogenized for 10 min,

centrifuged at 8000 rpm for 5 min and decanted in another tube. More acetonitrile (5 ml) was added to the precipitate, homogenized and centrifuged under previous conditions. The two supernatants were combined and 150 mg of C18 material added, the content stirred for 30 sec and centrifuged at 8000 rpm for 5 min. The resultant supernatant was placed in a water bath set at 40°C and evaporated to dryness under nitrogen before reconstitution with 10 mM potassium phosphate solution. This was then pressed through a 0.22 µm filter material into a vial before analysis on the LC–MS/MS.

3. RESULTS AND DISCUSSION

3.1.Linearity, matrix effect, selectivity and specificity

Good linearity was obtained with a regression coefficient (r^2) of 0.99 (Table 3). To determine the matrix effect, concentrations and peak areas of the calibration curves were compared in solution versus calibration curves in shrimp matrix. While comparing the concentrations of the curve in matrix and in solution by F Test for variances of two samples, there was no significant difference in the concentrations of the analytes. However, the analytical response was greater in matrix for sulfathiazole and sulfamethazine unlike sulfanilamide which required addition of 10 mM of potassium acid phosphate buffer. The method was suitable for detecting sulfaphenazole and the other sulfonamides at 50 µg/kg, although interference with leucomalachite green (at 1 µg/kg) was observed.

3.2.Decision limit (CC alpha) and detection capability (CC beta)

Blank (n=20) samples were spiked with the standards as reported elsewhere [5] and the findings are summarized in Table 3.

TABLE 3. SULFONAMIDE CALIBRATION CURVE AND OTHER PARAMETERS

	r^2	Syx	b(m)	% CV Syx	CCα (µg/kg)	CCβ
SDZ	0.992	6.51	0.996	0.139	9.657	16.404
SMZ	0.992	6.276	1.009	0.123	9.871	16.769
STZ	0.992	5.192	1.007	0.149	10.82	18.381
SND	0.991	7.372	1	0.131	11.311	19.214
STZe	0.993	3.589	1.002	0.142	5.8667	9.965

Coefficient of determination (r^2), Standard error (Syx), slope b (m), intercept (a)

3.3.Accuracy/recovery

To determine the accuracy of the method, the repeatability and reproducibility within the laboratory, blank samples were spiked at 3 levels: 50 µg/kg, 100 µg/kg and 150 µg/kg. The recoveries were determined in the range >80% – 110% (Table 4).

TABLE 4. REPEATABILITY, REPRODUCIBILITY AND RECOVERIES OF SULFONAMIDES.

Analyte	% CV Sr/% CV SR	% Rec (min)/max	% CV Sr/% CV SR	% Rec (min)/max	% CV Sr/% CV SR	% Rec (min)/max
SDZ	3.32/3.85	93.9/106.4	2.73/3.41	98.2/109.6	3.3/3.41	97.7/105.7
SMZ	3.82/3.99	93.7/107.8	3.32/3.36	89.5/104.7	1.27/1.42	93.6/106.5
STZ	4.82/5.91	92.5/105.7	4.5/7.47	92.6/107.6	3.07/3.67	92.1/103.7
SND	4.75/5.19	90.3/106.0	4.93/4.96	81.5/109.4	3.61/4.18	94.2/106.6
STZ	3.65/3.98	91.0/105.8	4.41/4.99	90.6/105.3	3.45/3.88	95.6/103.3

% CV Sr: coefficient of variation of the repeatability standard deviation. % CV SR: coefficient of variation of the reproducibility standard deviation.

4. CONCLUSIONS

An LC–MS/MS method was developed and validated for analysis of sulfonamides including sulfadiazine, sulfamethazine, sulfamerazine, sulfanilamide and sulfathiazole in shrimp. Very good recoveries of >80%–100% were obtained following fortification at 0.5×MRL, 1×MRL and 1.5×MRL. The method is available for use in routine testing and monitoring.

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SIMULTANEOUS DETECTION OF TEN LIPOPHILIC SHELLFISH TOXINS IN SHELLFISH BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY TANDEM MASS SPECTROMETRY

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Abstract

A method was developed for the simultaneous determination of 10 lipophilic shellfish toxins (LSTs) by using LC–MS/MS. To extract the LSTs, shellfish were treated with methanol and saponified further with sodium hydroxide solution and the extraction then purified by solid phase extraction (SPE). After all these pre-treatment processes, all the determine and were separated on a BEH C18 column (50 mm × 2.1 mm, 1.7 μm), and detected by electrospray ionization positive/negative multiple reaction monitoring (MRM) mode, then quantitated by matrix matching curve external standard method. The correlation coefficients of the linear equation of the 10 toxins are all above 0.999, the limits of quantifications are 0.7 μg/kg to 3.0 μg/kg, and the linear ranges are 1.0 μg/l to 96 μg/l. The average recovery rate of mussel, oyster and scallop were 90.6% ~ 123%, 79.2% ~ 126%, 88.2% ~ 125%, respectively and the relative standard deviation was 1.08% ~ 11.8%. The method was applied to the detection of 100 shellfish samples, 60 of which showed the positive expression of detected hYTX, and the highest content was 533.4 μg/kg. In addition, YTX and SPX1 were respectively detected in one mussel sample, with contents of 8.9 μg/kg and 2.8 μg/kg. This method is efficient, sensitive, fast and simple, and is suitable for daily monitoring and analysis of lipophilic shellfish toxins in bivalve shellfish.

1. INTRODUCTION

Lipophilic shellfish toxins are produced by toxic algae and microorganisms and are abundant in shellfish [1]. These toxins are common on the coasts of China, a major source of bivalve shellfish [2–7], and they have serious implications on export/trade [8]. The LSTs are divided into six groups — according to the chemical structure — namely, okadaic acid (OA), azaspiracid (AZA), brevetoxin (BTX), pectenotoxin (PTX), yessotoxin, (YTX) and cyclic imine (CI) [9]. These toxins have a half–life ranging from 15 days to several months [10, 11]. The risk of exposure is high in dehydrated aquatic products [9, 12, 13] with acute and chronic health implications [14, 15].

Detection methods for LSTs include mouse bioassay (MBA), enzyme linked immunosorbent assay (ELISA), HPLC, and LC–MS/MS [16–18]. The MBA can measure the total toxicity although specificity is poor [19]. While ELISA is fast, kits can be expensive and is prone to false positives unlike the HPLC which is more sensitive and accurate but requires sample pretreatment and derivatization where reagents can be unstable [20]. The LC–MS/MS has been recommended as an alternative to MBA due to its high specificity and high sensitivity among other advantages [21–23] although sensitivity and specificity could be better especially when analyzing multiple toxins. A study was undertaken to develop and validate a highly sensitive method suitable for 10 lipid–soluble shellfish toxins: okadaic acid (OA), dinophysis toxin–1, 2 (DTX1, DTX2), yessotoxin and homoyessotoxin (YTX and hYTX), azaspiracid–1, 2, 3 (AZA1, AZA2, AZA3), spirolides (SPX1), pectenotoxin–2 (PTX2).

2. MATERIALS AND METHODS

The following were used: API QTRAP 4500 Triple Quadrupole (AB SCIEX, USA); LC 20A HPLC (Shimadzu, Japan); Beckman Avanti J26XP Cryogenic High–speed centrifuge (Beckman Corporation, USA); XS205 Analytical Balance (Mettler Toledo, Switzerland); MilliQ IQ 7000 Ultrapure Water Machine (Millipore, USA); Centrifuge 5424R desktop high speed centrifuge (Eppendorf, Germany); Multireax oscillator (Heidolph, Germany); ED115 Binder, Oven (Binder, Germany). Methanol, acetonitrile, formic acid, ammonium formate (CNW, Germany); ammonia (25%, Beijing Dima Technology Company); NaOH, HCl (Guangzhou chemical company); ultrapure water (resistivity >18 MΩ·cm).

The following ten shellfish toxin standards and mussel quality control samples were used: OA, 8.4 μg/ml± 0.4 μg/ml, DTX1, 8.52 μg/ml ± 0.66 μg/ml, DTX2, 3.8 μg/ml ±0.2 μg/ml, PTX2, 4.40 μg/ml± 0.13 μg/ml, YTX, 4.92 μg/ml± 0.23 μg/ml, hYTX, 5.8 μg/ml ±0.3 μg/ml, (SPX1, 5.01 μg/ml ±0.24 μg/ml, AZA1, 1.30 μg/ml ±0.07 μg/ml, AZA2, 1.22 μg/ml ± 0.06 μg/ml, AZA3, 1.18 μg/ml ±0.05 μg/ml) (CRM FDMT1, National Research Council Canada). A mixed standard was prepared at 200 μg/l.

2.1.Sample preparation

This involved rinsing of the mussels, scallops and oysters under clean water, opening the shells, rinsing with clean water to remove sand and other foreign matter, removing intact shells, draining through a sieve, and then homogenizing. The homogenized samples (2.0 g) were weighed into 50 ml centrifuge tube, 10 ml methanol added, the content vortex mixed for 5 min, at 8000 r/min centrifuge 5 min, transferred to a fresh 50 ml centrifuge tube and the process repeated before combining the supernatant and storage at -20ºC. The content was then placed in a 1.5 ml centrifuge tube and spined at 15 000 r/min centrifuge for 10 min and 1.0 ml transferred to a sample vial. For measurement of OA, DTX1 and DTX2, addition of an alkali was required.

The extract was transferred into a 10 ml plastic tube with a stopper, 125 μl of sodium hydroxide solution (2.5 mol/l), added and the material mixed well. This was left to standard for 40 min at 76 ℃ before cooling to room temperature under cold running water followed by addition of HCl (125 μl, 2.5 mol/l) and 2.5 ml of water. The solution was mixed well and centrifuged at 8000 r/min for 10 min and the material then passed through C18 SPE column containing ~ 2 ml of methanol and 2 ml of pure water. Methanol:water (2 ml, 20%) was passed through the SPE column and the analytes eluted with 2 ml of 0.2% ammonia: methanol which was then pressed through a 0.22 μm filter before analysis.

The instrument conditions included use of an Acquity UPLC BEH C18 column (50 mm×2.1 mm, 1.7 m, Waters, USA), column temperature of 40ºC, flow rate of 0.4 ml/min, 5 μl injection volume. Mobile phase consisted of solvent A: 2 mmol/l ammonium formate and 50 mmol/l formic acid and B: 95% acetonitrile containing 2 mmol/l ammonium formate and 50 mmol/l acetic acid; with the elution gradient as follows: 0 to 1 min, 80%A; 1.1 min to 3 min, 80%–50% A; 3.1 min to 5.0 min, 50%–10% A; 5.1 min to 6 .0 min, 10% A; 6.1 min to 8.0 min, 10%–50% A; 8.1 min to 10 min, 50%–20% A;10.1 min to 12 min, 80% A.

For mass spectrometry, the conditions involved an electrospray ionization source (ESI both positive and negative), positive and negative modes at voltages of 5500 V and -4500 V, multi reaction monitoring (MRM), curtain gas pressure at 0.3 MPa, source gas at 55 psi, auxiliary gas

at 55 psi, collision gas flow rate medium, and ion source temperature of 550ºC. Additional parameters are reported in Table 1.

TABLE 1: MS PARAMETERS FOR THE TOXINS

Toxin	Precursor ion	Product ion	Retention time	DP	CE	Ionization mode
OA	803.4	255.0*/563.2	4.04	-10	-62/-58	Negative
DTX2	803.4	563.2*/255.0	4.17	-10	-62/-58	Negative
DTX1	817.4	255.2*/112.9	4.48	-10	-72/-85	Negative
YTX	570.4	467.4*/396.3	4.32	-10	-38/-38	Negative
hYTX	577.2	474.3*/509.0	4.34	-10	-39/-30	Negative
AZA1	842.5	824.5*/806.5	6.09	10	40/50	Positive
AZA2	856.5	838.5*/820.5	6.20	10	40/50	Positive
AZA3	828.5	810.4*/792.4	5.87	10	40/50	Positive
SPX1	692.4	674.4*/444.4	4.09	10	45/45	Positive
PTX2	876.5	823.4*/805.6	5.51	10	36/36	Positive

3. RESULTS AND DISCUSSION

The UPLC C18 chromatographic column used helped to shorten analytical time. The toxins OA, DTX1, DTX2, hYTX, and YTX showed sharper peaks in the mobile phase with ammonia water, and this also demonstrated high sensitivity. When the ammonia concentration was too high or too low, the sensitivity of hYTX and YTX was significantly reduced. A concentration of 0.01% ammonia was most appropriate for negative ionization. AZA1, AZA2, AZA3, SPX1 and PTX2 showed better peak shapes with ammonium formate/formic acid added to the mobile phase. The optimum combination was 2 mmol/l ammonium formate and 50 mmol/l formic acid aqueous solution and 95% acetonitrile aqueous solution (containing 2 mmol/l ammonium formate and 50 mmol/l formic acid) although minor adjustments were required for OA and DTX2.

The mixed standard solution for the 10 LSTs at a concentration of 200 µg/l were injected in the mass spectrometer using a pump syringe at a flow rate of 7 µl/min, and the ions were scanned in positive and negative ionization mode. The results show that AZA1, AZA2, AZA3, SPX–C and PTX2 have high molecular ion peaks in positive mode unlike OA, DTX1, DTX2, YTX and hYTX which were better in negative mode. The molecular ions response for YTX and hYTX

was intense at $[M-2H]^{2-}$ with a low response value at $[M-H]^{-}$. OA and DTX2 can be distinguished by fragmentary ions. The fragment ions for hYTX are mainly $[C_{47}H_{70}O_{20}S_2–2H]^{2-}$, $[C_{43}H_6O_{19}S_2–2H]^{2-}$, and $[C_{36}H_{56}O_{16}S_2–2H]^{2-}$, while the molecular ions for PT X2 are $[M+Na]^{+}$ and $[M+NH_4]^{+}$ although the latter was more stable and was therefore selected for analysis.

3.1.Matrix effect

Potential effect of matrix on responses was evaluated using matrix matched curves developed using the following volumes: 5 µl, 10 µl, 20 µl, 40 µl, 80 µl, 160 µl, 320 µl and 480 µl of a 200 µg/l mixed standard solution. Matrix enhancement was seen for ions in the negative mode compared to those in the positive mode. A ratio of the matrix matched curve: standard curve greater than one implied signal enhancement and below one was suppression [24]. The correlation coefficient values were above 0.999.

3.2.Optimization of the extraction of solvents

Organic solvents can improve the efficiency of extracting fat–soluble shellfish toxins. For the toxins in this study, use of methanol was previously reported [22, 25]. The effect of aqueous solutions such as 80% methanol [26] and 50% methanol aqueous solution along with acetonitrile was investigated using National Research Council Canada (NRC)–FDMT1 reference material consisting of the toxins domoic acid, okadaic acid, dinophysistoxins, azaspiracids, pectenotoxins, yessotoxin and spirolides. The mixed standards (6 µg/l) were added to the samples, extraction and clean up performed and recovery evaluated. The extraction efficiency of 80% methanol aqueous solution for YTX and hYTX was less than 70%, while for 50% methanol aqueous solution it was up to 40% only. Acetonitrile was not good for hYTX compared to methanol where overall recovery was 94.2%–113%. The recovery for multiple toxins in methanol was in the range 60%–80% when extraction was done once but improved to 85.5%–117% when the extraction step was repeated one more time.

3.3.Limit of detection, limit of quantification and linearity

A series of matrix matched standard curves were prepared using mixed standards (200 µg/l) taken in volumes of 5 µl, 10 µl, 20 µl, 40 µl, 80 µl, 160 µl, 320 µl and 480 µl. The detection limit was determined as three times the signal–to–noise ratio, and the quantification limit as 10 times the signal–to–noise ratio and the results are summarized in Table 2 below.

TABLE 2: SUMMARY OF LODS AND LOQS

Toxin	LOD (µg/kg)	LOQ (µg/kg)	Linear range (µg/l)	Slope
OA	0.5	1.5	1.0~96	1.24
DTX2	0.4	1.5	1.0~96	1.22
DTX1	0.3	1.0	1.0~96	1.22
YTX	0.3	1.0	1.0~96	1.22

Toxin	LOD (µg/kg)	LOQ (µg/kg)	Linear range (µg/l)	Slope
hYTX	0.2	0.7	1.0~96	1.30
AZA1	0.7	2.1	1.0~96	0.69
AZA2	0.4	1.3	1.0~96	0.65
AZA3	0.7	2.4	1.0~96	0.76
SPX1	0.6	2.0	1.0~96	0.54
PTX2	1	3.0	1.0~96	0.53

3.4.Accuracy and precision

These were determined by adding 0.6 ml, 1.2 ml and 6.0 ml of mixed standard solution to blank mussels, oysters and scallops (n=6) and content extracted to determine recovery levels and associated variations among replicates. The results are summarized Table 3 below.

TABLE 3: THE RECOVERY RATE AND RELATIVE STANDARD DEVIATION OF THREE KINDS OF SHELLFISH (N=6)

Toxin	Concentration (µg/l)	Mussels		Oyster		Scallop	
		% Recovery	RSD%	% Recovery	RSD%	% Recovery	RSD%
OA	6	114	4.68	92.3	3.34	113	2.94
	12	94.6	3.84	98.7	4.53	100	2.72
	60	90.6	4.22	99.7	1.49	94.9	6.10
DTX2	6	116	3.53	89.4	3.75	115	1.91
	12	97.1	3.13	90.8	3.84	95.4	1.73
	60	104	6.00	85.0	2.03	101	1.93
DTX1	6	120	3.52	95.6	5.51	125	2.53
	12	98.9	6.30	94.3	3.58	101	1.84

Toxin	Concentration (µg/l)	Mussels		Oyster		Scallop	
		% Recovery	RSD%	% Recovery	RSD%	% Recovery	RSD%
	60	98.3	3.20	105	2.08	105	2.48
YTX	6	115	2.37	93.0	1.93	117	1.61
	12	98.6	1.35	96.5	2.21	102	3.11
	60	97.8	1.55	94.7	3.44	108	1.21
hYTX	6	115	4.86	87.8	2.93	111	2.21
	12	94.7	2.39	79.2	3.64	104	1.23
	60	95.8	1.69	79.4	3.77	88.2	1.08
AZA1	6	99.7	3.86	97.5	5.10	113	3.71
	12	101	4.86	97.8	9.57	119	8.74
	60	93.1	8.08	97.4	8.17	110	5.62
AZA2	6	105	5.08	90.9	5.79	111	4.53
	12	109	5.16	92.9	8.55	115	7.28
	60	106	6.04	109	3.18	108	5.31
AZA3	6	94.4	7.25	101	9.07	117	7.05
	12	101	6.95	104	8.60	116	11.8
	60	96.7	8.23	108	3.18	123	4.56
SPX1	6	108	1.11	126	2.91	123	2.41
	12	105	2.17	113	2.82	110	1.37
	60	98.3	1.50	102	2.84	109	1.22
PTX2	6	123	1.84	115	2.84	122	3.62

Toxin	Concentration (µg/l)	Mussels		Oyster		Scallop	
		% Recovery	RSD%	% Recovery	RSD%	% Recovery	RSD%
	12	116	2.13	105	2.50	107	1.60
	60	99.1	2.43	96.8	5.10	94.8	2.18

3.5.Testing of field samples

Shellfish samples (n=100) consisting of 47 mussels, 29 oysters and 24 scallops were collected from the coasts of Guangdong, China. Sixty samples including 13 mussels, 27 oysters and 20 scallops contained hYTX in the range 3.0 µg/kg–533 µg/kg, although these were below the European Food Safety Authority limit of 1000 µg/kg for YTXs [27]. Also, YTX and SPX1 were detected in a mussel sample at 8.93 µg/kg, and 2.81 µg/kg, respectively, both below 10% of PTX2, YTX and SPX reported elsewhere [28].

4. CONCLUSIONS

A confirmatory analytical method has been developed, validated and applied for simultaneous determination of 10 lipophilic shellfish toxins at detection limits in the range 0.3 µg/kg–1 µg/kg, and quantification limits in the range 0.7 µg/kg–3.0 µg/kg. The method is fit–for–purpose and can be used for ensuring the safety of mussels, oysters and scallops before consumption.

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MIXTURES OF ANTIMICROBIALS IN EDIBLE FISH IN ARGENTINA, A CHALLENGE TO HEALTH RISK ASSESSMENT

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Abstract

A UHPLC–MS/MS method has been used to investigate the presence of 46 antimicrobials including amphenicols, cephalosporins, dihydrofolate reductase inhibitors, fluoroquinolones/quinolones, macrolides, nitrofurans, penicillins, sulfonamides and tetracyclines in Argentinean fish. All samples contained norfloxacin, clarithromycin, roxithromycin, doxycycline and oxytetracycline. Maximum residue levels for all antimicrobials except one, were exceeded in 82% of pacú, 57 % of shad, 57 % of trout and 50 % of salmon. Chloramphenicol, furazolidone and nitrofurantoin were detected in 41 %, 22 % and 4 % of the samples, respectively.

1. INTRODUCTION

Aquaculture is an economically important and expanding industry worldwide [1] although it is prone to the emergence and spread of infectious diseases [2] and thus requires prophylactic or therapeutic use of antimicrobials [3]. Improper use of the antimicrobials including parent compounds and their metabolites end up in the surrounding environment [4]. Wild or farmed aquatic fish may therefore be exposed to drugs including human pharmaceuticals because of their continuous release into aquatic systems [5]. These pharmaceuticals that may arise from inefficient municipal wastewater treatment plants. Developing countries are some of the most affected [6].

One of the effects of antimicrobials, whether arising from misuse (overuse) or underdosing, is antimicrobial resistance. Low levels such as those discharged into the environment, can result in resistance among bacteria which can in turn be spread around aquaculture production environments [7]. Antimicrobial residues in food are also associated with a range of other human health risks [8]. Resistance to drugs may as a result required unique and more expensive drugs [9, 10] and this has both health and economic implications. Addressing this challenge requires that further studies are performed on dose response assessment between resistance and the antimicrobial concentrations in the environment [11]. A critical area is aquaculture production where several antimicrobials are used [1] many of which are of importance to both humans and animals [12]. Resistance developed against such antimicrobial would impact both public health and agriculture.

Some studies have reported low levels of pharmaceutical/antimicrobial residues in certain food products in South America although there is limited information in wild fish and associated marketed products [6, 13]. Studies are therefore needed to assess antimicrobial levels in fish from contaminated areas and those from intensive farming systems exposed to environmental contamination. In this research, focus was placed on commercial fish including *Oncorhynchus mykiss*, *Prochilodus lineatus*, *Piaractus mesopotamicus* and *Oncorhynchus kisutch* sold in certain Argentinian markets.

2. METHODS AND MATERIALS

2.1. Chemicals and reagents

The following analytical standards (>90%) were procured from Sigma Aldrich (St Louis): beta lactams such as penicillin G sodium salt, penicillin V, amoxicillin, cephalexin, oxacillin sodium salt; amphenicols florfenicol and chloramphenicol; sulfonamides sulfabenzamide, sulfacetamide, sulfadiazine, sulfadimethoxine, sulfadoxine, sulfaguanidine, sulfamerazine, sulfamethazine, sulfamethizole, sulfamethoxazole, sulfamethoxypyridazine, sulfanitran, sulfapyridine, sulfaquinoxaline, sulfathiazole, sulfisomidin, sulfisoxazole; the tetracyclines oxytetracycline hydrochloride and tetracycline, doxycycline hyclate; quinolones ciprofloxacin, enrofloxacin, enoxacin sesquihydrate, norfloxacin, ofloxacin, moxifloxacin hydrochloride, marbofloxacin, oxolinic acid, nalidixic acid and flumequine; the macrolides erythromycin, roxithromycin, azithromycin and clarithromycin; nitrofurans furazolidone and nitrofurantoin. Other drugs were pipemidic acid, trimethoprim and tylosin phosphate. Labelled drugs included azithromycin–d3, enrofloxacin–d5, erythromycin–^{13}C and –d3, flumequine–$^{13}C_3$, trimethoprim–d3 and sulfamethazine–d4 (Santa Cruz Biotechnology, Dallas, USA).

Standard stock solution (500 mg/l) and isotope labelled standards solution (1000 mg/l) were prepared in solvent and stored at -20°C. Cephalexin, amoxicillin and furazolidone were dissolved in ultrapure water; penicillin V and penicillin G sodium salt in ultrapure water: acetonitrile (50:50, v/v) and oxacillin sodium salt in ultrapure water:methanol (50:50, v/v). Other compounds were dissolved in methanol. Fluoroquinolones and quinolones required addition of sodium hydroxide for proper dissolution. Mixed standard solutions were prepared at 1 mg/l and 10 mg/l using methanol while acetonitrile/aqueous 0.1 % formic acid (10:90, v/v) was use to prepare working standard solutions at 0.5 µg/l, 5 µg/l, 10 µg/l, 25 µg/l, 50 µg/l, 100 µg/l, 200 µg/l, 500 µg/l. Methanol and acetonitrile (HPLC grade, Baker, Philipsburg, NJ, USA); formic acid (98–100 %, Merck, Darmstadt, Germany); and ultrapure water (MilliQ water purification system, Millipore, Sigma, Bedford, USA) were used.

2.2.Sample preparation

Sampling and sample preparation were conducted as reported elsewhere [14]. Four fish species were purchased from, supermarkets and aquaculture facilities in Argentina between 2017 and 2019. These included seven samples each of trout and sábalo/shad: 11 samples of pacú and two of salmon. Unless immediately analyzed, the muscle tissues were freeze-dried and stored at -80°C. Samples were ground using a mortar and pestle before extraction and clean-up as reported elsewhere [15] with minor changes. The muscle tissues (0.5 g, dry weight) were spiked with 20 µl of 5 parts per million isotope-labelled antimicrobial standard mixture. The contents were the mixed and incubated at 4°C in the dark for one hour before adding 5 ml of acetonitrile: water (3:1, v/v) mixed further on a vortex mixer for 1 min, sonicated for 15 min, and spined on a centrifuge for 10 min at 3000 g.

This procedure was repeated twice, and the supernatants combined; 240 µl of 0.01 M EDTA added samples purified using StrataX cleanup cartridges (Phenomenex Corporation, Torrance, CA, USA), conditioned with 5 ml of methanol and then 10 ml of ultrapure water. Analytes were eluted from the sample extracts using 6 ml of methanol that was then evaporated to dryness (Concentrator plus/Vacufuge plus, Eppendorf, Hamburg, Germany) and reconstituted in 1 ml of mobile phase acetonitrile:aqueous 0.1% formic acid (10:90, v/v) before pressing through 0.22 µm PVDF filters (Durapore, Tullagreen, Ireland) into 2 ml sample vials (Agilent Technologies, Santa Clara, CA, USA) prior to analysis by the Waters Acquity UPLC Xevo

TQS–MS/MS (Waters Corporation, Milford, MA, USA) after separation on a Zorbax Eclipse Plus C–18 T3 analytical column (3.0 mm × 50 mm; 1.8 µm, Agilent, Santa Clara, CA, USA). Some samples were spiked with the antimicrobial mixtures at 50 µg/kg and 200 µg/kg to determine recoveries with a target of ≥50% or 25% up to 50% with good repeatability (RSD<25%). These methods would be quantitative while others with lower values were considered qualitative as indicated elsewhere [16].

2.3.Instrumental analysis

The antimicrobials were separated on the C18 column and analysed by UPLC–MS/MS equipped with an ESI source. The conditions included: 5 µl injection volume; a flow rate of 0.5 ml/min; 40°C column temperature; gradient mobile phase: acetonitrile (solvent A), and aqueous 0.1% formic acid (B) starting with 10 % A; increased linearly to 100% A (0 to 8.0 min); 100% A (1 min), initial conditions (9.5 min), equilibration (8.5 min). Blank samples consisting of acetonitrile: aqueous 0.1% formic acid (10:90, v/v) were also injected after every 9 sample runs to avoid sample carryover.

Following optimization of MS conditions determined by directly infusing 5 mg/l of each drug in methanol into the MS/MS. Two selected reaction monitoring (SRM) ion transitions were monitored for every antimicrobial with the most abundant used for quantification and the next intense used for confirmation. The relative abundances of the two transition ions were compared with the respective standards' (in triplicate) with acceptable variation of ± 20%. The retention time was also considered as acceptable within ± 2.5% of the associated standards' retention time.

All analytes were detected in the positive ESI source except chloramphenicol, florfenicol, nitrofurantoin, sulfacetamide, sulfanitran, sulfisoxazole and tylosin analyzed in negative mode. The ion transitions and mass parameters monitored are presented as reported by Griboff et al., [14]. Quantitation was performed using calibration curves (range 0.5 µg/l – 500 µg/l, $R^2 > 0.98$) of matrix matched fish samples including isotopically labelled internal standards. The limit of detection (LOD) and limit of quantification limits (LOQ) were used in the method and MassLynx V 4.1 software (Waters Corporation, Milford, MA, USA) facilitated data acquisition and processing.

3. RESULTS AND DISCUSSION

The validated method is suitable for analysis of 46 antimicrobials from ten families in single chromatographic run of 20 min following a published procedure [15]. Sample preparation was validated using four native and exotic fish species. The LODs ranged from 0.004 µg/kg to 7.8 µg/kg while LOQs varied from 0.01 µg/kg to 26.2 µg/kg as previously reported [3, 17, 18]. Good recoveries in the range 50% to 100% were attained except for enrofloxacin, moxifloxacin, norfloxacin, azithromycin, tylosin, amoxicillin, pipemidic acid, oxytetracycline and tetracycline where levels were much lower (26%–49%, RSD<25%) a common challenge associated with multianalyte methods [19].

The aquaculture fish samples contained residues of 42 antimicrobials. Only amoxicillin, pipemidic acid, sulfacetamide and sulfisoxasole were not detected. The highest residue levels were detected in pacú (n=40), followed by shad (n=37), trout (n=31) and salmon (n=14) although the salmon sample size was small. The maximum concentrations were in the ranges 0.50 µg/kg–11558 µg/kg for pacú; 0.41 µg/kg–1151.25 µg/kg for shad; 0 0.52 µg/kg–620/22 µg/kg for trout and 0.60 µg/kg–92.58 µg/kg for salmon.

The macrolides clarithromycin and roxithromycin, the quinolone enrofloxacin as well as the tetracyclines doxycycline and oxytetracycline were detected in all samples possibly due to extensive use in aquaculture production, or in human medicine [18]. This appears to be the first time clarithromycin is reported in edible fish muscle [14] an indication of poor wastewater treatment in Argentina [20]. Little is known about roxithromycin residues in edible fish although Li et al., [21] reported roxithromycin at levels as high as 1076 µg/kg d.w. in fish from Baiyangdian Lake. In a related study [22] researchers reported detection of up to 3.9 µg/kg d.w. of roxithromycin in two wild fish from urban rivers in Nanjing. Enrofloxacin levels in this study were lower than reported elsewhere [18, 23–25] in China, Brazil and Spain. The levels of oxytetracycline were similar to what Monteiro et al., [26] reported in Brazilian fish but higher than in reports by Liu et al., [24] in China; Done and Halden [27] in USA; and Grande–Martínez et al., [17] in Spain. The residue levels (including tetracyclines) reported are of major public health importance given reports that tetracycline resistance genes are some of commonest resistant genes, and that fluoroquinolones are also associated with bacterial resistance [28].

The other antimicrobials detected — although at lower frequencies — included nitrofurantoin (3.7%) to erythromycin/sulfaguanidine (85.2%). Florfenicol was only detected in trout and nitrofurantoin as well as oxacillin in pacú alone. Enrofloxacin was the most common fluoroquinolone while clarithromycin, roxithromycin and erythromycin were the most frequent macrolides, and azithromycin the least. Furazolidone (22.2%) was more frequent than nitrofurantoin (3.7%) among the nitrofurans. Penicillin G (51.9%) and oxacillin (7.4%) were the most common and least detected penicillins, respectively. Oxolinic acid was the most detected quinolone while nalidixic acid was the least frequent. Among the sulfonamides, sulfaguanidine was the most frequently detected and sulfathiazole the least. Doxycycline and oxytetracycline were more common than tetracycline (48.2%). The findings are in agreement with reports by Lulijwa et al., [29] who reported use of oxytetracycline, sulfadiazine and florfenicol, as well as sulfadimethoxine, erythromycin, amoxicillin and enrofloxacin in aquaculture production among the 11 major aquaculture producing countries namely Bangladesh, Chile, China, Egypt, India, Indonesia, Japan Norway, Philippines, South Korea and Vietnam. The usage levels are the in the range of 55%–73%.

The study noted that the antimicrobial residue levels exceeded MRLs established by European Commission (EC), Codex and the Ministério da Agricultura, Pecuária e Abastecimento from Brazil in 8% of pacú, 57% of shad, 57% of trout and 50% of salmon [14]. Levels of oxytetracycline above EU MRL have been reported elsewhere in tilapia cultured in Brazil [26], and catfish cultured in Nigeria [30]. The detection of forbidden substances such as chloramphenicol, furazolidone and nitrofurantoin in 41%, 22% and 4% of the fish samples, respectively, is an indication of poor management practices also as reported in China [31] Iran [32] and Nigeria [33]. Its toxicity notwithstanding [34], chloramphenicol is still used illegally in aquaculture facilities around the world due to the low cost and high effectiveness [35]. The presence of some of the residues in the current study can be attributed to wastewater plant treatment and discharges from production facilities [13, 36].

The risk associated with daily consumption of fish containing the drug residues was determined [14]. The estimated daily intake (EDI) for drug residues in shad, pacú, trout and salmon were lower than the ADI, suggesting low health risk, although low residue levels are associated with antimicrobial resistance [11, 28]. This risk may be higher due to combined exposure to antimicrobials used in production and from environmental/human pollutants. This is the first time such a study has reported both types of exposure in fish collected from the Argentinian

market [14]. While effects of chronic exposure to mixtures of residues remain unknown [36], the current study provides information to contribute a gap in knowledge on residues/contaminants in South America and Africa [37].

4. CONCLUSIONS

An isotopic UHPLC–MS/MS method was developed, validated and used to determine levels of residues of 46 antimicrobials in aquaculture trout, shad, pacú and salmon in Argentina. Levels of doxycycline, oxytetracycline and sulfamethazine were above MRLs while forbidden drugs such as chloramphenicol, furazolidone and nitrofurantoin were also detected. The residues were from environmental pollution and/or aquaculture production practices.

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DISTRIBUTION OF ANTIMICROBIALS IN WATER, SEDIMENTS AND BIOFILM IN RIVER CÓRDOBA, ARGENTINA

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Abstract

The distribution of 43 antimicrobials and four of their metabolites in water, sediment and biofilms associated with the Cordoba River were investigated. Sample preparation techniques including solid phase extraction, bead–beating disruption and pressurized liquid extraction were investigated followed by UPLC–ESI–MS/MS analysis. Samples from sites downstream of a wastewater treatment plant contained residues ranging from 0.003 µg/l to 652 µg/kg with biofilm and sediments containing the highest levels in the wet season. Fluoroquinolones, macrolides and trimethoprim were the most frequently detected. Different bioaccumulation factors were noted and those greater than 1000 l/kg dw. were associated with biofilms. This study adds to the body of knowledge on the fate and distribution of antimicrobials in urban rivers.

1. INTRODUCTION

Emerging contaminants are of concern to human and environmental health although regulatory criteria or norms for many of these are yet to be established [1]. Some of these contaminants are pharmaceuticals with vast biological effects and are difficult to remove from wastewater [2]. Over the last 20 years, antimicrobials have emerged as major group drawing attention due to challenges such as antimicrobial resistance [3, 4]. As part of the One Health concept [5] antimicrobial resistance control programmes have been initiated in Argentina since 2015, as in other Latin American countries. Continued investigation of environmental contamination is required.

The drugs have different mechanisms of action [6] and are widely used in animal or plant disease prevention and treatment [7]. While many are metabolized, it is estimated that between 10%–90% of antimicrobials used are excreted as parent compounds and are found in animal manure or sewage [6]. Thus, wastewater treatment plants (WWTPs) are considered a major source of these pharmaceuticals [8].

Freshwater resources are susceptible to urban and agricultural pollution with a range of contaminants including pharmaceuticals. Antimicrobials have been detected in the range of pg/l to µg/l in surface water and µg/kg to mg/kg in sediments [9, 10] suggesting therefore that sediments are reservoirs for a range of antimicrobials [11]. While biofilms such as those composed of bacteria, algae, archaea and fungi in submerged surfaces [12, 13] are good indicators of water pollution [14], there is limited information on the bioaccumulation of antimicrobials in urban fluvial biofilms although the levels in urban rivers may reach 0.276

mg/kg [15]. Biofilms may also be a source of pollutants for higher trophic levels of riverine food webs [9].

The presence of antimicrobials in the aquatic environment [8] requires control around the world including in Latin America as emerging pollutants in the urban water cycle become a concern [10]. In Argentina, antimicrobials have been reported in polluted rivers at concentrations of up to 0.97 µg/l [16]. Drugs such as monensin have been reported at concentrations as high as 4.670 µg/l in rivers due to agricultural effluents [17]. A level of 5.6 µg/kg of antimicrobials have been reported in fish by Ondarza et al., [18]. A multianalyte method covering a wider scope of drugs in difference aquatic matrices was required hence this study [19].

2. MATERIALS AND METHODS

2.1.Study site and monitoring campaigns.

The Suquía River was selected for the study because it is urban and associated with discharged from wastewater treatment, thus a potential source of pollution [20, 21]. River biofilm, surface water and sediment samples were collected in the wet and dry seasons. Water samples (n=2) collected 20 cm from the surface were placed in amber coloured glass bottles while sediment samples (n=2) were collected with a shovel (n=2). *In situ* biofilm colonization was performed between 45 and 60 days before sample collection [22, 23]. Samples were shipped to the laboratory on ice and protected from light.

2.2.Chemicals and materials

The following antimicrobials were included in the study: fluoroquinolones, quinolones, penicillins, cephalosporins, macrolides, tetracyclines, lincosamides, sulfonamides, dihydrofolate reductase inhibitors and nitroimidazoles. Isotopically labelled standards, chemicals and related material were used [19].

2.3.Physicochemical and microbiological parameters

A portable metre (WTW, Multiline F/Set 3) was used to measure water temperature, pH and conductivity of water *in situ* while water velocity (m/s) was determined by observing the rate at which a float travelled [24]. Other parameters measured according to the APHA [25] included alkalinity, dissolved oxygen, suspended and dissolved solids, nitrates, nitrites and ammonia, phosphates, chlorides, turbidity, total mesophyll aerobic bacteria. The parameters were integrated in a water quality index (WQI) [26]. Biofilm biomass and chlorophyll–a were measured, and autotrophic index calculated as indicated elsewhere [23]. Sediment texture and pH were determined according to Klute [27] while the organic matter was measured by wet combustion [28].

2.4.Antimicrobial analysis in river samples

For water extraction, 250 ml were pressed through a 0.45 µm filter material and 25 ng of sulfadimethoxine–d6 added before storage at 4°C and clean up by solid phase extraction [29]. Biofilm extraction involved removal of the material from glass surfaces with soft bristle brush and chlorine–free tap water and then prepared in 2 ml plastic tubes (n = 2), freeze dried and stored at -80°C until extraction using a procedure reported elsewhere [30]. For sediments, samples were freeze dried, sieved through 125 µm material and the finest portion prepared using a procedure slightly modified from a previous study [31].

Analysis was done on UPLC–ESI–MS/MS as reported elsewhere [29] and isotope labelled standards were used for quantification based on matrix matched calibration curves for all the study 3 matrices [19].

2.5.Bioaccumulation factors and pseudo partitioning coefficients.

Assuming active biological uptake or passive sorption as indicated elsewhere [12] this study used bioaccumulation to mean the concentration of antimicrobials in the biofilms (inside the cells and on surrounding matrix). Field–derived bioaccumulation factors (BAF) for biofilm in µg/kg and for water in µg/l, were calculated for each antimicrobial as in Eq. (1) below [19].

$$BAF\ \left(\frac{L}{kg_{d.w.}}\right) = \frac{biofilm\ AB\ concentration\ \left(\frac{\mu g}{kg_{d.w.}}\right)}{water\ AB\ concentration\ \left(\frac{\mu g}{L}\right)} \quad (1)$$

Where BAF = Field bioaccumulation factor; AB = antimicrobial/antibiotic

Pseudo partitioning coefficients (P–PC) were calculated for the residues [19] using the Eq. (2) [32].

$$P-PC\ \left(\frac{L}{kg_{d.w.}}\right) = \frac{sediment\ AB\ concentration\ \left(\frac{\mu g}{kg_{d.w.}}\right)}{water\ AB\ concentration\ \left(\frac{\mu g}{L}\right)} \quad (2)$$

2.6.Environmental risk assessment

The antimicrobial environmental risk in the water was assessed by estimation of the risk quotient (RQ), according to Eq. (3):

$$RQ = \frac{MEC}{PNEC} \quad (3)$$

where MEC = “Measured Environmental Concentration” of the highest drug residue level in river water; PNEC = Predicted No Effect Concentration. An approach by Tell et al., [33] was used where the lower PNEC is considered. This follows review of ecotoxicity data or minimum inhibitory concentrations [34]. The statistical analysis involved use of Infostat Software Package (2018), determining levels of significance ($\alpha = 0.05$) as reported elsewhere [35] Linear mixed models, LSD Fisher comparison test, principal component analysis (PCA) and multivariate analysis were used [19].

3. RESULTS AND DISCUSSION

3.1.Physicochemical and microbiological parameters

As detailed elsewhere [19], the conductivity and bacterial count associated with the Suquía River were high with levels of 754 µS/cm and total coliform count of 2. 3 million MPN 100/ml. The dissolved oxygen downstream of the WWTP was up to 7.6 mg/l. The quality of water worsened downstream, a trend observed over a period of 20 years [26, 36, 37]. Autotrophic biofilm communities were more upstream of the plant with Autotrophic index (AI) below 200, while heterotrophic communities predominated downstream (AI > 200). Sediment samples varied in organic carbon content and texture by seasons and sites. The percentage of sand

increased (67%–77%) during the dry season compared to the wet season (69%–39%) unlike for silk which was 31%–61% in the wet season and 33%–23% in the dry season. The percentage of organic carbon content ranged from 0.3% to 3.2% in the wet season and 0.35 to 2.29% in the dry season. The pH was generally between 6.1 and 7.5.

3.2.Antimicrobials in river samples.

Forty–three parent antimicrobials and four of their metabolites were analysed in river water samples, 36 in biofilm and 31 in sediments with detection limits ranging from 0.2 ng/l to 31 ng/l (water), 0.3 µg/kg and 28 µg/kg. (biofilm) and 0.1 µg/kg to 6 µg/kg (sediments). The percentage recoveries ranged from 21 % to 156%. All finding agreed with previous reported [12, 29, 31, 38].

Norfloxacin, ofloxacin, ciprofloxacin, cinoxacin, cephalexin, azithromycin, clarithromycin, doxycycline, clindamycin, sulfathiazole, trimethoprim and metronidazole were detected in 67% of the samples. Eight including fluoroquinolones, cephalosporins, macrolides, lincosamides, dihydrofolate reductase inhibitors and nitroimidazoles were found in 80% of the water at 0.003 µg/l–0.29 µg/l. Seven antimicrobials in the family of fluoroquinolones, macrolides and dihydrofolate reductase inhibitors were detected in 80% of the biofilm samples while the sediments contained six fluoroquinolones, macrolides, tetracyclines, sulfonamides and dihydrofolate reductase inhibitors ranging from 2 µg/kg to 34 µg/kg, in 40% of the samples. Quinolones and penicillins were not detected which is (partly) consistent with another study [39] were penicillins for instance were not detected. Sulfonamide metabolites and hydroxy metronidazole were also not detected, probably due to dilution as reported elsewhere [29].

Matrices obtained from control site S1 (El Diquecito–La Calera) did not contain antimicrobials. The number and concentration of the antimicrobials increased downstream. Overall cephalexin had the highest concentration (0.29 µg/l) in water at the downstream source S4; ciprofloxacin (up to 652 µg/kg) in biofilm and 34 µg/kg for ofloxacin in sediments, both at the source point S5. Cephalexin and clarithromycin were the most frequently detected in water, while ofloxacin and ciprofloxacin as well as clarithromycin and azithromycin were more common in biofilm. Doxycycline, sulfathiazole as well as the above fluroquinolones and macrolides were common in sediments. The residue levels in water were not different in the wet and dry seasons unlike in biofilm and sediment samples where the levels were higher in the wet season [19].

Valdés et al., [21] previously reported detection of ciprofloxacin in the range 0–0.036 µg/l in samples collected near Suquía River or downstream. The higher (0.078 µg/l) and more frequently detected levels in the current study could be attributed to proximity to the wastewater treatment plant. The findings on clarithromycin (0–0.145 µg/l) agree with other reports [21, 40], including levels of 0.008 µg/l found in samples from Córdoba province. The presence of these antimicrobials explains environmental degradation in the Suquía River due to anthropogenic uses and the wastewater treatment [20, 26, 35, 36].

Although few reports on presence of antimicrobial residues in aquatic ecosystems have been reported in Latin America [10], urban pollution with the substances studied here has been reported elsewhere [3, 6, 7, 40, 41]. Alcaraz et al., [42] reported ciprofloxacin (0.4 µg/l) and norfloxacin (1 µg/l) in the Las Prusianas stream, Argentina, while Teglia et al., [16] reported ciprofloxacin (0.74 µg/l –7.7 µg/l) and ofloxacin (0.71 µg/l–1.78 µg/l) in wastewater samples from livestock and poultry farms. Alonso et al., [17] also reported ionophores in the range 0.246 µg/l–1.222 µg/l in runoffs from animal farms. Mastrángelo et al., [43] reported the presence of sulfamethoxazole, ciprofloxacin, clarithromycin, metronidazole, ofloxacin, and trimethoprim

in the range 0.072 µg/l–0.326 µg/l in water from rivers Reconquista and Luján, Argentina. These findings are generally like what was found in the current study.

Locatelli et al., [44] found high levels of cephalexin in urban and sewage – impacted rivers in São Paulo, Brazil. Gros et al., [29] and Rodríguez–Mozaz et al., [39] reported the same antimicrobials in Ter River, Catalonia, Spain at a concentration of 0.200 µg/l. Mastrángelo et al., [43] reported ciprofloxacin in biofilms of the rivers Reconquista and Lujan at 179 µg/kg while in the current study the level was 659 µg/kg.

The drug concentrations in the fluvial biofilm in our study [19] were not different from other studies [15] that reported the same level of drugs in fluvial biofilms from Vienne River in France. Rodríguez–Mozaz et al., [45] and Huerta et al., [12] reported higher levels from studies in Spain. The high levels of antimicrobials in the biofilms indicate how important it is to consider them as sources/indicators of environmental pollution [13, 15, 45]. The presence of drugs (on biofilms) is not unusual [12, 15]. Other parameters such as biomass density, porosity and extracellular polymeric substances affect the sorption and intrabiofilm diffusion of residues/contaminants [46–49].

Fluoroquinolones, macrolides and trimethoprim, doxycycline and sulfathiazole were detected in sediments. Tetracyclines are associated with binding to suspended solids and sediment [50] which can explain doxycycline presence for instance. Meanwhile, high organic matter in the wet season can explain higher levels of the residues detected in this study [19]. Rains also tend to remove sediments that could attach to biofilms [51].

The drugs metronidazole, cephalexin and clindamycin were found in water only; doxycycline and sulfathiazole in sediments only and cinoxacin in biofilms only. This is unlike trimethoprim, ofloxacin and clarithromycin that were found in all matrices. While ciprofloxacin was detected in water and biofilms, recovery from sediments was poor. Azithromycin was only found in biofilm and sediment, but it is not very clear if this could be due to degradation [52]. Antimicrobial bioaccumulation factors in biofilms were in the range 66 l/kg dry weight and 12258 l/kg dry weight with trimethoprim being the lowest and ciprofloxacin the highest. Levels above 1000 l/kg are of significance [45].

Sediment pseudo partition coefficients were determined in the range 4 l/kg dry weight and 831 l/kg dry weight for ofloxacin, clarithromycin and trimethoprim. These levels suggest low tendency to partition into sediments. Some drugs such as azithromycin, doxycycline and sulfathiazole were found in sediments and not water and thus partitioning couldn't be determined. This agrees with previous findings [53] that absorption of most pharmaceuticals is not significant in freshwater and marine settings.

Trimethoprim showed a low–risk value (RQ <0.1) compared to norfloxacin, ofloxacin, clindamycin and metronidazole with moderate risk (RQs 0.14–0.41) and ciprofloxacin, cephalexin and clarithromycin with a high–risk value (RQ > 1). This could be associated with presence of drug resistance genes [54]. Rodríguez–Mozaz et al., [55] reported moderate risk associated with cephalexin, ciprofloxacin and azithromycin in water bodies of Portugal, Spain, Cyprus and Germany.

4. CONCLUSIONS

This study has found a range of antimicrobials in water, biofilm and sediment samples collected from the Suquía River in Argentina regardless of the season. The sources included wastewater

treatment plant discharges (mainly) and urban runoff. Fluoroquinolones, macrolides and trimethoprim were the most predominant although high levels of cephalexin were also noted in water. Accumulation was more in biofilms than sediments and as such biofilms are regarded excellent bioindicators of environmental contamination with antimicrobials.

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BIOACCUMULATION, BIOCONCENTRATION AND METABOLISM OF CARBAMAZEPINE AND OTHER PHARMACEUTICALS IN FISH UNDER FIELD AND CONTROLLED LABORATORY CONDITIONS

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Abstract

The presence of 20 pharmaceuticals, including carbamazepine and two metabolites in *Gambusia affinis* and *Jenynsia multidentate* fish from polluted areas of the Suquía River was studied. *G. affinis* contained all the 20 analytes and *J. multidentate* only 15. *J. multidentate* was exposed to carbamazepine under controlled laboratory conditions and the parent drug as well as its metabolites carbamazepine–10,11–epoxide (CBZ–EP) and 2–hydroxy carbamazepine (2–OH–CBZ) monitored in five organs. Carbamazepine and 2–OH–CBZ were found in gills, intestine, liver, brain and muscle of fish, while carbamazepine–10,11–epoxide (CBZ–EP) was detected in gills and muscle only. This study reports, for the first time, the biotransformation of carbamazepine to CBZ–EP and 2–OH–CBZ in fish, under controlled laboratory conditions.

1. INTRODUCTION

Many contaminants are emerging, not regulated and therefore a concern to humans and the environment [1]. These include a range of pharmaceuticals arising from incomplete removal in wastewater treatment [2]. Extensive work has now been undertaken to determine the occurrence, fate, effects, and risks of pharmaceuticals in the environment over the last 15 years [3]. A range of pharmaceuticals including psychiatric drugs, analgesics, anti–inflammatory agents, β–blockers, antiplatelets, antiasthma drugs, antihypertensive, antihistamines, lipid regulators, antimicrobials and contraceptives have been detected in fish and biota where they accumulate [4–14]. Effects of bioaccumulation of these chemicals may be chronic, multigenerational and are observed at higher levels of a food web [15].

Carbamazepine (CBZ) a drug used in treatment of various conditions such as epilepsy, trigeminal neuralgia, bipolar depression and mania [16] is one of the most detected drugs in urban surface waters and is potential marker of anthropogenic pollution [17]. The drug is well absorbed from the gastrointestinal tract, with most (72%) found in urine [18]. Most of the drug is excreted in urine as hydroxylated or conjugated metabolites with only 2% unchanged while ~ 28 % that is not absorbed is found in faeces with ~14% as metabolites [18]. Accumulation of CBZ has been noted in various organisms such as periphyton, algae, zooplankton, invertebrates, fish and birds [7–10, 13, 14, 19–34]. The presence of CBZ metabolites in biota especially marine mussels following experiment exposure to the parent drug has been investigated [30]. Such studies facilitate risk assessment of pharmacologically–active substances in biota [35].

Research reports on accumulation of pharmaceuticals in aquatic biota in South America are lacking even through wastewater treatment discharges in water bodies such as Suquía River in

Argentina are well known [36, 37]. Some of the drugs reported downstream of this river include CBZ (up to 113 ng/l), diclofenac (up to 145 ng/l) and atenolol (up to 581 ng/l) [38]. Research was undertaken to evaluate: 1) bioaccumulation of carbamazepine and other pharmaceuticals in *J. multidentata* and *G. affinis* wild fish from polluted areas in the Suquía River basin; 2) uptake, bioconcentration and biotransformation of experimental carbamazepine in *J. multidentata,* a native Argentinian fish [39].

2. MATERIALS AND METHODS

2.1.Chemicals/reagents

The following items were used: Diclofenac, codeine, carbamazepine, citalopram, diazepam, lorazepam, atenolol, sotalol, propranolol, nadolol, carazolol, hydrochlorothiazide, clopidogrel, salbutamol and levamisole (>95% Sigma Aldrich); sertraline and velafaxine (European Pharmacopeia); 2–hydroxycarbamazepine and carbamazepine–10, 11–epoxide (Toronto Research Chemicals); metoprolol (US Pharmacopeia); ibuprofen–d3, diazepam–d5, ronidazole–d3 and fluoxetine–d5 (Sigma Aldrich); Atenolol–d7, carbamazepine–d10, hydrochlorothiazide–d2, and citalopram–d4 (CDN isotopes); venlafaxine–d6 (Toronto Research Chemicals). Individual standard stock solutions (1000 mg/l) were prepared in methanol. Mixtures (20 mg/l and 1 mg/l) were prepared from the stock and then used to prepare working standards (0.1 µg/l, 0.5 µg/l, 1 µg/l, 5 µg/l, 10 µg/l, 50 µg/l, 100 µg/l) — for the calibration curves — using methanol/water (10:90, v/v) [39].

2.2.Sampling and monitoring campaigns

The sampling area was the Suquía River basin in Córdoba, Argentina, a drainage area and middle lower basin [40]. Thirty adult female fish (*G. affinis* and *J. multidentate*) each were captured in April (wet season) and July (dry season) [39]. These were obtained from two monitoring sites downstream of the city's wastewater treatment plant. *G. affinis* fish was found at a 3rd station, Capilla de los Remedios only during the wet season. The fourth station was Río Primero located 70 km downstream of the wastewater management plant and where both *G. affinis,* a 'foreign' fish [41] and *J. multidentata* a native fish [42] have been reported in polluted and unpolluted locations [43]. *J. multidentata* is thought to be a bioindicator of pollution [44, 45]. Following anaesthesia, fish samples were transported in aluminium film at 4ºC and later stored at -20ºC until analysis. Samples were freeze–dried, weighed and ground with a mortar/pestle to make one composite material of 10 *G. affinis* samples or 6 *J. multidentata* samples [39]. About 0.5 g was used for pharmaceutical analysis and the rest for lipid tests. The Guide for the Care and Use of Laboratory Animals [46] was closely followed.

2.3.Biota analysis

A procedure reported by Huerta et al., [9] was followed and it included extracting ~0.5 g of pooled triturated freeze–dried samples, by assisted solvent extraction (ASE 350®, Thermo Scientific Dionex, USA) using methanol (in 4 extraction cycles; 5 min, at 50ºC) [39]. Extracts were evaporated to dryness using nitrogen and dissolved in 1 ml of methanol before cleanup by gel permeation chromatography and HPLC–DAD (Agilent Technologies, USA, model 1260 Infinity). This included injection of 500 µl of the methanolic extract in a 300 mm × 21.2 mm × 10 µm pore size column connected to a 50 mm × 7.5 mm PLgel guard column. The mobile phase consisting of dichloromethane: methanol (90:10, v/v) was used at a flow rate of 5 ml/min. Fractions eluting at 13.5 min to 26.5 min contained the compounds of interest and were evaporated to dryness before reconstitution with 1 ml methanol/water (10:90, v/v).

A mixture of the labelled standards (50 µl, 1 mg/l) including ibuprofen–d3, diazepam–d5, ronidazole–d3, fluoxetine–d5, atenolol–d7, carbamazepine–d10, hydrochlorothiazide–d2, citalopram–d4 and venlafaxine–d6) was added and analysis done closely following another procedure [9]. Two SRM transitions were monitored, and quantification determined using a matrix matched calibration curve. Recovery studies were conducted using samples spiked at 100 ng/g. Percent recovery was calculated using Eq. (1):

$$Matrix\ effect\ (\%) = \left(\frac{A_{spiked\ matrix} - A_{matrix}}{A_{solvent}} - 1\right) \times 100 \quad (1)$$

$A_{spiked\ matrix}$ = area of the analyte in the spiked matrix; A_{matrix} = area of the analyte naturally occurring in the matrix; $A_{solvent}$ = area of the analyte in mobile phase. The concentrations of 5 µg/l and 12.5 µg/l were used to determine matrix effect and the detection limit/quantifications were as reported elsewhere [9]. The determination of total lipid content was according to an AOAC method [47].

The bioaccumulation factors (BAFs) for carbamazepine (in l/kg) in fish were estimated as the ratio of the concentration in whole fish (µg/kg), to the freely dissolved concentration in water (µg/l) [39]. The level of carbamazepine in river was as reported by Valdés et al., [38] . The estimated BAFs are only an indication of possible tendency for the drug to bioaccumulate [48].

2.4.Laboratory exposure conditions

Female adult *J. multidentata* (Cyprinodontiformes, Anablepidae) with a total weight of 0.5 (± 0.2) g and standard length of 29 (± 2) mm were used for laboratory bioassays. The fish was selected because it is regarded a bioindicator of water pollution [45, 49–54]. Collected fish were shipped in aerated 20 L water tanks then acclimatized at 21 (±1)°C, 12:12 h light: dark in 15 L fresh water in an aerated glass aquarium. This was done for 2 weeks prior to the experiments and fish fed with commercial feed pellets but also starved for 24 h prior to the experiments [39].

The bioconcentration assay was conducted at 100 µg/l of carbamazepine for 48 h to measure its accumulation and possible biotransformation in *J. multidentata*. This was guided by previous findings [25] where uptake of carbamazepine by mosquito fish (*G. holbrooki*) from reclaimed water was reported. The conditions and facilities included: 60 fish in 10 fish/glass aquarium about 1 L per fish; three control aquaria supplemented with 0.002% methanol, and three exposure aquariums with 100 µg/l. Fish were weighed after 48 h of exposure, anesthetized and sacrificed by transecting the spinal cord followed by removal of gills, brain, liver, intestine and muscle. Samples were pooled (10 organs in plastic tube) and stored at -20ºC [39].

2.5.Analysis of exposure water and exposed biota

Water was collected from each treatment before exposure and after 48 h and then analyzed for carbamazepine and metabolites [38, 39]. Analytical method optimization was performed closely following what others [9, 55] reported. Bioconcentration factors (BCFs) of the drug (in l/kg) in *J. multidentata* were estimated as the ratio of the concentration in fish organs (µg/kg) to the concentration in water (µg/l) [39].

2.6.Statistical analysis

For statistical analysis the Infostat Software Package [56] was used and included Friedman ANOVA analysis and Least Significant Difference (LSD) Fisher comparison test.

3. RESULTS AND DISCUSSION

3.1.Pharmaceuticals in fish from Suquía River

Twenty pharmaceuticals were detected in wild fish with seven of the drugs, namely atenolol, nadolol, diazepam, lorazepam, clopidogrel, salbutamol and hydrochlorothiazide the most frequent at levels ranging from 1 ng/g to 67 ng/g. Codeine showed the highest concentration 163 ng/g. Similar results have been reported by others [9] who found clopidogrel, carazolol, sotalol, salbutamol and diclofenac in fish from the Mediterranean Sea. What was different from the current study [39] were levels of propranolol, venlafaxine and citalopram which were higher in *G. affinis* and *J. multidentata*. The levels of carbamazepine in the current study [39] were like findings in *G. holbrooki* analyzed 7 days after contact with reclaimed water in Florida [25], common carp from Taihu lake [13], as well as mussels and clams from USA and Europe estuaries and coastal zones [7, 23, 24, 28, 57]. Alvarez–Muñoz et al., [28] also reported lower levels for half of the pharmaceuticals investigated, in mullet and flounder from estuaries in Tagus, Portugal and Scheldt Netherlands. Moreno et al., [14] found 90% of the same pharmaceuticals in golden grey mullet and black goby from Mar Menor lagoon, Spain. Unlike the current study [39], these scientists didn't detect carbamazepine in the same fish sample.

Carbamazepine seemed to accumulate in *G. affinis* during the dry season which could be attributed to low water flow rates although this was not observed in *J. multidentata*. The other pharmaceuticals showed a similar patter to CBZ's. The difference in concentration between these two fish is not clear as they share trophic level and similar diets [58]. *G. affinis* had a higher lipid content (4.6 ± 0.5 %) than *J. multidentata* (3.5 ± 0.5 %) and this could explain the different levels of certain hydrophobic drugs such as sertraline in *G. affinis.* However, it is still not clear why others such as atenolol and hydrochlorothiazide with low alcohol/water partition coefficient accumulated at a higher level. A possible reason could be a different drug interaction mechanism such as receptor binding on biotransformation for the polar pharmaceuticals [6, 27]. The pH level as studied elsewhere [59] influence the accumulation of weekly basic drugs such as diphenhydramine. Plasma pharmaceutical levels, protein content and differences in influx and efflux mechanisms at the blood brain barrier also influence the levels and differences in the drugs [33].

Carbamazepine and the metabolites (CBZ–EP, 2–OH–CBZ) were found in *G. affinis* collected from Capilla de los Remedios and Río Primero with BAFs ranging from 43 l/kg and 208 l/kg [39]. Other investigators have reported levels in the range 2.5 l/kg to 264 l/kg [2, 13, 22].

3.2.Carbamazepine water concentration and tissue distribution

There was no significant difference in the concentration of carbamazepine before and after 48 h of the experiments. There was also no appreciable drop in drug concentration in the water. Neither the metabolites nor the parent drug were detected at the respective LODS of 3 ng/l and 0.2 ng/l [39]. A method optimized for two metabolites and 16 parent pharmaceuticals was used to detected concentrations (10 mg–25 mg) in gills, intestine, liver, brain and muscle. Validation showed percent recoveries in the range of 40% to 128%. Ion suppression (-96%) and enhancement (164%) was observed [39]. Due to matrix effects the liver and intestine were difficult to handle and analyze as reported [6, 9, 27]. The method was suitable for analysis of all the pharmaceuticals except carazolol, sertraline and diclofenac. The LODs and LOQs ranged from 0.1 ng/g to 30.3 ng/g and 0.4 ng/g to 100 ng/g. Similar LOQs for pharmaceuticals in fish tissues are as reported elsewhere [8, 19, 60, 61]. The levels were higher than in other studies [9, 11, 27] .

Carbamazepine and metabolites, CBZ–EP and 2–OH–CBZ were detected in *J. multidentata* exposed to 100 µg/l carbamazepine for 48 h [39]. Brain and liver had the highest concentrations of 701 ng/g (± 206 ng/g) and 688 ng/g (± 83 ng/g), respectively. Higher levels in the brain could be due to respective mechanisms of action and use for psychiatry [62] while accumulation in liver is understandable since this is where detoxification largely occurs [15]. Accumulation of pollutants in the liver has been reported [4, 6]. The carbamazepine levels in muscle (430 ± 14 ng/g) and liver (688 ± 83 ng/g) were as reported by Garcia et al., [22] on bluntnose minnows (*Pimephales notatus*). In that study, accumulation of up to 324 ng/g–414 ng/g and 892 ng/g–1503 ng/g in muscle and liver, respectively, was reported. Tanoue et al., [27] recently reported distribution of pharmaceuticals, including CBZ, in *Carassius carassius* and *Cyprinus carpio* with CBZ concentration in the range 0.043 µg/l–0.12 µg/l.

While the metabolism of carbamazepine in humans is known to follow several pathways [18, 63] mainly mediated by the cytochrome P450, little is known about fish. Connors et al., [64] reported biotransformation in fish of 12 pharmaceuticals which are also substrates of specific human CYPs. The presence of the metabolites suggests breakdown from the parent CBZ in fish. The ratios of carbamazepine and metabolites in muscle and gills of *J. multidentata* resemble ratios observed in epileptic children [65]. Therefore, there appears to be some similarities between metabolism of the drug (s) in humans and the fish studied [39].

The metabolites 2–OH–CBZ and CBZ–EP have been reported by Moreno et al., [14] in wild fish collected from Mar Menor lagoon at 0.07 ng/g–0.3 ng/g and ~0.2 ng/g, respectively in muscle of golden grey mullet. Boillot et al., [30] also detected CBZ–EP and acridine in marine mussel and others [28] reported CBZ–EP and 2–OH–CBZ at ~1.3 ng/g in other species of mussels from the Ebro delta. The CBZ parent and metabolite levels were the same in liver and brain, which were both higher than in muscle, gills and intestine, a pattern that was reported in wild fish by others [6, 8, 33]. A similar trend was also reported for certain pharmaceuticals including antidepressants [4, 11, 65].

The BCFs for carbamazepine in *J. multidentata* were in the range 5 l/kg and 9 l/kg which is consistent with findings of < 10 l/kg reported elsewhere [26, 30, 66] for polar pharmaceuticals. Similar finding include a report by Garcia et al., [22] on BCFs of 2 l/kg–7 l/kg in plasma, liver, brain and muscle of *Pimephales notatus* and *Ictalurus punctatus* under experimental settings. Vernouillet et al., [21] reported BCF of 2.2 for *Pseudokirchneriella subcapitata* exposed to 150 mg/l of CBZ in 24 h, and 12.6 *Thamnocephalus platyurus* fed the algae treated with carbamazepine. Mussels (*Dreissena polymorpha*) exposed to low CBZ levels (0.236 µg/l) still show a low level of accumulation [67]. In their study, Lahti et al., [68] reported BCFs of ~0.4 in plasma of juvenile rainbow fish. Almeida et al., [26] also reported BCFs between 0.11 and 1.2 in *Venerupis decussate* and *Venerupis philippinarum*, exposed to CBZ below 10 µg/l for 96 h. Tanoue et al., [33] reported related findings for plasma, brain, liver, kidney, muscle and gills. It is still worth noting that BCF is good for estimating metabolic biotransformation rates and not necessarily a sole predictor of bioaccumulation potential [39, 69].

4. CONCLUSIONS

This study shows that up to 20 pharmaceuticals, including CBZ and two of its metabolites accumulated in wild *G. affinis* fish in the Suquía River and 15 of these compounds in wild *J. multidentata)* fish. Levels of the pharmaceuticals seemed to generally accumulate more in the dry season especially for *G. afiinis*. An isotope dilution LC–MS/MS method was established and used to evaluate the uptake and distribution of CBZ and its metabolites in different tissues

of *J. multidentata* following experimental exposure. The method is also suitable for detecting the drugs in naturally exposed fish.

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USING STABLE NITROGEN–ISOTOPE RATIOS TO DETERMINE SUITABILITY OF FRESHWATER SYSTEMS FOR FISH PRODUCTION

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Abstract

A study was undertaken to evaluate anthropogenic sources of pollution and causes of eutrophication in Córdoba, Argentina. Three reservoirs: San Roque Lake (SRL), Los Molinos Lake (LML) and Río Tercero Reservoir (RTR), were studied and stable nitrogen isotope ratios ($\delta^{15}N$) measured in samples including water, plankton, shrimp and fish muscle. The SRL samples had higher levels (14.9 ‰–20.0 ‰) than elsewhere, suggesting SRL is not suitable for fish production for human consumption.

1. INTRODUCTION

Aquatic reservoirs support fish production besides recreation [1] but these reservoirs now face the challenge of human population explosion, urbanization and heightened use of land including for agricultural services, all resulting in higher levels of nutrients and chemical contaminants that can flood the environment [2]. High levels of bioavailable nitrogen arising from agricultural fertilizers, livestock, domestic and industrial waste [3] for instance, have negative environmental consequences [4].

A common measure of the presence and impact of anthropogenic activities is the analysis of stable nitrogen isotope ratios ($\delta^{15}N$) [5]. $\delta^{15}N$ helps in monitoring pollution and assessing the impact of anthropogenic pollution on freshwater ecosystems [6]. Varying $\delta^{15}N$ levels have been reported such as -6‰ and +6 ‰ for NH_4^+ fertilizer, NO_3^- fertilizer and urea; 0‰ to 8‰ for soil nitrogen; -13‰ to +13‰; for atmospheric nitrogen deposition; +5‰ to +25‰ for manure and +4‰ to +19‰ for sewage [7]. $\delta^{15}N$ values from primary production can define an isotopic baseline for aquatic food webs [8].

Eutrophication and pollution of freshwater reservoirs has been reported as a major problem in Argentina [9, 10]. A study [11] was therefore undertaken to establish methods to help in understanding and estimating the impact of anthropogenic activities on aquatic ecosystems in Córdoba. This included evaluating $\delta^{15}N$ in water, plankton, shrimp (*Palaemonetes argentinus*) and omnivorous fish (*Odontesthes bonariensis*) from selected lakes. Additional work included determining if $\delta^{15}N$ is a useful chemical biomarker for determining varying levels of pollution and association with aquaculture food production. It has been determined that $\delta^{15}N$ is an indicator of freshwater systems for edible fish production [11].

2. MATERIALS AND METHODS

Samples were collected from SRL, LML and RTR. SRL. It has been reported that the quality of water around SRL is declining due to waste from the neighbourhood [12]. The LML supplies water for irrigation, recreational and farming [13] while RTR the largest artificial lake in Córdoba supplies water for a range of services including a nuclear power plant [14].

Samples were collected ensuring that they are of the same quality as the rest of the lake [15, 16]. Plankton (n=6) were collected by filtering 20 L of water using 50 µm nylon filter. *P. argentinus* (n=14) and *O. bonariensis* (n=24) samples were iced and transferred to the laboratory before the muscle tissue was separated. Biotic samples were dried, homogenized and fat removed with petroleum ether in a soxhlet apparatus. Selection of these samples was guided by previous work in SRL and LML [9, 10]. Lake water (n=6) was collected for determination of faecal coliform bacteria (FCB) following an established technique [17] and for determination of $\delta^{15}N$.

Samples were analyzed for the $\delta^{15}N$ composition using an Isotope Ratio Mass Spectrometer, and elemental analyzer (Thermo Fisher Scientific) with the isotopic abundance expressed as $\delta^{15}N$ parts per thousand (‰) relative to Atmospheric Air, from the following Eq. (1):

$$\delta 15N\ (‰) = (Rsample - Rstandard)/ R\ standard)x\ 1000 \quad (1)$$

where R = $^{15}N/^{14}N$.

3. RESULTS AND DISCUSSION

There was a significant difference ($p < 0.05$) in $\delta^{15}N$ values in samples from the same reservoir with fish muscle showing the highest levels, ahead of shrimp, plankton and water [11]. This trend was common among all reservoirs sampled. Fish and shrimps from SRL contained the same $\delta^{15}N$ values. This could be attributed to variations in water stress, body protein catabolism and urea recycling [18]. Changes in stable isotope value take time to reflect and can be influenced by diet, determined by species–specific isotopic turnover rate. The rate of turnover takes months in fish muscle and weeks in shrimps [19]. These trophic level–variations have been reported in literature [9, 10]. All SRL samples showed $\delta^{15}N$ values significantly higher than in the other reservoirs. While the $\delta^{15}N$ for water and plankton from RTR and LML were similar, the values were higher in shrimp and fish from RTR than LML's.

Changes in $\delta^{15}N$ provide information on origin and transformation of nitrogen in ecosystems [20, 21]. For instance, it has been determined elsewhere that in the upper reach of the East Tiaoxi River in China, soil is the major contributing source of nitrogen unlike in the middle and lower reaches where the sources are sewage/manure and chemical fertilizers, respectively [22]. In the current study by Griboff et al., [11] the $\delta^{15}N$ in water (SRL: 9.0 ± 0.4‰, LML: 4.0 ± 1.0 ‰ and RTR: 5.0 ± 2.0‰) were comparable to the findings by Jin et al., [22]. The findings suggest that the lakes receive nitrogen from various anthropogenic and natural sources. Biota from LML had $\delta^{15}N$ values between 8.0‰ – 14.0‰ and for RTR, this was between 9.0‰ – 17.0‰. The levels of 14.9‰ – 20.0 ‰ in organisms from SRL have been attributed to high sewage and manure exposures in part because of the faecal coliform bacteria detected [11]. High isotope ratio values with more ^{15}N are due to accumulation and degradation of human and animal waste [23]. The human ecosystem health is thus at a risk of exposure to pathogens, hydrocarbons, toxins, and endocrine disruptors [24]. Further, the SRL – where exposure to sewage has been demonstrated [25] – can be a source of contamination to food production [11] although the reservoir remains a critical resource of water in Argentina [26].

Faecal coliforms were determined in the range 3.6 MPN/100 ml – 93 MPN/100 ml in SRL, 0 – 9.3 MPN/100 ml in LML and 0–3.6 MPN/100 ml in RTR [11]. Although presence of sewage or manure in waterbodies is generally determined through detection of faecal indicator bacteria [27] this is inadequate or even challenging in determining long term impacts [28]. Measurement

of $\delta^{15}N$ can be an additional reliable indicator of the presence of nitrogen from sewage [11]. The high $\delta^{15}N$ levels determined in the current study suggest that SRL is deteriorating given previous reports where levels were low, between 9.4‰ – 17.2‰ and 9.9‰ –18.1‰ [9]. This deterioration could be attributed to human population growth near the lakeside. High $\delta^{15}N$ values in edible fish (20 ± 1‰) were observed [11] compared to what was measured earlier elsewhere [9]. The findings also compare favourably with those reported at 11.5 to 15.2‰ for fish thought to have been exposed to sewage [29, 30]. These findings reaffirm how, $\delta^{15}N$ is an important biomarker of faecal /sewage contamination [31, 32]. Also, fish appears to be a reliable indicator of sewage exposure than other aquatic biota [33] at all trophic levels [34].

4. CONCLUSIONS

Three water reservoirs SRL, LML and RTR were studied for levels of pollution by measuring $\delta^{15}N$ values. SRL samples showed the highest $\delta^{15}N$ values compared to the other two reservoirs suggesting sewage discharge and anthropogenic effects and therefore urgent need for corrective action at the affected reservoir. This study provides an important insight into use of $\delta^{15}N$ measurements to understand contamination of the food web in the lakes. Nevertheless, further research on the subject is recommended.

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TRANSFER OF METALS AND METALLOIDS FROM WATER TO EDIBLE FISH IN THE SAN ROQUE RESERVOIR, CÓRDOBA, ARGENTINA

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Abstract

Levels of 17 metals were analyzed in water, sediments, and aquatic organisms sampled from the San Roque Reservoir. Samples were divided into three trophic groups: plankton, shrimp (*Palaemonetes argentinus*) and fish (Silverside, *Odontesthes bonariensis).* Stable nitrogen isotope ($\delta^{15}N$) was used to investigate trophic interactions. The muscle of *O. bonariensis* contained Hg and As above known oral reference dose levels. Element trophic magnification factors (TMFs) were calculated using the slope of the regression line when trace element concentrations are plotted against the $\delta^{15}N$ values. The concentrations of Ni, Cd, Cr, Al, Mn, Fe, Mo, Ce, Nd, Pt and Pb during the wet and dry seasons, and Sr during the dry season were lower (TMF<1) with increasing trophic level. Overall, there was no significant relationship between levels of metals and trophic levels.

1. INTRODUCTION

Heavy metals are some of the common environmental pollutants affecting aquatic biota through domestic, industrial, and agricultural runoffs as well as atmospheric deposition [1]. Their persistence in the environment, bioaccumulation and high toxicity are a major concern [2]. Trophic transfer of these elements to higher organisms of the food web is one of the mechanisms through which humans are exposed [3] with fish being one of the main vehicles [4, 5]. Nonessential trace elements have been detected in edible fish tissues [6]. Trophodynamics of elements along a food chain can result in biomagnification and biodilution although there may not be a change in the components of the food web in some instances [7]. While Hg demonstrates biomagnification trends no clear pattern has been noted in the cases of Cd, Cr, As and Pb [8, 9]. Dietz et al., [8] reported levels of Cd increasing towards higher trophic levels in marine and freshwater ecosystems. Levels were higher in marine biota than in freshwater and terrestrial ecosystems unlike Pb. Nfon et al., [9] reported decreasing Pb and Cd concentrations with increasing $\delta^{15}N$ which suggests biodilution in the marine food chain. The transfer of trace elements in the aquatic biota is influenced by various factors including environmental conditions, how long the food chain is, the physicochemical properties and levels of the contaminants [10].

Stable isotope ratios $\delta^{13}C$ and $\delta^{15}N$, are used to elucidate biomagnification or biodilution in the trophic web [11, 12]. $\delta^{15}N$ for instance is a valuable biomarker used to assess trophic position because heavy nitrogen isotope enrichment occurs incrementally across trophic levels at a constant rate of 3‰–4‰ [13] unlike $\delta^{13}C$ where the enrichment is not so obvious (approx.1‰) [13]. Metals enter fish through different avenues and concentrates at different levels in organs [14]. The distribution in the fish tissues depends on the mode of exposure and can suggest pollution [15].

Accumulation of elements such as Hg, As, Cd, Cr, Pb, Cu in muscle tissue has been reported widely [16–18]. The bioaccumulation differs in tissues due to various physiological factors [19]

and therefore to obtain reliable results on, it is important to analyze multiple tissues as some such as muscle are poor indicators [20]. Higher accumulation can be attributed to levels of binding proteins such as metallothioneins in liver [21]. It is reported that distribution of the metals, among other contaminants, can also vary with species [19]. A common native fish, sensitive to pollution and used to a standardised acute toxicity test method in Argentina is the silverside *Odonthestes bonariensis* [18, 22–26].

The current study [10] aimed at determining the distribution and seasonal variation of the 17 metals Mn, Fe, Zn, Cu, Cd, Cr, Ni, Ag, Sr, Mo, Nd, Al, Ce, As, Pb, Pt and Hg in water, sediment and aquatic organisms from the San Roque Reservoir. It would also involve investigating the trophic transfer patterns of the metals in the aquatic food web: water, plankton, shrimp and Silverside fish; and estimating associated health risks of toxic elements present in Silverside fish. This entailed studying the biomagnification and biodilution of several metals and metalloids.

2. MATERIAL AND METHODS

2.1.Study site, sample collection and analysis

The San Roque reservoir, an artificial lake in the Punilla Valley, Córdoba, characterised by seasonal changes in water levels was studied. It is eutrophic–hypereutrophic with high levels of nutrients and high incidence of toxic cyanobacterial blooms [27]. Nevertheless, the reservoir remains an important source of drinking water for Córdoba city. It is also useful for irrigation, flood control, swimming, boating and fishing.

Samples were collected from easily accessible locations that were assumed to have the same water quality as the rest of the reservoir [27]. Two sampling campaigns were conducted at the end of the dry and wet seasons. Sampling, handling and transportation of the water, sediments, plankton, shrimp and various organs of silverside fish followed published guidelines [28] and analysis as reported elsewhere for Mn, Fe, Zn, Cu, Cd, Cr, Ni, Ag, Sr, Mo, Nd, Al, Ce, As, Pb, Pt and Hg [10]. Water (n=5) was collected into acid washed plastic bottles, ultrapure HNO_3 added, and the samples were stored at 4°C until analysis. Otherwise, water samples were pressed through 0.45 µm nitrocellulose filters (Sartorius, Göttingen, Germany) once ready for analysis. Sediment samples (n= 5) from depths of 0 cm –15 cm were collected using a plastic shovel, placed in clean 1 L plastic containers; dried at 40°C and sieved using acrylic meshes. The sieved sediment (200 mg) was digested using nitric, hydrochloric and hydrofluoric acid in a PTFE screw–capped container on a heating plate [10]. The analyses were performed using 1 g of < 63 µm dried materials as reported by Monferrán et al., [28]

Zoo/phytoplankton (n=5) were collected by filtering 20 L of water through a 50 µm nylon sieve. Shrimp (2.917 ± 0.032 cm) were captured using plastic nets and placed in 20 L waterfilled containers. The Silverside fish (n = 6 in the dry season and n = 12, wet season) caught weighing ~33 g and ~37 g were kept on ice and dissected to remove the liver, gills, brain, gonads and muscle. These samples were then dried at 40ºC (stored at -20°C when analysis was delayed) and ground and homogenized in mortar and pestle before analysis [10]. The various biological samples (20 g) were digested in triplicate using 8 ml of nitric acid and 1 mL of 30% H_2O_2 in a microwave digestion system set at 1600 W 75%, 15 min ramp to 150°C and 20 min at 150°C. An Inductively Coupled Plasma Mass spectrometer was used to analyses samples in triplicate. Certified reference materials including NIST 1646a, NIST 1573a, and NIST1515 were used for quality control. The percentage recoveries for the CRMs were 93 (± 15)%, 102 (± 17) %, 102 (± 18)% and 98 (± 11)%, respectively.

2.2.Stable isotopes and calculations of bioaccumulation factors

Isotope ratios $\delta^{13}C$ and $\delta^{15}N$ were determined in fish muscle as well as plankton and shrimp, using an EA IRMS (Costech Elemental Analyzer, Conflo III interface, and Delta Plus Advantage MS; (Thermo Corporation, USA). The stable isotope abundance (d) was determined according to Eq. (1) as follows:

$$\delta X‰ = (R\ sample/R\ standard) - 1)\ x\ 1000 \quad (1)$$

where X = ^{13}C or ^{15}N; R = $^{13}C/^{12}C$ or $^{15}N/^{14}N$.

R standard can be calculated according to the Vienna Pee Dee Belemnite reference for ^{13}C, and atmospheric N_2 (AIR) for ^{15}N.

For fish, invertebrates and plankton, trace element concentrations of total body homogenates were used for calculation. The bioaccumulation factor (BAF) is determined as reported elsewhere [29] in the Eq. (2):

$$BAF = \frac{Css}{Cw} \quad (2)$$

C_{ss} = element concentration at steady state (µg/g, dry weight), and C_w = element concentration in water (µg/ml).

3. RESULTS AND DISCUSSION

3.1.Multielement concentration in environmental compartments

The highest concentrations of the elements in water were during the dry season possibly due to low water volume compared to the wet season [10]. Related studies including work on physical and chemical parameters has been reported [30]. Regulatory levels have been established for the protection of the aquatic wildlife in Argentina [31]. In the current study [10], metals such as Al, Cu, Cr, Ni and Zn, during the dry season exceeded regulatory levels (100 µg/l, 2.87 µg/l, 2.5 µg/l, 4.2 µg/L, 4.54 µg/l, respectively). In the wet season, Cu and Zn were high (2.87 and 4.54 µg/l, respectively).

The upper layer of sediment (0–15 cm), had the highest metal concentrations in the dry season ($P < 0.05$). The high concentrations (relative to sandy sediments) could be due to the slow water flow which allows adequate time for the metals to deposit. The levels of Cr, Cu, Ni and Fe in sediments were lower than those reported elsewhere [28] in sediments of the Suquía River. The levels of Zn although higher in the San Roque Reservoir [10] than previously reported in La Calera, these didn't exceed 315 µg/g set limits set by the Canadian Guideline values for the Protection and Management of Aquatic Sediment Quality [32].

Levels of the elements in plankton, shrimp and fish were generally higher in the wet season than the dry season although this was not the case in water and sediment, the latter possibly due to increased levels and temperatures in the reservoir or due to rapid response to environmental changes by plankton or harsh conditions such as El Niño Southern Oscillation, seasonal changes, tsunamis and hurricanes [33].

The highest levels of Ni (98.6–9.1 µg/g), Al (8751–9052 µg/g), Fe (6277–6718 µg/g), Mo (0.39–0.53 µg/g), Mn (115–168 µg/g), Ce (7.9–4.8 µg/g), Cr (11.9–12.9 µg/g) and Nd (4.4–21.8 µg/g) were in plankton, while Cu (44–64 µg/g), Ag (1.9–0.2 µg/g) and Zn (70–105 µg/g)

were highest in shrimp; Hg was highest in fish and As in plankton in the wet season. Levels of As were the same in fish and shrimp in wet and dry seasons and in plankton (10.4 (µg/g) during the dry season. The concentration of Cd in plankton and shrimp ranged between 0.042 µg/g and 0.085 µg/g. Plankton may contain a variety of unicellular algae, rich in oligoelements and major elements such as Fe, Al, Mn and Ni, when filtered through a mesh net size of 50 µm [34]. These algae are useful bioindicators of environmental pollution. Essential and nonessential elements with a range of effects including competition with calcium for enzymatic sites [35] are common in the aquatic environment.

The findings of this study [10] agree with others [36] who reported that physiological requirements of organisms determine the final concentration of element in a body. Shrimps and certain/other invertebrates are net accumulators of metals such as Cu [37, 38] probably due to the presence of or need for hemocyanin [39]. The Cu levels (1.2 µg/g and 1.4 µg/g in dry and wet season, respectively) were not significantly different from what other researchers [40] at 1.6 µg/g in farmed fish, but lower than what Chale [41] reported at 4 µg/g in pelagic fish. Surprisingly, Cu concentration in the wild and farmed fish were the same yet the level in wild fish is expected to be low. Qiu et al., [40] argued that these levels could be attributed to lipid contents.

The Hg level in the San Roque fish (0.035 µg/g–0.07 µg/g) was lower than what Qiu et al., [40] reported (0.18 µg/g–0.22 µg/g) in pompano and snapper. This is possibly due to high levels in the surrounding water bodies. The Zn (57 µg/g–72 µg/g) and Cr (3.5 µg/g–5.5 µg/g) concentrations in the San Roque fish were higher than those reported by Qiu et al., [40] in farmed fish at 27.3 µg/g, and 0.54 µg/g for Zn and Cr, respectively. The concentration of As was 5.4 µg/g, and 3.5 µg/g in dry and wet seasons, respectively [10. This was higher than in the findings by Ikemoto et al., [42].

Higher concentrations of Ni, Al, Fe, Mo, Ce, Cr and Nd were found in plankton, while Cu, Ag, and Zn levels were highest in shrimp and Hg highest in fish [10]. The findings partly agree with Farag et al., [43] who reported Ag, Cu, and Zn bioaccumulated at high levels in invertebrates compared to fish from the Boulder River.

3.2.Trophic relationships; bioaccumulation of the elements.

The $\delta^{15}N$ levels in the dry season were 9.44 (± 0.20)‰; 15.94 (± 0.20)‰ and 17.22 (± 0.24) ‰ for plankton, shrimp and fish, respectively, and 9.92 (± 0.20)‰; 15.03 (± 0.20)‰ and 18.14 (± 0.20)‰ for plankton, shrimp and fish, respectively, in the wet season. The $\delta^{13}C$ values in the dry season were -18.97 (± 0.20)‰; -20.31 (± 0.20)‰ and -20.31 (± 0.24)‰ for plankton, shrimp and fish, respectively, and -22.13 (± 0.20)‰; -16.72 (± 0.20)‰ and -18.63 (± 0.22)‰ for plankton, shrimp and fish, respectively, in the wet season [10].

$\delta^{13}C$ can be used to identify primary production [13, 44] and determined association between levels in organisms such as herbivores and carnivores, correlate with the diet. A Silverside fish and shrimp showed higher $\delta^{13}C$ values than plankton in the dry season while there was no significant difference in the biota of the San Roque biota [10]. The $\delta^{15}N$ levels were significantly different ($P < 0.05$) among the different aquatic species in both the dry and wet seasons. The values increased in the pattern: plankton–shrimp–fish. A 5.8‰ difference between plankton and shrimp, and approximately 3.0‰ between shrimp and fish consistent with previous findings [13] were observed.

The bioaccumulation factors (BAFs) above 100 are recognized as significant [45]. The BAFs in the current study exceeded 100 in plankton for most elements, except Mo and Nd in the dry and Cd as well as Hg in the wet season. The same pattern (>100) was observed in *P. argentinus* for most elements, except Ce, Fe, Pb, Mo and Nd in the dry season as well as Cd and Hg in the wet season [10]. The levels were lower in fish (*O. Bonariensis*) for Al, Cd, Ce, Fe, Pb, Mn, Mo and Nd in the dry season, and Cd, Ce, Pb, Mo and Nd in the wet season. It was observed that Ag, As, Cr, Cu, Ni and Zn bioaccumulated throughout the dry and wet seasons in plankton, shrimp and fish. The bioaccumulation was lower for Al, Ce, Fe, Mo and Nd in shrimp and fish. The accumulation could be due to dietary uptake as this is the most common mechanism for fish [46]. Plankton BAFs were largely higher than in shrimp and fish which could be to the ability for algae to uptake metals directly from water, however even dead algae accumulate metals [34]. BAF levels for As, Cd, Cr, Hg and Zn in biota in this study were higher than those reported by Cui et al., [37].

3.3.Transfer of elements in the food chain

The relationship between concentrations of the elements and $\delta^{15}N$ was investigated and demonstrated on a curve. A positive slope indicated metal accumulation in the food web, while a negative slope implies elimination from the food web or interrupted trophic transfer [10]. A trophic level–dependent accumulation of metals in the studied food web was also observed. *O. bonariensis* is omnivorous with fish under 16 cm long feeding on plankton and invertebrates, while those above 20 cm long feed on other fish [47, 48].

Positive slopes were observed for Zn, As and Hg during the dry season, and Hg in the wet season [10]. The high levels of Hg suggest biomagnification in the food chain/web [49–53]. The regression slope for log Hg versus $\delta^{15}N$ (0.36) was higher than values reported elsewhere [52, 54]. These levels can be attributed to natural and anthropogenic sources of aquatic environment contamination [55] and a toxicological concern [56]. Biomagnification of Cu and Zn was not expected since these are essential elements [57, 58]. Revenga et al., [59] reported biodilution of As in a food web in Moreno Lake unlike in this study in the dry season where the levels were high. Meanwhile, previous studies [42] in Mekong Delta and in Yellow River Estuary Delta [37] did not determined a significant relationship between As and $\delta^{15}N$ in their respective food webs. Accumulation and trophic transfer of As in food chains was not reported as expected because of prompt excretion [9].

Significant negative slopes for Pb, Ni, Cr, Al, Mn, Fe, Mo, Ce, Nd, Pt and Cd were observed, an indication of biodilution [10]. Related findings for Al, Fe, Ni, Pb, Cr, Mn and Cd have been reported in freshwater and marine species [60, 61]. Campbell et al., [53] reported biodilution of Al, Fe, Ni, Pb and Cd in a pelagic Arctic marine food web. Watanabe et al, [62] reported how biodilution is not depended on body size. Biodilution has been reported in higher trophic levels due to factors such as homeostatic regulation, increased metabolism and reduced absorption of highly hydrophobic compounds [63]. The different distributions/levels of the metals including Cd, Fe, Mn, Ni, Pb and Al as observed in this study [10] could also be attributed to their regulation by proteins such as metallothionein and metallothionein–like proteins in both vertebrates and invertebrates [64]. Also, Ni, Cd and Pb which exist in poorly absorbed forms in aquatic media and aquatic invertebrates are generally stored in muscle and bones and this influences dilution levels [53, 54].

3.4.Distribution of elements in *O. bonariensis*

There was a significant difference ($P < 0.05$) in the levels of fish tissue–element accumulation. Cooper Cu and Mo had the highest levels in fish liver in both seasons, while As, Fe, and Cr were high in the wet season and Ag in the dry season [10]. The concentration of As was higher in plankton in the wet season, while Fe and Cr were higher in shrimps as reported elsewhere [47, 48] in studies involving *P. argentinus* and *O. bonariensis*. Findings of this study showed that Al, Ni, Zn, Mn, Nd and Ce levels were highest in gills in the wet and dry seasons as demonstrated elsewhere in a study of samples from the Danube River [66].

Liver and gills contained the highest levels of the metals while brain had the lowest, except for Ni, Cu and Cr as observed elsewhere [67, 68]. Muscles had low levels [19]. The high levels in the liver could be attributed to a large population of metallothioneins [68, 69]. The liver is thus an excellent indicator of both water pollution and chronic exposure to heavy metals [19, 69, 70]. Gills are another indicator due to direct contact with metals in the water [67, 71]. Accumulation of high metals in muscle could be attributed to chronic exposures [72].

The high Cu levels in *O. bonariensis* liver in the San Roque Reservoir [10] confirm previous reports [25] involving *Scarus gibbus* where gills had higher levels of the metal [73]. The gills had the highest levels of Zn, Ni, As and Al as reported by others [67, 74] who reported high Zn levels in fish gills. Others reported high Ni levels in liver [19] and As in muscle [20]. As reported elsewhere [19] the current study found the highest Cr, Ag, Fe and Mo concentrations in liver also attributable to metallothioneins [68, 69]. Accumulation of Cr in gills and liver has been reported in fish from the South Platte River basin in the USA at concentration range of 5 µg/g–7.6 µg/g [75, 76]. Lower levels have also been reported in *S. glanis* [77, 78].

The levels of Hg were highest in fish muscle although Regine et al., [79] reported highest levels in the liver of *Myleus rubripinnis* and *Semaprochilodus vari*. Levels of the metal in water were very low (below the LOD) as reported elsewhere [80]. The differences could be attributed to the different levels of metallothionein, sulfur amino acids in liver of different species [81]. No quantifiable levels of Pt and Cd were noted [10]. The levels of Hg, Ni and Cd were as reported by Avigliano et al., [18] while As, Cr, Fe, Mn and Zn levels in muscle of *O. bonariensis* were higher than other metals in the same species collected from Argentinian lakes and lagoons [18].

4. CONCLUSIONS

This study reports higher levels of metals and metalloids, mainly Al, Cu, Cr, Fe, Ni and Zn in water compared to sediment. There was a significant difference in the levels of certain trace elements among the organisms studied. Mercury demonstrated biomagnification in the food web, while most elements were bioconcentrated from water to plankton and then biodiluted from plankton to shrimp and fish. The metals accumulated largely in liver and gills since these are the main storage tissues for most of the elements. High levels of Hg and As were seen in muscle. Further studies are required to evaluate the transfer mechanisms of metals in the food web, where both bioaccumulation and biodilution factors that influence toxic metals–metalloids and other toxic pollutants in the food web, are considered.

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SPATIAL AND TEMPORAL DISTRIBUTION OF ANTIMICROBIALS IN WATER AND SEDIMENTS FROM CAGING FISH FARMS

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Abstract

A new chromatographic mass spectrometric technique has been validated for analysis of 12 antimicrobials in sediment and surface water and applied to investigate spatial and temporal distribution of the drug residues in four fish farms in Brazil over four seasons. The limits of quantification were below 9 ng/l and 16 mg/kg in water and sediment, respectively with recoveries in the range 80% –119%. Oxytetracycline, florfenicol, tetracycline and chlortetracycline were some of the drugs detected with oxytetracycline found in samples over the season while florfenicol, tetracycline and chlortetracycline were in samples collected in some months such as January, April, July and October.

1. INTRODUCTION

The rapidly growing aquaculture sector [1] inevitable involves the use of antimicrobials for various purposes [2, 3]. Residues of the parent drugs or metabolites may end up in water and sediment [4]. Some of these drugs may be easily degraded while other persist in the environment with negative consequences on biota [5] such as contributing to emergence of antimicrobial resistance [6] which in turn affects humans [2–7]. Up to 70%–80% of the antimicrobials in aquaculture end up in the surrounding environment [8]. Many are not completely absorbed by fish [9–11] and readily detected [12–14]. While there are efforts to reduce use of the drugs in aquaculture production [15] their use is still unavoidable because of diseases and stressful conditions requiring application of the drugs.

Reliable and sensitive techniques are required to control these chemicals although challenges such as complex matrices and ultra–low level targets are encountered [16]. One of these techniques is the liquid chromatography coupled to mass spectrometry (LC–MS/MS), where sample preparation may include offline solid phase extraction (SPE) albeit with some limitations [17–24]. An alternative is the use of online SPE–LC–MS/MS, with advantage such as smaller sample size, reduced sample preparation time and contamination [16, 25–30]. A study was undertaken in Brazil to develop and validate a sensitive and automated method using an online SPE–LC–MS/MS to determine 12 antimicrobials in surface water and sediment and to investigate spatial and temporal distribution [31].

2. MATERIALS AND METHODS

2.1. Chemicals, reagents, and equipment

The following were used: Methanol and acetonitrile (HPLC grade, Tedia Company Inc., Fairfield, OH, USA), Formic acid (99.5%, JT Baker, Phillipsburg, USA), Na_2EDTA (Sigma Aldrich, Dorset, UK), citric monohydrate acid, orthophosphoric acid, and sodium citrate dihydrate (analytical grade, JT Baker, PA, USA); ultrapure water (Milli Q system, Bedford, MA, USA); antimicrobials selected according to the Brazilian national residue monitoring programme for fish [32]. These included oxytetracycline (97%), tetracycline (97.5%), chlortetracycline (93%), ciprofloxacin (99.5%), enrofloxacin (99.0%), sarafloxacin (97.2%),

norfloxacin (99%), sulfathiazole (98.0%), sulfadimethoxine–d6 (99.4%), and florfenicol (98.0%) all from Sigma Aldrich (St. Louis, MO, USA); and sulfadimethoxine (99.5%) and sulfamethazine (99.5%) from ChemService (West Chester, PA, USA), and chloramphenicol (98.5%) from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

2.2. Procedure

The fluoroquinolones (10 mg) were dissolved in 0.5 ml NaOH 1 M, and 9.5 ml acetonitrile and the rest (10 mg) in acetonitrile only. Working standards of 1 mg/l prepared by dissolving in Milli Q water and river water collected upstream from the fish farms.

The online SPE–LC–MS/MS procedure involved an Agilent system equipped with a 1260 VL Infinity quaternary loading pump, a 1200 Series binary analytical pump, and other components, as well as 6430 tandem mass spectrometry (Agilent Technologies; Wilmington, DE, USA). Others were semipreparative Agilent Zorbax SB C8–80 (9.4 × 15 mm × 7 μm) column and Agilent Zorbax Eclipse Plus C18 (100 mm × 3 mm, 3.5 μm) column maintained at 30°C. The sample (900 ml) was loaded onto the SPE column and eluted with methanol (solvent C): water at pH 4 (solvent D) 5:95 (v/v) at a flow rate of 1 ml/min for 2 min; followed by 20:80 (v/v) of the solvent for 4 min. The mobile phase consisting of solvent A (acetonitrile + 0.1% formic acid) and B (water + 0.1% formic acid) was passed through the analytical column at the ratio 20:80 (v/v, A/B) at the flow rate of 0.4 ml/min for 5 min. The analyte was injected at the 4th min; the ratio was changed to 70:30 (v/v), A/B) from 5 min–8 min before the analyte was loaded after 10 min and the semipreparative column washed with 90:10 (v/v) of solvents C/D for 3 min [31].

The mass spectrometer was operated in electrospray ionization (ESI) with FF in the negative mode and most analytes in the positive mode. The rest of the conditions included gas temperature: 325°C; gas flow rate: 12 l/min; nebulizer/nitrogen gas pressure: 50 psi; and capillary voltage: 4000 V; collision–induced dissociation (CID) with N_2 (2 Torr). Additional parameters are summarized in Table 1.

TABLE 1: SPECTROMETRIC CONDITIONS

Drug	Retention time (min)	Precursor Ion	Product Ion	Cone voltage (V)	Collision Energy (eV)
CTC	11.26	479.1	462.2	125	12
		479.1	444.1	125	17
OTC	8.47	461.2	426.0	115	16
		461.2	201.1	115	41
TC	8.92	445.2	410.2	115	17
		445.2	154.2	115	30
SDM	12.36	311.1	156.0	120	16
		311.1	108.0	120	28
SMZ	9.86	279.1	186.0	115	12
		279.1	156.0	115	16
STZ	8.85	256.0	156.0	90	8
		256.0	108.0	90	20
CFX	8.46	332.1	288.1	125	13
		332.1	245.1	125	22
EFX	8.92	360.2	342.2	132	17
		360.2	316.2	132	16

Drug	Retention time (min)	Precursor Ion	Product Ion	Cone voltage (V)	Collision Energy (eV)
NFX	8.27	320.1	302.1	125	20
		320.1	231.0	125	44
SAR	10.04	386.1	342.1	119	15
		386.1	299.1	119	26
CAP	11.95	323.0	305.0	70	0
		323.0	275.0	70	8
FF	11.60	355.9	335.9	139	5
		355.9	185.1	139	13
SDM–d6	12.34	317.1	162.2	65	20
		317.1	108.1	65	28

2.3. Samples and sample manipulation

Water and sediment samples (n=3) were collected from locations 0 m, 100 m, and 1000 m downstream of fish farms at the Ilha Solteira hydroelectric dam reservoir. Water samples (100 ml) were collected 20 cm from the surface and stored in amber bottles with 0.1 g of Na_2EDTA to avoid photodegradation and control chelation [33]. Samples were filtered through a 0.45 µm Teflon membrane and stored at 3°C for 24 h before analysis. SDM–d6 (100 ng/ml) was added to the water, pH adjusted to 4, the material filtered through a 0.22 µm Teflon membrane and 900 µl used for online–SPE–LC–MS/MS study. The sediment samples (300 g) were also collected in clean amber coloured bottles, heated to 280°C, silanized, and 1 g of sodium azide added. The mixture was freeze dried and later sieved through a 60 mm mesh sieve.

Analytes were extracted from the sediments as recommended elsewhere [34] by weighing 2 (0.1) g into 25 ml Teflon bottles, spiking with SDM–d6 (100 mg/kg) 10 ml each of acetonitrile citric buffer (pH 3.0), vortexing for 1 min, ultrasonication for 15 min and spinning at 1350 g for 10 min on a centrifuge set at 5°C. The cumulative supernatants (following repeated extraction) were concentrated (R215 Buchi Rotavapor, Flawil, Switzerland at 40°C) and dissolved in acetonitrile and made up to10 ml with Milli Q water before purification by online SPE (Strata SAX cartridge, 500 mg/6 ml). The material was conditioned with methanol and water (10 ml each), drained after 30 min; 6 ml of the aqueous extract added. The material was filtered through 0.22 µm Teflon filter and 900 µl injected for the analysis. Surface water and sediments upstream of the fish farms were used for method development and validation [31].

2.4. Method validation

Water and sediment samples were collected upstream and evaluated for levels of the drugs at less than 30% of the limit of quantification. Validation was then performed using the following parameters: selectivity using blank samples and verifying absence of peaks and spectra of interest; linearity study, where matrix matched calibration curves were used involving all 12 antimicrobials in the range 10 ng/l–2000 ng/ for water and 10 mg/kg–500 mg/kg for sediment. Others were the limit of detection (LOD) and that of quantification (LOQ). Recoveries were determined at 100 ng/l to 200 ng/l (water) and 20 mg/kg, 50 mg/kg, and 200 mg/kg in sediment. Precision was also investigated, and the results analysed using the ASSISTAT program [35].

3. RESULTS AND DISCUSSION

An online SPE–LC–MS/MS was developed and validated and is useful for trace residue detection with low solvent consumption that should help reduce analytical costs [14, 16, 25–30]. For sediments, better results were seen when online SPE and offline SPE with HLB cartridges were used [31].

Seven columns were investigated and the Zorbax 80 SB C8 a precolumn in semipreparative systems yielded better results by retaining the antimicrobials for a longer period and improving sample cleaning. This is supported by previous studies [9–15, 17–20] that reported successful use of Agilent Zorbax Eclipse Plus C18 column for chromatographic separation. The pH of 4.0 was suitable for most of the drugs, namely: FF, OTC, TC, SDM, STZ, SMZ, EFX, CFX, NFX, and SAR with recoveries exceeding 70%, while CTC and CAP performed better at pH 7.0 [31].

3.1.Method validation and quality control

The method was selective as blank matrix spiked with the antimicrobials did not show interferences greater than 30% of the LOQ at their retention times. Good linearity was observed with regression coefficients above 0.99. The LODs ranged from 0.1 ng/l for FF to 2.6 ng/l for CAP while the LOQs ranged from 0.5 ng/l to 8.8. ng/l in the case of water. For sediments, the corresponding values were 0.4 µg/kg to 5.1 µg/kg (LODs) and 1.3 µg/kg to 16 µg/kg. The recoveries for water were in the range 70%–106% with RSD% 11%, and 89%–119% with the RSD of 7%. The method was also reproducible with RSD of 3%–20% for water and 2%–15% for sediment.

All farms that provided samples showed a certain level of residues. For example, fish farms 1, 2, and 4 had OTC levels in the range of 14 ng/l–993 ng/l; farms 1, 3, and 4 had FF (10 ng/l–425 ng/l. CT was found in farm 1 at 10 ng/l. The highest level of OTC was found in samples collected in July and October which could be attributed to a period of reduced rainfall and possibly concentration of the residues. Detection of TC in the same farm as OTC could be because they are related have the same metabolic pathway [36]. The OTC and FF levels in the present study were below those observed elsewhere [37]. The OTC levels were also lower than reported in a chronic toxicity study for *Danio rerio* [38]. The levels reported in the current study [31] are nevertheless worth noting based on a previous report [39] on environmental toxicity — including DNA damage — associated with these drugs. The levels of the antimicrobial residues such as OTC and FF decreased with the distance from the cages and this could be due various factors such as metabolism, dilution or sorption [40].

The level and pattern of OTC in water were like the sediments' although in some month OTC was not detected in water but in sediment. This is logical possibly due to sorption and partition factors. There was an outlier for samples obtained from farm 2 in October, as the levels where higher the further it was from the cage/farm. This could be due to contamination from the surrounding, rather than from the water body. Detection of residues over the year suggests continued use of the antimicrobials in farming. Tetracycline was found nearer to the cages, especially farm 1 and CTC was also detected in farm 4. This does correlate with the OTC levels. Chlortetracycline may be formed in the environment due to variations in pH, temperature, and exposure to light [33].

The findings in the current study [31] corroborate work done elsewhere [42] on Reda River in Poland where low levels (up to 41 ng/l) of EFX, CFX and NFX were detected in water samples

unlike in a separate study [13] where CFX or NFX were not detected in water, possibly due to sorption and binding in sediment [43]. Although the current study reported FF in water, the absence of the drug in sediment is not surprising as low frequency of the drug has been reported, for instance in a study conducted on the Elorn River, France [44]

4. CONCLUSIONS

An analytical method based on an online SPE–LC–MS/MS system has been established and is useful for analysis of 12 antimicrobials in water and sediment. Several antimicrobials were detected in water and sediment and showed seasonal distribution. Fish farming is demonstrated as the source of antimicrobial contamination in a reservoir in Brazil. The method facilitates environmental monitoring for contamination with antimicrobials.

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ASSESSMENT OF BETA–LACTAMS, SULFONAMIDES AND MACROLIDES IN FISHPONDS OF TWO AQUACULTURE FARMS IN CAMEROON

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Abstract

Small scale farmers in Cameroon practice aquaculture in earthen fishponds and inevitably use veterinary drugs to control disease among other purposes. Thus, there is a need to assess ponds (water and mud) for the presence of antimicrobial residues that could be dangerous for both fish and consumers. The results of this pilot study showed absence of macrolides residue in water and mud from the farming ponds of different fish species. Beta–lactams and/or sulfonamides were found water and liquid waste. Radio receptor assay (Charm II test) screening procedures were used [1]. The results demonstrate the need for regular monitoring of antimicrobial residues in fish as well as the immediate production environment.

1. INTRODUCTION

Aquaculture production is developing fast in Cameroon as a strategy to reduce fish importation and satisfy the increasing demand of the growing population [2, 3]. Veterinary drugs have been widely used in fish farming for therapeutic or disease preventive reasons and to improve yield etc [4, 5]. Recently, a cross sectional survey revealed that small scale farmers in Cameroon reared fish in earthen ponds and all of them used antimicrobials for different purposes [6]. The misuse of these antimicrobials could lead to the presence of residues in fish as well as in the environment (pond) where production is conducted. Potential effects include allergic reactions and antimicrobial resistance [4, 7]. The presence of antimicrobials in fish could result from water and mud pond. The present pilot study aimed to use Charm II tests to assess residues of some commonly used drugs (beta–lactams, sulfonamides and macrolides) in fishponds especially water and mud.

2. MATERIALS AND METHODS

2.1.Study areas and fish farming ponds

"Mfou"/Central and "Batié"/West of Cameroon was included as the study area. In Mfou, kanga and tilapia were farmed in the same pond while the catfish was farmed alone in another pond. Such separations are aimed at controlling reproduction and increasing yield [8]. In Batié, only carp was farmed in different ponds.

2.2. Detection of beta–lactams, sulfonamides and macrolides in water and mud ponds

No sample preparation was required for water while mud samples were reconstituted with deionized water and allowed to settle. The determination of beta–lactams, sulfonamides and macrolides in water and mud was adapted from the Charm II protocols for liquid waste and water [1]. In each case, a field sample or control sample (4 ml) was added to the test tube followed by the addition of 500 µl MSU extraction buffer (Charm Science Inc). A volume of

500 µl of Tissue Performance Negative Concentrate from Charm Sciences test kit was then added. Different tablets containing the binding reagent of each antimicrobial studied were then introduced in the test tubes and agitated on a mixer for 10s. The tablets were green (for beta-lactams, PMSU 050E), white (for sulfonamides, SMMSU 022H and macrolides, EMSU 024A). For beta–lactams and macrolides, the mixture was immediately incubated at 55°C for 2 min followed by addition of the tracer embedded in a yellow tablet for beta–lactams or green tablet for macrolides. For sulfonamides the pink tracer tablet was added and mixed before incubation at 65°C (3 min). Centrifugation was performed at 3300 rpm for 3 min. For beta–lactams and macrolides, an additional incubation step (55°C, 2 min) was required before centrifugation. After centrifugation, the supernatant was poured off completely and the pellet was broken up and dissolved in 300 µl of deionized water; 3 ml of scintillation fluid was added before mixing on vortex until mixture has uniformly cloudy in appearance. The mixture was counted for one minute in a scintillation counter (Charm II 7600 Analyzer) on the ^{14}C channel (for sulfa drugs) or on the ^{3}H channel (for macrolides and beta–lactams). The control point for water and mud was determined by subtracting a value of 30% from 6 negative controls (tap water) cpm average results. The results (cpm) for the sample were compared with the control point.

3. RESULTS AND DISCUSSION

Beta–lactams, sulfonamides and macrolides were detected in water and mud according to an established protocol for antimicrobial drugs in liquid waste and water [1]. The control points calculated were 1089, 1010 and 1443 for beta–lactams, sulfonamides and macrolides, respectively. The control point is the cutoff mark between a negative and a positive result. Test results greater than the control point indicate a negative sample, while results less than or equal to the control point suggest that the sample is presumptive positive. The average counts/values obtained from a triplicate of assays for the different samples and ponds are presented in Fig. 1. As shown in Table 1, no sample tested positive for macrolides while sulfonamides and beta–lactams were detected in water and mud from the pond containing carp and catfish. The pond used for tilapia and kanga contained beta–lactams while the mud contained sulfonamides at non–permissible levels. The results obtained suggest that water and mud can be a source of trace antimicrobials in fish potentially contributing to the development of drug resistance among other effects among consumers [7].

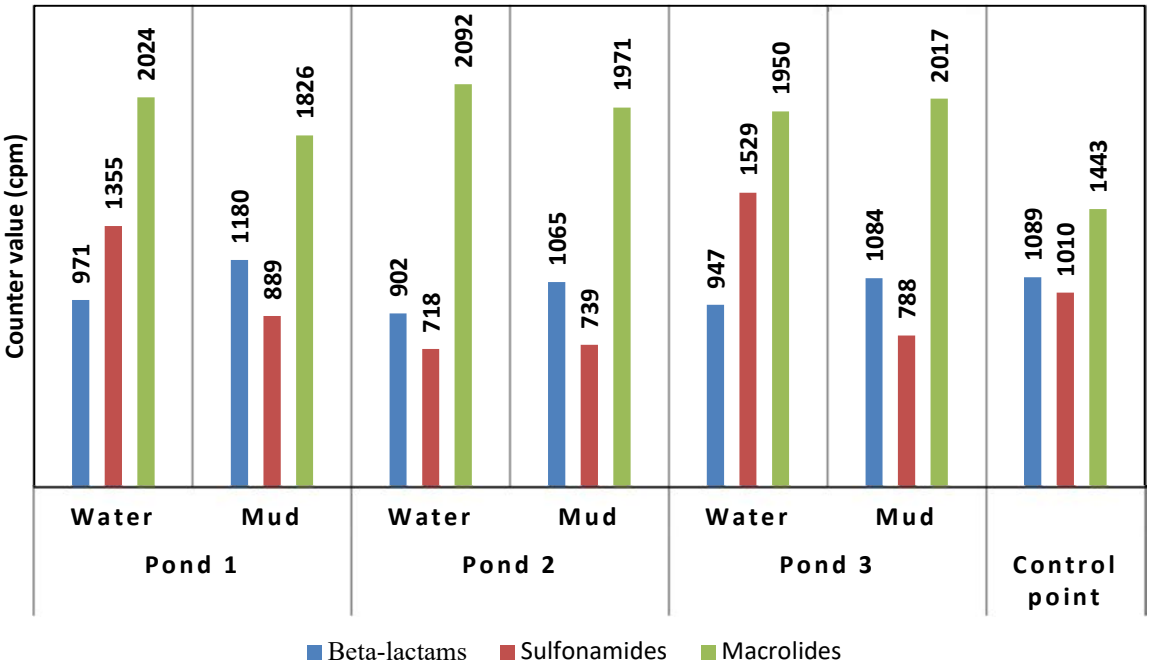

FIG. 1. *Detection of Beta–lactams, Sulfonamides and Macrolides in water and sediment ponds samples from a fish farm in Mfou, Cameroon, using Charm II radioreceptor screening test.*

Pond 1, Kanga and Tilapia; Pond 2, Carp; Pond 3, Catfish; Cpm=count per minute; cp=control point; Positive samples (cpm≤cp)

TABLE 1. ANTIMICROBIAL RESIDUES DETECTED IN WATER AND MUD FROM THREE PONDS

		Antimicrobial detected	
Pond	Fish species	Water	Mud
1	Tilapia, Kanga	β–lactams	sulfonamides
2	Carp	β–lactams, sulfonamides	β–lactams, sulfonamides
3	Catfish	β–lactams, sulfonamides	β–lactams, sulfonamides

4. CONCLUSIONS

This pilot study showed that water as well as mud in ponds where fish is farmed contains beta–lactams and sulfonamides at concentrations that could be harmful to fish and consumers. Further work is needed to conduct quantitative and confirmatory tests for the antimicrobial drugs in liquid waste and water and validate relevant screening methods. The studies should

also perform more robust correlation studies among the different matrices and determine seasonal patterns as well.

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AFLATOXINS IN FARMED FISH AND ENVIRONMENTAL SAMPLES

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Abstract

A study was undertaken to assess presence of aflatoxins in fish farmed in two Cameroonian localities and the potential source of the contaminants through analysis of feeds, water and mud. Samples of kanga, tilapia, catfish, and carp fish as well as water and mud (n=34) were collected from Mfou and Batié farming sites and analyzed by competitive ELISA. Fish contained total aflatoxins and aflatoxin B1 at levels higher than 20 parts per billion for catfish. Fish feed and mud also contained the mycotoxins.

1. INTRODUCTION

The demand for animal protein especially fish is on the rise globally due to high nutritional benefits [1]. In Cameroon fish represents 40% of the animal protein [2]. National efforts are in place to address production gaps including alternative fish farming [3] and as such almost 400000 tons of fish is expected to be farmed locally each year in Cameroon [4]. Increased production to address these needs requires inputs such as veterinary drugs, fertilizers, pesticides that could be misused [5]. Poor handling and storage of inputs such as cotton seeds, groundnut flour, maize, fish flour, that may be used in production can contribute to elaboration of mycotoxins [5–7].

Mycotoxins, secondary metabolites of fungi such as Aspergillus, Fusarium, and Penicillium [8] with diverse chemical structures [8, 9] can be harmful to consumers and animals [10, 11] affecting a range of tissues and systems [12, 13]. Aflatoxins known to be carcinogenic and mutagenic [14] are some of these mycotoxins of concern. Various levels occur in feed with some as high as 806.9 μg/kg as reported in Kenya, Tanzania, Rwanda, and Uganda by Marijani et al., [8]. The presence of mycotoxins in fish feed in sub–Saharan Africa have been reported [16, 17] and these could accumulate in fish tissue for human consumption [10, 18]. This pattern of distribution of the mycotoxins has never been reported in Cameroon hence the study that would include tissue, water and mud [19].

2. MATERIALS AND METHODS

2.1.Study areas, farming, sampling and sample processing

This study included Mfou in central Cameroon and "Batié [19]. Both locations have favourable climates for fish farming. Each site had more than four farming ponds (~ 600m^2 on average) with kanga and tilapia farmed together at Mfou and catfish. Only carp was farmed at Batie. This farming strategy was to help control reproduction and increasing yield [20].

Tilapia, African catfish, and kanga were collected from Mfou and common carp (*Cyprinus carpio*) from Batié. A standard sampling procedure established by the European Commission [21] was used with six fish (~500 g each) aged between 8 and 12 months collected and shipped in cold conditions to the laboratory. Fish feed (1 kg), pond water (1 L), and pond mud (1 kg) were also included. Fish were then descaled, eviscerated, and washed with tap water before

slicing them into fillets that were minced (Black & Decker®, England), homogenized and divided into several aliquots of 10 g each and stored at -20°C [19].

2.2.Determination of total aflatoxin (AFs) and aflatoxin B1 (AFB1)

The levels of AFs and AFB1 were assessed by quantitative ELISA (MaxSignal®, BIOO Scientific Corp, USA). Fish samples (2 g) were mixed with 8 ml of 87.5% methanol (HPLC grade, Sigma, Germany) and vortexed for 10 min (Vortex Genius 3, IKA, Germany) before centrifugation for 10 min at 4000 g for 10 min (Centrifuge Rotofix 32 A, Germany) and the supernatant used for analysis. Feed samples (5 g) were mixed with 25 ml of 70% methanol (HPLC grade, Sigma, Germany) mixed on a vortex for 10 min and centrifuged (4000 g, 10 min) and the supernatant used for analysis. Other samples (e.g., mud) were placed in tubes containing 1 ml of methanol/extraction buffer 1X: 6:4, v/v), vortexed for 1 min and centrifuged (4000 g, 5 min) [19].

2.3.Competitive ELISA.

Plates loaded with the supernatants were read at 450 nm (EL × 800, BIOTEK, Instruments Inc., Winooski, VT, USA). A good liner regression ($r^2 > 0.98$) was attained for AFs and AFB1 standards at 0 mg/kg, 0.05 mg/kg, 0.25 mg/kg, 0.75 mg/kg, 2.5 mg/kg, and 10 mg/kg. The reference limits of detection in the manufactured kit of 0.01 ppb for water and mud as well as 1 ppb for fish and fed were used. Statistical analysis involved the analysis of variance (ANOVA) and Duncan multiple range test Stat graphics Centurion XV version 16.1.18 (Stat-Point Technologies, Inc., USA) [19].

3. RESULTS AND DISCUSSION

3.1.Total aflatoxins and aflatoxins B1 in samples

As detailed elsewhere [19] both AFs and AFB1 varied significantly ($p < 0:05$) among the fish species with AFB1 levels in catfish in the range of 1.81 µg/kg to 15.69 µg/kg and 3.62 µg/kg to 31.38 µg/kg in the case of AFs. Kanga had the lowest levels (0.21 µg/kg for AFs 0.10 µg/kg for AFB1). Mud samples also contained AFs and AFB1 at varying levels ($p < 0:05$). Levels for the carp–pond mud were ~32 µg/kg and 16 µg/kg for AF and AFB1, respectively. The catfish pond had lower levels of 1.03 µg/kg AFB1 and 2.06 µg/kg AFs. Generally, the mud collected from Batié had higher toxin levels than Mfou's although there was no correlation between the presence of AFs and AFB1 in mud and in fish tissue. AFs and AFB1 were found in all water samples below 0.1 µg/kg with the highest level at 0.09 µg/kg. Fish from kanga and tilapia farms had more toxins than water and mud. Feed contained 1.82 µg/kg of AFB1 and 3.64 µg/kg of AFs but these levels were lower than in fish.

Aflatoxins, secondary metabolites of *Aspergillus flavus*, *A. parasiticus*, *A. nomius*, *A. arachidicola*, *Emericella astellata*, *E. venezuelensis,* and *E. olivicola* [22], accumulate in edible tissue and are therefore a human health risk [8, 10, 18, 23]. These toxins have been reported in fish [24–26] up to 40 µg/kg in catfish and 15.11 µg/kg in tilapia [25].

While aflatoxins are a consumer concern, these also affect fish health, productivity and development [27, 28]. Reduction of proteins in tilapia exposed to aflatoxins has been reported [29]. Carcinogenic effects of AFB1 in different fish species has also been reported [10]. The negative effective on fish productivity has economic implications [30, 31] besides consumer concern where the accepted levels of 20 µg/kg are exceeded [32]. Findings in this study [31] show that feeds were the main source of the aflatoxins although the levels found were below the 20 µg/kg limits set elsewhere [33]. The contamination of feed by mycotoxins has been attributed to poor storage practices [31, 34]. An improvement in storage practices and facilities can reduce contamination and consumer/fish exposure to the toxins.

4. CONCLUSIONS

Farmed fish from selected Cameroonian localities contain total aflatoxins and aflatoxin B1 with catfish containing high levels than tilapia. Some samples, mainly catfish showed toxin levels above 20 µg/kg. Different fish accumulated different levels of the toxins even in the same pond. Animal feed was the main source of the toxins while mud had very low levels. Testing and control of feed for fish production to reduce elaboration of the toxins in fish is required.

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ANTIMICROBIAL PROFILES IN WILD AND FARMED CHILEAN SALMONIDS

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Abstract

The occurrence of 46 antimicrobial residues in farmed salmon and wild trout from Chile has been investigated. Higher levels of the residues were observed in wild fish with almost the same residue types found in the cold and warm seasons. All farmed salmon samples contained five sulfonamides, one quinolone, one tetracycline and three macrolides. Levels were generally below the EU regulatory limits except for sulfaquinoxaline. Nitrofurantoin, a banned substance, was detected in one sample. The potential source of the different residues in farmed and wild fish requires thorough investigation.

1. INTRODUCTION

The global use of antimicrobials has increased by 30% in the last decade largely in low– and middle–income countries [1]. The drugs are some of the most widely used chemicals in animals, humans and plants [2]. Improper use, however, has potential human and environmental health risks [3] including the threat of antimicrobial resistance [4]. The antimicrobials are categorized into four groups: A (Avoid), B (Restrict), C (Caution) and D (Prudence) with risks decreasing from A to D [5]. Category A are those not authorized in veterinary medicine rather restricted human medicine. Category B is of importance in human medicine and their use in animals could contribute to resistance in humans. Antimicrobials in category C should be considered for use in the absence of effective category D drugs, generally the first option [5].

Parent pharmaceuticals and the metabolites may be excreted in urine and faeces [6]. Conventional wastewater treatment plants are not designed to remove such antimicrobials when discharged into the environment [7]. This can be a challenge to many countries especially the less developed countries [8–11]. The presence of antimicrobials in environmental matrices is on the rise [12] with fish being sensitive indicators of water contamination [13–15]. The antimicrobials can be attributed to both deliberate application and inadvertent discharge [16]. One of the potential avenues for discharge is aquaculture, a fast–growing industry with salmon one of the most dynamic production subsectors [17]. Chile is the second largest producer of salmonids in the world [18] and this requires use of antimicrobials. Use reached 334100 kg in 989.546 tons of salmon in 2019 [19].

This study aimed [20]: (i) to screen antimicrobials in farmed salmon and wild trout; (ii) to identify patterns of the antimicrobials and potential sources; and (iii) to assess temporal trends of the antimicrobials.

2. MATERIALS AND METHODS

2.1.Chemicals/reagents

The following were used: Antimicrobial standards (>90% purity; Sigma Aldrich (St. Louis, Missouri, USA) and azithromycin–d3, enrofloxacin–d5, erythromycin–^{13}C, trimethoprim–d3, flumequine–$^{13}C_3$, sulfamethazine–d4 all from Santa Cruz Biotechnology (Dallas, Texas, USA); HPLC grade methanol and acetonitrile (Baker, Philipsburg, NJ, USA); formic acid (98%–100 %, Merck, Darmstadt, Germany); and ultrapure water (from Milli Q water purification system, Sigma, Bedford, USA) [20].

2.2.Samples

Chilean farmed salmon (*Salmo salar,* juvenile specimens; n=5/6) were collected weekly between December 2018 and May 2019 from an inland fish farm located between two hills without identifiable sources of pollution. Wild trout (*Oncorhynchus mykiss,* n= 5/6) were collected from local recreational fishermen around Licanray and the Villarrica National Park (Araucanean Region, Chile) supplied by some streams flowing through a small agricultural area whose population also increases in summer due to tourists. Fish muscle samples (24 pools corresponding to 130 wild and 136 farmed fish), were collected, freeze–dried and kept at -80°C until analysis [20].

2.3.Determination of antimicrobial residues in salmonids

Forty–six antimicrobials including fluoroquinolones, tetracyclines, penicillins, macrolides, quinolones, amphenicols, nitrofurans, sulfonamides, dihydrofolate reductase inhibitor, and cephalosporins were analyzed [20] with sample preparation as reported elsewhere [21]. The tissue (0.5 g) spiked with isotope labelled standards, was extracted using 5 ml of acetonitrile/water (3:1) followed by cleanup with solid phase extraction (Strata X™, 500 mg, 6 ml) and the extracts evaporated to dryness before reconstitution in 1 ml of acetonitrile:aqueous 0.1% formic acid (10:90, v/v) and analysis on a Waters Acquity UPLC, Xevo TQS micro, ESI after separation using a Zorbax Eclipse Plus C18 column (3.0 × 50 mm; 1.8 µm, Agilent, USA). Acetonitrile (solvent A) and aqueous 0.1% formic acid (solvent B) were used as mobile phase based on the following gradient elution programme: 10% A; linearly to 100% A from the start to 8.0 min; 100% A for 1 min; back to initial conditions at 9.5 min, and equilibration at 8.5 min before another injection. The most abundant ion transition was used for quantification purposes and the second one to confirm the identity of the analytes. An acceptable retention time difference of ± 2.5% between standard and analyte was used to support confirmation. A set of samples were spiked at 50 µg/kg and 200 µg/kg of the analytes and recoveries in the range 26% to 94%, with detection limits from 0.004 µg/kg to 5.071 µg/kg [20].

2.4.Risk assessment for fish consumption

Consuming food containing residues of antimicrobials can be a health risk [22] whose exposure can be evaluated by calculating the Estimated Daily Intake (EDI) according to Eq. (1)

$$EDI = (C\ x\ IR) \div BW \ldots\ldots\ldots (1)$$

where C (µg/g) is the maximum antimicrobial concentration in the fish sample (wet weight), assuming a "worst case scenario"; IR = daily consumption of fish (150 g/day for adults and 75 g/day for children); BW = bodyweight (70 kg for adults and 20 kg for seven–year–olds). This

is according to a set procedure [23] with the Acceptable daily intake (ADI) as reported in China [24].

2.5.Data analysis

In order to assess residue variations in wild trout or farmed salmon between seasons, data from collected from December to February was characterized as warm season and from March to May as cold season. Shapiro Wilks and Levene tests were used to assess normality and homogeneity of variances, respectively. Residue concentrations in the two seasons and sources, (wild or farmed fish) were compared using the Kruskal Wallis test [25] and Dunn's multiple comparison test. Associations between residue levels and seasons/sources were determined using principal coordinate analysis (PCoA), principal component analysis (PCA) and Linear discriminant analysis (LDA, stepwise mode) of standardized values. The InfoStat [26] and Statistica 8.0 software [27] were used.

3. RESULTS AND DISCUSSION

As elaborated elsewhere [20], 32 of the 46 antimicrobial residues were detected in the wild trout and farmed salmon fish. The following were not detected: enrofloxacin, marbofloxacin, tylosin, chloramphenicol, florfenicol, furazolidone, sulfacetamide, sulfadiazine, sulfadoxine, sulfamerazine, sulfamethoxypyridazine, sulfanitran, sulfathiazole and sulfisoxazole. At least one (14 maximum) residue was found in each of the two matrices. Six of the residues were from category B/"Restrict" (fluoroquinolones and quinolones), one (macrolide) from category C/"Caution" and 7 from tetracyclines, sulfonamides and penicillins under category D/ "Prudence". There was no significant difference between the number of residue–types in the two fish. Overall, the residues were more frequent in the wild (18.5%) than in farmed fish (14.0%), mainly in the warm than in cold season. Levels were in the range of ~19 µg/kg to 113 µg/kg for wild fish and ~1.2 µg/kg and 48 µg/kg (cold wild), ~ 4 µg/kg to 21 µg/kg (farmed fish). The residues in wild fish could be attributed to discharges of industrial, livestock, hospital wastewater and domestic wastewater sources [28, 29] which can vary by season [30] with low levels reported in summer [31, 32].

For wild fish samples, norfloxacin, ciprofloxacin, clarithromycin, enrofloxacin and oxolinic acid concentrations showed significant differences between sources and seasons ($p<0.05$). These drugs are used for human and veterinary use [33] and could arise from wastewater, an important source of several pharmaceuticals, and these can bioaccumulate [34–36]. For Chilean salmon aquaculture, amoxicillin, doxycycline, florfenicol, oxytetracycline, flumequine, oxolinic acid and erythromycin are approved for use [37]. Their presence of these residues is therefore not a big surprise.

The study detected norfloxacin in wild fish samples and mainly in the warm season [20]. Levels of the same drug as high as 4.7 µg/kg have been detected in 18 fish species from the South China Sea [38]. Ciprofloxacin was most frequent in wild fish — compared to farmed salmon — at concentrations as high as ~48 µg/kg although lower levels have been reported elsewhere [22]. Clarithromycin was frequently detected in all culture samples as reported in aquaculture fish in Argentina [21]. Enrofloxacin levels in wild and farmed fish were comparable to other elsewhere [6, 21].

Enrofloxacin in Chile is only used in agriculture/aquaculture [39, 40], while oxolinic acid was widely used in aquaculture until 2010 when it was banned in Chile [36, 41]. Detection of oxolinic acid suggests limited degradation in the environment, and perhaps a long half–life [42]

assuming that there were no cases of violation. The five sulfonamides, erythromycin, roxithromycin, ofloxacin and tetracycline detected were found in a single pooled farmed fish sample. Ofloxacin, tetracycline and sulfonamides are authorized for use in cattle and poultry in Chile [43, 44]. Meanwhile, roxithromycin is widely used in human medicine [45]. Two other antimicrobials cephalexin (category C) and azithromycin, used in human medicine, were detected in farmed fish samples. Their presence could therefore be attributed to environmental contamination.

Oxytetracycline which along with florfenicol are the main antimicrobials used in salmon farming in Chile [33] was commonly detected with the highest concentration was in the cold season. The drug is used in treating systemic bacterial infections [46] while florfenicol is used to treat *Piscirickettsia salmonis* [37] although it was not detected in any pool sample. The detection of residues of pharmaceuticals used in human medicine or cattle in farmed fish is an indication of unintentional discharge [12].

Maximum residue levels (MRLs) of residues in animal products help ensure good production practices which in turn protects consumers [47]. Two pooled wild fish samples in the warm season had residue limits above EU MRLs [48]. Meanwhile one sample contained sulfaquinoxaline — a drug commonly used in Chile [44] — at a concentration of 112.51 µg/kg which is above the 100 µg/kg MRL. Residues of the drug have also been detected in Argentinian commercial shad and pacú samples [21]. Nitrofurantoin was detected in a pool sample although it is banned for animal use in several countries including Chile [49]. The Chilean Salmon Antibiotic Reduction Program (CSARP) has been established to help reduce the consumption and ecological impact of antimicrobial use in the Chilean salmon industry and attain 50% reduction in use by 2025 [50]. This study [20], however, demonstrates that the use of the drugs in food production is not the only source of residues and there is a need to pay attention to urban/anthropic origins of antimicrobials. Although none of the findings were outside acceptable daily intake [51], it is important to note that consumers often prefer wild fish over farmed fish and therefore exposure of wild fish to environmental contamination remains a concern [20].

4. CONCLUSIONS

Seasonal occurrence and variation of antimicrobial residues used in humans and veterinary production has been reported in Chilean wild trout and farmed salmon. For the first time, a comparison was made about antimicrobial levels in wild and farmed salmonids. Different sources of the residues were noted. Salmonids (especially wild) contained a range of residues including forbidden substances. The highest frequency and concentrations of the residues were in wild fish and environmental contamination is regarded as a major concern for South America. Nevertheless, most antimicrobial residues were below the EU MRL.

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PHYSICOCHEMICAL PROPERTIES OF POLYCYCLIC AROMATIC HYDROCARBONS (PAHS) AND DISTRIBUTION IN LEBANESE AQUATIC SYSTEMS

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Abstract

Seventeen polycyclic aromatic hydrocarbons (PAHs) were investigated in marine and continental Lebanese aquatic systems. The results showed that Lebanese seawater is more contaminated by PAHs compared to other sites on the Mediterranean Sea that were studied. On the marine side, the total concentration of PAHs ranged from 55.7 ng/l to 2683.8 ng/l in water and from 19.09 ng/l to 2025.03 ng/l in sediments. On the continental side, the total concentration ranged from 465.7 ng/l to 1399.9 ng/l in water and from 72.6 ng/l to 1074.7 ng/l in sediments. As statistical test was performed to determine the affinity of PAHs for the phases and this showed that when the number of structural rings in the molecule increases, the molecular mass and the log Ko/w increase, the PAHs accumulate in sediments. When the water solubility and the vapor pressure of the PAHs increase the compounds tend to remain in the aqueous phase. The PAHs in the sediments were thought to originate from combustion, industrial sites next to aquatic systems, and heavy traffic especially along the Lebanese coastline. The Effects Range Medium/Effects Range Low (ERL/ERM) approach was used to determine that few sites contained individual PAHs, whose presence may occasionally cause biological adverse effects to benthic organisms. The ecosystem–risk of exposure to PAHs in Lebanese sediments seems low.

1. INTRODUCTION

The PAHs are a class of the most common organic pollutants and are a subject of assessment in food and environmental matrices since they are associated with several toxic effects including carcinogenesis [1, 2]. In aquatic systems, the determination of these contaminants in different phases such as water and sediments has been a subject of great scientific attention as regards assessing the suitability of aquatic environment for biota and plant diversity. PAHs are discharged into the environment as complex mixtures [3] arising from anthropogenic activities involving pyrolysis or incomplete combustion of organic matter [4, 5]. They have been detected in different environmental matrices and are a toxicological concern.

Low molecular weight PAHs have demonstrated narcotic effects to marine organisms [6] while many high molecular weight PAHs are carcinogenic and/or mutagenic [6, 7]. PAHs end in the aquatic systems mainly through atmospheric deposition, surface runoff, industrial and municipal effluents, maritime transport and oil leakage [2, 8, 9] among others. Once in the aquatic systems, the physicochemical properties of PAHs including their solubility, vapor pressure and lipophilicity influence their distribution [6]. Ultimately PAHs are dissolved in aqueous phase, adsorbed to suspended solids and sediments or bioaccumulate in living aquatic organisms [6, 10].

The Mediterranean Sea is heavily contaminated [8] due to extensive anthropogenic activities and high population in the vicinity [11]. Nevertheless, there is limited information on the scope of contamination. A study was therefore carried out, to fill a gap in data on the presence of

PAHs on the eastern coast of the Mediterranean and contribute to a database for the environmental assessment [12].

2. MATERIALS AND METHODS

2.1.Chemicals, reagents, equipment and experimental design

The investigated PAHs, including several deuterated compounds as detailed elsewhere [12], were analyzed by GC–MS. On the marine side, water and sediments were sampled from 14 sites in the south of Beirut and 16 other sites in the north. Samples were taken from 6 coastal rivers and 8 sites on the two inner/inland rivers on the continental side.

3. RESULTS AND DISCUSSION

3.1.PAHs in water and sediments

A higher level of contamination with the 17 PAHs was detected in freshwater samples. The total average concentration was 874.1 ng/l although values ranged from 465.7 ng/l to 1399.9 ng/l [12]. High molecular weight (HMW) compounds such as benzo(b)fluoranthene [B(b)F], benzo(k)fluoranthene [B(k)F] and indeno (1,2,3, cd) pyrene (ICP) were the major contributors in marine sediments with average contribution to the total concentration of 13.6%, 15.5% and 12.9%, respectively [12]. Chrysene (Chry), B(k)F and benzo (g, h, i) perylene B [(ghi)P] were the major contributors in freshwater sediments with average contribution of 11.1%, 11.2% and 10.9%, respectively [12]. Moreover, 4– and 5–ringed PAHs were the dominant compounds in sediments. These two groups together accounted for more than 52% of the 17 PAHs in 11 freshwaters and 18 marine sediment samples. In freshwater sediments, the PAHs with 2 rings represented the lowest proportion (less than 10% of the total contamination). The 6 ringed PAHs accounted for less than 10% of the total contamination in marine sediments [12].

3.2.Partition and correlations; contamination sources

The number of rings, the molecular mass, the log Ko/w of the analytes showed moderate negative correlation with PAH concentrations in water (marine and freshwater). However, a high positive correlation in freshwater sediments and moderate positive correlation in marine sediments were observed [12]. The higher the number of rings, the higher is the molecular mass and the log Ko/w. Partitioning of the PAH into sediments therefore increases in this case. The average concentrations of PAHs in both freshwater and marine water strongly correlated with their water solubility, with correlation coefficients higher than 0.9. On the contrary, the average concentrations of PAHs in both marine and freshwater sediments showed moderate negative correlation with their water solubility. The vapor pressure showed very high positive correlation with PAH concentrations in both freshwater and marine water. As water solubility and the vapor pressure of the PAH increase, they accumulate more in the aqueous phase [12].

The ratios of fluoranthene Fln/(Fln+Pyr), LMW/HMW, B(a)A/[B(a)A+Chry] and Ant/(Ant+Phe) were used to investigate the origin of PAHs in Lebanese sediments. LMW/HMW ratios lower than unity in most of the marine and freshwater sediments samples and the strong correlation between them (correlation coefficient > 0.8 in both marine and freshwater sites) indicate the dominance of combustion sources of PAHs [12].

4. CONCLUSIONS

The quality of Lebanese surface water/sediment systems — regarding PAH contamination — on the eastern coasts of the Mediterranean Sea was evaluated. Different levels of PAHs were found in fresh and marine water and sediments. Low molecular weight PAHs were predominant in the aqueous phase, compared to the higher molecular weight compounds that were more in sediments.

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RESIDUES OF VETERINARY ANTIMICROBIALS IN WATER, SEDIMENT AND TROUT FROM LAKE TITICACA

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Abstract

The presence of nine antimicrobials including tetracyclines, sulfonamides and fluoroquinolones in drinking water, trout tissue and the sediment from Lake Titicaca has been investigated. Analysis of the randomly collected samples was conducted on a liquid chromatography coupled to mass spectrometer following cleanup by solid phase extraction. High levels of the residues were found in sediments and surface water samples. For example, sediments contained fluoroquinolones and tetracyclines below 4 mg/kg; surface water had fluoroquinolones between 408 ng/l and 653 ng/l and drinking water had ciprofloxacin between 188 ng/l and 222 ng/l. Ciprofloxacin, oxytetracycline, sarafloxacin and sulfathiazole were found in trout below 8 μg/kg.

1. INTRODUCTION

There is a growing demand for animal protein as human population grows [1] with aquaculture now contributing to this need worldwide [2] and is one of the fastest agricultural sectors [3] filling the gap in protein sources [4]. The annual production of trout fish in Peru exceeds 54000 tons mostly from the Puno region [5]. Increased aquaculture production is also associated with stresses and diseases requiring use of drugs with impacts natural resources [6].

The Coata River, a major tributary of Lake Titicaca in Puno flows through livestock production areas thus a potential source of veterinary pharmaceuticals [7]. Many of these are widely used in aquaculture including through feed [8, 9] and discharged in water or deposited in the sediments of lakes, lagoons or ponds used for aquaculture [10]. The drugs may remain active in the environment [11] with negative effects on soil, water ecosystems and humans including development of antimicrobial resistance [12, 13]. The different physicochemical properties of these drugs influence adsorption in sediments in the aquatic environment [14] due to their intrinsically hydrophobic nature [15] and affinity for organic matter in sediments [16, 17]. Other conditions such as oxygen deficiency and dark conditions reduce bio/photo–degradation [15, 18] thus influencing presence of the drugs. Several factors are known to influence discharge of contaminants/residues into the water [19, 20], although spatial and temporal distribution can be unpredictable [21, 22]. A relationship between size of the contaminant and the sediment content has been observed [15]. This is influenced by physical, chemical and biological factors [23, 24] as well as water and sediment properties [24]. Due to public health concerns about the

contamination of fish with antimicrobials [25], a study was undertaken to investigate the presence of nine antimicrobial residues in sediment, surface water and trout tissue from fish farms in the Lake Titicaca Bay and associated portable water from households in the city of Puno [26].

2. MATERIALS AND METHODS

The following were used: Uniscience Biovortex V1, Beckeman J2 HS centrifuge, Na_2EDTA, SPE– LC–MS/MS (Agilent, USA, with 1200 LC series), Marconi ultraturrax model MA102, Hitachi CF16RXII, (Agilent) Captiva cartridge, Supelco Visiprep collector system; methanol and acetonitrile (Tedia), formic acid (JT Baker), orthophosphoric acid (Mallinckrodt Chemicals), Na_2EDTA (Sigma Aldrich), citric acid monohydrate and dihydrate sodium citrate (JT Baker). The antimicrobials with respective purity levels included: chlortetracycline (93%), ciprofloxacin (99.5%), enrofloxacin (99.0%), oxytetracycline (97%), sarafloxacin (97.2%), sulfathiazole (98.0%) and sulfamethazine (99.5%) and sulfadimethoxine and tetracycline (97.5%) [26].

2.1.Sampling and extraction

Sixteen points were sampled for surface water and sediment, 15 points for trout and 4 points for drinking water. Samples were collected in the dry and rainy seasons. Water was collected 7 (± 2) m from the surface of the fish farms, and 0.1 g of Na_2EDTA added before pressed through a 0.22 µm filter material, adjusting pH to 4.0, refrigerating at 4°C prior to analysis SPE–LC–MS/MS system. Sediments (~ 300 g) were collected and placed in aluminium foil sachets, frozen and transferred for analysis. Trout (200 g – 250 g) were wrapped in aluminium foil containers, stored in a cold polystyrene thermal box, skin removed and either analyzed immediately or stored at 18°C.

Lyophilized sediment samples (2 g) were weighed into a 25 ml bottle, 10 ml each of acetonitrile and citrate buffer (pH 3) added, and the content mixed on a vortex for 1 min before sonication for 15 min and centrifugation at 5°C for 10 min. The resulting supernatant was transferred to a 250 ml round bottomed flask with 0.2 g Na_2EDTA and then filtered through a 0.22 µm material prior to analysis. Trout samples (5 g) were weighed into 50 ml Teflon tube, 50 µl of 1.0 µg/ml SDM–d6, 1 ml of 0.1 M Na_2EDTA and 24 ml of water:acetonitrile (70:30, v/v) with 0.1% formic acid added before the content was homogenized for 5 min and centrifuged for 5 min before 500 µl of the supernatant was eluted from captiva cleanup cartridges analyzed [26].

2.2.Antimicrobial analysis

As detailed elsewhere [26] after the preparative column, the antimicrobials were separated with a C18 analytical column (100 × 3 mm, 3.5 µm) maintained at 30°C. The solvents used on the SPE preparative column included methanol (solvent C):water and orthophosphoric acid solution at pH 4, (solvent D); attainment of a linear gradient of 20:80 (v/v) (C/D) in 4 min. This ensured transfer of the analytes onto the analytical column. The solvent programme on the latter included the following at a flow rate of 0.4 ml/min: 20:80 (v/v) (A/B) where A was acetonitrile + 0.1% formic acid and B water + 0.1% formic acid. The flow started with 5% B, increasing linearly to 95 % B in 13 min and maintained for 3 min and later equilibrated for 10 min at 5% of B. The LC–MS/MS was operated in the electrospray ionization (ESI) with multiple reaction monitoring, with nitrogen as nebulizer and collision gas.

2.3.Validation of the method

The method was validated for analysis of water, sediment and fish tissue matrices as reported elsewhere [27]. Matrix matched calibration curves were prepared for all the nine analytes at the following concentrations in triplicate: 20 ng/l, 50 ng/l, 200 ng/l, 1000 ng/l and 2000 ng/l for water; 10 ng/l, 20 ng/l, 50 ng/l, 100 ng/l, 200 ng/l, 500 ng/l and 1000 ng/l for sediment and 5 ng/l, 10 ng/l, 20 ng/l, 50 ng/l, 100 ng/l, 200 ng/l, and 500 ng/l for trout. The limits of detection (LOD) and limits of quantification (LOQ) were calculated as the minimum detectable amount of each analyte in the matrix at respective signal–to–noise ratio of 3 and 10 [26].

3. RESULTS AND DISCUSSION

Very good linearity (with linear regression coefficients above 0.99) was determined. The LODs and LOQs are summarized in Table 1 below [26].

TABLE 1. THE LODs AND LOQs FOR THE NINE ANTIMICROBIALS IN WATER, SEDIMENT AND TROUT MUSCLE SAMPLES.

Antimicrobial	Water		Sediment		Trout muscle	
	LOD ng/l	LOQ ng/l	LOD µg/kg	LOQ µg/kg	LOD µg/ kg	LOQ µg/ kg
Chlortetracycline	1.5	4.7	2.5	8.2	0.9	3.0
Oxytetracycline	1.2	4.0	1.3	4.3	1.2	4.0
Tetracycline	1.5	4.8	1.0	3.2	1.0	3.2
Sulfadimethoxine	1.0	3.2	0.6	1.9	0.3	0.9
Sulfamethazine	0.6	2.1	3.0	9.9	0.8	2.6
Sulfathiazole	1.2	4.1	4.0	13.0	1.3	4.0
Ciprofloxacin	1.2	4.1	5.1	16.0	0.4	1.2
Enrofloxacin	0.5	1.6	4.1	13.0	0.5	1.5
Sarafloxacin	2.6	8.5	3.4	11.0	0.6	1.9

Chlortetracycline (CTC), oxytetracycline (OXT), sulfadimethoxine (SDM), enrofloxacin (EFX), ciprofloxacin (CPX) and sarafloxacine (SAR) were above LOD or LOQ for the surface water samples with CPX as high as 201.0 (±91.1) ng/l in the dry season and 301.7 (±125.1) ng/l in the rainy season. Similar results were reported in Italy [28] although lower levels of 90 ng/l in Brisbane, Australia [29], 106 ng/l in waters draining into the Glatt River in Switzerland [30] have been reported.

Filtered tap water from four households contained CPX, 188 (±103) ng/l and 222 (± 116) ng/l in dry and rainy season, respectively an indication of the lack of effect on CPX [30]. The average concentration of the antimicrobials were as follows: 55.4 ng/l for CTC in surface water; 75.8 ng/l for TC; 12.8 ng/l for SDM; 61.7 ng/l for EFX and 73.8 ng/l for SAR [26]. Others were: 61.0 ng/l for CTC; 75.3 ng/l for TC, 12.9 ng/l for SDM and 75.5 ng/l for SAR in drinking water from selected homes in Puno city. Although fluoroquinolones feared for antimicrobial resistance were the most frequent, other drugs in smaller amounts can still contribute to the development of antimicrobial resistance [32]. The presence of drugs in drinking water and

treatment plants has been reported using different analytical tools [33] which supports this study [26]. The effects of the antimicrobial are wide–ranging. For instance, antimicrobials such as CTC could affect the macro and microfauna of irrigated soil [34, 35].

All sediment samples contained CTC at 0.36 (±0.15) mg/kg and 0.43 (±0.34) mg/kg in the dry and rainy seasons, respectively; and OTC at 1.57 (±2.13) mg/kg in the dry season as well as TC at 0.12 mg/kg. Three fluroquinolones EFX (1.62 ±1.52 mg/kg), CFX (1.98 ±1.45 mg/kg) and SAR (0.07 mg/kg) were detected in the fish farms [26]. STZ (1.58 ±2.13 mg/kg and SMZ (0.19 mg/kg) were also detected. The levels of residues detected is an indication of the adsorption capabilities as reported elsewhere [36].

The high frequency of CTC is possibly due to extensive use of the antimicrobials [37] including aquaculture [38] and can end up in water bodies such as Lake Titicaca [39, 40]. EFX had the highest concentration (2600.3 µg/kg). Although it is subject to photodegradation [41] it has a high affinity of sorption to sediment particles [42]. The levels of the residues in sediments were generally the same as in water and fish farms, except for OTC which though subject to natural photodegradation [43], adsorbs to sediments [44]. There is also a positive relationship between the molecular size and levels of the residues in the matrix [15].

Five of the nine monitored antimicrobials OTC, STZ, SMD, CFX and SAR were detected in trout tissue with the highest concentration in the dry and rainy season as follows: ~8.5 µg/kg STZ; ~7.85 µg/kg OTC; ~4.2 µg/kg CFX; ~3.55 (±0.1) µg kg SAR and 1.4 µg/kg SDM. Extensive use of these drugs in aquaculture production can explain the residues levels [45]. The drugs' presence at the farms could also be attributed to their stability in the water [44]. Constant use and disposal may lead to bioaccumulation and availability in various tissues and biological material [46, 47]. This has potential chronic toxicological disorders in fish [48, 49], humans [50] and aquatic ecosystem including algae [51] even at low concentrations [52–56].

Drugs such as SMZ can inhibit growth of certain microalgae [57] while OTC affects antioxidant activity of certain enzymes [58]. The effect on microalgae and cyanobacteria — that are thought to be more sensitive than algae [59, 60] — can in turn affect organisms at a higher trophic level [61]. Cases of antimicrobial resistance [61] have been in nontarget populations. Resistance has can compromise effective treatment of infectious diseases [29, 63] as in the case of CFX and *Staphylococcus aureus* [64]. Water is as significant a source of residues as is food [65– 67]. Besides resistance, drug residues may be associated with childhood obesity [68, 69], carcinogenicity [70], reduction in angiogenesis and therapeutic effects in mice [71], metabolic alterations [72] as well as cytotoxicity, neurotoxicity and genotoxicity [54] among others. Therefore, bioaccumulation of residues in fish (including its environment) and the food chain in general is a public health concern [13, 77–78].

4. CONCLUSIONS

A validated isotope–dilution liquid chromatography mass spectrometric analytical method was used to determine residues of antimicrobials including ciprofloxacin (653 ng/l), chlortetracycline (87 ng/l) and sarafloxacin (78 ng/l) in surface water from fish farms and drinking water in Puno city. Oxytetracycline (7.8 µg/kg), sulfathiazole (8.7 µg/kg), ciprofloxacin (4.2 µg/kg) and sarafloxacin (3.6 µg/kg) were detected in trout muscle. The findings in this study indicate that trout fish farming is contaminated with antimicrobials from agricultural runoff and wastewater. This calls for strengthened regular residue monitoring and control.

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PERFLUOROALKYL AND POLYFLUOROALKYL SUBSTANCES IN FARMED MARINE SHELLFISH IN SOUTH AFRICA

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Abstract

A UHPLC–MS/MS method was validated and used to study the concentration of 15 perfluoroalkyl substances (PFAS) in four species of farmed marine shellfish which were in turn used to determine the human daily intake of PFAS including perfluoropentanoic acid (PFPeA), perfluorooctanesulfonic acid (PFOS), perfluorohexanoic acid (PFHxA) and perfluorotetradecanoic acid (PFTeDA). These were detected at frequencies of 9471%, 8871%, 7671% and 71%, respectively. Farmed abalone, mussel, oyster and lobster contained concentrations of these substances in the range 0.12 to 0.49.22 ng/g, 4.83–6.43.22 ng/g, 0.64–0.66.22 ng/g and 0.22 ng/g ww, respectively. The estimated daily intake of PFAS through the consumption of marine shellfish was in the range 0.05 ng/kg bw/d – 1.58 ng/kg bw/d. The overall risk of exposure to these substances appears low based on this study.

1. INTRODUCTION

Perfluoroalkyl substances (PFAS) contain a fluorinated hydrophobic carbon chain bonded to variable hydrophilic head [1]. Almost 5000 PFAS registered worldwide [2] are used widely including in food packaging materials among others [3, 4]. The PFAS are stable, heat resistant and persist in the environment [5] where they do not biodegrade [6] but bioaccumulate [6, 7] or biomagnify in the food chain and end up consumers. Associated adverse effects include hepatotoxicity, immunotoxicity, neurotoxicity and developmental toxicity [8–10]. While there is limited data on the occurrence of PFAS in the South African environment, some information has been reported in South African milk [11] river water [6, 13–15], wild fish [13, 15–17] and wildlife [18–20].

Seafood forms an important part of human diet [21] and is important for food security as well as the growing aquaculture sector that is contributing 73% of the fish supply [22] as the global demand increases [23–25]. In South Africa the products include molluscs, namely, abalone (*Haliotis midae*), and mussel (*Mytilus galloprovincialis* and *Chromomytilus meridionalis*) and pacific oyster (*Crassostrea gigas*) [26]. It is important therefore to monitor a range of pollutants and evaluate risk of exposure [21] including the use of molluscs, such as mussel and oyster, as sentinel organisms for monitoring temporal and spatial distributions of persistent organic pollutants [22, 27]. Food and drinking water are major media of exposure to PFAS [6, 28–30]. Due to the absence of data on PFAS in marine shellfish from coastal environments a study was undertaken to investigate the concentrations of PFAS in farmed marine shellfish and estimate dietary intake [31].

2. MATERIAL AND METHODS

2.1.Chemicals, reagents and equipment

The following were used as detailed elsewhere [31]: Ammonium acetate and sodium dihydrogen orthophosphate monohydrate (Sigma Aldrich, South Africa); acetonitrile, 99.9% and methanol, 99.9% (Microsep South Africa); deionized water, 18.2 Ω m (Elgastat UHQ water purification system); QuEChERS extraction material including 4 g magnesium sulfate and 1 g sodium chloride (Agilent Technologies, Chemetrix, South Africa); High purity (97%–99.9%, Wellington Laboratories (Ontario, Canada) perfluorocarboxylic acid (PFCA): perfluorobutanoic acid (PFBA), perfluoropentanoic acid (PFPeA), perfluorohexanoic acid (PFHxA), perfluoroheptanoic acid (PFHpA), perfluorooctanoic acid (PFOA), perfluorononanoic acid (PFNA), perfluorodecanoic acid (PFDA), perfluoroundecanoic acid (PFUdA), perfluorododecanoic acid (PFDoA), pefluorotridecanoic acid (PFTrDA) and perfluorotetradecanoic acid (PFTeDA); perfluorosulfonic acid: linear perfluorobutanesulfonic acid (PFBS), perfluorohexanesulfonic acid (PFHxS), perfluorooctanesulfonic acid (PFOS) and perfluorodecanesulfonic (PFDS); $^{13}C_3$–PFOA and $^{13}C_3$–PFNA (Cambridge Isotope Laboratories, Inc. Massachusetts, USA. PerkinElmer LX50 ultrahigh performance liquid chromatography (Waltham, Massachusetts, USA); Kinetex® C18 1.7 µm particle size, 2.1×100 mm; PerkinElmer® QSight™ 220 triple quadruple mass spectrometer (Waltham, Massachusetts, USA) [31].

2.2.Sampling and sample preparation

Composite abalone (n=11) samples were collected from land–based culture tanks that use seawater from the South African west coast, while the lobster (n=1) sample was from semi recirculatory aquaculture system. Abalones were fed formulated high protein feed and seaweed. Mussel (n=3) and oyster (n=2) samples from different culture methods were also included. The samples were collected from 11 major marine shellfish farms in the Eastern Cape and Western Cape provinces in August and November 2019. Composite samples from each farm were prepared based on the size and age of the shellfish [31].

Shells were removed from the fish and the soft tissue homogenized (FASTH 21, ConsulAR SA, Switzerland). One gram was placed in a 50 ml polypropylene tube, spiked at 0.2 ng/g of $^{13}C_3$–PFOA and $^{13}C_3$–PFNA. Acetonitrile and water (10 ml each), 4 g of $MgSO_4$ and 1 g of NaCl were added before the content was mixed well to uniformity. This was spined on a centrifuge (8000 rpm, 10 min), 500 µl aliquot of the supernatant collected and pressed through a 0.22 µm nylon membrane filter before analysing 10 µl of the aliquot on a UHPLC–MS/MS following separation on the C18 1.7 µm particle size, 2.1×100 column. The mobile phase used was (A) 10 mM ammonium acetate in water and (B) methanol, at flow rate of 0.8 ml/min with 90% A held for 5 min and then decreased to 20% for 4.1 min; then 100% B for 3.1 min, reduced to 10% over 0.1 min. The run time was 12.3 min with 3.5 min used for equilibration time. The MS conditions included negative ESI, 4000 V, nitrogen used as drying (140) and nebulizer gas (400). Others were a desolvation temperature of 320°C and ion source temperature of 350°C [31].

2.3.Daily intake of PFAS

Assumptions were made in estimating daily intake of PFAS including that, 100% of the PFAS are absorbed, that the average daily fish consumption among South Africans is 16.4 g/day [25] for an average body weight of 70 kg [32]. The following were excluded due to low levels

or lack of detection: PFDA, PFuDA, PFDoA, PFTrDA and PFHpA. The determination of the estimated daily intake (EDI) of PFAS in ng/kg bw/d for South African adults through marine shellfish was based on three exposure scenarios: low intake, average intake and worse–case scenario by using the 5th percentile, mean and 95th percentile concentrations of each PFAS and Σ_{10}PFAS. The EDI was the product of concentration in ng/g and average daily fish consumption rate in grams per body weight (kg) of an adult South African. Meanwhile the hazard quotient was the ratio of the EDI to the reference dose [31].

2.4.Quality control/assurance and method development

Blank marine shellfish samples were used for the validation and preparation of calibration curves. Two $^{13}C_3$–labelled compounds were also used. Spiked quality control samples (n=3) were spiked and analysed for each batch of tests, to monitor method performance. Solvents were injected between samples. The method blank samples (n=6) were analysed for possible contamination and PFDoA, PFTrDA and PFTeDA detected although at concentrations lower than 0.5% of the least contaminated samples. PFPeA in mussel and oyster were externally validated and the results corrected for the recovery.

The method was validated using the following parameter/characteristics: recovery, repeatability and within laboratory reproducibility, linearity, decision limit, detection capability and limit of quantitation (LOQ). The LOQ was considered as the lowest detectable concentration with signal–to–noise ratio greater than 10. Recoveries and precisions were determined by spiking sample replicates (n=21) at three different concentrations over a period of three days. The linearity of the method was determined form eight–point matrix matched calibration curve at 0.005 ng/g to1 ng/g per analyte [31].

3. RESULTS AND DISCUSSION

3.1.Method validation

Reasonable linearity (r^2 >0.99 with the exception if PFOS that was at 0.9843) was attained in the range 0.005 ng/g to 1 ng/g for each analyte while good recoveries of 57% to 109% (abalone), 63%–119% (lobster), 82%–146% (mussel) and 63%–124% oyster) were obtained with reproducibility (within laboratory, n=63) of 8.1% to 27% and repeatability (n=21) of 5% to 12.4%. The CCα (30 pg/g–100 pg/g), CCβ (50 pg/g–160 pg/g) and LOQs (5 pg/g–50 pg/g) were determined [31].

3.2.Method application for analysis of PFAS in marine shellfish

The method [31] was found to be applicable to field samples with at least one PFAS found in every sample although PFPeA (94%), PFOS (88%), PFHxA (76%) and PFTeDA (71%) were more prevalent especially in mussel (> oyster > abalone > lobster) with the highest concentrations, ranging from 4.56 ng/g to 6.34 ng/g (i.e. PFPeA in mussels, 85% of the total PFAS) in all species. PFDA, PFUdA, PFDoA and PFTrDA were not detected above LOQs in all shellfish. The Σ_{11}PFAS concentrations were 0.12ng/g ww to 0.49 ng/g ww abalone; 4.83 ng/g ww to 6.43 ng/g ww in mussels; 0.64 ng/g ww to 0.66 ng/g ww in oysters and 0.22 ng/g ww in lobsters [31]. Abalones had the highest PFOA and PFOS levels with 93% PFDS. No PFDS was detected in most shellfish detected except for one lobster [31].

PFBA, PFBS, PFHxS and PFNA were also mostly in abalone with only PFBA in some mussel, oyster and lobster. In abalones, there was a positive correlation between the PFOS and PFBS (r^2 = 0.3538), PFHxA (r^2=0.70) and PFNA (r^2=0.4271). PFPeA correlated favourably with

PFBS (r^2=0.9948); while PFTeDA did with PFOA (r^2=0.90). This is an indication that the source of PFAS in abalone was the same [31]. Five of the PFAS were above the LOQ in mussel samples and in general PFPeA, PFTeDA and PFOS were the most prevalent. For mussel, a correlation was also observed between the PFOA and PFBeA (r^2=0.9902), PFOA and PFBA (r^2=0.419); PFOS vs PFPeA (r^2=0.9947); PFOS vs PFIA (r^2=0.9707) and PFOA vs PFNA (r^2 = 0.7559) [31].

The highest concentration of PFPeA in mussel agrees with previous studies [6] that reported high levels in water from Vaal River in South Africa. This is understandable since mussels can accumulate organic contaminants in seawater [33]. At least four PFAS were above the LOQ in oyster, mostly PFPeA (0.470 ng/g). Correlations were also observed as follows: PFOS vs PFTeDA (r^2=0.996); PFPeA vs PFOS (r^2=0.998) and PFTeDA vs PFOS (r^2=0.999). Meanwhile 5 PFAS were detected in lobster above the LOQ with the highest (0.094 ng/g) being PFPeA. The findings in this study [31] agree with previous observations on shellfish from the English Channel, and Atlantic and Mediterranean coasts of France [34] where concentrations of PFOS declined between 2013 and 2017 while PFCAs were stable over the same period in shellfish.

Additionally, short chain PFAS detected in the current study [31] have been found to be prevalent in Bangladesh [35]. Similar findings have been reported more in mussels than in fish [36]. The short chain compounds partition more in water [36] compared to the long chain chemicals which partition into sediments [37]. PFAS concentrations could also be attributed to formulated feed [38]. The predominance of short chain PFAS could be attributed to photodegradation of PFOA and PFOS [39] and defluorination [32, 40–43].

The PFOS levels in this study [31] were within the same range as in wild mussels reported in France [34], Spain [44] and Japan [45]. Similar PFOA levels in mussels were reported in Japan [45] and Korea [46]. The presence of PFPeA, the most abundant PFAS in oysters agreed with findings in Korea [46] while the concentrations of PFOS and PFHxA were much lower than levels reported in Japan [45] and Korea [46] for the seafood. Meanwhile the PFAS in lobster [31] were lower than in wild lobsters in China [47/8]. Generally, levels of PFAS in the environment and associated matrices vary with country [35, 49].

3.3.Estimating potential human exposure

The EDI of PFAS (Σ_{10}PFAS) in marine shellfish for the South Africans ranged from 0.05 ng/kg bw/d to 1.58 ng/kg bw/d predominantly short chain PFCAs with PFPeA up to 85% of the total EDI [31]. The levels for PFOA and PFOS were in the range 0.0006 ng/kg bw/d – 0.005 ng/kg bw/d, 0.003 ng/kg bw/d–0.009 ng/kg bw/d, respectively, while the average EDI for Σ_4 (PFOA, PFNA, PFHxS and PFOS) was 0.005 ng/kg bw/d [31]. Elsewhere total weekly intake of 6 ng/kg bw/w for PFOA, and 13 ng/kg bw/w for PFOS and 8 ng/kg bw/w for the Σ_4PFAS (PFOA, PFNA, PFHxS and PFOS) was reported in Europe [50]. While most of the PFAS were in small amounts and may not seem to pause a threat to humans, the long–term exposure even at small levels should draw attention of relevant authorities and scientists [31].

4. CONCLUSIONS

A study [31] was undertaken to investigate the levels and distribution of PFAS in farmed mussel, oyster, abalone and lobster. Short chain PFAS especially PFPeA (85%) predominated. PFAS were mostly found in abalone while mussels had the highest PFAS concentrations

followed by oyster, abalone and then lobster. The PFPeA, PFOA, PFBA and PFNA in mussel appear to originate from the same source just as PFPeA, PFBS and PFTeDA in oyster. A strong correlation was also observed between PFOS, PFBS, PFHxA, and PFNA in abalone. In general, the levels and occurrence of PFAS in the farmed shellfish doesn't seem to pose a threat to consumers although further studies including epidemiological investigations, are required to enhance understanding of the sources, levels over a long period of time as well as fate of the PFAS [31].

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FISH FARMING PRACTICES AND AGROCHEMICAL–USAGE IN PARTS OF CAMEROON

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Abstract

A cross-sectional study was conducted to understand aquaculture production practices in parts of Cameroon as the country started improving the fisheries section to reduce imports. This included 107 farmers in the central, southern, littoral, western regions of Cameroon, who practiced earthen fishpond farming (83.3%) and integrated fish farming (30%). Feeding involved the use of locally formulated feeds (~32%), animal manure, chicken droppings (~21%) and pig dung (~19%). Few farmers (~24%) use a prescription yet more than half of the farmers used agrochemical products including veterinary drugs such as tetracyclines the most used.

1. INTRODUCTION

Aquaculture by definition [1] has been growing tremendously since 1970 to 33% in 2005 [2] and has also been growing in Cameroon since the 1940s and [3] reaching 400000 tons in 2015 [4]. Conditions for fish farming in Cameroon are appropriate including natural inland waters of 40, 000 km^2 [5]. Studies have been conducted in the part to understand farming practices in the Western [6, 7], North–western [8] and Central [9–11] parts of Cameroon as the demand for fish farming increased in Africa [12] with intensive farming requiring a wide range of chemical inputs [13]. This results in residues in animal products in [14] Ghana [15, 16] Nigeria [17–19] and in Asia [20, 21] although this has not been done in Cameroon and this has potential risks to consumers [22, 23], hence this study [24].

2. MATERIALS AND METHODS

2.1.Study area, questionnaire and data analysis

The study included the central, southern, littoral and western regions with high populations and extensive farming [25, 26]. A questionnaire was used to collect data from 107 fish farmers between April and September 2016 [26]. Farmer–practices and production systems were evaluated according to Gobert [27]. A pilot survey was conducted with preliminary questionnaires tested among 10 fish farmers; questions on socioeconomics, fish farming practices, and use of agrochemicals were included [24]. Microsoft Excel software, the Statistical Package for the Social Science 20.0 (SPSS) and Epi info version 3.5.3 Windows Version were used to generate, process and report findings [24].

3. RESULTS AND DISCUSSION

3.1.Characteristics of participating farmers and constraints

The study included males (93%) mostly without formal training in fisheries (67%) and 46% with experience of 5–10 years. The participants mentioned that they encounter financial

constraints (64%) as the main challenge [24] which may influence the attitude towards use of agrochemicals, regulated or not.

3.2.Farming practices

Farms surveyed (n=726, averaging 200–500 m^2) were categorized into three. Almost 50% had between 1–4 ponds, 30% 5–10 ponds and ~21% 10 ponds. Earthen ponds (82.6%) are supplied by rivers. Government hatcheries supplied ~ 61% of the farmers while 10% of the fingerlings were from rivers. Most farmers (~68%) practiced polyculture involving tilapia, African catfish, common carp and kanga. Monoculture included Nile tilapia and African catfish in the four regions [26].

More than half (54%) of farmers reared fish only, while the rest included poultry, piggery, and crop farming. The feed used included vegetables, kitchen waste, chicken droppings and pig manure in addition to some locally manufactured and imported feed. The local feeds used by a good number (~32%) were made of maize grains, fish flour, soybean, groundnut and cotton waste. Some of the diseases encountered included white spot disease and epizootic ulcerative syndrome. This typically requires intervention by professionals. However, most of the farmers (~76%) reported that they do not refer to a veterinary for drug prescriptions [24].

3.3.Agrochemicals used farming

As detailed elsewhere [24], the commonest agrochemicals used included liming materials, fertilizers, veterinary drugs and pesticides. The liming material was used by 44% of fish farmers. Mono– and di–phosphate potassium permanganate, nitrogen, phosphorus, and potassium (NPK) and iodine were used as fertilizers and disease treatment. Only 10% of the farmers used the antimicrobials oxytetracycline and oxytetracycline HCl to treat white spot and fungal diseases in *Clarias gariepinus*. Others were motilium (Domperidone) and ovaprim used by 15% of the respondents for production of fingerlings. Very few farmers declared use of pesticides/herbicides including roundup 54 and pyriforce [24].

3.4.Farmer practices assessment

Using criteria elaborated elsewhere [24], findings on the practices of the farmers in the study results were scored in on a scale of 6–10 (bad), 11–14 (good) and 15–20 (very good). Few farmers demonstrated very good practices. The aquaculture sector in Cameroon is made of three types of farmers but those included in the study were mainly small–scale farmers producing 2–5 tons of fish per hectare unlike large scale farmers producing 16 tons per hectare [28]. Up to 125 farmers in the central and eastern regions had no prior knowledge on fish farming [24]. This potentially impacts general productivity and quality as reported elsewhere Ndah et al. [8] in southwest and northwest regions. The level of education and use of poor-quality water among farmers was also observed by Nsangou et al. [29].

It was determined [24] that the farmers faced challenges with obtaining quality fingerlings and feed. This is not uncommon through to Cameroon [30] and other developing countries [31–35]. Another challenge is the lack of opportunities for learning and improving farming skills [36–38]. Opportunities to enhance knowledge and experiences, including demonstrations on farm have been initiated [38–40]. Some of the farmers used water from natural water sources, potentially contaminated [24] which impacts the genera quality of the water and fish health as reported by Khoi [41].

There are benefits in polyculture such as combination of tilapia and catfish [24] where for instance catfish helps control reproduction of the tilapia and influences yield [42]. About 30% of the farmers surveyed [24] were involved in integrated fish farming most commonly in the west region of Cameroon. Similar practices in the southwest and northwest of the country were reported by Oshuware Oben et al., [43]. The farmers used chemicals and as reported elsewhere, this influences fish farming [44] and require proper use [30] and regulation [45] to safeguard consumers [26]. Use of veterinary drugs in aquaculture is global, including developed countries such as USA [44] or the EU to treat specific diseases and elsewhere [45].

The residues add to other contaminants of concern in aquaculture such as trace elements and pesticides [47]. The current study [24] identified sources of contamination including feed and animal manure [48], the latter being a common source of pathogenic microorganisms [49]. All these contribute to high risks associated with aquaculture products [50]. Exposure of fish to a range of contaminants in common in integrated systems where sources are diverse [51]. The finding of antimicrobials such as tetracyclines [24] is not a surprise because these are the most common in poultry and pig manures [52]. Similar findings associated with production of poultry in Cameroon and Tanzania [53, 54]. Integrated fish farming systems can be associated with antimicrobial resistance [55] with implications on human health [56]. Besides the drug residues, the study [24] also reported use of pesticides in ponds, and this has implication on foodstuffs including fish [57] among other human health outcomes [58].

4. CONCLUSIONS

Fish farming practices in Cameroon remain predominantly extensive, and semi–intensive in some regions. This is largely polyculture involving different species in earthen ponds. The use of agrochemicals was reported [24] with a risk of exposure to residues in fish products due in part to practices such as self–prescription. Interviews and interactions with most of the participants suggest that there is a need to improve fish production practices with safe and quality inputs as well as farmers working with experts in animal production such as veterinarians [24].

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ASSESSMENT OF SULFADIAZINE ACCUMULATION AND DEPLETION IN ZEBRA FISH USING ^{14}C–SULFADIAZINE

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Abstract

Research was undertaken to investigate the depletion of sulfadiazine, a sulfonamide used in a range of livestock and in aquaculture. The use and improper disposal of such drugs may result in bioaccumulation, bioconcentration and biomagnification in the aquatic environments. ^{14}C–sulfadiazine was administered into *Danio rerio* fish to evaluate the drug's bioconcentration and depuration after 20 days. A small amount of the drug was found, and no depuration observed.

1. INTRODUCTION

The use of pharmaceuticals in animal production and improper disposal results in contamination of the aquatic and terrestrial environments with parent compounds or their metabolites [1–3]. Sulfonamides are antibacterial derivatives of p–aminobenzene sulfonamide [4, 5]. A common example is sulfadiazine which doesn't persist much in the environment and is readily biotransformed/degraded [6, 7]. Pharmacologically active substances are associated with the risk of aquatic toxicity, acute [8, 9] or chronic [10] including nontargeted organisms [11–13]. The chronic effects may include disruption of fertility and reproduction [14] hence the need for preventive measures [12, 15].

Danio rerio was used to study the behaviour of sulfadiazine because it is a bioindicator organism, known for its sensitivity to a variety of chemical substances [15]. The fish was treated with ^{14}C–sulfadiazine for 20 days in a static water system with experimental details and findings reported [16].

2. MATERIALS AND METHODS

2.1.Reagents, material, test substance/organism, equipment

The following were used: sulfadiazine (Sigma Aldrich); ^{14}C– sulfadiazine [(3.58 MBq/mg, 9.25 MBq, 98.07% radiochemical, 98.57% chemical purity of and (IZOTOP—isotopes institute Co. Ltd., Budapest, Hungary]. *Danio rerio*/zebra fish (Cypiriformes, Cypirinidae); liquid scintillation counter (Packard, model 1600 TR); biological oxidizer (OX 500); liquid scintillation cocktail (Instagel, Packerd).

2.2.Experimental conditions

The conditions/environment included glass aquaria with specifications of 0.4 × 0.4 × 0.5 m (w × l × h). This received water: passed through 5 µm and 10 µm filters (FUSATI®), with 150 mg/l –300 mg/l $CaCO_3$, at 25 (±2)°C and pH of 7–7.5, constantly aerated [16]. Fish acclimated for a week were fed 9.32 mg of a commercial grain twice a day; the environment cleaned frequently, and healthy fish used for ecotoxicological tests following a standard procedure [17]. Treatment with sulfadiazine was conducted at 0.01 mg/l, 0.1 mg/l, 1.0 mg/l, 10 mg/l and 100 mg/l two replicates each [16].

2.3.Bioconcentration and depuration assays

^{14}C–Sulfadiazine (0.0947 µg, 4220.55 Bq) was administered for 11 days in 20–litre aquaria in triplicate, to determine bioconcentration guided by a previous study [18] where sulfadiazine was detected 100 ng/l. The drug's elimination was observed for 9 days. To each 4 L of water, 3.6 µl of the drug solution was added and three samples of 10 ng/l mixed with 10 ml of scintillator solution were analyzed using a liquid scintillator counter. The average level detected was 285.2 Bq [16]. Th control samples included the scintillator solution added to the water without the ^{14}C–sulfadiazine. Each tank with 4 L of cultivation water contained 20 fish, subjected to 12 h of day light, aerated regularly with the following parameters: 319 µS/cm, with 150–300 mg/l of $CaCO_3$, 25°C and 7.4 pH. Fish was collected daily, frozen and then prepared in a biological oxidizer (OX 500) before LSC analysis. Background material including remnant feed, excreta as well as water from the aquaria were also analyzed for background radioactivity [16].

3. RESULTS AND DISCUSSION

The highest possible concentration of sulfadiazine used did not cause death of the *Danio rerio* fish at 24h and 96 h [16]. This notwithstanding, sulfadiazine is thought to be toxic such as in the inhibition of chlorophyll in algae with LC50 of 7.8 mg/l –135 mg/l [19]. In a sperate study involving *Cladocera Daphnia magna* as a bioindicator, however, acute toxicity of sulfadiazine within 48 h was observed at the LC50 of 221 mg/l [20]. In this study [16] it was also not possible to increase the concentration of the drug above 77 mg/l for solubility reasons.

The depuration activity levels for the test samples were in the range 3.79 Bq–5.21 Bq (average of 4.47 in 9 days compared to an average of 1.34 for the control). The average activity per gram for the control and depuration experimental fish was 13.54 Bq/g and 4.62 Bq/g, respectively [16]. The corresponding values for the activity and activity per gram for the bioconcentration studies were 1.55 Bq and 4.84 Bq/g for the control and 4.92 Bq and 15.37 Bq/g for the treated experimental fish, respectively. Antimicrobial toxicity in organisms higher in the food chain, such as the indicator fish should be higher than in those at lower trophic levels such as algae where toxicity may be three times lower [21].

Exposure to drugs may be in smaller concentrations such as through municipal wastewater and surface waters [22] attributed to agriculture/veterinary and human use of pharmaceuticals and agrochemicals [23, 24]. These may be parent pharmaceuticals or their metabolites among different animal species [25, 26]. Some drugs such as sulfadiazine may interact with moieties such as sugars and appear inactive in the body although further breakdown in the environmental may release the parent drug or active metabolite [27].

The study [16] focussed on a sulfonamide because a previous survey of samples from a river in China found 12 antimicrobials with the sulfonamides in the range of 24 ng/l to 385 ng/l, the course attributed to use in pig and poultry farm nearby [28]. The current study was undertaken to determine if sulfadiazine can bioaccumulate in nontargeted organisms [16]. An equilibrium level was noted where administered radioactivity and that in water was constant [29].

Typically, organic compounds with log partition coefficient above 4.0 accumulate in lipid tissue [30] while acidic or weak basic compounds including antimicrobials such as sulfadiazine are ionized in the acidic conditions [31]. However, it is likely that such drugs may still bioaccumulate especially if the concentrations are above the environmental depuration levels as observed in this study [16]. Related findings have been reported in Chinese mitten crab (*Eriocheir sinensis*) following treatment with up to 1000 ng/l of sulfadiazine for 44 days [32]. Factors such as pH also affect bioconcentration and toxicity of drugs such as sulfadiazine in indicators like *D. magna* [33]. The effect of pH on sulfadiazine has been observed in chromatographic techniques involving the use of hollow–fibre liquid–phase microextraction, in which sulfadiazine levels in water samples decreased with increasing pH of the test solution used [34].

Radioactivity in the range 153.73 Bq–423.93 Bq was detected in background material from the bioconcentration phase. These were higher than in the depuration phase (13.40 Bq–19.41 Bq), and the control (12.89 Bq–15.69 Bq). There was therefore evidence of bioconcentration which agrees with findings involving flumequine, oxytetracycline, trimethoprim and sulfadiazine observed in sediment in 180 days [35]. There is also evidence that sulfadiazine once incorporated in the aquatic environment, it will not partition into solid or semisolid material and will therefore persist longer in the environment [36].

The levels of sulfadiazine in noncontaminated water analyzed at depuration was 201 Bq/l on average [16]. The bioconcentration was low although sulfadiazine, is not easily biodegraded, and can persist in water and soil [37]. The average radioactivity in water at bioconcentration was 1949.39 Bq compared to 185.75 Bq in the control [16]. In a related study involving sulfonamides such as sulfamethoxazole, the drugs underwent photo–transformation in shallow waters [38]. While veterinary drugs may bioconcentrate, lixiviation is also possible as reported elsewhere [39] for drugs frequently applied to soil at higher concentrations [39].

4. CONCLUSIONS

A study was conducted to determine if sulfadiazine bioconcentrates/bioaccumulates in a nontarget organism in an aquatic environment. ^{14}C–sulfadiazine was used, and low levels of the drug was observed in Zebra fish in a period of 20 days. While the bioaccumulation is low, long–term consumption of fish and related products originating from contaminated sources may pose a certain level of risk. Further research is, however, required to better understand the long–term effect of pharmaceuticals such as sulfadiazine may have on the environment and nontarget organism [16].

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DEPLETION OF RADIOLABELLED SULFADIAZINE IN RAINBOW TROUT

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Abstract

A study was undertaken to investigate the depletion of the sulfonamide sulfadiazine due to its frequent use and increased possibility for release into the aquatic environment. A labelled (^{14}C) molecule was used in rainbow trout (*Oncorhynchus mykkis*) at a specific activity of 3.5171 MBq/mg over a period of 14 days. The drug accumulated reached 5.10 x 10^{-4} mg/g fours at administration/exposure. The level was 1.0 x 10^{-4} mg/g after the purification phase.

1. INTRODUCTION

It is inevitable that antimicrobials are used in food production although there are health consequences including the development of antimicrobial resistance [1–3]. Several factors such as cost, efficiency, human safety, approval, environmental impact and ease of application, influence the choice of the drugs [4]. The drugs may include sulfonamides and florfenicol [5–7] beta–lactams, phenols, quinolones, fluoroquinolones, and oxytetracyclines [8] and choice as well as frequency of use varies with country [9]. The sulfonamides such as sulfadiazine are widely used and can end up in the aquatic environment posing various risks including development of resistance [10,11], against a range of pathogens [10]. The drug can accumulate up to 1150 mg/kg which would be a real human health risk [12, 13] potentially including carcinogenic and mutagenic effects [14], malformations in fish [15]. Microorganisms including algae and certain plants [13] are also affected. Maximum residue levels (MRLs) such as 100 µg/kg have been established in the United States, the European Union [16] and elsewhere.

Rainbow trout (*Oncorhynchus mykiss*) is widely produced in The Puno region of Peru with up to ~43290 tons of trout from artisanal producers and private companies [17]. Due to diseases, antimicrobials are used and possibly indiscriminately, contaminating waters of Lake Titicaca an important source of fresh water for Peru and Bolivia [18–20]. A better understanding of the dynamics of the antimicrobial in organisms using ^{14}C drugs [21, 22] is therefore required hence this study [23].

2. MATERIALS AND METHODS/EXPERIMENTS

2.1.Fish and experimental conditions

As detailed elsewhere [23] rainbow trout fry fish were used in a tank with 200 litres of chlorine–free water with a system to remove ammonia. Fish were acclimatized for 3 days during which they were given commercial fish feed (twice a day) consisting of: 49.5 (± 0.03) % protein, 12.5

(±0.02) % fat, 2.8 (±0.02) % fibre and 11 (±0.04) % ash; temperature maintained at ~ 14°C – 15° C, pH 6.65 –7.5 [23]. Stainless steel aquaria were used in triplicate.

Fry fish (n=14) of 4 cm – 5 cm length and weighing 1.0 g to 1.4 g were included in the exposure and depuration tests and grouped into two stages of exposure and depuration run for 7 days each [23]. During exposure, fish were placed in eight litres of water (at 13°C–15°C, pH 6.85 – 7.5; aerated at all times; exposed to light for 12 h and provided feed containing 0.0330 µg/mg sulfadiazine and 116203.407 Bq/g of radioactive ^{14}C–sulfadiazine (4–amine–N–2–Pyrimidinyl benzene sulfonamide), from Sigma Aldrich and specific activity of 3.5171 MBq/mg, activity of 9.25 MBq, > 98% purity as reported in other *Danio renio* studies [24]. For depuration, fish were transferred to clean aquaria containing four litres of water, provided commercial feed up to 12 mg per fish twice a day [23].

Fish were sacrificed (or frozen if not ready for analysis) and prepared in a biological oxidizer (OX500 R.J., Harvey Instrument Corporation) with the $^{14}CO_2$ absorbed in 10 ml of scintillation fluid and measured using a liquid scintillation counting (TriCarb 2910 TR LSA, Perkin Elmer). Water was filtered through a whatman filter paper size 42 on the 7th and final day of exposure and 14th and final day of depuration. Also 10 ml of the water was collected (in triplicate) and placed in containers with instagel plus scintillation solution and analyzed [23].

The absorption and depuration rates were determined following first order toxicokinetics according to Eq. (1) [25]:

$$\frac{dC_o}{dt} = (K_1 * C_a) - (K_2 * C_o) \quad (1)$$

C_a = concentration in water (ng/l), C_o = concentration in fish (ng/kg), K_1 = assimilation constant (L/kg/d), K_2 = elimination constant (d^{-1}) and t = time in day (d).

3. RESULTS AND DISCUSSION

Findings are detailed elsewhere [23] but briefly, no fish died among the treated and control groups during the experiment. The SDZ activity per gram decreased significantly up to 1.0 x 10^{-4} mg/g on day 7 of depuration, while the control sample had 0 mg/g. This difference could be attributed to the hydrophobic interaction and absorption of SDZ with slow [10, 27, 30]. The depuration levels between 3.79 Bq and 5.21 Bq were very close to the ones obtained in the bioconcentration phase, suggesting lack of depuration in 9 days in *Dario fish* [24]. In common carp, bioaccumulation of SDZ in the ranges 0.2 ng/g to 17 ng/g have been observed with a level of 3 ng/g noted at 48 days. It is reported that bioaccumulation in muscle tissue depends on the exposure concentrations of the chemical in exposure [30] although adsorption may not be complete [31, 32].

Elimination of SDZ occurred within seven days of the depuration phase [23]. Levels observed were as high as 131.67 µg/kg which is still above the MRL of 100 µg/kg [16]. Assuming an open bicompartmental model, the absorption rate constant (k_1) for SDZ was estimated to be 0.656 l/kg/d and the depuration speed constant (k_2) 0.160/d [23]. This compares with previous reports on hydrophobic compounds [33,30]. Up to 1.0 x 10^{-4} mg/g of SDZ was observed on the 7th day of depuration [23]. With such information, the rate of depuration can be estimated and this enhances the understanding of SDZ's depletion and biotransformation [30]; drug interactions as well as transport and reaction in a bioaccumulation model [34]. The information may also explain other characteristics such as the half–life of SDZ (4.33 days) known to be

lower than in other fish such as common carp. The dose of exposure [30] and the physicochemical properties [33] do have an effect as well.

The bioconcentration factor (BCF) for the drug was 4.1 l/kg [23] compared to 2.73 in the liver and 2.76 in muscle tissue when SDZ exposed at 6 µg/l in common carp [30]. The liver and kidney are target organs that bioconcentrate or bioaccumulate pharmaceuticals and other contaminants [35]. These phenomena are also influenced by properties such as hydrophobicity and partition coefficients [36, 37].

Antimicrobials are often discharged into the environment and this is a growing concern [28, 38] that requires regulation [28, 39, 40]. In this study [23] the drug was detected in water at levels of 35.27×10^{-4} mg/l and 5.21×10^{-4} mg/l after exposure and depuration phases, respectively. Such levels can in the long run have negative effects on the environment [11] especially where biodegradation is difficult and thus levels persist in water and sediment [41–43].

Drugs such SDZ could transfer to fish from the environment especially given the wide pH range [27]. Greater toxicity occurs at lower pH as a fraction of nonionized component of the drug [44] increase. Other parameters such as temperature also have an impact on the drugs' presence and distribution [45]. Appropriate mitigation measures are therefore needed to safeguard the environment and consumers [15, 46–49]. The presence of SDZ in fresh water has been reported [48] and that it could be associated with resistance genes [50]. The decrease in the relative abundance of nitrifying and denitrifying bacteria may also occur [48, 51].

Sulfadiazine was detected in sediment, but these decreased from 3.45×10^{-4} mg/g (after exposure) to 2.96×10^{-5} mg/g (after depuration) [23]. This suggests that SDZ cannot depurate in 7 days. Comparisons could be made with many antimicrobials like a range of organic pollutants and pharmaceuticals that are poorly adsorbed in the intestine but may be excreted unchanged [52, 53]. Persistence of SDZ in the environment due to a range of factors [23, 54, 55] and can be a further source of contaminants [56, 57] .

4. CONCLUSIONS

A study as detailed elsewhere [23] was successfully conducted to improve the understanding of how SDZ behaves in rainbow trout. Using a radiolabelled drug, it was observed that after 7 days of exposure and purification using trout fry, the activity was 5.10×10^{-4} mg/g following exposure and 1.0×10^{-4} mg/g after depuration. The levels detected were generally above established MRLs. The study provides a good foundation for further and more comprehensive depletion studies for commonly used antimicrobials in fish. Such studies from different parts of the world and environmental conditions are vital to setting of MRLs that are globally representative.

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ANTIMICROBIAL RESIDUES AND RESISTANT BACTERIA IN TILAPIA FROM A CAGE FARM

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Abstract

The relationship between antimicrobial residues in farmed *Oreochromis niloticus*, resistant bacteria, and sanitary practices of farmers in Ilha Solteira reservoir, Brazil was investigated. Small, medium and large fish (n=9) were collected from four cage farms every three months in a year; 10 antimicrobial residues analyzed by liquid chromatography mass spectrometry; and isolated bacteria tested for drug resistance. Oxytetracycline, tetracycline and florfenicol were detected in fish muscle. Correlation between the resistance index and antimicrobial concentration in the fish was established. Farm management practices that influence hygiene at the cages was also evaluated.

1. INTRODUCTION

The production and supply of nile tilapia (*Oreochromis niloticus*) is a critical sector in Brazil with government encouraging and growing the cage farming in federal waters and taking advantage of the country's abundant resources [1, 2]. However, these resources can be constrained hence the need to promote aquaculture in artificial reservoirs [3]. These nevertheless, require proper regulation to minimize negative environmental impact [4, 5]. Cage fish farming has intensified in Brazil and involves use of antimicrobials [6, 7] to control diseases and manage related stresses. Residues of these drugs could also contribute to the development of antimicrobial resistance [8] potentially negatively affecting the industry [9] and may increase medical costs in humans [10]. The monitoring of antimicrobial residues in tilapia from cage farms and has not been well studied [11]. A study was thus undertaken to: (1) determine the levels of ten drug residues in tilapia collected from cage farms in Ilha Solteira reservoir, Brazil. Measurement was against established maximum residue levels elsewhere [12–14]; (2) assess associated human health risks; (3) determine the multiple antibiotic resistance index; and (4) correlated relevant antimicrobial residues in the fish with farmer practice [15].

2. MATERIALS AND METHODS

2.1.Chemicals, reagents and equipment

The following were used as detailed elsewhere [15]: Aquaread AP 500 AgSolv sounder (Aquaread, Broad stairs, UK); Captiva ND cartridge (Agilent Technologies, Santa Clara, CA, USA); a Supelco Visiprep (Sigma Aldrich, St. Louis, MO, USA); Marconi MA102 ultraturrax (Piracicaba, SP, Brazil), Marconi MA102 ultraturrax (Piracicaba, SP, Brazil), A Triple Quadrupole 6430 mass spectrometer (Agilent Technologies, USA). The standards included the following from Sigma Aldrich were oxytetracycline (OTC, ≥97%), tetracycline (TC, ≥97.5%), chlortetracycline (≥93%), ciprofloxacin (≥99.5%), enrofloxacin (≥99%), sarafloxacin (≥97.2%), norfloxacin (≥99%), sulfathiazole (≥98%), sulfadimethoxine–d6

(99.4%), and florfenicol (FF, ≥98%). Sulfadimethoxine (99.5%) and sulfamethazine (99.5%) were from Chem Service (West Chester, PA, USA); and chloramphenicol [≥98.5%; Dr. Ehrenstorfer GmbH (Augsburg, Germany)]. Others were API 20 E and API 20 Strep Microbial Identification Strips (BioMerieux, Marcy L' Etoile, France); Culture media (Difco, MH).

The study [15] involved four georeferenced cage farms for tilapia located in Ilha Solteira reservoir, Brazil. A questionnaire was administered to characterize, among others: fish farms according to annual production, mortality rate, antimicrobial (s) used, how frequent and what the dose. Tilapia of different sizes: small fish (40 g–200 g); medium sized (200 g–500 g); and large fish (500 g–800 g) were collected and used to determine the drug residues.

2.2.Sample preparation and chromatographic conditions

Briefly, muscle samples were deskinned, chopped, and blended/ground in dry ice followed by analysis as described elsewhere [16]. The procedure involved addition (to 5 g of the fish sample) of 50 ml of sulfadimethoxine–d6 (1.0 mg/ml), 1 ml of 0.1 M disodium EDTA, 24 ml of acetonitrile: water both with 0.1% formic acid (70: 30, v/v). The mixture was homogenized (5 min, Marconi MA102 ultraturrax), centrifuged (1,370g for 5 min) and 500 ml of the supernatant eluted on Captiva ND cartridge (Agilent Technologies, Santa Clara, CA, USA) before injection of 10 µl in the LC–MS/MS. This followed separation on an Agilent Zorbax Eclipse Plus column C18 (3×100 mm; 3.5 µm). The column was kept at 30°C and the mobile phase (at a flow rate of 0.4 ml/min) included MilliQ water + 0.1% formic acid (A); acetonitrile + 0.1% formic acid (B). The gradient programme was as follows: 5% B linear gradient until 95% B in 13 min; maintained for 3 min before equilibration of the column with 5% B. Florfenicol was analyzed using different conditions [15].

Antimicrobials were selected based on the Brazilian National Plan of Residues and Contaminants [17]. The method was for parameters including limit of detection and quantification; linearity and recovery [15]. Risk assessment was determined based on the estimated daily intake in ng/d/ person, as a product of the maximum concentration of the antimicrobial (ng/g) in the adult biota and the amount consumed in fish per specific group [18]. Relevant data on fish consumption was obtained from local authorities [19] and compared against known MRLs [14].

Fish samples were disinfected with 70% alcohol for 10 min and kidney/brain samples swiped for the bacteria that were then investigated for colony morphology, Gram staining, haemolysis, oxidase and catalase behaviour, as well as phenotypic and genotypic profiling [15]. The bacteria identified were further analyzed on Muller Hinton agar Difco (MH) with 5% sheep blood [20]. The multiple antibiotic resistance (MAR) index was used to characterize each fish size and farm [21].

3. RESULTS AND DISCUSSION

It was determined that the tilapia produced in cages was in the range 120 t/year–1,800 t/year and drugs such as OTC or FF were used [15]. The fish (n=126) was analyzed for residues and resistant bacteria with levels for OTC (12 µg/kg to 1299 µg/kg, TC (12 µg/kg – 32.4 µg/kg), and FF (10 µg/kg – 525 µg/kg) detected [15]. The drug concentrations were significantly high in smaller ($P < 0.05$) than in medium and larger fish with OTC the most frequently detected. Higher levels were above EU and Brazilian MRLs of 100 µg/kg and 200 µg/kg, respectively. FF was largely in small fish at a concentration over 300 µg/kg [15].

Aeromonas burkholderia,Pasteurella, Pseudomonas and *Streptococcus* were detected, and the MAR index was in the range 0 to 0.86 suggesting bacteria was susceptible to 100% of tested antimicrobials but that some bacteria could be resistant to 86% of the antimicrobials. Further, the bacteria were resistant to STZ and TC (more) with FF to a small extent. The farmers reported use of antimicrobials in fish to prevent/control disease and this could explain the presence of FF and OTC in small fish [15]. The drugs detected in the study area [22] could be transported into the soil/environment [23, 24] and can contribute to resistance in surrounding aquaculture sites or the general aquatic environment [25–31].

Only one organism was isolated in farm 1 with an MAR index of zero unlike in the other farms, where more bacterial were isolated and the MAR index was directly related to the antimicrobials (OTC and FF) commonly used in aquaculture production of crustaceans, tilapia, catfish, lobster, and salmon [32]. It is not uncommon to detect TC and OTC together since TC is a by–product of OTC a fermentation product of *Streptomyces rimosus* [33]. Sulfathiazole was detected, possibly arising from use in human medicine and environmental contamination [29].

The presence of OTC could be attributed to therapeutic and prophylactic use [34]. Since the drug is thought to be less effective in aquaculture production due to poor intestinal absorption [35] its use would be expected to low. Indication of resistance to FF was the lowest and this understandable as the drug remans efficacious in treating pathogenic bacteria in salmon and catfish [36]. For all antimicrobials there was proper correlation between the MAR index and the concentration of the residues farm. Low levels of resistance could be attributed to good practices that may reduce stress or conditions resulting of infections and therefore requiring drug use in aquaculture [28, 37]. This is in addition to reduced use of antimicrobials for prophylaxis in many countries [37].

4. CONCLUSIONS

A study was undertaken to establish potential relationship between antimicrobials in cultured nile tilapia fish and antimicrobial resistance associated with the drugs, including oxytetracycline, tetracycline and florfenicol. Presence of the residues was reported due to use of the drugs to control diseases, especially at early stages of growth. Indices for multiple drug resistance were determined and the levels showed direct correlation with residue concentration. The method used for detecting the residues was validated and is applicable.

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MICROBES, ANTIBIOTIC SUSCEPTIBILITY AND RESISTANCE IN AQUACULTURE PRODUCTION

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Abstract

Integrated fish farming could be a source of resistant pathogenic bacteria in fishponds, which calls for regular assessment of the microbiological quality in such production sites. A study was conducted to assess the microbial contamination of an aquaculture production site in Mfou Cameroon and evaluate the associated antimicrobial resistance profiles. Water, sediment, fishmeal and African catfish, kanga and nile tilapia samples were collected to determine presence of eleven bacterial isolates and fungi, among others. Bacterial isolates from the skin of fish were subjected to penicillin G (10 µg), chloramphenicol (30 µg), sulfamethoxazole/trimethoprim (25 µg), erythromycin (15 µg) and tetracycline (30 µg), using the disk diffusion method. The microbial loads were above the recommended limits suggesting that the fishponds are another potential source of zoonotic pathogens. Multidrug resistance was observed for all isolates with an index above 0.2.

1. INTRODUCTION

Consumer demand for fresh fish continues to rise in Cameroon which has necessitated promoting intensive fish farming [1]. Integrated fish farming including poultry, piggery and crop farming is a common practice [2]. Locally formulated feeds and animal manure are used, although the latter could result in contamination of fishponds with microbial hazards. High bacterial levels in fishponds may impact the water quality and health/safety of the fish [3] as well as fish consumers and the environment in general [3, 4]. Injudicious use of veterinary drugs [2] can also lead to antimicrobial resistance [4–6]. The presence of antimicrobial residues in poultry products, chicken litter/manure confirm the risk associated with resistance [6]. Interventions are required to generate more data and mitigate the problem of antimicrobial resistance. A study [7] was thus undertaken to assess the microbiological quality of fresh fish, pond water, pond sediment and fish all associated with aquaculture production at Mfou in Cameroon.

2. MATERIALS AND METHODS

2.1.Study area, sample collection and processing

An intensive–privately owned fish production farm at Mfou, Mefou Afamba, located 60 km from Yaounde was included in the study [7]. The farm integrated fish farming with poultry, goat, and crop production and the ponds were drained into the surrounding environment. African catfish, kanga and nile tilapia (n = 36, each) were captured from three fishponds and placed in clean and sterile iceboxes containing pond water. The latter (n = 36; duplicates) was collected 10 cm to 15 cm from the surface of three different earthen ponds and placed in 250 ml presterilized bottles. Mud/sediment (n = 36) was collected from the bottom of the ponds and placed in 500 ml sterilized screw capped glass bottles. Fish meal (n=12) was collected from the farmers [7].

Fish were classified by species and source/fishponds, dipped in 70% alcohol for 2 min, then rinsed three times with sterilized distilled water as reported elsewhere [8]. The gills, intestines and skin were ground in a sterile stomacher while the sediment, and fish meal samples were mixed separately in sterile stomachers [7]. Water samples were also mixed in a sterile 1000 ml conical flask.

2.2.Serial dilution of samples and inoculation of media

As detailed elsewhere [7] each solid sample (25 g) and 25 ml of pond water were placed in 225 ml of sterile peptone water and homogenized on a vortex mixer and the solution left to stand at room temperature for 15 min–30 min before mixing 1 ml with 9 ml sterile distilled water to obtain a 10^{-1} dilution. Successive dilution was performed until 10^{-10} dilution. Various culture material including Plate Count Agar (PCA), Sabouraud Agar (SA), Mannitol Salt Agar (MSA), MacConkey Agar (MCA), Eosin Methylene Blue (EMB) were inoculated with the dilution. The pour plate method was used to determine Total Viable Aerobic Bacterial Count (TVAC), Fungal Count (FC), *Staphylococcus aureus* Count (SAC), Total Coliform Count (TCC) and Faecal Coliform Count (FCC). For TCC and FCC, brilliant lactose green broth was first inoculated with the solution and after incubation at 37°C and 44.5°C, respectively for 24 h, the material transferred into MCA and EMB, respectively. Meanwhile, SA was incubated at 30°C for 3–5 days after inoculation.

2.3. Isolation and identification of bacteria from the fish

Bacteria were isolated/identified from fish skin only [7]. Colonies from incubated plates were subcultured on fresh nutrient agar plates and presumptively identified based on morphology, and Gram staining before confirmation. For instance, *S. aureus* was confirmed using oxidase and catalase tests and enterobacteria by API–20 E kit (BioMérieux, France).

2.4. Antimicrobial susceptibility testing

Isolates from the fish were subjected to antimicrobials using the disk diffusion method on MHA (Mueller-Hinton Agar) according to guidelines Clinical and Laboratory Standards Institutes [9] to assess susceptibility [7]. The drugs included: penicillin (P, 10 µg), chloramphenicol (C, 30 µg), sulfamethoxazole–trimethoprim (SXT, 25 µg), erythromycin (E, 15 µg) and tetracycline (TE, 30 µg). Bacterial colonies (4–5) were transferred to test tubes containing 5 ml of sterile physiological water, forming a lawn [7]. The suspension was mixed and poured on to agar plates. Antimicrobial discs with known concentrations were gently pressed onto the agar, with the discs placed 24 mm from each other and from the edge of the plate. The plates were incubated at 37°C for 18–24 h and the zones of inhibition measured and classified as stated elsewhere [7, 9]. The Multiple Antimicrobial Resistance (MAR) index was determined by dividing the number of antimicrobials subject to resistance by the total number of antimicrobials tested [7, 10]. Values above 0.2 indicate isolates from high–risk sources [7, 11].

3. RESULTS AND DISCUSSION

3.1.Microbial load and bacterial isolation/identification

Microbial loads for all fish samples were generally high with TVAC in the range 4.70 log10 CFU/g to 8.49 log10 CFU/g; total FC from <log10 CFU/g to 6.58 log10 CFU/g; TCC: 3.6 log10 CFU/g–7.7 log10 CFU/g; FCC from 3.0 log10 CFU/g to 7.4 log10 CFU/g; SAC from 3.38 log10 CFU/g to 7.14 log10 CFU/g; fish meal: <log10 CFU/g to 7.06 log10 CFU/g. The

microbial loads from the different fish samples and sources were not significantly different ($p > 0.05$), but this was not the case for the total FC and FCC in the intestine gills and skin [7].

Also as detailed elsewhere [7], the mean total viable bacterial count of all fish was above 5.00 log10 CFU/g while the mean FC of water was 3.24 log10 CFU/ml and 2.72 to 3.24 log10 CFU/g for fish. The mean TCC for pondwater (5.13 log10 CFU/ml) and fish (4.47 to 5.06 log10 CFU/g) were above the respective recommended levels of 2–3.7 log10 CFU/ml and 2 log10 CFU/g. The mean FCC of the skin (3.7–3.9 log10 CFU/g) and pondwater (3.76 log10 CFU/ml) were also above respective set levels of 2 log CFU/g and 1–2 log CFU/ml.

The mean SAC for pondwater and skin levels were 4.09 log10 CFU/ml and 4.2–4.7 log10 CFU/g, respectively. The microbial levels in pond sediment (3.56–7.08 log10 CFU/g) and water (3.24–5.75 log10 CFU/ml) were not significantly different. For the fish meal, the mean TCC was 2.6 log10 CFU/g while the FCC was 2.13 log10 CFU/g [7]. The order of prevalence in increasing levels was as follows: *Aeromonas hydrophila*, *Kluyvera* spp., *Moraxella* spp., *Pasteurella multocida* and *Pseudomonas fluorescens* all at 2.4%; *Klebsiella oxytoca* and *Proteus* spp., at 4.7%; *Citrobacter freundii* and *Serratia fonticola* at 7%; *Enterobacter sakazakii* at 19%; *E. coli* at 12%; and *S. aureus* at 21.4% [7].

3.2.Antimicrobial susceptibility

Over 97% of bacteria were susceptible to chloramphenicol while 96% and 100% demonstrated resistance to penicillin G and erythromycin, respectively and 52% to sulfamethoxazole–trimethoprim and tetracycline, all with indices above 0.2 (0.4–1) *E. coli* and *Proteus vulgaris* being the highest at 1.0 [7].

3.3.Significance of microbial contamination of Mfou aquaculture site

The levels of microbes detected [7] indicate that the Mfou fish production farm is highly contaminated with TVAC (a quality indicator) irrespective of fish parts and species was above 5.00 log10 CFU/g the acceptable level [12]. The average TVAC for pond fish/water were higher than those reported elsewhere in Nigeria and Sudan [4, 13, 14]. Pond water and fish also contained unacceptable levels [15] an indication of faecal contamination, possibly due to the proximity of the ponds to residential areas. Such contamination is a common occurrence in integrated fish farming systems [16] and has negative implications to human health and aquaculture production [15]. Urgent action is required especially when acceptable levels 2 log10 CFU/g are exceeded [7, 18].

The diversity of the microbes in fish could be attributed to the high bacterial load in pond water, and as is commonly now observed in aquaculture fish [3]. The presence of enteric bacteria such as *E. coli* and *S. aureus* suggest multisource pollution including sewage effluents and animal/agricultural wastes. Humans are a source of *S. aureus* [18]. Presence of the zoonotic *Aeromonas hydrophila* is not unusual as it is associated with fish mortalities [19–21]. *Pasteurella multocida* in the fish skin is due to infection [21] and is of concern because it affects humans such as in cases of meningitis [22]. The study [7] also detected *Moraxella* sp. an opportunistic organism in fish [23] while *Serratia fonticola* associated with urinary tract infection and of public health relevance [24, 25] was also detected. *Citrobacter freundii*, and *Pseudomonas fluorescens* were also detected as reported before [24, 26]. The presence of *E. coli* and *S. aureus* [7] are of health concern especially when food is not properly cooked and could results in outbreaks [27]. Findings in the current study [7] resembled reports on coastal waters of Southwest Cameroon by Akoachere et al., [28].

3.4.Antimicrobial resistance profile and associated practices

Chloramphenicol showed antimicrobial activity of 97% [7]. The drug is prohibited in food production in many countries due to harmful effects [29]. In Cameroon the drug is not banned although its analogues florfenicol and thiamphenicol are preferred in veterinary practice [30, 31]. Detection of other drugs such as erythromycin was expected due to frequent use [5]. The high prevalence of antimicrobial resistance associated with fish skin could result from exposure to microbes in feed and/or environmental matrices such as sediment [32–35]. Animal waste runoff into the environment may contain antimicrobial residues [6] that could result in multidrug resistance.

The limited involvement of professionals/veterinarians by many farmers in Cameroon [7] may contribute to misuse of agricultural inputs and subsequently to development of antimicrobial resistance [4, 36]. Most of the drugs reported in this study are used in veterinary and human medicine in Cameroon [7, 30, 31].

4. CONCLUSIONS

High levels of microbes exceeding acceptable standards for TVAC, TCC, FCC and SAC were found in fish parts, mud and pond water. The pathogens were resistant to most of the drugs studied. The sources of such pathogens require proper attention. Sterile feed, good quality water, treated wastewater and manure with limited microbial loads should be used in aquaculture farming. Also, frequent assessment of antimicrobial resistance profiles is required to provide additional information on the magnitude of the problem. Further investigation on the possible causes of the high antimicrobial resistance is nevertheless recommended [7].

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ANNEX

MANUAL OF STANDARD OPERATING PROCEDURES (SOPS) FOR TARGETED CHEMICAL AND MICROBIAL CONTAMINANTS IN AQUACULTURE PRODUCTS AND PRODUCTION

Thirty–seven SOPs are reported to facilitate stepwise application analytical methodologies in food safety and control laboratories. These SOPs cover a range of chemical hazards such as veterinary drug residues, selected mycotoxins, toxic metals and some persistent organic pollutants as well as selected microbes. The material result from the methods developed, validated or adapted by participants in the CRP D52039.

The supplementary files for this publication can be found on the publication's individual web page at www.iaea.org/publications.

LIST OF ABBREVIATIONS

AHD	1–Amino Hydantoin
AMOZ	3–Amino–5–morpholino–methyl–1,3–oxazolidinone
AOZ	3–Amino–2–oxazolidinone
CCα	Decision limit
CCβ	Detection capability
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetra acetic acid
FAO	Food and Agricultural Organization of the United Nation
IAEA	International Atomic Energy Agency
LOD	Limit of detection
LOQ	Limit of quantification
LSC	Liquid Scintillation Counter
MRL	Maximum residue limit
MRM	Multiple reaction monitoring
NFs	Nitrofurans
QuEChERS	Quick easy cheap effective rugged and safe
SEM	Semicarbazide

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