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Title: Occurrence of antibiotics in mussels and clams from various FAO areas

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Corresponding Author: Dr. Sara Panseri, Ph.D

Corresponding Author's Institution: Faculty of Veterinary Medicine

First Author: Luca M Chiesa, Associate Professor

Order of Authors: Luca M Chiesa, Associate Professor; Maria Nobile, PhD student; Renato Malandra; Sara Panseri, Ph.D; Francesco Arioli, Assistant professor

Abstract: Filter feeders, like mussels and clams, are suitable bioindicators of environmental pollution. These shellfish, when destined for human consumption, undergo a depuration step that aims to nullify their pathogenic microorganism load and decrease chemical contamination. Nevertheless, the lack of contamination by drugs may not be guaranteed. Antimicrobials are a class of drugs of particular concern due to the increasing phenomenon of antibiotic resistance. Their use in breeding and aquaculture is a major cause of this. We developed a multiclass method for the HPLC-MS/MS analysis of 29 antimicrobials, validated according to the Commission Decision 2002/657/UE guidelines, and applied it to 50 mussel and 50 clam samples derived from various Food and Agricultural Organisation marine zones. The results obtained, indicate a negligible presence of antibiotics. Just one clam sample showed the presence of oxytetracycline at a concentration slightly higher than the European Union Maximum residue limit set for fish.

*Cover Letter after your requests

UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE VETERINARIE
PER LA SALUTE, LA PRODUZIONE ANIMALE
E LA SICUREZZA ALIMENTARE

Dear Sirs,

The topic of antibiotic resistance drives more and more the search for contaminants in food towards this class of pharmacological compounds. Although bivalve molluscs are bred offshore, where the antibiotics, eventually illicitly used, could undergo a drastic dilution, these filter-feeding animals, may constitute a means for the bio accumulation of antimicrobials and the distribution through the food chain from the aquatic environment to consumers. We used a validated multiclass method for the HPLC-MS/MS analysis of 29 antimicrobials, and the results of our work are very reassuring for the consumer indicating a very low presence and frequency of antibiotics in the edible tissue of mussels and clams; at the same time it could be hypothesized that the illegal treatment takes place in the purification stage prior to sale.

The provenience of the molluscs from various FAO marine areas, even if mostly from the Mediterranean Sea, could be an added value of the study, also accounting that the samples were collected at the Milan fishery market, which supplies all Italy, so being a verisimilar representation of the molluscs consumed by Italian people.

Before the submission, the British English was checked and revised by Proof-reading.com.

Kind regards

Sara Panseri

Department of Health, Animal Science and Food Safety

University of Milan

Via Celoria, 10

20133 Milan

Italy

PS; after your request we referenced the 10% similarities you found with iThenticate. The citations were two: all belonging to our research group and dealt with Materials and Methods. This was an imprecision but, as an excusatory, we did not try to auto-cite at any cost. Obviously we added in references the two works

***Response to Reviewers**

X-June 2017

Ms. Ref. No.: **FOODCHEM-D-17-02357**
Title: "Occurrence of antibiotics in mussels and clams from various FAO areas Food Chemistry"

Dear Professor,

Gordon Birch, Ph.D

Receiving Editor, *Food Chemistry*

We are very grateful to Editor for the opportunity to improve our research and to the reviewers for their helpful comments. We carefully followed their suggestions as well as the *Food Chemistry* author guidelines in order to make the manuscript more clear and as complete as possible. Therefore, the manuscript was managed as indicated by the reviewers and the editor. A professional English editor carefully revised the manuscript.

We hope that the reviewer's suggestions are satisfied and the manuscript is now suitable for publication in *Food Chemistry*.

The answers to the reviewer's questions are listed below according to each raised point and highlighted in red in the paper.

Best regards,

The Authors

Reviewers' comments:

Reviewer #1:

The authors developed a method using HPLC-MS/MS for analysis of 29 antimicrobials in mussel and clam samples and found a negligible presence of antibiotics. The method detection limits of the targets should better be listed. Also, the novelty of this paper should be made clear. Other comments are listed below:

Answer: All method detection's limits are reported in Table 2 as CCα and CCβ, and we corrected the ng g^{-1} by adding also wet weight.

As regards the novelty, the aim of the study was clarified in the last part of introduction and in the conclusions. Briefly, the present method is a multiclass protocol for the detection of 29 antibiotics of 8 different classes (in literature only few antibiotics, amongst other contaminants, or only a class of antibiotics are usually monitored), moreover our detection limits are much lower than the MRLs, so it is useful to increase the proportion of quantified data and accurately monitor the presence of antibiotics due to the antibiotic resistance matter.

1. Highlights: The first three highlights were not the findings of this paper. It should be recognized.

Answer: The first three highlights were modified in agreement with the study:

A multiclass LC-MS/MS method for 29 antibiotics was developed and validated.
Our detection limits were much lower than the maximum residue limits.
Pool of mussels and clams from different FAO zones were analysed.

2. Line 198: The 1 g aliquot is wet weight or dry weight? Please give more information on how the sample be homogenized.

Answer: The 1 g aliquot is referred to wet weight so we added this information on line 198. We also precised, at line 148, how the homogenization was done.

3. List the detected samples and specify the levels compared to previous publications.

Answer: The list of detected samples, their provenience and the calculated concentration in ng g^{-1} wet weight was reported in Table 3. Our detected levels were then compared at the end of Section 3.2, (line 324) by adding: "Low antibiotic concentrations were also reported in the study of Dodder et al. (2014), where they studied and found only few target antibiotics (lomefloxacin, enrofloxacin, sulfamethazine and erythromycin at the mean concentrations of 29, 1.3, 24 and 0.14 ng g^{-1} dry weight, respectively) but with a higher detection frequency from 17 to 94 % related to 68 mussel sampling stations of the coast of California collected from November 2009 and April 2010. Our results were reassuring if compared with the study of Li et al. (2012), where

all 22 target antibiotics of three classes, except tylosin were detected in the 190 molluscs samples of Bohai Sea of China. Their results, showed quinolones as the major compounds with concentrations of 0.71-1575.10 μg kg^{-1}, which were up to two orders of magnitude higher than those of sulphonamides (0-76.75 μg kg^{-1}) and macrolides (0-36.21 μg kg^{-1}). But in that study, they didn't discriminate the different antibiotics among the different molluscs analysed."

4. More publications should be cited to strengthen the research background: Rapid and sensitive determination of phytosterols in functional foods and medicinal herbs by using UHPLC-MS/MS with …….JOURNAL OF SEPARATION SCIENCE Volume: 40 Issue: 3 Pages: 725-732 Published: FEB 2017; 2.Simultaneous Determination of Food-Related Biogenic Amines and Precursor Amino Acids Using …….JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY Volume: 64 Issue: 43 Pages: 8225-8234 Published: NOV 2 2016; 3….Ultra-high-performance liquid chromatography-tandem triple quadrupole mass spectrometry, LIMNOLOGY AND OCEANOGRAPHY-METHODS Volume: 14 Issue: 10 Pages: 623-636 Published: OCT 2016; 4. Determination of six sulfonylurea herbicides in environmental water samples …….,JOURNAL OF CHROMATOGRAPHY A Volume: 1466 Pages: 12-20 Published: SEP 30 2016; 5.Graphene oxide-based microspheres for the dispersive solid-phase extraction of non-steroidal estrogens from water samples, JOURNAL OF CHROMATOGRAPHY A Volume: 1368 Pages: 18-25 Published: NOV 14 2014; 6.Salting-out assisted liquid-liquid extraction with the aid of experimental design for determination of …….TALANTA Volume: 106 Pages: 119-126 Published: MAR 15 2013.

Answer: we have added other citations for comparison with the results obtained in the "3.2 Investigation on clams and mussels from the food chain" Section to make the paper complete as possible.

Reviewer #2:

The Authors report the presence of antibiotics in shellfish specimens from various FAO areas in Italy. The topic is interesting because these findings give data of the diffusion and the use of drugs and pone problems of the risk for the environment and human health. However, the contribution is not completely clear and fluent. All the sections need to be better revised and focused on the real problem of the presence of the antibiotics. Although the contribution is interesting, the manuscript needs to be better review before it can be considered for publication.

SPECIFIC COMMENTS

Pages 3-7: The Introduction is very long and describes in a detailed way the antibiotic resistance story although the antibiotic resistance phenomenon is already known since years. Moreover the aim of the paper is not enough clear based of what written in the introduction. The Authors should carefully review the introduction, cut the paragraphs not useful and better focusing on the aim of the paper.

Answer: The introduction was carefully review, deleting some not useful paragraphs and clarifying the aim, as already made to answer Reviewer #1. Briefly, our method represent a multiclass protocol for the detection of 29 antibiotics of 8 different classes (in literature only few antibiotics, amongst other contaminants, or only a class of antibiotics is monitored), moreover we had detection limits much lower than the MRLs, so it's useful to increase the proportion of quantified data and accurately monitor the presence of antibiotics due to the antibiotic resistance matter.
The first part of Introduction was eliminated and from line 134 was deeply changed.

Page 8 line 183: The Authors have worked with two working solution (10 and 100 ng/ml). It is not clear why they have chosen these two concentrations. Is this the range of concentration they expected to find in the shellfish? Is 10 ng/ml the lowest concentration detected by HPLC?

Answer: the working solution at 10 and 100 ng/ml (now line 140) were chosen as the better concentration to spike blank samples during validation at the three validation levels reported in Table 2 and also for the construction of calibration curves for the samples quantification. We clarified this point in line 140. Moreover, the lowest concentrations detected in our case are indicated as C0 in 2.6. Method validation Section and showed for all analytes as the first validation levels in Table 2.

Pag 9 line 189: It is not clear if the number of 50 is refers to the total of collected mussels and/or clams or 50 are the number for each species of mussels and clams. How many specimens are wild and how many are farmed? The Authors should explain what was the criterion to select wild and farmed specimens (different geographical location, different antibiotics treatment, presence of fish and livestock farms). The Authors should better describe the marine zone and the relative specimens collected.

Answer: 50 is referred to the total of sample (500g each one) collected both for mussel and clam samples. Each sample was constituted of a pool of 200g of edible part, as written before. The samples were wild and farmed (50:50). The choice was based to evaluate the presence of antibiotics due to an eventual antibiotic treatment in farms and/or the presence of antibiotics due to the environmental pollution in case of wild shellfish. Moreover they were collected from various Food and Agricultural Organisation (FAO) marine zones (Fig. 1) to evaluate the antibiotic detection relatively to the different geographical location. The samples were also collected from different marine layers because mussels tend to grow on the surface of wave-washed rocks, while clams live in shallow water, so in depth. All these details were added in the "Sample collection" Section.

Page 3 line 198: How many pools were created? How many specimens for pool?

We created a total of 100 pools (50 for mussels and 50 for clams, each pool consisted of 200 g specimen). It's reported in Sample collection Section, better clarified.

Pag 11 line 243: it is not clear the meaning of the sentence " after the identification of blank

samples". Have the Authors studied the matrix effect working with the samples without added stardard? What are the criteria the Authors have chosen 20 blank samples?

Answer: "Blank samples" means that we have identified samples without presence of antibiotics, through a preliminary screening of pooled mussel or clam samples, as written before. We adjusted the sentence in this way: "After the identification of samples in which we checked the absence of antibiotics…".We used 20 blank samples as indicated in the guidelines for validation reported by Commission Decision 657/2002/CE. In fact, we specified the correct reference before.

Pages 14-16: The discussions should be better commented at the light of the obtained results. The finding of antibiotics in pools of clams and mussels in North Adriatic Sea should be correlated with the presence of fishing farms and/or livestock farms in that area. Moreover some comments on the potential risk for the environment and the human health should be added and discuss.

Answer: We commented the results compared to previous publications, as requested also by Reviewer #1.The finding of antibiotics in pools of farmed clams and mussels in North Adriatic Sea should be correlated to an intentional treatment, supported by Cabello (2006), about the well-known heavy prophylactic use of antibiotics in aquaculture, already reported before. We added in line 281 this sentence: "The finding of the four tetracyclines in this pool of farmed clams should be correlated with an intentional treatment."

As regards the human health we can say that the MRLs, are slightly exceeded only in one case, as already elucidated. Finally, in the light of our results, we can say that the MRLs, are slightly exceeded only in one clam sample, as already elucidated above. However, considering the annual Per capita consumption of 0.33 Kg clams (European Commission, 2016), the daily consumption is 0.91 g; the result of the multiplication of this value by the sum of the concentrations of the four tetracyclines (312.41 ng g^{-1}) found in the clam sample of North Adriatic Sea, is 0.29 µg day^{-1}. This datum could represents a risk mainly associated with the increase of antibiotic resistance phenomenon. Instead, due to the lack of detections, we cannot estimate a potential risk for the environment.

These last considerations are inserted into the manuscript.

OTHER COMMENTS

English
In general, the English is clear and correct, but the work still requires revision by a native speaker in order to eliminate some minor inaccuracies and stylistic errors e.g. incorrect in formal writing.

Answer: The work was sent to a proof reader before the submission, as usually done.

Occurrence of antibiotics in mussels and clams from various FAO areas

Luca Maria Chiesa [a], Maria Nobile [a], Renato Malandra [b], Sara Panseri [a,*], Francesco Arioli [a]

[a] Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy

[b] ATS Milano-Città metropolitana, Director of Veterinary Unit, Via Celoria 10, 20133 Milan, Italy

* Corresponding author: Sara Panseri, Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133 Milan, Italy. Phone: 0250317931; Fax: 00390250317941; E-mail: sara.panseri@unimi.it

ABSTRACT

Filter feeders, like mussels and clams, are suitable bioindicators of environmental pollution. These shellfish, when destined for human consumption, undergo a depuration step that aims to nullify their pathogenic microorganism load and decrease chemical contamination. Nevertheless, the lack of contamination by drugs may not be guaranteed. Antimicrobials are a class of drugs of particular concern due to the increasing phenomenon of antibiotic resistance. Their use in breeding and aquaculture is a major cause of this. We developed a multiclass method for the HPLC-MS/MS analysis of 29 antimicrobials, validated according to the Commission Decision 2002/657/UE guidelines, and applied it to 50 mussel and 50 clam samples derived from various Food and Agricultural Organisation marine zones. The results obtained, indicate a negligible presence of antibiotics. Just one clam sample showed the presence of oxytetracycline at a concentration slightly higher than the European Union Maximum residue limit set for fish.

Keywords: Antibiotics, Clam, HPLC-MS/MS, Mussel

Chemical compounds studied in this article

Amoxicillin (PubChem CID: 33613); Ampicillin (PubChem CID: 6249); Benzylpenicillin (PubChem CID: 5904); Cefalexin (PubChem CID: 27447); Cefquinome sulphate (PubChem CID: 9577261); Chloramphenicol (PubChem CID:5959); Chlortetracycline (PubChem CID: 54737570); Ciprofloxacin (PubChem CID: 2764); Cloxacillin (PubChem

CID: 6098); Dicloxacillin (PubChem CID: 18381); Doxycycline hyclate (PubChem CID: 54686183); Enrofloxacin (PubChem CID: 71188); Erythromycin (PubChem CID: 12560); Florfenicol (PubChem CID: 114811); Florfenicol amine (PubChem CID: 156406); Flumequine (PubChem CID: 3374); Lincomycin (PubChem CID: 3000540); Lomefloxacin hydrochloride (PubChem CID: 68624); Marbofloxacin (PubChem CID: 60651); Nalidixic acid (PubChem CID: 4421); Oxolinic acid (PubChem CID: 4628); Oxytetracycline (PubChem CID: 54675779); Sulphadiazine (PubChem CID: 441244); Sulphadimethoxine (PubChem CID: 5323); Sulphadimidine (PubChem CID: 5327); Sulphathiazole (PubChem CID: 5340); Tetracycline hydrochloride (PubChem CID: 54704426); Trimethoprim (PubChem CID: 5578); Tylosin (PubChem CID: 5280440).

1. Introduction

Antibiotics are among the most frequently detected group of potentially toxic pharmaceuticals; this underscores the following ecotoxicological concerns: 1) the cumulative toxic effects of antibiotics on aquatic animals are not well understood, 2) their continuous presence leads to the development of antibiotic-resistant bacteria, and 3) antibiotics can act, at very low concentrations, as signalling agents and change the natural microbial diversity in aquatic ecosystems (Fatta-Kassinos, Meric, & Nikolaou, 2011).An unknown amount of these drugs ends up either indirectly in the receiving waters, through sewer plants and land-fields, or directly as a result of intensive fish farming. For these reasons, organisms could also be exposed to a variety of compounds present in the environment at low concentrations. In recent years, pharmacological substances in the aquatic environment have become an increasing

concern. In this respect, municipal wastewater effluents represent the main source of pharmaceuticals in the environment (Kolpin et al., 2002).

Bivalves and the blue mussel (*Mytilus edulis*), in particular, are successfully used as indicator organisms for marine pollution monitoring (Baumard Budzinski, & Garrigues, 1998; O'Connor, 1998; Widdows et al., 1995). The general assumption is that mussel appears to be an appropriate sentinel organism because of its global distribution of large and accessible populations, its large size and sedentary adulthood, its tolerance to diverse environmental conditions, the ventilation of large volumes of water for nutrition, respiration and excretion (Krieger, Gee, & Lim, 1981), and its ability to accumulate numerous contaminants (Moy & Walday, 1996).

Hence, an increasing demand for biological studies of aquatic organisms has become a major impetus for the development and validation of high-performing analytical techniques capable of determining various antibiotics. Zouiten, Beltifa, Van Loco, Mansour and Reyns (2016) demonstrated the usefulness of ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to detect certain antibiotic residues in *Mytilus galloprovincialis* exposed to pharmaceutical wastewater in Tunisia. Li, Shi, Gao, Liu and Cai (2012) reported 22 antibiotics in molluscs obtained from the Bohai sea (China), based on accelerated solvent extraction pressurised liquid extraction, followed by a solid-phase extraction (SPE) clean-up. An enzymatic-microwave assisted extraction method with subsequent high-performance liquid chromatography (HPLC) was developed for the determination of 11 antibiotics in fish tissue and mussels of Spain (Fernandez-Torres, Lopez, Consentino, Mochon, & Payan, 2011). Conversely, Le Bris and Pouliquen (2004) studied the bioaccumulation of two

antibiotics, oxytetracycline and oxolinic acid, by the blue mussel, and stated that most veterinary and human antibiotics, such as tetracyclines and sulphonamides, should weakly accumulate in mussel.

In this context, the current study aimed to develop and validate (European Community, 2002; European Union, 2008) a sensitive, specific and robust HPLC-MS/MS multiclass method, for the determination of 29 antibiotics belonging to eight different chemical classes (penicillin, quinolones, tetracyclines, sulphonamides, macrolides, lincosamides, cephalosporins, amphenicols), in mussels and clams, both wild and farmed, collected from various geographic areas of the world and, particularly, Italy. The two types of shellfish were carefully selected for a comparison, considering that mussels tend to grow on the surface of wave-washed rocks, while clams live in shallow water. Hence, the development of a high sensitive multiclass method for antibiotics in this two edible organisms located from distinct areas and marine layers, and the differences in bioaccumulation between these organisms could be achieved to expand the knowledge from the point of view of food safety, relatively also to environmental contamination, to increase the proportion of quantified data and accurately monitor the presence of antibiotics due to the antibiotic resistance matter.

2. Material and methods

2.1. Chemicals and reagents

All solvents were of HPLC or analytical grade and were purchased from Fluka (Sigma-Aldrich, St. Louis, MO, USA). Formic acid (98–100%) was obtained from Riedel-

de Haën (Sigma-Aldrich, St. Louis, MO, USA). Trichloroacetic acid (TCA) crystals and the ingredients required to prepare EDTA-McIlvaine buffer solution, pH 4 (disodium hydrogen phosphate dihydrate, citric acid monohydrate and EDTA) were purchased from Fluka. Water was purified by a Milli-Q system (Millipore, Merck KGaA, Darmstadt, Germany). The extraction cartridges (Oasis HLB 3 mL, 60 mg) were provided by Waters (Milford, MA, USA). Amoxicillin, ampicillin, cloxacillin, dicloxacillin, benzylpenicillin, oxolinic acid, nalidixic acid, cefquinome sulphate, cefalexin, florfenicol, florfenicol amine, chloramphenicol, flumequine, lomefloxacin hydrochloride, ciprofloxacin, enrofloxacin, marbofloxacin, tetracycline hydrochloride, doxycycline hyclate, chlortetracycline hydrochloride, oxytetracycline, lincomycin, sulphathiazole, sulphadimidine, sulphadiazine, sulphadimethoxine, trimethoprim, erythromycin, tylosin and enrofloxacin d5 as the internal standards (IS) were purchased from Fluka.

2.2. Standard solutions

For each standard, stock solutions were prepared (1 mg mL^{-1}) in methanol and kept at -20 °C. Working solutions at 10 and 100 ng mL^{-1}, were prepared daily to spike the samples during the validation and to construct the calibration curves for the quantification of the real samples. Each working solution was maintained at 4 °C during the method validation procedures.

2.3. Sample collection

We collected a total 100 samples (500 g each one), and we created 100 pools obtained by dispersing 200 g of shellfish edible parts pooled by using an Ultraturrax (IKA®-Werke GmbH and Co. KG, Staufen, Germany) at 13500 rpm for 4 minutes. Mussels (a total of 50 pool samples of three species*: M. galloprovincialis*, *Mytilus edulis* and *Mytilus chilensis*) and clams (a total of 50 pool samples of six species*: Meretrix lyrata*, *Venerupis decussata*, *Venerupis philippinarum*, *Meretrix meretrix*, *Paphia textile* and *Venus gallina*), half wild and half farmed to evaluate the presence of antibiotics due to eventual antibiotic treatments in farms and/or the presence of these drugs due the environmental pollution in case of wild shellfish. Moreover they were collected from various Food and Agricultural Organisation (FAO) marine zones (Fig. 1) to evaluate the antibiotic detection relatively to the different geographical location. The samples were also collected from different marine layers because mussels tend to grow on the surface of wave-washed rocks, while clams live in shallow water so in depth. The samples were immediately frozen, transported to the laboratory and stored at -20 °C, until further analysis.

2.4. Sample extraction

An aliquot (1 g wet weight) of homogenised shelled mussel or clam, spiked with the IS at a final 2 ng mL^{-1}, 100 µl of 20% TCA for protein precipitation, and 5 mL McIlvaine buffer (pH 4.0), were combined. The samples were vortexed and sonicated for 15 min. After centrifugation (2500*g*, 4 °C, 10 min), the supernatant was transferred to a clean polytetrafluoroethylene centrifuge tube and defatted with 2 × 3 mL n-hexane.

Each time, the n-hexane layer was discarded after centrifugation at 2500*g*, 4 °C for 5 min. The obtained extracts were purified by SPE Oasis HLB cartridges under vacuum. The SPE cartridges were preconditioned with 3 mL methanol and 3 mL Milli-Q water. The samples were loaded, and then washed with 2 x 3 mL methanol:water (5:95 v/v). Finally, the analytes were eluted with 5 mL methanol and collected in a 15-mL glass tube. The eluate was evaporated in a rotary vacuum evaporator at 40 °C. The dried extract was reconstituted in 200 µL methanol:water (10:90 v/v), and then transferred to an auto-sampler vial. The injection volume was 10 µL.

2.5. HPLC-MS/MS analyses

The chromatographic separation was performed by a Surveyor MS quaternary pump with a degasser, a Rheodyne valve with a 20-µL loop and a Surveyor AS autosampler with a column oven (Thermo Fisher Scientific, San Jose, CA, USA). Chromatographic separation of the compounds was obtained using a Synergi Hydro-RP reverse-phase HPLC column (150 x 2.0 mm, internal diameter 4 µm), with a C18 guard column (4 x 3.0 mm; Phenomenex, Torrance, CA, USA). The mobile phase was a binary mixture of solvents A (aqueous formic acid 0.1%) and B (methanol). The run (0.2 mL min^{-1}) started with 98% A (5 min), which was then increased linearly to 50% (at 22 min). Next, mobile phase B was gradually increased to 95% (at 24 min) and remained constant for 5 min. The initial conditions were reached at 31 min, with an equilibration time that included the interval from 31–40 min. A triple-quadrupole TSQ Quantum MS (Thermo Fisher) equipped with an electrospray interface (ESI) set in the positive (ESI+)

mode was used to detect all analytes, except isoxazolyl penicillins and amphenicols, which were detected in the negative (ESI-) mode. Acquisition parameters were optimised by direct continuous pump-syringe infusion of the standard analyte solutions at 1 µg mL^{-1}. The flow rate was set at 20 µL min^{-1} flow rate, and the MS pump rate at 100 µL min^{-1}. The following conditions were used: capillary voltage 3.5 kV; ion transfer capillary temperature 340 °C; nitrogen as the sheath and auxiliary gases at 30 and 10 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr, and peak resolution 0.70 Da at full-width half-maximum (FWHM) (Chiesa et al., 2016). Three diagnostic product ions were chosen for each analyte and IS, as carried out in an our previous styudy about antibiotics in bovine urine (Chiesa et al., 2015). The acquisition was performed in multiple reaction-monitoring (MRM) mode. The selected diagnostic ions, one of which was chosen for the quantification, the collision energies and the relative intensities are reported in Table 1. Acquisition data were recorded and elaborated using Xcalibur™ software from Thermo Fisher.

2.6. Method validation

After the identification of samples in which we checked the absence of antibiotics, through a preliminary screening of pooled mussel or clam samples, the method was validated according to the Commission Decision 2002/657/EC criteria (European Community, 2002).

For each analyte, the method performance was evaluated by the determination of retention time (RT), transition ion ratios, recovery, accuracy (trueness), precision

(expressed as the intra- and inter-day repeatability), linearity, as well as the decision limit (CCα) and detection capability (CCβ), which were calculated as described in SANCO/2004/2726 revision 4 (European Union, 2008).

Twenty blank samples were used to evaluate the specificity and to check for any interference (signals, peaks, ion traces) in the region of interest where the target analytes were expected to elute. The selectivity was also tested by verifying a signal-to-noise ratio > 3 at the expected RT, and the ion abundance ratio associated with the different fragmentations. Validation was done by spiking the samples with all analytes at three concentration levels (C_0, 2 x C_0, 3 x C_0, validation levels Table 2) that were previously chosen according to a minimum detectable experimental concentration (C_0) in our conditions, considering that the maximum residue limits (MRLs) recommended by the Commission Regulation 37/2010 (European Union, 2010) for fish (but not for shellfish) range from 50–200 µg kg^{-1}. Each level had six replicates. The validation trials were repeated for three different days, resulting in three analytical series (matrix validation curves).

The instrumental linearity was also assessed through six-point calibration curves in the solvent containing a precise amount of IS (2 ng mL^{-1}), starting from the minimum detectable concentration for each group up to 100 ng mL^{-1}.

The recovery was calculated using the data from the validation points of the three, analytical series, expressed as a percentage of the measured concentration relative to the spiked concentration. The precision (intra- and inter-day repeatability) was evaluated by calculating the relative standard deviation of the results obtained for six replicates of each analyte at the three concentration levels of the three, analytical

series. Robustness was assessed using the approach of Youden (European Union, 2002), which is a fractional factorial design, based on minor modification (±10%) of seven experimental conditions of eight samples spiked at the minimum detectable concentrations.

Matrix effects was evaluated by Matuszewski, Constanzer and Chavez-Eng (2003) strategy, comparing the analytes of interest added post-extraction with pure solutions prepared in the mobile phase containing an equivalent amounts of the studied compounds.

3. Results and discussion

3.1 Validation performances

The selectivity of the method, assessed by injecting blank samples (20 mussel and 20 clam samples), did not show any interference (signals, peaks, ion traces) in the region of interest, i.e. where the target analytes were expected to be eluted. The selectivity also showed a good compliance with the relative RTs for each analyte, which were found to be within 2.5% tolerance, when compared with the standards, with peaks having a signal-to-noise ratio > 3. Moreover, the three chosen transitions showed an ion ratio within the recommended tolerances (European Union, 2002), when compared with the standards. The mean recoveries for all analytes ranged between 86–113%. The matrix validation curves also demonstrated a good fit for all analytes, with correlation coefficients > 0.99.

256 The intra- and inter-day repeatability values, which were calculated using one-
257 way analysis of variance and expressed as coefficients of variation, were below 14 and
258 20%, respectively. These values were lower than the variability of 22% indicated by
259 Thompson (2000). The CCα ranged from 0.51–5.76 ng g^{-1} wet weight, and CCβ values
260 from 0.65–5.93 ng g^{-1} wet weight (Table 2). Also, the method ruggedness was good in
261 the considered matrices. A modest matrix effect was found, with values ranging from
262 86–115% for the various compounds in the mussel and clam samples.
263
264 *3.2 Investigation on clams and mussels from the food chain*
265
266 The developed and validated method was applied to the analyses of 50 mussel
267 and 50 clam pooled samples, both wild and farmed, collected from various FAO zones
268 and locations within Italy. The samples were completely anonymous and randomly
269 collected from the food chain. Four tetracyclines (49.45 ng g^{-1} tetracycline, 125.03 ng g^{-1}
270 oxytetracycline, 60.45 ng g^{-1} doxycycline and 77.48 ng g^{-1} chlortetracycline) were
271 detected in one pool of farmed clams obtained from the Italian side of the North Adriatic
272 Sea. Figure 2 presents the chromatograms and the MS spectra of the four tetracyclines
273 detected in this pool, as an example. In this instance, the oxytetracycline concentration
274 was higher than the MRL of 100 ng g^{-1} (European Union, 2010) set for fish. The finding
275 of the four tetracyclines in this pool of farmed clams should be correlated with an
276 intentional treatment. Tetracycline was also found, at low concentration (0.55 ng g^{-1}) in
277 a pool of farmed mussels from Atlantic Spain, depurated in a plant in North Italy. The
278 quinolone, flumequine, was found in two other pools, one of mussels (3.59 ng g^{-1}) and

one of clams (0.84 ng g^{-1}), from two different Italian farms in the North Adriatic Sea. In these instances, the detection of antibiotics concerned only farmed mussels or clams. As stated by Cabello (2006), the heavy prophylactic use of antibiotics in aquaculture is well known.

Among the various antibiotics used in fish treatments, oxytetracycline is commonly prescribed against bacterial diseases for its wide antibacterial spectrum, its potency and its low cost. Doses usually administered by fish farmers are often higher than the recommended 50–100 mg kg^{-1} fish day^{-1}, for 7–10 d (Le Bris, Pouliquen, Debernardi, Buchet, & Pinault, 1995).

In the European Union, the cultivation methods of shellfish, with some minor differences, provide the distribution of juvenile molluscs on structures located in the open sea (Baylon, 1990). The use of antibiotics in these conditions would predictably lead to a dilution of these drugs, minimising their effect. After a period of about 20 months, before they are sold, the shellfish must undergo a depuration (few hours to days) in filtered and daily renewed seawater or in natural sites that meet the requirements of the EC Regulation No 853/2004 regarding the microbiological characteristics, chemical pollution and biotoxins present in the water of the culture area (European Union, 2004). The detection of four positive samples out of 100 (just one of which was non-compliant), seemed to confirm the previous statement on the possibility of antibiotic dilution in the open sea and the efficacy of the depuration treatment. It is moreover conceivable an illicit use of antimicrobials in the depuration step, to diminish or nullify the bacterial load in shellfish. The presence of tetracycline in a pool of mussels grown in Atlantic Spain and depurated in a plant of North Italy, suggested illegal practice

had occurred because the antimicrobial was only detected in the shellfish from Italy. Conversely, oxytetracycline and oxolinic acid are bioaccumulated by the blue mussels (Le Bris & Pouliquen, 2004) and this observation could provide an alternative explanation for the presence of tetracyclines in mussels. Moreover, the availability of oxytetracycline from sediment, the formation of complexes between this antibiotic and some mineral or organic components of the bivalves, and their low xenobiotic metabolism, as proved in the study of Le Bris et al. (1995) could explain the persistence of oxytetracycline in shellfish and consequently our results. The relatively stable oxytetracycline concentration in the clam *Scrobicularia plana* (up to 20 d) (Le Bris et al.,1995), supports the highest concentration of tetracyclines detected in one of our clam samples, particularly, considering they are grown “on land” between mud and sediments, a favourable environment for oxytetracycline accessibility, as above-mentioned and that the depuration of shellfish lasts around 48 h, explaining the persistence of this antibiotic. Finally, because of the scarcity of positive samples, no argumentation could be made about the differences between species and marine layer. Low antibiotic concentrations were also reported in the study of Dodder et al. (2014), where they studied and found only few target antibiotics (lomefloxacin, enrofloxacin, sulfamethazine and erythromycin at the mean concentrations of 29, 1.3, 24 and 0.14 ng g^{-1} dry weight, respectively) but with a higher detection frequency from 17 to 94 % related to 68 mussel sampling stations of the coast of California collected from November 2009 and April 2010. Our results were reassuring if compared with the study of Li et al. (2012), where all 22 target antibiotics of three classes, except tylosin were detected in the 190 molluscs samples of Bohai Sea of China. Their results, showed

quinolones as the major compounds with concentrations of 0.71-1575.10 µg kg^{-1}, which were up to two orders of magnitude higher than those of sulphonamides (0-76.75 µg kg^{-1}) and macrolides (0-36.21 µg kg^{-1}). But in that study, they didn't discriminate the different antibiotics among the different molluscs analysed.

Finally, in the light of our results, we can say that the MRLs, are slightly exceeded only in one clam sample, as already elucidated above. However, considering the annual Per capita consumption of 0.33 Kg clams (European Commission, 2016), the daily consumption is 0.91 g; the result of the multiplication of this value by the sum of the concentrations of the four tetracyclines (312.41 ng g^{-1}) found in the clam sample of North Adriatic Sea, is 0.29 µg day^{-1}. This datum could represents a risk mainly associated with the increase of antibiotic resistance phenomenon. Instead, due to the lack of detections, we cannot estimate a potential risk for the environment.

4. Conclusions

In this study we developed, optimised and validated a multiclass HPLC-MS/MS method for analysis of 29 antibiotics, belonging to eight different chemical classes, in mussel and clam samples. The aim was to monitor the eventual presence of antibiotics in various FAO marine zones, with particular attention on Italian seas, considering that antibiotic occurrence is available in wastewater. The two different matrices, mussels and clams never compared before, were chosen to study antibiotic bioaccumulation in distinct marine layers, given that the first grow, primarily, on the surface and the second in shallow. Even if the method had detection limits well lower than the MRLs, useful to

increase the proportion of quantified data and accurately monitor the presence of antibiotics due to the antibiotic resistance matter, only few detections had been registered, although, in one instance, the oxytetracycline content was higher than the MRL recommended for fish.

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Conflict of Interest

The authors confirm that there are no known conflicts of interest associated with this publication.

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List of figures

468 **Table 1.** MS/MS conditions for the MRM acquisitions of investigated antibiotics.

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Analyte	Precursor ion (m/z)	Product ions CE (m/z)	ESI
amoxicillin	366	**4(80)$_{20}$**, 134(21)$_{31}$, 349(100)$_{7}$	(+)
ampicillin	350	**106(100)$_{18}$**, 114(14)$_{29}$, 160(14)$_{14}$	(+)
cloxacillin	436	160(48)$_{13}$, 178(35)$_{33}$, **277(100)$_{14}$**	(-)
dicloxacillin	468	291(100)$_{21}$, **327(63)$_{16}$**, 424(32)$_{12}$	(-)
benzylpenicillin	335	114(61)$_{32}$, 160(92)$_{12}$, **176(100)$_{14}$**	(+)
oxolinic acid	262	160(5)$_{35}$, **216(10)$_{29}$**, 244(100)$_{18}$	(+)
nalidixic acid	233	159(22)$_{33}$, 187(69)$_{26}$, **215(100)$_{16}$**	(+)
cefalexin	348	158(63)$_{5}$, **174(100)$_{15}$**, 191(23)$_{6}$	(+)
cefquinome	529	**134(100)$_{15}$**, 324(43)$_{15}$, 396(44)$_{10}$	(+)
ciprofloxacin	332	(16)$_{22}$, **288(100)$_{17}$**, 314(94)$_{21}$	(+)
enrofloxacin	360	245(49)$_{26}$, **316(100)$_{18}$**, 342(29)$_{21}$	(+)
lomefloxacin	352	**265(100)$_{23}$**, 288(16)$_{19}$, 308(63)$_{16}$	(+)
marbofloxacin	363	72(83)$_{23}$, **320(100)$_{15}$**, 345(18)$_{21}$	(+)
florfenicol	356	169(1)$_{39}$, 185(35)$_{21}$, **336(100)$_{12}$**	(-)
florfenicol amine	248	130(24)$_{23}$, 134(8)$_{28}$, **230(100)$_{11}$**	(+)
chloramphenicol	321	152(65)$_{20}$, 194(35)$_{16}$, **257(100)$_{14}$**	(-)
flumequine	262	174(13)$_{39}$, **202(54)$_{32}$**, 244(100)$_{19}$	(+)
chlortetracycline	479	154(39)$_{27}$, **444(100)$_{21}$**, 462(69)$_{16}$	(+)
doxycycline	445	321(10)$_{31}$, 410(10)$_{24}$, **428(100)$_{19}$**	(+)
oxytetracycline	461	337(26)$_{29}$, **426(100)$_{19}$**, 443(52)$_{12}$	(+)
tetracycline	445	154(38)$_{30}$, **410(100)$_{19}$**, 427(43)$_{14}$	(+)
lincomycin	407	**126(100)$_{16}$**, 359(10)$_{18}$, 389(5)$_{28}$	(+)
sulphathiazole	256	92(50)$_{27}$, 108(45)$_{25}$, **156(100)$_{15}$**	(+)

sulphadimidine	279	$108(32)_{26}$, $124(39)_{265}$, **$186(100)_{18}$**	(+)
sulphadiazine	251	$92(58)_{27}$, **$108(62)_{23}$**, $156(100)_{16}$	(+)
sulphadimethoxin	311	$92(30)_{31}$, $108(34)_{28}$, **$156(100)_{20}$**	(+)
trimethoprim	291	$230(100)_{22}$, **$261(75)_{24}$**, $275(47)_{21}$	(+)
erythromycin	735	$116(32)_{36}$, **$158(100)_{30}$**, $576(37)_{19}$	(+)
tylosin	817	$156(12)_{42}$, **$174(100)_{37}$**, $772(38)_{29}$	(+)
enrofloxacin-d5	365	$245(49)_{32}$, **$321(100)_{27}$**, $347(46)_{19}$	(+)

Ions for quantification are in bold. The values in brackets represent the relative intensities (%). CE: collision energy, subscripted and expressed in volts.

474 **Table 2**. Validation parameters for all antibiotics.

Analyte	CCα (ng g^{-1})*	CCβ (ng g^{-1})*	Validation levels (ng g^{-1})*	Recovery (%) (*n*=18)	Repeatability intra-day (CV; *n*=6)	Repeatability inter-day (CV; *n*=18)
Amoxicillin	1.04	1.55	1.00	86	14	20
			2.00	92	9	16
			3.00	101	8	10
Ampicillin	1.10	1.62	1.00	90	14	20
			2.00	98	13	14
			3.00	100	9	9
Cloxacillin	5.05	5.56	5.00	95	14	17
			10.00	97	11	13
			15.00	98	9	10
Dicloxacillin	5.10	5.68	5.00	93	13	18
			10.00	97	12	17
			15.00	99	11	11
Benzylpenicillin	5.32	5.89	5.00	90	14	19
			10.00	92	13	17
			15.00	93	13	14
Oxolinic acid	1.11	1.64	1.00	88	14	20
			2.00	87	14	18
			3.00	92	12	13
Nalidixic acid	1.17	1.70	1.00	92	13	17
			2.00	95	11	15
			3.00	95	9	11
Cefalexin	5.53	5.80	5.00	102	14	20
			10.00	97	13	20
			15.00	101	13	18
Cefquinome	5.75	5.93	5.00	103	14	20
			10.00	91	11	15
			15.00	109	9	9
Ciprofloxacin	1.40	1.52	1.00	95	14	16
			2.00	105	14	16

			3.00	98	11	12
Enrofloxacin	1.13	1.17	1.00	100	8	15
			2.00	100	8	15
			3.00	100	7	8
Lomefloxacin	1.18	1.27	1.00	97	14	20
			2.00	103	13	20
			3.00	98	13	18
Marbofloxacin	1.44	1.58	1.00	103	14	20
			2.00	97	14	15
			3.00	101	8	10
Florfenicol	1.39	1.89	1.00	98	13	17
			2.00	101	12	17
			3.00	100	8	9
Florfenicol amine	1.37	1.48	1.00	92	6	12
			2.00	104	11	15
			3.00	97	10	11
Chloramphenicol	1.03	1.34	1.00	87	14	15
			2.00	91	11	13
			3.00	91	11	12
Flumequine	0.54	0.83	0.50	89	13	17
			1.00	89	11	15
			1.50	91	9	11
Chlortetracycline	1.26	1.48	1.00	92	7	11
			2.00	103	5	11
			3.00	98	7	10
Doxycycline	0.56	0.74	0.50	104	14	20
			1.00	96	13	20
			1.50	101	12	13
Oxytetracycline	0.51	0.72	0.50	102	10	16
			1.00	98	8	15
			1.50	101	9	9
Tetracycline	0.53	0.65	0.50	99	14	20
			1.00	113	10	12
			1.50	96	9	10
Lincomycin	1.15	1.29	1.00	101	14	20
			2.00	99	13	17
			3.00	100	11	12
Sulphathiazole	1.16	1.31	1.00	86	14	20
			2.00	96	10	17

			3.00	99	9	11
Sulphadimidine	1.13	1.25	1.00	101	8	11
			2.00	99	7	9
			3.00	100	7	7
Sulphadiazine	1.09	1.36	1.00	102	11	18
			2.00	102	9	15
			3.00	104	9	11
Sulphadimethoxine	1.14	1.45	1.00	87	12	19
			2.00	89	11	13
			3.00	93	10	11
Trimethoprim	1.11	1.39	1.00	90	12	19
			2.00	91	9	15
			3.00	91	7	12
Erythromycin	5.23	5.54	5.00	89	14	18
			10.00	87	10	11
			15.00	92	9	10
Tylosin	1.07	1.21	1.00	91	12	19
			2.00	94	11	13
			3.00	95	7	13

*The concentrations were expressed in ng g^{-1} wet weight.

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Table 3. List of the detected samples, their provenience and antibiotic concentration expressed in ng g^{-1} wet weight.

Sample and provenience	Tetracycline (ng g^{-1})	Oxytetracycline (ng g^{-1})	Doxycycline (ng g^{-1})	Chlortetracycline (ng g^{-1})	Flumequine (ng g^{-1})
Clams North Adriatic Sea	49.45	125.03	60.45	77.48	
Mussels Atlantic Spain	0.55				
Mussels North Adriatic Sea					3.59
Clams North Adriatic Sea					0.84

Highlights

- A multiclass LC-MS/MS method for 29 antibiotics was developed and validated.
- Our detection limits were much lower than the maximum residue limits.
- Pool of mussels and clams from different FAO zones were analysed.
- Antibiotic presence in the analysed shellfish is negligible.

Figure 1

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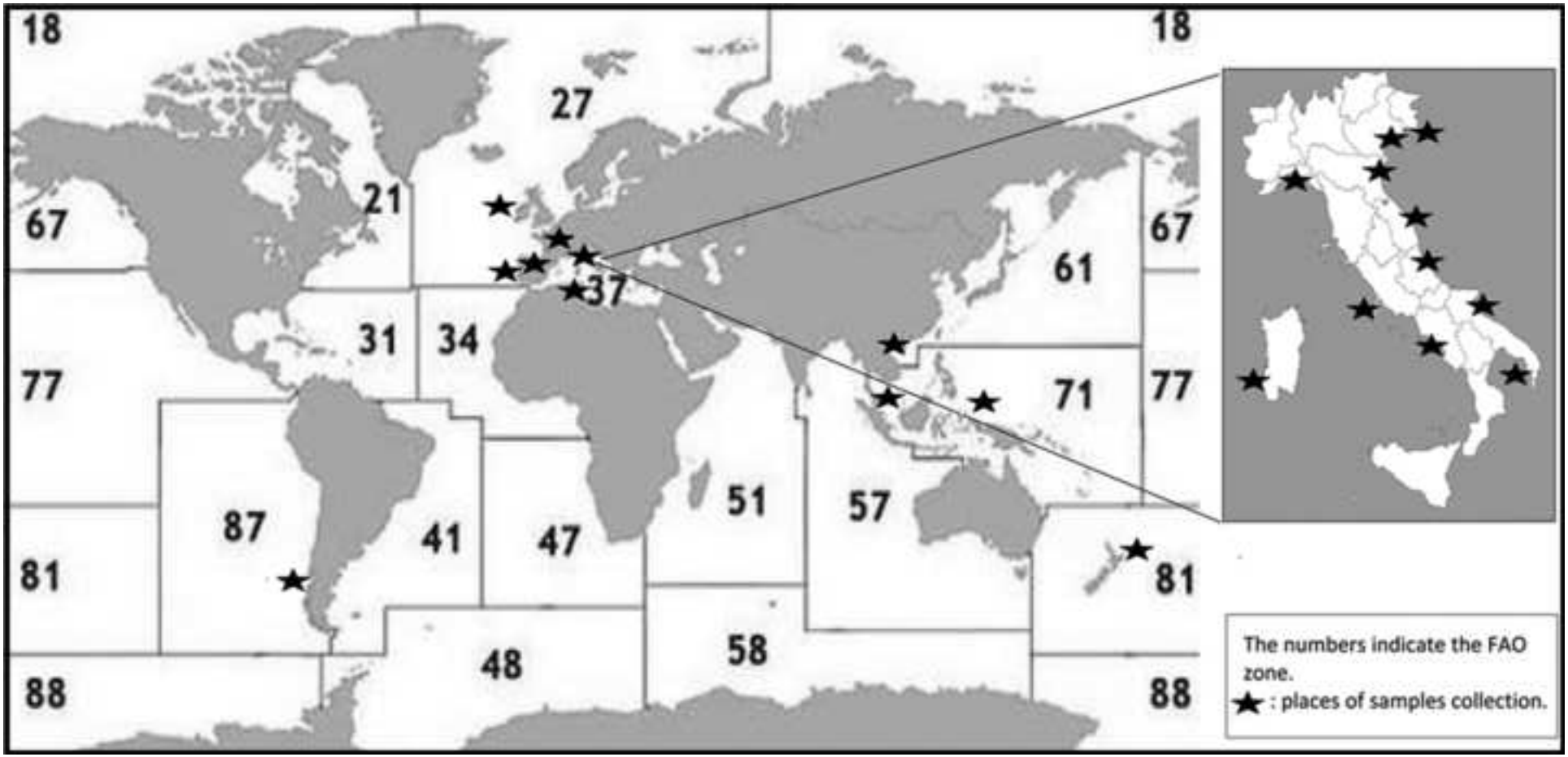

Figure 2

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