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

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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.



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

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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

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 $CC\alpha$ and $CC\beta$, 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 $\mu\text{g kg}^{-1}$, which were up to two orders of magnitude higher than those of sulphonamides (0-76.75 $\mu\text{g kg}^{-1}$) and macrolides (0-36.21 $\mu\text{g kg}^{-1}$). 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 withJOURNAL 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 UsingJOURNAL 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 ofTALANTA 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 pose 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 standard? 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 $0.29 \mu\text{g day}^{-1}$. 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.

1 **Occurrence of antibiotics in mussels and clams from various FAO areas**

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25 ABSTRACT

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27 Filter feeders, like mussels and clams, are suitable bioindicators of environmental
28 pollution. These shellfish, when destined for human consumption, undergo a depuration
29 step that aims to nullify their pathogenic microorganism load and decrease chemical
30 contamination. Nevertheless, the lack of contamination by drugs may not be
31 guaranteed. Antimicrobials are a class of drugs of particular concern due to the
32 increasing phenomenon of antibiotic resistance. Their use in breeding and aquaculture
33 is a major cause of this. We developed a multiclass method for the HPLC-MS/MS
34 analysis of 29 antimicrobials, validated according to the Commission Decision
35 2002/657/UE guidelines, and applied it to 50 mussel and 50 clam samples derived from
36 various Food and Agricultural Organisation marine zones. The results obtained, indicate
37 a negligible presence of antibiotics. Just one clam sample showed the presence of
38 oxytetracycline at a concentration slightly higher than the European Union Maximum
39 residue limit set for fish.

40

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

42

43 Chemical compounds studied in this article

44 Amoxicillin (PubChem CID: 33613); Ampicillin (PubChem CID: 6249); Benzylpenicillin

45 (PubChem CID: 5904); Cefalexin (PubChem CID: 27447); Cefquinome sulphate

46 (PubChem CID: 9577261); Chloramphenicol (PubChem CID:5959); Chlortetracycline

47 (PubChem CID: 54737570); Ciprofloxacin (PubChem CID: 2764); Cloxacillin (PubChem

48 CID: 6098); Dicloxacillin (PubChem CID: 18381); Doxycycline hyclate (PubChem CID:
49 54686183); Enrofloxacin (PubChem CID: 71188); Erythromycin (PubChem CID: 12560);
50 Florfenicol (PubChem CID: 114811); Florfenicol amine (PubChem CID: 156406);
51 Flumequine (PubChem CID: 3374); Lincomycin (PubChem CID: 3000540);
52 Lomefloxacin hydrochloride (PubChem CID: 68624); Marbofloxacin (PubChem CID:
53 60651); Nalidixic acid (PubChem CID: 4421); Oxolinic acid (PubChem CID: 4628);
54 Oxytetracycline (PubChem CID: 54675779); Sulphadiazine (PubChem CID: 441244);
55 Sulphadimethoxine (PubChem CID: 5323); Sulphadimidine (PubChem CID: 5327);
56 Sulphathiazole (PubChem CID: 5340); Tetracycline hydrochloride (PubChem CID:
57 54704426); Trimethoprim (PubChem CID: 5578); Tylosin (PubChem CID: 5280440).

58

59 **1. Introduction**

60

61 Antibiotics are among the most frequently detected group of potentially toxic
62 pharmaceuticals; this underscores the following ecotoxicological concerns: 1) the
63 cumulative toxic effects of antibiotics on aquatic animals are not well understood, 2)
64 their continuous presence leads to the development of antibiotic-resistant bacteria, and
65 3) antibiotics can act, at very low concentrations, as signalling agents and change the
66 natural microbial diversity in aquatic ecosystems (Fatta-Kassinos, Meric, & Nikolaou,
67 2011).An unknown amount of these drugs ends up either indirectly in the receiving
68 waters, through sewer plants and land-fields, or directly as a result of intensive fish
69 farming. For these reasons, organisms could also be exposed to a variety of
70 compounds present in the environment at low concentrations. In recent years,
71 pharmacological substances in the aquatic environment have become an increasing

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

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

82 Hence, an increasing demand for biological studies of aquatic organisms has
83 become a major impetus for the development and validation of high-performing
84 analytical techniques capable of determining various antibiotics. Zouiten, Beltifa, Van
85 Loco, Mansour and Reyns (2016) demonstrated the usefulness of ultra-performance
86 liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) to detect certain
87 antibiotic residues in *Mytilus galloprovincialis* exposed to pharmaceutical wastewater in
88 Tunisia. Li, Shi, Gao, Liu and Cai (2012) reported 22 antibiotics in molluscs obtained
89 from the Bohai sea (China), based on accelerated solvent extraction pressurised liquid
90 extraction, followed by a solid-phase extraction (SPE) clean-up. An enzymatic-
91 microwave assisted extraction method with subsequent high-performance liquid
92 chromatography (HPLC) was developed for the determination of 11 antibiotics in fish
93 tissue and mussels of Spain (Fernandez-Torres, Lopez, Consentino, Mochon, & Payan,
94 2011). Conversely, Le Bris and Pouliquen (2004) studied the bioaccumulation of two

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

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

112 2. Material and methods

113

114 2.1. Chemicals and reagents

115

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

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

130

131 *2.2. Standard solutions*

132

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

138

139 *2.3. Sample collection*

140

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

156

157 2.4. Sample extraction

158

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

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

172

173 *2.5. HPLC-MS/MS analyses*

174

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

187 mode was used to detect all analytes, except isoxazolyl penicillins and amphenicols,
188 which were detected in the negative (ESI-) mode. Acquisition parameters were
189 optimised by direct continuous pump-syringe infusion of the standard analyte solutions
190 at 1 $\mu\text{g mL}^{-1}$. The flow rate was set at 20 $\mu\text{L min}^{-1}$ flow rate, and the MS pump rate at
191 100 $\mu\text{L min}^{-1}$. The following conditions were used: capillary voltage 3.5 kV; ion transfer
192 capillary temperature 340 °C; nitrogen as the sheath and auxiliary gases at 30 and 10
193 arbitrary units, respectively; argon as the collision gas at 1.5 mTorr, and peak resolution
194 0.70 Da at full-width half-maximum (FWHM) (Chiesa et al., 2016). Three diagnostic
195 product ions were chosen for each analyte and IS, as carried out in an our previous
196 styudy about antibiotics in bovine urine (Chiesa et al., 2015). The acquisition was
197 performed in multiple reaction-monitoring (MRM) mode. The selected diagnostic ions,
198 one of which was chosen for the quantification, the collision energies and the relative
199 intensities are reported in Table 1. Acquisition data were recorded and elaborated using
200 Xcalibur™ software from Thermo Fisher.

201

202 *2.6. Method validation*

203

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

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

210 (expressed as the intra- and inter-day repeatability), linearity, as well as the decision
211 limit ($CC\alpha$) and detection capability ($CC\beta$), which were calculated as described in
212 SANCO/2004/2726 revision 4 (European Union, 2008).

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

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

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

233 series. Robustness was assessed using the approach of Youden (European Union,
234 2002), which is a fractional factorial design, based on minor modification ($\pm 10\%$) of
235 seven experimental conditions of eight samples spiked at the minimum detectable
236 concentrations.

237 Matrix effects was evaluated by Matuszewski, Constanzer and Chavez-Eng
238 (2003) strategy, comparing the analytes of interest added post-extraction with pure
239 solutions prepared in the mobile phase containing an equivalent amounts of the studied
240 compounds.

241

242 **3. Results and discussion**

243

244 *3.1 Validation performances*

245

246 The selectivity of the method, assessed by injecting blank samples (20 mussel
247 and 20 clam samples), did not show any interference (signals, peaks, ion traces) in the
248 region of interest, i.e. where the target analytes were expected to be eluted. The
249 selectivity also showed a good compliance with the relative RTs for each analyte, which
250 were found to be within 2.5% tolerance, when compared with the standards, with peaks
251 having a signal-to-noise ratio > 3 . Moreover, the three chosen transitions showed an ion
252 ratio within the recommended tolerances (European Union, 2002), when compared with
253 the standards. The mean recoveries for all analytes ranged between 86–113%. The
254 matrix validation curves also demonstrated a good fit for all analytes, with correlation
255 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 CC α ranged from 0.51–5.76 ng g⁻¹ wet weight, and CC β 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

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

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

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

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

325 quinolones as the major compounds with concentrations of 0.71-1575.10 $\mu\text{g kg}^{-1}$, which
326 were up to two orders of magnitude higher than those of sulphonamides (0-76.75 $\mu\text{g kg}^{-1}$)
327 and macrolides (0-36.21 $\mu\text{g kg}^{-1}$). But in that study, they didn't discriminate the
328 different antibiotics among the different molluscs analysed.
329 Finally, in the light of our results, we can say that the MRLs, are slightly exceeded only
330 in one clam sample, as already elucidated above. However, considering the annual Per
331 capita consumption of 0.33 Kg clams (European Commission, 2016), the daily
332 consumption is 0.91 g; the result of the multiplication of this value by the sum of the
333 concentrations of the four tetracyclines (312.41 ng g^{-1}) found in the clam sample of
334 North Adriatic Sea, is 0.29 $\mu\text{g day}^{-1}$. This datum could represents a risk mainly
335 associated with the increase of antibiotic resistance phenomenon. Instead, due to the
336 lack of detections, we cannot estimate a potential risk for the environment.

337

338 **4. Conclusions**

339

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

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

352

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363

364 **Conflict of Interest**

365

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

368

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459
460

461 **List of figures**

462

463 **Figure 1.** Map of sample collection sites and magnification of Italy (inset).

464

465 **Figure 2.** Chromatograms and MS spectra of the clams in which the four tetracyclines
466 were found.

467

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

469

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	166(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	(+)

470

471 Ions for quantification are in bold. The values in brackets represent the relative

472 intensities (%). CE: collision energy, subscripted and expressed in volts.

473

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

Analyte	CC α (ng g ⁻¹)*	CC β (ng g ⁻¹)*	Validation levels (ng g ⁻¹)*	Recovery (%) (n=18)	Repeatability	
					intra-day (CV; n=6)	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.

475

476

477

478 **Table 3.** List of the detected samples, their provenience and antibiotic concentration479 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})	Flumequinone (ng g^{-1})
<hr/>					
Clams					
North Adriatic Sea	49.45	125.03	60.45	77.48	
<hr/>					
Mussels					
Atlantic Spain	0.55				
<hr/>					
Mussels					
North Adriatic Sea					3.59
<hr/>					
Clams					
North Adriatic Sea					0.84
<hr/>					

480

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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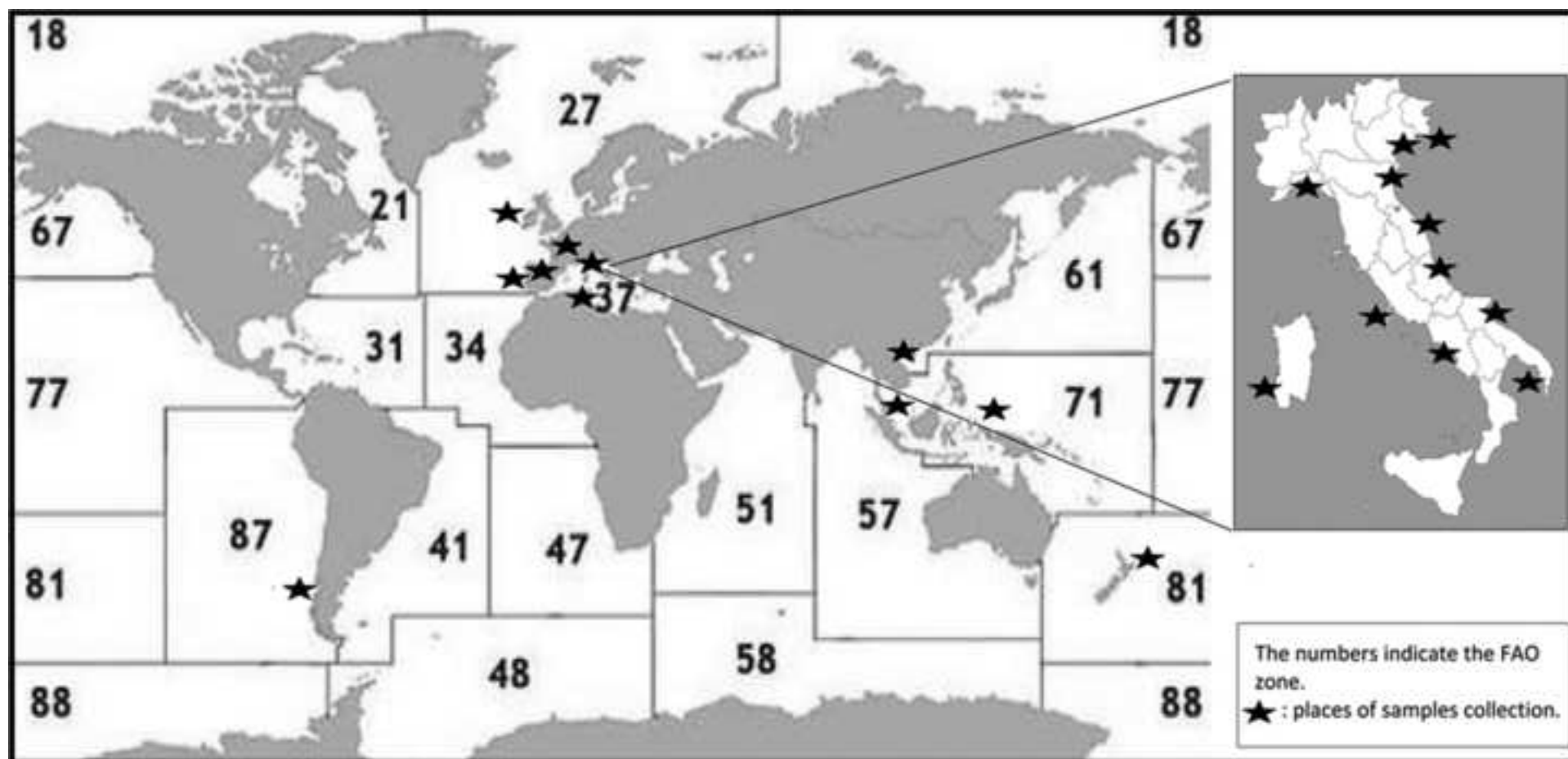


Figure 2
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