Simultaneous determination of antibiotic residues in edible tissue of farmed fish (*Oncorhynchus mykiss*) from the Umbria and Marche regions

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Abstract

Aquaculture is one of the fastest-growing sectors in global food production, and its expansion has driven the adoption and consolidation of intensive and semi-intensive production methods, which can increase the risk of infectious diseases. The use of various antibacterial compounds for therapeutic purposes has become increasingly common. Monitoring the presence of antimicrobial substances in aquaculture is of the utmost importance to prevent antibiotic resistance and ensure food safety. A multi-residue method was applied to investigate the incidence and the concentration of antibiotic residues in fish flesh collected from Umbria and Marche aquaculture farms over the 4-year period 2020-2023. Due to its rapidity and reliability, this method allowed for the evaluation of 70 antimicrobial molecules in fish flesh and the verification of European Union legislation compliance. Overall, 102 samples were analyzed, and only three antibiotic substances were detected, namely, sulfadiazine, oxytetracycline, and trimethoprim, with a variable presence of positive samples and residue concentration through different seasons, with higher values in winter. The highest value of positive samples was registered in 2021, with 63.2%, followed by 62.2% in 2022 and 51.7% in 2020; 11.1% was registered in 2023. Non-compliant samples were recorded for sulfadiazine (only one at the concentration of 222 µg/kg) and trimethoprim (the concentration ranged from 10 µg/kg to 226 µg/kg). The results indicated that 53.9% of the samples contained residues of authorized substances, with a 6.9% above the respective maximum residue limits.

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Introduction

The aquaculture industry is rapidly expanding and now accounts for nearly half of global fish production, making it the fastest-growing sector in food production (FAO, 2024). While aquaculture holds numerous theoretical benefits, the actual scenario is less optimistic, as this rapid growth has raised concerns about fish quality and safety. Similarly to other animal production sectors, the fish one incorporates intensive and semi-intensive breeding methods, contributing to heightened animal concentrations in confined spaces and a significant elevation in the risk of disease (EFSA, 2008). Furthermore, researchers anticipate a significant impact on aquaculture globally due to climate change (Cascarano *et al.*, 2021). Rising temperatures, indeed, will shift the balance in favor of either the host or pathogen, changing the frequency and distribution of disease. A number of endemic diseases of salmonids (*e.g.*, enteric red mouth, forunculosis, proliferative kidney disease, and white spot) will become more prevalent and difficult to control as water temperatures increase (Marcos-López *et al*., 2010). Albeit new strategies using natural substances to promote animal welfare have been proposed (Roila *et al.*, 2016; Torricelli *et al*., 2024), the use of antibiotics to manage fish diseases has become common practice and is on the rise, driven by the growing prevalence of diseases exacerbated by climate change (Defoirdt *et al.,* 2007; Defoirdt *et al.,* 2011). The use of antimicrobial drugs in aquaculture differs from their use in terrestrial animals. Specifically, antimicrobials are regularly added to animal feed and sometimes directly introduced into water through bath treatments (Samanidou and Evaggelopoulou 2007; Heuer *et al*., 2009). The presence of residues in edible tissues can occur due to various factors: failing to follow recommended label instructions or dosages or off-label usage (the use of a medicine for indications, age groups,

dosages, or routes of administration not approved); neglecting recommended withdrawal periods; administering excessive volumes at a single injection site; using equipment contaminated with antibiotics; or inadequately cleaning equipment used for drug mixing or administration (Rigos and Kogiannou, 2023; Jahan *et al.,* 2024). Additionally, chemical interactions between drugs, fluctuations in water temperature for aquatic species, and environmental contamination (CFIA, 2014). At the farm level, withdrawal periods are specified for drugs used in various animal species. These periods represent the duration post-administration during which edible products are deemed safe for consumption, meaning that residue levels are below the maximum residue limits (MRLs). Drug manufacturers establish withdrawal periods to ensure that treated animal products do not enter the food chain during this time. In cases where veterinarians allow the off-label use of antimicrobials, it is expected that the withdrawal period be adjusted accordingly, often extended to reduce the risk of residue accumulation in animal tissues. The withdrawal periods for antimicrobials used in fish are reported in *Supplementary Table* 1 (Vranic *et al.,* 2003). All medications legally utilized in aquaculture must receive approval from the government agency responsible for veterinary medicine, such as the European Medicine Agency and the European Parliament (European Parliament and Council of the European Union, 2019) in Europe and the Food and Drug Administration in the USA. These regulatory bodies establish guidelines for antibiotic usage, which may include approved routes of administration, forms of dosage, withdrawal periods, tolerance levels, and specifications for use in different species, including dosage rates and limitations (Burridge *et al.,* 2010). In the European Union (EU), legislation concerning residues of veterinary medicines and contaminants is standardized. Specifically, the primary legal framework governing this area is Commission Regulation (EU) 2017/625 (European Parliament and Council of the European Union, 2017), which outlines procedures for controlling veterinary drug residues in animals and their products in detail, while Commission Regulation (EU) 37/2010 (European Commission, 2010) governs pharmacologically active substances, classifying them and establishing MRLs in animal-derived food products. The EU has established safe MRLs for veterinary drugs and other substances used in veterinary medicine for animal products intended for human consumption. MRL values for antibiotics in fish, as determined by the EU, are summarized in *Supplementary Table 2*. Currently, in aquaculture, EU legislation allows the use of antibiotics such as tetracycline (TEA), oxytetracycline (OXY), flumequine (FLU), amoxicillin (AMO), trimethoprim (TRM), and the combination of TRM and sulfadiazine (SDZ). To verify compliance of food with established levels, the EU member states must implement an annual National Residue Sampling Plan adopting the following Commission Regulation (EU) 2017/625 (European Parliament and the Council of the European Union, 2017). The mandatory monitoring of antibiotic residues in edible animal tissues requires sensitive and selective analytical methodologies capable of verifying these legal demands and ensuring food safety and consumers' confidence, and health. The choice of antibiotics for analysis primarily hinges on regulatory policies (European Commission, 2010), but it is also influenced by scientific objectives related to food control and safety (Ministero della Salute, 2023). In the EFSA annual reports (2022, 2024), the monitoring data generated in the framework of the official control activities on the presence of residues of veterinary medicinal products and certain substances in live animals and animal products in the EU are summarized. For B1 group substances (antibacterial substances with an MRL), in 2020, on the overall number of 1570 samples analyzed, three non-compliant (Nc) samples were reported, in 2021, no Nc results were reported, and in 2022, three Nc samples were measured by different countries, equal to 0.18 % of the total 1678 samples analyzed. The B1 group substances for which non-compliances were found in the different years are SDZ, TRM, and OXY. Italy is the leading producer of freshwater salmonids in the EU, with an annual production of approximately 40,000 tons. Rainbow trout (*Oncorhynchus mykiss*) is the primary fish species farmed, positioning Italy as one of the top producers in Europe. Trout farming in Italy takes place in diverse aquatic environments, including basins with varying conditions, ranging from alpine and sub-alpine waters to lowland rivers and spring-fed streams (Camin *et al*., 2018). The Umbria and Marche regions, located in central Italy, account for the majority of trout production; therefore, it is crucial to monitor and control the presence of antibiotics if fish are produced in this area. This study aims to investigate the presence of antimicrobial residues in fish flesh analyzed within the context of the Italian official plan for the control of residues in the period 2020-2023 by applying a multi-class method using high-performance liquid chromatography (HPLC) coupled to high-resolution mass spectrometry.

Materials and Methods

Geographical location of trout farming

In the Umbria and Marche regions, trout farming is predominantly concentrated within the Nera River basin. Originating in the upper Valnerina Valley in Marche, Italy, the Nera River extends for a total length of 125 km, and its stream system drains an area of approximately 4200 km^2 (Fabrizi *et al.*, 2010). Umbria contributes significantly to national freshwater fish production, as previously mentioned, with over ten aquaculture facilities located along the Nera River. These facilities account for 7% of Italy's total freshwater fish production, primarily focusing on *Oncorhynchus mykiss* (rainbow trout), with an output of about 2800 tons per year. Figure 1 illustrates the distribution of aquaculture farms situated along the Nera River, highlighting the region's role in the nation's aquaculture industry (Sargenti *et al.,* 2020).

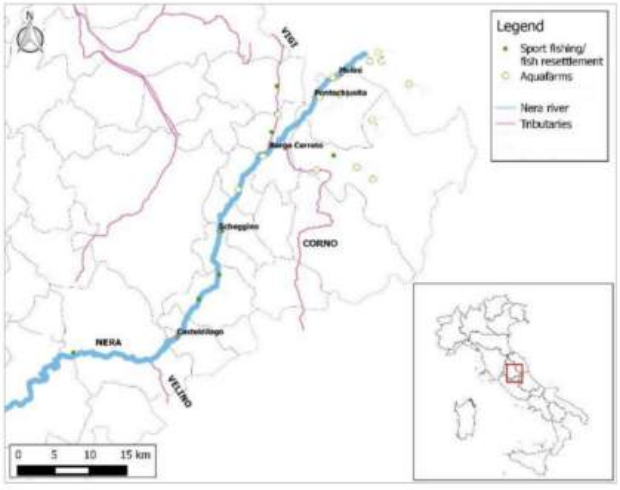

Figure 1. The course of the Nera River (blue line), trout farms (small spots).

Sample collection and preparation

A total of 102 samples of *Oncorhynchus mykiss* (rainbow trout) were collected by veterinary inspectors in the Umbria and Marche regions within the framework of official control, according to the national residue control plan over 4 years (2020-2023). The number of samples was calculated every year, taking into consideration animal production and the number of Nc samples detected within the preceding year. After sampling, the specimens were frozen (-20°C) and sent to the Experimental Zooprophylactic Institute of Umbria and Marche "Togo Rosati" to carry out analysis for antibiotic presence. Muscle and skin were homogenized and stored at -20°C until the day of the analysis.

Chemicals and reagents

Acetonitrile (ACN) and methanol (hypergrade for liquid chromatography mass spectrometry were supplied by Merck KGaA (Darmstadt, Germany). Formic acid 100% was purchased from Carlo Erba Reagents (Milano, Italy) N, N'-dimethylformamide was supplied from Fluka (Buchs, Switzerland). Ethylenediaminetetraacetic acid (EDTA) sodium salt dehydrate and ammonium acetate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was HPLC grade generated by a Milli-Q purification system (Millipore, Molsheim, France). AMO, ampicillin, penicillin G, oxacillin, penicillin V, cloxacillin, dicloxacillin, nafcillin, cefquinome, cefalonium, cefalexin, cefoperazone, ceftiofur, marbofloxacin, norfloxacin, ciprofloxacin, enrofloxacin, danofloxacin, difloxacin, sarafloxacin, oxolinic acid, nalidixic acid, FLU, erithromycin A, spiramycin I, tilmicosin, tylosin A, SDZ, sulfathiazole, sulfapyridine, sulfamerazine, TRM, sulfamethazine (sulfadimidine), sulfamethoxypyridazine, sulfamethoxazole, sulfadimethoxine, sulfaquinoxaline, methacycline, TEA, OXY, chlortetracycline, doxycycline, florfenicol amine, thiamphenicol, florfenicol, lincomycin, tiamulin, valnemulin, rifaximin, were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfamonomethoxine were obtained from Dr. Ehrenstorfer (Augsburg, Germany); desacetyl-cephapirin, cephapyrin, cefacetrile, cefazolin, tildipirosin, tulathromycin, neospiramycin, gamithromycin, 3-O-acetyltylosin, tylvalosin, 4-epi-tetracycline, 4-epi-OXY, 4-epi-chlortetracycline, florfenicol-d3, spiramycin I-d3, ceftiofur-d3 and tulathromycin marker (CP-60,300) were purchased from TRC Inc. (Toronto, Canada); sulfamethazine-^{13}C6 and enrofloxacin-d5 were obtained from WITEGA (Berlin, Germany).

Sample preparation

The sample was prepared as described by Moretti *et al.* (2016). Briefly, one-half grams of homogenate were weighed into a 50 mL polypropylene tube and spiked with 15 µL of β-lactams internal standard (IS) solution (ceftiofur-d_3 and penicillin G-d_7) and with 15 µL of the other four IS mixture (enrofloxacin-d_5, florfenicol-d_3, methacycline, and sulfamethazine-$^{13}C_6$). Later, 100 µL of 0.1 M of EDTA were added, and the sample was extracted with 3 mL of a mixture of ACN /water 4:1 (v/v) with 0.05% formic acid. The mixture was shaken for 10 min on a mechanical shaker and followed by centrifugation. The supernatant was collected in a 15 mL polypropylene tube, a second extraction with 3 mL of pure ACN was carried out, and the two extracts were combined and evaporated to dryness under nitrogen steam. Finally, the dry residues were redissolved in 1.5 mL of ammonium acetate 0.2 M, centrifuged at 14000 r.p.m. (30 min at 4 °C), and 5 µL of the final extract were injected into the LC-Q-Orbitrap system.

Chromatographic conditions

Chromatography analysis was performed on a Thermo Ultimate 3000 HPLC system (San Jose, CA, USA). Analytes were separated on a Poroshell 120 EC-C18 column (100×3.0 mm; 2.7 µm - Agilent Technologies, CA, USA) equipped with the Poroshell guard column (2.1×5 mm). Eluent A was a formic acid aqueous solution (0.1% v/v), and eluent B was methanol. The initial gradient was 95% A for 1 min, continued with a linear decrease to 5% A in 19 min. This condition was maintained for 11 min. The system returned to 95% A in 1 min and maintained these conditions for 4 min (total run time: 30 min). The column temperature was set at 30°C and the samples were kept at 16°C in the autosampler with temperature control. The injection volume was 5 µL, and the flow rate was 0.25 mL/min.

Mass spectrometry conditions

The mass spectrometer Q-Orbitrap (Thermo Scientific, San Jose, CA, USA) was equipped with a heated electrospray ionization (HESI-II) source. The HESI-II and capillary temperatures were set at 320°C and 300°C, respectively, and the electrospray voltage in positive ionization mode at 3.00 kV. Sheath and auxiliary gas were 35 and 15 arbitrary units. The mass spectrometer was controlled by the Xcalibur 4.4 software (Thermo Fisher Scientific, San Jose, CA, USA). The exact mass of the compounds was calculated using Qualbrowser in Xcalibur 4.4. After choosing the more intense product ions, fragmentation energy scans were carried out to obtain the optimal parameters for the complete fragmentation of precursor ions. All Q exactive parameters were optimized to improve selectivity and sensitivity. For the screening run, the acquisition was achieved in full scan mode. The screening run was applied to unknown samples, which, if suspect, were re-analyzed by applying the same chromatographic conditions, but focusing on the confirmatory parameters and acquiring two characteristic fragment ions. For the confirmatory step, a targeted selected ion monitoring method was prepared with an inclusion list that includes the m/z (mass-to-charge ratio) of interest and the retention times.

External quality control

To check its performance, in 2020-2023, the laboratory took part in interlaboratory studies. In particular, the laboratory participated in some proficiency tests on fish samples to verify the application and execution of the method. The results obtained in the exercises organized by different PT providers were fully satisfactory (z-score ≤|2|) (*Supplementary Table 3*).

Screening and confirmatory step

The analysis of real samples was carried out using a two-step approach. In Figure 2, the workflow exemplifying the routine application of the methodology (screening and confirmatory) is shown.

In the first step, the samples were analyzed using a semi-quantitative approach to determine the possible presence of the analytes. This phase involves the analysis of a single sample together with a blank sample and a fortified sample containing all analytes at the limit of quantification (LOQ, 10 µg/kg). In the above samples, also, IS were added at 10 µg/kg. If during the screening phase, the sample was suspected of the presence of one or more analytes above the LOQ, the confirmation phase is triggered. The confirmatory analysis was conducted by analyzing suspect samples in duplicate with the relative quality control and applying the appropriate dilution factor.

Results and Discussion

The method described was applied to the analysis of 102 samples of farmed rainbow trout (*Oncorhynchus mykiss*) collected in the Umbria and Marche regions. 62 samples out of 102 were found suspect at the screening analysis, so the confirmatory phase was triggered, and 55 samples were confirmed positive, thereby antibiotic residues were detected in 53.9% of the total samples. Detailed results on positive samples among the investigated years are reported in *Supplementary Table 4*.

Among the 70 molecules included in the method scope, only three antibiotic substances were detected: SDZ, OXY, and TRM. TRM in aquaculture is commonly used in combination with sulfonamides, specifically with SDZ. It should be emphasized that the selection of antibiotics included in the method scope was based on their high usage in Italian farms (Sargenti *et al.,* 2020). The detection frequencies were 31% for TRM, 4% for SDZ, and 1% for OXY. The measured concentrations ranged from 10 µg/kg to 50 µg/kg for OXY, from 10 µg/kg to 222 µg/kg for SDZ, and from 10 µg/kg to 226 µg/kg for TRM. Additionally, 0.1% of the total samples tested positive for both SDZ and TRM. Regarding Nc samples, the percentage registered was 3.4% in 2020 and 11.1% in 2022 and 2023. The Nc samples in 2020 and 2022 were refreed to TRM while for 2023, the only sample above the MRL was referred to SDZ. Currently, only four antibiotics are officially registered for use in Italian aquaculture: AMO, FLU, OXY, and TEA, along with one potentiated sulfonamide (a combination of SDZ and TRM) used in medicated feed formulations. It is also important to note that the non-compliance rate varies across different seasons. In 2020, the highest number of positive samples was detected during the winter period (January to April), in 2021 during the late spring and summer (from May to September), and in 2022 during the late autumn-winter period (from October to December). Figure 3 illustrates the number of positive samples, and Figure 4 reports the median concentrations of SDZ, TRM, and OXY detected throughout the months of each year. In 2020, the highest number of positive samples (9 samples) was recorded from January to April; in 2021, 12 samples were detected during the summer, and in 2022, 29 samples were identified from October to December. In 2023, only one Nc sample was detected.

The highest concentration of TRM was detected in the period from January to April in 2020 and in the period from October to December in 2022; the highest concentration of SDZ was detected in October 2023. The combination SDZ-TMP, instead, is mostly used in the fattening sector, for the treatment of enteric redmouth disease (ERM), a serious septicemic bacterial disease of salmonid fish species caused by *Yersinia ruckeri*. Young fish are most affected by the disease, while older/larger fish experience a more chronic condition with low-level mortality that persists over time, causing high cumulative stock losses (Kumar *et al.,* 2015). Pre-acute to acute ERM infection usually occurs in the spring and at the beginning of summer, during periods of rising water temperatures. Acute to subacute infections usually occur in yearling fish during the fall and early winter as water temperatures decrease (Zorriehzahra *et al.,* 2017).

Increasing water temperatures and the negative effects of extreme weather events are likely to alter the freshwater environment adversely for both wild and farmed salmonid populations, increasing their susceptibility to disease and the likelihood of disease emergence (Marcos-López *et al.,* 2010). In Figure 5, temperatures measured in Valnerina Valley from 2015 to 2022 show the trend of the decade's average temperature, indicating a progressive

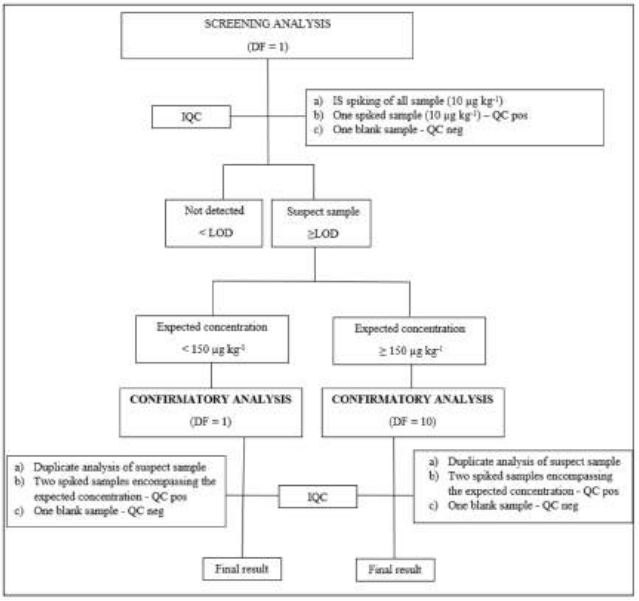

Figure 2. Application of the two-step approach for screening and confirmatory analysis of unknown fish samples. DF, dilution factor; LOD, limit of determination; QC, quality control; IQC, internal quality control.

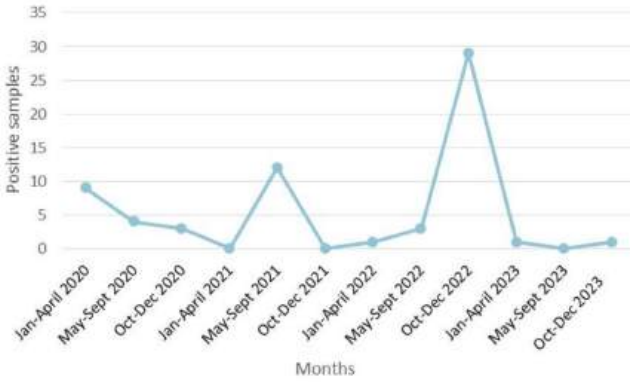

Figure 3. Positive samples detected during the months of the year.

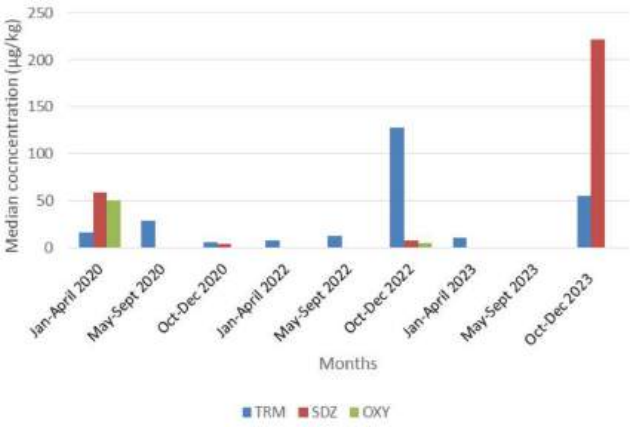

Figure 4. Antibiotic median concentration detected during the months from 2020 to 2023. TRM, trimethoprim; SDZ, sulfadiazine; OXY, oxytetracycline.

increase, except for occasional temporary fluctuations (ARPA Umbria, 2022). Between 2010 and 2022, the average temperature increased by 1.2 °C, data confirming the global temperature rise trend. In Figure 5, the summer's highest temperature has been reported; it is observed that the temperature rises from 26.8°C in 2015 to 29.0°C in 2022, with an increase of 2.2°C in just 7 years (Figures 5 and 6).

At high water temperatures, the dissolved oxygen content is lower, and *vice versa*. Specifically, when the temperature rises, the oxygen content of the water generally decreases, while the oxygen consumption of the fish increases. Climate change leads to a reduction in the minimum oxygen content (5 mg/L) and a decrease in the pH value of water (pH 3). This implies that the fish cannot feed effectively, causing health problems (Vasdravanidis *et al.,* 2022). The results obtained from the analysis of trout samples agree with the rising temperatures recorded in Valnerina. However, it has been observed that the largest number of positive samples and the highest average concentration were detected during the winter season. In 2016, Zonaras *et al.* calculated the withdrawal periods of the premix TRM-SDZ as 5 days at 24-26°C. In 2010, Romero-Gonzàlez *et al.* (2010) evaluated the different temperature-dependent depletion times of TRM and other veterinary drugs in fish. Since fish are poikilotherms, the temperature of their surroundings affects their metabolic rate. Withdrawal time is commonly understood to be the point at which the residues in all the tissues of all the animals have decreased below the MRL. As a result, to standardize the results obtained, withdrawal periods must be provided as degree-day values. In the study by Romero-Gonzàlez *et al.* (2010), two different water temperatures (14°C and 19.5°C) were used to calculate the elimination rates of the antibiotics, and the results indicate that the withdrawal period should be larger at lower temperatures. SDZ and TRM in combination are among the most used antibiotics in aquaculture for the treatment of the above-mentioned diseases. From the analysis of samples of this study, a higher frequency of finding for TRM was found in different years; in addition, most non-conforming samples were detected between October and December 2022 (Figure 4), when the water temperature was 10.3°C (Figure 6). This may be associated with the different rates of elimination of the two antibiotics in relation to temperature; at low temperatures, TRM needs a longer withdrawal time. Antibiotic elimination is significantly influenced by the temperature of the water. Periods of relatively low water temperatures necessitate an extended withdrawal time to prevent antibiotic residues in fish muscle. This study provides preliminary data to support a more prudent use of antibiotics in freshwater fish taking also in consideration the possibility to prolong the withdrawal period for TRM, furthermore, other environmental factors, such as water temperature, must be considered during the administration of these drugs to avoid the presence of residues in food of animal origin.

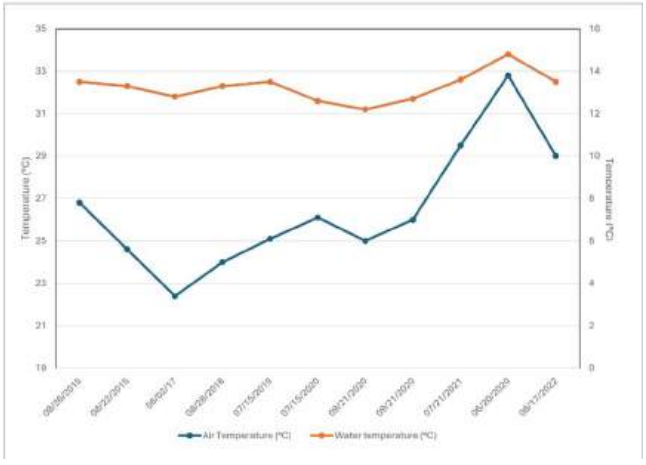

Figure 5. Air and water temperature recorded in Valnerina Valley during summers from 2015 to 2022.

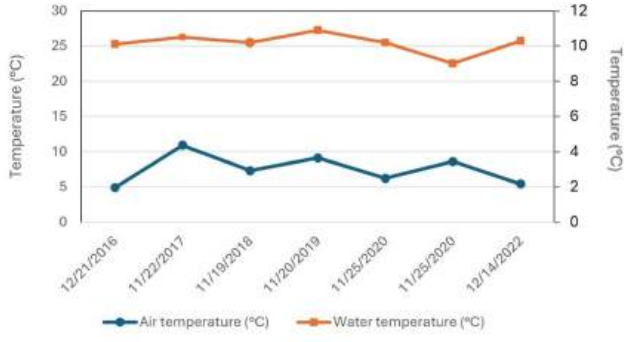

Figure 6. Air and water temperature recorded in Valnerina during winters from 2020 to 2023.

Conclusions

Routine control analysis must provide quick, accurate, and dependable results on the presence of antibiotic residues in edible fish tissues, preventing consumers from being supplied with fish containing antibiotic residues that are higher than permitted or perhaps including traces of drugs that are prohibited. The multiclass method allows for the simultaneous determination of 70 antibiotics from 10 different classes in fish flesh. The use of this procedure, for both screening and confirmatory purposes, significantly reduces the time needed for analysis and increases sample throughput. SDZ in association with TRM was the most detected molecule, followed by florfenicol and OXY in farmed rainbow trout collected in the Umbria and Marche regions. Even though most samples found positives complied with LMR, the multiclass analytical methods' sensitivity and ruggedness can assist the competent authorities in more precisely focusing their controls.

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Online supplementary material:
Supplementary Table 1. Withdrawal period of some antibiotics in rainbow trout.
Supplementary Table 2. Maximum residue limit value for antibiotics in fish according to Commission Regulation (EU) No 37/2010.
Supplementary Table 3. Results of participation in proficiency tests.
Supplementary Table 4. Prevalence of positive samples for antibiotic residues in Rainbow trout samples from Umbria and Marche aquaculture during 2020-2023.