IOP Conference Series:
Earth and
Environmental Science

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To cite this article: Mihaela Ilie *et al* 2023 *IOP Conf. Ser.: Earth Environ. Sci.* **1216** 012009

Evaluation of the Ecological Risk and the Development of Antimicrobial Resistance due to the Presence of the Macrolide Antibiotics Azithromycin and Clarithromycin in Romanian Aquatic Environment

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Abstract. The presence of antibiotics in the aquatic environment can result in the emergence of antibiotic-resistant pathogens. In this paper, our aim was to identify, quantify and distribute macrolide antibiotics in the aquatic environment in the river basins of Arges-Vedea, Buzau-Ialomita and Dobrogea-Litoral and of the Danube River. In the Arges-Vedea river basin area, clarithromycin was detected most frequently, i.e. 58.62%, in the Buzau-Ialomita river basin area with a frequency of 92.31% azithromycin was detected, and in the Dobrogea - Litoral river basin area clarithromycin was detected with a frequency of 35.48%. The highest concentration of azithromycin, 559 ng/L and of clarithromycin, 502 ng/L was recorded in the Buzau-Ialomita river basin. The ecotoxicological RQ_{ecotox} risk was also estimated, as well as the risk of developing antibiotic resistance of RQ_{AMR} pathogens, by the ratio of Measured Environmental Concentration (MEC) and Predicted No Effect Concentrations (PNEC). The study also aimed to investigate the prevalence of antibiotic resistance in potentially pathogenic bacteria isolated from aquatic environments. Thus, for β-lactam antibiotics, *E. coli* strains isolated from the Danube River exhibited a very high level of resistance to ampicillin (51%) and high level to azithromycin (43%), cefazolin (38%), amoxicillin+clavulanic acid (36%) and cefoxitin (26%). Low and respectively, low level resistance was noticed for aztreonam (6%) and imipenem (4%).

Keywords: macrolide antibiotics, antibiotic resistance bacteria (AMR), aquatic ecosystems environmental risk assessment;

1. Introduction

Emerging pharmaceutical pollutants from different classes such as antibiotics, analgesics, β-blockers etc., have been found in a wide variety of different matrices in the environment, i.e. in surface water, biota, sediments, groundwater and drinking water.

The occurrence of pathogens and emerging pollutants in water resources is one of the most serious risks to environment and is considered a major factor especially in the degradation of water quality. Antimicrobial resistant organisms and genes are now widely found throughout the environment and pose a serious threat to human health [1].

Antibiotics have different half-lives in the environment, some are highly persistent [2] and therefore their contamination levels in the environment have been increasing. Antibiotics and antibiotic-resistant pathogens enter the environment in different ways, including agricultural runoff, direct discharge of urban wastewater treatment or human waste, direct disposal of medical, veterinary industrial waste [3,4,5,6,7]. Antibiotic resistance involves the exchange of bacteria and gender between people, animals, and the environment [8], which can have a considerable impact on human health as well as unanticipated ecological impacts and responses. [9,10,11,12].

Antimicrobial resistance (AMR) is one of the ten threats identified by the World Health Organization (WHO) in 2019, since it affects modern healthcare and the effective prevention and treatment of an ever-increasing range of infections. Estimates of the burden of AMR are very impressive with a total of 671,689 cases of infections with selected antibiotic-resistant bacteria occurring in 2015 in Europe, out of which 63.5% were associated with healthcare [13]. There are numerous studies identifying specific genes or resistant bacteria in the environment and highlighting the role of the aquatic environment in dissemination and emergence of antibiotic resistance [14,15,16]. Still, little is known about the transmission dynamics of resistant bacteria and resistance genes in aquatic environments.

The EU Directive 2013/39/EU on priority substances in the field of water policy considers the contamination of water and soil with pharmaceutical residues an emerging environmental concern. Several pharmaceuticals, including three macrolide antibiotics (erythromycin, clarithromycin and azithromycin) are included on the first ‘watch list’ of the EU Priority Substances, with the aim to gather monitoring data for the purpose of facilitating the determination of appropriate measures to address the risk posed by these substances.

Antibiotics were proved to be directly toxic to fish causing oxidative stress, general stress response, histopathological lesions, hematological, metabolic, and reproductive disorders, as well as immunosuppressive and genotoxic effects. Environmentally realistic low concentrations of antibiotics also disturb aquatic bacterial communities causing alterations in fish symbiotic microbiota and induce emergence of antibiotic-resistant pathogenic bacteria by exerting selective pressure on spread of antibiotic-resistance genes [17].

Therefore, the presence of antibiotics in surface waters, even in low concentrations, disturbs the life cycle of aquatic organisms and the ecological balance, so evaluation of these substances in environment and setting up removal solutions at source have become a necessity [18].

In this paper, our aim was to identify, quantify and distribute the macrolide antibiotics azithromycin (AZM) and clarithromycin (CTM) in the aquatic environment in the river basins of Arges-Vedea (Area 1), Buzau-Ialomita (Area 2) and Dobrogea-Litoral (Area 3) and of the Danube River and to estimate the RQ_{ecotox} environmental risk as well as the risk of developing antibiotic resistance of RQ_{AMR} pathogens by the ratio of MEC and PNEC. The study also aimed to investigate the prevalence of antibiotic resistance in potentially pathogenic bacteria isolated from aquatic environments.

2. Materials and method

2.1. Sampling Locations

In order to achieve the proposed objectives, the main watercourses of the Arges-Vedea, Buzau-Ialomita and Dobrogea-Litoral hydrographic areas, upstream and downstream of the main urban agglomerations, as well as at the level of the Danube were investigated. The investigated river basins are located in the southern, south-east part of Romania being key basins that may influence the contamination of the Danube with emerging pharmaceutical pollutants and that require future intensive monitoring of the level of surface water contamination with antibiotics in order to establish protective measures to prevent the development of antibiotic resistance of pathogens and to reduce the ecotoxicological effects in the aquatic environment.

The monitoring sites from which the different categories of samples were taken were selected taking into account the following aspects: population density of the main cities in the river basin areas studied; the presence of facilities that can be major sources of pollution, such as hospitals; the existence of wastewater treatment plants that collect practically the entire load of pollutants generated in the area of urban agglomerations. There are about 4 million inhabitants, 80 hospitals and 19 wastewater treatment plants, in these regions.

The investigations were carried out on surface water samples collected from Dambovita R. – Budesti area, Glina WWTP area, Ialomita R. of Slobozia WWTP area, Arges R. - Hotarele and Clatesti area, Sabar R., Ciorogarla R. - upstream and downstream of Domnesti locality, Prahova R. - area of Campina WWTP, Dambu R. - area of Ploiesti WWTP, Danube River - Orsova area, Tulcea (upstream, downstream), Bastroe, Sulina, Sf. Gheorghe, Fetesti (km 43), Bala (km 9.5), Epurasu Branch, Izvoarele, Sulina. Determinations have also been made for identifying the antibiotics from biota samples (fish) from the area of Arges R. - Clatesti, Dambovita R. - Trout farm from Stoenesti (Figure 1).

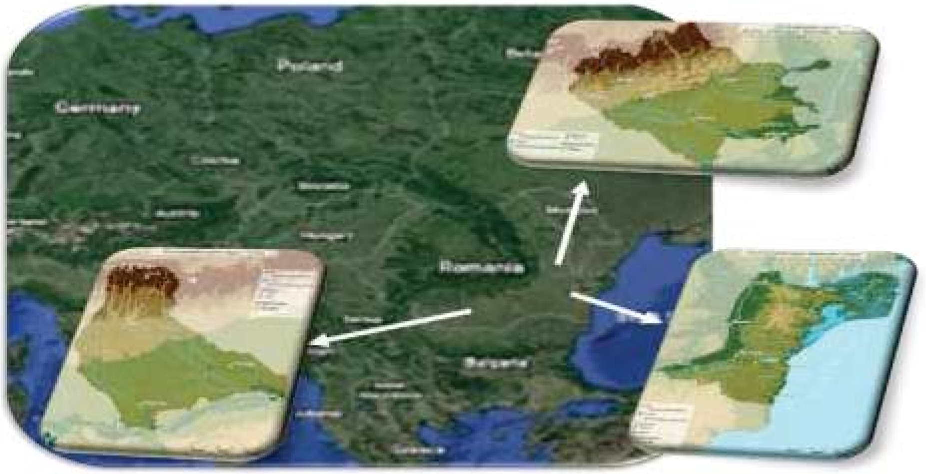


Figure 1. Sampling locations – river basins of Arges-Vedea, Buzau-Ialomita, Dobrogea-Litoral and the Danube River

2.2. *Methods*

The assessment of the presence of pharmaceutical residues belonging to macrolide antibiotics range in the aquatic environment at the level of the three investigated river basins and of the Danube, was carried out of 29 representative monitoring sections during June 2019 - May 2022, namely 22 sections for surface waters and seven sections in the area of the WWTP of cities such as Bucharest, Slobozia, Fundulea, Lehliu, Calarasi, Ploiesti and Campina.

Antibiotics chemical analysis. The method developed in this study for the identification of the macrolide antibiotics AZM and CTM by SPE-online-UHPLC-MS/MS Thermo Fisher Scientific meets the EU level requirements for the detection limit. Maximum acceptable method detection limits (ng/L) were set by the European Commission as Environmental Quality Standards (EQS) [19].

The reference standards used for this analysis were AZM and CTM, produced by Chiron AS (Norway) and LGC DR Ehrenstorfer (Germany). Ultra-pure water was obtained from a Millipore Milli-Q water purification system used in preparing all standard solutions. HPLC grades acetonitrile and formic acid for LC/MS were provided by Scharlau Chemie SA and VWR Chemicals.

Antibiotic susceptibility testing. The evaluation of antibiotic resistance profile was performed on strains of *Escherichia coli* (n = 47) isolated from the Danube River. Representative colonies were purified on Trypticase Soy Agar and the isolates were further identified by API 20E biochemical tests (bioMérieux).

The antibiotic susceptibility of isolates was tested by the standardized disc-diffusion method (Kirby-Bauer), according to the recommendations provided in CLSI 2020 (Clinical Laboratory Standard Institute). A panel of 16 antibiotics was used, namely: ampicillin (AMP), cefazolin (KZ), aztreonam (ATM), ceftriaxone (CRO), imipenem (IMP), cefoxitin (FOX), AZM, amoxicillin+clavulanic acid (AMC), CTM, erythromycin (E), sulfamethoxazole (RL), trimethoprim (W), doxycycline (DO), ciprofloxacin (CIP), tetracycline (TE) and penicillin (P) (Oxoid). Based on CLSI 2020 break points, after 24 h of incubation at 37^{0}C, the *E. coli* strains were classified as sensitive (S), intermediate (I) or resistant (R); intermediate strains were included in the resistant class.

Ecological risk assessment and the development of antimicrobial resistance to antibiotics of pathogenic agents. The estimated ecological risk quotient (RQ_{ecotox}) associated with the presence of AZM and CTM in aquatic environment was calculated for potential ecological risk assessment, using the ratio between the MEC of macrolide antibiotics detected in surface water and PNEC values, the environmental concentration at which no adverse effect on aquatic ecosystem function is to be expected [20,21].

The highest concentrations measured in surface waters (MECs) for AZM and CTM were considered, in order to calculate RQ_{ecotox} and RQ_{AMR}. The PNEC values are estimated based on available acute or chronic ecotoxicity data from the scientific literature for several aquatic organisms, where the ecotoxicity data is adjusted with an appropriate assessment factor, according to the European Technical Guidance Document and EMA (European Medicines Agency) [22,23]. Thus, the considered PNEC values for AZM are for *algae* - 1.8 ng/L; *invertebrate* - 440 ng/L and for *fish* - 84 000 ng/L, the PNEC values for CTM are for *algae* - 2 ng/L; *invertebrat*e - 8 160 and *fish* - 1 000 000 ng/L, respectively [24].

The calculated RQ value is then used to prioritize antibiotics that are likely to pose a high risk ($RQ \geq 1.0$); when $0.1 \leq RQ < 1$, moderate environmental risk is expected; compounds with $0.01 < RQ < 0.1$ have low risk and when $RQ < 0.01$ is no ecological risk to the aquatic ecosystem [25,26].

3. Results and Discussion

3.1. Identification and quantification of macrolide antibiotics AZM and CTM in the area of the studied river basins

Method development for the detection of AZM and CTM by UHPLC-MS/MS was carried out in accordance with the quantitative confirmation criteria described in Decision 2002/657/EC implementing Directive 96/23/EC concerning analytical methods and interpretation of results.

In the surface water samples analysed, the identification of the macrolide antibiotics AZM and CTM showed their presence in variable concentrations of ng/L level for AZM and CTM, of 288÷559 ng/L, 54÷502 ng/L, respectively. Distribution of AZM and CTM in aquatic environments is shown in Figures 2 and 3.

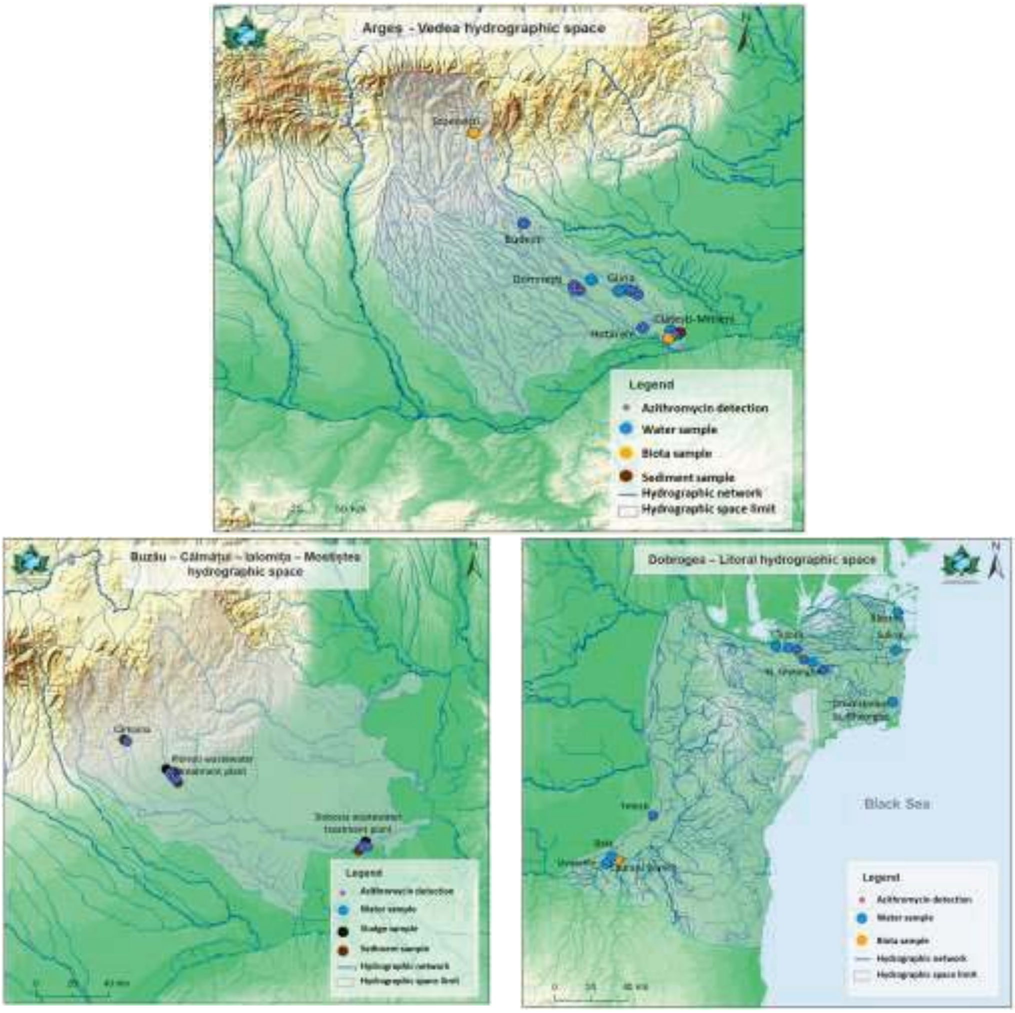
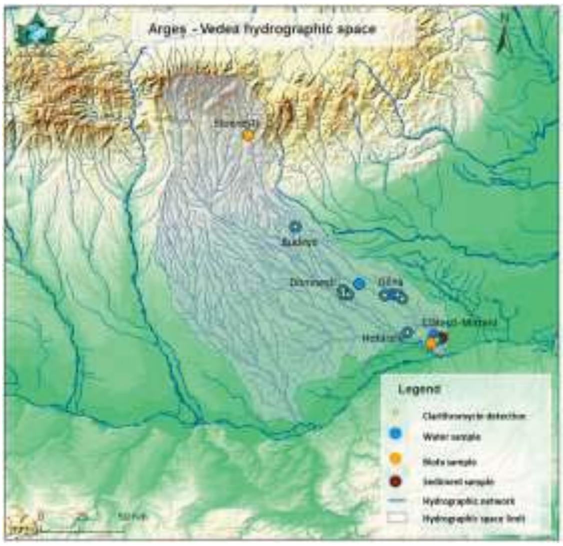


Figure 2. Distribution of AZM in aquatic environments from the areas under study



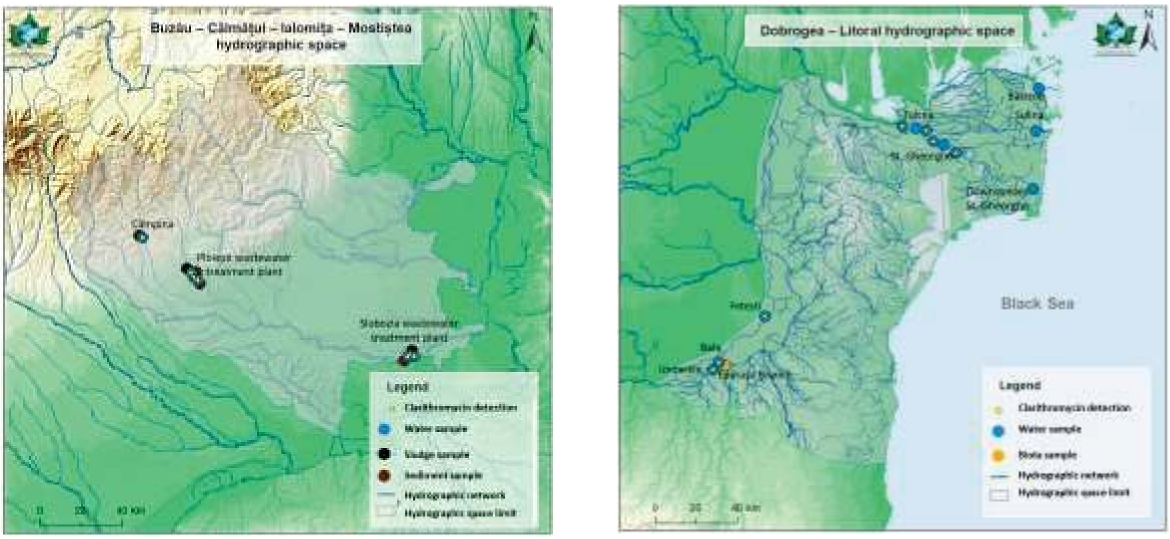


Figure 3. Distribution of CTM in aquatic environments from the areas under study

Identification of antibiotics macrolide AZM and CTM in the area of the studied river basins is shown in Figure 4.

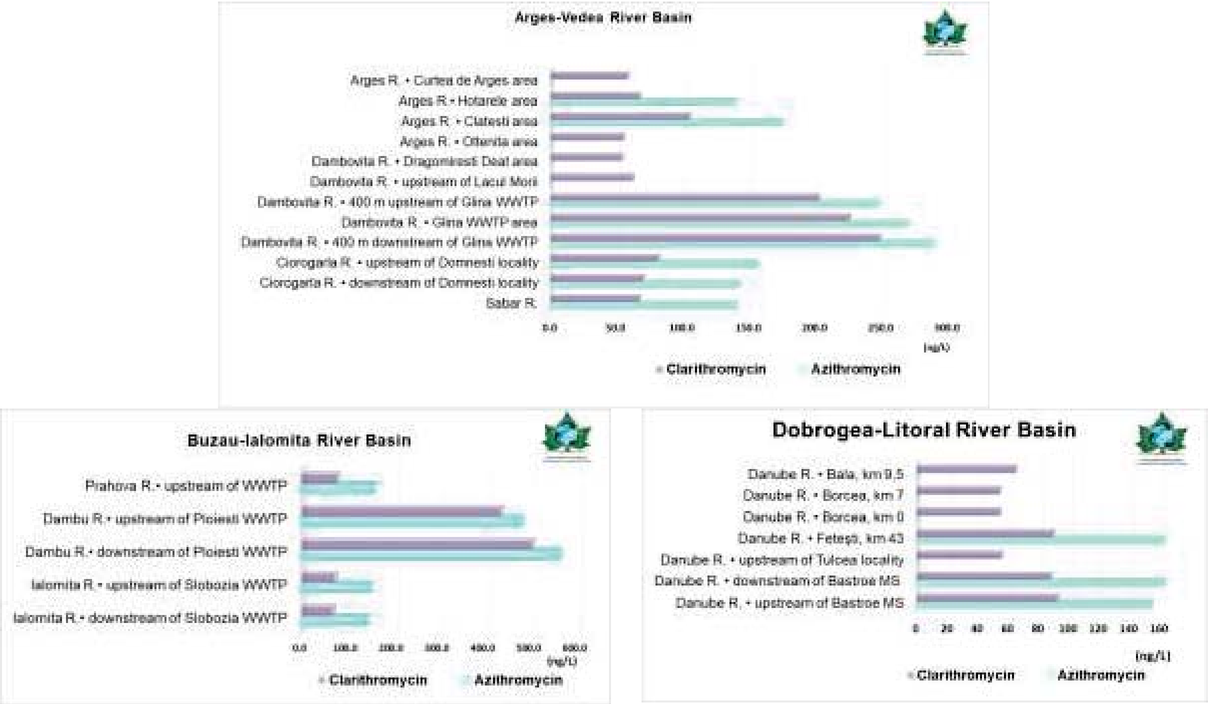


Figure 4. Identification of antibiotics in samples collected from the investigated areas

In the Area 1, CTM was detected most frequently, i.e. 58.62%, in the Area 2, AZM was detected with a frequency of 92.31%, and in the Area 3, CTM was detected with a frequency of 35.48%. AZM and CTM were detected downstream of the WWTP of Bucharest, Slobozia and Ploiesti, in Dambovita R., Ialomita R. and Dambu R., respectively. The highest concentration of AZM, of 559 ng/L and 502 ng/L CTM was recorded in the Buzau-Ialomita river basin. The ecotoxicological risk of RQ_{ecotox} as well as the risk of developing antibiotic resistance of RQ$_{ARM}$ pathogens was also estimated by the ratio of MEC and PNEC.

The results of the macrolide antibiotics AZM and CTM detection in the surface water and calculated ecological risk quotient RQ$_{ecotox}$ and RQ$_{AMR}$ are presented in Table no.1.

Studies carried out up to now have reported that CTM was occasionally detected at the level of Romania, having concentrations ranging between 1.2 and 23.2 ng/L, in the Danube basin (the Danube River) and the three main tributaries (Jiu, Arges and Olt) [27], additionally, no investigations were conducted in Romania's surface waters regarding the detection of azithromycin.

Table 1. Concentrations of AZM and CTM detected in surface water (MEC), their PNEC$_{ecotox(fish)}$ and PNEC$_{AMR}$, and calculated ecological risk quotient RQ$_{ecotox(fish)}$ and RQ$_{AMR}$

Macrolide antibiotics	PNEC$_{ecotox}$[19] / PNEC$_{AMR}$[16] (ng/L)	Location	MEC (ng/L)	RQ$_{ecotox}$ (MEC/ PNEC$_{ecotox}$)	RQ$_{AMR}$ (MEC/ PNEC$_{AMR}$)
AZM	8.4×10^4 / 250	Arges River - Clatesti	174	<0.01	>0.1
		Dambovita River - Budesti	154	<0.01	>0.1
		Dambovita River – downstream of Glina WWTP	247	<0.01	>0.1
		Sabar River	139	<0.01	>0.1
		Ciorogarla River	156	<0.01	>0.1
		Ialomita River – downstream of Slobozia WWTP	140	<0.01	>0.1
		Prahova River	154	<0.01	>0.1
		Dambu River- area Ploiesti WWTP	559	<0.01	>1.00
		Danube River – area Sulina	158	<0.01	>0.1
		Danube River – Fetesti (km 43)	162	<0.01	>0.1
CTM	1.0×10^6 / 250	Arges River - Hotarele	66	<0.01	>0.1
		Dambovita River – area Glina WWTP	202	<0.01	>0.1
		Sabar River	67	<0.01	>0.1
		Ciorogarla River	81	<0.01	>0.1
		Ialomita River - downstream of Slobozia WWTP	67	<0.01	>0.1
		Prahova River	77	<0.01	>0.1
		Dambu River -area Ploiesti WWTP	501	<0.01	>1.00
		Danube River –Fetesti (km 43)	89	<0.01	>0.1
		Danube River – area Sulina	86	<0.01	>0.1

The ratios of MEC to PNEC are very low, indicating that there is no significant RQ$_{ecotox}$ risk to fish health due to the presence in traces of the macrolide antibiotics AZM and CTM in surface water, but is of high risk to algae. Considering the risk of RQ$_{AMR}$ to develop bacterial resistance to these antibiotics, it is noted that there is a high risk in the Dambu River- Ploiesti WWTP area and a medium risk in all other investigated areas.

3.2. Antibiotic resistance phenotypic pattern in Escherichia coli strains isolated from the Danube River

The resistance rates of *E. coli* strains were evaluated in accordance with the intervals for resistance incidence proposed by European Centre for Infectious Disease Control (ECDC/ European Resistance Surveillance Network EARS-Net) for clinical bacterial strains, i.e:<1%= insignificant; 1 to<5%=very low; 5 to<10%=low; 10 to<25%=intermediate; 25 to<50%=high; ≥50%=very high [28].

In the case of β-lactam antibiotics, *E. coli* strains isolated from the Danube River exhibited a very high level of resistance to AMP (51%) and high level to AZM (43%), KZ (38%), AMC (36%) and FOX (26%). Low, and respectively, low level resistance was noticed for ATM (6%) and IMP (4%). Resistance to third generation cephalosporins was very low to CRO (4%) (Figure 5).

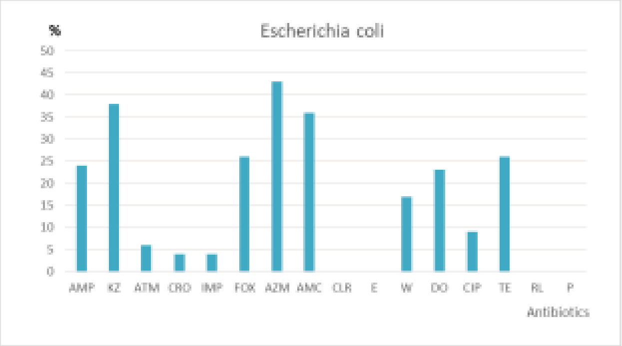


Figure 5. Distribution of antibiotic resistance markers (%) in *E.coli* strains isolated from the Danube River

Resistance to other classes of antibiotics (non-β-lactams) was high to tetracyclines (TE, 26%) and low to fluoroquinolones (CIP, 9%). The level of doxycycline resistance was intermediate (23%).

4. Conclusion

Quantification and distribution of the macrolide antibiotics AZM and CTM in the studied areas is presented in this study. The ecotoxicological RQecotox risk was estimated, as well as the risk of antibiotic resistance of RQAMR pathogens, which showed that there is no significant RQecotox risk for fish health due to the presence of the macrolide antibiotics AZM and CTM in traces, in surface water, but there is a high risk for algae and for the development of bacterial resistance to these antibiotics. It is noted that there is a high risk in the Dambu River- Ploiesti WWTP area and a medium risk in all other investigated areas. The study also aimed to investigate the prevalence of antibiotic resistance in potentially pathogenic bacteria isolated from aquatic environments. Thus, for β-lactam antibiotics, *E. coli* strains isolated from the Danube River exhibited a very high level of resistance to ampicillin (51%) and high level to azithromycin (43%), cefazolin (38%), amoxicillin+clavulanic acid (36%) and cefoxitin (26%). Low and respectively, low level resistance was noticed for aztreonam (6%) and imipenem (4%).

Given the potential risks due to the presence of the macrolide antibiotics AZM and CTM in the aquatic environment, there is a clear need for further knowledge on this type of contamination, especially in terms of monitoring ecological behaviour.

Author Contributions

Methodology, M.I., G.G. and F.M.; Chemical and microbiology analyses, M.I., G.G. and I.S.; Monitoring and Sampling, L.L. and P.I.G.; Data analysis, M.I., G.G., F.M. and G.D.; GIS maps, G.T.; Writing—original draft preparation, M.I., G.G. and F.M.; Writing—review and editing, M.I., G.G., F.M. and N.M.N.; All authors have read and agreed to the published version of the manuscript.

Acknowledgments

This paper was developed through Nucleu Programme – Contract number 39N/2019 carried out with the Romanian Ministry of Research and Innovation support, within the project ”Research on the ecotoxicity of pharmaceutical micropollutants on aquatic ecosystems”.

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