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Multiresistant bacteria: Invisible enemies of freshwater mussels☆

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ABSTRACT

Freshwater mussels are among the most endangered groups of fauna anywhere in world. The indiscriminate use of antibiotics has led to the emergence of resistant strains. These antibiotic-resistant bacteria play a key role in increasing the risk allied with the use of surface water and in spread of resistance genes. Two endangered freshwater mussel species, *Margaritifera margaritifera* and *Potomida littoralis*, were sampled at 4 sampling sites along a 50 km stretch of River Tua. Water samples were taken at same sites. Of the total of 135 isolates, 64.44% (39.26% from water and 25.19% from mussels) were coliform bacteria. Site T1, with the lowest concentration of coliform bacteria, and site T2 were the only ones where *M. margaritifera* was found. No *E. coli* isolates were found in this species and the pattern between water and mussels was similar. *P. littoralis*, which was present at T3/T4 sites, is the one that faces the highest concentration of bacterial toxins, which are found in treated wastewater effluents and around population centers. Sites T3/T4 have the isolates (water and mussels) with the highest resistance pattern, mainly to β-lactams. Water and *P. littoralis* isolates (T3/T4) showed resistance to penicillins and their combination with clavulanic acid, and to cephalosporins, precisely to a fourth generation of cephalosporin antibiotics. The analysis provides important information on the risk to water systems, as well as the need to investigate possible management measures. It is suggested that future studies on the health status of freshwater bivalves should incorporate measures to indicate bacteriological water quality.

1. Introduction

Freshwater bivalves (FB) are ubiquitous organisms in freshwater ecosystems. They are affected by various threats across the world, such as pollution (Goodchild et al., 2015), overexploitation of natural resources (Nobles and Zhang, 2011), competition by invasive alien species (Bódis et al., 2014; Pilotto et al., 2016; Sousa et al., 2019), loss of host fishes (Benaissa et al., 2019), habitat modifications and fragmentation (Sousa et al., 2020), and more recently by climate change (Atkinson and Vaughn, 2014; Santos et al., 2015; Strayer and Dudgeon, 2010). Freshwater mussels have a complex life cycle and are highly sensitive to anthropogenic pressures. They have been suffering global extinction and loss of biodiversity for decades and are among the most threatened animal groups on the planet (Downing et al., 2010). They are long-lived (some species can live for up to 100 years) and are responsible for important functions and vital ecosystem services (Vaughn and Hakenkamp, 2001). They are sensitive to environmental changes, making them excellent bioindicators (Grabarkiewicz and Davis, 2008). As benthic filter-feeders, freshwater mussels (the Unionida order) are constantly exposed to pollution and accumulated pollutants, sequestering heavy metals associated with suspended particles and deposited in bottom sediments (Naimo, 1995; Parra et al., 2021; Ribeiro Guevara et al., 2004) and even taking up pharmaceuticals, contaminants of emerging concern, and bacteria such as *Escherichia coli* (Ismail et al.,

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2015, 2014; Lara et al., 2002).

Despite being food for bivalves, bacteria can also be found in body tissues outside the gut in apparently healthy animals (Antunes et al., 2010; Starliper et al., 2008). According to Antunes et al. (2010) and Grizzle and Brunner (2009), mussels are capable of establishing mutualistic or antagonistic symbiotic relationships with bacteria. The bacterial flora characteristic of freshwater mussels is largely unknown, notwithstanding the increase in research on microbiomes examining correlations between bacterial communities and health and resilience in many different animal species (Leis et al., 2019; Trinh et al., 2018).

20 years after the introduction of the Water Framework Directive (WFD) (EC, 2000), monitoring tools still show weaknesses related to the poor linkage between pressures and effects on the ecosystem (Carvalho et al., 2019). Microbiota indicators of fecal contamination are a neglected area for assessing ecological status. Their inclusion and integration as a complementary tool for monitoring would be beneficial, considering the *One Health* approach. As a global strategy, One Health recognizes the interconnectedness of the health of people, animals, plants, and the environment from the local to the global levels and employs a holistic approach encouraging and expanding transdisciplinary collaborations, integrative research, capacity building, clinical practice, policy, and communication among many stakeholders (Aguirre et al., 2019). Moreover, the protection of water quality and water-related ecosystems is explicitly included in the UN Sustainable Development Goals (Goal 6 and Goal 14).

Many studies of FB have been published concerning biology, ecology, conservation (Denic et al., 2014; Ferreira-Rodríguez et al., 2019), on potential bioremediation tools (Lara et al., 2002; Soto and Mena, 1999), and on the effects of organic pollution, bacterial load, and chronic exposure to heavy metals on growth and oxidative stress variables in these animals (Rocha et al., 2015; Sabatini et al., 2011), but the dispersion of resistance to different types of antimicrobials has so far not been addressed. Bacteriological studies in FB have generally resorted to lethal sampling to obtain mixed fluids and tissues or samples of whole-body homogenates and to compare microbiota between specific tissues (Antunes et al., 2010; Chittick et al., 2001; Starliper, 2005; Starliper and Morrison, 1999).

On the other hand, highlight the presence and the role as a bioindicator that singular species may have. In this sense, Margaritifera margaritifera (Linnaeus, 1758) occurs in the upper reaches of water courses and is only present in a very few rivers of the Douro (Tuela, Mente, Rabaçal, Paiva, Beça and Terva) and Neiva Basins (Sousa et al., 2015). This species is currently listed as critically endangered in Europe (Cuttelod et al., 2011) and is included in Annexes II and V of the Habitats Directive (92/43/EEC) and in Annex III of the Berne Convention. It is classified as "Endangered" in the IUCN Red Book. *Potomida littoralis* (Cuvier, 1798) is an endangered freshwater mussel with a circum-Mediterranean distribution (Lopes-Lima et al., 2014) which is located exclusively in the middle and lower reaches of rivers.

In this context, the study reported here seeks to assess the resistance to antimicrobials of bacteria from two endangered freshwater mussels (*Margaritifera margaritifera* and *Potomida littoralis*) in the Tua River basin (northern Portugal). We explore whether site and mussel species are factors influencing the incidence of multidrug resistant (MDR) bacteria, and what potential risk they represent to freshwater ecosystems. The link between the location and species of mussels and the incidence of MDR is analyzed with a view to improving water resource conservation and management measures.

2. Material and methods

2.1. Study area

The Tua is one of the main tributaries of the River Douro. It runs through the Vila Real and Bragança District (northeastern Portugal). This river flows only through Portuguese territory and results from the merging of the Tuela and Rabaçal Rivers (both originating in Spain) 4 km upstream from the city of Mirandela. The surface area of the basin is 3122.80 km^2 in Portugal and 690.742 km^2 in Spain, making a total of 3813.540 km^2 (Fig. 1).

The study area covers the Montesinho Natural Park (≈75,000 ha) in its upper part located in the Terra Fria transmontana area and the end of the Regional Natural Park of Vale do Tua with about 25,000 ha (known as Baixo Tua). Around it there are a number of protected areas, namely the Natural Park of Douro Internacional, the Natural Park of Alvão, and the Protected Landscape of Albufeira do Azibo. The Rabaçal and Tuela sub-basins are characterized by deep, steep valleys with permanent meadows, large extensions of black oak (*Quercus pyrenaica*), chestnut groves, and fields of mainly wheat and rye. The main rock formations comprise basic (and ultrabasic) rocks, schists, and granites. In both rivers it is possible to find fish species like brown trout (*Salmo trutta*), Iberian barbel (*Luciobarbus bocagei*), Northern Iberian chub (*Squalius carolitertii*), loache (*Cobitis paludica*), Iberian straight-mouth nase (*Pseudochondrostoma duriense*) and *Iberocypris alburnoides,* this last two with the status of vulnerable (VU) by IUCN. In the end part of these two rivers, three more native bivalve species can be found, the *Anodonta anatina*, *Potomida littotalis* and *Unio delphinus* and the exotic species *Corbicula fluminea*.

The Baixo Tua region is characterized by considerable climate diversity, which is reflected in the plant landscape. There are woods of cork oak (Quercus suber) with a variable presence of holm oak and juniper as the most characteristic potential natural vegetation in the hottest and driest areas of the valley. Black oak forests occupy the coldest, rainiest areas of the plateau and the main mountain ranges. The geomorphology of this area is quite varied as a result of specific structural and lithological characteristics. It includes deep valleys and steep slopes, especially in the final reaches of the Tua and Tinhela Rivers, plus rocky outcrops of quartzite ridges and plateau areas, with little relief. In terms of its hydrological regime, the Tua River basin features significant year-on-year variability, with an average annual runoff of 988.1 hm3. There is also within-year variability, with high figures in winter and low figures in summer (varying on average between 4 hm3 in August and 277 hm3 in January). In this river, in addition to the species found in the Rabaçal and Tuela rivers, we can also find native fish species such as Cobitis calderoni, and the exotic ones (*Alburnus alburnus*, *Carassius carassius*, Cyprinus Carpio, Esox Lucius, Gobio lozanoi, *Gambusia holbrooki*, *Lepomis gibbosus*, and *Sander lucioperca*). Both rivers (Rabaçal, tuela and Tua) are attractive for fishing, and it is possible to find fishing concessions from the source to the mouth.

The Rabaçal and Tuela sub-basins (1867 Km^2) have a lower population (38,308 inhabitants) than the lower part of the Tua basin (48,255 inhabitants), which has a smaller surface area (1255 km^2). Thus, in the upper part of the basin agriculture is the main contributor to the high nutrient loads, while in the final reaches organic loads come mainly from agriculture, urban agglomerations, and industrial activities. The presence of several hydroelectric dams (2 in each sub-basin of the Rabaçal, Tuela and Tua) results in a loss of connectivity for aquatic communities and inflow regulation. This can lead, especially in the summer, to increased eutrophication with high mortality rates among aquatic organisms, situation that has been exacerbated by climate change (Haakonsson et al., 2020).

2.2. Sample processing, isolation, and identification

In this study, water (W) and freshwater bivalves (FB) were sampled at the same time in summer 2018 (Fig. 1). Water samples were collected at four sites in the Tua River basin (Rabaçal River T1 (Edral): 41°50′54.64″N, 7°7′57.34″W and T3 (Chelas): 41°30‘45.821″N, 7°12’32.92″W, and in the Tuela River T2 (Soeira): 41°51’43.78″N, 6°55’52.23″W, and the Tua River T4 (Barcel): 41°22’7.79″N, 7°14’20.88″W).

For each sampling site, two replicates of water were collected in 1 L

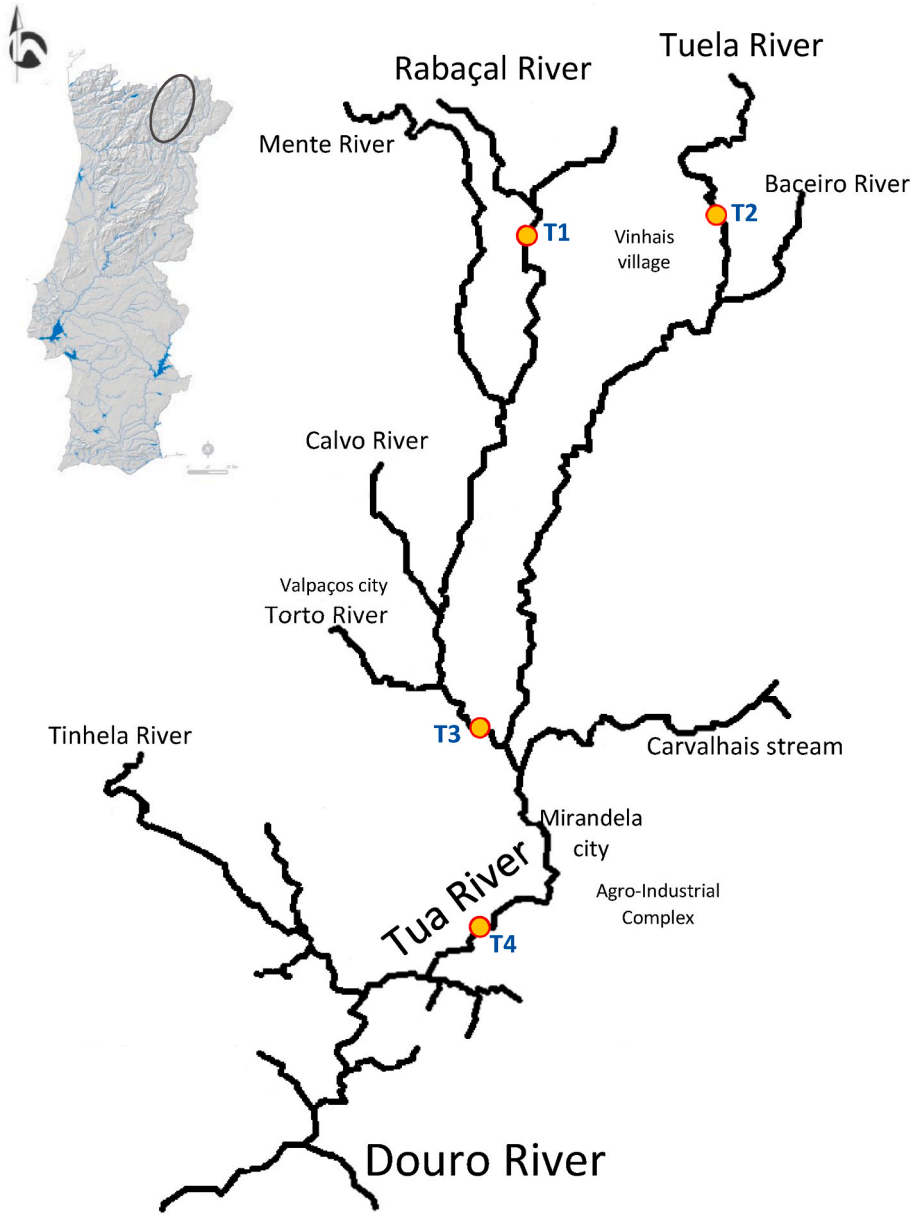

Fig. 1. Map of the study area and location of the four sampling sites (T1, T2, T3, and T4) in the Tua River basin.

sterile glass bottles, stored in cold containers, and transported for further analysis. Four individuals of *P. littoralis* were collected from T3 and T4, and two and three *M. margaritifera* were collected from T1 and T2, respectively. The samples were caught and maintained alive in a cooler with moist towels and taken to the University of Trás-os-Montes e Alto Douro (UTAD) for processing (travel time 50–100 min). Mussels were collected under a permit issued by the Institute for the Conservation of Nature and Forestry (ICNF). No ethics committee approval was needed, and no animal experiment has been performed in the scope of this research.

All the samples were analyzed at the Laboratory of Medical Microbiology - Antimicrobials, Biocides and Biofilms Unit, Department of Veterinary Sciences, UTAD. The strains were isolated from water by filter membrane method, where 100 mL were filtered with nitrocellulose membrane filters (0.45 μm pore size, Millipore, Watford, UK). The filters were placed on selective chromogenic media (Chromocult® Coliform Agar, Merck) and incubated for 24 h at 37 °C ± 1 °C.

Each bivalve was aseptically opened using sterile knifes and soft tissues were collected, weighed, and diluted in Buffered Peptone Water (1 g:9 mL) into sterile stomacher bags and homogenized for 1 min. Ten-fold serial dilutions were made up to 10^{-3} dilution in the same diluent/saline solution and 0.5 mL inocula from 10^{-1} and 10^{-2} dilutions were streaked onto the same selective chromogenic media, Chromocult and incubated for 24 h at 37 °C ± 1 °C. Chromocult Coliform Agar was used to detect and distinguish between *E. coli* and coliform bacteria in analyzing both water and bivalves samples.

A selection of presumptive colonies of *E. coli* (dark blue to violet color) and coliform bacteria (salmon red for other coliform bacteria colonies) were point-inoculated on a Brain Heart Infusion (BHI) medium and incubated for 24 h at 37 °C ± 1 °C, to obtain a pure culture for further susceptibility to antimicrobial agents screening.

2.3. Identification

Pure cultures were examined for colony and cell morphology, Gram stain, and oxidase and catalase activities. Additionally, the API 20 E commercial identification system (bioMérieux Inc., Hazelwood, MO, USA) was used in accordance with the manufacturer's instructions

(Geiss et al., 1985). Strains were kept frozen at −80 °C after re-suspension in tryptone soy broth (TSB, Oxoid) with 17% (v/v) glycerol.

2.4. Antimicrobial susceptibility screening

Isolates were subjected to antibiotic susceptibility test using 21 different antibiotics representing 5 classes of drugs, from which their antibiotic resistance profiles and multiple antibiotic resistance phenotypes were compiled. The isolates from water and the isolates from bivalves were tested for antibiotic susceptibility using a disk diffusion test (EUCAST, 2020). The protocol included standardized inocula of bacteria swabbed on Mueller-Hinton agar plates. An inoculum suspension of McFarland 0.5 was used, and plates were incubated at 35 °C ± 2 °C. Antimicrobial disks were used, and the samples were incubated for approximately 24–48 h. Zones of inhibition were measured, and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2020) and Clinical and Laboratory Standards Institute (CLSI, 2018) antimicrobial susceptibility tests were applied.

Each bacterial isolate was tested for 21 antibacterial agents (Oxoid disks, UK), representing 5 drug classes: β-lactams (amoxicillin - AML 10 μg, amoxicillin/clavulanic acid - AMC 30 μg, ticarcillin - TIC 75 μg, ticarcillin/clavulanic acid - TIM 85 μg, piperacillin - PRL 100 μg, piperacillin/tazobactam - TZP 110 μg, aztreonam - ATM 30 μg, imipenem - IMP 10 μg, meropenem- MEN 10 μg, cefoxitin - FOX 30 μg, ceftazidime- CAZ 30 μg, cefotaxime - CTX 30 μg, ceftriaxone- CRO 30 μg, cefoperazone- CFP 30 μg, and cefepime - FEP 30 μg), fluoroquinolones (ciprofloxacin - CIP 5 μg), aminoglycosides (amikacin - AK 30 μg, gentamicin - CN 10 μg, and tobramycin - TOB 10 μg),sulphonamides (sulfamethoxazole/trimethoprim - SXT 25 μg), and amphenicos (chloramphenicol - C 30 μg).

2.5. Statistical analysis

Multivariate statistical techniques were used (Hierarchical Cluster and Principal Component Analysis – PCA analyses) as exploratory data analysis techniques to identify groups of sites with similar profiles of antimicrobial susceptibility in water and freshwater bivalves. Cluster (unweighted pair group method average) and PCA analyses were performed using antimicrobial resistance data (%) for all *E. coli* and coliform isolates. Before the Hierarchical Cluster method was applied, the data was categorized using Bray-Curtis similarities. All statistical analysis was conducted using Primer version 7, with $p < 0.05$ (Plymouth Routines In Multivariate Ecological Research) software (Clarke and Gorley, 2015).

3. Results

3.1. Occurrence of E. coli and coliform bacteria in water and freshwater bivalves

From water and freshwater bivalves, 87 of the 135 isolates found (64.44%) were coliform bacteria- 53 of them (39.26%) came from water, compared with 34 (25.19%) from mussels. The species *M. margaritifera* was found only at sampling sites T1 and T2, while *P. littoralis* was found at T3 and T4.

From water samples were obtained 84 isolates: 31 *E. coli* and 53 coliform bacteria. 16 of the 31 *E. coli* isolates (51.61%) came from T4. 21 of the 53 coliform bacteria (39.62%) came from T3 and 20 (37.74%) from T4. In comparison, T1 had the lowest number of *E. coli* with just 3 of the 31 (9.68%) and of coliform bacteria with 5 out of 53 (9.43%).

The screening of 13 bivalves returned a total of 51 isolates: 17 *E. coli* and 34 coliforms. In fact, the sample included a total of 5 *M. margaritifera* (length = 7.95 cm ± 0.35; width = 3.45 cm ± 0.5). 2 specimens were from site T1 and 3 from T2. A total of 8 *P. littoralis* (8.75 cm ± 2.33; width = 5.30 cm ± 1.0) specimens were found: 4 each at sites T3 and T4.

These results reveal that most isolates were obtained from *P. littoralis*, with 14 out of 17 (82.35%) for *E. coli* and 28 out of 34 coliform bacteria. *M. margaritifera*, showed no *E. coli* isolates from site T1, but 6 of the 34 coliforms observed (17.65%) were from T1 and T2.

3.2. Antibiotic resistance data on different isolates from water and freshwater bivalves

Black blocks represent resistance and grey blocks indicate susceptibility to the five classes of antimicrobial agents, which included beta-lactams (aminopenicillins, ureidopenicillins cephalosporins, monobactam, carbapenems), fluoroquinolones, aminoglycosides amphenicols, and sulphonamides. White blocks indicate total susceptibility to antibiotics. Note that the profile of susceptibility to antimicrobials is higher for coliforms in both W and FB samples. It should also be noted that the susceptibility profile for water samples is similar to that of bivalve species in regard to the sampling sites; T1 and T2 (presence of *M. margaritif*era) show lower resistance values than T3 and T4 (presence of *P. littoralis*) (Fig. 2). Regarding *E. coli* isolates in water samples, T1 (66.7%) and T2 (50%) show the highest resistance to the aminoglycoside antibiotic. Also in water samples from T1 and T2, no isolates (*E. coli* and Coliforms) with resistance to the fluoroquinolone, amphenicol and sulphonamide groups were found. Moreover, there was no coliform resistance to fluoroquinolone at T3 and T4. For the FB samples from T1 and T2, no *E. coli* isolates with resistance to β-lactams, fluoroquinolones, aminoglycosides and sulphonamides were found. For coliforms from the same sampling sites it was found that there was no resistance to fluoroquinolones, amphenicols and sulphonamides.

An analysis of Fig. 3, which illustrates the hierarchical clustering of *E. coli* and Coliform isolates according to their phenotypical profiles, the two predominant clusters, cluster A and B, can he highlighted. Cluster A has approximately 50% of Bray-Curtis similarity, and represents the group of water samples collected at sites T1 and T2. Cluster B, brings together locations T3 and T4 for samples of W and FB with similarity values greater than 75%. Note that the FB isolates at sites T1 and T2 have low similarity levels with clusters A and B, and indeed with each other, with values of no more than 25%.

As reported in Fig. 4, among *E. coli* isolates multidrug resistant (MDR) was detected only at sampling site T4, in both water and FB, with 2cases out of 16 from water isolates and 3 out of 10 from FB (*P. littoralis*). Among coliform isolates, MDR was observed at T3 and T4 sampling sites, also in both water and *P. littoralis (*4/21 and 2/11 for T3 and 8/20 and 5/17 for T4). Twenty-four isolates (17.7% of the total isolates from W and FB) were found to be MDR (i.e. resistant to at least three different antibiotic classes). Multidrug resistance was higher for isolates from FB (19.6%) than from water (16.7%). Chloramphenicol and sulfamethoxazole/trimethoprim resistance was present in 100% of the MDR isolates from water (*E. coli* and coliforms). Ciprofloxacin resistance was also present in 100% of the MDR *E. coli* isolates from water.

A comparison of sampling sites shows that more *E. coli* and coliform isolates were found at T3 and T4 for both water and freshwater bivalves (in this case *P. littoralis*) than at T1 and T2. Also, MRD isolates (*E. coli* and Coliforms) were observed only at T3 and T4 sites, for water and *P. littoralis* (Fig. 5).

The first component accounted for 53.70% of the total variance and the second for 18.20%, with different groupings being observed due to the differences in antimicrobial patterns of resistance. Fig. 6 shows the sample projection on the space defined by the two principal components. Three groups can be seen: Group I with T3W, T3FB, T4W, T4FB; Group II with T2W, T2FB; and Group III with T1W, T1FB. The isolates in Group I include those with high patterns of resistance, mostly to β-lactams (AML, AMC, TIC, TIM, FOX, CAZ, CRO and FEP). Isolates from Group II include those with patterns of resistance to aminoglycosides (TOB and AK) and to β-lactams (PRL and ATM). Group III is interesting, with Coliform isolates showing a pattern of resistance to cefoperazone

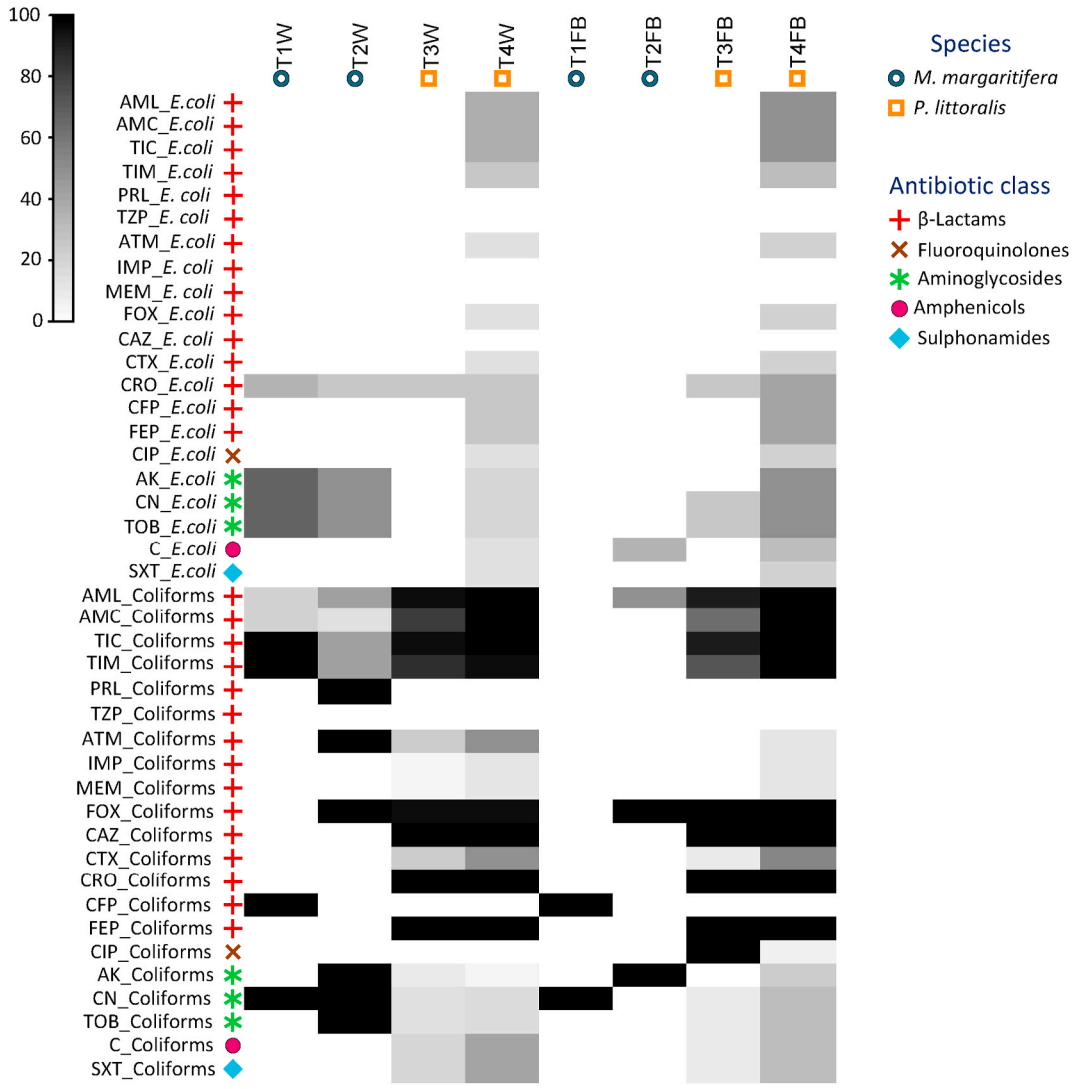

Fig. 2. Shadowmap showing the antimicrobial susceptibility profile of *E. coli* and coliforms isolates from water and freshwater bivalves. Rows represent individual antibiotics and columns represent the strains at sampling points. Black blocks represent resistance, grey and white blocks indicate different percentages of susceptibility to the five classes of antimicrobial agents. Circles and squares are the sites where *M. margaritifera* (T1 and T2) and *P. littoralis* (T3 and T4) were found, respectively.

(CFP).

4. Discussion

4.1. Impact of the presence of coliform bacteria in freshwater mussels

In Portugal, *M. margaritifera* populations still exist but only at sites with very low human pressure (Sousa et al., 2015). In fact, in this study the species was found only at sites T1 and T2, characterized by loads of anthropogenic disturbances, including altered flow regimes, habitat alteration, and pollution, much lower than those at T3 and T4. The *Salmo trutta*, a host fish for *M. margaritifera*, was also present at these sampling sites. Accordingly, site T1 was the one where fewest *E. coli* and coliform bacteria were found in water, followed by T2. An examination of the susceptibility profile of isolates shows that although there were no *E. coli* isolates from pearl mussels collected at site T1, in general there was a similar pattern between isolates from water and isolates from bivalve species, with susceptibility to antibiotics being higher in *M. margaritifera* than *P. littoralis* (Fig. 2). In this study, multidrug resistant (MDR) *E. coli* isolates were only detected at sampling site T4, located downstream from a wastewater treatment plant, in both water and FB (Fig. 4). The high capacity of mussels to filter and digest bacteria (Gomes et al., 2018; Lara et al., 2002) present in areas contaminated due to sewage discharge means that coliform bacteria constitute an important dietary source for this species. If this is the case, these mussels should be involved in reducing the number of bacteria and also in recycling the nutrients provided by domestic waste. As a negative result of this type of diet, the mussel population at T3 and T4 faces the highest concentration of bacterial toxins in the study area. The presence of the fecal indicator *E. coli* is related to the influence of wastewater. Zacharias et al. (2021) concur with this study in finding higher concentrations of *E. coli* in mussel samples than in water (Fig. 5), and further highlight that those high concentrations can influence the growth of the species. For their part, Bighiu et al. (2019) analyze the bacterial accumulation capacity of mussels at a laboratory scale and find the concentration of the indicator bacteria *E. coli* and enterococci to be 132 times higher than in water samples. This study also shows that bacteria can persist for up to 48 h in mussels before digestion, and thus concludes that this species makes a good bio-indicator, as it can indicate peaks of exposure to these bacteria.

In mammals, gram-negative bacteria, typically *E. coli,* produce toxic lipopolysaccharides (LPS or endotoxins) which trigger cytokine-

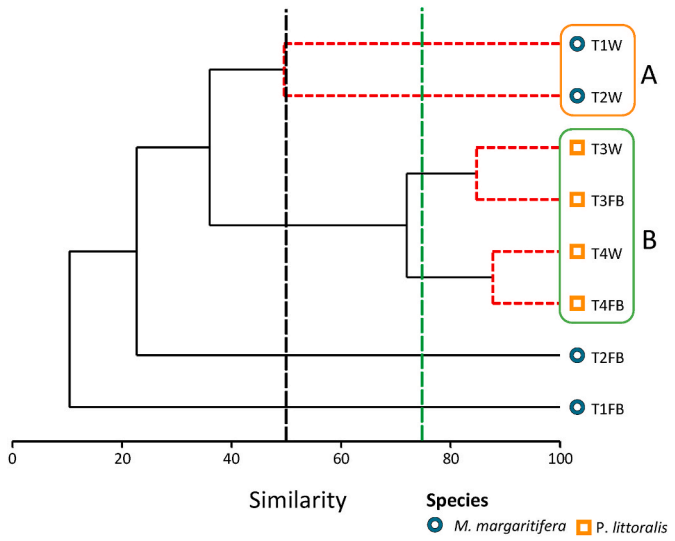

Fig. 3. Hierarchical clustering of *E. coli* and Coliform isolates according to their phenotypical profile (antimicrobial resistance). Circles and squares show the sites where *M. margaritifera* (T1 and T2) and *P. littoralis* (T3 and T4) were found, respectively.

mediated inflammatory reactions and lead to the production of reactive oxygen species (ROS) by phagocytic cells. The resulting oxidative stress is evidenced by increases in lipid peroxidation and decreases in reduced glutathione content, among other changes (Kheir-Eldin et al., 2001). In mussels, high bacterial loads have been associated with sick or dying animals (Starliper, 2011). Bacterial diseases such as vibriosis are studied more in marine bivalves than in freshwater mussels because the economic implications are greater in the former (Mateo et al., 2009; Pruzzo et al., 2005). However, there is a need to increase efforts in studies that check for the presence of these bacteria in freshwater mussel, and seek to determine how they affect the ecosystem and the animals that inhabit it.

4.2. Antibiotic resistant bacteria in mussel and water samples

This increase in antibiotic resistance observed at T3 and T4 sites in isolates from water and from *P. littoralis* could be attributed to high anthropogenic impact, resulting mainly from urban and industrial areas. Wastewater from hospitals, care facilities and from the agro-food industry are sources of human-pathogenic and/or multiresistant bacteria and various types of resistance (Gomes et al., 2021; Graham et al., 2019; Kaur et al., 2020; BIOHAZ et al., 2021; Voigt et al., 2020). Several studies show that antibiotics excreted into wastewater can lead to the spread of pathogenic antibiotic-resistant bacteria in the environment (Kümmerer, 2009; Zacharias et al., 2021).

Mussels living in surface waters that contain pathogenic bacteria and antibiotic-resistant bacteria may represent a reservoir of those microorganisms (Voigt et al., 2020), and thus can be used as indicators for their presence in a water environment. The results also show that the most impacted sampling sites, T3 and T4, have the isolates (from both water and bivalves) with the highest patterns of resistance, mostly to β-lactams (Fig. 2). β-lactam antibiotics are a family of structurally-related bactericidal drugs that contain the β-lactam ring in their chemical structure. Although they are one of the most important classes of antibacterial agents worldwide, their antibacterial efficacy has been restricted by the emergence of bacterial resistance (Lima et al., 2020). Carbapenems, which are part of the β-lactam group, show inhibitory microbiological activity against many infectious bacteria and are often used as *"antibiotics of last resort"* to treat multidrug resistance (Kattan et al., 2008). Our study finds resistance to carbapenems (IMP and MEM) in coliform isolates from water (T3 and T4) and mussels (T4) (Fig. 2).

In this study, isolates from water and *P. littoralis* (T3 and T4) show resistance to penicillins and their combination with clavulanic acid and to cephalosporins, specifically to a fourth generation of cephalosporin antibiotics. This species, found at the sites after the different spills, shows a very high resistance to multiple antibiotics. In addition, the samples collected at site T4 show a higher resistance than those from site T3 due to the presence of industrial plants and built-up areas between these two sites. This degradation, in both the water quality of the samples collected and the bivalve species, highlights the need to invest in measures to improve purification techniques and upgrade protection

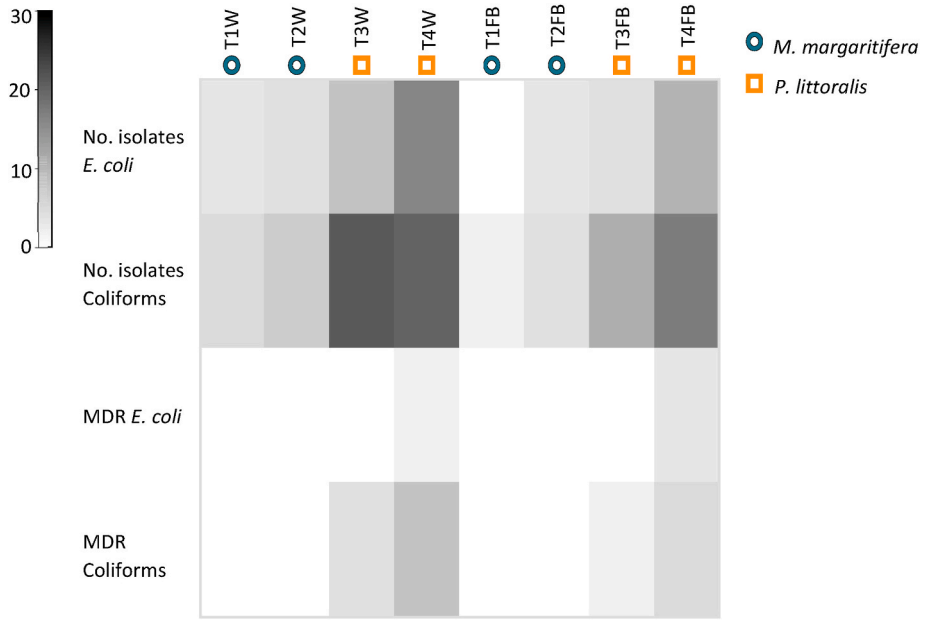

Fig. 4. Shade plot of total number of isolates and multidrug resistant-MDR isolates (*E. coli* and Coliforms) at each sampling site, for water and freshwater bivalve samples. Circles and squares show the sites where *M. margaritifera* (T1 and T2) and *P. littoralis* (T3 and T4) were found, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

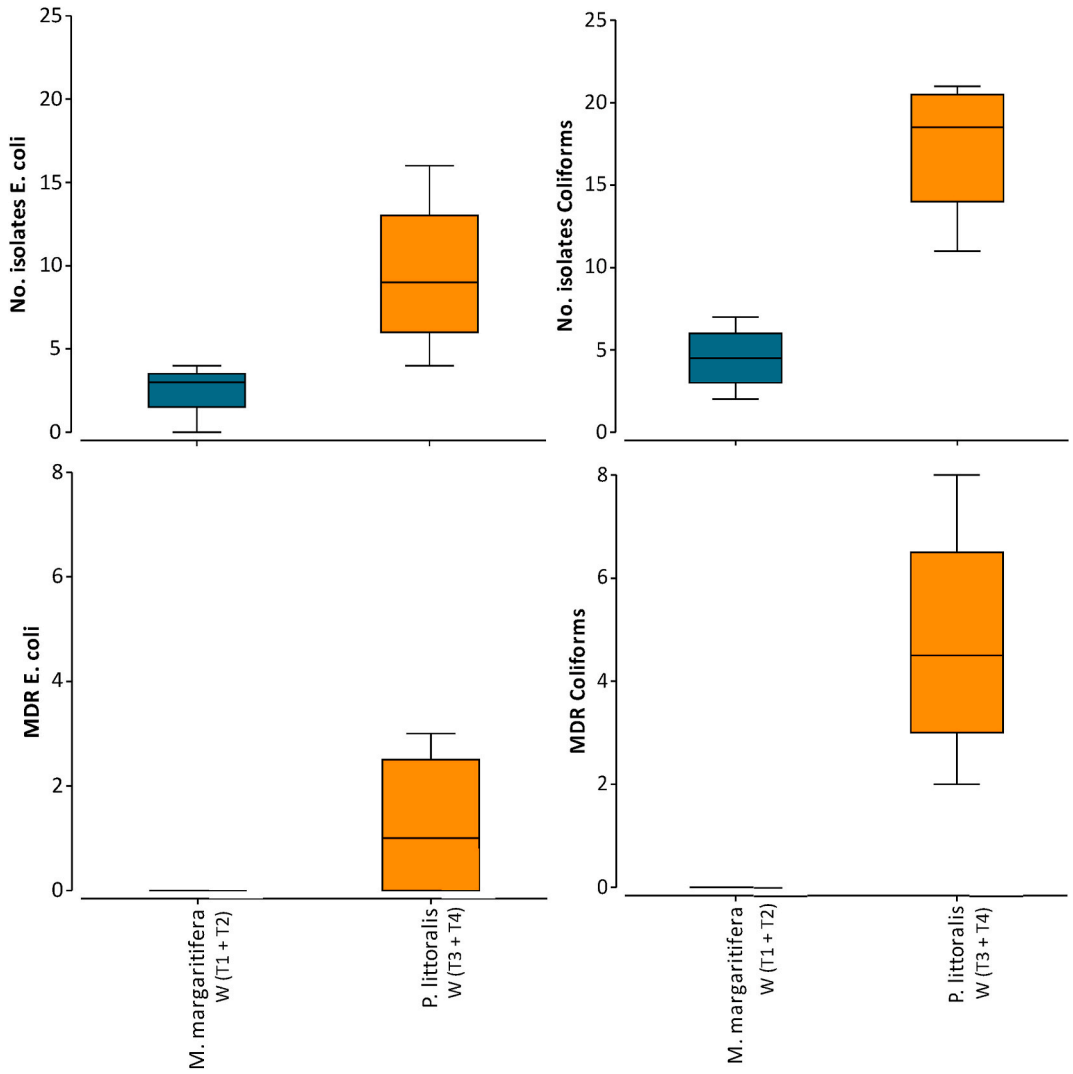

Fig. 5. Box and whisker plot of the number of total and multidrug resistant (MDR) isolates of *E. coli* and coliforms (water plus freshwater bivalves).

measures in water systems, e.g. by improving riverside vegetation (Acuña-Alonso et al., 2020). The results obtained are in line with those of Bighiu et al. (2019), who highlight that multi-resistant bacteria were found at 2 to 5 times higher concentrations in mussels (shell and tissue) than in their water sample. The results also suggest that *P. littoralis* seem to be display a major capability for surviving environmental injuries. Nevertheless, aggressions affecting the environment are exacerbated in these freshwater mussels because of bioaccumulation processes and their sessile lifestyle, so even if populations are managed and protected by local authorities their populations could face a downward trend.

The coliform isolates from *M. margaritifera* at T1 reflect their presence in water, with a similar pattern of resistance to ceftriaxone (CRO). Considering that this sampling site is upstream from the urban and food-producing environments, it is surprising to find that these isolates are resistant to a third generation of cephalosporin antibiotics. This reinforces the idea that aquatic environments may provide an ideal setting for the acquisition and dissemination of antibiotic resistance. Antibiotic-resistant bacteria also play an important role in increasing the risk associated with the use of surface waters (e.g. irrigation) and the spread of resistance genes (Graham et al., 2019). The diversity of antibiotic resistant bacteria is greater in the water samples than in the bivalves at T1 and T2, coinciding with Zacharias et al. (2021). However, at T3 and T4, where more variety was found, this difference is not so great. This could be due to the location of the sampling points, or to the low resistance and possibly low resilience of *M. margaritifera* as a species (Nogueira et al., 2021). On the other hand, the already limited distribution of *M. margaritifera* in Portugal could be under threat, so it could face decline.

4.3. General discussion

Identifying emerging risks is a priority for public health, so studies on mollusc-borne diseases as on finding prevention measures and mitigation strategies are key elements in ensuring environmental governance. To that end, an integrated approach is needed and the challenges of the water sector need to be addressed. Water resource plans have been proposed as planning instruments, and have indeed been used in several countries as an enabling tool for water resource management. Through them, stakeholders can prioritize water resource issues and seek the best solutions for all stakeholders, thus ensuring the effective management of water resources and adopting a process of continuous improvement.

This study reports an investigation into the antimicrobial resistance of bacteria obtained from two endangered freshwater mussels, *Margaritifera margaritifera* and *Potomida littoralis*, and from the water of the Tua River basin. Its secondary objective is to assess the current water plan for the basin and improve water planning there. *M. margaritifera* was only found at the upstream sites on the river (T1 and T2), so its habitat is limited (Nogueira et al., 2021). At sites T3 and T4, located downstream

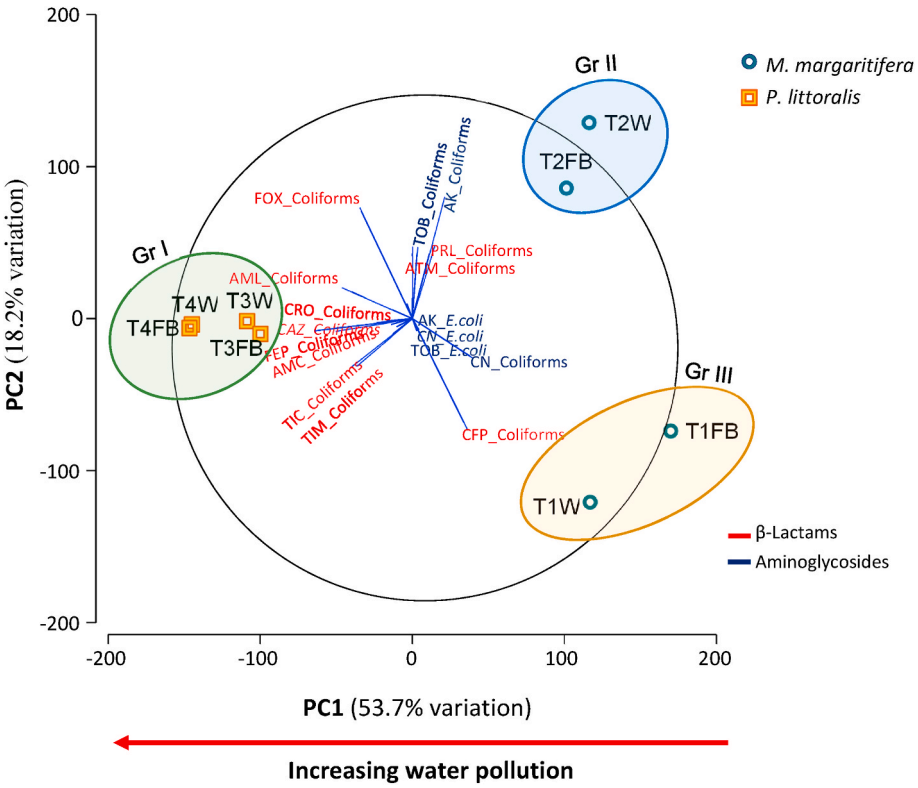

Fig. 6. PCA biplot of the links between samples (water and FB isolates) and tolerance to the five antimicrobial classes (β-lactams, aminoglycosides, fluoroquinolones, sulphonamides and amphenicos).

from wastewater treatment plants and other discharge points, including healthcare and agri-food facilities, higher numbers of *E. coli* and Coliform isolates were found in both water and freshwater bivalves (in this case *P. littoralis*) than at T1 and T2. MRD isolates (*E. coli* and Coliforms) were only found at sites T3 and T4 for both water and *P. littoralis* (Fig. 5). In addition, more multi-drug resistant isolates (*E. coli* and Coliforms) were found at T3 and T4 for water and *P. littoralis* (Fig. 4). The PCA score graphs revealed clear separations between infected and non-infected tissues, as well as in the water samples analyzed, showing a great difference between sampling points (Fig. 6).

The presence of these substances points to impact from treated wastewater on these species, whose biofiltration capacity increases the concentration of the substances in their organisms to levels higher than those found in the water. Serra-Compte et al. (2021) propose, for example, screening methods followed by chemical analysis as they would reduce the costs of antibiotic analysis, facilitating their implementation for environmental monitoring. They also stress that combined approaches would be beneficial to better understand and assess the risk of antibiotics in the environment and their potentially hazardous consequences for the environment and human health. These monitoring and assessment systems should be incorporated into water resource plans for areas at risk of pollution. This analysis seeks to provide constructive, practical suggestions for improving water monitoring, management, and governance across Europe, but many of our recommendations are generalizable to the future development of sustainable water management and policy worldwide.

5. Conclusions

The dispersion of resistance to several classes of antimicrobials is assessed simultaneously here in freshwater mussels and the water where they live, at sites subject to different levels of anthropogenic pressure, from an EcoHealth perspective under the One Health approach. 64.44% of all the samples were coliform bacteria, 39.26% were found in the water and 25.15% in the samples of mussels. *M. margaritifera*, a specially protected species, was only found at the sites free from anthropogenic pressure. The population of *P. littoralis* showed a pattern of high resistance with multiresistant bacteria. It is therefore inferred that the mussel species in the freshwater of the study area are being exposed to bacterial loads that could contribute to spread of pathogenic antibiotic-resistant bacteria in the environment, and to their decline. The results obtained can be used in the future to make decisions on preventive and corrective measures to improve water quality and mitigate the risk to human health. For example, measures such as improving water treatment at hospital sewage treatment plants, improving riverine vegetation protection (natural filter), increasing water quality monitoring, among others, may be pointed out.

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Author contributions

Conceptualization and methodology, M.J.S., C.F, X.A. and S.V.; sampling collection A.T, S.V., C.F. and M.J.S; performed the laboratory measurements. C.F; S.V. and M.J.S. writing—original draft preparation, M.J.S., C.F and S.V writing—review, editing and provided additional information M.J.S., C.F, S.V., A.T and X.A. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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