

Endangered freshwater bivalves as reservoirs of antimicrobial-resistant *Escherichia coli* and other species of the Enterobacteriaceae family

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INTRODUCTION

Freshwater unionoids are one of the most threatened animal groups worldwide, suffering dramatic regressions globally. The freshwater pearl mussel *Margaritifera margaritifera*, currently listed as critically endangered in Europe, and *Potomida littoralis*, listed as endangered, are both present in the River Tua basin (Portugal). These aquatic long-lived organisms, responsible for important functions and vital ecosystem services [1], are sensitive to environmental changes, making them excellent bioindicators [2]. According to Grizzle & Brunner [3] mussels are capable of establishing symbiotic mutualistic or antagonistic relationships with bacteria. The inappropriate use of antibiotics has led to the emergence and spread of resistant bacteria, recognized today as a serious public health problem. Based in the concept of "One health", the objective of this study was to examine the antibacterial resistance rates in *Escherichia coli* and other Enterobacteriaceae isolates obtained from water and the two freshwater bivalve species collected in the River Tua Basin.

METHODOLOGY

Water (W) and freshwater bivalves (FB) were sampled at same time in four sites, located in the Tua River basin, in summer of 2018 (Fig. 1). For each site, two replicates of water were collected in 1 L sterile glass bottles, stored in a cold container, and transported to further analyses. Four individuals of *P. littoralis* species were collected from T3 and T4 and two and three *M. margaritifera* species were collected from T1 and T2, respectively. The samples were transported live and maintained in a cooler with moist towels to the laboratory for processing. Mussels were collected under the authority of permit issued by the Institute for the Conservation of Nature and Forest (ICNF). The isolation of strains from water was performed by the filter membrane method. Each bivalve was aseptically opened using sterile knives and soft tissues were collected, weighted, and diluted on Buffered Peptone Water (1g:9 mL) into sterile stomacher bags and homogenized for 1 min. Chromocult® Coliform Agar, was used for *E. coli* and coliform bacteria detection. All the presumptive colonies of *E. coli* and coliform bacteria were point-inoculated on Brain Heart Infusion (BHI) medium and incubated 24 h at 37°C, to obtain pure culture, for susceptibility to antimicrobial agents screening by a disk diffusion test. Each bacterial isolate was tested for 21 antibacterial agents representing 5 drug classes: β - lactams, fluoroquinolones, aminoglycosides, sulphonamides and amphenicos. Hierarchical Cluster analyses (unweighted pair group method average) were performed as exploratory data analysis techniques, to identify groups of sites with similar profile of antimicrobial susceptibility in water and freshwater bivalves.

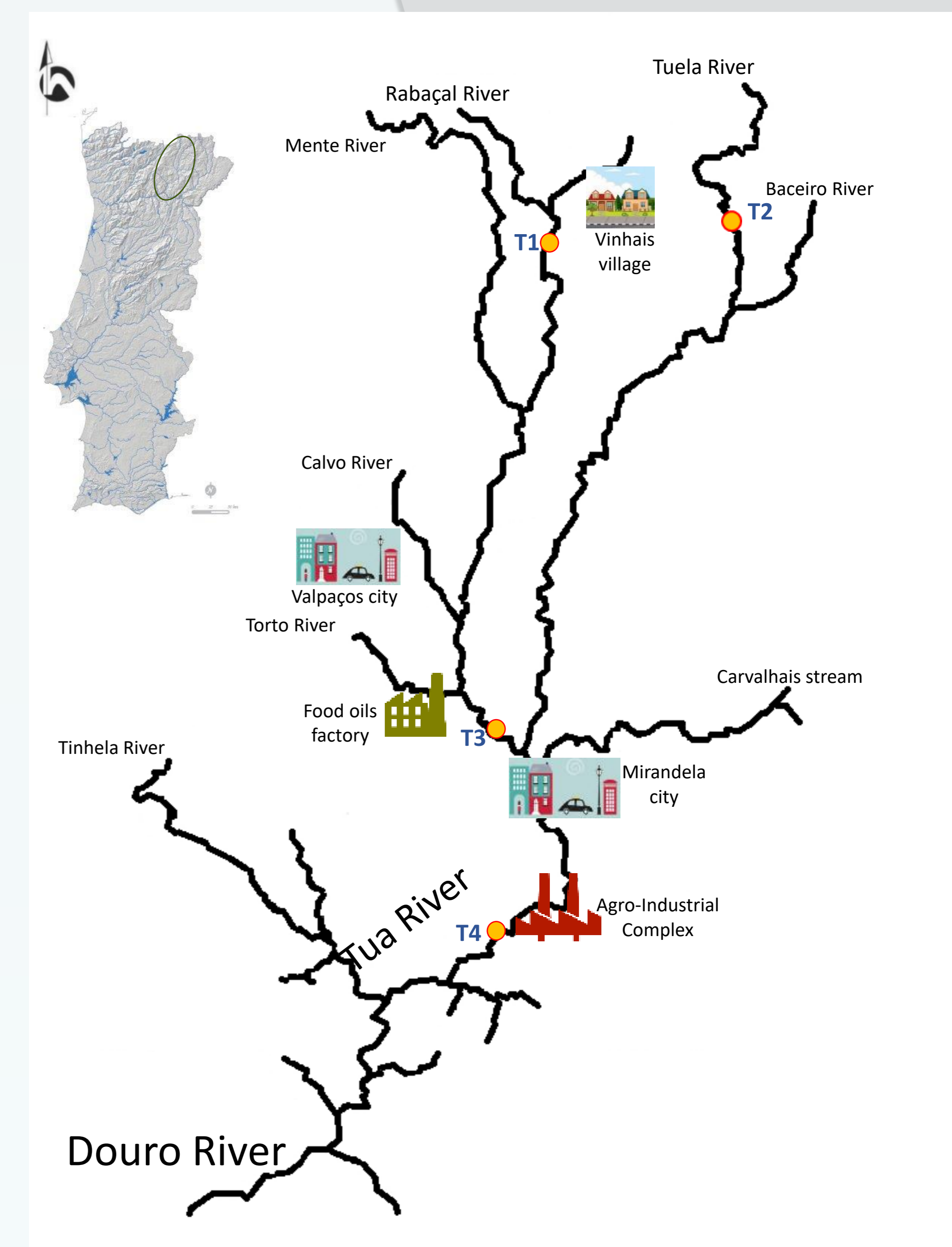


Fig. 1. Map of the study area and the location of the four sampling sites (T1, T2, T3 and T4) in Tua River basin.

RESULTS

A total of 135 isolates (W and FB) were found and 64.44 % (87/135) were coliforms bacteria and, among them, mostly came from water 39.26 % (53/135), comparing with coliforms bacteria from mussels 25.19 % (34/135). The species *M. margaritifera* was found only on the sampling sites T1 and T2, whereas the species *P. littoralis* was found on the locations T3 and T4. Among the total of 135 isolates (water and freshwater bivalves), 64.44 % (87/135) were coliforms bacteria and, among them, mostly came from water 39.26 % (53/135), comparing with coliforms bacteria from mussels 25.19 % (34/135). The species *M. margaritifera* was found only on the sampling sites T1 and T2, whereas the species *P. littoralis* was found on the locations T3 and T4. The profile susceptibility to antimicrobials was higher for coliforms for both W and FB samples (Fig. 2). The hierarchical clustering of *E. coli* and Coliforms isolates according to their phenotypical profile separates the sites in two predominant clusters. The first one represents the group of water samples collected at sites T1 and T2, and the second

one associates locations T3 and T4 for samples of W and FB with similarity values greater than 75%. FB isolates at sites T1 and T2 have low similarity with clusters A and B, and even between them, not exceeding the value of 25 % (Fig. 3). Comparing sampling sites, higher number of *E. coli* and Coliforms isolates was founded at T3 and T4, for both, water and freshwater bivalves, in this case *P. littoralis*, than at T1 and T2. Also, the presence of MRD isolates (*E. coli* and Coliforms) was observed only at T3 and T4 sites, for water and *P. littoralis* (Fig. 4).

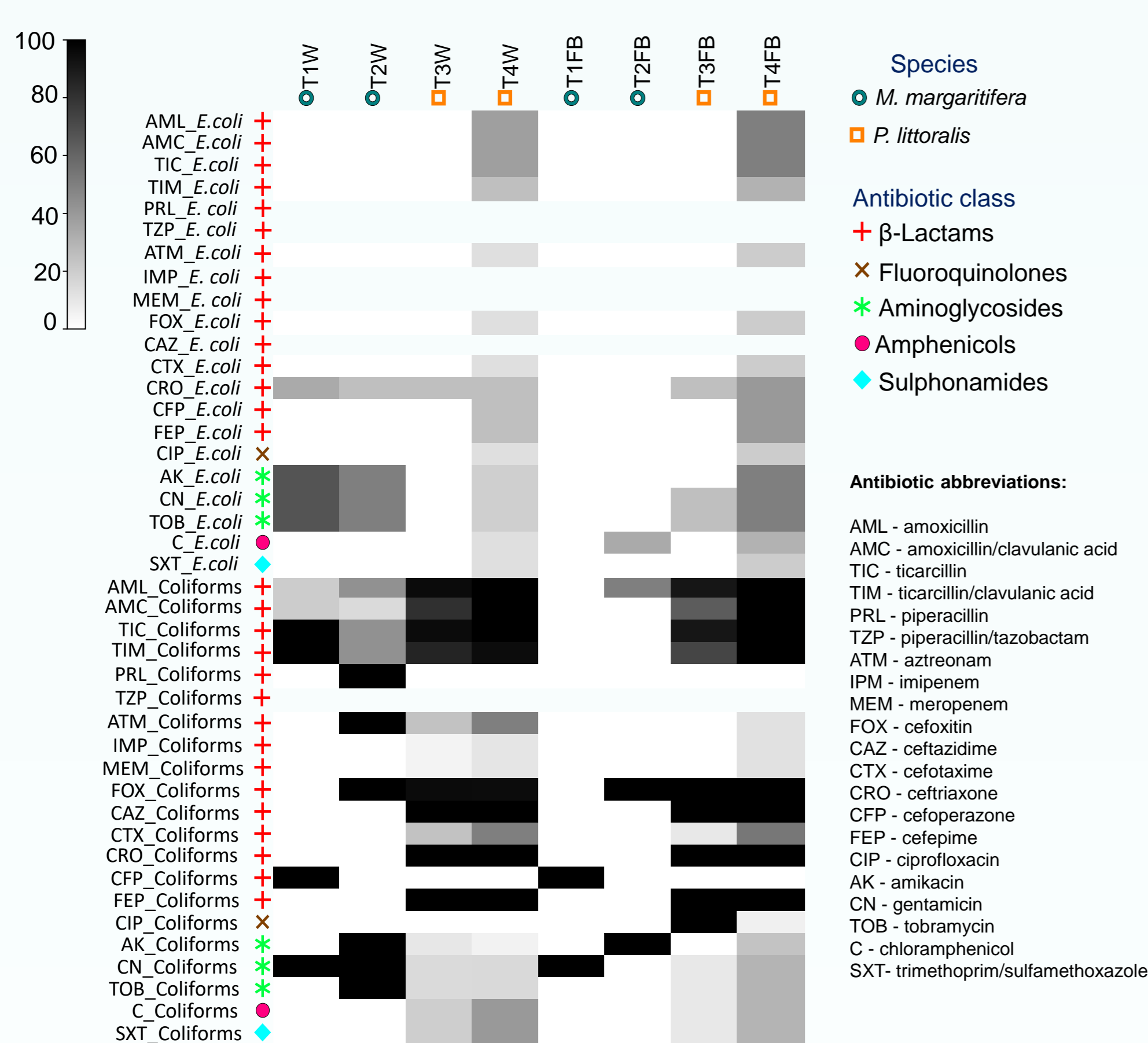


Fig. 2. Shadow map showing the antimicrobial susceptibility profile of *E. coli* and coliforms isolates from water and freshwater bivalves. Rows represent individual antibiotics, and columns represent the strains in sampling points. Black blocks represent the strains, grey and white blocks indicate different percentage 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 present, respectively.

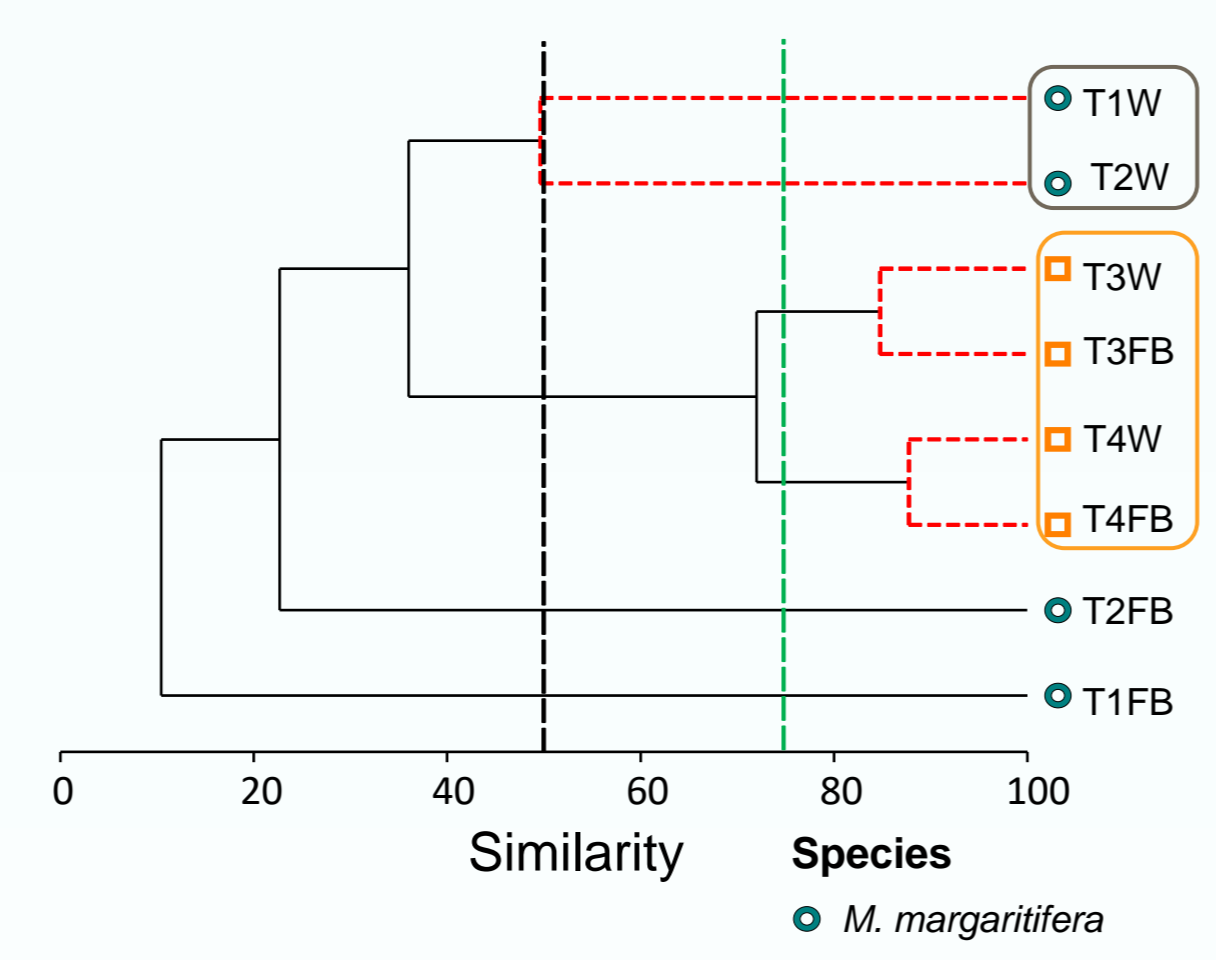


Fig. 3. Hierarchical clustering of *E. coli* and Coliforms isolates according to their phenotypical profile (antimicrobial resistance). Circles and squares are the sites where *M. margaritifera* (T1 and T2) and *P. littoralis* (T3 and T4) were present, respectively.

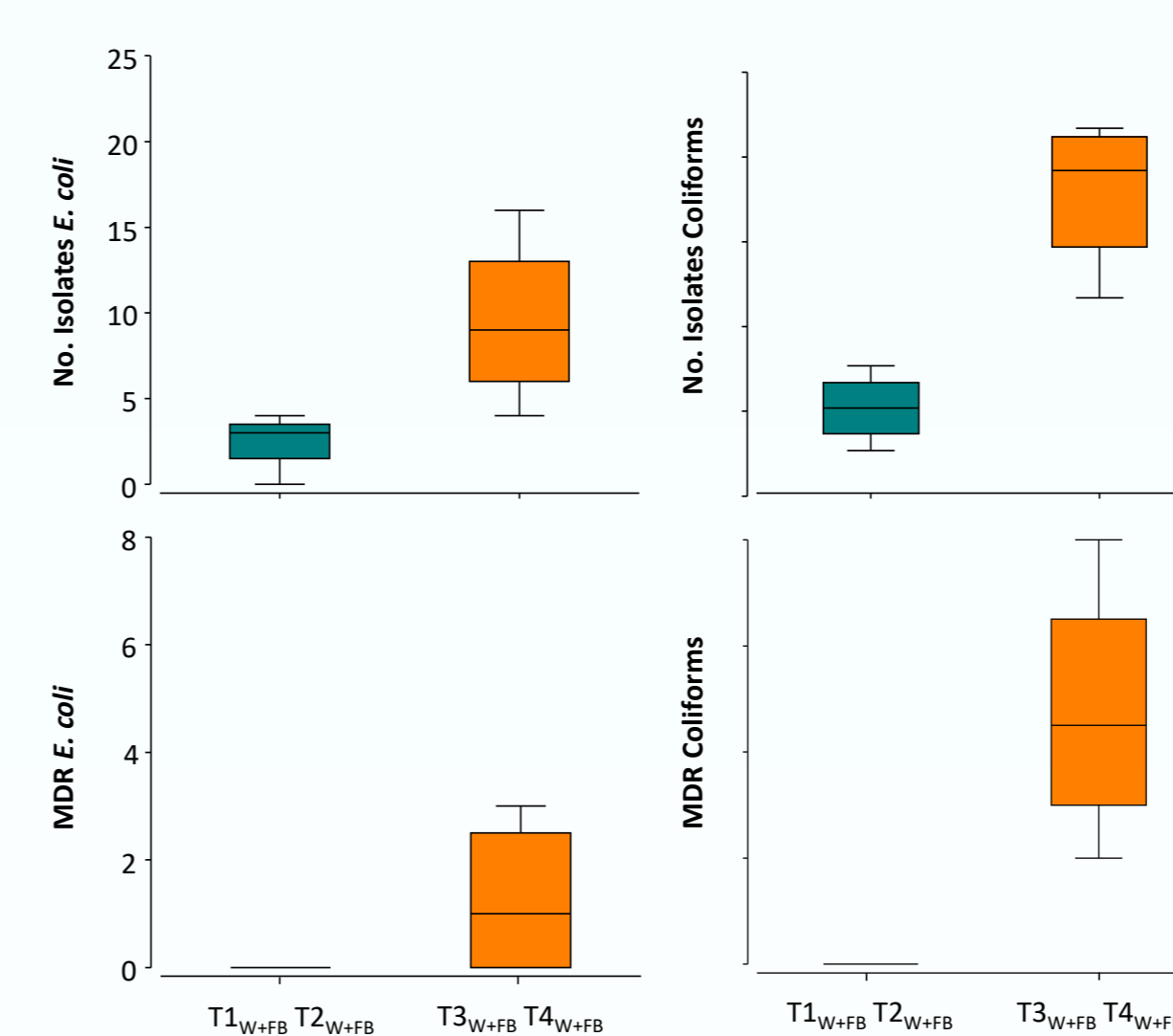


Fig. 4. Box and whisker plot of the number of total and multidrug resistant (MDR) isolates of *E. coli* and coliforms (water plus freshwater bivalves).

CONCLUSIONS

M. margaritifera inhabits in more pristine sectors of river not subject to the development of multidrug resistance, unlike *P. littoralis* which, is naturally found in lower stretches that are subjected to greater pressure and, as such, a greater probability of the appearance of multiresistant organisms. The presence of *E. coli* in freshwater bivalves can represent serious public health problems once bacteria are the most important biological contaminants in foodborne diseases. Given the conservation status of both studied species their protection against this silent/invisible enemy should be a priority, although work done with Unionid Mussels from Northern Portugal showed that they had a great Antimicrobial and Antibiofilm Activity.

REFERENCES

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