The meiofauna as neglected carriers of antibiotic resistant and pathogenic bacteria in freshwater ecosystems

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ABSTRACT

The World Health Organization considers antibiotic resistance as one of the main threats to human and other animals' health. Despite the measures used to limit the spread of antibiotic resistance, the efforts made are not enough to tackle this problem. Thus, it has become important to understand how bacteria acquire and transmit antibiotic resistant genes (ARGs), in particular in the environment, given the close connection between the latter and human and animal health, as defined by the *One-Health* concept. Aquatic ecosystems are often strongly impacted by anthropogenic activities, making them a source for ARGs and antibiotic resistant bacteria (ARB). Although freshwater meiofauna have been the object of active research, few studies have focused on the relationship between the spread of antibiotic resistance and these organisms. In this review, we investigated freshwater meiofauna as carriers of resistances since they play a central role in the aquatic environments and can harbor human and animal potential pathogens. We assessed if these animals could contribute to the spread of ARGs and of potentially pathogenic bacteria. Only four taxa (Rotifera, Chironomidae, Cladocera, Copepoda) were found to be the subject of studies focused on antibiotic resistance. The studies we analyzed, although with some limitations, demonstrated that ARGs and ARB can be found in these animals, and several of them showed the presence of potentially pathogenic bacteria for humans and animals within their microbiome. Thus, meiofauna can be considered a source and a reservoir, even if neglected, of ARGs and ARB for the freshwater environments. However, further studies are needed to evaluate the impact of the meiofauna on the spread and persistence of antibiotic resistance in these ecosystems.

INTRODUCTION

The development and spread of antibiotic resistance are emerging threats to human and animal health, due to the overuse and misuse of antibiotics in human healthcare and animal farming in the previous decades (MacLean and Millan, 2019). Urgent actions are needed to tackle the spread of antibiotic resistance and avoid the "worst case scenario" of 10 million dead people/year by 2050 because of antibiotic resistant bacterial infections (de Kraker *et al.*, 2016), as estimated by O'Neill in 2014. It is becoming clearer that the measures adopted until now to limit the spread of resistances are insufficient. It is essential to face this challenge using wider approaches, which focus on the role of the environment as a reservoir of antibiotic resist-

ance (Finley *et al.*, 2013) and apply the *One-Health* principles (Guardabassi *et al.*, 2020). The *One-Health* concept considers the health of humans closely connected to and dependent on that of other animals and the environment (Robinson *et al.*, 2016). To fulfill these criteria, it is pivotal to consider all the possible biotic and abiotic factors favoring the spread of the antibiotic resistance and all the ecological niches where the resistances can develop and persist in the environment (Finley *et al.*, 2013). Thus, investigating the characteristics and the dynamics of the antibiotic resistance genes (ARGs) and of the bacteria that harbor them (antibiotic resistant bacteria, ARB) in the different ecosystems plays a central role in this strategy (Finley *et al.*, 2013). The aquatic environments (and in particular freshwaters) could act as a natural pool of resistances (Olanrewaju *et al.*, 2019). Because often impacted by anthropogenic activities and subjected to the release of antibiotics, they can favor the acquisition and dissemination of ARGs and, even, stimulate their transfer into pathogenic bacteria of clinical interest (Marti *et al.*, 2014; Suzuki *et al.*, 2017; Nnadozie and Odume, 2019). The latter can come back to humans, *e.g.*, through recreational and fishery activities (Leonard *et al.*, 2018; Syrova *et al.*, 2018) and the consumption of raw seafood (Mughini-Gras *et al.*, 2019), leading to antibiotic resistant bacterial infections. Animals living in aquatic environments may exacerbate these issues offering refuge to ARB and allochthonous pathogenic bacteria, and providing a niche that allows for the persistence of bacteria carrying resistances (Fu *et al.*, 2017; Hong *et al.*, 2018). In this context,

the microbiome of freshwater meiofauna may constitute a neglected source of ARGs and pathogenic ARB for humans and animals.

The meiofauna designate a functional group of small animals living both in marine and freshwater environments with dimensions small enough to pass through a 1000-µm mesh net but bigger than 42-µm mesh net size (Fenchel, 1978; Higgins and Thiel, 1988; Schmid *et al.*, 2000), mostly from sediment-associated (benthic) habitats, but not only (Traunspurger and Majdi, 2017). On this basis, meiofauna are often divided in permanent (organisms spending their whole life cycle as meiofauna) or temporary (juvenile stages of animals, such as insects, that grow into macrofauna) (Stead *et al*; 2005; Schmid-Araya *et al.*, 2020), and constitute a trophic link between microbial primary production and higher trophic levels (Schmid-Araya and Schmid, 2000; Ptatscheck *et al.*, 2020). They can also be found on rocks, wood, algae, plants and animals (Pinto *et al.*, 2013). Meiofaunal organisms are ubiquitous and extremely diverse, comprising representatives from almost all the known animal phyla (with some phyla exclusive to meiofauna) (Giere, 2009; Fontaneto *et al.*, 2015), hence constituting useful biological models for comprehensive, modern ecological assessments (Giere, 2009). Meiofauna play a major role in aquatic ecosystem processes: decomposition of organic material, nutrient cycling and energy flow, bioerosion and bioturbation of bottom sediment (Adámek and Maršálek, 2013; Prather *et al.*, 2013). They are an important food source to a variety of animals that could not survive without them (Schmid-Araya and Schmid, 2000; Ptatscheck *et al.*, 2020), and respond rapidly to environmental changes, given their high turnover rates (Zeppilli *et al.*, 2015). For these reasons, they have been widely proposed as bio-indicators of the quality of aquatic ecosystems (Zeppilli *et al.*, 2015) and used to determine the impact of human activities and pollution in the environment (Schratzberger and Somerfield, 2020). In view of this, they could represent an interesting model for the study of the spread of the resistance traits in aquatic systems, but their role as carriers of ARB is still largely unexplored. Meiofauna have been extensively studied in the past decades with a conspicuous number of publications in recent years (Majdi *et al.*, 2020). Most of these studies investigated marine meiofauna, while little attention has been paid to the freshwater compartment (Majdi *et al.*, 2020). Similarly, the contribution of freshwater meiofauna to the spread of resistances in the environment is scarcely explored. The aim of this review is to summarize the actual knowledge about the role of freshwater meiofauna as carriers of ARB, in particular, of human and animal potentially pathogenic bacteria and ARGs. We selected the most studied taxa of freshwater meiofauna and analyzed the collected literature, finally highlighting what has been found and what has to be addressed by future research efforts to fill the still lasting knowledge gaps.

METHODS

As above stated, the term "meiofauna" defines a heterogeneous set of small animals, covering almost all animal phyla, with a broad range of functional groups that have key roles in different ecosystems (Schratzberger and Ingels, 2018). Given this, we decided to make a selection of taxa, associated to freshwater meiofauna, on the basis of the results obtained from field studies (Majdi *et al*., 2017; Ptatscheck *et al*., 2020), which investigated lake and streams, and the evidence from recent literature (for a summary of the main findings see Majdi *et al.*, 2020). Therefore, we chose the ten most studied taxa of freshwater meiofauna (Chironomidae, Cladocera, Copepoda, Gastrotricha, Nematoda, Oligochaeta, Ostracoda, Platyhelminthes, Rotifera, Tardigrada) (Tab. 1) and performed a survey on Clarivate Web of Science (WOS) on 16th July 2021, using the keywords (in all fields): [(*taxa** AND "antimicrobial resistance") OR (*taxa** AND "antibiotic* resistan*")], for example [(cladocer* AND "antimicrobial resistance") OR (cladocer* AND "antibiotic* resistan*")], in the case of antibiotic resistance, and [(*taxa** AND "microb*") OR (*taxa** AND "pathog*")], in the case of microbiome/pathobiome. Specifically, for *Daphnia* (which is a largely recognized model organism), the search was done as follow: [(daphnia AND "antimicrobial resistance") OR (daphnia AND "antibiotic* resistan*")] to show an example. We did the same for the terms "naupli*" and "turbellaria" (an improper word used in the past to refer to free-living flatworms). We limited the output to research articles provided with impact factor, while other documents (*e.g.*, review, book chapter, others) were discarded. Then, we evaluated the articles one by one and selected those of interest (*e.g.*, articles actually focused on the investigation

Tab. 1. List of the selected meiofaunal taxa and relative association with studies on antibiotic resistance.

Selected taxa	Association with ARGs/ARB
Chironomidae	Yes
Cladocera	Yes
Copepoda	Yes*
Gastrotricha	No
Nematoda	No
Oligochaeta	No
Ostracoda	No
Platyhelminthes	No
Rotifera	Yes*
Tardigrada	No

*As part of freshwater communities.

of the antibiotic resistance in freshwater meiofauna). Regarding the antibiotic resistome (total content of ARGs), we kept all the articles retrieved. For the microbiome/pathobiome (total content, in the meiofauna microbiome, of genera containing bacteria potentially pathogenic to humans and other animals), when the number of records associated with a specific taxon was scarce, we retained all the studies; in case of an elevated number of hits, we selected the most representative articles, preferring the ones focused on the whole microbiome rather than single pathways.

RESULTS

Meiofauna microbiome and pathobiome

The community of microorganisms inhabiting a defined environment, such as the body cavities of a multicellular organism, is defined as a microbiome (Marchesi and Ravel, 2015). Human and animal microbiomes are known to have a beneficial effect on the host (Sommer and Bäckhed, 2013). The microbiome affects the host's immunity, development, metabolism, nutritional habits, mating and behavior (Turnbaugh *et al.*, 2006; Rhee *et al.*, 2009; Sharon *et al.*, 2010; Shin *et al.*, 2011; Foster and McVey Neufeld, 2013; David *et al.*, 2014) and in some cases it can cause harm to the host by contributing to disease. The pathobiome represents the portion of the microbiome associated with a reduced health status in the host as a result of their interaction (Bass *et al.*, 2019; Vayssier-Taussat *et al.*, 2014). While microbial communities hosted by humans and other large vertebrates are complex and it is difficult to interpret their role on the host system (Waldor *et al.*, 2015), meiofauna offer a more convenient model to study microbiome-host interactions (*e.g.*, Olszewski *et al.*, 2020). Yet, little is known about host-microbiome interactions in freshwater especially for meiofauna. In this section, we focus on two main points: which microbial taxa are mainly represented in freshwater meiofauna and, among them, which bacterial genera might be potentially pathogenic to human and other animals and cross spread to the other food web levels.

Ostracods are small crustaceans, inhabiting both marine and freshwater ecosystems. They have the ability to hatch from eggs resistant to adverse environmental conditions, and rapidly populate available water bodies (Rossi *et al.*, 2012; Vandekerkhove *et al.*, 2013). Furthermore, some species show short life cycle and parthenogenetic reproduction and, therefore, are pioneering organisms of temporary freshwaters (Martins *et al.*, 2009; Olmo *et al.*, 2016). Olszewski and colleagues (2020) investigated the bacteria associated with two species of freshwater ostracods *Sclerocypris tuberculata* and *Potamocypris mastigophora* co-cultured in laboratory conditions after being collected from a lake sediment sample. Despite the fact they were grown in the same experimental conditions, each ostracod hosted distinct bacterial

communities. Especially the family *Comamonadaceae* (Betaproteobacteria) dominated in *P. mastigophora* and the *Aeromonadaceae* (Gammaproteobacteria) in *S. tuberculata*. The latter represented on average 38% of the total community composition of *S. tuberculata* while, in *P. mastigophora,* this family represented about 0.15% of the total microbiome. The genus *Acinetobacter,* which comprises potential pathogens for humans (Howard *et al.*, 2012), conversely was more abundant in *P. mastigophora* representing 0.42% towards 0.09% in *S. tuberculata*, suggesting that different species of ostracods within the same experimental system can diverge in the microbiome composition. In general, ostracod-associated bacterial communities (Jarett *et al.*, 2013; Schön *et al.*, 2019; Olszewski *et al.*, 2020), when looking at a higher taxonomic level (phylum), show a composition similar to that of the bacterial communities of other freshwater invertebrates (i.e., *Daphnia*) (Sison-Mangus *et al.*, 2015; Macke *et al.* 2017), while most differences are at lower diversity levels.

Another important group of small crustaceans in the meiofauna is the Cladocera, constituting the main secondary consumers in aquatic ecosystems, as they show the highest rate of filtration among small freshwater invertebrates (Dumont and Negrea, 2002). Organisms belonging to the genus *Daphnia* are filter feeders and play a central role in the food webs of lakes and ponds, as consumers of bacterioplankton and phytoplankton, and as preys of invertebrate and fish (Olanrewaju *et al.*, 2019). Furthermore, *Daphnia* has been extensively used as a model organism in biological research (Ebert, 2005). Several studies described the microbiome of *Daphnia* species (Freese and Schink, 2011; Sison-Mangus *et al.*, 2015; Macke *et al.*, 2017; Eckert *et al.*, 2021). These organisms harbor a beneficial and active microbiome, composed by both resident and transient bacterial species (Grossart *et al.*, 2009; Eckert and Pernthaler, 2014; Sison-Mangus *et al.*, 2015; Peerakietkhajorn *et al.*, 2016). Eckert and colleagues (2016) made an experiment adding *Daphnia* to a natural freshwater bacterial community. The authors found that the genus *Flavobacterium* composed over 50% of the daphnia-associated microbiome. *Flavobacterium* contains many opportunistically growing taxa and some potential fish pathogens (Nematollahi *et al.*, 2003; Neuenschwander *et al.*, 2015). Other human potentially pathogenic genera found were *Pseudomonas*, *Klebsiella*, *Brevundimonas* and *Acinetobacter* despite being less abundant compared to *Flavobacterium*. Similarly, Callens and colleagues (2020) found that the composition of the environmental bacterial community has a major influence on the *Daphnia* gut bacterial community, and described a similar pathobiome that included *Brevundimonas*, *Acinetobacter*, *Pseudomonas,* and *Flavobacterium*. Akbar and colleagues (2020) studied the effect of antibiotics on the gut microbiome of *Daphnia*, and observed that, in the presence of tetracycline, the abundance of *Pseudomonadaceae* and

Sphingobacteriaceae increased in the *Daphnia* microbiome. Qi and colleagues (2009) performed a metagenomic analysis of different species of *Daphnia*: one dataset from clones of *D. pulex* and *D. pulicaria* and two datasets from one clone of *D. magna*. Apart from *Comamonadaceae* (Proteobacteria), which represented the most part of the metagenomes, other taxa present in all datasets included the genera *Flavobacterium*, *Rhodobacter*, *Chromobacterium*, *Methylibium*, *Bordetella*, *Burkholderia,* and *Cupriavidus* whereas some sequences exclusive to *D. pulex* and the *D. pulicaria* datasets were classified within the *Aeromonas*, *Pseudomonas,* and *Delftia* genera (Qi *et al.*, 2009). Among the above mentioned taxa, some *Flavobacteria* are opportunistic fish pathogens (*e.g.*, of salmons) (Loch and Faisal, 2015), *Pseudomonas* and *Bordetella* include animal and human pathogenic species (Bergan, 1981; Hamidou Soumana *et al.*, 2017) while *Aeromonas* hits retrieved by the authors were attributable to *A. hydrophila*, which is an opportunistic pathogen of humans (Stratev and Odeyemi, 2016), and *A. salmonicida*, a fish pathogen (Dallaire-Dufresne *et al.*, 2014). Cooper and colleagues (2021) provided a differential analysis of *Daphnia* gut microbiome: they tested experimentally the effect of three antibiotics (aztreonam, erythromycin, sulfamethoxazole) on the gut microbiome. Among the treatments and the control, they found significant differences in the abundance of potentially pathogenic genera: *Pseudomonas*, *Aeromonas* and *Flavobacterium*.

Eckert and colleagues (2021) produced a large, comprehensive study of the microbiome associated with freshwater rotifers, cladocerans, and copepods and surrounding waters focusing on the core microbiome shared between specimens and considering the three groups of animals separately. They found that common families were *Flavobacteriaceae* (representing 3-21% of core zOTUs) and *Pseudomonadaceae* (representing 1-25% of core zOTUs).

Chironomidae larvae account for a high percentage of the total biomass of benthic microscopic fauna (Kornijów, 1997; Horppila *et al.*, 2000). They represent a very important item in the fish food of various ecological groups, and microbes are intrinsic to their guts and surfaces (Ding *et al.*, 2021). Halpern and Senderovich (2015) found that the microbiome of chironomid egg masses and larvae degraded various toxicants, enabling their host to live in polluted environments. Moore and colleagues (2003) described the survival of *Salmonella enterica*, serovar Typhimurium mr-DT-104 (a human and animal pathogen, Besser *et al.*, 2000), in freshwaters and freshwater sediments. The results obtained by the researchers indicated that *Salmonella enterica* could survive in aquatic sediments for several months (Moore *et al.*, 2003). Furthermore, they also demonstrated the uptake of salmonellae by chironomid larvae and adults suggesting they could be vectors of salmonellae in both aquatic and terrestrial environments. In 2007, *Aeromonas*

was discovered for the first time to be able to colonize chironomid egg masses, like *Vibrio cholerae* does (Halpern *et al.*, 2007; Senderovich *et al.*, 2008). Some authors highlighted the relationship between chironomids and some species of the *Aeromonas* genus; for instance, Laviad and Halpern (2016) investigated the complicated mutualistic relationship that seems to exist between chironomids and *Aeromonas* species. Indeed, *Aeromonas* species can be found in elevated numbers in the egg masses through all seasons of the year, and are able to control chironomid eggs viability both degrading the eggs and protecting them from toxic metals (Laviad and Halpern, 2016). Looking at human pathogenic species, Beaz-Hidalgo and colleagues (2012) isolated from chironomid egg masses *Aeromonas sanarellii* and *Aeromonas taiwanensis* previously described in hospitalized patients and harboring Shiga toxins (Alperi and Figueras, 2010). In conjugation experiments under the pressure of antibiotics, resistant (and potentially pathogenic) *Enterococcus*, *Escherichia* and *Flavobacterium* emerged in gut isolates of chironomid larvae (Ding *et al.*, 2021).

These studies show how the microbiome and its freshwater hosts may exhibit complex relationships. Meiofauna may play a critical role in maintenance and spread of microbes since, while transferring energy to the higher trophic levels (Schratzberger and Ingels, 2018), also components of the microbiome might be carried and transferred. However, information about the specificity (both host and environmental) of bacterial communities associated with different meiofaunal species is still lacking, and further studies are needed to answer questions about their role in carrying and spreading ARGs. The relevance of human and animal pathogens associated with meiofauna (Tab. 2) is greater in the presence of antibiotic resistance in the same microbiome, and even greater if the same pathogens are carriers of ARGs, as shown in the following section.

Antibiotic resistant bacteria and antibiotic resistance genes associated with meiofauna

Meiofauna represent a natural reservoir for bacteria (Laviad and Halpern, 2016) (including ARB and their associated ARGs). Furthermore, the physical and chemical conditions, inside and on the surface of some of these animals, differ from those of the surrounding water and, therefore, select for different bacterial communities that otherwise may not thrive in open water conditions (Tang *et al.*, 2010). Meiofauna can also enrich ARGs in their gastrointestinal tracts by preying on bacterioplankton (Kathol *et al.*, 2011) and phytoplankton (and its attached bacteria), potentially carrying ARGs, and, in this way, become a vector for their spread due to the long-range migration of these animals (Tang, 2005; Grossart *et al.*, 2010). Studies about ARGs and ARB in freshwater meiofauna are scarce. Our bibliography search led to only 8 published studies, most of them focused on Chironomidae or *Daphnia*.

Tab. 2. List of the research articles reporting potential pathogens in meiofauna.

Group	Taxa	Environment	Location	Sediment/ water column	Potentially pathogenic genera	Identification methodology	Reference
Ostracoda	*Sclerocypris tuberculata*	Temporary lake	Makgadikgadi depression, Botswana	Sediment	*Acinetobacter* (0.09%)	454 pyrosequencing	Olszewski *et al.*, 2020.
Ostracoda	*Potamocypris mastigophora*	Temporary lake	Makgadikgadi depression, Botswana	Sediment	*Acinetobacter* (0.42%)	454 pyrosequencing	Olszewski *et al.*, 2020.
Cladocera	*Daphnia*	Garden pond	Verbania, Italy	Water column	*Flavobacterium* (>50%), *Pseudomonas*, *Klebsiella*, *Brevundimonas*, *Acinetobacter*	Illumina MiSeq	Eckert *et al.*, 2016.
Cladocera	*Daphnia*	Laboratory maintained culture, inoculated with microbial communities deriving from: natural pond, fish pond, water reservoir, hay extract	Belgium	Water	*Brevundimonas*, *Acinetobacter*, *Pseudomonas*, *Flavobacterium*	Illumina MiSeq	Callens *et al.*, 2020.
Cladocera	*Daphnia pulex*	Laboratory maintained cultures, supplied with tetracycline	USA	Water	*Flavobacterium*, *Bordetella*, *Aeromonas*, *Pseudomonas*	454 pyrosequencing	Qi *et al.*, 2009.
Cladocera	*Daphnia pulicaria*	Laboratory maintained cultures, supplied with tetracycline	USA	Water	*Flavobacterium*, *Bordetella*, *Aeromonas*, *Pseudomonas*	454 pyrosequencing	Qi *et al.*, 2009.
Cladocera	*Daphnia magna*	Laboratory maintained cultures	Switzerland	Water	*Flavobacterium*, *Bordetella*	454 pyrosequencing	Qi *et al.*, 2009.
Cladocera	*Daphnia*	Farm harvested, laboratory maintained cultures	UK	Water	*Pseudomonas*, *Aeromonas*, *Flavobacterium*	Illumina MiSeq	Cooper *et al.*, 2021.
Chironomid larvae	*Chironomus tentans*	Experimental tanks maintained cultures	USA	Water and sediment	*Salmonella enterica*	Enumeration by spread-plate counts	Moore *et al.*, 2003.
Chironomid egg masses	Taxa not specified	Waste stabilization pond, river water	Israel	Water, artificial oviposition structure	*Vibrio cholerae*	3100 Genetic Analyzer	Halpern *et al.*, 2007.
Chironomid egg masses	Taxa not specified	Waste stabilization pond, river water	Israel	Water, artificial oviposition structure	*Aeromonas sanarellii*, *Aeromonas taiwanensis*	m-Aeromonas agar plating, genotyping by ERIC-PCR	Beaz-Hidalgo *et al.*, 2012.
Chironomid larvae	Chironomidae	River water	Panlong River, China	Water column and sediment	*Enterococcus*, *Escherichia*, *Flavobacterium*	Illumina MiSeq	Ding *et al.*, 2021.
Freshwater Rotifera	*Adineta vaga, Brachionous quadridentatus, Epiphanes senta, Euchlanis dilatata, Keratella quadrata, Keratella serrulata, Lecane elsa, Lecane inermis, Polyarthra* sp.*, Rotaria macrura, Rotaria rotatoria*	Lakes, ponds, followed by experimental cultures	Italy	Water column	*Pseudomonas* present in the core microbiome	Illumina MiSeq	Eckert *et al.*, 2021.

Regarding Chironomidae, as underlined in the previous section, there are some specific genera like *Vibrio* and *Aeromonas* widely reported to inhabit their surface or their gut. In particular, *Aeromonas* species are known for being resistant to β-lactams and have been associated with human diseases (Laviad and Halpern, 2016). In 2012, Beaz-Hidalgo and collaborators studied two isolates from chironomid egg masses, *Aeromonas taiwanensis* and *A. sanarellii*, testing the resistance of these two strains against 19 antimicrobials (Beaz-Hidalgo *et al.*, 2012). As expected, all strains were resistant to β-lactams (ampicillin, cefalotin and ertapenem), and 75% of the isolates were resistant to a combination of amoxicillin and clavulanic acid, too. The strains showed different susceptibility (25 to 78% sensitivity) to 12 of the 19 tested antibiotics (amikacin, aztreonam, cefepime, cefotaxime, ceftazidime, ciprofloxacin, gentamicin, piperacillin–tazobactam, tigecycline, tobramycin, trimethoprim–sulfamethoxazole, and imipenem) (Beaz-Hidalgo *et al.*, 2012; Laviad and Halpern, 2016). Similarly, Basu and collaborators (2010) studied the ARB load in chironomids, but in this case in larval isolates, testing eight antibiotics (ampicillin, chloramphenicol, colistin methanesulfonate, kanamycin, nalidixic acid, nitrofurantoin, streptomycin and tetracycline). They detected a wide incidence of antibiotic resistant bacteria in the larval isolates of an experimental pond and in sewage drain water. The strains isolated from the drain water were less susceptible than those isolated from the pond. The drain water isolates were found resistant to all the antibiotics except colistin methanesulfonate, and 100% of the isolated strains were resistant to ampicillin and chloramphenicol. While the isolates of the pond were 100% resistant to ampicillin, only 50% of them showed resistance to chloramphenicol and nitrofurantoin, and no resistances were found for the other tested antibiotics (Basu *et al.*, 2010). *sul*2 (a sulfonamide resistance gene), *tet*A (a tetracycline resistance gene), and *kan* (kanamycin resistance gene) were detected by quantitative Real Time PCR (qPCR), in the microbiome of chironomid larvae from Panlong River (China) (Ding *et al.*, 2021). In this case, the authors made the comparison with the water and sediment of the river, and performed a differential detection of *sul*2 in various parts of the larvae body, and at different stages of their development. The genes *sul*2, *tet*A, and *kan* were detected in the chironomid larvae, but the authors deepened the study only for *sul*2. This gene was more abundant in the microbiome of the larvae than in water and sediment. It was found in the head, chest, and abdomen of the larvae, with the highest concentration (9.1×10^{-4} copies/16S rRNA gene copy) found in the gut. Also, its content changed significantly during the different developmental stages of the larvae, increasing from the second (3.0×10^{-4} copies/16S rRNA gene copy) to the fourth (9.1×10^{-4} copies/16S rRNA gene copy) larval instars but decreasing in the adult stage (2.0×10^{-5} copies/16S rRNA gene copy) (Ding *et al.*, 2021).

In the case of *Daphnia,* their abundant and rare associated bacteria could be carriers of ARGs (e.g., *Flavobacterium, Pseudomonas, Klebsiella, Citrobacter, Acinetobacter, Serratia* and *Rhizobium*) (Eckert *et al.*, 2016). Olanrewaju and colleagues (2019) made a conjugation experiment with a vancomycin-resistant donor strain (*Enterococcus faecalis* carrying the *van*A resistance gene), and two vancomycin-susceptible and rifampicin-resistant recipient *E. faecalis* strains in the presence of *Daphnia magna* or *Daphnia pulex*. The isolates were prior tested for resistance to eight antibiotics (ciprofloxacin, imipenem, linezolid, rifampicin, streptomycin, trimethoprim, teicoplanin, and vancomycin) using the disk diffusion assay and showing resistance to two or three antibiotics. At the end of the experiment the presence of *van*A (evaluated by PCR) was used to confirm the transfer of vancomycin resistance between the donor and recipient strains in the treatment with only *D. magna*, *D. pulex*, or both *Daphnia* species. Successful conjugation was detected in all the cases within a 4-h feeding period with transconjugants expressing resistance to vancomycin and rifampicin. In another experiment, Eckert and colleagues (2016) tested whether the presence of *Daphnia* could modulate the abundance of ARGs, following the tetracycline resistance gene *tet*A, which is nearly constitutively present in the bacterial community of Lake Maggiore, in Italy (Di Cesare *et al.*, 2015). The presence of *Daphnia* resulted in lower abundances of *tet*A in the water, however, the gene was still detected within the animals in similar abundances as found in the water of the *Daphnia*-free treatment, suggesting a potential persistence or proliferation within the *Daphnia* microbiome (Eckert *et al.*, 2016). Cooper and Cressler (2020) used a metagenomic approach to characterize the microbiome present in *Daphnia magna* and get insight into the potential metabolic interactions between host and microbes. Even if it was not the main aim of the study, multiple genes and pathways involved in antibiotic resistance and detoxification were found in the bacterial metagenome-assembled genomes from the *Daphnia* isolates. The *Pedobacter* sp. genome encoded the multidrug efflux pump MdlAB/SmdAB and genes for macrolide export (*mac*A, *mac*B), while the *Polaromonas* sp. genome encoded the AcrAB-TolC/SmeDEF efflux pump (Cooper and Cressler, 2020).

Regarding other meiofaunal groups, studies on the presence of ARGs in their associated bacteria are even more difficult to find. Chaix and colleagues (2017) tested sixteen antibiotics (ampicillin, amoxicillin plus clavulanic acid, ticarcillin, ticarcillin plus clavulanic acid, piperacillin, piperacillin plus tazobactam, cefotaxime, cefoxitin, cefepime, ertapenem, imipenem, gentamicin, tobramycin, norfloxacin, ciprofloxacin, trimethoprim plus sulfamethoxazole) using the disc diffusion method, in *Aeromonas* populations associated with copepods and in the water of the Seine estuary. In this case, the researchers found no signif-

icant difference between *Aeromonas* antibiotic resistance phenotypes from the water column and copepods, except for the resistance to a combination of amoxicillin plus clavulanic acid and cefoxitin, which resulted to be higher in water. However at species level, the isolates of *A. salmonicida* from copepods were three times more resistant to ticarcillin plus clavulanic acid than the isolates from water (Chaix *et al.*, 2017). Xue and colleagues (2021) quantified (by qPCR) 26 ARGs encoding resistance to six classes of antibiotics (sulfonamides, tetracyclines, quinolones, macrolides, chloramphenicol and β-lactams), two integron integrase genes and seven transposases in phytoplankton-zooplankton (mainly rotifers, copepods and cladocerans) associated bacteria. *tet*Z, *tet*M, *tet*C (conferring resistance to tetracycline) and *flo*R (against florfenicol) were the dominant ARG subtypes (~ 70% relative abundance). In this study the authors also found a high abundance of mobile genetic elements, correlated with ARGs, suggesting that they may play a role in the propagation of ARGs in the aquatic communities (Xue *et al.*, 2021).

DISCUSSION

The spread of antibiotic resistance worldwide has led to the awareness of the central role played by the environment and aquatic ecosystems in the spread of resistant and/or pathogenic bacteria, with potentially harmful outcomes if reaching the clinical settings. It has been demonstrated that freshwaters and their biota could promote the development and persistence of resistances in different compartments (water column, sediment, suspended particles and aquatic animals) (Di Cesare *et al.*, 2020; Garner *et al.*, 2017; Jia *et al.*, 2018; Jia *et al.*, 2020; Sathicq *et al.*, 2021); however the contribution of freshwater meiofauna remains still largely unexplored.

In this review, we collected the data available up to now on the role of freshwater meiofauna as carriers of ARGs and potentially pathogenic bacteria and ARB. Meiofauna closely interact with bacteria (*e.g.*, increasing bacterial denitrification, reducing bacterial mineralization of organic pollutants, and contributing to macroalgae decomposition and nutrient recycling) (Näslund *et al.*, 2010; Bonaglia *et al.*, 2014; Herrera *et al.*, 2017; Schratzberger and Ingels, 2018), and several species feed on planktonic microorganisms (Kathol *et al.*, 2011).

The bacterial communities associated with meiofauna surfaces and gut have proven to be complex and may include human and animal potentially pathogenic bacteria that can find in meiofauna a refuge and sustaining conditions (Moore *et al.*, 2003; Beaz-Hidalgo *et al.*, 2012; Eckert *et al.*, 2016; Laviad and Halpern, 2016; Callens *et al.*, 2020; Olszewski *et al.*, 2020; Cooper *et al.*, 2021; Ding *et al.*, 2021). In addition, several of the reviewed studies reported that the meiofauna-derived isolates, in some cases potentially path-

ogenic strains, were resistant to a broad range of antibiotics (Halpern *et al.*, 2007; Basu *et al.*, 2010; Beaz-Hidalgo *et al.*, 2012; Laviad and Halpern, 2016; Chaix *et al.*, 2017); whereas others confirmed the role of meiofauna microbiome as carriers of ARGs (Eckert *et al.*, 2016; Cooper and Cressler, 2020; Ding *et al.*, 2021; Xue *et al.*, 2021).

Limitations of current researches

In this review, we proposed the freshwater meiofauna microbiome as an understudied carrier for antibiotic resistance. However, we could only focus on a small set of freshwater organisms and environments. Large gaps in the understanding of ARG dynamics in freshwater meiofauna are still present due to the variety of methodological approaches, target animals, and different levels of anthropogenic pollution of the investigated environments, which makes it difficult to arrive at a more general conclusion. Indeed, despite the fact that we selected 10 meiofaunal taxa, only for four of them (namely Chironomidae, Cladocera, Copepoda, Rotifera) we found studies concerning their association with antibiotic resistance (Tab. 3). However, meiofauna comprise a wide group of organisms that differ for morphology, trophic strategy and habitat (Fonseca *et al.*, 2018), and this might correlate with diverse features in carrying ARGs. Furthermore, it could be valuable addressing the differences in the antibiotic resistome composition and abundance between sediment and water column relatively to meiofaunal taxa, with a focus on benthic ones, since it has been shown that sediment could act as a long term reservoir of ARGs (Di Cesare *et al.*, 2020).

Comparison with other organisms

The limited numbers of studies and the lack of a wider metagenomic approach (*e.g.*, shotgun metagenomics) do not consent to draw a univocal picture of the ARGs specificity and trends in meiofauna bacterial communities. Nevertheless, some considerations can be made, in part comparing the results obtained in freshwater meiofauna to those observed in marine meiofauna or bigger invertebrates. For example, in the gut microbiome of a soil invertebrate, the earthworm *Metaphire guillelmi*, experimentally exposed to tetracycline, the resistance genes *tet*A, *tet*C, and *tet*W increased in concentration with growing antibiotic doses (Chao *et al.*, 2019). Likewise, Nasri and colleagues (2020) exposed an assemblage of marine nematodes to ciprofloxacin (a fluoroquinolone antibiotic) and observed the alteration of density and structure of their bacterial community, suggesting a selection due to the antibiotic and the possible presence of ARGs and ARB in the nematode microbiome. Similar findings could be observed also in freshwater meiofauna, since these animals have proven to be able to live in presence of antibiotics (Quinlan *et al.*, 2011) and in antibiotic polluted areas (Xue *et al.*, 2021).

Meiofauna microbiome flexibility

Eckert and colleagues (2021) proposed a pronounced flexibility of the microbiome composition as a common trait among the considered taxa (freshwater rotifers, cladocerans and copepods), finding similarity between the hosted microbiome and the water column community composition. Conversely, diverging results were found in both freshwater and marine benthic environments. Different freshwater species of ostracods were showing a host-specific microbiome (Olszewski *et al.*, 2020) and coexisting cryptic species of the *Litoditis marina* complex were also displaying substantial differences in their associated micro-

biomes, suggesting that different meiofaunal taxa might have diverse retention ability and flexibility towards the environmental microbiome, with a cascade effect on the pathobiome and antibiotic resistome. Meiofauna can accumulate ARGs from the surrounding environment (Eckert *et al.*, 2016) and this enrichment could follow an anthropogenic pollution gradient (Ding *et al.*, 2021), as also indirectly demonstrated by an higher level of resistance of bacteria isolated from chironomid larvae kept in sewage drain water in respect to the ones derived from animals grown in an experimental pond, suggesting that ARGs can find suitable conditions to spread within the meiofauna (Eckert *et al.*, 2016). The antibiotic pollution occurring in

Tab. 3. List of the research articles on antibiotic resistance in meiofauna.

Group	Environment	Location	Bacteria	Methodologies	Genes	Antibiotics	Reference
Chironomid egg masses	Waste stabilization pond	Northern Israel	*Aeromonas taiwanensis* and *Aeromonas sanarellii*	Antibiotic susceptibility test		19 antibiotics	Beaz-Hidalgo *et al.*, 2012.
Chironomid larvae	Experimental pond and drain water	Bardhaman, India	Aerobic heterotrophic bacterial population in the gut	Microbial count and antibiotic susceptibility test		8 antibiotics	Basu *et al.*, 2010.
Chironomidl arvae	River sediment and river water	Shandong, China	Intestinal bacteria of Chironomidae larvae	qPCR	*tet*A, *kan*, *sul*2, NDM-1, *mcr*-1		Ding *et al.*, 2021.
Daphnia magna and *Daphnia pulex*	Cultured continuously in filtered and autoclaved river water	—	*E. faecalis*	Antibiotic disk diffusion assay, PCR amplification of vancomycin resistance genes and DNA sequencing	*van*A	8 antibiotics	Olanrewaju *et al.*, 2019.
Daphnia obtusa	Surface lake water	Lake Maggiore, Italy	*E. coli*	qPCR	*tet*A		Eckert *et al.*, 2016.
Daphnia magna	Pond	—	Bacterial species present in the *Daphnia magna* microbiome	Shotgun sequencing	MdlAB/SmdAB, *mac*A, *mac*B, AcrAB-TolC/ SmeDE, BaeS-BaeR		Cooper and Cressler, 2020.
Phytoplankton-zooplankton communities	Urban river	Shannxi, China	Phytoplankton-zooplankton associated bacteria	qPCR	*sul*1, *sul*2, *sul*3, *tet*A, *tet*B, *tet*C, *tet*G, *tet*M, *tet*O, *tet*Q, *tet*W, *tet*Z, *gyr*A, *qnr*A, *qnr*B, *qnr*S, *erm*B, *erm*C, *erm*F, *cat*1, *flo*R, *cml*A, *bla*NDM1, *bla*IMP4, *bla*CTX-M, *bla*SHVintI1, intI2 tnpA-01, tnpA-02, tnpA-03, tnpA-04, tnpA-05, tnpA-07 and IS613		Xue *et al.*, 2021.
Copepods	Estuary	Seine, France	*Aeromonas*	Antibiotic susceptibility test		16 antibiotics	Chaix *et al.*, 2017.

human impacted areas can contribute to the above mentioned findings (Xue *et al.*, 2021).

Horizontal gene transfer of ARGs

Freshwater meiofauna can promote the spread and persistence of ARGs in the aquatic environments, via horizontal gene transfer, given the protective and suitable conditions within their body, especially in the gut, as demonstrated for other aquatic animals (Fu *et al.*, 2017). In particular, the microbiome of the gut of chironomid larvae showed to have the highest ARG abundances in respect to those of other body sections (head, chest) and stimulates horizontal gene transfer of the resistance traits by conjugation (Ding *et al.*, 2021). Other experimental findings supported that horizontal gene transfer of ARGs was enhanced in the presence of meiofauna and that it was a major mechanism for their spread, through mobile genetic elements (conjugative plas-

mids and transposons) and integron integrases (Olanrewaju *et al.*, 2019; Ding *et al.*, 2021; Xue *et al.*, 2021).

ARGs spread through meiofauna

We highlighted that the larval state can influence the antibiotic resistome: the microbiome of larval instars of chironomids was richer in ARGs than that of the adults (Ding *et al.*, 2021). This is of particular interest because larvae are preyed by fishes and, in this way, promote the spread and magnification of resistance along the food chain (Ding *et al.*, 2021), as reported for marine meiofauna, which demonstrated to be carriers of pathogenic ARB in aquaculture systems (Kalatzis *et al.*, 2016; Sahandi *et al.*, 2019; Hurtado *et al.*, 2020). As a consequence, the antibiotic resistance could be transferred to humans when fish and crustaceans or mollusks were consumed, especially as raw food (Mughini-Gras *et al.*, 2019) (Fig. 1). Meiofauna can also
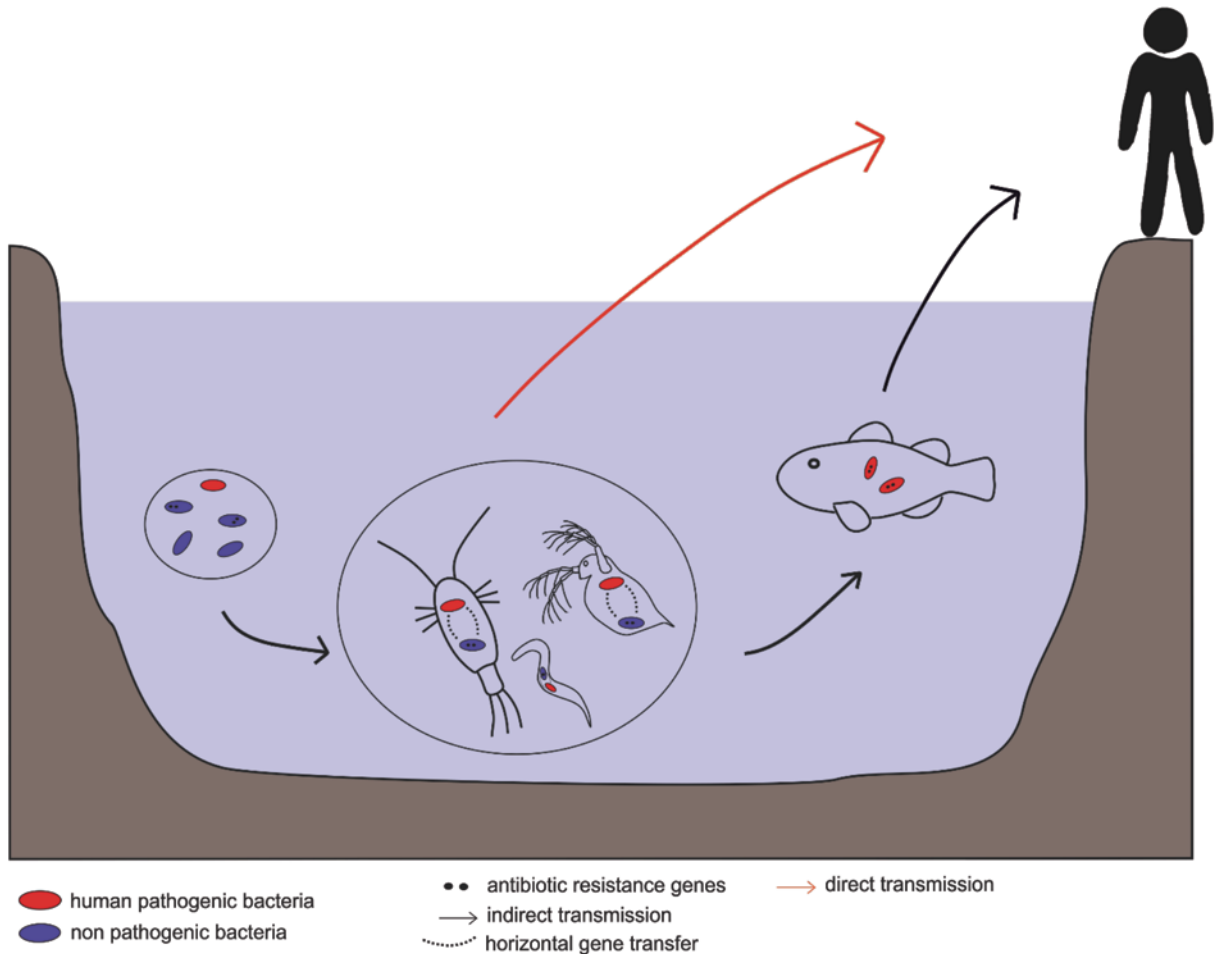

human pathogenic bacteria
non pathogenic bacteria

•• antibiotic resistance genes → direct transmission
→ indirect transmission
┄┄┄→ horizontal gene transfer

Fig. 1. Spread of ARGs within the meiofauna microbiome and along the food web. In meiofauna, ARGs could be transferred via horizontal gene transfer from non-pathogenic to pathogenic bacteria and their load magnified in the different hosts, potentially reaching humans (indirect transmission). In other cases, bathing activities, water consumption, and even inhalation of meiofauna can act as a direct transmission route.

reach people directly through bathing activities (Martinez et al., 2020), and even through the drinking water (when not properly treated) (Kos *et al.*, 2020) or the inhalation of suspended animals transported by the wind (Ptatscheck *et al.*, 2018; Fontaneto, 2019), offering further routes to the spread of resistances (Fig. 1).

Temperature effect on meiofauna resistome

Water temperature could drive the accumulation of ARGs in meiofauna microbiome. In particular, the scenarios of climate change and rising temperatures are closely linked with bacterial processes and infections, increased horizontal gene transfer and overall bacterial growth rates (Philipsborn *et al.*, 2016; reviewed in Burnham, 2021). Higher temperature was shown to lead to an increase in ARG abundances in the microbiome of freshwater meiofauna (Ding *et al.*, 2021; Xue *et al.*, 2021), probably due to the proliferation of ARB within these animals, even if some ARB seemed to be constitutively associated to them (*e.g.*, *Aeromonas* strains in chironomid eggs) (Beaz-Hidalgo *et al.*, 2012).

Biomonitoring of antibiotic resistance in environment

Meiofauna have been widely used as a proxy of water quality and some species have been the object of research for their resistance (or susceptibility) to the environmental pollution and potential implication in ecotoxicology (Schratzberger and Warwick, 1999; Alves *et al.*, 2013). Recently, Zeppilli and colleagues (2015) reviewed the relevance of the meiofauna as a good bioindicator for anthropogenic impact. In addition to this, it would be interesting and useful to investigate the application of meiofauna in the biomonitoring of antibiotic polluted areas, since, as demonstrated, they could rapidly respond to such anthropogenic impact with a changing of the ARGs load and assemblage (Xue *et al.*, 2021). Furthermore, the investigation of the meiofauna-hosted antibiotic resistome and pathobiome response in the scenario of climate change would be of great interest and the comparison of the antibiotic resistome and pathobiome of differentially tolerant meiofaunal species, along a gradient of anthropogenic impact, could help predicting ARGs enrichment in aquatic environments. 'Omics'-based approaches have been newly developed to test the health risk of ARGs and rely on the entire metagenome of the microbial communities to be assessed (*e.g.*, Oh *et al.*, 2018; de Nies *et al.*, 2021). Such methods could be used to test a range of meiofaunal taxa as bioindicators for antibiotic resistance pollution.

CONCLUSIONS

The data collected and analyzed in this review highlight the potential role of meiofauna as reservoirs of antibiotic resistances in freshwater. In summary:

- Human and animal potential pathogens were part of the microbiome of freshwater meiofauna;
- ARGs were found in Chironomidae, Cladocera, Copepoda and Rotifera microbiome;
- the genes *tet*A and *sul*2 were retrieved in multiple reviewed studies (however this might be biased by the detection method used).

The few studies found claim for a further effort from the scientific community to fill the current knowledge gaps. Possible directions for future research could be:

- the investigation of unstudied taxa;
- the study of the interactions between meiofauna and environmental factors, *e.g.*, temperature in the scenario of climate change;
- the study of the flexibility of the meiofauna microbiome and the horizontal gene transfer of ARGs using metagenomic approaches;
- the investigation of meiofauna microbiome as a bioindicator for antibiotic resistance in aquatic enviroments.

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