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Source: Journal of Shellfish Research, 37(1):121-129.
Published By: National Shellfisheries Association
URL: http://www.bioone.org/doi/full/10.2983/035.037.0110

Journal of Shellfish Research, Vol. 37, No. 1, 121–129, 2018.

ANTIMICROBIAL AND ANTIBIOFILM ACTIVITY OF UNIONID MUSSELS FROM THE NORTH OF PORTUGAL

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ABSTRACT The populations of freshwater mussels belonging to the family Unionidae have been facing drastic changes in terms of diversity and numbers caused by constant aggressions on their natural habitat. Nevertheless, bivalves are capable of developing strategies of defense to overcome potential aggressors. The present work aimed to assess the potential antibacterial capacity of different species of unionid mussels from the north of Portugal. For this purpose, circulating cells (hemocytes), fluids, and mucus were obtained by nonlethal methods from the species *Anodonta anatina* (Linnaeus), *Anodonta cygnea* (Linnaeus), *Potomida littoralis* (Cuvier), and *Unio delphinus* (Spengler), and tested against bacterial reference strains and multidrug-resistant isolates. The cellular fraction of *A. anatina*, *A. cygnea*, and *P. littoralis* showed antibacterial activity, detected by the agar disc diffusion method, against *Bacillus subtilis* ATCC 6683, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606. Circulating cells from *P. littoralis* and *A. anatina* also inhibited *Listeria monocytogenes* ATCC 19111 and *A. cygnea* has also inhibited a multidrug-resistant isolate of *Pseudomonas putida*. The plasma of all mentioned freshwater mussels, used directly or diluted, showed great ability to hamper or inhibit the biofilm formation of *Staphylococcus aureus* ATCC 25923, *P. aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922. *Anodonta cygnea* hampered the biofilm formation by *Enterococcus faecalis* ATCC 29212 as well. Overall, these results showed that not only cells play a relevant role in the immune system of these species but also the plasma, which likely contains antibiofilm substances. *Anodonta cygnea* stood out by presenting the best antibacterial inhibition potential.

KEY WORDS: antibacterial activity, freshwater bivalves, hemocytes, immune system, plasma

INTRODUCTION

In the last decades, freshwater mussels belonging to the superfamily Unionoidea have increasingly been endangered, yet the concern regarding their conservation is equally growing (Bogan 1993, Strayer 2000, Strayer & Malcom 2007, Geist 2015, Lopes-Lima et al. 2016). Their particular life cycle, in which before being autonomous, the larvae (glochidia) needs to fix to a specific fish host to develop to the next stage, limits greatly their dispersion and propagation. There are many reasons for their population decline: (1) pollution, which is one of the major threats, (2) the introduction of new species that can act as competitors (mussels and fish), (3) a reduction in their hosts' population, and (4) multiple other factors of anthropogenic origin (Bogan 1993, Strayer 2000).

Freshwater mussels are key elements in freshwater systems because their suspension-feeding behavior is fundamental for cleaning the water and helping the recirculation of particles in rivers, lakes, and streams. In addition, their shells can be used as support structures for other organisms' proliferation (animals, plants, and algae) (Bogan 1993, Strayer et al. 2004). Aggressions affecting the environment are potentiated in these animals because of bioaccumulation processes and to their sessile lifestyle, making freshwater mussels ideal monitoring species, very sensitive to environmental perturbations (Farris & Van Hassel 2006, Hartmann et al. 2015).

In a changing world where adaptation is a recurrent phenomenon, the increase of stressors in natural habitats raises the question whether environmental alterations may or may not have implications in the immune system of organisms. There have been reports suggesting that an immune modulation occurs (Mydlarz et al. 2006); however, in the long term, it is likely that the effects may cause deeper transformations. Freshwater mussels are not only being loaded with toxins because of the water pollution but are also being subjected to a microbial pool that is very labile.

Unionids, being suspension feeders, end up using large amounts of bacteria as a food source. Nevertheless, sometimes they are also able to establish symbiotic relationships either mutualistic or antagonistic with bacteria (Grizzle & Brunner 2009, Antunes et al. 2010). In the early stages of development (glochidia and juveniles), unionid mussels are prone to bacterial infectious diseases (Grizzle & Brunner 2009). Bacterial diseases such as vibriosis are much more studied in marine bivalves than in freshwater mussels because the economic implications are greater in the former case (Pruzzo et al. 2005, Mateo et al. 2009). When mussels reach the adult stage, they become much more resistant toward bacterial diseases, especially because they already have a mature innate immune system.

The immune system of unionid mussels, based on innate immunity (Danilova 2006, Mydlarz et al. 2006), comprises

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DOI: 10.2983/035.037.0110

exterior barriers such as the shell and mucus on the surface of the tissues, and the internal cellular component and respective circulatory fluids. Particularly, blood cells (hemocytes) play a fundamental role in defense, especially through mechanisms associated with phagocytosis (Blaise et al. 2002, Canesi et al. 2002, Hong et al. 2006) and detoxification processes (Soares-da-Silva et al. 2002), as well as the plasma, which is composed of humoral components.

The discovery of antimicrobial peptides (AMPs) and other molecules with antimicrobial properties from many organisms has been increasing (Cheng-Hua et al. 2009, Smith et al. 2010). Previous studies have already identified these peptides in invertebrates, such as crayfish (*Pacifastacus leniusculus*) (Jiravanichpaisal et al. 2007), but mostly in marine bivalves such as oysters (*Crassostrea gigas*) (Bacheré et al. 2015) and mussels (*Mytilus galloprovinciallis*) (Mitta et al. 1999, 2000). It is very likely that freshwater mussels may also produce natural products possessing antibacterial activities.

Therefore, the aim of the present study was to evaluate the potentiality of several species of Portuguese freshwater mussels to produce antibacterial compounds, by testing their fluids, (plasma and extrapallial fluid) cells, and mucus against reference and multidrug-resistant bacterial strains of well-known human pathogens. Four species within the family Unionidae were selected from two freshwater systems: *Anodonta cygnea* from a shallow lagoon (Barrinha - Mira), and *Anodonta anatina*, *Potomida littoralis*, and *Unio delphinus* from a river (Tâmega river).

MATERIALS AND METHODS

Location and Collection of Freshwater Mussels

The species of freshwater bivalves used in this study were collected in northern Portugal in the Spring of 2014: *Anodonta cygnea* was collected from the Mira lagoon (40°27′22″ N, 8°48′7″ W) and *Anodonta anatina*, *Unio delpinhus,* and *Potomida littoralis* from the Tâmega river (41°24′52″ N, 7°57′51″ W). They were kept in aerated tanks with dechlorinated water and acclimatized in these conditions for 2 wk before performing the assays, so the starting point of all tested species was the same, independent of their origin. These animals were fed daily with a microalgae diet (a mix of chlorophyte species). The organisms were considered healthy when the surface of the shell was smooth and shiny and when they were able to close the valves on disturbance.

Cells and Fluids Extraction

Hemolymph from three to six organisms of each species (*Anodonta cygnea*, *Anodonta anatina*, *Potomida littoralis*, and *Unio delphinus*) was carefully extracted using a 21G needle (Braun) attached to a 2-mL sterile syringe (Braun), by inserting it between the valves across the inner layer of the mantle, in the so-called intraepithelial space, avoiding contact with other surfaces. Each hemolymph sample was maintained on ice immediately after collection; no anti-aggregation solution was added to avoid introducing bias. The whole procedure was conducted using sterile material and in an aseptic environment.

The cells were isolated from the plasma by centrifugation at 200× g, 4°C for 10 min (Antunes et al. 2014), and resuspended in 50 mM phosphate buffer saline (PBS) for the susceptibility disc diffusion assay or in tryptic soy broth (TSB—Biokar Diagnostics, Allonne, France) for further use in the biofilm assays. The final concentration of cells was around 10^6 cells/mL. The plasma was filtered through a 0.22-µm membrane filter to eliminated eventual contaminants.

The extrapallial fluid could only be collected from the *Anodonta* species because this species is big enough to ensure a safe extraction by inserting the needle between the shell and the mantle; the fluid was filtered and kept on ice until use.

Mucus Collection

Mucus was collected from the foot surface using a sterile blade and further suspended in 50 mM PBS, vortexed, and kept on ice (it was not filtered because it was too viscous).

Bacterial Water Quality Analysis at the Collection Points

Water ($n = 19$) and biofilm ($n = 19$) samples were previously collected from 2009 to 2011, in a total of 10 surveys from Mira lagoon and nine surveys from Tâmega river to evaluate the natural habitat of the tested freshwater bivalves in terms of total mesophilic aerobic bacteria, *Escherichia coli* and *Enterococcus* spp., from the natural habitat. Biofilm was collected from the surface of rocks and macrophytes immersed in the water, by scraping it with a brush into a 50-mL falcon tube.

Water samples of 100 mL were filtered through 0.45-µm-pore-size membrane filters (Millipore Corporation, USA), which were then placed on tryptone bile X-glucuronide agar (TBX) (BioKar Diagnostics, Beauvais, France) and on Slanetz and Bartley agar (SB) (Oxoid, Basingstoke, UK) for *Escherichia coli* and *Enterococcus* spp. enumeration, respectively. The TBX plates were incubated at 37°C for 24 h and the SB plates at 37°C for 48 h. *Enterococcus* spp. colonies were confirmed in kanamycin aesculin azide agar (Liofilchem, Roseto degli Abruzzi, Italy), incubated at 44°C for 4 h.

For the determination of total aerobic mesophilic bacteria, dilutions of the water samples were made in tryptone salt and then 1 mL of each dilution was incorporated in plate count agar medium (Biokar Diagnostics) and incubated at 30°C for 72 h. The number of colony-forming units (CFUs) were counted and recorded.

The biofilm was diluted in tryptone salt and analyzed by incorporation in the abovementioned media (TBX, SB, and plate count agar) using the respective conditions of incubation.

Biochemical Quantifications on the Plasma from the Unionidae Species

Subsamples of the plasma from *Anodonta cygnea*, *Unio delphinus*, and *Potomida littoralis* were used for the quantification of total proteins (Bradford method), calcium (Quanti-Chrom Calcium Assay kit; Bioassays Systems), phosphates (PiBlue Phosphate assay kit; Bioassays Systems), and total lipids (Total lips Liquid kit; FAR Diagnostics, after extraction using methanol and chloroform).

Antibacterial Susceptibility Testing

Agar Disc Diffusion Method

For screening the potential antibacterial activity of cells, fluids, and mucus obtained from the four bivalve species, four

reference strains of bacteria (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6683, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922) were initially used. Fresh bacterial cultures were used to prepare an inoculum equivalent to 0.5 McFarland.

Mueller–Hinton agar (BioKar Diagnostics) plates were inoculated with the bacterial inocula. Then, small sterile paper discs (6 mm in diameter) were equidistantly attached to the agar (five per plate) and loaded with 15 µL of the sample (cells, fluids, or mucus). The plates were kept for 30 min at room temperature before incubation at 37°C for 18–24 h (Pereira et al. 2015). A negative control was made using PBS.

The plates were observed under a stereoscope (SZ61 stereoscope; Olympus America Inc., Center Valley, PA) and if zones of inhibition occurred around the discs, the diameter of such zones was measured. Photographs of zones of inhibition were also taken (PD70; Olympus America Inc.). The samples that have caused any bacterial inhibition around the discs were further studied against other potential pathogenic bacterial strains: *Salmonella enterica* Typhimurium CECT 443, *Klebsiella pneumoniae* ATCC 13883, *Acinetobacter baumannii* ATCC 19606, *Listeria monocytogenes* ATCC 19111, *Enterococcus faecalis* ATCC 29212, and multidrug-resistant clinical strains of *Pseudomonas putida*, and *Klebsiella* spp., following the same procedure.

Antibiofilm Activity Assay

Biofilm Biomass Quantification

The fractions that had given positive results in the previous assay, which were derived from the hemolymph, the cellular fraction, and the plasma fraction (pure or diluted in the ratio of 1:2 and 1:4 in TSB), were also tested for antibiofilm activity. Briefly, biofilms of the following strains were formed in the wells of 96-well plates: *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212. The initial inocula were at a concentration of 1×10^6 CFU/mL in TSB, to which the respective samples to be tested had been added. Six to eight replicate wells were used for each condition tested. The positive control consisted only the bacterial inoculum. The microplates were incubated at 37°C for 24 h. The biofilm biomass was quantified through the crystal violet staining, as described by Gomes et al. (2014).

Microscopic and Viability Analyses of Biofilms

The conditions that have hampered or decreased the biofilm formation were then also replicated for a qualitative assessment by microscopic visualization, after staining with the Live/Dead BacLight viability kit (Molecular Probes; Life Technologies, Carlsbad, CA). For this qualitative assay, three *Anodonta cygnea* were used.

Biofilms of *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, and *Escherichia coli* ATCC 25922 were formed in 35-mm-diameter polystyrene plates using TSB (control) and TSB supplemented with cellular fractions and plasma (pure or diluted in the ratio of 1:2 and 1:4 in TSB). The plates were incubated at 37°C for 24 h. After that, the supernatant phase was removed from each plate, the biofilms were washed with PBS, stained with 500 µL of the mixture of SYTO 9, and propidium iodide and incubated for 20 min at room temperature in the dark; then, the biofilms were rinsed and examined under a fluorescence microscope (BX41 Microscope; Olympus America Inc.) (Gomes et al. 2014).

Statistics

The statistical significance of differences between biofilms of controls and biofilms in the presence of hemolymph, the cellular fraction, or the plasma fraction were evaluated using Student's *t*-test. Probability levels <0.05 were considered statistically significant.

RESULTS

Microbial Quality of the Water at the Collection Points of the Bivalves

The annual variations of *Escherichia coli*, *Enterococcus* spp., and total mesophilic aerobic bacteria in the water and in the biofilm collected from Tâmega river and Mira lagoon are shown in Figure 1. It is possible to observe that counts in water and biofilm do not present the same profile of progression. Tâmega river, in comparison with Mira lagoon, presented higher fluctuations in all counts, as well as the highest values of *E. coli* and *Enterococcus* spp. in the water and biofilm samples and of total aerobic mesophilic bacteria in the biofilm. *Enterococcus* spp., *E. coli*, and total aerobic mesophilic bacteria maximums occurred in the biofilm fraction.

Plasma Composition

Regarding the quantification of the components of the plasma (Fig. 2), it was possible to observe that the plasma of the three species tested presented similar values in terms of calcium and total lipids. The species *Unio delphinus* presented higher differences in the quantity of the components tested, compared with the other species and the highest values concerning phosphates, whereas *Potomida littorallis* presented a lower content in terms of total proteins.

Antibacterial Activity

Regarding the results from the agar disc diffusion assay (Fig. 3), it was observed that the cellular fraction of some bivalve species could cause bacterial inhibition, but not against all bacteria tested. The zones of inhibition were generally small. The mucus did not cause any bacterial inhibition; in fact, greater bacterial proliferation around the disc was recorded, which probably means that the mucus harbors the bacteria itself. A similar result was observed with the extrapallial fluid samples.

The most promissory species was *Anodonta cygnea* from Mira lagoon because its cellular fraction caused greater zones of inhibition in *Bacillus subtilis* ATCC 6683 and *Pseudomonas aeruginosa* ATCC 27853, as well as multidrug-resistant isolates, such as *Pseudomonas putida* and *Acinetobacter baumannii* (Fig. 3A).

Potomida littoralis cellular fraction also inhibited *Bacillus subtilis* ATCC 66683, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 19111, *Acinetobacter baumannii* ATCC 19606, and *Salmonella enterica Typhimurium* CECT 443

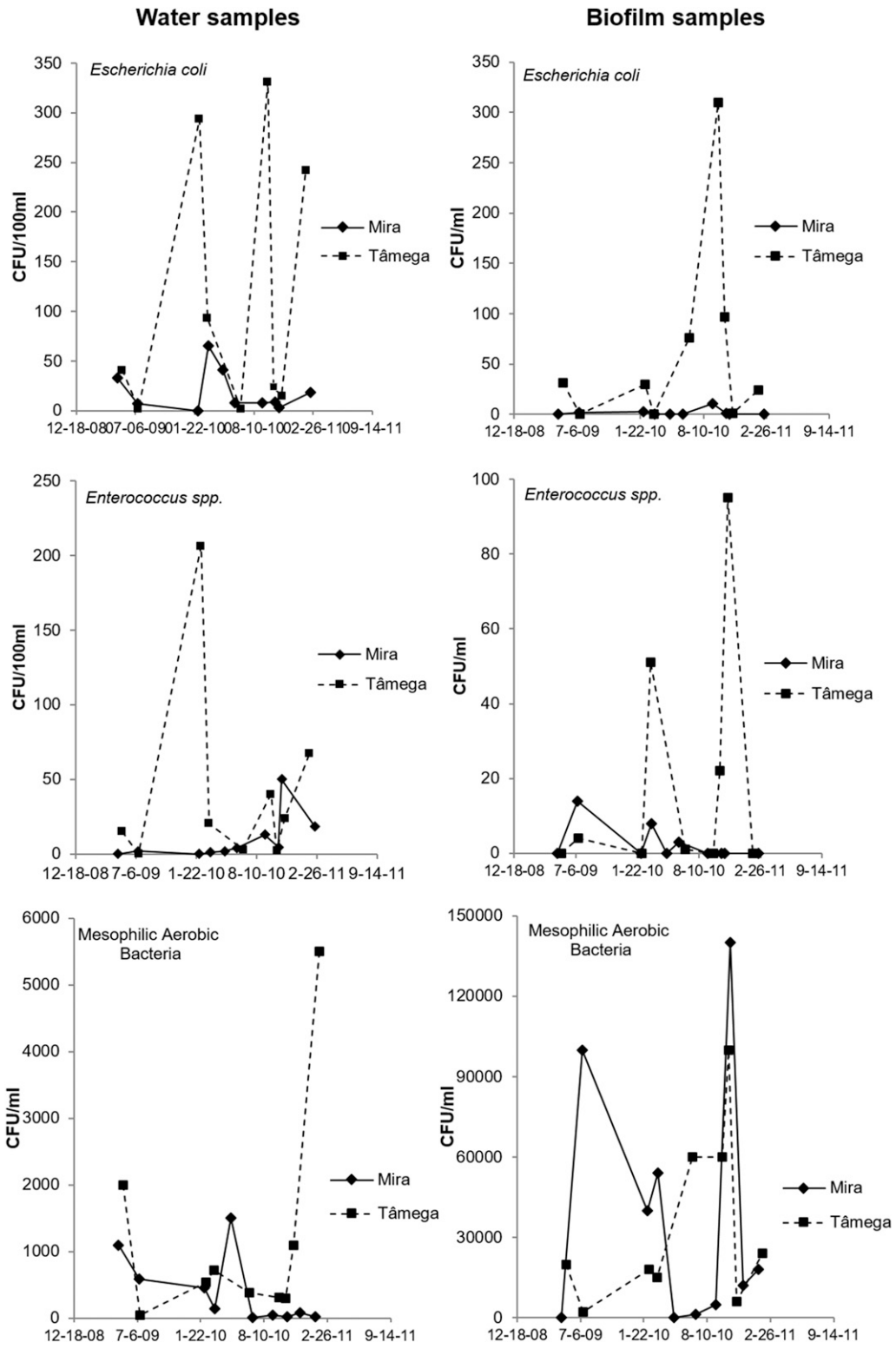

Figure 1. Annual variations (CFU/100 mL or CFU/mL) of *Escherichia coli*, *Enterococcus* spp., and mesophilic aerobic bacteria in samples of water and biofilm collected from the Tâmega river and Mira lagoon.

(Fig. 3B). The cellular fraction of *Anodonta anatina* showed similar results as the previous bivalve species, inhibiting the same strains; however, this inhibition was greater in the case of *S. enterica Typhimurium* CECT 443 and lesser in the case of *A. baumannii* ATCC 19606 (Fig. 3C). *Unio delphinius*, although being collected from the same place, at the Tâmega river, did not cause any bacterial inhibitions in this assay (results not shown).

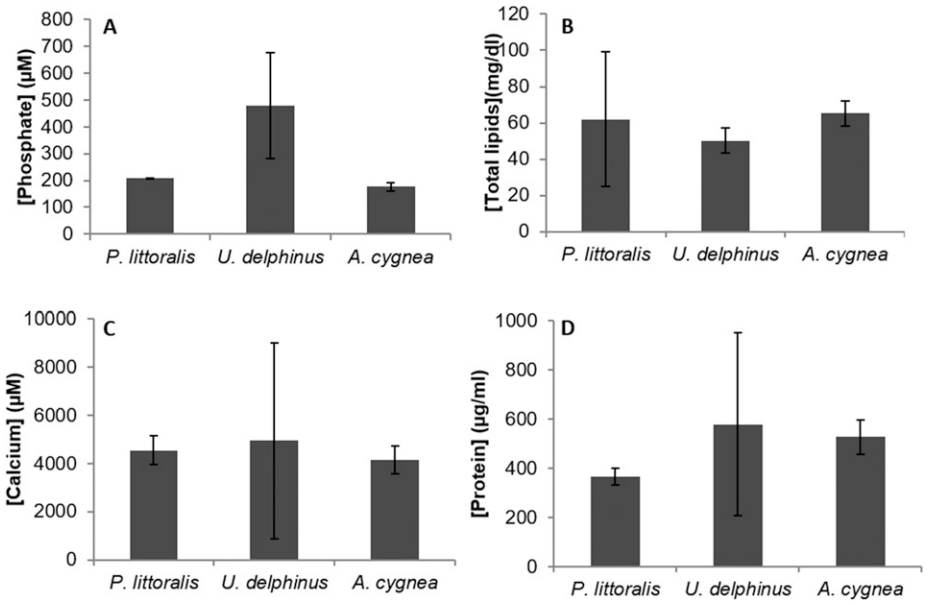

Figure 2. Quantification of several plasma constituents from Unionidae species, average numbers from three different periods of sampling (summer, spring, and autumn): *Anodonta cygnea*, *Potomida littoralis*, and *Unio delphinus*: (A) Total proteins, (B) Total lipids, (C) Phosphates, and (D) Calcium.

Antibiofilm Activity

Regarding the biofilm formation assay, the results were more evident, with almost all tested conditions being significantly different from the control ($P < 0.05$, Fig. 4). In the presence of the plasma of the bivalves, even when diluted, less biofilm was formed. Interestingly, the cellular fraction hampered the biofilm formation solely by *Escherichia coli* ATCC 25922, which was the bacterial strain tested that apparently had more difficulty to form a biofilm both in the presence of the cellular fraction and higher dilution of plasma of *Anodonta cygnea*. Moreover, *A. cygnea* showed great ability to also inhibit the biofilm formation of *Staphylococcus aureus* ATCC 25923, with

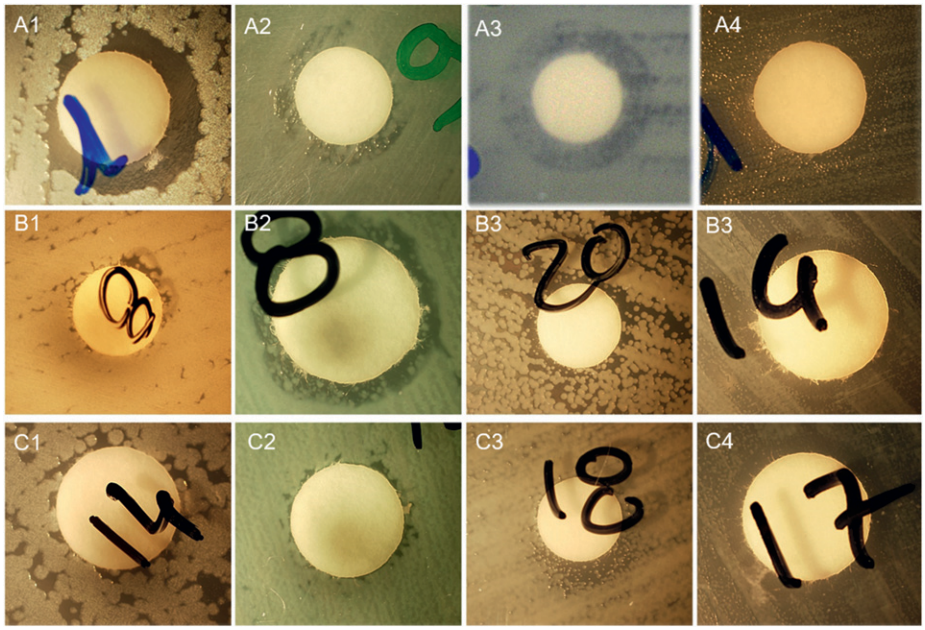

Figure 3. Antimicrobial activity (inhibition zones around the discs) of cellular fractions from freshwater bivalves against different strains of bacteria. In A, the *Anodonta cygnea* cellular fractions were tested against A1—*Bacillus subtilis* ATCC 6683, A2—*Pseudomonas aeruginosa* ATCC 27853, A3—*Pseudomonas putida*, and in A4—*Listeria monocytogenes* ATCC 19111. In B, *Potomida littoralis* cellular fractions against B1—*B. subtilis* ATCC 6683, B2—*P. aeruginosa* ATCC 27853, B3—*Acinetobacter baumannii* ATCC 19606, and in B4—*L. monocytogenes* ATCC 19111. In C, *Anodonta anatina* cellular fractions against C1—*B. subtilis* ATCC 6683, C2—*P. aeruginosa* ATCC 27853, C3—*Salmonella enterica* Typhimurium CECT 443, and in C4—*L. monocytogenes* ATCC 19111.

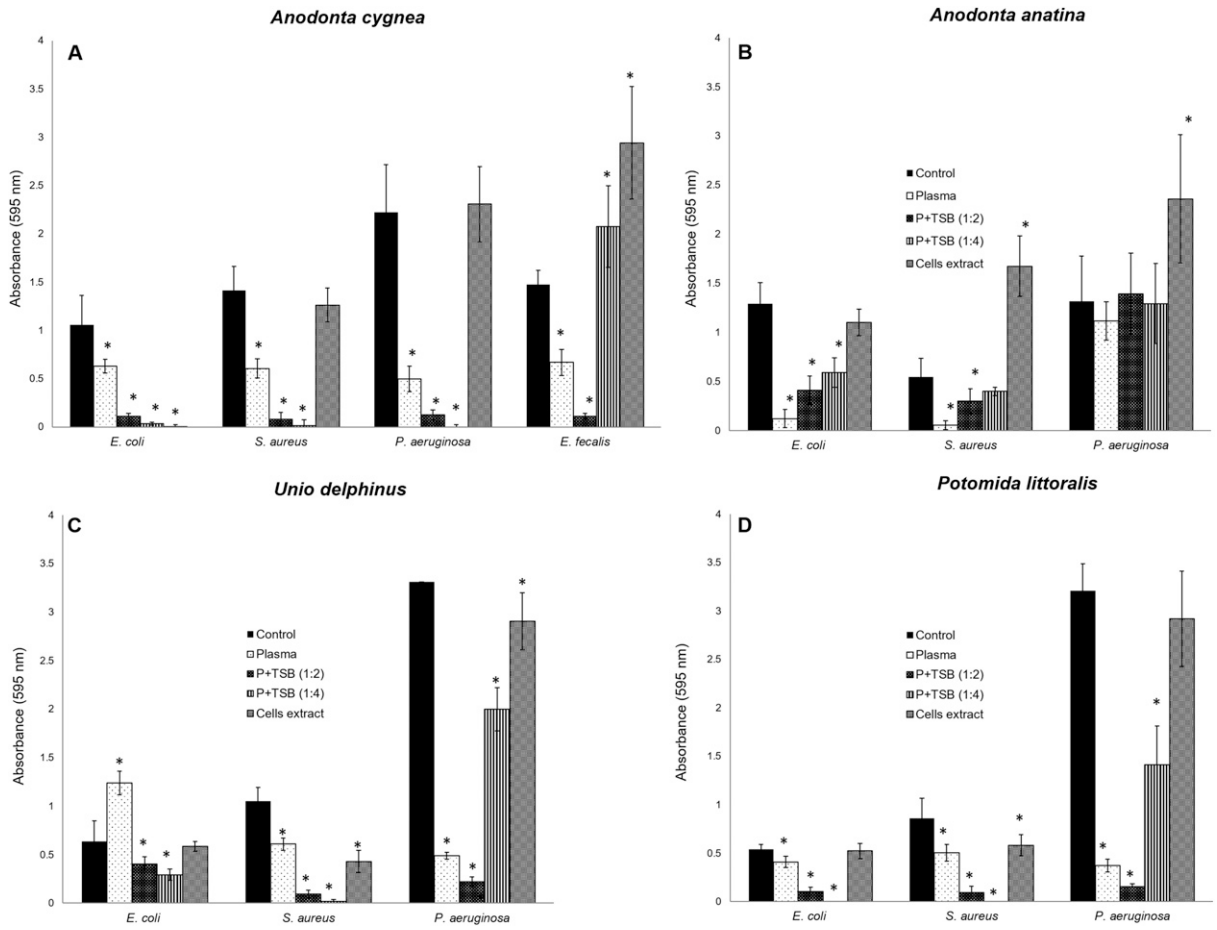

Figure 4. Biomass quantification of biofilms of *Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, and *Enterococcus faecalis* ATCC 29212 in the presence of different biological fractions from freshwater bivalves: plasma, plasma diluted in the ratio of 1:2, plasma diluted in the ratio of 1:4, and cells (cellular fractions) compared with a control situation (only the inoculum) tested with (A) *Anodonta cygnea*, (B) *Anodonta anatina*, (C) *Unio delphinus*, and in (D) *Potomida littoralis*. Differences statistically significant in relation to the control are marked with *, using Student's *t*-test with a $P < 0.05$.

all its components tested able to cause a lower biofilm production than the control. The biofilm of *Pseudomonas aeruginosa* ATCC 27853 was mostly inhibited when in contact with plasma; the diluted plasma caused the highest inhibition. Regarding *Enterococcus feacalis* ATCC 29212, *A. cygnea* caused inhibition only through pure plasma and plasma diluted in the ratio of 1:2; the higher dilution of the plasma and the cellular fraction caused higher biofilm production than the control (Fig. 4A). Although belonging to the same genus, *Anodonta anatina* showed slightly different results, presenting a significant capacity to inhibit the biofilm formation by *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, especially in the presence of undiluted and diluted 1:2 plasma, however, causing little inhibition in the biofilm formed by *P. aeruginosa* ATCC 27853. On the contrary, the cellular fraction of *A. anatina* was even able to significantly increase the biofilm biomass in respect to the control. The cellular fraction equally increased the biofilm biomass of *S. aureus* ATCC 25923 (Fig. 4B).

In terms of biofilm production, *Unio delphinus* and *Potomida littoralis* (Fig. 4C, D) caused a similar effect in the three different bacterial strains tested. *Staphylococcus aureus* ATCC 25923 suffered the highest inhibition in the biofilm formation (the highest value was recorded for the more diluted sample of plasma). *Pseudomonas aeruginosa* ATCC 27853 biofilm was inhibited in the presence of all fractions of these two species; nonetheless, the plasma diluted in the ratio of 1:2 caused the highest inhibition whereas the cellular fractions showed little effect on the biofilm formation. Regarding *Escherichia coli* ATCC 25922, the results were more inconsistent; although the diluted plasma produced a marked reduction on the biofilm formed, the undiluted plasma of *U. delphinus* led to a higher production of biofilm in comparison with the control. Hemocytes from *P. littorallis* and *U. delphinus* inhibited significantly the biofilm production by *S. aureus* ATCC 25923 ($P < 0.05$).

Viability Assay

The live/dead fluorescence assay (Fig. 5) allowed a qualitative analysis along with a viability assessment of the biofilms formed in the presence of different biological fractions of the freshwater bivalves. It was visible that no biofilm was formed in the presence of the plasma (nondiluted and at both dilutions) of *Anodonta cygnea*. The undiluted plasma and the plasma diluted

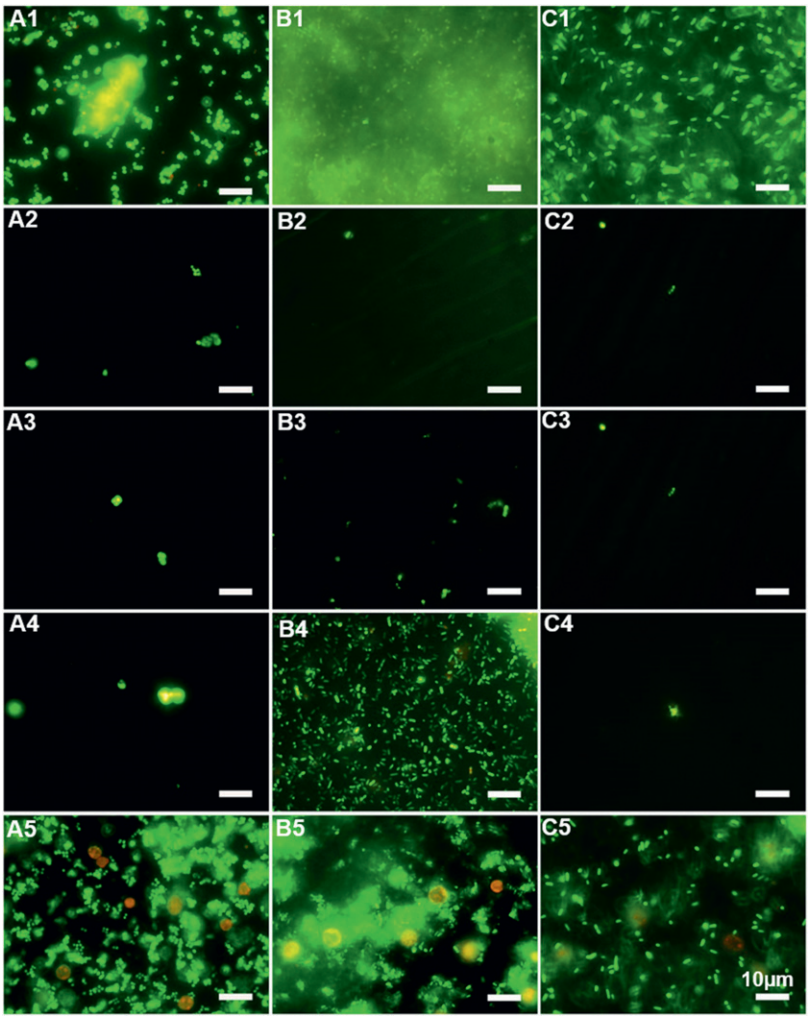

Figure 5. Evaluation of biofilm formation by different bacterial strains in the presence of cells and plasma fractions from the freshwater bivalve *Anodonta cygnea*. Live/dead viability staining images after 24 h of incubation. In A, *Staphylococcus aureus* ATCC 25923 was used; in B, *Pseudomonas aeruginosa* ATCC 27853 was used; and in C, *Escherichia coli* ATCC 25922 was used. A1, B1, and C1—control; A2, B2, and C2—presence of plasma; A3, B3, and C3—presence of plasma diluted in the ratio of 1:2 in TSB; A4, B4, and C4—presence of plasma diluted in the ratio of 1:4 in TSB; and A5, B5, and C5—presence of cellular fractions. Red dots observed in the number 5 situation (bottom line) refer to the cell nucleus that stained with the propidium iodide of the staining mixture. Scale bar corresponds to 10 µm.

in the ratio of 1:2 in TSB inhibited almost totally the growth of the three bacterial strains tested; even the more diluted fraction of the plasma could inhibit greatly the biofilm formation by *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. *Pseudomonas aeruginosa* ATCC 27853, despite being able to form a biofilm in the presence of the plasma, the biomass produced was lower than the one formed in the control condition. The cellular fraction showed almost no inhibition, the big red cells (Fig. 5A5, B5, C5) correspond to dead hemocytes, which may have been used as a substrate, favoring the proliferation of the three bacterial strains tested.

DISCUSSION

Invertebrate animals have an innate immune system mainly composed by the circulating cells in their body fluids, such as hemocytes, which are responsible for mechanisms such as phagocytosis (Mitta et al. 1999, Antunes et al. 2010, 2014). In previous studies, it was demonstrated that *Escherichia coli* and *Enterococcus faecalis* were not tolerated by *Anodonta* spp., being eliminated by the bivalve's granulocytes, which proved the action of the cellular immunity (Antunes et al. 2010, 2014); however, it is quite evident that humoral substances must also be present in the plasma, allowing it to play a significant role in the immunity defense, as shown by the present study, which revealed an antibiofilm activity of the plasma of all unionid species tested.

Cellular fractions could cause bacterial inhibition, although only to a relatively small extent, in some cases. Nevertheless, there were cases in which the cellular fractions stimulated the bacterial growth, which was probably due to the fact that the conditions used did not allow maintaining the hemocytes viable long enough. There is a gap in the literature in terms of the optimal conditions to maintain these cells viable. In the article by Hinzmann et al. (2013), some procedures were recommended such as the use of appropriate anti-aggregation solutions and

low temperature (keeping the cells on ice); still, the several attempts to put these cells into the culture failed (data not shown). Moreover, this work focused on the bacterial conditions, so nothing was added to avoid interference with the bacterial growth and the temperature selected was the optimal for the bacteria. Thus, the use of cellular fractions for assessing biological activities needs to be optimized. Nonetheless, the agar disc diffusion method showed that the cellular fraction from *Anodonta* spp. and *Potomida littoralis* inhibited *Bacillus subtilis* ATCC 6683 and *Pseudomonas aeruginosa* ATCC 27853, meaning that these fractions may comprise inhibitory components, which could have been originated from cells. Moreover, the agar disc diffusion assay showed that these species of freshwater mussels had also the potential to inhibit multidrug-resistant strains, such as multidrug-resistant isolates of *Pseudomonas putida* and *Acinetobacter baumannii*. Obviously, the agar disc diffusion assay is a basic screening assay that has several limitations, e.g., only a small volume of sample can be tested, and some substances do not diffuse in the agar medium. Probably, this can be one of the reasons explaining why in some cases no inhibition was recorded using the plasma fraction in the disc whereas very high inhibition was recorded in the biofilm quantification assay.

Taking into account the results regarding the effect of bivalves' biological fractions toward the biofilm formation by bacteria, it is likely that these bivalves developed molecular mechanisms capable of defending them from bacteria that could develop a biofilm inside them, putting at stake their survival. As no antibacterial effect on the bacterial growth was observed, the antibiofilm activity of plasma components was probably due to their interference in the communication/quorum-sensing system of the bacterial cells or due to specific physicochemical characteristics of that body fluid which do not favor the adhesion of bacteria to a surface. Moreover, because the diluted plasma still had the same effects, it may indicate that the components interfering with the biofilm formation are able to do it even at a low concentration. The fact that the inhibition of biofilm formation by these plasma components was found in diverse bivalve species and toward bacterial strains that are typically not present in their living water (e.g., *Staphylococcus aureus*) is suggestive of an innate nonspecific mechanism; otherwise, the bivalve species inhabiting waters with higher microbial contamination would have expressed a more pronounced inhibitory capacity against bacteria. The cellular fraction obtained from *Anodonta cygnea* was able to inhibit the biofilm formation by *Escherichia coli*, but not by the other bacterial strains, which means that it may have particular compounds targeting specifically the biofilm formation by *E. coli*. This hypothesis need to be further explored. Little is known on the chemical identification of these substances as well as on their mechanisms of action. Several antimicrobial compounds, namely AMPs, have been identified in cultivated species, e.g., mytilins in mussels (*Mytilus edulis* and *Mytilus galloprovicialis*) (Charlet et al. 1996, Mitta et al. 2000) or defensins from oysters (*Crassostrea virginica* and *Crassostrea gigas*) (Anderson & Beaven 2001, Bacheré et al. 2015) and big defensin from the scallop *Argopecten irradians* (Zhao et al. 2007). Thus, the studies on AMPs have been increasing because these peptides have the potential to replace actual antibiotics as new antimicrobial drugs with application on aquaculture (Cheng-Hua et al. 2009).

Similar studies on freshwater mussels were reported by Estari et al. (2011) regarding *Lamellidens marginalis* and by Santhiya and Sanjeevi (2014) using *Parreysia corrugatea*. Estari et al. (2011) tested fluids and tissue extracts from *L. marginalis*, which were diluted in solvents (water, chloroform, acetone, or methanol) and followed protein extraction protocols; among the different extracts tested, they obtained antimicrobial inhibition against *Staphylococcus aureus*, *Streptococcus pyroenes*, *Morganella morganii*, *Bacillus subtilis*, *Escherichia coli*, *Proteus vulgaris*, and *Crassostrea albicans*. The other study reported bacterial inhibition of tissue extracts through the diffusion agar method against pathogenic bacteria, such as *S. aureus*, *B. subtilis*, *E. coli*, *Pseudomonas aeruginosa*, and *Klebsiella pneumonia* (Santhiya & Sanjeevi 2014). Regarding freshwater bivalves, the molecules that may be involved in this antibacterial response are still unidentified; however, breakthroughs in this field may start to appear, because a big defensin gene was recently identified in the freshwater mussel *Hyriopsis cumingii* (Wang et al. 2014).

Herein, *Anodonta cygnea* gave more marked results in comparison with the other bivalve species, which, however, slightly followed the same pattern. The freshwater bivalve *A. cygnea* inhabits a more constant environment: the water from Mira lagoon, from where only *A. cygnea* was collected, presented lower fluctuations in the microbial counts in respect to the water samples analyzed from Tâmega river. The lagoon provides the appropriate conditions for this species to persist, allowing it to be integrated in the ''least concern'' conservation category. The species *Unio delphinus* and *Potomida littoralis* came from a river with higher fluctuation in terms of bacteria load (*Escherichia coli* and *Enterococcus* spp.) and this may explain the less-pronounced antibacterial results and the more fragile conservation status of these species, ''near threatened'' and ''endangered,'' respectively (IUCN red list). The species *Anodonta anatina*, equally with ''least concern'' conservation status, showed the lower antibacterial potential when compared with *A. cygnea*.

Although the plasma composition was not determined in the present study, it surely must be further explored to understand the immune system of this highly endangered group of species and to identify potential antimicrobial compounds. Because the bacterial burden is increasing in their natural habitat (Costa et al. 2013) and threatening even more their survival, it is expectable that freshwater bivalves may find strategies to overcome that hostile situation by producing antimicrobial substances; therefore, further studies in this field are fundamental to fully understand how humoral factors act in the innate immune response.

ACKNOWLEDGMENTS

This work was supported by the Portuguese Foundation for Science and Technology (FCT) with a research grant to M. H. (SFRH/BD/76265/2011). This article was also supported by the project INNOVMAR-Innovation and Sustainability in the Management and Exploitation of Marine Resources (NORTE-01-0145-FEDER-000035, within Research Line NOVELMAR), by North Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement, and through the European Regional Development Fund (ERDF).

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