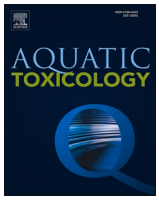
Aquatic Toxicology 265 (2023) 106779

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Motility of *Mytilus galloprovincialis* hemocytes: Sensitivity to paracetamol *in vitro* exposure

Gayatri Udayan [a], Maria Elena Giordano [a], Patrizia Pagliara [a], Maria Giulia Lionetto [a,b,*]

[a] Dept. Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Italy
[b] NBFC, National Biodiversity Future Center, Palermo 90133, Italy

ARTICLE INFO

Keywords:
Acetaminophen
Mussel
Cell motility
Hemocytes
Cell tracking
Directness
Velocity

ABSTRACT

Pharmaceuticals released into the environment (PiEs) represent an environmental problem of growing concern for the health of ecosystems and humans. An increasing number of studies show that PiEs pose a risk to aquatic organisms. The aim of the present work was to contribute to increasing the knowledge of the effects of PiE on marine biota focusing on the effect of paracetamol on the motility of hemocytes in *Mytilus galloprovincialis*, a bivalve mollusk species widely utilized as bioindicator organism. Hemocytes are the immunocompetent cells of bivalve mollusks. An early and key stage of mollusk immune response is represented by the recruitment and migration of these cells to the site of infection. Therefore, motility is an intrinsic characteristic of these cells. Here, we first characterized the spontaneous cell movement of *M. galloprovincialis* hemocytes when plated in a TC-treated polystyrene 96-well microplate. Two different cellular morphotypes were distinguished based on their appearance and motility behavior: spread cells and round-star-shaped cells. The two motility morphotypes were characterized by different velocities as well as movement directness, which were significantly lower in round-star-shaped cells with respect to spread cells. The sensitivity of the motility of *M. galloprovincialis* hemocytes to paracetamol at different concentrations (0.02, 0.2 and 2 mg/L) was investigated *in vitro* after 1h and 24h exposure. Paracetamol induced alterations in the motility behavior (both velocity and trajectories) of the hemocytes and the effects were cell-type specific. The study of hemocyte movements at the single cell level by cell tracking and velocimetric parameters analysis provides new sensitive tools for assessing the effects of emerging pollutants at the cellular levels in non-target organisms.

1. Introduction

Pharmaceuticals are globally used to support health and treat diseases. However, they can be released into the natural environment during their manufacture, use, and disposal, representing an increasing environmental concern for the health of ecosystems and humans (Wilkinson et al., 2022). These products might not be completely or effectively removed by wastewater conventional treatments (Pereira et al., 2016) and are often discarded directly into the environment through crude sewer systems. An increasing number of studies show that pharmaceuticals released into the environment (PiE) pose risks to aquatic organisms. This risk is evidenced not only by the sustained persistence of PiE in different aquatic compartments but also by their bioaccumulation in many species, as documented by Garcia et al. (2012), and Wang and Gardinali (2013). Coastal marine environments are at particular risk due to increasing inputs of human pharmaceuticals through sewage effluents (Gaw et al., 2014). Here, the PiE discharge represents an additional stressor for biota already impacted by other contaminants including trace metals, oil-based products, pesticides, fertilizers, antifouling compounds, whose effects on biota are known and have been studied for a longer time (Lionetto et al., 1998; Martinez et al., 2022).

As regards PiE detection frequencies and concentrations, acetaminophen (N-(4-hydroxyphenyl)acetamide) or paracetamol has been demonstrated to be one of the contaminants with the highest concentration and the highest detection frequencies in the river all over the world (Wilkinson et al., 2022). It is a non-steroidal anti-inflammatory drug, widely used as an analgesic and antipyretic. It is the world's most widely marketed over-the-counter drug (Warwick, 2008), mostly because of its non-prescription availability and low cost. The continuous high consumption and production of paracetamol as well as its occurrence in seawater give rise to concerns regarding impact on marine organisms, especially filter feeders (Koagouw et al., 2021).

* Corresponding author at: Dept. Biological and Environmental Sciences and Technologies (DiSTeBA), University of Salento, Italy.
E-mail address: giulia.lionetto@unisalento.it (M.G. Lionetto).

https://doi.org/10.1016/j.aquatox.2023.106779
Received 17 October 2023; Received in revised form 17 November 2023; Accepted 23 November 2023
Available online 24 November 2023

Mussels are efficient sessile filter feeders able to accumulate various chemical pollutants and are widely considered early warning biological systems in water quality monitoring and environmental pollution assessment (Caricato et al., 2019; Lionetto et al., 2021; Rosner et al., 2023) due to their ability to early develop detectable responses to chemical pollution exposure. In particular, mussel circulating immunocompetent cells known as hemocytes are widely used to study the toxicological impacts of environmental pollutants in marine organisms (Burgos-Aceves and Faggio, 2017; Cajaraville et al., 1996; Calisi et al., 2008; Lionetto et al., 2022), due to their role in the innate immunity of the organisms and the relative ease with which they can be extracted (Canesi et al., 2007). Hemocytes perform several important functions of cell-mediated innate immunity. These include cytotoxic production of oxygen and nitrogen reactive species (Costa et al., 2009; Novas et al., 2007), increased production of lysozymes (Li et al., 2008) and phagocytosis, as well as being involved in other important functions such as wound repair, shell repair, nutrient digestion and transport, and excretion (Beninger and Le Pennec, 2016). In addition, *Mytilus sp.* hemocytes display similar structure and function to mammalian immune cells and this property is useful for comparison in regulatory studies (Canesi et al., 2012). Primary hemocyte cultures have been proposed as a useful tool for the investigation of the biological effects and mechanisms of action of environmental pollutants (Barrick et al., 2018; Canesi et al., 2012). Alterations in the hemocytes abundance, cell viability, lysosomal destabilization, phagocytic activity, Reactive Oxygen species production, and genotoxicity represent some of the most investigated alterations induced by environmental pollutants on mussel hemocytes (Auguste et al., 2021; Calisi et al., 2023, 2008; Giannapas et al., 2012; Kaloyianni et al., 2009; Katsumiti et al., 2021). An early and key stage of mollusk immune response consists of hemocyte recruitment and migration to the site of infection. Therefore, motility is an intrinsic characteristic of these cells (Le Foll et al., 2010; Rioult et al., 2013). Although motility represents a basic feature of hemocytes fundamental for their functions, very few studies have investigated hemocyte motility in mussels (Gendre et al., 2023; Le Foll et al., 2010; Rioult et al., 2013) to date and particularly its sensitivity to environmental chemical pollutants (Gendre et al., 2023; Kaloyianni et al., 2009; Sendra et al., 2020). In general, cell motility plays a fundamental role in many physiological processes, such as embryogenesis, angiogenesis, wound healing, immune response, and several disease-related processes (SenGupta et al., 2021). In immune cells, motility is a fundamental characteristic of these cells enabling their immunosurveillance for the protection of the body. Immune cell motility has been widely characterized in humans and vertebrates (Jerison and Quake, 2020; Vesperini et al., 2021), but comparatively, much less information is available on invertebrates.

The aim of this study was to enhance understanding of the effects of PiE on marine biota by examining the impact of paracetamol on hemocyte motility in the bioindicator organism *Mytilus galloprovincialis*. In particular, the study addresses: (i) the functional analysis and characterization of spontaneous cell movement of *M. galloprovicialis* hemocytes when the cells were plated in a multi-well plate and cultured for 24h, and (ii) the response of hemocyte motility under *in vitro* exposure to increasing concentrations of paracetamol in the incubation medium. The choice of the *in vitro* approach for this study arises from the fact that *in vitro* testing on marine organisms has been widely used to assess pollutant toxicological effects and characterize the underlying toxicological mechanisms (Canesi et al., 2007); moreover, it is particularly useful for quickly pre-screening products for regulatory compliance.

2. Materials and methods

2.1. Materials

Mussels, *Mytilus galloprovincialis*, were purchased from a local farm (Ittica Demar, Lecce Italy). All experiments were performed in accordance with the Italian Animal Welfare legislation (D.L. 26/2014) that implemented the European Committee Council Directive (2010/63 EEC). Paracetamol of purity ≥99 % was purchased from Merck (Darmstadt, Germany).

Unless otherwise indicated, all chemicals were purchased from Merck and were of analytical grade. LysoSensor Green DND-189 was purchased from Thermo Fisher Scientific, Waltham, USA)

2.2. Methods

Mussels (*Mytilus galloprovincialis* Lam.) adult specimens (shell length: 6.6 ± 0.6 cm; shell width: 3.2 ± 0.3 cm) were purchased from a local supplier and immediately transferred to the laboratory and acclimated in static tanks containing aerated filtered natural seawater (1 L/animal), 37 PSU, at 15 °C in a thermostatic room for 24h prior to experimental use according to Auguste et al. (2021, 2020). During the acclimation period, animals were maintained without food.

2.2.3. Hemolymph withdrawal and culture

Hemolymph was withdrawn from the posterior adductor muscle and diluted in a disposable syringe 1:1 with filtered seawater FSW (0.2 μm filters). For each independent experiment ($n = 3$), the hemolymph was sampled from 3 to 4 animals and pooled in Falcon tubes at 15 °C.

A volume (50 μl) of diluted hemolymph (cell concentration = 1×10^6 cells/ml) was added into each well of Corning® 96-well solid black flat-bottom polystyrene TC-treated microplates. Hemocytes were allowed to adhere for 30 min at 15 °C forming a monolayer on the bottom of the well. Then, the excess hemolymph was removed, and the adherent cells were cultured for 24h with filtered natural seawater supplemented with 2 mM L-glutamine 40 IU/mL penicillin G and 100 μg/mL streptomycin.

2.2.4. Cell viability assessment

The viability of hemocytes was evaluated by trypan blue staining immediately after sampling. 0.4 % trypan blue osmotically adjusted to the osmolarity of mussel hemolymph was used according to Lionetto et al. (2022). The viability of the cells immediately after sampling corresponded to 100 %.

The vitality of the cells was also evaluated in the adherent cells cultured in Corning® 96-well solid black flat-bottom polystyrene TC-treated microplates. In this case, the MTT test was applied. The MTT test is widely used to assess cellular metabolic activity as an indicator of cell viability. The assay measures the reduction of the tetrazolium component MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) into an insoluble formazan product by mitochondrial dehydrogenase enzymes of viable cells. Briefly, hemocytes adherent to the bottom of 96-well cell culture plates were rinsed with FSW and incubated in FSW containing 0.5 mg/ml MTT. After 3 h at 15 °C, the MTT solution was removed and 100 μL of dimethyl sulfoxide was added to each well for the formazan crystals solubilization. The absorbance was measured at a wavelength of 570 nm using Cytation 5 imaging multimode reader (BioTek, Winooski, VT, USA), which combines conventional microplate detection with automated microscopy. Cell vitality at 24h was expressed as the percentage of OD at time 24 with respect to the OD recorded at time 0 immediately after seeding.

2.2.5. Cell motility assay

Cell motility was assessed by live cell time-lapse microscopy and trajectory analysis on adherent hemocytes cultured in Corning® 96-well solid black flat-bottom polystyrene TC-treated microplates. Time-lapse microscopy at a rate of 1 image every 1 min for 10 min was carried out to image hemocytes. The acquisition of cell images through time-lapse imaging was performed using the multimode reader Cytation 5. Hemocytes adherent to the bottom of the wells were imaged at time 0, 1h, and 24h into the culture. The use of the multimode reader facilitated the simultaneous acquisition of images from a high number of wells. Cell tracking was performed on single cells; at least 70 cells per well were analyzed and three well replicate per each experimental condition in

each independent experiment were performed. Cells escaping the recorded field were excluded from the analysis. Migration patterns and trajectories of hemocytes were determined using the ImageJ2 Manual Tracking plugin and the Chemotaxis and Migration Tool software (Ibidi GmbH).

For quantitative evaluation of spontaneous cell movement, several parameters of the trajectories were measured such as mean velocity, migrated distance, Euclidean distance, and directness, which were calculated using Chemotaxis and Migration Tool V2.0. Mean velocity (expressed in μm min^{-1}) is the absolute cell speed, whatever the direction, and was calculated by dividing the migrated distance by the time length of the cell tracking for each cell; migrated distance (expressed in μm) is the length of the migration path during the observation period (10 min); Euclidean distance (expressed in μm) is the length of the straight line between cell start and end point; directness is calculated from the ratio between the Euclidean distance and the accumulated distance for each trajectory. It is a measurement of how straightforward the cell trajectories were, whatever the directness of cell trajectories, and tends to 1 for straight tracks, but decreases to 0 for circular tracks.

In paracetamol exposure experiments, the plated cells were exposed for 24h to different drug concentrations (0.02, 0.2, and 2 mg/L), chosen on the basis of the paracetamol concentrations reported in seawater environments which vary from 3.2 ng/L (Benotti and Brownawell, 2007; Fairbairn et al., 2016) to more than 200 μg/L (Togola and Budzinski, 2008) in surface coastal waters. The paracetamol PEC value calculated by Stuer-Lauridsen et al. (2000) for the European Union waters is 0.65 mg/L. Therefore, the first two concentrations tested, 0.02 and 0.2 mg/L, are within the spectrum of environmentally relevant concentrations. In addition to this, we chose a third concentration, 2 mg/L outside this range, to possibly disclose, if any, potential pharmacological effects of the drug on cell survival and motility *in vitro*.

2.6.7. Statistical analysis

Values are given as the mean ± S.E.M of three independent experiments. Each experimental condition was replicated three times, and a minimum of 70 cells per replicate for each condition in every independent experiment were analyzed. Statistical tests utilized to evaluate the statistical significance of differences were One Way ANOVA, and Tukey multiple comparison post-test as indicated in the figures' captions.

3. Results

3.1. Characterization of spontaneous cell movement of hemocytes

Mussel hemocytes, when added to a TC-treated 96-well microplate, started attaching to the bottom of the well within a few minutes and acquired a spread appearance, showing single-cell movements based on lamellipodia activity and fast shape changes as shown in Fig. 1 (A–C) and in supporting video 1 (see supplementary material section) obtained from time-lapse imaging of hemocytes after 30 min attachment to the well bottom. Cell movements in the condition of random walk in a free field were monitored using brightfield microscopy at 20X magnification, capturing one image per minute over a 10 min period with the Cytation 5 Multimode reader. Fig. 1 (A–C) shows representative images of the same region of interest (ROI) acquired at times 0, 5 min, and 10 min. The numbering of the cells remains consistent across the three figures to illustrate their movement.

According to findings of Rioult et al. (2013) in *Mytilus edulis*, two main hemocyte morphotypes were observed also in *Mytilus galloprovincialis* based on cell motility analysis: spread cells (Fig. 2B) primarily exhibited a bipolar bean shape and sometimes a quite similar amoeboid outline, while smaller round-star-shaped cells (Fig. 2B) displayed narrow fast moving thick filopodia extending out from a condensed cell body. In both cell types, the movement was characterized by continuous shape changes through rapid extension and retraction of membrane protrusions. These two distinct motility morphotypes exhibited different

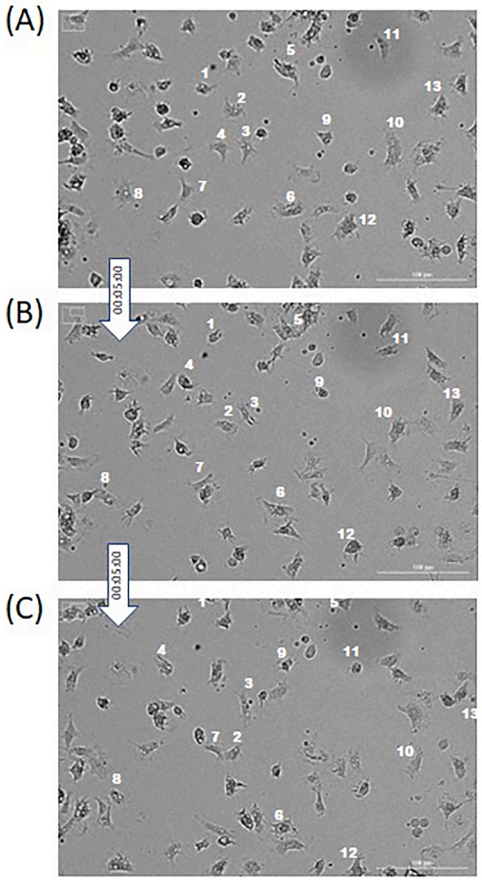


Fig. 1. Representative time-lapse imaging of hemocytes after 30 min attachment to the bottom of the well. Cell movements were monitored by brightfield microscopy at the rate of 1 image every 1 min for 10 min under 20X magnification using Cytation 5 Multimode reader; for brevity, Fig. A, B, and C show three of the 11 frames of the same region of interest (ROI) acquired at time 0, 5 min, and 10 min. The same cells were numbered in the three figures to show the movement. The arrows show the time intervals. Scale bar: 100 μm.

velocities, with round-star-shaped cells showing a significantly lower velocity compared to spread cells (Fig. 2A). Furthermore, the movement directness differed between the two morphotypes, being lower in round-star-shaped cells compared to spread cells. The representative trajectory plots are demonstrated in Fig. 2C and D.

3.2. Hemocyte viability and motility during 24h culture

In order to deepen the study of hemocyte motility over 24h, at first cell viability was assessed after 24h culture by MTT assay. As shown in Fig. 3, viability was about 97 % of the initial value (referred to time 0) after 24h culture, suggesting that hemocytes can be maintained easily in culture for at least 24h in a basal saline medium. Then, the spontaneous cell movement of *M. galloprovicialis* hemocytes was assessed during 24h culture period, showing a significant increase in cell velocity in both morphotypes (Fig. 4A). This increase commenced within the first hour for spread cells and continued to rise steadily over subsequent hours. Both the cell types showed a significant increase in velocity with time, with spread cells exhibiting a faster increase (where a significant increase in velocity with respect to time 0 was observed starting from the first hour) compared to round-star-shaped cells (where a significant increase in velocity with respect to time 0 was observed after 24h h). However, the magnitude of increase after 24h was similar for both morphotypes.

Regarding directness, while the two morphotypes typically exhibited

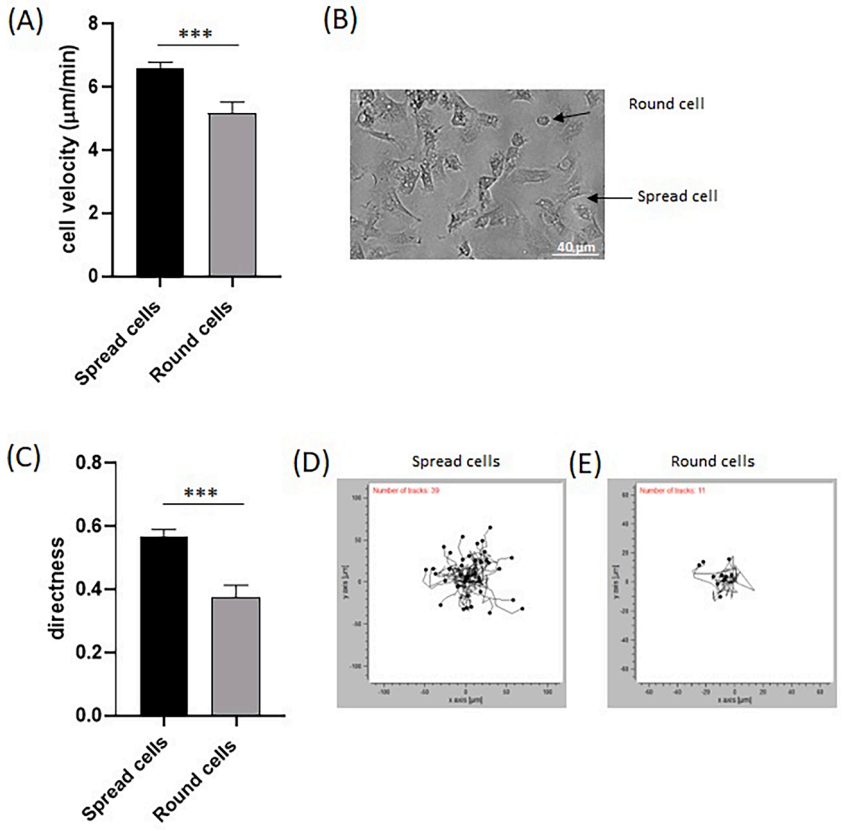


Fig. 2. (A, B, C, D). (A) Representative images of spread cells, and round cells. (B) Cell velocity expressed as µm/min; (C) Cell directness calculated from the ratio between accumulated distance and Euclidean distance; (D, E) Representative trajectory plots of spread and round cells respectively.

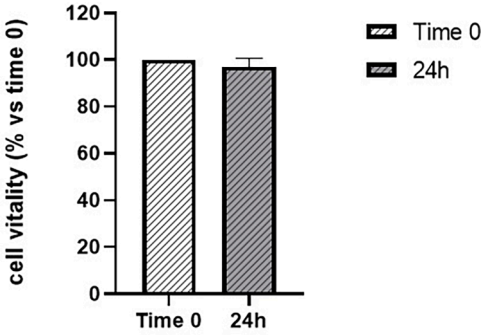


Fig. 3. Hemocyte vitality after 24h culture. Vitality was assessed by MTT assay and was expressed as a percentage relative to seeding values at day 0, which in turn was analyzed by trypan blue in suspended cell immediately after sampling.

different directness patterns, no statistically significant differences were observed over the 24 h culture period.

3.3. *Effect of in vitro exposure to paracetamol on hemocyte motility*

When the cells were exposed to increasing concentrations of paracetamol (range 0.02–2 mg/L) for 24h, no significant effect was observed on cell viability, as assessed by MTT test (Fig. 5). Regarding motility, Fig. 6 (A and B) shows the effect on spread cells velocity and directness after 1h and 24h exposure, respectively. After 1h exposure to paracetamol, no significant effects were detectable on cell velocity. However, after 24h exposure a slight but statistically significant effect was observed, even at the lowest tested concentration of 0.02 mg/L, suggesting a decrease in the activation of the cells after 24h. Increasing the concentration did not result in a further increase in the observed effect. In terms of directness, a slight significant decrease was observed after 24h exposure at the concentrations of 0.2 and 2 mg/L.

Fig. 7 (A and B) demonstrates the effect on round-star-shaped cells after 1h and 24h exposures. Velocity significantly increased after 1h exposure at the concentrations of 0.2 and 0.02 mg/L, suggesting the stimulation of the early activation of the cells. Simultaneously, a marked increase in the directness of the movement was recorded at all the three tested concentrations after 24h exposure. At the highest concentration of 2 mg/L, the effect was evident even after 1h exposure.

4. Discussion

Motility represents a fundamental cellular function that integrates several cellular processes, including cell adhesion, cell signaling, cytoskeleton activity, and cell volume changes. Despite its basic role in cellular physiology, it has been poorly investigated in relationship to its sensitivity to pollutants exposure. This work aimed to characterize the spontaneous motility of hemocytes in *Mytilus galloprovincialis,* a globally recognized sentinel species for pollution in coastal marine environments, and to explore *in vitro* the sensitivity of this fundamental cellular function to pollutant exposure in *M. galloprovincialis* hemocytes focusing on one of the most diffused PiE, paracetamol.

In our study, first, we characterized the spontaneous cell movement of *M. galloprovincialis* hemocytes when plated in a TC-treated polystyrene 96-well microplate. Immediately after plating,

M. galloprovincialis hemocytes showed spontaneous motile behavior, a random-like movement based on lamellipodia activity and fast shape changes, as already described for human immune cells (Pizzagalli et al., 2021) and also previously found in another mussel species, *Mytilus edulis* (Gendre et al., 2023; Le Foll et al., 2010; Rioult et al., 2013). In *M. galloprovincialis* hemocytes, we distinguished two different cellular

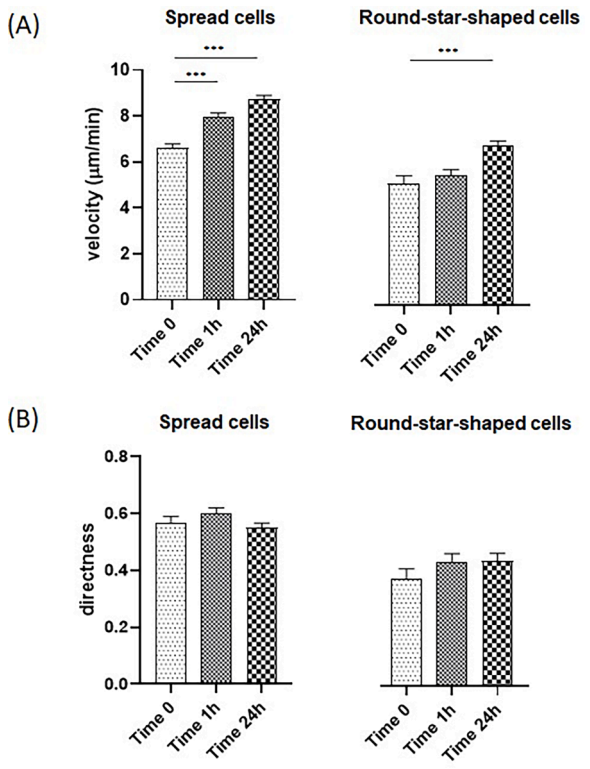


Fig. 4. (A,B,C) Velocity (expressed as μm min^{-1}) of spread and round-stars-shaped hemocytes assessed at time 0 (seeding value), after 1h and 24h culture; (C,D) directionality (expressed as the ratio between migrated distance and Euclidean distance) of spread and round-star-shaped hemocytes assessed at time 0 (seeding value), after 1h and 24h culture. Data are expressed as mean ± SEM. The statistical significance of data was assessed by One Way ANOVA and Dunnett's test.

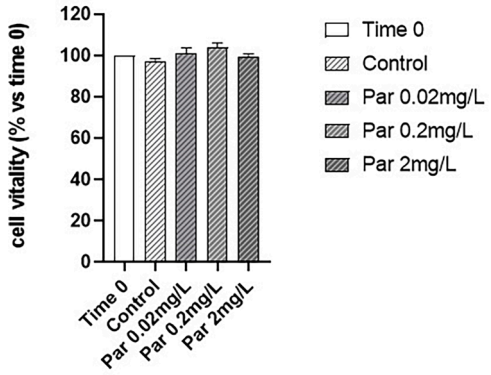


Fig. 5. Effect of paracetamol on hemocyte vitality after 24h culture. Vitality was assessed by MTT assay and was expressed as a percentage relative to seeding values at day 0, which in turn was analyzed by trypan blue in suspended cell immediately after sampling.

morphotypes on the basis of their appearance and motility behavior: spread cells and round-star-shaped cells. Both cell morphotypes showed rapid transitions between extended and shrunk cell morphologies with filopodia during their migration, but they exhibited cell-specific motility features with spread cells being faster than round-star-shaped cells and with a high intrinsic directionality of the movement. In general, the migration speed of the cells is believed to be controlled by dynamic regulation of the cytoskeleton and adhesion complex turnover (Graziano and Weiner, 2014; Sasaki et al., 2007), while the directionality of the cells is attributed to a restriction in lateral protrusions during lamellipodia generation with new protrusions preferentially generated from the pre-existing leading edge (Petrie et al., 2009). Therefore, the different speed and directionality observed in the two hemocyte morphotypes could reflect differences in the motility machinery of the two cell types including cytoskeleton and adhesion processes.

As regards the identification of the two motility-based morphotypes and their comparison with previous cytological classifications of mussel hemocytes, *M. galloprovincialis* is known to have two major types of hemocytes which have been described based on morphological characteristics detected by microscopic cytological observations of slide-attached cells (Cajaraville and Pal, 1995; Carballal et al., 1997) and by flow-cytometry analysis of dispersed cell suspensions (Andreyeva et al., 2019; Parrino et al., 2019): agranulocytes or hyalinocytes, which lack granules, and granulocytes, which contain granules. As described by Carballal et al., (1997), hyalinocytes are less abundant, smaller in size, have a high nuclear/cytoplasm ratio, and have a limited ability to spread on a glass slide, thus showing a round shape. On the other hand, granulocytes demonstrate a greater ability to spread on a glass slide, with distinct ectoplasm and endoplasm regions. The ectoplasm is rich in thin pseudopodia, while the endoplasm rich in dense granules. Some granulocytes contain abundant acidophilic granules, whereas others have basophilic granules while few of them contain both types of granules. These cells are likely professional phagocytes, which eliminate foreign microorganisms that invade the hemolymph and other tissues. As outlined by Le Foll et al. (2010), the rapid shape changes that characterize hemocyte motility in live cell preparations make it difficult for morphological classifications to be assessed in fixed cell preparations. Unambiguous identification of hemocyte subpopulations as classified according to cytological staining of fixed cells was not possible in our experimental systems, during the observation of vital cells in motion. However, according to the previous detailed cytological descriptions of the two different hemocyte cell types (Cajaraville and Pal, 1995; Carballal et al., 1997) granulocytes and hyalinocytes could correspond, respectively, to the spread cells and round-star-shaped cells described in this work, considering their similar motility behaviors and overlapping appearances. This is the first time that hemocytes are classified according to their motility behavior in *Mytilus galloprovincialis* and characterized by their velocimetric parameters. The velocity values recorded in our study (6.61 ± 0.17 μm/min for spread cells, 5.18 ± 0.34 μm/min for round-star-shaped cells immediately after plating) were comparable with the velocity of high-motile cells such as human neutrophyls (~8 μm/min) (Graham et al., 2009). This allows mussel hemocytes to be classified as high-speed cells. When compared with previous data on hemocyte motility in *Mytilus spp*, our values were higher than those reported for the entire hemocyte population by Sendra et al. (2020) for *M. galloprovincialis* (2.3 ± 1.5 μm/min) and by Gendre et al. (2023) for *M. edulis* (2.32 μm/min ± 1.57). The differences observed in comparison with literature data could be due to different experimental conditions, including different animal populations, cell density, experimental temperature, and the nature of the adhesion surface. As regards this last aspect, it could be crucial, considering the fundamental importance of cell-surface interaction on cell motility (Ziebert and Aranson, 2016). In our experiments, cells were plated on multiwell plates previously exposed to tissue culture treatment, a process that modify the hydrophobic plastic surface to make it more hydrophilic. The surface hydrophobicity/hydrophilicity is well known as a key factor in governing cell responses (Chang and Wang, 2011). Previous studies showed the more hydrophilic surface is the much more cell adhesion on the surface (Goddard and Hotchkiss, 2007; Xu and Siedlecki, 2007), although, as outlined by Vogler (1999), according to the cell type the relationship between hydrophilicity and cell adhesion is not always linear, especially at high hydrophilicity levels. Moreover, in our work, the cells were not labeled with nucleic acid stain which could interfere with intracellular cell signaling as demonstrated by Zhao et al. (2009).

When *M. galloprovincialis* hemocytes were cultured for 24h, the two cell types showed a significant increase in velocity with time according

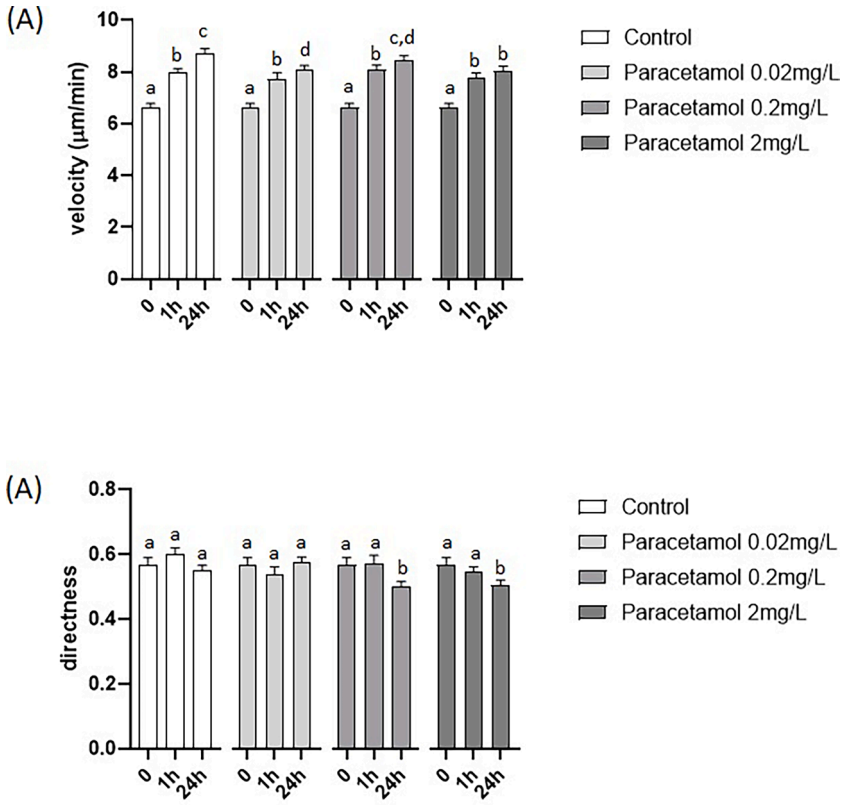


Fig. 6. (A,B) Velocity (expressed as µm min^{-1}) (A) and directness (expressed as the ratio between migrated distance and euclidean distance) (B) of spread hemocytes after 1h and 24h exposure to paracetamol. Data are expressed as mean ± SEM. The statistical significance of data was assessed by one way ANOVA and Tukey's post test. Different letters were used to show statistical significance ($p < 0.05$).

to previous observations of Rioult et al. (2013) in *M. edulis* hemocytes. However, the time course of activation was different in the two morphotypes, faster in spread cells with respect to round star shaped cells. The observed increase in cell velocity with time suggests activation of hemocytes during the 24h culture, presumably evocative of an inflammatory response after withdrawal and plating in the culture environment. Migration is presumably sensitive to stimulation factors released by hemocytes themselves in culture. The time course of activation was cell type specific, suggesting that spread cells are more prone and able to activate earlier in response to proinflammatory stimuli. Future work could further elucidate the nature of this phenomenon and explore the underlying signaling pathways.

In the second part of the study, the sensitivity of the motility of *M. galloprovincialis* hemocytes to paracetamol was investigated *in vitro* after 1h and 24h exposure, respectively. Our results indicate that exposure to paracetamol induced alterations in the motility of the haemocytes, and the effects were cell type-specific. In the case of spread cells, paracetamol exposure slightly reduced the activation of the hemocytes motility after 24h of exposure at all the three concentrations tested. The maximum velocity achieved after 24 h under paracetamol showed a slight but significant inhibition compared to the control when measured against the velocity at time 0. The effect was also evident on the trajectories, since the directness was slightly decreased at the concentrations of 0.2 and 2 mg/L after 24h exposure. Conversely, the effects were more pronounced for the round-star-shaped cells, involving the stimulation of the early activation of the cell motility within the first hour of exposure, at all the three tested concentrations, particularly at 0.2 mg/L. Also trajectories were influenced by the exposure to the drug, with a marked increase in the directness after 24h exposure. Overall, in round cells paracetamol exposure seems to evoke an early activation of cell motility.

In general, it is known that cell motility is governed by a complex network of signal transduction pathways that involve small GTPases, lipid second messengers, kinases, cytoskeleton-modifying proteins, and motor proteins (Welf and Haugh, 2011). In particular, several studies have highlighted the crucial role of members of the Rho GTPase family, notably Rac1, RhoA, and Cdc42, in the intracellular pathways regulating cell motility. CdC42 is involved in filopodia formation regulating the direction of migration, Rac interacts with the actin network regulating lamellipodia formations and membrane ruffles formation, while Rho interacts with myosin and helps the formation of stress fibers and focal adhesion (Raftopoulou and Hall, 2004). The presence of Rho GTPases in mussels have been demonstrated by previous works (Granger Joly de Boissel et al., 2017; Huang et al., 2022; Leprêtre et al., 2019; Li et al., 2015; Welf and Haugh, 2011) and Rho genes in bivalves have shown to exhibit great conservation than those in any other invertebrate (Li et al., 2015). Therefore, it is reasonable to argue that these signaling proteins can be involved also in Mytilus hemocyte motility. Considering the crucial role of Rho GTPases in controlling the signal transduction pathways that link cell surface receptors to a variety of intracellular responses including motility, it is possible to hypothesize that paracetamol exposure could interfere on upstream signals that could in turn influence the activity of Rho GTPases and the motility control in Mytilus hemocytes. Considering the different effects exerted by paracetamol exposure on the motility of the two different hemocyte morphotypes, the interference of paracetamol could involve the interaction with different upstream targets in the two cell types. The two motility parameters analyzed, velocity and directness, exhibited different sensitivity to the drug suggesting separate regulatory mechanisms on which the drug could act differently. In the case of directness, it was slightly inhibited in spread cells after 24h exposure while it was markedly increased in round shaped cells after 24h suggesting a possible different modulation on Cdc42 which is known to play a critical role in controlling the directionality of cell migration (Allen et al., 1998; Hind et al., 2014; Li et al.,

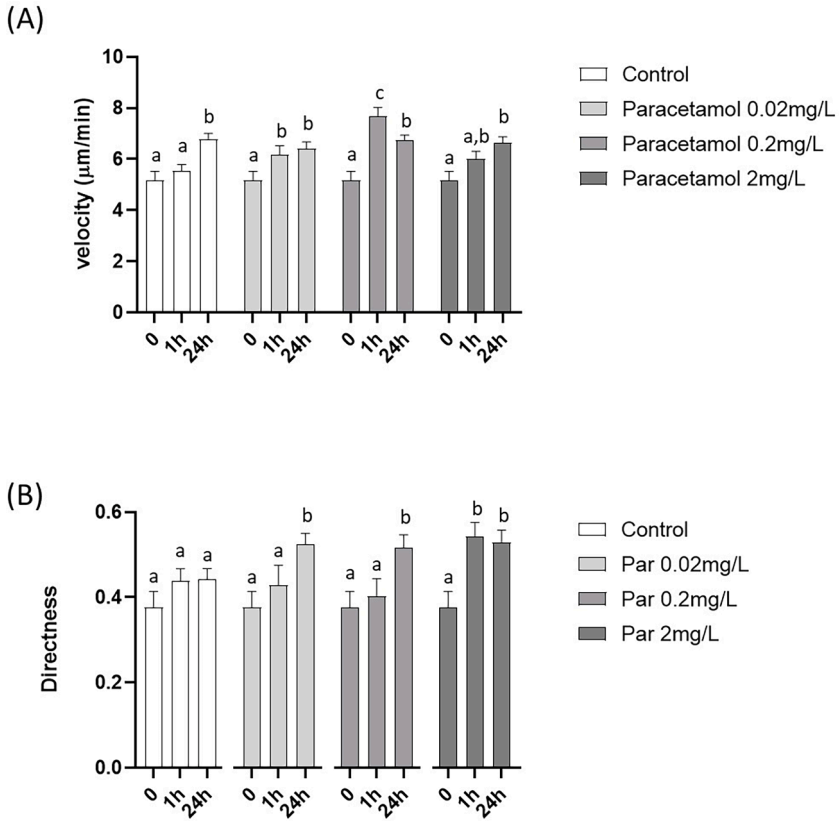


Fig. 7. (A,B) Velocity (expressed as µm min^{-1}) (A) and directness (expressed as the ratio between migrated distance and euclidean distance) (B) of round-star-shaped hemocytes after 1h (A,B) and 24h (C,D) exposure to paracetamol. Data are expressed as mean ± SEM. The statistical significance of data was assessed by one way ANOVA and Tukey's post test. Different letters were used to show statistical significance ($p < 0.05$).

2003; Srinivasan et al., 2003).

Moreover, the effects on both cell types were more marked after 24h exposure compared to 1h suggesting a time-dependence of the drug effects. Currently, paracetamol is considered a multidirectional drug, and several metabolic pathways are involved in its analgesic and antipyretic action: inhibition of cyclooxygenases, and involvement in the endocannabinoid system and serotonergic pathways (Mallet et al., 2023). Additionally, paracetamol influences transient receptor potential (TRP) channels and voltage-gated Kv7 potassium channels and inhibits T-type Cav3.2 calcium channels. It also exerts an impact on L-arginine in the nitric oxide (NO) synthesis pathway (Mallet et al., 2023). Future works will be addressed to investigate the specific molecular mechanisms underlying the effects observed on *M. galloprovincialis* hemocytes, the cell type specificity of the response, and its time dependence.

5. Conclusions

In conclusion, the obtained results contributed (a) to better characterize the motility of hemocytes in the sentinel organism *M. galloprovincialis* classifying two different hemocyte morphotypes on the basis of their velocimetric characteristics of the cells and (b) to increase the knowledge of the sensitivity of hemocyte motility to environmental chemical pollutants detecting significant effects of the *in vitro* exposure to paracetamol in terms of alteration in velocity and trajectories of the movements of the cells. The effects were cell type-specific suggesting the interaction with cell type-specific targets.

With future considerations, the study of hemocyte movements at the single cell level by cell tracking and velocimetric parameters analysis offers new sensitive tools for assessing the impacts of emerging pollutants at cellular levels in non-target organisms. This opens new perspectives for the development of novel biomarkers and/or bioassays suitable for environmental biomonitoring.

Ethics statement

M. galloprovincialis is not considered an endangered or protected species in any international species catalog and not included in the list of species regulated by EC Directive 2010/63/EU. No specific authorization is required to work on mussel samples.

CRediT authorship contribution statement

Gayatri Udayan: Conceptualization, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Maria Elena Giordano:** Investigation, Methodology, Writing – review & editing. **Patrizia Pagliara:** Conceptualization, Investigation, Writing – review & editing. **Maria Giulia Lionetto:** Conceptualization, Funding acquisition, Investigation, Supervision, Writing – original draft, Writing – review & editing.

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.

Data availability

Data will be made available on request.

Acknowledgments

This research was funded by the project "Dipartimento di Eccellenza" awarded to DiSTeBA by the Italian Ministry of University and Research, CUP: F85D18000130001 and by NBFC (National Biodiversiy Future Center) funded by European Union NextGenerationEU, PNRR, project n. CN00000033.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106779.

References

Allen, W.E., Zicha, D., Ridley, A.J., Jones, G.E., 1998. A role for Cdc42 in macrophage chemotaxis. J. Cell. Biol. 141, 1147–1157. https://doi.org/10.1083/jcb.141.5.1147.

Andreyeva, A.Y., Efremova, E.S., Kukhareva, T.A., 2019. Morphological and functional characterization of hemocytes in cultivated mussel (*Mytilus galloprovincialis*) and effect of hypoxia on hemocyte parameters. Fish Shellfish Immunol. 89, 361–367. https://doi.org/10.1016/j.fsi.2019.04.017.

Auguste, M., Balbi, T., Ciacci, C., Canonico, B., Papa, S., Borello, A., Vezzulli, L., Canesi, L., 2020. Shift in immune parameters after repeated exposure to nanoplastics in the marine bivalve Mytilus. Front. Immunol. 11, 426. https://doi.org/10.3389/fimmu.2020.00426.

Auguste, M., Mayall, C., Barbero, F., Hočevar, M., Alberti, S., Grassi, G., Puntes, V.F., Drobne, D., Canesi, L., 2021. Functional and morphological changes induced in Mytilus hemocytes by selected nanoparticles. Nanomaterials 11, 470. https://doi.org/10.3390/nano11020470.

Barrick, A., Guillet, C., Mouneyrac, C., Châtel, A., 2018. Investigating the establishment of primary cultures of hemocytes from *Mytilus edulis*. Cytotechnology 70, 1205–1220. https://doi.org/10.1007/s10616-018-0212-x.

Beninger, P.G., Le Pennec, M., 2016. Scallop structure and function. Developments in Aquaculture and Fisheries Science. Elsevier, pp. 85–159. https://doi.org/10.1016/B978-0-444-62710-0.00003-1.

Benotti, M.J., Brownawell, B.J., 2007. Distributions of pharmaceuticals in an urban estuary during both dry- and wet-weather conditions. Environ. Sci. Technol. 41, 5795–5802. https://doi.org/10.1021/es0629965.

Burgos-Aceves, M.A., Faggio, C., 2017. An approach to the study of the immunity functions of bivalve haemocytes: physiology and molecular aspects. Fish. Shellfish Immunol. 67, 513–517. https://doi.org/10.1016/j.fsi.2017.06.042.

Cajaraville, M.P., Olabarrieta, I., Marigomez, I., 1996. *In vitro* activities in Mussel hemocytes as biomarkers of environmental quality: a case study in the Abra Estuary (Biscay Bay). Ecotoxicol. Environ. Saf. 35, 253–260. https://doi.org/10.1006/eesa.1996.0108.

Cajaraville, M.P., Pal, S.G., 1995. Morphofunctional study of the haemocytes of the bivalve mollusc Mytilus galloprovincialis with emphasis on the endolysosomal compartment. Cell. Struct. Funct. 20, 355–367. https://doi.org/10.1247/csf.20.355.

Calisi, A., Giordano, M.E., Dondero, F., Maisano, M., Fasulo, S., Lionetto, M.G., 2023. Morphological and functional alterations in hemocytes of *Mytilus galloprovincialis* exposed in high-impact anthropogenic sites. Mar. Environ. Res. 188, 105988 https://doi.org/10.1016/j.marenvres.2023.105988.

Calisi, A., Lionetto, M.G., Caricato, R., Giordano, M.E., Schettino, T., 2008. Morphometric alterations in *Mytilus galloprovincialis* granulocytes: a new biomarker. Environ. Toxicol. Chem. 27, 1435–1441. https://doi.org/10.1897/07-396.

Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., Gallo, G., 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. Mar. Environ. Res. 76, 16–21. https://doi.org/10.1016/j.marenvres.2011.06.005. Emerging and persistent impacts on Marine Organisms: Detection methods and action mechanisms.

Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Rocchi, M., Pojana, G., Marcomini, A., 2007. Immunomodulation of *Mytilus* hemocytes by individual estrogenic chemicals and environmentally relevant mixtures of estrogens: *in vitro* and *in vivo* studies. Aquat. Toxicol. 81, 36–44. https://doi.org/10.1016/j.aquatox.2006.10.010.

Carballal, Mj, Mc, L., C, A., A, V., 1997. Hemolymph cell types of the mussel *Mytilus galloprovincialis*. Dis. Aquat. Org. 29, 127–135. https://doi.org/10.3354/dao029127.

Caricato, R., Giordano, M.E., Schettino, T., Maisano, M., Mauceri, A., Giannetto, A., Cappello, T., Parrino, V., Ancora, S., Caliani, I., Bianchi, N., Leonzio, C., Mancini, G., Cappello, S., Fasulo, S., Lionetto, M.G., 2019. Carbonic anhydrase integrated into a multimarker approach for the detection of the stress status induced by pollution exposure in *Mytilus galloprovincialis:* a field case study. Sci. Total Environ. 690, 140–150. https://doi.org/10.1016/j.scitotenv.2019.06.446.

Chang, H.-I., Wang, Y., 2011. Cell responses to surface and architecture of tissue engineering scaffolds. Regenerative Medicine and Tissue Engineering - Cells and Biomaterials. InTech. https://doi.org/10.5772/21983.

Costa, M.M., Prado-Alvarez, M., Gestal, C., Li, H., Roch, P., Novoa, B., Figueras, A., 2009. Functional and molecular immune response of Mediterranean mussel (*Mytilus galloprovincialis*) haemocytes against pathogen-associated molecular patterns and bacteria. Fish Shellfish Immunol. 26, 515–523. https://doi.org/10.1016/j.fsi.2009.02.001.

Fairbairn, D.J., Karpuzcu, M.E., Arnold, W.A., Barber, B.L., Kaufenberg, E.F., Koskinen, W.C., Novak, P.J., Rice, P.J., Swackhamer, D.L., 2016. Sources and transport of contaminants of emerging concern: a two-year study of occurrence and spatiotemporal variation in a mixed land use watershed. Sci. Total Environ. 551–552, 605–613. https://doi.org/10.1016/j.scitotenv.2016.02.056.

Garcia, S.N., Foster, M., Constantine, L.A., Huggett, D.B., 2012. Field and laboratory fish tissue accumulation of the anti-convulsant drug carbamazepine. Ecotoxicol. Environ. Saf. 84, 207–211. https://doi.org/10.1016/j.ecoenv.2012.07.013.

Gaw, S., Thomas, K.V., Hutchinson, T.H., 2014. Sources, impacts and trends of pharmaceuticals in the marine and coastal environment. Philosoph. Trans. R. Soc. B Biol. Sci. 369, 20130572 https://doi.org/10.1098/rstb.2013.0572.

Gendre, H., Palos Ladeiro, M., Geffard, A., Poret, A., Le Foll, F., Ben Cheikh, Y., 2023. Modulation of haemocyte motility by chemical and biological stresses in *Mytilus edulis* and Dreissena polymorpha. Fish Shellfish Immunol. 139, 108919 https://doi.org/10.1016/j.fsi.2023.108919.

Giannapas, M., Karnis, L., Dailianis, S., 2012. Generation of free radicals in haemocytes of mussels after exposure to low molecular weight PAH components: immune activation, oxidative and genotoxic effects. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 155, 182–189. https://doi.org/10.1016/j.cbpc.2011.08.001.

Goddard, J.M., Hotchkiss, J.H., 2007. Polymer surface modification for the attachment of bioactive compounds. Prog. Polym. Sci. 32, 698–725. https://doi.org/10.1016/j.progpolymsci.2007.04.002.

Graham, D.B., Zinselmeyer, B.H., Mascarenhas, F., Delgado, R., Miller, M.J., Swat, W., 2009. ITAM signaling by Vav family rho guanine nucleotide exchange factors regulates interstitial transit rates of neutrophils *in vivo*. PLOS One 4, e4652. https://doi.org/10.1371/journal.pone.0004652.

Granger Joly de Boissel, P., Fournier, M., Rodriguez-Lecompte, J.C., McKenna, P., Kibenge, F., Siah, A., 2017. Functional and molecular responses of the blue mussel *Mytilus edulis*' hemocytes exposed to cadmium - An *in vitro* model and transcriptomic approach. Fish Shellfish Immunol. 67, 575–585. https://doi.org/10.1016/j.fsi.2017.06.001.

Graziano, B.R., Weiner, O.D., 2014. Self-organization of protrusions and polarity during eukaryotic chemotaxis. Curr. Opin. Cell Biol. 0, 60–67. https://doi.org/10.1016/j.ceb.2014.06.007.

Hind, L.E., Mackay, J.L., Cox, D., Hammer, D.A., 2014. Two-dimensional motility of a macrophage cell line on microcontact-printed fibronectin. Cytoskeleton 71, 542–554. https://doi.org/10.1002/cm.21191 (Hoboken).

Huang, J., Huang, P., Lu, J., Wu, N., Lin, G., Zhang, X., Cao, H., Geng, W., Zhai, B., Xu, C., Sun, Z., 2022. Gene expression profiles provide insights into the survival strategies in deep-sea mussel (*Bathymodiolus platifrons*) of different developmental stages. BMC Genom. 23, 311. https://doi.org/10.1186/s12864-022-08505-9.

Jerison, E.R., Quake, S.R., 2020. Heterogeneous T cell motility behaviors emerge from a coupling between speed and turning *in vivo*. eLife 9, e53933. https://doi.org/10.7554/eLife.53933.

Kaloyianni, M., Dailianis, S., Chrisikopoulou, E., Zannou, A., Koutsogiannaki, S., Alamdari, D.H., Koliakos, G., Dimitriadis, V.K., 2009. Oxidative effects of inorganic and organic contaminants on haemolymph of mussels. Compar. Biochem. Physiol. Part C Toxicol. Pharmacol. 149, 631–639. https://doi.org/10.1016/j.cbpc.2009.01.006.

Katsumiti, A., Losada-Carrillo, M.P., Barros, M., Cajaraville, M.P., 2021. Polystyrene nanoplastics and microplastics can act as Trojan horse carriers of benzo(a)pyrene to mussel hemocytes *in vitro*. Sci. Rep. 11, 22396. https://doi.org/10.1038/s41598-021-01938-4.

Koagouw, W., Stewart, N.A., Ciocan, C., 2021. Long-term exposure of marine mussels to paracetamol: is time a healer or a killer? Environ. Sci. Pollut. Res. 28, 48823–48836. https://doi.org/10.1007/s11356-021-14136-6.

Le Foll, F., Rioult, D., Boussa, S., Pasquier, J., Dagher, Z., Leboulenger, F., 2010. Characterisation of *Mytilus edulis* hemocyte subpopulations by single cell time-lapse motility imaging. Fish Shellfish Immunol. 28, 372–386. https://doi.org/10.1016/j.fsi.2009.11.011.

Leprêtre, M., Almunia, C., Armengaud, J., Salvador, A., Geffard, A., Palos-Ladeiro, M., 2019. The immune system of the freshwater zebra mussel, *Dreissena polymorpha*, decrypted by proteogenomics of hemocytes and plasma compartments. J. Proteomics 202, 103366. https://doi.org/10.1016/j.jprot.2019.04.016.

Li, H., Parisi, M.-G., Toubiana, M., Cammarata, M., Roch, P., 2008. Lysozyme gene expression and hemocyte behaviour in the Mediterranean mussel, *Mytilus galloprovincialis,* after injection of various bacteria or temperature stresses. Fish Shellfish Immunol. 25, 143–152. https://doi.org/10.1016/j.fsi.2008.04.001.

Li, X., Wang, R., Xun, X., Jiao, W., Zhang, M., Wang, Shuyue, Wang, Shi, Zhang, L., Huang, X., Hu, X., Bao, Z., 2015. The Rho GTPase family genes in bivalvia genomes: sequence, evolution and expression analysis. PLOS One 10, e0143932. https://doi.org/10.1371/journal.pone.0143932.

Li, Z., Hannigan, M., Mo, Z., Liu, B., Lu, W., Wu, Y., Smrcka, A.V., Wu, G., Li, L., Liu, M., Huang, C.-K., Wu, D., 2003. Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. Cell 114, 215–227. https://doi.org/10.1016/s0092-8674(03)00559-2.

Lionetto, F., Lionetto, M.G., Mele, C., Corcione, C.E., Bagheri, S., Udayan, G., Maffezzoli, A., 2022. Autofluorescence of model polyethylene terephthalate nanoplastics for cell interaction studies. Nanomaterials 12, 1560. https://doi.org/10.3390/nano12091560.

Lionetto, M.G., Caricato, R., Giordano, M.E., 2021. Pollution biomarkers in the framework of marine biodiversity conservation: state of art and perspectives. Water 13, 1847. https://doi.org/10.3390/w13131847 (Basel).

Lionetto, M.G., Maffia, M., Cappello, M.S., Giordano, M.E., Storelli, C., Schettino, T., 1998. Effect of cadmium on carbonic anhydrase and Na+-K+-ATPase in eel, *Anguilla anguilla*, intestine and gills. Compar. Biochem. Physiol. Part A Mol. Integr. Physiol. 120, 89–91. https://doi.org/10.1016/S1095-6433(98)10014-4.

Mallet, C., Desmeules, J., Pegahi, R., Eschalier, A., 2023. An updated review on the metabolite (AM404)-mediated central mechanism of action of paracetamol

(Acetaminophen): experimental evidence and potential clinical impact. J. Pain Res. 16, 1081–1094. https://doi.org/10.2147/JPR.S393809.
Martinez, A.S., Underwood, T., Christofoletti, R.A., Pardal, A., Fortuna, M.A., Marcelo-Silva, J., Morais, G.C., Lana, P.C., 2022. Reviewing the effects of contamination on the biota of Brazilian coastal ecosystems: scientific challenges for a developing country in a changing world. Sci. Total Environ. 803, 150097 https://doi.org/10.1016/j.scitotenv.2021.150097.
Novas, A., Barcia, R., Ramos-Martínez, J.I., 2007. Nitric oxide production by haemocytes from *Mytilus galloprovincialis* shows seasonal variations. Fish Shellfish Immunol. 23, 886–891. https://doi.org/10.1016/j.fsi.2007.04.007.
Parrino, V., Costa, G., Cannavà, C., Fazio, E., Bonsignore, M., Concetta, S., Piccione, G., Fazio, F., 2019. Flow cytometry and micro-Raman spectroscopy: identification of hemocyte populations in the mussel *Mytilus galloprovincialis* (Bivalvia: mytilidae) from Faro Lake and Tyrrhenian Sea (Sicily, Italy). Fish Shellfish Immunol. 87, 1–8. https://doi.org/10.1016/j.fsi.2018.12.067.
Pereira, A.M.P.T., Silva, L.J.G., Lino, C.M., Meisel, L.M., Pena, A., 2016. Assessing environmental risk of pharmaceuticals in Portugal: an approach for the selection of the Portuguese monitoring stations in line with Directive 2013/39/EU. Chemosphere 144, 2507–2515. https://doi.org/10.1016/j.chemosphere.2015.10.100.
Petrie, R.J., Doyle, A.D., Yamada, K.M., 2009. Random *versus* directionally persistent cell migration. Nat. Rev. Mol. Cell Biol. 10, 538–549. https://doi.org/10.1038/nrm2729.
Pizzagalli, D.U., Pulfer, A., Thelen, M., Krause, R., Gonzalez, S.F., 2021. *In vivo* motility patterns displayed by immune cells under inflammatory conditions. Front. Immunol. 12, 804159 https://doi.org/10.3389/fimmu.2021.804159.
Raftopoulou, M., Hall, A., 2004. Cell migration: rho GTPases lead the way. Dev. Biol. 265, 23–32. https://doi.org/10.1016/j.ydbio.2003.06.003.
Rioult, D., Lebel, J.-M., Le Foll, F., 2013. Cell tracking and velocimetric parameters analysis as an approach to assess activity of mussel (*Mytilus edulis*) hemocytes *in vitro*. Cytotechnology 65, 749–758. https://doi.org/10.1007/s10616-013-9558-2.
Rosner, A., Ballarin, L., Barnay-Verdier, S., Borisenko, I., Drago, L., Drobne, D., Concetta Eliso, M., Harbuzov, Z., Grimaldi, A., Guy-Haim, T., Karahan, A., Lynch, I., Giulia Lionetto, M., Martinez, P., Mehennaoui, K., Oruc Ozcan, E., Pinsino, A., Paz, G., Rinkevich, B., Spagnuolo, A., Sugni, M., Cambier, S., 2023. A broad-taxa approach as an important concept in ecotoxicological studies and pollution monitoring. Biol. Rev. https://doi.org/10.1111/brv.13015.
Sasaki, A.T., Janetopoulos, C., Lee, S., Charest, P.G., Takeda, K., Sundheimer, L.W., Meili, R., Devreotes, P.N., Firtel, R.A., 2007. G protein–independent Ras/PI3K/F-actin circuit regulates basic cell motility. J. Cell Biol. 178, 185–191. https://doi.org/10.1083/jcb.200611138.
Sendra, M., Saco, A., Yeste, M.P., Romero, A., Novoa, B., Figueras, A., 2020. Nanoplastics: from tissue accumulation to cell translocation into *Mytilus galloprovincialis* hemocytes. Resilience of immune cells exposed to nanoplastics and nanoplastics plus Vibrio splendidus combination. J. Hazard. Mater. 388, 121788 https://doi.org/10.1016/j.jhazmat.2019.121788.
SenGupta, S., Parent, C.A., Bear, J.E., 2021. The principles of directed cell migration. Nat. Rev. Mol. Cell Biol. 22, 529–547. https://doi.org/10.1038/s41580-021-00366-6.
Srinivasan, S., Wang, F., Glavas, S., Ott, A., Hofmann, F., Aktories, K., Kalman, D., Bourne, H.R., 2003. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. J. Cell Biol. 160, 375–385. https://doi.org/10.1083/jcb.200208179.
Stuer-Lauridsen, F., Birkved, M., Hansen, L.P., Lützhøft, H.C., Halling-Sørensen, B., 2000. Environmental risk assessment of human pharmaceuticals in Denmark after normal therapeutic use. Chemosphere 40, 783–793. https://doi.org/10.1016/s0045-6535(99)00453-1.
Togola, A., Budzinski, H., 2008. Multi-residue analysis of pharmaceutical compounds in aqueous samples. J. Chromatogr. A 1177, 150–158. https://doi.org/10.1016/j.chroma.2007.10.105.
Vesperini, D., Montalvo, G., Qu, B., Lautenschläger, F., 2021. Characterization of immune cell migration using microfabrication. Biophys. Rev. 13, 185–202. https://doi.org/10.1007/s12551-021-00787-9.
Vogler, E.A., 1999. Water and the acute biological response to surfaces. J. Biomater. Sci. Polym. Ed. 10, 1015–1045. https://doi.org/10.1163/156856299x00667.
Wang, J., Gardinali, P.R., 2013. Uptake and depuration of pharmaceuticals in reclaimed water by mosquito fish (*Gambusia holbrooki*): a worst-case, multiple-exposure scenario. Environ. Toxicol. Chem. 32, 1752–1758. https://doi.org/10.1002/etc.2238.
Warwick, C., 2008. Paracetamol and fever management. J. R. Soc. Promot. Health 128, 320–323. https://doi.org/10.1177/1466424008092794.
Welf, E.S., Haugh, J.M., 2011. Signaling pathways that control cell migration: models and analysis. Wiley Interdiscip. Rev. Syst. Biol. Med. 3, 231–240. https://doi.org/10.1002/wsbm.110.
Wilkinson, J.L., Boxall, A.B.A., Kolpin, D.W., Leung, K.M.Y., Lai, R.W.S., Galbán-Malagón, C., Adell, A.D., Mondon, J., Metian, M., Marchant, R.A., Bouzas-Monroy, A., Cuni-Sanchez, A., Coors, A., Carriquiriborde, P., Rojo, M., Gordon, C., Cara, M., Moermond, M., Luarte, T., Petrosyan, V., Perikhanyan, Y., Mahon, C.S., McGurk, C.J., Hofmann, T., Kormoker, T., Iniguez, V., Guzman-Otazo, J., Tavares, J. L., Gildasio De Figueiredo, F., Razzolini, M.T.P., Dougnon, V., Gbaguidi, G., Traoré, O., Blais, J.M., Kimpe, L.E., Wong, M., Wong, D., Ntchantcho, R., Pizarro, J., Ying, G.-G., Chen, C.-E., Páez, M., Martínez-Lara, J., Otamonga, J.-P., Poté, J., Ifo, S. A., Wilson, P., Echeverría-Sáenz, S., Udikovic-Kolic, N., Milakovic, M., Fatta-Kassinos, D., Ioannou-Ttofa, L., Belušová, V., Vymazal, J., Cárdenas-Bustamante, M., Kassa, B.A., Garric, J., Chaumot, A., Gibba, P., Kunchulia, I., Seidensticker, S., Lyberatos, G., Halldórsson, H.P., Melling, M., Shashidhar, T., Lamba, M., Nastiti, A., Supriatin, A., Pourang, N., Abedini, A., Abdullah, O., Gharbia, S.S., Pilla, F., Chefetz, B., Topaz, T., Yao, K.M., Aubakirova, B., Beisenova, R., Olaka, L., Mulu, J. K., Chatanga, P., Ntuli, V., Blama, N.T., Sherif, S., Aris, A.Z., Looi, L.J., Niang, M., Traore, S.T., Oldenkamp, R., Ogunbanwo, O., Ashfaq, M., Iqbal, M., Abdeen, Z., O'Dea, A., Morales-Saldaña, J.M., Custodio, M., de la Cruz, H., Navarrete, I., Carvalho, F., Gogra, A.B., Koroma, B.M., Cerkvenik-Flajs, V., Gombač, M., Thwala, M., Choi, K., Kang, H., Ladu, J.L.C., Rico, A., Amerasinghe, P., Sobek, A., Horlitz, G., Zenker, A.K., King, A.C., Jiang, J.-J., Kariuki, R., Tumbo, M., Tezel, U., Onay, T.T., Lejju, J.B., Vystavna, Y., Vergeles, Y., Heinzen, H., Pérez-Parada, A., Sims, D.B., Figy, M., Good, D., Teta, C., 2022. Pharmaceutical pollution of the world's rivers. Proc. Natl. Acad. Sci. U. S. A. 119, e2113947119 https://doi.org/10.1073/pnas.2113947119.
Xu, L.-C., Siedlecki, C.A., 2007. Effects of surface wettability and contact time on protein adhesion to biomaterial surfaces. Biomaterials 28, 3273–3283. https://doi.org/10.1016/j.biomaterials.2007.03.032.
Zhao, H., Traganos, F., Dobrucki, J., Wlodkowic, D., Darzynkiewicz, Z., 2009. Induction of DNA damage response by the supravital probes of nucleic acids. Cytometry A 75, 510–519. https://doi.org/10.1002/cyto.a.20727.
Ziebert, F., Aranson, I., 2016. Computational approaches to substrate-based cell motility. NPJ Comput. Mater. 2, 16019.