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Highlights

- Cell detachment enhances the representativity of the analyzed cell population
- The triple labeling procedure improves the accuracy of measured endpoints
- Hemocyte adhesion ability is used to select functional cells for further analysis
- Hemocyte adhesion is a key property to consider before flow analyses

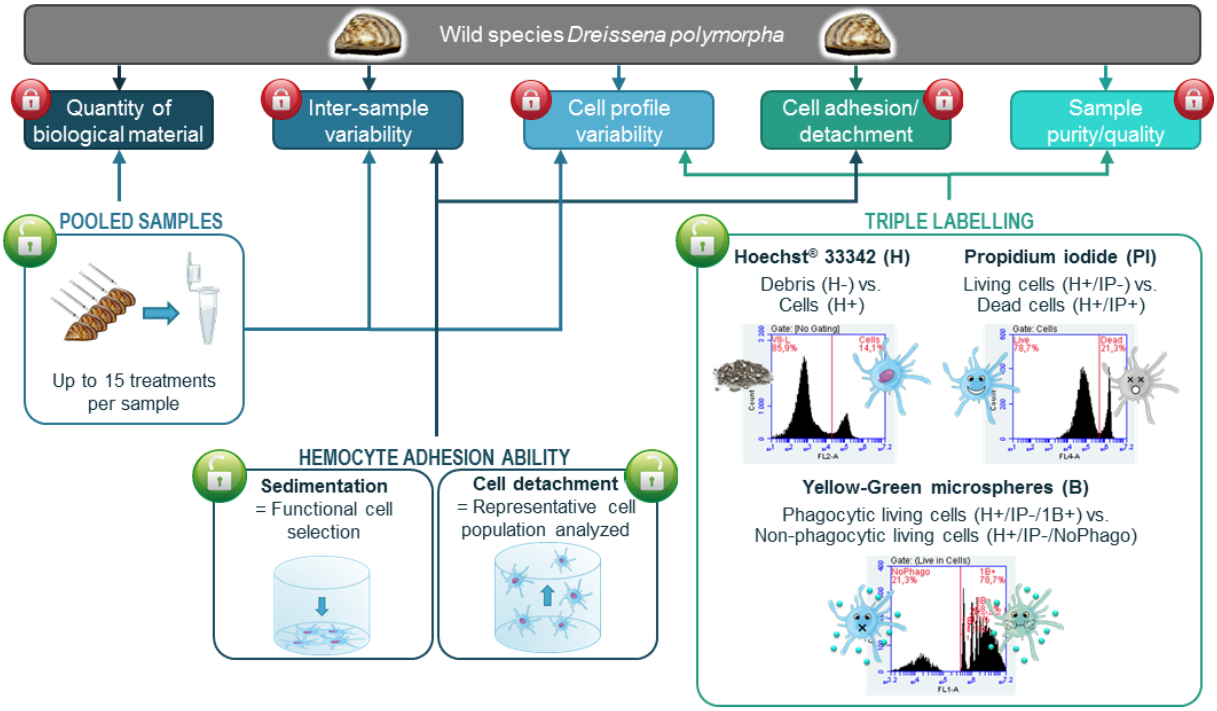
Abstract

Immunotoxicity analysis receives a strong interest in environmental *a priori* and *a posteriori* risk assessment procedures considering the direct involvement of the immune system in the health status of organisms, populations and thus ecosystems. The freshwater mussel *Dreissena polymorpha* is an invasive species widely used in ecotoxicology studies and biomonitoring surveys to evaluate the impacts of contaminants on aquatic fauna. Bivalve hemocytes are the immunocompetent cells circulating in the open circulatory system of the organism. However, there is nowadays no consensus on a protocol to evaluate the immunocompetent state of this particular cell type using flow cytometry. Wild species such as *D. polymorpha* present several technical barriers complicating their analyze including (i) the quality and the purity of the hemolymph sample, (ii) the controversial characterization of hemocyte subpopulations and their diversity, (iii) the quantity of biological material, and (iv) the high inter-individual variability of hemocyte responses. The present work proposes several technical and analytical improvements to control the above-mentioned issues. The inclusion of sedimentation and cell detachment steps in the pre-analytical phase of the protocol substantially ameliorate the quality of the hemolymph sample as well as the accuracy of the cytometric measurements, by selecting the analyzed cells on their adhesion ability and by increasing the concentration of the analyzed events. The development of an effective triple-labeling procedure including the cellular probe Hoechst® 33342, the membrane impermeant dye propidium iodide and yellow-green fluorescent microspheres allowed the simultaneous analysis of cytotoxicity and phagocytosis activity in hemocytes. It also significantly enhanced the accuracy of hemocyte endpoint measurements by eliminating non-target events from the analysis and allowing relevant gating strategies. Finally, the use of pooled samples of hemolymph noticeably reduced inter-sample variability while providing more plasticity in the experimental design and improving the discriminating potency between treatments. The developed protocol is suitable for *ex vivo* exposure of hemocyte in a chemical/environmental toxicity assessment as well as for *in vivo* exposure in the laboratory or *in situ* biomonitoring surveys with few adaptations.

Keywords:

Immune cells; wild species; triple labeling; bivalve; phagocytosis; adhesion

Graphical Abstract



1 Introduction

The evaluation of immunotoxicity is an important aspect of chemical hazard evaluation and environmental risk assessment processes. Several regulations support this status considering that immune system suppression has been associated with increased incidences of infections and neoplasia (e.g. European Commission (EC), 2006; US EPA, 2007). In invertebrates, which represent more than 90% of the extant aquatic species (Binelli et al., 2009), the immune defense system exclusively relies on innate humoral and cellular strategies principally ensured by the hemocytes, circulating cells in the hemolymph with high tissue infiltration capacities (Tryphonas et al., 2005; Le Foll et al., 2010). The open circulatory system of most of the invertebrates including bivalves implies that the hemolymph, and thus hemocytes, are directly exposed to environmental stressors or contaminants, and play a physiological role in toxic material transport as well as in defense mechanisms (Mersch et al., 1996). Evaluation of critical functions of the immune status in bivalves can thus help to determine the relationship between sublethal and/or chronic exposure to environmental contaminants and immunotoxicological effects (Blaise et al., 2002; Luengen et al., 2004).

The advantage of 'environmental sentinel' species in ecotoxicological studies is well recognized in the scientific community considering they can more reliably reflect natural ecosystem health status when compared to laboratory model organisms. Bivalves are used for several decades as valuable organisms in such context focused on aquatic environment considering their sedentariness, their large geographical distribution, their high filtering capacity, and their ability to accumulate and concentrate contaminants in a time- and dose-dependent manner (Ladeiro et al., 2017; Zuykov et al., 2013). As a result, several studies can be gathered from the literature demonstrating the sensitivity of immune-relative functions of bivalve hemocytes to environmental contaminants following *ex vivo, in vivo* and *in situ* exposures, highlighting their particular interest in the context of both (eco)toxicological studies and biomonitoring surveys (e.g. Brousseau et al., 1999; Fournier et al., 2000; Luengen et al., 2004; Tryphonas et al., 2005; Villela et al., 2006; Evariste et al., 2017).

Among bivalves, the zebra mussel *Dreissena polymorpha* has already demonstrated its relevance in such context and can nowadays be considered as the counterpart of *Mytilus* species for inland waters (e.g. Binelli et al., 2014; Palos Ladeiro et al., 2017). However, the use of wild species raises technical obstacles that must be unlocked, or at least controlled, before their proper use in such strategies. The limited knowledge about the immune physiology of some environmental species such as dreissenids (in comparison to laboratory model organisms) may represent a source of confusion and a barrier for the interpretation of the biological responses following exposure to contaminants. In particular, the incomplete characterization of their immune system limits the understanding and the control of the variability of the responses and the cellular profiles. The open circulatory system of bivalves often results in the omnipresence of undesirable material such as debris, impurities and non-target exogenous or endogenous cells in *D. polymorpha* hemolymph samples that strongly impact sample quality and make analyses even more complicated. There are no specific markers of *D. polymorpha* hemocytes available nowadays to circumvent this difficulty. The relatively small size of adult zebra mussels (20-40 mm, in comparison

to *Mytilus edulis*, up to 10 cm) limits the quantity of hemolymph that can be sampled (Jones, 1983), restricting the number of analyses performed and/or the experimental design (i.e. number of conditions tested per sample). Additionally, the adhesion and aggregation properties of hemocytes are often ignored in experimental and analytic procedures while they can be important factors to consider notably during flow analyses.

Here, we propose a protocol to perform the simultaneous analysis of cytotoxicity and phagocytosis activity on *D. polymorpha* hemocytes using flow cytometry. This technic provides the advantages of being rapid and allowing the immediate and quantitative acquisition of multiple morphological and functional endpoints on single cells. Flow cytometric analyses already proved to be a performant tool successfully applied to the study of bivalve immune system following *in vivo* or *ex vivo* exposures as well as in a physiological context (Goedken and De Guise, 2004; Le Foll et al., 2010; Rioult et al., 2014; Evariste et al., 2016, 2017, 2018). The present study focuses on several practical improvements in hemocyte suspension preparation and cytometric analysis (labeling and gating strategy) to overcome the above-mentioned technical barriers. The main objective is to result in an optimized protocol that can be routinely used for *ex vivo* exposure (i.e. chemical or environmental sample testing) with a possible adaptation to *in vivo* experiments (in the laboratory or in the field). The enhancements of the protocol focus on (i) the sample quality improvement, (ii) the optimization of the analyses performed regarding the quantity of available biological material, (iii) a better control of inter-sample variability (in terms of biological response and cellular profiles), and (iv) the proper consideration of the adhesion/detachment of hemocytes within the experimental procedures.

2 Materials and Methods

2.1 *Mussel origin and hemolymph collection*

Zebra mussels (1.8–2.5 cm length) were collected at the Lac du Der (Marne, France) between March and May 2016, and kept at least two weeks for acclimation in the laboratory before use. Mussels were maintained in aerated 30 L-tanks (250-300 indiv./tank) filled with 4.5 L of natural spring water (spring Aurèle, Cristalline) and equipped with a filtration system (TC-200, Aquatlantis). Water was 100 %-renewed weekly. Organisms were kept at 14 ± 1 °C in the dark and fed twice a week with fresh algae (*Chorella vulgaris* and *Scenedesmus obliquus*; 50:50) to reach a total feed supply rate of 2×10^6 cell.indiv^{-1}.j^{-1}.

Hemolymph was withdrawn from mussel posterior muscle using 0.3 mL-insulin syringes fitted with 29G × 1/2" needles (Myjector®, Terumo®). Syringes were rinsed using modified Leibovitz's L15-medium (L15-15%⊕: 15% (v/v) L15 medium, 85% (v/v) distilled water, phenol red 0.03 mM, 10 mM HEPES, 2 mM L-Glutamine, 0.1 mg.mL^{-1} Streptomycin, 100 U.mL^{-1} Penicillin, pH 7.5, 0.2 µm-filtered; Evariste et al. (Evariste et al., 2016)) and filled with 50 µL of medium before hemolymph sampling. Hemolymph samples can be individual or pooled samples from four or five mussels. Samples were then stored in sterile microtubes in a rack previously placed on ice. Cell count was performed on each sample using KOVA® Glasstic® slides with grids (Hycor Biomedical) according to the manufacturer's recommendations.

For the comparison of the results between pooled and individual samples, four individual samples of total hemolymph (i.e. hemocytes and plasma) were used to constitute a pooled sample. The adequate volume representing a quarter of the total number of cells needed (i.e. 50×10^3 cell.well^{-1}, for instance) of each individual sample was added to the pooled sample.

2.2 *Hemocyte* ex vivo *exposure*

Immediately after cell count, 50, 75 or 100×10^3 cell.well^{-1} were deposed in a 96-well microplate (U-bottom CellStar® 96 Well Culture Plate, Greiner Bio-One). Each volume was completed to 300 µL with L15-15%⊕ medium (or to 290 µL plus 10 µL of contamination solution for an exposure condition). 2.0-µm diameter yellow-green (YG) fluorescent polystyrene microspheres (Fluoresbrite® carboxylate microspheres, PolyScience) were also added with a 1:50 hemocytes-beads ratio to measure the phagocytic activity of hemocytes. Samples were conscientiously homogenized and then incubated for 4 h at 16 °C. Hemocyte mortality was then evaluated using propidium iodide (PI) solution (1.0 mg.mL^{-1}; Sigma-Aldrich) added at 1% (v/v final) just before cytometric analysis (Evariste et al., 2016, 2017).

2.3 Protocol optimized steps

2.3.1 Sedimentation step

To enhance the quality of the hemocyte deposit, a sedimentation step was realized as follows. Just after hemolymph deposition in a 96-well microplate, samples were kept for 20 min at 16 °C to allow the hemocytes to settle and adhere to the substrate (*i.e.* the bottom of the microplate well). The supernatant was removed and 190 µL of L15-15%⊕ medium were added. Then, the protocol continued as previously described in *part 2.2.*

2.3.2 Cell detachment step

A trypsin treatment was used to re-suspend hemocytes adherent to the microplate substrate at the end of the exposure period. Thus, 10 min (or 15 min when the cellular probe is used) before the end of the incubation period, each supernatant was transferred to a new 96-well microplate dedicated to cytometric analysis. Then, 100 µl of Trypsin-EDTA solution (Trypsin-EDTA 1 × solution purchased from Sigma-Aldrich and 4-fold diluted in 0.20-µm filtered demineralized water) were added in each emptied well used for cell exposure. After 10 min of incubation at room temperature, samples were thoroughly homogenized and transferred in the new microplate to obtain a total volume of 300 µL per sample containing both freshly detached hemocytes and supernatant. Afterward, dyes (PI and/or Hoechst) were added as described in *parts 2.2* and *2.3.3*.

2.3.3 Cellular probe

Hoechst® 33342 (Trihydrochloride trihydrate, ThermoFisher Scientific) is a nucleic acid stain commonly used as a vital cell-permanent nuclear counterstain emitting blue fluorescence when bound to double-strain DNA. The staining was performed on hemolymph samples adding 1% (v/v final) of Hoechst 500 µM solution (obtained from successive dilutions of the 20 mM-commercial solution in 0.20-µm filtered demineralized water) followed by 10 minutes of incubation at room temperature. This step was carried out just before PI addition before sample analysis and resulted in a triple-labeling (PI, YG beads and Hoechst) of the samples.

2.4 Flow cytometric analysis

Cytometric analyses were performed using Accuri™ C6 flow cytometer (Becton Dickinson) equipped with blue 473 nm- (on FL1 and FL3 channels) and UV 375 nm-lasers (on FL2 and FL4 channels). BD Accuri™ C6 software (v1.0.264.21) was used to analyze the data files. The hemocyte region (R1) was identified using cellular morphological parameters: forward scatter (FSC-A) for particle size and side scatter (SSC-A) for internal

complexity (Evariste et al., 2016). This region was deliberately chosen as wide enough to allow morphological modifications in the hemocyte cell population in particular due to phagocytic activity. Cellular events (H+) were identified using Hoechst® 33342 fluorescence recorded in the FL2 channel (427/10 nm filter). Hemocyte mortality (%PI+) was evaluated using PI fluorescence analyzed in the FL4 channel through a 670 LP–filter. Phagocytic parameters were determined using YG bead fluorescence in the FL1 channel equipped with a 530/30 nm filter 90% attenuated. The percentage of total phagocytic cells (%1B+) was defined as the percentage of hemocytes that engulfed one or more bead(s). The percentage of cells that engulfed three or more beads represented the percentage of efficient phagocytic cells (%3B+). The mean number of efficiently engulfed beads per cell was calculated by dividing the mean FL1 fluorescence relative to three or more beads by the mean FL1 fluorescence of one bead (Evariste et al., 2017). Each sample was analyzed for 10^4 events (or Hoechst-positive [H+] events when the cellular probe is used) in the R1 region at a flow rate of 66 µL.min^{-1}. To ensure the validity of data comparison between samples and tests during protocol optimization and validation, the fluorescence thresholds used for the analysis of each dye were defined superposing the fluorescence profiles of all the samples from the same test and kept similar for subsequent series of tests as far as possible.

2.5 Statistical analysis

Statistical analyses were conducted using Statistica 8.0 software (Statsoft, Inc.). The statistical procedure depended on the experimental design performed and the nature of the dataset. First, results were systematically tested for residual normality using Shapiro-Wilk's test with a 1%-risk.

For independent samples, homoscedasticity was tested with Brown-Forsythe's test (5% risk). The datasets which fulfill normality and homoscedasticity criteria were then analyzed with a one-way or two-way ANOVA followed by a posthoc Tukey's test ($\alpha = 0.05$). Otherwise, the Mann-Whitney U test ($\alpha = 0.05$) was used as a non-parametric alternative for two independent samples analysis.

For dependent samples, a *t*-test for dependent samples was performed to compare two samples showing normal distribution. When more than two groups were compared, repeated-measures ANOVA followed by posthoc Tukey's test was performed ($\alpha = 0.05$). Sphericity assumption was previously checked using the Mauchly test ($\alpha = 0.05$). For the comparison of two samples showing non-normal distribution, the Wilcoxon matched-pairs test ($\alpha = 0.05$) was performed.

3 Results and Discussion

3.1 Pool versus *individual samples*

One of the first obstacles that could be encountered working on *D. polymorpha* hemocytes is the quantity of biological material available per individual (from 1 to 3×10^5 cells per sample, or about 4 deposits of 75×10^3 cells at most). This can strongly limit the analysis performed on each sample and the number of tested conditions during *ex vivo* experiments. A simple way to solve these issues consists of pooling hemolymph samples from several individuals (e.g. Auffret and Oubella, 1997; Hégaret et al., 2003; Labreuche et al., 2006; Hinzmann et al., 2013; Tanguy et al., 2013). Besides it allows more flexibility in the experimental design particularly for *ex vivo* experiments, cellular profiles (SSC-FSC profiles) of pooled samples proved to be less variable than individual ones (Figure 1). The latter samples often exhibit several subpopulations of hemocytes that could be hardly identified as their shape and the position on the SSC-FSC profile could strongly vary (Figure 1A). These variations in hemocyte scattering profiles could generate an overlap with other endogenous (e.g. gill cells) or exogenous (e.g. microorganisms, algae) cells. Moreover, some cellular activities such as phagocytosis result in considerable disturbances of scattering parameters that prevent from identifying hemocyte subpopulations (Evariste et al., 2017). Cell mortality could also lead to morphological modifications which could be wrongly interpreted as a hemocyte sub-population (*Figure 2*), particularly when mortality is not simultaneously assessed. In the example proposed in *Figure 2,* cell mortality can be evaluated at 1.4% or 45.5% if the analyze focuses on P2- or P3-population, respectively. These values are both far from the average mortality of 24.7% evaluated in the global hemocyte region (R1). All these aspects could lead to variation in analyzed biological endpoints and/or skewed result interpretation considering that hemocyte functionalities are also significantly varying and are differentially modulated following chemical exposure according to the hemocyte subtype (Evariste et al., 2016, 2017). As a result, pooled samples could represent a valuable alternative in experiments where inter-individual variability may mask toxic responses following exposure dedicated to chemical or environmental samples toxicity assessment. For instance, the variability of the percentage of PI-positive events (%PI+) in the hemocyte region, evaluated with the coefficient of variation (CV), was shown to reach 53% in individual samples (N = 16) whereas a value of 26% was observed for pooled samples (N = 4) made from these individual samples (Supplemental Information *Figure S1A*). The use of pooled samples of hemolymph thus ensures that several conditions (e.g. different compounds or concentrations) could be tested with the same sample while increasing the power of discrimination between responses from various exposure conditions by limiting inter-sample variability. The major challenge of toxicity testing procedures is the global toxicity assessment of target substances, mixtures or effluents. Thus, the use of pooled samples in such an approach may not be a hindrance as it gives information on the toxic impact on the overall hemocyte population. In such kind of study, the differential toxicity among hemocyte subpopulations may represent a weaker interest in comparison to the improvement of an experimental design in which several compounds/concentrations can be tested on the same sample (thus allowing the

comparison of the toxicity among treatments). Hégaret et al. (2003) already validated the use of pools of hemolymph (instead of individual samples) to evaluate cell mortality and phagocytosis in oysters *Crassostrea virginica*. Individual samples are nonetheless of great interest in field and/or physiological studies where inter-individual variations and/or sub-population differential responses remained highly informative.

It must be noticed that the %PI+ observed in pooled samples is systematically (even insignificantly) higher than the mean value calculated from the corresponding individual samples (*Figure S1B*). This increase in cell mortality is concomitant with the non-negligible formation of cellular clumps (or small aggregates) which could be observed on the right upper part of the scatter profiles (*Figure 1B*). Cell-to-cell interactions and aggregate formation have been reported in hemocytes from various bivalve species including marine mussels and oysters. Hemocyte aggregation is known to be a spontaneous event occurring within minutes after removal of hemolymph from bivalve mollusks (Auffret and Oubella, 1997; Chen and Bayne, 1995; Le Foll et al., 2010). Le Foll et al. (Le Foll et al., 2010) demonstrated by time-lapse video microscopy that basophil hemocytes in *Mytilus edulis* species were involved in dynamic hemocyte-hemocyte interactions and the constitution of aggregate cores. Basophil (or Blast-*like*) cells have also been identified in *D. polymorpha* and share similar morphometric characteristics (Evariste et al., 2016). This may explain the systematic disappearing of this hemocyte sub-population characterized by small size (FSC) and low internal complexity (SSC) in pooled samples as shown on scatter profiles (*Figure 1B*). However, it must be emphasized that the classification of hemocyte subpopulations in bivalves remains largely controversial, impeded by a lack of consensus due to the use of different parameters and techniques to define their classification (Le Foll et al., 2010; Rebelo et al., 2013). The work of Rebelo et al. (Rebelo et al., 2013) supported the theory that the different subpopulations of hemocytes in the oyster *Crassostrea rhizophorea* are in fact different stages of one type of cells from immature cells (Blast-*like* or basophil cells) which, when mature, accumulates granules (granulocytes) or degranulates (hyalinocytes) in the event of environmental stress. In the present study, the main hemocyte population observed in pooled samples (*Figure 1B*) corresponds to 'hyalinocyte' sub-population. This can suggest that mixing hemolymph samples from different individuals generates mature hemocyte degranulation leading to one single population of agranular hemocytes. These overall observations could be interpreted as the first signs of some allorecognition in *D. polymorpha* hemocytes. However, further studies are needed to confirm such a hypothesis as allorecognition in bivalve has not been clearly reported nowadays (Costa et al., 2009). If confirmed, such phenomenon could also explain the slight increase of cell mortality observed in pooled samples when compared to individual ones.

Similarly, results showed that cell mortality significantly increased with the number of deposed cells (from 50×10^3 to 100×10^3, *Figure S2*) with a concomitant increase of value heterogenicity. These observations drew attention to the importance of keeping cell number identical to prevent skewing data interpretation, particularly in comparative studies such as chemical toxicity assessment. As a good compromise between cell mortality, data variability and the requirement to analyze 10,000 events (or H+ events) in the hemocyte region, we chose to deposit 75×10^3

cells per well in subsequent experiments. This size of deposit ensures an experimental design adapted to test at least 8 conditions on the same sample and up to 15 conditions on average (median value, N = 47).

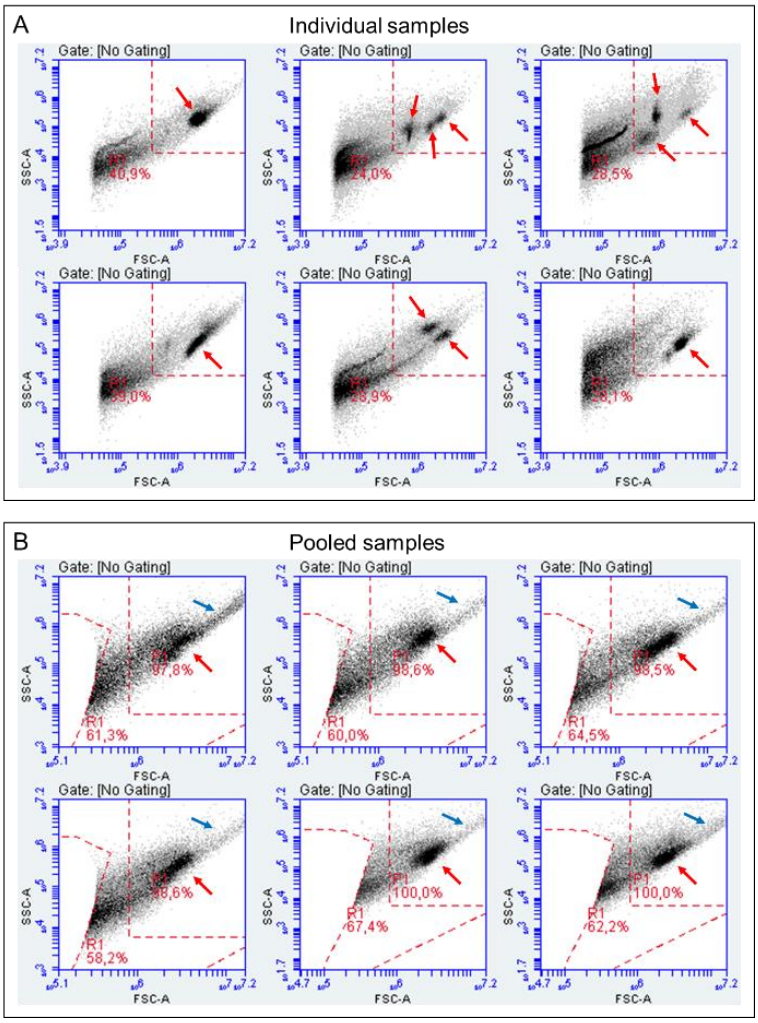


Figure 1. *Examples of cellular profiles (SSC-FSC; raw data voluntarily presented) of fresh individual (A) and pooled (B) samples (N = 5) of* Dreissena polymorpha *hemolymph analyzed by flow cytometry. Red and blue arrows point out cellular (sub)-populations and cellular clumps, respectively.*

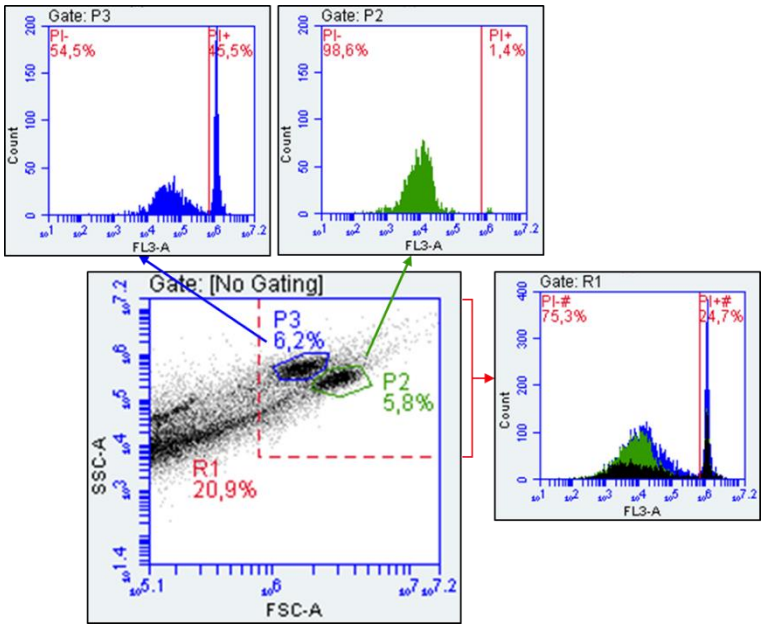


Figure 2. *Cell mortality (i.e. the percentage of PI+ events) measured in which* prima facie *could be considered as different hemocyte subpopulations from fresh individual samples of hemolymph (raw data voluntarily presented). The P2 and P3 regions surround two hemocyte subpopulations whereas the R1 region delimits the global hemocyte region.*

3.2 Protocol pre-analytical phase optimization

3.2.1 Cell detachment using Trypsin-EDTA: collecting the 'forgotten' cells

Hemocytes are circulating cells capable of fast tissue infiltration and strong adhesion to foreign surfaces. Cell sedimentation and adhesion to the substrate are systematically observed after hemolymph withdrawal, reflecting hemocyte activation (Rioult et al., 2013). This property is indubitably problematic when the analytical technics required that the cells remained isolated and in suspension such as for flow cytometry measurements. This aspect is unfortunately regularly neglected in studies analyzing bivalve hemocytes by flow cytometry (e.g. Sauvé et al., 2002; Labreuche et al., 2006; Tanguy et al., 2013; Le Guernic et al., 2015; Evariste et al., 2016, 2017, 2018). After the addition of a cell detachment step just before cytometric analyses using diluted Trypsin-EDTA solution, results showed that the %PI+ events detected in the R1 region are significantly lower (*Figure 3A*). The decrease in the %PI+ is concomitant to a significant increase in the event rate detected by the cytometer in the R1 region (*Figure 3B*), confirming that the trypsin treatment successfully detached adherent cells from the microplate wells. The increase in the number of adherent (and thus viable) cells in the acquisition gate resulted in an 'artefactual' diminution of the %PI+ in comparison to untreated samples. These results demonstrate that the %PI+ is significantly over-estimated in untreated samples where a non-negligible number of viable cells remains stick onto the plastic substrate. It proves the importance of accounting for the hemocyte adhesion ability during the preparation of the samples dedicated to flow cytometric analysis, otherwise the results may not be

representative of the whole hemocyte population present in the microplate well. It could be particularly true in functional analysis such as phagocytosis activity, considering that adhesion constitutes an initial step of phagocytosis process (Labreuche et al., 2006; Tanguy et al., 2013). In the literature, several antiaggregant/adhesive solutions are used during bivalve hemolymph preparation including heparin, EDTA, (modified) Alsever's solution, N-ethylmaleimide and calcium-free trypsin-EDTA (Grandiosa et al., 2016, 2018; Hégaret et al., 2003; Hinzmann et al., 2013; Huang et al., 2018; Le Foll et al., 2010; Rebelo et al., 2013). Both heparin and low temperature have been reported as ineffective in preventing invertebrate hemocyte aggregation (Grandiosa et al., 2018; Hinzmann et al., 2013). (Modified) Alsever's and EDTA solutions proved to be a suitable treatment in marine bivalves such as abalones, blue mussels or oysters (Grandiosa et al., 2016, 2018; Hégaret et al., 2003; Le Foll et al., 2010; Rebelo et al., 2013). However, these antiaggregant solutions are not applicable in freshwater species as they impact cell morphology and viability regarding the low osmolarity of freshwater bivalve internal fluids (Hinzmann et al., 2013). In particular, hemolymph osmolarity in *D polymorpha* species was measured at around 80 mOSM (Quinn et al., 2009). In our study, the commercial trypsin-EDTA solution had to be diluted by 4 before use to prevent hyperosmolarity issues. This treatment was the most efficient method to increase the number of functional cells analyzed by flow cytometry. However, the action of trypsin is rapidly inhibited when the enzyme is diluted at the end of the step #5 (see Table 1). Samples have thus to be quickly analyzed thereafter. To limit hemocyte sedimentation and re-adhesion to the substrate, cell suspensions are regularly and thoroughly mixed by pipetting before analysis. This homogenization method has been reported as efficient to mix cell suspension while maintaining high viability rates in abalone hemocytes even if vortexing should be preferred (Grandiosa et al., 2018). This latter method could not be performed with microplates.

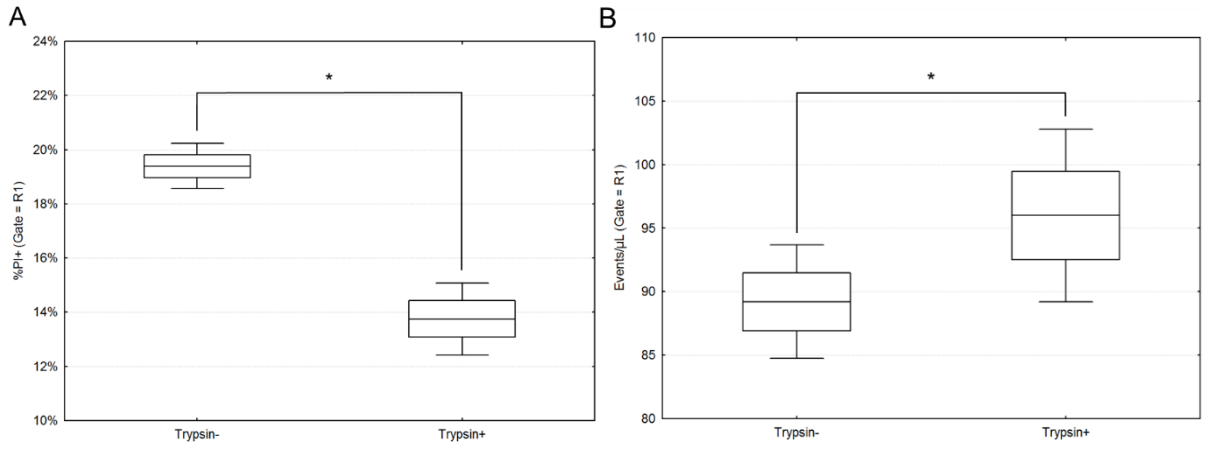


Figure 3. *Effects of Trypsin-EDTA treatment on the cell mortality (%PI+; A) and the cell rate (number of events detected per microliter; B) in the hemocyte region (R1) analyzed by flow cytometry. Data are presented as the mean value (horizontal line) ± SD (box). The whiskers represent the mean value ± 1.96*SD. Asterisks mark significant differences ($p < 0.05$) with (+) or without (-) trypsin-treatment, according to Mann-Whitney U test (N = 5 deposits from the same pool per treatment).*

3.2.2 Functional cell selection using a sedimentation step

The ability of hemocytes to strongly stick to a foreign surface in the minute range was the main difficulty encountered when performing flow cytometry analyses on such cell types. However, it is also possible to take advantage of hemocyte adhesion ability to increase the quality of the cell suspension before *ex vivo* exposure. A sedimentation step was proposed just before cell exposure, relying on the idea that a selection of functional (and thus viable) hemocytes could be performed based on their adhesion-to-substrate ability. Considering that adhesion constitutes an initial step in the phagocytosis process (Labreuche et al., 2006), this selection of cells based on their adhesion ability may be very appropriate to further perform a functional bioassay such as phagocytosis analysis.

Previous tests revealed that the %PI+ events in the hemocyte region were significantly higher at T0 than after 4h of incubation (*Figure S3*). These observations suggested that hemolymph withdrawal may result in a cellular shock that triggers cell death or at least a loss of membrane integrity. After 4h of incubation, cells may have recovered resulting in lower %PI+ events, considering that the cell concentration remained stable over time (p = 0.1, data not shown). It seems that the hemocyte population is not in a 'stable state' and that it takes time to recover from the effects generated by hemolymph withdrawal. Thanks to a sedimentation step, results showed that the %PI+ was not anymore significantly different between T0 and T4 (*Figure S3*) indicating that the hemocyte population status remained stable over the incubation time.

The *Figure 4* illustrates the effects of the sedimentation on the cell mortality for different pooled samples. A Factorial ANOVA was conducted to evaluate the influence of the sample (P1 to P3) and the treatment (sedimentation or not) on the %PI+ events in the R1 region. The analysis revealed that the main effects were statistically significant as well as the interaction. The significant interaction between the "Sample" and the "Treatment" factors ($F_{(2,12)} = 17.3$; $p < 10^{-2}$) indicated that the effect intensity of the sedimentation step on the %PI+ is dependent on the considered sample. In the present case, the effect is more important in P3-sample showing the highest %PI+ value without sedimentation. The sedimentation step thus provides two main advantages: first, it notably reduced the cell mortality level after 4h of incubation (< 10% in the present example) and secondly, it erased inter-sample variability with mean %PI+ values very similar and globally less variable in comparison to what is observed without sedimentation step (*Figure 4*).

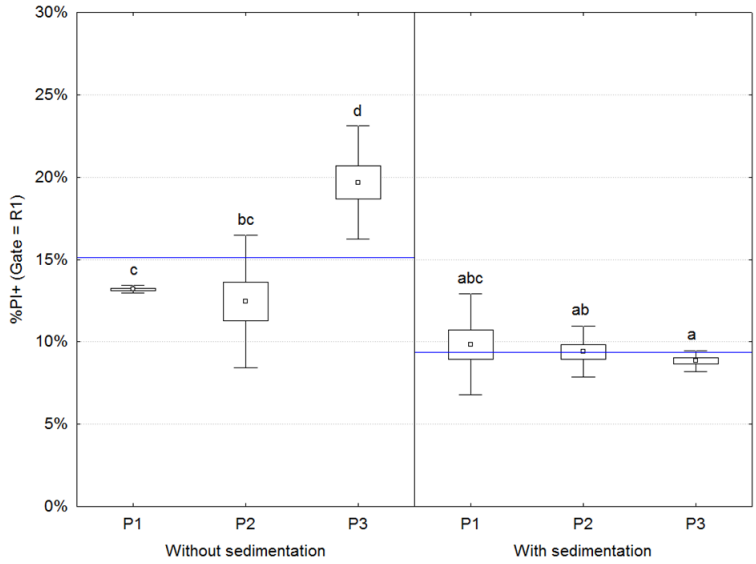


Figure 4. *Effects of the sedimentation step on the percentage of PI+ events in the hemocyte region (R1) measured in three pools of hemolymph after 4h of incubation. Data are presented as mean value (empty square) ± SE (box; N = 5). The whiskers represent the mean value ± 2*SD. A Factorial ANOVA followed by the Tukey HSD test was conducted to analyze the effects of the sample (P1 to P3) and the treatment (with or without the sedimentation step) on the %PI+. Groups with different letters are significantly ($p < 0.05$) different from each other within the interaction effect. Blue lines indicate the mean value of each treatment group for illustrative purposes.*

The effect of the sedimentation step was also analyzed on phagocytosis activity after 4h of incubation (Figure 5). A Factorial ANOVA was performed to analyze the effects of the sample and the sedimentation step on the percentage of the total (%1B+) and efficient (%3B+) phagocytic cells in the R1 region. This procedure gave similar results for both parameters with significant "Sample" ($F_{(2,12)} = 21.9$ with $p < 10^{-4}$ and $F_{(2,12)} = 16.2$ with $p < 10^{-3}$, respectively) and "Treatment" main effects ($F_{(2,12)} = 58.4$ with $p < 10^{-5}$ and $F_{(2,12)} = 122.8$ with $p < 10^{-6}$, respectively). The interaction effects were insignificant. The same analysis performed on the hemocyte avidity (i.e. mean number of efficiently engulfed beads per cell) proved to be insignificant and is thus not presented here. The results on the %1B+ and the %3B+ indicated that the inter-sample variability of these endpoints remained significant with or without a sedimentation step. Phagocytosis activity was lower in samples following the sedimentation and associated with a lower concentration of events detected in the R1 region in comparison to the unmodified protocol (data not shown). Hemolymphatic supernatant removal following the sedimentation step may explain these observations as many soluble humoral factors (such as pathogen recognition receptors) are present in the plasma of hemolymph (Leprêtre et al., 2019). Moreover, the beads used here have a non-specific coating with carboxylates and could thus bound to bacteria, viruses or debris which could, in turn, be recognized and stimulate phagocytosis activity of hemocytes. Their removal from the medium could hypothetically limit phagocytosis activation. As a result, the sedimentation step results in a phagocytic activity at a low or un-activated state of the cells. Further studies are needed to explore the reason for such an effect on phagocytosis measurements in *D. polymorpha* hemocytes.

The addition of a sedimentation step in the protocol nonetheless provides the advantage of focusing on functional cells during subsequent flow cytometry analysis while reducing cell mortality and inter-sample variability. These

aspects are of great interest notably for *ex vivo* exposure. The sedimentation step should however not be systematically performed in the case of *in vivo* exposure of mussels as the removal of dead and non-functional cells from the sample following sedimentation could biased results. The decision to include or not a selection of analyzed cells has to be well-considered in the light of the own objectives of the experiment.

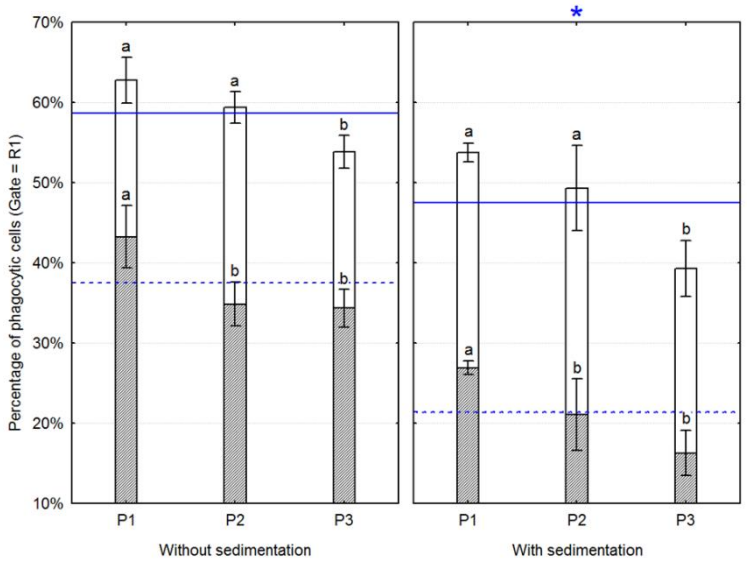


Figure 5. *Effects of the sedimentation step on the percentage of the total (white bars; %1B+) and efficient (hatched bars; %3B+) phagocytic cells in the hemocyte region (R1) measured in three pools of hemolymph after 4h of incubation. Data are presented as mean value ± SD (N = 5). The solid and dashed blue lines represent the mean group value for %1B+ and %3B+, respectively. A Factorial ANOVA was conducted to compare the main effects of the sample (P1 to P3), the treatment (with or without the sedimentation step) and the resulting interaction on the %1B+ and %3B+. Tukey HSD test was used as a posthoc test. Samples with different letters are significantly ($p < 0.05$) different from each other within the sample main effect. The blue asterisk illustrates the significant difference within the treatment main effect ($p < 10^{-3}$).*

3.3 Multi-labeling analysis in flow cytometry

The open circulatory system of bivalves and the omnipresence of debris and of (exo- or endogenous) non-target cells in *D. polymorpha* hemolymph strongly impact sample quality. The adequate positioning of the gate of interest (R1) helps to eliminate the main part of these undesirable events during the post-acquisition treatment of flow cytometry data. The addition of a cellular probe can nonetheless refine the results of hemocyte analysis by discriminating nucleated events from debris in the hemocyte region. Hoechst® 33342 was selected as non-specific nuclear counterstain considering that (*i*) no specific dye are available for *D. polymorpha* hemocytes nowadays, and (*ii*) its great cell-permeability allowing a short staining time. Hoechst® 33342 is excitable by an ultraviolet laser and emits a strong blue fluorescence making it compatible in a multi-labeling procedure using PI and YG beads (see discussion below). The combination of these three dyes resulted in a complete cytometric analysis focused on cellular (Hoechst-positive; H+) events in the hemocyte region with a simultaneous

assessment of cytotoxicity (PI staining) and phagocytic activity (YG beads), as illustrated in *Figure 6*. The main challenge in the choice of the trio of dyes relies on the compatibility of the cellular and mortality probes regarding the wide emission spectrum of YG beads. The trio of dyes was first selected according to their excitation/emission spectrum provided by the suppliers and the laser configuration of our cytometers. The dyes were then tested individually, by pairs and finally the trio Hoechst/PI/beads, on five pooled samples during a single experiment. The fluorescence profiles of each combination of probes were analyzed in comparison of the profile of the dye alone to evaluate the modification of the fluorescence profiles in the presence of several dyes.

For the probes alone, the most variable profiles were observed for Hoechst labeling (*Figure S4A*) sometimes requiring an adaptation of H+ fluorescence threshold for some samples or batch of samples. The addition of beads resulted in a slight increase of H+ fluorescence profile variability (*Figure S4C* and *D*) whereas no effect of PI was visible (*Figure S4B*). Nonetheless, no significant difference was shown in the %H+ events in the R1 region for the different combinations of probes ($p > 0.05$ according to repeated-measures ANOVA; data not shown). The PI fluorescence profiles were very similar among samples for the probe alone (*Figure S5*). The addition of the Hoechst probe increased PI fluorescence intensity as shown by a rightward shift of the whole PI profile (i.e. cell autofluorescence and PI+ peak; *Figure S5B*). This is probably the result of a slight overlap between Hoechst and PI fluorescence profiles (Hoechst® 33342 dye also emits in red wavelengths). The double-labeling using PI and beads also changed the FL4-fluorescence profile but only by increasing the cell autofluorescence resulting in a slight overlap of PI+ fluorescence peak (*Figure S5C*). The triple-labeling procedure resulted in a combination of these effects on PI fluorescence: an increase of cell autofluorescence and a rightward shift of the PI+ profile (*Figure S5C*). The PI+ fluorescence thresholds had thus to be adapted to coherently analyze PI labeling in triple- or double- (H+PI) labeled samples. With such adaptations, the percentage of PI+ cells in the R1 region did not significantly differ ($p > 0.05$ according to repeated-measures ANOVA) among the different labeling conditions (PI alone, PI+H, PI+B, and PI+H+B). No effect of PI or Hoechst was observed on the bead fluorescence profile whatever the conditions (*Figure S6*). Accordingly, no statistical differences between labeling conditions were highlighted on phagocytosis endpoints (i.e. %1B+, %3B+, and avidity) in the R1 region ($p > 0.05$ according to repeated-measures ANOVA; data not shown).

These results demonstrate the compatibility of the three selected probes for the simultaneous analysis of cytotoxicity and immunotoxicity in *D. polymorpha* hemocytes. PI and Hoechst® 33342 have been already used in a double-labeling procedure on hemocytes from silkworms and nematodes (Li et al., 2009; Okazaki et al., 2006). To our knowledge, this is the first time that a functional triple-labeling procedure is proposed for bivalve hemocytes. PI probe is classically excited in blue wavelength (at 480 nm) but it is also possible to excite it with a UV laser to limit the potential overlap with the broad YG bead excitation spectrum. Despite a partial overlap between Hoechst and PI emission spectrum, an adjustment of PI+ threshold eliminated the influence of Hoechst on the analysis of PI+ cells. The use of a cellular probe as the Hoechst® 33342 dye provides the substantial advantage of bypassing the problematic of the presence of untargeted events (i.e. debris etc.) in hemolymph samples. Focusing on H+ events eliminates the non-nucleated (H-) events from the analysis as well as algal cells

as *Chlorella vulgaris* (used to feed mussels) which did not integrate this dye whereas their FSC-SSC profile can partially overlap the R1 region (data not shown). Despite spermatozoids are generally observed out of the R1 region due to their lower size and complexity in comparison to hemocytes, the addition of Hoechst allows their discrimination from the cells of interest by the linear analysis of Hoechst emission profile to differentiate cell DNA content (*1n* versus *2n*). The best gating strategy is to focus on H+ events in the R1 region to tighten the data analysis on the hemocyte cell population. Even with an acquisition region eliminating the most part of the debris and small-sized events (e.g. the P1 region; see *Figure 6*), the percentage of H+ events only represents 45% of the total events on average (N = 12 pooled samples) with values ranging from 15% for the 'dirtiest" to 58% for the 'cleanest' sample. In the R1 region, the percentage of H+ events is much higher, with an average value equal to 77% ± 4.2%, indicating that cells are the main part of the detected events and thus validating the position of this region of interest on the FSC-SSC profile. It nonetheless also highlighted that more than 20% of the events detected in this region are not cells and may thus bias the endpoint acquisition if no cellular probe is used. In the absence of specific labeling of *Dreissena* hemocytes, the use of Hoechst dye enhances the quality of the cytometric analysis in such an organism for which the presence of debris in hemolymph samples is inevitable.

Besides the triple-labeling procedure reduces the quantity of biological material needed and allows the simultaneous analysis of cytotoxicity and immunotoxicity on the same sample, it also permits to have more complete and accurate measurements of the recorded biological endpoints. For instance, in our experiments, the mean %PI+ was equal to 16% when acquired in the R1 region as classically done, whereas it raised around 21% on average when analyzed in the cells (H+ events) in R1 (*Figure 7A*). Without this cellular probe, the hemocyte mortality could thus be underestimated by 5% on average. Similarly, the %PI+ is significantly higher when evaluated in overall cells without R1 gating in comparison with cells in R1 gating (*Figure 7A*). The mortality is thus overestimated of 3.6% on average when the population of analyzed cells is not restricted according to their FSC-SSC profile. More important differences were highlighted on the phagocytosis endpoints according to the gate of analysis (*Figure 7B*). In particular, the percentage of the total (%1B+) and efficient (%3B+) phagocytic cells were significantly 10%-higher on average when analyzed in cells in R1 (H+ in R1) when compared to acquisition on the R1 region, as classically done. An advantage of the triple-labeling procedure relies on the possibility to evaluate phagocytosis endpoints on viable cells (H+/IP-) only. Phagocytosis analysis can be done with no direct impact of potential cytotoxicity on the recorded data. In our example, %1B+ and %3B+ were more than 15% underestimated on average ($p < 0.05$) when classically analyzed in the R1 region in comparison with a focused analysis on viable cells in the same region (*Figure 7B*). Interestingly, the hemocyte avidity did not show any significant variation according to the gate of analysis, demonstrating that this endpoint is very robust whatever the gating strategy. Nonetheless, we recommend using a focused gating strategy on the viable cells present in the R1 region to assess the ability and the efficiency of the phagocytosis process in hemocyte without any impact of cell mortality on the generated data. It may be of great interest during chemical toxicity testing where acute cytotoxicity may occur and artefactually decrease the percentage of phagocytic cells. The comparison between the %PI+ acquired in the cells in R1 and in phagocytic (1B+) cells in R1 could inform on potential cytotoxicity

generated by phagocytosis activity during the incubation period. In our example, the mean %PI+ in cells in R1 was 22.2% whereas it was equal to 16.7% in phagocytic cells in R1 showing that no particular cytotoxicity was induced by phagocytosis activity. Finally, the analysis of the percentage of viable cells with no phagocytic activity (%1B- in viable cells in R1) can potentially highlight the presence of a phagocytosis inhibitor in the exposure medium.

The triple-labeling procedure presented here notably enhances the representativity of the generated data through the better discrimination between targeted and untargeted events. It also allows for the acquisition of additional data to describe more precisely the cytotoxicity and the immunotoxicity of the tested conditions by using an adequate post-gating strategy. The proposed trio of dyes required a cytometer equipped with an UV-laser to be functional and this may be a limiting factor considering that this feature is not very frequent in cytometer with a classic configuration. Works are in progress in our laboratory to propose another functional trio of probes with no need for such a specific feature. In view of current knowledge, it is nowadays impossible to propose a specific marker hemocyte cells. A recent study decrypted the proteome of *D. polymorpha* hemocytes using a proteogenomic approach (Leprêtre et al., 2019) which could be a first step towards the identification of such a specific probe but further studies are needed to achieve this objective. While awaiting the results of these researches, the use of a non-specific cellular probe such as Hoechst is recommended. A double-labeling procedure using PI and YG beads could nonetheless be performed on cytometers with a more classical laser configuration. The absence of a cellular probe induces some bias in the generated data as demonstrated in the present study, and this must be kept in mind when interpreting the results of such analysis. Indeed, each endpoint may include a slight inaccuracy in the value generated which may be considered in studies evaluating the physiological state of organisms, for instance. The double-labeling strategy remains valid in studies with a comparative objective (e.g. comparison of the toxicity of chemicals, etc.). PI was selected as a global probe for cytotoxicity assessment considering that it stains necrotic and late apoptotic cells (Sawai and Domae, 2011; Wallberg et al., 2016). But the developed protocol can also be easily applied to a more detailed analysis of cell mortality by combining PI measurement and caspase 3/7 analysis, as shown in a recent work from our laboratory (Le Guernic et al., 2019). The present study also demonstrated that the hemocyte avidity is a very interesting endpoint to record as it proved to be unaffected by protocol modifications or multi-labeling procedure.

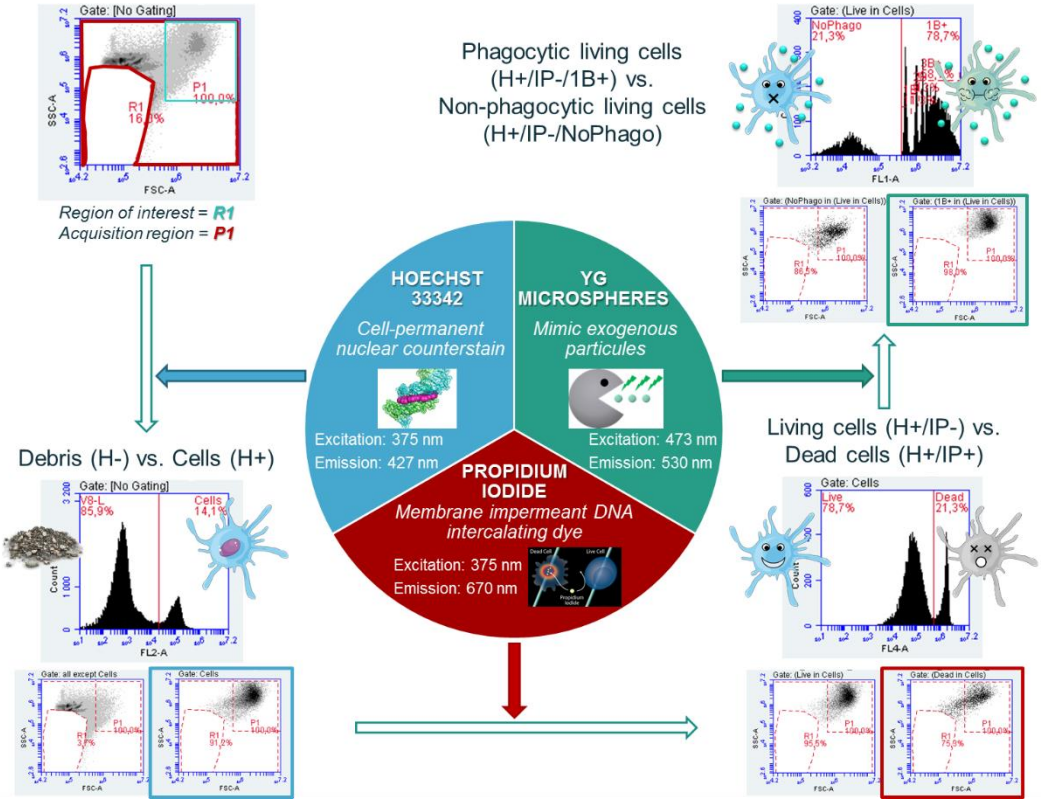


Figure 6. *The principle of the triple-labeling procedure proposed for* D. polymorpha *hemocyte analysis by flow cytometry and the associated gating strategy.*

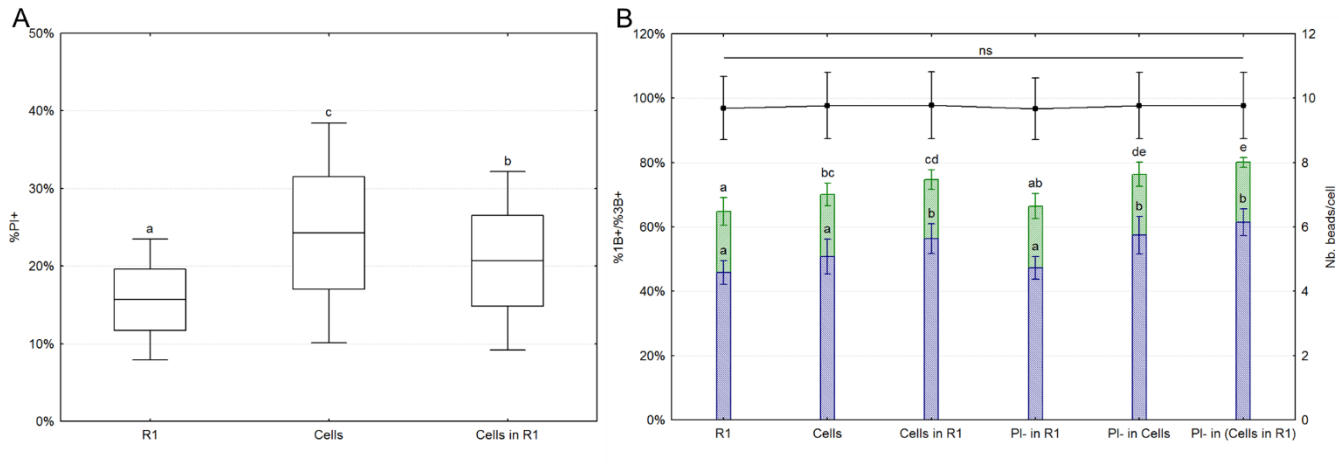


Figure 7. *Effects of the gating strategy on the mortality (%PI+; A) and phagocytosis endpoints (B). For mortality, data are presented as mean value (horizontal line) ± SD (box). The whiskers represent the mean value ± 1.96*SD. A repeated-measures ANOVA was performed to evaluate the effect of the gating strategy (R1, cells (H+) and cells in R1) and the treatment (PI+H and PI+H+B, N = 5 in each treatment) on the %PI+ events. No significant effect was observed for the treatment effect and the interaction Gate*Treatment ($p > 0.05$). Results are thus only presented for the significant main effect "Gate" ($p < 10^{-5}$; N = 10). Different letters indicate significant differences between gating strategies ($p < 0.05$ according to Tuckey posthoc test). For phagocytosis endpoints, data are presented as mean value ± SD. The percentage of the total (%1B+) and efficient %3B+) phagocytic cells are represented by the green hatched (to the right) and the blue hatched (to the left) bars (left axis). The hemocyte avidity is represented by the black line at the top of the graph (right axis). Different letters indicate*

significant differences between gating strategies ($p < 0.05$ according to Tuckey posthoc test following the repeated-measures ANOVA).

4 Conclusion

The present study was dedicated to the development of a new protocol to simultaneously analyze the cytotoxic and immunotoxic responses in *D. polymorpha* hemocytes using flow cytometry. To this end, some enhancements were proposed to solve the following issues: the quality and the quantity of hemolymph samples, the inter-sample variability of the responses and the cellular profiles, and the adhesion ability of hemocytes. The inclusion of several technical improvements gives rise to an optimized version of the hemocyte exposure protocol which is summarized in *Table 1*.

The use of pooled hemolymph samples ensures that several conditions (different compounds or concentrations) could be tested with the same sample allowing the deployment of relevant experimental design, in particular for *ex vivo* experiments dedicated to the evaluation of chemical or environmental sample toxicity. Simultaneously, the variability of the cellular profile and of the biological endpoints was reduced, increasing the inter-treatment discrimination potency of the bioassay. The consideration of the adhesion ability of hemocyte leads us to include an enzymatic detachment step followed by a proper homogenization of the cell suspension just before their analysis by flow cytometry to ensure that all the cells of interest (i.e. the functional cells with adhesion ability) were measured. It resulted in a decrease in the cell mortality associated with an increase in the concentration of analyzed events confirming that, without a cell detachment step, not all the hemocyte population is analyzed. The adhesion ability of hemocyte was also proposed to select the functional hemocytes for an *ex vivo* exposure followed by a functional analysis such as phagocytosis measurements. This step enhanced the sample quality by reducing the cell mortality after 4h of exposure as well as the inter-sample variability. It also ensures that only hemocyte potentially able to perform phagocytosis are present at the beginning of the exposure and that no non-functional cells initially present in the sample will biased phagocytosis analysis. However, the sedimentation step results in a selection of the analyzed cells that has to be deeply considered regarding the study objectives, particularly for *in vivo* experiments where such selection of the analyzed events may constitute an important bias. Finally, a triple-labeling procedure was proposed to include a cellular probe during cytometric data analysis and to simultaneously analyze cytotoxicity and immunotoxicity endpoints in the same sample. In the absence of a specific hemocyte probe, this labeling procedure is associated to a proper gating strategy to (i) limit the interference of un-target cells in the endpoint analysis, (ii) acquire complementary information (e.g. the mortality induced by phagocytosis activity), and (iii) refine the endpoint measurements (e.g. excluding dead cells for phagocytosis analysis).

All these modifications resulted in an operational and rapid protocol to analyze hemocyte mortality and phagocytosis activity in *D. polymorpha* hemocytes. This procedure enhanced the analysis quality as well as the accuracy of the analyzed endpoints. The proposed methodology is particularly adapted to *ex vivo* exposures such as chemical or environmental sample toxicity assessment, but it can also be applied to *in vivo* experiments such as biomonitoring surveys following minor adjustments.

539 ***Table 1.*** *Details of the protocol developed for hemocyte* ex vivo *or* in vivo *exposure followed by flow cytometry*
540 *analysis*

# Step	Step details
#1	***Hemolymph sampling.*** Withdrawn hemolymph from mussel muscle with insulin syringe previously rinsed and filled with 50 µL of L15-15%⊕. Store samples in sterile microtubes in a rack previously placed on ice.
#2	***Cell deposit.*** Determine cell concentration using KOVA® slide (or equivalent method). Depose 75×10^3 cell.well^{-1} in a 96-well microplate.
#3	***Sedimentation.*** For *ex vivo* exposure*:* Keep sample for 20 min at 16 °C for sedimentation. Remove supernatant and add 190 µL L15-15%⊕. For *in vivo* exposure*:* Complete volume in each well to 190 µL with L15-15%⊕.
#4	***Cell contamination and exposure.*** Add 10 µL of contamination solution or L15-15%⊕ medium (negative controls), and fluorescent beads to reach a 1:50 hemocytes-beads ratio. After homogenization, incubate at 16 °C for 4 h.
#5	***Cell detachment.*** 15 min before the end of the exposure period, transfer each sample supernatant in a new microplate. Add 100 µL of Trypsin-EDTA 1 × solution in each emptied well and incubate for 10 min at room temperature. Flush and transfer the detached cell suspension to the corresponding sample in the new microplate (i.e. 300 µL total for each sample)
#6	***Probe addition.*** Add 5 µM (final concentration) of Hoechst® 33342, homogenize and incubate for 10 min at room temperature. Add 10 µg.mL^{-1} (final concentration) of propidium iodide to each sample and thoroughly homogenize.
#7	***Flow cytometric analysis.*** Samples are analyzed by runs of 4 samples thoroughly mixed by 10-times pipetting just before analysis. Each sample is analyzed for 10,000 events (or Hoechst-positive events when the cellular probe is used) in the hemocyte region (R1) at a flow rate of 66 µL.min^{-1}. Hoechst staining is analyzed in FL2 channel (excitation: 375 nm) with a 427/10 nm filter; propidium iodide in FL4 channel (excitation: 375 nm) with a 670 LP filter; YG beads in FL1 channel (excitation: 473 nm) with a 530/30 nm filter attenuated by 90%.

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Acknowledgments

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References

Auffret, M., Oubella, R., 1997. Hemocyte aggregation in the oyster Crassostrea gigas: In vitro measurement and experimental modulation by xenobiotics. Comp. Biochem. Physiol. Part A Physiol. 118, 705–712. doi:10.1016/S0300-9629(97)00017-0

Binelli, A., Cogni, D., Parolini, M., Riva, C., Provini, A., 2009. In vivo experiments for the evaluation of genotoxic and cytotoxic effects of Triclosan in Zebra mussel hemocytes. Aquat. Toxicol. 91, 238–244. doi:10.1016/j.aquatox.2008.11.008

Binelli, A., Della Torre, C., Magni, S., Parolini, M., 2014. Does zebra mussel (Dreissena polymorpha) represent the freshwater counterpart of Mytilus in ecotoxicological studies? A critical review. Environ. Pollut. 196C, 386–403. doi:10.1016/j.envpol.2014.10.023

Blaise, C., Trottier, S., Gagné, F., Lallement, C., Hansen, P.-D.D., 2002. Immunocompetence of bivalve hemocytes as evaluated by miniaturized phagocytosis assay. Environ. Toxicol. 17, 160–169. doi:10.1002/tox.10047

Brousseau, P., Pellerin, J., Morin, Y., Cyr, D., Blakley, B., Boermans, H., Fournier, M., 1999. Flow cytometry as a tool to monitor the disturbance of phagocytosis in the clam Mya arenaria hemocytes following in vitro exposure to heavy metals. Toxicology 142, 145–156. doi:10.1016/S0300-483X(99)00165-1

Chen, J.-H., Bayne, C.J., 1995. Hemocyte adhesion in the California mussel (Mytilus californianus): regulation by adenosine. Biochim. Biophys. Acta - Mol. Cell Res. 1268, 178–184. doi:10.1016/0167-4889(95)00074-3

Costa, M.M., Dios, S., Alonso-Gutierrez, J., Romero, A., Novoa, B., Figueras, A., 2009. Evidence of high individual diversity on myticin C in mussel (Mytilus galloprovincialis). Dev. Comp. Immunol. 33, 162–170. doi:10.1016/j.dci.2008.08.005

European Commission (EC), 2006. Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and restriction of Chemicals (REACH), establishing a European Chemicals Agency, amending Directive 1999/4. Off. J. Eur. Communities 1–520. doi:2004R0726 - v.7 of 05.06.2013

574 Evariste, L., Auffret, M., Audonnet, S., Geffard, A., David, E., Brousseau, P., Fournier, M., Betoulle, S., 2016.
575 Functional features of hemocyte subpopulations of the invasive mollusk species Dreissena polymorpha.
576 Fish Shellfish Immunol. 56, 144–154. doi:10.1016/j.fsi.2016.06.054

577 Evariste, L., David, E., Cloutier, P.-L., Brousseau, P., Auffret, M., Desrosiers, M., Groleau, P.E., Fournier, M.,
578 Betoulle, S., 2018. Field biomonitoring using the zebra mussel Dreissena polymorpha and the quagga
579 mussel Dreissena bugensis following immunotoxic reponses. Is there a need to separate the two species?
580 Environ. Pollut. 238, 706–716. doi:10.1016/j.envpol.2018.03.098

581 Evariste, L., Rioult, D., Brousseau, P., Geffard, A., David, E., Auffret, M., Fournier, M., Betoulle, S., 2017.
582 Differential sensitivity to cadmium of immunomarkers measured in hemocyte subpopulations of zebra
583 mussel Dreissena polymorpha. Ecotoxicol. Environ. Saf. 137, 78–85. doi:10.1016/j.ecoenv.2016.11.027

584 Fournier, M., Cyr, D., Blakley, B., Boermans, H., Brousseau, P., 2000. Phagocytosis as a Biomarker of
585 Immunotoxicity in Wildlife Species Exposed to Environmental Xenobiotics. Am. Zool. 40, 412–420.
586 doi:10.1093/icb/40.3.412

587 Goedken, M., De Guise, S., 2004. Flow cytometry as a tool to quantify oyster defence mechanisms. Fish Shellfish
588 Immunol. 16, 539–552. doi:10.1016/j.fsi.2003.09.009

589 Grandiosa, R., Bouwman, M.-L., Young, T., Mérien, F., Alfaro, A.C., 2018. Effect of antiaggregants on the in vitro
590 viability, cell count and stability of abalone (Haliotis iris) haemocytes. Fish Shellfish Immunol. 78, 131–139.
591 doi:10.1016/j.fsi.2018.04.038

592 Grandiosa, R., Mérien, F., Pillay, K., Alfaro, A., 2016. Innovative application of classic and newer techniques for
593 the characterization of haemocytes in the New Zealand black-footed abalone (Haliotis iris). Fish Shellfish
594 Immunol. 48, 175–184. doi:10.1016/j.fsi.2015.11.039

595 Hégaret, H., Wikfors, G.H., Soudant, P., 2003. Flow cytometric analysis of haemocytes from eastern oysters,
596 Crassostrea virginica, subjected to a sudden temperature elevation. J. Exp. Mar. Bio. Ecol. 293, 249–265.
597 doi:10.1016/S0022-0981(03)00235-1

598 Hinzmann, M.F., Lopes-Lima, M., Gonçalves, J., Machado, J., 2013. Antiaggregant and toxic properties of
599 different solutions on hemocytes of three freshwater bivalves. Toxicol. Environ. Chem. 95, 790–805.
600 doi:10.1080/02772248.2013.818149

601 Huang, J., Li, S., Liu, Y., Liu, C., Xie, L., Zhang, R., 2018. Hemocytes in the extrapallial space of Pinctada fucata
602 are involved in immunity and biomineralization. Sci. Rep. 8, 4657. doi:10.1038/s41598-018-22961-y

603 Jones, H.D., 1983. The Circulatory Systems of Gastropods and Bivalves, in: Saleuddin, A.S.M., Wilbur, K.M.
604 (Eds.), The Mollusca, Vol. 5. Academic Press, pp. 189–238. doi:10.1016/B978-0-12-751405-5.50012-9

605 Labreuche, Y., Lambert, C., Soudant, P., Boulo, V., Huvet, A., Nicolas, J.L., 2006. Cellular and molecular

606 hemocyte responses of the Pacific oyster, Crassostrea gigas, following bacterial infection with Vibrio
607 aestuarianus strain 01/32. Microbes Infect. 8, 2715–2724. doi:10.1016/j.micinf.2006.07.020

608 Ladeiro, M.P., Barjhoux, I., Bigot-Clivot, A., Bonnard, M., David, E., Dedourge-Geffard, O., Geba, E., Lance, E.,
609 Lepretre, M., Magniez, G., Rioult, D., Aubert, D., Villena, I., Daniele, G., Salvador, A., Vulliet, E.,
610 Armengaud, J., Geffard, A., Palos Ladeiro, M., Barjhoux, I., Bigot-Clivot, A., Bonnard, M., David, E.,
611 Dedourge-Geffard, O., Geba, E., Lance, E., Lepretre, M., Magniez, G., Rioult, D., Aubert, D., Villena, I.,
612 Daniele, G., Salvador, A., Vulliet, E., Armengaud, J., Geffard, A., Ladeiro, M.P., Barjhoux, I., Bigot-Clivot,
613 A., Bonnard, M., David, E., Dedourge-Geffard, O., Geba, E., Lance, E., Lepretre, M., Magniez, G., Rioult,
614 D., Aubert, D., Villena, I., Daniele, G., Salvador, A., Vulliet, E., Armengaud, J., Geffard, A., 2017. Mussel as
615 a Tool to Define Continental Watershed Quality, in: Ray, S.B.T.-O. and M.M. (Ed.), Organismal and
616 Molecular Malacology. InTech, Rijeka, p. Ch. 03. doi:10.5772/67995

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

620 Le Guernic, A., Felix, C., Bigot, A., David, E., Dedourge-Geffard, O., Geffard, A., Betoulle, S., 2015. Food
621 Deprivation and Modulation of Hemocyte Activity in the Zebra Mussel (Dreissena polymorpha). J. Shellfish
622 Res. 34, 423–431. doi:10.2983/035.034.0226

623 Le Guernic, A., Geffard, A., Le Foll, F., Palos Ladeiro, M., 2019. Comparison of viability and phagocytic
624 responses of hemocytes withdrawn from the bivalves Mytilus edulis and Dreissena polymorpha, and
625 exposed to human parasitic protozoa. Int. J. Parasitol.

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

629 Li, X., Cowles, E.A., Cowles, R.S., Gaugler, R., Cox-Foster, D.L., 2009. Characterization of immunosuppressive
630 surface coat proteins from Steinernema glaseri that selectively kill blood cells in susceptible hosts. Mol.
631 Biochem. Parasitol. 165, 162–169. doi:10.1016/j.molbiopara.2009.02.001

632 Luengen, A.C., Friedman, C.S., Raimondi, P.T., Flegal, A.R.R., 2004. Evaluation of mussel immune responses as
633 indicators of contamination in San Francisco Bay. Mar. Environ. Res. 57, 197–212. doi:10.1016/S0141-
634 1136(03)00070-9

635 Mersch, J., Beauvais, M.-N., Nagel, P., 1996. Induction of micronuclei in haemocytes and gill cells of zebra
636 mussels, Dreissena polymorpha, exposed to clastogens. Mutat. Res. Toxicol. 371, 47–55.
637 doi:10.1016/S0165-1218(96)90093-2

638 Okazaki, T., Okudaira, N., Iwabuchi, K., Fugo, H., Nagai, T., 2006. Apoptosis and Adhesion of Hemocytes During

639 Molting Stage of Silkworm, Bombyx mori. Zool. Soc. Japan Zool. Sci. 23, 299–304. doi:10.2108/zsj.23.299

640 Quinn, B., Costello, M.J., Dorange, G., Wilson, J.G., Mothersill, C., 2009. Development of an in vitro culture
641 method for cells and tissues from the zebra mussel (Dreissena polymorpha). Cytotechnology 59, 121–34.
642 doi:10.1007/s10616-009-9202-3

643 Rebelo, M. de F., Figueiredo, E. de S., Mariante, R.M., Nóbrega, A., de Barros, C.M., Allodi, S., 2013. New
644 Insights from the Oyster Crassostrea rhizophorae on Bivalve Circulating Hemocytes. PLoS One 8, e57384.
645 doi:10.1371/journal.pone.0057384

646 Rioult, D., Lebel, J.-M., Le Foll, F., 2013. Cell tracking and velocimetric parameters analysis as an approach to
647 assess activity of mussel (Mytilus edulis) hemocytes in vitro. Cytotechnology 65, 749–758.
648 doi:10.1007/s10616-013-9558-2

649 Rioult, D., Pasquier, J., Boulangé-Lecomte, C., Poret, A., Abbas, I., Marin, M., Minier, C., Le Foll, F., 2014. The
650 multi-xenobiotic resistance (MXR) efflux activity in hemocytes of Mytilus edulis is mediated by an ATP
651 binding cassette transporter of class C (ABCC) principally inducible in eosinophilic granulocytes. Aquat.
652 Toxicol. 153, 98–109. doi:10.1016/j.aquatox.2013.11.012

653 Sauvé, S., Brousseau, P., Pellerin, J., Morin, Y., Senécal, L., Goudreau, P., Fournier, M., 2002. Phagocytic
654 activity of marine and freshwater bivalves: in vitro exposure of hemocytes to metals (Ag, Cd, Hg and Zn).
655 Aquat. Toxicol. 58, 189–200. doi:10.1016/S0166-445X(01)00232-6

656 Sawai, H., Domae, N., 2011. Discrimination between primary necrosis and apoptosis by necrostatin-1 in Annexin
657 V-positive/propidium iodide-negative cells. Biochem. Biophys. Res. Commun. 411, 569–573.
658 doi:10.1016/j.bbrc.2011.06.186

659 Tanguy, M., McKenna, P., Gauthier-Clerc, S., Pellerin, J., Danger, J.-M., Siah, A., 2013. Functional and molecular
660 responses in Mytilus edulis hemocytes exposed to bacteria, Vibrio splendidus. Dev. Comp. Immunol. 39,
661 419–429. doi:10.1016/j.dci.2012.10.015

662 Tryphonas, H., Fournier, M., Blakley, B.R., Smits, J.E.G., Brousseau, P. (Eds.), 2005. Investigative
663 immunotoxicology. Taylor & Francis.

664 US EPA, 2007. Pesticides; Data Requirements for Conventional Chemicals, Technical Amendments, and Data
665 Requirements for Biochemical and Microbial Pesticides; Final Rules.

666 Villela, I.V., de Oliveira, I.M., da Silva, J., Henriques, J.A.P., 2006. DNA damage and repair in haemolymph cells
667 of golden mussel (Limnoperna fortunei) exposed to environmental contaminants. Mutat. Res. - Genet.
668 Toxicol. Environ. Mutagen. 605, 78–86. doi:10.1016/j.mrgentox.2006.02.006

669 Wallberg, F., Tenev, T., Meier, P., 2016. Time-Lapse Imaging of Cell Death. Cold Spring Harb. Protoc. 2016,
670 pdb.prot087395. doi:10.1101/pdb.prot087395

671 Zuykov, M., Pelletier, E., Harper, D.A.T., 2013. Bivalve mollusks in metal pollution studies: From bioaccumulation
672 to biomonitoring. Chemosphere 93, 201–208. doi:10.1016/j.chemosphere.2013.05.001

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A new protocol for the simultaneous flow cytometric analysis of cytotoxicity and immunotoxicity on zebra mussel (*Dreissena polymorpha*) hemocytes

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Supplementary material

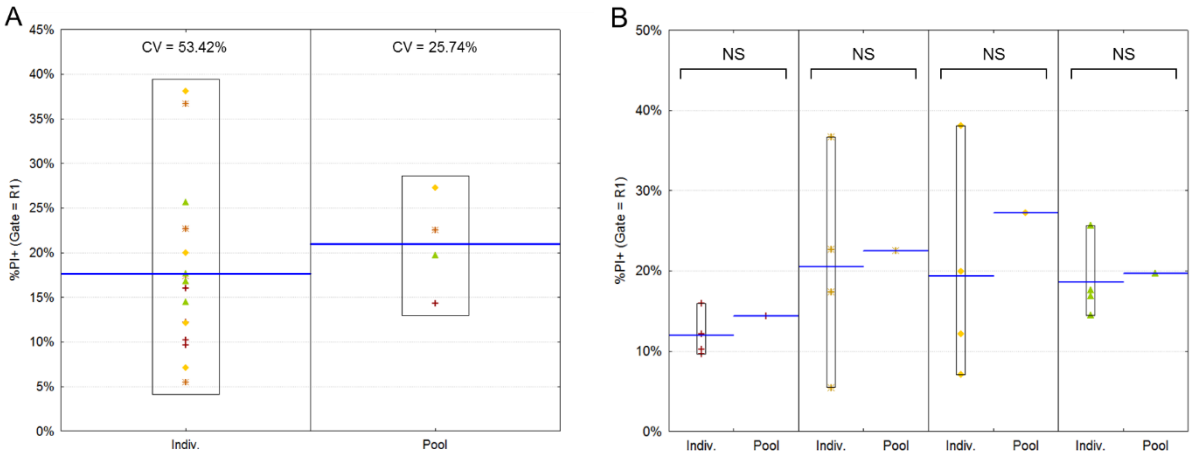


Figure S1. *Percentage of PI-positive events (%PI+) recorded in hemocyte region (R1) in fresh individual (left) and pooled (right) samples. (**A**) Global overview of value distribution for the individual and pooled samples. (**B**) Exploded view of the values observed for each pool and the corresponding individual samples. Individual samples used to make a pooled sample (N = 4) shared the same marker. Horizontal blue lines indicate the mean value for individual and pooled samples. No significant difference (NS) was observed between individual and pool values for each set of samples according to the Wilcoxon signed ranks test (p > 0.05).*

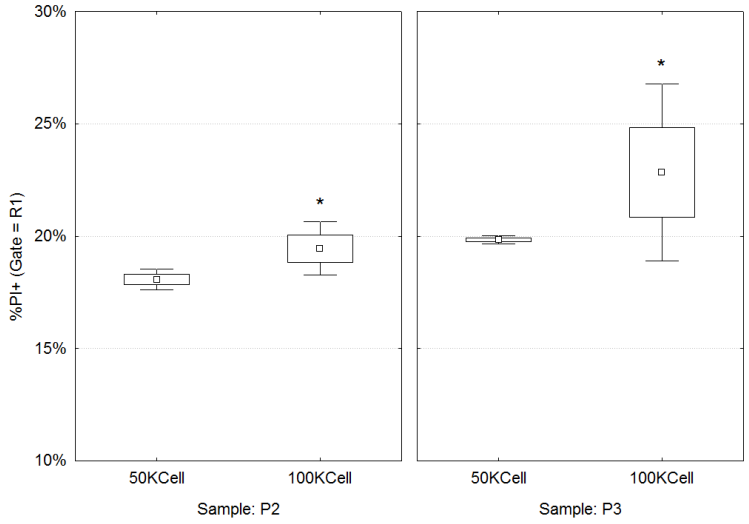
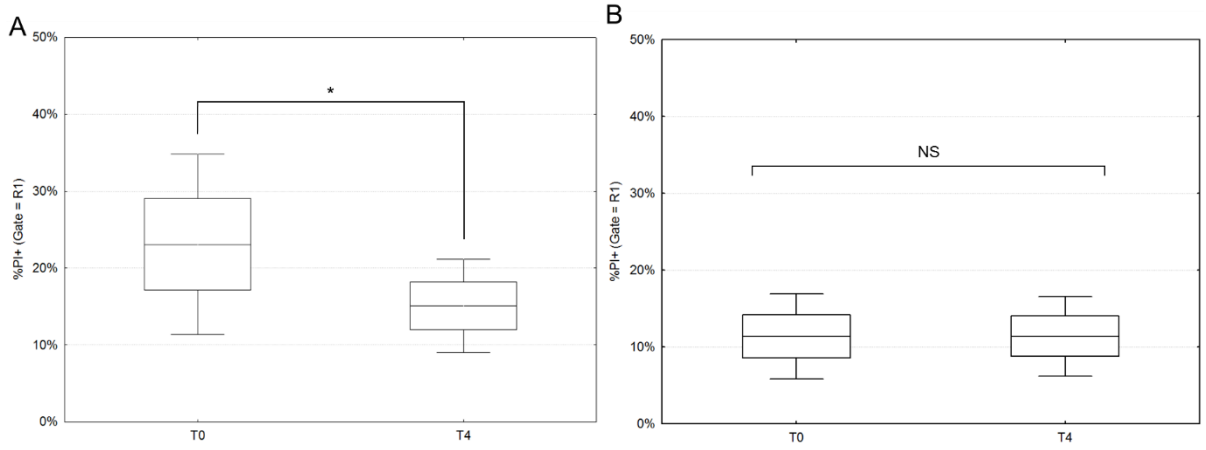


Figure S2. *Illustration of the effect of the number of cells deposed in microplate on the mortality rate (%PI+) analyzed by flow cytometry. For each pooled sample (P2 and P3), 50,000 and 100,000 cells were deposed four times and immediately analyzed for mortality (PI+ events) in hemocyte region (R1) by flow cytometry. Data are presented as mean value (empty square) ± standard deviation (box). The whiskers represent the mean value ± 1.96*standard deviation. Asterisks mark significant differences (p < 0.05) between %PI+ values observed for 50 KCell and 100 KCell for each sample, according to the non-parametric Mann-Whitney U test.*



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704 ***Figure S3.*** *Percentage of PI-positive events (%PI+) recorded in hemocyte region (R1) at T0 (fresh) and after 4h-*
705 *incubation at 16 °C without (**A**) or with (**B**) a sedimentation step before incubation (See* part 2.3.1 *and* Table 1 *for*
706 *details). Data are presented as mean value (horizontal line) ± SD (box). The whiskers represent the mean value*
707 *± 1.96*SD (N = 10 pools). Asterisks mark significant differences (p < 0.05) between T0- and T4-data, according*
708 *to the t-test for dependent samples. NS, not significant.*

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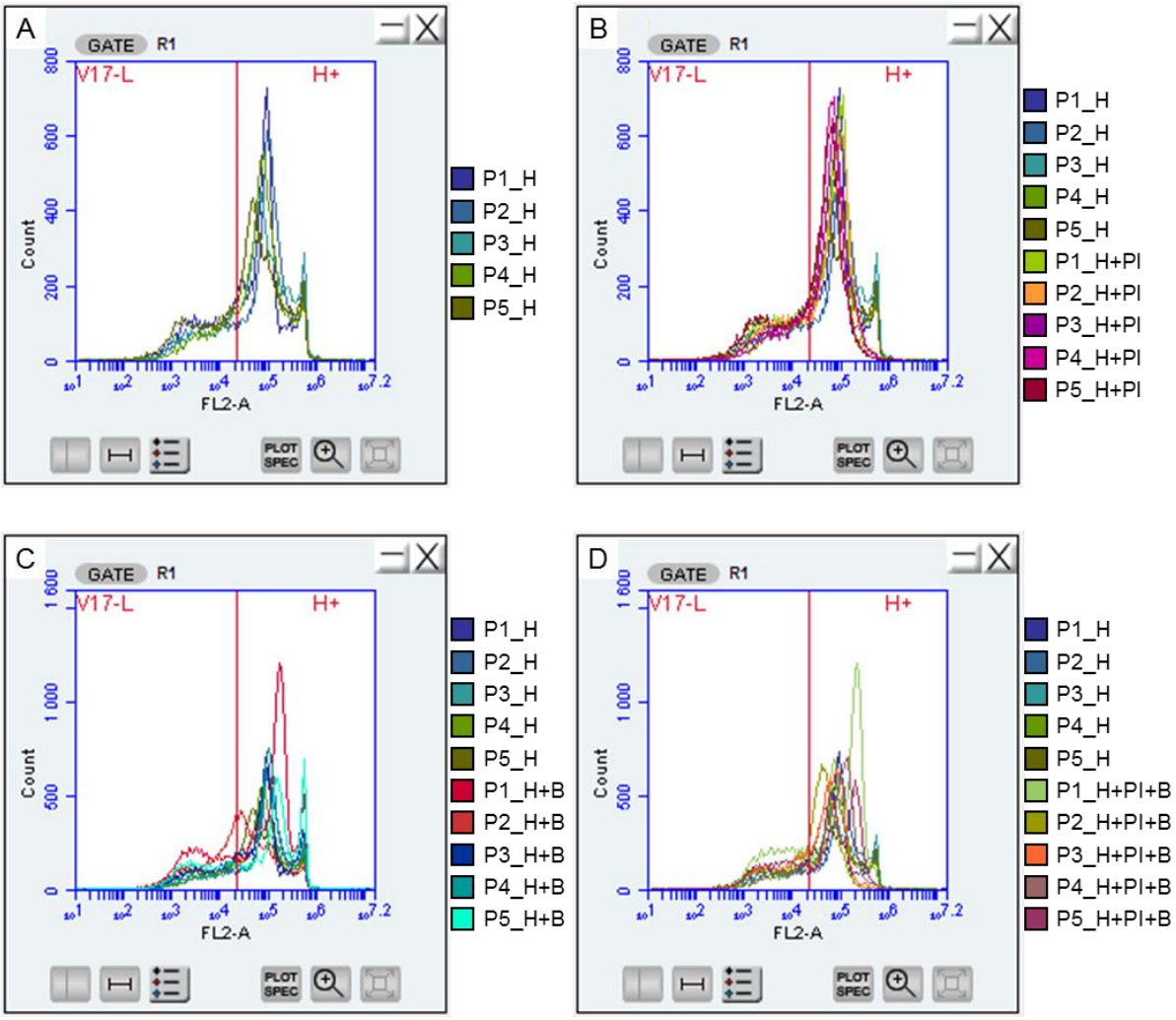

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Figure S4. *Fluorescence profiles of Hoechst® 33342 (H) observed in the hemocyte region (R1) after 4h-incubation at 16 °C for five pooled samples of hemolymph (P1 to P5). To see the effects of multi-labeling procedure on Hoechst fluorescence profile, the profiles of the samples were superimposed for each probe combination i.e. Hoechst alone (H, **A** to **D**), Hoechst and PI (H+PI, **B**), Hoechst and beads (H+B, **C**) and Hoechst, PI and beads (H+PI+B, **D**).*

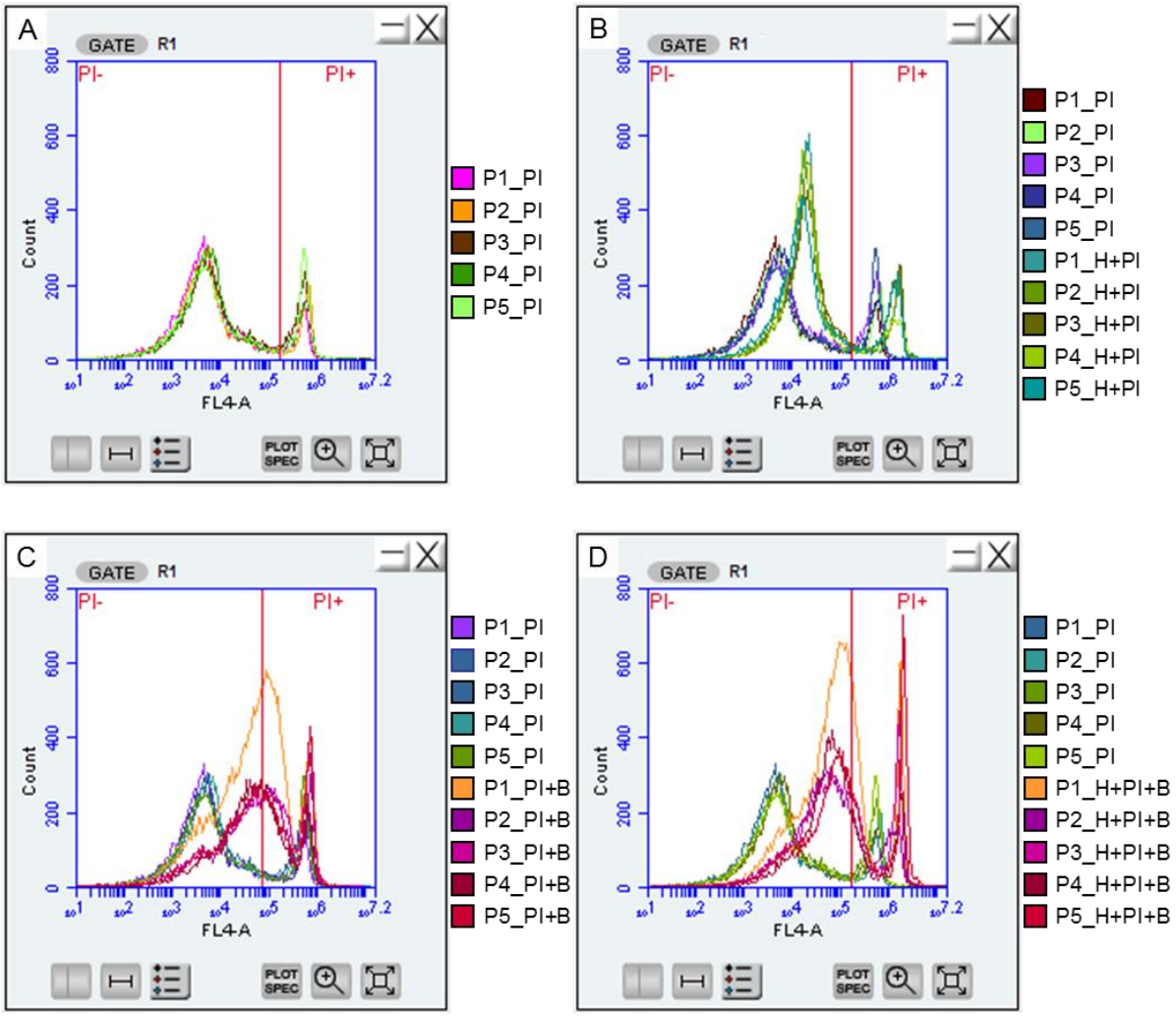
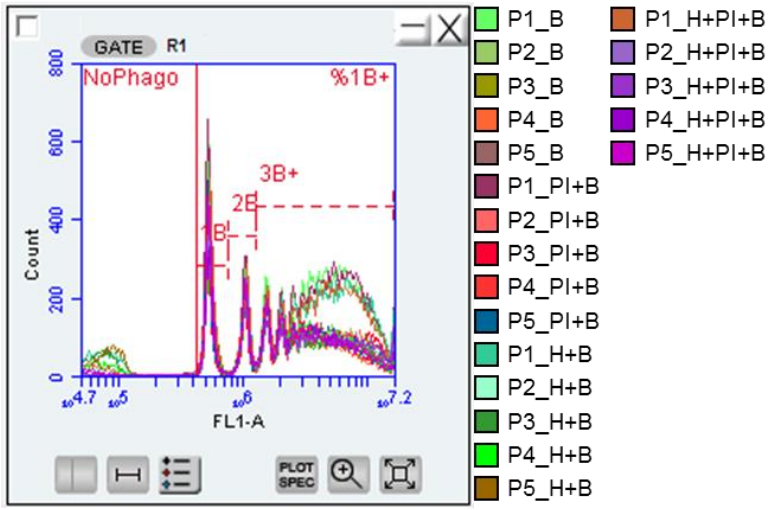


Figure S5. *Fluorescence profiles of propidium iodide (PI) observed in the hemocyte region (R1) after 4h-incubation at 16 °C for five pooled samples of hemolymph (P1 to P5). To see the effects of multi-labeling procedure on PI fluorescence profile, the profiles of the samples were superimposed for each probe combination i.e. PI alone (**A** to **D**), Hoechst and PI (H+PI, **B**), PI and beads (PI+B, **C**) and Hoechst, PI and beads (H+PI+B, **D**).*



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723 ***Figure S6.*** *Fluorescence profiles of YG beads (B) observed in the hemocyte region (R1) after 4h-incubation at*
724 *16 °C for five pooled samples of hemolymph (P1 to P5). To see the effects of multi-labeling procedure on beads*
725 *fluorescence profile, the profiles of the samples were superimposed for each probe combination i.e. beads alone*
726 *(B), beads and PI (PI+B), Hoechst and beads (H+B) and Hoechst, PI and beads (H+PI+B).*

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