Hemocytes of the Manila clam *Ruditapes philippinarum* (Adams et Reeve, 1850) as a potential biomarker of heavy metal pollution in marine environment monitoring

Anastasia V. Sinenko, Anna A. Anisimova, Irina A. Dmitrieva, Nadezhda K. Khristoforova
Far Eastern Federal University, Vladivostok, Russia, anisimova.aa@dvfu.ru

To estimate the potential of Manila clam hemocytes as a biomarker for heavy metal pollution, we studied the cellular composition of the hemolymph, hemocyte DNA content, and heavy metal content in Manila clams from Novik Bay.

Material and methods

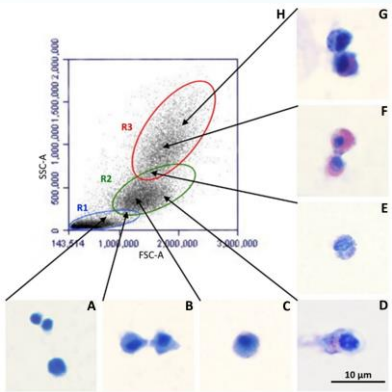
- Thirty-two clams *Ruditapes philippinarum* were collected in mid-July 2018 at four different sites in Novik Bay, 1) Uzkiy Cape, 2) Cape of Elagin, 3) Cape of Staritsky, and 4) a site near the settlement of Podnozhie (Fig. 1).
- For morphological analysis and classification of hemocytes, PFA-fixed cell suspensions were examined with a light AxioImager A1 microscope (Carl Zeiss) and a CytoFLEX flow cytometer (Beckman Coulter) using forward (FSC) and side (SSC) light scattering parameters (Fig. 2, A).
- For DNA content analysis, PFA-fixed cell suspension of *R. philippinarum*'s spermatozoa was used as a haploid standard. The mixed cell suspensions of hemocytes and spermatozoa were stained with DAPI solution and analyzed with a CytoFLEX flow cytometer (Beckman Coulter).

Figure 1 – Sites of molluscs collection in Novik Bay (Peter the Great Bay, Sea of Japan). 1 – Uzkiy Cape, 2 – Cape of Elagin, 3 – Cape of Staritsky, 4 – Podnozhie.

- The heavy metal (Fe, Mn, Cu, Zn, Cd, and Ni) content was determined in tissues of the Manila clams used in the hemolymph analysis, as well as of other bivalves sampled in the same sites. To do so, the molluscs were dried at a temperature of 85 °C for 2-3 days until no noticeable changes in weight could be detected. The tissue specimens were mechanically homogenized and analyzed with a Shimadzu AA-6800 atomic absorption spectrophotometer.

Results of the hemocyte structure analysis

- Proceeding from the microscopic analysis, five structural hemocyte types were identified, 1) blast-like cells, 2) hyalinocytes, 3) basophilic granulocytes, 4) neutrophilic granulocytes, and 5) eosinophilic granulocytes (Fig. 2, A—G).
- By means of flow cytometry, hemocytes were divided into three "subpopulations" according to their forward and side light scattering values, 1) region R1 (small cells of low complexity), 2) region R2 (large cells of intermediate complexity), and 3) region R3 (large cells of high complexity) (Fig. 2, H).



- The number of hemocytes assigned to different structural types was compared to the flow cytometry results using Spearman's correlation analysis. The comparison suggests that R1 is composed by blast-like cells and small hyalinocytes; R2, by a mix of large hyalinocytes, slightly granular basophilic granulocytes, and degranulated eosinophilic granulocytes; and R3, by the largest granulocytes of different affinity containing multiple and/or highly refractory inclusions.

Figure 2 – Structural types of Manila clam hemocytes (A—G) stained with azure-eosin after Romanowsky-Giemsa and their identification by flow cytometry (H). A – blast-like cells, B – hyalinocytes, C – slightly granulated basophilic granulocyte, D, E – neutrophilic granulocytes with different granularity levels, F – eosinophilic granulocytes, G – granulocytes with large refractive granules of different affinity; H – distribution of hemocytes by size (forward light scattering, FSC) and complexity (side light scattering, SSC): R1 – small cells of low complexity, R2 – large cells of intermediate complexity, R3 – large cells of high complexity.

The proportions of blast-like cells, hyalinocytes, and granulocytes in the hemolymph of Manila clams from all sites varied greatly and were not correlated to the heavy metal levels in mollusc tissues.

Results of the cell cycle analysis

- The analysis of DNA content showed that the distribution of cells in R1, unlike those in other regions, corresponds to that of the mitotic cycle (Fig. 3). Blast-like cells displaying high proliferative activity (as determined by flow DNA cytometry (Fig. 3, C)) were apparently divided into two size groups (Fig.2, A). The proportion of R1-cells in the active phases of the cell cycle (S, G_2, and M) was significantly positively correlated with the number of blast-like cells ($r = 0.518$, $p < 0.05$); however, none of these parameters were correlated with heavy metal content.

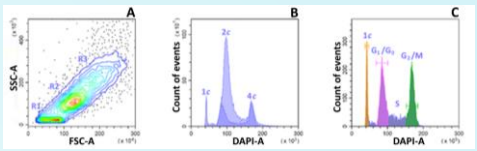


Figure 3 – Flow cytometric analysis of DNA content in Manila clam hemocytes with a haploid standard (spermatozoa of *R. philippinarum*). A – distribution of hemocytes by FSC and SSC parameters; B – distribution of all hemocytes by DNA content; C – distribution of cells in R1 by DNA content.

- Among all structural types, binucleated hemocytes were found, as well as cells with a pleomorphic nucleus (Fig. 4). Some nuclei contained heterogeneous chromatin or displayed signs of karyorexis and karyolysis.

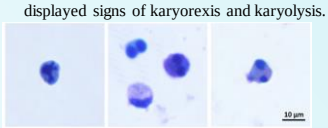


Figure 4 – Hemocytes with doubled and pleomorphic nuclei in Manila clams from Novik Bay.

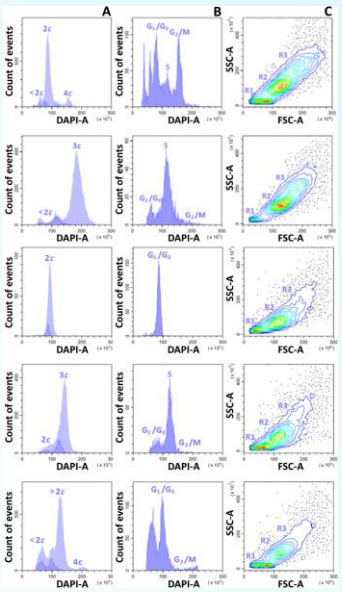


Figure 5 – DNA cell cycle alterations in hemocytes of Manila clams from Novik Bay. A – distribution of all hemocytes by DNA content; B – distribution of cells in R1 by DNA content; C – distribution of hemocytes by FSC and SSC parameters.

- Deviant DNA profiles were regularly observed in samples from all sites (Fig. 5). In some specimens, a portion of cycling R1-cells showed a decrease in the DNA content. Their frequency of occurrence histogram contained an additional peak closed to 3 *c* position in the middle of the S-phase plateau and demonstrated a selective loss of the G_1/M-cells. This pattern may indicate either aneuploidy or apoptosis; both phenomena are known to be associated with genotoxic heavy metal effects and are widely used as a tool for detecting continuous water pollution.
- These two scenarios are complementary rather than mutually exclusive. Hemocytes can receive an altered number of chromosomes as a result of mitotic errors; afterwards, these cells are eliminated by apoptosis or differentiate ignoring the cell cycle checkpoints.
- When aligning DNA profiles of blast-like cells (region R1) and differentiated cells (regions R2 and R3), the peak of differentiated cells was occasionally shifted "to the right" (relative to the diploid G_1-value of blast-like cells) or even reached tri- and tetraploid values. In such specimens, the proportion of hemocytes with doubled and pleomorphic nuclei increased; they comprised both young hyalinocytes and mature eosinophilic granulocytes.
- All these genetic abnormalities may be due to toxic effects of heavy metals. In Novik Bay, *R. philippinarum*, as well as *Callista brevisiphonata*, showed low potential for bioaccumulation compared with *Modiolus kurilensis* and *Panopea abrupta*. However, the level of bioaccumulated metals in mollusc tissues does not always correlate directly with their content in the environment.
- In *R. philippinarum*, aneuploidy displayed stronger correlation with sediment pollution than with bioaccumulation level.

The examination of aneuploidy and other DNA cell cycle alterations in Manila clam hemocytes may be used for marine environmental monitoring in impact waters of the Far East.