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CELL BIOLOGY

Fluorescent *in vitro* Phagocytosis Assay Differentiates Hemocyte Activity of the Bivalve Molluscs *Modiolus kurilensis* (Bernard, 1983) Inhabiting Impacted and Non-Impacted Water Areas

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Abstract—Hemocyte phagocytosis assay in vitro revealed no significant differences in the average values of phagocytic activity (PA) and phagocytic index (PI) in samples of the molluscs *Modiolus kurilensis* that inhabit non-impacted water areas (Kievka, Vostok, and Troitsa bays, the Sea of Japan). The only exception was an insignificantly lower value of PI for individuals from Troitsa Bay. The average values of PA for hemocytes of the total population of bivalves from non-impacted water areas were 52.0 ± 1.0%. On the basis of the inter-annual analysis of PA, the bivalve population from Vostok Bay was chosen as an appropriate reference group that is characterized by relatively stable PA values. In comparison with molluscs from the reference group and other investigated samples, *M. kurilensis* from the impacted water area (Sportivnaya Gavan, Amursky Bay) had considerably lower values of average PA (36.0 ± 2.0%) and PI (2.1 ± 0.05). The presented cell-mediated immunity assay can serve as an effective method for estimation of the physiological state of the bivalve in natural and aquacultural populations in order to differentiate molluscs from impacted and non-impacted water areas.

Keywords: innate immunity, phagocytosis, bivalve molluscs, physiological condition, hemolymph

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INTRODUCTION

The active development of aquaculture and biomonitoring studies of bivalve molluscs have led to the necessity to find and develop simple and effective methods to assess the physiological states of marine organisms. The central system that is responsible for the formation of physiological adaptations of molluscs to environmental changes for the maintenance of homeostasis and the immune defense is hemolymph, which is a transport and protective tissue of the internal environment. Therefore, the activity rates of cellular immunity factors in molluscs can be used as objective diagnostic criteria of their physiological state. Accordingly, phagocytosis, as a fundamental component of the protective function of the body, can serve as the main parameter for assessing the immune status. The results of the study of the physiological state of marine organisms under the influence of various factors, including those of an anthropogenic origin, were summarized by Mydlarz et al. [31]. It has been shown that different types of pollution have a negative effect on the body, suppressing and exhausting its protective function [16, 18, 20, 22, 24, 32, 33]; however, in rare cases, a reverse reaction of the immune response is observed [14]. It has been reported by many authors that a low level of pollution tends to produce an immunostimulatory effect [18, 20, 32, 33], while high concentrations have a suppressive effect [20, 28, 32, 33]. It should be noted that the experiments are usually carried out mainly with a short-term exposure to artificially simulated pollutions; thus, the experiment does not always show the true picture of the body's immune response that corresponds to the reaction of natural populations in their natural habitat. Moreover, there are only few studies (and none at all for the Russian waters of the Sea of Japan) on the activity of cellular immunity factors for bivalves from waters with varying degrees of anthropogenic load [11, 15, 21, 22, 26, 27, 29]. Despite the good knowledge of the morphological and functional parameters of hemolymph in such mussels as *Mytilus edulis* [11, 21, 30, 37], *M. galloprovincialis* [35], *M. trossulus* [21], and *Perna perna* [12], only very limited data are available in the literature for representatives of the widespread genus *Modiolus* [1, 36]. Therefore, the aim of this work was to reveal the natural variability of phagocytosis as the main mecha-

nism of cellular immunity in the bivalve molluscs *Modiolus kurilensis* from non-impacted and impacted areas of the Sea of Japan.

MATERIALS AND METHODS

Collection of animals and sampling area. The studies of the immune status were carried out on mature individuals of the bivalve molluscs *Modiolus kurilensis* with a shell length of 65–100 mm, which were collected in Peter the Great Bay (viz., Troitsa and Vostok bays) and Kievka Bay of the Sea of Japan. The animals were collected in May–June 2006 in accordance with the peak of their physiological activity [5] in non-impacted water areas (water areas that are not exposed to the direct impacts of human activities, natural and technical catastrophes, and that are dependent on the influence of local natural sources): Troitsa Bay, Vostok Bay, and Kievka Bay (28 individuals from each water area). In these areas, human economic activities are negligible and the geochemical conditions and the content of pollutants in water and soil correspond to natural pollution [3, 4, 8]. In June 2009, the bivalves were collected in Vostok Bay and in an impacted area of Amursky Bay that is located near the point source of emissions of pollutants and subject to the action of the local toxic load from the source, viz., in the Sportivnaya Gavan Bay (30 ind. from each area), and in June 2010 and 2011, in Vostok Bay (30 ind. from each area). According to the physico-chemical monitoring of water areas of the Far East, Sportivnaya Gavan Bay, located within the city of Vladivostok, does not meet the sanitary and epidemiological norms and the requirements for natural environment indicators for phenols, petroleum products, and for the number of pathogenic bacteria, such as Enterococci, the concentrations of oxygen, phosphorus, heavy metals, and a number of other parameters that vary significantly in different years [6–8, 9]. The animals were used in the experiment on the day of collection.

Hemolymph sampling. To assess the activity of cell immunity two indicators of phagocytosis were determined: phagocytic activity (PA), viz., the proportion of phagocytic hemocytes and the phagocytic index (PI), viz., the average number of bacteria that are ingested by one active phagocyte. For this purpose, hemolymph (300 μL) was taken from the hemal sinus of the posterior adductor muscle of each animal and centrifuged for 12 min at 800 g in a refrigerated centrifuge at a temperature of 15°C, followed by washing with a calcium- and magnesium-free salt solution (CMFSS) of the following composition: 436 mM NaCl, 10 mM KCl, 22 mM $Na_2HPO_4 \cdot 7H_2O$, 16 mM glucose, 12 mM N-(2-hydroxyethyl) piperazine-N'-2-ethanesulfonic acid (HEPES), containing 0.45 M ethylenediaminetetraacetic acid (EDTA). To obtain the primary cell culture, the cells were centrifuged again under the same conditions and resuspended in 300 μL of artificial sea water (ASW) with 1090 mOsm osmolality containing 460 mM NaCl, 9.4 mM KCl, 48.3 mM $MgCl_2 \cdot 6H_2O$, 6 mM $NaHCO_3$, 10.8 mM $CaCl_2 \cdot 2H_2O$, and 10 mM HEPES. To initiate the phagocytic reaction, a suspension from each animal, which was composed of 10000–15000 hemocytes, was transferred onto glass slides. The samples were incubated in a moist chamber at 17°C for 40 minutes for maximum cell adhesion to the substrate.

Preparation of the bacterial suspension. A strain of *Staphylococcus aureus* 636, which was previously isolated from marine aquatic organisms and stored in the collection of bacterial cultures of the Zhirmunsky Institute of Marine Biology (IMB), Far Eastern Branch, Russian Academy of Sciences in a deep freeze at –85°C was used as the biotic particles for initiating the phagocytic process *in vitro*. The bacteria were grown on solid Chapman medium (pH 7.0 ± 0.2) at room temperature to form well-defined colonies of a rounded shape; the cell mass was then washed with ASW. The resulting bacterial suspension was heat inactivated for 1 h at 72°C and then incubated with a 0.01% solution of fluorescein-5-isothiocyanate (FITC, MP Biomedicals) prepared in 0.1 M carbonate-bicarbonate buffer (pH 9.3). After 1 h, the labeled bacteria were washed three times by centrifugation at 1200 g for 20 minutes and resuspended in ASW. After the last centrifugation, the bacterial suspension was diluted in ASW to a concentration of 20 million CFU/mL. The cell concentration was determined using a Goryaev's hemocytometer.

Phagocytosis assay. The resulting bacterial suspension was added to the hemocytes of the primary cell culture in the amount of 15–20 cells per hemocyte. Fluorescence quenching of the non-internalized bacteria was carried out by incubating preparations with a 0.1% trypan blue solution in ASW for 12 min. Preparations were washed three times with ASW. At 1 hour and 20 minutes after the addition of bacteria to the cells, the reaction of phagocytosis was stopped by fixation for 1 hour with a 4% paraformaldehyde solution prepared with ASW. The phagocytosis reaction was carried out in two parallel replicates.

Data analysis. The resulting preparations were photographed in a Zeiss Axio Imager A1 fluorescence microscope (Germany) with an AxioCam MRc5 camera using a fluorescent filter (Zeiss BP 450-490/FT 510/LP 520). The images were analyzed in the AxioVs40 and V4.6.3.0 package of computer programs. For better visualization of cells borders, photos were taken using the technique of phase contrast. To assess the phagocytic status of hemocytes, the bacteria were counted in 200 hemolymph cells of one animal. To calculate the PI, the average number of bacteria was calculated that were ingested by one active phagocyte. The PA (in %) was calculated by the formula: $PA = Nph/Nh \times 100\%$, where *Nph* is the number of phagocytic hemocytes and *Nh* the total number of analyzed hemocytes. Statistical analysis was per-

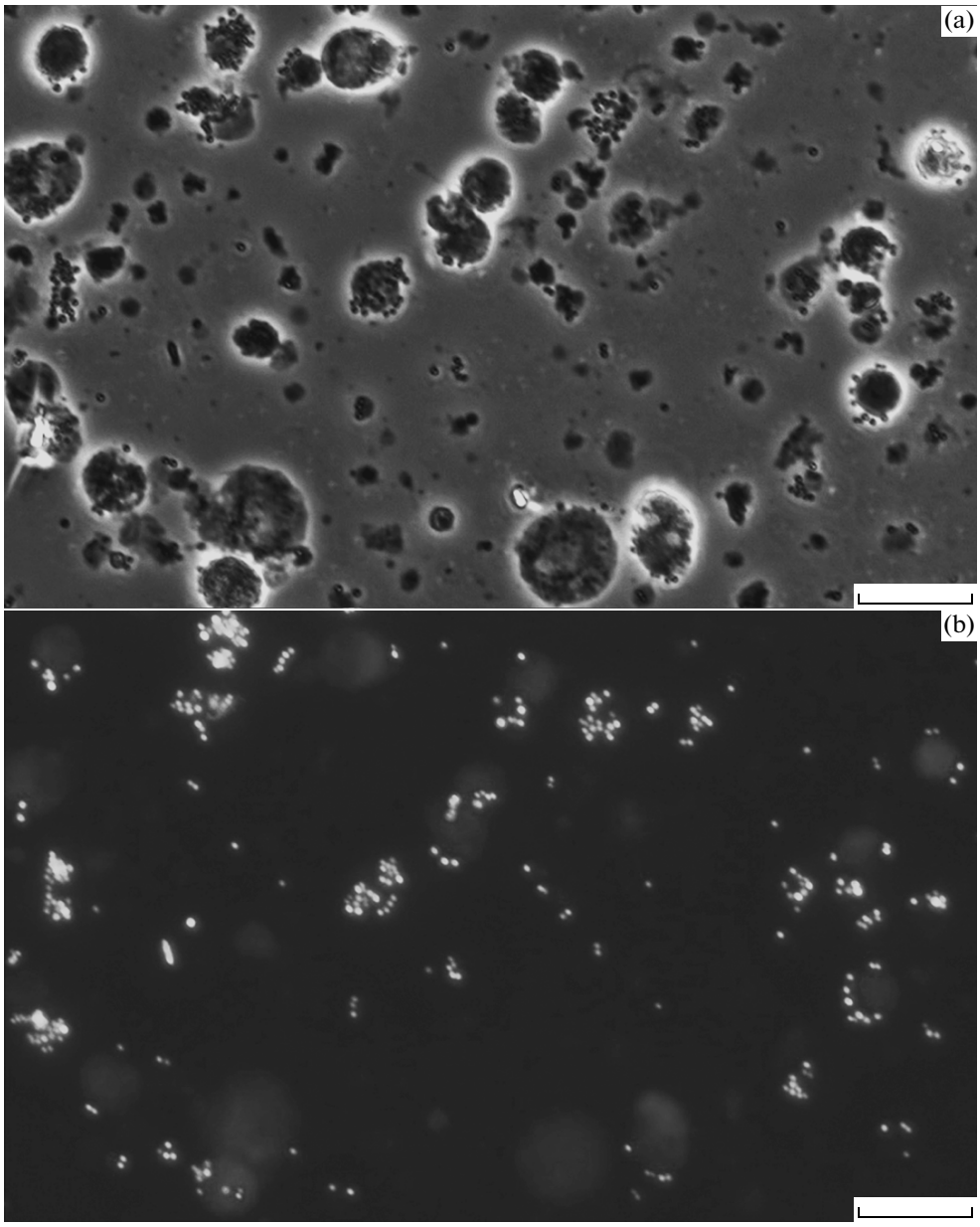

Fig. 1. The typical view of the *Modiolus kurilensis* hemocytes in the reaction *in vitro* phagocytosis with the heat-inactivated and FITC-labeled bacteria *Staphylococcus aureus*. (a) Phase contrast, (b) fluorescence. Scale 20 μm.

formed using the Microsoft Excel 2007 software package and Statistica 6.0. The Kolmogorov–Smirnov test showed that the distributions of PA and PI values obey a normal distribution law with significance levels $p > 0.05$ for all samples; therefore, further statistical analysis was performed using the parametric criteria. To test the significance of the hypothesis of either the absence or the presence of differences between the studied samples we used the one-factor dispersion analysis (ANOVA) and the paired *t*-test. All data in the work are presented as the mean value ± confidence interval.

RESULTS

The fluorescent analysis of *in vitro* phagocytic reactions showed that not all hemocytes of *Modiolus kurilensis* possessed phagocytic activity; phagocytes also varied in the number of ingested bacteria (Fig. 1). In the first phase of the study, the reaction of *in vitro* phagocytosis was quantitatively assessed; the ranges of variability of the phagocytic status of hemocytes of *M. kurilensis* were determined for non-impacted water areas, viz., the Vostok, Kievka, and Troitsa bays (Fig. 2). It was found that the average PA in molluscs that were caught in Kievka Bay was 48.2 ± 5%, the

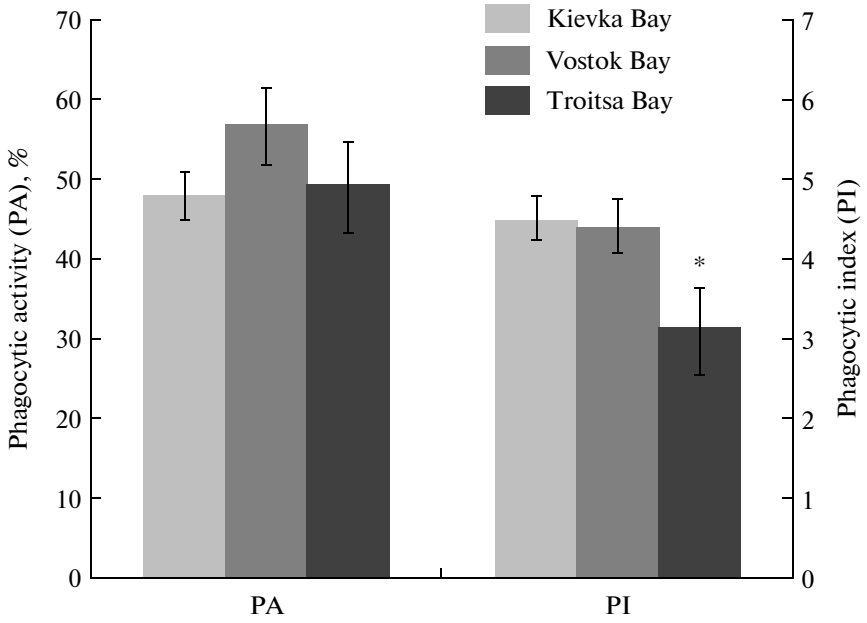

Fig. 2. A comparative analysis of the phagocytic status of the *Modiolus kurilensis* hemocytes in samples from non-impacted water areas. The histogram shows the mean values ± confidence intervals. * The significance of differences of average independent samples ($p < 0.05$).

average PI was 4.5 ± 0.3; the PA values ranged from 36 to 68% and the PI varied from 2.6 to 5.8. In bivalves that were caught in Vostok Bay, the average PA was 56.5 ± 4.6%, the average PI was 4.4 ± 0.36; the PA values ranged from 32 to 75% and PI ranged from 2.7 to 5.9. It is noteworthy that the values of phagocytosis parameters were average in a majority of the animals that were caught in Vostok Bay and in Kievka Bay, while the mollusc population from Troitsa Bay was dominated by individuals with a PI below 4.0 and with a PA less than 50%; their share reached 75% of the entire sample. On the average, the PA of the animals from Troitsa Bay was 49.3 ± 6.7%, while the PI did not exceed 3.1 ± 0.46. In this case, the range of variation of the phagocytosis parameters increased: the PA ranged from 27 to 82% and the PI ranged from 1.8 to 5.0.

A comparison of the PI values of three samples from the "clean" water areas using the one-way ANOVA test showed significant differences with a significance level of $p < 0.05$. Further analysis using the paired t-test revealed a significant decrease in the PI of animals from Troitsa Bay ($p < 0.05$) and the absence of significant differences ($p > 0.05$) between samples from Vostok Bay and Kievka Bay (Fig. 2). In the case of the PA values, application of the criteria showed no significant differences at a significance level of $p > 0.05$ (Fig. 2). The average value of the PA of hemocytes for the total sample of the animals from non-impacted waters areas was 52.0 ± 2.8%.

The second objective of the study was to assess the interannual level of the phagocytic status of hemocytes of *M. kurilensis* from Vostok Bay on the basis of PA monitoring. The average PA of animals that were collected in the water area was 56.5 ± 4.6% in 2006,

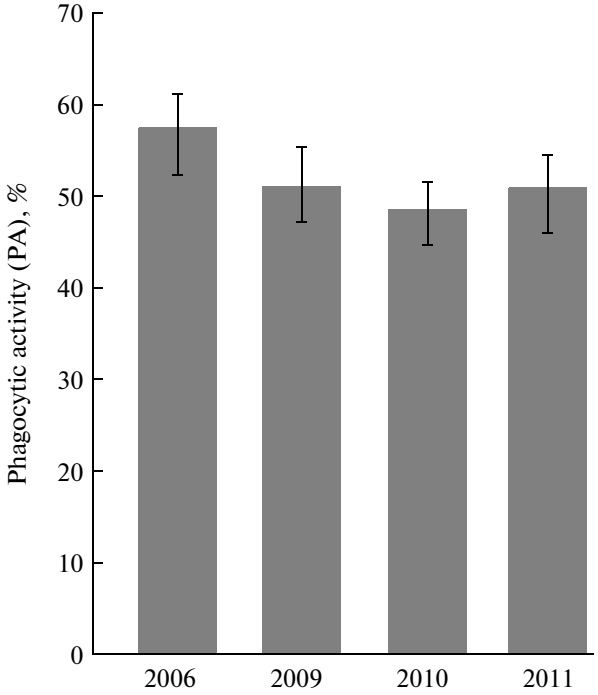

Fig. 3. An interannual estimation of the phagocytic status of *Modiolus kurilensis* hemocytes on the basis of analysis of the mean PA (Vostok Bay). The histogram shows the mean values ± confidence intervals.

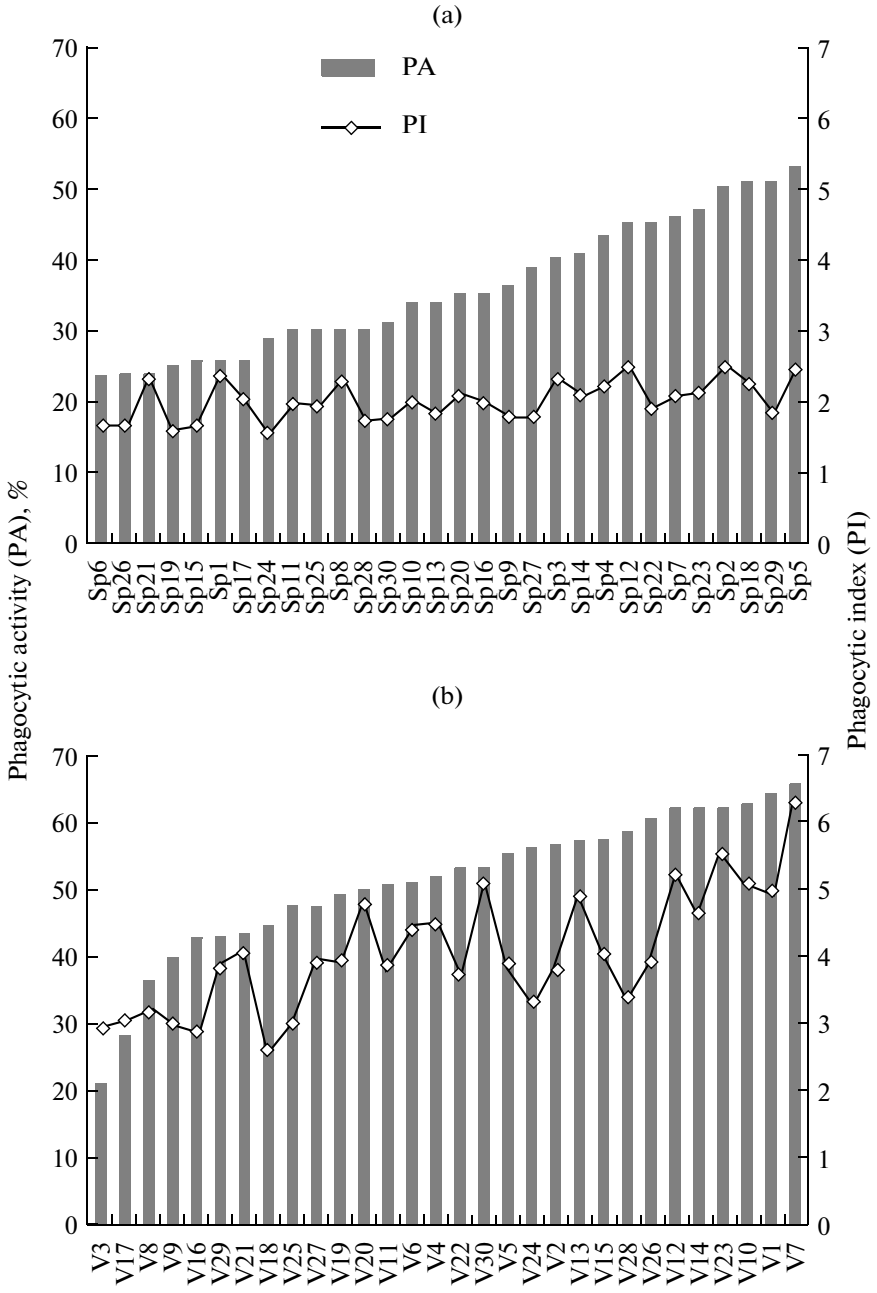

Fig. 4. A diagram of the phagocytosis of the *Modiolus kurilensis* hemocytes that were collected in Sportivnaya Gavan Bay (a) and Vostok Bay (b), in the range of phagocytic activity (PA). Codes of the number of test animals: Sp1–Sp30 and V1–V30.

51.0 ± 3.7%, in 2009, 49.0 ± 3.5% in 2010, and 51.0 ± 4.3% in 2011. The ANOVA and the *t*-test showed no significant differences ($p > 0.05$) in all the comparisons (Fig. 3). Therefore, at the next stage of the experiment, the molluscs from Vostok Bay, which were characterized by relatively stable values of the phagocytosis parameters that are representative of hemocytes for molluscs from non-impacted waters areas, were chosen as the reference group for a comparative analysis with the group from the impacted water area.

The final stage of the work involved a comparative analysis of the phagocytosis status of hemocytes of the *M. kurilensis* that were collected in 2009 in impacted and non-impacted water areas. As the diagrams (Fig. 4) of the ranked series, 77% of the molluscs from Sportivnaya Gavan Bay had PA values lower than 40% (Fig. 4a); while such values were recorded only in 10% of the molluscs from Vostok Bay (Fig. 4b). In this case, the PA of specimens from Sportivnaya Gavan ranged from 24 to 53%, and PI varied from 1.6 to 2.6. The maximum number of hemocytes involved in phagocy-

tosis in molluscs from Vostok Bay reached 65%, the minimum number of those was 21%, with PI ranging from 2.6 to 6.4.

In the reference group, the mean values of phagocytosis in molluscs collected in 2009 in Vostok Bay were 51.0 ± 3.7% for PA and 4.1 ± 0.32 for PI (Fig. 5). Bivalves caught in the area of Sportivnaya Gavan had lower average values of PA (36.0 ± 3.2%) and PI (2.1 ± 0.1). The t-test confirmed the significance of the differences ($p > 0.001$) in both the PA and the PI. It is noteworthy that the average values of both phagocytosis parameters were significantly lower ($p > 0.001$) for animals from Sportivnaya Gavan as compared with those for all the other samples that were investigated in this study (see the table).

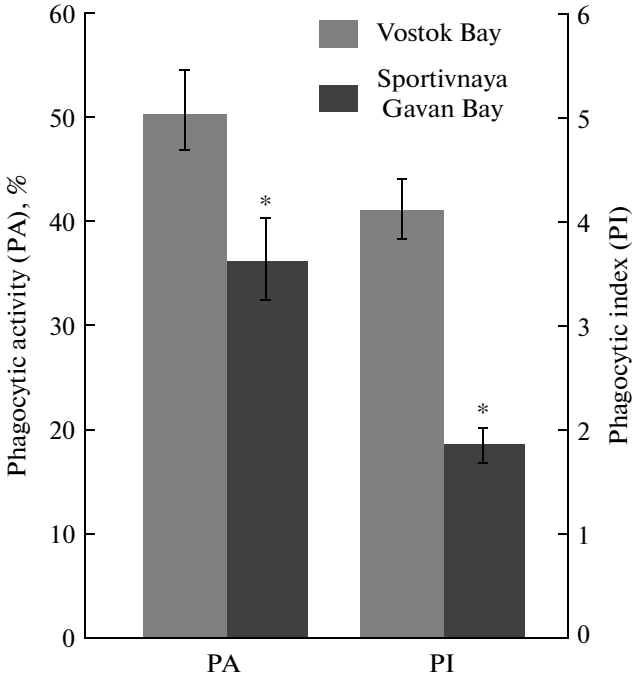

Fig. 5. A comparative analysis of the phagocytic status of *Modiolus kurilensis* hemocytes in the samples from non-impacted (Vostok Bay) and impacted (Sportivnaya Gavan Bay) water areas. The histogram shows the mean values ± confidence intervals. * The significance of differences of average independent samples ($p < 0.001$).

DISCUSSION

Disorder of the phagocytic function of hemocytes significantly weakens the entire system of protective mechanisms of marine invertebrates [35, 37]. It is known that not all types of mollusc hemocytes possess phagocytic activity; acidophilic granulocytes are most effective in the reaction [13, 25]. A number of studies have shown that the phagocytic activity is revealed on average by 40 to 90% of hemocytes [13, 18, 34]; while the PI varies significantly depending on the type of particle that is used as an antigen [37]. In the studied individuals of *Modiolus kurilensis* from non-impacted water areas of the Sea of Japan, 82% of the hemolymph cells performed phagocytosis. We found no significant differences of the mean PA and PI values between the samples from the studied water areas, except for the animals from Troitsa Bay, which showed a statistically significant reduction of the mean PI in comparison with that of the molluscs from Kievka Bay and Vostok Bay. The PI values of animals from Troitsa Bay may be caused by some fluctuations in hydrological parameters and many other factors, such as the number and composition of the microphytes, by the pattern of the currents, which could bring pollutants, and by the development of parasitic invasions [19, 31]. Despite this, the study enabled us to obtain a range of variation of the mean values of PA (52.0 ± 2.8%) for the animals that inhabit the waters of the Sea of Japan, which are not subject to extreme impacts of any environmental factors.

The hemocyte phagocytic status of *Modiolus kurilensis* from non-impacted and impacted water areas of the Sea of Japan in 2006, 2009, 2010, and 2011

Year	Station	Phagocytosis parameter	
		phagocytic activity (%) (mean value ± confidence interval)	phagocytic index (mean value ± confidence interval)
2006	Kievka Bay	48.2 ± 5	4.5 ± 0.3
	Vostok Bay	56.5 ± 4.6	4.4 ± 0.36
	Troitsa Bay	49.3 ± 6.7	3.1 ± 0.46*
2009	Sportivnaya Gavan Bay	36.0 ± 3.2**	2.1 ± 0.1**
	Vostok Bay	51.0 ± 3.7	4.1 ± 0.32
2010	Vostok Bay	49.0 ± 3.5	–
2011	Vostok Bay	51.0 ± 4.3	–

* The significance of the differences of average independent samples ($p < 0.05$).

** The significance of the differences of average independent samples ($p < 0.001$).

Different types of pollution can alter the immune status of molluscs; the resulting effect depends on the type and concentration of pollutants. Thus, in the initial periods of the experiment, the PA of hemocytes of the genus Mytilus either did not change or increased only insignificantly in the presence of low concentrations of copper (0.2 ppm) [32, 33], cadmium (40 μg/L) [20], estrogen (0.1–5 mM) [17], and wastewater (0.1–5 mM) [11, 21, 22]. At further exposure or with increased doses of the agent, phagocytosis was suppressed, the release of free radicals was inhibited, and the resistance of the animals to bacterial infections accordingly decreased [20, 28, 32, 33]. A reduced PA was recorded in the hemolymph of *Crassostrea virginica* under pulsed exposure to cadmium (1 μg/L), but a 2-week exposure to this metal in the same concentration induced an increase in the content of circulating cells and caused the activation of phagocytosis [18]. A complicated pattern of the immune response was also detected in *Crassostrea gigas*: a 500 ng/L cadmium concentration caused a rise in the number of dead hemocytes and increased mortality of individuals; however, at a longer exposure (21 days), hemocyte viability and functional activity grew and animal mortality was reduced as a result of the suppression of the activity of pathogenic organisms [14]. As well, heavy metals may not have a toxic effect, for example, *C. virginica* hemocytes accumulated copper (0.2 and 0.5 ppm) for antimicrobial protection [23]. Data on the inhibition of hemocyte phagocytosis in different species of molluscs during long-term exposure to pollutants have been reported by different authors [22, 24, 26].

Studies of the influence of anthropogenic factors on the marine ecosystem of Sportivnaya Gavan Bay have previously shown that a change in the chemical composition of the seawater and pollution of sediments on the sea bottom cause disorders of the reproductive function of benthic organisms [2, 10, 38]. Moreover, our investigation of the activity of the cell immunity of *M. kurilensis* from the same waters revealed deviations in the phagocytosis parameters as compared with those of animals from non-impacted waters areas. Molluscs from the impacted water area had significantly reduced average values of PA (36.0 ± 3.2%) and PI (2.1 ± 0.1). The average values of the phagocytosis parameters of hemocytes in molluscs that were collected in the same year in Vostok Bay were 4.1 ± 0.32 (PI) and 51.0 ± 3.7% (PA); these parameters did not significantly differ from those in the interannual dynamics.

An overview of studies in this direction leads to the conclusion that the process of adaptation to extreme factors is accompanied by profound changes in the immune system. The first stage of stress can occur in different ways: depending on the strength and type of exposure, as well as on individual properties of an organism, stress either stimulates or suppresses the immune response. At the transition to the second stage, the hemolymph response to stress is expressed in increased hemocyte phagocytic activity and higher production of reactive oxygen species, which leads to a short-term increase in the resistance of the immune system in molluscs. When molluscs that inhabit impacted waters are exposed to a long-term or chronic stress, we observe a persistent suppression of the immune protection and its transition to a qualitatively new level of function, which is also indicated by the results of this study.

Interpretation of the effects of environmental factors on the immune parameters of molluscs still causes some difficulties because it requires the integration of a wide range of parameters of the environment and the organism *per se*. Taking the individual characteristics of the particular mollusc species that live in non-impacted water conditions with a certain hydrological regime into account, we can determine the limits of the normal variability of various parameters and develop criteria on this basis for assessing the physiological state; a significant deviation can be regarded as a reaction to stress. Based on these results, we can recommend the phagocytic activity of hemocytes, which is a relatively stable parameter under normal environmental conditions and significantly decreases under conditions of prolonged stress, as a measure that objectively differentiates the physiological state of *M. kurilensis* from non-impacted and impacted water areas.

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