Motility of adherent hemocytes as a potential marker of immunocompetence and immunotoxic effects in bivalves

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Introduction

Hemocytes are the formed elements of hemolymph, found as circulating immunocompetent cells in the open vascular system of bivalve molluscs. In a first phase of response to microbial threat, hemocytes migrate toward infected sites[1] to deliver, subsequently, effective immune responses associating reactive oxygen and NO production, antimicrobial peptides secretion and phagocytosis. In marine mussels, while exclusively based onto innate mechanisms, recruitment and activation of hemocytes have been suggested to be pathogen-specific.[2]

Mussels are well adapted to fluctuant coastal and estuarine environment, being tolerant to large-scale variations of temperature, salinity and oxygen levels.[3] Therefore, mussel beds extend from intertidal rocky shores and paralic locations to subtidal oceanic waters. Since 2011 however, abnormal episodes of mortality are described in various mussel-rearing sites of the Atlantic coast, reminiscent of those repeatedly affecting oysters since the beginning of 1960s.[4] These increased incidence and prevalence of mussel lethal diseases are of multiple etiology, resulting from the combined action of various stressors, including elevated temperature and turbidity, microbial and chemical contamination, physiologic stress associated to reproduction. The pathogenic bacterium *Vibrio splendidus* is supposed to be involved in mussel mortalities.[5] To find answers to this issue, Ifremer (*Institut Français de Recherche pour l'Exploitation de la Mer*) has proposed the Mytilobs program.[6]

In the present study, we communicate preliminary results focused on the effects of temperature and water contamination by xenobiotics onto the first phase of hemocyte immune response, *e.g.* cell migration. To quantify individual cell velocities *in vitro*, we have used an approach based on nuclei tracking under time-lapse microscopy imaging.[7] The hemocytes were collected from individuals at two distinct sampling sites differentially impacted by pollution, highly contaminated embankments in the largest seaport of west Europe (Le Havre) and a less contaminated area corresponding to the rocky shore of Yport.[8] The main objective of the work was to determine if mussel hemocyte migration velocity is affected by temperature or sampling site.

Materials and Methods

Adult mussels (4-5 cm shell length), *Mytilus sp.*, were collected between December and February in 2012 on the intertidal rocky shore of Yport, France (49°44 N, 0°18 E) or from the sea harbor of Le Havre, in a subtidal bed of basin Vétillard (49°74 N, 0°31 E). Mussels were transported to the laboratory for immediate analysis. Hemolymph was aseptically withdrawn, in a BSL2 laminar flow cabinet, from the posterior adductor muscle sinus, by gentle aspiration with a 1 mL syringe equipped with a 22G needle.

Volumes of crude hemolymph corresponding to 100 µL or 200 µL were deposited in 35-mm culture dishes (Corning, New York, NY, USA). After 30 min of sedimentation, hemolymph was removed and each well or dish were washed twice by using marine physiological saline solution (MPSS) containing (in mM): 470 NaCl, 10 KCl, 10 CaCl2, 10 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), 48.7 MgSO4, pH 7.8, 0.2 µm sterile filtered. Osmolality of MPSS was checked to be close to local seawater osmolality (963 mOsmol.kg^{-1}) by using a vapor pressure osmometer (WescorElitech, Logan, UT, USA).

To quantify velocity, cells in culture were incubated with 10 µM of the nuclei-specific fluorescent probe Hoechst 33342 for 30 minutes at 14°C. A culture dish was placed on the stage of a TE-2000 inverted microscope (Nikon, Champigny-sur-Marne, France) equipped for epifluorescence excitation (HBO arc lamp with 377/50 nm bandpath filter) and time-lapse imaging. A Peltier temperature controller (PDMI-2 and TC-202A; Harvard Apparatus, Holliston, MA, USA) keeps preparation at 14°C for extended live cell imaging. Wild-field epifluorescence time-lapse imaging was performed with a x10 objective (numerical aperture 0.3). A VCM-D1 shutter (Uniblitz, Vincent Associates, NY, USA) was added in the illumination pathway to cut off the excitation light between two image recordings. Camera and shutter were controlled by Metamorph (Molecular Device, Sunnyvale, CA, USA) as acquisition software. A CCD Coolsnap EZ camera (Photometrics, Tucson, AZ, USA) captured 12-bit digital of 1392×1040 pixels greyscale images every 30 s for 30 min (409 nm long path emission filter). Camera and software were calibrated to express distance in microns. Time-series image stacks were imported into Metamorph Analysis software. The track Objects application (available with Multi-Dimensional Motion Analysis option) was started. Typically, for each biological replicate, 20 nuclei were randomly chosen to be tracked.

Results and Discussion

Velocity of hemocyte migration was followed by dynamic microcopy *in vitro*. After plating, cells were allowed to adhere 20 min and then imaged in MPSS, by using time-lapse recordings under controlled temperature. As described earlier,[9] the cell population was extremely motile. Individual hemocytes exhibited fast outstanding morphology changes. This feature renders difficult image-to-image single cell tracking because cells escaped to automatic shape recognition. This difficulty was overcome by using nuclei tracking with the DNA fluorescent probe Hoechst 33342[7] since nuclei shapes

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remained remarkably constant and were easy to follow over time. After 30 min of wide field epifluorescence recording at a rate of 1 image/30 s, off-line tracking was carried out on a set of 20 nuclei, selected randomly in the microscopic field (Figure 1A). Figure 1B shows the resulting determined trajectories. Mussel hemocytes migrated by random taxis (Figure 1C). Cells escaping the recorded field or belonging to aggregates were excluded from the analysis.

In order to determine the influence of temperature elevation on hemocyte migration speed, the bath temperature was changed in steps of 30 min from 14 to 22°C. This resulted in a speed up of mussel hemocyte velocity. This response was evidenced at the single cell level and was also found when migration speed was expressed as a mean populational velocity, computed from the average migration of twenty individual cells randomly sample in multiplicate experiments (Figure 2A).

In a previous work, we have studied the level of multi xenobiotic resistance (MXR)-efflux activity in hemocytes collected from the same contrasted sites, in terms of xenobiotic contamination, than in the present work. It was concluded that, in *Mytilus sp.*, the MXR phenotype is mediated by an ABCC/MRP-type transporter, induced by chemical contamination in eosinophilic granular hemocytes.[8] Herein, we found that motility of hemocytes from Le Havre harbor is significantly diminished par comparison to migration speed of cells obtained from mussels sampled from the reference site, Yport (Figure 2B).

These results have some physiological implications. Water temperature and levels of chemical contamination are determinants of immune cell migration speed in *Mytilus edulis*. In particular, we provide the first evidence for a decrease of immune cell migration activity in organisms collected from polluted areas. This observation may be understood as a decrease of immunocompetence in conditions of high water chemical contamination. Further experiments are necessary to confirm these data and to investigate the regulations of mussel hemocytes motility in the presence of both microbial and chemical contaminants.

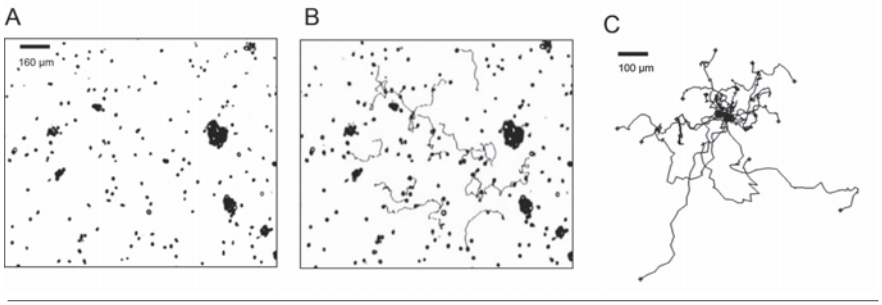
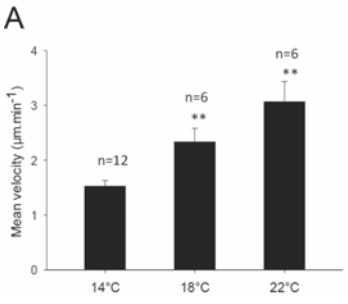


Figure 1. *Mytilus edulis* hemocyte tracking by time lapse imaging of Hoechst 33342-stained nuclei. A) 500 µL of freshly withdrawn hemolymph were plated on glass bottom 35-mm dishes and covered with 2 mL marine physiological saline solution. Nuclei were stained with Hoechst 33342. Last wide field epifluorescence inverted micrograph from a 30-min 0.033-Hz time-lapse sequence under x10 magnification used to track nuclei (HBO arc lamp, excitation 377/50 nm, emission 409 nm). B) Twenty hemocyte tracks computed from nuclei in motion for 30 min in the microscopic field and distinct from aggregates are superimposed to the image shown in A. C) Overlay of twenty hemocyte trajectories displayed from a common origin. The point reached by each cell after 30 min recording is indicated by a small cross.



B

	Mean Velocity (µm.min^{-1})	*n*
Yport	2.72**	5
Le Havre	1.89	4

Figure 2. Effects of bath temperature or mussel sampling site on hemocyte velocity. A) Dependence of mean hemocyte velocity to temperature. Freshly collected hemocytes were deposited in a temperature-controlled recording chamber filled with marine physiological saline solution. Replicated cell cultures obtained from individuals n=6-12 collected from a mussel bed of basin Vetillard (seaport of Le Havre). Mean cell velocities were determined from 20 trajectories recorded during 30 min. B) Dependence of mean hemocyte velocity to mussel origins. Hemocytes were collected from mussels originating from the rocky shore of Yport or from the seaport of Le Havre, placed at 14°C and used to 30-min endpoint mean migration velocity recordings. Migration speeds are expressed as mean±SEM. Values significantly different from velocity of hemocytes from Le Havre at 14°C are indicated (*P<0.05, **P<0.01, ***P<0.001, Student's *t*-tests).

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