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The mass mortality of blue mussels (*Mytilus* spp.) from the Atlantic coast of France is associated with heavy genomic abnormalities as evidenced by flow cytometry

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ABSTRACT

Since 2014, France's blue mussel industry has been facing heavy mortality outbreaks (90–100%) affecting both juveniles and adults. This report presents evidence of heavy genomic abnormalities associated with mortality outbreaks in blue mussels, *Mytilus edulis-galloprovincialis*, from the Atlantic coast of France. In this study, ploidy characteristics of hemic cells were investigated using Flow CytoMetry (FCM), revealing an unusual, broad continuum of ploidy distribution from hypodiploidy to tetraploidy. FCM was additionally used to evaluate, at individual and populations levels, different thresholds of genomic abnormality (GA%) using the percentage of non-diploid nuclei. Individual mussels were considered to be abnormal when more than 10% of hemocytes in S–G2/M phase were present. At the population level, a threshold of 6% for the mean intensity of the abnormality is proposed, which means in the population, more than 6% of individual mussels have to present with more than 10% of their hemocytes in S–G2/M phase. GA% was found to be significantly predictive of the final mortality. Based on the established thresholds, only two mussel stocks analyzed in this study were considered to have good cytogenetic quality, while all other stocks appeared to be affected. FCM offers a very powerful tool to help manage current blue mussel mortality in France. We also believe that annual and extensive determination of cytogenetic quality of wild and cultivated mussel beds along with exclusive use of FCM-qualified mussel seeds should be a priority.

1. Introduction

Mussels are of great importance in the marine ecosystem as they actively filter the water and are a food source for many organisms including humans. Like other bivalves, mussels are frequently used in biomonitoring programs due to their biological characteristics and wide geographic distribution (Viarengo et al., 2007). Hence, the use of naturally occurring mussels is a very direct approach to *in situ* biomonitoring programs for environmental effects related to municipal, agricultural and industrial effluents. In France, in addition to this eco-sentinel action, mussels are also of great economic importance, and the blue mussel industry (*Mytilus edulis* and *Mytilus galloprovincialis*) produces more than 77,000 metric tons each year (FAO, 2014). This industry relies completely on natural spatfall limited to the Atlantic coast, principally in the Pertuis Charentais zone and the Bourgneuf Bay (Goulletquer and Héral, 1997). However, since 2014, the production of French blue mussels decreased dramatically because of sudden and atypical mass mortality outbreaks (90–100%) for which the causes remained uncertain, and both juvenile and adult blue mussels were affected (Béchemin et al., 2015).

Mortality of both naturally occurring and cultured blue mussels has been reported in many other locations around the world (Fuentes et al., 1992, 1994; Myrand and Gaudreault, 1995; Tremblay et al., 1998; Myrand et al., 2000). The extent of mortalities can be highly variable, and several stress-related factors have been investigated, including pathogens, pollution, temperature, food depletion and reproduction, leading to a preliminary conclusion that none of these factors can alone explain the observed mortalities. Several studies have already shown that the exposure of mussels to environmental contaminations gives rise to various DNA damages, including abnormalities in DNA content and distribution as well as progressive development of circulating aneuploid-polyploid cells in the hemolymph. Additionally, in several marine mollusks species including mussels, circulating aneuploid-polyploid cells in the hemolymph are also particularly observed during the development of a frequently fatal leukemia-like cancer disease called hemic (or disseminated) neoplasia (Elston et al., 1992; Landsberg, 1996; Bihari et al., 2003; Vassilenko and Baldwin, 2014). This malignant disease has been reported in 15 species of marine bivalves with a world-wide distribution including 4 species of oysters, 6 species of

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clams and 5 species of mussels (Elston et al., 1992; da Silva et al., 2005; Delaporte et al., 2008; Galimany and Sunila, 2008; Le Grand et al., 2010; Diaz et al., 2011). The disease is characterized by enlarged, circulating, continuously dividing neoplastic cells in the hemolymph, which in late stages, completely replace normal hemocytes and penetrate various tissues such as connective tissue, gonads, the mantle and foot (Barber, 2004; Carballal et al., 2015). This disease is commonly fatal and causes significant mortalities and decreases in market harvests in economically important species such as mussels, clams and cockles (Bower, 1989; Elston et al., 1992; Barber, 2004; Carballal et al., 2015). As reviewed by Barber (2004) and more recently by Carballal et al. (2015), different causes have been suggested regarding etiology of hemic neoplasia including genetic alterations, virus, retrotranspons, and contaminants. Although the exact role of chemical contamination is still controversial, several studies addressed the role of anthropogenic pollution and some of them finally suggested that exposures to environmental contaminants may be triggers for the disease by inducing rapid genetic instability promoting or at least accelerating disease progression (Arriagada et al., 2014). Whatever their origin, i.e., linked to genotoxic effect of pollutants and/or to a leukemia-like disease, the genomic abnormalities of circulating cells in the hemolymph of mussels can be reliably studied by FCM which is the most valuable technique for measuring the DNA content and for cell cycle analysis by permitting the characterization of cells in the G0/G1, S and G2/M phases and additionally detecting populations that have abnormal amounts of DNA (polyploid or aneuploid). Comparatively to other available methods (histology and hemocytology), this approach is now recognized as a non-tedious, non-subjective, cost-effective and high precision technique to study the observed DNA damage, cell cycle alterations and ploidy changes (Elston et al., 1990; Moore et al., 1991; Reno et al., 1994; da Silva et al., 2005).

In the context of mass mortality outbreaks affecting blue mussels in France since 2014, we hypothesized that the observed mortality was probably linked to their poor cytogenetic quality, particularly in terms of ploidy variations and cell cycle DNA alterations affecting circulating cells in the hemolymph. Indeed, in mussels experiencing the loss of normal hemocytes and their replacement by abnormal (DNA-damaged, aneuploid, polyploid, neoplastic) cells would at least leave the animal immunocompromised and, therefore, more susceptible to mortality. Additionally, besides their implication in defense and tissue repair, hemocytes are responsible for digestion, absorption and transportation of nutrients within the animal. Hence, any genomic abnormality concerning circulating cells of hemolymph would greatly decrease the overall fitness of the animal leading to starvation and then inducing its death (or at least increasing its susceptibility for mortality) (Barber, 2004; Carballal et al., 2015). To explore this hypothesis, we used FCM to study the DNA content and cell cycle characteristics of hemocytes collected from various wild and cultivated blue mussels stocks along the French Atlantic coast. Biomonitoring of various areas situated in the Pertuis Charentais zone and Bourgneuf Bay was conducted by comparative FCM analysis of hemolymph cells before and after the mortality events that affected blue mussels (*M. edulis/galloprovincialis*) stocks during the winter-spring of 2015. Additionally, hemocytological analyses were performed on some selected contrasting samples to visually identify potential diseased cells based on their inability to adhere and their characteristic morphology.

2. Materials and methods

2.1. Field sampling

In the present work, the studied blue mussels are named *M. edulis* and *M. galloprovincialis*. Indeed, mussel aquaculture in France is based on these two very closely related species which can interbreed in areas of co-occurrence and produce fully fertile hybrids that are morphologically and physiologically intermediate between the parental taxa (Bierne et al., 2003). Adult specimens of 4–6 cm long were collected before the mortality in January and February of 2015 from 7 different mussel stocks distributed in four areas across the Pertuis Charentais zone and three others across the Bourgneuf Bay (Fig. 1). Each mussel stock was constituted of 300 adult mussels and was maintained alone in a separate tank and provided with 250-L/h of unheated and UV-filtered seawater. The mortality events were declared finished when no dead mussels were recorded in hatchery condition and three months after the peak of mortality in the field. For each mussel stock, the final mortality level was calculated after counting dead and alive mussels in the hatchery condition. This final mortality level was also compared to that estimated in open field conditions by mussel farmers and state services. Finally, at this time, the same analyses accomplished before the mortality were applied again to surviving mussels collected from the same open field sites and also from some surviving mussels maintained in the experimental hatchery.

2.2. Hemolymph collection, FCM and hemocytology

Hemolymph samples were individually taken from 80 to 120 mussels per site and immediately used for FCM and hemocytological analyses. Hemolymph was withdrawn in a non-destructive manner from the adductor muscle of each individual mussel with sterile 1 mL syringe fitted with a 26 gauge needle. For each animal, a volume of approximately 0.2 mL of hemolymph was collected and stored temporarily in an Eppendorf microcentrifuge tube on ice to prevent clumping.

For FCM analysis, hemolymph samples were transferred into microtubes maintained on ice. For each individual mussel, 0.1 mL of hemolymph was used, without any fixation step, for nuclei extraction and staining. Each hemolymph sample was first diluted in 1 mL of ice-cold nuclei extraction buffer (5 mM $MgCl_2$, 85 mM NaCl, 10 mM Tris, 0.1% Triton X100, pH 7). Extraction of nuclei was realized by detergent (Triton X-100) action and facilitated by several pipetting. Extracted nuclei were then purified by filtration through a 30 µm nylon sieve (Celltrics, Sysmex) to eliminate membranes and large clumps. Samples were then simultaneously treated with DNase-free RNase A (Sigma R4875, 50 µg mL^{-1}) and stained at room temperature for 45 min in the dark with propidium iodide (PI, Sigma, P4170) at a concentration of 50 µg mL^{-1} in a 2 mL final solution. For some selected samples showing contrasting FCM patterns, ploidy level estimations were also done by using nuclei from trout red blood cells (TRBC, Beckman Coulter, DNA Reference Calibrator, 629972) as an internal standard. In this case, 2 µl of TRBC were mixed into each hemolymph sample and then the nuclei of both internal standard and sample were subjected to the same extraction and staining procedures as described above.

For each mussel stock, FCM analyses started right after the PI-staining period and lasted for 60–90 min where the PI staining was observed to be stable (da Silva et al., 2005). Analysis was performed on a CyFlow® Ml flow cytometer equipped with a 532 nm, 30 mW green laser (Sysmex, Sainte Geneviève des Bois, France). PI fluorescence, which is related to the DNA content of each nucleus, was detected on the FL3 detector (orange-red fluorescence detector at 550–600 nm). For each sample, 10,000 nuclei were counted at low flow rate (15 µl min^{-1}). Cell-cycle estimates were done using the method described in (da Silva et al., 2005) for removing doublets and debris. Thus, in order to distinguish nuclei in the G2/M phase from doublets of G0/G1 nuclei that have the same DNA content, FL3-area vs. FL3-width dot-plots were used to gate single nuclei. Thus a region (R1) was drawn on these dot-plot representations to discriminate single nuclei from doublets. After gating them on R1, single nuclei were next plotted on a FL3-area