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Oyster's cells regulatory volume decrease: A new tool for evaluating the toxicity of low concentration hydrocarbons in marine waters

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ABSTRACT

Human activities require fossil fuels for transport and energy, a substantial part of which can accidentally or voluntarily (oil spillage) flow to the marine environment and cause adverse effects in human and ecosystems' health. This experiment was designed to estimate the suitability of an original cellular biomarker to early quantify the biological risk associated to hydrocarbons pollutants in seawater. Oocytes and hepatopancreas cells, isolated from oyster (*Crassostrea gigas*), were tested for their capacity to regulate their volume following a hypo-osmotic challenge. Cell volumes were estimated from cell images recorded at regular time intervals during a 90 min-period. When exposed to diluted seawater (osmolalities from 895 to 712 mosm kg^{-1}), both cell types first swell and then undergo a shrinkage known as Regulatory Volume Decrease (RVD). This process is inversely proportional to the magnitude of the osmotic shock and is best fitted using a first-order exponential decay model. The Recovered Volume Factor (RVF) calculated from this model appears to be an accurate tool to compare cells responses. As shown by an about 50% decrease in RVF, the RVD process was significantly inhibited in cells sampled from oysters previously exposed to a low concentration of diesel oil (8.4 mg L^{-1} during 24 h). This toxic effect was interpreted as a decreased permeability of the cell membranes resulting from an alteration of their lipidic structure by diesel oil compounds. In contrast, the previous contact of oysters with diesel did not induce any rise in the gills glutathione S-transferase specific activity. Therefore, this work demonstrates that the study of the RVD process of cells selected from sentinel animal species could be an alternative bioassay for the monitoring of hydrocarbons and probably, of various chemicals in the environment liable to alter the cellular regulations. Especially, given the high sensitivity of this biomarker compared with a proven one, it could become a relevant and accurate tool to estimate the biological hazards of micropollutants in the water.

1. Introduction

The marine environment appears to be the final receptacle of many chemicals by the way of terrestrial runoffs, atmospheric depositions and inadvertent releases. Among these contaminants, besides natural seeps (Kvenvolden and Cooper, 2003), 0.4 million tons a year of petrochemical compounds are discharged in natural waters through spills from vessels, underwater pipes, offshore platforms and oil refineries (Schwarzenbach et al., 2006). The final concentration of hydrocarbons found in the ocean results from both microbial activity (Leahy and Colwell, 1990) and abiotic weathering processes such as spreading, evaporation, emulsification, dissolution, photo-oxidation and sedimentation (National Research Council (US) report, 2003). After an early period of considerable degradation, the hydrocarbon concentration tends to stabilize at a low concentration (Nocentini et al., 2000). Analytical techniques are used to determine the levels of the numerous compounds of hydrocarbons in water (Stenstrom et al., 1986). However, they do not report the synergic and antagonist effects of complex mixtures on the ecosystems. Therefore, the quantification of their biological hazards requires the use of relevant integrative biomarkers. The effects of contaminants at lower levels on biological organizations occur more rapidly than those at higher levels (Schettino et al., 2012). Therefore, the measurement of subcellular and cellular biomarkers to pollutants exposure is frequently used in environmental monitoring. Until now, ecotoxicology research essentially focused on biochemical and molecular processes (*e.g.* detoxifying and antioxidant enzymatic activities, lipid/protein oxidation, metallothioneins expression and DNA damages). In contrast, studies of cellular physiology alterations due to chemical pollutions are scarce (e.g. Droguet et al., 2012; Haberkorn, 2014 in

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the *Crassostrea gigas* oyster's cells). The complex cell working, based on rapid and standardized regulations, is yet liable to be impaired by pollutions.

This work aims to describe an original and sensitive cellular biomarker based on the capacity of all animal cells to regulate their volume under anisosmotic conditions. Cell volume regulation is of major physiological importance since it takes part in the integrity and functions of the cell by maintaining an optimal concentration of intracellular enzymes and metabolites. Their membrane being highly permeable to water (Hoffmann and Simonsen, 1989), all animal cells can potentially experience swelling or shrinkage episodes, causing complex mechanisms of volume restoration. These mechanisms have been extensively reviewed in vertebrates, essentially mammals (Wehner et al., 2003; Hoffmann et al., 2009; Koivusalo et al., 2009; Hoffmann and Pedersen, 2011). In brief, they include sensors of cell volume, intracellular signalling systems coupled to these sensors and finally, membrane transporters mediating the release/uptake of osmotically active compounds to compensate for cell swelling/shrinkage and to restore optimal cell function. For example, the regulatory volume decrease (RVD) process caused by a hypo-osmotic cell swelling results in intracellular osmolytes and osmotically obliged water efflux. The cell volume regulation mechanism has seldom been the target of applied research (Morabito et al., 2013). The objective of this preliminary study is to test whether the volume regulation capacity of the oyster *C. gigas'* cells, can help in the biomonitoring of coastal seawaters. Zhang et al. (2011) then Cappello et al. (2013) already reported perturbations in cellular osmoregulatory mechanism caused by exposure to toxic compounds in *Rudipapes philippinarum* and *Mytilus galloprovincialis*, respectively. Thus, we hypothesize that the RVD process could be impaired after the chronic exposition of the organism to a low concentration of diesel oil in water.

Diesel oil (CAS n° 68334-30-5) is one of the most relevant sources of petroleum by-products in coastal waters because it is the main fuel used in boats. It is a complex combination of hydrocarbons, produced by distillation of crude oil, consisting of paraffin's primarily with up to 25% aromatic compounds (Scheider, 2011). An exposition to diesel can produce a harmful impact on ecosystems (Grande et al., 2012) and cause multi-organ failures in humans through cutaneous exposure, vapour inhalation and incidentally, the gastrointestinal tract (Li et al., 1999). Sea- or freshwater pollution monitoring studies employing biomarkers in bivalves have been widely reported in the literature (Dondero et al., 2011; Canesi et al., 2012; Hamza-Chaffai, 2014; Lacroix et al., 2015; Maisano et al., 2016). Indeed, bivalves are sessile and filter-feeder organisms, able to accumulate contaminants in their tissues. Moreover, bivalve species are reared in estuarine and intertidal zones liable to be impacted by hydrocarbons. The oyster, being worldwide extensively bred, appears as a common model, potentially adaptable to many experimental areas. Although bivalves are osmoconformers, their cells are able to regulate their volume as demonstrated with the hemocytes of *Neotia ponderosa* (Amende and Pierce, 1980a, 1980b, Pierce, 1982; Smith and Pierce, 1987; Pierce and Warren, 2001) or the gill cells in *Geukensia demissa* (Deaton, 1987; Neufeld and Wright, 1996; Deaton, 2001).

In order to assess the sensitivity of this potential new cellular biomarker, the results will be compared with those of a proven one: the oyster gill's glutathione S-transferases (GSTs) specific activity. GSTs are cytosolic enzymes involved in the phase II xenobiotics biotransformation (Sheehan et al., 2001). Generally, the detoxification mechanism of GSTs relies on the conjugation of reduced glutathione with toxic compounds or their metabolites to increase their hydrophilicity and hence, facilitating their excretion (Hoarau et al., 2002). The pollution is revealed by an increased activity of these enzymes. For example, Zanette et al. (2011); Lüchmann et al. (2011) reported a significant increase in GSTs activity in gills of *C. gigas* and digestive gland of *Crassostrea brasiliana*, respectively, exposed for few days to diesel fuel. This study focuses on the validation of a new sensitive biomarker in biomonitoring programs of marine environments.

2. Material and methods

2.1. Animals and tissues samples collection and treatment

This study was performed during the reproductive period of the Pacific oyster (*C. gigas*): from the beginning of May to the end of July. Cultivated diploid and triploid oysters (90–100 mm mean shell length) were obtained from a local oyster-farm (Séné, Brittany). They were stored in the laboratory under natural photoperiod for at least two days in recirculating 10-L aquaria filled with aerated natural seawater (salinity: 34‰, temperature: 18 °C). The water was changed daily. Following this acclimation period, oysters were randomly divided into two groups: control animals were maintained in acclimation conditions, whereas treated ones were transferred for 24 h to seawater 10-L tanks containing diesel oil (8.4 mg L^{-1}). This dose was chosen to enable comparison with the effects of the lowest diesel oil concentration used by Zanette et al. (2011).

The fragments of three different organs, in close contact with the environment, were excised from control and diesel-exposed oysters. The gills were systematically taken from all oysters and immediately frozen in liquid nitrogen, and then stored at −20 °C for further GSTs specific activity determination. The gonad was taken from diploid oysters. A sample of it was microscopically observed (magnification 40 X) to determine the oyster's sex. Only the ovaries were used for their oocytes. Their dissociation was easily obtained by gentle shaking of the organ suspension in filtered seawater. The hepatopancreas (accumulation and detoxication of various toxic substances) was taken at first from diploid oysters and later from triploid ones which are sterile, because the oocytes becoming more and more intrusive in the pallial cavity prevented the isolation of other cell types. The hepatopancreas cells dissociation was performed by laceration of the tissue with scissors in filtered seawater. Oocytes and hepatopancreas cells suspensions were kept under slight shake, at room temperature, for at least one hour before experimentation to ensure that they reached a steady state with respect to ion and water contents (Guizouarn and Motais, 1999). The viability of cell populations, as determined by the Trypan blue exclusion test was at least 85% for oocytes and 95% for hepatopancreas cells.

2.2. Measurement of cells volume variations

A Malassez cell was loaded by capillary action with 50 μL of an oocytes or hepatopancreas cells suspension then put down on the stage of an Olympus BX60 microscope. About a minute was required to allow the sample to settle. The dilution of the suspensions was adjusted to observe at least 6 cells at magnification 40X. Cells images were obtained using a video camera (Olympus XC30) connected to the microscope and stored on a PC computer. By scanning the Malassez cell, using its grid as a landmark to avoid repetitions, the diameters of twelve cells were measured using the image analysis software Cell©. As isolated cells in suspension adopt a spherical shape, their volumes were estimated from their diameters:

$$volume = \left[4\pi(diameter/2)^3\right]/3.$$

According to this method, cells measurements were performed