The cell cultures and the use of haemocytes from marine molluscs for ecotoxicology assessment

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Abstract Among aquatic organisms suitable for biological monitoring, molluscs occupy a prominent place due to their wide geographic distribution, their abundance and accessibility in the field as well as in aquaculture. Molluscs reflect the degree of environmental contamination and are the most useful bioindicator tools. The study of modulation of immune system or immunomodulation in marine molluscs has become one of the privileged ways for evaluating the physiological effects of environmental factors. Physiological responses of molluscs to environmental stresses could be mediated by haemocytes. These cells are continually exposed to the external environment due to the open circulatory system of molluscs and are affected by pollutants. In fact, several studies showed the effects of different environmental contaminants on haemocyte functions (viability, phagocytosis, ROS production) as well as on proteins involved in cytoskeletal structure maintenance using the *in vitro* approaches. In ecotoxicology, *in vitro* approach is an alternative to animal testing due to the reduced use of experimental animals, low cost and rapid performance. Although several studies showed the importance of using *in vitro* cell models to determine the effects of different environmental contaminants on haemocyte parameters in marine

molluscs, a few reviews highlight these effects. The main purpose of this paper is to summarize the recent data on the effect of some xenobiotics on haemocyte parameters in some mollusc species and then suggest future research prospects.

Keywords Contamination · Haemocytes · Immunity · *In vitro* · Molluscs · Pollutants

Introduction

For the last decades, large quantities of environmental pollutants were introduced into terrestrial and aquatic ecosystems by human activities and may possess an important toxic potential. Immunotoxic effects of environmental exposure to chemical contaminants could be evaluated by monitoring cellular immune parameters of sentinel species (Wong et al. 1992). During the last decade, *in vitro* approaches were used to study physiological processes (Lebel et al. 1996; Poncet et al. 2000; Serpentine et al. 2000; Riinkevich 2005; Auzoux-Bordenave and Domart-Coulon 2010) and the effects of pollutants (Domart-Coulon et al. 2000; Canesi et al. 2007a; Binelli et al. 2009; Matozzo et al. 2011). In fact, *in vitro* approaches are very useful, sensitive and selective for the detection, evaluation and monitoring of anthropogenic contaminants in aquatic environments. They provide important data to determine mechanisms at the molecular and the

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cellular levels and allow the use of specific endpoints to determine the targets of toxic effects with great precision and reproducibility.

Marine invertebrate cells, especially haemocytes, are routinely used to understand the impact of pollutants with accurate precision (Brousseau et al. 2000; Olabarrieta et al. 2001; Gagnaire et al. 2004; Duchemin et al. 2008; Mottin et al. 2010). These cells play a key role in digestion, metabolite transport, shell repair (Armstrong et al. 1971; Pollero et al. 1985; Cheng 1996; Mount et al. 2004) and immune system (Adema et al. 1991; Gopalakrishnan et al. 2009). They are able of executing several immune functions including phagocytosis (Tripp 1961; Cheng 1981; López et al. 1997a) and cytotoxicity through the production of reactive oxygen intermediates (Pipe 1992). The central role of phagocytosis in immune defense and the sensitivity of this biological function to environmental xenobiotics in several species emphasize the usefulness of this approach (Krzystyniak et al. 1989; Voccia et al. 1994; Fugere et al. 1996). The use of haemocytes and the monitoring of their phagocytic activity may represent an ideal biological endpoint to determine the level of toxicity linked to aquatic contaminants. Haemocytes are also involved in detoxification through the accumulation of metallic and organic xenobiotics in their well-developed endolysosomal system (Cajaville and Pal 1995). Some of these xenobiotic molecules are known to give rise to highly reactive oxygen species (ROS) (Kehrer 1993). In certain circumstances of ROS overproduction the protection afforded by antioxidant defense mechanisms might be overcome, thereby leading to oxidative damage to tissue macromolecules including DNA, proteins and lipids. Xenobiotic-enhanced oxyradical generation can be a possible mechanism of pollution toxicity (Livingstone et al. 1990). On the other hand, oxyradicals are involved in cell-mediated antimicrobial defense mechanisms; hence impairment of the ability of haemocytes to produce ROS can be taken as an indication of immunosuppression (Anderson et al. 1994). The aim of the present paper is to summarize the current knowledge about the effect of some xenobiotics on haemocyte parameters like cell morphology/behavior, cell viability, phagocytosis activity and ROS production of some marine mollusc species. However other parameters (Fig. 1) such as apoptosis, enzymes activities, proteins involved in cytoskeletal structure

maintenance etc. were already studied and could be the subject of another review.

Mollusc haemocytes as models

Haemocyte characterization has been, and is, one of the most debated problems encountered by researchers. Since the 1970s, various classification criteria, mainly based on cell morphological aspects and enzyme cytochemistry, have been used to identify circulating haemocyte types (Nakahara and Bevelander 1969; Foley and Cheng 1974). In those studies, differing terminologies were adopted for haemocytes, such as lymphocytes, granulocytes, agranular and granular leukocytes, macrophages, and fibrocytes. More recent studies concerning mollusc haemocytes hypothesized the existence of two circulating haemocyte types, granulocytes and hyalinocytes (Cheng 1981; Hine 1999). Granulocytes generally contain many cytoplasmic granules (granular haemocytes), whereas hyalinocytes have few or no granules (agranular haemocytes) (Hine 1999) (Fig. 2). Both cell types (hyalinocytes and granulocytes) were described in a lot of bivalve species like *Mya arenaria* (Huffman and Tripp 1982), *Mercenaria mercenaria* (Moore and Eble 1977), *Venerupis philippinarum* (Oubella et al. 1996; Cima et al. 2000), *Ruditapes decussatus* (López et al. 1997b), *Meretrix lusoria* (Chang et al. 2005), *Mytilus edulis* (Pipe 1990), *M. galloprovincialis* (Carballal et al. 1997), *Perna perna* (Barracco et al. 1999), *Ostrea edulis* and *Crassostrea gigas* (Auffret 1989) and *C. rhizophorae* (Barth et al. 2005). Subpopulations of agranulocytes were observed in some bivalve species, such as small and large hyalinocytes in *C. virginica* and *O. edulis* (Ashton-Alcox and Ford 1998; Xue et al. 2001; Hégaret et al. 2003; Bigas et al. 2006), intermediate cells in *C. virginica* (Goedken and DeGuise 2004), small agranulocytes in *C. gigas* (Lambert et al. 2003, 2007; Labreuche et al. 2006) and blast-like cells in *R. philippinarum* (Cima et al. 2000), in the Sydney rock oyster *Saccostrea glomerata* (Aladaileh et al. 2007) and in the Suminoe oyster *C. ariakensis* (Donaghy et al. 2009). Subpopulations of granulocytes were also observed. In fact, three types of *R. decussatus* granulocytes were identified, using light microscopy, in accordance with the presence of basophilic or acidophilic granules or a mixture of both in the cytoplasm (López et al. 1997a). Similarly, Cima

Fig. 1 Effect of the main contaminants in aquatic ecosystems on the immune system of molluscs (Girón-Pérez 2010)

Fig. 2 **a** Photomicrograph from *Ruditapes decussatus* hemocyte cytoplasm showing granuloocytes (Gr) with cytoplasmic granules (gr) and nucleus (n) and hyalinocytes (Hy) with hyaline cytoplasm and nucleus (n). ($\times 1325$). **b** Electronmicrograph

showing thin sections of a granuloocyte (Gr) and hyalinocyte (Hy). N nucleus, gr electron-dense particles, mi mitochondria, g Golgi complex, ps pseudopods, v electron-lucid vesicles ($\times 5800$) (López et al. 1997a)

et al. (2000) characterized the granuloocytes of *R. philippinarum* into three subpopulations: basophils, acidophils and neutrophils. Cheng (1981) suggested that the occurrence of various types of granules might

be related to differentiation and maturation processes; specifically, basophilic granules were hypothesized to be immature granules which mature and become acidophilic. Recently, Rioult et al. (2013) proposed a

novel approach which helped to propose a functional classification of cell subtypes. This approach was based on fluorescence staining and nuclei-tracking to determine migration velocity of haemocytes *in vitro*. It allowed the characterization of two types of *M. edulis* haemocytes: small star-shaped cells, which were less motile, and spread granular cells with faster migrations.

In the marine gastropod *H. tuberculata* it is shown that only very few basophilic granulocytes (<1 % of cytocentrifuged cells and <8 % of spontaneously adhered cells) could be found and that the large majority of cells was constituted of hyalinocytes (more than 99 % of cyto-centrifuged cells, and more than 92 % of spontaneously adhered cells), which could be sorted by flow cytometry into small (5–7 μm) and large cells (8–12 μm) (Travers et al. 2008a) (Fig. 3a–d). The large cells contained big cytoplasmic vacuoles that did not stain, similar to those observed in living adherent haemocytes (Fig. 3e). Small hyalinocytes had scarce cytoplasm, which gave them a very high N/C ratio. They correspond to the so-called blast-

like cells (Travers et al. 2008a). In other gastropod species only a single type of mature cells was described: agranular cells in *H. diversicolor* (Chen et al. 1996), *H. discus discus* (Donaghy et al. 2010), *Littorina littorea* (Gorbushin and Iakovleva 2006), *Aplysia californica* and *Megathura crenulata* (Martin et al. 2007). However, Sahaphong et al. (2001) showed the presence of hyalinocytes and granulocytes in *H. asinina* haemolymph using the transmission electron microscopy. To elucidate the haemocyte lineage, Cheng (1981) and Auffret (1988), based on the observation of granules in some blast-like cells, proposed two types of cell precursors which differentiate into granulocytes and hyalinocytes. Those statements were reviewed by Hine (1999) who suggested, in the absence of granuloblasts, a model with only one precursor cell type giving rise to hyalinocytes which further mature into granulocytes. Granules were never observed in blast-like cells from disk abalone, spiny top shell (Donaghy et al. 2010), and *V. philippinarum* (Cima et al. 2000; Matozzo et al. 2011) supporting the hypothesis presented by Hine (1999).

Fig. 3 Light micrographs of haemocytes of *H. tuberculata*. Bar 10 μm . **a** Haemocytes prepared by spontaneous adhesion and stained with Giemsa. **b** Spontaneously adhered haemocytes stained with May Grunwald Giemsa (MGG). **c**, **d** Cytospined haemocytes stained with MGG; note the bilobulated nucleus

(arrow) and blast-like cells (BL). **e** Haemocytes of *H. tuberculata* prepared by spontaneous adhesion and stained with Hemacolor[®]; note the unstained vacuoles (arrowheads) and the bilobulated nucleus (arrow). GR granulocytes, BL blast-like cell, all other cells are large hyalinocytes (Travers et al. 2008a)

Effect of xenobiotics on haemocytes parameters

Cells preparation for in vitro exposure

For many applications, e.g., morphological or functional testing, several authors showed that stimulation of fresh (Gagnaire et al. 2004, 2006; Höher et al. 2012) or primary cultured haemocytes in an artificial medium for a short time is sufficient (Mottin et al. 2010; Ladhar-Chaabouni et al. 2014; Astuya et al. 2015; Katsumiti et al. 2015). However, to determine the effect of low-level environmental toxicants on haemocyte viability or function, establishment of longer-term cultures would be required. To date, only abalone *Haliotis* spp. (Latire et al. 2012; Van der Merwe et al. 2010) and the freshwater mussel *Dreissena polymorpha* (Parolini et al. 2011) have yielded haemocytes that appear capable of long-term survival under in vitro conditions. The maintenance of haemocyte primary cultures for 7–10 days has been attained for *H. tuberculata* and *H. midae*, and 15 days for *D. polymorpha*. Nevertheless, to ensure the success of such cultures, the most appropriate medium should be formulated especially based on the commercial media developed for use in mammalian cell culture. These commercial media may be used as a foundation and adapted in terms of osmolarity, pH and nutritional components to simulate the marine environment (Table 1). Although it is generally not recommended, antibiotics were added to culture media. The trade-off when doing this is that cell integrity is challenged and that resistant bacterial strains, which are difficult to eradicate, may develop (Barracco et al. 1999; Domart-Coulon et al. 1994). Thus, in immunotoxicity studies, attaining culture conditions, including appropriate medium, optimal pH, osmolarity and temperature that support cell viability while keeping contamination is necessary. Nevertheless, modifications of cellular activities have to be due only with pollutant tested but not to culture conditions (Gagnaire et al. 2006).

Morphology and behavior

In the absence of xenobiotics, several studies showed the elongated shape with large pseudopods of mollusc haemocytes (Olabarrieta et al. 2001; Gomez-Mendikute and Cajaraville 2003; Chang et al. 2005; Mottin et al. 2010; Latire et al. 2012; Gaume et al. 2012; Ladhar-Chaabouni et al. 2014; Halm-Lemeille et al.

2014). In the presence of cadmium (Cd), copper (Cu) and organic xenobiotics (paraquat and benzo[a]pyrene), Gomez-Mendikute and Cajaraville (2003) showed that *M. galloprovincialis* haemocytes adopted a round shape with no cell extensions. After exposure to 1000 μM Cd, Olabarrieta et al. (2001) showed that *M. galloprovincialis* haemocytes lose their characteristic distribution of the actin cytoskeleton and become rounded, compared to well spread control haemocytes. No cytoplasmic prolongations can be distinguished and actin filaments appear disorganized, with disperse actin aggregates inside the cells. Katsumiti et al. (2014) showed that an exposure to the three forms of Cd (ionic Cd, CdS quantum dots and bulk CdS) does not affect the cytoskeleton integrity of *M. galloprovincialis* haemocytes. In the presence of zinc, Mottin et al. (2010) showed that *H. tuberculata* haemocytes became isolated and more rounded and that cell areas decreased by 36 and by 52 % in the presence of 100 and 1000 μM zinc, respectively. Similarly, Latire et al. (2012) and Ladhar-Chaabouni et al. (2014) (Fig. 4) showed that after exposure to cadmium, haemocytes became more rounded and a reduction of contact with neighbouring cells was detected beginning at a concentration of 500 $\mu\text{g L}^{-1}$ of CdCl_2 during 10 days and at 1 $\mu\text{g mL}^{-1}$ of CdCl_2 during 24 h, respectively. *H. tuberculata* haemocytes morphology was also affected after exposure to triclosan (TCS: antibacterial agent which presents moderate water solubility of 12 mg L^{-1} and high hydrophobicity: TCS is highly adsorbed to particulate and bioaccumulated in fatty tissues in various aquatic organisms). Indeed, Gaume et al. (2012) showed that 8 μM TCS induced a total loss of haemocytes adherence to the bottom of the culture plates due to cell shape changes. Morphological modifications of *H. tuberculata* haemocytes were not observed after an exposure to PCB28 (congener of polychlorinated biphenyls: PCBs, which are compounds of anthropogenic origin that possess 209 congeners) during 10 days. However, in the presence of PCB153 (congener of PCBs), the haemocytes became isolated and morphological modifications could be observed (Halm-Lemeille et al. 2014). These morphological modifications due to contaminants exposure are generally associated with a disturbance of cytoskeleton organization, as demonstrated by fluorescence microscopy (Olabarrieta et al. 2001; Gomez-Mendikute and Cajaraville 2003) or proteomic analysis (Chora et al.

Table 1 Examples of culture media formulations: A and B for haemocyte primary culture from *H. midae* (Van der Merwe et al. 2010), C for haemocyte primary culture from *H. tuberculata* (Latire et al. 2012)

Culture medium A	Culture medium B	Culture medium C
NaCl (10.1 g)	NaCl (6.15 g)	NaCl (250 mM)
KCl (0.27 g)	KCl (0.1675 g)	KCl (10 mM)
CaCl ₂ (0.3 g)	CaCl ₂ ·2H ₂ O (0.34 g)	CaCl ₂ (2.5 mM)
MgSO ₄ ·7H ₂ O (0.5 g)	MgSO ₄ ·7H ₂ O (1.5725 g)	MgSO ₄ (25 mM)
MgCl ₂ ·6H ₂ O (1.95 g)	MgCl ₂ ·6H ₂ O (1.165 g)	Hepes (10 mM)
Dissolve in 500 mL Leibovitz-L15	NaHCO ₃ (0.045 g)	Adjust pH to 7.4
Add Penicillin/streptomycin (1 % v/v)	Dissolve in 250 mL dH ₂ O	Dissolve in Hank's 199 medium
Gentamycin (0.5 % v/v), Amphotericin B (0.4 % v/v)	Adjust pH to 7.6	Add
Adjust pH to 7.6	Add	L-glutamine (2 mM)
Add glutamax (or L-glutamine) (2 % v/v)	Penicillin/streptomycin (1 % v/v)	Penicillin (60 µg mL ⁻¹) Streptomycin (100 µg mL ⁻¹) concavaline A (2 mM)
osmolarity ~1000 mmol/kg	Gentamycin (0.5 % v/v)	

2009). Further studies should be performed to confirm this hypothesis in mollusc haemocytes.

Cell viability

Primary cultured haemocytes from *H. tuberculata* exposed to heavy metals such as cadmium and zinc, showed a significant decrease of their viability using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay (Latire et al. 2012; Mottin et al. 2010). In fact, Mottin et al. (2010) showed that at zinc concentrations of 100 µM and higher, haemocyte viability decreased, and maximum toxicity was observed when cells were exposed to 1000 µM zinc. The LC₅₀ (24 h) for Zinc was approximately 314 µM for haemocytes in culture (Table 2). Similarly, Latire et al. (2012) showed a significant decrease of haemocyte viability in a concentration-dependent manner beginning at a cadmium chloride concentration of 500 µg L⁻¹ after 10 days of exposure. The maximum toxicity was observed when cells were exposed to 50,000 µg L⁻¹ of cadmium chloride (93 % of cells died at this concentration). The in vitro Cytotoxicity of Cd was also assessed in *M. galloprovincialis* haemocytes using the neutral red (NR) and XTT assays (similar to the MTT assay) (Fig. 5) (Olabarrieta et al. 2001). Results showed that cell viability expressed as LC₅₀ (24 h) was 750 µM when using the NR assay and 400 µM with the XTT assay (Table 2), indicating a higher sensitivity of the XTT

assay for the assessment of haemocyte viability. In the same species, Gomez-Mendikute and Cajaraville (2003) showed a significant decrease (according to XTT test) on haemocytes viability after exposure to Cd ($1120 \times 10^5 \mu\text{g mL}^{-1}$) and Cu ($12.72 \times 10^5 - \mu\text{g mL}^{-1}$) during 24 h. Similar results were observed in *V. philippinarum* haemocytes exposed to 10, 50 and 250 µg mL⁻¹ of sodium fluoride for 60 min according to the Trypan Blue exclusion assay. This assay evidences the alteration of the cell plasma membrane permeability after the NaF exposure (Ballarin et al. 2014). The Cytotoxicity of three types of nanoparticles (NPs): Au, ZnO and SiO₂ NPs, with their ionic and bulk forms and additives used in the NPs preparations (Na-citrate, Ecodis P90 and L-arginine), was also evaluated (Table 2). Results showed that Au, ZnO and SiO₂ NPs were less toxic for mussel haemocytes than the corresponding ionic forms but more toxic than the bulk forms (according to NR and MTT assays). ZnO NPs were the most toxic NPs (Katsumiti et al. 2015). Nevertheless, Ladhar-Chaabouni et al. (2014) showed no effect of Cd on *H. tuberculata* haemocyte viability after in vitro exposure to 1 and 100 µg mL⁻¹ of CdCl₂ during 24 h (according to NR and MTT assays). Authors explained the last results by the presence of physiological means to immobilize heavy metals. In fact, it was shown that gastropod haemocytes accumulate metals within lysosomes to reach intracellular concentrations over 20,000 µg/g (Mason 1983). In addition to intralysosomal accumulation, another

Fig. 4 Light microscopy images showing the morphology of *Haliotis tuberculata* haemocytes. Cells were seeded on coverslips at a density of 0.5×10^6 cells per well and grown at 17 °C

in culture medium for 24 h in the absence (a) or presence of $1 \mu\text{g mL}^{-1}$ CdCl_2 (b) and $100 \mu\text{g mL}^{-1}$ CdCl_2 (c) (Ladhar-Chaabouni et al. 2014)

physiological means to immobilize heavy metals is to scavenge them with metal-binding proteins or metallothioneins (MT). Molluscan haemocytes are known to synthesize MT after Cd exposure as a protection mechanism against metal toxicity (Roesijadi et al. 1997; Langston and Bebianno 1998). Another study carried out with pesticides showed no significant effect of a mixture of 14 pesticides and methaldehyde alone tested at different concentrations (according to flow cytometry) on Pacific oyster haemocytes maintained in vitro for short-term periods (2, 4 and 21 h)

(Moreau et al. 2014). Gagnaire et al. (2003) reported no effect on cell viability in Pacific cupped oyster haemocytes exposed to atrazine in vitro. However, in vitro exposure to PCBs showed a decrease by 27 % of *H. tuberculata* cell viability after exposure to $1.25 \mu\text{M}$ of PCB 153 during 10 days. When *H. tuberculata* haemocytes were exposed to $2.5 \mu\text{M}$ and higher of PCB153, a drastic growth inhibition was recorded. PCB153 was reported as the most toxic for *H. tuberculata* haemocytes and PCB28 the least toxic (Halm-Lemeille et al. 2014). Gaume et al. (2012)

Table 2 Concentrations and toxic effects of some xenobiotics on mollusc haemocytes

Assay/value	Species	Xenobiotic	Period	Effect	References
LC ₅₀ (MTT)/314 mM	<i>Haliotis tuberculata</i>	ZnCl ₂	24 h	Reduction of cell viability to 50 %	Mottin et al. (2010)
LC ₅₀ (MTT)/4100 µg L ⁻¹		ClCl ₂	10 days	Reduction of cell viability to 50 %	Latire et al. (2012)
IC ₅₀ (XTT)/4.00 µM		Triclosan	24 h	Reduction of cell metabolic activity	Gaume et al. (2012)
LC ₅₀ (MTT)/45.24 mg L ⁻¹		Amitriptyline	48 h	Reduction of cell metabolic activity	Minguez et al. (2014)
LC ₅₀ (MTT)/4.76 mg L ⁻¹		Clomipramine		Amitriptyline less cytotoxic than Clomipramine	
LC ₅₀ (MTT)/32.94 mg L ⁻¹		Citalopram		Citalopram less cytotoxic than Paroxetine	
LC ₅₀ (MTT)/4.16 mg L ⁻¹		Paroxetine			
EC ₅₀ />40 µM		PCB3	72 h	PCB153 is the most toxic for the abalone haemocytes	Halm-Lemeille et al. (2014)
EC ₅₀ /89.41 µM		PCB28			
EC ₅₀ /21.17 µM	<i>Mytilus galloprovincialis</i>	PCB107			
EC ₅₀ /20.81 µM		PCB136			
EC ₅₀ /1.67 µM		PCB153			
EC ₅₀ /47.67 µM		PCB77			
EC ₅₀ /4.31 µM		PCB169			
LC ₅₀ (NR)/750 µM		CdCl ₂	24 h	Reduction of cell viability to 50 %	Olabarrieta et al. (2001)
LC ₅₀ (XTT)/400 µM					
LC ₅₀ (MTT)/14.93 mg L ⁻¹		Ionic Au	24 h	Au, ZnO and SiO ₂ NPs were less toxic than the corresponding ionic forms but more toxic than the bulk forms	Katsumiti et al. (2015)
LC ₅₀ (MTT)/>100 mg L ⁻¹		Bulk Au			
LC ₅₀ (MTT)/76.35 mg L ⁻¹		Au5-Cit			
LC ₅₀ (MTT)/81.66 mg L ⁻¹		Au15-Cit		ZnO NPs were the most toxic NPs	
LC ₅₀ (MTT)/83.47 mg L ⁻¹		Au40-Cit			
LC ₅₀ (MTT)/411.76 mg L ⁻¹		Na citrate			
LC ₅₀ (MTT)/6.7 mg L ⁻¹		Ionic Zn			
LC ₅₀ (NR)/7.17 mg L ⁻¹		Bulk Zn			
LC ₅₀ (MTT)/53.87 mg L ⁻¹					
LC ₅₀ (NR)/61.29 mg L ⁻¹		ZnO < 130-EcoP90			
LC ₅₀ (MTT)/13.41 mg L ⁻¹					
LC ₅₀ (NR)/12.56 mg L ⁻¹					
LC ₅₀ (MTT)/39.48 mg L ⁻¹		ZnO < 280-EcoP90			
LC ₅₀ (NR)/38.01 mg L ⁻¹					
LC ₅₀ (MTT)/3.87 mg L ⁻¹		Ecodis P90			
LC ₅₀ (NR)/3.65 mg L ⁻¹					
LC ₅₀ (MTT and NR)/>100 mg L ⁻¹		Ionic Si			
LC ₅₀ (MTT and NR)/>100 mg L ⁻¹		Bulk Si			
LC ₅₀ (MTT and NR)/>100 mg L ⁻¹		SiO ₂ 15-Larg			

Table 2 continued

Assay/value	Species	Xenobiotic	Period	Effect	References
LC ₅₀ (MTT and NR)/≥100 mg L ⁻¹	(Au5-Cit, Au15-Cit and Au40-Cit NPs: Na-citrate stabilized Au NPs of 5, 15 and 40 nm in size, respectively); (Zn5130-EcoP90 and Zn5280-EcoP90: Ecodis-P90 ZnO NPs of 20–70 and 500 × 260 × 260 nm, respectively); (SiO215-Larg, SiO230-Larg and SiO270-Larg: L-arginine stabilized SiO2 NPs of 15, 30 and 70 nm, respectively)	SiO ₂ 30-Larg			
LC ₅₀ (MTT and NR)/≥100 mg L ⁻¹		SiO ₂ 70-Larg			
LC ₅₀ (MTT and NR)/≥250 mg L ⁻¹		L-arginine			

(Au5-Cit, Au15-Cit and Au40-Cit NPs: Na-citrate stabilized Au NPs of 5, 15 and 40 nm in size, respectively); (Zn5130-EcoP90 and Zn5280-EcoP90: Ecodis-P90 ZnO NPs of 20–70 and 500 × 260 × 260 nm, respectively); (SiO215-Larg, SiO230-Larg and SiO270-Larg: L-arginine stabilized SiO2 NPs of 15, 30 and 70 nm, respectively)

showed that an exposure of *H. tuberculata* haemocytes to 8 µM of TCS and to 4 µM methyl-triclosan (MTCS: product of methylation of triclosan) during 24 h caused a significant decrease of cell metabolic activity. In the same species, Minguez et al. (2014) studied the in vitro cyto-toxicity of four common antidepressants, namely Amitriptyline, Clomipramine, Citalopram and Paroxetine, on primary cultured haemocytes after 48 h of exposure (Table 2). Using the MTT assay, the results showed that two compounds of the same family of antidepressant displayed different levels of toxicity: In the family of TriCyclic Antidepressants (TCAs), Amitriptyline was less cytotoxic than Clomipramine, and in the family of Selective Serotonin Reuptake Inhibitors (SSRIs), it was Citalopram which was less cytotoxic than Paroxetine. Using NR assay, the results showed that antidepressants induced a significant permeabilization of lysosomes in almost 100 % of the haemocytes at LC₅₀. A decrease in the lysosomal membrane stability was also observed in *M. galloprovincialis* haemocytes exposed in vitro to different concentrations of chromium (0.1, 1, 10 and 100 µM) (Ciacci et al. 2011).

Phagocytosis activity

Phagocytosis by haemocytes is an integral aspect of immune defense, occurring prior to the intracellular killing of an invasive pathogen. In molluscs, engulfment of foreign particles by haemocytes occurs by invagination of the cell membrane followed by pseudopod formation and particle internalization into an endocytic vacuole, also called the primary phagosome. Cytoplasmic lysosomal granules then migrate and fuse with the primary phagosome. Contents of granules, i.e. numerous hydrolases, including phosphatases, esterases, amidases, as well as carbohydrate hydrolases, and oxidative enzymes such as peroxidase and cytochrome c oxidase (López et al. 1997b; Cima et al. 2000), are subsequently discharged in the so-called secondary phagosome, accomplishing the enzymatic degradation of engulfed foreign material (López et al. 1997c). In bivalves phagocytic capability was described for both granulocytes and hyalinocytes. However, granulocytes are the more efficient phagocytic cells (Cajaraville and Pal 1995; López et al. 1997c; Pipe et al. 1997; Carballal et al. 1997; Hégaret et al. 2003; Terahara et al. 2006). In abalone where granulocytes are absent or very under-represented, the

Fig. 5 Cytotoxicity of Cd to haemocytes assessed by the neutral red assay (a) and XTT assay (b). Haemocytes were exposed to indicated Cd concentrations for 24 h. Data are means of 11 measurements (Olabarrieta et al. 2001)

phagocytosis is clearly done by hyalinocytes (Travers et al. 2008a). After exposure to different contaminants, the phagocytic capability of haemocytes from different mollusc species was described. Xenobiotics effects can range from total immunosuppression (suppression of the immune responses like phagocytosis) to immunostimulation (stimulation of the immune responses like phagocytosis), including no effect. Flow cytometric evaluation of phagocytosis of fluorescent beads showed a decrease of phagocytic activity of *H. tuberculata* haemocytes after an exposure to high concentrations of antidepressants, zinc and CdCl₂ (for a long period: 10 days) (Mottin et al. 2010; Latire et al. 2012; Minguez et al. 2014). In this case, authors attributed the immunosuppression to the cytoskeleton disorganization mediated directly by the interaction of metals with structural proteins of the cytoskeleton or indirectly by dysfunctions in calcium homeostasis mechanisms. Immunosuppression was also observed by Sauvé et al. (2002a) who showed that an exposure to a concentration higher than 10⁻⁴ M

of CdCl₂ induced a significant dose related inhibition of marine and freshwater bivalve haemocyte phagocytosis. Similarly, Sauvé et al. (2002b) showed that Cd, Cu, Ni, and Zn caused significant immunosuppressive effects with concentrations inducing 50 % inhibition ranging from 10⁻⁵ to 10⁻⁴ M in *Eisenia fetida*, *Lumbricus terrestris*, *Aporrectodea turgida*, and *Tubifex tubifex*. Bado-Nilles et al. (2008) investigated the in vitro effects of different polycyclic aromatic hydrocarbons (PAHs) on haemocyte and haemolymph parameters of the Pacific oyster *C. gigas*. The phagocytosis activity was significantly reduced in presence of fluorene, pyrene and heavy fuel oil (HFO). Such reduction was determined by Höher et al. (2012) who showed that the lowered phagocytic activity (uptake of NR stained zymosan particles) of *M. edulis* haemocytes was determined in mussels with the highest PCB body burden. Similarly, Gagnaire et al. (2006) showed that a mixture of eight pesticides during 24 h decreased phagocytotic activity of *C. gigas* haemocytes using flow cytometry. Marisa et al. (2015) observed that the pretreatment of *R. philippinarum* haemocytes with two concentrations of titanium dioxide nanoparticles: n-TiO₂ (1 and 10 μg mL⁻¹) before incubation with yeast caused a significant decrease in the phagocytic activity. Nevertheless, the concomitant incubation of haemocytes with n-TiO₂ and yeast caused a decrease in the phagocytic activity only at the highest concentration tested. However, cells from *M. galloprovincialis* exposed in vitro to n-TiO₂ (1, 5 and 10 μg mL⁻¹) showed a biphasic response: phagocytosis of NR conjugated zymosan particles increased at the lowest concentration, whereas it decreased at the highest concentrations (Ciacci et al. 2012). In that study, a slight increase in phagocytic activity was also observed in haemocytes treated with 5 μg mL⁻¹ n-SiO₂. A biphasic effect was also observed on *M. galloprovincialis* phagocytic activity after in vitro exposure to estrogenic compounds. Thus at lower concentrations (0.1–5 μM) a significant stimulation was detected, while at 25–100 μM an inhibitory effect was observed (Canesi et al. 2007b). At low concentrations of antidepressants, Minguez et al. (2014) showed an increase of phagocytic activity of *H. tuberculata* haemocytes. This stimulation may be explained by a hormetic effect. Immunostimulation was previously demonstrated in several species such

as rodents, reptiles, birds, fishes, bovines, whales and primates exposed to mercury and cadmium (Bernier et al. 1995; De Guise et al. 1997; Dieter et al. 1983; Voccia et al. 1994). For bivalves, it was proposed that the stimulation of phagocytosis by divalent cations might be the result of decreased surface charge through binding on negatively charged groups, leading to an enhanced binding and subsequent phagocytosis on nonpolar particles (Cheng and Sullivan 1984). Ladhar-chaabouni et al. (2014) showed no significant variations of phagocytosis of abalone haemocytes after the exposure to 1 and 100 $\mu\text{g mL}^{-1}$ of CdCl_2 during 24 h. The same results were observed in *M. edulis* haemocytes exposed to 400 $\mu\text{g L}^{-1}$ of cadmium (Coles et al. 1995). Dyrinda et al. (1998) did not observe any difference in phagocytic activity from mussels originating from several sites characterized by various levels of contamination in the UK. Katsumiti et al. (2014) showed that phagocytosis was not modified in *M. galloprovincialis* haemocytes exposed to ionic Cd. Similarly, no significant effect of a mixture of 14 pesticides, tested at different concentrations, on the phagocytic activity of *C. gigas* haemocytes was observed (Moreau et al. 2014). Thus, for a given contaminant, the responses of immunological biomarkers like phagocytosis are sometimes contradictory since the effects can range from almost total immunodepression to immunostimulation, including no effect (Fournier et al. 2000). This variability in responses could be the result of different parameters like (i) the concentration of the pollutant. In fact, and in general, it has been reported that a low level of pollutant tends to produce an immunostimulatory effect, while high concentrations have a suppressive effect. (ii) The nature of pollutant, since among the twenty-three pollutants (belonging to the most important groups of xenobiotics: PAHs, PCBs, and pesticides), only pesticides (mixture of eight pesticides) caused a significant decrease of phagocytotic activity of *C. gigas* haemocytes (Gagnaire et al. 2006). Similarly, Brousseau et al. (2000) showed that the sensitivity of clam haemocyte's phagocytosis to the different metals varied considerably with the metals. The immunotoxic potential of metals can be ranked in the following increasing order: $\text{ZnCl}_2 > -\text{CdCl}_2 > \text{AgNO}_3 > \text{HgCl}_2 > \text{CH}_3\text{HgCl}$. (iii) Duration of exposure could explain (for example) the differences observed by Ladhar-Chaabouni et al. (2014) and Latire et al. (2012) concerning

phagocytotic activity of *H. tuberculata* haemocytes after short-term and long-term (respectively) exposure to high concentrations of CdCl_2 . (iv) From species to species, the haemocyte phagocytic activity varies. For example, Sauv et al. (2002b) showed that haemocyte phagocytosis varied with species after in vitro exposure to different metals (Ag, Cd, Hg and Zn) (Fig. 6). (v) Acclimatization appears to have an effect on haemocyte phagocytosis as shown by Hurtado et al. (2011). These authors studied the changes in the haemocyte characteristics of the Pacific oyster *C. gigas* and the carpet shell clam *R. decussatus* acclimated 1 or 2 days under emersed conditions and for 1, 2, 7, or 10 days to flowing seawater conditions (submerged), when compared to hemolymph withdrawn from organisms sampled in the field and immediately analyzed in the laboratory (unacclimated). Results showed that phagocytosis was higher in unacclimated oysters and those acclimated 1 and 2 days in emersed conditions and 1 day in submerged conditions. Phagocytosis significantly decreased until reaching the lowest values in oysters acclimated 10 days under submerged conditions. (vi) Intrinsic parameters such as sex and reproductive stage should be taken into account when studying the impact of pollutants on haemocyte functions like phagocytosis. Indeed, Matozzo and Marin (2010) reported sex-dependent differences in phagocytosis efficiency, with females more efficient than males. Female triploids had a higher phagocytic index than male triploids or male and female diploids (Duchemin et al. 2007). During gametogenesis Travers et al. (2008b) showed a decrease of phagocytosis activity in *H. tuberculata* haemocytes which could be a consequence of depleted energy reserves.

ROS production

Reactive oxygen species (ROS) generation is generally described as a cascade of enzymatic reactions that starts with the production of the superoxide anion (O_2^-) by NADPH oxidase (NOX) complexes (Drge 2002; Bedard and Krause 2007; Segal 2008). Hydrogen peroxide (H_2O_2) may then be produced from superoxide either spontaneously or catalysed by the superoxide dismutase (SOD) enzyme (Donaghy et al. 2009). In the presence of reduced transition metals, hydrogen peroxide can be converted into the highly-reactive hydroxyl radical ($\cdot\text{OH}$). Hydrogen peroxide may also

Fig. 6 Phagocytic response of various bivalves species as a function of nominal additions of metals Ag, Hg (inorganic and organic), Cd and Zn (Sauvé et al. 2002b)

give rise to highly toxic hypochlorite (HOCl), catalysed by myeloperoxidase (MPO) in the presence of chloride ion (Donaghy et al. 2009). An increase of ROS production in *H. tuberculata* haemocytes beginning at a concentration of $5 \mu\text{g L}^{-1}$ (environmental concentration reported in coastal water) of CdCl₂ was observed by flow cytometry using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a fluorescent probe after 10 days of exposure (Latire et al. 2012). At the environmental concentration (0.001 mg L^{-1}) of three antidepressant compounds (Amitriptyline, Clomipramine and Paroxetine) a significant increase of ROS production was detected in *H. tuberculata*

haemocytes. This increase was detected at LC₁₀ (7.5 mg L^{-1}) for Citalopram (Minguez et al. 2014). Through a series of functional in vitro assays, Katsumiti et al. (2014) showed a significant increase of ROS production in *M. galloprovincialis* haemocytes after exposure to a wide range of concentrations of CdS quantum dots (QDs) (0.001 – 100 mg Cd L^{-1}) used for treatment and diagnosis of cancer and for targeted drug delivery. Superoxide anions (O_2^-) production was measured in *M. galloprovincialis* haemocytes after treatment with Zn ($50 \mu\text{M}$) (Kaloyianni et al. 2006) and Cd ($50 \mu\text{M}$) (Vouras and Dailianis 2012), since these anions take part in all other

reactions that lead to the production of H_2O_2 , OH^- , $\text{OH}^\cdot$ and HNO_2^- radicals. The results showed a significant enhancement of O_2^- production in haemocytes exposed to Zn and Cd compared with those measured in control cells. Winston et al. (1996) showed that ROS generation within the lysosomal compartment of *M. edulis* haemocytes is enhanced by the uptake of iron-EDTA or purpurin, respectively, a model for metal chelate-mediated redox cycling and a redox cycling anthraquinone. The authors suggested that in *M. edulis* haemolymph, ROS generated by haemocyte lysosomes are, at least in part, associated with the defenses of this organism against endocytosed pathogens. A combination of two mechanisms could explain the increase of ROS production in response to contaminants exposure: contaminants act directly on the mitochondrial respiratory chain, generating overproduction of ROS, or contaminants are able to interact directly with glutathione peroxidase and catalase, decreasing their efficiency in detoxifying peroxides and H_2O_2 , with both of these processes leading to an increase in ROS level (Nzengue et al. 2008). However, an inhibition of ROS generation was observed after the in vitro exposure of *C. virginica* haemocytes to sub-lethal concentrations (up to $15\text{ }\mu\text{M}$) of CdCl_2 (Butler and Roesijadi 2000). The decrease of ROS production was also observed in *M. galloprovincialis* haemocytes exposed in vitro to Cu (Gomez-Mendikute and Cajaraville 2003) and in *H. tuberculata* haemocytes exposed to high concentrations of antidepressant compounds (Minguez et al. 2014) and Zn (Mottin et al. 2010). Such decrease could be explained by a disorganisation of the cytoskeleton. As suggested by Thiagarajan et al. (2006), the disturbance of the cytoskeleton could be correlated to the NADPH-oxidase complex contained in the plasma membrane. This complex is responsible for the formation of O_2^- in haemocytes and could be inhibited in response to contaminants exposure. To stimulate ROS production in haemocytes, several authors used chemicals such as phorbol myristate acetate (PMA). PMA is an activator of intracellular C kinase protein (Li et al. 2000), which catalyses the phosphorylation of cytosolic proteins of NOX2 (the NADPH oxidase complex involved in ROS production during the oxidative burst in animal cells) (Toreilles et al. 1996), allowing them to migrate and activate the protein complex. In some bivalve species,

however, PMA stimulation did not induce an increase in ROS production. In contrast, the addition of this compound even resulted in decreased oxidative activity in *C. gigas* (Lambert et al. 2003, 2007) and in the Manila clam (Lambert et al. 2005; Rifi 2005), supporting the hypothesis of a bivalve NOX different than NOX2 expressed in bivalve haemocytes. That is why care has to be taken with knowledge and techniques derived from vertebrate biology.

Conclusion

For the last decades, the impact of human activities on the environment increased, in association with population increase and the major technological upheavals of the industrial revolution. The release of contaminants into the environment causes instability, disorder, harm, or discomfort to the ecosystem (physical systems or living organisms). That is why the major problem at the end of the twentieth century is the preservation of environmental health since it has become clear that the health of human populations depends to a large extent on the quality of their environment. The monitoring of ecosystems quality is largely based on the evaluation of the ecotoxicological impact of pollutants through simple and reliable methods, specifically adapted to the study of xenobiotic effects on living organisms. In this context, in vitro approaches were used to understand the impact of pollutants on marine mollusc haemocytes. In vitro tests could be considered as a useful, sensitive and selective tool for the detection, evaluation and monitoring of anthropogenic contaminants in aquatic environments. In addition, the variations of haemocyte parameters could be considered as potential biomarkers of the physiological status of the mollusc species. Nevertheless, to reach higher sensitivity and specificity of the haemocytes as biomarkers, the influence of endogenous factors (gametogenesis and gender for example) that may modify the responses of biomarkers to environmental toxicants should be taken into consideration. Moreover, the interactions between immunocompetence, disease susceptibility and pollutants in marine molluscs should be developed, since animals with disturbed defense mechanisms due to pollutants may be more susceptible to infectious diseases.

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