ISJ 7: 149-156, 2010 ISSN 1824-307X

REVIEW

Relationships between innate immunity in bivalve molluscs and environmental pollution

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Accepted May 25, 2010

Abstract

The immune system of invertebrates, such as molluscs consists of innate mechanisms very effective against antigens commonly present in the environment. However, these defense strategies could be altered by pollutants. This review is focused mainly on the effect of metals, PCB, pesticides, PAHs, and others environmental pollutant on immune response of molluscs.

Key Words: molluscs; immune system; environmental pollution; metals; pesticides; PAHs; PCB

Introduction

Immune system is strongly influenced by environmental conditions. Successful host resistance is a major determinant in whether a pathogen will result in a disease outbreak. Altered environmental conditions can affect immunity directly, by changing the concentration and efficiency of components including cytokines, cytokine receptors and cells of the immune response, or indirectly by inducing general stress response. Subsequently, the relationship immunity-environment is complex, but is an essential comprehended mechanistic aspect of it, and thus allow predictions on the potential effect of environmental factor on immune response (Mydlarz *et al*., 2006).

The bivalve molluscs have characteristics such as high distribution worldwide, sedentary and filter-feeding habits; hence these organisms accumulate large number of bacteria and chemical pollutants, which are both a source of nourishment and an immune challenge (Bernal-Hernandez *et al*., 2010).

The immune response of molluscs has an important defense function against bacteria, fungi, and parasites. The immune system is constituted for a first line defense including physicochemical barriers as the cuticle, shell and mucus layer. Moreover, in bivalves, cellular and humoral components are present and operate in a coordinated way (Galloway and Depledge, 2001).

Cellular response is carried out by circulating hemocytes that can kill microbes through phagocytosis and citotoxic reactions that include the release of lysosomal enzymes and anti-microbial peptides, and the respiratory burst which involves the production of oxygen metabolites, meaning superoxide anion, hydrogen peroxide, and intermediated compounds with high bactericidal activity (Pruzzo *et al*., 2005). Hemocytes are also involved in other physiological functions, such as wound and shell repair, digestion and transport of nutrients. Hemocytes classification is controversial, but has hypothesize the existence of two circulating hemocytes cells: granulocytes (containing many citoplasmatic granules) and hyalinocytes (containing few or no granules). Granulocytes are generally the most abundant cell type with higher phagocytic activity, while hyalinocytes are usually smaller than granulocytes, and have a high nucleus/cytoplasm ratio (Hine *et al*., 1999; Matozzo *et al*., 2007).

The humoral components present in hemolymph are lectins, lysosomal enzymes and antimicrobial peptides. The presences of lectins have been shown in marine bivalves such as mussels, oyster, and clams. The role of lectins is induced agglutination of bacteria and act as a molecular bridge between the surface of bacteria and hemocytes (Pruzzo *et al*., 2005).

In spite of the efficiency of the immune system of molluscs in normal conditions, it may be altered by external factors (Fig. 1). Thus, this review is focused mainly on the effect of metals, PCB, pesticides, PAHs, and others environmental pollutants on immune response of molluscs.

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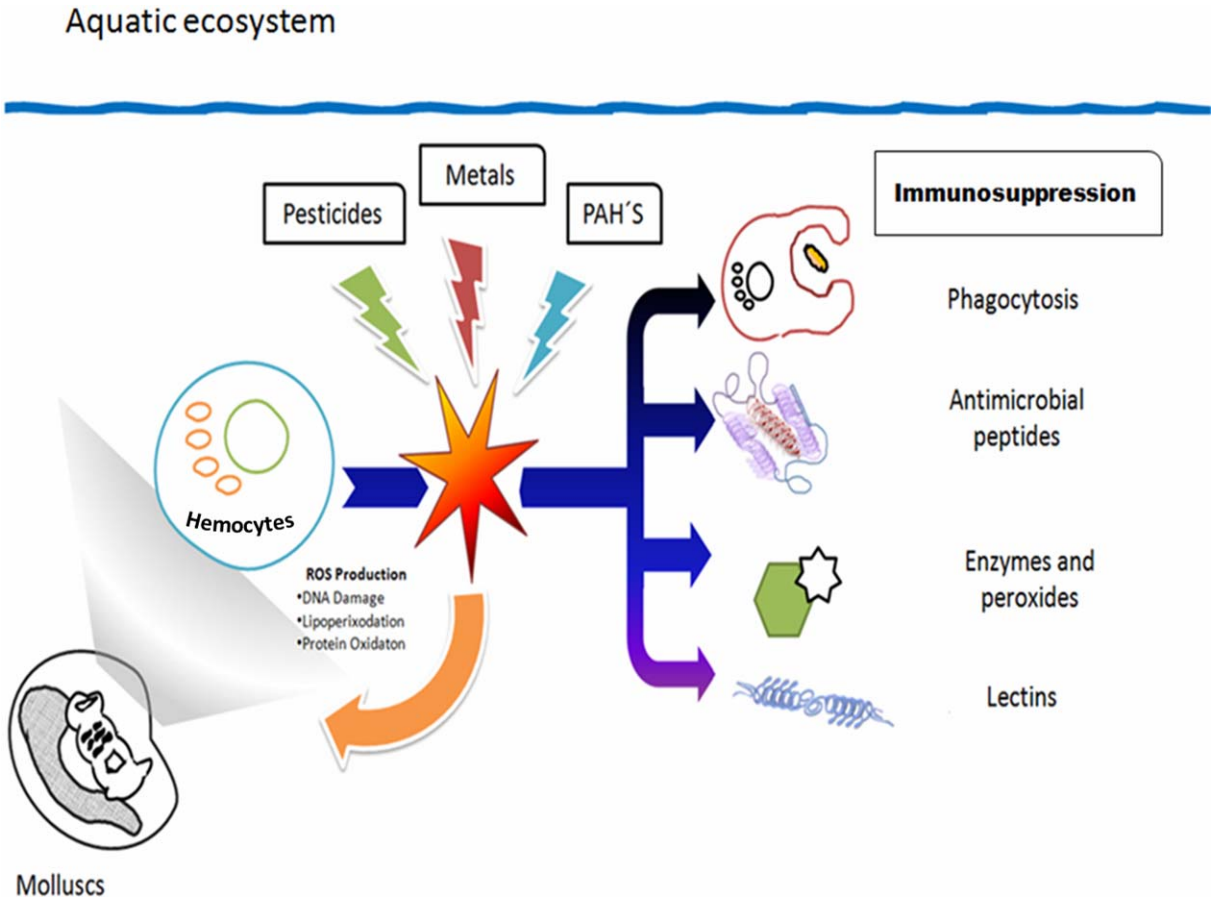

Fig. 1 Effect of the main contaminants in aquatic ecosystems on the immune system of molluscs

Xenobiotics and immune system of molluscs

The presence of chemical contaminants in water is a major subject of concern, since many of these molecules are potent immunosupressors, even at a low concentration (Table 1). A possible consequence for immunodeficient oyster could be an increased susceptibility to parasites and other pathogenic microorganisms (Auffret *et al*., 2002).

Metal effects

Studies performed to understand the relationship between metals and immunotoxicity have been showed that *in vitro* Cd exposure of hemocytes at sub-lethal concentrations up to 15 µM $CdCl_2$ induce significantly increase in metallothionein (MT) and inhibition of ROS generation (Butler *et al.,* 2000).

Studies realized with oyster (*Ostrea edulis*) showed that exposure to $CdCl_2$ (1, 10, 50, 100 µM) and co-exposure to $CdCl_2$ and $CuCl_2$ (0.75 µM), induced non significant changes in the serum total protein level. Moreover, the level of serum acid phosphatase, and hydrolytic enzymes remained unaltered. But a dose-dependent increase in total hemocytes was found in oyster exposed to $CdCl_2$. On the other hand, the exposure to 1, 10 or 50 µM $CdCl_2$ resulted in a dose-dependent decreased in the cell membrane potential, probably related to membrane alterations. Phagocytic activity of *O. edulis* exposed to 1 or 10 µM CdCl*2*, or to 1 µM $CdCl_2$/0.75 µM $CuCl_2$ showed a severe decrease compared with control group (Auffret *et al*., 2002).

The effect of copper exposure (0.02 and 0.05 ppm), alone or simultaneously with *Vibrio tubiashii* at different temperature, was evaluated on mussels (*Mytilus edulis*). Results showed that 0.05 ppm copper induced a significant reduction on cellular content in hemolymph. When co-exposed mussels to bacterial challenge, the reduction in cell number was higher, compared with the effect of metal alone. In addition, intracellular superoxide decreased significantly by exposure to 0.02 and 0.05 ppm of copper to 10 °C. However, there was an increase of this parameter when analyzed at 15 °C. While, phagocytosis was increment by exposure at 0.02 ppm compared with control group (Parry and Pipe, 2004).

Another study evaluated the effect of cadmium, copper on ROS production, and hemocyte viability from *Mytilus galloprovincialis*. Results showed a significantly decrease on viability of hemocytes (according XTT test) exposed to Cd ($1{,}120 \times 10^{-5}$ µg/ml). Exposure to Cu (12.72 µg/ml) also induced

Table 1 Immunotoxic effect of pollutants on molluscs

Xenobiotic	Effect	Species	Reference
Metals			
Cd	Hemocyte counts ↑ Cell membrane potential ↓	*Ostrea edullis*	Auffret *et al.*, 2002
Cd alone or Cd/Cu	Phagocytic activity ↓	*Ostrea edullis*	Auffret *et al.*, 2002
Cd (*in vitro*)	Methalothionein↑ ROS ↑	*Crassostrea Virginica*	Butler *et al.*, 2000
Cd, and Cu	Hemocyte viability ↓ Phagocytosis ↓	*Mytilus galloprovincialis*	Gómez-Mandikute *et al.*, 2003
Cu	Hemocyte counts ↓ Superoxide anion ↓	*Elliptio complanata*	Gangé *et al.*, 2008
Cu	Phagocytosis ↑	*Mytilus edulis*	Parry *et al.*, 2004
Gas			
O_3	Cellular viability ↓ COX-activity ↑ NO_2^- ↑	*Elliptio complanata*	Gangé *et al.*, 2008
Estrogenic substances			
Nonylphenol, monoethoxilate carboxylate, and 17α-ethynyl estradiol	Lysosomal enzyme release ↑ Phagocytosis ↑↓	*Mytilus galloprovincialis*	Canesi *et al.*, 2007
17β-estradiol	Phagocytosis ↓	*Corbicula fluminea*	Champeau *et al.*, 2006
Pharmaceutical drugs			
Benzafibrinate, gembibrozil, and trimetophin	Phagocytosis ↑	*Elliptio complanata*	Gagné *et al.*, 2006
Novobiocin, and morphin	Phagocytosis ↓	*Elliptio complanata*	Gagné *et al.*, 2006
Zulfamethazole, novobiocin, gemfibrocil, benzafibrate, and carbamazepine	Esterase activity ↓	*Elliptio complanata*	Gagné *et al.*, 2006
Oxytetracycline, novobiocine, naproxen	Cell adherence ↓	*Elliptio complanata*	Gagné *et al.*, 2006
Gemfibrozil, bezafibrate	Cell adherence ↑	*Elliptio complanata*	Gagné *et al.*, 2006
Novobiocine,and sulfapyridine	Lipoperoxidation ↑	*Elliptio complanata*	Gagné *et al.*, 2006
Coprostanol, and naproxen	Lipoperoxidation ↓	*Elliptio complanata*	Gagné *et al.*, 2006

PAHs			
Benzo[a]pyrene, and phenanthrene	Granulocyte cell (%) ↑ Cell mortality, esterase and lysosome-positive cells ↓	*Crassostrea gigas*	Gagnaire *et al.*, 2006
Benzo[a]pyrene	Hemocytes viability ↓	*Mytilus galloprovincialis*	Gómez-Mandikute *et al.*, 2003
Benzo[a]pyrene	Lysozyme activity ↓ Phagocytic activity ↓ Adhesion capability ↓	*Chamelea gallina*	Matozzo *et al.*, 2009
Phenanthrene	Hemocyte mortality ↑ Phagocytic cells ↓ Superoxide generation ↓	*Cerastoderma edule*	Wootton *et al.*, 2003
Phenanthrene	Hemocyte number ↑ Cell membrane stability↓ Phagocytosis↓	*Pecten maximus*	Hannam *et al.*, 2010
PCB			
PCB 77	lysosome-positive cells ↓	*Crassostrea gigas*	Gagnaire *et al.*, 2006
Pesticides			
2.4D	Cell mortality ↑	*Crassostrea gigas*	Gagnaire *et al.*, 2006
Paroxon	Esterase and lysosome positive-cell (%) ↓	*Crassostrea gigas*	Gagnaire *et al.*, 2006
Chlorothalonil	ROS positive-cell (%) ↑ Cell mortality and granulocyte (%)↑	*Crassostrea gigas*	Gagnaire *et al.*, 2006
Pesticide mixture (atrazine, gliphosate, alachlor, metalachlor, fosetyl-aluminum, terbuthilazine, diuron, carbaryl)	Phagocytosis ↓ Cell mortality, and ROS production ↑ Genes relationship with immune response (e.g., lyzozyme, defensines) ↓ Susceptibility to bacteria challenge ↑	*Crassostrea gigas*	Gagnaire *et al.*, 2007
Paraquat	Hemocytes viability	*Mytilus galloprovincialis*	Gómez-Mandikute *et al.*, 2003
Other			
Fuel Oil No. 6	Cellular viability↓ GST and CAT↑ GPx ↓	*Pinctada imbricate*	Nusseti, et al. 2004
4-Nonylphenol	Lysozyme concentration ↓ Apoptitic Index ↑	*Tapes philippinarum*	Matozzo, et al. 2005

Symbol: Reduction (↓), increased (↑), biphasic effect (↑↓)

decrease in this parameter. The superoxide anion production (using NBT reduction test) in hemocytes was evaluated. The results indicated that Cd exposure induced no changes, but Cu exposure decrease the NBT reduction (Gómez-Mandikute *et al.*, 2003).

The studies have been showed that metals could modulate different immunologic parameters on molluscs. However, the immunomodulation is influenced by metal concentration, and other factors such as presence of potential pathogens and environmental variables, like temperature for instance.

Estrogenic substances effects

Others pollutant substances frequently present in aquatic ecosystem are estrogenic chemical. In this context, Canesi, *et al.* (2007), evaluated the *in vitro* effect of endocrine disruptor compounds on *Mytilus* hemocytes. The results showed that hemocytes incubated during 30 minutes, with estrogenic compounds, such as nonylphenol monoethoxylate carboxylate (NP1EC) and 17α-ethynyl estradiol, increased the lysosomal enzyme release in 65 % and 45 %, respectively compared with control hemocytes. On the other hand, a biphasic effect was observed on phagocytosis, thus to lower concentrations (0.1 - 5 µM) a significant stimulations was detected, while to 25 - 100 µM a inhibitory effect was observed.

The effect of 17β-estradiol (20, 200 and 2000 ng/l) was evaluated on Asian clam *Corbicula fluminea,* exposure during 15 or 30 days. Results showed that this estrogenic substance did not affect the cell viability. However, the exposure to 200 and 2000 ng/l significantly inhibit the phagocytosis, in both evaluated times (Champeau *et al.*, 2006).

The effects of endocrine disrupters, such as natural or synthetic steroids, on immune system of molluscs are not well known yet, in part by limited knowledge on invertebrate endocrine system and immunendocrine network. However, results suggest that immune system represents an important target of estrogenic compounds. Thus, the study of these compounds and their effect on invertebrate physiology is necessary.

Pharmaceutical products effects

Municipal effluents represent a major source of pollution. These effluents could contain pharmaceutical products, xenobiotics that could modulate immune response of aquatic organisms. Studies on mussels (*E. complanata*) hemocytes exposure *in vitro* to pharmaceutical drugs (benzafibrate, carbamazepine, fluoxetine, gemfibrozil, morphine, naproxen, novobiocin, oxytetracycline, sulfamethazole, sulfapyridine and trimethoprim) and urban waste (coprostanol, caffeine, cotinine) at 0, 2.5, 25, 50 and 100 µM, showed that some products as benzafibrate, gemfibrozil and trimethoprim, increased phagocytosis, while novobiocin and morphine reduced its activity. Intracellular esterase activity was reduced with sulfamethazole, novobiocin, gemfibrozil, benzafibrate and carbamazepine. Cellular adhesion was decreased by oxytetracycline, novobiocin and naproxen, and increased by gemfibrozil, bezafibrate and sulfapyridine. Exposure to these products also modulated lipoperoxidation (LPO) in hemocytes. Coprostanol and naproxen were more potent to reduce LPO while novobiocin and sulfapyridine were the most potent to induce LPO. On the other hand, on a parallel experiment, mussels were placed in aeration lagoons for the treatment of domestic wastewaters during 60 days. In mussels, a decrease of intracellular esterase and phagocytic activity was observed (Gagne *et al., 2006*).

PAHs effects

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous class of organic contaminants generated as results of anthropogenic sources or natural constituents of crude oil. The effect of phenanthrene (50, 100, 200 or 400 µg/l) on immunological parameters of *Mytilus edulis*, *Cerastoderma edule*, and *Ensis siliqua*, were evaluated after exposure during 7 and 14 days. Phenanthrene exposure at 400 µg/l resulted in 100 % mortality of *C. edule* after 14 days exposure, while total mortality of *E. siliqua* was observed 7 days after exposure. Nevertheless, no mortality on *M. edulis* was reported. In general terms, results of immunologic parameters showed that acid phosphatase concentration was increase in *M. edulis* after 7 days exposure to phenanthrene 50, 100 and 200 µg/l, but a diminish in this parameter was observed 14 days after exposure at same concentrations. Phagocytic cells percentage and superoxide generation were significantly reduce in *C. edule* after 14 days exposure to 100 and 200 µg/l. Comparative analysis between three different species suggests that *E. siliqua* is less sensible to alterations by exposure to phenanthrene (Wootton *et al.,* 2003).

Studies realized with scallop *Pecten maximus,* showed that exposure during 7 days at 100 and 200 µg/l phenanthrene, increase hemocyte number. Nevertheless, cell membrane stability, and phagocytosis were reduced when organism were exposed to 200 µg/l. In addition, oxidative stress parameters were evaluated, indicating that 200 µg/l phenanthrene provoke diminish of GSH activity, but significantly increased lipoperoxidation index (Hannam *et al*., 2010).

Another PAH is Benzo(a)pyrene, effect of 0.5 mg/l of this substance was evaluated on immune response of clam *Chamelea gallina*. Exposure to this xenobiotic during 7 and 12 days significantly decreased lysozyme activity, phagocytic activity and adhesion capability (Matozzo *et al.*, 2009). Another study showed that benzo(a)pyrene did not have significant effect on viability of hemocytes of *Mytilus galloprovincialis*. But this substance induced a significant increased in superoxide anion production (Gómez-Mandikute *et al.*, 2003).

Studies made with Pacific oyster *Crassostea gigas* exposed *in vitro* to benzo(a)pyrene and phrenanthrene showed that these substances significantly increased granulocyte percentage, but decreased cell mortality and esterase and lysosome-positive cells at doses of 200 and 300 µmol/l, respectively (Gagnaire *et al.,* 2006).

Atlantic pearl oyster (*Pinctada imbricata)* exposure to Fuel oil N^{o} 6 during 7 days, showed no

significantly changes in immunological parameters, such as hemocyte number, phagocytosis, and lysozyme concentration. However, cellular viability was reduced when oyster were exposed to this xenobiotic. Antioxidant enzymes such as, glutathione S-transferase (GST), and catalase (CAT) were significantly higher in the digestive gland. While in mantle, an increase of glutathione peroxidasa (GPx), and decrease GST activity was detected. This report suggests that these enzymes should be considered as potential tools for biomonitoring marine environmental contamination (Nusetti *et al.,* 2004).

Other studies have focused their efforts to study the immune system in organisms living at low temperature (-1 to 5 °C). Camus *et al.* (2002), evaluated the effect of benzo(a)pyrene on oxyradical scavenging capacity (TOSC), and cell membrane stability of hemocytes from Arctic scallops (*Chlamys islandicus*). Results indicated a reduction of TOSC, and cellular membrane stability when benzo(a)pyrene was administrated at 74 and 90.6 mg/Kg. These alterations should be negative for the cellular immunity of bivalves by reducing the phagocytosis ability of hemocytes.

PAHs exposure could increase susceptibility to infections. Some studies suggest that reduction in immunocompetence is related to stimulation of ROS production induced by PAHs.

Pesticides effects

Pesticides are often used in successful agriculture. However, the pesticide use leads to severe environmental pollution. This way, aquatic organisms are frequently affected by these xenobiotics.

Studies realized with Pacific oyster *Crassostea* exposure to pesticide, showed that 2,4-Dichlorophenoxyacetic acid (2,4D), increased cell mortality at 450 µmol/l after a 4 h incubation period. While, paroxon exposure induced decrease in percentage of esterase-positive cells after 4 and 24 h of incubation at 400 µmol/l. But paroxon at 40 and 400 µmol/l, after 24 h incubation period, decreased lysosome-positive cells percentage; in contrast ROS-positive cells were significantly increased at 400 µmol/l after 4 h incubation period. The fungicide chlorothalonil at 2 µmol/l, significantly increased cell mortality and granulocyte percentage at 200 µmol/l after 4 h incubation period. In addition, a pesticide mixture (alachlor, metolachlor, terbutilazina, glyphosate, diuron, atrazine, carbaryl, and fosteyl aluminium) was realized, but interestingly enough none of this eight compounds generated significantly effect when tested individually on *C. gigas,* but the mixture indeed decreased phagocytic activity (Gagnaire *et al.,* 2006).

Other studies carried out with pesticides, showed that paraquat on *Mytilus galloprovincialis* hemocytes, showed a significantly decrease on viability of hemocytes (according XTT test) exposed to paraquat (10 µg/ml). On the other hand, paraquat exposure, induced a significantly increase in superoxide anion (Gómez-Mandikute *et al.*, 2003)

In order to know the effect of pesticide on bacteria challenge, Pacific oyster *C. gigas* were exposed to a mixture of pesticides (atrazine, glyphosate, alachlor, metolachlor, fosetyl-alumimium, terbuthylazine, diuron and carbaryl) at environmental relevant concentration over a 7-days period. As a first step, hemocyte parameters (cell mortality, enzymes activities, and phagocytosis) were evaluated. The results showed that phagocytosis was significantly reduced, while cell mortality, esterase and ROS production, were not altered. However, real-time PCR analyses showed that 19 genes (involved with cell signaling, cytoskeleton function, phagocytosis and other defense mechanisms) were down-regulated in treated animals. Moreover an increased susceptibility to a bacteria challenge was observed. As a second step, the interaction between pesticide exposure and bacteria challenge (*Vibrio splendidus,* $4x10^7$ UFC/oyter) was evaluated. In this co-exposure condition, was observed that 10 of 19 genes (focolin, galectin, LBP, c-Src, ankyrin, ProCL, SOD, TMP, lysozyme, defensin) was up-regulated. The authors suggested that up-regulated genes could induce damage in host-tissue (Gagnaire *et al.,* 2007).

Ozone effects

On the other hand, the municipal effluents then sometimes undergo disinfection. A common process involved is ozonation. However, ozone treatment might generate toxic products. Studies carried out by Gagné *et al*. (2008), showed that freshwater mussels (*Elliptio complanata*) exposed to ozone (range 1 - 20 %), in laboratory condition for 7 weeks, significantly diminished phagocytosis and cellular viability. However, cell adherence suffered no changes when compared to control group. In contrast, COX-activity and nitrite levels were significantly increased. According to results, O_3, at concentration evaluated, reduce microbial loading and completely remove citotoxicity, but increased inflammatory properties of the effluents. The observed effect could be related to the formation of carboxylic acid, aldehydes, and ketones which modifies the redox status of treated wastewaters.

Others Substances

The effect of 4-nonylphenol (NP), final product of nonylphenol eyhoxylates, substances used as stabilizer, was evaluated on clam *Tapes philippinarum*. Results showed that exposure at sublethal concentrations (0.05 - 0.2 mg/l) during 7 days, significantly reduced lysozyme concentration and SOD activity. In contrast, apoptotic index was increasing at same concentrations (Matozzo *et al.*, 2005).

In another research, hemocytes from the Pacific oyster *Crassostea gigas* were exposed *in vitro* to polyclorinated byphenyls (PCB), such as PCB 77. The results showed that this substance significantly decreased lysosome-positive cells at 6 and 60 µmol/l after 4 h incubation (Gagnaire *et al.,* 2006).

Field studies

Laboratory studies showed some advantages, the principal being the experiments performed in controlled conditions. However, field researchers in ecotoxicology, permit to analyze parameters

according to conditions present at one particular moment, and with all the factors that have influence over an ecosystem. Furthermore, these type of studies are more suited to distinguish a correlationship between factors present on specific sites.

Recently, our research group evaluated the presence and concentration of PAHs (pyrene, naphtalene, and benzo(a)pyrene), metals (Cu, Pb, Zn, Mn, As, Fe), and organophosphorus pesticide (acetylcholine inhibition) on Mexican Pacific estuarine zone, and the relationship of pollutants with immunological and oxidative stress parameters in oyster *Crassostrea corteziensis.* Results indicated that the main xenobiotic detected were Cu and naphthalene. Furthermore, the acethylcholine inhibition tests, suggest the presence of organophosphorus pesticide in the estuary. Microbicidal activity was not altered, but a significantly decrease in hemocyte number was detected. On oxidative stress parameters, an increase of superoxide anion, hydrogen peroxide, catalase activity and lipoperoxidation were observed in gills from oyster (unpublished data).

In other studies, a positive correlation between xenobiotic concentration presents in ecosystem and increase in defense mechanisms of molluscs has been reported (Fisher *et al.,* 2000; Oliver *et al.*, 2003).Thus, experiment designing have been made to show if the deployment of eastern oyster (*Crassostrea virginica*) from uncontaminated through contaminated sites would increase immune response parameters and vice versa. The results showed that hemocytes count and bactericidal activity were significantly elevated after 12-week deployment at contaminated sites (metals, PAHs and PCB) from Florida. However, when similar experiment were realized inverted, the results were ambiguous, thus lysozyme concentration was reduced, but hemocyte activities (principally bacteria killing index and hemocyte count) were not challenged. Authors suggest that these results could be indicative of an acclimatization response with adaptative consequences of oyster to chronically polluted sites (Fisher *et al.,* 2003).

Conclusion

The data presented here suggests that all groups of pollutants may be hazardous to molluscs defense system. In general terms, there are many examples of links between xenobiotic and susceptibility to diseases in wildlife species, principally vertebrates with economical importance. Also laboratory tests allowed identifying potential hazard, mainly anthropogenic chemicals with immunosupressor properties. However, invertebrate organisms have ecological relevance besides only economical importance, as they represent around 95 % of all animal species. In this matter is very important to understand the immunologic mechanisms invertebrate as molluscs and relationship with environmental condition. This will allow acknowledging the susceptibility of each species to antigen challenges, mainly infectious agents, and if such conditions affects the intrinsic resistance of each organism.

Acknowledgments

This review was conducted under FOMIX (CONACyT-Nayarit government) project (number 131614), and PROMEP-SEP founding. Thanks to MSc C Rodríguez-Cervantes for English correction style, and MD student I Parrao for figure design.

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