Echinoderm Reactive Oxygen Species (ROS) Production Measured by Peroxidase, Luminol-Enhanced Chemiluminescence (PLCL) as an Immunotoxicological Tool

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Abstract. The importance of reactive oxygen species (ROS) production in invertebrate immunity prompted the use of this response in immunotoxicological studies in several taxa including marine organisms. In this chapter, we review the effects of environmental factors and contaminants such as heavy metals and polychlorinated biphenyls (PCBs) on the production of ROS by the main immune effector cells of echinoderms, the so-called amoebocytes. ROS production was measured by the peroxidase, luminol-enhanced chemiluminescence (PLCL) method. This method was found to predominantly reflect the production of superoxide anions and peroxides, among which hydrogen peroxide and peroxynitrite are the main species detected. Exogenous factors such as water temperature and salinity can influence this immune response in echinoderms. However, gender, handling stress and parasitism by a castrating ciliate apparently did not affect it. The impact of metals on ROS production differed greatly according to the duration and routes of exposure; in vitro and short-term in vivo exposures to metals caused an inhibition of this immune response, while the opposite effect was observed in a long-term in vivo exposure study. On the other hand, PCBs systematically had a stimulatory effect on ROS production independent of the echinoderm species or exposure routes. From the study of complex field contaminations, it appeared that contaminants released in the environment, such as metals, modulate starfish amoebocyte ROS production. This impact potentially represents a threat to the sustainability of natural populations of echinoderms and thereby to the stability of benthic ecosystems.

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1 Introduction

In contrast to vertebrates, invertebrates defend themselves against offending microorganisms only by means of non-specific, innate mechanisms. The efficiency of this primitive immune system is witnessed by the fact that 95 % of existing animals are invertebrates. This system relies on both cellular and humoral components. The immune cells are involved in phagocytosis, encapsulation, hydrolytic enzyme secretion and the respiratory burst (Chia and Xing 1996). The latter is a mechanism by which a phagocytic cell drastically increases its oxygen consumption upon encounter with foreign material. The oxygen is converted into the very reactive superoxide anion O_2– by a membrane-bound enzyme, the NAD(P)H-oxidase (Babior 1984). This free radical in turn yields several radical or non-radical oxidants such as the hydroxyl radical (·OH–), hydrogen peroxide (H_2O_2) or singlet oxygen ($^1O_2$). The reduction of hydrogen peroxide by the myeloperoxidase (MPO) enzyme may also lead to the formation of hypochlorous acid (HClO). All these oxidants are collectively called reactive oxygen species (ROS). The transduction of the stimulatory signal from the membrane to the NAD(P)H-oxidase involves phospholipase C (PLC) and protein kinase C (PKC) and requires the presence of calcium ions (Lennartz 1999; Torreilles et al. 1999). ROS are thought to destroy foreign particles by oxidising macromolecules such as lipids, proteins or nucleic acid, resulting in lipid peroxidation, enzymatic activity disruption and DNA damage (Buechter 1988). They might also act by inducing an osmotic imbalance in the lysosomes, ending in the activation of hydrolytic enzymes (Reeves et al. 2002).

The importance of ROS production in invertebrate immunity prompted the use of this response in immunotoxicological studies in several taxa including marine organisms. Most of these studies are dedicated to bivalve molluscs or arthropods (Baier-Anderson and Anderson 2000). In these groups, metals generally inhibit ROS production, although, in some instances, an increase in this immune response was observed after both in vitro (Anderson et al. 1997) and in vivo (Dyrynda et al. 1998) contaminations. Decreased ROS production was also observed in bivalves after experimental polycyclic aromatic hydrocarbons (PAH) exposure and in mussels collected in the field after an oil spill (Larson et al. 1989; Dyrynda et al. 1997). Similarly, Pipe et al. (1995) determined the production of ROS by immune cells of mussels collected in contaminated sites and found a negative correlation between the immune response and the level of organic contamination. The observed effects appear to vary greatly according to the contaminant and the invertebrate species studied and the exposure mode. The mechanism by which the production of ROS is affected remains unclear and probably depends on the contaminant studied. Metals are known to take part in the formation of certain reactive species (Kehrer 2000) but can also act by inactivating divalent cation-requiring enzymes involved in ROS production. Other contaminants

such as pentachlorophenol are uncouplers of oxidative phosphorylation and may act by interfering with the activity of the NAD(P)H-oxidase (Baier-Anderson and Anderson 1997).

Despite their ecological and economical importance, echinoderms have been largely ignored in the field of immunotoxicology. Numerous representatives of this phylum are key species of marine benthic ecosystems. By their abundance or their trophic role, they often act as structuring species of their ecosystem. Moreover, since the marine sediments represent a reservoir for many contaminants, echinoderms that live within or upon the sediments are particularly at risk concerning the impact of marine pollution. ROS production in this phylum has been reported by a few authors (Ito et al. 1992, Wheatley et al. 1998). Therefore, we investigated the effects of environmental factors and contaminants such as heavy metals and polychlorinated biphenyls (PCBs) on the production of ROS by the main immune effector cells of echinoderms, called amoebocytes. These results are reviewed and discussed in the present work along with a description of the method used to measure ROS production.

2 Measurement of Reactive Oxygen Species (ROS) Production

Luminol-enhanced chemiluminescence (LCL) has been used for a long time for the measurement of the respiratory burst of vertebrate phagocytic cells. This method is considered to be sensitive to both the formation of oxygen radicals and the activity of the endogenous peroxidase, myeloperoxidase (MPO). The signal obtained using invertebrate immune cells is often much lower compared to their vertebrate counterparts (Torreilles et al. 1996). This can be improved by adding an exogenous peroxidase (horseradish peroxidase, HRP) to the measuring medium (Coteur et al. 2002). Adding HRP increases the signal intensity and makes it more specific to the presence of peroxides. We used this system to measure ROS production by echinoderm amoebocytes and determined the specificity of this method. It was found that peroxidase, luminol-enhanced chemiluminescence (PLCL) is predominantly affected by superoxide anions and peroxides, among which hydrogen peroxide and peroxynitrite appear to be the main species detected (Coteur et al. 2002).

The detailed protocol of this method is as follows. Experimental animals are wrapped in a towel to absorb external seawater and then bled by cutting the tip of one arm and draining 3 ml of coelomic fluid in an equal volume of sterile anticoagulant buffer [Ca^{2+}, Mg^{2+}-free artificial seawater (CMFASW) (Noble 1970) with 1.2×10^{-2} M EDTA] at 4 °C. The amoebocyte concentration of this suspension is determined as follows: 200 µl of the cell suspension from each animal is distributed in wells of a UV-transparent microplate (96-wells, UVstar, Greiner), cells are mildly agitated for 5 s and the absorbance at 280 nm

is determined using a microplate reader (Spectrafluor Plus, TECAN; three flashes per well). This is related to the cell concentration by using the following predetermined relation (Coteur et al. 2002): A_{280}=0.157 × cell concentration (×106 cells ml^{-1})+0.067. The suspension is then centrifuged (400 *g*, 10 min, 4 °C) and the supernatant replaced by CMFASW (without EDTA), the volume of which is adjusted to obtain a concentration of $1\pm0.25 \times 10^6$ cells ml^{-1}. This concentration is used for normalising chemiluminescence measurements. For PLCL measurements, 100 µl of a luminol/HRP solution [freshly prepared by 100-fold dilution in artificial seawater (ASW) of a stock solution of luminol 10 mg ml^{-1}, HRP 5 mg ml^{-1}, dimethylsulfoxide (DMSO) 1 M in ASW] is added to 20 µl of a bacteria suspension (*Micrococcus luteus*, 5×10^9 bacteria ml^{-1} in ASW) or an equivalent volume of ASW (unstimulated controls) in replicate wells of an opaque white microplate (96 wells, Lumitrac, Greiner). The chemical background (i.e. the chemiluminescence of the bacteria and the solutions alone) is then measured using a microplate reader (Spectrafluor Plus, TECAN) with the following settings: luminescence mode, integration time = 0.5 s, photomultiplier gain = 180. The reading time for 80 wells is about 75 s. Subsequently, 80 µl of amoebocyte suspension in CMFASW is added (resulting in a final amoebocyte concentration of $4\pm1\times10^5$ cells ml^{-1}). (Thus, the resulting final solution contains 60 % of the nominal Ca^{2+} and Mg^{2+} concentrations of seawater.) The microplate is stored at 13±1 °C (i.e. the average field seawater temperature) and PLCL is measured every 10 min over a 2-h period. Measurements are normalised with the actual amoebocyte concentration in each sample and expressed as the sum of all 10-min interval measurement [in relative light units (RLU), an internal scale of the instrument] for 10^6 cells ml^{-1} (total chemiluminescence) for resting or stimulated amoebocytes.

3
Modulation of ROS Production by Environmental Factors

In order to distinguish the influence of environmental pollution from natural variability of echinoderm immune responses, it is crucial to delineate the endogenous or exogenous factors that affect these responses. For instance, injury, acute temperature shock, acidic pH or heavy metal shock were shown to affect the sea urchin immune system (Matranga et al. 2000, 2002). Moreover, a year-round study of a particular immune response is necessary in order to identify periods that might be characterised by increased variability compared to others. Therefore, we investigated the effects of environmental factors and the annual variability of ROS production by amoebocytes of the starfish *Asterias rubens* (Coteur et al. 2004).

The ROS production by amoebocytes is efficiently stimulated by the presence of bacteria or bacterial wall components such as lipopolysaccharide (LPS) but not by the soluble stimulant phorbol-myristate-acetate (PMA, a

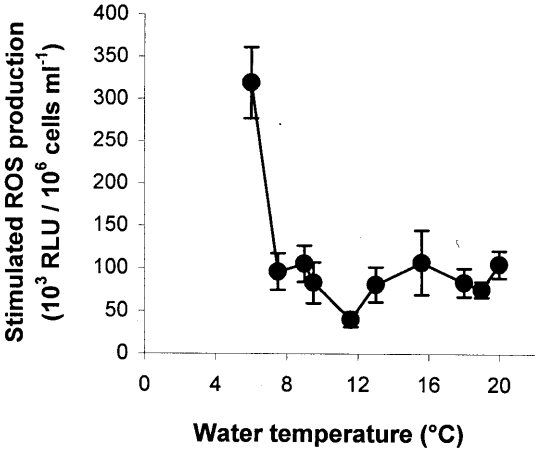

Fig. 1. Bacteria-stimulated reactive oxygen species (*ROS*) production (mean ± SE, n=10) in starfishes collected monthly in Ambleteuse (France) in function of the water temperature measured at each sampling occasion. *RLU* Relative light units

protein kinase C activator) (Coteur et al. 2002). Other factors such as water temperature affected the production of ROS both in experimental conditions and in the field. The ROS production in starfish maintained in very cold waters (≤ 6 °C) was dramatically increased compared to starfish held at (even slightly) higher temperatures and this effect was also observed in the field during the coldest month of the year (Fig. 1).

It is thus a threshold-type effect that precludes the use of ROS production in the field during this period since very high natural variations can occur in limited time. Another factor that partially determines the immune response is the water salinity as ROS production decreases with increasing salinity. However, handling stress, gender and parasitism by a castrating ciliate did not influence amoebocyte ROS production in our experimental setup (Coteur et al. 2004).

4 Impact of Metal Contaminations

In order to have a comprehensive picture of the effects of metals on ROS production, this immune response was measured after in vitro exposure of *A. rubens* amoebocytes or after in vivo experimental contamination of starfishes. Furthermore, natural populations exposed to these contaminants were monitored in the field.

A first experiment was designed to screen the potential of several metals to affect ROS production in vitro. Amoebocytes were exposed to different concentrations of selected metals prior to measuring the unstimulated ROS production. Among all metals tested, only mercury and silver were shown to modulate the resting amoebocyte ROS production (linear regressions:

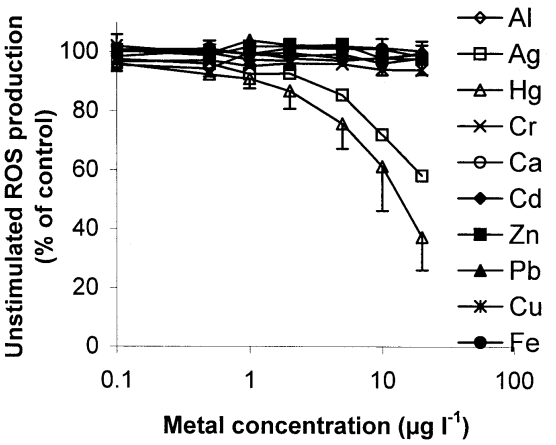

Fig. 2. Reactive oxygen species (*ROS*) production by unstimulated starfish amoebocytes exposed to different metal solution (percentages of control, means and SD are indicated for Hg and Ca as an example; n=6)

$p<0.001$, $R^2=0.277$ and $p=0.004$, $R^2=0.165$, for Hg and Ag respectively) (Fig. 2). The lack of ROS production inhibition by other metals (Al, Cr, Cd, Zn, Pb, Fe and Cu) is somewhat surprising since Larson et al. (1989) found that, among several types of xenobiotics, Cu was the most immunosuppressive in vitro. However, these authors used a contaminating copper concentration range of 200–2,500 µg l^{-1}. In our hands, the maximal copper concentration tested was 2 µg l^{-1} which is closer to environmentally relevant concentrations. In order to compare the sensitivity of *A. rubens* amoebocytes with that of bivalve hemocytes, we calculated the IC50 values (the metal concentration inducing 50 % of response suppression) for mercury and silver. These values (14.8 and 23.0 µg l^{-1} for Hg and Ag respectively) are 60–600 times lower than those obtained using the phagocytic activity of hemocytes of the clams *Spisula polynyma* and *Mya arenaria* (Brousseau et al. 2000; Fournier et al. 2000). It thus seems that starfish amoebocyte ROS production is highly sensitive to in vitro contamination by silver and mercury but not to other metals (at least at or close to environmentally relevant concentrations).

The impact of in vivo metal contaminations was studied along a metal contamination gradient occurring in a Norwegian fjord, the Sørfjord, where wastes of three smelters, built in the innermost part of the fjord, were discharged in this area for more than 60 years. Concentrations of mercury in fish and of cadmium and lead in mussels from this fjord resulted in public advisories to regulate human consumption (North Sea Task Force 1993).

ROS production was measured both in starfishes sampled along the gradient (long-term – life-long – contaminations) and in starfishes transferred up the gradient (short-term experimental contamination) (Coteur et al. 2003a). The production of ROS in starfishes from field populations increased along the pollution gradient in direct relation with the contamination of the starfishes by cadmium, lead and zinc (e.g. zinc, Fig. 3).

In contrast, when starfishes were transferred from the control site to the contaminated head of the fjord, the temporary accumulation of some metals such as zinc or cadmium in starfishes was accompanied by an inhibition of ROS production (Fig. 4).

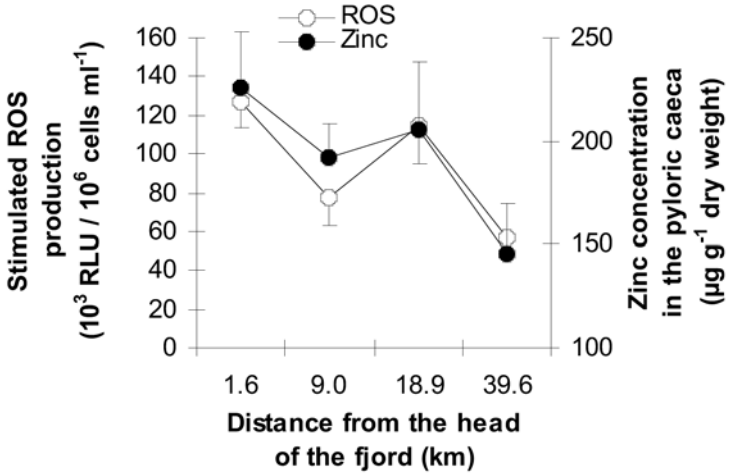

Fig. 3. Bacteria-stimulated reactive oxygen species (*ROS*) production (mean ± SE, *n*=10) and zinc concentration in the pyloric caeca (mean ± SE, *n*=5) of starfishes collected along a metal pollution gradient in a Norwegian fjord. *RLU* Relative light units

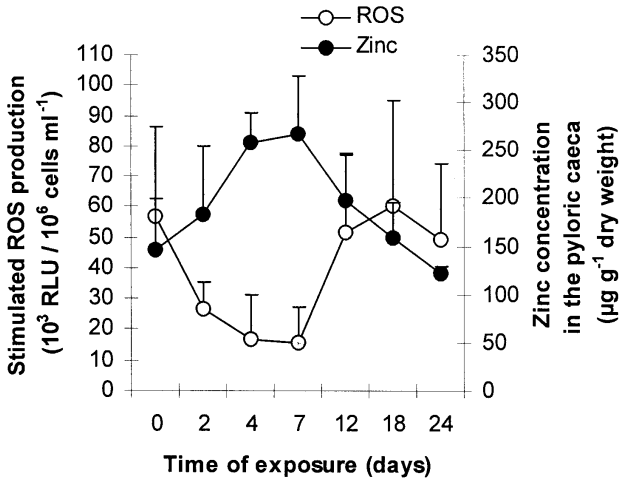

Fig. 4. Effects of short-term transfer experiments up the gradient of metal contamination in a Norwegian fjord. Bacteria-stimulated reactive oxygen species (*ROS*) production (mean ± SE, *n*=10) and zinc concentration in the pyloric caeca (mean ± SE, *n*=5) of starfishes transferred from the uncontaminated site (opening of the fjord) to the most contaminated site (head of the fjord) over time. *RLU* Relative light units

Thus, it seems that, depending on the duration of exposure (several days or life-long), the effects of the same contaminants can range from a severe inhibition to a marked increase in ROS production. We hypothesised that the impact of metals in field conditions would occur in three phases: short-term inhibitory effects exerted by direct action of metals on the immune cells are followed by a recovery due to the induction of protective mechanisms and, eventually, when these mechanisms are overwhelmed, indirect stimulatory effects on the immune responses appear due to a global disruption of the animal physiology.

For studies on the impact of metal contamination on stress marker production, see Matranga et al., (this Vol.).

5 Impact of PCB Contaminations

Polychlorinated biphenyls are persistent organic contaminants of human origin which accumulate in the environment. These contaminants are highly toxic, particularly the non-ortho-substituted and mono-ortho-substituted congeners, the so-called coplanar PCB congeners (cPCBs) that can display a configuration very close to that of the highly toxic 2,3,7,8-tetrachlorodibenzodioxin (2,3,7,8-TCDD).

The effects of PCBs were tested by injecting coplanar or non-coplanar congeners in the coelomic cavity of the sea urchin *Paracentrotus lividus* (Coteur

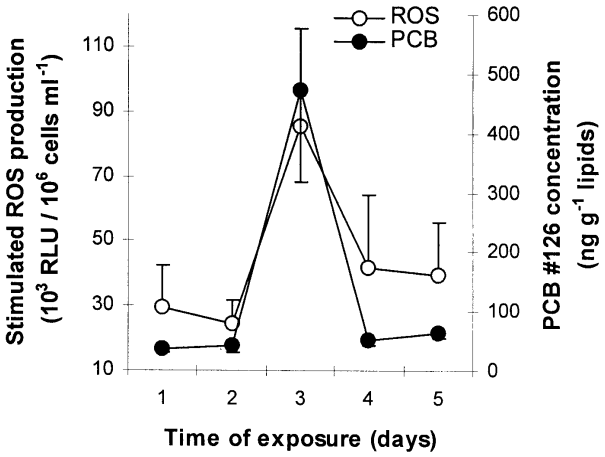

Fig. 5. Bacteria-stimulated reactive oxygen species (*ROS*) production (mean ± SE, *n*=4) and PCB congener #126 (mean ± SD, *n*=4) concentrations in the body wall of starfishes maintained on sediments spiked with a mixture of PCB congeners, over exposure time. *RLU* Relative light units

et al. 2001) and of the starfish *A. rubens* (Danis et al., pers. comm.). In both studies, PCBs were found to increase the production of ROS; coplanar congeners being the most effective in this respect. At very high concentrations, however, a steep drop in ROS production was observed probably due to direct cellular toxicity (Danis et al., pers. comm.). Similarly, when starfishes were maintained on a sediment spiked with a mixture of PCB congeners, the amoebocyte ROS production followed closely the transient accumulation of coplanar congeners, such as congener #126, in the starfish tissues (Fig. 5).

Likewise, the contamination by coplanar congener #77 was found to increase the ROS production of starfishes exposed through water, sediments or food (Danis et al. 2003).

6
Impact of Complex Contaminations

We investigated amoebocyte ROS production in natural populations of starfishes exposed to complex, multi-elemental contaminations in order to assess the general impact of contaminants on the immune function of

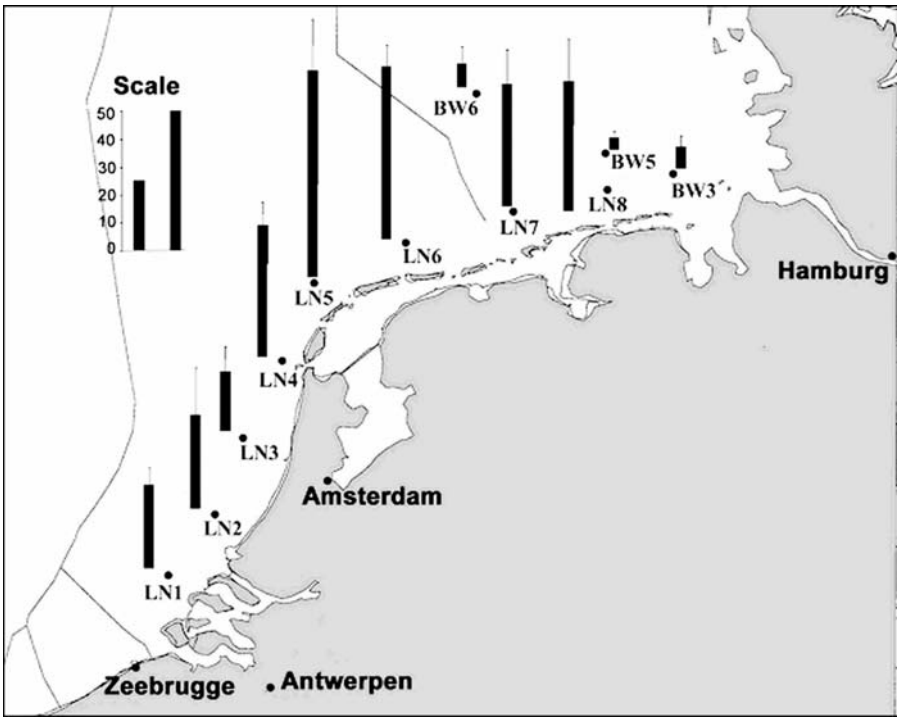

Fig. 6. Bacteria-stimulated reactive oxygen species (ROS) production (10^3 RLU/10^6 cells ml^{-1}; mean ± SE, n=10) in starfishes collected from different stations in the North Sea. *RLU* Relative light units

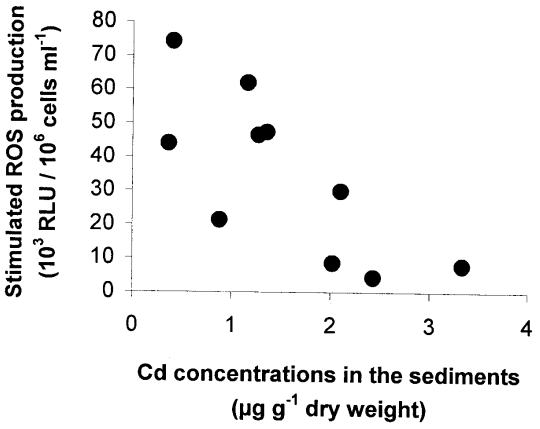

Fig. 7. Bacteria-stimulated reactive oxygen species (*ROS*) production (mean ± SE, n=10) in starfishes collected from different stations in the North Sea in function of the cadmium concentration in the sediments of these stations. *RLU* Relative light units

starfishes in the field. Starfishes were collected at different North Sea stations presenting a large range of metal and PCB contamination levels (Coteur et al. 2003b). Significant differences were found in ROS production of starfishes collected at the different stations. In particular, ROS production was significantly inhibited at the stations located off the Elbe/Weser estuaries (BW stations) and off the North Sea Canal (LN3) (Fig. 6).

Moreover, ROS production was negatively linked with the levels of metal contamination in the starfish tissues and in the sediments (Fig. 7). However, no link was found between the immune response and the non-coplanar PCB concentrations in the samples.

From this study it can be concluded that the contamination levels observed in field conditions are sufficient to induce immunomodulation in echinoderms and that this modulation is particularly linked with metal contamination of the environment.

7 Discussion

The production of reactive oxygen species constitutes one of the main mechanisms of destruction of foreign material in invertebrates. In echinoderms, it was first demonstrated by Ito et al. (1992) who found that sea urchin amoebocytes produce hydrogen peroxide when stimulated by erythrocytes. From our studies, bacteria or bacterial wall components also appear as efficient stimulators of ROS production. On the other hand, the parasitism of starfishes by a castrating ciliate or the challenge of sea urchins by the amoeba *Paramoeba invadens* did not modulate this oxidative activity (Wheatley et al. 1998; Coteur et al. 2004). Exogenous factors such as water temperature and salinity can influence this immune response in echino-

derms. It has been hypothesised that this increased ROS production is linked to the elevated concentration of dissolved oxygen, a major source of ROS, at low water temperature (Regoli et al. 2000). Sea urchin coelomocytes react by overexpressing a heat shock protein (Hsp70-like protein) when subjected either to a rise or to a drop in temperature, the latter inducing a greater stress response than the former (Matranga et al. 2000). The sharp rise in Hsp70 expression in coelomocytes at low temperatures might represent a protective response towards the ROS produced by these cells, since Hsp70 is an efficient antioxidant (Chong et al. 1998). From our studies, it is concluded that if a particular period is to be selected for comparing ROS production in the field, we would suggest either October to December, which is characterised by constant (but low) resting and stimulated ROS production (the latter being around 50×10^3 RLU/10^6 cells ml^{-1}), or the period between April and August, during which stimulated ROS production is high and constant (around 100×10^3 RLU/10^6 cells ml^{-1}) but somewhat offset by an increased variation in resting ROS production.

When these precautions are taken, ROS production appears as a very efficient tool for immunotoxicological studies since it is modulated by the most abundant and toxic contaminants. However, concerning metals, the effect differs greatly according to the exposure type: the duration and routes of exposure appear crucial for determining the impact on ROS production. This variability in the impact of metals makes it necessary to always include background-exposed starfishes whether in experimental conditions or in the field in order to work on relative instead of absolute grounds.

In contrast to metals, PCBs have systematically the same effect on ROS production independent of the echinoderm species or exposure routes in experimental conditions. Either the stimulatory effect of PCB could be explained by a direct action of PCBs on the intrinsic mechanism of ROS production by amoebocytes as suggested by Coteur et al. (2001), or PCB detoxification mechanisms could take place in amoebocytes; these processes lead to the production of a certain amount of oxygen free radicals, adding to the ROS produced spontaneously by the immune cells.

A contaminant-induced increase or decrease in ROS production both represent a hazard to the health of individuals. Decreased production will lead to recurrent infections since invading microorganisms will not be efficiently destroyed. This was illustrated in molluscs in which contaminants that reduced ROS production by oyster hemocytes exacerbated the severity of infections (see Baier-Anderson and Anderson 2000). It was also shown that some mollusc pathogens have the ability to inhibit ROS production and consequently to survive in their host (LaPeyre et al. 1995). On the other hand, increased ROS production can lead to damages to self tissues since ROS are, by their very nature, totally non-specific and are able to alter a wide range of self-macromolecules such as DNA and enzymes (Buechter 1988).

From the study of complex field contaminations, it appears that contaminants released in the environment, such as metals, modulate starfish amoebo-

cyte ROS production. This impact potentially represents a threat to the sustainability of natural populations of echinoderms and thereby to the stability of benthic ecosystems.

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