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Ecotoxicology and Environmental Safety 63 (2006) 393–405
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

A multiparametric approach for monitoring immunotoxic responses in mussels from contaminated sites in Western Mediterranea

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Received 29 October 2004; received in revised form 14 September 2005; accepted 10 October 2005
Available online 13 December 2005

Abstract

As a part of the multidisciplinary program Biological Effects of Environmental Pollution in Marine Coastal Ecosystems of the European Commission, this study aimed to validate immunological alterations as biomarkers of exposure to chemical contamination in polluted areas of Western Mediterranea. The status of the immune system has been assessed in mussels (*Mytilus galloprovincialis*) by measuring several immunopathological and immunocompetence parameters. Alterations of total hemocyte counts, lysosomal stability, and phagocytosis were among the most reliable effects observed in polluted sites and suggested immunosuppressive conditions in contaminated mussels. An immunotoxicological index was calculated from the set of individual data. By providing a single value per sampling station to score immunological alterations in mussels, this novel approach allowed recognition of a gradient of perturbation correlated to pollution intensity in two of the three sites monitored. Processing a set of biological parameters by this method was found to increase the ecotoxicological relevance of such multiparametric studies for the assessement of chemical contamination in coastal waters.

Keywords: Immunotoxicology; Bivalve mollusks; Environmental contamination; Population survey; Immunotoxicological index

1. Introduction

Various procedures for environmental surveys, including monitoring programs, networks for ecological watch, and institutional observatories, have been set up and are now operational for medium- and long-term monitoring of marine ecosytems exposed to chemical contamination. It is necessary to acquire and study biological variables as signals produced by biota in response to habitat disturbance to increase knowledge of mechanisms of action of pollutants in coastal ecosystems and to provide better tools for site management.

Many chemicals released in the environment are able to generate toxicity in aquatic organisms. Recent knowledge on biochemical, cellular, and other genotoxic effects has allowed development of useful so-called exposure biomarkers (Depledge, 1994). Among alterations identified in physiological systems, chemically induced immunological disorders have been well documented in an increasing number of species. Immunotoxicology is now a fertile domain to develop biomarkers of effect able to detect probably less specific but sensitive signals for deleterious effects of

Abbreviations: ACPS, serum acid phosphatase; BEEP, biological effects of environmental pollution in marine coastal ecosystems; DW, dry weight; EC, European commission; HAAS, hemocyte antiaggregant solution; MT, metallothionein; NR, neutral red; NBT, nitro blue tetrazolium; OD, optical density; PAHs, polycyclic aromatic hydrocarbons; PC, phagocytic capacity; PCBs, polychlorinated biphenyls; PI, phagocytic index; PRTC, total hemolymph cell proteins; PRTH, total hemolymph proteins; PRTS, total serum proteins; ROS, reactive oxygen species; RT, room temperature; THC, total hemocyte counts; WP3; work package 3

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0147-6513/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2005.10.016

environmental contamination (Luster et al., 1989; Bernier et al., 1995; Zeeman, 1996; Burchiel et al., 1997). Any application of such biomarkers in monitoring programs, however, needs preliminary field studies for validation.

Several benthic species of annelids, mollusks, and echinoderms are interesting biological models useful to research in ecotoxicology. As filter-feeders, bivalve mollusks bioaccumulate for most environmental pollutants, including heavy metals and organics (Zatta et al., 1992; Phillips, 1995, Gunther et al., 1999). The process of bioconcentration is obviously an aggravating factor in toxicity. Furthermore, from an ecological point of view, this phenomenon leads to a real integration of chronic contamination in sites usually classified as moderately polluted. Oysters and mussels, which constitute large populations with rapid turnovers in intertidal or subtidal layers of estuarine ecosystems, are now considered ecosentinels in ecotoxicological monitoring programs. As demonstrated by exposure of mussels in the laboratory, several classes of xenobiotics are able to induce severe alterations of both structure and function in their immune system, even at low concentration (Coles et al., 1994, 1995; Pipe and Coles, 1995). In complement, investigations on individuals sampled in the field demonstrated that immunotoxicity could be used to monitor adverse biological effects in polluted areas (Pipe et al., 1995; Cajaraville et al., 2000).

The present study aimed to validate the use of immunological alterations in the mussel *Mytilus galloprovincialis* as biomarkers of chemical contamination within the framework of the EC program "Biological Effects of Environmental Pollution in Marine Coastal Ecosystems" (2001–2004). Three polluted sites of Western Mediterranean coasts were selected by the Work Package 3 (WP3) because of their differences in contamination profile. The main issues to be adressed here were (i) the ability of immunological biomarkers to detect slight to marked differences in contamination among sampling stations within these sites and (ii) the sensitivity to other factors such as sampling period, other environmental factors, and biological cycles. For that purpose, a standardized set of immunopathological and functional parameters was measured in mussels sampled twice a year to assess possible seasonal changes in either the environment or the biota. The former category of parameters included hemocyte counts, cell viability, and lysosomal stability. In complement, cellular detoxication activity based on the expression of metallothioneins in hemocytes has been investigated. The phagocytic activity of hemocytes and the production of intracellular ROS were evaluated as functional parameters.

2. Material and methods

2.1. Animals and sampling sites

Adult mussels, *M. galloprovincialis*, with shell length ranging from 40 to 60 mm ($n = 30$), were collected by scuba divers from natural beds settled on rocky shores and harbor piers. The sampling stations were located on maps during the first campaign in May 2001 (BEEP 01) and resampled during following campaigns in September 2001 (BEEP 02), September 2002 (BEEP 04), and May 2003 (BEEP 05). The sites were not sampled in May 2002 because of technical problems.

The BEEP program included three sites of the Western Mediterranean sea, in France, Italy and Spain (Fig. 1), all considered "hot spots" of marine coastal pollution. The extreme variety of molecules presumably released by human activities in these areas made the chemical charge an unsatisfactory picture of the ecotoxicological risk. Consequently, it was decided by the WP3 component of BEEP to study several stations in each of the three sites to provide an extensive range of toxic potential (Beliaeff and Bocquené, 2004). The stations were located with respect to the main identified pollution sources to follow a suspected contamination gradient. The most distant from the hot spot was considered the reference station (L label in the text below for low pollution level). Others were located either at the hot spot (H label for high pollution level) or near a source of moderate contamination (M label for medium pollution level). When a distinct toxicological profile appeared for a given contamination level, an additional station was sampled (sublabels a and b). For example, the French station Ha was located near petrochemical plants when station Hb received urban sewage. The Italian station Ha was located inside Genova harbor and station Hb outside. The actual contamination level of the sites was assessed through a chemical analysis for heavy metals and selected organics provided to BEEP WP3 by other participants, namely IFREMER-Nantes and the University of Bordeaux.

2.2. Collection of hemolymph and cell preparation

The mussels were processed on board the oceanographic research vessel "L'Europe" chartered for BEEP campaigns. Hemolymph was withdrawn from the adductor muscle sinus of each individual as previously described by Auffret and Oubella (1995) and immediately processed. All steps of assays requiring live cells were performed on board the vessel. Cell extracts or hemolymph fluids were fixed or frozen as described below and stored on board in appropriate conditions (refrigerator, freezer, or liquid nitrogen). All final analyses were performed in the laboratory under standardized conditions. Since the total volume of hemolymph obtained in a single mussel ranged from 0.5 to 1 mL, all assays and measurements could not be performed for each individual. Consequently, at each station, subsamples of 10 mussels were processed as following: one for THC, one for NR uptake and NBT assays, and one for the phagocytosis assay. The hemolymph fluid (serum) and the cell fraction were obtained from this last batch.

2.3. Determination of cell counts

THC were performed in flow cytometry (FACSCalibur, Becton–Dickinson) from paraformaldehyde-fixed hemocyte suspensions ($n = 10$). The flow cytometer was operated with phosphate-buffered saline (400 mOsm kg^{-1}) as sheath fluid. The flow rate was measured to allow absolute cell counts and the number of events set to 10,000. The results were expressed in 10^6 cells mL^{-1}.

2.4. Protein measurement

The total protein contents of hemolymph were measured using the Bio-Rad DC assay (Bio-Rad Laboratories) in samples ($n = 10$) prepared on board the vessel from freshly withdrawn hemolymph. The hemolymph fluid and the cell fraction were obtained by centrifugation (5 min, 5000*g*) and stored frozen. Their protein contents, PRTS and PRTC were expressed in mg mL^{-1} serum or in mg mL^{-1} supernatant. Since direct measurement of cell numbers could not be achieved on board, protein measurement in total hemolymph (PRTH) allowed indirect quantification of the number of cells in this fluid.