Molecular Ecology and Biotechnology

Technical procedures for stimulation with organic and inorganic pollutants, in a controlled environment, of model organism *Mytilus galloprovincialis* (Lamarck 1819)

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1 Introduction

1.1 CISAS project

The procedures described have been carried out within the project CISAS "International Centre of advanced study in environment, ecosystem and human health".

The present research program aims at investigating environmental pollution and its connection with the ecosystem and human health, starting from a significant number of case studies (SIN's of Augusta, Milazzo and Crotone) as true natural laboratories suitable for modern multidisciplinary investigation.
The widespread development of human activities in the industrial field, since the post-war period, in addition to the creation of an economy that places our country in the eighth place among the world's major industrialized Countries has been causing an important and complex phenomenon of environmental contamination, with crucial effects on terrestrial and marine ecosystems and human health. A decisive action of the Government and the Ministries in charge of environmental protection allowed to identify a number of contaminated areas in the Italian territory in need of an urgent and important recovery action. They are the so-called contaminated Sites of National Interest (SIN), characterized by severe environmental degradation and human impact phenomena of various origin, substantially caused by the development of major industrial activities. In relation to the environmental characterization of these areas, it has recently been considered crucial and necessary to understand the specific impact of these factors on the health of the people living in proximity of the above mentioned sites. The environment and human health are generally observed and investigated with a sectorial approach: rarely synergic actions have been carried out to study quantitatively cause-effect relationships between pollution and population health conditions. The collection of impact on the environment and on human health is a mandatory step for the development of modern technologies in the fields of chemistry, system biology, biotechnology, medicine, etc.

1.2 Ecosystem and contaminant WP3

This WP is focused on pollutants toxicity and related molecular response mechanisms in marine ecosystem. Biological and toxicological responses to different contaminants, including emerging ones, will be investigated in the three study areas (Priolo-Augusta, Crotone, Milazzo), with the aim to highlight possible relationships with human diseases. Biological responses will be investigated through a wide panel of bio-markers in model marine organisms with different complexity. In order to analyze toxic effects at various levels of biological organization, a systemic ecotoxicologycal approach will be employed. Transcriptomic and epigenetic analyses will be carried out in order to unveil responses and modifications induced by exposure to selected pollutants. Moreover, edible, benthic and nectobenthic marine species will be analyzed, with the aim to investigate pollutants uptake routes by humans through seafood consumption. Data obtained by the analyses of natural samples will be validated under controlled conditions in mesocosm.

1.3 In vivo experiments, Task 3.3

Task 3.3 The use of model organisms to unveil novel toxicity mechanisms (IFC) Based on critical issues recognized in the three study areas, possible mechanisms involved in emergent diseases will be investigated in model systems (i.e. *Danio rerio, Paracentrotus lividus, Dicentrarchus labrax, Octopus vulgaris,* etc.) (Schnitzler et al. 2011; Campinho et al. 20\13, 2014; Couderc et al. 2016; Marelli et al. 2016). The strategy is based on the widely recognized and accepted knowledge of the evolutionary conservation of the most molecular mechanisms involved in basal functions, in both embryonal and adult tissues (like stress response, cell cycle regulation, embryo development and morphogenesis, etc.) (Picone et al. 2016). Moreover, the overlapping between many molecular mechanisms involved in tumoral and endocrine diseases and

development/differentiation is well known. Thus, embryonal/larval stages of selected species (easy to manage and manipulate, and well characterized at molecular level) are well suitable both for the preliminary detection of phenotypical effects (i.e. teratogenicity, etc.) (Kinch et al. 2016) of the exposure to pollutants and for further analyses focused to unveil molecular mechanisms involved (Zhuo et al. 2014; Guo et al. 2014). Furthermore, transcriptomic analyses (carried out by means of Next Generation Sequencing) will provide global transcriptional profiles, suitable to detect variations involving any RNA species, including ncRNAs, induced by environmental pollutant found in the three study areas (Cao et al. 2016; Chen et al. 2016).

2 Mussels as a model organism

Bivalves can filter large volumes of water, processing microalgae, bacteria, sediments, particulates, and natural nanoparticles, potentially accumulating different chemicals in their tissues. These organisms have been long recognized as valuable indicators of pollution, and extensive background information is now available on their biological responses to a wide range of both inorganic and organic chemicals (Dagnino et al., 2007; Moore et al., 2006). In particular, the mussel *Mytilus galloprovincialis* (Fig. 1), abundant in coastal marine and estuarine environments, can represent a suitable model organism for characterizing the potential impact in marine Mediterranean ecosystem.

Figure 1 *Mytilus galloprovincialis*

Food particles trapped by the gill sieve are moved towards the labial palps and the mouth thus entering the gut, and reaching the digestive gland, where digestion occurs. Digestive cells have an extremely developed lysosomal system for intra-cellular digestion and nutrient accumulation for gametogenesis (Gosling, 1992).

Bivalve hemocytes resemble in structure and function the mammalian monocyte/macrophage lineage: they are responsible for cell-mediated immunity through phagocytosis and various cytotoxic reactions (such as lysosomal enzyme and antimicrobial peptide release and oxyradical production); hemolymph serum contains humoral defense factors, such as soluble lectins, hydrolytic enzymes and antimicrobial peptides (Canesi et al., 2002). Although bivalve hemocytes are extremely heterogeneous, in *Mytilus spp*. granular hemocytes represent the dominant cell type; they are characterized by a low nucleus/cytoplasm ratio, high phagocytic activity and capacity for oxyradical production (Garcia-Garcia et al., 2008). The responses of *Mytilus galloprovincialis* hemocytes to bacterial signals, cytokines, hormones (Canesi et al., 2006a), and to a number of environmental contaminants has been largely characterized (Canesi et al., 2003, 2006b, 2007a, 2007b, 2007c, 2007d). In these cells, like in mammalian immunocytes, the immune response is modulated by the

activation state of rapid pathways involving tyrosine-mediated cell signalling, with a key role of Mitogen Activated Protein Kinases (MAPKs), in particular the stress-activated MAPKs p38 and JNKs, and of PKC (Canesi et al., 2006b). Conservation of basic cellular processes of innate immunity from invertebrates to mammals represents an useful basis for studying common biological responses to environmental exposure to different family of contaminants.

The use of Mytilus in the context of the CISAS project will follow two main ways:

1-. Validation of stressed conditions through widely accepted response markers

2- Indentification of novel mechanisms of molecular response by means of comprehensive omic tools

3 In vivo experimental design

Mediterranean mussels (*M. galloprovincialis*) with a maximum shell length of 5 cm were obtained from a commercial shellfish farm from Ganzirri e Faro lake (Messina). They were acclimated in aquarium with artificial sea water (36 per thousand salinity) for 3 days at 18°C with continuous aeration and they were fed daily whit commercial microalgae frozen mixture.

Fugure 2: Group of six *M. galloprovincialis* in experimental condition

Individuals were maintained in the laboratory for 3 days before testing, in separate aquaria, in order to release metals and microorganisms (Freitas *et al*., 2012 ; Maffei *et al*., 2009). During this period the organisms were maintained at temperature 17.0 ± 1.0 °C; pH 7.80 ± 0.10, 12 light: 12 dark photoperiod and continuous aeration, in artificial seawater (salinity 35 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center). Seawater was renewed every two days (Coppola et al., 2017).
After this period, organisms were distributed in different glass aquarium (6 L seawater, salinity 35), with 6 individuals per container and 3 concentration corresponding to low, intermediate and high level of pollution (Biblio) for 7 contaminants (Cd, Mn, Ni, Pb, Zn, Cu, all the precedent in mixture), as reported in Table 1.
During the exposure period containers were continuously aerated, temperature and salinity (35 ± 1) were daily checked with a thermometer and refractometer. When necessary salinity values were adjusted adding water to the containers, according to the above stated conditions. Mortality was daily checked and organisms were considered dead when their shells gaped and failed to shut again after external stimulus. Aeration was not intensive and it was similar among aquaria.

Mortality was recorded.

The following table lists the used contaminants and the relative concentrations.

	Low [µg/l]	Medium [µg/l]	High [µg/l]
Cd^{2+}	0.01µg/l	0.1µg/l	1µg/l
Mn^{2+}	0.01µg/l	0.1µg/l	1µg/l
Ni^{2+}	0.01µg/l	0.1µg/l	1µg/l
Pb^{2+}	5µg/l	10µg/l	20µg/l
Cu^{2+}	5µg/l	10µg/l	20µg/l
Zn^{2+}	5µg/l	10µg/l	20µg/l
Mix*	Mix1Σlow concentration	Mix2 ΣMed concentration	Mix3 ΣHigh concentration

Table 1.(*Mix composition: Cd^{2+}; Mn^{2+}; Ni^{2+}; Pb^{2+}; Cu^{2+} ; Zn^{2+}.)

At each sampling period (12h, 2h and 5 days), organisms were dissected in different anatomical tissues (gills, adductor muscle, digestive gland, gonads, haemocytes) and mainteined at -80°C for subsequent analysis and posteriorly manually homogenized with a mortar and a pestle under ice in presence of trizol lysis buffer.

Each homogenized tissues was divided in different aliquots, from a maximum of 0.5 g for muscle to 0,05 mg for haemocytes, used for molecular and biomarker analyses.

6. Acknowledgments

Project CISAS “International Centre of advanced study in environment, ecosystem and human health”.

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