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# Effects of petrochemical contamination on caged marine mussels using a multi-biomarker approach: Histological changes, neurotoxicity and hypoxic stress

Maria Maisano [a, *], Tiziana Cappello [a], Antonino Natalotto [a], Valeria Vitale [a], Vincenzo Parrino [a], Alessia Giannetto [a], Sabrina Oliva [a], Giuseppe Mancini [b], Simone Cappello [c], Angela Mauceri [a], Salvatore Fasulo [a, **]

[a] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Messina, Italy
[b] Department of Industrial Engineering, University of Catania, Catania, Italy
[c] Institute for Coastal Marine Environment, National Research Center, Messina, Italy

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ABSTRACT

This work was designed to evaluate the biological effects of petrochemical contamination on marine mussels. *Mytilus galloprovincialis*, widely used as sentinel organisms in biomonitoring studies, were caged at the "Augusta-Melilli-Priolo" industrial site (eastern Sicily, Italy), chosen as one of the largest petrochemical areas in Europe, and Brucoli, chosen as reference site. Chemical analyses of sediments at the polluted site revealed high levels of PAHs and mercury, exceeding the national and international guideline limits. In mussels from the polluted site, severe morphological alterations were observed in gills, mainly involved in nutrient uptake and gas exchange. Changes in serotonergic and cholinergic systems, investigated through immunohistochemical, metabolomics and enzymatic approaches, were highlighted in gills, as well as onset of hypoxic adaptive responses with up-regulation of hypoxia-inducible factor transcript. Overall, the application of a multi-biomarker panel results effective in assessing the biological effects of petrochemical contamination on the health of aquatic organisms.


## 1. Introduction

Great concern has been raised over the impact of oil refinery wastewaters. The petrochemical industries and oil refining activities generate different kinds of wastes, released to the environment in the form of gases, particles, sludge, and liquid effluent. These wastewaters are characterised by the presence of significant amounts of polycyclic aromatic hydrocarbons (PAHs), phenols, heavy metals and their derivatives, sulphides, naphthenic acids and other chemicals (Dorris et al., 1972), most of them known to be toxic even at low concentrations (Long et al., 1995). Therefore, environmental petrochemical contamination poses a serious threat to marine ecosystems and human health (Fasulo et al., 2012a; Sureda et al., 2011), especially close to petroleum handling facilities such as harbours and refineries.

One of the largest and most complex petrochemical sites in Europe is the "Augusta-Melilli-Priolo" industrial area, located in the Augusta Bay and extended about 20 km along the eastern coastline of Sicily (south Italy). The industrial area, established in the 1950s, presents a number of chlor-alkali plants and other industrial installations including oil refineries, petrochemical and chemical industries, cement plants and electric power stations. Based on previous reports published by ICRAM (2005), as well as on more recent studies performed in this area (De Domenico et al., 2011, 2013; Di Leonardo et al., 2014), among the pollutants present in sediments, PAHs and mercury are found at alarmingly elevated concentrations, exceeding the standard limit reported by national

* Corresponding author. Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy.
** Corresponding author. Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres 31, 98166 Messina, Italy.
*E-mail addresses:* mmaisano@unime.it (M. Maisano), sfasulo@unime.it (S. Fasulo).

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and international sediment quality guidelines (SQGs) (Ministerial Decree No. 260/2010; Dutch SQGs (De Domenico et al., 2013)). Moreover, Sprovieri et al. (2011) documented the key role played by the Augusta basin in the mercury contamination of the entire Mediterranean Sea. Owing to the high state of environmental degradation, the Augusta area has been recognized as a site of high environmental risk by the Italian Government (Law No. 426/1998) and World Health Organization (Martuzzi et al., 2002), and included in the National Remediation Plan by the Italian Environmental Protection Ministry in 2002.

Within the Programmes of Relevant National Interest (PRIN), the 36 month project "SYSTEMS BIOLOGY", started in February 2013 and still ongoing, was funded by the Italian Ministry of Education, University and Research with the purposes, among the others, to assess the biological effects of petrochemical contamination on mussels caged at the Augusta Bay, as well as to validate the potential actions for an environmentally sustainable remediation of the area (Fasulo et al., 2015). Bivalve molluscs, particularly *Mytilus* spp., have been widely used as bioindicators in environmental monitoring programmes due to their broad geographical distribution, filter-feeding and sedentary lifestyle, and ability to tolerate and bioaccumulate high amounts of pollutants in their tissues (D'Agata et al., 2014; Fasulo et al., 2012b; Lacroix et al., 2015; Sureda et al., 2011). In addition, mussels are suitable to conduct caging experiments in the field (active biomonitoring). This approach allows a reduction of physiological variables by using mussels from a single population, more control over the experiment, and therefore an accurate evaluation of the effects of pollutant mixtures in the natural environment (Cappello et al., 2013a, 2013b, 2015; Fasulo et al., 2012b; Lacroix et al., 2015; Marigomez et al., 2013).

In the present work, some of the results from the "SYSTEMS BIOLOGY" project are presented. In order to assess the potential adverse effects of petrochemical contamination on mussels caged at the Augusta Bay, a multi-biomarker approach was applied on the gills of mussels. The gills, mainly involved in filter-feeding, gas exchange and neuronal signalling, are the first organ to be affected by environmental stressors, and therefore have been frequently used as a model system in ecotoxicological studies (Cappello et al., 2013b, 2015; Ciacci et al., 2012; Lacroix et al., 2015). The histology of the gills, as a tool for monitoring the general health status of organisms (Auffret, 1988), was evaluated. A panel of biomarkers indicative of neuronal alterations was applied on gills in order to investigate on the serotonergic (i.e. serotonin, 5-HT, and its receptor, 5-$HT_3R$) and cholinergic (i.e. acetylcholinesterase, AChE, choline acetyltransferase, ChAT, acetylcholine, choline, acetate) systems. Indeed, it is well documented for aquatic invertebrates that impairment in the nervous systems may occur after exposure to toxicant compounds (Cappello et al., 2015; Lionetto et al., 2013; Maisano et al., 2015). Moreover, onset of hypoxic adaptive responses was assessed in the mussel gills by measuring the expression of the hypoxia-inducible factor 1 (HIF-1), which plays a key role in hypoxia signal transduction and transcriptional regulation in aquatic invertebrates (Giannetto et al., 2015; Kawabe and Yokoyama, 2012). The concentrations of PAHs and metals in sediments, as well as water physico-chemical parameters, were also measured.

## 2. Materials and methods

### 2.1. Study area

The petrochemical area of "Augusta-Melilli-Priolo", located in the Augusta Bay (eastern Sicily, Italy), has been recognized as site of high environmental risk owing to the heavy industrialization and high level of pollution (Law No. 426/1998). Conversely, the hamlet of Brucoli, located at about 15 km from Augusta, was selected as a reference site because it is considered to be unpolluted by petrochemical contamination (Fig. 1).

At both sites, water physico-chemical parameters such as conductivity, temperature (T), redox potential (Eh), pH, and dissolved oxygen (DO) concentration, were measured in triplicate samples *in situ* using a Waterproof CyberScan PCD 650 multiparameter (Eutech Instruments).

Sediment samples were collected at the depth of 6 m in twice using sterile Plexiglas cores (50 cm long, 10 cm diameter). After collection, samples were immediately transported to the laboratory in a cool box (4 ± 1 °C), where samples were used for immediate analysis or aliquots were stored at −80 ± 5 °C and at −20 ± 1 °C with glycerol (20% final concentration).

### 2.2. Chemical analysis in sediments

#### 2.2.1. Determination of hydrocarbons

The composition of the Total Extracted and Resolved Hydrocarbons and theirs derivates (TERHCs) was analyzed following the US EPA procedures 3540C and 8015D for hydrocarbons, and 3580A, 3640A, and 8270D for PAHs in sediment samples (US EPA, 2007). Briefly, a mixture of $CH_2Cl_2$:$CH_3COCH_3$ (1:1, v/v) was added to samples. The mixture was sonicated for 2 min in ultrasound bath (Branson 1200 Ultrasonic Cleaner, Branson USA). Samples were further shaken at 150 *g* for 30 min, centrifuged at 5000 *g* for 10 min and supernatant was passed through a ceramic column filled with anhydrous $Na_2SO_4$ (Sigma-Aldrich, Milan). Residues were re-suspended in $CH_2Cl_2$ prior the gas chromatography (GC) analysis. All measures were performed using a Master GC DANI Instruments (Development Analytical Instruments), equipped with SSL injector and FID detector. Samples (1 μl) were injected in splitless mode at 330 °C. The analytical column was a Restek Rxi-5 Sil MS with Integra-Guard, 30 m × 0.25 mm (ID × 0.25 μm film thickness). Helium carrier gas was maintained at a constant flow of 1.5 mL $min^{-1}$. Total heavy hydrocarbons and PAHs were also calculated for each sample (Genovese et al., 2014).

#### 2.2.2. Determination of metals

For determination of metals, prior to analysis, sediment samples were digested in concentrated nitric acid and concentrated hydrochloric acid using microwave heating (Ethos easy digestion system, Millestone) according to the US EPA 3051A sample preparation method (US EPA, 2007). The sample and acids were placed in a fluorocarbon polymer (PFA) microwave vessel, sealed and heated in the microwave unit. After cooling, the vessel contents were filtered and then diluted to volume and analyzed by Inductively coupled plasma-mass spectrometry (PerkinElmer Nexion 350X Spectrometer) according to the EPA 6020A method. Reagent-grade chemicals were used in all the preparations.

### 2.3. Experimental design

Mussels *Mytilus galloprovincialis* (5.2 ± 0.4 cm shell length) were obtained from the Consortium of Fishermen aquaculture farm (Ferrara, Italy) in October 2013. Mussels were maintained in aerated seawater in large flow-through holding tanks for two weeks, and then transferred to Augusta (37°10′ 39.201″N, 15°12′ 26.286″E) and Brucoli (37°17′ 23.67″N, 15°12′ 40.68″E) for 60 days in stainless steel cages deployed by scuba-diving. Then, mussels were retrieved and gills from fifteen mussels per site were rapidly excised and flash-frozen in liquid nitrogen for metabolomics, enzymatic, and molecular measurements, transferred to the laboratory and stored at − 80 °C prior to analysis. Further, small pieces of each dissected tissue were taken for histological and