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^{1}H NMR-based metabolomics investigation on the effects of petrochemical contamination in posterior adductor muscles of caged mussel *Mytilus galloprovincialis*

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ABSTRACT

Environmental metabolomics is a high-throughout approach that provides a snapshot of the metabolic status of an organism. In order to elucidate the biological effects of petrochemical contamination on aquatic invertebrates, mussels *Mytilus galloprovincialis* were caged at the "Augusta-Melilli-Priolo" petrochemical area and Brucoli (Sicily, south Italy), chosen as the reference site. After confirming the elevated concentrations of polycyclic aromatic hydrocarbons (PAHs) and mercury (Hg) in Augusta sediments in our previous work (Maisano et al., 2016a), herein an environmental metabolomics approach based on protonic nuclear magnetic resonance (^{1}H NMR), coupled with chemometrics, was applied on the mussel posterior adductor muscle (PAM), the main muscular system in bivalve molluscs. Amino acids, osmolytes, energy storage compounds, tricarboxylic acid cycle intermediates, and nucleotides, were found in PAM NMR spectra. Principal Component Analysis (PCA) indicated that mussels caged at the polluted site clustered separately from mussels from the control area, suggesting a clear differentiation between their metabolic profiles. Specifically, disorders in energy metabolism, alterations in amino acids metabolism, and disturbance in the osmoregulatory processes were observed in mussel PAM. Overall, findings from this work demonstrated the usefulness of applying an active biomonitoring strategy for environmental risk assessment, and the effectiveness of metabolomics in elucidating changes in metabolic pathways of aquatic organisms caged at sites differentially contaminated, and thus its suitability to be applied in ecotoxicological studies.

1. Introduction

Metabolomics is a high-throughout approach that focuses on the comprehensive study of endogenous low-molecular-weight metabolic entities (< 1000 Da) present in cells, tissues, biofluids, or even whole individuals (Lin et al., 2006). The value of metabolomics lies in the fact that it profiles simultaneously a wide range of metabolites involved in a variety of metabolic pathways, providing thus a snapshot of the biological processes that are considered most proximal to a specific phenotype or disease. Among the analytical technologies well established and frequently applied in metabolomics studies, proton nuclear magnetic resonance (^{1}H NMR) spectroscopy has been successfully applied in the field of ecotoxicology. NMR is rapid and delivers rich structural and quantitative information, and it has demonstrated great potential in elucidating the interactions between organisms and environment, and in discovering metabolite biomarkers upon exposure to contaminants, both in fish (Brandão et al., 2015; Cappello et al., 2016a, 2016b; Iacono et al., 2010; Lankadurai et al., 2013) and aquatic invertebrates, including bivalve molluscs (Cao and Wang, 2016; Cappello et al., 2017, 2015, 2013a; Fasulo et al., 2012a; Hines et al., 2010; Wu et al., 2011).

Bivalve molluscs, especially mussels, are routinely used as sentinel species in environmental monitoring programs and ecotoxicological studies. This is mainly because they are widely distributed in marine, estuarine and freshwater environments, are sedentary filter-feeder organisms, and are able to bioaccumulate and tolerate high amounts of contaminants in their tissues (Cappello et al., 2013b; Ciacci et al., 2012; D'Agata et al., 2014; Giannetto et al., 2015; Lacroix et al., 2015; Maisano et al., 2016b; Viarengo et al., 2007). Additionally, mussels have demonstrated to be suitable for active biomonitoring studies, which are based on the use of caged organisms from a reference site. This strategy allows field surveys to be conducted at sites where native organisms are absent or rare, as well as more control over the experiment since the use of animals from a single population minimizes confounding factors such as age and reproductive status of organisms, which may influence both contaminant bioaccumulation and biomarker

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responses. Therefore, caging approaches have been largely applied for an accurate evaluation of the effects of pollutant mixtures in natural environments (Cappello et al., 2013b; Fasulo et al., 2012a; Lacroix et al., 2015; Maisano et al., 2016a; Marigomez et al., 2013).

A very recent example of the application of an active biomonitoring strategy for environmental risk assessment is provided by the 36-month project "Systems Biology", (Fasulo et al., 2015). Within this research project, one of the main purposes was to assess the impact of petrochemical contamination on mussels *Mytilus galloprovincialis* caged at the "Augusta-Melilli-Priolo" industrial area (Sicily, south Italy). This is one of the largest and most complex petrochemical sites in Europe because of the numerous chlor-alkali plants, oil refineries and chemical industries present in the area, which 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). As largely documented by previous studies (Cappello et al., 2015, 2013a, 2013b; De Domenico et al., 2011; Di Leonardo et al., 2014; Fasulo et al., 2012a), the "Augusta-Melilli-Priolo" petrochemical site is characterized by alarmingly elevated concentrations of polycyclic aromatic hydrocarbons (PAHs) and mercury (Hg), exceeding the limit established in national and international sediment quality guidelines (SQGs) (Ministerial Decree No. 260/2010; Dutch SQGs (De Domenico et al., 2013)). This was also confirmed by our recent work (Maisano et al., 2016a), in which the levels of total heavy hydrocarbons and PAHs were found to be 37-fold and 14-fold greater, respectively, in sediment samples collected at Augusta compared to those from a reference site, namely Brucoli (Sicily, Italy). Noteworthy, the level of Hg recorded at Augusta was $78.1 \pm 20.5\ mg\ kg^{-1}$ dw, whereas at Brucoli it was $0.05 \pm 0.01\ mg\ kg^{-1}$ dw (Maisano et al., 2016a).

Therefore, the present study was designed to elucidate the biological effects of petrochemical contamination in marine mussels caged at the "Augusta-Melilli-Priolo" industrial area, with the aim to characterize at metabolite level the adverse effects of petrochemical pollution on mussel posterior adductor muscle (PAM). The choice of PAM as a target organ is due to the fact that adductor muscle is the main muscular system in bivalve molluscs, and has been successfully employed in mechanistic environmental research (Hines et al., 2010; Leung et al., 2014; Wu et al., 2011). Most bivalve species have two adductor muscles, which are located near the anterior and posterior margins of the shell valves. The posterior muscles allow the mussel to open and or close its shell. The regulation of muscle contraction was studied in the adult mussel *M. trossulus*, and it was reported that adductor muscles can be locked in the contracted state, called 'catch', and maintain tension for a long time with very little energy consumption (Ruegg, 1971). In light of the important physiological role played by mussel PAM, a NMR-based metabolomics approach coupled with multivariate statistics was applied in order to evaluate the potential alterations induced by petrochemical contaminants on this organ.

2. Material and methods

2.1. Study area characterization

As reported previously (Cappello et al., 2013b; Fasulo et al., 2012a; Maisano et al., 2016a), the "Augusta-Melilli-Priolo" is a petrochemical area located in the Augusta Bay (eastern Sicily, Italy), considered to be a site of high environmental risk owing to the high level of industrialization and pollution (Law No. 426/1998). Conversely, the hamlet of Brucoli, at about 15 km from Augusta (Fig. 1), was selected as a reference site because it is considered to be not affected by petrochemical contamination, as previously documented (Maisano et al., 2016a).

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). Data were previously described in Maisano et al. (2016a), and reported herein as mean ± standard deviation (SD) in Table 1.

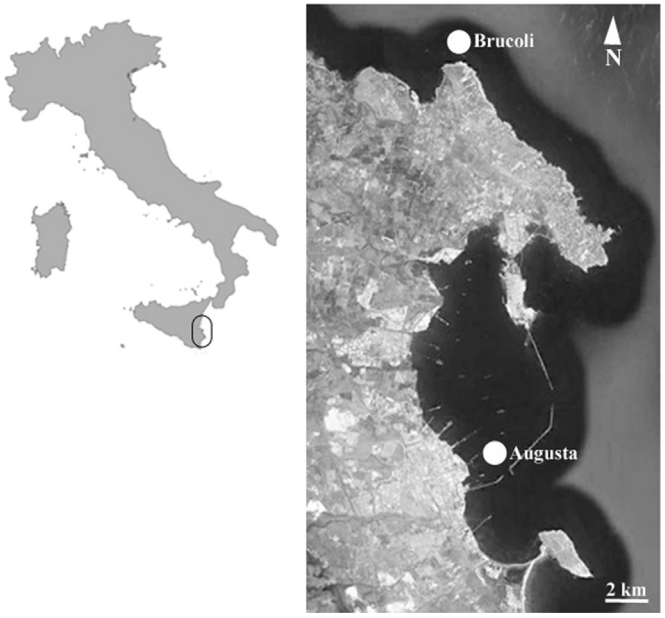

Fig. 1. Map showing location of the mussel caging sites, Augusta (37°10' 39.201"N, 15°12' 26.286"E) and Brucoli (37°17' 23.67"N, 15°12' 40.68"E).

Table 1
Water temperature (T), salinity, dissolved oxygen (DO), pH and redox potential measured at the reference site, Brucoli, and at the polluted site, Augusta. Mean and associated standard deviation are presented.

Site	T (°C)	Salinity (PSU)	DO (mg/L)	pH	Redox potential (mV)
Brucoli	20 ± 0.3	38.1 ± 0.1	5.1 ± 0.3	7.70.02	200
Augusta	21 ± 0.2	38.1 ± 0.1	3.6 ± 0.2	7.8	210

Sediment samples were also collected at the depth of 6 m in twice using sterile Plexiglas cores (50 cm long, 10 cm diameter), and then transported to the laboratory in cool boxes for the determination of hydrocarbons and metals, which levels are reported in Maisano et al. (2016a).

2.2. Experimental design

Mussels *Mytilus galloprovincialis* (mean shell length: 5.2 ± 0.4 cm) were purchased in October 2013 from the Consortium of Fishermen aquaculture farm (Ferrara, Italy). After maintaining in aerated seawater in large flow-through holding tanks for two weeks, mussels were 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) in stainless steel cages by scuba-diving. After 60 days, mussels were retrieved and posterior adductor muscle (PAM) tissues from fifteen mussels per site were rapidly excised and flash-frozen in liquid nitrogen, transferred to the laboratory, and then stored at – 80 °C prior to the metabolomics analysis.

2.3. ^{1}H NMR-based metabolomics

2.3.1. Tissue metabolite extraction

Extraction of polar metabolites from PAM tissues of mussels (n = 15 per site) was performed using a "two-step" methanol/chloroform/water protocol, as described in previous works (Fasulo et al., 2012a; Cappello et al., 2013a; Wu et al., 2008). Briefly, 150 mg sub-sample of PAM tissues were homogenized in 4 mL/g of cold methanol and 0.85 mL/g of cold water by a TissueLyser LT bead mill (Qiagen) with 3.2 mm stainless steel beads, for 15 min at 50 vibrations/s. The homogenates