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Histopathology and stress biomarkers in the clam *Venerupis philippinarum* from the Venice Lagoon (Italy)

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ABSTRACT

The aim of this study was to evaluate the histomorphology and the stress response in the bivalve *Venerupis philippinarum* sampled in four differently polluted sites of the Venice Lagoon (Palude del Monte, Marghera, Ca' Roman and Val di Brenta). This species is often used as bioindicator of environmental pollution since it can bioaccumulate a large variety of pollutants because of its filter feeding. Chemical analyses for heavy metals (Cd, Cu, Hg and Pb) and polycyclic aromatic hydrocarbons (PAHs) were performed on whole soft tissues of *V. philippinarum*. The histological evaluation of clams revealed the presence of *Perkinsus* sp. infection in animals from all sites, although a very high prevalence of parasites was evidenced in clams from Ca' Roman. *Perkinsus* sp. were systemically distributed in the mantle, in the intestine and digestive gland, in gonads and gills. The trophozoites of *Perkinsus* sp. were found isolated or in cluster surrounded by a heavy hemocitical response. Haemocytes always exhibited an immunopositivity to cytochrome P4501A (CYP1A), heat shock protein 70 (HSP70), 4-hydroxy-2-nonenal (HNE) and nitrotyrosine (NT) antibodies. The digestive gland of animals from Palude del Monte showed the highest malondialdehyde (MDA) concentration, whereas clams from Ca' Roman exhibited the highest quantity of metallothioneins.

1. Introduction

The Venice Lagoon is a transitional environment on the Adriatic coast influenced by such human activities as agriculture, industry and tourism. The sludge of Venice and the rivers from the hinterland pour into the lagoon, where sediments trap pollution.

For this reason, in recent years numerous national and international projects have been carried out in order to evaluate the quality status of the lagoon environment. Biomonitoring programmes usually involve the use of biomarkers, which represent biochemical, physiological or behavioural variations measured in tissues, biological fluids or whole organisms [1,2]. Several vertebrate and invertebrate species are employed in marine monitoring programmes as sentinel models to evaluate environmental quality. Among invertebrates, the Manila clam *Venerupis philippinarum* has largely been used to investigate the water/sediment pollution in coastal lagoon ecosystems since it is a filter-feeding bivalve living in soft bottoms [3–8]. Moreover, it represents an important economic resource for fisheries in North Adriatic lagoons, where this species is fished and farmed.

At the cellular level, the metabolism of environmental stressors frequently results in the formation of reactive oxygen species (ROS) [9]. They are produced naturally during metabolism and their toxic effects are usually prevented by antioxidants, both molecular and enzymatic ones. During oxidative stress conditions, the production of ROS is greater than the ability of cells to remove them, leading to lipid peroxidation, protein carbonils formation and cell death [10,11]. Lipid peroxides are unstable indicators of oxidative stress in cells that decompose to form more complex and reactive compounds such as malondialdehyde (MDA), which is a natural byproducts of lipid peroxidation [12]. MDA originates from the oxidative degradation of PUFAs and represents a highly toxic aldehyde with a specific affinity to proteins and DNA [12].

4-hydroxy-2-nonenal (HNE), the most abundant and toxic α,β-unsaturated aldehyde, originates from the β-cleavage of hydroperoxides from ω-6 PUFAs and is mainly involved in the inhibition of protein and DNA synthesis, in the inactivation of enzymes, and is also a potent mutagen agent [12]. Moreover, one of the most

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important ROS is the superoxide radical, which reacts with nitric oxide giving rise to peroxynitrite, a potent oxidant that may oxidize proteins, lipids and DNA [13]. Nitrotyrosine (NT) is a relatively stable marker for peroxynitrite production [13].

Heat shock proteins (HSPs), also called *stress proteins*, are a family of highly conserved cellular proteins that are present in all cells in all life forms [14–16]. An evident biomarker role was shown to be played by Heath Shock Protein 70 (HSP70) that protects cells against harmful conditions by binding and refolding damaged proteins. There are constitutive members (HSC70) of the heat shock proteins, which play important chaperoning role in unstressed cells, and inducible (HSP70) forms, which are expressed in detectable levels after acute stressor insults [14,17]. In aquatic species, the expression of HSP70 has been studied in fish after exposure to heat shock, pesticides, virus, metals and other toxic compounds [18–21]. In mussel, an increased expression of HSP70 has been detected after exposure to contaminants [22,23]. Moreover, an upper regulation of HSP70 has been observed in the clam *Venerupis decussata* upon *Perkinsus olseni* infection [24].

The cytochrome P4501A (CYP1A) subfamily is involved in the biotransformation of a variety of contaminants like polychlorodibenzo dioxins (PCDDs), polycyclic aromatic hydrocarbons (PAHs), polyhalogenated aromatic hydrocarbons (PHAHs) and polychlotobiphenils (PCBs). Its induction plays a central role in transforming pesticides in aquatic organisms [25].

Metallothioneins (MTs) are ubiquitary proteins that have been found in a very wide range of organisms, including vertebrates, invertebrates, plants and bacteria [26]. MTs exhibit high affinity for metals, giving rise to their important role in metal metabolism, detoxification of heavy metals, immune response and the antioxidant process [27,28]. The use of (MTs) seems to be recognized as the most valid method to indicate metal exposure. In fact MTs are induced by metals residues and measurements of their levels are at present used in both vertebrates and invertebrates.

The aim of this study was to evaluate the histomorphology of the different tissues and organs of the bivalve *V. philippinarum* sampled in four differently polluted sites of the Venice Lagoon: Palude del Monte, Marghera, Ca' Roman and Val di Brenta [29]. We also used an immunohistochemical approach to detect the localization of the following oxidative stress biomarkers: CYP1A, HSP70, HNE and NT. Chemical analyses for heavy metals (Cd, Cu, Hg and Pb) and PAHs were performed on whole soft tissues of clams. Moreover, to test the amount of lipid peroxidation, we evaluated the MDA concentration in digestive gland using the thiobarbituric acid reactive substances (TBARS), a well-established assay for screening and monitoring lipid peroxidation. The amount of metallothioneins, as a biomarker of heavy metal exposure, was estimated in digestive gland by a spectrophotometric method.

2. Materials and methods

2.1. Organisms

The clams *V. philippinarum* were collected at four sites of Venice Lagoon (northern terminus of the Adriatic Sea) characterized by different pollution levels. All the clams were adult organisms with a shell size of 4.03 ± 0.3 cm. The four sites were: Palude del Monte [45.28.59 N; 12.21.15 E], Marghera [45.25.55 N; 12.16.09 E], Ca' Roman [45.14.28 N; 12.16.55 E] and Val di Brenta [45.11.50 N; 12.15.38 E] (Fig. 1). For each site, 50 animals were caught, immediately transferred in portable fridge at 4 °C to the laboratory and processed for the analysis. For histology and immunohistochemistry, the whole body from 40 animals (10 clams/site) was fixed in 4% paraformaldehyde prepared in phosphate-buffered saline (PBS, 0.1 M, pH 7.4) at 4 °C overnight, washed in PBS, dehydrated through a graded series of ethanol and embedded in paraffin. Consecutive sections were cut at a thickness of 4 μm using a microtome.

For the chemical analysis the whole soft body from 40 animals (10 clams/site) was immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

For the TBARS assay and metallothionein determination, digestive glands were grouped in three pools of 10 clams per site and stored at −80 °C.

2.2. Chemical analysis

2.2.1. PAH determination

Chemical analysis was performed on whole soft tissues of *V. philippinarum*. Extraction was carried out using SampliQ Buffered QuEChERS AOAC Extraction kit (Agilent, CA, USA). An amount of 3 g of homogenized clam sample was weighed in a 50 ml centrifuge tube; before extraction an Agilent Ceramic Bar Homogenizer was added. To sample 10 ml deionized water and 12 ml acetonitrile were added; after each solvent addition the sample was shaked for 15 min. After addition of salts (6 g anhydrous $MgSO_4$ and 1.5 g anhydrous NaOAc) tubes were vigorously shaked for 1 min, and then centrifuged at 4000 rpm for 10 min. Clean-up was performed by means of Agilent Dispersive 15 ml SPE Fatty Sample AOAC kit. A 4 ml aliquot of the previous upper organic extraction layer was transferred into a dispersive SPE 15 ml tube containing salts (400 mg PSA/400 mg C18 EC/1200 mg anhydrous $MgSO_4$). Subsequently tubes were vigorously shaked for 1 min and then centrifuged at 4000 rpm for 10 min. After filtration with disposable syringe filter (Millipore) the extract is ready for analytical determination.

HPLC-FLD analyses were carried out by means of an Alliance Empower HPLC system equipped with a 2475 Multi λ fluorimetric detector a sample manager and a quaternary solvent manager (Waters, MA, USA). The column was a Supelcosil LC-PAH 150 × 3 mm 5 μm HPLC column with a Supelguard LC-18 20 × 3mm guard column (Supelco, PA, USA), kept at 30 °C. The flow rate was 0.5 ml min^{-1} with an injection volume of 30 μl. The sample manager was maintained at 30 °C.

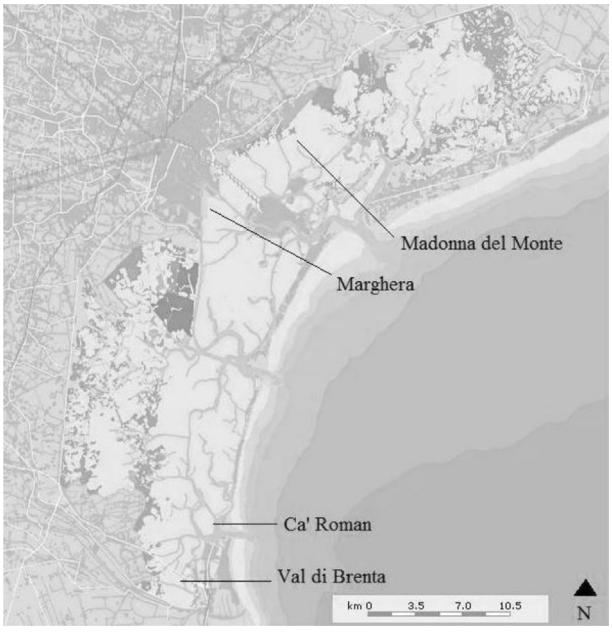

Fig. 1. Map of the Venice Lagoon indicating the location of sampling station: Palude del Monte [45.28.59 N; 12.21.15 E], Marghera [45.25.55 N; 12.16.09 E], Ca' Roman [45.14.28 N; 12.16.55 E] and Val di Brenta [45.11.50 N; 12.15.38 E].