Environmental Pollution 177 (2013) 82–89

Contents lists available at SciVerse ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Biomonitoring study of an estuarine coastal ecosystem, the Sacca di Goro lagoon, using *Ruditapes philippinarum* (Mollusca: Bivalvia)

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ARTICLE INFO

Article history:
Received 10 April 2012
Received in revised form
3 January 2013
Accepted 22 January 2013

Keywords:
Micronucleus test
Catalase
Superoxide dismutase
Sediment contamination
Reburrowing

ABSTRACT

Coastal lagoons are constantly subjected to releases of chemical pollutants, and so organisms may be exposed to such toxicants. This study investigated through a multivariate approach the physiological status of bivalve *Ruditapes philippinarum*, farmed in Sacca di Goro lagoon. Biomarkers at different levels of biological organization (catalase, superoxide dismutase, genotoxicity, reburrowing behavior) were evaluated at three sites exposed to different environmental conditions. A seasonal trend was observed, and micronucleus frequency was significantly lowest at the relatively pristine reference site. Enzymatic activity toward oxyradicals be quite efficient since variations in responsiveness were not consistent. However, behavioral impairment was observed in reburrowing rates. Sediment concentrations showed low PAH levels and high natural levels of trace metals Cr and Ni. DistLM statistical analysis revealed a non-significant relationship between selected biomarkers and xenobiotics. Therefore other potentially toxic compounds in admixture at low doses may be involved in driving differing spatial distribution of physiological impairment.

1. Introduction

About 100 000 chemicals are currently in widespread use and, since a high proportion of them reach aquatic environments such as estuarine and coastal lagoons through riverine inputs, they may become contaminants with potentially hazardous effects on ecosystems (Schwarzenbach et al., 2006; Sumpter, 2009). In recent years, the EU has become increasingly aware of gaps in its environmental policy, and so it has implemented regulatory approaches and developed instruments to improve environmental risk assessment of chemicals in relation to potentially impacted ecosystem functions and the associated protection goals (EFSA, 2010; Water Framework Directive EU2000/60).

The river Po flows through one of the most densely populated and productive agricultural and industrial regions of Italy, influencing the quality of freshwater inputs into the aquatic ecosystems of the coastal lagoons of the delta (Camusso et al., 2002). Sacca di Goro, located along the North East Adriatic coast is a highly productive lagoon considered extremely important for its ecological and economic value and moreover a case study for highly impacted coastal environments (Zaldivar et al., 2003). Pollutants carried in from branches of the river Po and from several freshwater drainage canals (Po di Volano and the Po di Goro) lead to anthropogenic eutrophication, frequent summer anoxia and chemical contamination with pesticides. Contamination with trace metals (Locatelli and Torsi, 2001; Viaroli et al., 2006) and eutrophication (Bartoli et al., 2001) together with pesticides (Carafa et al., 2007), have been reported and show a seasonal pattern, peaking in spring as this is the main period of agrochemical applications. Monitoring programs of ARPA (Regional Environmental Agency) in 2011 for surface water from the Ferrara area have mostly reported concentrations below the acceptable pesticide limit of 0.1 $\mu g\ l^{-1}$ for a single active substance (Directive EU 98/83) but with a few point exceedances for terbuthylazine, oxadiazon, metolachlor (not in Annex I of the Directive EU 91/414/EC), 3,4-dichloroaniline and azoxystrobin (all $<2\ \mu g\ l^{-1}$) during the spring and summer periods.

Low concentrations of pollutants come from many sources of anthropogenic releases and their behavior is complex because of their physicochemical interaction with seawater and sediment and

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http://dx.doi.org/10.1016/j.envpol.2013.01.042

since their concentrations change over time. The sensitivity to concentration of the biological responses to acute or chronic exposure may differ across species (Brian et al., 2005) indicating possible additive and cumulative effects of mixtures of pollutants at concentrations that give no response to the individual chemicals.

The ecotoxicological approach with biomarkers in sentinel species has been proposed for environmental monitoring programs (Allan et al., 2006), combining chemical analyses with laboratory and field based biological endpoints (Viarengo et al., 2007; Binelli et al., 2010). It aims to evaluate the consequences of exposure to low doses of contaminants at different levels of biological organization (Galloway et al., 2004), such as ecological consequences on population and communities (Durou et al., 2007; Forbes et al., 2006) whereas chemical analyses do not necessarily reveal potential biological effects. Studies have been successful in different taxonomic groups of aquatic organisms such as, on clams *Scrobicularia plana* (Bonnard et al., 2009) and *Ruditapes philippinarum* (Matozzo et al., 2006; Moschino et al., 2011), an infaunal polychaete *Nereis diversicolor* (Mouneyrac et al., 2010) and crabs (Dissanayake et al., 2010).

At sub-organism levels, one of the most investigated pathways in the exposure of organisms to xenobiotics is the induction of oxidative stress through the formation of reactive oxygen species (ROS) acting as an additional coinducer in mutagenic actions (Wiseman and Halliwell, 1996). The exposure of bivalves to contaminants activates their defensive system with antioxidant enzymes (Pellerin Massicotte, 1994; Greco et al., 2010). Catalase (CAT) is one of the earliest enzymes to be induced, and is not specific for contaminant classes (Van der Oost et al., 2003; Tlili et al., 2010).

Another reliable biomarker used in biomonitoring studies is micronuclei frequency (MN), a test that is able to detect genetic alteration such as chromosomal damage in aquatic invertebrates (Bolognesi and Hayashi, 2011; Izquierdo et al., 2003). The chromosomal and genomic damage caused by a genotoxic compound shows a clear dose- and time-dependent response (Bolognesi et al., 1996; Jha et al., 2005).

At the individual level, behavioral endpoints are sensitive tools to assess the impact of contaminants with concentrations far below lethal effects (Amiard Triquet, 2009), and so this is a suitable approach to potentially link the physiological function of the individual with important ecological processes.

The present field study investigates the reliability of ecotoxicological endpoints as behavioral and biochemical tests in the Manila clam (*Ruditapes philippinarum*) in order to evaluate its physiological status in the Sacca di Goro lagoon of the Po river; this clam is a sediment-dwelling bivalve, native to Indo-Pacific areas and distributed worldwide for farming, and often recognized as a sensitive indicator for monitoring toxic substances in water (Moraga et al., 2002; Matozzo et al., 2012). This lagoon, with a surface area of 26 km^2 and an average depth of 1.5 m, has highly variable salinity, and is one of the most important aquaculture systems in Italy for *R. philippinarum* and *Mytilus galloprovincialis*. About 10 km^2 is used for farming *R. philippinarum*, with a mean density of 500 individuals per m^2, giving an average production of about 15 000 ton $year^{-1}$ with the clams reaching commercial size in approximately 18 months. Italy is the main producer of *R. philippinarum* in the EU, totaling 50 000 ton $year^{-1}$ with an annual revenue of 50–100 million €.

To examine the effects of concentrations of pollutants on clams, biological parameters accounting for the physiology of the animals (i.e. behavior response) were investigated, together with cellular/molecular parameters (i.e. biochemical and genotoxic) accounting for cumulative exposure. A multivariate approach was performed, including chemical analysis of sediment (trace metals, PAHs) with the assessment of biomarkers at three sites exposed to different environmental conditions.

2. Materials and methods

2.1. Study area

The sampling stations, located in the licensed farming area of the Sacca di Goro lagoon, were selected along a spatial pollution gradient: site G is close to the port of Goro (N 44°50′27.4″ EO 12°17′45.1″), site P (N 44°48′57.6″ EO 12°17′09.4″) is next to the freshwater inflow of Po di Volano coming from watersheds mainly dedicated to agriculture, and the third sampling site N (N 44°47′36.4″ EO 12°19′20.7″) was located in the nursery area near the southern sand barrier close to the sea, with a high hydrodynamism and salinity similar to that of the Adriatic sea. Site N, considered to be the reference site, is a natural collector of *R. philippinarum* larvae, and is not exploited since it is used by fishermen for the repopulation program during the year.

2.2. Sampling

Clams were collected from three sampling sites in the farmed area of the Sacca di Goro lagoon in May and December 2009 using a manual rake (Nursery, N; Goro port, G; Po di Volano, P). Clams homogeneous in size (25–30 mm) were sampled and stored immediately in a cooler box for transport to the CRIM Lab facilities in Goro within 2 h. At both samplings 180 clams (20 clams from each site with three replicates) were used for experiments on behavioral kinetics at the CRIM laboratories, and another 45 were transported the day after sampling under refrigeration (at 4 °C) to the Environmental Carcinogenesis Unit of the National Cancer Research Institute in Genoa, where they were processed for enzymatic analysis and the MN assay. The gametogenesis and spawning process of the individual farmed clams could have an influence on biomarkers, and so we designed the sampling in order to avoid this phase (Meneghetti et al., 2004).

Sediment was sampled for analysis of trace metals and PAHs in February, May, August and December 2009, with five random samples being taken from each site. The upper layer of superficial sediments (0–10 cm) was collected using an Eijkelcamp multisampler, placed in glass jars covered by aluminum foil, and immediately transferred to a portable freezer and stored at −20 °C until analysis.

2.3. Biochemical analysis

The digestive glands of clams were rapidly dissected whilst frozen in liquid nitrogen and then stored at −80 °C. Three sub-samples were prepared by pooling digestive glands from three to five clams, for each site and sampling campaign. Hepatopancreatic tissues were homogenized with a Potter Elvehjem tissue grinder in ice cold buffer, comprising 10 mM of Tris–HCl (pH 7.6) containing 150 mM KCl and 0.5 M sucrose. The homogenate was centrifuged for 15 min at 1480 rpm and at 4 °C, and then the supernatant solution was centrifuged for 45 min at 9800 rpm at 4 °C. The resulting mitochondrial pellet was resuspended in the homogenizing buffer (3 mL). The supernatant solution obtained after centrifugation at 28 000 rpm for 90 min was considered as the cytosolic fraction (Orbea et al., 2002). Aliquots were stored at −80 °C until analysis. Total protein contents of all fractions were determined spectrophotometrically at 595 nm according to the method of Lowry et al. (1951) using bovine serum albumin (BSA, fraction V) as a standard. The progress of Catalase (CAT) and Superoxide dismutase (SOD) enzymatic reactions was measured using a UV VIS spectrophotometer (Beckman DU 6400, USA), maintained at room temperature. Reactions were measured for 120 s and a linear portion of the decline curve was used to calculate reaction rates.

Reaction conditions for activity assays with selected antioxidant enzymes were carried out as follows:

CAT activity was measured in the cytosolic and mitochondrial fractions (Orbea et al., 2002) by determining the decrease in H_2O_2 at 240 nm using 150 mM H_2O_2 as a substrate in 80 mM of phosphate buffer (pH 7), and then summing the activities of these two fractions to give the total CAT activity calculated as mM H_2O_2 degraded (expressed in mmol min^{-1} mg^{-1} protein).

The activity of SOD was analyzed in the cytosolic fraction of the homogenate, being indirectly assayed by measuring cytochrome *c* reduction in potassium phosphate buffer 50 mM, EDTA 0.1 mM, pH 7.8 containing 0.3 mM cytochrome *c*, hypoxanthine 1.5 mM, and xanthine oxidase 56 mUnit ml^{-1} ($\Delta A_{550} = 0.025$ min^{-1}), followed spectrophotometrically at 550 nm. Results were expressed as SOD units U mg^{-1} protein (1 U = amount which causes 50% inhibition of the initial rate of cytochrome *c* reduction by common substrate $O_2^{\bullet -}$ radicals produced by enzymatic activity of xanthine oxidase on hypoxanthine).

2.4. Biomarker of genotoxicity: the micronucleus test

The MN frequency assays were performed according to Bolognesi and Fenech (2012). Gills were removed, and the cells isolated by enzymatic digestion for 7 min at 37 °C with a solution of 0.1 mg ml^{-1} dispase I (neutral protease, grade I,