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Physiological responses of the European cockle *Cerastoderma edule* (Bivalvia: Cardidae) as indicators of coastal lagoon pollution

Jeamylle Nilin [a], João Luís Teixeira Pestana [b], Nuno Gonçalo Ferreira [b], Susana Loureiro [b], Letícia Veras Costa-Lotufo [a,*], Amadeu M.V.M. Soares [b]

[a] Laboratory of Marine Ecotoxicology, Instituto de Ciências do Mar (LABOMAR), Universidade Federal do Ceará, 60165-081 Fortaleza-Ceará-Brazil
[b] Department of Biology and CESAM, University of Aveiro, 3810-193 Aveiro-Portugal

HIGHLIGHTS

► Physiological responses as indicators of contamination in cockles.
► Clearance rate, air survival and energy reserves show a high correlation with Hg content.
► Oxygen consumption is not a good parameter to access Hg contamination in cockles.

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ABSTRACT

Physiological responses can be used as effective parameters to identify environmentally stressful conditions. In this study, physiology changes such as oxygen consumption, clearance rate, survival in air, condition index and energy reserves were measured on natural populations of cockles collected from different sites at Ria de Aveiro, Portugal. At those sites, sediment samples were collected for Hg concentration analysis. Cockles were used for the evaluation of both the Hg concentration and physiological response. Mercury was detected in the cockle tissue and in the sediment collected from the sampling points both nearby and distant from the main mercury contamination source. The energy content was negatively correlated with both Hg concentration in cockle tissues and survival in air. Nonetheless, the energy content was positively correlated with the condition index, and there was a positive correlation between the survival in air test and the tissue mercury concentration. A PCA-factor analysis explained 86.8% of the total variance. The principal factor (62.7%) consisted of the air survival, the Hg in soft tissues (positive) and the condition index (negative). The second factor (24.1%) consisted of a negative correlation between the oxygen consumption and the clearance rate. Due to their sensitivity to environmental conditions, the physiological responses of cockles can be used to assess the ecological status of aquatic environments. More effort should be invested in investigating the effects of environmental perturbations on cockle health once they are a good reporter organism.

1. Introduction

Quality assessments of coastal environments have been traditionally conducted using chemical analyses (Astudillo et al., 2005; Kehrig et al., 2006, Coelho et al., 2007) and structural measurements of biological communities (Astudillo et al., 2005; Carvalho et al., 2011; Coelho et al., 2007; Kehrig et al., 2006). Field studies have been conducted to assess the effects of different xenobiotics on native organisms under real exposure scenarios, including tidal variations, changes in salinity and temperature and exposure to chemicals (Järnegren and Altin, 2006; Nicholson, 1999).

Bivalves have often been used as sentinel organisms in environmental assessments due to their characteristics as filter feeders, their sessile life style, their wide distribution, their easy handling and collection, and their sensitivity to pollutants (Amaral et al., 2005; Domingos et al., 2007; Leinio and Lehtonen, 2005; Montaudouin et al., 2010; Pereira et al., 2012). In addition, bivalves play an important role in the economic and social activities of communities near estuarine and coastal areas worldwide. Biochemical and physiological responses have also been used as early indicators of potential ecosystem damage caused by metals and organic contaminants. These studies have been conducted in a variety of bivalves, but mainly with commercial species such as *Cerastoderma edule*, *Mytilus edulis*, *Macoma baltica*, *Perna perna*, *Perna viridis* and *Scrobicularia plana* (Ahmad et al., 2011; Bergayou et al., 2009; Leinio and Lehtonen, 2005; Nicholson, 1999; Paul-Pont et al., 2010; Pereira et al., 2012; Pessatti et al., 2002; Resgalla et al., 2009; Schiedek et al., 2006; Verlecar et al., 2007; Wang et al., 2005).

The European cockle *C. edule* (Linnaeus, 1758) is a suspension-feeding bivalve living in the first few centimetres of the sediment. These bivalves are found along the European Atlantic coast from the Barent Sea and the southern Baltic to Mauritania, West Africa (FAO,

* Corresponding author. Tel.: +55 85 3366 7029.
E-mail address: costalotufo@gmail.com (L.V. Costa-Lotufo).

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2011). Cockles are commercialized both fresh and canned mainly in the British, Dutch and French markets, representing a total capture production of 11.809 t in 2009 (FAO, 2011). In Portugal, cockles are collected and marketed throughout the year and have the legal capture size allowed above 2.5 cm. They are sold mainly to the Spanish market. Of the 3.881 t registered in Portugal commercial ports, only 300 t are produced by aquaculture in marine or brackish water (DGPA, 1998, 2010). The Aveiro region has a key role in the Portuguese fisheries market, contributing 10,695 t of fishes, crustaceans and mollusks. Cockles remain one of the most commercially valuable resources in this market (DGPA, 2010).

Ria de Aveiro is the most important coastal lagoon in the northwest region of Portugal, with a high economic, tourist and ecological relevance. The lagoon is connected to the Atlantic Ocean by a narrow channel. The edge of the lagoon is delimited by a sand bar, and the major freshwater contribution comes from Vouga River (29 $m^3.s^{-1}$) (Dias et al., 2000). One important source of contamination in this region is the chemical industrial complex situated in Estarreja, which produces fertilizers, mineral acids, chlorine and soda, plastics and other substances (IDAD, 2006). Mercury, the primary contaminant, is discharged by a chloralkali production process. Despite the reduction in the amount of mercury waste outflow in the last two decades, the accumulation in the sediments and animals, in addition to other environmental damage, can still be observed (Abreu et al., 2000; Coelho et al., 2007; Ramalhosa et al., 2006). There are other sources of contamination in the nearby areas: several small marinas, deep sea and coastal fishing ports, chemical and commercial ports, dockyards, and agricultural and domestic sewage disposal. All of these are potential sources of organotins, metals and polycyclic aromatic hydrocarbons (PAHs) (Castro et al., 2006; Sousa et al., 2007).

In the present study, we evaluated the physiological responses of *C. edule*, such as oxygen consumption and clearance rate, survival in air, condition index and total energy content, and assessed their use as indicators of mercury pollution. Cockles were collected from several sites along Ria de Aveiro, Portugal, to sample populations with different levels of mercury exposure. Our goal was to identify the best physiological parameters, in terms of sensitivity and relevance, for use in future monitoring programs.

2. Materials and methods

2.1. Study area and sampling

The animals and sediments were collected during the low tide, in areas submerged or recently exposed to air, at six different sites at Ria de Aveiro, Portugal, in July 2010 (Fig. 1). The cockles were removed from the sediment using a rake and a net, and their valves were cleaned in situ. The surface sediment was collected with a plastic shovel (approximately 8–10 samples) from approximately 3 cm below the surface. These samples were stored in a plastic bag for later chemical analysis. The sampling sites were chosen to cover a vast area of the lagoon, and they were selected based on previous studies of the sources of contamination and ecotoxicological and chemical data (Coelho et al., 2007; Monterroso et al., 2003b; Ramalhosa et al., 2006). Barra is located near the seaward entrance of the lagoon. Despite having only a moderate amount of naval traffic, previous studies indicated low levels of contamination with metals, organotins and estrogenic contaminants. Therefore, Barra can be considered a reference site (Abreu et al., 2000; Galante-Oliveira et al., 2009; Rodrigues, 2011; Sousa et al., 2009). The São Jacinto channel is located in the central part of the lagoon, far from direct contamination sources, in front of the natural reserve of the São Jacinto dunes. The Ílhavo channel is the southern site, and it receives domestic and agricultural effluents. The Cais do Bacalhoeiro site is located in a sand–mud bank on the opposite side of a deep-sea fishing port. Rio Novo do Príncipe is situated in the confluence of the Vouga River with the lagoon, and it is exposed to effluents from a pulp/paper plant located in Cacia. Bico do Laranjo is located near the mouth of the lagoon, between the central channel of Ria de Aveiro and the Laranjo Basin. The latter is the site with the highest levels of mercury contamination because it is close to the chloralkali plant in the Estarreja industrial complex (Fig. 1).

C. edule specimens (26 ± 1.4 mm long and C.V. = 5.47%) and sediment samples were collected on three different days at the same tide level. Due to sampling logistical constraints, the samples were collected from Barra and Bico do Laranjo on the first day, São Jacinto and Rio Novo do Príncipe on the second day and Cais do Bacalhoeiro and Ílhavo on the third day. The samples were transported to the laboratory within 2 h of collection. Between 70 and 80 animals were randomly collected at each site, and divided in sub-samplings to physiological and chemical analyses. Animals used to analyze oxygen consumption, clearance rates and survival in air test were acclimated in artificial seawater (salinity 34 ± 2; pH 8.0 ± 0.3; 20 ± 2 °C) with continuous aeration for 2 h prior to the beginning of the experiments. The main purpose of acclimatization was to restart the respiration and filtration, and also purge the sediment inside animal that could interfere with experimental procedures. For the measurement of energy reserves, condition index and mercury body burdens, the animals were dissected, and their tissues were separated and stored frozen (−20 °C) until analysis.

2.2. Mercury analysis

The cockle soft tissue samples (n = 10 per site) and sediment (composite samples) from each sampling site were dried at 50 °C and analyzed by pyrolysis atomic absorption spectrometry with gold amalgamation. We used a LECO AMA-254 (Advanced Mercury Analyzer) with no pre-treatment of the samples (Costley et al., 2000). The analyses were conducted with certified reference material TORT-2 (lobsters hepatopancreas) and PACS-2 (marine sediment) purchased from the National Research Council Canada and were carried out in parallel with experimental measurements. The mercury recovery from the sediment samples was 94.6 ± 1.7% of that of PACS-2, and the mercury recovery from the cockle tissue was 93.9 ± 2.3% of that of TORT-2.

2.3. Physiological responses

2.3.1. Measurement of oxygen consumption

The oxygen consumption was determined by simple static respirometry using cockles held for 1 h in 50 mL gastight syringes (Hamilton, USA). To measure oxygen consumption in *C. edule*, ten syringes per site were employed, each holding one organism. The syringes were filled with artificial seawater and organisms, the remaining air was expelled from each syringe and cockles were then left in the dark in a water bath (20 °C).

After 1 h, the initial oxygen concentrations were measured with an oxygen meter (model 782, with an oxygen electrode model 1302, Strathkelvin Instruments, Glasgow). The samples were taken using a 0.5 mL gastight syringe, and the test solutions were injected manually into the electrode chamber (volume = 70 μL) at a constant rate (3 mL min^{-1}). The readings were taken after 1 min. After 2 h, the final oxygen concentrations were measured in the same way. The organisms were then dried for 96 h at 60 °C and weighed. The oxygen consumption was determined by the differences between the oxygen content of water before ($T_{initial}$ = 0 h) and after (T_{final} = 1 h) the exposure period, and the respiration rate was expressed as mg oxygen consumed per g of dry soft tissues. Three blank controls (syringes with no organisms) were used to correct for the ambient oxygen depletions due to factors other than organism respiration (e.g., microbial respiration).

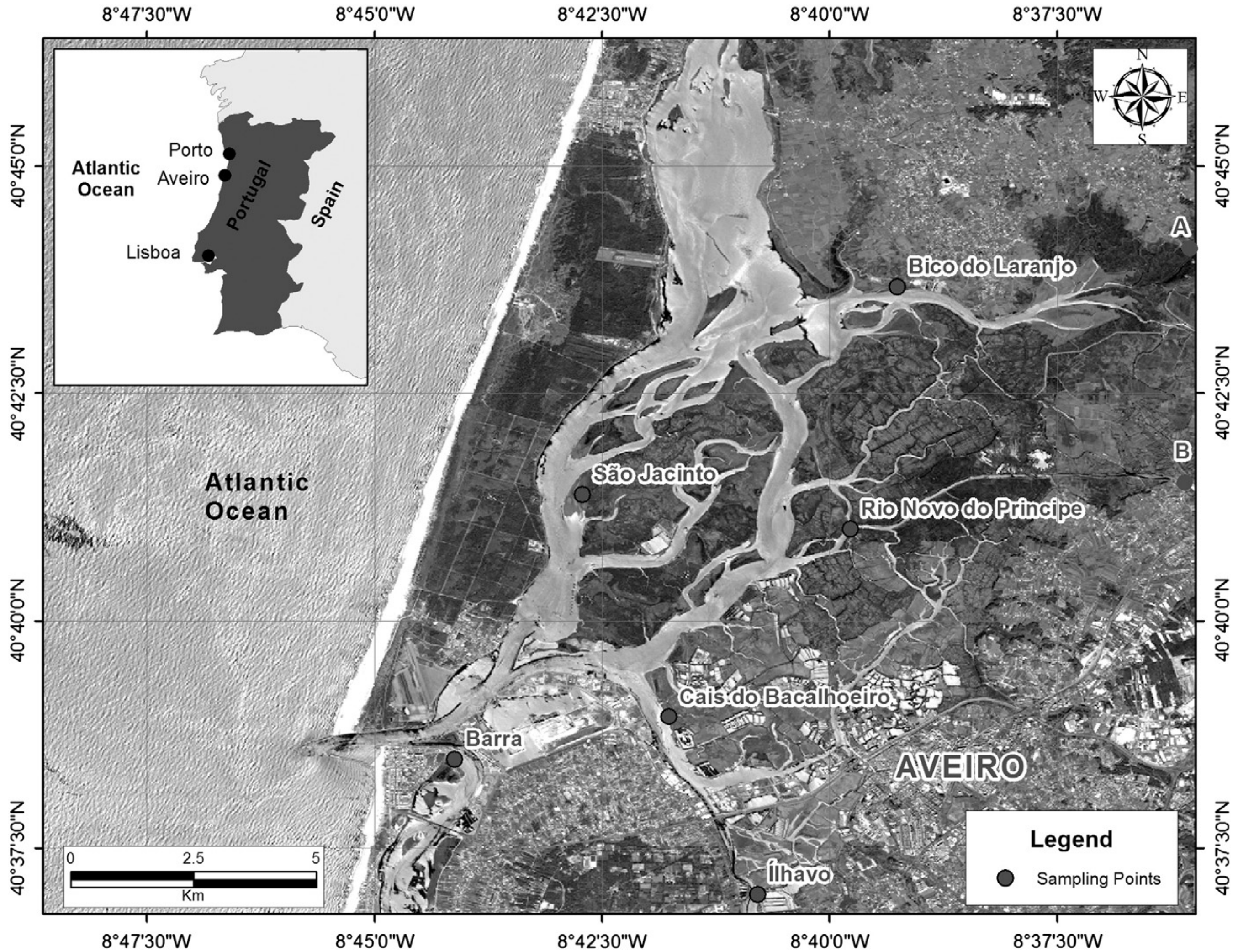

Fig. 1. Sampling sites in Ria de Aveiro, Portugal. WorldView1 satellite Panchromatic image from August 2010, Datum WGS-84. A—industrial Complex of Estarreja. B—pulp/paper mill at Cacia.

2.3.2. Clearance rate

The clearance rate was estimated following a previously developed method (Coughlan, 1969) that is based on the rate of absorption of a neutral red dye by animals (Masilamoni et al., 2002). The initial solution (1.5 mg L^{-1}) was prepared in artificial seawater. One organism was introduced into a chamber filled with 150 mL of neutral red solution (n = 5 for each site). After observing the valve aperture and siphon display, the dye concentration in 3 mL aliquots of the solution was measured every 15 min for 45 min at 530 nm in a spectrophotometer (Jenway model UV–vis 6505). There were three readings per sample. The rate of clearance (L h^{-1}) was calculated by using the equation (Coughlan 1969):

$$CR = V(\ln C_i - \ln C_f)/t,$$

where V is volume in the chamber (L), C_i is the initial concentration, and C_f is the final concentration after time t (hour). This rate was normalized by the dry weight of soft tissues (g) for each animal after 96 h drying at 60 °C.

In addition, a treatment with no animals containing the initial solution (1.5 mg.L^{-1}) was used as a control.

2.3.3. Survival in air test

The animals were placed in chambers lined with filter paper that was soaked with artificial seawater to maintain the moisture content. The chambers were covered with perforated plastic film and held at a constant temperature (20 ± 2 °C) and photoperiod (14 h light: 10 h dark). For each site, three replicates of 7 organisms were used. Their survival was checked daily by an inspection of valve closure. When the open valves did not close after mechanical stimulation, the animals were considered to be dead. This procedure was repeated daily until the death of all organisms was recorded.

2.3.4. Condition index

The soft tissues (n = 10 per site) were removed from the shell and blotted dry on a paper towel to remove the excess water; wet tissue and air-dried shells were then weighed. The condition index was calculated following the formula developed by Lucas and Beninger (1985):

$$CI = \text{soft tissues weight}/\text{shell weight} \times 100.$$

2.3.5. Total energy content

For each sampling site, all soft tissue from five cockles was homogenized individually in 4 mL of ultrapure water and divided in two aliquots (300 µL) for analysis of the following measurements: (1) lipidic content and (2) protein and sugar content.

The total lipid quantification was based on the method described by Bligh and Dyer (1959). Briefly, after adding 500 µL chloroform (spectrophotometric grade), each sample was vortexed, and 500 μL methanol (spectrophotometric grade) and 250 μL ultrapure water were added. Afterwards, the samples were centrifuged (1000 *g*, 5 min, 4 °C), and the top phase was discarded; the remaining phase was used for lipid measurement. 100 μL of lipid extract plus 500 μL H_2SO_4 were heated for 15 min (200 °C). After the samples cooled down, 1.5 mL of ultrapure water was added, and the total lipid content was determined by measuring the absorbance at 370 nm using tripalmitin as a standard.

To determine the total protein and carbohydrate content, 100 μL of trichloroacetic acid (TCA 15%) was added to the homogenized aliquot samples and incubated at −20 °C for 10 min (method adapted from De Coen and Janssen, 1997). The supernatant, representing the carbohydrate fraction, was separated after centrifugation (1000 *g*, 10 min, 4 °C). The total carbohydrate content was determined by adding 50 μL of 5% phenol and 200 μL sulfuric acid (H_2SO_4) to 50 μL of each sample in a 96-multiwell microplate and then incubating the samples for 30 min at 20 °C; the absorbance was measured at 492 nm using glucose as a standard. The remaining pellet was resuspended in 1.25 mL sodium hydroxide (NaOH), incubated at 60 °C for 30 min, neutralized with 750 μL hydrochloric acid (HCl) and then used to measure the protein fraction. The total protein content was then determined using the Bradford's reagent (Bradford, 1976), and the absorbance was measured at 590 nm using bovine serum albumin as a standard.

The total energy content was calculated by summing the lipid, protein and carbohydrate contents, and the data were expressed as $J*mg\ org^{-1}$ (wet weight).

2.4. Data analysis

The allometric relationship between body size (W, in g of dry weight) and the physiological components (e.g., clearance rate and oxygen consumption) was calculated according to the expression: $Y' = Y\,W^{-b}$ (Smaal et al., 1997). In the equation, Y' denotes the allometric relationship, Y denotes the measured physiological component and b denotes the mass exponent. The mass exponent value (b) was $b = 0.7$ and $b = 0.5$ for the respiration and clearance rates, respectively (Smaal et al. 1997).

The comparison of the physiological responses and mercury content between the reference site (Barra) and all other sites was analyzed by one-way ANOVA, which was followed by Dunnett's multiple comparison test whenever significant differences were observed ($p<0.05$). The D'Agostino–Pearson omnibus test was used to check the data normality. The correlation matrix was applied to identify correlations between variables ($p<0.01$). The parameters were integrated by factor analysis using a principal components analysis (PCA) as the extraction method with a varimax rotation procedure. The statistical analyses were carried out using the GraphPad Prism (Intuitive Software for Science, San Diego, CA, USA) and STATISTICA (version 7) software.

3. Results and discussion

3.1. Mercury contamination

Many studies have reported metal contamination in the water, sediment and biota around Ria de Aveiro; the most prevalent metal detected has been mercury (Abreu et al., 2000; Coelho et al., 2007; Monterroso et al., 2003b; Ramalhosa et al., 2006). In this study, the presence of mercury was confirmed in all sediment and cockle tissue samples (Table 1). The highest levels of Hg in sediment were from Cais do Bacalhoeiro (37.0 ± 1.47 $ng.g^{-1}$) and Rio Novo do Príncipe (34.77 ± 0.18 $ng.g^{-1}$), followed by Bico do Laranjo (28.54 ± 0.13 $ng.g^{-1}$), Ílhavo (18.79 ± 0.61 $ng.g^{-1}$), São Jacinto (10.49 ± 0.47 $ng.g^{-1}$) and Barra (7.25 ± 0.09 $ng.g^{-1}$). However, the pattern of mercury contamination was not the same in the cockles samples; organisms from Bico do Laranjo (341.3 ± 3.38 $ng.g^{-1}$) and Ílhavo (144.5 ± 5.37 $ng.g^{-1}$) showed a higher mercury body burden than bivalves from the other sampling sites (São Jacinto > Rio Novo do Príncipe > Barra > Cais do Bacalhoeiro). Other studies have shown that the sediments around Bico do Laranjo are the most contaminated in the Ria de Aveiro due to the proximity to the Estarreja industrial complex, which is the primary source of mercury contamination (Abreu et al., 2000; Ramalhosa et al., 2006). Ramalhosa et al. (2006) detected higher mercury concentrations in the deeper sediment layers compared to the surface layers, indicating that Hg discharges were higher in the past. However, a substantial mercury concentration was also detected in suspended particles; this phenomenon could be explained by remobilization processes or a recent Hg discharge (Coelho et al., 2005; Monterroso et al., 2003b). The mercury present in particulate fractions at the Laranjo Basin can be exported to other regions by tidal exchanges, although the mercury levels decrease as these waters merge with the ocean (Abreu et al., 2000; Monterroso et al., 2003b).

Our results do not agree with those of Figueira et al. (2011) who only found Hg in cockle tissues and sediments from surrounding areas near the Laranjo Basin. This lack of concordance between these studies is most likely due to differences in our methods of metal analysis; Figueira et al. used a weak extraction methodology with 1 M HNO_3 prior to taking measurements using inductively coupled plasma-mass spectrometry (ICP-MS). Surprisingly, the second highest Hg body burdens were found in the cockles from Ílhavo, the farthest site from the main source of mercury (Fig. 1). This result indicates that there may be another source of mercury contamination near this area or even that this is an area with great deposition of contaminated particulate matter. The Ílhavo, Rio Novo do Príncipe and Cais do Bacalheiro sites are located in the southern of Laranjo Basin, indicating that there may be fluctuations in the deposition that are controlled by tidal dynamics and river drainage flow (from the Vouga and Antuã rivers) in this direction. The tidal dynamics can alter the patterns of particle deposition and disturb the superficial bottom layer, thus explaining the incongruity between the sediment and tissue Hg concentrations found in our study. Coelho et al. (2007) also found high concentrations of mercury in sites near the

Table 1
Physico-chemical parameters measured at the different sampling sites in Ria de Aveiro, Portugal, and Hg content in sediments and cockle tissues in July, 2010.

Sampling site	Temperature (°C)	Salinity	Dissolved oxygen ($mg.L^{-1}$)	pH	Sediment Hg content ($ng.g^{-1}$) ± SD	Cockle Hg content ($ng.g^{-1}$) ± SD
Bico do Laranjo—BL	28	32	7.4	7.4	28.54 ± 0.13[a]	341.3 ± 3.38[a]
Barra—BA	20	37	8.4	7.9	7.25 ± 0.09	82.48 ± 1.68
São Jacinto—SJ	20	37	8.0	7.9	10.49 ± 0.47[a]	98.6 ± 0.31[a]
Rio Novo do Príncipe—RP	21	31	7.6	7.7	34.77 ± 0.18[a]	90.14 ± 4.17
Cais do Bacalhoeiro—CB	23	35	8.2	8.1	37.0 ± 1.47[a]	78.29 ± 1.30
Ílhavo—IL	22	31	7.2	7.9	18.79 ± 0.61[a]	144.5 ± 5.37[a]

[a] Denotes significant differences compared with reference site: Barra (Dunnett test, $p<0.05$).

lagoon entrance with higher bioaccumulation values in invertebrates compared with those from the Laranjo Basin, which is historically thought to be the most contaminated site. The transport of fine particles was considered to be the main explanation for this metal contamination in that study. Consequently, suspension and surface deposit feeders are prone to uptake and bioaccumulate more mercury.

Several other forms of metal contamination, such as copper, chromium, cadmium, nickel, lead and zinc, were found in the Ria de Aveiro ecosystem (Figueira et al., 2011; Monterroso et al., 2003a). This contamination was also associated with industrial activities along the lagoon, resulting in the presence of organic compounds, such as tributyltin (TBT) (Galante-Oliveira et al., 2009; Sousa et al., 2007) and polycyclic aromatic hydrocarbons (PAHs) (Oliveira et al., 2009b; Vidal et al., 2011). This contamination was attributed to port activities and to the steroid hormones and phenolic compounds (Sousa et al., 2009) from the domestic and industrial sewage treatment plant. All of these factors can alter the physiological functions of organisms within the region of contamination.

3.2. Physiological responses

The allometric relationship between the oxygen consumption, clearance rate and body weight of *C. edule* collected at Ria de Aveiro was achieved by using the b-coefficients (b = 0.7 and 0.5) for respiration and clearance rate obtained by Smaal et al. (1997). Ibarrola et al. (2008) also determined a similar mass exponent to evaluate these rates in cockles.

The oxygen consumption was lower in organisms collected in Barra, with the lowest reported value (0.89 ± 0.13 mg O_2 h^{-1} g^{-1}). The next lowest value of oxygen consumption was recorded for animals from Bico do Laranjo (1.06 ± 0.34 mg O_2 h^{-1} g^{-1}). No other significant differences were observed in the cockles from the other sampling sites (Fig. 2). Smaal et al. (1997) found similar respiration rates in the summer months (approximately 1.5 mg O_2 h^{-1} g^{-1}), with an annual mean of 0.83 mg O_2 h^{-1} g^{-1}.

Respiration rate has been used as a reliable parameter for a variety of freshwater and marine invertebrate species that have potential uses as indicators of the effects of different contaminants on organismal metabolism (Pestana et al., 2009, 2010; Willows, 1994). In the present study, respiration results should be interpreted bearing in mind that experiment duration is short which might decrease the sensitivity of respiration rate as an indication for physiological status of organisms.

Some studies have also shown that the respiration rate is less sensitive than other physiological responses, such as the clearance rate, absorption efficiency, scope for growth, length or weight (Wang et al., 2005). Furthermore, Resgalla et al. (2009) considered low respiration rates to be an indication that the animals were exposed to a less impacted environment. However, the second lowest respiration rate they measured was in samples from the nearest site of mercury source (Bico do Laranjo), suggesting that other factors beyond those that they considered may contribute to their results.

The animals from Cais do Bacalhoeiro showed the lowest clearance rate (0.88 ± 0.33 L h^{-1} g^{-1}). The results from the organisms collected from all other sites showed no significant differences in comparison to the rate measured in organisms collected at the reference site at Barra (1.75 ± 0.37 L h^{-1} g^{-1}) (Fig. 3). As shown previously, higher rates were measured during the summer months, with average of 1.52 L h^{-1} g^{-1} (Smaal et al., 1997).

Burt et al. (2007) showed a CR reduction (<0.4 L h^{-1} g^{-1}) after 90 days of exposure in cockles that were transplanted to metal-contaminated areas. In addition, an increasing level of oxygen consumption was recorded, suggesting that the animals were under stressful conditions. Although, the Hg contamination in the sediment samples from Cais do Bacolheiro was higher than in the samples from the other sites, the Hg levels in the cockle tissue did not differ from those of the reference site (Table 1). Therefore, the metal was not the main contributor to the CR reduction. Jones et al. (2011) showed a strong relationship between a high bed density and CR reduction in cockles. However, this variable was not considered in the present study.

The survival in air test showed that the Bico do Laranjo animals can withstand greater air exposure, as they had highest LT_{50} values (5.75 ± 0.02 days), while the Barra and São Jacinto animals had LT_{50} values of 3.53 ± 0.01 and 3.66 ± 0.02 days, respectively. The animals from Ílhavo, Cais do Bacalhoeiro and Rio Novo do Príncipe had LT_{50} values of 3.80 ± 0.01, 3.51 ± 0.01 and 3.49 ± 0.01 days, respectively (Fig. 4). These results suggest that the cockles at Bico do Laranjo are more capable of withstanding anoxic conditions than the cockles from the other sampling sites around the lagoon. This type of tolerance test, commonly referred as stress on stress (SOS), has been demonstrated to be a sensitive indicator because high levels of contamination were associated with a low capacity for air exposure (Hellou and Law, 2003; Ivanina et al., 2010; Matozzo et al., 2003; Pampanin et al., 2005). However, the results form Bico do Laranjo showed the opposite relationship, most likely due to some adaptations to low tidal amplitude and high phase lag in the high and low water cockles observed in the inner sites at Ria de Aveiro (Dias et al., 2000). This type of natural confounding factor seems to be an important limitation of survival in air test in transitional environments, as highlighted by Nesto et al. (2007).

Since condition index may reflect the energy expenditure for growth, the present results showed that cockles collected from Bico do Laranjo ($51.9 \pm 10.6\%$) and Ílhavo ($54.7 \pm 6.6\%$) were more affected in terms of growth than the cockles from the reference site (65.5 ±

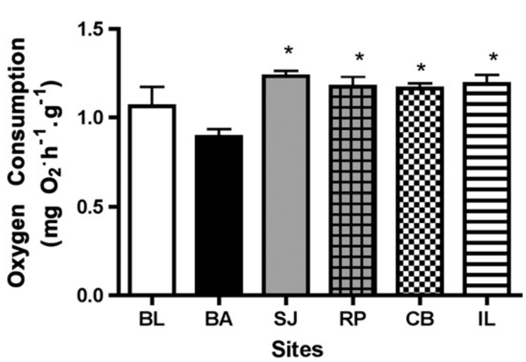

Fig. 2. Oxygen consumption of *C. edule* collected from sampling sites at Ria de Aveiro, Portugal. BL—Bico do Laranjo; BA—Barra; SJ—São Jacinto; RP—Rio Novo do Príncipe; CB—Cais do Bacalhoeiro; IL—Canal de Ílhavo. All results are expressed as the mean values ± standard deviation (n = 10); *indicates a significant difference from the reference site, Barra (Dunnett test, p<0.05).

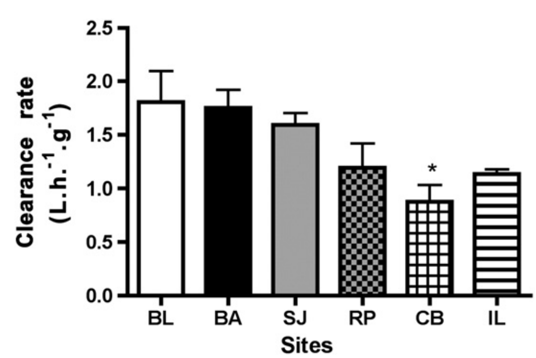

Fig. 3. Clearance rate of *C. edule* collected from sampling sites at Ria de Aveiro, Portugal. BL—Bico do Laranjo; BA—Barra; SJ—São Jacinto; RP—Rio Novo do Príncipe; CB—Cais do Bacalhoeiro; IL—Canal de Ílhavo. All results are expressed as the mean values ± standard deviation (n = 5); *indicates a significant difference from the reference site, Barra (Dunnett test, p<0.05).

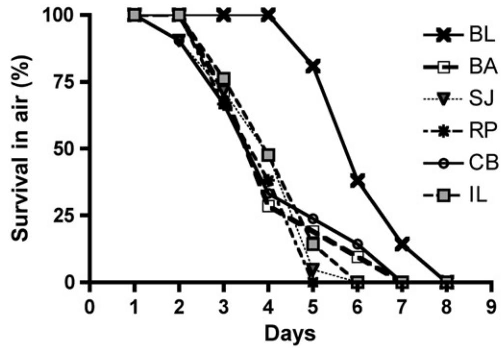

Fig. 4. Survival in air of *C. edule* collected from sampling sites at Ria de Aveiro, Portugal. BL—Bico do Laranjo; BA—Barra; SJ—São Jacinto; RP—Rio Novo do Príncipe; CB—Cais do Bacalhoeiro; IL—Canal de Ílhavo. All results are expressed as percentage of survival in air exposure (n = 21).

8.0%) (Fig. 5a). This result may indicate that these animals are under stressful conditions, possibly due to natural (e.g., low food supply) or even anthropogenic factors (e.g., the presence of pollutants) that could influence their energy balance. In the present study, the local food supply was not verified. However, according to measurements by Lopes et al. (2007) of chlorophyll-a, dissolved nitrogen and phosphate concentrations, Ria de Aveiro has moderately low overall eutrophic conditions. The inner stations (e.g., Cais do Bico do Laranjo and Ílhavo) are considered eutrophic, and the outer stations (e.g., Barra) are classified as mesotrophic. The main source of nutrients in this system seemed to be deposited by freshwater sources, including precipitation and runoff from agricultural land. The high concentrations of chlorophyll-a in the inner areas of the lagoon during low tide suggest the export of phytoplankton to the ocean.

Nevertheless, the present results demonstrated that the energy content of the cockles at Bico do Laranjo (850 ± 247.2 mJ.mg org^{-1})

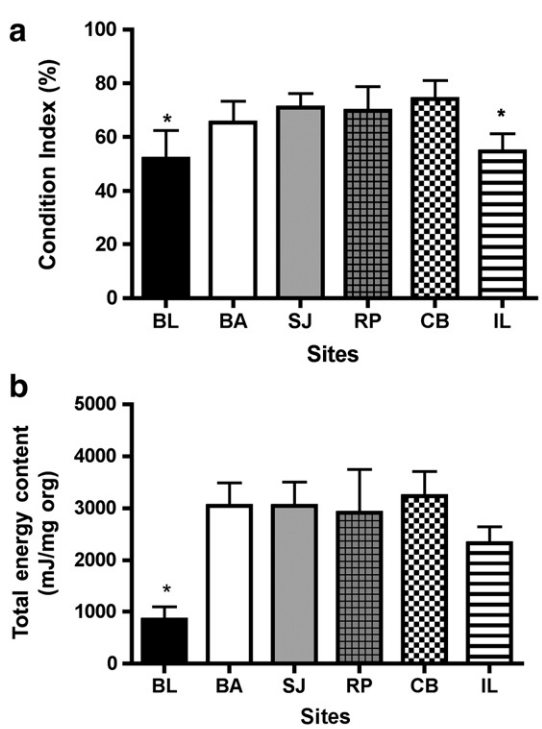

Fig. 5. Condition index (n = 10) (a) and total energy content (n = 5) (b) of *C. edule* collected from sampling sites at Ria de Aveiro, Portugal. BL—Bico do Laranjo; BA—Barra; SJ—São Jacinto; RP—Rio Novo do Príncipe; CB—Cais do Bacalhoeiro; IL—Canal de Ílhavo. All results are expressed as the mean values ± standard deviation; *indicates a significant difference from the reference site, Barra (Dunnett test, $p < 0.05$).

was about three-fold less than the energy content of the cockles collected in Barra (3045 ± 443.4 mJ.mg org^{-1}).

Energy balance is modulated by differences between absorbed energy and metabolic losses. Thus, energy can only be assigned for growth and reproduction when the amount absorbed is greater than the amount lost (Gosling, 2003). Nicholson (1999) applied condition index analysis of green mussel *P. viridis* to monitor contaminated sites in Hong Kong, China. He observed lower indices at contaminated sites than at the reference sites. Changes in the energy balance and condition index are not always representative of anthropogenic stress because they can also vary with the seasons and reproductive periods. However, Cardoso et al. (2009) showed that *C. edule* body mass continued to increase even after the beginning of the spawning period in April, indicating that the investment in somatic tissue was prioritized over reproduction. The results of the present study show that mercury contamination was strongly correlated with energy content and condition index, as shown below.

Ecotoxicological parameters based on energetic budgets and on the feeding and respiration rates of a variety of organisms have been currently evaluated to validate their robustness in the environmental quality assessment (Järnegren and Altin, 2006; Pessatti et al., 2002; Sukhotin et al., 2003; Willows, 1994). Although these parameters have an intrinsic natural variation, they are somehow related to metabolic demands that can be due to stressors' exposures. Traits will bring higher ecological relevance to toxicity studies and therefore to risk assessment procedures. The difficulties behind measurements are overlapped by its importance. Intrinsic sensitivity of a species will be directly related to the environmental exposure and can be evaluated by the bioconcentration that will be linked to several traits like the mode of respiration, size of the organism or its integument permeability (Rubach et al., 2011). In addition, it has been recognized that species-specific physiological and ecological factors can influence the vulnerability to chemical stress, thus differentially affecting population and community level. For this reason it has been proposed that a better understanding should be implemented on specie traits in order to permit a better prediction of the potential adverse effects of chemicals, bridging also the gap between laboratory controlled studies with model species and real scenarios and/with several species.

It is worthwhile to mention that physiological parameters are good indicators of the general population healthy, and the related natural variability could lead to discrepancies between the presence of environmental stressors and bivalve responses (Montaudouin et al., 2010). However, the impairment of individual healthy can have consequences for growth, reproduction and survival. These effects can, in turn, propagate the effects of the contaminants throughout the higher levels of the biological hierarchy (Maltby, 1994). Indeed, these parameters have been assessed to determine the sub-lethal effects of different types of contaminants, including metals (Maltby, 1994; De Coen and Janssen, 2003).

3.3. Data multivariate analysis

Based on the simple correlation matrix ($p < 0.01$), it was evident that the correlations between energy content, mercury in cockle tissues and air survival were significantly negative. Also, their correlations with the condition index were positive. The correlation between the survival in air test and mercury in the tissues was positive but was the least significant. The sediment mercury content did not have as clear a trend as the soft tissue mercury content (Table 2). Due to this strong correlation, neither energy content nor mercury content in sediment was used in the PCA factor analysis. All other physiological parameters, including the Hg content in the cockle tissue, were integrated into the PCA-factor analysis, and these variables explained 86.8% of the total variance (Table 3). The principal factor (F1) contributed 62.7% of the total variance,

Table 2
Correlation matrix of *C. edule* physiological responses and mercury content in cockle soft tissue and sediment from Ria de Aveiro, Portugal. Marked (bold) correlations are significant at $p<0.01$.

	Oxygen consumption	Clearance rate	Energy content	Survival in air	Condition index	Hg cockle	Hg sediment
Oxygen consumption	1.00						
Clearance rate	−0.56	1.00					
Energy content	0.12	−0.45	1.00				
Survival in air	−0.16	0.52	**−0.97**	1.00			
Condition index	0.22	−0.40	**0.87**	−0.75	1.00		
Hg cockle	−0.12	0.48	**−0.99**	**0.99**	−0.81	1.00	
Hg sediment	0.36	−0.61	0.15	0.16	0.13	0.17	1.00

exhibiting a clear positive correlation among survival in air and Hg in the soft tissues and a negative correlation with the condition index. The secondary factor (F2) accounted for 24.1% of the variance, and oxygen consumption was inversely correlated to clearance rate. When the associations for all parameters and cases (studied sites) with the PCA factors were analyzed, it was possible to identify different trends. The Barra animals had significant alterations in oxygen consumption and clearance rates. The Bico do Laranjo cockles were different in terms of survival in air, condition index and Hg contamination. Inversely, the cockles from Bico do Laranjo, São Jacinto, Rio Novo do Príncipe and Cais do Bacalhoeiro were all associated with factor 1 (Fig. 6). These results agree with other studies performed at Ria de Aveiro that found that the Laranjo Basin has the highest mercury contamination and that other inland areas, such as the Ílhavo channel and Rio Novo do Príncipe, had the next highest contamination levels (Abreu et al., 2000; Castro et al., 2006; Coelho et al., 2007; Oliveira et al., 2009a).

In Ria de Aveiro, mercury contamination has been detected in the sediment, water, plankton, macroalgae, polychaetes, crabs, bivalves and fishes (Pereira et al., 2008). Due to their filter feeding, bivalves bioaccumulate mercury in their tissues and experience long-term exposure to contaminants on the order of years. Inside the bivalves' bodies, this metal can be redistributed into different organs, mainly the gills and digestive gland. The redistribution affects cellular and biochemical mechanisms, such as lipid peroxidation, antioxidant protection and esterase activity (Ahmad et al., 2011; Roméo et al., 2006; Verlecar et al., 2007). Although a correlation has been demonstrated between mercury contamination and physiological parameters in cockles, the influence of other contaminants on the observed response cannot be excluded. Monterroso et al. (2003a) demonstrated that, in addition to mercury, there were higher concentrations of Cu, Cd, Zn and Pb at the same locations in Ria de Aveiro basin, based on samples such as sediments, pore water and biota. It is important to include the analysis of these metals in further studies to assess their contributions to the observed physiological alterations.

4. Conclusions

In this study, the physiological responses of *C. edule* collected from six different sites in Ria de Aveiro were measured and compared to the mercury body burdens in the cockles.

Table 3
Factor loadings (extraction by principal components analysis with varimax rotation) of physiological and chemical variables on the two factors. Marked (bold) loadings are >0.70.

	Factor 1	Factor 2	Communalities
Oxygen consumption	0.002159	**0.934555**	0.747763
Clearance rate	0.406452	**−0.783556**	0.601710
Survival in air	**0.954263**	−0.171609	0.998846
CI	**−0.862262**	0.171752	0.966575
Hg cockle	**0.981171**	−0.120120	0.999078
Eigenvalues	3.1355	1.2071	
Prp. totl (%)	62.71	24.14	

Mercury analysis of the soft tissue from *C. edule* showed mercury bioaccumulation from all of the sampling sites, with higher levels at Bico do Laranjo and Ílhavo. The sediment samples from Cais do Bacalhoeiro and Rio Novo do Príncipe showed the highest Hg concentrations. This finding suggests that the sediment can be remobilized and that fine particles may be redistributed at sites distant from the main mercury sources at the Estarreja Industrial complex (Laranjo Basin). These results show that studies of metal contamination cannot reflect the real impact on organisms through only sediment analyses; they must also consider the constant sediment dynamics generated by the tides and river flow.

The physiological responses of indigenous organisms, such as *C. edule*, can be considered good tools for the evaluation of environmental contamination. These indicator species exhibit clear and reproducible responses to contamination, and they link environmental levels of the contaminants to the ecological status of the ecosystems. Survival in air, condition index and energy content appear to be the most contaminant-sensitive parameters for sentinel organisms, such as cockles. These factors provide information about the environmental health status as well as the cockle health status, which is important as they are consumed by humans.

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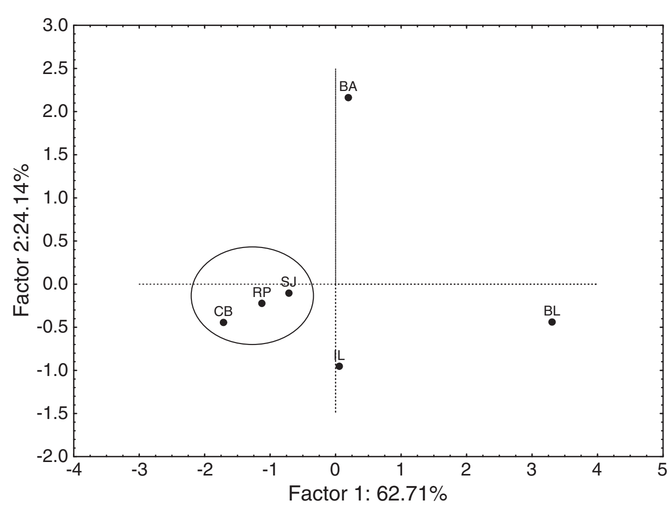

Fig. 6. Representation of six sampling sites at Ria de Aveiro, Portugal, obtained from PCA—factor analysis of physiological and chemical parameters. BL—Bico do Laranjo; BA—Barra; SJ—São Jacinto; RP—Rio Novo do Príncipe; CB—Cais do Bacalhoeiro; IL—Canal de Ílhavo.

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