Original Research

Potential Impact of UWWT Effluent Discharges on *Ruditapes decussatus*: An Approach Using Biomarkers

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Abstract

The use of biomarkers in bivalves has gained significance as a reliable method for the assessment of the presence and effect of contaminants in aquatic ecosystems. However, it is important to note that the biomarkers respond not only to contaminant loadings and bioavailability but also to environmental stress. Therefore, the association between biomarkers and contamination/pollution should be conducted cautiously as the environmental factors also affect their response. These factors should be integrated into the assessment of the response of the biomarkers. The potential impact of effluents from an

urban wastewater treatment plant (UWWTP) on the *Ruditapes decussatus* clam specimens located 1.5 Km away in a surrounding area was evaluated. After one month of exposure, three biomarkers were analyzed, namely lipid peroxidation (LPO), acetylcholinesterase (AChE) and electron transport system (ETS). These parameters were also determined for a control group purchased from a local nursery, which had no influence from UWWTP, in order to compare the results obtained from both groups. *The in situ* physicochemical characterization of the exposure site (temperature, salinity, pH and dissolved oxygen) was evaluated together with nutritional parameters, morphometric measurements and condition index. The biochemical analysis showed that the activity levels of AChE fluctuated from 4.0 to 4.6 nmol/min g protein and that of LPO from 101.5 to 248.9 nmol MDA/g protein. Also, the ETS activity levels were in the range of 27.2 to 30.2 nmol O_2/min g protein. The lipid peroxidation was found to be the most responsive biomarker toward the damage caused by environmental conditions on the clams.

Keywords
Ruditapes decussatus; biomarkers; lipid peroxidation; acetylcholinesterase; electron transport system

1. Introduction

The environmental monitoring of air, soils, and aquatic ecosystems is of great importance. Several methods have been implemented to address this concern, either by the direct measurement of the level of potential contaminants or using biological surrogates for the simultaneous estimation of the bioaccumulated concentrations and effects of the substances [1]. In this context, one of the most significant approaches is the use of biomarkers on wildlife communities called sentinel species, which ultimately leads to the early identification and warning of any potential risks to the human population [2, 3]. Biomarkers are valuable tools that can be used to determine the interactions between biological systems and potential contaminants (chemical, physical or biological) [4] by measuring the appropriate biological response at molecular, cellular, biochemical, physiological or behavioral levels [5]. Among the biological effects of the pollutants, biochemical changes occur more quickly and sensitively. Therefore, biomarkers allow the integrated measurement of bioavailable contaminants causing biochemical responses [6].

These biomonitoring methods evaluate not only the presence, but the more significant response of the organisms to these contaminants by the assessment of biomarkers, i.e., parameters that reflect their effects at a molecular, cellular, organ, and organism level. This approach does not replace chemical monitoring or population studies but integrates them in determining the toxic effects of the pollutants even under trace or sub-lethal concentrations [7].

Several systems have been described for the classification of biomarkers. According to the World Health Organization [4], biomarkers are divided into three categories: exposure, effect, and susceptibility. A more descriptive approach was established by van der Oost et al. [8], who classified biomarkers into ten different categories on the basis of the type of monitored response as follows: biotransformation enzymes, oxidative stress, biotransformation products, amino acids and proteins,

hematological, immunological, reproductive and endocrine, neuromuscular, genotoxic, physiological and morphological types.

The selection of each biomarker or group of biomarkers is specific to some organisms or exposure conditions. Some of the most frequently used biomarkers for the detection of marine pollution through the local biota analysis are cytochrome P450A1 enzymatic induction, metallothioneins (proteins), DNA integrity, acetylcholinesterase activity, electron transport system activity, and lipid peroxidation [9-11]. The latter three biomarkers were selected for this study.

Lipid peroxidation (LPO) is an oxidative stress biomarker considered to have great potential for the assessment of environmental risks [12]. LPO is attributed to the presence of some pollutants, such as polycyclic aromatic hydrocarbons (PAHs), metals, polychlorobiphenyls (PCBs), and pharmaceuticals [13]. Among them, caffeine has been found [9] to attack the membrane lipids and initiate an autocatalytic chain reaction that leads to the oxidation of the polyunsaturated fatty acids that constitute the membrane [8, 14]. The degradation of the lipid membrane results in the formation of peroxides and aldehydes. The extent of this process is evaluated by the quantification of one of the byproducts of lipid degradation, namely malondialdehyde (MDA) [15]. Despite being a relatively renowned mechanism and an easier assay technique, other factors such as nutrition and environmental stress can be sources of oxidative stress, which can influence the obtained concentration of MDA. Therefore, the influence of external factors on the MDA concentration should be considered for the application of the biomarker [16].

Acetylcholinesterase (AChE) activity is a neuromuscular biomarker [12]. It is one of the critical enzymes involved in neural functions responsible for maintaining the transmission of nerve impulses by the hydrolysis of the acetylthiocholine neurotransmitter. The regular activity of acetylcholinesterase can be inhibited by the presence of contaminants, such as organophosphates, carbamates [8], pharmaceuticals [6], metals (Cd, Cu, and Pb) [17] or surfactants [18]. This inhibition increases the concentration of acetylthiocholine that causes overstimulation of the nervous system, which consequently leads to paralysis and death of the organism in question [19]. Additionally, there may be a variation in the activity of this enzyme due to environmental factors, such as *in situ* temperature and salinity. It was reported that this parameter is directly correlated to temperature and inversely proportional to salinity [20].

The electron transport system (ETS) activity is a physiological and morphological biomarker [12]. ETS is a complex enzyme system (found in the cell's mitochondrial and microsomal systems) responsible for controlling the utilization of oxygen [21]. The measurement of ETS activity provides information on the potential metabolic activity, which is the theoretically supported maximum respiration rate [21-23]. This method is an indirect approach for the estimation of respiration rates and physical conditions. However, ETS can also be used as an estimate for energy consumption, while the lipid, protein, and carbohydrate contents provide an estimation of the available energy reserves. The exposure to contaminants or other stress factors is documented to have a negative impact on metabolism, which conduces to the depletion of energy reserves and therefore affects the growth [23].

One of the common biological groups used as sentinels for monitoring the water quality and biomarker responses are molluscs. This is attributed to their wide geographic distribution, abundance, and accessibility in the field as well as aquaculture. Bivalves are the most representative ones within the group [24, 25]. The clams within the group are highlighted by their low mobility and filtration feeding habits that allow the determination of localized pollution sources in both water

and sediments. Furthermore, these molluscs have the potential for bioaccumulation due to their metabolic rates and capacity to filter up to 5 L/h of water. Therefore, they are used to detect the contaminants in the environment [2, 26]. Thus, the three biomarkers mentioned above (LPO, AChE and ETS) were monitored in the *Ruditapes decussatus* clam species.

Ruditapes decussatus is cultured in the Atlantic coast of France, Spain, Portugal and the Mediterranean basin [10, 27]. In Portugal, it is one of the most important commercial shellfish species of inshore waters, where around 90% of these clams are harvested in the south coast of Algarve, Ria Formosa coastal lagoon, with a production of 3,261 tons in 2017 [28]. The Ria Formosa lagoon (south coast of Portugal) is a highly productive shallow mesotidal system with high water renewal through six permanent connections to the ocean [29].

Since a majority of the studies on biomarkers were performed in the laboratory under artificially simulated exposure conditions [9, 30], the results may be affected by the unrealistic settings as it is not possible to mimic the real environmental surroundings. Therefore, it is important to perform these studies in the real environment [31, 32].

In order to evaluate the potential impact of the complex mixture of compounds derived from an urban wastewater treatment plant (UWWTP) within the Ria Formosa lagoon under environmental stress conditions in its vicinity, the clams were exposed to realistic conditions in an area influenced by the discharge of effluents. The profile of potential environmental contaminants is a long, expensive and laborious process, which may not reflect the harmful effects on biota. The evaluation of changes in the selected biomarkers at biochemical, cellular and physiological levels is an early and effective control tool for the assessment of the health of aquatic organisms and, ultimately, the ecosystem [33]. Therefore, the clams from the control site (group control of clam from a nursery) were exposed to the potential impact of effluent dispersal from the UWWTP (exposure group located 1.5 km away from UWWTP) during 1 month in summer. This period is characterized by an increase in the anthropogenic touristic pressure in Ria Formosa with a higher volume of effluents and a high demand of this species. Due to this approach, the response of clams to the environmental conditions was evaluated by the quantification of three biomarkers, namely lipid peroxidation (LPO), acetylcholinesterase (AChE) activity, and electron transport system (ETS) activity.

To complement the information of the biomarkers, the protein and lipid concentrations, condition index and morphometric measurements were also determined for the *Ruditapes decussatus* clams. Also, the *in situ* environmental characterization (temperature, salinity, pH and dissolved oxygen) was performed. The integration of the overall parameters aims to gather information relative to the potential contamination by UWWTP in order to evaluate the need to study the best treatment approach to decrease this problem.

2. Materials and Methods

2.1 Sampling and Exposure Conditions

Two kilograms of clam samples of *Ruditapes decussatus* with a "large" size (mean *ca.* 35 mm) were purchased from a local nursery away from direct anthropogenic impact, which was close to the main inlet in Ria Formosa. One kilogram of these clams was used as the control group (Cg). The other kilogram was used as the exposure group (Eg).

The control group was depurated for 24 h and frozen at –20 °C immediately after the purchase.

The exposure group (1 kg) was put in cages and exposed to a location ca. 1.5 km downstream from the Faro Northwest UWWTP to establish the potential impact of the UWWTP effluent discharges away from the zone of the direct impact of those effluents, as reported by Cravo et al. [34]. After one month, the clams from the exposure group were recovered, depurated for 24 h in the lab and frozen at –20 °C.

In the exposure site, the temperature, salinity, pH, and dissolved oxygen of water were measured at the beginning and end of the exposure periods using a multiparametric probe YSI 6820.

In the control site (clam nursery), these physicochemical parameters were not directly evaluated as there was no permission for water sampling and water monitorization.

However, the typical physicochemical values in the Ria Formosa lagoon close to the clam nursery were as follows: salinity $\geq$ 36, pH around 8 and dissolved oxygen close to saturation [35, 36].

2.2 Faro Northwest (NW) UWWTP

The Faro NW UWWTP located in the South of Portugal (Algarve) is a facility that receives influents from domestic and hospital sources. It has an installed capacity of 44530 population equivalents or 10 000 m^3 per day. The UWWTP was estimated to discharge 5000 m^3 per day into the sensitive Ria Formosa Lagoon region. The plant uses biological treatment with activated sludges and UV disinfection of the effluent in order to comply with the discharge requirements of Ria Formosa Lagoon.

2.3 Condition Index

The condition index of the clams was calculated per site, as described by Aafaf et al. [37] using equation 1:

$$CI\ (\%) = \frac{wet\ weight}{total\ weight} \times 100 \tag{1}$$

The total weight (g) was obtained by weighing the whole clam, and wet weight (g) was determined by weighing the soft tissues of the clam (edible part) separately from the shell.

2.4 Protein Concentration and Lipid Content

The total protein was determined using five replicates by the Kjeldahl and AOAC methods 928.08 [38]. The results were expressed in g protein/100 g as the wet weight (ww). For the calculation of AChE activity, the protein concentration was evaluated by the Bradford method [39] using bovine serum albumin (BSA) as standard. The protein concentration was expressed in mg/ml.

The lipid content of the clams was determined by the Soxhlet method (AOAC 960.39) using four replicates. The results were expressed as g/100 g ww [38].

2.5 Biochemical Analysis

The samples were homogenized using the Ika Ultra-Turrax Tube Drive Control. Due to different sample volume, two centrifuges were used, namely the Sigma 2K15 centrifuge and Hermle Z 100M

minicentrifuge. The Hitachi U-2000 spectrophotometer was used for the measurement of absorbance.

The following reagents with at least 95% purity were provided by different suppliers: acetylthiocholine iodide, 5,5'-dithiobis-(2-nitrobenzoic acid) and 2-p-iodo-phenyl 3-p-nitrophenyl 5-phenyl tetrazolium chloride (Acros Organics), tris-(hydroxymethyl)aminomethane, Pierce™ Coomassie (Bradford) Protein Assay Kit (Fisher Scientific), butylated hydroxytoluene (Sigma), 1-methyl-2-phenylindole (Alfa Aesar), polyvinylpyrrolidone and Triton X-100 (VWR), malondialdehyde bis(dimethyl acetal) (Merck), and nicotinamide adenine dinucleotide reduced (NADH) and NADPH) (AppliChem Panreac).

2.5.1 ETS

The ETS activity was determined using the method described by Simcic and Brancelj [22], except that the individually whole clams were considered rather than pools of specimens. Briefly, each soft tissue clam was homogenized in 4 ml of ice-cold homogenization buffer [0.1 M phosphate buffer, pH 8.4; 15% (w/v) polyvinylpyrrolidone; 153 µM MgSO4; 0.2% (v/v) Triton X-100] for 2 min at 1000 rpm. This procedure was followed by 20 s of sonication and centrifugation at 10000 rpm/4 min under 0 °C.

The absorbance of the samples was measured as follows: About 0.05 ml of the supernatant, 1.5 ml of substrate solution [0.1 M phosphate buffer, pH 8.4; 1.7 mM NADH; 0.25 mM NADPH; 0.2% (V/V) Triton X-100] and 0.5 ml of the coloring solution [2-p-iodo-phenyl 3-p-nitrophenyl 5-phenyl tetrazolium chloride, 8 mM] were taken for the analysis. The mixture was incubated at 20 °C for 40 min, and 0.5 ml of the stopping solution [formalin(conc.):H_3PO_4(conc.) = 1:1] was added. The absorbance was then recorded at 490 nm against the blank sample. The samples were analyzed thrice.

The activity was calculated using equation 2 [40]:

$$ETS \left(nmol \frac{O_2}{\min g\, ww}\right) = \frac{Abs \times V_H \times \frac{1}{t} \times V_A}{V_{inc} \times ww \times 31.8} \times 10^3 \qquad (2)$$

where Abs is the corrected absorbance of the samples, V_H is the total homogenate volume (ml), t is the incubation time (min), VA is the total assay volume (ml), V_{inc} is the volume of the incubated homogenate, and ww stands for the sample wet weight (g) (Silva et al., 2020). The constant 31.8 is a conversion factor that allows the determination of the equivalent oxygen utilization, assuming that 2 µmol of INT-formazan are equivalent to 1 µmol of oxygen [41].

2.5.2 AChE

The AChE activity was determined by an adapted method on the basis of the procedures described by Ellman et al. [42] and Porte et al. [43]. The hydrolyzation of acetylthiocholine led to the formation of thiocholine that, in turn, reacted with 5,5-dithiobis-2-nitrobenzoate ion (DTNB), forming a yellow-colored anion 5-thio-2-nitrobenzoic acid. The principle of this method is based on the monitoring of the formation rate of this anion [42].

The whole soft tissues of the clams were individually homogenized in a mixture of Tris-HCl (100 mM, pH 8) buffer at a ratio of 1:5 [sample (g):buffer (ml)] and 10% Triton X-100 at a ratio of 1:10

[buffer (ml):Triton (μl)]. The homogenization was performed for 2 min at 1000 rpm. The samples were then centrifuged for 30 min at 15000 rpm under 4 °C. The supernatant was divided in half, where one of the aliquots stored at –60 °C to later be used to determine the protein content of the samples, and the other was used to determine the AChE activity immediately after homogenization.

For the determination of activity, the absorbance of the three blanks and two duplicate were registered for each sample at 405 nm against a blank sample of Tris-HCl buffer. In order to determine the activity of the blank sample, 1.6 ml of the buffer and 200 μL of DTNB (1 mM) were stabilized at room temperature for 5 min. This was followed by the addition of 200 μL of AChI (10 mM), and the absorbances were measured at 405 nm for 60 s. A similar procedure was used to determine the activity of the samples, except that 1.5 ml of the buffer, 200 μL of DTNB and 100 μL of the sample were incubated.

The initial (0 s) and final (60 s) absorbances of both the samples and blanks were registered, and ΔA (min^{-1}) was calculated.

The AChE activity was calculated using equation 3 [40]:

$$AChE \left(\frac{nmol}{min} mg\ prot\right) = \frac{\Delta A}{\varepsilon} \times \frac{V_C}{V_S} \times \frac{10^3}{[P]} \times 10^6 \tag{3}$$

where, ΔA (min^{-1}) is the difference between the initial and final absorbances, ε is the extinction coefficient (1.36×10^4 ml/nmol), V_C is the cuvette volume (ml), V_S is the sample volume (μl), and $[P]$ is the total protein concentration (mg/ml).

2.5.3 LPO

The methods described by Gerard-Monnier et al. [44] and OxisResearchTM [45] for LPO determination were modified in this study.

For the LPO method, the whole soft tissues of the clams were individually homogenized in a mixture of tris-HCl (0.02 M) buffer at a ratio of 3:1 [buffer (ml): sample (g)] (with a minimum of 5 ml per sample) and BHT (0.5 M) at a ratio of 1:10 [buffer (mL): BHT (μl)]. The homogenization of the sample was performed at 1000 rpm for 2 min. The samples were then centrifuged for 45 min at 15000 rpm under 4 °C. Unlike the two previous biomarkers, the absorbance of the samples was determined, given that the supernatants were stored stably at –80 °C.

A calibration curve with the following concentrations was prepared and read once before any samples: eight MDA levels between 0 μM (blank) and 20 μM was prepared by the dilution of several volumes (0, 50, 125, 250, 500, 1000, 1500 and 2000 μL) of a standard solution of MDA at 20 μM mg/L to 2 ml using Tris-NaCl.

After this procedure, 300 μL of each MDA standard was pipetted and transferred to an Eppendorf tube (in triplicate) followed by the addition of 975 μl of diluted R1 (18 ml of stock N-methyl-2-phenylindole solution prepared in acetonitrile and 6 mL of methanol) and 225 μl of methanesulfonic acid (15.4 M), which were mixed thoroughly. The Eppendorf tubes were incubated under 45 °C for 60 min. Thereafter, the absorbance values were recorded in 2 ml of cuvettes at 586 nm against a Tris-NaCl (pH 8.6) blank sample, and a calibration curve of the absorbance versus concentration was plotted.

The analysis of the experimental samples was performed using the same procedure with the exception that 300 μl of previously prepared supernatant was pipetted to the Eppendorf tubes (in

duplicate). After the incubation period, the samples were centrifuged at 6000 rpm for 1 min due to the formation of precipitates.

The surrogate for LPO determination, namely MDA concentration, was calculated using equation 4:

$$MDA \left(nmol \frac{MDA}{g} \, protein \right) = \frac{\frac{Abs - b}{a} \times V_T}{ww \times [P]} \times 10^3 \tag{4}$$

where, Abs is the recorded absorbance of the samples, the terms a and b are obtained from the linear equation corresponding to the calibration curve (y = ax + b); V_T is the volume of Tris-HCl used to homogenize the sample (ml), ww is the wet weight of the sample (g), and [P] is the protein concentration (mg/g).

2.6 Statistical Analysis

All the presented results are expressed as mean value ±standard deviation (SD).

The results obtained from the clam samples (control and exposure groups) and biomarkers were analyzed to detect the possible statistical differences using the Kruskal-Wallis test and one-way ANOVA (analysis of variance). Both the tests were evaluated to show a significance level of 5% (α = 0.05).

The principal component analysis (PCA), an unsupervised multivariate analytical method, was built using MetaboAnalyst 4.0 [46-49] to discriminate the main variables responsible for the variance of biomarkers, morphometric and nutritional factors. The variables considered for the analysis were ETS, AChE and LPO biomarkers, proteins, lipids, and condition index (CI). All the variables were presented as their mean values.

3. Results and Discussion

3.1 Environmental Characterization Context

The *in situ* water characteristics were measured at the beginning and end of the exposure experiments, as shown in Table 1.

Table 1 Temperature, salinity, pH, and dissolved oxygen (%) at the exposure site in the beginning and at the end of the exposure period of the clams.

T (°C)		Salinity		pH		Dissolved Oxygen mg/L; % saturation)	
June	July	June	July	June	July	June	July
22	28	36.8	37.1	7.7	8.1	5.6; 79	8.3; 140

All the parameters taken for the study showed higher values at the end of the exposure period. Globally, the water temperature followed the typical seasonal pattern of atmospheric temperature, with higher values in July that mostly exceeded 25 °C.

The water salinity of both the samplings showed the typical values corresponding to that of marine waters. Therefore, the exposure site showed no direct influence from the effluents/freshwater (S > 36) [50].

The pH values were in the range of 7.7 to 8.1. This was also similar to the pH values found in marine waters [51].

The percentage of dissolved oxygen varied with months. The hour of the sampling showed a significant impact on the resultant values. The minimum value (79%) was achieved at the exposure site in the early morning of June 2016, while the maximum (140%) was recorded in the period from noon to 2 pm.

3.2 Morphometric Characteristics, Condition Index and Concentration of Proteins and Lipids

Table 2 shows the morphometric characteristics of the clams. Significant correlations were observed between the length and total mass of the clams: r = 0.89 (p < 0.01) for the control group and r = 0.88 (p < 0.01) for exposure group.

The CI was lower in the clams of the exposure group. This index reflects the physiological status of the organisms. It showed that the exposed bivalves might be under more stress after a month than those observed in the control site, where they live in natural environmental conditions, as reported by other authors [12]. The clams from the exposure group showed a lower value of CI, edible mass and percentage of lipids (Table 2) compared to those from the control group, which indicated a decrease in body weight and lipid content in the former after a month. This may suggest the emission of gametes, as reflected by the loss of lipid content. The variation of CI may be associated with fluctuations in the reproductive cycle of the clams. Generally, the spawning of the species occurs during the summer [52]. Although the values of CI, edible mass and lipids were lower in the clams from the exposure group, the differences were not significant (p > 0.05).

3.3 Biomarker Response

The use of several biomarkers is the best approach to achieve an integrated overview of the biological responses to the mixtures of contaminants even under low concentrations [53].

There is an allometric dependency of the rate of energy metabolism on the body weight of the bivalves [54]. This change in energy metabolism during the growth of the animals is strongly influenced by the habitat conditions, mainly environmental factors such as temperature, salinity and dissolved oxygen, as well as bioavailability of the contaminants [12, 30, 54].

Table 2 Summary of the length, width, thickness, total mass, shell mass, edible mass, condition index (n = 30), and percentage of total lipids and proteins (n = 5) in *Ruditapes decussatus* from the control and exposure groups.

Clams' parameters	Control group	Exposure group
Total mass (g)	6.7 ±2.33	6.8 ±1.78
Edible mass (g)	2.7 ±0.93	2.4 ±0.63
Shell mass (g)	3.9 ±1.38	4.2 ±1.11
CI (%)	39.3 ±2.25	34.5 ±3.43
Length (mm)	33.0 ±3.27	33.7 ±3.05

Width (mm)	23.7 ±2.52	24.3 ±2.28
Thickness (mm)	13.1 ±5.57	15.6 ±1.55
Total lipids (%)	3.07 ±2.15	1.06 ±0.07
Total protein (%)	9.61 ±0.008	10.09 ±0.002

The impact of these conditions (environmental and/or chemical stresses) upon bivalves can be evaluated by the quantification of the target biomarkers, such as electron transport system (ETS), acetylcholinesterase (AChE) and lipid peroxidation (LPO) as determined in the present study.

Table 3 shows the profile of the three selected biomarkers (ETS, AChE and LPO) in Ruditapes decussatus clams exposed to potential contamination from the effluent of the UWWTP in Ria Formosa located at a distance of 1.5 km away from the exposure site. The results were obtained after one month of exposure.

Among the three biomarkers, there were significant statistical differences ($p < 0.05$) in the LPO results obtained from the two sites (control and exposure sites). The spatial statistical differences might suggest that the site of exposure does produce a noticeable variation corresponding to the biomarker response, where a particular type of stress is responsible for the spatial fluctuation. This may be attributed to the fluctuations in the environmental factors (such as water temperature, salinity, dissolved oxygen) or the presence of some target contaminants [12, 55, 56].

Table 3 Electron transport system (ETS), acetylcholinesterase (AChE) and lipid peroxidation (LPO) contents in the *Ruditapes decussatus* clams from the control and exposure group. The statistical tests used were ANOVA and Kruskal-Wallis (n = 30).

Biomarker	Control group	Exposure group
ETS (nmol O_2/min g ww)	27.2 ±0.73	30.2 ±1.80
AChE (nmol/min mg prot	4.0 ±0.47	4.6 ±0.96
LPO (nmol MDA/g prot)	248.9 ±14.78 [a,b]	101.5 ±15.15 [a,b]

[a] significant statistical for the same biomarker by the ANOVA test
[b] significant statistical for the same biomarker by the Kruskal-Wallis test

Significant statistical differences between the control and exposure groups ($p < 0.05$) were only observed in the LPO results evaluated by the ANOVA and Kruskal-Wallis tests relative to the AChE and ETS analysis (mean: 4.0 and 4.6 nmol/min mg protein and 27.2 and 30.2 nmol O_2/min g ww, respectively; $p > 0.05$).

The ETS assay is a useful metabolic indicator illustrating an allometric relationship of a typical metabolic function with body size. However, it is not a simple conservative measure of body mass. The variation in the dry weight and organic N are also important factors. Nevertheless, the mass of organic N is a better parameter for scaling the ETS activity, as it is closely related to organic matter and hence, to metabolically active tissues than the total dry weight [57]. Other studies also demonstrated that oxygen consumption is closely dependent on the size of the organism [58].

The ETS activity is closely related to the salinity of the bivalves. The specimens from the sites with higher salinity showed lower ETS activity. This correlation observed for mussels may also be applicable to clams [56]. The combined effect of increased salinity may be responsible for the lower and similar activity values recorded for both groups ($p > 0.05$). This might provide evidence for the

significance of environmental factors as sources of stress that influence the clams, as explained by the relevant changes in the water temperature and dissolved oxygen.

Other studies conducted on bivalves confirmed the positive correlation between ETS activity and growth in clams [59].

The increased metabolic activity is expressed by the ETS levels, representing an attempt to fight against the oxidative stress upon the expenditure of their energy storage to prevent LPO for metabolic defenses involved in the detoxification processes [9, 60]. Although clams were able to increase their metabolic activity, the defense mechanisms might not be sufficiently efficient to fight against LPO [9] as it will be shown afterwards.

The AChE activity (Table 3) presents some variations between the groups. The clams in the control group were presumably from a site with low contamination, and those in the exposure group were far from the zone of influence of the UWWTP ($\approx$ 1.5 km).

From Table 1, it was observed that the amount of dissolved oxygen can cause stress due to the saturation of 140% attained during the day, suggesting a decrease in the values recorded at night. The variation of *in situ* temperature, salinity, and dissolved oxygen caused fluctuations in the AChE activity. The AChE activity correlated negatively with the salinity and dissolved oxygen in different bivalve species [20, 61], while it was directly proportional to the temperature [20, 61, 62]. These results noticeably demonstrated the need to consider the potential influence of abiotic factors such as temperature and salinity on AChE activity upon the application of this biomarker to monitor the exposure in places with salinity gradients. The water temperatures of the studied exposure sites (Table 1) were similar, ranging from 25 °C to 27 °C between June and July. The highest variation in salinity was observed between the first day of exposure and the time of recovery of the clams. The AChE activity values (Table 3) were integrative and representative measurements recorded upon one month of exposure against the water characteristics (Table 1) that represented only a snapshot of the environmental conditions, which was highly variable in time and space.

Furthermore, the obtained AChE activity levels in the same species from a study conducted in the Ria Formosa during 2007 and 2008 were compared [63]. The studies showed similar yet higher values. This suggested a lower impact on the activity of the present samples relative to that reported in 2007–2008. For example, the highest activity registered during the same months (June and July) was 2.6 nmol/min mg protein which was lower than that obtained in the present study upon consideration of the two groups. The saturation of dissolved oxygen, pH and accumulation of organic matter on the sediments could also influence the biomarker and alter its response to some extent. This discrepancy may be responsible for the lower activity levels reported during the study conducted in 2007–2008.

There were significant differences in the LPO values between the control and exposure groups. The LPO values were surprisingly higher in clams from the control group (248.9 nmol MDA/g protein) than those from the exposure group (101.5 nmol MDA/g protein).

The higher LPO concentration registered in the control group may likely be attributed to some form of stress other than the contaminants, as stated for AChE activity, such as environmental conditions (ex: nutritional factors or dissolved oxygen) or biotic sensitivity before the spawning process that might negatively influence the MDA concentrations. This phenomenon was reported in a previous study considering the same species of clams from Ria Formosa [63]. This reinforces the statement by Vidal et al. [64] on the consideration of the effects of abiotic factors for the

environmental studies using biomarkers. However, the nutritional factors were irrelevant for these specimens, as indicated by the LPO data in Table 3.

The comparison of the LPO data obtained in this study with those conducted in 2007–2008 [63], showed an increase in the MDA concentrations for the former. For example, the highest MDA concentration was registered in July 2008 (204.3 nmol MDA/g protein), which was equivalent to that obtained for clams in the control group before the spawning period.

Thus, a very important aspect of the multi-biomarker approach is the detailed knowledge of the basal biomarker response and its seasonal variation to distinguish the pollution-induced effects from those induced by the natural biological cycle of the clams, including the reproductive cycle and spawning period [65, 66].

There are limited studies on the multi-biomarker univariate approach without an integrated overview that allows the global assessment of the influence of contaminants and environmental conditions at each site of exposure, allowing the overview of biomarker responses and corresponding processes. Therefore, a principal component analysis (PCA) model was built to integrate the data (Figure 1). To perform the PCA analysis, the biomarkers, morphometric characteristics and nutritional parameters were considered. Since there were only two studied sites (control vs. exposure groups), only one principal component (PC1) was calculated, explaining 100% of the variance of the data, enabling the spatial separation of the variables (biomarkers, morphological and nutritional parameters) by their relevance on the separation between the two exposure sites. The PC1 showed a separation between the exposed (positive PC1) and control sites (negative PC1), as shown in Figure 1A.

From the corresponding loadings 1 plot (Figure 1B), the variable LPO was identified as the most relevant factor for the separation of the two sites, which was elevated in the control group. The LPO variable was relevant to the observed separation (Figure 1B). In fact, the PCA model highlights the association of higher amounts of LPO with high condition index (CI) and lipids among the clams from the control site. In contrast, the clams from the exposure site showed slightly higher protein content, AChE activity and ETS biomarker activity (Figure 1B).
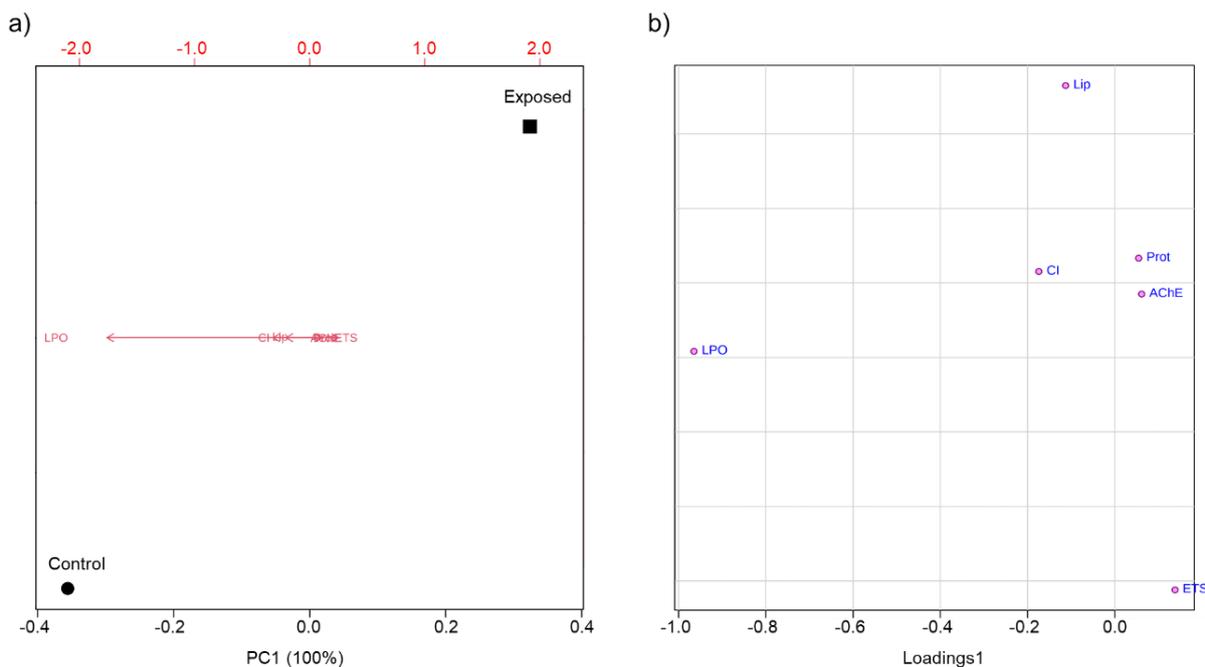
Figure 1 Principal component analysis (PCA) of the nutritional and biochemical parameters used to evaluate the condition of the *Ruditapes decussatus* clam. The considered parameters were the electron transport system (ETS), lipid peroxidation (LPO), and acetylcholinesterase (AChE), morphometric characteristics such as condition index (CI), nutritional factors such as lipids (Lip) and proteins content (Prot). (A) bi-plot with the scores (separation between the groups) and loadings1 (in red, projection of the influence of each variable on the separation) representation; (B) loadings1 plot.

From this data, the clams from the control site were mostly influenced by the high levels of the damage biomarker LPO. This confirms the difficulty of establishing the baseline levels of the biomarkers in less contaminated regions due to the range of natural variability. However, the maximum LPO recorded for the clams in the control group can be attributed to their higher sensitivity before the spawning period than those observed after one month of exposure. Consequently, the alterations in the environmental (Table 1) or physiological parameters (Table 2) can have a relevant effect on the biomarker responses, as reported by other authors [62, 67]. The variability of the biomarkers may also depend on the variation of environmental parameters (e.g., high seawater temperature, changes in salinity, intense solar irradiance, oxygen decrease, etc.) that could induce higher physiological stress, as already observed in other bivalve species [62, 65, 66]. This data confirmed that the temporal and spatial clams at those locations in the Ria Formosa lagoon were differently impacted by the contaminants, environmental and/or physiological factors, which distinctively influenced the biomarker responses.

In summary, the PCA of the overall data (Figure 1) revealed that the biomarker responses changed spatially with the variation in the environmental conditions. During the summer season, high water temperatures exceeding 25 °C (high solar radiance) and the potential increase in organic matter within the sediment may lead to a decrease in the oxygen level, pH of the water and interstitial water in the sediments particularly during the night that may also affect the biomarker responses and undermine the health of the clams.

4. Conclusions

Different biomarker responses were observed during this study. The influence of environmental factors or other stressors were studied. The LPO is a damage biomarker, which appears as a secondary factor for the interpretation of the data. The LPO value was maximum in clams from the control group due to their high sensitivity toward the detoxification of compounds (organic and inorganic) or environmental stress before the spawning period. Moreover, the loss of body mass and increased environmental pressure during summer may confound the response of the biomarker. This study can be considered as an adequate starting point toward the study. In order to draw definitive conclusions, a more target-specific approach regarding the site characterization, such as an analysis of the prevalent contaminants responsible for the lipid peroxidation results coupled with a broader battery of the biomarker analysis, may be required.

Considering the complex mixtures of the present contaminants, the biomarker responses provided valuable information for environmental assessment. Nevertheless, it is important to identify the interactions (synergistic, addition or antagonistic effects), including the environmental and biological factors induced by the natural biological cycle of clams, including the reproductive cycle and spawning period that might complicate the general interpretation of the biomarker response. Abiotic factors can confound the biomarker response and find significance in a multi-biomarker approach.

Author contributions

Conceptualization, A.C. and C.M.M.A; Methodology, A.C., C.M.M.A. and S.S.; Sampling, A.C. and C.C; Formal Analysis, S.S.; Writing-Original Draft Preparation, S.S.; Writing-Review & Editing, C.M.M.A, J.R. and A.C.; Statistical Analysis, J.R.

Competing Interests

Authors declare no conflict of interest.

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