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Environmental Health Assessment of the Northwest Portuguese Coast—Biochemical Biomarker Responses in the Marine Gastropod *Phorcus lineatus*

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Abstract: Coastal areas are frequently impacted by anthropogenic pollution, due to intense human activity in these zones. Our study aimed to monitor the impacts of anthropogenic pollution in four Portuguese locations on the northwest coast, and to identify the most affected areas and/or seasons by applying a multi-biomarker approach. Water and specimens of *Phorcus lineatus* were collected on the rocky shore during low tide in four sites along the northwest Portuguese coast (1. Amorosa; 2. Cabo do Mundo; 3. Homem do Leme; 4. S. Félix da Marinha) with different anthropogenic pressures, including an industrial maritime shipyard; an oil refinery; an international airport; and an area with high human population density. The collection took place over two seasons: the summer of 2021 and the winter of 2022. Several biochemical biomarkers, including reactive oxygen species; protein carbonyl content; lipid peroxidation (LPO); carboxylesterase (CE); and antioxidant (superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and neurotoxicity—acetylcholinesterase (AChE)) enzymes were measured. The results showed seasonal variation, with the ROS, LPO, CE, and GST activities depending particularly on the season, but the SOD and CAT activities being similar between summer and winter. CAT showed lower activity in Site 1 than in the other sites during both seasons ($p < 0.05$). The Integrated Biomarker Response (IBR) index showed that biomarker responses were higher in winter. The multivariate analysis confirmed the higher contribution of the factor season to the *P. lineatus*' response to pollutants, compared to the spatial variation in the northwest Portuguese coast. Overall, this study shows that *P. lineatus* can be a suitable bioindicator species for environmental biomonitoring, and that the IBR index allows the identification of temporal contamination patterns.

Keywords: marine pollution; gastropod; oxidative stress; biomarkers

Citation: Santos, D.; Varandas, S.; Carrola, J.S.; Saavedra, M.J.; Luzio, A.; Monteiro, S.M.; Cabecinha, E. Environmental Health Assessment of the Northwest Portuguese Coast—Biochemical Biomarker Responses in the Marine Gastropod *Phorcus lineatus*. *Water* **2024**, *16*, 5. https://doi.org/10.3390/w16010005

Academic Editor: José Luis Sánchez-Lizaso

Received: 8 November 2023
Revised: 11 December 2023
Accepted: 13 December 2023
Published: 19 December 2023

1. Introduction

Coastal areas, particularly near densely populated cities, are frequently subjected to anthropogenic pollution from urban, industrial, and agricultural activities. Among such contamination, heavy metals are priority pollutants that have gained special attention due to their persistence, bioaccumulative potential, and toxicity at low levels of exposure [1,2]. The marine environment—particularly coastal areas—receives a large amount of heavy metal from agricultural and industrial activities, sewage treatment discharges, urban stormwater runoff, and antifouling paints used in marine structures [2,3]. In turn,

it has been reported that heavy metals may cause distinct toxic effects such as embryotoxicity, oxidative stress, increased apoptosis, and/or histological alterations in fish [4,5], bivalves [6–8], polychaetes [9], and algae [10]. In addition, due to their bioaccumulative and biomagnification potential, heavy metals can enter the food web and be transferred to higher trophic levels, which represents a critical environmental issue with implications for the ecological sustainability and economic management of aquatic ecosystems, including wildlife and human health [1].

According to a report by the European Environment Agency (EEA), about 75–96% of European seas are still contaminated with heavy metals [11]. For instance, in the Vigo estuary—the most important in Galicia (Spain)—it was reported that zinc (Zn) concentrations in the seawater surface are between 0.2 and 10.3 µg/L [12]. In several sites on the northwest coast of Portugal, concentrations of cadmium (Cd), chromium (Cr), copper (Cu), and Zn in seawater ranged from 1.2–35 ng/L, 15–87 ng/L, 126–1819 ng/L, and 2889–16,867 ng/L, respectively [13]. More recently, a spring campaign in the Douro River estuary (Portugal) assessed the content and distribution of several trace elements (e.g., Cr, Cu, Zn, Cd) in three matrices (water, sediments, and native flora) collected at five sampling points [14]. In both the water and sediment samples, the mean concentrations of some trace elements, particularly Cu and lead (Pb), exceeded the levels considered safe for aquatic organisms. In this regard, in compliance with the Water Framework Directive (2000/60/EC) and the Marine Strategy Framework Directive 2008/56/EC, it is crucial to regularly assess the pollution levels and potential ecological risks of heavy metals in coastal ecosystems, in order to achieve a healthy and productive marine environmental status [15,16]. In this context, for a comprehensive understanding of the impact of marine pollution, it is critical to measure abiotic factors and chemical parameters (e.g., pollutant concentration), but also to determine their adverse biological effects on the biota through biomarkers [17,18]. Linking these physical, chemical, and biological parameters provides valuable data for an effective and integrated risk assessment of the anthropogenic impact on coastal areas.

Over the last decades, gastropods have been exploited as seafood but also used as suitable bioindicators of heavy metal pollution, since these organisms can accumulate metals from all environmental compartments (i.e., water, sediment, food, and inorganic particulate material) [19]. *Phorcus lineatus* (also known as *Monodonta lineatus*; da Costa, 1778) is a herbivorous marine gastropod broadly distributed along the Eastern Atlantic and is usually found from the upper to the mid-eulittoral zone [20]. It is a species tolerant of environmental alterations; has reduced mobility; is abundant and easy to sample; and is available all year long [20]. *P. lineatus* becomes sexually mature around the second year of life, with the breeding cycle occurring between June and September [21]. As for bivalves, the genera *Phorcus* or *Monodonta* have been reported to be strong accumulators of Cd, Cu, and Zn, even in poorly contaminated sites [19], thus being valuable bioindicators in monitoring metal contamination. In this conceptual framework, the present study aimed to (1) assess the general health status and contamination status of *P. lineatus* collected along four sites on the Northwest Atlantic coast of Portugal by applying an enzymatic multi-biomarker approach; (2) monitor the impacts of heavy metal pollution on these sites; (3) identify possible seasonal behavior of metals along the Northwest Atlantic coast of Portugal.

2. Materials and Methods

2.1. Study Area and Sampling

Sampling was performed at four sites along the northwest Portuguese coast (S1—Amorosa, 41°39′34.3″ N 8°49′29.8″ W; S2—Cabo do Mundo, 41°13′15.8″ N 8°42′58.0″ W; S3—Homem do Leme, 41°09′31.8″ N 8°41′10.0″ W; S4—S. Félix da Marinha, 41°02′8.91″ N 8°38′55.1″ W) (Figure 1) during two seasons: summer of 2021 (August) and winter of 2021/2022 (February). The selected sites comprise areas with different anthropogenic pressures, known for their proximity to potential sources of pollution. Amorosa is a coastal area located in the North of Portugal (Viana do Castelo), near the Lima River and adjacent to the Viana do Castelo Sea Port, which comprises a maritime shipyard industry with a high population density. Cabo do

Mundo is located in the city of Porto (the second largest city of Portugal), in the vicinity of important industrial settlements, such as an oil refinery and an international airport. Homem do Leme, also located in Porto, is close to the mouth of the Douro River and to an industrial and mercantile harbor with intensive traffic of vessels (Leixões harbor). S. Félix da Marinha, located south of Vila Nova de Gaia, is affected by urban and industrial settlements.

Figure 1. Map showing the sampling locations of the study area on the northwest Portuguese coast. S1—Amorosa (41°39′34.3″ N 8°49′29.8″ W); S2—Cabo do Mundo (41°13′15.8″ N 8°42′58.0″ W); S3—Homem do Leme (41°09′31.8″ N 8°41′10.0″ W); S4—S. Félix da Marinha (41°02′8.91″ N 8°38′55.1″ W).

Thirty adult specimens of *P. lineatus* were randomly collected at each site by handpicking at low tide in the intertidal zone, and transported alive, in refrigerated containers filled with local seawater, to the laboratory for further processing. At each site, three replicates of surface water samples were collected in glass bottles, transported to the laboratory at a low temperature in thermoboxes, and stored at 4 °C until analysis. The physical and chemical parameters—namely pH, temperature, and dissolved oxygen (D.O.)—were also measured at each site using multiparameter probes (HQ40d Multi HACH; YSI Ecosense EC300).

The sampling of specimens was authorized by the Instituto da Conservação da Natureza e das Florestas—the Portuguese Institute for Nature Conservation and Forests (ICNF).

2.2. Tissue Preparation for Biochemical Assays

The size (cm) and total weight (g) of each individual was measured, and the soft tissue was excised from the shell and weighed (g). The condition factor (CF) of each animal was estimated according to the formula CF = total weight/size^3 [22]. For each sample, the whole organism was then homogenized for 90 s at 30 Hz using a TissueLyser II (Qiagen, Venlo, The Netherlands), in an ice-cold buffer (pH 7.4, sucrose 0.32 mM, HEPES 20 mM, $MgCl_2$ 1 mM, and phenylmethylsulphonyl fluoride 0.5 mM). Subsequently, each sample was centrifuged (15,000× *g*, 20 min, 4 °C, Sigma 3K30, Osterode, Germany), and the homogenate supernatant was collected and frozen at −20 °C until the biochemical biomarker assessments.

2.3. Biochemical Biomarker Measurements

The enzymatic activity of the samples was measured using a PowerWave XS2 microplate scanning spectrophotometer (Bio-Tek Instruments, USA) or a Varian Cary Eclipse (Varian, Palo Alto, CA, USA) spectrofluorometer. All the determinations were performed in duplicate. Protein concentration in each sample was determined at 280 nm using the Take3 Multi-Volume plate (Take3 plate, BioTek Instruments, Vermont, WI, USA) and used to normalize enzyme activities.

Total reactive oxygen species (ROS) were determined following the methodology described by [23], using the fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA), at 485 nm (excitation) and 530 nm (emission) wavelengths. ROS concentration was estimated based on a DCF standard curve and expressed as µmol DCF/mg protein.

Superoxide dismutase (SOD) activity was measured through the inhibition of the nitroblue tetrazolium (NBT) reduction, at 560 nm [24]. Catalase (CAT) activity was determined using a solution containing 100 mM sodium buffer and 20 mM hydrogen peroxide (H_2O_2), by measuring H_2O_2 consumption at 240 nm [25]. SOD and CAT were quantified with standard curves (0–60 U/mL) and expressed as U/mg protein. For glutathione S-transferase (GST) activity determination, the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (GSH), in a 100 mM phosphate buffer, was measured at 340 nm [26] and expressed as µmol/min.mg protein. Carboxylesterase (CE) activity was measured by monitoring the reaction product of 4-nitrophenol at 405 nm [27], and expressed as µmol 4-nitrophenol/min.mg protein.

The content of malondialdehyde (MDA), an indicator of lipid peroxidation (LPO), was determined through the quantification of the MDA–thiobarbituric acid (TBA) adducts at 530 nm, with a correction for non-specific adducts at 600 nm [28], and expressed as µmol MDA/mg protein. The protein carbonyl content (PCO) was measured as dinitrophenylhydrazine (DNPH) derivatized protein at 450 nm [29] and expressed as nmol DNPH/mg protein.

Acetylcholinesterase (AChE) activity was determined by measuring the conjugation of thiocholine with 5,5-dithio-bis-(2-nitrobenzoic acid) (DTNB) at 405 nm [30], and expressed as µmol TNB/min.mg protein.

2.4. Water Collection and Analysis

In the water samples collected at each site, the levels of nitrite (NO_2^-), nitrate (NO_3^-), fluorides (F^-), chlorides (Cl^-), phosphates ($P_2O_5^-$), sulfates (SO_4^{2-}), ammonium (NH_4), calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), alkalinity (HCO_3^-), biochemical oxygen demand (BOD), chemical oxygen deficiency (COD), hardness ($CaCO_3$), and total suspended solids (TSS) (expressed in mg/L) were determined. For the determination of metal levels (expressed in µg/L), water samples were acidified with 65% nitric acid (HNO_3, Merck, Darmstadt, Germany), and the concentrations of arsenic (As), cadmium (Cd), lead (Pb), copper (Cu), manganese (Mn), zinc (Zn), nickel (Ni), chromium (Cr), and iron (Fe) were analyzed using atomic absorption spectrophotometry and electrochemistry (UNICAM 939 AA Spectrometer Furnace UNICAM GF90, Cambridge, UK). All samples were analyzed in duplicate. Each run of samples was calibrated using aqueous mixed standards prepared with HNO_3. The analytical methods used to determine the physicochemical parameters are described in Supplementary File S1.

2.5. Integrated Biomarker Response (IBR)

To integrate the effect of all biomarkers and establish a straightforward interpretation of the contamination level at each sampling site, the IBR index was calculated according to the method described by [31] and later adapted by [32]. For the calculation procedure, data were normalized ($Y = (X - \mu)/s$, where X is the mean value of the biomarker at a given sampling site, and µ and *s* are the general mean and the standard deviation, respectively, of all data for a given biomarker). Subsequently, a score for each biomarker ($S = Y + |Min|$,

where S $\geq$ 0 and |Min| is the absolute minimum value for all calculated Y) was obtained to calculate the IBR index:

$$\text{IBR} = \sum_{i=1}^{n} A_i, \text{ where } A_i = S_i \times S_{i+1} \times sin\frac{\frac{2\pi}{n}}{2}.$$

S_i and S_{i+1} represent two consecutive clockwise biomarker scores; A_i is the area that connects two scores; and n is the number of biomarkers. Biomarkers were arranged clockwise according to their biological organization, as follows: ROS, SOD, CAT, GST, CE, LPO, PCO, and AChE.

2.6. Statistical Analysis

ANOVA statistical analysis was performed using Prism 9.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data were checked for assumptions of homogeneity of variance and normality with the Brown–Forsythe and Kolmogorov–Smirnov tests, respectively. Then, to compare sites and seasons, a two-way ANOVA followed by Tukey's multiple comparison post-hoc test was performed for each biomarker. For the data that did not fulfill a normal distribution, the non-parametric Kruskal–Wallis test on ranks, followed by the post-hoc Dunn's multi-comparison test, was used. Statistical differences were considered significant when $p < 0.05$. The results are expressed as means and standard deviations (means $\pm$ SD).

To explore spatial and/or temporal patterns of biological data (enzymatic activities and biometric variables) between sites, a non-metric Multidimensional Scaling (nMDS) analysis—an ordination method based on a rank order of Bray–Curtis similarities—was used. The nMDS was computed from transformed (square root) biological data using the package PRIMER 7 [33,34]. nMDS is a very flexible technique for analyzing many different types of data, especially highly dimensional data that exhibit strong deviations from assumptions of normality. Contrasting with some other ordination techniques, the nMDS method fits data to several axes that are determined a priori to the analysis, and does not contain hidden axes of variation.

In order to understand the effects of a pollution gradient and the time—namely the season—on the enzymatic activities and biometric variables of *P. lineatus*, a constrained ordination was applied to the studied variables. Response data are not compositional, so a linear method was used. A redundancy analysis (RDA) was applied to extract and summarize the variation in the response variables that can be explained by the explanatory variables. These effects were tested for the different sites and seasons. A partial RDA (pRDA) was applied in order to clarify the variations in biometric variables and enzymatic activities of the gastropod, which were explained by the two groups of co-variables, testing the simple effects of (1) time (season) and (2) space (sites). Additionally, to identify which environmental variables better explained the variance in the enzymatic activities and biometric variables analyzed, other pRDA tested the simple effects of (1) physicochemical parameters (PC) and (2) specific pollutants (SP).

Variables were standardized before all analyses to preserve the original scale. The significance of the variables was tested (both individually and altogether) with 999 Monte Carlo permutations. RDA and pRDA were carried out using CANOCO 5 (version 5.14, Biometrics, Wageningen, The Netherlands).

3. Results and Discussion

3.1. Physicochemical Parameters

The physicochemical parameters measured at all sites, during both seasons, are presented in Table 1.

The comparison of the physicochemical parameters between the seasons showed that the major concentrations of NO_3^-, Cl^-, $P_2O_5^-$, NH_4, and K, and of the metals Cd, Cr, Cu, Mn, Ni, and Zn occurred in summer, particularly in Site 3; while during the cold season, the parameters F^-, SO_4^{2-}, Ca, and Mg, and the metals As, Fe, and Pb showed higher levels,

mainly at Sites 1, 3, and 4. It has been suggested that the increase of Cd and Zn during warm periods and of Pb during cold periods, as observed in the present study, could be related to the solubility of metals in the water and their complexation with Cl^- ions [35].

Table 1. Physicochemical parameters of water sampled during the summer of 2021 and the winter of 2022 from sampling sites (Sites 1, 2, 3, and 4) along the northwest Portuguese coast.

		Summer				Winter			
	Parameter	**Site 1 Amorosa**	**Site 2 Cabo do Mundo**	**Site 3 Homem do Leme**	**Site 4 S. Félix Marinha**	**Site 1 Amorosa**	**Site 2 Cabo do Mundo**	**Site 3 Homem do Leme**	**Site 4 S. Félix Marinha**
	pH	7.73	7.81	7.87	8	8.43	8.13	8.15	7.89
	Temperature (°C)	17	17.3	18	18.2	13	13.1	14.4	14.2
mg/L	**Nitrite (NO_2^-)**	0.02	0.16	0.1	0.03	0.91	0.01	0.01	0.02
	Nitrate (NO_3^-)	2.1	6.05	2.58	1.79	1.06	5.81	0.94	0.7
	Fluorides (F^-)	0.89	0.82	0.81	0.79	0.91	0.9	0.9	0.92
	Chlorides (Cl^-)	15,161.5	17,173.3	16,345.8	15,061.7	14,985.7	13,839.6	15,285.3	14,373.6
	Phosphate ($P_2O_5^-$)	0.12	0.13	0.14	0.12	n.d.	n.d.	n.d.	n.d.
	Sulfates (SO_4^{2-})	2496.1	2713.9	2768.3	2779.2	2920.8	2659.4	2920.8	2822.7
	Ammonium (NH_4)	0.26	0.16	0.21	0.1	0.01	0.01	0.01	0.01
	Calcium (Ca)	184.5	181.2	181.2	187.8	598.4	589.9	598.4	589.9
	Potassium (K)	473.8	507.6	494.8	507.6	397.3	349.2	299.4	457.3
	Magnesium (Mg)	1127	1179	1165	1125	1517	1406	1434	1734
	Sodium (Na)	7318.36	7540.16	7643.64	7747.83	7074.66	6653.86	6685.77	6911.29
	Alkalinity (HCO_3^-)	146.4	140.3	146.4	146.4	134.2	152.5	152.5	131.15
	Biochemical oxygen demand (BOD)	6.25	12.5	6.25	6.25	0	0	0	0
	Chemical oxygen deficiency (COD)	426.14	337.06	663.69	218.28	1964	1828	1988	2000
	Total Suspended Solids (TSS)	125	141	130	128	126	134	132	137
	Total hardness ($CaCO_3$)	5099	5305	5248	5099	7741.5	7261.3	7399.4	8610.5
µg/L	**Arsenic (As)**	43.08	39.39	40.97	35.69	99.87	41.76	76.22	94.23
	Cadmium (Cd)	7.168	3.813	9.508	3.784	0.635	1.03	1.656	1.772
	Copper (Cu)	6.232	6.634	14.62	12.74	7.439	5.816	19.24	2.078
	Chromium (Cr)	0.818	0.936	3.411	0.398	0.271	0.503	0.463	0.209
	Iron (Fe)	0.35	0.59	1	0.39	0.92	4.32	2.44	0.55
	Manganese (Mn)	1.326	2.352	5.292	0.56	0.486	1.527	1.376	0.296
	Nickel (Ni)	122.6	46.8	75.4	35	n.d.	1.463	n.d.	n.d.
	Lead (Pb)	n.d.	0.156	0.129	0.154	3.846	6.159	5.389	5.039
	Zinc (Zn)	2.6	2.51	2.7	10.49	1.052	0.453	1.244	0.454

The spatial and temporal variations observed in this study confirm the different inputs of urban, industrial, and marine traffic effluents. For instance, the proximity of some of the sites to large and small rivers that cross several agricultural areas may contribute to a potential increased influx of As, Cd, Cu, Fe, Pb, Ni, and Zn, since these metals are components of pesticides and fertilizers and, therefore, commonly leached from agricultural lands [36–38]. Similarly, other studies recorded a spatiotemporal variation of metal concentrations in the northwest coast of Portugal's seawaters [13,39,40], as well in other coastal regions [35,41].

3.2. Biometric Parameters

The biometric parameters of the sampled *P. lineatus* along the Northwest Atlantic coast of Portugal are shown in Table 2. The gastropods sampled at Site 2 presented a higher total length and weight in both seasons compared to Sites 1 and 4 ($p < 0.01$). Both total length and weight increased significantly during the winter season ($p < 0.001$), unlike the summer season. The lower length and weight in the summer may be related to the spawning period of *P. lineatus*, which causes a loss of nutrient reserves. Regarding the condition factor (K), during the summer season, it was significantly lower in Site 2 compared to the remaining sites ($p < 0.05$) and to the winter season ($p < 0.001$). This difference may be related to the higher biochemical oxygen demand and TSS observed in Site 2, which translates into low

dissolved oxygen levels and, consequently, physiological changes in *P. lineatus* in order to maintain homeostasis.

Table 2. Biometric parameters of the snails (*Phorcus lineatus*) collected at the sampling sites, along the northwest Portuguese coast, during the summer of 2021 and winter of 2022.

	Seasons/Sites	Total Length (cm)	Total Weight (g)	Weight of the Soft Tissues (g)	Condition Factor (CF)
Summer	**Site 1—Amorosa**	1.73 ± 0.12 ac	2.38 ± 0.48 ab	0.68 ± 0.18 a	0.46 ± 0.06 a
	Site 2—Cabo do Mundo	1.91 ± 0.15 b	2.76 ± 0.61 a	0.90 ± 0.23 b	0.39 ± 0.07 b
	Site 3—Homem do Leme	1.84 ± 0.21 ab	2.90 ± 1.11 a	0.76 ± 0.29 ab	0.44 ± 0.04 a
	Site 4—S. Félix da Marinha	1.70 ± 0.11 c	2.17 ± 0.47 b	0.49 ± 0.14 c	0.44 ± 0.05 a
Winter	**Site 1—Amorosa**	1.88 ± 0.16 a*	3.07 ± 0.99 a*	0.74 ± 0.20 a	0.45 ± 0.08 ab
	Site 2—Cabo do Mundo	2.16 ± 0.18 b*	4.65 ± 1.34 b*	1.44 ± 0.47 b*	0.45 ± 0.04 a*
	Site 3—Homem do Leme	2.00 ± 0.14 c*	3.41 ± 0.84 a*	1.09 ± 0.29 bc*	0.42 ± 0.03 ab
	Site 4—S. Félix da Marinha	1.93 ± 0.15 ac*	2.99 ± 1.05 a*	0.95 ± 0.26 c*	0.41 ± 0.11 b

Notes: Data are expressed as mean ± S.D. The statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test. Different lowercase letters indicate significant differences between sites during each season ($p < 0.05$); asterisks (*) indicate differences between seasons for each site ($p < 0.05$).

Overall, this variability between sites and seasons could be related to the physiological and reproductive status of the gastropods and/or food availability. However, it can also be related to adaptative strategies against low oxygen levels and/or exposure to pollution on these sites, which leads the species to invest more energy in detoxification mechanisms [35].

3.3. Biochemical Biomarkers

Biochemical biomarkers are important tools to evaluate and understand linkages between pollutant exposure and potentially associated impacts on the ecosystem's health status.

In the present study, the levels of ROS (Figure 2A) were similar among all sites during the summer season ($p > 0.05$), but higher compared to the winter season ($p < 0.01$). In turn, during the cold period, *P. lineatus* of Site 2 presented significantly lower levels of ROS than those of Site 4 ($p = 0.012$). Considering the LPO levels (Figure 2B), there were significant spatial and seasonal variations in their levels. During the summer season, Site 1 showed higher levels of LPO in comparison to the other sites ($p < 0.001$), while in the winter season, both Sites 1 and 2 presented lower LPO levels than Sites 3 and 4 ($p < 0.001$). In addition, Site 3 also showed higher LPO levels than Site 4 ($p < 0.001$). PCO (Figure 2C), another biomarker of oxidative stress, showed similar levels among all sites in the summer season ($p > 0.05$); however, during the cold period, it showed higher levels in Site 1 in comparison to Site 4 ($p = 0.016$). The above results indicate both spatial and temporal variation of biochemical responses, and consequently the exposure to contaminants that caused the induction of oxidative stress in *P. lineatus*. In response to such oxidative stress, the antioxidant enzymes can be induced as a protective mechanism, inhibited, or show no response. Indeed, SOD did not show any significant changes between sites and seasons ($p > 0.05$, Figure 3A), while the difference in the enzymatic activity of CAT was significant between Site 1 and the remaining sites ($p < 0.05$), but not between seasons ($p > 0.05$, Figure 3B). These results suggest an adaptive response of *P. lineatus* or the activation of other detoxification mechanisms to deal with the potential contamination of the sampling sites. GST (Figure 3C), a phase II biotransformation enzyme involved in the detoxification of several xenobiotic compounds, showed similar activity in the summer season across all sites ($p > 0.05$); but, in the winter season, the gastropods of Site 1 showed higher GST activity in comparison to the other sites ($p < 0.001$). This suggests that GST was more responsive to the type of contamination in Site 1, with its increase showing the activation of detoxification mechanisms in *P. lineatus*. Besides, in all sites, the GST showed higher values of activity in the winter season compared to the summer ($p < 0.001$). Overall, considering the oxidative stress, antioxidant system, and detoxification biomarkers, the above results suggest that *P. lineatus* had a higher response in the winter season compared to the warm period, at least for most of the biomarkers. This

could be related to a variation in contaminant inputs at the sampling sites; nevertheless, it is important to consider that the gastropod's response may also depend on abiotic factors, food availability, and its physiological state [42,43].

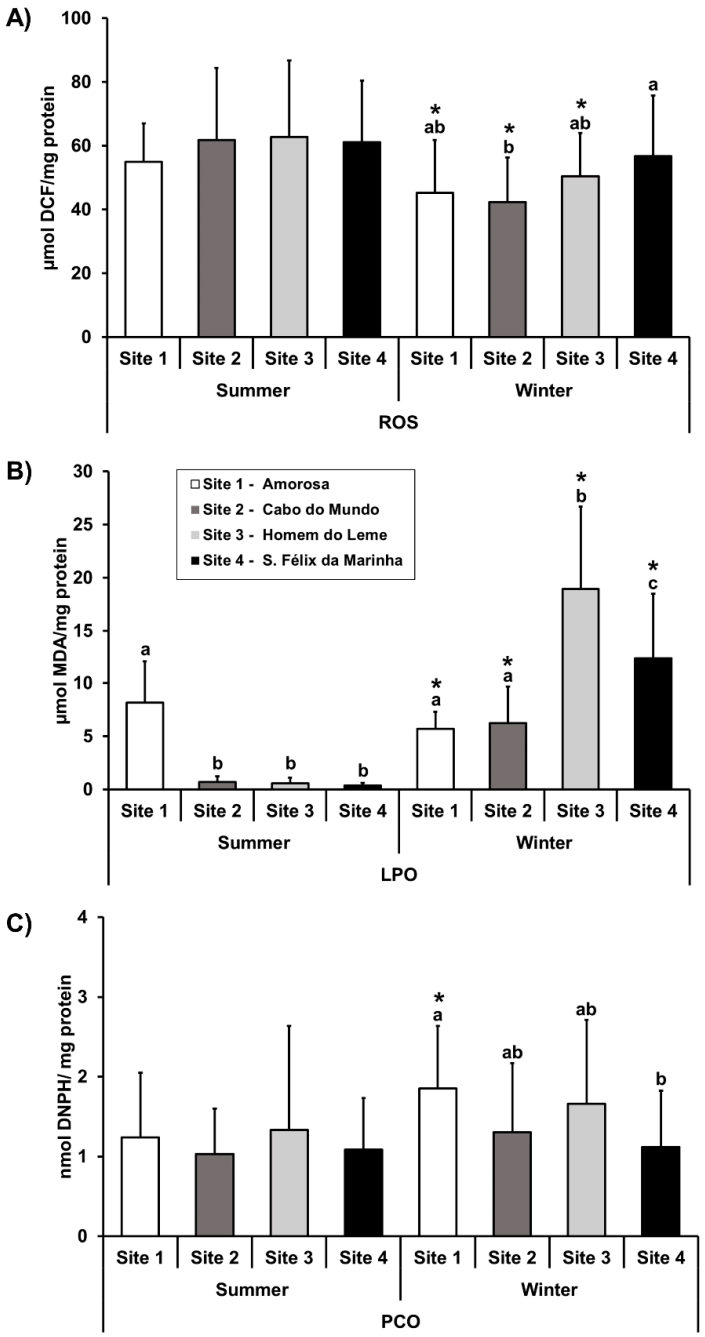

Figure 2. Spatiotemporal variation of (**A**) reactive oxygen species (ROS), (**B**) lipid peroxidation (LPO), and (**C**) protein carbonyl content (PCO) levels in *Phorcus lineatus* sampled during summer and winter from four sites on the northwest Portuguese coast (S1: Amorosa; S2: Cabo do Mundo; S3: Homem do Leme; S4: S. Félix da Marinha). Data are expressed as mean ± S.D. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test. Different lowercase letters indicate significant differences between sites during the same season ($p < 0.05$), and asterisks (*) indicate significant differences between seasons for each site ($p < 0.05$). Absence of superscript indicates no significant differences.

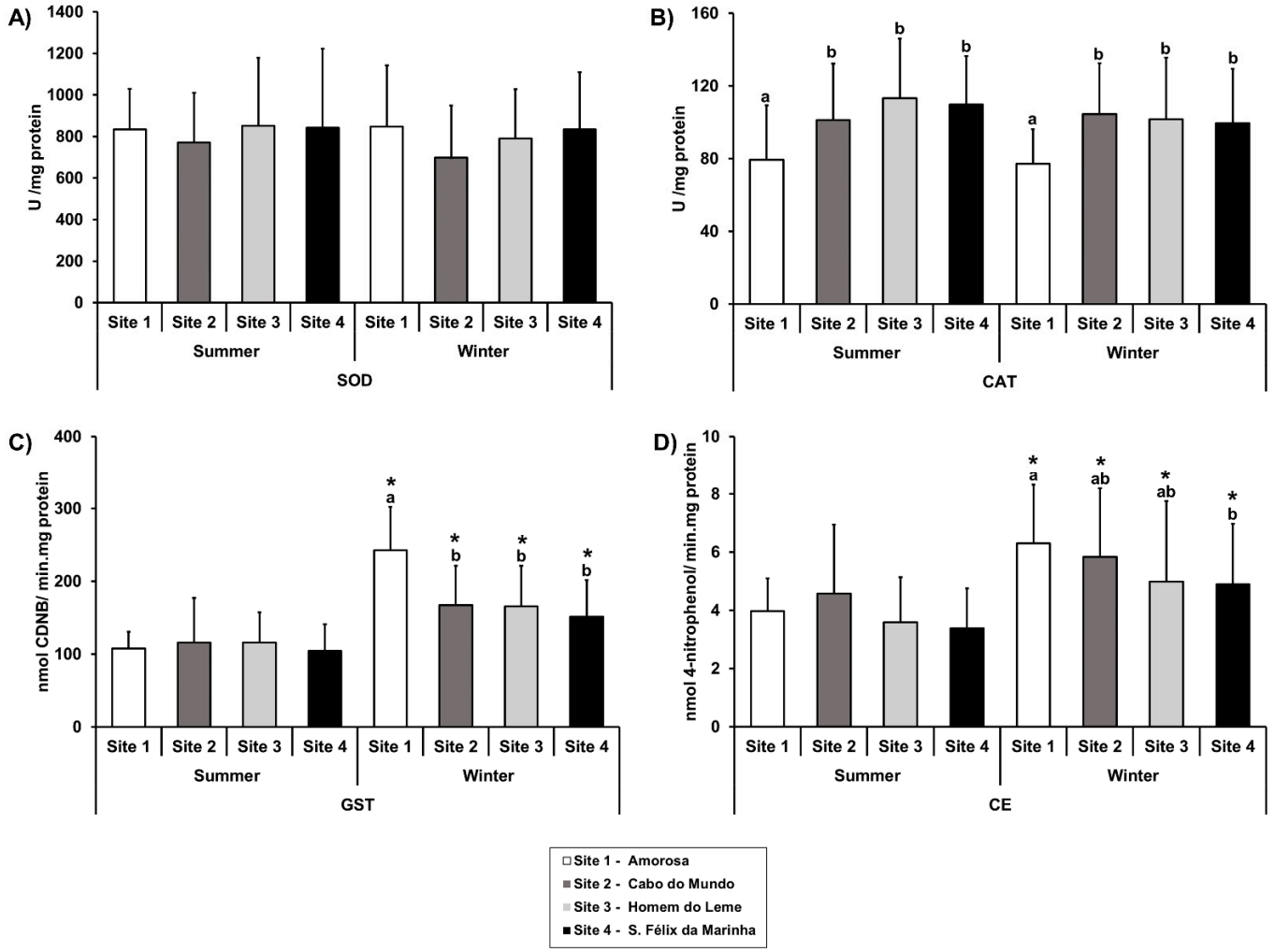

Figure 3. Spatiotemporal variation of (**A**) superoxide dismutase (SOD), (**B**) catalase (CAT), and (**C**) glutathione-S-transferase (GST), and (**D**) carboxylesterase (CE) activity in *Phorcus lineatus* sampled during summer and winter from four sites on the northwest Portuguese coast (S1: Amorosa; S2: Cabo do Mundo; S3: Homem do Leme; S4: S. Félix da Marinha). Data are expressed as mean ± S.D. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test. Different lowercase letters indicate significant differences between sites during the same season ($p < 0.05$), and asterisks (*) indicate significant differences between seasons for each site ($p < 0.05$). Absence of superscript indicates no significant differences.

CE catalyzes the hydrolysis of a wide range of exogenous and endogenous carboxylesterases and plays a protective role against organophosphate insecticides [44], and therefore qualifies as a suitable enzyme for pollution monitoring. AChE, which catalyzes the hydrolysis of the neurotransmitter acetylcholine in the cholinergic neurons, is also a common biomarker of neurotoxicity [45]. Both cholinesterases and carboxylesterases belong to the group of hydrolases [44]. In the present study, CE (Figure 3D) showed similar activity in all the sites during the warm season ($p > 0.05$), but its activity increased significantly during the winter season ($p < 0.01$). During the cold season, the gastropods of Site 1 presented higher CE activity than Site 4 ($p = 0.040$). Contrarily, AChE (Figure 4) showed significant variation in the summer season, with the gastropods of Site 4 having a higher AChE activity than those of Sites 1 and 2 ($p < 0.001$) and Site 3 ($p = 0.007$). However, in the winter period, the gastropods exhibited similar levels of AChE activity in all sites ($p > 0.05$). It has been suggested that the combination of CE and AChE in biomonitoring programs may provide useful information about the potential exposure and effects of

pesticides on aquatic invertebrates [44]. In the present study, a distinct seasonal profile between CE and AChE was observed, with CE presenting higher activity in the cold season and AChE with higher activity in the warm period. In field studies, seasonal variations in CE and AChE activity were demonstrated, mostly related to the contamination inputs in the sampling areas [35,45–48]. Nonetheless, it has been suggested that is also necessary to consider the effects of environmental variables, particularly temperature, on the seasonal variation of CE and AChE activity [44,47,49]. For instance, higher AChE activity at higher temperatures (summer) have been demonstrated in the gills of *Mytilus* sp. [47]. Besides, the seasonal variation observed in this study could also be related to the reproductive cycle of *P. lineatus*. Najimi et al. [50] found that the periods of maximal and minimal activity of AChE coincided, respectively, with the spawning and sexual rest of the mussel *Perna perna*.

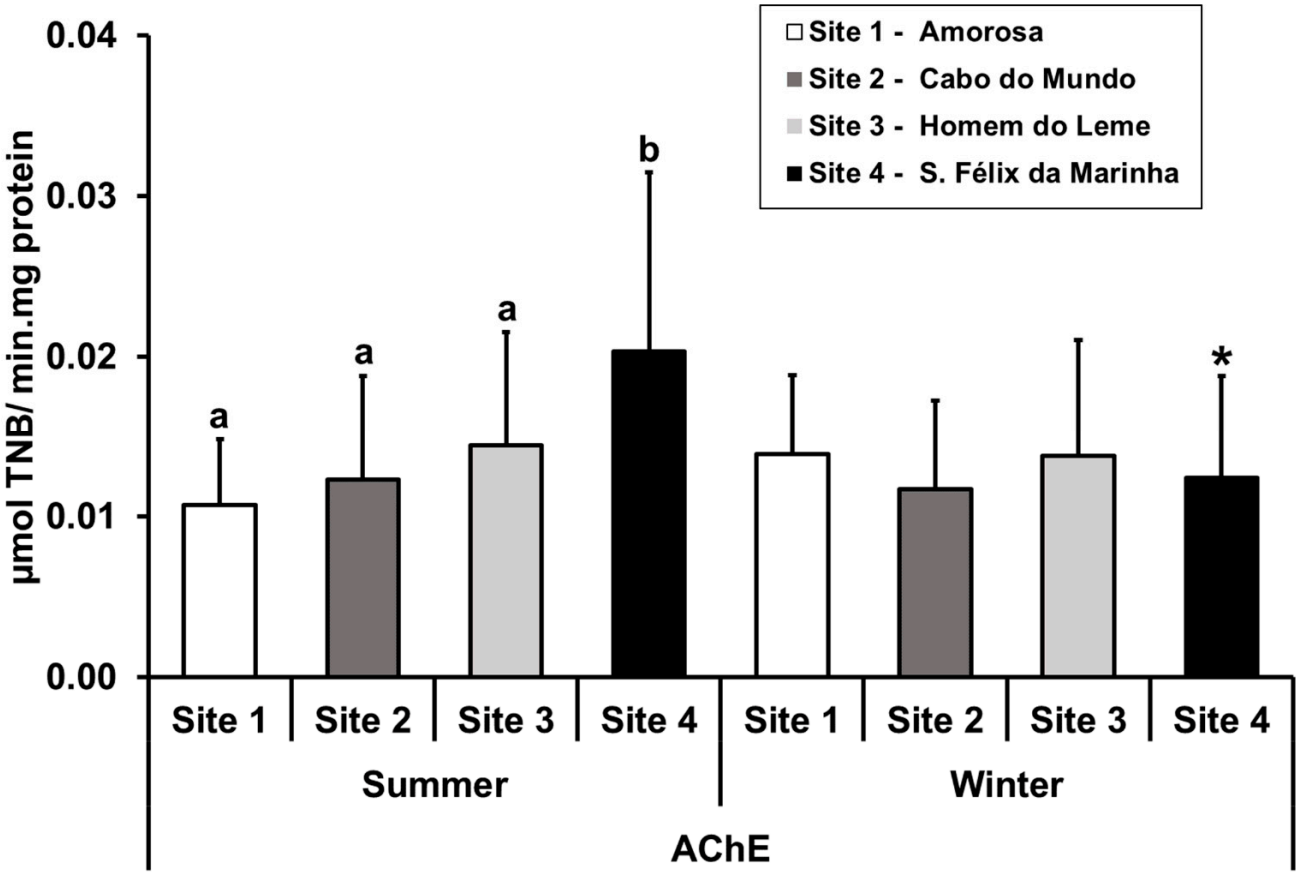

Figure 4. Spatiotemporal variation of acetylcholinesterase (AChE) activity in *Phorcus lineatus* sampled in summer and winter from four sites on the northwest Portuguese coast (S1: Amorosa; S2: Cabo do Mundo; S3: Homem do Leme; S4: S. Félix da Marinha). Data are expressed as mean ± S.D. Statistical analysis was performed using two-way ANOVA followed by Tukey's multiple comparison test. Different lowercase letters indicate significant differences between sites during the same season ($p < 0.05$), and asterisks (*) indicate significant differences between seasons for each site ($p < 0.05$). Absence of superscript indicates no significant differences.

Thus, considering the present results, chemical analysis regarding contaminant levels—particularly pesticides, fertilizers, and polycyclic aromatic hydrocarbons (PAHs)—would be needed in order to further clarify whether the variation of enzymatic biomarkers is related to abiotic factors, *P. lineatus'* physiology, heavy metals, or the presence of other contaminants.

3.4. Integrated Biomarker Response (IBR)

The IBR index is a practical tool to integrate multiple biomarker responses and evaluate the susceptibility of the organisms to pollutants [31]. In the present study, the inter-site and seasonal comparison of the IBR values (Figure 5) showed both spatial and temporal variation. In both seasons, Sites 1 and 3 showed the highest IBR values, reflecting that the gastropods in these locations were under higher stress in comparison with Sites 2 and 4 (Figure 5A,B). Indeed, these two sites (1 and 3) are subject to high industrial and urban stressors, including a maritime shipyard and an industrial and mercantile harbor supporting intensive traffic of vessels (Leixões harbor), respectively.

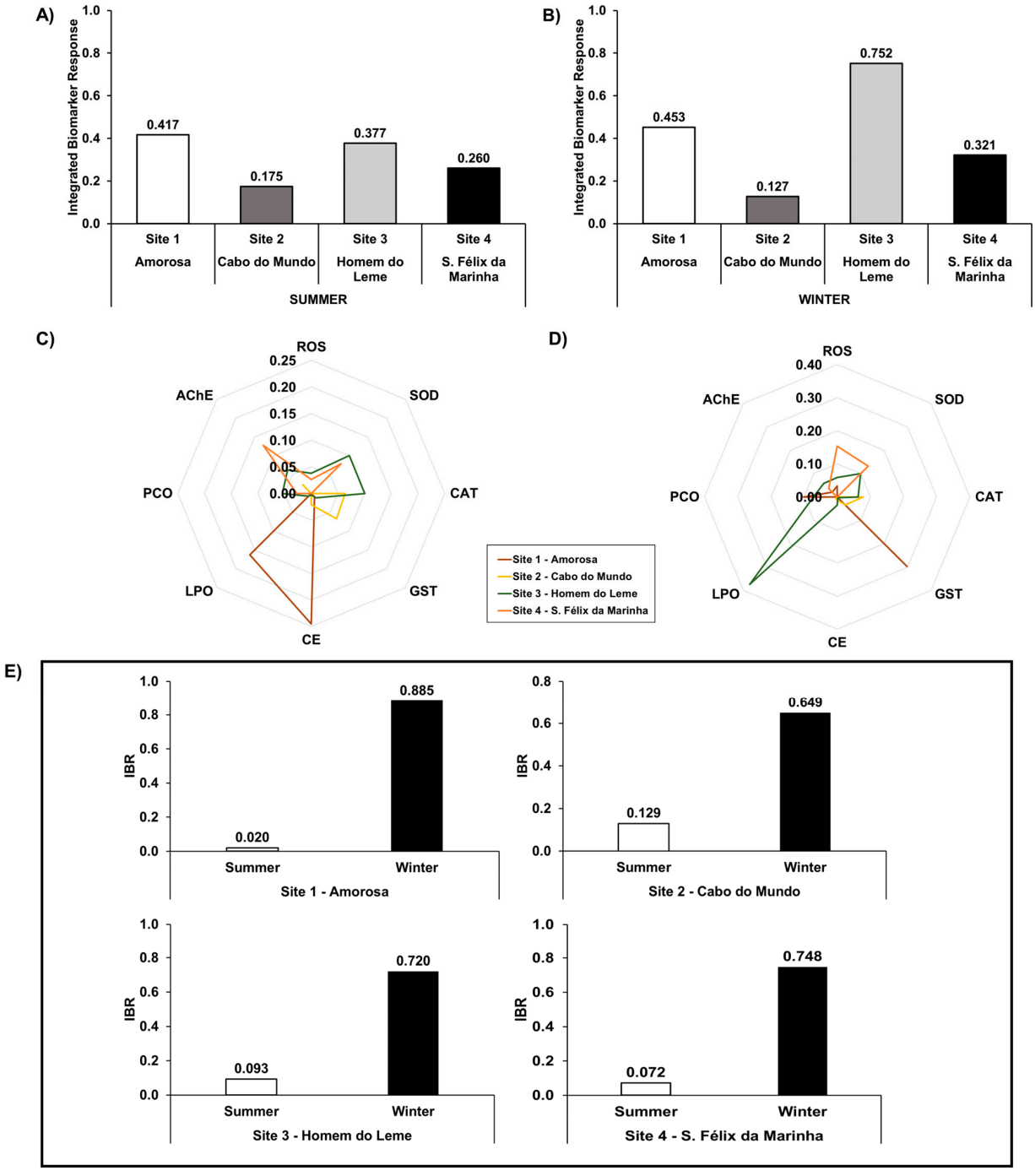

Figure 5. Integrated biomarker response (IBR) values and corresponding star plots for (**A**,**C**) summer and (**B**,**D**) winter in the four sampling sites on the northwest Portuguese coast. (**E**) IBR values for each site.

When considering the summer season, the biomarkers CE, LPO, and AChE were the most discriminant factors, while in the winter, it was the LPO and GST biomarkers (Figure 5C,D). Moreover, the IBR index showed that gastropods were under higher stress during the cold season in all sites (Figure 5E), corroborating the biochemical data. These results substantiate the value of the IBR index in biosurveillance programs, by summarizing information from a set of multiple biomarkers and avoiding misinterpretation of data in a given ecosystem [51].

3.5. Multivariate Analysis

The resultant ordination (non-metric multidimensional scaling, or n-MDS) achieved a stress value of 0.14, not exceeding the commonly accepted limit of 0.2 for the interpretative power [52–54], and thus the major trends could be interpreted. The n-MDS ordination of *P. lineatus*' enzymatic activities and biometric variables displayed a separation into two groups according to the sampling season (Figure S1), confirming that this species displays different activity and fitness in each season.

The redundancy analysis (RDA) results showed which studied variables related to enzymatic activities and biometric variables contributed more to the explained variance in response to physicochemical parameters (PC) and specific pollutants (SP) (Figure 6). Monte Carlo permutations were highly significant for all axes ($p < 0.001$; 95.06% for the first two axes), where physicochemical parameters and specific pollutants account for 14.88% of the total variance (Figure 6). The variables most positively correlated with axis 1 were the biometric variables (the weight of the soft tissues (WST), length (Leng), and total weight (TW)), and the most negatively correlated variable was ROS, which was also positively correlated with the increased concentration of SO_4 and Cu, but negatively correlated with Cd. AChE, CE, and GST were positively correlated with axis 2, and increased with higher concentrations of Cu and SO_4, conversely to ROS. LPO was negatively correlated with axis 2, along with the increased concentration of F^- and Pb.

To analyze the effect of spatial and temporal scales, a pRDA of two groups of variables, testing simple effects, was conducted (group 1, season; group 2, sites) (Figure 7A–C). The results from this partial constrained ordination estimated the fraction of variance in the biometric variables and enzymatic activities of *P. lineatus*, explained by each subset of variables, e.g., [55] (Figure 7). The results showed that the temporal factor (season, $p < 0.001$, 58.5%) and the spatial factor (sampling sites, $p < 0.001$, 43.0%) contribute greatly and significantly to the variation explained by the *P. lineatus* in a pollution gradient. The shared variation between the two groups was not significant (Figure 7D).

The pRDA biplot related to season (Figure 7B) shows that axis 1 separates summer from winter. The higher enzymatic activities LPO, GST, and CE are positively related to winter and negatively to summer, therefore corroborating the individual biochemical data. The data also show that higher biometric values are strongly correlated to axis 1 and Site 2 (Figure 7C), located near the oil refinery outfall. The enzymatic activity of CAT is negatively correlated to axis 2, presenting the highest values in Site 3 and the lowest in Site 1. Contrary to this, Site 1 is characterized by higher values of GST and PCO. AChE, LPO, and ROS presented higher values in Site 4.

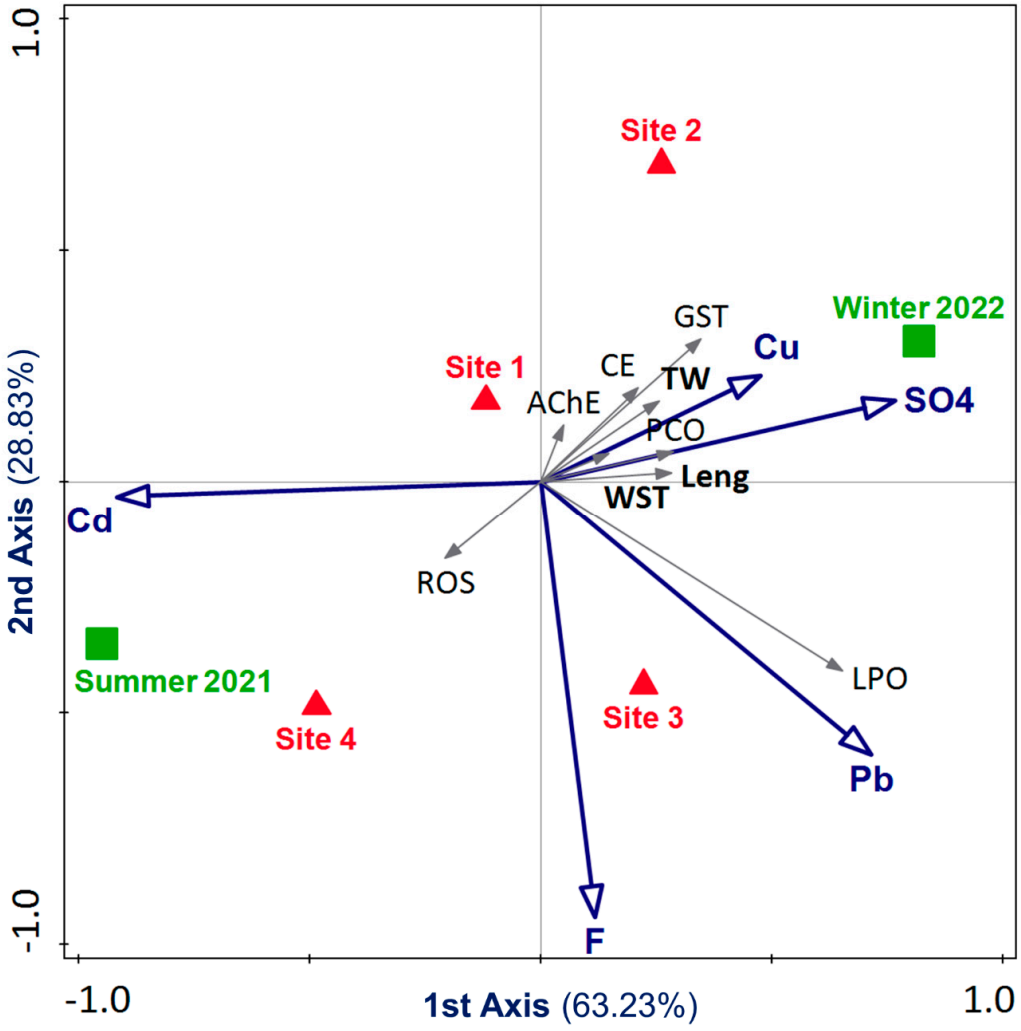

Figure 6. Redundancy analysis (RDA) results showing the total variance explained by all the studied variables in *Phorcus lineatus* from the four sampling sites (Site 1: Amorosa; Site 2: Cabo do Mundo; Site 3: Homem do Leme; and Site 4: S. Félix da Marinha) during summer and winter. Dark blue: physicochemical parameters and specific pollutants; grey: enzymatic activities; and black: biometric variables. The first two axes explain 95.06% of the observed variation, respectively. Abbreviations: AChE: acetylcholinesterase; Cd: cadmium; CE: carboxylesterase; Cu: copper; F: fluorides; GST: glutathione S-transferase; Leng: length; LPO: lipid peroxidation; Pb: lead; PCO: protein carbonyl content; ROS: reactive oxygen species; SO4: sulfates; TW: total weight; WST: weight of the soft tissues.

A pRDA of two groups of variables, testing simple effects, was processed (group 1, the PC, with three variables—TSS, NH_4, and Na; and group 2, the SP, with three variables—Cd, Pb, and Cu) (Figure 8A–C). The results reflected a lower contribution to the explained variation from PC (5.8%) and SP (1.1%) compared to the shared variation between the two groups (93.1%) (Figure 8D). The PC pRDA biplot (Figure 8B) highlights that axis 1 splits ROS (positively associated with NH_4) from the other enzymatic activities, except for LPO, which is positively associated with TSS and negatively with Na. A similar pattern was observable in the SP pRDA indicating that, once again, ROS shows a behavior contrary to the other enzymatic activities (positively related to Cu concentration). Also, in this analysis, LPO stands out, presenting activities associated with high Pb concentrations. The biometric parameters, in particular WST and length, are negatively correlated with high concentrations of Cd.

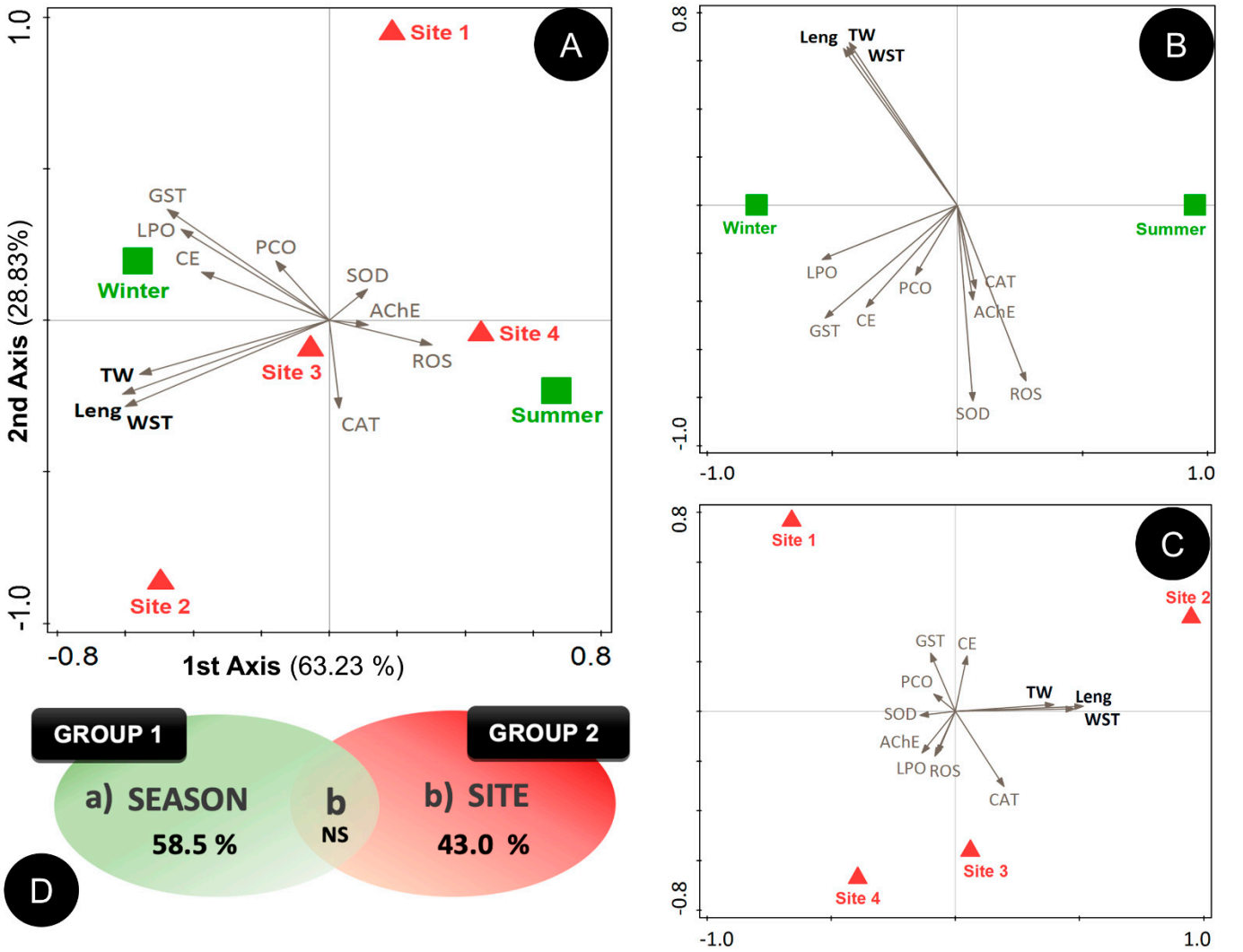

Figure 7. Partial redundancy analysis (pRDA) for (**A**) sites and season of the studied variables; (**B**) group 1—season; and (**C**) group 2—sites. (**D**) Partition of the variation in enzymatic activities and biometric variables explained by the two groups, testing the simple effects. Abbreviations: Site 1: Amorosa; Site 2: Cabo do Mundo; Site 3: Homem do Leme; Site 4: S. Félix da Marinha; AChE: acetylcholinesterase, CAT: catalase, CE: carboxylesterase, GST: glutathione S-transferase, Leng: length, LPO: lipid peroxidation; PCO: protein carbonyl content; ROS: reactive oxygen species; SOD: superoxide dismutase; TW: total weight; WST: weight of the soft tissues.

Overall, the redundancy analysis confirms the higher contribution of the factor season in the response of *P. lineatus* to pollutants, compared to the spatial variation in the northwest Portuguese coast. Indeed, data indicates that gastropods had a higher enzymatic response during the winter, being under higher stress during the cold season. It has been suggested that during winter, due to the rainfall's increase, there could be an exacerbation in the transport of higher amounts of sediments and pollutants from land to rivers and, consequently, to coastal regions [56]. Besides, the increase of pollutants—including metals—during the winter and the consequent higher stress of gastropods could also be related to the perturbation of the seabed by the longshore current, the upwelling phenomenon, and waves during the rainy season [56]. The proximity of the sampling sites to rivers that cross vast urban and agricultural areas (e.g., Site 3 is close to the Douro River) may therefore explain the temporal variation between sites and the response of *P. lineatus* in the present study.

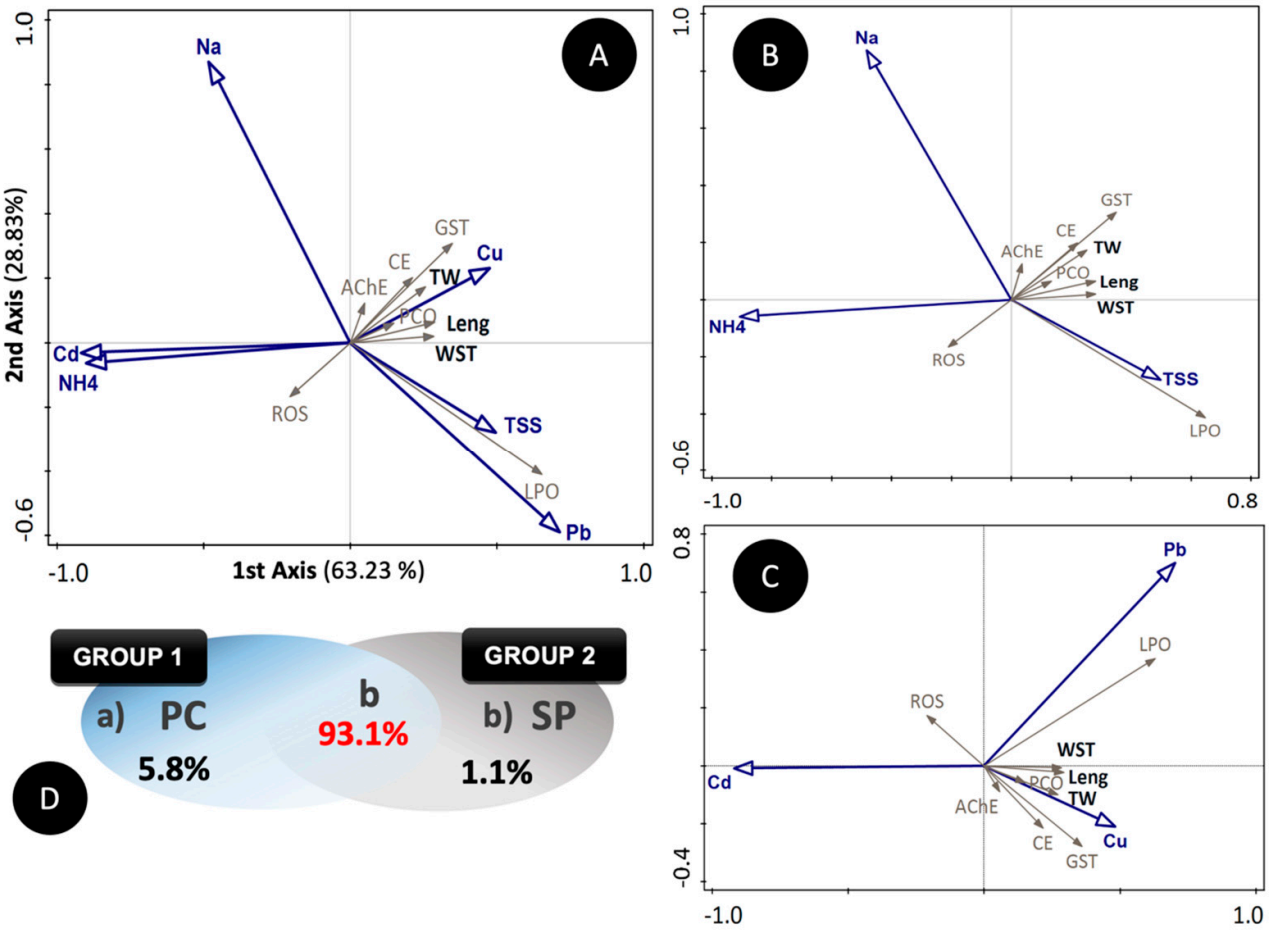

Figure 8. Partial redundancy analysis (pRDA) for (**A**) two groups of the studied variables: (**B**) group 1—physicochemical parameters (PC); and (**C**) group 2—specific pollutants (SP). (**D**) Partition of the variation in enzymatic activities and biometric variables explained by the two groups and respective shared variation testing the simple effects.

4. Conclusions

Based on the results obtained, this study shows that the contamination in the northwest Portuguese coast and the enzymatic responses of *P. lineatus* therein are mostly correlated to the season, but also to a spatial pollution gradient. This reflects an interaction between contaminant inputs and physicochemical variables. The long-term exposure and persistence of heavy metals in the sampling sites can result in risks to both the ecosystems and human health.

Overall, the present study indicates that *P. lineatus* can be used as a bioindicator species for environmental biomonitoring. It also reinforces that the implementation of different methodologies, such as chemical, biological, and statistical, can offer a better and safer perspective for marine biomonitoring programs.

These results reinforce the need for further research on coastal areas, implementing more complete pollution monitoring programs (e.g., including pesticides, PAHs, etc.) alongside additional bioindicators—like fishes—and abiotic factors, as well as investing more time, with—for example—monthly monitoring to confirm the spatial and temporal variation of contamination. This is crucial for ensuring better monitoring and assessment of the environmental status of national marine waters, in addition to the achievement of the objectives set out in the environmental targets and the Programme of Measures of the Marine Strategy Framework Directive (MSFD).

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/w16010005/s1.

Author Contributions: D.S.: investigation, validation, writing—original draft, review, and editing. S.V.: field sampling, results validation, writing—review and editing. J.S.C.: field sampling, writing—review and editing. M.J.S.: writing—review and editing. A.L.: results validation, writing—review and editing. S.M.M. and E.C.: conceptualization, resources, writing—review and editing, supervision. All authors have read and agreed to the published version of the manuscript.

Funding: This work is supported by national funds from the FCT (Portuguese Foundation for Science and Technology), under the project UIDB/04033/2020; by the ATLANTIDA project (ref. NORTE-01-0145-FEDER-000 040); and it was also supported by the Norte Portugal Regional Operational Programme (NORTE 2020), under the PORTUGAL 2020 Partnership Agreement and through the European Regional Development Fund (ERDF).

Institutional Review Board Statement: The use of gastropods is not regulated by the European Directive for the Protection of Animals Used for Scientific Purposes (2010-63-EU Directive). However, the capture of gastropods, as wild animals, was formally authorized by the Instituto da Conservação da Natureza e das Florestas—the Portuguese Institute for Nature Conservation and Forests (ICNF), ensuring that the sampling of these organisms complied with legal standards and followed ethical, environmental, and conservation considerations.

Data Availability Statement: Data are contained within the article and Supplementary Materials.

Conflicts of Interest: The authors declare no conflict of interest.

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