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Oxidative Stress-Related Biomarkers in Tissues of the Euryhaline Guppy *Poecilia Vivipara* Exposed In Situ to a Coastal Water Environment with a Long History of Metal Contamination

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How To Cite: Mathias, A.J.C.; Machado, A.A.d.S.; Jorge, M.B.; et al. Oxidative Stress-Related Biomarkers in Tissues of the Euryhaline Guppy *Poecilia Vivipara* Exposed In Situ to a Coastal Water Environment with a Long History of Metal Contamination. *Earth: Environmental Sustainability* **2025**, *1*(1), 84–101. https://doi.org/10.53941/eesus.2025.100007

Received: 5 August 2025
Revised: 27 August 2025
Accepted: 3 September 2025
Published: 10 September 2025

Abstract: We aimed to select reliable biomarkers of metal exposure in the eurhalyne guppy *Poecilia vivipara*. Individuals were exposed to three different sites in a coastal bay (i.e., Linguado Channel, Babitonga Bay, Southern Brazil), a coastal environment with a long history of metal contamination. Temperature, salinity, pH, dissolved oxygen, and dissolved organic carbon) were measured in seawater from the exposure sites. After exposure, fish were anesthetized and their tissues (i.e., gill and liver) were dissected to evaluate the Ag, Cd, Cr, Cu, Ni, Pb, and Zn concentrations, as well as a suite of biomarkers: antioxidant capacity against peroxyl radicals (ACAP), antioxidant enzyme activities (catalase and glutathione S-transferase), metallothionein-like protein (MTLP) concentration, and lipid peroxidation level (LPO). Seawater physicochemical conditions were similar in the exposure sites. Metal concentrations in tissues did not differ significantly between exposure sites. Principal component analysis indicated close correlations between Ni and ACAP, Ag/Cd and MTLPs, and Zn and LPO in the gills. In the liver, there was a close correlation between Pb and LPO. These findings highlight the importance and need for selecting relevant and suitable tissues and biomarkers for biomonitoring programs that aim to assess and monitor fish exposure to metal contamination in coastal waters. The findings also point to the need for future research focused on the response of oxidative stress–related biomarkers to long-term in situ exposure of fish to coastal waters contaminated with metals and other inorganic and organic pollutants.

Keywords: biomarkers; ecotoxicology; fish; metals; oxidative stress; pollution

1. Introduction

The coastal zones are characterized by littoral fringes, high biological diversity, and environmental fragility [1,2]. They are under the influence of several natural processes, such as waves, tides, and marine streams. However, they are also subjected to anthropogenic climate and non-climate drivers [3,4]. Human activities are generating and discharging chemical contaminants in coastal zones at harmful environmental

concentrations that may be toxic [5–8]. As in other regions of the world [9,10], coastal management in Brazil is a true political riddle [11].

Coastal environments are becoming sinks for a large variety of anthropogenic residues and chemical contaminants. Costal ecosystems are subjected to intense degradation, contamination, and pollution by several classes of chemical contaminants, including metals [12–16]. Metals are well known as toxic contaminants to aquatic animals because they are environmentally persistent, bioavailable, and easily bioaccumulated [17–19]. Excessive concentrations of metals cause adverse effects in many estuarine and marine animals, including fish [20–22].

Only trace amounts of essential metals (e.g., Cr, Cu, Ni, and Zn) are needed for the normal functioning of marine fish [23,24]. However, excessive levels of essential metals and even trace amounts of non-essential metals can induce sublethal and lethal effects in marine fish, leading to changes in populations and altering the ecosystem structure [25,26]. In addition to other sublethal effects, increased concentrations of tissue metals can induce oxidative stress in fish through several mechanisms, including the inhibition of antioxidant enzymes, such as catalase (CAT) and glutathione S-transferase (GST) [27,28]. In turn, the organism's defense against the harmful action of reactive oxygen species (ROS) is associated with the antioxidant capacity against ROS, such as peroxyl radicals (ACAP). This capacity involves enzymatic antioxidants, such as CAT and GST [27,28], as well as non-enzymatic antioxidants, such as metallothioneins [29,30]. When the total antioxidant capacity is overwhelmed by ROS, an oxidative stress condition is established, and oxidative damage to biomolecules, such as lipoperoxidation (LPO), can be observed [31–33]. As well as their role as antioxidants, metallothioneins play another important protective mechanism against metal toxicity in fish. These macromolecules bind metals and then mitigate the potential negative effects of these contaminants [34,35].

Several approaches and techniques have been employed to assess and monitor the environmental quality, including physicochemical and biological aspects involving water, sediment, and biota [16,36,37]. Among these approaches, biomarkers have been widely used in aquatic systems [37–42]. In this context, fish have been widely used as a biological model in ecotoxicological studies [43–46]. In addition, the oxidative stress–related parameters mentioned above are considered useful biomarkers of fish exposure to metals [47–52], including in the euryhaline guppy *Poecilia vivipara* [53,54]. However, more field studies are needed to identify the most reliable and adequate biomarkers and biomonitors (tissues and organisms) to be used in environmental programs aiming to evaluate and monitor metal contamination and risk in coastal waters [34,35,55].

The guppy *P. vivipara* (Cyprinodontiformes, Poeciliidae) is an endemic, euryhaline, and live-bearing fish. It has an opportunistic feeding behavior and lives among macrophytes in calm and shallow water bodies; it is commonly found in lentic and semi-lentic coastal environments of the Southern Atlantic Ocean [56]. This guppy species is a valuable model organism in ecological and toxicological studies due to its widespread distribution, small size, high tolerance to variations in water salinity and temperature, and clear biochemical and physiological responses to environmental stressors, including chemical pollutants. Furthermore, *P. vivipara* is easy to handle and culture in the laboratory, and shows fast reproduction with a large number of offspring [57,58]. These characteristics make it an ideal candidate for environmental quality biomonitoring and ecotoxicological studies, especially within the Neotropical region.

The guppy *P. vivipara* has been indicated as a promising biological model to assess the impact of several classes of chemical contaminants. Several genes associated with different metabolic functions, such as biotransformation, membrane transport, and the immune system, were reported to indicate the susceptibility and/or molecular responses of *P. vivipara* to the toxic effects induced by exposure to the diesel oil water accommodated fraction [59]. Histopathological alterations in the liver of *P. vivipara* were shown to be an important tool for the environmental quality assessment of rivers [60]. Oxidative stress and DNA damage were shown to be responses of *P. vivipara* to atrazine [53] and phenanthrene [53,61] exposure. More recently, biotransformation-, endocrine-, and antioxidant pathway–related genes were shown to be differentially affected in the liver of guppies exposed to sanitary sewage [62]. Regarding metals, the guppy *P. vivipara* was shown to have high tolerance to these elements, suggesting the presence of defense mechanisms ensuring its survival in aquatic environments contaminated with these elements [63,64]. Oxidative stress–related biomarkers were reported to be reliable biomarkers of guppy exposure to Cu [53,54]. However, it is worth noting that the biomarker responses in *P. vivipara* were only evaluated under laboratory-controlled conditions and testing only one metal (Cu) [53,54]).

Babitonga Bay (26°02′–26°28′ S and 48°28′–48°50′ W) encompasses an area of 160 km^2 in the state of Santa Catarina (Southern Brazil). The bay has an average depth of 6 m and is connected to the South Atlantic Ocean through a 1.7-km wide channel, with a maximum depth of 28 m. It receives water flows from many rivers and is characterized by tidal flats and islands in the inner waters. This bay hosts the most southern large mangrove forest in South America. It is a vital estuarine complex, showing high biodiversity, including typical

native mangroves, sandy beaches, rocky shores, and islands. This bay supports critical aquatic life, providing essential habitat for vulnerable species like the Guiana dolphin, La Plata dolphin, and Atlantic goliath grouper. This bay also supports local livelihoods because it is a geographically significant major port and navigation area for cities like Joinville, the largest city of the Santa Catarina state (Southern Brazil). In addition, it is a rich historical site marked by indigenous and immigrant settlement. The drowned valley morphology and protected waters of the Babitonga Bay make it a unique ecosystem, which is facing environmental pressures from nearby industrial and domestic pollution [65–71].

The Linguado Channel is part of the Babitonga Bay estuarine complex Because it is located in the southern region of the bay. Since 1938, this channel has been closed, and it has undergone an intense process of siltation that has affected the whole bay. Since the middle of the twentieth century, human settlement around this area has generated and released a variety of chemical residues into the bay: (1) the Itapoá harbor operates important port activities; (2) the city of Joinville is an important industrial center with intense metallurgical activity; (3) the cities of Garuva and Araquari contain small chemical industries, as well as agricultural and tourism activities; and (4) the city of Balneário Barra do Sul is known for its fishing and shipbuilding activities. As a result of the large variety of anthropogenic activities developed around the Babitonga Bay over decades, this coastal environment is reported as having a long history of chemical contamination, with the Linguado Channel being especially associated with metal (e.g., As, Cd, Cr, Cu, Ni, Pb, and Zn) pollution [72–75].

In light of the description above, we assessed the response of oxidative stress-related biomarkers in tissues (i.e., gill and liver) of the euryhaline guppy *P. vivipara* acutely (96 h) exposed in situ to different sites in the Linguado Channel (i.e., Babitonga Bay, Southern Brazil). We hypothesize that the biomarker responses will be dependent on the metal and fish tissue considered. We also hypothesize that the biomarker responses observed in situ here will be comparable to those previously reported under laboratory-controlled conditions with increasing Cu concentrations in salt water. Acute (96 h) exposure to increasing Cu concentrations in salt water has been shown to affect gill ROS, ACAP, and LPO and liver CAT activity [54]. The analysis of the responses of these biomarkers in situ would reinforce and strengthen the adequacy and reliability of these biomarkers as an alternative and complementary tool to assess and monitor metal contamination in coastal zones. They will also highlight the need for selecting the most reliable tissues and biomarkers when using fish in ecotoxicological studies, as well as to assess and monitor metal contamination in coastal waters.

2. Materials and Methods

2.1. Fish Collection, Acclimation, and In Situ Exposure

Individuals of the euryhaline guppy *P. vivipara* were collected (Brazilian Ministry of Environment; permit SISBIO # 37129-1) in Arroio do Gelo, a stream running into the Cassino Beach, city of Rio Grande (Rio Grande do Sul State, Southern Brazil). Collected fish were transferred to the laboratory and kept in seawater under controlled conditions (photoperiod: 12L:12D; temperature: 20 ± 3 °C, and salinity: 24 ± 5 PSU) for 2 weeks. This period of acclimation was proven to be adequate for testing oxidative-stress related biomarkers in the guppy *P. vivipara* exposed to metal (Cu) in saline water [54]. The acclimated fish were then transferred to the exposure sites in the Linguado Channel, Babitonga Bay (Santa Catarina State, Southern Brazil) using dark boxes containing seawater that was continuously aerated.

Fish were in situ exposed in three different sites (n = 40 fish each site) along the Linguado Channel, in the Babitonga Bay (Site 1: 26°22′11.1″ S 48°40′00.8″ O; Site 2: 26°26′24.1″ S 48°37′44.2″ O; and Site 3: 26°27′21.9″ S 48°36′09.4″ O; Figure 1). Fish were kept in 6 cages (2 cages in each exposure site; 20 fish in each cage; fish density: ~1 g/L). Cages were maintained completely immersed in seawater for 4 days. One day before and every day during the experiment, seawater physicochemical parameters (i.e., temperature, salinity, pH, and dissolved oxygen) were measured in situ in each exposure site, using a multiparameter analyzer (HI98194, Hanna Instruments, Carrollton, TX, USA). Water samples were also collected from each exposure site for dissolved organic carbon measurement in the laboratory, using a total organic carbon analyzer (TOC-VCPH, Shimadzu, Japan).

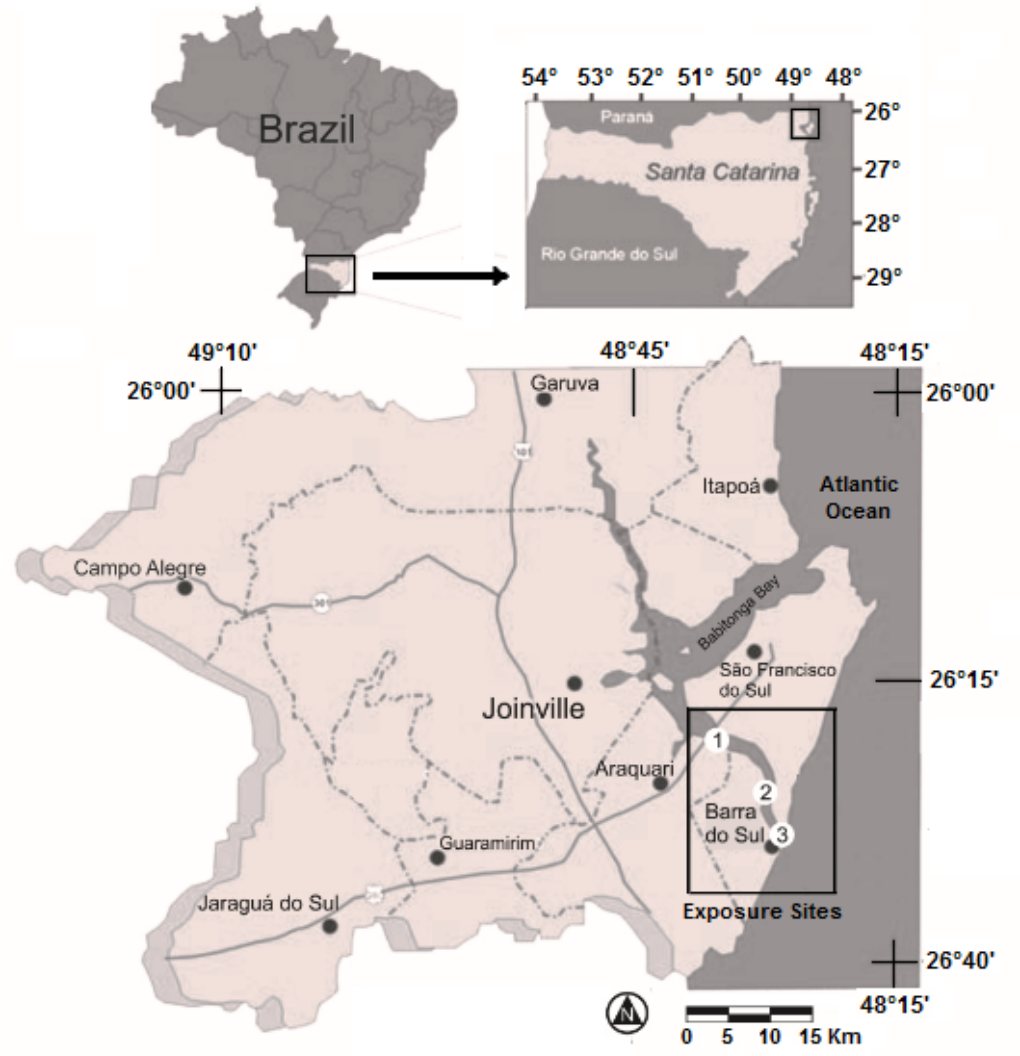

Figure 1. Map indicating the three sites (Sites 1–3) of in situ exposure of the euryhaline guppy *Poecilia vivipara* in the Linguado Channel (Babitonga Bay, Southern Brazil). Site 1 (26°22′11.1″ S 48°40′00.8″ O), Site 2 (26°26′24.1″ S 48°37′44.2″ O), and Site 3 (26°27′21.9″ S 48°36′09.4″ O).

2.2. Collection and Analysis of Biological Samples

After 4 days of exposure in the field, guppies were anesthetized (benzocaine; 0.1 g L^{-1}). Then, their wet body weight (WBW) and length (TBL: total body length; SBL: standard body length) were measured and their tissues (i.e., gill, and liver) were dissected. Samples were split into two aliquots for the analysis of metal concentrations and biomarkers. Sample aliquots were immediately weighed and frozen in liquid nitrogen. They were transferred to the laboratory in dry ice. Upon arrival in the laboratory, sample aliquots were kept in an ultrafreezer (–80 °C) until their analysis.

For measurements of tissue metal concentrations, samples were dried (~70 °C) for 96 h, weighed (tissue dry weight; dw), and digested in 65% HNO_3 (Suprapur, Merck, USA) until complete digestion. Digested samples were diluted with MilliQ water. Concentrations of essential (Cr, Cu, Ni, and Zn) and non-essential (Ag, Cd, and Pb) metals were measured (atomic absorption spectrometry coupled to a graphite furnace; GF-AAS, AAnalyst 700, Perkin-Elmer, USA). Metal concentrations in the digested sample were determined based on calibration curves. A multi-elemental standard solution (Merck, Darmstadt, Germany) was employed to build these curves. Their coefficient of determination (r^2) ranged from 0.993 to 0.999. Results are expressed as µg g^{-1} dw. Analysis of blanks, spiked matrices, and a certified reference material (CRM; DORM-4, National Research Council Canada, Ottawa, ON, Canada) were performed and used as quality control and assurance procedures for metal determination. Metal recovery rates based on the CRM analysis ranged from 92 to 105%. The limits of detection (LOD) corresponded to 1.6, 0.6, 1.3, 2.2, 15.8, and 0.5 µg g^{-1} dry weight for Ag, Cd, Cr, Cu, Ni, Pb, and Zn, respectively. The limits of quantification (LOQ) corresponded to 4.7, 1.9, 3.8, 1.3, 6.6, 47.7, and 1.5 µg g^{-1} dry

weight, respectively. All determinations performed on the equipment and method blanks showed values below the LOQ, indicating that there was no contamination during the sample preparation and analysis.

Oxidative stress–related biomarkers were measured in homogenates of gills and liver samples. Selected biomarkers included antioxidant capacity against peroxyl radicals (ACAP), activities of catalase (CAT) and glutathione S-transferase (GST), concentration of metallothionein-like proteins (MTLPs), and lipid peroxidation. These biomarkers were chosen because they were found to be responsive to acute metal (Cu) exposure in *P. vivipara* in laboratory conditions [54]. As for other fish [27–33], ACAP, CAT, GST, and MTLPs play essential roles in the antioxidant defense system, while LPO is a typical response of the oxidative stress condition in the guppy *P. vivipara* [54]. ACAP was determined according to Amado et al. [76]. Results are expressed as 1/relative area. CAT activity was evaluated based on the H_2O_2 degradation, according to the method described by Beutler [77]. The method described by Keen et al. [78] was used to measure the GST activity. MTLP concentration was measured as described by Viarengo et al. [79]. The amount of total proteins was determined in the tissue homogenates using the Bradford method (Microprote, Doles, Goiânia, GO, Brazil) and used to normalize the CAT (μmol H_2O_2 mg protein^{-1} min^{-1}), GST (mmol CDNB mg protein^{-1} min^{-1}), and MTLPs (nmol GSH mg^{-1} protein) results. LPO was measured as described by Oakes and Van Der Kraak [80] and results are expressed as nmol MDA mg^{-1} wet weight.

2.3. Data Presentation and Statistical Analyses

Data on seawater parameters (n = 6 days per exposure site), tissue (gill and liver) concentrations of essential (Cr, Cu, Ni, and Zn) and non-essential (Ag, Cd, and Pb) metals (n = 10 samples per exposure site), and biomarkers (n = 10 samples per exposure site) are expressed as means (± standard error). For each parameter analyzed in seawater, mean values were compared between exposure sites using analysis of variance (ANOVA) with exposure site as the categorical predictor (One-Way ANOVA). For each parameter analyzed in fish tissues, mean values were compared using ANOVA with fish tissue and exposure site as categorical predictors (Two-Way ANOVA). In both cases, ANOVA assumptions were previously validated. For this, the normal probability plot for raw residuals was used to check data normality, while the Cochran C test was employed to verify the homogeneity of variances. Data on seawater physicochemical parameters, biomarkers, and the tissue concentrations of Cd and Pb were statistically analyzed without mathematical transformation. Data on tissue Ag, Cr, Cu, Ni, and Zn did not meet the ANOVA assumptions (i.e., data normality and homogeneity of variance). Therefore, they were decimal logarithm transformed to meet these assumptions. In all cases, significant differences were considered when $p < 0.05$ ($\alpha = 0.05$). When metal concentrations were <LOQ, data were replaced by the corresponding LOQ/2 to be statistically analyzed. Significantly different mean values were compared with the Tukey HSD test. Due to the high complexity, data on seawater physicochemical parameters, tissue metal concentrations, and biomarker responses were subjected to a principal component analysis (PCA). This analysis was employed to reduce the dimensionality of data, thus allowing for better identification and exploration of the relationships and importance of the variables to the data matrix. The statistical analyses were performed using the Statistica software 7.0 (StatSoft, Tulsa, OK, USA), while graphs were built using the SigmaPlot software (Systat Software, Chicago, IL, USA).

3. Results

Data on physicochemical parameters of the seawater collected at the three different exposure sites are shown in Table 1. For all parameters analyzed, there was no significant difference among the exposure sites (Table S1).

Table 1. Physicochemical parameters of seawater samples collected in the exposure sites of the euryhaline guppy *Poecilia vivipara* in the Linguado Channel (Babitonga Bay, Southern Brazil). Data are expressed as mean values (±standard error; n = 6). For each parameter analyzed, there was no significant difference between exposure sites (One-Way ANOVA; $p > 0.05$).

Physicochemical Parameter	Exposure Site		
	Site 1	Site 2	Site 3
Temperature (°C)	24.2 ± 1.1	22.5 ± 2.6	24.0 ± 1.1
Salinity	31.7 ± 1.6	31.3 ± 1.3	27.7 ± 2.6
pH	8.0 ± 0.2	7.9 ± 0.2	8.1 ± 0.1
Dissolved O_2 content (mg/L)	7.1 ± 0.4	7.3 ± 0.2	7.4 ± 0.2
Dissolved organic carbon (mg/L)	3.4 ± 0.3	3.3 ± 0.3	2.7 ± 0.4

The biometric data of exposed fish were as follows: Site 1 – WBW = 0.76 ± 0.04 g, TBL = 40.1 ± 0.8 mm, and SBL = 32.4 ± 0.6 mm; Site 2 – WBW = 0.84 ± 0.05 g, TBL = 40.8 ± 0.7 mm, and SBL = 33.6 ± 0.6 mm; and Site 3 – WBW = 0.77 ± 0.05 g, TBL = 39.1 ± 0.8 mm, and SBL = 33.5 ± 0.9 mm).

In fish gills, Ag and Cd concentrations were lower than their respective LOD. The ranges of Cr, Cu, Ni, Pb, and Zn concentrations were 2.3–12.7, 2.1–19.6, 0.10–1.42, 23.4–36.5, and 6.3–248.2 μg g^{-1} dw, respectively. In fish livers, Cd concentration was lower than its respective LOD. The ranges of Ag, Cr, Cu, Ni, Pb, and Zn concentrations were 0.92–11.5, 1.9–17.0, 353.9–4,565.9, 0.37–2.14, 23.5–41.1, and 14.0–691.8 μg g^{-1} dw, respectively. In both tissues (gill and liver), no significant differences were found between exposure sites for all analyzed metals (Figure 2; Table S2). Furthermore, no significant differences were found in gill and liver Cd, Cr, and Pb concentrations. This finding was independent of the exposure site (Table S2). However, depending on the sampling site, Ag, Cu, Ni, and Zn concentrations were higher in livers than in gills (Figure 2; Table S2).

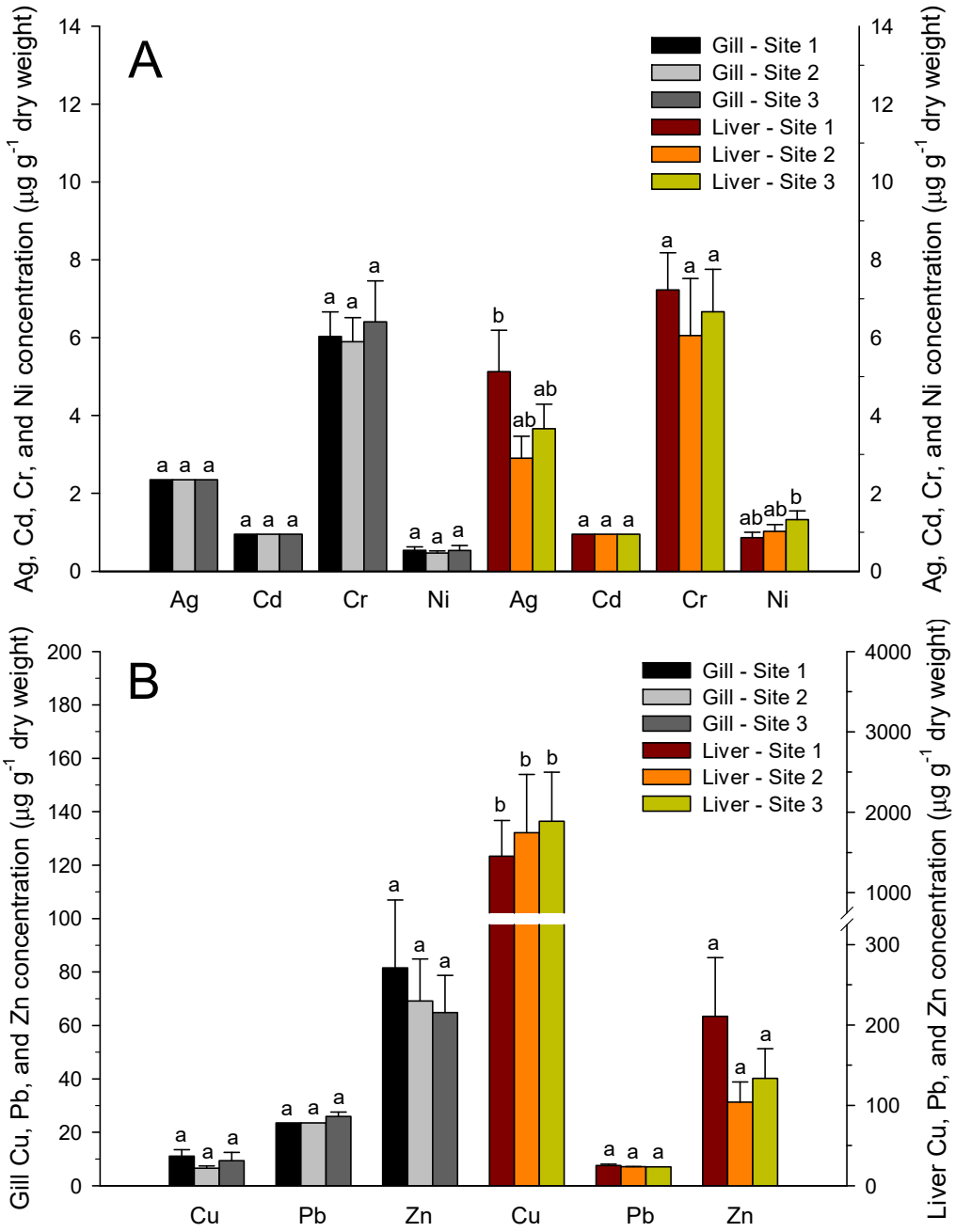

Figure 2. Concentrations of Ag, Cd, Cr, and Ni (**A**) and Cu, Pb, and Zn (**B**) in guppy (*Poecilia vivipara*) tissues (gills and liver). Fish were acutely (4 days) exposed in situ to three different sites (Sites 1–3) in the Linguado Channel, Babitonga Bay (Santa Catarina State, Southern Brazil). Data are expressed as mean ± standard error. Different letters indicate significantly different mean values between exposure sites and fish tissues for each metal analyzed (Two-Way ANOVA; post hoc Tukey HSD test; $p > 0.05$).

The biomarker responses were influenced by the exposure site (MTLP), tissue (CAT), or both factors (ACAP and GST). An interactive effect of both factors (exposure site and tissue) was also observed in the ACAP. No influence of these factors was observed on LPO. ACAP was higher in gills than in livers, while CAT and GST activities were higher in livers than in gills, regardless of the exposure site. ACAP was higher in the gills of fish exposed to Site 1 than in the gills of those exposed to Sites 2 or 3. ACAP was higher in the liver of fish exposed to Site 2 than in the liver of those exposed to Sites 1 or 3. For gills, GST was higher in fish exposed to Sites 1 or 3 than in those exposed to Site 2. For both tissues, fish exposed to Sites 2 or 3 showed higher MTLP concentration than those exposed to Site 1 (Figure 3; Table S3).

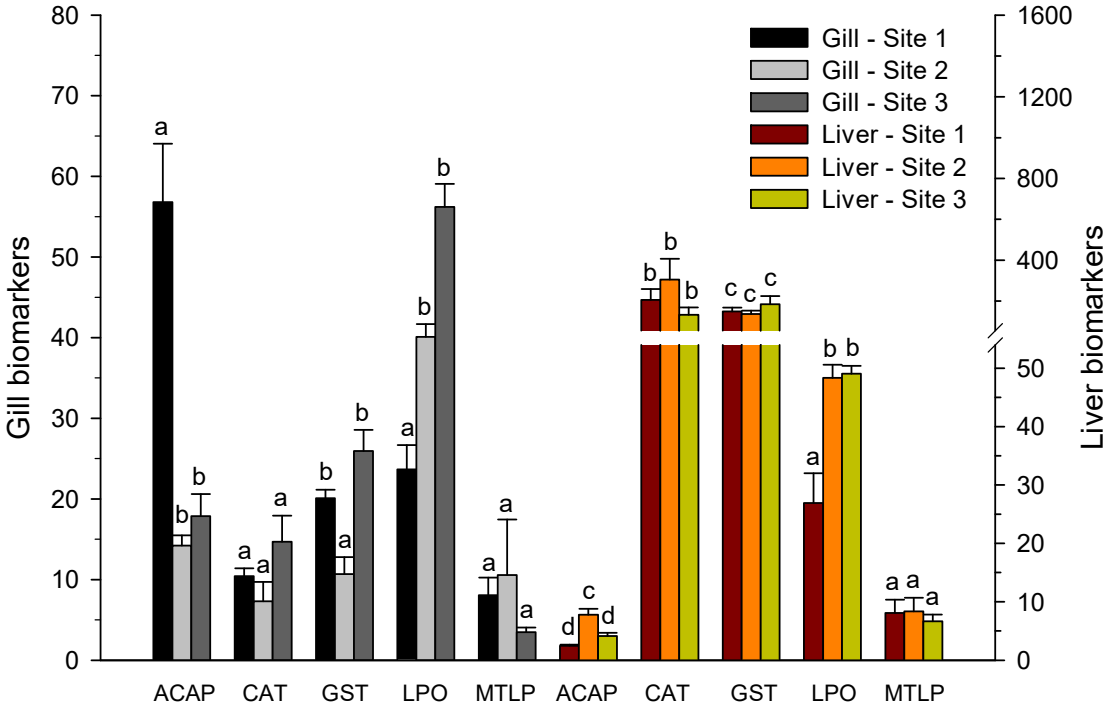

Figure 3. Biomarker responses in guppy (*Poecilia vivipara*) tissues (gills and liver). Fish were acutely (4 days) exposed in situ to three different sites (Sites 1–3) in the Linguado Channel (Babitonga Bay, Southern Brazil). ACAP: (1 relative area^{-1}) × 100; CAT: µmol H_2O_2 mg^{-1} protein min^{-1}; GST: (mmol CDNB mg^{-1} protein min^{-1}) × 100; MTLP: nmol GSH mg^{-1} protein, and LPO: nmol MDA mg^{-1} wet weight. Data are expressed as mean values (± standard error). For each parameter analyzed, mean values significantly different between exposure sites and fish tissues are represented by different letters (Two-Way ANOVA; post hoc Tukey HSD test; $p > 0.05$).

For the gill data, first two axes of the PCA explained 45% of the data variance. Approximately 29% of the data variance was explained in the first axis. This was particularly influenced by Ni, DOC, and ACAP, which were closely and positively correlated. In turn, MTLP was positively influenced by Ag and Cd. ACAP and MTLP were negatively correlated, with ACAP being strongly and negatively correlated with Ag and Cd. CAT and GST also contributed to the explanation of data variance in this axis, and they were closely and positively correlated with each other. However, they were strongly and negatively correlated with Ni and, to a lesser extent, with Cr. Essential (Cr and Ni) and non-essential (Ag and Cd) metals were negatively related. The second axis accounted for 17.8% and was influenced by LPO, which was closely and positively correlated with Zn and, to a lesser extent, with Cu. The explanation for data variance in the second axis was also associated with Cu and Pb, as well as seawater salinity and temperature. In this case, Pb was closely and positively correlated with seawater temperature. Essential (Cu and Zn) and non-essential (Pb) metals were negatively related (Figure 4A).

For the liver, the first two axes of the PCA accounted for 47% of the data variance. Approximately 25% of the data variance was explained in the first axis. This was mostly influenced by Cr and Zn, which were closely correlated. Ni and seawater salinity were also closely correlated and showed an important contribution to the data variance explanation in the first axis. In turn, GST was closely and negatively correlated with Pb. Essential metals (Cr, Ni, and Zn) were positively related. The second axis accounted for approximately 22% of the data variation and was especially influenced by CAT and DOC, which were closely and positively correlated. CAT was also

positively correlated with Ag, but strongly and negatively correlated with MTLPs and, to a lesser extent, with ACAP. This axis also had the contribution of MTLPs, Pb, and LPO. Despite the lower contribution of LPO, it was closely correlated with Pb. ACAP and MTLPs were positively correlated. GST and Cd, as well as seawater pH and dissolved oxygen content, also had an important contribution to the second axis. GST was primarily influenced by Cd and seawater pH. No clear relationship was observed between essential and/or essential metals (Figure 4B).

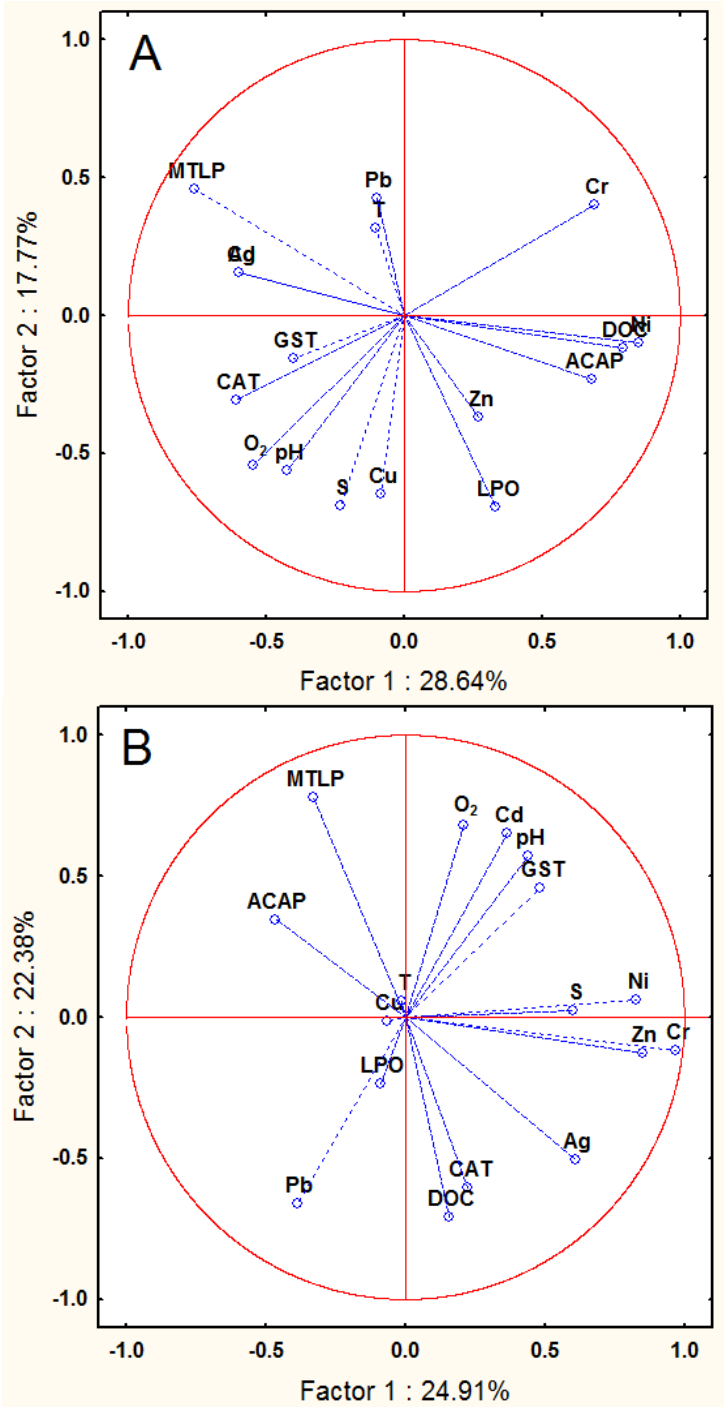

Figure 4. Principal component analysis (PCA) conducted with data on bay water parameters (T: temperature; S: salinity; O_2: dissolved O_2 content; DOC: dissolved organic carbon; and pH), concentrations of essential (Cr, Cu, Ni, and Zn) and non-essential (Ag, Cd, and Pb), and biomarker responses (ACAP: total antioxidant capacity; CAT: catalase activity; GST: glutathione S-transferase activity; LPO: lipid peroxidation; and MTLPs: metallothioneins-like proteins) in tissues (**A**): gills; and (**B**): liver) of the euryhaline guppy *Poecilia vivipara* exposed (96 h) in situ to three different sites (Sites 1–3) in the Linguado Channel, Babitonga Bay (Santa Catarina State, Southern Brazil).

4. Discussion

Frequent and marked changes in seawater conditions are typically observed in coastal zones. Changes in water conditions, especially pH, temperature, salinity, and dissolved organic matter, can alter the bioavailability, bioaccumulation, and consequently the toxicity of metals [52,81]. Historically, the Babitonga Bay faces threats from industrial and domestic effluents, particularly from Joinville city. Industrial and domestic effluents reach the bay via the Saguaçu Lagoon and Cubatão River, which significantly impacts water quality [66]. In the present study, physicochemical parameters measured in seawater samples along the experimental period were similar in the three sites of *P. vivipara* exposure in the Linguado Channel, Babitonga Bay. This finding is in line with the fact that, even though the Babitonga Bay receives water flows from many rivers, it is characterized as a homogeneous estuary with respect to its physicochemical parameters [65]. In turn, biotic factors affecting metal uptake and bioaccumulation by marine fish are the species' physiological traits and individual body condition [52,82,83]. In the present study, only one fish species was used, the euryhaline guppy *P. vivipara*. Furthermore, the biometric data of individuals exposed in the three different sites were very similar. Therefore, as observed here, no significant differences in tissue (gills and liver) concentrations of the analyzed metals (Ag, Cd, Cr, Cu, Ni, Pb, and Zn) would be expected among fish from the three exposure sites.

Regarding the distribution of metals in *P. vivipara*, when significant differences between tissues were observed, metal (Ag, Ni, and Cu) concentrations were always higher in the liver than in the gill. This finding agrees with the results of previous studies with other fish species [24,84–86]. Also, as expected, fish liver showed much higher CAT and GST activities than the gills. In fish liver, CAT is involved in metal detoxification by breaking hydrogen peroxide (H_2O_2) into water and oxygen. This catalytic process is crucial to avoiding metal-induced ROS generation and consequently oxidative stress and damage to biomolecules [87]. In turn, ACAP was much higher in the gills than in the liver. Peroxyl radicals are generated during the normal metabolic process in fish, but their formation can be enhanced in the presence of contaminants, such as metals. As a type of ROS, peroxyl radicals can cause oxidative damage to biomolecules, such as lipids. Protection against the potential oxidative damage from peroxyl radicals is provided by ACAP [76,88]. Therefore, the findings reported here on metal distribution and biomarker responses can be explained because the liver is a primary site for metal storage and detoxification in marine fish, while gills are mainly involved in gas exchange and are more prone to metal contamination from the surrounding water [24,87].

Unlike what was observed for metals, some oxidative stress–related parameters (ACAP, GST, and LPO) varied according to the location where the fish were exposed in the Linguado Channel, Babitonga Bay. As no significant spatial changes in the seawater physicochemical parameters and fish body condition were observed between the three sites of exposure, it can be assumed that the observed spatial changes in the biomarker responses are associated with other factors. These variations are likely associated with the responses of biomarkers to the temporal and spatial changes observed in the concentrations of several metals in sediments and water samples from the Linguado Channel [72–75,89]. Another potential explanation is the fact that contamination in the Babitonga Bay is characterized by the presence of chemical contaminants other than metals [72–75], such as polycyclic aromatic hydrocarbons [89]. Therefore, further studies focused on the spatial variation of other classes of chemical contaminants would help to explain the differences observed in ACAP, GST, and LPO between the fish exposure sites. In this context, it is important to consider the differential spatial responses observed for these oxidative stress-related biomarkers in both gill and liver.

As observed for the physicochemical seawater conditions and fish body condition, a lack of spatial variation in the average concentrations of metals in fish tissues was also found. However, metal concentrations in each fish tissue (gill and liver) varied considerably within each site of fish exposure in the Linguado Channel. In fish gills, minimum and maximum average values varied 5.5-, 9.3-, 14.2-, 1.6-, and 1.6-fold for Cr, Cu, Ni, Pb, and Zn, respectively. In fish liver, they varied 12.5-, 8.9-, 12.9-, 5.8-, 1.7-, and 49.4-fold for Ag, Cr, Cu, Ni, Pb, and Zn, respectively. As mentioned above, temporal and spatial changes in the concentrations of several metals have been reported in sediments and water samples from the Linguado Channel [72–75,89]. This could explain, at least in part, the wide ranges of metal concentrations observed in tissues within each exposure site. Tissue bioaccumulation generally reflects the bioavailability and impact of metals in their surroundings [13,90]. For this reason, we employed the PCA approach to identify the most important parameters that explained the variance in the original data. This analysis included the data on the physicochemical seawater parameters, tissue metal concentrations, and biomarker responses.

Results indicated that the first two components of the PCA explained almost half of the variation in the original data for both tissues (gill: 46.41%; liver: 47.29%). This finding confirms the expected impact of metal contamination in the Linguado Channel (Babitonga Bay) on key biomarkers associated with fish oxidative status. As hypothesized,

metal influence on the biomarker responses was also dependent on the fish tissue. Overall, gill biomarkers were responsive to all analyzed metals (essentials and non-essentials), except for Pb. In turn, liver biomarkers were responsive only to non-essential metals (Ag, Cd, and Pb). Furthermore, different profiles of biomarker responses were observed in the gill and liver. In gill, all oxidative stress–related biomarkers were responsive to tissue metal concentrations. In turn, only the enzymatic antioxidants (CAT and GST) and the biomarker of oxidative damage to biomolecules (LPO) were responsive to tissue metal concentrations in the liver. In this context, it is interesting to note that PCA results indicated that essential and non-essential metals were negatively related in the guppy gills, indicating a potential competition between these two classes of metals. In turn, no clear relationship was observed in the guppy liver. These findings can be explained considering the different patterns of accumulation, storage, and excretion of essential and non-essential metals. As for other fish, *P. vivipara* accumulated metals from their environment and food and showed different patterns of storage and excretion that varied by metal and the specific organ [91–94]. Another possible explanation for our findings is the different roles played by gills and liver in fish, which result in varying capacities of their antioxidant systems to protect against oxidative stress. The gills are involved in gas exchange; therefore, they are more sensitive to metal contamination from the surrounding water, which is important for uptake and essential for excretion. In turn, the liver is the primary site for metal storage and detoxification [24,87]. Accordingly, different profiles of the antioxidant system between tissues were observed in the guppy *P. vivipara*, with the gills showing a much higher ACAP than the liver. In turn, the liver presented higher activities of antioxidant enzymes (CAT and GST) than the gills.

The PCA results also pointed out several close (positive or negative) and interesting correlations between metal concentrations and biomarker responses. ACAP and MTLPs were positively correlated in the guppy liver, thus reinforcing the potential role of metallothioneins as non-enzymatic antioxidants in this organ [29,30]. The protective role of these macromolecules by binding metals was also clearly observed in the gills of *P. vivipara*, which are in close and direct contact with metals present in the surrounding seawater [34,35]. A close and positive correlation was observed between the gill concentrations of MTLPs and Ag and Cd concentrations. Another interesting relationship was observed between DOC and ACAP in the gills of *P. vivipara*. The close and positive relationship found between these parameters, highlights the key role of DOC against metal toxicity to fish gills. DOC can complex with metals, thus reducing their bioavailability and absorption [95]. It is expected that a lower metal absorption by the gills would lead to a lower use of non-enzymatic antioxidants, thus resulting in a higher gill ACAP, as observed here. It is worth noting that this relationship was not observed for liver ACAP.

Regarding the enzymatic antioxidants, gill CAT and GST showed a close and positive correlation. In the gills of marine fish, this positive correlation often indicates an antioxidant response to metal-induced oxidative stress [96]. In this context, it is worth noting the negative relationship observed between CAT and GST with ACAP in the guppy gills, as well as the negative correlation observed between CAT and ACAP in the guppy liver. These findings can be explained by considering the increased activity of these enzymes and a higher consumption of non-enzymatic antioxidants to counteract the harmful effects of ROS [31,32].

The PCA results also indicated important relationships between metal concentrations and the oxidative stress–related parameters in both fish tissues. In the guppy gill, the strong positive correlation observed between MTLPs and Ag and Cd, as well as the lower influence of Pb on MTLPs, can be explained by considering that these non-essential metals are known inducers of metallothionein synthesis in fish [96–101]. In turn, the positive influence of Ag and Cd in CAT and GST activities is in line with the fact that these non-essential metals are inducers of ROS generation and antioxidant enzyme activities in fish tissues [102–108]. On the other hand, the strong negative correlation between Cr concentration and CAT and GST activities reinforces the potential inhibitory effect of this metal on the activity of antioxidant enzymes [109,110]. In turn, the strong positive influence of Zn and the lower positive influence of Cu on LPO agree with the known oxidative stress induced by fish exposure to these essential metals [52,54,111–113].

In the guppy liver, PCA results indicated a positive influence of Ag on CAT activity, while Cd was strongly correlated with GST. As already mentioned for gills, these findings can be explained by considering the inducing effect of these non-essential metals on ROS generation and antioxidant enzymes inhibition [102–108]. While LPO in gills was positively influenced by Cu and Zn, this oxidative stress parameter was positively and less influenced by Pb. This non-essential metal can induce oxidative damage to biomolecules, including lipids, in fish liver [114,115]. Finally, a negative correlation was observed between several essential metals (Cr, Ni, and Zn) and ACAP, indicating an augmented consumption of antioxidants with increasing concentrations of these elements. Aquatic contamination with essential metals is widely reported to deplete antioxidant capacity in fish liver [28,115,116].

Our findings obtained with *P. vivipara* exposed to a coastal bay (average salinity 28–32 ppt) with a long history of metal contamination, including Cu, are comparable to those obtained with this estuarine guppy acutely exposed to Cu in saline water (24 ppt) under laboratory-controlled conditions [53,54]. In the laboratory, almost all

tested biochemical parameters [antioxidant enzyme activities (CAT, GST, superoxide dismutase, and glutathione reductase)], MTLPs, ROS, ACAP, and LPO] were influenced by Cu exposure. However, CAT (liver) and ROS, ACAP, and LPO (gills and liver) were the most responsible biomarkers for increasing Cu levels in saline water [53,54]. Considering that a strong correlation was observed here between LPO and Cu and Zn concentrations in the guppy gills, this biological parameter seems to be a good biomarker of fish exposure in the field to excessive concentrations of essential metals. Regarding the liver, no marked influence of Cu concentration was observed on the oxidative stress-related biomarkers evaluated here. Despite this, ACAP was strongly and negatively influenced by the concentrations of several other essential metals (Cr, Ni, and Zn). Therefore, ACAP seems to be a reliable biomarker of fish exposed to excessive concentrations of essential metals in the field.

Regarding non-essential metals, our findings suggest that gill CAT and GST activities are reliable biomarkers for fish exposure to Cr. In contrast, MTLPs and ACAP in gills appear to be effective biomarkers for environmental exposure to Ag and Cd. In turn, liver CAT, MTLP, and ACAP were shown to be responsive to environmental exposure to Ag, while liver GST was a reliable biomarker of environmental exposure to Cd and LPO was a promising biomarker of environmental Pb contamination.

As observed in the present study with the euryhaline guppy *P. vivipara*, oxidative stress–related parameters, such as LPO, CAT, GST, and ACAP, are reported as reliable biomarkers of freshwater and coastal fish exposure to essential and non-essential metals, both under controlled-laboratory conditions and in the field [38,47,48,50,117,118]. Therefore, our findings support and reinforce the idea that these biomarkers provide sensitive indicators of metal contamination and its associated health impacts on coastal fish. Certainly, monitoring these biomarkers can help assess the potential risks of metal pollution in aquatic ecosystems, as well as inform biomonitoring programs and ecotoxicological studies. As already explored by our research group using fish and other coastal organisms [37,48,119,120], data from a multi-biomarker approach associated with metal bioaccumulation analysis can help to identify and demonstrate the impacts of environmental pollution involving essential and non-essential metals in coastal ecosystems. In this context, the seasonal variations in the response of biomarkers need to be taken into account. It is also essential to consider and integrate the potential synergistic effects of water physicochemical parameters, as well as other inorganic and organic contaminants, on the responses of the biomarkers analyzed here [121–125]. This certainly will improve the adequacy and reliability of using oxidative-stress related biomarkers as alternative tools in future biomonitoring programs in coastal waters.

5. Conclusions

Our findings reinforce the reliability of using oxidative stress–related biomarkers to evaluate and monitor metal contamination in coastal waters. Furthermore, they highlight the need for considering the most reliable biomarkers in the different fish tissues to evaluate and monitor the biological impacts of metal contamination in coastal waters. Our findings also strengthen the need for combining chemical and biological approaches in the scope of environmental health monitoring programs. Furthermore, they highlight the importance of this combination for a better integration and interpretation of data from ecotoxicological studies. Finally, future studies using oxidative stress–related biomarkers in the guppy *P. vivipara* after long-term in situ exposure to coastal waters contaminated with metals are encouraged. Results from these studies would help increase the suitability and usefulness of these biomarkers in coastal management and ecotoxicological evaluations. They could also help to better understand the spatial and temporal variations in the biological impact of metals, as well as other inorganic and organic contaminants, which is crucial in future biomonitoring efforts.

Supplementary Materials

The additional data and information can be downloaded at: https://media.sciltp.com/articles/others/250910102159509 3/112-revised_Supplementary_Material.pdf. Table S1: Comparison between the mean values of the physicochemical parameters of seawater (T: temperature; S: salinity: pH; O_2: dissolved oxygen content; and DOC: dissolved organic carbon) in the three exposure sites (Sites 1–3) of the euryhaline guppy *Poecilia vivipara* in a coastal water environment with long history of metal contamination. Table S2: Comparison (Two-Way ANOVA followed by the Tukey HSD test) between mean concentrations of metals (Ag, Cd, Cr, Cu, Ni, Pb, and Zn) in tissues (1: gills; 2: liver) of the euryhaline guppy *Poecilia vivipara* exposed (96 h) to three different sites (Sites 1–3) in a coastal water environment with long history of metal contamination. Table S3: Comparison (Two-Way ANOVA followed by the Tukey HSD test) between mean values of biomarkers (ACAP, CAT, GST, LPO, and PSMT) in tissues (1: gill; 2: liver) of the euryhaline guppy *Poecilia vivipara* exposed (96 h) to three different sites (Sites 1–3) in a coastal water environment with long history of metal contamination.

Author Contributions

A.J.C.M.: methodology, investigation, formal analysis, data curation, validation, writing—reviewing and editing; A.A.d.S.M.: methodology, investigation, formal analysis, data curation, validation, writing—reviewing and editing; M.B.J.: methodology, investigation, formal analysis, data curation, validation, writing—reviewing and editing; J.d.S.F.: methodology, investigation, formal analysis, data curation, validation, writing—reviewing and editing; M.M.L.: methodology, investigation, formal analysis, data curation, validation, writing—reviewing and editing; C.B.d.R.M.: conceptualization, methodology, investigation, formal analysis, resources, writing—reviewing and editing, project administration, funding acquisition; A.B.: conceptualization, methodology, investigation, formal analysis, resources, data curation, validation, writing—reviewing and editing, supervision, project administration, funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the International Development Research Centre (IDRC, Ottawa, Canada; grant # 451-PR-08), International Copper Association (ICA, New York, NY, USA; grant # 25686 A-07 2013), 'Coordenação de Aperfeiçoamento de Pessoal de Ensino Superior' (CAPES – Programa Ciências do Mar, Brasília, DF, Brazil; grant # 84/2010), and 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' (CNPq – Instituto Nacional de Ciência e Tecnologia de Toxicologia Aquática, Brasília, DF, Brazil; grant # 573949/2008-5). A.B. is a research fellow from the Brazilian CNPq (Proc. # 311410/2021- 9) and was supported by the International Canada Research Chair Program from IDRC.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Institutional Ethics Committee (CEUA) of Universidade Federal do Rio Grande—FURG (protocol code 23116.001413/2014-13; date of approval: 16 June 2014).

Informed Consent Statement

Not applicable.

Data Availability Statement

Data you be available upon request.

Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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