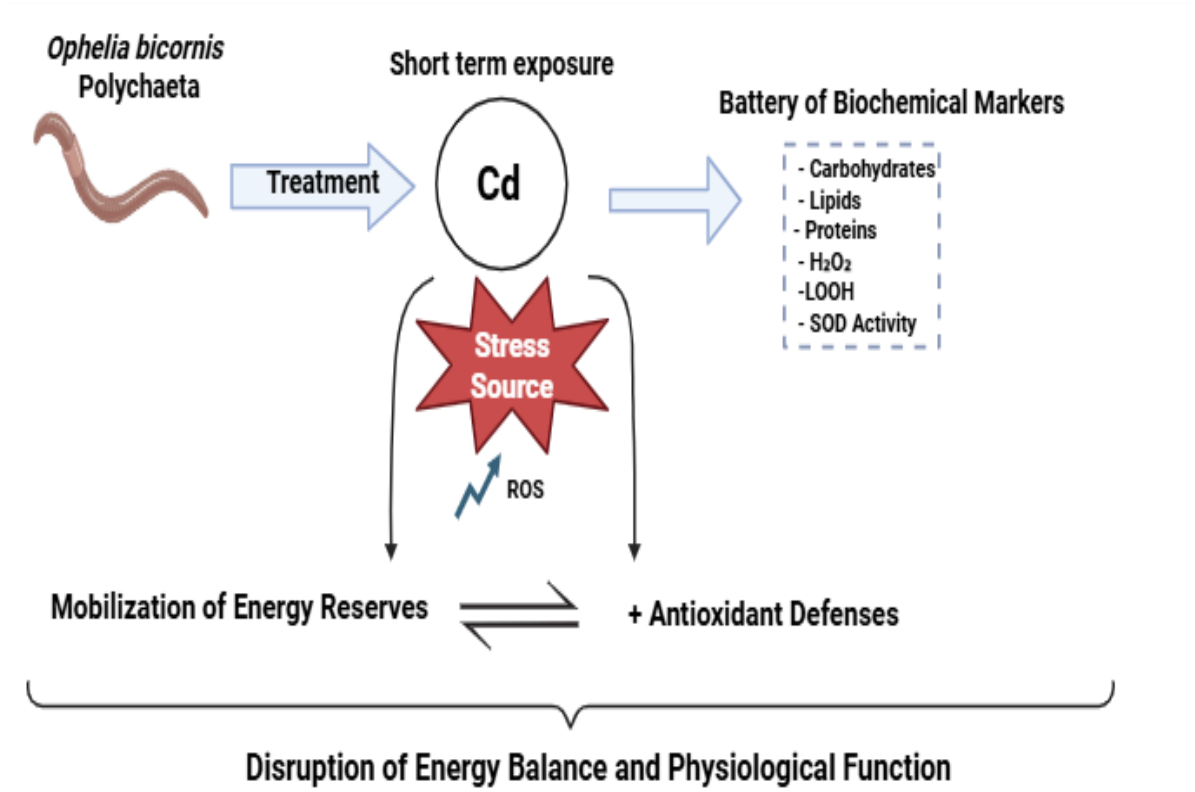
Graphical abstract



Short-term effects of cadmium exposure on energy reserves and stress markers on *Ophelia bicornis* Savigny, 1822 (Polychaeta: Opheliidae)

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Abstract

Ophelia bicornis (Polychaeta: Opheliidae) has been identified as a potential bioindicator for ecotoxicological studies. This study evaluates the short-term physiological and biochemical effects of cadmium (Cd) exposure on *O. bicornis* by examining energy reserves (lipids, carbohydrates, proteins) and oxidative stress biomarkers (hydrogen peroxide, lipid peroxide (LOOH), and Superoxide dismutase (SOD). Cd treatment was conducted at a sublethal concentration LC_{10} obtained after 48h of exposure (31.11 mg/L) as previously determined. Statistical analyses revealed significant interactions between Cd treatment and exposure time affecting several biochemical parameters. Amounts of Carbohydrates increased notably after 48h in Cd-exposed individuals, indicating a time-dependent energy mobilization. Lipid contents were also affected by treatment and exposure duration, reflecting energy reserve use under metal stress. In contrast, protein remained stable, with no significant variation across treatment or time. Oxidative stress biomarkers were markedly influenced by Cd exposure. LOOH levels increased significantly with treatment and time, peaking at 48h. Similarly, H_2O_2 increased with Cd and exhibited a significant time-dependent interaction, highlighting a progressive oxidative challenge. SOD activity was primarily modulated by exposure duration, reaching its maximum at 48h. Overall, the present experiment shows that sublethal Cd disrupts energy metabolism and triggers oxidative stress response in *O. bicornis*.

Keywords*:* *Ophelia bicornis*, Bioindicator, Toxicity, Energy metabolism, Oxidative stress, Biomarkers.

1. Introduction

In recent years, it has become evident that, to fully assess the impact of anthropogenic trace metal inputs on aquatic ecosystems, it is essential to understand the chemical and physiological processes that govern metal accumulation in organisms, as well as how these metals influence energy reserves and antioxidant activities. Many toxicants produce a sublethal effect by binding to or interfering with essential cellular components such as enzymes and metabolites, thereby disrupting

fundamental processes within the organism (Holmstrup *et al.* 2010). They can disrupt energy metabolism, deplete energy stores, and induce oxidative stress, ultimately affecting overall health and survival of aquatic organisms (Song *et al.* 2023). These substances are particularly known for their toxic effects, persistence, and tendency to transfer and accumulate through all the trophic levels (Di Salvatore *et al.* 2013). Trace metals are among the most extensively studied groups of xenobiotics (Pan and Wang 2012). Besides, various chemical substances, including cadmium, can exert multiple mechanisms of toxicity, each contributing to the overall harmful effects (Valavanidis *et al.* 2006; Regoli and Giuliani 2014). Cadmium (Cd) is a heavy metal hazardous to all living organisms, and its effects have been studied on various organisms (Sharma *et al.* 2015). It is a non-essential element that can be toxic even at trace levels for aquatic species. Cadmium is a highly toxic environmental pollutant that poses a significant threat due to its tendency to accumulate in marine ecosystems, its ability to easily cross cell membranes, and its persistence in the environment, which can be found at high concentrations in seawater (Amira *et al.* 2018). Polychaetes play an important role in the functioning of benthic communities (Hutchings 1998). They represent 35 to 50% of benthic macrofauna and are often the numerically dominant macrobenthic taxon. Found in coastal and littoral ecosystems, where the distribution of species in soft-bottom habitats is primarily influenced by sediment particle size (Gambi and Giangrande 1986) and organic matter content, they are frequently exposed to various contaminants. Due to their sensitivity and ecological significance, polychaetes serve as excellent indicators for studying the effects of pollutants and the transfer of xenobiotics through food chains (Dean 2008).Consequently, they have been extensively used in coastal studies for environmental monitoring and are considered a key group in ecotoxicological research (Scaps 2000).Furthermore, Polychaete Annelids are a major link in food webs and are widely consumed by many species, such as crustaceans, fishes, and birds. Among them, the endobenthic species *Ophelia bicornis* (Savigny 1818) plays a crucial role in evaluating the structure and functioning of estuarine ecosystems. This polychaete is particularly notable for its physiological tolerance to various environmental stressors, especially its resistance to

sediment-bound contaminants. Accordingly, reduced energy reserves and elevated metabolic rates in animals have associated to metal toxicity, especially in the case of cadmium, Zinc and copper (Pook *et al.* 2009). Recent studies have explored advanced analytical and computational approaches for environmental pollution assessment. Deep learning and optimization-based models have been applied to classify and predict air pollution levels and analyze environmental data (Mohandas *et al.* 2025b, 2025a). Additionally, hybrid recurrent neural network models combined with Internet of Things (IoT) data have been proposed for urban air pollution prediction (Mohandas *et al.* 2025c). Beyond atmospheric pollution, graph-based and remote sensing-driven approaches have been developed for environmental monitoring (Sivasubramanian *et al.* 2025), and early detection of ecological stress, such as pest disease identification in agricultural systems (Maruthai *et al.* 2025; Mohandas *et al.* 2025a). While these studies provide valuable large-scale insights, experimental ecotoxicological investigations remain essential for understanding species-specific biochemical responses to metal exposure. *Ophelia bicornis*, which commonly inhabits organic-rich environments, is considered a key species for ecological assessments and a reliable bioindicator for monitoring heavy metal pollution (Bat *et al.* 2016). In this context, *Ophelia bicornis* could be effectively used as a bioindicator species in ecotoxicological studies of marine and coastal ecosystems along the eastern Algerian coast to assess metal contamination (Labiod and Belabed 2024).This species is distributed across the North, Mediterranean, Black Sea, and Atlantic coasts of North Africa and Europe, typically inhabiting high-energy intertidal zones and fine to medium sediments (Maltagliati *et al.* 2005).

The presence of a xenobiotic in the environment always poses a risk to living organisms. However, the relationship between toxic concentrations and the toxic response is fairly complex and difficult to predict, as it depends on several factors, including toxicokinetics and genetic variability. One method of quantifying xenobiotic interaction and its potential impact on living organisms is through biomarker monitoring. Therefore, biomarkers measure exposure, toxicity, and individual susceptibility to environmental chemicals, making them valuable tools for assessing and monitoring

the risk of long-term effects associated with exposure to xenobiotics(Depledge 2020). Generally, biochemical biomarkers are frequently used to identify early signs of stress or toxicity in benthic fauna (Mayer *et al.* 2018; Depledge 2020). One of the main consequences of metal toxicity is additional energy costs, and the resulting metabolic load can lead to disruption in oxidative metabolism and increased anaerobic activity (Gashkina 2024). Furthermore, metal toxicity mainly involves changes at biological levels of cellular activities, including reactive oxygen species (ROS) generation, which can cause damage associated with the different pathological processes (Makhdoumi *et al.* 2020). Likewise, Cd accumulation has also been reported to alter key biochemical and physiological functions (Wright and Welbourn 1994)and to induce the production of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2), and lipid peroxide (LOOH) (Benedetti *et al.* 2015). A significant increase in the tissue concentration appeared 48h after exposure in *Donax trunculus*, following treatment with LC_{50} (2.59 mg/L) and LC_{25} (1.32 mg/L) of Cd (Belabed and Soltani 2018). Moreover, the presence of cadmium chloride ($CdCl_2$) in seawater led to mortality in the marine benthic species *Donax trunculus* at concentrations corresponding to LC_{10} (0.72 mg/L) and LC_{50} (2.59 mg/L) after 96h of exposure, and also affected the biomarker responses such as acetylcholinesterase (AChE) and Catalase (CAT) activities (Belabed and Soltani 2022). Thus, some biomarkers such as Superoxide dismutase, catalase, glutathione peroxidase, glutathione, and glutathione reductase are indicative of oxidative stress caused by exposure to pro-oxidants (Demirci-Çekiç *et al.* 2022), whereas metallothionein contents are widely used as biomarkers of metal contamination by binding and removing toxic metals (Cosson and Amiard 2021). These antioxidant defenses aim to protect cells and tissues from oxidative damage by neutralizing the toxicity of ROS (Singh *et al.* 2024).

In previous studies (Belabed and Soltani 2018, 2022; Merad *et al.* 2024), Cd was tested on a Molluscan species, *Donax trunculus*, widely used as bioindicator of marine pollution. The present study aimed to investigate the short-term effects (24, 48h) of cadmium exposure at a sublethal concentration LC_{10} (31.11 mg/L) (Labiod and Belabed 2024) in the marine organism *O. bicornis*, to

assess the extent of metal contamination. Acute toxicity data on *O. bicornis* and its responses to toxic substances remain scarce in the scientific literature. In light of the limited data available, a battery of stress indices was employed, including SOD activity, the generation of free radicals, and the biochemical composition. Characterization of the response of stress indices could determine the possible adverse effects of cadmium on worms and evaluate the potential harmful impacts on aquatic environments.

2. Materials and methods

2.1 . Sampling and contamination protocol

The annelid *O. bicornis* was collected at low tide by hand from littoral sediments in the intertidal zone (depth greater than 20 cm) at El Hennaya Beach (36°54’24.00" N, 08°7’43.62" E), that is located in the district of Berrihane, which belongs to the province of El Tarf (Northeast Algeria).This site is not exposed to any source of pollution because of its location, which is quite remote from the various discharges.Once in the laboratory, the samples were transported in plastic buckets with sediment from the collection site, and they were acclimatized for 48 hours. Afterwards, the collected worms were separated from the residue, cleaned from debris with seawater from the site of origin of worms, and they were transferred to the glass Petri dishes containing filtered natural seawater for another period of acclimatization (24h) in the dark at a temperature of 16°C. This acclimatization phase establishes stable experimental conditions and enhances the accuracy of toxicity test results, reflecting the actual effect of the toxic substance on organisms. Sublethal concentrations of Cd (LC_{10}) were administered to lots (n = 30 for each treatment). Thus, every 24 hours throughout the exposure period, worms (0.3 g in weight) were sampled andimmediately homogenized (10% w/v) in Tris-HCl buffer (100 mM; pH = 7.4). The homogenates were centrifuged at 9000×g for 20 min at 4 °C. The supernatant was then preserved at -80°C until biochemical analysis. Analyses were performed to assess the total lipid, carbohydrate, and protein contents as well as oxidative stress responses by measuring Superoxide dismutase (SOD) activity and quantifying Hydrogen peroxide (H_2O_2) and Lipid hydroperoxides (LOOHs)

levels. Assays were conducted on six individuals in both the treated and control groups, spanning a treatment period of 48 hours.

2.2 . Energy reserve analysis

In this experimental procedure, biochemical analyses concerned only the animals that survived the two days of Cd exposure. The extraction of protein, carbohydrate and lipid contents is determined according to Shibko *et al.*(1966). The method involves treating a homogenate by adding 1 ml of 20% trichloroacetic acid (TCA) to the sample. After grinding with ultrasound (Sonifier B-30), centrifugation at 5000 × g for 10 minutes at 4°C yields a supernatant 1 that is collected use in the total carbohydrate analysis, while the pellet 1 undergoes washing with 1 ml chloroform ether (1V/1V), centrifuged again (5000 g for 10 min). To determine the carbohydrate content of the supernatant fraction, 4 ml of anthrone was added to 100 µl of supernatant, and quantification was performed by measuring absorbance at 620 nm against a standard curve of glucose standard (Sigma, USA) (Duchateau and Florkin 1959). The supernatant 2 recovered will be used for the determination of the total lipids based on the vanillin method of (Goldsworthy *et al.* 1972) and absorbance of the samples was measured at 530 nm. The remaining pellet 2 was re-suspended in 1ml NaOH (0,1 N) used for assessing the total protein content (Bradford, 1976a) (Sigma, USA). The absorbance was measured at 595 nm using bovine serum albumin (Sigma, USA) as a standard. The contents of carbohydrates, proteins and lipids were expressed in µg/mg of body weight.

2.3 . Superoxide dismutase (SOD) activity

The superoxide dismutase (SOD) activity was determined by monitoring the photochemical reduction of NBT according to the method of Beauchamp and Fridovich (1971). One unit of SOD activity corresponded to the amount of enzyme required to cause 50% inhibition of NBT reduction at 560 nm measured by a spectrophotometer. The SOD activity was expressed as U/mg of protein.

2.4 . Hydrogen peroxide (H_2O_2)

Analysis of hydrogen peroxyde (H_2O_2) levels were carried out by the method described by Ou and Wolff (1996), using the ferrous ion oxidation xylenol orange assays (FOX1). The amount of H_2O_2 in the supernatant was determined at 560 nm using a spectrophotometer. Values were expressed as mmol/mg of protein.

2.5 . Lipid hydroperoxides (LOOHs) content

Lipid hydroperoxides (LOOH) formation was estimated according to the method described by Jiang *et al.* (1992) using the ferrous oxidation in xylenol orange assay. Hydroperoxides content was colorimetrically determined at 560 nm. Results were expressed as mmol/ mg of protein.

2.6 . Total Protein quantification

Protein content was done by the method of Bradford (1976), which is based on the binding of Coomassie brilliant blue G -250, present in the Bradford reagent, to the total protein present in the sample, yielding a stable staining compound. The presence of this compound can be spectrophotometrically quantified at 595 nm.

2.7 . Statistical analysis

All statistical analyses were conducted using R software (version 4.5.0; R Core Team 2025). Prior to hypothesis testing, data normality and homogeneity of variances were assessed using the Shapiro–Wilk test and Levene's test, respectively. For carbohydrate content, which met normality and homogeneity assumptions ($p > 0.05$), two-way ANOVA was applied to evaluate the effects of Exposure Time, Treatment, and their interaction. Significant effects were followed by Tukey's HSD post hoc test for pairwise comparisons while controlling for multiple testing. For all other measured biomarkers (lipids, proteins, LOOH, H_2O_2, and SOD), which did not meet normality assumptions, the non-parametric Scheirer–Ray–Hare test was used as a factorial alternative to ANOVA, with significant effects further analyzed using Dunn's test for multiple comparisons. Exploratory data analysis was performed to visually assess variable distributions across experimental groups defined by Exposure Time and Treatment. Boxplots summarizing median, interquartile range, overall

dispersion, and potential outliers were generated using the *ggplot2* package (version 3.4.2; Wickham 2016), supporting the evaluation of model assumptions. Bar plots with error bars (mean ± SD) were used to facilitate visual comparison of central tendencies and variability. To explore overall variation patterns and relationships among biomarkers, Principal Component Analysis (PCA) was performed using the *FactoMineR* package (version 2.7; Lê *et al.* 2008), and results were visualized with the *factoextra* package (version 1.0.7; Kassambara and Mundt 2020). Redundancy Analysis (RDA) via the *vegan* package (version 2.6-4; Oksanen *et al.* 2022) quantified the proportion of variance in biomarker responses explained by Exposure Time and Treatment. All results are presented as mean ± standard deviation (SD), and the number of biological replicates used in each assay is reported in the Results section. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Changes in the energy reserves

All energy reserve components exhibited large variations. Generally, changes in energy reserve responses are detected after prolonged exposure, over several hours or days. Proteins were quantitatively the most important energy fractions in the biochemical composition of *O. bicornis* (28,88 µg/mg of tissue), whereas carbohydrates and lipids represented respectively only 3,93 and 1,93 µg/mg of tissue.

3.1.1. Carbohydrates contents

The values (µg/mg of Tissue) obtained in *Ophelia bicornis* are presented in figure 1. The two-way ANOVA revealed a marginally significant interaction between the factors Time and Treatment ($p = 0.0569$), suggesting that the effect of the treatment on Carbohydrates contents depends on the duration of exposure. No significant main effects were detected for Time ($p = 0.0672$) or for Treatment ($p = 0.1534$), indicating that these factors alone do not account for the observed variations (Figure 1, A). However, the interaction between these two factors highlights a combined influence on the measured response.

Post hoc analysis using Tukey's test showed a significant increase in Carbohydrates after 48 hours in the Cd treated series compared to the initial time point (0 hours) ($p = 0.0287$), indicating a Time-dependent effect of the treatment (Figure 1, B). In contrast, no significant differences were observed within the controls group over time, confirming that the variations in concentration over time are primarily attributable to the treatment.

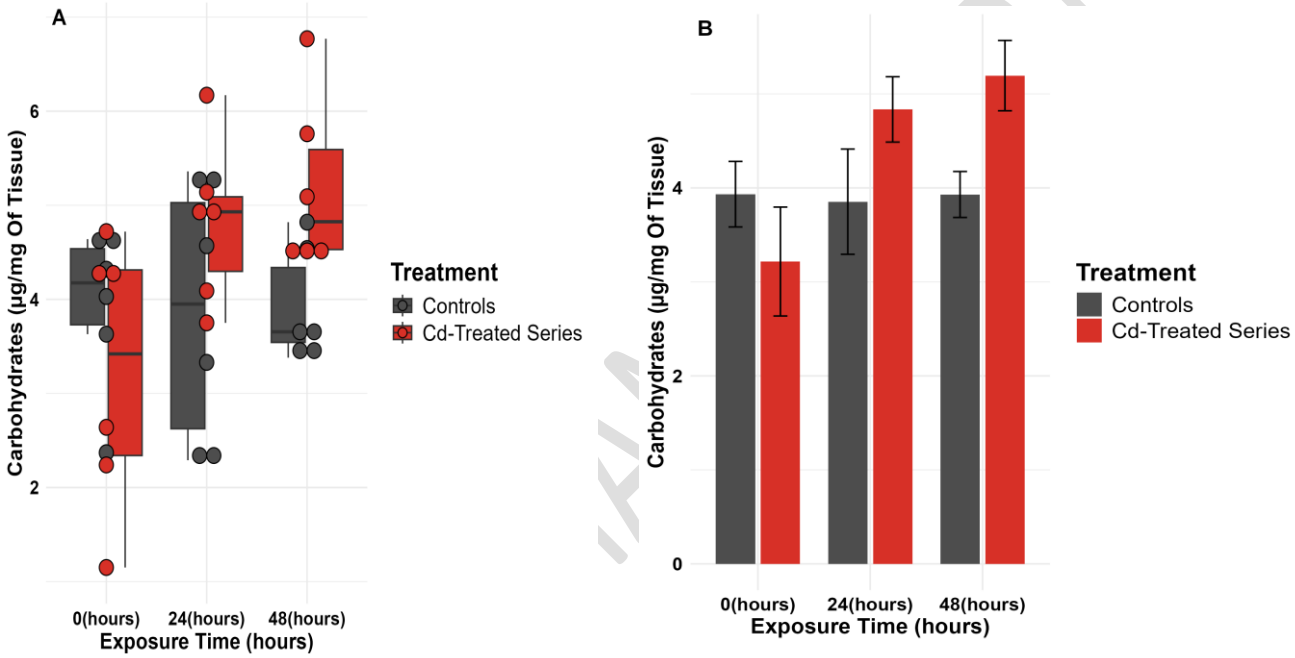

Figure 1. Effect of cadmium on carbohydrate content (µg/mg Of Tissue) in *O. bicornis* (n = 6). **(A)** Boxplot showing mean carbohydrate content after exposure to cadmium (mean ± SD). **(B)** Error bar plot representing the standard deviation of carbohydrate content (mean ± SD).

3.1.2. Lipids contents

The Scheirer-Ray-Hare test, was used to assess the effects of Exposure Time and Treatment on Lipid contents (µg/mg of Tissue) in 36 observations. The test revealed a statistically significant effect of Treatment ($H = 4.91$, $p = 0.0267$) and a significant Time × Treatment interaction ($H = 7.26$, $p = 0.0265$), suggesting that the impact of treatment depends on exposure duration (Figure 3). However, the main effect of Time alone was not statistically significant ($H = 4.51$, $p = 0.1048$) (Figure 2, C).

Post-hoc Dunn tests with Bonferroni correction showed a significant difference in lipids between Cd-treated series and Control groups (Z = -2.22, p = 0.0267), while no significant pairwise differences were found between time points, including between 24 and 48 hours (*adjusted* p = 0.1197) (Figure 2, D).

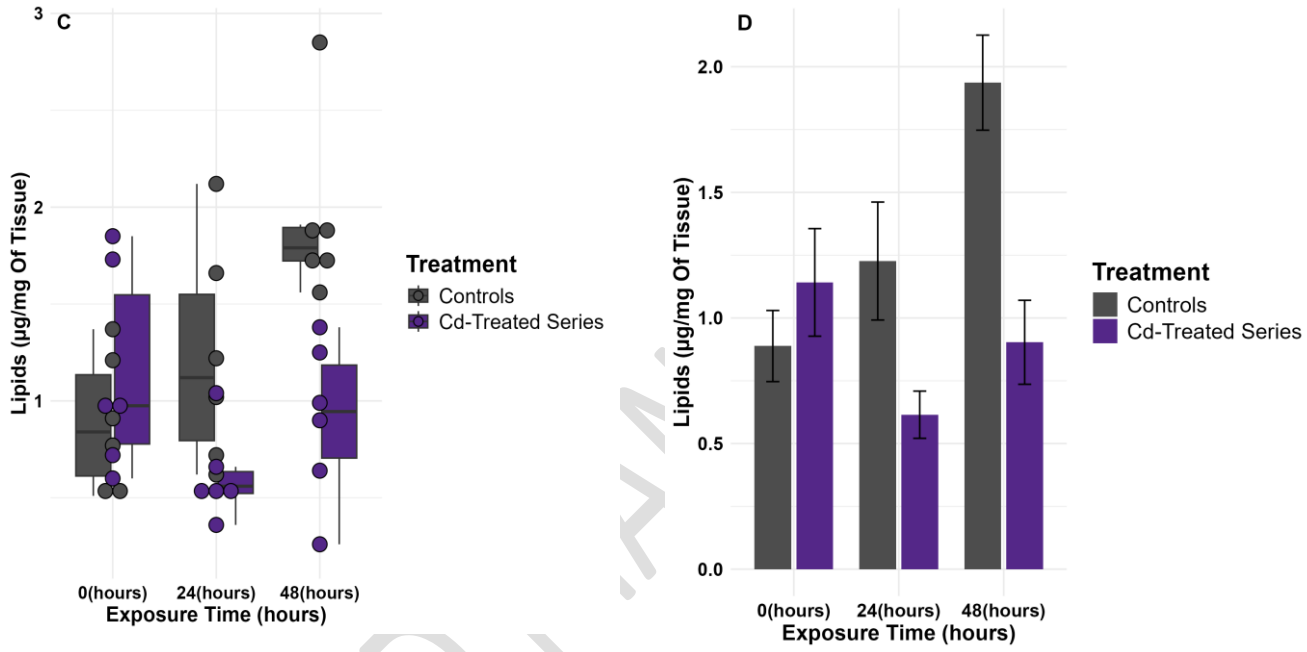

Figure 2. Effect of cadmium on lipid content (µg/mg Of Tissue) in *O. bicornis* (n = 6). **(C)** Boxplot showing mean lipid content after exposure to cadmium (mean ± SD). **(D)** Error bar plot representing the standard deviation of lipid content (mean ± SD).

3.1.3. Proteins contents

The Scheirer-Ray-Hare test, was used to examine the effects of Time**,** Treatment, and their interaction on Proteins contents (µg/mg of Tissue) across 36 observations. The results showed that none of the factors reached statistical significance at the 0.05 level (Figure 3, E). The effect of Time on Proteins (µg/mg of Tissue) was not significant (H = 1.7643, p = 0.41388), indicating that Protein's expression did not vary significantly across the different Time points.

Similarly, the Treatment factor was also not significant (H = 2.6043, p = 0.10658), suggesting no strong evidence that the treatment alone influenced protein levels. The interaction between Time

and Treatmentwas likewise non-significant (H = 3.5013, p = 0.17366), meaning there was no combined effect of Time and Treatment on Proteins (µg/mg of Tissue) (Figure 3, F).

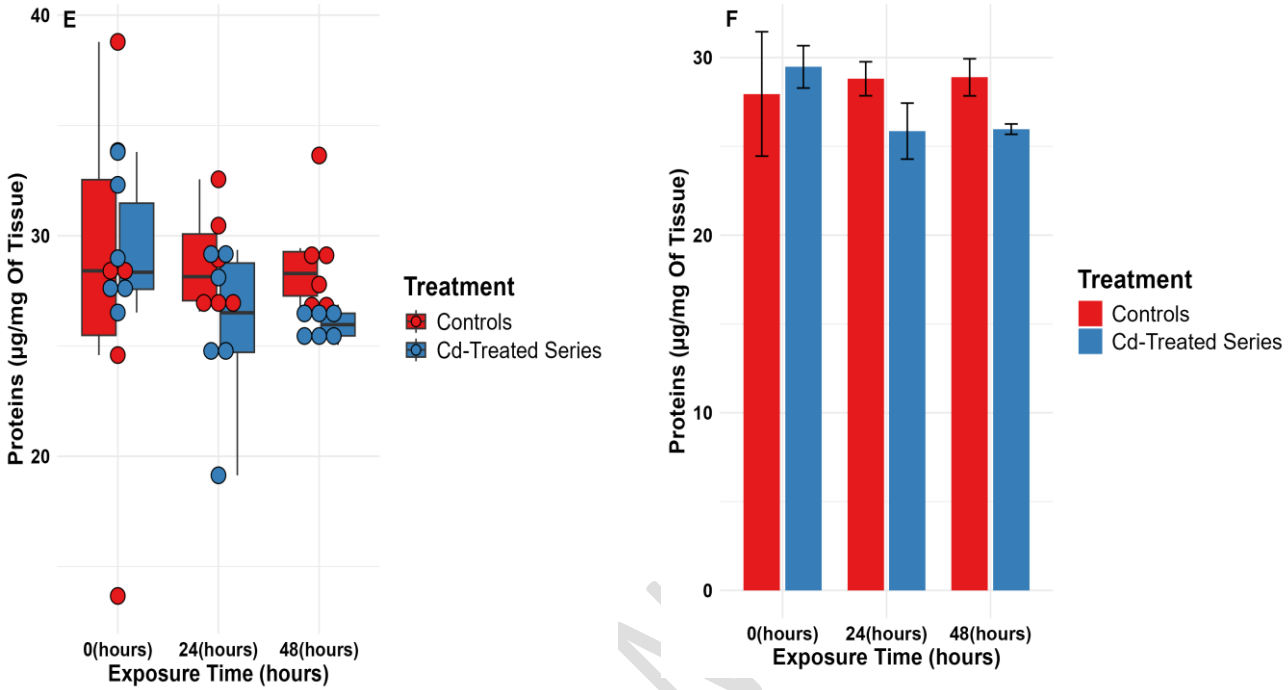

Figure 3. Effect of cadmium on protein content (µg/mg Of Tissue) in *O. bicornis* (n = 6). **(E)** Boxplot showing mean protein content after exposure to cadmium (mean ± SD). **(F)** Error bar plot representing the standard deviation of protein content (mean ± SD).

3.1.4. H_2O_2 levels (mmole/mg of Proteins)

The Scheirer-Ray-Hare test revealed a marginally non-significant effect of Timeon H_2O_2 Levels (mmole/mg of Proteins) (H = 5.39, p = 0.0676), a significant effect of Treatment (H = 8.02, p = 0.0046), and a significant Time × Treatment interaction (H = 9.33, p = 0.0094) (Figure 4, G).

Post-hoc Dunn tests with Bonferroni correction showed that H_2O_2 Levels (mmole/mg of Proteins) were significantly higher in the Cd-treated group compared to controls (p = 0.0046). Regarding Time, no pairwise comparisons reached significance after correction, although the difference between 0 and 48 hours approached significance (p = 0.0945). These results indicate that treatment and the interaction between Time and Treatment significantly influence H_2O_2 levels (mmole/mg of

Proteins), with Cd treatment increasing levels overall and a time-dependent variation depending on treatment (Figure 4, H).

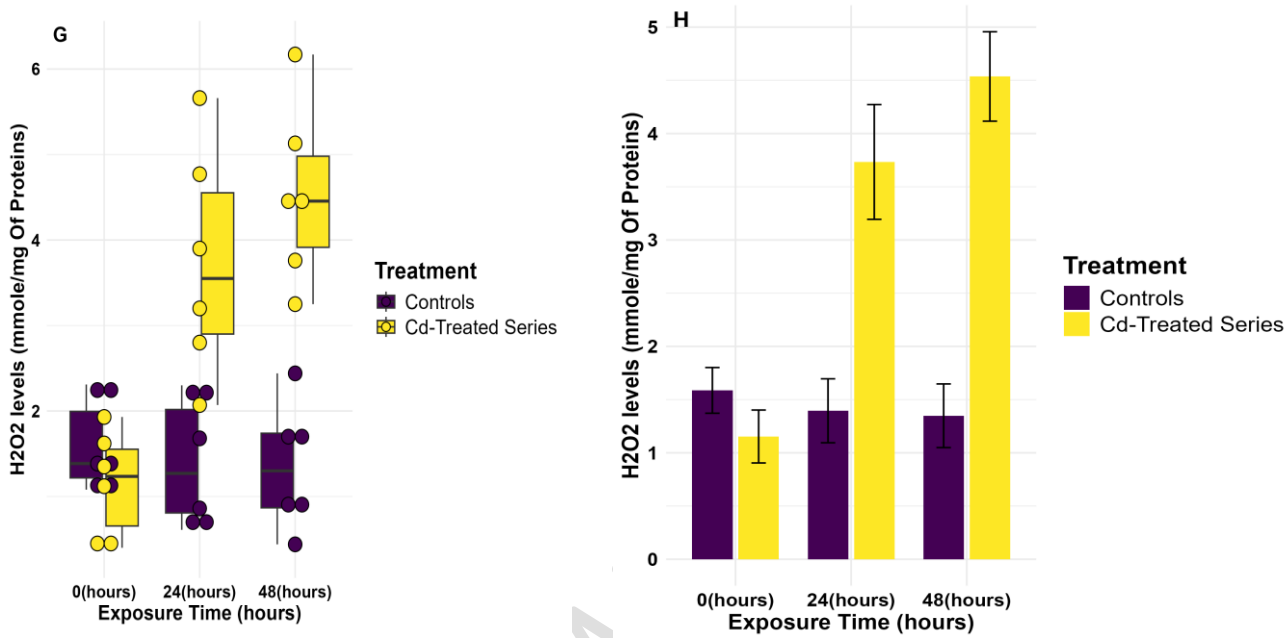

Figure 4. Effect of cadmium on H_2O_2 levels (mmole/mg Of Proteins) in *O. bicornis* (n = 6). **(G)** Boxplot showing mean H_2O_2 levels after exposure to cadmium (mean ± SD). **(H)** Error bar plot representing the standard deviation of H2O2 levels (mean ± SD).

3.1.5. LOOH levels (mmole/mg of Proteins)

The Scheirer-Ray-Hare test showed significant effects of Time (H = 14.45, p = 0.00073) and treatment (H = 5.71, p = 0.01690) on LOOH levels (mmole/mg of Proteins), while the interaction between Time and Treatment was not significant (H = 1.31, p = 0.52) (Figure 5, I).

Post-hoc Dunn tests with Bonferroni correction revealed that LOOH levels (mmole/mg of Proteins) at 48 hours were significantly higher than at 0 hours (p = 0.0049) and 24 hours (p = 0.0019), with no difference between 0 and 24 hours. Additionally, Cd-treated samples showed significantly higher LOOH levels (mmole/mg of Proteins) compared to controls (p = 0.0169). These results indicate that both Time and Treatment independently affect LOOH levels (mmole/mg of Proteins), with increases observed at 48 hours and following Cd exposure (Figure 5, J).

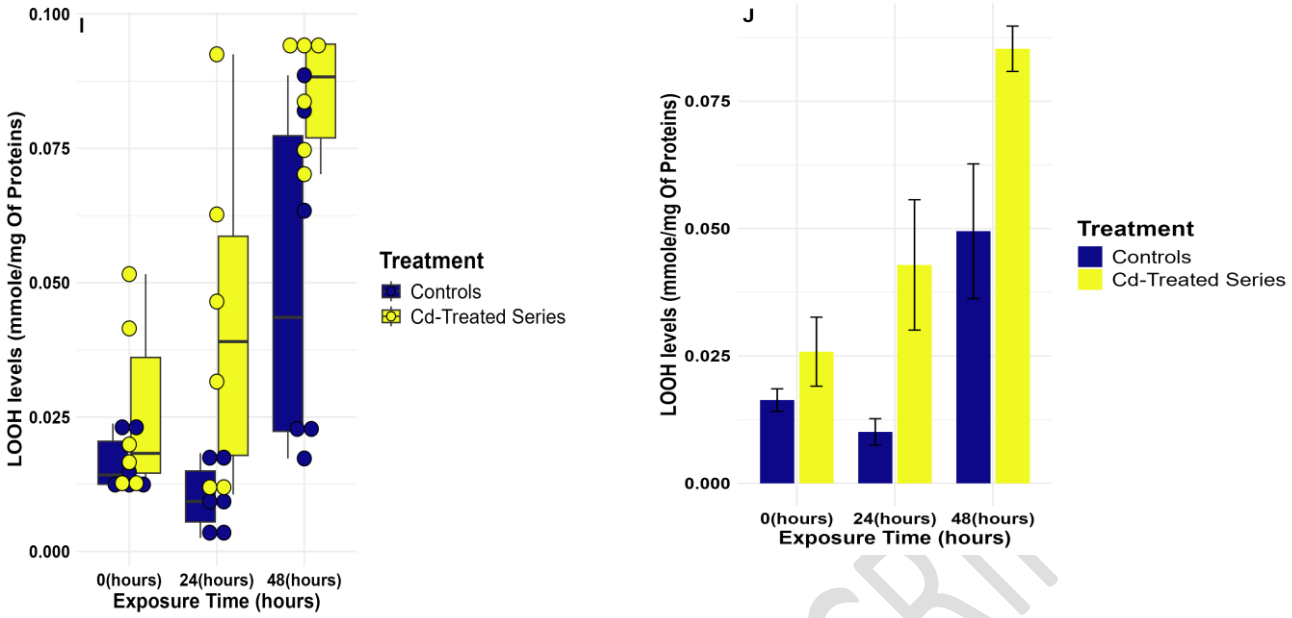

Figure 5. Effect of cadmium on LOOH levels (mmole/mg Of Proteins) in *O. bicornis* (n = 6). **(I)** Boxplot showing mean LOOH levels after exposure to cadmium (mean ± SD). **(J)** Error bar plot representing the standard deviation of LOOH levels (mean ± SD).

3.1.6. SOD Activity (U/mg of Proteins)

The Scheirer-Ray-Hare test showed a significant effect of Timeon SOD Activity (U/mg of Proteins) ($H = 12.71$, $p = 0.00174$), while neither Treatment ($H = 2.26$, $p = 0.13286$) or the Time × Treatment interaction ($H = 1.99$, $p = 0.36975$) were significant (Figure 6, K).

Post-hoc Dunn tests with Bonferroni correction indicated that SOD activity at 48 hours was significantly different from 0 hours ($p = 0.0012$), while differences between 0 and 24 hours and between 24 and 48 hours were not statistically significant after adjustment ($p = 0.51$ and $p = 0.09$, respectively). No significant difference was found between Cd-treated series and control groups ($p = 0.13$). These results suggest that SOD Activity (U/mg of Proteins) changes significantly over Time, particularly between 0 and 48 hours, but is not significantly affected by treatment or the interaction of treatment and Time (Figure 6, L).

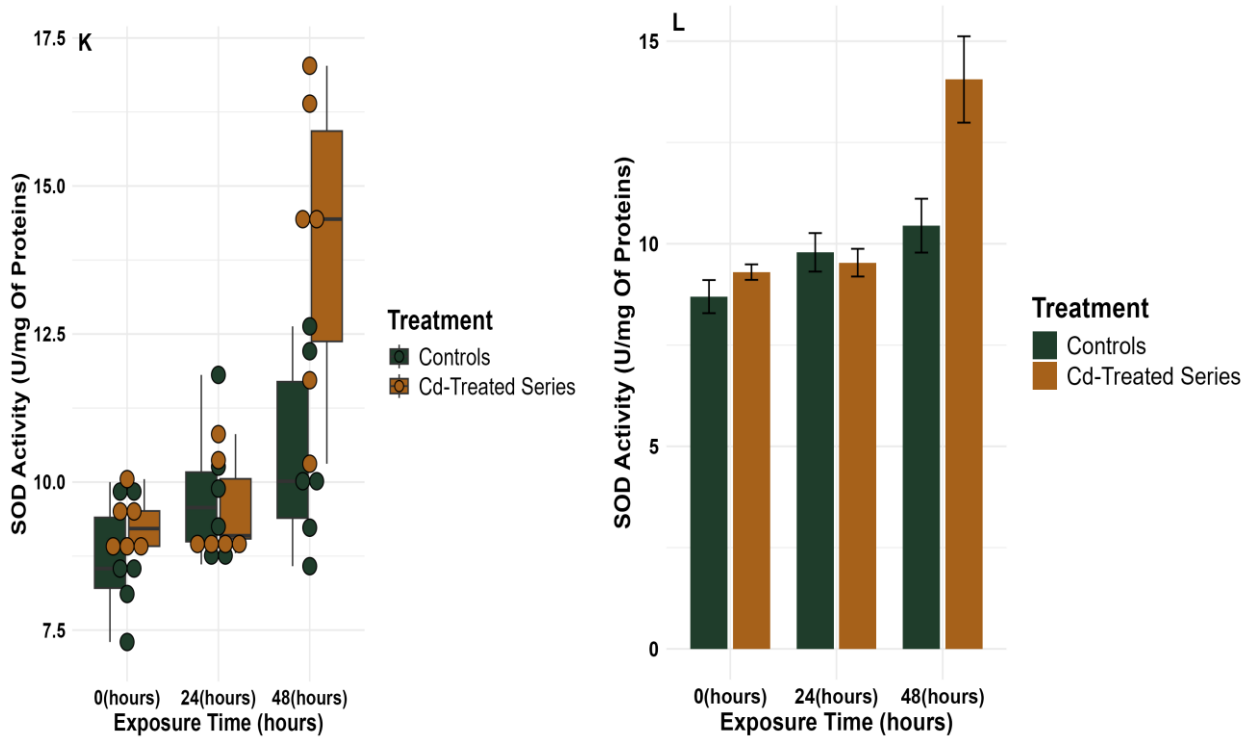

Figure 6. Effect of cadmium on SOD activity (U/mg Of Proteins) in *O. bicornis* (n = 6). **(K)** Boxplot showing mean SOD activity after exposure to cadmium (mean ± SD). **(L)** Error bar plot representing the standard deviation of SOD activity (mean ± SD).

3.2. Principal Component Analysis (PCA) and Redundancy Analysis (RDA) of Biochemical Biomarkers in Ophelia bicornis: Effects of Exposure Time and Treatment

The combined application of PCA and RDA provided complementary insights into the biochemical responses to treatment and exposure time. PCA, an unconstrained ordination method, revealed that the first two principal components explained 62.82% of the total variance (PC1: 43.3%; PC2: 19.5%). PC1 was showing strong positive correlations with H_2O_2 levels ($r = 0.89$, $p < 0.001$), Carbohydrate ($r = 0.71$, $p < 0.001$), and LOOH levels ($r = 0.70$, $p < 0.001$). This axis was positively associated with the Cd-treated group, particularly at 48 hours. PC2 was mainly correlated with Lipid ($r = 0.74$, $p < 0.001$) and SOD activity ($r = 0.49$, $p = 0.003$) and was significantly associated with the 48-hour Exposure Time (Figure 7).

In parallel, RDA (constraining the ordination by Treatment and Exposure Time explained 47.3% and 6% of the variance on Axis 1 and Axis 2, respectively. Axis 1 was positively associated with H_2O_2 levels ($r = 0.33$, $p < 0.001$) and SOD activity ($r = 0.12$, $p < 0.01$), and negatively with protein ($r = -0.19$) and lipid ($r = -0.08$), indicating increased oxidative stress in exposed samples. Axis 2 was primarily correlated with Lipid ($r = 0.13$, $p < 0.01$) and SOD activity ($r = 0.09$, $p < 0.05$), reflecting Time-dependent metabolic responses (Figure 8).

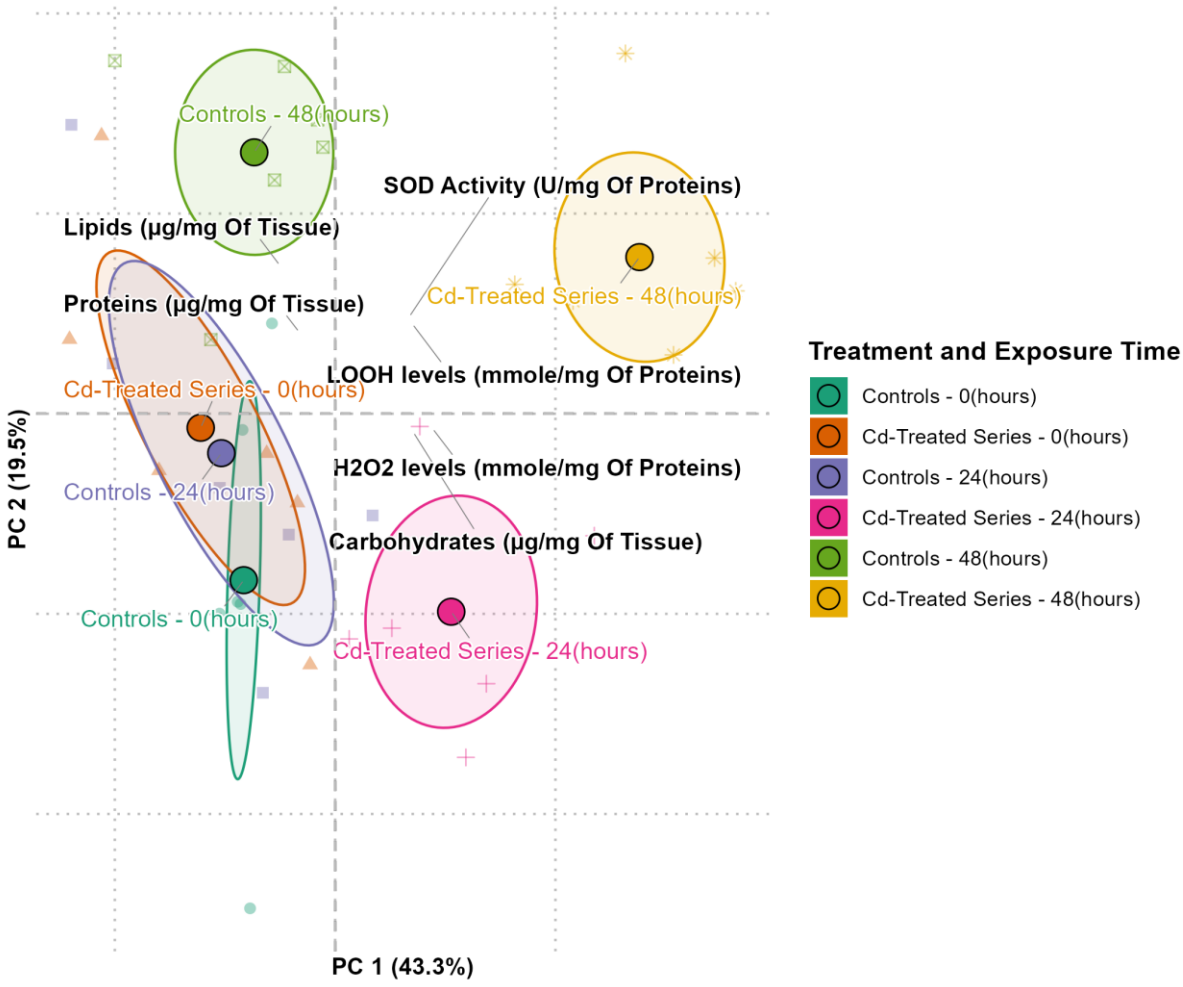

Figure 7. Principal Component Analysis (PCA) Biplot of Biochemical Biomarkers in Relation to Treatment and Exposure Time in *Ophelia bicornis*. **Legend:** The PCA Biplot illustrates the distribution of biochemical parameters (n = 6) measured in Tissue samples exposed to Cd-Treated series and control conditions over varying Exposure Times. Variables include Carbohydrates contents (µg/mg Of Tissue), Lipids (µg/mg Of Tissue), Proteins (µg/mg Of Tissue), H_2O_2 levels (mmole/mg Of Protein), LOOH levels (mmole/mg Of Protein), and SOD activity (U/mg Of Protein). These variables are represented as vectors, where their direction and length indicate the

magnitude and contribution to the principal components. Sample groups, defined by Treatment (Controls vs. Cd-Treated series) and Exposure Time (0, 24, 48 hours), are displayed as points with colored 95% confidence ellipses delineating group clustering. Points oriented in the direction of a vector suggest higher values of the corresponding biomarker. (PC1: 43.3%; PC2: 19.5%).

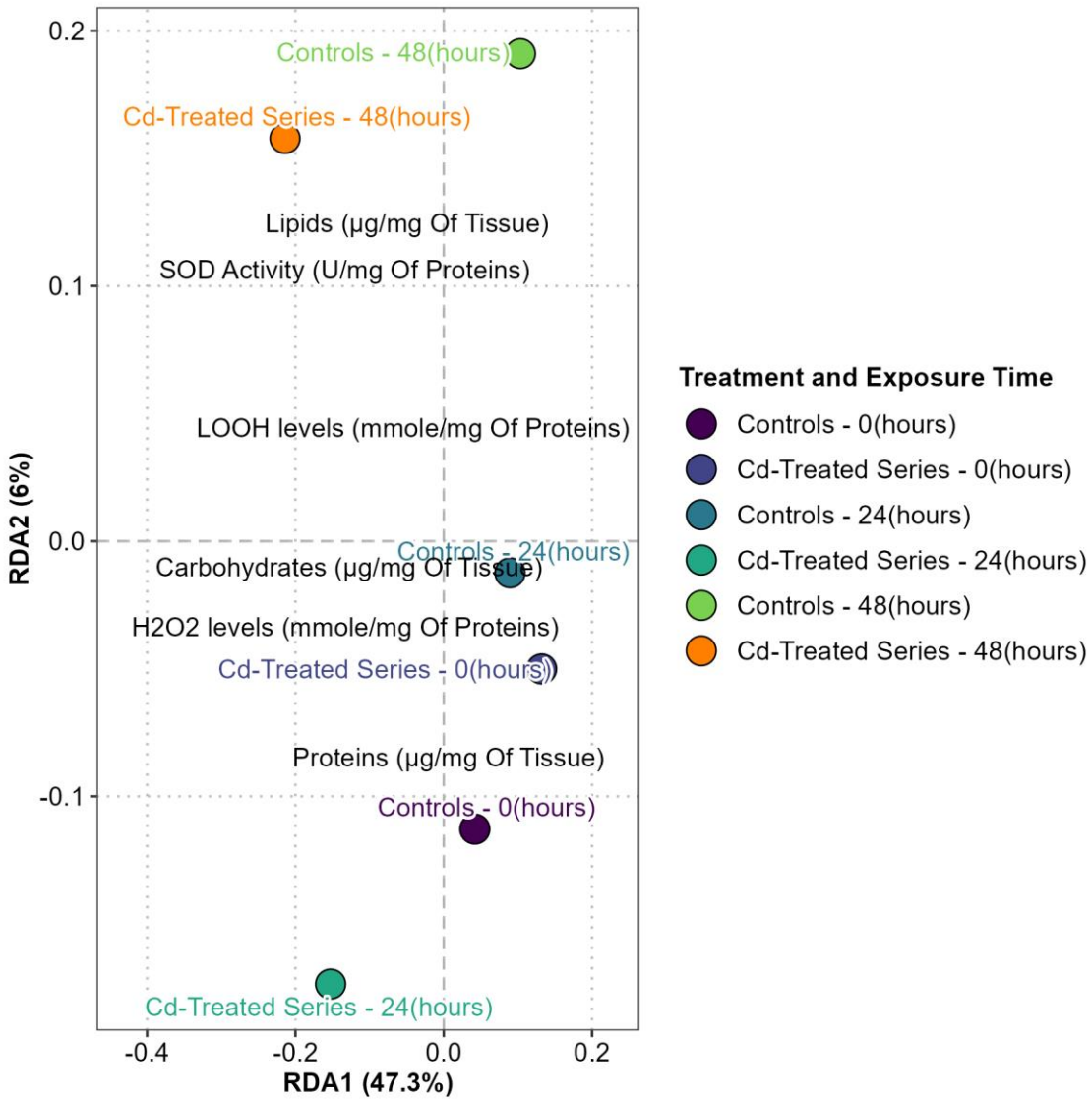

Figure 8. Redundancy Analysis (RDA) Biplot of Biochemical Biomarkers Constrained by Cadmium Treatment and Exposure Time. **Legend:** The RDA Biplot shows the relationship between biochemical parameters (Carbohydrates, Lipids, Proteins, H_2O_2 levels, LOOH levels, and SOD activity) and the explanatory variables Treatment (Controls vs. Cd-Treated series) and Exposure Time (0, 24, 48 hours). Biochemical variables are represented as vectors indicating their strength and direction of influence on the ordination axes. Sample points are grouped according to treatment and exposure duration, with 95% confidence ellipses highlighting clustering patterns. The first two RDA axes explain 27% and 5.1% of the variance, respectively. Vectors pointing toward sample clusters indicate higher biomarker expression under those conditions.

4. Discussion

Short-term tests were designed to measure not only mortality rates, but also to determine tolerance ranges of metabolic systems most sensitive to metal stress. The toxicity tests are necessary in water pollution evaluation, as chemical and physical parameters are insufficient to determine potential effects on aquatic organisms. Studying the responses of organisms to environmental stressors plays a key role in ecotoxicological studies. The established lethal concentration values could be used as key indicators in toxicity tests to demonstrate the effects of toxic pollutants, particularly for ecotoxicological evaluation of marine ecosystems in risk assessments. This work aims to evaluate the toxic effects of cadmium and its capacity to alter several variations in organism function. Namely, the estimation of biochemical contents, and the evaluation of enzymatic activities in *O. bicornis* species. The most toxicants exert their effects at basic level of the organism by reacting with enzymes or metabolites and other functional components of the cell (Ho Yu 2000). Thus, organisms' energy reserves provide a valuable indicator of their ability to cope with stress long before negative effects appear at more complex biological levels (Smolders *et al.* 2003). To examine energy metabolism perturbations, many bioenergetic parameters are available such as levels of energy reserves as carbohydrates, lipids and proteins (Meyer *et al.* 2002), cellular energy allocation (De Coen and Janssen 2003), scope for growth (Widdows *et al.* 2002). This research represents the first attempt to evaluate cadmium toxicity using *Ophelia bicornis* as a test organism. In this context, the energy status of *O. bicornis* was studied to gain insight into Cd-induced lethal effects and the ability of cadmium to induce disturbances on different levels of organism function. Cadmium is a very toxic metal, and even though it is unable to undergo Fenton-type reactions and therefore is considered a non-redox metal, and it has nevertheless been associated to increased production of reactive oxygen species (ROS) to marine organisms (Unsal *et al.* 2020; Liu *et al.* 2022).

Our results revealed that cadmium exposure and duration both influenced the biochemical responses in *O. bicornis*. Energy-related compounds such as carbohydrates and lipids were mobilized over time in the cadmium-treated series compared to the control group, reflecting a physiological

adjustment to metal-induced stress. However, protein levels remained relatively constant, suggesting that they were not significantly affected by treatment or exposure time. These findings are consistent with the notion that the earliest toxic effects of chemicals manifest primarily at the cellular and biochemical levels. As highlighted by Zhou *et al.* (2004), recent studies have increasingly focused on measurement at these levels to assess whether exposure to pollutants causes harmful effects. The observed changes in carbohydrate and lipid contents, along with the stability of protein levels, reflect such early biochemical responses to cadmium-induced stress. The biochemical composition (carbohydrates, lipids and proteins) indeed varies between taxonomic groups, but also among species, developmental stages, analyzed tissues, and environmental conditions. Moreover, this study suggests that the main storage materials in *O. bicornis* are proteins, whereas carbohydrates and lipids were present at a relatively low level.

The increase in carbohydrates levels after 48h oh exposure likely reflects a stress-induced mobilization of glycogen or other carbohydrate stores to provide readily available energy for detoxification possesses. The observed increase in carbohydrate levels in cd-exposed *Ophelia bicornis* may reflect a typical stress response in marine invertebrates. However, the utilization of carbohydrates by aquatic animals is relatively low compared to that of terrestrial animals. In marine invertebrates, carbohydrates are often the first source of energy utilized during acute stress, as they can be quickly metabolized through glycolysis to produce ATP (Wang *et al.* 2016).Increases in circulating glucose in the hemolymph have been reported in response to a variety of stressors such as salinity, temperature, hypoxia, or exposure to heavy metals (Wang *et al.* 2012). Notably, exposure to cadmium induced in the crayfish *Procambarus clarkii* a significant increase in hemolymph glucose concentration (Reddy *et al.* 1994).Carbohydrates and lipids play an important role in building up reserves for embryo development in Annelids. At the same time, the reproductive effort is high with up to 80% of the total energy allocated to gamete biomass (Hoeger and Schenk 2024). With increasing levels of stress, the maintenance of physiological integrity becomes more challenging for organisms. As an immediate response to the toxicity, organisms tend

485 to reduce their metabolic rate (Hand and Hardewig 1996). Some studies have suggested that the
486 lipid contents of the exposed animals were significantly decreased compared to the control (Lucia *et*
487 *al.* 2010; Yang *et al.* 2013), and this was confirmed by our study. In addition to carbohydrates, total
488 lipid content significantly decreased in *Ophelia bicornis* under cadmium exposure, particularly after
489 48h, suggesting a depletion of lipid reserves in response to metal-induced stress. This finding is
490 consistent with previous studies in crustaceans such as crabs, where lipid levels in the
491 hepatopancreas and ovary decreased after prolonged cadmium exposure compared to controls (Liu
492 *et al.* 2016). Lipid reserves are mobilized or degraded under metal-induced stress, likely to meet
493 elevated energy demands or due to oxidative damage. Luis and Passos (1995) found that lipids are
494 crucial during the life cycle of *N. diversicolor*, particularly for reproduction. Lipids accumulate as
495 an energetic reserve in oocytes and support metabolism during gametogenesis when the animals
496 stop feeding. In aquatic invertebrates, reductions in the sequestration of reserves have been shown
497 to affect the number or quality of gametes produced (Mathieu and Lubet 1993).

498 In contrast, protein contents remained relatively stable. The stability in protein levels suggests that
499 *O. bicornis* reserves proteins, avoiding their breakdown unless under severe or prolonged stress.
500 Proteins were not preferentially mobilized as energy sources under these exposure conditions.
501 Similarly, according to the hypothesis proposed by Ketata *et al.*(2007), proteins are not primarily
502 involved in energy metabolism in the clam *Ruditapes decussatus* exposed to cadmium. This
503 observation aligns with our results, which also revealed no significant change in protein levels
504 across treatment conditions or exposure durations. This contrasts with the findings of Barber and
505 Blake (2006) reported that proteins can serve as a significant energy source, particularly when other
506 reserves are depleted. Bivalves store energy primarily as carbohydrates (mainly glycogen) and
507 lipids to maintain physiological integrity, during reproductive periods, whereas annelids tend to rely
508 more on proteins to support their fundamental metabolic activities, it has been shown that proteins
509 are the main components of muscle tissue. According to the research of (Tripp-Valdez *et al.* 2019)
510 proteins, complex macromolecules described as biopolymers, are the most abundant of cellular

511 organic molecules. Under severe stress conditions, proteins may serve as an alternative energy
512 source. However, this process compromises their structural and functional roles, since proteins are
513 not primarily synthesized or stored for energy supply (Le Gal *et al.* 1997). On the other hand, Cd
514 exposure has been shown to alter protein metabolism in bivalves, affecting both synthesis and
515 degradation pathways in various tissues. The effects of cadmium on protein levels were tissue-
516 specific and varied with the duration or exposure (Ivanina *et al.* 2008). Cadmium is known to affect
517 reproduction and energy metabolism in marine organisms (Baudou *et al.* 2017; Louis *et al.* 2021).
518 Concurrently, oxidative stress-induced protein oxidation can result in structural alterations and loss
519 of function, disrupting cellular processes dependent on properly functioning proteins(Boguszewska-
520 Mańkowska *et al.* 2015). A significant decrease in lipids, proteins and carbohydrates contents in
521 muscles and gills of *Carcinus Aestuarii* as compared to *Portunus segnis* has been observed (Chetoui
522 *et al.* 2021). Thus, seasonal changes can influence the physiology of crabs by affecting their
523 metabolic activity and response, leading to disturbances in biochemical components such as lipids,
524 proteins and carbohydrates. These alterations appear to be linked to the accumulation of trace
525 elements, environmental factors and the reproductive cycles of aquatic organisms (Chetoui *et al.*
526 2021).

527 At the cellular level, oxidative stress plays an important role in the development of damage.
528 Reactive oxygen species (ROS) can be free radicals derived from oxygen, such as superoxide anion
529 (O_2^-) and hydroxyl radicals (OH), or non-radical derivatives such as hydrogen peroxide (H_2O_2).
530 These molecules are produced through several mechanisms (Fouzai *et al.* 2020).While ROS play
531 essential roles in cellular functions, their excessive accumulation beyond the antioxidant capacity of
532 the organism causes oxidative damage to biomolecules, leading to disruptions in cellular functions
533 and structures (Sinenko *et al.* 2021). The degradation of these radicals is controlled by antioxidants
534 systems, which adapt to the level of radicals present. The antioxidant system of marine organisms
535 consists of low molecular weight scavengers and antioxidant enzymes which interact in a
536 sophisticated network. Several studies on aquatic organisms have demonstrated the importance of

antioxidant enzymes in protecting cellular systems from oxidative damages induced by xenobiotic. The impact of oxidative stress has been shown to extend beyond individual cellular processes, influencing various downstream effects of metal toxicity (Chen *et al.* 2018). The deleterious effects of metals like cadmium are responsible for oxidative damage in living organism. Previous investigations indicated that cadmium induces the formation of oxygen free radicals in tissues and inhibits the activity of some enzymes of the antioxidant defense system (Unsal *et al.* 2020). When organisms are exposed to elevated levels of trace metals, oxidative stress can damage to essential biomolecules such as proteins, lipids will be happened, impairing physiological function (Singh *et al.* 2019; Haidar *et al.* 2023).In response, organisms have developed a variety of defense systems to mitigate the toxic effects of metals and maintain internal homeostasis. One of these mechanisms is the production of antioxidant enzymes such as superoxide dismutase (SOD), catalase, and glutathione, which work synergistically to neutralize reactive oxygen species (ROS) generated during metal exposure (Jomova *et al.* 2023). Cope with oxidative stress produced by ROS generation, SOD as the most powerful and the primary detoxification enzyme, removes the superoxide radical through the process of dismutation to oxygen and hydrogen peroxide. In the present study, changes in the activity of SOD were observed in *O. bicornis* under cadmium treatment. Similar to findings in other marine invertebrates exposed to Cu (Brown *et al.* 2004), or reported in field studies (Pérez *et al.* 2004), Cd may induce oxidative damage in *O. bicornis*, potentially through the generation of ROS. In response to oxidative stress, organisms exposed to various organic and metal contaminants often exhibit increased activity of antioxidant enzymes. However, these responses vary depending on the species, the enzymes involved, and whether the exposure is to a single or a mixture of contaminants(Regoli and Giuliani 2014). SOD activity in *O. bicornis* exposed to cadmium remained constant at 24h but showed an increase after 48h of exposure, with values still higher than those of the control at day 0. Our results are in full agreement with those reported in literature. Similar results were obtained in previous investigations of several polychaete species. According to (Sun and Zhou 2008), the activity of SOD in *N. diversicolor*

exposed to Cd and petroleum hydrocarbons (PHCs) increased after a 6 day exposure, and (Moreira *et al.* 2006) also reported a significant induction of this enzyme when the species was exposed to contaminated sediments. The increase in SOD activity suggested that SOD was induced as consequence of generation of $O_2^{\cdot-}$This response likely reflects the role of the antioxidant enzyme SOD in removing excess free radicals, mainly $O_2^{\cdot-}$ and H_2O_2 generated to counteract Cd-induced stress and mitigate their toxicity. However, (Chaâbane *et al.* 2020) showed that SOD activity enhanced in the gills and digestive gland of chromium (VI)-exposed *Venus verrucosa*. ROS accumulation is a widely documented effect of cadmium exposure in marine organisms leading to oxidative damage and cellular dysfunction (Liu *et al.* 2022). Cd does not induce ROS production directly; however, it can cause indirect damage and generate free radicals (Patra *et al.* 2011). This indirect damage was elucidated in the present study by a significant increase of H_2O_2 levels confirming the metal potential to boost pro-oxidants' production. The enhanced H_2O_2 generation in *O. bicornis* was associated with an increase in LOOH levels, as compared to the control group. Hydrogen peroxide is a central redox signaling molecule, capable of serving as messenger to carry a redox signal from the site of its generation to a target site (Sies *et al.* 2017).The levels of H_2O_2 were found to be increased in *D. magna* adults exposed to the metal mixture (Cu/Cd), oxidative stress was found as a common mechanism underlying the toxicity of these metals (Majid 2024). These finding are in line with those of (Chetoui *et al.* 2022), and (Chaâbane *et al.* 2020). Our study found that Cd exposure at a sublethal concentration (LC_{10}) induced significant oxidative stress responses in *Ophelia bicornis*. Lipid hydroperoxides (LOOH) and hydrogen peroxide (H_2O_2) increased significantly over time, indicating heightened ROS production and lipid peroxidation. Concurrently, superoxide dismutase (SOD) activity increased after 48h, suggesting an enzymatic attempt to counteract ROS accumulation. However, the sustained elevation of H_2O_2 and LOOH, despite SOD activation, highlights an oxidative imbalance. This underscores the vulnerability of *O. bicornis* tissues to damage caused by Cd exposure. These findings regarding energy metabolism and stress responses may help predict the effects of Cd on marine organisms, particularly annelids such as *O.*

589 *bicornis*, which are highly exposed to metal contaminants in their habitats. *O. bicornis* is especially
590 important in ecotoxicological studies, living in direct contact with contaminated sediments.

591 **5. Conclusion**

592 The physiological and biochemical responses of the annelid *Ophelia bicornis* were investigated
593 under laboratory conditions after short exposure to cadmium (Cd), focusing on antioxidant enzyme
594 activity and the content of major biochemical components. These findings provide insight into the
595 molecular responses triggered by Cd exposure and the underlying mechanisms in *O. bicornis*,
596 emphasizing the significant role of oxidative stress in mediating the cellular response to cadmium-
597 induced damage. responses of the antioxidant system and biochemical parameters in this species
598 are not well-documented; thus, our study provides the first evidence addressing these aspects
599 regarding cadmium toxicity. This research highlights the ecotoxicological relevance of *O. bicornis*
600 as a sensitive bioindicator of cadmium exposure. Even at sublethal concentrations, Cd disrupted the
601 energy balance in exposed organisms and induced oxidative stress. this is evidenced by increased
602 carbohydrates mobilization, alterations of lipid contents, elevated levels lipid hydroperoxides
603 (LOOH) and hydrogen peroxide (H_2O_2), and a time dependent modulation of superoxide dismutase
604 (SOD) activity. These physiological and biochemical changes indicate that *O. bicornis* relies on its
605 energy reserves and antioxidant defenses to cope with metal-induced stress. The consistent
606 correlation between of oxidative stress biomarkers and cadmium exposure further underscores their
607 value as early warning indicators. Overall, these findings enhance our understanding of how benthic
608 invertebrates respond to metal-contaminated environments and confirm the sensitivity of *O.*
609 *bicornis*. This establishes its use as a bioindicator in environmental monitoring andprovides a
610 baseline for evaluating its physiological responses to metal exposure, and opens a new perspective
611 for its application in future ecotoxicological and biomonitoring studies.

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