https://doi.org/10.48047/AFJBS.6.15.2024.11542-11554

African Journal of Biological Sciences

Journal homepage: http://www.afjbs.com

Research Paper Open Access

Assessing the effectiveness and biochemical consequences of depuration in *Mytilus galloprovincialis* Lamark 1819

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Volume 6, Issue 15, Sep 2024

Received: 15 July 2024

Accepted: 25 Aug 2024

Published: 25 Sep 2024

doi: 10.48047/AFJBS.6.15.2024.11542-11554

Abstract

Despite the growing body of research, definitive evidence on the effectiveness of depuration in detoxifying aquatic organisms and its associated biochemical impacts remains limited. This study evaluates the potential of depuration to mitigate anthropogenic pollution in *Mytilus galloprovincialis* (Mollusca, Bivalvia) by examining various biomarkers, including glycogen reserves, and protein metabolism, as well as their correlation with lactate dehydrogenase activity (LDH) and protein carbonyls (PCs). Adult *M. galloprovincialis* specimens were collected from Sidi Salem, a site heavily polluted with a range of contaminants, notably heavy metals, were transferred to aquaria containing seawater from a clean reference site (El Hnaya) for an 8-day depuration period. Samples were taken at 0, 2, 4, 6, and 8 days. Depuration had a significant effect on all biomarkers, which remained unchanged between non-depurated and depurated individuals. Glycogen and protein levels increased substantially after 8 days of depuration, while LDH activity content and PCs showed significant reductions after 6 and 8 days, respectively. These results suggest that depuration can effectively reduce oxidative stress in contaminated bivalves, contributing to improved physiological health.

***Keywords** : Depuration, Mussels, Glycogen, Protein, Lactate dehydrogenase, Protein carbonyls.*

Introduction

Coastal areas and estuaries are among the most susceptible regions to chemical pollution due to their proximity to anthropogenic activities (Rabei et al., 2018; Hamdani et al., 2020; Zhong et al., 2024). Increasing industrialization and intensive agricultural practices in many countries have led to a significant rise in the discharge of pollutants, such as heavy metals, pesticides, and microplastics, into aquatic ecosystems, severely affecting both freshwater and marine environments (Amira et al., 2018; Yezli et al., 2022; Sebih et al., 2023; Kneel et al., 2024). Bivalve molluscs are known to readily accumulate contaminants from sediments, suspended particulates, water, and food due to their sedentary nature, filter-feeding behavior, and high bioaccumulation potential (Laffon et al., 2006). Consequently, they have been extensively used as bioindicators to monitor environmental

contaminants in marine, estuarine, and freshwater environments (Amamra et al., 2019; Ladouali et al., 2022; Castro et al., 2023; Della Torre et al., 2024). Their effectiveness as bioindicators is further enhanced by their ease of collection, widespread distribution, suitable size for biochemical analyses, and high tolerance to pollution, supported by an active immune system (Sifi et al., 2018; Daas et al., 2022; Merad et al., 2023). In addition to their ecological significance, bivalves are a valuable source of seafood, providing essential nutrients such as minerals, vitamins, proteins, carbohydrates, and lipids (Chaâbane et al., 2020). Within the European Union, depuration is a mandated process designed to reduce levels of pathogenic microorganisms, such as *Escherichia coli*, in bivalves harvested from contaminated waters, thereby ensuring their safety for human consumption (Regulation EC Nos. 853/2004 and 854/2004; EC, 2004a, 2004b). This process involves holding bivalves in clean seawater for up to 48 hours to allow them to purge contaminants (Lee et al., 2008). However, while some studies suggest that depuration may reduce toxic metal levels (Belabed & Soltani, 2018; Sami et al., 2020; Chinnadurai et al., 2022), its effectiveness in removing a broader range of chemical pollutants remains poorly understood.

Biochemical responses of bivalves to depuration are also not well characterized. Indicators such as glycogen content are valuable metrics for assessing bivalve health. Glycogen, the primary energy reserve in marine bivalves, reflects their energetic status; depleted reserves can lead to protein catabolism (Merad & Soltani, 2017; Lu et al., 2019; Sun et al., 2024). Additional biomarkers, such as lactate dehydrogenase (LDH) and protein carbonyls (PCs), offer insights into oxidative stress and exposure to chemical pollutants. LDH supports anaerobic metabolism during glycolysis, while PCs are products of protein oxidation (Reid et al., 2020; Jiang et al., 2023; Mendela et al., 2024). Both biomarkers are indicative of chemical stress exposure (Trabelsi et al., 2019; Hani et al., 2021; Falconi et al., 2024).

This study aims to evaluate the effectiveness of depuration in mitigating the adverse effects of pollution on the health of *Mytilus galloprovincialis* (Lamark, 1819) collected from Sidi Salem beach in northeastern Algeria, a region heavily impacted by industrial activities, including fertilizer and pesticide production and a major steel complex, contributing to significant heavy metal contamination in local waters and sediments (Belabed et al., 2017; Amira et al., 2018; Larba et al., 2023). We will assess the reversibility of pollution effects by monitoring changes in various biochemical biomarkers, including glycogen, proteins, LDH, and PCs, over a depuration period of 2, 4, 6, and 8 days.

1. Materials and Methods

2.1. Sampling collection: Specimens of *M. galloprovincialis* were collected in November 2023 from Sidi Salem beach, approximately 1 km east of Annaba city (coordinates: 368500N–78470E). This beach, which receives industrial and domestic wastewater, is considered a polluted site (Fig. 1). The mussels were transported to laboratory in cool boxes containing ice packs to keep the temperature low and to avoid further stress. Dead or damaged specimens were eliminated and individuals meeting specified criteria (shell length 5 ± 1 cm) underwent a meticulous cleaning and selection process. This included the removal of epibionts, such as barnacles and algae.

Figure. 1. Map of Annaba gulf showing sampling sites Sidi Salem and El Hnaya

2.2. Depuration experiment: The experiment was commenced within 3 h since the shellfish collection and conduced in the laboratory for 2, 4, 6, and 8 days. Two groups were established: one was exposed to seawater from the Sidi Salem site (non-depurated group), while the other was placed in seawater obtained from the El Hnaya site (36° 54′ 26.76″ N, 8° 07′ 17.22″ E) (depurated group). This beach is located approximately 30 km east of Annaba, between the villages of Berrihane and Kantra El-Hamra, and 18 km east of Oued El Mafragh (Fig. 1). The El Hnaya site is considered a reference site due to its distance from industrial and urban areas (Drif et al., 2019). For this reason, we chose to use clean seawater from this site for bivalve depuration. At four days, depuration water was changed and the aquaria were cleaned every day to avoid refiltration of depurate contaminants, no food was added during the depuration.

The experimental conditions were measured during the sampling period and about: dissolved oxygen 8.2 mg/L, pH 8.0–8.3, salinity 33.6 g/L, temperature 14°C, and a 12-h light/dark cycle.

2.3. Glycogen and protein quantification: Individual fractions of soft tissue of four mussels were used for biochemical compound quantification. Glycogen analysis was performed following the method of Van Handel (1965). The sample was homogenized in 1 mL of 20% trichloroacetic acid (TCA) and centrifuged at 5000 × g for 10 minutes. The resulting pellet was resuspended in 50 mL of a saturated sodium solution with 2 mL of absolute ethanol, followed by a second centrifugation at 4000 × g for 10 minutes. The pellet was again resuspended in 1 mL of a saturated sodium solution in 60% ethanol and subjected to a third centrifugation at 4000 × g for 10 minutes. The supernatant obtained was used for glycogen quantification using the anthrone reagent and a standard glycogen solution (0.1 mg/mL in distilled water). Absorbance was measured at 620 nm, and glycogen content was expressed as grams per 100 grams of fresh tissue.

Protein extraction was performed following the method of Shibko et al. (1966). The samples underwent two centrifugation steps (5000 × g for 10 minutes each), first in 20% TCA and then in a mixture of ether and chloroform (1:1, v/v). The resulting pellet was resuspended in 1 mL of 0.1% NaOH. The supernatant obtained was used for protein quantification according to the Bradford assay (1976), utilizing the Coomassie Brilliant Blue G-250 reagent (Merck) and bovine serum albumin (Sigma) as a standard (1 mg/mL in distilled water). Absorbance was measured at a wavelength of 595 nm, and protein content was expressed as grams per 100 grams of fresh tissue.

2.4. Lactate dehydrogenase (LDH) activity and Protein Carbonyls (PCs) Assay: Visceral mass fractions from four individual mussels were analyzed for biomarker measurements. The LDH activity was assessed using the method of Hill and Lévi (1954), which involves the enzyme's reversible conversion of lactate to pyruvate. This assay is based on the continuous monitoring of the oxidation rate of NADH (nicotinamide adenine dinucleotide), which is consumed during the enzymatic reaction. A tissue fragment was homogenized in 1 mL of Tris-HCl buffer (0.1 M, pH 7.2), and the resulting homogenate was centrifuged

at 3000 rpm for 5 minutes. The supernatant obtained was used as the enzyme source. The assay was performed by adding 50 µL of the supernatant to 675 µL of substrate buffer (0.2 M, pH 10) and 50 µL of an NAD coenzyme solution (10 mg NAD dissolved in 1 mL of distilled water). Absorbance was measured at 340 nm every minute over a 5-minute period. The LDH activity was expressed as µmol/min^{-1}/mg^{-1} protein.

The PCs levels were measured following the method of Levine et al. (1990). Indeed, after incubation, the assay mixture was centrifuged at 10,000g during 10 min at 4 °C and the supernatant was precipitated with TCA and then centrifuged at 10,000g during 3 min. The pellet was resuspended in 2,4-dinitrophenylhydrazine and incubated at 25 °C for 60 min. Proteins were then precipitated by adding TCA. The resulting mixture was centrifuged and the obtained pellet was washed three times with acetone. The absorbance was read at 360 nm and the carbonyl content was calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}. Results were expressed as nmol carbonyl/mg protein. Protein content was estimated by the Bradford method (Bradford, 1976), using bovine serum albumin (BSA).

2.5. Statistical analysis: Results were presented as mean ± Standard Error of the Mean (SEM). Statistical analyses were conducted using IBM SPSS Statistics 26. Data normality was assessed using the Kolmogorov-Smirnov test. Statistically significant differences ($p < 0.05$) were established using the analysis of variances (two-way ANOVA) and Tukey's multiple comparison test was applied.

2. Results

2.1. *Glycogen and protein content:* Maximum glycogen content (6.56 ± 0.13 g/100 g ww) was observed at the beginning of the experiment (days 0). A significant decrease was recorded in depurated individuals compared to non-depurated individuals at 2, and 4 days of depuration ($p < 0.01$), followed by a significant increase in depurated individuals by day 8 ($p < 0.05$) (Figure 2). A two-way ANOVA revealed a highly significant depuration days effect ($p < 0.01$), experimental phase effect (non-depurated vs depurated) ($p < 0.01$), and a significant depuration days x experimental phase interaction ($p < 0.001$).

Similarly, protein content exhibited higher protein levels compared to non-depurated individuals ($p < 0.01$) in the end of the experiment (8 days) (Figure 3). A two-way ANOVA (depuration days and experimental phase) indicated a significant of depuration days, experimental phase and depuration days, experimental phase interaction ($p < 0.001$).

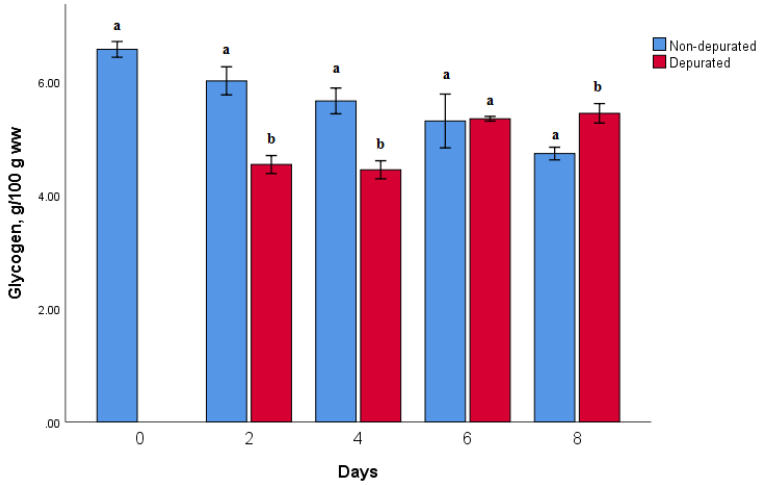

Figure 2. Glycogen content in *M. galloprovincialis* thought the depuration period. Different letters represent significant differences between non-depurated and depurated mussels (Tukey's test, $p < 0.05$); Mean ± SEM, n = 4

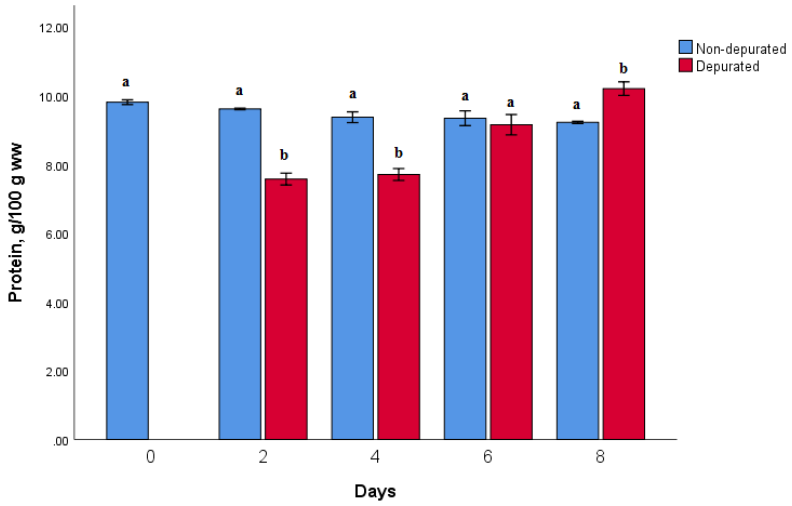

Figure 3. Protein content in *M. galloprovincialis* thought the depuration period. Different letters represent significant differences between non-depurated and depurated mussels (Tukey's test, $p < 0.05$); Mean ± SEM, n = 4

2.2. Lactate dehydrogenase (LDH) activity and protein carbonyls levels (PCs):

Figure 4 shows the specific activity of LDH. Depurated individuals exhibited significantly lower LDH levels than non-depurated individuals after 6 and 8 days of depuration ($p < 0.05$ and $p < 0.001$, respectively). A two-way ANOVA revealed a highly significant effect of experimental phase (non-depurated vs. depurated) and its interaction with depuration days ($p < 0.001$). For PCs levels, no significant differences were observed between non-depurated and depurated groups up to day 8 ($p > 0.05$) (Figure 5). However, by day 8, depurated mussels exhibited a significant reduction in PCs concentrations compared to non-depurated mussels ($p < 0.01$). Both experimental phase and depuration days had significant effects ($p < 0.001$), as revealed by a two-way ANOVA analysis. Additionally, there was a significant interaction between experimental phase and depuration days ($p < 0.01$).

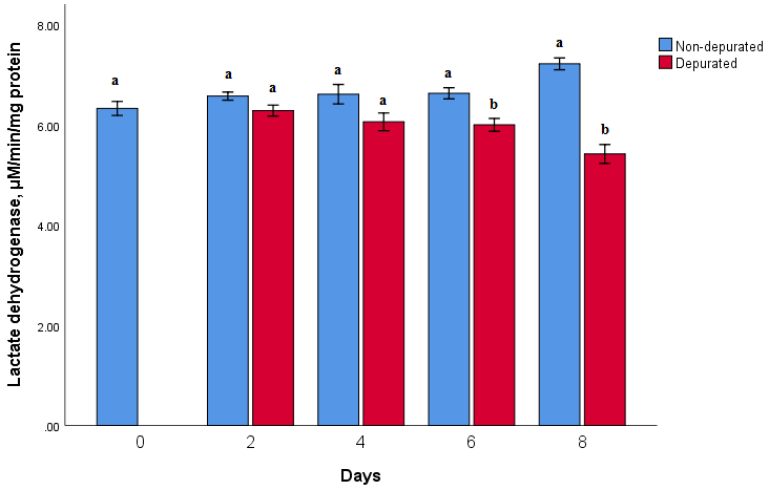

Figure 4. Lactate dehydrogenase activity in *M. galloprovincialis* thought the depuration period. Different letters represent significant differences between non-depurated and depurated mussels (Tukey's test, $p < 0.05$); Mean ± SEM, n = 4

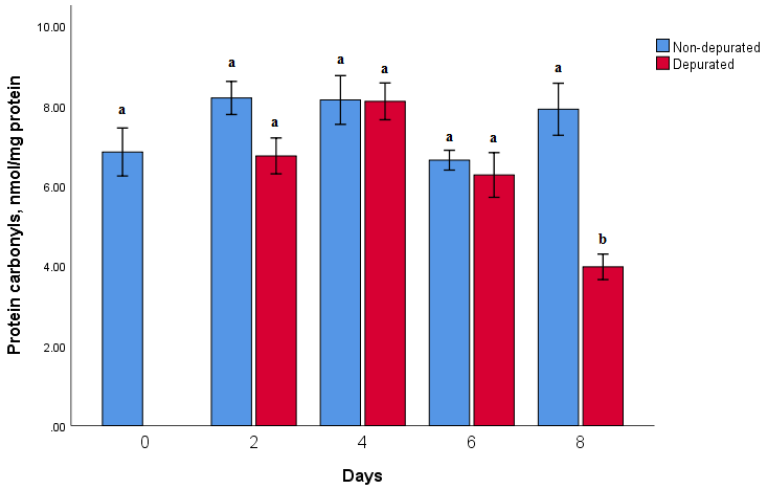

Figure 5. Protein carbonyls levels in *M. galloprovincialis* thought the days period. Different letters represent significant differences ($p < 0.05$) between non-depurated and depurated mussels (Tukey's test, $p < 0.05$); Mean ± SEM, n = 4

2.3. *Correlation test:* Pearson correlation analysis revealed a negative correlation between glycogen content and LDH activity (r = -0.100), as well as between protein concentration and levels of protein carbonyls (r = -0.314) (Table 1).

Table 1. Pearson correlation test between Glycogen levels and Lactate dehydrogenase activity (n = 36).

Parameters	r	*p*
Glycogen - Lactate dehydrogenase	-0.090	0.583
Protein - Protein carbonyls	-0.056	0.731

3. Discussion

Marine pollution has a profound impact on aquatic organisms, leading to observable changes in various biological responses, including biometric, biochemical, and cellular markers (Carrier-Belleau et al., 2021; Nadia et al., 2023; Olenin et al., 2024). These biological responses serve as ecotoxicological biomarkers, indicating specific or generalized stress in sentinel species when exposed to pollutants (Tlili et al., 2020; Qu et al., 2022; Martínez-Morcillo et al., 2024). Among these sentinel species, bivalve mollusks, such as *Mytilus galloprovincialis*, are of particular importance due to their ability to bioaccumulate pollutants, resulting from continuous exposure to water, sediments, and contaminated food (Abderrahmani et al., 2021; Pazi et al., 2023; Barboza et al., 2024). At the sub-individual level, physiological responses in bivalves serve as early warning indicators for ecotoxicological risk assessments (Lozano-Bilbao et al., 2018). A key management strategy for reducing contaminant levels in bivalves is depuration, where the organisms are placed in clean seawater tanks, allowing them to purge toxic substances (El-Shenawy, 2004; El-Gamal, 2011; Chinnadurai et al., 2023). This process effectively removes contaminants from their gills and intestinal tracts, preventing recontamination. Depuration has been shown to reduce the levels of heavy metals (Belabed & Soltani, 2018; Chinnadurai et al., 2022; Sami, 2024), petroleum hydrocarbons (Al-Saad et al., 2011; El-Gamal, 2011; Al-Saad et al., 2022), pesticides (Ivorra et al., 2019; Hamoudi et al., 2024), and microplastics (Ribeiro et al., 2022; Liu et al., 2023; Soubaneh et al., 2023).

Several factors affect the efficiency of the depuration process, including water quality, oxygenation and flow rates, salinity, temperature, shellfish-to-water ratios, and the nature of the pollutants (Manfra and Accorneo, 2005; Birnstiel et al., 2019; Chinnadurai et al., 2022). Our findings demonstrate that

depuration restored glycogen, protein, lactate dehydrogenase (LDH), and protein carbonyl levels (PCs) in *M. galloprovincialis* collected from the polluted Sidi Salem site (Ouali et al., 2018; Amira et al., 2018; Drif et al., 2019). Notably, the effectiveness of the process was dependent on the duration of depuration.

Glycogen is the primary metabolic reserve in mussels (Ke & Li, 2013; Vodáková & Douda. 2019; Wu et al., 2024), serving as a glucose source to sustain tissue function (Martinez-Pita et al., 2012). It is considered a reliable physiological marker for evaluating the health and stress levels of bivalves (Sim-Smith & Jeffs, 2011; Teixeira et al., 2017; Liu et al., 2023). Environmental stressors can rapidly alter glycogen levels, which are linked to nutritional status, stress, life cycle stages, and sexual maturity (Anacleto et al., 2015; Yang et al., 2023). Our study observed a decrease in glycogen levels during the first 2 to 4 days of depuration, followed by an increase after 6 to 8 days, a pattern consistent with observations in *Oreochromis mossambicus* after lead exposure and subsequent depuration (James et al., 1996). This initial glycogen reduction may be attributed to dietary restrictions during the depuration process, as previously noted (Oliveira et al., 2022; Bi et al., 2023; Chen et al., 2024). Other studies, including Anacleto et al. (2015) and Liu et al. (2021), have reported sustained reductions in glycogen levels during depuration to heavy metals in different bivalve species. The increase in glycogen levels observed in our study on the last day of depuration (day 8) can be explained by the efficiency of the detoxification system in mussels as described in previous studies (Freitas et al., 2012).

Proteins are essential for growth, reproduction, and overall maintenance, making them valuable biomarkers for assessing the health of aquatic organisms (Merad & Soltani, 2017; McCartney et al., 2021; Bai et al., 2023). In our study, we observed a reduction in protein levels during the first 4 days of depuration, likely due to stress-induced proteolysis. However, after 6 to 8 days of depuration, protein levels increased, a recovery that has also been reported in *Ruditapes decussatus* and *Venus verrucosa* (Soliman et al., 2015; Çağlak et al., 2020) treated by metals. Long-term depuration has been shown to restore protein levels, as observed in *Perna viridis* exposed to zinc (Narváez et al., 2005). The post-depuration increase in protein synthesis may be related to metal detoxification (Çağlak et al., 2017; 2020).

Lactate dehydrogenase (LDH), a key enzyme in anaerobic glycolysis, is a well-known biomarker of chemical stress and cellular damage (Deng et al., 2022; Gagné et al., 2023; Zhong et al., 2024). In our study, LDH activity significantly decreased during the depuration period, particularly after 6 to 8 days, indicating reduced anaerobic metabolism. This decrease suggests a compromised defense mechanism and reduced glycolysis, aligning with findings from previous studies (Rao, 2006; Tabane et al., 2024). Similar results have been observed in *Lamellidens marginalis* exposed to oil (Dharani Chakravarthy & Balamurugan, 2023), and in *Perna perna* after a 4-week depuration period following exposure to contaminated water (Tabane et al. 2024)**.** The reduction in LDH activity in our study corresponds with increased glycogen levels, suggesting a shift away from anaerobic metabolism.

Protein carbonyls (PCs) are reliable markers of protein oxidation, which occurs early during oxidative stress (Weber et al., 2015; Capó et al., 2021; Tang et al., 2022). Our results show a significant reduction in PCs levels after 8 days of depuration, consistent with previous studies on *Meretrix meretrix* and *Donax trunculus* exposed to sublethal concentrations of Cd (Zhou et al., 2024; Merad et al., 2016). Similarly, extended depuration in *Ruditapes philippinarum* exposed to polycyclic aromatic hydrocarbons, as well as on mullets (*Mugil cephalus*) collected polluted environment has been shown to reduce PCs levels (Li et al., 2020; Ferreira et al., 2007). The decrease in PCs is consistent with the increase in protein levels observed in our study, reinforcing the hypothesis that metal ions can inactivate proteins via non-specific binding, leading to reversible denaturation (Çağlak et al., 2017; 2020). Overall, depuration in *M. galloprovincialis* facilitated detoxification and reduced oxidative stress, confirming its potential as a vital tool in ecotoxicological assessments and pollution mitigation strategies (Freitas et al., 2012; Belabed & Soltani, 2022; Pizzurro et al., 2023; Tabane et al., 2024).

4. Conclusion

Overall, the results of this study demonstrated that *Mytilus galloprovincialis* inhabiting the polluted environment of Sidi Salem exhibited lower energy reserves, including glycogen and protein levels, and elevated biomarkers of oxidative stress such as LDH activity and protein carbonyls.

Furthermore, this research revealed that the restoration of biochemical effects caused by pollution at the Sidi Salem site in the bivalve mollusk *M. galloprovincialis* during the depuration process is time-dependent. Our findings showed that glycogen and protein levels significantly increased after eight days of depuration, while LDH activity and protein carbonyls showed significant reductions within six and eight days, respectively.

This study significantly contributes to our understanding of the mechanisms underlying depuration in the elimination of chemical toxins, as well as the biochemical and physiological status of bivalves. It underscores the benefits of depuration as a mitigation tool to reduce pollution levels in bivalves.

5. Acknowledgements

The authors wish to thank anonymous referees for useful discussions and critical reading of the manuscript. This study was conducted as part of a PRFU project (N° D01N01UN230120230005) of Pr. S. Yezli-Touiker), and was supported by the National Fund allocated to Research The authors wish to thank anonymous referees for useful discussions and critical reading of the manuscript. This study was conducted as part of a PRFU project (N° D01N01UN230120230005) of Pr. S. Yezli-Touiker), and was supported by the National Fund allocated to Research Laboratory of Excellence laboratory of Applied Animal Biology (University Badji Mokhtar, Annaba).

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