

## Research Article

# Integrative Biomarker Indices in a Benthic Indicator Species *Modiolus Modiolus* (L.) Under a Simulated Oil Spill

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**Abstract.** The horse mussel *Modiolus modiolus* (L.) is a sentinel bivalve species used in monitoring programs to assess potential biological exposure to anthropogenic contaminants, including oil hydrocarbons, in benthic environments. In an aquarium-based experiment, these mussels were exposed to a simulated oil spill where crude oil at realistic concentrations (from low to high) interacted with an inert environment (seawater, gravel surface, etc.) and the biota. Using a combination of endpoints that included tissue contaminant load, protease activity, antioxidant enzyme activity, and low-molecular antioxidant profiles, we characterized *M. modiolus* responses to this simulated crude oil spill. Significant differences were observed in tissue protein reserves, protease activity, and oxidative stress markers including glutathione content and glutathione-S-transferase activity in the hepatopancreas and gill tissues of the bivalves treated with oil. Total concentrations of oil-derived polycyclic aromatic hydrocarbons in *M. modiolus* tissues were generally highest under the highest ambient oil concentration with much lower levels at the lowest. This general pattern does match the activity of protein quality control proteases but not the antioxidant enzyme activity profiles. Glutathione-S-transferase activity in bivalves showed decreased activity under the high compared to the lower oil load and oil-free animals, while glutathione content and calpain activity were positively correlated with oil uptake. The data suggest that these benthic organisms were exhibiting biological responses to the oil polycyclic aromatic hydrocarbons and support the bioindicator value of the species.

**Keywords:** *M. modiolus*, oil spill, PAHs, integrative biomarker approach, protein quality control, GSH, GST

## 1. Introduction

The bivalve mollusks are widely used as indicator species in ecotoxicological biomonitoring programs [1-3]. Mollusk health status assessment through integrative biomarker indices is widely used in the biomonitoring programs as such a 'Mussel Watch' developed to detect nature consequences of the 'Prestige' oil spill [4]. Horse mussels *Modiolus modiolus* (L.) are a deep-sea benthic species readily accumulating oil and associated

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polycyclic aromatic hydrocarbons (PAHs) [2, 5] from contaminated marine and coastal environments including due to sporadic spills [6]. Mussels respond to pollutant uptake through activating molecular mechanisms maintaining cell viability under pollutant-induced oxidative stress, toxicity, cellular stress threatening the structural and functional integrity of macromolecules, organelles, and tissues. Cell viability depends on antioxidant and detoxification response involving intracellular glutathione/glutathione-S-transferase (GSH/GST) system and cell fate is under the control of proteolytic systems which recognize abnormal proteins and organelles, target and proteolytically eliminate them via the mechanism of protein quality control, as well as regulate cell death pathways. All the executor enzymes of the response reactions could be applied as pollutant biomarkers [7-10].

An oil spill was simulated an aquarium-based experiment by providing crude oil interaction with marine water and gravel and gradual decline in concentration due to dissolution. In order to study the effects of oil on horse mussels in a realistic environment, a panel of biomarkers specifically indicating organic contamination and oxidative stress were assessed in *M. modiolus* gills and hepatopancreas. Integrative biomarker indices included the antioxidant and detoxification systems and protein quality control enzymes. The studied parameters were as follows: glutathione-S-transferase (GST), cathepsin B, cathepsin D, and calpain peptidase activities, as well as the reduced glutathione (GSH) and tissue protein contents.

## 2. Materials and Methods

### 2.1. Scheme of the experiment

*M. modiolus* individuals were collected from a near-shore site of the Chupa Bay of the White Sea (66° 33' N, 33° 64' E) using a 0.1 m<sup>2</sup> Van Veen grab at depth of 8-10 m. Only individuals of a similar shell length (70.2±3.9 mm) and age (6-7 years) and of reproductive resting were included in the study. *M. modiolus* were maintained at 10 °C and 12h light: 12h dark regimen in aerated 20 L plexiglass tanks (25 individuals per tank) filled with seawater partially (10 L) replaced daily. After 7-day acclimation, the mussels were randomly assigned to either oil-free or oil-treated groups. In a semi-static exposure setup, oil was added to tanks filled with 5 kg of shore gravels (particle size 3-5 mm) as 1, 5, and 50 mL of 10% Surgut oil emulsion in seawater, corresponding low (L), medium (M), and high (H) oil treatments. At the start of the experiment, 10L of L, M, or H oil-contaminated water was added to tanks with animals assigned to oil treatments

instead of a regular water replacement; since then, a half of a tank water volume (10L) was replaced daily with oil-free seawater to simulate the dissolution of spilled oil. Water samples for PAH quantification (mg per L) were taken in triplicates from each tank with oiled gravel (initial point) and at 1, 3, and 10 days of mussel exposure. *Modiolus hepatopaneas* and gill tissues were sampled before the oil exposure (0d) and at 1 (1d), 3 (3d), and 10 (10d) days following exposure. The organs were excised with a lancet and immediately frozen in liquid nitrogen.

## 2.2. Dissolved PAHs content

Water-soluble PAH concentrations in water treatments were estimated after extraction and concentration of the PAHs in hexane followed by its determination by fluorescence at Ex 310 nm, Em 360 nm wavelengths according to the protocol [11]; results were expressed as total PAH content (mg/L).

## 2.3. Biomarker analyses

The soluble fraction of cellular proteins used as a source of the enzymes was obtained after the homogenization of a tissue sample (0.1 g) in 0.125 M PBS and centrifugation at 100,000 g for 60 min. GST activity was determined by the GSH-CDNB (nM/min/mg) production through the reaction of reduced glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) [12]. The concentration of reduced glutathione was determined using 0.01% o-phthalaldehyde after precipitation of the soluble proteins by 5% TCA with fluorescence (Em 420 nm, Ex 350 nm) [13]. Cathepsin B (CatB) peptidase activity (AU) was measured by the hydrolysis of 65 mM ethyl ether D-benzoyl L-arginine hydrochloride (BAEE) [14]. Cathepsin D (CatD) proteolytic activity (AU) was assessed with 1.0% bovine serum hemoglobin hydrolysis [14]. Calpain activity (AU) was measured by amino acid content produced via calcium-dependent casein hydrolysis [15]. Water-soluble protein concentration (mg per g of wet weight) was determined according to the Bradford technique [16].

## 2.4. Statistical analyses

Statistical analyses were carried out using MS Excel and StatGraphics software. A one-way analysis of variance (ANOVA) of the biochemical parameters was performed to determine the effects of environmental factors with water hydrocarbon concentrations

TABLE 1: Calculated and real concentrations of dissolved PAHs (mg/L) in tank water before, at 1-, 3-, and 10-days of mussel exposure and PAH content in the soft body of mussels (mg/g wet weight) exposed for 10 days.

		Oil concentration		
		Low	Medium	High
Tank water				
Calculated		9.00	45.00	450.00
Initial		2.20 ± 0.64	3.50 ± 0.17	68.00 ± 1.80
Day 1		0.80 ± 0.10	0.17 ± 0.04	18.60 ± 1.31
Day 3		0.47 ± 0.07	0.09 ± 0.05	21.90 ± 0.30
Day 10		0.07 ± 0.03	0.29 ± 0.08	4.80 ± 0.93
Mussel	soft			
body				
Day 10		0.4 ± 0.07	1.8 ± 0.40	9.6 ± 0.16

as a fixed factor. A multiple comparison post-hoc test (Tukey's HSD) was used to determine significance, with  $p$ -values less than 0.05 considered significant. All data are presented as mean ± standard deviation.

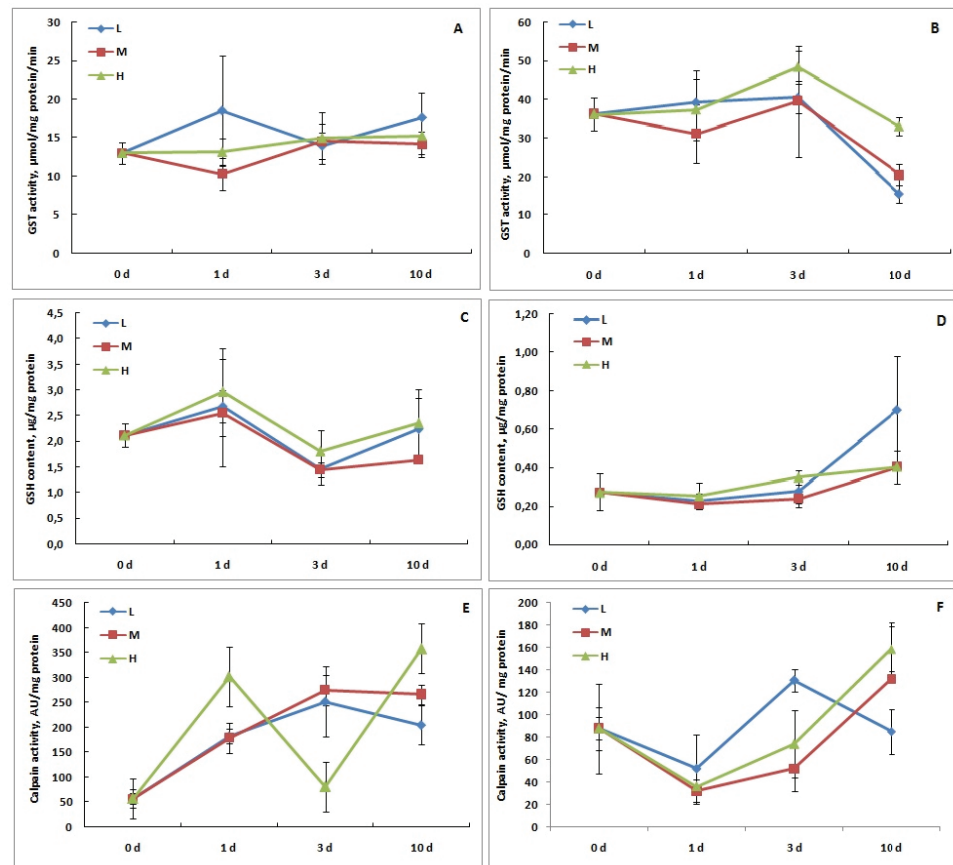
### 3. Results

#### 3.1. PAH concentrations in tank water and *M. modiolus*

Water from the tanks with oiled gravel and tanks with experimental animals has been sampled on the same dates as tissue sampling and then analyzed for PAHs (Table). Initial PAH concentrations in H, M, and L water treatments were 68.0, 3.5, and 2.2 mg/L respectively, and gradually declined over the experimental period. By the end of the 10-days experiment, total PAHs content in the soft body of oil-exposed mussels was higher than in the surrounding water.

#### 3.2. The biotransformation system in oil-treated *M. modiolus*

The trend to GST induction in gill and hepatopancreas of mussels subjected to L and M oil contamination, while substantial downregulation in hepatopancreas at maximal (H) oil load were observed (Fig. A, B). One-way ANOVA analysis shows the significant effect ( $F = 2.09$ ,  $p < 0.01$ ) of water hydrocarbon level on the activity of GST in mussel hepatopancreas while failing to indicate an oil-induced response in gill. GSH content in mussel gill was higher at acute oil exposure (1d) while exhausted with the time of exposure; increase in GSH content in hepatopancreas was demonstrated at prolonged



**Figure 1:** Integrative biomarker indices in gills (A, C, E) and hepatopancreas (B, D, F) of *M. modiolus* before the experiment (0d) and exposed to ambient water with low (L), medium (M), and high (H) oil concentrations during 1, 3, and 10 days. Bars represent mean  $\pm$  SD ( $n = 7$ ); significant differences between oil-treated and control groups (Tukey's test,  $p < 0.05$ ) are discussed in the text.

10-days exposure (Fig. C, D). ANOVA analysis revealed a weak effect of water hydrocarbon concentration on peptide-bound-thiol content ( $F = 1.53$ ,  $p < 0.05$ ).

### 3.3. The protein quality control related protease activities in oil-treated *M. modiolus*

Proteolytic activities of autophagic pathway of proteolysis, such as cathepsin B and cathepsin D, did not show any relevant patterns with oil uptake or exposure time (data not shown), whereas cytoplasmatic  $\text{Ca}^{2+}$ -dependent calpains have been activated ( $p < 0.05$ ) since acute (1d) oil treatment in gill and at 3 to 10 days in hepatopancreas depending on dosage (Fig. E, F).

## 4. Discussion

Similar to other observations [9, 17, 18], the total PAH concentration in water treatments was lower than the calculated one because of hydrocarbon sorption on shore gravel and incomplete solubilization of crude oil on seawater. Total PAH content gradually diminished throughout the experiment due to degradation of low molecular weight hydrocarbons, evaporation, and biosorption of PAHs; although it is evident that a 10-day period is not enough to completely exclude a contaminant from the water. Based on our observations, soluble oil fractions are readily accumulated by the mussels with PAH content in their tissues exceeding those in the surrounding water.

Our results show that the bivalve biotransformation system was influenced by oil which primarily affected GSH/GST (reduced glutathione/glutathione-S-transferase) system. Besides the role of GST and GSH in phase II xenobiotic biotransformation, they recognize to be oxidative stress markers [4, 10, 19]. The activity of GST is often induced and correlates with PAH levels, though in certain cases (as in our observation), inhibition of activity following exposure to contaminants has also been reported [20]. In our study, though no apparent dose-dependent GST activity response in *M. modiolus* gills was shown, there was significant suppression of hepatopancreas enzyme activity induced by M and H oil dosages at maximal exposure. A similar effect on bivalve *Crassostrea gigas* treated by dissolved diesel fuel (up to 1.0 mL/L) [18] the authors assigned to GST synthesis impairment due to the contaminant. Partial depletion of cellular GSH under the 3-day oil treatment likely indicates the downregulation of antioxidant defense mechanisms leading to oxidative stress [21]. Probably due to gradual pollutant elimination according to semi-static experimental design the relative abundance of tissue GSH reserves recovers at 10-day exposure. The GSH content patterns in the gill and hepatopancreas of *M. modiolus* are consistent with those in *Mytilus edulis* at similar experimentation [9]. The GST activation and GSH accumulation in *M. modiolus* gill at short-time oil exposure indicate the upregulation of detoxification reactions [21], whereas an inadequate increase in GSH content at GST activity suppression in hepatopancreas could be a sign of compromised health status.

Lysosomal autophagy is considered a second-level protective process in conferring resistance to toxicant-induced oxidative stress and proteostasis maintenance in bivalves [22]. Catheptic proteases in both the gill and hepatopancreas tissues were found not to be significantly affected by oil PAHs in *M. modiolus*, and the activation of calpains revealed a nonspecific stress response under oil uptake. Unlike *M. modiolus*, the oil exposure was found to have a significant influence on autophagy proteases in *Mytilus*

*edulis* gill [9]. Calpains are pleiotropic proteases with extremely high hydrolytic capacity in invertebrates [23, 24] specifically contributing to both the integrity and destruction of the tissues due to xenobiotic load. Oil-induced calpain activation developed earlier in *M. modiolus* gill than in hepatopancreas. An increase in total calcium-dependent proteolytic capacity is considered a non-specific response to pollutants and natural stressors; similar patterns of calpain activity were found in mussels *M. edulis* experimentally treated with oil PAHs [9] and inhabiting the White Sea coastal zones with complex anthropogenic pollution, particularly oil contamination [14]. Specific calpain activity patterns correlating with accumulated oil have not been shown; however, calpain activity generally responds in a complicated manner, as calpains are involved in various intracellular functions.

Though the oil components were a sole factor affecting mussel health, the bivalve defense mechanisms responded more complex than could be predicted. In general, oil treatment stimulated defense responses in the mussels with primary effects on GST and calpain-mediated reactions; mostly, biomarkers responded to oil in a dose- and time-dependent manner. Although the hepatopancreas is recognized as a main hydrocarbon storage site in mussels [25], the defense responses in gill develop earlier than in hepatopancreas apparently indicating a primary path of contaminant uptake through the gill. The results obtained indicate that a benthic species *M. modiolus* responds to crude oil less than littoral *M. edulis* to the same exposure [14]. Coping with multiple stressors, a marine littoral species, *M. edulis*, has well-developed compensatory mechanisms to tolerate xenobiotics as well; in contrast, the adaptive response occurs in a relatively long-time frame in benthic species, like *M. modiolus*.

## 5. Conclusion

The integrative multi-biomarker approach highlights the responsiveness of antioxidant enzymes and protein quality control proteases to organic contamination in *Modiolus modiolus* (L.). Among the studied biomarkers, GST and calpain activity were the most responsive towards an experimental oil spill. Despite responding less than littoral bivalves, last decade *M. modiolus* has gained importance as sentinel species of marine pollution in numerous biomonitoring programs.

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## 8. Conflict of Interest

The authors declare no conflict of interest.

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