Evaluation of the long-term effects of formaldehyde on the physiology of the Mediterranean mussel, *Mytilus galloprovincialis*

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Abstract

Formalin baths are the most widely used treatment for ectoparasitic fish diseases. Nonetheless, their use in fish cages has been blamed for a number of problems. Although a considerable amount of literature has been produced on the short-term toxic effects of formaldehyde, there is virtually no data on the long-term side effects of the compound on non-target organisms. Therefore, the purpose of this research was to assess the long-term formaldehyde toxicity in Mediterranean mussel, *Mytilus galloprovincialis*, a common sentinel species that inhabits the area surrounding cage farms. Mussels were kept in a laboratory microenvironment at 20±1°C for 21 days and exposed to two different formaldehyde concentrations during experimentation: a low dose (L; 40 ppb) based on formaldehyde field measurements in the vicinity of Mediterranean cages, and a high dose (H; 400 ppb) generated by a factor of 10 of the previous dose. A multi-biomarker approach that included antioxidant enzymes such as catalase (CAT) and superoxide dismutase (SOD), lipid peroxidation (MDA), lysosomal stability (NRRT), genotoxicity tests, condition index (CI), and stress on stress (SoS), was used to evaluate the toxicity of formaldehyde on mussels. The results of the selected tests indicate that formaldehyde does not cause chronic toxicity in mussels subjected to commonly measured concentrations in the aquatic environment following formalin bath treatments. Despite being defined as reversible, the stress brought by the high dose used seems to reduce the antioxidant activity of the tested organism. The significance of this research lies in its contribution to understanding the wider ecological effects of formaldehyde exposure. Moreover, the results highlight the need for further research on other non-target marine organisms to fully understand the cumulative effects of formaldehyde on marine ecosystems.

Keywords: Formaldehyde, formalin, toxicity, Mediterranean mussel, *Mytilus galloprovincialis*

1.Introduction

The intensive use of fish cages has undoubtedly accelerated the global growth of marine aquaculture. However, poor hygiene and management combined with adverse environmental conditions, favour the appearance of ectoparasitic diseases that can occasionally be devastating to farmed fish. Formalin baths are arguably the most common treatment to battle ectoparasites worldwide (Boyd & McNevin, 2015). They are extremely effective against protozoans and helminths invading the gills, skin, and fins of fish. Formalin is an aqueous solution containing 37%-40% formaldehyde and 10%-15% methanol, as a stabilizer to prevent the polymerization of formaldehyde to paraformaldehyde (Leal et al., 2018). The application of formaldehyde baths in cages is carried out with the inclusion of tarpaulin bags used to seal the shifted nets. Such baths last from 30 to 60 min with concentration levels adjusted (150-250 ppm), depending on the water temperature (Hodkovicova et al., 2019). After the completion of the bath, the bags are removed, and formalin is released into the environment. These laborious and clearly weather-dependent practices have also raised considerable concerns regarding the safety of workers, divers, consumers, and more importantly, the environment.

Consequently, plenty of research including main toxicity and degradation studies (Leal et al., 2018), has evaluated the possible side effects of formalin use in aquaculture environments. Although formaldehyde has been well-studied in terms of its acute toxic effects, most existing research focuses on its immediate impacts, leaving a significant knowledge gap regarding its long-term environmental effects, especially on organisms other than the target fish species. A unique attempt to measure formaldehyde residues in the aquatic environment during the summer period after formalin bath treatments showed that negligible amounts of the

substance remained in the seawater (Kotsiri et al., 2023). However, the fate of formaldehyde may depend on the environment subjected to different treatment schedules. Regarding the effects on exposed organisms, there are studies with controversial conclusions on the possible effects of formaldehyde and the results always vary with the experimental conditions used. In particular, some studies have reported potential stress events in sea bream, *Sparus aurata*, and sea bass, *Dicentrarchus labrax* (Yildiz and Ergonul, 2010) and adverse effects on rainbow trout, *Oncorhynchus mykiss* (Buchmann et al., 2004). The adverse effects of formaldehyde on treated fish are mainly associated with gill damage (Leal et al., 2018).

In contrast, Speare et al. (1997) reported limited significant adverse effects on Atlantic salmon, *Salmo salar,* when exposed to recommended doses of formalin.

The Mediterranean mussel, *Mytilus galloprovincialis*, is generally considered an ideal bio-indicator organism (Curpan et al., 2022; Mikhailov et al., 1998; Özden et al., 2010) which is commonly found in marine ecosystems near fish farms. Specifically, mussels serve as effective indicators for monitoring coastal environments due to several advantageous biological characteristics, as noted by Orescanin et al. (2006) and Kristan et al. (2014). They are sedentary filter-feeders, meaning they remain in one location and filter water for food (Wu et al., 2017). This way of life provides them with a large capacity to accumulate pollutants in their tissues. Moreover, mussels exhibit a strong and specific response to pollutants, making them valuable for detecting environmental contamination (Catsiki and Florou, 2006). Their low decontamination kinetics further contribute to their suitability as indicator organisms for monitoring pollutants over extended periods. Additionally, mussels are easy to collect, and provide sample tissue for chemical analysis (Wangl et al., 1996). These characteristics make mussels highly suitable as sentinel species for monitoring coastal environments, providing valuable insights into environmental health and pollution levels.

Therefore, this study addresses the critical need to assess the long-term toxicity of formaldehyde, particularly in the context of aquaculture practices that frequently introduce this compound into marine ecosystems, by evaluating the effects of low and high concentrations of formaldehyde on the Mediterranean mussel over 21 days. Understanding the chronic effects on sentinel species such as the Mediterranean mussel will help to fill the gap in the current literature, which overlooks these longer-term effects.

In addition, this research makes a unique contribution to the field by using a multi-biomarker approach to provide a comprehensive assessment of stress responses in mussels exposed to formaldehyde. The results highlight the need for further research on other non-target organisms to understand the cumulative and potentially ecosystem-wide effects of formaldehyde exposure.

2.Material and methods

2.1 Experimental setup

Adult Mediterranean mussels (60±5 mm length, 33±3.3 mm height, 22±2.6 width, 12.3±2.3 g net weight) were obtained from a mussel farm located in the Saronic Gulf of Attica, Greece. The mussels were all scrap-cleaned and kept for 5 days for acclimatization in an aerated tank containing artificial seawater (ASW, Red Sea Coral Pro Salt) without algae feeding. Approximately 32 g of salt per litre of water was used to create the artificial seawater and the salinity was measured using the HQ14d Portable Salt Meter (HACH).The acclimation procedure was performed to allow the mussels to gradually adapt to new culture conditions. A microenvironment with nine Plexiglas tanks (13 L) was used (Fig. S1). Mussels were equally distributed into the tanks (2 mussels/L) giving a total of 28 mussels per tank and maintained at 20°C at a 12:12 L: D photoperiod on a daily diet of Phytomaxx (NYOS) live phytoplankton

provided at a density of 500m cells/ml. Two concentrations of formaldehyde were tested in triplicate against a control (C). The low concentration (L) was based on field formaldehyde measurements around cages (Kotsiri et al., 2023) and the high dose (H) was produced by a factor x10 of the low dose. Eventually, the L and H groups were exposed to 40 and 400 ppb formaldehyde, respectively. There was a 100% replacement of seawater every other day with re-dosing of formaldehyde concentrations. Seawater quality was analyzed daily by measuring temperature (20±0.5°C), dissolved oxygen levels (93.5±2.7%), and salinity (29.2±0.4‰). The experiment lasted 21 days, excluding the adaptation period, during which no mussel mortality was observed in formaldehyde-exposed and non-exposed mussel groups.

2.2 Sampling

At each sampling, gills, mantle and digestive glands were collected from tested mussels and immediately stored at 80°C until analysed for oxidative stress (superoxide dismutase - SOD, catalase - CAT) and damage (lipid peroxidation - LPO). Haemolymph samples were collected for genotoxicity and neutral red retention time (NRRT) assays (Fig. 1). Whole mussels were also collected for condition index (CI) and the remaining mussels at the end of the experiment were used for stress-on-stress (SoS) biomarker determination (Fig. 1). After each mussel removal, the volume of water was adjusted to the number of mussels in each tank to maintain the desired ratio steadily. The detailed sampling plan for the following analyses is shown in Figure 1.

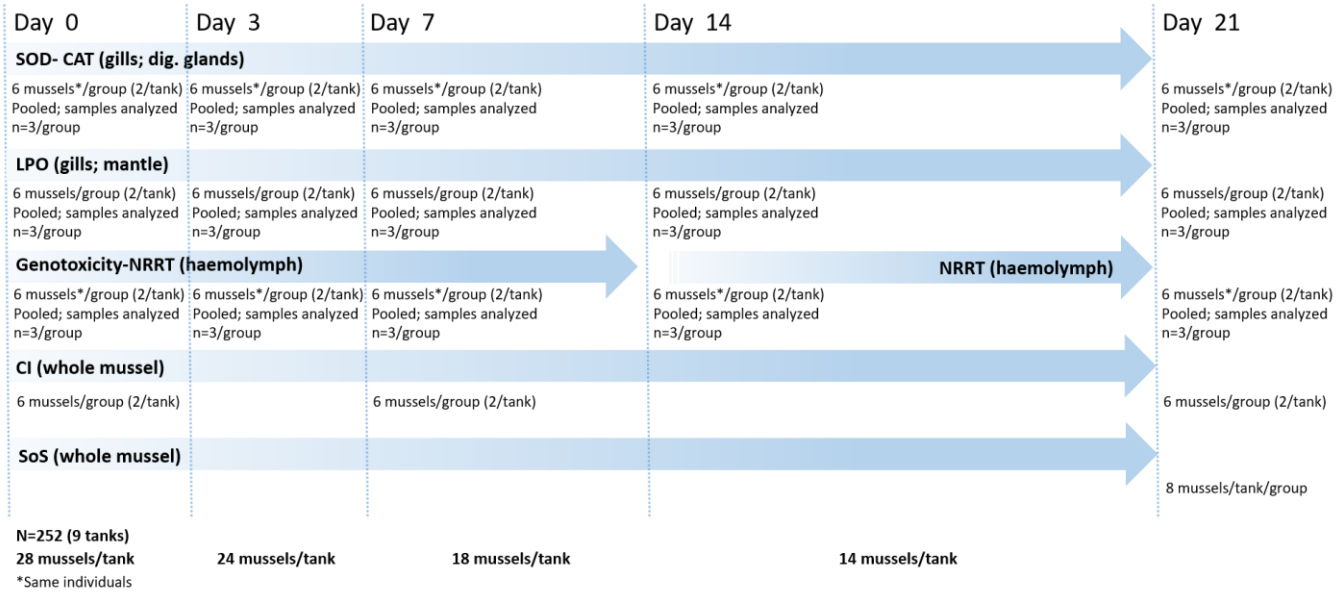

Fig. 1. Sampling diagram

2.3 Antioxidant status

Gills and digestive gland homogenates (supernatants) were used to assess the activity of total SOD (cytosolic and mitochondrial) and CAT by an enzymatic assay kit brought from Cayman Chemical (Cat.706002 and 707002, Ann Arbor, MI, USA) based on manufacturer's instructions. In addition, protein concentration in samples was determined with the *RC DC*™ Protein Assay kit II (Bio-Rad) using bovine serum albumin (BSA) as standard (Bradford, 1976).

2.4 Oxidative damage

To assess the oxidative stress in pathophysiological processes, the levels of LPO in gill and mantle were measured using a thiobarbituric acid reactive substances (TBARS) kit from BioVision Inc (Cat. K739, Abcam, USA) according to the manufacturer's protocol.

2.5 Neutral red retention time assay

The NRRT assay is based on the incorporation of the cationic dye-neutral red probe within lysosomes of mussel haemocytes and was applied according to a modified method of Miller et al. (2015). The detailed protocol is documented in the Supporting Information.

2.6 Single Cell Gel Electrophoresis (Comet Assay)

Haemolymph, gills and digestive system were collected from mussels. At the termination of each trial (sampling days 0, 3, and 14), 1 ml of haemolymph was collected and mixed with 5 ml phosphate-buffered saline (PBS). Gills and digestive gland were firstly dissected and placed in 10 ml cold HBSS-balanced solution (Mg^{++}, Ca^{++} free) on ice, then injected with collagenase solution (Biochrom AG, Germany), and finally minced into small pieces. The filtered cell suspensions were filtered and centrifuged (3000 rpm, 5 min), while the centrifugation-resuspension processes were repeated twice for the remaining pellet, in 10 ml PBS. The alkaline comet assay protocol is provided in the Supporting Information.

2.7 Condition Index

The CI was assessed in mussels to determine their physiological status at the beginning and after 7 and 21 days of exposure. The CI was calculated as the percentage (%) of the ratio between the drained weight of the soft tissues (g) and the whole mussel weight (tissue and shell) (g)(Gomes et al., 2013; Gonçalves et al., 2022).

2.8 Stress-on-Stress

SoS response is characterized as an integrated tolerance test in air exposure and a contaminant-biological effect monitoring tool. At the end of the experiment, eight mussels from each treatment were used for the LT_{50} (the median survival time or the time (days) in which 50% of mussels have died) estimation. The extensive SoS protocol is provided in the Supporting Information.

2.9 Statistical analysis

Two-way analysis of variance (ANOVA) was applied for statistical comparisons between treatments and time followed by Tukey's multiple comparisons test after verification of the normality of the data (Kolmogorov-Smirnov; Shapiro-Wilk test). LT_{50} values were calculated using the Kaplan-Meier method for Survival Analysis (Kaplan and Meier, 1992), and survival curves were compared with the Log-rank (Mantel-Cox) test. Results were considered significant when $P< 0.05$ and analysis was performed with GraphPad Prism v.9. Principal component analysis (PCA) was carried out using XLSTAT 2021 (Addinsoft, New York, NY, USA) and used to evaluate the relationship between treatments and the antioxidant enzyme activities in the gills and digestive glands of mussels over the experimental period.

3.Results and Discussion

3.1 Antioxidant status

SOD and CAT are categorized as crucial first-line defense antioxidants and are generated mainly through the mitochondrial energy production pathway (Kim et al., 2017; Morad et al., 2023). SOD is an enzyme that plays a crucial role in protecting organisms by converting superoxide radicals into oxygen and hydrogen peroxide. This process eliminates excess

reactive oxygen species (ROS) and helps maintain the redox balance of the immune system (Wu et al., 2017). CAT, on the other hand, plays an important role in the defense against oxidative stress through the detoxification of hydrogen peroxide which is a toxic product and the major precursor of hydroxyl radical (Revel et al., 2019; Soldatov et al., 2013). The catalytic activity of this enzyme breaks down hydrogen peroxide into water and oxygen, thus ensuring the elimination of ROS from the system (Chakraborty et al., 2013). Different antioxidant enzymatic activities (SOD & CAT) were found in the mussel gills and digestive glands following exposure to formaldehyde for 21 days, compared to the control group (Fig. 2).

SOD activity in both tissues of unexposed mussels remained at a similar level over time ($P > 0.05$; Fig. 2A-B). In the mussel gills, no significant differences were encountered in exposed mussels compared to unexposed mussels, and this pattern persisted regardless of the duration of exposure ($P > 0.05$; Fig. 2A). This suggests that the oxidative stress induced by formaldehyde exposure did not significantly alter the SOD activity and did not disrupt the balance of the antioxidant system in the mussels. On the other hand, in exposed mussels at high concentration (H), SOD activity increased significantly after 7 and 21 days in the digestive gland when compared to day 0 ($P < 0.05$; Fig. 2B). However, insignificant changes were noticed in the other groups during the experimental period. The observed increase in SOD activity in the digestive gland of mussels could be attributed to its increased synthesis in response to oxidative stress. Specifically, it is necessary to catalyze the excess superoxide radical into the less destructive hydrogen peroxide and molecular oxygen to prevent cellular oxidative damage (Jo et al., 2008; Wu et al., 2017). It appears that exposure to formaldehyde may generate superoxide radicals in the tissue. However, the increase in SOD activity on days 7 and 21 suggests that these radicals were counteracted. A similar pattern was observed in the

228 blue mussels *Mytilus* spp., which were exposed to microplastics (MPs) for 10 days (Revel et
229 al., 2019) and polystyrene MPs for 14 days (Paul-Pont et al., 2016), respectively.
230 The CAT activity in mussel gills that were exposed to formaldehyde remained at the same
231 level as in unexposed mussels during the entire exposure period ($P > 0.05$; Fig. 2C), except on
232 days 7 and 21, where the CAT activities significantly increased ($P < 0.05$). This increase in CAT
233 activity could be attributed to the protection against lipid peroxidation on these specific days
234 (Fig. 3A) (De Almeida et al., 2004; Vlahogianni et al., 2007). Our results suggest that CAT acts
235 as a defense mechanism against formaldehyde and that mussel exposure at high
236 concentration affected antioxidant enzymatic defenses compared to control animals. In line
237 with the current findings, previous studies on blue mussels, and clams, *Scrobicularia plana,*
238 have demonstrated an increase in CAT activity when exposed to polyethylene (PE) and
239 polypropylene (PP) plastic particles (Revel et al., 2019) and to polystyrene (PS) MPs,
240 respectively (Ribeiro et al., 2017). Moreover, these results reflect those of Khessiba et al.
241 (2005) and Natalotto et al. (2015) who also found that the enzymatic activity of CAT increased
242 in Mediterranean mussel and noble pen shell, *Pinna nobilis* exposed to gamma-
243 hexachlorocyclohexane (γ-HCH, lindane) and a polluted area, respectively.
244 The present study revealed significant fluctuations and varying trends in CAT activity in the
245 digestive gland based on the concentration of formaldehyde exposure. This considerable
246 variability may suggest that in animals, following the initial decline in CAT activity, CAT
247 production is triggered to counteract the impacts of oxidative stress. Our results are
248 consistent with those of De Almeida et al. (2004), who observed similar fluctuations of CAT
249 activity in the digestive glands of the brown mussel, *Perna perna* exposed to Cd and Cu for
250 120 h. CAT activity in the digestive gland of mussels exposed to formaldehyde was
251 significantly different from that of unexposed mussels throughout the exposure period ($P <$

252 0.05; Fig. 2D) except for H group on days 3 and 21. In the L group, the values were higher
253 compared to the control group throughout the exposure period, showing a significant
254 increase after 3, 7, and 14 days of exposure ($P< 0.05$) (Fig. 2D). As previously mentioned, the
255 elevated levels of CAT activity can be attributed to protection against lipid peroxidation (De
256 Almeida et al., 2004; Vlahogianni et al., 2007) (Fig. 3). This finding aligns well with previous
257 studies that demonstrated increased CAT levels in Mediterranean mussels exposed to $CdCl_2$
258 (Viarengo et al., 1999), in clam, *S. plana*, blue mussels, *Mytilus* spp., and Korean mussel,
259 *Mytilus coruscus* exposed to various sizes and types of MPs (Revel et al., 2019; Ribeiro et al.,
260 2017; Wang et al., 2020).
261 On the contrary, CAT activity was overwhelmed in H-exposed mussels at 3 and 21 days of
262 exposure, compared to the other groups ($P< 0.05$), suggesting a heightened sensitivity of
263 mussels to oxidative stress (Fig. 2D). This interesting result suggests that the mussels' ability
264 to eliminate H_2O_2 generated as a result of formaldehyde exposure, seemed to decline. Various
265 factors could account for this observation. Initially, the inhibition of CAT at the highest
266 concentration of the experiment might be due to a decline in NADPH levels, as this coenzyme
267 is essential for CAT activity, or an increase in the inhibitor superoxide anion (Al-Brakati et al.,
268 2021; Morad et al., 2023). An alternative explanation for this decline may be either a delay in
269 protein synthesis or a direct inhibitory effect of formaldehyde on CAT (Wang et al., 2011). The
270 results mentioned align with other research demonstrating that exposure to sodium arsenite
271 in bivalves can inhibit the activity of the detoxification enzyme CAT (Chakraborty et al., 2013)
272 and that marine mussels *Mytilus edulis*, *M. galloprovincialis*, and *S. plana* exposed to MPs
273 may also exhibit inhibition in their digestive glands (Avio et al., 2015; Paul-Pont et al., 2016;
274 Ribeiro et al., 2017). These results further support the notion that antioxidant activity may

increase at low concentrations of xenobiotics or during short-term exposure. However, it may decrease at higher concentrations or long-term exposure (Wang et al., 2011).

Moreover, the CAT activity measured in both L and H groups followed a bell-shaped increase in response to formaldehyde. Several reports have mentioned that an organism exposed to relatively high concentrations may elicit a higher modulation, while a bell-shaped increase in enzymatic activity is predictable at low concentrations of xenobiotics (Kim et al., 2017; Parolini et al., 2016). A possible explanation for this could be that the initial increase caused by the activation of enzyme synthesis is followed by a decrease in activity due to the direct inhibitory effect and/or the increased catabolic rate of the chemical on the enzyme molecules (Parolini et al., 2016; Viarengo et al., 2007).

Unexpectedly, higher CAT activity was observed in the digestive glands compared to gills, although they are the most important organ exposed to seawater due to their respiratory function. The antioxidant system of the digestive gland is more influenced by internal (e.g. reproductive period and nutrition) and less by external factors (e.g. environmental pollutants) (Box et al., 2009; Santovito et al., 2005). On the other hand, the digestive gland is involved in several processes, including molecular biotransformation, metabolic regulation and detoxification of xenobiotics (Multisanti et al., 2024; Tresnakova et al., 2023). The increase in CAT activity in the digestive gland suggests that the degree of oxidative stress is higher in this tissue and may be related to the detoxification of formaldehyde. In this context, it was mentioned that gills have a fast and efficient enzymatic mechanism against increased levels of oxygen radicals (Irato et al., 2007). This result is consistent with other studies showing that the digestive gland has the highest CAT activity, while the gills are the organ with the highest GST, GPx and AChE enzyme activities (Vidal-Liñán and Bellas, 2013). In addition, Irato et al. (2007) showed similar results of CAT responses to changes in dissolved oxygen in the bivalves

Unequal arc, *Scapharca inaequivalvis* and Manila clam, *Tapes philippinarum*. Thus, the antioxidant defense system of mussels exposed to formaldehyde shows tissue-specific responses indicating the different functions of the tissues during stress responses. The observed tissue-specific response to xenobiotics is not uncommon in Mediterranean mussel. In particular, the herbicide metabolite propachlor ESA can activate different intracellular oxidative pathways of protein carbonylation in a tissue-specific manner (Tresnakova et al., 2023), and polyethylene glycol (PEG) exposure affected SOD activity and LPO in the gills, whereas only LPO was affected in the digestive gland (Multisanti et al., 2024). Furthermore, Matozzo et al. (2018) mentioned that exposure of Mediterranean mussel to the herbicide glyphosate affected SOD activity in a tissue-specific manner, with a reduction in the digestive gland and no effect in the gills.

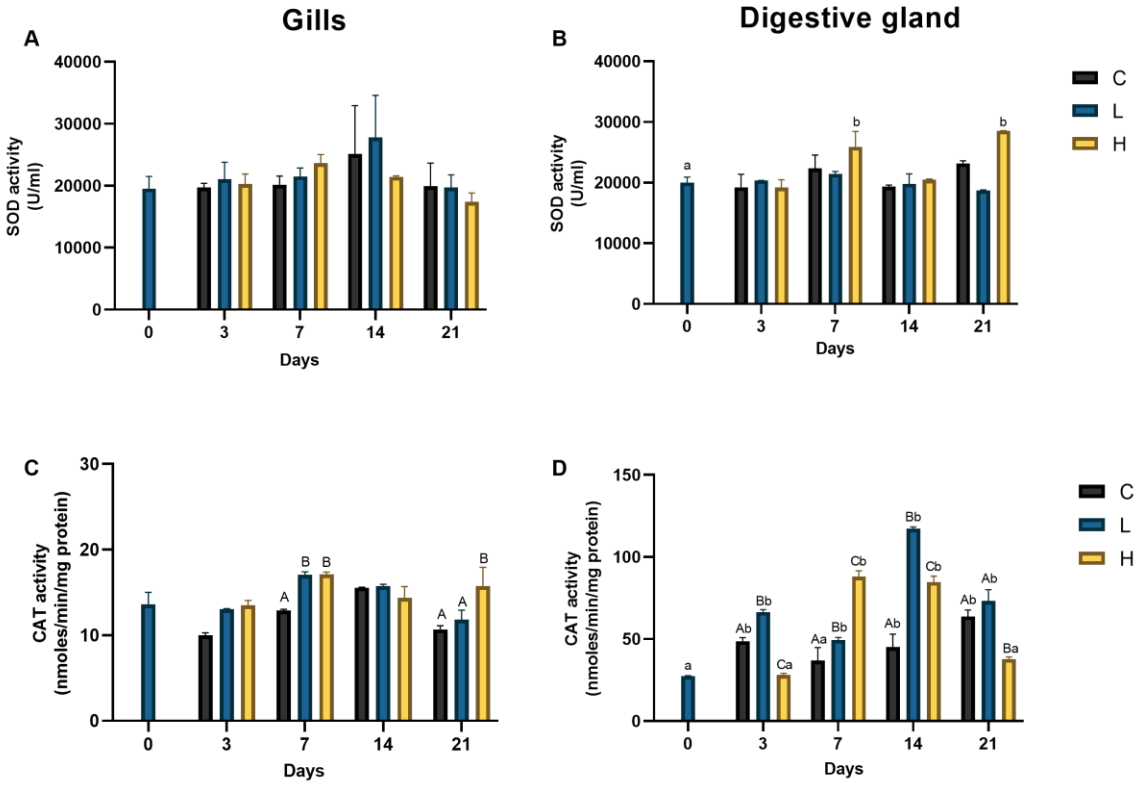

Fig. 2. SOD (A–B) and CAT (C–D) activities in the gills and digestive gland of *M. galloprovincialis* from unexposed and exposed to different concentrations of formaldehyde for 21 days. C: Unexposed

mussels, L: Low concentration, H: High concentration. Data are expressed as the mean ± standard deviation (S.D.). Different capital and lower-case letters indicate significant differences between treatments for the same day and between days and day 0, respectively ($P < 0.05$).

3.2 Oxidative damage

LPO is a free radical-related process and occurs in response to the oxidative degradation of polyunsaturated fatty acids (PUFAs) (Fahmy and Sayed, 2017; Reiter et al., 2014). In gills, MDA values showed significant changes between day 0 and the other groups throughout the experimental period (except for unexposed mussels on day 3, $P > 0.05$) and between unexposed and exposed mussels ($P < 0.05$; Fig. 3A). The MDA content was significantly increased at the high concentration on day 3, while the levels on day 21 decreased significantly compared to those of the control and L ($P < 0.05$) groups.

In the mantle, MDA values also showed significant changes between day 0 and the other groups throughout the period, except for the unexposed mussels on day 3 ($P > 0.05$). In addition, MDA values of the unexposed and exposed mussels differed significantly ($P < 0.05$) throughout the experimental period, except for day 7 (Fig. 3B). Overall, steady MDA levels, without large fluctuations, were observed in the mantle compared to the gills. Thus, in Mediterranean mussel, gill tissue appeared to be more susceptible to peroxidation, even at lower formaldehyde concentrations, compared to mantle tissue. MDA content of mantle also showed a similar trend to that of gills on days 3, 7, and 14 and a reverse trend on day 21, where a significant increase after long-term exposure to the highest concentration was observed (Fig. 3).

The significantly elevated MDA levels in both tissues examined indicate that the antioxidant defense system is overwhelmed and cannot compete with the formation of hydroxyl radicals

by the Fenton reaction, leading to lipid peroxidation (Box et al., 2009; Gomes et al., 2011; Gonçalves et al., 2022). Nevertheless, the increase in CAT activity on day 21 suggests that the mussels' gills were strong enough to prevent lipid peroxidation of the membranes (Fig. 2C), and had a robust mechanism to counteract oxidative stress. By eliminating H2O2, CAT prevented the formation of highly reactive hydroxyl radicals, which are known to cause significant damage to cell membranes and other cellular components (Bebianno et al., 2005). These results seem to agree with other studies on lipid peroxidation in response to organic pollutants in other aquatic bivalves such as the Japanese oyster *Crassostrea gigas*, duck mussel, *Anodonta anatine,* and blue mussel, *M. edulis* (Falfushynska et al., 2013; Niamul Haque et al., 2019; Park et al., 2016). Similarly, LPO showed a significant increase after exposure to heavy metals. Bonnail et al. (2016) observed an increase in LPO levels in the whole soft bodies of the Asian clam *Corbicula fluminea* exposed to > 1 mg L^{-1} Cu. Likewise, in the gills and digestive glands of the bivalve *Chambardia rubens* exposure to CuONPs or Cu^{2+} triggered a significant increase in lipid peroxidation (Morad et al., 2023).

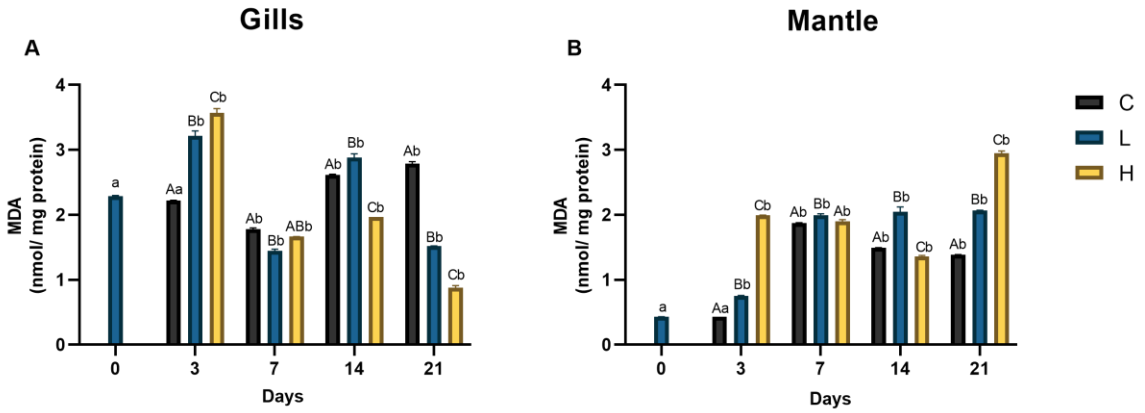

Fig. 3. MDA levels in the gills (A) and mantle (B) of *M. galloprovincialis* from unexposed and exposed to different concentrations of formaldehyde for 21 days. C: Unexposed mussels, L: Low concentration, H: High concentration. Data are expressed as the mean ± standard deviation (S.D.).

Different capital and lower-case letters indicate significant differences between treatments for the same day and between days and day 0, respectively (P< 0.05).

3.3 Neutral red retention time assay

The NRRT assay is an effective indicator to assess the health status for possible impaired immunocompetence and cell injury of marine biota in laboratory and field conditions (Mamaca et al., 2005; Regoli et al., 2004). The time period between the NR probe application and the appearance of the first evidence of dye loss from the lysosomes to the cytosol on at least 50% of the examined cells, represents the NRR time for each mussel. Statistical difference was observed in NRRT values in haemocytes of day 0 and treated mussels on day 14 and 21 (70±17.3 and 60±0.0 min, respectively) (Fig. 4), suggesting that time has probably affected the physiological and health conditions in this species after the exposure. Additionally, significantly lower NRRT values were found in the H group on day 7 compared to non-treated (C) and L-treated groups. However, according to Martínez-Gómez et al. (2017) and Vethaak et al. (2017), mussels are classified as “moderately stressed” (120 min> NRRT≥ 50 min) as stated in the individual capacity to cope with environmental stress. After day 14, the mussels were found to be unaffected by the presence of formaldehyde, as evidenced by the widespread reduction in NRR time in all groups.

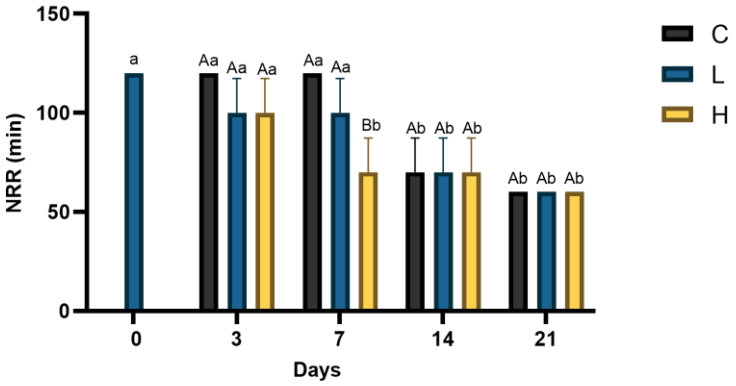

Fig. 4. Determination of NRR values in haemocytes of formaldehyde-exposed mussels for 21 days. Values are the mean NRR time expressed as min ±SD obtained by the analysis of each slide (n=2x3 for each group of mussels): Unexposed mussels, L: Low concentration, H: High concentration. Different capital and lower-case letters indicate significant differences between treatments for the same day and between days and day 0, respectively (P< 0.05).

3.4 Comet Assay

The comet assay is used to assess the DNA damage of cells with applications in genotoxicity testing, ecotoxicology, and fundamental research in DNA damage and repair (Collins and Dusinská, 2002). Herein, this assay aimed to determine the DNA damage in individual cells caused by the application of formaldehyde. Thus, upon examining the haemolymph of the formaldehyde-exposed groups, no statistical differences were observed. Tail DNA showed an increasing pattern during days 7 and 14, where the amount of damage per group was relatively equal, with H showing the highest DNA damage (Fig. 5).

In mussel gills, similar levels of DNA damage (~10%) were found between the control and L groups at all sampling points (Fig. 5B). On the contrary, higher genotoxicity was evident in the H group, on day 3 (P< 0.05), with approximately 14% tail DNA, which was then reduced to ~10.3% on day 7. It can therefore be concluded that the application of formaldehyde at the highest concentration (H) caused an initial shock to the mussels, which was alleviated in the following days.

Regarding the results in the digestive gland, a similar trend to the gills was apparent. In detail, H demonstrated the most significant level of genotoxicity resulting in 12.3% tail DNA on day 3 (P< 0.05) followed by a downward trend in the subsequent days (Fig. 5C).

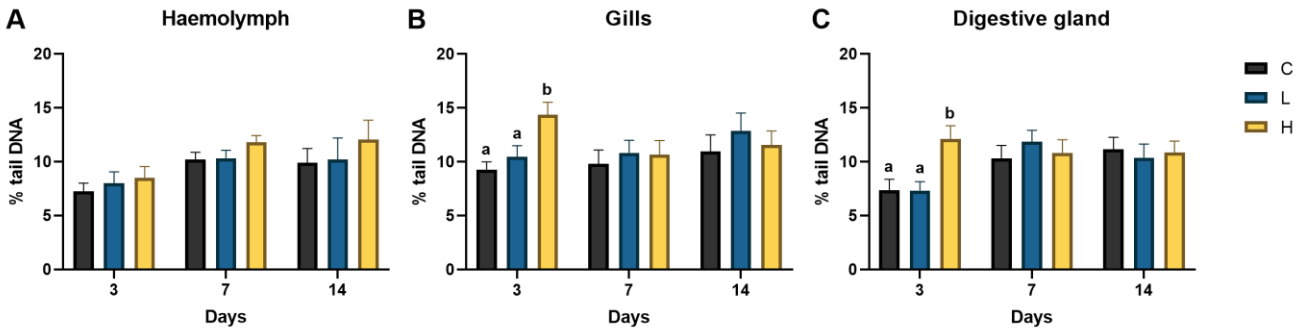

Fig. 5. The percentage of genotoxicity in % tail DNA for mussels exposed (L and H) and unexposed (C) to formaldehyde per sampling day (3, 7, 14) in haemolymph (A), gills (B), and digestive glands (C). Data are expressed as the mean ± standard error (SEM). Different lower-case letters indicate significant differences between treatments for the same day (P< 0.05).

3.5 Condition Index

The CI index in mussels is a measure of their physiological health and overall well-being, often used to assess the state of mussel populations in both aquaculture and ecological studies. It provides insights into the mussels' growth, reproductive status, and adaptation to environmental conditions such as chemical exposure (Brooks et al., 2015; Pampanin et al., 2005). No statistically significant changes were found in the comparison of the CI values between the unexposed and exposed mussels, and across different times of exposure. The values ranged between 31.5± 9.1% and 41.4± 3.4 (P> 0.05) (Table 1).

Overall, the absence of statistically significant changes in the CI values suggests that despite exposure to a potential stressor, the overall health, growth, and tissue development in the mussels remained stable throughout the study.

Table 1. Condition index (mean± S.D.) (%) of mussels exposed to formaldehyde at the beginning, in the middle, and at the end of the exposure.

Days	C	L	H
0		41.4± 3.4	
7	34.4± 6.5	32.8±5.5	31.5± 9.1
21	33.5± 6.8	32.6± 6.8	34.6± 6.7

417

418 *3.5 Stress on Stress*

419 The SoS biomarker in mussels is a tool used to assess the sensitivity of these organisms to
420 pollutants and other environmental changes (Thain et al., 2019). It is widely known that when
421 marine organisms are exposed to xenobiotics, they become more vulnerable and less tolerant
422 of environmental fluctuations (Viarengo et al., 1995). Mussels have the remarkable ability to
423 withstand being out of the water for many days by keeping their shells tightly closed. This
424 survival mechanism is dependent on the amount of adenosine triphosphate (ATP) available
425 to fuel their adductor muscles. However, if the mussels remain exposed to air for too long,
426 their ATP reserves will eventually be depleted, leading to their death (Thain et al., 2019). To
427 assess the impact of formaldehyde on mussel survival in the air and in the organism's
428 physiology, the median survival time (LT_{50}) was determined. This is the point in time when
429 the probability of survival equals 50% and is measured in days (Fig. 6). The LT_{50} was assessed
430 against the developed background (BAC) and environmental assessment (EAC) (Martínez-
431 Gómez et al., 2017; Thain et al., 2019).
432 Thus, according to SoS assessment criteria unexposed mussels, and mussels exposed in low
433 concentration were identified as “non-stressed” (>BAC) which means that these mussels were
434 healthy (Table 2, Fig. 6). Mussels exposed to high concentrations were classified as
435 "moderately stressed" (BAC > x ≥ EAC), indicating that although these mussels are stressed,
436 they are still capable of compensating (Table 2, Fig. 6).

437

Table 2: BAC, and EAC levels for SoS measurements and median survival for all treatments.

Days	BAC	EAC	C	L	H
LT_{50}	10	5	11	12	8.5

Bivalves when subjected to natural or chemical stress increase their metabolism and require extra energy which is drawn from the glycogen (de Zwaan and Wijsman, 1976; Matozzo et al., 2003). Therefore, laboratory and field studies have demonstrated that mussels exposed to different contaminants were more sensitive to the stress of aerial exposure. Marcheselli et al. (2011) noted an excessively decreased tolerance to anoxic conditions in *M. galloprovincialis* exposed to the higher tested concentration of the anti-fouling biocide zinc pyrithione (ZnPT) (LT_{50} = 5.4 days) compared to the control animals (LT_{50} = 10.7 days). Also, a significant reduction of survival in the air in the same species was observed by Viarengo et al. (1995) after a 3-day short-term assay to dimethyl-benzo anthracene (DMBA), copper (Cu^{2+}), and a mixture of DMBA and Cu^{2+}. Data demonstrating that short-term exposure to pentachlorophenol (PCP) and tributyltin (TBT) reduced tolerance to air exposure in *M. edulis* mussels have been also reported (Wang et al., 1992). Moreover, laboratory experiments have indicated that exposure to 4-nonylphenol, an environmental pollutant, can significantly impact the survival rate of *T. philippinarum* clams when subjected to air exposure. Specifically, LT_{50} values reduced from 8 days in control to 5 days in the highest concentration (Matozzo et al., 2003), which is in agreement with a previous study for *M. edulis* exposed to polychlorinated biphenyls (PCBs) and cadmium (Veldhuizen-Tsoerkan et al., 1991).

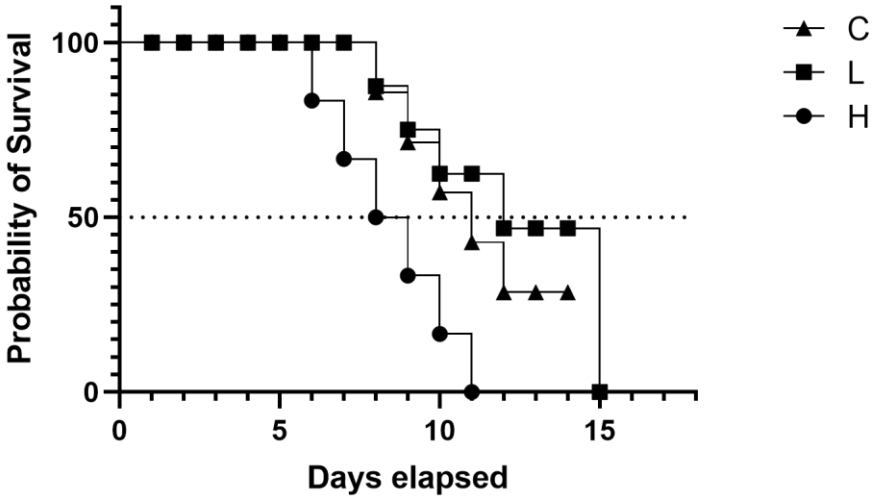

Fig. 6. Probability of survival on air condition (SoS) of unexposed (C) and exposed (L and H) mussels.

3.6 PCA analysis

PCA analysis was conducted on the data for gills and digestive gland to compare their spatial distribution differences. The PCA biplot was used to visualize correlations between different concentrations and days of exposure, as well as between the different antioxidant enzymatic systems.

Regarding gills, F1 and F2 explained 89.13% of the total variation in the data, indicating that they sufficiently represented most of the data (F1 = 49.38% and F2 = 39.74%) (Fig. 7A). The results showed a clear division between unexposed and exposed mussels, with the former appearing on the negative side of the F1 axis and most of the latter on the positive side where the MDA content, SOD, and CAT enzymes were present. On day 21, gill recovery effects were observed as L and H groups were found on the same side of the axis as unexposed mussels. In contrast, on days 7 and 14, the mussels were found on the opposite side and were closely related to the tested biomarkers.

The two principal components of the digestive gland account for 100% of the total variance, with F1 representing 51.88% and F2 48.12% (Fig. 7B). It seems that there was no clear distinction between the unexposed and exposed mussels, or the duration of their exposure. The observations that were actively collected were somewhat clustered towards the center of the F1 and F2 axes, except for L group on day 14, which was located near CAT, and H group on both days 7 and 21, which were in the same quadrant as SOD.

In conclusion, the results of PCA were markedly different between gills and digestive glands, reflecting the distinct physiological and metabolic functions of these two tissues.

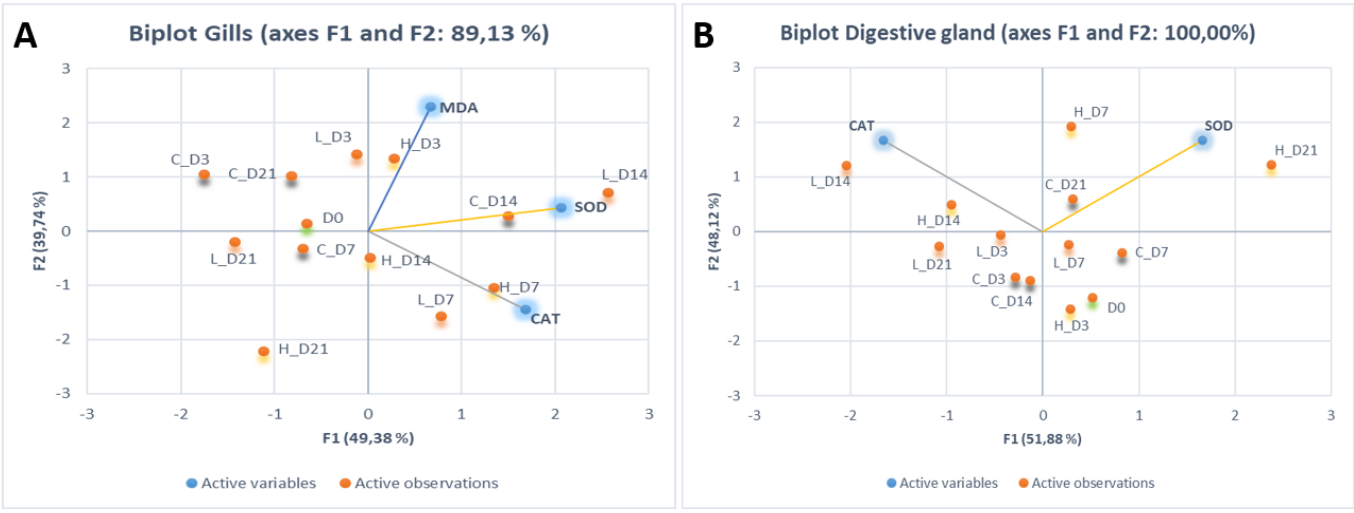

Fig. 7. Graphical representation of PCA conducted on biochemical parameters in mussels *M. galloprovincialis* after 21 days of formaldehyde exposure. A. PCA of MDA content, CAT, and SOD activities in the gills of mussels from unexposed (C) and exposed (L and H) groups. B. PCA of CAT and SOD activities in the digestive gland of mussels from unexposed (C) and exposed (L and H) groups.

4.Conclusions

The present study revealed that commonly measured concentrations (40 ppb) of formaldehyde in the vicinity of fish cages after formalin bath treatments, do not seem to provoke chronic toxicity in mussels. Extremely higher dosages used (400 ppb) seemed to

impair the antioxidant activity of mussels and cause stress, which is, however, described as reversible. It appears that exposure to high formaldehyde levels may generate superoxide radicals in the digestive gland which were counterbalanced by the increase in SOD activity. Additionally, it was found that the enzyme CAT acts as a defense mechanism against formaldehyde and contributes to protection against lipid peroxidation. The results show tissue-specific responses that reflect the different physiological and metabolic functions of mussel's tissues during stress responses. The mantle appeared to be more resistant to peroxidation, while the increased CAT activity in the digestive gland suggests that the degree of oxidative stress is higher in this tissue than in the gills, leading to the conclusion that the latter have a rapid and efficient enzymatic mechanism against increased levels of oxygen radicals. This study provides a much-needed assessment of the long-term effects of formaldehyde exposure on non-target marine organisms, in particular Mediterranean mussels, which are commonly found around fish cage farms. The important contribution of this study lies in its pioneering assessment of the long-term effects of formaldehyde on sentinel species, which advances the understanding of the wider ecological effects of formaldehyde. The results highlight the need for further research on other non-target marine organisms to fully understand the effects of formaldehyde in marine ecosystems. In addition, extending the analysis to longer exposure periods beyond 21 days and testing a wider range of formaldehyde concentrations are essential to gain a more comprehensive understanding of how mussels respond to formaldehyde exposure.

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