NOVA SCHOOL OF SCIENCE & TECHNOLOGY

DEPARTAMENT OF CHEMISTRY

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BSc in Biochemistry

EVALUATION OF THE EFFECTS OF EXPOSURE TO URBAN PARTICULATE MATTER (PM) ON MARINE ORGANISMS

MASTER IN BIOCHEMISTRY

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"*Science is neat, but I'm afraid it's not very forgiving*."
(Mr. Clarke, from *Stranger Things*)

"*I am on a curiosity voyage, and I need my paddles to travel.*
These books…these books are my paddles."
(Dustin, from *Stranger Things*)

ABSTRACT

Atmospheric particulate matter (PM) is a significant threat, as it contains pollutants that can cause damage to the environment and especially to the aquatic biota. This thesis falls within the scope of the research project "AMBIEnCE" and explores how pollutants from wet precipitation of atmospheric PM cause toxicity in marine organisms (*Dicentrarchus labrax*, *Mytilus galloprovincialis*, and *Palaemon varians*). The assays included exposure to different concentrations of suspended PM (5.7 and 11.4 mg/L). After 7, 14 and 21 days, for analyses, selected organs were sampled (muscle, liver/ digestive gland, gills, and intestines/viscera), depending on the target species. An additional trial was performed to study the eventual trophic transfer of PM by feeding seabass with PM-contaminated *Polychaetes*

After the exposure assays, several oxidative stress biomarkers were analysed (CAT, SOD, GPX, and GST), lipid peroxidation (MDA content) and total ubiquitin, which allowed the assessment of the response to exposure to the different concentrations of PM-tested. Overall, seabass showed increased enzyme activities in the exposed animals exposed, especially those exposed to 5.7 mg/L compared to respective controls. In the seabass contaminated via diet, higher enzymatic activity was observed in muscle and liver compared to the respective controls. An increase in oxidative stress enzymes was also observed in mussels and shrimp exposed to PM. The MDA and ubiquitin levels provided information on cellular and protein damage. Although variable results, an increase in MDA and ubiquitin levels was evident in the gills of exposed mussels. Nonetheless, the higher mortality observed in fish and shrimps exposed to the highest PM concentration suggests a severe effect on these species.

Overall, these results show that PM exposure can significantly impact marine organisms with increasing PM concentration and exposure time. Additionally, it provides valuable information for the risk assessment of marine ecosystems.

Keywords: Particulate Matter (PM), Estuarine organisms, Oxidative stress, Biomarkers

RESUMO

O material particulado atmosférico (*PM*) é uma ameaça significativa, pois contém poluentes que podem causar danos no ambiente e principalmente no biota aquático. Esta tese enquadra-se no âmbito do projeto de investigação "AMBIEnCE" e explora como os poluentes da precipitação húmida de PM atmosféricos causam toxicidade em organismos marinhos (*Dicentrarchus labrax, Mytilus galloprovincialis* e *Palaemon variantes*). Os ensaios incluíram a exposição a diferentes concentrações de PM em suspensão (5,7 e 11,4 mg/L). Após 7, 14 e 21 dias, para as análises, foram amostrados vários órgãos (músculo, fígado/glândula digestiva, brânquias e intestinos), selecionados de acordo com a espécie alvo. Um ensaio adicional foi realizado para estudar a eventual transferência trófica de PM alimentando robalos com *Polychaetes* contaminados com *PM*.

Após os ensaios de exposição, vários biomarcadores de stress oxidativo foram analisados (CAT, SOD, GPX e GST), peroxidação lipídica (níveis de MDA) e ubiquitina total, o que permitiu avaliar a resposta à exposição às diferentes concentrações de *PM* testadas. No geral, o robalo mostrou atividades enzimáticas aumentadas nos animais expostos, especialmente naqueles expostos a 5,7 mg/L em comparação com os respetivos controlos. Nos robalos contaminados através da dieta, observou-se maior atividade enzimática no músculo e fígado em comparação com os respetivos controlos. Um aumento nas enzimas do estresse oxidativo também foi observado nos mexilhões e camarões expostos às diferentes concentrações de *PM*. Os níveis de MDA e ubiquitina forneceram informações sobre danos celulares e proteicos. Embora se tenha observado que os resultados obtidos apresentaram alguma variabilidade, um aumento nos níveis de MDA e ubiquitina foi evidente nas brânquias dos mexilhões expostos a PM. No entanto, foi o aumento da mortalidade observada em peixes e camarões expostos à maior concentração de PM que sugeriu um efeito severo da exposição a PM.

No geral, os resultados mostram que a exposição ao *PM* tem um impacto significativo nos organismos marinhos com o aumento da concentração de *PM* e do tempo de exposição. Além disso, fornece informações valiosas de avaliação de risco para os ecossistemas marinhos.

Palavras chave: Material Particulado (*PM*), Organismos de estuários, Stress Oxidativo, Biomarcadores.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF REACTIONS

GLOSSARY

Ad libitum	Latin term meaning as much or as often as necessary
Bioaccumulation	The accumulation of a chemical within the tissue of an organism either directly (from the medium, usually water), or indirectly (from consumption of contaminated food).
Biomagnification	Process by which a compound (such as a pollutant or pesticide) increases its concentration in the tissues of organisms as it travels up the food chain.
Biomarkers	Naturally occurring molecule, gene, or characteristic by which a particular pathological, physiological process, disease… can be identified.
Biomonitoring	EEA describes as "*The use of a biological entity as a detector and its response as a measure to determine environmental conditions. Toxicity tests and biological surveys are common biomonitoring methods*"
Biotransformation	Biochemical modification (enzymatic or non-enzymatic) of a xenobiotic that may result in either reduced or increased toxicity. This process generally gives rise to compounds that are more readily excreted in the urine and faeces and thus serves as a detoxification process. However, some xenobiotics are turned into more toxic metabolites.
Carcinogen	A chemical which induces cancer.
Chronic toxicity	Describes adverse effects manifested after a long period of uptake of small quantities of a toxicant. The dose is small enough that no acute effects are manifested, and the time is frequently a significant part of the expected normal lifetime of the organism. The most serious manifestation of chronic toxicity is carcinogenesis, but other types of chronic toxicity are also known (*e.g.*, reproductive effects, behavioural effects).

Comparative toxicology	The study of the variation in toxicity expression of exogeneous chemicals towards organisms of different taxonomic groups or different genetic strains.
Control group	Consists of a group of experimental organisms that are not exposed to the treatment or chemical used in the main testing. They are compared to the exposed experimental groups to observe whether the resulting effects in the experimental groups are significant.
e.g.,	*exempli gratia* (Latin for 'for example')
Ecology	Branch of biology that deals with the relations of organisms to one another and to their physical surroundings.
Ecosystem	The total of all interactions linking organisms in a community with each other and their environment. Comprises different communities along with the physical and chemical characteristics of the environment.
Ecotoxicology	Branch that deals with the nature and effects of toxic chemicals on biological organisms.
Exposure	Contact of an external chemical, physical, or biological agent with the outer boundary of an organism. Exposure is quantified as the concentration of the agent in the medium integrated over the time duration of contact.
Free radicals	A type of unstable molecule created during normal cell metabolism (chemical changes that take place in a cell), which can build up in cells and cause damage to other molecules, such as DNA, lipids, and proteins
Genotoxic	Chemical which induces DNA or chromosome damage. This can be determined directly (*i.e.*, measuring mutations or chromosome abnormalities) or indirectly (*i.e.*, measuring DNA repair, sister-chromatid exchange, etc).
Hypoxia	A state in which oxygen is not available in sufficient amounts at the tissue level to maintain adequate homeostasis.
i.e.,	*id est* (Latin for 'that is')
Model organism	Non-human species studied to understand a particular biological phenomenon, which may provide insight into the workings of other organisms.
Mutagenic	Chemical which induces changes in the genetic material in the cell nucleus in ways that allow the changes to be transmitted during cell division.

PAHs Naturally occurring chemicals formed because of incomplete combustion. Several of the chemicals in this group are carcinogenic.

PCBs A manufactured group of chemicals used mainly for their indiscriminate ability of biocide. Several of the chemicals in this group are carcinogenic.

Pesticides Any substance used to destroy or inhibit the action of plant or animal pests.

Phase I reactions In animals, this phase acts as a first step in the drug metabolism for the elimination of foreign compounds from the body. The main reactions involved oxidation, reduction, and hydrolysis. The aim of phase I is to add or unmask a reactive functional group to which phase II metabolic enzymes can add a highly water-soluble molecule. This is desired because the more water soluble a compound is, the more readily it is excreted by the kidneys. Although some compounds can be eliminated solely by phase I mechanisms, most go on to be involved in the phase II metabolism. Also, phase I is not a necessary precursor to phase II metabolism for some foreign molecules. The products of Phase I reactions may be potent electrophiles that can be conjugated and detoxified in Phase II reactions or that may react with nucleophilic groups on cellular constituents, thereby causing toxicity.

Phase II reactions In animals, this phase involves conjugation reactions with endogenous substrates of Phase I products and other xenobiotics that contain functional groups such as hydroxyl, amino, carboxyl, epoxide, or halogen. The endogenous metabolites include sugars, amino acids, glutathione, and sulphate. The conjugation products, with rare exceptions, are more polar, less toxic, and more readily excreted than their parent compounds. There are two general types of conjugations: type I (*e.g.*, glycoside and sulphate formation), in which an activated conjugating agent combines with substrate to yield the conjugated product; and type II (*e.g.*, amino acid conjugation), in which the substrate is activated and then combines with an amino acid to yield a conjugated product.

Reactive oxygen species Reactive oxygen species are formed in vivo, either during, or because of, aerobic metabolism, which are linked to a few toxic endpoints, resulting many times in a phenomenon known as oxidative stress. These include species such as superoxide anion, hydrogen peroxide, singlet oxygen, and the highly reactive hydroxyl radical are also known.

Resistance Refers to the ability of an organism to show decreased sensitivity to a chemical that normally causes deleterious effects. The term resistance refers to the situation where a change in the genetic constitution of an individual is present. This alteration may spread within the population in response to the stressor chemical, and enable a greater number of individuals to resist the toxic action than in the previous unexposed population. Thus, an essential feature of resistance is selection and then inheritance by subsequent generations. In microorganisms, this frequently involves mutations and induction of enzymes by the toxicant; in higher organisms, it usually involves selection for genes already present in the population at low frequency.

Tolerance The terms resistance and tolerance are closely related and have been used in several different ways. However, the term tolerance is reserved for situations in which individual organisms acquire the ability to resist the effect of a toxicant, usually because of prior exposure.

Toxicity The intrinsic degree to which a chemical may cause adverse effects.

Xenobiotics Compounds present in the environment (natural or synthetic) at levels, which can cause adverse effects at the biological level.

ACRONYMS

CAT	Catalase
BSA	Bovine Serum Albumin
EEA	European Environmental Agency
EPA	Environmental Protection Agency
ETC	Electron Transport Chain
EU	European Union
GPX	Glutathione Peroxidase
GST	Glutathione-S-Transferase
LPO	Lipid Peroxidase
MDA	Malondialdehyde
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NIST	National Institute of Standards and Technology
Nitro-PAH	Nitro substituted Polycyclic Aromatic Hydrocarbons
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyls
PM	Particulate Matter
ROS	Reactive Oxygen Species
SOCs	Soil Organic Carbon

SOD	Superoxide Dismutase
sp	Species
SPM	Suspended Particulate Matter
UBI	Ubiquitin
USA	United States of America
WHO	World Health Organization

1

INTRODUCTION

1.1 Particulate Matter (PM)

The European Environment Agency (EEA) describes Particulate Matter (PM) as [1]:

"A collective name for fine solid or liquid particles added to the atmosphere by processes at the Earth's surface. Particulate matter includes dust, smoke, soot, pollen, and soil particles."

The previous definition refers to Particulate Matter (PM) or Suspended Particulate Matter (SPM) as a broad term used to describe a group of suspended particles which does not entail hazardous components. However, PM's morphology (*e.g.*, isometric spheres, platelets, and fibres), size, and elemental composition (**Figure 1**) are critical components in understanding the particulates' distribution, permanency patterns, ability to incorporate harmful substances and in what ways these components and their interactions may affect the environment and the organisms directly associated [2]. Three main categories can be employed to classify particulates according to size. Coarse particulates refer to agglomerates where particles' size ranges between 2.5 to 10 μm (PM10- PM2.5), fine particulates are smaller with sizes that can reach 2.5 μm (PM2.5), and the ultrafine particulates refers to particles smaller than 0.1 μm [2].

Depending on the source, the proportion of significant components in PM may differ. Sulphate, nitrates, ammonia, sodium chloride, black carbon, mineral dust, and water are frequent constituents of these mixtures. Additionally, according to the National Institute of Standards and Technology (NIST), organic compounds such as polycyclic aromatic hydrocarbons (PAHs), chlorinated pesticides, polychlorinated biphenyls (PCBs), and heavy metals are among the most common classes of toxic components associated with PM [3, 4, 5].

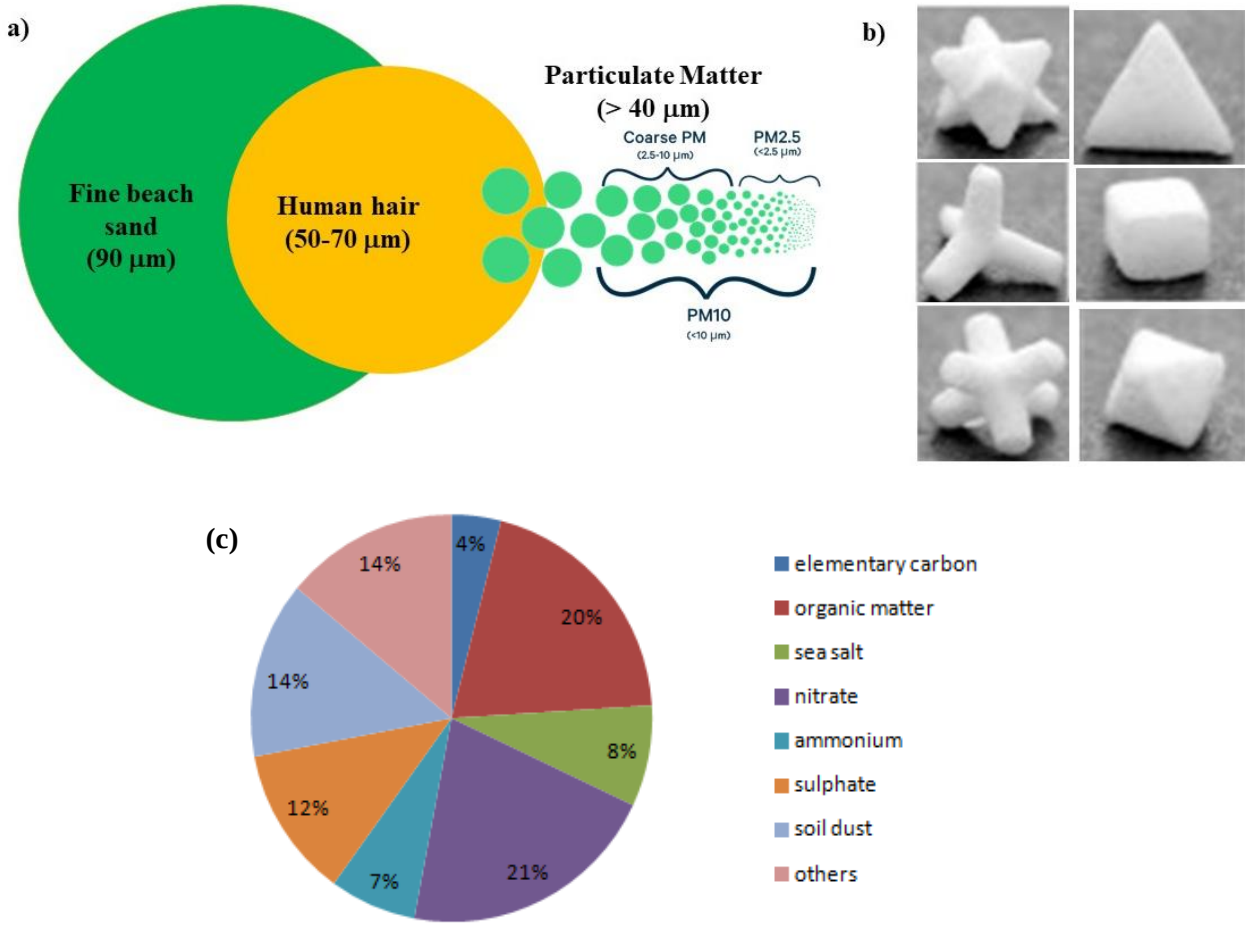


Figure 1 | Characterization of Particulate Matter (PM) according to size **(a)**, shape **(b)**, and elemental composition **(c)**. Adapted from EPA and Air quality Documentation [6, 7].

Over the years, PM concentrations have increased significantly due to urbanisation and industrialisation, according to WHO (World Health Organization). This organisation also defines "pollution" as a condition where substances exist in concentrations exceeding environmental levels, causing harm to fauna, flora, and the ecosystem [8]. Moreover, as shown in **Figure 2**, further data suggests a recent stagnation of PM levels.

In Europe, fine carbonaceous particles are the main component of main PM emissions, with household biomass burning and diesel vehicle engines being the most prominent sources of organic and black carbon. However, in contemplation of reducing PM emissions, EPA (Environmental Protection Agency) has set EU limit values primarily targeted at industrial processes, road transport, and other significant point sources [2, 9, 10].

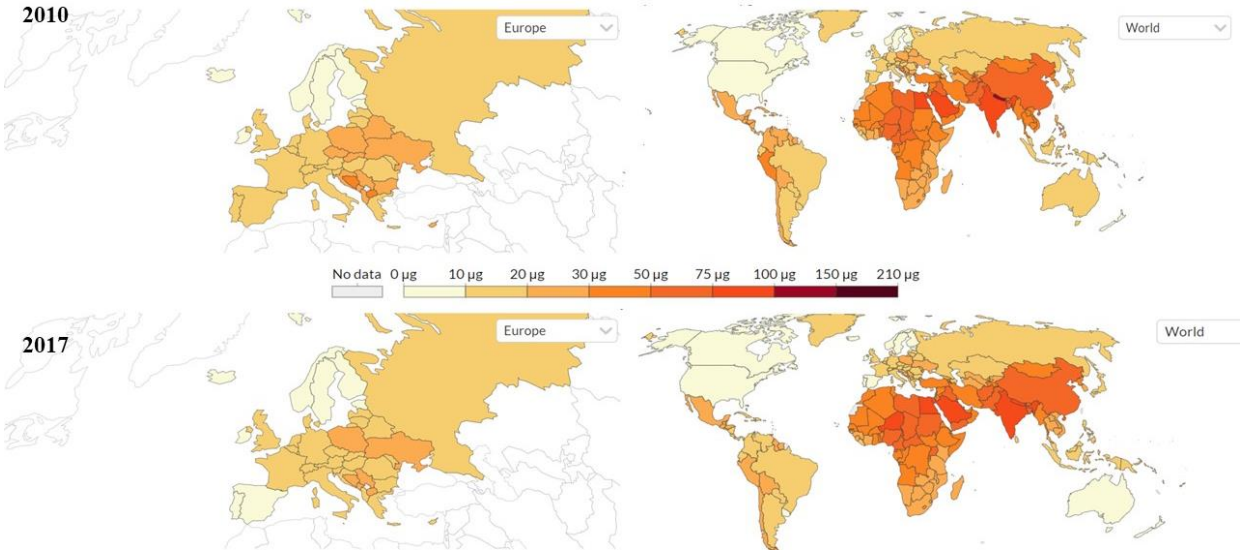


Figure 2 | PM distribution in Europe and worldwide. The maps illustrate the PM levels in Europe and the world in 2010 (upper) and 2017 (lower). Source: *Global Change Data Lab* [11].

1.1.1 Main sources

As previously stated, PM's size, chemical content and composition depend strongly on its source and formation processes (**Figure 3**). This group of substances can act as primary or secondary pollutant, which means it can be emitted directly from a source (primary) or created *in situ* through intermediate atmospheric reactions (secondary). Primary pollutants (*e.g.*, coarse particulates) are typically easier to regulate than secondary by limiting their known sources. Contrarily, secondary pollutants have a wide variety of sources and do not always represent environmental degradation and harm, with some even originating from natural organic matter produced by the metabolism of certain algae species [12]. For instance, fine particles are most often created in the atmosphere as a result of gas-to-particle conversions and chemical interactions, which cause the particles to expand and change composition. [2].

On the other hand, PM may be classified as both natural and anthropogenic. The former refers mainly to mineral particles from dry or semi-dry areas (*e.g.*, deserts) and originates by physical processes (*e.g.*, aeolian weathering of soils, sea spray, volcanic activity, and plant release). The latter (anthropogenic) arises largely from combustion and high-temperature procedures (*e.g.*, smelting and industrial welding procedures, or mechanical disruption actions causing the particles suspension). The main contributors vary depending on the particle's characterisation. For instance, diesel-fuelled vehicle engines mainly release fine particulates, and biomass combustion increases ultrafine particulate concentration. Lastly, others, such as traffic suspension, mining, construction operations, and agricultural land management activities, increase the circulation of coarse particulates [13].

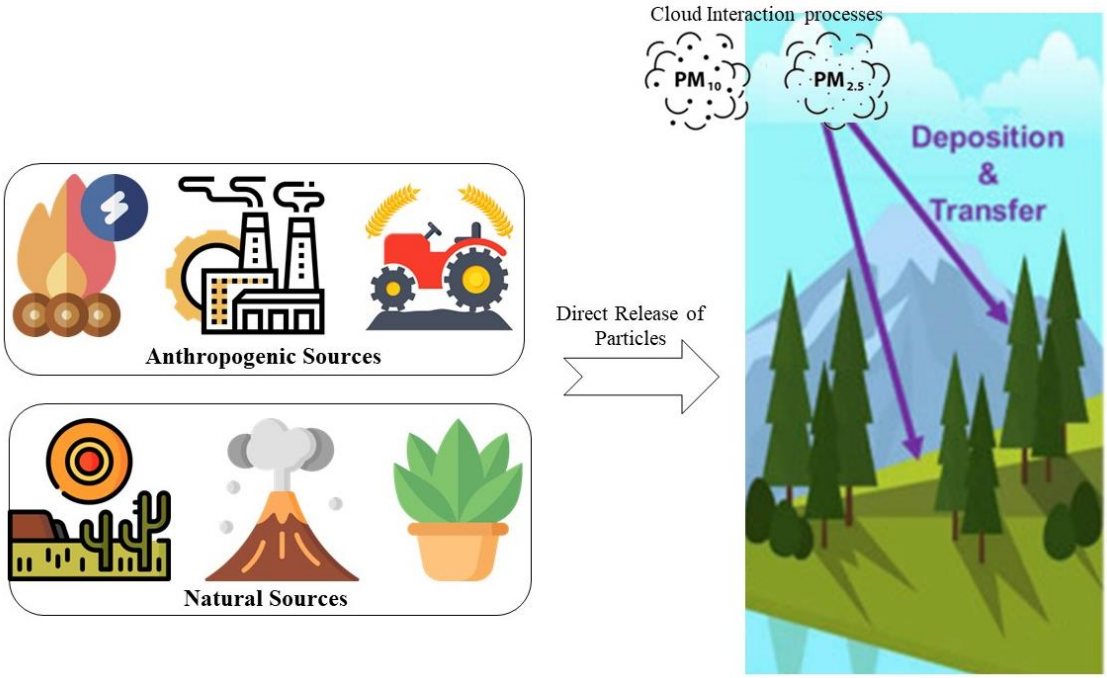


Figure 3 | Particulate Matter (PM) characterization according to origin. Adapted from *Luo et al.* [14].

1.1.2 Distribution pathways

Suspended Particulate Matter (SPMs) can be used to measure water quality, trace sediment transport (that may include pollutants or not), determine hydrodynamic models, and may also convey information about carbon cycles [8, 15].

The atmosphere is fundamental in transferring and depositing nutrients in an ecosystem through the naturally occurring processes of dry and wet deposition, as shown in **Figure 4** [16]. Wet deposition is the input of substances in the dissolved state, usually occurring through rain and snowfall, and dry deposition occurs in the particulate state (*i.e.*, dust foil). However, the higher pollutant concentrations caused by increased human activity result in harmful contaminants finding their way through dry and wet processes into soil, marine and freshwater. These processes may lead to a considerable impact on human health and biodiversity [17, 18].

Several factors can influence PM's transfer into marine environments. For example, according to the literature, dry deposition onto wet surfaces appears to be a more critical nutrient transport than dry deposition on dry surfaces since most impose high aerodynamic resistance, leading to lower deposition rates [16]. Another critical factor that substantially affects the dry particulate removal phenomenon is the particulate composition, which is directly related to size (*i.e.*, most SOCs, soluble inorganic species, and many trace metals are too minuscule to settle by gravity or rain, and dry deposition removes them slower. Consequently, their atmospheric durability and long-distance transmission ability increase), shape (*i.e.*, particles with heterogeneous shape can acquire a spherical form when humidified,

which means that in aquatic environments, particles will acquire a smaller distribution of shapes). Finally, hydrophobicity also influences the transfer process, as hydrophilic particles have an increased ability to penetrate the humid deposition layer above the water surface [19, 20].

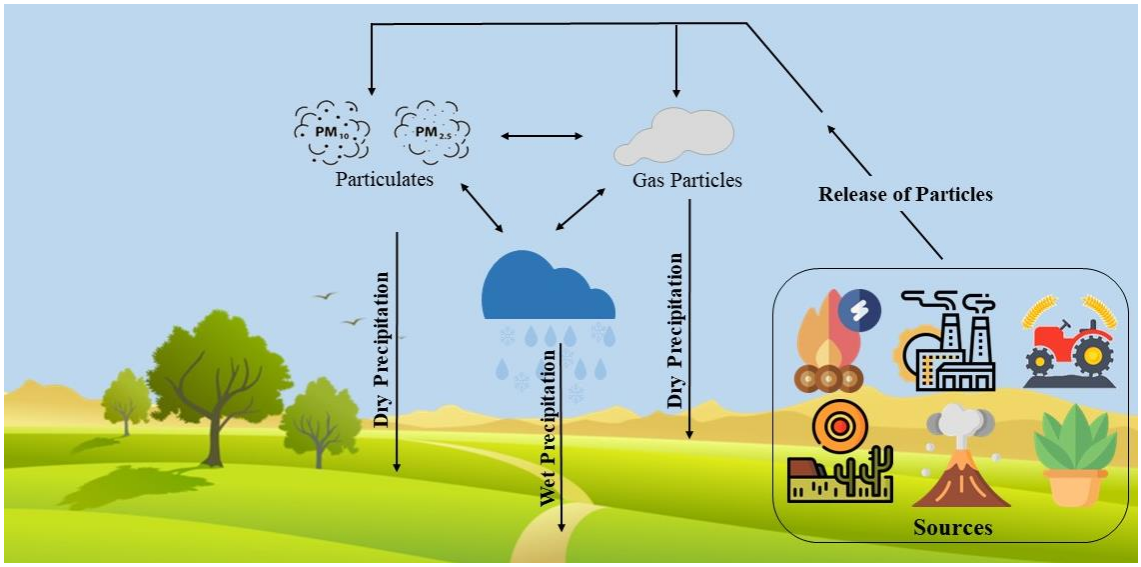


Figure 4 | Particulate Matter (PM) division according to its source. Adapted from *Jia et al.* [21].

1.1.3 Toxicological studies

Previous epidemiological studies have shown that PM generally induces inflammatory responses in the respiratory and cardiovascular systems [22]. However, its toxic effect varies according to chemical composition, size, air concentration and exposure time [2]. PM2.5 is a prevalent and dangerous constituent of air pollution, which due to its miniature size, penetrates deeper skin layers than other size particulates [23, 24]. On the other hand, it contains highly genotoxic, mutagenic, and carcinogenic substances, which may lead to various problems for the organisms exposed to the particulates [25].

Despite extensive studies on the PM effects on human health, there is a lack of awareness of its influence on aquatic organisms, which is critical since aquatic ecosystems can be very susceptible to PM toxicity due to their accumulative ability in water bodies via wet and dry deposition [26]. An example of research on the effect of PM on marine biota involved an acute toxicity study on the embryonic stages of zebrafish (*Danio rerio*). Here, the authors found evidence that PM2.5 triggered toxicity in various fish organs, with the cardiovascular, hepatic, and neurological systems being the most affected [27].

1.2 Biological Models

1.2.1 Seabass (*Dicentrarchus labrax*)

The European Sea bass (*Dicentrarchus labrax;* Linnaeus, 1758) is a marine species inhabiting the shallow coastal waters (< 100 m), particularly along the Mediterranean coasts and Northeast Atlantic (**Figure 5**), where the conditions allow for better survival [28]. *D. labrax* is a euryhaline (30‰ to total strength seawater) and eurythermal (5 to 28 ºC) specie, and according to its natural life cycle, juveniles are more often found in estuaries [29, 30].

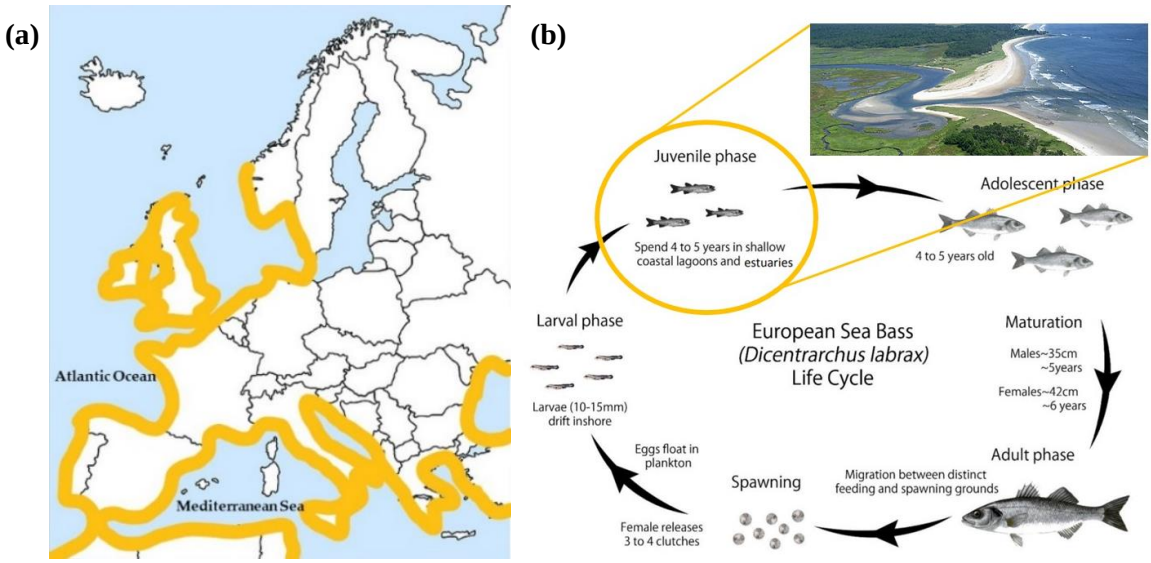


Figure 5 | General distribution range and habitats of the European sea bass in the Northeast Atlantic **(a).** Adapted from *López et al.* [31, 32]. Sea bass' specific distribution in function to its life cycle **(b)**. Adapted from *Carrol et al.* [33].

Seabass has a high commercial interest, being cultured in many European countries, with Mediterranean aquaculture representing above 96% of the total production in 2016 [34]. Additionally, its commercial production is rapidly expanding, being, in Portugal, the third most produced species in aquaculture systems [35]. According to the FAO Cultured Aquatic Species Information Programme guide, sea bass can be cultured in three major production systems: sea cages, raceways, and larval tanks (the last one mainly used in the primary life stages of the animal) [36]. Despite being a robust species, seabass is constantly surrounded by elements that drastically impair its survival rates, such as abiotic factors (temperature, pH, salinity, and others), stress factors, and environmental contaminants [37, 38]. These can result in dynamic modifications in estuarine systems, especially in their nursery role, that may, in turn, affect the early stages of growth essential for the renovation of fisheries resources in aquatic ecosystems. Accordingly, due to its resistance, economic relevance, and wide prevalence in estuary habitats, *D. labrax* is considered a critical marine fish model for research [34, 39, 40, 41].

Additionally, depending on the entry route, many contaminants, including PM, involve assimilation directly from the aquatic ecosystem, mainly via the gills, skin, or intestine (**Figure 6**). These can lead to different levels of stress. Stress is an essential factor to consider in fish, such as sea bass, primarily when referring to aquaculture. This phenomenon causes a decrease in muscular activity due to a quick loss in energy reserves (*i.e.*, adenosine triphosphate, ATP) and lactic acid accumulation, resulting in a post-mortem pH drop and softening of the muscle texture [42].

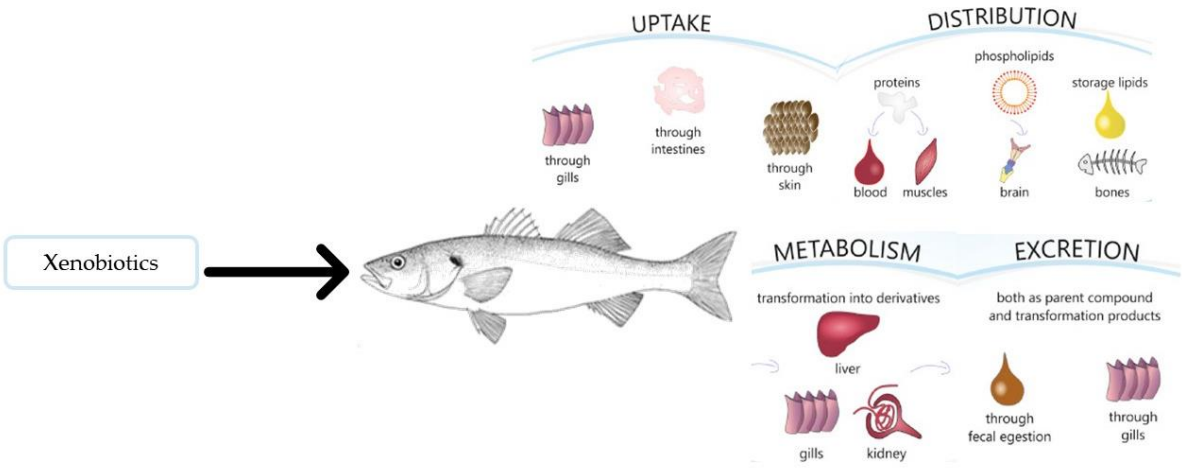


Figure 6 | Scheme representing the organism's general responses to xenobiotic exposure. Adapted from *Ross et al*. and *Barton et al.* [43, 44].

1.2.1.1 Morphology

Regarding their anatomy, sea bass is an elongated silver-grey fish with blue and green reflections on its scales [28, 36]. Typically, gender assessment of bass is visually possible, as females are larger than males, have a pointed head, and have larger pre-dorsal and pre-anal lengths. However, sex identification is only possible during the spawning season, which differs between wild (December and March in the Mediterranean and between March and June in the Atlantic) and captive-bred individuals (between February and July) [45].

1.2.1.2 Uptake ability of PM through gills

The skin, gills, and intestine are the three leading uptake pathways for aquatic contaminants that ultimately determine PM's effect on fish. The gills, however, constitute the principal target for wet-deposited xenobiotics [34, 46].

Fish gills (**Figure 7**) are organs with multiple functions involved in ion transport, gas exchange, acid-base regulation, and waste elimination [47]. However, the same highly efficient features that allow gas exchanges (*e.g.*, counter-current high flow of blood and water, a thin membrane separating blood and water, a large surface area ratio, and a high rate of water flow and blood perfusion) also provide suitable conditions for the uptake of xenobiotic chemicals.

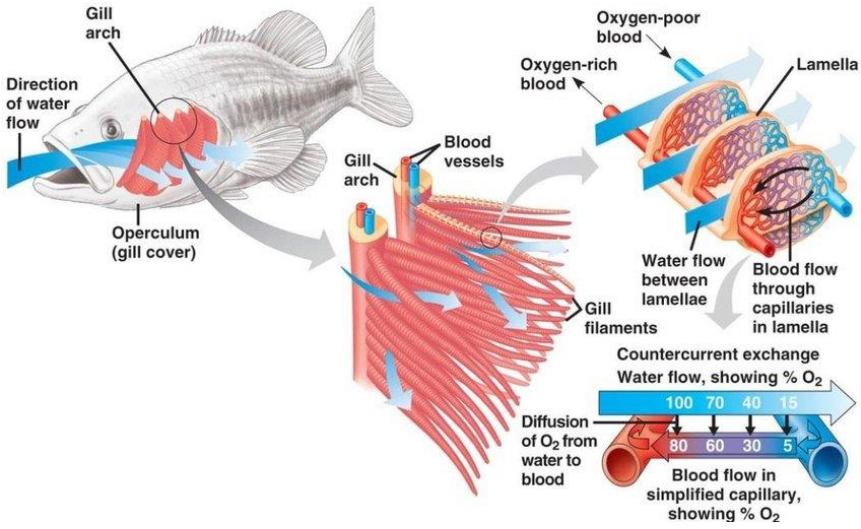


Figure 7 | Scheme representing the general responses to xenobiotic exposure in fish gills. Source: *Kumar et al.* [48].

Other factors such as environmental factors (*e.g.*, temperature, salinity, pH), physical and chemical properties of the particulate components (*e.g.*, molecule charge, molecular weight, lipid solubility, molecular volume, concentration in the water), and the state of the gills, can influence the flow rate of water, and consequently the uptake of small hazardous substances [49].

1.2.1.3 Contamination of the food chain with PM

In ecology, a food chain is a linear succession of organisms through which nutrients and energy move from primary producers to major consumers. However, there is a significant concern regarding cross-contamination with PAHs, PCBs, heavy metals, and other xenobiotic substances along the chain as they undergo biomagnification, reaching higher levels of contaminants at the highest consumers (**Figure 8**) [50].

Within aquatic environments, since estuaries and coastal areas constitute the interface between terrestrial and marine habitats, they generally have substantial pollution inputs from land, making them the most susceptible to accumulation. In this context, organisms that live within coastal food webs are more likely to ingest a more significant number of contaminants than those living offshore [51]. Still, other determinants such as intrinsic characteristics of the particles (*e.g.*, size, composition, and shape) and the organism's ability to deal with ingested compounds during uptake, distribution, metabolism, and excretion steps are also involved in determining the amount of damage a contaminant has caused on the organism [52, 53].

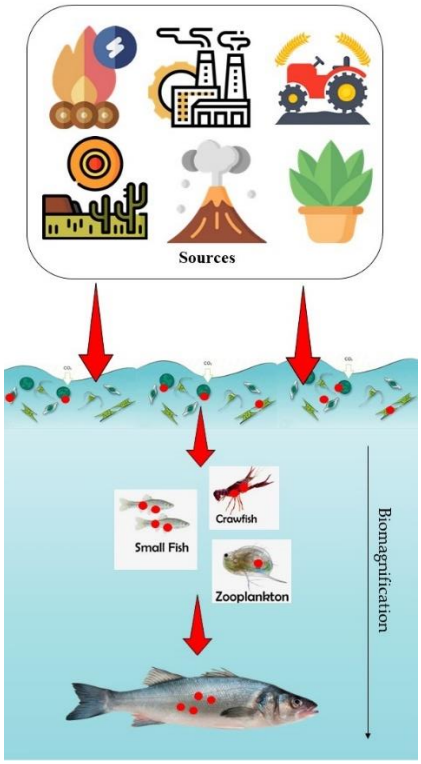


Figure 8 | Biomagnification within the food chain. Adapted from *Unuofin et al* [54].

The European Sea bass (*Dicentrarchus labrax*) is a carnivorous heterotroph, feeding on small invertebrates, molluscs, and other small fish in the wild [55]. Previous studies on the assessment of seabass feeding are directed towards estuarine systems, where younger life stages are more often found [29, 30]. Here, the trophic level of seabass was found to increase from 3 to 4.6 according to parameters such as size, and prey availability [55]. Since seabass is at a high consumer level in their food chain, environmental compounds that reach lower trophic levels are likely to biomagnify along the trophic chain, increasing the risk of exposure and toxic effects on top predators and, consequently, humans consuming them [56, 57].

Polychaete sp. is a marine invertebrate distinguished by its segmented soft body with parapodia and bristles, occurring in a wide range of marine habitats, including estuaries, lagoons, backwaters, and coastal inshore waters [58]. They are sometimes identified in the sea bass' diet and are widely employed as fishing bait, with *Marphysa sanguinea*, *Hediste diversicolor*, and *Diopatra neapolitana* being the most harvested species in Portugal [59]. Regarding their particle collection strategies, these organisms' feeding modes are surface and subsurface deposit-feeding and suspension or filter-feeding. The *Polychaete sp*. is known for its high selectivity with smaller particles clinging easier onto the tentaculate deposit-feeders [60, 61].

1.2.2 Mussel (*Mytilus galloprovincialis*)

The European mussel (*Mytilus galloprovincialis*; Lamarck, 1819) is native of the Mediterranean and the eastern Atlantic regions (from Ireland and the United Kingdom to northern Africa) [62]. This organism is found in multiple regions at intertidal zones to depths of 40 m, mainly corresponding to marine inshore, sheltered harbours, and estuaries. Mussels quickly spread, displace, and outcompete native species in response to climate change. Its primary forms of invasion are through aquaculture or via vectors such as ship ballast water and sediment or ship hull fouling (**Figure 9**) [63, 64].

M. galloprovincialis has a heightened tolerance to factors such as salinity and water temperature (< 24 ºC). This remarkable resistance, together with its feeding-filtration activity, extensive native range, high adaptability, and low mobility, allows the mussel to be considered a notable model organism used for many purposes (*e.g.*, biomonitoring, pollution indicator, research model, human food, and beverage) [65, 66].

Figure 9 | General distribution and habitats of mussel (*Mytilus sp.*) in Europe. Adapted from *Gaitán-Espitia et al.* [67].

1.2.2.1 Contamination of the food chain with PM

Mussels, under optimal conditions, ingest a considerable amount of water when feeding on suspended particles, which are filtered by the gills and absorbed by the organism. These particles can include natural feeding components, like plankton and other microscopic free-floating sea creatures. However, these can also involve water-suspended contaminants [68]. For example, suppose the water has suspended PM traces, which can be associated with multiple toxic substances (organic compounds, inorganic ions, and heavy metals). In this case, these will bioaccumulate in the mussel's tissues in large amounts, mainly due to the organism's high filtration rate. Also, since mussels are almost at the bottom of the food chain, it can lead to further bioaccumulation up the food chain.

On the other hand, under sub-optimal conditions, the mussels can reduce or completely close their inhalant aperture, and consequently, lower water filtration occurs in the gills. Thus, surviving for a period with minimal energy consumption. Even though this defence mechanism exists, it is mainly associated with food shortages and not as much with contamination [69].

1.2.3 Shrimp (*Palaemon varians*)

Palaemon varians (leach, 1814) is a widespread migratory species inhabiting from the Baltic Sea and Great Britain to the western Mediterranean, as depicted in **Figure 10.** They are often found in shallow salt marsh pools, other brackish pounds, and lagoons, which have an indirect link to the sea without having fully marine conditions [70, 71]. Here a higher tolerance of hypoxia is required due to being environments mostly stagnant, made up of highly turbid water, and even susceptible to a broad seasonal variation in salinity and temperature, which leads to *P. varians* being highly adaptable and resistant to environmental stresses [72].

Its highly adaptable and resistant features, low maintenance under laboratory conditions and ability to accumulate make *Palaemon varians* a suitable non-fish model organism. Thus, being commonly used in ecotoxicological studies to, for instance, reflect the amount of contamination in its surrounding environment [73, 74, 75].

Figure 10 | General distribution range and functional habitats of the *Palaemon varians*. Adapted from *Christodoulou et al* [70].

1.2.3.1 Morphology and behavior

P. varians' maximum carapace length reported stands at 5 cm, and the individual's colour is generally transparent. Shrimp exhibit sexual dimorphism as well, with females having longer bodies

than males, who are more similar to juvenile females. However, such distinctions are insufficient for precise identification [76, 77].

These shrimps usually have a varied diet ranging from molluscs, crustaceans, organisms' remains, plant material, and unidentified organic debris (varying according to the shrimp size) [78, 79]. Previous ecological studies have described *P. varians*' behavioural mechanisms in response to external environmental changes, including exposure to contaminants [80, 81]. For starters, moult cycle disruptions occur more often when organisms are under significant stress. Over-moulting leads to shrimp being vulnerable to predators or another bigger shrimp. Additionally, this can harm the organisms since it can bring them to hide in locations with suboptimal survival conditions, including high competition or inadequate availability of resources and predation [82, 83].

1.3 Oxidative Stress

Organisms with aerobic metabolism will inevitably produce reactive oxygen species (ROS), which, under normal conditions, play essential roles in operating the immune system, maintaining the redox balance, and activating cellular signalling pathways [84]. ROS can appear in the chloroplasts, peroxisomes, and mainly in the mitochondria during energy synthesis in the final step of the electron transport chain (ETC). Here, the molecular oxygen can often be reduced into a superoxide radical ($\bullet O_2^-$). Under low concentrations, ROS are promptly neutralised by the antioxidant enzymes (*e.g.*, Superoxide Dismutase (SOD), catalase (CAT), Glutathione-S-Transferase (GST)), and/or non-enzymatic (*e.g.*, amino acids, tocopherol, and vitamins A, E, K, and C) antioxidant defence mechanisms, which restore cell homeostasis [85, 86, 87, 88]. Nevertheless, if the disturbances occur on a larger scale or for a prolonged period, it will lead to a state of oxidative stress.

Oxidative stress (**Figure 11**) is a condition in which a disruption in the equilibrium between the production and elimination of ROS is triggered by exogenous or endogenous factors. Under these conditions, the organism produces a more significant amount of ROS, leading to tissue impairment, with particular emphasis on the damage of cellular components (*e.g.*, lipids, proteins, nucleic acids, membranes, and organelles), which can lead to activation of cell death processes such as apoptosis [89]. Lipid peroxidation is a typical example of damage induced by ROS, which results in cell membranes rigidity and loss of permeability and integrity. Here, polyunsaturated fatty acids are attacked by free radicals, and a chain reaction is triggered [90]. On the other hand, protein oxidation reactions are also prevalent and involve ROS propagation, resulting in modified amino acid side chains, cleaved peptides, and the generation of other oxidations products [89].

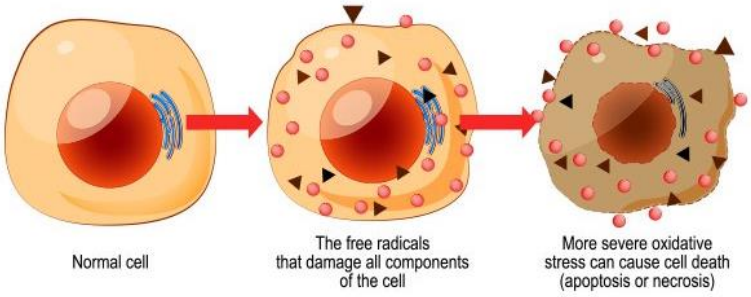


Figure 11 | Schematic representation of oxidative stress and its consequences at the cellular level. Source: *Nuzzo et al.* [91].

1.3.1 Marine organisms

Marine organisms are not exempt from oxidative stress (**Figure 12**). Thus, it is critical to assess the organism's reaction to a range of external pressures and stresses, such as extreme environmental changes (*e.g.*, pH, temperature, salinity) and the presence of a variety of hazardous chemicals (*e.g.*, pesticides and heavy metals) that can lead to free radical build-up, which can, in turn, affect the organism's homeostasis [92, 93, 94, 95, 96, 97].

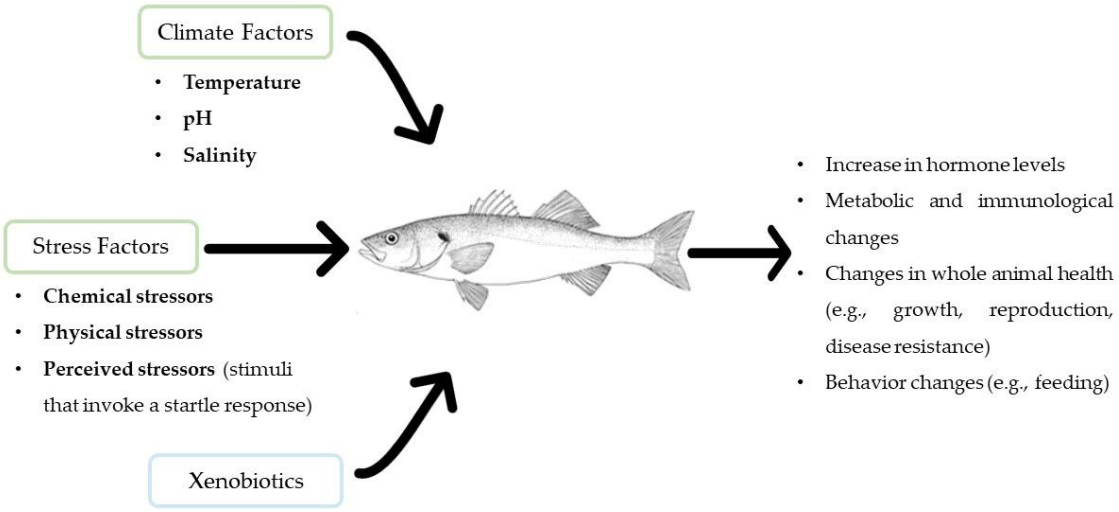


Figure 12 | Schematic representation of the exposure to distinct stresses and their consequences on fish. Adapted from *Maculewicz et al.* [87].

Previous studies have also positively correlated the amount of antioxidant activity and contaminants. Thus, pollutants associated with PM (*e.g.*, PAHs, PCBs, and heavy metals) which enhance ROS formation and lead to oxidative stress, can be assessed through biomonitoring studies [98].

1.3.2 Antioxidant enzymes

Organisms usually have a very intricate antioxidant defence mechanism, which collectively acts by stopping the oxidation process of free ROS in excess and their consequential harmful effect on biomolecules and tissues. According to their specific responses, antioxidants can be characterised into three distinct levels of defence, as depicted in **Figure 13**, which may include radical preventive, radical scavenging, and repair of damage induced by radicals. The first line of antioxidant defence may include catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPX), which act mainly by neutralising any free radical or molecule with the potential to turn into one. The second line of defence is glutathione-s-transferase (GST), which inhibits chain initiation and breaks chain propagation reactions. The third line of defence includes the reactions after free radical damage (damage of DNA, proteins, and lipids) [88, 99].

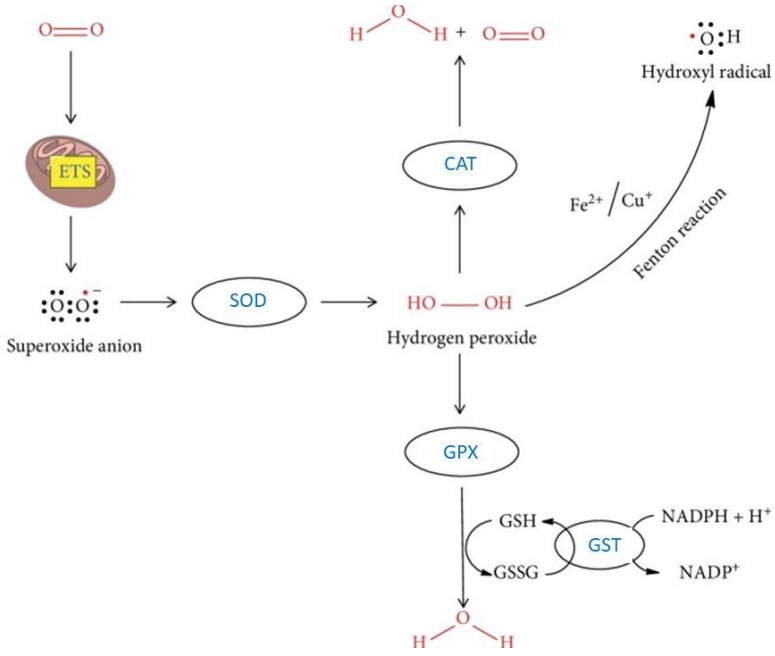


Figure 13 | Schematic representation of the enzymatic antioxidant defense mechanism in response to external contaminators. Source:*Nandi et al.* [100].

Superoxide dismutase (SOD) is an oxidoreductase enzyme found in most living organisms, including marine species. It catalyses the dismutation of the harmful superoxide anion into hydrogen peroxide (H_2O_2) and oxygen, thus, preventing further damage to the surrounding tissues. This oxidiser agent (*i.e.*, H_2O_2), when accumulated in excessive amounts in the tissues or cells, is also toxic, so it must be decomposed directly by catalase [101, 102].

Catalase (CAT) is an a enzyme found in all aerobic organisms, predominantly in the peroxisome but also in the mitochondria and cytoplasm of cells, with an antioxidant action. This enzyme plays a crucial role in inflammation and suppression of apoptosis, which are both recognized to be linked to

oxidative stress. Its main action depends on neutralising and decomposing hydrogen peroxide (nonradical ROS coming from SOD's activity) into one molecule of oxygen and two molecules of water in a two-step reaction represented below (**Expressions 1.a and 1.b**). Here, a reduction of the hydrogen peroxide molecule results in a spectroscopically active intermediate covalently bonded to the metal in the enzyme's structure. Finally, the intermediate compound goes through a couple of redox reactions, producing oxygen and water as final products [103, 104].

$$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$$ **Reaction 1- Haber - Weiss**

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + HO^-$$ **Reaction 2 - Fenton**

Glutathione Peroxidase (GPX) is an intracellular antioxidant enzyme found predominantly in cells' peroxisomes and cytosol. When CAT occurs in lower concentrations, GPX performs the reduction of H_2O_2 to water and the lipid peroxides into their corresponding alcohols [105].

$$H_2O_2 + 2GSH \rightarrow 2H_2O + GSSG$$ **Reaction 3**

Glutathione-S-Transferase (GST) is part of a detoxification phase II process that catalyses the conjugation of glutathione (GSH) to various electrophilic compounds (**Figure 14**). This versatility comes from its innate polymorphism, which contributes to the inter-individual differences in response to a wide variety of xenobiotics, primarily heavy metals [106]. This enzyme is ubiquitous in aerobic cells, and its biotransformation of exogenous harmful compounds allows the organisms to eliminate origin products more efficiently. However, even after conjugation, some products are still reactive, indicating that they must follow additional pathways to eliminate them [107].

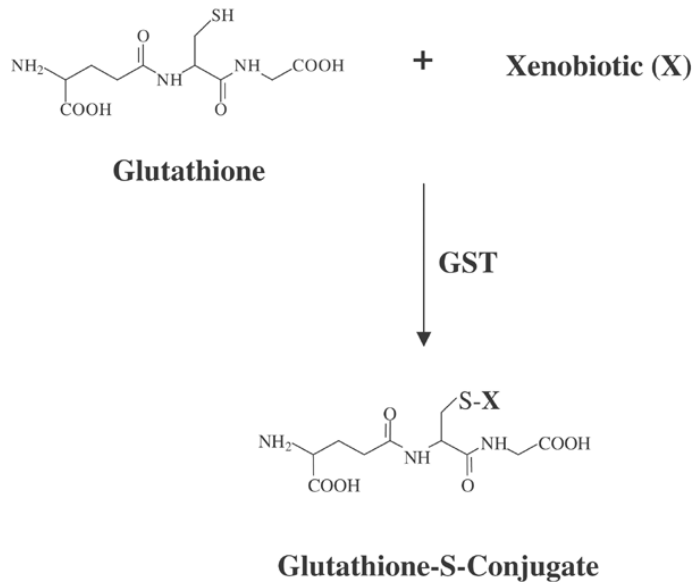


Figure 14 | Schematic representation of the glutathione's conjugation with a xenobiotic via the enzyme Glutathione-S-Transferase. Sourced from *Townsend et al.* [106].

1.4 Toxicity Assays

1.4.1 Stress biomarkers

Many basic comparative ecotoxicological studies using tissues from model organisms are based on selecting a group of molecular biomarkers, which can elucidate the underlying mechanisms of action of a specific xenobiotic or mixtures and give insight into the level of toxicity after chronic exposures to sublethal concentrations. These molecular biomarkers mainly monitor primary or secondary products of ROS and the status of antioxidant defence mechanisms activated intending to reduce or avoid oxidative stress [108, 109].

The methodologies for assessing antioxidant defence system biomarkers entail measuring enzyme activity, with some of the most monitored being Catalase (CAT), Superoxide Dismutase (SOD), Glutathione Peroxidase (GPX), and Glutathione-S-Transferase (GST). These are ubiquitous in many animal models (including aquatic organisms) and tissues and can have two distinct possible responses when exposed to a contaminant for an extended period. Their activity can be depleted or rise under specific conditions, allowing for an understanding of the toxic effect occurring [109]. On the other hand, other biomarkers may include several cellular damages triggered by the xenobiotic, including the oxidation of proteins and lipids [86]. For lipid damage, the most followed methodology involves reactions of the lipid peroxidation process, and for protein damage, many methodologies can be used, including specific ELISAs [110, 111].

1.5 Objectives

This work aims to provide a valuable contribution to the knowledge of the multiple traits related to atmospheric PM effects on the aquatic environment, namely its impact on marine organisms, more specifically seabass (*Dicentrarchus labrax*), mussel (*Mytilus galloprovincialis*), and shrimp (*Palaemon varians*). The assessment of specific biomarkers (oxidative stress enzymes, lipid peroxidation and total Ubiquitin) in various species allows comparing the responses to the exposure of atmospheric-derived PM and to understand the risk to the marine biota.

2

MATERIALS AND METHODS

2.1 Characterization and preparation of the PM solution

The atmospheric particulate matter used (**Figure 15**) in the exposure assays was a standard reference material (NIST, SRM® 1648a, USA) containing a variety of contaminants, with the most prevalent of which being polycyclic aromatic hydrocarbons (PAHs), nitro-substituted (nitro- PAHs), polychlorinated biphenyls (PCBs), chlorinated pesticides, and heavy metals, as presented in **Table 1**. Here, the PMs described have 5.85 μm, 1.35 μm, and 30.1 μm, which are the dimension below which 50 %, 10 %, and 90 % of the volume are present, respectively [112].

This project included two distinct concentrations of PM: 11,4 mg/L, to match the levels of atmospheric coarse PM over the North Atlantic, as forecasted by Copernicus, which is the European Union's Earth Observation Programme [113] and 5,7 mg/L, to evaluate if the same effects still occur at half the concentration. Both solutions were prepared by weighing the proper amount of solid in microtubes (1.5 mL) and adding 1 mL of artificial seawater. Finally, for improved particle dispersion, the microtubes were homogenized for 1 minute and sonicated for 10 minutes at 25 ºC in an ultrasonic bath (J-P Selecta Ultrasounds HD, Barcelona, Spain).

The artificial seawater was prepared by diluting artificial sea salt (Red Sea salt, Israel) into the amount of water necessary until the salinity reached the desired value, which varied according to the organism.

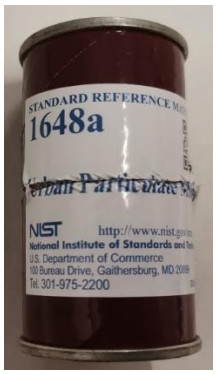


Figure 15 | Representative image of Standard urban Particulate Matter (1648a) solid mixture.

Table 1 | Mass Fraction Values (Dry-Mass Basis) of the contaminants in higher percentages in each component category of the 2g particulate matter standard reference material (NIST, SRM® 1648a, USA; with certificate analysis) during the exposure assay.

Main Components	Top element fractions	MFV (mg/ kg)	MFV (μg/ kg)	MFV (%)*
Elements	Zinc (Zn)	4800 ± 270	-	-
	Chlorine (Cl)	4543 ± 47	-	-
	Sodium (Na)	4240 ± 60	-	-
Carbon	Total	-	-	12.7
	Organic	-	-	10.5
	Element	-	-	2.3
PAHs	Benzo[*b*]fluoranthene**	8.89 ±0.05	-	-
	Fluoranthene	8.07 ± 0.14	-	-
	Chrysene	6.12 ± 0.06	-	-
	Pyrene	5.88 ± 0.07	-	-
	Benzo[*ghi*]perylene	5.00 ± 0.18	-	-
	Phenanthrene	4.86 ± 0.17	-	-
Nitro- PAHs	2-Nitrofluoranthene	-	257 ± 26	-
	9-Nitroanthracene	-	178 ± 24	-
	1-Nitropyrene	-	85.7 ± 7.3	-
	7-Nitrobenz[*a*]anthracene	-	83.0 ± 1.1	-
	2-Nitropyrene	-	48.7 ± 2.0	-
	3-Nitrophenanthrene	-	23.4 ± 0.4	-
PCBs***	PCB 180	-	45.9 ± 2.0	-
	PCB 138	-	41 ± 12	-
	PCB 153	-	40.0 ± 4.9	-
	PCB 149	-	38.9 ± 2.6	-
	PCB 95	-	34.6 ± 3.3	-
	PCB 101	-	34 ± 15	-
Chlorinated Pesticides	4,4'-DDT	-	76 ± 18	-
	Trans-chlordane	-	29.6 ± 1.5	-
	2,2,5,5,8,9,10-heptachlorobornane	-	23.2 ± 1.5	-
	4,4'-DDE	-	20.8 ± 9.6	-
	Cis-chlordane	-	19.1 ± 6.8	-
	Trans-nonachloride	-	14.9 ± 3.0	-
Toxic Metals	Titanium (Ti)	4021 ± 86	-	-
	Manganese (Mn)	790 ± 44	-	-

	Copper (Cu)	610 ± 70	-	-
	Chromium (Cr)	402 ± 13	-	-
	Vanadium (V)	127 ± 11	-	-
	Nickel (Ni)	81.1 ± 6.8	-	-

* Dry-mass percentage.

** Based on extraction metho and conditions.

*** PCB 153 (2,2',4,4',5,5'-Hexachlorobiphenyl); PCB 149 (2,2',3,4',5',6-Hexachlorobiphenyl); PCB 138 (2,2',3,4,4',5'-Hexachlorobiphenyl); PCB 95 (2,2',3,5',6-Pentachlorobiphenyl); PCB 101 (2,2'4,5,5'-Pentachlorobiphenyl); PCB 180 (2,2',3,4,4',5,5'-Heptachlorobiphenyl).

2.2 Biological Models

2.2.1 Seabass (*Dicentrarchus labrax*)

Seabass (*Dicentrarchus labrax*) served as the biological model for the trials and further biomarker evaluation of selected key tissues. These organisms were collected from aquaculture producers (IPMA, Olhão), as shown in **Figure 16**, and were transferred into the fish facilities at the FCT-NOVA, considering the parameters necessary to avoid mortalities during transport [114].

Upon arrival, the fish were housed for one week in tanks (20 L volume capacity) containing water from Guicho's (Farol do Cabo Raso, Cascais, Lisbon, Portugal), considered pristine seawater, to reduce stress during the acclimatization period and before starting exposure trials. These tanks were under continuous aeration (> 6 mg/L dissolved oxygen) and a photoperiod of 12 h light-dark.

Figure 16 | Representative image of the organism employed and collection facilities. **(a)** The model organism of seabass (*Dicentrarchus labrax*). **(b)** Representation of the path taken by the organisms sourced from Google maps [115]. **(c)** IPMA aquaculture facilities at Olhão [114].

2.2.2 Mussel (*Mytilus galloprovincialis*)

The Mussels (*M. galloprovincialis)* was another model organism used for the exposure trials. These were manually gathered from Cabo Raso in Guincho beach (Cascais, Lisbon, Portugal), as depicted in **Figure 17. a**, and then transported (**Figure 17. b**) to the laboratory facilities in a refrigerated thermal container. After arrival, the organisms were immediately placed in tanks containing seawater from the sampling site (33 ‰, pH= 8.0), considered clean water, for acclimatization purposes before starting the exposure trials. The same water also corresponded to the one used during the exposure trials. The acclimatization period lasted for three days in a tank (20 L volume capacity) with continuous aeration (> 6 mg/L dissolved oxygen) and a photoperiod of 12 h light-dark.

Figure 17 | Representative image of the mussel used in exposure trials and its collecting site. **(a)** Image of the biological model (*Mytilus galloprovincialis*) sourced from *Paiva et al.* [116]. **(b)** Collection site. **(c)** The satellite image of the collection site was obtained from Google maps [115].

2.2.3 Shrimp (*Palaemon varians*)

The third and last biological model selected to perform the exposure assays with the suspended PM was the shrimp (*P. varians*). The individuals were collected from a shrimp farm at Alcochete (Portugal) and transported to the UCIBIO (FCT-UNL) facilities (**Figure 18**). Upon arrival, the shrimp were immediately transferred to one acclimatization tank (6 L volume capacity) containing water from the same collecting place (salinity: 37.70 ppm, and temperature: 24 ºC). Here, the tank was equipped with a water filter, an oxygen continuous aeration system, and a timer programmed with a photoperiod of 12h light-dark. These conditions were also carried out to the tanks prepared afterwards for exposure (apart from the water filter).

Figure 18 | Representative image of the shrimp used in exposure trials and its collecting site. (a) Image of the biological model (*Palaemon varians*). (b) The satellite image of the collection site was obtained from Google maps [115].

2.3 Exposure Assays

2.3.1 Seabass exposed to suspended Particulate Matter (PM)

During the exposure trials, the seabass fish were kept in tanks filled with double-filtered seawater with a constant pH (8.09 ± 0.10), salinity (35.05 ± 0.17 ppm), and temperature (21.6 ± 0.31 ºC), weekly monitored, and the organisms were fed daily with pellets *ad libitum*.

The exposure experiment included 45 subjects (2.45 ± 1.32 g of weight and 5.73 ± 0.82 cm of length) randomly distributed through three tanks (15 each). Each tank, with constant oxygenation and water circulation, contained 0 mg/L (control group), 5.7 mg/L and 11.4 mg/L of the standard particulate matter, as represented in **Figure 19**. After 7, 14, and 21 days of exposure, the seabass were collected and sacrificed to remove three organs (muscle, gills, and liver). Each organ was homogenized (Tissue Master 125, Omni, Kennesaw, GA, USA) in 1 mL (liver) or 2 mL (muscle and gills) of phosphate-buffered saline solution (PBS: 140 mM NaCl (Panreac, Barcelona, Spain), 10 mM Na_2HPO_4, (Sigma-Aldrich, St. Louis, MO USA), 3 mM KCl,(Merck, Darmstadt, Germany), 2.0 mL KH_2PO_4, pH 7.40, (Sigma-Aldrich)) and centrifuged for 10 minutes at 15,000 × g and 4ºC (VWR, model CT 15RE, Hitachi Koki Co., Ltd., Tokyo, Japan) to obtain only the cytosolic fraction. Finally, the supernatants were collected, transferred to microtubes (1.5 mL), and stored at – 45 ºC until further analysis [117].

The contamination process occurred every 48h, corresponding also to water exchanges during which, the organisms were only placed in the assay tanks after sufficient time had passed for complete homogenizing of the suspended particles.

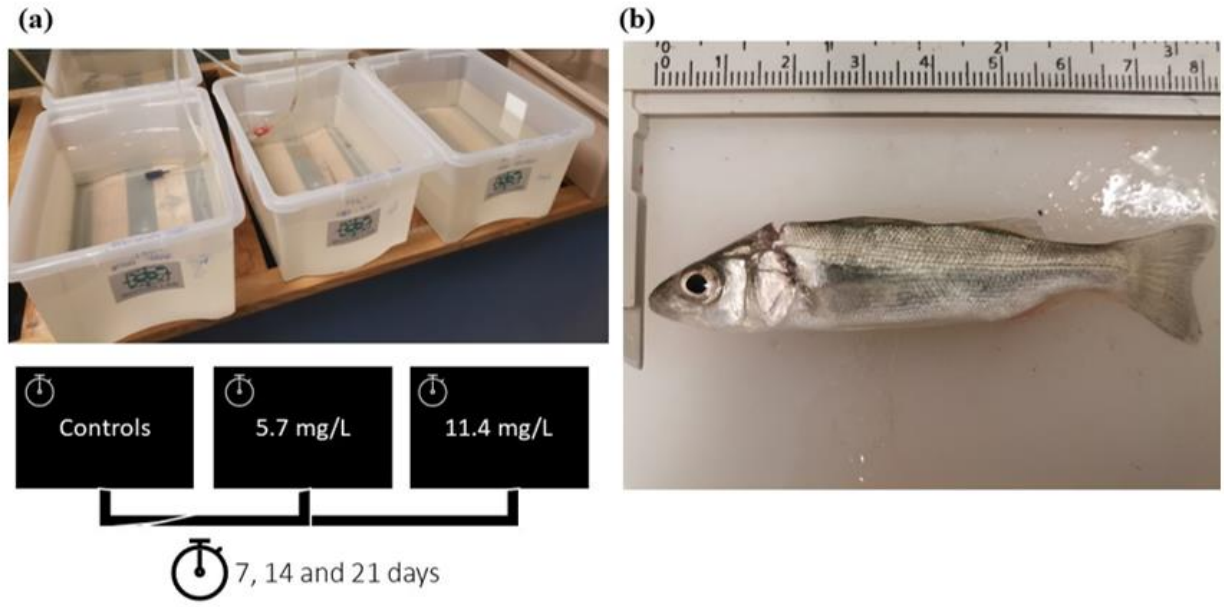


Figure 19 | Representative image of the exposure trials. **(a)** Tank's layout with continuous aeration. **(b)** Juvenile seabass (*Dicentrarchus labrax*).

2.3.2 Seabass fed with *Polychaetes* previously exposed to Particulate Matter (PM)

The organisms were divided into two tanks filled with sea water from Guincho's beach (Cascais, Lisbon, Portugal), as previously referred, renewed every two days. The water parameters such as pH (8.09 ± 0.10), salinity (35.05 ± 0.17 ppm), and temperature (21.6 ± 0.31 ºC) were monitored daily.

The exposure assay was divided into two parts (**Figure 20 a**). The first part was carried out at the University of Aveiro (Portugal). Here, 60 *Polychaete sp*. were divided into the control group (30 individuals with an average weight of 72.91 ± 14.91 mg) and a group of organisms exposed to a concentration of 11.4 mg/L of the PM standard compound (30 individuals with an average weight of 70.04 ± 45.44 mg). Afterwards, the contaminated and non-contaminated *Polychaetes* were lyophilized and dispatched in hermetically sealed tubes to FCT-NOVA (Costa da Caparica, Portugal).

The second part of the exposure assay consisted of 12 individuals divided through two aquariums, 5 in the control group (average weight: 2.80 ± 0.75 g; average size: 5.82 ± 0.56 cm) and 7 in the exposed group (average weight: 2.57 ± 0.73 g; average size: 5.10 ± 0.74 cm) as represented in the **Figure 20 b**. The control group was fed non-contaminated *Polychaetes*, and the exposed group was fed contaminated *Polychaetes* After 4 days of exposure, the fish were sampled, and a selection of organs were removed (muscle, liver, gills, and intestine). Each organ was homogenized (Tissue Master 125, Omni, Kennesaw, GA, USA) in 1 mL (liver) or 2 mL (muscle, gills, and intestines) of phosphate-buffered saline solution (PBS: 140 mM NaCl (Panreac, Barcelona, Spain), 10 mM Na_2HPO_4, (Sigma-Aldrich, St. Louis, MO USA), 3 mM KCl,(Merck, Darmstadt, Germany), 2.0 mL KH_2PO_4, pH 7.40, (Sigma-Aldrich)) and centrifuged for 10 minutes at 15,000 × g, 4ºC (VWR, model CT 15RE, Hitachi Koki Co., Ltd., Tokyo, Japan) to obtain only the cytosolic fraction. Finally, the supernatants were collected, transferred to microtubes (1.5 mL), and stored at – 45 ºC until further analysis [117].

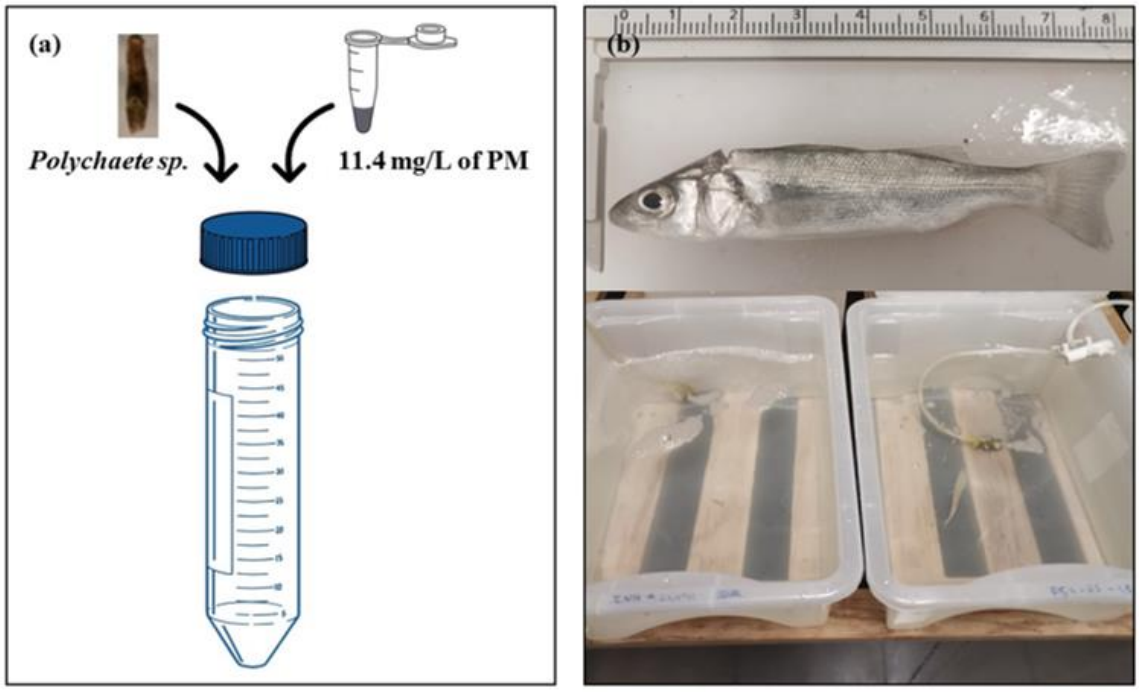


Figure 20 | Representative image of the exposure trials. **(a)** Schematic representation of the *Polychaetes* assay performed at Aveiro University. **(b)** The biological model (*Discentrarchus labrax*). **(c)** Tank's layout with continuous aeration.

2.3.3 Mussels exposed to suspended Particulate Matter (PM)

The mussels employed for the exposure assay were first washed with tap water to remove impurities, sand, other residues and attached algae before initiating the trials. Afterwards, the organisms were assorted into four tanks filled with water from Guincho's beach (Cascais, Lisbon, Portugal) with parameters of pH (8.06 ± 0.07), salinity (35.10 ± 0.17 ppm), temperature (17.60 ± 0.40 ºC), total dissolved Solids (TDS: 35.10 ± 0.17 ppm), and electric conductivity (59.26 ± 0.99 mS/cm), monitored daily.

Additionally, the water changes were carried out every 48h, along with the PM contamination, and the feeding with 1.5 mg/L of an algae supplement (*Chlorella algae* from Shine superfood, Portugal) previously dissolved in the same exposure water occurred daily.

The assay involved 48 organisms in total (shell length 3.6 ± 0.39 cm), randomly distributed through three tanks (16 mussels each). As displayed in **Figure 21**, the tanks consisted of a control group (0 mg/L of PM) and two exposure concentrations (5.7 mg/L and 11.4 mg/L of PM). After 7, 14 and 21 days, mussels were sampled and sacrificed to remove gills and digestive glands. Each organ was then homogenized (Tissue Master 125, Omni, Kennesaw, GA, USA) in 2 mL of phosphate-buffered saline solution (PBS: 140 mM NaCl (Panreac, Barcelona, Spain); 10 mM Na_2HPO_4, (Sigma-Aldrich, St. Louis, MO USA); 3 mM KCl,(Merck, Darmstadt, Germany); 2.0 mL KH_2PO_4, pH 7.40, (Sigma-Aldrich)) and centrifuged for 10 minutes at 15,000 × g (VWR, model CT 15RE from Hitachi Koki Co., Ltd., Tokyo, Japan) to obtain only the protein portion present in the cytosol. Finally, the supernatants were collected and stored at – 45ºC until further analysis [117].

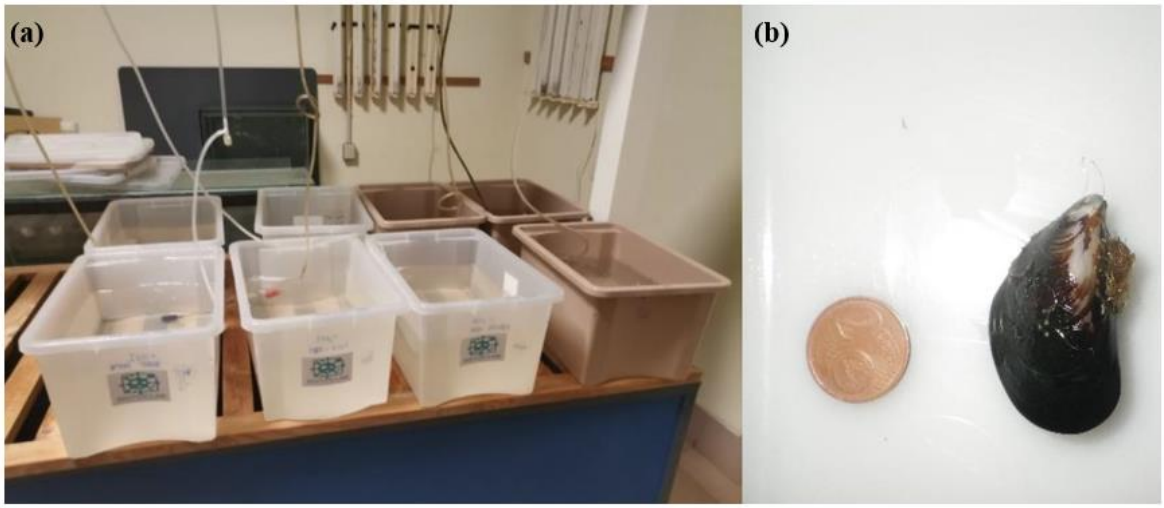


Figure 21 | | Representative image of the exposure trials. **(a)** Tank's layout with continuous aeration. **(b)** The biological model (*M. galloprovincialis*).

2.3.4 Shrimp exposed to suspended Particulate Matter (PM)

In the last exposure assays the organisms (*P. varians*) were divided into three separate tanks (2 mL each) with water from Guincho's beach (Cascais, Lisbon, Portugal). Here, physicochemical parameters as salinity (34.00 ± 0.87 ppm), temperature (22.08 ± 0.62 ºC), conductivity (55.39 ± 1.12 mS), and total suspended matter (27.63 ± 0.52 mg/L) were monitored daily.

The water and contamination conditions were renewed every 48h, and the shrimp were fed daily with the same number of fish pellets (Premium gold flake-mix, Vitakraft, Germany).

As depicted in **Figure 22**, the first tank contained 21 individuals with an average size of 1.53 ± 0.48 cm and a weight of 0.30 ± 0.21 g in water without any contaminant (control). The second tank held 21 individuals with an average size of 1.27 ± 0.23 cm and a weight of 0.22 ± 0.13 g exposed to 5.7 mg/L of PM. Finally, the third tank took 21 individuals with an average size of 1.32 ± 0.30 cm and a weight of 0.24 ± 0.16 g exposed to 11.4 mg/L of PM.

Before starting the acclimatization period, a group of 6 shrimp (T0) were collected and stored at a temperature of -45ºC until further analyses. After 7, 14 and 21 days, individuals were collected from each tank, and two selected organs (muscle and viscera) were removed and homogenized (Tissue Master, Omni, Kennesaw, GA, USA) in 1 mL (muscle) or 0.5 mL (viscera) of phosphate-buffered saline solution (PBS: 140 mM NaCl (Panreac, Barcelona, Spain), 10 mM Na_2HPO_4, (Sigma-Aldrich, St. Louis, MO USA), 3 mM KCl, (Merck, Darmstadt, Germany), 2.0 mL KH_2PO_4, pH 7.40, (Sigma-Aldrich)). Each sample was centrifuged for 10 minutes at 15,000 × g, 4ºC (VWR, model CT 15RE from Hitachi Koki Co., Ltd., 132 Tokyo, Japan, and the resulting supernatants were transferred to new microtubes (1.5 mL) and stored at – 45 ºC until further analysis [117].

Figure 22 | Representation of the PM exposure trial. **(a)** Tank's layout with air circulation. The controls and individuals exposed to the standard PM concentrations (5.7 mg/L and 11.4 mg/L, respectively) were placed from left to right. **(b)** Organisms (*Palaemon varians*) in the acclimatization tank.

2.4 Total Protein determination

The Bradford assay was performed following the *Bradford method* (1976), which allows for the quantification of the soluble protein concentration present in the cytosol portion of the samples after interacting with the Bradford reagent (Coomassie G-250) [118]. The protein contents of each sample were quantified by comparing its absorbance value with those obtained from the standard concentration curve (0 to 4 mg/L) prepared from a stock solution of Bovine albumin (BSA, Nzytech, Portugal). Additionally, the blank consisted of the same phosphate-buffered saline solution (PBS ~7.3) used during the homogenization of the samples.

Absorbance values were measured at 595 nm using a microplate reader (Synergy HTX, BioTek, USA) after placing 20 μL of the sample or standard in a 96-well microplate in duplicate (Greiner, BioOne GmbH, Frickenhausen, Germany), followed by 180 μL of the Bradford reagent.

The total protein values were used to normalize the biomarker's results.

2.5 Antioxidant Biomarkers

2.5.1 Catalase (CAT)

The Catalase assay allowed indirect monitoring of hydrogen peroxide (H_2O_2) conversion into oxygen (O_2) and water (H_2O) [119].

The procedure started by preparing a standard concentration curve made from a formaldehyde stock solution (4.25 mM; Sigma- Aldrich; USA) and then making serial dilutions to obtain various concentration values from 0 to 75 mM. Afterwards, in a 96-clear Plate (Greiner, BioOne GmbH, Frickenhausen, Germany), 20 μL of the samples or blank solution (PBS~7.3) were placed in duplicate, together with 100 μL of assay buffer (100 mM potassium phosphate, pH 7.0), and 30 μL of pure methanol (~ 99.8%; Honeywell; Seelze; Germany). Afterwards, the reaction started by adding 20 μL of hydrogen peroxide (0.035 M; H_2O_2 35% Pure; PanReac AppliChem; Barcelona; Spain). After a 20-minute incubation period at room temperature, with the plate covered in foil and under constant agitation (Optic rymen System, Spain), 30 μL of potassium hydroxide (KOH 10 M) and 30 μL of 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (purpald; ≥ 99%; Sigma- Aldrich Germany; dissolved in 34.2 mM in 0.5 M HCl, and water) were added to the wells. An additional 10-minute incubation under the same conditions took place and 10 μL of potassium periodate (KIO_4; 65.2 mM in 0.5 M KOH) was added to stop the reaction. In the end, a last 5-minute incubation period was carried out and the formaldehyde values were obtained spectrophotometrically at 540 nm using a microplate reader (Synergy HTX, BioTek, USA) and then compared to those from the standard curve.

The activity results obtained here were normalized through the total protein concentrations obtained by the Bradford assay.

2.5.2 Superoxide Dismutase (SOD)

The superoxide dismutase (SOD) assay was based on the protocol *SOD assay kit (Elabscience, E-BC-K020)* adapted to 96-well microplate (Greiner, BioOne GmbH, Frickenhausen, Germany) readings [120]. Here, the activity was determined by following the absorbance changes at 560 nm every minute for 26 minutes using a microplate reader (Synergy HTX, BioTek, USA). In the end, the results were also normalized by the total protein concentration determined by the Bradford assay.

The order in which the reagents are added to the assay solution is a critical component. In the first round, 200 μL of potassium phosphate buffer (50 mM; pH 8.0), 10 μL of Ethylenediaminetetraacetic acid (EDTA; 3mM; Riedel-Haën, Germany), 10 μL of xanthine (3 mM; Sigma Aldrich), 10 μL of Nitro-blue tetrazolium (NBT; 0.75 mM; Sigma-Aldrich) were added. Only after the 10 μL of samples in duplicate and the blanks (PBS ~ 7.3) in quadruplicate may be combined. The enzyme Xanthine oxidase (XOD, 0.4 U/ mg of protein; Sigma-Aldrich) was only added in the ends, right before the plate reading.

2.5.3 Glutathione Peroxidase (GPX)

The glutathione peroxidase (GPX) assay was determined based on the spectrophotometric measurement (λ = 340 nm) of the disappearance of β-NADPH every minute for a period of 5-minutes. The activity results obtained were normalized by the total protein concentration of each sample previously determined [121].

First, 20 μL of the sample in duplicate and the blank (PBS~7.3) in quadruplicate were placed in the wells of a 96-well microplate (Greiner, BioOne GmbH, Frickenhausen, Germany). Afterwards, the assay was carried out by mixing phosphate buffer (50 mM; pH 7.4) with Ethylenediaminetetraacetic acid (EDTA; 5 mM; Riedel-Haën, Germany) and adding 120 μL into each well. Then, 50 μL of the co-substrate solution, consisting of 18.7 mg NADPH (~98%; Sigma- Aldrich; Germany), 24.6 mg reduced glutathione (GSH; 200 mM; Sigma-Aldrich; USA), a pinch of sodium azide (4 mM NaN_3; Sigma- Aldrich; Germany), and 5 μL of Glutathione Reductase (GSSH reductase from S. cerevisiae; (Sigma- Aldrich; USA) were mixed and added into the microplate wells. Finally, Cumene Hydroperoxide ($C_9H_{12}O_2$; 80%; Aldrich Chemistry; Germany) was diluted to 15 mM, and 20 μL was added to the wells just before the measurements.

2.5.4 Glutathione-S-Transferase (GST)

The Glutathione-S-Transferase (GST) activity was based on the protocol by *Habig et al*. (1974), adapted to a 96-well microplate (Greiner, BioOne GmbH, Frickenhausen, Germany). During the assay, conjugates are formed between glutathione (GSH) and 1-Chloro-2,4-dinitrobenzene (CDNB) [122].

First the reaction mixture was prepared containing 9.6 mL of phosphate buffered saline solution (PBS ~ 7.3), 0.1 mL of reduced glutathione (GSH; 200 mM; Sigma-Aldrich; USA), and 0.1 mL of 1-chloro-2,4-dinitrobenzene (CDNB; 100 mM; Sigma-Aldrich; USA). In each microplate well, 20 μL of the sample and blank volume (PBS buffer used for homogenization) were added, in duplicate, followed by 180 μL of the reaction mixture. In the end, the reaction was measured using a microplate reader (Synergy HTX, BioTek, USA) every minute for 6 minutes.

The GST activities were calculated using CDNB's extinction coefficient of 5.3 mM^{-1}, and the results were normalized by the total protein concentration of each sample obtained from the Bradford assay.

2.6 Cellular damage biomarkers

2.6.1 Lipid Peroxidation (LPO)

The lipid peroxidation (LPO) process consists of a free radical chain reaction mechanism, which was followed by employing the thiobarbituric acid assay based on the *Uchiyama et al*. (1978) protocol [123, 124]. The results were obtained by comparing the absorbance obtained for each sample to the standard curve (0 to 0.100 mM) and normalized with the protein concentration previously determined during the Bradford assay.

First, a reaction mixture was prepared by combining 2.29 mL of phosphate-buffered saline solution (PBS: 140 mM NaCl (Panreac, Barcelona, Spain), 10 mM Na_2HPO_4, (Sigma-Aldrich, St. Louis, MO USA), 3 mM KCl, (Merck, Darmstadt, Germany), 2.0 mL KH_2PO_4, pH 7.40, (Sigma-Aldrich)), 0.64 mL of Sodium lauryl sulfate (SDS 8.1 %)), 4.75 mL of thiochloroacetic acid (10 mg/ mL TCA 20 %, pH 3.5; PanReac AppliChem; Barcelona Spain), 4.75 mL of 4,6-Dihydroxy-2-mercaptopyrimidine,4,6-Dihydroxypyrimidine-2-thiol (0.2 mg/ L TBA 1 %; Sigma- Aldrich; Germany), 0.64 mL of Sodium Dodecyl Sulfate (SDS; 8.1% m/v), and 2.57 mL of ultra-pure water.

Afterwards, 295 μL of the reaction mixture and 5 μL of sample or standard curve were added to a microtube (1.5 mL). The microtubes were mixed for 1 minute and placed on a heating plate (100 ºC; Labret, made in CHN) to start the reaction. After 10 minutes, every microtube was moved into ice to slow the reaction, and 62.5 μL of ultra-pure water was added to each microtube. Finally, 150 μL of each microtube was placed (in duplicate) on a 96-well microplate (Greiner, BioOne GmbH, Frickenhausen,

Germany), and the absorbance was read at 530 nm using a microplate reader (Synergy HTX, BioTek, USA).

2.6.2 Ubiquitin Assay (UBI)

Ubiquitination was carried out by following a method described by *Crowther et al*. (2009) and adapted to a 96-well microplate (Greiner, BioOne GmbH, Frickenhausen, Germany) [125]. The results were obtained by comparing the absorbance obtained for each sample to a standard curve (0 to 0.8000 mg/mL) and then expressed as total protein concentration. The curve was built by making serial dilutions from a standard ubiquitin solution (5 mg/mL).

First, 50 μL of sample and standard curve were added to a 96-well microplate and left for an overnight incubation, covered in aluminium foil at 4ºC. Afterwards, the microplate was washed twice with PBS containing 0.05% Tween-20 (PanReact ApliChem) and incubated (2 hours, 37 °C, covered in aluminium foil) with 100 μL of a blocking solution (1% BSA in PBS ~ 7.3).

After a new washing with PBS and 0.05% Tween-20 twice, 50 μL of the primary antibody in PBS with 1% BSA (200 mg/mL anti-Ub mouse monoclonal IgG; OriGene) was added to the microplate wells and left to incubate overnight (covered in aluminium foil at 4ºC). Then, after washing the microplate following the same procedure, 50 μL of secondary antibody with a 1:1000 dilution in PBS 1%BSA (Anti -Mouse Ig - Fc specific- Alkaline Phosphatase antibody produced in goat; Sigma- Aldrich) was added and left to incubate overnight (covered in aluminium foil at 4ºC). After the incubation period, the microplate was rewashed (three times), and 50 μL of the substrate solution (10 mg of 4-Nitrophenyl phosphate disodium salt hexahydrate (PnPP ≥ 99%; Sigma- Aldrich), 157 mg trizma hydrochloride (Tris HCl; Sigma- Aldrich; USA), 58.4 mg NaCl, and 50 mL magnesium chloride hexahydrate (5 mM $MgCl_2$; BioChemika; Japan), pH: 8.5-9.0) was added to the microplate wells followed by about 30 minutes of incubation. Then, 50 μL of STOP solution (3M NaOH) was added, and the absorbance was read at 405 nm using a microplate reader (Synergy HTX, BioTek, USA).

2.7 Statistical Analysis

The statistical analysis was carried out using GraphPad Prism (Version 8.0.1). Multiple comparison tests were performed using one-way ANOVA followed by Tukey's test or the non-parametric Mann-Whitney test (when statistic assumptions were not achieved) to assess significant differences ($p < 0.05$) among tested organisms.

3 RESULTS

3.1 Seabass exposed to suspended Particulate Matter (PM)

3.1.1 Mortality rate

A mortality increase was observed according to the different tested PM concentrations and exposure time (**Figure 23**), which was higher (88%) in fish exposed to 11.4 mg/L of PM after 14 days of exposure when all fish died.

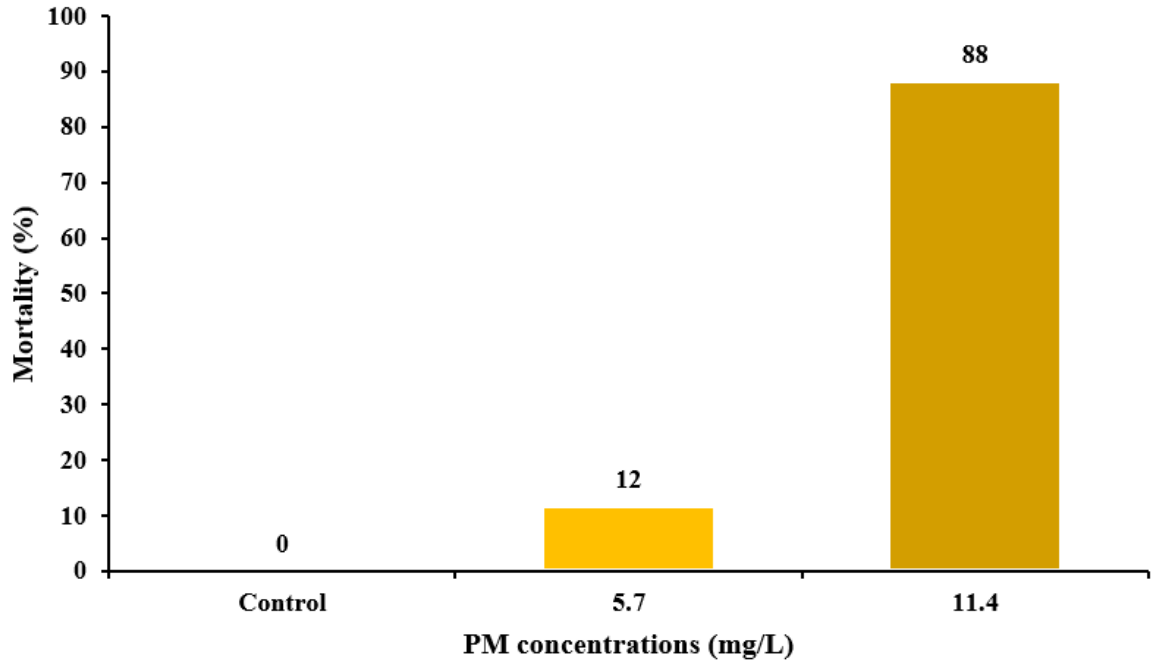


Figure 23 | *Dicentrarchus labrax* mortality rate (% of the total number of individuals deceased) observed during the exposure assay of the organism to PM.

3.1.2 Antioxidant Biomarkers

3.1.2.1 Catalase (CAT)

CAT's activity was assessed in seabass' organs (muscle, liver, and gills) following exposure to PM for 7, 14, and 21 days, as depicted in **Figure 24**. The gills presented the highest values, while the lowest activities were observed in the liver and muscle.

In the muscle (**Figure 24 a**), a general reduction in CAT's activity is observed with a concomitant increase in PM concentration and exposure time. First, an overall increase is observed when comparing the activity values of the PM concentration of 5.7 mg/L with the control group in individuals collected after 7 and 14 days of exposure. The opposite is observed in individuals exposed to the same concentration (5.7 mg/L) but after 21 days or for the group subjected to a PM concentration of 11.4 mg/L after 7 days of exposure, whose CAT's activity seems to be diminished compared to their respective control groups.

After a more thorough examination, the highest mean CAT activity (22.32 ± 10.80 nmol/min/mg of total protein) was determined in the muscles of animals collected after 7 days of exposure to 5.7 mg/L of PM. In contrast, the lowest mean activity (5.62 ± 3.89 nmol/min/mg of total protein) occurred in the control organisms collected after 14 days.

No statistically significant differences ($p > 0.05$) between the exposed seabass and controls. However, significant differences ($p < 0.05$) were found between exposure periods in fish muscle exposed to 5.7 mg/L of PM.

Regarding the exposure time, CAT activity in the gills follows the same pattern as in the muscle (**Figure 24 b**). In the organisms sampled after 7 days of exposure, both PM concentrations (5.7 and 11.4 mg/L) increased compared to their control groups. Meanwhile, the opposite (decrease in activity) occurred in the groups exposed to 11.4 mg/L PM, collected after 14 and 21 days of exposure.

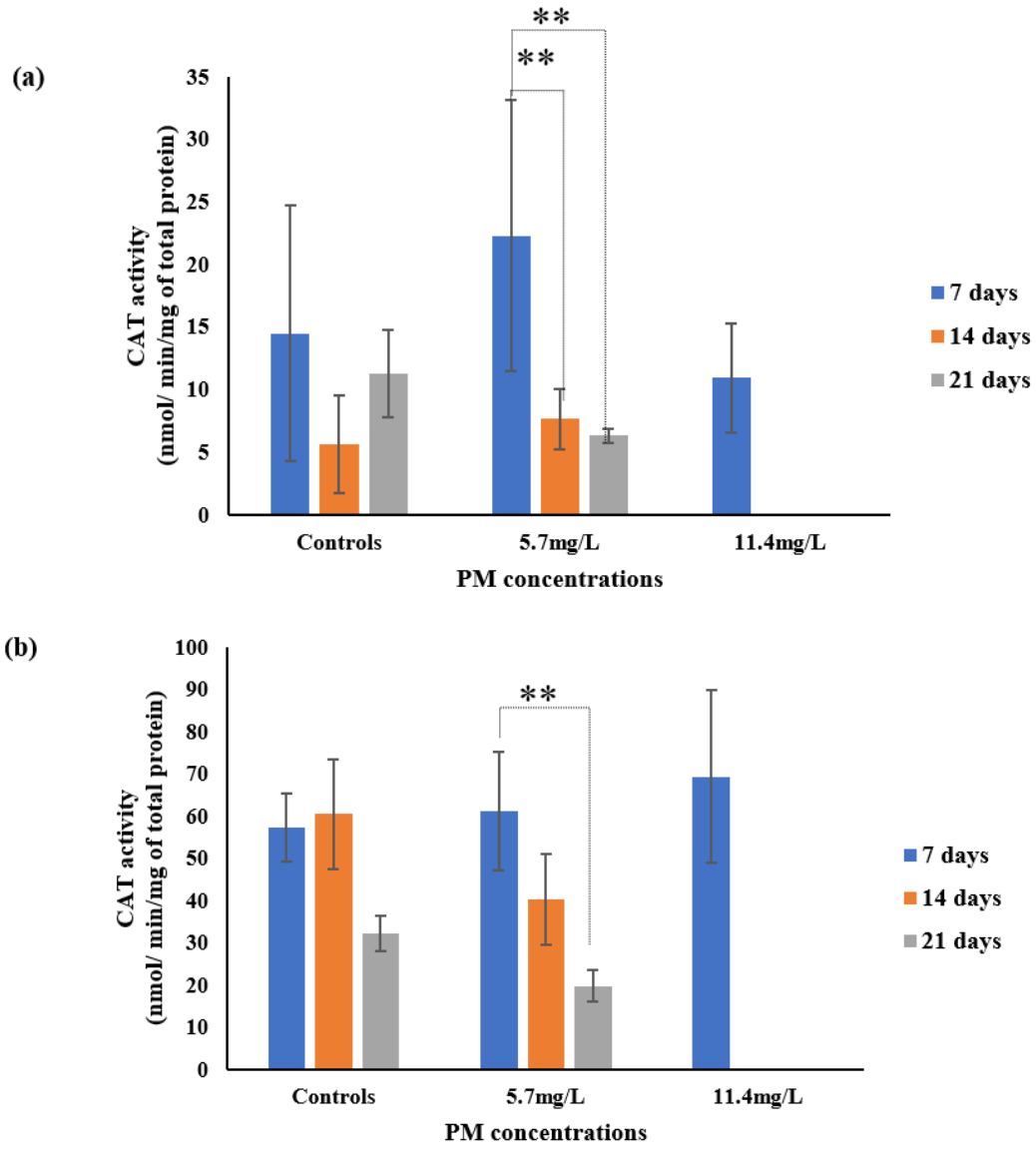
After a more detailed analysis, it is observed that the highest average CAT activity (69.43 ± 20.32 nmol/min/mg of total protein) was observed in fish exposed to 11.4 mg/L of PM after 7 days of exposure. On the other hand, the lowest (19.87 ± 3.73 nmol/min/mg of total protein) was observed in organisms exposed to a PM concentration of 5.7 mg/L after 21 days of exposure.

No significant differences ($p > 0.05$) were detected between the controls and gills of exposed fish. However, significant differences ($p < 0.05$) were found between exposure periods in fish gills exposed to 5.7 mg/L of PM collected after 7 and 21 days of exposure.

CAT's activity in the liver follows a similar behaviour to the one seen in the gills (**Figure 24 c**). First, the activity increased in the organisms collected after 7 and 14 days in both PM concentrations (5.7 and 11.4 mg/L). Meanwhile, after the last 21 days, the activity proceeded to decrease.

The highest mean activity (32.27 ± 16.13 nmol/min/mg of total protein) was observed in fish exposed to 5.7 mg/L after 7 days of exposure, and the lowest activity (5.80 ± 3.85 nmol/min/mg of total protein) occurred after 21 days of exposure at the concentration of 5.7 mg/L.

Significant differences ($p < 0.05$) were detected between fish livers exposed to 5.7 mg/L and respective controls after 7 days of exposure. Further significant variations occurred between the livers of fish exposed to a PM concentration of 5.7 mg/L for 7 days and those for 14 and 21 days. Significant differences were also found between the fish livers collected after 7 days and those from 14 and 21 days.



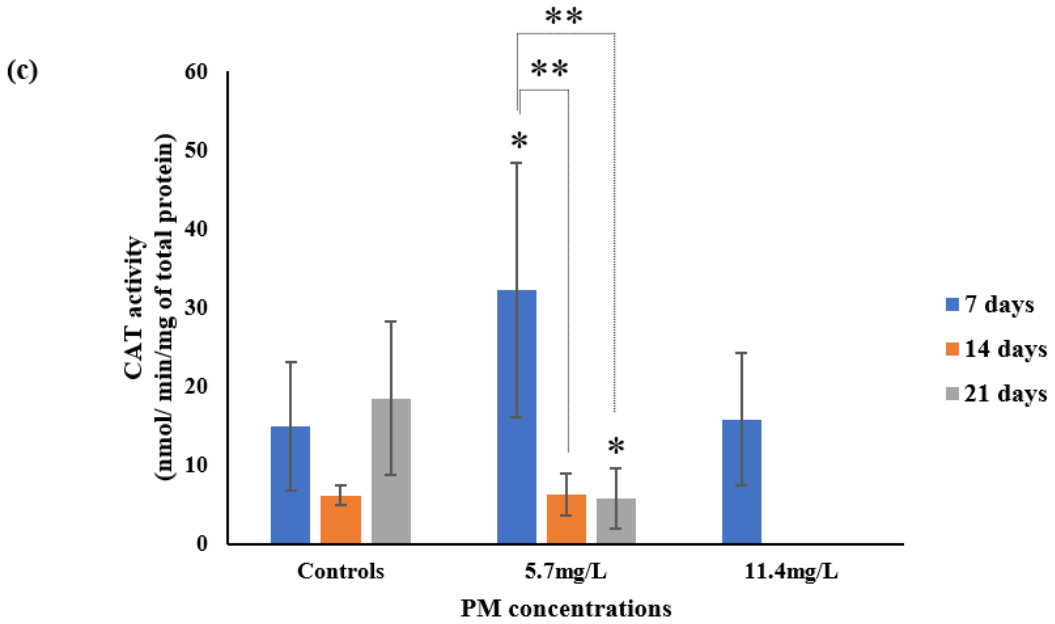


Figure 24 | CAT activity (mean ± SD) in the seabass' organs after 7, 14 and 21 days of exposure to PM; (**a)** muscle; (**b)** gills; (**c)** liver. Significant differences ($p < 0.05$) comparing to the control groups (*****). Significant differences ($p < 0.05$) between the exposure periods (******).

3.1.2.2 Superoxide Dismutase (SOD)

The SOD's activity was assessed in seabass organs (muscle, liver, and gills) following exposure to PM for 7, 14, and 21 days, as shown in **Figure 25**. The gills presented the highest values, while the lowest activities were observed in the livers.

Figure 25 a shows that when the exposure period and concentration increase, the SOD activity in the seabass muscle tends to decrease. That is, after 7 days, SOD activity increased in organisms exposed to the lowest PM dose of 5.7 mg/L compared to the control group. However, when the exposure period increased, the activity began to show decreasing levels. The same occurred in animals exposed to the maximum concentration (11.4 mg/L) after 7 days.

The highest average activity (134.53 ± 5.98 U/mg of total protein) was observed in the muscle of seabass exposed to 5.7 mg/L of PM, sampled after 7 days, and the lowest activity (2.51 ± 2.07 U/mg of total protein) was registered in seabass exposed to 5.7 mg/L after 14 days of exposure.

Statistical analyses revealed significant differences ($p < 0.05$) between seabass exposed to 5.7 mg/L of PM for 7 and 14 days and their respective controls. Additionally, significant differences ($p < 0.05$) were also seen in the group exposed to a PM concentration of 5.7 mg/L when comparing the organisms collected after 7 days of exposure and those from 14 and 21 days.

SOD activity exhibits the same general behaviour in gills (**Figure 25 b**) and liver (**Figure 25 c**), according to PM concentrations and exposure period.

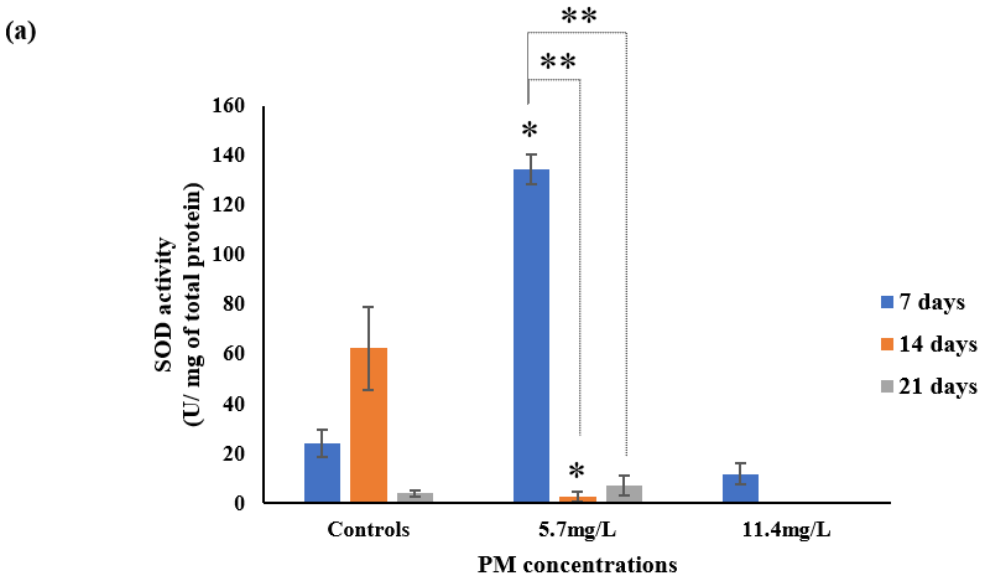
SOD values in the gills of organisms exposed to the lowest PM concentration (5.7 mg/L) increased until the 21st day and then decreased. However, organisms exposed to the highest PM concentration (11.4 mg/L) show reduced activity up to 7 days of exposure.

In seabass gills, the highest average activity (134.53 ± 5.98 U/mg of total protein) was determined in seabass exposed to 5.7 mg/L of PM after 7 days of exposure, and the lowest activity (2.68 ± 1.76 U/mg of total protein) was observed in seabass exposed to 11.4 mg/L of PM after 7 days of exposure.

Statistical analyses revealed significant differences ($p < 0.05$) between the seabass exposed to 5.7 mg/L of PM after 7 and 21 days and between seabass exposed to 11.4 mg/L after 7 days and their respective controls. Significant differences were also found between the seabass livers collected after 7 days of exposure and those from 14 and 21 days.

In seabass livers, the highest average activity (77.60 ± 59.02 U/mg of total protein) was found in seabass exposed to 5.7 mg/L of PM after 7 days of exposure. Moreover, the lowest activity (4.46 ± 1.96 U/mg of total protein) value was determined after 14 days of exposure to 5.7 mg/L.

Significant differences ($p < 0.05$) were found between seabass exposed to 5.7 mg/L of PM after 7 days and its respective controls. Significant differences were also found between the seabass livers collected after 7 days of exposure and those from 14 and 21 days.



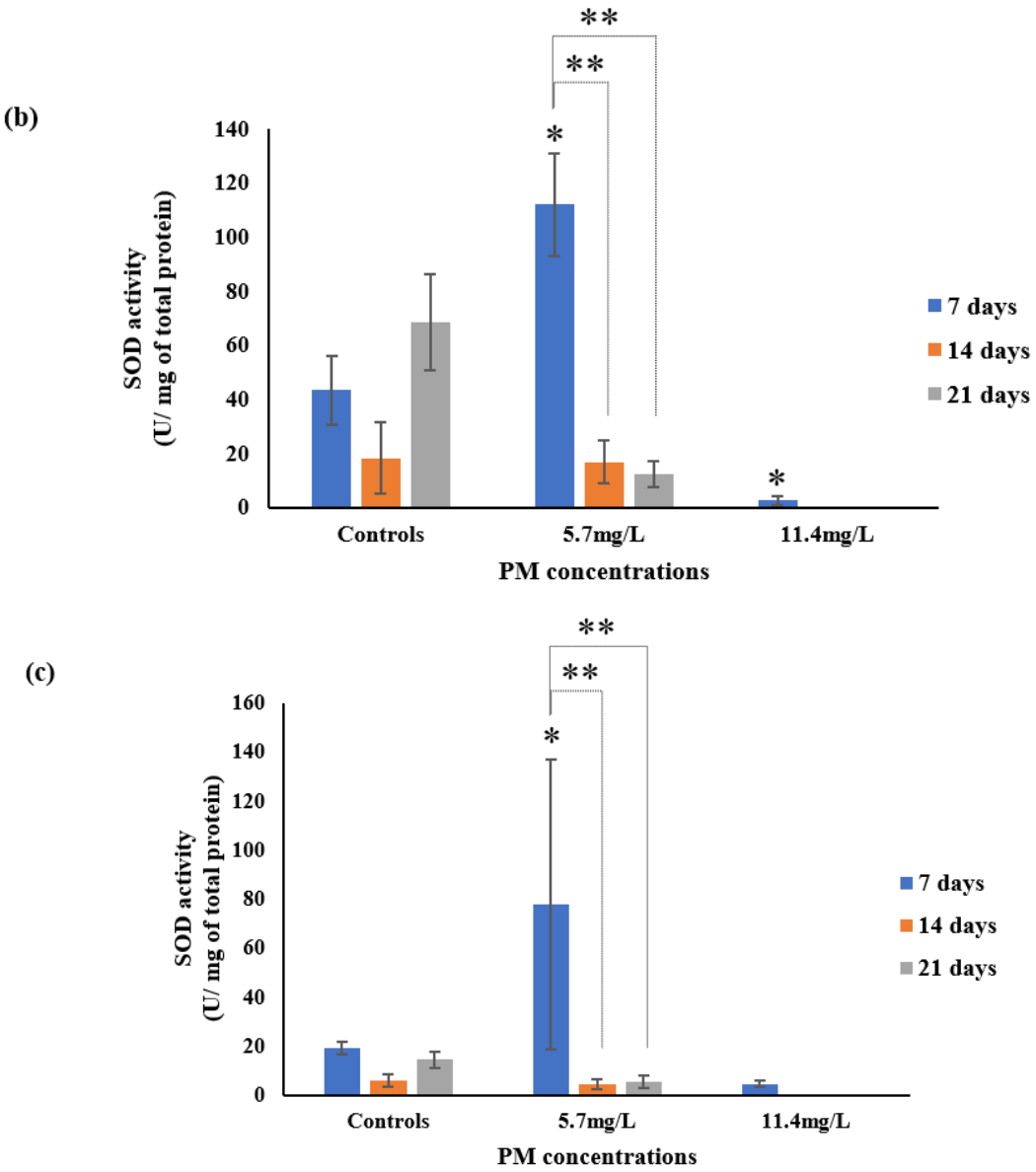


Figure 25 | SOD activity (mean ± SD) in the seabass' organs after 7, 14 and 21 days of exposure to PM; (**a)** muscle; (**b)** gills; (**c)** liver. Significant differences ($p < 0.05$) comparing to the control groups (*****). Significant differences ($p < 0.05$) between the exposure periods (******).

3.1.2.3 Glutathione Peroxidase (GPX)

GPX's activity measured in seabass organs (muscle, liver, and gills) exposed to the different concentrations of PM for 7, 14, and 21 days are presented in **Figure 26**. The livers presented the highest values, while the lowest activities were observed in the gills. Furthermore, a similar trend was observed in all three organs analysed. All GPX activities increased after the first 7 days of exposure in organisms exposed to the lowest PM concentration (5.7 mg/L) compared to their control group. However, organisms exposed to the greatest PM concentration (11.4 mg/L) exhibited lower activities. Finally, compared

to the control groups, there is an overall reduction in SOD activity after 14 and 21 days in animals exposed to the different PM concentrations.

In the seabass's muscle (**Figure 26 a**), the highest average activity ($2.97x10^{-2} \pm 8.24x10^{-3}$ nmol/min/mg of total protein) was observed in seabass exposed to 5.7 mg/L of PM after 7 days of exposure, and the lowest average activity ($4.14x10^{-4} \pm 5.28x10^{-5}$ nmol/min/mg of total protein) was determined in controls after 14 days of exposure.

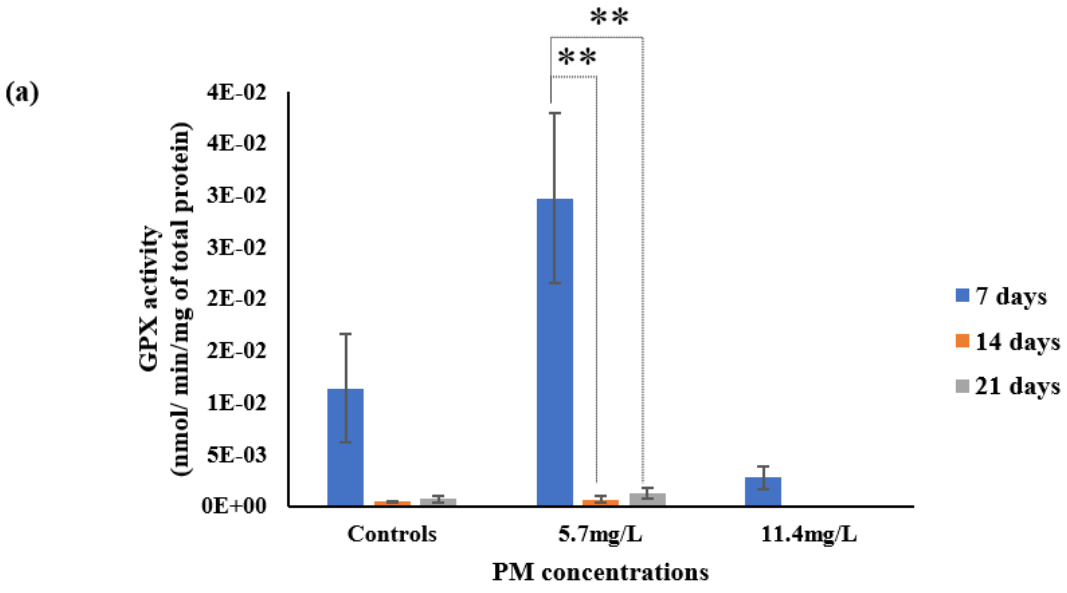
Statistical analyses revealed significant differences ($p < 0.05$) between the results obtained for seabass collected after 7 days of exposure and those from 14 and 21 days, respectively.

In seabass' gills (**Figure 26 b**), he highest average activity ($3.33x10^{-2} \pm 1.28x10^{-3}$ nmol/min/mg of total protein) was observed in seabass exposed to 5.7 mg/L of PM after 7 days of exposure, and the lowest average activity ($7.75x10^{-4} \pm 7.82x10^{-4}$ nmol/min/mg of total protein) was determined in seabass exposed to 5.7 mg/L of PM after 14 days of exposure.

Statistical analyses revealed significant differences ($p < 0.05$) between the results obtained for seabass after 7 days of exposure and those exposed after 14 and 21 days.

In seabass's livers (**Figure 26 c**), the highest average activity ($3.61x10^{-2} \pm 1.23x10^{-3}$ nmol/min/mg of total protein) was found in seabass exposed to 5.7 mg/L of PM after 7 days of exposure, and the lowest average activity ($9.03x10^{-4} \pm 3.89x10^{-4}$ nmol/min/mg of total protein) was determined after 21 days of exposure to 5.7 mg/L of PM.

Significant differences ($p < 0.05$) were detected between seabass exposed to 5.7 mg/L in each exposure period and their respective controls. Additionally, significant differences ($p < 0.05$) are also seen in the seabass collected after 7 days of exposure and those from 14 and 21 days, respectively.



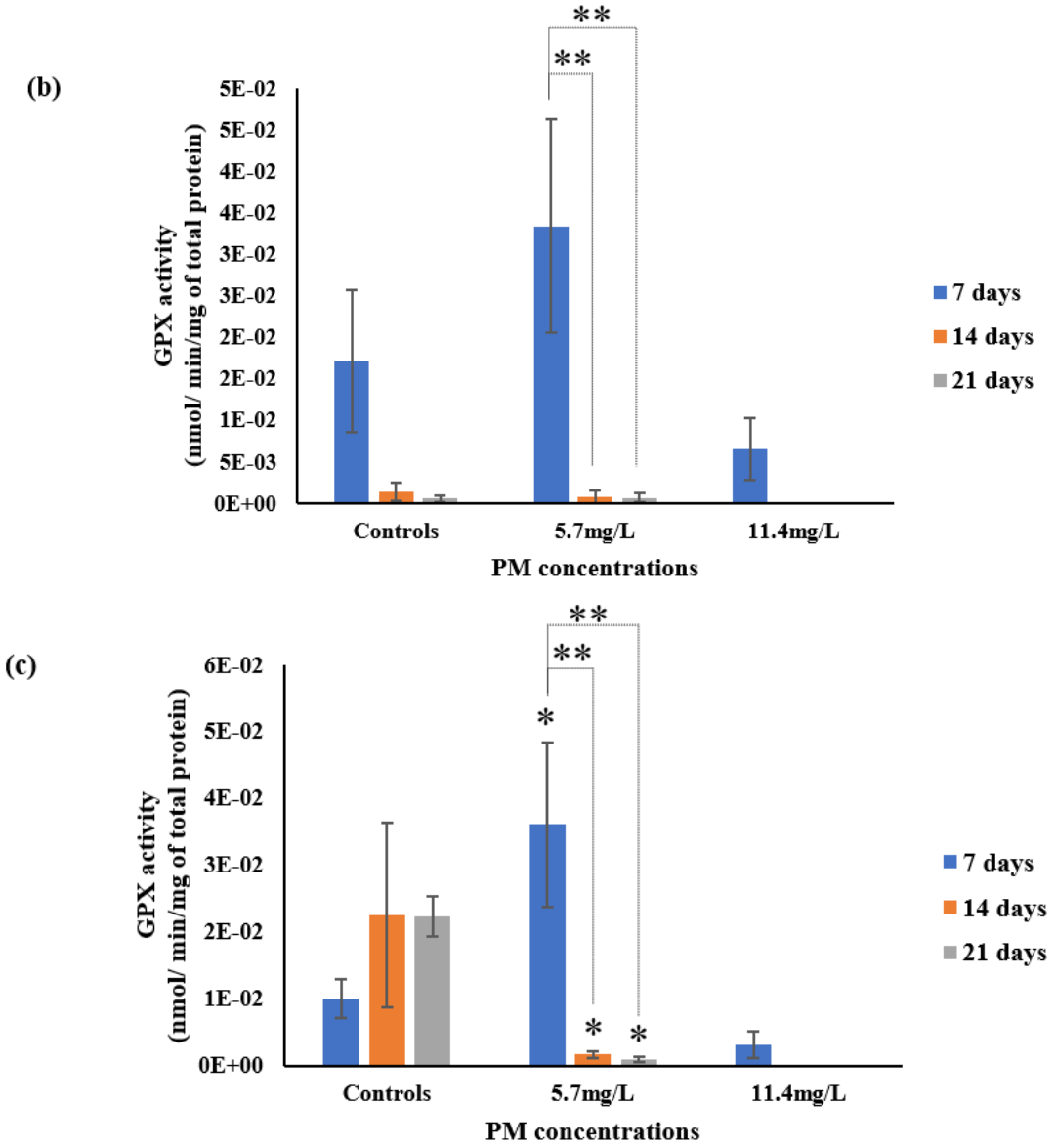


Figure 26 | GPX activity (mean ± SD) in the seabass' organs after 7, 14 and 21 days of exposure to PM; **(a)** muscle; **(b)** gills; **(c)** liver. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.1.2.4 Glutathione-S-Transferase (GST)

The GST results measured in seabass organs (muscle, liver, and gills) after exposure to PM for 7, 14, and 21 days are presented in **Figure 27**. The most prominent activity levels were observed in the gills and livers. Like previous biomarkers, the overall increase in GST's activity is consistent across all organs studied. Except for organisms collected on day 21 of treatment (exposed to a PM concentration of 5.7 mg/L), GST activity tends to increase.

In seabass' muscle (**Figure 27 a**), the highest average activity was determined in seabsss exposed to 5.7 mg/L of PM after 7 days of exposure (12.56 ± 4.04 nmol/min/mg of total protein), and the

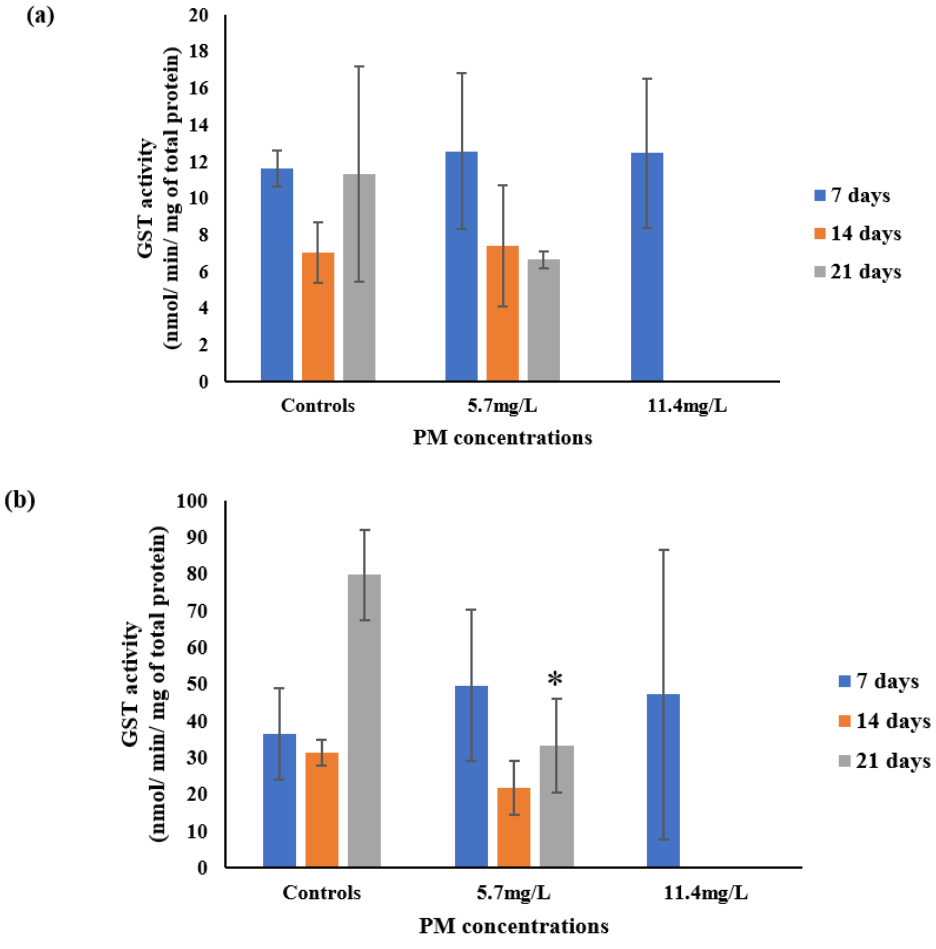
lowest average activity was observed in seabass exposed to 5.7 mg/L of PM after 21 days of exposure (6.66 ± 0.44 nmol/min/mg of total protein).

In seabass gills (**Figure 27 b**), the highest average activity (79.78 ± 12.26 mmol/ min/ mg of total protein) was determined in controls after 21 days of exposure. Moreover, the lowest average activity (21.82 ± 7.48 mmol/ min/ mg of total protein) was determined in seabass exposed to 5.7 mg/L after 14 days of exposure.

Statistical analyses revealed significant differences ($p < 0.05$) between the seabass exposed to 5.7 mg/L of PM collected after 21 days and its respective controls.

In seabass livers (**Figure 27 c**), the highest average activity (80.98 ± 19.04 mmol/ min/ mg of total protein) was observed in seabass exposed to 5.7 mg/L after 7 days of exposure, and the lowest average activity (41.45 ± 11.89 mmol/ min/ mg of total protein) was observed after 21days of exposure to 5.7 mg/L of PM.

Statistical analyses revealed significant differences ($p < 0.05$) between the seabass exposed to 5.7 mg/L of PM collected after 7 days and its respective controls.



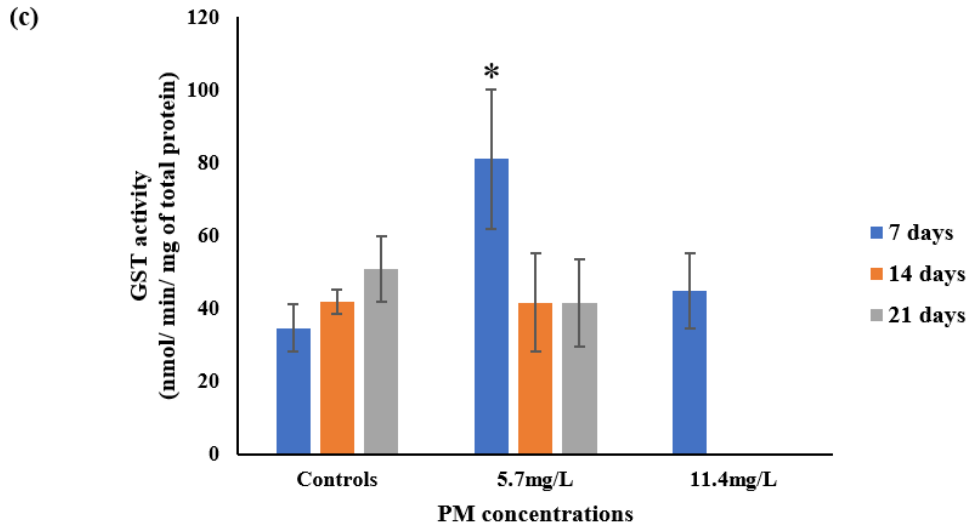


Figure 27 | GST activity (mean ± SD) in the seabass' organs after 7, 14 and 21 days of exposure to PM; **(a)** muscle; **(b)** gills; **(c)** liver. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.1.3 Cellular damage biomarkers

3.1.3.1 Lipid Peroxidation (LPO)

The lipid peroxidation results (expressed as MDA content) measured in the organs (muscle, liver, and gills) after exposure to PM for 7, 14, and 21 days are shown in **Figure 28**. Overall, the highest MDA values were detected in the gills, and the lowest values were determined in the muscle.

In the muscles (**Figure 28 a**), a rise in MDA concentrations can be observed in seabass exposed to the different concentrations, except for those exposed to 5.7 mg/L of PM after 21 days, which show a decrease.

The highest average concentration (63.94 ± 4.70 pmol/mg of total protein) was determined in the muscle of seabass exposed to 5.7 mg/L of PM after 7 days, and the lowest concentration (16.31 ± 10.87 pmol/mg of total protein) was determined in controls collected after 7 days of exposure.

Statistical analyses revealed significant differences ($p < 0.05$) between the individuals sampled after 7 days of exposure and their respective controls. Additionally, significant differences ($p < 0.05$) are also seen in the seabass exposed to a PM concentration of 5.7 mg/L collected after 7 days of exposure and those from 14 and 21 days.

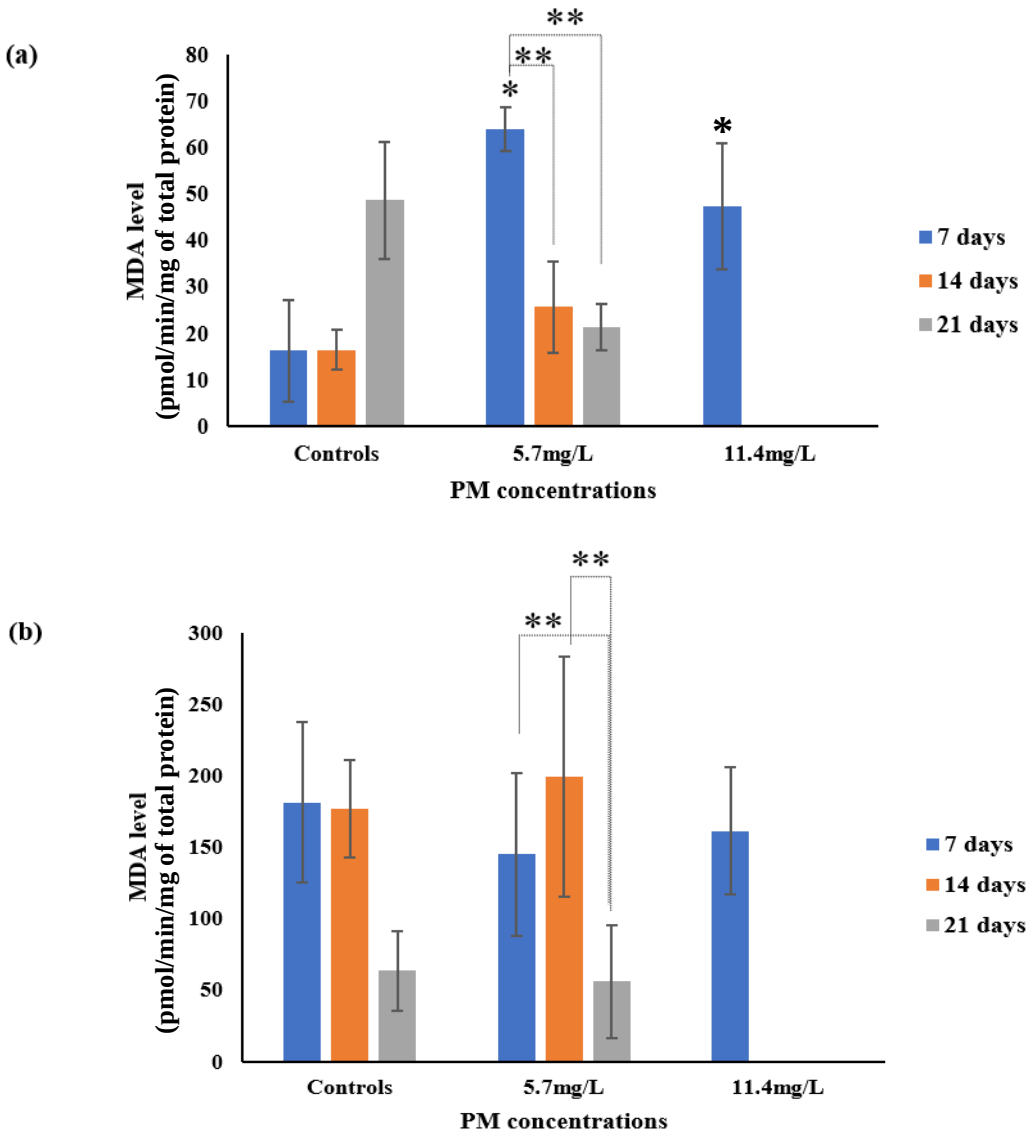
In the seabass gills (**Figure 28 b**), the highest average concentration (199.36 ± 83.65 pmol/mg of total protein) was observed in seabass exposed to 5.7 mg/L of PM after 14 days of exposure, and the

lowest value (56.08 ± 39.61 pmol/mg of total protein) was detected in seabass exposed to 5.7 mg/L of PM after 21 days.

Significant differences ($p < 0.05$) are seen between the seabass exposed to a PM concentration of 5.7 mg/L collected after 7 and 14 days of exposure and those from 21 days.

In seabass livers (**Figure 28 c**), the highest average activity (98.17 ± 26.92 pmol/mg of total protein) was determined in the control group collected after 7 days, and the lowest average activity (21.39 ± 6.99 pmol/mg of total protein) was detected in seabass exposed to 5.7 mg/L after 14 days.

Significant differences ($p < 0.05$) are observed between the organisms exposed to a PM concentration of 5.7 and 11.4 collected after 7 days compared to their respective control groups. Additionally, significant differences ($p < 0.05$) are seen between the seabass exposed to a PM concentration of 5.7 mg/L collected after 7 and 14 days of exposure and those from 21 days.



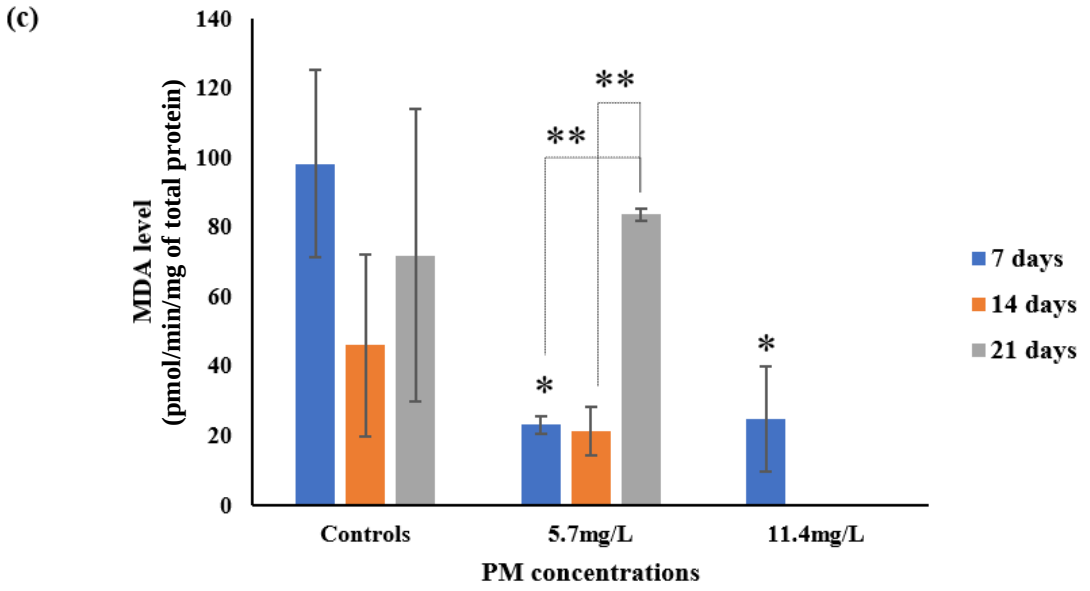


Figure 28 | MDA concentration (mean ± SD) in seabass' organs after 7, 14 and 21 days of exposure to PM; **(a)** muscle; **(b)** gills; **(c)** liver. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.1.3.2 Total ubiquitin levels (UBI)

The total ubiquitin results for the analysed organs are presented in **Figure 29**.

In seabass muscle (**Figure 29 a**), an increase in ubiquitin levels was observed in the muscle of exposed animals.

The highest average value was detected in seabass exposed to 5.7 mg/L of PM after 14 days of exposure (0.76 ± 0.44 µg/mg of total protein), and the lowest was determined in the controls, after 7 days of exposure (0.008 ± 0.005 µg/mg of total protein).

Significant ($p < 0.05$) differences were detected between seabass exposed to 5.7 mg/L and controls after 7 days of exposure. Additionally, considerable differences ($p < 0.05$) are observed in the seabass exposed to a PM concentration of 5.7 mg/L collected after 7 days of exposure and those from 14 days.

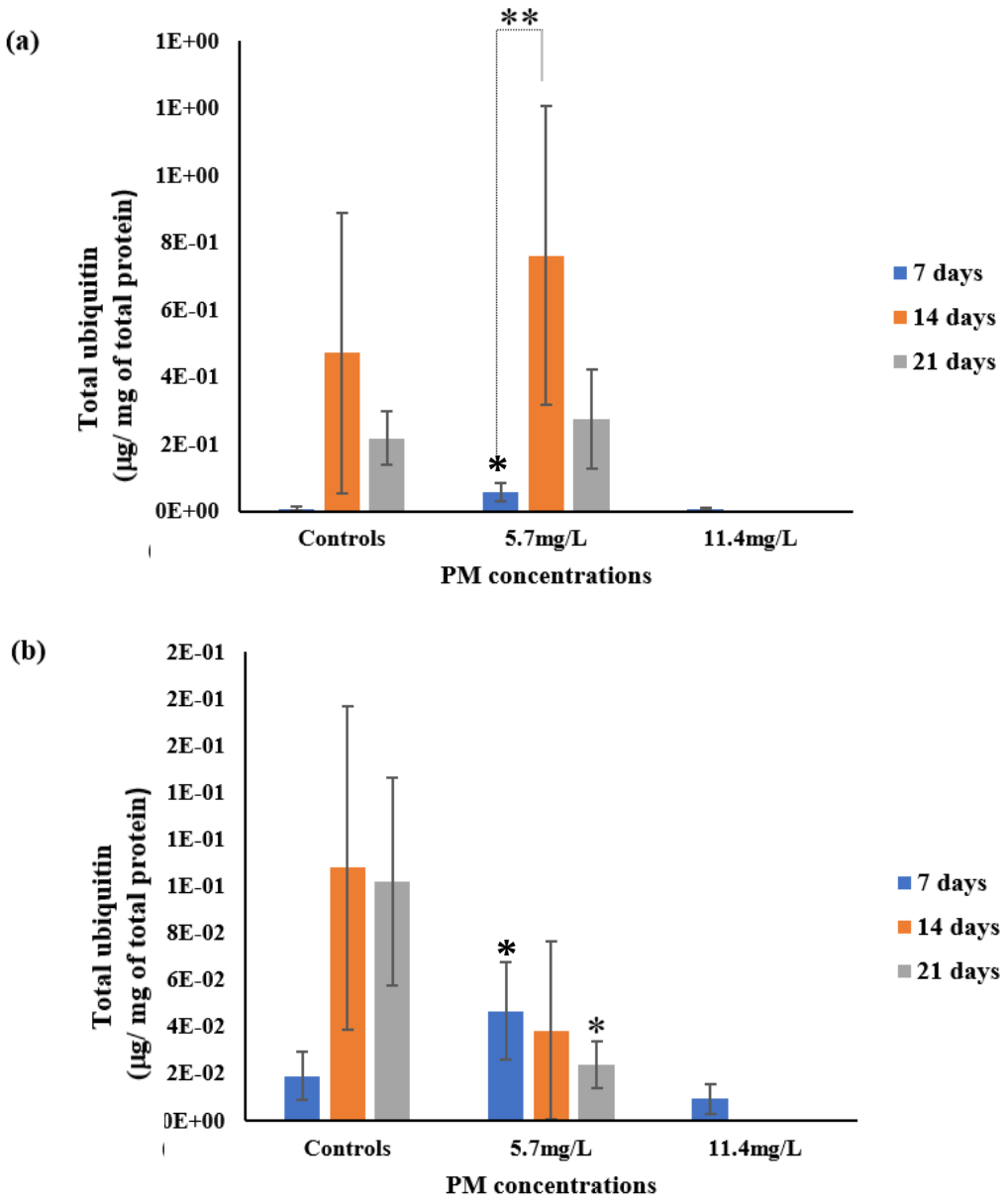
In the gills (**Figure 29 b**). a general decrease can be observed, except for those exposed to 5.7 mg/L after 7-day of exposure.

The highest average concentration was determined in seabass exposed to 5.7 mg/L of PM after 14 days of exposure (0.108 ± 0.069 µg/mg of total protein), and the lowest was observed in seabass exposed to 11.4 mg/L of PM after 7 days of exposure (0.009 ± 0.006 µg/mg of total protein).

Statistical analyses revealed significant differences ($p < 0.05$) between the gills of seabass exposed to 5.7 mg/L and respective controls after 7 and 21 days of exposure.

In seabass livers (**Figure 29 c**), the highest average value of total protein was determined in seabass exposed to 5.7 mg/L of PM after 21 days of exposure (1.56 ± 0.11 µg/mg of total protein), and the lowest was observed after 7 days of exposure to 11.4 mg/L of PM (0.007 ± 0.004 µg/mg of total protein).

Significant ($p < 0.05$) differences were detected between seabass exposed to 5.7 mg/L and controls after 7, 14 and 21 days of exposure.



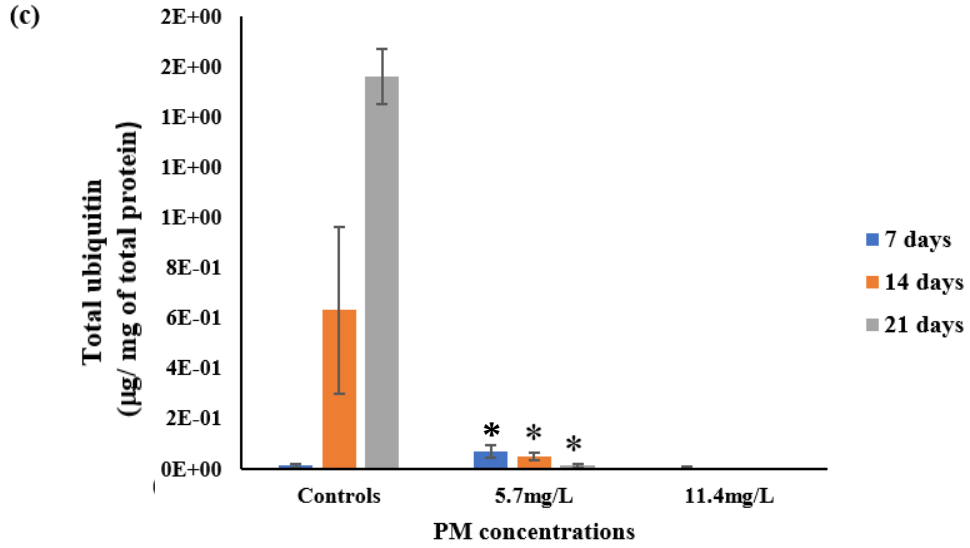


Figure 29 | Ubiquitin level (mean ± SD) in seabass' organs after 7, 14 and 21 days of exposure to PM; **(a)** muscle; **(b)** gills; **(c)** liver. Significant differences ($p < 0.05$) comparing to the control groups (*).

3.2 Seabass fed with *Polychaetes* previously exposed to Particulate Matter (PM)

3.2.1 Mortality rate

In this assay, no mortalities were observed.

3.2.2 Antioxidant Biomarkers

After seabass exposure via feeding with Polychaete sp. (previously exposed to a PM concentration of 11.4 mg/L), several biomarkers (*e.g.*, oxidative stress enzymes, lipid peroxidation, total ubiquitin) were evaluated in selected seabass organs (muscle, liver, gills, and intestines).

3.2.2.1 Catalase (CAT)

As shown in **Figure 30**, the maximum average activity (107.54 ± 40.66 nmol/min/mg of total protein) was recorded in the gills of control seabass, whereas the lowest average activity (1.58 ± 0.91 nmol/min/mg of total protein) was observed in seabass intestines.

There were no significant changes ($p > 0.05$) between exposed and control animals. However, CAT appears to increase in the muscles and livers of exposed seabass and decrease in the gills and intestines of exposed seabass.

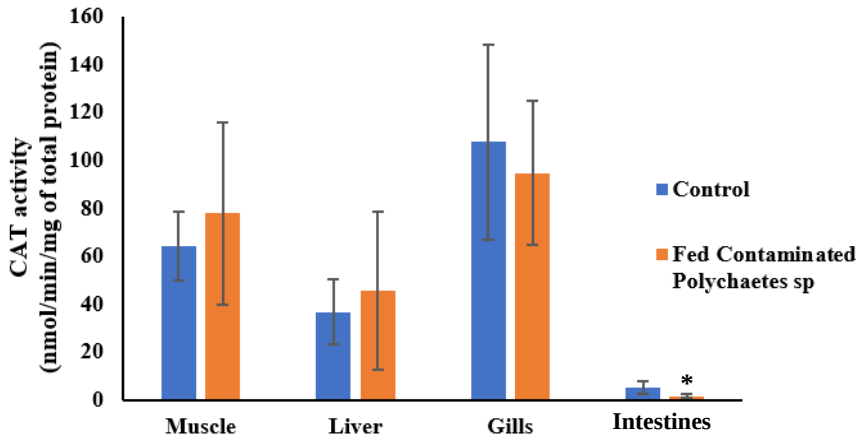


Figure 30 | CAT activity (mean ± SD) in seabass' organs (muscle, liver, gills, and intestines) after 4 days of feeding with contaminated food (*Polychaete sp.*).

3.2.2.2 Superoxide Dismutase (SOD)

Regarding SOD activity in seabass analysed organs (**Figure 31**), the highest averages value was detected in seabass intestines from the organisms fed contaminated *Polychaetes* (83.02 ± 63.36 U/mg of total protein), and the lowest value was determined in seabass livers from the organisms fed contaminated Polychaetes (4.90 ± 2.36 U/mg of total protein).

Statistical analyses revealed significant changes ($p < 0.05$) between the intestines of control seabass and those exposed to PM.

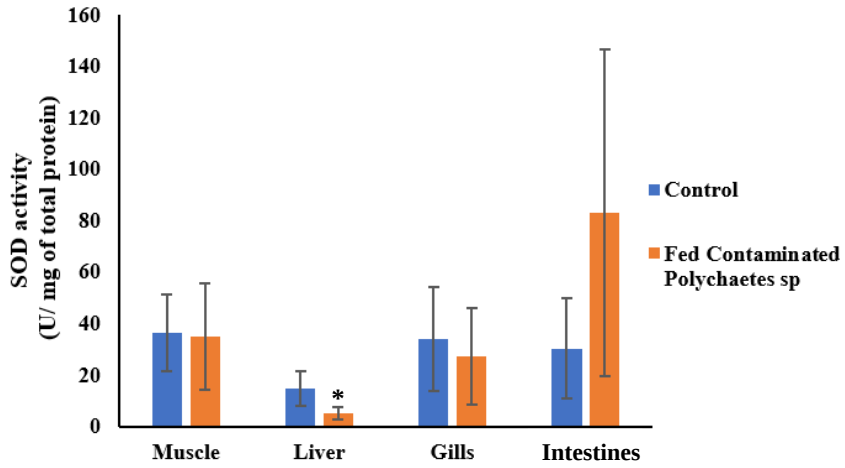


Figure 31 | SOD activity (mean ± SD) in seabass' organs (muscle, liver, gills, and intestines) after 4 days of feeding with contaminated food (*Polychaete sp.*).

3.2.2.3 Glutathione Peroxidase (GPX)

GPX's activity results are presented in **Figure 32**. The highest average activity was found in the seabass gills of the respective control (0.66 ± 0.36 nmol/min/mg of total protein), and the lowest was determined in the seabass livers of the respective control (0.06 ± 0.03 nmol/min/mg of total protein).

Statistical analyses revealed no significant changes ($p > 0.05$) between seabass feed with contaminated *Polychaete sp.* and their respective controls.

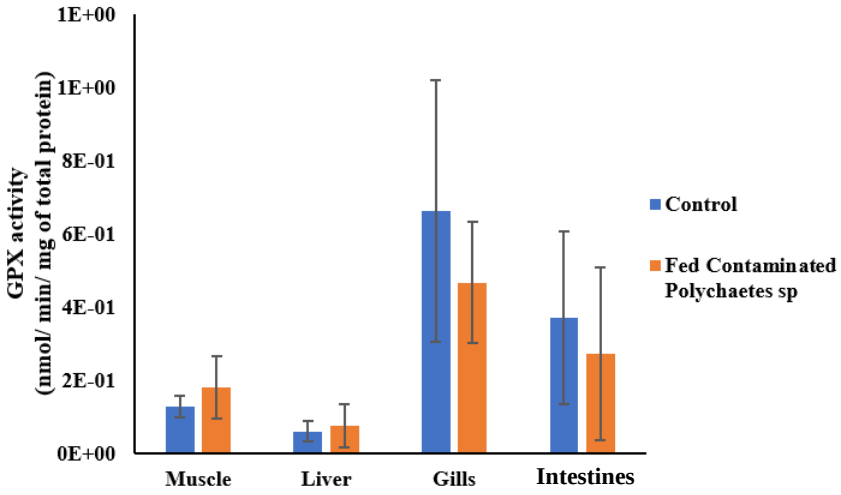


Figure 32 | GPX activity (mean ± SD) in seabass' organs (muscle, liver, gills, and intestines) after 4 days of feeding with contaminated food (*Polychaete sp.*).

3.2.2.4 Glutathione-S-Transferase (GST)

Regarding the GST results (**Figure 33**), the highest average activity (63.76 ± 22.71 nmol/min/mg of total protein) was observed in the gills of the control seabass, and the lowest was determined in the muscle of the control seabass (16.86 ± 3.40 nmol/min/mg of total protein).

Although no significant ($p < 0.05$) changes were detected, the overall GST activity in most organs is higher in exposed groups than in their controls, except for the gills.

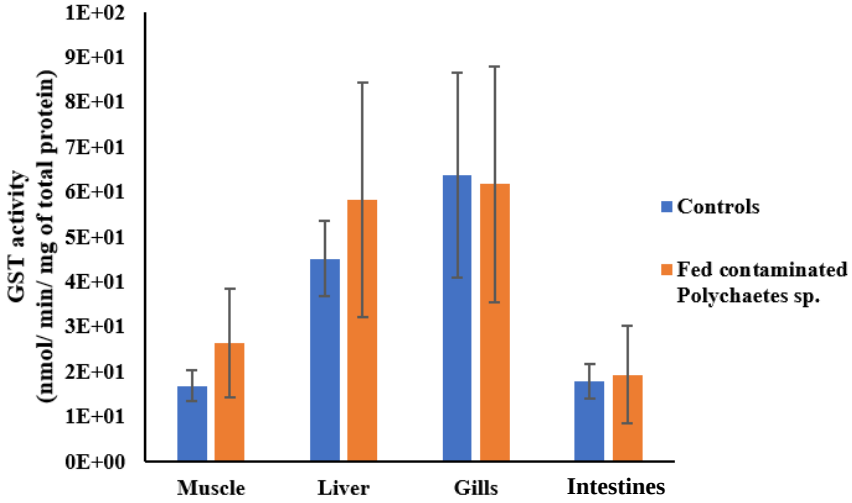


Figure 33 | GST activity (mean ± SD) in seabass' organs (muscle, liver, gills, and intestines) after 4 days of feeding with contaminated *Polychaete sp*.

3.2.3 Cellular damage biomarkers

3.2.3.1 Lipid Peroxidation (LPO)

Lipid Peroxidation (MDA concentration) results are presented in **Figure 34**. The highest average MDA concentration was found in the intestines of control seabass (98.76 ± 19.22 pmol/mg of total protein). In contrast, the lowest values were determined in the seabass gills of the control group (1.17 ± 0.86 pmol/mg of total protein).

Statistical analyses revealed significant ($p < 0.05$) differences between exposed seabass livers, gills and intestines and their respective controls.

The MDA concentrations in muscles, livers and gills show an increase in animals fed *Polychaete sp.* previously contaminated with PM. However, MDA decreases in contaminated organisms in the seabass intestines compared to its control group.

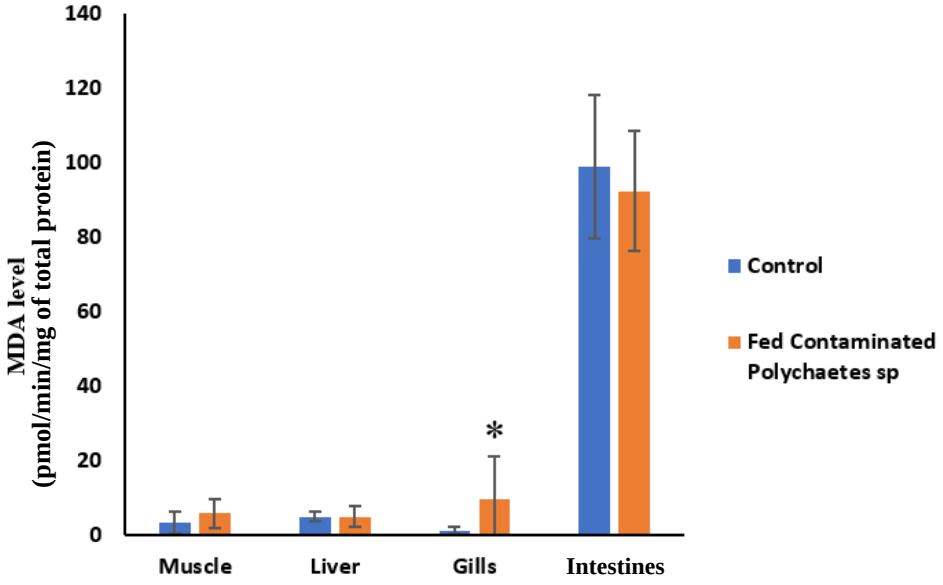


Figure 34 | MDA concentration (mean ± SD) in seabass' organs (muscle, liver, gills, and intestines) after 4 days of feeding with contaminated food (*Polychaete sp.*). Significant differences ($p < 0.05$) comparing to the control groups (*****).

3.2.3.2 Total ubiquitin levels (UBI)

The total Ubiquitin results are presented in **Figure 35**. The highest average Ubiquitin values (0.11 ± 0.0 7 µg/mg of total protein) were observed in intestines of the controls, and the lowest average levels (0.002 ± 4.5 $x10^{-04}$ µg/mg of total protein) were determined in livers of control seabass.

Regarding statistics, the only significant differences ($p < 0.05$) were found in the liver and intestines when compared to their respective controls.

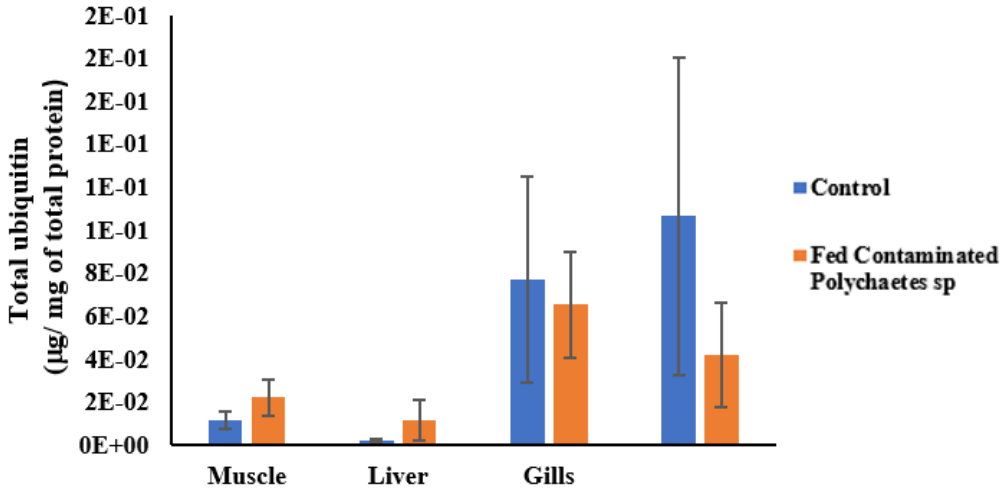


Figure 35 | Ubiquitin level (mean ± SD) in seabass' organs (muscle, liver, gills, and intestines) after 4 days of feeding with contaminated food (*Polychaete sp.*).

3.3 Mussels exposed to suspended Particulate Matter (PM)

3.3.1 Mortality rate

During this assay, no mortality was recorded. Further, mussel parasites (*e.g.*, small crabs, *Arcotheres atrinae*) were found in animals after 21 days of exposure, when dissecting the animals [126].

3.3.2 Antioxidant Biomarkers

After exposure to two different PM concentrations (5.7 and 11.4 mg/L), several biomarkers (*e.g.*, oxidative stress enzymes, lipid peroxidation, total ubiquitin) were assessed in selected mussel's organs (gills, and digestive glands).

3.3.2.1 Catalase (CAT)

The CAT assay's results assessed are presented in **Figure 36**.

In the gills (**Figure 36 a**), the overall outcome was increased in CAT activity.

The highest average activity was determined in the control group collected after 7 days (0.08 ± 0.04 nmol/min/mg of total protein). In contrast, the lowest average value (0.03 ± 0.01 nmol/min/mg of total protein) was observed in the PM concentrations of 5.7 mg/L in the organisms collected after 14 days. Regarding statistics, no significant differences ($p > 0.05$) were found between the organs of exposed seabass and their respective controls.

CAT activity in the digestive gland (**Figure 36 b**) increased up to 14 days of exposure and decreased at 21 days.

The highest average activity levels (0.10 ± 0.03 nmol/min/mg of total protein) were determined in mussels exposed to a PM concentration of 5.7 mg/L for 14 days. In contrast, (0.04 ± 0.04 nmol/min/mg of total protein) the lowest values were observed in the control group sampled after 7 days of exposure. Regarding statistics, no significant differences ($p > 0.05$) were found between the organs of exposed seabass and their respective controls.

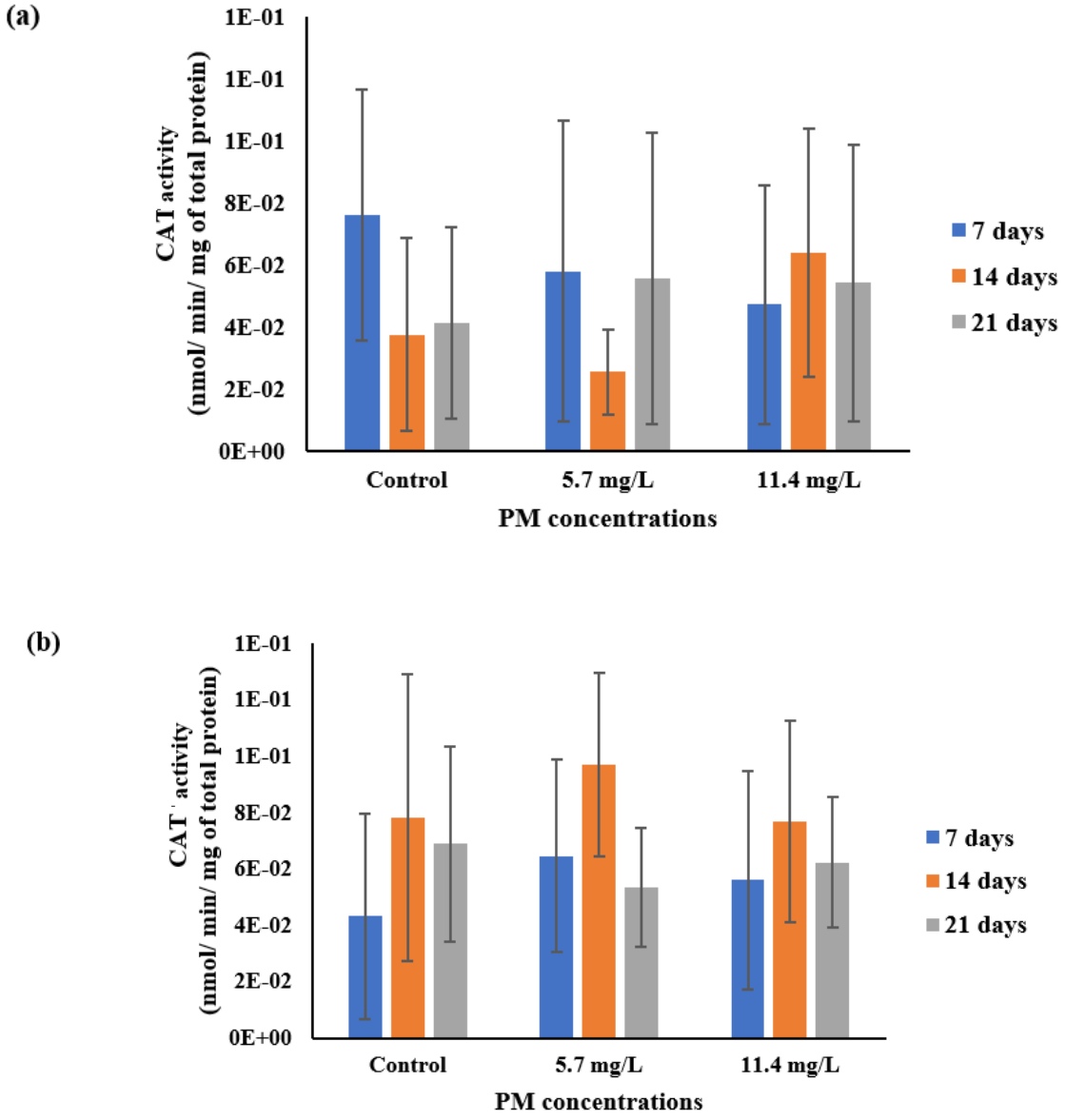


Figure 36 | CAT activity (mean ± SD) in mussel's organs after 7, 14 and 21 days of exposure to PM. **(a)** Gills; **(b)** Digestive Glands.

3.3.2.2 Superoxide Dismutase (SOD)

The results of SOD activity are shown in **Figure 37**. The highest average activity levels (13.58 ± 2.99 U/mg of total protein) were observed in the gills of mussels exposed to 11.4 mg/L of PM for 21 days. On the other hand, the lowest average value (3.74 ± 1.32 U/mg of total protein) was observed in the digestive gland of individuals exposed to 11.4 mg/L of PM for 7 days.

No statistical differences ($p > 0.05$) were found among controls in the gills. Nonetheless, a general trend towards increasing SOD activity was found in both the gills (**Figure 37 a**) and the digestive glands (**Figure 37 b**). However, statistical analysis of digestive glands revealed a significant increase ($p < 0.05$) between animals collected after 7 days and those exposed for 21 days in the PM concentrations of 11.4 mg/L.

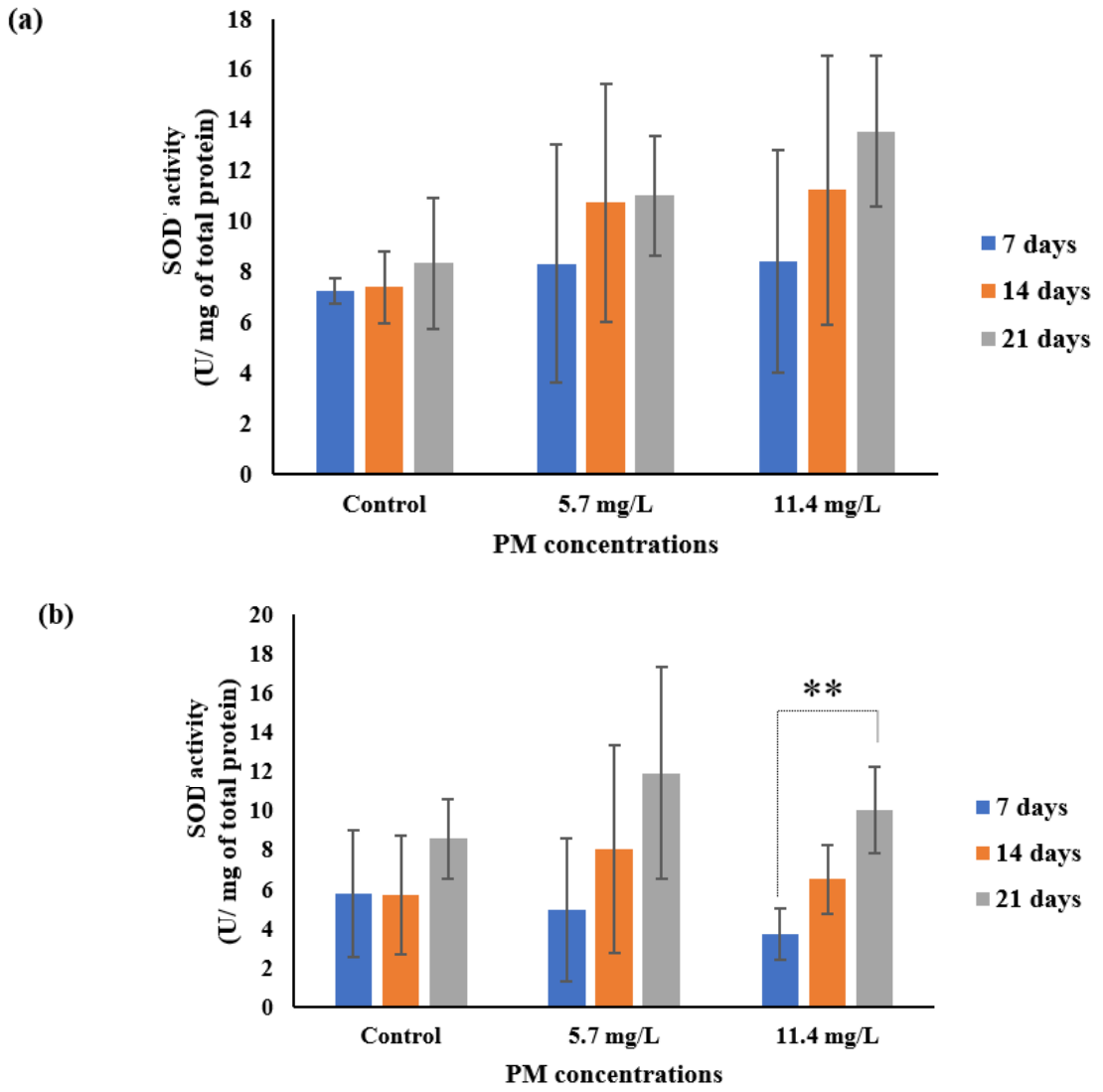


Figure 37 | SOD activity (mean ± SD) in mussel's organs after 7, 14 and 21 days of exposure to PM. **(a)** Gills. **(b)** Digestive Glands. Significant differences ($p < 0.05$) between the exposure periods (**).

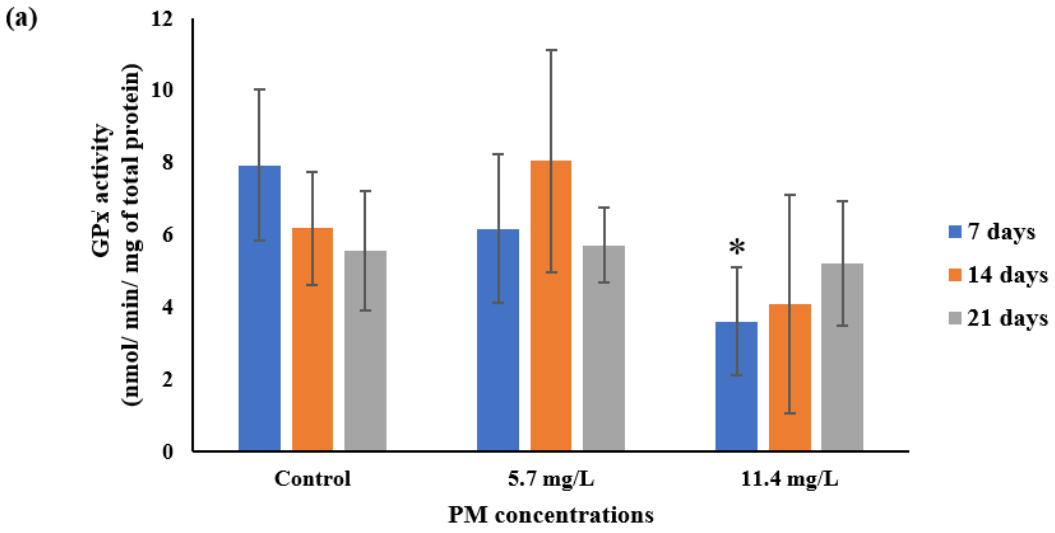
3.3.2.3 Glutathione Peroxidase (GPX)

The GPX results are shown in **Figure 38**. The highest average activity levels (8.04 ± 3.09 nmol/min/mg of total protein) were found in the gills of mussels exposed to 5.7 mg/L of PM for 14 days. On the other hand, the lowest average activities (3.49 ± 2.44 nmol/min/mg of total protein) were observed in the digestive gland of individuals exposed to 5.7 mg/L of PM after 7 days.

Significant GPX activities ($p < 0.05$) were observed in the gills of animals exposed to 11.4 mg/L after 7 days compared to their respective controls.

Regarding the gills (**Figure 38 a**), a slight increase is evident in mussels exposed to 5.7 mg/L after PM after 14 and 21 days compared to their respective controls. A decrease in activity appears to occur at the highest concentration (11.4 mg/L) throughout the exposure periods compared to their respective controls.

On the digestive glands (**Figure 38 b**), an increase in GPX's average activity (5.7 mg/L and 11.4 mg/L) was observed in animals exposed to both PM-tested concentrations according to the exposure time. The individuals exposed to a concentration of 5.7 mg/L showed lower activity values after 7 and 14 days, but activity increased after 21 days compared to respective controls. Additionally, in mussels exposed to 11.4 mg/L of PM, lower levels are also observed after 7 days of exposure. However, after 14 days, an increase seems to occur compared to the respective controls.



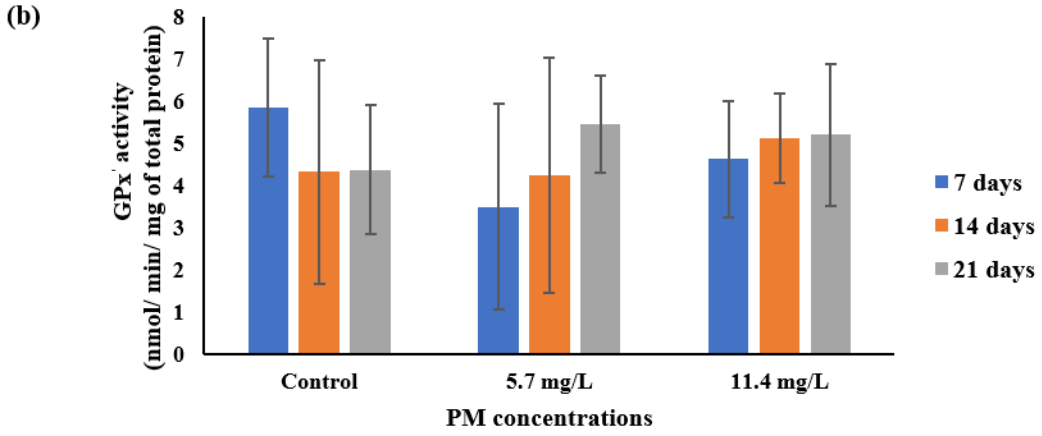


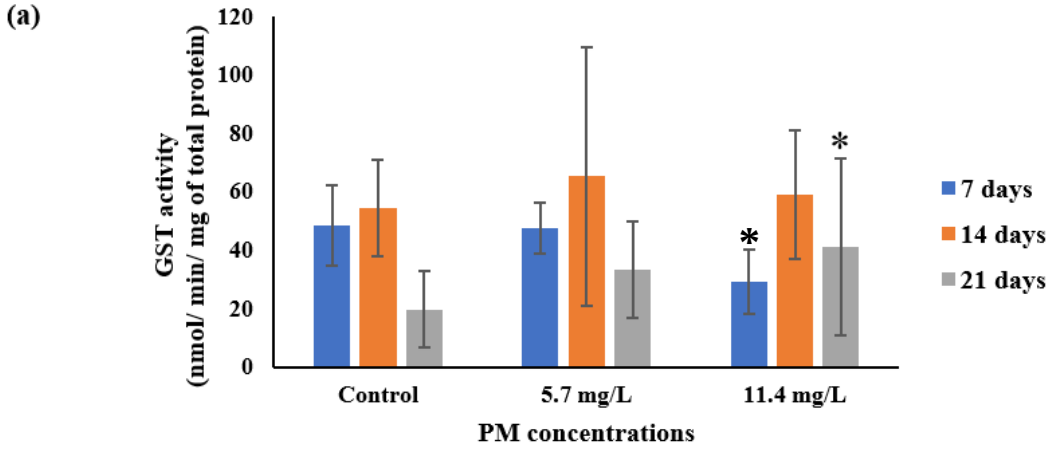
Figure 38 | GPX activity (mean ± SD) in mussel's organs after 7, 14 and 21 days of exposure to PM. **(a)** Gills. **(b)** Digestive Glands. Significant differences ($p < 0.05$) comparing to the control groups (*****).

3.3.2.4 Glutathione-S-Transferase (GST)

GST activity results are shown in **Figure 39**. The highest GST average activity levels were found in mussel's gills, and among them, the mussels exposed to 11.4 mg/L of PM for 21 days had the highest average activities (87.65 ± 30.23 nmol/min/mg of total protein). On the other hand, the lowest activities (29.21 ± 10.87 nmol/min/mg of total protein) were observed in the digestive glands of mussels exposed to 11.4mg/L of PM for 7 days.

Statistical analysis of the gills' results (**Figure 39 a**) found significant differences ($p < 0.05$) in the mussels exposed to a PM concentration of 11.4 mg/L collected after 7 and 21 days when compared to their respective controls.

Concerning the GST activity in the digestive gland (**Image 39 b**), significant differences ($p < 0.05$) were found in the mussels exposed to a PM concentration of 11.4 mg/L collected after 7 and 21 days. Additionally, the same was verified on the concentration of 5.7 mg/L collected after 21 says when compared to their respective controls ($p < 0.05$). Similarly, the same was observed between animals exposed to 5.7 mg/L of PM after 7 and 21 days of exposure.



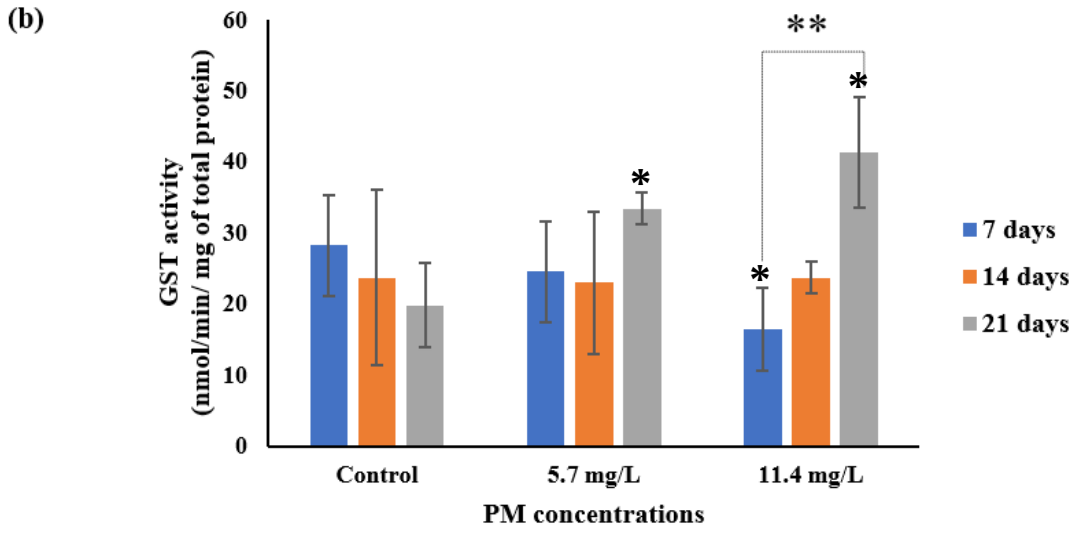


Figure 39 | GST activity (mean ± SD) in mussel's organs after 7, 14 and 21 days of exposure to PM. **(a)** Gills. **(b)** Digestive Glands. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.3.3 Cellular damage biomarkers

3.3.3.1 Lipid Peroxidation (LPO)

The Lipid Peroxidation was evaluated by calculating the MDA concentration in mussels' organs (gills and digestive gland), as shown in **Figure 40**. The gills (**Figure 40 a**) and digestive glands (**Figure 40 b**) presented higher MDA concentrations according to increasing PM concentrations and exposure time.

The highest average MDA concentrations were found in the gills of animals exposed to the highest concentration (11.4 mg/L) after 7 days of exposure (111.65 ± 8.15 pmol/mg of total protein). The lowest values were observed in mussels exposed to 5.7 mg/ L after 21 days (41.21 ± 13.10 pmol/mg of total protein).

Significant differences ($p < 0.05$) were found between the exposed organisms (PM concentration of 11.4 mg/L) collected after 21 days and their respective controls. Additionally, the groups (exposed to a PM concentration of 5.7 mg/L) collected after 7 days and those from 21 days in the mussel gills.

The highest average MD concentration in the digestive glands was found in the control group collected after 7 days (109.05 ± 11.04 pmol/mg of total protein). In contrast, the lowest values were observed in the controls sampled after 21 days (54.54 ± 29.27 pmol/mg of total protein). Even though no statistically significant differences were found concerning the digestive glands, the results still

suggest a general increase in MDA concentration, especially over extended exposure periods (14 days and 21 days), compared to their respective controls.

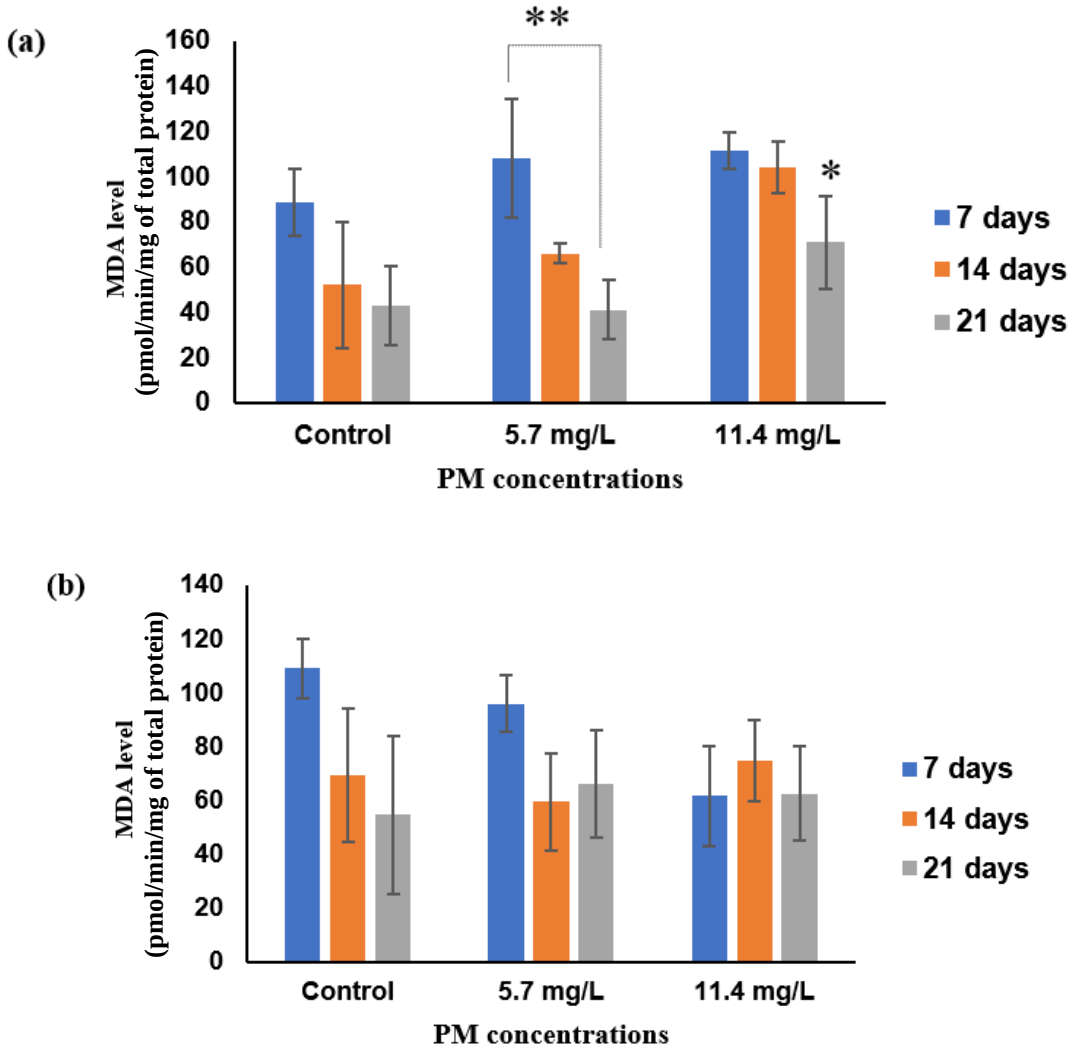


Figure 40 | MDA concentration (mean ± SD) in mussel's organs after 7, 14 and 21 days of exposure to PM; **(a)** Gills; **(b)** Digestive glands. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.3.3.2 Total ubiquitin levels (UBI)

The total Ubiquitin measured in the gills and digestive gland is presented in **Figure 41**. The highest average levels of total ubiquitin were observed in the gills exposed to a PM concentration of 11.4 mg/L after 21 days (0.87 ± 0.27 µg/mg of total protein). On the other hand, the lowest levels were observed in the digestive glands exposed to a PM concentration of 11.4 mg/L for 7 days (0.27 ± 0.06 µg/mg of total protein). Generally, a slight increase in total Ubiquitin concentration is observed mainly at the highest exposure concentration (11.4 mg/L) in mussel's gills (**Figures 41 a**) and digestive glands (**Figures 41 b**).

Statistical analysis indicated significant differences ($p < 0.05$), in the gills, between mussels exposed to a PM concentration of 11.4 mg/L compared to its controls after 21 days of exposure. On the other hand, in the digestive glands, no significant differences ($p > 0.05$) were found.

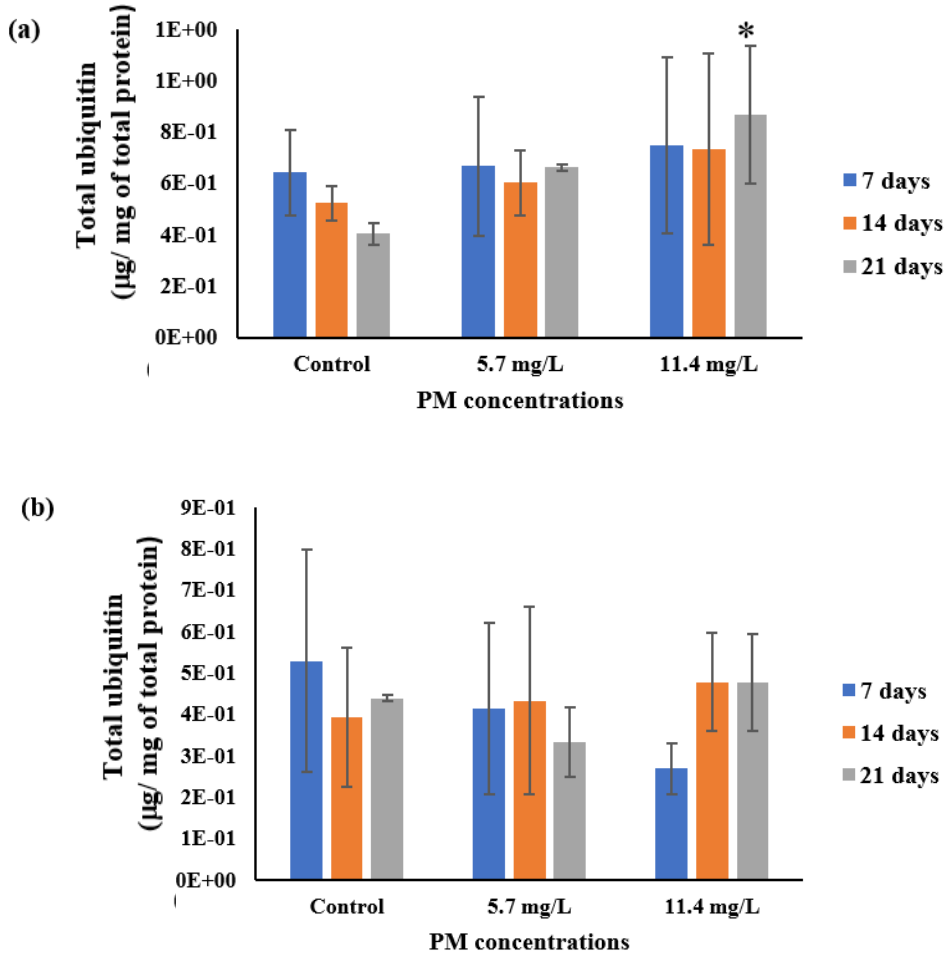


Figure 41 | Ubiquitin level (mean ± SD) in mussel's organs after 7, 14 and 21 days of exposure to PM. **(a)** Gills. **(b)** Digestive glands. Significant differences ($p < 0.05$) comparing to the control groups (*****).

3.4 Shrimps exposed to suspended Particulate Matter (PM)

3.4.1 Mortality rate

The cumulative mortality was recorded during the exposure assays and is shown in **Figure 42**. There were 11 deaths (64%), 2 in controls and animals exposed to 5.7 mg/L of PM, and 7 deaths (18%) were recorded in animals exposed to 11,4 mg/L of PM.

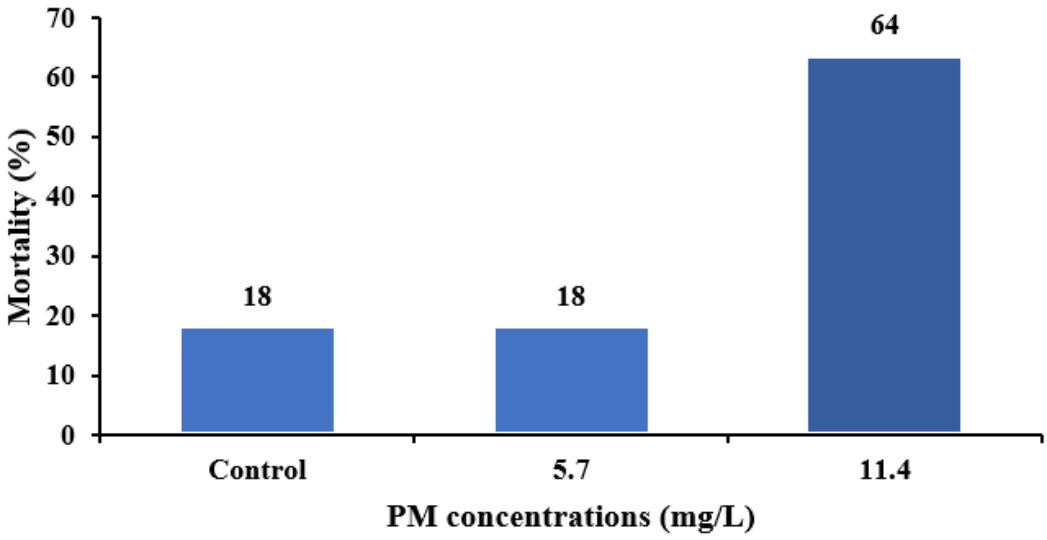


Figure 42 | *Palaemon varians* mortality rate (% of the total number of individuals deceased) observed during the exposure assay to PM.

3.4.2 Antioxidant Biomarkers

The biomarkers' activities were assessed in selected shrimp organs (muscle and viscera) following exposure to different PM concentrations (5.7 and 11.4 mg/ mL) for 7, 14, and 21 days. The results show lower values in the organisms kept under controlled conditions than those from the control groups collected after 7, 14, and 21 days, as shown in **Table 2** in the **Appendix A**.

3.4.2.1 Catalase (CAT)

CAT activities determined in both organs (muscle and viscera) of the exposed shrimps are shown in **Figure 43**.

In muscle (**Figure 43 a**), results show a decrease in CAT activities following 7 days of exposure, which is most prominent in animals exposed to 5.7 mg/L of PM. The opposite occurs in the organisms collected after 14 and 21 days (an increase in CAT activity is observed).

The highest average value (0.506 ± 0.407 nmol/min/mg of total protein) occurred in animals exposed to 5.7 mg/L of PM after 14 days of exposure, and the lowest (0.045 ± 0.011 nmol/min/mg of total protein) was determined in controls after 21 days of exposure.

No significant differences ($p > 0.05$) were detected between each exposed group and their respective controls throughout all the exposure times (7, 14 and 21 days). However, statistically, significant differences are observed when comparing the organisms collected after 7 and 14 and 21 days, respectively, in the organisms exposed to a PM concentration of 11.4 mg/L.

Regarding the viscera (**Figure 43 b**), an increase in CAT activity is observed after 7 and 14 days of exposure. However, the opposite is observed in animals collected after 21 days of exposure.

The highest average activities (1.664 ± 0.733 nmol/min/mg of total protein) occurred in animals exposed to 11.4 mg/L of PM after 7 days of exposure, and the lowest activities (0.333 ± 0.083 nmol/min/mg of total protein) were found in animals exposed to 5.7 mg/L of PM after 21 days of exposure.

Additionally, statistical analyses revealed no significant differences ($p > 0.05$) when comparing exposed animals to their respective controls.

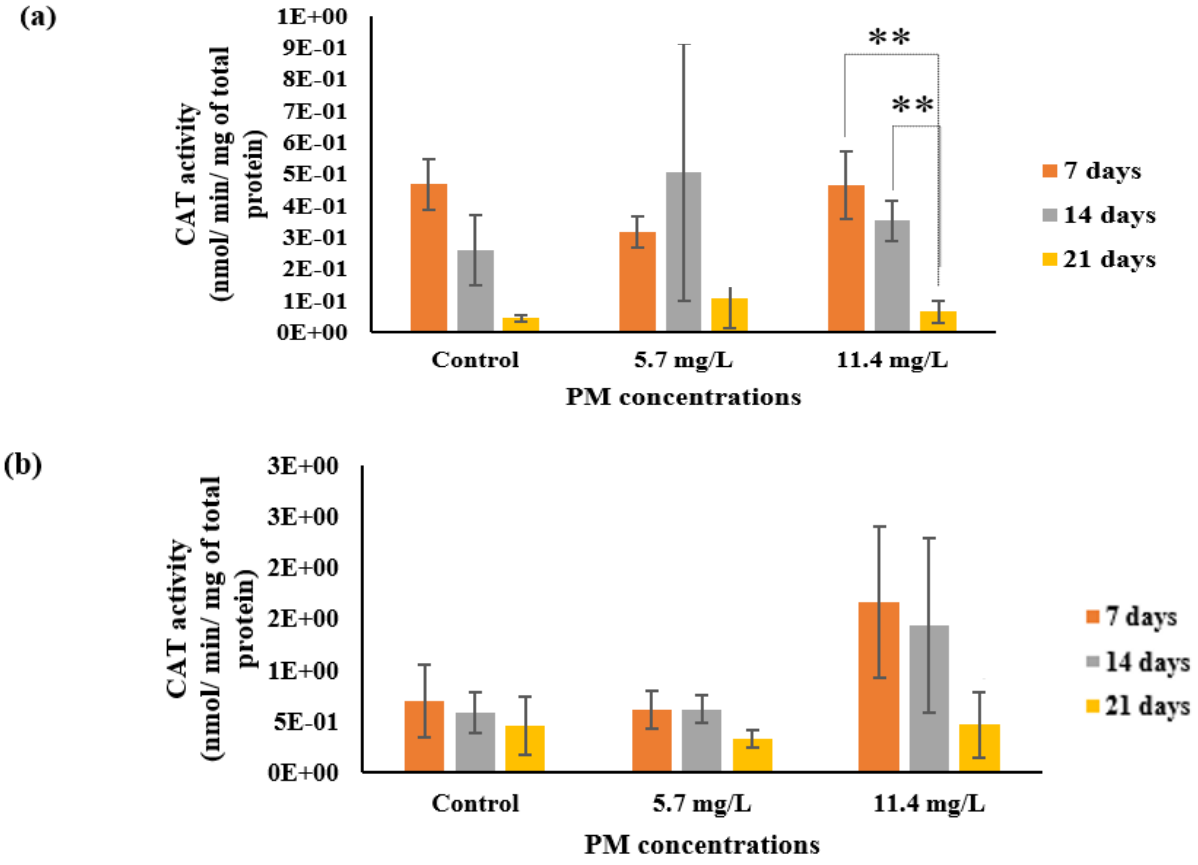


Figure 43 | CAT activity (mean ± SD) in shrimp organs after 7, 14 and 21 days of exposure to PM. **(a)** Muscle. **(b)** viscera. Significant differences ($p < 0.05$) between the exposure periods (**).

3.4.2.2 Superoxide Dismutase (SOD)

SOD results determined in shrimp exposed to the different concentration of PM are shown in **Figure 44**.

The SOD activities measured in the shrimp muscle (**Figure 44 a**) remained constant during 7 and 14 days when comparing control animals with those exposed to different PM concentrations. However, the shrimp sampled after 21 days had a noticeable decrease when comparing both exposed groups to their respective controls.

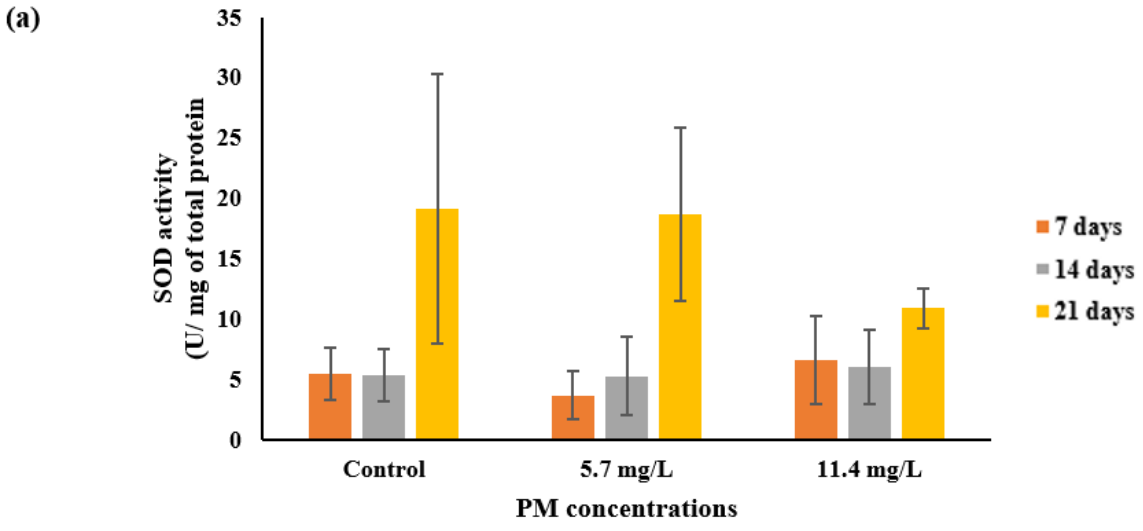
The highest average activity (19.16 ± 11.19 U/mg of total protein) was determined in control animals, and the lowest (3.710 ± 1.981 U/mg of total protein) was observed in animals exposed to 5.7 mg/L of PM after 7 days.

No significant differences ($p > 0.05$) were detected between exposed animals and their respective controls.

Overall, the activity in the viscera remained quite similar (**Figure 44 b**). The most noticeable differences are evident in organisms exposed to a PM concentration of 5.7 mg/L sampled after 21 days.

Shrimps exposed to 5.7 mg/L of PM for 21 days showed the highest SOD activities (67.18 ± 33.46 U/mg of total protein). At the same time, the lowest activities (11.44 ± 3.89 U/mg of total protein) were determined in animals exposed to 5.7 mg/L of PM after 7 days.

No significant differences ($p > 0.05$) were detected between exposed shrimp and their respective controls.



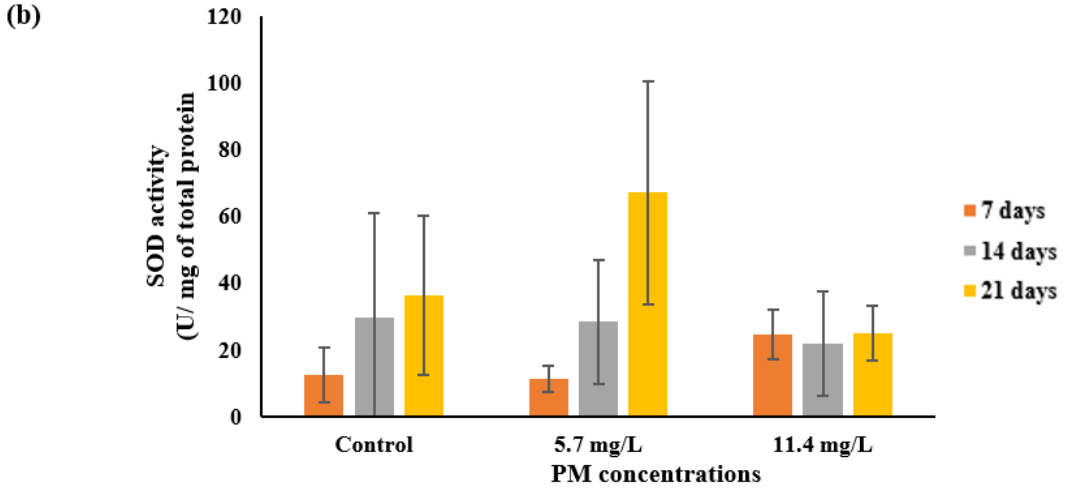


Figure 44 | SOD activity (mean ± SD) in shrimp organs after 7, 14 and 21 days of exposure to PM. **(a)** Muscle. **(b)** viscera. Significant differences ($p < 0.05$) between the exposure periods (**).

3.4.2.3 Glutathione Peroxidase (GPX)

GPX activity results are presented in **Figure 45**.

In shrimp muscle (**Figure 45 a**), the highest average values ($4.05x10^{-3} \pm 4.00x10^{-3}$ nmol/min/mg of total protein) were determined in controls after 14 days of exposure, and the lowest average activity values ($2.65x10^{-4} \pm 9.97x10^{-5}$ nmol/min/mg of total protein) were determined in animals exposed to 11.4 mg/L of PM after 14 days.

A general decrease, mainly after the 14th day of exposure was observed.

Significant differences ($p < 0.05$) are seen in the shrimp exposed to a PM concentration of 5.7 mg/L collected after 7 days of exposure and those from 14 and 21 days.

Regarding the viscera (**Figure 45 b**), except for the exposed groups sampled during the first 7 days of exposure, a general increase in GPX activity is evident compared to the control group.

Here the highest average values ($2.85x10^{-3} \pm 1.44x10^{-3}$ nmol/min/mg of total protein) were observed in the controls after 7 days of exposure, and the lowest activity value ($2.57x10^{-4} \pm 9.19x10^{-5}$ nmol/min/mg of total protein) was determined in the control group after 14 days of exposure.

The statistically significant differences ($p < 0.05$) found are between animals exposed to the highest contaminant concentration (11.4 mg/L) and their respective controls on the 14th day of exposure. Additionally, statistically significant differences are seen in the shrimp exposed to a PM concentration of 5.7 mg/L collected after 7 days of exposure and those from 14 days.

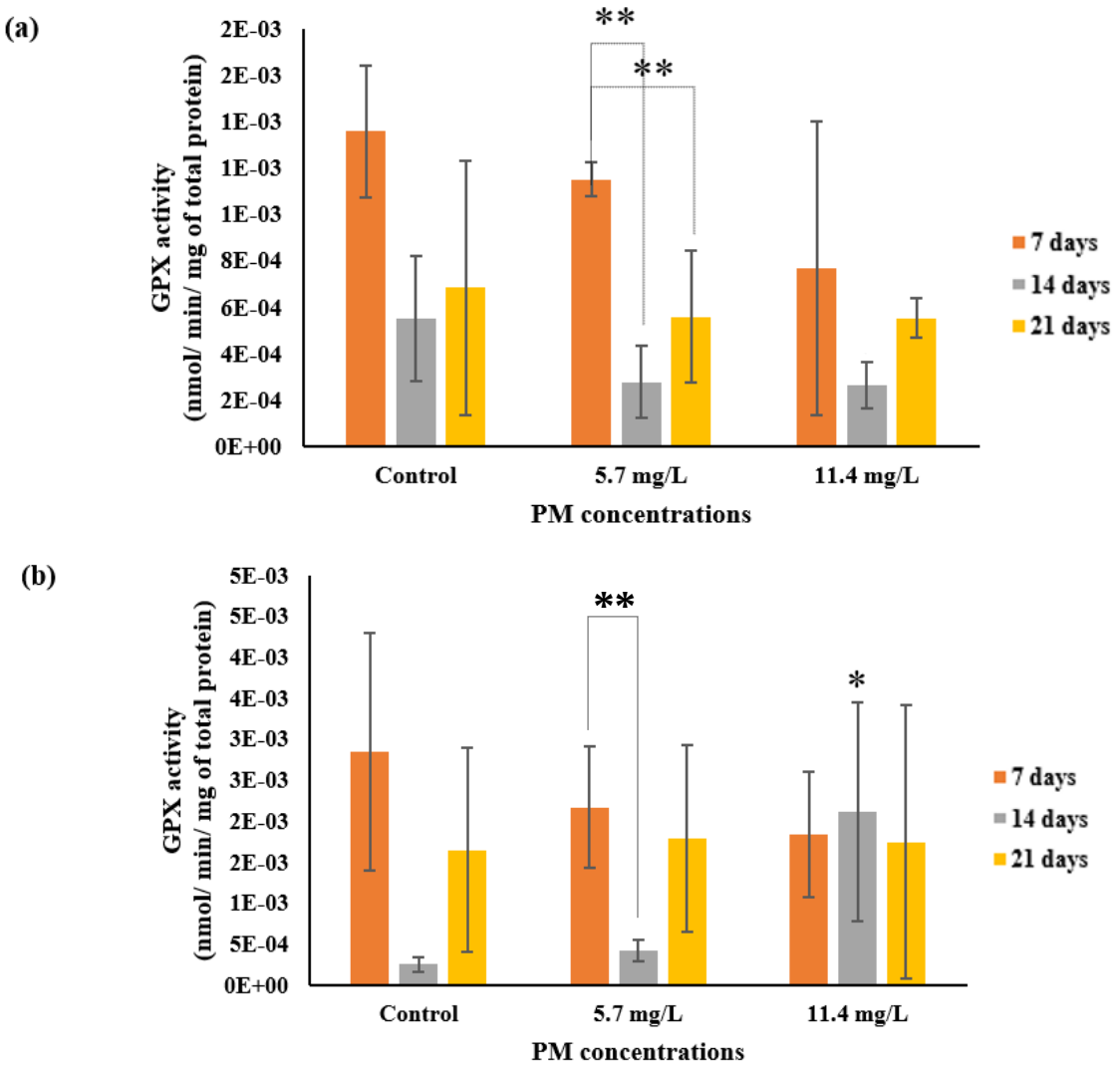


Figure 45 | GPX activity (mean ± SD) in shrimp organs after 7, 14 and 21 days of exposure to PM. **(a)** Muscle. **(b)** viscera. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.4.2.4 Glutathione-S-Transferase (GST)

The GST activity results in shrimp exposed to different concentrations of PM are presented in **Figure 46**.

A general decrease in GST activity in the muscle is most noticeable in the organisms after 21 days of exposure compared to the respective controls (**Figure 46 a**).

The highest average activity (8.911 ± 1.168 nmol/min/mg of total protein) was determined in individuals exposed to 11.4 mg/L of PM after 14 days, and the lowest average activity (3.285 ± 1.986 nmol/min/mg of total protein) was determined in animals exposed to 11.4 mg/L of PM after 21 days.

Significant differences ($p < 0.05$) are seen in the shrimp exposed to a PM concentration of 11.4 mg/L collected after 14 days of exposure and those from 21 days.

GST activity increases in the viscera of exposed animals compared to their respective controls (**Figure 46 b**).

The highest average activity (81.036 ± 39.638 nmol/min/mg of total protein) was observed in animals exposed to 11.4 mg/L of PM after 7 days, and the lowest average activity (15.963 ± 7.291 nmol/min/mg of total protein) was observed in animals exposed to 5.7 mg/L of PM after 7 days.

Statistical analyses revealed significant differences ($p < 0.05$) between the viscera of shrimp exposed to 11.4 mg/L of PM and their respective controls.

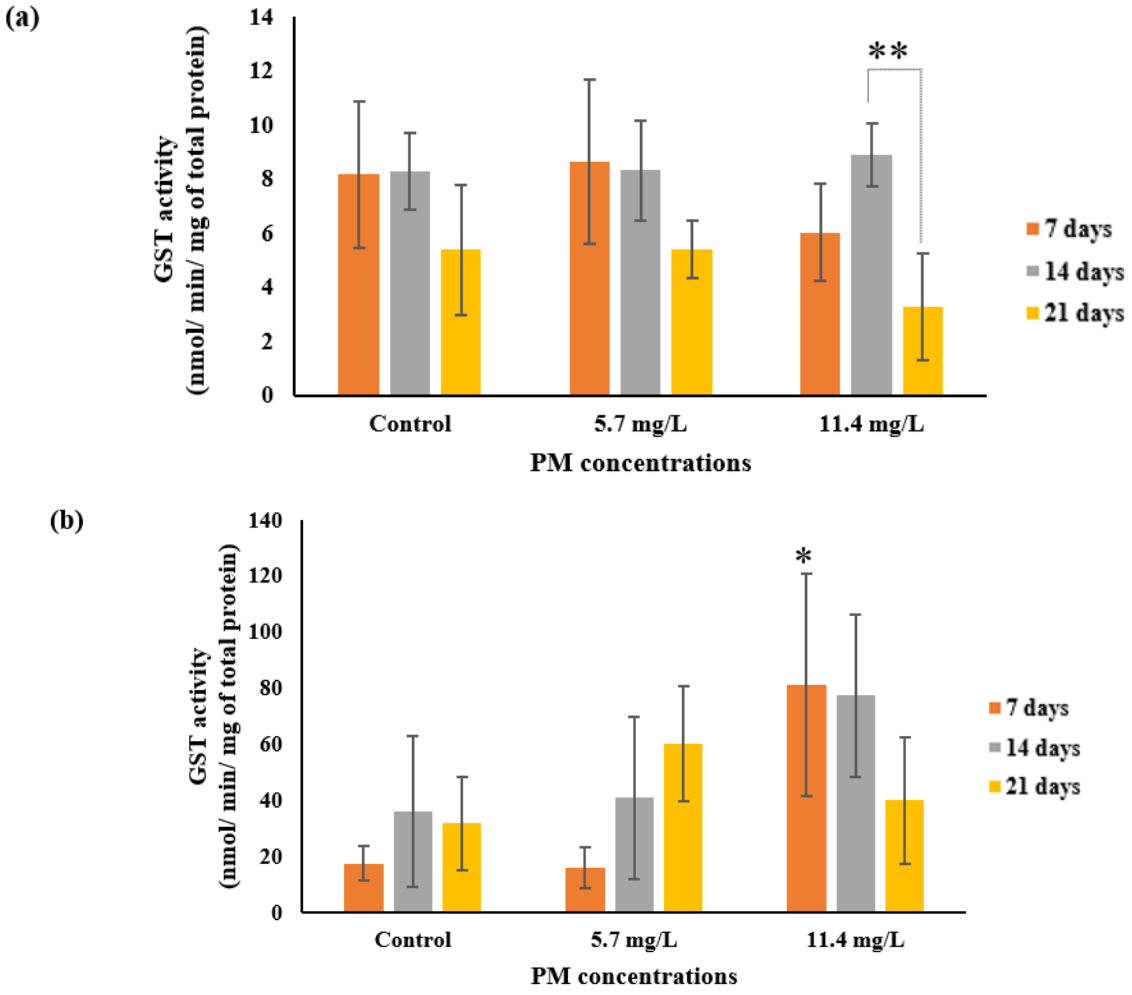


Figure 46 | GST activity (mean ± SD) in shrimp organs after 7, 14 and 21 days of exposure to PM. a) Muscle. b) viscera. Significant differences ($p < 0.05$) comparing to the control groups (*****). Significant differences ($p < 0.05$) between the exposure periods (******).

3.4.3 Cellular damage biomarkers

3.4.3.1 Lipid Peroxidation (LPO)

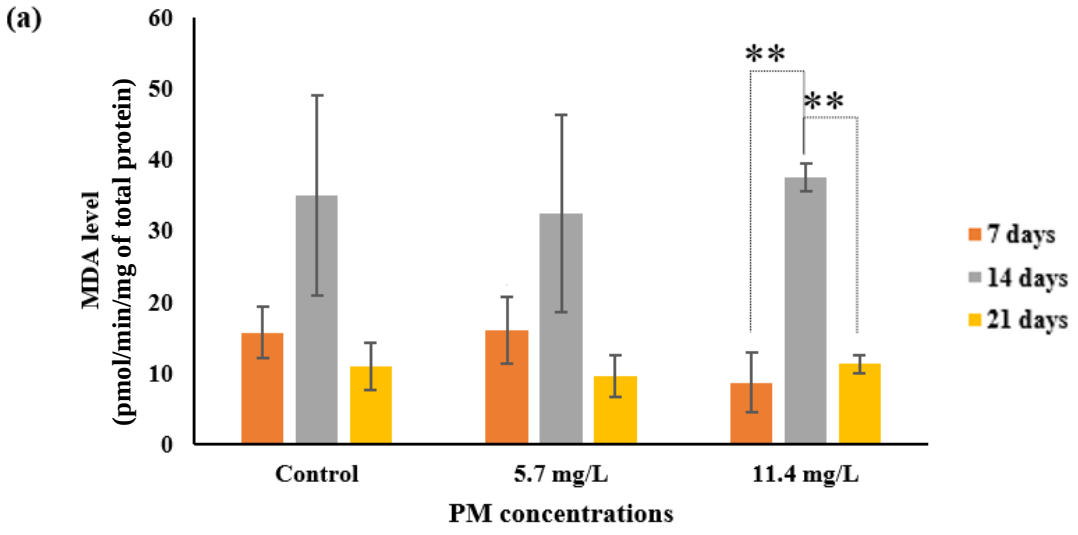
Lipid peroxidation results (expressed as MDA content) in shrimp muscle and viscera are presented in **Figure 47 a** and **b**, respectively**.** The MDA remains constant throughout the exposure assay in both analysed organs. However, increased levels were detected in the muscle and viscera of shrimps collected after 14 days of exposure.

In the shrimp muscles, the highest average levels of MDA (37.53 ± 1.92 pmol/mg of total protein) were found in the organisms exposed to a PM concentration of 11.4 mg/L collected after 14 days. The lowest average levels (8.77 ± 4.16 pmol/mg of total protein) occurred group exposed to a PM concentration of 11.4 mg/L collected after 7 days.

Significant differences ($p < 0.05$) are seen in the shrimp exposed to a PM concentration of 11.4 mg/L between exposure times, as represented in **Figure 47 a**.

In the viscera, the highest average MDA levels (79.71 ± 58.17 pmol/mg of total protein) were found in controls after 14 days of exposure, and the lowest average values (8.99 ± 4.16 pmol/mg of total protein) were observed in the shrimp exposed to 11.4 mg/L of PM and collected after 7 days of exposure.

Significant differences ($p < 0.05$) are seen in the shrimp exposed to a PM concentration of 5.7 and 11.4 mg/L between exposure times, as represented in **Figure 47 b**.



(b)

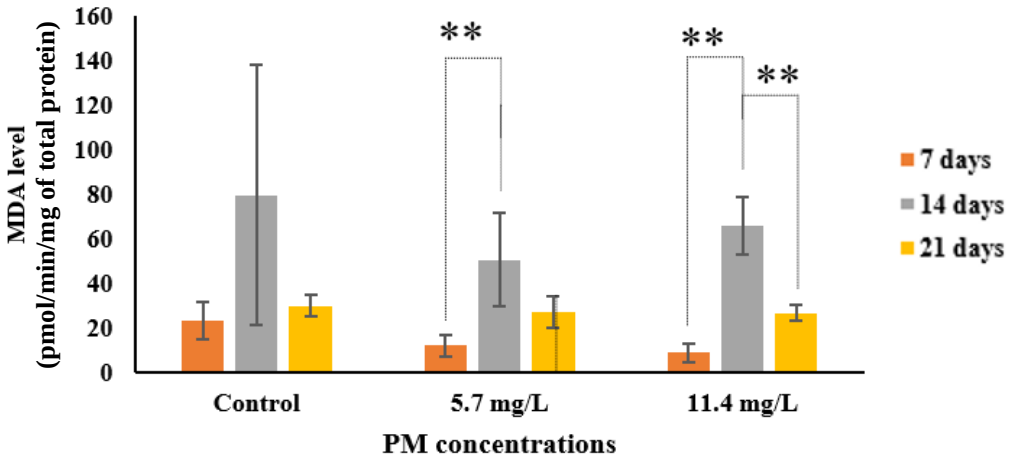


Figure 47 | MDA concentration (mean ± SD) in shrimp's organs after 7, 14 and 21 days of exposure to PM; a) Muscle; b) viscera. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

3.4.3.2 Total ubiquitin levels (UBI)

Total ubiquitin concentration measured in shrimps' muscle and viscera is shown in **Figure 48**. Overall, no statistically significant results were found ($p > 0.05$).

In the muscles (**Figure 48 a**), total ubiquitin concentration seems to increase when comparing exposed animals with their respective controls, except for individuals exposed for 7 days to 11.4 mg/L of PM, which show higher ubiquitin values than the controls from the same exposure time.

The highest average value was measured in the muscle of shrimps exposed to 11.4 mg/L of PM after 14 days (0.036 ± 0.020 µg/ mg of total protein), and the lowest average value was measured in animals exposed to 11.4 mg/L of PM collected after 21 days (0.010 ± 0.007 µg/ mg of total protein).

Concerning the viscera, the highest average value (0.130 ± 0.146 µg/ mg of total protein) was determined in animals exposed to 11.4 mg/L PM after 7 days of exposure, and the lowest average levels (0.015 ± 0.008 µg/ mg of total protein) were found in animals exposed to 11.4 mg/L of PM after 14 days of exposure.

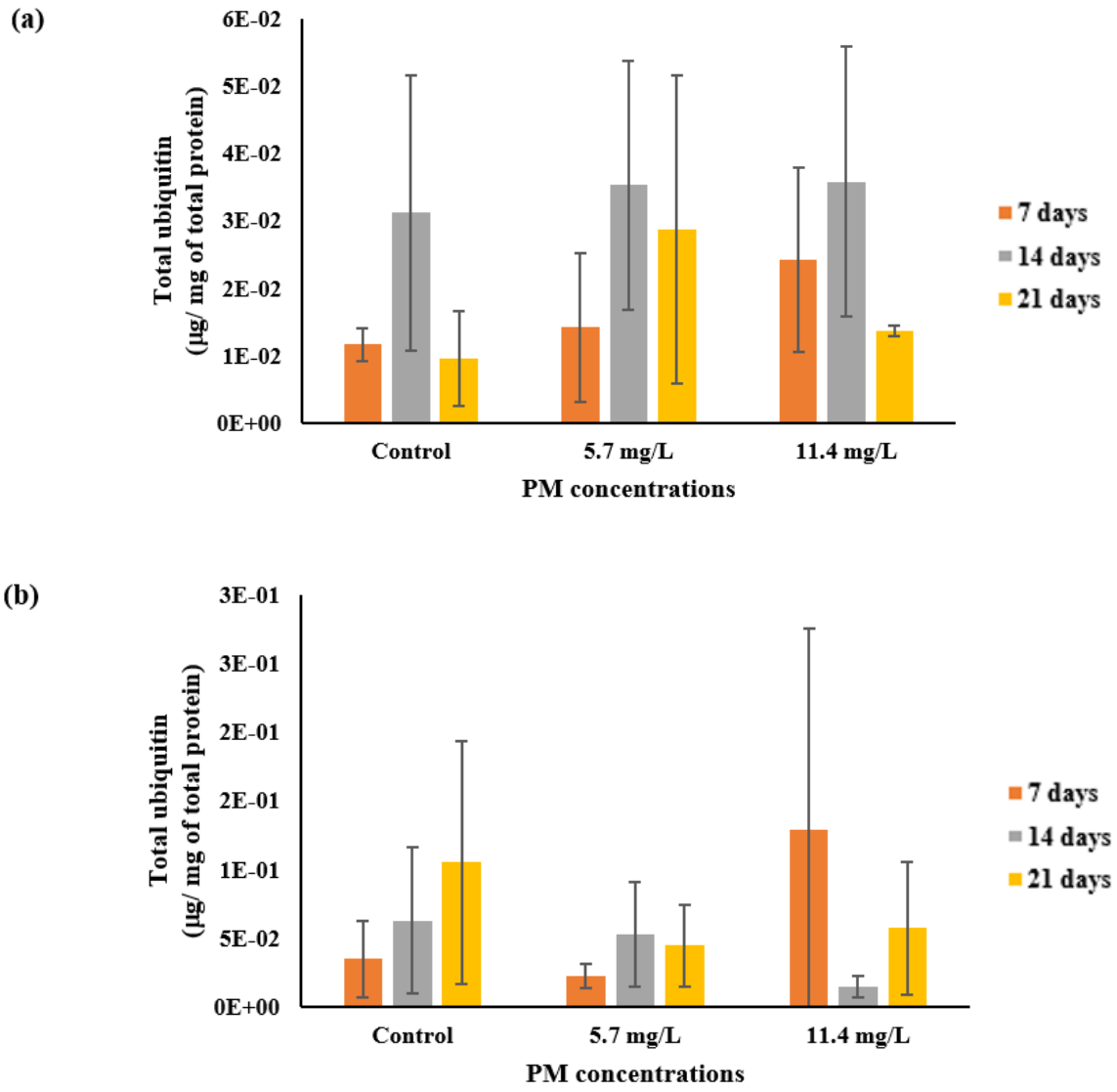


Figure 48 | Ubiquitin level (mean ± SD) in shrimp's organs after 7, 14 and 21 days of exposure to PM. a) Muscle. b) viscera. Significant differences ($p < 0.05$) comparing to the control groups (*). Significant differences ($p < 0.05$) between the exposure periods (**).

4
DISCUSSION

Suspended particulate matter (SPM) is a highly heterogeneous and complex material combined with contaminants resulting from particulate matter (PM) deposition in waterbodies such as oceans, rivers, or lakes. The uptake of these pollutants and the damage they can cause are highly determined by the physico-chemical features of particulates [127]. In terms of size, the smaller the particulates, the easier they tend to penetrate deeper and faster into the respiratory tract and migrate into the systemic circulation, reaching multiple organs and inducing additional damage [128, 129, 130]. Hence, the particulates employed in the exposure assays described in this work are mostly made of particles in the smallest size range. On the other hand, the general composition of these particulates can vary when in solid form or when in liquid suspensions, presenting a plethora of pollutants as those present in the PM used in the present study, such as PAHs, N-PAHs, PCBs, chlorinated pesticides, heavy metals [112]. These major components are known to bioaccumulate through the food web [131, 132, 133, 134]. Thus, even though extensive research has been conducted on SPM's toxic effects, this study provided a deeper insight into the condition of several antioxidant defence systems against ROS and their consequences on cellular components in aquatic organisms exposed to SPM. Using aquatic organisms as model species for toxicological studies based on chronic exposure to sublethal concentrations of specific xenobiotics is a common approach. This is mainly owing to their minimal upkeep, ease of handling and being more vulnerable to exposure and toxicity than terrestrial species, making them good indicators of toxicity [135]. These characteristics of aquatic model systems allow several investigations on cellular response to ROS, repair mechanisms, and assessment of oxidative stress.

The continuously produced oxygen free radicals in living cells are essential for biological functions. However, the unregulated production of ROS can lead to oxidative damage, which is a state with increasing interest in toxicological studies as it is one of the main signs of existing stresses [85]. Stress, in general, does not necessarily entail a final detrimental effect on organisms because most animals, including marine organisms, have a set of adaptative responses to restore their homeostatic state. However, one of the causes of the stress of most research interests is exposure to xenobiotics [136, 135]. Considering PM is a leading hazardous mixture, a more in-depth analysis of its toxic effect on organisms

becomes crucial, especially regarding aquatic organisms [137]. In this sense, the state of oxidative stress can be monitored using molecular biomarkers. The most used to monitor the condition of the antioxidant defence mechanisms against ROS include catalase, superoxide dismutase, glutathione peroxidase and glutathione-s-transferase activities [138].

Sea bass exposed to suspended Particulate Matter (PM)

Sea bass is considered a paramount marine fish model for research in estuarine environments [34, 39, 40, 41], as they may reflect exposure to environmental contaminants, for example, after ingesting previously contaminated prey. In fish like D. *labrax*, the primary mucosal surfaces that work as entry routes for SPM into the body are the skin, gills, and mouth. When exposed to xenobiotics, the gills are a main organ in direct contact with marine suspended contaminants and are considered highly susceptible to these [135, 139]. Thus, regarding the present study, evidence of oxidative stress was expected in the gills, as reflected by the increased enzymatic biomarker activities shown by this organ after 7 days of exposure. Additionally, according to the LPO results, it is observed that the secondary product (MDA) resultant from lipid peroxidation is produced in higher amounts in the gills, which may indicate more significant cellular damage. Fish gills are known to accumulate bioavailable pollutants very quickly, which allows them to be used as valuable tools for assessing the bioavailability of the suspended contaminants in water, particularly when referring to metal bioaccumulation, which is indeed one of the major contaminants also present in the standard particulate matter used in this study [46]. Additional validation on the remarkable ability of gills to bioaccumulate pollutants were reported in previous *in vitro* studies comparing cell lines from different organs, showing that cytotoxicity in gills cell lines is significantly greater than that observed in liver cells [140].

Once passing through the body's membranes into the bloodstream, pollutants such as those present in suspended PM reach other organs in the body where they can be retained. The amount of bioaccumulation varies according to the organ, with the liver, kidney, and muscle currently being the most used tissues in ecological and toxicological studies once they are highly metabolic active tissues capable of accumulating xenobiotics at elevated levels (liver > kidney > muscle). The liver is the main target of toxic effects due to its sizeable standard blood supply, leading to increased exposure to toxicants in this organ [46]. According to results obtained during this study, after the seabass's exposure to suspended PM, after gills the liver shows the highest oxidative stress enzyme activities and lipid peroxidation values. Regarding the total ubiquitin, a general decrease was recorded. This finding is unclear and must be further investigated.

Additionally, regarding the biomarkers, when an increase in enzyme activities is observed, this may indicate that cellular antioxidant defence systems responded to the imbalance caused by exposure to PM contaminants. On the other hand, the decrease in activity values, due to PM being composed of a complex mixture of contaminants, may indicate that compounds with an inhibitory effect on the

antioxidant enzymatic defences are overcoming the others with a synergistic effect [141]. Regarding the situations where a rise in activity is firstly observed, followed by a reduction at higher exposure concentrations or a longer exposure period, it may be because cells first respond by increasing the enzymatic activity but then, later, with the continuation of severe stress, they reach a threshold where they are no longer capable of fighting exposure to contaminants and therefore avoiding harmful effects.

Seabass fed with *Polychaetes* previously exposed to Particulate Matter (PM)

The assay assessing the toxicological effects on the European sea bass through feeding with contaminated *Polychaetes* helped to assess the trophic transfer of PM and risks associated with potential bioaccumulation in aquatic biota.

The intestinal mucosa of fish, like sea bass has an essential immunological role in regulating the uptake of substances, preventing harmful substances, and simultaneously allowing the passage of nutrients and water [142]. However, its function can decrease in response to environmental stressors [143].

The oxidative stress induction in fish can be triggered by many factors, including aquatic pollutants, and is of great importance when assessing the risk of aquatic contamination and the safety of fish for human nutrition [136]. The impact on the organism's equilibrium leads to repercussions in its system *(i.e.*, increased ROS production, which can affect biomolecules and cause an inflammatory response, followed by tissue damage and even death), which can be evaluated through biomarkers of oxidative stress. An essential factor to consider is the substantial variability often found when evaluating antioxidant responses in wild fish, which may be associated with a high degree of genetic variations in wild animals [136]. However, as the population used for this study came from an aquaculture producer, this variability may have been reduced. Additionally, aquaculture has shown that diet is vital for individuals' health, primarily affecting immunity.PM contamination did not lead to significant organism mortality, indicating that the organism's defence against oxidative stress is sufficient to prevent more severe effects [114].

On the other hand, the individual response of each organ varies since the amount of contaminant reaching each organ can be different due to volume variances in fish tissues [144]. Previous research has revealed that carnivorous fish are expected to accumulate higher lipidic content in their tissues. Additionally, as the significant SPM components are mainly lipophilic, they are expected to be found in higher quantities in tissues with higher lipid percentages [142, 145]. As a result, it stands to reason that the muscle and liver, which naturally have a more extensive lipidic content, would accumulate higher percentages of the contaminants, leading to higher ROS generation and, as a result, increased enzymatic activity. This explanation supports the results obtained in the present study since the activity of most enzymatic antioxidant biomarkers were higher in the muscle and liver of fish, compared to their respective controls. The increased SOD activity in the intestines suggested that PM was uptook mostly by these, resulting in a response to decrease ROS generation and prevent oxidative stress. Concerning

the gills, all enzymatic antioxidants reveal a general reduction in their activities. This result entails a lack of effect of the PM compounds in this organ, which can be linked to the entry route as gills were not the primary exposure route. Nonetheless, lipid peroxidation (MDA level) showed a significant increase in fish gills, compared to their respective controls, suggesting a negative effect on this organ. However, the link to ingestion with contaminated *Polychaetes* is unclear.

Still, a general increase in the enzyme biomarkers determined in the intestines would be expected since it is the organ directly exposed to PM via feeding. However, that is only true in SOD. The behaviour observed in this organ can be explained by the fact that being the entry route of the compound does not necessarily entail a greater counteract since it will mainly depend on the compound's persistence in the organ [146]. Another reason may be that the intestines possess different immunologic, enzymatic, and non-enzymatic mechanisms that assist in distinguishing between nourishing and hazardous substances, which were not evaluated during this assay [142]. Additionally, as previously mentioned, intestines activity decreases its functions in response to environmental stresses [142, 143]. After observing how each organ reacts to PM exposure through its enzymatic protective strategies, it is possible to determine its effectiveness by evaluating the effects of high ROS production. Muscle and liver, which exhibited increased enzymatic responses when exposed to the particulate, also appear to have greater biomarker levels, which may suggest lipid and protein damage. This may imply that while the cells could counteract ROS production, they did not successfully prevent it, resulting in cellular damage. Concerning gills and intestines, enzyme activities in fish exposed to PM were low, and no apparent protein or lipid peroxidation damage was evident. As seen earlier, this may imply that the contaminants' concentration in these tissues was insufficient to cause significant ROS production leading to harmful effects. As such, this resulted in a diminished antioxidant enzymatic response.

Mussels exposed to suspended Particulate Matter (PM)

Mussels, due to their heightened tolerance, feeding-filtration capability, extensive native range, high adaptability, and low mobility, it is considered notable model organism [65,66]. When exposed to external xenobiotics, *M. galloprovincialis* increases ROS production, which can affect biomolecules and cause an inflammatory response, followed by tissue damage. Like other marine species, mussels have also developed ROS removal mechanisms to protect from these outcomes. However, when unable to deal with the amount of ROS formed, cells undergo oxidative stress, resulting in injury or even cell death [147]. In the assay involving the *M. galloprovincialis* exposure, no mortality was observed after exposure to the different PM concentrations, and no significant morphological changes were observed during organ sampling, further emphasising the mussel's innate resistance to external contaminants [148, 149]. In general, the biomarker results showed that exposure to PM causes an increase in biomarkers levels, although not statistically significant in many cases. However, this will depend on the specific biomarker analysed in a plethora of investigations. For instance, antioxidant enzymes activities (*e.g.*,

SOD, CAT, GST, and GPX) are used to evaluate the efficacy of antioxidant protection against oxidative stress, as when organisms are exposed to xenobiotics [150]. These enzymes can behave in three distinct ways increasing their activity to remove ROS by decreasing their activity, indicating that oxidative stress is too extreme for the enzyme to oppose the toxic effect or that the contaminants are enzymatic inhibitors. Ultimately, cell death is the final stage when the enzymatic activity and the other defence mechanisms are no longer effective against ROS and subsequent injury. CAT, SOD, and GPX are first-stage enzymes that will act directly in neutralising ROS formation. Thus, they are expected to increase in activity when in the presence of contaminants [88, 99]. In this study, SOD and GPX activity behaved very similarly in gills of animals exposed to the highest PM concentrations tested, indicating a more prominent toxic effect in this organ.

Furthermore, both in the gills and digestive glands, the antioxidant enzyme activities increased significantly over the exposure time (7 to 21 days), and mainly in animals exposed to 11.4 mg/L of PM, suggesting apparent oxidative stress. On the contrary, although presenting a general rise with increasing concentration and exposure period, CAT's activity, as expected, does not exhibit considerable differences between the two organs evaluated. GST is ubiquitous in body's cells but is mainly present in an animal's liver; it is also in the mussel's digestive gland, which functions similarly to the liver. As a result, GST exists in more significant amounts in the digestive gland; consequently, this organ is expected to have higher activity, which may also indicate an elevated capability of cells to remove xenobiotics [151]. However, this is not noticeable in this study, and higher results are observed in the gills, which may be explained by the filtration capacity of *M. galloprovinciallis*', as they are filter feeders, which means that they ingest a large amount of water containing suspended particles, which may include some contaminants [68].

Previous studies have suggested that mussels only efficiently retain particles larger than 3 to 7 mm (depending on the species' feeding structures) and can distinguish between nutritious and non-edible particles, rejecting the latter [152, 153]. Therefore, digestive glands' activity may be lower if they cannot uptake particles (eventually containing contaminants) under these conditions. On the other hand, the activity in the gills is higher since this organ is in direct contact with water-containing contaminants. Furthermore, in this study, the GST activity, in both organs (gills and digestive glands), increased significantly over exposure time (7 to 21 days), especially in organisms exposed to the greatest PM concentration (11.4 mg/L), which suggests a response to fight oxidative stress. Additionally, GST is an antioxidant enzyme involved in phase II detoxification of xenobiotics, including heavy metals [154, 155]. Since one of the main components of the PM suspension used for exposure trials is heavy metals, this increase in activity was anticipated. When a cell cannot neutralise the ROS formed, it may result in several harmful effects, such as lipid peroxidation and protein damage. The first one is a reactional cascade that leads to the oxidation of lipids and is significantly associated with the disruption of lipids

that constitute the cellular membranes, which will, in turn, compromise their structural integrity [90]. On the other hand, protein damage is linked to ubiquitination which is a process that involves protein labelling, more specifically, proteins whose structure and function are compromised. These are labelled with a ubiquitin molecule to be later eliminated [111]. Concerning the results obtained in this study, a more prominent effect on the mussels' membrane lipids is suggested by the increase in MDA concentrations (especially in animals exposed to the highest PM concentrations (11.4 mg/L), which can lead to cellular damage. This phenomenon indirectly entails that the enzymatic activity, despite a general increase, is not efficient enough to remove the ROS produced, consequently leading to cellular damage. On the other hand, the total Ubiquitin levels also suggest slight protein damage in animals exposed to the highest exposure concentration.

Shrimps exposed to suspended Particulate Matter (PM)

The shrimp also have features that cause them to be a suitable model organism, such as a highly adaptable and resistant to environmental stresses, low maintenance under laboratory conditions and the ability to accumulate xenobiotics [72, 73, 81]. The assay consisted of exposing *P. varians* to different concentrations of particulate matter (PM), which, coupled with the determination of oxidative stress enzymes, allowed for assessing the toxicity in this species.

The mortality rate was the first parameter assessed during the exposure assay since it is a valuable indicator of the PM's toxicity in exposed organisms. Here, the highest mortality was seen in the highest PM concentration of 11.4 mg/L. In addition, behavioural changes were observed in previous studies with *P. varians* when animals were subjected to extreme stress conditions or after exposure to individual contaminants such as benzo(a)pyrene, fenitrothion, and anthracene [82, 155, 156]. Moreover, exposure assay with shrimps reveals that, apart from GPX, most enzymatic biomarker levels are more substantial in viscera than in the muscle samples. The higher activation of the antioxidant defence system suggests a more significant impact on exposure to PM in viscera. On the other hand, total ubiquitin was significantly elevated in the viscera of exposed shrimps compared to the respective controls, suggesting more significant damage at the protein level in these tissues.

5

FINAL REMARKS

Aquatic ecosystems are constantly threatened by the entry of contaminants, mainly from terrestrial discharges. These findings indicate, to some degree, a decline on the aquatic environment health and biodiversity, particularly when referring to those organisms with high economic value like the *Dicentrarchus labrax*, *Mytilus galloprovinicalis*, and *Palaemon varians* [156].

There is an impetus to enhance environmental and ecotoxicological studies on the effects of PM in aquatic ecosystems. Thus, the use of marine model organisms allows for monitoring the presence and effects of pollutants in aquatic ecosystems. Many studies also focus on assessing the toxicity of contaminants alone or combined in groups of two or three. However, organisms in their natural habitat are more likely to be exposed to a highly complex mixture of contaminants, such as the particulate matter used in this assay. Naturally, the results here may not directly correlate with the ones obtained from individually evaluated components, leading to an under or over-assessment of the potential toxicological effect than those observed in complex mixtures [157, 96].

The results obtained after exposing the seabass to two distinct concentrations of a standard particulate mixture (5.7 mg/L and 11.4 mg/L) presented a general toxic response, as shown by oxidative stress enzymes, LPO and total ubiquitin.

The seabass was also contaminated through feeding with exposed *Polychaetes* (PM concentration of 11,4 mg/L) in order to evaluate the PM's ability for trophic transfer. Here, generally, the enzymatic biomarkers did not show significant differences ($p > 0.05$) between the organs of the exposed animals and the respective controls, except for the CAT determined in the fish intestines, and the SOD determined in the fish liver. Regarding the evaluation of the cellular damage, the gills show a significant difference ($p < 0.05$) in the total ubiquitin levels compared to their respective controls, suggesting some protein damage in this organ. The overall results suggest little effect in the animals fed with contaminated *polychaetes*. However, this may be due to the experimental period (4 days) being to short to perceive more pronounced effects.

Regarding *M. galloprovincialis*, the overall results suggest that exposure to suspended PM, containing several known contaminants, affects antioxidant enzymes activities in the organism, showing some sublethal effects at the cellular level, as LPO and protein damage. However, due to the known high tolerance of *Mytilus sp*. to pollution, a low mortality rate was observed, confirming that they are an "early warning" indicator of environmental contamination.

Finally, regarding shrimp, CAT and GPX presented the most significant results indicating a response of the antioxidant defence mechanisms of the organisms to fight oxidative stress.

6

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A

APPENDIX

A.1 Biomarker values of the t0 individuals

Table 2 | Biomarker´s levels determined in shrimp collected prior to the beginning of the exposure assays (T0).

Conditions	**t0**	
Organs	**Muscle**	**Viscera**
CAT	0.33 ± 0,12	3.17 ± 2.78
SOD	19.49 ± 14.34	62.44 ± 37.06
GPX	1.04E-03 ± 4.26E-04	3.78E-03 ± 1.42E-03
GST	5.75 ± 1.07	18.14 ± 2.94
LPO	0.008 ± 0.003	0.023 ± 0.011
UBI	0.016 ± 0.010	0.055 ± 0.024

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