Nanoparticles influence the herbicide diuron mediated toxicity on marine mussel *Mytilus galloprovincialis*: single and mixture exposure study

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Abstract

The exposure of habitats to the herbicide diuron, one of the most effective and highly used antifouling agents worldwide, leads to serious environmental toxicity, mainly for marine invertebrates. Moreover, nanoparticles (NPs) act as carriers of organic pollutants in marine ecosystems, thereby influencing their bioaccumulation and toxicity in exposed organisms. This study aimed to investigate the individual and combined toxicity of diuron and two NPs (ZnO NPs and TiO2 NPs) at sub-lethal doses on the marine mussel *Mytilus galloprovincialis*.

The results showed that the diuron alone and diuron- ZnO NPs exposure at 100 μg l\(^{-1}\) significantly decreased the filtration capacity and respiration rates compared to the control group for the mussel *M. galloprovincialis*. However, no significant effects were observed following the exposure to TiO2 NPs and diuron- TiO2 NPs co-exposure. Further, diuron alone and diuron- ZnO NPs co-exposure at 100 μg l\(^{-1}\) significantly increased the levels of antioxidant entities, such as malondialdehyde (MDA), catalase (CAT), superoxide dismutase (SOD), and glutathione S-transferase (GST) in the digestive gland and gills of this mollusk. The diuron- TiO2 NPs co-exposure reduced the activities of antioxidant enzymes compared to exposure of diuron alone. The diuron, in the presence of NPs, induced physiologic and oxidative pathways that led to various responses of the antioxidant’s entities in *Mytilus galloprovincialis*.

1. Introduction

Marine ecosystems receive daily numerous chemicals as a result of anthropogenic activities, reducing their ecologic and economic value, as well as for humans themselves. Pesticides comprise natural or synthetic substances intended to deplete unwanted pests, considered undesirable for agriculture and public health. They are used on a large scale, comprising under this generic name the insecticides, herbicides, fungicides and various other substances [1]. Nowadays, the use of pesticides has gradually increased worldwide to meet the needs of the growing human population. However, their misapplication has become a major problem, mainly in developing countries. The pesticides are organic toxic pollutants because they induce severe unwanted effects on humans and environment [2, 3]. Following their use, the pesticides reach various compartments of the biosphere, where they interfere with different types of ecosystems, affecting their integrity and functionality. These products are often persistent and their long-term undesirable effects are highly visible at community, individual and molecular levels. At community level, the pesticides impact negatively the animals by altering their behavior and
physiological functions [1]. This usually leads to increased mortality rates, decrease in biodiversity, including the meiobenthic communities in marine habitats [5–11]. At molecular level, the pesticides have a negative impact too on the nervous system, causing serious health issues, such as carcinogenicity [12] and neurotoxicity [13], as well as the alteration of oxidative enzymes, leading to oxidative damage of tissues [14].

Among pesticide, the diuron [3-(3, 4-dichlorophenyl)-1, L-dimethyl urea] is a widely used pesticide for weeds control [15], very effective and a worldwide antifouling agent [16]. The diuron is highly stable and persistent, mainly in aquatic environments. This compound causes kidney disease, haemolytic anaemia, compensatory haematopoiesis, endocrine problems and it is also potentially cancerous [17, 18].

The diuron threats severely aquatic organisms, especially the sensitive species [16]. Thus, scientific efforts are needed to highlight better the toxicity of this chemical compound and to overcome its persistence and recalcitrance.

Recently, nanoparticles (NPs) became a class of contaminants of interest in marine ecosystem that attract the attention of scientists worldwide, given their widespread use in many industrial applications [19]. NPs are defined as small objects that behaves unitary with respect to their transport and properties. NPs comprise particles with a size between 1 and 100 nanometers and of great scientific interest given they act as a bridge between bulk materials and atomic and molecular structures [19].

Among NPs, metal oxides nanoparticles are widely used as basic materials in various industrial applications. Among metal oxides, the TiO₂ and ZnO have drawn too consideration given their even more extensive production compared to other products. The annual estimated production of zinc oxide (ZnO) nanoparticles (NPs) is over 60, 000 tons a year worldwide [20] and it is estimated that the worldwide production of TiO₂ will reach 2.5 million tons by 2025 [21].

TiO₂ and ZnO NPs are used as inorganic UV filters in numerous personal care products (ICCCR, 2011). They are commonly used in sunscreen lotions, covering a broad spectrum for UV blocking, without sacrificing transparency. Products that use NPs provide better texture, spread ability, and UV protection [22].

One of the main issues, however, with the NPs is their release in various types of ecosystems and the toxic interactions with biota as well as with other pollutants.

Previous studies demonstrated that both TiO₂ and ZnO NPs are widespread in various ecosystems [23–25], being discharged into aquatic environments as a result of the extensive use [24, 26].

Proper risk assessments for TiO₂ and ZnO NPs release in marine habitats is thus paramount to assure the environmental safety. Previous studies showed that NPs deleteriously impact bacteria [27, 28], echinoderms [29] and algae [30, 31]. Moreover, their interaction, as well as with other contaminants could occur in marine ecosystems, leading to different toxicities profiles in marine organisms. Due to their high surface-volume area, the NPs can absorb other contaminants and form nanoparticle-toxin complexes [32], acting as accumulators and carriers of these toxic end-products in marine biota [33, 34].

The interaction between chemicals and various cells compartments were considered in previous toxicological studies, based on the production level for reactive oxygen species (ROS) and antioxidant enzymes. Thus, the equilibrium between the production and elimination of ROS determines the toxicity potential of pollutants and the enzymes activities status is crucial in a proper risk evaluation. The activities of enzymatic antioxidants such as superoxide dismutase (SOD), catalase (CAT) and glutathione S transferase (GST) offer a versatile mechanism to control the intracellular ROS in maintaining the redox homeostasis, hence protecting the organisms from the oxidative stress [35]. Therefore, the evaluation of the level of antioxidants enzymes can help environmental scientists to monitor closer the level of oxidative stress induced in organisms exposed to single or a mixture of pollutants.

To date, there are limited data that have been reported about the influence of different NPs types and the toxicity of pesticides to marine invertebrates. Therefore, such investigations represent a promising avenue for further research. To our knowledge, no previous laboratory experiments focused on diuron, in presence of different NPs were undertaken. The purpose of the present study was: (1) to evaluate the potential toxicity of diuron alone or in combination with ZnO and TiO₂ NPs in mussels; (2) to compare the sensitivity of two mussels organs (i.e. digestive gland and gills) to this herbicide and nanoparticles exposure; (3) to explore the enzymatic pathways involved in the toxicity of this mixture of contaminants and (4) to screen out if nanoparticle could effectively reduce the toxicity of diuron in real life scenarios.

2. Material and methods

2.1. Chemicals

Diuron; the 3-(3, 4-Dichlorophenyl)-1, 1-dimethylurea. Purity ≥ 98% was purchased from sigma Aldrich (USA).

TiO₂ and ZnO nanoparticles were synthesized according to [36, 37]. Briefly, to synthesize TiO₂ NPs, Titanium (IV) butoxide [Ti(OCH₂CH₂CH₂CH₃)₄], Aldrich, AR grade] (5 ml) were dissolved in 50 ml of
Dimethyl Sulfoxide (DMSO) [(CH$_3$)$_2$SO, Sigma] and then heated to 190 °C and kept at this temperature for 2 h under mechanical agitation. At the end of reaction, the precipitate was separated by centrifugation, washed several times with ethanol/acetone (2:1), and then dried in vacuum at 50 °C for 12 h to yield a white dry TiO$_2$ powder. The as-prepared TiO$_2$ sample was calcinated at 400 °C. The subsequent annealing of TiO$_2$ NPs after the solvothermal treatment induces crystallinity and completely removes the solvent molecules entrapped inside the particles.

For ZnO NPs, zinc acetate dehydrate [(Zn (OAc)$_2$ 2H$_2$O), Aldrich, AR grade] (60 mg) were dissolved in 50 ml of 1,3-propanediol (ACROS Organics, 98%) and then heated at 160 °C and kept at this temperature for 1 h under continuous mechanical agitation. At the end of the reaction, the precipitate was centrifuged, washed several times with ethanol and acetone, and then dried in vacuum at a 50 °C for 12 h to yield a white dry ZnO powder.

2.2. Structural and optical characterizations
The crystallographic structure of the TiO$_2$ and ZnO nanoparticles was characterized by x-ray diffraction patterns (XRD) (STOE-STADI P) (an INEL diffractometer using a cobalt K$_\alpha$ radiation ($\lambda = 1.5418$ Å). Morphological details of the nanoparticles were characterized by transmission electron microscopy (TEM) (JEOL-ARM 200F). Energy-dispersive X-ray spectrometry (EDX) attached to the TEM was used for elemental analysis.

Particle size measurement in distilled water and in seawater (0.1 mg ml$^{-1}$) using Dynamic light scattering (DLS) were carried out using a Zetasizer Ultra (Malvern Panalytical Ltd., Malvern, UK) equipped with a He-Ne laser at a wavelength of 632.8 nm and a maximum power of 10 mW. All experiments were performed at 25 °C using a sample volume of 1 ml and disposable cuvettes (DTS0012, Malvern Panalytical Ltd., UK). The instrument settings were optimised automatically by means of the ZS XPLORER software (Malvern Panalytical Ltd., UK).

2.3. Test animals
Mediterranean mussels (M. galloprovincialis) of similar shell length (40 ± 2 mm) were collected from Menzel Jemil at Bizerte lagoon, Tunisia. Upon arrival to laboratory, the periostracum was polished with polypropylene plaques to remove epiphytes. The mussels were then distributed in 3L glass tanks and acclimated for a week on a 12 h light/dark cycle prior to exposure. After the acclimatisation, 12 experimental conditions were set up in 04 replicates of ten individuals per tank (table 1).

A stock of diuron solution was prepared by dissolving diuron in dimethylsulfoxide (DMSO), before dilution in seawater, as the DMSO has no significant effect on the biomarker responses [38]. Moreover, it was showed that exposure to DMSO at concentrations less or equal to 0.005% V/V (50 μl$^{-1}$) did not affect adversely the metabolic profiles of marine bivalves [39, 40]. The stock solution was first prepared by mixing 20 mg of diuron in 1 ml of DMSO. A volume of 15 μl from the stock solution was added to experimental tanks of each diuron prepared in DMSO (20 mg ml$^{-1}$), to allow a concentration of 100 μg diuron. l$^{-1}$ seawater and 7.5 μl representing the volume added to tanks from the stock solution to provide a concentration of 50 μg diuron. l$^{-1}$ seawater.

The NPs-treated group comprise the mussels exposed to TiO$_2$ and ZnO nanoparticles, at concentrations of 50 μg l$^{-1}$/seawater (ZnONP1 and TiO$_2$ NP1) and 100 μg l$^{-1}$/seawater (ZnONP2 and TiO$_2$ NP2), respectively.

The NPs concentrations used in the current study were selected based on relevant concentrations of metal oxide nanoparticles found in marine ecosystems, ranging from nanograms to milligrams per liter [41, 42].
stock of NPs solutions (20 mg ml$^{-1}$) were prepared by dissolving each NPs in distilled water and before proceeding with the experiment, the stock solutions were kept at room temperature for two hours under mechanical agitation. 7.5 μl and 15 μl volumes were added to tanks from the NPs stock solutions to provide concentrations of 50 μg l$^{-1}$ and 100 μg l$^{-1}$ NPs/seawater, respectively.

The mixture-treated group comprised the mussels exposed to a mixture of (1): 50 μg l$^{-1}$/seawater of TiO$_2$ NPs and ZnO NPs with 50 μg l$^{-1}$/seawater of diuron (ration 1:1, at a final concentration of 100 μg l$^{-1}$) and (2): 100 μg l$^{-1}$/seawater of TiO$_2$ and ZnO nanoparticles with 100 μg l$^{-1}$/seawater of diuron (ration 1:1, at a final concentration of 200 μg l$^{-1}$). The DMSO-treated group (15 μl: less than 0.005% V/V) was established to examine the effects of the solvent and comprising the highest volume of DMSO used in this experiment.

The exposed mussels were subject to daily concentrations of DMSO, NPs, diuron and mixture of NPs + diuron in seawater, supplied altogether for a period of 14 days.

At the end of the experiment no mortality was noticed and all individuals were apparently feeding normal. During the experiment, the salinity, temperature, dissolved oxygen and pH were measured daily with a thermo-salinity meter (LF196; WTW, Weilheim, Germany), an oximeter (OXI 330/SET; WTW) and a pH meter (pH 330/SET-1, WTW), respectively. Temperature was maintained at 19 ± 2 °C, oxygen at 6.2 mg l$^{-1}$ and the salinity was 32%o. Tanks were filled with natural seawater to live and changed every two days.

### 2.4. Physiological parameters determination

One replicate (n = 10 mussels) was considered for physiological parameters establishment.

The measurement of the filtration rate (FR) was performed based on the loss of neutral red dye particles from the water column, according to [43] in closed chambers. Immediately following exposure, five mussels from each treatment were placed in 200 ml beakers (one mussel per beaker), containing 100 ml of neutral red solution (1 mg l$^{-1}$). Prior to the placement of mussels in solution, an aliquot of water was removed from each beaker to measure the initial concentration, C0. After two hours, the mussels were removed and the remaining solution, (Ct), along with the initial aliquot (C0), were acidified to pH 5 with HCl 5%. Neutral red concentrations were determined by measuring the absorbance at 550 nm. Standards of neutral red were measured along with the samples and used to establish a standard curve, from which the dye concentrations were extrapolated.

FR was calculated using the following equation:

$$ FR = \left[ \frac{M}{nt} \right] \log \left( \frac{CO}{Ct} \right) $$

Where FR is the clearance rate (mg indiv$^{-1}$ h$^{-1}$), M is the water volume, n the number of individuals employed, t the time in h and C0 and Ct the concentrations at the beginning and at the end of the experiment, respectively.

The respiration rate (RR) was measured on five mussels with calibrated oxygen electrodes, connected to an oximeter, according to [44]. The decline in oxygen concentration was performed every half an hour for three hand calculated, using the following equation:

$$ RR = \frac{[Cti-C0]}{V} \times \frac{V}{(t_i - t_0)} $$

Where RR is the respiration rate (mg O$_2$ h$^{-1}$), Ct is the concentration of oxygen in the water (mg O$_2$ l$^{-1}$) at time t (t0: initial time and ti: end time h) and V is the total volume of the solution from the sealed chamber.

### 2.5. Biomarkers measurements

The mussels were removed at the end of experiment from each tank and fixed with liquid nitrogen. The digestive glands and the gills were dissected on ice and homogenized with a polytron homogenizer in 10 mM Tris-HCl, pH 7.2, containing 500 mM sucrose, 1 mM EDTA and 1 mM PMSF, supernatants were collected by centrifugation at 20,000g (4 °C for 30 min).

The supernatant, containing the cytosol and the microsomes, was removed and used to determine the activities of Superoxide dismutase (SOD), Catalase (CAT) and glutathione S-transferase (GST), as well as of lipid peroxidation level (LPO) and protein content.

The SOD activity was assessed following the conjugation of the GSH with 1-chloro-2,4-dinitrobenzene at 450 nm [45].

The CAT activity was measured through absorbance at 240 nm in interaction with H$_2$O$_2$, according to [46]. The reaction volume and time were 1 ml and 1 min, respectively. The reaction solution contained 80 mM phosphate buffer, pH 6.5 and 50 mM H$_2$O$_2$; the activity was measured as μMol/min/mg protein.

The GST activity was assessed following the conjugation of the GSH with 1-chloro-2,4-dinitrobenzene at 340 nm [47].

The lipid peroxidation (LPO) was estimated according to [48], in terms of thiobarbituric acid reactive species (TBARS), by using malondialdehyde (MDA) as standard. The absorbance was read at 535 nm and the lipid peroxidation was expressed as nMol of malondialdehyde (MDA)/mg protein.

The protein content was measured according to [49], using bovine serum albumin (BSA) as standard.
2.6. Statistics
Statistical analysis was carried out with STATISTICA 8.0. Each parameter was reported as mean ± standard deviation. Prior to statistical analyses, each parameter was checked for normality and homogeneity of variance. The variation of each parameter among concentration and contaminant was tested with one-way ANOVA tests, followed by post hoc Tukey’s tests.

3. Results

3.1. Nanoparticles morphology under environmental change
The XRD pattern related to TiO2 NPs (figure 1(A)) of the powder revealed well-crystallized and pure TiO2 particles. All the diffraction peaks of TiO2 were indexed to tetragonal anatase phase (space group 41/amd, lattice constants a = 3.7845 Å, c = 9.5143 Å, (figure 1(A)) with the strongest characteristic peak. The peak broadening in the XRD pattern clearly indicated that small particles were present in samples. The XRD pattern related to ZnO NPs (figure 1(B)) of the synthesized powder revealed well-crystallized and pure ZnO particles with the hexagonal wurtzite phase.

The TEM images (figure 2) showed that the quasi-spherical TiO2 nanoparticles with a good dispersal capacity were formed, with a size ≤40 nm. The EDX spectrum (figure 2) indicates that the nano-particles are of high purity, given that only Ti and O elements were detected. In addition, the TEM image (figure 2) showed that ZnO nanoparticles with a quasi-spherical shape were formed and their size was ≤40 nm. The presence of C and Cu was due to the copper grid used for the TEM/EDX experiments.
According to the DLS results (figure 3), the size of the TiO$_2$ and ZnO nanoparticles showed small changes between the dispersed mediums: distilled water (figure 3(A)) or sea water (figure 3(B)). The TiO$_2$ nanoparticles size varied from 37 nm in distilled water to 59 nm in sea water (figure 3). Similarly, figure 3 shows that ZnO nanoparticles varied from 27 to 43 nm. In addition, the zeta potential was raised to be $-46$ mV for ZnO nanoparticles and $-43$ mV for TiO$_2$ nanoparticles, indicating high stability of nanoparticles (figure 4).

### 3.2. Impact of diuron and nanoparticles in mussels

#### 3.2.1. Filtration and respiration capacities response

Filtration and respiration capacities showed response-dependent reaction to the type and concentration of contaminants (figures 5(A) and (B)).

Following diuron exposure, the mean ± SD filtration capacity of mussels in control was 115 ± 4.58 mg/animal/h and decreased significantly to 53.6 ± 3.2 and 28.2 ± 2.07 mg/animal/h, respectively, in treatments with Di1 = 50 μg l$^{-1}$ and Di2 = 100 μg l$^{-1}$ (figure 5(A)). Similarly, ZnONP2 decreased significantly ($p = 0.0013$) the filtration capacity from 115 ± 4.58 mg/animal/h to 88.8 ± 4.14 mg/animal/h. In contrast, no significant modifications were observed after exposure to ZnONP1, TiO2NP1 and TiO2NP2, respectively.

The exposure to ZnONP2 combined with Di2 decreased significantly ($p = 0.0002$) the filtration capacity from 115 ± 4.58 mg/animal/h to 24.2 ± 2.07 mg/animal/h. In contrast, no significant modifications were observed following exposure to ZnONP2 combined with Di1, TiO2NP1 combined with Di1 and TiO2NP2 combined with Di2.

The respiration rate (RR) showed similar a similar pattern; it decreases following exposure to diuron in a concentration-dependent manner, from 0.69 ± 0.06 mg O$_2$/h detected in control to 0.035 ± 0.021 mg O$_2$/h.
after exposure to Di1 and Di2, respectively (figure 5(B)). The RR decreased also, by about 20% after exposure to ZnONP2 and 63% after exposure to ZnONP2 combined to Di2.

No significant modifications of RR were observed for mussels exposed to ZnONP1, TiO2NP1 and TiO2NP2 and the binary mixture of ZnONP1 + Di1, TiO2NP1 + Di1 and TiO2NP2 + Di2, respectively, compared to the control group.

3.2.2. Oxidative stress biomarkers

The activities of antioxidant enzymes (i.e., SOD, CAT and GST) measured in digestive gland and gills showed contaminant concentration-dependent responses (figure 6). The SOD activity increased in both tissues directly with the exposure to diuron (figure 6(A)). The activity of this biomarker increased to 3.67 ± 0.2 U mg⁻¹ protein after exposure to Di1 = 50 µg l⁻¹ and to 4.68 ± 0.2 U/mg protein after Di2 = 100 µg l⁻¹ in the digestive gland showing significant difference (p = 0.003 and p = 0.00013, respectively). Similarly, the SOD activity increased to 2.19 ± 0.06 U/mg protein after Di1 and to 2.85 ± 0.1 U/mg protein after Di2 in gills,
resulting in significant differences ($p = 0.0013$ and $p = 0.00012$, respectively) compared to control groups. Figure 5(B) shows that diuron impacts also the CAT activity which increased significantly ($p < 0.05$) by about 48% and 49% in digestive gland and gills, respectively at Di1 exposure. The highest value of CAT activity was observed in the digestive gland of mussels exposed to Di2, significantly ($p = 0.0012$) compared to control (figure 6(B)).

The GST activity significantly increased after diuron exposure in the digestive gland and gills, reaching a maximum concentration of 5.57 ± 0.14 nmol/min/mg protein (figure 6(C)). To check the potential effect of diuron on cell membrane damage, we analysed the effect of Diuron in LPO level (figure 6(D)). After 14 days of treatment with 50 and 100 $\mu$g l$^{-1}$ of diuron, a significant increase in TBARS was observed in the digestive gland and gills, starting from 50 $\mu$g l$^{-1}$ and reaching a maximum of about 5.37 ± 0.07 nmol TBARS/mg proteins in the mussel’s digestive gland exposed to Di2.

To compare the redox status of mussels exposed to nanoparticles and of those exposed to diuron, the activities of SOD, CAT and GST, respectively, were measured in the digestive gland and gills after exposure to similar concentrations of TiO$_2$ and ZnO NPs. Both nanoparticles did not affect significantly the activities of biomarkers in both tissues of interest when exposed to a concentration of 50 $\mu$g l$^{-1}$ compared with control. In contrast, the SOD and CAT activities increased significantly after exposure to 100 $\mu$g l$^{-1}$ of TiO$_2$ and ZnO NPs in the digestive gland and no significant effect was observed in the gills exposed to 100 $\mu$g l$^{-1}$ of TiO$_2$ NPs compared to control.

The GST activity and LPO level increased in the digestive gland and gills only after exposure to 100 $\mu$g l$^{-1}$ of ZnO NPs. However, no significant modification were observed after exposure to similar concentrations of TiO$_2$ NPs compared to control.

The combined effects of diuron and the two NPs in different redox markers showed concentration-dependent effects. The mixture of diuron with TiO$_2$ and ZnO NPs at 50 $\mu$g l$^{-1}$ did not show a significant effect for all tested markers in the digestive gland and gills. Similarly, no significant changes were observed after exposure to the mixture of 100 $\mu$g l$^{-1}$ of diuron with TiO$_2$ NPs (TiO$_2$ NP2 + Di2) in digestive gland and gills compared with control. Nevertheless, the mixture of 100 $\mu$g l$^{-1}$ of diuron with ZnO NPs (ZnONP2 + Di2) increased significantly the activities of SOD, CAT and GST and level of LPO in the digestive gland and gills compared to control (figures 5(A)–(D)).

### 4. Discussion

The current experiment provided the first experimental evidence of a physiological response and oxidative stress-related effect initiated by diuron herbicide, either as a single contaminant or in combination with two NPs type; TiO$_2$ and ZnO NPs, in the Mediterranean mussel *M. galloprovincialis*.

The physiologic measurements provided a clue of the likely consequences of these environmental stressors within a marine population and considered as successful for further quantifying the impact of chemicals on bivalve molluscs [50, 51]. The focus on the feeding activity of bivalves is trendy in laboratory tests, including parameters such as the filtration capacity and respiration rate as indicators of chemical toxicity [52]. Moreover, the filtration capacity and respiration rate cover crucial physiological parameters to explain the short-term adaptations of mussel feeding in a stressful environment.

In the current experiment the diuron impacts adversely the physiologic status of the mediterranean mussel *M. galloprovincialis*. Furthermore, the reduction was potentially related to the accumulation of diuron in the tissues of the targeted mussel, leading to a decrease in oxygen uptake aerobic cellular energy production, as it was hypothesized for other organisms exposed to environmental stressors [53, 54]. Our results are in good agreement with a previous study, which reported a reduction of feeding rate in the mussel *Mytilus edulis* directly with the exposure to pesticides [51].

In the present study, exposure to ZnONP2 and the combination of ZnO NPs with diuron at 100 $\mu$g l$^{-1}$ decreases the filtration capacity and respiration rates. Similarly, [52] observed a decrease in filtration capacity and respiration rate in clams exposed to TiO$_2$ NPs and AuTiO$_2$ NPs. This author suggested that NPs can reduce the capacity of gills to consume oxygen and to clear food particles. Additionally, the physiological changes could be correlated to the accumulation of NPs in tissues, thus impeding the normal beat rhythm of cilia and muscular changes in gills, which in turn are controlled by the nervous system.

Our observations indicated that *M. galloprovincialis* was not capable to maintain its normal aerobic metabolism following exposure to diuron and ZnO NPs, potentially due an energy-saving mechanism employed to reduce their activity whenever exposed to environmental stressors. However, the normal physiologic response observed after exposure to TiO$_2$ NPs and the mixture of TiO$_2$ NPs with diuron, confirms that the toxic effect is intimately dependent to the type of contaminant employed, suggesting toxicologic interactions between...
the diuron and TiO$_2$ NPs. Furthermore, the physiological response observed in the current experiment could be related to the size of NPs, as suggested previously by [55].

Because of their chemical proprieties, the pesticides accumulate in numerous marine species, comprising invertebrates and leading to oxidative stress [56, 57]. In the present work, the relation between the enzymatic activities and diuron concentrations could explain the bioavailability of this herbicide in the digestive system and the gills of this mussel.

Concerning the oxidative stress markers, the exposure to diuron triggered an oxidative stress in the digestive gland and gills, in a concentration–response manner, given that the activities of SOD, CAT and GST increased significantly after the exposure to diuron, reaching a maximum after the exposure to the highest concentrations of the pesticide. These results emphasise a potentia increase in ROS production, leading to modified antioxidant responses, given that both SOD and CAT are involved in the detoxification of free radicals [58]. Similarly, [59] observed an increase in antioxidant enzymes’ activities after exposure to diuron, suggesting that the contaminant interfered with the antioxidant defense enzymes, triggering the ROS production. The concentration–response of antioxidant enzymes observed in the digestive gland and gills to the tested contaminant observed in this experiment indicate that diuron could exert similar toxic mechanisms related to oxidative stress pathway in the analyzed organs.

The ROS over-production in mussels could lead to oxidative damage caused by the imbalance of reactive oxygen. The ROS, if not properly scavenged, can disturb the cellular redox balance, leading to oxidative damage of lipids and membranes [60]. In the present experiment, increased LPO induction after short-term exposure of mussels to diuron may be caused by excessive amounts of ROS and non-compensatory action of the antioxidant system against the oxidative stress.

Alternatively, the LPO induction after diuron exposure could be related to the direct toxic action on membranes. Thus, the contaminants impact cells’ membranes, by dissolving the portion of lipid from within [61] or by intercalating with the lipidic bilayer [62].

Another finding of this laboratory experiment is that the characterisation of NPs proved that both TiO$_2$ and ZnO NPs have sizes within those previously reported [36, 37]. The addition of NPs in seawater induced small changes in the aggregation status of these NPs due to the large negative charge in seawater compared to distilled water, associated with the presence of (in) organic ligands such as polysaccharides, sulphides, and NOM [63, 64]. In addition, the increasing salinity, and therefore ionic strength in seawater, reduces the negativity of electrophoretic mobility of the particles to encourage aggregation. Moreover, the zeta potential results of TiO$_2$ and ZnO NPs are in agreement with [26] findings, suggesting the stability of NPs in the environment. The stability of colloidal solutions, depending to the surface charge of nanoparticles, was determined through the zeta potential analysis. In general, the particles stay away each to each other, without forming aggregates, whenever the particles from a colloidial solution have large negative or positive zeta potential values (i.e. greater than +50 mV or smaller than −50 mV) [65].

Furthermore, the exposure of mussels to NPs showed different responses directly with the concentrations, organs and types of nanoparticles employed. The SOD, CAT and GST activities showed the ability of mussels to avoid oxidative damage through the antioxidant defense system at 50 µgl$^{-1}$. The antioxidant defense system can scavenge reactive oxygen free radicals and antioxidant enzymes, including SOD, CAT and GST, hence playing an important role in this process.

The concentration 100 µgl$^{-1}$ of TiO$_2$ NPs induced oxidative stress, but did not led to lipid oxidative damage. This result is related to the induction of anti-oxidant enzymes that likely are produced in enough concentration to prevent the occurrence of lipid oxidative damage. These results confirm a previous finding, related to the response of zebrasfish Danio rerio larvae exposed to 100 µgl$^{-1}$ of TiO$_2$, which did not damaged the biomolecules [26]. In contrast, the exposure to 100 µgl$^{-1}$ of ZnO NPs induced anti-oxidant enzymes and lipid damage, suggesting that toxicity is dependent to the type of NPs used. Similarly, elevated enzymatic activity of catalase and superoxide dismutase were found in the gills and the digestive gland of the carpet clam Ruditapes philippinarum after 7 days of exposure to ZnO NPs [66]. These findings indicate that the up-regulation of the antioxidant system of marine bivalves might counteract the ROS production and to prevent the oxidative injury following exposure to nano-ZnO. Our results are concurrent with previous studies, which indicated that the response of biomarkers to NPs contamination was contaminant type and concentration specific [52, 67]. Moreover, the response of mussel was dependent to the size of NPs; lower dimensions of ZnO NPs proved to be the most toxic for mussels. This finding was also discovered by [68], which demonstrated that smaller sizes induced the highest contents of intracellular reactive oxygen species, resulting in membrane damage and internalization. Reduced size means more surface/volume area and more bonding sites for molecules attachments at the surface of any given type of nanoparticle [69, 70]. This aspect increases their reactivity and makes them more miscible compared to the bulk material. The size effect governs not only the penetration process, but also the distribution of particles within tissues.
The GST was previously associated with the metabolism of pollutants in bivalves and identified as a potential metabolic pathway for NPs [71], concurrent to the increase in GST activity as a response to response to ZnO NP2 = 100 μg l−1 exposure.

Alternatively, the exposure to a mixture of diuron with ZnO NPs and TiO2 NPs showed different interaction pathways in gills and digestive gland. This result is in agreement with [72], which reported that the toxicity of ZnO was modulated by the presence of organic pollutants.

In parallel, the mixture of diuron with ZnO NPs at 50 μg l−1 and with TiO2 NPs at 50 μg l−1 and 100 μg l−1 potentially caused a mutual cancelation of toxic effects compared to diuron alone. Overall, the results for the mixture group suggest potential interactions between these substances in the tissues of this species of mussel. Similar findings to ours, namely that co-exposure induced mutual cancelation for the freshwater mussel Unio tumidus in response to titanium oxide nanoparticles and Bisphenol A suggest that antagonistic relationship between these two substances [73].

5. Conclusions

The findings of this laboratory experiment comprise the first reported evidence of a toxic effect induced by diuron in the presence of ZnO and TiO2 nanoparticles. From a physiologic perspective, the exposure to diuron alone impacted negatively the filtration rate and the respiration capacity. In contrast, this response was not visible following exposure neither to TiO2 NPs, nor to the combination of diuron with TiO2 NPs, suggesting antagonistic interactions between these two compounds.

From the redox perspective, our results confirm that the antioxidant defense mechanisms is complex and a potential early biomarker of the damage induced by exposure to this herbicide and nanoparticles. These observations open a window for further studies, aiming to deepen the understanding of the efficiency degradation mechanisms of herbicides via nanoparticles. Consequently, the development of remediation technologies NPs based calls for further studies focused on the effects of NPs alone and mixed with organic pollutants in different sentinel species.

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Data availability statement

The data generated and/or analysed during the current study are not publicly available for legal/ethical reasons but are available from the corresponding author on reasonable request.

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