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Original article

Impacts of nanoparticles and phosphonates in the behavior and oxidative status of the mediterranean mussels (*Mytilus galloprovincialis*)

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ABSTRACT

The current study investigated the exposure of the Mediterranean mussel (*Mytilus galloprovincialis*) to gold nanoparticles decorated zinc oxide (Au-ZnO NPs) and phosphonate [Diethyl (3-cyano-1-hydroxy-1-phenyl-2-methylpropyl)] phosphate (PC). The mussels were exposed to concentrations of 50 and 100 μg L^{-1} of both compounds alone, as well as to a mixture of both pollutants (i.e. Mix). The singular and the combined effect of each pollutant was investigated by measuring the concentration of various metals (i.e., Cu, Fe, Mn, Zn and Au) in the the digestive glands and gills of mussels, their filtration capacity (FC), respiration rate (RR) and the response of oxidative biomarkers, respectively, following 14 days of exposure. The concentrations of Cu, Fe, Mn, Zn and Au increased directly with Au-ZnO NPs in mussel tissues, but significantly only for Zn. In contrast, the mixture of Au-ZnO100 NPs and PC100 did not induce any significant increase in the content of metals in digetsve glands and gills, suggesting antagonistic interactions between contaminants. In addition, FC and RR levels decreased following exposure to Au-ZnO100 NPs and PC100 treatments and no significant alterations were observed after the exposure to 50 μg.L^{-1} of both contaminants and Mix. Hydrogen peroxide (H_2O_2) level, GSH/GSSG ratio, superoxide dismutase (SOD), catalase (CAT) and acetylcholinesterase (AChE) activities showed significant changes following the exposure to both Au-ZnO NPs and PC, in the gills and the digestive glands of the mussel. However, no significant modifications were observed in both organs following the exposure to Mix. The current study advances the understanding of the toxicity of NPs and phosphonates on *M. galloprovincialis* and sets the path for future ecotoxicological studies regarding the synergic effects of these substances on marine species. Moreover, the current experiment suggests that the oxidative stress and the neurotoxic pathways are responsive following the exposure of marine invertebrates to both nanoparticles and phosphonates, with potential antagonist interactions of these substances on the physiology of targeted species.

1. Introduction

Most human settlements are based within a 100 km range from coastlines. Coastal ecosystems represent as important endpoint for contaminants generated by human activities. In the absence of suitable legislation, these habitats experienced significant degradation in time, leading to a reduction of their productivity (Madeira et al., 2018; Hou et al., 2019; Sutton-Grier and Sandifer, 2019; Vieira et al., 2020; Saidani et al., 2021).

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Many pollutants induce molecular and structural changes within living biota, with a negative impact on the structure and functionality of marine ecosystems (Geret et al., 2003). The nanoparticles (NPs), for example, comprise an emerging class of anthropogenic pollutants, with multifaceted interactions within the marine realm (Hou et al., 2019; Saidani et al., 2019). NPs are widely employed in many industrial sectors, such as in the production of biosensors, electronic catalysts, paints, and cosmetics. The production of NPs at industrial scale and their heavy usage raised many concerns regarding the ecologic impact following their release into marine habitats. ZnO nanoparticles are among the most widely used type of NPs (Yung et al., 2015). Their chemical stability and presence under various forms and surfaces contributes to their persistence in ecosystems (Montes et al., 2012). In addition, the decoration of ZnO NPs with gold is considered non-toxic, given their molecular stability (Choi et al., 2008). Nanoparticles end up in marine ecosystems through various pathways, such as via aerial deposition, effluents, dumping or transported by rivers. Following their arrival in marine ecosystems, they accumulate further in sediment and biota, inducing negative effects (Gottschalk et al., 2009; Vale et al., 2016; Li et al., 2017; Hou et al., 2019). Recently, the assessment of their toxicity on aquatic organisms was extensively investigated, revealing that the oxidative stress is a common endpoint as response to nanotoxicity (Fkiri et al., 2018; Saidani et al., 2019). The phosphonates (PC) are widely used for various industrial applications due to their high stability, corrosion inhibition and dispersing properties (Gledhill et al., 1992; Knepper, 2003). They are used in paper and textile industries and comprise an essential ingredient for many industrial cleaning products. Furthermore, they are used in membrane technology, as hardness stabilizers in cooling systems, in metal industry, oil production, medical applications, and cement modification. It was estimated that the industrial production of phosphonates reached 94,000 t in 2012 (EPA, 2013). The hydroxyphosphonates are well-known for their antioxidant, antiviral and anticancer properties. These products are characterized by their ability to complex with metal compounds and to develop a high antiradical capacity, by reducing the activity of Diphenyl picrylhydrazyl (DPPH) (Aouani et al., 2015). Although the understanding of both direct and indirect ecotoxicological effects of NPs and PC for marine biota was improved recently, most studies tested the effects of these two compounds alone, but not their combined impact.

The bivalve mollusks comprise excellent sentinel species in ecotoxicological assessments. They are abundant, of sufficient size to carry out analyzes, easy to sample and important from an economic and ecologic perspective. Given their sessile or sedentary life-style, as well as their suspension feeding strategy, the bivalve mollusks represent ideal candidates for studying the toxic effects induced by exposure to organic and metal pollutants (Canesi et al. 2012; Corsi et al., 2014). Bivalves concentrate various dissolved and suspended contaminants from water, that otherwise are found at very low concentrations in environment (Gomes et al., 2011, Canesi et al., 2012, Thio et al., 2012). Moreover, bivalves are routinely used to estimate the quality of coastal ecosystems during biomonitoring programs (Zuykov et al., 2013; Sellami et al., 2017).

In general, the first important impact of a pollutant occurs at molecular level, by altering the functions of biomolecules, such as in the case of fatty acids (e.g., in biomembranes, nucleic acids and proteins). Recent ecotoxicological studies measured the molecular changes or the response of various biomarkers in sentinel species. The biomarkers comprise observable and/or measurable changes at molecular, biochemical and cellular level, and usually they emphasize, at an early stage, the toxic effects of pollutants (Lagadic et al., 1997, Long et al., 2004). The most frequently measured biomarkers in bivalves involved in ecotoxicological studies are i) the antioxidant defense biomarkers, which are related to oxidative stress (Monserrat et al., 2007) and ii) the cholinesterases (ChE), mainly the acetylcholinesterase (AChE), which is an indicator of neurotoxic effects (Matozzo et al., 2005). The oxidative stress occurs whenever the amount of reactive oxygen species (ROS) exceeds the antioxidant defense capacity. Excessive ROS production induces direct oxidation of proteins and lipids, altering the redox status of cells and influencing the capacity for gene expression (Livingstone, 2003). The enzymatic defense system comprises the catalase (CAT) and superoxide dismutase (SOD), emphasizing their suitability as stress biomarkers for various aquatic communities (Lionetto et al., 2003). The measurement of the AChE activity is important for assessing the effects caused by neurotoxic compounds for marine biota. The AChE plays a key role in the neuromuscular system, by preventing incontrollable muscle contractions. This enzyme catabolizes the transformation of the neurotransmitter acetylcholine into choline in the cholinergic synapses; its inhibition causes acetylcholine accumulation, the hyperpolarization of the postsynaptic membrane, and interrupts the neural transmission. For these reasons, the inhibition of this enzyme can cause physiologic changes, leading in extreme cases to paralysis and death for marine organisms (Fulton and Key, 2001). In the current study we evaluated for the first time the toxicity of NPs (i.e. gold nanoparticles decorated zinc oxide, hereafter Au-ZnONPs) and phosphonate (i.e. diethyl (3-cyano-1-hydroxy-1-phenyl-2-methylpropyl) phosphate (PC) on the mussel *M. galloprovincialis*. Both chemical and biological approaches were employed to deepen the understanding of their toxic effects, as well as of their interactions.

2. Material and methods

2.1. Synthesis of Au-ZnO nanoparticles

The production of Au-ZnO nanoparticles was achieved with one-pot chemical synthesis according to Fkiri et al. (2017), in which $HAuCl_4{}^*3H_2O$ gold (III) chloride (Sigma-Aldrich, $\geq$ 99.9%), $Zn(OAc)2{}^*2H_2O$ zinc acetate dihydrate (Sigma-Aldrich, ACS reagent, $\geq$ 98%) were mixed in 50 mL of 1.3-propandiol solvent ($C_3H_8O_2$, Aldrich, 98%). The mixture was kept under reflux at 160 °C for 60 min. The sequential reactions were thermally controlled and the obtained precipitate was centrifuged (3000 $\times$ *g*), washed several times with ethanol, and dried at 100 °C overnight to produce a solid powder.

2.2. Sample collection and contamination

Mussels were collected from Bizerte lagoon (Tunisia, between latitude 37° 8′ and 37° 16′ N and longitude 9° 46′ and 9° 56′ E). This lagoon covers an area of approximately 150 km^2 and has an average depth of 8 m. A total of 150 mussels (*M. galloprovincialis*) of similar shell length (45–55 mm) were collected, followed by the polishing of the periostracum with polypropylene plaques to remove epiphytes. They were then distributed in 3L glass tanks and acclimated for seven days on a 12 h light/dark cycle prior to exposure. During the experimental period, 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 at 32‰. Tanks were filled up to level with sea water and changed every 48 h. After seven days of acclimatization, six experimental conditions were set up in five replicates, each replicate comprising five mussels: control, Au-

ZnONP50, Au-ZnONP100, PC50, PC100 and Mix (Au-ZnONP100 + PC100). The control mussels were not exposed to any stressor, whereas the exposed mussels were subjected to daily concentrations of NPs at 50 $\mu g.L^{-1}$ Au-ZnONPs, 100 $\mu g.L^{-1}$ Au-ZnO NPs, 50 $\mu g.L^{-1}$ PC, 100 $\mu g.L^{-1}$ PC and 100 $\mu g.L^{-1}$ of both Au-ZnO NPs and PC in seawater, supplied altogether for a period of 14 days. Au-ZnO NPs and PC concentrations were selected based on environmentally relevant concentrations of NPs and PC (Gottschalk et al., 2009; Luo et al., 2011; Wang et al., 2019; Li et al., 2017; Saidani et al., 2021). After 14 days of exposure, no mortality was observed and all specimens were seen to be feeding normally.

2.3. The characterization of nanoparticle

The crystallographic structure of the obtained powders was investigated by X- ray diffraction patterns (XRD) (i.e. an INEL diffractometer using a cobalt Kα radiation (λ = 1.7890 Å)). The morphological details of the synthesized gold particles were characterized by transmission electron microscopy (TEM) (JEOL). Energy-dispersive X-ray spectrograph (EDX) attached to the TEM was used for elemental analysis. Dynamic light scattering (DLS) of Au-ZnO NPs in the seawater after 14 days of exposure was measured using an Amtec SM 200 Zetasizer operating with a He–Ne laser (632.8 nm).

2.4. Filtration capacity (FC) and respiration rates (RR)

Measurements of filtration rate *(FR)* were performed according to Coughlan, (1969) (i.e. loss of neutral red dye particles) in closed chambers. Following the exposure, five mussels from each treatment were placed in 200 mL beakers (1 clam per beaker) containing 100 mL of neutral red solution (1 $mg.L^{-1}$). Prior to placing the mussels in the solution, an aliquot of water was removed from each beaker to determine the initial concentration, *C0*. After 2 h the mussels were removed and the remaining solution, *(Ct),* along with the initial aliquot *(C0),* were acidified to pH 5 with HCl 5%. The concentrations of neutral red were measured through 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:

$$FR = [M/nt]\log(C0/Ct) \quad (1)$$

where *FR* is the filtration rate ($mg.indiv^{-1}.h^{-1}$), M is the total volume of water, *n* the number of employed mussels, *t* the time in h and *C0* and *Ct* the concentration values between two sampling times. The respiration rate (RR) was measured on five mussels with calibrated oxygen electrodes connected to an oximeter, Basti et al. (2016). The decline in oxygen concentration was measured every half hour for three hours and calculated using the following equation:

$$RR = [Ct0 - Cti] \times V/(ti - t0) \quad (2)$$

where *RR* is the respiration rate (mg O_2 h^{-1}), *Ct* is the concentration of dissolved oxygen (mg O_2 l^{-1}) at time *t* (t0: initial time and ti: end time h) and *V* is the volume of the total solution in the sealed chamber.

2.5. Metals analyses

Mussels (n = 5) were collected from each experimental condition after 14 days of exposure and the concentration of metals measured. Samples were dried at 105 °C with a microwave to constant mass, and then their dry mass was determined gravimetrically with a balance. The digestion was performed by adding 69% HNO_3 (3 mL), 30% H_2O_2 (3 mL) and H_2O (1 mL) to each sample. The digestion was operated in microwave at 720 W for 20 min. After digestion, extracts were transferred into a graduated polypropylene test tube and diluted with ultrapure water to 50 mL (Talarico et al., 2014). The metal content (i.e., Cu, Fe, Mn, Zn and Au) was analyzed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) using reference material of mussel tissue (CRM 278). The detection limits for Cu, Fe, Mn, Zn and Au were in the 0.002–25 $mg.L^{-1}$ range. Values reported were corrected for background levels determined in blank sterile filtered seawater.

2.6. Biomarker measurements

Mussels (n = 10) were removed at the end of experiment from each experimental condition and fixed in liquid Nitrogen. Gills and digestive glands were removed 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 both cytosol and microsomes, was removed and used to determine H_2O_2 level, GSH/GSSG ratio, SOD, CAT and AChE activities·H_2O_2 levels were measured following the method of Wolff (1994): a volume of 0.1 mL of the supernatant was added to 900 mL of FOX1 reagent (100 mM xylenolorange, 100 mM sorbitol, 250 mM ammonium ferrous sulfate and 25 mM H_2SO_4), vortexed and incubated at room temperature for 30 min. The sample was then centrifuged at 3.000g for 3 min and the absorbance of the supernatant read at 560 nm.

The GSH/GSSG ratio was measured spectrofluorimetric, according to Hissin and Hilf (1976). The GSH and GSSG were calculated from a calibration curve and standards. The fluorescence at 420 nm was assessed with excitation at 350 nm and SOD activity measured as the ability of the enzyme to inhibit auto-oxidation of pyrogallol (Marklund and Marklund, 1974). CAT activity was measured by the decrease in absorbance at 240 nm, due to H_2O_2 consumption, according to Aebi (1974). The reaction volume and reaction time were 1 mL and 1 min, respectively. The reaction solution contained 80 mM phosphate buffer, pH 6.5 and 50 mM H_2O_2. Specific SOD and CAT activities are given in text as $nMol.min^{-1}.mg^{-1}$ protein.

AChE activity was determined by measuring the absorbance for 5 min at 412 nm in the presence of 1 mM acetylthiocholine (Ellman et al., 1961) and its activity expressed as $\mu mol.min^{-1}.mg^{-1}$ protein. The protein content was measured according to the (Bradford, 1976), by using bovine serum albumin (BSA) as standard.

2.7. Statistics

Statistical analysis was carried with software STATISTICA 8.0. The measured parameters were reported as mean ± standard deviation. The variation of each parameter among control and treatments was tested with one-way ANOVA ($p < 0.05$), following data transformation and checking for their normality and homogeneity of variances, followed by *post-hoc* Tukey's tests.

3. Result

3.1. The structure of nanoparticles

The crystalline phase of the synthesized powder was determined by XRD. Fig. 1a shows the XRD patterns of Au-ZnO nanoparticles. The diffraction peaks of Au-ZnO can be indexed to hexagonal wurtzite ZnO with the strong (100), (002) and (101) characteristic peaks (space group P63mc, JCPDS No. 36–1451) (Mezni et al., 2014, Fkiri et al., 2017). For the Au–ZnO nanocomposites, besides the diffraction peaks of ZnO nanoparticles, two additional small-intensity diffraction peaks were observed at 2θ = 38.3° and 44.2°

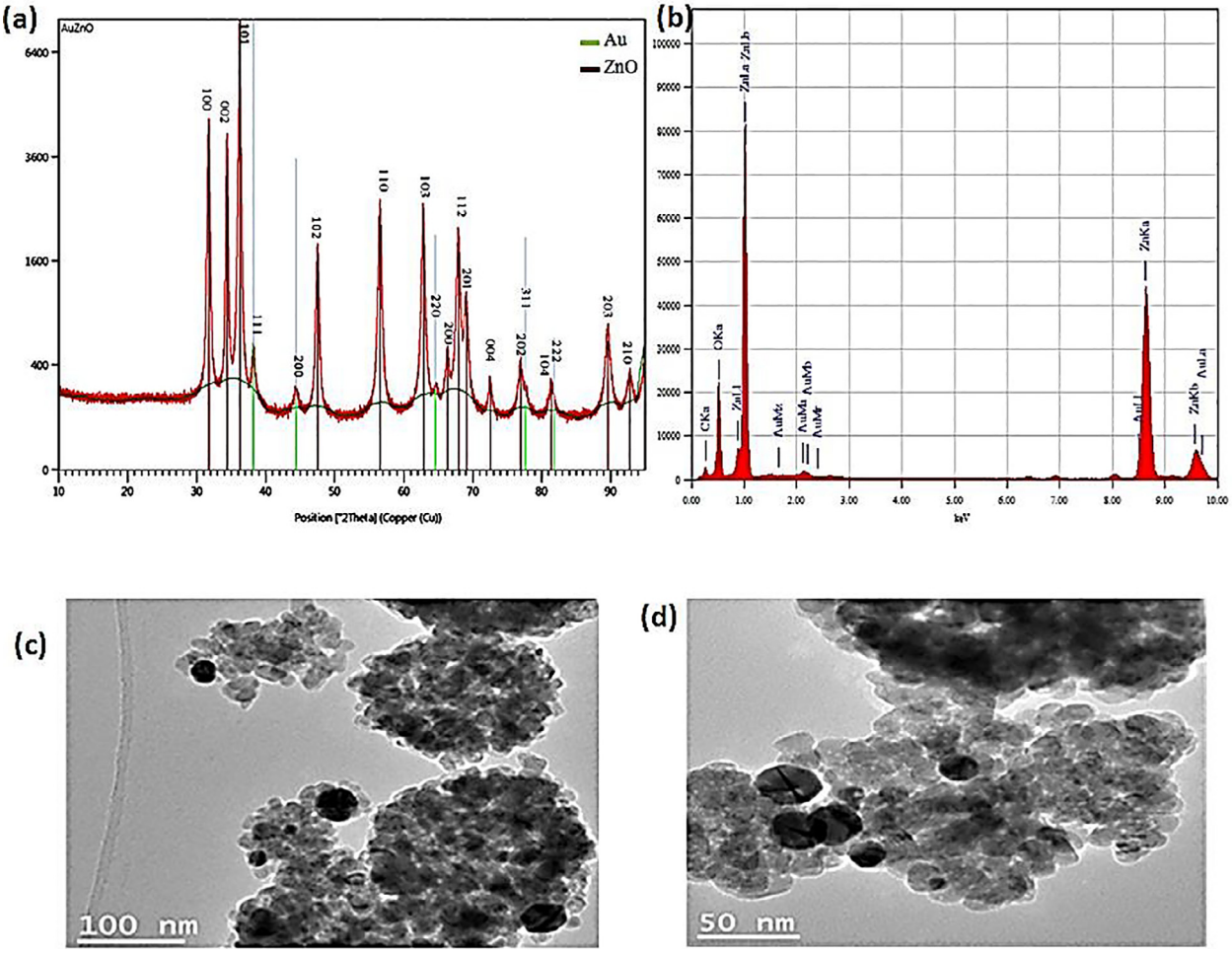

Fig. 1. XRD patterns (a), EDX spectrum (b), TEM image in distilled water (c), and TEM image in sea water (d) of ZnO-decorated AuNPs.

and assigned to the diffraction of (1 1 1) and (2 0 0) planes of face-centered cubic Au NPs, respectively (JCPDS No. 65–2870).

The morphology of the as-synthesized powder nanoparticles was determined by TEM in distilled water and sea water, respectively (Fig. 1c, d). Fig. 1 shows also that the Au-ZnO NPs were composed mainly of faceted quasi-spherical Au cores (around 20–50 nm) surrounded by closely packed ZnO NPs (8–15 nm), so that the materials could be seen as small-size ZnO NPs decorating large-size Au NPs (Au-ZnO). The high purity of the synthesized NPs was confirmed by the EDAX spectrum (Fig. 1b).

According to DLS data (Fig. 2), the Z-average particle diameter in seawater is Diam = 82.62 nm and the average relative half width of the distribution is $\sigma = 10 \pm 1$ nm. The position of the major peak of the scattered intensity distribution ($d \approx 82$ nm) does not exceed the size dispersion obtained from TEM images.

3.2. Metal levels in contaminated mussels

The Cu, Fe, Mn, Zn and Au concentrations in solution increased directly with the addition of the Au-ZnO NPs (Table 1). The Zn level increases significantly ($p < 0.001$), from 0.07122 ± 0.02 $\mu g.kg^{-1}$ to 1.208 ± 0.043 $\mu g.kg^{-1}$ after exposure to Au-ZnONP50 and reaching a maximum of 2.312 ± 0.129 $\mu g.kg^{-1}$ after exposure to Au-ZnONP100. Similarly, Au concentration increases directly with Au-ZnONPs concentration and reached a maximum of 0.436 ± 0.015 $\mu g.kg^{-1}$ after exposure to Au-ZnONP100.

Similar trends were observed for other metals, like iron, manganese and copper after exposure to both Au-ZnONP50 and Au-ZnONP100. However, no significant modification ($p > 0.05$) of metals was observed in mussels treated with PC, whatever the employed concentrations. Overall, the results seem to indicate that the presence of PC significantly affected the accumulation of essential metals by bivalves following exposure to NPs.

3.3. Physiological parameters and biomarkers responses

The filtration capacity (FC) and respiration rate (RR) of mussels exposed to Au-ZnO NPs and PC are presented in Fig. 3. No significant modifications ($p > 0.05$) were noticed for both FC and RR, after the exposure to Au-ZnONP50 and PC50. In contrast, a significant reduction was observed on FC and RR after 14 days of exposure to Au-ZnONP100. FC decreased from 120.8 ± 2.58 $mg.ind^{-1}.h^{-1}$ in the control group to 67.6 ± 7.92 $mg.ind^{-1}.h^{-1}$ after exposure to Au-ZnONP100. RR decreased significantly ($p = 0.0001$) from 0.754 ± 0.01 mg $O_2.h^{-1}$ to 0.248 ± 0.025 mg $O_2.h^{-1}$ after exposure to Au-ZnONP100.

The exposure to both Au-ZnONPs and PC at 100 $\mu g.L^{-1}$ did not significantly; ($p > 0.05$) affect the physiological parameters.

The antioxidant parameters of control and treated mussels are presented in Fig. 4. Significant differences were observed in H_2O_2 levels, GSH/GSSG ratios, SOD and CAT activities, after NPs treatment (Fig. 4).

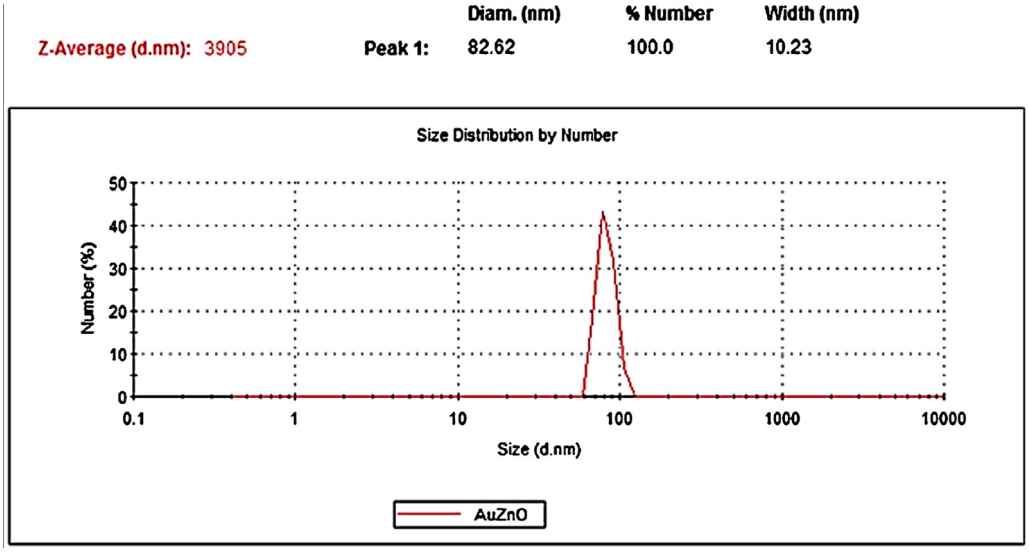

Fig. 2. Dynamic light scattering (DLS) of Au-ZnO nanoparticles dispersed in seawater.

Table 1
Metals level (mg/Kg DW) in the mediterranean mussels *Mytilus galloprovincialis* (n = 5) untreated (Control) and treated with two concentrations of Au-ZnO nanoparticles (Au-ZnONP50 = 50 μg/L and Au-ZnONP100 = 100 μg/L), two concentrations of phosphonates (PC50 = 50 μg/L and PC100 = 100 μg/L) and the mixture (MIX = Au-ZnONP100 + PC100). * and ** indicate significant effect at $p < 0.05$ and $p < 0.01$, respectively. ND: not detected.

Metals	Control	Au-ZnONP50	Au-ZnONP100	PC50	PC100	MIX
Cu	0.025 ± 0.017	0.031 ± 0.015 *	0.036 ± 0.013 *	0.025 ± 0.002	0.028 ± 0.003	0.030 ± 0.004
Fe	0.058 ± 0.016	0.083 ± 0.049*	0.0842 ± 0.018*	0.055 ± 0.022	0.057 ± 0.027	0.056 ± 0.019
Mn	0.075 ± 0.001	0.086 ± 0.015*	0.0876 ± 0.012*	0.074 ± 0.004	0.074 ± 0.003	0.071 ± 0.087
Zn	0.07122 ± 0.025	1.208 ± 0.043*	2.312 ± 0.129**	0.0716 ± 0.011	0.0816 ± 0.16	1.174 ± 0.068*
Au	ND	0.175 ± 0.001*	0.436 ± 0.015**	ND	ND	0.412 ± 0.018**

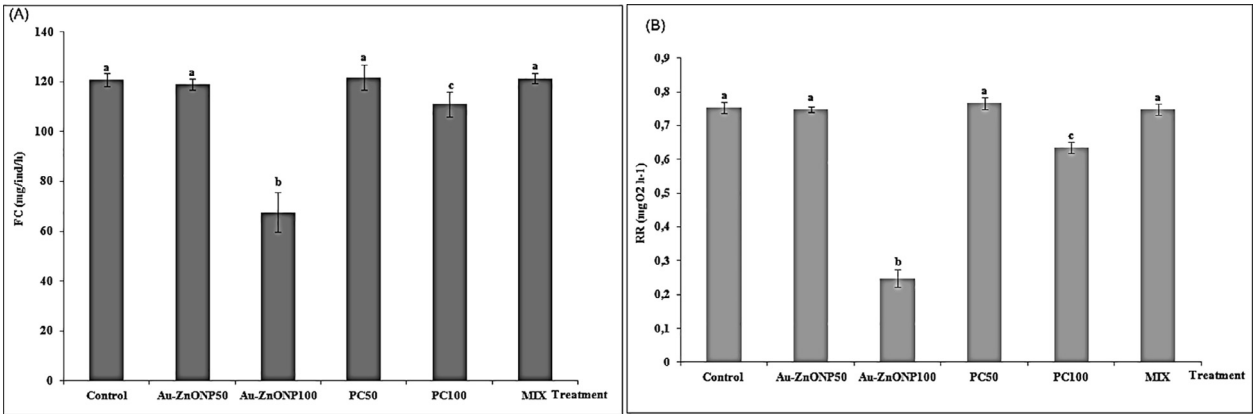

Fig. 3. Filtration capacity (FC) and respiration rates (RR) in the Mediterranean mussels *Mytilus galloprovincialis* (n = 5) untreated (Control) and treated with two concentrations of Au-ZnO nanoparticles (Au-ZnONP50 = 50 μg/L and Au-ZnONP100 = 100 μg/L), two concentrations of Hydroxyphosphonates (PC50 = 50 μg/L and PC100 = 100 μg/L) and the mixture (MIX = Au-ZnONP100 + PC100). a, b and c: different letters indicate significant effect at $p < 0.05$.

The H_2O_2 level increased significantly ($p < 0.001$) from 0.09 ± 0.001 mM to 0.27 ± 0.028 mM in gills after Au-ZnONP50 and reached a maximum of 0.86 ± 0.02 mM in the same organ after Au-ZnONP100 treatment. However, no significant modification was observed in the digestive gland after exposure to 50 $\mu g.L^{-1}$ of Au-ZnONPs (Fig. 4A).

Additionally, the PCA ordination suggests a correlation between metals and biomarker responses, including the H_2O_2 level (Fig. 5). According to the PCA ordination plot, the NPs toxicity is related to the concentration responsible for metallic deregulation in gills and digestive gland (Fig. 5).

The SOD activity in the mussels' tissues increased significantly in the 50 and 100 $\mu g.L^{-1}$ Au-ZnONPs groups (Fig. 4B). Moreover, the GSH content in the treatments with an Au-ZnONPs and PC concentration of 100 $\mu g.L^{-1}$ was found to increase significantly () in gills (Fig. 4).

The exposure of mussels to 50 and 100 $\mu g.L^{-1}$ of Diethyl (3-cyano-1-hydroxy-1-phenyl-2-methylpropyl) phosphate (PC) did not affect the GSH/GSSG ratio, SOD and CAT activities and the H_2O_2 level in the digestive gland (Fig. 4). However, the concentration 100 $\mu g.L^{-1}$ induced oxidative stress in gills, showing the tolerance threshold of this organ to this product.

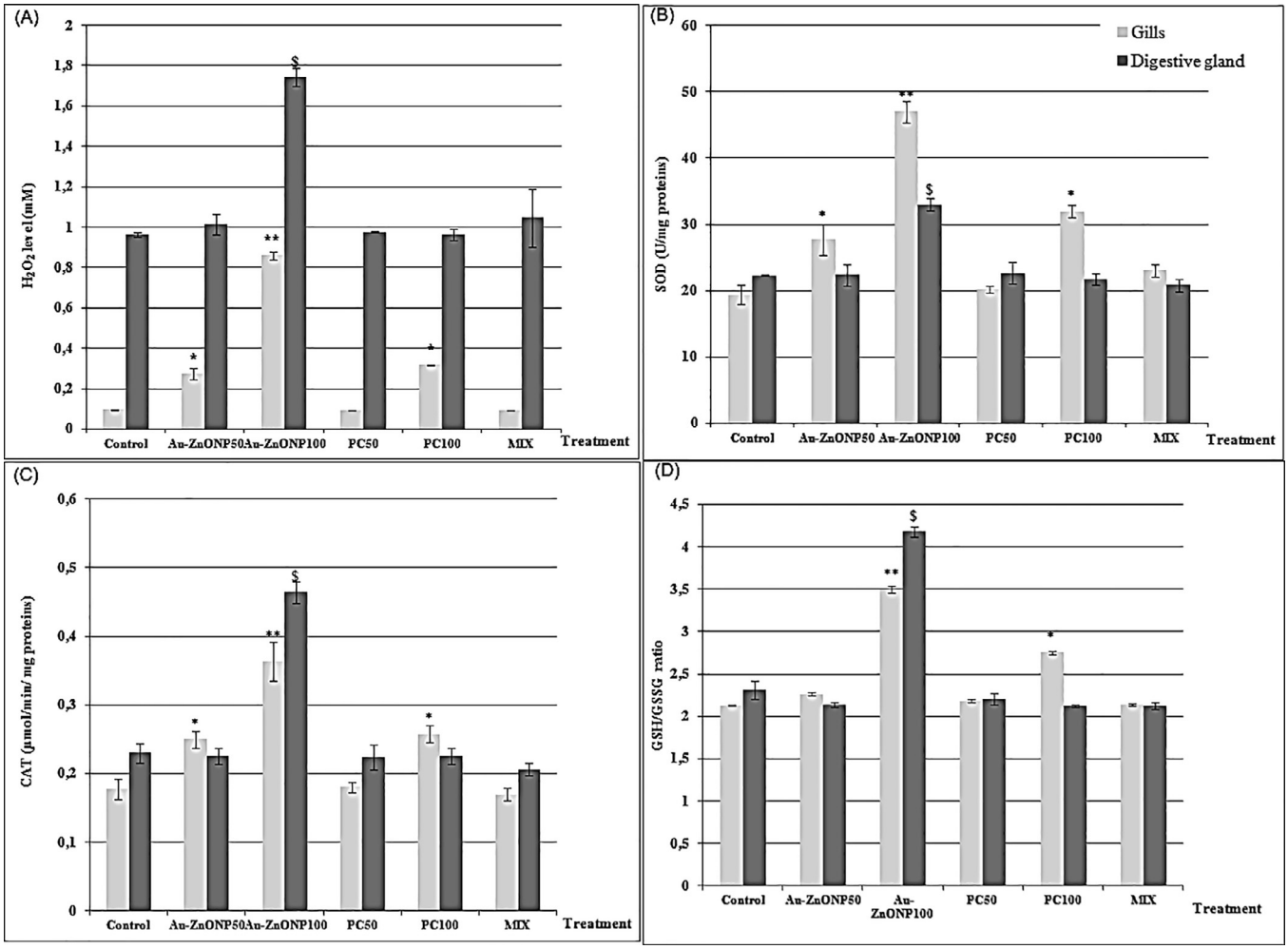

Fig. 4. (A) H_2O_2 level, (B) superoxide dismutase (SOD), (C) catalase (CAT) and (D) GSH/GSSG ratio in the gills and digestive gland of the Mediterranean mussels *Mytilus galloprovincialis* (n = 10) untreated (Control) and treated with two concentrations of Au-ZnO nanoparticles (Au-ZnONP50 = 50 µg/L and Au-ZnONP100 = 100 µg/L), two concentrations of Hydroxyphosphonates (PC50 = 50 µg/L and PC100 = 100 µg/L) and the mixture (MIX = Au-ZnONP100 + PC100). * and **: indicate significant effect respectively at $p < 0.05$, $p < 0.01$ in the gills. $: indicate significant effect at $p < 0.05$ in the digestive gland.

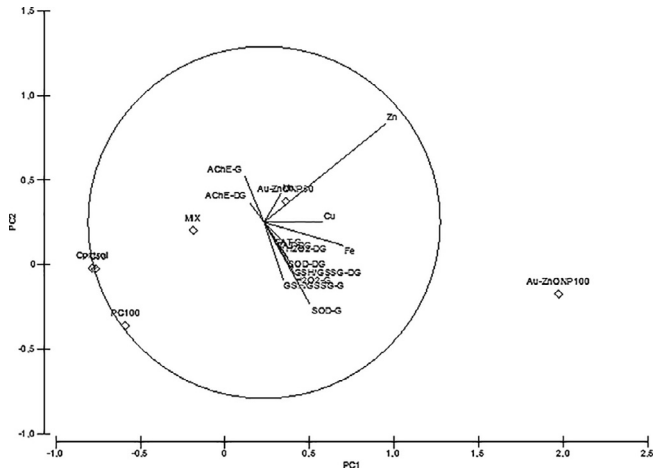

Fig. 5. Variable distribution plots assayed by the principal component analysis (PCA) between essential metals, concentration of contaminants and all measured biomarkers.

The co-exposure to 100 µg.L^{-1} of Au-ZnONPs and 100 µg.L^{-1} of PC (Mix) did not affect the H_2O_2 level, GSH/GSSG ratio, SOD and CAT activities.

AChE activity was modulated in response to AuZnONP100 and PC100 in the gills (Fig. 6). The activity of this biomarker decreased significantly ($p < 0.001$) from 8.13 ± 0.03 µmol.min^{-1}.mg^{-1} protein to 5.49 ± 0.08 µmol.min^{-1}.mg^{-1} protein and to 7.43 ± 0.015 µmol. min^{-1}.mg^{-1} protein ($p < 0.001$) respectively.

4. Discussion

The physiological and biochemical effects induced by the interaction of nanoparticles (NPs) with phosphonate (PC) in aquatic habitats was previously described (Canesi et al., 2012; Pan et al., 2012; Wegner et al., 2012; Trevisan et al., 2014; Cid et al., 2015). In the current study, the main emphasis was on Au-ZnO NPs and phosphonates (PC), respectively, as such as to clearly relate the potential chemical interactions between these compounds and the roles they play in PCs in NPs bioavailability, accumulation and toxicity. The morphologic determination, by using transmission electron microscopy (TEM) and Dynamic light scattering (DLS), confirms the stability of Au-ZnO NP's under the environment parameters change including pH and salinity.

Due to the role played by the essential metals as precursors in most enzymatic activities, they are carefully regulated by physiologic mechanisms in most organisms and thus the knowledge of their concentrations in marine organism is important (Caglar

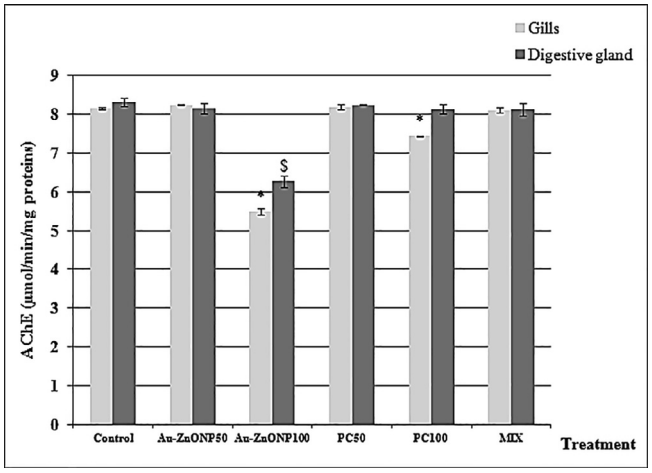

Fig. 6. Acetylcholinesterase (AChE) activity in the gills and digestive gland of the Mediterranean mussels *Mytilus galloprovincialis* (n = 10) untreated (Control) and treated with two concentrations of Au-ZnO nanoparticles (Au-ZnONP50 = 50 µg/L and Au-ZnONP100 = 100 µg/L), two concentrations of Hydroxyphosphonates (PC50 = 50 µg/L and PC100 = 100 µg/L) and the mixture (MIX = Au-ZnONP100 + PC100). * and $ indicate significant effect respectively in the gills and digestive gland at $p < 0.05$.

et al., 2019). In the current study, the measurement of essential metals following exposure to Au-ZnO NPs showed an increase in zinc and gold concentrations, possibly due to their bonding with NPs and their bioaccumulation in bivalves. This finding is in accordance to previous studies, which employed the leech *E. complanata* exposed to nanoZnO, leading to increased zinc concentration (Gagné et al., 2015). In addition, the presence of Au-ZnONPs significantly affected the accumulation of other essential metals, such as iron, manganese and copper by bivalves. These findings are in accordance to previous studies, which observed that $nTiO_2$ nanoparticles affected the metal bioavailability and its accumulation in *P. subcapitata* (Hartmann et al., 2010) and *Daphnia magna* (Hartmann et al., 2012). Moreover, the exposure of the mussels *M. galloprovincialis* to copper nanoparticles led to enhanced bioaccumulation of copper (Gomes et al., 2011) and the exposure of the oyster *Crassostrea gigas* to nanoZnO, to zinc bioaccumulation (Trevisan et al., 2014).

The treatment of mussels with PC and the mixture of NPs and PC did not affect the concentration of metals, possibly due to the synergic interaction between the used compounds. The PCs are known for their high complexation capacity with metallic ions (Möller et al., 2010). The Au-ZnONPs affected the physiology of this mussel at concentrations > 50 $\mu g.L^{-1}$, explicable through the interaction between NPs and the targeted tissues. The decrease of FC and RR values indicates that the mussels detected the presence of these stressors early during the process (Wegner et al., 2012), in accordance to previous studies equally carried on mussels, which observed a decrease of their filtering activity following exposure to nanopolystyrene (Wegner et al., 2012) and nanoZnO (Trevisan et al. 2014).

The mixture of Au-ZnONPs and PC at 100 $\mu g.L^{-1}$ did not affect the measured physiologic endpoints, suggesting potential interactions between contaminants at this biological level (Aouani et al., 2015).

We suspect that at cellular level, the interaction among NPs and PC with biological components could have occurred, leading to metabolic disturbances. The antioxidant molecules, including the H_2O_2 and antioxidant enzymes, are frequently measured to highlight the defensive mechanisms employed by organisms against toxic effects. In the current study, the H_2O_2 level increase could potentially be related to the metallic disturbance occurred after NPs treatment. It is known that the zinc represents an essential element within cells and a crucial component in various enzymes (Lushchak, 2011). This metal is involved in more than 70 different metallo-enzymes responsible for several chemical, metabolic, and immunological reactions (Valko et al., 2005). Equally, the zinc is responsible for the increase of hydrogen peroxide (H_2O_2) at cellular level, leading to oxidative stress (Valko et al., 2005). The oxidative stress is induced by the presence of molecules with unpaired electrons, usually derived from oxygen and various reactive intermediates (Talas et al., 2008). The increase in H_2O_2 concentrations in gills and digestive gland of mussels may therefore be linked to the increase in zinc concentration. However, this increase could also be linked to the increase in iron and copper concentrations. Thus, these two essential elements led to an increase in H_2O_2, according to the following reactions:

$$M(II) - SOD + O_2^- \rightarrow M(I) - SOD + O_2 \quad (3)$$

$$M(I) - SOD + O_2^- + 2H^+ \rightarrow M(II) - SOD + H_2O_2 \quad (4)$$

where M represents the copper or the iron; hence, the level of H_2O_2 is linked to Fe and Cu concentrations (Valko et al., 2005).

The level of antioxidant enzymes is a good indicator for the impacts of pollutants (Ates et al., 2008). In addition, stimulation of antioxidant enzyme can be caused by an effective antioxidant defense mechanism acting against the oxidative stress (Ates et al., 2008; Kakoolaki et al., 2013). In our experiment, the oxidative stress status was confirmed by the SOD activity and GSH/GSSG ratio. SOD and GSH were suspected since two decades ago to be involved in the antioxidant defense system (Van der Oost et al., 2003; Zhu et al., 2008); thus, they can be induced by a mild oxidative stress as a compensatory response.

The GSH content can increase due to the mild oxidative stress associated with an increase in its synthesis ratio (Zhu et al., 2008). Therefore, these results indicate that the oxidative stress of Au-ZnONPs and PC might occur in the gills of mussels following the exposure to a concentration higher than 100 $\mu g.L^{-1}$.

The modification of the SOD activity observed in the current experiment could be related to the deregulation of metals after contamination, mainly for Fe, Zn and Cu. This metalloenzyme exists as various isotypes, characterized by the redox active metals at various catalytic sites. In mussel, the Cu/Zn–SOD was found in gills and digestive glands and all three isoforms usually contribute to the cytosolic activity of this enzyme, one of them inducible by pollutants in experimental conditions. We conclude that the SOD modulation observed in the current experiment could represent a direct consequence of Cu/Zn–SOD activation.

The hydrogen peroxide plays an important role in various biological processes at cellular level and its concentration is controlled by CAT (Valko et al., 2005).

The CAT is involved in detoxification and plays an important role in the antioxidant defense mechanisms. This enzyme is located in peroxisomes and catalyzes the conversion of hydrogen peroxide (H_2O_2) to molecular oxygen (O_2) and water (Daoud et al., 2012). An increase in CAT activity observed in the current study may therefore be linked to the increase of H_2O_2 in gills and digestive glands of mussels. The experiment confirms that the degree of oxidative stress is probably a consequence of the ROS-derived lophocladine detoxification. It is well-known established that ROS can regulate the expression of antioxidant enzymes (Takano et al., 2003). Moreover, it was also showed that CAT is partially affected by the oxidation to an inactive Fe (IV) form. The superoxide anion formed during detoxification may reverse the formation of this inactive compound by reducing the inactive Fe (IV) compound to the active Fe (III) (Zamocky and Koller, 1999).

The results for CAT activity and H_2O_2 levels highlighted the oxidative stress induced by Au-ZnONPs in mussels, in accordance to a previous study that showed that AuNPs are responsible for the occurrence of oxidative stress in the gills and the digestive gland of the clam *Corbicula fluminea* (Renault et al., 2008). Cid et al. (2015) showed that the CAT activity increased in a concentration-dependent manner in the clam *C. fluminea* exposed to NPs, suggesting that this increase is linked to the production of ROS in the digestive gland and to the NPs penetration within the digestive cells. Pan et al. (2012) also showed that the CAT activity increased in the clam *Scrobicularia plana* exposed to a concentration of 100 $\mu g.L^{-1}$ of AuNPs, linking this increase to the defense system activation.

The increased activities of all markers of oxidative stress, in both tissues of the mussel exposed to Au-ZnONPs, indicate that NPs induce oxidative stress in marine biota. In addition, the antioxidant enzymes induction may contribute to maintain a relatively low level of the ROS, which in turn is generated through the Haber–Weiss reaction (Orun et al., 2008).

The gills are sensitive to pollutants and are routinely employed as target organ in ecotoxicologic experiments (Alazemi et al., 1996). Moreover, the gills perform many biological functions, such as respiration, osmoregulation, and excretion of waste and in the acid-base balance (Alazemi et al., 1996). Our findings indicate that gills are more sensitive compared to digestive glands, as was reflected by the significant increase in SOD and CAT activities following Au-ZnONP50 exposure, corroborated with the lack of any effect in the digestive gland. The sensibility of gills could be related to the fact that they exposed to contaminants at a higher rate compared to other internal organs. The gills in mussels are ciliated and behave like a primordial mouth, carrying food particles towards the digestive gland during the feeding process.

The co-exposure to 100 $\mu g.L^{-1}$ of Au-ZnONPs and 100 $\mu g.L^{-1}$ of PC (Mix) did not affect significantly the oxidative biomarkers. These results were likely linked to the chelating and antioxidant proprieties of PC's against metallic nanoparticles (Aouani et al., 2015). These products were characterized by their high ability to complex with metal compounds and to develop an efficient antiradical capacity, along with the reduction of the DPPH radical activity (Aouani et al., 2015). In addition, the complexation action of phosphonates with metallic ions was previously confirmed, thus supporting the interaction between metallic nanoparticles and the PC (Möller et al., 2010).

The measurement of the AChE activity in marine organisms was previously investigated, given their crucial role in living organisms (Holas et al., 2012; Dhull et al., 2013; Akrami et al., 2014; He et al., 2014). The AChE hydrolyzes acetylcholine in the central nervous system (He et al., 2014) and is routinely used for the detection of toxic effects induced by organophosphates (Dhull et al., 2013) and heavy metals (Gioda et al., 2013). In the current experiment, the AChE activity was induced in the gills following exposure to a concentration of 100 $\mu g.L^{-1}$ of both AuZnONPs and PC, respectively. This finding highlights the toxic effect and the potential threshold for the induction of neurotoxicity in mussels, following their exposure to both AuZnO NPs and PC. Moreover, the AChE inhibition could be also linked to indirect effects of H_2O_2, which are generated in the presence of both compounds in gills. The activity of AChE is regulated by the level of hydrogen peroxide, which is responsible for the alteration of this enzyme active site (Schallreuter et al., 2006; Schallreuter et al., 2007). The absence of any effect on this biomarker recorded in mussels treated with a mixture of AuZnONP100 and PC100 confirms the link with H_2O_2 level, which decreased as a response to contamination with the mixture, highlighting the potential interaction between these two pollutants. Schallreuter et al. (2007) also showed that essential metals could influence the AChE activity and Zhu et al. (2007) showed that metals plays an essential role in the regulation of this enzyme *via* calcium dependent proteins, which inhibits in turn the expression of this biomarker. During the current experiment, this essential metal was strongly induced in the gills exposed to both compounds, revealing the complex nature among essential metals, concentration of pollutants and the AChE activity (Fig. 6). Our findings are in accordance with Chakraborty et al. (2013) that emphasized also the inhibition of the AChE activity in the gills of *Lamellidens marginali* exposed to sodium arsenite. Vidal-Liñan et al. (2015) also reported the inhibition of AChE activity in the gills of *M. galloprovincialis* mussel after 30 days of exposure to Nonylphenol. The complexation of PC with metals and its ability to attenuate free radicals was previously demonstrated by Aouani et al. (2015), justifying the lack of any synergic effect of both compounds on the AChE level in mussels.

5. Conclusion

The current experiment contributes to a better understanding of the toxic mechanisms of metallic NPs, mainly Au-ZnO NPs, potentially beneficial for future risk management strategies that assess the metallic NPs in nanotechnology applications. The results of this work contribute to the advancement of knowledge on three fundamental aspects of the toxicology of metallic NPs: i) the toxic mechanisms of Au-ZnO NPs in bivalves; ii) the effect of the change in concentration on the toxicity of Au-ZnO NPs and iii) the potential to reduce the effect of NPs using α-hydroxyphosphonates. We conclude that Au-ZnO NPs are toxic for *M. galloprovincialis* and can induce biochemical changes in the tissues of this mussel at a concentration equal or higher than 100 $\mu g.L^{-1}$. These changes were mainly reflected through an increase in the activities of oxidative enzymes, paralleled by the formation of ROS in cells and the inhibition of the acetylcholinesterase activity. However, the addition of Diethyl (3-cyano-1-hydroxy-1-phenyl-2-methylpropyl) phosphate (PC) corrected the biochemical and physiological effects in mussels. We have also showed that the modification of all these parameters seems to be a consequence of the indirect effect of metallic elements induced after contamination with these pollutants. The mechanism of toxicity of Au-ZnO NPs in bivalves was thus better understood, leaving room for further investigations. More long-term studies are required to properly investigate the chemical and metabolic interaction mechanisms in marine invertebrates.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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