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Research article

Histological and biochemical evidence of Cr_2O_3 and Al_2O_3 nanoparticles toxicity in the marine gastropod *Stramonita haemastoma*: A preliminary application of integrated biomarker response (IBR)

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ABSTRACT

Nanoparticles (NPs) have actively contributed to nanotechnologies advancement over the last years, due to the unique properties they possess compared to their pristine counterparts. Consequently, NPs found wide applications in various fields such as the medical, biomedical, chemical, agro-food industries, and cosmetology. NP's extensive uses could lead to their release into the environment, especially in the marine ecosystems, considered as NPs sink, resulting in harmful effects on organisms. Concerns regarding NPs' toxicity in aquatic organisms have emerged, however, several points remain unexplored. In the present study, the toxicity of chromium oxide (Cr_2O_3 = 42 nm) and aluminum oxide (Al_2O_3 = 38 nm) NPs (1 mg/L, 2.5 mg/L, and 5 mg/L) in the gills of the marine gastropod *Stramonita haemastoma* was assessed through time (7, 14, and 28 days) by a multi-biomarker, Integrated biomarkers response (IBR), and Histological analysis. Both NPs induced varied changes in the antioxidant system, suggesting the onset of oxidative stress marked by superoxide dismutase (SOD), catalase (CAT), acetylcholinesterase (AChE), metallothionein (MT), and malondialdehyde (MDA) levels imbalance. Varied histological alterations in the gills of *S. haemastoma* were also observed including inflammation, hypertrophy, and lamellar fusion, IBR proved to be a promising tool for assessing NPs toxicity in gastropods. In this study results indicated the co-response of reduced glutathione (GSH), glutathione S-transferase (GST), glutathione peroxidase (GPx), CAT, SOD, and MT after 28 days of exposure. *S. haemastoma* showed sensitivity to all exposure concentrations of NPs thus validating this species as a suitable indicator of NPs contamination and toxicity.

1. Introduction

The advances in nanotechnologies over the last decade have contributed to extending the interest given to Nanoparticles (NPs) in a wide range of application areas. NPs are typically defined as particles with at least one dimension varying from 1 to 100 nm, allowing them to exhibit specific functional characteristics superior to their bulk elements (Abdel-Latif et al., 2020; Abramenko et al., 2021). Consequently, NPs

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have been utilized in various sectors including medical, biomedical, chemical industries, environmental applications, and cosmetics (Sánchez et al., 2011; Corsi et al., 2020). According to economic data, the global market size of metallic nanoparticles (Me-NPs) is predicted to reach 4.2 billion USD with an annual growth rate of nearly 11.5 % (focus on catalysis, 2023).

The increasing use of NPs in various applications raises concerns about their release into the environment, especially in the aquatic ecosystems, which are considered their major sink (Baker et al., 2014; Bour et al., 2015). For instance, processes such as atmospheric deposition, environmental dumping, effluents, and others could lead to the introduction of NPs in the marine ecosystem as their final destination (Matranga and Corsi, 2012; Xia et al., 2017). Once in seawater, NPs can form agglomerations and settle in marine sediments, leading to their accumulation over time (Canesi et al., 2015; Peng et al., 2017). Studies have shown that NPs can reach concentrations in marine sediments in the range of mg/kg (Nam et al., 2014; Fan et al., 2018; Tou et al., 2021). These NPs are considered pollutants that can potentially harm effects to marine species and compromise the integrity of the aquatic ecosystem (Canesi et al., 2017). Consequently, concerns have emerged regarding NPs' potential toxicity (Blasco et al., 2015). Despite the recent expansion of ecotoxicological research, previous investigations primarily focused on freshwater species and specific NPs types such as TiO_2, CuO, and ZnO_2. Likewise, these studies were mainly limited to bivalves regarding marine species (Minetto et al., 2014 and 2016).

The characteristics that make NPs profitable, including their small size, high reaction surface, shape, and optical ones are crucial factors in their potential toxicity to aquatic organisms (khan et al., 2019; Turan et al., 2019). Several studies reported their ability to interact with biological barriers, affecting their bioavailability and causing harmful effects, particularly by inducing oxidative stress, which is considered the main toxicity mechanism of NPs (De Felice and Parolini, 2020; Hichem et al., 2022). Exposure to NPs can alter cellular antioxidant defenses, leading to various effects including the modification of cellular components such as proteins, lipids, and DNA, mainly through indirect oxidation pathways due to ions released in aquatic organisms (Handy et al., 2008; Deng et al., 2016; Hou et al., 2019).

Chromium oxide (Cr_2O_3) and Aluminum oxide (Al_2O_3) are among the most widely used NPs. Due to their high functional properties, both NPs found application in numerous sectors, namely as green pigments, catalysts, semiconductors, chemical and agro-industrial industries, biomedical, medical, cosmetics, antibacterial agents, and others (Becker et al., 2016; Mui et al., 2016; Sharma et al., 2022; Adnan and Mohammed, 2024). Several studies have reported their toxicity to various biological models. Cr_2O_3 was reported to induce cytotoxicity in the green alga *Chlamydomonas reinhardtii* and L929 cells (Alarifi et al., 2016; da Costa et al., 2015), genotoxicity in rats (Singh et al., 2016), high acute toxicity in *Daphnia magna* (Puerari et al., 2016), and oxidative stress induction in the freshwater fish *Labeo rohita* (Kanwal et al., 2019). Although Al_2O_3 NPs have been considered less toxic than most common NPs, numerous studies reported also toxic effects induced by Al_2O_3 (García-Saucedo et al., 2011). For instance, cytotoxicity in mouse neuroblastoma cells (Nogueira et al., 2019), oxidative stress induction in the mussel *Unio tigridis* (Canli and Canli, 2021), histological alterations in the fish *Oreochromis mossambicus* (Murali et al., 2018), neurotoxicity on dopaminergic neurons in rats (Liu et al., 2020), and accumulation in *Artemia salina* larvae (Ates et al., 2015). Despite the extensive research on NPs toxicity assessment, the existing literature concerning both NPs remains limited, especially on aquatic organisms (Załęska-Radziwiłł et al., 2020). For instance, to the best of our knowledge, no prior study has explored the toxicity of Cr_2O_3 NPs on marine organisms, with limited existing studies on freshwater species.

The increasing population density and economic activities are putting pressure on coastal ecosystems. Therefore, it is becoming increasingly important to evaluate NPs fate and toxicity on benthic organisms (Mouneyrac et al., 2014). Mollusks have been widely used as bioindicators for assessing NPs aquatic contamination and toxicity by several relevant studies. For instance, marine bivalves have been recognized as particularly affected by NPs contamination, leading to a focus on bivalves in many studies, while research focused only on other species is still lacking (Canesi et al., 2010a; Canesi et al., 2012; Rocha et al., 2015).

Stramonita haemastoma is a coastal marine gastropod, with a wide geographic distribution. These marine snails are principal predators of bivalves and other small gastropods. They have been used as indicators of Tributyltin (TBT) pollution and imposex induction; besides owning economic importance in purple dye extraction and human consumption (Bouzahouane et al., 2018; Madeira et al., 2018; El Ayari et al., 2018). Although *S. haemastoma* has received little attention as a bioindicator, recent limited *in situ* studies have judged this marine snail as a promising indicator of marine metallic contamination (Di Bella et al., 2018; Bouzahouane et al., 2024). However, to the extent of our knowledge, this marine gastropod has not been used as an indicator of NPs contamination and toxicity. Considering the earlier key points, *S. haemastoma* could be a new promising bioindicator of NPs toxicity assessment, since gastropods are usually suitable biological models as they offer several practical advantages namely their abundance, breading resistance, and sensitivity to pollutants exposure (Caixeta et al., 2020). To our best knowledge, No prior study apart from ours regarding the use of *S. haemastoma* as a bioindicator of NPs toxicity same as *in vivo in situ* studies was done. Key biochemical and histological biomarkers linked to critical physiological functions were selected for this study. Biochemical indicators include the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), reduced glutathione (GSH), and glutathione peroxidase (GPx), all essential for defense against reactive oxygen species (ROS) generated by xenobiotic exposure. Oxidative damage was assessed by measuring malondialdehyde (MDA) levels. Additionally, Metallothionein (MT) levels were evaluated as a biomarker for metal detoxification. The activity of glutathione-S-transferase (GST), a phase II biotransformation enzyme involved in xenobiotic detoxification, and acetylcholinesterase (AChE) activity, associated with cholinergic neurotransmission, was also evaluated as biomarkers for neuromuscular toxicity. Additionally, a detailed histological study was conducted to observe structural alterations in the gill tissues, providing insights into tissue-level damage caused by NPs exposure.

Integrated biomarker response (IBR) is a useful tool that allows the visualization of multivariate data of numerous biomarker response trends (Beliaeff and Burgeot, 2002). This method has been widely used, especially for *in situ* investigations, including ecotoxicological studies to better understand the correlations among biomarkers and contamination levels in field studies (Devin et al., 2014). However, the application of IBR in *in vivo* and *in vitro* NPs toxicological studies is still limited. For example, in marine species, the use of IBR is mainly limited to bivalves in some studies (Xia et al., 2013; Devin et al., 2017; Xia et al., 2017; Li et al., 2021).

This study aims to investigate the potential *in vivo* toxicity of Cr_2O_3 and Al_2O_3 NPs, with increasing exposure concentrations through time in the gills of *S. haemastoma*. This will be accomplished using a multi-biomarkers approach and the first application of IBR to a range of biomarkers over time, along with histological analysis.

2. Materials and methods

2.1. Nanoparticle synthesis

The solvothermal method was used to prepare both Al_2O_3 and Cr_2O_3 NPs, by dissolving 0.4 mol of Al $(NO_3)_3$, $9H_2O$ in 40 mL of deionized water (Dw) and Cr $(NO_3)_3$, $9H_2O$ in 60 mL of Dw under stirring, respectively. For Al_2O_3 preparation, the main mixture was centrifuged then the precipitate was dispersed in distilled water, heated at 400 °C, and washed and finally dried for 8 h at 90 °C. The Cr_2O_3 NPs mixture

was heated at 140 °C for 24 h, the resulting precipitates were washed with Dw, dried, and calcined at 500 °C to obtain green Cr_2O_3 NPs.

2.2. Nanoparticle characterization

The structural characterization of both samples was performed using X-ray diffraction (XRD) with a Siemens D-5000 diffractometer in 20° – 90° 2θ range with Cu-Kα radiation (λ ¼ 1.5418 Å). UV–Vis spectrophotometer was used to investigate UV–Vis absorption spectra for both NPs and Morphological analysis using Scanning Electron Microscopy (SEM: Thermo Fisher Scientific) was also performed **(**Bouarroudj et al., 2023**)**.

Malvern Zetasizer Nano-ZS (Malvern Panalytical) equipped with DTS 1070 disposable folded capillary cells and MPT-2 Titrator, was used to assess the zeta potential and the zeta average diameter of both NPs in function of pH using NaOH (0.03 M) and HCl (0.03 M) solutions, by dynamic light scattering (DLS) and calculation of 20 measurements points. Energy-dispersive spectroscopy (EDS) was also used to investigate the purity of both further prepared Al_2O_3 and Cr_2O_3 NPs and the optical properties of both NPs were also evaluated by calculating the optical band gap (Eg) from UV–Visible spectra for Cr_2O_3 and Al_2O_3 NPs (supplementary).

2.3. Animal sampling and conservation

Stramonita haemastoma sampling (4 to 10 cm) followed previous work by Sedrati et al. (2024). Briefly, after collection from Annaba city, Algeria in the Cap de Garde (36°96′N, 7°78′E) by SCUBA. This area is an isolated natural site, characterized by the absence of industrial activity or human habitation **(**Bouzahouane et al., 2024**)**. Natural seawater (NSW) was collected from the same sampling site, filtered at 50 μm, and stored in the dark. The preliminary average water parameters were monitored by a multiparameter (®Horiba) and were maintained during all experimental periods (salinity: 34.2 ppt, Temperature: 23 °C, pH: 8.2, dissolved oxygen: 9.22 mg/L). The collected snails were acclimated for 7 days and kept in laboratory conditions with a photoperiod of 12:12 h dark/light in NSW-contained aquariums with constant aeration, water was renewed daily during the acclimation period, and then every 48 h in exposure periods.

2.4. Experimental design

S. haemastoma snails were randomly divided into groups of 20 individuals each, and each group was placed in identically sized boxes containing 3 L of NSW. The snails were maintained in constant aeration in bubbling conditions, mimicking their natural conditions. Marine snails were exposed for a total of 28 days, with three assessment endpoints at 7, 14, and 28 days to either Cr_2O_3 or Al_2O_3 NPs. Nano-powders of Cr_2O_3 and Al_2O_3 were added to MilliQ water with stirring to prepare stock solutions (1 g/L) of both NPs using a bath sonicator (50 kHz) for 30 min. Exposure was made by diluting equal volumes of stock solutions into each box. The snail groups were set and exposed to nominal concentrations as follows: group 1 as control, group 2 exposed to 1 mg/L Al_2O_3 NPs, group 3 exposed to 2.5 mg/L Al_2O_3 NPs, group 4 exposed to 5 mg/L Al_2O_3 NPs, group 5 exposed to 1 mg/L Cr_2O_3 NPs, group 6 exposed to 2.5 mg/L Cr_2O_3 NPs, and group 7 exposed to 5 mg/L Cr_2O_3. Gastropods were not fed during the exposure term, and experiments were conducted with 3 independent replicates. NPs nominal concentrations in this study were selected following the previously applied concentrations in prior studies, and the predicted concentrations in sediments **(**Canesi et al., 2010a, 2010b**;** Hao and Chen, 2012**;** Gornati et al., 2016**;** Xia et al., 2017**;** Caixeta et al., 2020**)**. Exposure duration was selected according to some criteria as follows: - The average exposure periods reported in related previous studies and reviews, – periods were selected to assess the potential effects evolution in a sub-acute term **(**Canesi et al., 2012**;** Abdel-Latif et al., 2020**;** Roma et al., 2020**)**.

2.5. Biochemical analysis

2.5.1. Preparation of tissue homogenates

At the end of each exposure duration point (7, 14, and 28 days), snails from the control and the exposed groups were euthanized by instant freezing and carefully dissected. The gills were obtained for oxidative stress biomarkers analysis, and 03 individuals were used for analysis at each period from different replicates. Immediately, aliquot samples were homogenized using an ultrasonicator bath and a vortex, using 3 buffers, each specific to biomarkers. Briefly, aliquots samples were homogenized in Tris-HCl buffer (10 mM, pH: 7.3, 1:3 *w/v*) for reduced glutathione (GSH), glutathione peroxidase (GPx), glutathione-S-transferase (GST), superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) assays **(**Zaidi et al., 2021**)**. Aliquot samples were homogenized in phosphate buffer (100 mM, pH, 7, 1:3 *v/w*) for acetylcholinesterase (AChE) analysis. Homogenates were then centrifuged at 11250 ×*g* for 15 min (4 °C) and supernatants were immediately stored at −80 °C.

Tris-HCl (20 mM), saccharose (0.5 M), and leupeptin (0.006 mM) were used as a buffer for the metallothionein (MT) assay, homogenates were centrifuged at 20,000 ×*g* for 30 min (4 °C) and instantly conserved at −80 °C **(**Bouzahouane et al., 2018**)**.

2.5.2. Protein quantification

The total protein rates were determined according to the Bradford (1976) assay at 595 nm using Bovine serum Albumin (BSA) as a standard (Sigma®).

2.5.3. Oxidative stress biomarkers

The Weckbecker and Cory (1988) method was used to monitor GSH levels, based on the 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) reduction by thiol group (-SH) of glutathione absorbance assessment at 412 nm. The GSH content was expressed as nanomole per milligram of protein (nmol mg^{-1} protein).

The GST activity was assessed using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate for reduced glutathione resulting in the formation of molecules from the conjugation reaction then monitored the absorbance changes for 2 min at 340 nm according to **Habig et al.** (1974) method, GST activity was expressed in units (U) of GST per milligram of protein ((U) GST mg^{-1} protein).

The GPx activity was measured following the Chiu et al. (1976) method, by absorbance assessment at 412 nm and GPx activity was defined as the amount of enzyme oxidizing 1 μmol of GSH. GPx was expressed as nanomoles of GSH oxidized mg^{-1} protein **(**Thirupurasundari et al., 2009**;** Wu et al., 2011**)**.

The **Marklund and Marklund** (1974) method based on the pyrogallol oxidation inhibition by SOD in the presence of EDTA assay was employed to assess SOD enzymatic activity by absorbance monitoring for 5 min at 420 nm, SOD concentrations were represented in (U) SOD mg^{-1} protein.

CAT enzymatic activity was measured using the Hydrogen peroxide (H_2O_2) rates decrease and monitored optical densities variation at 240 nm according to **Beers and Sizer** (1952) assay, one unit (U) of CAT is equivalent to the amount of enzyme catalyzing the degradation of 1 μmol of H_2O_2 per min and was expressed as U min^{-1} mg^{-1} protein.

2.5.4. Lipid peroxidation biomarker (LPO)

The evaluation of MDA rates in the gills of the marine snails was measured as an indicator of Lipid peroxidation (LPO), MDA levels were assessed by the thiobarbituric acid (TBA) reaction assay at 535 nm in a final mixture composed of 1 mL of the supernatant, 1 mL TBA (0.67 %), and 1 mL trichloroacetic acid (TCA, 5 %) according to the initial protocol reported by Fatima et al. (2000) with some modifications by Zhao et al. (2009). An extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used to calculate the MDA rates which was expressed as nmol MDA mg^{-1} protein. More detailed procedures of the biomarkers analysis were

previously reported by Zaidi et al. (2021).

2.5.5. Neurotoxicity biomarker

The AChE enzymatic activity was determined following the Ellman et al. (1961) assay, based on acetylcholine (ACh) as a substrate for (AChE) and the formation of yellow color molecules due to thiocholine (SCh) reaction with DTNB, which can be monitored at 412 for 5 min for the estimation of enzymatic activity, AChE activity was expressed in nmol min^{-1} mg^{-1} protein.

2.5.6. Metallothionein (MT) analysis

Metallothionein (MT) content was evaluated according to the method proposed by Viarengo et al. (1997) specifically adapted for marine invertebrates and based on the monitoring of absorbance at 412 nm with Ellman's reagent (DTNB) for the determination of the thiols (-SH) after obtaining a partially purified MT fraction from the cytosolic content using an ethanol/chloroform treatment. The MT content was measured by 1 mol Mt. = 20 mol GSH as an equation and was expressed as nmol g^{-1} tissue.

2.6. Histological study

The gills samples from each group (control, exposed to Cr_2O_3 and Al_2O_3 NPs) were dissected immediately for histological observations after 28 days of exposure, then preserved in 10 % formaldehyde. 2 individuals were pulled from different replicates for all groups and then histological samples were dehydrated with a succession of graduated ethanol and cleared using xylene, 3 to 5 μm sections were obtained by a microtome after embedding gills samples in prepared paraffin wax (®Sigma). Tissue cuts were then directly mounted in microscopic slides and colored using Eosin (e) and Hematoxylin (H). Finally, the prepared histological slides of gills samples were observed for alteration determination in a Leica DM1000 LED optical microscope, and photographic shots of the histological slides were recorded **(**Hould, 1984**;** Zaidi et al., 2021**).**

2.7. IBRv2 analysis

IBR is a method used to integrate multiple biomarker responses and experimental factors into one single index. This helps to better frame the key impacts and reactions of biomarkers. The method has been successfully used to evaluate the effects of NPs on oxidative stress biomarkers in bivalves **(**Beliaeff and Burgeot, 2002**;** Marigómez et al., 2013**).** The term "v2" in IBRv2 typically stands for "version 2," which indicates an updated version of the original Integrated Biomarker Response (IBR) index and is referred to as IBR in this study. This version incorporates adjustments to improve its applicability, accuracy, or sensitivity in environmental biomonitoring. In this study, IBRv2 was applied to various biomarkers and exposure concentrations for each exposure period to identify the potential impacts of NPs. The IBRv2 calculations are based according to Sanchez et al. (2013) modifications to adapt IBR in oxidative stress biomarkers as follows:

Individual biomarkers data (X_i) is compared to average biomarkers references (X_0) to obtain (Yi), and log transformation is applied to reduce variations.

$$Yi = log\ (X_i/X_0)$$

Standardized biomarkers response (Z_i) is obtained by estimating the global mean (μ) and standard deviation (σ) of Yi.

$$Z_i = (Y_i - \mu)/\sigma$$

A deviation index (Ai) is calculated to establish a basal line of 0 as a center in which biomarkers' response changes are represented, by applying the average reference biomarker data (Z_0) and (Z_i).

$$Ai = (Z_i - Z_0)$$

Finally, the values of Ai parameters for each biomarker are calculated to obtain the IBR values.

$$IBR = \sum |Ai|$$

2.8. Statistical analyses

All statistical data analyses were conducted using R software (R Core Team, 2023), and a two-way ANOVA permutation was performed to compare groups, NPs concentrations, and duration. Exposure was compared by applying the *Post hoc* test of Dunnett for Multiple Comparisons of Means; followed by the Dunn multiple comparison test with *p*-values adjusted with the Bonferroni method. Descriptive statistics are expressed as mean and standard deviation and statistical significance was determined at a significance level of $p < 0.05$ for all analyses . The percentage variation rates of biomarker activity in the gills of snails were calculated to quantify the relative changes between the control and exposed groups for each time duration. The formula used to calculate the percentage increase or decrease is as follows:

$$\text{Percentage Change}\ (\%) = ((\text{X exposed} - \text{X control}) \times 100\ )/\text{X control})$$

Where:

X exposed represents the biomarker activity measured in the gills of snails exposed to nanoparticles (NPs) at a given concentration and time.

X control represents the biomarker activity measured in the gills of snails from the control group (not exposed to NPs).

The resulting percentage values indicate an increase (positive percentage) or decrease (negative percentage) in biomarker activity relative to the control.

3. Results

3.1. Cr_2O_3 and Al_2O_3 NPs characterization

3.1.1. XRD analysis

The crystal structures and purity of both NPs were assessed by X-ray diffraction (XRD) analysis., The diffraction peaks observed at 2θ values correspond to the lattice plane (012), (104), (110), (006), (113), (202), (024), (116), (122), (214), (300), (119), (220), (306), (312), (0210) and (134), indicating the rhombohedra phase for Cr_2O_3 (Fig. 1A) (space group, R-3c). For α-Al_2O_3, the peaks correspond to the lattice plane (012), (104), (110), (006), (024), (116), (211), (018), (214), (300), (119), (220), (131), (128) and (0 2 10) (Fig. 1B) (space group, R-3c). Both phase observations agree with the literature values **(**Kafi-Ahmadi et al., 2022**).**

The average grain size (D) of Al_2O_3 NPs was determined to be 38 nm, while for Cr_2O_3 NPs, it was calculated to be 42 nm, (D) using the Scherer formula **(**Messai et al., 2018**).**

3.1.2. SEM analysis

SEM analysis was performed to investigate the morphology and structure of both Al_2O_3 NPs and Cr_2O_3 NPs. Both NPs samples exhibited remarkably consistent sizes indicating a successful synthesis process. The SEM images revealed that Cr_2O_3 NPs exhibit a spherical shape (Fig. 1C), while Al_2O_2 NPs showed plate-like α-Al_2O_3 (Fig. 1D).

3.1.3. EDS analysis

The presence of Cr_2O_3 and Al_2O_3 NPs in each sample was confirmed along with high purity by the characteristic peaks corresponding to Cr, O, and Al, O elements in the EDS analysis (supplementary).

3.1.4. DLS analysis

The zeta potential and the zeta average diameter of the NPs in this study showed high variations depending on pH. The isoelectric points (IEP) of Al_2O_3 and Cr_2O_3 were 2.33 and 2.95, respectively, indicating an

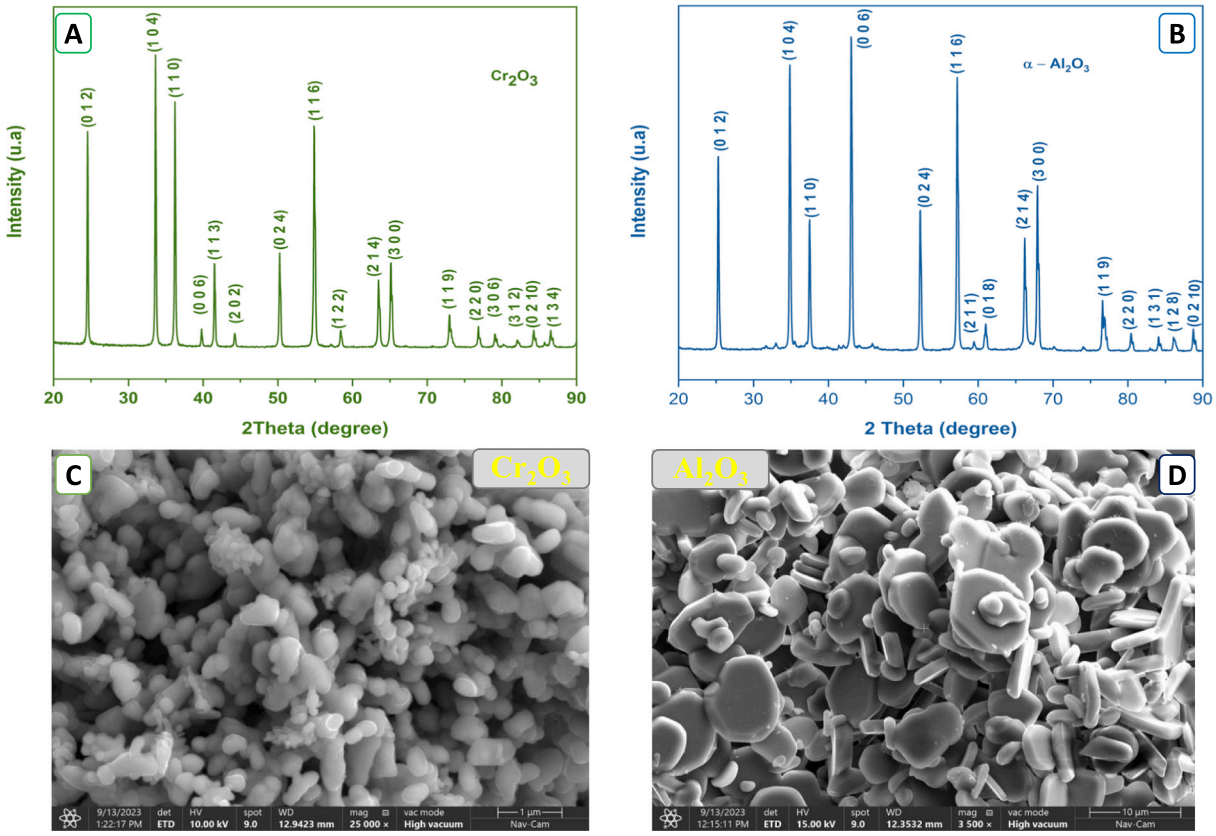

Fig. 1. XRD of Cr_2O_3 (A) and Al_2O_3 (B) nanoparticles, SEM of spherical shape Cr_2O_3 (C) and α-Al_2O_3 (D) NPs.

acidic character. Suspensions of Al_2O_3 and Cr_2O_3 NPs are stable only at pH higher than 6.5, and optimal stability was noticed in a pH range of 7 to 7.6 (Fig. 2). Additionally, the optical properties of both NPs were also evaluated and presented in the supplementary materials.

3.2. Biochemical biomarkers

The percentage variation rates among the control and the groups exposed to both NPs in the gills of snails were calculated at each time duration. The percentage variation rates of biomarker activity in the gills of snails were calculated to quantify the relative changes between the control and exposed groups for each time duration, the multi-biomarkers approach results will be presented by exposure periods. The variations trends of all the studied biomarkers for the control groups and the exposed groups (Cr_2O_3 or Al_2O_3 NPs) are represented in Fig. 11, in the gills of *S. haemastoma* the levels and activities of GSH, CAT, AChE, MT, and MDA shoed significant difference ($p < 0.05$) among exposure periods (7, 14, and 28 days). GST and SOD activities showed significant differences ($p < 0.05$) among concentrations and control groups for both NPs.

3.2.1. Following 7 days

The changes in GSH, GST, GPx, and CAT activity in the gills of snails are represented in Fig. 3 and Fig. 4 for both NPs. After 7 days, exposure to 1 mg/L ($p < 0.05$) and 2.5 mg/L of Al_2O_3 NPs led to 55.9 % and 42.6 % increase in GSH, while 1 mg/L of Cr_2O_3 caused 34.08 % GSH depletion. GST showed an increase of 145 % and 290 % ($p < 0.05$) in groups exposed to 1 mg/L and 5 mg/L of Al_2O_3 and approximately 133.01 % in the group exposed to 5 mg/L Cr_2O_3. GPx activity tended to increase in the gills of all exposed groups, ranging from 105 to 170 % for Al_2O_3 NPs and 117 to 385 % for groups treated with Cr_2O_3. CAT activity was reduced by 70.9 % following exposure to 1 mg/L of Cr_2O_3, while groups exposed to 2.5 mg/L and 5 mg/L of Al_2O_3 showed higher ($p < 0.05$) CAT activity, ranging from 37.4 % to 65.4 %. SOD, MDA, MT, and AChE variations in the gills of snails for both NPs are represented in Fig. 5 and Fig. 6. A significant decrease ($p < 0.05$) ranging from 50 % to 66 % in SOD activity was observed in all groups treated with Al_2O_3, with similar reduction ($p < 0.05$) of 71 % in the group exposed to 2.5 mg/L of Cr_2O_3. MDA in all exposed groups showed higher levels ranging from 156 % to 300 % for Al_2O_3 and significant depletion ($p < 0.05$) in MT rates was observed in the gills of all groups exposed to Cr_2O_3 ranging from 45 % to 49 %. Exposure to 2.5 and 5 mg/L of Al_2O_3 induced significantly lower MT levels ($p < 0.05$), ranging from 44 % to 109 %. AChE activity in the gills of *S. haemastoma* was significantly ($p < 0.05$) decreased by 263.28 % due to 2.5 mg/L exposure to Cr_2O_3, while a 215 % significant increase and a 75 % decrease at 2.5 mg/L and 1 mg/L was observed, respectively, for Al_2O_3.

3.2.2. Following 14 days

After 14 days, the levels of GSH significantly increased ($p < 0.05$) by 65 % at 1 mg/L and 2.5 mg/L of Al_2O_3, while GSH depletion was observed in all groups exposed to Cr_2O_3 with a significant ($p < 0.05$) decrease of 46 % observed at 2.5 mg/L. GST activity showed significantly decreased concentrations in all groups, ranging from 33 % to 74 % for Al_2O_3 and, 6 % to 45 % for Cr_2O_3. GPx activity decreased by 24 % (1 mg/L) ($p < 0.05$) and 35 % (5 mg/L) due to Cr_2O_3 exposure. On the other hand, GPx activity increased by 15.5 % (2.5 mg/L) and 24 % (5 mg/L) in gills exposed to Al_2O_3. CAT activity increased by 125 % at 1 mg/L and a significant decrease ($p < 0.05$) by 64 % at 5 mg/L was noted in groups exposed to Cr_2O_3 with decreases ranging from 46 % to 56 % at 2.5 mg/L and 5 mg/L of Al_2O_3. After 14 days, SOD was highly affected by Al_2O_3 ($p < 0.05$) NPs, with a significant decrease ranging from 45 % to 55 % in all treated groups. In the same way, MDA levels were significantly ($p < 0.05$) higher (95 %) at 5 mg/L of Al_2O_3. The exposure to 1 mg/L and 2.5 mg/L of Cr_2O_3 NPs caused significant ($p < 0.05$) depletion of MT levels (76 % and 83 %, respectively), while 1 mg/L of

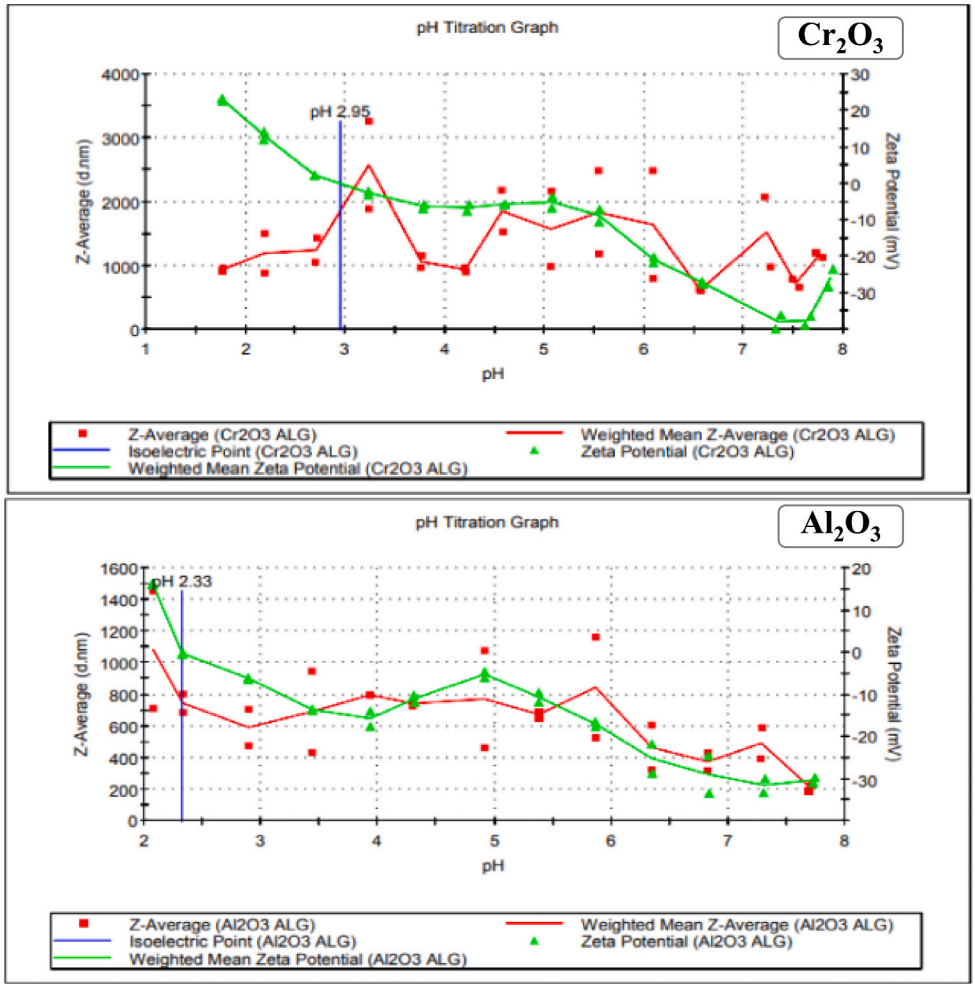

Fig. 2. zeta potential And The Zeta-average diameter (nm) of Cr_2O_3 and Al_2O_3 Nps as a function of pH.

Al_2O_3 induced reduced (183 %) MT rates. There was a significant inhibition (60 % to 81 %) of AChE activity in all groups treated with Cr_2O_3, and similarly, all groups exposed to Al_2O_3 presented decreased enzymatic activity (36 % to 63 %).

3.2.3. *Following 28 days*

At the end of exposure duration (28 days), both NPs increased the levels of GSH in all groups, with significant ($p < 0.05$) induction at 5 mg/L of Cr_2O_3 (239 %) and Al_2O_3 (268 %). There was a pronounced induction in GST activity in all treated groups, with the most significant increase ($p < 0.05$) at 2.5 mg/L of Cr_2O_3 (310.1 %) and 5 mg/L of Al_2O_3 (351 %). Similarly, all groups for both NPs showed increased GPx activity with the most significant ($p < 0.05$) variations at 2.5 mg/L of Cr_2O_3 (382 %) and 5 mg/L of Al_2O_3 (272 %). Likewise, CAT activity increased for all groups at day 28, with significant ($p < 0.05$) induction at 1 mg/L of Cr_2O_3 (65 %) and 5 mg/L of Al_2O_3 (132 %). In contrast, SOD activity was reduced in all exposed groups for both NPs with significant inhibition observed at 5 mg/L of Cr_2O_3 (73 %). MDA levels in snails' gills were highly affected by Al_2O_3 NPs, with increased rates ranging from 214 % to 41. Similarly, Cr_2O_3 exposure resulted in increased ($p < 0.05$) MDA levels at 2.5 mg/L (81 %) and 5 mg/L (227 %). There was a significant ($p < 0.05$) depletion of MT rates at 1 mg/L Cr_2O_3 (53 %) and a significant ($p < 0.05$) induction at 1 mg/L of Al_2O_3. AChE activity was induced by both NPs, with a dose-dependent increase ranging from 5 % to 46 % due to Cr_2O_3 exposure. An increase ranging from 152 % to 203 % was also observed after Al_2O_3 exposure. Significant ($p < 0.05$) among exposure duration points was observed for GSH, CAT, MDA, MT, and AChE in the gills of snails treated with Cr_2O_3, and GSH, GST, CAT, and MDA in individuals exposed to Al_2O_3.

3.3. *IBR v2*

Applying the multi-biomarkers approach is challenging for the comparative aspects among biomarkers or even pollutants effects. During this study, IBR v2 was used to analyze various biomarkers and present their responses in graphical summaries with standardized numeric values, allowing better identification of the impacts (Liu et al., 2017; Li et al., 2021). IBR positive values indicate biomarker induction, while negative ones are inhibition, following the recommendation of IBR v2 conception (Sanchez et al., 2013). IBR values have been plotted and, organized into two categories: 1- enzymatic biomarkers, and 2- non-enzymatic biomarkers, providing a clearer understanding of the results and specific identification. IBR v2 results are represented in Fig. 7 for individuals exposed to Cr_2O_3 and Fig. 8 for individuals exposed to Al_2O_3. IBR v2 global evolution according to exposure periods and concentrations for each and both NPs are represented in Fig. 10 and could serve as a summary tool to identify the potential biomarkers sensitivity trend and thus biomarkers behavior and response to both NPs exposure.

3.3.1. *At 7 days*

After 7 days of exposure to Cr_2O_3 NPs at 1 mg/L, the IBR values showed negative responses for SOD, CAT, MT, and positive responses for MDA. At 2.5 mg/L, CAT and SOD had a more negative impact along with MT. At 5 mg/L, IBR values highlighted negative AChE, GSH, and positive

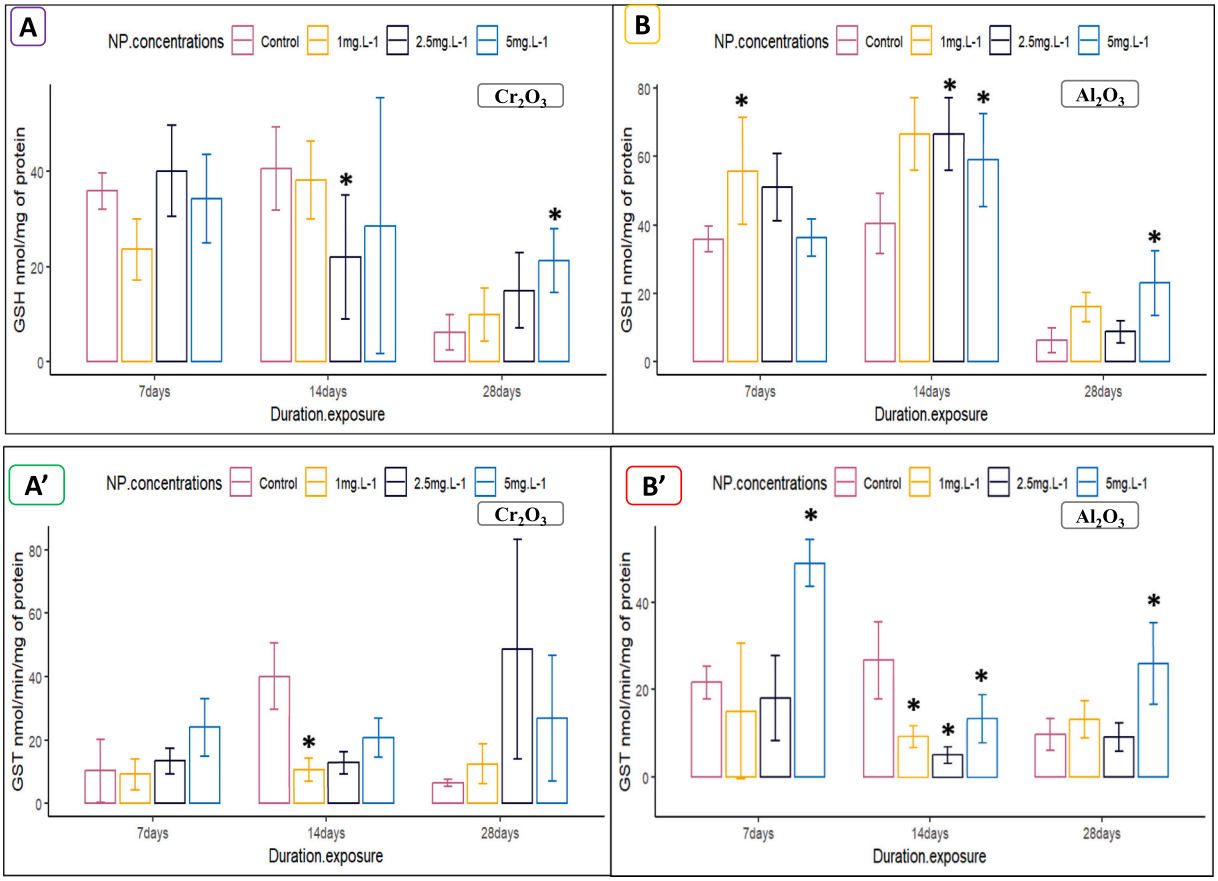

Fig. 3. GSH, and GST vrates in the Gills of *Stramonita haemastoma* treated with Cr_2O_3 (A, A') and Al_2O_3 (B,B'). The values are expressed as mean ± S.d. $n = 3$ (* $p < 0.05$).

responses for GST and MDA. The IBR values highlighted positive values for MDA and GPx, and negative responses for SOD and MT responses in all groups exposed to Al_2O_3.

3.3.2. At 14 days

On day 14, IBR levels at 1 mg/L (Cr_2O_3) were related to CAT, negative AChE, and MT response, and further highlighted negative GST, MDA, and GSH response. SOD and GPx were the most affected biomarkers at 5 mg/L. On the other hand, in individuals exposed to 1 mg/L of Al_2O_3 the IBR values reflected SOD inhibition and GSH induction. At 2.5 mg/L, GST and SOD were mainly inhibited and GSH was inducted. The IBR index represented positive MT, MDA, and negative AChE response at 5 mg/L of Al_2O_3. Overall, when setting the mean values of the IBR index per NPs concentration, the trend was mostly dose-dependent at all periods.

3.3.3. At 28 days

After 28 days, the IBR showed the co-action of all biomarkers in a dose-dependent trend, showing high responses for all biomarkers for both NPs. CAT < GST < GPx < AChE< MDA induction with SOD inhibition represented the main response for both NPs with the higher IBR values observed for Al_2O_3.

3.4. Histological analysis

The respiratory system anatomy of *Muricidae* like *Stramonita haemastoma* is considered far-developed. However, there is limited information available in the literature. According to our observations and literature (Beesley et al., 1998), *S. haemastoma* gills are mainly composed of a left ctenidium as a primary respiratory organ suspended by membranes to the mantle cavity and a sensitive organ called ospharidium which primarily plays a water-taster role. Gills are composed mostly of leaflets (filaments) containing running blood vessels. Histological shots as shown in Fig. 9, revealed that the control gills exhibited a regular structure, normal filament organization, and a central cavity (Fig. 9. A, B). However, after 28 days of exposure to 1 mg/L of Cr_2O_3. There was a pluri-stratification of the goblet cells in the gills filaments extremities. Exposure to 1 mg/L of Al_2O_3 and 2.5 mg/L of both NPs resulted in focal thickening of the gills epithelium and filaments, with a progressive loss in structure organization (Fig. 9. G, H, I). Lamella fusion was also observed (Fig. 9. F, I). On the other hand, exposure to 5 mg/L of Cr_2O_3 led to a high proliferation of goblet cells throughout the whole gill blades and filaments, along with partial regression of the filaments and signs of inflammation (Fig. 9. D, E). Exposure to 5 mg/L of Al_2O_3 induced focal thickening of the ctenidium epithelium and filaments (Fig, 9. H, F). At all NPs exposure concentrations, there was partial hypertrophy of the central cavity of the ctenidium and/or ospharidium, accompanied by an increase in mucus cells compared to the control. (See Fig. 9)

4. Discussion

In the present study, the toxicity of both NPs was assessed over time in the gills of the marine snail *Stramonita haemastoma* using various indicators. These indicators were employed to evaluate antioxidant responses, oxidative stress, cellular damage, disruption of neuronal activity, and structural changes at the tissue level, providing a comprehensive understanding of NPs toxicity in this marine snail. Additionally, the size, shape, zeta potential, and other characterization of Cr_2O_3 and Al_2O_3 NPs were investigated as they are important factors

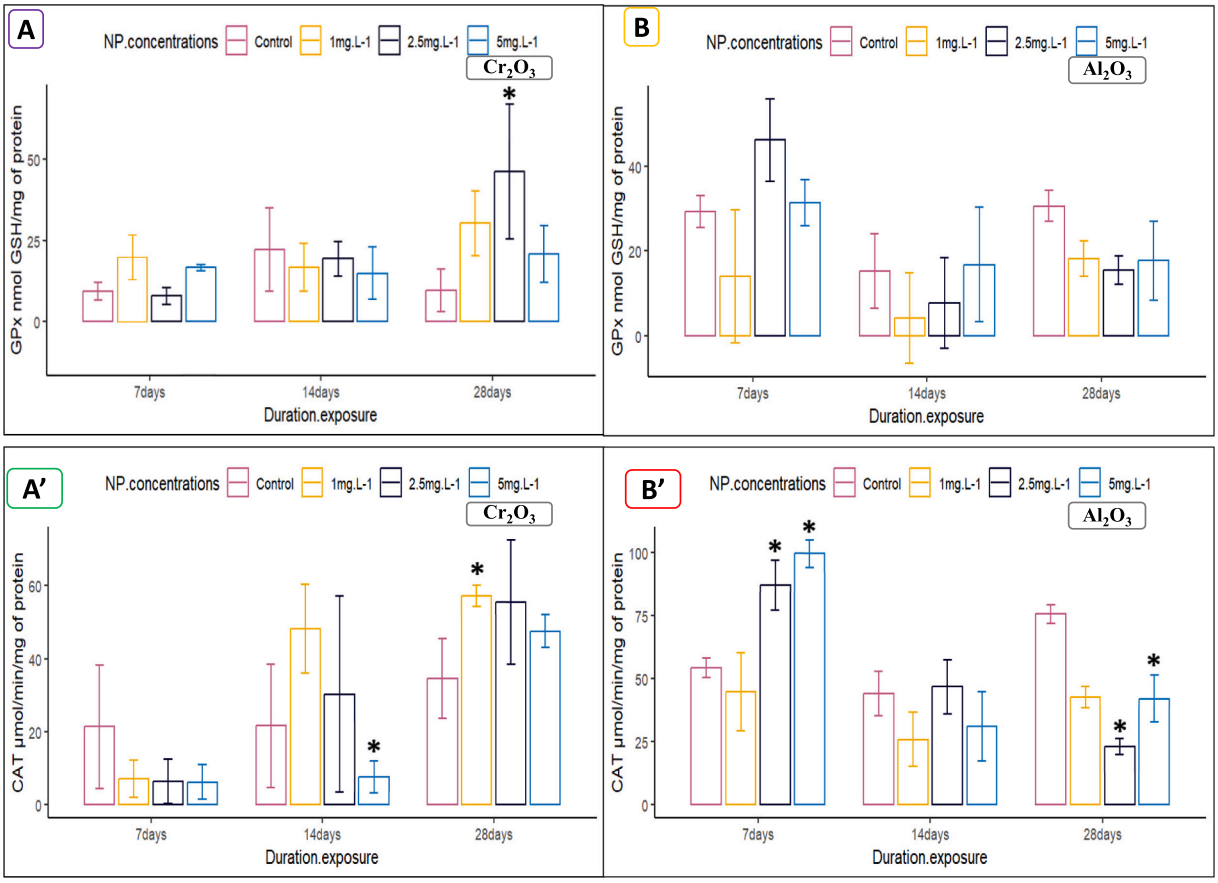

Fig. 4. GPx, and CAT rates in the Gills of *Stramonita haemastoma* treated with Cr_2O_3 (A, A') and Al_2O_3 (B,B'). The values are expressed as mean ± S.d. n = 3 (* $p < 0.05$).

that could enhance or reduce NPs stability in the medium and thus their toxicity (Mezni et al., 2018; Arvidsson et al., 2020). Both NPs showed zeta values allowing them to be more likely stable in the NSW with a zeta average size ranging from 200 nm to 700 nm for Cr_2O_3, and relatively lower at a maximum of 300 nm for Al_2O_3 which could suggest their higher bioavailability. NSW in this study was used to mimic natural conditions and thus take into account the impact of natural organic matter (NOM) (Canesi et al., 2017).

Enzymatic and non-enzymatic biomarkers are crucial cellular antioxidant defenses against oxidative damages caused by NPs, they play a key role in the detoxification process which could be by their direct interaction with xenobiotic in biotransformation pathway, or indirectly *via* maintaining the redox system hemostasis and elimination of ROS (Valko et al., 2007; Forman et al., 2009; Board and Menon, 2013). Non-enzymatic biomarkers like GSH and MT play an important role in NPs detoxification by scavenging ROS as anion superoxide O^{2-} and other radicals like HO, exhibiting metallic binding proprieties, and NPs modulation, especially in the case of MT. GSH serves as an important subtraction for antioxidants. Any imbalance in these biomarkers serves as an indicator of NPs toxicity (Saad et al., 2016; Gulcin, 2020; Roma et al., 2020). Enzymatic biomarkers that trigger or inhibit are considered indicators of NPs toxicity occurrence mainly through oxidative stress induction. The primary function of GST, GPx, CAT, and SOD is the removal of many types of ROS, with each antioxidant targeting specific types of ROS. Furthermore, AChE is crucial in neurotransmission by inhibiting ACH and has been used as a marker of neurotoxicity in several studies (Sedrati et al., 2024). In the same way, MDA is a molecule resulting from the lipidic peroxidation process triggered by oxidative stress, and it is also been widely applied as an effective marker of NPs toxicity (Hu and Gao, 2010; Shaw and Handy, 2011; Brigelius-Flohé and Maiorino, 2013; Vale et al., 2016).

The exposure of *S. haemastoma* to both types of NPs had varied effects on biomarkers in the gills, depending on the concentrations and duration of exposure. Overall, after 7 days, Cr_2O_3 NPs caused MT, AChE, and SOD activities to decrease with MDA levels to increase, on the other hand, Al_2O_3 NPs induced GST, CAT activities, GSH, MDA rates to increase, SOD activity and MT rates to decrease. Suggesting the response of marine snails to NPs exposure by activation of antioxidant defenses to counteract the mitigated oxidative stress indicated by MDA rates increase due to Al_2O_3 NPs (Fig. 5) as additionally, IBR results confirmed the high induction of MDA rates (Fig. 8&9) and AChE inhibition due to Cr_2O_3 NPs (Fig. 6) exposure. MT levels reduction point also the detoxification process of NPs. Puerari et al. (2016) reported the induction of ROS-mediated toxicity by Cr_2O_3 in *Daphnia Magna*. Our results observed in the IBR analysis indicating the sensitivity of SOD, CAT activities and MDA rates to Cr_2O_3 NPs (Fig. 8) were similar to the findings of Kanwal et al. (2019) in fish *Labeo rohita* by CAT, SOD decrease, and MDA induction due to 25 mg/L of Cr_2O_3. Exposure to high concentrations of Al_2O_3 was found to decrease the levels of SOD while increasing the levels of CAT and MDA in *D. magna* (Nogueira et al., 2020a, 2020b). Additionally, the induction of GST, MDA, CAT, and SOD was reported in the fish *Carassius auratus*, at low concentrations (10 and 100 µg/L) after 7 days, while our results indicated that SOD activity was mostly decreased at all exposure concentrations for both NPs (Benavides et al., 2016). Furthermore, in zebrafish, *Danio rerio,* and clam *Ruditapes decussatus*, the levels of CAT activity, MDA rates, and ROS content increased due to exposure to Ag, ZnO, SiO_2, and TiO_2 NPs (Saidani et al., 2019; Rashidian et al., 2023). The study by Nunes et al. (2020) reported an increase in GST, CAT activities, and MDA contents accompanying the formation of NPs- mucus filaments in the gills of the mussel *Perna perna* following

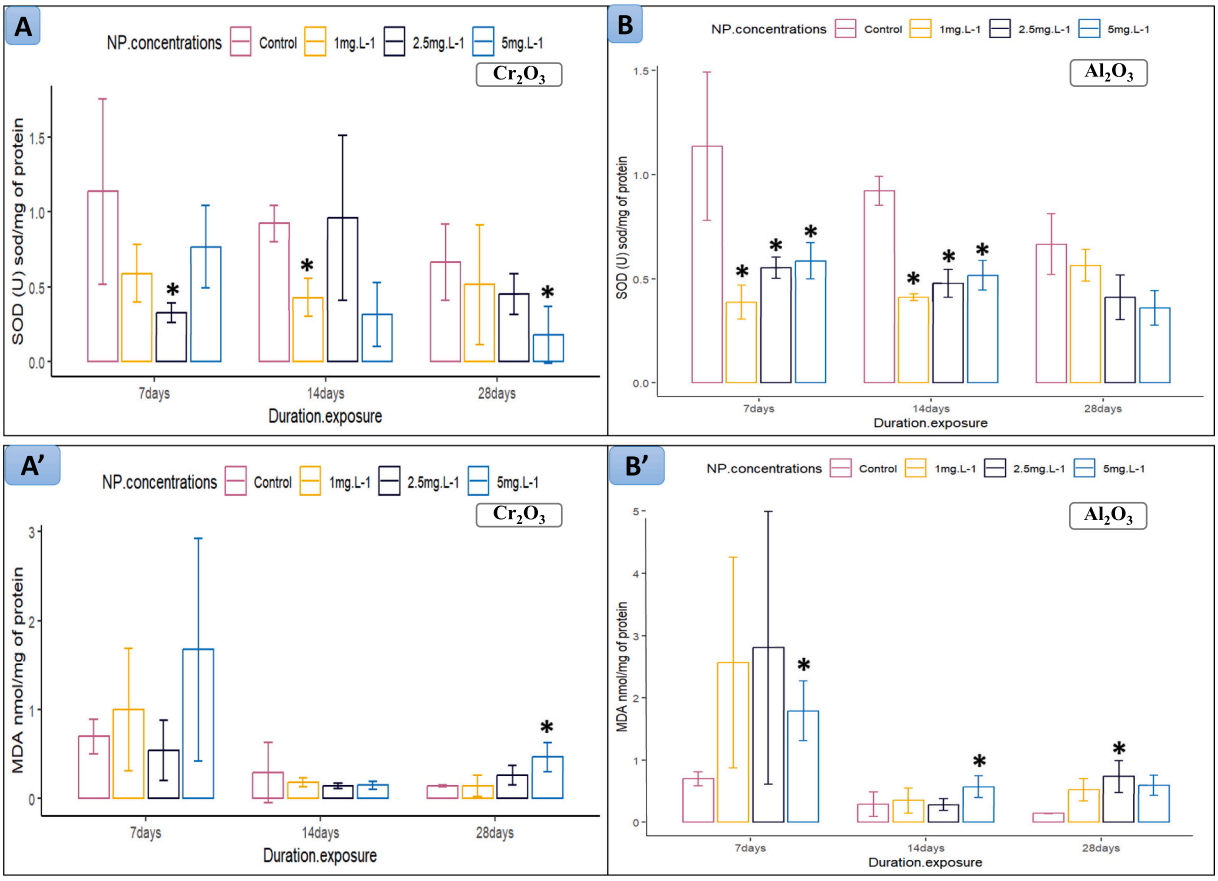

Fig. 5. SOD, and MDA rates in the Gills of *Stramonita haemastoma* treated with Cr_2O_3 (A, A') and Al_2O_3 (B,B'). The values are expressed as mean ± S.d. n = 3 (* $p < 0.05$).

exposure to TiO_2 (48 h). In this study, similar phenomena were observed with microfilaments with NPs accumulation, which was clearer in the case of Cr_2O_3 potentially due to their green pigmentation. This observation is, to our best knowledge, the first report of such a case in gastropods **(**Fig. 5 **in supplementary)** and could indicate NPs bioavailability. Similar to the observed results in this study, De Oliveira et al. (2014) **and** Katuli et al. (2014) reported AChE activity inhibition in zebrafish *D. rerio* after acute exposure to high doses (20–200 mg/kg) of iron oxide NPs (IOs NPs) and sub-acute exposure to Ag NPs. Moreover, Nelson et al. (2018) reported neurotoxic effects on the gills functions of bivalves due to metallic traces.

On day 14, both NPs induced a decrease in GST, SOD activities (Fig. 4&5), and MT rates with AChE activity decrease due to Cr_2O_3 exposure (Fig. 6). GSH levels increased due to Al_2O_3 exposure while Cr_2O_3 caused GSH content depletion at 2.5 mg/L (Fig. 3), MDA rates showed lower rates (Figs. 5, 11). IBR results for the same identified the higher response of CAT activity, GSH, and MT rates to Cr_2O_3 exposure (Fig. 7), IBR results indicated also the sensitivity of MT rates and AChE activity to Al_2O_3 exposure (Fig. 8) which further could refer to the limitation of standard classical biomarkers evaluation. These results could suggest the onset of adaptive mechanisms in response to oxidative damage. Continuous decreases in AChE activity and MT levels, variations in GSH, rates, and decrease in GST activity could indicate enzymatic consumption and detoxification process of NPs, while the decrease in SOD further indicates the high production of O^{2-} resulting in cellular redox instability. The differential response of biomarkers for each NP exposure highlights the complexity of gastropods' interaction in response to pollutant contamination. The up and down-regulation of antioxidant system genes were reported, with similar findings in the digestive gland of *Mytilus galloprovincialis* in response to TiO_2 exposure **(**Gornati et al., 2016**)**. Marine organisms possess a diversity of complex mechanisms and biochemical cascades as an adaptation to oxidative stress, from gene expression to catalytic activities that could vary in function of time and exposure doses **(**Regoli and Giuliani, 2014**)**. Federici et al. (2007) reported GSH rates induction and NPs accumulation after 14-day exposure to 1 mg/L of TiO_2 NPs in the gills of Rainbow trout *Oncorhynchus mykis*, consistent with the finding of Ray et al. (2020) which reported SOD and CAT activities reduction after 7 and 14 exposure days to CuO NPs in the gills of the mussel *Lamellidens marginalis* at 0.5 to 5 mg/L. Cordeiro et al. (2021) reported the higher impact of TiO_2 NPs (1 mg/L) on the digestive gland of the shrimp *Litopenaeus vanname* compared to their gills, where GST activity decreased and GSH content induction was reported. Ramsden et al. (2013) reported GSH rate induction in the gills of *D. rerio* following 1 mg/L exposure to TiO_2 NPs for 14 days.

Overall, at the end of the exposure period (28 days), both types of NPs induced various changes in biomarkers response, showing some similarities and differences. For instance, Cr_2O_3 NPs caused increased GSH content, GPx, CAT (Fig. 3&4), and AChE activities (Fig. 6) compared to the control, especially at higher exposure concentrations. The group exposed to Al_2O_3 NPs had increased activities of GSH, GST (Fig. 3), MT (Fig. 6), and MDA (Fig. 6) rates in some exposure concentrations, along with a decrease in CAT activity (Fig. 4). IBR results indicated the sensitivity and response of GST, CAT, GPx, AChE, MT and MDA contents with the highest response factor recorded at 2.5 mg/L for Cr_2O_3 (Fig. 7) and in a dose-dependent manner for Al_2O_3 (Fig. 8). These results suggest that oxidative damages accumulated over time, leading to instability in the redox system. The imbalance in antioxidants and MDA levels further confirms that the defensive systems were unable to preserve the cellular stability from the action of ROS and further

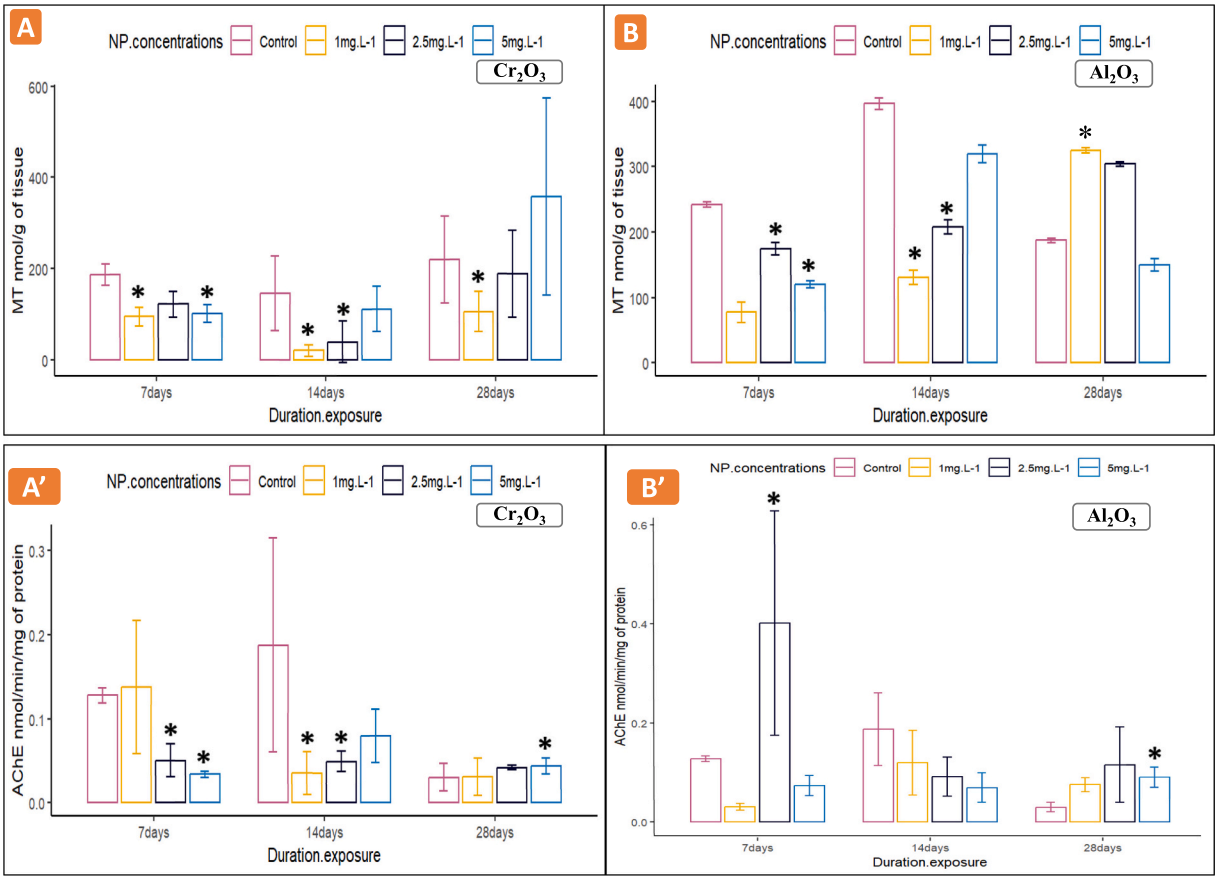

Fig. 6. MT, and AChE rates in the Gills of *Stramonita haemastoma* treated with Cr_2O_3 (A, A') and Al_2O_3 (B,B'). The values are expressed as mean ± S.d. n = 3 (* $p < 0.05$).

indicates that gills are a potential target of NPs toxicity. The induction of GST activity and GSH rates suggests the continuous presence of NPs and their uptake in gills thus acting in the detoxification process which could potentially contribute to the release of NPs ions (Caixeta et al., 2020; Dube and Okuthe, 2023). Huang et al. (2018) found that exposure to TiO_2 exposure (2.5 mg/L- 10 mg/L) in the mussel *Mytilus coruscus* caused CAT, GPx, GST activity induction, and an increase in MDA rates, along with SOD activity inhibition. Similarly, another study by Canesi et al. (2010b) reported that TiO_2 exposure (0.05–5 mg/L) caused CAT and GST activity induction in the gills of *M. galloprovincialis*. Consistent with our findings, other studies have reported MT level induction, depletion, and gene-up regulations in bivalves due to NPs exposure, indicating that MT imbalance is a key marker of NPs toxicity, as our results indicated the ability of both studied NPs to disturb MT rates (Buffet et al., 2011 and 2012; Gomes et al., 2011 and 2012). The exposure to TiO_2, CuO, and Al_2O_3 induced similar effects in biomarkers with lesser impact for Al_2O_3. This included CAT, SOD activity decrease, and GST activity induction in the digestive gland and gills of the mussel *Unio tigridis* (Canli and Canli, 2021). Al_2O_3 accumulation into gills was also reported in *U. tigridis* (Canli et al., 2022), while Al_2O_3 was reported to induce higher toxicity than other NPs, including SOD activity decrease and GPx activity induction in rats (Canli et al., 2019). Additionally, neurotoxic effects were reported in *D. rerio* due to Al_2O_3 NPs, including (ACh) inhibition, Al_2O_3 NPs accumulation, and memory capacity alteration resulting from oxidative damage (Chen et al., 2020). The different responses observed of some biomarkers to each type of NPs for instance SOD, GPx and, CAT could be interpreted as due potentially to the intracellular pathways and locations in which those NPs could induce oxidative stress and consequently unbalancing biomarkers activities and rates.

Histological assessment is a crucial and reliable tool that could provide evidence of NPs toxicity and impacts on the cellular to tissue level (Cid et al., 2015; Leite et al., 2020). In the present study, the histological evaluation results showed clear evidence of alterations in the gills of *S. haemastoma* for both NPs. Various histological damage was observed, ranging from lamella fusion to gills central cavities hypertrophy with signs of inflammation and tissue destruction. The gills of the marine snails were sensitive to both NPs concentrations and, after 28 days, showed strong evidence of progressive histological alteration. The findings further imply the failure of the redox system defenses in the preservation from oxidative damage and suggest that both Cr_2O_3 and Al_2O_3 NPs were uptake and translocated through the gills of *S. haemastoma*, thus, inducing harmful effects by direct interaction along cell membranes or by their internalization (Canesi et al., 2008; Bonany et al., 2022). The present results are consistent with previously reported histological findings as several studies noted gills histological damage in bivalves (Cuevas et al., 2015; Canli et al., 2022), gastropods (Arrighetti et al., 2022), fish (Xiong et al., 2011), and *Daphnia magna* (Puerari et al., 2016) due to NPs contamination. Cr_2O_3 NPs were reported to induce inflammation and edema in the liver and kidneys of *Labeo rohita* after 21 days of exposure (**Kanwal et al., 2019**), and cellular dilatation and degeneration were also reported in rats (**Fatima and Ahmad, 2019**). Al_2O_3 NPs induced Lipofuscin aggregates, lamellar fusion, and hemolytic infiltration following 96 h exposure in the gills of *M. galloprovincialis* (Ertürk Gürkan and Gürkan, 2021). Similarly to our findings, Al_2O_3 was reported to induce lamella fusion and hyperplasia in the gills of *Carassius auratus*, at low concentrations (10 and 100 μg/L) (Benavides et al., 2016), necrosis, hyperplasia, and inflammation were also observed in freshwater fish *Oreochromis mossambicus* (Murali et al., 2018). The results obtained in the present studies pointed to the ability

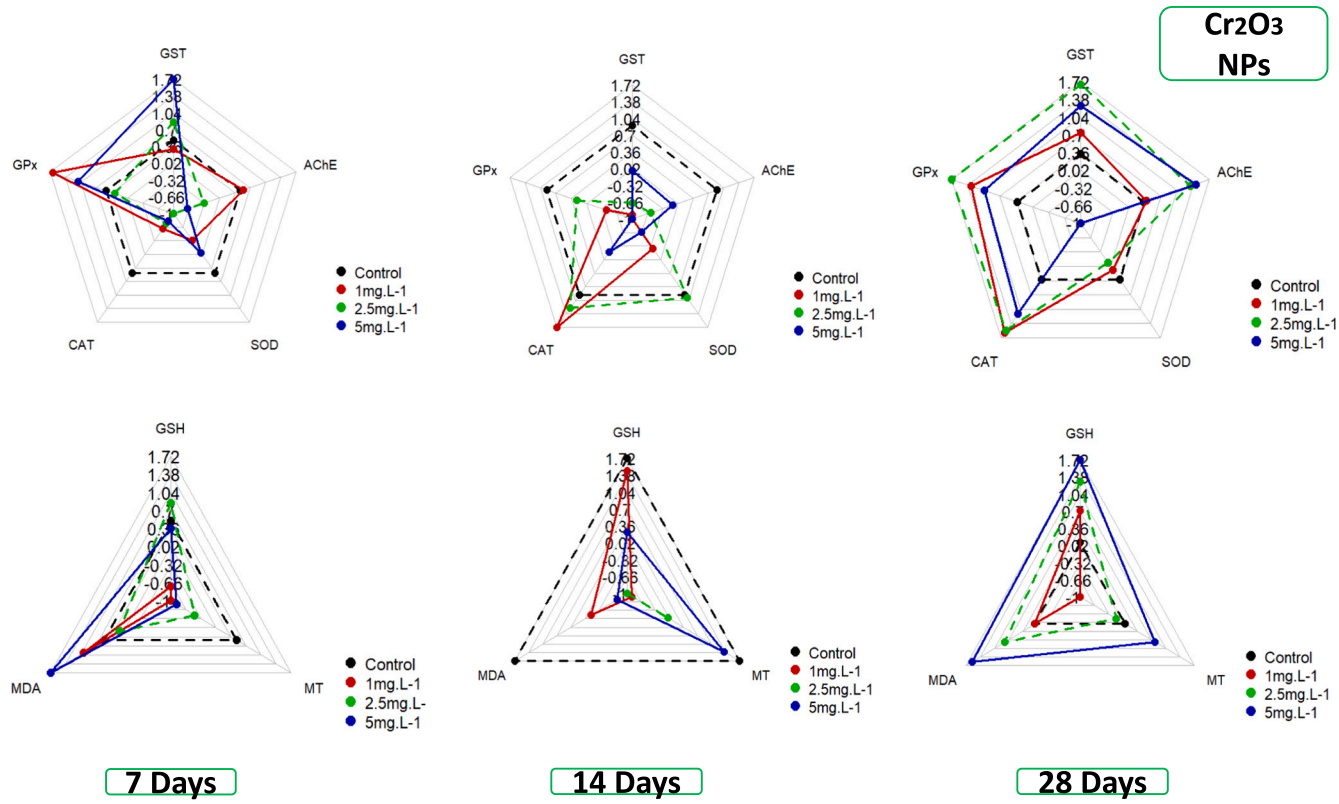

Fig. 7. IBR v2 Star plots of GST, GPx, CAT, SOD,and AChE activities (enzymatic biomarkers) and GSH, MT, MDA Contents (Non enzymatic biomarkers) in gills of of *Stramonita haemastoma* treated with Cr_2O_3. For 7, 14, and 28 exposure days.

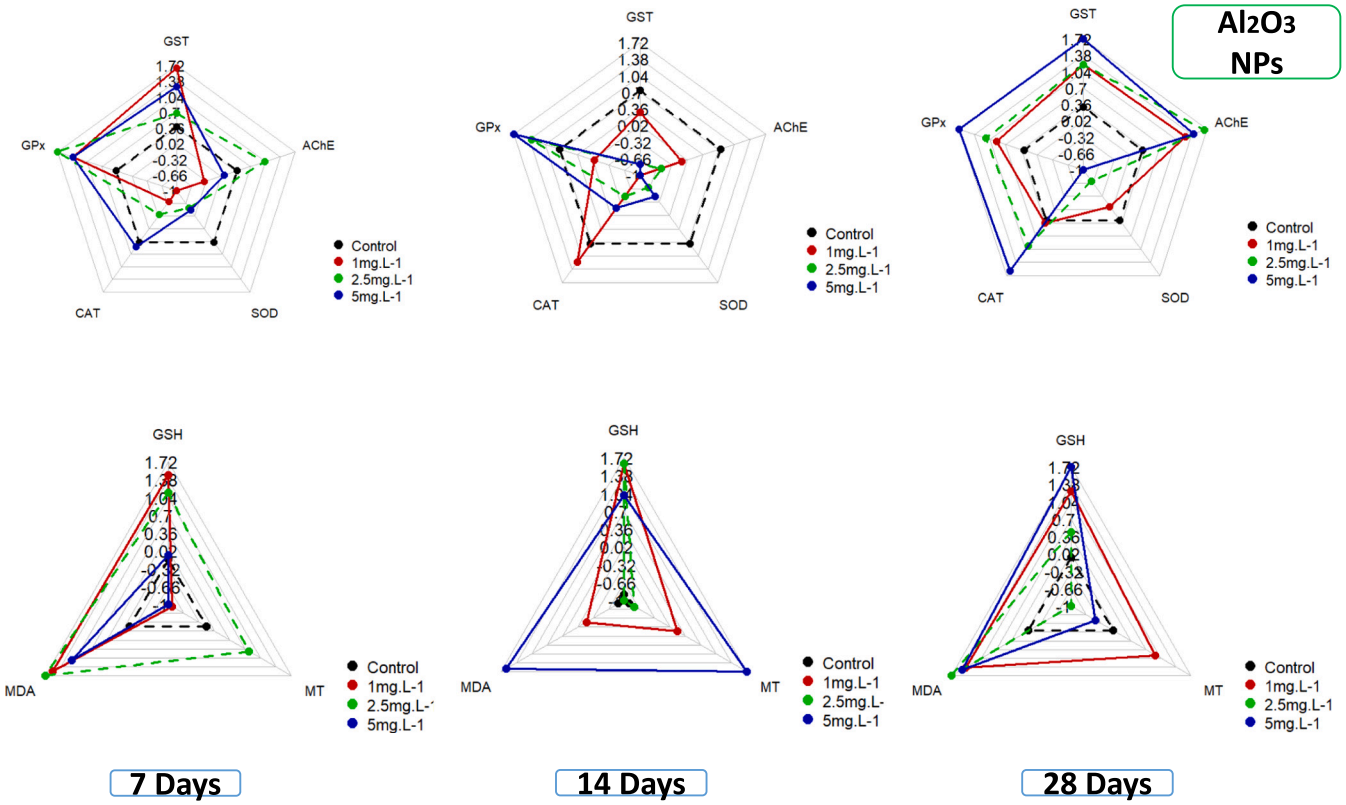

Fig. 8. IBR v2 Star plots of GST, GPx, CAT, SOD,and AChE activities (enzymatic biomarkers) and GSH, MT, MDA Contents (Non enzymatic biomarkers) in gills of of *Stramonita haemastoma* treated with Al_2O_3. For 7, 14, and 28 exposure days.

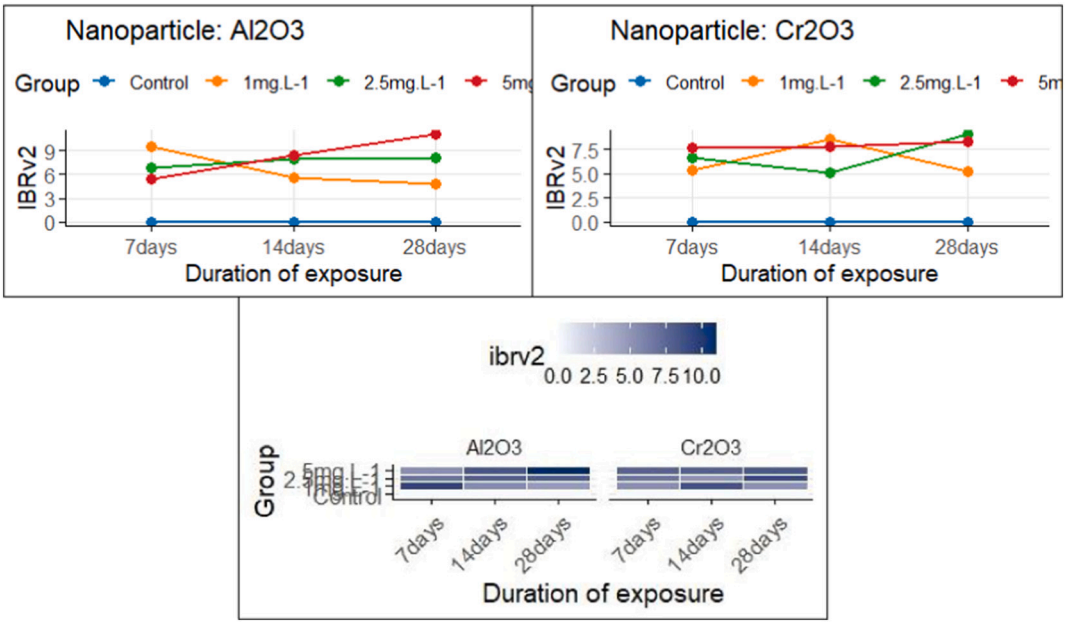

Fig. 10. IBR v2 values evolution trough exposure periods (7, 14, and 28 days) and both Cr_2O_3, and Al_2O_3 NPs concentrations (control, 1, 2.5, and 5 mg/L) in *Stramonita haemastoma* gills.

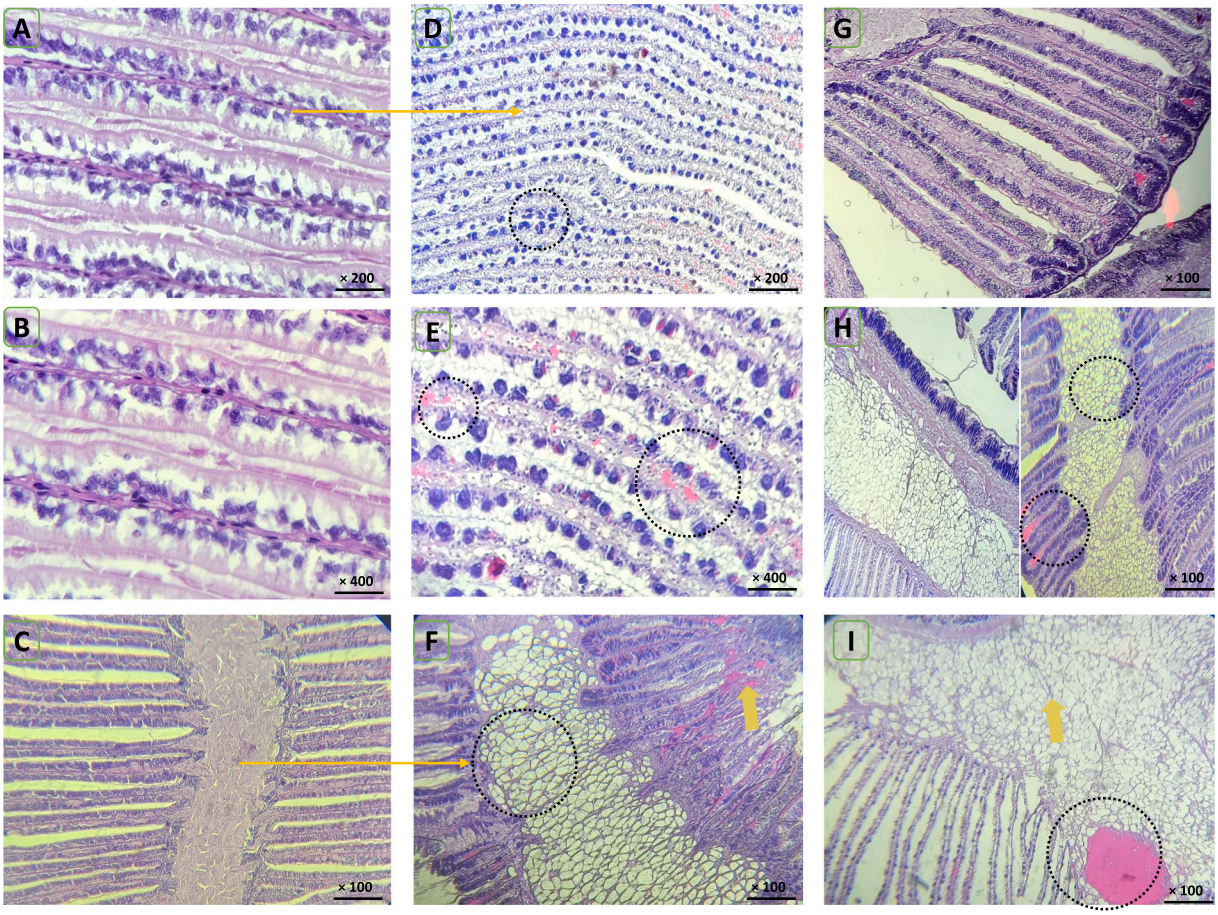

Fig. 9. Histological alterations observed in the Gills of *Stramonita haemastoma* exposed to 28 days of $Cr_2O_3$and Al_2O_3. A,B: Control gills filaments, C: Control ospharidium, D (Cr_2O_3 exposure), E (Al_2O_3 exposure): Goblet cells proliferations, F (Cr_2O_3 exposure), H (both NPs), I (Al_2O_3 exposure): central cavity hypertrophy, lamella fusion G (Al_2O_3 exposure): filaments thickening (observed in both NPs exposure), I (Cr_2O_3 exposure): filaments alteration. coloration H&E X100, X200, X400 low power.

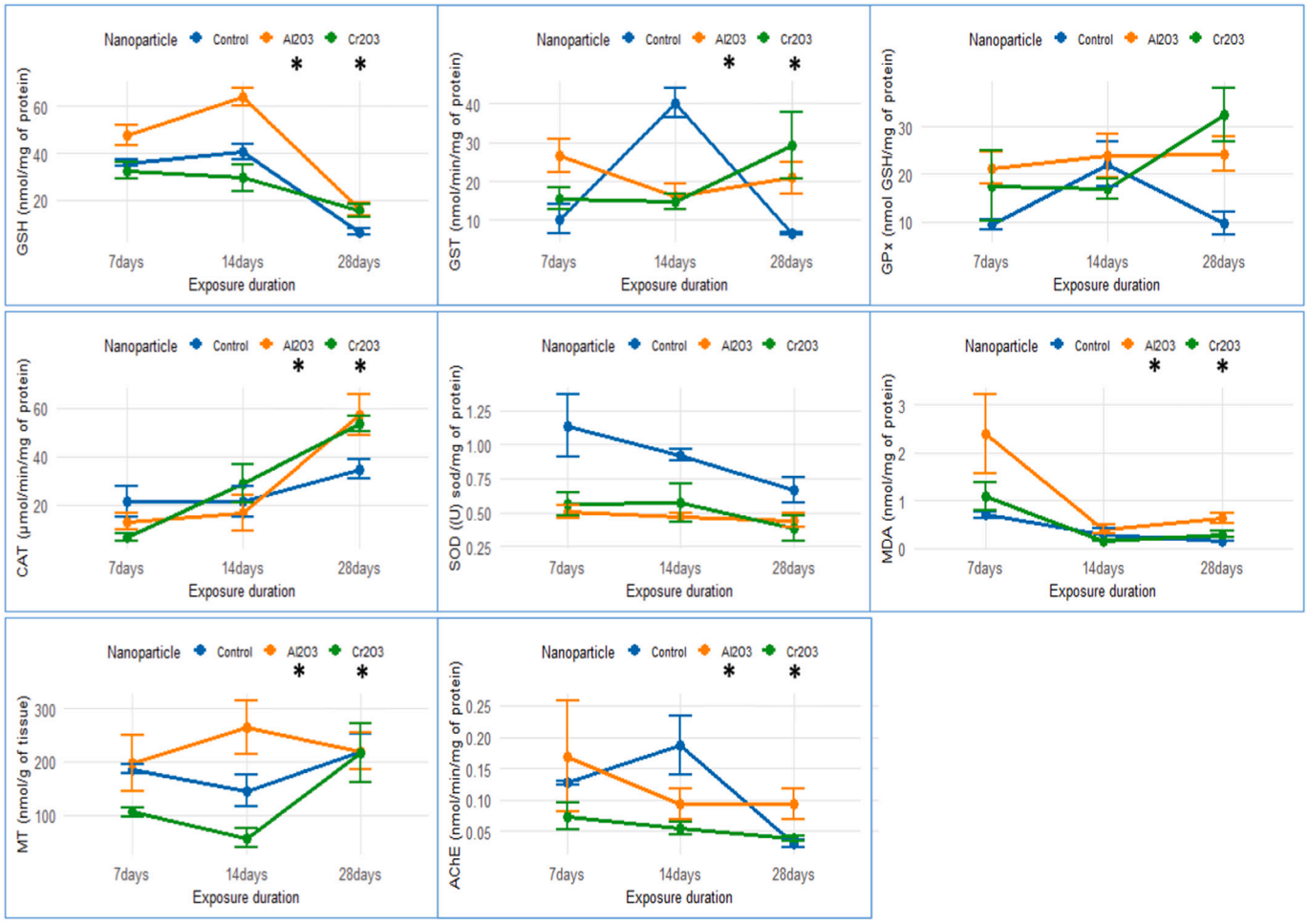

Fig. 11. The trend variation of biomarkers (GSH, GST, GPx, CAT, SOD, MDA, AChE, and MT in the gills of *Stramonita haemastoma* exposed to Al_2O_3 and Cr_2O_3 NPs thought exposure durations (* $p < 0.05$).

of both NPs to induce a large diversity of permanent histological damages and further prove that NPs have the potential to decrease the health status of aquatic ecosystems including marine ones, for instance, NPs concentrations will inevitably rise with their increased applications by time thus, may cause direct harmful effects to aquatic organisms or indirect one as in the case of trophic web translocations.

The usage of a multi-biomarkers approach to evaluate the pollutant's toxicity is crucial. However, interpreting various biomarkers responses to contamination independently can be challenging, and researchers face a dilemma in achieving proper results interpretation.

The integrated biomarkers response (IBR) is a valuable tool for evaluating trends in oxidative stress biomarkers (Beliaeff and Burgeot, 2002; Sanchez et al., 2013; Roma et al., 2024). The IBRv2 analysis provides an integrative assessment of multiple biomarkers, allowing us to evaluate the overall stress response of snail organisms to nanoparticle (NPs) exposure in a comprehensive manner. Unlike the individual biomarker analyses, which focus on specific biochemical pathways or enzymatic activities, the IBRv2 combines these responses into a single index, offering a broader perspective on the cumulative effects of stressors. This holistic approach adds significant value by facilitating comparisons across experimental groups and identifying patterns that may not be apparent from individual biomarker data alone. In this study, IBR was used to further clarify and validate results obtained from assessing numerous biomarkers. Briefly, the results of IBR showed clear responses of biomarkers to different concentrations for both NPs and exposure periods. Specifically at day 7 and day 14, IBRv2 indicated that both NPs have a significant impact on biomarkers, with higher response observed in 2 to 3 biomarkers, namely after 7 days SOD, AChE, and MDA due to Cr_2O_3 NPs exposure while the most sensitive biomarkers due to Al_2O_3 exposure were SOD, MT, and MDA. Following 14 exposure days, IBRv2 results indicated that AChE and MT were the most sensitive to Cr_2O_3 NPs exposure, and SOD, MT, MDA due to Al_2O_3 exposure. At 28 days, there was a higher global response in nearly all biomarkers, indicating the onset of oxidative stress and the activation of mechanisms to reduce damage. The results for both NPs confirm that effects triggered at 7 days were slightly reduced at day 14, suggesting the early response, with the activation of defensive mechanisms, and induced higher values at day 28, indicating cumulative oxidative damages causing co-regulation of all biomarkers' action IBRv2 global evolution according to exposure periods and concentrations are shown in Fig. 10. Such results could not be detected when evaluating biomarkers individually, highlighting the usefulness of IBR in the evaluation NPs toxicity. To our best knowledge, the present study is the first to assess NPs toxicity using IBR in marine gastropods. While IBR remains poorly used in the literature, several related studies have reported its successful application, primarily to bivalves (Xia et al., 2013; Devin et al., 2017; Liu et al., 2017; Li et al., 2021; Gonçalves et al., 2022; Roma et al., 2024). Previous studies have reported time and dose-dependent impacts on IBR of SOD, CAT, AChE, and MDA Xia et al. (2017) following exposure to 1 mg/L of TiO_2 NPs in the gills and digestive glands of the clam *C. farreri*. The exposure to high concentrations of ZnO_2 NPs (1 to 100 mg/L) showed high values after 5 days and then reduced IBR values in fluvial biofilm bacteria of GPx, CAT, SOD, and GR (Hou et al., 2016). The present study differs from previous ones as we use a variety of biomarkers, including

AChE and MT responses.

Several studies have identified ion release as a primary mechanism for increasing ROS production (Yung et al., 2014; Peng et al., 2017). Previous studies indicate that the toxicity of Cr_2O_3 NPs is attributed to the liberation of Hexavalent Cr ions in A549 cells and other models, leading to constantly increased intra and extra-cellular ROS levels (Horie et al., 2013; Alarifi et al., 2016). In the same way, da Costa et al. (2015) reported higher ROS production due to Cr_2O_3 NPs exposure in the green alga *Chlamydomonas reinhardtii*. The results observed in this study indicate the uptake of both NPs in the gills of *S. haemastoma*, as gills are responsible for ensuring respiratory function in marine snails, the contamination of both NPs caused their direct contact with gills. The uptake of NPs agglomeration mainly through the gills of bivalves and then their translocation to the digestive gland was previously identified as a main pathway of NPs accumulation (Koehler et al., 2008; Ciacci et al., 2012; Volland et al., 2015). Internalization of NPs could occur and be influenced by several factors, including NPs size, zeta average diameter, shape, and concentration (Barmo et al., 2013). Furthermore, NPs toxicity could be amplified in the environment, as interaction with other pollutants and their absorption on NPs' surface, significantly increases their toxicity. Conversely, this aspect should also be considered in future studies. The present study addressed this divergence by using NSW, as potential interactions could be important factors (Canesi et al., 2017; Latchere et al., 2023a, 2023b).

5. Conclusion

In this present work, the toxicity of Cr_2O_3 and Al_2O_3 NPs was investigated over time in the gills of the marine gastropod *Stramonita haemastoma*. The findings provided important novel insights into the induction of oxidative stress by NPs and the adaptive mechanisms of marine snails. Both NPs were able to induce various changes in the antioxidant system, with the most marked effects observed in SOD, CAT, AChE, MT, and MDA. In the same way, histological evaluation showed clear evidence of both NPs' bioavailability in the gills of snails as dose-dependent alterations including inflammation, hypertrophy, and lamella fusion signs suggesting that Cr_2O_3 and Al_2O_3 NPs could translocate through gills in marine gastropods. The use of IBR in this study was crucial for identifying NPs toxicity patterns and further provided evidence of the time-dependent impact of exposure on biomarkers and induction of oxidative stress. IBR was applied to a battery of biomarkers and appeared to be a potentially ideal tool to assess NPs toxicity and compare impact trends in marine gastropods. The methodological contribution of this paper could be extended to future related studies. Additionally, the sensitivity of *S. haemastoma* to NPs exposure validates this gastropod as a promising bioindicator of NPs contamination in marine ecosystems. Future extended toxicological evaluations are required to elucidate NPs uptake, translocation, and toxicity mechanisms at the molecular level, especially for Cr_2O_3 which has received less attention compared to other NPs in ecotoxicological studies. We strongly suggest and recommend a more expanded assessment of the actual coastal NPs contamination especially regarding specific NPs as Cr_2O_3 due to the lack of studies involving this type of NPs and others compared to their economic importance. Another important crucial point to consider in perspective is as discussed the combined impacts and interactions among several types of NPs and other pollutants and toxins.

CRediT authorship contribution statement

Fateh Sedrati: Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Hana Bouzahouane:** Visualization, Validation, Supervision, Methodology. **Mohcen Menaa:** Software, Formal analysis, Data curation. **Fadila Khaldi:** Validation, Supervision. **Tayeb Bouarroudj:** Software, Investigation, Formal analysis. **Lassaad Gzara:** Visualization, Formal analysis. **Mounira Bensalem:** Resources, Investigation. **Omar Laouar:** Methodology, Investigation. **Noomene Sleimi:** Resources. **Hichem Nasri:** Resources. **Carla O. Silva:** Writing – review & editing, Writing – original draft, Visualization. **Kheireddine Ouali:** Validation, Resources.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbpc.2025.110159.

Data availability

Data will be made available on request.

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