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ORIGINAL ARTICLE



# Toxicity of gamma aluminium oxide nanoparticles in the Mediterranean mussel (*Mytilus galloprovincialis*): histopathological alterations and antioxidant responses in the gill and digestive gland

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**ABSTRACT**

**Purpose:** Accumulation of Gamma aluminium oxide nanoparticles γ-$Al_2O_3$ NPs significant impact on aquatic ecosystems. However, the toxicity of γ-$Al_2O_3$ NPs in aquatic organisms has been limited investigated. This study investigated histopathological changes and antioxidant responses induced by different concentrations of γ-$Al_2O_3$ NPs in *Mytilus galloprovincialis*.

**Material and methods:** In this study, mussels were exposed to different concentrations of 5 nm γ-$Al_2O_3$ NPs (0, 5, 20 and 40 mg/L) for 96 h under controlled laboratory conditions. Gill and digestive gland from mussels were assessed to histopathological (light microscopy, histopathological condition indices, digestive gland tubule types), SOD, CAT, GPx activities.

**Results:** Histopathological indices calculated higher, and significantly different in all exposure groups compared to the control group in gill and digestive gland ($p < 0.05$). Atrophic phase tubules proportion very high in 20 and 40 mg/L γ-$Al_2O_3$ NPs exposure groups. No significant changes in CAT activities in the gill and digestive gland ($p > 0.05$). Superoxide dismutase (SOD) activities significantly different ($p \leq 0.05$) in the digestive gland from 20 mg/L γ-$Al_2O_3$ NPs exposures, and GPx activities significantly different ($p < 0.05$) in gill from 40 mg/L γ-$Al_2O_3$ NPs exposures.

**Conclusion:** These results indicate that contamination of γ-$Al_2O_3$ NPs negatively affects the aquatic organism.

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**KEYWORDS**
Histopathology; oxidative stress; gamma aluminium oxide nanoparticles; nanotoxicity; Mediterranean mussel

## Introduction

The development of nanotechnology has led to the widespread of a new formation called nanomaterials (NMs). NMs are defined as structures designed to a minimum size of less than 100 nm and contain features that allow successful applications in a wide range of fields from electronics to medicine (Gornati *et al*. 2009, 2016). It has been pointed out that the toxicity of these materials, which are the product of technology to facilitate daily life, can potentially affect organisms and environmental safety (Jacobs *et al*. 2010), moreover, many substances that are considered harmless can become toxic at the nanoscale through their higher reactivity and easier penetration through biological barriers (Cattaneo *et al*. 2010, 2014, Gornati *et al*. 2016). Due to their large surface area, these nanoparticles (NPs) can directly lead to the formation of harmful oxyradicals (ROS) and even cell damage by attacking DNA, proteins, and membranes (Brown *et al*. 2001). Also, many NPs are thought to increase their existing toxicity by their tendency to bind transition metals and organic chemical pollutants in the environment they enter (Cheng *et al*. 2004, Gilliland *et al*. 2004). Thus, the high affinity of the particles to penetrate the body and cells makes it a potential vehicle for the transport of toxic contaminants associated with the nanoparticle to regions in organisms where they would normally not enter (Pelkmans and Helenius 2002, Na *et al*. 2003, Panyam *et al*. 2003, Panyam and Labhasetwar 2003, Berry *et al*. 2004, Lacava *et al*. 2004, Moore 2006). Especially in aquatic animals, entry of NPs into cells can be directly from the gills and other outer surface epithelium, as well as entry by endocytotic pathways, is defined as the main mechanisms assumed (Moore 2006, Bareford and Swaan 2007, Ivanov 2008, Doherty and McMahon 2009, Bouallegui *et al*. 2017).

Aluminium oxide nanoparticles ($Al_2O_3$ NPs), which is a metal oxide, is used in explosive-derived formations (Kaste and Rice 2004) such as protective paints (Khanna 2008), the construction of some filtration membranes (DeFriend *et al*. 2003), various vehicle fuels (Luca *et al*. 2005, Lewis *et al*. 2011), and the content of heat-raising nanofluids (Wong and Kurma 2008). It is inevitable that $Al_2O_3$ NPs, the use of which has increased with the increase of such engineering applications, will enter the water, terrestrial and atmospheric environment and have possible negative effects for organisms, just like other NPs (Maynard *et al*. 2006, Nowack and Bucheli 2007, Sadiq *et al*. 2011, Baker *et al*. 2014, Corsi *et al*. 2014).

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Related to this, some studies have been planned to determine the toxicity of $Al_2O_3$ NPs and their possible harmful effects on various organisms. For example, it has been determined that worms (Heckmann *et al*. 2011), nematodes (Li *et al*. 2012), and some prokaryotic organisms (Pakrashi *et al*. 2011) are negatively affected by $Al_2O_3$ NPs. Studies on the accumulation of this nanoparticle, which is characterized by its insolubility in water, in the sediment structure and its effects on aquatic organisms, are also limited (Zhu *et al*. 2008, Zhu *et al*. 2009, Sadiq *et al*. 2011, Pakrashi *et al*. 2013). Besides, the effect of $Al_2O_3$ NPs exposure on some living groups in the aquatic habitat is also revealed; for example, histological and physiological effects of $Al_2O_3$ NPs have been determined in studies conducted on some fish species (Benavides *et al*. 2016, Bai and Tang 2020, Canli and Canli 2020) and amphibians (Ismail *et al*. 2019). However, only one study was found on the effects of gamma form, one of the two forms of $Al_2O_3$ NPs, on aquatic organisms (Ateş *et al*. 2013).

Mussels have long been recognized as valuable biomarkers of environmental pollution. The reason for this can be shown to be their high capacity to tolerate and accumulate various pollutants, sedentary lifestyle, wide geographical distribution, and abundance situations (Wang *et al*. 1996, Viarengo *et al*. 2007, Banni *et al*. 2014, Leite *et al*. 2020). All together make mussels a good model for monitoring environmental status in coastal ecosystems and planning in vitro studies as they are easy to collect and maintain (Box *et al*. 2007, Sureda *et al*. 2011, 2018). Recently, it is accepted as the main target group to examine the effects of NPs due to these properties (Canesi *et al*. 2012, Sendra *et al*. 2018).

*Mytilus galloprovincialis* Lamarck, 1819 (Mediterranean mussel or black mussel), one of the most preferred species as an indicator of pollution among marine mussels, is an organism that is frequently used in scientific studies due to its large number in our country, its easy sampling, its distribution in a wide geographical area and its relatively low diagnostic problem (Aksoy *et al*. 1999). In addition to these features, it is also of ecological importance as it is at the bottom of the food web and is a food source for many other living species (Baskın 2019).

Antioxidants are chemical substances that play a role in free radicals in biological systems. They can reduce the energy of free radicals, help them stabilize by making them use their own electrons, stop them in the first place they are formed, and reduce damage from free radicals by interrupting an oxidation chain reaction (Otitoju and Onwurah 2011). The enzymes involved in antioxidant reactions; superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) (Valavanidis *et al*. 2006). Because of these properties, these three enzymes can be considered as potential biomarkers or bioindicators in revealing the risks of contaminants (Otitoju and Onwurah 2011).

As a result of the literature search, we did not find a study evaluating the possible toxic effects of $\gamma$-$Al_2O_3$ NPs on *M. galloprovincialis*, a powerful bioindicator species for the aquatic ecosystem. This study aims to reveal the possible toxic effects of gamma aluminium oxide nanoparticles ($\gamma$-$Al_2O_3$ NPs), which is a widely used area, on organisms if it enters the aquatic ecosystem. For this purpose, histopathological changes and antioxidant responses were examined in the digestive gland and gill tissues of individuals belonging to the *M. galloprovincialis*, which were exposed to different concentrations of $\gamma$-$Al_2O_3$ NPs for 96 h under laboratory conditions.

## Clinical significance

- Histopathological indices were calculated higher in both gill and digestive gland tissues in all exposure groups, and these differences were statistically significant.
- The SOD activities measured in the digestive gland of mussel samples exposed to 20mg/L γ-Al2O3 NPs differ significantly.
- The GPx efficiencies in gill tissue differ significantly at 40mg/L γ-Al2O3 NPs exposure.

## Material and methods

### *Experimental design*

Mediterranean mussels (62 ± 4 mm shell length) obtained from a local farm (Gelibolu Seafood Import Export Industry, Turkey), were transferred to the laboratory and acclimatized for 4 days in stock aquariums containing 15 L of seawater (about 1 L per mussel) and was changed daily (first 2 days all changed, other 2 days 50% changed). The study was designed as 10 individuals for each group and a three replicate trial setup was created for 96 h. The exposure concentrations (0, 5, 20 and 40 mg/L) were determined based on the information provided in the literature (Sadiq *et al*. 2011). The groups have been preserved in an aerated environment with the necessary water quality qualities. Temperature and dissolved oxygen levels of the water were measured by a YSI MPS 556 probe and were 18–21 °C and 7.8–8.3 mg/L, respectively. The pH of water was measured by a HANNA C 200 (HI 83200) photometer daily and maintained at pH: 7.7–7.9. All experiments were conducted in accordance with ethical rules.

### *Preparation of $Al_2O_3$ NPs suspension*

$\gamma$-$Al_2O_3$ NPs (5 nm and, 99.5% pure) were purchased, as uncoated nanomaterials, from Skyspring Nanomaterials, Inc. (Houston, USA) and stored at room temperature. Stock suspensions of $\gamma$-$Al_2O_3$ NPs were prepared by dissolving in seawater in 10% (mass/volume). The solution was sonicated in an ultrasonic bath for approximately 30 min to achieve maximum nanoparticle distribution (Wang *et al*. 2009). The stock suspensions were transferred to the aquarium units in appropriate volumes with an automatic pipette depending on the concentration ratio of each experimental group and the mussels were exposed to the nanoparticles for 96 h.

### Sampling

In the experiment, ten mussels from each aquarium were dissected at the end of the exposure time. The five samples of each group were used for histopathologic findings and five samples were dissected for the determination of antioxidant enzyme analyses. The target tissues were identified as gill and digestive gland for both histopathology and enzyme activity analyses.

### Histopathological assessment

Gills and digestive glands of 5 mussels per γ-$Al_2O_3$ NPs exposures (0, 5, 20 and 40 mg/L) were excised and immersed in Davidson's fixative. Fixation lasted 24 h at room temperature. Afterward, the specimens were dehydrated in progressive series of ethanol and embedded in paraffin. Tissues were cut with a 5 μm thickness in a Leica rotary microtome. Sections were obtained from each treatment group, then stained with hematoxylin-eosin (Wilson and Gamble 2002). Histological alterations were examined, and micrographs were taken using a CX31 Olympus light microscope equipped with a digital camera by using DP2-BSW software.

### Morphological phases of digestive gland tubules

To identify the morphological phases of digestive gland tubules, the epithelium and tubular lumen modifications were examined. The histopathological changes in the digestive tubules were classified three main phases (type I: holding phase; type II: absorptive phase; type III: atrophic phase) (Marigomez *et al*. 1990, Owen 1996, Carella *et al*. 2015, Rocha *et al*. 2016, Bouallegui *et al*. 2018). The proportion of each digestive type per animal was analyzed by 300 visual random digestive tubules per exposure group.

### Histopathological condition indices

Histopathological changes were identified in gills and digestive tubules of the mussels for each γ-$Al_2O_3$ NPs exposure. Individual semi-quantitative histopathological condition indices ($I_h$) for the gills and digestive tubules were estimated for mussels, according to weighed indices originally proposed by Bernet *et al*. (1999) for fish and modified by Costa *et al*. (2013) for clam and Cuevas *et al*. (2015) for mussels. The $I_h$ was estimated according to the concepts of the differential significance of each surveyed alteration (weight) and its degree of dissemination. The weight value ranges between 1 (minimum severity) and 3 (maximum severity), and the score value between 0 (none) and 6 (diffuse). The $I_h$ was calculated following formula, as described by Costa *et al*. (2013) as follows:

$$I_h = \frac{\sum_l^j w_j a_{jh}}{\sum_l^j M_j}$$

$I_h$ is the histopathological condition indices for individual $h$; $w_j$ the weight of the $j$th histopathological alteration; $a_{jh}$ the score attributed to the $h$th individual for the $j$th alteration and $M_j$ is the maximum attributable value for the $j$th alteration.

Weight for the histopathological changes and their importance weight ($w$) in the examined tissues of the mussels reported in Table 1.

Table 1. Histopathological changes and their importance weight ($w$) in gills and digestive tubules of *M. galloprovincialis* exposed for 96 h under different γ-$Al_2O_3$ NPs concentrations.

| Tissue | Histopathological alteration | $w$ |
|---|---|---|
| Gills | Lipofuscin aggregates | 1 |
| | Haemocyte infiltration | 1 |
| | Enlarged central vessel | 1 |
| | Loss of cilia | 2 |
| | Lamellar fusion | 3 |
| Digestive tubules | Lipofuscin aggregates | 1 |
| | Haemocyte infiltration | 1 |
| | Hyperplasia | 2 |
| | Hypertrophy | 2 |
| | Tubule regression | 2 |
| | Necrosis | 3 |

Importance weights according to Costa *et al*. (2013) and Pinto *et al*. (2019).

### Antioxidant responses

Within the scope of the study, the activities of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) enzymes of the antioxidant system were investigated in the gill and digestive gland tissues of *M. galloprovincialis* samples exposed to γ-$Al_2O_3$ NPs. Digestive gland and gill tissues were maintained at −45 °C and kept in 96% ethanol until enzyme analyses were performed.

Cytosolic fractions were obtained by preparing homogenate with phosphate buffer (0.05 M, pH: 7.4, 1: 5 w/w) from the digestive gland and gill tissues to measure the enzymes SOD, CAT, GPx which are used as biological markers of oxidative stress. The homogenate was centrifuged 2 times for 20 min at 4 °C at 10,000 *g*, and the supernatants were stored at −45 °C until antioxidant enzyme analysis. The amount of protein in each homogenate was determined by the method of Bradford (1976) and the enzyme-specific activities measured spectrophotometrically were calculated as mU/mg $protein^{-1}$.

SOD enzyme activity was measured based on the method of Flöhe and Ötting (1984) and Gilliland *et al*. (2004). SOD activity as a basic principle; determined by the formation of colour by reducing Nitro Blue tetrazolium (NBT) by the superoxide radical produced by the xanthine oxidase/hypoxanthine system. The reduction of the produced superoxide radical to NBT ends with the formation of blue-colored formazon that gives maximum absorbance at 550 nm.

Catalase activity was measured utilizing Clairborne (1985), based on the decomposition of $H_2O_2$. The samples were measured at 240 nm for 90 s, with measurement every 15 s. Any decrease in absorbance was observed; noted that it was directly proportional to CAT enzyme activity. GPx activity was determined following the method described by Wendel (1980). The basic principle is that as long as GR degradation continues, a decrease in absorbance occurs. This decrease in absorbance is directly proportional to the GPx activity in the environment. Measurements were recorded at a wavelength of 340 nm.

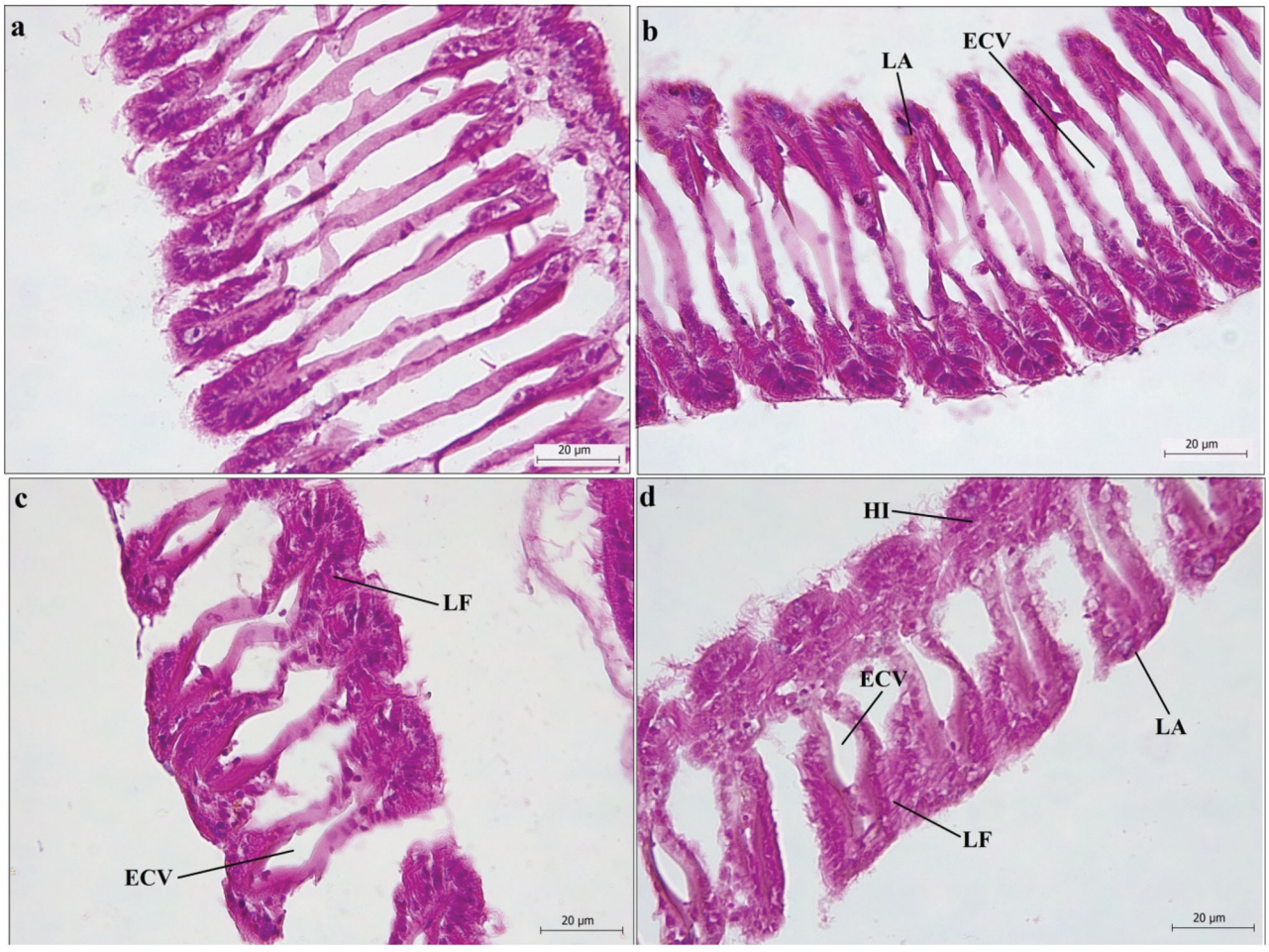


Figure 1. Micrographs of the gills of *M. galloprovincialis* from different concentrations of γ-$Al_2O_3$ NPs exposures for 96 h, stained with H&E. (a) Control (0 mg/L), (b) 5 mg/L, (c) 20 mg/L, (d) 40 mg/L. (LA: lipofuscin aggregation, ECV: enlargement of the central vessel, LF: lamellar fusion, HI: haemocytes infiltration).

### *Statistical analyses*

The statistical analyses were carried out in SPSS 21.0 software. Normality of data and homogeneity of variances was tested using Kolmogorov–Smirnov and Levene tests, respectively. The results were compared using parametric tests (One-Way ANOVA and Tukey's sub-test) and/or non-parametric tests (Kruskal–Wallis test). The correlation between enzyme levels measured at dose concentrations was calculated by Pearson correlation. The significant differences among concentrations were presented with different letters or figures. Discriminant analysis was employed to determine the significance of non-redundant variables (histological indices and antioxidant responses for individuals that were exposed to each dose concentration) to segregate dose concentrations after verification of non-linearity between means and variances. A significance level α was set to 0.05 for all analyses.

## Results

### *Characterization of γ-$Al_2O_3$ NPs*

The characterization of the size and shape and γ-$Al_2O_3$ NPs was measured in seawater and performed by XRD, FT-IR, TEM analysis, and as reported previously by Ateş *et al.* (2013), TEM results showed that γ-$Al_2O_3$ NPs with small and large spherical shapes. Zeta potential was measured negatively charged for γ-$Al_2O_3$ NPs (5 nm).

### *Histopathological assessment*

Histopathological observation of gill sections from the control group (0 mg/L γ-$Al_2O_3$ NPs) mussel revealed normal microanatomy. In this group, mussels were identified that gill filaments in a regular arrangement, epithelial cells with a normal distribution of lateral frontal cilia, and central vessels width is normal to measure (Figure 1(a)). Gill sections of *M. galloprovincialis* exposed to γ-$Al_2O_3$ NPs evidenced different histopathological alterations. The histopathological findings induced by γ-$Al_2O_3$ NPs exposures included lipofuscin aggregate, haemocyte infiltration, enlarged central vessel, loss of cilia, lamellar fusion (Figure 1(b–d)). Dramatic histopathological changes were seen in the gills of mussels exposed to 20 and 40 mg/L γ-$Al_2O_3$ NPs. Especially in these experimental groups significant lamellar fusion, and enlarged central vein were identified (Figure 1(c,d)).

In the control group, the normal phasic activity of the digestive glands was observed, which showed more proportion of holding, absorbing phase tubules compare to athropic phase tubule. Also in this group reduced the number of lipofuscin aggregates and haemocyte infiltration (Figure 2(a)). However, the histopathology of digestive glands from mussels exposed to different concentrations of γ-$Al_2O_3$ NPs was

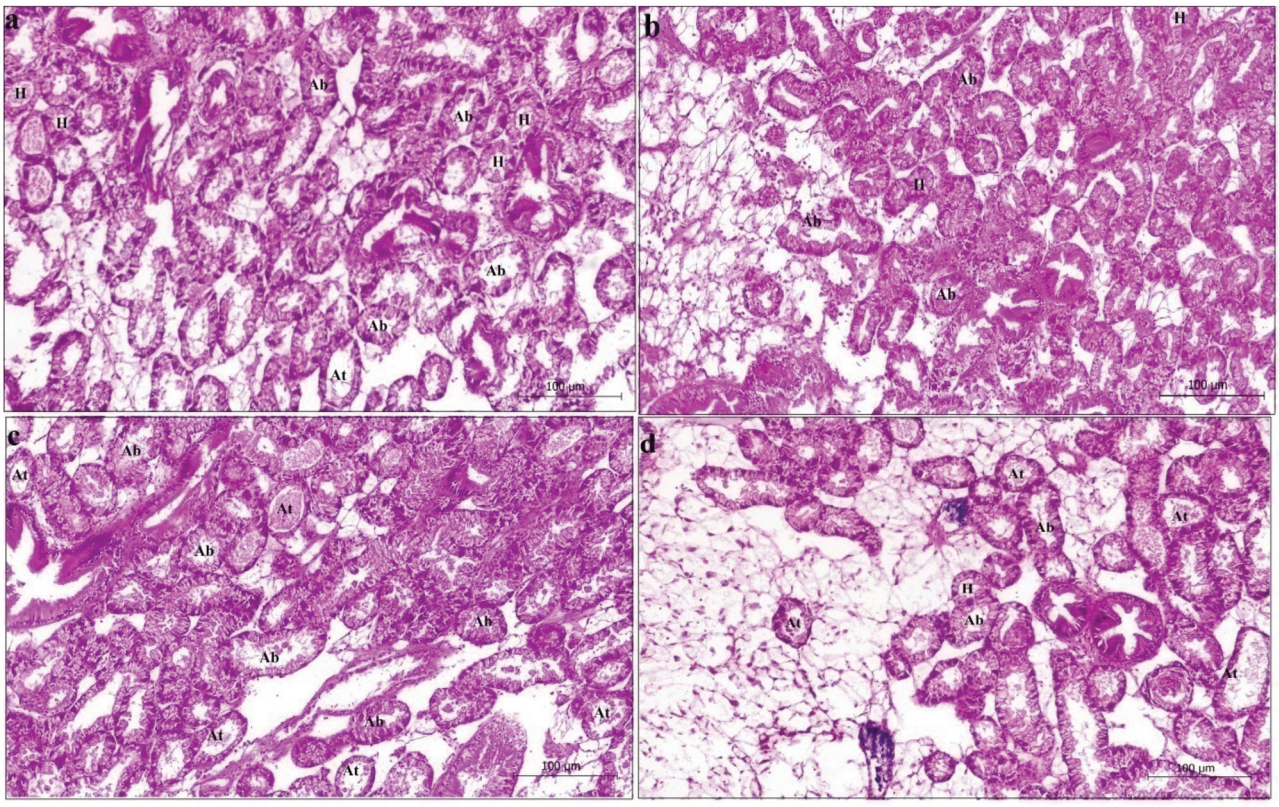


Figure 2. Micrographs of the digestive tubules of *M. galloprovincialis* from different concentrations of γ-$Al_2O_3$ NPs exposures for 96 h, stained with H&E. (a) Control (0 mg/L), (b) 5 mg/L, (c) 20 mg/L, (d) 40 mg/L. (H: holding phase, Ab: absorbing phase, At: athropic phase).

different from the control group (Figure 2(b–d)). The proportion of digestive tubules in athropic phase increased significantly in 40 mg/L γ-$Al_2O_3$ NPs. Digestive gland sections of NPs exposure groups observed that several histopathological changes; hyperplasia, hypertrophy, tubule regression, lipofuscin aggregation, haemocyte infiltration, and necrosis.

The inflammatory response in the digestive gland characterized by focal haemocytic infiltration (in 20 mg/L γ-$Al_2O_3$ NPs Figure 3). In exposed to 20, 40 mg/L γ-$Al_2O_3$ NPs mussels digestive gland showed increases of lipofuscin aggregation.

## Morphological phases of digestive gland tubules

Mussels digestive gland tubules showed that three different types (Type 1: holding phase, Type 2: Absorbing phase, and Type 3: Athropic phase) (Figure 4). The proportion of tubule types of control and other exposure groups showed in Figure 4. In the control group mussels, showed more than 90% of tubules in the holding and absorbing phase (88 ± 4% and 7 ± 3% respectively). In other exposure groups (5, 20 and 40 mg/L γ-$Al_2O_3$ NPs), the proportion of athropic phase tubules increasing remarkably (18 ± 2%, 34 ± 4% and 54 ± 4% respectively). The increasing of the athropic phase tubules counts negatively affects some vital activities (digestion, immune defense, metabolic and homeostatic regulation) of the mussels.

## Histopathological condition indices

Six histopathological alterations in the digestive gland and five histopathological alterations were identified in the gill

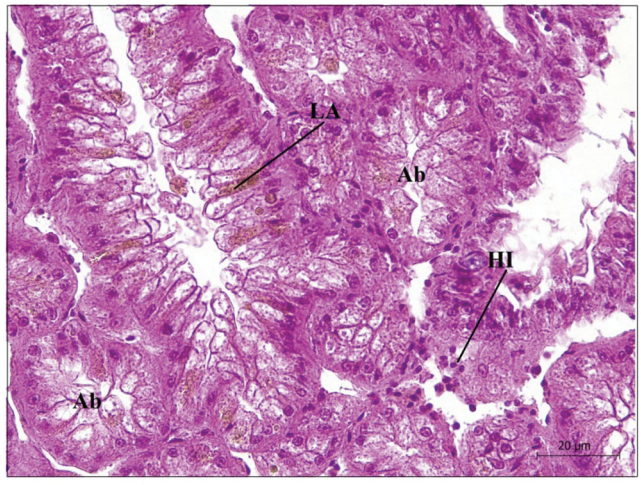


Figure 3. Micrographs of the digestive tubules of *M. galloprovincialis* from 20 mg/L γ-$Al_2O_3$ NPs exposures for 96 h, stained with H&E. (Ab: absorbing phase, LA: lipofuscin Aggregation, HI: haemocyte infiltration).

considered for the weighed indices approach (Table 1). The results of histopathological condition indices ($I_h$) in digestive gland and gill exposed under different concentrations of γ-$Al_2O_3$ NPs (0, 5, 20 and 40 mg/L) for 96 h illustrated in Figure 5. Control group (0 mg/L γ-$Al_2O_3$ NPs) mussels showed low alteration $I_h$ in the digestive gland and gill. In these tissues; comparing to control, exposure to γ-$Al_2O_3$ NPs different concentrations leads to an increase of $I_h$ ($p < 0.05$). Especially increasing of the $I_h$ significantly different from control and other exposures groups ($p < 0.05$) in the digestive glands of 40 mg/L γ-$Al_2O_3$ NPs group.

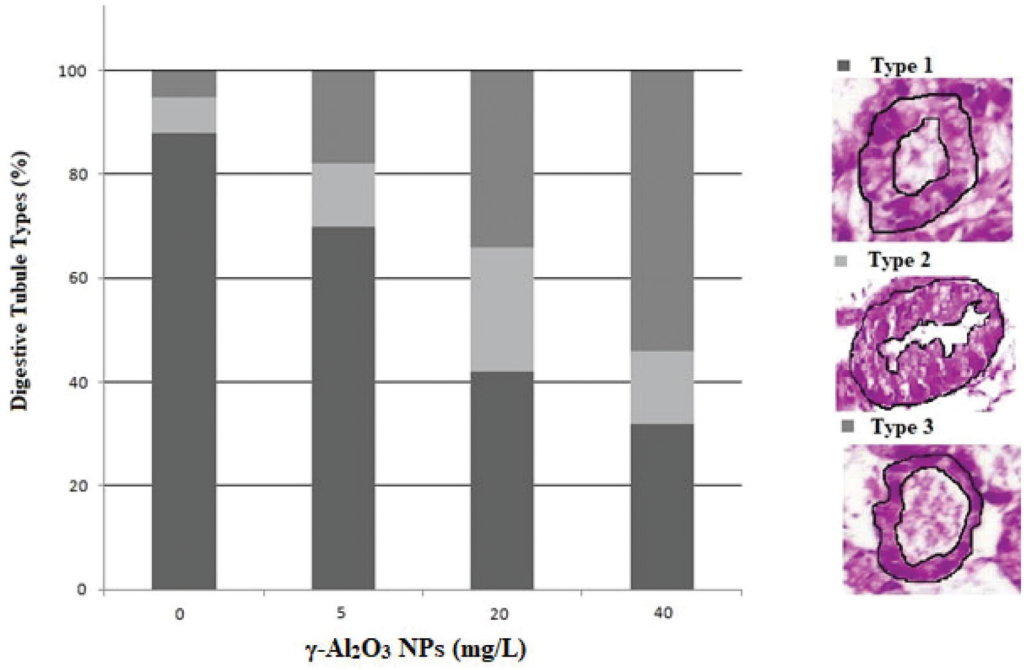


Figure 4. Digestive tubule types (%) from *M. galloprovincialis* exposed under different concentrations of γ-$Al_2O_3$ NPs (0, 5, 20 and 40 mg/L) for 96 h. Type 1: holding phase, type 2: absorbing phase, type 3: atrophic phase.

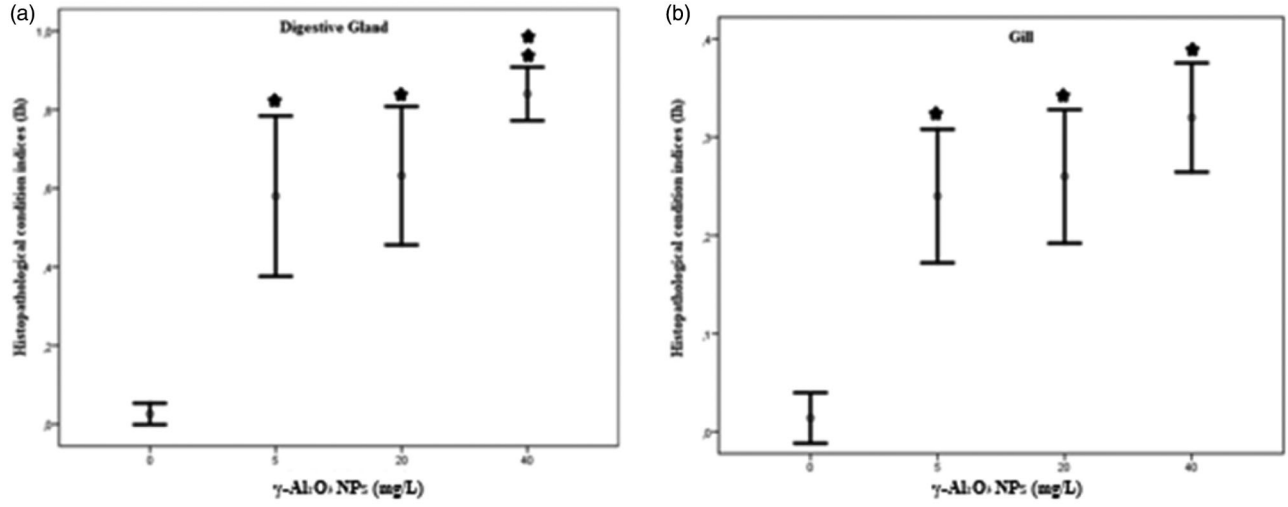


Figure 5. Histopathological condition indices ($I_h$) from *M. galloprovincialis* exposed under different concentrations of γ-$Al_2O_3$ NPs (0, 5, 20 and 40 mg/L) for 96 h. (a) Digestive gland, (b) gill. Results are mean ± standard deviation. Significant differences between exposure groups were represented by *, $p \leq 0.05$.

## Antioxidant responses

### Gills

The measured SOD activities in the gill tissues of the control group not exposed to any dose of γ-$Al_2O_3$ NPs ranged between 14.8 and 38.8 $Umg^{-1}$. It was found that the SOD activity of mussels exposed to different concentrations of γ-$Al_2O_3$ NPs increased however, this increase in SOD activity compared to control organisms was not statistically significant ($F = 0.8$, df = 3, $p > 0.05$). CAT activity increased depending on the dose compared to the control group. No significant differences in terms of CAT activity were observed among all the tested conditions ($F = 1.3$, df = 3, $p > 0.05$). A single homogeneous subset was calculated by Tukey's test with the 3.0 harmonic mean sample size. The activity of GPx was higher in organisms exposed to γ-$Al_2O_3$ NPs compared with organisms control group (0 mg/L) mussels. However, these differences and the differences observed between dose groups were not statistically significant ($F = 2.7$, df = 3, $p > 0.05$) but also the difference between the 40 mg/L concentration and the control group is statistically significant. It was observed that only dose groups formed a different subset from the control group (Figure 6(a–c)). When the correlations between antioxidant enzyme activities measured in gill tissue were examined, it was determined that the correlation between SOD and CAT enzymes in individuals exposed to 5 mg/L doses of γ-$Al_2O_3$ NPs and the correlation between CAT and GPx enzymes in individuals exposed to a dose of 40 mg/L were found to be significant (Table 2).

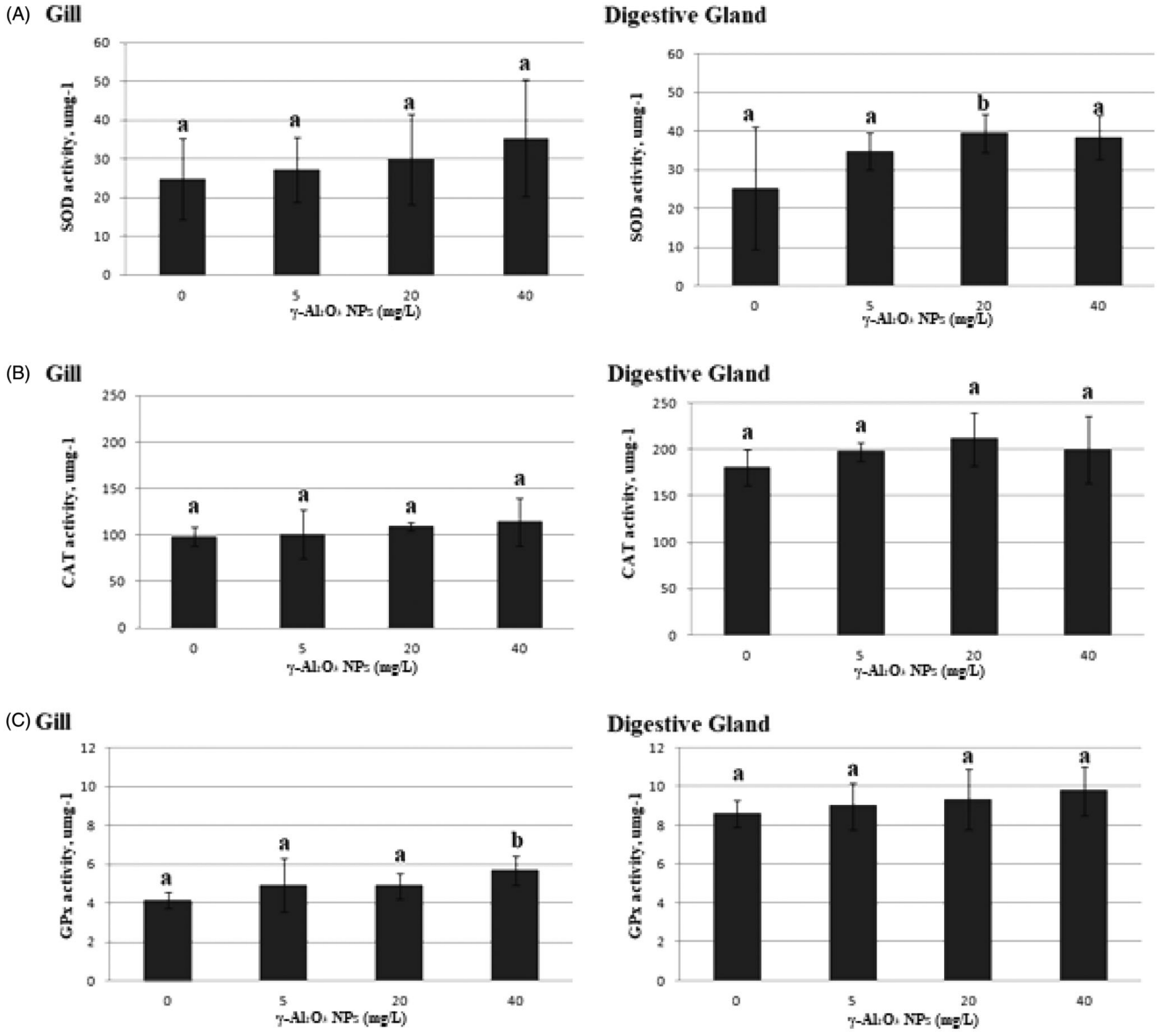


Figure 6. (a) SOD, (b) CAT and (c) GPx activities in *M. galloprovincialis* exposed to different concentrations of γ-$Al_2O_3$ NPs (0, 5, 20 and 40 mg/L). Values are presented as mean and standard deviation. Significant differences ($p \leq 0.05$) among concentrations are represented with different letters.

### *Digestive glands*

The digestive gland SOD activities of the samples determined as the control group and not dosed vary between 5.3 and 40.2 $Umg^{-1}$. SOD activity also increased with increasing dose administration and the highest value was recorded at 20 mg/L dose administration as 47.4 $Umg^{-1}$. This increase in enzyme activity is statistically significant compared to the control group. The level of SOD activity affected by a dose increase is at the limit of statistical significance ($F = 3.1$, df = 3, $p \leq 0.05$). Similar to the SOD activity, the measured CAT activity values increased depending on the dose, and the highest value was recorded as 244.3 especially at the dose of 20 mg/L. The difference between groups in terms of CAT activity is not significant ($F = 0.9$, df = 3, $p > 0.05$). The activity of GPx was higher in organisms exposed to increased doses of γ-$Al_2O_3$ NPs compared with organisms under control conditions, with no significant differences among contaminated organisms ($F = 1.0$, df = 3, $p > 0.05$) (Figure 6(a–c)). The correlations between antioxidant enzyme activities measured in digestive gland tissue showed that no correlation between antioxidant enzyme activities exposed to different doses of γ-$Al_2O_3$ NPs was found to be significant (Table 2).

The discriminant analysis included dose concentration as a grouping (independent) variable plus the histological indices for each antioxidant enzyme capacity. As a result of these analyzes, when the histological index values and antioxidant enzyme levels are evaluated together according to the dose concentration exposed, it is possible to say that the exposed groups, especially in the digestive gland, are separated from the control group. This distinction was not so clear for gill tissue (Figure 7).

## Discussion

The present study estimated the potentially toxic effects (histopathological and antioxidant responses) of γ-$Al_2O_3$ NPs on the gill and digestive gland of the Mediterranean mussel

for the first time. Similar to our study toxic effects of $Al_2O_3$ NPs were evaluated in the different tissues and organisms (Chen *et al*. 2008 Cheng *et al*. 2004, Lin *et al*. 2008, Stanley *et al*. 2010, Sadiq *et al*. 2011, Burklew *et al*. 2012, Ateş *et al*. 2013, Pakrashi *et al*. 2013, Benavides *et al*. 2016, Rajiv *et al*. 2016, Murali *et al*. 2017, Vidya *et al*. 2017, 2018, Canli *et al*. 2018, Abdel-Khalek *et al*. 2020, Nogueira *et al*. 2020). The general belief about nanoparticles is that as the size of the particle gets smaller, the toxicity increases, but the relationship between the intrinsic properties of the particles and their toxicity is more complex than surface chemistry. Although various factors affecting the behaviour of NPs in water, physico-chemical properties seem to be the most important factor in determining the fate and ecotoxicity of NPs, especially in the aquatic environment (Martins Gomes 2012).

Table 2. Pearson correlation coefficients between the specific activity of antioxidant defence enzymes (SOD, CAT, GSH-Px) in the gills and digestive glands of the exposure group mussels.

| | $\gamma$-$Al_2O_3$ NPs Concentrations (mg/L) | | SOD | CAT | GPx |
|---|---|---|---|---|---|
| Gill | Control | SOD | | −0.07 | −0.01 |
| | | CAT | −0.07 | | −0.66 |
| | | GPx | −0.01 | −0.66 | |
| | 5 | SOD | | 0.93* | 0.07 |
| | | CAT | 0.93* | | 0.08 |
| | | GPx | 0.07 | 0.08 | |
| | 20 | SOD | | −0.54 | 0.08 |
| | | CAT | −0.54 | | 0.6 |
| | | GPx | 0.08 | 0.6 | |
| | 40 | SOD | | 0.22 | 0.24 |
| | | CAT | 0.22 | | 0.9* |
| | | GPx | 0.24 | 0.9* | |
| Digestive Gland | Control | SOD | | 0.17 | 0.78 |
| | | CAT | 0.17 | | 0.06 |
| | | GPx | 0.78 | 0.06 | |
| | 5 | SOD | | 0.02 | −0.03 |
| | | CAT | 0.02 | | 0.66 |
| | | GPx | −0.03 | 0.66 | |
| | 20 | SOD | | −0.45 | −0.65 |
| | | CAT | −0.45 | | 0.12 |
| | | GPx | −0.65 | 0.12 | |
| | 40 | SOD | | 0.04 | 0.19 |
| | | CAT | 0.04 | | −0.62 |
| | | GPx | 0.19 | −0.62 | |

*Correlation is significant at the 0.01 level (2-tailed).

Histopathological findings are important to the assessment of the health status of the organisms. After 96 h of exposure, $\gamma$-$Al_2O_3$ NPs-exposed mussel showed significant histopathological alterations in the gill and digestive gland. Histological alterations in the gill could serve as a model for studying interactions between environment and mussel (Pinto *et al*. 2019). Our results showed an increase in the accumulation of lipofuscin aggregation in the gill of mussels exposed to $\gamma$-$Al_2O_3$ NPs. Previous studies have demonstrated lipofuscin accumulation within pathologically altered lysosomes of epithelial cells of the digestive diverticula following exposure to xenobiotics (Moore 1990). According to different studies, exposure to NPs caused haemocyte infiltration in different tissues (Costa *et al*. 2013, Cuevas *et al*. 2015, Bouallegui *et al*. 2017). In our study, we observed haemocyte infiltrations in the gill and digestive tubule is related to the inflammatory response. Histological alterations in inflated gill a quantitative model is proposed to explain the inflammatory intensity (Bouallegui *et al*. 2017). The present study showed dose-dependent histopathological alterations in gills associated with an inflammatory response. Also, we observed loss of cilia, epithelial deformation, fusion, enlarged central vessels in gill filaments. Such histological alterations may be the result of $\gamma$-$Al_2O_3$ NPs. The digestive gland is the main organ for accumulation, metabolism, immune defense system, homeostasis, and detoxification of xenobiotics in mussel (Bouallegui *et al*. 2017). After 96 h. of exposure, a significant increase in the haemocytic infiltration, lipofuscin aggregation, hyperplasia, hypertrophy and tubular deformations in the digestive gland. Histological changes in the digestive tubule is considered to initial process of necrosis in exposure to different xenobiotics (Rocha *et al*. 2016). Also, we observed focal necrosis in some parts of the digestive gland. Our

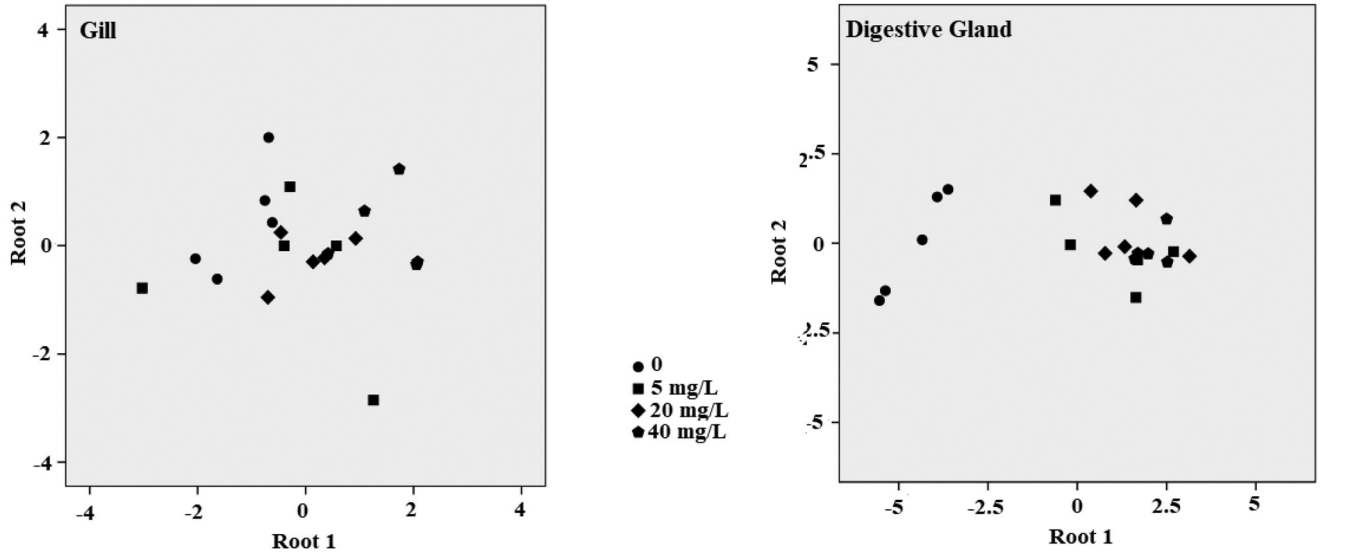


Figure 7. Discriminant analysis scatterplot of individuals exposed to different concentrations of $\gamma$-$Al_2O_3$ NPs. The models include the histopathological indices and the antioxidant responses of the gill and digestive gland tissues for each concentration (for gill twelve variables, Wilk's $\lambda = 0.381$, $p > 0.05$; for digestive gland twelve variables, Wilk's $\lambda = 0.095$, $p \approx 0$).

results agree with previous studies revealed that the toxicological response of exposure of different NPs in mussels (Ruiz *et al*. 2015, Rocha *et al*. 2016, Bouallegui *et al*. 2017). Digestive tubule atrophy appears to occur after the loss of digestive cells leading to a digestive epithelium reduced in heigh (Zorita *et al*. 2006, Rocha *et al*. 2016, Bouallegui *et al*. 2017). In this study, we calculated the significant increase in Type 3 (athropic phase) digestive tubules. The histological alterations and the changes in the frequency of digestive tubule types are defined as important markers of mussel metabolism.

NPs are possible sources of oxidative stress in organisms by being integrated into the cytoplasm and nucleus through their ability to pass through biological membranes (Jeng and Swanson 2006, Shrivastava *et al*. 2014, Canli *et al*. 2019). Due to these potentials, NPs toxicity is being studied intensively on different organisms at both histological, physiological, and molecular levels all around the world. There are also studies on $Al_2O_3$ NPs toxicity which is the subject of this study; however, there are not many studies on different forms of this nanoparticle, which has two forms.

This study aims to observe the histological damage and a measurable physiological response that $\gamma$-$Al_2O_3$ NPs, a nanoparticle with a wide area of use, can cause in the mussel *M. galloprovincialis*, which is commercially important and consumed as food if it is included in the aquatic ecosystem. For this purpose, the first findings of a measurable physiological response and tissue damage were obtained as a result of this study, which was planned at similar concentrations found in nature. However, its higher concentrations can trigger oxidative damage, which can ultimately endanger the survival of these affected organisms. Acute exposure to $\gamma$-$Al_2O_3$ NPs causes a gradual increase in the concentration of this particle in mussel tissues, especially in gill and we observed the highest oxidative damage at the moderate dose in the digestive gland. This may suggest that antioxidant defense mechanisms are active in an attempt to protect cells against the harmful effects of $\gamma$-$Al_2O_3$ NPs. However, the average values of all the measured antioxidant enzyme activities were higher in the digestive glands than in the gills.

Various studies have also revealed an increase in SOD activity as a defense mechanism against increased oxidative stress (Serdar *et al*. 2018, Yildirim *et al*. 2018, Yildirim and Yaman 2019) and also, due to nanoparticles in aquatic organisms (Tremblay *et al*. 2011, Benavides *et al*. 2016, Gornati *et al*. 2016, Sendra *et al*. 2018). Besides, SOD is the first enzyme of the antioxidant chain reaction, and with this property, it can be used as an oxidative stress signal as an indicator of environmental pollution. The results showed an increase in SOD enzymatic activity in the gill of mussels exposed to different $\gamma$-$Al_2O_3$ NPs concentrations, while in the digestive gland tissues again tended to increase until moderate dose exposure, slightly decreased at the highest dose, however, as a result of this decrease, the activity was also higher than in the control group. The increase in SOD activity seen in tissues may be due to the synthesis of new enzymes or increased levels of pre-existing enzymes at lower concentrations. It can be assumed that the downward trend observed in SOD is the result of the depletion of detoxification mechanisms (Jacobson and Reimschuessel 1998).

CAT and GPx are two enzymes, both of which eliminate hydrogen peroxide, although they are located in different places in the cell. The activities specific to CAT and GPx were higher at each dose concentration than control and higher in the digestive gland than in the gills. The results may show that mussels are struggling with oxidative stress caused by $\gamma$-$Al_2O_3$ NPs exposed to different doses. A possible explanation for the reduction of the CAT enzyme in the digestive gland of mussels exposed to the highest dose concentration may be that exposure to pollutants causes increased enzyme activity, but higher levels or sustained exposure may inhibit enzyme activity due to cellular depletion caused by oxidative stress, reducing defense mechanisms (Jacobson and Reimschuessel 1998).

Studies with $Al_2O_3$ NPs are generally planned to expose various living groups in binary mixtures with different metal oxides. Among these studies, some examples that investigated the effects of $Al_2O_3$ NPs and different metal oxide mixtures on the mitotic index and chromosomal aberration changes of the bulb root cells (Ahmed *et al*. 2018), the antioxidant responses it creates in rats (Canli *et al*. 2019) and the effects on the nervous system of Nile tilapia (Canli and Canli 2020). Some studies have evaluated the single and combined effects of $Al_2O_3$ NPs and ZnO metal oxide NPs. The cytotoxic effects of mixtures in various proportions and individual exposures on rat fibroblasts (Köerich *et al*. 2020) and antioxidant responses in *Carassius auratus* (Benavides *et al*. 2016) were determined. There are also studies with some aquatic organisms that are only exposed to $Al_2O_3$ NPs. For example, its effects on embryonic development and mortality in zebrafish and *Xenopus laevis* larvae which is a model organism (Ismail *et al*. 2019), its intake and toxicity by cladocerans (Pakrashi *et al*. 2013), its toxicity on a crustacean, *Hyalella azteka* (Stanley *et al*. 2010), some microalgae genus (*Chlorella sp.* and *Scenedesmus sp*.) (Sadiq *et al*. 2011), on *Artemia salina* larvae (Ateş *et al*. 2013) and on zebrafish (Bai and Tang 2020) were investigated. The general opinion in studies investigating $Al_2O_3$ NPs toxicity is that this nanoparticle increases the toxicity and in turn, antioxidant responses occur. The results evaluated within the scope of this study also support previous studies. Histopathological and enzyme activity findings, which are evaluated as toxicological markers as a result of $Al_2O_3$ NPs exposure, are that $Al_2O_3$ NPs also creates tissue damage and physiological response in mussels. Toxicity was also evaluated in different NPs exposure studies in mussels. The histological and enzymatical effects of $TiO_2$ NPs on *M. provincialis* tissues (Gornati *et al*. 2016), the effects of polystrene NPs on gene expression level at mussel embryos (Balbi *et al*. 2017), cytotoxic effects of $CeO_2$ NPs on mussel haemocytes and Ag NPs on *M. provincialis* tissues (Bouallegui *et al*. 2018) has been demonstrated and negative effects of different nanoparticles on both physiological, histological and developmental characteristics of the target species were observed.

## Conclusion

The present study revealed that histopathological changes and antioxidant responses in *M. galloprovincialis*, which is fed

as filter-feeding and has a widespread, under the condition that γ-$Al_2O_3$ NPs, which is a widely used area, enters the aquatic ecosystem. The study results indicated γ-$Al_2O_3$ NPs exposure caused significant histological and physiological alterations in mussels, which are also used as a food source by other living groups. Considering the properties of this nanoparticle in a wide range of industrial areas, we think that its use in the test environment at the rates determined by considering adequate preparation, dosage, maintenance, and characterization will be beneficial in terms of ecotoxicological results.

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## Disclosure statement

No potential conflict of interest was reported by the author(s).

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