Toxicity of silver nanoparticles on the clam Ruditapes decussatus assessed through biomarkers and clearance rate

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Abstract

The current bioassay aims the evaluation of the ecotoxicity of the silver nanoparticles (NPs) (100 and 200 μg l⁻¹) on Ruditapes decussatus after 48 h and 7 days of its exposure. The biochemical analyses performed at gills and digestive glands showed a disturbance of the antioxidant system following the exposure of clams to Ag NPs. The catalase activity was induced and varied significantly depending on the concentration of Ag NPs and the exposure time, as well as the organ analysed. Simultaneously, a decrease in Gluthation S-Transferase activity was observed at all concentrations tested and organs considered. The acetylcholinesterase activity results confirmed that the threshold of Ag NPs able to disrupt the cholinergic system was less than 100 μg l⁻¹. In overall, silver nanoparticles had a significant caused antagonistic effect on clams in both oxidative- and cholinergic states. Furthermore the findings obtained in this study demonstrated that Ruditapes decussatus can employed as a potential bioindicative species in the assessment of the toxicity of such an emerging material as Ag NPs.

1. Introduction

The increased efforts in current and recent research led to the development and the marketing of new nanomaterials [1]. Nanotechnology advances and innovations provide to the scientists and industrialists a promising opportunity to improve the quality and production system of their products. There is a wide range of the use of silver nanoparticles (Ag NPs) in many areas and industries because of their bactericidal effects and they result in aqueous environments from inadequate spills of their manufacturing, shipments and improper handling or their disposal [2–4]. The Ag NPs have unique characteristics that differ from those of stable form of silver and its Ag⁺ ions, and as a result, they show different toxicity. As Ag NPs are designed to be highly reactive, they are expected to be far more toxic than Ag⁺ ions, in addition these nanoparticles have high ability to penetrate the cell membrane [5]. According to [6], it is hypothesized that once inside the cell, Ag NPs binds to ligands containing sulphide, thus allowing the denaturation of the proteins, which would cause cell death. This could explain several harmful effects of this types of nanoparticles on human health, such as inflammation and cancer risks [7, 8]. Humans may be exposed to Ag NPs through the consumption of sea food such as shrimps or mollusks grown in the AgNPs polluted waters, however little is known and proven about the effects of silver nanoparticles on these aquatic organisms [2, 9].

Various compartments of the aquatic ecosystem can be affected by the toxicity of these nanomaterials, especially if they are quickly adsorbed on the substrate [10, 11]. To assess the short-term ecotoxic impact of Ag NPs, we considered three criteria: (1) results should be derived from quick experiments involving taxa with a
significant position in the food web in order to provide early warning signals of such emerging contaminant’s ecological effects; (2) the organisms tested should be of benthic lifestyle so the impact of pollution could be clearly demonstrated; and (3) the organism used as model species in the study must have a wide geographical distribution in order to enable the generalization of the obtained findings.

The above listed criteria could be meet among the aquatic taxa by *Ruditapes decussatus*, which is endemic from the northeastern coasts of the Atlantic Ocean, Northern Europe to Africa, including the North Channel and Sea (https://fr.wikipedia.org/wiki/Palourde_commune). It is also found along the Mediterranean coast, including Tunisia, the Red Sea, the Adriatic Sea, Tyrrenhenian Sea and the Aegean Sea (https://fr.wikipedia.org/wiki/Palourde_commune). The common clam lives, along the coast, depressed on 15 centimeters in the substrate of the infra-littoral zone, on the eutras, the estuaries, etc. They can be found at depths ranging from 1 to 3 m. These characteristics made the *Ruditapes decussatus* suitable to be used as a model species to assess the quality of aquatic ecosystems, in biomonitoring programs, as well as in the assessment of the impact of numerous classic pollutants such as metals or Polycyclic Aromatic Hydrocarbons in laboratory conditions [10, 11]. In the same context, these organisms could be used to assess the toxicity of Ag NP.

The current bioassay was designed to investigate the response of the clam *Ruditapes decussatus* to the Ag NPs exposure. Our findings would be useful to enhance the global knowledge and understanding of the impact of Ag NPs on aquatic ecosystems. Herein, we aimed to address the following questions:

1. Are Ag NPs harmful to the common clam *R. decussatus*?
2. If so, how does *R. decussatus* respond at biomarker level and clearance rate?
3. What are the thresholds of Ag NPs with discernible effects, and which organs are they targeting?

## 2. Material and methods

### 2.1. Structural and optical characterizations

The silver nanoparticles used in this study had a purity of 99.99% (*Aldrich Prod. No. 730793 Sigma-Aldrich, St. Louis, Mo, USA*). To characterize the crystallinity and the morphology of silver nanoparticles (AgNPs), XRD, TEM and SEM measurements were performed. The microstructure of Ag nanoparticles was examined by x-ray diffraction patterns (XRD) (STOE-STADI P) (an INEL diffractometer using a copper Kα radiation (λ = 1.5406 Å). To go insight the shape NPs, transmission electron microscopy (TEM) and SEM microscopy were carried out using JEOL 2011 instrument working at 100 kV. To study the stability and the size distribution of the sample, dynamic light scattering (DLS) and zeta potential technique were used. The measurement of particle size in distilled water and in seawater (0.1 mg ml\(^{-1}\)) using Dynamic light scattering (DLS) were carried out by a Zetasizer Ultra (Malvern Panalytical Ltd, Malvern, UK) equipped with a He-Ne laser at a wavelength of 632.8 nm and a maximum power of 10 mW. Finally, TEM and SEM images were taken to prove the stability of AgNPs in buffer solution.

### 2.2. The clam collection

On May 23, 2018, clams (*Ruditapes decussatus*) of similar size (shell lengths 3.5 ± 0.4 cm, [12]) were collected at Menzel Jemil from the Bizerte lagoon (37°13’11.14”, 9°56’6.80”). This biotope is located in Northern Tunisia and is connected with the Mediterranean Sea via an artificial canal and Ichkeul Lake via ‘Oued Tinja’. The samples collected are transferred to the laboratory and placed in two-liter glass bottles known as ‘microcosms’ for a week of acclimatization. The water at microcosms was changed every couple of days, they were also ventilated with air pumps and monitored in order to ensure the clam’s survival.

### 2.3. Treatments and laboratory conditions

After the acclimatization phase, the samples were treated with Ag NPs. The experiment was designed in three replicates for each treatment, with 5 individuals in each replicate. During this bioassay, three Ag NPs treatments were considered (C1 = 100 \(\mu\)g l\(^{-1}\) and C2 = 200 \(\mu\)g l\(^{-1}\)) along with an untreated control. In order to investigate the effect of exposure length, samples were exposed to AgNPs for 2 and 7 days in order to investigate the effect of the longevity of exposure. The study was carried out under controlled conditions with a temperature of 18 °C and a photoperiod of 12 h/12 h, light/darkness cycle.

### 2.4. Biochemical analyses

At the end of the experiment, the clams were opened by incision of their adductive muscles with a scalpel. The digestive gland and gills were then separated and stored on ice at 4 °C to prevent protein denaturation. The total protein content was extracted in TBS buffer (Tris 50 mM, NaCl 150 mM) (pH 7.4). The digestive glands and gills...
were ground in Ultra Turrax (IKA) than the samples were centrifuged at 9000g for 30 min at 4 °C. The supernatant was stored at −80 °C until further biochemical analysis was performed.

The protein concentration was determined spectrophotometrically at 595 nm using Bradford’s colorimetric method (1976) following the reaction between the proteins and coomassie blue. The catalase (CAT) activity was determined according to Aebi’s protocol [13], which is based on a spectrophotometric method, by measuring the optical density at 240 nm for 3 min in relation with the degradation of hydrogen peroxide (H₂O₂). The specific activity of CAT is given in μ mol/min/mg proteins.

The Glutathione S-Transferase (GST) activity was measured as described Habig et al [14]. According to these authors, the cytosolic extract reacts in a phosphate medium with 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate in the presence of a cofactor, the Glutathione (GSH). The reaction between these two products results in the formation of the complex (CDNB-GSH), which absorbs at wavelength of 340 nm. The GST specific activity is given in n mol/min/mg of protein.

In the assessment of the acetylcholinesterase (AChE) activity the protocol proposed by Ellman et al [15] was adopted. This method is based on the hydrolysis of acetylthiocholine by acetylcholinesterase. The thiocohene released reacts with 5−S′-Dithio-bis (2-Nitrobenzoate) to form 5-Thio-2-NitroBenzoate (TNB), which absorbs at wavelength of 412 nm. The specific activity of AChE is given in μ mol/min/mg proteins.

2.5. Clearance rate determination
The clearance rate was measured based on the disappearance of the Neutral Red dye introduced into the water column in presence of the clams [16]. After being exposed to different Ag NPs concentrations (100 and 200 μg l⁻¹), three bivalves from each replicate were placed into 200 ml beakers (one bivalve/beaker) containing 100 ml of a Neutral Red solution (1 mg ml⁻¹) sheltered from light. Then, the purity measurement were carried out in intervals of 30, 60, 90, and 120 min. After each time slot, a water sample of 10 ml was taken from each beaker and undergone an acid attack (HCl 37%). The remaining Neutral Red concentration were determined by measuring it at the optical density of 530 nm. The clearance rate is expressed in mg/animal/h.

2.6. Statistical analysis
The homogeneity and normality (Gaussian distribution) of all data series obtained were tested by using Bartlett and Kolmogorov-Smirnov tests, respectively, in Statistica v.8.0 software. The one-factor ANalysis Of VAriances (1-ANOVA) has been employed to highlight a significant global difference between treatments. Multiple comparisons were performed thereafter to compare pairs using the Tukey’s HSD (honestly significant difference) post-hoc test. A p-value less than 0.05 is considered significant.

3. Results

3.1. Silver nanoparticles morphology
As can be seen in figure 1, AgNPs exhibited spherical shape with an average size of 20 nm (figure 1(a)). This observation was further confirmed by SEM image (figure 1(b)). On the other hand, AgNPs displayed good distribution and no agglomeration can be detected. Figure 1(c) showed the XRD pattern of the sample which indicated the high crystallinity of the obtained nanoparticles; all diffraction peaks of AgNPs are indexed and no impurities can be detected. The obtained results in size distribution from DLS analysis showed coherent data (figure 2). Indeed, the Z-average size was found to be in the range of 20–30 nm. Indeed, this is an expected result, because TEM measured the diameter of pure AgNPs while DLS technique measured the hydrodynamic diameter of particles (AgNPs + surfactant or stabilizer). In our case AgNPs contains sodium citrate as stabilizer at its surface which increase the real size of AgNPs observed by TEM microscopy. On the other hand, the zeta potential exhibited negative peak which generated high repulsion between silver nanoparticles and also between AgNPs and hard anions (such as Cl⁻) and therefore increased the stability of the particles long time in water or saline solutions. This observation was confirmed after incubation of AgNPs in buffer solution. As shown in figure 3, the size, and the shape of AgNPs not affected after incubation in buffer solution; no agglomeration can be observed, which prove the high stability of the utilized AgNPs even in buffer solution.

3.2. Biochemical biomarkers
3.2.1. Catalase activity
Ag NPs caused a significant increase in CAT activity in gills after 48 h and 7 days of clam exposure compared to controls (Tukey’s HSD test: \( p < 0.001 \)) at both concentrations tested (figure 4). It should be noted that induction of catalase increased with time and concentration. No significant change in the CAT activity was
observed after introducing nanoparticles ($p$-value = 0.615) for 48 h, at digestive glands. On the contrary, a period of 7 days resulted sufficient to reach the significance threshold after the exposure to C1 and C2 ($p < 0.001$).
3.2.2. Glutathion S-Transferase activity

The resulting GST changes as a response of *R. decussatus* to Ag NPs are given in figure 5. In gills, GST activity showed significant decrease only when clams were exposed to C2 for 48 h. However, no significant fluctuation of GST activity was observed, even at the highest Ag NPs concentrations when tested after 7 days of exposure (1-ANOVA: *p*-value = 0.149). In the digestive glands, however, there was observed a significant decrease of GST activity compared to control, following the exposure of clams to C1 and C2 for 48 h and 7 days (*p* < 0.05). While, after 7 days of exposure, it was not observed a significant difference from controls (1-ANOVA: *p*-value = 0.302).

3.2.3. Acetylcholinesterase activity

The AChE activity in the gills did not showed significant differences in comparison to controls, at both time slots fixed, 48 h (1-ANOVA: *p*-value = 0.583) and 7 days (1-ANOVA: *p*-value = 0.247) after the Ag NPs exposure (figure 6). However, a different trend was observed in the digestive gland, where the presence of Ag NPs resulted in a discernible increase of AChE activity for C1 and C2, 2 days after exposure (*p* < 0.05). There was no
difference in the AChE activity measured a week after the Ag NPs exposure at both concentrations, where its values were statistically similar to the control (figure 6).

3.3. Clearance rate

The clearance rate was evaluated at different intervals of time, after 30, 60 and 90 min. It was observed that the clearance rate increased with the increasement of the Ag NPs concentration were measured 30 min after the silver nanoparticles exposure (figure 7).

After 60 min of exposure, a decrease in the clearance rate was observed in both concentrations tested, in addition a significant variation of it was observed in the C2 when compared to the control. The results obtained after 90 min of exposure showed that the clearance rate decreased significantly by 70% in the C1 and 40% in the C2 when compared to controls.

4. Discussion

4.1. The potential of biochemical markers as AgNPs toxicity indicator

Humans may be exposed to a wide range of contaminants absorbed from aquatic organisms consumed as seafood. Emerging contaminants, particularly nanomaterials, are of high concern since their toxicity is not fully studied and documented. Herein, the bioaccumulation of NPs of silver resulted in an excess production of Reactive Oxygen Species (ROS) which may affect in the long term the viability of organisms [17]. ROS are oxygen derivatives with a very short life span due to their high reactivity [18]. Such toxic effect of Ag NPs has been highlighted by a modulation of the antioxidant defense. When the ROS amount in the body increases, the organism responds by a multitude of non-enzymatic substances (GSH, vitamins (A, C and E), ubiquinone, carotenoids, flavonoids, uric acid etc) which help in neutralization and the reduction of ROS levels. Once the capacity of these trappers is exceed, antioxidant enzymes are mobilized. The results obtained demonstrated that Ag NPs have an inductor effect on the CAT activity in dose- and time-depending manner (figure 4). Indeed, such a response evolved in a linear way throughout the experiment. Similar effects have been reported for the...
freshwater snails *Lymnaea luteola* and *Biomphalaria alexandrina* exposed to ZnO NPs by [19] and [20], respectively, for the freshwater bivalve *Corbicula fluminea* exposed to Ag NPs by [21], all reporting an increase of CAT activity under stress.

The comparison of the induction thresholds showed that they depend on the organ in question. Evidently, in both AgNPs concentrations tested there was not observed an induction of CAT activity in digestive glands, after two days of exposure. Previously, it was reported that the metallic ions interact with certain enzymatic cofactors inactivating the catalase [22]. In overall, the comparison of the biochemical responses of *R. decussatus* to Ag NPs, revealed that the CAT activity was at higher levels at digestive glands, but the time of its response was shorter at gills.
Regarding the GST levels changes in response of the silver nanoparticles treatment, after two days of exposure, a significant decrease of GST was observed in both analyzed organs, gills and digestive glands (figure 5). The GST is a phase II biotransformation enzyme, that detoxifies the cell by conjugating a thiol group to metabolites or pollutants reducing their liposoluble nature and thus facilitating their removal by excretion or degradation [23]. GST induction indicates the presence of relatively high oxidizing stress, whereas its reduction in relation to the control treatment suggests an alteration in the body’s physiological state [24, 25]. The reduced glutathione appears to have a protective role in cells against Ag NPs. The previously published data on GST responses to NPs were different from our findings. Buffet et al [26, 27] reported an increase of GST activity in the burrowing bivalve, generally present in estuaries Scrobiralia plana when it was exposed to ZnO NPs and Ag NPs. The reduction of GTS observed in our study as a response to AgNPs exposure, could be due to increased consumption of free reduced glutathion (GSH), which is used as the substrate of the GST, or due to the closure of shells in clams as a change in their behavior to isolate themselves when exposed to NPs. A different pattern resulted in GST activity after 7 days of exposure at both organs, the digestive gland and gills, where no difference with controls was observed.

There are very few neurotoxicity markers used to detect the neurotoxic effects of xenobiotics, especially in the aquatic models. The most used marker in ecotoxicological studies is the monitoring of AChE activity [28]. The present study revealed no significant difference in AChE activity in gills in silver nanoparticles treatments when compared to the control (figure 6), the same result was observed also in the AChE activity in digestive glands after 2 two days of AgNPs exposure. A similar finding was observed by [26, 27] in Scrobirala plana after being exposed to copper, zinc and silver nano-oxides. [28], on the other hand showed a stimulation of AChE activity, after the exposure of this species to 100 mgL$^{-1}$ of gold nanoparticles, suggesting that such response might be related to the development of a compensatory response of these bivalves that acquired the ability to produce new AChE isoforms in response to stress [29]. Similarly, a significant induction of the AChE activity was observed in our study at the digestive glands after 48 h of Ag NPs exposure at both concentrations applied, C1 and C2 (figure 6). The increased of AChE activity observed in our study could be explained by an overexpression of genes encoding this enzyme and by the decrease of the expression of genes encoding for the vesicular carrier of acetylcholine, in order to reduce neuronal hyperexcitation as proposed by [30] and [31]. In addition, the results obtained for AChE activity could be also explained by the role this enzyme plays not only in nervous transmission but also in the apoptosis mechanisms against xenobiotics such as Ag NPs in our case [32, 33]. The cholinergic system activation causes an inhibition of cell proliferation, inducing apoptosis [32, 34, 35].

4.2. Clearance rate alterations and AgNPs toxicity

The present study showed that Ag NPs significantly affected the clearance rate of the clam, R. decussatus (figure 7). The clearance rate parameter, increased at the beginning of the Ag NPs exposure (30 and 60 min) and then decreased with the increasement of the exposure time (90 and 100 min), when compared to controls. The exposure to the NPs serves as a stressor in clams, which tends to increase the clearance rate as a defense mechanism against this harmful agent. However, this protective mechanism cannot be efficient for long periods, of time, therefore after 90 min of exposure, the clams end up reducing the filtration rate. Our results are in line with those reported by [36], who found that contamination of the blue mussel, Mytilus edulis to CuO NPs resulted in bioaccumulation of these nanoparticles in a concentration-dependent manner, but also in a significant decrease in the clearance rate.

The balance between the activity of the mitochondrial Electron Transport System and the energy reserves has also been reported to be relevant and can explain the changes in filtration capacity [37, 38]. In fact, as stress intensity increases, the activity of Electron Transport System decreases, slowing the metabolism and clearance rate, as well as the mobilization of stored energy, glycogen [38, 39].

5. Conclusions

Bivalves are still the mostly commonly used bioindicators in ecotoxicology due to their diversity and omnipresence in coastal ecosystems, as well as their position in the aquatic food web [40]. That is the reason clams R. decussatus, were chosen in the current study to assess the toxicity of the silver nanoparticles as emerging contaminants.

The Ag NPs affected significantly the biochemical responses of clams, resulting in changes in the CAT, GST and AChE enzyme activities. The activity of CAT was silver nanoparticles time and concentration dependent, it showed the lowest activity at the beginning of the clam exposure, while its highest level was recorded when the highest concentration of Ag NPs was applied, after 7 days of exposure. The GST activity decreased as the result of AgNPs exposure suggesting its protective role against toxicity inside cells. The AChE showed particularly high sensitivity after two days of exposure at 200 μgL$^{-1}$ of concentration. Our findings suggests that the contaminants
affect clams in a differently than other aquatic species for which a classic decrease in AChE activity was observed following their exposure to classic or emerging pollutants such as NPs. The activation of the cholinergic system was only observed at digestive glands and is most likely related to the tissue homeostasis deregulation. The employ of genomic and proteomics approaches could be essential to further investigate the differences observed between organs and the mechanism of cholinergic activation only at digestive glands. It would be also interesting the evaluation of additional parameters, such as temperature and pH, that might influence the effect of Ag NPs in seawater.

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Data availability statement

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

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