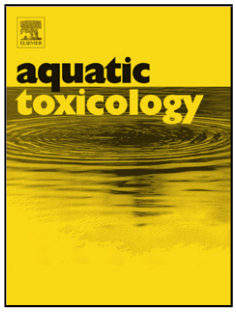

Title: Role of endocytotic uptake routes in impacting the ROS-related toxicity of silver nanoparticles to *Mytilus galloprovincialis*: A redox proteomic investigation

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Role of endocytotic uptake routes in impacting the ROS-related toxicity of silver nanoparticles to *Mytilus galloprovincialis*: A redox proteomic investigation

Short title: Impact of Uptake Routes to Mussels' Toxicity by Silver Nanoparticles

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Highlights:

- Oxidative stress has been described as a major mechanism of nanoparticle toxicity.

- AgNPs were able to generate protein thiol oxidation and/or carbonyl formation.
- ROS toxicity caused by AgNP seemed to be linked to tissue-specific function requirements.
- Endocytotic uptake routes were found to be strongly underlying nanotoxicity.
- Redox-sensitive proteins were revealed as an additional onset of NP-caused oxidative stress.

Abstract

Oxidative stress is often implicated in nanoparticle toxicity. Several studies have highlighted the role of internalization routes in determining nanotoxicity. Here, we investigate how two endocytotic mechanisms (clathrin- and caveolae-mediated) impact on redox balance in gill and digestive gland of the mussel, *Mytilus galloprovincialis.* Animals were exposed (for 3, 6 and 12 h) to two sizes of silver nanoparticles (AgNP: < 50 nm and < 100 nm) prior to and after blockade of two endocytic pathways (amantadine blocks clathrin-mediated endocytosis while nystatin blocks caveolae-mediated endocytosis). Redox-proteomic tools were used to determine effects. Our results demonstrate the ability of both sizes of AgNP (<50 and < 100 nm) to cause protein thiol oxidation and/or protein carbonylation. However, blockade of endocytotic routes mitigated AgNP toxicity. Differential ROS-related toxicity of AgNP to mussel tissues seemed to be linked to tissue-specific mode of action requirements. Cell uptake mechanism strongly influences toxicity of AgNPs in this filter-feeder.

Keywords: oxidative stress; carbonyls; thiols; AgNPs; endocytosis; mussel

1. Introduction:

Oxidative stress-related effects on cell structure, protein alteration/misfolding, cell membrane and DNA damage have been identified as major aspects of nanoparticle (NP) toxicity in bivalves (Katsumiti et al, 2015; Rocha et al, 2016). Many studies proposed as a paradigm that the most adverse NP-mediated effects are due to excessive production of reactive oxygen species (ROS) resulting in oxidative stress. Using a proteomic analysis, it has been shown that silver nanoparticle (AgNP) toxicity in the mussel, *Mytilus galloprovincialis*, is oxidative stress-related (Gomes et al., 2013). Other studies reported changes in antioxidant enzyme activities in response to NPs (McDonagh & Sheehan, 2006). Such activities have been shown also to be dependent on the animal's physiological status, reproductive phase and maturity (Guerriero et al, 2004). Other factors affecting NP toxicity include exposure time, chemical composition, final NP concentration and target organ (McDonagh & Sheehan, 2006; Katsumiti et al, 2015). NPs can decrease antioxidant defenses, cause cytoskeletal effects, protein oxidation, mitochondrial disruption or DNA strand breaks (D'Agata et al, 2014; Gornati et al, 2016; Rocha et al, 2016). Canesi et al, (2008, 2010, 2012) reported that exposure to different NP types (carbon black, C60 fullerenes, TiO_2 and SiO_2) induced production of oxyradicals and nitric oxide even at lower (1-100µg/L) concentrations (Barmo et al, 2013). Tedesco et al, (2010) found redox-related proteomic changes in response to gold nanoparticles (AuNPs) in mussel tissues. Other studies have suggested the potential toxicity of NPs to be related to their ability to catalyze Haber-Weiss and Fenton reactions on their surfaces during interactions with biological structures (Canesi et al, 2012; Buffet et al, 2014; Katsumiti et al, 2015). Proteomic tools are a promising way to gain better understanding of NP toxicity mechanisms. Proteins are quantitatively the most important ROS targets in biological systems, and their direct oxidation can cause changes in metabolism and related intracellular signaling (Rainville et al, 2015). Redox-based changes in the proteome include carbonylation of amino acid side-chains (Dalle-Donne et al., 2003). This occurs when side

chains are directly oxidized or react with lipid and sugar oxidation products, forming aldehyde or ketone groups (Sheehan et al, 2010; Valko et al, 2007, 2016). Protein carbonyl levels are sensitive markers of oxidative stress in sentinel organisms. Carbonylation affects cell signal transduction (Dowling & Sheehan, 2006; Sheehan & McDonagh, 2008; Sheehan et al, 2010; Wong et al, 2010; Valko et al, 2007, 2016). Carbonyl groups are especially common in proline, lysine, arginine and threonine residues. Because of the abundance of these residues in proteins, carbonylation is the most quantitatively important redox lesion in biological systems. Sulfur-containing amino acids such as cysteine and methionine can also be directly oxidized into structural variants such as disulfides, sulfoxides or sulfones (Winterbourn, 2008). Their modification can also be studied with redox proteomic tools (Sheehan et al., 2010). The thiol groups of cysteines are particularly sensitive to oxidation and thiols react significantly faster than other amino acid side-chains with ROS thus contributing to antioxidant defense by redox buffering (Sheehan et al, 2010; Tedesco et al, 2010; Bruschi et al, 2011; Rainville et al, 2014; Valko et al, 2007, 2016). Whilst not very abundant in proteins, cysteines are often conserved in evolution because of their functional properties (notably in catalysis and redox signaling). Therefore, their oxidation can impact disproportionally on cell function (Winterbourn, 2008).

Previous studies reported that the toxicity of NPs depends, in part, on physico-chemical attributes of the material such as chemical composition, size, shape, surface charge and final concentration, which also influence NP behavior and fate in the water column (Tedesco &Sheehan, 2010; Rocha et al, 2016 and Canesi & Corsi, 2016). However, relatively few studies have investigated the potential for uptake mechanisms to affect toxicity (Moore, 2006; dos Santos et al, 2011 and Khan et al, 2015).

Uptake by endocytosis has been described as a potential mechanism for NP entry into cells (Moore, 2006). Clathrin-dependent endocytosis, involves assembly of clathrin (a

specific coat protein) to form clathrin-coated pits. This leads to formation of an endo-lysosomal compartment (conventional endocytosis). Alternatively, caveolae-dependent endocytosis occurs *via* cell-surface flask-shaped invaginations that contain caveolin-coated proteins in the plasma membrane (Nichols and Lippincott-Shwartz 2001; Pelkmans and Helenius 2002; Razani and Lisanti 2002; Moore 2006; Puthenveedu et al. 2006; Bareford and Swaan 2007; Khan et al. 2015).

In the present study we assess how cell uptake mechanisms influence NP toxicity. While uptake mechanisms undoubtedly facilitate NP entry into cells (Moore 2006, Ivanov, 2008; dosSantos et al, 2011; Khan et al., 2015), it is unclear whether or not uptake mechanisms influence cellular oxidative balance. We blocked clathrin- and caveolae-mediated endocytosis, respectively, and then assessed the toxicity of AgNPs (< 50 nm and < 100 nm) following exposure for 3, 6 and 12 h.

2. Material and Methods:

2.1. Chemicals and silver nanoparticles AgNP

Protein assay dye reagent concentrate was purchased from Bio-Rad (CA, USA). Unstained protein molecular weight markers for SDS-PAGE were from Thermo Scientific (Rockford, IL, USA). 5-Iodoacetamido-fluorescein (IAF), 5-fluorescein thiosemicarbazide (FTSC) and all other general reagents suitable for electrophoresis were purchased from Sigma–Aldrich Ireland Ltd. (Arklow, Co. Wicklow).

Poly-vinyl-pyrrolidone (PVP)-coated AgNP (< 100 nm; 99.5 % trace metal based) was purchased from Sigma (Steinheim, Germany). PVP-coated AgNP (< 50 nm; 99.1% purity) was produced locally using a modified polyol process (Mezni et al. 2014a,b) by the chemistry department (FSB, Bizerte, Tunisia). AgNP stock solutions were then prepared and suspended in artificial sea water (ASW; salinity = 35%, pH 8.0), prepared as previously

described (Bouallegui et al., 2017b). Briefly, AgNP stock solution was mixed several times by inversion and an aliquot removed as a working solution that was sonicated for 15 min in alternating cycles (2 x 30 s) in an ultrasonic bath (100 W; 40 KHz; VWR, Strasbourg, France). Transmission electron microscopy (TEM) analysis was performed on a TECNAI G20\ instrument (Ultra-Twin, FSB, Bizerte, Tunisia). X-ray diffraction (XRD) analysis was performed with a D8 Advance diffractometer (Bruker, Bizerte, Tunisia). UV-Vis spectrometry was performed in a PG-instruments T60 spectrophotometer (PG-instruments, Leicestershire, UK).

Endocytotic uptake can be selectively blocked with amantadine (clathrin-mediated pathway) and nystatin (caveolin-mediated pathway), respectively (Ivanov, 2008; Khan et al, 2015). A stock solution of amantadine (3 mg/mL; Sigma, Steinheim, Germany) was prepared in ultrapure water. Nystatin (Sigma) stock solution (5 mg/mL; Sigma) was prepared in dimethyl-sulfoxide (DMSO, Sigma); the final concentration of DMSO in all Nystatin exposures was 0.05% (v/v) (Ivanov 2008, Khan et al. 2015).

2.2. Mussels and experimental design

Mussels (*M. galloprovincialis*) of average shell length 75 [± 5] mm were collected from Bizerte Lagoon (Northeast Tunisia), immediately transported to the laboratory and maintained in oxygenated ASW (salinity 35%, pH 8) in static tanks under standard conditions (aeration, photoperiod: 12/12 h; T= 16°C). They were acclimated for 48 h with change of water each 12h prior to exposure. Mussels were separately exposed to 100 µg AgNP/L <50nm and <100nm before and after uptake route inhibition for 3, 6 and 12 h (treatment group: N=10; exposure rate=1 mussel/0.5 L ASW/tank) (Bouallegui et al., 2017b, Katsumiti et al. 2015; Canesi & Corsi 2016). For inhibitor-treated groups, effective concentration ranges used were selected based on a previous study (Khan et al., 2015). Mussels were incubated for 3 h with 100 µM amantadine (Sigma, Steinheim, Germany), then placed in

AgNP exposure solutions (without amantadine) for the required times. For nystatin, mussels were exposed with 50 µM nystatin/ 0.05% (v/v) DMSO for 1 h and then exposed to AgNP (Ivanov 2008; Angel et al. 2013; Khan et al. 2015). Control groups (N=10) of mussels were maintained in oxygenated tanks of only ASW and/or ASW with the inhibitors exactly as above with the AgNP treatments. Exposures to vehicle alone or in the presence of AgNP of differing sizes were conducted to ensure effects were not caused by any carrier modulation of NP behavior or by the carrier itself. All exposures were performed in triplicate.

2.3. Animal dissection and homogenate preparation

Digestive gland and gill tissues were dissected from control and treated groups of mussel and were then homogenized (10mM Tris-HCL, pH 7.2; 0.5 M sucrose; 1 mM EDTA; 1 mM PMSF), centrifuged at 20,000x*g* for 1h and supernatants were stored at -80°C until required.

2.4. Protein quantification

Protein concentration was measured by the method of Bradford (1976). Protein estimation was performed in quadruplicate in a microplate reader at a wavelength of 595 nm using bovine serum albumin as a standard. Protein content for each tissue (digestive gland and gill) was calculated and recorded.

2.5. Labeling protein thiols

Protein thiols were labeled with IAF (Baty et al., 2005) (stock solution 20mM IAF in DMSO) by adding prepared tissue homogenates (40µg protein) to a final concentration of 200 µM (Chaudhuri et al, 2006). Proteins were exposed to IAF for 2 h at 4°C in the dark before precipitation of proteins with a final concentration of 20% (w/v) trichloroacetic acid (TCA) for 5 min at 4° C and centrifuged at 11,000*g* for 3 min at 4°C. Pellets were resuspended in 40 µl of water and 500µl of ice cold acetone (incubated for 2h up to overnight at -20°C) before centrifugation at 11,000*g* for 3 min at 4°C. The pellet was dried for 5-10 min

to ensure there was no acetone present and then resuspended in sample buffer (deionised water; 0.5 M Tris-HCl, pH 6.8; glycerol; 10% (w/v) SDS; 0.5% (w/v) bromophenol blue). (Laemmli, 1970).

2.6. Labeling protein carbonyls

Protein carbonyls were labeled by adding FTSC to tissue homogenates (40µg) to a final concentration of 1 mM (Chaudhuri et al, 2006). Samples were incubated for 2h in the dark at 4°C before precipitation of proteins with a final concentration of 10% (w/v) TCA. Pellets were washed twice with 500 µl of ice cold 1:1 ethanol-ethylacetate. Prior to resuspension and electrophoresis as above, pellets were centrifuged and dried to make sure no solvent remained. All subsequent steps were performed with minimal exposure of samples to light.

2.7. One-dimensional electrophoresis (1DE)

The IAF/FTSC labeled proteins were run on discontinuous polyacrylamide gels (12% resolving gel and 4.5% stacking gel; Laemmli et al., 1970). Protein samples and markers were denatured by heating at 95°C for 5 min before loading into wells at 20µg per lane, with three replicate lanes per sample. After electrophoresis at 90V for 1-1.5 h and 120V for 2h, the gels were scanned using a Typhoon scanner (9410, Amersham Biosciences), with an excitation wavelength of 488 nm and emission wavelength of 520 ±20 nm (bandpass filter). After acquisition of fluorescence images, gels were stained with colloidal coomassie blue, and gel images were acquired with a GS-800 calibrated densitometer (BioRad, Herculus, CA, USA).

2.8. Data analysis

For each 1DE separation, all bands were subsequently analyzed by Quantity One image analysis software (BioRad, Hercules, CA, USA) measuring the total intensity for each lane quantified as arbitrary unit (a.u.). Fluorescence values were normalized for loading by

normalizing them with coomassie staining intensity for the same lane. An average of three replicates from three different extracts for each treatment and for each tissue was determined. Results are presented as means ±SD of protein thiol/carbonyl. Normal distribution and homogeneity of variance were tested using Shapiro-Wilk and Bartlett tests prior to statistical analysis. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a Tukey's HSD *post-hoc* test and significance determined at $p<0.05$.

3. Results

3.1. Properties of Nanoparticles

TEM analysis showed a homogeneous spherical shape for both sizes of NP, with an approximate primary size of 90 nm and 50 nm for AgNP <100 nm and AgNP <50nm, respectively. Size distribution revealed median sizes of 85±32.57 nm and 41.6±18.82 nm, respectively. XRD analysis revealed the crystalline nature of the AgNPs with diffraction peaks matching the face centered cubic (fcc) phase of silver. The UV-Vis spectrum (T60; PG-instruments, Leicestershire, UK) of the colloidal solution (λ_{max} = 400 nm) performed prior to exposure was consistent with a homogenous dispersion of AgNP in aqueous solution (Leopold & Lendl, 2003).

3.2. Redox-based changes in proteins after 3h of exposure

The free thiol content of gill tissue showed a significant increase when exposed to AgNP<100nm for 3h in the presence of nystatin (a caveolae-mediated endocytosis blocker) (Fig. 1A). In the presence of amantadine (a clathrin-mediated endocytosis blocker) a significant decrease in free thiols (thiol oxidation) with either AgNP<50 nm and AgNP<100 nm was observed (Figure 1A). In the same context, protein carbonyl content showed a significant increase only with exposure to AgNP<100 nm, whilst significant decreases were noticed when exposed to AgNP<50 nm and AgNP<100 nm in the presence of amantadine

(Figure 1A). Otherwise, digestive gland free thiol content increased when exposed to AgNP<50 nm either in the presence of DMSO or amantadine, whilst the carbonyl content increased with AgNP< 50 nm alone, and in the presence of nystatin with both sizes of AgNPs and a decrease for AgNP < 50 nm in the presence of DMSO (Figure 1B).

3.3. Redox-based changes in proteins after 6h of exposure

After 6h exposure, gill free thiol content increased significantly with AgNP<50 nm whilst thiol oxidation was observed in the presence of DMSO with either AgNP size (<50nm or <100nm; Figure 2A).An increase in protein carbonyl content was evident only when exposed to AgNP< 50nm in the presence of nystatin, whilst carbonyl content decreases were evident with both sizes of AgNP in the presence of DMSO, and with AgNP< 100nm in the presence of amantadine (Figure 2A). In the digestive gland, at this time-point (6h), free thiol content increased with AgNP< 50nm and AgNP< 100nm alone and in the presence of DMSO. Significant protein thiol decreases (thiol oxidation) were observed with both sizes of AgNP in the presence of nystatin (Figure 2B). In this same context, protein carbonyl content increased only in the presence of amantadine with both sizes of AgNP used in this study (Figure 2B).

3.4. Redox-based changes in proteins after 12h of exposure

Decreased free thiol content coupled with an increase in protein carbonyls was observed in gills of mussels exposed for 12h to AgNP< 50 nm (Figure 3A). However, only an increase in the carbonyl content was found on exposure to AgNP< 100 nm (Figure 3A). In the presence of DMSO with AgNP< 100 nm, free thiol content increased, whilst both sizes of AgNP increased the protein carbonyl content in the presence of DMSO (Figure 3A). Also, in the presence of nystatin AgNP< 100 nm caused a significant decrease in free thiols. However, in the presence of amantadine, AgNP< 50 nm caused increased protein thiols. Low levels of carbonyl content were observed with both sizes of AgNP in the presence of either nystatin or

amantadine (versus nystatin and amantadine treatment alone, respectively) (Figure 3A). Furthermore, in the digestive gland, free thiol content showed significant decreases when mussels were exposed to either size of AgNP in the presence of DMSO (Figure 3B). Also, an increase in free thiols was evident with both NP sizes in the presence of amantadine. Protein carbonyl content was found to have increased on exposure to AgNP <50 nm in the presence of nystatin (Figure 3B).

4. Discussion

Oxidative stress-related changes have been identified as a major component of NP toxicity in marine organisms. Several studies have found toxicity to be strongly related to physicochemical attributes of NPs such as chemical composition and particle size (Tedesco & Sheehan, 2010; Gomes et al, 2011, 2013; Tedesco et al, 2008; Hu et al, 2014; Rainville et al, 2014; Katsumiti et al, 2015). Tedesco and Sheehan (2010) highlighted the necessity of further studies to probe the dependence of nanoparticle toxicity on cell/tissue uptake with the aim to understand better their role in determining the potential toxicity of NPs. The present study aimed to investigate redox changes in gill and digestive gland, and to probe the relationship between such changes and cell uptake route. Our results reveal that a decrease in reduced thiols (free thiol oxidation) is evident in gills of mussels exposed to AgNP< 50 nm for 12h without any uptake pathway inhibition. This showed that these NPs were triggering oxidative stress under the experimental conditions used. Moreover, longer exposure times gave greater levels of protein oxidation (Gomes et al, 2013). AgNPs have previously been suggested to induce leakage of electrons from the mitochondrial electron transport chain promoting ROS generation (Tedesco and Sheehan, 2010). Ag ions released from AgNPs in this timeframe (12h) could directly oxidize protein thiols (Dobias & Bernier-Latmani, 2013; Park et al, 2013). Differential redox-based changes (i.e., oxidized thiols, carbonyls formation, or both)

in different tissues might be related to tissue-specific functional requirements associated with their individual physiological roles (Guerriero et al, 2003; Limon-Pacheco & Gonsebatt, 2009; Guerriero et al, 2014). Paradoxically, protein free thiol content (reduced thiols) actually increased in gills and digestive gland after exposure to AgNP< 50 nm for 6h, and only in the digestive gland after exposure to AgNP< 100 nm for 6h. This might represent an attempt by mussels to decrease the NP toxicity by reducing proteins. These findings agree with observations on exposure of *Daphnia magna* to AgNPs (Rainville et al., 2014). In fact, increased protein thiol content can be considered as increasing cellular redox buffering capacity in an attempt to restore the redox balance of the cell. Protein disulfide bond formation and reduction depend on the availability of electrons donors or acceptors, which might be impacted by the presence of Ag ions leached from AgNP and/or be due to NP interactions with cell structures (Deneke, 2001). AgNPs increased protein carbonyl content in some instances where no thiol oxidation was evident (Figure 1 A&B; Figure 3A) which, again, was observed in *D. magna* exposure (Rainville et al., 2014). It is expected that irreversible protein side-chain modifications (i.e., protein carbonyls and hyper-oxidized cysteines and methionines) lead to partially unfolded and inactivated proteins that are rapidly cleared from cells (Dowling and Sheehan, 2006; Regoli and Giuliani, 2014).

mTOR is an evolutionarily conserved serine/threonine protein kinase that senses and integrates a variety of cellular physiological and environmental signals to regulate cell growth (Jung et al., 2010). The phosphorylated active form of mTOR is involved in various cellular processes, such as activation of protein translation and inhibition of autophagic activity (Sforzini et al, 2017). Since protein carbonyls are often formed on proline, lysine, arginine and threonine residues (Dalle-Donne et al., 2003; Sheehan et al, 2010; Tedesco et al, 2010), it is possible that mTOR-induced autophagic sequestration is a response to AgNP stress. Autophagy is a well-documented general mechanism of cytoplasmic protein turnover in

eukaryotic cells that plays an essential role in cell survival of toxic insults. It involves sequestration of cytoplasmic components, removal of damaged organelles and protein aggregates and their subsequent degradation within lysosomes. Excessive autophagy has deleterious consequences for tissue/organism health as lysosomal cytotoxicity decreases in circulating hemocytes and is associated with enhanced inflammatory response and tissue damage in mussel gill and digestive gland (Bouallegui et al., 2017a, b, c and d). Such damage is related with either over-expression of cytokines or over-production of ROS, which will inhibit the mTOR signaling pathway and activate stress-induced autophagy (Moore et al., 2015).

We aimed to assess the involvement of uptake pathways by blocking such routes with pharmaceutical inhibitors. DMSO did not cause any evident carrier-mediated effect but, in the presence of AgNPs, it influenced the redox-sensitivity of the surrounding medium, causing significant redox-based changes (e.g., oxidation of protein thiols in the gills and digestive gland, with both sizes of AgNP at different time points).Unusually, in the digestive gland increases in carbonyl content with either size of AgNP were found. It has previously been shown that formation of endocytotic coated pits in digestive cells is important in digestive cells during nutrient uptake (Dimitriadis et al., 2004). Therefore, digestive cells would be expected to contain a well-developed lysosomal vacuolar system for uptake and digestion of nutrients. Metal accumulation in vesicles is prevented by uptake blockers such as nystatin. Late-exposure observations (6h and 12h), showed altered redox equilibrium, despite the blockade of caveolae-mediated uptake route (e.g., increase of carbonyls in gill extracts [AgNP<50nm] and thiols oxidation in digestive gland [both sizes]). These results could be due to the ability of cells to internalize AgNPs by activating other uptake pathways (e.g., phagocytosis, macro-micro-pinocytosis) (Ivanov, 2008).

Our data are in good agreement with previous investigations (Bouallegui et al., 2017a) assessing the role of uptake pathways in determining lysosomal cytotoxicity of AgNP to mussel immune cells. More interestingly, inhibition of clathrin-mediated endocytosis may decrease deleterious effects of excessive autophagic rate, by inhibiting the turnover mechanism of AgNP uptake.

A clear relationship linking NP size to redox-based changes in the *M. galloprovincialis* proteome was not demonstrated in this study. Overall, the role of clathrin-mediated endocytosis, as a major mechanism of sorting cargos including NPs to the endo-lysosome vacuolar system merits more detailed investigation.

5. Conclusion

We demonstrated that NP uptake pathways play a major role in affecting the redox balance and in determining redox-based changes in cells. Blockade of clathrin-mediated endocytosis had a beneficial role in preventing ROS-related disturbance. There is also a possible role for the mTOR pathway in responding to NP toxicity. Our results also reveal differential tissue-specific redox changes. Overall, uptake routes impact NP toxicity more than NP size.

Ethical statement

All mussel experiments were carried out in accordance with the declaration of Helsinki and associated guidelines for animal experiments.

Disclosure statements

The authors have no potential conflict of interest to declare. All authors materially participated in the research and/or article preparation, so roles for all authors is described as below: YB: planned, designed the experimental sets, collected, analyzed the results and drafted, edited and revised the final version of the manuscript; RB: helped in the experimental sets; RO: helped in planning the experimental sets; DS: planned, analyzed,

edited and approved the final version of the paper. All authors have approved the submission of the final copy of the manuscript.

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Figure captions

Figure 1. Thiol and carbonyl content in the gills (A) and the digestive gland (B) from mussels exposed to different experimental conditions for 3h. Data are presented as Means ±SD in arbitrary units (n=3). P value is set to *< 0.05 and **<0.01; Ag50: AgNPs<50 nm; Ag100: AgNPs<100 nm; Cont: control; Amant: Amantadine; Nyst: Nystatine.

Figure 2. Thiol and carbonyl content in the gills (A) and the digestive gland (B) from mussels exposed to different experimental conditions for 6h. Data are presented as Means ±SD in arbitrary units (n=3). P value is set to *< 0.05 and **<0.01; Ag50: AgNPs<50 nm; Ag100: AgNPs<100 nm; Cont: control; Amant: Amantadine; Nyst: Nystatine.

Figure 3. Thiol and carbonyl content in the gills (A) and the digestive gland (B) from mussels exposed to different experimental conditions for 12h. Data are presented as Means ±SD in arbitrary units (n=3). P value is set to *< 0.05 and **<0.01; Ag50: AgNPs<50 nm; Ag100: AgNPs<100 nm; Cont: control; Amant: Amantadine; Nyst: Nystatine.

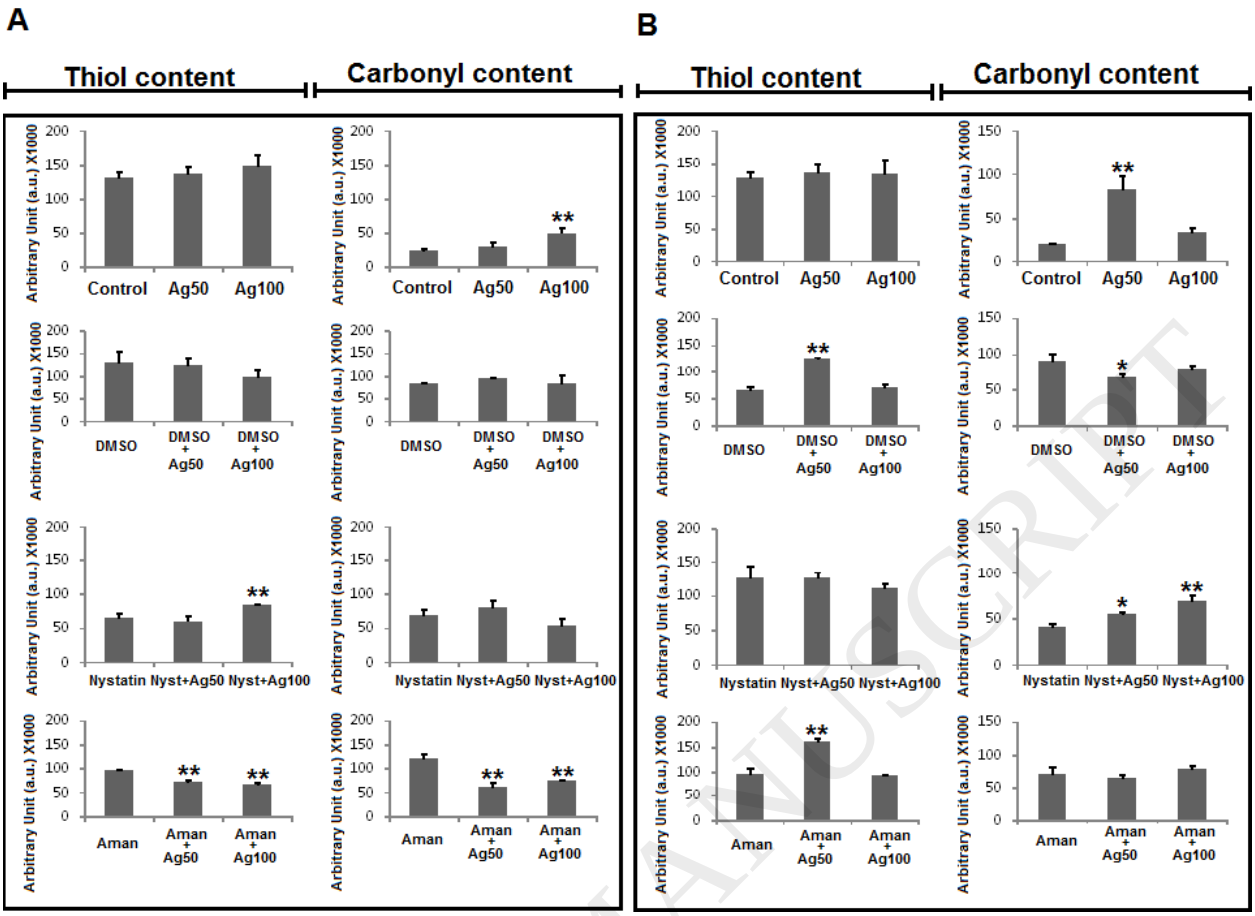

Figure 1.

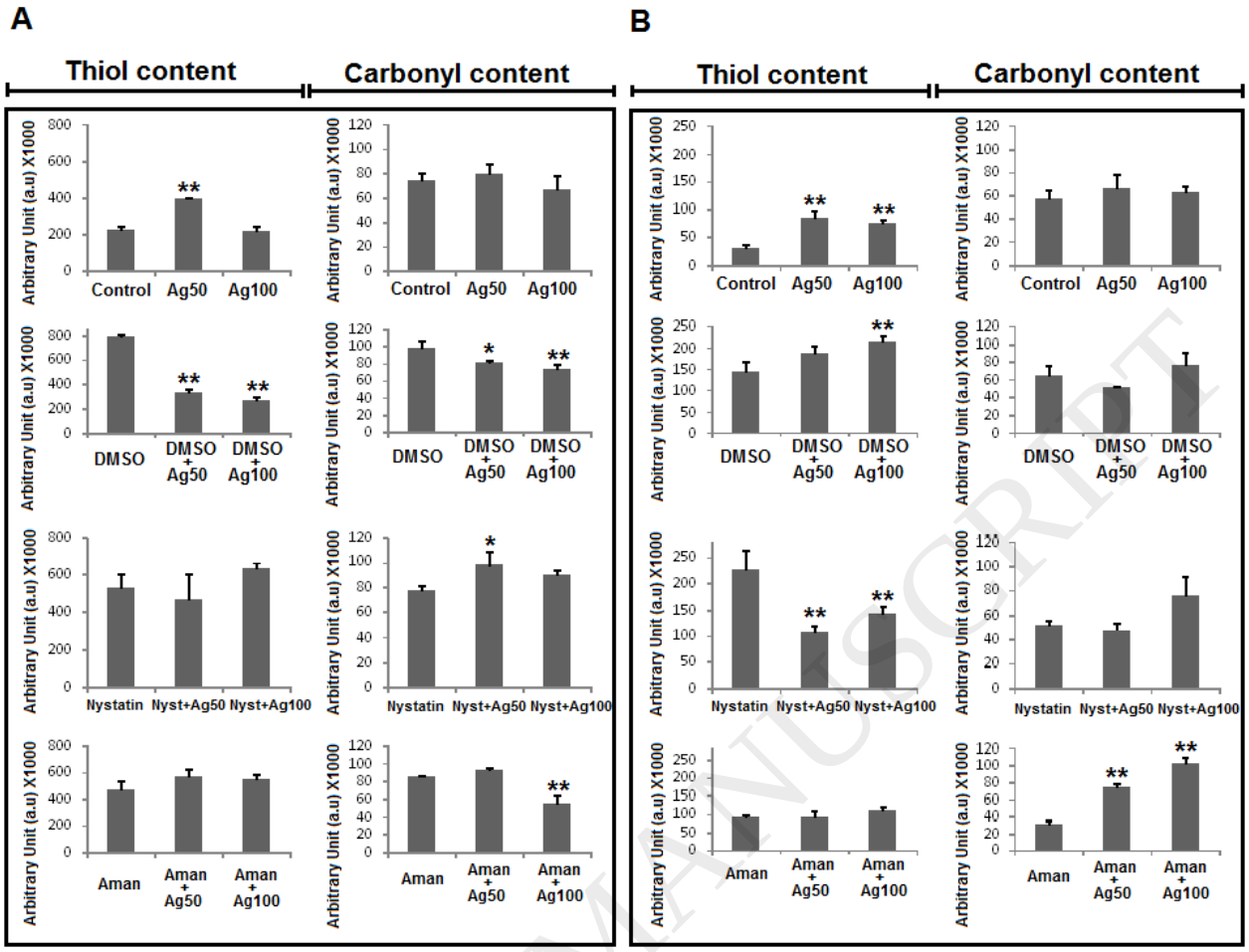

Figure 2.

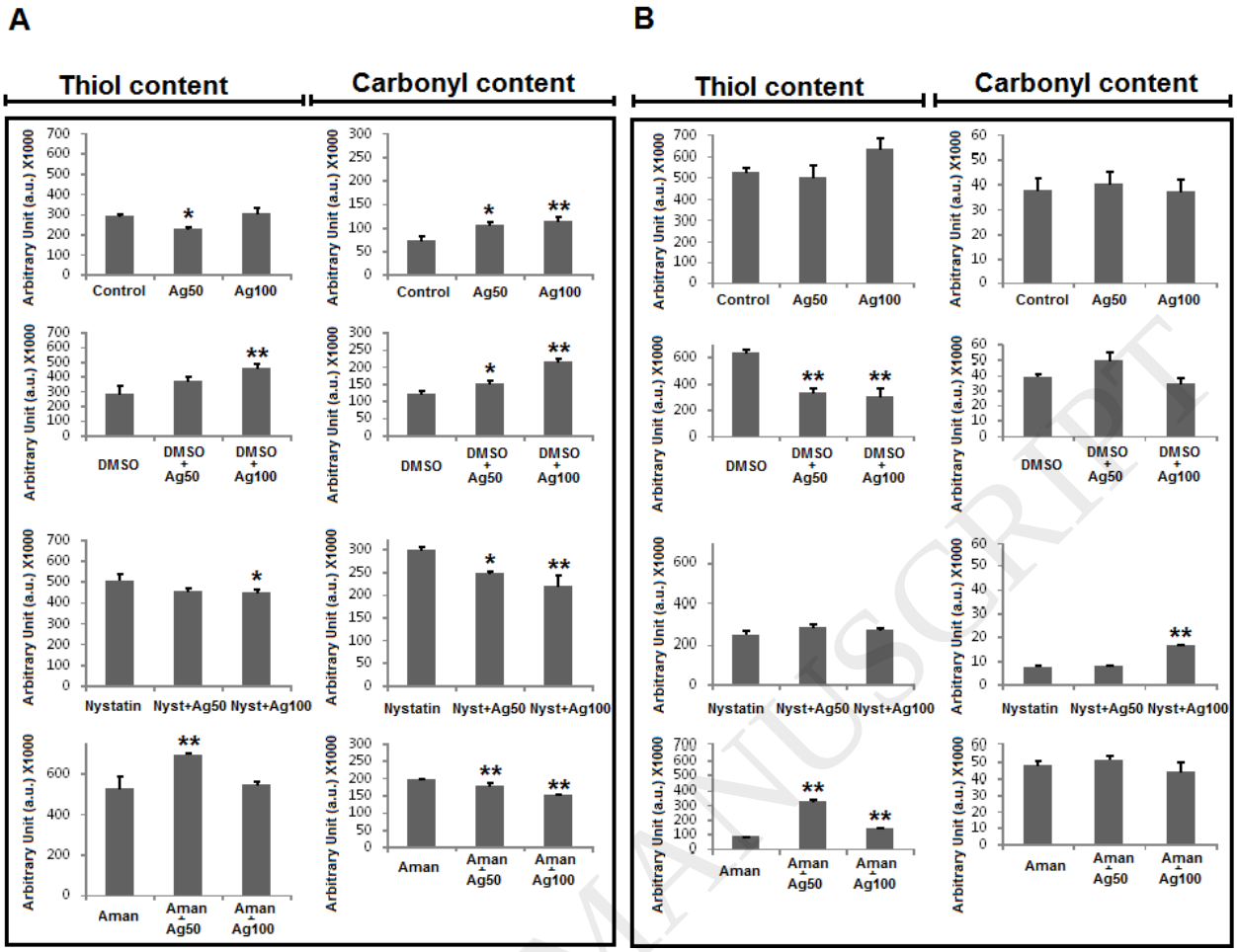

Figure 3.