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1 **Effects of triangular gold nanoparticles in the clams *Ruditapes decussatus***

2
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33 **Abstract**

34 **Context:** Many reports link nanoparticles with adverse environmental effects. However,
35 relatively little information is available on how these compounds interact with marine
36 organisms.

37 **Objective:** Our aim was to examine the effects of triangular gold nanoparticles (Tr-Au NPs) in
38 the clam, *Ruditapes decussatus*.

39 **Materials and methods:** Clams were exposed to Tr-Au1 = 5 µg/L and Tr-Au2 = 10 µg/L for
40 2 and 7 days to study effects of these nanoparticles. Effects on shell structure were investigated.
41 Superoxide dismutase (SOD), catalase (CAT), glutathione transferase (GST) activities, protein
42 carbonyl levels and malondialdehyde content were used to assess biochemical status.

43 **Results:** Transmission electron microscopy and electron dispersive X-ray microanalysis (EDX)
44 showed that gold leads to modification of shell structure and morphology. Indeed, Tr-Au NPs
45 size were altered forming aggregate particles. Triangular gold NPs increased SOD, CAT and
46 GST activities in gill and digestive gland in a concentration- and time-dependent manner
47 indicating defense against oxidative stress. Lipid peroxidation and protein carbonyl levels
48 indicated that Tr-Au NPs caused significant cellular damage.

49 **Conclusion:** This study provides a framework for understanding of toxicological effects of
50 nanoparticles on a filter feeding organism. It explores also novel interactions between Tr-Au
51 NPs and bivalve species.

52

53 **Keywords:** Nanoparticles; *Ruditapes decussatus*; Oxidative stress; Biomarkers; Biomonitoring

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65 **Introduction**

66 Environmental pollutants, such as nanoparticles (NPs) increasingly used in consumer
67 products and human activities, can be released into marine ecosystems where they may interact
68 with aquatic organisms and induce toxic effects at different levels of biological organization
69 (Li et al. 2013; Maurer-Jones et al. 2013; Baker et al. 2014; Corsi et al. 2014; Minetto et al.
70 2014; Cid et al. 2015; Grillo et al. 2015). Indeed, NPs can penetrate biobarriers and move easily
71 into and through organisms which can cause toxic effects (Nowack et al. 2007). Oxidative stress
72 histological and morphological changes are often associated with NP toxicity (Li et al. 2013;
73 Cid et al. 2015).

74 Gold NPs (Au NPs) are used in the medical sector as a contrast agent (Azzay and
75 Mansour, 2009) and in drug delivery systems (Yih and Al-Fandi, 2006). Au NPs are generally
76 regarded as being non-toxic, inert and biocompatible. However, they have also been shown to
77 have some biological effects on the nanoscale (Clough, 2009). Because of their extensive use,
78 Au NPs could potentially represent a significant novel anthropogenic input to the aquatic
79 environment (Lapresta Fernández et al. 2012).

80 Bivalves are considered prime candidates for uptake of pollutants during environmental
81 contamination scenarios (Galloway et al. 2002; Livingstone, 2001). NP ecotoxicity has been
82 reported in several sentinel species including *Mytilus edulis*, *Ruditapes philippinarum* and
83 *Daphnia magna* (Tedesco et al. 2010; Khan et al. 2014; García Negrete et al. 2015). The
84 Mediterranean clam *Ruditapes decussatus* has been used as a sentinel species in aquatic
85 toxicology due to its tolerance for chemical contaminants (Dellali et al. 2001; Sellami et al.
86 2015). These organisms are abundant and farmed commercially around the Mediterranean Sea
87 (Mohamed et al. 2003). They are relatively resistant to a wide variety of pollutants and
88 environmental stressors, making them especially suitable in marine biomonitoring. They filter
89 large volumes of pollutants and may represent a significant target for NPs in the aquatic
90 environment (Canesi et al. 2012).

91 Oxidative stress is recognized as one of the most common effects of nanotoxicity
92 (Klaine et al. 2008). Au NPs are able to induce reactive oxygen species (ROS) production in
93 bivalves triggering oxidative stress. In redox homeostasis, ROS are detoxified by antioxidant
94 defenses which include antioxidant enzymes such as superoxide dismutase (SOD), catalase
95 (CAT) and glutathione transferase (GST). These protect important cellular components such as
96 lipids, proteins, and DNA from oxidative damage. Thus, antioxidant enzyme activity levels can
97 provide valuable information regarding the effects of NPs on an organism's biology (Cid et al.
98 2015). In addition, shells of bivalve's offer important practical advantages for monitoring

99 chemical contamination of the aquatic environment (Cravo et al. 2004; Yang et al. 2011;
100 Sellami et al. 2015).

101 **Despite the links between NP exposure and** adverse environmental effects **in sentinel**
102 **species such as clams, relatively** little information is available on how these compounds might
103 interact with bivalve shells. The present study aims, for the first time, **to establish if there is a**
104 **correlation between activities of antioxidant enzymes and triangular gold NP (Tr-Au NPs) -**
105 **associated effects on shells of *R. decussatus*.**

106

107 **Materials and methods**

108 *Tr-Au NPs Preparation*

109

110 Tr-Au NPs were produced by a modified polyol process involving a surface regulating polymer,
111 polyvinylpyrrolidone (PVP). Briefly, 25 mL of triethylene glycol (ACROS Organics, 98%)
112 solution, containing 0.038 mMol of hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄·3H₂O)
113 (Sigma-Aldrich), and 0.05 molar ratio of PVP (K30; Sigma-Aldrich) to HAuCl₄ were mixed
114 and heated to 150°C. The mixture **was held** at the same temperature for 30 min under constant
115 agitation. The final colloidal solution had a blue color. The product was separated by
116 centrifugation, washed several times with ethanol/acetone (2:1), and dispersed in MilliQ-water.
117 From this solution, dilutions were prepared directly without allowing the colloid to age. In order
118 to investigate effects of Tr-Au NPs on *R. decussatus*, many Tr-Au NPs suspensions with
119 different concentrations were prepared. **For a low-level concentration**, the 280 mg L⁻¹Tr-Au
120 NPs suspension was diluted to a 5 µg L⁻¹ concentration in standard sea water (SW), and in
121 MilliQ-water, for comparison purposes. **For a high-level concentration**, the 280 mg L⁻¹ Tr-Au
122 NPs suspension was diluted to 10 µg L⁻¹, again in both standard SW and MilliQ-water,
123 according to the required concentration in each particular case throughout this study.

124

125 *Characterization*

126

127 **Tr-Au NPs characteristics** were studied using transmission electron microscopy (TEM) in a
128 JEOL-JFC 1600 microscope operating at 200 keV and an energy-dispersive X-ray spectrograph
129 (EDX). Selected area electron diffraction patterns (SAED) were also acquired in order to
130 determine the crystallographic orientation of NP facets. The optical absorption spectra of
131 diluted Tr-Au NPs solution were performed on a Perkin-Elmer Lambda 11 UV/VIS
132 spectrophotometer.

133

134 *Animals and treatments*

135

136 Adult clams (*R. decussatus*) of 2.5-3 cm shell length (maximum axis) were obtained from a site
137 in Bizerte lagoon (37°13'18.54''N, 9°55'59.61''E). Acclimation occurred in free-flow tanks
138 for one week before commencing exposures. For the course of the experiment, 5 individuals
139 were placed in each tank with 3 L of SW obtained from the sampling site (salinity 37,
140 temperature 18°C, oxygen at 6.5 mg/L), containing 5 and 10 µg/L of Tr-Au NPs respectively.
141 A control series without Tr-Au NPs was run in parallel. Each experiment was performed in
142 triplicate. Exposure treatments are denoted as follows: Tr-Au1 for 5 µg/L gold concentration
143 and Tr-Au2 for 10 µg/L gold concentration. The concentrations selected, while higher than
144 environmental levels, were chosen on the basis of previous results considered for Au NP effects
145 on bivalves and because they have previously shown mild oxidative stress responses (Tedesco
146 et al. 2008, 2010). Aeration at the bottom of the tank was used to minimize agglomeration and
147 subsequent sedimentation of the contaminant, and SW was changed every 48 h. For the duration
148 of the experiment (2 and 7 days), Tanks were filled with natural SW changed every 48 h and
149 the environmental parameters were the same as those used for the acclimation period. Test
150 animals were checked daily. No mortality was observed and all animals were seen to be feeding
151 normally. Unexposed and Tr-Au NP-exposed clams were collected after 2 and 7 days. Animals
152 were dissected. Gill and digestive gland were collected, immediately frozen in liquid nitrogen
153 and stored at -80 °C until required. Shells of control and treated clams were retained after 2 and
154 7 days, dried, crushed manually to obtain a powder with grain size of 80-200 µm and stored at
155 4°C prior to chemical characterization.

156

157 *Biochemical analysis*

158

159 Digestive gland and gill were homogenised by a polytron homogenizer in 10 mM
160 Tris/HCl, pH 7.2, containing 500 mM sucrose, 1mM EDTA and 1 mM PMSF, supernatants
161 were collected by centrifugation at 20,000 × g (4°C for 30 min). Antioxidant enzymatic
162 activities were measured in the cytosolic fraction of 15 clams from controls and groups exposed
163 to Tr-Au NPs. Changes in optical density were quantified using a Beckman DU500
164 spectrophotometer. Protein content was estimated by the method of Bradford (1976) using
165 bovine serum albumin (BSA) as a standard. SOD activity was assessed by the ability of the
166 enzyme to inhibit auto-oxidation of pyrogallol. We used 0.2 mM pyrogallol in air-equilibrated

167 50 mM Tris- buffer pH 8.20, containing 1 Mm EDTA (Marklund and Marklund, 1974) and is
168 expressed in $\mu\text{mol}/\text{min}/\text{mg}$ of total protein. CAT activity was measured by the decrease in
169 absorbance at 240 nm due to H_2O_2 consumption (Aebi,1974). The reaction volume and reaction
170 time were 1 mL and 1 min, respectively. The reaction solution contained 80 mM phosphate
171 buffer, pH 6.5 and 50 mM H_2O_2 (Ni et al., 1990) and CAT activity is given as nmol/min/mg
172 protein. GST activity was measured by a modification of the method of Habig et al.1974. There
173 action mixture contained 200 μL supernatant, 2mLphosphate buffer (0.125 M, pH
174 7.7,containing Na_2 EDTA ,0.05 M, 2–4 °C), H_2O 400 μL , 200 μL 15mM 1-chloro-2,4-
175 dinitrobenzene (CDNB) dissolved in 95% ethanol and 200 μL 15mM of reduced glutathione
176 (GSH).GST activity was determined following the conjugation of GSH with CDNB at 340 nm.
177 A unit of GST activity was defined as the amount of glutathione conjugate formed using 1nM
178 GSH and CDNB/min per mg protein (nM 2,4-dinitrophenyl glutathione/mg protein/min).
179 Lipid peroxidation was determined in terms of thiobarbituric acid reactive species (TBARS),
180 by malondialdehyde (MDA; Buege and Aust, 1978). Briefly, 1mL sample extract was mixed
181 with 2mL of trichloroacetic acid-thiobarbituric acid-HCl (TCA–TBA–HCl) reagent (15% (w/v)
182 TCA, 0.375% (w/v) TBA and 0.25 N HCl). Mixture was boiled for 15 min, cooled and
183 centrifuged at 10,000g to remove precipitate. Absorbance was measured at 535 nm and MDA
184 content was calculated using an extinction coefficient of $156 \text{ mM}^{-1} \text{ cm}^{-1}$. Lipid peroxidation
185 was expressed as nMol MDA/mg protein.

186 Protein carbonyl levels were also determined (Levine et al., 1990). After incubation, the
187 assay mixture was centrifuged at 10,000g for 10 min at 4 °C and the supernatant precipitated
188 with TCA followed by centrifugation at 10,000g for 3 min. The pellet was resuspended in 2, 4-
189 dini-trophenyl hydrazine and incubated at 25 °C for 60 min.
190 Proteins were then precipitated with TCA. The mixture was then centrifuged and the pellet was
191 washed with acetone three times. Absorbance was measured at 360 nm and carbonyl content
192 was calculated using a molar extinction coefficient of $22 \text{ mM}^{-1} \text{ cm}^{-1}$. Results were expressed as
193 nMol carbonyl/mg protein.

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197

198 *Statistical analyses*

199

200 Statistical analysis **was performed** using STATISTICA 8.0. Results of enzymatic activities were
201 reported as mean \pm standard deviation. Variation of each enzyme **across** time and concentration
202 was tested by one-way ANOVA ($p < 0.05$). Previously, we tested the pre-requisites for analysis
203 of variance (normality and homogeneity of variances). When significant differences were
204 found, Tukey's test was applied to determine which values differed significantly.

205

206 **Results**

207

208 *Morphological and elemental analysis*

209

210 **A representative TEM image of the synthesized NPs is shown in Fig. 1a.** The majority of
211 NPs were equilateral triangular prisms with an average edge length of 150 nm (Fig. 1b). Energy
212 dispersive spectrum (EDX) analysis for the sample confirmed that Tr-Au NPs consist of only
213 gold (Fig. 1c, the copper element originated from copper grid). **An inset** to Fig.1a, gives typical
214 selected area electron diffraction (SAED) pattern obtained by directing the electron beam
215 perpendicular to a single gold nanoplate deposited flat on the TEM grid. The hexagonal
216 symmetrical spots of the SAED pattern show that gold nanoplates are single crystals and the
217 incident electron beam is perpendicular to the {111} facet of the tested plate. **The absorbance**
218 **spectrum** of the as-prepared colloidal solution (Fig.1.D) show an extinction band with a
219 maximum intensity around 720 nm due to the contribution of in-plane dipole SPR absorption
220 of anisotropic products in which the oscillation of free electrons was strongly restricted in the
221 planar structure. Structural characterization of shell and the nanosize distribution of unexposed
222 and Tr-Au exposed clams determined by TEM and EDX are depicted in Fig 2. The detailed
223 structure of the pure shell is seen in **Fig 2. (a,b)** while TEM images show an alternation of
224 homogeneous light and dark layers with an average length of 1 μ m as indicated by the scale bar
225 of 300 nm.

226 Shell structure seems to resemble a complex cross-lamellar structure. This is in good
227 agreement of the findings of Yang et al. (2011). Indeed, the different CaCO₃ polymorphs can
228 form diverse types of structure, such as prismatic, sheet nacreous, lenticular nacreous, foliated,
229 cross-lamellar, complex cross lamellar and homogeneous structure (Taylor and Layman, 1972;
230 Currey and Taylor, 1974). The EDX spectrum confirms the components and the purity of the
231 shell which is composed essentially of calcium carbonate (CaCO₃), in general about 95 wt.%
232 and less than 5 wt.% organic materials (Currey., 1977). The two principal polymorphs of CaCO₃
233 in shells are aragonite and calcite (Sellami et al. 2015). **Figure 2-d**, shows the influence of Tr-

234 Au NPs exposure on the structure and morphology of the shell. The EDX spectrum (Fig 2-e)
235 confirms the presence of Au NPs (Au element is detected) in the shell after exposure. The Ca
236 and O elements refer to the aragonite composition of the shell. The presence of Cu is due to the
237 copper grid used for the TEM/EDX experiments. As shown in Fig 2-d, and compared to the
238 TEM images of pure clam shell (Fig.2-a) and pure Tr-Au NPs (Figure 1a), the size of gold and
239 clam shell were changed. Indeed, the size of pure Tr-Au NPs as shown in Figure 1-a, is in the
240 range of 150 nm however it was approximately 300 nm (Fig.2-d) after exposure. The same
241 phenomenon was observed for the shell itself; the size was changed from ~1 μm in unexposed
242 clams (Fig.2-d) to a few nanometers (100 - 400 nm) in the Tr-Au₂ NPs-exposed animals. To
243 explain this phenomenon, it should be noted that clam shell are porous structures (Yang et al.
244 2011). Indeed, based on the work of Yang et al. (2011), the pore size can range from 300 to 500
245 nm. On the other hand, it was previously found that Au NPs can bioaccumulate in clams,
246 forming aggregate particles (Pan et al. 2012). Consequently, **it is likely** that the size increase
247 from 150 (pure Tr-Au NPs) to 300 nm (in the Tr-Au NPs exposed clams) **on** bioaccumulation
248 of Tr-Au NPs **through clam shell pores** leads to modification of the shell structure **when the**
249 **pores become saturated**. This can explain also the behavior of the clam shell observed in Fig.2-
250 d and the Tr-Au NPs **-induced alteration of size** from ~1 μm to the nanometer scale (~ 100 to
251 300 nm). These results are very important **in understanding** the reactivity of NPs with clams in
252 environmental contexts. García-Negrete et al. (2013) showed that agglomerates can be formed
253 for Au NPs in artificial SW and this result is related to NP concentration in the SW medium.

254

255 *Biomarker responses to Tr-Au NPs exposure*

256

257 **SOD, CAT and GST activities of clams treated with Tr-Au₁ and Tr-Au₂ NPs for 2 and 7**
258 **days, respectively, were determined (Figs. 3 and 4). Tr-Au NPs induced an overall**
259 **concentration- and time-dependent** increase in antioxidant enzyme activity **in both gill and**
260 **digestive gland**. SOD activity in digestive gland after 2 days exposure increased by 28%
261 compared to controls with no effect **on gill SOD** activity after this exposure time. After 7 days
262 of exposure, SOD activity increased in both gill and digestive gland (Fig. 4). A different pattern
263 of variation in CAT activity was observed between gills and digestive gland after 48 h exposure
264 (Fig.3). Indeed, **2 days of exposure did not alter CAT activity in gills**. In contrast, **digestive**
265 **gland CAT activity** increased in groups treated with Tr-Au₁ and Tr-Au₂, respectively. Exposure
266 to Tr-Au₁ and Tr-Au₂ for 7 days caused a significant ($p < 0.05$) increase of CAT activity in
267 both tissues (Fig. 4). CAT activity increased by approximately 30% and 42% in gill of treated

268 groups exposed respectively to Tr-Au1 and Tr-Au2 and by about 18% and 47% in digestive
269 gland. The pronounced effect of Tr-Au NPs in gill was supported by morphological changes
270 visible after 7 days of exposure. **Gill GST activity** increased from 0.74 ± 0.1 nmol/min/mg
271 protein to 1.26 ± 0.28 nmol/min/mg protein in Tr-Au1-treated groups and to 1.33 ± 0.42
272 nmol/min/mg protein in Tr-Au2-treated groups ($p = 0.045$; $p = 0.023$). **Digestive gland GST**
273 **activity** increased in a concentration-dependent manner by approximately 9% and 20%,
274 respectively, on exposure to Tr-Au1 and Tr-Au2. Lipid peroxidation determined by measuring
275 MDA content, and protein carbonyl levels of clams exposed to Tr-Au NPs were similar to the
276 control after 2 days of exposure (Fig. 5). **However, after 7 days of exposure, Tr-Au NPs**
277 **increased protein carbonyl and MDA levels significantly ($p < 0.05$) for both concentrations and**
278 **in both tissues (Fig. 6).**

279

280 **Discussion**

281

282 **Ecotoxicological effects of gold nanoparticles have so far not been extensively studied.**
283 **This study aimed to assess the impact of Tr-Au NPs on key antioxidant biomarkers and to**
284 **explore potential risks posed by this NP to the sentinel species *R. decussatus*. SOD, CAT and**
285 **GST are involved in the defense against oxidative stress (Regoli et al. 2011). Induction of these**
286 **enzyme activities are consistent with production of ROS (reactive oxygen species) in response**
287 **to Tr-Au NPs exposure** since it is known that NPs are capable of crossing cell membranes,
288 leading to cell damage (Li et al. 2010). In addition, the pronounced effect in the digestive gland
289 even of short-term exposure may suggest that Tr-Au NPs may be concentrated in digestive
290 gland rather than gill (Tedesco et al. 2008; García-Negrete et al. 2013). Exposure to Tr-Au2
291 NPs increased SOD activity after 2 days in both tissues. This result suggests that SOD responds
292 to Tr-Au2 NPs stress effects generated in both tissues after 7 days **most likely** to protect stressed
293 cells. This finding underlines the importance of exposure time in the biochemical response of
294 *R. decussatus* to NPs. **A similar pattern was previously reported in mussels upon exposure to**
295 **Ag NPs with a significant increase along the exposure period** (Gomes et al. 2014).

296 Our data are also in agreement with a previous study showing that Au NPs cause
297 oxidative stress in bivalves, especially in digestive gland (Tedesco et al. 2008). **Presumably,**
298 **NPs are ingested more efficiently** when clams are exposed to Au NPs forming aggregate
299 particles (Pan et al. 2012). NPs are also known to interact with thiol-groups that are found in
300 many antioxidant proteins **and which, when oxidized, can result in inhibition/inactivation of**
301 **enzymes (SOD and CAT) as oxidative stress develops** (Bar-Ilan et al. 2009; Lapresta-Fernandez

302 et al. 2012). Tedesco et al. (2008) showed that Au NPs caused oxidative stress in mussel
303 digestive gland (induction of CAT) but also in gill. **On the other hand**, GST is known to protect
304 cells against ROS during oxidative processes **as part of phase II biotransformation** (Livingstone,
305 2001). GST has already been associated with the metabolism of pollutants in bivalves (Sheehan
306 and Power, 1999) and identified as a metabolic pathway for NP metabolism (Wiegand et al.
307 2001). Our data also agree well with a previous study showing a significant increase of GST
308 activity **in a time- and concentration-dependent manner** (Cid et al. 2015).

309 Malondialdehyde (MDA) and protein carbonyl levels **are often altered in NP-induced**
310 **toxicity** (Ma et al. 2010; Tedesco et al. 2010) **and they are regarded as predictive biomarkers**
311 **for oxidative stress** (Xu et al. 2011). Our results are in good agreement with Chandurvelan et
312 al. (2013) who found a significant increase in CAT activity in digestive gland of the mussel
313 *Perna canaliculus*, while lipid peroxidation levels remained unchanged. Vale et al. (2014)
314 obtained similar results in the freshwater bivalve *Corbicula fluminea* when studying the effect
315 of TiO₂ NPs on the MDA content after 10 days of exposure. TiO₂ NPs showed significant
316 effects on MDA generation. These authors suggest that the presence of the NPs in the medium
317 contributes to cell damages as confirmed by lipid peroxidation data. Additionally, hydrophobic
318 interactions between proteins and lipids might alter protein conformation, thus affecting
319 enzyme activities. **Both lipid peroxidation and increased protein carbonyl levels** are
320 biochemical perturbations resulting from oxidative stress (Khazri et al. 2015). Changes in
321 carbonyls were previously reported in *M. edulis* in response to Au NPs-citrate and CuO NPs
322 (Tedesco et al. 2008; Hu et al. 2014). These authors correlated increased protein carbonylation
323 with oxidative stress related to ROS production on exposure to NPs.

324

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328

329 **Conclusions**

330 The present study contributes new information about the nano-toxicity of Tr-Au NPs in the
331 marine bivalve *R. decussatus*. Several toxicological endpoints **were altered** by Tr-Au NPs
332 exposure: (i) morphological changes to shells and NPs (ii) significant increases in SOD,
333 CAT and GST activities in digestive gland and gill in concentration- and time-dependent
334 manner and (iii) increased lipid peroxidation and protein carbonyl levels in both organs after 7
335 days of NP exposure. Exposure to Tr-Au NPs disrupts the oxidant/antioxidant balance in the

336 organs of *R. decussatus*, leading to induction of oxidative stress and cellular damage.
337 Furthermore, considering **the increasing prevalence** of nanotechnology, the present study
338 provides valuable information regarding the interaction between NPs **and biota** suggesting
339 potential risks for mollusks bivalves, **notably to their shell structure**. Further investigation
340 **focusing in more depth on the mechanisms of nanomaterial incorporation and interaction with**
341 **marine organisms are required. This will help us understand** the toxicity of NPs and can be used
342 on the programs of environmental risk assessments.

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348

349 **Declaration of interest**

350 Authors do not have any potential conflict of interest

351 **Ethical statement**

352 The study complied with the declaration of Helsinki.

353

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Figure legends:

Fig. 1. (a) TEM images, (b) particle size distribution, (c) EDX spectrum of triangular gold NPs. (d) Plasmonic response of Tr-Au NPs dispersed in ethanol. The inset in (a) shows typical selected area electron diffraction (SAED) pattern from a single NP.

Fig. 2. TEM images (a, b) and EDX spectrum (c) of pure clam shells (chemical formula; CaCO_3), (d, e) TEM image and EDX spectrum of Tr-Au NPs of exposed clams. The inset in (d) shows typical selected area electron diffraction (SAED) pattern from Tr-Au NP-exposed clams.

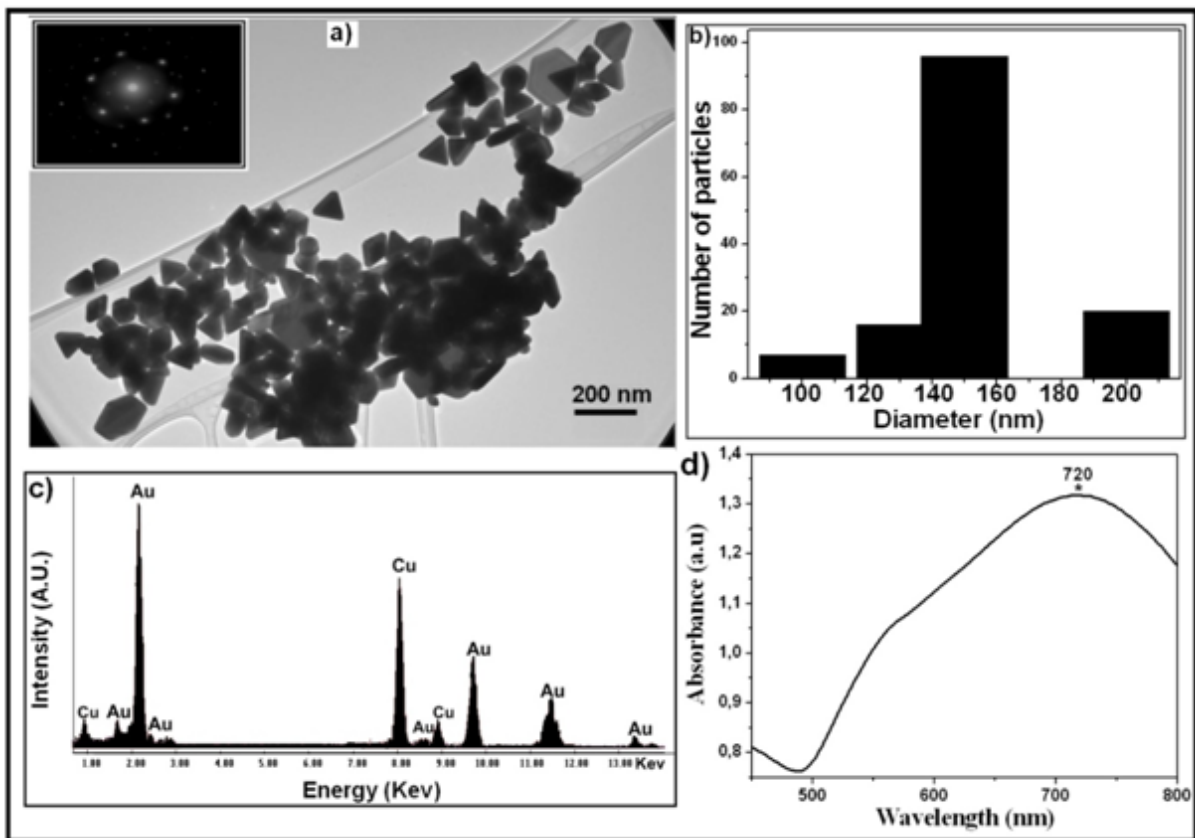
Fig. 3. SOD, CAT and GST activities after two days exposure. Specific activities in gill and digestive gland of untreated (Control) and treated (Tr-Au1 = 5 $\mu\text{g/L}$ and Tr-Au2 = 10 $\mu\text{g/L}$) *R. decussatus* after 2 days exposure to Tr-Au NPs. Different letters indicate statistical differences ($p < 0.05$) compared to control. Data are mean \pm SD.

Fig. 4. SOD, CAT and GST activities after seven days exposure. Specific activities in gill and digestive gland of untreated (Control) and treated (Tr-Au1 = 5 $\mu\text{g/L}$ and Tr-Au2 = 10 $\mu\text{g/L}$) *R. decussatus* after 7 d of exposure to Tr-Au NPs. Different letters indicate statistical differences ($p < 0.05$) compared to control. Data are mean \pm SD.

Fig. 5. Effect of 2 days Tr-Au NPs treatment on MDA and protein carbonyl levels in gill and digestive gland of *R. decussatus*. All values are given as means ($n = 5$). Groups with different letters are significantly different (ANOVA, post-hoc, Tukey HSD test, STATISTICA 8.0, $p < 0.05$).

Fig. 6. Effect of 7 days Tr-Au NPs treatment on malondialdehyde and protein carbonyls levels in gill and digestive gland of *R. decussatus*. All values are given as means ($n = 5$). Groups with different letters are significantly different (ANOVA, post-hoc, Tukey HSD test, STATISTICA 8.0, $p < 0.05$).

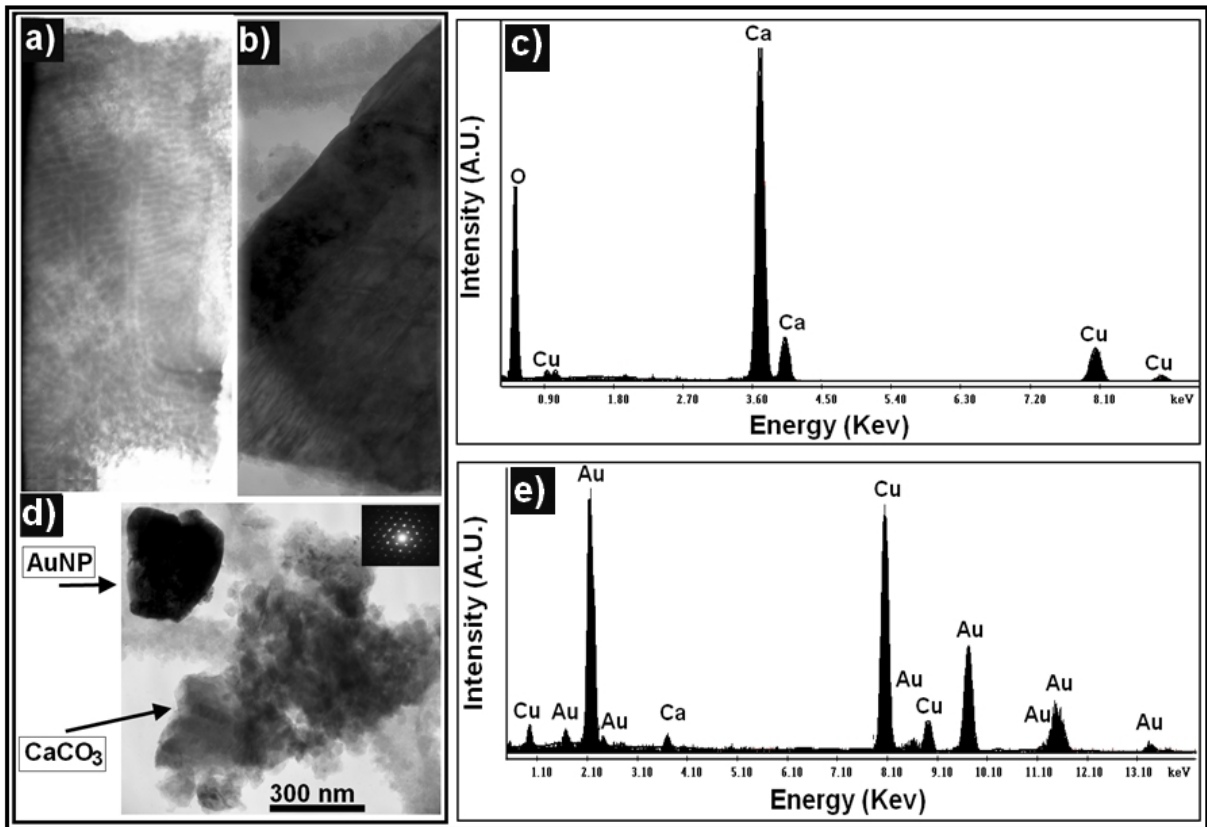
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519 Fig. 1

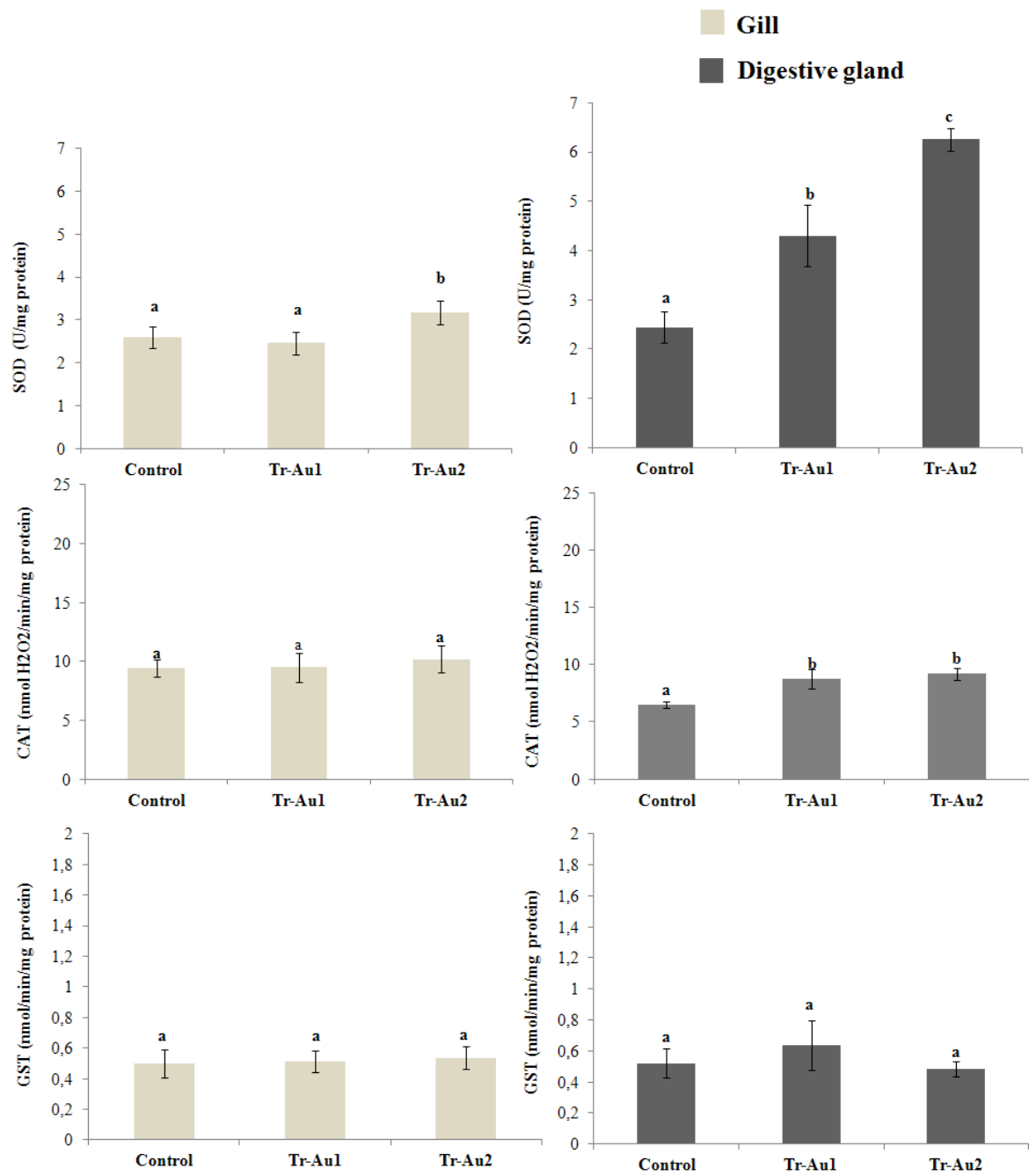
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522 Fig. 2

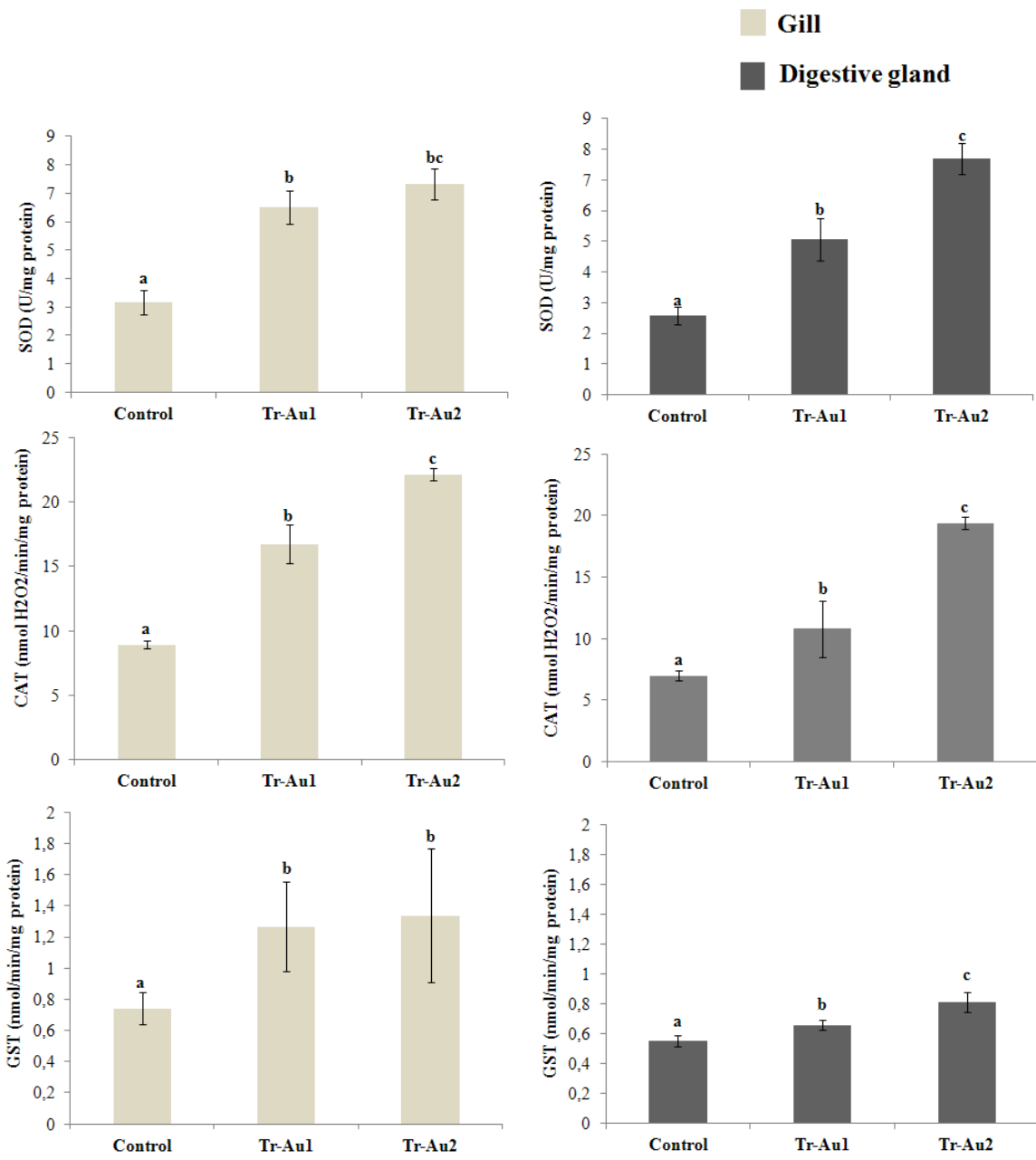
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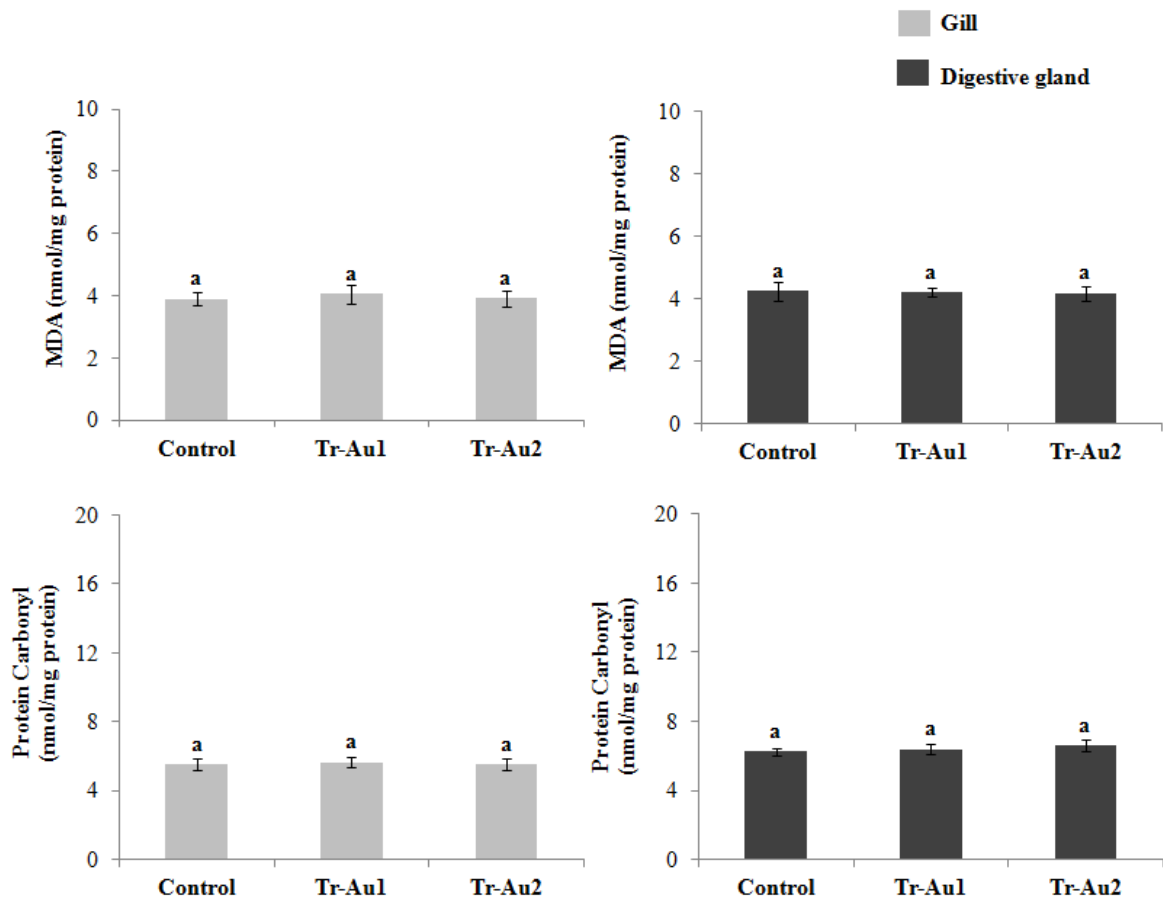
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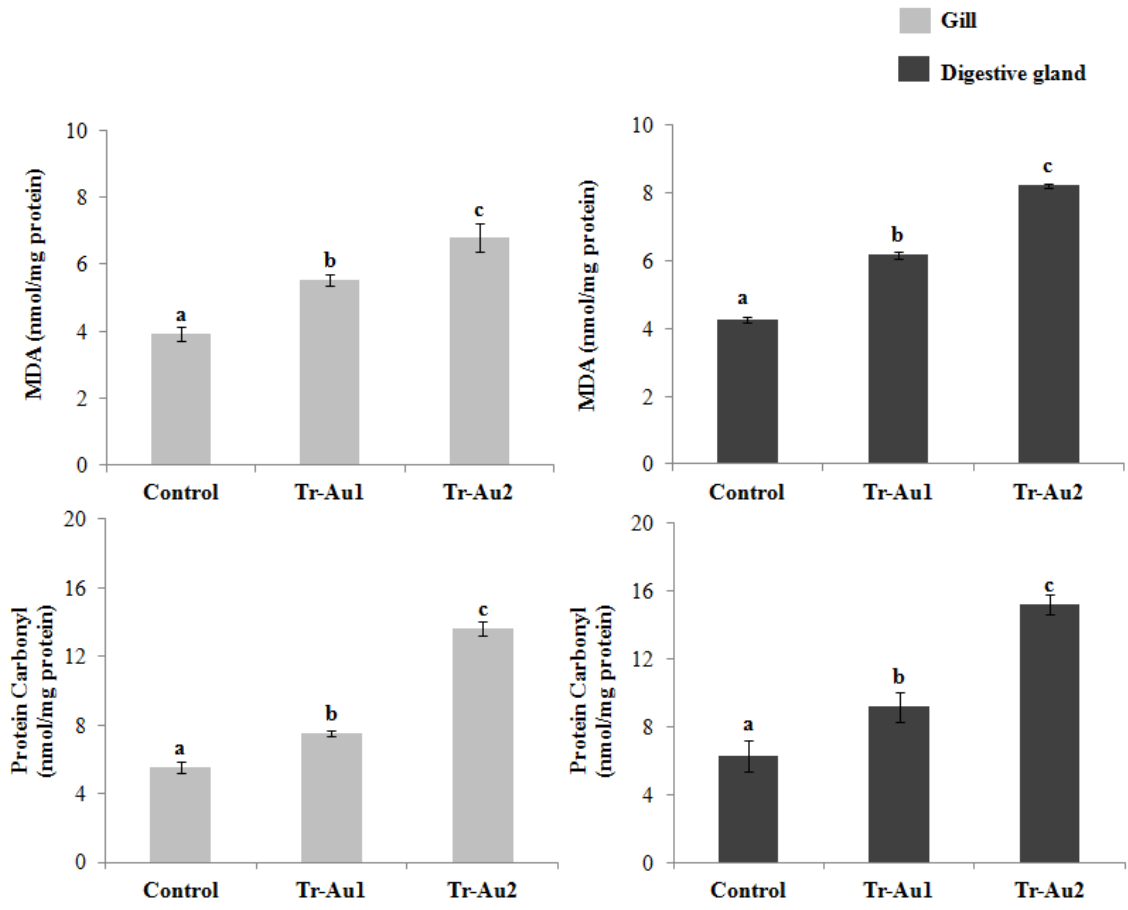
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 528 Fig. 4
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