1 # Exposure of juvenile turbot (*Scophthalmus maximus*) to silver

2 # nanoparticles and 17α-ethinylestradiol mixtures: Implications

3 # for contaminant uptake and plasma steroid hormone levels

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Abstract

Combined exposure to engineered nanoparticles (ENPs) and anthropogenic contaminants can lead to changes in bioavailability, uptake and thus effects of both these two groups of contaminants. In this study we investigated effects of single and combined exposures of silver (Ag) nanoparticles (AgNPs) and the synthetic hormone 17α-ethinylestradiol (EE2) on tissue uptake of both these contaminants in juvenile turbot (*Scophthalmus maximus*). Silver uptake and tissue distribution (gills, liver, kidney, stomach, muscle and bile) were analyzed following a 14-day, 2-h daily pulsed exposure to AgNPs (2 µg L^{-1} and 200 µg L^{-1}), Ag$^+$ (50 µg L^{-1}), EE2 (50 ng L^{-1}) and AgNP+EE2 (2 or 200 µg L^{-1}+50 ng L^{-1}).) Effects of the exposures on plasma vitellogenin Vtg levels, EE2 and steroid hormone concentrations were investigated. The AgNP and AgNP+EE2 exposures resulted in similar Ag concentrations in the tissues, indicating that combined exposure did not influence Ag uptake in tissues. The highest Ag concentrations were found in gills. For the. Ag$^+$ exposed fish, the highest Ag concentrations were measured in the liver. Our results show dissolution processes of AgNPs in seawater, indicating that the tissue concentrations of Ag may partly originate from ionic release. Plasma EE2 concentrations and Vtg induction were similar in fish exposed to the single contaminants and the mixed contaminants, indicating that the presence of AgNPs did not significantly alter EE2.uptake Similarly, concentrations of most steroid hormones were not significantly altered due to exposures to the combined contaminants versus the single compound exposures. However, high concentrations of AgNPs in combination with EE2 caused a drop of estrone (E1) and androstenedione (AN) levels in fish plasma below quantification limits. Our results indicate that the interactive effects between AgNPs and EE2 are limited, with only high concentrations of

41   AgNPs triggering some synergistic effects on plasma steroid hormone concentrations in juvenile

42   turbots.

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1. Introduction

Due to their antibacterial properties, silver (Ag) nanoparticles (AgNPs) are currently the most frequently used engineered nanoparticles (ENPs) in consumer products, particularly in textiles and health care items (Nanotechproject, 2009). Release of AgNPs from consumer products such as clothes, paints and washing machines have been documented and thus AgNPs will inevitably find their way into the environment (Kaegi et al., 2010; Benn and Westerhoff, 2008; Farkas et al., 2011). Silver is a known toxicant for aquatic organisms and AgNP toxicity has been demonstrated in algae, crustaceans and fish (Navarro et al., 2008; Farmen et al., 2012; Ribeiro et al., 2014). Despite intense research within the field of nanoecotoxicology, studies investigating AgNP effects in the marine environment are scarce.. While some recent studies have shown Ag uptake and toxic effects in marine invertebrates following AgNP exposure, information on AgNP bioavailability and effects on benthic marine fish is limited (Wang et al., 2014; Garcia-Alonso et al., 2014).

Engineered nanoparticles have been shown to co-occur with other anthropogenic contaminants of concern such as polycyclic aromatic hydrocarbons (PAHs), persistent organic pollutants (POPs) or synthetic hormones. ENP-contaminant interactions have already been demonstrated for contaminants such as PAHs, causing concerns on potential co-transport of adsorbed contaminants, and a resulting increase in contaminant toxicity ((Baun et al., 2008; Farkas et al., 2012: Tedesco et al., 2010; Hull et al., 2013). However, recent studies show that ENP-contaminant co-exposure can cause both amplified and alleviated effects (Canesi et al. 2015). TiO_2NPs were shown to increase uptake and toxic effects of arsenic and cadmium in the common carp (*Cyprinus carpio*) (Sun et al., 2007; Zhang et al., 2007). In marine mollusks,

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increased toxicity of benzo(a)pyrene (B(a)P) and tributyltin (TBT) was reported in the presence

of TiO$_2$NPs (Zhu et al., 2011; Farkas et al., 2015). However, both synergistic and antagonistic

effects were reported for TiO$_2$NP-tetrachlorodibenzodioxin (TCDD) co-exposures in

Mediterranean mussels (*Mytilus galloprovincialis*) *in vitro* and *in vivo*, depending on the

investigated endpoint (Canesi et al., 2014; Canesi et al. 2015

So far, most studies investigating combined effects of ENPs and other contaminants have used

TiO$_2$NPs and carbon based ENPs, while few data are available on combined effects of AgNP-

contaminant mixtures (Volker et al., 2014). Recently, a study on combined effects of 17α-

ethinylestradiol (EE2), a synthetic estrogen used in contraceptive pills, and AgNPs on the

freshwater mudsnail (*Potamopyrgus antipodarum*) showed that AgNPs reduced the stimulating

effects of EE2 on the estrogen receptor and on the gene expression of the egg yolk precursor

protein vitellogenin (Vtg) at environmentally relevant concentrations of EE2 (Volker et al.,

2014). Vitellogenin is only present in females, and the presence of Vtg in males is thus caused by

exposure to estrogenic compounds (Sumpter and Jobling, 1995).

Synthetic hormones are ubiquitous pollutants in the aquatic environment and EE2 is, along with

the natural estrogens 17β-estradiol (βE2) and estrone (E1), considered as the main estrogenic

compound in sewage treatment plant effluents (Routledge et al., 1998; Kolpin et al., 2002).

Endocrine disruption such as induction of Vtg production and decreased fertility in male fish are

documented effects of EE2 exposure (MacLatchy et al., 2003; Thorpe et al., 2003; Schultz et al.,

2003; Kidd et al., 2007). Moreover, EE2 induced changes in sex steroid hormone concentrations

have been described in different fish species. Schultz et al. (2003) observed reduced plasma

concentrations of 11-ketotestosterone (11-KT) in male rainbow trout (*Oncorhynchus mykiss*) following EE2 exposure. Similarly, MacLatchy et al. (2003) also observed decreased 11-KT levels in plasma from male mummichog (*Fundulus heroclitus*) (Schultz et al 2003; MacLatchy et al 2003). It has also been documented that exposure of male juvenile turbots (*Scophthalmus maximus*) to EE2, caused decreased plasma androgen levels (Labadie and Budzinski, 2006). Due to the importance of steroid hormones for reproduction and thus fitness, disturbances of steroid hormone levels by anthropogenic contaminants such as EE2 are considered a major threat to fish (Evans-Storms and Cidlowski., 1995).

In the present study we investigated uptake and endocrine effects in juvenile turbot, a marine benthic fish of commercial importance, following exposure to AgNPs and EE2, and to exposure of these two contaminants in combination (i.e. in a mixture). The fish were exposed for 14 days in a pulsed exposure mode with daily exposures of 2 h. To investigate the role of AgNP dissolution and ionic release of Ag^+ into the marine environment, Ag uptake and organ distribution after both AgNP and Ag^+ exposure were studied. The influence of AgNPs on EE2 uptake was studied by determining plasma EE2 concentrations and Vtg levels. In addition to a control, a group was also exposed to polyvinylpyrrolidone (PVP), which is the coating material of the used AgNPs. Effects of the single contaminants and contaminant mixtures on plasma steroid hormone homeostasis were determined by gas chromatography tandem mass spectrometry (GC-MS/MS). To our knowledge this is the first study investigating the single and combined effects of AgNPs and EE2 on a benthic marine fish species.

2. Material and Methods

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2.1 Nanoparticles

Polyvinylpyrrolidone coated AgNPs with a nominal diameter of approximately 15 nm were purchased from Particular GmbH (Hannover, Germany). PVP is a water-soluble polymer providing steric stabilization for ENPs. The particles were delivered as aqueous dispersions at a concentration of 100 mg L^{-1} (1 wt. % L^{-1} PVP). Nanoparticle shape and size was determined by transmission electron microscopy (TEM). The stock dispersion (100 µl) was applied on carbon coated copper grids (200 nm mesh), particle attachment was allowed for several minutes and the remaining liquid carefully removed to prevent drying artefacts. TEM images were recorded with a Zeiss Libra 120 EF TEM (Carl Zeiss AG, Oberkochen, Germany).

The hydrodynamic diameter of AgNPs in ultrapure water (Milli-Q, Merk Millipore, Darmstadt, Germany) was determined using dynamic light scattering (DLS) with a N5 submicron Particle Size Analyzer (Beckman Coulter Inc, Brea, USA). The samples were filtered through a 200 nm filter prior to DLS analysis. Nanoparticle aggregation and dissolution in seawater was analyzed by determining changes in surface plasmon resonance at a λ_{max} of 414 nm by UV-VIS spectrometry (Lambda 40 UV/VIS Spectrometer, Perkin Elmer, Waltham, USA). AgNP dispersions in seawater and ultrapure water (10 mg L^{-1}) were prepared and the surface plasmon resonance determined after 0, 1, 2, 3 and 20 h.

2.2 Ionic release

The release of Ag^+ from AgNPs dispersed in seawater was determined by both ultracentrifugation with subsequent inductively coupled plasma high resolution mass spectrometry (ICP-HR-MS) analysis, and by ion selective electrode (ISE) measurements.

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138   Dispersions of 200 μg L^{-1} AgNPs in seawater (*n*=3) were slowly agitated at 15°C for 2 h,

139   equaling the exposure duration. Subsequently, the suspensions were centrifuged for 1 h at 20,000

140   rpm in a Sorvall ultracentrifuge (Thermo Fisher Scientific, Waltham, USA). Supernatant samples

141   of 10 mL were collected, stabilized with 0.1 M HNO$_3$ and analyzed with ICP-HR-MS with a

142   Finnigan Element 2 instrument (Thermo Fisher Scientific, Waltham, USA) applying a SC-FAST

143   flow injection analysis system (ESI, Elemental Scientific, Inc. Omaha, USA).

144

145   For ISE analysis, 80 mL of AgNP dispersions were analyzed with an Ag ion selective electrode

146   (ELIT 8211 crystal membrane; Nico2000, London, UK) coupled with a potassium nitrate (ELIT

147   002) reference electrode in a dual electrode head (ELIT 201). A five point calibration curve was

148   prepared with AgNO$_3$ according to the manufacturer guidelines.

149

150   **2.3 Fish husbandry**

151   Fish husbandry and the exposure experiments were conducted according to the standards of the

152   Norwegian Animal Welfare Act and were approved by the Norwegian Animal Research

153   Authority. Juvenile turbot with an average length of 23 ± 0.7 cm and an average weight of 204 ±

154   12 g were purchased from Stolt Sea Farm Norway AS (Kvinesdal, Norway). The fish were kept

155   in 60 L polypropylene (PP) tanks (5 fish per tank). The tanks were set up as a flow-through

156   system with a water inflow of 340 ± 7 mL min^{-1}, resulting in a calculated turnover of the tank

157   volume in 3 h. Before entering the tanks, the seawater (salinity 33.5 ppt) was filtered through 5

158   and 1 μm Cuno filters (3 M, St. Paul, USA). The water temperature was 14 °C and exposure

159   tanks were aerated with pressurized air to ensure constant oxygen saturation. The exposure tanks

160   were covered with semi see-through lids and the light was dimmed to reduce stress for the fish.

161   The light:dark regime was set to 16:8 h. The fish were allowed to acclimate for 2 weeks before

162   the start of the experiment and were fed *ad libitum* with commercial fish food in the pre-

163   exposure period.

164

165   **2.4 Exposure**

166   To mimic a realistic environmental exposure scenario in a coastal area, the fish were exposed in

167   a pulsed exposure mode in which the fish were exposed every day for 2 h for 14 days. Exposure

168   groups are presented in Fig 1. Briefly, fish were exposed to two concentrations of AgNPs (low

169   concentration: LC-AgNP, 2 µg L^{-1}, high concentration: HC-AgNP, 200 µg L^{-1}), to Ag$^+$ (50 µg L$^-$

170   ^{1} Ag; prepared from AgNO$_3$), EE2 (50 ng L^{-1}), and to mixtures of AgNPs and EE2 (LC-

171   AgNP+EE2; HC-AgNP+EE2). A control group, receiving only water was included. Since the

172   AgNPs used in the present study were surface stabilized with PVP, a PVP treatment group

173   exposed to the maximum PVP concentrations expected in AgNP groups was also included.

174   Exposure solutions were prepared daily from stock solutions prior to exposure. For the combined

175   exposure groups, AgNPs and EE2 were mixed together shortly before each exposure start. Each

176   treatment was conducted in triplicates (3 tanks, n=15 fish per treatment), except for PVP which

177   was performed in duplicates (2 tanks, n=10 fish per treatment), resulting in a total number of

178   n=115 fish in the experiment (Fig 1). The water inflow was cut off temporarily during the 2 h

179   exposure duration and re-opened thereafter. Oxygen saturation and ammonia concentrations in

180   the water were monitored to ensure good water quality and avoid additional stress to the fish (Fig

181   S1, S2).

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183   **2.5 Exposure validation**

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184   In order to determine Ag concentrations in the exposure tanks during and after the treatments,

185   and to monitor potential Ag accumulation in the tanks during the experimental period of 14 d,

186   water samples for Ag quantification were taken at days 2, 6 and 10 of the experiment from

187   control and HC-AgNP exposure tanks. Ag^+ tanks were sampled once at day 10. Samples (10 mL)

188   were taken both at the start (h=0) and at the end (h=2) of the exposure, and further on 1 h, 2 h

189   and 22 h after re-starting the water flow (Fig 2). The samples were taken from the bottom of the

190   tanks, preserved in 0.1 M HNO_3 and subsequently analyzed with ICP-HR-MS (Thermo Fisher

191   Scientific, Waltham, USA). Similarly, EE2 concentrations in exposure tanks were analyzed at

192   day 2 and day 9 during the experiment. At each time point (start and end of the exposure and +1

193   h, +2 h and +24 h) 1L of water was sampled and immediately spiked with 50 µl of 0.4 ng $µL^{-1}$

194   d_4-ethinylestradiol (d_4-EE2) as internal standard. Water samples were extracted with 500 mg

195   solid phase Chromabond C18 cartridges (Macherey Nagel, Düren, Germany) (see supporting

196   information for more detail). The EE2 was eluted from the cartridges, derivatized and analyzed

197   by GC-MS/MS using a fully validated method described previously (Hansen et al., 2011). The

198   following SRM ion transitions were used: m/z 425.20 to m/z 231.00 (target) and m/z 193.00

199   (qualifier) for EE2 and m/z 425.20 to m/z 233.00 (target) and m/z 195.00 (qualifier) for d_4-EE2.

200

201   **2.6 Sampling**

202   After 14 d of exposure the fish were anaesthetized with tricaine methanesulfonate (MS-222) and

203   length and weight of each fish were measured. For determination of steroid hormones and Vtg in

204   plasma, blood samples were taken with heparinized syringes from the caudal vein. Thereafter

205   fish were killed by severing the spinal cord. Liver and gonads were weighed and samples of gills,

10

206 stomach, liver, kidney, brain and bile were taken to determine Ag tissue concentrations. Tissue

207 samples were stored at –20°C until further processing, plasma samples for Vtg analysis at –80°C.

208

209 **2.7 Silver concentration in tissues**

210 For each exposure group, tissues (gills, liver, kidney, stomach, muscle) from 7 fish were

211 analyzed for Ag concentration. In addition, bile samples were analyzed, but only from

212 individuals in the high and low AgNP and Ag^+ exposure groups (n=3). The tissue samples were

213 freeze-dried, and 50% v/v HNO_3 was added for microwave digestion in a high-pressure

214 microwave system (Milestone UltraClave, EMLS, Leutkirch, Germany). The digested samples

215 were analyzed with ICP-HR-MS (Thermo Fisher Scientific, Waltham, USA). Detection limits

216 (LOD) for Ag tissue concentrations were derived either from the IDL-25% taking sample weight

217 into consideration, or were calculated based on the 3x standard deviation of blank samples. The

218 more conservative value was used as LOD for each sample. The IDL-25% was calculated from

219 the subsequent analysis of solutions, containing decreasing, low concentrations of the element.

220 Finally, the concentration resulting in a relative standard deviation (RSD) of approximately 25%

221 (n=3 scans) were selected as IDL with baseline corrections applied for these values. All Ag

222 concentrations in the tissues are presented as dry weight (dw) concentrations.

223

224 **2.8 Vitellogenin analysis**

225 Vitellogenin concentrations in turbot plasma samples were determined quantitatively using an

226 indirect competitive enzyme-linked immuno-sorbent assay (ELISA).Briefly, 1:1000 diluted CS-2

227 polyclonal rabbit anti-turbot Vtg (Biosense, Bergen, Norway) was used as primary antibody and

228 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit as secondary antibody (Sigma-

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229 Aldrich, Steinheim, Germany) to determine Vtg concentration in plasma samples diluted 1:500

230 in triplicate on 96-well microtiter plates pre-coated with purified turbot Vtg (100 ng/ml).

231 Concentrations of Vtg were determined colorimetrically at 405 nm using an Ultra Microplate

232 Reader (ELX 808 IU model, Biotek Instruments Inc., Winooski, VT, USA) 30 min after adding

233 ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid;) Fluka, Buchs, Switzerland).

234 Purified Vtg used for standards and microtiter plate coating was isolated and prepared from the

235 plasma of turbot repeatedly injected with EE2 according to Silversand et al. (1993).

236

237 **2.9 Steroid hormone analysis**

238 To determine steroid hormone concentrations, blood samples were spiked with a mixture of

239 deuterated standards (50 µl of a 0.4 ng/µl in methanol) containing the following deuterated

240 analogues: d_7-androstendione (dAN), d_4-estrone (dE1), d_5-17β-estradiol (dE2), d_4-pregnenolone,

241 d_9-progesterone, (dPRO), d_3-testosterone (dT) and d_3-dihydrotestosterone (dDHT). dAN, dE1

242 and dE2 were purchased from CDN isotopes (Pointe-Claire, QC, Canada) and dPRE, dPRO, dT

243 and dDHT were purchased from Toronto Research Chemicals (North York, ON, Canada). All

244 deuterated steroid analogues were above 98% purity.

245 Concentrations of androstenedione (AN), dehydroepiandrosterone (DHEA), dihydrotestosterone

246 (DHT), estrone (E1), 17β-estradiol (βE2), pregnenolone (PRE), progesterone (PRO), 17-

247 hydroxypregnenolone (OH-PRE), 17-hydroxyprogesterone (OH-PRO) and testosterone (T) were

248 determined with GC-MS/MS using a Bruker Scion™ gas chromatograph (GC) coupled to a

249 SCION TQ GC triple quadrupole tandem mass spectrometry (MS/MS) system (Bruker Daltonik,

250 Bremen, Germany) (see supporting information for more detail).

251 A detailed description of the method used for steroid analysis, including quality criteria, is

252 described in Poulsen et al. (2015) and Nossen et al. (2016). Limits of detection (LOD) and limits

253 of quantification (LOQ) were determined as recommended by the ICH guideline (2005) equation

254 1 and 2. σ is the standard deviation of the area ratio obtained from the least concentrated mixture

255 of each analyte above LOQ. S is the slope of the calibration curve for each analyte.

256 $$LOD = 3.3 \times \frac{\sigma}{S} \qquad (1)$$

257 $$LOQ = 10 \times \frac{\sigma}{S} \qquad (2)$$

258 Method limit of detection (LOD) was steroid hormone specific and ranged between 0.006 and

259 0.28 ng mL^{-1} plasma, and internal standard absolute recoveries were between 69 and 94% across

260 plasma samples.

261

262 **2.10 Data analysis**

263 Statistical analyses were performed with Statistica 12 (StatSoft, Tulsa, USA). Data were tested

264 for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test, $p<0.05$) and were log

265 transformed where required. Differences between treatment groups were evaluated by ANOVA

266 and subsequent post hoc analysis (Tukey test, $p<0.05$). Data sets that did not fulfill the criteria

267 for ANOVA after transformation were analyzed by a nonparametric test (Kruskal-Wallis one

268 way analysis on ranks, $p<0.05$). For statistical analysis values below LOD (tissue Ag, steroid

269 hormones) and LOQ (steroid hormones) were replaced by half the LOD or LOQ, respectively.

270 Groups with 50% or more of the samples featuring values below the LOD/LOQ were excluded

13

271 from statistical analysis. Graphs were prepared with SigmaPlot 12.0 (Systat Software Inc.,

272 Chicago, USA) and Adobe Illustrator CS5 (Adobe Systems, San Jose, USA).

273

3. Results and Discussion

3.1 Exposure validation

276 To mimic an environmentally relevant exposure scenario in a coastal area affected by river

277 runoff and tidal waters, turbot were subjected daily to a 2 h exposure to AgNPs, Ag^+, EE2, PVP

278 and mixtures of AgNP+EE2 followed by a depuration period. At the start of the static exposure,

279 the measured exposure Ag concentrations (i.e. in the water) were 131 ± 10 µg L^{-1} and 33 µg L^{-1},

280 for the HC-AgNP and Ag^+ groups, respectively (Fig 2 a). This was approximately 66 % lower

281 than the nominal exposure concentrations. During the 2 h static exposure the Ag water

282 concentrations in the exposure tanks dropped to 95% of the starting concentration in both groups,

283 likely due to Ag adhesion to tank walls (Fig 2 a,b). After re-installing the water flow,

284 concentrations decreased further to about half of the starting concentrations within 2 hours, in

285 both the AgNP and Ag^+ exposure tanks. At the start of the static exposure, the measured water

286 concentrations of EE2 were on average 10 ng L^{-1}, which is approximately 20% of the nominal

287 concentration, and it decreased to approximately 90% of the starting concentration during the 2 h

288 static exposure (Fig.1a). Following this period, the pattern of the decrease in the EE2

289 concentrations in the exposure water followed the same concentration decrease pattern as Ag

290 (Fig 2 a,b). Thus, 24 h after the start of the exposure, exposure (or water?) concentrations were <

291 1 µg L^{-1} (AgNP) or 1 ng L^{-1} (EE2). The concentrations in the control tank were always below the

292 LOD (Fig 2 a,b). Although exposures were repeated daily, no increases of the contaminant levels

293 were observed in the exposure tanks during the 14 d of exposure, indicating that the

14

294 contaminants were removed during depuration phases. Oxygen saturation and ammonium

295 concentrations during exposure are shown in the supporting information (Fig S1, S2).

296

297 **3.2 Nanoparticle characteristics and behavior in seawater**

298 TEM-imaging showed that the PVP-coated AgNPs had a spherical shape and an average size of

299 13 nm (Fig 3a). The average hydrodynamic diameters (DLS; nr. based; 10 mg L^{-1}) were 69±0.4

300 nm in MilliQ water and 69.6±5 nm in seawater at the start of the exposure (time point 0).. The

301 AgNPs remained stable during 2 h in MilliQ water (65±5 nm), while the hydrodynamic diameter

302 of AgNPs in seawater increased slightly over time to 76.6±0.6 nm after 1 h and to 83±2.2 nm

303 after 2 h. In ultrapure water the surface plasmon resonance at λ_{max} (414 nm) decreased to 95%

304 within the first 2 h, and to approximately 70% after 20 h (data not shown). In seawater, the

305 surface plasmon resonance was reduced to around 68% after 2 h and further decreased to

306 approximately 15% after 20 h (Fig 3c). No spectral shift of λ_{max} was detected (Fig 3c). The ionic

307 (Ag+?) release from AgNPs after 2 h in seawater within 2 h was about 15%, as determined by

308 removing the AgNPs using ultracentrifugation, and between 20% and 25% according to ISE

309 measurements. Our results show that despite their PVP coating, AgNPs underwent aggregation

310 and dissolution processes and ionic release in seawater, which occurred, however, relatively

311 slow.

312

313 Ionic release from AgNPs in different exposure media and the role in AgNP toxicity have been

314 extensively discussed (Yang et al., 2012; Behra et al., 2013; Sharma et al., 2014). ENPs coated

315 with PVP, a molecule providing steric surface stabilization, are reported to be more stable in

316 high ionic strength seawater compared to non-stabilized or charge stabilized ENPs (Christian et

15

317 al., 2008; Huynh and Chen, 2011; Levard et al., 2012). However, findings on AgNP-PVP

318 stability differ between studies, and reported dissolution rates vary from 3-50% depending on

319 particle size and physicochemical characteristics of the media (Behra et al., 2013; Sharma et al.,

320 2014; Angel et al., 2013; Misra et al., 2012).

321

322 **3.3 Distribution of AgNPs and Ag^+ in tissues**

323 In AgNP and Ag^+ exposed fish, Ag was detectable in most tissues despite the relatively short

324 daily exposure duration (2 h), each followed by a depuration period that occurred within the

325 static exposure time, and after the exposure was ended (Fig 4, Tab S1). In the AgNP exposed

326 groups Ag tissue concentrations were slightly, however not significantly, affected by the

327 presence of EE2 in the exposure water. In the fish exposed to HC-AgNP and HC-AgNP+EE2,

328 respectively, Ag concentrations were highest in gills (1250±748 ng g^{-1}; 733±378 ng g^{-1}) > liver

329 (390±346 ng g^{-1}; 453±300 ng g^{-1}) > bile (374±442 ng g^{-1}; n.a) > kidney (228±216 ng g^{-1}; 75±42

330 ng g^{-1}) > stomach (114±42 ng g^{-1}; 87±34 ng g^{-1}) > muscle (12±5 ng g^{-1}; 9±7 ng g^{-1}) (Fig 4; Tab

331 S1). Both the high AgNP exposure, and the combined high AgNP+EE2 exposure resulted in

332 significantly higher (HC-AgNP, p=0.00017; HC-AgNP+EE2, p=0.00084) Ag concentrations in

333 gills compared to the group exposed to Ag^+ (Fig 4).In contrast, Ag accumulation was higher in

334 the stomach of the Ag^+-exposed group as compared to the HC-AgNP+EE2 (p=0.033) treatment

335 group. In liver, Ag concentrations were slightly, however insignificantly higher in fish exposed

336 to HC-AgNP and HC-AgNP+EE2 as compared to the Ag^+-exposed group. No differences

337 between these groups (or all groups?) were detected in kidney, bile and muscle (Tab S1). In

338 kidney and muscle, Ag was below the LOD in both the low- concentration AgNP exposure

339 groups (LC-AgNP, LC-AgNP+EE2; Tab S1), and in most of the brain samples Ag

340 concentrations were marginally below the LOD (data not shown). This can be at least partly

341 attributed to the low sample weight of brain tissues and the conservative detection limits applied.

342 However, an uptake of Ag in brain following prolonged or continuous exposure, as reported in

343 previous studies, seems possible (Kwok et al., 2012; Jang et al., 2014). Similar concentrations of

344 Ag in bile and liver in the AgNP and Ag^+ exposure groups indicate that Ag was at least partially

345 excreted via the intestinal tract. Excretion of Ag via bile after Ag^+ exposure has previously been

346 documented in marine fish (Wood et al., 2010).

347

348 In previous studies, both liver and gills have been described as the predominant organs for AgNP

349 accumulation (Kwok et al., 2012; Jang et al., 2014; Scown et al., 2010). However, Ag uptake and

350 tissue distribution may depend on AgNP speciation. In this study, the high Ag concentrations in

351 gills of AgNP exposed fish indicate that AgNPs are mostly attached to gill surfaces or trapped in

352 gill mucus. Gill tissues were sampled after a depuration phase of 22 h, which indicates that the

353 AgNPs were not removed from gill surfaces within that timeframe, suggesting that effects of

354 AgNPs on gill function should be investigated in marine fish. AgNP-gill associations were

355 previously reported in zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*) (Kwok et

356 al., 2012; Griffitt et al., 2009). The similar Ag accumulation in internal organs in the high AgNP

357 and the Ag^+ groups, despite the 75% lower Ag exposure concentration suggest that internal Ag

358 uptake derives from ionic release, which was approximately 15-25% in our study. Previous

359 studies showed that dissolved Ag readily forms complexes with Cl resulting in different $AgCl_n$

360 species, mostly accumulating in livers and not in the gills in two flatfish species, the English sole

361 (*Parophrys vetulus*) and the starry flounder (*Platichthys stellatus*) (Ferguson and Hogstrand,

362 1998; Webb and Wood, 2000; Hogstrand et al., 2002).

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363

3.4 Plasma EE2 and vitellogenin concentrations

In control fish, AgNP, Ag^+ and PVP exposed fish, EE2 concentrations in plasma were below the limit of detection (0.003 ng g^{-1}; Fig 5a). In fish exposed to EE2, plasma EE2 concentrations were between 0.2 ng g^{-1} and 0.5 ng g^{-1}, and did not differ between males and females. Labadie and Budzinski (2006) reported similar EE2 plasma concentrations of about 1 ng g^{-1} in both sexes of juvenile turbot following exposure to 3.5 ng L^{-1} EE2 for 14 days. The slightly lower concentrations in the present study can be explained by the pulsed exposure regime, which was followed by a daily depuration phase. Average plasma EE2 levels were similar in HC-AgNP+EE2 (0.31 ng g^{-1}) compared to LC-AgNP+EE2 (0.26 ng g^{-1}) and EE2 exposed fish (0.27 ng g^{-1}) (Fig 5a).

Induction of the egg yolk precursor protein Vtg is often used as a biomarker of exposure to estrogenic substances such as EE2 in oviparous organisms such as teleosts (Heppell et al., 1995). In the present study induction of Vtg, determined as plasma Vtg levels, was similar in male and female fish. Concentrations of Vtg were significantly higher in the HC-AgNP+EE2, LC-AgNP+EE2 and EE2 exposure groups compared to the control and PVP exposed groups (Fig 5b). In contrast to plasma EE2 concentrations, Vtg concentration was not significantly higher in fish exposed to only EE2 compared to Vtg concentrations in fish exposed to the AgNP+EE2 mixtures (Fig 5b).

These results indicate that the AgNP concentrations employed in the present study did not significantly alter EE2 uptake and accumulation in juvenile turbot suggesting that the AgNPs used herein did not bind or otherwise interact with EE2 under our experimental conditions

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(salinity, pH, temperature, exposure duration). A previous study investigating interactions between two metal ENPs and the PAH phenanthrene (PHE), showed that while gold ENPs had a strong binding capacity for PHE, neither citrate-, nor PVP-coated AgNPs interacted with PHE (Farkas et al., 2012). Thus, this suggests that organic compounds such as EE2 and PHE are not associated with PVP coated AgNPs, at least not in a manner that makes them more available for uptake in fish and possibly also in other aquatic organisms.

3.5 Effects on plasma steroid hormone levels

Concentrations of the steroid hormones AN, DHEA, DHT, E1, βE2, PRE, PRO, OH-PRE, OH-PRO and T were determined in plasma of control and exposed fish. In control fish, AN levels were on average 0.13 (LOQ-0.20) ng g^{-1} in males and 0.07 (LOQ-0.16) ng g^{-1} in females, and were thus approximately two times higher in males than in females (Fig 6). In controls, T was only detectable in male fish at an average concentration of 0.12 (LOD-0.28) ng g^{-1} (Tab S2). Previously reported androgen levels (AN, T) in juvenile turbot were slightly higher than in the present study but in a comparable range (Labadie and Budzinski, 2006). DHEA was approximately 2.5 times higher in females compared to males, while E1, PRE and PRO were only slightly higher in female control fish than in the males (Tab S2, S3). Concentrations of DHT, βE2, OH-PRE and OH-PRO were below the LOQ in more than 90% of all fish (data not shown).

In the PVP exposed turbots, the plasma AN concentrations in male and female fish were notably, although insignificant 2 times higher than in the control fish (Fig 6b). In addition, average T concentrations were 4.5 times higher in PVP exposed male fish (0.64 ng g^{-1}) compared

19

to controls (0.14 ng g^{-1}), although this increase was only marginally significant (p=0.0587) due to a single outlier in the PVP group. Furthermore, TS levels were even higher in PVP exposed females (1.44 ng g^{-1}) (Tab S2, S3). This indicates an androgenic potential of PVP, which should be further investigated as PVP is a common coating material for ENPs and is a common component in water additives used in research, tropical fish industries and aquaculture (Harnish et al., 2011).

In female fish, the group exposed to EE2 had significantly lower DHEA concentrations compared to controls (p=0.031) and PVP exposed fish (p=0.037) (Fig 6 a). Following EE2 exposure, AN was slightly lower in male fish than in the control controls (Fig 6 b), although no difference in plasma T was detected compared to the control fish. This is in agreement with previous reports of unaltered free T plasma levels following EE2 exposure in male juvenile turbot, but a decrease in the androgens AN and 11-KT (Labadie and Budzinski, 2006).

Even though the results of the present study show no significant impact of AgNPs on EE2 uptake (Fig 5), combined exposures to EE2 and HC-AgNP caused deviation from homeostasis in two plasma steroid hormones. Similarly to females exposed only to EE2, DHEA concentrations in females exposed to the HC-AgNP+EE2 mixture were significantly lower than in the control females (p=0.007) and the PVP exposed females (p=0.01) (Fig 6a). In addition, in fish exposed to HC-AgNP+EE2, AN concentrations were < LOQ in 67% of the male fish and 100% of the female fish (Fig 6b). While no significant effect on E1 concentrations was observed in EE2-only exposed fish, levels of E1 were < LOQ in HC-AgNP+EE2 exposed females, while there were no effects in males (Tab S2, S3). PRO and PRE plasma concentrations were not significantly

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432 affected by any of the exposures. This suggests that the cytochrome P_{450} 17 (CYP17) responsible

433 for the transformation of the progestagens PRE and PRO into the androgens DHEA and AN may

434 be a potential target for the effects of AgNP+EE2 mixtures...Effects of Ag and AgNPs on

435 different enzymes of the steroidogenic CYP family are not well studied, but Garcia et al. (2014)

436 observed increased transcription of CYP11A1 and 3β-HSD (transforming Δ-5 steroids into Δ-4

437 steroids in the steroidogenesis) mRNA in male rats exposed to AgNPs. This aspect needs further

438 investigation.

439

440 In this study we found no significant effects of exposure to combined mixtures of AgNPs and

441 EE2 on contaminant uptake and accumulation of either of the two contaminants,, and only slight

442 changes in steroid hormone levels deriving from the AgNP and -EE2 co-exposures. Differences

443 in ENP-contaminant mixed toxicity will strongly depend on the ENP-contaminant interactions

444 deriving from the properties of ENPs and contaminants. As AgNPs are amongst the most

445 frequently used ENPs, further research on interactive effects with different contaminant groups is

446 needed. Furthermore, the role of Ag^+ deriving from Ag dissolution in AgNP-contaminant

447 mixtures should be investigated in more detail.

448

452

453 **Supporting information**

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454   More detailed information on the methods used for EE2 extraction from water and steroid

455   hormone analysis are given in the supporting information. Results reporting oxygen and

456   ammonia concentrations in water samples, tissue Ag and plasma steroid hormone concentrations

457   are shown in figure FigS1, FigS2 and tables TabS1-S3.

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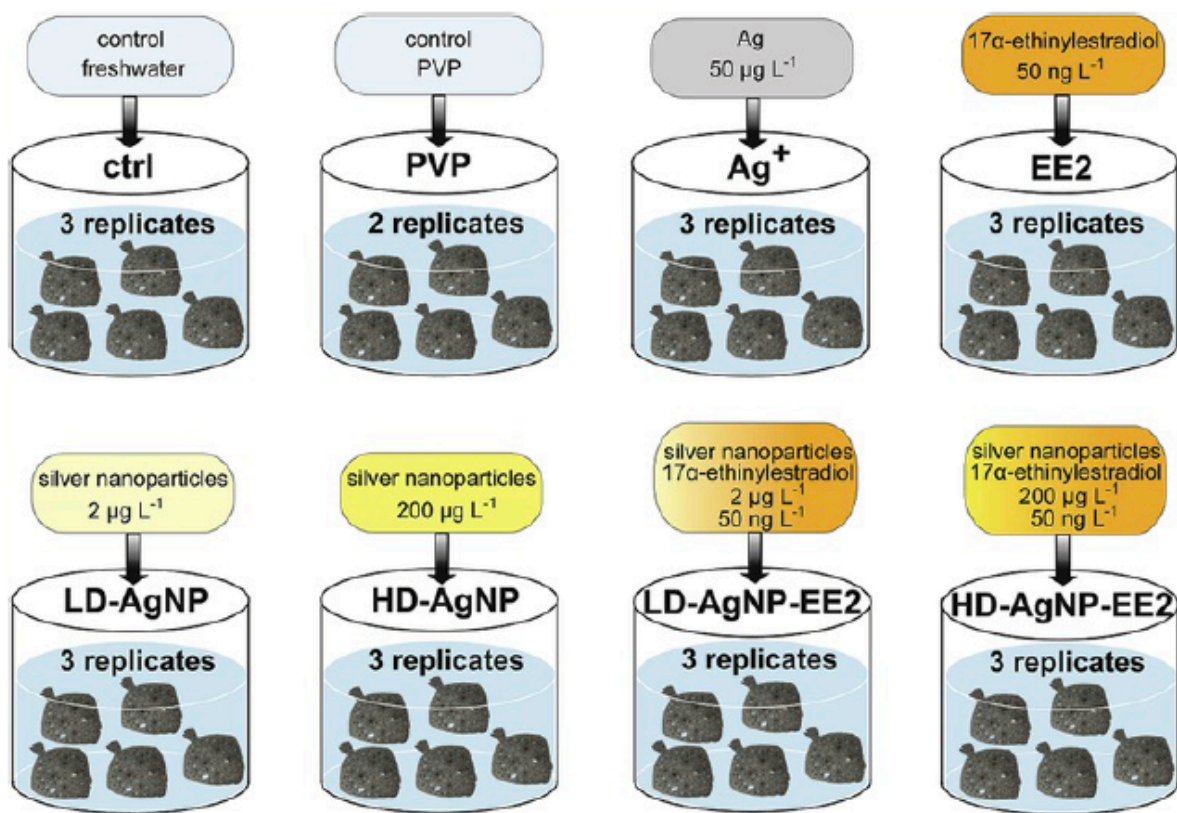
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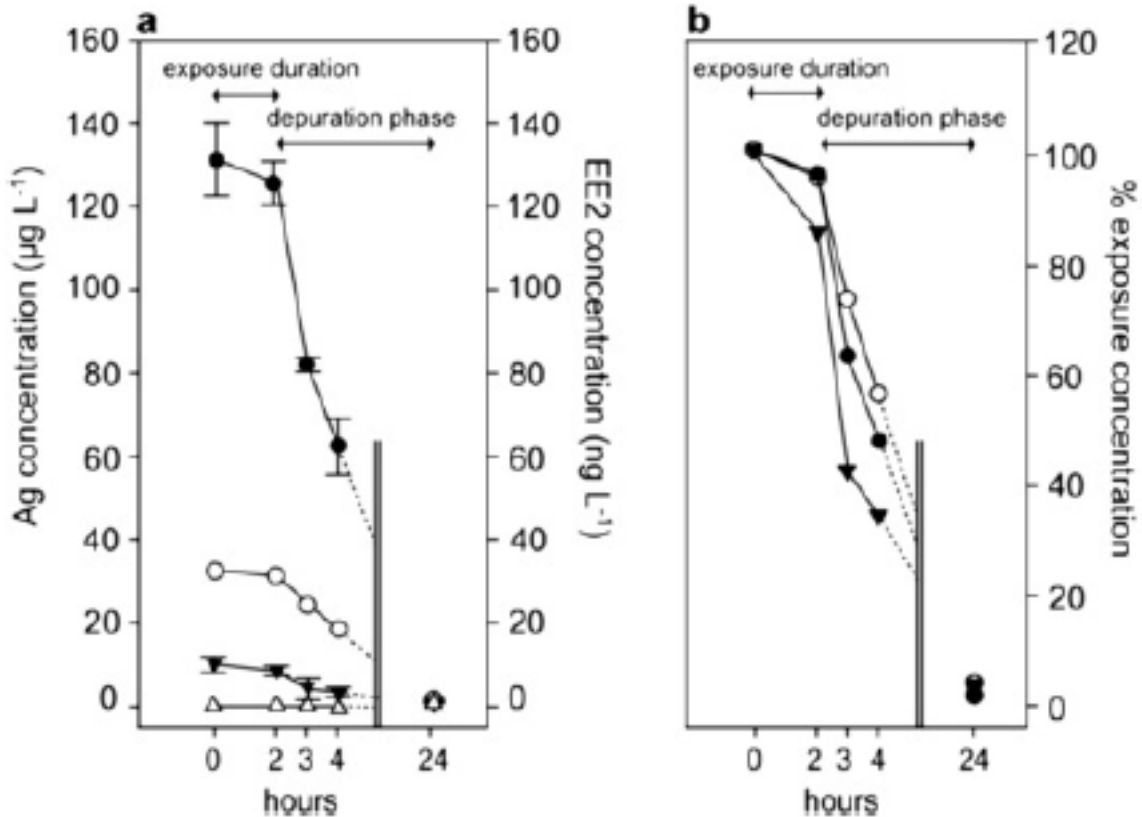
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666 **Figures**

667

668 Figure 1: Scheme of the exposure setup showing the different exposure groups and respective

669 exposure concentrations. Each exposure group, except for the PVP control group (replicates),

670 consisted of 3 replicate tanks with 5 fish in each tank, resulting in n=15.

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672
673 Figure 2: Absolute (a) and relative (b) contaminant concentrations of HC-AgNP (closed circles;

674 µg L^{-1}), Ag^{+} (open circles; µg L^{-1}), controls (open triangles; µg L^{-1}) and EE2 (closed triangles; ng

675 L^{-1}) during the daily exposures. The samples were analyzed at the exposure start (0 h), end (2 h),

676 and after 1 h (i.e. 3 h after exposure start), 2 h (4 h after exposure start) and 22 h (24 h after

677 exposure start) of depuration. Dotted lines show the approximated concentration decline.

678 Vertical lines indicate x-axis breaks from 5.5 h to 22 h.

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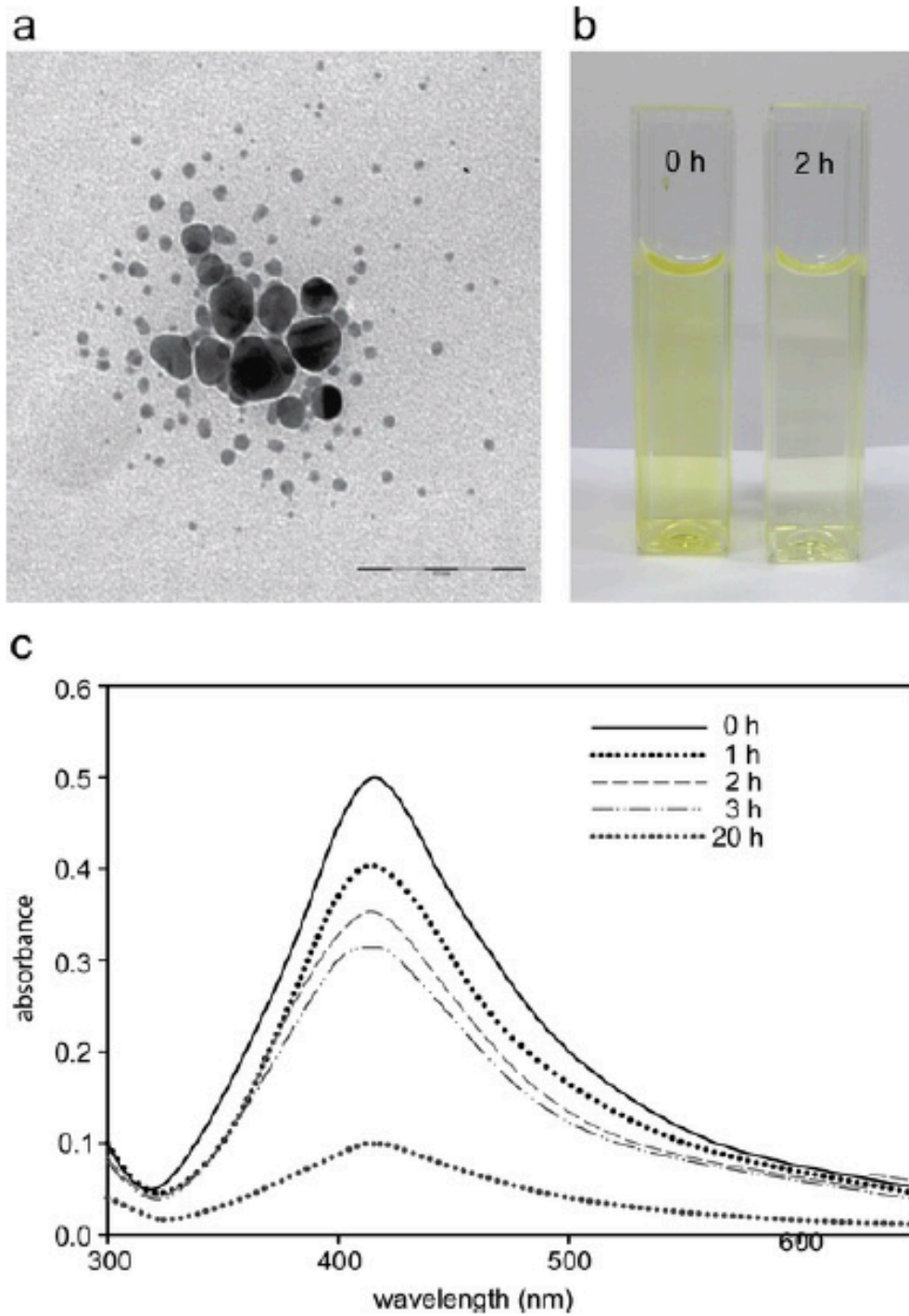
Figure 3: a) representative TEM image showing AgNPs; scale bar: 100 nm; b) AgNP solutions in

seawater after 0 and 2 h; c) changes of surface plasmon resonance (414 nm) of AgNPs in

seawater over time.

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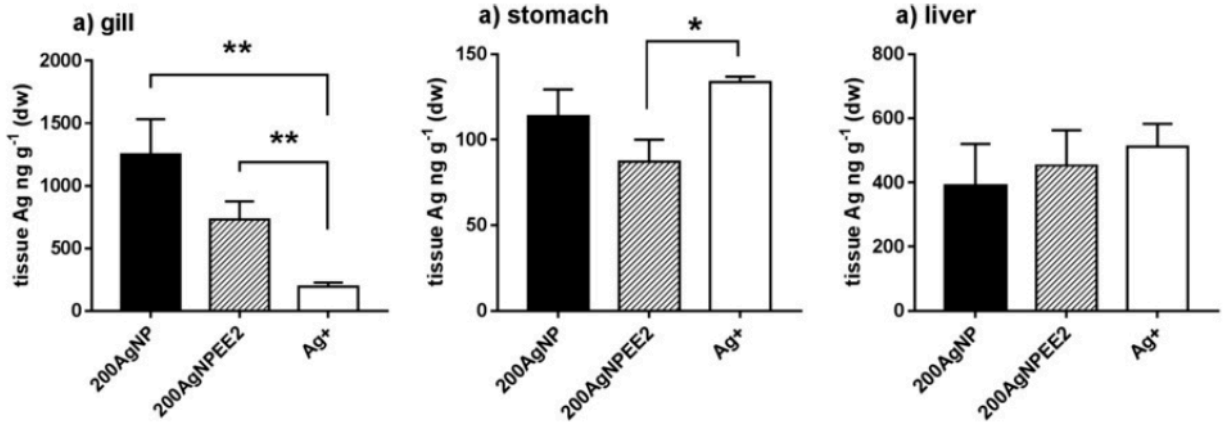
685
686 Figure 4: Ag tissue concentrations in HC-AgNP, HC-AgNP+EE2 and Ag$^+$ exposed juvenile

687 turbot. . Data are shown as mean±SEM.   Stars denote significant differences in Ag

688 concentrations (p<0.05).

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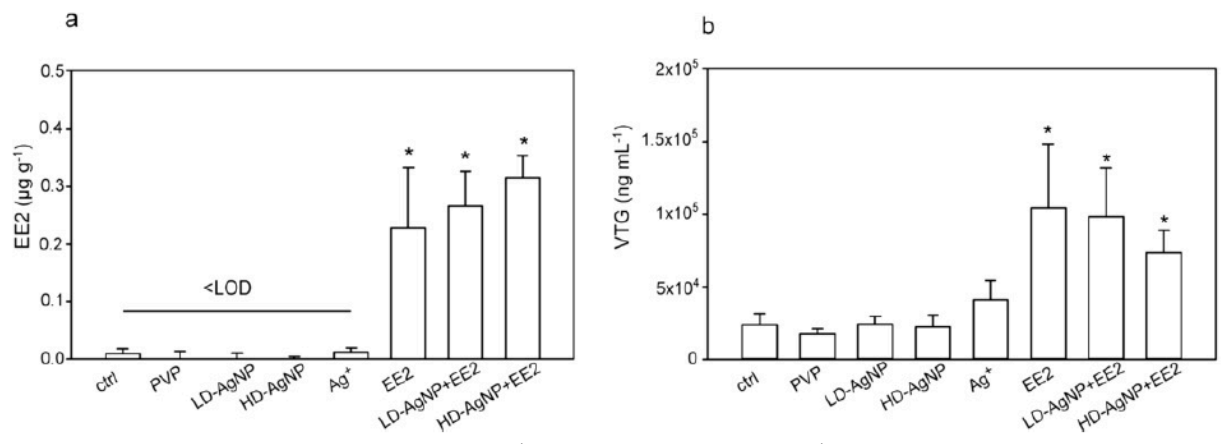
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691 Figure 5: Concentrations of EE2 (ng g^{-1}) a) and Vtg (ng mL^{-1}) b) in plasma of exposed and

692 unexposed juvenile turbot (data from male and female fish pooled). Data are presented as

693 median±75th percentile. Stars denote significant differences from control (p<0.05).

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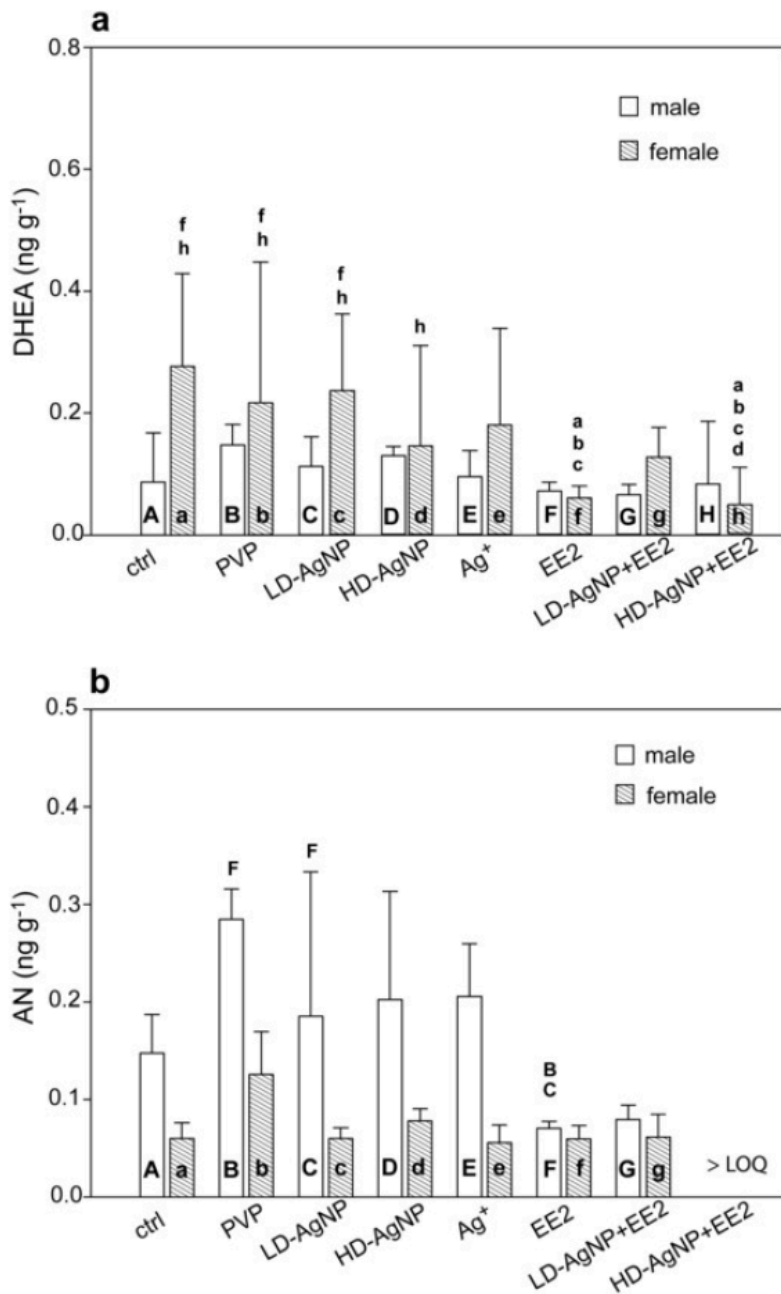
Figure 6: Effects of AgNPs, Ag$^+$, EE2 and AgNP+EE2 co-exposures on dehydroepiandrosterone

(DHEA) (a) and androstenedione (AN) (b) concentrations in plasma of male (white bars) and

female (striped bars) juvenile turbot. Data are presented as median±75th percentile. Letters

above bars show significant differences between treatment groups (p<0.05). Capital letters are

used for male fish, whilst small letters are used for female fish.