Exposure of juvenile turbot (*Scophthalmus maximus*) to silver nanoparticles and 17α-ethinylestradiol mixtures: Implications 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
AgNPs triggering some synergistic effects on plasma steroid hormone concentrations in juvenile turbots.

**Keywords:** Androgens, Estradiol, Silver nanoparticles, Mixed exposure, Vitellogenin, Testosterone
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$_2$NPs 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,
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$_2$NPs-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 heteroclitus*) (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
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 \(\lambda_{\text{max}}\) 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.
Dispersions of 200 µg L\(^{-1}\) AgNPs in seawater \((n=3)\) were slowly agitated at 15°C for 2 h, equaling the exposure duration. Subsequently, the suspensions were centrifuged for 1 h at 20,000 rpm in a Sorvall ultracentrifuge (Thermo Fisher Scientific, Waltham, USA). Supernatant samples of 10 mL were collected, stabilized with 0.1 M HNO\(_3\) and analyzed with ICP-HR-MS with a Finnigan Element 2 instrument (Thermo Fisher Scientific, Waltham, USA) applying a SC-FAST flow injection analysis system (ESI, Elemental Scientific, Inc. Omaha, USA).

For ISE analysis, 80 mL of AgNP dispersions were analyzed with an Ag ion selective electrode (ELIT 8211 crystal membrane; Nico2000, London, UK) coupled with a potassium nitrate (ELIT 002) reference electrode in a dual electrode head (ELIT 201). A five point calibration curve was prepared with AgNO\(_3\) according to the manufacturer guidelines.

2.3 Fish husbandry

Fish husbandry and the exposure experiments were conducted according to the standards of the Norwegian Animal Welfare Act and were approved by the Norwegian Animal Research Authority. Juvenile turbot with an average length of 23 ± 0.7 cm and an average weight of 204 ± 12 g were purchased from Stolt Sea Farm Norway AS (Kvinesdal, Norway). The fish were kept in 60 L polypropylene (PP) tanks (5 fish per tank). The tanks were set up as a flow-through system with a water inflow of 340 ± 7 mL min\(^{-1}\), resulting in a calculated turnover of the tank volume in 3 h. Before entering the tanks, the seawater (salinity 33.5 ppt) was filtered through 5 and 1 µm Cuno filters (3 M, St. Paul, USA). The water temperature was 14 °C and exposure tanks were aerated with pressurized air to ensure constant oxygen saturation. The exposure tanks were covered with semi see-through lids and the light was dimmed to reduce stress for the fish.
The light:dark regime was set to 16:8 h. The fish were allowed to acclimate for 2 weeks before the start of the experiment and were fed *ad libitum* with commercial fish food in the pre-exposure period.

### 2.4 Exposure

To mimic a realistic environmental exposure scenario in a coastal area, the fish were exposed in a pulsed exposure mode in which the fish were exposed every day for 2 h for 14 days. Exposure groups are presented in Fig 1. Briefly, fish were exposed to two concentrations of AgNPs (low concentration: LC-AgNP, 2 µg L⁻¹, high concentration: HC-AgNP, 200 µg L⁻¹), to Ag⁺ (50 µg L⁻¹ Ag; prepared from AgNO₃), EE2 (50 ng L⁻¹), and to mixtures of AgNPs and EE2 (LC-AgNP+EE2; HC-AgNP+EE2). A control group, receiving only water was included. Since the AgNPs used in the present study were surface stabilized with PVP, a PVP treatment group exposed to the maximum PVP concentrations expected in AgNP groups was also included. Exposure solutions were prepared daily from stock solutions prior to exposure. For the combined exposure groups, AgNPs and EE2 were mixed together shortly before each exposure start. Each treatment was conducted in triplicates (3 tanks, *n*=15 fish per treatment), except for PVP which was performed in duplicates (2 tanks, *n*=10 fish per treatment), resulting in a total number of *n*=115 fish in the experiment (Fig 1). The water inflow was cut off temporarily during the 2 h exposure duration and re-opened thereafter. Oxygen saturation and ammonia concentrations in the water were monitored to ensure good water quality and avoid additional stress to the fish (Fig S1, S2).

### 2.5 Exposure validation
In order to determine Ag concentrations in the exposure tanks during and after the treatments, and to monitor potential Ag accumulation in the tanks during the experimental period of 14 d, water samples for Ag quantification were taken at days 2, 6 and 10 of the experiment from control and HC-AgNP exposure tanks. Ag\(^+\) tanks were sampled once at day 10. Samples (10 mL) were taken both at the start (h=0) and at the end (h=2) of the exposure, and further on 1 h, 2 h and 22 h after re-starting the water flow (Fig 2). The samples were taken from the bottom of the tanks, preserved in 0.1 M HNO\(_3\) and subsequently analyzed with ICP-HR-MS (Thermo Fisher Scientific, Waltham, USA). Similarly, EE2 concentrations in exposure tanks were analyzed at day 2 and day 9 during the experiment. At each time point (start and end of the exposure and +1 h, +2 h and +24 h) 1L of water was sampled and immediately spiked with 50 µl of 0.4 ng µL\(^{-1}\) d\(_4\)-ethinylestradiol (d\(_4\)-EE2) as internal standard. Water samples were extracted with 500 mg solid phase Chromabond C18 cartridges (Macherey Nagel, Düren, Germany) (see supporting information for more detail). The EE2 was eluted from the cartridges, derivatized and analyzed by GC-MS/MS using a fully validated method described previously (Hansen et al., 2011). The following SRM ion transitions were used: m/z 425.20 to m/z 231.00 (target) and m/z 193.00 (qualifier) for EE2 and m/z 425.20 to m/z 233.00 (target) and m/z 195.00 (qualifier) for d\(_4\)-EE2.

2.6 Sampling

After 14 d of exposure the fish were anaesthetized with tricaine methanesulfonate (MS-222) and length and weight of each fish were measured. For determination of steroid hormones and Vtg in plasma, blood samples were taken with heparinized syringes from the caudal vein. Thereafter fish were killed by severing the spinal cord. Liver and gonads were weighed and samples of gills,
stomach, liver, kidney, brain and bile were taken to determine Ag tissue concentrations. Tissue samples were stored at –20°C until further processing, plasma samples for Vtg analysis at –80°C.

2.7 Silver concentration in tissues

For each exposure group, tissues (gills, liver, kidney, stomach, muscle) from 7 fish were analyzed for Ag concentration. In addition, bile samples were analyzed, but only from individuals in the high and low AgNP and Ag⁺ exposure groups (n=3). The tissue samples were freeze-dried, and 50% v/v HNO₃ was added for microwave digestion in a high-pressure microwave system (Milestone UltraClave, EMLS, Leutkirch, Germany). The digested samples were analyzed with ICP-HR-MS (Thermo Fisher Scientific, Waltham, USA). Detection limits (LOD) for Ag tissue concentrations were derived either from the IDL-25% taking sample weight into consideration, or were calculated based on the 3x standard deviation of blank samples. The more conservative value was used as LOD for each sample. The IDL-25% was calculated from the subsequent analysis of solutions, containing decreasing, low concentrations of the element. Finally, the concentration resulting in a relative standard deviation (RSD) of approximately 25% (n=3 scans) were selected as IDL with baseline corrections applied for these values. All Ag concentrations in the tissues are presented as dry weight (dw) concentrations.

2.8 Vitellogenin analysis

Vitellogenin concentrations in turbot plasma samples were determined quantitatively using an indirect competitive enzyme-linked immuno-sorbent assay (ELISA). Briefly, 1:1000 diluted CS-2 polyclonal rabbit anti-turbot Vtg (Biosense, Bergen, Norway) was used as primary antibody and 1:2000 diluted horseradish peroxidase-conjugated goat anti-rabbit as secondary antibody (Sigma-
Aldrich, Steinheim, Germany) to determine Vtg concentration in plasma samples diluted 1:500 in triplicate on 96-well microtiter plates pre-coated with purified turbot Vtg (100 ng/ml). Concentrations of Vtg were determined colorimetrically at 405 nm using an Ultra Microplate Reader (ELX 808 IU model, Biotek Instruments Inc., Winooski, VT, USA) 30 min after adding ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; Fluka, Buchs, Switzerland). Purified Vtg used for standards and microtiter plate coating was isolated and prepared from the plasma of turbot repeatedly injected with EE2 according to Silversand et al. (1993).

2.9 Steroid hormone analysis

To determine steroid hormone concentrations, blood samples were spiked with a mixture of deuterated standards (50 µl of a 0.4 ng/µl in methanol) containing the following deuterated analogues: d7-androstendione (dAN), d4-estrone (dE1), d5-17β-estradiol (dE2), d4-pregnenolone, d9-progesterone, (dPRO), d3-testosterone (dT) and d3-dihydrotestosterone (dDHT). dAN, dE1 and dE2 were purchased from CDN isotopes (Pointe-Claire, QC, Canada) and dPRE, dPRO, dT and dDHT were purchased from Toronto Research Chemicals (North York, ON, Canada). All deuterated steroid analogues were above 98% purity.

Concentrations of androstenedione (AN), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT), estrone (E1), 17β-estradiol (βE2), pregnenolone (PRE), progesterone (PRO), 17-hydroxypregnenolone (OH-PRE), 17-hydroxyprogesterone (OH-PRO) and testosterone (T) were determined with GC-MS/MS using a Bruker Scion™ gas chromatograph (GC) coupled to a SCION TQ GC triple quadrupole tandem mass spectrometry (MS/MS) system (Bruker Daltonik, Bremen, Germany) (see supporting information for more detail).
A detailed description of the method used for steroid analysis, including quality criteria, is described in Poulsen et al. (2015) and Nossen et al. (2016). Limits of detection (LOD) and limits of quantification (LOQ) were determined as recommended by the ICH guideline (2005) equation 1 and 2. \( \sigma \) is the standard deviation of the area ratio obtained from the least concentrated mixture of each analyte above LOQ. \( S \) is the slope of the calibration curve for each analyte.

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LOD = 3.3 \times \frac{\sigma}{s} \quad (1)
\]

\[
LOQ = 10 \times \frac{\sigma}{s} \quad (2)
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Method limit of detection (LOD) was steroid hormone specific and ranged between 0.006 and 0.28 ng mL\(^{-1}\) plasma, and internal standard absolute recoveries were between 69 and 94% across plasma samples.

2.10 Data analysis

Statistical analyses were performed with Statistica 12 (StatSoft, Tulsa, USA). Data were tested for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett test, \( p < 0.05 \)) and were log transformed where required. Differences between treatment groups were evaluated by ANOVA and subsequent post hoc analysis (Tukey test, \( p < 0.05 \)). Data sets that did not fulfill the criteria for ANOVA after transformation were analyzed by a nonparametric test (Kruskal-Wallis one way analysis on ranks, \( p < 0.05 \)). For statistical analysis values below LOD (tissue Ag, steroid hormones) and LOQ (steroid hormones) were replaced by half the LOD or LOQ, respectively. Groups with 50% or more of the samples featuring values below the LOD/LOQ were excluded.
from statistical analysis. Graphs were prepared with SigmaPlot 12.0 (Systat Software Inc., Chicago, USA) and Adobe Illustrator CS5 (Adobe Systems, San Jose, USA).

3. Results and Discussion

3.1 Exposure validation

To mimic an environmentally relevant exposure scenario in a coastal area affected by river runoff and tidal waters, turbot were subjected daily to a 2 h exposure to AgNPs, Ag\(^+\), EE2, PVP and mixtures of AgNP+EE2 followed by a depuration period. At the start of the static exposure, the measured exposure Ag concentrations (i.e. in the water) were 131 ± 10 µg L\(^{-1}\) and 33 µg L\(^{-1}\), for the HC-AgNP and Ag\(^+\) groups, respectively (Fig 2 a). This was approximately 66 % lower than the nominal exposure concentrations. During the 2 h static exposure the Ag water concentrations in the exposure tanks dropped to 95% of the starting concentration in both groups, likely due to Ag adhesion to tank walls (Fig 2 a,b). After re-installing the water flow, concentrations decreased further to about half of the starting concentrations within 2 hours, in both the AgNP and Ag\(^+\) exposure tanks. At the start of the static exposure, the measured water concentrations of EE2 were on average 10 ng L\(^{-1}\), which is approximately 20% of the nominal concentration, and it decreased to approximately 90% of the starting concentration during the 2 h static exposure (Fig.1a). Following this period, the pattern of the decrease in the EE2 concentrations in the exposure water followed the same concentration decrease pattern as Ag (Fig 2 a,b). Thus, 24 h after the start of the exposure, exposure (or water?) concentrations were < 1 µg L\(^{-1}\) (AgNP) or 1 ng L\(^{-1}\) (EE2). The concentrations in the control tank were always below the LOD (Fig 2 a,b). Although exposures were repeated daily, no increases of the contaminant levels were observed in the exposure tanks during the 14 d of exposure, indicating that the
contaminants were removed during depuration phases. Oxygen saturation and ammonium concentrations during exposure are shown in the supporting information (Fig S1, S2).

### 3.2 Nanoparticle characteristics and behavior in seawater

TEM-imaging showed that the PVP-coated AgNPs had a spherical shape and an average size of 13 nm (Fig 3a). The average hydrodynamic diameters (DLS; nr. based; 10 mg L⁻¹) were 69±0.4 nm in MilliQ water and 69.6±5 nm in seawater at the start of the exposure (time point 0). The AgNPs remained stable during 2 h in MilliQ water (65±5 nm), while the hydrodynamic diameter of AgNPs in seawater increased slightly over time to 76.6±0.6 nm after 1 h and to 83±2.2 nm after 2 h. In ultrapure water the surface plasmon resonance at λ_{max} (414 nm) decreased to 95% within the first 2 h, and to approximately 70% after 20 h (data not shown). In seawater, the surface plasmon resonance was reduced to around 68% after 2 h and further decreased to approximately 15% after 20 h (Fig 3c). No spectral shift of λ_{max} was detected (Fig 3c). The ionic (Ag⁺?) release from AgNPs after 2 h in seawater within 2 h was about 15%, as determined by removing the AgNPs using ultracentrifugation, and between 20% and 25% according to ISE measurements. Our results show that despite their PVP coating, AgNPs underwent aggregation and dissolution processes and ionic release in seawater, which occurred, however, relatively slow.

Ionic release from AgNPs in different exposure media and the role in AgNP toxicity have been extensively discussed (Yang et al., 2012; Behra et al., 2013; Sharma et al., 2014). ENPs coated with PVP, a molecule providing steric surface stabilization, are reported to be more stable in high ionic strength seawater compared to non-stabilized or charge stabilized ENPs (Christian et
al., 2008; Huynh and Chen, 2011; Levard et al., 2012). However, findings on AgNP-PVP stability differ between studies, and reported dissolution rates vary from 3-50% depending on particle size and physicochemical characteristics of the media (Behra et al., 2013; Sharma et al., 2014; Angel et al., 2013; Misra et al., 2012).

3.3 Distribution of AgNPs and Ag⁺ in tissues

In AgNP and Ag⁺ exposed fish, Ag was detectable in most tissues despite the relatively short daily exposure duration (2 h), each followed by a depuration period that occurred within the static exposure time, and after the exposure was ended (Fig 4, Tab S1). In the AgNP exposed groups Ag tissue concentrations were slightly, however not significantly, affected by the presence of EE2 in the exposure water. In the fish exposed to HC-AgNP and HC-AgNP+EE2, respectively, Ag concentrations were highest in gills (1250±748 ng g⁻¹; 733±378 ng g⁻¹) > liver (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 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 S1). Both the high AgNP exposure, and the combined high AgNP+EE2 exposure resulted in significantly higher (HC-AgNP, p=0.00017; HC-AgNP+EE2, p=0.00084) Ag concentrations in gills compared to the group exposed to Ag⁺ (Fig 4). In contrast, Ag accumulation was higher in the stomach of the Ag⁺-exposed group as compared to the HC-AgNP+EE2 (p=0.033) treatment group. In liver, Ag concentrations were slightly, however insignificantly higher in fish exposed to HC-AgNP and HC-AgNP+EE2 as compared to the Ag⁺-exposed group. No differences between these groups (or all groups?) were detected in kidney, bile and muscle (Tab S1). In kidney and muscle, Ag was below the LOD in both the low- concentration AgNP exposure groups (LC-AgNP, LC-AgNP+EE2; Tab S1), and in most of the brain samples Ag
concentrations were marginally below the LOD (data not shown). This can be at least partly attributed to the low sample weight of brain tissues and the conservative detection limits applied. However, an uptake of Ag in brain following prolonged or continuous exposure, as reported in previous studies, seems possible (Kwok et al., 2012; Jang et al., 2014). Similar concentrations of Ag in bile and liver in the AgNP and Ag$^+$ exposure groups indicate that Ag was at least partially excreted via the intestinal tract. Excretion of Ag via bile after Ag$^+$ exposure has previously been documented in marine fish (Wood et al., 2010).

In previous studies, both liver and gills have been described as the predominant organs for AgNP accumulation (Kwok et al., 2012; Jang et al., 2014; Scown et al., 2010). However, Ag uptake and tissue distribution may depend on AgNP speciation. In this study, the high Ag concentrations in gills of AgNP exposed fish indicate that AgNPs are mostly attached to gill surfaces or trapped in gill mucus. Gill tissues were sampled after a depuration phase of 22 h, which indicates that the AgNPs were not removed from gill surfaces within that timeframe, suggesting that effects of AgNPs on gill function should be investigated in marine fish. AgNP-gill associations were previously reported in zebrafish (Danio rerio) and Japanese medaka (Oryzias latipes) (Kwok et al., 2012; Griffitt et al., 2009). The similar Ag accumulation in internal organs in the high AgNP and the Ag$^+$ groups, despite the 75% lower Ag exposure concentration suggest that internal Ag uptake derives from ionic release, which was approximately 15-25% in our study. Previous studies showed that dissolved Ag readily forms complexes with Cl resulting in different AgCl$_n$ species, mostly accumulating in livers and not in the gills in two flatfish species, the English sole (Parophrys vetulus) and the starry flounder (Platichthys stellatus) (Ferguson and Hogstrand, 1998; Webb and Wood, 2000; Hogstrand et al., 2002).
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.
(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
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
affected by any of the exposures. This suggests that the cytochrome P<sub>450</sub> 17 (CYP17) responsible for the transformation of the progestagens PRE and PRO into the androgens DHEA and AN may be a potential target for the effects of AgNP+EE2 mixtures...Effects of Ag and AgNPs on different enzymes of the steroidogenic CYP family are not well studied, but Garcia et al. (2014) observed increased transcription of CYP11A1 and 3β-HSD (transforming Δ-5 steroids into Δ-4 steroids in the steroidogenesis) mRNA in male rats exposed to AgNPs. This aspect needs further investigation.

In this study we found no significant effects of exposure to combined mixtures of AgNPs and EE2 on contaminant uptake and accumulation of either of the two contaminants,, and only slight changes in steroid hormone levels deriving from the AgNP and -EE2 co-exposures. Differences in ENP-contaminant mixed toxicity will strongly depend on the ENP-contaminant interactions deriving from the properties of ENPs and contaminants. As AgNPs are amongst the most frequently used ENPs, further research on interactive effects with different contaminant groups is needed. Furthermore, the role of Ag<sup>+</sup> deriving from Ag dissolution in AgNP-contaminant mixtures should be investigated in more detail.

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**Supporting information**
More detailed information on the methods used for EE2 extraction from water and steroid hormone analysis are given in the supporting information. Results reporting oxygen and ammonia concentrations in water samples, tissue Ag and plasma steroid hormone concentrations are shown in figure FigS1, FigS2 and tables TabS1-S3.


Figure 1: Scheme of the exposure setup showing the different exposure groups and respective exposure concentrations. Each exposure group, except for the PVP control group (replicates), consisted of 3 replicate tanks with 5 fish in each tank, resulting in \( n=15 \).
Figure 2: Absolute (a) and relative (b) contaminant concentrations of HC-AgNP (closed circles; µg L$^{-1}$), Ag$^+$ (open circles; µg L$^{-1}$), controls (open triangles; µg L$^{-1}$) and EE2 (closed triangles; ng L$^{-1}$) during the daily exposures. The samples were analyzed at the exposure start (0 h), end (2 h), and after 1 h (i.e. 3 h after exposure start), 2 h (4 h after exposure start) and 22 h (24 h after exposure start) of depuration. Dotted lines show the approximated concentration decline. Vertical lines indicate x-axis breaks from 5.5 h to 22 h.
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.
Figure 4: Ag tissue concentrations in HC-AgNP, HC-AgNP+EE2 and Ag⁺ exposed juvenile turbot. Data are shown as mean±SEM. Stars denote significant differences in Ag concentrations (p<0.05).

Figure 5: Concentrations of EE2 (ng g⁻¹) a) and Vtg (ng mL⁻¹) b) in plasma of exposed and unexposed juvenile turbot (data from male and female fish pooled). Data are presented as median±75th percentile. Stars denote significant differences from control (p<0.05).
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.