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Combined Effects of Silver Nanoparticles and 17α-ethinyl estradiol on Turbot *(Scophthalmus maximus)*

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Environmental Toxicology and Chemistry
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Contents

List of Figures

List of Tables

Acronyms

Ag^+	Silver ion
$AgNO_3$	Silver nitrate
AgNPs	Silver nanoparticles
ANOVA	Analysis of variance
BBB	Blood-brain barrier
BSA	Bovine serum slbumin
DLS	Dynamic light scattering
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
dw	Dry weight
E1	Estrone
E2	Estradiol
E3	Estriol
ER	Estrogen receptors
EDCs	Endocrine disrupting chemicals
EE2	17α-ethinyl estradiol
ELISA	Enzyme-linked immunosorbent assay
ENPs	Engineered nanoparticles
EPA	Environmental protection agency
GC-MS	Gass chromatography-mass spectrometry
H_2O_2	Dihydrogen peroxide
HD	Hydrodynamic diameter
HNO_3	Nitric acid
HR-ICP-MS	High resolution inductively coupled plasma-mass spectrometry
ICP-MS	Inductively coupled plasma-mass spectrometry
IDL	Instrument detection limit
ISE	Ion selective electrode

K_{ow}	Octanol-Water Partition Coefficient
LOD	Limit of detection
LOEC	Lowest observed effect concentration
LOQ	Limit of quantification
MDL	Method detection limit
MES	Mestranol
MS222	Tricaine mesylate
nC60	Fullerene nanoparticles
NOEC	No observed effect concentration
NSB	Non-specific binding
OD	Optical density
PBS	Phosphate buffer saline
PBS-T	Phosphate buffer saline + 0.05% Tween
PTF	Polymer thick films
PVP	Polyvinylpyrrolidone
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SE	Standard error
SEM	Standard error of the mean
TEM	Transmission electron microscopy
TiO_2	Titanium oxide
UV-vis	Ultra violet visible spectrometry
Vtg	Vitellogenin
ww	Wet weight
WWTP	Waste water treatment

Acknowledgments

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Radka Staňková

Abstract

Due to their antimicrobial properties silver nanoparticles are one of the most used nanomaterials in the commercially available products nowadays. The exponential increase in production of nanomaterials raises concerns about the environmental hazards to aquatic organisms. Previous exotoxicological studies have shown that silver nanoparticles can be toxic to fish. However, combined effects of silver nanoparticles with other pollutants have not been studied in depth so far.

In this study, the uptake and effects of low and high concentrations of silver nanoparticles (AgNPs) and 17α-ethinyl estradiol (EE2), both alone and in combination on juvenile turbots *(Scophthalmus maximus)*, were investigated. A solution of silver nitrate ($AgNO_3$) containing 50 μg/L Ag ions was included in order to differentiate the effects of AgNP from that of ionic silver. The AgNP agglomerate dissolution was determined by ultracentrifugation followed by ICP-MS analysis of the supernatant and independently by ion selective electrode. The amount of ionic silver within undiluted particle dispersions was determined to be 15 -25 %. The body distribution of Ag was measured by ICP-MS. Results show that after 13 days of pulsed exposure to AgNPs and $AgNO_3$ silver was detected in all organs that were evaluated (gills, stomach, liver, kidney, muscle) except for brain. The uptake of Ag into the tissues was dose dependent. In all analyzed tissues except for gills there was no statistically significant difference between high Ag exposure groups (AgNP200, AgNP200EE2 and Ag^+). High concentration of Ag in the gills suggests that the AgNPs were trapped in the mucus layer or adsorbed on the gill surface. Detectable concentrations of Ag in organs and tissues indicate that Ag was probably taken up via the gastrointestinal route, crossed the epithelial barrier, accumulated in the liver and was excreted via the bile.

The ability of 17α-ethinyl estradiol to induce vitellogenin (Vtg) in plasma was measured with enzyme-linked-immunosorbent assay. The average measured vitellogenin concentrations in the control fish were 24 000 ng/mL. Fish exposed to EE2 treatment (EE2, AgNP2EE2, AgNP200EE2) showed significant induction of Vtg compared with the control group. The Vtg concentrations were 105 000 ng/mL, 98 000 ng/mL and 73 000 ng/mL, respectively. However, there was no

statistically significant difference among these groups. The findings of this study showed that AgNPs probably do not enhance the effects of 17α-ethinyl estradiol in *Scophthalmus maximus*.

Chapter 1

Introduction

Nanotechnology is a rapidly growing industry over the recent years. Among consumer products that include nanomaterials, silver nanoparticles (AgNPs) are one of the most widely used materials due to their antimicrobial and physico-chemical properties. It has been shown, that during their production, usage or after disposal, some of these products may release nanoparticles into the environment. Aquatic environment is particularly vulnerable as it is a known sink for many pollutants. Even though numerous studies reported the toxicity of various engineered nanomaterials to aquatic organisms (Gagné et al., 2012; Asghari et al., 2012; Hu et al., 2011; Scown et al., 2010b; Federici et al., 2007), the information on their behaviour in the aquatic environment, their bioavailability or uptake routes are still scarce. Moreover, all of those processes are likely to be different from the conventional pollutants due to the extremely small size of nanomaterials and their unique properties. It is unclear whether the toxicity of AgNPs is caused by ionic form of Ag that is released from the nanoparticles under oxic conditions or by the nanoparticle-specific mechanisms, or whether there are any combined effects of the two forms. There is a lack of data and understanding about the effects of AgNPs in combination with other pollutants. The high surface to volume ratio of nanoparticles is a desired property in the medical sector, but it can affect the fate and effects of co-ocuring substances in the aquatic environment. Among such compounds are also endocrine disrupting chemicals (EDCs). Their occurance in the effluents from waste water treatment plants (WWTPs) is the main cause of the induction of vitellogenin synthesis in the male or juvenile fish and occurance of intersex conditions.

1.1 Nanoparticles

Nanoparticles are defined as particulate matter with at least one dimension that is smaller than 100 nm (Christian et al., 2008). However, aggregates that are nanoparticles held together by weak chemical bounds (van der Walls, capillary, electrostatic or other physical forces) and agglomerates that are bound together by strong chemical bounds (metallic, ionic, covalent bounds) can be larger than 100 nm (Eggersdorfer and Pratsinis, 2014). According to their origin, nanoparticles can be divided into natural, that derive from biological or geological processes (fires, volcano eruptions, erosion) or anthropogenic that derive from industrial manufacturing. Anthropogenic nanoparticles have also been unintentionally created for centuries as byproducts of combustion, chemical manufacturing or ore smelting, while engineered nanoparticles (ENPs) for advanced technologies and consumer products have became a new source of exposure in recent years.

Engineered nanoparticles include fullerenes and carbon nanotubes, metals, metal oxides, ceramics semiconductors and polymeric nanoparticles. Metal nanoparticles and metal oxide nanoparticles such as Ag, Au, Ni, Pt, Al, Si, ZnO and TiO_2 are implemented in electronics (Zaniewski et al., 2013), sensing (Guerrini et al., 2009), environmental clean-up (**?**) or drug delivery (Mudshinge et al., 2011). Ceramic nanoparticles such as silicon nitride (Si_3N_4) and silicon carbide (SiC) can be used in many applications such as drug delivery, electronics, mining, food industry etc. Carbon nanotubes are also widely used in electromagnetic shielding as polymer composite, for hydrogen storage and in batteries due to their good conductive properties (Collins and Avouris, 2000).

1.1.1 Properties of nanoparticles

Properties of ENPs are influenced by many factors including the composition, morphology, surface area, charge, solubility, coating etc. Due to their small size and consequently very high surface area to volume ratio the chemical, mechanical, electrical or optical properties of nanomaterials significantly differ from the bulk materials.

Several studies have reported the influence of the size of nanoparticles on their toxicity, with the smaller particles being more toxic than the large ones. The size of ENPs affects interactions with living cells, uptake efficiency, internalization pathway selection, intracellular localization and cytotoxicity (Shang et al., 2014). Nanotoxicity threshold at about 10 nm was experimentally demonstrated by De Jong et al. (2008), who exposed rats to gold NPs with size of 10, 50, 100 and 250 nm. The results showed that only 10 nm particles were distributed in almost all organs including blood, liver, spleen, kidney, testis, thymus, heart, lung and brain, whereas the larger particles were only detected in blood, liver and spleen. The particle size is an important factor in the cellular uptake processes. Kim et al. (2012) reported that the 10 nm AgNPs had a greater ability to induce apoptosis in the mouse osteoblastic cell line (MC3T3-E1) than the 50 and 100 nm AgNPs. Another study showed, that single AgNPs (5-46 nm) can be passively transported into and out of zebrafish embryos through chorion pore canals (Kim et al., 2012).

The surface of nanoparticles can be modified by coating it with ultrathin inorganic films, which may be negatively or positively charged, or may be stabilized based on steric effects. For example AgNP suspensions are usually stabilized by charged polymers (e.g. polyvinylpyrrolidone PVP), tannic acid, or citrate (figure 1.1). Citrate is weakly bound to the nanoparticle surface and is readily displaced by a range of other molecules (thiols, polymers, amins). Tannic acid is often used as a coating material in applications where high particle concentrations are required. PVP binds strongly to the AgNP surface, therefore it provides greater stability than citrate or tannic acid, but is more difficult to remove. Coating has been found to be of particular interest for the controlled release of bioactive agents such as drugs or genes, because it provides protection from rapid degradation and prolonged duration of bioactive agents. It can be used to improve the microstructure and mechanical properties of nanoparticles and prevent formation of microcracks in high temperatures. It also modifies the photoactive properties of nanoparticles by either enhancing photocatalytic activity of particles for environmental remediation use or by hindering them in cosmetic products (Hakim et al., 2007). Surface coating is also used to prevent aggregation and ensure higher stabilization of colloids (Mine et al., 2003; Kobayashi et al., 2005). Various studies have shown that toxicity of nanoparticles is dependent on surface coating (Suresh et al., 2012; Ahn et al., 2014; Nguyen et al., 2013; Asghari et al., 2012). Nguyen

et al. (2013) compared toxic effects of uncoated (20, 40, 60 and 80 nm), citrate- and PVP-coated (10, 50, and 75 nm) AgNPs in murine macrophage J774A.1 and human HT29 epithelial cells. Their results showed that uncoated AgNPs were more toxic than coated AgNPs. Additionally their results suggest that uncoated AgNPs suppress inflammatory responses and enhance oxidative stress in the test cells, while coated AgNPs induce toxic effects through up-regulation of cytokines. In contrast, Suresh et al. (2012) reported that poly(diallyldimethylammonium)-coated AgNPs were found to be the most toxic to mouse macrophage and lung epithelial cells, followed by biogenic-Ag and oleate-Ag nanoparticles, whereas uncoated or colloidal AgNPs were found to be the least toxic. That indicates that the cytotoxicity of AgNPs is dependent not only on coating materials but also on the cell-types.

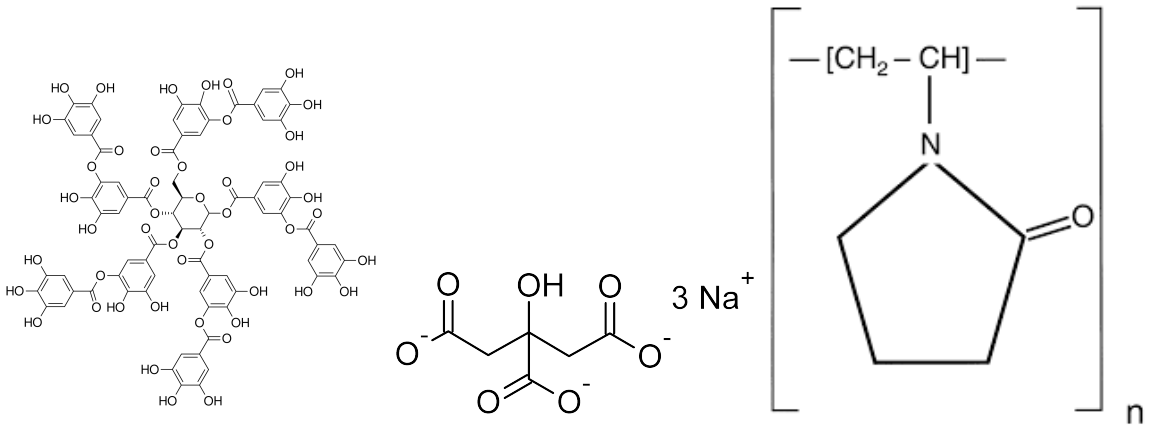


Figure 1.1: Molecular structure of different materials used for surface coating of silver nanoparticles. a) tannic acid b) citrate c) polyvinylpyrrolidone (PVP)

1.1.2 Transport and fate of nanoparticles in the aquatic environment

Due to increased production of ENPs, there is also the increased possibility of potential release into the environment. Waste from domestic sources and industrial activities, accidental spillages, atmospheric emissions and agricultural run-offs might be a few examples of different exposure routes. Several studies modelling the behaviour of nanoparticles in WWTPs have been published (Brar et al., 2010; Yang et al., 2013; Adams and Kramer, 1999). Kaegi et al. (2011) investigated the behaviour of AgNPs in a pilot WWTP fed with municipal wastewater. A AgNP suspension was spiked into the influent at a concentration of 2 400 μg/L (first 24 h) and 130 μg/L (for following 24 days). After 43 days cca 5% of the added silver nanoparticles left the WWTP via

the effluent, cca 85% ended up in the excess sludge and cca 5% remained in the WWTP. Despite some available experimental data, the behaviour of ENPs in the WWTPs remains unclear.

Both agglomeration and aggregation of NPs lead to bigger particles that can easily sink to the sediments. Particle agglomeration and aggregation are influenced by a number of factors, including primary particle characteristics (surface charge, particle shape and size) as well as the properties of the medium that the particles are suspended in (ionic strength, pH) (Tolaymat et al., 2010). Agglomeration and aggregation of AgNPs occur particularly at low pH, which neutralizes the negative surface charged coatings of the ENPs or at high ionic strenght (Gao et al., 2009). The newly formed aggregates may consist solely of AgNPs, but according to Andy et al. (2008) it is more likely that nanoparticles will form aggregates with existing natural colloids. Aggregation and agglomeration of AgNP also depends on their surface coating. El Badawy et al. (2010) showed that while the citrate, sodium borohydride ($NaBH_4$) coated and uncoated AgNP aggregated at higher ionic strengths and/or acidic pH, the ionic strength or pH had no impact on aggregation of PVP coated AgNPs.

Dissolution is another important process influencing the fate of AgNPs in the aquatic environment. Both nanoparticle properties and environmental parameters influence how AgNPs dissolve and consequently impact their toxicity as Ag^+ may be released from the NP by oxidation of zero-valent silver (Tolaymat et al., 2010). In their study Wang et al. (2014) measured the release of Ag^+ from AgNPs of various sizes with different coatings exposed to river and lake water for up to 4 months. They observed that Ag^+ release correlated to AgNP size and that PVP and tannic acid coated AgNPs incline more to Ag^+ release than citrate coated AgNPs. Dissolution rate also depends on the shape of the nanoparticle (Baalousha et al., 2012). Gao et al. (2009) highlined an important role of water chemistry on the dissolution of AgNPs, showing negligible AgNP dissolution over 24 h in presence of high concentrations of chloride, sulfide, and dissolved organic carbon (DOC) (1 mg/L Cl^-,1 mg/L S^{2-} and 20 mg/L DOC) and slight decreases in particle diameter with lower concentrations. The mechanism of Ag^+ release from the AgNP is not clear. Innovation and Island (2010) suggest that it is an oxidative dissolution process with O_2, which lead in the scale of their laboratory experiment to full reactive dissolution and disappearance of

the particle phase. Other studies show formation of partially oxidized AgNP with chemisorbed Ag^+ due to extreme sensitivity to oxygen (Lok et al., 2007; Henglein, 1998). The study of Dobias and Bernier-Latmani (2013) confirms the later hypothesis. Even though the small AgNPs (< 10 nm) in their study lost more than 80% of the initial silver content, complete dissolution of AgNPs was not observed even after 4 months. It supports the theory that dominant silver release process is desorption of chemisorbed Ag^+ from the surface of AgNPs. They suggest however, that oxidative dissolution occurs as a parallel, but much slower process.

1.1.3 Uptake and distribution of nanoparticles in fish

The fish gill is a multi purpose organ serving for aquatic gas exchange, osmotic and ionic regulation, acid-base regulation and excretion of nitrogenous wastes (Evans et al., 2005). Each gill arch consists of thin filaments that are subdivided into highly folded lamellae. Their large surface area together with counter-current flow of water to the flow of blood is crucial for gas exchange and maintaining homeostasis (Evans et al., 1999). Gills in marine fish are also responsible for the secretion of salt. The gill surface (and similarly the skin and gastrointestinal tract) is covered by mucus, which consists of water and high-molecular weight, gel-forming macromolecules. It serves as a natural semipermeable barrier that enables the exchange of nutrients, water, gases, odorants, hormones, and gametes (Ángeles Esteban, 2012). Several models have been developed to predict metal toxicity to aquatic organisms. These models calculate the amount of a metal binding to a fish gill, which equates with metal toxicity (Playle, 2004; Macdonald et al., 2002; Pagenkopf, 1983). The metal ions usually move through the cell using the ion transport pathways. Organic lipophillic chemicals can enter the blood either through transcellular diffusion or through paracellular diffusion. Handy et al. (2008a) speculate that the uptake mechanism of ENPs by gill epithelial cells will be driven by endocytosis, rather than by diffusion through the cell membranes or by ion transfer. The authors also hypothesize that given the size of ENPs, they are not likely to be taken up by the ion transporters and they will sooner aggregate in the presence of Ca^{2+} and Mg^{2+} ions rather than diffuse. Uptake of ENPs via endocytosis was previously observed for mammalian cells (Conner and Schmid, 2003). On the other hand Lee et al. (2007) reported, that the diffusion via chorion pore canals facilitated the uptake and depuration of small AgNPs (5 - 46 nm) into zebrafish embryo cells.

The other possible uptake route for the ENPs to enter the fish body is through gastrointestinal absorption. Since the fish are capable of taking much larger materials by endocytosis across the gut than the mammals, they might be in greater danger from ENPs uptake via this route. Federici et al. (2007) showed Ti accumulation in gut of rainbow trout from ingested water containing TiO_2 NPs. Xenobiotics taken up from the gut are transported by a single blood vessel (vena porta) via the intestinal epithelium to the liver, which is followed by endocytosis into hepatocytes (Smedsrud et al., 1984). Metabolites can be excreted via the kidneys or via the bile.

The skin of fish together with the protective mucus layer serves as an effective barrier to ENPs uptake. However, if the skin is damaged by infection or inflammation, xenobiotics may simply diffuse directly into the blood through the damaged tissue and quickly access internal organs (Handy et al., 2008a). The nerve endings in the buccal cavity, olfactory openings, eyes and urinary/genital openings may serve as other possible entry points for ENPs. It has been demonstrated, that manganese oxide nanoparticles can reach the brain of rats through the upper respiratory tract via the olfactory bulb (Elder et al., 2006). Borg-Neczak and Tjälve (1996) showed that fish are capable of acummulating chemicals via the olfactory bulb as well. It is uncertain, if ENPs can pass through the brain-blood barrier (BBB). It has however been demonstrated that AgNP can pass the BBB in rats (Trickler et al., 2010; Tang et al., 2010)

1.1.4 Toxic effects of nanoparticles

Many ENPs have been proved to be toxic to various organisms. The most investigated aquatic species are freshwater crustaceans *(Daphnia magna)* (Asghari et al., 2012; Rosenkranz et al., 2009). Other studies showed negative effects of ENPs on fish (Gagné et al., 2012; Zhang et al., 2007; Farmen et al., 2012) or algae (Baun et al., 2008). Toxicity of nanoparticles is very dependant on physical and chemical properties such as the composition, surface area, size, charge, coating etc. Moreover, the reactivity of NPs and consequently their toxicity also depends on the environmental conditions such as pH or salinity. To date, the majority of research of ENPs toxicity has been focusing on fresh water species, reporting various sub-lethal effects including reduced feeding (Croteau et al., 2011), growth and reproduction (Zhao and Wang, 2011), swim-

ming (Asghari et al., 2012), or decreased hatch rate (Hu et al., 2011). However, little research has been done on marine organisms. Even though the mechanisms of action are still poorly understood, ENPs can pass through cell walls and membranes. Possible toxic effects include disruption of cell membranes, formation of reactive oxygen species (ROS) (Fu et al., 2015), chromosomal aberrations and DNA damage (Vignardi et al., 2015), protein oxidation or decreased mitochondrial activity (Ahn et al., 2014).

1.1.5 Silver nanoparticles

Silver nanoparticles (AgNP) are among the most widely used nanoparticles. They have attracted increased interest due to their physical-chemical properties including a high electrical and thermal conductivity, chemical stability or fungicidal and herbicidal properties. Due to their anti-bacterial properties the silver nanoparticles can be used effectively by coating them on the surfaces that require antimicrobial functions, for instance, in the medical sector (implantable devices, polymethylmetacrylate bone cement, dental materials, hydrogel for wound dressing, coating of hospital textile etc.) The antimicrobial effects of silver ions on micro-organisms are very well known. However, the bactericidal mechanism is only partially understood. It is generally believed that heavy metals react with proteins by combining the thiol groups, which leads to the inactivation of the proteins. Feng et al. (2000) suggest, that as a reaction against the denaturation effects of silver ions, DNA molecules become condensed and lose their replication abilities. Silver nanoparticles have been proved to have potential antimicrobial effects against various infectious organisms, including *Escherichia coli, Staphylococcus aureus, Bacillus subtilis, Staphylococcus epidermidis and Streptococcus pyogenes* (Cho et al., 2005; Yoon et al., 2007; Raja and Singh, 2013; Shahverdi et al., 2007). For instance, Morones et al. (2005) reported size dependent bactericidal properties of the silver nanoparticles, where particles mainly in the range of 1–10 nm attached to the surface of the cell membrane of *Escherichia coli* and drastically disturb its permeability and respiration. Yoon et al. (2007) observed, that *Bacilus subtilis* showed higher sensitivity than *Escherichia coli* to silver nanoparticles suggesting a selective antimicrobial effect. Combined synergetic effects of silver nanoparticles and different antibiotics such as penicillin, amoxicillin, imipenem, trimethoprim, gentamycin, vancomycin and ciprofloxacin was also reported (Naqvi et al., 2013; Shahverdi et al., 2007). The antifungal effect

of silver NPs has received only marginal attention. Panácek et al. (2009) reported that AgNPs antifungal activity is comparable with ionic silver and that AgNPs exhibit high antifungal activity against pathogenic *Candida spp.* at the concentrations around 1 mg/L of Ag. Similarly (Kim et al., 2008) showed antifungal activities of AgNPs against *Trichophyton mentagrophytes* and *Candida albanicans*.

The electrochemical and optical properties of AgNPs have been incorporated in nanoscale sensors, catalysts, nanoelectronic devices, biochemical tagging reagents, optical switches etc. (Jiang et al., 2005). Silver nanoparticles are extremely efficient at absorbing and scattering light due to the collective oscillation of conduction electrons excited by light at specific wavelengths known as a surface plasmon resonance (Abbasi et al., 2014).

There are several methods that are currently being used for AgNPs synthesis, including chemical, photochemical, physical and biological synthesis. The most common method for synthesis of AgNP is chemical reduction of silver ions to metallic silver by organic and inorganic reducing agents. This step is followed by agglomeration into oligomeric clusters and eventuelly formation of metallic colloidal silver particles (Wiley et al., 2005). Another widely used approach is physical synthesis that utilizes several methods such as evaporation/condensation and laser ablation.

In order to understand and control the nanoparticle synthesis, it is important to determine different parameters of the particle such as size, shape, surface area, crystallinity etc. Transmission and scanning electron microscopy, atomic force microscopy, dynamic light scattering (DLS), powder X-ray diffractometry, infrared spectroscopy and UV–vis spectroscopy are some of the most common techniques used for characterisation of nanoparticles (Abou El-Nour et al., 2010). Among them DLS and UV-vis are the most commonly used ones. UV-visible spectroscopy monitors how the nanoparticles change over time. When AgNPs aggregate, the metal particles become electronically coupled. This coupled system has a different surface plasmon resonance than the individual particles. In case the particles undergo aggregation, the plasmon resonance will be red-shifted to a longer wavelength than the resonance of an individual nanoparticle, and aggregation is observable as an intensity increase in the red/infrared region of the spectrum (Amendola and Meneghetti, 2009). DLS is a method that depends on the interaction of light with particles. It measures the light scattered from the laser that passes through a colloid. The

modulation of the scattered light intensity as a function of time is analyzed, and the hydrodynamic size of particles can be determined (Tomaszewska et al., 2013).

1.2 Endocrine disruptors

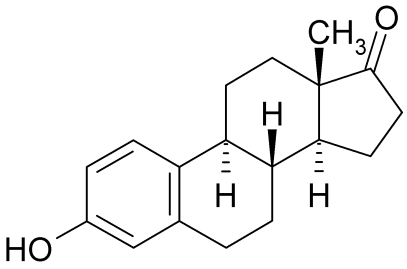
Endocrine disrupting chemicals (EDCs) were defined by the U.S. Environmental Protection Agency (EPA) as 'exogenous agents that interfere with synthesis, secretion, transport, metabolism, binding action, or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction and developmental process.' Although the term 'endocrine disruptor' was coined at the Wingspread conference in Wisconsin (1991), the effects of substances that would be labeled nowadays as EDCs have been studied since the 1940's. Presently, there are nearly 1 000 chemicals that have been classified as EDCs or potential EDCs on the TEDX (The Endocrine Disruption Exchange, Inc) list of potential endocrine disruptors.

- organochlorine pesticides (Guillette, 2000; LI et al., 2008; Andersen et al., 2002)
- phthalates (Swan et al., 2005; Fisher, 2004)
- heavy metals (Gore, 2007; Henson and Chedrese, 2004)
- non-steroidal synthetic estrogenic compounds (nonylphenol, octylphenol, benzophenone, bisphenol A) (Oehlmann et al.; Laws, 2000; Xi et al., 2013)
- steroidal estrogens (estradiol (E2), estriol (E3), estrone (E1) and ethinyl estradiol (EE2), mestranol (MES)) (Aris et al., 2014; Versonnen and Janssen, 2004; Dussault et al., 2009)

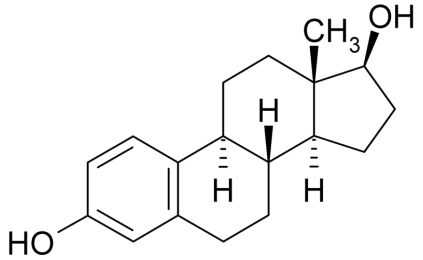
EDCs have been found in aquatic ecosystems worldwide (Fossi et al., 2004; Scott et al., 2007; Wang et al., 2014; Allen et al., 1999) and investigation of their effects on fishes have been described in numerous studies and reviews (Kime, 1999; Segner, 2009; Mills and Chichester, 2005). The mechanisms of action of EDCs involves binding to genomic and non-genomic steroid receptors (Tollefsen, 2002; Thomas and Dong, 2006) and steroid binding proteins (Gale et al., 2004), alternation of steroid homeostasis or induction of vitellogenesis (Arukwe, 2000). Other

examples of EDCs effects on fish in particular are masculinization, occurance of intersex conditions, changes in sex ratio, gonad growth and sexual maturity or altered eggs size.

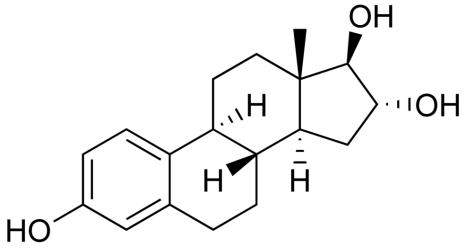
Environmental estrogens (xenoestrogens) are anthropogenic or natural compounds that can mimic the function of the natural steroid 17β-estradiol (E2) by binding with estrogen receptors (ER) or influencing cell signalling pathways (Marino, 2014). Both synthetic (EE2, MES) and natural steroidal estrogens (E1, E2, E3) are very biologically potent substances and therefore of particular concern for human and wildlife health. In mammalian species E2 is the most potent estrogen synthetised from cholesterol in female ovaries, E1 is its main degradation product produced in liver. E3 is only synthetised in significant amounts during pregnancy by placenta. (Marin and Matozzo, 2004).



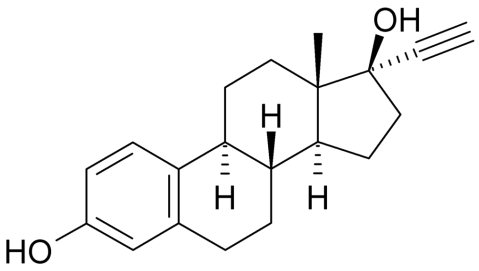
(a) Estrone E1



(b) Estradiol



(c) Estriol



(d) Ethinyl estradiol

Figure 1.2: Molecular structure of steroidal estrogens

1.2.1 17α-ethinyl estradiol (EE2)

The 17α-ethinyl estradiol (EE2) is a synthetic estrogen that is predominantly being used in oral contraceptives, for treating breast and prostate cancer, pre-and postmenopausal disorders or hypogonadism. It is also used in agriculture to control ovulation, treat reproductive disorders or promote growth in livestock or to develop mono-sex fish populations (Beardmore et al., 2001; Connolly, 2009; Marin and Matozzo, 2004). It is one of the most active EDCs, being 10 times more potent than the natural estrogens. The potency compared to the other estrogens can be expressed as EE2 > E2 > E1 (Andrew et al., 2010). It is a moderate hydrophobic compound (log K_{ow} = 4.15) (Lai et al., 2000) with low water solubility (4.8 mg/L at 20 °C) and low vapour pressure (4.5×10^{11} mm Hg at 20 °C) (table 1.1). These properties are indicating relatively high affinity of EE2 to a solid phase, which has an influence on the transport and fate of EE2 in the aquatic environment.

	Molecular weight	**Water solubility** (mg/L at 20 °C)	**Vapour pressure** (mm Hg)	**log** K_{ow}
Estrone (E1)	270.4	13	2.3×10^{-10}	3.43
17β-ethinyl estradiol (E2)	272.4	13	2.3×10^{-10}	2.94
Estriol (E3)	288.4	13	6.7×10^{-15}	2.81
17α-ethinyl estradiol (EE2)	296.4	4.8	4.5×10^{-11}	4.15
Mestranol (MeEE2)	310.4	0.3	7.5×10^{-10}	4.67

Table 1.1: Physicochemical properties of steroidal estrogens (Lai et al., 2000)

1.2.2 Sources of environmental estrogens and their transport to the aquatic environment

Human and animal excretion by urine have been identified as the main source of estrogens in waste water treatment plants (WWTPs). The quantity of excreted estrogens depends on the sex, hormonal status, stage of menstruation, use of contraceptives and pregnancy. It is estimated that between 10 and 100 μg of E1 and E2 are excreted daily by women. Pregnant women may

excrete up to 30 000 μg of estrogen per day, primarily in the form of E3 (Baronti et al., 2000). Prior to excretion in urine, EE2 is metabolized in human body into biologically inactive, water-soluble conjugates of sulfuric and glucuronic acids. However, many microorganisms (e.g., *Escherichia coli*) exhibit glucuronidase and sulfatase activity, and are therefore able to convert the non-active conjugates into biologically active unconjugated estrogens (Aris et al., 2014). Another sources of EE2 contamination in aquatic environment are agricultural run-off, as sewage and manure are used as fertilisers and cattle feedlot effluents. Supplemental hormones are used in beef cattle and sheep for synchronizing the heat cycles of females and planed reproduction. In beef cattle, they are also used for temperament control, milk production or growth stimulation. Recently, some studies showed that the amount of estrogen excreted by livestock was of the same order of magnitude or even higher as by humans (Lange et al., 2002; Liu et al., 2012; Johnson et al., 2006). Lange et al. (2002) reported that the total daily excretion mass of estrogen ranged from 120 – 2 300 μg per swine, which excreted more than one order of magnitude more estrogen than a normalized human excretes daily.

The ability of WWTPs to remove steroid estrogens and other EDCs from effluent is not sufficient and it results in presence of EDCs in surface waters. The occurrence of estrogens in surface waters and effluents from the WWTPs has been investigated in numerous studies (Baronti et al., 2000; Chang et al., 2011; Lundström et al., 2010; Yoshimoto et al., 2004). Published studies broadly conclude that conventional wastewater treatment is efficient in the removal of E2 (85 - 99%), but E1 removal is relatively poor (25 - 80%) (Khanal et al., 2006). Estrogen removal efficiency is highly dependent on WWTPs methods. Kirk et al. (2002) examined the occurance and fate of estrogens in five WWTPs in the UK and reported that most of the removal occurred in the secondary treatment with some additional removal associated with tertiary treatment. Huang and Sedlak (2001) found that effluent filtration, using sand filtration or microfiltration, removes approx. 70% of the hormones from secondary effluent, while advanced treatment, using reverse osmosis, removes more than 95% of hormones. Insufficient treatment of estrogen compounds by the WWTP results in detectable environmental concentrations of estrogens in surface water, sediments or biota. Water concentrations of EE2 within the low ng/L range have been observed worldwide. In Germany, concentrations of EE2 in rivers were up to 5 ng/L (Kuch

and Ballschmiter, 2001). The United States Geological Survey measured EE2 concentrations in 139 streams across 30 states during 1999 and 2000 and reported EE2 concentrations from 5 to 273 ng/L.

1.2.3 Fate of EE2 in the aquatic environment

The removal of estrogens from water and sediments is expected to be largely the result of a combination of physical sorption to bed sediments, photolysis, binding to particles, and biodegradation. The biodegradation of estrogens depends on many factors, such as physical-chemical properties of the chemicals, presence of bacteria, temperature, aeration and sludge-retention times (Kuster et al., 2004). Studies have shown that due to its properties (high lipophilicity, persistance, low polarity) EE2 is highly resistant to biodegradation and therefore the sorption to sediments is predominant process in the aquatic environment (Wang et al., 2014; Kuster et al., 2004; Dussault et al., 2009; Aris et al., 2014). However, (**?**) reported that aerobic degradation in urban and industrially affected rivers in UK was much faster in summer than in winter, suggesting that photodegradation might be an important aspect contributing to EE2 removal from the surface waters. A halflife of 17 days was determined for EE2, whereas E2 was transformed by heterotrophic microorganisms to E1 with halflife of only 0.2 to 9 days under the same aerobic conditions.

Sorption to organic matter is an important factor influencing fate of EE2 in the aquatic environment. Organic matter in the aquatic phase can be either dissolved or colloidal. Sorption to organic matter is dependent on the octanol-water partition coefficient (K_{ow}), which is an indicator of compound hydrophobicity. Relatively low K_{ow} of EE2 indicates high affinity for the solid phase (Kuster et al., 2004). However, a few studies suggested that the partition of EDCs onto colloids is relatively independent of their K_{ow}, and the sorption may be attributed to the physical-chemical properties of colloidal materials (Zhou et al., 2007). The sorption to the colloidal phase is an important transport route to the environment, since colloidal phase of WWTPs effluents is often difficult to remove from the aqueous phase without advanced treatment technologies. The study of Zhou et al. (2007) showed that 20 - 29% of EE2 present in the water phase was es-

timated to be associated with aquatic colloids. Another study shows that between 1 and 50% of the aqueous E2 and EE2 concentrations associated with colloidal material (Holbrook Richard David, 2003). According to **?**, between 13% and 92% of the estrogens entering a river system would end up in the sediments with the majority of sorption occurring within the first 24 h. Due to its previously described resistance for biodegradation, EE2 accumulates in sediments. Degradation under anaerobic conditions that occur in sediments is mostly slower than under aerobic conditions or degradation is even impossible. Whereas EE2 concentrations in the surface waters are in the ng/L range, concentrations in sediments have been reported in μg/kg range (**?**). That supports a hypothesis that sediments are the major sink of estrogenic contaminants and that benthic organisms may be exposed to much higher concentrations of those contaminants than free-swimming fauna.

1.2.4 Uptake of EE2

Estrogenic chemicals from WWTP effluents discharged into the aquatic environments can enter the fish through different routes, such as gills, skin and also gastrointestinal absorption from food (Lien and McKim, 1993). Recently, it has been shown that EE2 is primary taken up via the gills due to its affinity to sex hormone binding globulin, which is a carrier glycoprotein located on the filament arteries (Scott et al., 2005). Blewett (2011) reported tissue-specific pattern of EE2 accumulation after 2h exposure to 100 ng/L of radio-labeled EE2, with highest accumulation in the liver and gall bladder that accounted for 40% - 60% of the total accumulation. The carcass showed 20-30% accumulation while the gut accounted for 10 - 20% and spleen and gills for less than 10% of the total burden.

Even though there are numerous studies available on the effects of EE2 in fish (Versonnen and Janssen, 2004; Peters et al., 2007; Orn et al., 2006; Andrew et al., 2010), bioaccumulation has been to date subject of only a few studies. Al-Ansari et al. (2010) measured EE2 concentrations in shorthead redhorse suckers *(Moxostoma macrolepidotum)* collected near a WWTP in the St. Clair River in Ontario, Canada. Concentration of 1.6 ± 0.6 ng/g (wet weight) was measured in males and 1.43 ± 0.96 ng/g in females. No EE2 was detected in the samples from the reference site (Port Lambton). The authors hypothesise that possible source of EE2 in *Moxostoma macrolepidotum* is feeding on benthic invertebrates. These results are in agreement with

Dussault et al. (2009) that suggest that consumption of invertebrate food items could provide an additional source of exposure to estrogenic substances in vertebrate predators.

1.2.5 Effects of EE2 in the aquatic environment

The toxic potential of estrogen compounds to various organisms has been extensively studied. A number of biomarkers have been developed and used as early warning signals to provide an overview of EE2 toxicity on aquatic organisms. Both *in vitro* and *in vivo* studies have reported evidence concerning the effects of exposure to estrogens, such as the induction of plasma vitellogenin in male and juvenile fish, occurance of intersex conditions, decreased egg and sperm production, reduced gamete quality, reduced fertility and fecundity and also behavioral changes (Peters et al., 2007; Versonnen and Janssen, 2004; Orn et al., 2006; Andrew et al., 2010; Ferreira et al., 2009).

Vitellogenin (Vtg) is a phosphoglycolipoprotein and the egg yolk precursor, that is synthetized in the liver of sexually matured female teleost, after binding of E2 to estrogen receptors (ER) in the hepatocytes. It results in secretion of Vtg into the blood stream and its transportation to the ovaries, where it is cleaved into developing oocytes and forms the egg yolk proteins. Males and juveniles also posses the Vtg genes, but they are under normal circumstances inactive. However, the expression of Vtg by male and juvenile fish may be triggered after exposure to E2 or other estrogenic substances (Luo et al., 2011). Several studies demonstrated high levels of plasma Vtg in the male fish caught in the rivers and estuaries (Madsen et al., 2013; Hashimoto et al., 2000; Allen et al., 1999; Purdom et al., 1994b). Vtg production by juvenile and male fish has been first proposed as an effective biomarker to indicate exposure to EDCs in 1994 by Purdom et al. (1994c) and is used extensively ever since. In recent years, numerous methods for Vtg detection have been developed. Those methods include various immunoassay techniques such as radioimmunoassay (RIA), immunodiffusion, immunoblotting or enzyme-linked immunosorbent assay (ELISA) (Lomax et al., 1998). ELISA is the most favoured method widely used for detection and quantification of proteins due to its relative simplicity, good sensitivity and use of non-radioactive agents. Vtg purification is an essential step for development of ELISA. Several methods have been described for VTG purification e.g. anion exchange chromatography

(Kishida et al., 1992), size exclusion (Matsubara et al., 1999) chromatography or simple precipitation (Sherry et al., 1999).

1.3 Combined effects of nanoparticles and other pollutants - "The Trojan horse" effect

Due to the large reactive surface to volume ratio and subsequent high sorption capacity and reactivity, ENPs can interact with anthropogenic contaminants of concern and influence their fate and effects in aquatic environment. This property of ENPs is used for environmental remediation of contaminated sites (Karn et al., 2009) and is expected to bring benefits to the medical sector as nanoparticle-mediated delivery systems for preventive treatment of the oxidative damage occurring in neurodegenerative diseases like Alzheimer's, Wilson's and Parkinson's (Brigger et al., 2012; Cui et al., 2005; Roney et al., 2005; Choi et al., 2007b). However, interactions between ENPs and anthropogenic contaminants could affect behaviour and distribution of the anthropogenic contaminants in the environment. The presence of ENPs can therefore enhance impacts of contaminants, highlighting research needs especially at pollution hot spots like waste water treatment plants and harbours. Evidence for this facilitated transport of associated molecules or chemicals, the so called "Trojan horse" transport mechanism, which can be coupled to increased toxicity, has been found in recent studies (Baun et al., 2008; Zhang et al., 2007; Park et al., 2010).

1.4 Pollution in the Trondheimsfjord, Norway

The Trondheimsfjord is situated between 63°18.9'N, 9°50.9'E and 64°07 N, 11°18.9 E and with the lenght of 130km it is the third longest fjord in Norway. The Trondheimsfjord is rich with fish species, with more than a hundred fish species present, including many of the most important Norwegian commercial fish (cod, saithe, haddock, whiting, blue whiting, hake, herring, sprat, and several flatfish species). Over the years it has been intensively studied and investigations of the pelagic and benthic ecosystems revealed different sources of anthropogenic pollution including shipping, industrial activities, agricultural runoffs or discharged effluents

Figure 1.3: The bottom topography of the receiving waters near the Høvringen and Ladehammeren WWTPs

from WWTPs. With a population of 181 513 (October 1, 2013) the Trondheim municipality is the third most populous municipality in Norway. The town is served by two WWTPs at Høvringen and Ladehammeren. The larger Høvringen wastewater plant was constructed in 1970's and implemented a primary tratment of the sewage in 2004. The discharges from Høvringen pass through a diffuser system through two obliquely positioned pipes ending about 180 m offshore with diffusers at 49-65 m depth in order to mix the wastewater effluent with fjord water and prevent the pollutants from reaching the surface or risking an exposure of the shorelines with faecal bacteria and other potential harmful organisms. Previous studies showed elevated levels of contaminants (such as polyaromatic hydrocarbons, heavy metals, polychlorinated biphenyls, pesticides, and brominated flame retardants) in organisms from Munkholmen, which is an island close to Trondheim city and harbour (Rygg, 2002; Berge, 2003a,b) (figure 1.3). Numerous studies have shown presence of EE2 in WWTPs effluents (Dussault et al., 2009; Wang et al., 2014; Routledge et al., 1998; Aris et al., 2014) and its harmful effects on the marine organisms (e.g. feminization of male fish). However, to date no study on EE2 and Ag occurance in the WWTPs in Trondheim has been conducted.

1.5 Turbot as a study species

Due to EE2 occurance in aquatic environment and its accumulation in marine sediments numerous studies have used fish species as research animals for studying the effects of estrogenic compounds. In this study, turbot *(Scophthalmus maximus)*, a valuable species for commercial fishing in Europe and Asia, were selected. Turbot is a marine demersal carnivorous fish naturally abundant in European waters, along all European coasts to Morocco. Adult turbots feed mainly on other bottom-living fishes, while the juvenile's diet is based on crustaceans.

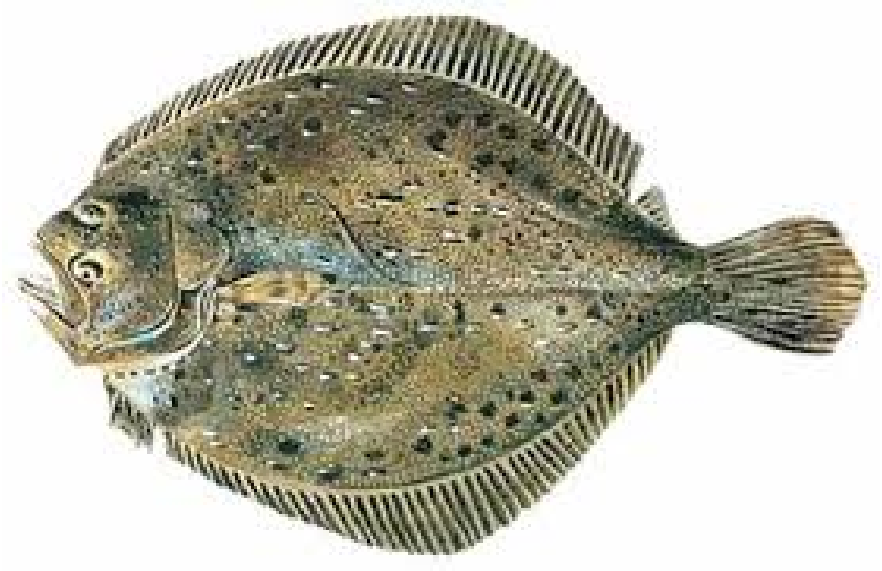


Figure 1.4: Study species: *Scophtalmus maximus*

1.6 Aim of the study

The aim of this study was to investigate exposure and potential effects of AgNP and 17α-ethinyl estradiol (EE2), both alone and in combination on turbot *(Scophthalmus maximus)* as a representative benthic vertebrate.

The main objectives of this master thesis were:

1. To gain a better understanding of the potential routes of exposure for AgNPs and Ag^{+} in *Scophthalmus maximus* and possible enhanced uptake of EE2 by the so called 'Trojan horse effect'.

2. To investigate possible toxic effects of EE2 in the presence of AgNPs in *Scophthalmus maximus*.

3. To determine vitellogenin levels and Ag concentrations in wild flat fish sampled at the outlet of Høvringen waste water treatment plant in Trondheim.

Chapter 2

Materials and methods

2.1 Nanoparticles

Polyvinylpyrrolidone (PVP) coated nanoparticles (1%) with a nominal diameter of approximately 15 nm were purchased from Particular GmbH (Hannover, Germany). The particles were delivered as aqueous dispersions at a concentration of 100 mg/L. To determine particle shape and size, 100 μl of the stock dispersion 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. Transmission electron microscope images were taken with a Zeiss Libra 120 EF TEM (Carl Zeiss AG, Germany). The hydrodynamic diameter of the particles was determined with dynamic light scattering (DLS) in MilliQ water with a N5 submicron Particle Size Analyzer (Beckman 150 Coulter Inc, CA, USA). The samples were filtered through a 200 nm filter prior to DLS analysis. Nanoparticle aggregation 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 suspensions in seawater (10 mg/L) were prepared and the absorbance was determined after 0, 1, 2, 3 and 20 h.

2.2 Experimental setup

Juvenile turbot with an average size of 23 ± 0.7 cm and an average weight of 204 ± 12 g were purchased from Stolt Sea Farm Turbot Norway AS (Kvinesdal, Norway). The fish were divided randomly into 23 different 60L polymer thick films (PTF) tanks with 5 fish per tank. The exposure tanks were divided into 8 different exposure groups: control, AgNP200, AgNP2, AgNP200EE2, AgNP2EE2, EE2, Ag+ (with 15 individuals per exposure group) and PVP (10 fish per exposure group). The nominal concentrations for each exposure group are given in (table 2.1). The seawater was filtered through 200 μm and 100 μm filters and water temperature was set to 14 °C. The water inflow was set to 340 ± 7 mL/min, resulting in a turnover of the tank volume in 3h. Exposure tanks were aerated with pressurized air and covered with semi see trough lids, that dimmed the light to reduce stress for the fish. The light and dark period was 12h/12h. The fish were allowed to acclimatize and fed *ad libitum* for 2 weeks before the start of the experiment. Feeding was stopped during the exposure.

	AgNP (μg/L)	EE2 (ng/L)	Ag^+ (μg/L)	PVP (mg/L)
Control	0	0	0	0
PVP	0	0	0	10
AgNP2	2	0	0	10
AgNP200	200	0	0	10
Ag+	0	0	50	0
AgNP2EE2	2	50	0	10
AgNP200EE2	200	50	0	10
EE2	0	50	0	0

Table 2.1: Nominal exposure concentrations of AgNP, Ag^+, EE2 and PVP in different exposure groups

2.3 Exposure and sampling

The fish were exposed daily to a 3h pulsed exposure for 13 days. The water flow was shut down during the exposure duration. Oxygen saturation and ammonia concentrations were monitored to ensure a good water quality. To determine Ag exposure concentrations due to Ag changes in over time and potential Ag accumulation in the tanks, samples for Ag quantification were taken at different time points in control tanks, AgNP200 and Ag^+ tanks (figure 2.1). Water samples for Ag analysis were preserved in 0.1 M nitric acid (HNO_3) and analyzed with high resolution inductively coupled plasma-mass spectrometry (HR-ICP-MS) (Thermo Fisher Scientific, Waltham, USA).

In order to determine EE2 concentrations in EE2, AgNP2EE2 and AgNP200EE2 exposed tanks, water samples were taken before, during and after the exposure period at 2 days of the experiment. One L of water was taken for each time point and immediately 50 μL of 0.4 ng/μL deuterated d4-ethinylestradiol (d4-EE2) was added as internal standard. The samples were extracted with solid phase extraction columns (Chromabond® C18, Macherey Nagel, Düren, Germany). Columns were stored at – 20 °C until further analysis. Concentrations of EE2 were determined by gas chromatography–mass spectrometry (GC-MS).

Figure 2.1: Experimental setup for the AgNP and EE2 exposure with twenty-three 60L PTF tanks containing 5 fish per tank.

The AgNP agglomerate dissolution was determined by ultracentrifugation followed by ICP-MS analysis of the supernatant. The AgNP samples of nominal concentration 200 μg/L AgNP were centrifuged at 20,000 rpm for 1h in order to let the undissolved AgNPs sink down. Then, the supernatant was taken and diluted with HNO_3 for measurement by ICP-MS (Thermo Fisher Scientific, Waltham, USA). To verify this result, the free Ag ion activity was also determined by the use of ion selective electrode (ISE).

After 13 d of pulsed exposure, the fish were anesthetized with tricaine mesylate (MS222) and measured and weighted individually. Blood samples were taken from anesthetized fish from the caudal vein using heparinized syringe. To obtain plasma samples for Vtg analysis, the blood was centrifuged at 13 000 rpm for 5 min (Eppendorf MiniSpin Plus) and plasma aliquots were stored at -80 °C until vitellogenin analysis. Fish were killed by severing the spinal cord. Samples from gills, stomach, liver, kidney, brain, muscle and bile were taken to determine Ag tissue distribution. Samples were stored at -20 °C until further processing.

2.4 Field investigations of pollution in Trondheimsfjord

Sixteen individuals from Pleuronectidae family *(Pleuronectes platessa, Limanda limanda, Platichthys flesus)* were caught close to the outlet of Høvringen WWTP in Trondheimsfjord, Norway in September and October 2012. Another seven individuals were caught in September 2014 at Sørburøy, Norway, that was chosen as the clean reference site. All of the fish were transported to the laboratory and were sampled the same day as described in section 2.3.

2.5 ELISA analysis

2.5.1 ELISA development

Purified Vtg was required for the standard curves of the ELISA and to coat the 96-well microtiter plates. It was obtained by concentration of Vtg from plasma of turbot injected repeatedly with the natural estrogen 17β-estradiol. This was achieved by precipitating Vtg in turbot plasma by adding EDTA, centrifugation, resuspension, addition of $MgCl_2$ etc. as described by Silversand and Haux (1989). The final Vtg precipitate was resuspended in phosphate buffered saline, aliquoted and stored at -80 °C until analysis. A chessboard titration was used to select the dilution factor of primary and secondary antibodies for use in the ELISA analysis. Two-fold serial dilutions of purified Vtg (400 ng/mL to 0.4 ng/mL were prepared in a coating buffer (0.05 M carbonate – bicarbonate buffer of pH 9.6 at 25°C) and used for coating each well column-wise (150 μl per well) on 96-well microtitration plate (Nunc-Immuno™ MicroWell™ 96 well solid plates, M9410 SIGMA, SIGMA-ALDRICH, Co., USA). The coated plates were sealed with an aluminium foil and incubated overnight (16 h) at 4 °C after which the plates were washed 3 times with 300 μL washing buffer (PBS-T phosphate buffered saline + 0.05 % Tween) to remove all unbound antigens and blocked with 250 μL blocking buffer (1% Bovine Serum Albumine (BSA) in PBS-T) to reduce non-specific binding (NSB). After 1 hour incubation at 37 °C the plates were washed again three times with 300 μL washing buffer.

A dilution series of primary antibody (rabbit anti-turbot Vitellogenin polyclonal antibody CS-2, Biosense Laboratories AS, Bergen, Norway) (1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000) was added row-wise to the plate (150 μL per well). The plates were sealed and incubated for 1h at 37 °C and then washed three times with PBS-T. A secondary antibody (Anti-Rabbit IgG, A6154, SIGMA-ALDRICH, Co., USA) was diluted 1:2000 in blocking/dilution buffer and added to the plate (150 μL per well). The plates were sealed and incubated again for 1h at 37 °C, after which the secondary antibody was removed and plates were washed for three times with PBS-T.

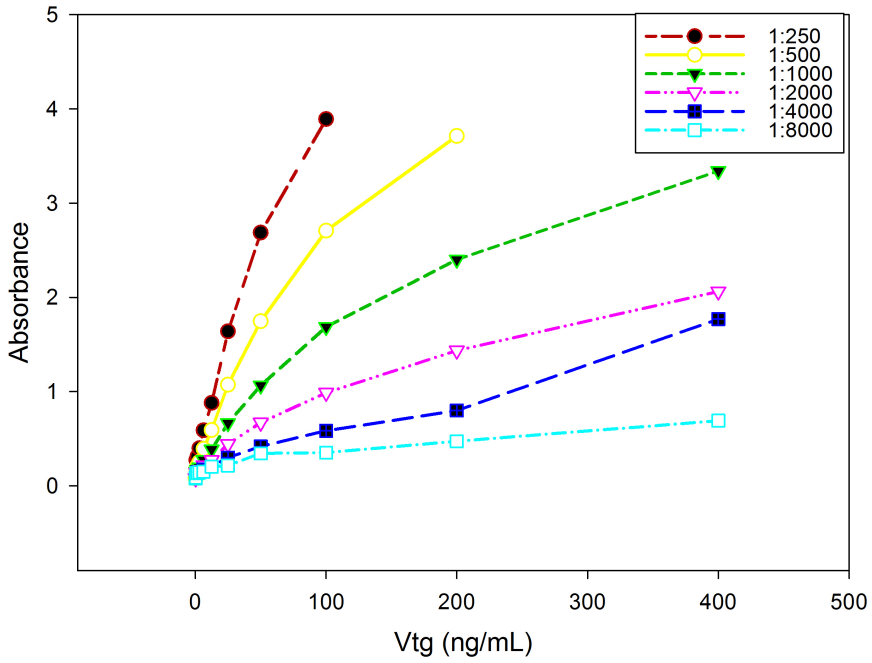


Figure 2.2: ELISA calibration curves of coating Vtg and primary antibody obtained by the chessboard titrations

The enzyme substrate solution (10 mg of ABTS [-$(NH_4)_2$], (2,2' –azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) in 20 ml of substrate buffer (sodium acetate trihydrate with adjusted pH to 4.1 with citric acid), added 8 μL of H_2O_2) was pipetted to each well (150 μL per well) and the absorbance (expressed as optical density, OD) of the wells was read after 15, 30 and 45 minutes at 405 nm using a microplate reader (ELx808™ Absorbance Microplate Reader, BioTek, USA). Combinations of coating Vtg dilutions and diluted primary antibody that had absorbance values 1.2 – 1.8 within 30 min were noted and evaluated for further use through preparation of calibration curves (figure 2.2).

Based on the results of chessboard titration the 96-wells plates were coated with purified Vtg (100 ng/mL) in 0.05 M carbonate-bicarbonate buffer (150 μL per well) and incubated overnight at 4 °C. The same procedure was used as for previously described chessboard titrations. A two-fold dilution series of primary antibody in PBS (1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000) was added column-wise to the plate and dilution series of secondary antibody (1:1000, 1:2000, 1:3000, 1:4000) was added row-wise. Combinations of primary and secondary antibodies that yielded an OD of 1.2 – 1.8 within 30 minutes were noted. Coating Vtg of concentration 100

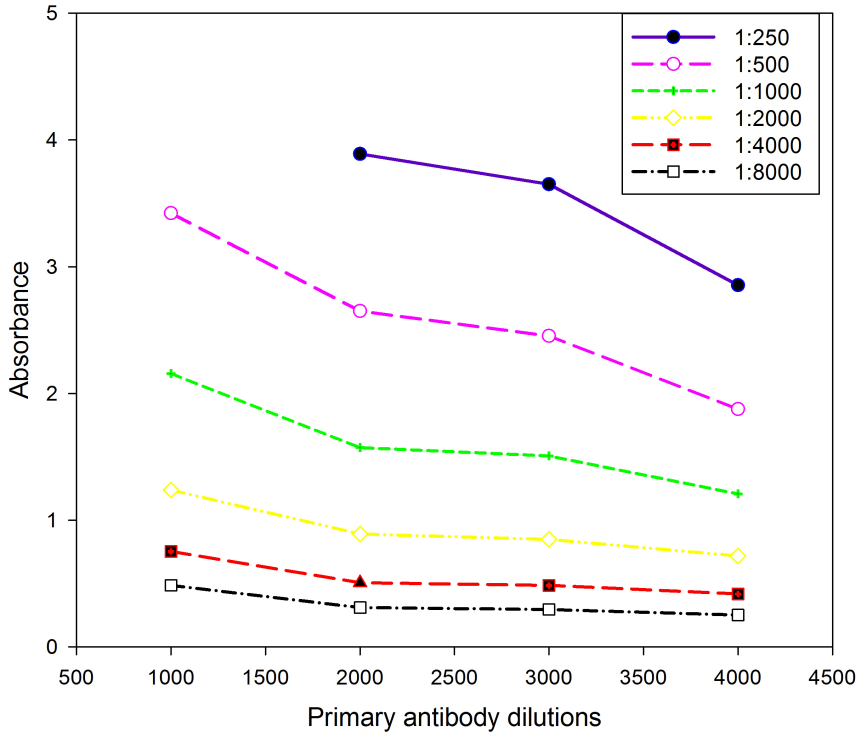


Figure 2.3: ELISA calibration curves of primary and secondary antibody dilution obtained by the chessboard titrations

ng/mL , primary antibody dilution of 1:1000 and secondary antibody dilution of 1:2000 were selected for ELISA analysis based on ELISA's working range and preservation of reagents (figure 2.3).

2.5.2 ELISA procedure

Purified Vitellogenin was used to coat the 96-well microtitration plates (100 ng/mL) and for preparation of the standard curve. Three wells were coated with BSA in coating buffer and used for non-specific binding (NSB). The coated plates were incubated overnight at 4 °C. A 1000 ng/mL standard Vtg in PBS-T was serially diluted in a two-fold series to 0.98 ng/mL. The samples were diluted 500× in PBS-T. Both samples and Vtg standards were mixed (1:1) with diluted primary antibody (1:1000 in PBS-T) and incubated in glass tubes for 1 hour at 37 °C. Triplicate aliquots of standards and samples (150 μL) were transferred to the plates. The ELISA procedure was identical to chessboard titrations from this point.

Vtg absorbance values read after 30 min (expressed as OD) were converted to the proportion of antibody bound (B) expressed as a percentage of that in the zero standard by the following equation:

$$B = (OD - NSB / OD_0 - NSB) \times 100 \tag{2.1}$$

where OD is the absorbance of a given sample or standard, OD_0 is the absorbance of the zero standard and NSB is the non-specific binding absorbance value. Binding percentage values were logit transformed by the following equation:

$$LogitB = LOG_{10} \times (B/(1-B)) \tag{2.2}$$

and plotted againts log Vtg concentration to achieve a linear transformation of standard and plasma dilution curve.

2.5.3 ELISA validation

A calibration curve, prepared from serial two-fold dilution of purified Vtg (10000, 8000, 6000 ng/mL) was made. A log-log curve fit was used to define the relationship between the concentration of Vtg and absorbance. Vtg dilution series generated standard curves with a working range between 23.44 and 5000 ng/mL with 50% binding around 180 ng/mL (figure A.3). Broad working range of ELISA was important because of predicted variety in Vtg concentrations in the samples.

Determination of the limit of detection (LOD) and limit of quantitation (LOQ) was performed according to the guidelines of International Standards Organization (ISO) and International Union of Pure and Applied Chemistry (IUPAC) (ICH Steering Committee, 1996). LOD is the smallest amount of concentration that can be reliably distinguished from zero. LOQ is a concentration above which the analytical method can operate with an acceptable precision (Shibahara et al., 2013) LOD was calculated as the mean measured content of blank samples plus 3.3 times the standard deviation (SD) of the mean value and the LOQ as the mean mea-

sured content of blank samples plus 10 times the SD of the mean value.:

$$LoD = NSB + 3.3 \times SD(NSB) \tag{2.3}$$

$$LoQ = NSB + 10 \times SD(NSB) \tag{2.4}$$

Both values were determined from NSB-corrected absorbance levels and recalculated into concentration using the relevant standard curve equations.

The within-day and between-day precisions were calculated based on standard curves from 8 assays. Two standard curves were freshly prepared each day and used on two plates for four days. The average within-day precision was 5.4% (table A.2) and the average between-day precision was 12.2% (table A.3). Sample from the highest EE2 exposure group was also used as a positive control on each plate, that was measured. The average within-day precision for this positive control was 6.8% and the average between-day precision was 12%.

2.6 ICP-MS

Samples from gills, stomach, liver, kidney, brain and bile were taken to determine Ag tissue distribution. Samples were stored at -20 °C until further processing. Before digestion in a high-pressure microwave system (Milestone UltraClave, EMLS, Leutkirch, Germany), the tissue samples were freeze dried and weighted. Samples or certified reference materials were transferred to PTFE-Teflon vials and 2 mL of 50 %(v/v) HNO_3 was added. Digestion was carried out according to a temperature profile which increases gradually from room temperature up to 250 °C within 1 h. In addition there was a cooling step which allowed temperature to return back to the initial value within ca 1 h. Then, the digested samples were diluted with ultrapure water to 60 mL in polypropylene vials to achieve a final HNO_3 concentration of 0.6 M. Inductively coupled plasma mass spectrometry (ICP-MS) analyses was performed using a Thermo Finnigan model Element 2 instrument (Bremen, Germany). To assess possible contamination during sample preparation, blank samples of HNO_3 and ultrapure water were prepared by the same procedure as the samples.

Samples were divided according to their weight into 7 different size classes (0.5, 0.4, 0.3, 0.2, 0.1, 0.05 and 0.02 g). The method detection limits (MDLs) were calculated for each size class separately. MDLs were either based on 3 times the standard deviation of the blanks, or on the instrument detection limits (IDLs) depending on which method resulted in higher values. The IDLs were estimated from the subsequent analysis of solutions, containing decreasing, low concentrations of the element. Finally, the concentration resulting in a relative standard deviation of approximately 25% (n = 3 scans) were selected as IDL with baseline corrections applied for these values (table 2.2).

Method detection limits							
Size class (g)	0.5	0.4	0.3	0.2	0.1	0.05	0.02
Ag (μg/g)	0.0024	0.003	0.004	0.006	0.012	0.03	0.06

Table 2.2: Method detection limits for ICP-MS analysis of Ag

2.7 Statistics

2.7.1 ELISA

Statistical analysis was performed using STATISTICA (version 12, StatSoft). Weighted mean and standard errors (SE) were calculated for each exposure group. All data were log transformed and tested for normality using Shapiro-Wilk test and for equality of group variances using Brown-Forsythe test. The data showed normality in log-transformed form and were tested using one-way analysis of variance (ANOVA) followed by a Turkey post-hoc test. The level of significance was set as $p < 0.05$. Graphs were prepared with SigmaPlot 12.0 (Systat Software Inc., Chicago, USA).

2.7.2 ICP-MS

For statistical puprposes the values that were under the LOD were substituted by 50 % of the detection limit for the respective weight group. Data were log transformed and checked for normality using Shapiro-Wilk test and for equality of group variances using Brown-Forsythe test. Assumptions of ANOVA were not satisfied. Linear regression analysis with discrete (i.e. categorical) dependant variables (covariates) was performed to compare Ag content in different exposure groups within each organ. The level of significance was set as $p < 0.05$. The appropriateness of the model was verified by examining residual plots (figure A.1). Statistical analysis was performed using R (version 3.1.2). Graphs were prepared with SigmaPlot 12.0 (Systat Software Inc., Chicago, USA).

Chapter 3

Results

3.1 Aqueous Ag and EE2 concentrations

The average water concentrations of Ag measured in test aquaria during the flow-through experiment are shown in figure 3.1. The actual mean concentration of AgNP in AgNP200 group was 131 μg/L AgNP at the beginning of exposure and 125.6 μg/L at the end of 3 hours exposure period. Ag concentration of 31-33 μg/L was measured in the Ag^{+} exposed tanks at the beginning and at the end of the 3 hours exposure period. The dissolved EE2 concentrations in the EE2 exposure tank were 10.11± 1.8 ng/L at the beginning and 8.61± 1.1 ng/L at the end of the exposure period.

The amount of dissolved ionic silver from the high AgNP exposure group (200 μg/L) determined by ultracentrifugation followed by ICP-MS analysis of the supernatant was 15%. The amount of ionic silver within undiluted particle dispersions determined by ISE was 20 -25 % (nominal concentration 200 μg/L AgNP). The purchased nanoparticles had a nominal diameter of 15nm according to manufacturer. As shown by TEM, AgNPs were mostly present as single particles or loose agglomerates and were not seen to undergo aggregation in seawater (figure 3.2).

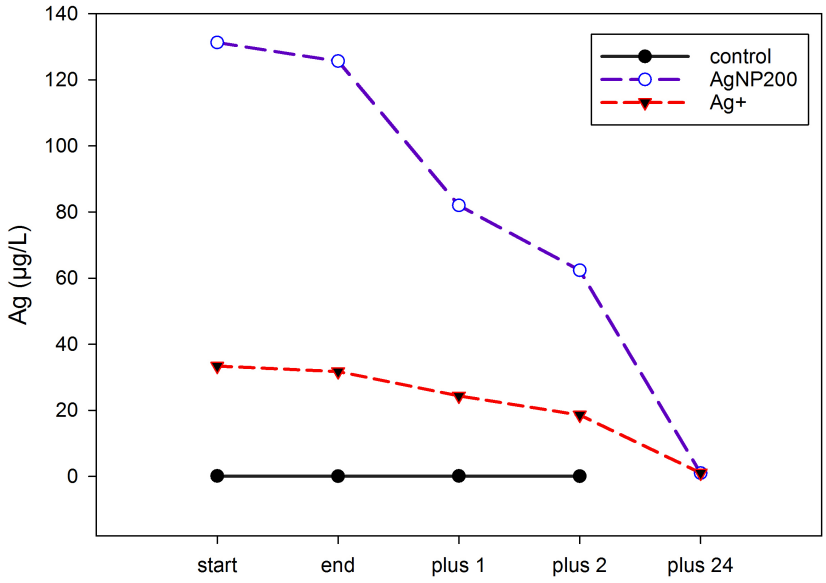


Figure 3.1: Measured concentrations of Ag in the seawater at the beginning and at the end of the 3 h exposure, and further at 1 h, 2 h and 24 h after re-opening the water.

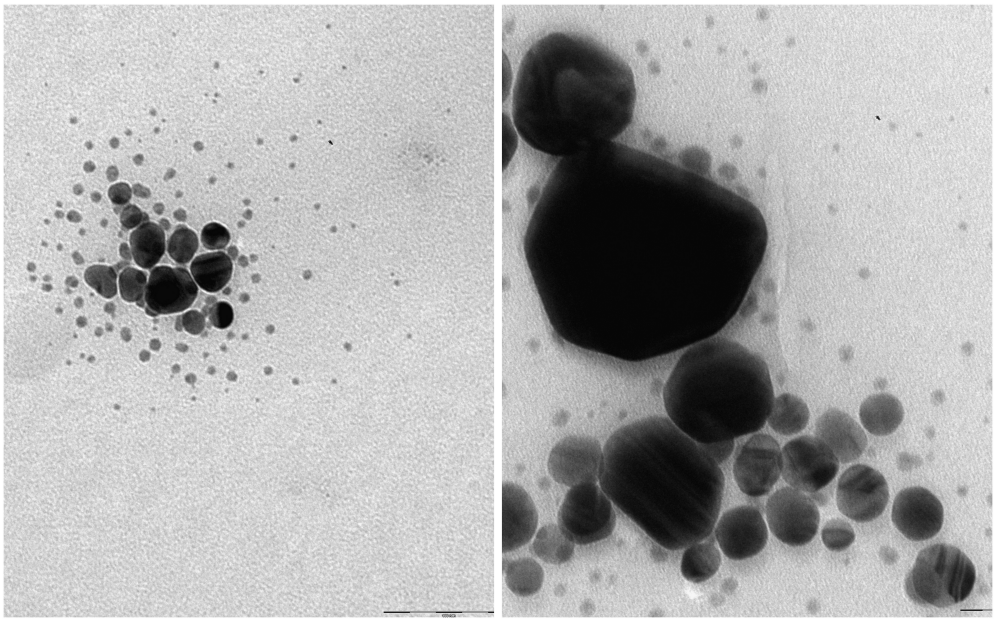


Figure 3.2: Transmission electron microscopy (TEM) images of silver nanoparticles in seawater. Scale bars are 100 nm and 1 μm

3.2 Ag uptake and accumulation

The average concentrations in the control groups were low (0.004±0.001 μg/g in liver, 0.007±0.002 μg/g in bile and 0.018±0.008 μg/g in gill)(mean+SE), or no Ag was detected (in stomach, muscle, kidney and brain tissue). Very low concentrations of Ag or values under the limits of detection were also measured in the PVP exposed group. Levels of silver in the brains of exposed fish were below the detection limit for all treatments. In all of the investigated organs the concentration of Ag was significantly higher in AgNPs and Ag ions exposed fish than in the control group or fish exposed to PVP. The uptake of Ag into tissues was exposure dependent (table A.1).

In all exposure groups there were significantly enhanced levels of silver in/on the gills compared with controls, except for the PVP exposure group. The highest concentrations of Ag were measured in gills of fish from the AgNP200 group (1.250±0.262 μg/g) (mean+SE) and there was no statistically significant difference between AgNP200 and AgNP200EE2 (0.733±0.132 μg/g) exposure groups (figure 3.3). The Ag concentrations in the AgNP200 and AgNP200EE2 groups were significantly higher compared to the Ag concentrations in the gills of fish from the low AgNP treatment, where the concentrations were about ten times lower (0.100 ±0.030 in AgNP2 and 0.063 ±0.012 in the AgNP2EE2 exposure group). Similar mean Ag concentration were observed in the gills of fish from the Ag+ exposure group (0.196±0.028 μg/g).

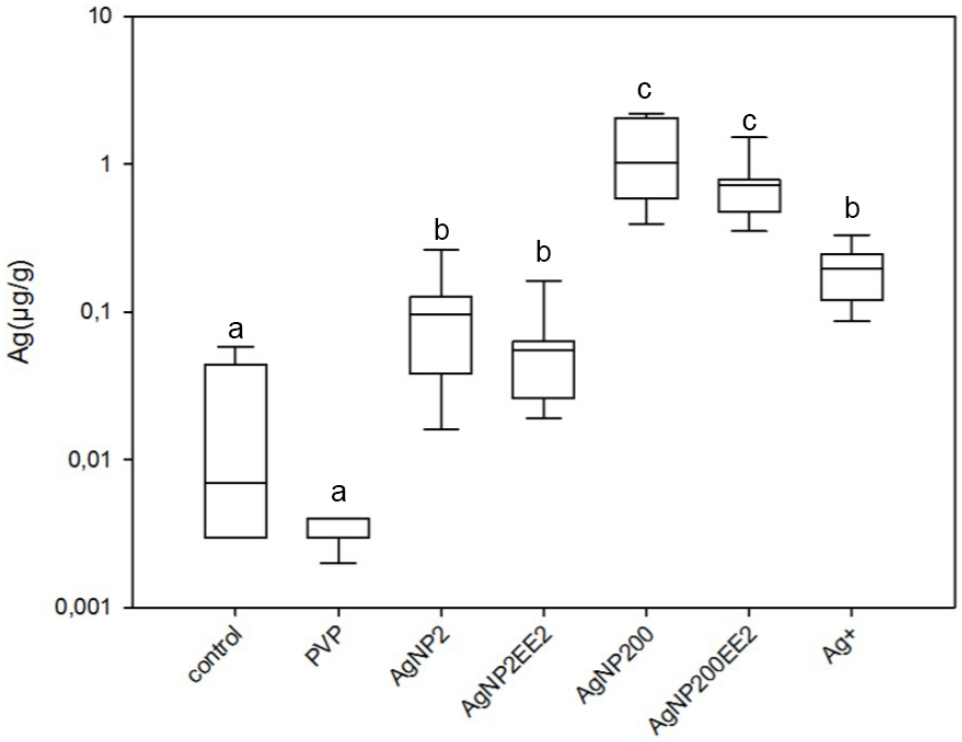


Figure 3.3: Concentration of Ag (μg/g) in gills of *Scophthalmus maximus* in different exposure groups as determined by ICP-MS. Data are displayed as median ± 75th and 25th percentiles (box boundaries) and 10th and 90th percentiles (whiskers). Experimental groups consisted of seven fish. The same letters indicate no statistically significant differences between exposure groups $p < 0.05$ (Linear regression)

In all exposure groups, except for the PVP group, the concentrations of Ag in the liver were significantly higher than in the control group. The highest uptake occurred in the liver of fish exposed to AgNP200EE2, AgNP200 and Ag^+ (0.453±0.102, 0.389±0.121 and 0.512±0.066 μg/g respectively). These groups were not significantly different among each other. The uptake of Ag into the liver was significantly higher in AgNP200 and AgNP200EE2 exposure groups (0.389±0.121 μg/g and 0.453±0.102 μg/g, respectively) and Ag^+ exposure group (0.512±0.066 μg/g) than the Ag uptake in the AgNP2 and AgNP2EE2 treatment groups (0.019±0.004 μg/g and 0.022±0.004 μg/g, respectively) (figure 3.4). In the high AgNP exposure groups the silver content was two times lower than the levels in the gill tissue in those fish. However, in the Ag^+ exposure group, the hepatic concentration of silver was more than two times higher than in the gill tissue.

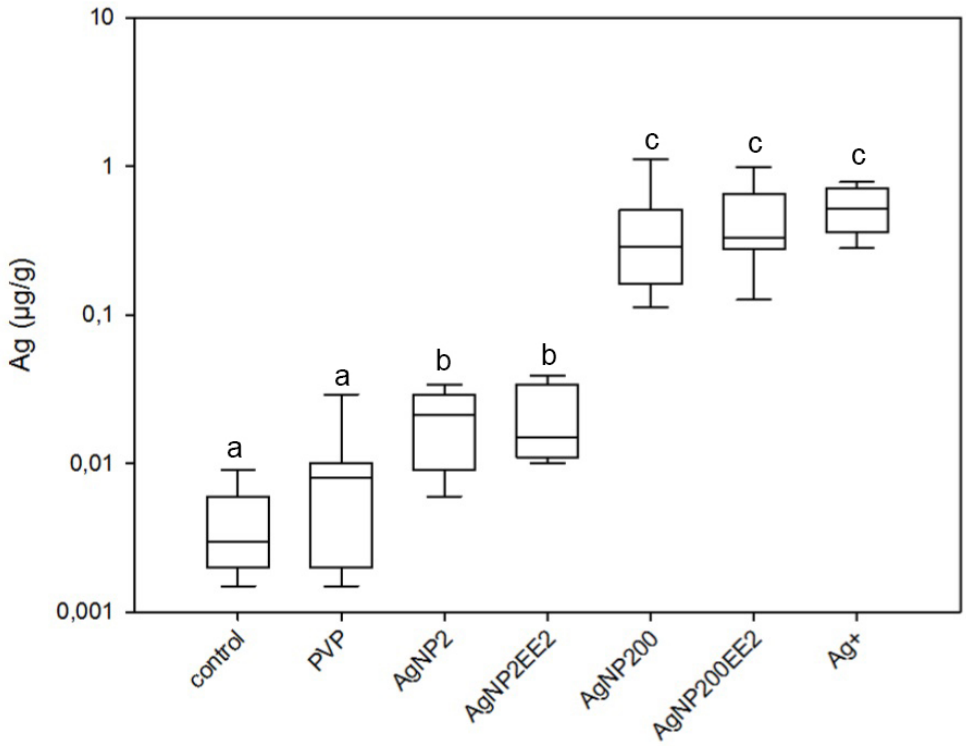


Figure 3.4: Concentration of Ag (μg/g) in liver of *Scophthalmus maximus* in different exposure groups as determined by ICP-MS. Data are displayed as median ± 75th and 25th percentiles (box boundaries) and 10th and 90th percentiles (whiskers). Experimental groups consisted of seven fish. Same letters indicate no statistically significant differences between exposure groups $p < 0.05$ (Linear regression).

The concentrations of Ag in stomach, muscle and kidneys were much lower than the concentration in the gills or liver. The same pattern with high AgNP and Ag^{+} exposure groups being significantly different from the low AgNP exposure groups was observed (table A.1).

3.3 Plasma vitellogenin

Figure 3.5 shows the Vtg induction in plasma of juvenile turbots after 13 days exposure. Fish exposed to EE2 treatment (EE2, AgNP2EE2, AgNP200EE2) showed significant ($p < 0.05$) induction of Vtg compared to the control group. The highest concentration of Vtg protein was measured in the EE2 treated group (104 558 ng/mL) followed by AgNP2EE2 (98 392 ng/mL) and AgNP200EE2 (73 456 ng/mL). There was no statistical difference between those groups, even though there was a tendency to lower concentration of induced Vtg with higher concentration of AgNPs. In the absence of EE2, the fish exposed only to AgNP2, AgNP200 and PVP had mean Vtg concentration below 25 000 ng/mL. The measured concentration of Vtg were slightly higher in Ag^+ treated group (40 805 ng/mL). The Ag^+ exposed group showed statistically significant difference from the control and PVP exposed group, but no statistically significant difference from the AgNP2 and AgNP200 exposed group was observed. No gender differences were observed.

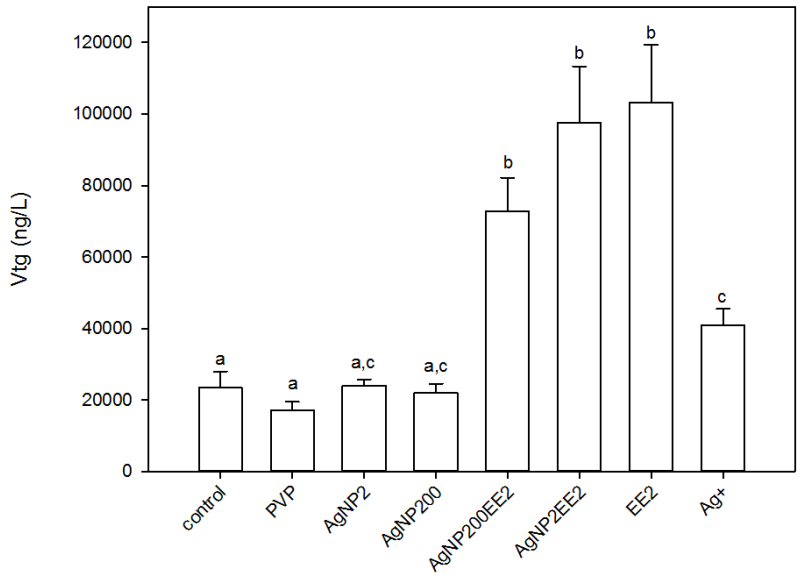


Figure 3.5: Mean plasma vitellogenin concentration in juvenile turbots after 13 days of exposure. Error bars represent SEM (for control, AgNP2, AgNP200, AgNP200EE2, AgNP2EE2, EE2, Ag+ n=15, for PVP exposed group n=10). Same letters indicate no statistically significant differences between exposure groups $p < 0.05$ (one-way ANOVA and Turkey post-hoc test)

3.4 Free-ranging fish

The fish sampled at the outlet of the Høvringen WWTP in Trondheimsfjord had mean Vtg plasma concentrations between 22 000 and 63 000 ng/mL. The mean Vtg concentration measured in males was 36 279 ± 1 308 ng/mL (n = 3) and in females: 44 044 ± 3 980 ng/mL (n= 10) (mean ± SEM). The fish from reference site at Sørburøya were not sampled for plasma. The mean Ag concentration in fish from Høvringen were 0.107 ± 0.033 μg/g (dw), 0.0284 ± 0.019 μg/g (dw), 0.300 ± 0.087 μg/g (dw) (mean ± SEM) in liver, kidney and brain, respectively. In muscle the concentration of Ag were under the limit of detection. The highest Ag concentration measured in the fish from Sørburøya was in the liver, followed by brain, kidneys and gills. The measured values were 0.610 ± 0.175 μg/g (dw), 0.226 ± 0.032 μg/g (dw), 0.012 ± 0.004 μg/g (dw) and 0.010 ± 0.001 μg/g (dw), respectively.

Chapter 4

Discussion

There is a lack of information in the literature regarding the uptake and biodistribution of silver nanoparticles into internal organs or the potential toxicity of silver nanoparticles in see-water fish. Moreover there are very few studies that would investigate combined effects of AgNPs and other pollutants. The main aims of this master thesis were, therefore, to identify the target organs for acummulation of silver nanoparticles as a result of laboratory exposure to different concentrations of silver nanoparticles alone and in combintation with EE2 in *(Scophthalmus maximus)*.

4.1 Exposure and uptake of AgNPs

It is important to measure the actual water concentration in order to avoid under- or overestimation caused by e.g overdosing or adhesion of particles to aquaria surfaces. The analysis of water samples from AgNP200 and Ag^+ exposure tanks showed decreasing concentrations of Ag over time. The actual mean concentration of AgNP in the high AgNP exposure group (AgNP200) was 131 μg/L AgNP at the beginning of exposure and 125.6 μg/L at the end of 3 hours exposure period. Because it is unclear whether AgNP toxicity is specifically related to nanoparticle properties or is caused due to the effects of dissolved Ag^+ ions, the turbots were exposed to Ag^+ in addition to AgNP. It was determined that 15-25% of the AgNP was present as Ag^+ ions by measuring the ionic activity and by ultracentrifugation followed by ICP-MS. Thus, the concentration of dissolved Ag^+ from AgNP during the exposure was approximately 19-33 μg/L in the

high exposure group. These results correspond to 31-33 μg/L of Ag that were measured at the beginning and at the end of the 3 hours exposure period in the Ag^+ exposed tanks.

The knowledge of body distribution of AgNPs in organisms is limited due to lack of methods for the direct measurements of ENPs in the tissues and organs. Current approaches for the measurement of ENPs involve electron microscopy and special microscopic techniques such as scanning electron microscopes or transmission electron microscopes (Handy et al., 2008a). These techniques allow to obtain images at nanometre resolution. Moreover scanning probe microscopy offers atomic resolution (Madhavan, 2004). However, electron microscopy is very labour consuming. Furthermore it deals with a need to develop suitable radiolabelled metal ENPs of environmental relevance. There is also a need for validation that the label does not become detached from the ENPs by dissolution once it enters the organism (Shaw and Handy, 2011). In this study, acid digestion followed by ICP-MS was used to analyze Ag concentrations in different fish tissues. The advantage of ICP-MS is its elemental specificity, good resolution and low detection limits.

After repeated exposure of turbots to AgNPs and Ag^+, Ag was detected in all organs evaluated (gills, stomach, liver, kidneys) except for brain. This is in agreement with previous exposure studies reporting distribution of silver nanoparticles to multiple organs of fish (Hawkins et al., 2014; Farmen et al., 2012; Scown et al., 2010a; Gaiser et al., 2012). In all analyzed tissues, except for gills, no statistically significant difference was observed between the high AgNP exposure groups (AgNP200, AgNP200EE2) and the group that was exposed just to silver ions (added as $AgNO_3$). The amount of ionic silver within undiluted particle dispersions from the high AgNP exposed group determined by ultracentrifugation and ISE was 15-25%. Thus, the concentration of dissolved ionic Ag from the high AgNP exposed group corresponds to the actual concentration ionic silver in the Ag^+ exposed group. In gills the concentration of Ag was significantly higher in high AgNP exposure groups (AgNP200, AgNP200EE2) than in Ag^+ exposure group. The elevated levels of Ag were observed in all groups except for the control and PVP exposed group, thus verifying that AgNPs or Ag ions are accumulating in or being adsorbed on the gill surface. Gills are in direct contact with toxicants from aquatic environment. That, together with their physiological function makes them a primary target for waterborne chemicals (Reid and

McDonald, 1991). The gills are protected by a mucus layer that is able to chelate cations (Handy and Eddy, 1991). The surface charge of ENPs and their electrostatic properties are not fundamentally different from charged ions even though the speed and strength of the ENPs binding to the mucoproteins in the fish mucus may be different from the metal ions (Handy et al., 2008b). The contaminated mucus will subsequently slough in order to protect the epithelial cells. Eventually, the supply of mucus will get exhausted, which might occur faster in the presence of ENPs compared to other substances (SMITH et al., 2007). In addition, the ENPs undergo localised dissolution in the mucus layer. Ionic Ag has been proved to cause damage to gill lamellae of the rainbow trout (Scown et al., 2010b; Farkas et al., 2011). The high concentration of Ag in the gills in the groups exposed to AgNPs compared to Ag^+ exposed group suggests that AgNPs were trapped in the mucus layer on the surface of the gills. Similar phenomenon was observed with single walled carbon nanotubes that associated with gill mucus in rainbow trout *(Oncorhynchus mykiss)* (SMITH et al., 2007). Farkas et al. (2011) reported that PVP and citrate coated AgNPs accumulate in gill epithelial cells of rainbow trout and consequent transport of ionic Ag through these layers. The authors also showed that PVP coated particles caused a higher silver transport over the epithelium compared to citrate coated AgNPs. The low Ag concentration from the fish exposed to Ag ions only might be explained by high concentration of Cl^- in the seawater and consequent complexation of Ag ions. In addition cationic competition with Na^+ and Ca^2+ is likely to occur (Byrne, 2002). In contrast to this study, Farkas et al. (2011) observed the higher amount of silver in gill cells of rainbow trout exposed to Ag^+ than to AgNPs (10 and 20 mg/L). The authors reported more silver being transported over the cultured gills epithelium following AgNP exposure compared to Ag^+ exposure, pointing towards the possible transport of AgNPs through the epithelium rather than of ions released from the particles. Absorption of AgNP into the blood via the gills is therefore a possible uptake route. However, the species differences in the importance of gill as an uptake route were reported by Wood et al. (1999). While tidepool sculpin *(Oligocottus maculosus)* and seawater adapted rainbow trout *(Oncorhynchus myksis)* accumulated more Ag on the gills than in the intestines, the English sole *(Parophrys vetulus)* and the starry flounder *(Platichthys stellatus)* accumulated less Ag on the gills compared to the intestine. Present study shows higher accumulation of Ag on the gills compared to intestines.

Marine fish must drink to replace water lost through osmosis and thus avoid dehydration. As a result of drinking, the fish take up a large amount of monovalent ions across the gastrointestinal tract that are excreted across the gills by basolateral Na^+, K^+ -ATPase. The accumulation of Ag in the gut was highest in the fish exposed to ionic Ag^+, but AgNPs taken up orally may possibly undergo dissolution in the digestive tract and release free Ag ions. Since 15-25% of the total silver in the AgNP suspension was present in form of Ag ions, it is possible that only this fraction was absorbed by the intestine. It has been shown that Ag inhibits drinking in marine fish by an unknown mechanism. It has been suggested that Ag can suppress the drinking reflex by disrupting the renin-angiotensin system (Wood et al., 1999). Translocation of ENPs through the intestinal barrier is driven by endocytosis. Large particles tend to stay trapped in the mucus layer, while smaller particles enter the bloodstream and accumulate in liver. A study on rats showed that 14 nm latex particles, which were slightly negatively charged, crossed the distal colon mucus gel layer within 2 min. Particles with a size of 415 nm crossed it in 30 min, whereas 1 μm large ones did not cross at all (Szentkuti, 1997). The diameter of AgNP particles used for turbot exposure was 15 nm, therefore it is probable that the particles crossed the epithelial barrier and accumulated in the liver, where the Ag concentration was 3 to 9 times higher than in the stomach.

The major ways for removal of xenobiotics are renal, hepatic, biliary and branchial excretion. The branchial excretion through the gills depends on the diffusion gradient that would have to be reversed to the outward direction. Therefore excretion by vesicular trafficking from the basolateral membrane to the apical memmbrane of the gill seems unlikely (Handy et al., 2008a). The average concentration of Ag in the liver was 0.39 μg/g (AgNP200), 0.45 μg/g (AgNP200EE2) and 0.51 μg/g (Ag^+), which is 3 to 6 times higher than concentrations measured in kidneys. Glomerular filtration is a process that is highly dependent on molecule size of ENPs. Choi et al. (2007a) proposed that in mammals quantum dots can be cleared via the kidneys when the hydrodynamic diameter (HD) of nanoparticles is decreased to 5.5 nm. In the same study quantum dots with HD > 8 nm did not demonstrate renal filtration but instead exhibited uptake in the reticuloendothelial system and lungs. It is therefore unlikely that AgNPs with HD of 15 nm that were used for turbot exposure would pass the glomerular filter and hepatic excretion seems

more likely. However, no statistically significant difference between the high AgNPs exposure groups and Ag^+ exposed group suggests that only the Ag ions dissolved from the particles were detected in the liver.

The hepatobiliary clearance is the primary route of excretion for particles that do not undergo renal clearance. It relies on endocytosis and vesicular trafficking to form the bile. Most tissue distribution studies of nanoparticles have demonstrated that nanoparticles are transported to the liver and spleen (De Jong et al., 2008; Li and Huang; Xie et al., 2009). It has been suggested that particles smaller than the pore size of liver fenestrae (< 100 nm) are taken up by liver while larger particles lead to acummulation in spleen. Lankveld et al. (2010) reported that 20 nm silver nanoparticles were mainly distributed to liver of intravenously exposed rats, whereas the 80 and 110 nm particles mainly acummulated in spleen. Similarly, Choi et al. (2007a) suggests that liver is capable of capturing 10-20 nm particles for the clearance of viruses and other small objects. It has been shown, that metals can form deposits in the liver (Jones et al., 1989) and it can not be excluded that the same deposit formation can occur in the case of nanoparticles. However, several studies have shown that many metals are excreted from the liver to the bile (Hauser-Davis et al., 2012; Furumoto et al., 2001). The amount of Ag measured in liver was 0.39, 0.45 and 0.51 μg/g for AgNP2, AgNP200 and Ag^+ exposed group, respectively. The Ag concentration in bile was measured only in the fish exposed to AgNP2, AgNP200, Ag^+ and control group. The concentration of Ag in the AgNP200 and Ag^+ exposed groups was 0.37 μg/g and 0.39 μg/g, respectively. That confirms, that AgNPs or more likely Ag^+ ions were taken up by hepatocytes in the liver and excreted from the body via the bile.

4.2 Effects of EE2

Measured EE2 water concentration was 20% of nominal concentration 50 ng/L. Decrease in concentration was expected due to the hydrophobic properties of EE2 (low K_{ow}), partitioning to surfaces and uptake by the fish and is in agreement with other studies. Peters et al. (2007) reported measured EE2 water concentrations 10-20% of nominal values for the 100 ng/L EE2 treatment over a 24 h static exposure.

Over the past decades, several biomarkers have been developed for the detection and assessment of the estrogenic potential of EDCs. In fish, as representatives of ovarious species, induction of vitellogenin and zona radiata-proteins (Meucci and Arukwe, 2005), secondary sex characteristics, and sex ratio (Dang, 2014) are indicatives of chemicals interfering with steroidogenesis pathways (Hutchinson et al., 2006). Numerous *in vivo* and *in vitro* studies have shown the value of Vtg as a rapidly inducible biomarker for estrogens in both adult and juvenile fish (Hutchinson et al., 2006; Marin and Matozzo, 2004; Madsen et al., 2013; Dang, 2014).

The present study investigated the effects of EE2 on induction of Vtg in plasma of juvenile turbots. After 13 days of pulsed exposure to 0.05 μg/L EE2 alone and in combination with low and high concentrations of AgNPs (2μg/L and 200 μg/L, respectively) no mortality was observed in any of the treatments, indicating that the fish were not unduly stressed. Likewise, Versonnen and Janssen (2004) reported that exposure to 0.001, 0.01 and 0.1 μg/L EE2 for 2 weeks caused no mortality in eight-month-old zebrafish. Among different species, there have been reported considerable differences on Vtg response. The study of Orn et al. (2006) showed a difference in response between zebrafish and medaka after exposure to EE2. While exposure of zebrafish to 100 ng/L EE2 resulted in 100% mortality within 14 days, no mortality occurred in medaka. Vtg induction in carp *(Cyprinus carpio)* was reported to be much lower than in trout *(Salmo trutta)* (Purdom et al., 1994a). In the carp a Vtg induction has been observed at 10 ng EE2/L (Purdom et al., 1994a), while in the sheepshead minnow *(Syprinodon variegatus)* the Vtg induction occured at a concentration of 100 ng EE2/L (Folmar et al., 2000). For assessing the potential of species as test organisms, sensitivity is often ranked according to no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) values. For Vtg induction in

flounder *(Platichthys flesus)* NOEC of 2.7 ng/L and LOEC of 5.1 ng/L was reported by Madsen et al. (2013), which was in accordance with the LOEC of 14.5 ng/L found by Allen et al. (1999). Therefore, given the nominal exposure of 50 ng/L used in this study, significant induction of vitellogenin was expected.

The Vtg concentrations in juvenile turbots were significantly higher in EE2 exposed groups (EE2, AgNP2EE2 and AgNP200EE2) than in control group and non EE2 treated groups. The findings of EE2 induced vitellogenin as described in the present study are consistent with previous studies. However, no statistically significant difference was found between the Vtg levels in EE2 treated groups. Several studies have been conducted to assess xenoestrogen effects in fish. Allen et al. (1999) exposed male flounders caught offshore to 10 EE2 ng/L. The mean Vtg concentration at the beginning of the experiment was 68.8 ± 12.3 ng/mL in males and had risen to 1.37 mg/mL after 21 days of exposure. After the end of exposure period the fish were transferred to clean water and linear elimination of Vtg from the blood was observed. Mean plasma Vtg concentrations had decreased to 19 424 ng/mL approximately 3 weeks after the transfer to clean water. In another study, the ability of EE2 to elevate Vtg levels was investigated in male flounders exposed to EE2. Average Vtg concentrations in the control fish ranged between 25 and 100 ng/mL. After three weeks of exposure to 5.1, 8.1 and 16.8 EE2 ng/L (actual concentration of EE2 in water) the vitellogenin concentrations measured were 0.01, 2.1 and 17 mg/mL, respectively (Madsen et al., 2013). It should be noted, that relatively high concentrations of Vtg in exposed groups reported by Madsen et al. (2013); Allen et al. (1999) are due to differences in exposure setting. While most of the laboratory studies use continuous exposure, 3h/day pulsed exposure was used in this study.

Interestingly, statistically significant induction of Vtg was observed in the Ag^+ exposed group compared to control group, suggesting a possible effect of Ag^+ on estrogenic pathways. There was no statistically significant difference between Ag^+ exposed group and both low AgNPs treatments (AgNP2, AgNP200), even though the measured Vtg concentration in the AgNP exposed groups were much lower. Pham et al. (2012) observed induction of Vtg in the liver of male Japanese Medaka *(Oryzias latipes)* exposed to 1 μg/L and 25 μg/L AgNPs for 28 days leading

them to conclude that AgNPs either mimic or trigger receptors in estrogenic pathways and/or AgNPs might bind with proteins/antibodies in bloodstream of the fish and consequently trigger the metabolism pathways of estrogenic chemicals. However, given the partial dissolution of the AgNPs in present study, similar values for AgNP200 and Ag^+ exposed group would be expected. On the contrary, Gagné et al. (2012) reported decreased inducion of Vtg-like proteins in rainbow trout *(Oncorhynchus mykiss)* after 96 h exposure to 0.06, 0.6 and 6 μ/L of AgNP (20 nm) and silver nitrate ($AgNO_3$). The results of this study do not permit a definite conclusion on whether Ag^+ or nanosilver can induce Vtg and further research on this topic is needed.

4.3 Combined effects of AgNPs and EE2

In addition to the intrinsic toxicity of AgNPs, the interactions between AgNPs and other co-occuring contaminants may influence their environmental fate, bioavailability, and toxicity. However, the knowledge of the effects of nanosilver in combination with other pollutants is limited. The exposure of juvenile turbots to 50 ng/L of EE2 alone and in combination with high (200 μg/L) and low (2μg/L) concentrations of AgNP resulted in significant induction of plasma vitellogenin. However, there was no statistically significant difference in between the exposures, which indicates that the presence of AgNPs does not affect EE2 effects on Vtg expression. Due to different properties and behaviour in the test media it is difficult to compare effects of different ENPs.

To the author's best knowledge there is only one study that has investigated influence of nanosilver on the effects of EE2 in marine organisms. In this study Völker et al. (2014) investigated the combined effects of AgNPs on the freshwater mudsnail *Potamopyrgus antipodarum*. Their results show decreased Vtg expression observed in presence of the AgNPs at 50 and 12.5 ng/L EE2 after 28 days exposure. However, at 25 ng/L EE2 the low concentration of AgNP (1.25 μg/L Ag) enhanced EE2 effects on the Vtg expression. Authors hypothesize that it might be caused by a low-dose stimulation or hormesis. However, the results do not allow conclusions on whether the presence of AgNP reduced or enhanced bioavailability of EE2. In the study of Park et al. (2011) on zebrafish, combined exposure to EE2 and fullerene nanoparticles (nC60) resulted

in decreasing Vtg expression and therefore reduced bioavailibility of EE2 with increasing concentration of nC60. In the present study, higher concentration of AgNPs resulted in lower Vtg induction in juvenile turbots. The mean plasma Vtg concentration was slightly higher in the low AgNP exposure group than in the group exposed to higher concentration of AgNP, even though there was no statistically significant difference between these groups. However, the nC60s have an extremely low water solubility ($< 10^{-9}$ mg/L) (Lyon and Alvarez, 2008) compared to AgNPs and form aggregates in the aqueous phase. Thus, they are more likely to adsorp EE2, while the AgNP in this study were shown to undergo dissolution rather than aggregation.

4.4 Field survey of pollution in the Trondheimsfjord

Discharges of treated sewage from the WWTPs have been identified as a major cause of estrogenic effects on fish in the freshwater environment (Ternes et al., 1999). Among the most common pollutants that are being released into the aquatic environment after passing municipal wastewater treatment are estrogens, especially the synthetic 17α-ethinylestradiol that is used in oral contraceptives. The first studies reported elevated Vtg levels in freshwater species, however recently, the findings of the same conditions in marine fish brought concerns that coastal marine organisms might be also at risk. Ferreira et al. (2009) showed significant induction of Vtg in *Lipophrys pholis* collected at the Porto coast in the north of Portugal, which receives effluents from the cities of Matosinhos and Porto. Similarly, elevated levels of Vtg and zona radiata-proteins were found in some adult male swordfish *(Xiphias gladius)* caught in the Strait of Messina, Sicily, Italy (Fossi et al., 2004).

The mean Vtg concentration in plasma of male flatfish sampled at the outlet of Høvringen WWTP was 36 279 ± 1 308 ng/mL. The fish from Sørburøya were not sampled for plasma and therefore the information of the Vtg induction from the reference site is lacking. However, if compared to a control group from the exposure experiment, there was 75% increase in Vtg induction in the Høvringen fish. For comparison Allen et al. (1999) investigated Vtg levels in plasma of flounder *(Platichthys flesus)* in UK estuarine and marine waters. The concentration range was from 23.8 ng/mL at the reference site to 100 000 ng/mL at contaminated sites.

Kleinkauf et al. (2004) revealed a seasonal cycle in Vtg concentrations in male flounders caught in Mersey estuary, UK. In late spring and summer the fish have relatively low concentrations of Vtg in μg/mL range, while in mid-winter mean concentrations were as high as 5 mg/mL. Elevated levels of plasma Vtg in male flounders were also found in Japanese flounders *(Pleuronectes yokohamae)* collected in Tokyo Bay that ranged from 25 ng/mL to 2 200 ng/mL (Hashimoto et al., 2000). In the study of Madsen et al. (2013) the majority of flounders sampled at nine Danish coastal sites had Vtg concentrations less than 100 ng/mL, which is fairly low value compared to this study or study of Allen et al. (1999).

It has been shown, that estrogen exposure is often accompanied by the presence of intersex conditions. (Stentiford and Feist, 2005) reported cases of intersex in flatfish species dab *(Limanda limanda)* collected from the North Sea in 2003. No ovotestis conditions have been observed in any of the fish from Høvringen or Sørburøya. One of the reasons might be that ovotestis is induced through exposure of flounder larvae. Sexual differentiation in the larvae which is a very estrogen sensitive process probably does not occur in the estuaries but offshore under relatively uncontaminated conditions. Also based on the findings of Allen et al. (1999) it seems that ovotestis only occurs in flounder populations when mean plasma Vtg levels in male adults exceed 100 000 ng/mL.

Silver occurs in open ocean waters typically at very low concentrations (0.05 - 5.0 ng/L) due to its high reactivity and low crustal abundance (Ratte, 1999). Silver ions have been reported to be highly toxic to fish. However, Ag is present in the seawater mainly as silver sulfide and chloride complexes rather than in the ionic form. DOC might be another important variable for silver speciation in the seawater due to its ability to bind silver. The concentrations of Ag measured in the tissues of sampled field fish were generally low with the highest values found in liver. This is in agreement with other field surveys and laboratory experiments (Webb and Wood, 2000; Galvez et al., 2001). Greig and Wenzloff (1977) reported Ag concentration up to 0.8 μg/L (ww) in winter flounder *(Pleuronectes americanus)* and 0.09 μg/L (ww) in yellowtail flounder *(Pleuronectes ferruginea)* collected in New York Bight, US. The concentration in brain, kidney and gills in wild-ranging flat fish in Trondheimsfjord were generally very low. Interestingly, Ag

was detected in the brain of field fish, even though no Ag was detected in brains of the fish from the exposure experiment indicating that silver has the ability to penetrate blood brain barrier in the fish after a chronic exposure.

Over the years, several environmental quality surveys of the Trondheimsfjord areas have been conducted. The comprehensive survey from 2000-2003 aimed to identify potential impacts of effluents of Høvringen and Ladehammeren WWTP on eutrophication. It has been demonstrated that the water quality in general was good within the objectives that have been defined for natural conditions by the Norwegian authorities and the quality was falling within the definition of "good ecological status" in accordance with the Water Framework Directive. The same results have been confirmed by the report evaluating the conditions in the receiving waters for effluents from Høvringen and Ladehammeren WWTPs in 2011-2012 (Sintef, 2012). However, the levels of EE2 in the influents and effluents from the Trondheim WWTPs and receiving waters have not been measured. The induction of Vtg in male fish caught at the outlet of the Høvringen WWTP indicate exposure to EDCs. However, due to the small sample size and a need to collect additional samples from a reference site, further research on this topic is needed.

Chapter 5

Conclusion

In the present study the uptake and effects of AgNPs and EE2 both alone and in combination on *Scophthalmus maximus* were investigated. After 13 days pulsed exposure to high (200μg/L) and low concentration (2μg/L) of AgNP alone and in combination with EE2, or silver ions added as $AgNO_3$, Ag was detectable in all exposure groups except for the control and PVP exposed ones. Detectable Ag concentrations in gills and intestines imply that both branchial and intestinal uptake occurred. However, the relatively high concentration of Ag from the AgNP exposure groups in the gills compared to Ag^+ suggests that the nanoparticles were trapped in the gill mucus layer. The rest of the investigated organs showed similar Ag distribution pattern with no statistically significant difference within the AgNP200, AgNP200EE2 and Ag^+ exposed group. The lowest silver concentrations were observed in muscle, suggesting a low potential of this tissue for trophic transfer of silver.

Induction of plasma vitellogenin was observed in juvenile turbots exposed to EE2 alone and in combination with low and high concentration of AgNPs. The combined exposure to EE2 and AgNPs did not result statistically significant enhanced or decreased expression of vitellogenin, even though there was a non-significant tendency toward lower Vtg concentration with increased Ag concentrations. Statistically significant induction of vitellogenin was observed in Ag^+ exposed group, suggesting a possible effect of Ag^+ on estrogenic effects. However, the results of this study do not permit a definite conclusion on whether Ag^+ or AgNPs can cause vitellogenin induction and further research on this topic is needed.

Wild flatfish sampled at the outlet of the Høvringen WWTP in Trondheimsfjord, Norway had

elevated concentrations of Vtg compared to control fish from the exposure experiment suggesting possible exposure of the fish to EDCs. The concentration of Ag measured in the tissues of the fish caught at Høvringen and Sørburøya were generally low. However, due to the small sample size and a need to collect additional samples from a reference site, further research on free ranging fish in Trondheimsfjord is needed.

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Appendix A

Additional Information

A.1 Exposure Experiment Turbot Biometry

Sample ID	Exposure	Tank	Weight (g)	Length (cm)	Sex
1A1	control	1A	182.1	23	m
1A2	control	1A	216.03	23.5	f
1A3	control	1A	218.27	22.3	f
1A4	control	1A	211.2	23.2	f
1A5	control	1A	211.34		m
1B1	control	1B	217		f
1B2	control	1B	208.08	23.2	f
1B3	control	1B	202.88		m
1B4	control	1B	205.81	23	m
1B5	control	1B	211.63	23.1	m
1C1	control	1C	225.82	24.1	f
1C2	control	1C	212.75	23.2	m
1C3	control	1C	185.45	23	m
1C4	control	1C	200.11	21.9	m
1C5	control	1C	205	23	f

Sample ID	**Exposure**	**Tank**	**Weight (g)**	**Length (cm)**	**Sex**
2A1	AgNP200	2A	191.5		f
2A2	AgNP200	2A	206.5	23	m
2A3	AgNP200	2A	205.41	23.7	f
2A4	AgNP200	2A	200.98	22.8	f
2A5	AgNP200	2A	198.6		m
2B1	AgNP200	2B	222.67	24.2	m
2B2	AgNP200	2B	215.39	23.7	m
2B3	AgNP200	2B	225	24	m
2B4	AgNP200	2B	204.26	22.5	m
2B5	AgNP200	2B	204.57	22.6	m
2C1	AgNP200	2C	191.6	23.3	f
2C2	AgNP200	2C	213.7	22	m
2C3	AgNP200	2C	220.4	23.2	m
2C4	AgNP200	2C	196.6	23	m
2C5	AgNP200	2C	218.8	22.4	f
3A1	AgNP2	3A	195.4	23.3	m
3A2	AgNP2	3A	207	23.4	m
3A3	AgNP2	3A	206.7	21.9	f
3A4	AgNP2	3A	190	22.4	m
3A5	AgNP2	3A	202		m
3B1	AgNP2	3B	211	23.2	m
3B2	AgNP2	3B	187.6	22.1	m
3B3	AgNP2	3B	201.9	23.5	m
3B4	AgNP2	3B	183.3	23.8	m
3B5	AgNP2	3B	185.1		f
3C1	AgNP2	3C	197.7	22	m
3C2	AgNP2	3C	198.5	23	f
3C3	AgNP2	3C	207.3	22.5	m
3C4	AgNP2	3C	228	24.2	f
3C5	AgNP2	3C	190.8	23.2	m

Sample ID	Exposure	Tank	Weight (g)	Length (cm)	Sex
4A1	AgNP200EE2	4A	187.7		m
4A2	AgNP200EE2	4A	220	23.9	m
4A3	AgNP200EE2	4A	187.6	20.6	f
4A4	AgNP200EE2	4A	214.47	24	f
4A5	AgNP200EE2	4A	198.4	22.7	f
4B1	AgNP200EE2	4B	215.9	23.4	f
4B2	AgNP200EE2	4B	230.9	24.5	f
4B3	AgNP200EE2	4B	197.9	23.3	m
4B4	AgNP200EE2	4B	198.8	23	m
4B5	AgNP200EE2	4B	223.4	23.4	f
4C1	AgNP200EE2	4C	198.53	23.4	m
4C2	AgNP200EE2	4C	201.45	22.5	f
4C3	AgNP200EE2	4C	196.33	24	m
4C4	AgNP200EE2	4C	198	23.4	f
4C5	AgNP200EE2	4C	198.92	22.2	f
5A1	AgNP2EE2	5A	222.32	23.5	f
5A2	AgNP2EE2	5A	191	22.3	f
5A3	AgNP2EE2	5A	199.8	23.5	f
5A4	AgNP2EE2	5A	198.5	21.5	f
5A5	AgNP2EE2	5A	209.4	23.3	f
5B1	AgNP2EE2	5B	202.7	23.5	f
5B2	AgNP2EE2	5B	194.6	23	f
5B3	AgNP2EE2	5B	192.2	23	m
5B4	AgNP2EE2	5B	208.7	23.5	m
5B5	AgNP2EE2	5B	211.7	23.5	m
5C1	AgNP2EE2	5C	192.98		m
5C2	AgNP2EE2	5C	214		f
5C3	AgNP2EE2	5C	206.7	21.5	f
5C4	AgNP2EE2	5C	225.5	23.5	f
5C5	AgNP2EE2	5C	220.3	24	f

Sample ID	**Location**	**Species**	**Weight (g)**	**Length (cm)**	**Sex**
1	Høvringen	P. flesus or L. limanda			
2	Høvringen	P. platessa	1032	45	f
3	Høvringen	P. platessa	743	39.5	m
4	Høvringen	L. limanda	482.2	35.5	f
8	Høvringen	L. limanda	369.7	33.5	f
16	Høvringen	P. platessa	1276.9	49	f
18	Høvringen	L. limanda	516.9	36.2	f
19	Høvringen	L. limanda	544.2	36.7	f
20	Høvringen	P. platessa	910	42.5	f
21	Høvringen	P. flesus or L. limanda	362.5	32	m
22	Høvringen	L. limanda	540	35.5	f
23	Høvringen	P. platessa	1140	47	f
24	Høvringen	P. platessa	989.5	43.5	m
25	Høvringen	P. platessa	1529.3	49.5	f
26	Høvringen	P. flesus or L. limanda	466	35.7	f
27	Høvringen	L. limanda	349.9	32	m
28	Høvringen	L. limanda	578.3	35.1	f

A.2 ICP-MS

	Control	AgNP200	AgNP2	AgNP200EE2	$^{*}AgNP2EE2$	$^{*}Ag^{+}$	PVP
Liver	0.004±0.001	0.389±0.121	0.019±0.004	0.453±0.102	0.022±0.004	0.512±0.066	0.003±0.003
Gill	0.018 ± 0.008	1.250 ± 0.262	0.100 ± 0.030	0.733 ± 0.132	0.063 ± 0.012	0.196 ± 0.028	0.003 ± 0.0002
Stomach	n/d	0.114±0.015	0.006±0.001	0.087±0.012	0.007±0.002	0.134±0.003	n/d
Muscle	n/d	0.012±0.002	n/d	0.009±0.003	n/d	0.010±0.001	n/d
Kidney	n/d	0.123±0.028	n/d	0.065±0.009	0.005±0.001	0.085±0.010	n/d
Brain	n/d	n/d	n/d	n/d	n/d	n/d	n/d
Bile	0.007±0.002	0.374±0.221	0.010±0.002	n/a	n/a	0.391±0.152	n/a

Table A.1: Ag concentrations measured in different tissues μg/g (dw). Data are given as mean ± SE (n = 7, * n = 11), n/d: more than 50% values were under the limit of detection, n/a: data not analyzed

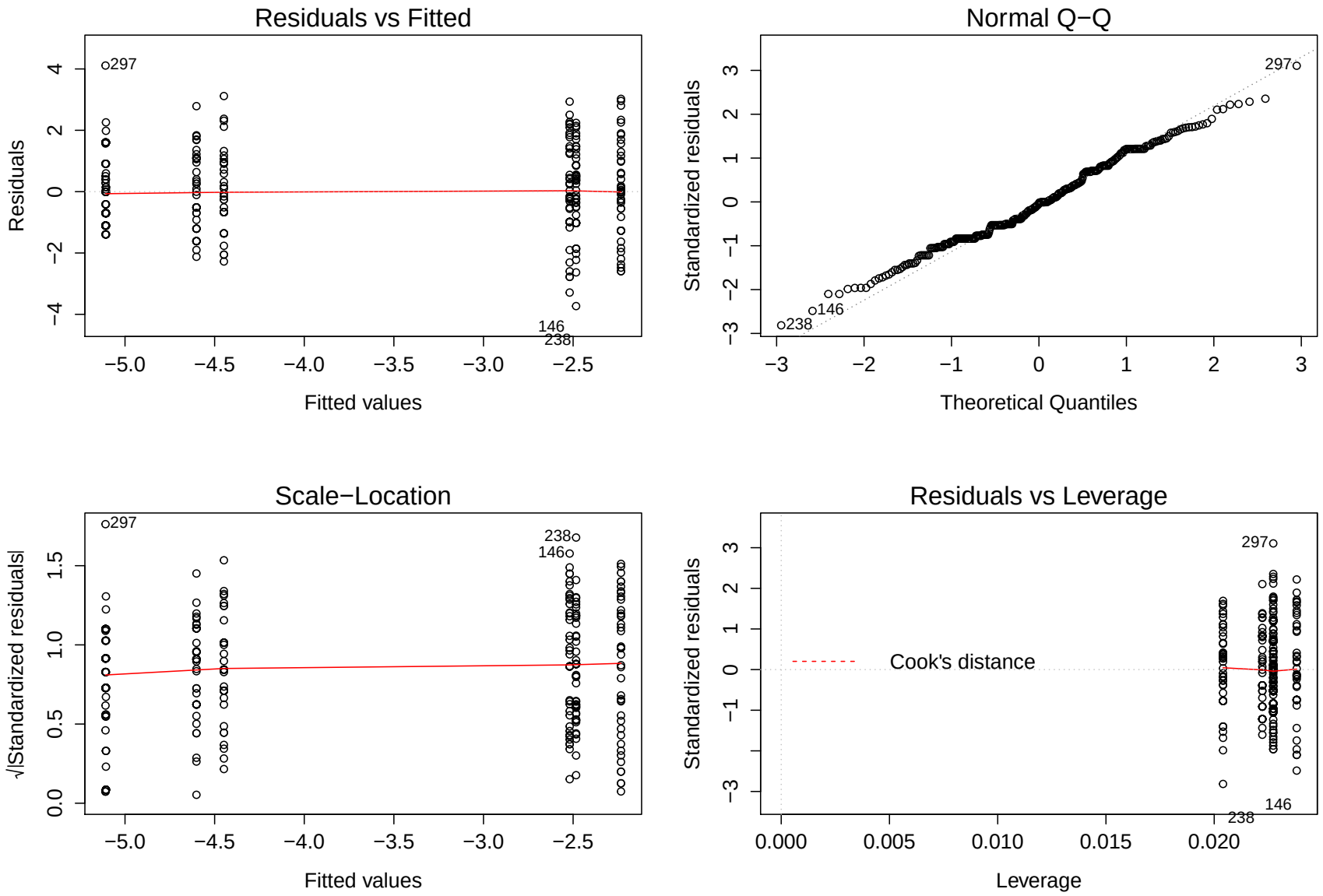

Figure A.1: Residual plots for linear regression diagnostics.

A.3 ELISA

A.3.1 Standard curves used for calculations of Vtg concentrations

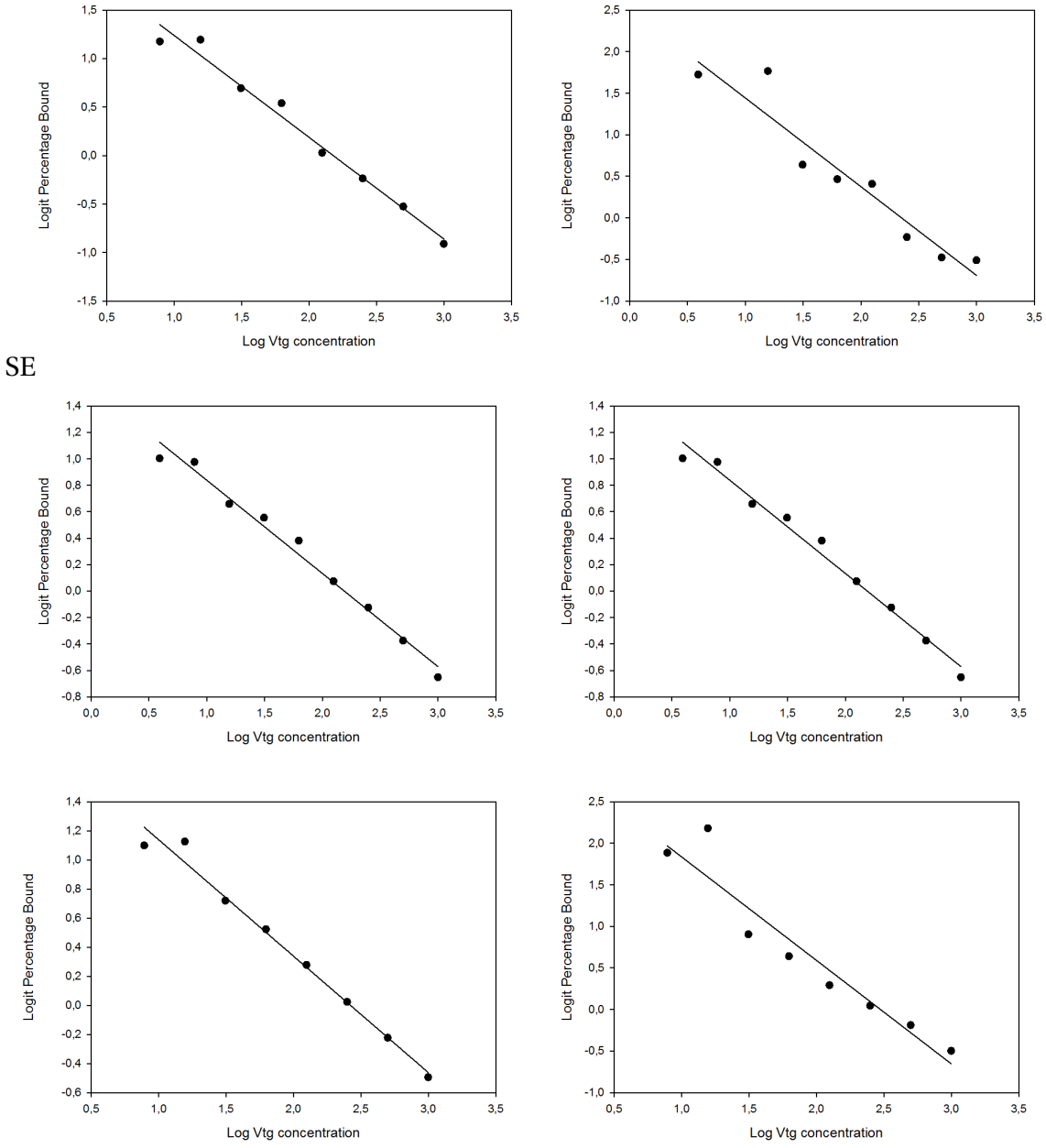

Figure A.2: Standard curves for the ELISA assay. Data were obtained as an average of three absorbance measurements and converted into Vtg concentration using respective equations.

A.3.2 ELISA validation

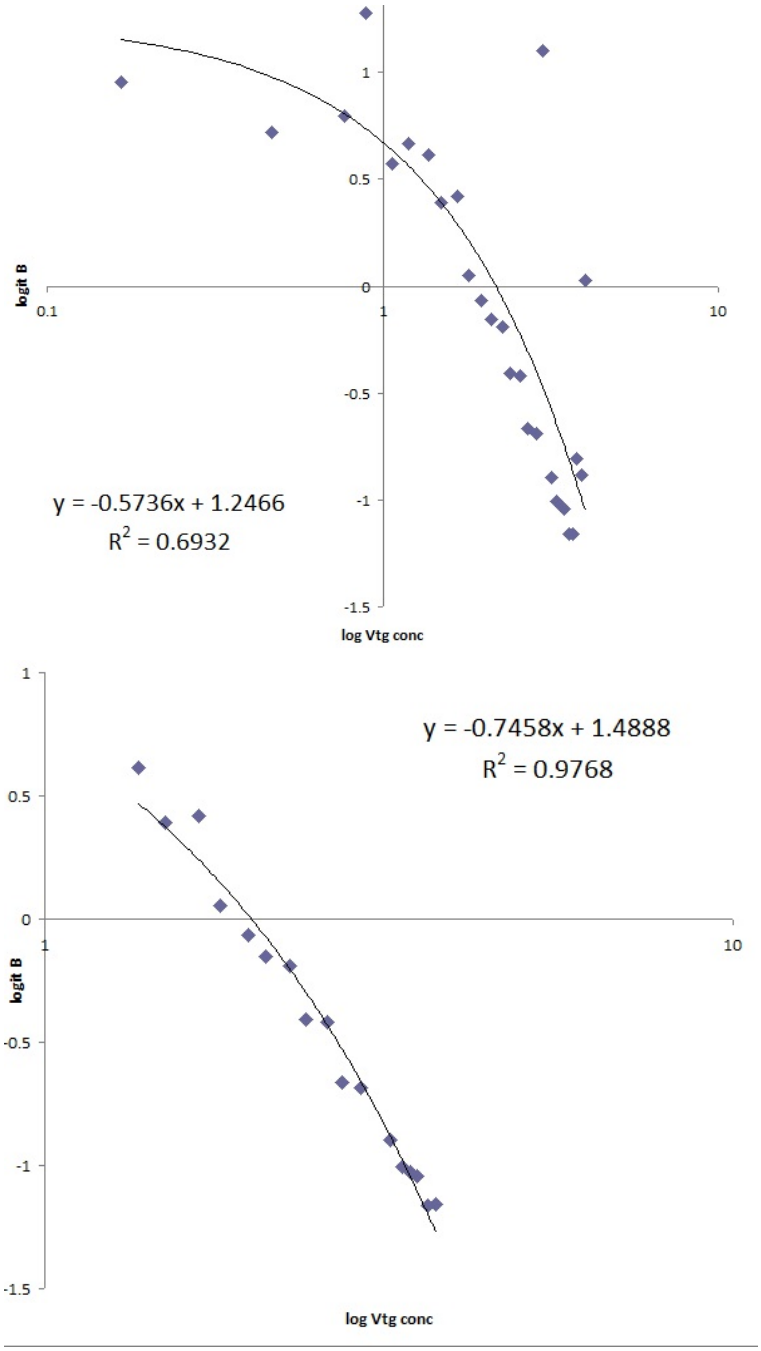

Figure A.3: Logit binding percentage values plotted against log Vtg concentration for ELISA standard curve. The working range between 23.44 and 5000 ng/mL with 50 percent binding around 180 ng/mL was based on the linear part of the curve.

	Plate ID	Conc (ng/ml)	NSB	0.00	1.95	3.91	7.81	15.63	31.3	63	125	250	500	1000	POS
Day 1	1	**Average Absorbance (450 nm)**	0.118	1.368	1.421	1.390	1.278	1.282	1.133	1.059	0.741	0.573	0.428	0.308	99452
	2	**Average Absorbance (450 nm)**	0.129	1.536	1.558	1.510	1.440	1.513	1.274	1.177	1.141	0.650	0.481	0.461	147104
		mean	0.123	1.452	1.490	1.450	1.359	1.397	1.204	1.118	0.941	0.612	0.455	0.385	123278
		std dev	0.005	0.084	0.068	0.060	0.081	0.116	0.070	0.059	0.200	0.038	0.026	0.076	
		CV	6.1	8.2	6.5	5.9	8.4	11.7	8.3	7.5	30.1	8.9	8.2	28.1	
Day 2	3	**Average Absorbance (450 nm)**	0.000	1,184	1.153	1.134	1.065	1.020	0.956	0.859	0.651	0.493	0.321	0.173	104839
	4	**Average Absorbance (450 nm)**	0.000	1.297	1.316	1.180	1.173	1.064	1.014	0.917	0.703	0.556	0.385	0.236	98904
		mean	0.000	1.240	1.234	1.157	1.119	1.042	0.985	0.888	0.677	0.524	0.353	0.205	
		std dev	0.000	0.057	0.082	0.023	0.054	0.022	0.029	0.029	0.026	0.032	0.032	0.031	
		CV		4.6	6.6	2.0	4.8	2.1	3.0	3.2	3.9	6.0	8.9	15.3	
Day 3	5	**Average Absorbance (450 nm)**	0.072	1.278	1.314	1.305	1.189	1.194	1.085	1.000	0.862	0.691	0.524	0.365	148207
	6	**Average Absorbance (450 nm)**	0.074	1.243	1.360	1.338	1.228	1.236	1.114	1.026	0.849	0.688	0.534	0.256	213709
		mean	0.073	1.261	1.337	1.321	1.209	1.215	1.100	1.013	0.855	0.690	0.529	0.360	
		std dev	0.001	0.017	0.023	0.017	0.020	0.021	0.015	0.013	0.007	0.001	0.005	0.005	
		CV	1.1	1.4	1.7	1.2	1.6	1.7	1.3	1.3	0.8	0.2	1.0	1.2	
Day 4	STD CURVE	**Average Absorbance (450 nm)**	0.088	1.275	1.320	1.349	1.225	1.215	1.064	0.931	0.717	0.577	0.421	0.299	185036
	HOVRINGEN	**Average Absorbance (450 nm)**	0.081	1.217	1.244	1.186	1.144	1.030	1.015	0.906	0.769	0.597	0.443	0.316	73968
		mean	0.084	1.246	1.282	1.267	1.184	1.123	1.040	0.919	0.743	0.587	0.432	0.308	
		std dev	0.003	0.029	0.038	0.081	0.040	0.093	0.025	0.013	0.026	0.010	0.011	0.008	
		CV	4.0	2.3	3.0	6.4	3.4	8.2	2.4	1.4	3.5	1.7	2.5	2.7	
		Overall Within-Day Precision (working range 3.91 - 1000 ng/mL)	3.7	4.1	4.4	3.9	4.6	5.9	3.7	3.3	9.6	4.2	5.2	11.8	
		Overall Within-Day Precision	**5.8**												

Table A.2: Within-day repeatability precision for turbot Vtg standard curves. Within-day repeatability precision was calculated as % CV between the six aliquots analysed in one day. Overall within-day repeatability precision was calculated as average of the individual precision values for four days.

conc (ng/mL)	NSB	0.00	1.95	3.91	7.81	15.63	31.3	63	125	250	500	1000
Average absorbance (450nm) 4 days	0.070	1.300	1.336	1.299	1.218	1.194	1.082	0.984	0.804	0.603	0.442	0.314
std dev	0.002	0.026	0.023	0.027	0.022	0.042	0.021	0.019	0.079	0.015	0.011	0.029
CV	2.9	2.0	1.7	2.0	1.8	3.5	2.0	1.9	9.8	2.5	2.4	9.1

Overall between day precision (working range 3.91 - 1000 ng/mL)	CV = 3.9

Table A.3: Between-day repeatability for turbot Vtg standard curves. Between-day repeatability precision was calculated as % CV between the average measured absorbances from each day. Overall Between-day repeatability precision was calculated as average precision for all absorbances.