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Marko Klaic, BSc MSc

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Declaration of Academic Integrity

I hereby confirm that I prepared this Master Thesis independently and on my own, by exclusive reliance on the tools and literature indicated therein. The sources of other people's work have been appropriately referenced. Quotes from other sources are marked appropriately. The thesis has not been submitted to any other examination board.

Vienna, 16 March 2021

Marko Klaic

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1 Introduction

Medicinal products for human and veterinary use are applied in large quantities. In 2001, 31000 tonnes of pharmaceuticals were used only in Germany. Looking at the active pharmaceutical ingredient, 100 000 tons per year of different synthetic active chemicals are produced in countries all over the world. Approximately 4000 active pharmaceutical ingredients, the substances which are supposedly accountable for the desired effect, have been available worldwide in 2014 (Weber *et al.*, 2014). And there are more to come: The European Medicines Agency (EMA) released 66 positive opinions (which are recommendations for an EU-wide marketing authorization for the European Commission) on human medicines and 15 on veterinary medicines in 2019, of which in total were 35 new active substances for that year (European Medicines Agency, 2020a; European Medicines Agency, 2020b).

After the application of medicinal products, components can be absorbed and pharmacokinetics lead in the end to the excretion of the substances, either metabolized or in its original form. Human and animal excretions can reach wastewater treatment plants (WWTP) and can be used as natural fertilizers, if untreated by WWTP manure are sometimes directly used as fertilizers. Raw sewage containing pharmaceuticals or their metabolites reach the WWTP and might, or might not, reduce the content of the various contaminants of the influent. It is a known problem that several substances, including constituents of medicinal products, are only reduced limitedly in WWTP influents and therefore released more or less untreated into the aquatic environment through the effluents. Among those chemicals are substances of categories like antibiotics, analgesics, anti-inflammatory drugs, disinfectants or antihypertensive drugs. Measurable levels of those substance categories have been found not only in WWTP effluents, but also in surface waters (Fent, 2013). Should this fact raise concern?

1.1 Medicinal products in the environment

In 2018, Greenpeace published a report, in which they documented testing results from samples taken from canals and rivers in intensive livestock farming regions across the EU. The samples were tested for pesticides, nutrients, metals and veterinary drugs. In 23 out of 29 samples, veterinary drugs were found. Altogether, 21 drugs were detected, of those were 17 antimicrobials and of those antimicrobials 12 were antibiotics (Regelsberger *et al.*, 2018). The key question is, if the detected substances are likely to have an impact on biota in the aquatic environment and if so, if there is a

threshold below which the occurrence of the substance is negligible, the so called “no observed effect level“ (NOEL).

Let us look at some medicinal products, which have already been reported to cause problems in the environment. One of the probably most talked-about issues, caused by the release of antibiotics into the environment is increasing antibiotic resistances (ABR) in bacteria. Since bacteria are ubiquitous, the release of antibiotics into the environment is one possible pathway of developing ABR. Mechanisms like hydrolysis, efflux or an altered target in bacteria are responsible on molecular scale to handle the otherwise toxic antibiotics and cause ABR. In 1937, the first antimicrobial substances were introduced (Davies and Davies, 2010) and are until today in therapeutic use. The use of antibiotics lead in the course of time to increasing resistances which have been identified, for instance there were over 1000 identified β-lactamases which are related to β-lactamase resistances (Davies and Davies, 2010). This leads to the question, how humankind will overcome this obstacle, if at some point multiantibiotic-resistant bacteria become predominant. In 2008 for example, 3.6% (in numbers 440000) of the approximated tuberculosis cases worldwide were accounted to multi-drug-resistant tuberculosis (*i.e.* an ABR to either rifampicin or isoniazid) (Gandhi *et al.*, 2010).

In 2004, researchers were able to pinpoint the reason of a population decline of the Oriental white-backed vulture *Gyps bengalensis* of over 95% in an Indian national park. Since these vultures feed mainly on carcasses of domestic livestock in Pakistan, researchers tried to narrow probable drugs used to treat animals in the region, which might cause deaths in the birds. The non-steroidal anti-inflammatory drug (NSAID) 2-[2-(2,6-Dichlorophenylamino)phenyl]acetic acid – „Diclofenac”, an antipyretic, anti-inflammatory and analgesic medicinal product, was identified as the most probable substance. An experiment was conducted in which ten specimen of vultures received food which contained diclofenac, all ten exposed vultures died of renal failure (Oaks *et al.*, 2004).

Another substance class which is causing problems when released into the environment, but probably less commonly known than the two examples before, are antihypertensive substances. Measurable levels of these medicinal products were detectable in different surface fresh waters at levels, which partially are already toxic by *e.g.* having an impact on reproduction, like it was for instance correlated for the crustacean *Ceriodaphnia dubia* in the British Tyne estuary (Godoy *et al.*, 2015).

WWTP are designed to remove all, or the major part of pollutants from waste water. The overall operating principle of WWTPs consists of mechanical separation, biological treatment using micro-organisms, chemical treatment and polishing. A big problem might develop, if the efficacy of the

WWTPs is inhibited by exactly the substances, which the WWTP is supposed to treat. In 2008, researchers conducted a study to determine whether influents containing disinfectants could have an inhibiting impact on WWTP and therefore reduce the efficacy – and they were able to show in a model that especially disinfectants on a sodium hypochlorite basis have a high effect on WWTPs and therefore the use in households should be discussed (Bodík *et al.*, 2008).

As shown in the passages before, medicinal products in the environment may cause a huge variety of different problems, of which most probably many have not been detected yet. Therefore, broad minded approaches are necessary to address this every part of this issue. There is a need for research in this area, as well as governmental actions following this research. Legislative authorities have been aware of these problems and try to deal with them in different ways, as depicted in the following chapter.

1.2 Regulation of medicinal products

The European Union (EU) is aware of harming effects of medicinal products in the environment. In 2015, the first watch list for emerging water pollutants was published by the European Commission which contained various substances to be monitored by the European member states (EC, 2015), in 2018 followed an update of the watch list (EC, 2018). Decision 2018/840/EU (EC, 2018) states in particular:

> "The substances in the watch list are to be selected from amongst those for which the information available indicates that they may pose a significant risk, at Union level, to or via the aquatic environment, but for which monitoring data are insufficient to come to a conclusion on the actual risk posed. Highly toxic substances, used in many Member States and discharged to the aquatic environment but not or rarely monitored, should be considered for inclusion in the watch list. […]" (European Commission, 2018, p.1)

and

> "The monitoring of the substances in the watch list should generate high-quality data on their concentrations in the aquatic environment, fit for the purpose of supporting, in a separate review exercise […] the risk assessments that underpin the identification of priority substances. In that review, substances found to pose a significant risk should be considered for inclusion in the priority substances list. [...]" (European Commission, 2018, p.1)

Substance types like neonicotinoids, herbicides or sunscreen ingredients have been included in the watch list, as well as substances of pharmaceutical relevance like hormones, macrolide antibiotics and the previously mentioned NSAID Diclofenac. The previously mentioned update, which the watch list has received, led regarding the pharmaceutically relevant substances to the removal of Diclofenac due to "sufficient high-quality monitoring data available" (quote from European Commission, 2018, L 141/10) but at the same time to the addition of antibiotics (European Commission, 2018).

At the same time, the European Medicines Agency (EMA) requires from applicants in order to receive a marketing authorization for medicinal products in the EU to provide an environmental risk assessment (ERA). This applies both for medicinal products for human use as well as for veterinary use. Two different guidelines explain the process on how to conduct an ERA in both cases.

For medicinal products for human use, the ERA is divided in two phases, while the second phase itself is divided in two tiers (EMA, 2006). The objective of phase I is to estimate the exposure, of phase II tier A to predict the initial risk and of phase II tier B the substance compartment-specific clarification and risk assessment.

In phase I a formula is suggested to estimate the local surface water concentration, defined as the Predicted Environmental Concentration (PEC). If the value is below 0.01 µg/L and other environmental concerns are not evident, the ERA can stop at this point. Otherwise the ERA enters phase II Tier A to determine the environmental fate and conduct an effect analysis. Several aquatic effect studies following protocols by the Organisation for Economic Co-operation and Development (OECD) are recommended, including experiments on adsorption, biodegradability, aerobic and anaerobic transformation, growth inhibition, reproduction, toxicity and respiration inhibition tests. At the same time, the Predicted No Effect Concentration (PNEC) for different biological compartments is determined. Depending on the outcomes of the various studies which are put in relation to determine the risk as a value, a substance which poses a potential risk to the environment is required to enter phase II tier B. Depending on the specific potential risk which has been identified in tier A, additional tests are conducted depending on which organisms might be affected by the substance or regarding the terrestrial environmental fate, if there is an indication and the substance is not readily biodegradable (EMA, 2006).

If environmental risks can't be excluded, measures must be taken like *e.g.* labelling of the product to prohibit the emission into the environment by additional information how to handle the product

or to include the risks the product might cause when released into the environment. It is required to provide an ERA for every medicinal product for human use, even if the content of the ERA is a justification for no submission of data, because for instance the substance is highly unlikely to cause any risk when released into the environment (EMA, 2006).

For veterinary medicinal products, an environmental impact assessment (EIA) has to be provided just like the ERA for human medicinal products. The process of the evaluation is overall similar, in detail there are some differences. In contrast to the previously described process for the evaluation of an ERA for human medicinal products in the EU, the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) published guidelines which have been adopted in that form in both the US by the Food and Drug Administration (FDA) and the EU by the EMA (VICH, 2001; VICH, 2005). Phase I consists of an assessment regarding the environmental exposure, phase II assesses the risk of these substances to have an impact on so-called "non-target" species in the environment.

In phase I, a decision tree consisting of a series of questions must be answered. The answers to questions might differ depending on the regulatory agency, which is already shown in the first question: "Is the VMP (Veterinary Medicinal Product) exempt from the need for an EIA by legislation and/or regulation?" (VICH, 2001, p. 3) – the products, which are excluded by the FDA, will be discussed a few passages further below. Another question which leads to differing answers is "Is the VMP intended for use in a minor species that is reared and treated similarly to a major species for which an EIA already exists?" (VICH, 2001, p. 3). For the EMA, major species are dogs, cats, salmon, chickens, pigs, sheep and cattle (EMA, 2019) while the FDA defines major species as chickens, turkeys, pigs, cattle, cats, dogs and horses (FDA, 2020). After several questions, the decision tree splits into two branches, one for species in the aquatic environment and the other for species in the terrestrial environment. In the end, Phase II is triggered if the VMP is an endoparasiticide and/or ectoparasiticide (in both branches), the recalculated predicted environmental concentration of the VMP is over 100 µg/kg (in the terrestrial branch) or the recalculated environmental introduction concentration is over 1 µg/L (in the aquatic branch). Otherwise, the EIA ends in phase I (VICH, 2001).

Phase II for VMPs depends heavily on both, the substance and the species the product is administered to. This phase is generally split in tier A, consisting of simple and inexpensive tests in comparison to tier B which is required to enter, if the achieved data are insufficient to complete the EIA. Three branches are distinguished in general: The aquaculture branch, the intensively reared animals branch and the pasture animals branch – independently of the branch the substance might enter,

several studies (following OECD guidelines) are recommended for every substance entering phase II, which are: Water solubility, dissociation constants in water, UV-Visible absorption spectrum and melting point/melting range studies regarding physical-chemical properties and soil adsorption/desorption studies regarding the environmental fate. Additional studies must be conducted depending on the previously mentioned branches the substances enter in phase II, tailored specifically to elaborate on potential issues (VICH, 2005). If after the conduction of all required studies the risk is not eliminated, according to the guideline "[…] the applicant is recommended to discuss their dossier and proposals for further data or risk mitigation with the regulatory authority" (VICH, 2005, p. 22).

As previously mentioned, in the US the EIA for VMPs is in general the same as in the EU with *e.g.* the exceptions regarding the classification of species. The FDA equivalent to the EMA ERA is the Environmental Assessment (EA) and in contrast, according to the 21 CFR § 25.30 (Environmental Impact Considerations, 1996) and § 25.31 (Environmental Impact Considerations, 1997) in several cases a reduced EA is sufficient. This reduced EA requires to assess any specific action which could have an impact on the quality of the human environment. Exceptions are for instance states as follows:

> Action on an [New Drug Application], abbreviated application, application for marketing approval of a biologic product, or a supplement to such applications, or action on an [Over-the-counter] monograph, for substances that occur naturally in the environment when the action does not alter significantly the concentration or distribution of the substance, its metabolites, or degradation products in the environment (Environmental Impact Considerations, 1997, 21 C.F.R. §25.31 (c)).

Another exception would be any action on an investigational new drug application.

Nonetheless, if no exclusion is in place, an EA must be performed. At first, the substances which should be assessed have to be identified, considering that *e.g.* metabolites might be of interest. Next is the chemical and physical characterization by the determination of the water solubility, dissociation constants, octanol/water partition coefficient and vapor pressure or henry's law constant. The aim of this characterization is to assess if the compound tends to accumulate in the atmospheric, terrestrial or aquatic compartment. The following step is to investigate if depletion mechanisms exist in the environment. If fast mechanisms deplete the substance completely, the only required test is for microbial inhibition. If the depletion is incomplete, it is required to assess the environmental effects of the substance in a tiered approach: In tier 1, acute ecotoxicity is evaluated on at least one organism. Depending on the outcome, either the EA can stop, continues to tier 2 or

skips tier 2 and start immediately with tier 3 testing. In tier 2, acute ecotoxicity test should be performed on a fish species, an aquatic invertebrate species and an algal species to form the minimum aquatic base set. For the terrestrial base set which also should be performed, a soil microbial toxicity test, an earthworm toxicity test and a plant early growth test are the minimum requirements. Again, depending on the outcome, either the EA can stop or it has to enter the final tier 3 to assess chronic toxicity. If tier 3 testing must be performed, it is recommended to ask for the FDA's guidance for appropriate design (FDA, 2018).

At the same time, the US Environmental Protection Agency (EPA) is also regulating substances of interest in the environment. Several acts and regulations were passed: In 1948 the "Clean Water Act", the basis for the regulation of disposal of pollutants was passed and included 126 substances. The law which made it possible for the EPA to set standards for public water was passed in 1974 as the "Safe Drinking Water Act". This act divides contaminants into two groups: Substances which might cause adverse effects are regulated by the "National Primary Drinking Water Regulation", while substances with organoleptic or aesthetic effects on water are regulated by the "National Secondary Drinking Water Regulation". The "Unregulated Contaminant Monitoring" on the other hand is an unregulated system which is nevertheless collecting data, leading to the publishing of the "Contaminant Candidate Lists", which are updated every few years and is the list on which 17α-Ethinylestradiol (EE2) is stated. The first contaminant candidate list was published in March 1998, 50 chemicals and 10 microbiological contaminants were on the list. EE2 was added onto the list in 2009. The current list is the "Contaminant Candidate List 4" containing 112 contaminants, consisting of 100 chemicals and 12 microbiological contaminants. A fifth version is as of December 2020 under evaluation of the EPA (da Cunha *et al.*, 2016).

In contrast to the EU and the US, which seem heavily regulated, some countries struggle with the implementation of these regulations. Brazil for example often orientates towards the EPA in the US: Resolutions on waterbodies of surface water classifications and environmental standards were implemented, but emission control is still an issue and almost no river meets the standards (da Cunha *et al.*, 2016). At the same time, even though environmental quality standards for many pollutants exist, limits for substances like EE2 are lacking – this is also the case for drinking water. Looking at the overall picture, according to da Cunha *et al.* (2016), in 2015 only 13.3% of Brazil's population had access to safe drinking water, below 50% were connected to the sewage system and below 40% of sewage waters were treated. – These basic needs are of higher priority before other issues can be tackled (da Cunha *et al.*, 2016), and this might be the case for many other countries with similar problems regarding water supply. From the view on the Brazilian authorization of

medicinal products, no evaluation of environmental risks has to be submitted (Brazilian National Health Surveillance Agency, 2019) in contrast to the European ERA or the American EA which are required to be part of the "Module 1" of the Common Technical Document, the format in which the dossier has to be submitted to receive a marketing approval in case of a positive evaluation. Nonetheless, the Brazilian guideline is under revision as of December 2020 and could be updated regarding environmental assessments in the future.

1.3 17α-Ethinylestradiol

Even though many countries have a different approach on the issue of various substances in the environment, it is a fact that a problem is existing and must be dealt with. One of these substance groups, which have caused concern in the environment, are hormonally active substances, better known as Endocrine Disrupting Chemicals (EDC). The World Health Organization (WHO) published an assessment titled "State of the Science of Endocrine Disrupting Chemicals" in 2012 to discuss effects of these substances on organisms. EDCs in general are substances, which interact with the endocrine system and cause negative effects on health. According to the assessment, in 2012 approximately 800 substances were suspected or known of their potential to act as EDCs. Simultaneously, illnesses related to disturbances in the endocrine system were increasing: Almost 40% of young men had reduced semen quality, male babies faced increasing numbers of genial malformations, numbers of babies with reduced birth weight and preterm birth were increasing, as well as increasing cases of type 2 diabetes and obesity to name a few. It is known that EDCs can affect other biota than humans negatively resulting in numerous ways, *e.g.* causing population declines, increase in endocrine-related disorders etc. (Bergman *et al.*, 2013). One of the substances, which is without a doubt an endocrine disrupting chemical, is EE2.

The chemical structure of EE2 is depicted in Fig. 1; the substance was described for the first time in 1938 by Inhoffen and Hohlweg, who were conducting studies on the efficacy of orally administered, supposedly estrogenically active substances. Studies on a castrated rat, a female baboon and a rabbit delivered positive results on the oral absorption of estrogenic substances, which was a big deal at that time, since hormonally active substances had to be administered via injections (Inhoffen and Hohlweg, 1938) and it is known that patient compliance can correlate with more pleasant applications. It took several more years until the development of orally applied contraceptives started in 1950 and it took more than ten more years, until in the 1960s sufficiently functioning oral contraceptives were available, also commonly known as "the pill". Nowadays usually EE2 is combined with a second substance with a progestogenic effect, leading to products known as "combined oral

contraceptive" to enhance the contraceptive effect of EE2. The first pills contained doses of up to 150 µg EE2 but this was reduced to 30 µg/pill in the 1970s (Lammers *et al.*, 1998). Products with an even lower content of *e.g.* 20 µg EE2 per pill are also available. During the course of time other beneficial effects of products containing EE2 were observed, *e.g.* using a combined product with Drosiprenone resulted in reduced menstruation pain or reduced forming of acne (Machado *et al.*, 2011).

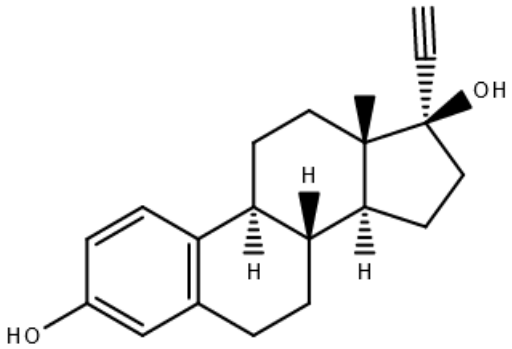

Figure 1: Structure of EE2.

As many active ingredients in pharmaceuticals, EE2 is metabolized in the human body after uptake, before it is excreted. It has been reported that major human metabolic pathways of EE2 are hydroxylation via CYP enzymes, glucuronidation or sulfation (Zhang *et al.*, 2007). In all three cases it is visible that the core structure is still existing and that the chemical group which was added onto the core structure of EE2 could be separated again (Fig. 2).

The wide-spread use of drugs containing EE2 as affective ingredient was followed by a massive release of EE2 and its metabolites into the environment. A previously published review by Aris *et al.* (2014) named the main sources of EE2 in the environment: Human urine, livestock wastewater and runoffs of manure and sewage sludge which was used previously agriculturally (Fig. 3).

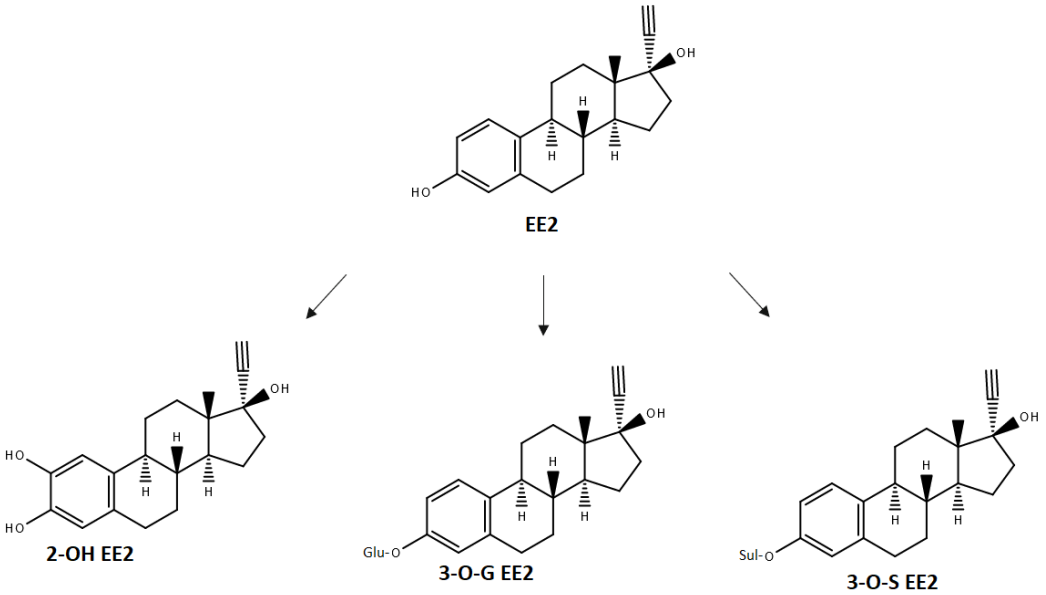

Figure 2: Metabolic pathways of EE2, redrawn after Zhang et al. (2007).

Once in the environment adverse effects have been observed in biota. A drastic example was given by Hoffmann and Kloas (2012) showed the harming potential of EE2 on the frog species *Xenopus laevis*. Adult males which were exposed to various concentrations of EE2 displayed lowered sexual arousal, which was clearly shown by analyzing their calls (Hoffmann and Kloas, 2012). This result is an obvious indication of how EE2 can have an impact on biota in the environment, which could lead in the worst case to extinction because of mating loss. But are there also other implications that EE2 could harm in other ways the environment?

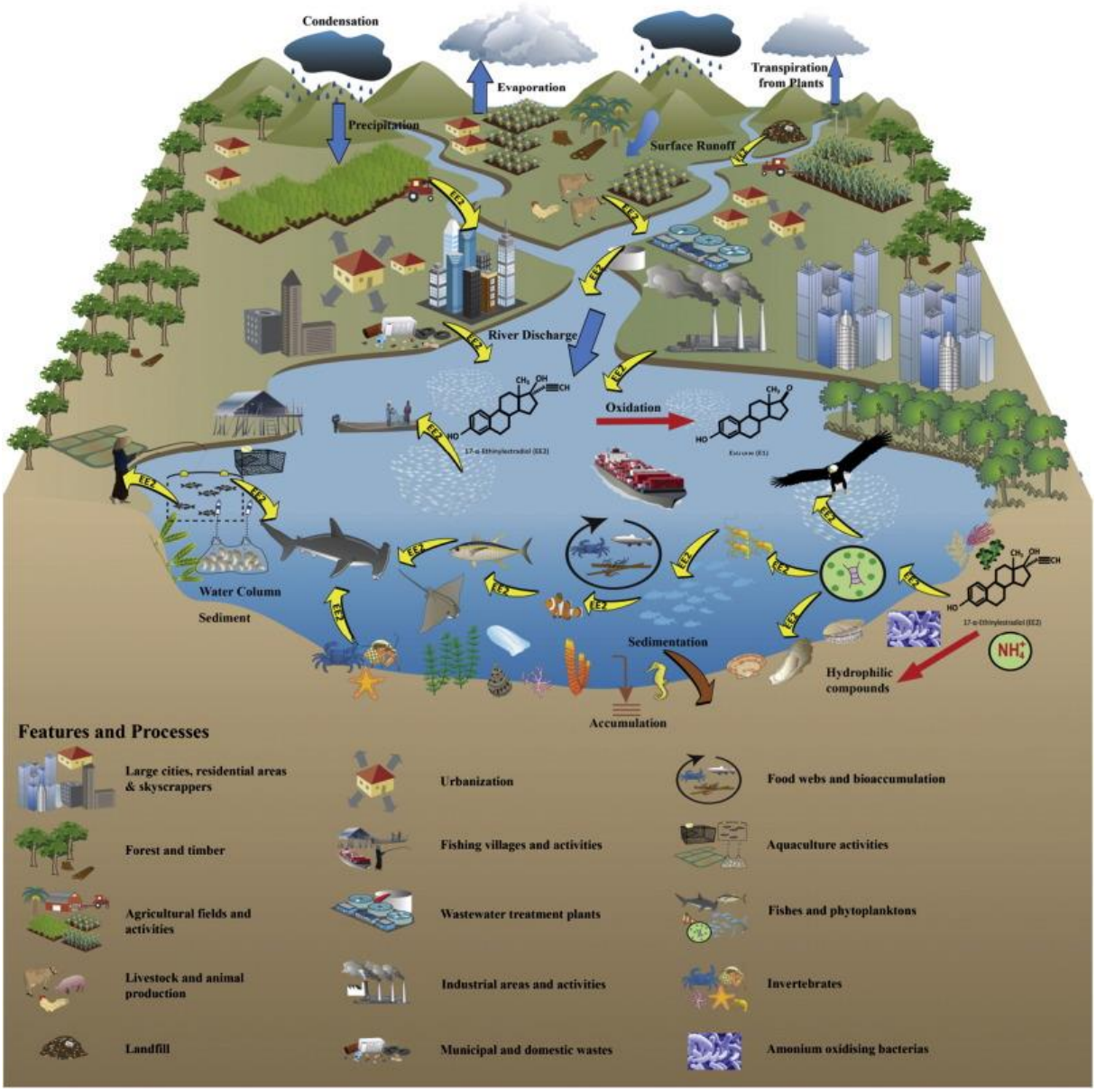

Figure 3: Sources, fate and transport of EE2 in the environment. Reprinted from Aris et al. (2014), with permission from Elsevier for this thesis. Symbols in the figure are Courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/symbols/).

In the review of Aris *et al.* (2014) topics like EE2 levels found in the environment, effects of EE2 on exposed organisms and the possible removal of EE2 were discussed. Environmental levels of EE2 were detectable at various levels, different effects on various species were reported and the need to propose a specific design to eliminate EE2 in the environment was expressed (Aris *et al.*, 2014). Six years have passed since the review was accepted by the publishing journal, and the status quo could have changed in this period of time as well.

Therefore, the aim of this work is to discuss the following questions:

- Where and in which concentrations does EE2 occur in the environment?
- What are the effects of EE2 on biota?
- How can the environmental concentrations of EE2 be reduced effectively?

2 Levels of EE2 in the environment

2.1 Studies on levels of EE2 in the environment

The EU is currently monitoring several substances in their member states which are considered as emerging pollutants (European Commission, 2018). In April 2018, a document containing the 1st review of this "Watch List by the European Commission´s science and knowledge service", the Joint Research Centre was published. 25 member states have submitted data for this compilation, while Spain, Greece and Malta did not submit any data at all. Of the submitted data, 98.3% were river samples, 1.2% lake samples and 0.5% coastal/transitional water samples (Loos *et al.*, 2018). One of the substances which were monitored was EE2.

Without looking at the measured concentrations yet, one parameter catches the attention of the reader: The desired Limit of Quantitation (LOQ) is set equally to the predicted no effect concentration (PNEC), below which no effects are expected. For EE2 the PNEC is estimated to be 0.035 ng/L, a concentration which many laboratories delivering data for the Watch List review could not achieve as the LOQ. Four countries reported that the commissioned laboratories reached an LOQ of 0.03 ng/L, another 4 countries reported 0.035 ng/L, all other countries reported values above these levels. 82 samples were therefore to be quantified and of those, in 75 the PNEC of 0.035 ng/L was exceeded. Nonetheless, the authors tried to utilize the data with higher LOQs than 0.035 ng/L and developed two scenarios to interpret all available data: In both scenarios, samples which were not able to be quantified are set to half of the respective reported LOQ. For further prediction, two cases are developed: In one case, all records are taken into account, in the other case only records are taken into account if the recalculated value is equal to or below to the PNEC. Only in the latter case, the median concentration was 0.015 ng/L and below the PNEC. With the first scenario (0.05 ng/L) as well as with considering only the quantitated results (0.1 ng/L), both median concentrations exceed the PNEC. Eventually the authors concluded that the available data is insufficient and therefore the substance shall remain on the Watch List (Loos *et al.*, 2018).

One of the EU member states which has provided data is Austria, and this member state has also published its own report regarding the presence of hormones and pharmaceuticals in surface waters. The report states that 20 measuring points were selected in Austria and from each, a sample was taken in fall/winter 2017 and another sample in spring 2018. The samples were analyzed using a method which detected multiple substances at the same time using ultra high performance liquid chromatography connected with a tandem mass spectrometer (UPLC-MS/MS). The outcome is that

EE2 was not detected in any sample, but the method showed at the same time a LOQ of 0.1 ng/L and a limit of detection (LOD) of 0.05 ng/L. Both limits are well above the PNEC of 0.035 ng/L. Additionally, the estrogenic effect of the samples was determined using a bioassay and the results of this assay lead to the assumption that even if EE2 was not detectable chemically, it was probably present at low concentrations in the samples (Clara *et al.*, 2019).

Not only governmental organizations are investigating the environmental concentrations of EE2, there are also several researching groups dealing with this topic. The following studies have been published between January 2014 and August 2020:

In January 2010, the team around Valdés *et al.* (2015) took samples from sewage effluents and their respective receiving waters in the Pampas region and the Río de la Plata estuary in Argentina to determine the concentration of estrone, 17β-estradiol and EE2. In total seven samples were analyzed using HPLC-MS/MS with a LOD of 15 ng/L and LOQ of 45 ng/L for EE2. EE2 was detected in every sample of sewage effluent accounting to 80 ng/L, 65 ng/L and 187 ng/L respectively. In the surface waters only one sample had detectable levels of EE2 (43 ng/L) - the concentration in the three other samples was below the LOD. The authors interpret the results as a probable threat on aquatic organisms (Valdés *et al.*, 2015).

The determination of the distributions of estrogens and bisphenol A in the Yangzte River Estuary in China and the East China Sea was the aim of Shi *et al.* (2014), who took water and sediment samples in the wet season in 2010 and in the dry season in 2011. Four municipal WWTPs discharge their effluents into the Yangtze River Estuary, this was considered in the sampling strategy by distribution of the sampling points. Samples were analyzed via LC-MS/MS and the method detection limits were ranging from 0.02 to 0.05 ng/g for sediment samples and from 0.02 to 0.1 ng/L for water samples. In the wet season, EE2 levels were not detectable in all 15 water samples and 30 sediment samples. In the dry season, EE2 was detected in only one out of 30 water samples (0.11 ng/L) and in two out of 30 sediment samples (0.06 ng/g and 0.72 ng/g). In a separate recalculation to determine the estrogenicity of a drawn sample where the sampling location is close to a livestock farm, the high estrogenic potential was assigned to high EE2 levels in the sample (Shi *et al.*, 2014).

Nie *et al.* (2014) took samples in August 2011 from the upper Huangpu River, a large river in Shanghai to analyze EE2 as well as the other estrogenic compounds estrone, estradiol, estriol, bisphenol A and 4-tert-octyphenol. While five sampling sites were located at five tributaries of the Huangpu River, six sampling sites were located at the main river itself and additional four sampling sites were

taken from receiving streams of animal feeding operations. Suspended particulate matter and colloidal samples, obtained by filtration, were analyzed as well to identify if there was a tendency for adsorption. The chosen method for the analysis of EE2 was GC-MS using a prior derivatization step. The authors report LODs of 0.10 – 0.49 ng/L and LOQs of 0.30 – 1.97 ng/L in aqueous samples and LODs of 0.15 – 0.44 ng/g and 0.93 – 3.15 ng/g in suspended particulate matter samples for all analyzed substances. In the aqueous samples, EE2 was detected at concentrations up to 20.1 ng/L from animal feeding operation receiving streams, while except for one single sample of the main river, EE2 was not detectable. This was also the case for colloidal samples, EE2 was detected in only one sample. On the contrary, EE2 was detected in all suspended particulate matter samples with levels of up to approximately 120 ng/g. In this case the highest values were observed in the samples taken at the receiving stream of animal feeding operations (Nie *et al.*, 2014).

The northern parts of the Taihu Lake in China were the study area of Wang *et al.* (2014) in May 2013 in which various estrogenous compounds were investigated on, and one of those was EE2. Eight sampling sites were chosen and water, sediment and biota (fish, river snail and clam) samples were collected and analyzed using HPLC-MS/MS. The LODs were 0.8 ng/L for water samples and 0.5 ng/g for sediment samples as well as for fish samples, a specific LOD for other biota samples was not stated, assuming same limits due to the identical sample preparation. In water samples, EE2 was only detectable in two samples with 21.1 ng/L and 33.5 ng/L. EE2 was detectable in every sediment and biota sample and ranged from 4.32 to 184 ng/g in sediment samples and from 21.3 to 417 ng/g (dry weight) in biota samples. In direct comparison of the three biota species, river snails displayed with a bioaccumulation factor of 25,033 the highest for EE2 (for clams were 6061 and for fish were 4115 reported) (Wang *et al.*, 2014).

Prior to the previously described study, the team around Yan *et al.* (2014) took samples from the same waterbody, Taihu Lake in China. In contrast to Wang *et al.* (2014) the samples were taken earlier, that is from November to December in 2011. The focus in this study were in general emerging organic contaminants, among those was also EE2. With the analyzing system of an UHPLC/MS/MS which was used they achieved an MDL of 1 ng/L for EE2. At two out of eight sampling points, EE2 was not detectable. The concentration range of the other substances was between 1.64 ng/L and 4.00 ng/L. An additional calculation of the hazard quotient lead to the result that EE2 is one of the greatest hazards in the lake (Yan *et al.*, 2014).

Avar *et al.* (2016) published a study, in which samples from rivers in the Carpathian Basin in Slovenia and Hungary were measured to determine concentrations of estradiol and EE2. Unfortunately, it

was not stated when the samples were taken. The authors used HPLC-MS and HPLC-MS/MS as the analyzing system, the LOQ for EE2 was for HPLC-MS at 0.001 ng/L and for HPLC-MS/MS 0.2 ng/L. Both systems were used to determine the EE2 concentration and regarding the HPLC-MS method, several samples had concentrations ranging from 0.002 ng/L to 0.175 ng/L. The presence of EE2 using HPLC-MS/MS was only confirmable at two sampling sites, river Zala at Balatonhidvég (0.62 ng/L) and the canal Héviz-Páhoki at Alsópáhok (0.436 ng/L). The presence of EE2 could not be confirmed in the other 21 samples using HPLC-MS/MS (Avar *et al.*, 2016). The data which were obtained using the HPLC-MS method were summarized in table 1.

Another study which was conducted in an EU member state was by Garrido *et al.* (2016), who monitored emerging pollutants in the Guadiamar River basin in southern Spain. Among several other substances, EE2 was analyzed in water samples which were collected in June 2014 at six sampling sites which were located at the main river and tributaries. The analysis was performed using a LC-MS/MS system with a method specific for the determination of hormones and industrial pollutants besides two other methods which were used for pharmaceuticals in human use and for veterinary pharmaceuticals. They achieved for EE2 a method detection limit of 15.0 ng/L and a method quantification limit of 49.5 ng/L. In the studied area, EE2 was not detected in any sample (Garrido *et al.*, 2016).

Barber *et al.* (2019) conducted a large-scale study with various aims to assess topics like fish endocrine disruption, exposure risk and wastewater reuse in the Shenandoah River Watershed in eastern West Virginia and northern Virginia. Another target was to verify if the model which was used to predict environmental concentrations was close to reality and therefore equal measured environmental concentrations. For this verification, samples were taken in 2014, 2015 and 2016 over a period of 4 weeks every 7 days, while the number of samples which was drawn varied between 4 and 12 at each site. The samples were analyzed using 21 different analytical methods, one of those measured among other substances EE2. The method detection limit was at 0.1 ng/L and the authors reported the following results: EE2 was measured above the method detection limit at only one out of nine locations at the South Fork Shenandoah River (2.4 ng/L), the concentrations in all other samples were below the method detection limit (Barber *et al.*, 2019).

The Hawkesbury River in Australia was the point of interest in a study published by Uraipong *et al.* (2017). The authors developed an ELISA for the specific simultaneous detection of EE2 and mestranol, which was used in a second step to determine the concentration of those substances in water supply. Eight samples were taken along the Hawkesbury River in Emigrant Greek, Northern New

South Wales and South Greek, Sydney upstream, at the discharge point and downstream of the discharge point of WWTPs stating that the samples were "fresh" but no sampling period was stated. The researchers were able to achieve a LOD of 0.04 ± 0.02 µg/L and an LOQ of 0.05 ± 0.01 ng/L for EE2 and similar results for mestranol. With their method were the following results obtained: Upstream samples of the WWTPs had concentrations of 15 ng/L, downstream samples had concentrations of 28 – 29 ng/L for EE2 and mestranol. In a catchment nearby the WWTP, the combined concentrations were in the catchment at 5.5 ng/L, the farer away upwards the samples were taken the concentrations were lower with 8.3 ng/L at 3 km, 6.1 ng/L at 9 km and 4.1 ng/L at 11 km. According to the scientists, although no separation of the combined concentrations of EE2 and mestranol was conducted, they expect that almost 100% of the detected substances were EE2 residues. Another statement that was made was the expectation that agriculture and urbanization are the main contributors to the high EE2 levels (Uraipong *et al.*, 2017).

The aim of the study which was conducted by Pereira *et al.* (2017) was to give an insight on the impact of surface water flow rates and WWTPs on environmental concentrations of various substances, among them EE2. The samples were taken across Portugal from Tâmega River, Tua River, Mondego River, Trancão River, Tagus River, Xarrama River, Guadiana River and Álamo Creek. From 20 different sites in 2014 from September to November and in 2015 from February to March, altogether 72 samples were collected. The analysis was performed using a LC-MS/MS system, for EE2 the method detection limit was at 6.82 ng/L and the method quantification limit at 20.65 ng/L. EE2 was not detected in any analyzed sample (Pereira *et al.*, 2017).

Another China-based study was conducted by Niu and Zhang (2017) regarding the Huai River and its potential pollutants, in January 2010 were water samples taken at four different sites. The samples were analyzed in this study using an HPLC system with a diode array detector and reached for EE2 a LOD of 0.12 pg/L. With this comparably low LOD, EE2 was detectable in 11 out of 12 samples in total and the concentration in the samples in which EE2 was detected was ranging from 0.048 ng/L to 0.174 ng/L. The authors pointed out that EE2 had the tendency to decrease in its concentrations from sampling points upstream compared to downstream in contrast to other substances which they analyzed, leading to the assumption that for these chemicals had additional sources of emission (Niu and Zhang, 2017).

Griffero *et al.* (2019) published a study about South American Atlantic coastal lagoons in Uruguay. At 23 points of the Laguna de Castillos and the Laguna de Rocha samples were taken along streams, lagoons and coastal sea zones. The drawing of the samples took place in 2017 in February, May,

August and November, which resulted in total in 92 samples. The analysis was performed using LC combined with high resolution mass spectrometry, the LOQ for EE2 was 0.1 µg/L. EE2 was detectable only in four samples: In May at one site with 0.24 µg/L, in August at the same site with 0.13 µg/L and in winter at two different sites with 0.42 µg/L and 45.51 µg/L. Even though in general temporal distribution for the other substances was not observed, EE2 levels increased in winter at a site close to an urban area according to the authors (Griffero *et al.*, 2019).

For the study of Coelho *et al.* (2020), the researchers took samples in São Paulo waters in Brazil, namely in one of the Billings reservoir branches. In total eight sampling campaigns took place from June 2017 to February 2018, four in the dry period from June to August 2017 and four in the wet period from October 2018 to February 2019. The analysis was performed according to a method published by the US EPA via LC-MS with a limit of detection of 30 µg/L and limit of quantification of 100 µg/L. The authors state that by evaporation of the solvent and suspension of the residue, a concentration factor of 1000 was achieved leading to a quantification limit of 100 ng/L. In the dry period, EE2 concentrations were ranging from < LOQ to 1200 ± 140 ng/L while in the wet period the concentrations were ranging from < LOQ to 300 ± 90 ng/L. At one sampling site with low anthropogenic impact no sampling campaign EE2 was detectable, the other three sampling sites were probably impacted by WWTP according to the researchers (Coelho *et al.*, 2020).

2.2 Discussion of the studies on levels of EE2 in the environment

The first fact that draws attention is that both governmental measurements and measurements conducted by researchers in academic institutions failed in lots of cases to establish analytical limits which were low enough to be able to discuss if in cases, in which EE2 could not be quantified, a risk is posed to the environment according to the PNEC or not. Nonetheless, an interpretation of the reported data in several cases is possible, because even if the method limits were above the PNEC, EE2 could still be measured in several cases. Before the EE2 emissions into the environment are not lowered in these places, there is no urgent need to improve the analytical parameters of the used methods.

Regarding the locations in which the samples were taken, every continent except for the Antarctica and Africa was represented. If the presented studies were the data set to evaluate if actions regarding the EE2 emissions are required, a recommendation would be that additional data have to be gathered with lower method limits. It would be necessary, and this statement is also a recommendation for studies to be published in the future, to use methods with lower limits, maybe even

below the PNEC to be certain that environmental concentrations which tend to fluctuate for various known or unknown reasons are below the PNEC even if a concentration peak occurs. There were only three studies in the reviewed period which reported analytical limits below the PNEC namely Shi *et al.* (2014), Avar *et al.* (2016) and Niu and Zhang (2017).

As reported by Wang *et al.* (2014), EE2 showed a tendency to accumulate in sediment with a concentration of up to 184 ng/g and in biota up to levels of 417 ng/g. A simple consideration can explain this observation: EE2 is a highly lipophilic substance which is excreted mainly via conjugation with hydrophilic groups (two of those are shown in Fig. 2 in the introduction). If those groups are separated from the mother substance, the highly lipophilic behavior returns and could display its affinity to *e.g.* substances in sediment or fat containing compartments in living organisms.

The highest EE2 level in water samples was measured in Brazil with 1200 ± 140 ng/L, which above the PNEC with a factor of over 300,000. The authors of this study (Coelho *et al.*, 2020) propose that the dry season in which this sample was taken has an influence on environmental concentrations of EE2 This effect was also observed by Shi *et al.* (2014) in China, although the concentration range in this study was between not detectable and 0.11 ng/L (Shi *et al.*, 2014). The previously stated observation of EE2 levels by Coelho *et al.* (2020) closes the circle to the described situation regarding EE2 regulation in Brazil: No limits for EE2 in the environment (da Cunha *et al.*, 2016) are set on the one hand and on the other, the national water treatment system has to catch up to the standards of other countries like the EU member states or the USA.

The results obtained from Wang *et al.* (2014) and Yan *et al.* (2014) are the results which can be compared the best since the waterbody was the same and many sample drawing locations were close. The sampling took place approximately one and half years apart and therefore it is possible to compare the course. One result draws the attention of the reader: The concentration at the inlet to the Wangyu river was in 2011 at 2.28 ng/L and in 2013 at 21.1 ng/L, which means that the concentration has risen approximately nine fold in only 1.5 years if these results are not outliers. The highest reported value in 2011 was 4.00 ng/L, and the highest reported value in 2013 reached 33.5 ng/L. Although a trend regarding rising EE2 concentrations is visible, it must be kept in mind that singular points in time are not able to portray the whole picture, since various regular and irregular, known and unknown impacts can influence the EE2 concentration. Known impact has been reported for instance by Coelho *et al.* (2020) like increased concentrations when flow rates decrease, which can occur naturally during dry periods.

Aris *et al.* (2014) divided the results they observed into the categories “water”, “sediment” and “biota” which was due to the low number of sediment and biota results not done in this review. Biota samples in this review were analyzed only by Wang *et al.* (2014) from Taihu Lake in China, which can be compared location-wise to the results obtained from Zhang *et al.* (2011): They collected in August 2008 biota samples in the Yundang Lagoon in Xiamen City, which is an urban area. The measured levels (based on the lipid weight) in this study were for the short-necked clam *Ruditapes philippinarum* sample 3.42 ng/g, for the black seabream *Acanthopagrus schlegel* 3.03 ng/g and for the yellow fin seabream *Sparus latus* 2.71 ng/g, whereas in the tilapia sample the level was below the detection limit of 0.54 ng/g (Zhang *et al.*, 2011). Wang *et al.* (2014) reported concentrations in biota ranging from 21.3 to 417 µg/kg dry weight.

As a first approximation, the obtained EE2 concentrations by Zhang *et al.* (2011) were recalculated for this work into dry weight using data on total lipids and water provided by the United States Department of Agriculture FoodData Central Database (available at https://fdc.nal.usda.gov/). According to this approximation, the EE2 content in *R. philippinarum* is 0.16 µg/kg dry weight, in *A. schlegel* 0.34 µg/kg dry weight and in *S. latus* is 0.30 µg/kg dry weight. These recalculated values are below the values which were reported by Wang *et al.* (2014). Just like it was previously described for the Wangyu river, these singular measurements are just snapshots of a moment, nonetheless a direct comparison shows an increase in EE2 concentration.

Other previously reviewed studies reported EE2 levels in mussels up to 38 ng/g dry weight (Pojana *et al.*, 2007) and in fish of up to 78.15 ng/g based on lipid weight or 2.30 ng/g based on wet weight (Al-Ansari *et al.*, 2010). Recalculation of the latter result as described in the previous chapter lead to 11.34 µg/kg dry weight. These contents of EE2 in dry weight are either in or close to the measured range in biota reported by Wang *et al.* (2014).

At this point it should be noted that the need to recalculate the EE2 contents in biota shows the need in this field of science to establish either a common reporting method as dry weight, lipid fraction or another reference value. Another way would be to provide enough data to enable other researchers to recalculate the reported data into the reference value they need for their own work.

In this review, Wang *et al.* (2014) and Shi *et al.* (2014) analyzed sediment samples in Taihu Lake (China) and the Yangtze River Estuary (both located in China) and detected up to 184 ng/g dry weight and 0.72 ng/g dry weight, respectively. In the review by Aris *et al.* (2014), several studies measured the levels in sediment. The highest reported concentration was ca. 130 ng/g dry weight

(Froehner *et al.*, 2012), while the measurement of other several sediment samples in China did not exceed 10 ng/g dry weight as summarized by Aris *et al.* (2014). The highest concentration of EE2 in sediment that was reported by Wang *et al.* (2014) (417 ng/g dry weight) exceeds all reported concentrations of the reviewed publications by Aris *et al.* (2014), which should raise concern – again it has to be kept in mind that measurements at one timepoint do not necessarily display the whole picture of environmental levels of EE2.

Finally, the analysis of water samples was also summarized by Aris *et al.* (2014) with a highest reported EE2 level of 34 ng/L at the Venice Lagoon (Pojana *et al.*, 2007). The highest EE2 concentration in waterbodies that was presented in this review reached tremendous 1200 ng/L in Brazil (Coelho *et al.*, 2020), while in Europe the highest measured concentration reached 0.68 ng/L (Avar *et al.*, 2016). The latter result should be taken with precaution, since some other European laboratories operated with method detection limits of 6.82 ng/L (Pereira *et al.*, 2017) or 15.0 ng/L (Garrido *et al.*, 2016). Looking at China, which is in both reviews the country with the most results, it can be observed that while Aris *et al.* (2014) also happened to review publications in which no EE2 was detected in water samples, in this review were no studies which reported that EE2 was exclusively below the analytical limits in that country.

Another fact which should be considered during further studies is that although EE2 was not detected, this does not mean that degradation products do not possess estrogenic activity and can still disrupt the endocrine system. To obtain a whole picture it would be therefore necessary to identify potential degradation products of EE2 which still pose a risk to the environment, whether the source of these are natural or sewage treatment processes and to screen for those products.

3 Effects of EE2 on biota

A summary of shown effects of EE2 on biota is given in table 2 in the appendix. If partial results of the presented studies were that EE2 had no impact on specific investigated parameters, these partial results will not be summarized here. Also, if a study investigated on more combined substances including EE2, these results will be excluded from this work. If studies report results on more than one species, these studies will not be divided but will be retained as one. Due to simplification, the nominal concentrations which were used in the experiments are stated in these summaries. Where possible, the units were unified to ng/L for better comparability.

3.1 Zebra fish *Danio rerio*

A study which focused on the zebrafish *Danio rerio* was conducted by Luzio *et al.* (2016). They investigated the effect of 4 ng/L EE2 on the gonad development and sex development at 23, 28 and 33 °C (and additional control groups for each temperature) with the exposure starting 2 hours post fertilization until 60 days post fertilization. Regarding biometric parameters, while no effect on the mortality was observed, an increase in temperature and in EE2 resulted in weight and length increase, while the length increase was significant in almost every group. Differences in gonad classification were observed in the 23°C and 33°C group when compared to the respective control group: At 23°C, the gonad differentiation was promoted, while at 33°C male gonad development was delayed. For male fish, at 28 and 33 °C testis maturity was decreased, while for females all EE2 exposed groups were further developed in shorter time (Luzio *et al.*, 2016).

Zebrafish were exposed to EE2 and 17β-trenbolone in a study which was published by Örn *et al.* (2016) investigating the impact on gonad maturation, sex ratio and vitellogenin production. Fish were exposed to all possible mixtures of 2 or 5 ng/L EE2 and 1, 10 or 50 ng/L 17β-trenbolone as well as only 2 or 5 ng/L EE2. The exposure started 20 days post hatching and ended 60 days post hatching, while vitellogenin levels were determined in fish 40 days post hatching. Following significant results in comparison to the control were obtained: Higher vitellogenin levels in the 5 ng/L EE2 group, a drift towards females in all groups which contained 5 ng/L EE2 as well as the 2 ng/L EE2 group, while in contrast 2 ng/L EE2 combined with 50 ng/L 17β-trenbolone caused a male shift. Ovary maturation was significantly inhibited in every group that contained 5 ng/L EE2, while testis maturation was inhibited in the 2 ng/L EE2 + 1 ng/L 17β-trenbolone and 5 ng/L EE2 + 50 ng/L 17β-trenbolone exposure group. Also, 2 ng/L EE2 + 50 ng/L 17β-trenbolone accelerated testis maturation (Örn *et al.*, 2016).

Goundadkar and Katti (2017) published a study on zebrafish *D. rerio* and swimming behavioral impact of various substances, EE2 was one of the substances. Fish were exposed to a concentration of 5 ng/L for 75 days, while tests were conducted on day 15, 30, 60 and 75. The researchers were monitoring the total swimming duration, immobility and erratic swimming phases. Significant results when compared to the control group were on day 30 decreased swimming activity, increased immobility and freezing episodes. On day 60 and 75 they observed a decreased swimming activity and an increase in immobility, freezing and erratic movements (Goundadkar and Katti, 2017).

In a study conducted by Valcarce *et al.* (2017), male zebrafish were exposed to EE2 in order to assess effects on sperm and on the offspring. The fish were exposed to 2.5, 5 and 10 ng/L EE2 for 14 days. Regarding the malformation rate in the F1 generation, a statistically significant increase was observed in the 2.5 and 5 ng/L EE2 group. Five days post fertilization, lymphoedema in the 5 ng/L group significantly increased, as well as the areas of the otoliths (biomineral crystals located in the inner ear, support balance and hearing). In testes of male fish which were exposed to 5 ng/L EE2, the expression of the genes *bdnf* and *dmrt1* (both genes linked to breeding quality) was significantly lower when compared to the control group. Analysis of mRNA in semen of male fish exposed to 5 ng/L showed a significant increase in transcription of *esr2b* (encodes estrogen receptors). The authors also analyzed the gene expression of F1 larvae 25 hours post fertilization in the anterior embryonic trunks showed that *esr1* (encodes estrogen receptors) and *vegfc* (lymphatic development) were significantly downregulated. Analysis of the motor function showed for F1 generation larvae of males in the 5 ng/L group that the available space was less crossed and that they screen the testing arena less than the control group. In summary the results indicate that paternal EE2 exposure can have an impact on following generations (Valcarce *et al.*, 2017).

Another study which was conducted on zebrafish was published by Fenske *et al.* (2020), who investigated on the impacts of EE2 exposure on levels of hormonal steroids and behavior. Fish were exposed to EE2 at concentrations of 0.5, 1.5, 5.0, 50 and 75 ng/L for either 1 h (acute exposure) or 15 d (long-term exposure). Summarizing behavioral responses, significant differences were observed regarding acute exposure at 5 and 50 ng/L with decreased anxiety-like behavior while chronic exposure displayed increased social behavior at 1.5, 5 and 50 ng/L. Also, an increase in anxiety-like behavior at 75 ng/L was observed while aggression decreased at 1.5 and 5 ng/L. Hormonal profiles of long-term exposed fish showed significant decrease in testosterone levels (all exposed groups), in estradiol levels (1.5, 5, 50 and 75 ng/L EE2 groups) and cortisol levels (1.5, 5 and 75 ng/L EE2 groups) (Fenske *et al.*, 2020).

D. rerio is the species on which the most studies regarding impact of EE2 were published in the reviewed period. The lowest initial EE2 concentration was 1.5 ng/L, at which still an impact was reported while the same study by Fenske *et al.* (2020) reported also the highest EE2 concentration experiments were conducted at 75 ng/L (Fenske *et al.*, 2020). That study was also the only one to address acute toxicity with an exposure period of 1 h, while the other studies in this group addressed long-term exposure over the duration of up to 75 d. The reported effects of all studies in this group can be summarized as impacting growth, maturation, hormone levels and behavior.

In the review published by Aris *et al.* (2014), *D. rerio* was also the target species in several reported studies. The shortest reported exposure period at that time was 24 h and the longest was 90 days, with exposure concentrations ranging from 0.5 ng/L to 50 ng/L. Reported effects can be summarized as impact on behavior, reproduction, gene expression, growth and maturation (Aris *et al.*, 2014). Altogether these results are complementary and display wide range of effects on *D. rerio* over various EE2 concentrations and long-term exposure periods.

3.2 Japanese rice fish *Oryzias latipes*

The transgenerational effects of EE2 and BPA on Japanese rice fish, also known as medaka *Oryzias latipes* were investigated by Bhandari *et al.* (2015), who exposed fertilized eggs to EE2 at a concentration of 50 ng/L for 7 days starting in the time window which determines the sex of the adult fish. Subsequent generations (F1 to F4) were not exposed to EE2. The results showed that the EE2 uptake was 1.2 pg/mg egg on the first day and 4.0 pg/mg egg in 7 days. Significant differences to the control group were observed in the F1 generation with a higher fertility, the F2 generation which displayed a reduction in the fertilization rate and in the F3 and F4 generation the embryo survival was lower (Bhandari *et al.*, 2015).

Anderson *et al.* (2020) conducted a study regarding the impact on the heart rate of Japanese medaka caused by EE2. Embryos were exposed to various EE2 concentrations. In a preliminary range-finding experiment which lasted from 6 h post fertilization to 120 h post fertilization, the heart rate of embryos decreased at 50 and 500 ng/L, while it increased at 50000 ng/L (interestingly, no effect was observed at 5000 as well as at 5 ng/L when compared to the control group). Further experiments with embryos using 0.1, 1, 10, 100 and 1000 ng/L EE2 showed significant decreases in the heart rate 144, 168 and 192 h after fertilization occurred, while 120 h and 216 h after fertilization the pattern was more complex, although a decrease in heart rate still occurred. Another experiment assessing the heart rate was performed with various estrogen modulators: These

modulators were mixed at either 10 or 100 ng/L with 10 ng/L EE2 and exposed to embryos 120 hours post fertilization, ending the experiment after hatching. A complex response pattern with each modulator was observed, which was interpreted by the authors as a link that the G protein-coupled estrogen receptor plays a role in estrogen-induced bradycardia (Anderson *et al.*, 2020).

A study on impacted gene expression by EE2 and bisphenol a in male *O. latipes* was conducted by Bhandari *et al.* (2020). Fertilized eggs were exposed to 10 ng/L EE2 for 50 days. Comparing the testes to the control group, in the EE2 group the expression of 813 genes decreased while that of 653 increased significantly. Additional analysis of gene ontology showed enrichment of differentially expressed genes and was categorized in various groups (Alzheimer disease-presenilin pathway, cadherin signaling pathway, integrin signaling pathway) which were induced (Bhandari *et al.*, 2020).

The reported studies on *O. latipes* were ranging regarding the exposure duration from several days to weeks and covered a broad range of EE2 concentrations. Especially the study by Anderson *et al.* (2020) contributed to this with concentrations as low as 0.1 ng/L up to 50,000 ng/L. Interestingly, the heart rate decreased up to 1000 ng/L EE2, while an increase in the heart rate was observed at 50,000 ng/L causing at high levels the opposite effect (Anderson *et al.*, 2020). Reported effects were in short impact on the fertility of subsequent generations, heart rate and regulation of genes.

Previously, Scholz and Gutzeit (2000) exposed *O. latipes* for 2 months at concentrations of 1, 10 and 100 ng/L and reported several effects: In females effects on reproduction and gene expression and in males feminization was observed (Scholz and Gutzeit, 2000). The results from Scholz and Gutzeit (2000) cover especially with the duration of 2 months a longer period than the studies which were reviewed in this work and show the effects of EE2 on this species.

3.3 Siamese fighting fish *Betta splendens*

Dzieweczynski *et al.* (2014) exposed female Siamese fighting fish *Betta splendens* to EE2 at concentrations of 10 ng/L for 5 hours. Statistical evaluation of the behavior which was observed in boldness assays showed that EE2 had an impact on boldness behavioral syndrome by decreased overall behavior and no consistent shyness or boldness (Dzieweczynski *et al.*, 2014).

Another study which was conducted on *B. splendens* was published by Cram *et al.* (2019) to investigate on the mating choice. Male fish were exposed to 5 ng/L EE2, while female fish were exposed

to either 5 or 10 ng/L for 2 weeks. Statistical analysis of the behavior showed that (in comparison to the control group) the highest responses from males towards females were achieved when exposed females were used, and this response was higher in the 5 ng/L than in the 10 ng/L group. Additionally, exposed males showed a lower response than females (Cram *et al.*, 2019).

The two studies on *B. splendens* show that both acute and long-term exposure can have an impact on behavior and in particular reproduction at levels of 5 or 10 ng/L EE2. The results also make clear that in some cases it is necessary to use advanced statistics to analyze interactions between parameters which can affect complex mechanisms like the stated, impacted areas. In general it can be stated that the behavior and reproductive behavior were impacted. Studies conducted with species could in future focus on topics like gene expression and feminization since effects on these areas have been reported on other species as it was stated.

3.4 Inland Silverside *Menidia beryllina*

The effects of bifenthrin and EE2 on egg and offspring production by the inland silverside *Menidia beryllina* were investigated on by Decourten *et al.* (2017). Adult fish were exposed to a concentration of 1 ng/L EE2 for 14 days, embryos of those were exposed for 21 days post hatching. Both experiments were conducted at 22 and 28°C. When compared to the control group, EE2 exposure lead to the following significant results: Reduced number of eggs in the F0 generation in the 22°C group, increased number of eggs of the F0 generation in 28°C and reduced number of eggs of the F1 generation in the 28°C group. The proportion of female fish in the F1 generation was in the 28°C group larger and the proportion of deformed larvae in the F2 generation was in the 28°C group larger (six-fold higher than in the 22°C group) (DeCourten *et al.*, 2017).

Another study was conducted by DeCourten (2019) focusing on effects regarding reproduction and development by bifenthrin and EE2 at 22 and 28°C on inland silverside *M. beryllina*. The parental generation was exposed to 1 ng/L EE2 for 14 days. Larvae of the F1 generation were exposed starting at fertilization until 21 days post hatching, while F2 generation larvae were raised in water containing no EE2. The authors chose to analyze genes which take a role in development, growth and reproduction. They found that in the F1 generation group which was kept at 28°C, for nine out of ten selected genes the expression was significantly reduced. In the F2 generation, the expression of GPR30 (G-protein coupled estrogen receptor, only observed in larvae) was reduced in the 22°C group while the expression of FSHR (follicle stimulating hormone receptor) was reduced in the 28°C group (DeCourten *et al.*, 2019).

DeCourten *et al.* (2017; 2019) decided to conduct experiments with the same species, initial concentration of EE2 and exposure duration in both studies and used this to deepen the knowledge which was generated in the first study in 2017. The altered expression of various genes was observed in 2017 and in 2019 it was reported that the affected genes play a role in reproduction, therefore EE2 is impacting the offspring in the long run (DeCourten *et al.*, 2017; DeCourten *et al.*, 2019). Additional data on acute exposure and behavioral changes caused by EE2 could be investigated areas in the future.

3.5 Argentinian silverside *Odontesthes bonariensis*

Gárriz *et al.* (2015) conducted a study on the reproductive process in Argentinian silverside *Odontesthes bonariensis* affected by EE2, E2 and a mixture of those. Exposure samples with concentrations of 22.5 (175), 45 (350), 90 (700) or 180 (1400) ng/L EE2 and mixtures which had additional E2 (respective E2 concentration in brackets) were tested. Sperm samples from the fish were extracted, mixed with the exposure samples and observed immediately. The motility in all except the lowest concentration mixture exposure samples was lower, but no significant changes regarding the control group were observed. The embryo-larval survival was monitored over a mean duration of 8 days for embryos and 16 days for larvae, resulting in a significantly reduced embryo survival in the highest concentration group and in mixtures an adverse effect was observed for the 90 ng/L EE2 mixed with 700 ng/L E2 group. A significantly reduced larvae survival was observed for 45 and 90 ng/L EE2 and in all mixture groups except the 45 ng/L EE2 mixed with 350 ng/L E2 group. In a fertilization assay treated sperm samples were used to *in vitro* fertilize eggs with 180 ng/L EE2, 1800 ng/L or the previously mentioned exposure mixture with the highest concentration. The results showed a significant decrease in fertilization only observed in the highest mixture group (Gárriz *et al.*, 2015).

Effects of EE2 and E2 on the endocrine-reproductive axis in *O. bonariensis* were investigated on by Gárriz *et al.* (2017) in a consecutive study. Fish were exposed to 45 ng/L EE2 and a mixture with additional 350 ng/L E2 for 14 days. Significant results compared to the control group were: Increased expression of *gnrh-III* (related to the synthesis and release of gonadotropins) in the 45 ng/L EE2 group, increased expression of *cyp19a1b* (related to brain aromatase) in the group exposed to the mixture and an increase in pyknotic nuclei. Decreased were the *fshr* and *lhcgr* (related to gonadal gonadotropin receptors) expressions in both groups and the length of spermatogenic lobules. The authors interpret that the overall danger which these results indicate in the end is sterility (Gárriz *et al.*, 2017).

Like the previously mentioned studies by DeCourten *et al.* (2017; 2019), Gárriz *et al.* (2015; 2017) decided to conduct studies on one species in two consecutive studies with similar exposure durations. The amount of initial exposure concentrations was reduced in the second study by Gárriz *et al.* (2017), but the results were more detailed: While in 2015 they reported in general an impact on survival of embryos and larvae, the reason for the observed reduced fertilization rate was specified in 2017 with the alteration of the expression of various genes (Gárriz *et al.*, 2015, Gárriz *et al.*, 2017). Similar to the studies conducted by DeCourten *et al.* (2017; 2019), additional data regarding behavioral changes could extend the pattern on species-specific effects caused by EE2.

3.6 Gilthead seabream *Sparus aurata*

Capolupo *et al.* (2018) conducted a study on three species: Mediterranean mussel *Mytilus galloprovincialis* and sea urchin *Paracentrotus lividus* were investigated on embryo-larval development and fertilization success, while sea bream *Sparus aurata* was investigated on survival of post-hatch larvae. Various substances were tested, among them EE2 at a concentration of 5, 50 or 500 ng/L. Gamete fertilization assays were performed for mussels by addition of sperm into solutions containing EE2 for 60 min, following an addition of eggs for 30 min while for sea urchins the initial duration of the sperm exposure was the same, but the time after egg addition was reduced to 15 min. In mussels, a significant reduction of fertilization success by 24.0% was observed in the 500 ng/L group, while in sea urchin the fertilization success decreased correlating with increasing EE2 concentration from 19.9%, over 29.5% to 32.0% at 5, 50 and 500 ng/L EE2, respectively. Embryotoxicity assays were performed on mussels after eggs and sperm were mixed and after 30 min put into plates, into which EE2 solutions were added. For sea urchins the waiting period for this assay were after mixing 15 min, the eggs were added afterwards into vials containing EE2. The total exposure time was 48 h for both species. The percentage of normally developed embryos was reduced in mussels at 5, 50 and 500 ng/L EE2 by 19.9%, 29.5% and 32.0% significantly, while in sea urchin significant reductions were observed in the 50 ng/L (22.4%) and 500 ng/L (28%) group. The last conducted test was fish larvae mortality on *S. aurata*, by exposure of larvae to the previously stated EE2 concentrations for 96 h, which resulted in significant decrease of survival in the 50 and 500 ng/L EE2 group by 45.9% and 40.4%, respectively (Capolupo *et al.*, 2018).

Valero *et al.* (2020) conducted a study on gilthead seabream *S. aurata* regarding the impact of EE2 and tamoxifen on antimicrobial humoral activities. In the first experiment, males were fed for 28 days a diet containing 5 or 50 µg/g EE2. In the second experiment, fish were fed a diet containing 5 µg/g EE2 for 50 days and received afterwards for 22 days food which was not enriched with EE2.

In the third experiment, fish were exposed to 2.5 or 5 µg/g EE2 via food for 83 days and received afterwards for 91 days a diet without EE2. Summarized the results showed that exposure duration, stage of development and levels of EE2 exposure had a complex impact on the activities of peroxidase, protease, antiprotease and the bactericidal property of serum (Valero *et al.*, 2020).

Previously, Cabas *et al.* (2012) performed experiments on *S. aurata*. These lasted 15 and 29 days and fish were exposed to 5 or 50 ng/L EE2. They reported increased testosterone levels, expression of the vitellogenin gene and mRNA levels of the estrogen receptor alpha gene (Cabas *et al.*, 2012). These previously reported results complement the spectrum of documented impact which EE2 has on the gilthead seabream, since the focus by Capolupo *et al.* (2018) and Valero *et al.* (2020) was on different parameters as previously described. Both studies presented in this work can be specified as long-term exposure studies which assessed a broad range of initial EE2 concentrations. They reported an impact on the mortality of larvae, various enzymes and the bactericidal activity of serum which in the long run also affects the mortality with a lowered protection. Acute exposure and studies on the behavior of *S. aurata* could lead to significant results, if the pattern which EE2 shows continues also in this species.

3.7 African clawed frog *Xenopus laevis*

The frog species *X. laevis* and the effects of EE2 regarding heme metabolism observed via mRNA was the focus of Garmshausen *et al.* (2015). 4-years-old male frogs were exposed to EE2 levels of 0.3, 29.6 or 2960 ng/L for 28 days. Increase of the EE2 dose compared to a control group showed that the relative expression of vitellogenin (known as a biomarker for estrogenic substances) increased and that of heme oxygenase 1 and 2 (both form biliverdin). The researchers concluded that the dorsal axis development could be affected because biliverdin takes part in the development of this axis in embryos (Garmshausen *et al.*, 2015).

Three species were part of the study conducted by Tamschick *et al.* (2016): African clawed frog *X. laevis*, green toad *Bufo viridis* and European tree frog *Hyla arborea*. EE2 levels of 50, 500 and 5000 ng/L were used, unfortunately the authors did not explicitly state the duration of exposure. Nonetheless, since the data of the EE2 concentration monitoring end after 10 weeks, it can be assumed that the exposure duration was 10 weeks. The results indicate that EE2 induced genetic male to phenotypic female sex reversal in all three species (whereas in the control groups no sex reversal was observed): For *X. laevis*, an increasing concentration lead to an increased sex reversal ratio of up to 100% at 5000 ng/L. Contrary to *X. laevis*, for both *B. viridis* and *H. arborea* no sex reversal was

observed in the 50 ng/L group, while in the 500 ng/L group the sex reversal ratio was at 36.4% and 31.6%, respectively. At 5000 ng/L, the ratio decreased slightly in *Bufo viridis* to 33.3%, while in *Hyla arborea* the decrease reached 15.0%. Additionally, in groups which were treated with EE2, mixed sex gonads were observed in all species (Tamschick *et al.*, 2016).

Both studies which were conducted on *X. laevis* were of long-term duration and covered a range from 0.3 to 5000 ng/L EE2. The impact which was observed were different expression levels of several enzymes, and impact on the sex ratio and gonads which can cause in the long run a reproduction problem for this species. Additional studies on acute exposure and behavioral changes would also broaden the knowledge in this species regarding the impact of EE2.

3.8 Sprague Dawley® rat

Experiments were performed by Cecarrelli *et al.* (2015) on 8-month-old Sprague Dawley rats, which were exposed to EE2 perinatally via the rat mother. These were fed with peanut oil containing EE2 in two doses (low dose group: 4 ng/kg/day, high dose group: 400 ng/kg/day) or peanut oil without EE2, starting on the day of mating, during pregnancy and lactation. The researchers observed the following significant results: Lower weight in the high dose group, pain responses in male rats were higher in both dose groups, higher grooming duration in both groups and impact on both estradiol serum levels and the estradiol/testosterone ratio (Cecarrelli *et al.*, 2015).

Another study which was conducted on Sprague Dawley rats regarding play behavior and the impact of EE2 on this behavior was conducted by Zaccaroni *et al.* (2017). Female rats were fed daily with 4 ng/kg/day or 400 ng/kg/day EE2 during gestation day 5 to 20 or postnatal day 1 to 21. Significant results in comparison to a control group were a delayed vaginal opening in the 400 ng/kg/day EE2 postnatal group, increasing social activity proportional to higher EE2 doses (increasing with treatment duration), while this impact was stronger in the gestational groups than in the postnatal groups. The social activities were statistically assigned to various groups and further analyzed. Aggressive-like play was affected significantly by EE2 correlating to the exposed dose. Also, exposure in the gestational group showed a larger impact than in the postnatal group resulting in aggressive neck grooming and pounce. Pinning increased also with increasing EE2 exposure (Zaccaroni *et al.*, 2017).

Even though the two studies on Sprague Dawley rats were conducted by two different groups of researchers, the exposure concentration of EE2 was exactly the same (Cecarrelli *et al.*, 2015;

Zaccaroni *et al.*, 2017). Since these rats do not live in water, the EE2 exposure had to occur through the diet and showed various effects like impacted social activity and alteration in hormone levels. Both studies reported behavioral changes after long-term exposure, therefore data on acute exposure and reproduction could complement the data situation on this species. Sprague Dawley rats are laboratory rats and the results generated using EE2 on the one hand might be informative in case of further research using this species, on the other hand could be used for the establishment of parallels to other rat species.

3.9 Studies on other species

Impact of EE2 on crucian carp *Carassius auratus* was assessed by Zhou *et al.* (2019), who exposed fish to 17,100 ng/L EE2 for 9, 18 or 27 days. Determination of the gonadosomatic index showed that exposed male fish had after 18 days a significant decrease in this parameter when compared to the control group, which according to the authors can be interpreted as an inhibition of the testis development. The hepatosomatic index increased after 27 days and was interpreted as enhanced liver metabolism. Analysis of muscle, gill and liver tissue showed bioaccumulation of EE2 and an increase in estrone, which the authors interpret as a disrupted homeostasis. Statistical analysis of metabolites showed that exposure to EE2 lead to alterations of metabolites in kidneys and gonads in affected fish (Zhou *et al.*, 2019).

The sublethal effects of EE2 on gonadal and liver histology on male and adult fish of the species *Cnesterodon decemmaculatus* were investigated on by Young *et al.* (2016). A preliminary experiment was conducted using 20 or 200 ng/L EE2 for 8 and 12 weeks, while in a second experiment 20, 100 or 200 ng/L EE2 for 8 and 16 weeks were tested. A significant effect was observed on the condition factor (indicates adversely affected health), which was in the 200 ng/L EE2 group higher after 8 and 16 weeks as well as in the 100 ng/L group after 16 weeks. Gonadal histology was not impacted by EE2 in the control and 20 ng/L group, while in every other group testis histoarchitecture alterations and testis-ova were observed. Regarding liver histology, alterations were observed in the 100 and 200 ng/L EE2 groups, while no alterations were visible in the 20 ng/L group (Young *et al.*, 2016).

EE2 and a mixture of EE2 and fluoxetine on the crustacean *Daphnia magna* was investigated on by Luna *et al.* (2015) over a period of 40 days. The concentrations which were tested were 10 (10), 100 (1000) and 1000 (100,000) ng/L EE2, the fluoxetine concentration in the additional mixture solutions is added in brackets. The results show that produced neonates in all EE2 groups were lower

than in the control group after 21 days, while after 40 days less neonates were produced in the 100 and 1000 ng/L group compared to the control group. The population growth rate in all EE2 groups was also lower when compared to the control after 21 days. After 40 days, the population growth rate was lower in the 100 and 1000 µg/L EE2 group compared to the control. *D. magna* exposed to the mixture groups showed an increase in the time period until first reproduction took place except for the 10 ng/L EE2 mixture with 100 ng/L fluoxetine group. Additionally, in the 1000 ng/L EE2 mixture with 100,000 ng/L fluoxetine group death occurred earlier than in the other groups after already 21 days and also fewer neonates were produced at the end of the experiment. After 21 and 40 days, the 10 ng/L EE2 mixture with 10 ng/L fluoxetine group and the 1000 ng/L EE2 mixture with 100,000 ng/L fluoxetine group showed a lower population growth rate than the control and 100 ng/L EE2 and 1000 ng/L fluoxetine group (Luna *et al.*, 2015).

Genotoxic and cytotoxic studies were conducted by Belhaj *et al.* (2017) on the nanophytoplankton *Dunaliella salina* using 10, 100 and 1000 ng/L EE2 for 11 days. The growth was inhibited significantly in the 100 and 1000 ng/L group, when compared to the control group, while growth promotion was observed for 10 ng/L EE2. Chlorophyll a and b contents were reduced in the 100 and 1000 ng/L group, while they were increased in the 10 ng/L group when compared to the control. Regarding carotenoids, an increase in the content was observed in the 10 and 100 ng/L groups, while 1000 ng/L EE2 caused a decrease when compared to the control group. Total protein was increased in the 100 and 1000 ng/L group, no effect was observed in the 10 ng/L group compared to the control group. Regarding carbohydrates, the authors state that the levels increased like the protein results. Polyphenol and flavonoid concentrations as well as the antioxidant capacity were significantly increased in the 100 and 1000 ng/L group compared to the control group, whereas no significance was observed for the other groups. Antioxidant responses via the enzymes superoxide dismutase, catalase and glutathione peroxidase varied significantly: Superoxide dismutase activity increased in the 100 and 1000 ng/L group, while a decrease for catalase in the 1000 ng/L group and for glutathione peroxidase in the 100 and 1000 ng/L group was observed. The fatty acid composition differed significantly in the 100 and 1000 ng/L groups, in which polyunsaturated fatty acids decreased whereas monounsaturated fatty acids and saturated fatty acids increased (Belhaj *et al.*, 2017).

Dang *et al.* (2017) conducted a study on the behavior of mosquitofish *Gambusia affinis* towards Japanese medaka *O. latipes* after EE2 exposure at concentrations of 0.5, 5 and 50 ng/L for 2 days. Significant results in female mosquitofish were increased durations of approaching attempts in the 50 ng/L group which were after additional 2 days in water free of EE2 reversed. In adult male

mosquitofish, all groups which were exposed to EE2 displayed increased approach attempts and approach attempt durations when compared to the control group, while the ability to reverse this behavior was again achieved by treatment in EE2 free water for 2 additional days (Dang *et al.*, 2017).

Yang *et al.* (2016) exposed Chinese rare minnow *Gobiocypris rarus* to EE2 concentrations of 1, 5, 25 or 125 ng/L for 3 and 6 days. They studied the gene expression of *kiss1*, *kiss2*, *GPR54a* (*kiss1ra*) and *GPR54b* (*kiss1rb*), which play a role in pubertal development and reproduction. The results showed complex patterns of increase and decrease of the expression of the respective mRNA in tested tissues, concluding that EE2 has the potential to have an impact on neuroendocrine homeostasis (Yang *et al.*, 2016).

The effects of EE2 on least killifish *Heterandria Formosa* were investigated on by Jackson *et al.* (2019). Fish were exposed to 5 and 25 ng/L EE2 started six days after birth and was interrupted after half of each group reached sexual maturity and lasted therefore for 12 to 23 weeks. In male fish, the exposure to 5 ng/L EE2 lead to significant intersex alterations, whereas no significance was observed in the 25 ng/L group. Gonadal histology in female fish showed that with increasing EE2 concentration, the maturing of ova slowed down. In male fish, gonadal histology showed that increasing EE2 exposure lead to more sperm in the intermediate stage. Additional gonadal histology showed also that in these groups all males were intersex and sperm immaturity increased with increasing EE2 concentration. With increasing EE2 concentration, also liver damage and disrupted morphology were observed (Jackson *et al.*, 2019).

Lined seahorses *Hippocampus erectus* were exposed to EE2 and progesterone in a study conducted by Qin *et al.* (2020). Seahorses were exposed to 5 or 50 ng/L EE2 for 60 days and lead to the following significant results: Increased mortality (males 99%, females 83%) and ventilation rate in the 50 ng/L group, increased feeding rate in females in both exposure groups and improved ovary development in females. Following significant results were obtained only from the seahorses in the 5 ng/L after the experiment ended: Smaller brood pouches in males exposed to 5 ng/L EE2 and impacted expression of genes via up- and downregulation which also lead to arresting spermatogenesis (Qin *et al.*, 2020).

D´Alvise *et al.* (2020) conducted a study on two-month old long-snouted seahorses *Hippocampus guttulatus*, which were exposed to 21 ng/L EE2 for 30 days. When compared to the control group, exposed seahorses showed a significantly lower mass and size and higher mortality. Also observed were slower development of brood pouches in males, increased testosterone levels as well as an

increased free androgen index (bioavailable testosterone). 17β-estradiol levels in both genders and decreased FSH and vitellogenin levels in females were also increased (D´Alvise *et al.*, 2020).

Voisin *et al.* (2016) conducted a study on the self-fertilizing mangrove rivulus *Kryptolebias marmoratus* focusing on the delayed impacts of EE2 regarding developmental exposure. After hatching, exposed fish were transferred into solutions containing either 4 ng/L or 120 ng/L EE2 for 28 days and afterwards raised in water containing no EE2. EE2 caused a slower growth in fish, which aligned in the course of time and therefore did not significantly differ from the control group after 56 days post hatching in the 4 ng/L group and after 91 days post hatching in both groups. Regarding weight, 28 days post hatching only the 120 ng/L group weighed less than the control group 28 days post hatching, after 56 days no significant difference was visible. Fish exposed to 4 ng/L EE2 laid significantly less eggs. A significant effect of 120 ng/L EE2 was also visible on 11-ketotestosterone levels, resulting in an increase. Testosterone levels showed in the 120 ng/L group an increase after both 91 and 168 days after (Voisin *et al.*, 2016).

Experiments were conducted on the bullfrog tadpole *Lithobates catesbeianus* by Salle *et al.* (2016) which were exposed for 96 h to a concentration of 10 ng/L EE2. Significant results which were observed are a higher heart rate, higher contraction strength by ventricle strips and higher pumping capacity. The researchers conclude that EE2 has an impact on the cardiac muscle and on the energy expenditure by *L. catesbeianus* (Salla *et al.*, 2016).

Lee *et al.* (2014) investigated on the brackish medaka *Oryzias melastigma* using initial concentrations of 1, 10, 50 or 100 ng/L EE2 for 14 days. The number of spawned eggs was significantly reduced in the 50 and 100 ng/L and group. A trend in the reproductive behavior was observed in the 10 ng/L group on the duration of the following behavior, while in the 50 and 100 ng/L group dancing behavior decreased significantly. No copulation took place in the 100 ng/L group, while the researchers pointed out that in the 50 ng/L group only one of five fish pairs copulated (Lee *et al.*, 2014).

Leet *et al.* (2015) published a study on fathead minnows *Pimephales promelas* which reported to test the effect of 17β-trenbolone and EE2 on their sex differentiation focusing on molecular response. The experiment was executed using larvae starting 10 days post hatching which were exposed to 5 ng/L EE2 for 10 days. Survival decreased to 80% compared to 97% in the control group, and although no significance regarding the growth compared to the control group was observed, compared to the group which was treated with 5 ng/L 17β-trenbolone there was a growth increase in the EE2 group. In the EE2 group, in females the genes *star, cyp17* and *cyp19a* (all three part of

steroidgenesis) were downregulated while *dmrt1* (part of testicular development) was upregulated. The gene *esr1* (ovarian development) was upregulated in both sexes. As stated by the authors, the timeframe in which the larvae were used in their development is crucial for the development of gonads, while females showed to have a higher impact on the investigated genes than males (Leet *et al.*, 2015).

The effects of EE2 exposure on guppies *Poecilia reticulata* on their chemical and visual communication was investigated on by Saaristo *et al.* (2019). Male and female guppies were exposed to 14 ng/L EE2 for 28 days and afterwards behavioral assays were conducted by grouping of exposed and unexposed fish. Regarding female visual cues, the total time of performance and frequency of sigmoid displays (male mating strategy) were increased in both control and exposed males significantly toward control female fish. An additional experiment was performed in the same setting, but the researchers added manually chemical cues from either exposed or control females into the used tank. In this experiment it was displayed that males preferred control female fish with exposed cues and exposed female fish with control cues. The authors concluded that EE2 exposure is complex regarding attractiveness towards males (Saaristo *et al.*, 2019).

Hu *et al.* (2017) published a study on the embryonic development of clearhead icefish *Protosalanx hyalocranius*, which were exposed to EE2 and estradiol. Embryos were exposed to EE2 at concentrations of 0.05, 10, 1600, 8000, 40,000, 200,000 and 1,000,000 ng/L for 30 days. Significant results when compared to the control group were for the 1,000,000 ng/L group a decreased number of survived embryos/larvae after 27 days, an increase in teratogenesis rate starting at 1600 ng/L and all higher EE2 concentrations and a longer hatching time at 200,000 and 1,000,000 ng/L (Hu *et al.*, 2017).

The team of Islam *et al.* (2020) published a study on the impact of EE2 on Sydney rock oyster *Saccostrea glomerata.* The parental generation was exposed to EE2 at a concentration of 50 ng/L for 25 days during gonadal development alongside to an unexposed control group. Embryo production was induced between every possible pairing of exposed and unexposed males and females. Additionally, post-fertilized larvae which were obtained were exposed to 5 or 50 ng/L EE2 starting on day 2 and ending on day 9. Results, which were statistically significant, were on the second day a lower number of D-veliger (development level) larvae and the amount of actively swimming larvae in this stage in pairs, in which both parents were exposed to EE2 when compared to pairs of unexposed parents. After 9 days, the survival rate of larvae in the F1 generation was in both tested EE2 concentrations lower when compared to larvae which were kept in medium without EE2 for every

possible parental pair combination. Almost the same observation was made regarding the shell length, but in this case additionally to the larvae exposure it was also visible that if both parents were exposed to EE2, the shell growth was in all groups inhibited significantly (Islam *et al.*, 2020).

Juvenile turbot *Scopthalmus maximus* were exposed by Farkas *et al.* (2017) to EE2 and silver nanoparticles. The fish were exposed for 14 days, exposing them daily for 2 hours, while the concentration of EE2 was in every experiment containing EE2 at 50 ng/L. In combined experiments the silver nanoparticle concentration was either at 2000 or 200,000 ng/L. In all groups which were exposed to EE2, significant increase in plasma EE2 levels and vitellogenin levels were observed. An effect based on the gender was visible regarding androstenedione (androgen precursor) and dehydroepiandrosterone (steroid hormone precursor) levels: In all groups which were treated with EE2, female fish had lower dehydroepiandrosterone levels when compared to the control, while male fish had lower androstenedione levels when compared to the control (Farkas *et al.*, 2017).

3.10 Further discussion

The first fact that stands out that no reported study exposed the species to initial EE2 concentrations which were at or below the PNEC of 0.035 ng/L. The lowest initial EE2 concentration was reported by Anderson *et al.* (2020), who exposed Japanese medaka *Oryzias latipes* to 0.1 ng/L and were able to report a decreased heart rate in embryos. Therefore, there is at the moment no verification by these studies that the PNEC is equal to the actual no effect concentration. Future studies should aim to include also concentrations below 0.035 ng/L to visualize by data generated in real experiments at which threshold an applicable environmental EE2 concentration lies. Nonetheless, every study in this review reported an impact on biota caused by EE2 at various concentrations of up to 50,000 ng/L (Anderson *et al.*, 2020).

With the classification of acute exposure equaling durations of below 24 h, only a few studies were published in the considered publication period. The lowest exposure period which had an impact on biota was reported by Fenske *et al.* (2020) with 1 h, who exposed Zebrafish *D. rerio* and observed at a concentration of 1.5 ng/L of impact on monitored parameters (Fenske *et al.*, 2020). On the other side of this exposure spectrum, the study with the longest exposure was conducted by Jackson *et al.* (2019) lasted 23 weeks and reported impact on reproduction and organs (Jackson *et al.* 2019). According to the reviewed literature, it is clear that long term exposure at concentrations above the PNEC has an impact on many different species.

As it was made clear with several studies, the impact of EE2 is in many cases not easily describable. Increasing concentrations do not result automatically in a predictable effect following a simple dose response curve like it was shown *e.g.* on the expression of mRNA (Yang *et al.* 2016). The impact of hormones and substances which can act like hormones depends often on interactions with each other, and therefore the interpretation of such data is in many cases not straightforward like results show for the interaction of EE2 with 17β-trenbolone in Zebrafish *D. rerio* (Örn *et al.*, 2016).

When it comes to complex interactions, it also has to be kept in mind that not only chemical substances can affect biota. This fact was considered by DeCourten *et al.* (2017; 2019) in two consecutive studies conducted on Inland silverside (*M. beryllina*). While they did not let go of the initial concentration of 1 ng/L EE2 in both studies, experiments were also conducted at both 22°C and 28°C and showed that results were significantly different depending on the temperature. With the emerging climate crisis, this result could have extensive impact on various species in the upcoming future with consequences which might not have been considered yet in this debate. In this review, the only other study which investigated also on the impact of temperature was conducted by Luzio *et al.* (2016) on *D. rerio* and has also reported that the temperature impacted the results. With the climate crisis, temperature as a factor which impacts development will have to be considered in future studies.

Taking a similar line when considering the climate crisis and the impact on following generations, transgenerational effects on more than the F0 and F1 generation were conducted by Bhandari *et al.* (2015) and DeCourten *et al.*, (2017; 2019). While the latter exposed the F0 and F1 generation of *M. beryllina* to EE2 and reported impact, Bhandari *et al.* (2015) exposed only the parental generation of *O. latipes* to EE2 and was still able to report a reduced embryo survival in the F3 and F4 generation. The possibility to impact later generations, even if no exposure takes place, exists and should be considered in future studies.

Chinese rare minnow (*G. rarus*) is the only species from the group of "other species" in this thesis, which Aris *et al.* (2014) has also reviewed. While here the reported impact was an alteration in the expression of genes which are involved in reproduction and pubertal development (Yang *et al.*, 2016), in the previous review impact on tissue somatic indices, mortality, growth and maturation that was caused by EE2 was reported (Zha *et al.*, 2008).

Aris *et al.* (2014) reviewed studies on species, which were not included in this work. These species were the three-spined stickleback *Gastrosteus aculeatus*, juvenile Atlantic salmon *Salmo salar,* gulf

pipefish *Syngathus scovelli* and seawater fish sand gobies *Pomatoschistus minutus*. The effects which were summarized fit well into the pattern of observations reported in this work: Impact on reproduction, maturation, gene expression and levels of vitellogenin (Aris *et al.*, 2014).

The vast majority of areas which were affected in the stated studies can be summarized as reproduction, maturation, behavior and levels of hormones, proteins and mRNA. This red thread runs through the review and is not surprising: EE2 is used as a substitute to natural estrogenic compounds and shows an impact on exactly the areas, which one would expect to have an impact on at first. Nonetheless, if the EE2 concentration reaches a specific species-dependent level, an increase in mortality was reported by several authors (Luna *et al.* 2015, Capolupo *et al.* 2018, Qin *et al.* 2020, D´Alvise *et al.* 2020). Altogether, the reviewed studies depict a larger picture of effects on EE2 on biota and are not contradictory.

4 Methods to reduce EE2 levels in the environment

Due to comparable mechanisms, methods can be grouped in “Chemical treatment”, “Biological treatment” and “Adsorption and Ion Exchange”. A list of treatment methods which lead to the best reported removal efficiency is available in table 3 in the appendix. If a study investigated on more substances than EE2, the results for these other substances will be excluded in this work.

4.1 Chemical treatment

The aim of the study conducted by Deng *et al.* (2015) was to try a combined oxidation and ultrasound treatment on steroid estrogen mixtures containing E1, E2 and EE2 using potassium permanganate $KMnO_4$ as oxidant. The researchers conducted preliminary experiments testing the degradation efficacy of either only KMnO4 or ultrasound. The best reduction efficiency for EE2 (at an initial concentration of 25 µg/L) was observed in 6 mg/L $KMnO_4$ solutions at 70.5% after 120 min. Further experiments were conducted using ultrasound and a lower $KMnO_4$ concentration than 6 mg/L due to its property to colorize solutions, since the researchers aim was to deepen the knowledge on the removal characteristics. The researchers generated many results, which can be summarized as follows: The removal efficiency increased in combined $KMnO_4$ and ultrasound systems, the removal efficiencies were higher in binary estrogen systems compared to the tertiary estrogen system and removal efficiencies were higher in a natural water matrix compared to pure water (Deng *et al.*, 2015). Unfortunately, the researchers decided not to use a higher concentration of $KMnO_4$ for additional studies although they reported better removal at higher concentrations.

Frontistis *et al.* (2015) published a study to investigate the degradation of EE2 by solar radiation, UVA and UVC. In one of the experiments which was performed, the researchers used UVC and varying hydrogen peroxide H_2O_2 contents leading to the result that in a solution which contains initially 100 µg/L EE2, 10 mg/L H_2O_2 were enough to reduce the EE2 content by 100% after 15 minutes. Using the same initial concentration, various water matrices were tested (ultrapure water, secondary-treated wastewater, 10 mg/L humic acid solution and a mixture containing same parts of secondary treated wastewater and ultrapure water). For UVC combined with 10 mg/L H_2O_2, complete removal was achieved after 15 min in every matrix except for ultrapure water which achieved 100% removal efficiency after 10 min. Interestingly, although the researchers reported EE2 removal efficiencies of up to 100% at concentrations of 100 µg/L, which can be considered regarding potential environmental concentrations high, the estrogenicity could still be present according to the stated potential degradation products (Frontistis *et al.*, 2015).

The effect of nanoscale zero-valent iron (nZVI) on E2 and EE2 concentrations was the aim of a study published by the team around Jarošová *et al.* (2015). They prepared solutions which contained E2 at 60 µg/L and EE2 at 120 µg/L and added nZVI particles. The best result generated using nZVI particles was generated at 6 g/L particles removing 93% of EE2 after 1 h and was close to 100% after 5 h. Additional bioassays were used to assess the estrogenic activity and revealed the activity decreased until after 1h no significant decrease was measurable (Jarošová *et al.*, 2015).

In a study published by Zhou *et al.* (2015), experiments were conducted on the reduction and removal of different substances using ozonation, ultrasonic ozonation and photocatalytic ozonation, one of these substances was EE2. The researchers constructed an experimental setup to conduct experiments on effluent sewage which was prefiltered by the previously mentioned methods and spiked to reach an initial concentration of 5 µg/L, the duration of every experiment was 12 min. The highest removal efficiency of 86.0% was achieved using ultrasonic ozonation with a supply of 30 µg/L O_3 at pH 9.5 and 240 W. In additional experiments, humic acid caused a reduction of the removal efficiency (Zhou *et al.*, 2015).

The capability of Fe^{IV}, Fe^{V} and Fe^{VI} to remove different estrogens, one of them EE2, was assessed by Machalová Šišková *et al.* (2016). Effluent water of a WWTP was used and the estrogens were added to reach final concentrations of 100 µg/L, while different concentrations of Na_4FeO_4 (Fe^{IV}), K_3FeO_4 (Fe^{V}) and K_2FeO_4 (Fe^{VI}) were used at 1, 10 and 100 mg/L. After 5 min, no additional significant removal of EE2 was observed and when comparing the iron species between each other, Fe^{IV} was less efficient. While Fe^{V} and Fe^{VI} species were able to reduce the EE2 content by 100% after 5 min at 10 mg/L, when using 100 mg/L Fe^{IV} it reached only approximately 80% (Machalová Šišková *et al.*, 2016).

The team around Yang at al. (2017) investigated on the influence of natural organic matter (NOM) and horseradish peroxidase (HRP) on photodegradation of EE2. NOM improved the EE2 removal efficiency compared to solutions containing no NOM, which could be even more increased when HRP was present simultaneously. After 8 h, of initially 500 µg/L EE2 35.1 % were removed in a solution containing 5 mgC/L NOM and 0.01 U/mL HRP, while only 23.1% of EE2 were removed in a solution which contained only 5 mgC/L NOM (Yang *et al.*, 2017).

The aim of the study conducted by de Liz *et al.* (2017) was the degradation of E1, E2 and EE2 using so-called glass Raschig rings (hollow cylinders with almost identical diameter and length) which were TiO_2 coated. Photolytic and photocatalytic reactions were tested by using a mercury vapor

lamp (providing UVA and UVC) at an initial concentration of 20 µg/L for 60 min. The addition of the glass rings, which corresponded to ca. 200 mg/L TiO_2 and the use of UVA resulted after 60 min removal efficiencies of up to 98% for EE2. Additional experiments using treated WWTP samples which were spiked with EE2 showed that the removal efficiency was inhibited and reached 50% at an initial concentration of 50 µg/L after 60 min. The researchers also assessed the degradation products and were not able to detect any after 30 min (de Liz *et al.*, 2017).

The removal of pharmaceutical pollutants which are included in the EU Watch List, therefore including also EE2, using modified magnetite (Fe_3O_4-R400) as a catalyst and H_2O_2 was the aim of a study published by Serrano *et al.* (2019). In the experiments, 0.2 g/L of the catalyst and an amount of H_2O_2 which corresponded stoichiometrically to the amount of the substance to be removed at initial concentrations of 1000 µg/L were added. 100% removal efficiency for EE2 was observed at 50°C after already 15 min, while complete removal was also observed at lower temperatures resulting in a longer required reaction time. An additional experiment using the effluents of WWTPs, which were spiked to reach a concentration of 1000 µg/L EE2, was performed with an increased catalyst quantity of 2 g/L and resulted in a complete removal of EE2 after 60 to 90 min (Serrano *et al.*, 2019).

A study which was published by He *et al.* (2020) dealt with photosensitive cellular polymeric substances (CPS) to accelerate the photodegradation of EE2. The highest removal efficiency of 75.5% was reported for intracellular polymeric substances from anaerobic cultures (obtained from previously cultured bacteria) at a concentration of 10.0 mgC/L and an initial concentration of 0.5 mg/L EE2 after 5h. Analysis of the degradation products of this reaction showed that the basic structure of EE2 was still intact and either a double bond, a hydroxyl group or a ketone group was added onto the structure. Further performed experiments showed that not every fraction of the CPS promoted the degradation of EE2, that the contribution of hydroxyl radicals on the degradation mechanism was little, while that of $^1O_2$ was high, that the ionic strength of the CPS had an impact on the degradation rate and either proteins or amino acids accelerated the degradation (He *et al.*, 2020).

Long *et al.* (2020) prepared a photocatalyst consisting of AgI/BiOI/$BiPO_4$ and tested its capability regarding the removal of EE2. Solutions containing 3 mg/L EE2 were prepared, 5 mg of the catalyst were added and left for 30 min in the dark to reach an adsorption equilibrium, after reaching of the equilibrium the reaction was started using a Xe lamp. After 8 min, the removal efficiency for AgI/BiOI/$BiPO_4$ reached 100%. Additional experiments showed that the catalyst still had a removal efficiency of 82% after a 5th reuse (Long *et al.*, 2020).

Six of the ten studies of the chemical treatment group report removal efficiencies of close to 100%, namely Frontistis *et al.* (2015), Jarošová *et al.* (2015), Machalová Šišková *et al.* (2016), de Liz *et al.* (2017), Serrano *et al.* (2019) and Long *et al.* (2020). When these removal efficiencies are compared to the following studies, the highest ratio of almost complete removals was reported in this group. At the same time, the highest initial concentrations with complete EE2 removal was reported by Serrano *et al.* (2019). The duration to achieve the best removal efficiency did not exceed 8 h (Yang *et al.*, 2017) and can be viewed as a generally faster treatment mechanism.

Used in the methods are in general catalysts, chemicals, radiation and soundwaves or combinations of those to achieve the removal of EE2. Not only the degradation products of EE2 should be considered, but also byproducts coming from the treatment agents. If these methods have a future in the upscaled treatment of EE2, it should be monitored if *e.g.* catalysts dissolve into the treated sample and if so, if these byproducts pose a threat to the environment. If a realistic threatening scenario is possible, a follow-up treatment of the byproducts might be necessary. Also, sufficient supply of the required chemicals should be guaranteed to ensure a seamless treatment process for a probable upcoming application.

Aris *et al.* (2014) grouped the studies differently and summarized advantages and disadvantages of the various treatment methods. Regarding the "chemical treatment" group, advantages were the possibility to react with a broad range of contaminants and removal of stable contaminants, while disadvantages were expensive treatment methods, production of precipitates and risk to the environment arising from the used materials in the treatment process (Aris *et al.*, 2014). These arguments apply also for the chemical treatments discussed in this thesis. For the expected mostly oxidizing treatment methods, it can be assumed that a broad spectrum of contaminants would react with the used materials, even if seemingly stable molecules are among these contaminants. At the same time, the production of some catalysts can require an investment on material, equipment and personnel, while precipitates can also be expected especially if hardly soluble byproducts are produced. The removal of precipitates could be carried out mechanically, while soluble byproducts can (as already previously already stated) pose a threat to the environment, independent of the treatment material used or the contaminant.

4.2 Biological treatment

In total 38 different fungal strains regarding the EE2 removement capability were tested by Różalska *et al.* (2015). Pretreated cultures were supplemented with EE2 at a concentration of 10 mg/mL to

determine the reduction efficiency. Also, mineral media were supplemented with various amounts of NaCl to investigate on the effect of NaCl on EE2 removal by *Aspergillus versicolor* IM 2161 and *Aspergillus fumigatus* IM 6510. After 72 h, eighteen of the 38 fungal strains showed a removal efficiency of over 50%, three of those strains (IM 6464, IM 6446 and IM 879, all of those were isolated from soils) did not reduce the EE2 within the first 24 h, but after this initial period the removal efficiency rose close to 100% after 72 h, while for other strains almost 100% removal were achieved after already 24 h (IM 2161, IM 6510). The experiments with the *Aspergillus* strains showed that both 0.8% and 1.4% NaCl didn't affect the EE2 removal significantly for *A. versicolor*, but 2.8% NaCl caused an inhibition leading to only 17.7% after 24 h (below 5%), catching up after 48h to the same amount which were displayed at lower NaCl concentrations. *A. fumigatus* showed already at the lowest NaCl concentration an inhibitory effect but was statistically significant for only 1.4% and 2.8%. After 72 h, at the concentration of 2.8% the removal efficiency was at approximately 10% for this strain (Różalska *et al.*, 2015).

Hofmann and Schlosser (2016) conducted a study to test the removal of various substances, one of them EE2, using *Phoma* sp. strain UHH 5-1-03. Pretreated cultures were used to evaluate the removal efficiency in solutions containing 74.1 mg/L EE2 as well as supernatants obtained from *Phoma* sp. which contained Laccase. After 4 h of incubation, the EE2 content was reduced by 95%, while approximately complete removal was achieved after 24 h for cultures. The supernatant displayed a reduction efficiency of 82% after 4 h of incubation, while added syringaldehyde did not affect the EE2 removal. Additional characterization experiments were conducted and showed that EE2 dimers were produced (Hofmann and Schlosser, 2016).

Cupuaçu, the residue of *Theobroma grandiflorum*, was tested on its capability regarding EE2 removal via induction of Laccase produced by *Pycnoporus sanguineus* (ATCC 4518) by Golveia *et al.* (2018). Experiments were conducted in solutions containing 5 mg/L EE2 for 24 h, which contained Laccase equal to 200 U regarding the removal of EE2. The highest removal efficiency was measured after 4 h and reached 86.18%, every value measured from 8 h onwards was below the detection limit of 0.39 µg/mL and therefore reached over 86.18% removal efficiency. Analysis of the degradation products revealed that EE2 dimers and most probably a hydroxylated product was formed in the removal process (Golveia *et al.*, 2018).

Electrochemically modified dissolved organic matter (DOM) was investigated on by He *et al.* (2018a) regarding the efficiency of EE2 removal. The researchers modified DOM, separated the fractions based on the molecular weight and tested its efficiency on EE2 removal in solutions containing

0.5 mg/L EE2 as well as the impact of irradiation causing photodegradation and *Shewanella oneidensis*, a quinone-reducing bacterium. After 132 h, the highest removal efficiency of 41.6% was reported using $2.9*10^9$ CFU/mL *S. oneidensis* MR-1 and 5.0 mgC/L fulvic acids with a molecular weight of below 3 kDa and 1 mmol/L sodium anthraquinone-2-sulfonate. The authors also stated that additional photodegradation increased removal rates for EE2 and that the products of the EE2 removal reaction are less toxic than EE2 itself (He *et al.*, 2018a).

He *et al.* (2018b) conducted experiments using the bacterium *Hyphomicrobium* sp. GHH and the grass *Lolium perenne* on the EE2 removal in soil. Experiments were conducted after spiking soil with EE2 to reach an initial concentration of 23.5 mg/kg. The highest removal efficiency (98.7%) was reported after 42 days using both *L. perenne* (200 plants were cultivated) and *Hyphomicrobium* sp. GHH simultaneously. EE2 was mainly stored in the roots of *L. perenne* (He *et al.*, 2018b).

He *et al.* (2019a) conducted an additional study on the EE2 removal using DOM and microorganisms. The highest EE2 removal efficiency was reported with 98.4% after 90 min at an initial concentration of 0.5 mg/L EE2 using 5.0 mgC/L fulvic acids (< 3 kDa) and 3% (v/v) long-term electro-domesticated microorganisms (pretreated using electric current), which were obtained from anaerobic activated sludge at a Chinese purification plant. Further conducted microbial degradation experiments showed that the removal of EE2 was increased after electrical stimulation. A characterization of the degradation products was also performed and showed that EE2 was partially transformed into hydroxylated products as well as estrone and estradiol (He *et al.*, 2019a).

He *et al.* (2019b) conducted a study on the remediation of soil which has been co-contaminated with EE2 and Cd using ryegrass (which was not further specified in the publication) and *Hyphomicrobium* sp. GHH bacteria. Soil, which has been spiked with 25 mg/kg EE2 was used and treated with ryegrass, bacteria or both combined for 28 days. Removal rates in combined treatments reached up to 90% when no Cd was present in soil while removal decreased with increasing Cd concentration. Additional analyses showed that EE2 was mainly stored in the root of the ryegrass (He *et al.*, 2019b).

In the biological treatment group, even though only one study was able to report a removal efficiency of approximately 100% (Hofmann and Schlosser 2016), four other studies reported removal efficiencies of 90% and above, namely Różalska *et al.* (2015), He *et al.* (2018b), He *et al.* (2019a) and He *et al.* (2019b) with a shortest treatment period 90 min reported by He *et al.* (2019b). The treatment duration of biological treatment method is in the range of hours to days.

Regarding biological treatment, Aris *et al.* (2014) summarized advantages as major pollutant removal in WWTP, the requirement of using ammonia oxidizing bacteria and effective under anaerobic and aerobic conditions. Disadvantages were the amounts of generated toxic sludge and the environmental risk this sludge poses and instable removal of some compounds (Aris *et al.*, 2014). As it was shown in this thesis, the use of ammonia oxidizing bacteria is not necessary, it is also possible to use other organisms like fungi or plants for treatment. Fungi could be cultivated and maintained similarly to bacteria, in contrast to grass like *L. perenne* with several other requirements. The production of toxic sludge and its risk towards the environment plays definitely a role in the reviewed studies, especially looking at the issue of degradation products which were reported as *e.g.* EE2 dimers (Hofmann and Schlosser 2016, Golveia *et al.*, 2018) or hydroxylated products (He *et al.*, 2019a).

Aris *et al.* (2014) summarized on biological degradation the used organisms together with the operating condition and the removal efficiencies. The species which were specified were not used in the studies which were reviewed in this thesis. Aris *et al.* (2014) reviewed several studies which reported that EE2 was often not degradable using biological treatment, whereas from 2014 onwards no study was published that reported that EE2 was not degradable via biological treatment. The question that comes up in this case is if either all researching groups were extremely successful in this field or if studies which were unsuccessful regarding the degradation of EE2 were not published. Nonetheless, it can be summarized that some researchers were able to report removal efficiencies of close to 100%.

4.3 Adsorption and Ion exchange

Wang *et al.* (2017) assessed the potential of magnetic ion exchange resin (MIEX) to reduce EE2 concentrations. Adsorption was performed for 1 h and after 15 min of settling, the EE2 concentration in the supernatant was determined. 75.3% removal efficiency were observed for the lowest initial concentration of 20 µg/L at a dosage of 10 mL/L MIEX, which did not improve with higher MIEX concentrations. The researchers state that EE2 molecules diffusing into the inside of the MIEX are in an alkaline environment, are ionized and form negative charged molecules which are removed from water via ion exchange (Wang *et al.*, 2017).

De Castro *et al.* (2018) conducted experiments on the removal of EE2 and other substances using inexpensive materials like sand, vermiculite, non-activated charcoal and granular activated carbon while the latter three all were mixed with sand in polishing units. The effluents of a WWTP were

used directly for experiments, which were running without interruption for 2 months with a flow rate of 0.33 m^3/day. After 15 days of running of the experimental setup a biofilm has formed, which supposedly was important for the removal efficiency. Samples were taken on ten different days within each experimental run, which lasted 30 days. The initial mean concentration for EE2 was approximately 10 ng/L and resulted in a removal efficiency of over 99% for every experimental setup (de Castro *et al.*, 2018).

Tang *et al.* (2018) conducted a study focusing on gamma- and beta-cyclodextrin polymers regarding the removal of estradiol, bisphenol A and EE2. The removal of EE2 was tested with an initial concentration of 11.9 µg/L and the researchers reported for 0.4 mg/L beta-cyclodextrin polymers saturation after 10 min and for 0.4 mg/L gamma-cyclodextrin polymers after 5 min. Additionally, approximately 100% removal efficiency after five consecutive regeneration cycles were achieved for both polymers (Tang *et al.*, 2018).

Another study which focused on the adsorption of EE2 using soil was conducted by de Oliveira *et al.* (2019). 250 mg of soil was tested regarding its capability to adsorb 30 mL of 2 mg/L EE2 for 1440 min. The authors report an adsorption peak after 45 min corresponding to 27% removal efficiency, which followed a decrease of the removal rate explained through the adsorption rate that was varying and sometimes below the desorption rate. An equilibrium between adsorption and desorption was reported after 720 min, which equals approximately 40 % of EE2 removal (de Oliveira *et al.*, 2019).

Two of the four studies which are in the “adsorption and ion exchange” group show a removal efficiency of approximately 100%, while one achieved this efficiency after already 5 min (Tang *et al.*, 2018) and the other after 30 days (de Castro *et al.*, 2018). The two other studies by Wang *et al.* (2017) and de Oliveira *et al.* (2019) showed lower removal efficiencies of 75.3% and ca. 40%, respectively and the period was in between the two previously stated studies with high removal efficiencies.

The used material is of different origin – while soil (de Oliveira *et al.*, 2019) and sand (de Castro *et al.*, 2018) can be collected easily in the environment and are comparably low in effort regarding preparation, MIEX resin (Wang *et al.*, 2017) and Gamma-cyclodextrin polymers (Tang *et al.*, 2018) have to be manufactured and require more preparation prior to the use as adsorption or ion exchange material.

The arguments concerning adsorption and ion exchange by Aris *et al.* (2014) were regarding advantages high efficiency and ability to remove substances with various properties and regarding disadvantages the waste which was generated by activated carbon, a decrease in removal efficiency over time, non-selective removal or expensive treatments (Aris *et al.*, 2014). The stated advantages apply in this work for Gamma-cyclodextrin polymer, which removed almost all of the EE2 and is also able to adsorb other contaminants (Tang *et al.* 2018) and also for the materials tested by de Castro *et al.* (2018) as reported. The problem regarding generation of waste was addressed previously, while a reduced removal efficiency can be expected after either several cycles of adsorption and stripping of the contaminant due to change in properties of the treatment material or irreversible binding to the adsorbent. The financial expense in this group varies depending on the material from low (soil (de Oliveira *et al.*, 2019); sand (de Castro *et al.*, 2018)) to high (Gamma-cyclodextrin polymer (Tang *et al.*, 2018); magnetic ion exchange (Wang *et al.* 2017)), although this should be calculated after consideration of the profit of environmental protection.

Previously, Snyder *et al.* (2007) conducted experiments on the removal efficiency of membranes and activated carbon of pharmaceuticals and endocrine disruptors. EE2 was one of the investigated substances and reported for powdered activated carbon a removal efficiency of approximately 95% after 4 h at an initial concentration of 100 ng/L. The removal efficiency for granular activated carbon after 30 d was over 99% at an initial concentration of 10 µg/L as reported by de Castro *et al.* (2018). The two teams used different experimental setups as it is visible in their respective studies and were able to obtain satisfying results with removals of close to 100%. Looking at the results generated by Snyder *et al.* (2007), it would have been interesting to check by de Castro *et al.* (2018) if lower contact time would result in similar results.

The studies in the "adsorption and ion exchange" group did not degrade EE2 itself but adsorbed or adhered the substance onto a material. In this case the disposal of the material or in case of stripping of EE2 of the material, either for reuse of the material or the degradation of EE2, the stripping matrix should be treated additionally in a way which secures that EE2 is not re-emitted into the environment. Nonetheless, adsorption of EE2 onto material can be classified as a cheaper remediation method if the used material is not expensive, like it has been for instance for soil (de Oliveira *et al.*, 2019) and sand (de Castro *et al.*, 2018) which adsorbed EE2 and therefore countries which invest less into protection of the environment could implement these methods to prevent the emission of EE2. In contrast to the two other groups, when it comes to adsorption it is clear that EE2 still has its estrogenic property as long as no change in the molecular structure takes place. One option

after the stripping of EE2 off the adsorbent would be the treatment with a method from the chemical or biological treatment group to make sure that EE2 is not reemitted into the environment.

4.4 Further discussion

The issue of degradation products which in some cases still possess an estrogenic property was addressed by some studies (for described structures see Frontistis *et al.* (2015); Rozálska *et al.* (2015); Hofmann and Schlosser (2016)). For further studies, a first step could be the measurement of the TOC before and after the experiment. If for instance EE2 is oxidized completely into CO_2, this would result in a lower TOC value in the treated solution. If the TOC value indicates that EE2 was transformed into another organic species, a test on the estrogenic property of the solution could be a second step, since the product could have lost its endocrine property and not necessarily every laboratory owns the equipment to determine the structure of degradation products immediately. If the test result shows no estrogenic activity with a LOD that is low enough (and for this, the PNEC would be an appropriate value), no further tests would be necessary. If the LOD is above the PNEC or the test result shows estrogenic activity, further identification of the products would be interesting so that further studies could focus on the removal of the degradation products.

Overall, the initial concentrations of EE2 were ranging over several orders of magnitude as it is depicted in table 3 and therefore a two-step approach could be used if WWTP or other preliminary purification equipment are supposed to be expanded with any method focusing on EE2 removal: In a first step, a method which has proven to remove EE2 at higher initial concentrations could be used and afterwards a second method focusing on lower concentrations could be established so that the final concentration in the treated sample is below the PNEC so able to state that no estrogenic impact is to be expected. The duration, how long it takes to remove EE2 in order to reach a specific concentration could in this case also be crucial regarding the transition from the “high-concentration” method to the “low-concentration” method. Experiments for optimization in WWTPs should be conducted to generate data outside of laboratories.

The study which was stated by Aris *et al.* (2014) to achieve the highest removal efficiency was conducted by Vader *et al.* (2000) who used nitrifying activated sludge to degrade EE2 at an initial concentration of app. 50 µg/L. Vader *et al.* (2000) report that after six days a removal efficiency of 100% (Vader *et al.*, 2000). A removal rate of 100% or close to 100% was reported by several studies as it is displayed in table 3 with no further information of the LOD and therefore the precision of “100%” is not defined.

For future studies, removal experiments should be conducted using real environmental matrices, since the composition of the matrix can have an impact on the removal efficiency. This impact can either result in enhancement of the removal by *e.g.* supportive mechanisms or in hinderance of the removal by *e.g.* competitive reactions. Another fact that always has to be kept in mind for future studies is that factors like the amount of used catalyst, chemical or microorganism or initial concentration of the pollutant can impact the removal efficiencies of the stated studies.

5 Conclusion

EE2 is present in the environment around the globe. Unfortunately, the available data is in many cases not able to verify whether EE2 levels are above or below the PNEC of 0.035 ng/L in waterbodies. Even the attempt of governmental organizations to generate reliable data fails in many cases on the establishment of analytical limits, which allow to make a statement regarding the PNEC. In the reviewed literature, which reported detectable levels, it was reported that EE2 is present both at levels over and below the PNEC.

Reviewed literature on effects of EE2 on biota report summarized effects on development, maturation, reproduction, behavior, gene expression and levels of several endogenous substances. The vast majority of conducted studies exposed the respective species over a long-term period, acute exposure studies are underrepresented although the few studies of the latter duration have also shown an impact. No study conducted experiments below or equal to the PNEC. Therefore, it is recommended for future studies to consider these two areas of acute exposure and low EE2 concentrations.

Methods to reduce levels of EE2 in the environment are of different origin and can be grouped in general into chemical treatment, biological treatment, adsorption and ion exchange. Every method has its benefits and disadvantages but shows in general its potential to prevent emissions of EE2 into the environment. Depending on the initial concentration of EE2, the treatment period and expected removal efficiency various methods are available for use.

6 Abstract

17α-ethinylestradiol (EE2) is one of the two active compounds in combined oral contraceptives and was described for the first time in 1938. It is an Endocrine Disruptive Substance and therefore in the focus of research regarding its potential negative impact in the environment. The European Union included EE2 in 2015 in a watch list of priority substances to be monitored in the environment. Additionally, several countries require assessments on the risk of medicinal products towards the environment prior to the marketing authorization.

The last review on EE2 in the environment was published in 2014. Since then, well above 70 studies on the topic have been published. The aim of this thesis was therefore to bring together recent data with earlier published ones. The topics that were emphasized were environmental levels of EE2, effects of EE2 on biota and methods to reduce EE2 levels in the environment. This should give an overview of the recent status of knowledge and developments regarding environmental aspects of this frequently used drug.

Sources of EE2 in the environment are human urine, livestock wastewater and runoffs of manure and sewage sludge. EE2 levels are still detectable in many countries and were both above and below the predicted no effect concentration of 0.035 ng/L. In several cases it is questionable how safe the measured levels in the environment are due to analytical limits which were above the predicted no effect concentration. Effects on several species caused by EE2 levels above the predicted no effect concentration, in particular after long term exposition, were reported by every reviewed study and impacted especially the areas development, maturation, reproduction, behavior, gene expression and levels of several endogenous substances.

To support the degradation of EE2 prior to the entry into the environment, appropriate treatment methods could help to control the emissions of EE2. Several methods for the reduction of EE2 levels of up to 100% removal efficiency were reported and are of chemical, biological, adsorptive or ion-exchange nature. Depending on the required properties like initial EE2 concentration or treatment duration, several promising methods are available.

7 Zusammenfassung

17α-Ethinylestradiol (EE2) ist eine der zwei aktiven Komponenten in kombinierten oralen Kontrazeptiva und wurde das erste Mal 1938 beschrieben. Es ist eine endokrin wirksame Substanz und daher im Fokus der Forschung hinsichtlich ihres Potentials eines negativen Einflusses in der Umwelt zu haben. Die Europäische Union inkludierte EE2 im Jahr 2015 in einer Watchlist von Substanzen, die prioritär in der Umwelt überwacht werden sollen. Zusätzlich verlangen einige Staaten Einschätzungen bezüglich des Risikos von Arzneimitteln gegenüber der Umwelt vor der Marktzulassung.

Der letzte Review zu EE2 in der Umwelt wurde im Jahr 2014 publiziert. Seitdem wurden weit über 70 Studien zu diesem Thema publiziert. Das Ziel dieser These war es rezente und früher veröffentlichte Daten zusammenzubringen. Themen hierfür waren EE2 Konzentrationen in der Umwelt, Effekte von EE2 auf Biota und Methoden um EE2 Konzentrationen in der Umwelt zu reduzieren. Dies soll einen Überblick über den aktuellen Wissensstand und Entwicklungen bezüglich Umweltaspekten dieser häufig verwendeten Substanz geben.

Quellen von EE2 in der Umwelt sind menschlicher Urin, Abwasser von Tierbeständen und Abflüsse von Dünger und Klärschlamm. EE2 Konzentrationen sind immer noch in vielen Ländern messbar und waren sowohl über als auch unter der vorhergesagten Konzentration ohne Effekt (Predicted No Effect Concentration, PNEC) von 0,035 ng/L. In einigen Fällen ist es fragwürdig, wie sicher die gemessenen Konzentrationen in der Umwelt sind, da die analytischen Grenzen oft nahe dem PNEC lagen. Effekte auf einige Spezies durch EE2 Konzentrationen, die über dem PNEC lagen, insbesondere nach Langzeit Exposition, wurden in jeder betrachteten Studie berichtet und hatten einen Einfluss auf die Bereiche Entwicklung, Reifung, Fortpflanzung, Verhalten, Genexpression und Konzentrationen von einigen endogenen Substanzen.

Um den Abbau von EE2 zu unterstützen, bevor die Substanz in die Umwelt gelangt, könnten geeignete Behandlungsmethoden helfen die Emission von EE2 zu kontrollieren. Einige Methoden zur Reduktion von EE2 Konzentrationen von bis zu 100% Entfernungseffizienz wurden berichtet und wirkten auf Basis chemischer, biologischer, adsorptiver oder Ionen-Tausch Mechanismen. Abhängig von den geforderten Eigenschaften wie die initiale EE2 Konzentration oder Behandlungsdauer sind einige vielversprechende Methoden verfügbar.

8 References

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9 Tables

Table 1: EE2 levels in the environment (n.d. = not detected).

Sampling location or waterbody	Sampling period	EE2 concentration	Reference
Wulka, Burgenland, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Leitha, Burgenland, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Raab, Burgenland, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Pinka, Burgenland, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Drau, Carinthia, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Glan, Carinthia, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Schwechat, Lower Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Danube, Lower Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Thaya, Lower Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
March, Lower Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Zaya, Lower Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Antiesen, Upper Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Danube, Upper Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Krems, Upper Austria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Salzach, Salzburg, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Lafnitz, Styria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Mur, Styria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Mürz, Styria, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Inn, Tyrol, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Lauterach, Vorarlberg, Austria	4th quarter 2017 and 2nd quarter 2018	< LOD (0.05 ng/L)	Loos *et al.*, 2018
Huangpu River receiving streams (aqueous samples), Shanghai, China	August 2011	n.d. - 20.1 ng/L	Nie *et al.*, 2014
Huangpu River receiving streams (colloidal samples), Shanghai, China	August 2011	app. 5 - 120 ng/g	Nie *et al.*, 2014
Surface Waters, Pampa Region, Argentina	January 2010	43 - 187 ng/L	Valdés *et al.*, 2015

Sampling location or waterbody	Sampling period	EE2 concentration	Reference
Surface water, Río de la Plata estuary, Argentina	January 2010	< LOD (15 ng/L)	Valdés *et al.*, 2015
Taihu Lake (water samples), China	May 2013	n.d. - 33.5 ng/L	Wang *et al.*, 2014
Taihu Lake (sediment samples), China	May 2013	4.32 - 184 ng/g	Wang *et al.*, 2014
Taihu Lake (biota samples), China	May 2013	21.3 - 417 ng/g (dry weight)	Wang *et al.*, 2014
Taihu Lake, China	November to December 2011	n.d. - 4.00 ng/L	Yan *et al.*, 2014
Danube, Budapest, Hungary	not stated	0.124 ng/L	Avar *et al.*, 2016
Danube, Dunaföldvár, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Danube, Solt, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Danube, Paks, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Danube, Mohács, Hungary	not stated	0.005 ng/L	Avar *et al.*, 2016
Drava, Maribor, Slovenia	not stated	0.006 ng/L	Avar *et al.*, 2016
Drava, Drávaszabolcs, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Sava, Ljubljana, Slovenia	not stated	0.002 ng/L	Avar *et al.*, 2016
Ljubjanica, Ljubljana, Slovenia	not stated	0.003 ng/L	Avar *et al.*, 2016
Mur, Murarátka, Hungary	not stated	0.008 ng/L	Avar *et al.*, 2016
Zala, Balatonhídvég, Hungary	not stated	0.68 ng/L	Avar *et al.*, 2016
Hévíz-Páhoki canal, Alsópáhok, Hungary	not stated	0.52 ng/L	Avar *et al.*, 2016
Imremajori canal, Balatonfenyves, Hungary	not stated	0.018 ng/L	Avar *et al.*, 2016
Sió, Szekszárd-Palánk, Hungary	not stated	0.097 ng/L	Avar *et al.*, 2016
Kapos, Kaposvár, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Zagyva, Szolnok, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Tisza, Szolnok, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Tisza, Tiszakécske, Hungary	not stated	0.099 ng/L	Avar *et al.*, 2016
Tisza, Csongrád, Hungary	not stated	0.143 ng/L	Avar *et al.*, 2016
Lake Balaton, Balatonlelle, Hunary	not stated	0.133 ng/L	Avar *et al.*, 2016
Lake Balaton, Balatonszárszó, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016
Lake Balaton, Tihany, Hungary	not stated	< LOD (0.001 ng/L)	Avar *et al.*, 2016

Sampling location or waterbody	Sampling period	EE2 concentration	Reference
Pécsi víz total, Pécs, Hungary	not stated	0.175 ng/L	Avar *et al.*, 2016
Guadiamar River, Spain	June 2014	< MDL (15.0 ng/L)	Garrido *et al.*, 2016
Hawkesbury River, Australia	not stated	n.d. - 29 ng/L	Uraipong *et al.*, 2017
Tâmega River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Tua River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Mondego River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Trancão River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Tagus River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Xarrama River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Guadiana River, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Álamo Creek, Portugal	September to November 2014	< MDL (6.82 ng/L)	Pereira *et al.*, 2017
Huai River, China	January 2010	n.d. - 0.174 ng/L	Niu and Zhang, 2017
Laguna de Castillos, Uruguay	February, May, August, November 2017	n.d. - 45 µg/L	Griffero *et al.*, 2019
Laguna de Rocha, Uruguay	February, May, August, November 2017	< LOQ 0.1 µg/L	Griffero *et al.*, 2019
Billings Reservoir Branch, Brazil	June 2017 - February 2018, June 2017 - August 2017, October 2018 - February 2019	n.d. - 1200 ng/L	Coelho *et al.*, 2020
Yangtze River Estuary (water samples), China	Wet season 2010 and dry season 2011	n.d. - 0.11 ng/L	Shi *et al.*, 2014
Yangtze River Estuary (sediment samples), China	Wet season 2010 and dry season 2011	n.d. - 0.72 ng/g	Shi *et al.*, 2014
Shenandoah River Watershed, USA	2014 - 2016	n.d. - 2.4 ng/L	Barber *et al.*, 2019

Table 2: Effects on biota caused by EE2.

Species	Nominal EE2 concentration	Exposure period	Effects	Reference
Zebrafish *Danio rerio*	4 ng/L	60 d	Length increase, impact on gonad development (temperature-dependent) and on maturation	Luzio *et al.*, 2016
	5 ng/L	20 d	Higher vitellogenin levels	Örn *et al.*, 2016
	2, 5 ng/L (+ 17β-trenbolone in some cases)	40 d	Impact on sex ratio and gonad maturation	
	5 ng/L	30 d	Decreased swimming activity, increased immobility and freezing episodes	Goundadkar and Katti, 2017
		60 and 75 d	Decreased swimming activity, increased immobility, freezing episodes and erratic movement	
	2.5 ng/L, 5 ng/L	14 d	Increased malformation rate	Valcarce *et al.*, 2017
	5 ng/L		Impact on the expression of several genes; increased lymphoedema, otolith areas; lower locomotion	
	1.5 - 75 ng/L	1h, 15 d	Impact on behavior and levels of cortisol, estradiol and testosterone	Fenske *et al.*, 2020
Japanese medaka *Oryzias latipes*	50 ng/L	7 d	Increased fertility in the F1 generation, decreased fertilization rate in the F2 generation, reduced embryo survival in the F3 and F4 generation	Bhandari *et al.*, 2015
	0.1, 1, 10, 50, 100, 500, 1000 ng/L	Various	Decreased heart rate in embryos	Anderson *et al.*, 2020
	50000 ng/L	114 h	Increased heart rate in embryos	
	10 ng/L (+ estrogen modulators)	Various	Decreased heart rate in embryos	
	10 ng/L	50 d	Up- and downregulation of genes	Bhandari *et al.*, 2020
Siamese fighting fish *Betta splendens*	10 ng/L	5 h	Decreased overall behavior, no cosistent shyness and boldness	Dzieweczynski *et al.*, 2014
	5, 10 ng/L	14 d	Impact on mate choice	Cram *et al.*, 2019

Species	Nominal EE2 concentration	Exposure period	Effects	Reference
Inland silverside *Menidia beryllina*	1 ng/L	14 - 21 d	Higher proportion of female fish, deformed larvae, altered number of eggs	DeCourten *et al.*, 2017
	1 ng/L	14 - 21 d	Decreased expression of genes involved in development, growth and reproduction, Decreased expression of GPR30 and FSHR	DeCourten *et al.*, 2019
Argentinian silverside *Odontesthes bonariensis*	180 ng/L, 90 ng/L (+ 700 ng/L E2)	8 d	Reduced embryo survival	Gárriz *et al.*, 2015
	22.5 ng/L to 180 ng/L (in some cases + E2)	16 d	Reduced larvae survival	
	180 ng/L (+ 1400 ng/L E2)	Immediate	Reduced fertilization%	
	45 ng/L (+ in some cases 350 ng/L E2)	14 d	Increased expression of gnrh-III and cyp19a1b, decreased expression of fshr and lhcgr, increase in pyknotic nuclei, decrease in the length of spermatogenic lobules	Gárriz *et al.*, 2017
Gilthead seabream *Sparus aurata*	50, 500 ng/L	96 h	Increased mortality of larvae	Capolupo *et al.*, 2018
	2.5, 5, 50 µg/g	28 d, 50 d, 83 d	Impact on peroxidase, protease, antiprotease activities and bactericidal activity of serum	Valero *et al.*, 2020
African clawed frog *Xenopus laevis*	0.3, 29.6, 2960 ng/L	28 d	Relative expression increase of vitellogenin and decrease of heme oxygenase 1 and heme oxygenase 2	Garmshausen *et al.*, 2015
	50, 500, 5000 ng/L	70 d	Male-to-female sex reversal, mixed sex gonads	Tamschick *et al.*, 2016
Sprague Dawley rat	4 ng/kg/day	Pregnancy, lactation	Higher pain response in male rats, higher grooming duration, impact on estradiol serum levels and estradiol/testosterone ratio	Cecarrelli *et al.*, 2015
	400 ng/kg/day		Lower weight, higher pain response in male rats, higher grooming duration, impact on estradiol serum levels and estradiol/testosterone ratio	
Sprague Dawley® rat	4 ng/kg/day	16 - 21 d	Impact on social activity	Zaccaroni *et al.*, 2017
	400 ng/kg/day		Delayed vaginal opening, social activity impacted	
Green toad *Bufo viridis*	50, 500, 5000 ng/L	70 d	Mixed sex gonads	Tamschick *et al.*, 2016
	500, 5000 ng/L		Male-to-female sex reversal	

Species	Nominal EE2 concentration	Exposure period	Effects	Reference
Crucian carp *Carassius auratus*	17100 ng/L	18 d	Decreased gonadosomatic index in males	Zhou *et al.*, 2019
		27 d	Increased hepatosomatic index, alterations of metabolites in kidneys and gonads	
Ten spotted live-bearer *Cnesterodon decemmaculatus*	100, 200 ng/L	8 -16 weeks	Higher condition factor, impacted gonadal and liver histology	Young *et al.*, 2016
Water flea *Daphnia magna*	10 ng/L to 1000 ng/L (+ additional mixtures with fluoxetine)	40 d	Impact on reproduction and mortality	Luna *et al.*, 2015
Green microalgae *Dunaliella salina*	10 ng/L	11 d	Growth promotion; increased chlorophyll a, chlorophyll b and carotenoid content	Belhaj *et al.*, 2017
	100 ng/L		Growth inhibition; decreased chlorophyll a and chlorophyll b content, glutathione peroxidase activity, polyunsaturated fatty acids; increased carotenoid content, total protein, carbohydrates, polyphenol concentration, flavonoid concentration, antioxidant capacity, superoxide dismutase activity, monounsaturated fatty acids, saturated fatty acids	
	1000 ng/L		Growth inhibition; decreased chlorophyll a, chlorophyll b, carotenoid content, catalase activity, glutathione peroxidase activity, polyunsaturated fatty acids; increased total protein, carbohydrates, polyphenol concentration, flavonoid concentration and antioxidant capacity, superoxide dismutase activity, monounsaturated fatty acids, saturated fatty acids	
Mosquitofish *Gambusia affinis*	50 ng/L	2 d	Increased duration of approaching attempts in females	Dang *et al.*, 2017
	0.5, 5, 50 ng/L		Increased approach attempts and duration of approaching attempts in males	
Chinese rare minnow *Gobiocypris rarus*	1, 5, 25, 125 ng/L	3, 6 d	Impact on expression of genes involved in pubertal development and reproduction	Yang *et al.*, 2016

Species	Nominal EE2 concentration	Exposure period	Effects	Reference
Least killifish *Heterandria Formosa*	5, 25 ng/L	12 - 23 weeks	Intersex alterations, liver damage and disrupted morphology, higher sperm immaturity in males, slower ova maturation in females	Jackson *et al.*, 2019
Lined seahorses *Hippocampus erectus*	5 ng/L	60 d	Increased feeding rate, improved ovary development, smaller brood pouches, impact on gene expression leading to arrested spermatogenesis	Qin *et al.*, 2020
	50 ng/L		Increased mortality, ventilation and feeding rate, improved ovary development	
Long-snouted seahorses *Hippocampus guttulatus*	21 ng/L	30 d	Decreased mass, size, FSH levels and vitellogenin levels; increased mortality, testosterone levels, free androgen index, 17β-estradiol levels; slower development of brood pouches in males	D´Alvise *et al.*, 2020
European tree frog *Hyla arborea*	50, 500, 5000 ng/L	70 d	Mixed sex gonads	Tamschick *et al.*, 2016
	500, 5000 ng/L		Male-to-female sex reversal	
Mangrove rivulus *Kryptolebias marmoratus*	4 ng/L	28 d	Slower growth, less layed eggs	Voisin *et al.*, 2016
	120 ng/L		Slower growth, slower weight increase, increased levels of testosterone and 11-ketotestosterone	
Lithobates catesbeianus *Lithobates catesbeianus*	10 ng/L	96 h	Higher heart rate, higher contraction strength by ventricle strips and higher pumping capacity	Salla *et al.*, 2016
Mediterranean mussel *Mytilus galloprovincialis*	500 ng/L	90 min	Reduced fertilization success	Capolupo *et al.*, 2018
	5, 50, 500 ng/L	48 h	Less normally developed embryos	
Brackish medaka *Oryzias melastigma*	50 ng/L	14 d	Reduced number of spawned eggs, reproductive behavior changes	Lee *et al.*, 2014
	100 ng/L		Reduced number of spawned eggs, reproductive behavior changes, no copulation	
Sea urchin *Paracentrotus lividus*	5, 50, 500 ng/L	75 min	Reduced fertilization success	Capolupo *et al.*, 2018
	50, 500 ng/L	48 h	Less normally developed embryos	
Fathead minnow *Pimephales promelas*	5 ng/L	10 d	Lower survival, impacted regulation of genes involved in gonad development	Leet *et al.*, 2015

Species	**Nominal EE2 concentration**	**Exposure period**	**Effects**	**Reference**
Guppies *Poecilia reticulata*	14 ng/L	28 d	Impacted male mating choices	Saaristo *et al.*, 2019
Clearhead icefish *Protosalanx hyalocranius*	1000000 ng/L	27 d	Less surviving embryos/larvae	Hu *et al.*, 2017
	1600 ng/L, 8000 ng/L, 40000 ng/L, 200000 ng/L, 1000000 ng/L	30 d	Increased teratogenesis	
	200000 ng/L, 1000000 ng/L		Longer hatching time	
Sydney rock oyster *Saccostrea glomerata*	5, 50 ng/L	8 d, 25 d	Less actively swimming larvae and slower development of those, lower survival, lower shell length	Islam *et al.*, 2020
Turbot *Scopthalmus maximus*	50 ng/L	14 d	Increase in EE2 and vitellogenin plasma levels, decrease in dehydroepiandrosterone levels in female and androstenedione levels in males	Farkas *et al.*, 2017

Table 3: Methods to reduce EE2 levels.

Treatment method which lead to the best reported removal efficiency	Best reported removal efficiency, treatment-period in brackets	Initial EE2 concentration	Reference
KMnO4 and Ultrasound	70.5% (120 min)	25 µg/L	Deng *et al.*, 2015
UVC, H2O2 and ultrapure water	100% (10 min)	100 µg/L	Frontistis *et al.*, 2015
Nanoscale zero-valent iron	ca. 100% (300 min)	120 µg/L	Jarošová *et al.*, 2015
Ultrasonic Ozonation	86.0% (12 min)	5 µg/L	Zhou *et al.*, 2015
K_3FeO_4 or K_2FeO_4	100% (5 min)	100 µg/L	Machalová Šišková *et al.*, 2016
Natural organic matter and horseradish peroxidase	35.1% (8 h)	500 µg/L	Yang *et al.*, 2017
TiO2 coated glass rings	ca. 98% (60 min)	20 µg/L	de Liz *et al.*, 2017
Modified magnetite, H2O2	100% (15 min)	1000 µg/L	Serrano *et al.*, 2019
Intracellular polymeric substances from anaerobic cultures	75.5% (5 h)	0.5 mg/L	He *et al.*, 2020
AgI/BiOI/$BiPO_4$	100% (8 min)	3 mg/L	Long *et al.*, 2020
Fungal transformation	98.6% (72 h)	10 mg/mL	Różalska *et al.*, 2015
Phoma sp. strain UHH 5-1-03	Ca. 100% (24 h)	74.1 mg/L	Hofmann and Schlosser 2016
Laccase (from *Pycnoporus sanguineus*)	86.18% (4 h)	5 mg/L	Golveia *et al.*, 2018
Shweanella oneidensis, fulvic acids, sodium anthraquinone-2-sulfonate	41.6% (132 h)	0.5 mg/L	He *et al.*, 2018a
Lolium perenne and *Hyphomicrobium* sp.	98.7% (42 d)	23.5 mg/kg	He *et al.*, 2018b
Long-term electro-domesticated microorganisms, fulvic acids	98.4% (90 min)	0.5 mg/L	He *et al.*, 2019a
Ryegrass and *Hyphomicrobium* sp. GHH	90% (28 d)	25 mg/kg	He *et al.*, 2019b
Magnetic ion exchange	75.3% (75 min)	20 µg/L	Wang *et al.*, 2017
Sand, vermiculite, charcoal, granulated activated carbon	>99% (30 d)	10 µg/L	de Castro *et al.*, 2018
Gamma-cyclodextrin polymer	ca. 100% (5 min)	11.9 µg/L	Tang *et al.*, 2018
Soil	ca. 40% (720 min)	2 mg/L	de Oliveira *et al.*, 2019