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In vitro bioassays to screen for endocrine active pharmaceuticals in surface and waste waters

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ABSTRACT

In the context of the European Water Framework Directive (WFD) it is fully recognized that pharmaceuticals can represent a relevant issue for the achievement of the good chemical and ecological status of European surface water bodies. The recent European Directive on the review of priority substances in surface water bodies has included three pharmaceuticals of widespread use (diclofenac, 17α-ethinylestradiol (EE2), 17β-estradiol (E2)) in the European monitoring list, the so-called watch list. Endocrine active pharmaceuticals such as EE2 and E2 (also occurring as natural hormone) can cause adverse effects on aquatic ecosystems at very low levels. However, monitoring of these pharmaceuticals within the watch list mechanism of the WFD and national monitoring programs can be difficult because of detection problems of most routine analytical methods. With proposed annual average Environmental Quality Standards (AA-EQS) of 0.035 ng/L and 0.4 ng/L, respectively, the estrogenic pharmaceutical EE2 and the natural hormone E2 are among those substances. Sensitive *in vitro* bioassays could reduce the current detection problems by measuring the estrogenic activity of environmental samples. In a short review article the application of this approach to screen and assess the risks of endocrine active pharmaceuticals with a focus on estrogenic pharmaceuticals in environmental waters is discussed.

1. European legislation with regard to endocrine active pharmaceuticals

The European Directive 2013/39/EU [1] on the review of priority substances in surface water bodies has included three substances with pharmaceutical use and of emerging concern (diclofenac, 17α-ethinylestradiol (EE2), 17β-estradiol (E2)) in the European monitoring list, the so-called "watch list". This inclusion represents a first step toward a more integrative approach for the assessment and management of pharmaceuticals in the aquatic environment.

It is highlighted in several scientific studies (*e.g.* [2,3]) that endocrine active pharmaceuticals can cause adverse effects on aquatic ecosystems also at extremely low levels with consequent reduction of the biodiversity of sensitive aquatic species (*e.g.* fish, amphibians). The convincing evidence derived from these studies that EE2 and E2 are environmentally relevant steroids with the ability to elicit negative effects on the population level resulted in their initial listing in the European Commission proposal as candidate priority substances under the European Water Framework Directive (WFD) [4]. The very high biological activity of EE2 and E2 in the environment leads to chronic environmental quality criteria (Annual-Average Environmental Quality Standards, AA-EQS) of less than 1 ng/L. These AA-EQS have been derived on the basis of species sensitivity distribution (SSD) studies based on data of the most sensitive taxonomic groups (fish and amphibians) applying an assessment factor of 2. For surface waters this results in a proposed AA-EQS of 0.035 ng/L for EE2 and 0.4 ng/L for E2, respectively, representing concentrations that should not be exceeded in order to protect the aquatic environment and human health. After a long discussion at the European Council, it was decided that EE2 and E2 are to be included in the first EU-wide watch list monitoring program foreseen by the Directive 2013/39/EU.

The WFD sets out stringent quality criteria for the analytical methods intended to monitor the water status of priority substances and substances on the watch list. All analytical methods applied by member states for the purposes of chemical monitoring have to meet certain minimum performance criteria, including rules on the measurement uncertainty and on the limit of quantification (LOQ) (Directive 2009/90/EC) [5]. The minimum performance criterion for the LOQ is LOQ ≤ EQS/3. For some priority substances, current or proposed EQS values are lower than the

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current LOQ, as described in the CIS guidance no. 19 and a recent JRC report [6].

The detection of these substances at very low levels requires a great analytical effort and there is the need to improve the use of bioanalytical methods, such as *in vitro* bioassays, in support or in replacement of chemical methods for the detection and assessment of endocrine active pharmaceuticals. *In vitro* assays to detect E2 and EE2 in environmental water samples could be applied in the context of the implementation of the Directive 2013/39/EU and the Water Framework Directive (WFD) [4].

2. Pharmaceutical use of 17α-ethinylestradiol (EE2) and 17β-estradiol (E2) and their occurrence in surface and waste waters

There is increasing evidence that steroidal estrogens can disrupt fish population dynamics at low concentrations. Among those compounds the synthetic pharmaceutical EE2 and its natural counterpart E2 are of special relevance because of their use, estrogenic potency and pseudopersistence in the environment.

The most frequent use of EE2 is as the estrogen component of combined oral contraceptives, but it is also added to pharmaceutical products such as hormonal replacement therapies, and used for the treatment of menopausal and post-menopausal symptoms (especially the vasomotor effects). Additionally, it is applied as a palliative treatment in breast and prostate cancer and is found in lotions against diffuse androgen-dependent hair loss in women. In veterinary pharmaceuticals, EE2 is used in livestock to treat reproductive disorders and to control ovulation [7,8]. Its natural counterpart, the primary female sex hormone E2, is also used in pharmaceuticals, mostly in hormone replacement therapy [8], but also to treat infertility in women or advanced prostate cancer, as well as to relieve symptoms of breast cancer.

Excess E2 and EE2 are excreted *via* urine in the form of water-soluble conjugates and either enter the aquatic environment directly (veterinary pharmaceuticals) or, due to their incomplete removal, *via* waste water treatment plants (WWTP) [9,10]. Besides the natural estrogens E1, E2 and estriol (E3), EE2 is the synthetic estrogen most commonly found in waste water [9]. EE2 appears to be mainly stable under aerobic conditions of the activated sludge process of WWTP, indicating higher stability under environmental conditions than the natural steroids E2 and E1. Interestingly, mestradiol (MeEE2), another common synthetic contraceptive component, is rapidly eliminated and small portions of EE2 are formed by demethylation [11], contributing to the EE2-load of WWTP effluent. In English rivers, transformation of E2 and EE2 by microorganisms and the resulting half-lives of E2 and EE2 were investigated. Microorganisms transformed E2 to E1 with half-lives of 0.2 to 9 days, EE2 on the contrary was found to be considerably more resistant to biodegradation (half-life of 17 days) [12].

Only a few studies exist on measured environmental concentrations of E2 and EE2. In the Tonghui river, which receives water from a WWTP in Beijing (China), E2 concentrations of 0.2 ng/L were measured [13]. In the same river, in Beijing itself, much higher concentrations of 32–68 ng/L EE2 were measured at two different sampling sites [14]. Unexpectedly high concentrations of 11.1 ng EE2/L were also found in lake surface water (Massachusetts, US), possibly due to pollution by a hospital in the vicinity of the sampling site [7]. In a groundwater source located in a livestock zone, E2 and EE2 were detected at concentrations of 0.3 and 0.5 ng/L, respectively, whereas in deep phreatic groundwater downstream of an agglomeration higher concentrations of 1.3 ng E2/L and 3 ng EE2/L were measured [15]. At the Cerro Colorado spring near the Mexico city metropolitan area, concentrations of 0.02 ng E2/L and 0.06 ng EE2/L were measured [16]. In the Paris area (France), E2 was present in river water in concentrations between 1.1 and 3.0 ng/L. The biologically more potent EE2 was found in similar concentrations (1.1–2.9 ng/L), contributing 18-27% to the total estrogen concentrations [17]. In some Dutch surface waters, E2 and EE2 were measured at concentrations of 0.8–5.5 ng E2/L and 0.6–3–4 ng EE2/L [18].

For the majority of measurements in water bodies polluted with pharmaceutical estrogens, however, routine analytical methods are not sensitive enough to detect E2 and EE2 in the low ng/L or pg/L range, resulting in no detects (*e.g.* [11,18,19]). In addition, EE2, as well as its natural counterpart E2 do not tend to bioaccumulate in biota or sediment, thus the option of analyzing those compartments will not reduce LOQ problems (as it is the case for several of the current priority substances).

In order to circumvent these detection problems, environmental concentrations are either modeled (*e.g.* [20]), or, instead of concentrations, the estrogenic activities of these compounds are measured with *in vitro* bioassays and reported in E2-equivalent (EEQ) concentrations, indicating the overall estrogenicity of a water sample (*e.g.* [21,22]). The use of such *in vitro* assays for effect monitoring can identify total estrogenic activity present in environmental samples taking into consideration also some antagonistic or synergistic interactions [23]. This is of special relevance as pharmaceutical estrogens (*e.g.* E2 and EE2, discussed in this paper) enter the aquatic environment *via* the same routes than anthropogenic estrogens and estrogens from livestock (*e.g.* E2 and E1), resulting in a potent mixture of (xeno-)estrogens which increases the estrogenic load in water bodies (Fig. 1, modified from [24]). According to Aerni et al. [25], mainly natural and synthetic hormones originating from urban wastewater are responsible for the estrogenic activity in environmental water samples. A contamination with estrogenic substances can be especially problematic if the treated wastewater is not sufficiently diluted in the receiving river [26].

3. Effects of steroidal estrogens on aquatic ecosystems

Comprehensive and convincing studies showed that EE2, E2, and E1 lead to estrogenic effects at concentrations found in the environment [27,28]. Additionally, there is strong evidence that EE2 and E2 are among the steroidal estrogens found in WWTP effluents, which are responsible for the estrogenic effects in fish found in the receiving waters (*e.g.* [25,29]). Models to predict the exposure of riverine fish to EE2, E2 and E1 correlated well with observations of the impact on fish populations in the field [26]. In English rivers endocrine disruptive effects were detected in fish placed downstream of WWTPs [30,31]. In Swiss rivers elevated vitellogenin levels in male brown trout near WWTPs correlated well with concentrations of 1 ng EEQ/L [32]. Wild fish living downstream of pharmaceutical manufacture discharges exhibited strong signs of endocrine disruption including vitellogenin induction, intersex and a male-biased sex-ratio, resulting in a decrease of population densities and sensitive fish species [33]. Concentrations as low as 0.1 ng/L provoked feminization in some species of male wild fish [2].

The exposure of a whole lake with 5–6 ng EE2/L over a time span of two years lead to a collapse of a native fathead minnow (*Pimephales promelas*) population due to missing juvenile fish [34]. A long-term study (aquatic mesocosms and laboratory aquaria) over three generations of fathead minnows at environmentally relevant concentrations of EE2 (3.2 ng/L) resulted in disrupted population dynamics due to direct and transgenerational effects on survival and fecundity [35]. The authors pointed out that fish populations, in particular short-lived highly fecund fishes, exposed to environmentally relevant EE2 concentrations may not recover from exposure. Additionally, from reliable laboratory studies it is known

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