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Steroid Hormones in the Aquatic Environment

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Abstract

Steroid hormones are extremely important natural hormones in all vertebrates. They control a wide range of physiological processes, including osmoregulation, sexual maturity, reproduction and stress responses. In addition, many synthetic steroid hormones are in widespread and general use, both as human and veterinary pharmaceuticals. Recent advances in environmental analytical chemistry have enabled concentrations of steroid hormones in rivers to be determined. Many different steroid hormones, both natural and synthetic, including transformation products, have been identified and quantified, demonstrating that they are widespread aquatic contaminants. Laboratory ecotoxicology experiments, mainly conducted with fish, but also amphibians, have shown that some steroid hormones, both natural and synthetic, can adversely affect reproduction when present in the water at extremely low concentrations: even sub-ng/L. Recent research has demonstrated that mixtures of different steroid hormones can inhibit reproduction even when each individual hormone is present at a concentration below which it would not invoke a measurable effect on its own. Limited field studies have supported the conclusions of the laboratory studies that steroid hormones may be environmental pollutants of significant concern. Further research is required to identify the main sources of steroid hormones entering the aquatic environment, better describe the complex mixtures of steroid hormones now known to be ubiquitously present, and determine the impacts of environmentally-realistic mixtures of steroid hormones on aquatic vertebrates, especially fish. Only once that research is completed can a robust aquatic risk assessment of steroid hormones be concluded.

Keywords: Surface Water, Steroid Hormones, Environmental Concentrations, Effects of Steroid Hormones, Mixture Effects, Environmental Risk Assessment

1.0 Introduction:

The number of chemicals in widespread use, and hence potentially present in the environment, is unknown. It is often said to be around 100,000, but a recent comprehensive analysis of chemical inventories of many major countries suggested that the number of chemicals and chemical mixtures registered, and hence quite likely to be in use, is around 350,000 (Wang et al., 2020). Although the proportion of these chemicals likely to be present in the environment is also

unknown, it is quite likely to be high, because it is not easy to use a chemical without it ultimately ending up in the environment. As a consequence of the widespread use of chemicals, the introduction of novel entities “chemical pollution” has been recognised as one of the 'planetary boundaries' within which humanity can hopefully operate safely (Steffen et al., 2015). However, no boundary is presently identified because of uncertainty about the degree of risk and disruptive effects. Thus, two key unresolved questions will be 'which chemicals present in the environment pose significant risks and which species are most vulnerable?' It is possible that the majority of chemicals present in the environment are usually present at concentrations too low to constitute any significant risk to the environment by themselves, although some definitely do when considered as mixtures with other compounds (Carvalho et al., 2014). For example, the biocide tributyl tin (TBT) has had major adverse effects on mollusc populations across the world (Carson, 2002; Matthiessen and Gibbs, 1998). However as a consequence of the lack of ecotoxicological information on the vast majority of chemicals known to be present in the environment, determining which chemicals pose the greatest risk to the environment has proved very difficult. Some initial efforts to identify the chemicals of most concern have recently been attempted (e.g. Johnson et al., 2017), but these are limited by the lack of relevant information on most chemicals.

One group of chemicals that has recently received a lot of attention from environmental scientists is pharmaceuticals. The concern about these chemicals is based primarily on the fact that they are all biologically active, and hence at least in theory all of them could cause effects on non-target species exposed to them. This concern was highlighted when it was discovered that the veterinary use of the anti-inflammatory drug diclofenac, was responsible for the deaths of millions of vultures in Asia (Oaks et al., 2004). This discovery raised the issue of which pharmaceuticals were causing harm to organisms present in the environment. To answer that question requires the approximately 3,000 pharmaceuticals in everyday use to be ranked by their degree of risk, an exercise that has yet to be attempted , although some are known to be more potent than others. The realisation that oestrogens, both natural and synthetic, adversely affected the reproduction of fish when present at very low concentrations, led Runnalls et al. (2010), to propose that steroid hormones were a class of pharmaceuticals of high concern. The topic was subsequently reviewed by Caldwell et al. (2012). Subsequent research has demonstrated that some synthetic steroid hormones, particularly one oestrogen (ethynyl estradiol) and some

progestogens (e.g. levonorgestrel), maybe the human pharmaceuticals of most environmental concern (Gunnarsson et al., 2019), at least in the aquatic environment.

Worldwide, steroids are increasingly being detected in the water environment as a consequence of their increased use in human and veterinary medicine, awareness of their significance and improved analytical methodology (Weizel et al., 2018; Ma et al., 2016). Certain classes of environmental (exogenous) steroids are a major water quality concern because of their reported adverse effects on aquatic organisms (most especially fish) at very low concentrations (1 ng/L or less) (Gunnarsson et al., 2019; Jobling and Tyler, 2003; Runnalls et al., 2010). Natural and synthetic homologs of steroids have been demonstrated, by laboratory experiments and in-situ studies, to alter (disrupt) the optimal function of the endocrine system in an intact organism, resulting in adverse health effects (Hinfray et al., 2010; Jobling and Tyler, 2003; Sanchez et al., 2011; Thrupp et al., 2018; Willi et al., 2019; Zhang et al., 2011). Progeny of exposed parents have been reported to show altered reproductive ability (Cripe et al., 2009; Guedes-Alonso et al., 2014; Raimondo et al., 2009). The reported modes of action of exogenous steroids include; blocking the synthesis of their endogenous homologs, blocking the synthesis of specific hormone target receptors, mimicking the biological actions of endogenous hormones or antagonizing their effects (Aris et al., 2014; Dzieweczynski and Hebert, 2013). A recent environmental risk ranking of drugs by the pharmaceutical industry (Gunnarsson et al., 2019) reports that some synthetic steroidal drugs present the highest risk of all drugs.

To date, all reviews of steroid hormones in the environment have focussed exclusively on single classes of steroids (e.g., oestrogens: Caldwell et al., 2012; progestogens: Fent, 2015; Kumar et al., 2015). However, the environment is contaminated with complex mixtures of steroid hormones, and hence it is these mixtures that organisms are exposed to. Here, we aim to provide a representative, balanced account of what is known currently about sex steroid hormones in the aquatic environment, thus allowing the degree of risk, both individually and as mixtures, they might pose to be assessed. Further, we aim to stimulate more research on mixtures of steroid hormones, to enable better estimates of the risks they pose to be determined. The emphasis in this work is on the aquatic environment because that is where the highest concentrations of sex steroid hormones are found, as a consequence of the main route of entry of steroid hormones into the environment being effluents of wastewater treatment plants discharging into rivers.

This paper focuses on mammalian hormones, covering their presence in the aquatic environment

and their effects - if any, of course - on aquatic organisms. Mammalian steroid hormones reach the aquatic environment via effluent discharges from wastewater treatment plants (WwTPs). Hence humans are likely the main source of the steroid hormones present in the aquatic environment. Farm animals also produce and excrete a range of steroid hormones, which are either identical to, or very similar to, those excreted by people (Yang et al., 2021). In fact, it has been estimated that the farm animal population of the UK (primarily cattle, pigs, sheep and chickens) probably excretes a greater amount of steroid hormone than the human population (Johnson et al., 2006). However, those authors state that very little of it is likely to reach the aquatic environment. That conclusion was based on the agricultural set-up in the UK, where animal farms tend to be relatively small. In countries that have large, intensive, animal-rearing facilities, as many do, a different conclusion might be reached. For example, Zhang et al. (2021), studied two large swine (pig) farms in China. They found that a wide variety of different steroid hormones, both natural and synthetic, were present in both the liquid and solid wastes produced by the farms. Despite both farms being equipped with sophisticated integrated wastewater treatment systems, the effluent released into the receiving environment contained relatively high concentrations of steroid hormones. The occurrence of progestins in surface waters has also been linked to animal waste (Fent et al., 2015) and the overall significance of the animal industry is that it may contribute more to the load of steroids that human waste (Liu et al., 20115c; 2012b). Research on the steroid hormone contribution to the environment of animal farms that do not have on-site wastewater treatment facilities (the majority in most of the world), and hence discharge directly to the environment, is urgently needed.

In addition to mammalian steroid hormones, other classes of steroid hormones are synthesized by other groups of organisms. For example, arthropods (a group of invertebrates including insects, arachnids and crustaceans) produce ecdysteroids, hormones that play major roles in moulting and development, and to a lesser extent in reproduction. Plants produce brassinosteroids, hormones that play roles in numerous processes (e.g., stress), including reproduction (Nolan et al., 2020). Both invertebrate ecdysteroids and plant brassinosteroids are structurally very similar to mammalian steroid hormones. There appear to be no data on the presence of these compounds in the aquatic environment. If they are present, they are likely to have been present for a very long time, and seem unlikely to be responsible for any effects on aquatic organisms. The situation with synthetic mammalian steroid hormones is very different, as they will have been present in

the aquatic environment for no more than a few decades at most.

We have also excluded discussion of steroid hormone antagonists, such as the estrogen receptor antagonists tamoxifen and fulvestrant (both used to treat breast cancer), the anti-mineralocorticoid spironolactone, and the progesterone antagonists used as an abortifacient, for the early termination of pregnancy, such as mifepristone. We are unaware of any evidence to date that steroid hormone antagonists are present in the aquatic environment at concentrations that would cause concern.

1.1 Classes of Natural Steroids

A characteristic feature of all steroids is their common sterane (cyclopentane-perhydro-phenanthrene structure) backbone with a side chain at carbon C-17(Cuedes-Alonso et al., 2014; Ojoghoro et al., 2017). Based on structural characteristics, vertebrate steroids can be subdivided into five classes, namely androgens, estrogens, glucocorticoids, mineralocorticoids and progestogens (also known as gestagens or progestins) (Table 1 and Figure 1). Glucocorticoids and mineralocorticoids are collectively referred to as corticosteroids. Partial removal of the sterane side chain at C-17 produces the 21 carbon progestogens and corticosteroids, while the complete removal of the sidechain results in androgens (19 carbon products). The 18-carbon compounds (estrogens) are derived from the androgens by the additional removal of the methyl group at C-10.

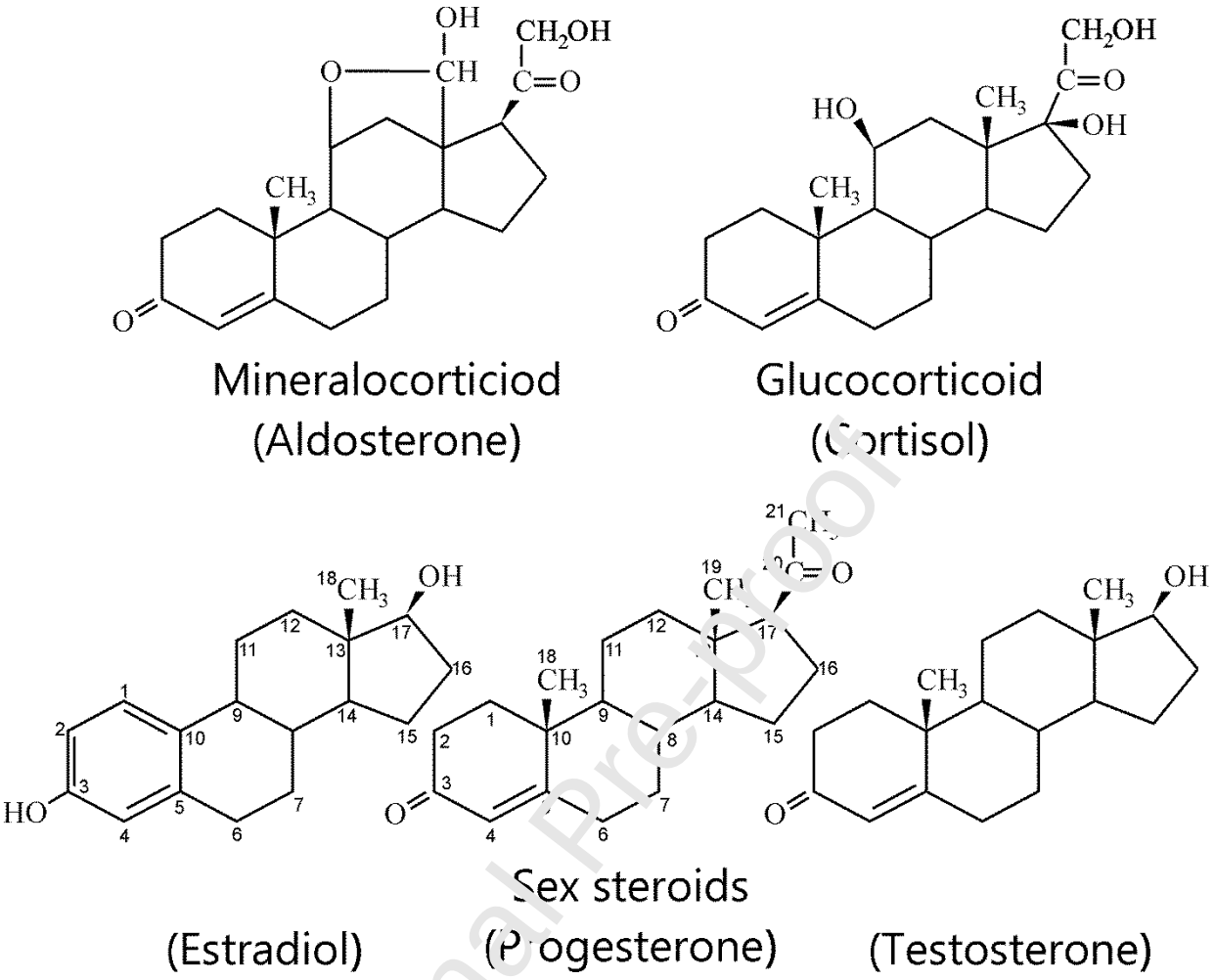

Figure 1: Structure of representative members of steroid hormone classes with carbon numbers shown on Estradiol and Progesterone

Table: 1: Classes of Steroids with examples of compounds and references to their occurrence in the aquatic environment

Class	Description	Examples	References
Androgens	These compounds are commonly known as male sex hormones, because they control the development and maintenance of sexual characteristics in male vertebrates. They are however, also produced at low levels in females. Natural synthesis of these compound occurs	**Natural**: Testosterone, Androsterone and Dihydrotestosterone **Synthetic**: Methyltestosterone	Havens et al., 2020; Ankley et al., 2018; Liu et al., 2015a, b; Guedes-Alonso et al., 2014; Liu et

	in the adrenal glands, testes and ovaries. Synthetic analogous of these compounds have been developed and used in veterinary medicine and human medicine.		al., 2011a; Juntti et al., 2010
Estrogens	Estrogens are a group of chemically similar compounds that are key in the development of the female reproductive system and the regulation of female secondary sexual characteristics. Commonly known as female sex hormones, they also occur in males, but at low levels relative to females. The 21st century has seen a significant increase in the use of these compounds in both human and veterinary medicine.	**Natural**: Estrone (E1), Estradiol (E2) and Estriol (E3) **Synthetic**: ethynyl estradiol (EE2)	Hu et al., 2019; Zhang and Fent, 2018; Ma, Yates and Ashworth, 2016; Aris, Shamsuddin and Praveena, 2014; Lombardi et al., 2001
Glucocorticoids	Glucocorticoids are compounds naturally produced in vertebrates by the adrenal gland. They control energy supply through generation of glucose from non-carbohydrate carbon substrates (glucogenesis). Glucocorticoids are important in reducing stress-related inflammation and allergies caused by an overactive immune system. Synthetic glucocorticoids have been developed for the treatment of immune	**Natural:** Cortisol and Cortisone **Synthetic:** Prednisone, Dexamethasone, Beclomethasone and Triamcinolone	Shen et al., 2020; Weizel et al., 2018; Isobe et al., 2015; Liu et al., 2015b; Ammann et al., 2014; Guedes-Alonso et al., 2014; Löwenberg

	hypersensitivity-related diseases.		et al., 2008
Mineralocorticoids	Mineralocorticoids are produced in the cortex of the adrenal gland. They control blood pressure by regulating the salt and water balances in the body.	**Natural:** Aldosterone **Synthetic:** Spironolactone and Fludrocortisone	Weizel et al., 2018; Zhao, Zhang and Fent, 2016; Ammann et al., 2014
Progestogens	Progestogens are a class of steroidal hormones that play important role in the oestrous and menstrual cycle and in maintaining pregnancy. Like the other classes of steroids, several synthetic forms of progestogens have been developed and used in human and veterinary medicine	**Natural:** Progesterone **Synthetic:** Norethisterone and Levonorgestrel	Ojoghoro et al., 2017; Fent, 2015; Zhang et al., 2014; Fayad et al., 2013; Runnalls et al., 2013

1.2 Synthetic Steroids

Besides the many natural steroid hormones present in the aquatic environment, many synthetic steroid hormones are also present. The picture is currently nowhere near complete, but given that a lot of synthetic steroid hormones, especially synthetic glucocorticoids and synthetic progestogens, are in widespread use, it is very likely that many synthetic steroid hormones are present in wastewater effluent, and hence in all rivers receiving effluent (see, for example, Shen et al., 2018). In fact, some synthetic hormones are amongst the most prescribed drugs. For example, the synthetic glucocorticoid fluticasone, which is used primarily to treat asthma, was the 15th most prescribed medication in the United States in 2017, with more than 32 million prescriptions. The oral contraceptive formulation of drospirenone (a synthetic progestogen) with ethinyl estradiol (EE2) was the 98th most prescribed medication in the United States, with more than eight million prescriptions.

A reason for some concern about the presence of synthetic steroid hormones in the environment (fully discussed in Section 2.1) is that they are likely to be considerably more resistant to degradation in the environment, and hence will be more persistent, than natural steroid

hormones. Synthetic steroid hormones have been designed to be more resistant to metabolism in patients, enabling them to be taken less frequently, in lower doses, and be more bioavailable, hence more potent. For example,EE2 has greatly improved bioavailability, and is more resistant to metabolism, when taken orally by patients compared to estradiol (bioavailability is the amount of a drug absorbed into the systemic circulation in a pharmacologically active form). It is the ethinyl group at carbon 17 (C17) that blocks oxidation of the C17-hydroxy group, a structural feature found on a substantial number of other synthetic steroid hormones. This steric hindrance of metabolism is the primary factor responsible for the dramatically increased potency of EE2 relative to E2. This increased potency also occurs in fish, presumably for the same reason: Thorpe et al., (2003) reported that EE2 was between 11 and 27 times (95% confidence limits) more potent than E2 at stimulating vitellogenin synthesis in fish when present at similar water concentrations (see also the section on effects of steroid hormones).

Many synthetic steroid hormones contain halogen groups. Most often the halogen inserted into the steroid hormone is fluorine (Wang et al., 2014), but it can be chlorine. The very first fluoro-pharmaceutical was fludrocortisone (Florinef), a corticosteroid with potent mineralocorticoid activity, which was first marketed in 1954. In fact, some synthetic steroid hormones contain more than one halogen atom (e.g., the synthetic glucocorticoid fluticasone propionate contains 3 fluorine atoms: Figure 2), and sometimes both fluorine and chlorine atoms (e.g., the synthetic glucocorticoid clobetasol propionate contains both a fluorine and a chlorine atom). There are several factors that explain the high prevalence of the inclusion of fluorine in pharmaceuticals, including many synthetic steroid hormones. One is that the incorporation of fluorine, the second-smallest atom after hydrogen, into the steroidal structure does not drastically change the parent structure, thus maintaining the biological activity of the molecule. But perhaps the factor most relevant to the environmental presence of fluorinated steroid hormones is that metabolism is often slowed down in comparison to the non-fluorinated parent compound, as a consequence of the C-F bond being the strongest bond that carbon can form.

Some of the synthetic steroid hormones are prodrugs that need to be metabolised before becoming biologically active. For example, the synthetic progestogen desogestrel is a prodrug that is rapidly converted in the body to 3-ketodesogestrel, the biologically active metabolite. Similarly, the synthetic glucocorticoid beclomethasone dipropionate is metabolised in people firstly to the monopropionate, then beclomethasone, which are the two biologically active

molecules. In the case of prodrugs, it seems likely that very little, if any, of the parent prodrug would reach the environment; instead, environmental chemists should monitor for the active metabolites.

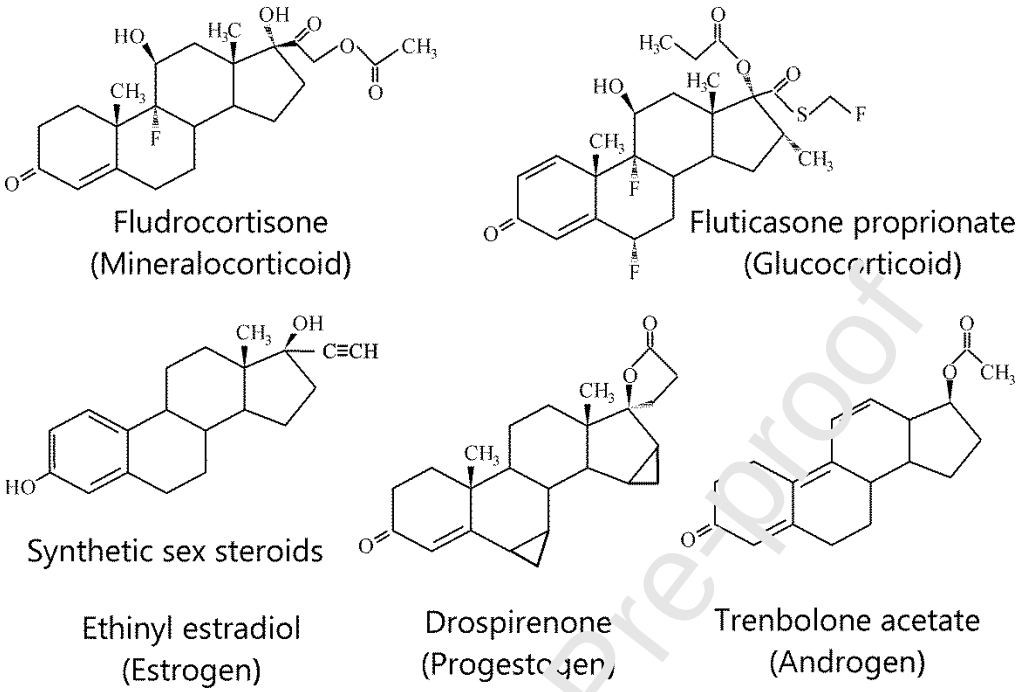

Figure 2: Examples of the structures of some synthetic steroid hormones in the classes shown in Figure 1

It is also possible that some synthetic anabolic steroid hormones are present in the aquatic environment as a consequence of their use by people (as opposed to their use in agriculture to fatten animals). There is a vast range of anabolic androgenic steroids illegally available and used primarily by men seeking to improve their physical appearance (e.g., bodybuilders). Their true composition can be difficult to determine, although most are, unsurprisingly, derived from various forms of testosterone, predominantly testosterone enanthate. Milder effects can be obtained with stanozolol, oxandrolone and nandrolone, and hence these anabolic steroids can be preferred by females. Despite their widespread, and possibly increasing, use, the proportion of the overall population taking anabolic steroid hormones at any one time is low - perhaps 0.2% of all men - and hence it is likely that their concentrations in the environment will be extremely low. In fact, Shimko et al., (2019) could not detect any anabolic steroids in quantifiable concentrations in wastewater when searching specifically for them, whereas they could easily

detect and quantify the concentrations of many endogenous androgens and their metabolites.

2.0 Occurrence and Concentrations in the Environment

A wide range of steroid hormones, both natural and synthetic, from the five groups mentioned above, have been identified as being present in the aquatic environment at concentrations that range from sub nanogram per litre to tens of nanograms per litre (ng/L). Tables 2 and 3 present concentrations of some representative members (natural and synthetic) of each steroidal class used in human and veterinary medicine.. The data were obtained from recently published articles that reported the quality control checks performed for their work that were in line with recommendations (Loos et al. 2018; Kase et al., 2018; Könemann et al., 2018; Vanderford et al., 2014). Table 4 presents examples of some unusually high concentrations reported in the water environment.

Table 2 reports what we have termed 'representative concentrations' of various steroid hormones in surface waters, primarily rivers. However, it is difficult, and potentially impossible, to know how truly representative these concentrations are. For example, often the river water samples were intentionally taken a short distance downstream of a wastewater treatment works, and hence they might represent a worse-case scenario in that river. This is a major problem when spot samples are collected and analyzed. A truly representative picture can be obtained only when samples are taken at regular distances throughout an entire river or river catchment. Such a comprehensive study has not yet been reported for any steroid hormone in any river. Probably the closest study to the ideal one is that reported by Williams et al., (2012), in which predicted steroid estrogen concentration in two UK river catchments were compared to measured values. However, given that caveat, much available literatures suggest that concentrations of most, and perhaps all, steroid hormones in surface waters are generally in the low ng/L range, and sometimes even below 1 ng/L; that is, in the pg/L range.

Despite the general message being that concentrations of steroid hormones in surface waters are in the low, or sub, ng/L range, significantly higher values have often been reported, even recently when the most modern equipment has been used. Table 3 presents information on some of the 'high' concentrations that have been reported. Often these high values are reported as the maximum concentration found in just one of all the samples analyzed in a particular study (i.e., the high concentration occurred in just one sample, and hence is not representative), but sometimes the 'high' concentrations are median or average concentrations, and hence are

presented as representative. The issue of how reliable these concentrations might be is discussed in a following section.

Steroid hormones, both natural and synthetic, are used extensively in veterinary practise, most often to aid fattening of beef cattle and controlling reproduction in a range of farm animals. They are also increasingly used in the expanding aquaculture industry, again to regulate reproduction. Reported concentrations of the most widely used veterinary steroid hormones are given in Table 4. In general, it appears that reported concentrations are higher than those shown in Tables 2 and 3, which cover steroid hormones originating from people. A number of papers report concentrations of individual veterinary steroid hormones above 1 µg/L, and occasionally above 10 µg/L. Those concentrations were obtained from samples collected at, or very close to, sites where the steroids were being used, such as intensive agriculture facilities (e.g., lagoons, runoffs from cattle feedlots in the US) or aquaculture sites. They are unlikely to be representative of the wider aquatic environment.

Table 2: Representative concentrations of Steroid Hormones in surface water

Class	Steroid	Conc. (ng/L)	Reference	Type
Estrogen	E1	nd – 2.3	Hu et al., 2019	Natural
Estrogen	E1	0.21 - 0.91	Zhang and Fent, 2018	Natural
Estrogen	E1	0.2-3.0	Cargouët et al., 2004	Natural
Estrogen	E2	<0.1 – 7.3	Avar et al., 2016	Natural
Estrogen	E2	0.2-3.2	Cargouët et al., 2004	Natural
Estrogen	E3	0.4-2.5	Cargouët et al., 2004	Natural
Estrogen	EE2	nd - 0.64	Hu et al., 2019	Synthetic
Estrogen	EE2	0.1-2.9	Cargouët et al., 2004	Synthetic
Androgen	Testosterone	nd - 1.2	Liu et al., 2011a	Natural
Androgen	Testosterone	nd - 0.4	Liu et al., 2015b	Natural
Androgen	Methyltestosterone	<0.21 - 0.3	Tölgyesi et al., 2010	Synthetic
Progestogens	Progesterone	0.3 - 30.0	Liu et al., 2012a	Natural
Progestogens	Progesterone	<0.1 - 1.2	Šauer et al., 2018	Natural
Progestogens	Norgestrel	0.3 - 22.2	Liu et al., 2011a	Synthetic
Progestogens	Norgestrel	2.2 - 18.5	Liu et al., 2012a	Synthetic
Glucocorticoids	Cortisol	1.2 - 11.0	Shen et al., 2020	Natural
Glucocorticoids	Cortisol	0.2 - 1.3	Weizel et al., 2018	Natural

Glucocorticoids	Prednisolone	2.7 – 94.0	Shen et al., 2020	Synthetic
Glucocorticoids	Prednisolone	nd – 40.0	Liu et al., 2015b	Synthetic
Mineralocorticoids	Aldosterone	<1.0 – 2.0	Ammann et al., 2014	Natural
Mineralocorticoids	Spironolactone	1.0 – 4.0	Ammann et al., 2014	Synthetic

nd: not detected

Table 3: Some high concentrations of Steroid Hormones reported to be present in surface water

Class	Steroid	Conc. (ng/L)	Reference	Type
Estrogen	E2	38.1	Wang et al., 2015	Natural
Glucocorticoids	20α-Dihydrocortisone	54.0 – 138.0	Shen et al., 2020	Natural
Glucocorticoids	3β, 5β -Tetrahydrocortisol	1.3 – 69.0	Shen et al., 2020	Natural
Glucocorticoids	3α, 5β Tetrahydrocortisol	3.9 – 103.0	Shen et al., 2020	Natural
Glucocorticoids	Tetrahydrocortisone	2.3 – 115.0	Shen et al., 2020	Natural
Glucocorticoids	3α, 20β-Cortol	0.33 – 93.0	Shen et al., 2020	Natural
Glucocorticoids	α-Cortolone	1.4 – 127.0	Shen et al., 2020	Natural
Glucocorticoids	β-Cortolone	4.1 – 161.0	Shen et al., 2020	Natural
Progestogens	Norgestrel	253 - 677	Liu et al., 2012b	Synthetic
Progestogens	Progesterone	375	Dequattro et al., 2012	Natural

Table 4: Representative Concentrations of Veterinary Steroid Hormones in Animal Farms Effluent and Surrounding Receiving Surface Water

Class	Steroid	Conc (ng/L)	Reference	Type
Androgen	17α -Trenbolone	<0.1 – 1720 (rff)	Ankley et al., 2018	Metaboli te
Androgen	17b-Trenbolone	<0.1 – 270 (rff)	Ankley et al., 2018	Metaboli te
Androgen	Androsta-1,4- diene-3,17-dione	11.6 – 82.1 (sw)	Liu et al., 2012a	Natural
	Androsta-1,4- diene-3,17-dione	75.2 – 142.8 (sw)	Liu et al., 2012b	Natural
Androgen	Testosterone	2.6 – 3.9 (sw)	Liu et al., 2012a	Natural

Androgen	Testosterone	13.8 – 15.8	Liu et al., 2012b	Natural
Estrogen	E1	19.7 - 22.1 9 (sw)	Liu et al., 2012a	Natural
Estrogen	EE2	281.5 – 437.0 (sw)	Liu et al., 2012a	Natural
Progestogens	Dydrogesterone	9.2 – 10.0 (sw)	Liu et al., 2014	Synthetic
Progestogens	Medroxyprogesterone acetate	13.7 - 15.1 (sw)	Liu et al., 2012b	Synthetic
Progestogens	Medroxyprogesterone acetate	13.6 - 99.7 (eff)	Liu et al., 2012a	Synthetic
Progestogens	Melengestrol acetate	16.5 – 500 (rff)	Bartelt-Hunt et al., 2012	Synthetic
Progestogens	Norgestrel	253.0 – 667.0 (sw)	Liu et al., 2012b	Synthetic
Progestogens	Norgestrel	17.2 – 18.5 (sw)	Liu et al., 2012a	Synthetic
Progestogens	Progesterone	25.5 - 35.5 (sw)	Liu et al., 2012b	Natural
Progestogens	Progesterone	27 – 31.0 (sw)	Liu et al., 2012a	Natural

sw: surface water; eff: effluent; rff: runoff

2.1 Human Metabolism and Transformation in the Environment

Humans and animals produce many transformation products (TPs) of steroidal compounds due to the extensive metabolism that these compounds undergo in the liver, changing them to varying extents. Then as metabolites, conjugates or the unchanged parent, depending on the individual compound, they are all excreted (Evgenidou et al., 2015; Cwiertny et al., 2014). Hepatic metabolism of steroids and other pharmaceuticals occurs in two main phases. Oxidation, reduction and hydrolytic cleavage (phase I) and in phase II, glucuronidation, acetylation and sulphation, depending on the compound (Figure 3) (Evgenidou et al., 2015; Cwiertny et al., 2014; López-Serna et al., 2012). Further structural transformation of some excreted metabolites occurs in sewers and in other sewage-collecting systems such as cesspits, cesspools and septic tanks (Jelic et al., 2015; Rudelle et al., 2011; Schilperoort et al., 2012) prior to engineered treatment in wastewater treatment plants (WWTPs), where the bulk of transformation happens. Transformation and not mineralisation of steroids is potentially a common phenomenon in the

environment (Liu et al., 2020) and begins well before WWTPs. Surface waters are, therefore, exposed not only to the parent compounds but to their human metabolites and transformation products (TPs), which can potentially be more potent relative to parent molecules (Guo et al., 2016; Ojoghoro et al., 2017). For example, the prodrug beclomethasone dipropionate (BDP) (mentioned earlier), a glucocorticoid steroid used mostly by inhalation for treatment of respiratory disorders e.g., asthma (Foe et al., 1998; Wilcox and Avery, 1973), is administered pharmacologically inactive, and only become active following metabolism. The hydrolytic breakdown of the compound's ester side chain yields a much more potent monopropionate derivative, beclomethasone monopropionate (BMP) (Zawilska et al., 2013; Huttunen et al., 2011; Rautio et al., 2008). Monitoring BDP alone, the focus of traditional risk assessment paradigms, will result in underestimating the risk associated with the compound, as it is not the major excretion product.

Microbial back-transformation of TPs to parent compounds have been reported in the environment (Qu et al., 2013; Su et al., 2016), a process that potentially increases environmental exposure to parent compounds downstream of effluent discharge points or point of initial transformation. Qu et al., (2013) reported the back-transformation of animal metabolites and TPs of the growth promoter trenbolone acetate in conditions representative of surface waters. Phase II metabolites can be back-transformed (deconjugated) to their respective parent compound by the enzymes β-glucuronidase (in the case of glucuronidation) or arylsufatase for sulfation, both of which are secreted by microorganisms (Evgenidou et al., 2015; Liu et al., 2015c; Gomes et al., 2009). For example, Gomes et al., (2009) reported the preferential deconjugation of glucuronides conjugates of estrogens relative to sulfate conjugates in crude sewage. Furthermore, there is evidence in the literature of the ready deconjugation of glucuronated estrogens in sewers, due to the large amounts of the β-glucuronidase enzyme produced by faecal bacteria (*Escherichia coli*) (Baronti et al., 2000; D'Ascenzo et al., 2003; Heberer, 2002). Based on the daily human excretion of conjugated estrogens, Baronti et al., (2000) noted that deconjugation of estrogens preferentially occurs in sewers. Additionally, two glucuronides of estradiol (17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide) were reported to be cleaved when in contact with diluted activated sludge solution, resulting in release of estradiol (Ternes et al., 1999a; Ternes et al. 1999b).

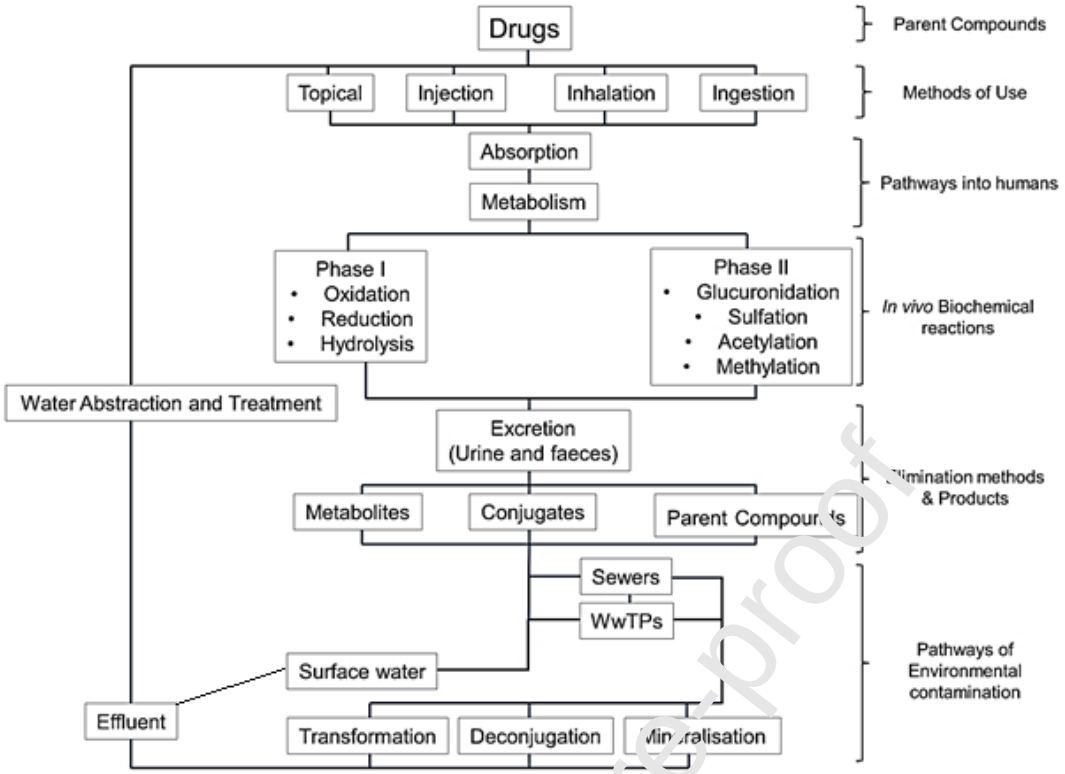

Figure 3: General pathways of environmental occurrence of pharmaceuticals (Evgenidou et al., 2015; Liu et al., 2011b; Pérez and Barceló, 2007)

The shape and structure of steroids have an important role in their activity and subsequent effects, which has been recognised for many years in evaluating structure-activity relationships (Marshall and Cramer, 1988). Structural conservation in TPs is ecotoxicologically important, especially if a parent's potency is structure dependent. The implication is that the given parent's inherent biological acitivity(ies) can be conserved in TPs or new toxicity potency can be created across multiple biological end points (Cwiertny et al., 2014; Forsgren et al., 2014). To illustrate, TPs of steroids have been reported to retain the structural backbone of the biologically active parent compounds and have exhibited equal or enhanced biological activity relative to the parent compound (Diniz et al., 2015; Cwiertny et al., 2014; Haddad and Kümmerer, 2014). The latter point is especially relevant with TPs of steroidal hormones as they can exhibit distinct biological activities though differing only by slight structural modification to their side chains. Thus, retention of the basic structure of the parent steroid in TPs often provides empirical evidence of potential conservation of biological potency. The term 'transformation', as used in this work, refers conservatively to minor modification in the molecular structure of parent compounds

involving the structural reorganisation of composite peripheral elements, functional groups and/or cleavage of side-chain or the substitution of a functional group and/or element with an external one. Steroid transformation as used in the present work would therefore convey the idea of substantial conservation of core parent structural backbone in products.

All four steroid classes (progestogens, estrogens, androgens and glucocorticoids) making up this group of compounds have a common parent tetracyclic backbone (see section 1.1). The target nuclear receptors that each steroid – progesterone, estrogen, androgen or glucocorticoid - are designed to interact with, to elicit their intended biological activity, is achieved by slight alterations of their chemical structure. Thus, the slightest modification in the arrangements of the peripheral atoms and/or functional groups of these compounds due to environmental transformation can modify their pharmacophore (electronic and spatial arrangement of a compound's atoms), and alter their optimal binding with a target biological site to trigger or block biological signalling (Ojoghoro et al., 2017; Overington et al., 2006). Put simply, modification of the pharmacophore caused by transformation could make resultant TPs bind to different ligands (nuclear receptors) and elicit different biological responses (e.g. activation or deactivation of certain gene expression) (Cwiertny et al., 2014; Jenkins et al., 2004; Runnalls et al., 2013). For example, the C-19 mammalian metabolite of cortisol (a glucocorticoid), 5α -androstan-3,11,17-trione, formed by side chain cleavage of cortisol, was reported to possess the biological activity of a different class of steroid (Grillitsch et al., 2010). Grillitsch et al., (2010) reported the masculinisation (anal fin characteristics) of the Japanese medaka (*Oryzias latipes*) by 5α -androstan-3,11,17-trione in a dose-dependent manner similar to the reference androgen 11-ketotestosterone.

Similarly, structural modification to specific locations in progesterone have been shown to not only modify its progestagenic activity, but resultant TPs may also demonstrate activity in other steroid pathways (e.g., androgenicity) (Besse and Garric, 2008, 2009; Ojoghoro et al., 2017). Most transformation reported in the literature occurred in these positions. Progesterone differs from its androgenic counterpart testosterone only by having a –COCH3 functional group at carbon 17 (C17). Thus, transformation involving cleavage of the –COCH3 and subsequent hydroxylation (-OH) at the C17, will yield testosterone, an androgen with differing biological activity relative to progesterone. Another example is the reported bacteria and algae transformation of progesterone to known androgens (Carson et al., 2008; Jenkins et al., 2004).

Peng et al., (2014) reported four (4) androgens (including androsta-1,4-diene-3,17-dione and 4-androstene-3,17-dione) following breakdown of progesterone by two freshwater algae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa*. The androgen 4-androstene-3,17-dione was formed by the cleavage of the –COCH3 side chain of progesterone at C17. Subsequent dehydrogenation of 4-androstene-3,17-dione at C1 resulted in androsta-1,4-diene-3,17-dione. Similar transformation and androgenic products had been previously reported by Carson et al., (2008) and Jenkins et al., (2004) in a biotransformation reaction mediated by the bacterium *Mycobacterium smegmatis*.

A recent study has established the potential biotransformation of a relatively weak oestrogen (E1) to a much stronger one (E2) by the enzyme 17β-hydroxysteroid dehydrogenases (17βHSDs) (Tapper et al., 2020). Tapper et al., (2020) reported the *in vivo* transformation of E1 to E2 by rainbow trout (*Oncorhynchus mykiss*) and lake trout (*Salvelinus namaycush*). The lesson; fish exposure to relatively less potent steroids in the aquatic environment does not necessarily translate to low risk. Understanding biotransformation is important when evaluating the potential adverse effects that exposure to environmental steroids could have.

In summary, metabolism and transformation are widespread and occurring regularly, leading to a likely multitude of TPs, most of which await identification in the environment (many may never be identified). At least some of these TPs will possess biological activity, although most will probably be less active, and hence of less concern, than the parent molecules. As shown above, some TPs could possess completely different biological activities compared to the parent molecules. A very recent example (Weizel et al., 2021) illustrates this point extremely well with the behaviour of progestogens in wastewater treatment. Those derived from 19-nortestosterone, as many early synthetic progestogens were, can undergo aromatisation, leading to estrogen-like products. For example, the degradation of norethisterone acetate led to the formation of EE2, an extremely potent synthetic estrogen. The situation is very complex, difficult to predict based on current knowledge, and may never be fully known. Lessons learnt from the adverse environmental impacts of organochlorine pesticides due to the prioritization of their economic benefits over and above understanding their environmental fate (Carson, 2002; Krebs et al., 1999) stress the need for understanding the identity and toxicity of human metabolites and environmental TPs (resulting from natural and engineered degradation processes). The inclusion of TPs in traditional surface and groundwater monitoring programmes will not only protect and

preserve the quality of these water systems, but will be useful in understanding their ubiquity and facilitating effective risk assessments.

3.0 Effects of Steroid Hormones on Aquatic Organisms

3.1 Mechanisms Behind Effects

The effects of steroid hormones, both natural and synthetic, in humans are extremely well documented, as are the mechanisms behind these effects. So, do they produce the same effects in aquatic species? In mammals, steroid hormones act through specific receptors that act as transcription factors, binding to genes that contain the appropriate response elements. Thus, for example, estrogens bind to the estrogen receptor (ER) that is then translocated to the nucleus, where it binds to estrogen response elements (EREs) and regulates gene transcription. There are specific receptors for the natural estrogens (ER), progesterones (PR), androgens (ARs), glucocorticoids (GR) and mineralocorticoids (MR). These receptors are found throughout the vertebrates (Baker, 2011; 2019), and hence both natural and synthetic steroid hormones present in the aquatic environment almost certainly act via these receptors. However, two factors complicate the situation. One is that during the evolution of teleost (bony) fish - but not cartilaginous fish - the entire genome was duplicated twice, accounting for why in teleost fishes there are at least three ERs and two ARs (some duplicated receptors were probably lost during evolution). At least in theory the duplicated receptors within a species could possess different specificities, as their sequences diverged during evolution. The other factor is that during evolution the protein sequence of a receptor can change; thus, for example, the sequence of the human PR is not identical to the sequences of any fish PRs. These fairly subtle changes in sequence can, and sometimes do, change the ligand specificities of receptors. Put another way, steroid hormones that bind strongly to the human PR may not do so to any fish PR. This makes extrapolating effects from humans to fish (so-called read across; see Rand-Weaver et al., 2013) potentially problematic. However, in general it is fair to say that the specificities of steroid hormone receptors have been relatively well-maintained during evolution. For example, Tohyama et al., (2016) reported that the ERs of ray-finned fishes (all teleosts) showed similar responses to both endogenous and synthetic steroid hormones. However, this is not always the case, as recent studies with synthetic progestogens and fish have shown. Initial evidence from in vivo reproduction studies showed that some synthetic progestogens were extremely potent, whereas other synthetic progestogens were much less potent (Runnalls et al., 2013; Zeilinger et

al., 2009), results supported by subsequent assessments of transcriptional effects in fish exposed to various synthetic progestogens (Zhao et al., 2015; Zucchi et al., 2014). It now appears that the first-generation synthetic progestogens, such as levonorgestrel, are extremely potent in fish by acting through the AR, not the PR (Bain et al., 2015), which is, in retrospect, not surprising as they are derivatives of 19-nortestosterone. In contrast the latest synthetic progestogens (termed 4th generation) show relatively low activity in fish (Garoche et al., 2020; Runnalls et al., 2013s; Schmid et al., 2020a), possibly because they have relatively low affinity for the fish PR. The situation can be summarised by saying that, unlike the ER, human and fish PRs have different ligand specificities, thus making read-across problematic. The situation with the other steroid receptors (AR, GR and MR) is less clear presently, as a consequence of them being less well studied in fish compared to the ER and PR. However, there is strong evidence that the natural corticosteroid cortisol is less potent that the synthetic glucocorticoid clobetasol propionate, although probably due to the efficient metabolism of cortisol by fish rather than anything to do with receptor specificity (Faltermann et al., 2020). Such studies once again highlight the fact that at least some of the synthetic steroid hormones present in the aquatic environment are very potent to fish, and hence justify thoughtful and comprehensive study.

3.1.1 The Importance of Hydrophobicity

To have effects on aquatic organisms, chemicals have to get into those organisms. The main factor controlling the uptake of chemicals is their hydrophobicity, which is driven by the pH of the surrounding water environment (Kovacs et al., 2006). The more hydrophobic a chemical is, the more readily it is taken up. And conversely, the more hydrophilic a chemical is, the less well it is taken up. Hydrophobic chemicals are not only readily taken up, they bioconcentrate in organisms, such that the internal concentration is higher - sometimes much higher - than the external (water) concentration (Rand-Weaver et al., 2013). For example, Rand-Weaver et al., (2013) predicted a plasma bioconcentration factor (BCF_{plasma}) of 132 when they applied the 'Fish Plasma Model' to a fish exposed to a representative EE2 concentration of 0.26ng/L at typical surface water pH of 7.4. The measured BCF for EE2 in the fathead minnow is likely to be around 500 (Länge et al., 2001), and EE2 has been shown to bioconcentrate in the plasma of fish (Runnalls et al., 2015). Since steroid hormones are reasonably hydrophobic, they can get into organisms easily, where they can bioconcentrate. This is a major reason behind their potency. The behaviour of chemicals, in terms of partitioning, is also driven by their hydrophobicity. This

in turn can influence their biodegradation and hence long term persistence. Natural estrogens have been observed to degrade when adsorbed to sediments, more rapidly when conditions are aerobic, rather than anaerobic (Bradley et al., 2017). It seems likely that along with the availability of oxygen, other factors such as light (Bradley and Writer, 2014) and the characteristics of the organic matter (Ma and Yates, 2018) influence the fate of steroids in the aqueous environment.

3.2 Laboratory Studies

3.2.1 Invertebrates

The vast majority of animals are invertebrates, including in the aquatic environment. Hence it is imperative to know if the steroid hormones present in the aquatic environment have adverse effects on aquatic invertebrates. Many laboratory studies have been conducted in which various species of aquatic invertebrates (molluscs and crustaceans in particular) have been exposed to various steroid hormones, most often estrogens. The results have been highly variable; many authors have reported that the hormones caused effects but their results have very rarely, in our opinion, been convincing. For example, it is extremely rare for authors to demonstrate that any apparent effects they report were concentration-dependent: no sigmoidal dose-response relationship is provided. Claims that invertebrates respond to estrogens continue to be published (Jones et al., 2017), but such studies usually suffer from many problems, as has been very carefully and intelligently demonstrated by Scott (Scott, 2018). He argues, for example, that as many of the key enzymes required for the biosynthesis of vertebrates' steroid hormones, such as the enzyme aromatase, do not appear to be present in invertebrates, their absence therefore questions the results of many studies claiming that endogenous steroid hormone concentrations are associated with reproductive cycles (Scott, 2018). Similarly, although there have been claims that steroid hormone receptors are present in invertebrates, recent genetic evidence suggest otherwise. Based on complete genome sequences of two species of mollusc, Kaur et al., (2015) could not identify any convincing homologues of the AR, GR, PR or MR. These same authors subsequently demonstrated that, as would be expected based on the absence of the AR, steroid androgen exposure of a mollusc had no effect on reproductive physiology (Kaur et al., 2016). Further, Fernández-González et al., (2020) found no evidence that an estrogen could induce the egg-yolk protein vitellogenin in mussels. Collectively, as Scott 2018, argues, it seems unlikely that the mammalian steroid hormones are functionally active on molluscs - and quite possibly all

other invertebrates.

3.2.2 Vertebrates

It has been known for quite some time that fish are very sensitive to the presence of steroid hormones in the water, mainly as a consequence of research related to aquaculture (Donaldson, 1996; Hunter and Donaldson, 1983). However, it took the discovery of feminised fish in British rivers (see 'Field Studies' section below) to kick-start research on the responses and sensitivities of aquatic organisms, particularly fish, to the presence of steroid hormones in the water. Purdom et al., (1994) were the first to demonstrate the extraordinary sensitivity of fish to some steroid hormones, most notably EE2. They showed that concentrations of EE2 below 1 ng/L stimulated vitellogenin synthesis in a concentration-dependent manner. Many subsequent studies, utilising a wide variety of species of fish, have confirmed that exposure to very low concentrations of EE2 causes a range of molecular, biochemical and physiological effects, leading to reduced egg production by females and feminisation of males (Länge et al., 2001; Runnalls et al., 2015). Caldwell et al., (2008, 2012) have twice comprehensively reviewed the effects of oestrogenic steroid hormones on fish, confirming that EE2 is the most potent oestrogen, although it is important to realise that the natural oestrogens E1 and E2 are also quite potent (Panter et al., 1998; Thorpe et al., 2003). One intriguing recent finding was that E1 is more potent *in vivo* than might be expected based on its receptor binding affinity, because once internalised it is metabolised to E2 by fish (Tapper et al., 2020).

Table 5 provides some examples of laboratory studies with representatives of other classes of steroid hormones. Some synthetic progestogens adversely affect reproduction of fish at very low concentrations. For example, the synthetic progestogen levonorgestrel - a first generation progestogen - inhibits reproduction to some degree even when present at a concentration below 1 ng/L (Runnalls et al., 2015; Zeilinger et al., 2009). It is of equivalent potency to EE2 (Runnalls et al., 2015). As a first-generation synthetic progestogen, it was known to exhibit significant androgenic effects in women taking the drug as a contraceptive. In fish and amphibians, it is thought to act primarily through the androgen receptor, rather than the progesterone receptor (Svensson et al., 2013). However, more recently developed synthetic progestogens, the so-called third and fourth generation progestogens, such as desogestrel, appear to be considerably less potent to fish (Runnalls et al., 2013). They are more specific to the human PR than the older generation progestogens such as levonorgestrel, but probably have lower affinities for the fish

PR.

Regarding androgens, probably the best data are concerned with the anabolic androgen trenbolone (these studies are reviewed by Ankley et al., 2018). Laboratory studies have shown that 17-trenbolone is a potent androgen receptor agonist in fish, masculinising female fish when present at relatively low ng/L concentrations. This synthetic androgen is used widely in some countries (e.g., USA) to fatten livestock, particularly cattle. Runoff from beef feedlots can contain various metabolites of trenbolone acetate as well as the parent compound, leading to contamination of receiving waters. However, it remains unknown if trenbolone is affecting wild fish populations.

It appears that both natural and synthetic glucocorticoids must be present in the water at higher concentrations (tens to hundreds of ng's/L) before effects on fish are manifest (Table 5). Less research has been devoted to studying any effects of glucocorticoids on fish than has been the case with both estrogens and progestogens. The first study was not published until 2011 (Kugathas and Sumpter, 2011), which showed that the synthetic glucocorticoid beclomethasone dipropionate caused the same effects in fish as it does in humans, such as anti-inflammatory responses and raised plasma glucose concentrations. Subsequent research has confirmed, and expanded on, those initial findings (e.g., Willi et al., 2018).

Amphibians (frogs, toads, newts and salamanders) spend varying amounts of time living in water, and hence could potentially also be affected by exogenous steroid hormones. This possibility has been investigated, and some researchers have demonstrated in laboratory experiments that the presence of some synthetic steroid hormones in the water can, for example, disrupt reproduction (Hoffmann and Kloas, 2012; Säfholm et al., 2014) and affect gene expression in endocrine glands (Lorenz et al., 2016). However, we consider that wild amphibians are much less likely to be affected by environmental exposure to steroid hormones compared to fish, as a consequence of their different life histories compared to fish. Many amphibians spend only their egg and larval stages in water; adults are terrestrial. Also, almost all adult amphibians have lungs, and breathe air, rather than extract oxygen from water continually passing over gills, as fish do. These, and other, factors suggest that the exposure of amphibians to steroid hormones in their environment will be much lower than that of fish, and hence adverse effects on wild amphibian populations seem much less likely to occur (but see section 3.4 for some limited evidence that challenges that opinion).

3.3 Field Studies

Given that much of the freshwater environment throughout the world is contaminated with steroid hormones, and that many of these hormones are extremely potent, it might be expected that there would be many documented examples of adversely affected fish populations. Yet this is not the case. There are two reasons for this. One is that hormone-specific biomarkers are scarce, although imposex in molluscs caused by exposure to TBT and intersexuality in fish caused by exposure to oestrogenic chemicals could be considered biomarkers. However, that first condition is not caused by a hormone, and the second condition can be caused by many chemicals, not only steroidal oestrogens. Therefore knowing what endpoint to measure in order to be able to confidently link changes in that endpoint to exposure to a particular class of steroid hormones, or even steroid hormones in general, is not straightforward. The other is that even if hormone-specific endpoints have been identified, the magnitude of change that those endpoints undergo is often, although not always, relatively small, making them difficult to use in uncontrolled environments such as field studies. However, in the case of estrogens a unique endpoint, vitellogenin induction, has been identified. Estrogens stimulate fish to synthesize vitellogenin, and plasma concentrations can increase by up to a million-fold, making it an extraordinarily powerful biomarker (Sumpter and Jobling, 1995). As a consequence, it has been very widely used in both laboratory and, to a somewhat lesser extent, field studies. But currently no such unique and powerful biomarkers are known for androgens, progestogens, glucocorticoids and mineralocorticoids. Each of these classes of hormone probably does elicit unique responses (as well as some common ones), but the magnitude of these effects is not large, making them difficult to utilise with confidence. For example, glucocorticoids increase plasma glucose concentrations in fish (Kugathas and Sumpter, 2011), and also increase the expression of genes that play pivotal roles in gluconeogenesis (Kugathas et al., 2013; Willi et al., 2018), but the magnitude of these changes is relatively small, being 5-fold at most. In theory these endpoints could be utilised as biomarkers to investigate whether or not wild fish populations are being exposed to glucocorticoids at high enough concentrations to cause effects, but so far, they have not been utilised in this way.

There is strong, and widespread, evidence that concentrations of estrogenic steroid hormones can be high enough in at least some rivers to trigger physiological effects. The first evidence for this was provided by Purdom et al., (1994), who showed that male fish placed in WwTP effluents in

England had extremely high plasma vitellogenin concentrations, indicating exposure to estrogenic chemicals. Purdom et al., (1994) speculated that the causative chemical was likely to be EE2, although they did not provide conclusive evidence to support their suggestion. Their discovery was followed up by Harries et al., (1997), who reported using caged fish to demonstrate estrogenic activity in a number of rivers in the UK, and by Jobling et al., (1998), who not only reported elevated plasma vitellogenin concentrations in wild fish living downstream of WwTPs in many UK rivers, but also reported that intersexuality was widespread in these fish. Collectively, these studies demonstrated that many rivers in the UK were contaminated with estrogenic chemicals, present at concentrations high enough to induce major reproductive effects in wild fish. Chemical analysis of WwTP effluent, using state-of-the-art instrumentation at that time, strongly suggested that EE2 was a major contributor to the estrogenic activity of effluent and, presumably, rivers receiving effluent (Desbrow et al., 1998). These UK studies, covering more than a decade of both laboratory and field research, are reviewed in Sumpter and Jobling, (2013). Subsequently, biological and chemical studies in many other countries reported very similar results (e.g., Hinck et al., (2009). Although it has never been possible to definitively prove that estrogenic steroid hormones alone, probably present as mixtures of both natural and synthetic chemicals, are solely responsible for the widely reported feminisation of male fish - and probably never will be - the evidence is considered very strong. Specific effect based monitoring approaches have been utilised by many workers to assess overall activity of samples from the environment. Samples from the Danube river, screened with progestogen and glucocorticoid receptors, indicated that effect based monitoring is a powerful tool, although mixtures of components in the real world can influence outcomes (Hashmi et al., 2019).

Besides the widespread phenomenon of estrogenic WwTP effluents feminising wild fish, we are aware of only one other convincing example of steroid hormones adversely affecting wild fish. This occurred downstream from a pharmaceutical manufacturing facility in France. Sanchez et al., (2011) reported that wild gudgeon (a small, common, teleost fish) collected downstream of a facility manufacturing pharmaceuticals exhibited strong signs of endocrine disruption, including elevated plasma vitellogenin concentrations, intersexuality, and a male-biased sex ratio. These effects were associated with a decrease in fish density and a lack of sensitive fish species, suggesting population-level effects. A bioanalytical analysis of the contaminants that were

present at this site revealed the presence of a number of steroid hormone activities, including very high concentrations of glucocorticoid, antimineralocorticoid and progestogenic activities, as well as weak estrogenic activity (Creusot et al., 2014). Chemical analysis detected 60 out of 118 targeted steroids and other pharmaceuticals. Steroid hormones positively identified at high concentrations included the glucocorticoids 6 alpha-methylprednisolone and dexamethasone, the antimineralocorticoid spironolactone and the androgen androstenedione. Lower concentrations (but up to 33 ng/L) of the potent progestogen levonorgestrel were also reported. Overall, the results of the chemical analysis of the effluent and river water downstream of the pharmaceutical manufacturing facility adequately explained the masculinisation of female gudgeon reported by Sanchez et al., (2011).

It is quite possible that the complex mixtures of steroid hormones known to be present in rivers receiving WwTP effluent are affecting aquatic organisms, especially fish, quite widely but demonstrating this with a high degree of confidence is extremely difficult.

Table 5: Biological Effects of Steroids on Aquatic Organisms

Test compound	Class	Concentration (ng/L)	Test organism	Exposure time (days)	Observed effects	References
Androstenedione	Androgen	15, 74, 700	*Pimephales promelas*	26	Male: Increased Vtg mRNA expression, reduced GSI. Females: tubercle development	Dequattro et al., 2015
11-ketotestosterone & Dihydrotestosterone	Androgen	20, 200	*Pimephales promelas*	45	Both sex: Somatic growth, tubercle formation, dorsal fin spots (female at	Margiotta-Casaluci and Sumpter, 2011

					200 ng/L). Male: spermatogenic process induction; Female: severe disruption ovarian physiology and morphology, intersex	
17α-trenbolone	Androgen	7 - 11	*Pimephales promelas*	21	Reduced testosterone, estradiol, fecundity & Vtg levels; female masculinisation	Jensen et al., 2006
17β-trenbolone	Androgen	3 - 10	*Pimephales promelas*	21	Vtg levels & fecundity inhibition	Miller et al., 2007
17β-trenbolone	Androgen	10	*Danio rerio (embryo)*	-	Irreversible masculinisation	Ankley et al., 2018
17β-trenbolone	Androgen	3	*Danio rerio (larva and embryo)*	60	Increased weight	Ankley et al., 2018; Baumann et al., 2014
Cortisol	Glucocorticoid	100 - 700	*Danio rerio*	1 - 5	Increased heart rate; decreased spontaneous muscle contraction and accelerated hatching	Willi et al., 2018
Cortisone	Glucocorticoid	100	*Danio rerio*	4 - 5	Down regulation of Vtg gene	Willi et al., 2018
BDP	Glucocorticoid	100	*Pimephales promelas*	21	Elevated blood	Kugathas and Sumpter, 2011;

					glucose conc. in both sexes. Females: reduced Vtg levels; presence of dorsal fin black spots	Kugathas et al., 2013
Clobetasol propionate	Glucocorticoid	91	*Danio rerio*	2 - 5	Increased heart rate, hatching & decreased muscle contraction	Willi et al., 2018
E1	Estrogen	10 - 100	*Oryzias latipes*	100	Intersex (testis-ova)	Metcalfe et al., 2001
E2	Estrogen	5 - 25	*Danio rerio* (Adult male)	21	Vtg induction; modification of male sexual characteristic	Brion et al., 2004
EE2	Estrogen	0.1	*Oryzias latipes*	100	Testis-ova (intersex)	Metcalfe et al., 2001
EE2	Estrogen	0.4	*Pimephales promelas*	21	Reduced spawning	Runnalls et al., 2015
Norethindrone	Progestogen	1 - 10	*Pimephales promelas*	21	Female masculinisation (presence of dark spot)	Paulos et al., 2010
Progesterone	Progestogen	2	*Danio rerio*	2 - 6	Embryo: 4-fold induction of *pgr*, *ar*, *mr*, and *hsd17b3* genes	Zucchi et al., 2012
Gestodene	Progestogen	1	*Pimephales promelas*	21	Inhibition of spawning; female masculinisa	Runnalls et al., 2013

					tion	
Levonogestrel	Progestogen	0.5 - 25	*Pimephales promelas*	21	Reduced egg production. Masculinisation of females.	Runnalls et al., 2015; Zeilinger et al., 2009

Vtg: vitellogenin; BDP: Beclomethasone dipropionate; T: Trenbolone; LNG: Levonorgestrel; Norethindrone: NTD; GSI: Gonadosomatic index

3.3.1 An Artificial Field Study

In an ambitious and rightfully influential experiment, Kidd and his colleagues dosed an entire lake with EE2 for 3 consecutive years. They achieved an EE2 concentration of between 5 and 6 ng/L throughout those 3 years. They then assessed the effects of the EE2 on the aquatic species living naturally in the lake.

Recruitment (i.e., successful breeding) of fathead minnow failed soon after addition of the EE2, leading to a near extinction of this small species of fish from the lake (Kidd et al., 2007). The populations of both lake trout and white sucker, both larger, longer-lived species of fish, declined only slightly, although their body condition was reduced (Kidd et al., 2007), probably as a consequence of reduced food availability: both species feed on fathead minnows. In complete contrast, algal, microbial, zooplankton and benthic invertebrate (e.g., leech) communities showed absolutely no evidence of declines in abundance during the 3 years when EE2 was added to the lake (Kidd et al., 2020, 2014). Thus, there were no direct toxic effects on any of these groups of organisms. Those results support the conclusions of most of the laboratory studies that utilised invertebrates (see above), which also suggested that invertebrates are, relatively speaking, very insensitive to EE2.

Potential effects on amphibians living in and/or around the lake were also assessed. In addition, laboratory studies investigated the effects of EE2 on the same amphibian species. Effects were observed in both the laboratory and field studies, although those effects were very much less dramatic than the effects on the fish communities in the lake. No effects on sex ratios were found, although some, but not all, mink frog tadpoles exposed to EE2 in both the laboratory experiments and the dosed lake were intersex (Park and Kidd, 2005).

Overall, the results are consistent with the results of the many laboratory experiments that investigated the effects of EE2 on vertebrates (fish and amphibians) and invertebrates. However,

it should be kept in mind that the concentration of EE2 in the dosed lake (5-6 ng/L) was considerably higher than the concentration expected to be present in a typical river receiving wastewater plant effluent. Nevertheless, the whole-lake experiment investigated the ecological effects of EE2, which no laboratory experiment can do, and in doing so it demonstrated that laboratory experiments focusing only on direct effects might underestimate the true environmental impacts of steroid hormones.

3.4 Effects of Mixtures of Steroid Hormones

As summarised above, it is now clear that the aquatic environment can be simultaneously contaminated by many different steroid hormones, both natural and synthetic, of all classes of steroids. The full picture is currently not known, and may well be significantly more complex. It is clear that if we are going to understand what effects - if any, of course - environmental concentrations of steroid hormones have on aquatic organisms, then it is necessary to know how those organisms respond to complex mixtures of steroid hormones. Only in the last few years has significant effort been devoted to address this important issue.

3.4.1 Mixtures containing only one class of steroid hormones

Thorpe and colleagues were the first to investigate how fish respond to mixtures of steroid hormones. Fish were exposed to relatively simple mixtures containing only oestrogens, which showed that they acted additively in inducing vitellogenin synthesis (Thorpe et al., 2003, 2001). By collaborating with mathematicians, it was demonstrated that the model of concentration addition (C.A.) best predicted the additive effects observed. Follow-up research demonstrated that mixtures of environmental oestrogens containing both steroidal oestrogenic hormones and weakly oestrogenic industrial chemicals that are not steroids (e.g., nonylphenol) also produced additive effects that could be accurately predicted based on C.A. (Brian et al., 2005). These results were perhaps not surprising, although they are important. More recently it has been demonstrated that fairly simple mixtures of glucocorticoids act additively on gene expression in embryonic zebrafish (Willi et al., 2019), and simple mixtures of progestogens also act additively (Zucchi et al., 2014; Zhao et al., 2015; Rossier et al., 2016) on both embryos and adult fish.

3.4.2 More complex mixtures

As oestrogens and some synthetic progestogens both directly affect reproduction in all vertebrates, including fish, understandably a number of researchers have studied the effects of mixtures of these two classes of steroid hormones on reproduction of fish. Most of these studies

have utilised binary mixtures containing just one oestrogen (almost always ethinyl estradiol) and one progestogen. Various studies have focussed on gene transcription as the main endpoint (e.g., Liang et al., 2019) or the apical endpoint egg production (e.g., (Hua et al., 2016; Runnalls et al., 2015), and routinely reported additive effects. In one of these studies (Runnalls et al., 2015), mathematical modelling again demonstrated that the additive effects were best predicted by the C.A. model.

Very recent research has begun to tackle the question of how aquatic organisms respond to complex mixtures of steroid hormones containing one or more representatives of the different classes of steroid hormones. These are difficult experiments to conduct, especially if the endpoint employed is egg production (i.e., the ability to reproduce successfully), because adult, sexually mature fish are used and exposure to the steroid hormone mixtures needs to occur for extended periods of time before effects become convincing. To date there is only one report of the effects of complex mixtures of steroid hormones on the ability of fish to reproduce (Thrupp et al., 2018). This paper showed that the components of a mixture of one synthetic oestrogen, two synthetic progestogens, one synthetic androgen and one synthetic glucocorticoid acted additively in inhibiting egg production. In this case mathematical modelling showed that the model of Independent Action (I.A.) better predicted the additivity than did the model of C.A. (Thrupp et al., 2018). Using mainly gene expression as the endpoint, Fent and co-workers have shown that both embryo and adult zebrafish respond additively when exposed to mixtures of steroid hormones containing various combinations of the different classes of steroid hormone (e.g., Faltermann et al., 2020; Schmid et al., 2020; Willi et al., 2019). By analysing the expression of many genes, Fent and his co-workers have been able to determine whether or not the individual steroid hormones still produce their unique transcriptional responses when present in mixtures with steroids of other classes. For example, their studies demonstrated that in steroid hormone mixtures containing representatives of the different classes, the synthetic glucocorticoid in that mixture regulated its target genes independently of the presence of the other steroids. Put another way, the transcriptional signature of each individual steroid hormone was visible. Only very occasionally did interactive (additive) effects occur at the gene expression level. This occasional crosstalk was a consequence of a few genes, such as the vitellogenin gene, possessing genetic response elements to both oestrogens and glucocorticoids (Schmid et al., 2020a).

4.0 The environmental risk posed by steroid hormones

The first attempt at a thorough environmental risk assessment of a steroid hormone, namely EE2, was undertaken in 2008 (Caldwell et al., 2012, 2008). This included all relevant published literature in the assessment, as well as conducting an analysis of the quality of each paper. The work concluded that the predicted no-effect concentration of EE2 was 0.35 ng/L. After applying a further assessment factor of 10, to cover the possibility that different species of fish than those used in the relevant laboratory experiments may be more sensitive to EE2, they derived an environmental quality standard (EQS) of 0.035 ng/L. As discussed above, such a concentration would be exceeded in many rivers across the world. It is not difficult to understand why the European Union under the Water Framework Directive, placed EE2 (along with E2) on a "Watch List" of chemicals of potential environmental concern (EU, 2015). Subsequent research has shown that concentrations of EE2 below 0.035 ng/L do cause adverse effects to fish, but nevertheless, the suggested EQS should be fully protective (Rennalls et al., 2015).
Thorough analyses, such as those conducted by Caldwell et al., (2008, 2012), have not been undertaken on any other group of steroid hormones. Nevertheless, two reviews (Fent, 2015; Kumar et al., 2015) covering both the presence of progestogens in the aquatic environment and their effects in laboratory studies have concluded that synthetic progestogens in particular might pose a threat to wild fish. No similar analysis has been conducted as yet for any other class of steroid hormones. Thus, presently the available evidence suggest that oestrogens and progestogens are of more environmental concern than are the other groups of steroid hormones.
Probably the most important issue from the risk assessment perspective is whether or not a mixture of steroid hormones can cause effects when each individual component of the mixture is present at a concentration that, by itself, would not cause an effect. A few studies (Gómez et al., 2021; Thrupp et al., 2018; Carvalho et al., 2014) have tackled this extremely relevant and important issue. These studies above, demonstrate that if each steroid hormone in a mixture is present at a concentration that would not produce a significant effect on its own, that is, a concentration lower than the lowest observed effect concentration (LOEC), nevertheless the mixture can produce a significant adverse effect: a phenomenon now known as 'something from 'nothing''. The Thrupp et al., (2018) study shows that when each steroid hormone in a mixture is present at a concentration that would produce a small, but nevertheless measurable, effect (say between 10% and 20% of the maximum effect), the mixture produces a very pronounced effect, a phenomenon they termed 'a lot from a little'. These results provide an important warning; just

because each individual steroid hormone in a river is present at a concentration below which it would cause an effect (i.e., be a risk) does not mean that steroid hormones are not of environmental concern. If many are present simultaneously, then their mixture might be capable of causing adverse effects, and hence present an environmental risk. Given that some of the steroidal pharmaceuticals are considered to present the highest risk of any human pharmaceutical (Gunnarsson et al., 2019), even when considered individually, the possibility that the very complex mixtures of steroid hormones known to be present in probably all rivers receiving effluent from WwTPs needs to be taken seriously. However, demonstrating this convincingly (if true, of course) will be a major scientific challenge. The first step towards addressing this challenge has been recommended by Kase et al., 2018. The effect-based *in-vitro* method was demonstrated to be highly specific and sensitive in predicting the risk posed by mixtures of steroids.

The majority of mixtures studies conducted to date however, have not involved using mixtures in which all the steroid hormones were present at environmentally representative concentrations, which are generally very low (Table 5). That is primarily because the studies were intended to address the issue of whether or not steroid hormones could interact additively, thus producing effects of greater magnitude that the individual steroids, at the concentrations present in the mixture, would cause. Hence concentrations shown to cause effects in single hormone experiments were used in the experiments summarised above. Often experiments involved mixtures containing one or more steroid hormones at concentration that were environmental relevant, but other steroids in the mixture were present at concentrations above those reported to be present in the environment. It would be extremely useful for future mixture experiments to be conducted in which all the steroid hormones in the mixture were present at concentrations representative of those in a river receiving WWTPs effluent.

5.0 Reliability of Published Environmental Data

The quality (reliability) of published data is sometimes questioned for various reasons. Research data quality is often discussed in terms of its reproducibility - the extent to which similar results are obtained when a given experiment is repeated (Baker and Penny, 2016; Begley, 2013; Mebane et al., 2019). The fact is that not all published research, in any field, but possibly especially so in the sciences, is robust. Some will not be repeatable. When questioned, more than 70% of scientists were reported to be unable to reproduce work done by their peers, and over half

of the scientists were unable to always reproduce their own research (Baker and Penny, 2016; Begley, 2013). Natural systems are inherently very complex, and variability in their response to the influence of often unknown factors may result in irreproducibility of a given challenge (Mebane et al., 2019). Still, confidence in the reliability of a robust finding is enhanced when that finding can be repeated. This section of the present work is not intended to continue the 'reproducibility crisis' debate, but instead highlight certain experimental red flags that could undermine the integrity of published data in environmental chemistry and ecotoxicology.

Poor experimental designs, along with complex analytical and weak statistical methods, selective reporting, deliberate data omission and manipulation are fundamental data reliability issues affecting the quality of published scientific research (Begley, 2013; Ioannidis, 2005). It is often very difficult to fully evaluate how reliable published scientific data are. As a consequence, headline results from poor research can become quite influential, sometimes triggering national media discussions involving trade unions, NGOs and regulators, especially if the data appear to show that there is a major problem (Ellis et al., 2020).

In environmental analytical chemistry, multi-step extraction and clean-up methods, often using different equipment for final quantification, are frequently published for the same analytes. Small differences in the performance of methods can result in very different outcomes when the chemicals under investigation are present at low concentrations close to the LOD. This is certainly true for the determination of steroid hormones, frequently present in the sub ng/L range (Lundstedt et al., 2014; Vanderford et al., 2014; Yttri et al., 2015). Examples of analytical quality control checks include; (a) the use of blanks, (b) spiked samples (to assess and quantify matrix effects), (c) radioactive labelled internal standards and (d) multiple analysis of samples for statistically robust determination of method performance and robustness. A good publication reporting the concentrations of environmental pollutants should always contain both the analytical method limits of detection and quantification (LOD and LOQ). It should be noted, however, that analytical data are still being published that cannot possibly be true, even though the above quality checks are reported to have been followed (Ellis et al., 2020; Griffero et al., 2019). Amongst suggestions to improve reliability of scientific data is the call to develop standardized analytical methods robust enough to make for improved analytical testing that facilitates quality data reporting (next to zero false positives and negatives) and increase the comparability between different studies (Vanderford et al., 2014). There is a need to

independently validate reported levels of steroids hormones in aquatic environment (Ellis et al., 2020), because of potential errors associated with measuring the low concentrations present. Compared to analytical environmental chemistry, there may be more variability in the quality of ecotoxicology research. This is for at least two reasons.

1) Ecotoxicologists rarely include internal standards, such as positive controls, in their experimental design. The impact is that ecotoxicologists are often unaware of how good or bad their results are, due to them not having a baseline to compare their result against (Harris et al., 2014). Some exposure studies do not measure the concentration of the test chemical in the exposure medium (Harris and Sumpter, 2015). In such situations, there is no way of establishing if the said chemical was ever present and at what concentration (if present). Furthermore, it is often difficult to establish the repeatability of some works as only results of single experiments or a number of separate, different experiments, are reported.
2) There is a lack of standardised approaches. Much ecotoxicology research is done with 'unique' animals about which very little is known (Harris et al., 2014; Moermond et al., 2016). In addition, a wide variety of endpoints are utilised, many of which have not been well studied. Thus, baseline values are often not well established. Though the OECD provide detailed guidelines on a range of validated ecotoxicity tests, such guidelines are used mainly by industry; academic researchers rarely conduct validated tests based on OECD guidelines. Many of the limitations of the existing regulatory guidelines on how to assess the possible environmental impact of pharmaceuticals on aquatic organisms are discussed in Coors et al. (2021). Although inter-laboratory validation studies are often conducted by groups of environmental chemists, they are rarely conducted by ecotoxicologists investigating effects of chemicals.

Besides these often-fundamental problems, the reliability of ecotoxicology results is often compromised by poor experimental design (Harris et al., 2014; Harris and Sumpter, 2015). To provide just one example, quite often only a single concentration of a test chemical is used, and thus nothing is learnt about the concentration-response relationship (if effects occur), such as establishing the no-observed-effect-concentration (NOEC) or the LOEC, which are crucial values to determine if regulation is deemed appropriate. It would be extremely helpful if academic scientists collaborated with regulators, but this rarely occurs. The current situation

often leads to academic studies being of little, if any, use to regulators, as they try to determine safe environmental concentrations of chemicals, including steroid hormones.

CRediT authorship contribution statemen

Jasper Ojoghoro: Data curation, Formal Analysis, Investigation, Visualization, Writing - original draft and editing. **Mark Scrimshaw:** Data curation, Project Administration, Visualisation, Writing - review and editing. **John Sumpter:** Conceptualization, Data curation, Methodology, Project Administration, Writing - original draft and editing

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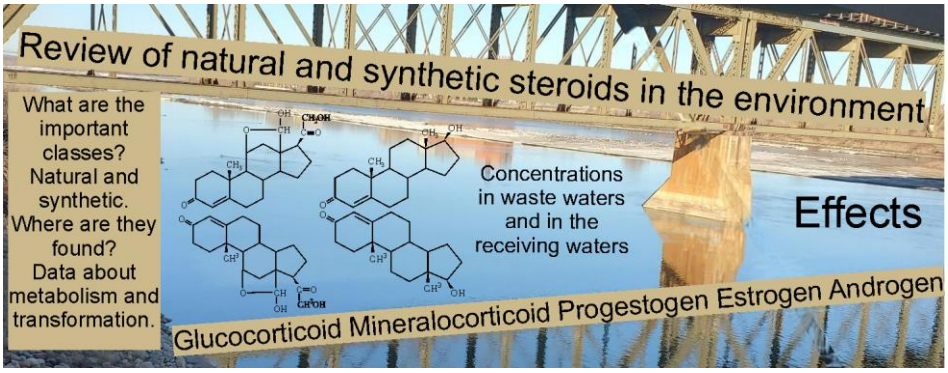
Declaration of competing interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:



Graphical abstract

HIGHLIGHTS

- A wide variety of different steroid hormones are present in surface waters across the world.
- They are usually present at very low concentrations.
- Transformation in the aquatic environment is important, but adds further complexity.
- Extremely low concentrations of some steroid hormones can cause adverse effects.
- Environmental risk assessments of steroid hormones must incorporate mixture effects.