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Trigger values for investigation of hormonal activity in drinking water and its sources using CALUX bioassays

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ABSTRACT

To screen for hormonal activity in water samples, highly sensitive *in vitro* CALUX bioassays are available which allow detection of estrogenic (ERα), androgenic (AR), progestagenic (PR), and glucocorticoid (GR) activities. This paper presents trigger values for the ERα, AR, PR, and GR CALUX bioassays for agonistic hormonal activities in (drinking) water, which define a level above which human health risk cannot be waived *a priori* and additional examination of specific endocrine activity may be warranted. The trigger values are based on 1) acceptable or tolerable daily intake (ADI/TDI) values of specific compounds, 2) pharmacokinetic factors defining their bioavailability, 3) estimations of the bioavailability of unknown compounds with equivalent hormonal activity, 4) relative endocrine potencies, and 5) physiological, and drinking water allocation factors. As a result, trigger values of 3.8 ng 17β-estradiol (E2)-equivalents (eq)/L, 11 ng dihydrotestosterone (DHT)-eq/L, 21 ng dexamethasone (DEX)-eq/L, and 333 ng Org2058-eq/L were derived. Benchmark Quotient (BQ) values were derived by dividing hormonal activity in water samples by the derived trigger using the highest concentrations detected in a recent, limited screening of Dutch water samples, and were in the order of (value) AR (0.41) > ERα (0.13) > GR (0.06) > PR (0.04). The application of trigger values derived in the present study can help to judge measured agonistic hormonal activities in water samples using the CALUX bioassays and help to decide whether further examination of specific endocrine activity followed by a subsequent safety evaluation may be warranted, or whether concentrations of such activity are of low priority with respect to health concerns in the human population. For instance, at one specific drinking water production site ERα and AR (but no GR and PR) activities were detected in drinking water, however, these levels are at least a factor 83 smaller than the respective trigger values, and therefore no human health risks are to be expected from hormonal activity in Dutch drinking water from this site.

1. Introduction

The presence of contaminants in (aquatic) environments has become a topic of much concern, especially for those compounds with hormonal activity. With respect to human health these potentially endocrine disrupting compounds (EDCs) may affect biological processes and therefore may cause a threat to human health (Guillette and Iguchi, 2012; Vandenberg et al., 2012). In addition to chemical methods which are intended to detect individual compounds, *in vitro* bioassays (also called effect-directed bioassays or bioanalytical tools) are now recognized as sensitive monitoring tools to screen for contaminants based on their biological action. As

Abbreviations: ADI, Acceptable Daily Intake; ADME, Absorption, Distribution, Metabolism, Excretion; AR, Androgen Receptor; BQ, Benchmark Quotient; DEX, Dexamethasone; DHT, Dihydrotestosterone; DMSO, Dimethylsulfoxide; E1, Estrone; E2, 17β-Estradiol; EE2, 17α-Ethinylestradiol; EDCs, Endocrine Disruptive Compounds; Eq, Equivalent; ERα, Estrogen Receptor alpha; fu_p, Fraction unbound to protein; GR, Glucocorticoid Receptor; JECFA, Joint Expert Committee on Food Additives (FAO/WHO); LOEL, Lowest Observed Effect Level; NOEL, No Observed Effect Level; Org2058, 16α-ethyl-21-hydroxy-19-norpregn-4-ene-3,20-dione; P4, Progesterone; PR, Progestogen Receptor; T, Testosterone; TDI, Tolerable Daily Intake; TTC, Threshold of Toxicological Concern.

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the specific chemical composition of a sample is often unknown, and mixture effects cannot be detected by chemical methods, *in vitro* bioassays are highly suitable tools to examine the presence of complex mixtures of low concentrations (Escher and Leusch, 2011; Leusch et al., 2010; van der Linden et al., 2008).

A number of *in vitro* bioassays have been developed for estrogenicity, including mammalian cell-, or yeast-based reporter gene assays, or proliferation assays of estrogen-responsive cells. The ERα CALUX reporter gene bioassay has good sensitivity and reproducibility (Leusch, 2008; Leusch et al., 2010), and recently other CALUX *in vitro* bioassays for the detection of endocrine activity have been developed, able to detect androgenic, glucocorticoid or progestagenic activity (Houtman et al., 2009; Leusch et al., 2010, 2012; Sonneveld et al., 2005). These recent CALUX bioassays consist of U2OS human osteosarcoma (bone cancer) cells, which are transfected with a luciferase gene under control of a specific endocrine receptor, such as the estrogen (ERα), androgen (AR), glucocorticoid (GR), and progesterone (PR) receptors (Sonneveld et al., 2005).

Chemical (compound-directed) analysis provides a method of absolute quantification of certain compounds in water samples, but the toxicological properties of these compounds are not always known (Schriks et al., 2010a). CALUX bioassays do not discriminate between different specific compounds but detect the total specific endocrine activity, and so the concentration of endocrine activity is expressed as an equivalent (eq) to a potent reference compound, *e.g.* 17β-estradiol (E2) for ERα-, dihydrotestosterone (DHT) for AR-, dexamethasone (DEX) for GR-, and Org2058 for PR-mediated activity (Sonneveld et al., 2005). Some examples of compounds with specific endocrine activity and their potencies (expressed relative to E2, DHT, DEX or Org2058) are given in Table 1. By using *in vitro* bioassays, the combined biological activities of the mixture can be quantified and expressed as ng eq of a reference compound per L. This enables unknown compounds to be detected by their activity and provides toxicological relevance (*i.e.* specific endocrine activity) of this mixture. Together with the sensitivity and robustness, these properties make the use of *in vitro* bioassays as the CALUX bioassay battery very suitable as a screening tool for endocrine activity in water samples (Escher and Leusch, 2011). Application of these CALUX bioassays demonstrated different specific hormonal activities in specific samples of waste- and/or surface water (Schriks et al., 2010b; van der Linden et al., 2008).

By using *in vitro* bioassays, the combined biological activities of the mixture can be quantified, which enables unknown compounds to be detected. Compounds causing endocrine effects are the subject to many processes after oral intake, including limited uptake and first pass metabolism (reducing absorption), binding to protein (reducing distribution), biotransformation reactions (metabolism) by *e.g.* the liver, and excretion. As these absorption, distribution, metabolism and excretion (ADME) processes are only marginally included by the CALUX bioassays, the quantitative translation of *in vitro* results from *in vitro* bioassays to the human health risks, is seriously hampered. Therefore, relative endocrine potencies determined in *in vitro* bioassays cannot directly be used to predict risks or effects in humans.

The generation of data by means of these *in vitro* bioassays requires the establishment of limits of maximum tolerable (drinking) water concentrations for hormonal activity by which the cellular responses from water samples can be judged. As limit values for drinking water are aimed at the protection of human health, such limits should be sufficiently conservative to serve as a warning signal. On the other hand, such limits should not be too conservative, to avoid unnecessary and costly additional protection measures. A trigger value of 7 ng E2-eq/L has been derived for estrogenic activity in water samples earlier (Mennes, 2004). In the present study, the method used to derive that trigger value is extended to derive trigger values for a selection of other CALUX bioassays for hormonal activity based on Acceptable or Tolerable Daily Intake (ADI or TDI) values reported for some well-known, potent endocrine compounds. These ADI/TDI reference values are combined with realistic worst-case pharmacokinetic factors representing different ADME characteristics and exposure assumptions in order to derive trigger values for hormonal activities in drinking water. The trigger values can be used to define levels below which health risks are not expected, and above which a more detailed examination of endocrine activities in water sample is warranted. Next to the derivation of these specific trigger values for the selected CALUX bioassays for agonistic hormonal

Table 1
Relative potencies of a selection of compounds measured using the U2OS-based ERα, AR, GR, and PR CALUX bioassays. The underlined values have been used for the calculations in the present study.

Compound	Relative potency	Reference(s)	Compound	Relative potency	Reference(s)
Estrogens			Glucocorticoids		
17β-estradiol (E2)	<u>1.00</u>	(Houtman et al., 2009; Sonneveld et al., 2006)	Dexamethasone (DEX)	<u>1.00</u>	(Houtman et al., 2009; Schriks et al., 2010b)
17α-ethinylestradiol (EE2)	1.86	(Houtman et al., 2009; Sonneveld et al., 2006)	Cortisol	0.08, 0.07	(Houtman et al., 2009), (Schriks et al., 2010b)
Estrone (E1)	0.02	(Houtman et al., 2009; Sonneveld et al., 2006)	Cortisone	0.00	(Houtman et al., 2009; Schriks et al., 2010b)
Estriol	0.04	(Sonneveld et al., 2006)	Prednisolone	0.09, 0.2	(Houtman et al., 2009), (Schriks et al., 2010b)
Bisphenol A	2.5×10^{-5}	(ter Veld et al., 2006)	Prednisone	0.00	(Schriks et al., 2010b)
Nonylphenol	4.6×10^{-5}	(ter Veld et al., 2006)	6α-methylprednisolone	0.4	(Schriks et al., 2010b)
Genistein	5.0×10^{-5}, 2×10^{-4}	(van der Woude et al., 2005), (Sonneveld et al., 2006)	Triamcinolone acetonide	2.3	(Schriks et al., 2010b)
Androgens			Progestagens		
Dihydrotestosterone (DHT)	<u>1.00</u>	(Houtman et al., 2009; Sonneveld et al., 2006)	Org2058	<u>1.00</u>	(Houtman et al., 2009)
Testosterone	0.21, <u>0.15</u>	(Houtman et al., 2009), (Sonneveld et al., 2006)	Progesterone (P4)	<u>0.07</u>	(Houtman et al., 2009)
Trenbolone	0.94	(Houtman et al., 2009)	Levonorgestrel	0.46	(Houtman et al., 2009)
Androstenedione	0.06	(Houtman et al., 2009)	Norethisterone	0.08	(Houtman et al., 2009)
Androstanediol	0.01	(Houtman et al., 2009; Sonneveld et al., 2006)	Medroxyprogesterone acetate	0.59	(Houtman et al., 2009)
Androstenediol	0.02	(Houtman et al., 2009)			
Androsterone	0.01	(Houtman et al., 2009)			
Epi-androsterone	0.00	(Houtman et al., 2009)			

DEX = dexamethasone; DHT = dihydrotestosterone; E2 = 17β-estradiol; eq = equivalent; Org2058 = 16a-ethyl-21-hydroxy-19nor-4-pregnene-3,20-dione.

activity in water, this paper is an illustration of the strategy of deriving trigger values for *in vitro* bioassays, the application of which could encourage development of other effect-directed bioassays and adherent trigger values (Bhattacharya et al., 2011).

2. Theory/calculation

Several steps were involved in deriving the trigger values (Fig. 1). As point of departure, recognized (*i.e.* defined by the FAO/WHO Joint Expert Committee on Food Additives (JECFA)) ADI values of specific reference compounds were chosen. To compensate for differences in uptake and first pass metabolism, the specific ADI (ng/kg bw/day) is multiplied by the estimated oral bioavailable fraction of the reference compound, *i.e.* the fraction that passes the intestinal transport barriers and that escapes first pass metabolism by the intestine and liver. Subsequently, the outcome is multiplied by the fraction unbound to plasma proteins (fu_p), yielding the internal, available concentration of the reference compound which does not elicit adverse effects to the body (internal ng ADI reference compound/kg bw/day). In the next step, the internal, available concentration of the reference compound is divided by the assumed maximum of the orally bioavailable fraction and by the maximum fu_p, estimated for other compounds with the same endocrine activity, that could potentially be present in water samples.

The estimations for bioavailability were based on values reported in literature. The estimations for fu_p values were based on literature or on calculations by the Simcyp *in silico* prediction tool fu_p (Simcyp, 2007), based on the *LogP* and *pKa* values of the compounds acquired with Chemicalize (Chemicalize, 2012), to provide a set of more uniform data. The Simcyp prediction model is parameterized using a large and diverse dataset of compounds (Lobell and Sivarajah, 2003; Turner et al., 2006) and performs more accurately than the widely used model by Austin et al. or the Hallifax–Houston model for estimating binding to plasma protein (Austin et al., 2002; Emoto et al., 2009; Hallifax and Houston, 2006). The pharmacokinetic factors considered in this document are summarized in Table 2.

The highest oral bioavailability (if sufficient information was available) and fu_p values of relevant compounds were used to estimate the safe, external equivalent dose that does not elicit effects (ng ADI reference compound-eq/kg bw/day). In case the ADI reference compound differed from the reference compound in which the specific endocrine activity is normally expressed (*i.e.* testosterone (T) instead of dihydroxytestosterone (DHT), and progesterone (P4) instead of Org2058), this was compensated by the relative potency of these compounds (Table 1). By multiplying the external equivalent dose by 60 kg bw as a default, average body weight (WHO, 2011), and dividing it by a default, average water consumption of 2 L water/day (WHO, 2011), and applying the 20% default allocation factor set for drinking water (WHO, 2011), a trigger value in equivalents of a specific hormonal activity (ng reference compound-eq/L) is derived. These trigger values can be used for the specific adherent CALUX bioassays. The derived trigger values are summarized in Table 3, and the factors used to calculate the various trigger values are explained in detail below.

2.1. Relevant endocrine compounds in water

The following compounds are regarded as relevant for their potential occurrence in water samples. The natural estrogens E2 and estrone (E1), as well as the synthetic 17α-ethinylestradiol (EE2) are considered the dominant estrogenic compounds potentially present in surface water samples (Snyder et al., 2001), as well as estriol, and the industrial compounds nonylphenol, bisphenol A, 4-*t*-octylphenol and benzyl butyl phthalate (Leusch, 2008). The natural androgens T, DHT, androstenedione, androstanediol, androstenediol, androstanedione, androsterone, and epi-androsterone can be expected to occur in environmental water samples (Liu et al., 2009, 2011; Thomas et al., 2002), although synthetic compounds as trenbolone and boldenone could also potentially be present (Liu et al., 2011, 2012). The glucocorticoids cortisol, cortisone, prednisolone, prednisone, dexamethasone (DEX), 6α-methylprednisolone, triamcinolone acetonide have been detected

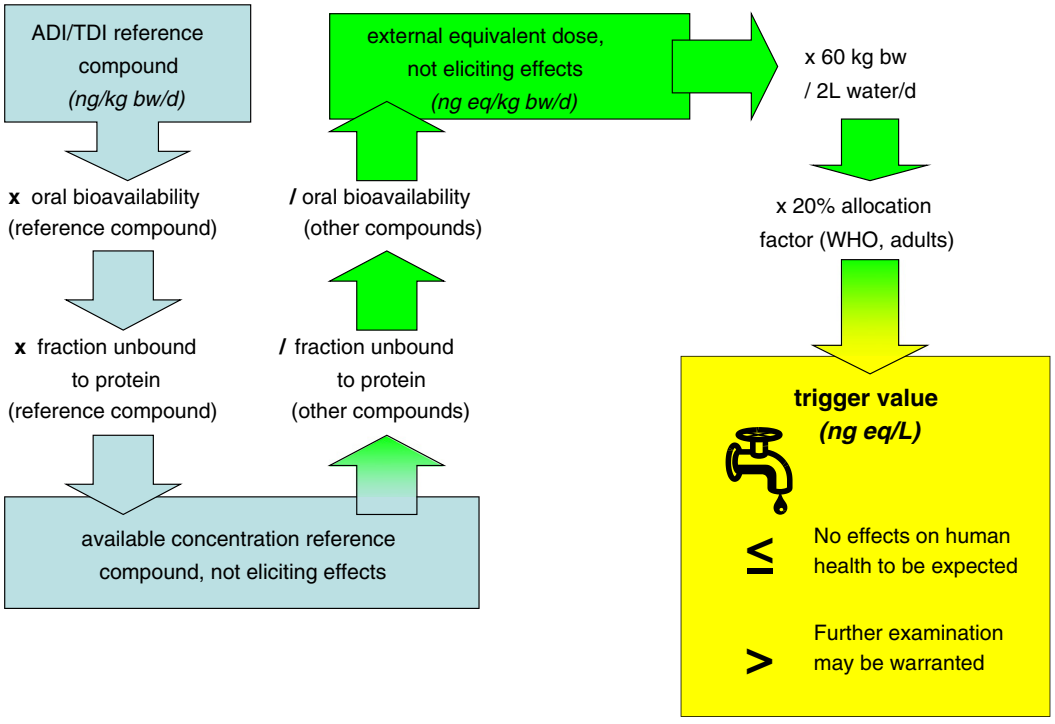

Fig. 1. Flow diagram of deriving trigger values for additional investigation for hormonal activities in water from acceptable or tolerable daily intake (ADI or TDI) values of reference compounds, as conducted in the present study.

Table 2
Pharmacokinetic factors of endocrine compounds. The underlined values have been used for the calculations in the present study.

Compound	CAS number	Bioavailability fraction	fu_p [a]	Reference(s)
Estrogens				
17β-estradiol (E2)	50-28-2	~5%	2% (5.9%)	(Dunn et al., 1981; Fotherby, 1996; Kuhnz et al., 1993; O'Connell, 1995)
Ethinylestradiol (EE2)	57-63-6	~50%	2% (5.1%)	(Back et al., 1979; Back et al., 1981; Back et al., 1987; Düsterberg et al., 1986; Fotherby, 1996; Grimmer et al., 1986; Hammond et al., 1982; Orme, 1982)
Estrone (E1)	53-16-7	~2–10%	4% (3.4%)	(Dunn et al., 1981; O'Connell, 1995)
Estriol	50-27-1	~10%	8–14% (16%)	(Dunn et al., 1981; Head, 1998; Moutsatsou and Oakey, 1988)
Bisphenol A	80-05-7		5% (4.4%)	(Csanady et al., 2002)
Nonylphenol (4-*n*-)	104-40-5		(0.8%)	
4-*t*-octylphenol	140-66-9		(2.3%)	
Benzyl butyl phthalate	85-68-7		(1.6%)	
Genistein	446-72-0		(3.4%)	
Androgens				
Testosterone (T)	58-22-0	~3.5%	2% (8.5%)	(Dunn et al., 1981; Södergård et al., 1982; Täuber et al., 1986)
Dihydrotestosterone (DHT)	521-18-6		1% (8.2%)	(Dunn et al., 1981)
Androstenedione	63-05-8		8% (4.9%)	(Dunn et al., 1981)
Androstanediol	1852-53-5		(3.9%)	
Androstenediol	2-75-8		3% (14.3%)	(Dunn et al., 1981)
Androstanedione	2-27-2		(4.7%)	
Androsterone	53-41-8		4% (5.8%)	(Dunn et al., 1981)
Epi-androsterone	481-29-8		(5.8%)	
Trenbolone	10161-33-8		(22.7%)	
Boldenone	846-48-0		(8.6%)	
Glucocorticoids				
Cortisol	50-23-7		4% (44.5%)	(Dunn et al., 1981)
Cortisone	53-06-5		16% (35.1%)	(Dunn et al., 1981)
Prednisolone	50-24-8	~78–85%	~20% (44.8%)	(Bergrem et al., 1983; Rose et al., 1981)
Prednisone	53-03-2			
Dexamethasone (DEX)	50-02-2	~70%	~23% (34.7%)	(Duggan et al., 1975; O'Sullivan et al., 1997; Rose et al., 1981; Sweetman, 2009)
6α-methylprednisolone	83-43-2		(37.5%)	
Triamcinolone acetamide	124-94-7		(70.1%)	
Progestagens				
Progesterone (P4)	57-83-0	<10%	2.5% (4%)	(Dunn et al., 1981; Fotherby, 1996; Simon et al., 1993)
Norethisterone	68-22-4	~60%	(9.8%)	(Back et al., 1978; Fotherby, 1996)
Levonorgestrel	797-63-7	~94%	2% (6.4%)	(Back et al., 1981; Back et al., 1987; Fotherby, 1996; Grimmer et al., 1986; Humpel et al., 1978)
Medroxyprogesterone	520-85-4	<15%	(6.2%)	(Fotherby, 1996)

[a] Fraction unbound to protein (fu_p) values reported in literature, or estimated using the Simcyp prediction tool (between parenthesis).

in surface or waste water (Chang et al., 2007; Liu et al., 2011; Schriks et al., 2010b), and could therefore potentially be present in surface water. The same can be expected for the progestagenic compounds P4, norethisterone, levonorgestrel, and medroxyprogesterone (Besse and Garric, 2009; Tolgyesi et al., 2010).

2.2. Acceptable Daily Intake (ADI) values

The JECFA has derived an ADI of 50 ng/kg BW/day for E2, because it is used as oestrus regulator in cattle and consequently may be present in (increased) quantities in food (FAO/WHO, 2000). This ADI was based on E2-induced hormone-dependent changes in 23 post-menopausal women, who have low endogenous E2 production. Such changes did not occur at a dose level of 0.3 mg/day E2 (equivalent to a NOEL of 5 μg/kg BW/day), which did not relieve symptoms of menopause (FAO/WHO, 2000). The derived ADI of 50 ng/kg BW/day for E2 by the JECFA was chosen as the point of departure for the trigger value for estrogenic activity, as E2 is considered one of the two dominant environmental estrogens, together with EE2 (Snyder et al., 2001). The JECFA did not derive ADI/TDI values for EE2 and E1.

Several compounds can contribute to the androgenic activity in environmental samples, including DHT and T. For the AR reference compound DHT no ADI has been reported, however, for T the JECFA did derive an ADI of 2 μg/kg BW/day (FAO/WHO, 2000). Testosterone is allowed to be used as growth regulator in cattle in Canada, the USA and Australia, and as a result may be present in (increased) quantities in food. In a trial involving five male patients having eunuchoidism, resulting in very low testosterone levels and impaired sexual functioning,

Table 3
Trigger values for estrogenic (ERα), androgenic (AR), progestagenic (PR), and glucocorticoid (GR) activities in drinking water, ADIs and pharmacokinetic factors used to derive them.

	ADI/TDI reference (μg/kg bw/day)[a]	Safety factor ADI/TDI[a]	Bioavailability reference	fu_p reference	Bioavailability other compounds	fu_p other compounds	External equivalent dose	Trigger value
ERα	0.050 (E2)	100	5%	2%	50%	16%	0.625 ng E2-eq/kg bw/day	3.8 ng E2-eq/L
AR	2 (T)	1000	3.5%	2%	50%	23%	12.17 ng T-eq/kg bw/day = 1.826 ng DHT-eq/kg bw/day	11 ng DHT-eq/L
GR	0.015 (DEX)	100	70%	23%	100%	70%	3.450 ng DEX-eq/kg bw/day	21 ng DEX-eq/L
PR	30 (P4)	100	10%	2.5%	100%	10%	750 ng P4-eq/kg bw/day = 55.5 ng Org2058-eq/kg bw/day	333 ng Org2058-eq/L

[a] Acceptable Daily Intake (ADI) values reported by the JECFA (FAO/WHO, 1995, 2000). DEX = dexamethasone; DHT = dihydrotestosterone; E2 = 17β-estradiol; eq = equivalent; Org2058 = 16a-ethyl-21-hydroxy-19nor-4-pregnene-3,20-dione, P4 = progesterone, T = testosterone.

an oral dose of 100 mg T/day had no effect on sexual functioning, while an oral dose of 400 mg T/day was effective in restoring full sexual function (Johnsen et al., 1974). Therefore, an oral dose of 100 mg T/day (equivalent to 1.7 mg/kg BW/day) was used as NOEL in the calculation of the ADI of 2 μg/kg BW/day, which was used as point of departure for deriving the trigger value for androgenic activity.

Several natural and synthetic glucocorticoids have been detected in environmental water samples including the GR reference compound DEX. For DEX the JECFA has derived an ADI of 15 ng/kg bw/day because this substance dose-dependently increases tyrosine amino transferase activity in the supernatant of rat liver homogenates (FAO/WHO, 1995). This ADI was used as point of departure for deriving the trigger value for glucocorticoid activity.

The natural P4, and the synthetic norethisterone and levonorgestrel are among the most relevant progestagenic compounds in environmental samples (Besse and Garric, 2009; Tolgyesi et al., 2010). For P4, the JECFA derived an ADI of 30 μg/kg bw/day because a single oral dose of 200 mg of fine-particle progesterone (equivalent to 3.3 mg/kg bw) to women provides blood concentrations similar to those found during the luteal phase of the ovulatory cycle (FAO/WHO, 2000). This dose was considered to be the LOEL and used in the calculation of the ADI for progesterone, which was used as a point of departure for deriving the trigger value for progestagenic activity.

2.3. Pharmacokinetic factors and external equivalent dose

For E2 it is necessary to take into account that it is well absorbed from the gut, but that most of an oral dose (~95%) does not reach the circulation due to first pass metabolism in the liver (Fotherby, 1996; Kuhnz et al., 1993; O'Connell, 1995). The estimated oral bioavailability factor therefore is 5% (Table 2). Only ~2% of this remaining 5% E2 in the blood is not bound to plasma proteins (Dunn et al., 1981; Fotherby, 1996; Kuhnz et al., 1993; O'Connell, 1995), and therefore available to elicit a biological response. Synthetic estrogens can be highly bioavailable. EE2, is also well absorbed but less affected by first pass metabolism, resulting in an estimated oral bioavailability fraction of ~50% (Düsterberg et al., 1986; Fotherby, 1996; Orme, 1982). However, EE2 is also extensively bound to plasma protein, similar as E2 (Hammond et al., 1982). As estimation for other estrogenic compounds which could potentially occur in (drinking) water, a maximum oral bioavailability fraction of 50% is set, assuming that no estrogenic compounds have a higher bioavailability compared to EE2. Among the relevant estrogenic compounds, estriol is least bound to plasma protein with a fu_p up to 14% reported in literature (Moutsatsou and Oakey, 1988), and a Simcyp fu_p estimation of 16% (Table 2). The latter, highest value is taken as maximum fu_p for all the estrogenic compounds potentially present in water.

T is well absorbed but has a low oral bioavailability of ~3.5% due to first pass metabolism (Täuber et al., 1986), and ~98% of the remaining testosterone in the blood is bound to plasma proteins and unavailable to produce a biological response (Dunn et al., 1981; Södergård et al., 1982). The estimated oral bioavailability factor therefore is 3.5%, of which only 2% is not bound to plasma proteins (Table 2). As little information is available on the oral bioavailability of the other androgenic compounds potentially present in water including synthetic androgens, 50% is set as the maximum oral bioavailability fraction for androgens, assuming that these compounds do not have a higher oral bioavailability than the structurally similar estrogens. According to the Simcyp fu_p estimation, trenbolone is the androgenic compound least bound to plasma protein (Table 2), and therefore the estimated 23% is assumed as maximum fu_p for all the androgenic compounds present in water.

The glucocorticoid DEX is well absorbed, with little first-pass metabolism and with ~70% reaching the circulation, of which ~77% is bound to plasma protein (Duggan et al., 1975; O'Sullivan et al., 1997; Rose et al., 1981; Sweetman, 2009). The estimated oral bioavailability factor therefore is 70%, of which 23% is not bound to plasma proteins (Table 2). As glucocorticoids can be highly bioavailable, *e.g.* ~85% for prednisolone (Bergrem et al., 1983; Rose et al., 1981), but little information is available on the bioavailability of other relevant, synthetic glucocorticoid compounds, a conservative maximum bioavailability fraction of 100% is assumed for other glucocorticoids which could potentially occur in (drinking) water. According to the Simcyp fu_p estimation triamcinolone acetamide is the glucocorticoid estimated to be the least bound to plasma proteins (Table 2), and therefore 70% is assumed as maximum fu_p for all the glucocorticoid compounds present in water.

P4 has an oral bioavailability of less than 10% as it is deactivated in the gastrointestinal tract and metabolized due to first pass metabolism (Fotherby, 1996; Simon et al., 1993). Subsequently ~98.5% is bound to plasma proteins in the circulation (Dunn et al., 1981; Fotherby, 1996). The estimated oral bioavailability factor of P4 therefore is 10%, of which only 2.5% is not bound to plasma proteins (Table 2). As other progestagenic compounds can be highly bioavailable, *i.e.* ~94% for levonorgestrel (Back et al., 1981, 1987; Fotherby, 1996; Grimmer et al., 1986; Humpel et al., 1978), a conservative maximum bioavailability of 100% is assumed for other progestagens which could potentially occur in water. According to the Simcyp fu_p estimation norethisterone is the progestagenic compound estimated with the lowest plasma protein binding (Table 3), and therefore 10% is assumed as maximum fu_p for the total progestagenic compounds present in water.

2.4. Relative potencies

Because the androgenic and progestagenic activities are usually expressed equivalent to other reference compounds than the compounds of which the ADIs were used (*i.e.* DHT instead of T, and Org2058 instead of P4), the external equivalent doses for androgenic and progestagenic activities need to be multiplied by the relative potencies (Table 1). For testosterone the lowest reported relative potency (0.15) was chosen (Sonneveld et al., 2006).

2.5. Physiological, and drinking water allocation factors

As shown in Fig. 1, by multiplying the external equivalent dose by an average bw of 60 kg and dividing it by 2 L water/day as an average water consumption as defaults (WHO, 2011), and applying the default 20% allocation factor set for drinking water (WHO, 2011), a drinking water concentration in equivalents of a specific hormonal activity (ng reference compound-eq/L) is derived (Table 3).

2.6. Trigger values

As an illustration, the calculation of the trigger value for estrogenic activity is shown in Eq. (1) below:

$$\text{Trigger value} = 1000 \times \frac{BW \times Af}{V} \times RP \times \frac{ADI_{E2} \times fa_{E2} \times fu_{PE2}}{fa_x \times fu_{Px}} \text{ng E2}-\text{eq/L} \quad (1)$$

in which:

BW	Default adult body weight (60 kg)
Af	Default drinking water allocation factor (0.2)
V	Default adult daily drinking water consumption (2 L/d)
ADI_{E2}	ADI of E2 (0.050 μg/kg bw/d)
fa_{E2}	Fraction absorbed of E2 (0.05)
fu_{PE2}	Fraction unbound to protein of E2 (0.02)
fa_x	Fraction absorbed of unknown estrogenic substance in water samples (0.5)

fu_{Px} Fraction unbound to protein for unknown estrogenic substance in water samples (0.16)

RP Relative potency of ADI reference substance compared to the CALUX bioassay reference substance (for E2 this is equal to 1).

2.7. Measured activities in water samples

As a case study, the battery of ERα, AR, GR and PR CALUX bioassays was used to screen drinking water and surface water samples, as well as water samples taken at different stages of drinking water production from ground or surface water at different sites, according to methods reported earlier (van der Linden et al., 2008). Samples were taken and screened from 3 different sites: S1, S2 (both during April 2010) and S3 (during November 2011). S1 is a public supply well field with former landfill, industrial area and a military camp within the recharge area, S2 is a public supply well field with a former landfill and urban area (small industries, sewages) within the recharge area, and S3 is an artificial recharge site, at which the infiltrated water is a (pretreated) mixture of water from the river Rhine and the river Meuse. In addition we also included the activities measured in drinking and surface water as reported by van der Linden et al. (2008) in the data reported.

2.8. Benchmark Quotient (BQ) values and detection limits

Concentrations of endocrine activities in water can be expressed as a BQ value (concentration in water divided by a provisional guideline value, *i.e.* the derived trigger value) (de Jongh et al., 2012; Schriks et al., 2010a). Conservatively, at a BQ value of ≥1 in drinking water, a potential human health concern cannot be waived if the water was to be consumed over a lifetime period.

The standard detection limits for the CALUX bioassays using standard protocol for analyzing water samples are 0.01 ng E2-eq/L for the ERα CALUX bioassay, 0.081 ng DHT-eq/L for the AR CALUX bioassay, 4.1 ng DEX-eq/L for the GR CALUX bioassay, and 0.34 ng Org2058-eq/L for the PR CALUX bioassay. In this standard method the content of 1 L of water sample is concentrated into 50 μL dimethylsulfoxide (DMSO), to which the cells of the CALUX bioassay are exposed in a final concentration of 0.1%. Thus, the cells are exposed to a 20-fold higher concentration of the compounds than present in the water itself. By concentrating more water into less DMSO and exposing the cells to higher concentrations the detection limit of the CALUX bioassay can be easily improved 4 to 16-fold.

3. Results

By using Eq. (1), a trigger value for estrogenic activity of 3.8 ng E2-eq/L was calculated. The trigger values for the other hormonal activities were derived in a similar manner (Table 3). For instance, for androgenic activity a trigger value of 11 ng DHT-eq/L can be derived taking into account a relative potency factor of 0.15 to correct for the higher potency of DHT compared to the ADI reference substance T; for glucocorticoid activity a trigger value of 21 ng DEX-eq/L can be derived; and for progestagenic activity a trigger value of 333 ng Org2058-eq/L, taking into account a relative potency factor of 0.07 to correct for the higher potency of Org2058 compared to the ADI reference substance P4. In a similar manner, the trigger values can be translated into other references compounds, *e.g.* the trigger value of 333 ng Org2058-eq/L for progestagenic activity can be translated into 724 ng levonorgestrel-eq/L, taking into account the relative potency of levonorgestrel compared to Org2058 of 0.46 (Table 1).

Although based on the ADI of one reference compound, these trigger values apply to the mixture of specific agonistic hormonal activities, assuming doses addition. Any synergistic effects of individual compounds have not been taken into account, but are not to be

Table 4
Ranges of benchmark quotient (BQ) values (measured activity divided by trigger value) and levels of hormonal activity (in parentheses) detected at different sample sites (S). For a more detailed description of the sample sites see Section 2.7. Note the reported activities and subsequent BQ values are not corrected for sample preparation recoveries.

Sample site/study	BQ-values ERα CALUX (ng E2-eq/L)	BQ-values AR CALUX (ng DHT-eq/L)	BQ-value GR CALUX (ng DEX-eq/L)	BQ-value PR CALUX (ng Org2058-eq/L)
S1				
Monitoring wells	0.002–0.283 (0.008–1.074)	<0.010–0.023 (<LOD[b] –0.25)	<0.10 (<LOD[c])	<0.0002 (<LOD[d])
Pumping wells	0.003–0.021 (0.012–0.079)	<0.010–0.012 (<LOD[b] –0.13)	<0.10 (<LOD[c])	<0.0002 (<LOD[d])
Drinking water	0.006 (0.022)	0.012 (0.13)	<0.10 (<LOD[c])	<0.0002 (<LOD[d])
S2				
Monitoring wells	0.016–0.055 (0.060–0.186)	<0.010 (<LOD[b])	<0.10 (<LOD[c])	<0.0002 (<LOD[d])
Pumping wells	0.007–0.010 (0.027–0.037)	<0.010 (<LOD[b])	<0.10 (<LOD[c])	<0.0002 (<LOD[d])
Drinking water	0.008 (0.032)	<0.010 (<LOD[b])	<0.10 (<LOD[c])	<0.0002 (<LOD[d])
S3				
Infiltration pond	0.031 (0.118)	<0.007 (<LOD[f])	<0.20 (<LOD[g])	<0.001 (<LOD[h])
Monitoring wells	0.007[i]–0.020 (0.028[i]–0.077)	<0.007 (<LOD[f])	<0.20 (<LOD[g])	<0.001 (<LOD[h])
Raw water before treatment	0.014 (0.054)	<0.007 (<LOD[f])	<0.20 (<LOD[g])	<0.001 (<LOD[h])
Horizontal well	0.009[i] (0.035[i])	<0.007 (<LOD[f])	<0.20 (<LOD[g])	<0.001 (<LOD[h])
Drinking water	<0.003 (<LOD[e])	<0.007 (<LOD[f])	<0.20 (<LOD[g])	<0.001 (<LOD[h])
van der Linden et al. (2008)				
Surface water	0.047–0.132 (0.18–0.50)	<0.010–0.409 (<LOD[b]–4.5)	0.019–0.062 (0.39–1.3)	<0.0002–0.036 (<LOD[d]–12)
Drinking water	<0.004 (<LOD[a])	<0.010 (<LOD[b])	<0.10 (<LOD[c])	<0.0002 (<LOD[d])

[a] LOD = 0.014 ng E2-eq/L.
[b] LOD = 0.11 ng DHT-eq/L.
[c] LOD = 2.0 ng DEX-eq/L.
[d] LOD = 0.051 ng Org2058-eq/L.
[e] LOD = 0.010 ng E2-eq/L.
[f] LOD = 0.081 ng DHT-eq/L.
[g] LOD = 4.1 ng DEX-eq/L.
[h] LOD = 0.34 ng Org2058-eq/L.
[i] Below limit of quantification, estimate value is provided.

expected at the low levels as detected in drinking water (Pieters and Könemann, 1997).

In the study by van der Linden et al. (2008) levels up to 0.50 ng E2-eq/L, 12 ng DHT-eq/L, 1.3 ng DEX-eq/L and 4.5 ng Org2058-eq/L were detected in specific surface (brook or river) water samples (van der Linden et al., 2008), resulting in BQ values of 0.13, 0.06, 0.41, and 0.04, respectively (Table 4).

In the final drinking water produced at S1, 0.022 ng E2-eq/L, and at S2 0.032 ng E2-eq/L, were detected, resulting in BQ values of 0.006 and 0.008, respectively. No estrogenic activity was detected in the drinking water produced at S3 (Table 4). Interestingly, at S2 androgenic activity was detected at a specific monitoring and at a specific pumping well (Table 4). In the drinking water produced at S2, also 0.13 ng DHT-eq/L was detected, resulting in a BQ value of 0.012. In the samples taken at S1, S2 and S3 no glucocorticoid and progestagenic activities were detected (Table 4). The levels of endocrine activity measured in drinking water are at least 83 times smaller than the respective trigger values, and therefore there is no reason for concern with respect to human health.

4. Discussion

In the present paper, trigger values for endocrine activity in drinking water were derived, based on acceptable or tolerable daily intake (ADI) values for some well-known endocrine reference compounds, combined with pharmacokinetic factors representing the ADME characteristics for absorption and distribution as well as exposure assumptions. These trigger values can be used to judge whether endocrine activity in water samples, as determined *e.g.* with the ERα, AR, GR and PR CALUX bioassays, are of no concern, or whether they raise a need for additional examination of specific endocrine activity to decide in more detail if the respective activities would raise a concern for human health. Thus, exposure to a level of endocrine activity above the trigger values does not necessarily mean that a health effect is to be expected, as the chance that adverse health effects would occur at exposures to levels of endocrine activity below the respective trigger values is at least very little, if not negligible. When water samples contain endocrine active substances at levels above the trigger values, an additional investigation focused on identification of the substances responsible for the activity is recommended, which could ultimately result in a full risk assessment. As an example, in the present paper the trigger values were compared with data obtained with the CALUX bioassays (Table 4). As the trigger values are directly derived from the ADIs of the reference compounds, they can be used with other bioassays for these agonistic hormonal activities as well, although the sensitivity of many *in vitro* bioassays is much lower, or even too low.

In general no endocrine activity is detected in Dutch drinking water, although very low estrogenic and androgenic activities in water from specific drinking water production sites have been reported in the present study (Table 4). Even the highest concentrations of hormonal activities found in the surface, ground and raw water samples in this limited study are presumed to present no appreciable concern to human health if these were detected in drinking water, according to the BQ-values (Schriks et al., 2010a).

The point of departure for deriving a trigger value is the ADI (or TDI) value of a specific reference compound, the quality and validity of which therefore is of great impact. We have chosen to use uniformal, recognized ADIs (*i.e.* all defined by the JECFA) of reference compounds relevant for their contribution to hormonal activity in surface water, for deriving the trigger values for total endocrine activity in (drinking) water samples. However, in addition to the safety factor of 100, the ADI for T contains an extra safety factor of 10 (Table 3), because of the limited number of subjects in the study (FAO/WHO, 2000).

As ADIs already contain safety and uncertainty assumptions, including for example inter-individual differences, realistic predictions for absorption and protein binding were used rather than worst-case assumptions for these ADME factors whenever possible (although the bioavailability of glucocorticoid and progestagenic compounds was set at 100% as explained in Section 2.3). With respect to the exposure assumptions, one can envisage that smaller body weights relative to water consumption, as well as differences in the susceptibility of developmental stages during embryogenesis and further postnatal development, can result in an increased susceptibility to endocrine disrupting compounds (Vandenberg et al., 2012). With respect to the body weight and water consumption, other values could be used, *e.g.* 1 L water consumption per 10 kg bw for children and 0.75 L water consumption per 5 kg bw for bottle-fed infants (WHO, 2011). These values would lower the trigger values by a factor of 3.3 or 4.9, respectively. However, for deriving the trigger values we have assumed that these differences are covered by the uncertainty factor of 10 for inter-individual differences that is used to derive an ADI. Additionally, these higher exposures only last for a short time, not a whole lifetime, while the ADI is derived from studies with near to lifetime exposures. As the consumption of 2 L water per day covers also the consumption of water after the heating or boiling of foods and liquids, possibly part of the endocrine compounds present in water can be degraded, although these compounds are generally regarded to possess high thermal stability (Bowden et al., 2009).

By using effect-directed bioassays, the combined biological activities of the agonists (or antagonists) in a mixture can be quantified. The application of CALUX bioassays with the trigger values derived in the present paper regards only agonistic activity for these hormonal endpoints. In the study by van der Linden et al. (2008), no antagonistic endocrine activities were detected, but in other studies, anti-androgenic activity was measured at high levels (Leusch et al., 2012). Many estrogenic compounds express also anti-androgenic activity, which likely explains most of the anti-androgenic activity in certain water samples (Jobling et al., 2009; Leusch et al., 2012; Weiss et al., 2009). With respect to the different specific endocrine activities, in mixtures this is further complicated by the fact that also progestagenic compounds may express anti-androgenic and/or estrogenic activity, as well as that a measured effect in one of the CALUX bioassays can be a sum of agonistic and antagonistic activity (Besse and Garric, 2009). Although the role of antagonistic activity is important (Jobling et al., 2009), the trigger values derived in the present paper are only meant for total added agonist activity.

One assumption that is made, is that metabolism is a process yielding deactivated or less active metabolites, whereas examples are known of metabolites that have higher estrogenic activity than parent compounds and of compounds which are bioactivated by metabolism (Legler et al., 2002; Sotoca et al., 2010). In addition, differences between compounds with respect to the ADME factors metabolism and excretion were not taken into account as well, which could be a topic of future refinement; currently we are performing studies to investigate and quantify the role of metabolism on the *in vitro–in vivo* correlation of estrogenic effects (Punt et al., 2013). Another point of consideration is the potential loss of bioactive compounds during sample preparation. Firstly, the reproducibility of the sample preparation method, and secondly, the recovery of the applied sample preparation procedure should be taken into account with the use of the CALUX bioassay trigger values. As an illustration of the latter, the average sample extraction recovery percentages by an ethylacetate extraction method were determined for specific compounds by van der Linden et al. (2008). For the specific estrogenic and androgenic compounds these recoveries were acceptable, ranging from 94% (estrone) to 120% (EE2), and from 88% (testosterone, trenbolene) to 92% (DHT, testosterone, trenbolene). However, with recovery percentages ranging from 68% (prednisolone) to 89% (DEX) for glucocorticoid activity, or even from 40% (progesterone) to 89% (levonorgestrel)

Table 5
Indication of the relevance of endocrine activity in drinking water or surface water compared to other sources.

Endocrine activity	Other selected sources of exposure	Relevance of uptake via surface water[a]	Relevance of uptake via drinking water[a]	Selected references for endocrine activity in other sources
Estrogenic	Milk, dairy, soy products, vegetables and other plant-derived food-stuffs, meat, eggs, pharmaceuticals	Negligible	Negligible	(Behr et al., 2011; Malekinejad et al., 2006)
Androgenic	Meat	Relevant	Negligible	(Kootstra et al., 2004; Stephany, 2010)
Glucocorticoid	Milk, dairy, meat, eggs, pharmaceuticals	Low	Negligible	(Bovee et al., 2011)
Progestagenic	Milk, dairy, meat, eggs	Negligible	Negligible	(Waldmann et al., 1999)

[a] Amount of endocrine activity in 2 L water divided by an estimation of the amount of exposure to endocrine activity via other sources (amount in water divided by the amount in other sources corrected for assumptions for average daily consumption): relevant = ~1–0.1 fold, low = ~0.1–0.01 fold, very low = ~0.01–0.001 fold, negligible = ~<0.001 fold.

for progestagenic activity (van der Linden et al., 2008), using the measured levels directly to compare to the trigger values could therefore underestimate the presence of compounds with such activity. This also applies to the endocrine activities in the occurrence data as reported in the present paper. As different sample preparation methods are applied, the recoveries were not included in the derived trigger values. It would be of interest to study whether a generic correction for the loss of a specific activity during sample preparation could be applied, *e.g.* the lowest recovery of a compound from a selection of representative compounds with a certain method. This would be a relevant topic for further studies.

Recently Mons et al. (2013) set drinking water target values for individual steroid endocrine chemicals at 10 ng/L, using the threshold of toxicological concern (TTC) approach (Mons et al., 2013). The TTC is a risk assessment tool for evaluating substances with little or no toxicity data and which have an extremely low level of exposure, and is based on the concept that an exposure threshold value can be established below which a very low probability of an appreciable risk to human health exists (Hennes, 2012). The TTC can be used as a first pragmatic approach, also to base trigger values for *in vitro* bioassays upon. This is done by applying a high potency reference compounds for the respective bioassay at TTC level. Effects as measured in an unidentified mixture should not exceed this level. However, this does not take different ADME characteristics between different compounds in the mixture into account. In addition, the European Food Safety Authority (EFSA) excludes endocrine-active compounds from the TTC approach (EFSA, 2012). Therefore, we prefer the more data intensive use of trigger values for CALUX bioassays as these differentiate between different classes of hormones, these are based on specific toxicity data of these hormones, and these take ADME characteristics into account. It should be noticed, however, that the trigger values derived for ERα, AR and GR activity in the current study differ less than 3-fold compared to the TTC approach based target value of 10 ng/L for individual steroid endocrine chemicals. Only the trigger value for PR activity is 33-fold higher than this TTC approach based target values.

The default WHO allocation factor of 20% for drinking water, which is used in the present study for deriving the trigger values, assumes 80% of the total exposure to endocrine compounds to be contributed via other sources than drinking water. This allocation factor, however, could have been less conservative, since the exposure to endocrine activity via other sources than drinking water is evident (Table 5). For instance, the diet can contain high amounts of estrogenic compounds: natural hormones in milk, dairy products and meat, as well as phytoestrogens in soy products, vegetables, and other plant derived-products (Caldwell et al., 2010; Leusch et al., 2009; Wise et al., 2011). According to an inventory by Leusch et al. (2009) the exposure to estrogenic activity is 600 to 1390 times higher via other sources than surface water containing 1 to 16 ng E2-eq/L (Caldwell et al., 2010; Leusch et al., 2009). In addition to dietary exposure, people can also be exposed to high amounts of endocrine activity via drugs, such as estrogenic compounds in contraceptives or to glucocorticoids which are frequently prescribed and applied against numerous pathologies (Table 5). Although the type of compounds with endocrine activity can differ, also with respect to the pharmacokinetic factors, even if trace amounts of compounds with estrogenic, glucocorticoid and progestagenic activity are ingested via drinking water, this appears unlikely to be a significant route of exposure compared with other, dietary sources. Thus, one can argue about the relevance of analyzing endocrine activity in drinking water. However, the possible presence of chemical contaminants in drinking water is a major concern for citizens (Novak et al., 2011; Schwarzenbach et al., 2006), and compounds with endocrine activity do not belong in impeccable drinking water, justifying monitoring for the presence of these compounds and the efforts made to remove these compounds.

5. Conclusions

1) Trigger values of 3.8 ng 17β-estradiol-equivalents/L, 11 ng dihydrotestosterone-equivalents/L, 21 ng dexamethasone-equivalents/L, and 333 ng Org2058-equivalents/L were derived for estrogenic (ERα), androgenic (AR), glucocorticoid (GR) and progestagenic (PR) activity in drinking water, respectively.
2) These trigger values can be used with the ERα, AR, GR and PR CALUX bioassays, as well as with other suitable bioassays, taking the sample preparation recoveries of the specific bioassays into account.
3) Based on the highest concentrations detected in surface water in a limited screening study of Dutch water samples, the order of Benchmark Quotient (BQ) values (concentration in water divided by the derived trigger value) was AR (0.41) > ERα (0.13) > GR (0.06) > PR (0.04), showing that no health risks are to be expected if consumed as drinking water.
4) At a specific drinking water production site ERα and AR (but no GR and PR) activities were detected in drinking water, however, these levels are at least 83 times smaller than the respective trigger values, and therefore no human health risks are to be expected from hormonal activity in Dutch drinking water.
5) Trigger values for drinking water are useful screening tools to decide whether examination of specific endocrine activity may be warranted, followed by a subsequent safety evaluation, or whether concentrations of such activity are of low priority with respect to health concerns in the human population.

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