Detection of Multiple Hormonal Activities in Wastewater Effluents and Surface Water, Using a Panel of Steroid Receptor CALUX Bioassays

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It is generally known that there are compounds present in the aquatic environment that can disturb endocrine processes, for example via interaction with the endogenous hormone receptors. Most research so far has focused on compounds that bind to the estrogen and/or androgen receptor, but ligands for other hormone receptors might also be present. In this study, a newly completed panel of human cell derived CALUX reporter gene bioassays was utilized to test water extracts for estrogen (ER), as well as androgen (AR), progesterone (PR), and glucocorticoid (GR) receptor mediated transactivation activity. Effluents from industry, hospital, and municipal sewage treatment plants, as well as tap water and different sources of surface water were tested. The CALUX reporter gene panel showed high sensitivity and specificity to known agonists, enabling discrimination between different receptor based endocrine responses present in the aquatic environment. Our results clearly showed the presence of agonistic activity on the ER, as well as on the AR, PR, and GR in the raw and wastewater and surface water extracts. However, no hormone receptor-mediated transactivation was detected in the drinking water or in the blank water. The levels of estrogenic activity were 0.2−0.5 ng E2-equiv/L for surface water and 0.4−1.0 ng E2-equiv/L for municipal effluents, which was consistent with previous studies. Surprisingly, the other hormonal activities were found to be present in similar or much higher levels. Most notably, glucocorticoid-like activity was detected in all samples, at surprisingly high levels ranging from 0.39−1.3 ng Dex-equiv/L in surface water and 11−243 ng Dex-equiv/L in effluents. When regarding the fact that dexamethasone in the GR CALUX bioassay is a factor 12 more potent than the natural hormone cortisol, results expressed as cortisol equivalents would range up to 2900 ng cortisol equiv/L. Further studies are needed to establish the identity of the active compounds and to understand the significance of the level of activities with regard to human and ecotoxicological risks.

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Introduction

Aquatic ecosystems are exposed to complex mixtures of compounds, of both natural and anthropogenic origin. Since the discovery that compounds in the aquatic environment can alter reproductive sexual characteristics, many projects have been conducted concerning the presence, identity, and activity of compounds that can act on the endocrine pathway. Most research has been focused on feminization or masculinization of fish present in the aquatic environment. However, the potential impact of these adverse effects on a population level still remains unclear.

The compounds that have been identified to be at least partially responsible for the disruption of the reproductive development of e.g., fish, can act as agonists or antagonists on the estrogen receptor (female sex hormone) and/or the androgen receptor (male sex hormone). The natural hormones estradiol (and related metabolites) and synthetic hormones such as ethinylestradiol (used in birth control pills) are thought to be mainly responsible for the observed effects. These natural and synthetic hormones are excreted by humans and animals in their conjugated (inactive) form and enter the aquatic environment, either untreated or via sewage treatment plants (STPs). However, at the STP and in the environment the inactive hormones can be deconjugated (i.e., reactivated), thereby disrupting the normal endocrine function of aquatic organisms. In addition to the known estrogens, other (anthropogenic) compounds such as bisphenol A and tributyltin were also identified as endocrine-disrupting compounds. Very recently, it was demonstrated that long-term exposure to a low ng/L range of estrogenic compounds can have devastating effects on fitness and survival of aquatic species (*1, 2*). Compounds interfering with other hormonal systems if present at equipotent levels could equally pose a risk to aquatic organisms, and possibly even to humans, if they survive drinking water treatment.

The estrogen receptor and the androgen receptor belong to a larger group of nuclear hormone receptors (*3*). Other members of this nuclear receptor super family are also involved in endocrine processes that may similarly be disrupted if active receptor−ligands are present in the environment. Relatively little attention is given to possible ligands for these other receptors in endocrine-disruptor research. This is surprising, since, e.g., approximately 13% of the over 20 000 FDA approved drugs target nuclear receptors specifically (*4*). Some of these natural and synthetic hormones have already been identified in the aquatic environment (*5, 6*) and research has shown that pharmaceuticals and personal care products can survive drinking water treatment (*7, 8*). Several of these drug residues, as well as natural and synthetic analogues, can act in concert on the same nuclear hormone receptor (*9*), resulting in an integrated response of complex mixtures. The total agonistic or antagonistic activity of complex mixtures can be measured by applying nuclear receptor specific reporter gene assays. Once the total endocrine activity of extracts or samples from certain sites or wastewater streams is established, the identification of culprit chemicals can be achieved by an activity directed identification process.

To be able to aid in the identification of compounds that can act on the different hormone receptors, bioassays have to be sensitive and respond only to a well defined mode of action (*10*). We have established a panel of nuclear receptor specific chemically activated luciferase expression (CALUX) reporter gene cell lines, expanding a previously described (*11, 12*) panel of estrogen (ERα), androgen (AR), and

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TABLE 1. Water Sample Coding and Specifications

water sample	sample code	specifications
blank	B	Evian mineral water, glass bottle
drinking water	DW	tap water
raw industrial effluent	I	sample taken at point of discharge to surface water
raw hospital effluent	H	1:1:1 mixture of three locations (same hospital), taken at point of discharge to sewage system
treated paper mill effluent	PM	sample taken at point of discharge to surface water
treated municipal effluents	S1, S2	sample taken at point of discharge to surface water
surface water	W1	brook, sample taken just beneath the surface
surface water	W2, W3	river, sample taken just beneath the surface

progesterone (PR) bioassays with a bioassay detecting glucocorticoid (GR) receptor activation and/or inhibition. These cell lines allow sensitive and specific measurements of hormone receptor action by complex mixtures of compounds. Since these cell lines all use the same human (U2OS) cell line, and basal plasmids for stable transfection are carefully matched, results among assays can be easily and reliably compared. Our aim is to study the utility of this panel of reporter gene assays in aquatic monitoring for hormonal activity in extracts of wastewater effluents and surface waters.

Materials and Methods

Chemicals. The compounds 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), 17α-ethinylestradiol (EE2), dexamethasone (Dex), estrone (E1), cortisol, norethynodrel, testosterone (Tes), progesterone (Prog), trenbolone, levonorgestrel, and prednisolone were purchased from Sigma/Aldrich (Zwijndrecht, The Netherlands). Org2058 was kindly provided by Dr. W. Schoonen (Organon, The Netherlands). Betamethasone was obtained from Steraloids Inc. (Newport, RI). Dimethyl sulfoxide (DMSO) was purchased from Acros (Geel, Belgium), neomycin (G148) was obtained from Life Technologies (Breda, The Netherlands), and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) came from Merck (Amsterdam, The Netherlands). The solvent ethyl acetate was obtained from Baker (Deventer, The Netherlands). Cell culture media (DF) and fetal calf serum were purchased from Invitrogen (Breda, The Netherlands).

Sample Collection. Water samples were collected from different sources throughout The Netherlands (see Table 1). The different sampling sites were not connected hydrologically. All samples collected were stored in glass 1 L bottles. After collection, samples were cooled to 4 °C for transport to KIWA Water Research, where they were filtered and extracted within 72 (effluent within 24) hours of arrival. Samples were not allowed to come into contact with plastics to prevent possible false positives and/or contamination.

Sample Preparation. To prevent any contamination during the extraction procedure, only glass, Teflon, and stainless steel equipment was used. All glassware was extensively cleaned prior to use with sodium hydroxide in ethanol absolute and all materials were rinsed with distilled acetone and distilled petroleum ether before use. Before extraction, suspended particles were removed from the water samples by filtering each sample using 0.45 μm cellulose nitrate filters (Sartorius).

Sample Extraction. From each sample, 1 L of water was extracted three times with ethyl acetate (200, 50, and 50 mL). All extracts were pooled in a 500 mL flask and the volume was reduced to around 200 mL by distillation. The volume was further reduced to 5−10 mL in a Kuderna Danish setup, at moderate speed to allow full evaporation of coextracted water. The remaining ethyl acetate fraction was transferred to a glass test tube. All extracts were evaporated under a gentle stream of nitrogen at 56 °C to a volume of 0.5 mL and transferred to a preweighted glass conical vial. The test tubes were rinsed with 0.5 mL of ethyl acetate, which was added to the extract. The ethyl acetate was further evaporated to approximately 3 μL under a nitrogen stream at 56 °C; the last microliters were left to evaporate spontaneously. The extracts were redissolved in 50 μL of DMSO. All extracts were stored at −18 °C until analysis.

TABLE 2. Recoveries of Selected Estrogens, Glucocorticoids, Progestagens, and Androgens Using Ethyl Acetate LLE Extraction

compound	class	Evian (%)	surface water (%)
estradiol	estrogen	95	95
estrone	estrogen	94	107
ethinylestradiol	estrogen	110	120
dexamethasone	glucocorticoid	77	89
cortisol	glucocorticoid	75	79
prednisolone	glucocorticoid	68	73
progesterone	progestagen	40	44
norethynodrel	progestagen	56	76
levonorgestrel	progestagen	88	89
dihydrotestosterone	androgen	92	89
testosterone	androgen	88	92
trenbolone	androgen	92	88

Recovery Determination. To determine the recovery of the extraction procedure for typical endocrine-active compounds, samples of Evian mineral water and river water were spiked with a mixture of compounds (Table 2) at 4 μg/L. These compounds are reference compounds for the CALUX tests and/or compounds that have been identified in the environment and are known to have hormone receptor mediated activity. These samples, together with unspiked samples of these waters, were filtered and extracted as described above. The DMSO extracts were subsequently diluted 10 times with ultrapure water containing the deuterated standards and subsequently 2 times with acetonitril (total volume 1 mL with 5% DMSO) for analysis by LC-MS.

A hybrid LTQ-Orbitrap mass spectrometer (Thermo Electron, Bremen, Germany) provided with an ESI interface was interfaced to a Surveyor HPLC system (Thermo Electron) for the chromatographic separation. The LTQ-Orbitrap was automatically tuned and calibrated according to the factory tuning and calibration procedure. The capillary used was a metal needle maintained at a temperature of 275 °C. The sheat and sweep gas were set to arbitrary units of 30 and 5, respectively. A source voltage of 3.6 kV and a capillary voltage of 35 V were used only in the positive mode. The tube lens was set to 70 V. Full scan high-accuracy mass spectra were acquired in the range of 150−800 m/z with the resolution set at 30000. Mobile phases consisted of acetonitril and 0.05% formic acid in water, and the injection volume used was 5 μL. The separation was performed on an Omnispher C18 column (100 × 2.0 mm i.d., 3 μm).

CALUX Bioassays. All water extracts were tested for hormone receptor agonists or antagonists using the previ-

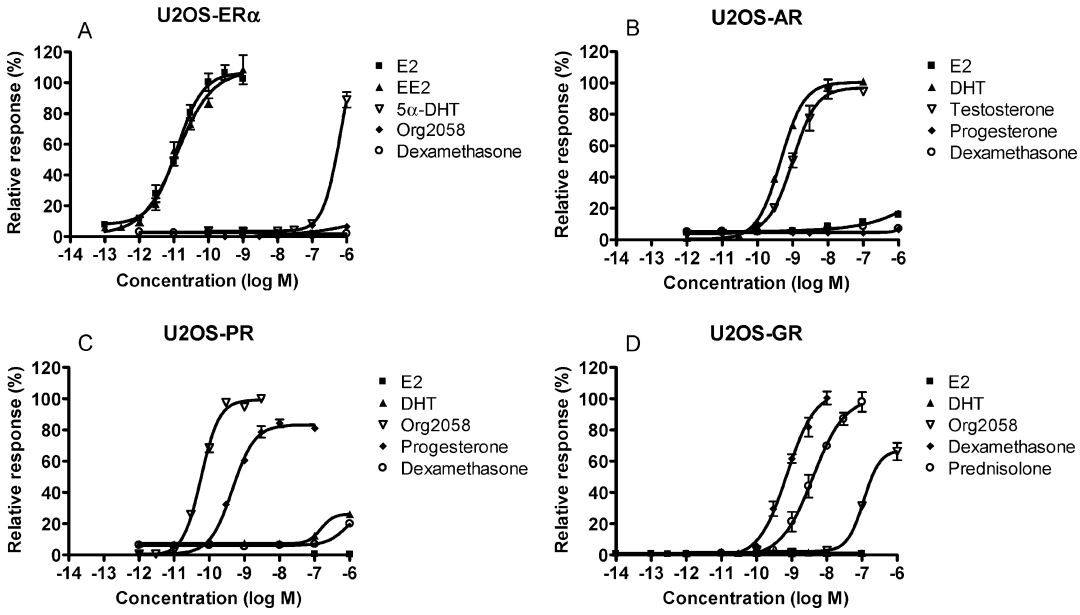

FIGURE 1. Transcriptional activity in a panel of CALUX cell lines, measuring estrogens (A), androgens (B), progestins (C), and glucocorticoids (D). Each cell line is exposed to a series of reference compounds for different bioassays. The response is expressed as % relative response to the reference agonist, i.e., estradiol (A, EC_{50} $1.2e^{-11}$), DHT (B, EC_{50} $3.2e^{-10}$), Org2058 (C, EC_{50} $5.8e^{-11}$), and dexamethasone (D, EC_{50} $7.3e^{-10}$).

ously described ERα, GR, and PR CALUX bioassays (*11, 13*). The AR CALUX bioassay used in this study is an improved version (pGL3 instead of pGL2 based) of the bioassay previously described by Sonneveld et al. (*11*), resulting in higher responses. All CALUX bioassays used utilize the U2OS human cell line with a luciferase gene under the transcriptional control of responsive elements for activated hormone receptors. The CALUX bioassays were performed as described previously (*13, 11*). In short, cells were seeded into 96-well plates with DF medium (without phenol red) that was supplemented with stripped (dextran-coated charcoal treated) serum. After 24 h of incubation (37 °C, 7.5% CO_2), the medium was replaced by medium containing the water extracts (0.1% DMSO) for agonistic activity testing (in triplicate). After 24 h of incubation, the medium was removed and the cells were lysed in 30 μL of Triton-lysis buffer. The amount of luciferase activity was quantified using a luminometer (Lucy 2, Anthos, Austria). On all plates, a dose-response curve of the reference compound was included for quantification of the response, which was estradiol, dihydrotestosterone, dexamethasone, or Org2058 for the ER, AR, GR, or PR CALUX respectively. All extracts were analyzed in triplicate. Only dilutions that were negative in the cytotoxicity test were used for quantification of the response.

For antagonistic activity testing, the same procedure was followed as for agonistic activity, with the exception that the exposure medium was also supplemented with the reference compound at EC_{50} level. In case of an antagonistic response, less luciferase is produced compared to the medium that is supplemented with the reference compounds only, resulting in a lower light production. The decrease in response is interpolated in an antagonist reference curve. As an extra control that the antagonistic effect observed is specific and not due to nonspecific effects like cytotoxicity, a positive control is included using the 1000 × EC_{50} level of the agonistic reference compound. Truly antagonistic extracts should show no decease in response when coexposed to such a high dose of reference compound.

Cytotoxicity. Cells were seeded into 96-well plates as for the CALUX bioassays. After 24 h, the medium was replaced with medium containing water extracts (0.1% DMSO). After 24 h exposure to the extracts, the medium was replaced by a mixture of PBS and medium (1:5) containing 1 mg/mL 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). The cells were incubated for approximately two hours at 37 °C, until formazan crystal formation became evident under the microscope. After incubation, the formed blue formazan crystals were dissolved by adding 100 μL of DMSO and the amount of formazan formed was quantified using a spectrophotometer (λ 595 nm). Results were expressed as percentage of color formation compared to cells exposed to DMSO.

Results and Discussion

CALUX Response Characterization. To be able to discriminate between different hormonal activities in the aquatic environment, bioassays applied have to be sensitive enough to measure at the low (or sub) ng/L range. Importantly, they also have to respond selectively to a nuclear receptor pathway. The CALUX cell lines showed good selectivity and a highly inducible response (fold induction >30 for all cell lines) by the relevant reference compounds (see Figure 1). Reference compounds for the different receptors generally do not induce responses on the other receptors, or only at very high concentrations (>10^{-6} M), indicating that the measured response is likely to be caused by inherent cross reactivity at high concentrations. Previous research has shown that the ER, PR, GR, and AR are not endogenously present at detectable levels in the wild type U2OS cell lines (*11, 14*, Sonneveld et al. (unpublished results)), making cross reactivity by binding to other hormone receptors unlikely. Results also show that the synthetic progestin Org2058 showed activity both in the PR and the GR CALUX, reflecting the known affinity of some progestins for the GR receptor, both in humans (*15, 16*) and in other mammalian species (*17*). The currently applied AR CALUX (pGL3 based instead of pGL2 based) was found to be more responsive than the one previously described (*12*). This androgen responsive bioassay was found to respond specifically to androgens only, whereas other literature described androgen responsive cell lines, utilizing endogenously present androgen receptors, also respond to glucocorticoids (*18–20*) and/or progestins (*21*).

Performance of Extraction Method. Since the cells in the bioassays are exposed to extracts of the water and not to the water itself, recovery of the active compounds is a major determinant for the outcome of the tests. The

TABLE 3. Estrogen (ERα), Progestin (PR), Glucocorticoid (GR), and Androgen (AR) Receptor Activity Determined in Different Types of Water Samples[a,b]

bioassay	ERα (ng E2-equiv/L)	PR (ng Org-eq/L)	GR (ng Dex-equiv/L)	AR (ng DHT-equiv/L)
industry	3.4 ± 0.4	2.2 ± 0.4	243 ± 32	81 ± 13
hospital	24 ± 2	n.d.	96 ± 13	86 ± 2
paper mill (PM)	0.16 ± 0.02	n.d.	11 ± 2	n.d.
STP 1 (S1)	1.0 ± 0.1	0.78 ± 0.15	38 ± 13	0.75 ± 0.09
STP 2 (S2)	0.39 ± 0.08	0.86 ± 0.30	11 ± 2	0.83 ± 0.21
surface water 1	0.50 ± 0.2	4.5 ± 1.7	1.3 ± 0.2	12 ± 2
surface water 2	0.22 ± 0.03	n.d.	0.39 ± 0.10	n.d.
surface water 3	0.18 ± 0.02	n.d.	1.0 ± 0.2	n.d.
drinking water	n.d.	n.d.	n.d.	n.d.
blank (Evian)	n.d.	n.d.	n.d.	n.d.

[a] CALUX determinations were carried out in triplicate. Activities are reported as ng of reference compound equivalents per L of water (± SD). [b] n.d. = not detected; STP = sewage treatment plant; E2 = 17β-estradiol; Org = synthetic progestin Org2058; Dex = dexamethasone; DHT = dihydrotestosterone.

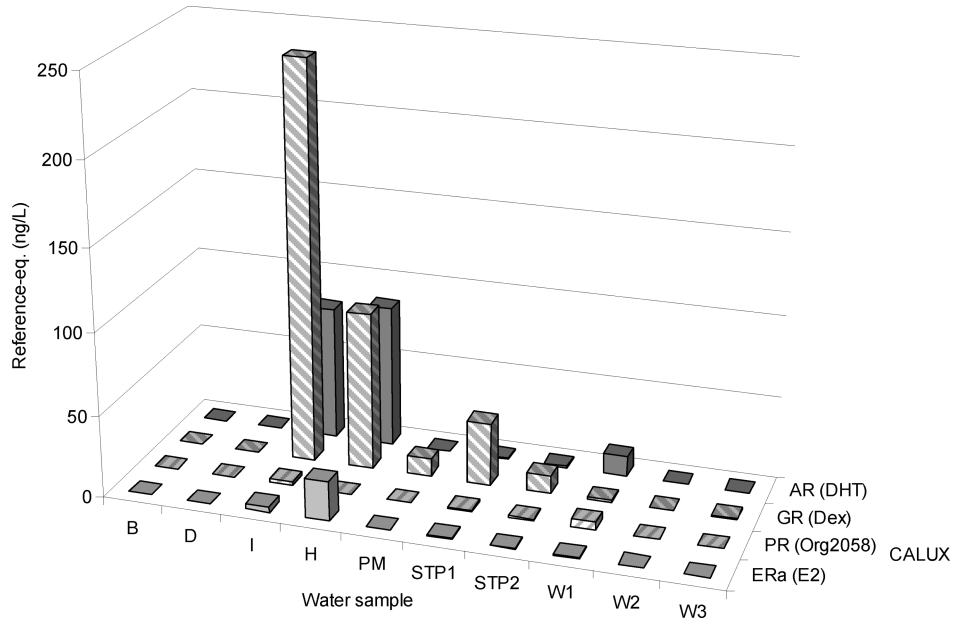

FIGURE 2. CALUX panel response for different types of water, aimed at determining estrogen-, androgen-, progestagen-, and glucocorticoid-like activity. Cells were seeded in 96-well plates and exposed to water extracts in DMSO. Results are expressed as ng of the reference compound equivalents per L of water. Water types tested are drinking water (D), procedure blank (B), raw industrial effluent (I), raw hospital effluent (H), treated paper mill effluent (PM), municipal sewage treatment plant effluents (STP1 and STP2), and surface waters (W1, W2, and W3).

recoveries of the extraction method applied for estrogens, androgens, and glucocorticoids that were spiked to mineral water and river water ranged between 68 and 120% (Table 2). The recovery of progestagenic compounds was generally lower compared to the other steroid hormones, ranging from 40−89%. Possibly, progesterone was partially lost during the filtration (sand filtration gave higher recovery, unpublished results). The higher recoveries in the surface water compared to mineral water for some compounds (e.g., norethynodrel and prednisolone) might be caused by background levels of these compounds in the surface water. Overall, similar recoveries were obtained for the two water types.

Biological Activity in Water Extracts. Some environmental contaminants are known to be hormone receptor antagonists. Therefore, in addition to measuring agonistic activity in the water sample extracts, all extracts were analyzed for antagonistic activity by coexposure with a known concentration of reference compound. However, no antagonistic activity was detected in any of the water sample extracts (results not shown).

Water sample extracts were tested for estrogenic, androgenic, progestagenic, and glucocorticoid-like activity. All types of activity were detected in the environmental water extracts (see Table 3 and Figure 2), although water sample 1 (sample from a small brook) contained significantly higher levels of androgenic and progestagenic activity than the two other (river) water samples, possibly due to local discharge of untreated wastewater. No hormonal activity was detected in the blank or in the drinking water sample. Validation of the CALUX bioassays showed that reproducibility was 20−30%. Overall, the highest activities were found in the industrial effluent and in the raw hospital effluent. The high activities in the raw hospital effluent are not surprising, since it consists largely of untreated wastewater. Probably a large portion of the activity in the hospital effluent might be explained by excreted (natural) hormones and administered drugs.

Estrogenic activity was detected in all water samples except the blank and the drinking water. The estrogenic activity found in the water samples ranged between 0.39 and 1.0 ng E2-equiv/L for the municipal STP samples and 0.18−0.50 ng E2-equiv/L for the surface water samples. These levels are consistent with levels reported previously for the Dutch aquatic environment (*22, 23*). The highest level of estrogenic activity was detected in the raw hospital effluent,

at a level of 24 ng E2-equiv/L. Relatively low estrogenic activity was detected in the municipal effluents. Although these values are still in the normal range for Dutch effluents (*24, 25*), these levels are only up to five times higher than those detected in the surface waters tested in this study. Depending on location, the relative contribution of effluent to the total water body in Dutch surface waters can vary between 0 and almost 100% (*24, 26*). It should be noted however, that these surface water samples were not taken in the vicinity of the STPs.

In five out of the eight water samples, androgenic activity was detected, with again the highest activity in the raw hospital effluent (86 ng DHT-equiv/L), followed by the industrial effluent (81 ng DHT-equiv/L). Activity in the municipal STP effluents was detected at a level of 0.8 ng DHT-equiv/L, but no activity was detected in the surface water, except in surface water 1, which contained androgenic activity far exceeding the concentrations detected in the municipal effluents. The androgenic activity levels in the STP effluent are consistent with levels reported in effluents elsewhere using YAS, a yeast based reporter gene assay (*27*), although higher values up to 600 ng DHT-equiv/L have also been reported, depending on the type of sewage treatment applied (*28*). Androgenic activity found in these studies could be almost completely explained by the presence of known (natural) androgens, which are found regularly in effluent and surface waters (*29*) in the ng/L range, although the origin of the androgenic activity in surface water 1 remains to be discovered. No androgenic activity was detected in the paper mill effluent. Previously, paper mill effluents have been attributed to being a source of environmental androgens in several studies (*30, 31*). Possibly, the extensive treatment that is applied at this particular plant decreased the androgenic activity to a level below the AR CALUX LOQ of 0.1 ng DHT-equiv/L.

Progestagenic activity was detected in four out of the eight extracts, ranging from 0.78 to 4.5 ng Org2058-equiv/L, with highest PR agonistic activity being detected in the industrial effluent sample and surface water 1. Both STP effluents contained progestagenic activity in the same range: 0.78 and 0.86 ng Org2058-equiv/L. Previous research on the presence of progestins in effluent showed that progesterone is degraded very rapidly in sewage treatment plants and even in the effluent after sampling if no preservation technique is applied (*32*). However, natural steroids, synthetic progestins, and metabolites have been detected in surface waters at concentrations up to several ng/L for progesterone and medroxyprogesterone (*5, 33*). Since in the PR CALUX, progesterone is about 13 times less potent than our reference compound Org2058 and medroxyprogesterone is approximately as potent as Org2058, these reported concentrations are in the same range as our findings for total progestagenic activity. Part of the progestagenic activity found in the aquatic samples might be attributed to natural hormones. Even though progesterone, unlike the synthetic progestins, appears to break down more easily than estrogens in wastewater (*34*), progestin excretion by farm animals far exceeds androgen and estrogen excretion (by a factor of 45 and 10, respectively, (*35*)). Part of the excreted hormones can enter the surface water without passing any treatment plant, e.g. via direct runoff from cattle pastures.

The most striking finding of the study is the extremely high glucocorticoid-like activity found in the samples. Although by far the highest level of glucocorticoid receptor activity was detected in the industrial effluent (243 ng Dex-equiv/L), all environmental water samples showed a considerable amount of glucocorticoid receptor agonistic activity. The second highest level measured was in the raw hospital effluent, with a glucocorticoid-like activity of 96 ng Dex-equiv/L, almost 3-fold lower than the industrial effluent. The municipal STPs still had a surprisingly high concentration of 38 and 11 ng Dex-equiv/L in the final effluent, possibly contributing to the glucocorticoid concentration in the surface water. The concentrations in the surface waters tested were between 0.39 and 1.3 ng Dex-equiv/L. It should be noted that the use of the relatively strong glucocorticoid dexamethasone as a reference compound in our studies does not fully reflect the biological potency of the observed glucocorticoid-like activities in waste and surface waters. Dexamethasone is a factor 12 more potent in the GR CALUX bioassay than the major endogenous hormone cortisol (Sonneveld, unpublished data). Therefore, when expressing the GR CALUX results as cortisol equivalents, all GR CALUX values should be multiplied by this factor, meaning that levels up to 2900 ng cortisol equiv/L have been detected.

To the best of our knowledge, only one study has reported glucocorticoid activity in effluents and surface water samples (*36*). However, the levels of glucocorticoids found by Chang et al. are much lower than the glucocorticoid receptor activity determined in our study. The identity of the compounds responsible for the GR response is currently unknown. It is likely that next to the natural glucocorticoid hormones, such as cortisol, also widely used immunosuppressive drugs (e.g., beclometasone as asthma inhaler) and ointments (e.g., betamethasone in topical cream against skin irritation) contribute to the observed responses through urine and faeces excretion, since glucocorticoids belong to the most frequently prescribed drugs worldwide (*37*). For example, a metabolite of the immunosuppressive drug prednisolone has recently been identified in Dutch groundwater contaminated by leaking sewage water (Heringa et al., unpublished results). The high GR CALUX activity levels found in the effluent after treatment (and the even higher levels in the untreated hospital effluent) also suggest a human origin of at least part of the environmentally present glucocorticoids. Again, the relatively high level of glucocorticoid activity in surface water 1 suggests the discharge of untreated or uncontrolled effluent, since this location is not located in the vicinity of an STP.

Glucocorticoids play a pivotal role in the regulation of glucose metabolism and inflammation, and the homology of the GR among mammalian species is relatively high (*38*). In humans, chronic exposure to glucocorticoids is linked to a multitude of biological effects including obesity, type 2 diabetes, and inflammatory diseases (*39, 40*), especially in pre- and neonatals (*41, 42*). Chronic exposure should therefore be avoided and the presence of significant levels of glucocorticoids in the environment may be reason for concern.

Results thus far indicate that, although environmental antagonists have been identified for almost all nuclear receptors, the measured total endocrine activity in water samples is mostly agonistic. Possibly, the presence of both agonists and antagonists in the same extract may mask each individual contribution. As pointed out by Sohoni and Sumpter (*43*), several environmental estrogens are antiandrogens and since estrogens are quantified in all water samples the measured androgenic activity could be an underestimation. This makes it important to determine all biological responses at different locations, since compounds responsible for the different biological responses observed might have different degradation speeds (*34*) or different distribution between water and sediment (*44*). Therefore, effort should be made to separate these compounds using approaches like effect directed analysis, to get a more clear picture about the identity of the compounds involved.

This study shows that it is useful to apply a broad range panel-approach for monitoring EDC type activity in wastewater and surface water. All four hormone receptor bioassays applied showed quantifiable responses for the water extracts: estrogens, androgens, progestins, and glucocorticoids. Of all

the responses tested, only estrogenic activity is currently occasionally monitored in the aquatic environment. The levels of androgenic, progestagenic, and glucocorticoid activity are mostly higher than estrogenic levels in this study, however, it is stressed that this study represents a pilot survey of a broad range of endocrine activities in the aquatic environment. The extraction methods applied can be optimized for progestagen extraction and adding a deconjugation step might possibly increase the activities found even further, especially in the raw effluents. Further investigations on these activities are necessary in order to evaluate the biological significance of the androgenic, progestagenic, and glucocorticoid activities with respect to human and environmental health.

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Literature Cited

(1) Kidd, K. A.; Blanchfield, P. J.; Mills, K. H.; Palace, V. P.; Evans, R. E.; Lazorchak, J. M.; Flick, R. W. Collapse of a fish population after exposure to a synthetic estrogen. *PNAS* **2007**, *21*, 8897–8901.

(2) Sumpter, J. P. Endocrine disruptors in the aquatic environment: an overview. *Acta Hydrochim. Hydrobiol.* **2005**, *33* (1), 9–16.

(3) Nuclear Receptors Nomenclature Committee. A unified nomenclature system for the nuclear receptor superfamily. *Cell* **1999**, *97*, 161–163.

(4) Overington, J. P.; Al-Lazikani, B.; Hopkins, A. L. How many drug targets are there? *Nat. Rev. Drug Discov.* **2006**, *5* (12), 993–996.

(5) Kolodziej, E. P.; Sedlak, D. L. Rangeland grazing as a source of steroid hormones to surface waters. *Environ. Sci. Technol.* **2007**, *41*, 3514–3520.

(6) Durhan, E. J.; Lambright, C. S.; Makynen, E. A.; Lazochak, J.; Hartig, P. C.; Wilson, V. S.; Gray, L. E.; Ankley, G. T. Identification of metabolites of trenbolone acetate in androgenic runoff from a beef feedlot. *Environ. Health Perspect.* **2006**, *114* (1), 65–68.

(7) Westerhof, P.; Yoon, Y.; Snyder, S.; Werts, E. Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. *Environ. Sci. Technol.* **2005**, *39* (17), 6649–6663.

(8) Versteegh, J. F. M.; van der Aa, N. G. F. M.; Dijkman, E. Geneesmiddelen in drinkwater en drinkwaterbronnen; report no. 703719016/2007; RIVM, 2007.

(9) Silva, E.; Rajapakse, N.; Kortenkamp, A. Something from "nothing"−eight weak estrogenic chemicals combined at concentrations below NOECs produce significant mixture effects. *Environ. Sci. Technol.* **2002**, *15*, 1751–1756.

(10) Eggen, R. I. L.; Behra, R.; Burkhardt-Holm, P.; Escher, B. I.; Schweigert, N. Challenges in ecotoxicology. Mechanistic understanding will help overcome the newest challenges. *Environ. Sci. Technol.* **2004**, *38*, 59A–64A.

(11) Sonneveld, E.; Jansen, H. J.; Riteco, J. A. C.; Brouwer, A.; Van der Burg, B. Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid responsive bioassays. *Toxicol. Sci.* **2005**, *83*, 136–148.

(12) Sonneveld, E.; Riteco, J. A. C.; Jansen, H. J.; Pieterse, B.; Brouwer, A.; Schoonen, W. G.; Van der Burg, B. Comparison of in vitro and in vivo screening models for androgenic and estrogenic activities. *Toxicol. Sci.* **2006**, *89*, 173–187.

(13) Hamers, T.; Kamstra, J. H.; Sonneveld, E.; Murk, A. J.; Kester, M. H.; Andersson, P. L.; Legler, J.; Brouwer, A. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. *Toxicol. Sci.* **2006**, *92*, 157–173.

(14) Quaedackers, M. E.; Van den Brink, C. E.; Wissink, S.; Schreurs, R. H. M.; Gustafsson, J. A.; van der Saag, P. T.; van der Burg, B. 4-Hydroxytamoxifen Trans-represses nuclear factor-κB activity in human osteoblastic U2-OS cells through estrogen receptor (ER) α and not through ERβ. *Endocrinology* **2001**, *142*, 1156–1166.

(15) Koubovec, D.; Ronacher, K.; Stubsrud, E.; Louw, A.; Hapgood, J. P. Synthetic progestins used in HRT have different glucocorticoid agonist properties. *Mol. Cell. Endocrinol.* **2005**, *242* (1−2), 23–32.

(16) Issar, M.; Sahasranaman, S.; Buchwald, P.; Hochhaus, G. Differences in the glucocorticoid to progesterone receptor selectivity of inhaled glucocorticoids. *Eur. Respir. J.* **2006**, *27*, 511–516.

(17) Giannopoulos, G.; Keicline, D. Species-related differences in steroid-binding specificity of glucocorticoid receptors in lung. *Endocrinology* **1981**, *108*, 1414–1419.

(18) Wilson, V. S.; Bobseine, K.; Lambright, C. R.; Gray, L. E., Jr. A novel cell line, MDA-kb2, that stably expresses an androgen- and glucocorticoid-responsive reporter for the detection of hormone receptor agonists and antagonists. *Toxicol. Sci.* **2002**, *66*, 69–81.

(19) Soto, A. M.; Maffini, M. V.; Schaeberle, C. M.; Sonnenschein, C. Strengths and weaknesses of in vitro assays for estrogenic and androgenic activity. *Best Pract. Res. Clin. Endocrinol. Metab.* **2006**, *20*, 15–33.

(20) Kim, H. J.; Park, Y. I.; Dong, M. S. Comparison of prostate cancer cell lines for androgen receptor-mediated reporter gene assays. *Toxicol. In Vitro* **2006**, *20*, 1159–1167.

(21) Willemsen, P.; Scippo, M-L.; Kausel, G.; Figueroa, J.; Maghuin-Rogister, G.; Martial, J.; Muller, M. Use of reporter cell lines for detection of endocrine disruptor activity. *Anal. Bioanal. Chem.* **2004**, *378*, 655–663.

(22) Puijker, L. M. Oestrogene activiteit in oppervlaktewater van de Rijn; RIWA: The Netherlands, 2007.

(23) Vethaak, A. D.; Lahr, J.; Schrap, S. M.; Belfroid, A. C.; Rijs, G. B.; Gerritsen, A.; de Boer, J.; Bulder, A. S.; Grinwis, G. C.; Kuiper, R. V.; Legler, J.; Murk, T. A.; Peijenburg, W.; Verhaar, H. J.; de Voogt, P. An integrated assessment of estrogenic contamination and biological effects in the aquatic environment of The Netherlands. *Chemosphere* **2005**, *59* (4), 511–524.

(24) Vethaak, A. D.; Rijs, G. B. J.; Schrap, S. M.; Ruiter, H.; Gerritsen, A.; Lahr, J. *Estrogens and Xeno-estrogens in the Aquatic Environment of The Netherlands: Occurrence, Potency and Biological Effects*; Report 2002.001; Dutch National Institute of Inland Water Management and Waste Water Treatment: The Netherlands, 2002; available from http://www.rijkswaterstaat.nl/rws/riza/home/publicaties/index.html.

(25) Kinnberg, K. *Evaluation of in vitro assays for determination of estrogenic activity in the environment*; Report 43; Danish Environmental Protection Agency: Denmark, 2003; available from http://glwww.mst.dk/homepage/.

(26) Gerritsen A. A. M.; Rijs, G. B. J.; Klein Breteler, J. G. P.; Lahr, J. Oestrogene effecten in vissen in regionale wateren, Report no. 2003.19 (in Dutch). Dutch National Institute of Inland Water Management and Waste Water Treatment. 2003. The Netherlands. Available from: http://www.rijkswaterstaat.nl/rws/riza/home/publicaties/index.html.

(27) Kirk, L. A.; Lye, C. M.; Tyler, C. R.; Sumpter, J. P. Changes in estrogenic and androgenic activities at different stages of treatment in waste water treatment works. *Environ. Toxicol. Chem.* **2002**, *21*, 972–979.

(28) Thomas, K. V.; Hurst, M. R.; Smith, A.; McHugh, M.; Matthiessen, P.; Waldock, M. J. An assessment of androgenic activity and the identification of environmental androgens in United Kingdom estuaries. *Environ. Toxicol. Chem.* **2002**, *21*, 1456–1461.

(29) Kolodziej, E. P.; Gray, J. L.; Sedlak, D. L. Quantification of steroid hormones with pheromonal properties in municipal wastewater effluents. *Environ. Toxicol. Chem.* **2003**, *22*, 2622–2629.

(30) Parks, L. G.; Lambright, C. S.; Orlando, E. F.; Guillette, L. J., Jr.; Ankley, G. T.; Gray, L. E., Jr. Masculinization of female mosquitofish in Kraft mill effluent-contaminated Fenholloway river water is associated with androgen-receptor agonist activity. *Toxicol. Sci.* **2001**, *58*, 339–349.

(31) Ellis, R. J.; van den Heuvel, M. R.; Bandelj, E.; Smith, M. A.; McCarthy, L. H.; Stuthridge, T. R.; Dietrich, D. R. In vivo and in vitro assessment of the androgenic potential of a pulp and paper mill effluent. *Environ. Toxicol. Chem.* **2003**, *22*, 1448–1456.

(32) Labadie, P.; Budzinski, H. Determination of steroidal hormone profiles along the Jalle d'Eysines River (near Bordeaux, France). *Environ. Sci. Technol.* **2005**, *39* (14), 5113–5120.

(33) Kuster, K.; Lopez de Alda, M. J.; Barcelo, D. Analysis and distribution of estrogens and progestogens in sewage sludge, soils and sediments. *Trends Anal. Chem.* **2004**, *23* (10), 790–798.

(34) Labadie, P.; Budzinski, H. Development of an analytical procedure for determination of selected estrogens and progestagens in water samples. *Anal. Bioanal. Chem.* **2005**, *381*, 1199–1205.

(35) Lange, I. G.; Daxenberger, A.; Schiffer, B.; Witters, H.; Ibaretta, D.; Meyer, H. H. D. Sex hormones originating from different

livestock production systems: fate and potential disrupting activity in the environment. *Anal. Chim. Acta* **2002**, *473*, 27–37.

(36) Chang, H.; Hu, J.; Shao, B. Occurrence of natural and synthetic glucocorticoids in sewage treatment plants and receiving river waters. *Environ. Sci. Technol.* **2007**, *41*, 3462–3468.

(37) Lu, N. Z.; Wardell, S. E.; Burnstein, K. L.; Defranco, D.; Fuller, P. J.; Giguere, V.; Hochberg, R. B.; McKay, L.; Renoior, J-M.; Weigel, N. L.; Wilson, E. M.; McDonnell, D. P.; Cidlowski, J. A. International union of pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralcorticoid, progesterone, and androgen receptors. *Pharmacol. Rev.* **2006**, *58*, 782–797.

(38) Yudt, M. R.; Cidlowski, J. A. The glucocorticoid receptor: coding a diversity of proteins and responses through a single gene. *Mol. Endocrinol.* **2002**, *16* (8), 1719–1726.

(39) Saag, K. H. Resolved: Low-dose glucocorticoids are neither safe nor effective for the long-term treatment of rheumatoid arthritis. *Arthritis Care Res.* **2001**, *45*, 468–471.

(40) Dallman, M. F.; la Fleur, S. E.; Pecoraro, N. C.; Gomez, F.; Houshyar, H.; Akana, S. F. Mini review: glucocorticoids - Food intake, abdominal obesity, and wealthy nations in 2004. *Endocrinology* **2004**, *145* (6), 2633–2638.

(41) Drake, A. J.; Tang, J. I.; Nyirenda, M. J. Mechanisms underlying the role of glucocorticoids in the early life programming of adult disease. *Clin. Sci. (London)* **2007**, *113* (5), 219–232.

(42) Seckl, J. R.; Holmes, M. C. Mechanisms of disease: glucocorticoids, their placental metabolism and fetal "programming" of adult pathophysiology. *Nat. Clin. Pract. Endocrine Metab.* **2007**, *3* (6), 479–488.

(43) Sohoni, P.; Sumpter, J. P. Several environmental oestrogens are also anti-androgens. *J. Endocrinol.* **1998**, *158* (3), 327–39.

(44) Kuster, M.; López de Alda, M. J.; Barceló, D. Analysis and distribution of estrogens and progestogens in sewage sludge, soils and sediments. *Trends Anal. Chem.* **2004**, *23* (10−11), 790–798.

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