

**Temporal variation in multiple
hormonal activities of surface
waters located in the Dutch part
of the Rhine basin**

RIWA
Rhine Water Works
The Netherlands



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Association of Rhine Waterworks - RIWA

September 2009

¹ KWR, Watercycle Research Institute, Nieuwegein, the Netherlands
² BioDetection Systems b.v., Amsterdam, the Netherlands

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Foreword

This study was commissioned by RIWA-Rhine and performed by KWR in collaboration with BioDetection Systems b.v. (BDS). The following organizations contributed to the present report:



RIWA-Rhine is the Association of River Water Companies using water from the Dutch part of the Rhine river as the source for drinking water production. RIWA-Rhine aims to achieve a water quality that allows drinking water to be produced using only simple, near-natural treatment.

One of the instruments to achieve this goal is a water quality monitoring network. Results from this network are used to underpin demands regarding quality improvements to water authorities and polluters. In addition, specific research is carried out. RIWA-Rhine works together, e.g. in drinking water policy issues with the German, Swiss and French colleagues in the IAWR, the International Association of Water works in the Rhine basin. This umbrella organisation which was set up in 1970 by RIWA, ARW (Arbeitsgemeinschaft Rhein-Wasserwerke) and AWBR (Arbeitsgemeinschaft Wasserwerke Bodensee-Rhein), covers the whole Rhine catchment basin.



Having started out as Kiwa Water Research, a section of KIWA NV, KWR Watercycle Research Institute has been an independent entity since 2007. It has built up a solid foundation from over 60 years of research and development for the Dutch drinking water sector companies, its current shareholders. Unique in the world, this collaboration of the water companies in the Netherlands has resulted in a powerful knowledge base and an extensive collective memory for the drinking water sector. KWR performs a broad range of research projects around drinking water, including drinking water quality. Since 2008, KWR has extended its field of activities to include the entire watercycle.



BioDetection Systems

BioDetection Systems b.v. (BDS) is a Dutch company providing bioassays for a wide range of compounds or mixtures of compounds from natural and man-made origin, such as dioxin and dioxin-like compounds, like PCBs (DR CALUX[®]), estrogens or pseudo estrogens (ER CALUX[®]), androgen hormones (AR CALUX[®]) and other persistent organic pollutants (POPs). BDS' bioassays find particular applications for analyzing very low levels of contaminants in food, feed and environmental samples. Other applications include chemical testing and biological investigation for activity profiling of bio-potent compounds such as drugs, metabolites and residues, and use and abuse of growth promoters, steroids etc.



Abstract



Presently, most research in the field of endocrine disruption has focused on the estrogenic compounds. However, increasing emission of unknown compounds into the environment, may also affect other important hormone dependent processes. Recently, a suite of novel sensitive bioassay techniques has become available to look further than estrogenic effects only. These so-called CALUX bioassays may provide a valuable contribution to the assessment of water quality. The latter is important for drinking water companies, and in this context RIWA-Rhine commissioned a study to utilize these new bioassays in a novel monitoring study carried out at two important locations of the Dutch part of the Rhine basin (Lobith and Nieuwegein). The results clearly show the presence of multiple types of hormonal activity at both sample locations over the one-year sampling campaign. Estrogenic activity at both sample locations was in the same range as reported earlier and androgenic/progestagenic/thyroidogenic activities were very low (or \lt LOD). An interesting finding as observed in the present study is the glucocorticogenic activity in water samples from both sample locations. Glucocorticoids have important physiological functions and they are applied against a great number of human diseases. Apart from other important aspects, more research should be devoted to chemical identification of glucocorticogenic compounds, a proper human health based risk assessment and a broader (temporo)spatial impression of glucocorticogenic activity in Dutch surface waters.

List of abbreviations

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AR-CALUX	Androgenic responsive CALUX
CALUX	Chemically Activated LUciferase gene eXpression
DMSO	Dimethylsulfoxide
ER-CALUX	Estrogenic responsive CALUX
GR-CALUX	Glucocorticogenic responsive CALUX
PR-CALUX	Progestagenic responsive CALUX
RLU	Relative Light Units
SPE	Solid Phase Extraction
TR-CALUX	Thyroid hormone response CALUX

Introduction and background

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The presence of xenobiotic compounds in our environment has become a world-wide concern, especially since some of these compounds may disrupt hormone dependent (physiological) processes, such as vertebrate fetal development (Cooper and Kavlock 1997). As such, over the last decades a large research effort is being directed towards the occurrence, effects and risks of these compounds. One group of compounds that received much attention are the estrogenic compounds, such as ethinylestradiol, which is commonly used in the oral contraceptive pill. The last few decades it has become clear that estrogenic compounds may enter the aquatic environment through sewage treatment plants (STPs) (Ankley et al., 2008). The occurrence and risks of these estrogenic compounds for humans and wildlife have been extensively discussed elsewhere (e.g. Sumpter 1998; Belfroid et al., 1999; Mennes, 1996). However, the increasing emission of unknown potentially hormone active compounds, such as pharmaceuticals and personal care products (GWRC 2004; Schwarzenbach et al., 2006) into the aquatic environment may also affect other important hormone dependent physiological processes in humans and wildlife. Recently, a suite of new sensitive bioassay techniques has become available to look further than estrogenic effects only. These so-called CALUX bioassays detect either glucocorticoid-, androgen-, progesterone- or thyroid hormone receptor activation and/or inhibition. Since these bioassays provide an integrated (biologically relevant) effect-orientated response, they are ideally suited to provide a valuable contribution to the assessment of (surface) water quality. The latter is important for drinking water companies and in this context the Association of Rhine Water works in the Netherlands (RIWA-Rhine) commissioned a study to utilize these bioassays in a novel surface water monitoring study. In this study, a first impression of the temporal variation in multiple hormonal (CALUX) activity was obtained in two important locations of the Dutch part of the Rhine basin, namely the Rhine river at the German-Dutch border (Lobith) and at a major drinking water intake site (Lekkanaal in Nieuwegein). The results of this research are described in the present report.

Materials and methods

The methodology of the present research comprises four basic steps as illustrated in figure 1. Firstly, water samples were collected and transferred to the laboratory. Secondly, water samples were cleaned and extracted on a solid phase extraction (SPE) column. The eluates were subsequently evaporated and taken up in the solvent dimethylsulfoxide (DMSO). Thirdly, in the CALUX bioassays, transgenic U2OS cells seeded on 96 wells plates were exposed to sample extracts and their response was measured accordingly (step 4). Each of these steps is described in more detail below.

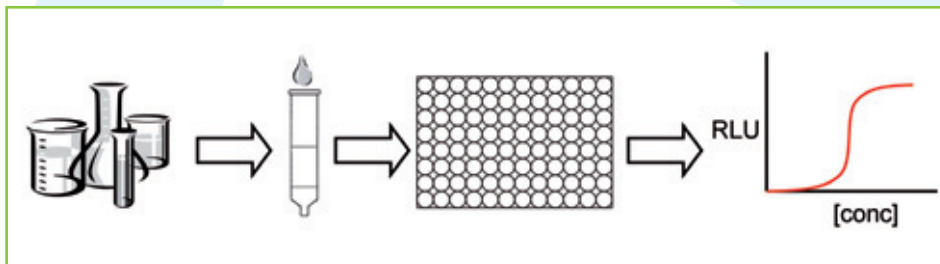


Figure 1. Schematic overview of the methodological approach. 1) sample collection; 2) sample cleanup/SPE extraction; 3) CALUX bioassay; 4) quantification of the response/analysis of the results.

Chemicals

Chemicals were obtained as described by van der Linden et al. (2008). Briefly, dexamethasone (dex), dihydrotestosterone (DHT), estradiol (E2) and 3,3',5-triiodo-L-thyronine (T3) were purchased from Sigma/Aldrich (the Netherlands). Org2058 was kindly provided by dr. W. Schoonen (Schering-Plough, the Netherlands). DMSO was purchased from Acros (Belgium). The solvents ethylacetate and acetonitril were obtained from, respectively, Baker (the Netherlands) and Boom (the Netherlands).

Cell culture

The human U2OS osteosarcoma1 cell line (figure 2) was cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (DF, Gibco) supplemented with 7.5% FCS at 37°C and 7.5% CO₂ as described by Sonneveld et al. (2005).

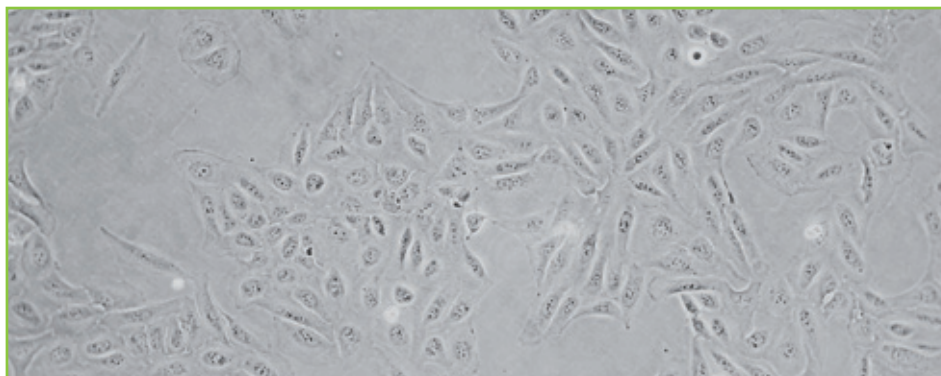


Figure 2. Photo of the human U2OS osteosarcoma cell line. Photo courtesy of BioDetection Systems b.v., NL

Samples

Sample collection and preparation

Water samples of both sites (Lobith-Rhine and Nieuwegein-Lekkanaal) (figure 3) were collected twice a month in the timeframe August 2007 to August 2008. At the day of sampling, surface water samples were collected by immersion of 1 liter ultra-cleaned dark glass bottles approximately 25 cm below the water surface (picture 1 and 2 appendix 1). After collection, the samples were immediately stored at 4°C until extraction (within 24 hrs). Prior to sample treatment, all glassware was extensively washed with distilled acetone followed by petroleum ether and dried under ambient circumstances.



Figure 3. Location of the sample sites in the Netherlands.

Sample extraction

200 mg Oasis HLB solid phase extraction columns (Waters) were conditioned twice with ethylacetate, followed by conditioning with methanol (once) and Evian mineral water (twice). A glass filtration column filled with ignited sea sand (Mallinckrodt Baker) was placed on top of the extraction column and the samples (1 L) were isolated at a flow rate of approximately 10 mL/min. After drying, the columns were eluated three times with 2.5 mL ethylacetate. The ethylacetate fraction was transferred

into a glass tube and subsequently evaporated at 56°C under a gentle stream of nitrogen to a volume of approximately 3 µL. The last microliters were left to evaporate spontaneously and the extracts were redissolved in 50 µL DMSO. All extracts were stored at -18°C until further analysis. Evian mineral water was used as a procedure blank.

CALUX bioassays

The corresponding reference compounds of the ER-CALUX, GR-CALUX, AR-CALUX, PR-CALUX and TR-CALUX are, respectively, estradiol (E2), dexamethasone (dex), dihydrotestosterone (DHT), the synthetic progestin Org2058 and thyroid hormone (T3). The principle of the CALUX bioassay is relatively uniform for all types of hormonal action and is schematically illustrated in figure 4.

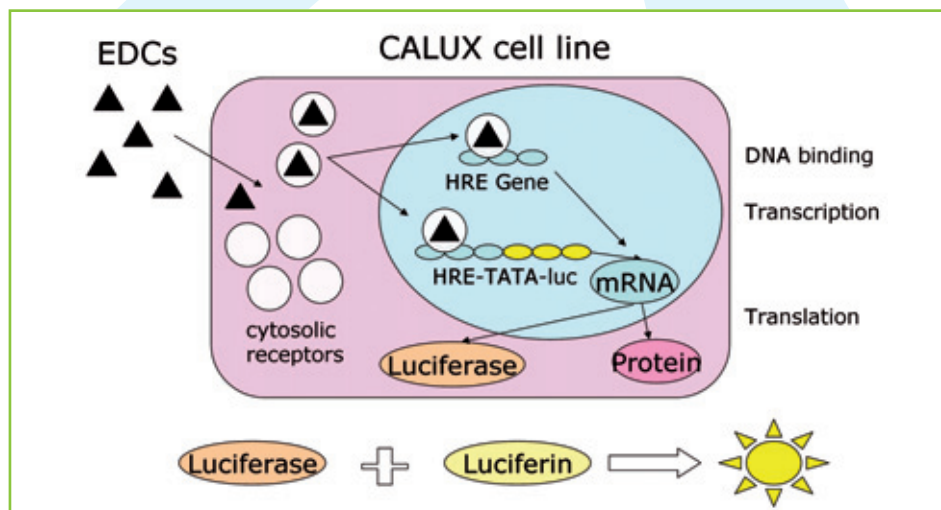


Figure 4. Schematic illustration of the CALUX bioassay.

Briefly, hormones or hormone-like compounds enter the target cell, and bind to cytosolic receptors. Upon dimerization of ligand-bound receptors, and/or formation of receptor dimers, translocation of the complex to the nucleus will occur. In the nucleus the complex binds to hormone response elements (HRE) on the DNA. Under normal physiological conditions, these HREs are able to initiate transcription of genes, thereby resulting in mRNA synthesis, translation and production of proteins that cause physiological effects. In the transgenic CALUX bioassay, the translocated complex binds to the specifically engineered HRE-Luc gene, ultimately leading to luciferase protein synthesis. Luciferase production can be measured by addition of the substrate luciferin, producing a photonic reaction. The amount of photons is a measure for exposure to hormones or hormone-like compounds.

In the framework of the present research, CALUX bioassays for multiple types of hormonal action were carried out as described by van der Linden et al. (2008). Briefly, U2OS human cells with a HRE-Luc gene were seeded into 96 wells plates with DF medium (w/o phenol red and supplemented with DCC stripped serum). After 24 hours of incubation (37°C, 7.5% CO₂), the medium was replaced by medium containing sample extracts (max. 0.4% DMSO) for activity testing. After 24 hours of exposure, the medium was removed and the cells were lysed in 30 µL 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 corresponding reference compounds was included for adequate quantification of the response. To rule out any confounding influences due to toxicity of the water extracts, cells were monitored for signs of cytotoxicity by means of light microscopy.

Data analysis

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Data analysis was performed as described by van der Linden et al. (2008). Briefly, dilution series of water extracts in DMSO were analyzed in triplicate. Dose response curves of the reference compounds were fitted using the sigmoidal fit with variable Hill slope. Fold induction was calculated by dividing the mean value light units from exposed and nonexposed (solvent control) wells. The amount of reference compound equivalents (EQ) in the sample extract was determined by interpolating the response of the water extract into the dose response curve of the reference compound. Results were expressed as nanograms of reference compound equivalents per liter of water.

Results

CALUX bioassay standard curves

The CALUX bioassays showed typical dose response curves, with highly inducible responses (fold induction ≥ 30 for all cell lines) by the relevant reference compounds (figure 5). The CALUX bioassay response characterization and performance evaluation of the extraction method, were performed earlier by van der Linden et al. (2008) and Heringa et al. (unpublished results) and are not within the scope of the present study. It needs to be noted that the SPE recovery of the reference compound T₃ was not satisfactory. Therefore, results regarding thyroidogenic responses should be interpreted with caution.

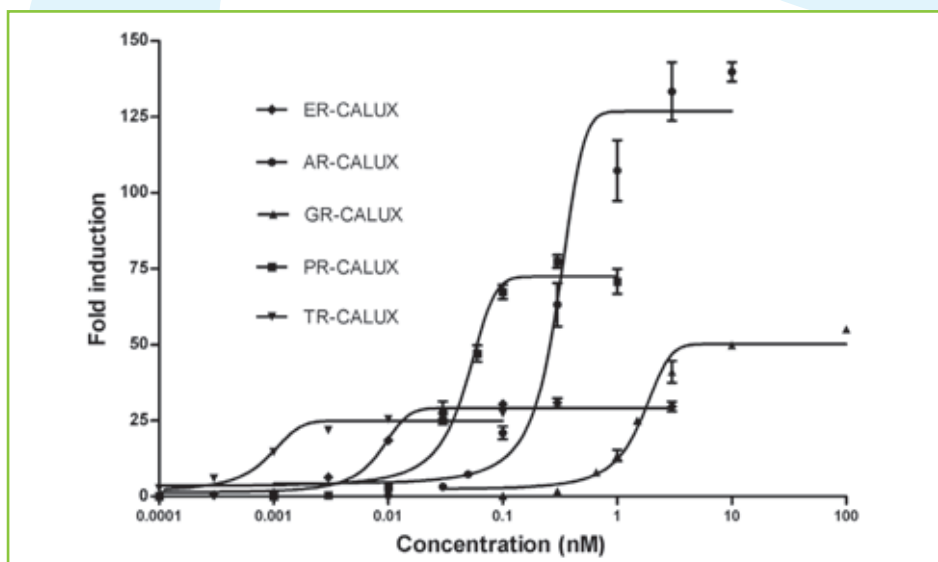


Figure 5. Typical example of dose response curves from the applied suite of CALUX bioassays, measuring reference compounds, i.e., estradiol (E₂, estrogens, ER-CALUX), dexamethasone (dex, glucocorticoids, GR-CALUX), dihydrotestosterone (DHT, androgens, AR-CALUX), Org2058 (Org2058, progestins, PR-CALUX) and 3,3',5-triiodo-L-thyronine (T₃, thyroidogens, TR-CALUX). Responses are fitted (log(agonist) vs. response - variable slope) using GraphPad Prism 5 and expressed as fold induction relative to the solvent control. The corresponding Log EC₅₀ values \pm SD are respectively 0.008 ± 0.0004 nM (E₂), 1.6 ± 0.09 nM (dexamethasone), 0.3 nM ± 0.02 nM (DHT), 0.05 ± 0.002 nM (Org2058) and $0.0008 \pm 5.2 \times 10^{-19}$ nM (T₃).

Temporal variation in CALUX bioassay activity of water extracts

The results clearly show the presence of agonistic hormonal activity on the ER, PR, AR and GR at both sample locations (Lobith and Nieuwegein) over time (figure 6). Only thyroidogenic activity was $<$ LOD during the whole sampling period (data not shown). The estrogenic activity found in the water samples ranged between 0.02 and 0.73 ng E₂ equivalents/L at sample location Lobith, whereas estrogenic activity found at sample location Nieuwegein was somewhat lower, namely between 0.025 and 0.4 ng E₂ equivalents/L. In the timeframe September 11th-12th a peak in estrogenic activity of 0.73 ng and 0.4 E₂ equivalents/L was measured at sample location Lobith and Nieuwegein,

respectively. Progestagenic activity was detected in all samples at both sample locations in the same range (~0.02 to ~0.09 ng Org2058 equivalents/L) but, surprisingly, this activity decreased to below detection levels after May 20th 2008 throughout the rest of the sampling period. Androgenic activity was low in all samples at both sample locations (<0.05 ng DHT equivalents/L) and was absent in respectively 78% (Lobith) or 89% (Nieuwegein) of the water samples.

Glucocorticogenic activities in the surface waters tested were between 0.75 and 2.7 ng dex equivalents/L for sample location Lobith and between 0.41 and 2.4 ng dex equivalents/L for sample location Nieuwegein. Sample concentrations at location Lobith were generally higher (44.4% of the samples above 1.5 ng dex equivalents/L) as compared to location Nieuwegein (25.9% of the samples above 1.5 ng dex equivalents/L). Glucocorticogenic activity was absent in 18.5% of the samples taken at Lobith and in 44.4% of the samples taken at Nieuwegein. After July 2nd 2008 until the end of the sampling period only estrogenic activity was detected.

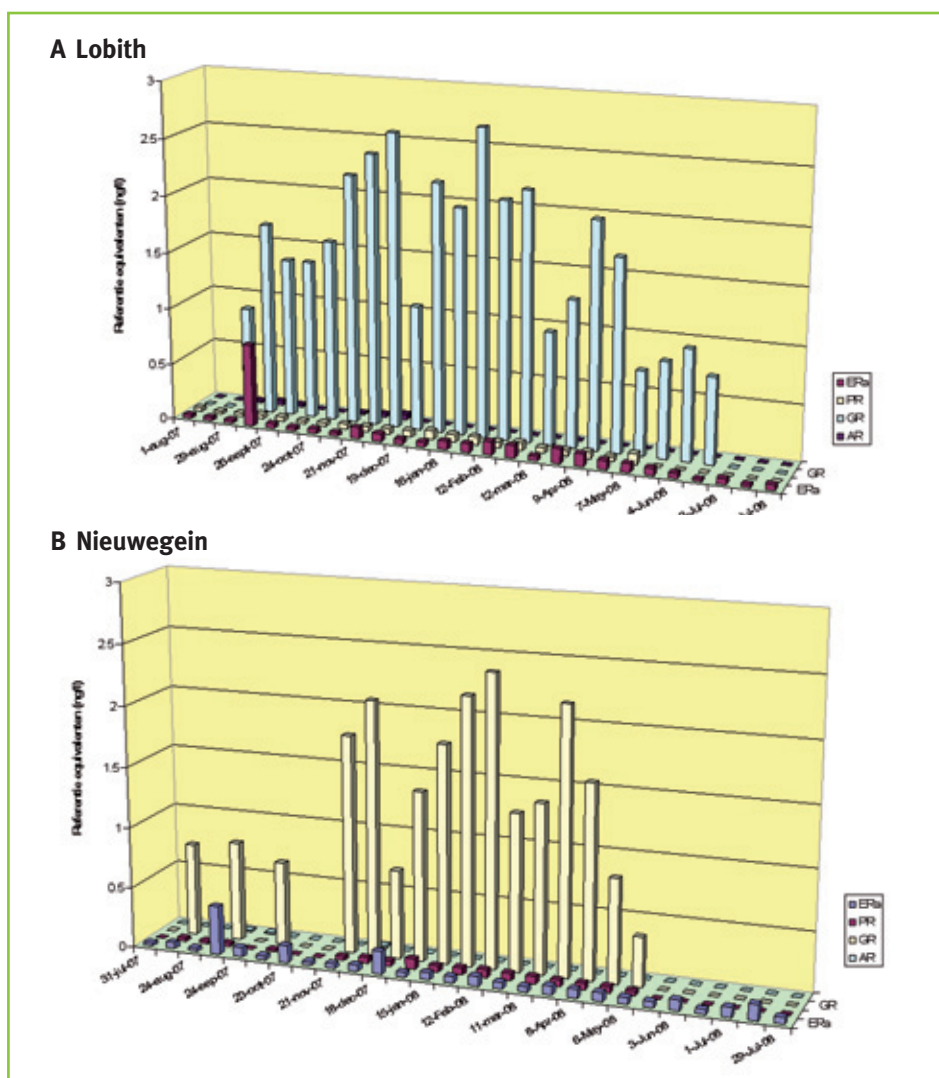


Figure 6. Time series of sample location (A) Lobith (Rhine) and (B) Nieuwegein (Lekkanaal) illustrating temporal variation in androgenic (AR), glucocorticogenic (GR), progestagenic (PR) and estrogenic (ER) activity as measured by CALUX bioassays through the sampling period August 2007 until August 2008. Thyrogenic activity was < LOD during the whole sampling period (data not shown). Zero value means < LOD. For raw data see appendix 2 and 3.

Yearly trends of hormone active compounds in the River Rhine

Hormone active compounds might reveal a yearly trend due to seasonal changes in use or river flow, as seen for some pharmaceuticals (ter Laak et al., in preparation). The last factor can be eliminated by calculating a daily load by multiplying the concentrations of the reference compounds (as reference equivalents (EQs)) by the Rhine flow (m³/sec) on the day of sampling (obtained from the Dutch Ministry of Public Works (Rijkswaterstaat). To facilitate comparison of the multiple types of hormone activity, the daily load was subsequently calculated as a percentage of the maximum daily load during the sampling period. If this is done, figure 7 shows that no clear trend can be observed for the various types of hormone activity. Maximum loads can be observed in late summer (estradiol EQs), winter (dihydrotestosterone and dexamethasone EQs) and spring (org2058 EQs).

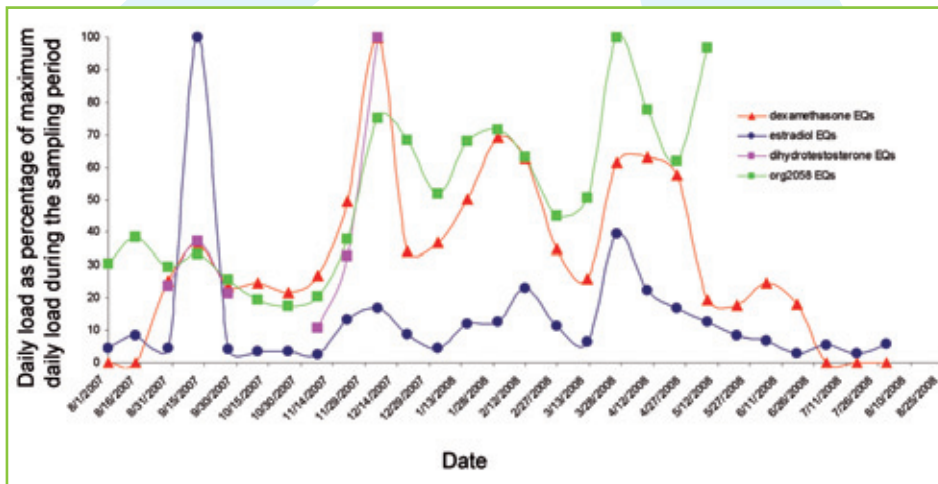


Figure 7. Yearly trend of daily loads of reference compounds (set as percentage of the maximum load during the sampling period). Absence of measured data represents a value < LOD.

Discussion

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New sensitive technologies have become available to detect multiple types of hormonal activity in various matrices such as surface waters. The present study shows for the first time a temporal variation in multiple hormonal activities of two surface waters located in the Dutch part of the Rhine basin, as determined with a novel suite of CALUX bioassays. The results illustrate that estrogenic activity at both sample locations is in the same range as earlier observed by Puijker (2007). Androgenic and progestagenic activities were both very low (or $<$ LOD) and in the same range as described earlier for a number of Dutch surface waters by van der Linden et al. (2008). The absence of thyroid hormone activity in surface water samples taken during the sampling campaign may be attributed to poor SPE recovery of thyroid hormone and/or thyroidogenic compounds. However, the results could also indicate a total absence of thyroidogenic compounds. If the latter is true, the results are of particular interest since Meulenberg and Marchesini (2006) showed potent interaction of various Dutch surface water extracts (including Rhine) on binding of thyroid hormones to transport proteins. This discrepancy could suggest a differential underlying mode of action of thyroidogenic compounds. However, this remains speculative and clearly more research is required to be able to provide definite conclusions regarding thyroidogenic activity in surface waters.

The daily load of reference compounds was highly variable during the sampling period and no clear trends could be observed. This suggests that several important variables exist that influence the concentration of hormone active compounds in Rhine water. It can be speculated that these variables include application/consumption of hormone active compounds as shown for pharmaceuticals (van der Aa et al., 2009) and amounts subsequently discharged to the environment. However, more research is necessary to underpin this hypothesis. As illustrated in figure 6, a new interesting finding of the present study is the presence of glucocorticogenic activity in water samples from both sample locations. These results should not be directly compared to the other types of hormonal activities, since the individual reference compounds have differential hormonal activities. For practical reasons the various hormonal activities are plotted in one 3D-graph, as also done by van der Linden et al (2008). These authors found concentrations up to 235 ng dex equivalents/L in raw industrial effluent.

In this study the chemical structure of the responsible compounds was not investigated in detail, and an attempt to do this by means of LC-MS/MS is currently in progress. Those results will be published elsewhere, and may shed a light on the identity of glucocorticogenic compounds as present in the Dutch part of the Rhine basin. A first indication of glucocorticoid identity may be found in a publication of Chang et al. (2007). In this study the authors report the occurrence of six glucocorticoids (prednisone, prednisolone, cortisone, cortisol, dexamethasone, and 6-methylprednisolone) in sewage treatment plants (STPs) and receiving rivers in China. Analysis with LC-MS/MS revealed that surface water samples contained glucocorticoids in the low ng/L range, which is in the same order of magnitude as the concentration of glucocorticogenic compounds found in the present study.

Glucocorticoids have important physiological functions such as an anti-inflammatory and immunosuppressive action and they are applied in human medication against a great number of human diseases including severe allergies, skin problems, asthma and arthritis. Some illustrative representatives are dexamethasone, triamcinolon, prednisone and cortisone (Lowenberg et al., 2008).

As hypothesized by Shore and Shemesh (2003) a quantity of glucocorticoids (excreted by humans and cattle) can be released into the environment through the effluents of sewage treatment plants. Hence, more quantitative research should be carried out to trace the origins of glucocorticoid emission. As shown in the present study, the concentration of glucocorticogenic compounds becomes lower downstream the Rhine, as illustrated by the results of sample site Nieuwegein. This may be explained by breakdown processes, metabolism or sorption of hormone active compounds to sediment or suspended matter.

Presently, the potential human risks accompanying (chronic) exposure to a low concentration of (synthetic) glucocorticoids are unknown. In 1998 the JECFA¹ derived an acceptable daily intake (ADI) for dexamethasone of ~1 µg/person/day, based on tyrosine aminotransferase induction in rat liver. In the present in vitro CALUX study, the maximum concentration of glucocorticogenic compounds was 2.7 ng dexamethasone equivalents/L. Hence, an adult person that would drink 2 liters of unpurified surface water per day would be exposed to 5.4 ng dexamethasone equivalents per day. If it would be assumed that this concentration results from dexamethasone alone, still a large margin of safety would exist. In fact, the margin of safety may be even larger in practice, since (i) ADME² in vivo may even lead to lower plasma concentrations of dexamethasone and (ii) drinking water purification steps are likely to remove relatively lipophilic compounds such as steroid hormones (Nghiem et al., 2004). However, other compounds than dexamethasone alone may be responsible for the results as obtained in the present study, thereby greatly complicating an adequate human benchmark based risk assessment. Again, this stresses the need for chemical compound identification in the water samples and further investigations are necessary to evaluate the biological significance of glucocorticogenic activity for human/environmental health. In addition, glucocorticogenic compounds may be found in higher concentrations in other Dutch surface waters, which are also under the impact of wastewater discharge. Therefore, further work should be devoted to temporal-spatial variation of glucocorticogenic compounds in the Dutch aquatic environment including a life cycle assessment (metabolism) and determination of removal efficiency of specific compounds in drinking water purification plants.

¹ Joint FAO/WHO Expert Committee on Food Additives

² Absorption, distribution, metabolism, excretion

Conclusions and recommendations

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The present study shows normal estrogenic activity, low levels of androgenic, progestagenic and an absence of thyroidogenic activity. The latter type of hormonal activity deserves more attention, and the SPE procedure should be optimized to fit T₃ extraction.

An interesting finding of the present study is the presence of glucocorticogenic activity in water samples from both sample locations. Although it is not very likely that the concentrations of glucocorticogenic compounds will pose a threat for drinking water production and/or human health, further work should be devoted to the following aspects:

1. Further research should elucidate the contribution of glucocorticogenic compounds delivered by municipal and industrial sources in relation to veterinary (diffuse) emission.
2. For a proper human health based risk assessment, it is recommended to elucidate the chemical structure of potential glucocorticogenic compounds in potent water extracts.
3. In the present study, only surface waters in the Dutch part of the Rhine basin were sampled for multiple types of hormone activity. However, other surface water in the Netherlands may also be under the impact of glucocorticogenic compounds. It is therefore important to get a broader spatial impression of glucocorticogenic activity in Dutch surface waters.
4. A chemical analysis in potent glucocorticogenic water extracts should lead to a prioritization of glucocorticogenic compounds that should be further tested for removal efficiency in drinking water treatment steps (such as activated carbon).
5. Hormone (like) compounds are most likely mainly excreted as glucuronidated products due to phase II biotransformation. It needs to be noted that a major part of these conjugated products is deglucuronidated during sewage treatment, however a part may still reach surface waters in a conjugated form. Therefore, addition of a deglucuronization step (see Belfroid et al., 1998) as a part of sample preparation may lead to even higher CALUX activity of water extracts.
6. Results as obtained by Houtman and co-workers (2004) have shown that both synthetic and natural estrogen exposure may lead to feminization of certain fish species. Since glucocorticoids play a pivotal role during vertebrate development, the ecological significance of glucocorticoid exposure for wildlife species such as fish needs further investigation.

Acknowledgements

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Appendices

- Appendix 1: Picture 1. sample bottle.
 Picture 2. Sampling of Lekkanaal (Nieuwegein).
- Appendix 2: Raw data of the present study.



Appendix 1. Picture 1. sample bottle.



Appendix 1. Picture 2. sampling of the river Lekkanaal (Nieuwegein)

Appendix 2. Raw data of the present study (location Lobith), as equivalents of the given reference compound.

CALUX	ERa	PR	GR	AR	TRB
LOBITH	E2 (ng/L)	Org2058 (ng/L)	Dex (ng/L)	DHT (ng/L)	T3 (ng/L)
1-aug-07	0.029	0.031	<LOD	<LOD	<LOD
14-aug-07	0.027	0.020	<LOD	<LOD	<LOD
29-aug-07	0.026	0.027	0.92	0.017	<LOD
12-sept-07	0.73	0.039	1.7	0.034	<LOD
26-sept-07	0.04	0.038	1.4	0.025	<LOD
10-oct-07	0.032	0.028	1.4	<LOD	<LOD
24-oct-07	0.041	0.032	1.6	<LOD	<LOD
7-nov-07	0.031	0.042	2.2	0.017	<LOD
21-nov-07	0.1	0.046	2.4	0.031	<LOD
5-dec-07	0.068	0.049	2.6	0.051	<LOD
19-dec-07	0.044	0.055	1.1	<0.05	<LOD
2-jan-08	0.042	0.078	2.2	<0.05	<LOD
16-jan-08	0.075	0.068	2	<0.05	<LOD
30-Jan-08	0.076	0.07	2.7	<LOD	<LOD
12-Feb-08	0.12	0.053	2.1	<LOD	<LOD
27-feb-08	0.11	0.071	2.2	<0.05	<LOD
12-mar-08	0.039	0.049	1	<0.05	<LOD
26-Mar-08	0.13	0.053	1.3	<LOD	<LOD
9-Apr-08	0.11	0.062	2	<LOD	<LOD
23-Apr-08	0.078	0.046	1.7	<LOD	<LOD
7-May-08	0.076	0.094	0.75	<LOD	<LOD
21-May-08	0.062	<LOD	0.85	<LOD	<LOD
4-Jun-08	0.042	<LOD	0.98	<LOD	<LOD
18-Jun-08	0.02	<LOD	0.76	<LOD	<LOD
2-Jul-08	0.047	<LOD	<LOD	<LOD	<LOD
16-Jul-08	0.024	<LOD	<LOD	<LOD	<LOD
30-Jul-08	0.048	<LOD	<LOD	<LOD	<LOD

Appendix 3. Raw data of the present study (location Lekkanaal), as equivalents of the given reference compound.

CALUX	ERa	PR	GR	AR	TRB
LEKKANAAL	ERa (ng/L)	Org2058 (ng/L)	Dex (ng/L)	DHT (ng/L)	T3 (ng/L)
31-jul-07	0.025	<LOD	<LOD	<LOD	<LOD
14-aug-07	0.051	0.025	0.76	<LOD	<LOD
24-aug-07	0.027	0.022	<LOD	<LOD	<LOD
11-sept-07	0.4	0.028	0.82	0.013	<LOD
24-sep-07	0.078	<LOD	<LOD	0.013	<LOD
10-oct-07	0.033	0.021	0.69	<LOD	<LOD
23-oct-07	0.14	<LOD	<LOD	<LOD	<LOD
6-nov-07	0.031	<LOD	<LOD	<LOD	<LOD
21-nov-07	0.037	0.033	1.8	<LOD	<LOD
5-dec-07	0.053	0.045	2.1	0.020	<LOD
18-dec-07	0.19	0.044	0.73	<0.05	<LOD
2-jan-08	0.04	0.085	1.4	<0.05	<LOD
15-jan-08	0.061	0.051	1.8	<0.05	<LOD
29-Jan-08	0.053	0.059	2.2	<LOD	<LOD
12-Feb-08	0.08	0.067	2.4	<LOD	<LOD
26-feb-08	0.057	0.061	1.3	<0.05	<LOD
11-mar-08	0.044	0.06	1.4	<0.05	<LOD
25-Mar-08	0.076	0.056	2.2	<LOD	<LOD
8-Apr-08	0.076	0.055	1.6	<LOD	<LOD
23-Apr-08	0.071	0.051	0.86	<LOD	<LOD
6-May-08	0.058	0.038	0.41	<LOD	<LOD
20-May-08	0.051	<LOD	<LOD	<LOD	<LOD
3-Jun-08	0.091	<LOD	<LOD	<LOD	<LOD
17-Jun-08	0.041	<LOD	<LOD	<LOD	<LOD
1-Jul-08	0.082	<LOD	<LOD	<LOD	<LOD
15-Jul-08	0.13	<LOD	<LOD	<LOD	<LOD
29-Jul-08	0.054	<LOD	<LOD	<LOD	<LOD

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