



# High resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in The Netherlands

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*Watercycle Research Institute*





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High resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in The Netherlands

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# Abstract

In the past two decades much research effort has focused on the occurrence, effects and risks of estrogenic compounds. However, increasing emissions of new emerging compounds may also affect the action of hormonal pathways other than the estrogenic hormonal axis. Recently, a suite of novel CALUX<sup>®</sup> bioassays has become available that enables to look further than estrogenic effects only. By employing these bioassays, we recently showed high glucocorticogenic activity in wastewaters collected at various sites in The Netherlands. However, since bioassays provide an integrated biological response, the identity of the responsible biological compounds remained unknown. Therefore, our current objective was to elucidate the chemical composition of the wastewater extracts used in our previous study by means of LC-high resolution Orbitrap<sup>®</sup> MS/MS and to determine if the compounds quantified could account for the observed glucocorticoid responsive (GR) CALUX bioassay response. The mass spectrometric analysis revealed the presence of various glucocorticoids in the range of 13-1900 ng/L. In extracts of hospital wastewater - collected prior to sewage treatment - several glucocorticoids were identified (cortisol: 275-301 ng/L, cortisone: 381-472 ng/L, prednisone: 117-545 ng/L, prednisolone: 315-1918 ng/L and triamcinolone acetonide: 14-41 ng/L) which are used to treat a great number of human pathologies. A potency balance calculation based on the instrumental analyses and relative potencies (REPs) of the individual glucocorticoids, supports the conclusion that triamcinolone acetonide (REP=1.3), dexamethasone (REP=1) and prednisolone (REP=0.2) are the main contributors to the glucocorticogenic activity in the investigated wastewater extracts. The action of these compounds is concentration additive and the overall glucocorticogenic activity can be explained to a fairly large extent by their contribution.



# Contents

|   |           |
|---|-----------|
| <b>Abstract</b>   | <b>1</b>  |
| <b>Contents</b>   | <b>3</b>  |
| <b>1 Introduction</b>   | <b>5</b>  |
| <b>2 Experimental section</b>   | <b>7</b>  |
| 2.1 Chemicals   | 7         |
| 2.2 Samples   | 7         |
| 2.3 Recovery determination of glucocorticoid standards                                    | 8         |
| 2.4 Mass Spectrometric conditions   | 8         |
| 2.5 GR CALUX bioassay and determination of relative potencies (REPS)                      | 8         |
| <b>3 Results and discussion</b>   | <b>9</b>  |
| 3.1 Characteristics of instrumental and biological procedures for glucocorticoid analysis | 9         |
| 3.2 Occurrence of glucocorticoids in wastewater extracts                                  | 12        |
| <b>4 Acknowledgements</b>   | <b>17</b> |
| <b>5 References</b>   | <b>19</b> |





# 1 Introduction

The presence of xenobiotic compounds in our environment has become a topic of world-wide concern, especially since some of them may disrupt hormone dependent (physiological) processes, such as vertebrate fetal development (1). Much attention has been directed to anthropogenic compounds that target the estrogen receptor such as 4-nonylphenol, bisphenol A, phthalate plasticizers (2, 3) and the natural and synthetic hormones 17 $\alpha$ -(ethynyl) estradiol, 17 $\beta$ - estradiol, estriol and estrone (4, 5). In the last decades it has become clear that these compounds may enter the aquatic environment through effluents of sewage treatment plants (STPs) (6-8) and their presence has been linked to adverse population-level effects on aquatic vertebrates such as fish (9, 10). As such, during the last decades a large research effort is directed towards the occurrence, effects on physiological processes and risks of (xeno)estrogenic compounds which have been extensively discussed elsewhere (2, 4, 11, 12). However, increasing emissions of new and unknown hormonally active compounds, such as pharmaceuticals and personal care products (13, 14) into the aquatic environment may also affect other important hormone dependent physiological processes in humans and wildlife. Therefore, we recently carried out a comprehensive study on the occurrence of hormonally active compounds in various Dutch surface and waste waters using novel CALUX bioassays for estrogens, progestins, glucocorticoids and androgens (15). We found significant levels of all activities, and particularly high levels of glucocorticogenic activity. However, since CALUX bioassays provide an integrated biological response, the identity of the responsible glucocorticogenic compounds remained to be established. A first indication of compound identity can be found in a report in which Chang and co-workers (16) demonstrate the occurrence of several natural and synthetic glucocorticoids (cortisol, cortisone, dexamethasone, 6 $\alpha$ -methylprednisolone, prednisolone and prednisone) in sewage treatment plant effluents and receiving waters in China. The glucocorticoids most frequently detected in effluent were cortisol, cortisone and prednisolone. Glucocorticoids are essential endocrine hormones involved in the regulation of nearly every physiological process and their effects are largely mediated through binding to the glucocorticoid receptors (GRs) (17, 18). They have been among the most frequently prescribed drugs and are applied against a great number of human pathologies including allergies, skin problems, asthma and arthritis (19). Recently more attention has been drawn to interference of xenobiotics with targets of the glucocorticoid pathway such as the function of 11 $\beta$ -hydroxysteroid dehydrogenase (11 $\beta$ -HSD) or disruption of GR function (20). Interference with these delicate mechanisms has been associated with e.g. impaired gluconeogenesis, reproductive toxicity, hypertension and altered vascular function (21). In addition, recent findings by Hillegass and co-workers (22), show that matrix metalloproteinase-13 is a target for dexamethasone leading to several developmental abnormalities in Zebra fish (*Danio rerio*). However, the environmental effects of glucocorticoid exposure (in the ng/L range) on other aquatic species are poorly understood at present and deserve more research attention.

Partially based on the results obtained by Chang and co-workers (16, 23), we hypothesize that the glucocorticogenic (GR) CALUX bioassay response we have observed in wastewater effluents (reported in 15) could have been caused by (combinational) effects of such natural and synthetic glucocorticoids. Since chemical analysis was not performed in our previous study, the identity of the compounds responsible for the then reported GR response remained unknown. Therefore, the objectives of the present research were (i) to identify and quantify the concentration of glucocorticoid compounds in wastewater extracts stored from our previous study and (ii) determine whether the compounds quantified could account for the magnitude of GR CALUX bioassay response observed. To this end, a chemical analysis of a selection of glucocorticoid compounds was performed in several potent wastewater extracts stored from our previous study on glucocorticogenic activity of wastewaters (15) by means of LC-high resolution Orbitrap MS/MS. An inventory was made of existing glucocorticoid compounds/medicines; to this end scientific publications, pharmaceutical handbooks, and the internet were searched and the availability of reference standards was assessed. Finally, a potency balance analysis was conducted and a mixture activity experiment was carried out to evaluate if the detected glucocorticoid compounds act in an additive or synergistic manner.



## 2 Experimental section

### 2.1 Chemicals

The inventory led to 18 relevant glucocorticoids for which reference standards were available. Aldosterone (CAS 52-39-1), amcinonide (CAS 51022-69-6), betamethasone (CAS 378-44-9), cortisone (CAS 53-06-5), dexamethasone (CAS 50-02-2), flunisolide (CAS 3385-03-3), fluorometholone (CAS 426-13-1), hydrocortisone (cortisol) (CAS 50-23-7), 6 $\alpha$ -methylprednisolone (CAS 83-43-2), prednisolone (CAS 50-24-8), prednisone (CAS 53-03-2), triamcinolone (CAS 83474-03-7) and triamcinolone acetonide (CAS 8054-16-8) were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). The compounds desoximetasone (CAS 382-67-2), prednicarbate (CAS 73771-04-7), rimexolone (CAS 49697-38-3) and triamcinolone hexacetonide (CAS 5611-51-8) were purchased from Chemos (Regenstauf, Germany). Paramethasone (CAS 53-33-8) was obtained from the Schering-Plough Corporation (Oss, The Netherlands). These standards represent compounds that are known to be present in drugs and have GR mediated activity. Glucocorticoid standards were purchased in the highest purity available (96% or higher). Dimethylsulfoxide (DMSO) was at least 99.7% pure and purchased from Merck (Darmstadt, Germany). The solvent ethyl acetate was obtained from Baker (Deventer, the Netherlands). Water used for LC-MS/MS analysis was generated from an ultra pure water system from Millipore (Bedford, MA, USA) with a specific resistance of 18 M $\Omega$ .cm. Ultra gradient acetonitrile (Mallinckrodt Baker, Deventer, The Netherlands) was used as an organic modifier. Chemicals to strip wastewater of glucocorticogenic compounds, including 4C Norit A charcoal and dextran T-70, were obtained from Sigma-Aldrich. Stock solutions of the individual glucocorticoid standards were prepared at 1 mM in DMSO and stored at -20°C in brown glass vials (Thermo Electron corporation, Breda, The Netherlands) in the dark.

### 2.2 Samples

For the present study, various potent wastewater extracts in DMSO, stored from our previous study, were chemically analyzed (15). We selected an industrial wastewater (from a factory producing veterinary medicines), a hospital wastewater (*further referred to as hospital wastewater 1*), a paper mill effluent and a municipal sewage treatment plant effluent. These extracts had been stored for approximately two years at -20°C in conical glass vials (Omnilabo, Breda, The Netherlands) in the dark. Furthermore, a new batch of 25 L of hospital wastewater was collected on 8/19/2009 in an ultra-cleaned stainless steel drum to (i) to get an impression of the identity/quantity of glucocorticoids in hospital wastewater at a different point in time (*further referred to as hospital wastewater 2*) and (ii) to provide a suitable wastewater matrix to perform recovery experiments of individual glucocorticoid standards. In order to obtain wastewater devoid of glucocorticoid compounds, hospital wastewater 2 was stripped with activated charcoal as described for the removal of steroids in fetal bovine serum (24). Briefly, freshly collected (hospital) wastewater was filtered over a 5  $\mu$ m Whatman grade no. 3 paper filter ('s Hertogenbosch, The Netherlands) and stirred overnight with 2.5 g dextran coated activated charcoal (DCC) per liter of wastewater at 4 °C. Next, wastewater was centrifuged at 500 g for 10 minutes to pellet the charcoal and subsequently the stripped wastewater was filtered over a 1  $\mu$ m Envirocheck HV cartridge (Pall Gelman Laboratory, Ann Arbor, MI, USA) prior to 0.45  $\mu$ m filtration on cellulose nitrate filters (Sartorius). All glassware was extensively cleaned with sodium hydroxide in ethanol absolute and all materials were rinsed with distilled acetone and distilled petroleum ether prior to use. Finally, stripped wastewater was also utilized to perform an additional experiment to assess the combined action of glucocorticoids in a mixture. To this end, 6 glass bottles with 1 L of stripped wastewater were spiked in duplicate with stock solutions (1 mM in DMSO) of 5 individual glucocorticoid standards (cortisol, cortisone, prednisolone, prednisone and triamcinolone acetonide) at concentration levels of 210, 290, 230, 90 and 30 ng/L, and with a mixture of the same set of glucocorticoid standards, respectively. The rationale for choosing these compounds is to mimic a true environmental sample being hospital wastewater 1 (*cf.* Table 2 for instrumentally detected concentration levels of glucocorticoids). After a three-fold extraction with ethyl acetate (according to ref. 15), the samples were redissolved in 50  $\mu$ L DMSO followed by subsequent LC-MS/MS and GR CALUX bioassay analysis. Potency balance analyses were conducted by comparing the observed GR CALUX bioassay response magnitudes to those predicted based on the concentrations of known glucocorticoid standards present in the extract. Concentrations of individual glucocorticoids as determined by means of LC-MS/MS were multiplied by their GR CALUX bioassay specific relative potencies (REPs) (calculated by dividing the EC<sub>50</sub> of the reference compound dexamethasone by the EC<sub>50</sub> of compound x; 25). The sum of the products for all

target compounds present in an extract provides an estimate of the dexamethasone equivalents (dex EQs) in the extracts.

### 2.3 Recovery determination of glucocorticoid standards

To determine the recovery of the extraction procedure for a set of 18 glucocorticoid standards, duplicate samples of 1 liter of surface water and stripped (hospital) wastewater were spiked with a mixture of compounds at a concentration level of 1 µg/L. An exception was made for cortisol, cortisone, prednisolone, prednisone and triamcinolone acetonide which were spiked at realistic environmental concentration levels being 210, 290, 230, 90 and 30 ng/L, respectively. Spiked surface waters / stripped wastewaters were extracted thrice with ethyl acetate and extracts were evaporated to dryness and redissolved in 50 µL of DMSO. The extracts were next diluted 1:9 (v:v) in ultrapure water and subsequently 1:1 (v:v) in acetonitrile (final volume 1 mL containing 5% DMSO) upon LC-MS/MS analysis.

### 2.4 Mass Spectrometric conditions

The LC-LTQ-FT Orbitrap MS system consisted of a Surveyor autosampler model Plus, a Surveyor quaternary gradient LC pump and an LTQ-FT Orbitrap mass spectrometer (Thermo Electron GmbH, Bremen, Germany). The linear ion trap (LTQ) part of the hybrid MS system was equipped with an Ion Max Electrospray Ionization (ESI) probe and operated in the positive ion mode. Full-scan accurate mass spectra (mass range from 50 to 600 Da) were obtained at high resolution (30,000 FWHM) and processed using Xcalibur v.2.0 software. The Electrospray source conditions were: capillary voltage 4.5 kV, heated capillary temperature 275°C, capillary voltage 30 V and tube lens 65 V. The mass spectrometer was operated in a data-dependent-acquisition (DDA) mode in which both MS and MS<sup>2</sup> spectra were acquired. In this mode, the acquisition software probes the MS spectra in real-time on a scan-by-scan basis to select the most intense parent ions for MS<sup>2</sup> analysis. The instrument is initially set to operate in full-scan ('survey') mode until an ion exceeds a preset threshold at which point the instrument switches into the product-ion mode (MS<sup>2</sup>). The product ions were generated in the LTQ trap at a normalized collision energy setting of 35% and using an isolation width of 2 Da. Five microliters of the final extract was injected into the LC system consisting of a 100 mmx2.0 mm i.d. column packed with 3-µm Omnisphere C<sub>18</sub> material (Varian-Chrompack, Middelburg, the Netherlands). The guard column was 4.0x2.0 mm i.d. packed with pellicular C<sub>18</sub> material (Phenomenex). The analytical column and the guard column were maintained at a temperature of 10°C in a column thermostat. A linear gradient of acetonitrile (5 to 60%) and water with 0.05% formic acid was used in 40 min and increased to 100 % acetonitrile in 5 min and held at this composition for an additional 10 min. The analytical column was re-equilibrated for 15 minutes between consecutive runs. The flow rate of the mobile phase was 0.3 mL/min (for further details, see 26).

### 2.5 GR CALUX bioassay and determination of relative potencies (REPS)

Human U2OS osteosarcoma cells, stably transfected with a GR-controlled luciferase reporter gene construct were cultured as described previously (27). GR CALUX bioassays were performed as described by Van der Linden *et al* (15). Briefly, cells were seeded into 96 wells plates with DF medium (without phenol red and supplemented with DCC stripped serum). After 24 hrs of incubation (37°C, 7.5% CO<sub>2</sub>), the medium was replaced by medium containing sample extracts (max 0.4% DMSO) for activity testing. After 24 hrs of exposure in triplicate, 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 (Berthold, Germany). To rule out any confounding influences due to toxicity of the extracts, cells were monitored for signs of cytotoxicity by means of light microscopy. On all plates, a dose response curve of the reference compound dexamethasone was included for adequate quantification of the response to nanograms dexamethasone equivalents per litre (ng dex EQs/L). Dose response curves of all individual glucocorticoid standards and dexamethasone were fitted using a sigmoidal fit with variable hill slope (log(agonist) vs. response – variable slope) in Graphpad Prism 5. Relative potencies (REPs) of all glucocorticoid standards were calculated as described.

### 3 Results and discussion

The DCC stripping of (hospital) wastewater as described resulted in a response < LOD in both the GR CALUX bioassay and the LC-MS/MS procedure, respectively (*data not shown*). Hence, we conclude that the stripping procedure with activated charcoal does remove glucocorticoid compounds to a sufficient extent, enabling a proper assessment of individual compound recoveries.

#### 3.1 Characteristics of instrumental and biological procedures for glucocorticoid analysis

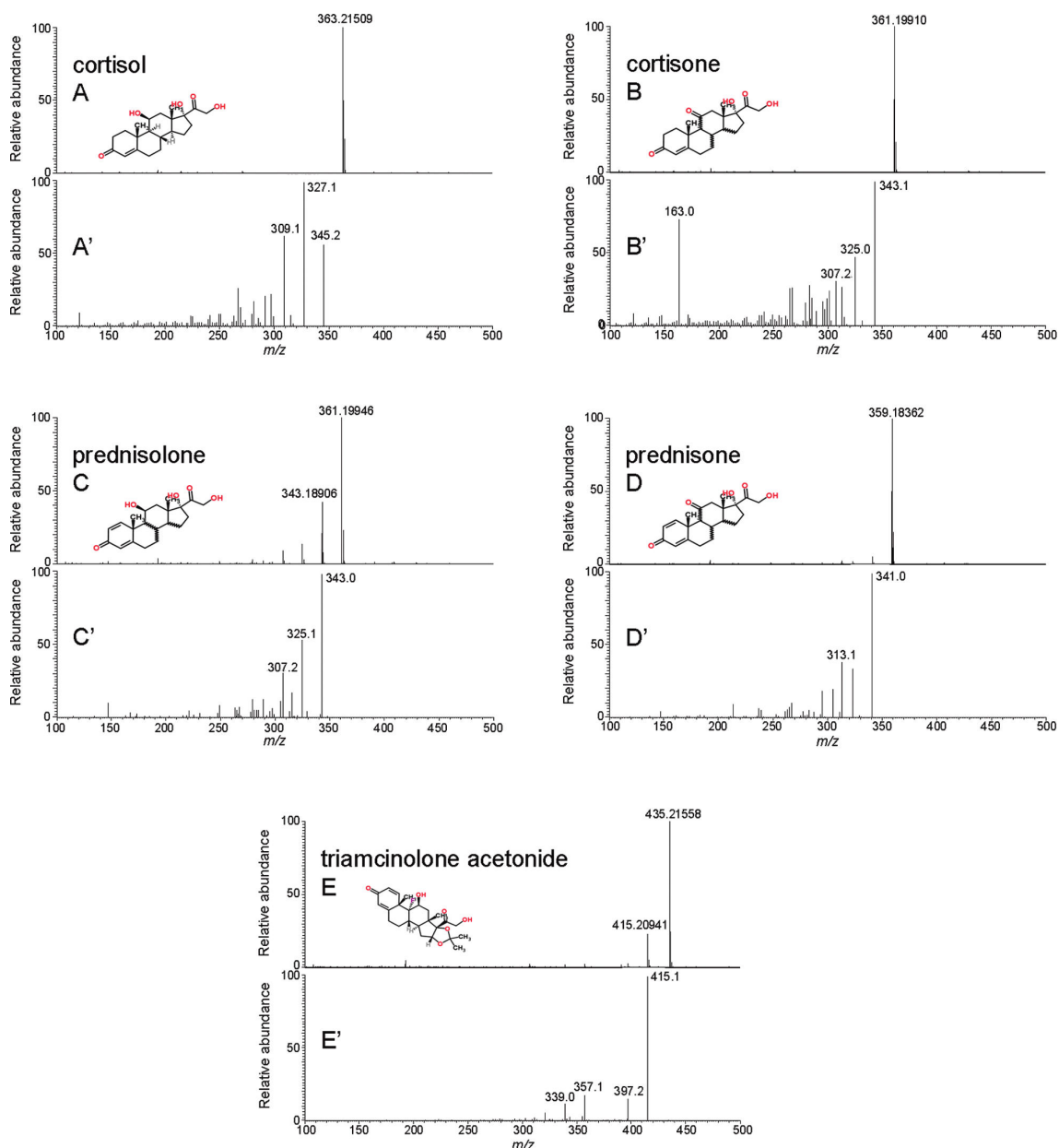
An overview of the analytical and GR CALUX bioassay detection characteristics of the 18 glucocorticoid standards is presented in Table 1.

**Table 1. Glucocorticoid standards investigated in this study, and characteristics of GR CALUX bioassay with LC-MS/MS**

| compound                   | CAS no.    | elemental composition | GR CALUX EC50 (nM) | REP <sup>a</sup>  | retention time on LC-MS/MS (min.) | m/z (theoretical) [M + H] <sup>+</sup> | product ion mass 1 (Da) | product ion mass 2 (Da) (relative abundance to product ion mass 1) |
|----------------------------|------------|-----------------------|--------------------|-------------------|-----------------------------------|--|-------------------------|--|
| aldosterone                | 52-39-1    | C21H28O5              | 112.2 ± 4.84       | 0.008 ± 0.06      | 20.97                             | 361.20095                              | 343                     | 315 (25%)  |
| amcinonide                 | 51022-69-6 | C28H35FO7             | 0.49 ± 0.04        | 1.7 ± 0.09        | 40.33                             | 503.24396                              | 483                     | 399 (90%)  |
| betamethasone <sup>b</sup> | 378-44-9   | C22H29FO5             | 1.02 ± 0.05        | 0.8 ± 0.06        | 25.71                             | 393.20718                              | 373                     | 355 (20%)  |
| cortisol                   | 50-23-7    | C21H30O5              | 11.4 ± 0.87        | 0.07 ± 0.08       | 22.56                             | 363.21660                              | 327                     | 309 (70%)  |
| cortisone                  | 53-06-5    | C21H28O5              | >1000 <sup>c</sup> | <0.0008 ± 0.00006 | 23.19                             | 361.20095                              | 343                     | 163 (80%)  |
| desoximetasone             | 382-67-2   | C22H29FO4             | 0.66 ± 0.03        | 1.3 ± 0.06        | 28.99                             | 377.21226                              | 357                     | 339 (30%)  |
| dexamethasone              | 50-02-2    | C22H29FO5             | 0.84 ± 0.03        | 1 ± 0.05          | 25.94                             | 393.20718                              | 373                     | 355 (30%)  |
| flunisolide                | 3385-03-3  | C24H31FO6             | 0.49 ± 0.03        | 1.7 ± 0.07        | 27.32                             | 435.21774                              | 321                     | 397 (100%)   |
| fluorometholone            | 426-13-1   | C22H29FO4             | 0.59 ± 0.03        | 1.4 ± 0.06        | 29.14                             | 377.21226                              | 357                     | 399 (50%)  |
| 6α-methylprednisolone      | 83-43-2    | C22H30O5              | 2.25 ± 0.14        | 0.4 ± 0.07        | 24.88                             | 375.21660                              | 357                     | 339 (60%)  |
| paramethasone <sup>b</sup> | 53-33-8    | C22H29FO5             | 1.14 ± 0.04        | 0.7 ± 0.05        | 25.71                             | 393.20718                              | 337                     | 355 (90%)  |
| prednicarbate              | 73771-04-7 | C27H36O8              | 4.75 ± 0.20        | 0.2 ± 0.06        | 39.80                             | 489.24829                              | 381                     | 471 (80%)  |
| prednisolone               | 50-24-8    | C21H28O5              | 3.68 ± 0.34        | 0.2 ± 0.1         | 22.40                             | 361.20095                              | 343                     | 325 (60%)  |
| prednisone                 | 53-03-2    | C21H26O5              | >500 <sup>c</sup>  | <0.002 ± 0.0004   | 22.68                             | 359.18530                              | 341                     | 313 (50%)  |
| rimexolone                 | 49697-38-3 | C24H34O3              | 0.83 ± 0.04        | 1 ± 0.06          | 41.58                             | 371.25807                              | 353                     | 295 (20%)  |
| triamcinolone              | 83474-03-7 | C21H27FO6             | 5.67 ± 0.23        | 0.2 ± 0.05        | 19.37                             | 395.18644                              | 375                     | 357 (30%)  |
| triamcinolone acetonide    | 8054-16-8  | C24H31FO6             | 0.37 ± 0.01        | 2.3 ± 0.04        | 27.23                             | 435.21774                              | 415                     | 397 (20%)  |
| triamcinolone hexacetonide | 5611-51-8  | C30H41FO7             | 3.40 ± 0.17        | 0.3 ± 0.06        | 46.94                             | 533.29091                              | 513                     | 397 (90%)  |

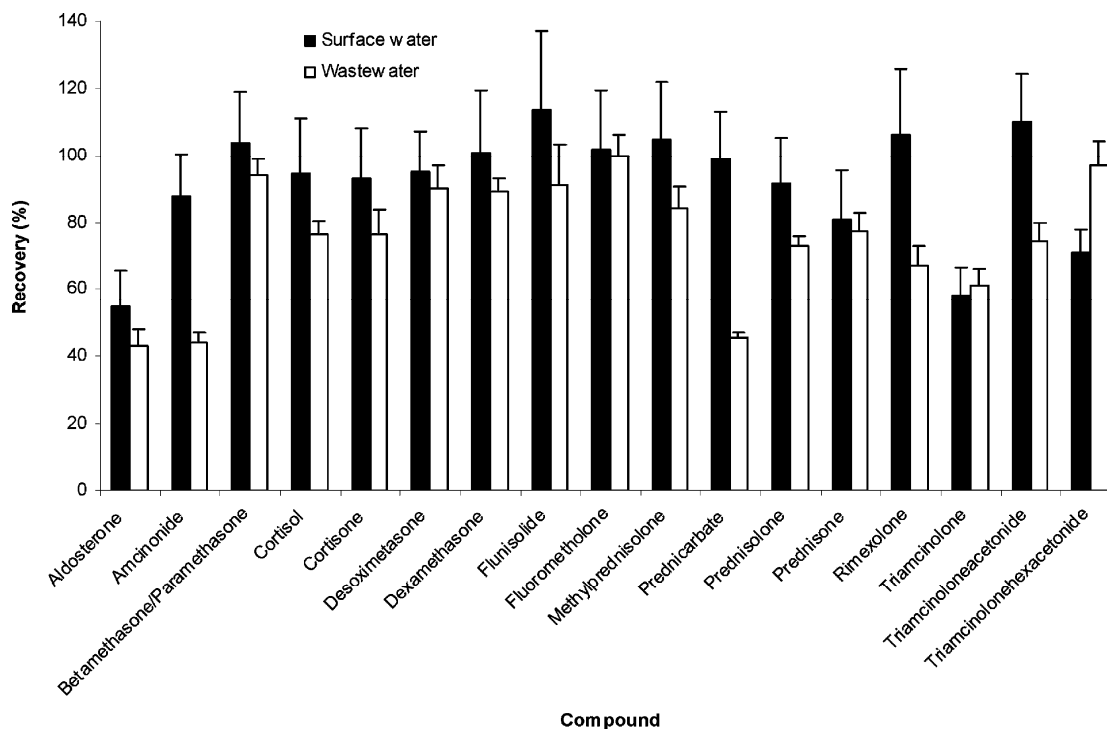
<sup>a</sup> Relative potencies (REPs) expressed as  $EC50_{\text{dexamethasone}}/EC50_{\text{compound}}$ . <sup>b</sup> Similar molecular weight and retention time. <sup>c</sup> Estimated; maximum induction was not reached.

The six point calibration curves of glucocorticoid standards recorded by means of LC-MS/MS were fitted quadratically ( $R^2 > 0.999$ ) in the 10-2000 ng/L range (final water concentrations). All samples were concentrated or diluted in such a way that concentrations fell within this range. The estimated instrumental limit of detection (LOD) was 10 ng/L for all glucocorticoid standards with the exception of aldosterone and prednisolone (20 ng/L). Glucocorticoid compounds could not be detected reliably below the given LODs. For mass spectrometric conditions initially both positive and negative ionization modes were tested on their suitability for the types of wastewater sampled. In the positive ionization mode we obtained better signal-to-noise ratios/sensitivities and therefore this mode was used to produce the final data as shown in the present study. Pseudo-molecular ion accurate masses and product ion masses recorded in the Orbitrap MS/MS are presented in Table 1. Figure 1A-E shows the high resolution MS spectra for a selection of 5 glucocorticoids. For confirmation purposes, MS<sup>2</sup> mass experiments (Figure 1A'-E') were performed with the LTQ to construct low resolution product ion spectra with nominal masses.



**Figure 1. High-Resolution full scan MS spectra (A-E) and low-resolution MS product ion spectra (A'-E') for a selection of glucocorticoid standards.**

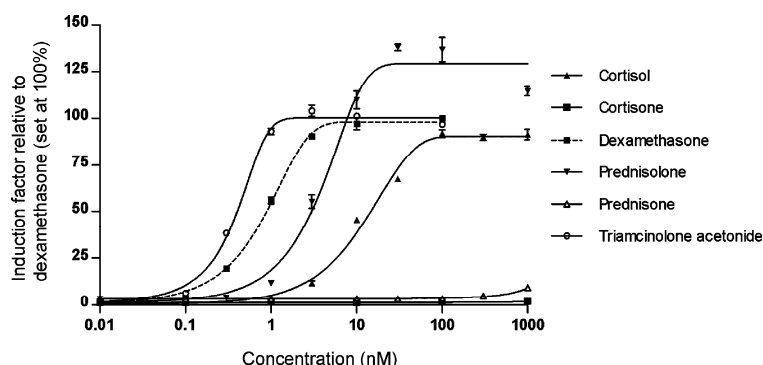
The recoveries of the extraction procedure for 18 glucocorticoid standards spiked to surface water ranged between  $55\% \pm 11\%$  (aldosterone) and  $114\% \pm 23\%$  (flunisolide) with an overall average of  $92\% \pm 17\%$  (Figure 2). The recovery of glucocorticoid standards spiked to stripped wastewater was generally somewhat lower, ranging from  $43\% \pm 5\%$  (aldosterone) to  $100\% \pm 6\%$  (fluorometholone) with an overall average of  $75\% \pm 18\%$ . This might be the result of ion suppression due to the more complex matrix of wastewater.



**Figure 2. Recoveries of glucocorticoids standards spiked into surface water and stripped wastewater. Bars represent mean values  $\pm$  standard deviation of two independent extractions at a spiked concentration level of  $1 \mu\text{g/L}$ .**

To investigate the potential effect of the stripping method on the recovery of glucocorticoids standards, a non-stripped wastewater extract was spiked with glucocorticoid standards. Table S1 (Supporting Information) shows that the “spike” recovery of spiked glucocorticoid compounds to a non-stripped wastewater extract is sufficient ( $>50\%$ ) with the exception for triamcinolone hexacetonide. However, the latter compound is less relevant for the present study since it was not detected in any sample extract. The relative potencies (REPs) of all glucocorticoid standards tested are listed in Table 1. Figure 3 presents GR CALUX concentration response curves for six major glucocorticoids found in the various wastewater extracts. From the total number of 18 glucocorticoid standards tested, five (amcinonide, desoximetasone, flunisolide, fluorometholone and triamcinolone acetonide) were more potent than the reference agonist dexamethasone. The main reason for choosing the latter compound as a reference standard is to allow comparison of the results to historical data. The least potent glucocorticoid standards were prednisone and cortisone, with REPs of  $< 0.002$  and  $< 0.0008$ , respectively. For the latter glucocorticoid standards, the REP was estimated since maximum induction in the GR CALUX bioassay was not reached. The method Limit of Detection (LOD) for the GR CALUX bioassay was  $1.35 \text{ ng dex EQs/L}$ .





**Figure 3. Concentration response curves for a selection of glucocorticoid standards obtained with the GR CALUX bioassay. The responses are expressed as induction factor relative to the reference agonist dexamethasone which is set at 100%. Each point represents a triplicate measurement  $\pm$  SD.**

### 3.2 Occurrence of glucocorticoids in wastewater extracts

In total, six different glucocorticoids were found in the various extracts (Table 2). Figure 4 shows a typical LC-high resolution Orbitrap MS chromatogram of hospital wastewater 2 including extracted-ion chromatograms (positive-ion mode) for the detected glucocorticoids. Overall, the highest concentration ( $\sim 2 \mu\text{g/L}$ ) was observed for prednisolone in hospital wastewater 2, while this compound was also found at lower concentrations in the extracts of industry- and hospital wastewater 1 from our previous study (Table 2). The synthetic glucocorticoid triamcinolone acetonide was found at relatively low concentrations ( $<40 \text{ ng/L}$ ) in hospital wastewaters 1-2 and in sewage treatment plant effluent. The fact that this compound was the only one found in treated sewage treatment effluent at a concentration level equal to raw hospital wastewater, may suggest that triamcinolone acetonide is poorly degraded or insufficiently removed during sewage treatment. However, this is an assumption and more research on the removal efficiency of this potent glucocorticoid compound is necessary.

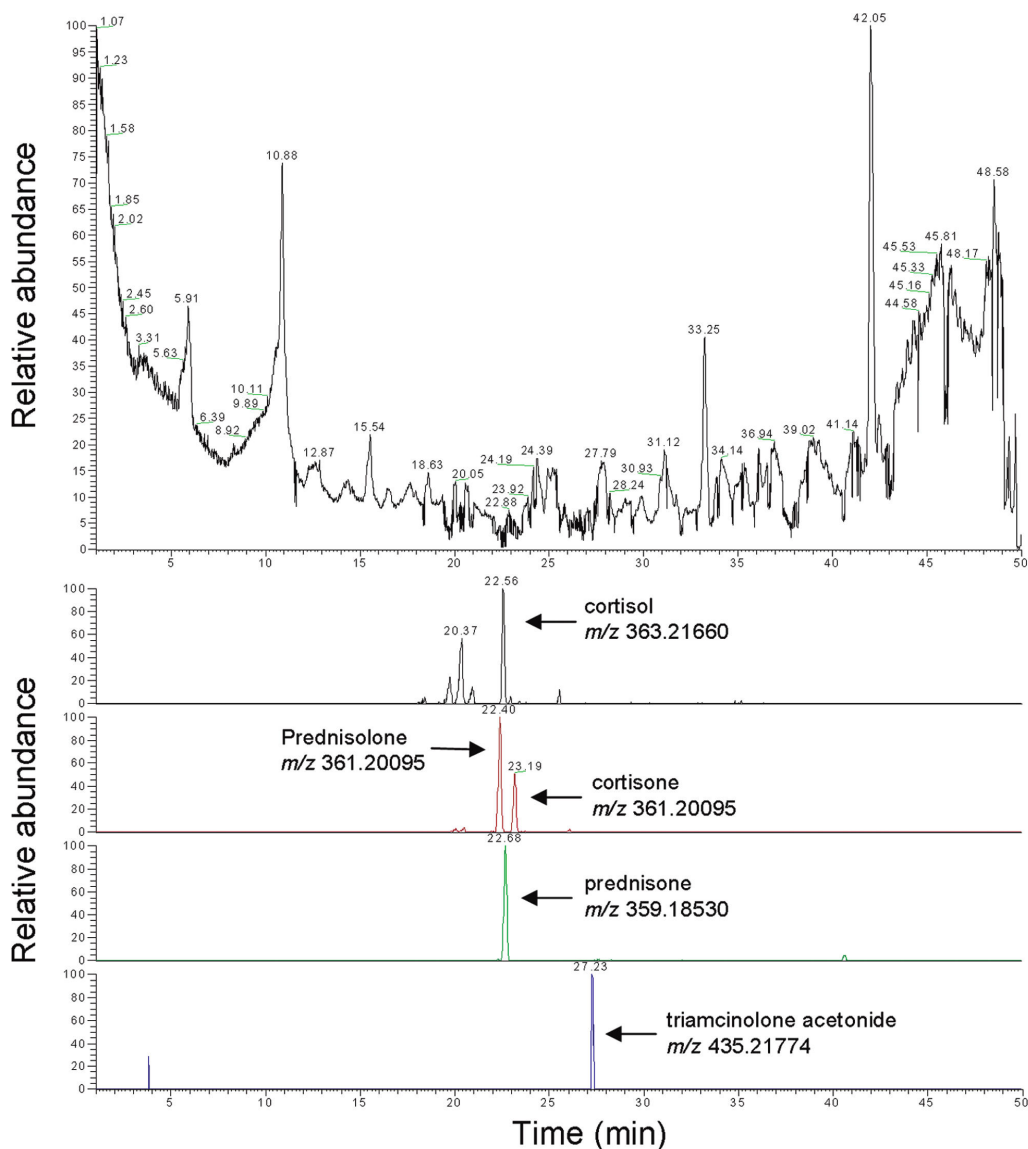
**Table 2. Concentration of hormones in extracts of various wastewaters collected in The Netherlands.**

| sample location<br>(sampling date)  | GR CALUX<br>(ng dex EQs/L) | glucocorticoids          | LC-MS/MS measured<br>concentration <sup>a</sup><br>(ng/L) | relative<br>potency (REP) <sup>b</sup> | predicted<br>(total) dex EQs <sup>c</sup><br>(ng dex EQs/L) |
|---|----------------------------|--------------------------|---|--|---|
| industry wastewater<br>(12/05/2006;<br>Van der Linden<br>et al., 2008)                | 243 $\pm$ 32               | cortisol                 | 13 $\pm$ 1 (80%)  | 0.07 $\pm$ 0.08                        | 0.92 $\pm$ 1.1  |
|   |                            | cortisone                | 26 $\pm$ 3 (80%)  | <0.0008 $\pm$ 0.00006                  | max. 0.02 $\pm$ 0.1   |
|   |                            | dexamethasone            | 90 $\pm$ 9 (89%)  | 1 $\pm$ 0.05                           | 90.0 $\pm$ 0.1  |
|   |                            | prednisolone             | 247 $\pm$ 28 (73%)  | 0.2 $\pm$ 0.1                          | 49.3 $\pm$ 0.5  |
|   |                            | prednisone               | N.D. (77%)  | -                                      | -   |
|   |                            | triamcinolone acetonide  | N.D. (74%)  | -                                      | -   |
|   |                            | $\Sigma$ 140.2 $\pm$ 1.3 |   |  |   |
| hospital wastewater 1<br>(12/11/2006;<br>Van der Linden<br>et al., 2008)              | 96 $\pm$ 13                | cortisol                 | 275 $\pm$ 27 (80%)  | 0.07 $\pm$ 0.08                        | 19.2 $\pm$ 1.1  |
|   |                            | cortisone                | 381 $\pm$ 44 (80%)  | <0.0008 $\pm$ 0.00006                  | max. 0.03 $\pm$ 0.8   |
|   |                            | dexamethasone            | N.D. (89%)  | -                                      | -   |
|   |                            | prednisolone             | 315 $\pm$ 30 (73%)  | 0.2 $\pm$ 0.1                          | 63.0 $\pm$ 0.5  |
|   |                            | prednisone               | 117 $\pm$ 12 (77%)  | <0.002 $\pm$ 0.0004                    | Max. 0.2 $\pm$ 0.2  |
|   |                            | triamcinolone acetonide  | 41 $\pm$ 4 (74%)  | 2.3 $\pm$ 0.04                         | 93.2 $\pm$ 0.1  |
|   |                            | $\Sigma$ 175.7 $\pm$ 1.5 |   |  |   |
| hospital wastewater 2<br>(8/19/2009)  | 609 $\pm$ 79               | cortisol                 | 301 $\pm$ 29 (80%)  | 0.07 $\pm$ 0.08                        | 21.1 $\pm$ 1.1  |
|   |                            | cortisone                | 472 $\pm$ 54 (80%)  | <0.0008 $\pm$ 0.00006                  | max. 0.4 $\pm$ 0.1  |
|   |                            | dexamethasone            | N.D. (89%)  | -                                      | -   |
|   |                            | prednisolone             | 1918 $\pm$ 182 (73%)                                      | 0.2 $\pm$ 0.1                          | 383.6 $\pm$ 0.5   |
|   |                            | prednisone               | 545 $\pm$ 58 (77%)  | <0.002 $\pm$ 0.0004                    | 1.1 $\pm$ 0.2   |
|   |                            | triamcinolone acetonide  | 14 $\pm$ 1 (74%)  | 2.3 $\pm$ 0.04                         | 31.1 $\pm$ 0.1  |
|   |                            | $\Sigma$ 437.2 $\pm$ 1.3 |   |  |   |
| paper mill treated<br>wastewater<br>(01/15/2007;<br>Van der Linden<br>et al., 2008)   | 11 $\pm$ 2                 | cortisol                 | N.D. (80%)  | -                                      | -   |
|   |                            | cortisone                | N.D. (80%)  | -                                      | -   |
|   |                            | dexamethasone            | N.D. (89%)  | -                                      | -   |
|   |                            | prednisolone             | N.D. (73%)  | -                                      | -   |
|   |                            | prednisone               | N.D. (77%)  | -                                      | -   |
|   |                            | triamcinolone acetonide  | N.D. (74%)  | -                                      | -   |
|   |                            | 0                        |   |  |   |
| sewage treatment<br>plant effluent<br>(11/15/2006;<br>Van der Linden<br>et al., 2008) | 38 $\pm$ 13                | cortisol                 | N.D. (80%)  | -                                      | -   |
|   |                            | cortisone                | N.D. (80%)  | -                                      | -   |
|   |                            | dexamethasone            | N.D. (89%)  | -                                      | -   |
|   |                            | prednisolone             | N.D. (73%)  | -                                      | -   |
|   |                            | prednisone               | N.D. (77%)  | -                                      | -   |
|   |                            | triamcinolone acetonide  | 14 $\pm$ 1 (74%)  | 2.3 $\pm$ 0.04                         | 31.1 $\pm$ 0.1  |
|   |                            | $\Sigma$ 31.1 $\pm$ 0.1  |   |  |   |

**High resolution mass spectrometric identification and quantification of glucocorticoid compounds in various wastewaters in The Netherlands**

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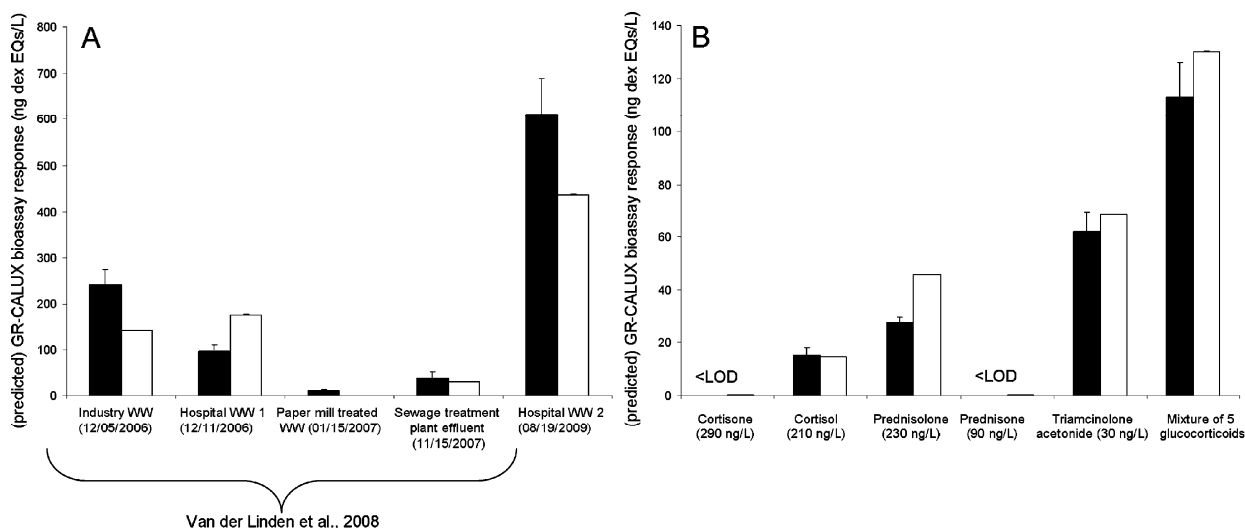
Interestingly, it was observed that hospital wastewater 1 contained the same set of glucocorticoids as found in hospital wastewater 2. However, the concentration levels of prednisone and prednisolone determined in hospital wastewater 2 were much higher than those observed in hospital wastewater 1. It is unlikely that this discrepancy can be explained by degradation of glucocorticoid compounds during storage, since repeated measurements show that concentration levels of glucocorticoids standards stored during 1.5 y (at  $-18^{\circ}\text{C}$ ) remain relatively constant (Supporting Information, Table S2). Possibly, a higher periodical prescription of glucocorticoid containing medicines to hospitalized patients during the time of collection of hospital wastewater 2 has led to the higher concentration levels observed.



**Figure 4. Full scan LC-Orbitrap MS chromatogram (top) of hospital wastewater 2 and extracted-ion chromatograms (positive-ion mode; bottom panels) for a selection of 5 glucocorticoids based on accurate mass with a mass window of 5 ppm.**

The compounds prednisolone and prednisone belong to frequently applied glucocorticoids and their prescribed use in The Netherlands amounts to respectively 19.2 kg and 3.3 kg per 1,000,000 inhabitants in 2007 (Van der Aa, personal communication based on data obtained from the Foundation for Pharmaceutical Statistics, SFK). These data cover oral consumption and injections (prednisolone only) of sale quantities of prescribed drugs delivered by public pharmacies. Total consumption in The Netherlands will be higher, since sale quantities by hospitals are not included. For comparison, Besse and co-workers (28) present an estimated total annual consumption in France of 57.6 kg and 23.8 kg per

1,000,000 inhabitants for prednisolone and prednisone in 2004, respectively. These data cover sale quantities of all prescribed drugs delivered in France by both hospitals and pharmacies. Considering the fact that glucocorticoids such as cortisol, cortisone, prednisone and dexamethasone are relatively well removed during sewage treatment (% removal > 98%) (16), only low concentrations of these compounds in surface waters are expected. Chang and co-workers (16) indeed found low concentrations for a set of six glucocorticoids (cortisol, cortisone, prednisolone, prednisone, dexamethasone and 6 $\alpha$ -methylprednisolone) in the range of 0.02-4.2 ng/L in samples taken from the Tonghui and Qing rivers in China. This is further corroborated by the data we have reported earlier on GR activity measured in three Dutch surface waters which ranged from 0.39 to 1.3 ng dex EQs/L (15). When chemically determined concentrations of the individual glucocorticoids present in the sample extracts are multiplied by their corresponding relative potencies (REPs) and subsequently summed (Figure 5A), it was found that for most extracts the so-called (chemically) “predicted” GR CALUX response was lower than the actually measured GR CALUX response. In extracts of industry wastewater, hospital wastewater 2 and sewage treatment plant effluent the predicted response explaining the experimentally obtained GR CALUX response was in the range of approximately 60%-80%. As hypothesized by others in the case of estrogenic activity of water samples, this may suggest the presence of unidentified agonists present in the extracts (29, 30). Indeed, several other glucocorticoids were identified (with unknown REPs) in the extracts by means of an accurate mass based screen (flucortin, fluprednidene and hydrocortisone aceponate; *results not shown*), however compound identities could not be confirmed due to a lack of analytical standards. In addition, various glucocorticoid compounds could have been present in the extracts in concentration levels below the given LOD. However, at a level below 10 ng/L their contribution to the overall glucocorticoid activity in potent wastewater extracts such as hospital wastewater 1 and 2 would only be marginal, given their REPs. In hospital wastewater 1 the predicted GR CALUX response amounted to approximately 170% of the experimentally obtained GR CALUX response. The reason for this observation is poorly understood at present, but an explanation may be sought in unknown antagonistic compounds present in the mixture. To investigate the combined activity of mixtures of glucocorticoids in the GR CALUX bioassay in more detail, stripped wastewater was spiked with five individual glucocorticoid standards and a mixture of the same set of glucocorticoid standards followed by extraction and subsequent GR CALUX bioanalysis of the extracts. Figure 5B illustrates that low-potency glucocorticoids such as cortisol and prednisone did not contribute to the overall response of the mixture. Furthermore, it can be observed that the predicted GR CALUX bioassay response is in good agreement with the experimentally obtained GR CALUX bioassay response.



**FIGURE 5. Measured GR CALUX bioassay response (black bars) versus “predicted GR CALUX bioassay response” (white bars, corrected for individual compound recoveries in wastewater) elicited by (A) the various wastewater (WW) extracts and (B) by extracts of five individual glucocorticoid standards and a mixture (at the same concentration levels as the individual glucocorticoid standards). GR CALUX bioassay responses of the extracts were interpolated into a dexamethasone standard curve (0.03-100 nM) and response magnitude is presented as nanogram of dexamethasone equivalents/L (ng dex EQs/L).**

Therefore, it can be concluded that the combined behavior of the set of selected glucocorticoids can be best described by the concept of concentration addition. For chemicals - such as (xeno)estrogens and androgens - that interact with well-defined molecular targets concentration additivity has been observed before (25, 31, 32, 33). However, these results cannot explain the discrepancy between the predicted GR CALUX bioassay response and the experimental GR CALUX bioassay response that is observed in hospital wastewater 1. Therefore, alternative explanations must be sought for this observation, such as (i) interexperimental variation in the REP leading to a deviation in the predicted GR CALUX bioassay response (25) (ii) the presence of unidentified compounds that might have modulated the activity of the identified GR agonists and finally (iii) the presence of unidentified agonists that were not taken into account while calculating the predicted GR CALUX bioassay response.

At present, there are insufficient data to predict the environmental concentrations of glucocorticoid compounds in surface waters. However, as shown by Chang and co-workers (16), glucocorticoids seem to be relatively well removed in sewage treatment plants. This is consistent with the fact that environmental concentrations of glucocorticogenic activity in surface waters measured so far were in the (low) ng/L range (15, 16). The significance for aquatic biota of (chronic) exposure to low concentrations of glucocorticoids is unknown at the present and deserves more research attention. From a technological perspective, the removal efficiency of specific glucocorticoids in drinking water purification plants needs further attention. Studies addressing this issue are underway in our laboratory.



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