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Final report

Steroid hormones in the aquatic environment - Insights from new analytical methods for corticosteroids and progestogens

von:
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On behalf of the German Environment Agency

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Abstract: Steroid hormones in the aquatic environment - Insights from new analytical methods for corticosteroids and progestogens

Endocrine active therapeutics such as steroid hormones gain increasing focus in environmental research as it was reported that they cause endocrine disruption in aquatic organisms exposed to environmentally relevant concentrations. Data about occurrence and fate of steroid hormones are scare. In this study occurrence of about 60 steroids in wastewater treatment plant effluents and surface waters as well as the fate and biodegradability of corticosteroids and progestogens in activated sludge treatment were investigated. Corticosteroids and progestogens were prioritized in views of their high prescription and potency to impact endocrine-related functions. A reliable analytical method for the simultaneous determination of mineralocorticoids, glucocorticoids and progestogens in the aquatic environment was developed. Utilizing this method three mineralocorticoids, 23 glucocorticoids and 10 progestogens could be detected in the analyzed samples, many of them were detected for the first time in the environment, particularly in Germany and the EU.

Moreover, biodegradation studies were designed to enable the comparison of the biodegradability and transformation processes in activated sludge treatment for structure-related steroids under standardized aerobic conditions. Half-lives ranged from <0.5 h to >14 d suggesting large variations in the biodegradability. Transformations products were identified by LC-HRMS and transformation pathways were proposed. Based on these data relationships between structure and stability were elaborated. Several of the newly identified transformation products were persistent and some of them could be detected in wastewater treatment plant effluents and surface waters. Hence, these transformation products should be also included in environmental monitoring.

Kurzbeschreibung: Steroidhormone in der aquatischen Umwelt – Erkenntnisse aus neuen analytischen Nachweiserfahren für Corticosteroide und Progestagene

Endokrin aktive Substanzen wie Steroidhormone stehen vermehrt im Interesse der Umweltwissenschaften, da endokrine Effekte in Wasserorganismen bereits bei sehr niedrigen Umweltkonzentrationen beschrieben sind. Allerdings ist die Datenlage über das Vorkommen und das Verhalten von Steroidhormonen in der Umwelt sehr lückenhaft. In dieser Studie wurde das Vorkommen von ca. 60 Steroiden im Kläranlagenablauf und in Oberflächengewässern untersucht. Des Weiteren wurde das Verhalten und die Bioabbaubarkeit von Corticosteroiden und Progestagenen, die beide hohe Verschreibungszahlen und ein hohes endokrines Potential aufweisen, während einer Aktivschlammbehandlung betrachtet. Zu diesem Zweck wurde eine Analysenmethode für die simultane Bestimmung von Mineralcorticoiden, Glukokortikoiden und Progestagenen in wässrigen Proben entwickelt. Mit Hilfe dieser Methode konnten drei Mineralcorticoide, 23 Glukokortikoide und 10 Progestagene in den analysierten Proben nachgewiesen werden. Viele der Substanzen wurden zum ersten Mal in der Umwelt, insbesondere in Deutschland und der EU, nachgewiesen. Die Abbaustudien wurden so konzipiert, dass diese einen strukturabhängigen Vergleich der Abbaubarkeit und der Transformationsprozesse unter standardisierten aeroben Bedingungen ermöglichten. Halbwertzeiten reichten von <0,5 Stunden bis >14 Tagen, was die große Bandbreite in der Stabilität innerhalb der untersuchten Steroide verdeutlicht. Die entstandenen Transformationsprodukte wurden mittels LC-HRMS identifiziert und entsprechende Abbauwege postuliert. Basierend auf diesen Ergebnissen wurden Zusammenhänge zwischen der Stabilität der Substanzen und deren Molekülstruktur ausgearbeitet. Viele der neu identifizierten Transformationsprodukte waren persistent und manche von ihnen konnten im Kläranlagenablauf und in Oberflächengewässern nachgewiesen werden. Daher sollten Transformationsprodukte in zukünftige Umweltstudien mit einbezogen werden.

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List of abbreviations

ASE	Accelerated solvent extraction
BfG	Bundesanstalt für Gewässerkunde
CE	Collision energy
DP	Declustering potential
ESI	Electrospray ionization
EWG	Einwohnergleichwerte
GPC	Gel permeation chromatography
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
IGB	Leibniz-Institut für Gewässerökologie und Binnenfischerei
LOQ	Limit of quantification
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS-MS	Tandem mass spektrometry
PMT	Pharmaceuticals and related metabolites and transformation products
PSI	Pound-force per square inch
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe
RPLC	Reversed-phase liquid chromatography
SOC	Synthetic oral contraceptive
SPE	Solid phase extraction
SPM	Suspended particulate matter
TOC	Total organic carbon
PMT	Pharmaceuticals and related metabolites and transformation products
TP	Transformation product
GC	Glucocorticoid
PG	Progestogen
MC	Mineralocorticoid

Summary

Aims

The aim of the project was the development of a highly sensitive analytical method for determination of steroidal pharmaceuticals and their transformation products in aquatic systems. For prioritization and identification of the main transformation products biodegradability and transformation processes were investigated by biodegradation studies.

Content

Selection of analytes

In a first step steroidal pharmaceuticals were prioritized by consumption data and known risk potentials. Glucocorticoids, mineralocorticoids, and progestogens are widely used synthetic steroids in human therapy. Despite their relative low production volumes and expectable low environmental concentrations these substances pose an ecotoxicologically high risk potential due to strong endocrine activity. Highly sensitive methods for quantification of these steroid hormones in surface water are still lacking. Furthermore, degradation products of steroid hormones formed during biological wastewater treatment are widely unknown.

Method development

Synthetic steroids pose a threat on aquatic organisms at very low concentrations down to the pg/L range. Consequently, extreme sensitive analytical methods are necessary to detect such low concentrations. In this project the analytical method developed by Weizel et al. [1] was optimized for 47 glucocorticoids (GC), 6 mineralocorticoids (MC), 20 progestogens (PG) including metabolites and transformation products providing limits of quantification down to 0.02 ng/L. These were realized by high volume solid phase enrichment and a following silica gel clean-up. After chromatographic separation the analytes were detected by a highly sensitive and selective tandem-mass spectrometric approach (LC-MS/MS).

Degradation of steroid hormones

During biological wastewater treatment steroid hormones are transformed into various transformation products. To date, no comprehensive studies are available dealing with the (bio)transformation processes of a broad range of steroid hormones with activated sludge. To close this knowledge gap, lab scaled treatment experiments according to Wick et al. [2] were included in the project. Degradation behavior of the investigated steroid hormones ranged from very fast degradation to recalcitrance. Presumably initial degradation processes were inhibited by the engineered structure of the synthetic GCs. Therefore, the desired benefits of the designed structures for medicinal treatment go along with the disadvantage of a limited elimination in the municipal wastewater treatment. In this context, structural features that led to a distinct increase in stability could be characterized. Thus, with the results obtained in this study, the (bio)degradability of the synthetic steroids in biological wastewater treatment can be predicted to some extent from their chemical structure. Also, a more mechanistic understanding of the persistence of steroid hormones can improve the interpretation of their monitoring data in general, since the resistance of the steroid compounds against degradation in WWTP seems to have a larger effect on the environmental levels in the receiving waters than the consumption pattern. Most of the identified primary TPs can be expected as still active and since they could be found in WWTP effluent and surface water a threat for aquatic life. Thus, TP formation must be considered for the environmental risk profile. Especially, the evidence of the conversion of progestogenic to estrogenic compounds and the formation of potentially hazardous TPs emphasizes the need of a more comprehensive risk assessment of synthetic steroids. TP

formation should be considered as a strong assessment criterion for industrial discharge permissions into municipal WWTPs with biological treatment and in the discussion about the necessity of a fourth treatment stage.

Occurrence of synthetic steroid hormones

73 synthetic steroids including glucocorticoids, mineralocorticoids and progestogens and their identified TPs were quantified in WWTP effluents and surface water. The occurrence of dienogest, mometasonefuroate, flumethasone pivalate, and the metabolites 6β-hydroxy dienogest, 6β-hydroxy triamcinolone acetonide, 7α-thiomethyl spironolactone, and 11α-hydroxy canrenone could be shown for the first time. In addition, a ubiquitous presence of GC monoesters, betamethasone propionate, betamethasone valerate, and 6α-methylprednisolone propionate was found in WWTP effluents and surface water. Highest concentrations were found for triamcinolone acetonide ranging from 5.5 ng/L to 28 ng/L in WWTP effluents.

Conclusions

This study demonstrates synthetic steroids used in medication are despite their rather low production volumes a substance class of emerging concern and should be added to current regulatory monitoring activities for estrogens such as ethinylestradiol (EE2). Due to chemical modifications such as fluorination or introduction of acetals, biodegradation of synthetic steroids in WWTP can be rather low. This stands in contrast to natural steroids, which are considered as biodegradable. This reluctance leads to an appreciable emission and widespread occurrence of some synthetic steroids in aquatic systems. In this project several synthetic steroids have been detected in WWTP effluent and surface waters. Thus, the investigated synthetic hormones could be responsible for elevated endocrine activities in WWTP effluent and contaminated surface water. However, there is still a lack of data regarding the eco-toxicological risk of this substance class. This is especially true for TPs and for mixtures of synthetic steroids. Changes in receptor selectivity caused by transformation reaction should be intensely investigated. However, since effect levels reported so far are in the range of the concentrations found in surface water samples in this study, a comprehensive monitoring of both the parent steroid hormones as well as their human metabolites and environmental TP is recommended.

Results of this project have been published in form of three peer-reviewed articles:

Weizel et al. (2018): Occurrence of Glucocorticoids, Mineralocorticoids, and Progestogens in Various Treated Wastewater, Rivers, and Streams (https://doi.org/10.1021/acs.est.7b06147)

Weizel et al. (2020a): Analysis of the aerobic biodegradation of glucocorticoids: Elucidation of the kinetics and transformation reactions (https://doi.org/10.1016/j.watres.2020.115561)

Weizel et al. (2020b): Fate and behavior of progestogens in activated sludge treatment: Kinetics and transformation products (https://doi.org/10.1016/j.watres.2020.116515)

These provide technical details of the analytical methods and a detailed description of method development.

Zusammenfassung

Ziel

Ziel des Projekts war die Entwicklung von hochsensitiven analytischen Methoden zur Bestimmung von Steroidwirkstoffen und deren Transformationsprodukte in Gewässern. Durch Abbaustudien sollten erfolgte eine Priorisierung auf Basis der Stabilität der Wirkstoffe und die Identifizierung der wichtigsten Transformationsprodukte.

Inhalt

Auswahl der Analyten

In einem ersten Schritt erfolgte eine Priorisierung der bekannten Steroidwirkstoffe nach Verbrauchsmengen und einem ggfs. bekannten Umweltrisiko. Glucocorticoide, Mineralcorticoide und Gestagene sind viel eingesetzte synthetische Steroide in diversen therapeutischen Ansätzen. Trotz ihrer vergleichsweisen geringen Produktionsmengen und damit einhergehend geringen zu erwartenden Umweltkonzentrationen beinhalten diese Substanzen auf Grund ihrer hohen endokrinen Wirksamkeit ein hohes ökotoxikologisches Risikopotential. Hochsensitive Analysenmethoden für die Quantifizierung dieser Steroidhormone in Oberflächengewässern stehen zurzeit nicht zur Verfügung. Des Weiteren sind die Abbauprodukte von Steroidhormonen, die während der biologischen Abwasserbehandlung entstehen, weites gehend unbekannt.

Methodenentwicklung

Synthetische Steroide stellen bereits bei sehr geringen Konzentrationen im pg/L Bereich eine Gefahr für aquatische Organismen dar. Dementsprechend sind extrem sensitive Methoden erforderlich, um diese hochwirksamen Stoffe in diesen geringen Spuren nachweisen zu können. In diesem Projekt wurde die Methode von Weizel et al. [1] für den Nachweis von 47 Glucocorticoiden (GC), 6 Mineralcorticoiden (MC), 20 Gestagenen inklusive Metaboliten und Transformationsprodukten mit einer Nachweisgrenze von 0,02 ng/L optimiert. Erreicht wurde dies durch eine Festphasenanreicherung von großen Probenvolumina mit einem anschließenden Silikagel-Clean-Up. Die Detektion erfolgte durch eine hochsensitive und selektive LC.MS/MS-Methode.

Abbauverhalten von Steroidhormonen

Während der biologischen Abwasserbehandlung werden Steroidhormone in verschiedenste Produkte umgewandelt. Bisher gab es keine Abbaustudien in Belebtschlamm für eine große Bandbreite an Steroidhormonen. Um diese Wissenslücke zu schließen wurden Laborexperimente zum Abbauverhalten gemäß Wick et al. [2] durchgeführt. Die Abbaubarkeit der untersuchten Steroidhormone reichte von einer sehr schnellen Degradation bis hin zu einer hohen Stabilität. Der Grund dafür liegt vermutlich in der Inhibierung initialer Abbaureaktionen bedingt durch chemische Modifikation in der Molekülstruktur in den synthetischen Steroiden. Die durch das Wirkstoffdesign gewonnenen therapeutischen Vorteile gehen folglich einher mit einer stark reduzierten biologischen Abbaubarkeit in Kläranlagen. Vor diesem Hintergrund wurden Zusammenhänge zwischen Strukturmerkmalen und der Stabilität der Verbindungen abgeleitet. Somit ermöglichen die Studienergebnisse eine grobe Abschätzung der Bioabbaubarkeit von synthetischen Steroiden in Kläranlagen anhand der chemischen Struktur. Des Weiteren ermöglicht das mechanistische Verständnis des Abbauverhaltens von Steroidhormonen eine bessere Interpretation von Monitoringdaten, da der Einfluss der Bioabbaubarkeit auf die Umweltkonzentrationen einen größeren Einfluss hat als die Verbrauchsmengen. Viele der identifizierten Transformationsprodukte (TPs) können als

weiterhin biologisch aktiv angesehen werden und das Vorkommen dieser in Oberflächengewässern als eine Gefahr für aquatische Lebewesen angesehen werden. Folglich muss die Bildung von TPs bei der Umweltbewertung berücksichtigt werden. Insbesondere die Umwandlung von Gestagenen in östrogenartige Strukturen bedarf einer genaueren Risikobewertung. Die Bildung von TPs sollte auch ein wichtiges Bewertungskriterium bei der Erteilung von Einleitungsgenehmigungen von Industrieabwässern in Kläranlagen mit biologischer Reinigungsstufe sein und bei der Diskussion über die Notwendigkeit einer vierten Reinigungsstufe berücksichtigt werden.

Vorkommen von synthetischen Hormonen in der Umwelt

73 synthetische Steroide inclusive Glucocorticoide, Mineralcorticoide und Gestagenen sowie deren identifizierten TPs wurden in Kläranlagenabläufen und Oberflächengewässern analysiert. Dienogest, Mometasonefuroat, Flumethasonepivalat und die Metaboliten 6β-Hydroxydienogest, 6β-Hydroxytriamcinolonacetonid, 7α-Thiomethylspironolacton und 11α-Hydroxycanrenon konnten erstmalig nachgewiesen werden. Zusätzlich wurde ein ubiquitäres Vorkommen von GC-Monoestern, Betamethasonpropionat, Betamethasonvalerat und 6α-Methylprednisolonpropionat in Kläranlagenabläufen und Oberflächengewässern festgestellt. Die höchsten Konzentrationen wurden für Triamcinoloneacetonid im Bereich von 5,5 bis 28 ng/L in Kläranlagenabläufen gefunden.

Schlussfolgerungen

Diese Studie belegt, dass synthetische Steroide trotz ihrer eher geringen Produktionsmengen eine besorgniserregende Substanzklasse darstellen. Daher sollten sie im Gewässermonitoring neben Östrogenen wie Ethinylestradiol (EE2) berücksichtigt werden. Auf Grund von chemischen Modifikationen wie der Einbau von Fluoratomen oder Acetalgruppen kann die Bioabbaubarkeit von synthetischen Steroiden in Kläranlagen im Vergleich zu natürlichen Steroiden stark herabgesetzt sein. Diese erhöhte Stabilität führt zu einer nachweisbaren Emission und damit zu einem großflächigen Vorkommen von manchen synthetischen Steroiden in Gewässern. In diesem Projekt konnten mehrere synthetische Steroide, teilweise erstmalig, in Kläranlagenabläufen und Oberflächengewässern nachgewiesen werden. Damit könnten die hier untersuchten synthetischen Hormone für die erhöhten endokrinen Aktivitäten in Kläranlagenabläufen und belasteten Oberflächengewässern verantwortlich sein. Auf jeden Fall gibt es noch große Wissenslücken bezüglich des ökotoxikologischen Risikos durch diese Substanzklasse. Dies trifft vor allem auf TPs und Mischungen synthetischer Steroide zu. Änderungen in der Rezeptorselektivität bedingt durch Transformationsreaktionen sollten in weiteren Studien untersucht werden. Da bisher berichtete Effektkonzentrationen im Bereich der in dieser Studie gefundenen Umweltkonzentrationen liegen, ist ein umfangreiches Monitoring für synthetische Hormone und deren Humanmetaboliten sowie TPs zu empfehlen.

Die Projektergebnisse wurden in Form von peer-reviewed Artikeln veröffentlicht:

Weizel et al. (2018): Occurrence of Glucocorticoids, Mineralocorticoids, and Progestogens in Various Treated Wastewater, Rivers, and Streams (https://doi.org/10.1021/acs.est.7b06147)

Weizel et al. (2020a): Analysis of the aerobic biodegradation of glucocorticoids: Elucidation of the kinetics and transformation reactions (https://doi.org/10.1016/j.watres.2020.115561)

Weizel et al. (2020b): Fate and behavior of progestogens in activated sludge treatment: Kinetics and transformation products (https://doi.org/10.1016/j.watres.2020.116515)

1 Introduction

The project ran from March 2018 until April 2020 as a follow-up project of the project "Method development for analysis of pharmaceuticals in environmental samples" (Forschungskennzahl: 3715674130, final report https://www.umweltbundesamt.de/publikationen/method-development-for-analysis-of-pharmaceuticals). Results of this project have been published in form of three peer-reviewed articles [1], [3], [4]. These provide technical details of the analytical methods and a detailed description of method development.

1.1 Project background

Since the last decades the increasing findings of pharmaceuticals in the aquatic environment and their potential risk for the aquatic life has become an urgent issue. The number of pharmaceutical residues detectable in the low µg/L range in the environment is still increasing. Endocrine active therapeutics such as steroid hormones can cause adverse effects in aquatic organisms even at very low environmental concentrations [5]. Until now the focus of the environmental concern and consequently the research focus is lying on the estrogenic compounds. In contrast, environmental data about occurrence and fate of the other steroidal hormone classes glucocorticoids, mineralocorticoids, progestogens and androgens in aquatic systems are rare and data are insufficient for a risk assessment [6]. This project will fill this data gap for the 3 hormone classes glucocorticoids, mineralocorticoids and progestogens.

After uptake and distribution pharmaceuticals can be metabolized within the human body. In most cases oxidative biotransformation reactions lead to more polar metabolites, which are excreted after conjugation to glucuronic acid, sulfate or amino acids via urine. The excretion pattern varies strongly between the different pharmaceuticals and even individuals. So, there are pharmaceuticals which are excreted mostly unchanged and others which are completely metabolized such as allopurinol to its metabolite oxipurinol. Hence, the high number of potential contaminants resulting from the various steroid pharmaceuticals on the market can be expected to be further increased by metabolization reactions leading to a high number of human metabolites.

For most pharmaceuticals effluents of municipal wastewater treatment plants (WWTPs) are indicated as the major source in the aquatic environment. Parent pharmaceuticals and their human metabolites are continuously discharged into the WWTPs by domestic and hospital wastewater, after they were excreted, washed off from the human skin or directly disposed. With few exceptions biological wastewater treatment is not able to mineralize organic micropollutants, but rather a high variety of transformation products (TPs) are formed (reviewed in [7]). These TPs were discharged together with the remaining parent pharmaceuticals and metabolites by WWTPs. Metabolites and TPs might have similar activities or in a few cases even an enhanced eco-toxicological potential as reported in [8]and [9]. Thus, it is crucial to know which stable TPs are formed in biological WWTP processes and are discharged by municipal WWTPs into rivers and streams.

However, so far both metabolites and transformation products of steroid pharmaceuticals are scarcely considered in environmental studies and consequently there is a lack of data concerning occurrence, distribution and fate.

Therefore, the aim of this project was to investigate the fate and behavior of selected steroid hormones in activated sludge treatment. Identified TPs were included into a highly sensitive analytical method for determination of glucocorticoids, mineralocorticoids and progestogens as

well as several human metabolites[1]. The method was applied to WWTP effluent and surface water samples in order to investigate occurrence of the newly identified TPs.

1.2 Conceptual design

To reach the project aims, a project concept was elaborated including an in-depth review of scientific literature, innovative method development, biodegradation experiments and extensive monitoring campaigns. In a first step a selection of metabolites and already identified TPs of prioritized steroidal pharmaceuticals was done (ref. chapter 2). For these a highly sensitive analytical method for quantification in water was developed and validated (ref. chapter 3). Biodegradation experiments were carried out to identify major transformation products since biotransformation reactions of many steroid hormones were not well characterized so far (ref. chapter 4). Based on the study results transformation reactions and pathways were deviated and rules for a prediction of stabilities by molecular structure established. The newly developed analysis method was utilized to evaluate the occurrence and distribution of the analytes in environmental samples from different aquatic systems.

2 Selection of metabolites and transformation products

Due to their mode of action as modulators for the nucleus steroid receptors, steroid hormones pose a high potential risk to the aquatic environment [10] and conform to the requirements of the indicative list of the main pollutants in the European Water Framework Directive [11]. However, the watch list of substances for an EU-wide monitoring [12] currently contains only the estrogenic steroids 17β-estradiol, estrone and 17α-ethinylestradiol whereas other steroid types, such as progestogens or corticosteroids are not considered so far. Among the steroid family, corticosteroids and progestogens were prioritized in views of high prescription and potency to impact endocrine-related functions.

Progestogens and corticosteroids are used in many fields of modern medicine. In contrast to estrogens, a huge number of steroid derivatives are applied, which makes the regulation and environmental assessment more difficult. Even though a few studies documented the presence of synthetic corticosteroids [13], [14], [15], [16], [17] and progestogens [6], [18], [19], [20] in the environment, there is little consistent and comprehensive knowledge about their occurrence, fate and biodegradability in the wastewater treatment systems and finally in the aquatic environment. Recalcitrant compounds or still active transformation products could be responsible for so far unexplainable endocrine activities in wastewater effluents. To close this gape of knowledge and to estimate the risk potential there is an urgent need to elucidate biodegradation and transformation reaction of these synthetic steroid hormones.

In a first step steroidal pharmaceuticals were prioritized by consumption data and known risk potentials. Furthermore, already known human metabolites and TPs were selected from literature. Since this literature search revealed an extensive gape of knowledge, biodegradation experiments were conducted for identification of the main TPs.

3 Analytical method

For quantification of steroidal pharmaceuticals, their metabolites and transformation products in environmental samples highly sensitive and selective analytical methods are required. Liquid chromatographic methods with tandem mass spectrometric detection (LC-MS-MS) are state-of-the-art. Due to the very low environmental concentrations an enrichment step is necessary. The analytical methods described in the final report of the precursor project were complemented by the metabolites and transformations products or optimized and finalized, if necessary.

For analysis of water samples and water phases of aerated incubation experiments with activated sludge the method described by Weizel et al. [1], [3],[4] [21] was used. In brief:

- 500 mL of filtered WWTP effluent (<1µm glass fiber GF6, Whatmann) or 100 mL water phase from degradation experiments were spiked with 2 ng of each isotope labeled internal standard and enriched by SPE (6 mL, 500 mg, C18ec Chromabond, Macherey-Nagel).
- Cartridges were then eluted 3 times with 3 mL methanol and evaporated to dryness by a gentle nitrogen stream at 40°C.
- After reconstitution with 0.3 mL n-hexane and 0.7 mL acetone, the extracts were loaded onto dried and pre-conditioned silica gel cartridges (1 g, 6 mL, Chromabond SiOH, Machery-Nagel) for sample clean up.
- The cartridges were eluted 3 times with 2 mL acetone/n-hexane (7:3).
- Afterwards, the extracts were evaporated via a gentle nitrogen stream at 40°C and were finally dissolved in 250 µL methanol and 250 µL Milli-Q prior to LC-MS/MS analysis.

It has to be noted that the quantification of carboxylic TPs was achieved without silica gel extraction, since these polar TPs were not desorbed from silica gel cartridges under the used conditions.

Extracts were analyzed via liquid chromatography with high resolution mass spectrometry (LC-HRMS) for compound identification and tandem mass spectrometry (LC-MS/MS) for quantification. The analytical method was optimized and validated for the analysis of 73 steroid hormones in surface water and WWTP effluents [1], [21]. The recoveries of all steroids ranged from 70 % to 115 % in WWTP effluents and from 73 % to 112 % in surface water. While the limits of quantification (LOQ) ranged from 0.05 ng L^{-1} to 6.2 ng L^{-1} in WWTP effluents and from 0.02 ng L-1 to 0.5 ng L^{-1} in surface waters.

4 (Bio)Transformation of steroid hormones

During biological wastewater treatment steroid hormones can be transformed into various transformation products. To date, no comprehensive studies are available dealing with the (bio)transformation processes of a broad range of steroid hormones with activated sludge, even though steroids were the first organic molecules manufactured by industrial fermentative processes. Numerous microbiological transformation reactions for specific steroid substrates are already well known (reviewed in [22], [23]). However, the microbial community of wastewater treatment systems is quite diverse [24] and thus, an identification of TPs in controlled lab scale incubation experiments with activated sludge can be an expedient approach to understand the removal processes of steroid hormones. Altogether, there is a major deficit in knowledge about the fate and behavior of this chemical group. To close this gap of knowledge lab-scaled treatment experiments were conducted within the project.

4.1 Microcosm experiments with activated sludge

Activated sludge was sampled from the aeration tank of a municipal WWTP (WWTP Koblenz, capacity: 320,000 population equivalents, sludge retention time: approx. 12 d, hydraulic retention time: approx. 6 h, total suspended solids: approx. 4.0 $g_{ss}L^{-1}$). Within six hours after sampling, the inoculum was aliquoted and diluted ten-fold with WWTP effluent. Dilution was utilized in order to minimize sorption and matrix effects in chemical analysis. The concentrations of suspended solid C_{ss} in the prepared microcosms were approx. 0.4 $g_{ss}L^{-1}$ and were analyzed for every experimental sequence accordingly. During the incubation, the sludge slurries were continuously aerated with synthetic air and stirred over the complete experiments. In addition, temperature and pH were controlled during incubation. The elucidation of the kinetics was utilized at steroid concentration of 200 ng L^{-1} in triplicate. Steroids were spiked in groups and the remaining steroid concentration was monitored at each sampling point. Equivalent sterile experiments (activated sludge autoclaved at 121 °C for 60 min) were run in parallel to differentiate between biotic/abiotic degradation and to assess the impact of sorption. Experiments for TP identification were conducted at 2 µg L^{-1} for glucocorticoids, and at 500 µg L^{-1} for the progestogens without sample enrichment. Each experiment was spiked with one individual steroid and additionally, one negative control batch without the analyte spike was run in parallel (for further details please refer [4], [3]).

4.2 Identification of transformation products

High resolution mass spectrometry (via a hybrid quadrupole time of flight mass spectrometer, QTOF, TripleTOF 5600, Sciex), equipped with ESI as the ion source was used for the TP identification. Both polarization modes were applied in separate measurements. For analyte separation, the same chromatography as described in section 3 was used.

The acquired data were processed by a non-target approach. Automated peak-picking and alignment procedure were assessed via a data evaluation script in R [25]. Briefly, the XICs (extracted ion chromatogram) were extracted using the xcms package [26]. The XICs were then checked for local maxima. After signal-to-noise calculation (SN ≥ 3) and further filtering, a peak list was generated. Finally, the features were automatically aligned between the samples by comparing m/z and retention time (RT) of all generated features in all samples. The aligned peak lists were obtained by grouping those within a tolerance window of m/z = 5 ppm and RT = 10 s.

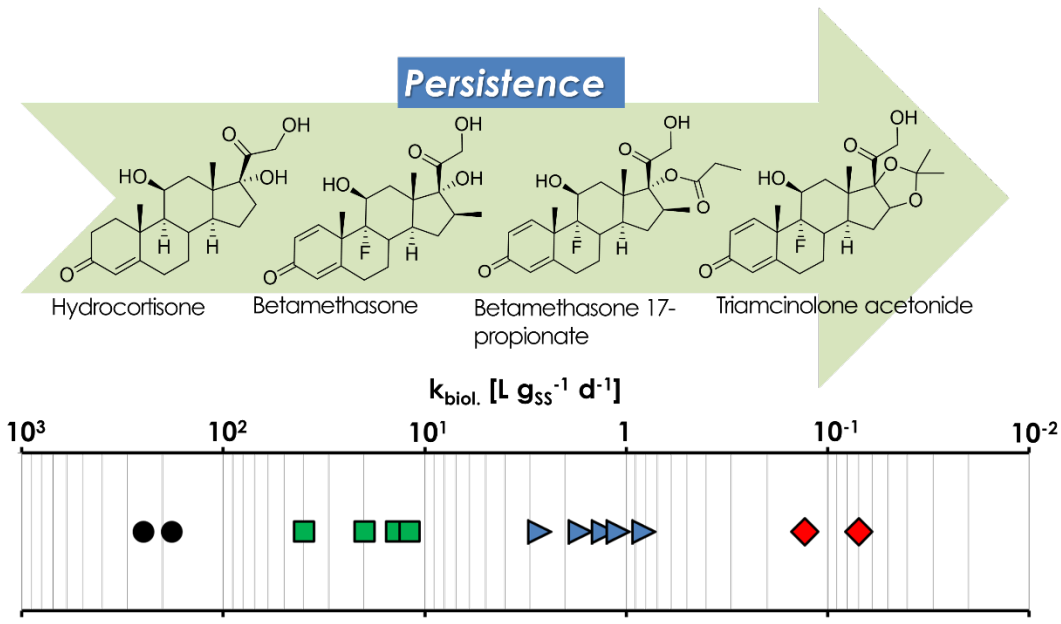
Afterwards, the aligned peak lists were searched for clear differences in the time courses between spiked batches and non-spiked controls for potential TPs. As criteria for prioritization

of TPs, an increasing or decreasing trend or an intermediate maximum of the intensity within the incubation time was defined. In addition, only features detected in at least three consecutive samples were considered. The tentatively identified TPs were verified by the measurements of reference standards through a comparison of RT, accurate masses and MS^2 fragmentations. In cases without commercially available standards, chemical structures of the TPs were proposed by combining the analytical information and by analogy to the MS^2 fragmentation of authentic results obtained from other experiments in this study (for further details please refer [4],[3]).

4.3 Fate and behavior of glucocorticoids in activated sludge treatment

13 GCs were incubated in the aerobic lab scale degradation experiments with activated sludge as described above and kinetic parameters were evaluated. For 12 GCs, main TPs were identified and transformation pathways proposed. As shown in Table 1 and Figure 1, the rate constants of the biodegradation range over four orders in magnitude. For hydrocortisone and prednisolone, a very fast and complete removal was observed (DT_{50}<0.5 h). Diesters such as betamethasone dipropionate or methylprednisolone aceponate and their alcohol steroids (betamethasone, methylprednisolone, beclomethasone) showed an efficient removal (DT_{50}: 1 to 5 h). An increase in stability was observed for monoester GCs such as betamethasone 17-valerate, beclomethasone –17-propionate, clobetasol propionate and fluticasone propionate. In addition, budesonide, a non-halogenated GC with a cyclic ketal moiety showed a moderately removal (DT_{50}: 10 to 72 h). Most recalcitrant steroids were the fluorinated acetonides triamcinolone acetonide and fluocinolone acetonide (DT_{50}: > 14 d).

Figure 1: Increasing stability of glucocorticoids in biological wastewater treatment with activated sludge. The degradation rate constants of the 13 analyzed steroids ranged over four orders of magnitude (Source: own figure, BfG).



The biodegradation of GCs in contact with activated sludge seems to be inhibited up to ten-fold (in comparison to hydrocortisone), if a halogen substituent is located at ring B, as it is the case for beclomethasone and betamethasone. Therefore, fluorine and chlorine at C9 substantially enhanced the stability of the steroids in activated sludge treatment. Dodson and Moir [27] described the generalized degradation route of steroids by the initial hydroxylation at C9, which subsequently leads to the ring fission between C9and C10 following the 9,10-seco pathway [28].

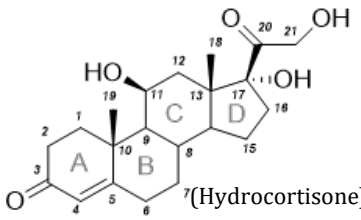
It can be assumed that this reaction pathway is inhibited in 9α-halo steroids and led to the observed microbiological stability. Particularly for clobetasol propionate and fluticasone propionate an elevated stability was observed, thus it can be concluded that structural modifications at the C21 position lead to a higher stability due to their missing hydroxyl group at the C21 position which is decisive for the migration of the ester moiety. Therefore, for both GCs, the C17-ester hydrolysis can be excluded as the initial degradation step. Furthermore, steroids with cyclic ketal groups exhibited elevated stability towards microbiological degradation, while combinations of structural characteristics led to even higher persistency (e.g. triamcinolone acetonide). Overall, these results are useful to assess the biodegradability of GCs since structure moieties could be indicated which lead to higher stability in the treatment with activated sludge.

Table 1: Kinetic parameter for the biodegradation of GCs in contact with activated sludge

Substance	Abbreviation	$k_{biol.}$ [L g_{ss}^{-1} d^{-1}]	DT_{50} [d]	Correlation coefficient r
Prednisolone	PNL	250±20	<0.02	0.999±0.002
Hydrocortisone	HCOR	180±10	<0.02	0.998±0.003
Betamethasone 17,21-dipropionate	BMSdiprop	40±1	0.06	0.953±0.023
Betamethasone	BMS	20±1	0.11	0.999±0.001
Beclomethasone	BEC	14±1	0.17	0.999±0.001
6α-Methylprednisolone	MPNL	12±1	0.19	0.999±0.001
Beclomethasone 17-propionate	BECprop17	2.7±0.4 [b)]	0.26	0.997±0.001
Betamethasone 17-valerate	BMSval17	1.7±0.1 [b)]	0.41	0.995±0.006
Budesonide	BDN	1.3±0.1	1.8	0.987±0.005
Fluticasone propionate	FLUprop	1.1±0.1	2.1	0.952±0.016
Clobetasol propionate	CLOprop	0.82±0.15	2.8	0.993±0.001
Fluocinolone acetonide	FCNact	0.13±0.03	>14	0.921±0.053
Triamcinolone acetonide	TRIact	0.07±0.03	>14	0.811±0.135

The elucidation of the TPs revealed similar reactions as well as specific transformation reaction for several GCs, depending on their structural characteristics. Based on the identified TPs, a variety of enzymatically mediated reactions were postulated. An overview of the suggested reactions that were observed for the individual GCs is shown in Table 2.

Table 2: Detected transformation reactions of GCs in contact with activated sludge. Propose of the association of TP formation and involved structure moieties.



[7](Hydrocortisone)

Transformation reaction	Identified transformation products	Structural Moieties
Δ^1-dehydrogenation	Hydrocortisone, 1,2-dihydro fluticasone propionate, 1,2-dihydro clobetasol propionate	3-one-4-ene
Δ^1-hydrogenation	Fluticasone propionate, budesonide, fluocinolone acetonide	1,4-diene-3-one
Δ^4-hydrogenation	Clobetasol propionate, fluticasone propionate, budesonide, triamcinolone acetonide, fluocinolone acetonide, 1,2-dihydro steroids	1,4-diene-3-one, 3-one-4-ene
Oxidative side-chain degradation	Beclomethasone, betamethasone	17α-hydroxy-17β-(2-hydroxyacetyl)
Ester hydrolysis	Beclomethasone 21-propionate, beclomethasone dipropionate, betamethasone 21-propionate, betamethasone dipropionate, betamethasone 21-valerate, 6α-methylprednisolone 21-propionate, 6α-methylprednisolone aceponate	C21-ester
Ester isomerization (Acyl-migration)	Beclomethasone 17-propionate, betamethasone 17-propionate, betamethasone 17-valerate, 6α-methylprednisolone 17-propionate	21-hydroxy-C17α-ester
9,11-Epoxidation (dechlorination)	Beclomethasone propionate, beclomethasone	9α-chloro-11β-hydroxy
Oxidative hydroxylation at C21	Budesonide, fluocinolone acetonide, triamcinolone acetonide	C16, C17-ketals
Sulfation	Beclomethasone, betamethasone, 17-oxo betamethasone	-
Hydroxylation at ring A	Clobetasol propionate	-
Hydroxylation at ring B	Beclomethasone, betamethasone, 17-oxo betamethasone	-

For betamethasone and beclomethasone, oxidative side chain degradation at C17 could be identified as the major degradation pathway, leading to potential androgenic structures (17-oxo/17-hydroxy TPs). GCs with a ketal moiety showed the formation of ketoacids at C17 and fluticasone propionate was found to hydrolyze to its 17β-carboxylic acid.

It has to be noted that the incubation of the TPs triamcinolone acetonide 21-carboxylic acid and fluticasone 17β-carboxylic acid propionate revealed pronounced stability towards biodegradation in activated sludge treatment. Thus, these TPs were indicated as persistent in the lab experiments.

Further identified transformation reactions were regioselective hydrogenation at ring A, hydroxylations (presumably at ring A and B) and dechlorination in case of 9α-chloro GCs such as beclomethasone.

From a more general view, the elucidation of the main TPs of a broad range of GCs showed that initial degradation processes, responsible for the biodegradation of hydrocortisone, were inhibited by the engineered structure of the synthetic GCs. Therefore, the desired benefits of the designed structures for medicine go along with the disadvantage of a limited elimination in the municipal wastewater treatment.

The range of investigated compounds enabled new insights into the biodegradation process of GCs. Together with the elucidation of TPs and transformation reactions the results allowed the following conclusions:

- Steroid hormones, in general, are considered as biodegradable. Within the group of GCs the results highlights that certain synthetic steroids are recalcitrant in aerobic wastewater processes, especially triamcinolone acetonide and further ketal steroids. Therefore, there is a strong need for efficient removal strategies, in particular for persistent hormones with elevated potencies.
- The spectrum of the analyzed GCs showed a pronounced variability in aerobic degradation. In this context, structural features that led to a distinct increase in stability could be characterized. Thus, on the basis of the current results, the (bio)degradability of GCs in biological wastewater treatment can be predicted by means of the chemical structure. Also, the results from previous studies can be better interpreted, as differences in stability primarily lead to particular glucocorticoid burden in the environment and not exclusively the consumption pattern.

A more detailed discussion can be found in Weizel et al. [3].

4.4 Fate and behavior of progestogens in activated sludge treatment

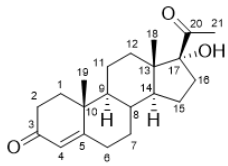
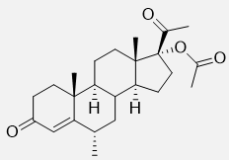
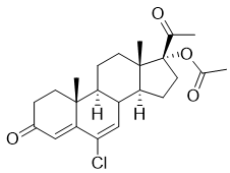
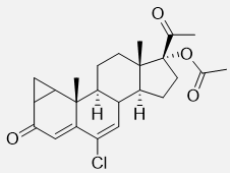
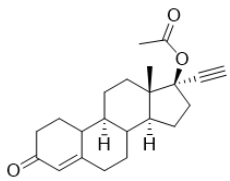
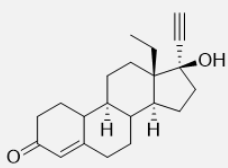
Nine commonly used synthetic oral contraceptives (SOCs) (17α-hydroxyprogesterone, medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, levonorgestrel, dienogest, norethisterone acetate, etonogestrel, drospirenone) were incubated in aerobic lab scale degradation experiments with activated sludge as described in chapter 4.1. Further experiments were conducted for the identification of the main TPs of six SOCs (medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, dienogest, etonogestrel, norethisterone acetate).

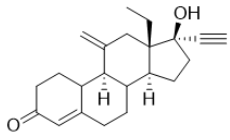
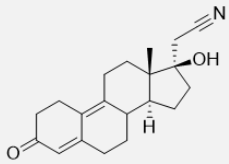
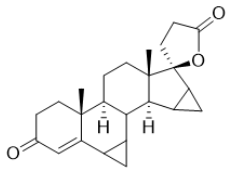
As shown in Table 3, the degradation kinetics revealed a fast and complete removal after 48 h for most of the SOCs. Cyproterone acetate and dienogest were the most recalcitrant steroids of the analyzed SOCs with half-lives of 8.65 h and 4.55 h, respectively. Thus, only moderate removals of these SOCs can be predicted in full scale WWTPs. In contrast to the analyzed GCs, a

clear cluster of structure and stability was not observed. But a closer look reveals relationships of structure moieties and biodegradability within the specific groups of the SOCs.

The stability observed for the 17α-hydroxyprogesterone derivatives increases from endogenous 17α-hydroxyprogesterone to medroxyprogesterone acetate, to chlormadinone acetate and to cyproterone acetate. Such behavior could not be derived for the 19-nortestosterone derivatives. Their degradation rates seemed to be determined by a more complex combination of different factors. This is most likely due to the larger differences in their structure, which lead to more divergent degradation pathways of the individual 19-nortestosterone SOCs.

Table 3: Kinetic parameter for the biodegradation of SOCs in contact with activated sludge. Steroids are grouped by types and sorted in ascending order of stability. The uncertainty is expressed as the standard deviation of the replicates. Experimental conditions were as follows: n= 3, c_0= 200 ngL^{-1}, c_{ss} = 0.37 g_{ss}L^{-1}.

Type	Substance	Structure	$k_{biol.}$ [L g_{ss}^{-1} d^{-1}]	DT_{50} [h]	Pearson coefficient
17α-Hydroxyprogesterone	17α-Hydroxyprogesterone		-*)	<0.5	-
	Medroxyprogesterone acetate		35 ± 3	1.31 ±0.13	0.998
	Chlormadinone acetate		25 ± 2	1.77 ± 0.11	0.995
	Cyproterone acetate		5.2 ± 0.1	8.65 ± 0.21	0.998
19-Nortestosterone	Norethisterone acetate		-*)	<0.5	-
	Levonorgestrel		94 ± 5	0.48 ± 0.03	0.999

Type	Substance	Structure	$k_{biol.}$ [L g_{ss}^{-1} d^{-1}]	DT_{50} [h]	Pearson coefficient
	Etonogestrel		47 ± 2	0.96 ± 0.05	0.852
	Dienogest		9.9 ± 0.2	4.55 ± 0.11	0.998
Spironolactone	Drospirenone		110 ± 10	<0.5	0.920

*) Not enough points above LOQ for calculation. Minimum of points for calculation was set to 3.

Regarding the overlap of analyzed PGs, the kinetic behavior is in good accordance with previously reported results from a lab scale degradation study [29], since the calculated half-lives were in the same range. In addition, the concentrations in German WWTP effluents underline the behavior of the analyzed SOCs, as only dienogest and cyproterone acetate could be frequently detected in the effluents up to 4.4 ng L^{-1} and 3.7 ng L^{-1}, respectively [1].

According to the observed kinetics, it is very unlikely that PGs with half-lives below 1 h (in this study: 17α-hydroxyprogesterone, levonorgestrel, norethisterone acetate, etonogestrel, drospirenone) are present in municipal WWTP effluents in appreciable concentrations, since the total consumption of the individual PGs are relatively low in comparison to other pharmaceuticals. However, limited studies reported scattered detections of these PGs [30], [18], [31], [32]. On the other hand, endogenous PGs such as progesterone (not included in this study) or 17α-hydroxyprogesterone, were frequently detected in WWTP effluents in spite of their high degradation rates [33], [1]. These findings can be linked to the natural origin leading to much higher influent concentrations in comparison to other PGs [34].

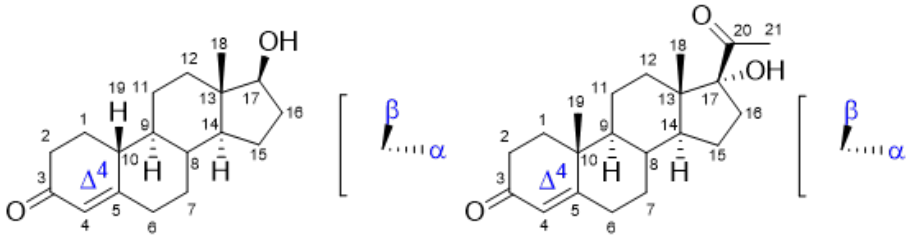
Moreover, numerous TPs were detected via HRMS. As the major transformation reactions hydrogenation/dehydrogenation of ring A and non-selective hydroxylations were identified for the 17α-hydroxyprogesterone derivatives (medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate) as well as for 19-nortestosterone derivatives (dienogest, norethisterone acetate, etonogestrel). Seven of the identified TPs were confirmed by reference standards.

The biodegradation of cyproterone acetate revealed an almost quantitative transformation to 3α-hydroxy cyproterone acetate which is reported to be genotoxic [35]. A comparative evaluation of the TPs formed and the steroid structure, displayed the inhibition of several transformation reactions which led to increasing stability, especially for cyproterone acetate.

For the 19-nortestosterone derivatives, the aromatization of the steroid ring A was identified, leading to the formation of estrogen-like TPs. For instance, the degradation of norethisterone acetate revealed the formation of 17α-ethinylestradiol, a well-known and very potent synthetic estrogen (Fig. 2).

In addition, the identified Δ^4-hydrogenation of norethisterone revealed the formation of the active TP 5α-dihydro norethisterone in appreciable quantity. Therefore, the project results highlighted that wastewater treatment of 19-nortestosterone SOCs with activated sludge lead to the formation of active steroid TPs. TP formation can cause an interaction with other steroidal receptors.

Table 4: Detected transformation reactions of SOCs in contact with activated sludge. Propose of the association of TP formation and involved structure moieties.



19-Nortestosterone Derivatives 17α-Hydroxyprogesterone Derivatives

Transformation reaction	Identified transformation products	Structural Moieties
Δ^1-dehydrogenation	Delmadinone Acetate, 1,2-Dehydro-Medroxyprogesterone Acetate	3-one-4-ene
Δ^1-hydrogenation	4,5-Dihydro- Medroxyprogesterone Acetate, 4,5-Dihydro-Chlormadinone Acetate, 4,5-Dihydro-Cyproterone Acetate	1,4-diene-3-one
Δ^4-hydrogenation	5α-dihydro norethisterone	1,4-diene-3-one, 3-one-4-ene
Aromatisation	17α-ethinylestradiol	1,4-diene-3-one
Reduction of carbonyl group at ring A	3a-Hydroxy-cyproterone Acetate, 3a-Hydroxy-3,4-dihydro-delmadinone Acetate	3-one
Ester hydrolysis	Norethisterone	C21-ester
Sulfation	17α-ethinylestradiol	-

The main scientific outcomes for the degradation study of SOCs were:

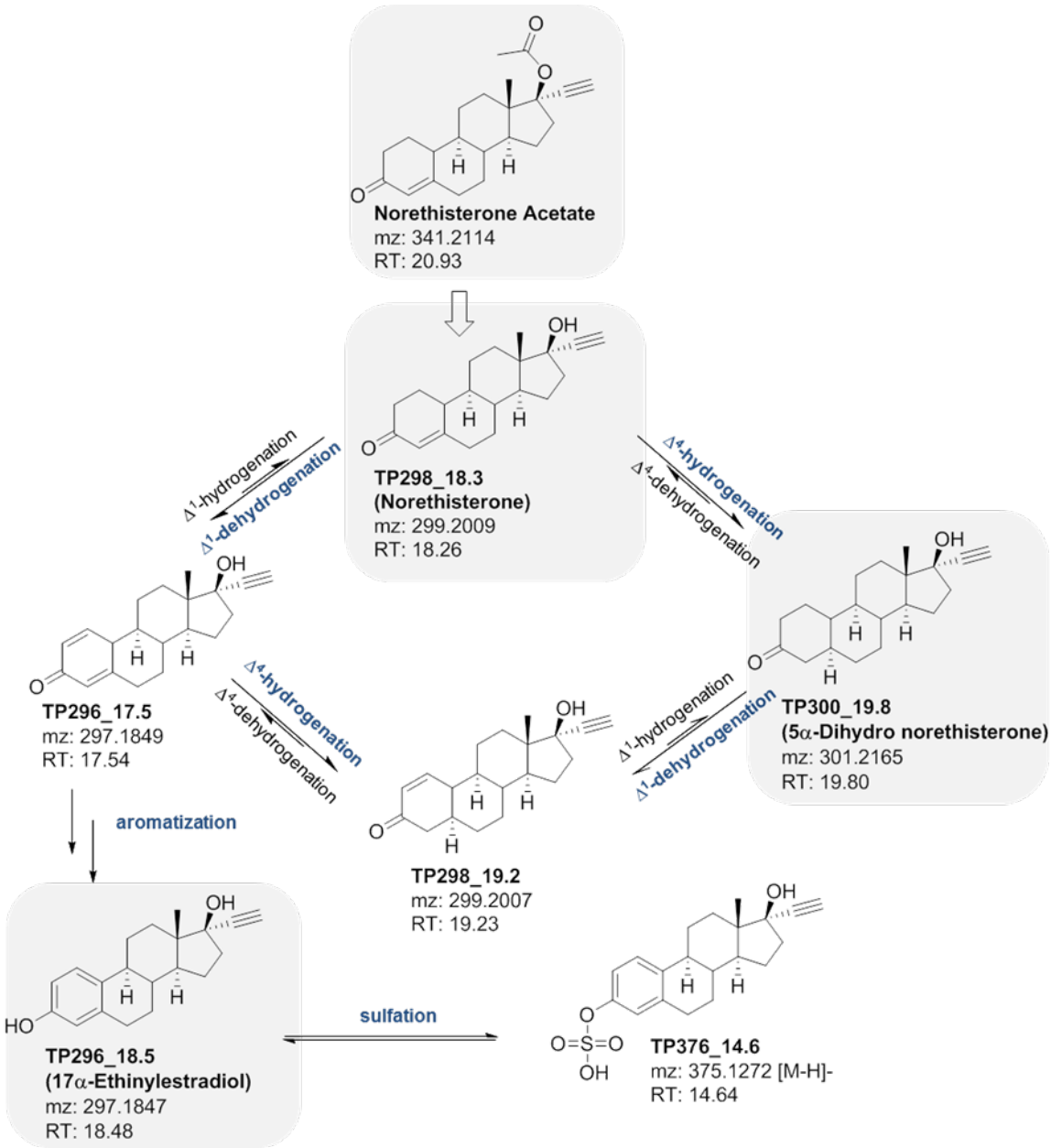
- Relatively good biodegradability of most SOCs was found, except for cyproterone acetate and dienogest.
- 17α-hydroxyprogesterone derivatives were degraded initially at ring A via carbon double bond (de)hydrogenations. Δ^6-Double bond forces 3-keto reduction, especially for cyproterone acetate.
- 19-Nortestosterone derivatives showed the formation of estrogen-like TPs by the aromatization of ring A, leading to a shift in the receptor binding capability.
- Most of the primary TPs can be expected as still active. Thus, TP formation must be considered for the environmental risk profile. These outcomes reinforce that it is mandatory

to consider TP formation of steroids as a strong assessment criterion for industrial discharge permissions into municipal WWTPs with biological treatment trains.

- The evidence of the conversion of progestogenic to estrogenic compounds and the formation of potentially hazardous TPs indicates the need of a more comprehensive risk assessment of synthetic steroids.

A more detailed discussion can be found in Weizel et al. [4].

Figure 2: Scheme of the proposed initial degradation pathway of norethisterone acetate in contact with activated sludge. Structures highlighted in grey were confirmed by the measurement of their reference standards. (Source: own figure, adapted from Weizel et al. 2020B [4], BfG)



5 Occurrence of steroid hormones, metabolites and TPs in WWTP effluents and receiving surface waters

In order to analyze the synthetic steroid pollution in the aquatic environment, a robust, comprehensive and highly sensitive analytical method for the quantification of a broad range of natural and synthetic steroids including glucocorticoids (GCs), mineralocorticoids (MCs) and progestogens (PGs) as well as their main TPs, which were commercially available, was developed (ref. Table 5). For method details see Weizel et al. [1].

Table 5: Overview on all analyzed hormones including metabolites and identified transformation products

Glucocorticoids (GC)		**Glucocorticoids (GC)**	
Abbr.	**Substance**	**Abbr.**	**Substance**
BEC	Beclomethasone	FMS	Flumethasone
BECprop	Beclomethasone 17-propionate	FMSpiv	Flumethasone 21-pivalate
BECdiprop	Beclomethasone 17,21-dipropionate	FCNact	Fluocinolone acetonide
BMS	Betamethasone	FML	Fluorometholone
BMS17oxo	17-Oxo betamethasone	FLUfur	Fluticasone 17-furoate
BMSac	Betamethasone 21-acetat	FLUprop	Fluticasone 17-propionate
BMS17val	Betamethasone 17-valerat	FLUcarb	Fluticasone 17β-carboxylic acid
BMS21val	Betamethasone 21-valerat	FLUA	Fluocinolone acetonide
BMS21prop	Betamethasone 17-propionat	HC	Hydrocortisone
BMS17prop	Betamethasone 21-propionat	HAL	Halcinonide
BMSdiprop	Betamethasone 17,21-dipropionate	HLM	Halometasone
BDN	Budesonide	MPNL	Methylprednisolone
BDN-m1	6ß-Hydroxy budesonide	MPNLacp	Methylprednisolone 21-acetate 17-propionate
BDN-m2	Budesonide 21-carboxylic acid	MPNLprop	Methylprednisolone 21-propionate
CIC	Ciclesonide	MOM	Mometasone
CIC-m1	Desisobutyryl ciclesonide	MOMfur	Mometasone 17-furoate
CLO	Clobetasol	MOMepofur	9,11-Epoxy mometasone furoate
CLOprop	Clobetasol 17-propionate	PNL	Prednisolone
HCOR	Cortisol (Hydrocortisone)	PNS	Prednisone
COR	Cortisone	TRIact	Triamcinolone acetonide
DMS	Dexamethasone	TRIact-m1	6ß-Hydroxy triamcinolone acetonide
DMS-m1	6ß-Hydroxy dexamethasone	TRIactm2	Triamcinolone acetonide 21-carboxylic acid
DMSac	Dexamethasone 21-acetate	TRIactm3	6β-Hydroxy21-oic triamcinolone acetonide
DFCval	Diflucortolone 21-valerate		
Progestogens (PG)		**Mineralocorticoids (MC)**	
Abbr.	**Substance**	**Abbr.**	**Substance**
CLM	Chlormadinone	CAN	Canrenone
CLMac	Chlormadinone acetate	CAN-m1	11α-Hydroxy canrenone
CYP	Cyproterone	FLC	Fludrocortisone

CYPac	Cyproterone acetate	FLCac	Fludrocortisone 21-acetate
CYPac-OH	3α-Hydroxy cyproterone acetate	SPL	Spironolactone
DIE	Dienogest	SPL-m1	7α-Thiomethyl spironolactone
DIE-m1	6ß-Hydroxy dienogest		
DIE-m2	Δ9,11-dehydro-17α-cyanomethyl estradiol		
DPN	Drospirenone		
ETG	Etonogestrel		
GES	Gestodene		
HPG	17α-Hydroxy progesterone		
LNG	Levonorgestrel		
MPR	Medroxy progesterone		
MPRac	Medroxy progesterone acetate		
MPRac-m1	6ß-Hydroxy medroxy progesterone acetate		
MEG	Megestrol		
MEGac	Megestrol acetate		
NES	Norethisterone		
NESac	Norethisterone acetate		

In a monitoring campaign steroid hormone loads in WWTP effluents and surface waters affected by different amounts of wastewater were evaluated. Water samples from five German WWTP effluents and surface waters of Saar (Rehlingen), Rhine (Koblenz), and Teltow Canal were analyzed (ref. Figure 3). The Teltow Canal is highly affected by effluent of waste water treatment plants and industrial discharge. At Koblenz, the Rhine has a wastewater proportion estimated to 5% [3] whereas at Rehlingen wastewater proportion is estimated to 15%.

As shown in Fig. 1a, the MCs canrenone and 7α-thiomethyl spironolactone (both are human metabolites of spironolactone) were detected in all WWTP effluents in concentrations up to 19 ng L-1and 3.8 ng L-1, respectively. In addition, both steroids were frequently found in surface waters receiving elevated percentage of WWTP effluent.

Figure 3: Sampling sites. Different colors show the river catchment areas according to German Wasserrahmenrichtlinie. (Source: Umweltbundesamt; map is based upon Länderarbeitsgemeinschaft Wasser (LAWA) and Bundesamt für Kartographie und Geodäsie (BKG))

23 of 37 GCs were detected in at least one sample. The predominant GCs in WWTP effluents and surface waters are shown in Figure 4. Triamcinolone acetonide showed by far the highest concentrations. In WWTP effluents its concentration was up to 28 ng L-1. Further frequently discharged GCs were clobetasol propionate, the betamethasone monoesters (propionate and valerate) and mometasone furoate. Endogenous hydrocortisone was found in all analyzed samples in relatively low concentrations around 1 ng L-1. Notably, the increasing ratio of the total GCs concentration in surface waters emphasizes other sources, such as wild life and animal farming. However, the detected concentrations in less charged surface waters were lower.

From the analyzed PGs, dienogest and cyproterone acetate (Figure 5) were identified as the predominant steroids in Germany, since they were detected in all analyzed WWTP effluents. For a detailed view of the steroid burdens, surface water samples with different WWTP effluent ratios were sampled. As expected, concentrations of the steroids correlate with the content of treated wastewater in the receiving waters. Therefore, municipal WWTPs seem to be the main point-source for synthetic steroids in the environment. Cyproterone acetate and dienogest as well as their main TPs were ubiquitously discharged into the environment from German WWTPs. Both TPs could be significant drivers of anthropogenic induced endocrine activity in the environment. The detected concentrations of SOCs and TPs emphasize a risk for aquatic organisms and their reproductive system. Within the identified TPs the carboxylic acids of fluticasone propionate and triamcinolone acetonide were found to be persistent, and as a consequence, they were detected in all WWTP effluents. Also, ester isomerization of

C17-monoester GCs (6α-methylprednisolone propionate, beclomethasone propionate, betamethasone propionate, betamethasone valerate) needs to be considered for monitoring, since both esters (C17-monoesters and C21-monoesters) were present in treated wastewater at elevated concentrations, contrarily to their diesters. In addition, the determined transformation reactions were successfully transferred to other GCs which could then be detected in the effluents of several WWTPs. Therefore, the identified TPs emphasize that similar structural moieties lead to equivalent biotransformation and thus, similar TPs are generated. Moreover, the analysis of effluent samples taken from eight conventional full-scale WWTPs revealed the occurrence of several of the TPs in appreciable concentrations and frequencies, which emphasize the need of an in-depth ecotoxicological evaluation and a careful consideration of the TPs formed from frequently used synthetic GCs (summarized in Table 6).

Finally, two of the newly identified TPs (3α hydroxy cyproterone acetate and Δ9,11-dehydro-17α-cyanomethyl estradiol) were frequently detected in effluent samples taken from eight conventional full-scale WWTPs (summarized in Table 6). These results showed that cyproterone acetate and dienogest as well as their main TPs were ubiquitously discharged into the environment from German WWTPs. Both TPs could be significant drivers of anthropogenic induced endocrine activity in the environment. The detected concentrations of SOCs and TPs emphasize a risk for aquatic organisms and their reproductive system.

Table 6: Overview of the monitoring results of the analysis of glucocorticoids and progestogens as well as their newly identified TPs in German WWTP effluents (n=8). The limits of quantification (LOQ) were calculated from WWTP effluents as the signal-to-noise ratio ≥ 10. Precursor steroids are highlighted in bold.

Type	Precursor and TPs	LOQ [ng L^{-1}]	n > LOQ	Median [ng L^{-1}]	Min [ng L^{-1}]	Max [ng L^{-1}]
Glucocorticoids	**Betamethasone dipropionate**	0.3	0	<0.3	<0.3	<0.3
	Betamethasone 17-propionate	0.4	7	0.8	0.6	1.6
	Betamethasone 21-propionate [a)]	0.4	8	0.9	0.5	1.3
	Betamethasone 17-valerate	0.5	8	1.2	0.6	3.1
	Betamethasone 21-valerate [a)]	0.5	6	0.6	0.4	1.1
	Betamethasone	0.2	8	0.8	0.2	1.4
	17-Oxo betamethasone	0.3	2	0.3	0.3	0.3
	TP332 [b)]	0.3	3	0.5	0.3	0.5
	Budesonide	1.2	0	<1.2	<1.2	<1.2
	Budesonide 21-carboxylic acid	0.4	4	0.6	0.4	0.8
	Clobetasol propionate	0.4	8	1.2	0.5	3.8

Type	Precursor and TPs	LOQ [ng L^{-1}]	n > LOQ	Median [ng L^{-1}]	Min [ng L^{-1}]	Max [ng L^{-1}]
	Fluticasone propionate	0.4	4	0.8	0.4	1.6
	Fluticasone 17β-carboxylic acid	0.5	8	1.4	0.5	2.8
	Fluocinolone acetonide	0.2	3	0.4	0.2	0.4
	Hydrocortisone	0.2	8	1.6	0.9	2.8
	Mometasone furoate	0.4	8	1.2	0.6	1.9
	9,11-Epoxy mometasone furoate	0.2	3	0.3	0.2	0.5
	Triamcinolone acetonide	0.4	8	12	1.0	20
	6β-Hydroxy triamcinolone acetonide	0.2	8	1.5	0.7	2.2
	Triamcinolone acetonide 21-carboxylic acid	0.3	8	1.8	0.5	3.7
	6β-Hydroxy21-oic triamcinolone acetonide	0.4	8	0.9	0.5	1.5
Progestogens	**Chlormadinone acetate**	0.4	3	0.5	0.4	0.7
	Cyproterone acetate	1.0	8	3.8	1.2	7.7
	3α-Hydroxy cyproterone acetate [c]	-	8	2.6 [c]	0.3 [c]	8.1 [c]
	Dienogest	0.3	8	1.2	0.3	3.7
	6β-Hydroxy dienogest	0.5	8	1.2	0.6	1.6
	Δ9,11-dehydro-17α-cyanomethyl estradiol [c]	-	8 [c]	6.7 [c]	1.2 [c]	16 [c]
	17α-Hydroxyprogesterone	0.4	8	0.6	0.4	1.0

a. Related C17-ester was used as calibration standard for quantification

b. 17-Oxo betamethasone was used as calibration standard for quantification. Proposed structure

c. Precursor steroids were used for an estimation of the concentration. Proposed structure

Figure 4: Overview of the monitoring results for selected mineralocorticoids in German WWTP effluents (a) and surface waters (b). The illustrated surface water samples represent three examples of different wastewater impacted rivers (Source: own figure, BfG).

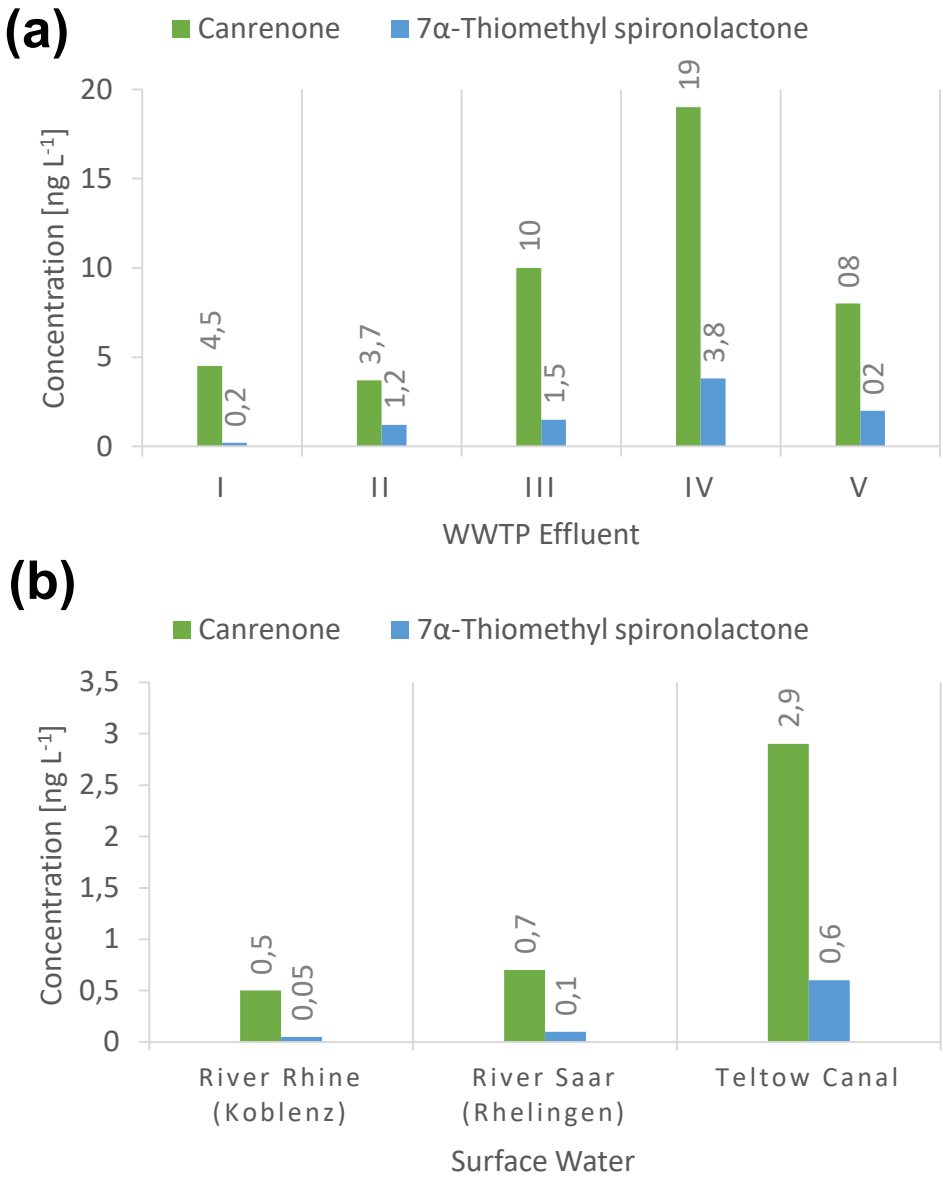


Figure 5: Overview of the monitoring results for selected glucocorticoids in German WWTP effluents (a) and surface waters (b). The illustrated surface water samples represent three examples of different wastewater impacted rivers (Source: own figure, BfG).

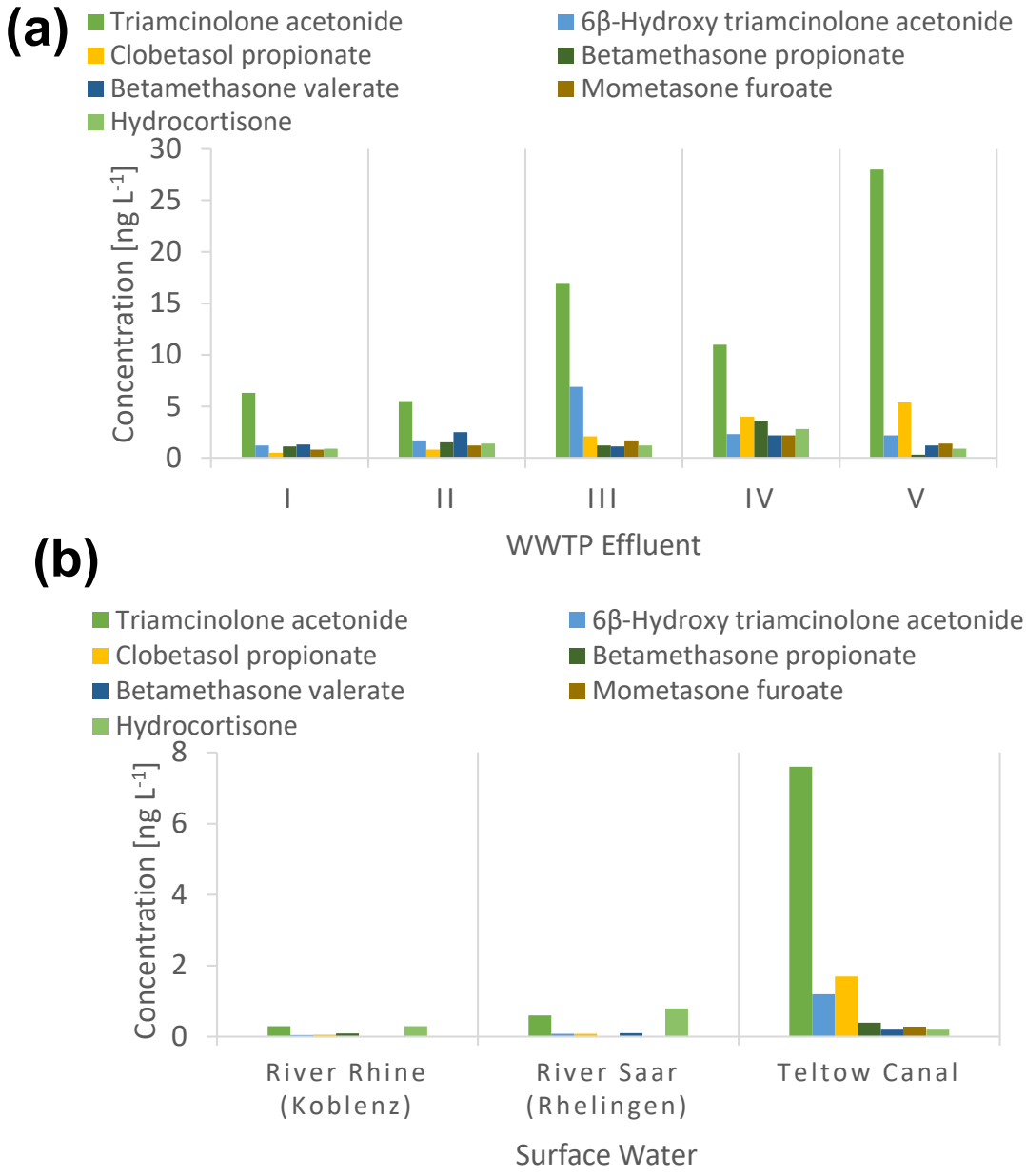
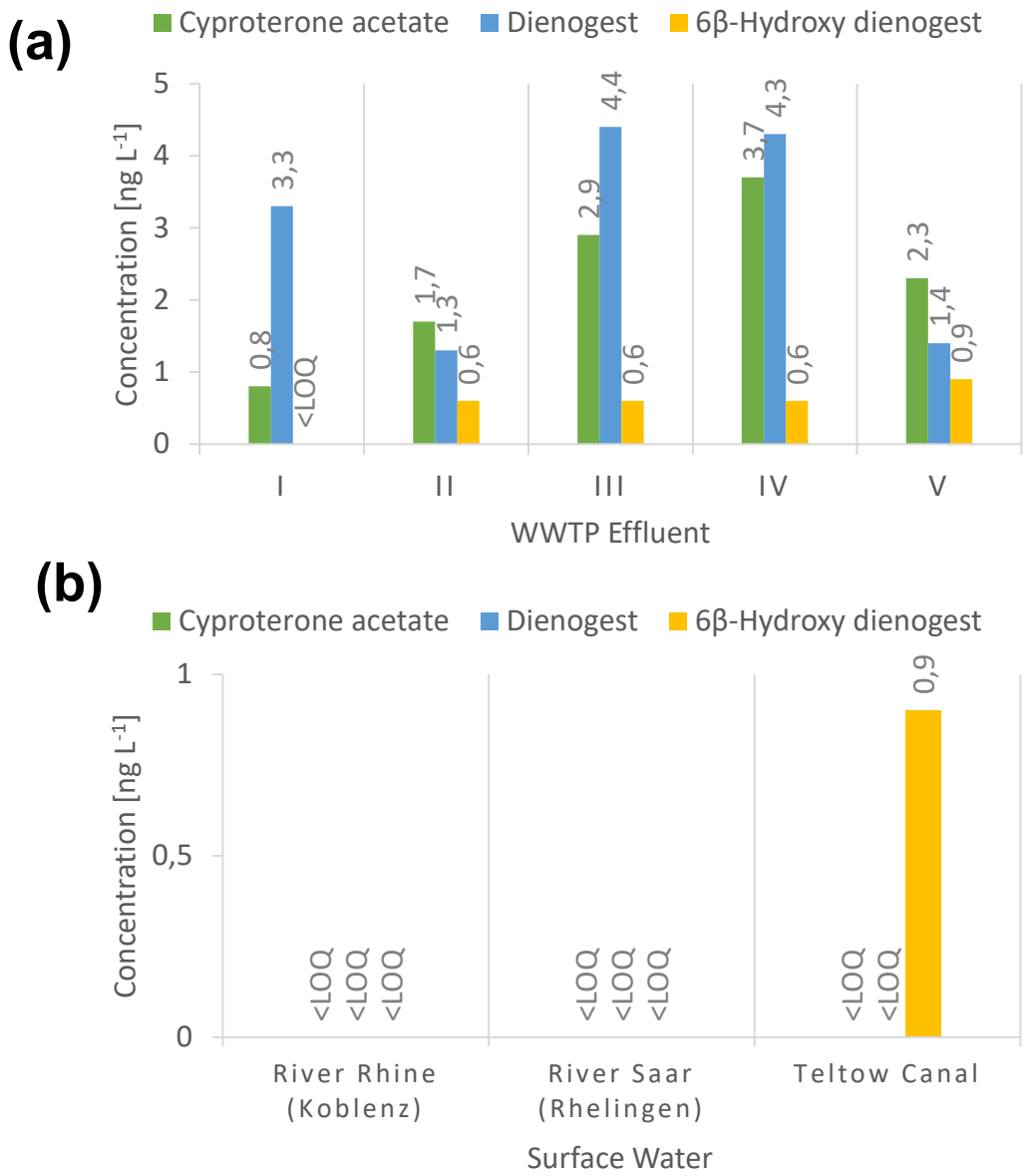


Figure 6: Overview of the monitoring results for selected progestogens in German WWTP effluents (a) and surface waters (b). The illustrated surface water samples represent three examples of different wastewater impacted rivers (Source: own figure, BfG).



Based on these results, recommendations for analytes for a nationwide steroid monitoring were derived. The prioritized steroids are summarized in Table 7. Together with previous results, a prioritization of substances for ecotoxicological tests is needed. Furthermore, for an assessment of steroids in the environment it is necessary to consider the activity of single substances as well as the additive effects of steroids.

6 Conclusion

The controlled lab incubation experiments with activated sludge successfully enabled the comparison of the stability of synthetic steroids and revealed profound insights into the fate and behavior of glucocorticoids and progestogens in the biological wastewater treatment. It could be shown that several glucocorticoids and some of their TPs are rarely biodegradable and hence they are discharged into the aquatic environment via the WWTP effluents in appreciable concentrations. Other steroids were found to be rapidly removed and therefore they are inappropriate for environmental monitoring. The incubation experiments revealed that the individual synthetic steroid burdens in the environment were primarily driven by stability and not by the consumption pattern exclusively. Consequently, prioritization and risk assessments should consider stability of steroid hormones and should not base on consumption data alone.

A large number of common as well as more specific transformation reactions were identified for the analyzed glucocorticoids and progestogens. In this context, structural features that led to a distinct increase in stability could be characterized. On the basis of the project results, the (bio)degradability of synthetic steroid hormones in biological wastewater treatment can be predicted by means of the chemical structure, whereas the relationship of structure and stability to enzymatic degradation requires more research for a better understanding.

The identification of TPs and degradation pathways for a broad range of steroid hormones revealed a large number of so far unknown compounds with potentially endocrine disrupting potential. Moreover, the conversion of progestogenic to estrogenic compounds could be shown for 19-nortestosterone type steroids. Thus, changes in the endocrine mode of action should be considered in the risk assessment and industrial discharge permissions.

The findings of the monitoring campaigns revealed the presence of numerous steroids in the WWTP effluents. The transformation products such as 3α hydroxy cyproterone acetate, Δ9,11-dehydro-17α-cyanomethyl estradiol, 17-oxo betamethasone and 6β-hydroxy triamcinolone were detected for the first time. The sensitive and comprehensive analytical methods allowed a good overview of single steroid burdens in the environment and enabled the indication of prevalent steroids from each steroid type (glucocorticoids, mineralocorticoids and progestogens). In addition, the detection of several TPs in the effluents of full scale WWTPs illustrated that TPs should be considered in environmental monitoring.

This study clearly demonstrates that this substance class is of emerging concern and monitoring activities should be expanded beyond estrogens such as ethinylestradiol (EE2). Table 3 gives an overview about the most important hormonal substances based on the results obtained in this project. Besides monitoring there is an urgent need of effect-based studies considering additive effects of mixtures and changes in receptor selectivity caused by the formation of TPs.

Table 7: Recommendations for corticosteroids and progestogens for environmental monitoring

Class	Name	CAS-No.	Comment
Mineralo-corticoids	Canrenone	976-71-6	active metabolite of spironolactone, frequently detected
	7α-Thiomethyl spironolactone	38753-77-4	metabolite of spironolactone, frequently detected

Class	Name	CAS-No.	Comment
Glucocorticoids	Beclomethasone 17-propionate	5534-18-9	OTC pharmaceutical in Germany, active metabolite/TP of beclomethasone dipropionate, no detection in Germany
	Beclomethasone	4419-39-0	OTC pharmaceutical in Germany, active metabolite/TP of beclomethasone dipropionate, no detection in Germany
	Betamethasone	378-44-9	active metabolite/TP of betamethasone ester, frequently detected
	Betamethasone 17-valerate	2152-44-5	transformation to the 21-ester and betamethasone, frequently detected
	Betamethasone 17-propionate	5534-13-4	transformation to the 21-ester and betamethasone, frequently detected
	17-Oxo betamethasone	3109-01-1	TP of betamethasone with proposed androgenic activity, transformation to the isomer TP332, detected in WWTP effluents
	Budesonide	51333-22-3	non-halogeneded ketal, moderate removal in WWTPs
	Budesonide 21-carboxylic acid	-	(Budesonide impurity 1, racemic), TP of budesonide
	Clobetasol propionate	25122-46-7	moderate removal in WWTPs, frequently detected
	Cortisone	53-06-5	endogenous glucocorticoid, frequently detected, can be used as marker for untreated wastewater
	Dexamethasone	50-02-2	isolated detections in Germany
	Fluticasone propionate	80474-14-2	OTC pharmaceutical in Germany, moderate removal in WWTPs, frequently detected
	Fluticasone 17β-carboxylic acid propionate	65429-42-7	metabolite/TP of fluticasone propionate, persistent in activated sludge treatment, frequently detected
	Fluocinolone acetonide	67-73-2	low consumption in Germany, hardly biodegradable, isolated detections in low concentrations in German WWTP effluents

Class	Name	CAS-No.	Comment
	Fluocinolone acetonide 21-caroxylic acid	106931-78-6	metabolite/TP of fluticasone propionate, persistent in activated sludge treatment,
	Hydrocortisone	50-23-7	endogenous glucocorticoid, frequently detected, can be used as marker for untreated wastewater
	Mometasone furoate	83919-23-7	OTC pharmaceutical in Germany, moderate removal, frequently detected
	9,11-Epoxy mometasone furoate	83881-09-8	metabolite/TP of mometasone furoate- isolated detections in German WWTP effluents,
	Triamcinolone acetonide	76-25-5	Fluorinated ketal, hardly biodegradable, frequently detected in high concentrations
	6β-Hydroxy triamcinolone acetonide	3869-32-7	metabolite of triamcinolone acetonide, frequently detected
	Triamcinolone acetonide 21-carboxylic acid	53962-41-7	metabolite/TP of triamcinolone acetonide, persistent in activated sludge treatment, frequently detected
	6β-Hydroxy 21-oic triamcinolone acetonide	68263-02-5	metabolite/TP of triamcinolone acetonide, persistent in activated sludge treatment, frequently detected
Progestogens	Chlormadinone acetate	302-22-7	low consumption in Germany, isolated detections in low concentrations in German WWTP effluents
	Delmadinone acetate	13698-49-2	main TP of chlormadinone acetate, active steroid
	Cyproterone acetate	427-51-0	high consumption in Germany, moderate removal in activated sludge treatment, frequently detected in appreciable concentrations
	3α-Hydroxy cyproterone acetate	167356-55-0	metabolite and main TP of cyproterone acetate, frequently detected
	Dienogest	65928-58-7	high consumption in Germany, moderate removal in activated sludge treatment, frequently detected in appreciable concentrations
	Δ9,11-dehydro-17α-cyanomethylestradiol	86153-38-0	TP of dienogest, proposed estrogenic activity, frequently detected in German WWTP effluents
	Levonorgestrel	797-63-7	potent synthetic progestogen, isolated detections in German WWTP effluents

Class	Name	CAS-No.	Comment
	5α-Dihydro levonorgestrel	78088-19-4	metabolite and proposed TP of levonorgestrel
	Medroxyprogesterone acetate	71-58-9	synthetic progestogen, not detected in Germany
	1,2-Dehydro medroxyprogesterone acetate	-	main TP of medroxyprogesterone acetate
	Megestrol acetate	595-33-5	synthetic progestogen, isolated detections in German WWTP effluents
	1,2-Dehydro megestrol acetate	982-89-8	proposed TP of megestrol acetate
	Norethisterone	68-22-4	active metabolite/TP of its ester derivatives (enanthate, acetate), not detected in German WWTP effluents
	5α-Dihydro norethisterone	52-79-9	active metabolite/TP of norethisterone
	17α-Ethinylestradiol	57-63-6	synthetic estrogen, TP of norethisterone

6.1 Future issues

Based on the results of this study the following future issues and questions arise

a) development of environmental monitoring strategies for steroid hormones

Several of the investigated hormones could be detected in WWTP effluent suggesting a widespread occurrence in the aquatic environment. Due to effect levels in the range of concentrations found in the environment a comprehensive monitoring of these compounds is recommended. Besides ecotoxicological studies addressing adverse effects on organisms, studies on bioavailability and bioaccumulation are needed. However, analytical methods for the determination of synthetic steroids in biota are currently missing and a big challenge, since extreme low detection limits have to be reached in complex matrices. Selective enrichment is hampered by an enormous excess of natural steroids. Furthermore, distribution within tissues and metabolism of synthetic steroids is mostly unknown.

b) evaluation of the risk of steroid hormones

The findings of this study suggest the risk of an adverse effect of synthetic steroids in aquatic environment. The (eco)toxicological risks of individual parent compounds, metabolites and TPs as well as their mixtures should be evaluated.

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