STEROID HORMONES IN THE AQUATIC ENVIRONMENT

ANALYSIS, OCCURRENCE AND FATE OF CORTICOSTEROIDS AND PROGESTOGENS

von

Alexander Weizel
aus Kiseljowsk (Russland)

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Universität Koblenz-Landau

Gutachter:
Prof. Dr. Thomas A. Ternes
Prof. Dr. Joachim Scholz
Prof. Dr. Torsten C. Schmidt

Prüfungskommission:
Prof. Dr. Thomas A. Ternes
Prof. Dr. Joachim Scholz
Prof. Dr. Christian Fischer
Prof. Dr. Torsten C. Schmidt

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„Jeder ist ein Genie! Aber wenn Du einen Fisch danach beurteilst, ob er auf einen Baum klettern kann, wird er sein ganzes Leben glauben, dass er dumm ist."

(Albert Einstein)

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Summary

Thousands of chemicals from daily use are being discharged from civilization into the water cycle via different pathways. Ingredients of personal care products, detergents, pharmaceuticals, pesticides, and industrial chemicals thus find their way into the aquatic ecosystems and may cause adverse impacts on the ecology. Pharmaceuticals for instance, represent a central group of anthropogenic chemicals, because of their designed potency to interfere with physiological functions in organisms. Ecotoxicological effects from pharmaceutical burden have been verified in the past. Therapeutic groups with pronounced endocrine disrupting potentials such as steroid hormones gain increasing focus in environmental research as it was reported that they cause endocrine disruption in aquatic organisms even when exposed to environmentally relevant concentrations. This thesis considers the comprehensive investigation of the occurrence of corticosteroids and progestogens in wastewater treatment plant (WWTP) effluents and surface waters as well as the elucidation of the fate and biodegradability of these steroid families during activated sludge treatment.

For the first goal of the thesis, a robust and highly sensitive analytical method based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed in order to simultaneously determine the occurrence of around 60 mineralocorticoids, glucocorticoids and progestogens in the aquatic environment. A special focus was set to the compound selection due to the diversity of marketed synthetic steroids. Some analytical challenges have been approved by individual approaches regarding sensitivity enhancement and compound stabilities. These results may be important for further research in environmental analysis of steroid hormones. Reliable and low quantification limits are the perquisite for the determination of corticosteroids and progestogens at relevant concentrations due to low consumption volumes and simultaneously low effect-based trigger values. Achieved quantification limits for all target analytes ranged between 0.02 ng/L and 0.5 ng/L in surface water and 0.05 ng/L to 5 ng/L in WWTP effluents. This sensitivity enabled the detection of three mineralocorticoids, 23 glucocorticoids and 10 progestogens within the sampling campaign around Germany. Many of them were detected for the first time in the environment, particularly in Germany and the EU. To the best of our knowledge, this in-depth steroid screening provided a good overview of single steroid burden and allowed for the identification of predominantly steroids of each steroid type analyzed for the first time. The frequent detection of highly potent synthetic steroids (e.g. triamcinolone acetonide, clobetasol propionate, betamethasone valerate, dienogest, cyproterone acetate) highlighted insufficient removal during conventional

wastewater treatment and indicated the need for regulation to control their emission since the steroid concentrations were found to be above the reported effect-based trigger values for biota.

Overall, the study revealed reliable environmental data of poorly or even not analyzed steroids. The results complement the existing knowledge in this field but also provided new information which can be used particularly for compound prioritization in ecotoxicological research and environmental analysis.

Based on the data obtained from the monitoring campaign, incubation experiments were conducted to enable the comparison of the biodegradability and transformation processes in activated sludge treatment for structure-related steroids under aerobic and standardized experimental conditions. The compounds were accurately selected to cover manifold structural moieties of commonly used glucocorticoids, including non-halogenated and halogenated steroids, their mono- and diesters, and several acetonide-type steroids. This approach allowed for a structure-based interpretation of the results. The obtained biodegradation rate constants suggested large variations in the biodegradability (half-lifes ranged from < 0.5 h to > 14 d). An increasing stability was identified in the order from non-halogenated steroids (e.g. hydrocortisone), over 9α-halogenated steroids (e.g. betamethasone), to C17-monoesters (e.g. betamethasone 17-valerate, clobetasol propionate), and finally to acetonides (e.g. triamcinolone acetonide), thus suggesting a strong relationship of the biodegradability with the glucocorticoid structure. Some explanations for this behavior have been received by identifying the transformation products (TPs) and elucidating individual transformation pathways. The results revealed the identification of the likelihood of transformation reactions depending on the chemical steroid structure for the first time. Among the identified TPs, the carboxylates (e.g. TPs of fluticasone propionate, triamcinolone acetonide) have been shown persistency in the subsequent incubation experiments. The newly identified TPs furthermore were frequently detected in the effluents of full-scale wastewater treatment plants. These findings emphasized i) the transferability of the lab-scale degradation experiments to real world and that ii) insufficient removals may cause adverse effects in the aquatic environment due to the ability of the precursor steroids and TPs to interact with the endocrine system in biota.

For the last goal, the conceptual study for glucocorticoids was applied to progestogens. Here, two sub-types of the steroid family frequently used for hormonal contraception were selected (17α-hydroxyprogesterone and 19-norstestosterone type). The progestogens showed a fast and complete degradation within six hours, and thus empathizes pronounced biodegradability. However, cyproterone acetate and dienogest

have been found to be more recalcitrant in activated sludge treatment. This was consistent with their ubiquitously occurrence during the previous monitoring campaign. The elucidation of TPs again revealed some crucial information regarding the observed behavior and highlighted furthermore the formation of hazardous TPs. It was shown that 19-nortestosterone type steroids are able to undergo aromatization at ring A in contact with activated sludge, leading to the formation of estrogen-like TPs with a phenolic moiety at ring A. In the case of norethisterone the formation of 17α-ethinylestradiol was confirmed, which is a well-known potent synthetic estrogen with elevated ecotoxicological potency. Thus, the results indicated for the very first time an unknown source of estrogenic compounds, particularly for 17α-ethinylestradiol.

In conclusion, some steroids were found to be very stable in activated sludge treatment, others degrade well, and others which do degrade but predominantly to active TPs depending on their chemical structure. Fluorinated acetal steroids such as triamcinolone acetonide and fluocinolone acetonide are poorly biodegradable, which is reflected in high concentrations detected ubiquitously in WWTP effluents. Endogenous steroids and their most related synthetic once such as hydrocortisone, prednisolone or 17α-hydroxyprogesterone are readily biodegradable. Regardless their high influent concentrations, they are almost completely removed in conventional WWTPs. Steroids between this range have been found to form elevated quantities of TPs which are partially still active, which particularly the case for betamethasone, fluticasone propionate, cyproterone acetate or dienogest.

The thesis illustrates the need for an extensive evaluation of the environmental risks and carried out that corticosteroids and progestogens merit more attention in environmental regulatory and research than it is currently the case.

Zusammenfassung

Unzählige Chemikalien des täglichen Gebrauchs werden von der Zivilisation über verschiedene Eintragspfade in den Wasserkreislauf eingetragen. Inhaltsstoffe aus Körperpflegeprodukten, Waschmitteln, Arzneimitteln, Pestiziden und Industriechemikalien finden so ihren Weg in die aquatischen Ökosysteme und können negative Auswirkungen auf die dortige Ökologie bewirken. Pharmazeutika z.B. stellen eine zentrale Gruppe anthropogener Chemikalien dar, die durch ihre konzeptionierte Wirkung zu einer empfindlichen Störung physiologischer Funktionen in aquatischen Organismen führen können. Ökotoxikologische Auswirkungen, induziert durch Arzneimittelrückstände in der Umwelt, wurden in der Vergangenheit vermehrt aufgezeigt. Dabei geraten endokrine Disruptoren, wie z.B. Steroidhormone, immer häufiger in den Fokus der Umweltforschung, da sie negative Auswirkungen bereits bei sehr niedrigen Konzentrationen verursachen.

Die vorliegende Dissertation befasst sich mit der umfassenden Untersuchung des Umweltvorkommens von Corticosteroiden und Progestagenen in Klärwerksabläufen (KWA) und Oberflächengewässern, sowie mit der Untersuchung des biologischen Abbauverhaltens dieser Steroidklassen während der biologischen Abwasserbehandlung.

Zu Beginn der Arbeiten wurde eine robuste und sensitive Multikomponenten-Analysemethode entwickelt, um das Vorkommen von rund 60 Mineralocorticoiden, Glucocorticoiden und Progestagenen in der aquatischen Umwelt zu analysieren. Bedingt durch die Vielzahl an vermarkteten synthetischen Steroiden wurde bei der Methodenentwicklung ein besonderer Schwerpunkt auf die Auswahl der Analyten gelegt.

Es konnten individuelle Ansätze für analytische Herausforderungen erarbeitet werden, wie z.B. die Optimierung von Nachweisgrenzen und die Stabilität der Analyten während der Probenvorbereitung. Eine der wichtigsten Voraussetzungen für die Bestimmung von Steroidhormonen in der Umwelt, sind niedrige und verlässliche Quantifizierungsgrenzen. Zum einen da die Effektkonzentrationen sehr niedrig sind, und zum anderen, weil ihre geringen Verbrauchsmengen niedrige Umweltkonzentrationen vorhersagen. Die Bestimmungsgrenzen der entwickelten Methode für alle Analyten lagen im Bereich von 0,02 ng/L bis 0,5 ng/L in Oberflächengewässer und von 0,05 ng/L bis 5,0 ng/L in KWAs. Dies ermöglichte den Nachweis von insgesamt drei Mineralocorticoiden, 23 Glucocorticoiden und 10 Progestagenen innerhalb der Probenahmekampagne in ganz Deutschland. Das Umweltvorkommen vieler der detektierten Steroide wurden zum ersten

Mal nachgewiesen, insbesondere mit Blick auf deutsche und europäische Gewässer. Das umfassende Steroidscreening lieferte einen guten Überblick über die Einzelstoff-konzentrationen und ermöglichte so die Identifizierung der Hauptkontaminanten aus jeder Steroidklasse. Weiterhin zeigen die Ergebnisse das ubiquitäre Vorkommen von hochpotenten synthetischen Steroiden (z.B. Triamcinolonacetonid, Clobetasolpropionat, Betamethasonvalerat, Dienogest, Cyproteronacetat) und damit ihre unzureichende Entfernung durch die konventionelle Abwasserbehandlung in Kläranlagen. Die ermittelten Konzentrationen vieler Einzelsubstanzen lagen z.T. oberhalb der bekannten Effektkonzentrationen. Ihr ubiquitäres Vorkommen verdeutlicht einen Handlungsbedarf in der Regulierung der Steroidhormonemissionen durch geklärtes Abwasser zum Schutz der Ökosysteme.

In Summe lieferte diese Studie zum ersten Mal belastbare Umweltdaten von schlecht bzw. gar nicht untersuchten Steroiden. Es konnten vorangegangene Studienergebnisse bestätigt werden, aber auch viele neue Erkenntnisse gewonnen werden, welche insbesondere für die Analytenpriorisierung in der ökotoxikologischen Forschung und dem Umweltmonitoring genutzt werden können.

Auf Grundlage dieser Monitoringergebnisse wurden Laborabbaustudien mit Belebtschlamm konzeptioniert, mit dem Ziel, Rückschlüsse zwischen biologischer Abbaubarkeit, dem Transformationsverhalten und der chemischen Struktur zu erzielen. Hierfür wurden die Steroide unter aeroben und standardisierten Bedingungen mit Belebtschlamm inkubiert. Die Modellsteroide wurden so ausgewählt, um die vielfältigen Strukturen der verwendeten Glucocorticoiden abzudecken, darunter nicht-halogenierte und halogenierte Steroide, ihre Mono- und Diester sowie mehrere Steroide vom Acetonid-Typ. Der Ansatz ermöglichte anschließend eine strukturbasierte Interpretation der Ergebnisse. Die kinetischen Konstanten zeigten große Unterschiede in der biologischen Abbaubarkeit der untersuchten Glucocorticoide (Halbwertszeiten von < 30 min bis > 14 Tage). Eine zunehmende Stabilität wurde in der Reihenfolge von nicht-halogenierten Steroiden (z.B. Hydrocortison) über 9α-halogenierten Steroiden (z.B. Betamethason), zu C17-Monoestern (z.B. Betamethason-17-valerat, Clobetasolpropionat) und schließlich zu Acetoniden (z.B. Triamcinolonacetonid) festgestellt. Diese Ergebnisse lassen auf eine starke Beziehung zwischen Bioabbau und chemischer Struktur schließen. Erklärungen für diese Beobachtungen wurden anschließend durch die Identifizierung von Transformationsprodukten (TPs) und Transformationspfaden erhalten. Auf Basis dieser Ergebnisse konnten Transformationsreaktionen verschiedenen Strukturmerkmalen zugeordnet werden, was in Zukunft die Vorhersage der biologischen

Stabilität und TP-Bildung von Glucocorticoiden erleichtert. Weiterhin konnte in Laborversuchen gezeigt werden, dass einige der neu identifizierten TPs ein persistentes Verhalten während der Inkubation aufweisen (u.a. Caboxy-TPs von Fluticasonpropionat, Triamcinolonacetonid) und entsprechend ubiquitär in den KWAs nachweisbar sind. Diese Ergebnisse verdeutlichen die Übertragbarkeit der im Labormaßstab identifizierten Transformationsreaktionen auf reale Kläranlagen und betonen erneut, dass eine unzureichende Eliminierung der Vorläufersteroide und ihren TPs zu adversen Effekten in der aquatischen Umwelt führen kann.

Das Studienkonzept für Glucocorticoide wurde anschließend auf die Gruppe der Progestagene angewendet. Dabei wurden zwei Steroidtypen ausgewählt, welche häufig als aktive Wirkstoffe in der Antibabypille eingesetzt werden (17α-Hydroxyprogesterontyp und 19-Norstestosterontyp). Die untersuchten Steroide zeigten insgesamt einen schnellen und vollständigen Abbau innerhalb der ersten sechs Stunden und wiesen damit eine ausgeprägte biologische Abbaubarkeit auf. Nur Cyproteronacetat und Dienogest zeigten eine höhere Stabilität in den Laborversuchen. Dies steht im Einklang mit ihrem ubiquitären Umweltvorkommen und den relativ hohen detektierten Konzentrationen in den untersuchten Kläranlagenabläufen. Die Aufklärung der TPs ergab erneut Erklärungsansätze für das beobachtete Degradationsverhalten dieser Substanzklasse. Darüber hinaus wurde die Bildung von potenziell gefährlichen TPs nachgewiesen. Es konnte gezeigt werden, dass Steroide des 19-Nortestosterontyps in Kontakt mit Belebtschlamm konjugierte Ringsysteme bilden, was zur Bildung östrogenähnlicher TPs mit einem phenolischen Ring A führt. Am Beispiel von Norethisteron wurde die Bildung des starken synthetischen Östrogens 17α-Ethinylestradiol verifiziert. Damit konnten diese Ergebnisse erstmalig zeigen, dass östrogen-ähnliche Verbindungen durch die Transformation von Vorläufersubstanzen in der Abwasserbehandlung entstehen und eine bisher unbekannte Quelle für 17α-Ethinylestradiol identifizieren.

Zusammengefasst konnte diese Dissertation aufzeigen, dass einige Steroide bei der Belebtschlammbehandlung sehr stabil sind, andere gut abgebaut werden und andere, die zwar abgebaut werden, jedoch in Abhängigkeit von ihrer chemischen Struktur überwiegend zur Bildung von aktiven TPs führen. Fluorierte Acetalsteroide wie z.B. Triamcinolonacetonid oder Fluocinolonacetonid sind biologisch schlecht abbaubar, was sich in hohen umweltkonzentrationen widerspiegelt. Natürliche Steroide und ihre engsten synthetischen Analoga wie z.B. Hydrocortison, Prednisolon oder 17α-Hydroxyprogesteron sind gut

biologisch abbaubar. Ungeachtet ihrer hohen Konzentrationen im Zulauf, werden sie in konventionellen Kläranlagen fast vollständig entfernt und nur in sehr geringen Mengen über die Klärwerke emittiert. Steroide mit moderater biologischer Abbaubarkeit bilden z.T. hohe Mengen an TPs, die z.T. noch eine endokrine Wirkung besitzen. Dies ist insbesondere der Fall bei Betamethason, Fluticasonpropionat, Cyproteronacetat und Dienogest.

Die Ergebnisse dieser Dissertation illustrieren die Notwendigkeit einer umfassenden Umweltrisikobewertung und verdeutlichen eindrucksvoll, dass Corticosteroide und Progestagene in der Umweltregulierung und Forschung mehr Beachtung geschenkt werden muss als dies derzeit der Fall ist.

1 General Introduction

Therapeutic groups with pronounced endocrine disrupting potentials such as steroid hormones gain increasing focus in environmental research (KIDD ET AL., 2007; ZEILINGER ET AL., 2009; KUGATHAS AND SUMPTER, 2011; FENT, 2015; JIA ET AL., 2016). Although the first effects labeled as endocrine disruption were reported in the 1930s (DODDS ET AL., 1938; MATTHIESSEN, 2003) there is currently some growing evidence that steroid hormones from anthropogenic origin can adversely impact the aquatic wildlife at environmentally relevant concentrations (KUMAR ET AL., 2015; FENT, 2015).

The continuously growing number of chemicals detected in water bodies is contrasted by a small number of analytes that are considered in routine analysis in Germany and the European Union (EU). From a regulatory level, steroid hormones are not mentioned that much as it might be expected due to their high potential risk, although the European Water Framework Directive (WFD, 2000) contains the paragraph:

> *"Substances and preparations, or the breakdown products of such, which have been proved to possess carcinogenic or mutagenic properties or properties which may affect steroidogenic, thyroid, reproduction or other endocrine-related functions in or via the aquatic environment"*

Steroid hormones do indeed meet the requirements for this categorization, due to their mode of action as modulators for nucleus steroid receptors. However, currently the watch list of substances for union-wide monitoring (EU DECISION, 2018) contains only the estrogenic steroids 17β-estradiol, estrone, and 17α-ethinylestradiol while progestogens and corticosteroids are not considered.

This *status quo* can be explained by two facts. i) Over the last few decades, environmental research focused mainly on estrogens as they were associated with disregulation of reproductive functions at relatively low concentrations. As a consequence, reliable data on occurrence, behavior, and impacts for corticosteroids and progestogens in the aquatic environment are lacking which is a significant knowledge gap. On the other hand, ii) corticosteroids and progestogens are used in many fields of modern medicine. In contrast to estrogens, this is particularly affecting the number of applied steroid derivatives and makes the regulation and environmental risk assessment more difficult.

Even though isolated studies determined the presence of corticosteroids and progestogens in the environment (details in Section 1.3.4), little consistent and comprehensive knowledge

exist about their occurrence, fate and biodegradability. More research is therefore needed to close the current knowledge gaps. Finally, synthetic corticosteroids and progestogens are compounds with an "engineered" toxicological potential for the aquatic ecology.

This general introduction discusses the properties of progestogens and corticosteroids, their main sources & sinks as well as the possible risks based on current scientific knowledge from an analytical and environmental perspective.

1.1 Classification of Steroid Hormones

Steroids are a group of natural products possessing a condensed four-cyclic skeleton. The steroid core is arranged strictly stereospecific which leads to the characteristic molecular configuration and pronounced bioactivity. The basic structure of all steroids is derived from that of sterane with a planar steroid core (Fig. 1.1).

The terminology of steroid hormones is not always consistent in terms of the numbering of carbon atoms, labeling of substituent configurations or referring to double bonds. Many steroids are annotated by their trivial names, which again can lead to confusion since different trivial names for the same compound are used (e.g. cortisol, hydrocortisone, etc.) (MOSS, 1989; SULTAN AND RAZA, 2015; KUMAR ET AL., 2015). This is most likely due to the special importance of steroids within various areas in science, medicine, and technology leading to different notations in those individual fields. Therefore, IUPAC (International Union of Pure and Applied Chemistry) nomenclature is often chosen to avoid this problem. However, the IUPAC names for steroids and other complex organic molecules are very long and require a well-trained eye to handle them quickly. According to IUPAC, hydrocortisone (Fig. 1.1) is named as follows: "(8S,9S,10R,11S,13S,14S,17R)-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-2,6,7,8,9,11,12,14,15,16-decahydro-1H-cyclopenta[a]phenanthren-3-one".

To remove the complexity, trivial names of the steroids are used in this thesis and specific discussions related to the chemical structure are terminated as shown in Fig. 1.1, which is the most common notation in the literature.
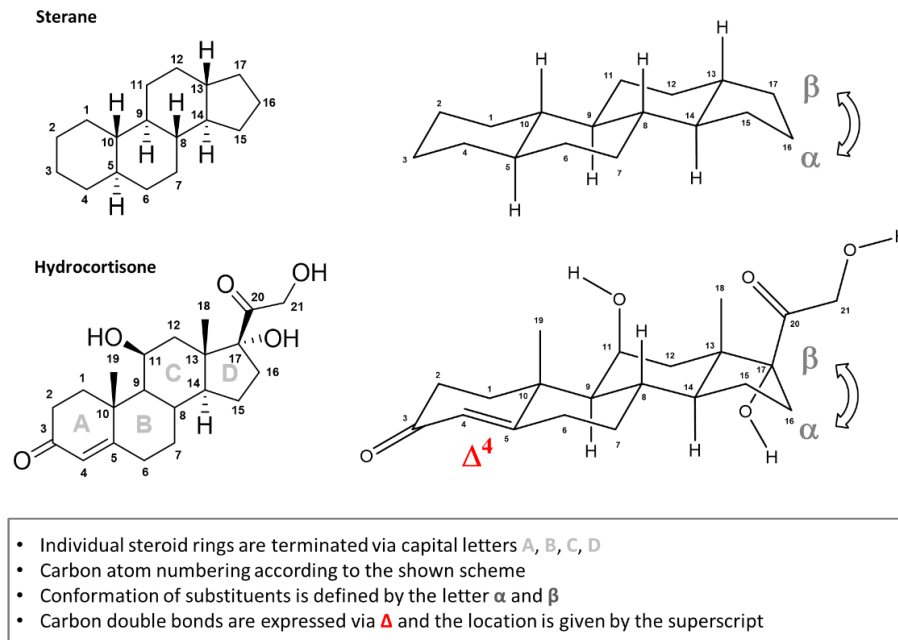
- Individual steroid rings are terminated via capital letters A, B, C, D
- Carbon atom numbering according to the shown scheme
- Conformation of substituents is defined by the letter α and β
- Carbon double bonds are expressed via Δ and the location is given by the superscript

Figure 1.1. Numbering, chemical structure and configuration of the basic sterane core and hydrocortisone.

1.2   Properties of Corticosteroids and Progestogens

1.2.1   Steroid Biosynthesis

The function of steroids in mammalians is based on the formation of so-called ligand-receptor complexes (Fig. 1.2), leading to expression of specific gene sequences involved in various physiological processes. In mammalians, steroid hormones can be divided into five groups. Androgens, estrogens, and gestagens (sex steroids) are formed in the gonads, while mineralocorticoids and glucocorticoids (corticosteroids) are produced by the adrenal cortex (LEMKE, 2013). These individual steroids are synthesized from the parent compound cholesterol by numerous enzymatically catalyzed reactions (HANUKOGLU,1992). The chemical structure of the steroid leads to a pronounced affinity for the target receptor of the nuclear receptor superfamily, according to the key-lock principle. The binding of a steroid to the receptor protein causes a specific conformational change. Steroids may act as agonist (activation) and antagonists (deactivation). Thus, depending on their structure, steroids can differ in their pattern of hormonal activities.
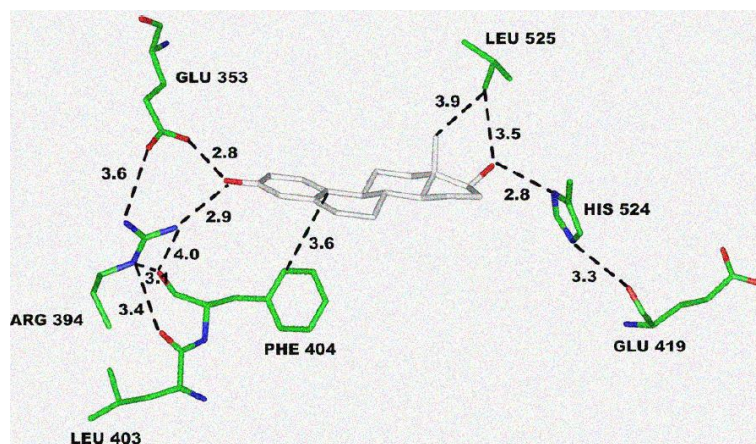
Figure 1.2. Ligand-receptor complex of the human estrogen receptor alpha (ER$_\alpha$) and 17α-estradiol. Hydrogen bonds between amino acids and steroid are shown in dashed lines. The 3-hydroxy moiety interacts with the γ-carboxylate of GLU353. Steroid receptors for 3-keto steroids (androgen, progestogen, mineralocorticoid, glucocorticoid) consist of an amido NH$_2$ group that formed a hydrogen bond with the 3-oxo moiety. In green: carbon; in red: oxygen; in blue: nitrogen (adopted from BAKER, 2011).

1.2.2 Corticosteroids

Corticosteroids are subdivided into two different classes. The differentiation into glucocorticoids and mineralocorticoids is based on their physiological role in mammalians. Mineralocorticoids are responsible for the regulation of the electrolyte and fluid balance (e.g. potassium and sodium retention) and therefore for the regulation of the blood pressure. The primary mineralocorticoid is aldosterone, although other endogenous steroids have mineralocorticoid functions (BODOR AND BUCHWALD, 2006).

Glucocorticoids are involved in glucose metabolism. They mediate the availability of glucose in the organism under stressful and non-stressful conditions. This is why cortisone and hydrocortisone are occasionally called the stress hormone. Besides their role in glucose metabolism, glucocorticoids are important for fetal development, such as in the maturation of the respiratory tracks during pregnancy. Another important role is its immunological function. The primary endogenous glucocorticoid is hydrocortisone (BODOR AND BUCHWALD, 2006).

These multiple physiological functions of corticosteroids in general, make them one of the most prescribed pharmaceutical group and led to the diversity of medical indications. Historically, the first characterization of the adrenal cortex steroid cortisone is assigned to Reichstein and Kendall (1935), which was the beginning of modern endocrinology. After the

structural characterization of cholesterol (1932) and the discovery of the basic structure of steroids, the structure of cortisone was proven.

At the same time, the first specific steroids were used for medicinal therapies. The use of cortisone in the treatment of rheumatoid arthritis and its peerless therapeutic benefits was honored with the Nobel prize in medicine (KENDALL, 1950). Within these decades the physiological role of adrenal cortex steroids was studied and became globally the center of attraction within the pharmaceutical industry and medicine.

1.2.3 Progestogens

Progestogens (also called progestins, progestagens and gestagens) are a group of steroids comprising the natural progesterone and various synthetic steroids. They were defined as compounds that maintain pregnancy (KUHL, 2011). Progesterone, as the primary endogenous progestogen, binds to the progesterone receptor and is essential for the function of the cervix, uterus, endometrium, the central nervous system, pituitary, and the breast. Besides the use in hormonal contraception, progestogens are indicated for the treatment of cancer, gynecological disorders, menopausal symptoms and hormonal replacement (KUHL, 2011; KUMAR ET AL., 2015). The most commonly used progestogens are synthetic derivatives based on the parent steroid types 19-nortestosterone, 17α-hydroxyprogesterone and spironolactone. Although all synthetic progestogens have progestogenic activity, they differ largely in their hormonal pattern. As members of the 3-keto steroid family, progestogens are known to act as weak androgens or anti-androgens, glucocorticoids or anti-mineralocorticoids and exert wanted and unwanted partial clinical effects (KUHL, 2011).

1.2.4 Structural Diversity of Synthetic Steroid Hormones

After the discovery of several steroids from natural origin, the development of synthetic derivatives of such was in the focus of organic chemists. Over the decades of steroid research, structure-related activity relationships were figured out and, on that basis, numerous active derivates were developed and marketed (KUHL, 2011).

The activity and receptor selectivity of glucocorticoids can be improved by the introduction of carbon double bonds and substituents at specific sides (Fig. 1.3). The first developed synthetic glucocorticoid was prednisolone, which is the 1,2-dehydro (Δ^1) derivative of hydrocortisone. Prednisolone showed a four-fold higher activity than hydrocortisone. Further increase of the activity was achieved by introducing fluorine at C9 in α-position, while a

methyl substituent at C16 lead to a significant reduction of the mineralocorticoid receptor binding affinity and thus to a reduction of unwanted side effects in glucocorticoid therapy (BODOR AND BUCHWALD, 2006). These early studies on synthetic steroids, revealed how the selective introduction of fluorine improves the properties of pharmaceuticals for the first time (reviewed in SHAH AND WESTWELL, 2007).

Glucocorticoids are primarily used as esterified derivatives. The esterification of the terminal hydroxyl groups (at C17 and C21) with short-chained carboxylates (e.g. acetate, propionate) reveals a favorable ratio of the target and side effect profile. For instance, esterified steroids show better skin penetration when used in creams and ointments for dermal diseases. The benefit of diesters in dermal applications is the low receptor binding affinity, which significantly increases after the enzymatic hydrolysis within the skin adsorption. These prodrug concepts promote the wanted interaction of the pharmaceutical with the steroid receptor at the targeted tissue (BODOR AND BUCHWALD, 2006). Another example of glucocorticoid "engineering" is the inhibition of endogenous metabolism by blocking the active sides of the steroids. This leads to longer-acting glucocorticoids with higher plasma half-lifes, such as 6α-methylprednisolone and clobetasol propionate (BODOR AND BUCHWALD, 2006). Overall, during the development of new corticosteroid therapies, numerous different synthetic glucocorticoids with specific characteristics were designed. As a consequence, a broad range of prodrugs, active metabolites, and glucocorticoids can be potentially discharged into the receiving waters by the WWTPs.

In addition, norethisterone (developed in 1951) was the first well-tolerated progestogen (DJERASSI ET AL., 1954). Further derivatives were synthesized and marketed in the following years. Structural moieties that are associated with the progesterone receptor binding affinity are the 3-keto and the Δ^4-double bond (KUHL, 2011).

The development of 17α-hydroxyprogesterone type oral contraceptives was reported first in 1954 (KUHL, 2011). Progesterone was found to be ineffective after oral administration due to its extensive inactivation via metabolism. In search of active progesterone derivatives, compounds with space-filling substituents at the C17 position showed pronounced oral efficacy, due to the inhibitory effect on the reduction of the 20-keto moiety (NEUMANN, 1994). This discovery was the beginning of an entire substance class and decades later, there are about 20 different progestogens available with progesterone-like activity (FENT 2015, KUMAR ET AL., 2015).
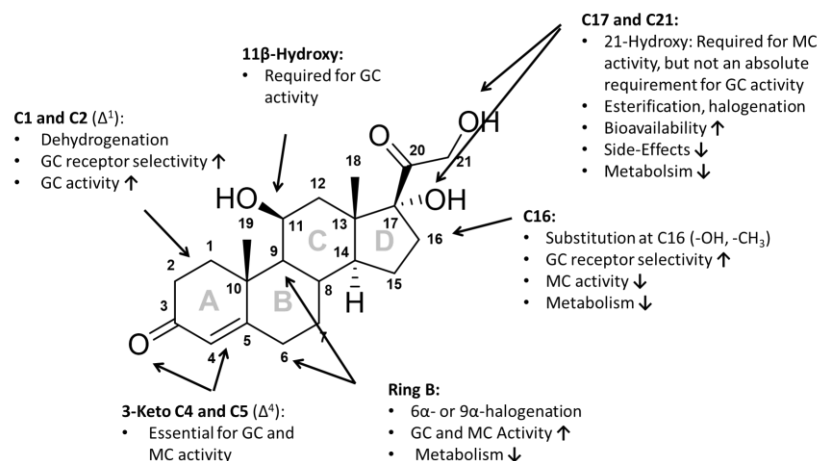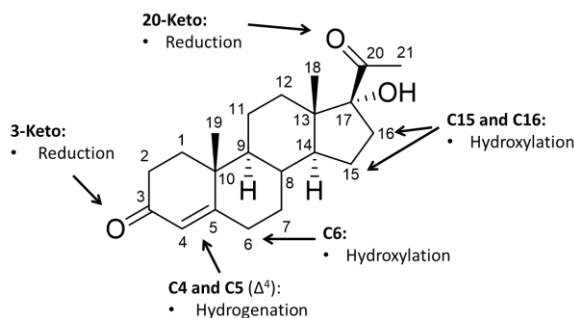
Figure 1.3. Structure-activity relationships of glucocorticoid and metabolic deactivation of 17α-hydroxyprogesterone structures according to current knowledge. (modified from BODOR AND BUCHWALD, 2006; NEUMANN, 1994).

1.3 Steroids in the Aquatic Environment

1.3.1 Sources and Pathways into the Environment

Steroid hormones may originate from households, industry, agriculture, or wildlife and be discharged into the environment. An overview of the potential emission routes of steroid hormones into surface waters is shown in Fig. 1.4.

Similar to other pharmaceuticals, incomplete elimination during wastewater treatment leads to continuous discharges of steroids through municipal WWTP effluents (LIU ET AL., 2011B; RICHARDSON AND TERNES, 2018). Especially, wastewater from in-patient healthcare facilities was figured out as highly contaminated with frequently prescribed steroids (SCHRIKS ET AL., 2010; ZHANG ET AL., 2017). Considerable quantities of endogenous steroids are excreted by humans,

animals, and plants due to their natural origin and are transported directly or indirectly into the environmental compartments (ZHANG ET AL., 2014).

Untreated wastewater, in general, contains high levels of steroids (CHANG ET AL., 2009; HERRERO ET AL., 2012; WU ET AL., 2019). Chang *et al.* (2009) analyzed the source apportionment of steroids in urban rivers from China and found that about 60% of the steroid hormones detected were discharged into the river by untreated wastewater sources. Although, untreated wastewater is discussed as a significant source of emerging contaminants in low- and middle-income countries (WILLIAMS ET AL., 2019), leaking canalizations and combined sewer overflow discharges may contribute to the release of steroid contaminations in high-income countries as well (GASPERI ET AL., 2008; PHILLIPS ET AL., 2012; MUTZNER ET AL., 2019). Phillips *et al.* (2012) reported that the annual steroid loads caused by combined sewer overflow discharges are significant (40-90%), although they represent only 10% of the total annual water discharge. This is true for steroids with high removal efficiencies in WWTPs, while hardly degraded compounds showed a contrary behavior caused by the dilution with runoff after heavy rainfalls. However, at present, to which this impacts the overall hormonal activities in the receiving water bodies is difficult to assess, as the potency of the individual steroids varies greatly (LIU ET AL., 2011A; JIA ET AL., 2016).

Another pathway of steroids into the aquatic environment is the agriculture sector (SHORE AND SHEMESH, 2003; JENKINS ET AL., 2006). Endogenous steroids and their transformation products in manure from meat and dairy plants can be washed off and transported to rivers and streams after spreading manure onto the agricultural landscapes. Such events can lead to spatiotemporal high steroid loads (TERNES ET AL., 2004; KÜMMERER 2010). Besides these, synthetic steroids are known to be improperly utilized as growth promoters in meat production or as sex control agents in aquacultures (PIFERRER 2001; LIU ET AL., 2015 & 2017; LUCKENBACH ET AL., 2017).

In addition, paper mill effluents are related to cause endocrine disruption since they are known as local point sources of phytosterols. The biotransformation of phytosterols into progesterone and potent androgens in the receiving water has been shown to cause masculinization of female fish in wild mosquitofish populations (PARKS ET AL., 2001, JENKINS ET AL., 2004). Moreover, industrial discharges may pose emission hot spots of pharmaceuticals and steroids in surface waters and cause severe adverse effects on local wildlife (CUI ET AL., 2006; SCHRIKS ET AL., 2010; CREUSOT ET AL., 2014; reviewed in LARSSON 2014). A particularly serious example related to the release of elevated loads of synthetic steroids was reported in a French river (SANCHEZ ET AL., 2011; CREUSOT ET AL., 2014). Wild fish populations downstream of

the discharge site of pharmaceutical industry were seriously disrupted and showed reproductive alterations as well as body deformations.

All those sources and events can lead either to continuous emissions or to short-time locally bounden discharges. However, the effluents of municipal WWTPs are a common source particularly for synthetic steroids.
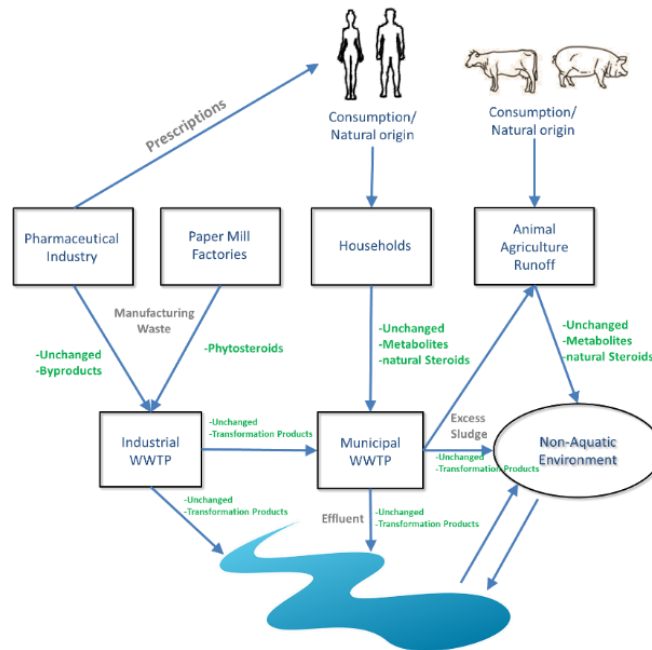

Figure 1.4. Routes and sources of corticosteroids and progestogens into the environment (modified from KÜMMERER, 2010; LIU ET AL., 2011A).

1.3.2   Biological Wastewater Treatment

State-of-the-art WWTPs using conventional activated sludge (CAS) treatment consist of several principal stages as simplified in Fig. 1.5. The raw wastewater influent is treated in a mechanical stage to remove solids and smaller particles. After a pre-clarification, the biological treatment with activated sludge is conducted for the removal of nutrients and other soluble bulk organic compounds. Typically, the biological stage consists of a non-aerated (denitrification) and an aerated sector (nitrification). The anoxic sector is primarily designed to remove nitrogenous nutrients via denitrification, while the efficient microbial elimination of bulk organic matter takes place in the aerobic sector. After the biological stage, the treated wastewater is passed through a secondary clarifier for final sedimentation. CAS treatment plants operate with secondary sludge return flow since this supports a more diverse

microbiological community and constant sludge concentrations in the basins (TERNES AND JOSS 2006; MARGOT ET AL., 2015; GRANDCLEMENT ET AL., 2017).
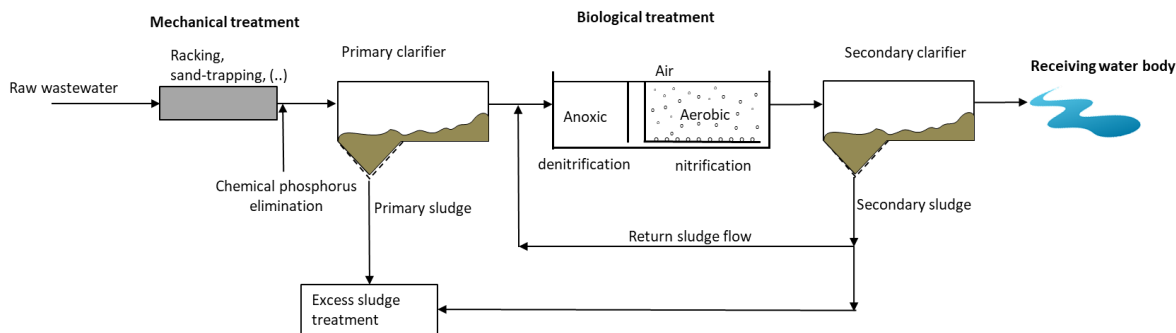
Figure 1.5. Scheme of a WWTP operating with conventional activated sludge (CAS) treatment (adopted from MARGOT ET AL., 2015).

As WWTPs are not primarily designed for the efficient removal of micropollutants (and steroids) they may pass through the biological treatment and subsequently, their constant discharge leads to a quasi-persistent state in the receiving water bodies (GRANDCLEMENT ET AL., 2017). Due to the higher biological activity in the aerobic sector, it is expected that most transformations of micropollutants occur under aerobic conditions (RICHARDSON AND TERNES, 2018).

Although, several operational factors in biological wastewater treatment have been discussed as relevant for the removal, a consensus on which of them are the main drivers and how the interaction of factors affect the elimination is still lacking (FALAS ET AL., 2016). The main operational parameters affecting the removal are the sludge retention time (SRT), the hydraulic residence time (HRT), redox condition and the pH (TERNES AND JOSS 2006; KOH ET AL., 2009; MAENG ET AL., 2013; PETRIE ET AL., 2014A & 2014B; GRANDCLEMENT ET AL., 2017). However, further conditions may affect the removal efficiencies as well, such as temperature or the precipitation rate (GRANDCLEMENT ET AL., 2017). Besides the technical factors, compound removal generally depends on the physicochemical property of the micropollutant and also to the composition of the feed wastewater.

Sludge Retention Time (SRT). A crucial parameter commonly connected to the efficiency of micropollutant removal is the SRT (MAENG ET AL., 2013; PETRIE ET AL., 2014A & 2014B; GRANDCLEMENT ET AL., 2017). It is defined as the main residence time of the microorganisms in the biological treatment stage and is related to the growth rate of the biomass (CLARA ET AL.,

2005; GRANDCLEMENT ET AL., 2017). CAS treatment plants usually operate with excess sludge return flow which allows the enrichment of slowly growing microorganisms. It has been shown that micropollutants can only be removed above a critical SRT. If the SRT is below this value, the effluent concentrations of micropollutants are expected to be in the range of the influent concentrations (CLARA ET AL., 2005). Ternes *et al.* (2004) recommended a minimum SRT for medium-sized and larger WWTPs of at least 12-15 days. Clara *et al.* (2005) proposed a minimum SRT of 10-15 days. However, the correlation of SRT increase and removal efficiency is not clearly understood, since the effects may vary significantly depending on the analyzed compounds (JOSS ET AL., 2005; VIENO ET AL., 2005; FALAS ET AL., 2016). The operating SRTs in modern CAS treatment plants are in the range of 12-15 days. Much higher SRTs are not practicable, since high sludge ages may create a favorable environment for the evolution of antibiotic resistance (KÜMMERER ET AL., 2019).

Hydraulic Residence Time (HRT). The HRT is defined as the residence time of the wastewater in the biological stage. From the actual knowledge, the micropollutant removal efficiencies are less affected by HRT then by the SRT. However, recent studies carried out that the removal of several readily and moderate degradable micropollutants increases by longer HRTs, while the removal of recalcitrant substances was not or marginally affected (GRANDCLEMENT ET AL., 2017). The improvement of the micropollutant removal efficiency in systems with longer HRT is attributed to substrate limitation which led to the metabolism of trace compounds and less-favorable substances (such as micropollutants) as carbon or energy source and increased contact time between micropollutants and the sludge flocs (KOH ET AL., 2009).

1.3.3   Removal and Biodegradation of Steroids

The removal of steroids (and other micropollutants) in CAS treatment is based on two general principles. Either they are removed by sorption onto suspended solids (and assimilation into biomass) or biotic and abiotic degradation leads to their removal (TERNES AND JOSS, 2006; MARGOT ET AL., 2015). In special cases, volatilization during the aeration of the wastewater is a distinct elimination process. However, volatilization can be expected as negligible for steroid removal due to their low volatility.

Sorption to Activated Sludge. Sorption of trace compounds to suspended solids occurs via two different mechanisms. Uncharged and hydrophobic substances are mainly absorbed by the hydrophobic interaction of a compound with the cell membrane of the bacteria and fat fraction of the sludge flocs. Electrostatic interaction of polar groups and positively charged compounds with the negatively charged sludge surface is called adsorption. Both ways lead to the removal of the compounds by the sedimentation of suspended solids. Particularly, for very apolar (log K_d > 4) and cationic substances sorption can be the dominant removal process and has to be considered (JOSS ET AL., 2005).

Although the mechanisms involved in sorption processes are complex (MacKay and Vasudevan 2012), in a first approximation the quantity of the sorbed fraction (c_{sorbed}) of uncharged compounds (e.g. steroid hormones) can be described by a simplified linear equation:

$$(1) \quad c_{sorbed} = K_d * c_{ss} * c_{dissolved}$$

where K_d is the sorption coefficient, c_{ss} the concentration of suspended solids, and $c_{dissolved}$ the concentration of the compound in the liquid phase (TERNES ET AL., 2004). The partitioning of uncharged compounds between solid and liquid phases is often approximated using the octanol-water coefficient K_{OW}. Thus, the compounds' log D values can be used for a rough estimation of the sorbed fraction.

Joss *et al.* (2005) concluded that for compounds with sorption coefficients of below 300 L/kg (log D < 2.48) sorption onto secondary sludge is not relevant for the overall removal. Values of steroid hormones are usually around log D = 3 (WU, 2016) which indicates that sorption has to be considered for steroids. In contrast to this, Andersen *et al.* (2005) concluded that sorption is not significant for estrogens in typical CAS WWTPs under equilibrium conditions (equilibrium of partitioning between solid and liquid phase) due to the low excess sludge production. Liu *et al.* (2011A) showed that sorption of androgens, progestogens, estrogens, and glucocorticoids is less than 20% of the influent mass loads in Chinese WWTPs and indicated biodegradation as the main removal mechanism of steroids in CAS treatment. These results are consistent with further observations (FAN ET AL., 2011; CHANG ET AL., 2011; YU ET AL., 2019). However, comprehensive studies addressing the sorption behavior of steroids other than estrogens are limited and covered mostly natural steroid hormones. The majority of the detected steroids in the particulate matter were endogenous steroids which is most likely rather a consequence of high influent concentrations than of their sorption ability. So far, there is not much evidence that sorption is a significant removal process for corticosteroids and progestogens.

Biodegradation of Micropollutants and Steroids. The degradation (or transformation) of micropollutants in activated sludge treatment occurs via abiotic reactions (e.g. chemical oxidation, hydrolysis, photocatalyzed processes) and microbiologically mediated reactions. Biotransformation by microorganisms in general results from catabolic and co-metabolic mechanisms (GRANDCLEMENT ET AL., 2017; RICHARDSON AND TERNES, 2018). Either the micropollutants are involved in the primary metabolism and are directly used as carbon and energy source for cell growth by specific microorganisms (catabolism), or the transformation occurs indirectly via the metabolism of a primary substrate as the nutrient source by non-specific microorganisms or enzymes (coincidental transformation of the compound) (MICHAEL ET AL., 2014).

Due to the low concentrations of micropollutants, usually between few ng/L to µg/L, the kinetics of the biotransformation in activated sludge treatment can be described by the biodegradation rate constant $k_{biol.}$ (L/(g$_{ss}$*d)) using the following pseudo first-order equation (SCHWARZENBACH ET AL., 2005; JOSS ET AL., 2006):

$$(2) \quad -\ln\frac{c}{c_0} = k_{biol.} * c_{ss} * t$$

where c_0 is the initial concentration of the compound, c_{ss} the concentration of suspended solids, and t the incubation time. Joss *et al.* (2006) suggested a general classification scheme for the biodegradability according to the derived biotransformation rate constants:

- Compounds with $k_{biol.}$ < 0.1 L/(g$_{ss}$*d) are not removed (<20%)
- Compounds with $k_{biol.}$ > 10 L/(g$_{ss}$*d) are readily removed (>90%)
- Compounds between 0.1 L/(g$_{ss}$*d) < $k_{biol.}$ < 10 L/(g$_{ss}$*d) are moderately removed

According to this classification, biodegradation rate constants can be useful to characterize the biodegradability of micropollutants. However, biodegradation kinetic studies of progestogens and corticosteroids are scarce. Chang *et al.* (2011) compared the kinetics of 18 androgens and progestogens in aerobic incubation experiments with activated sludge. The analyzed steroids were completely removed within 24 h of treatment. The obtained degradation half-lives between 0.6 h and 3.3 h suggested a rapid removal. However, most of the included steroids were endogenous androgens and progestogens, respectively.

In addition, Miyamoto *et al.* (2014) analyzed the removal of 10 glucocorticoids in contact with activated sludge and found a high variation in the biodegradability. The results of both

studies revealed higher stability of synthetic steroids in comparison to natural steroids albeit there is not enough reliable data for a generalized conclusion.

In almost all cases micropollutants are not completely mineralized during microbial degradation. They are rather partially degraded leading to the formation of TPs. There are a couple of studies indicating that TPs can be even more toxic than their parent compounds and thus TPs should be carefully evaluated as well (NALECZ-JAWECKI ET AL., 2008; CELIC ET AL., 2009; KOSJEK ET AL., 2009; CWIERTNEY ET AL., 2014).

In the recent years the biotransformation of micropollutants in activated sludge treatment was extensively studied and TPs were identified for pharmaceuticals, fragrances, pesticides, and personal care products (QUINTANA ET AL., 2005; HELBLING ET AL, 2010A, 2010B & 2012; WICK ET AL., 2011; reviewed in PETRIE ET AL., 2015; GULDE ET AL., 2016; RICHARDSON AND TERNES, 2018). On that basis, rule-based biotransformation prediction tools were implemented which became more and more capable and have been greatly improved the prediction of TPs as well as preferences in biodegradation pathways over time.

Contrarily to this, there is only limited research dealing with the transformation processes of corticosteroids and progestogens in CAS treatment (LIU ET AL., 2013; WANG ET AL., 2018; YU ET AL., 2018), although steroids were one of the first pharmaceutical group manufactured via microbial fermentation. The transformation of specific steroids by pure microorganism cultures is well documented (e.g. in BHATTI AND KHERA 2012; CHARNEY AND HERZOG, 1967). On the other hand, the biological wastewater treatment systems are characterized by a mixed microbiological community and thus results cannot be easily transferred onto activated sludge treatment. Due to the very limited data available, such rule-based prediction models are not applicable for steroids and illustrate the need for research in this field.

Biodegradation of steroid hormones under aerobic conditions is often connected with two general degradation pathways, which were characterized so far (DODSON AND MUIR, 1961; CHEN ET AL., 2016B, 2017 & 2018, CHIANG ET AL., 2020, OLIVERA AND LUENGO, 2019). Androgens are degraded following the *9,10-seco* pathway (CHEN ET AL., 2016B; CHIANG ET AL., 2020) while for estrogens the *4,5-seco* pathway was identified as the preferred degradation pathway (CHEN ET AL., 2018; reviewed in OLIVERA AND LUENGO, 2019). As shown in Fig. 1.6, both pathways start via hydroxylation either at ring B for androgens or at the aromatic ring A for estrogens and subsequently lead to the breakdown of the steroid structure. Microorganisms which promote the biodegradation were recently identified in activated sludge and the detection of several TPs in WWTPs revealed that the biodegradation most likely occurs following these pathways (CHEN ET AL., 2017 & 2018). As a marker for the characterization of the *4,5-seco* pathway

pyridinestrone acid can be used (Chen et al., 2018) since it was found to accumulate in the degradation experiments and was exclusively detected during the degradation following this pathway.

However, it is not known to what extent primary, secondary or higher TPs are discharged by WWTP effluents and if there are environmental risks from the exposure to these TPs.
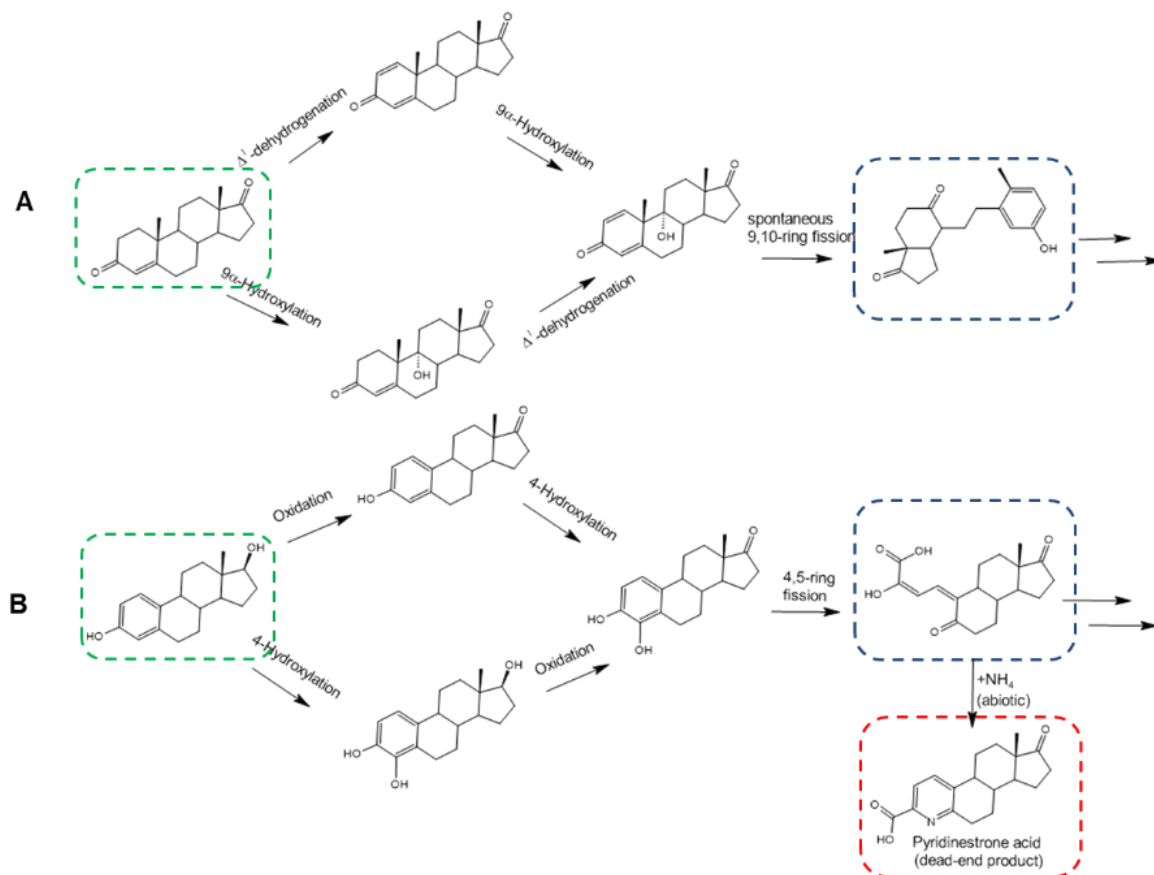
Figure 1.6. Initial steps of the general biodegradation pathways of androgens and estrogens following the 9,10-seco pathway (**A**) and the 4,5-seco pathway (**B**), exemplified for androstenedione and 17β-estradiol (framed green). The biodegradation leads to the breakdown of the steroid core (framed blue). Pyridinestrone acid as a dead-end product (framed red) is often used as an indicator of the degradation following the 4,5-seco pathway.

Regardless the important findings, the transferability of these degradation pathways to other steroid classes is not clear. So far, it has not been investigated if other steroid classes such as corticosteroids and progestogens are degraded by a similar route. Moreover, steroid degradation was mainly studied for natural hormones such as testosterone and 17β-estradiol. Impacts of the steroid structure onto the degradation pathways and the biodegradability are very likely as it is known form synthetic estrogens (TERNES ET AL., 2004; YI AND HARPER, 2007; RICHARDSON AND TERNES, 2018). The insertion of the 17α-ethinyl group to

17β-estradiol for example significantly decreases the biodegradability in comparison to 17β-estradiol (Yi and Harper, 2007). In contrast to estrogens, many of the used corticosteroids and progestogens contain substituents such as halogens to enhance pharmacokinetic and physicochemical properties. The influence of fluorine substituents onto the biodegradability of organic compounds has been shown in various examples (Kümmerer 2010). It can be assumed to elicit impacts on the degradability and biodegradation pathways of synthetic steroid hormones.

Unfortunately, there are very limited studies addressing TP identification of corticosteroids and progestogens (Yu et al., 2018; Liu et al., 2013; Wang et al., 2018). Liu *et al.* (2013) investigated the biodegradation of progesterone and norgestrel in aerobic batch systems inoculated with activated sludge. Norgestrel was found to degrade into 4,5-dihydronorgestrel, 6,7-dehydronorgestrel, and 3α,5β-tetrahydronorgestrel. About 60% of the initial compound was transformed into 4,5-dihydronorgestrel, indicating that fission of the steroid rings might not be necessarily a significant degradation pathway.

In addition, the incubation of progesterone revealed the formation of eight TPs. Here, the authors proposed that the degradation preferably occurs at the C17-side chains, leading to the formation of 17-hydroxy and 17-oxo compounds, or in other words, to the formation of androgens. This hypothesis is supported by other observations as it was verified that plant steroids undergo a cascade of side-chain degradation reactions yielding into progesterone and subsequently to androgens by bacteria in surface waters (Jenkins et al., 2004). Also, Wang *et al.* (2018) proposed that biodegradation of hydrocortisone in river-based aquifer media occurs via a similar mechanism since they detected 17-hydroxy and 17-oxo TPs of hydrocortisone. Oxidative degradation of the steroid C17-side chain is a vital metabolic process particularly known form the catabolism of cholesterol (Olivera and Luengo, 2019). Thus, aliphatic substituents at C17-position, as the case for glucocorticoids and progesterone-type steroids, seem to be a preferred target site for microbiologically mediated degradation. After this, they are likely degraded according to the pathway of androgens or estrogens as it was recently hypothesized (Yu et al., 2018).

1.3.4 Occurrence of Corticosteroids and Progestogens

The first detection of progestogens was reported in 1989. Norethisterone was found in eight WWTP effluent samples from England with concentrations ranging between 8 and 20 ng/L as well as up to 17 ng/L in river samples (AHERNE AND BRIGGS, 1989). Later, Kuch and Ballschmiter (2000) detected levonorgestrel (1 ng/L) in the effluent of a German WWTP with activated sludge treatment. The occurrence of various progestogens has been shown in the following decades by different researchers and almost the complete water cycle. Although most research focused on the determination of norethisterone, levonorgestrel and the natural steroid progesterone, there are a couple of studies indicating further progestogens in the aquatic environment (Tab. 1.1). For instance, medroxyprogesterone acetate and megestrol acetate were detected up to 17 ng/L and 1 ng/L in Chinese WWTP effluents (CHANG ET AL., 2008 & 2011). Golovko *et al.* (2018) found dienogest (1 ng/L), cyproterone acetate (2.8 ng/L), mifepristone (0.5 ng/L) and further progestogens in the effluents of Czech and Slovakian WWTPs. With few exceptions, all these studies have been determined that the concentrations are usually in the lower ng/L-range in WWTP effluents and also in wastewater receiving surface waters. Thus, it can be assumed that various progestogens are not completely removed in WWTPs and consequently discharged into receiving water bodies. However, a comparison of the individual studies is difficult, in particular, due to the analysis of limited or different progestogens and the specificity of consumption volumes in the regions.

Glucocorticoids in WWTP effluents were reported firstly in 2007. Chang *et al.* (2007) detected cortisone, dexamethasone, hydrocortisone, prednisone, and prednisolone in Chinese WWTP effluents. The concentrations were all in the sub-ng/L range. Much higher concentrations were found in the river samples, thus the authors assumed untreated wastewater discharges into the rivers which could be verified in later studies (CHANG ET AL., 2009).

These very first studies analyzed a limited number of glucocorticoids. In further progress of research, the occurrence of highly potent synthetic glucocorticoids has been revealed. In instance, Schriks *et al.* (2010) detected triamcinolone acetonide (14 ng/L) in one WWTP effluent in the Netherlands and betamethasone 17-valerate was detected up to 7.6 ng/L as well as clobetasol propionate up to 4.9 ng/L in Japanese WWTP effluents (KITAICHI ET AL., 2010; ISOBE ET AL., 2015; NAKAYAMA ET AL., 2016). Recently, Jia *et al.* (2016) found that even more synthetic glucocorticoids are discharged by WWTP effluents in the USA and detected 12 different steroids in at least one sample. This could be confirmed by Wu *et al.* (2019). Similar

to progestogens, the concentrations are usually in the lower ng/L-range in WWTP effluents, while triamcinolone acetonide was consistently found at tens of ng/L in the WWTP effluents. An overview of the concentrations of corticosteroids and progestogens in wastewaters of industry, hospitals, municipal WWTPs and surface waters detected so far is shown in Tab. 1.1.

Despite the results summarized below, there is still insufficient and inconsistent knowledge about the occurrence and fate compared to other steroid classes, especially in surface waters. In recent years, however, the number of reports has been increased, particularly due to the development of highly sensitive analytical techniques and methods for environmental steroid monitoring. This growing number of recent studies emphasizes the actuality of the topic.

Tab. 1.1. Occurrence of glucocorticoids, mineralocorticoids, and progestogens in hospital wastewater (WW$_H$), industry wastewater (WW$_I$), municipal wastewater treatment plant influents (Inf) & effluents (Eff), and surface water (SW). CLLE= Continuous Liquid-Liquid Extraction, SPE= Solid Phase Extraction, GC= Gas Chromatography, LC= Liquid Chromatography, MS= Mass Spectrometry, HRMS= High-Resolution Mass Spectrometry.

Compound	Analytical Method	Location	Matrix	Concentration [ng/L]	Reference
Corticosteroids (Glucocorticoids)					
Betamethasone	SPE-LC-MS/MS	France	Inf	15 (+dexamethasone)	Piram et al., 2008
			Eff	7 (+dexamethasone)	
	SPE-LC-MS	Japan	Inf	9.4 (+dexamethasone)	Kitaichi et al., 2010
	SPE-LC-MS/MS	Switzerland	WW$_H$	1720 (+dexamethasone)	Ammann et al., 2014
			Inf	106 (+dexamethasone)	
			Eff	15 (+dexamethasone)	
			SW	8-13 (+dexamethasone)	
	SPE-LC-MS/MS	Japan	Eff	0.29-1.3 (+dexamethasone)	Isobe et al., 2015
	SPE-LC-MS/MS	Japan	Eff	<0.075-1.7 (+dexamethasone)	Nakayama et al., 2016
	SPE-LC-MS/MS	USA	Eff	0.18-0.66	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	1.26	Wu et al., 2019
			Eff	0.11	
Betamethasone 21-acetate	SPE-LC-MS/MS	Switzerland	Eff	4	Ammann et al., 2014
			SW	<1-13	
Betamethasone 17-valerate	SPE-LC-MS	Japan	Inf	8.6	Kitaichi et al., 2010
			Eff	1.3	
	SPE-LC-MS/MS	Japan	Eff	0.84-4.7	Isobe et al., 2015
	SPE-LC-MS/MS	Japan	Eff	<0.21-7.6	Nakayama et al., 2016
Budesonide	SPE-LC-MS/MS	France	Eff	3	Piram et al., 2008
	SPE-LC-MS/MS	Switzerland	WW$_H$	4	Ammann et al., 2014
			Inf	1	
			SW	1-4	
	SPE-LC-MS/MS	USA	Eff	0.29-0.36	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	4.97	Wu et al., 2019
			Eff	<0.21	
Clobetasol	SPE-LC-MS/MS	Switzerland	SW	<0.5-1	Ammann et al., 2014
Clobetasol propionate	SPE-LC-MS/MS	Switzerland	WW$_H$	7	Ammann et al., 2014
			Inf	7	
	SPE-LC-MS/MS	Japan	Eff	0.91-3.0	Isobe et al., 2015
	SPE-LC-MS/MS	Japan	Eff	<0.21-4.9	Nakayama et al., 2016
	SPE-LC-MS/MS	USA	Eff	1.04-2.35	Jia et al., 2016

	SPE-LC-MS/MS	USA	Inf	4.68	Wu et al., 2019
			Eff	2.22	
Cortisone	SPE-LC-MS/MS	China	Inf	4.6-86	Chang et al., 2007
			Eff	0.13-0.58	
			SW	0.06-4.2	
	SPE-LC-MS/MS	France	Inf	174	Piram et al., 2008
			Eff	229	
	SPE-LC-MS/MS	China	Eff	0.26-0.88	Chang et al., 2009
			SW	0.05-29	
	SPE-LC-HRMS	The Netherlands	WW$_i$	26	Schriks et al., 2010
	SPE-LC-MS/MS	China	Inf	15.6	Fan et al., 2011
			Eff	0.24	
	SPE-LC-MS/MS	China	Inf	14.5-45.8	Liu et al., 2011b
			Eff	<0.38	
			SW	0.6-1.9	
	SPE-LC-MS/MS	Spain	Inf	122-285	Herrero et al., 2012
			Eff	<3	
	SPE-LC-MS/MS	Switzerland	WW$_H$	378 (+Hydrocortisone)	Ammann et al., 2014
			Inf	160 (+Hydrocortisone)	
			Eff	26 (+Hydrocortisone)	
			SW	7-10 (+Hydrocortisone)	
	SPE-LC-MS/MS	USA	Eff	<0.12-0.51	Jia et al., 2016
	SPE-LC-MS/MS	The Netherlands	Inf	115-261	Houtman et al., 2018
	SPE-LC-MS/MS	USA	Inf	389	Wu et al., 2019
			Eff	0.21	
Dexamethasone	SPE-LC-MS/MS	China	Inf	0.11-0.16	Chang et al., 2007
			Eff	0.02-0.09	
			SW	0.02-0.31	
	SPE-LC-MS/MS	China	Eff	0.05	Chang et al., 2009
			SW	0.05-8.0	
	SPE-LC-MS/MS	Hungary	SW	<0.01-0.06	Tölgyesi et al., 2010
	SPE-LC-HRMS	The Netherlands	HH$_I$	90	Schriks et al., 2010
	SPE-LC-MS/MS	China	Inf	0.81	Fan et al., 2011
			Eff	0.03	
	SPE-LC-MS/MS	China	Inf	3.8-22.6	Liu et al., 2011b
			Eff	<0.83	
			SW	<0.13	
	SPE-LC-MS/MS	Spain	Inf	<7.5	Herrero et al., 2012

			Eff	<3	
			SW	<0.5	
	SPE-LC-MS/MS	Japan	Eff	<0.075-1.7	Nakayama et al., 2016
	SPE-LC-MS/MS	USA	Eff	<0.06-0.16	Jia et al., 2016
	SPE-LC-MS/MS	The Netherlands	Inf	7.0-21.0	Houtman et al., 2018
			Eff	0.0-2.0	
	SPE-LC-MS/MS	USA	Inf	0.47	Wu et al., 2019
			Eff	<0.04	
Fluocinolone acetonide	SPE-LC-MS/MS	USA	Eff	0.91-3.69	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	0.83	Wu et al., 2019
			Eff	0.37	
Fluorometholone			WW$_H$	2	
	SPE-LC-MS/MS	Switzerland	Inf	3	Ammann et al., 2014
			SW	<0.5-1.0	
Fluocinonide	SPE-LC-MS/MS	USA	Eff	<0.2-0.27	Jia et al., 2016
Fluticasone propionate	SPE-LC-MS/MS	Switzerland	WW$_H$	5	Ammann et al., 2014
			Inf	5	
	SPE-LC-MS/MS	USA	Eff	0.34-1.43	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	4.03	Wu et al., 2019
			Eff	1.48	
Flumetasone	SPE-LC-MS/MS	Hungary	SW	<0.06-1.43	Tölgyesi et al., 2010
	SPE-LC-MS/MS	Switzerland	WWH	5	Ammann et al., 2014
			Inf	6	
			Eff	3	
			SW	1-2	
Hydrocortisone	SPE-LC-MS/MS	China	Inf	7.6-120	Chang et al., 2007
			Eff	0.25-1.9	
	SPE-LC-MS/MS	France	Inf	53	Piram et al., 2008
			Eff	63	
	SPE-LC-MS/MS	China	Eff	0.19-0.57	Chang et al., 2009
			SW	0.11-20	
	SPE-LC-MS/MS	Hungary	SW	<0.17-2.67	Tölgyesi et al., 2010
	SPE-LC-HRMS	The Netherlands	WW$_I$	13	Schriks et al., 2010
			WW$_H$	275-301	
	SPE-LC-MS/MS	China	Inf	22.1	Fan et al., 2011
			Eff	0.13	
	SPE-LC-MS/MS	China	Inf	12.7-28.8	Liu et al., 2011b

			Eff	<0.47	
			SW	<0.2	
	SPE-LC-MS/MS	Spain	Inf	136-270	Herrero et al., 2012
			Eff	<3	
	SPE-LC-MS/MS	Japan	Eff	0.42-1.36	Isobe et al., 2015
	SPE-LC-MS/MS	Japan	Eff	0.28-6.6	Nakayama et al., 2016
	SPE-LC-MS/MS	USA	Eff	0.22-1.57	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	249	Wu et al., 2019
			Eff	0.74	
6α-Methylprednisolone	SPE-LC-MS/MS	China	Inf	<0.08-2.0	Chang et al., 2007
			Eff	<0.02	
	SPE-LC-MS/MS	China	SW	0.2-0.41	Chang et al., 2009
	SPE-LC-MS/MS	China	Inf	0.2	Fan et al., 2011
			Eff	0.03	
	SPE-LC-MS/MS	Switzerland	WW_H	36	Ammann et al., 2014
			Inf	8	
			Eff	1	
			SW	3-5	
	SPE-LC-MS/MS	Japan	Eff	0.13-3.4	Nakayama et al., 2016
	SPE-LC-MS/MS	USA	Eff	<0.03-1.53	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	14.7	Wu et al., 2019
			Eff	0.24	
Prednicarbat	SPE-LC-MS/MS	The Netherlands	Inf	57-65	Houtman et al., 2018
Prednisolone	SPE-LC-MS/MS	China	Inf	1.5-7.5	Chang et al., 2007
			Eff	0.47-0.72	
			SW	0.03-0.64	
	SPE-LC-MS/MS	China	Eff	0.47-0.72	Chang et al., 2009
			SW	0.25-1.8	
	SPE-LC-MS/MS	Hungary	SW	<0.04-0.58	Tölgyesi et al., 2010
	SPE-LC-HRMS	The Netherlands	WW_I	247	Schriks et al., 2010
			WW_H	315-1918	
	SPE-LC-MS/MS	China	Inf	1.7	Fan et al., 2011
			Eff	0.07	
	SPE-LC-MS/MS	Spain	Inf	24-33	Herrero et al., 2012
			Eff	<3	
			SW	<0.5	
	SPE-LC-MS/MS	Japan	Eff	0.51-1.6	Isobe et al., 2015
	SPE-LC-MS/MS	Japan	Eff	<0.075-1.7	Nakayama et al., 2016

	SPE-LC-MS/MS	USA	Eff	<0.03-1.43	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	19.7	Wu et al., 2019
			Eff	0.16	
Prednisone	SPE-LC-MS/MS	China	Inf	0.44-8.4	Chang et al., 2007
			Eff	0.18	
			SW	0.12-0.86	
	SPE-LC-MS/MS	China	SW	0.04-2.4	Chang et al., 2009
	SPE-LC-HRMS	The Netherlands	WW$_H$	117-545	Schriks et al., 2010
	SPE-LC-MS/MS	China	Inf	0.57	Fan et al., 2011
			Eff	0.06	
	SPE-LC-MS/MS	China	Inf	8.5	Liu et al., 2011b
			Eff	<0.32	
			SW	<0.18	
	SPE-LC-MS/MS	Spain	Inf	21-45	Herrero et al., 2012
			Eff	<3	
			SW	<0.5	
	SPE-LC-MS/MS	Switzerland	WW$_H$	1221 (+prednisolone)	Ammann et al., 2014
			Inf	336 (+prednisolone)	
			SW	10-12 (+prednisolone)	
	SPE-LC-MS/MS	The Netherlands	Inf	50	Houtman et al., 2018
	SPE-LC-MS/MS	USA	Inf	4.02	Wu et al., 2019
			Eff	<0.06	
Triamcinolone	SPE-LC-MS/MS	France	Inf	31	Piram et al., 2008
			Eff	30	
	SPE-LC-MS/MS	Hungary	SW	<0.5	Tölgyesi et al., 2010
Triamcinolone acetonide	SPE-LC-MS/MS	France	Inf	40	Piram et al., 2008
			Eff	3	
	SPE-LC-MS/MS	Hungary	SW	<0.02	Tölgyesi et al., 2010
	SPE-LC-HRMS	The Netherlands	WW$_H$	14-41	Schriks et al., 2010
			Eff	14	
	SPE-LC-MS/MS	Switzerland	WW$_H$	14	Ammann et al., 2014
			Inf	6	
			Eff	1	
	SPE-LC-MS/MS	USA	Eff	5.75-14.0	Jia et al., 2016
	SPE-LC-MS/MS	USA	Inf	22.1	Wu et al., 2019
			Eff	17.9	

Corticosteroids (Mineralocorticoids)					
Spironolactone	SPE-LC-MS/MS	Switzerland	WW_H	130	Ammann et al., 2014
			Inf	36	
			Eff	2	
			SW	1-4	
Fludrocortisone acetate	SPE-LC-MS/MS	Switzerland	WW_H	82	Ammann et al., 2014
			Inf	36	
			Eff	12	
			SW	5-14	
Progestogens (17α-Hydroxyprogesterone Type)					
Chlormadinone acetate	SPE-LC-HRMS	Czech Republic	Inf	1.5	Golovko et al., 2018
Cyproterone acetate	SPE-LC-MS/MS	China	SW	0.36 [a)]	Schen et al., 2018
			Eff	0.23 [a)]	
	SPE-LC-HRMS	Czech Republic	Inf	0.23-6.7	Golovko et al., 2018
			Eff	2.8	
	SPE-LC-HRMS	Czech Republic	Inf	2.9-12	Sauer et al., 2018
			Eff	0.50	
	SPE-LC-MS/MS	The Netherlands	Inf	20	Houtman et al., 2018
			Eff	5	
Medroxyprogesterone	SPE-GC-MS/MS	USA	Eff	<0.4-14.9	Kolodziej et al., 2003
	SPE-LC-MS/MS	China	Inf	0.58	Fan et al., 2011
			Eff	0.73	
	SPE-LC-MS/MS	Switzerland	WW_H	42	Ammann et al., 2014
			Inf	6	
			SW	1-5	
	SPE-LC-MS/MS	Czech Republic	Eff	2.0 [a)]	Macikova et al., 2014
		Switzerland	SW	2.7 [a)]	
	SPE-LC-HRMS	Czech Republic	Eff	0.23	Golovko et al., 2018
	SPE-LC-HRMS	Czech Republic	Inf	0.19	Sauer et al., 2018
			Eff	0.95	
			SW	0.12	
Medroxyprogesterone acetate	SPE-LC-MS/MS	Japan	Inf	0.21-2.42	Chang et al., 2008
			Eff	0.03-0.42	
	SPE-LC-MS/MS	China	Inf	18-58	Chang et al., 2011
			Eff	0.1-1.1	
	SPE-LC-MS/MS	China	Inf	1.08	Fan et al., 2011
			Eff	0.06	

	SPE-LC-MS/MS	China	Inf	2.4	Liu et al., 2014
			Eff	0.9	
	SPE-LC-MS/MS	Switzerland	Inf	4.2-120	Zhang et al., 2017
	SPE-LC-HRMS	Czech Republic	Inf	2.6-4.4	Golovko et al., 2018
			Eff	0.21-0.58	
Megestrol acetate	SPE-LC-MS/MS	Japan	Eff	0.35	Chang et al., 2008
	SPE-LC-MS/MS	China	Inf	1.9-9.3	Chang et al., 2011
			Eff	0.1-0.7	
	SPE-LC-MS/MS	China	Inf	4.6	Fan et al., 2011
			Eff	0.2	
	SPE-LC-MS/MS	China	Inf	3.0	Liu et al., 2014
			Eff	1.2	
			SW	0.6	
	SPE-LC-MS/MS	Switzerland	Inf	11	Zhang et al., 2017
			Eff	0.8	
			SW	0.14-4.6	
	SPE-LC-HRMS	Czech Republic	Inf	4.8-6.3	Golovko et al., 2018
			Eff	0.23-0.4	
	SPE-LC-HRMS	Czech Republic	Inf	0.52-13	Sauer et al., 2018
			Eff	0.13-1.0	
		Slovakian Republic	Inf	4.2	
	SPE-LC-MS/MS	China	Inf	0.15 [a]	Schen et al., 2018
			Eff	0.14 [a]	

Progestogens (19-Nortestosterone Type)					
Dienogest	SPE-LC-HRMS	Czech Republic	Inf	1.9-11.0	Golovko et al., 2018
			Eff	0.14-1.0	
	SPE-LC-HRMS	Slovakian Republic	Inf	3.9	Sauer et al., 2018
Dydrogesterone	SPE-LC-MS/MS	China	Inf	35.1	Liu et al., 2014
			SW	3.6	
	SPE-LC-HRMS	Czech Republic	Inf	0.28	Golovko et al., 2018
			Eff	0.51	
	SPE-LC-MS/MS	China	Eff	3.1 [a]	Shen et al., 2018
			SW	4.4 [a]	
Gestodene	SPE-LC-MS/MS	China	Eff	1.6 [a]	Shen et al., 2018
			SW	1.8 [a]	
	SPE-LC-HRMS	Czech Republic	Inf	5.5-7.7	Golovko et al., 2018
			Eff	0.71	
	SPE-LC-HRMS	Czech Republic	Inf	5.0-6.3	Sauer et al., 2018

Levonorgestrel	SPE-GC-HRMS	Germany	Eff	1.0	Kuch and Ballschmiter 2000
	SPE-LC-MS	Spain	Inf	<0.2-16.1	Petrovic et al., 2002
			Eff	<0.2-4.0	
	SPE-LC-MS/MS	France	Eff	0.9-17.6	Vulliet et al., 2007
	SPE-LC-MS/MS	France	SW	5.3-7.0	Vulliet et al., 2008
	SPE-LC-MS/MS	Canada	Inf	150-170	Viglio et al., 2008
			Eff	30	
	SPE-LC-MS/MS	China	Eff	6.7-9.2	Liu et al., 2011b
			SW	3.7	
	SPE-LC-MS/MS	Hungary	SW	0.85-3.4	Avar et al., 2016
	SPE-LC-MS/MS	The Netherlands	Inf	8.0	Houtman et al., 2018
Norethisterone	Immunoassay	England	Eff	8-20	Aherne and Briggs 1989
			SW	<2-17	
	SPE-LC-MS	Spain	Inf	<0.2-8.9	Petrovic et al., 2002
			Eff	<0.2-17.4	
	CLLE-GC-MS	USA	SW	48 [a]	Kolpin et al., 2002
	SPE-LC-MS/MS	France	Eff	5.2-41	Vulliet et al., 2007
	SPE-LC-MS/MS	France	SW	2.7-2.8	Vulliet et al., 2008
	SPE-LC-MS/MS	Canada	Inf	70-205	Viglio et al., 2008
			Eff	53	
	SPE-LC-MS/MS	China	Inf	4.6-12	Chang et al., 2011
	SPE-LC-MS/MS	China	SW	3.6-3.7	Liu et al., 2014
	SPE-LC-MS/MS	Switzerland	SW	5.9-14.0	Zhang et al., 2017
	SPE-LC-MS/MS	Hong Kong	Eff	7.7 [a]	Wu et al., 2017
	SPE-LC-MS/MS	China	SW	0.11-0.78	Shen et al., 2018
	SPE-LC-MS/MS	The Netherlands	Inf	33	Houtman et al., 2018
	SPE-LC-HRMS	Czech Republic	Eff	0.85	Golovko et al., 2018
Norgestrel	SPE-LC-MS/MS	China	Inf	28-59	Liu et al., 2011b
			Eff	6.7-9.2	
			SW	3.7-22.2	
	SPE-LC-MS/MS	China	Inf	5.5	Liu et al., 2014
Progestogens (Spironolactone Type)					
Drospirenone	SPE-LC-MS/MS	Hungary	SW	0.26-4.30	Avar et al., 2016

a) Mean values are given.

1.4 Risks Associated with the Presence of Corticosteroids and Progestogens in the Environment

Progestogens. Steroids classified to this group have been shown to induce adverse effects in invertebrates and amphibians at very low concentrations (KUMAR ET AL., 2015; FENT, 2015). The effect-based trigger values of several progestogens in fish were found to be in the range of reported environmental concentrations (KUMAR ET AL., 2015). Ecotoxicological studies were predominantly done in fish while effects have been observed for different life stages, including transcriptional changes, decreasing fecundity, affected sex ratio and growth as well as reduction of the mating activity (FENT, 2015; STEINBACH ET AL., 2019).

With regard to the adverse effects on aquatic organisms, cyproterone acetate, levonorgestrel, norethisterone and gestodene have been found to reduce fecundity of female fish at concentrations around 1 ng/L (SHARPE ET AL., 2004; ZEILINGER ET AL., 2009; PAULOS ET AL., 2010; RUNNALLS ET AL., 2013). The exposure to progestogens also affects the reproductive system of male fish, although these effects were observed at higher concentrations (KROUPOVA ET AL., 2014; SVENSSON ET AL., 2014). Further studies have been shown that amphibians can be affected by low levonorgestrel concentrations (1.3 ng/L) as well (HOFFMANN AND KLOAS, 2012; SÄFHOLM ET AL., 2012). Until now, many progestogens were not analyzed for their ecotoxicological potential, whereas levonorgestrel, norethisterone and natural progesterone have been most frequently studied (FENT, 2015).

Despite the potential risk on aquatic ecosystem health, which is certainly a serious problem, there is no evidence of effects on human endocrine systems from these low environmental emissions so far (LEUSCH ET AL., 2018). A couple of early studies hypothesized the connection between exposure to environmental steroids and declining semen quality, increasing incidence of testicular cancer and breast cancer in human population (SHARPE AND SKAKKEBAEK, 1993; CARLSEN ET AL., 1995; HANDELSMAN, 2001; SAFE, 2008). However, linking empirically health development to the chronic exposure to low steroid levels seems epidemiologically almost impossible.

The accumulation of progestogens in aquatic organisms was discussed as a potentially risk on humans from dietary of fish and seafood from aquacultures (LIU ET AL., 2015 & 2017). Norethisterone, medroxyprogesterone acetate, and cyproterone acetate were detected in different fish tissues originated from the improperly use in Chinese aquacultures. Due to the moderate biological accumulation factors in filet muscle in comparison to liver and bile, it was concluded that there are no human health risks from consumption.

Corticosteroids. Ecotoxicological effects on aquatic organisms which can be caused through corticosteroid exposure are not well analyzed. The first results on potential impacts were reported in 2011 (KUGATHAS AND SUMPTER, 2011). Increased plasma glucose levels and decreased leucocytes have been detected in adult fathead minnows exposed to 1 µg/L beclomethasone dipropionate for 21 days. However, corticosteroids are involved in various physiological functions and they are known to act on every organ in fish. Hence, numerous gene expressions could therefore be affected by corticosteroid exposure as described by Kugathas (2011) and Hidasi (2016). Reported effects on fish *in vivo* include impacts on territorial and breeding behavior, morphological development, gluconeogenesis, regeneration, osmoregulation, and the immune response of fish (details in HIDASI, 2016). In most cases the tested concentrations were relatively high, thus it remains unclear whether environmentally relevant corticosteroid burdens may cause similar effects.

Nevertheless, recent studies from the Fent group have been indicating that potent synthetic corticosteroids likely induce physiological and transcriptional effects even at environmentally relevant concentrations. For instance, altered plasma glucose levels, decreased blood leukocyte numbers, and strong transcriptional changes have been documented for fludrocortisone acetate in adult zebrafish at 6 ng/L and 42 ng/L (ZHAO ET AL., 2016). Another study revealed that the exposure to 0.05 nM (23.3 ng/L) clobetasol propionate suppresses the innate immune system in zebrafish embryo models (HIDASI ET AL., 2017). The high ecotoxicological potential of clobetasol propionate on developing zebrafish embryos and adult zebrafish was confirmed by further studies (WILLI ET AL., 2018 & 2019; FALTERMANN ET AL., 2020; SCHMID ET AL., 2020). Besides this, more endpoints were found to be significantly affected by clobetasol propionate, including decreases in spontaneous muscle contractions, increased heart beating, accelerated hatching and changes of gene expressions related to glucose metabolism, immune system and development (WILLI ET AL., 2018 & 2019; SCHMID ET AL., 2020). Similar effects were observed for fluticasone propionate and triamcinolone acetonide (WILLI ET AL., 2019).

However, more realistically scenarios are those where aquatic organisms are exposed to a number of steroid hormones in the environments and thus likely leading to combined activities of the steroids. Effects of steroid mixtures have been analyzed *in vivo* by the same working groups. The results suggested that corticosteroids act additively on the individual endpoints (WILLI ET AL., 2019 & 2020; SCHMID ET AL., 2020) while combinations of weak (hydrocortisone) and potent corticosteroids (clobetasol propionate) showed similar effects as the most potent steroid alone (FALTERMANN ET AL., 2020).

As highlighted by Margiotta-Casaluci *et al.* (2016), the evaluation of the risks to aquatic organisms requires the consideration of the compounds' uptake dynamics and metabolism in the organism particularly for steroids. Weak *in vitro*-activities do not necessarily result to weak *in vivo*-risks, when the physicochemical properties of the compound lead to high uptake dynamics.

In conclusion, there are several indications that corticosteroids can have impacts on aquatic organism, particularly in contaminated sites of surface waters receiving elevated loads of WWTP effluent. Although the mechanism of the molecularly interaction of steroids and steroid mixtures in non-target organisms is not well understood, in the recent years many new biomarker endpoints were identified which attempted to detect ecotoxicological consequences of corticosteroid exposure sensitively.

Regarding to progestogens, cyproterone acetate, levonorgestrel, norethisterone and gestodene have been found to impact fish at concentrations which are reported in WWTP effluents as well as surface waters. However, for many further steroids effect-based trigger values were not available.

1.5 Analytical Challenges

The quantification of steroid hormones in the environment requires robust and sensitive analytical methods, since the predicted concentrations in the aquatic environment are in the lower ng/L range (KUGATHAS AND SUMPTER, 2013; FENT, 2015). The analysis of structure derivatives can cause interferences which may lead to errors or misinterpretation of analytical results. Some aspects and considerations should be taken into account during the development of analytical methods for the chemical analysis of steroid.

Most of the analytical methods used in environmental monitoring of corticosteroids and progestogens are based on liquid chromatography tandem mass spectrometry (LC-MS/MS). Prior to the instrumental analysis, the steroids are enriched via solid phase extraction, typically with reversed phase columns. To achieve lower detection limits and acceptable ion suppression several clean-up treatments has been developed, mostly based on normal phase extraction techniques such as silica gel extraction (CHANG ET AL., 2007 & 2008; LIU ET AL. 2011A; JIA ET AL., 2016).

The chromatographic separation of isomers and epimers is an important issue in the multi-component analysis of steroids, since there are several synthetic steroids with the same

monoisotopic mass (e.g. triamcinolone acetonide and flunisolide, betamethasone and dexamethasone, cortisone and prednisolone, norethisterone acetate and megestrol and canrenone). In addition, similarities in the fragmentation do often not allow co-elution of such pairs in the MRM mode (multiple reaction monitoring). It was reported that the choice of the mobile phase in LC can improve the chromatographic resolution of betamethasone and dexamethasone (HERRERO ET AL., 2012). A complete base-line separation was achieved by using acetonitrile instead of methanol as the mobile phase and allowed the quantification of the single steroids. Stable retention times, narrow peak widths and high chromatographic resolution minimize furthermore analytical errors caused by unknown steroids in real samples, in particular for natural steroids. A prominent example is that of the human metabolites of progesterone, since various hydroxylated metabolites are excreted by humans which can be detected in the environment (ZHANG AND FENT, 2018; SHEN ET AL., 2018). Steroids tend to in-source fragmentation in ESI (CHANG ET AL., 2007) and thus has to be considered when co-elution of compounds cannot be avoided (as the case for spironolactone and canrenone, discussed in detail in Chapter 2). A further aspect is that heavy isotopes may cause quantification errors in the analysis of large molecules. For example, the ^{37}Cl-isotope of beclomethasone propionate has the same mass as clobetasol propionate. Such cross interferences from heavy isotopes should also be taken into account in the development of the chromatography and detection.

Steroids in general show high fragmentation even at low collision energies, resulting in the formation of many fragments and consequently to less intense MRM transitions. Improvements of the detection sensitivity were found when using adduct ions as precursors in ESI (CHANG ET AL., 2007; HERRERO ET AL., 2012). Glucocorticoids form different precursor ions depending on the mobile phase and ionization mode used (HERRERO ET AL., 2012). For many glucocorticoids it has been reported that formiate and acetate adducts in ESI negative mode showed the best results in sensitivity and are therefore appropriate for environmental analysis. Their tendency for adduct formation was attributed to the ketone at position C20 as well as the hydroxyl groups at position C17 and C21, respectively (HERRERO ET AL., 2012). Progestogens, on the other hand, were mainly measured in ESI positive using the [M+H]$^+$-ion.

In addition, as mentioned in chapter 1.2.4, the diversity of potential candidates challenges the selection of target steroids. Previous analytical methods covered often only a limited number of steroids, thus it remained unclear which steroids from the individual steroid types are present in the environment. This gap limits the comparability of study results.

1.6   Objectives

The main objective of this thesis was to analyze the **occurrence** of a broad range of synthetic steroid hormones in the aquatic environment and to elucidate the **fate** (transformation) and the **behavior** (overall removal, kinetic) of such high priority micropollutants in biological wastewater treatment.

Due to the limited studies focusing on the occurrence of corticosteroids and progestogens in the environment, the first crucial issue of this thesis was the development, optimization, and validation of a comprehensive and **highly sensitive multi-residue analytical method** for the simultaneous quantification of corticosteroids and progestogens in WWTP effluents as well as in rivers and streams (*Chapter 2*). A special focus was set to synthetic compounds, that were not or rarely addressed in environmental research so far. In addition, several human metabolites were considered and their presence in the aquatic environment was tracked as well. The developed method was then applied to various samples from German WWTP effluents and surface waters to indicate i) which of them are the prevalent steroids from each steroid type (glucocorticoids, mineralocorticoids, and progestogens) and ii) what concentrations of these occurred in the aquatic environments.

Another important aim was to take a closer look at their **fate** and **behavior** during **biological wastewater treatment**. Controlled and standardized aerobic degradation experiments with activated sludge taken from a municipal WWTP were conducted to **elucidate** the **biodegradability** of 13 **glucocorticoids** (*Chapter 3*). These experiments should give new insights into the transformation and stability of synthetic glucocorticoids during biological wastewater treatment. A special emphasis was placed on structure-stability relationships. By the investigation of a large number of steroids, a structure-based interpretation of the results was carried out. Together with the identification of transformation products, this approach is promising to improve the understanding of the degradation processes responsible for the removal of synthetic glucocorticoids. The analysis of WWTP effluents was finally conducted to analyze whether the identified transformation products in the lab can be detected in full-scale plants and consequently be possible drivers for endocrine activities in the environment.

A further concern of this thesis was to elucidate the aerobic **biodegradation** of **progestogens** (*Chapter 4*). Similar to the approach described above, nine different progestogens were incubated in comparable degradation batches. The kinetic of the

removal as well as the main transformation products formed were elucidated. Two different structural types of progestogens (17α-hydroxyprogesterone type and 19-nortestosterone type) were selected and the results were then compared. To estimate the transferability of the lab results to full-scale WWTPs, effluent samples of eight WWTPs were analyzed for the presence of transformation products.

1.7 Thesis Outline

Development and application of a robust and highly sensitive quantification method.
Chapter 2 describes the development, optimization, and validation of a highly sensitive multi-residue analytical method based on solid-phase extraction, silica clean-up, and liquid chromatography coupled to tandem mass spectrometry for the simultaneous quantification of corticosteroids and progestogens in the aquatic environment. The developed method was applied to analyze the occurrence of over 60 steroids in German wastewater treatment plant effluents, rivers, and streams.

Kinetic and transformation of glucocorticoids in biological wastewater treatment.
Chapter 3 focuses on the elucidation of the biodegradation of 13 glucocorticoids in lab-scale incubation experiments under aerobic conditions with contact to activated sludge. Biodegradation kinetics were determined and the transformation products formed were identified via high-resolution mass spectrometry and reference standards. The occurrence of the newly identified TPs was analyzed in WWTP effluents.

Biodegradation of 17α-hydroxyprogesterone and 19-nortestosterone type steroid hormones.
Chapter 4 reports the elucidation of the aerobic biodegradation of progestogens (17α-hydroxyprogesterone and 19-nortestosterone type) with activated sludge from a municipal WWTP as inoculum. In analogy to *Chapter 3*, biodegradation kinetics of nine progestogens were determined and the main transformation products for six steroids were identified by high-resolution mass spectrometry measurements and reference standards. The occurrence of progestogens and the newly identified TPs has been analyzed in WWTP effluents.

Final conclusions.
Chapter 5 discusses the key results from a more general view, emphasizes how the gained data in these studies can be interpreted and outlines some further research needs.

2 Occurrence of Glucocorticoids, Mineralocorticoids, and Progestogens in Various Treated Wastewater, Rivers, and Streams
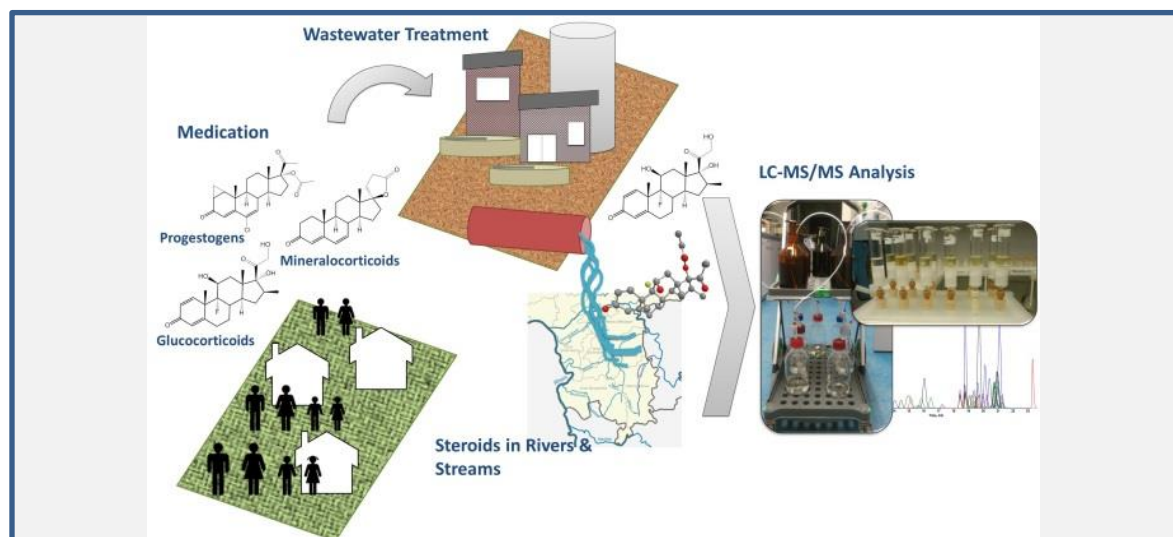
ABSTRACT

In the current study, a highly sensitive analytical method was developed for the determination of 60 steroids including glucocorticoids (GC), mineralocorticoids (MC), and progestogens (PG) in WWTP effluents and surface water using liquid chromatography with tandem mass spectrometry detection (LC-MS/MS). The limits of quantification (LOQ) ranged between 0.02 ng/L (cortisone) to 0.5 ng/L (drospirenone) in surface water and from 0.05 ng/L (betamethasone) to 5.0 ng/L (chlormadinone) in treated wastewater. After optimization, the developed method was applied to WWTP effluents, rivers and streams around Germany. Numerous steroids have been detected during the sampling campaign and predominant analytes from all steroid types were determined. Moreover, the occurrence of dienogest, mometasone furoate, flumethasone pivalate, and the metabolites 6β-hydroxy dienogest, 6β-hydroxy triamcinolone acetonide, 7α-thiomethyl spironolactone, and 11α-hydroxy canrenone is reported for the first time. In addition, this study revealed the ubiquitous presence of topically applied GC monoesters betamethasone propionate, betamethasone valerate, and 6α-methylprednisolone propionate in WWTP effluents and surface water.

2.1   Introduction
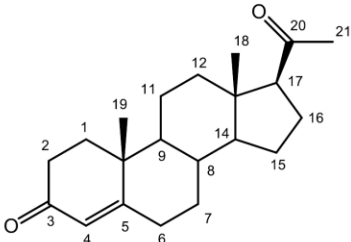
Steroid hormones are widely used in human therapy. As a consequence, the number of approved synthetic hormones is still increasing and thus, the loads entering the wastewater treatment plants (WWTPs) are appreciable (LEMKE ET AL., 2013). Glucocorticoids, for instance, are crucial steroid hormones. Similar to other hormones they are administered as tablets, inhalation-powders, nasal sprays, eye and ear drops, injections, shampoos, creams, ointments, foams or lotions (ROTE LISTE, 2016). Since they are partially metabolized, their metabolites are excreted together with the unchanged compounds via urine and feces. Additionally, non-metabolized steroid hormones can be washed off from skin for topically utilized products. Therefore, a mixture of steroid hormones enters the municipal WWTPs. It has to be noted that several natural steroid hormones are also excreted in substantial quantities (BEISEL ET AL., 1964) without medicinal therapies.

If steroidal hormones, or their metabolites, pass the WWTPs they are discharged into the receiving waters. Induced endocrine disruption in wildlife by natural and synthetic steroids has been known for decades (PURDOM ET AL., 1994; SUMPTER AND JOBLING, 1995; TERNES ET AL., 1999; MATTHIESSEN, 2003) and hence became an important topic in environmental research. In mammals and fish for instance, endogenous steroid hormones are involved in various essential physiological processes by binding on intracellular steroid receptors. Due to structural similarities the majority of hormones exhibit cross receptor binding affinities, and therefore they can act as agonists or antagonists on different receptor types (HOWELL ET AL., 1994; LALONE ET AL., 2013; KUMAR ET AL., 2015). Despite their importance in many physiological regulations, exogenous steroids are known to cause adverse effects on aquatic biota. Multiple effects have been reported so far on fish exposed to steroids in laboratory experiments (HOWELL ET AL., 1994; ZEILINGER ET AL., 2009; KUGATHAS AND SUMPTER, 2011; KUGATHAS 2011; LALONE ET AL., 2013; KUGATHAS ET AL., 2013; ZHAO ET AL., 2015 & 2016; MCNEIL ET AL., 2016; THRUPP ET AL., 2018; WILLI ET AL., 2018) and even on wild fish populations (JOBLING AND TYLER, 2003; SANCHEZ ET AL., 2011; GILBERT, 2011). Moreover, various bioactivities were determined in different water bodies by bioassays (VAN DER LINDEN ET AL., 2008; ZHAO ET AL., 2011; SCHRIKS ET AL., 2013; CHEN ET AL., 2016A; JIA ET AL., 2016; CONLEY ET AL., 2017). Once steroids reach rivers and streams, they are likely to impact the endocrine system of aquatic organisms and are known to trigger adverse effects. Recently studies showed already the occurrence of several progestogens (PG) (CHANG ET AL. 2008, 2009 & 2011; SUN ET AL., 2009; TÖLGYESI ET AL., 2010; LIU ET AL., 2011A & 2011B), mineralocorticoids (MC) (CREUSOT ET AL., 2014, AMMANN ET AL., 2014) and glucocorticoids (GC) (CHANG ET AL., 2007, PIRAM ET AL., 2008, SCHRIKS ET AL., 2010, VULLIET AND CREN-OLIVE, 2011, HERRERO ET AL., 2012, ISOBE ET AL., 2015, NAKAYAMA ET AL., 2016) in the environment.
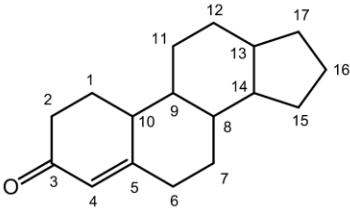
For their determination in environmental matrices, sensitive analytical methods are essential as the steroidal hormones pose a threat on aquatic organism at very low concentrations down to the ng/L range (ZEILINGER ET AL., 2009; KUGATHAS AND SUMPTER, 2011; KUGATHAS ET AL., 2013; ZHAO ET AL., 2015; MCNEIL ET AL., 2016). Comprehensive analytical methods for the multi-residue determination of steroid hormones in environmental matrices are mainly missing. The published methods monitored a limited number of steroids (CHANG ET AL., 2009; TÖLGYESI ET AL., 2010; LIU ET AL., 2011A) focused on natural compounds (CHANG ET AL., 2008 & 2011) or investigated individual steroid hormone classes (CHANG ET AL., 2007; SUN ET AL., 2009; LIU ET AL., 2011A; JIA ET AL., 2016). This study aimed to develop a robust, comprehensive, and highly sensitive analytical method for the quantification of natural and anthropogenic steroids of different classes (PG, MC, GC) as well as their human metabolites in WWTP effluents and surface waters.

In total, 60 target hormones (Tab. 2.1) were selected according to i) elevated usage in human therapy, ii) known excreted metabolites, iii) reported potency on aquatic biota, iv) lack of occurrence data in European rivers and streams and v) topically applied hormones such as diester and monoester derivatives. Finally, another aim of this study was to determine the occurrence of selected steroids and their metabolites in WWTP effluents and several German rivers and streams using the developed multi-method.

Table 2.1. Abbreviation and chemical structures of the target steroid hormones considered in the study.

Basic structure of Gluco- and Mineralocorticoids	Basic structure of Progestogens

Glucocorticoids

Abbreviation	Name	1 + 2	6	9	11	16	17	21
COR	Cortisone				=O		-OH	-OH
HCOR	Cortisol				-OH		-OH	-OH
PNS	Prednisone	C=C			=O		-OH	-OH
PNL	Prednisolone	C=C			-OH		-OH	-OH
MPNL	6α-Methylprednisolone	C=C	α-CH₃		-OH		-OH	-OH
MPNLacp	6α-Methylprednisolone aceponate	C=C	α-CH₃		-OH		-O-COC₂H₅	-O-COCH₃
MPNLprop	6α-Methylprednisolone 21-propionate	C=C	α-CH₃		-OH		-OH	-O-COC₂H₅
BDN	Budesonide	C=C			-OH	-O-HC(C₃H₇)-O-		-OH
BDN-m1	6ß-Hydroxy budesonide	C=C	ß-OH		-OH	-O-HC(C₃H₇)-O-		-OH
BMS	Betamethasone	C=C		-F	-OH	ß-CH₃	-OH	-OH
BMSdiprop	Betamethasone dipropionate	C=C		-F	-OH	ß-CH₃	-O-COC₂H₅	-O-COC₂H₅
BMSprop	Betamethasone 17-propionate	C=C		-F	-OH	ß-CH₃	-O-COC₂H₅	-OH
BMSval	Betamethasone 17-valerate	C=C		-F	-OH	ß-CH₃	-O-COC₄H₉	-OH
BMSac	Betamethasone 21-acetate	C=C		-F	-OH	ß-CH₃	-OH	-O-COCH₃
DMS	Dexamethasone	C=C		-F	-OH	α-CH₃	-OH	-OH
DMS-m1	6ß-Hydroxy dexamethasone	C=C	ß-OH	-F	-OH	α-CH₃	-OH	-OH
DMSac	Dexamethasone 21-acetate	C=C		-F	-OH	α-CH₃	-OH	-O-COCH₃
TRIact	Triamcinolone acetonide	C=C		-F	-OH	-O-C(CH₃)₂-O-		-OH
TRIact-m1	6ß-Triamcinolone acetonide	C=C	ß-OH	-F	-OH	-O-C(CH₃)₂-O-		-OH
FCNact	Fluocinolone acetonide	C=C	-F	-F	-OH	-O-C(CH₃)₂-O-		-OH
FLUfur	Fluticasone furoate	C=C	-F	-F	-OH	α-CH₃	-O-COC₄H₃O	SCH₂F [a]
FLUprop	Fluticasone propionate	C=C	-F	-F	-OH	α-CH₃	-O-COC₂H₅	SCH₂F [a]
FMS	Flumethasone	C=C	-F	-F	-OH	ß-CH₃	-OH	-OH
FMSpiv	Flumethasone pivalate	C=C	-F	-F	-OH	ß-CH₃	-OH	-O-COC(CH₃)₃
FML	Fluorometholone	C=C	α-CH₃	-F	-OH		-OH	
BEC	Beclomethasone	C=C		-Cl	-OH	ß-CH₃	-OH	-OH
BECdiprop	Beclomethasone dipropionate	C=C		-Cl	-OH	ß-CH₃	-O-COC₂H₅	-O-COC₂H₅
BECprop	Beclomethasone 17-propionate	C=C		-Cl	-OH	ß-CH₃	-O-COC₂H₅	-OH
CLO	Clobetasol	C=C		-F	-OH	ß-CH₃	-OH	-Cl
CLOprop	Clobetasol propionate	C=C		-F	-OH	ß-CH₃	-O-COC₂H₅	-Cl
MOM	Mometasone	C=C		-Cl	-OH	α-CH₃	-OH	-Cl
MOMfur	Mometasone furoate	C=C		-Cl	-OH	α-CH₃	-O-COC₄H₃O	-Cl
CIC	Ciclesonide	C=C			-OH	-O-HC(C₆H₁₁)-O-		-O-COCH(CH₃)₂
CIC-m1	Desisobutyryl ciclesonide	C=C			-OH	-O-HC(C₆H₁₁)-O-		-OH
DFCval	Diflucortolone valerate	C=C	-F	-F	-OH	α-CH₃		-O-COC₄H₉
HAL	Halcinonide			-F	-OH	-O-C(CH₃)₂-O-		-Cl
HLM	Halometasone	C(1)=C(2)-Cl	-F	-F	-OH	α-CH₃	-OH	-OH

Mineralocorticoids

Abbreviation	Name	1 + 2	7	9	11	16	17	21
FLC	Fludrocortisone			-F	-OH	-OH	-OH	-OH
FLCac	Fludrocortisone acetate			-F	-OH	-OH	-OH	-O-COCH₃
SPL-m1	7α-Thiomethyl spironolactone		α-S-CH₃				-O-COC₂H₄- [b]	
CAN	Canrenone		C(6)=C(7)				-O-COC₂H₄- [b]	
CAN-m1	11α-Hydroxy canrenone		C(6)=C(7)		-OH		-O-COC₂H₄- [b]	

Progestogens

Abbreviation	Name	1 + 2	6	10	11	13	16	17
CLM	Chlormadinone		Cl-C(6)=C(7)	ß-CH₃		ß-CH₃		-COCH₃, -OH
CLMac	Chlormadinone acetate		Cl-C(6)=C(7)	ß-CH₃		ß-CH₃		-COCH₃, -O-COCH₃
CYP	Cyproterone	-CH₂-	Cl-C(6)=C(7)	ß-CH₃		ß-CH₃		-COCH₃, -OH
CYPac	Cyproterone acetate	-CH₂-	Cl-C(6)=C(7)	ß-CH₃		ß-CH₃		-COCH₃, -O-COCH₃
DIE	Dienogest			C(9)=C(10)				-OH, -CH₂-CN
DIE-m1	6ß-Hydroxy dienogest		-ß-OH	C(9)=C(10)				-OH, -CH₂-CN
DPN	Drospirenone		(6)-CH₂-(7)				(15)-CH₂-(16)	-O-COC₂H₄-
ETG	Etonogestrel			ß-CH₃	=CH₂	ß-C₂H₅		-CCH, -OH
GES	Gestodene					ß-C₂H₅	(15)-CH₂-(16)	-CCH, -OH
HPG	17α-Hydroxy progesterone			ß-CH₃		ß-CH₃		α-OH, -COCH₃
LNG	Levonorgestrel					ß-C₂H₅		-CCH, ß-OH
MRP	Medroxy progesterone		α-CH₃	ß-CH₃		ß-CH₃		α-OH,-COCH₃
MRPac	Medroxy progesterone acetate		α-CH₃	ß-CH₃		ß-CH₃		α-O-COCH₃,-COCH₃
MRPac-m1	6ß-Hydroxy medroxy progesterone acetate		α-CH₃	ß-CH₃, α-OH		ß-CH₃		α-O-COCH₃,-COCH₃
MEG	Megestrol		CH₃-C(6)=C(7)	ß-CH₃		ß-CH₃		α-OH, -COCH₃
MEGac	Megestrol acetate		CH₃-C(6)=C(7)	ß-CH₃		ß-CH₃		α-O-COCH₃,-COCH₃
NES	Norethisterone					ß-CH₃		-CCH, ß-OH
NESac	Norethisterone acetate					ß-CH₃		-CCH, ß-O-COCH₃

[a] sulfur instead of C(21)
[b] without -COCH₃ group at pos. C17

2.2   Materials and Methods

2.2.1   Reagents and Materials

HPLC grade methanol and n-hexane were obtained from Sigma-Aldrich (Seelze, Germany) and Pico grade acetone was purchased from Promochem® (LGC Standards, Wesel, Germany). Milli-Q water was obtained from Millipore (18.2 MΩ, Merck, Darmstadt, Germany). Reference standards and isotope-labeled substances (Tab. A.1) were all purchased from Sigma-Aldrich, Santa Cruz Biochemical (Dallas, USA) or Toronto Research Chemicals Inc. (Ontario, Canada).

2.2.2   Sampling of Wastewater Effluents and Surface Water

Treated wastewater was collected from five conventional municipal German WWTPs. The sample locations of river and surface water were chosen in the instance to get a broad spectrum of river types. All water samples were taken as grab samples either from the WWTP effluent discharge or below the water surface close to the river bank. Sampling dates, surface water dimensions, WWTP capacities and locations can be found in appendix A (Tab. A.2, Fig. A.1). In addition, river water was collected upstream and downstream of three WWTPs discharges.

2.2.3   Target Compound Selection

The synthetic steroid hormones were selected based on the application quantity prescribed in Germany in 2014 (SCHWABE AND PAFFRATH, 2015) (number of prescribed daily dose x defined daily dose). In case of glucocorticoids, further criteria were considered, such as their relative potencies according to the ATC-codes (WHOCC, 2017) (Anatomical Therapeutic Chemical). The steroid hormone types progestogens (PG), glucocorticoids (GC), mineralocorticoids (MC) and some of their main commercially available metabolites were included in the developed method. Androgens were not considered due to their limited use in medicinal therapy. In total, 60 analytes comprising 18 PG, 37 GC and five MC were integrated into one analytical method. Detailed chemical structures of the selected compounds, calculated consumption volumes further information regarding analytes can be found in appendix A (Tab. A.3). Optimized MS parameters of the target analytes are summarized in Tab. A.1.

2.2.4 Sample Preparation

All samples were collected in cleaned and baked (at 550 °C for 8 h) amber glass bottles. If samples could not be extracted within 24 h, acidification to pH 3 with sulfuric acid was performed to prevent biodegradation. The water samples were cooled down to 4 °C during transport to the laboratory and afterwards filtered using a 1 µm glass fiber filter (Whatman, GF6, Maidstone, United Kingdom). The filtered samples were finally adjusted to pH 7-8.5 with diluted ammonia solution or sulfuric acid before enrichment. For sample enrichment, 500 mL filtered WWTP effluent and 1000 mL surface water were spiked with 1 ng of each surrogate standard from IS-mix 1 prior to SPE. The water samples were loaded onto end-capped C18 cartridges (C18ec, 6 mL, 500 mg, Macherey-Nagel, Düren, Germany), which were preconditioned with 3 x 2 mL methanol followed by 3 x 3 mL Milli-Q. Water samples were passed through the cartridges by gravity within 12 h. The cartridges were rinsed with 3 x 2 mL Milli-Q and dried by nitrogen for approximately 2 h. For elution of the extracted analytes, 3 x 3 mL methanol was used. Subsequently, the extracts were evaporated to dryness under a gentle stream of nitrogen at 40 °C and were re-dissolved with 300 µL n-hexane and 700 µL acetone for further clean up. If the cartridges were not eluted immediately, they were stored at -20 °C in the dark after drying. The schematic workflow of the developed method is shown in Fig. 2.1. Purification was achieved by commercially available silica gel glass cartridges (1 g, 6 mL, Macherey-Nagel). The silica gel was dried for 2 h at 100 °C before usage. The polarity and composition of the elution solvent were optimized for the target analytes. The cartridges were preconditioned with 3 x 3 mL n-hexane/acetone (3:7). Afterwards, the sample extracts were loaded onto the cartridges and were eluted three times with 2 mL n-hexane/acetone (3:7). Since, several esterified internal standards (e.g. betamethasone dipropionate-d10, betamethasone propionate-d5) hydrolyzed during the sample treatment, we spiked 4 deuterated internal standards after the sample clean-up (IS-mix 2) to prevent the hydrolysis of these surrogates. Otherwise, it would lead to contaminations of the samples with non-labeled steroids. Therefore, 1 ng of each surrogate standard from IS-mix 2 was spiked to the extracts after clean-up. Then, the extracts were evaporated under a gentle stream of nitrogen at 40 °C to dryness and reconstituted with 250 µL methanol and 250 µL Milli-Q for LC-MS/MS analysis.

Sampling of surface and wastewater

Stabilizing of samples by acidifing to pH 3 with 2.5 mmol/L H_2SO_4

Sample preparation

Filtration of samples by 1 µm glass fiber filter.

a) 1 L surface water (conc.factor= 2000:1)

b) 0.5 L WWTP effluent (conc.factor= 1000:1)

-Spike of IS-mix 1 (1 ng each IS)

-pH adjustment to 7.0-8.5

Sample enrichment

Solid phase extraction:

Machery-Nagel Chromabond® C18ec (6 mL, 500 mg)

-Preconditioning: 3 x 3 mL methanol

-Equilibration: 3 x 3 mL Milli-Q

-Washing: 3 x 2 mL Milli-Q

-Elution: 3 x 2 mL methanol

Evaporation of extracts to dryness with N_2 at 40 °C

Reconstitution in 1 mL acetone/n-hexane (70:30)

Sample purification

Silica gel clean-up:

Macherey-Nagel Chromabond® SiOH (6 mL, 1 g)

-Preconditioning: 3 x 3 mL acetone/n-Hexan (70:30)

-Elution: 3 x 2 mL acetone/n-hexane (70:30)

-Spike of IS-mix 2 (1 ng each IS)

Evaporation of extracts to dryness with N_2 at 40 °C

Reconstitution in 250 µL methanol followed by 250 µL Milli-Q

LC-ESI-MS/MS

Chromatograpy: MN Nucleoshell RP 18plus (3 x 150 mm, 2.7 µm)

Detection: MS/MS (Sciex API 6500, QTRAP)

Flow: 0.3 mL/min, Inj.-vol: 10 µL

DM 1: ESI(+)/ESI(-), eluent A- Milli-Q + 0.1% HCOOH, eluent B- ACN + 0.1% HCOOH, sMRM-Mode

DM 2: ESI(-), eluent A- Milli-Q, eluent B-ACN, post column: 3 % NH_4OH in methanol, flow isokratic pump: 60 µL/min, MRM-Mode

Figure 2.1. Workflow of the developed method for the trace analysis of steroid hormones by LC-MS/MS.

2.2.5 Chemical Analysis

The analysis was performed with an HPLC system, consisting of a G1367E autosampler, a G1330B cooling thermostat for the autosampler, a G1312B binary HPLC pump, a G1310B isocratic HPLC pump, a G1379B membrane degasser and a G1316A column oven (all Agilent 1260Infinity Series, Waldbronn, Germany). Separation was achieved with a MN Nucleoshell RP 18plus column (3 x 150 mm, 2.7 μm) (Macherey-Nagel) with a flow rate of 0.3 mL/min. The injection volume was 10 μL and column oven temperature was set to 25 °C. Sensitive quantification was achieved by splitting LC-MS/MS analysis into two chromatographic runs. Milli-Q with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used as mobile phases for detection-method 1 (DM 1, ESI^{+/-}). Detection-method 2 (DM 2, ESI[-]) operates with non-acidified eluents Milli-Q (C) and acetonitrile (D). To increase the ionization efficiency of the targeted analytes in DM 2, a post-column addition of a 3% ammonia solution in methanol with a flow rate of 0.06 mL min^{-1} was applied by using an isocratic pump and a mixing tee according to previous literature (GENTILI ET AL., 2002; SCHLÜSENER ET AL., 2005).

To avoid a co-elution of interfering substances, the LC gradient for both detection methods was optimized as follows: from 0 to 0.5 min 10% B or D; from 0.5 min to 15 min gradual increase to 47% B or D; then B or D was linearly increased up to 98% in 5 min and held for 10 min; finally returning to 10% B or D in 0.1 min and held for 5 min for equilibration at the end of each chromatographic run, in total 35 min. The HPLC system was coupled to a triple-quadrupole mass spectrometer system (QqQ-LIT-MS, API 6500 QTrap, Sciex, Darmstadt, Germany) equipped with an IonDrive™ ion source for electrospray ionization (ESI). The general MS parameters for both detection-methods were: ion source gas 1 (GS1) and ion source gas 2 (GS2) 35 psi; curtain gas (CUR) 45 psi; collision gas (CAD) medium; source temperature (TEM) 400 °C; ion spray voltage for negative and positive ionization mode -4500 V/5500 V; entrance potential (EP) -10 V/10 V; collision cell exit potential (CXP) -14 V/ 14 V.

DM 1 was performed with switching polarities within the chromatographic runs using scheduled multiple reaction monitoring (sMRM) mode. The specific parameters in DM 1 were as follows: MRM detection window 50 s; target scan time 0.6 s and settling time 4 ms. DM 2 operates only in negative ionization mode using multiple reaction monitoring (MRM) with adjusted dwell times of 20 ms for all MRM transitions.

MS data acquisition was controlled with Analyst 1.6.3 (Sciex). For identification and quantification, the two most sensitive MRM transitions of each analyte were monitored (Tab. A.1).

2.2.6   Quantification and Quality Control

A calibration curve in the concentration range of 0.005–50 ng/mL was used for quantification. Isotope-labeled internal standards (IS-mix 1 and 2) were added to every calibration standard to reach a concentration of 2 ng/mL of each surrogate. Linear regression was applied to the calibration curves with a weighting factor 1/x. The peak areas of the analytes were corrected by one of the 19 surrogate standards (Tab. A.1) for the compensation of the loss during the sample preparation and the matrix-induced ion-suppression in the environmental samples. Furthermore, a calibration standard was measured every tenth sample within a sequence for quality control and method blank samples (non-spiked Milli-Q) were run in parallel every batch to control the contaminations and carryover effects. No significant contaminations or carryovers were obtained during the sample treatment. Finally, data were processed with the software MultiQuant 3.0.2 (Sciex).

2.2.7   Method Validation

To validate the developed method, recoveries and repeatability were examined over the complete concentration range for river water and WWTP effluent. All samples were processed in quadruplicate. Surface water was spiked with 0.05, 0.25, 0.5, and 5.0 ng/L of each analyte, while WWTP effluent was spiked with concentrations of 0.5, 1.0, 10, and 50 ng/L. Due to partial hydrolysis of several glucocorticoid esters, the validation of betamethasone, dexamethasone, beclomethasone, and methylprednisolone was conducted in surface water at two concentration levels (0.5 and 5.0 ng/L) in separate experiments, for their i) diesters, ii) monoesters and iii) free alcohols. For the determination of the limit of detection (LOD) and limit of quantification (LOQ), the Software PeakView® 2.2.0 (Sciex) was used. By definition, the calculations were based on a signal-to-noise (SN) ratio of 3 (LOD) and 10 (LOQ) either using the background concentration or a total spike amount in the smoothed (smoothing factor: 2.0) chromatograms of environmental samples. Noise area was selected manually from the background that bordered on the chromatographic peak. For the determination of LOD and LOQ, the 3σ SN values were used and extrapolated

accordingly. Matrix effects were calculated from surface water samples (c=0.5 ng/L) and WWTP effluents (c=10 ng/L) spiked after the sample treatment. Detailed description and results of the determination of matrix effects are provided in the appendix (Appendix A).

2.3 Results and Discussion

2.3.1 Method Performance

The calibration curves of all analytes showed good linearity (R >0.99) in the defined concentration range. The peak widths were approximately 0.3 min for all analytes. LC gradient was optimized to separate the interfering analyte pairs with similar or even identical molar masses (Fig. A.2). Most synthetic steroids consist of a similar steroid structure and the same functional groups. Thus, for quantification, it is essential to achieve an appreciable chromatographic separation (i.e. for epimers beta- and dexamethasone or cortisone/prednisolone). The developed chromatographic method showed no interfering substances and all critical steroid pairs were base-line separated.

In recent studies (AMMANN ET AL., 2014; JIA ET AL., 2016) spironolactone was monitored using as a precursor the in-source fragment m/z 341. As canrenone forms the same precursor and fragments, insufficient chromatographic separation of canrenone and spironolactone lead to incorrect evaluations. Unfortunately, the separation of these two compounds needs a slowly increasing gradient (VLASE ET AL., 2011) that leads to very long retention times and expanded peak widths with the column used (RP C18ec). Hence, we decided to exclude spironolactone from the quantification and exclusively monitoring the m/z 417 → m/z 341 transition for its qualitative detection. However, spironolactone was not detected in any water sample.

Moreover, to achieve low LOQs we compared the detection sensitivities of formiate adducts [M+HCOO]- with those of [M+H]+ ions in surface waters with acidified eluents (detection method 1) since GC and MC preferentially form carboxylic adducts (formiate and acetate) in ESI (Fig. A.4). It was already reported that the analysis of these adducts might increase the sensitivity of detection for steroids (CHANG ET AL., 2007; HERRERO ET AL., 2012; JIA ET AL., 2016). However, several steroids (e.g. flumethasone pivalate, halcinonide) showed low LOQs when [M-H]- ions were considered for fragmentation in non-buffered eluents and addition of ammonia solution after the chromatography (detection method 2) according to Gentili *et al.* (2002) and Schlüsener *et al.* (2005). Finally, the analytical method was split into two chromatographic runs, to reach low LOQs. For 12 steroids the [M+HCOO]- adduct ions were

used for quantification (solely un-esterified GC). For 39 analytes a higher sensitivity was observed when using [M+H]+ ions and for nine steroids most suitable results were achieved when [M-H]- ions were used for the fragmentation in detection method 2 (Tab. A.1).

For further increase of sensitivity, a silica gel clean-up was used after SPE, to reduce matrix impurities as described in previous studies (Fig. A.5 - A.6) (TERNES ET AL., 1999; CHANG ET AL., 2009; JIA ET AL., 2016). In this framework, we determined the matrix effects in surface water and WWTP effluent (Fig. A.7 - A.8). For surface water the matrix effects ranged from 6% (megestrol) to 37% (ciclesonide) and in WWTP effluents from -10% (flumethasone pivalate) to 40% (ciclesonide). Moreover, by these improvements, LOQs in the range of 0.02 ng/L (e.g. cortisone) to 0.5 ng/L (e.g. drospirenone) in surface water and from 0.05 ng/L (e.g. betamethasone) to 5.0 ng/L (chlormadinone) in treated wastewater could be achieved (Tab. A.4).

2.3.2   Method Validation

As shown in Fig. 2.2, relative recoveries ranged from 73 ± 3% (prednisone) to 112 ± 8% (gestodene) in river water and from 70 ± 10% (cortisone) to 113 ± 2% (mometasone furoate) in WWTP effluents (details provided in Tab. A.5). The recoveries of the analytes were similar at all spiked concentrations and showed no significant scattering or trends. Moreover, the results were comparable for all steroid types as well as for river water and treated wastewater in the considered concentration range.

For validation of the analysis of the diesters of betamethasone, beclomethasone, and 6α-methylprednisolone we chose a different approach for the determination of the recoveries since these diesters are known to hydrolyze rapidly to their active monoester metabolites. Moreover, the spontaneous isomerization of these C17-monoesters to the C21-esters as well as a continuing ester cleavage is already known from several pharmacokinetic studies (BUNDGAARD AND HANSEN, 1981; CHEUNG ET AL., 1985; TÄUBER, 1994). This phenomenon of isomerization is described in the literature as acyl-migration (TÄUBER, 1994) and was observed at neutral pH (CHEUNG ET AL., 1985; TÄUBER, 1994) for several glucocorticoid monoesters. Thus, these rearrangements are likely to occur in the aquatic environment.
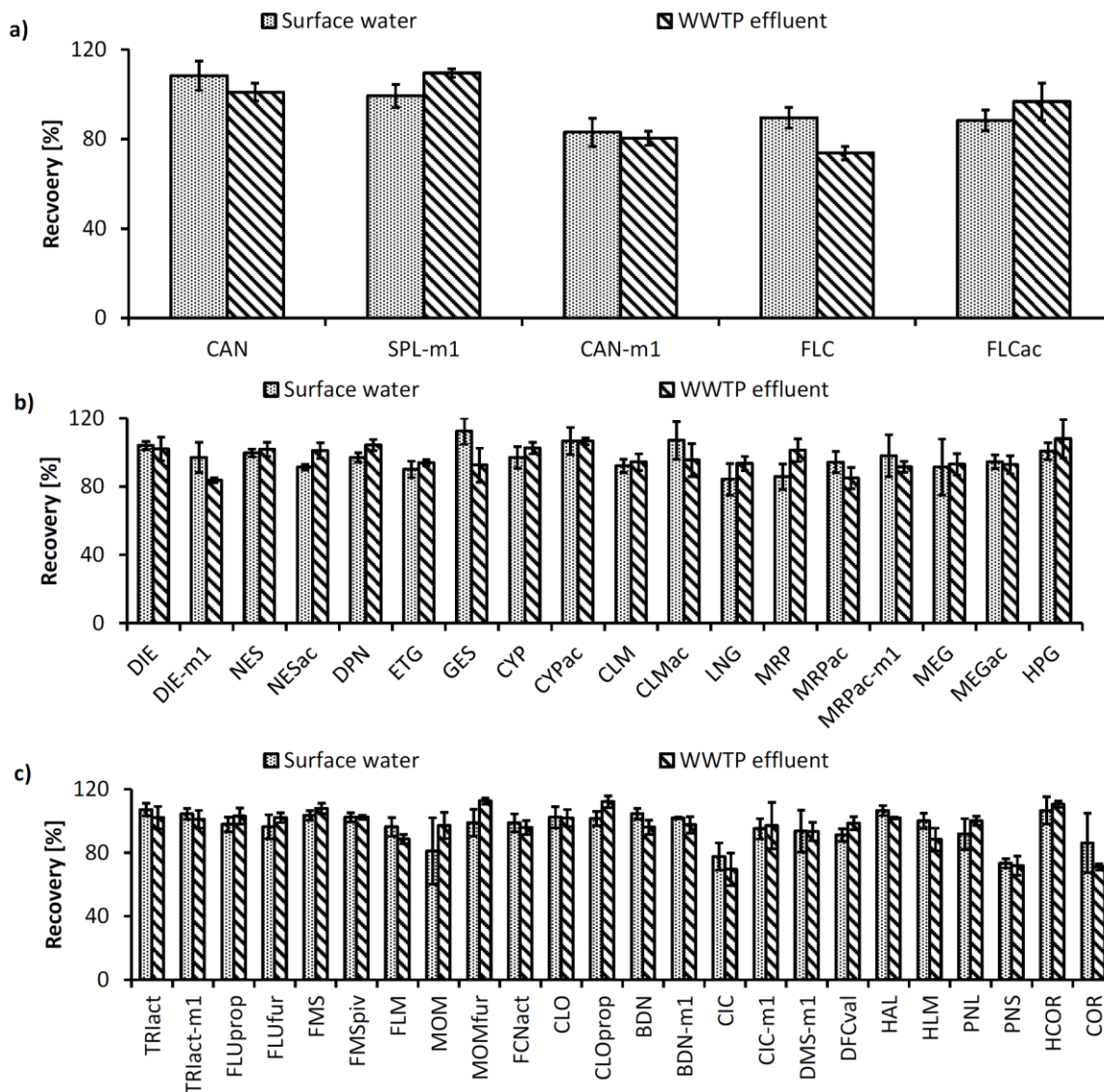
Figure 2.2. Recoveries (corrected by isotope-labeled surrogates) of a) mineralocorticoids, b) progestogens and c) glucocorticoids in surface water and WWTP effluents. Recovery rates were averaged over four concentration levels and error bars express the relative standard deviation (RSD%).

C17-monoesters of further target compounds did not show any isomerization, due to structural barriers. For instance, the substitution of the C21-hydroxyl group with chlorine as present in clobetasol propionate and mometasone furoate hinders an isomerization leading to more stable esterified GC (TÄUBER, 1994). As a consequence of the acyl-migration, two chromatographically separated peaks were detected for both transitions of betamethasone propionate, betamethasone valerate, beclomethasone propionate and 6α-methylprednisolone propionate which were confirmed by high-resolution mass spectrometry and finally quantified as the sum of both peaks (C17/C21-monoester) as

shown exemplarily for betamethasone propionate in Fig. 2.3 (for other esters, see Fig. A.9 - A.11). Differences in the MS²-spectra of both esters could be attributed to the secondary hydroxyl group at position C17 in the C21 monoester, which leads to a loss of H_2O in the fragmentation. Furthermore, to compare the sensitivity for the isomeric monoesters, we determined the sum of peak areas in water samples that were spiked at different sample preparation steps, since the ratios of C17/C21-monoesters differ depending to their dwell times in aqueous media. The summed peak areas of C17/C21-esters were almost constant, regardless of the extent of migration, thus their detection sensitivities were comparable. It should be noted that the corresponding deuterated internal standards (e.g. betamethasone dipropionate-d10, betamethasone propionate-d5) hydrolyzed in the same way during the sample treatment. As the hydrolysis of the deuterated standards leads to contamination with non-labeled steroids, we spiked this group of deuterated internal standards after the sample clean-up (IS-mix 2). The esters were stable in the methanolic standards as well as in the final diluent (methanol/Milli-Q 1:1).
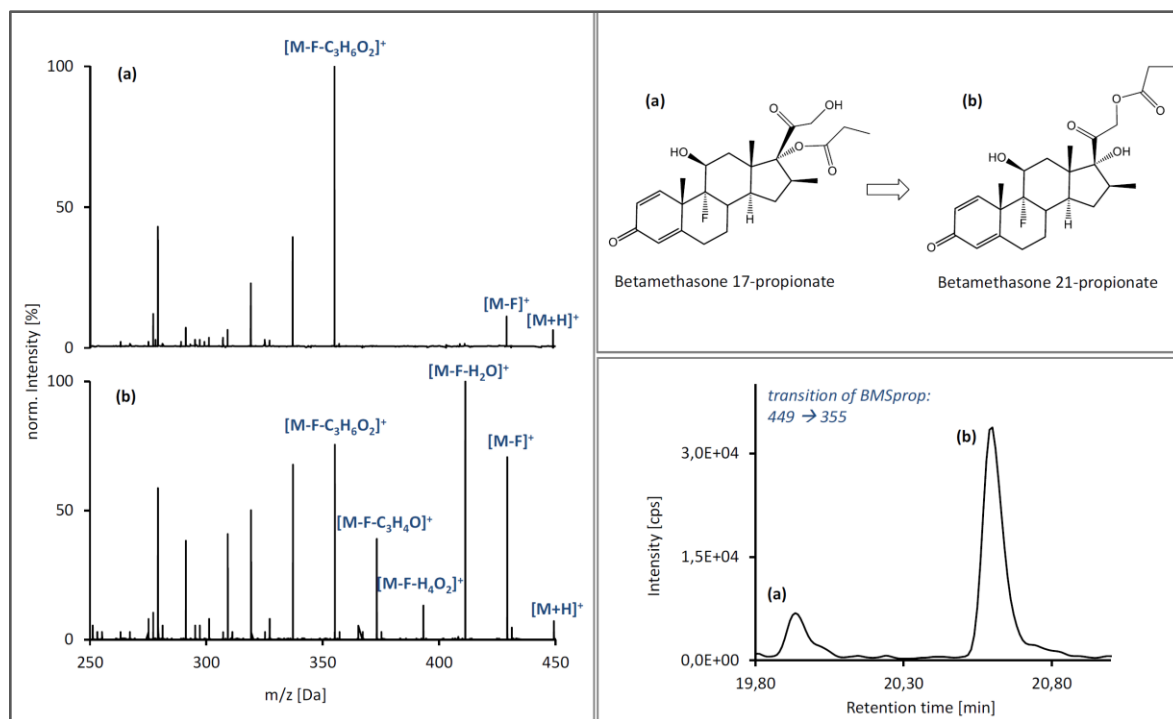
Figure 2.3. Chemical structures, extracted ion chromatogram of non-spiked WWTP effluent and high-resolution MS²-spectra of (a) betamethasone 17-propionate (accurate mass= 449.2329 Da, Δppm=-1.1) and (b) betamethasone 21-propionate (accurate mass= 449.2321 Da, Δppm=-2.9). MS²-spectra was recorded with Triple TOF® 6600 Quadrupole Time-of-Flight (QTOF) mass analyzer (Sciex) at similar conditions as adjusted for the quantification method (CE= 20 eV, Cone voltage= 5500 V).

The recovery rates and reproducibility of analysis of i) diesters, ii) monoesters, and iii) steroid alcohols were determined separately for surface water at two different concentrations (0.5 ng/L, 5.0 ng/L). Recoveries were calculated as the sum of the spiked compound and its formed hydrolysis products. As shown in Tab. 2.2, this validation approach revealed reproducible and almost closed recoveries in all experiments. Therefore, we were able to verify that all target steroids and metabolites were quantitatively recovered. Total recoveries of the diesters ranged from 90 ± 9% (BECdiprop) to 108 ± 6% (BMSdiprop) and for the steroid alcohols from 86 ± 2% (BEC) to 110 ± 7% (BMS). The monoesters of betamethasone, dexamethasone, and 6α-methylprednisolone revealed good recoveries close to 100%. Lower recoveries of beclomethasone propionate might be caused by unknown degradation reactions (Fig. A.10).

Table 2.2. Steroid ester decomposition during the sample treatment. Errors representing the reproducibility (expressed as the 95%-confidence intervals) of glucocorticoid i) diester, ii) monoester and iii) alcohols.

Substance	Recovery [%], c=0.5 ng/L					Recovery [%], c=5.0 ng/L				
	n1	n2	n3	n4	Mean ± 95%-CI	n1	n2	n3	n4	Mean ± 95%-CI
i) diesters										
BMSdiprop	87	91	82	88	87 ± 6	93	88	93	93	92 ± 4
BMSprop	12	11	14	13	12 ± 2	6	8	8	14	9 ± 6
BMS	11	5	13	10	9 ± 6	<1	1	<1	2	<1
Σ	111	107	109	110	108 ± 6	99	97	101	109	102 ± 8
MPNLacp	69	72	62	68	68 ± 7	80	73	73	66	73 ± 9
MPNLprop	37	27	31	38	33 ± 8	26	29	30	47	33 ± 15
MPNL	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Σ	105	99	93	106	101 ± 10	105	102	103	113	106 ± 8
BECdiprop	94	89	82	86	88 ± 9	94	88	89	93	91 ± 5
BECprop	4	<1	2	4	3 ± 3	2	3	3	5	3 ± 2
BEC	<1	<1	<1	<1	<1	<1	<1	<1	<1	<1
Σ	98	89	84	89	90 ± 9	96	91	92	98	94 ± 5
ii) monoesters										
BMSac	92	105	84	119	100 ± 24	105	94	101	98	99 ± 7
BMSprop	82	87	80	81	82 ± 5	94	91	97	93	94 ± 4
BMSval	63	71	62	86	71 ± 18	69	62	63	65	65 ± 4
BMS	18	32	21	24	24 ± 10	21	14	13	12	15 ± 6
Averaged Σ	85	98	83	103	95 ± 17	96	87	91	89	91 ± 6
MPNLprop	80	80	85	80	81 ± 4	81	79	83	83	82 ± 3
MPNL	18	21	17	22	19 ± 4	22	16	16	15	17 ± 5
Σ	98	101	102	102	101 ± 3	104	95	99	97	99 ± 6
BECprop	37	46	41	63	47 ± 18	44	41	42	44	43 ± 3
BEC	8	12	10	11	10 ± 2	12	9	9	8	10 ± 2
Σ	45	57	50	74	57 ± 20	56	50	51	53	52 ± 4
DMSac	89	98	83	92	91 ± 13	95	93	96	93	94 ± 4
DMS	11	16	13	10	13 ± 6	13	13	13	12	13 ± 1
Σ	100	114	97	102	103 ± 17	107	106	109	105	107 ± 4
iii) alcohols										
BMS	96	109	113	100	105 ± 13	104	113	111	114	110 ± 7
MPNL	89	99	96	93	94 ± 7	95	93	97	99	96 ± 4
BEC	86	86	87	84	86 ± 2	98	94	100	103	98 ± 6
DMS	98	96	101	91	97 ± 7	101	106	104	108	105 ± 5

2.3.3 Occurrence of Steroid Hormones in Environmental Samples

Mineralocorticoids (MC). The developed analytical method was applied to several effluents from municipal WWTPs and various rivers and streams to monitor the discharge and occurrence of different types of steroidal pollutants. Concentrations of the most frequently detected analytes are summarized in Tab. 2.3. Among MC, the spironolactone metabolites canrenone and 7α-thiomethyl spironolactone were commonly present in WWTP effluents, rivers and streams. Measured concentrations of canrenone ranged up to 19 ng/L in WWTP effluents and up to 8.3 ng/L in the rivers and streams containing an elevated percentage of treated wastewater. The concentrations of 7α-thiomethyl spironolactone were lower, ranging up to 2.3 ng/L in WWTP effluents and up to 1.3 ng/L in surface waters. Both metabolites were found to be ubiquitously present in nearly all analyzed water samples and hence should be considered in further monitoring campaigns of steroid hormones. In addition, 11α-hydroxy canrenone was detected in WWTP effluent 1 and the receiving surface water SW-1b (Tab. A.4). In contrast to its metabolites, spironolactone was not detected at all, because spironolactone is rapidly metabolized in humans to canrenone, 7α-thiomethyl spironolactone as well as to other metabolites (SADEE ET AL., 1973). In Germany, its annual consumption accounted for 9.2 t in 2014 (SCHWABE AND PAFFRATH, 2015, WHOCC, 2017). The instability of spironolactone in contact with activated sludge and in aqueous solutions was shown elsewhere (SULAIMAN ET AL., 2015). Despite the high metabolism and fast degradation, the environmental relevance of spironolactone and its major metabolite canrenone has been revealed, after abnormal fishes were spotted in the vicinity of a chemical plant producing the steroidal compound (SANCHEZ ET AL., 2011; GILBERT, 2011). Chemical analysis confirmed the high concentrations of both pollutants (spironolactone, canrenone) in the river water downstream of a pharmaceutical manufacturer (CREUSOT ET AL., 2014). In addition, LaLone *et al.* (2010) observed androgenic effects on fish that were exposed to spironolactone. Therefore, spironolactone and its active metabolite canrenone may pose a potential risk to biota.

Table 2.3. Occurrence of most commonly detected steroid hormones in various municipal WWTP effluents and surface waters in Germany. (< = below detection limit, <LOQ = above detection limit, below quantification limit).

	Wastewater treatment plant effluent Concentration [ng/L]						Surface water Concentration [ng/L]											
	1	2	3	4	5*	LOD/LOQ	Mühlenbach (downstream WWTP 1), SW-1b	River Nahe (downstream WWTP 2), SW-2b	Schwelme (downstream WWTP 3), SW-3b	River Wupper (downstream Schwelme), SW-4b	Teltow canal, SW-5*	Landgraben (downstream WWTP), SW-6	River Neckar SW-7	River Main, SW-8	River Lahn, SW-9a	River Rhine SW-10d	River Ahr, SW-11	LOD/LOQ
Mineralocorticoids (MC)																		
Canrenone	4.5	3.7	10	19	8.0	0.4/1.4	3.0	1.6	8.3	1.2	2.9	1.8	0.6	0.4	0.8	0.5	0.2	0.08/0.2
7α-Thiomethyl spironolactone	0.2	1.2	1.5	3.8	2.0	0.05/0.2	0.1	0.3	1.3	0.2	0.6	0.2	0.07	0.08	0.3	0.05	<LOQ	0.01/0.03
Glucocorticoids (GC)																		
Triamcinolone acetonide	6.3	5.5	17	11	28	0.1/0.5	4.4	1.0	12	1.5	7.6	8.5	0.3	0.6	0.3	0.3	0.05	0.01/0.04
6β-Hydroxy triamcinolone acetonide	1.2	1.7	6.9	2.3	2.2	0.06/0.2	0.9	0.2	5.1	0.6	1.2	0.8	<	0.1	0.08	0.05	<	0.03/0.05
Fluticasone propionate	<LOQ	0.1	0.5	1.0	0.9	0.05/0.1	<LOQ	<LOQ	0.4	0.06	0.3	0.2	<LOQ	<	<LOQ	<	<	0.05/0.10
Mometasone furoate	0.8	1.2	1.7	2.2	1.4	0.08/0.3	0.6	<LOQ	1.0	<LOQ	0.2	0.8	<	<	<LOQ	<	<	0.05/0.2
Fluocinolone acetonide	0.1	0.1	0.1	0.2	0.2	0.03/0.1	0.09	<LOQ	0.1	<LOQ	0.09	0.1	<	<	<LOQ	<	<	0.02/0.05
Clobetasol propionate	0.5	0.8	2.1	4.0	5.4	0.08/0.3	0.4	0.2	3.4	0.3	1.7	0.2	0.05	0.1	0.1	0.06	<	0.02/0.05
Betamethasone propionate	1.1	1.5	1.2	3.6	0.3	0.08/0.2	0.9	0.2	0.6	0.07	0.4	1.2	<LOQ	<	0.07	0.09	<	0.02/0.05
Betamethasone valerate	1.3	2.5	1.1	2.2	1.2	0.08/0.3	0.9	0.2	0.7	<LOQ	0.2	1.3	<	<LOQ	<LOQ	<LOQ	<	0.03/0.2
Betamethasone	0.6	0.4	0.05	0.2	0.6	0.02/0.05	0.5	0.2	0.4	<LOQ	1.0	0.3	0.1	0.1	0.1	<	<LOQ	0.02/0.05
Sum of BMS derivatives	*3.0*	*4.4*	*2.4*	*6.0*	*2.1*		*2.3*	*0.6*	*1.7*	*0.1*	*1.6*	*2.8*	*0.1*	*0.1*	*0.17*	*0.09*	*<LOQ*	
Budesonide	<	<	1.2	2.0	<	0.5/1.0	<	<	0.7	<LOQ	<	<LOQ	<	<	<	<	<	0.2/0.5
Methylprednisolone propionate	1.4	<LOQ	2.4	0.5	4.2	0.2/0.5	0.9	<	1.3	<LOQ	0.9	0.6	<	<	<	<	<	0.06/0.2
Methylprednisolone	<LOQ	<	0.1	1.0	0.2	0.02/0.06	<LOQ	<LOQ	0.2	0.05	0.2	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<	0.01/0.05
Sum of MPNL derivatives	*1.4*	*<LOQ*	*2.5*	*1.5*	*4.4*		*0.9*	*<LOQ*	*1.5*	*0.05*	*1.1*	*0.6*	*<LOQ*	*<LOQ*	*<LOQ*	*<LOQ*	*<*	
Prednisolone	<LOQ	<LOQ	0.3	0.6	<LOQ	0.06/0.2	0.05	0.07	0.4	0.06	<LOQ	0.05	0.1	0.07	0.05	0.08	0.05	0.02/0.05
Prednisone	<LOQ	<LOQ	0.2	0.4	<LOQ	0.06/0.2	<LOQ	<LOQ	<LOQ	<LOQ	<	0.05	<	<LOQ	<LOQ	<LOQ	<	0.03/0.05
Cortisol	0.9	1.4	1.2	2.8	0.9	0.06/0.2	0.7	1.3	1.3	0.4	0.2	1.0	0.6	0.7	1.3	0.3	0.7	0.02/0.08
Cortisone	0.2	0.3	0.4	0.9	0.2	0.1/0.2	0.2	0.4	0.7	0.3	0.08	0.2	0.6	0.7	1.0	0.1	0.2	0.01/0.02
Progestogens (PG)																		
Dienogest	3.3	1.3	4.4	4.3	1.4	0.2/0.3	2.3	0.2	2.0	0.3	<	0.1	0.05	0.05	0.09	<LOQ	<	0.02/0.05
6ß-Hydroxy dienogest	<LOQ	0.6	0.6	0.6	0.9	0.2/0.4	<	<	0.4	<	<	0.5	<	<	<	<	<	0.05/0.1
Cyproterone acetate	0.8	1.7	2.9	3.7	2.3	0.3/0.8	0.6	0.2	2.6	0.3	0.9	0.6	<	<	<	<	<	0.05/0.2
17α-Hydroxy progesterone	1.1	0.7	0.7	1.0	1.3	0.3/0.7	0.6	<LOQ	<LOQ	<	<	0.6	<	<	<	<	<	0.25/0.5

*) Acidified after sampling for transport

Glucocorticoids (GC). Due to the structural diversity of synthetic GC used and the wide range of medicinal applications, this hormone class is the largest group of target hormones in this study. Concentrations of the most frequently detected GCs are summarized in Tab. 2.3. Results in detail are provided in appendix A (Tab. A.4).

In total, 23 of 37 GC were found in at least one sample and 14 of them were present in all five WWTP effluents above the LODs. Triamcinolone acetonide and its metabolite 6β-hydroxy triamcinolone acetonide were found as the predominant GC compounds in our sampling campaign since they accounted for 39-66% of the total GC concentration in the WWTP effluents. The concentrations of triamcinolone acetonide ranged from 5.5 ng/L to 28 ng/L in WWTP effluents and its metabolite was found with concentrations between 1.2 ng/L and 6.9 ng/L, respectively. Furthermore, the measured concentrations of triamcinolone acetonide are in good agreement with studies analyzing a Dutch WWTP effluent (14 ng/L) (Schriks et al., 2010) and WWTP effluents in the U.S. (6-14 ng/L) (Jia et al., 2016). Triamcinolone acetonide was detected in our study in 20 of 22 rivers and streams above the LOQ (0.04 ng/L) ranging from 0.04 ng/L to 12 ng/L. This indicates the ubiquitous presence of triamcinolone acetonide in rivers and even streams with a relatively low percentage of treated wastewater. Furthermore, triamcinolone acetonide as well as its bi-fluorinated analogue fluocinolone acetonide were reported to be relatively stable during laboratory degradation experiments with activated sludge (Miyamoto et al., 2014). Although, concentrations of fluocinolone acetonide were in all cases below 1 ng/L due to low consumption in Germany (12 kg in 2014) (Schwabe and Paffrath, 2015; WHOCC, 2017).

Residues of mometasone furoate and fluticasone propionate have been detected in all WWTP effluents at concentrations up to 2.2 ng/L and 1.0 ng/L, respectively. The detection frequency of fluticasone propionate was comparable to WWTP effluents in the U.S. (Jia et al., 2016). However, mometasone furoate was detected above LOQ in treated wastewaters and 4 rivers and streams (0.2 - 1.0 ng/L). It should be noted that in Germany fluticasone propionate and mometasone furoate are over-the-counter drugs for the treatment of seasonal rhinitis. Therefore, the total used quantities might be higher than calculated, caused by their additional usage in non-prescription products, thus their discharge into water bodies may vary from season to season.

Traces of further GC were detected less frequently such as beclomethasone (0.07 ng/L) and flumethasone pivalate (0.05 ng/L) above LOQ in several surface waters (Tab. A.4).

The natural steroids cortisone and cortisol were detected in all water samples. In WWTP effluents the concentrations of cortisol and cortisone were measured up to 2.8 ng/L and 0.9 ng/L. Surface water samples contained both steroids in concentrations up to 1.3 ng/L

(cortisol) and 1.0 ng/L (cortisone). Moreover, in particular, their percentage on the overall GC concentration was found to increase with decreasing wastewater ratios. Both analytes could be detected above LOQ in surface waters upstream of the WWTPs (SW-1a, SW-3a; Tab. A.4) without receiving wastewater. This finding indicates there are other sources such as wildlife or agriculture runoff, although these inputs are low compared to the WWTP discharges.

Prescribed volumes of non-halogenated GC are significantly higher in Germany than those of halogenated steroids (Tab. A.3). The measured concentrations of prednisolone and prednisone in treated wastewaters and surface waters did not reflect this consumption quantity. Both analytes were detected in the effluents of WWTP 3 and WWTP 4, whereby concentrations of prednisolone with 0.3 ng/L and 0.6 ng/L found to be slightly higher than prednisone with 0.2 ng/L and 0.4 ng/L. Prednisolone was detected in 17 of 22 rivers and streams above LOQ ranging from 0.05 ng/L to 0.4 ng/L, whereas only two streams (SW-6, SW-9a) contained prednisone above LOQ. Furthermore, budesonide was also found in the effluent of WWTP 3 (1.2 ng/L) and WWTP 4 (2.0 ng/L) and additionally in the corresponding surface water taken downstream from WWTP 3 (0.7 ng/L).

The environmental abundance and application quantities of halogenated and non-halogenated GC suggest divergent degradation during wastewater treatment. Literature data for the removal efficiencies of most GC are rare (CHANG ET AL., 2007; FAN ET AL., 2011; HERRERO ET AL., 2012). However, laboratory degradation experiments for a limited number of GC in contact with activated sludge support this hypothesis (MIYAMOTO ET AL., 2014). Halogenated GC were designed to enhance glucocorticoid potency. The insertions of halogen substituents lead to enhanced receptor binding affinities and higher persistency in the human body (PHILLIPPS, 1990). Thus, the inhibition of the enzymatic reactions could also affect the behavior during the municipal wastewater treatment and result in more persistent pollutants with lower degradation rates.

Among other steroids, the ester derivatives of betamethasone and 6α-methylprednisolone are mainly utilized topically in ointments and creams for the medicinal therapy of diverse skin diseases (MORI ET AL., 1994; ROTE LISTE, 2016; PHARMANET, 2017). Although these esters are known to metabolize extensively, researches could show the presence of betamethasone valerate in WWTP effluents (ISOBE ET AL., 2015; NAKAYAMA ET AL., 2016). To investigate their presence in water samples, we included the diesters and monoesters of betamethasone and 6α-methylprednisolone in the analytical method. In all WWTP effluents betamethasone propionate, betamethasone valerate and 6α-methylprednisolone propionate exhibited higher concentrations than their alcohols. Measured concentrations of the betamethasone

propionate and valerate ester ranged from 0.3 ng/L to 3.6 ng/L and from 1.1 ng/L to 2.5 ng/L, respectively. Non-esterified betamethasone concentrations were between 0.05 ng/L and 0.6 ng/L. The profile of the detected derivatives of 6α-methylprednisolone was similar to that of betamethasone. Concentrations of 6α-methylprednisolone and its propionate monoester were found up to 1.0 ng/L and 4.2 ng/L in WWTP effluents. Therefore, the results indicated higher abundances of the monoester derivatives than parent steroid alcohols, thus these monoesters should be considered in further studies. Moreover, esterified steroids are reported to be more potent (CHEUNG ET AL., 1985) due to faster diffusion and uptake into the cell, so this might be also affecting the uptake in waterborne organisms. Nevertheless, single betamethasone concentrations were in good agreement to those found in U.S. WWTP effluents (0.18-0.66 ng/L) (JIA ET AL., 2016) and Japanese wastewaters (0.29-1.3 ng/L) (ISOBE ET AL., 2015). However, a substantially higher concentration was measured in one French WWTP effluent (7 ng/L) (PIRAM ET AL., 2008). Betamethasone valerate concentrations in the literature (0.84-4.7 ng/L) (ISOBE ET AL., 2015; NAKAYAMA ET AL., 2016) are comparable to our detected values in WWTP effluents.

Similar to betamethasone valerate, clobetasol propionate is administered topically (PHARMANET, 2017). In WWTP effluents clobetasol propionate concentrations ranged from 0.5 ng/L to 5.4 ng/L. Furthermore, clobetasol propionate was found in 12 of 22 surface water samples above the LOQ, in concentrations ranging up to 3.4 ng/L (SW-3b).

Nonetheless, to our knowledge, this study reveals the first reported concentrations of betamethasone valerate in European water bodies and the occurrence of betamethasone propionate and 6α-methylprednisolone propionate in notable frequencies and concentrations in treated wastewater as well as rivers and streams is reported for the first time.

Progestogens (PG). Cyproterone acetate and dienogest were found to be the most common detected PG. Highest concentrations were obtained for dienogest, ranging from 1.3 ng/L to 4.4 ng/L in WWTP effluents (Tab. 2.3) and in 10 of 22 surface water samples from 0.05 ng/L to 2.3 ng/L, respectively. Its metabolite 6β-hydroxy dienogest was present in 4 of the 5 WWTP effluent samples above LOQ (0.6-0.9 ng/L). Concentrations of cyproterone acetate ranged from 0.8 ng/L to 3.7 ng/L in WWTP effluents and 6 of the 22 surface water samples from 0.2 ng/L to 2.6 ng/L. Moreover, further 7 of the investigated 18 PG were found in at least one sample above the detection limits (Tab. A.4, e.g. chlormadinone acetate, levonorgestrel, medroxyprogesterone acetate). During our sampling campaign levonorgestrel was found in 2 surface waters up to 0.7 ng/L. Available data of synthetic PGs

in environmental waters are limited (LIU ET AL., 2011B), especially for the newer generation of oral contraceptives (LIU ET AL., 2011B; KUMAR ET AL., 2015; FENT, 2015). For example, in 71 French surface water samples, the mean concentration of levonorgestrel was 3.6 ng/L (detection frequency of 47%) (VULLIET AND CREN-OLIVE, 2011), in Spanish effluents it was frequently found in concentrations up to 4 ng/L (PETROVIC ET AL., 2002), whereas only one effluent (1 ng/L) (KUCH AND BALLSCHMITTER, 2000) contained levonorgestrel in Germany. In addition, the PG norethisterone and megestrol acetate were found in Swiss surface waters in concentrations up to 4.6 ng/L and 14 ng/L, respectively (ZHANG ET AL., 2017). These differences might be the results of the country-depending consumption figures of synthetic oral contraceptives.

Impacts of PG on the endocrine systems of aquatic organisms cannot be excluded even if concentrations were found to be in the sub-ng/L range since toxicological studies described inhibition of reproduction in fathead minnow exposed to levonorgestrel traces (0.8 ng/L) (ZEILINGER ET AL., 2009). Further studies showed decreasing testosterone plasma levels in fish when exposed to 1 ng/L cyproterone acetate (SHARPE ET AL., 2004) as well as decreasing fecundity in fathead minnow after exposure to 1 ng/L norethisterone (PAULOS ET AL., 2010).

In particular, no information concerning the occurrences and ecotoxicological potentials of dienogest and its hydroxylated metabolite was found in the literature. The ecotoxicological risks to the aquatic environment need to be evaluated in further investigations since reasonable concentrations were detected in all WWTP effluents (1.3-4.4 ng/L) as well as in rivers and streams (0.05-2.3 ng/L).

Supplemental interactions of steroid mixtures on endocrine systems should be expected as it is known for several steroidal compounds (SILVA ET AL., 2002; ZHAO ET AL., 2015 & 2016; THRUPP ET AL., 2018; WILLI ET AL., 2018). The assessment of single concentrations may underestimate the total adversary effects of steroidal micropollutants on aquatic organisms. However, the knowledge about single compounds and steroid compositions of concern is still lacking. Mixtures of endocrine active substances are known to acting additive, less-than-additive or even show synergistic interactions (BERGERON ET AL., 1999, THROPE ET AL., 2001, SILVA ET AL., 2002, ZHAO ET AL., 2015 AND 2016, THRUPP ET AL., 2018, WILLI ET AL., 2018) depending on the organism and composition. Therefore, determining a broad number of steroids by comprehensive and sensitive analytical methods is an important tool for prioritizing compounds of concern and identifying hormone mixtures reaching water bodies. The developed ultra-sensitive multi-method has enabled to successfully identify the predominant

steroids and revealed a large number of known, and more importantly, several unknown steroidal pollutants in various surface waters and WWTP effluents.

3 Analysis of the Aerobic Biodegradation of Glucocorticoids: Elucidation of the Kinetics and Transformation Reactions in Activated Sludge Treatment
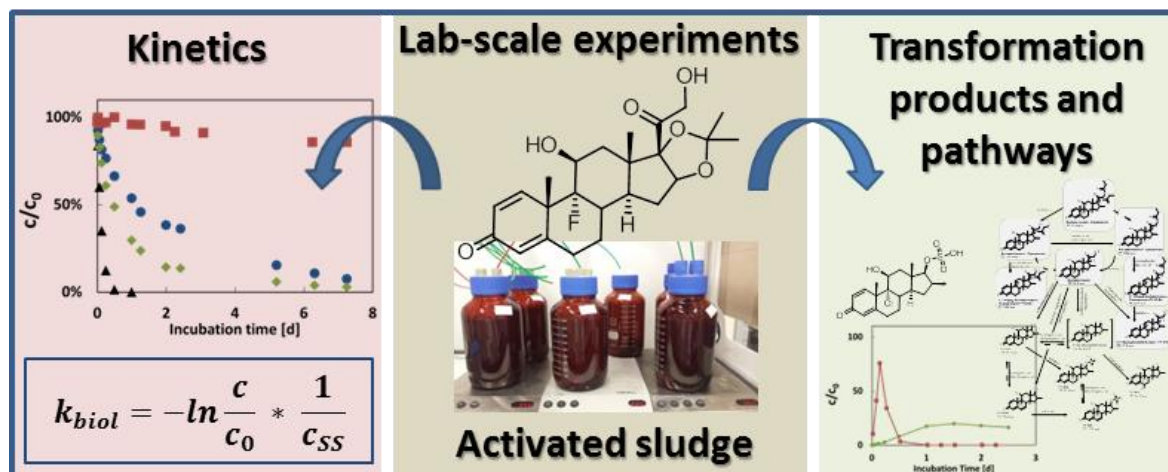
ABSTRACT

Glucocorticoids (GCs) are one of the most prescribed pharmaceutical classes worldwide. They have been reached a high focus as environmental pollutants in the current scientific research, due to their potential risks to aquatic organisms even in the lower ng/L range. The objective of this study was to determine the kinetic behavior of selected GCs and to identify their main transformation products (TPs) in lab-scaled biodegradation experiments. Therefore, we analyzed the removal of 13 GCs in aerated incubation experiments with activated sludge taken from a German municipal wastewater treatment plant (WWTP) as inoculum. For all steroids, an exponential decrease of the concentrations was observed, which was modeled by pseudo-first order kinetics. Overall, the rate constants $k_{biol.}$ ranged from 0.07 $L/(g_{ss}·d)$ (triamcinolone acetonide) to 250 $L/(g_{ss}·d)$ (prednisolone). These results emphasize the broad variation in the biodegradability of GCs. The selection of the studied GCs enabled a deduction of microbiological stability related to functional groups. Based on the identified TPs, a variety of enzymatically mediated reactions were postulated. Moreover, the identified TPs exhibited their intact steroid core. The main observed reactions were regioselective hydrogenation of carbon double-bonds, degradation of the steroid C17 side-chain, ester hydrolysis and oxidative hydroxylation. In total, 41 TPs were tentatively identified from 12 GCs. Exact 22 TPs were unambiguously confirmed by authentic reference standards. Additionally, 12 TPs were detected in the effluents of municipal WWTPs, and to the best of our knowledge, the occurrence of eight of these TPs has been shown for the first time.

3.1 Introduction

The diversity of medical indications of Glucocorticoids (GCs) leads to annually growing prescriptions. Also, new therapies with GCs are still under development (Bodor and Buchwald, 2006). Due to the range of therapeutic properties, approx. 16 basic GCs are registered in Germany. This number is increasing, since a variety of esterified and functionalized derivatives of the basic GCs are applied (Rote Liste, 2016). Thus, GCs are one of the most prescribed pharmaceutical classes worldwide.

The fate of these GCs depends on their dosage forms (e.g. ointments, powders, shampoos, tablets, sprays) and their route of administration (e.g. oral, dermal, inhalation). Several GCs are designed to be applied on the skin or are used systemically in oral dosage forms. They are either washed-off from the skin unchanged or are excreted via urine and feces after metabolism in the human body. Afterwards, the compounds reach, more or less continuously, the municipal wastewater treatment plants (WWTPs).

Recent studies indicated an incomplete removal of certain GCs during biological wastewater treatment. Especially synthetic compounds such as fluocinolone acetonide, triamcinolone acetonide or clobetasol propionate, were found to be poorly removed (Fan et al., 2011; Wu et al., 2019) and fluticasone propionate was reported as moderately degradable (63%) (Wu et al., 2019). In addition, Miyamoto *et al.* (2014) analyzed the fate of ten GCs during the incubation with activated sludge and showed the recalcitrant behavior of triamcinolone acetonide and fluocinolone acetonide in their lab-scale incubation experiments. On the other hand, enhanced biodegradability was reported for hydrocortisone, prednisolone, and betamethasone (Wu et al., 2019; Miyamoto et al., 2014). Therefore, it is not surprising that particularly synthetic GCs have been detected in WWTP effluents and receiving surface waters worldwide (Chang et al., 2007, Schriks et al., 2010, Jia et al., 2016, Sonavane et al., 2018., Weizel et al., 2018).

The detected concentrations of triamcinolone acetate in WWTP effluents in the USA were as high as 17.9 ng/L (Wu et al., 2019) while clobetasol propionate concentrations ranged from 1.04 ng/L to 2.35 ng/L (Jia et al., 2016). Trimacinolone acetonide was found in WWTP effluents in the Netherlands (Schriks et al., 2010) and Germany (Weizel et al., 2018) in similar concentrations to the USA. In WWTP effluents from Japan, betamethasone valerate and clobetasol propionate were identified as the predominant GCs (Isobe et al., 2015).

Moreover, recent studies highlighted that GCs are potentially harmful to aquatic organisms (Kugathas and Sumpter, 2011; LaLone et al., 2011; Kugathas et al., 2013; Chen et al., 2016a; Willi et al., 2018 & 2019). *In vivo* effects have been shown for a variety of end-points. For instance, Kugathas *et al.* (2013) detected increased plasma glucose concentrations in adult

fathead minnows exposed to 100 ng/L beclomethasone dipropionate. McNeil *et al.* (2016) and Willi *et al.* (2018) analyzed the effects of exposure to different GCs during the development of zebrafish embryos. Multiple end-points that are mediated by endogenous GCs were triggered significantly during the exposure, including decreases in spontaneous muscle contractions, increases in heart rates, and accelerated hatching (WILLI ET AL., 2018). LaLone *et al.* (2011) highlighted significant morphological changes of the gonads in male fathead minnows exposed to 100 ng/L dexamethasone. At higher dexamethasone concentration (500 µg/L) operculum deformities were observed. Therefore, active GCs are known to mimic endogenous hormones and potentially cause severe adverse effects in aquatic organisms.

WWTP effluents are the main point source of synthetic GCs into rivers and streams. With few exceptions biological wastewater treatment is not able to mineralize organic micropollutants, but rather a high variety of transformation products (TPs) are formed and discharged by WWTPs (reviewed in EVGENIDOU ET AL., 2015). These TPs might have similar activities or in a few cases even an enhanced ecotoxicological potential as reported in Celiz *et al.* (2009) and Cwiertney *et al.* (2014).

Furthermore, it has to be noted that GCs and their TPs are likely to induce additive effects (WILLI ET AL., 2019 & 2020). 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. To date, no comprehensive studies are available dealing with the (bio)transformation processes of GCs in municipal WWTPs, even though GCs were one of the first organic molecules manufactured by industrial fermentative processes. Numerous microbiological transformation reactions for specific GC substrates are already well known (reviewed in BHATTI AND KHERA, 2012 AND CHARNEY AND HERZOG, 1967). However, the microbial community of wastewater treatment systems is quite diverse (WOLFF ET AL., 2018) and thus, identification of TPs in controlled lab-scale incubation experiments with activated sludge can be a promising approach to understand the removal processes of GCs. Altogether, there is a major deficit in knowledge about the fate and behavior of GCs in WWTPs.

In that context, the current study was initiated to elucidate and predict the biodegradability and transformation of a range of synthetic GCs. Therefore, we analyzed the fate of 13 GCs during aerobic incubation with activated sludge from municipal WWTPs and we elucidated the biological degradation pathways of 12 synthetic GCs.

3.2 Materials and Methods

3.2.1 Chemicals and Compound Selection

Details on chemical reference materials used in this study are summarized in appendix B (Tab. B.1). The GCs were selected based on i) their occurrence in WWTP effluents and ii) structural similarities to elucidate the importance of certain moieties for their biodegradability and the formation of stable TPs. The GCs considered can be roughly subdivided into four classes: a) non-halogenated GCs (hydrocortisone, prednisolone, 6α-methylprednisolone aceponate, b) halogenated GCs (beclomethasone dipropionate, beclomethasone, betamethasone dipropionate, betamethasone 17-valerate, betamethasone), c) halogenated GCs with altered side chains (clobetasol propionate, fluticasone propionate, and d) C16, C17-ketal GCs (budesonide, fluocinolone acetonide, triamcinolone acetonide).

3.2.2 Biodegradation Batch Systems with Activated Sludge

For the lab-scale experiments, 200 mL of a homogenized activated sludge slurry was diluted ten-fold with WWTP effluents to a final batch volume of 2 L. Dilution was utilized to minimize sorption on suspended solids and to reduce matrix effects during chemical analysis.

Activated sludge was freshly sampled from the aeration tank of a conventional WWTP (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). The diluted slurry was continuously aerated with synthetic air and stirred during inoculation to maintain aerobic conditions. In addition, temperature and pH were controlled over the experiments. No significant drifts were observed within the incubation experiments (T ± 1.6 °C, pH ± 0.2). An overview of all conducted experiments and the experimental conditions is provided in Tab. 3.1.

After an equilibration time of approx. 12 h target steroids were spiked into the diluted activated sludge slurry. Kinetic experiments were spiked in triplicate with an analyte concentration of 200 ng/L, and for TP identifications 2 µg/L were spiked. As a reference (negative control), one non-spiked experiment was included in each experimental sequence. In addition, sterile (inactive) control experiments (autoclaved for 60 min at 121 °C) were used to differentiate between the biotic and abiotic transformation. Samples of the batches were taken at defined times.

For analysis, 100 mL of filtered samples (< 1µm glass fiber GF6, Whatmann, Maidstone, UK) were spiked with 2 ng of each isotope-labeled internal standard and were subsequently enriched via SPE (C18ec, Machery-Nagel, Düren, Germany) according to the protocol shown in Fig. 2.1.

Table 3.1. Overview of the conducted experiments. All experiments were performed with freshly sampled activated sludge from the aeration tank of a conventional WWTP and were diluted with WWTP effluent (1:10).

Experiment	GCs spiked	c_{Target} [ng/L]	c_{ss} [g_{ss}·L]	pH	Temperature [°C]	Incubation time [d]
K1 (n=3)	Hydrocortisone, beclomethasone, betamethasone, 6α-methylprednisolone, clobetasol propionate, fluticasone propionate	200	0.42	7.7 ± 0.1	24.2 ± 1.2	8.08
S1 (n=2)	Hydrocortisone, beclomethasone, betamethasone, 6α-methylprednisolone, clobetasol propionate, fluticasone propionate	200	0.42 (autoclaved at 121°C)	7.7 ± 0.1	24.2 ± 1.2	8.08
K2 (n=3)	Prednisolone, beclomethasone 17-propionate, betamethasone 17-valerate, betamethasone dipropionate, budesonide, fluocinolone acetonide, triamcinolone acetonide	200	0.30	7.8 ± 0.2	24.9 ± 1.4	14.08
S2 (n=2)	Prednisolone, beclomethasone 17-propionate, betamethasone 17-valerate, betamethasone dipropionate, budesonide, fluocinolone acetonide, triamcinolone acetonide	200	0.30 (autoclaved at 121°C)	7.8 ± 0.2	24.9 ± 1.4	14.08
T1 (n=1)	Beclomethasone dipropionate	2000	0.39	8.0 ± 0.2	22.3 ± 0.6	6.21
T2 (n=1)	Betamethasone dipropionate	2000	0.33	8.1 ± 0.1	26.2 ± 1.6	5.0
T3 (n=1)	Betamethasone 17-valerate	2000	0.33	8.1 ± 0.1	26.2 ± 1.6	5.0
T4 (n=1)	Betamethasone	2000	0.33	8.1 ± 0.1	26.2 ± 1.6	5.0
T5 (n=1)	17-Oxo betamethasone	2000	0.42	7.7 ± 0.1	24.2 ± 1.2	7.25
T6 (n=1)	Clobetasol propionate	2000	0.35	8.2 ± 0.1	20.0 ± 0.6	8.08
T7 (n=1)	1,2-Dihydro	2000	0.35	8.0 ± 0.2	24.5 ± 1.0	13.11
T8 (n=1)	Fluticasone propionate	2000	0.38	8.0 ± 0.2	24.0 ± 1.0	8.08
T9 (n=1)	1,2-Dihydro (TP502b)	2000	0.33	8.1 ± 0.1	25.0 ± 1.2	6.0
T10 (n=1)	17β-carboxylic acid (TP452)	2000	0.33	8.1 ± 0.1	25.0 ± 1.2	7.1
T11 (n=1)	6α-Methylprednisolone aceponate	2000	0.39	8.0 ± 0.2	22.3 ± 0.6	6.21
T12 (n=1)	Hydrocortisone	2000	0.33	8.1 ± 0.1	25.0 ± 1.2	6.0
T13 (n=1)	Prednisolone	2000	0.33	8.1 ± 0.1	25.0 ± 1.2	6.0
T14 (n=1)	Budesonide	2000	0.39	8.0 ± 0.2	22.3 ± 0.6	6.21
T15 (n=1)	Fluocinolone acetonide	2000	0.39	8.0 ± 0.2	22.3 ± 0.6	14.21
T16 (n=1)	Triamcinolone acetonide	2000	0.39	8.0 ± 0.2	22.3 ± 0.6	14.21
T17 (n=1)	21-carboxylic acid (TP448)	2000	0.42	7.7 ± 0.1	24.2 ± 1.2	17.3

3.2.3 High-Resolution Mass Spectrometry for TP Identification and Data Handling

MS² spectra were recorded by a hybrid quadrupole time of flight mass spectrometer (QTOF) (SCIEX TripleTOF 5600, Darmstadt, Germany). The QTOF system was equipped with a DuoSpray ion source and a TurboIonSpray probe for electrospray ionization (ESI) experiments.

For TP identification both polarization modes were applied in separate runs. The parameters for positive and negative ionization were as follows (values for ESI(-) in parenthesis): ion source gas (GS) 1 and 2, 35 and 45 psi; curtain gas (CUR), 40 psi; source temperature (TEM) 500°C; ion spray voltage floating (ISVF), 5500 eV (-4500 eV); declustering potential (DP), 60 V (−100 V); ion release delay (IRD), 67 ms; ion release width (IRW), 25 ms.

Full scan experiments (100-700 Da) were performed with an accumulation time of 0.2 s in the high sensitivity mode. Eight independent data acquisition (IDA) experiments were

acquired for MS² spectra accumulation (accumulation time: 0.05 s). The fragmentation conditions were as follows: mass range, 30-700 Da; CE, 40 eV (-40 eV); collision energy spread (CES), 15 eV (-15 eV).

The mass spectrometer was automatically re-calibrated after four runs using an automated calibrant delivery system (CDS) via atmospheric pressure chemical ionization (APCI).

Chromatographic separation was achieved using reversed-phase liquid-chromatography (2 x 150 mm, 2.7 µm, MN Nucleoshell RP 18puls, Macherey-Nagel) before detection by QTOF.

The acquired data were finally processed by a non-target approach. For automated peak-picking and alignment procedure, a data evaluation script in R was used. Further information regarding the data processing algorithm is provided elsewhere (DIETRICH AND TERNES, 2020 IN PREP.).

The final peak lists were searched for clear differences in the time courses between spiked batches and non-spiked controls for potential TPs. The tentatively identified TPs were verified by authentic standards through the comparison of retention times (RT), accurate masses and MS² fragmentations. In those cases, without commercially available standards, chemical structures of the TPs were proposed by combining the information of retention time shifts, isotopic patterns and MS² fragmentation spectra. In addition, MS² spectra of tentatively identified TPs were assessed manually by comparing to parent steroids or their related TPs.

3.2.4 Sampling and Analysis of WWTP Effluent Samples

The target GCs and their TPs were quantified in eight effluents from WWTPs in Germany. Details regarding the sample locations are provided in appendix B (Tab. B.2). The monitored WWTPs are equipped with conventional biological treatment trains. Grab samples were taken directly from WWTP effluents and were immediately cooled before the transport to the laboratory. The samples were treated within 24 h after sampling according to the protocol shown in Fig. 2.1.

In brief, 500 mL of filtered WWTP effluent (<1µm glass fiber GF6, Whatmann) 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 before detection. 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.

3.2.5   Quantification of Target Steroids and TPs

Quantitative analysis was carried out with TripleQuad-LIT-MS (API 6500 QTrap, Sciex) using the sMRM (scheduled multiple reaction monitoring) mode. Details regarding chemical analysis are provided in appendix B (Tab. B.1).

A calibration curve ranging from 0.05 to 200 ng/mL was used for quantification. Linear regression was applied with a weighting factor 1/x for each analyte. The peak areas were corrected by isotope-labeled surrogate standards. Furthermore, for quality control, a control standard was measured every tenth sample within a sequence and procedural blanks were analyzed within every experimental sequence to determine possible contaminations during the sample treatment. The limits of quantification (LOQs) and recoveries were evaluated from spiked WWTP effluents (10 ng/L) in triplicate. The recoveries for all analytes ranged from 71 ± 5% to 107 ± 9%. Results of the method performance are provided in appendix B (Tab. B.4).

3.3   Results and Discussion

3.3.1   Kinetic Analysis

Since all studied steroids revealed an exponential decrease in concentration, a pseudo-first order kinetic was modeled (Tab. 3.2). The degradation rate constants $k_{biol.}$ were calculated according to Schwarzenbach *et al.* (2005). The chemical structures of the analyzed GCs are provided in appendix B (Tab. B.3).

The applied kinetic model revealed good linearity for the analyzed steroids (r > 0.95), except for triamcinolone acetonide (0.898) and the rate constants $k_{biol.}$ varied over four orders of magnitude (10^{-2} to 10^2 L/(g_{ss}·d)). The natural hormone hydrocortisone and its most similar

synthetic derivative prednisolone were rapidly degraded within three hours in the aerated batch experiments, with DT_{50} values (dissipation time for 50% removal) below the maximum resolution of the experiment (< 30 min).

Furthermore, the prodrug betamethasone dipropionate showed a complete removal after 12 h of incubation. Degradation rates of the corresponding steroid alcohols (6α-methylprednisolone, beclomethasone, and betamethasone) were found to be in the same range. However, significantly lower rates were observed for the C17-monoesters. Because diesters and monoesters represent precursors of steroid alcohols, their removal leads primarily to active GCs. However, the elevated degradation rate constants of the diesters indicate a high removal in full-scale WWTP and thus a low discharge into aquatic environments. In contrast, the monoesters and their related steroid alcohols were frequently found in WWTP effluents (ISOBE ET AL., 2015; WEIZEL ET AL. 2018).

Fluticasone propionate and clobetasol propionate are fluorinated GCs with altered side chains. Both GCs exhibited much lower rate constants ($k_{biol.}$ < 1.2 L/(g$_{ss}$·d), DT_{50} > 2 d) indicating an insufficient removal in WWTPs. These results are in agreement with the very few reported data (FAN ET AL., 2011, WU ET AL., 2019).

The lowest degree of biodegradation was observed for cyclic ketal steroids. Especially the fluorinated ketals triamcinolone acetonide and fluocinolone acetonide were found to be persistent in contact with activated sludge (recovery after 14 days > 70%), while budesonide was the most recalcitrant non-halogenated GC in this study.

Synthetic GCs were primarily designed for enhanced glucocorticoid potency. For instance, the insertion of halogen substituents leads to increased persistency in the human body (BODOR AND BUCHWALD, 2006), whereas the introduction of ester groups increases the lipophilicity and thus its bioavailability through absorption (TÄUBER, 1994).

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 placed 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 Muir (1961) described the generalized degradation route of steroids by the initial hydroxylation at C9, which subsequently leads to the ring fission between C9 and C10 following the 9,10-*seco* pathway (OLIVERA AND LUENGO, 2019). It can be assumed that this reaction pathway is inhibited in 9α-halo steroids and led to the observed microbiological stability. Moreover, a further decrease in biodegradability was observed for steroids containing a C17-ester moiety.

Particularly for clobetasol propionate and fluticasone propionate elevated stability was observed, thus it can be concluded that structural modifications at the C21 position lead to higher stability due to their missing hydroxyl group at the C21 position which is decisive for the migration of the ester moiety (ester hydrolysis is discussed in detail in section 3.2.2). 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. In particular, cyclic ketal steroids which are frequently administered in the USA or Switzerland such as halcinonide, flunisolide or desonide should receive more attention in environmental monitoring campaigns due to expectable limited removals in biological wastewater treatment.

Table 3.2. Summary of the kinetic results from experiment K1 and K2. Glucocorticoids are sorted by increasing stability. The uncertainty is expressed as the standard deviation of the replicates. Experimental conditions were as follows: activated sludge diluted with effluent (1:10), c_0= 200 ng/L, n= 3).

Substance	Abbreviation	Recovery at t_0 (c=200 ng/L) [%]	$k_{biol.}$ [L/(g_{ss}*d]	DT_{50} [d]	Pearson coefficient
Prednisolone	PNL	75 [a]	250±20	<0.02	0.999±0.002
Hydrocortisone	HCOR	92 [a]	180±10	<0.02	0.998±0.003
Betamethasone 17,21-dipropionate	BMSdiprop	106±8	40±1	0.06	0.976±0.011
Betamethasone	BMS	94±3	20±1	0.08	0.999±0.001
Beclomethasone	BEC	76±3	15±1	0.11	0.998±0.001
6α-Methylprednisolone	MPNL	105±2	12±1	0.14	0.999±0.001
Beclomethasone 17-propionate	BECprop17	80±3	2.7±0.3	0.25	0.999±0.003
Betamethasone 17-valerate	BMSval17	72±3	1.7±0.1	0.41	0.997±0.004
Budesonide	BDN	84±3	1.8±0.1	1.3	0.997±0.001
Fluticasone propionate	FLUprop	73±2	1.1±0.1	1.5	0.962±0.008
Clobetasol propionate	CLOprop	84±3	0.82±0.15	2.0	0.993±0.002
Fluocinolone acetonide	FCNact	98±4	0.13±0.02	>14	0.959±0.028
Triamcinolone acetonide	TRlact	106±4	0.07±0.03	>14	0.898±0.074

[a] The recovery of the first sample is shown, due to fast degradation during the sampling of the replicates.
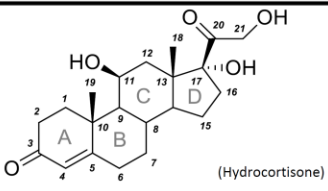
3.3.2 Identification of TPs and Characterization of Transformation Reactions

In total, 41 TPs were tentatively identified from 12 GCs. For 22 TPs the suggested chemical structure was verified by authentic reference standards. Structural proposals of the TPs without commercially available reference standards were based on the acquired high-resolution mass spectrometry (HRMS) data, RT shifts and by the analogy to the proved structures. Chemical structures (tentatively) and further information of the detected TPs are

provided in appendix B (Tab. B.3). The analytical results (chromatograms, mass spectra and time courses) can be found in Fig. B.1.

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 Tab. 3.3. The following chapters discuss the main outcomes in detail.

Table 3.3. Detected transformation reactions of glucocorticoids in contact with activated sludge. Propose of the association of involved structure moieties on TP formation.

(Hydrocortisone)

Transformation	Steroids	Structural Moieties
Δ¹-dehydrogenation	Hydrocortisone, 1,2-dihydro fluticasone propionate, 1,2-dihydro clobetasol propionate	3-one-4-ene
Δ¹-hydrogenation	Fluticasone propionate, budesonide, fluocinolone acetonide	1,4-diene-3-one
Δ⁴-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	secondary hydroxyl group at C17
Hydroxylation at ring A	Clobetasol propionate	-
Hydroxylation at ring B	Beclomethasone, betamethasone, 17-oxo betamethasone	-

Natural and Related Glucocorticoids. This group of natural and related GCs is characterized by a non-halogenated core structure. In general, they have a rather "weak" potency due to short plasma half-lives and low receptor binding affinities (MUTSCHLER, 1997). Although biodegradation of hydrocortisone and prednisolone was observed, no clear TP formation was detected in the analyzed time series. It has to be noted, that small amounts of prednisolone were detected as an intermediate by LC-MS/MS during the incubation of hydrocortisone, which was rapidly degraded again. Therefore, it can be assumed that a fast degradation led either to TPs which are directly incorporated into metabolic pathways of the microorganisms or which have a high polarity and thus cannot be covered by the analytical method used. The enrichment techniques were based on reversed phases which are limited

in the retention of extreme polar compounds. However, it has to be noted that in this study only TPs with concentrations down to about 5% of the initial concentration could be detected. Based on these results it can be assumed that the biodegradation of these three compounds might not lead to large amounts of recalcitrant TPs in contact with activated sludge and hence, it is unlikely they can be detected in WWTP effluents.

Glucocorticoid Diesters and Monoesters. Synthetic GCs are often esterified with carboxylic acids such as propionic acid to reinforce therapeutic properties (BODOR AND BUCHWALD, 2006). These GC esters are used primarily for topical treatments. Several ester steroids were considered in our study since their fate and the behavior during wastewater treatment is unknown so far.

6α-Methylprednisolone aceponate, beclomethasone 17-propionate, beclomethasone dipropionate, betamethasone 17-valerate, betamethasone dipropionate, clobetasol 17-propionate and fluticasone 17-propionate were incubated in independent experiments, to enable a clear assignment of the TPs identified to the parent GCs. A summary of the results is provided in Fig. 3.1.

The diesters were rapidly removed within 12 h and as their initial degradation reaction, the ester hydrolysis was observed. As the main TPs, the monoesters at C17 and C21 positions were identified, which were then further hydrolyzed to the corresponding steroid alcohols (Fig. 3.1 c). The hydrolysis of the diester was also detected in the sterile control, but the rate of hydrolysis was more than a factor of ten lower (Fig. 3.1 a).

The experiments with the C17-monoesters of beclomethasone and betamethasone as initial steroids revealed the formation of the corresponding C21-monoesters as intermediates which can be explained by an intramolecular migration of the ester moiety (BUNDGAARD AND HANSEN, 1981; TÄUBER, 1994). Sterile and regular experiments showed identical rates for the migration (Fig. 3.1 b). Thus, it can be concluded that the ester migration is mediated by one or more abiotic reactions that are controlled by pH and temperature (BUNDGAARD AND HANSEN, 1981; TÄUBER 1994). In contrast, the hydrolysis of the C21-monoesters was found to be enzymatically catalyzed, since in the autoclaved samples the C21 monoesters were found to accumulate over a period. During the incubation of the aceponate diester (6α-methylprednisolone-17-propionate-21-acetate) only the formation of the propionate monoester was identified. Thus, the favored enzymatic hydrolysis of ester moieties occurs obviously at position C21.

Moreover, clobetasol propionate and fluticasone propionate are characterized by a missing hydroxyl group at position C21. Since the 21-hydroxy moiety is crucial as a target for the

migration of the propionate ester, both GCs were not hydrolyzed or rearranged in the experiments T6 and T8. It can therefore be concluded that immediate ester hydrolysis cannot be the initial degradation reaction of C17 ester GCs.
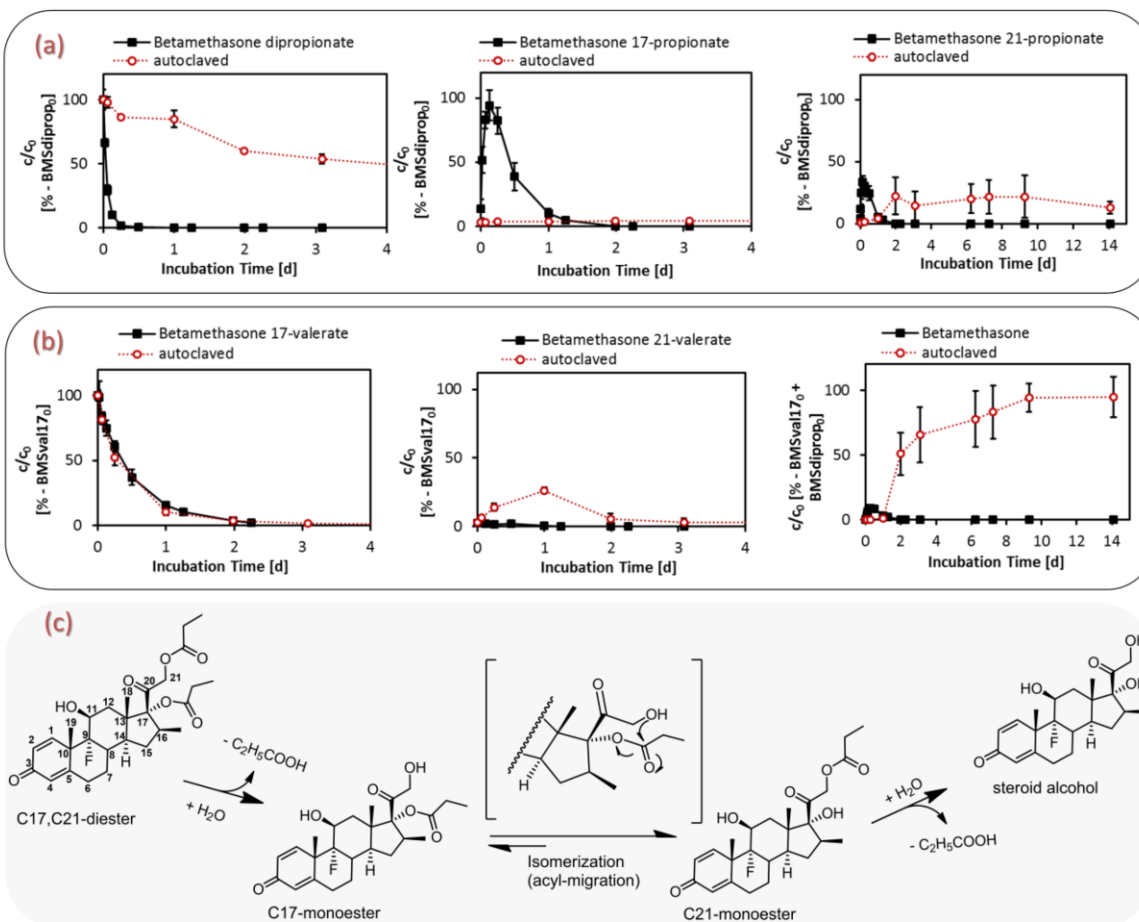
Figure 3.1. Time trends of the ester hydrolysis of the betamethasone derivatives from experiments K2 and S2. Experimental conditions were as follows: c_0= 200 ng/L, c_{ss}= 0.30 g_{ss}/L. The concentrations of all steroids were quantified by authentic reference standards. Error bars indicate the uncertainty as standard deviations (n= 3). The dotted lines (red) rep-resent sterile control batches with autoclaved activated sludge (n= 2). (a): Betamethasone dipropionate (BMSdiprop). (b): Betamethasone 17-valerate (BMSval17). (c): Scheme of ester hydrolysis and isomerization of betamethasone dipropionate according to Bundgaard and Hansen (1981).

Formation of Glucocorticoid Carboxylic Acids. The analyzed ketal GCs (budesonide, fluocinolone acetonide, triamcinolone acetonide) showed significant stability towards microbiological degradation as outlined in section 3.3.1. Supplementary to the kinetic results, similar TPs were identified for all studied ketal steroids. Ketal GCs are characterized by their α hydroxyl groups at C16 and C17, which are acetalized by aliphatic ketones to form

cyclic ketals (BUNDGAARD AND HANSEN, 1981). GCs with such chemical moieties show strong binding affinity to the GC receptor and are denoted as GCs with an enhanced potency (MUTSCHLER, 1997).

During the individual incubation experiments, the analyzed ketals showed one characteristic transformation reaction. The major TPs identified were the C21-carboxylic acids whose formations were confirmed by authentic reference standards (Fig. B.1). The formation of C21 carboxylic acids can be explained by oxidation of the primary alcohol at C21 and was found only for this group of steroids. The time course of the TP formation is shown exemplarily for budesonide in Fig. 3.2.

Fluticasone propionate is a steroid used for the treatment of asthma and rhinitis. Several pharmaceuticals with fluticasone propionate as the active ingredient were recently awarded a non-prescription status and hence are sold as over-the-counter drugs in Germany (BUNDESRAT, 2016). As a consequence, the growing consumption of fluticasone propionate can be forecast for Germany. In addition, the chemical structure of fluticasone propionate was designed to reduce the side-effects in the therapy of lung diseases, which is attributed to the beneficial ability of the thioester hydrolysis at C17. The metabolic hydrolysis decreases the systemic side-effects during corticosteroid therapy significantly (JOHNSON, 1998).

As the main reaction and initial degradation step of fluticasone propionate, the enzymatically mediated hydrolysis of the thioester was identified which finally led to the formation of TP452, the respective 17β-carboxylic acid (Fig. 3.2).
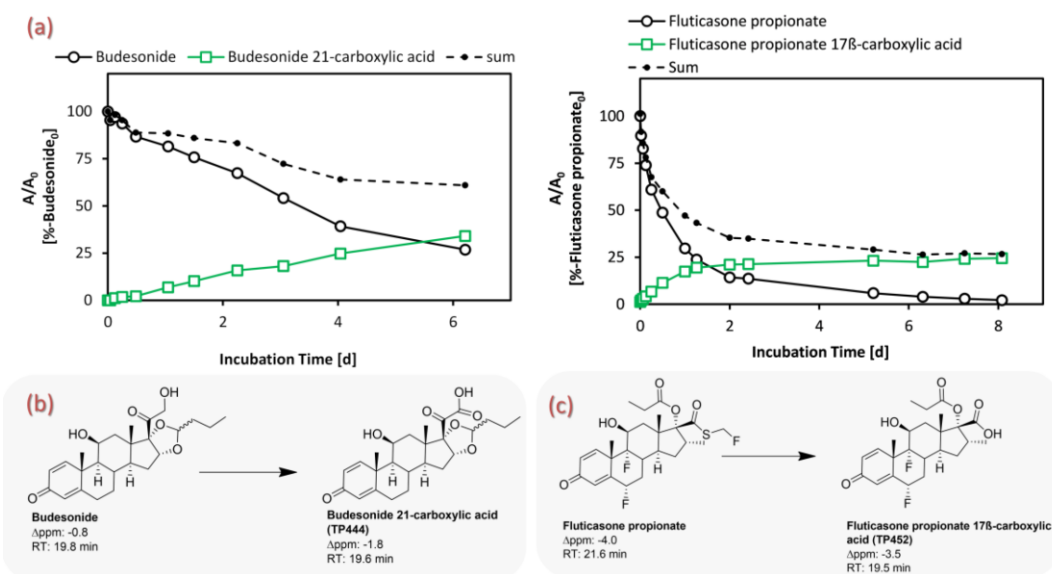
Figure 3.2. Time trends of the degradation of budesonide (exemplarily for all ketal steroid) and fluticasone propionate. The formation of their carboxylic acid TPs (TP444, TP452) is shown for the experiments T14 and T8 (**a**). Chemical structures of the precursor glucocorticoids and carboxylic acid TPs are illustrated in (**b**) and (**c**).

For further insights into biodegradability and fate, the carboxylic acids of triamcinolone acetonide and fluticasone propionate were analyzed in separate incubation experiments. As shown in Fig. 3.3, both tested carboxylic acid TPs were not degraded at all during the incubation periods and further expected transformation reactions, such as hydrogenations or side-chain degradation were not observed. Therefore, these results indicate a persistent behavior of the carboxylic TPs in biological wastewater treatment.
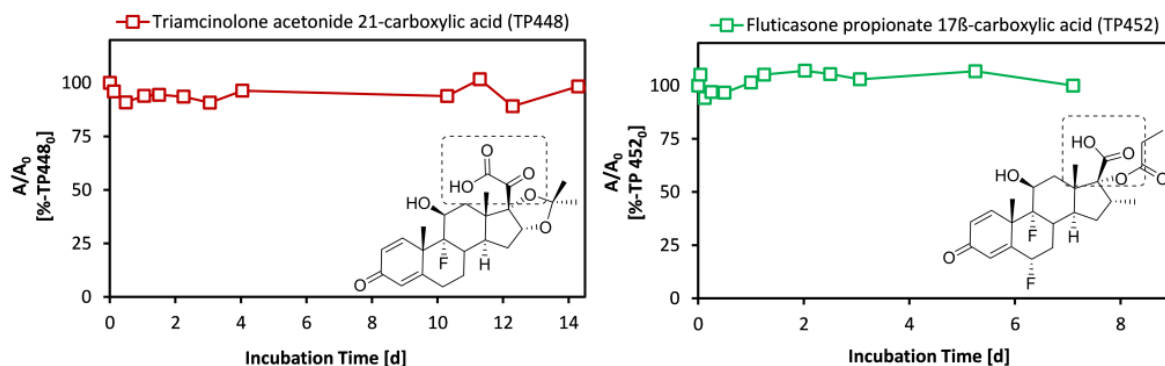
Figure 3.3. Microbiological stability of the carboxy-TPs of triamcinolone acetonide (TP448) and fluticasone propionate (TP452) during the incubation with activated sludge from the experiments T10 and T17.

Betamethasone and Beclomethasone. The structural characteristic of betamethasone and its related steroids is the 9α-halo moiety (e.g. dexamethasone, flumethasone and beclomethasone). Betamethasone (9α-fluoro) and beclomethasone (9α-chloro) were selected as initial substances for tracking the fate during biodegradation since both GCs are in widespread use in Germany. The time courses of TP formation and the tentative pathway of the observed betamethasone biotransformation in activated sludge are shown in Fig. 3.4. The proposed degradation pathway of beclomethasone (dipropionate) is illustrated in Fig. 3.5. Detailed information to the verified or proposed TPs is provided in the appendix B. During the incubation experiments of both GCs the formation of four similar TPs has been detected (betamethasone: TP332, TP348a, TP412, and TP414; beclomethasone TP348b, TP364, TP428, and TP430). Thus, comparable degradation pathways were identified for betamethasone and beclomethasone.

The biodegradation of betamethasone revealed the formation of 17-oxo betamethasone (OxoBMS), whose retention time and MS² matched that of an authentic reference standard. However, OxoBMS was detected to a minor extent (Fig. 3.4 a), but appreciable quantities were found for an isomeric TP (TP332). For beclomethasone an analogic TP (TP348b) was

formed. Both, TP332 and TP348b, were found as the major TPs formed during the biodegradation of the analyzed betamethasone and beclomethasone derivatives. The comparison of the MS² spectra of OxoBMS and TP332 revealed a high structural similarity since the fragmentation pattern for both compounds were comparable (Fig. B.2). However, the RT shift between TP332 and OxoBMS (ΔRT: 3.1 min) suggested a more polar compound and thus a 17β-hydroxy-6,7-dehydro derivative was tentatively proposed for TP332 and TP348b as the most plausible structure, whereas the exact position of the carbon double bond could not be determined since reference standards of these TPs were not commercially available. For confirmation of the RT shift between 17β-hydroxy and 17-oxo steroids, two commercially available steroid pairs (estrone/17β-estradiol and androstenedione/testosterone) were analyzed under the same chromatographic conditions (data not shown). The RT shifts were found to be similar to those for TP332 and OxoBMS, which supported the proposed 17β-hydroxyl moiety of TP332 and TP348b.

For further insights, OxoBMS was spiked as the initial compound in a separate experiment. A fast and nearly complete enzymatically mediated isomerization to TP332 (Fig. 3.4 b) was found, revealing the 17-oxo TPs as intermediates in the biodegradation of betamethasone and related GCs. Such behavior was not observed for the ketal steroids, clobetasol propionate and fluticasone propionate. Therefore, it is likely that the 17α-hydroxyl group is crucial as a reactive site for the transformation since in all these GCs the 17α-hydroxyl group is protected and the side chain degradation has not been observed. On the other hand, the steric effects of the 17α-substituents might be responsible for the inhibition of the side-chain cleavage.

The enzymatic conversion of the steroid side chain to yield 17-oxo and 17β-hydroxy steroids was proposed to occur in the biodegradation of hydrocortisone (WANG ET AL., 2018) and progesterone (YU ET AL., 2018). This transformation is an important reaction in the biosynthesis of androgens in mammals, which is catalyzed by Cytochrome P450 17A1 (YOSHIMOTO AND AUCHUS, 2015). On the other hand, the reversible reduction of the steroid 17-oxo moiety constitutes an important control mechanism for nuclear receptor ligands (LUKACIK ET AL., 2006). For example, the enzymatic conversion of estrone to 17β-estradiol (KHANAL ET AL., 2006) and androstenedione to testosterone (BHATTI AND KEHRA, 2012) regulate the ratio of the active 17β-hydroxy steroid and the less active 17-oxo analogue in the endogenous endocrine system in humans.

Nevertheless, TP332 and TP348b were degraded to TP348a (TP of betamethasone) and TP364 (TP of beclomethasone), which were tentatively proposed as hydroxylated derivatives. For both GCs, the amount of their formation was below 15%. According to

previous studies the hydroxyl group was suspected to be at ring B, since hydroxylation of steroids in human metabolism revealed usually C6-hydroxylated products (BUTLER AND GRAY, 1970). During further incubation, TP412 and TP414 (TPs of betamethasone) as well as TP428 and TP430 (analogic TPs of beclomethasone) were formed and could be identified as O-sulfated TPs. The MS² spectra of the TPs indicated the presence of sulfate (Fig. B.1). Sulfation is a known enzymatic reaction of hydroxylic moieties occurring in biological wastewater treatment, as reported in El Sharkawy *et al.* (1991) and Jewell *et al.* (2015).

In contrast to the analogy of the biodegradation of betamethasone and beclomethasone, the beclomethasone derivatives were found to undergo intramolecular epoxidations between the C9 and C11 position, which led to the formation of three epoxy TPs (TP428a, TP428b and TP372). These epoxy TPs could be unambiguously confirmed via authentic reference standards and their formation was observed in the sterile control as well. The epoxidation of chlorohydrins (trans-1-chloro-2-hydroxy-hydrocarbons) can be enzymatically catalyzed (FAUZI ET AL., 1996) or be promoted by alkaline conditions. Such intramolecular epoxidation was not observed for 9α-fluoro steroids since fluorine is not a good leaving group due to its elevated basicity.
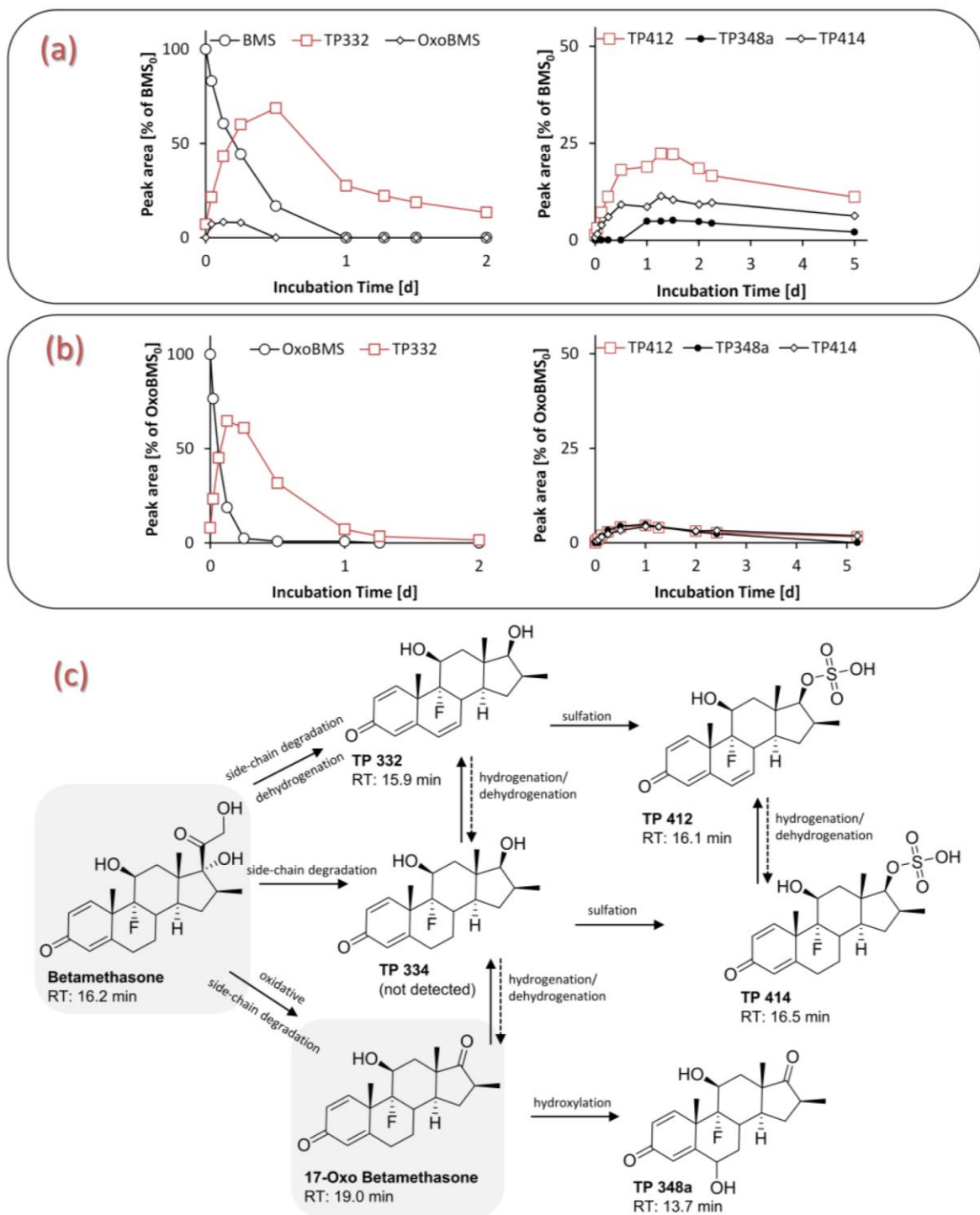
Figure 3.4. Time courses of the microbial degradation of (**a**) betamethasone (BMS) and (**b**) 17-oxo betamethasone (OxoBMS) in contact with activated sludge (diluted with WWTP effluent (1:10), c_0= 2 µg/L, n= 1). The lower scheme (**c**) illustrates the proposed degradation route for betamethasone. Structures highlighted in grey were verified by authentic reference standards. The dashed lines indicate minor pathways.
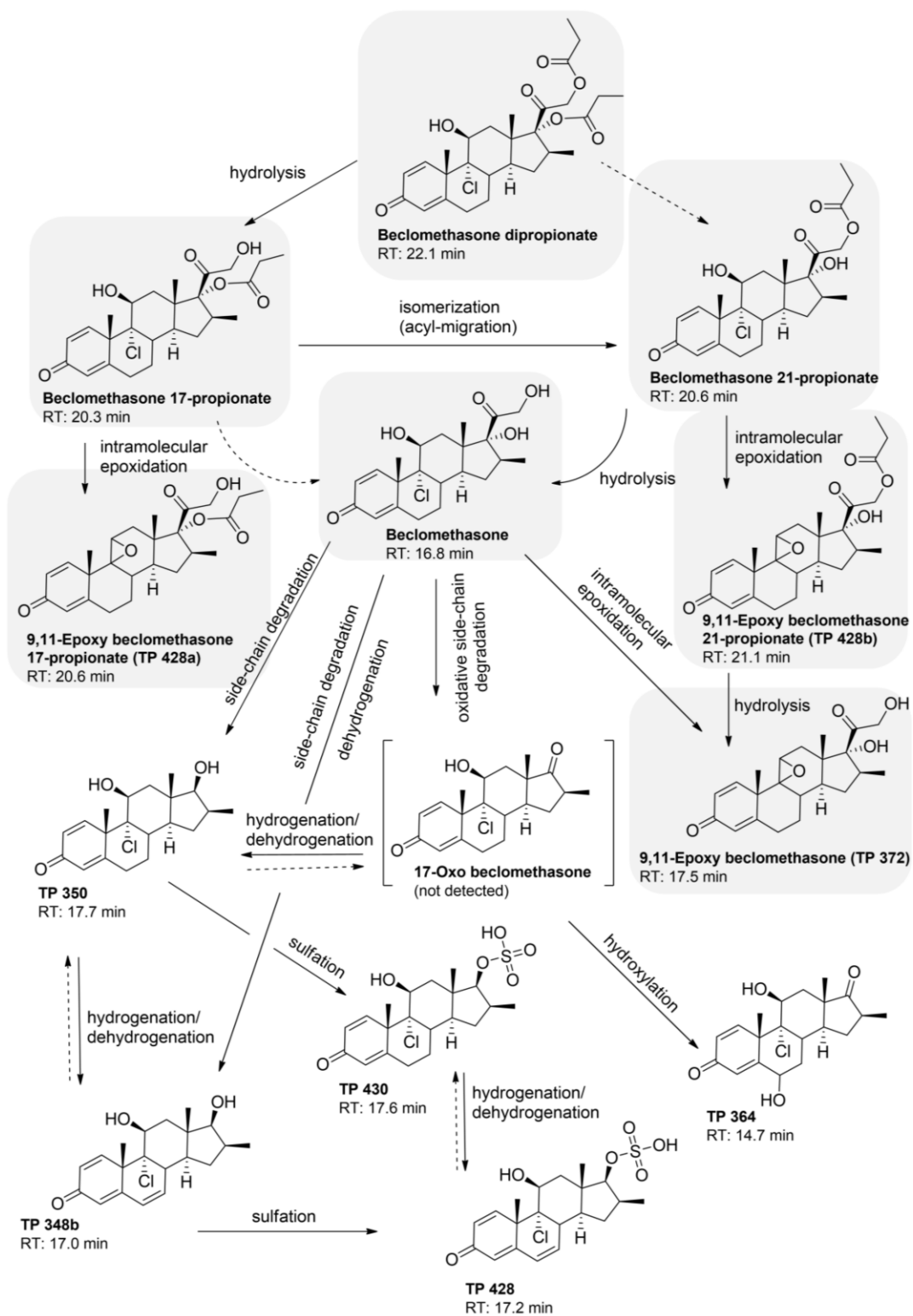
Figure 3.5. Proposed degradation pathway of beclomethasone dipropionate based on the TPs identified. Structures highlighted in grey were verified by authentic reference standards. The dashed lines indicate minor pathways. (Time courses of TP formation can be found in the appendix B).

Hydrogenation and Dehydrogenation of Steroid Ring A. The regulation of the steroid activity in humans is often coupled to hydrogenation/dehydrogenation of carbon bonds in steroid ring A (Hanukoglu, 1992). For instance, the weak androgen testosterone is activated by the enzyme 5α reductase and leads to the formation of the potent androgen 5α-dihydro testosterone (DHT). Moreover, the Δ^1-dehydrogenation (dehydrogenation between C1 and C2) is associated with the four-fold enhancement of the GC activity of prednisolone in comparison to hydrocortisone (Mutschler, 1997). On the other hand, hydrogenation can also lead to an increased affinity to other steroid receptors, as it was previously reported for norethisterone and its hydrogenated metabolites (Larrea et al., 2001). Therefore, steroid activity is impacted by the presence and position of double bonds.

In this study, hydrogenation of the carbon double bond at position C4 and C5 (Δ^4-hydrogenation) was identified as the primary transformation reaction of several GCs and was identified for budesonide, clobetasol propionate, fluticasone propionate, fluocinolone acetonide and triamcinolone acetonide (Tab. B.3, Fig. B.1). In addition, the formation of two hydrogenated TP isomers (Δ^1- and Δ^4-dihydro) was detected for budesonide, fluocinolone acetonide and fluticasone propionate, although one TP was in all cases dominant. The comparison with authentic reference standards of the corresponding 1,2-dihydro compounds confirmed that the Δ^1-hydrogenated TPs (1,2-dihydro) were formed to a minor extent, since both isomers could be clearly distinguished by their RTs. It should be noted that the Δ^4-hydrogenated TPs (4,5-dihydro) were not commercially available for reference. However, the MS² spectra showed considerable similarities (MS² spectra are provided in appendix B) and the reduction of the carbonyl could be excluded since all TPs showed a positive RT shift in comparison to their parent GCs.

When 1,2-dihydro fluticasone propionate (TP502b) was incubated as the initial compound, the formation of appreciable amounts of fluticasone propionate and TP502a were detected. In other words, Δ^1-dehydrogenation of TP502b results in the formation of fluticasone propionate which was then further converted to TP502b via Δ^4-hydrogenation. Similar behavior was noted during the incubation of (1,2-dihydro) clobetasol propionate, and hydrocortisone was found to be converted to prednisolone, which is the Δ^1-dehydro derivative of hydrocortisone. The time courses of the TP formation for the fluticasone propionate and the 1,2-dihydro fluticasone propionate (TP502b) experiments are illustrated in Fig. 3.6. Chromatographic separation of the hydrogenated TPs is shown exemplarily for the incubation of 1,2 dihydro fluticasone propionate (TP502b) in Fig. 3.6 (a).

As Δ^4-hydrogenation of fluticasone propionate leads to the formation of TP502a, while Δ^4- hydrogenation of 1,2-dihydro fluticasone propionate (TP502b) yields TP504, the tetra-

hydrogenated derivative, Δ^4-hydrogenation seems to be the dominant reaction. In both experiments, the main TP was the product of the enzymatic reduction of the C4-C5 double bond. The preferred formation of 4,5-dihydro fluticasone propionate (TP502a) suggests therefore strong regioselectivity of the enzymatic hydrogenation and was likewise noted for other GCs in this study. On the other hand, 1,2-hydrogenated steroids showed a considerable level of Δ^1-dehydrogenation. The enzymatically mediated Δ^1-dehydrogenation was previously reported during the biodegradation of progesterone (YU ET AL., 2018) and hydrocortisone (BREDEHÖFT ET AL, 2012) and seems to be a prevalent transformation reaction of steroids.
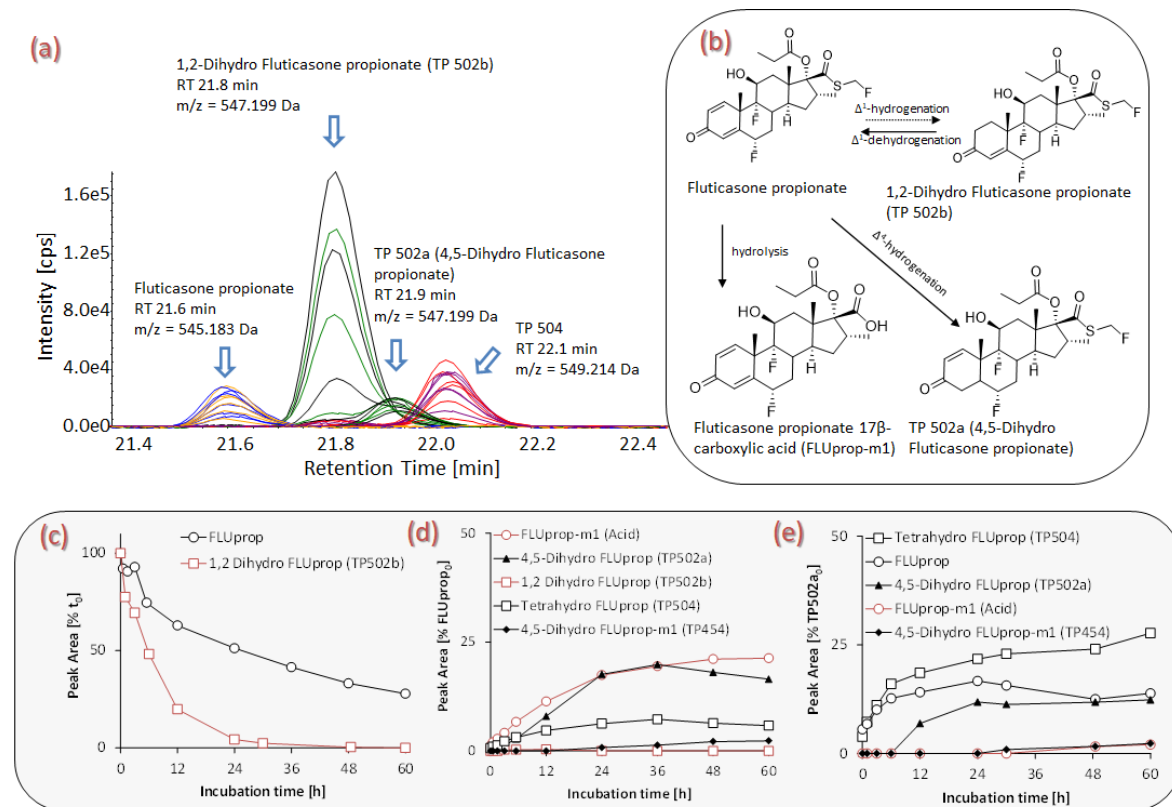
Figure 3.6. Results from the individual fluticasone propionate (FLUprop) incubation experiments T8 and T9. (**a**): Chromatographic differentiation of the detected TPs during the incubation of 1,2-dihydro fluticasone propionate (TP502b). Extracted masses represent the formate adducts in ESI negative mode. (**b**): Chemical structure of fluticasone propionate and related TPs. (**c**): Time course of the microbiological degradation of fluticasone propionate and TP502b. (**d**): Time courses of TP formation for fluticasone propionate. (**e**): Time courses of TP formation for TP502b.

The hydrogenation of the double bond at C4 and C5 (Δ^4-hydrogenation) can yield 5α- or 5β-hydrogenated TPs and thus to a significant change in the geometry of the steroid core. As the results indicated the formation of one major Δ^4-hydrogenated TP for a broad range of GCs, it can be expected that the enzymatic Δ^4-hydrogenation is stereospecific, although identification of the stereochemistry was impossible due to the missing reference materials. These hypotheses need to be addressed in further research. Finally, a proposal of a general scheme of the favored sites and reactions was derived and illustrated in Fig. 3.7, since the evaluation of the detected TPs, revealed a strong regio- and stereoselectivity of the transformation reactions during the incubation of GCs with activated sludge.
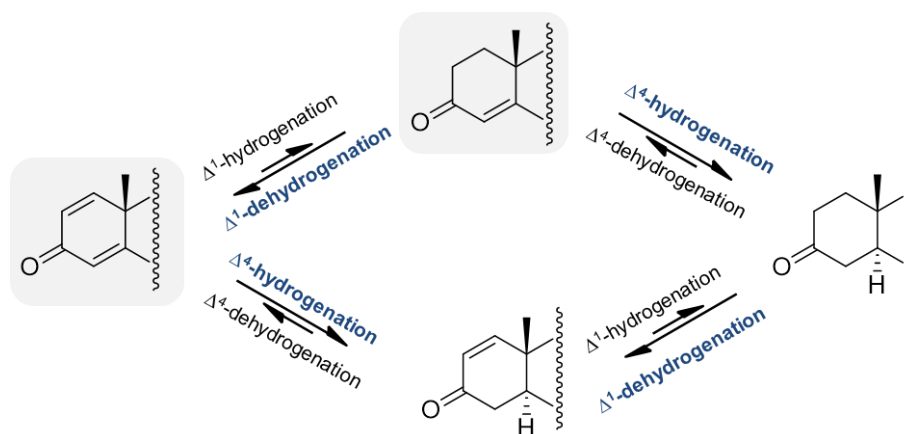
Figure 3.7. Likelihood of the hydrogenation/dehydrogenation sites in the biodegradation of 3-oxo-4-ene and 3-oxo-1,4-diene steroids according to the results from the degradation experiments.

3.3.3 Occurrence of Glucocorticoids and TPs in WWTP Effluents

The occurrence of the target GCs and TPs was investigated in eight German WWTPs equipped with conventional biological treatment units. For quantification, the previously reported method was optimized for the TPs (Weizel et al., 2018). Details of the applied analytical method (Tab. B.4), results of the analyzed WWTP effluents (Tab. B.5, Fig. B.4-B.6), and detailed sample information (Tab. B.2) can be found in the appendix B. Furthermore, the results of the degradation study were transferred to related GCs which were not investigated in the degradation experiments mentioned above. These were TPs derived for the steroids 6β-hydroxy triamcinolone acetonide (TP: 6β-hydroxy-21-oic triamcinolone acetonide) and mometasone furoate (TP: 9,11-epoxy mometasone furoate),

since both compounds were recently found in elevated concentrations in WWTP effluents (Weizel et al., 2018) and authentic reference standards of the proposed TPs were available. As shown in Tab. 3.4, six of the 13 parent GCs were detected in all WWTP effluents. Triamcinolone acetonide was found with the highest concentrations (up to 20 ng/L) besides clobetasol propionate (up to 3.8 ng/L), the betamethasone monoesters (up to 3.1 ng/L), and mometasone furoate (up to 1.9 ng/L), respectively.

Moreover, 11 TPs could be detected and quantified via their authentic reference standards. All of the analyzed WWTP effluents contained the carboxylic acid TPs of triamcinolone acetonide (TP448), its metabolite 6β-hydroxy triamcinolone acetonide and fluticasone propionate (TP458). The concentration of TP458 was in all cases higher than its parent GC fluticasone propionate with a maximum concentration of 2.8 ng/L. The results therefore indicate that these carboxylic acid TPs are widely present in WWTP effluents and thus discharged in rivers and streams. It has to be noted, that these TPs are also discharged into the WWTPs by domestic wastewater since they are human metabolites (Pearce et al., 2006; Argenti et al., 2013). Although carboxy-TPs are rather inactive GC receptor agonists in comparison to the parent steroids (Johnson et al., 1998), further research is recommended to rule out a possible adverse effect on waterborne organisms by cross-receptor affinities.

Furthermore, the sampling campaign revealed the ubiquitous occurrence of the betamethasone monoesters. In addition to 17-monoesters, all analyzed WWTP effluents contained relatively high concentrations of betamethasone 21-valerate and betamethasone 21-propionate. Since these monoesters can be predicted as precursors of betamethasone, the exclusion of such GCs leads to a considerable underestimation of the overall concentration of betamethasone, not at least because GC mixtures induce additive effects (Willi et al., 2019).

The secondary betamethasone TPs 17-oxo betamethasone and TP332 could be detected in two and three WWTP effluents, respectively. 17-Oxo and 17-hydroxy steroids are known to trigger the androgen receptor, thus the TPs could contribute to residual bioactivities in WWTP effluents as was recently reported (Houtman et al., 2018). Moreover, the conjugated TP of 17-oxo betamethasone (TP412) was found in all WWTP effluents (Fig. B.4). Since the conjugation of steroids is a reversible process, TP412 can be assumed as a precursor of TP332 (Cwiertny et al., 2014). However, due to the missing reference standard for TP412 its quantification was not possible.

On the other hand, the hydrogenated TPs were all below the limit of quantification (LOQ). It can be assumed that the average hydraulic retention time in the biological treatment areas

is not high enough for the extensive generation of hydrogenated TPs and thus, the formed concentrations might too low for analytical detection.

Table 3.4. Concentrations of GCs and 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 and TPs	LOQ [ng/L]	n > LOQ	Median [ng/L]	Minimum [ng/L]	Maximum [ng/L]
Triamcinolone acetonide	0.4	8	12	1.0	20
TP448 (CBX)	0.3	8	1.8	0.5	3.7
Fluocinolone acetonide	0.2	3	0.4	0.2	0.4
Budesonide	1.2	0	<1.2	<1.2	<1.2
TP444 (CBX)	0.4	4	0.6	0.4	0.8
Fluticasone propionate	0.4	4	0.8	0.4	1.6
TP452 (CBX)	0.5	8	1.4	0.5	2.8
Clobetasol propionate	0.4	8	1.2	0.5	3.8
Hydrocortisone	0.2	8	1.6	0.9	2.8
Betamethasone 17-valerate	0.5	8	1.2	0.6	3.1
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
TP 332 [b]	0.3	3	0.5	0.3	0.5
Betamethasone dipropionate	0.3	0	<0.3	<0.3	<0.3
17-propionate	0.4	7	0.8	0.6	1.6
21-propionate [a]	0.4	8	0.9	0.5	1.3
Further GCs and suspected TPs					
6β-Hydroxy triamcinolone acetonide	0.2	8	1.5	0.7	2.2
6β-hydroxy-21-oic triamcinolone acetonide (CBX)	0.4	8	0.9	0.5	1.5
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

[a] Related C17-ester was used as reference standard for quantification
[b] 17-Oxo betamethasone was used as reference standard for quantification

3.4   Conclusions

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:

•   The spectrum of the analyzed GCs showed a pronounced variability in aerobic degradation. In this context, structural features that led to a distinct increase of stability could be characterized. Thus, based on the current results, the (bio)degradability of GCs in biological wastewater treatment can be predicted using the chemical structure. Also, the results from previous studies can be better interpreted, as this shows that higher stability primarily leads to the glucocorticoid burden in the environment and not exclusively the consumption pattern.

•   Steroid hormones, in general, are considered as biodegradable. Within the group of GCs the results highlight 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.

• 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.

• Tracking the main TPs is seen as important, because for many of the occurring TPs residual endocrine activity cannot be ruled out. The TPs might therefore significantly contribute to the detected residual endocrine activities in the aquatic environments.

4 Fate and Behavior of Progestogens in Activated Sludge Treatment: Kinetics and Identification of Transformation Products
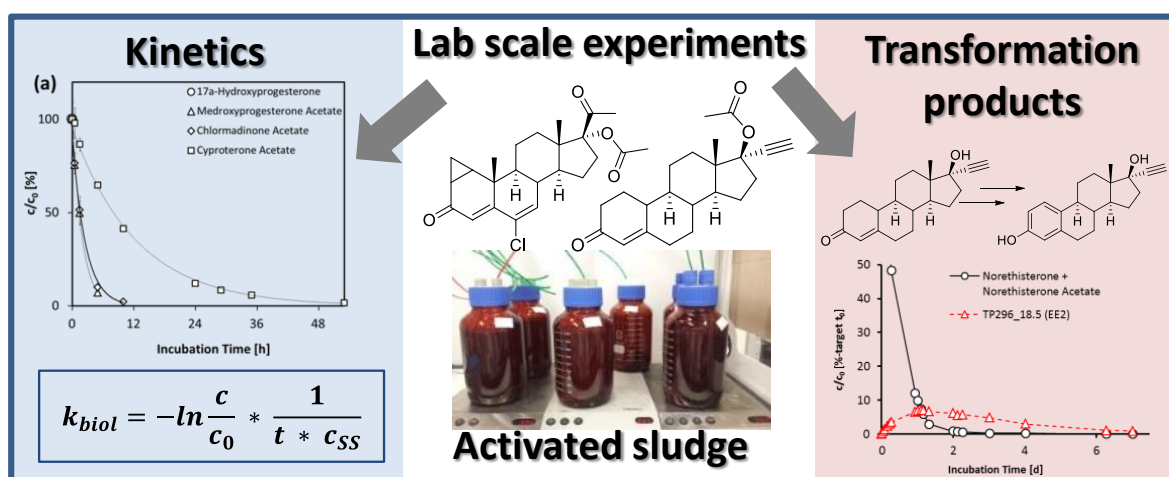
ABSTRACT

Previous studies have shown the high ecotoxicological potential of progestogens (PGs) on the reproductive system of aquatic organisms. Yet the ubiquitous presence of several PGs in wastewater treatment plant (WWTP) effluents indicates an incomplete removal during treatment. To investigate the fate and behavior of PGs during biological wastewater treatment, nine commonly used PGs were incubated in aerobic lab-scale degradation experiments with activated sludge taken from a municipal WWTP. The degradation kinetics revealed a fast removal after 48 h for most of the compounds. Cyproterone acetate and dienogest were the most recalcitrant of the analyzed steroids with half-lives of 8.65 h and 4.55 h, respectively. Thus, only moderate removals of these PGs can be predicted in full-scale WWTPs. Moreover, numerous transformation products (TPs) were detected via high-resolution mass spectrometry. Hydrogenation or dehydrogenation of ring A and non-selective hydroxylations of 17α-hydroxyprogesterone derivatives (medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate) as well as for 19-nortestosterone derivatives (dienogest, norethisterone acetate, etonogestrel) were observed as major transformation reactions. 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. In a comparative evaluation of the TPs formed and the steroid structure, it was observed that molecular structure played a role in the inhibition of several transformation reactions, explaining the increased recalcitrance of these compounds. In addition, aromatization of the steroid ring A was identified for the 19-nortestosterone derivatives leading to the formation of estrogen-like TPs. For instance, the degradation of norethisterone acetate led to the formation of 17α-ethinylestradiol, a well-known and very potent synthetic estrogen. The evidence of the conversion of progestogenic to estrogenic compounds and the formation of potentially hazardous TPs indicates the need of a more comprehensive environmental risk assessment for synthetic steroids. Two of the newly identified TPs (3α-hydroxy cyproterone acetate and Δ9,11-dehydro-17α-cyanomethyl estradiol) were detected in WWTP effluents for the first time.

4.1   Introduction

From today's perspective, the introduction of hormonal contraception in the early sixties can be regarded as historical for the development of society. Regardless of the social and medical importance, endogenous steroid hormones can cause adverse effects when released into environmental compartments (reviewed in FENT, 2015; KUMAR ET AL., 2015; and LIU ET AL., 2011B).

Progestogens (PGs) used in cancer treatment, contraception and replacement therapies are synthetic steroid hormones that were designed to mimic natural progesterone. The properties and potency of active steroids used in hormonal contraception were optimized by alterations of the structure of natural hormones. For instance, the introduction of substituents into the progesterone (e.g. 17α-hydroxy progesterone) skeleton resulted in increased hormonal potency and slower inactivation rates via the endogenous metabolism (KUHL, 2011). It has been shown that esterification at C17 leads to an inhibition of the 20-keto reduction, for which the steric hindrance of the space-filling 17α-ester was found to be responsible. In addition, the metabolic hydroxylation at the C6 position was significantly inhibited by introducing a methyl or chlorine at C6 (NEUMANN, 1994). The result of these observations was the development of potent synthetic progesterone derivatives such as medroxyprogesterone acetate, chlormadinone acetate or cyproterone acetate.

19-Nortestosterone derivatives are another class of steroids frequently used for hormonal contraception. They are derived from the anabolic compound nandrolone (19-nortestosterone). Modifications of the steroid skeleton led to various derivatives which differ in their potency and pattern of hormonal activities (KUHL, 2011). The introduction of a 17α-ethinyl moiety caused a shift from the androgenic to the progestogenic activity owning to the inhibition of the 17β-oxidation (KUHL, 2011).

Over the last decades, it was shown that PGs induce diverse adverse effects in fish at concentrations down to the low or sub-nanogram per litre range (reviewed in FENT, 2015; KUMAR ET AL., 2015; and LIU ET AL., 2011B). For instance, levonorgestrel inhibited the reproduction in fathead minnows at 0.8 ng/L (ZEILINGER ET AL., 2009). Cyproterone acetate was found to decrease the testosterone plasma level in adult mummichog after exposure to 1.0 ng/L (SHARPE ET AL, 2004) and an exposure to 1.0 ng/L norethisterone caused a decrease in fecundity in fathead minnows (PAULOS ET AL., 2010). The exposure of 3.2 ng/L etonogestrel was responsible for a reduction of the mating activity in Endler guppy males and completely suppressed the reproduction ability of the females (STEINBACH ET AL., 2019). In addition, the additive effects of PG mixtures might be expected as recently reported (ZHAO ET AL., 2015).

These multiple effects onto different indicator organisms highlighted serious ecotoxicological consequences for fish and other aquatic wildlife from steroid burdens at environmentally relevant concentrations. Since most of the effects reported showed significant impacts for reproduction, this property makes them the most important pharmaceutical group of concern after 17α-ethinylestradiol (KUMAR ET AL., 2015).

Even though it was shown that different PGs were discharged mainly by municipal wastewater treatment plants (WWTPs) (CHANG ET AL., 2008 & 2011; LIU ET AL., 2011B; GOLOVKO ET AL., 2018; WEIZEL ET AL., 2018; YU ET AL., 2019), knowledge about their behavior during biological wastewater treatment is scarce. The consumption profile of PGs varies between countries and regions, thus different regions reported different dominant PGs occurring in the municipal WWTP effluents. Chang *et al.* (2011) found progesterone as the predominant PG in seven WWTP effluents from Beijing, while Yu *et al.* (2019) reported progesterone, drospirenone, dydrogesterone and levonorgestrel as the most frequently detected PGs in 21 WWTP effluents in China. In Swiss WWTP effluents progesterone and medroxyprogesterone acetate were most prominent (ZHANG ET AL., 2017). Golovko *et al.* (2018) detected dienogest, medroxyprogesterone acetate and megestrol acetate most often in Czech WWTP effluents. On the other hand, dienogest and cyproterone acetate could be identified ubiquitously in German WWTP effluents (WEIZEL ET AL., 2018).

Some studies reported that PGs were partially removed in WWTPs (FAN ET AL., 2011; CHANG ET AL., 2008 & 2011), and were still discharged by the WWTP effluents. However, these studies covered a limited number of target steroids and the fate of many commonly used PGs is unknown, which could explain the detected progestogenic activity of WWTP effluents in further studies (BAIN ET AL., 2014; VAN DER LINDEN ET AL., 2008; HOUTMAN ET AL., 2018). In addition to the incomplete removal of PGs, their biological transformation products (TPs) formed during the wastewater treatment can contribute to the endocrine activity of the WWTP effluents (YU ET AL., 2018). The TPs of common PGs are mainly unknown, since only a very few studies investigated the fate and the formation of TPs. For instance, the analysis of the microbiological degradation of progesterone with activated sludge results in the formation of 12 TPs (YU ET AL., 2018) and for levonorgestrel four TPs were identified (LIU ET AL., 2013). The main transformation reactions include hydrogenations/dehydrogenations as well as side-chain degradation in the case of progesterone. The side-chain degradation results in the formation of potent androgens such as 4-adrostene-3,17-dione and 5α-dihydrotestosterone (YU ET AL., 2018). It is noted that the postulated TPs exhibit the intact steroid core. Thus, there is some evidence that the degradation of PGs still leads to endocrine active compounds which can be discharged into receiving waters. A prominent example of adverse effects in

the aquatic environment caused by steroid transformation was reported by Jenkins *et al.* (2004). It was found that androgens can be formed from progesterone by the bacterium *Mycobacterium smegmatis*. The researchers hypothesized that environmentally induced masculinization among natural populations of female mosquitofish was caused by the microbiological transformation of plant steroids into progesterone and subsequently to potent androgens. These plant steroids were emitted into the rivers by the waste streams of wood pulping processes at local paper mills. Therefore, the transformation of steroids can cause a shift in the steroid selectivity and has to be addressed through the consideration of the TPs formed.
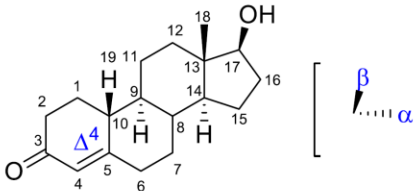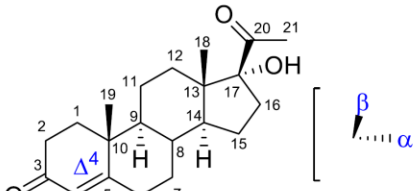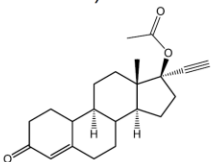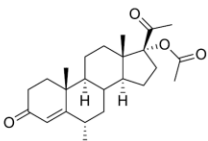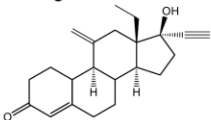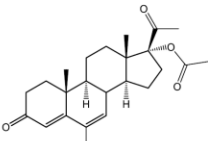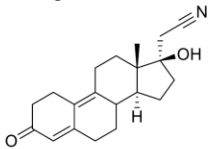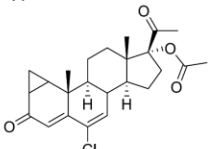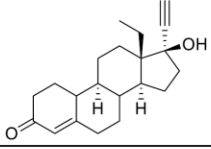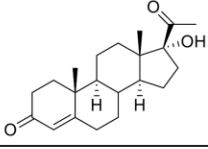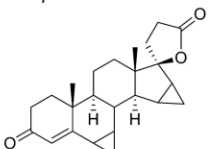
Overall, there is a major lack of understanding the transformation processes of PGs in wastewater treatment. In this context, this study was initiated to elucidate the removal of common PGs during biological wastewater treatment. Therefore, we analyzed the biodegradability of nine PGs (17α-hydroxyprogesterone, medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, levonorgestrel, dienogest, norethisterone acetate, etonogestrel, drospirenone) in aerated lab-scale incubation experiments with activated sludge taken from a municipal WWTP. Furthermore, experiments were conducted for the identification of the main TPs of six PGs (medroxyprogesterone acetate, chlormadinone acetate, cyproterone acetate, dienogest, etonogestrel, norethisterone acetate). Finally, the identified TPs and the parent PGs were analyzed in eight German municipal WWTP effluents.

4.2   Materials and Methods

4.2.1   Chemicals and Compound Selection

Chemical suppliers and further information for the reference standards used in this study are provided in the Appendix C (Tab. C-1). The parent PGs analyzed in this study as well as their annual consumption volumes in Germany for 2018 are shown in Tab. 4.1. The analyzed steroid substrates were selected based on i) the consumption level in Germany, ii) the reported ecotoxicological potency and iii) the chemical structure.

Table 4.1. Selected PGs and their annual consumption in Germany in 2018. *Consumption data: Internal calculations by UBA based on IQVIA MIDAS (2019), sales data.*

19-Nortestosterone Derivatives		17α-Hydroxyprogesterone Derivatives	
Compound	**Consumption [kg in 2018 in GER]**	**Compound**	**Consumption [kg in 2018 in GER]**
Norethisterone (Acetate + Enanthate)	53.4	Medroxyprogesterone Acetate	131.2
Etonogestrel	38 (+25.2 as Desogestrel)	Chlormadinone Acetate	254.3
Dienogest	761.1	Cyproterone Acetate	648.4
Levonorgestrel	58	17α-Hydroxyprogesterone	not marketed in Germany according to hhtps://pharmanet-bund.de
		Drospirenone	175.1

4.2.2   Removal Experiments for the Elucidation of the Kinetic Behavior

Volumes of 200 mL of freshly sampled activated sludge slurry was filled into 2 L brown glass vessels and were immediately diluted with WWTP effluent to a final volume of 2 L. Dilution (1:10) was utilized to reduce sorption on suspended solids and to minimize matrix effects

during chemical analysis. The activated sludge was sampled from the aeration tank of a conventional WWTP (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). The activated sludge slurries were stirred and aerated by synthetic air over the incubation time to maintain aerobic conditions and temperature and pH were controlled as well. No significant drifts and changes were observed within the incubation experiments. Prior to the addition of the steroids, the preparations were allowed to equilibrate for approximately 12 h. The kinetic experiments were conducted in triplicate at an initial concentration of 200 ng/L of each analyte. Inactive sterilized control experiments (autoclaved at 121 °C for 60 min) were run in parallel to differentiate between biotic/abiotic degradation and to assess the impact of sorption. All conducted experiments and their experimental conditions are summarized in Tab. 4.2. For analysis, samples were taken at defined times across the incubation experiment and were immediately filtered (< 1 µm glass fiber, GF6 Whatman, Maidstone, UK). Afterwards, 100 mL of the filtered sample was spiked with 2 ng of each isotope-labeled internal standard and subsequently enriched via SPE (Chromabond C18ec, Macherey-Nagel, Düren, Germany) according to the protocol described in Weizel *et al.* (2018).

4.2.3   Degradation Experiments for the Identification of Transformation Products

For the degradation experiments, 20 mL of freshly sampled activated sludge was filled into 250 mL brown glass vessels and diluted by WWTP effluent to a final volume of 200 mL. Experiments for TP identification were conducted at an initial analyte concentration of 500 µg/L in individual experiments. As a negative control, one non-spiked experiment was included in each experimental sequence. Samples were taken at defined times during the incubation experiment and were immediately filtered with glass fiber syringe filters (< 1 µm, GF6, Whatman). Afterwards, aliquots of 1 mL were spiked with 2 ng of each isotope-labeled internal standard and were stored at 4 °C until HRMS analysis. The samples were not enriched via SPE and TP identification was carried out by direct injection of 80 µL filtered sample.

Table 4.2. Overview of the conducted experiments. All experiments were performed with activated sludge sampled from the aeration tank of a conventional WWTP and were diluted with WWTP effluent (1:10). c_{Target}: *nominal spike concentration, c_{ss}: total concentration of suspended solids, SPE: solid-phase extraction*

Experiment	SOCs spiked	c_{Target} [µg/L]	Sample pretreatment	c_{ss} [g_{ss}/ L]	pH	Temperature [°C]	Incubation time [d]
K	17α-Hydroxyprogesterone, chlormadinone acetate, cyproterone acetate, dienogest, drospirenone, etonogestrel, levonorgestrel, medroxyprogesterone acetate, norethisterone acetate	0.2	SPE (100 mL)	0.37	8.2±0.1	26.3±0.2	7.1
K$_{sterile}$	17α-Hydroxyprogesterone, chlormadinone acetate, cyproterone acetate, dienogest, drospirenone, etonogestrel, levonorgestrel, medroxyprogesterone acetate, norethisterone acetate	0.2	SPE (100 mL)	0.37	8.2±0.1	26.3±0.2	7.1
D1	Dienogest	500	direct injection	0.33	8.1±0.1	23.6±0.3	
D2	Etonogestrel	500	direct injection	0.33	8.1±0.1	23.6±0.3	7.03
D3	Norethisterone acetate	500	direct injection	0.33	8.1±0.1	23.6±0.3	7.03
D4	Chlormadinone acetate	500	direct injection	0.43	8.2±0.2	28.2±0.5	3.02
D5	Cyproterone acetate	500	direct injection	0.33	8.1±0.1	23.6±0.3	7.03
D6	Medroxyprogesterone acetate	500	direct injection	0.43	8.2±0.2	28.2±0.5	3.02

4.2.4 Sampling of WWTP Effluents

The target PGs and their TPs were quantified in eight effluents from WWTPs in Germany. Details regarding the sample locations are provided in the Appendix C (Tab. C-2). All sampled WWTPs are equipped with conventional activated sludge treatment trains with denitrification and nitrification. For quantification and verification of PGs and TPs, grab samples were taken directly from the WWTP effluents and were immediately cooled with ice during the transport to the laboratory.

4.2.5 Analytical Methods

A hybrid quadrupole time of flight mass spectrometer (QTOF) (SCIEX TripleTOF 5600, Darmstadt, Germany) was used for TP identification. It was equipped with a DuoSpray ion source and a TurboIonSpray probe for electrospray ionization (ESI). TOF-MS mass resolution was at least 35,000 at 956 Da. Both polarization modes (negative ESI(-) and positive ESI(+)) were applied in separate measurements. The source parameters in positive and negative ionization mode were as follows (values for ESI(-) in parenthesis): ion source gas (GS) 1 and 2, 35 and 45 psi; curtain gas (CUR), 40 psi; source temperature (TEM) 500°C; ion spray voltage floating (ISVF), 5500 eV (-4500 eV); declustering potential (DP), 60 V (−100 V); ion release delay (IRD), 67 ms; ion release width (IRW), 25 ms. Full scan experiments (100–700 Da) were applied with an accumulation time of 0.2 s in the high sensitivity mode. Eight information dependent acquisition (IDA) experiments were included

to acquire MS² spectra (accumulation time: 0.05 s). The fragmentation conditions were as follows: mass range, 30 700 Da; CE, 40 eV (-40 eV); collision energy spread (CES), 15 eV (-15 eV).

The QTOF was automatically recalibrated after four runs using an automated calibrant delivery system (CDS) via atmospheric pressure chemical ionization (APCI).

For chromatography, a reversed-phase column was utilized (2 x 150 mm, 2.7 µm, MN Nucleoshell RP 18puls, Macherey-Nagel). The chromatographic conditions and the gradient used were previously reported in Weizel *et al.* (2020).

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 (R DEVELOPMENT CORE TEAM, 2008). More details regarding the data processing algorithm are provided in Dietrich and Ternes (2020, IN PREP.). Briefly, the XICs (extracted ion chromatogram) were extracted using the xcms package (SMITH ET AL., 2006). 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 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 the 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 retention times (RT), accurate masses and MS² fragmentations. In those cases, without commercially available standards, chemical structures of the TPs were proposed by characterizing the spectra and comparing these to the MS² fragmentation of related compounds obtained from other experiments in this study.

Quantitative analysis of the analytes was carried out with a TripleQuad-LIT-MS (triple quadrupole-linear ion trap mass spectrometer) (API 6500 QTrap, Sciex) utilizing the sMRM (scheduled multiple reaction monitoring) mode. For sample extraction, 500 mL of filtered WWTP effluent (<1µm glass fiber GF6, Whatman) was spiked with 2 ng of each isotope-labeled internal standard and enriched via SPE (6 mL, 500 mg, C18ec Chromabond, Macherey-Nagel). The elution of the target analytes was achieved with methanol. The extracts were then 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 re-dissolved 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 with 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 water before detection. The WWTP effluent samples were prepared within 24 h after sampling. More details regarding the chemical analysis used in this study can be found in Weizel *et al.* (2018).

A calibration curve ranging from 0.05 to 500 ng/mL was used for quantification. All standard solutions and mixes were dissolved in methanol and were diluted accordingly. Stable-isotope dilution analysis was used for the correction of ion-suppression. Recoveries were evaluated from spiked WWTP effluents (10 ng/L) in quadruplicate. The calculations of the limits of quantification (LOQs) were based on a signal-to-noise ratio of 10 either using the detected peaks in the non-spiked WWTP effluent samples or the peaks in the spiked samples. Noise area was selected manually from the baseline that bordered on the chromatographic peak. For the determination, the 3σ-values were used and were extrapolated accordingly. Results of the method performance are provided in the Appendix C (Tab. C-5).

4.3   Results and Discussion

4.3.1   Kinetical Behavior of Progestogens

As model substances, four 17α-hydroxyprogesterone derivatives (17α-hydroxy-progesterone, medroxyprogesterone acetate, chlormadinone acetate, and cyproterone acetate) and four 19-nortestosterone derivatives (dienogest, norethisterone acetate, etonogestrel, and levonorgestrel) were selected and analyzed. In addition, drospirenone, a spironolactone derivative used in hormonal contraception was considered as well.

The microbiological removal of PGs can be described by pseudo-first order kinetics (SCHWARZENBACH ET AL., 2005). The determined rate constants $k_{biol.}$ were normalized to the total concentration of suspended solids. The rate constants for the aerobic biodegradation ranged from 5.2 L/(g_{ss}*d) (cyproterone acetate) to 110 L/(g_{ss}*d) (drospirenone) (Tab. 4.3). For 17α-hydroxyprogesterone and norethisterone acetate, a very fast degradation was observed. Rate constants for these PGs were not calculated since only very few sampling points exhibited concentrations above the LOQ.

The concentrations of the PGs during the incubation experiments are shown in Fig. 4.1. The comparison of the sterile control and non-sterile experiments indicated that biodegradation

(e.g. transformation) was the major removal process since sorption on suspended solids was negligible (Fig. 4.1). Overall, PGs were degraded below their LOQs within six hours of incubation, except dienogest and cyproterone acetate. Both PGs were the most recalcitrant steroids in the study. However, DT_{50} values (dissipation time needed for 50% removal) of these were still below 12 h.

Regarding the comparison to literature studies of PGs, the kinetic behavior is consistent with previously reported results from a lab-scale degradation study (CHANG ET AL, 2011), since the calculated half-lives were in the same range. In addition, the concentrations in German WWTP effluents underscore the behavior of the analyzed PGs, as only dienogest and cyproterone acetate could be frequently detected in the effluents up to 4.4 ng/L and 3.7 ng/L, respectively (WEIZEL ET AL., 2018).

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, a few studies did report sporadic detections of these PGs (VULLIET ET AL., 2007; LIU ET AL., 2011A; LIU ET AL., 2014; PETROVIC ET AL., 2002). Endogenous PGs such as progesterone (not included in this study) or 17α-hydroxyprogesterone were frequently detected in WWTP effluents despite their high degradation rates (ZHANG ET AL., 2017; WEIZEL ET AL., 2018). These findings can be linked to the natural origin leading to much higher influent concentrations in comparison to other PGs (SCHÖNESHÖFER ET AL., 1986). This is also true for other steroid hormones, since Weizel *et al.* (2020) showed that hydrocortisone persists in WWTP effluents, despite the rapid microbial degradation of this compound.

Concerning cyproterone acetate, the knowledge that this compound can adversely impact fish at concentrations as low as 1 ng/L (SHARPE ET AL., 2004) and the observed moderate removal, it represents a risk for aquatic organisms in surface waters receiving WWTP effluent. This is also true for dienogest, though reliable data of its impact on aquatic biota are scarce (SCHMID ET AL., 2020; FENT, 2015; KUMAR ET AL., 2015).

Table 4.3. Overview of the kinetic results. PGs 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 ng/L, c_{ss} = 0.37 g_{ss}/L.

$k_{biol.}$: pseudo first-order rate constant of the biological degradation in contact with activated sludge

Type	Substance	$k_{biol.}$ [L/(g_{ss}·d)]	DT$_{50}$ [h]	Pearson coefficient
17α-Hydroxyprogesterone	17α-Hydroxyprogesterone	- [a)	<0.02	-
	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	- [a)	<0.02	-
	Levonorgestrel	94 ± 5	0.48 ± 0.03	0.999
	Etonogestrel	47 ± 2	0.96 ± 0.05	0.852
	Dienogest	9.9 ± 0.2	4.55 ± 0.11	0.998
Spironolactone	Drospirenone	110 ± 10	<0.02	0.920

[a) Not enough points above LOQ for calculation. Minimum of points for calculation was set to 3.
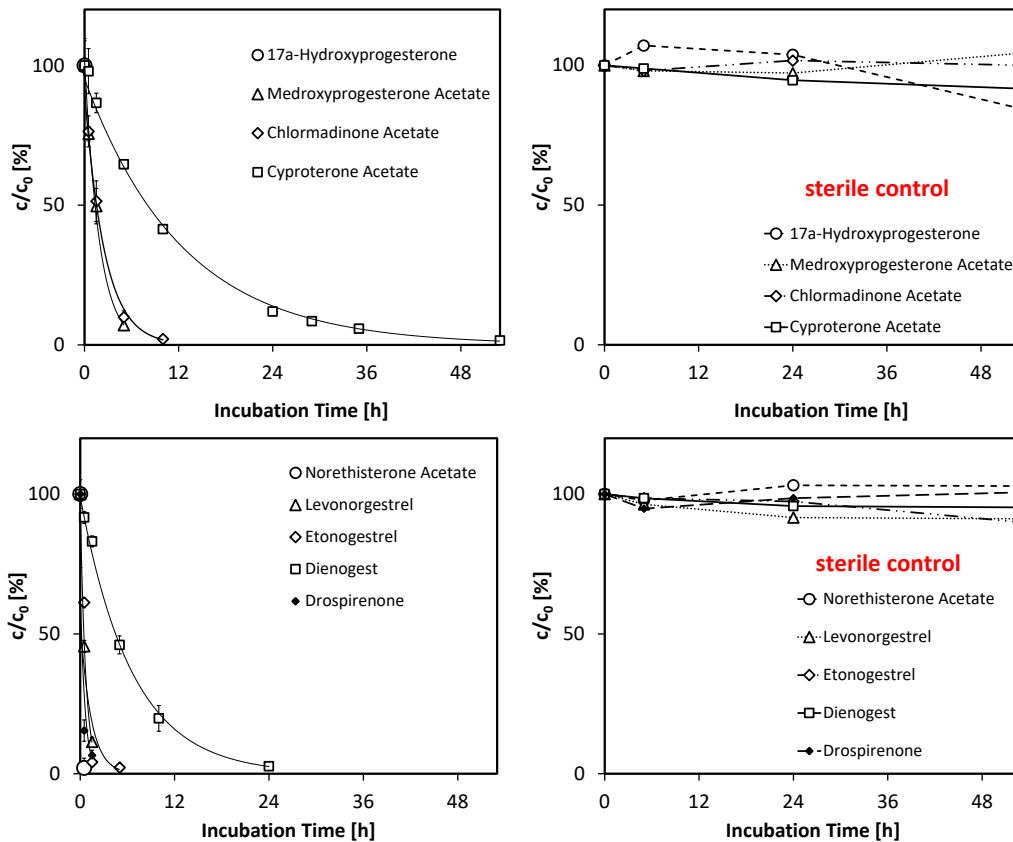
Figure 4.1. Degradation of (**a**) 17α-Hydroxyprogesterone derivatives and (**b**) 19-nortestosterone derivatives from experiments K and K$_{sterile}$ expressed as the percentage of the total concentration at t$_0$ as a function of the incubation time with activated sludge. Experimental conditions were as follows: n=3, c_0 = 200 ng/L, c_{ss}= 0.37 g_{ss}/L. The fitted lines in the regular experiment (left) indicate the exponential degradation according to the pseudo-first-order kinetic model. Data points below the LOQs were removed. The error bars represent the standard deviation of the replicates. Results of the corresponding autoclaved control experiments (sterile) are shown on the right.

4.3.2 Fate of 17α-Hydroxyprogesterone Derivatives

Three 17α-acetate esters (chlormadinone acetate, medroxyprogesterone acetate, cyproterone acetate) were used as steroid substrates for the degradation study. An overview of the detected TPs is provided in the Appendix C (Tab. C-3). It should be noted that due to the high initial concentrations used for the TP identification experiments (500 µg/L), numerous minor TPs were detected as well. Therefore, only the main TPs are discussed in the following sections. Moreover, a consistent terminology was used for the TPs, consisting of their monoisotopic mass and retention time.

During the biodegradation of medroxyprogesterone acetate 18 TPs were detected, of which two of them were confirmed by the measurement of authentic reference standards (TP402_18.1, TP386_21.3). Chlormadinone acetate showed the formation of 21 TPs. One of these was unambiguously identified as the active steroid delmadinone acetate (TP402_20.5) via a reference standard. For cyproterone acetate, 10 TPs were detected.

The main identified TPs of medroxyprogesterone acetate were the Δ^1-dehydro TP (TP384_20.4) and the Δ^1-isomer (TP386_21.3) (Fig. 4.2 a). Similar TPs (TP402_20.6 and TP404_21.0) were found for chlormadinone acetate (Fig. 4.2 b). In addition to (de)hydrogenation of the carbon double-bonds, the reduction of the 3-keto moiety was observed for chlormadinone acetate, which led to the considerable formation of TP404_20.2 and TP406_20.7. These TPs were tentatively proposed as the 3α-hydroxy derivatives of TP402_20.6 and TP404_21.0, while cyproterone acetate showed only the formation of the main TP 3α-hydroxy cyproterone acetate (TP418_20.4) to an appreciable extent (Fig. 2c). The formation of 3β-hydroxy chlormadinone acetate can be excluded, as confirmed by the measurement of a reference standard (Fig. 4.2 b). Since the stereochemistry of the C3-hydroxy group is crucial for the activity and binding affinity of steroids (Sᴄʜɴᴇɪᴅᴇʀ ᴇᴛ ᴀʟ., 2009), the verification of the chlormadinone TPs and other proposed TPs should be a subject of further research.

As stated, TP418_20.4 (Fig. 4.2 c) was found as the major TP of cyproterone acetate. The elevated formation of this TP indicates that the treatment with activated sludge does not eliminate the endocrine activity, since C3-reduced TPs of the 17α-hydroxyprogesterone derivatives are known to still be endocrine active (Sᴄʜɴᴇɪᴅᴇʀ ᴇᴛ ᴀʟ., 2009). In addition, 3α-hydroxy steroids (TP404_20.2 and TP406_20.7, TP418_20.4 and TP420_20.6) can bind covalently to DNA leading to the formation of DNA adducts and pronounced genotoxicity (Kᴇʀᴅᴀʀ ᴇᴛ ᴀʟ., 1995; Mᴀʀᴛᴇʟʟɪ ᴇᴛ ᴀʟ., 1996; Kᴀsᴘᴇʀ, 2001). However, of the identified TPs only a few of them exhibited more than 10% of the initial peak area of their parent steroid at t_0.

Based on the obtained results, we proposed initial degradation pathways for the three analyzed 17α-hydroxyprogesterone derivatives Fig. 4.3.
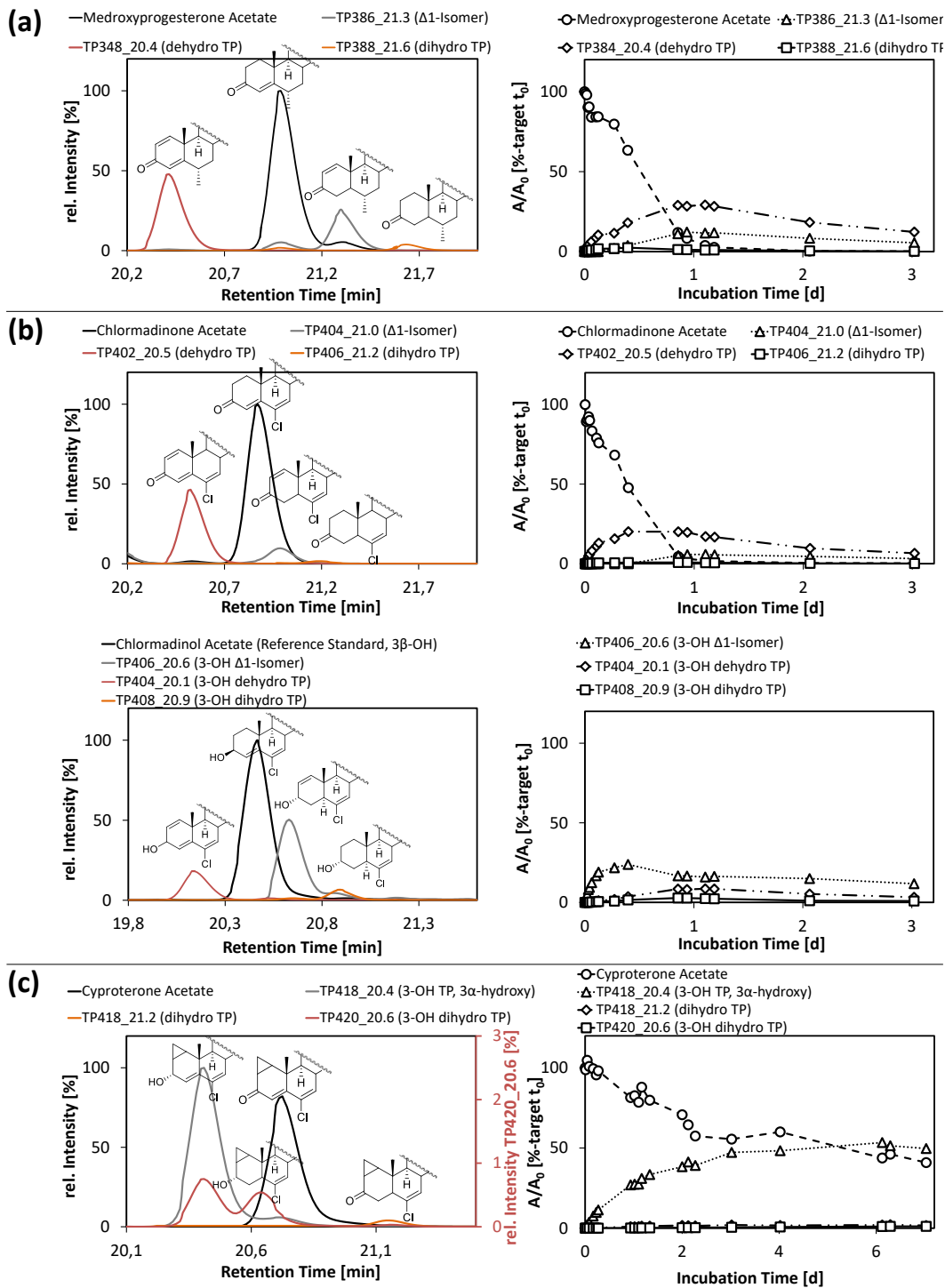
Figure 4.2. Chromatographic separation and time courses of the (de)hydrogenated TPs detected during the incubation of a) medroxyprogesterone acetate (D6) b) chlormadinone acetate (D4) and c) cyproterone acetate (D5) in contact with activated sludge. Extracted ion chromatograms represent the individual m/z of the steroids detected with LC-QTOF in ESI(+).

(a)

major TP

Medroxyprogesterone Acetate
mz: 387.2537 Da
RT: 20.99 min

TP384_20.4
mz: 385.2378 Da
RT: 20.42 min

TP386_21.3
mz: 387.2538 Da
RT: 21.29 min

TP388_21.6
mz: 389.2690 Da
RT: 21.64 min

(b)

major TP

Chlormadinone Acetate
mz: 405.1830 Da
RT: 20.89 min

TP402_20.5 (Delmadinone Acetate)
mz: 403.1677 Da
RT: 20.52 min

TP404_20.1
mz: 405.1834 Da
RT: 20.14 min

TP404_21.0
mz: 405.1834 Da
RT: 20.98 min

TP406_21.2
mz: 407.1975 Da
RT: 21.18 min

TP408_20.9
mz: 409.2131 Da
RT: 20.89 min

major TP

TP406_20.6
mz: 407.1988 Da
RT: 20.63 min

(c)

Cyproterone Acetate
mz: 417.1836 Da
RT: 20.72 min

(not detected)

(not detected)

(not detected)

(not detected)

TP418_21.2
mz: 419.1989 Da
RT: 21.16 min

TP420_20.6
mz: 421.2136 Da
RT: 20.64 min

major TP

TP418_20.4
mz: 419.1990 Da
RT: 20.41 min

Figure 4.3. Proposed initial degradation pathways of (a) medroxyprogesterone acetate, (b) chlormadinone acetate and (c) cyproterone acetate. Structures highlighted in grey were confirmed via authentic reference standard.

Overall, the comparative evaluation of the primary TPs permits some interesting conclusions. The Δ^1-dehydrogenation was significant for medroxyprogesterone as well as for chlormadinone, while it was completely inhibited for cyproterone as the initial reaction. Hence, the cyclopropane moiety in cyproterone leads strictly to the inhibition of the Δ^1-double bond formation, most likely due to the extended ring tension. The 3-keto reduction to a hydroxyl group was significant when a Δ^6-double bond (as well as chlorine at C6) was present.

Martelli *et al.* (1996) proposed similar structure relationships for the observed reactivity of cyproerone acetate, chlormadinone acetate and megestrol acetate with DNA, due to the different extent of metabolic 3-keto reduction. Both results in combination, suggest that the activated sludge treatment of megestrol acetate (not included in this study) might lead to a lesser extent of 3-keto reduction and a higher rate of Δ^1-dehydrogenation, as observed for chlormadinone acetate in this study. The specific structural moiety responsible for the 3-keto reduction of steroids is most likely the Δ^6-double bond. This hypothesis should be addressed in further degradation studies.

The ring A (de)hydrogenations of progesterone derivatives varied significantly from those observed for glucocorticoids (WEIZEL ET AL., 2020), since glucocorticoids were preferably reduced at the Δ^4-double bond in α-position. As shown in Fig. 4.2, Δ^4-hydrogenated TPs were detected, but with less intensity than the dehydrogenated TPs. This behavior might be explained by the inhibitory effect of C6 substituents on the reduction of the Δ^4-double bond as described by Kuhl (2011). Hydrogenation in α-position is hampered by the steric configuration (e.g. C6α-methyl for medroxyprogesterone). The hydrolysis of the 17α-ester during the incubation with activated sludge was not observed. Recently it has been shown, that the ester cleavage at position C17 occurs via isomerization, while direct hydrolysis is sterically hindered at this position (WEIZEL ET AL., 2020). Thus, the ester hydrolysis can be excluded as a major degradation reaction in the biological wastewater treatment of such PGs.

In addition, several minor hydroxylated TPs have been identified. The hydroxylation was not selective, since several isomers were detected for the analyzed steroids (Fig. C-3). The hydroxylated TPs were formed from all primary (de)hydrogenated TPs and *vice versa*. These TPs can be expected as intermediates in the biodegradation route of steroids which can be further transformed into carboxylates and compounds with a broken steroid skeleton (e.g. 9,10-seco pathway). Within the evaluation of the results, minor TPs containing a carboxylic moiety were indicated by the characteristic loss of carbon dioxide during MS² fragmentation.

In addition, dechlorinated TPs of chlormadinone acetate and cyproterone acetate were identified. Since these TPs were formed to a small degree, dechlorination seems not to be a significant degradation pathway of chlormadinone acetate and cyproterone acetate in microbiological wastewater treatment. However, the dechlorination of cyproterone acetate was previously reported in fish tissue (LIU ET AL., 2019).

The elucidation of the exact structures of these secondary, tertiary, or higher TPs was out of the scope of this study since our work focused on the main primary TPs which are formed to an appreciable degree.

4.3.3   Fate of 19-Nortestosterone Derivatives

Steroids of this type are characterized by a 17α-ethinyl group (cyanomethyl in case of dienogest) and the missing methyl group at C19. The identified TPs of the 19-nortestosterone derivatives dienogest, norethisterone acetate, and etonogestrel are summarized in the Appendix C (Tab. C-4).

Three main TPs (TP309_11.9, TP309_16.4 and TP327_13.0) were detected for dienogest (Fig. 4.4 a). Further 20 TPs were detected with a maximum formation of less than 5% of the initial peak area of dienogest. As shown in Fig. 4.4 b etonogestrel was degraded to four main TPs (TP326_20.8, TP324_20.6, TP344_16.4, and TP374_15.7). Here, 22 minor TPs with maximum peak areas below 5% of the initial peak area of the precursor were tentatively identified. The degradation of norethisterone acetate revealed the formation of three major TPs (norethisterone, TP300_19.8, TP318_13.8, Fig. 4.4 c) and further 30 TPs with less than 5% of the initial peak area of the precursor.

The main TPs observed for all analyzed model compounds were 5α-hydrogenated derivatives (TP326_20.8, TP300_19.8) and also hydroxylated TPs of the parent steroids (TP327_13.0, TP344_16.4, TP318_13.8) were detected.

Moreover, the large number of different TPs emphasized that many transformation reactions are responsible for the removal of the steroids during wastewater treatment with activated sludge. For instance, the insertion of one or more hydroxyl groups was not regioselective, since in those cases several isomers could be detected and thus numerous TPs were formed (Fig. C-4). Similar to the 17α-hydroxy progesterone derivatives, several carboxylates were tentatively identified (Fig. C-2) by their characteristic loss of carbon dioxide in the MS² fragmentation.

In the lab-scale experiments, norethisterone acetate (prodrug) was rapidly hydrolyzed to its active human metabolite norethisterone (TP298_18.3) (KUHL, 2011). In contrast to the 17α-acetate esters discussed in Section 4.3.2, the 17β-position is favored for the initial ester hydrolysis. On the other hand, the 17α-ethinyl and the nitrile group of dienogest were not degraded, as was already consistently reported (CIRJA ET AL., 2007; CAJTHAML ET AL., 2009).

In addition, the initial transformation via Δ^4-hydrogenation was confirmed, since TP300_19.8 was identified as 5α-dihydro norethisterone via the measurement of an authentic reference standard. This TP is known to interact with the progestogen and estrogen receptors (LARREA ET AL., 2001). Analogous to the confirmed results of norethisterone acetate, the major TPs of etonogestrel was proposed as the Δ^4-hydrogenated product (TP326_20.8) and the Δ^1-isomer of etonogestrel (TP324_20.6). Final confirmation of the etonogestrel TPs was impossible since reference compounds were not available. Recently, similar transformation was found during the microbial degradation of levonorgestrel in soil (TANG ET AL., 2019), activated sludge (LIU ET AL., 2013) and by freshwater microalgae (PENG ET AL., 2014).

Although tetrahydrogenated products (3α,5α/3β,5α-tetrahydro TPs) are known as human metabolites (LEMUS ET AL., 2009), such TPs were not detected in our experiments.

In addition, the Δ^1-dehydrogenated derivatives of norethisterone (TP296_17.5) and etonogestrel (TP322_19.6) were detected to a lesser extent (< 5%). The behavior was consistent with the observed (de)hydrogenations of glucocorticoids (WEIZEL ET AL., 2020).
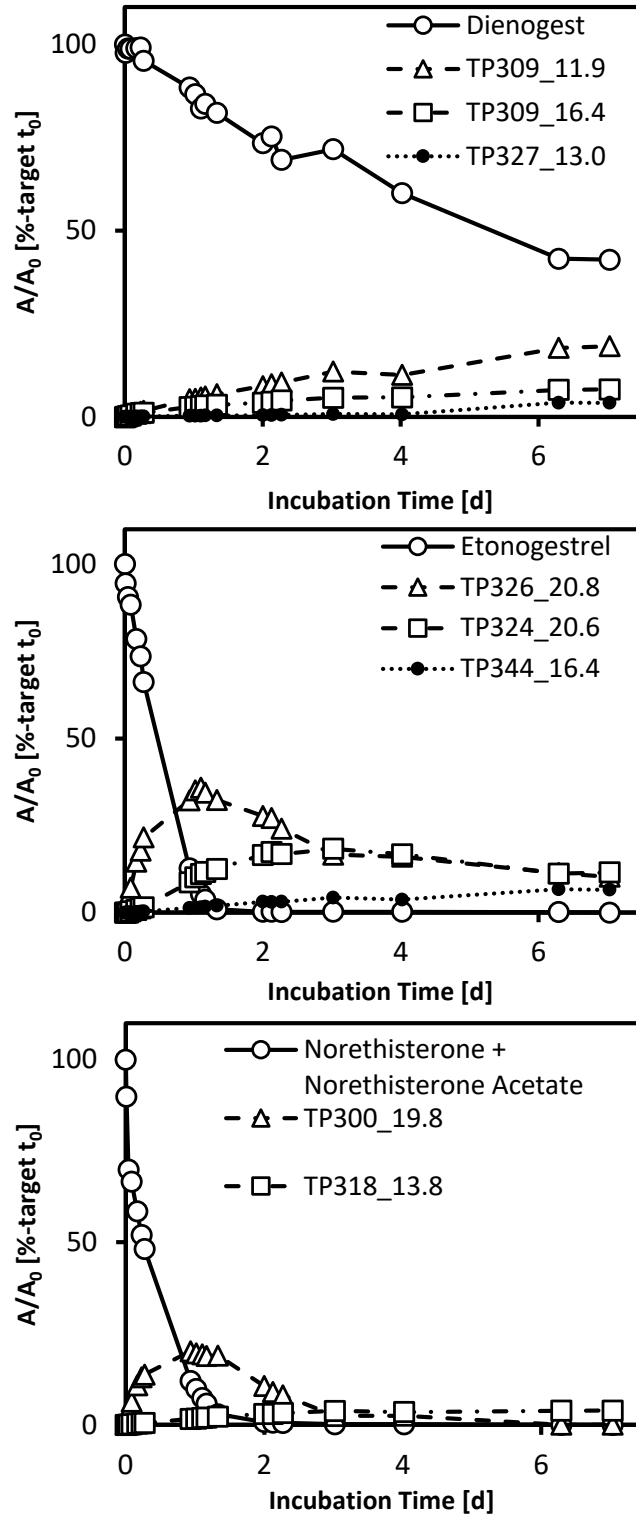
Figure 4.4. Time courses of the degradation and formation of the main TPs detected expressed as the percentage of the parent steroid peak area at t_0 during the incubation of 19-nortestosterone derivatives with activated sludge found in experiments D1-D3.

4.3.4   Aromatization of Ring A

In addition to the TPs mentioned above, the formation of an aromatized ring A was found for all 19-nortestosterone steroids. The elucidation of the TPs revealed the formation of 17α-ethinylestradiol (TP296_18.5, EE2) by the aromatization of norethisterone (Fig. 4.5), which was unambiguously confirmed via its reference standard. For dienogest and etonogestrel, similar TPs were detected (TP309_16.4, TP322_20.0, Fig. C-5) and thus the obtained results indicate that 19-nortestosterone steroids can be converted to highly potent estrogenic steroids during biological wastewater treatment.

Moreover, EE2 reached concentrations as high as 7% (after 24 h) of the initial concentration of the precursor. This suggests that the transformation of 0.5 ng/L norethisterone can lead to the formation of 0.035 ng/L EE2 (worst-case). Concerning the proposed European environmental quality standard of the Water Framework Directive (EU DECISION, 2015) for EE2 (0.035 ng/L), the biotransformation of elevated norethisterone concentrations might be significant for environmental EE2 concentrations and especially when determining the removal of EE2 in WWTPs.
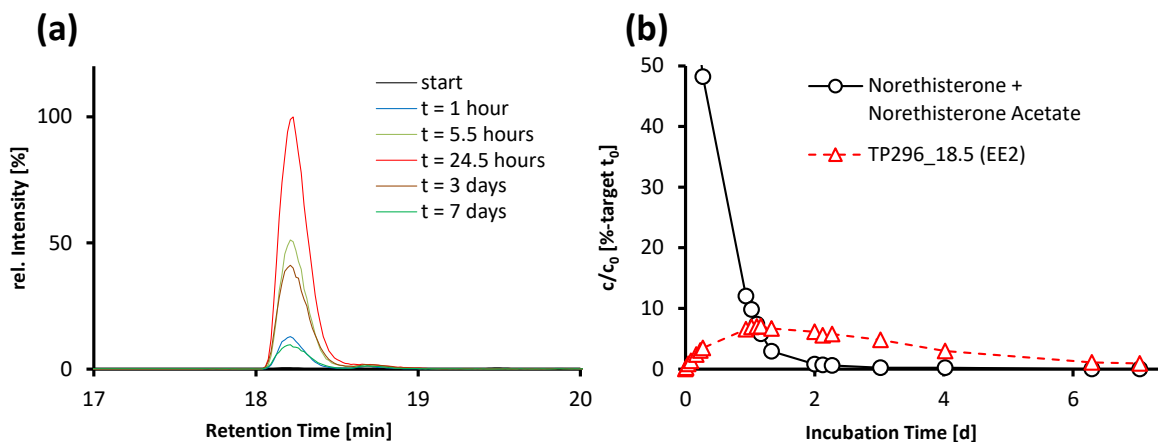
Figure 4.5. Aromatization of norethisterone (acetate) in contact with activated sludge found in experiment D3 verified by quantitative LC-MS/MS analysis (method details are provided in the appendix C). (a) The relative intensity of 17α-ethinylestradiol (TP296_18.5, EE2) depending on the incubation time. (b) Time course of the relative EE2 concentration during the incubation.

The aromatization leading to estrogen-like TPs was not detected for the 17α-hydroxyprogesterone derivatives. Due to the C19-methyl group, an oxidative elimination of the methyl group is needed for the formation of the phenolic ring A. The

enzymatically mediated conversion of C19-methyl steroids into estrogens requires specific enzymes such as aromatase *cyp19* (KAO ET AL., 2001). In the case of 19-nortestosterone derivatives, dehydrogenation followed by enolisation of the 3-oxo moiety leads to the formation of the phenolic ring A. This transformation reaction can likely be mediated by bacteria from activated sludge as it was identified in this study. 19-Nortestosterone derivatives were found to metabolize to estrogens in the liver of human adults without the presence of aromatase. Thus, a similar mechanism of the aromatization was assumed (KUHNZ, 1997, KUHL AND WIEGRATZ, 2007).

In addition, sulfate conjugates of the corresponding aromatized TPs were detected (TP376_14.6, TP389_12.9, TP402_16.7) which is consistent with the reported behavior of EE2 and other estrogens during activated sludge treatment (YI AND HARPER, 2007). The consideration of the fragmentation spectra (MS² spectra are provided in the Appendix C) indicated that the sulfate groups are bonded to the aromatized moiety (phenolic sulfate conjugate). Thus, these findings indicated the formation of an aromatized site, as only the cleavage of $SO_3^{\cdot-}$ (m/z: 79.9568 Da) could be observed without the presence of HSO_4^- (m/z: 96.9596 Da) which is characteristic for phenolic sulfate conjugates (WEIDOLF ET AL., 1988). These results supported the proposed structure for the estrogen-like TPs indirectly. Based on the identified TPs, an initial degradation pathway was proposed and illustrated exemplarily for norethisterone acetate in Fig. 4.6.
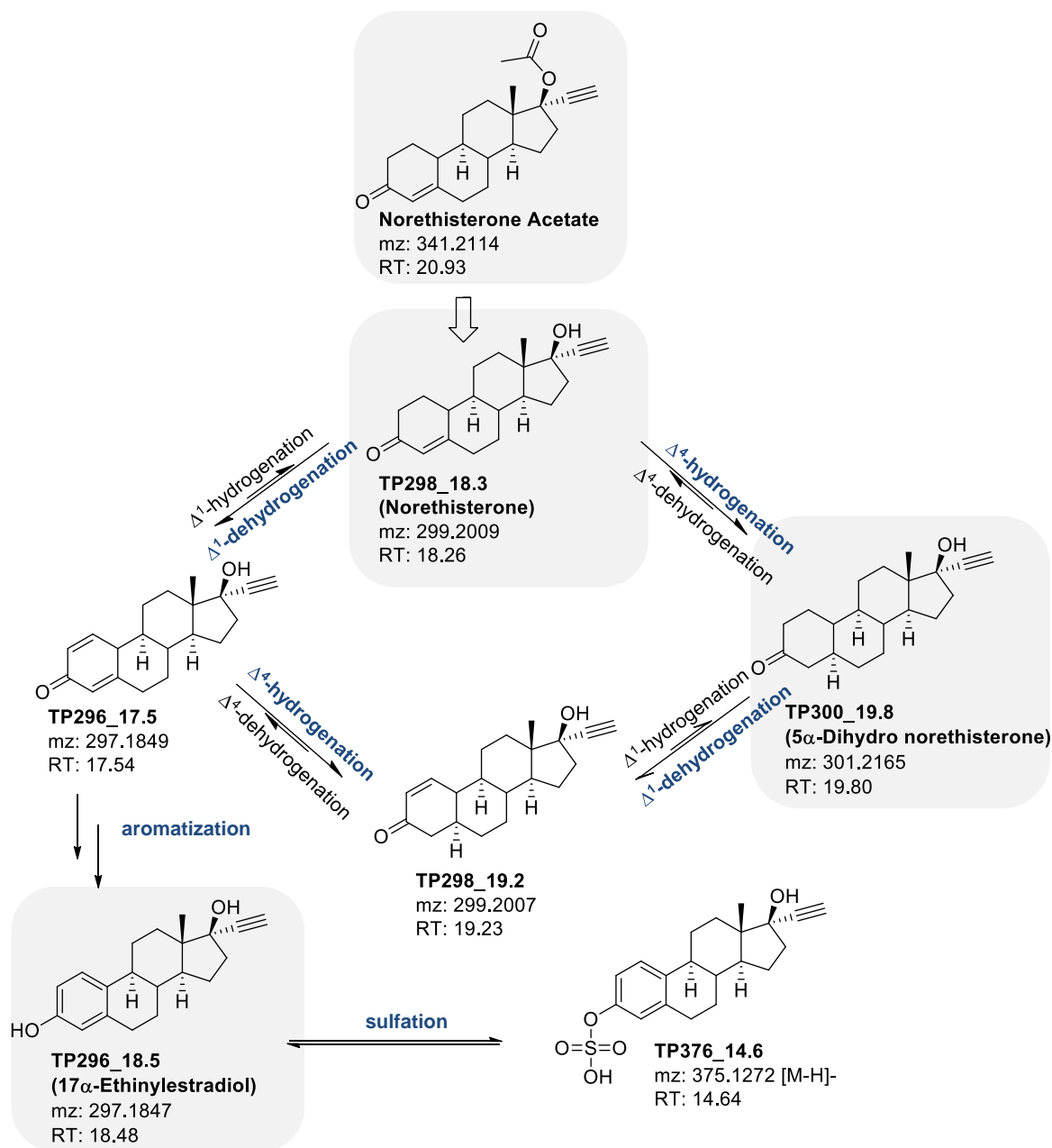
Figure 4.6. Scheme of the proposed initial degradation pathway of norethisterone acetate in contact with activated sludge. Structures highlighted in grey were confirmed by the measurement of their reference standards.

4.3.5 Occurrence of PGs and their TPs in WWTP Effluents

The occurrence of the PGs from Tab. 4.1 and their main TPs was analyzed in eight WWTP effluents. Before the analysis, recoveries and detection limits were determined for the targeted steroids. Results of the method performance are provided in the Appendix C (Tab. C-5). The recoveries ranged between 84% (TP388_21.6) and 115% (drospirenone), while the LOQs ranged from 0.3 ng/L (dienogest) to 5.6 ng/L (chlormadinol

acetate). As recently indicated (WEIZEL ET AL., 2018), cyproterone acetate and dienogest were ubiquitously detected in the WWTP effluents (Tab. 4.4). In addition to these, chlormadinone acetate (3/8) and endogenous 17α-hydroxyprogesterone (8/8) were detected most often. These results are consistent with the relative consumption of PGs in Germany (Tab. 4.1) as dienogest and cyproterone acetate have the highest consumption levels. Furthermore, both compounds were only moderately degraded in the lab experiments. As a consequence, relatively high concentrations were found in German WWTP effluents.

The cyproterone acetate concentrations were above 7 ng/L in two WWTP effluents and dienogest was detected up to 3.7 ng/L. Especially the high cyproterone acetate concentrations are of ecological relevance since the concentrations were significantly higher than the compound's lowest observed effect concentration of 1 ng/L (SHARPE ET AL., 2004).

In addition, the screening for the identified TPs revealed the occurrence of two dienogest and one cyproterone acetate TP. TPs of other PGs were not detected, likely due to the low consumption of these steroids in Germany. The 3-oxo reduced TP of cyproterone acetate (TP418_20.4) was detected in all WWTP effluents. An estimation of the concentration using the cyproterone acetate calibration revealed elevated concentrations up to 8.1 ng/L (Fig. C-6). TPs of dienogest (TP309_16.4 and TP327_11.3) were detected in all WWTP effluents. The 6β-hydroxy TP (TP327_11.3) was found with concentrations up to 1.6 ng/L. Since 6β-hydroxy dienogest is a human as well the observation that only a limited quantity of this TP was formed in the degradation experiments, its occurrence might be attributed to the incomplete removal in the WWTPs rather than by its formation.

TP309_16.4 was tentatively identified as the ring A aromatized TP of dienogest with an estrogen-like structure. This TP was detected in all WWTP effluents. Similar to the TP of cyproterone acetate, an estimation of the concentration was made via the calibration of dienogest. This approach revealed partially very high concentrations up to 16 ng/L and concentrations of TP309_16.4 were in all cases higher than those of dienogest (Fig. C-6). However, an accurate quantification of the detected TPs has to be addressed in further research.

Table 4.4. Concentrations of PGs and 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 and TPs	LOQ [ng/L]	n > LOQ	Median [ng/L]	Minimum [ng/L]	Maximum [ng/L]
17α-Hydroxyprogesterone	0.4	8	0.6	0.4	1.0
Medroxyprogesterone Acetate	0.6	0	<0.6	<0.6	<0.6
TP388_21.6	3.8	0	<3.8	<3.8	<3.8
Chlormadinone Acetate	0.4	3	0.5	0.4	0.7
Delmadinone Acetate	0.9	0	<0.9	<0.9	<0.9
Chlormadinol Acetate	5.6	0	<5.6	<5.6	<5.6
Cyproterone Acetate	1.0	8	3.8	1.2	7.7
TP418_20.4 [a]	-	8	2.6[a]	0.3[a]	8.1[a]
Dienogest	0.3	8	1.2	0.3	3.7
TP327_11.3	0.5	8	1.2	0.6	1.6
TP309_16.4 [a]	-	8	6.7[a]	1.2[a]	16[a]
Norethisterone Acetate	1.0	0	<1.0	<1.0	<1.0
Norethisterone	1.0	0	<1.0	<1.0	<1.0
TP300_19.8 (5α-dihydro)	1.2	0	<1.2	<1.2	<1.2
TP296_18.5 (EE2)	2.9	0	<2.9	<2.9	<2.9
Etonogestrel	1.2	0	<1.2	<1.2	<1.2
Levonorgestrel	1.0	0	<1.0	<1.0	<1.0
Drospirenone	0.8	0	<0.8	<0.8	<0.8

[a] Precursor steroids were used for an estimation of the concentration

4.4 Conclusions

- The results of PG analysis enabled new insights into the structure/stability relationship of synthetic steroids.

- 17α-hydroxyprogesterone derivatives were degraded initially at ring A via carbon double bond (de)hydrogenations. A Δ^6-double bond promotes 3-keto reduction, which was particularly pronounced for cyproterone acetate due to the inhibition of the Δ^1-dehydrogenation by the cyclopropane moiety. 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.

- There is evidence that many TPs are still biologically active. Thus, TP formation must be taken into account for the environmental risk profile. These outcomes reinforce that it is mandatory to consider TP formation of steroids as an assessment criterion for industrial discharge permissions into municipal WWTPs with biological treatment trains.

- Most PGs were degraded within six hours in contact with activated sludge. Only cyproterone acetate and dienogest showed moderate stability (half-lives of 8.65 h and 4.55 h).

- 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 anthropogenically induced endocrine activity in the environment.

- Detected concentrations of PGs and TPs constitute a risk for aquatic organisms and their reproductive system.

5 Final Conclusions

Most research addressing steroid hormones in the environment focuses on estrogens and androgens, while steroid types from corticosteroids and progestogens getting only minor attention in disciplines of environmental research so far. Their consumption volumes and ability to interact with the endocrine system of aquatic organisms at very low concentrations are arguable concerns for the need of the evaluation of environmental distributions and ecotoxicological risks for biota. The current thesis complemented existing knowledge and provided new information on the occurrence, the fate, and the behavior of corticosteroids and progestogens in the aquatic environment.

For the first goal of the thesis, a robust and highly sensitive analytical method was developed in order to simultaneously determine the occurrence of around 60 mineralocorticoids, glucocorticoids and progestogens in the aquatic environment. Some analytical challenges have been approved by individual approaches regarding to sensitivity enhancement and compound stabilities. These results may be important for further research in environmental analysis of steroid hormones. Reliable and low quantification limits are the perquisite for the determination of corticosteroids and progestogens at relevant concentrations due to low consumption volumes and simultaneously low effect-based trigger values. Achieved quantification limits for all target analytes ranged between 0.02 ng/L and 0.5 ng/L in surface water and 0.05 ng/L to 5 ng/L in WWTP effluents. This sensitivity enabled the detection of three mineralocorticoids, 23 glucocorticoids and 10 progestogens within the sampling campaign around Germany. Many of them were detected for the first time in the environment. This in-depth steroid screening provided furthermore a good overview of single steroid burden and allowed the identification of predominantly steroids of each steroid type analyzed. Elevated concentrations of the active spironolactone metabolites canrenone (up to 19 ng/L) and 7α-thiomethyl spironolactone (up to 2.3 ng/L) were found in all WWTP effluents, suggesting their frequently occurrences in the environment.

Moreover, the broad range of analyzed glucocorticoids confirmed recent findings and provided new insights into occurrences of further potent steroid pollutants, such as mometasone furoate and 6β-hydroxy triamcinolone acetonide. High effluent concentrations of triamcinolone acetonide (up to 28 ng/L) and clobetasol propionate (up to 5.4 ng/L) were confirmed to be similar in German WWTPs. Both compounds were also found in 91% and 55% (n=22) of the surface waters analyzed. Our results suggest furthermore that monoesters of betamethasone and 6α-methylprednisolone must be considered in target

analysis and risk evaluation, since they showed elevated concentrations in WWTP effluents (e.g. up to 4.2 ng/L for 6α-methylprednisolone propionate). Upon now, it was assumed that such monoesters (betamethasone 17-propionate, betamethasone 17-valerate, and 6α-methylprednisolone 17-propionate) hydrolyze during the wastewater treatment and thus they were frequently not considered in the chemical analysis as well as ecotoxicological evaluation. This is a major gap, since it can be assumed that the dynamics of the uptake into cell membranes of fish are accelerated for esterified compounds in comparison to the corresponding alcohols. Our study suggests that in WWTP effluents betamethasone monoester concentrations (sum of propionate and valerate, 1.5 – 5.8 ng/L) are significantly higher than those of the hydrolysis product betamethasone (0.05 – 0.6 ng/L), although the betamethasone concentrations are very similar to other results.

Our study also showed the ubiquitous occurrences of the progestogens dienogest (up to 4.4 ng/L) and cyproterone acetate (up to 3.7 ng/L) in WWTP effluents and receiving surface waters, thus highlighted the presence of such potent synthetic sex steroids above their effect concentrations. In a national context, these are the very first results identifying the predominant progestogens in the environment.

Overall, our study revealed reliable environmental data of poorly or even not analyzed steroids. The results complement the existing knowledge in this field but also provide new information which can be used particularly for compound prioritization in ecotoxicological research and environmental analysis.

Further goals of this thesis were derived after the evaluation of the data obtained from the monitoring campaign and have been addressed the biodegradation of glucocorticoids and progestogens. Experiments were designed to enable the comparison of the biodegradability and transformation processes of structure-related steroids during activated sludge treatment under standardized experimental conditions.

In the first experiments, the kinetic behavior of 13 glucocorticoids has been investigated in lab-scaled aerated activated sludge slurries. The compounds were accurately selected to cover manifold structural moieties of commonly used synthetic glucocorticoids, including non-halogenated and halogenated steroids, their mono- and diesters, and several acetonide-type steroids. This approach allowed a structure-based interpretation of the results.

The obtained rate constants (from 10^2 to 10^{-2} L/(g_{ss}*d)) were found to vary over four orders of magnitude, and thus suggesting great variations in the biodegradability (half-lifes ranged from < 0.5 h to > 14 d). Related to the structures these results suggested increasing stability

in the order non-halogenated steroids (e.g. hydrocortisone), < 9α-halogenated steroids (e.g. betamethasone), < C17-monoesters (e.g. betamethasone 17-valerate, clobetasol propionate), << acetonides (e.g. triamcinolone acetonide). Some fundamental explanations for this behavior have been received by the elucidation of the TPs formed for 12 glucocorticoids.

We showed that steroids containing substituents in ring B (betamethasone, beclomethasone, 6α-methyl-prednisolone), were primarily degraded via oxidative side-chain degradation, since 17-oxo/17-hydroxy TPs have been detected to significant extents. In contrast, for non-halogenated steroids (hydrocortisone, prednisolone) similar TPs were not detected, leading to the conclusion that both groups were degraded following different pathways. We hypothesized that the *9,10-seco* pathway, which initially starts with C9-hydroxylation, is inhibited due to the substituents in ring B and results to lower degradation rates. The degradation experiments demonstrated furthermore that there is no evidence for the direct hydrolysis of the 17α-ester group, while C21-esters have been found to degrade rapidly through microbial mediated ester hydrolysis. This behavior was underlined by different investigations of 17α-monoester derivatives and in addition one mixed diester (6α-methylprednisolone 17-propionate 21-acetate). By the comparison of experiments, it was concluded that 17α-esters undergo migration to the hydroxylic group at C21, leading to the formation of 21-monoesters. Hence, ester hydrolysis is not found for monoesters without a hydroxylic group at this side, as the case for clobetasol propionate and fluticasone propionate. This assumption was supported by the fact that there was no detectable 6α-methylprednisolone 21-acetate formation at any time, while 17-propionate and 21-propionate were the major primary TPs during the degradation of the corresponding mixed diester. Another interesting observation for monoesters was made by the comparison of abiotic (autoclaved activated sludge slurries) and regularly experiments as those results suggested that ester migration is not driven by microbial activity, while the ester hydrolysis at C21 was found to be enhanced in biologically active experiments. This behavior most likely leading to the observed higher stability of C17-monoester in comparison to the corresponding alcohols.

Moreover, since acetonide steroids showed pronounced stabilities, further experiments were conducted to elucidate their TPs. The experiments (budesonide, fluocinolone acetonide, triamcinolone acetonide) showed the formation of 21-carboxylic acids, which are likely formed via oxidative hydroxylation at C21. However, the data suggested that particularly the fluorinated acetonides were recalcitrant in activated sludge treatment. Besides the common transformation reactions identified within glucocorticoid types

mentioned above, more specific reactions were observed, such as the hydrolysis of fluticasone propionate to its 17β-carboxylic acid.

Within the third research topic, the strategy for glucocorticoids was applied on progestogens. Here we selected two types of progestogenic steroids frequently used in hormonal contraception and analyzed the kinetical behavior (nine steroids) as well as their fate (six steroids) during activated sludge treatment. The fast and complete degradation within 6 h suggested pronounced biodegradability for progestogens (k_{biol}: 25 to > 110 L/(g$_{ss}$*d)). However, two compounds have been found to stuck out since they revealed lower degradation rates, suggesting only moderate removals for both, cyproterone acetate and dienogest (5.2 and 9.9 L/(g$_{ss}$*d)).

The elucidation of TPs again revealed some crucial information regarding the observed behavior. Experimental results for three 17α-hydroxyprogesterone type progestogens carried out that esters in α-position do not hydrolyze directly since hydrolysis products were not detected for such type of steroids. This is consistent with our observations made during the biodegradation of glucocorticoid esters. The initial degradation pathway was found to starts by ring A dehydrogenation and hydrogenation, where the Δ^1-dehydro TP was dominant besides the formation of Δ^1-dehydro-Δ^4-dihydro and Δ^4-dihydro TP to lesser extent. Interestingly, such likelihood of saturation and desaturation of carbon double bonds have been observed also for glucocorticoids.

In addition, the structure-based interpretation suggested a reduction of the 3-keto moiety when steroids contain a Δ^6-carbon double bond. This was the case for chlormadinone acetate and particularly for cyproterone acetate but not for medroxyprogesterone acetate (no Δ^6-carbon double bond). It was concluded that Δ^1-dehydrogenation is inhibited strictly due to the cyclopropane moiety between C1 and C2 in cyproterone acetate, leading to an almost quantitatively reduction of the 3-keto moiety. Such behavior could explain its higher stability in comparison to the other analyzed compounds. Overall, these experiments highlighted the formation of elevated quantities of TPs which have the potential to possess endocrine-related functions in biota, both the 3-hydroxy TPs as well as the Δ^1-dehydro TPs.

In complementation, the fate of three 19-nortestosterone type progestogens (dienogest, etonogestrel, norethisterone) was analyzed. Although the results indicated saturation/desaturation at ring A similar to those of 17α-hydroxyprogesterone derivatives, one structure specific transformation reaction was identified. Our results carried out that progestogens from this group are able to undergo aromatization at ring A in contact with activated sludge, thus leading to the formation of estrogen-like TPs. In the case of

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norethisterone, we confirmed the formation of 17α-ethinylestradiol. We further attributed the comparatively low formation of the Δ^1-dehydrogenated TPs during these experiments to the possibility of aromatization of the 19-nortestosterone derivatives. Therefore, it was hypothesized that Δ^1-dehydro TPs consecutively enolized to the corresponding estrogen-like TPs due to their missing methyl group at C10. The results have been marked an unknown source for estrogens, particularly for 17α-ethinylestradiol for the first time.

Moreover, the frequently detection of several of the identified TPs in full-scale plants (6 TPs) confirmed the transferability of the lab-scale degradation experiments. These findings highlighted that WWTPs are point sources of many steroids and TPs with intact steroid core. As a consequence, such compounds are able to cause adverse effects in the aquatic environment due to their ability to interact multitude with the endocrine system. The study furthermore shows that the majority of the analyzed steroids and TPs rarely or even not occur in WWTP effluents in Germany, which is most likely due to low consumption volumes. Therefore, in a globally context this may lead to differences and thus country-specific consumption pattern has to be taken into account.

This thesis showed that the biodegradation of corticosteroids and progestogens cannot be generalized in terms of degradability and preferences in degradation pathways. It has been shown that some steroids are very stable in activated sludge treatment, others degrade well, and others which do degrade but predominantly to active TPs. From a molecularly perspective, this work provided some structure-depending relations to stability and transformation reactions for two important classes of steroid hormones. These findings can be used for the improvement of biodegradation pathway prediction tools for steroids. The gained knowledge about occurrence and behavior is an excellent basis to evaluate wastewater treatment processes regarding efficiency in complementation to existing indicator micropollutants such as estrogens.

The results of this thesis furthermore demonstrate the need for action on that field, as it is very unlikely that the discharge of steroidal micropollutants will decrease in the short term.

Based on the investigations and the knowledge gained, we recommended

- i) *the development of environmental monitoring strategies for steroid hormones,*
- ii) *the evaluation of the environmental risk of individual steroid hormones in general,*
- iii) *and elaboration of a strategy for the risk assessment containing the consideration of additive effects and changes in the receptor selectivity caused by the formation of TPs.*

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Overall, the ubiquitous occurrence of the individual steroids has been emphasized by behavioral factors/elements during activated sludge treatment and brought new results, particularly for the situation in Germany. From a holistic perspective the results suggest that low biodegradability primarily leads to the individual steroid hormone burdens in the environment and is not driven by the consumption pattern alone. It was shown that biodegradation of progestogens can lead to the formation of estrogenic TPs and thus indicating the consideration of cross receptor activities in steroid risk assessment.

The need for an extensive evaluation of the environmental risks results from their ability to interact with the endocrine system of aquatic organisms. They truly merit more attention in environmental research and regulatory than it is currently the case. The results of this work provide a good basis to initiate further research and hopefully provide some substantial arguments for decision makers and legislators.

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Appendix A-Supplementary Data for Chapter 2

Occurrence of Glucocorticoids, Mineralocorticoids and Progestogens in Various Treated Wastewater, Rivers and Streams

OUTLINE

Table A.1. LC-MS/MS detection method and further information of steroid hormones investigated. (TRC= Toronto Research Chemicals, Canada Ontario; SC= Santa Cruz Biotechnology, USA Texas; SA= Sigma-Aldrich, Germany Munich).

Abbreviation	Substance	Supplier	CAS-No.	Chemical formula	Application quantity in GER [kg in 2014]	logD (pH7) [6]	Internal standard used for correction	Retention time [min]	Adduct	Precursor [Da]	Fragment mass [Da]	Collision energy [V]	Declustering potential [V]
	Progestogens (PG)												
CLM	Chlormadinone	TRC	1961-77-9	$C_{21}H_{27}ClO_3$	-	3.28	d4-E1	21.2	[M-H]⁻	361	333/287	-27/-30	-40
CLMac	Chlormadinone acetate	TRC	302-22-7	$C_{23}H_{29}ClO_4$	99	3.72	d5-CLOprop	22.3	[M+H]⁺	405	309/267	22/32	90
CYP	Cyproterone	SC	2098-66-0	$C_{22}H_{27}ClO_3$	-	3.20	d5-CLOprop	20.9	[M+H]⁺	375	321//293	28/32	110
CYPac	Cyproterone acetate	SA	427-51-0	$C_{24}H_{29}ClO_4$	99	3.64	d3-CYPac	22.0	[M+H]⁺	417	357/321	23/27	100
DIE	Dienogest	SA	65928-58-7	$C_{20}H_{25}NO_2$	278	2.31	d8-DIE	17.5	[M+H]⁺	312	161/135	38/40	160
DIE-m1	6ß-Hydroxy dienogest	SC	-	$C_{20}H_{25}NO_3$	-	1.08	d8-DIE	12.7	[M+H]⁺	328	107/251	33/33	60
DPN	Drospirenone	SA	67392-87-4	$C_{24}H_{30}O_3$	61	3.37	13C3-DPN	20.6	[M+H]⁺	367	97/197	30/30	90
ETG	Etonogestrel	SA	54048-10-1	$C_{22}H_{28}O_2$	0.4	3.60	d6-LNG	21.2	[M+H]⁺	325	257/197	25/27	80
GES	Gestodene	SA	60282-87-3	$C_{21}H_{26}O_2$	-	3.46	13C3-DPN	20.3	[M+H]⁺	311	109/201	32/26	100
HPG	17α-Hydroxy progesterone	SA	68-96-2	$C_{21}H_{30}O_3$	-	3.40	d6-LNG	20.6	[M+H]⁺	331	109/97	34/28	80
LNG	Levonorgestrel	SA	797-63-7	$C_{21}H_{28}O_2$	17	3.66	d6-LNG	20.9	[M+H]⁺	313	245/109	25/32	120
MPR	Medroxy progesterone	SA	520-85-4	$C_{22}H_{32}O_3$	-	3.69	d6-LNG	21.4	[M+H]⁺	345	123/97	33/50	100
MPRac	Medroxy progesterone acetate	SA	71-58-9	$C_{24}H_{34}O_4$	570	4.13	d3-CYPac	22.3	[M+H]⁺	387	327/123	20/40	100
MPRac-m1	6ß-Hydroxy medroxy progesterone acetate	TRC	984-47-4	$C_{24}H_{34}O_5$	-	2.89	d4-E1	19.7	[M-H]⁻	401	359/341	-25/-36	-75
MEG	Megestrol	TRC	3562-63-8	$C_{22}H_{30}O_3$	-	3.28	d4-E1	21.0	[M-H]⁻	341	313/255	-26/-25	-90
MEGac	Megestrol acetate	TRC	595-33-5	$C_{24}H_{32}O_4$	-	3.72	d3-CYPac	22.1	[M+H]⁺	385	224/267	40/26	80
NES	Norethisterone	SA	68-22-4	$C_{20}H_{26}O_2$	12	3.22	d6-NES	19.8	[M+H]⁺	299	231/109	25/32	110
NESac	Norethisterone acetate	SA	51-98-9	$C_{22}H_{32}O_4$	9	3.66	d10-BMSdiprop	22.2	[M+H]⁺	341	281/109	20/40	110
	Glucocorticoids (GC)												
BEC	Beclomethasone	SA	4419-39-0	$C_{22}H_{29}ClO_5$	-	2.15	d3-FMS	17.2	[M+HCOO]⁻	453	377 / 297	-20 / -34	-10
BECprop	Beclomethasone 17-propionate	TRC	5534-18-9	$C_{25}H_{33}ClO_6$	-	3.29	d5-CLOprop	20.4	[M+H]⁺	465	355 / 337	16 / 20	40
BECdiprop	Beclomethasone 17,21-dipropionate	TRC	5534-09-8	$C_{28}H_{37}ClO_7$	158	4.43	d10-BECdiprop	22.4	[M+H]⁺	521	411 / 319	15 / 25	70
BMS	Betamethasone	SA	378-44-9	$C_{22}H_{29}FO_5$	7	1.68	d5-DMS	16.4	[M+HCOO]⁻ /[M+H]⁺	437 /393	361 /373	-23 /17	-10 /70
BMSac	Betamethasone 21-acetat	SA	987-24-6	$C_{24}H_{31}FO_6$	7	2.12	d3-BMSac	19.7	[M+H]⁺	435	415 / 397	12 / 15	40
BMSval	Betamethasone 17-valerat	SA	2152-44-5	$C_{27}H_{37}FO_6$	98	3.71	d5-CLOprop	21.3	[M+H]⁺	477	355/337	18/20	60
BMSprop	Betamethasone 17-propionat	TRC	5534-13-4	$C_{25}H_{33}FO_6$	-	2.82	d5-BMSprop	19.9	[M+H]⁺	449	429/355	11/16	70
BMSdiprop	Betamethasone 17,21-dipropionate	SA	5593-20-4	$C_{28}H_{37}FO_7$	116	3.96	d10-BMSdiprop	22.1	[M+H]⁺	505	411/485	17/14	50
BDN	Budesonide	SA	51333-22-3	$C_{25}H_{34}O_6$	354	2.73	d8-BDN	19.9 (20.0)	[M+H]⁺	431	323/147	20/35	30
BDN-m1	6ß-Hydroxy budesonide	SC	88411-77-2	$C_{25}H_{34}O_7$	-	1.50	13C3-TRIact	15.4	[M+H]⁺	447	339/357	17/17	50
CIC	Ciclesonide	TRC	126544-47-6	$C_{32}H_{44}O_7$	1	5.32	d10-BECdiprop	24.8	[M+H]⁺	541	323/305	25/30	80
CIC-m1	Desisobutyryl ciclesonide	TRC	161115-59-9	$C_{28}H_{38}O_6$	-	3.64	d5-CLOprop	21.8	[M+H]⁺ /[M+HCOO]⁻	471 /515	323 /357	25 /-20	80 /-40
CLO	Clobetasol	SA	25122-41-2	$C_{22}H_{28}ClFO_4$	-	3.04	d6-LNG	20.4	[M+H]⁺	411	373/171	20/29	60

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CLOprop	Clobetasol 17-propionate	SA	25122-46-7	C$_{25}$H$_{32}$ClFO$_5$	89	4.18	d5-CLOprop	21.8	[M+H]$^+$	467	373/355	16/20	50
HCOR	Cortisol (Hydrocortisone)	SA	50-23-7	C$_{21}$H$_{30}$O$_5$	605	1.28	d8-PNL	14.5	[M+HCOO]$^-$ /[M+H]$^+$	407 /363	331 /121	-23 /32	-20 /110
COR	Cortisone	SA	53-06-5	C$_{21}$H$_{28}$O$_5$	-	1.66	d8-PNL	14.8	[M+HCOO]$^-$	405	329/301	-15/-27	-10
DMS	Dexamethasone	SA	50-02-2	C$_{22}$H$_{29}$FO$_5$	277	1.68	d5-DMS	16.6	[M+HCOO]$^-$ /[M+H]$^+$	437 /393	361 /373	-23 /17	-10 /70
DMS-m1	6ß-Hydroxy dexamethasone	TRC	55879-87-3	C$_{22}$H$_{29}$FO$_6$	-	0.45	d5-DMS	10.8	[M+HCOO]$^-$	453	377/308	-24/-45	-40
DMSac	Dexamethasone 21-acetate	SA	1177-87-3	C$_{24}$H$_{31}$FO$_6$	3	2.12	d3-BMSac	20.0	[M+H]$^+$	435	415/397	12/15	40
DFCval	Diflucortolone 21-valerate	TRC	59198-70-8	C$_{27}$H$_{36}$F$_2$O$_5$	3	4.04	-	22.5	[M-H]$^-$	477	457/373	-14/-23	-40
FMS	Flumethasone	SA	2135-17-3	C$_{22}$H$_{28}$F$_2$O$_5$	-	1.34	d3-FMS	16.8	[M+HCOO]$^-$	455	379/305	-25/-50	-40
FMSpiv	Flumethasone 21-pivalate	SA	2002-29-1	C$_{27}$H$_{36}$F$_2$O$_6$	1	3.58	-	21.9	[M-H]$^-$	493	371/101	-23/-55	-90
FCNact	Fluocinolone acetonide	SA	67-73-2	C$_{24}$H$_{30}$F$_2$O$_6$	12	1.60	13C3-TRIact	18.2	[M+H]$^+$	453	413/433	17/13	80
FML	Fluorometholone	SA	426-13-1	C$_{22}$H$_{29}$FO$_4$	3	1.34	d4-E1	18.7	[M-H]$^-$	375	355/255	-12/-20	-50
FLUfur	Fluticasone 17-furoate	TRC	397864-44-7	C$_{27}$H$_{29}$F$_3$O$_6$S	2	4.13	d5-FLUprop	21.8	[M+H]$^+$	539	313/293	17/29	80
FLUprop	Fluticasone 17-propionate	SA	80474-14-2	C$_{25}$H$_{31}$F$_3$O$_5$S	80	3.72	d5-FLUprop	21.8	[M+H]$^+$	501	313/293	20/25	80
HAL	Halcinonide	TRC	3093-35-4	C$_{24}$H$_{32}$ClFO$_5$	-	3.30	-	21.7	[M-H]$^-$	453	433/309	-33/-44	-120
HLM	Halometasone	TRC	50629-82-8	C$_{22}$H$_{27}$ClF$_2$O$_5$	1	1.73	d4-E1	19.2	[M-H]$^-$	443	413/362	-12/-35	-20
MPNL	Methylprednisolone	SA	83-43-2	C$_{22}$H$_{30}$O$_5$	157	1.56	d3-FMS	15.9	[M+HCOO]$^-$	419	343/294	-23/-47	-10
MPNLacp	Methylprednisolone 21-acetate 17-propionate	TRC	86401-95-8	C$_{27}$H$_{36}$O$_7$	98	3.14	d10-BECdiprop	21.5	[M+H]$^+$	473	381/101	16/22	60
MPNLprop	Methylprednisolone 21-propionate	TRC	138804-88-3	C$_{25}$H$_{34}$O$_6$	-	2.70	d5-CLOprop	20.6	[M+H]$^+$	431	339/253	15/32	50
MOM	Mometasone	TRC	105102-22-5	C$_{22}$H$_{28}$Cl$_2$O$_4$	-	3.50	d6-LNG	20.8	[M+H]$^+$ /[M+HCOO]$^-$	427 /471	373 /435	16 /-15	60 /-30
MOMfur	Mometasone 17-furoate	SA	83919-23-7	C$_{27}$H$_{30}$Cl$_2$O$_6$	63	5.06	d5-FLUprop	21.9	[M+H]$^+$	521	355/373	23/17	50
PNL	Prednisolone	SA	50-24-8	C$_{21}$H$_{28}$O$_5$	3175	1.27	d8-PNL	15.3	[M+HCOO]$^-$	405	359/329	-15/-23	-10
PNS	Prednisone	SA	53-03-2	C$_{21}$H$_{26}$O$_5$	354	1.66	d8-PNL	14.5	[M+HCOO]$^-$	403	357/327	-12/-19	-20
TRIact	Triamcinolone acetonide	SA	76-25-5	C$_{24}$H$_{31}$FO$_6$	1155	1.94	13C3-TRIact	17.6	[M+H]$^+$	435	415/397	14/20	80
TRIact-m1	6ß-Hydroxy triamcinolone acetonide	TRC	3869-32-7	C$_{24}$H$_{31}$FO$_7$	-	0.71	13C3-TRIact	13.4	[M+H]$^+$	451	387/329	13/20	90

Mineralocorticoids (MC)

CAN	Canrenone	SA	976-71-6	C$_{22}$H$_{28}$O$_3$	-	3.60	d6-CAN	20.8	[M+H]$^+$	341	107/187	35/32	110
CAN-m1	11α-Hydroxy canrenone	TRC	192569-17-8	C$_{22}$H$_{28}$O$_4$	-	2.29	d4-E1	17.0	[M-H]$^-$	355	311/267	-20/-25	-130
FLC	Fludrocortisone	TRC	127-31-1	C$_{21}$H$_{29}$FO$_5$	-	1.32	d5-DMS	14.7	[M+H]$^+$	381	361/343	28/28	130
FLCac	Fludrocortisone 21-acetate	SA	514-36-3	C$_{23}$H$_{31}$FO$_6$	0.4	1.76	13C3-TRIact	18.9	[M+H$^+$]$^+$	423	343/325	30/31	120
SPL	Spironolactone	SA	52-01-7	C$_{24}$H$_{32}$O$_4$S	9150	3.64	d6-CAN	20.8	[M+H]$^+$	417	341	20	40
SPL-m1	7α-Thiomethyl spironolactone	TRC	38753-77-4	C$_{23}$H$_{32}$O$_3$S	-	4.18	d7-SPL-m1	20.9	[M+H]$^+$	389	341/323	25/23	110

Surrogates

Internal standard mix 1 (IS-mix 1)

d8-BDN	Budesonide-d8	TRC	-	C$_{25}$H$_{26}$D$_8$O$_6$	-	-	-	19.8 (19.9)	[M+H]$^+$	439	323	19	40
d6-CAN	Canrenone-d6	TRC	-	C$_{22}$H$_{22}$D$_6$O$_3$	-	-	-	20.7	[M+H]$^+$	347	107	37	110
d5-CLOprop	Clobetasol 17-propionate-d5	TRC	-	C$_{25}$H$_{27}$D$_5$ClFO$_5$	-	-	-	21.7	[M+H]$^+$	472	373	17	70
d3-CYPac	Cyproterone acetate-d3	TRC	-	C$_{24}$H$_{26}$D$_3$ClO$_4$	-	-	-	21.9	[M+H]$^+$	420	357	25	100

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d5-DMS	Dexamethasone-d5	TRC	-	C$_{22}$H$_{24}$D$_5$FO$_5$	-	-	-	16.6	[M+HCOO]⁻ /[M+H]⁺	442 /398	364 /378	-25 /17	-10 /70
d8-DIE	Dienogest-d8	TRC	-	C$_{20}$H$_{17}$D$_8$NO$_2$	-	-	-	17.4	[M+H]⁺	320	167	38	160
13C3-DPN	Drospirenone-13C3	TRC	-	C$_{21}$^{13}C$_3$H$_{30}$O$_3$	-	-	-	20.6	[M+H]⁺	370	97	35	100
d3-FMS	Flumethasone-d3	TRC	-	C$_{22}$H$_{25}$D$_3$F$_2$O$_5$	-	-	-	16.8	[M+HCOO]⁻	458	382	-24	-30
d6-LNG	Levonorgestrel-d6	TRC	-	C$_{21}$H$_{22}$D$_6$O$_2$	-	-	-	20.8	[M+H]⁺	319	251	25	120
d6-NES	Norethisterone-d6	TRC	-	C$_{20}$H$_{20}$D$_6$O$_2$	-	-	-	19.7	[M+H]⁺	305	237	27	100
d8-PNL	Prednisolone-d8	TRC	-	C$_{21}$H$_{20}$D$_8$O$_5$	-	-	-	15.3	[M+HCOO]⁻	413	367	-16	-10
d5-FLUprop	Fluticasone 17-propionate-d5	TRC	-	C$_{25}$H$_{26}$D$_5$F$_3$O$_5$S	-	-	-	21.7	[M+H]⁺	506	313	20	80
13C3-TRIact	Triamcinolone acetonide-13C3	TRC	-	C$_{21}$^{13}C$_3$H$_{31}$FO$_6$	-	-	-	17.6	[M+H]⁺	438	418	15	80
d7-SPL-m1	7α-Thiomethyl spironolactone-d7	TRC	-	C$_{23}$H$_{25}$D$_7$O$_3$S	-	-	-	20.8	[M+H]⁺	396	348	25	110
d4-E1	Estrone-d4	SA	-	C$_{18}$H$_{18}$D$_4$O$_2$	-	-	-	20.2	[M-H]⁻	273	147	-50	-100
	Internal standard mix 2 (IS-mix 2)												
d10-BECdirop	Beclomethasone 17, 21-diropionate-d10	TRC	-	C$_{28}$H$_{27}$D$_{10}$ClO$_7$	-	-	-	22.3	[M+H]⁺	531	319	25	30
d3-BMSac	Betamethasone 21-acetate-d3	TRC	-	C$_{24}$H$_{28}$D$_3$FO$_6$	-	-	-	19.6	[M+H]⁺	438	418	12	50
d5-BMSprop	Betamethasone 17-propionate-d5	TRC	-	C$_{25}$H$_{28}$D$_5$FO$_6$	-	-	-	19.8	[M+H]⁺	454	434	12	70
d10-BMSdiprop	Betamethasone 17,21-dipropionate-d10	TRC	-	C$_{28}$H$_{27}$D$_{10}$FO$_7$	-	-	-	22.0	[M+H]⁺	515	416	17	50

Table A.2. Measured environmental samples, sampling dates, locations and capacities of wastewater treatment plants. The efflux values were obtained from online sources of the federal agencies and base on modelling approaches or on measured data, whereas dates and sampling location may differ.

Abbreviation	Name/capacity	Efflux [m³/s]	Sampling Date	Location
WWTP effluent samples				
WWTPeff 1	25,000 citizens (person equivalents not known)		17/05/23	Groß-Gerau (Hessia)
WWTPeff 2	68,000 person equivalents (size: 80,000 pe)		17/05/23	Bingen (RLP)
WWTPeff 3	26,487 person equivalents (size: 48,000 pe)		17/05/26	Schwelm (NRW)
WWTPeff 4	220,000 person equivalents (size: 320,000 pe)		17/03/14	Koblenz (RLP)
WWTPeff 5	105,000 m³/day wastewater (dry weather conditions)		17/05/30	Wandlitz (Brandenburg)
Rivers and streams				
SW-1a	Mühlenbach (upstream WWTP)	<1	17/05/23	Groß-Gerau
SW-1b	Mühlenbach (downstream WWTP)	~1	17/05/23	Groß-Gerau
SW-2a	River Nahe (upstream WWTP)	~12	17/05/23	Bingen
SW-2b	River Nahe (downstream WWTP)	~12	17/05/23	Bingen
SW-3a	Schwelme (upstream WWTP)	~0.3	17/05/26	Schwelm
SW-3b	Schwelme (downstream WWTP, immediately for entry in river Wupper)	~1	17/05/26	Wuppertal
SW-4a	River Wupper (upstream entry Schwelme)	~8	17/05/26	Wuppertal
SW-4b	River Wupper (downstream entry Schwelme)	~9	17/05/26	Wuppertal
SW-5	Teltow canal	~9	17/08/14	Berlin
SW-6	Landgraben (downstream industrial WWTP)	<1	17/05/23	Weiterstadt
SW-7	River Neckar	~110	17/05/23	Mannheim
SW-8	River Main	200	17/05/23	Wiesbaden
SW-9a	River Lahn	~20	17/05/23	Limburg a.d.Lahn
SW-9b	River Lahn	~34	17/05/24	Lahnstein
SW-10a	River Rhine (km 432)	~1600	17/05/23	Frankenthal
SW-10b	River Rhine (km 434)	~1600	17/05/23	Frankenthal
SW-10c	River Rhine (km 482)	~1400	17/05/23	Trebur
SW-10d	River Rhine (km 590)	1860	17/03/06	Koblenz
SW-10e	River Rhine (km 590)	1010	17/04/25	Koblenz
SW-10f	River Rhine (km 590)	1340	17/06/01	Koblenz
SW-11	River Ahr	~1	17/05/22	Sinzig
SW-12	River Rur	~13	17/05/21	Kreuzau

Figure A.1. Map sections of sampling locations.
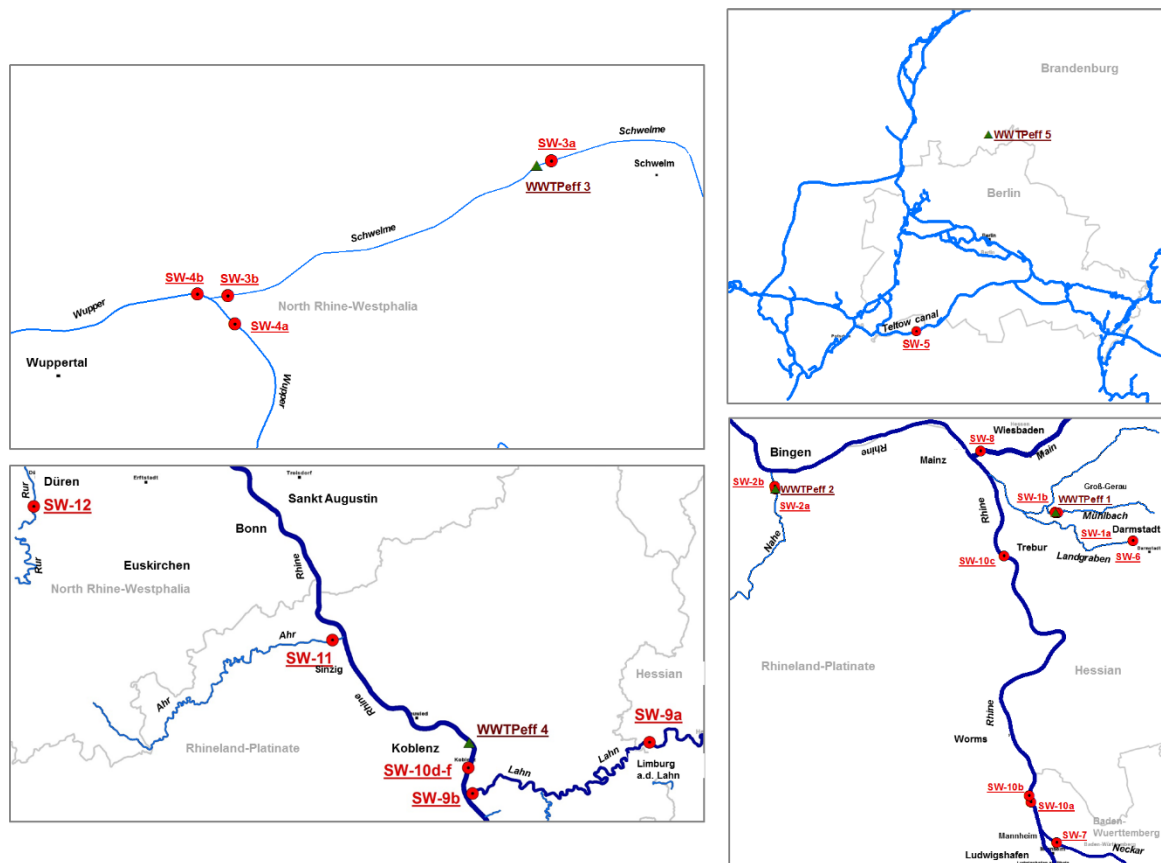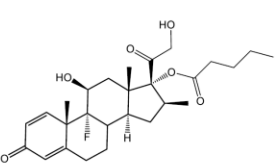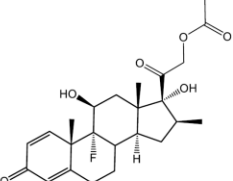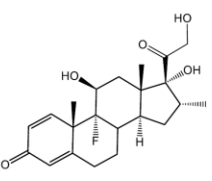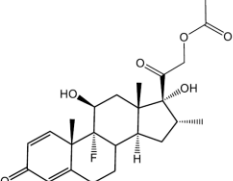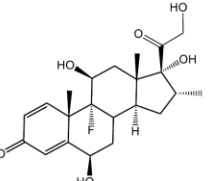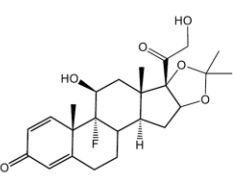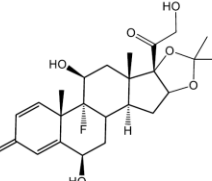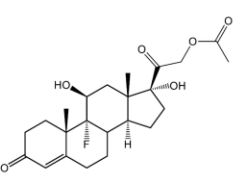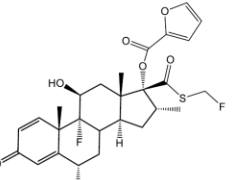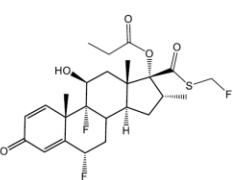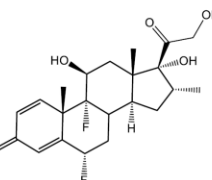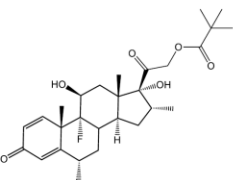
Appendix A

Table A.3. Chemical structures and properties of target steroid hormones. Dosage forms were assembled from pharma-bund.de.[1] Application quantities prescribed in Germany 2014 were calculated based on the number of prescribed daily doses[2] x defined daily doses.[3] Therapeutic use was summarized from different references.[4-5]

Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure
Glucocorticoids (GC)							
Cortisone (COR) **CAS:** 53-06-5 **Appl. Quantity:** -		**Cortisol (HCOR)** **CAS:** 50-23-7 **Appl. Quantity:** 605 kg		**Prednisone (PNS)** **CAS:** 53-03-2 **Appl. Quantity:** 354 kg		**Prednisolone (PNL)** **CAS:** 50-24-8 **Appl. Quantity:** 3175 kg	
Dosage Forms: **Therapeutic Use:** Prodrug of HCOR, natural hormone		**Dosage Forms:** Injection, tablet, drops, ointment, spray **Therapeutic Use:** Natural hormone, replacement therapy in patients with adrenocortical insufficiency, treatment of rheumatic disorder, allergic conditions, dermatitis, veterinary medicine, active metabolite of COR		**Dosage Forms:** Tablet, suppository **Therapeutic Use:** Prodrug of PNL, allergic disorders, Morbus Crohn, dermatitis		**Dosage Forms:** Cream, tincture, injection, tablet, ointment, suppository **Therapeutic Use:** Treatment of lung diseases, allergic disorders, Morbus Crohn, dermatitis, veterinary medicine, active metabolite of PNS	
Methylprednisolone (MPNL) **CAS:** 83-43-2 **Appl. Quantity:** 157 kg		**Methylprednisolone aceponate (MPNLacp)** **CAS:** 86401-95-8 **Appl. Quantity:** 98 kg		**Methylprednisolone 21-propionate (MPNLprop)** **CAS:** 138804-88-3 **Appl. Quantity:** -		**Budesonide (BDN)** **CAS:** 51333-22-3 **Appl. Quantity:** 354 kg	
Dosage Forms: Tablet, injection **Therapeutic Use:** Allergic conditions, Morbus Crohn, treatment of rheumatic disorder and multiple sclerosis, veterinary medicine, metabolite of MPNLacp		**Dosage Forms:** Cream, ointment, injection **Therapeutic Use:** Allergic conditions, dermatitis, veterinary medicine		**Dosage Forms:** **Therapeutic Use:** Metabolite of MPNLacp		**Dosage Forms:** Inhalation powder, spray, tablet, foam, ointment **Therapeutic Use:** Treatment of lung and bronchial diseases, allergic rhinitis, Morbus Crohn	
6ß-Hydroxy budesonide (BDN-m1) **CAS:** 88411-77-2 **Appl. Quantity:** -		**Betamethasone (BMS)** **CAS:** 378-44-9 **Appl. Quantity:** 7 kg		**Betamethasone dipropionate (BMSdiprop)** **CAS:** 5593-20-4 **Appl. Quantity:** 116 kg		**Betamethasone 17-propionate (BMSprop)** **CAS:** 5534-13-4 **Appl. Quantity:** -	
Dosage Forms: **Therapeutic Use:** Metabolite of BDN		**Dosage Forms:** Tablet, solution, injection **Therapeutic Use:** Allergic disorders, treatment of rheumatic disorder, autoimmune diseases, veterinary medicine, metabolite of BMSdiprop/BMSac/BMSval		**Dosage Forms:** Ointment, cream, gel, foam, transdermal patch, injection **Therapeutic Use:** Dermatitis, therapy of Morbus Crohn and Colitis ulcerosa, allergic disorders		**Dosage Forms:** **Therapeutic Use:** Active metabolite of BMSdiprop	
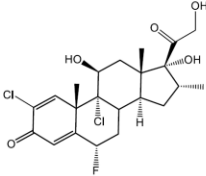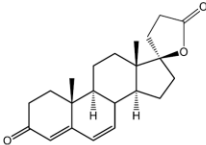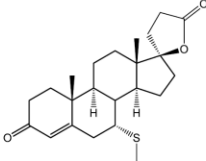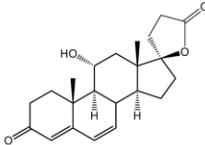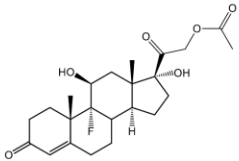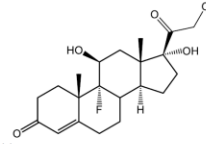
Appendix A

Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure
Glucocorticoids (GC)							

Betamethasone 17-valerate (BMSval)
CAS: 2152-44-5
Appl. Quantity: 98 kg
Dosage Forms: Ointment, cream, solution, foam
Therapeutic Use: Treatment of dermatitis, allergic disorders, veterinary medicine (only topically administration)

Betamethasone 21-acetate (BMSac)
CAS: 987-24-6
Appl. Quantity: 7 kg
Dosage Forms: Injection
Therapeutic Use: Treatment of rheumatic disorder, autoimmune diseases, veterinary medicine

Dexamethasone (DMS)
CAS: 50-02-2
Appl. Quantity: 277 kg
Dosage Forms: Tablet, solution, drops, injection, nasal spray, cream, intravitreal implant
Therapeutic Use: Allergic disorders, treatment of rheumatic disorder, treatment of eye diseases, autoimmune diseases, veterinary medicine

Dexamethasone 21-acetate (PNL)
CAS: 1177-87-3
Appl. Quantity: 3 kg
Dosage Forms: Drops, injection
Therapeutic Use: Allergic disorders, treatment of rheumatic disorder, autoimmune diseases, veterinary medicine

6β-Hydroxy dexamethasone (DMS-m1)
CAS: 55879-87-3
Appl. Quantity: -
Dosage Forms:
Therapeutic Use: Metabolite of DMS/DMSac

Triamcinolone acetonide (TRIact)
CAS: 76-25-5
Appl. Quantity: 1155 kg
Dosage Forms: Spray, drops, tablet, cream, ointment, injection, dental powder, nasal spray, tincture
Therapeutic Use: Treatment of seasonal rhinitis, dermatitis, therapy of arthrosis and rheumatic disorder, veterinary medicine, OTC drug (as tablet)

6β-Hydroxy triamcinolone acetonide (TRIact-m1)
CAS: 3869-32-7
Appl. Quantity: -
Dosage Forms:
Therapeutic Use: Metabolite of TRIact

Fluocinolone acetonide (FCNact)
CAS: 67-73-2
Appl. Quantity: 12 kg
Dosage Forms: Cream, ointment, intravitreal implant, drops, suppository
Therapeutic Use: Treatment of inflammatory and allergic skin diseases

Fluticasone furoate (FLUfur)
CAS: 397864-44-7
Appl. Quantity: 2 kg
Dosage Forms: Nasal spray, powder for inhalation
Therapeutic Use: Allergic disorders, treatment of lung and bronchial diseases

Fluticasone propionate (FLUprop)
CAS: 80474-14-2
Appl. Quantity: 80 kg
Dosage Forms: Inhalation powder, nasal spray, cream
Therapeutic Use: Dermatitis, allergic disorders, treatment of lung and bronchial diseases, OTC drug (nasal spray for seasonal rhinitis)

Flumethasone (FMS)
CAS: 2135-17-3
Appl. Quantity: -
Dosage Forms:
Therapeutic Use: Metabolite of FMSpiv

Flumethasone 21-pivalate (FMSpiv)
CAS: 2002-29-1
Appl. Quantity: 1 kg
Dosage Forms: Cream, tincture, ointment
Therapeutic Use: Treatment of inflammatory and allergic skin diseases, actinic dermatitis

Appendix A

Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure
Glucocorticoids (GC)							
Fluorometholone (FML) **CAS:** 426-13-1 **Appl. Quantity:** 3 kg		**Beclomethasone (BEC)** **CAS:** 4419-39-0 **Appl. Quantity:** -		**Beclomethasone dipropionate (BECdiprop)** **CAS:** 5534-09-8 **Appl. Quantity:** 158 kg		**Beclomethasone 17-propionate (BECprop)** **CAS:** 5534-18-9 **Appl. Quantity:** -	
Dosage Forms: Eye drops **Therapeutic Use:** Therapy of inflammatory eye diseases		**Dosage Forms:** **Therapeutic Use:** Metabolite of BECdiprop		**Dosage Forms:** Solution/powder for Inhalation, nasal spray **Therapeutic Use:** Treatment of lung and bronchial diseases, allergic disorders, OTC (nasal spray for seasonal rhinitis)		**Dosage Forms:** **Therapeutic Use:** Active metabolite of BECdiprop	
Clobetasol propionate (CLOprop) **CAS:** 25122-46-7 **Appl. Quantity:** 89 kg		**Clobetasol (CLO)** **CAS:** 25122-41-2 **Appl. Quantity:** -		**Mometasone furoate (MOMfur)** **CAS:** 83919-23-7 **Appl. Quantity:** 63 kg		**Mometasone (MOM)** **CAS:** 105102-22-5 **Appl. Quantity:** -	
Dosage Forms: Cream, ointment, solution, foam, shampoo **Therapeutic Use:** Treatment of inflammatory and allergic skin diseases (only topical administration)		**Dosage Forms:** **Therapeutic Use:** Metabolite of CLOprop		**Dosage Forms:** Drops, nasal spray, powder for inhalation, cream, ointment **Therapeutic Use:** Therapy of rhinitis, asthma, treatment of inflammatory and allergic skin diseases, OTC (nasal spray for seasonal rhinitis), Veterinary medicine		**Dosage Forms:** **Therapeutic Use:** Metabolite of MOMfur	
Ciclesonide (CIC) **CAS:** 126544-47-6 **Appl. Quantity:** 1 kg		**Desisobutyryl ciclesonide (CIC-m1)** **CAS:** 161115-59-9 **Appl. Quantity:** -		**Diflucortolone valerate (DFCval)** **CAS:** 59198-70-8 **Appl. Quantity:** 3 kg		**Halcinonide (HAL)** **CAS:** 3093-35-4 **Appl. Quantity:** -	
Dosage Forms: Solution for Inhalation **Therapeutic Use:** Prodrug of CIC-m1, treatment of lung and bronchial diseases		**Dosage Forms:** - **Therapeutic Use:** Active metabolite of CIC		**Dosage Forms:** Ointment, cream **Therapeutic Use:** Treatment of inflammatory and allergic skin diseases (only topical administration)		**Dosage Forms:** Cream, ointment, solution **Therapeutic Use:** No drug approval in Germany (but manufacturer of HAL in Germany), permitted in bordering countries, treatment of inflammatory and allergic skin diseases (only topically administration)	

Appendix A

Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure

Glucocorticoids (GC)

Halomethasone (HLM)
CAS:
50629-82-8
Appl. Quantity:
1 kg

Dosage Forms: Cream, ointment
Therapeutic Use: Treatment of inflammatory and allergic skin diseases (only topical administration)

Mineralocorticoids (MC)

Canrenone (CAN)

CAS:
976-71-6
Appl. Quantity:
9150 kg
(Spironolactone)

Dosage Forms: Tablet, injection
Therapeutic Use: Active metabolite of spironolactone, treatment of high blood pressure, chronic heart failure, liver and kidney diseases, hormone therapy

7α-Thiomethyl spironolactone (SPL-m1)
CAS:
38753-77-4
Appl. Quantity:
-

Dosage Forms:
Therapeutic Use: Metabolite of SPL, treatment of high blood pressure, chronic heart failure, liver and kidney diseases, hormone therapy

11α-Hydroxy canrenone (CAN-m1)
CAS:
192569-17-8
Appl. Quantity:
-

Dosage Forms:
Therapeutic Use: Metabolite of SPL, treatment of high blood pressure, chronic heart failure, liver and kidney diseases, hormone therapy

Fludrocortisone acetate (FLCac)
CAS:
514-36-3
Appl. Quantity:
0.4 kg (all FLC derivatives)

Dosage Forms: Tablet, ear drops, solution, emulsion
Therapeutic Use: Treatment of Addison disease, therapy of ear infections, veterinary medicine, (prodrug of FLC)

Fludrocortisone (FLC)
CAS:
127-31-1
Appl. Quantity:
0.4 kg (all FLC derivatives)

Dosage Forms: Tablet
Therapeutic Use: Active metabolite of FLCac

Appendix A

Progestogens (PG)

Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure
Chlormadinone acetate (CLMac) **CAS:** 302-22-7 **Appl. Quantity:** 99 kg **Dosage Forms:** Tablet, Injection **Therapeutic Use:** Hormonal contraception, hormone replacement therapy, treatment of gynecological disorders, veterinary medicine		**Chlormadinone (CLM)** **CAS:** 1961-77-9 **Appl. Quantity:** - **Dosage Forms:** **Therapeutic Use:** Metabolite of CLMac		**Cyproterone acetate (CYPac)** **CAS:** 427-51-0 **Appl. Quantity:** 99 kg **Dosage Forms:** Tablet, injection **Therapeutic Use:** Hormonal contraception, hormone replacement therapy, hormone therapy, treatment of dermatological conditions, treatment of cancer		**Cyproterone (CYP)** **CAS:** 2098-66-0 **Appl. Quantity:** - **Dosage Forms:** **Therapeutic Use:** Metabolite of CYPac	
Dienogest (DIE) **CAS:** 65928-58-7 **Appl. Quantity:** 278 kg **Dosage Forms:** Tablet **Therapeutic Use:** Hormonal contraception, hormone replacement, treatment of gynecological disorders and dermatological conditions		**6β-Hydroxy dienogest (DIE-m1)** **CAS:** n.a. **Appl. Quantity:** - **Dosage Forms:** **Therapeutic Use:** Metabolite of DIE		**Drospirenone (DPN)** **CAS:** 67392-87-4 **Appl. Quantity:** 61 kg **Dosage Forms:** Tablet **Therapeutic Use:** Hormonal contraception, treatment of dermatological conditions		**Etonogestrel (ETG)** **CAS:** 54048-10-1 **Appl. Quantity:** 0.4 kg **Dosage Forms:** Implant, intrauterine devices **Therapeutic Use:** Long-term contraception	
Gestodene (GES) **CAS:** 60282-87-3 **Appl. Quantity:** n.a. **Dosage Forms:** Tablet, transdermal patch **Therapeutic Use:** Hormonal contraception		**17α-Hydroxy progesterone (HPG)** **CAS:** 68-96-2 **Appl. Quantity:** n.a. **Dosage Forms:** Injection (as its caproate ester) **Therapeutic Use:** Natural hormone, prevention of preterm birth		**Levonorgestrel (LNG)** **CAS:** 797-63-7 **Appl. Quantity:** 17 kg **Dosage Forms:** Tablet, transdermal patch, implant, intrauterine devices **Therapeutic Use:** Hormonal contraception, emergency contraception, hormone replacement therapy		**Medroxy progesterone acetate (MRPac)** **CAS:** 71-58-9 **Appl. Quantity:** 570 kg **Dosage Forms:** Injection, tablet **Therapeutic Use:** Long-term hormonal contraception, hormone replacement therapy, treatment of cancer, veterinary medicine	

Appendix A

Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure	Properties	Chemical structure
Progestogens (PG), Estrogens and Finasteride							
6β-Hydroxy progesterone acetate (MRPac-m1) **CAS:** 984-47-4 **Appl. Quantity:** - **Dosage Forms:** **Therapeutic Use:**	Metabolite of MRPac	**Medroxy progesterone (MRP)** **CAS:** 520-85-4 **Appl. Quantity:** - **Dosage Forms:** **Therapeutic Use:**	Metabolite of MRPac	**Megestrol acetate (MEGac)** **CAS:** 595-33-5 **Appl. Quantity:** n.a. **Dosage Forms:** Tablet **Therapeutic Use:** Treatment of cancer, veterinary medicine		**Megestrol (MEG)** **CAS:** 3562-63-8 **Appl. Quantity:** - **Dosage Forms:** **Therapeutic Use:**	Metabolite of MEGac
Norethisterone acetate (NESac) **CAS:** 51-98-9 **Appl. Quantity:** 21 kg (all NES derivatives) **Dosage Forms:** Tablet, injection, transdermal patch **Therapeutic Use:** Hormonal contraception, hormone replacement therapy, treatment of gynecological disorders		**Norethisterone (NES)** **CAS:** 68-22-4 **Appl. Quantity:** 21 kg (all NES derivatives) **Dosage Forms:** Tablet, injection (in part as enantate ester) **Therapeutic Use:** Hormonal contraception, hormone replacement therapy, treatment of gynecological disorders (Metabolite of NESac)					

Method Optimization and Determination of Matrix Effects.

Chromatography. Chromatographic separation of beta-/dexamethasone and cortisone/cortisol/prednisone/prednisolone was achieved by a flat increasing gradient and the use of acetonitrile as organic eluent. In addition, further optimization of the chromatography was conducted in order to reduce the signal background and ion-suppression (matrix effects). Both gradients in Fig. A.2 revealed appropriate separations of critical analytes. For the final method we have chosen a longer chromatographic run time since higher sensitivities for most of our analytes in environmental matrices (Fig. A.2+A.3) were achieved. The total ion chromatograms (TIC) shown in Fig. A.2 illustrate the "reduction" in the signal background, due to more eligible distribution of the matrix constituents. As a consequence, the detection sensitivities for the majority of the monitored steroids increased as exemplary shown in Fig A.3, whereas the ion-suppression for ciclesonide (analyte, which elute last) increased by these improvements.
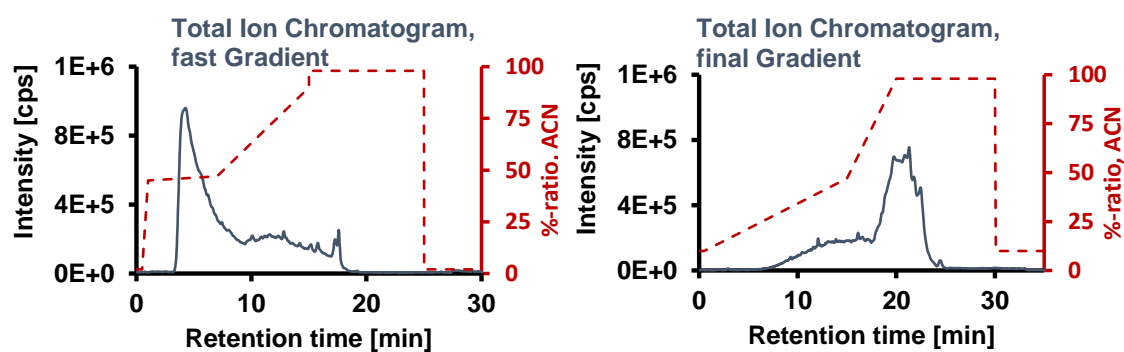
Figure A.2. Comparison of gradients and distribution of the matrix constituents. For both gradients comparable analyte separations were achieved. Sample: 1L surface water spiked with 0.25 ng of each analyte.

Comparison of Analyte Precursor for Sensitivity. For the decision making process which precursor reveals the highest sensitivity, a comparison of [M+FOR]⁻, [M+H]⁺ and [M-H]⁻ in spiked surface water samples was performed. As shown exemplary for the analytes halcinonide, budesonide and cortisol (Fig.A.4), the differences in sensitivity were significant depending to the monitored adduct. In addition, several analytes showed partly different sensitivities in standard solution and in the presence of matrix constituents. Typically, the precursor ions are selected at beginning of the method development in matrix-free standards, which could lead to the choice of less sensitive transitions, due to different ion-suppression and/or signal background. Therefore, precursor ions and MRM transitions should be selected in matrix loaded samples for a most sensitive analysis.

At least, the detection mode scheduled MRM was selected, due to higher dwell times for the monitoring of the transitions as described for multi-methods in Hermes et al..[7]
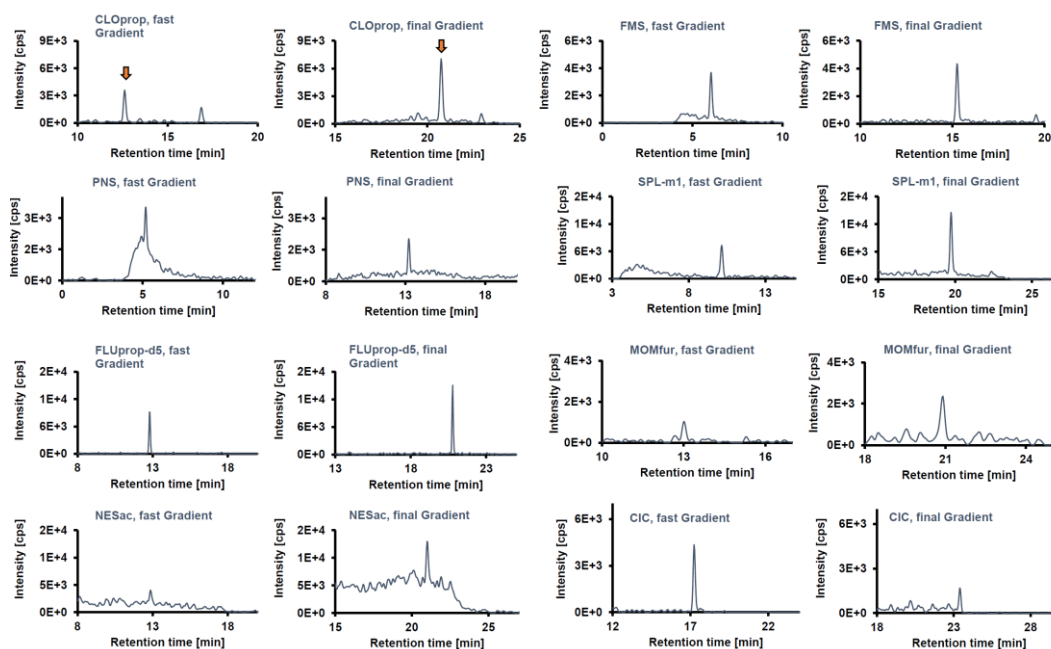
Figure A.3. Increasing of detection sensitivity by gradient optimization. Exemplary shown for clobetasol propionate (CLOprop), flumethasone (FMS), prednisone (PNS), thiomethyl spironolactone (SPL-m1), fluticasone propionate-d5 (FLUprop-d5), mometasone furoate (MOMfur) and norethisterone acetate (NESac). In contrast, ciclesonide (CIC) showed decreasing detection sensitivity. Sample: 1L surface water spiked with 0.25 ng of each analyte (1 ng of each surrogate).
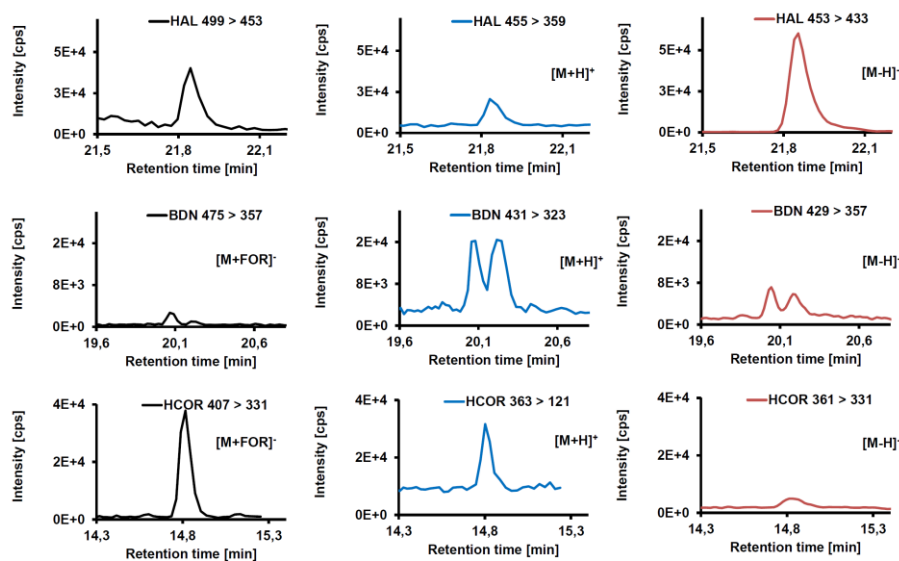
Figure A.4. Comparison of analyte precursor for quantification of glucocorticoids in environmental matrices. Exemplary shown for halcinonide (HAL), budesonide (BDN) and cortisol (HCOR). Sample: 1L surface water spiked with 0.5 ng of each analyte.

Optimization of the Sample Clean-Up.
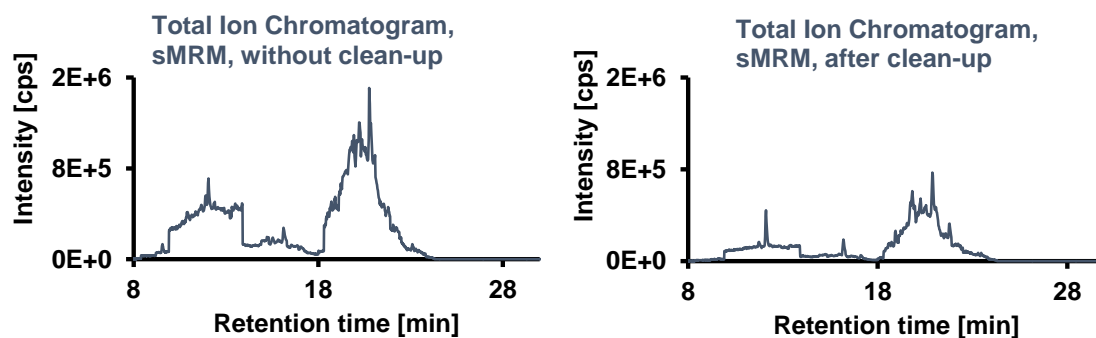
Figure A.5. Reduction of matrix effects by the silica gel clean-up. Sample: 1L surface water spiked with 0.5 ng of each analyte (1 ng of each surrogate).
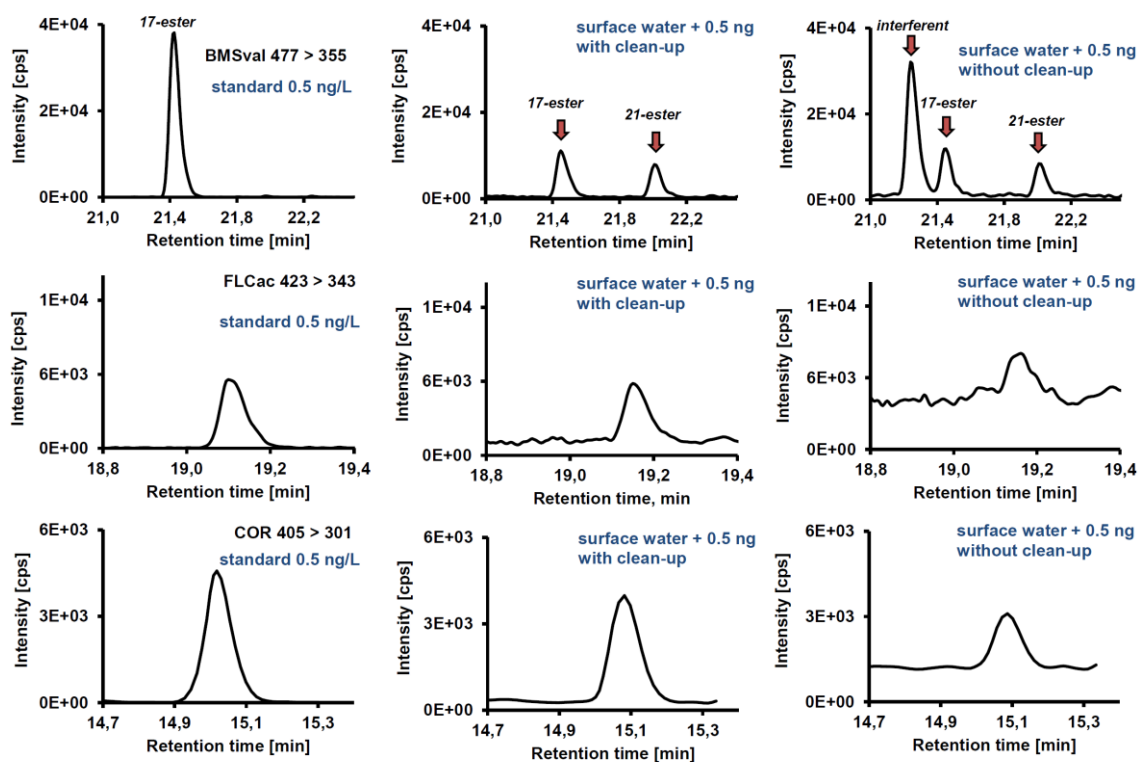
Figure A.6. Increasing of detection sensitivity by silica gel clean-up. Exemplary shown for betamethasone valerate (BMSval), fludrocortisone acetate (FLCac) and cortisone (COR). In contrast, ciclesonide (CIC) showed decreasing detection sensitivity. Sample: 1L surface water spiked with 0.5 ng of each analyte (1 ng of each surrogate).

Matrix Effects. Surface water and WWTP effluent were spiked in duplicate after the silica gel clean-up to determine the ion-suppression and the loss of the analytes during the sample treatment. For the calculation of the matrix effect, the background concentrations in the water samples were measured and subtracted from the spiked samples, accordingly. For these experiments, the concentrations were not corrected by internal standards.

$$Matrix\ Effect\ [\%] = 100\% - \left[\frac{(c_{spike\ after\ clean-up} - c_{background})}{c_{target}} * 100\%\right]$$

Positive values for the matrix effects indicate decreasing peak areas (ion-suppression) and negative values are results of ion-enhancement. The loss of the analytes during the sample treatment can be described, as a first approximation, as the discrepancy of the sum of matrix effects and absolute recovery from 100%.

$$loss\ [\%] = 100\% - [abs. Recovery + Matrix\ Effect]$$
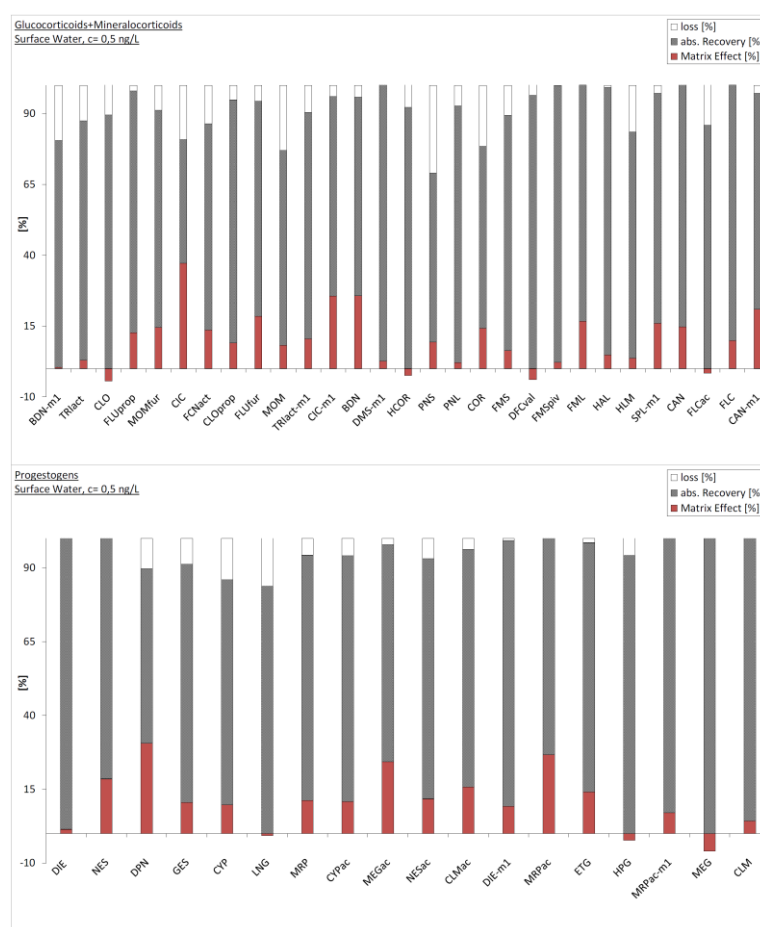
Figure A.7. Matrix effects of the target steroids in surface water separately for corticosteroids (above) and progestogens (below), determined at c= 0.5 ng/L. (without diesters, monoesters and alcohols of BMS, DMS, BEC and MPNL)

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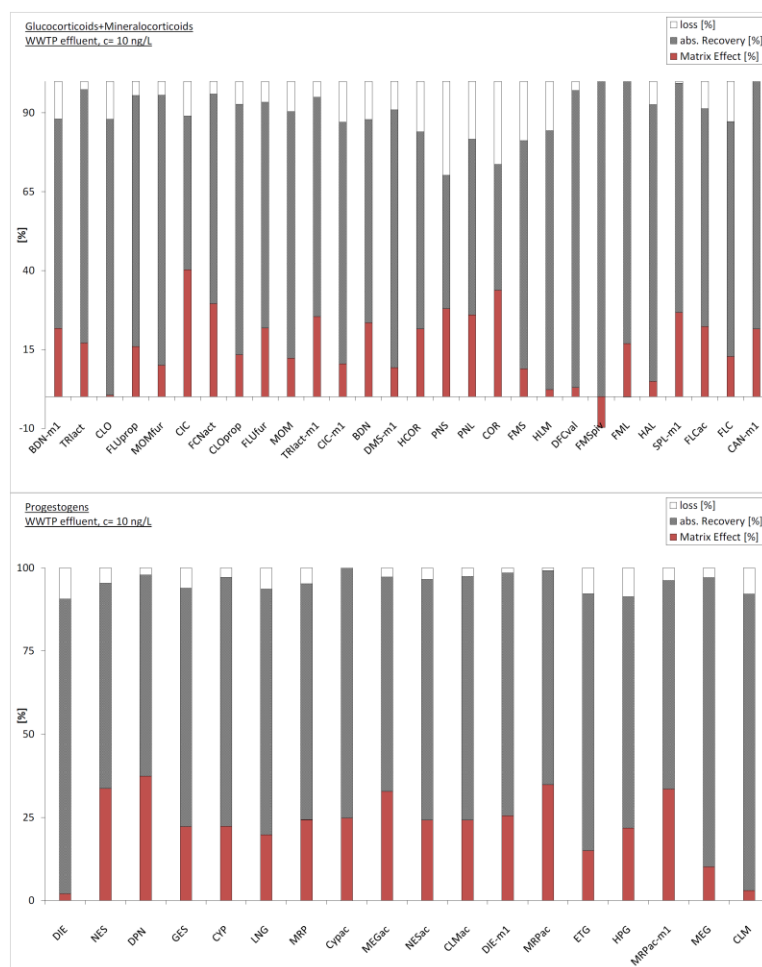
Figure A.8. Matrix effects of the target steroids in WWTP effluent separately for corticosteroids (above) and progestogens (below), determined at c= 10 ng/L. (without diesters, monoesters and alcohols of BMS, DMS, BEC and MPNL. CAN is not shown since its high background concentration in the WWTP effluent)

Appendix A

Table A.4. Concentrations of target analytes in German WWTP effluents and rivers/ streams. LOD and LOQ calculations were based on a signal-to-noise ratio of 3 (LOD) and 10 (LOQ) either using the background concentration or a total spike amount in the smoothed (smoothing factor: 2.0) chromatograms of environmental samples. (< = below detection limit, <LOQ= above detection limit, below quantification limit).

Substance	Concentration [ng/L] WWTP effluent					LOD/LOQ
	1	2	3	4	5	
Ciclesonide	<	<	<	<	<	0.06 / 0.3
Desisobutyryl ciclesonide	<	<	<	<	<	0.5 / 1.0
6ß-Hydroxy dexamethasone	<	<	<	<	<	0.07 / 0.2
Diflucortolone valerate	<	<	<	<	<	0.02 / 0.05
Halcinonide	<	<	<	<	<	0.02 / 0.3
Halomethasone	<	<	<	<	<	0.1 / 0.5
Prednisolone	<LOQ	<LOQ	0.4	0.6	<LOQ	0.06 / 0.2
Prednisone	<LOQ	<LOQ	0.2	0.4	<LOQ	0.06 / 0.2
Cortisol (Hydrocortisone)	0.9	1.4	1.2	2.8	0.9	0.06 / 0.2
Cortisone	0.2	0.3	0.4	0.9	0.2	0.1 / 0.2
Progestogens (PG)						
Dienogest	3.3	1.3	4.4	4.3	1.4	0.2 / 0.3
6ß-Hydroxy dienogest	<LOQ	0.6	0.6	0.6	0.9	0.2 / 0.4
Norethisterone	<	<	<	<	<	1.0 / 1.5
Norethisterone acetate	<	<	<	<	<	0.5 / 1.0
Drospirenone	<	<	<	<	<	0.5 / 1.0
Etonogestrel	<	<	<	<	<	0.5 / 2.0
Gestodene	<	<	<	<	<	1.0 / 2.5
Cyproterone	<	<	<	<	<	0.5 / 1.0
Cyproterone acetate	0.8	1.7	2.9	3.7	2.3	0.3 / 0.8
Chlormadinone						1.5 / 5.0
Chlormadinone acetate	<	<LOQ	<LOQ	<LOQ	<	0.1 / 0.3
Levonorgestrel	<	<LOQ	<	<	<	0.3 / 1.0
Medroxy progesterone	<	<	<	<	<	0.08 / 0.3
Medroxy progesterone acetate	<LOQ	<LOQ	<LOQ	<	<	0.08 / 0.3
6ß-Hydroxy medroxy progesterone acetate	<	<	<	<	<	0.2 / 0.5
Megestrol	<	<	<	<	<	0.5 / 1.0
Megestrol acetate	<	<LOQ	<	<	<	0.06 / 0.3
17α-Hydroxy progesterone	1.1	0.7	0.7	1.0	1.3	0.3 / 0.7

Substance	Concentration [ng/L] WWTP effluent					LOD/LOQ
	1	2	3	4	5	
Mineralocorticoids (MC)						
Canrenone	4.5	3.7	10	19	8.0	0.4 / 1.4
7α-Thiomethyl spironolactone	0.2	1.2	1.5	3.8	2.0	0.05 / 0.1
11α-Hydroxy canrenone	<LOQ	<	<	<	<	0.5 / 3.0
Fludrocortisone	<	<	<	<	<	0.5 / 0.8
Fludrocortisone acetate	<	<	<	<	<	0.5 / 1.5
Glucocorticoids (GC)						
Beclomethasone	<	<LOQ	<LOQ	<	<	0.02 / 0.07
Beclomethasone propionate	<LOQ	<LOQ	<LOQ	<	<	0.1 / 0.3
Beclomethasone dipropionate	<	<	<	<	<	0.1 / 0.5
Betamethasone	0.6	0.4	0.05	0.2	0.6	0.01 / 0.05
Betamethasone 21-acetate	<	<	<	<	<	0.05 / 0.2
Betamethasone valerate	1.3	2.5	1.1	2.2	1.2	0.08 / 0.3
Betamethasone propionate	1.1	1.5	1.2	3.6	0.3	0.08 / 0.2
Betamethasone dipropionate	<	<	<	<	<	0.08 / 0.3
6α-Methylprednisolone	<LOQ	<	0.1	1.0	0.2	0.02 / 0.06
6α-Methylprednisolone propionate	1.4	<LOQ	2.4	0.5	4.2	0.2 / 0.5
6α-Methylprednisolone aceponate	<	<	<	<	<	0.3 / 0.5
Dexamethasone	<	<	<	<	<	0.05 / 0.1
Dexamethasone 21-acetate	<	<	<	<	<	0.3 / 0.5
Triamcinolone acetonide	6.3	5.5	17	11	28	0.1 / 0.5
6ß-Hydroxy triamcinolone acetonide	1.2	1.7	6.9	2.3	2.2	0.06 / 0.2
Fluticasone 17-propionate	<LOQ	0.1	0.5	1.0	0.9	0.05 / 0.1
Fluticasone 17-furoate	<LOQ	<	<	<	<LOQ	0.05 / 0.2
Flumetasone	<	<	<	<	<	0.05 / 0.1
Flumetasone 21-pivalate	<	<	<	<	<	0.04 / 0.1
Fluorometholone	<	<	<	<	<	0.05 / 0.3
Mometasone	<	<	<	<	<	1.0 / 2.0
Mometasone 17-furoate	0.8	1.2	1.7	2.2	1.4	0.08 / 0.3
Fluocinolone acetonide	0.1	0.1	0.1	0.2	0.2	0.03 / 0.1
Clobetasol	<	<	<	<	<	0.2 / 0.5
Clobetasol propionate	0.5	0.8	2.1	4.0	5.4	0.08 / 0.3
Budesonide	<	<	1.2	2.0	<	0.5 / 1.0
6ß-Hydroxy budesonide	<	<	<	<	<	0.2 / 0.5

Substance	Concentration [ng/L] Surface water											LOD/LOQ
	SW-1a	SW-1b	SW-2a	SW-2b	SW-3a	SW-3b	SW-4a	SW-4b	SW-5	SW-6	SW-7	
Mineralocorticoids (MC)												
Canrenone	<	3.0	1.6	1.6	<	8.3	0.5	1.2	2.9	1.8	0.6	0.08 / 0.2
7α-Thiomethyl spironolactone	<	0.1	0.2	0.3	<	1.3	0.03	0.2	0.6	0.2	0.07	0.01 / 0.03
11α-Hydroxy canrenone	<	0.4	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Fludrocortisone	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Fludrocortisone acetate	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Glucocorticoids (GC)												
Beclomethasone	<	<	<	<	<	<	<	<	0.07	<	<	0.02 / 0.05
Beclomethasone propionate	<	<LOQ	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Beclomethasone dipropionate	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.2
Betamethasone	<	0.5	0.2	0.2	<	0.4	<LOQ	0.05	1.0	0.3	0.1	0.02 / 0.05
Betamethasone 21-acetate	<	<	<	<	<	<	<	<	<	<	<	0.03 / 0.1
Betamethasone valerate	<	0.9	<LOQ	0.2	<	0.7	<	<LOQ	0.2	1.3	<	0.03 / 0.2
Betamethasone propionate	<	0.9	0.05	0.2	<	0.6	<LOQ	0.07	0.4	1.2	<LOQ	0.02 / 0.05
Betamethasone dipropionate	<	<	<	<	<	<LOQ	<	<	<	<	<	0.02 / 0.2
6α-Methylprednisolone	<	<LOQ	<LOQ	<LOQ	<	0.2	<LOQ	0.05	0.2	<LOQ	<LOQ	0.01 / 0.05
6α-Methylprednisolone propionate	<	0.9	<	<	<	1.3	<	<LOQ	0.9	0.6	<	0.06 / 0.2
6α-Methylprednisolone aceponate	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.1
Dexamethasone	<	<	<	<	<	<	<	<	<	<LOQ	<	0.02 / 0.05
Dexamethasone 21-acetate	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.07
Triamcinolone acetonide	0.04	4.4	0.7	1.0	<	12	0.09	1.5	7.6	8.5	0.3	0.01 / 0.04
6ß-Hydroxy triamcinolone acetonide	<	0.9	0.1	0.2	<	5.1	<LOQ	0.6	1.2	0.8	<	0.03 / 0.05

Appendix A

| Substance | Concentration [ng/L] Surface water | | | | | | | | | | | LOD/LOQ |
	SW-1a	SW-1b	SW-2a	SW-2b	SW-3a	SW-3b	SW-4a	SW-4b	SW-5	SW-6	SW-7	
Fluticasone 17-propionate	<	<LOQ	<LOQ	<LOQ	<	0.4	<LOQ	<LOQ	0.3	0.2	<LOQ	0.05 / 0.1
Fluticasone 17-furoate	<	<LOQ	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Flumetasone	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.05
Flumetasone 21-pivalate	<	<	<	<	<	<	<	<	<	<LOQ	<	0.02 / 0.05
Fluorometholone	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.03
Mometasone	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Mometasone 17-furoate	<	0.6	<LOQ	<LOQ	<	1.0	<	<LOQ	0.2	0.8	<	0.05 / 0.2
Fluocinolone acetonide	<	0.09	<LOQ	<LOQ	<	0.1	<LOQ	<LOQ	0.09	0.1	<	0.02 / 0.05
Clobetasol	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Clobetasol propionate	<	0.4	0.1	0.2	<	3.4	<LOQ	0.3	1.7	0.2	0.05	0.02 / 0.05
Budesonide	<	<	<	<	<	0.7	<	<LOQ	<	<LOQ	<	0.3 / 0.5
6ß-Hydroxy budesonide	<	<	<	<	<	<	<	<	<	<LOQ	<	0.05 / 0.1
Ciclesonide	<	<	<	<	<	<	<	<	<	<	<	0.03 / 0.05
Desisobutyryl ciclesonide	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
6ß-Hydroxy dexamethasone	<	<	<	<	<	<	<	<	<	<	<	0.01 / 0.02
Diflucortolone valerate	<	<	<	<	<	<	<	<	<	<	<	0.01 / 0.02
Halcinonide	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.1
Halomethasone	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Prednisolone	0.2	0.05	0.2	0.07	0.07	0.4	0.1	0.06	<LOQ	0.05	0.1	0.02 / 0.05
Prednisone	<	<LOQ	<LOQ	<LOQ	<	<LOQ	<	<LOQ	<	0.05	<	0.03 / 0.05
Cortisol (Hydrocortisone)	0.2	0.7	1.3	1.3	0.2	1.3	0.3	0.4	0.2	1.0	0.6	0.02 / 0.08
Cortisone	0.1	0.2	0.3	0.4	0.2	0.7	0.2	0.3	0.08	0.2	0.6	0.01 / 0.02
Progestogens (PG)												
Dienogest	<	2.3	0.08	0.2	<	2.0	<	0.3	<	0.1	0.05	0.02 / 0.05
6ß-Hydroxy dienogest	<	<	<	<	<	0.4	<	<	<	0.5	<	0.05 / 0.1
Norethisterone	<	<	<	<	<	<	<	<	<	<LOQ	<	0.1 / 0.3
Norethisterone acetate	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Drospirenone	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5

Appendix A

Substance	Concentration [ng/L] Surface water											LOD/LOQ
	SW-1a	SW-1b	SW-2a	SW-2b	SW-3a	SW-3b	SW-4a	SW-4b	SW-5	SW-6	SW-7	
Etonogestrel	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Gestodene	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Cyproterone	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Cyproterone acetate	<	0.6	<LOQ	0.2	<	2.6	<	0.3	0.9	0.6	<	0.05 / 0.2
Chlormadinone	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.5
Chlormadinone acetate	<	<	<	<	<	0.1	<	<	<	<	<	0.05 / 0.1
Levonorgestrel	<	<	<	<	<	0.5	<	<	<	0.7	<	0.05 / 0.3
Medroxy progesterone	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Medroxy progesterone acetate	<	0.1	<LOQ	<	<	0.1	<	<	<	<	<	0.05 / 0.1
6ß-Hydroxy medroxy progesterone acetate	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Megestrol	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.4
Megestrol acetate	<	<	<	<LOQ	<	<	<	<	<	<	<	0.05 / 0.2
17α-Hydroxy progesterone	<	0.6	<	<LOQ	<	<LOQ	<	<	<	0.6	<	0.3 / 0.5

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	Concentration [ng/L] Surface water											
Substance	SW-8	SW-9a	SW-9b	SW-10a	SW-10b	SW-10c	SW-10d	SW-10e	SW-10f	SW-11	SW-12	LOD/LOQ
Mineralocorticoids (MC)												
Canrenone	0.4	0.8	1.0	<LOQ	0.2	0.2	0.5	0.2	<LOQ	0.2	0.2	0.08 / 0.2
7α-Thiomethyl spironolactone	0.08	0.3	0.2	<LOQ	<LOQ	<LOQ	0.05	0.03	<LOQ	<LOQ	<LOQ	0.01 / 0.03
11α-Hydroxy canrenone	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Fludrocortisone	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Fludrocortisone acetate	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Glucocorticoids (GC)												
Beclomethasone	<	<	<LOQ	<	<	<	<	<	<	<	<	0.02 / 0.05
Beclomethasone propionate	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Beclomethasone dipropionate	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.2
Betamethasone	0.1	0.1	0.09	<	<	<	<	<LOQ	<	<LOQ	<LOQ	0.02 / 0.05
Betamethasone 21-acetate	<	<	<	<	<	<	<	<	<	<	<	0.03 / 0.1
Betamethasone valerate	<LOQ	<LOQ	<LOQ	<	<	<	<LOQ	<	<	<	<	0.03 / 0.2
Betamethasone propionate	<	0.07	<LOQ	<	<	<LOQ	0.09	<LOQ	<	<	<	0.02 / 0.05
Betamethasone dipropionate	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.2
6α-Methylprednisolone	<LOQ	<LOQ	<LOQ	<	<	<	<LOQ	<	<	<	<	0.01 / 0.05
6α-Methylprednisolone propionate	<	<	<	<	<	<	<	<	<	<	<	0.06 / 0.2
6α-Methylprednisolone aceponate	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.1
Dexamethasone	<	<	<LOQ	<	<	<	<	<	<	<	<	0.02 / 0.05
Dexamethasone 21-acetate	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.07
Triamcinolone acetonide	0.6	0.3	0.3	<LOQ	0.06	0.06	0.3	0.07	0.08	0.05	0.1	0.01 / 0.05
6ß-Hydroxy triamcinolone acetonide	0.1	0.08	0.08		<LOQ	<LOQ	0.05		<LOQ			0.03 / 0.05

Appendix A

Substance	Concentration [ng/L] Surface water											
	SW-8	SW-9a	SW-9b	SW-10a	SW-10b	SW-10c	SW-10d	SW-10e	SW-10f	SW-11	SW-12	LOD/LOQ
Fluticasone 17-propionate	<	<LOQ	<	<	<	<	<LOQ	<	<	<	<	0.05 / 0.1
Fluticasone 17-furoate	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Flumetasone	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.05
Flumetasone 21-pivalate	<	0.05	<	<	<	<	<	<	<	<	<	0.02 / 0.05
Fluorometholone	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.03
Mometasone	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Mometasone 17-furoate	<	<LOQ	<	<	<	<	<	<	<	<	<	0.05 / 0.2
Fluocinolone acetonide	<	<LOQ	<LOQ	<	<	<	<	<	<	<	<	0.02 / 0.05
Clobetasol	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Clobetasol propionate	0.1	0.1	0.09	<LOQ	<	<	0.06	<LOQ	<	<	<	0.02 / 0.05
Budesonide	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
6ß-Hydroxy budesonide	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Ciclesonide	<	<	<	<	<	<	<	<	<	<	<	0.03 / 0.05
Desisobutyryl ciclesonide	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
6ß-Hydroxy dexamethasone	<	<	<	<	<	<	<	<	<	<	<	0.01 / 0.02
Diflucortolone valerate	<	<	<	<	<	<	<	<	<	<	<	0.01 / 0.02
Halcinonide	<	<	<	<	<	<	<	<	<	<	<	0.02 / 0.1
Halomethasone	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Prednisolone	0.07	<LOQ	0.05	<LOQ	<LOQ	0.05	0.08	0.05	<LOQ	0.05	0.09	0.02 / 0.05
Prednisone	<LOQ	0.05	<LOQ	<	<	<	<LOQ	<	<	<	<	0.03 / 0.05
Cortisol (Hydrocortisone)	0.7	1.3	1.2	0.3	0.2	0.5	0.3	0.1	0.2	0.7	0.2	0.02 / 0.08
Cortisone	0.7	1.0	0.8	0.3	0.1	0.4	0.1	0.02	0.06	0.2	0.4	0.01 / 0.02
Progestogens (PG)												
Dienogest	0.05	0.09	0.06	<	<	<	<LOQ	<LOQ	<	<	<	0.02 / 0.05
6ß-Hydroxy dienogest	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Norethisterone	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Norethisterone acetate	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Drospirenone	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5

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Substance	Concentration [ng/L] Surface water											
	SW-8	SW-9a	SW-9b	SW-10a	SW-10b	SW-10c	SW-10d	SW-10e	SW-10f	SW-11	SW-12	LOD/LOQ
Etonogestrel	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Gestodene	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5
Cyproterone	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.3
Cyproterone acetate	<	<	<LOQ	<	<	<	<	<	<	<	<	0.05 / 0.2
Chlormadinone	<	<	<	<	<	<	<	<	<	<	<	0.1 / 0.5
Chlormadinone acetate	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Levonorgestrel	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.3
Medroxy progesterone	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Medroxy progesterone acetate	<	<LOQ	<	<	<	<	<	<	<	<	<	0.05 / 0.1
6ß-Hydroxy medroxy progesterone acetate	<	<	<	<	<	<	<	<	<	<	<	0.05 / 0.1
Megestrol	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.4
Megestrol acetate	<	<	<LOQ	<	<	<	<	<	<	<	<	0.05 / 0.2
17α-Hydroxy progesterone	<	<	<	<	<	<	<	<	<	<	<	0.3 / 0.5

Appendix A

Table A.5. Recovery rates (corrected by internal standards) and reproducibility (expressed as 95%-confidence intervals) for the target steroid hormones in surface water at concentration levels 0.05, 0.25, 0.5 and 5 ng/L and WWTP effluent at concentration levels 0.5 ng/L, 1.0 ng/L, 10 ng/L and 50 ng/L. When the initial concentrations of the analytes were higher than the spike level, the recoveries were not determined. (- = spike below initial conc., <LOD= below limit of detection).

Abbreviation	Substance	Surface water (1 L river Rhine) Recovery [%], (n=4)				WWTP effluent (0.5 L WWTP 4) Recovery [%], (n=4)			
		c=0.05 ng/L	c=0.25 ng/L	c=0.5 ng/L	c=5 ng/L	c=0.5 ng/L	c=1.0 ng/L	c=10 ng/L	c=50 ng/L
Mineralocorticoids (MC)									
CAN	**Canrenone**	-	102±8	107±8	116±11	-	-	-	101±4
SPL-m1	**7α-Thiomethyl spironolactone**	-	95±7	98±8	105±9	-	-	111±12	108±10
CAN-m1	**11α-Hydroxy canrenone**	<LOD	89±13	80±16	80±5	<LOD	78±9	80±6	83±8
FLC	**Fludrocortisone**	86±32	86±23	94±17	92±10	73±16	71±7	76±5	75±8
FLCac	**Fludrocortisone acetate**	<LOD	87±11	93±6	85±10	85±9	99±8	103±7	100±7
Glucocorticoids (GC)									
TRIact	**Triamcinolone acetonide**	-	-	104±11	110±12	-	-	-	102±7
TRIact-m1	**6ß-Hydroxy triamcinolone acetonide**	-	101±5	104±7	108±9	-	-	105±6	93±6
FLUprop	**Fluticasone 17-propionate**	98±24	94±12	95±10	104±7	-	97±17	107±8	105±7
FLUfur	**Fluticasone 17-furoate**	106±26	93±12	89±8	97±10	101±11	105±11	98±5	104±7
FMS	**Flumetasone**	103±13	102±4	101±8	108±10	109±4	106±8	112±9	104±8
FMSpiv	**Flumetasone pivalate**	103±10	106±6	99±14	101±5	101±6	102±6	104±4	102±10
FLM	**Fluorometholone**	103±14	99±6	91±7	92±6	92±6	86±9	88±3	88±6
MOM	**Mometasone**	<LOD	<LOD	69±19	93±8	<LOD	89±14	97±9	105±10
MOMfur	**Mometasone 17-furoate**	111±28	97±8	95±7	92±13	-	-	114±11	111±14
FCNact	**Fluocinolone acetonide**	107±13	97±2	95±5	96±11	96±10	95±6	101±3	91±4
CLO	**Clobetasol**	109±13	102±21	93±16	105±21	100±5	95±11	105±9	107±8
CLOprop	**Clobetasol propionate**	-	97±9	101±8	106±6	-	-	109±13	115±11
BDN	**Budesonide**	<LOD	108±23	101±10	104±3	-	-	93±9	99±4
BDN-m1	**6ß-Hydroxy budesonide**	102±17	102±13	101±10	102±18	99±9	100±10	101±5	90±8
CIC	**Ciclesonide**	71±10	85±14	73±8	81±19	62±4	65±5	74±11	77±6
CIC-m1	**Desisobutyryl ciclesonide**	<LOD	99±8	88±11	98±10	77±21	97±7	107±11	107±9
DMS-m1	**6ß-Hydroxy dexamethasone**	75±9	99±26	99±20	101±14	88±6	100±17	95±16	90±6
DFCval	**Diflucortolone valerate**	93±12	95±23	89±13	87±13	93±12	98±15	102±5	101±3

HAL	**Halcinonide**	106±2	111±6	106±14	103±9	102±2	97±11	102±9	101±4
HLM	**Halomethasone**	104±25	101±15	93±14	102±19	87±6	80±21	82±9	97±16
PNL	**Prednisolone**	-	86±2	87±23	102±11	-	91±16	98±5	102±9
PNS	**Prednisone**	72±19	76±6	71±7	74±31	68±12	72±16	75±11	76±12
HCOR	**Cortisol (Hydrocortisone)**	-	-	100±8	113±11	-	-	109±9	112±8
COR	**Cortisone**	-	103±9	84±7	71±14	-	68±2	70±13	72±10
Progestogens (PG)									
DIE	**Dienogest**	106±14	106±5	103±17	101±12	-	-	97±13	107±9
DIE-m1	**6ß-Hydroxy dienogest**	108±17	99±17	88±21	93±16	-	83±5	83±6	85±5
NES	**Norethisterone**	<LOD	101±19	97±16	101±13	<LOD	97±5	103±15	105±9
NESac	**Norethisterone acetate**	<LOD	93±11	90±10	91±7	102±10	96±7	99±4	107±1
DPN	**Drospirenone**	<LOD	98±17	94±21	99±7	100±14	107±7	107±4	103±9
ETG	**Etonogestrel**	<LOD	85±4	93±15	92±13	<LOD	95±19	92±9	95±10
GES	**Gestodene**	<LOD	106±29	109±18	122±17	<LOD	<LOD	86±11	99±9
CYP	**Cyproterone**	<LOD	93±15	94±14	104±11	101±22	99±17	103±5	107±11
CYPac	**Cyproterone acetate**	-	97±10	110±13	113±3	-	-	105±9	108±7
CLM	**Chlormadinone**	<LOD	96±3	91±8	89±5	<LOD	97±21	89±7	97±9
CLMac	**Chlormadinone acetate**	122±22	99±6	96±12	111±11	90±11	86±13	100±10	106±7
LNG	**Levonorgestrel**	<LOD	81±12	80±18	96±11	95±8	92±23	89±8	98±7
MRP	**Medroxy progesterone**	77±35	88±16	86±17	92±4	110±11	95±6	97±9	103±5
MRPac	**Medroxy progesterone acetate**	101±24	91±8	88±10	97±3	84±8	78±6	88±9	90±4
MRPac-m1	**6ß-Hydroxy medroxy progesterone acetate**	115±16	98±18	89±15	90±8	92±12	95±11	91±2	88±3
MEG	**Megestrol**	<LOD	77±10	90±21	107±10	89±19	98±10	87±6	98±5
MEGac	**Megestrol acetate**	89±24	96±8	95±6	98±4	99±8	92±10	87±7	93±9
HPG	**17α-Hydroxy progesterone**	<LOD	103±10	104±2	95±15	-	97±13	106±5	121±5
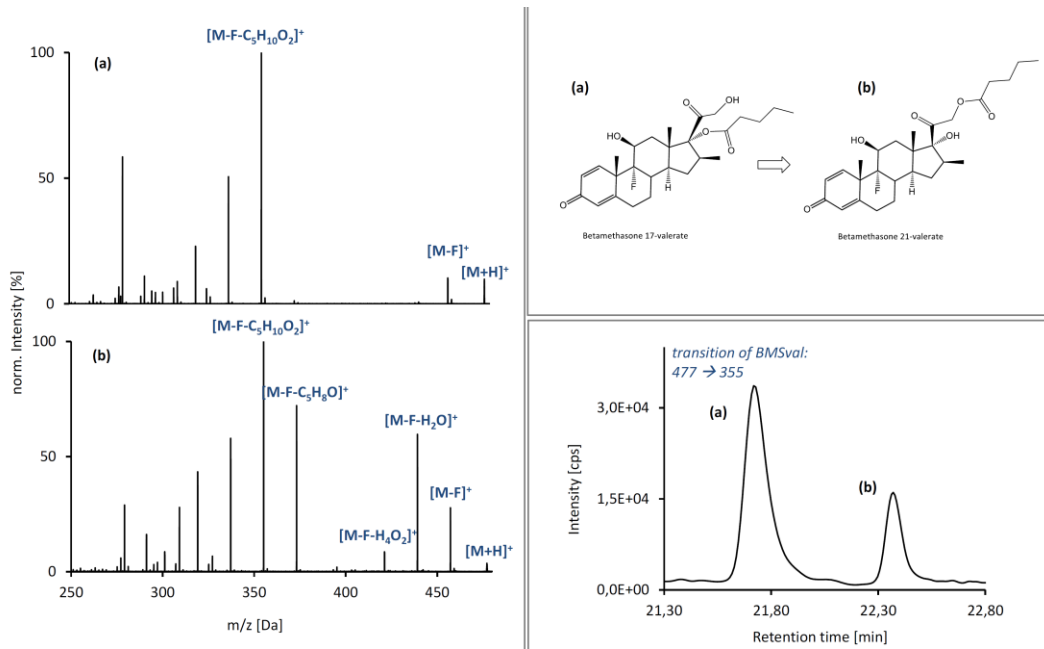
Figure A.9. Chemical structures, extracted ion chromatogram of non-spiked WWTP effluent and high-resolution MS²-spectra of (a) betamethasone 17-valerate and (b) betamethasone 21-valerate.
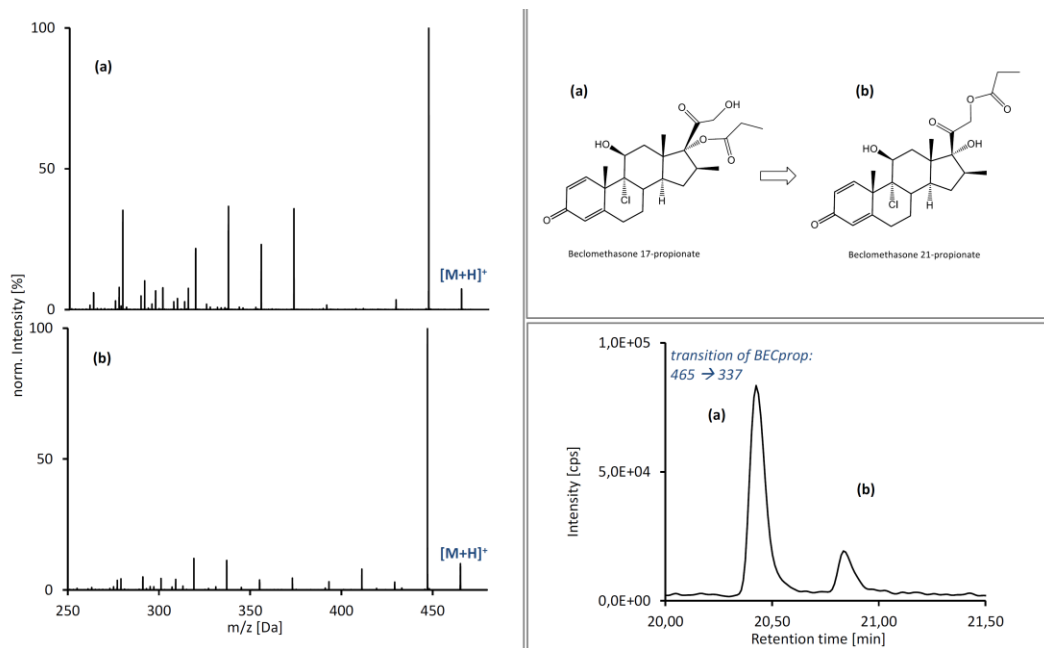
Figure A.10. Chemical structures, extracted ion chromatogram of WWTP effluent (spike-level =5 ng) and high-resolution MS²-spectra of (a) beclomethasone 17-propionate and (b) beclomethasone 21-propionate.
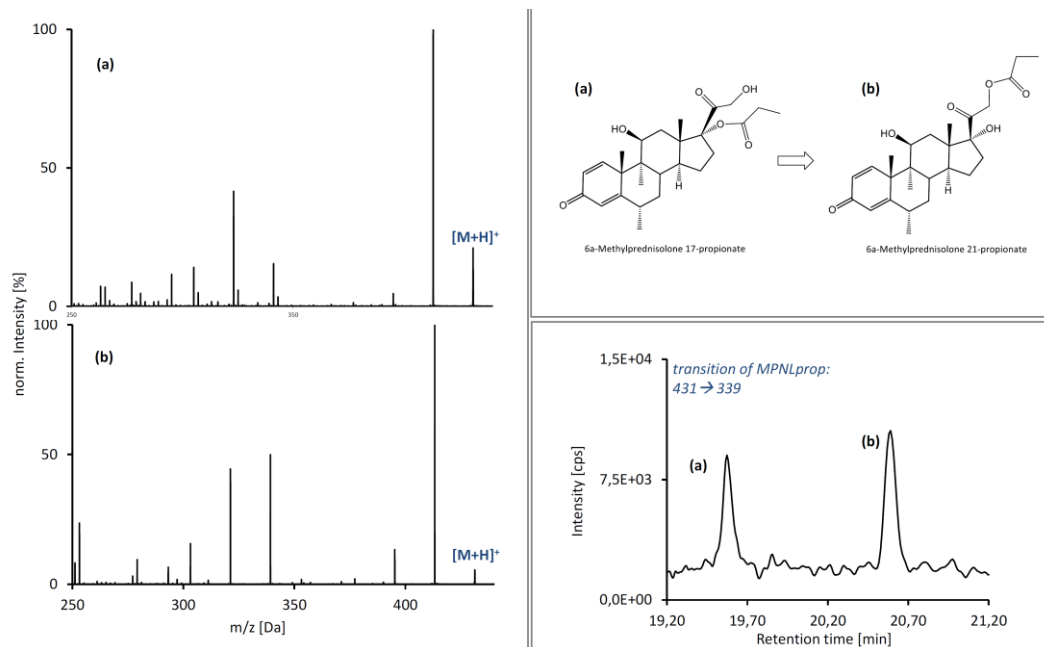
Figure A.11. Chemical structures, extracted ion chromatogram of non-spiked WWTP effluent and high-resolution MS²-spectra of (a) 6α-Methylprednisolone 17-propionate and (b) 6α-Methylprednisolone 21-propionate.

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Appendix B-Supplementary Data for Chapter 3

Analysis of the Aerobic Biodegradation of Glucocorticoids: Elucidation of the Kinetics and Transformation Reactions

OUTLINE

Sample Preparation

All samples were collected in cleaned and baked (at 550 °C for 8 h) amber glass bottles. The WWTP effluent samples were cooled down to 4 °C during transport to the laboratory and afterwards filtered using a 1 µm glass fiber filter (Whatman, GF6, Maidstone, United Kingdom). For sample enrichment, 500 mL filtered WWTP effluent was spiked with 2 ng of each surrogate standard prior to SPE. The water samples were loaded onto end-capped C18 cartridges (C18ec, 6 mL, 500 mg, Macherey-Nagel, Düren, Germany), which were preconditioned with 3 x 3 mL methanol followed by 3 x 3 mL Milli-Q. The samples were passed through the cartridges by gravity within 12 h. The cartridges were rinsed with 3 x 2 mL Milli-Q and dried by nitrogen for approximately 2 h. For elution of the extracted analytes 3 x 3 mL methanol was used. Subsequently, the extracts were evaporated to dryness under a gentle stream of nitrogen at 40 °C and were re-dissolved with 300 µL n-hexane and 700 µL acetone for further clean up. If the cartridges were not eluted immediately, they were stored at -20 °C in the dark after drying. Purification was achieved by commercially available silica gel glass cartridges (1 g, 6 mL, Macherey-Nagel). The silica gel was dried for 2 h at 100 °C prior to usage. Polarity and composition of the elution solvent was optimized for the target analytes. The cartridges were preconditioned with 3 x 3 mL n-hexane/acetone (3:7). Afterwards, the sample extracts were loaded onto the cartridges and were eluted three times with 2 mL n-hexane/acetone (3:7). Then, the extracts were evaporated under a gentle stream of nitrogen at 40 °C to dryness and reconstituted with 250 µL methanol and 250 µL Milli-Q for LC-MS/MS analysis.

Chemical Analysis

Target and HRMS analysis were performed with an HPLC system, consisting of a G1367E autosampler, a G1330B cooling thermostat for the autosampler, a G1312B binary HPLC pump, a G1310B isocratic HPLC pump, a G1379B membrane degasser and a G1316A column oven (all Agilent 1260Infinity Series, Waldbronn, Germany). Separation was achieved with a MN Nucleoshell RP 18plus column (2 mm x 150 mm, 2.7 µm) (Macherey-Nagel) with a flow rate of 0.25 mL/min. The injection volume was 10 µL and column oven temperature was set to 40 °C. As mobile phases, Milli-Q water with 0.1% formic acid (A) and acetonitrile with 0.1% formic acid (B) were used.
In order to avoid a co-elution of interfering substances, the LC gradient was optimized as follows: from 0 to 0.5 min 10% B ; from 0.5 min to 15 min gradual increase to 47% B; then

B was linearly increased up to 98% in 5 min and held for 10 min; finally returning to 10% B in 0.1 min and held for 5 min for equilibration at the end of each chromatographic run, in total 35 min. The HPLC system was coupled to either a triple-quadrupole mass spectrometer system (QqQ-LIT-MS, API 6500 QTrap, Sciex, Darmstadt, Germany) for target analysis or to a hybrid quadrupole time of flight mass spectrometer (QTOF) (SCIEX TripleTOF 5600, Sciex).

The general MS parameters for the target analysis in both polarizations were: ion source gas 1 (GS1) and ion source gas 2 (GS2) 35 psi; curtain gas (CUR) 45 psi; collision gas (CAD) medium; source temperature (TEM) 400 °C; ion spray voltage for negative and positive ionization mode -4500 V/5500 V; entrance potential (EP) -10 V/10 V; collision cell exit potential (CXP) -14 V/ 14 V.

Target analysis was performed with switching polarities within the chromatographic runs using scheduled multiple reaction monitoring (sMRM) mode. The specific parameters were as follows: MRM detection window 50 s; target scan time 0.6 s and settling time 4 ms.

MS data acquisition was controlled with Analyst 1.6.3 (Sciex). For identification and quantification, the two most sensitive MRM transitions of each analyte were monitored.

Appendix B

LC-MS/MS parameter for target analysis

Table B.1. LC-MS/MS detection method and further information of steroid hormones investigated. (TRC = Toronto Research Chemicals, Canada Ontario; TLC = Santa Cruz Biotechnology, USA Texas; SA = Sigma-Aldrich, Germany Munich)

Abbreviation	Substance	Supplier	CAS-No.	Chemical formula	Internal standard used for correction	Adduct	Precursor [Da]	Fragment mass [Da]	Collision energy [V]	Declustering potential [V]
BEC	Beclomethasone	SA	4419-39-0	$C_{22}H_{29}ClO_5$	d5-DMS	$[M+HCOO]^-$	453	377 / 297	-20 / -34	-10
BECprop17	Beclomethasone 17-propionate	TRC	5534-18-9	$C_{25}H_{33}ClO_6$	d5-CLOprop	$[M+H]^+$	465	355 / 337	16 / 20	40
BECprop21	Beclomethasone 21-propionate	TRC	69224-79-9	$C_{25}H_{33}ClO_6$	d5-CLOprop	$[M+H]^+$	465	355 / 337	16 / 20	40
BECdiprop	Beclomethasone 17,21-dipropionate	TRC	5534-09-8	$C_{28}H_{37}ClO_7$	d5-CLOprop	$[M+H]^+$	521	411 / 319	15 / 25	70
TP428a	Beclomethasone 9,11-epoxy 17-propionate	TRC	79578-39-5	$C_{25}H_{32}O_6$	d5-CLOprop	$[M+H]^+$	429	355 / 279	16 / 26	10
TP428b	Beclomethasone 9,11-epoxy 21-propionate	TRC	205105-83-5	$C_{25}H_{32}O_6$	d5-CLOprop	$[M+H]^+$	429	355 / 279	16 / 26	10
TP372	Beclomethasone 9,11-epoxide	TRC	981-34-0	$C_{22}H_{28}O_5$	d5-DMS	$[M+HCOO]^-$	417	341 / 121	-17 / -45	-10
BMS	Betamethasone	SA	378-44-9	$C_{22}H_{29}FO_5$	d5-DMS	$[M+HCOO]^-$ / $[M+H]^+$	437 / 393	361 / 373	-23 / 17	-10 / 70
BMSval17	Betamethasone 17-valerat	SA	2152-44-5	$C_{27}H_{37}FO_6$	d5-CLOprop	$[M+H]^+$	477	355 / 337	18 / 20	60
BMSval21	Betamethasone 21-valerat	TRC	2240-28-0	$C_{27}H_{37}FO_6$	d5-CLOprop	$[M+H]^+$	477	355 / 337	18 / 20	60
BMSprop17	Betamethasone 17-propionat	TRC	5534-13-4	$C_{25}H_{33}FO_6$	d5-CLOprop	$[M+H]^+$	449	429 / 355	11 / 16	70
BMSprop21	Betamethasone 21-propionat	TRC	75883-07-7	$C_{25}H_{33}FO_6$	d5-CLOprop	$[M+H]^+$	449	429 / 355	11 / 16	70
BMSdiprop	Betamethasone 17,21-dipropionate	SA	5593-20-4	$C_{28}H_{37}FO_7$	d5-CLOprop	$[M+H]^+$	505	411 / 485	17 / 14	50
OxoBMS	17-Oxo betamethasone	TRC	3109-01-1	$C_{20}H_{25}FO_3$	d6-NES	$[M+H]^+$	333	295 / 313	15 / 12	60
BDN	Budesonide	SA	51333-22-3	$C_{25}H_{34}O_6$	d8-BDN	$[M+H]^+$	431	323 / 147	20 / 35	30
TP432b	1,2-Dihydro budesonide	TRC	137174-25-5	$C_{25}H_{36}O_6$	d8-BDN	$[M+H]^+$ / $[M+HCOO]^-$	433 / 477	325 / 359	25 / -19	40 / -40
TP444	Budesonide impurity 1 (budesonide 21-carboxylic acid)	TLC	-	$C_{25}H_{32}O_7$	d3-FLUprop-m1 / d8-BDN	$[M-H]^-$ / $[M+H]^+$	443 / 445	299 / 226	-35 / 35	-20 / 50
CLOprop	Clobetasol 17-propionate	SA	25122-46-7	$C_{25}H_{32}ClFO_5$	d5-CLOprop	$[M+H]^+$	467	373 / 355	16 / 20	50
DH-CLOprop	1,2-Dihydro clobetasol propionate	TRC	25120-99-4	$C_{25}H_{34}ClFO_5$	d5-CLOprop	$[M+H]^+$	469	395 / 375	27 / 27	150
HCOR	Cortisol (Hydrocortisone)	SA	50-23-7	$C_{21}H_{30}O_5$	d8-PNL	$[M+HCOO]^-$ / $[M+H]^+$	407 / 363	331 / 121	-23 / 32	-20 / 110
FCNact	Fluocinolone acetonide	SA	67-73-2	$C_{24}H_{30}F_2O_6$	13C3-FCNact	$[M+H]^+$	453	413 / 433	17 / 13	80
TP454b	1,2-Dihydro fluocinolone acetonide	TRC	1178-54-7	$C_{24}H_{32}F_2O_6$	13C3-FCNact	$[M+H]^+$	455	415 / 435	15 / 13	120
TP466	Fluocinolone acetonide 21-carboxylic acid	TRC	106931-78-6	$C_{24}H_{28}F_2O_7$	13C3-FCNact	$[M+H]^+$	467	447 / 427	15 / 19	60
FLUprop	Fluticasone 17-propionate	SA	80474-14-2	$C_{25}H_{31}F_3O_5S$	d5-FLUprop	$[M+H]^+$	501	313 / 293	20 / 25	80
TP502b	1,2-Dihydro fluticasone propionate	TRC	105613-90-9	$C_{25}H_{33}F_3O_5S$	d5-FLUprop	$[M+H]^+$	503	335 / 315	27 / 30	160
TP452 (FLUprop-m1)	Fluticasone 17β-carboxylic acid propionate	TRC	65429-42-7	$C_{24}H_{30}F_2O_6$	d3-FLUprop-m1	$[M-H]^-$ / $[M+H]^+$	451 / 453	395 / 433	-25 / 13	0 / 70
MPNL	Methylprednisolone	SA	83-43-2	$C_{22}H_{30}O_5$	d8-PNL	$[M+HCOO]^-$	419	343 / 294	-23 / -47	-10
MPNLacp	Methylprednisolone 17-propionate, 21-acetate	TRC	86401-95-8	$C_{27}H_{36}O_7$	d5-CLOprop	$[M+H]^+$	473	381 / 101	16 / 22	60
MPNLprop17	Methylprednisolone 17-propionate	TLC	79512-61-1	$C_{25}H_{34}O_6$	d5-CLOprop	$[M+H]^+$	431	339 / 253	15 / 32	50
MPNLprop21	Methylprednisolone 21-propionate	TRC	138804-88-3	$C_{25}H_{34}O_6$	d5-CLOprop	$[M+H]^+$	431	339 / 253	15 / 32	50
MOMfur	Mometasone 17-furoate	SA	83919-23-7	$C_{27}H_{30}Cl_2O_6$	d3-MOMfur	$[M+H]^+$	521	355 / 373	23 / 17	50
EpoxyMOMfur	Mometasone 9,11-epoxy furoate (mometasone furoate EP impurity D)	TRC	83881-09-8	$C_{27}H_{29}ClO_6$	d3-MOMfur	$[M+H]^+$	485	373 / 355	16 / 20	0
PNL	Prednisolone	SA	50-24-8	$C_{21}H_{28}O_5$	d8-PNL	$[M+HCOO]^-$	405	329 / 295	-23 / -42	-10
TRIact	Triamcinolone acetonide	SA	76-25-5	$C_{24}H_{31}FO_6$	13C3-TRIact	$[M+H]^+$	435	415 / 397	14 / 20	80
DH-TRIact	1,2-Dihydro triamcinolone acetonide	TRC	1524-86-3	$C_{24}H_{33}FO_6$	d5-DMS / 13C3-TRIact	$[M+HCOO]^-$ /	481 / 437	377 / 341	-35 / 30	0 / 120

Abbreviation	Name	TRC	CAS	Formula	Internal standard	Ionization	Precursor	Product ions		
						$[M+H]^+$				
TP448	Triamcinolone acetonide 21-carboxylic acid	TRC	53962-41-7	$C_{24}H_{29}FO_7$	d3-FLUprop-m1	$[M+H]^+$ / $[M-H]^-$	449 / 447	429 / 249	15 / -65	60 / -50
TRIact-m1	6ß-Hydroxy triamcinolone acetonide	TRC	3869-32-7	$C_{24}H_{31}FO_7$	13C3-TRIact	$[M+H]^+$	451	387 / 329	13 / 20	90
TRIact-m3	6β-Hydroxy 21-oic triamcinolone acetonide	TRC	68263-02-5	$C_{24}H_{29}FO_8$	d7-SPL-m1	$[M+H]^+$	465	427 / 401	15 / 18	50
Internal Standards										
d8-BDN	Budesonide-d8	TRC	-		-	$[M+H]^+$	439	323	19	40
d5-CLOprop	Clobetasol 17-propionate-d5	TRC	-	$C_{25}H_{27}D_5ClFO_5$	-	$[M+H]^+$	472	373	17	70
d5-DMS	Dexamethasone-d5	TRC	-	$C_{22}H_{24}D_5FO_5$	-	$[M+HCOO]^-$ / $[M+H]^+$	442 / 398	364 / 378	-25 / 17	-10 / 70
d8-PNL	Prednisolone-d8	TRC	-	$C_{21}H_{20}D_8O_5$	-	$[M+HCOO]^-$	413	367	-16	-10
d5-FLUprop	Fluticasone 17-propionate-d5	TRC	-	$C_{25}H_{26}D_5F_3O_5S$	-	$[M+H]^+$	506	313	20	80
13C3-TRIact	Triamcinolone acetonide-13C3	TRC	-	$C_{21}{}^{13}C_3H_{31}FO_6$	-	$[M+H]^+$	438	418	15	80
13C3-FCNact	Fluocinolone acetonide-13C3	TRC	-	$C_{21}{}^{13}C_3H_{30}F_2O_6$	-	$[M+H]^+$	456	416	17	90
d3-MOMfur	Mometasone furoate-d3	TRC	-	$C_{27}H_{27}D_3Cl_2O_6$	-	$[M+H]^+$	524	355	23	85
d7-SPL-m1	7α-Thiomethyl spironolactone-d7	TRC	-	$C_{23}H_{25}D_7O_3S$	-	$[M+H]^+$	396	348	25	110
d6-NES	Norethisterone-d6	TRC	-	$C_{20}H_{20}D_6O_2$	-	$[M+H]^+$	305	237	27	100
d3-FLUprop-m1	Fluticasone 17β-carboxylic acid propionate-d3	TRC	-	$C_{25}H_{26}D_5F_3O_5S$	-	$[M+H]^+$ / $[M-H]^-$	456 / 454	436 / 395	13 / -25	40 / -90

Sample Information
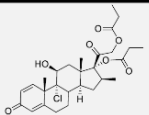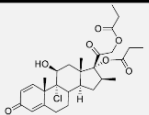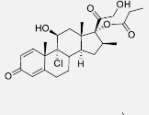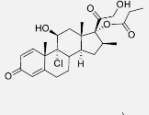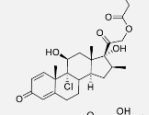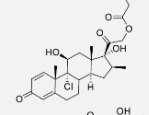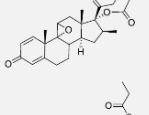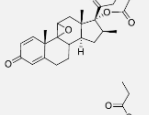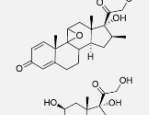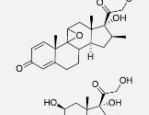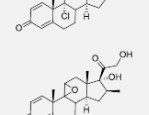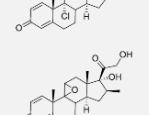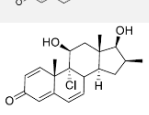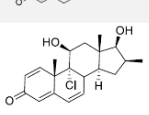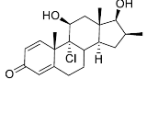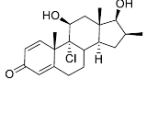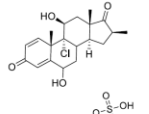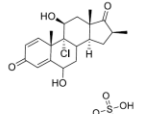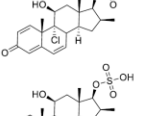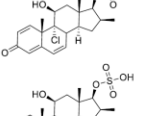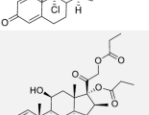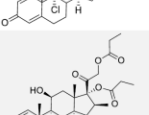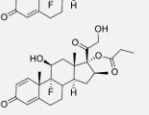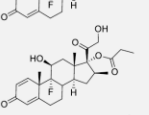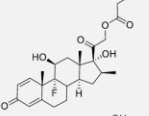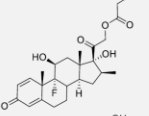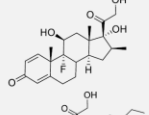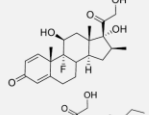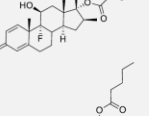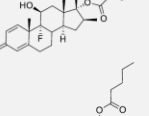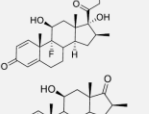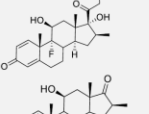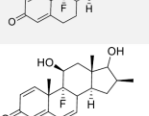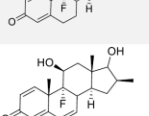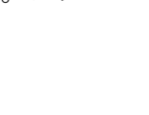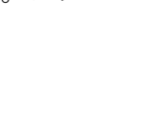
Table B.2. Location, capacities and sampling dates of the analyzed WWTP effluent samples.

Abbreviation	Location, postal code	Capacity (population equivalents)	Sampling Date
WWTP_A	*Kalt, 56294*	*35,000*	*21.03.2019, 18:15*
WWTP_B	*Daxweiler, 55442*	*8,550*	*21.03.2019, 17:15*
WWTP_C	*Groß-Gerau, 64521*	*45,000*	*21.03.2019, 10:30*
WWTP_D	*Griesheim, 64347*	*50,000*	*21.03.2019, 12:15*
WWTP_E	*Bingen, 55411*	*80,000*	*21.03.2019, 15:30*
WWTP_F	*Mayen, 56727*	*30,000*	*21.03.2019, 18:15*
WWTP_G	*Frankfurt, 60528*	*1,350,000*	*01.04.2019, 12:30*
WWTP_H	*Koblenz,56070*	*320,000*	*01.04.2019, 16:00*

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List of Detected TPs and Parent GCs
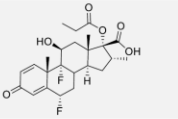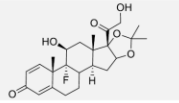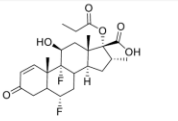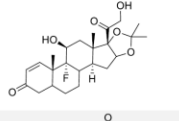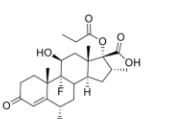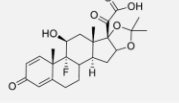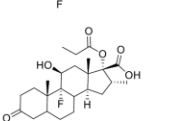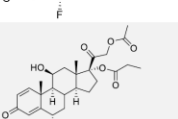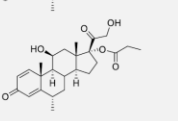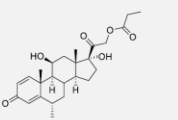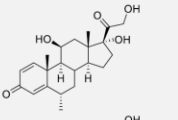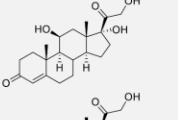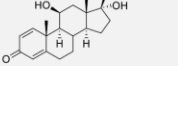
Table B.3. Overview of the detected TPs. Steroid structures highlighted in grey are confirmed by authentic reference standards. The conducted experiments are bolded in the table.

Name, chem. Formula, accurate mass, RT	Structure	Identification	Detected in Experiment
Beclomethasone dipropionate C28H37ClO7 520.221 Da (Δ 3.6 ppm) RT 22.1 min		Reference standard	T1
Beclomethasone 17-propionate C25H33ClO6 464.196 Da (Δ 1.8 ppm) RT 20.3 min		Reference standard	T1, K2, S2
Beclomethasone 21-propionate C28H37ClO7 464.196 Da (Δ 2.0 ppm) RT 20.6 min		Reference standard	T1, K2, S2
TP428a C25H32O6 428.220 Da (Δ 0.9 ppm) RT 20.6 min		Reference standard	T1, K2, S2
TP428b C25H32O6 428.222 Da (Δ -4.0 ppm) RT 21.1 min		Reference standard	T1, K2, S2
Beclomethasone C22H29ClO5 408.170 Da (Δ 1.2 ppm) RT 16.8 min		Reference standard	T1, K1, K2, S1, S2
TP372 C22H28O5 372.194 Da (Δ 0.1 ppm) RT 17.5 min		Reference standard	T1, K1, K2, S1, S2
TP348b C20H25ClO3 348.150 Da (Δ -2.9 ppm) RT 17.0 min		Isotopic pattern, MS², RT shift (proposed structure)	K1, K2, T1
TP350 C20H27ClO3 350.166 Da (Δ -1.9 ppm) RT 17.7 min		Isotopic pattern, MS², RT shift (proposed structure)	T1
TP364 C20H25ClO4 364.145 Da (Δ -1.3 ppm) RT 14.7 min		Isotopic pattern, MS², RT shift (proposed structure)	T1
TP428c C20H25ClO6S 427.099 Da (Δ -0.8 ppm) RT 17.2 min		Isotopic pattern, MS² (proposed structure)	T1
TP430 C20H27ClO6S 431.127 Da (Δ -1.6 ppm) RT 17.6 min		Isotopic pattern, MS² (proposed structure)	T1
Betamethasone dipropionate C28H37FO7 504.252 Da (Δ 1.6 ppm) RT 21.9 min		Reference standard	K2, S2, **T2**
Betamethasone 17-propionate C25H33FO6 448.225 Da (Δ 2.0 ppm) RT 19.8 min		Reference standard	K2, S2, T2
Betamethasone 21-propionate C25H33FO6 448.226 Da (Δ 1.1 ppm) RT 20.4 min		Reference standard	K2, S2, T2
Betamethasone C22H29FO5 392.200 Da (Δ 0.5 ppm) RT 16.2 min		Reference standard	K1, K2, S1, S2, **T3**, T4
Betamethasone 17-valerate C27H37FO6 476.257 Da (Δ 1.2 ppm) RT 21.2 min		Reference standard	K2, S2, **T4**
Betamethasone 21-valerate C27H37FO6 476.256 Da (Δ 2.7 ppm) RT 21.7 min		Reference standard	K2, S2, T4
17-Oxo betamethasone C20H25FO3 332.179 Da (Δ -2.0 ppm) RT 19.0 min		Reference standard	T2, T3, T4, **T5**
TP332 C20H25FO3 332.178 Da (Δ 2.5 ppm) RT 15.9 min		Isotopic pattern, MS², RT shift, comparison with reference standard of 17-oxo betamethasone (proposed structure)	K1, K2, T2, T3, T4, T5

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TP348a C20H25FO4 348.173 Da (Δ 1.0 ppm) RT 13.6 min	Isotopic pattern, MS², RT shift (proposed structure)	T2, T3, T4, T5
TP412 C20H25FO6S 412.135 Da (Δ 0.4 ppm) RT 16.1 min	Isotopic pattern, MS² (proposed structure)	T2, T3, T4, T5
TP414 C20H27FO6S 414.151 Da (Δ 0.8 ppm) RT 16.5 min	Isotopic pattern, MS² (proposed structure)	T2, T3, T4, T5
Budesonide C25H34O6 430.236 Da (Δ -0.9 ppm) RT 19.8 min	Reference standard	K2, S2, **T14**
TP432a (4,5-dihydro budesonide) C25H36O6 432.251 Da (Δ 0.4 ppm) RT 20.9 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro isomer	T14
TP432b (1,2-dihydro budesonide) C25H36O6 432.251 Da (Δ -0.1 ppm) RT 20.2 min	Reference standard	T14
TP434 C25H38O6 434.267 Da (Δ 0.2 ppm) RT 21.1 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro budesonide	T14
TP444 C25H32O7 444.216 Da (Δ -1.8 ppm) RT 19.6 min	Reference standard	K2, T14
Clobetasol propionate C25H32ClFO5 466.192 Da (Δ 1.3 ppm) RT 21.6 min	Reference standard	K1, S1, **T6**, T7
TP468 (4,5-dihydro clobetasol propionate) C25H34ClFO5 468.206 Da (Δ 3.1 ppm) RT 22.3 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro isomer	K1, T6, T7
TP470 C25H36ClFO5 470.224 Da (Δ -1.9 ppm) RT 22.5 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro clobetasol propionate	K1, T6, T7
TP486 C25H36ClFO6 486.220 Da (Δ -3.2 ppm) RT 21.8 min	Isotopic pattern, MS², RT shift (proposed structure)	T6, T7

1,2-Dihydro clobetasol propionate C25H34ClFO5 468.207 Da (Δ 2.3 ppm) RT 21.9 min	Reference standard	**T7**
Fluocinolone acetonide C24H30F2O6 452.201 Da (Δ 0.5 ppm) RT 17.7 min	Reference standard	K2, S2, **T1**
TP454a (4,5-dihydro fluocinolone acetonide) C24H32F2O6 454.216 Da (Δ 2.6 ppm) RT 18.7 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro isomer	K2, T15
TP454b (1,2-dihydro fluocinolone acetonide) C24H32F2O6 454.217 Da (Δ 0.4 ppm) RT 18.3 min	Reference standard	T15
TP456 C24H34F2O6 456.233 Da (Δ -1.0 ppm) RT 18.8 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro fluocinolone acetonide	T15
TP466 C24H28F2O7 466.180 Da (Δ 1.5 ppm) RT 17.6 min	Reference standard	K2, T15
Fluticasone propionate C25H31F3O5S 500.182 Da (Δ 4.0 ppm) RT 21.6 min	Reference standard	K1, S1, **T8**
TP502a (4,5-dihydro fluticasone propionate) C25H33F3O5S 502.198 Da (Δ 4.3 ppm) RT 22.0 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro isomer	T8, T9
TP502b (1,2-Dihydro fluticasone propionate) C25H33F3O5S 502.198 Da (Δ 4.7 ppm) RT 21.8 min	Reference standard	T8, **T9**
TP504 C25H35F3O5S 504.206 Da (Δ 0.6 ppm) RT 22.1 min	Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro fluticasone propionate	T8, T9

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TP452 (Fluticasone propionate 17β-carboxylic acid) C24H30F2O6 452.199 Da (Δ 3.7 ppm) RT 19.5 min		Reference standard	K1, S1, T8, T9, **T10**
TP454a (4,5-dihydro fluticasone propionate 17β-carboxylic acid) C24H32F2O6 454.218 Da (Δ -3.3 ppm) RT 20.0 min		Isotopic pattern, MS², RT shift, comparison with fluticasone propionate 17β-carboxylic acid reference standard	T8, T9
TP454b (1,2-dihydro fluticasone propio-nate 17β-carboxylic acid) C24H32F2O6 454.217 Da (Δ -0.8 ppm) RT 19.7 min		Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro isomer	T9
TP456 C24H34F2O6 456.232 Da (Δ -1.2 ppm) RT 20.1 min		Isotopic pattern, MS², RT shift,	T9
6α-Methylprednisolone aceponate C27H36O7 472.245 Da (Δ 1.7 ppm) RT 21.3 min		Reference standard	**T11**
6α-Methylprednisolone 17-propionate C25H34O6 430.235 Da (Δ 1.0 ppm) RT 19.4 min		Reference standard	T11
6α-Methylprednisolone 21-propionate C25H34O6 430.235 Da (Δ 2.4 ppm) RT 20.4 min		Reference standard	T11
6α-Methylprednisolone C22H30O5 374.209 Da (Δ 0.5 ppm) RT 15.8 min		Reference standard	K1, S1, T11
Hydrocortisone C21H30O5 362.209 Da (Δ -0.3 ppm) RT 14.4 min		Reference standard	K1, S1, **T12**
Prednisolone C21H28O5 360.194 Da (Δ -1.7 ppm) RT 14.2 min		Reference standard	K2, S2, T12, **T13**

Triamcinolone acetonide C24H31FO6 434.211 Da (Δ -0.8 ppm) RT 17.3 min		Reference standard	K2, S2, **T16**
TP436 (4,5-dihydro triamcinolone acetonide) C24H33FO6 436.226 Da (Δ -0.5 ppm) RT 19.3 min		Isotopic pattern, MS², RT shift, comparison with reference standard of 1,2-dihydro triamcinolone acetonide	T16
TP448 (Trimacinolone acetonide 21-carboxylic acid) C24H29FO7 448.190 Da (Δ -0.6 ppm) RT 17.4 min		Reference standard	K2, T16, **T17**

Method Performance

Table B.4. Recovery rates (corrected by internal standards), precision (expressed as 95 %-confidence intervals) and limits of quantification (LOQ) for the target steroid hormones in WWTP effluent spiked with 10 ng/L (n = 4).

Abbreviation	Compound	LOQ [ng L^{-1}]	Recovery [%]	Precision (95 %-CI) [%]
BEC	Beclomethasone	0.7	94	± 4
BECprop17	Beclomethasone 17-propionate	0.9	92	± 10
BECprop21	Beclomethasone 21-propionate	0.9	-	-
BECdiprop	Beclomethasone 17,21-dipropionate	0.5	94	± 5
TP428a	Beclomethasone 9,11-epoxy 17-propionate	1.0	87	± 16
TP428b	Beclomethasone 9,11-epoxy 21-propionate	1.3	-	-
TP372	Beclomethasone 9,11-epoxide	0.5	89	± 1
BMS	Betamethasone	0.2	90	± 1
BMSval17	Betamethasone 17-valerat	0.5	88	± 8
BMSval21	Betamethasone 21-valerat	0.5	-	-
BMSprop17	Betamethasone 17-propionat	0.4	94	± 4
BMSprop21	Betamethasone 21-propionat	0.4	-	-
BMSdiprop	Betamethasone 17,21-dipropionate	0.3	92	± 4
OxoBMS	17-Oxo betamethasone	0.3	76	± 2
BDN	Budesonide	1.2	96	± 2
TP432b	1,2-Dihydro budesonide	1.5	82	± 7
TP444	Budesonide impurity 1 (budesonide 21-carboxylic acid)	0.4	107	± 9
CLOprop	Clobetasol 17-propionate	0.4	90	± 7
DH-CLOprop	1,2-Dihydro clobetasol propionate	1.6	87	± 6
HCOR	Cortisol (Hydrocortisone)	0.2	96	± 4
FCNact	Fluocinolone acetonide	0.2	98	± 5
TP454b	1,2-Dihydro fluocinolone acetonide	0.9	87	± 12
TP466	Fluocinolone acetonide 21-carboxylic acid	0.8	87	± 5
FLUprop	Fluticasone 17-propionate	0.4	86	± 2
TP502b	1,2-Dihydro fluticasone propionate	0.8	94	± 11
TP452 (FLUprop-m1)	Fluticasone 17β-carboxylic acid propionate	0.5	94	± 10
MPNL	Methylprednisolone	0.1	91	± 2

MPNLacp	Methylprednisolone 17-propionate, 21-acetate	0.5	106	± 8
MPNLprop17	Methylprednisolone 17-propionate	1.2	-	-
MPNLprop21	Methylprednisolone 21-propionate	1.2	105	± 6
MOMfur	Mometasone 17-furoate	0.4	95	± 15
EpoxyMOMfur	Mometasone 9,11-epoxy furoate (mometasone furoate EP impurity D)	0.2	95	± 4
PNL	Prednisolone	0.4	97	± 8
TRIact	Triamcinolone acetonide	0.4	99	± 8
DH-TRIact	1,2-Dihydro triamcinolone acetonide	6.0	86	± 9
TP448	Triamcinolone acetonide 21-carboxylic acid	0.3	102	± 5
TRIact-m1	6ß-Hydroxy triamcinolone acetonide	0.2	105	± 6
TRIact-m3	6β-Hydroxy 21-oic triamcinolone acetonide	0.4	±	± 5

Appendix B

Occurrence of Glucocorticoids and TPs in WWTP Effluents

Table B.5. Detected concentrations of GCs and their TPs in WWTP effluents. Values in grey were below the LOQ.

Abbreviation	Compound	Concentration [ng L⁻¹]							
		WWTP_A	WWTP_B	WWTP_C	WWTP_D	WWTP_E	WWTP_F	WWTP_G	WWTP_H
BEC	Beclomethasone	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7
BECprop17	Beclomethasone 17-propionate	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9
BECprop21	Beclomethasone 21-propionate	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9
BECdiprop	Beclomethasone 17,21-dipropionate	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
TP428a	Beclomethasone 9,11-epoxy 17-propionate	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
TP428b	Beclomethasone 9,11-epoxy 21-propionate	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3	< 1.3
TP372	Beclomethasone 9,11-epoxide	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
BMS	Betamethasone	0.4	0.2	1.0	1.0	1.4	0.5	1.1	0.2
BMSval17	Betamethasone 17-valerat	1.0	0.6	0.7	0.9	1.6	1.3	1.3	3.1
BMSval21	Betamethasone 21-valerat	0.4	< 0.4	0.4	0.5	1.1	0.6	< 0.4	0.7
BMSprop17	Betamethasone 17-propionat	0.7	< 0.4	0.8	0.8	0.6	1.0	1.6	1.1
BMSprop21	Betamethasone 21-propionat	1.0	0.7	1.3	1.3	1.1	0.6	0.5	0.8
BMSdiprop	Betamethasone 17,21-dipropionate	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3
OxoBMS	17-Oxo betamethasone	< 0.3	< 0.3	< 0.3	< 0.3	< 0.3	0.3	< 0.3	0.3
TP332	TP 332	< 0.3	< 0.3	< 0.3	0.5	< 0.3	0.3	< 0.3	0.5
BDN	Budesonide	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
TP412	Peak area →	(37550)	(27170)	(42950)	(5937)	(10120)	(31930)	(9759)	(10010)
TP432b	1,2-Dihydro budesonide	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5	< 1.5
TP444	Budesonide impurity 1 (budesonide 21-carboxylic acid)	0.6	< 0.4	0.5	< 0.4	0.4	< 0.4	< 0.4	0.8
CLOprop	Clobetasol 17-propionate	1.3	0.5	2.0	1.1	0.7	1.3	1.1	3.8
DH-CLOprop	1,2-Dihydro clobetasol propionate	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6	< 1.6
HCOR	Cortisol (Hydrocortisone)	2.8	1.2	1.3	0.9	2.2	2.6	1.3	1.9
FCNact	Fluocinolone acetonide	< 0.2	< 0.2	< 0.2	< 0.2	0.4	< 0.2	0.4	0.2
TP454b	1,2-Dihydro fluocinolone acetonide	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9
TP466	Fluocinolone acetonide 21-carboxylic acid	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8
FLUprop	Fluticasone 17-propionate	0.9	< 0.4	0.4	< 0.4	< 0.4	0.6	< 0.4	1.6
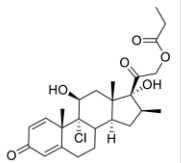
Appendix B

TP502b	1,2-Dihydro fluticasone propionate	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8
TP452 (FLUprop-m1)	Fluticasone 17β-carboxylic acid propionate	2.8	0.5	1.0	1.6	1.1	1.9	1.0	2.0
MPNL	Methylprednisolone	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1	< 0.1
MPNLacp	Methylprednisolone 17-propionate, 21-acetate	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
MPNLprop17	Methylprednisolone 17-propionate	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
MPNLprop21	Methylprednisolone 21-propionate	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
MOMfur	Mometasone 17-furoate	1.5	0.6	1.2	1.1	1.4	0.7	1.1	1.9
EpoxyMOMfur	Mometasone 9,11-epoxy furoate (mometasone furoate EP impurity D)	0.3	< 0.2	< 0.2	< 0.2	0.2	< 0.2	< 0.2	0.5
PNL	Prednisolone	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4
TRIact	Triamcinolone acetonide	18	1.0	7.5	20	6.3	9.6	14	16
DH-TRIact	1,2-Dihydro triamcinolone acetonide	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0	< 6.0
TP448	Triamcinolone acetonide 21-carboxylic acid	1.8	0.8	2.2	3.7	1.8	1.5	0.5	3.3
TRIact-m1	6ß-Hydroxy triamcinolone acetonide	1.4	0.7	1.7	2.2	1.5	1.4	0.9	2.1
TRIact-m3	6β-Hydroxy 21-oic triamcinolone acetonide	0.9	0.5	0.9	1.5	0.8	0.8	1.1	1.1

Analytical Results and Further Remarks

Figure B.1. Overview of the analytical results and further remarks to all GCs and detected TPs.

Name: Beclomethasone 21-propionate	Formula: C25H33ClO6	Precursor: [M+H]$^+$	RT Shift: -1.5 min	Atomic Modification: - C3H4O
Structure:		Identification: Reference Standard	Comments: TP of beclomethasone dipropionate,	

XIC of 465.203±0.005 Da ESI(+)
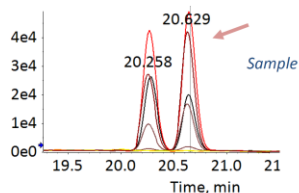
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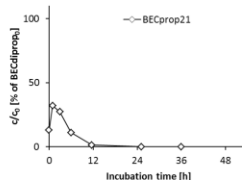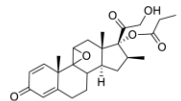
MS/MS Spectra, m/z 465.2 Da, ESI(+)

Isotope Pattern

Name: TP428a (9,11-epoxy beclomethasone 17-propionate	Formula: C25H32O6	Precursor: [M+H]$^+$	RT Shift: -1.5 min	Atomic Modification: - C3H5ClO
Proposed Structure:		Identification: Reference Standard	Comments: TP of beclomethasone dipropionate, formation occurred likewise in the sterile batch, interference by the in-source fragment of beclomethasone 21-propionate might be possible	

XIC of 429.227±0.005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 429.2 Da, ESI(+)

Isotope Pattern

164
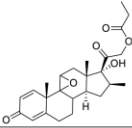
Name: TP428b (9,11-epoxy beclomethasone 21-propionate	Formula: C25H32O6	Precursor: [M+H]⁺	RT Shift: -1.0 min	Atomic Modification: - C3H5ClO
Structure:		Identification: Reference Standard	Comments: TP of beclomethasone dipropionate, formation occurred likewise in the sterile batch	

XIC of 429.227±0.005 Da ESI(+)

Time Course
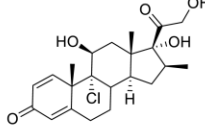
MS/MS Spectra, m/z 429.2 Da, ESI(+)

Isotope Pattern

Name: Beclomethasone	Formula: C22H29ClO5	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: -5.3 min	Atomic Modification: -C6H8O2
Structure:		Identification: Reference Standard	Comments: TP of beclomethasone dipropionate, active compound	

XIC of 409.177±0.005 Da ESI(+)
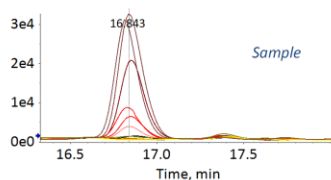
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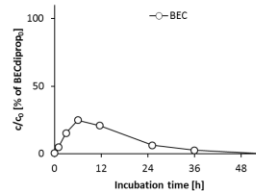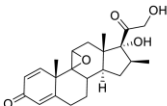
MS/MS Spectra, m/z 409.2 Da, ESI(+)

Isotope Pattern

Name: TP372 (9,11-epoxy beclomethasone)	Formula: C22H28O5	Precursor: [M+H]⁺	RT Shift: -4.6 min	Atomic Modification: -C6H9ClO2
Structure:		Identification: Reference Standard	Comments: TP of beclomethasone dipropionate, formation occurred likewise in the sterile batch	

XIC of 373.201±0.005 Da ESI(+)
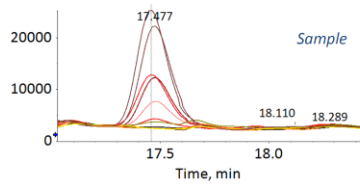
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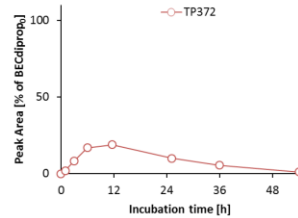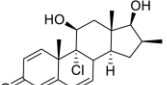
MS/MS Spectra, m/z 373.2 Da, ESI(+)

Isotope Pattern

Name: TP348b	Formula: C20H25ClO3	Precursor: [M+H]⁺	RT Shift: -5.1 min	Atomic Modification: -C8H12O4
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS²	Comments: TP of beclomethasone dipropionate, comparison to 17-oxo betamethasone standard revealed similarity, alternative structures might be possible, reference standard not available	

XIC of 349.158±0.005 Da ESI(+)
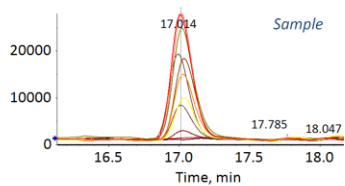
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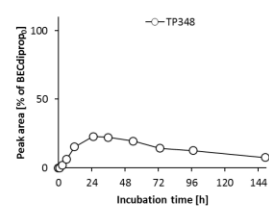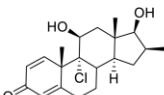
MS/MS Spectra, m/z 349.2 Da (TP348b) and 333.2 Da (17-oxo betamethasone), ESI(+)

Isotope Pattern

Name: TP350	Formula: C20H27ClO3	Precursor: [M+H]⁺	RT Shift: -4.4 min	Atomic Modification: -C8H10O4
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS²	**Comments:** TP of beclomethasone dipropionate, alternative structures might be possible, reference standard not available	

XIC of 351.173±0.005 Da ESI(+)
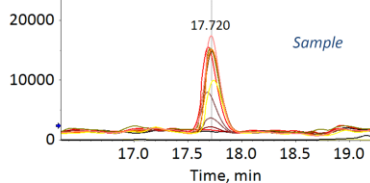
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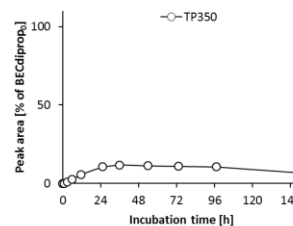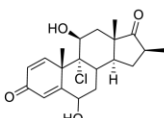
MS/MS Spectra, m/z 351.2 Da (TP350) and 349.2 Da (TP348b), ESI(+)

Isotope Pattern

Name: TP364	Formula: C20H25ClO4	Precursor: [M+H]⁺	RT Shift: -7.4 min	Atomic Modification: -C8H12O3
Proposed Structure:		**Identification: :** RT shift, isotopic pattern, accurate mass, comparison to TP348b, MS²	**Comments:** TP of beclomethasone dipropionate, alternative structures might be possible, reference standard not available	

XIC of 365.151±0.005 Da ESI(+)
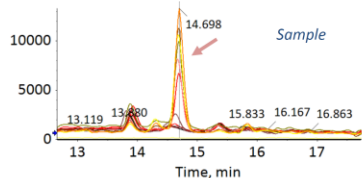
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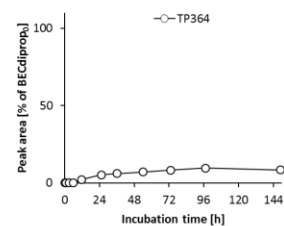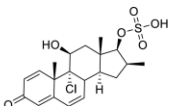
MS/MS Spectra, m/z 365.2 Da (TP364) and 349.2 Da (TP348b), ESI(+)

Isotope Pattern

Name: TP428c	Formula: C20H25ClO6S	Precursor: [M-H]⁻	RT Shift: -4.9 min	Atomic Modification: -C8H12O, +S
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS²	**Comments:** TP of beclomethasone dipropionate, alternative structures might be possible, reference standard not available	

XIC of 427.099±0.005 Da ESI(-)
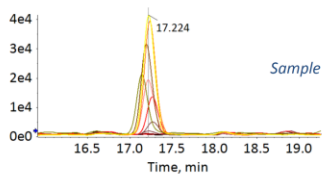
Time Course
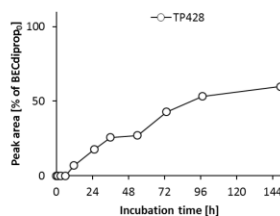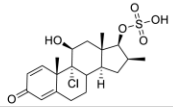
MS/MS Spectrum, m/z 427.1 Da, ESI(-)

Isotope Pattern

Name: TP430	Formula: C20H27ClO6S	Precursor: [M-H]⁻	RT Shift: -4.6 min	Atomic Modification: -C8H10O, +S
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS², comparison to TP428c	**Comments:** TP of beclomethasone dipropionate, alternative structures might be possible, reference standard not available	

XIC of 429.115±0.005 Da ESI(-)
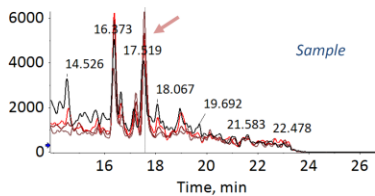
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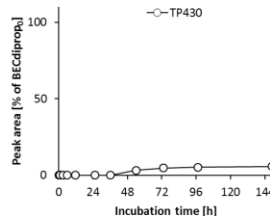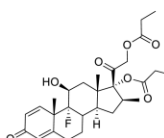
MS/MS Spectra, m/z 429.1 Da (TP430) and 427.1 Da (TP428c), ESI(-)
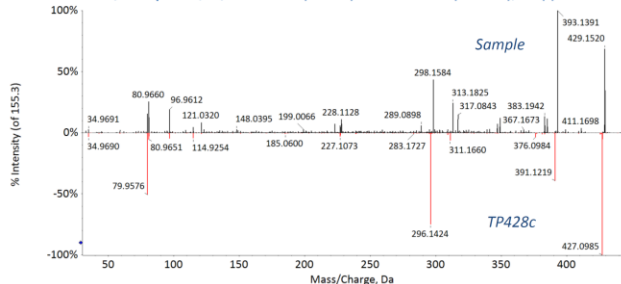
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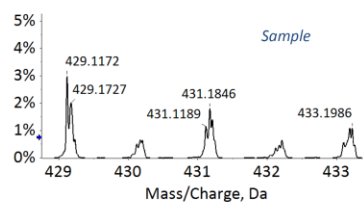
Name: Betamethasone dipropionate	Formula: C28H37FO7	Precursor: [M+H]+	RT Shift: -	Atomic Modification: -
Structure:		Identification: Reference standard	Comments: Compound is a prodrug	

XIC of 505.259±0.005 Da ESI(+)
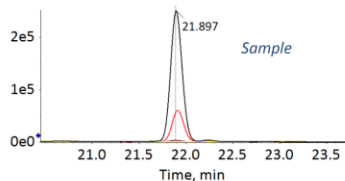
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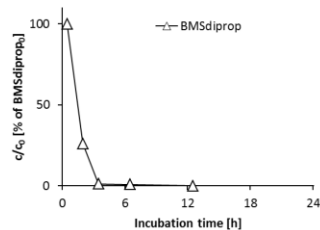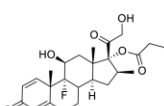
MS/MS Spectra, m/z 505.3 Da, ESI(+)

Isotope Pattern

Name: Betamethasone 17-propionate	Formula: C25H33FO6	Precursor: [M+H]+	RT Shift: -2.1 min	Atomic Modification: -C3H4O
Structure:		Identification: Reference standard	Comments: TP of betamethasone dipropionate, active compound, formation occurred likewise in the sterile batch	
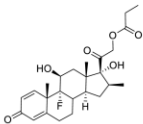
XIC of 449.233±0.005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 449.2 Da, ESI(+)

Isotope Pattern

Name: Betamethasone 21-propionate	Formula: C25H33FO6	Precursor: [M+H]⁺	RT Shift: -1.5 min	Atomic Modification: -C3H4O
Structure:		Identification: Reference standard	Comments: TP of betamethasone dipropionate and betamethasone 17-propionate, formation occurred likewise in the sterile batch	

XIC of 449.233±0.005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 449.2 Da, ESI(+)
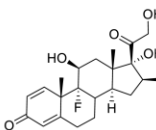
Isotope Pattern
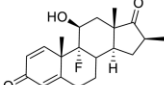
Name: Betamethasone	Formula: C22H29FO5	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: -5.7 min	Atomic Modification: -C6H8O2
Structure:		Identification: Reference standard	Comments: TP of betamethasone dipropionate and betamethasone 17-valerate, formation occurred likewise in the sterile batch	

XIC of 393.206±0.005 Da ESI(+)

Time Courses

MS/MS Spectra, m/z 393.2 Da, ESI(+)

Isotope Pattern

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Name: 17-Oxo betamethasone	Formula: C20H25FO3	Precursor: [M+H]⁺	RT Shift: +2.8 min (with regard to BMS)	Atomic Modification: -C2H4O2
Structure:		Identification: Reference standard	Comments: TP of betamethasone dipropionate, betamethasone 17-valerate and betamethasone, detection in very low intensities	

XIC of 333.186±0.005 Da ESI(+)

Time Courses
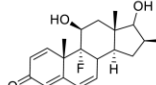
MS/MS Spectra, m/z 333.2 Da, ESI(+)

Isotope Pattern

Name: TP332	Formula: C20H25FO3	Precursor: [M+H]⁺	RT Shift: -0.3 min (with regard to BMS)	Atomic Modification: -C2H4O2
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 17-oxo betamethasone	Comments: Major TP of betamethasone dipropionate, betamethasone 17-valerate, betamethasone and 17-oxo betamethasone, alternative structure might be possible,	

XIC of 333.186±0.005 Da ESI(+)
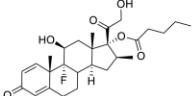
Time Courses

MS/MS Spectra, m/z 333.2 Da, ESI(+)

Isotope Pattern

Name: Betamethasone 17-valerate	Formula: C27H37FO6	Precursor: [M+H]+	RT Shift: -	Atomic Modification: -
Structure:		Identification: Reference standard	Comments: -	

XIC of 477.264±0.005 Da ESI(+)
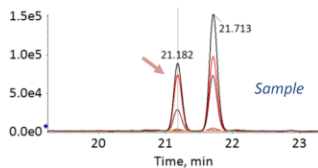
Time Course
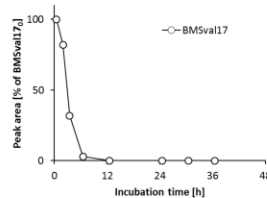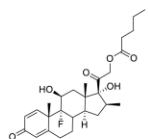
MS/MS Spectra, m/z 477.3 Da, ESI(+)
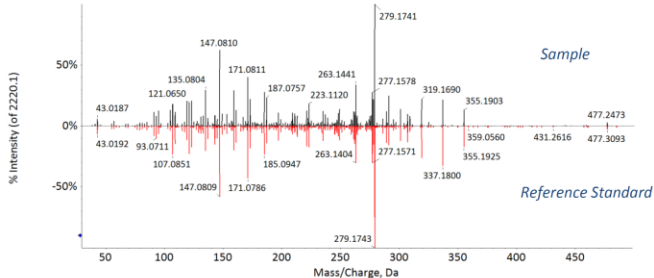
Isotope Pattern
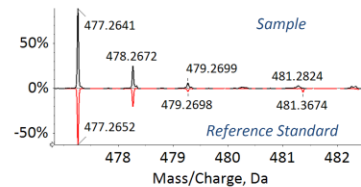
Name: Betamethasone 21-valerate	Formula: C27H37FO6	Precursor: [M+H]+	RT Shift: +0.5 min	Atomic Modification: -
Structure:		Identification: Reference standard	Comments: TP of betamethasone 17-valerate	

XIC of 477.264±0.005 Da ESI(+)

Time Course
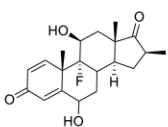
MS/MS Spectra, m/z 477.3 Da, ESI(+)
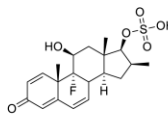
Isotope Pattern

Name: TP348a		Formula: C20H25FO4	Precursor: [M+H]⁺	RT Shift: -2.6 min (with regard to BMS)	Atomic Modification: -C2H4O
Proposed Structure:			Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 17-oxo betamethasone	Comments: TP of betamethasone dipropionate, betamethasone 17-valerate, betamethasone and 17-oxo betamethasone, detection in low intensities, alternative structures might be possible	

XIC of 349.181±0.005 Da ESI(+)

Time Courses
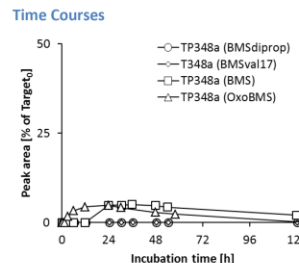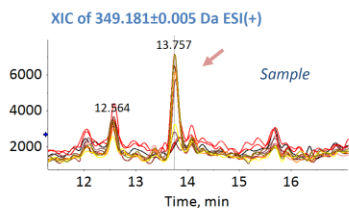
MS/MS Spectra, m/z 349.2 Da (TP348a) and 333.3 Da (17-oxo betamethasone), ESI(+)
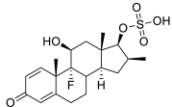
Isotope Pattern

Name: TP412		Formula: C20H25FO6S	Precursor: [M-H]⁻/(M+H)⁺	RT Shift: -0.2 min (with regard to BMS)	Atomic Modification: -C2H4, +H2S
Proposed Structure:			Identification: RT shift, isotopic pattern, accurate mass, MS²,	Comments: High intensities in ESI(-) due to the sulfate conjugation, alternative structures might be possible	

XIC of 411.129±0.005 Da ESI(-)

Time Courses
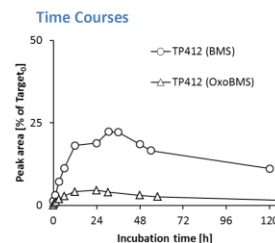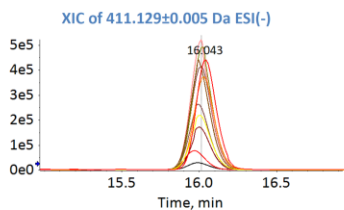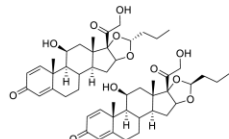
MS/MS Spectra, m/z 411.1 Da, ESI(-)
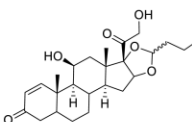
Isotope Pattern

Name: TP414		Formula: C20H27FO6S	Precursor: [M-H]⁻/(M+H)⁺	RT Shift: +0.3 min (with regard to BMS)	Atomic Modification: -C2H4, +H4S
Proposed Structure:			Identification: RT shift, isotopic pattern, accurate mass, MS²,	Comments: High intensities in ESI(-) due to sulfate conjugation, alternative structures might be possible	

XIC of 413.144±0.005 Da ESI(-)

Time Courses

MS/MS Spectra, m/z 413.1 Da, ESI(-)

Sample_ Betamethasone Batch

Sample_17-Oxo betamethasone Batch

Isotope Pattern

Name: Budesonide	Formula: C25H34O6	Precursor: [M+H]⁺	RT Shift:-	Atomic Modification:-
Structure:		Identification: Reference Standard	Comments: Racemic budesonide was used for incubation	

XIC of 431.244±0.005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 431.2 Da, ESI(+)

Sample

Reference Standard

Isotope Pattern

174

Name: TP432a (4,5-Dihydro budesonide)	Formula: C25H36O6	Precursor: [M+H]$^+$	RT Shift: +1.1 min	Atomic Modification: +H2
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro budesonide	Comments: No double peak! Formation seems to be depended on stereochemistry, alternative structures might be possible	

XIC of 433.259±0.005 Da ESI(+)
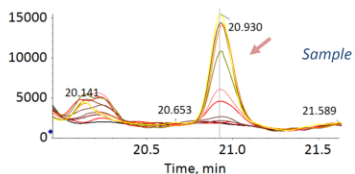
Time Course
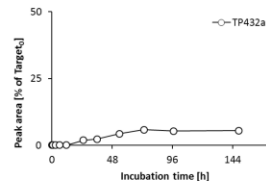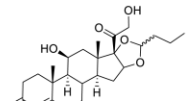
MS/MS Spectra, m/z 433.2 Da, ESI(+)

Isotope Pattern

Name: TP432b (1,2-Dihydro budesonide)	Formula: C25H36O6	Precursor: [M+H]$^+$	RT Shift: +0.4 min	Atomic Modification: +H2
Structure:		Identification: Reference Standard	Comments: No MS/MS spectra recorded, confirmation via target analysis, very low intensities were detected.	

XIC of 433.259±0.005 Da ESI(+)
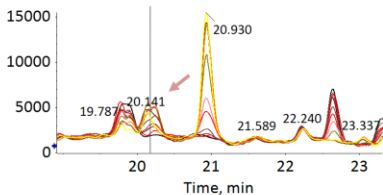
Time Course
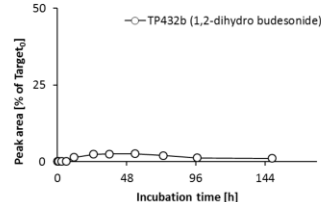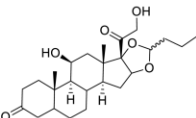
XIC of MRM 432.2 < 343.2 Da, ESI(+)

Isotope Pattern

Name: TP434	Formula: C25H38O6	Precursor: [M+H]+	RT Shift: +1.3 min	Atomic Modification: +H4
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro budesonide	**Comments:** No double peak! Formation seems to be depended on stereochemistry, alternative structures might be possible	

XIC of 435.274±0.005 Da ESI(+)
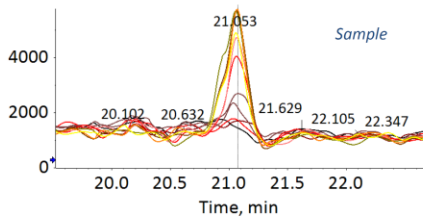
Time Course
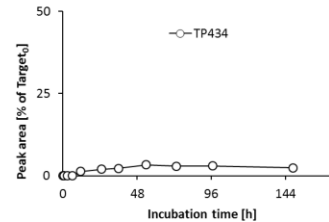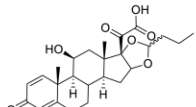
MS/MS Spectra, m/z 435.2 Da (TP434) and 433.2 Da (1,2-dihydro budesonide), ESI(+)

Isotope Pattern

Name: TP444	Formula: C25H32O7	Precursor: [M+H]+, [M-H]-	RT Shift: -0.2 min	Atomic Modification: -H2, +O
Structure:		**Identification:** Reference Standard	**Comments:** No double peak! Formation seems to be depended on stereochemistry. Racemic reference standard was used	

XIC of 445.222±0.005 Da ESI(+)
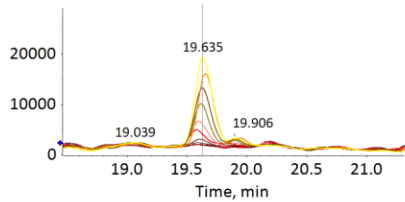
Time Course
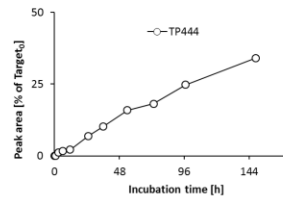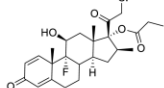
MS/MS Spectra, m/z 445.2 Da, ESI(+)
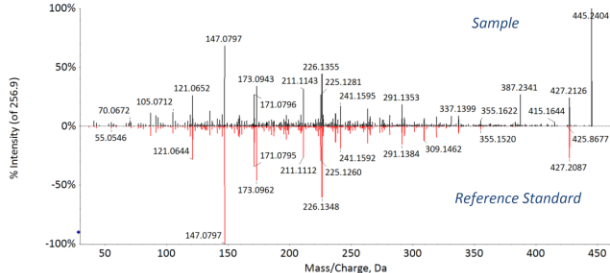
Isotope Pattern
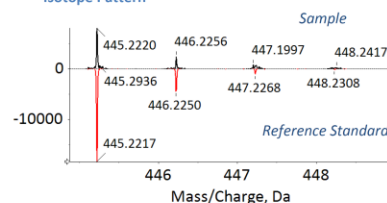
Name: Clobetasol propionate	Formula: C25H32ClFO5	Precursor: [M+H]⁺	RT Shift: -	Atomic Modification: -
Structure:		Identification: Reference standard	Comments: Formation of clobetasol propionate during the incubation of 1,2-dihydro clobetasol propionate.	

XIC of 467.200±0.005 Da ESI(+)
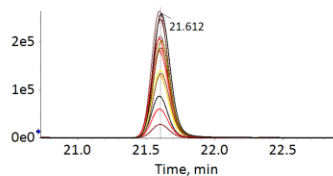
Time Courses
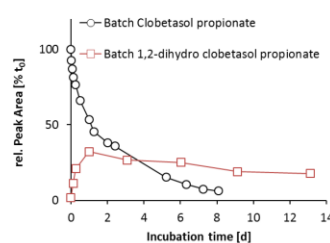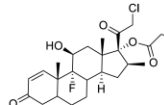
MS/MS Spectra, m/z 467.2 Da, ESI(+)

Isotope Pattern

Name: TP468	Formula: C25H34ClFO5	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: +0.7 min	Atomic Modification: +H2
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro clobetasol propionate	Comments: Formation of TP468 during the incubation of 1,2-dihydro clobetasol propionate. Interference in ESI(+), differentiation via formate adduct in ESI(-). Alternative structures might be possible.	

XIC of 469.215±0.005 Da ESI(+)   **XIC of 513.206±0.005 Da ESI(-)**
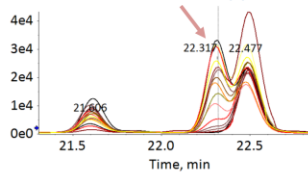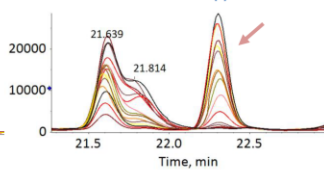
Time Courses
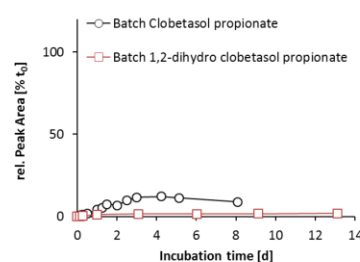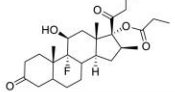
MS/MS Spectra, m/z 469.2 Da, ESI(+)

Isotope Pattern
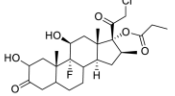
Name: TP470	Formula: C25H36ClFO5	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: +0.9 min	Atomic Modification: +H4
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro clobetasol propionate	Comments: Low intensity of MS2 signal. Formation of TP470 during the incubation of 1,2-dihydro clobetasol propionate. Alternative structures might be possible	

XIC of 471.231±0.005 Da ESI(+)

XIC of 515.222±0.005 Da ESI(-)

Time Courses
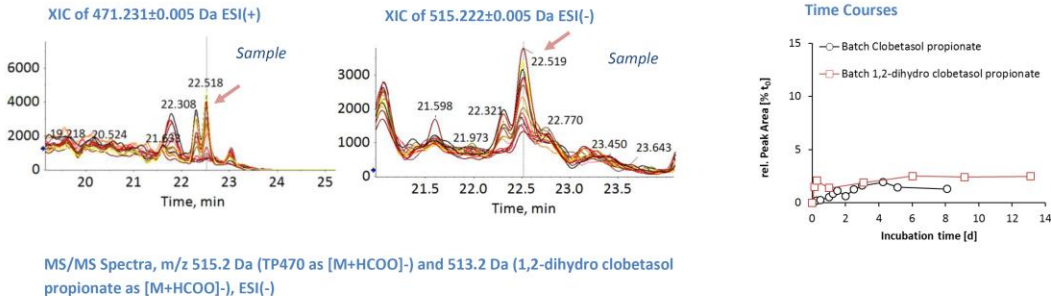
MS/MS Spectra, m/z 515.2 Da (TP470 as [M+HCOO]-) and 513.2 Da (1,2-dihydro clobetasol propionate as [M+HCOO]-), ESI(-)
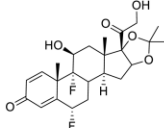
Isotope Pattern

Name: TP486	Formula: C25H36ClFO6	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: +0.2 min	Atomic Modification: +H4O
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS²	Comments: Positive RT shift although hydroxylation is suggested. Maybe due to the interaction of keto and vicinal hydroxyl group (intramolecular H-bonding interaction) (Stensen and Jensen, 1994). Formate adduct formation was conspicuously high, which emphasizes the vicinal position of the hydroxyl group. Low intensity of MS2 signal. Alternative structures might be possible (e.g. Bayer-Villiger oxidation product)	

XIC of 531.217±0.005 Da ESI(-)

Time Courses

MS/MS Spectra, m/z 531.2 Da (as [M+HCOO]⁻), ESI(-)

Isotope Pattern

Name: Fluocinolone acetonide	Formula: C24H30F2O6	Precursor: [M+H]+	RT Shift: -	Atomic Modification: -
Structure:		**Identification:** Reference Standard	**Comments:** Very low consumption figure in Germany.	

Structure diagram of Fluocinolone acetonide.

XIC of 453.208±0.005 Da ESI(+)

Sample, peak at 17.708

Time Course

Fluocinolone acetonide
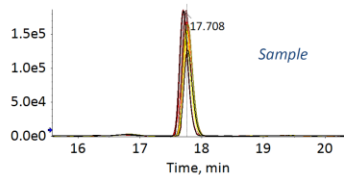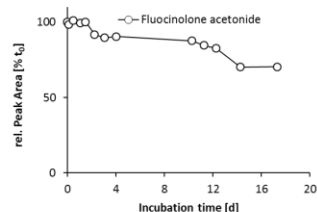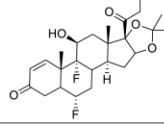
MS/MS Spectra, m/z 453.2 Da, ESI(+)

Sample / Reference Standard

Isotope Pattern

Name: TP454a (4,5-Dihydro fluocinolone acetonide)	Formula: C24H32F2O6	Precursor: [M+H]+, [M+HCOO]-	RT Shift: +1.0 min	Atomic Modification: +H2
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro fluocinolone acetonide	**Comments:** Alternative structures might be possible. Reference Standard was commercially not available.	

Proposed structure diagram of TP454a.

XIC of 499.215±0.005 Da ESI(-)

Peaks at 17.781, 18.297, 18.732

Time Course

TP454a (4,5-dihydro fluocinolone acetonide)

MS/MS Spectra, m/z 499.2 Da as [M+HCOO]-, ESI(-)
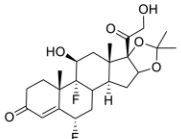
Sample / Reference Standard of 1,2-Dihydro Fluocinolone Acetonide
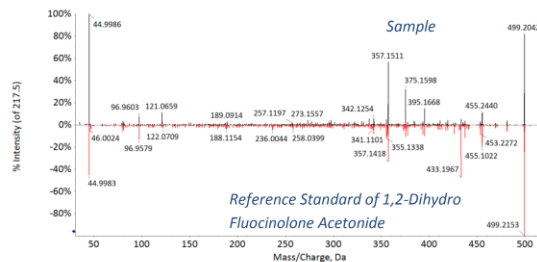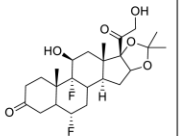
Isotope Pattern, ESI(+)
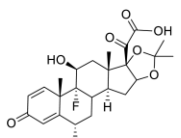
Name: TP454b (1,2-Dihydro fluocinolone acetonide)	Formula: C24H32F2O6	Precursor: [M+H]+, [M+HCOO]-	RT Shift: +0.6 min	Atomic Modification: +H2
Structure:		Identification: Reference Standard	Comments: Detected at very low intensities.	

XIC of 499.215±0.005 Da ESI(-)

Time Course

MS/MS Spectra, m/z 499.2 Da as [M+HCOO]-, ESI(-)

Name: TP456	Formula: C24H34F2O6	Precursor: [M+H]+, [M+HCOO]-	RT Shift: +1.1 min	Atomic Modification: +H4
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro fluocinolone acetonide	Comments: -	

XIC of 501.231±0.005 Da ESI(-)

Time Course

MS/MS Spectra, m/z 501.2 Da (TP456) and 499.2 Da (1,2-dihydro fluocinolone acetonide) as [M+HCOO]-, ESI(-)

Isotope Pattern

Name: TP466	Formula: C24H28F2O7	Precursor: [M+H]⁺, [M-H]⁻	RT Shift: -0.1 min	Atomic Modification: -H2, +O
Structure:		Identification: Reference Standard	Comments: -	

XIC of 467.188±0.005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 467.2 Da, ESI(+)

Sample
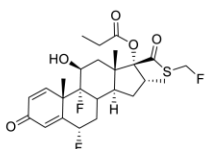
Reference Standard

Isotope Pattern

Name: Fluticasone propionate	Formula: C25H31F3O5S	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: -	Atomic Modification: -
Structure:		Identification: Reference Standard	Comments: Formation of fluticasone propionate during the incubation of 1,2-dihydro fluticasone propionate.	

XIC of 501.192±0.005 Da ESI(+)
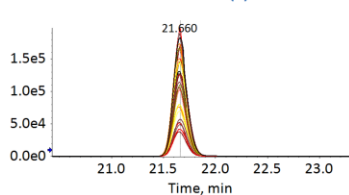
Time Course
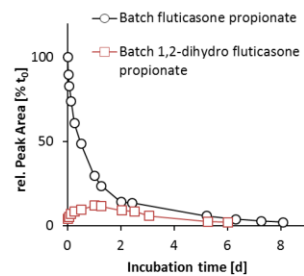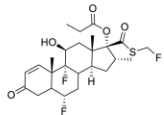
MS/MS Spectrum, m/z 501.2 Da, ESI(+)
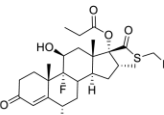
Isotope Pattern

Name: TP502a (4,5-Dihydro fluticasone propionate)	**Formula:** C25H33F3O5S	**Precursor:** [M+H]⁺, [M+HCOO]⁻	**RT Shift:** +0.4 min	**Atomic Modification:** +H2
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro fluticasone propionate	**Comments:** Formation of TP502a during the incubation of fluticasone propionate and 1,2-dihydro fluticasone propionate. Alternative structure might be possible	

Name: TP502b (1,2-Dihydro fluticasone propionate)	**Formula:** C25H33F3O5S	**Precursor:** [M+H]⁺, [M+HCOO]⁻	**RT Shift:** +0.2 min	**Atomic Modification:** +H2
Structure:		**Identification:** Reference Standard	**Comments:** Very low quantities detected. Minor hydrogenated TP of fluticasone propionate. No MS/MS recorded.	

Name: TP504		Formula: C25H35F3O5S	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: +0.4 min	Atomic Modification: +H4
Proposed Structure:			Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro fluticasone propionate	Comments: Very low quantities detected. Alternative structure might be possible.	
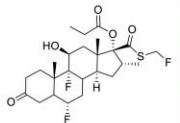
Batch Fluticasone Propionate: XIC of 505.223 Da ESI (+)
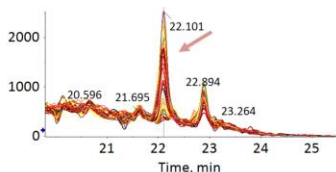
Batch 1,2-Dihydro Fluticasone Propionate: XIC of 505.223 Da ESI (+)
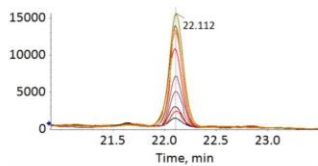
Time Course
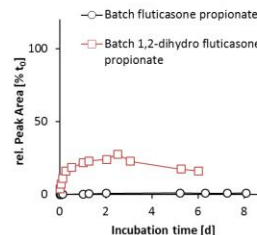
MS/MS Spectra, m/z 549.2 Da, ESI(-)
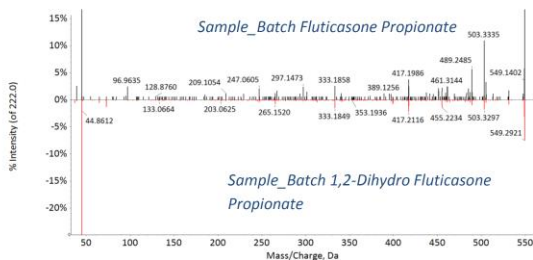
Isotope Pattern

Name: TP452 (Fluticasone propionate 17β-carboxylic acid)		Formula: C24H30F2O6	Precursor: [M+H]⁺, [M-H]⁻	RT Shift: -2.2 min	Atomic Modification: -CHFS, +O
Structure:			Identification: Reference Standard	Comments: TP showed persistency in single incubation experiment!	
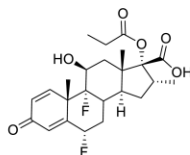
Batch fluticasone propionate: XIC of 453.208 Da ESI(+)
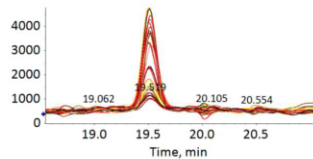
Time Courses
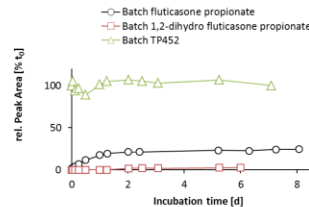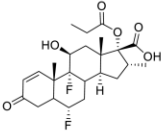
MS/MS Spectra, m/z 451.2, ESI(-)

Isotope Pattern

Name: TP454a (4,5-Dihydro fluticasone propionate 17β-carboxylic acid)	Formula: C24H32F2O6	Precursor: [M+H]⁺, [M-H]⁻	RT Shift: -1.7 min	Atomic Modification: -CFS, +HO
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of fluticasone propionate 17β-carboxylic acid	Comments: -	

Name: TP454b (1,2-Dihydro fluticasone propionate 17β-carboxylic acid)	Formula: C24H32F2O6	Precursor: [M+H]⁺, [M-H]⁻	RT Shift: -2.0 min	Atomic Modification: -CFS, +HO
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of fluticasone propionate 17β-carboxylic acid	Comments: Detected exclusively during the incubation of 1,2-dihydro fluticasone propionate (experiment T9)	

Name: TP456	Formula: C24H34F2O6	Precursor: [M+H]⁺, [M-H]⁻	RT Shift:	Atomic Modification: -CFS, +3HO
Proposed Structure:		**Identification:** RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of fluticasone propionate 17β-carboxylic acid	**Comments:** Detected exclusively during the incubation of 1,2-dihydro fluticasone propionate (experiment T9)	

Batch_1,2-Dihydro fluticasone propionate:
XIC of 455.225±0.005 Da ESI(-)
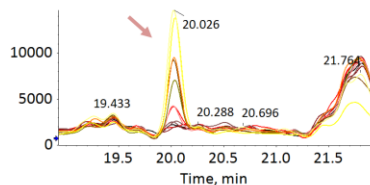
Time Course
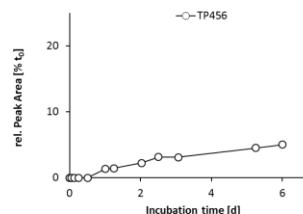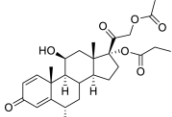
MS/MS Spectra, m/z 455.2 Da (TP456) and 453.2 Da (TP454a), ESI(-)

Isotope Pattern

Name: 6α-Methylprednisolone aceponate	Formula: C27H36O7	Precursor: [M+H]⁺	RT Shift: -	Atomic Modification: -
Structure:		**Identification:** Reference Standard	**Comments:** Compound is a prodrug	

XIC of 473.253±0.005 Da ESI(+)
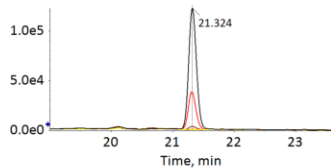
Time Course
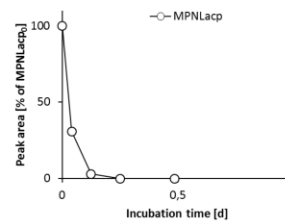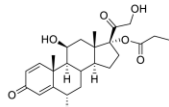
MS/MS Spectra, m/z 473.2 Da, ESI(+)

Isotope Pattern

Name: 6α-Methylprednisolone 17-propionate		Formula: C25H34O6	Precursor: [M+H]⁺	RT Shift: -1.9 min	Atomic Modification: -C2H2O
Structure:			Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 6α-methylprednisolone 21-propionate	Comments: TP of 6α-methylprednisolone aceponate, active compound	

XIC of 431.243±0.005 Da ESI(+)
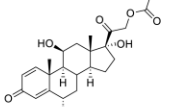
Time Course

MS/MS Spectrum, m/z 431.2 Da, ESI(+)

Isotope Pattern

Name: 6α-Methylprednisolone 21-propionate		Formula: C25H34O6	Precursor: [M+H]⁺	RT Shift: -0.9 min	Atomic Modification: -C2H2O
Structure:			Identification: Reference standard	Comments: TP of 6α-methylprednisolone aceponate	

XIC of 431.243±0.005 Da ESI(+)

Time Course
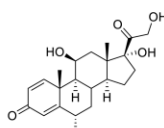
MS/MS Spectrum, m/z 431.2 Da, ESI(+)

Isotope Pattern

Name: 6α-Methylprednisolone	Formula: C22H30O5	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: +5.5 min	Atomic Modification: -C5H6O2
Structure:		Identification: Reference standard	Comments: TP of 6α-methylprednisolone aceponate, active compound	

XIC of 375.217±0.005 Da ESI(+)
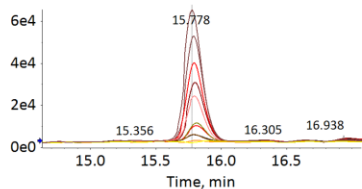
Time Course
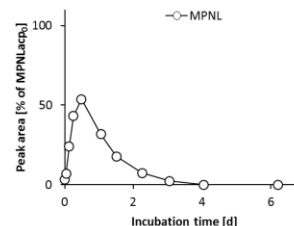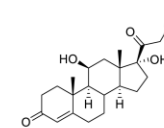
MS/MS Spectrum, m/z 375.2 Da, ESI(+)
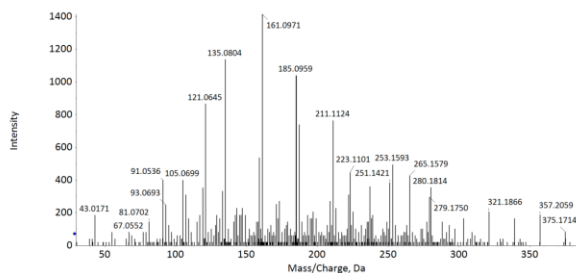
Isotope Pattern
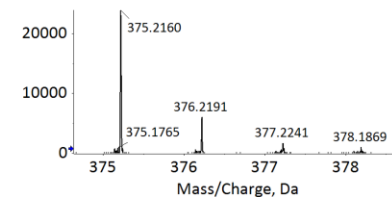
Name: Hydrocortisone	Formula: C21H30O5	Precursor: [M+H]⁺, [M+HCOO]⁻	RT Shift: -	Atomic Modification: -
Structure:		Identification: Reference standard	Comments: -	

XIC of 407.208±005 Da as [M+HCOO]⁻ in ESI(-)
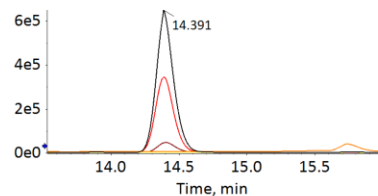
Time Course
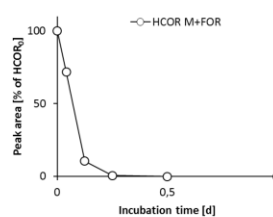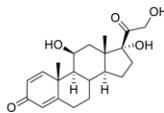
MS/MS Spectrum, m/z 363.2, ESI(+)

Isotope Pattern

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Name: Prednisolone	**Formula:** C21H28O5	**Precursor:** [M+H]+, [M+HCOO]-	**RT Shift:** -0.2 min (in comparison to hydrocortisone)	**Atomic Modification:** -
Structure: 		**Identification:** Reference standard	**Comments:** Formation of prednisolone was detected during the incubation of hydrocortisone	

XIC of 405.192±005 Da as [M+HCOO]- in ESI(-)
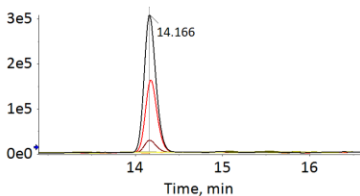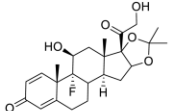
Time Course
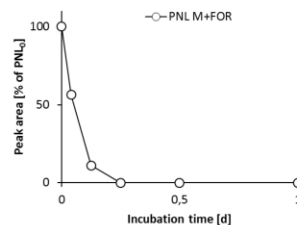
MS/MS Spectrum, m/z 361.2, ESI(+)
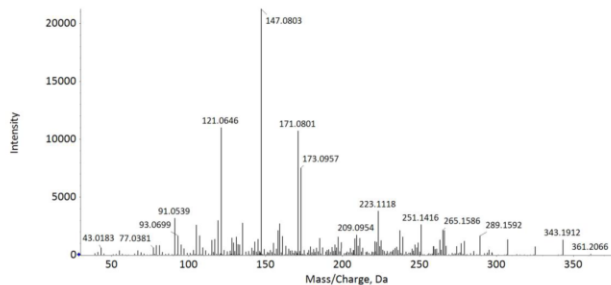
Isotope Pattern

Name: Triamcinolone acetonide	**Formula:** C24H31FO6	**Precursor:** [M+H]+	**RT Shift:** -	**Atomic Modification:** -
Structure: 		**Identification:** Reference standard	**Comments:** -	

XIC of 435.218±005 Da ESI(+)
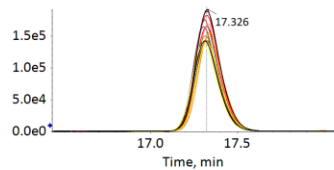
Time Course
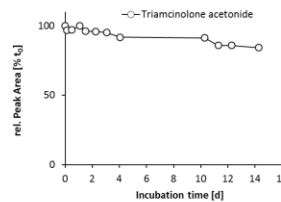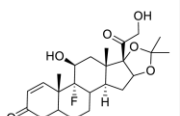
MS/MS Spectrum, m/z 435.2, ESI(+)

Isotope Pattern

Name: TP436	Formula: C24H33FO6	Precursor: [M+H]+	RT Shift: +2.0 min	Atomic Modification: +2H
Proposed Structure:		Identification: RT shift, isotopic pattern, accurate mass, MS², comparison to reference standard of 1,2-dihydro triamcinolone acetonide	Comments: RT shift of 2.0 min is conspicuous	

XIC of 437.233±005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 437.2, ESI(+)

Sample

Reference Standard of 1,2-Dihydro Triamcinolone Acetonide
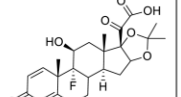
Isotope Pattern

Name: TP448	Formula: C24H29FO7	Precursor: [M+H]+, [M-H]-	RT Shift: +0.1 min	Atomic Modification: +O, -2H
Proposed Structure:		Identification: Comparison to reference standard	Comments: TP showed persistency in single incubation experiment!	

XIC of 449.197±005 Da ESI(+)

Time Course

MS/MS Spectra, m/z 449.2, ESI(+)

Sample

Reference Standard
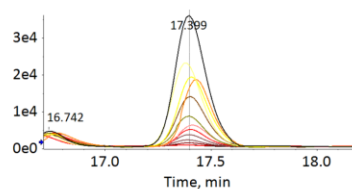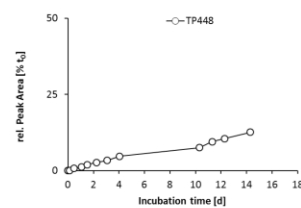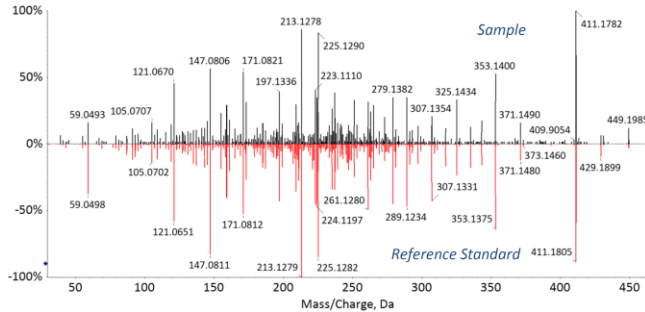
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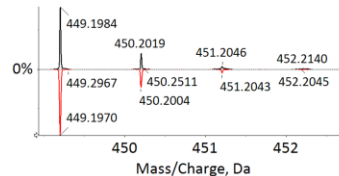
Additional Figures
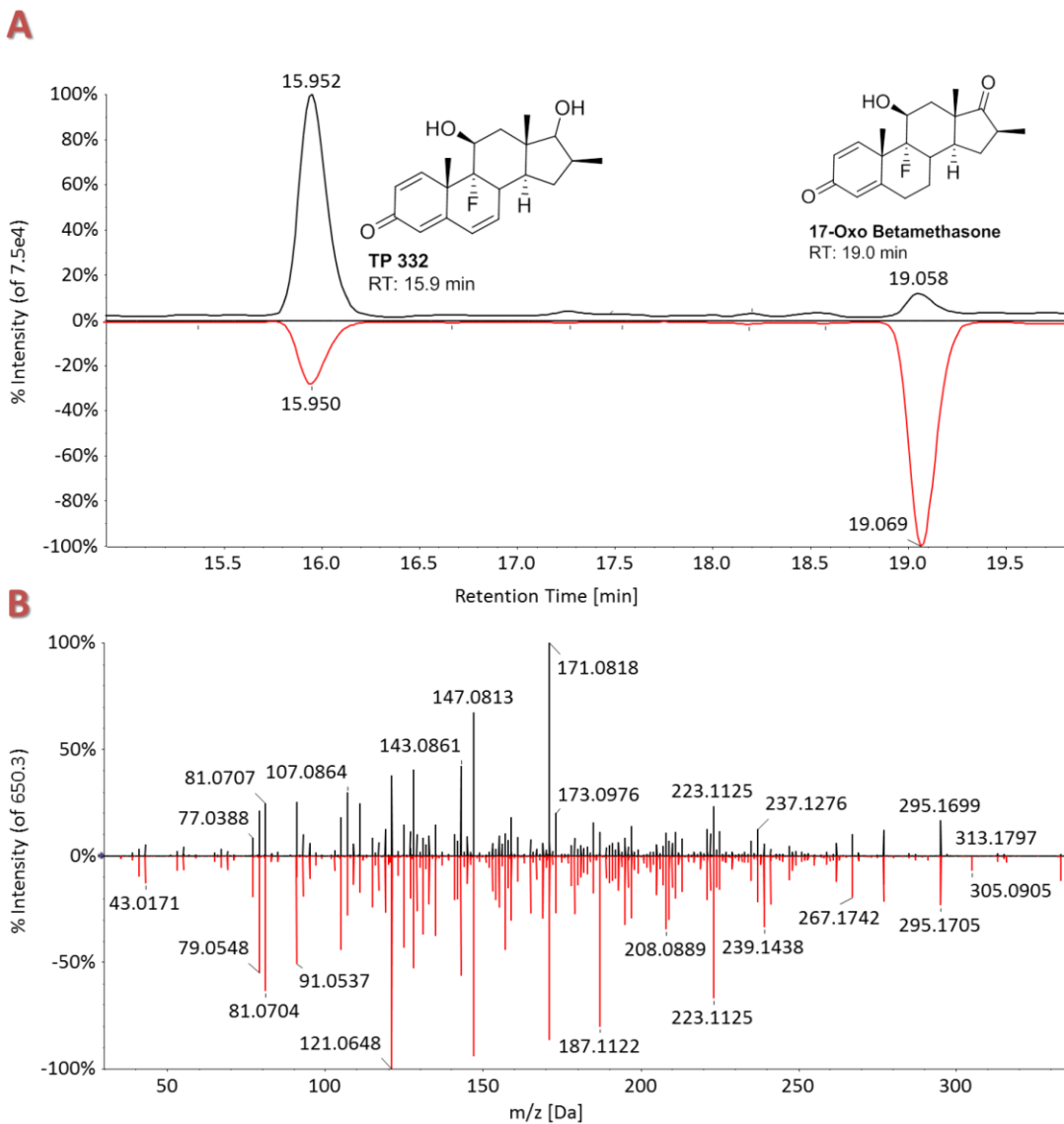
Figure B.2. Comparison of 17-oxo betamethasone (OxoBMS) and TP332. **A**: Extracted ion chromatogram of m/z 333.186 Da from Experiment T4 (in black) and experiment T5 (in red). **B**: MS² spectra of m/z 332.2 Da of OxoBMS (in black, RT 19.1 min) and TP332 (in red, 15.9 min).
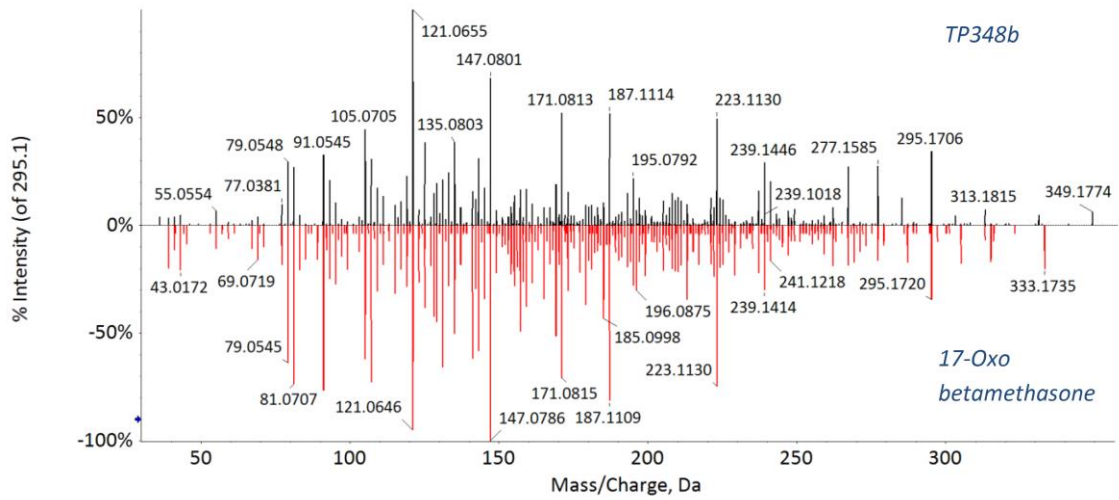
Figure B.3. Comparison of the MS² spectra of the beclomethasone TP348b (m/z 349.2 Da) and 17-oxo betamethasone (m/z 332,2 Da).
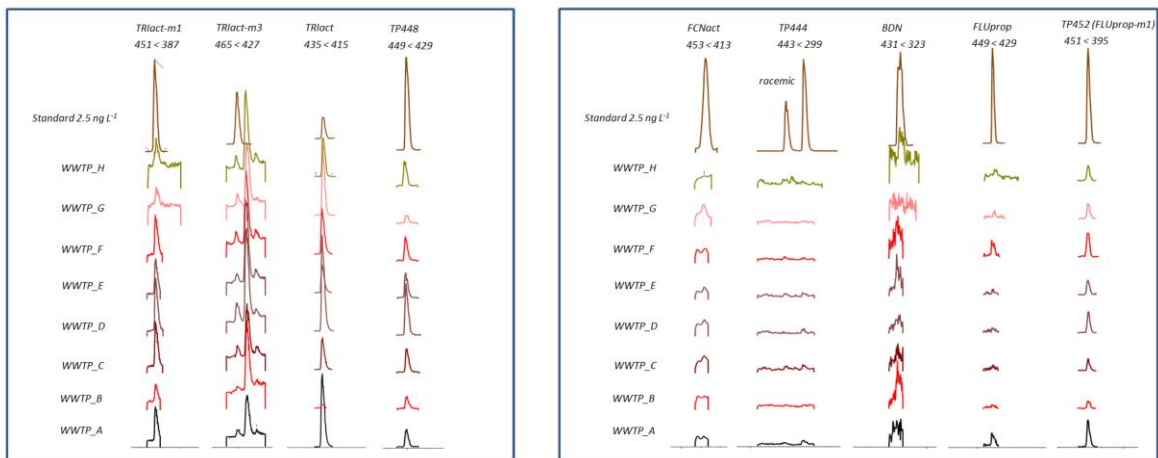
Figure B.4. LC-MS/MS chromatograms of the detected analytes in the analyzed WWTP effluents (A).
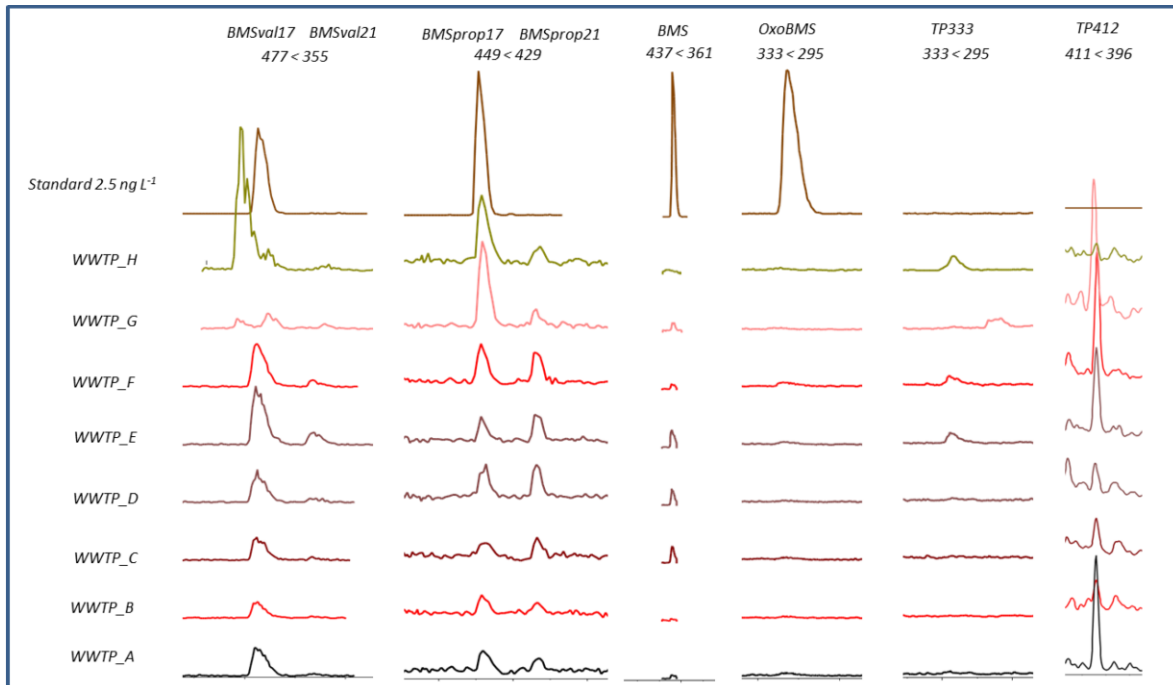
Figure B.5. LC-MS/MS chromatograms of the detected analytes in the analyzed WWTP effluents (B).
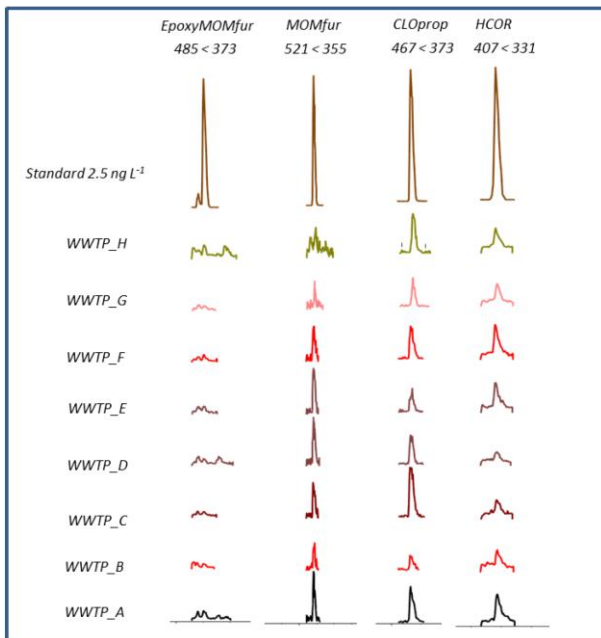
Figure B.6. LC-MS/MS chromatograms of the detected analytes in the analyzed WWTP effluents (C).
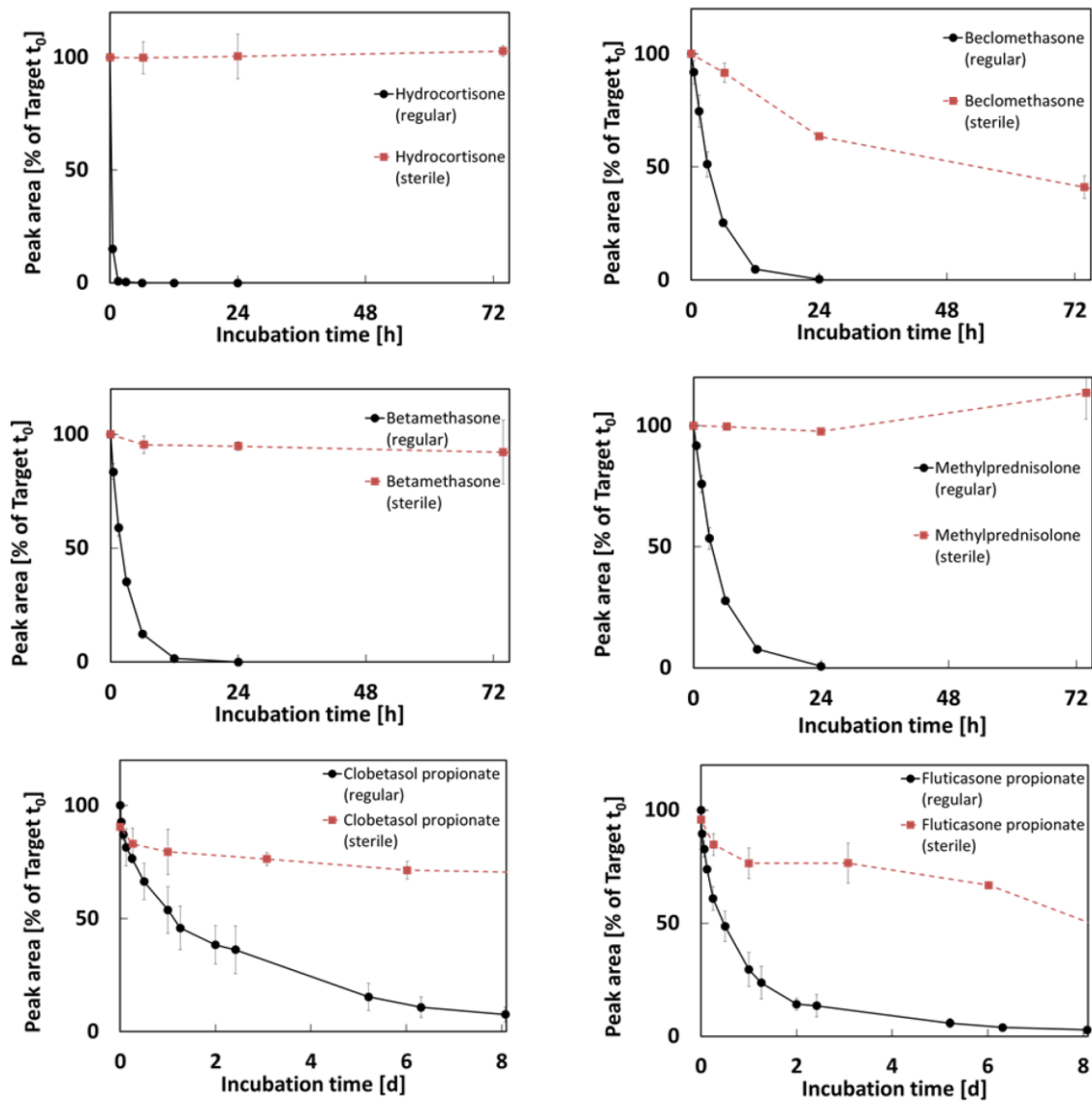
Figure B.7. Time courses of target steroids from the sterile (red) and regular (black) kinetic experiments K1/S1. Errors are expressed as the standard deviation (sterile n=2, regular n=3).
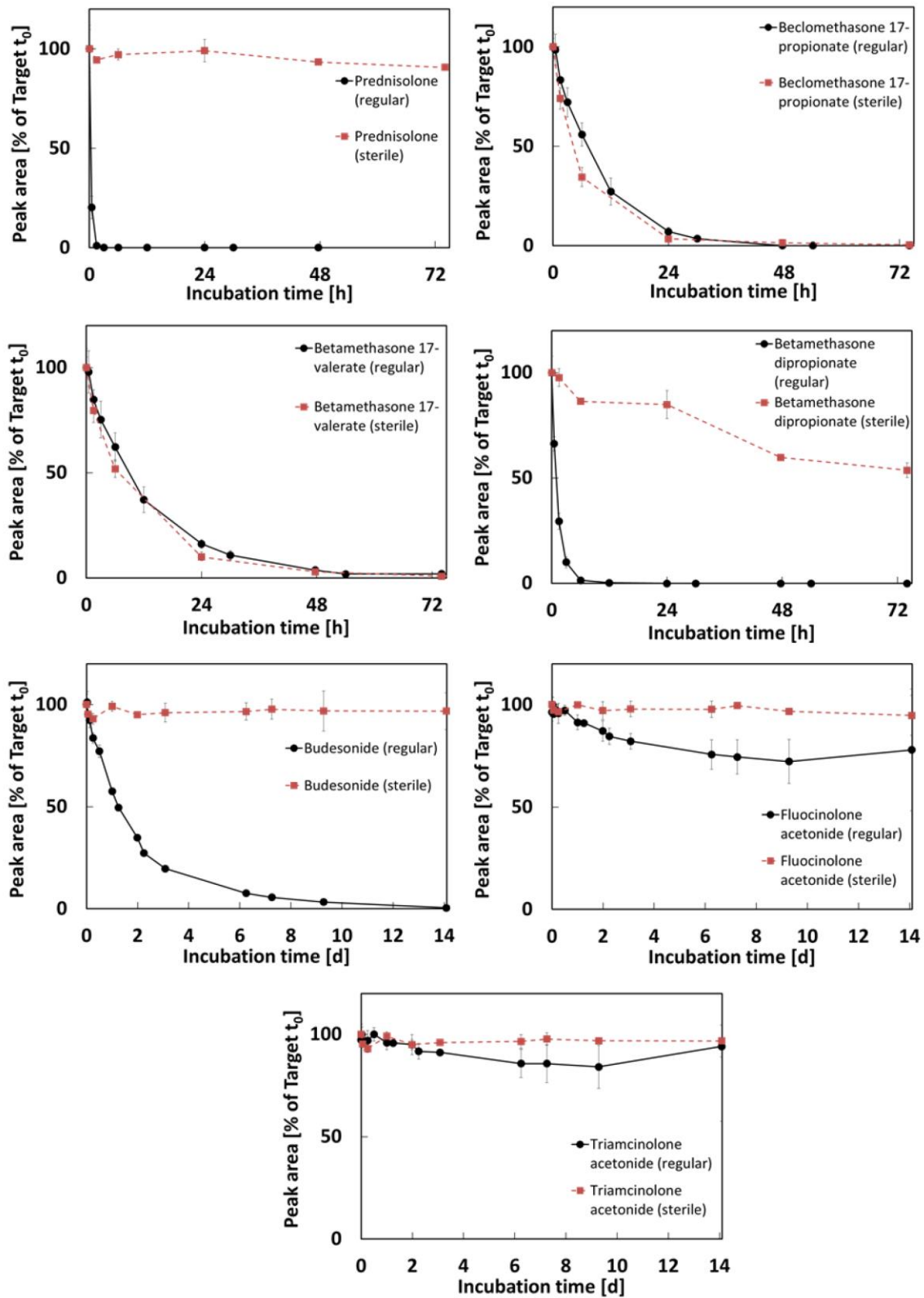
Figure B.8. Time courses of target steroids from the sterile (red) and regular (black) kinetic experiments K2/S2. Errors are expressed as the standard deviation (sterile n=2, regular n=3).

References

Stensen, W., Jensen, E., **1994**. High-performace liquid chromatographic separation of naphtoquinones and their derivatives: Effect of hydrogen bonding on retention. *Journal of Chromatography A* 659 (1), 87-93.

Appendix C-Supplementary Data for Chapter 4

Fate and Behavior of Synthetic Oral Contraceptives (SOCs) in Activated Sludge Treatment: Elucidation of the Kinetics and Transformation Products

OUTLINE

LC-MS/MS parameter for target analysis

Table C.1. LC-MS/MS detection method and further information. (TRC = Toronto Research Chemicals, Canada Ontario; TLC = TLC Pharmaceutical 2 Standards, Canada Ontario, SC = Santa Cruz Biotechnology, USA Texas; SA = Sigma-Aldrich, Germany Munich)

Abbreviation	Substance	Supplier	CAS-No.	Chemical formula	Internal standard used for correction	Adduct	Precursor [Da]	Fragment mass [Da]	Collision energy [V]	Declustering potential [V]
HPG	17α-Hydroxyprogesterone	SA	68-96-2	$C_{21}H_{30}O_3$	13C3-HPG	$[M+H]^+$	331	109 / 97	34 / 28	80
CLMac	Chlormadinone acetate	TRC	302-22-7	$C_{23}H_{29}ClO_4$	d5-CLOprop	$[M+H]^+$	405	309 / 267	22 / 32	90
TP402_20.5	Delmadinone acetate	TRC	13698-49-2	$C_{23}H_{27}ClO_4$	d5-CLOprop	$[M+H]^+$	403	343 / 265	18 / 28	90
TP406_20.6	Chlormadinol acetate	TRC	3114-44-1	$C_{23}H_{31}ClO_4$	d5-CLOprop	$[M+H]^+$	407	347 /329	12 / 16	50
CYPac	Cyproterone acetate	SA	427-51-0	$C_{24}H_{29}ClO_4$	d3-CYPac	$[M+H]^+$	375	321//293	28 / 32	110
CYP	Cyproterone	SC	2098-66-0	$C_{22}H_{27}ClO_3$	d5-CLOprop	$[M+H]^+$	417	357/321	23 / 27	100
TP418_20.4	(3α-Hydroxy 3-deoxocyproterone acetate)	-	167356-55-0	$C_{24}H_{31}ClO_4$	d3-CYPac	$[M+H]^+$	419	317 / 359	40* / 40*	100*
DIE	Dienogest	SA	65928-58-7	$C_{20}H_{25}NO_2$	d8-DIE	$[M+H]^+$	312	161/135	38 / 40	160
TP327_11.3	6β-Hydroxy dienogest	SC	-	$C_{20}H_{25}NO_3$	d8-DIE	$[M+H]^+$	328	107/251	33 / 33	60
TP309_16.4	(Δ9,11-dehydro-17α-cyanomethyl estradiol)	-	86153-38-0	$C_{20}H_{23}NO_2$	d8-DIE	$[M+H]^+$	310	159 / 133	40* / 40*	100*
DPN	Drospirenone	SA	67392-87-4	$C_{24}H_{30}O_3$	13C3-DPN	$[M+H]^+$	367	97 / 197	30 / 30	90
ETG	Etonogestrel	SA	54048-10-1	$C_{22}H_{28}O_2$	d6-LNG	$[M+H]^+$	325	257 /1 97	25 / 27	80
LNG	Levonorgestrel	SA	797-63-7	$C_{21}H_{28}O_2$	d6-LNG	$[M+H]^+$	313	245 / 109	25 / 32	120
MRPac	Medroxyprogesterone acetate	SA	71-58-9	$C_{24}H_{34}O_4$	d3-CYPac	$[M+H]^+$	505	411 / 485	17 / 14	50
MRP	Medroxyprogesterone	SA	520-85-4	$C_{22}H_{32}O_3$	d6-LNG	$[M+H]^+$	345	123 / 97	33 / 50	100
TP388_21.6	4,5(β)-Dihydro medroxyprogesterone acetate	TLC	69688-15-9	$C_{24}H_{36}O_4$	d3-CYPac	$[M+H]^+$	389	329 / 311	13 / 22	60
NESac	Norethisterone acetate	SA	51-98-9	$C_{22}H_{32}O_4$	d6-NES	$[M+H]^+$	341	281/109	20 / 40	110
TP298_ (NES)	Norethisterone	SA	68-22-4	$C_{20}H_{26}O_2$	d6-NES	$[M+H]^+$	299	231/109	25 / 32	110
TP300_19.8	4,5(α)-Dihydro norethisterone	TRC	52-79-9	$C_{20}H_{28}O_2$	d6-NES	$[M+H]^+$	301	265 / 215	21 / 25	140
TP296_18.5 (EE2)	17α-Ethinylestradiol	SA	57-63-6	$C_{20}H_{24}O_2$	d6-NES	$[M+H]^+$	297 / 279	107 / 133	35 / 25	150 / 120

Internal Standards

13C3-HPG	17α-Hdroxyprogesterone-2,3,4-$^{13}C_3$	SA	1356154-92-1	$^{13}C_3C_{18}H_{30}O_3$	-	$[M+H]^+$	334	112	32	110
d5-CLOprop	Clobetasol 17-propionate-d5	TRC	-	$C_{25}H_{27}D_5ClFO_5$	-	$[M+H]^+$	472	373	17	70
d3-CYPac	Cyproterone acetate-d3	TRC	-	$C_{24}H_{26}D_3ClO_4$	-	$[M+H]^+$	420	357	25	100
d8-DIE	Dienogest-d8	TRC	-	$C_{20}H_{17}D_8NO_2$	-	$[M+H]^+$	320	167	38	160
13C3-DPN	Drospirenone-$^{13}C_3$	TRC	-	$C_{21}{}^{13}C_3H_{30}O_3$	-	$[M+H]^+$	370	97	35	100
d6-LNG	Levonorgestrel-d6	TRC	-	$C_{21}H_{22}D_6O_2$	-	$[M+H]^+$	319	251	25	120
d6-NES	Norethisterone-d6	TRC	-	$C_{20}H_{20}D_6O_2$	-	$[M+H]^+$	305	237	27	100

*) Standard CE and DP values from the used HRMS method were used for quantification due to missing reference standard.
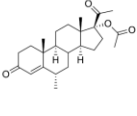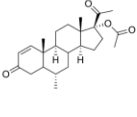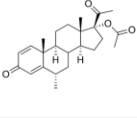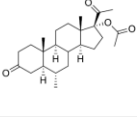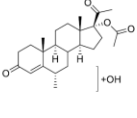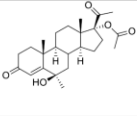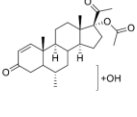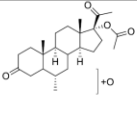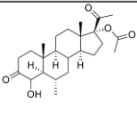
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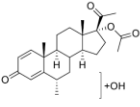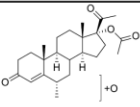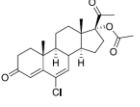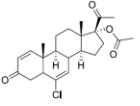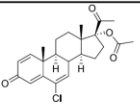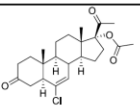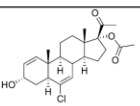
Table C.2. Location, capacities and sampling dates of the analyzed WWTP effluent samples.
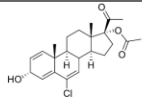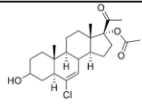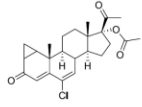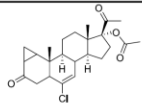
Abbreviation	Location, postal code	Capacity (population equivalents)	Sampling Date
WWTP_A	*Daxweiler, 55442*	*8,550*	*21.03.2019, 17:15*
WWTP_B	*Kalt, 56294*	*35,000*	*21.03.2019, 18:15*
WWTP_C	*Groß-Gerau, 64521*	*45,000*	*21.03.2019, 10:30*
WWTP_D	*Bingen, 55411*	*80,000*	*21.03.2019, 15:30*
WWTP_E	*Griesheim, 64347*	*50,000*	*21.03.2019, 12:15*
WWTP_F	*Frankfurt, 60528*	*1,350,000*	*01.04.2019, 12:30*
WWTP_G	*Koblenz,56070*	*320,000*	*01.04.2019, 16:00*
WWTP_H	*Mayen, 56727*	*30,000*	*21.03.2019, 19:00*

List of detected TPs and parent progestogens

Table C.3. Main transformation products detected for the 17α-hydroxyprogesterone derivatives. Precursor masses and retention times represent the average of all samples along the incubation experiments.

M+H Precursor mass [Da] (Error in ppm)	Chemical Formula	Changes	RT [min]	Name	Structure	Identification Level
387.2537 (-1.84)	C24H34O4	-	20.99	Medroxyprogesterone acetate		Reference standard
387.2538 (-2.10)	C24H34O4	-	21.29	TP386_21.3		Reference standard
385.2378 (-1.20)	C24H32O4	-H2	20.42	TP384_20.4		(proposed)
389.2690 (-0.94)	C24H36O4	+H2	21.64	TP388_21.6		Reference standard
403.2487 (-1.98)	C24H34O5	+O	17.04	TP402_17.0		(proposed)
403.2483 (-0.99)	C24H34O5	+O	18.09	TP402_18.1		Reference standard
403.2486 (-1.73)	C24H34O5	+O	19.13	TP402_19.1		(proposed)
403.2480 (-0.25)	C24H34O5	+O	19.50	TP402_19.5		(proposed)
405.2639 (-0.86)	C24H36O5	+H2O	20.56	TP404_20.6		(proposed)

401.2329 (-1.62)	C24H32O5	-H2, +O	16.00	TP400_16.0		(proposed)
401.2327 (-1.12)	C24H32O5	-H2, +O	16.71	TP400_16.7		(proposed)
331.2268 (-0.09)	C21H30O3	-C3H4O	20.08	TP330_20.1	no final structure	
361.2378 (-1.28)	C22H32O4	-C2H2	19.73	TP360_19.7	no final structure	
363.2539 (-2.52)	C22H34O4	-C2	18.69	TP362_18.7	no final structure	
415.2114 (0.28)	C24H30O6	-H4, +O2	15.91	TP414_15.9	no final structure	
417.2278 (-1.52)	C24H32O6	-H2, +O2	17.09	TP416_17.1	no final structure	
419.2435 (-1.63)	C24H34O6	+O2	17.88	TP418_17.9	no final structure	
405.1830 (-0.71)	C23H29ClO4	-	20.89	Chlormadinone acetate		Reference standard
405.1834 (-1.69)	C23H29ClO4	-	20.98	TP404_21.0		(proposed)
403.1677 (-1.58)	C23H27ClO4	-H2	20.52	TP402_20.5		Reference standard
407.1975 (2.12)	C23H31ClO4	+H2	21.18	TP406_21.2		(proposed)
407.1988 (-1.07)	C23H31ClO4	+H2	20.63	TP406_20.6		(proposed)

405.1834 (-1.69)	C23H29ClO4	-	20.14	TP404_20.1		(proposed)
409.2131 (2.23)	C23H33ClO4	+H4	20.89	TP408_20.9		(proposed)
401.2332 (-2.37)	C24H32O5	+H3CO, -Cl	20.38	TP400_20.4	no final structure	
373.2381 (-2.05)	C23H32O4	+H3, -Cl	20.36	TP372_20.4	no final structure	
371.2228 (-3.00)	C23H30O4	+H, -Cl	20.11	TP370_20.1	no final structure	
349.1942 (-3.77)	C21H29ClO2	-C3O2	20.43	TP348_20.4	no final structure	
351.2097 (-3.32)	C21H31ClO2	+H2,-C3O2	20.32	TP350_20.2	no final structure	
399.2168 (-0.50)	C24H30O5	+CHO, -Cl	19.98	TP398_20.0	no final structure	
425.2094 (-1.11)	C23H33ClO5	+H4O	20.02	TP424_19.9	no final structure	
387.2165 (0.26)	C23H30O5	+HO, -Cl	14.99	TP386_15.0	no final structure	
389.2322 (0.13)	C23H32O5	+H3O, -Cl	16.21	TP388_16.2	no final structure	
389.2325 (-0.64)	C23H32O5	+H3O, -Cl	15.99	TP388_16.0	no final structure	
417.1836 (-2.12)	C24H29ClO4	-	20.72	Cyproterone acetate		Reference standard
419.1989 (-1.28)	C24H31ClO4	+H2	21.16	TP418_21.2		
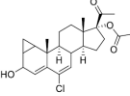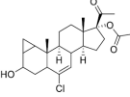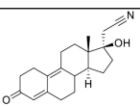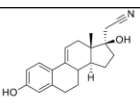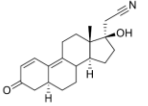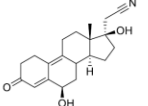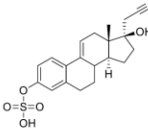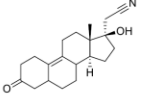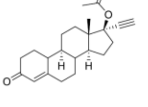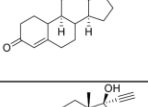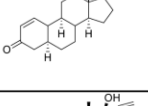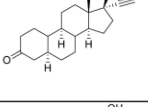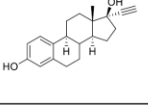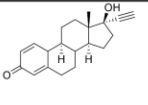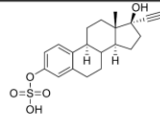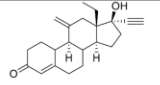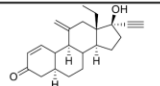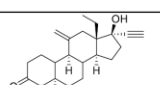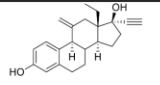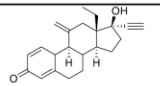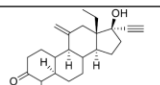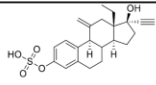
[M+H]+ Precursor mass [Da] (Error in ppm)	Chemical Formula	Changes	RT [min]	Name	Structure	Identification
419.1990 (-1.52)	C24H31ClO4	+H2	20.41	TP418_20.4		Reference standard
421.2136 (0.98)	C24H33ClO4	+H4	20.64	TP420_20.6		
385.2370 (0.87)	C24H32O4	-Cl, +H3	20.09	TP384_20.1	no final structure	
383.2221 (-1.08)	C24H30O4	-Cl, +H	19.92	TP382_19.9	no final structure	
401.2323 (-0.12)	C24H32O5	-Cl, +H3O	16.50	TP400_16.5	no final structure	
401.2328 (-1.37)	C24H32O5	-Cl, +H3O	15.80	TP400_15.8	no final structure	
403.2482 (-0.74)	C24H34O5	-Cl, +H5O	16.15	TP402_16.2	no final structure	

Table C.4. Main transformation products detected for the 19-nortestosterone derivatives. Precursor masses and retention times represent the average of all samples along the incubation experiments.

[M+H]+ Precursor mass [Da] (Error in ppm)	Chemical Formula	Changes	RT [min]	Name	Structure	Identification
312.1961 (-0.94)	C20H25NO2	-	15.61	Dienogest		Reference standard
310.1806 (-1.43)	C20H23NO2	-H2	16.43	TP309_16.4		
310.1807 (-1.76)	C20H23NO2	-H2	11.10	TP309_11.1	no final structure	

312.1967 (-2.87)	C20H25NO2	-	16.75	TP311_16.8		(proposed)
328.1908 (-0.24)	C20H25NO3	+O	13.32	TP327_13.3	no final structure	
328.1910 (-0.85)	C20H25NO3	+O	13.04	TP327_13.0	no final structure	
328.1909 (-0.55)	C20H25NO3	+O	11.31	TP327_11.3		Reference standard
[M-H]-, 388.1219 (1.34)	C20H23NO5S	+O3S, -H2	12.94	TP389_12.9		
314.2121 (-2.05)	C20H27NO2	+H2	17.91	TP313_17.9		(proposed)
341.2114 (-0.82)	C22H28O3	-	20.93	Norethisterone acetate		Reference standard
299.2009 (-1.15)	C20H26O2	-C2H2O	18.26	TP298_18.3 (Norethisterone)		Reference standard
299.2007 (-0.48)	C20H26O2	-C2H2O	19.23	TP298_19.2		
301.2165 (-0.98)	C20H28O2	-C2O	19.80	TP300_19.8		Reference standard
297.1847 (0.69)	C20H24O2	-C2H4O	18.48	TP296_18.5 (Ethinylestradiol)		Reference standard
317.2114 (-0.88)	C20H28O3	-C2,+H2	17.99	TP316_18.0	no final structure	
297.1849 (0.02)	C20H24O2	-C2H4O	17.54	TP296_17.5		

319.1905 (-0.36)	C19H26O4	-C3H2, +O	13.81	TP318_13.8	no final structure	
[M-H]-, 331.1920 (-1.56)	C20H28O4	-C2, +O	16.32	TP332_16.3	no final structure (carboxylic acid moiety)	
[M-H]-, 333.2075 (-1.10)	C20H30O4	-C2, +H2O	13.85	TP334_13.9	no final structure (carboxylic acid moiety)	
[M-H]-, 347.1868 (-1.15)	C20H28O5	-C2, +O2	13.70	TP348_13.7	no final structure (carboxylic acid moiety)	
[M-H]-, 375.1272 (-0.08)	C20H24O5S	-C2H4, +SO2	14.64	TP376_14.6 (Ethinylestradiol sulfate)		
325.2163 (-0.29)	C22H28O2	-	19.93	Etonogestrel		Reference standard
325.2167 (-1.52)	C22H28O2	-	20.59	TP324_20.6		
327.2323 (-1.36)	C22H30O2	+H2	20.82	TP326_20.8		
323.2008 (-0.75)	C22H26O2	-H2	19.96	TP322_20.0		
323.2007 (-0.45)	C22H26O2	-H2	19.61	TP322_19.6		
343.2272 (-1.25)	C22H30O3	+H2O	19.73	TP342_19.7		

[M-H]-, 373.2024 (-0.94)	C22H30O5	+H2O3	15.64	TP374_15.6	no final structure (carboxylic acid moiety)	
[M-H]-, 357.2071 (0.10)	C22H30O4	+H2O2	17.53	TP358_17.5	no final structure (carboxylic acid moiety)	
[M-H]-, 357.2076 (-1.30)	C22H30O4	+H2O2	18.52	TP358_18.5	no final structure (carboxylic acid moiety)	
[M-H]-, 401.1431 (-0.70)	C22H26O5S	-H2, +O3S	16.66	TP402_16.7		

Method performance

Table C.5. Recovery rates (corrected by internal standards), precision (expressed as the 95%-confidence intervals) and limits of quantification (LOQ) for the target steroid hormones in WWTP effluents spiked 10 ng/L (n=4).

Abbreviation	Compound	LOQ [ng/L]	Recovery [%]	Precision (95 %-CI) [%]
HPG	17α-Hydroxyprogesterone	0.4	102	± 4
CLMac	Chlormadinone acetate	0.4	101	± 1
TP402_20.5	Delmadinone acetate	0.9	103	± 6
TP406_20.6	Chlormadinol acetate	5.6	94	± 11
CYPac	Cyproterone acetate	1.0	105	± 2
CYP	Cyproterone	0.9	99	± 2
DIE	Dienogest	0.3	107	± 2
TP327_11.3	6β-Hydroxy dienogest	0.5	106	± 3
DPN	Drospirenone	0.8	115	± 6
ETG	Etonogestrel	1.2	97	± 3
LNG	Levonorgestrel	1.0	104	± 4
MRPac	Medroxyprogesterone acetate	0.6	88	± 4
MRP	Medroxyprogesterone	0.4	108	± 3
TP388_21.6	4,5(β)-Dihydro medroxyprogesterone acetate	3.8	99	± 10
NESac	Norethisterone acetate	0.6	94	± 4
TP298_ (NES)	Norethisterone	0.7	103	± 5
TP300_19.8	4,5(α)-Dihydro norethisterone	1.2	99	± 7
TP296_18.5 (EE2)	17α-Ethinylestradiol	2.9	103	± 3

Occurrence of progestogens and TPs in WWTP effluents

Table C.6. Detected concentrations of SOCs and their TPs in WWTP effluents. Values in grey are below the LOQ.

		Concentration [ng/L]							
Abbreviation	Compound	WWTP_A	WWTP_B	WWTP_C	WWTP_D	WWTP_E	WWTP_F	WWTP_G	WWTP_H
HPG	17α-Hydroxyprogesterone	0.4	1.0	0.5	0.6	0.6	0.6	0.7	0.9
CLMac	Chlormadinone acetate	< 5.6	< 5.6	< 5.6	< 5.6	< 5.6	< 5.6	< 5.6	< 5.6
TP402_20.5	Delmadinone acetate	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9
TP406_20.6	Chlormadinol acetate	< 0.4	0.7	< 0.4	0.5	< 0.4	< 0.4	< 0.4	0.4
CYPac	Cyproterone acetate	2.8	4.8	2.0	7.1	2.3	1.2	7.7	4.7
CYP	Cyproterone	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9	< 0.9
TP418_20.4	(3α-Hydroxy 3-deoxocyproterone acetate)*	2.8*	2.7*	1.3*	2.6*	4.9*	2.4*	0.3*	8.1*
DIE	Dienogest	0.9	2.0	0.3	2.2	0.5	1.2	1.2	3.7
TP327_11.3	6β-Hydroxy dienogest	0.6	1.4	0.6	1.6	1.2	1.4	1.1	1.1
TP309_16.4	(Δ9,11-dehydro-17α-cyanomethyl estradiol)*	1.2*	8.7*	5.4*	7.8*	16*	6.6*	2.8*	4.2*
DPN	Drospirenone	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8	< 0.8
ETG	Etonogestrel	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
LNG	Levonorgestrel	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0	< 1.0
MRPac	Medroxyprogesterone acetate	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
MRP	Medroxyprogesterone	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4	< 0.4
TP388_21.6	4,5(β)-Dihydro medroxyprogesterone acetate	< 3.8	< 3.8	< 3.8	< 3.8	< 3.8	< 3.8	< 3.8	< 3.8
NESac	Norethisterone acetate	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6	< 0.6
TP298_ (NES)	Norethisterone	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7	< 0.7
TP300_19.8	4,5(α)-Dihydro norethisterone	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2	< 1.2
TP296_18.5 (EE2)	17α-Ethinylestradiol	< 2.9	< 2.9	< 2.9	< 2.9	< 2.9	< 2.9	< 2.9	< 2.9

*) Calibration of the precursor steroids were used for an estimation of the concentration.

Analytical results and further remarks

Figure C.1. Analytical results and further remarks for 17α-hydroxyprogesterone derivatives and their detected TPs. a) XIC of the corresponding m/z, b) HRMS spectra, c) MS/MS spectra, d) time course of the relative intensity, e) information and comments.

Name:	Formula:	Precursor:	RT Shift: -	Identification:
Medroxyprogesterone Acetate	C24H34O4	[M+H]+		Reference standard
Structure:			**Comments:**	

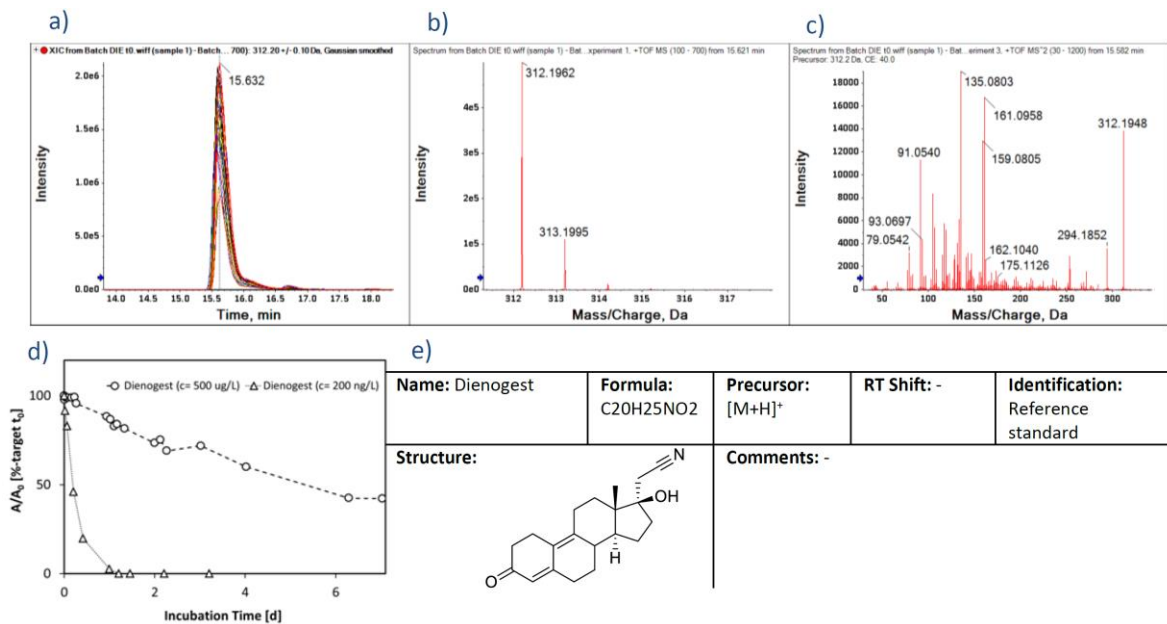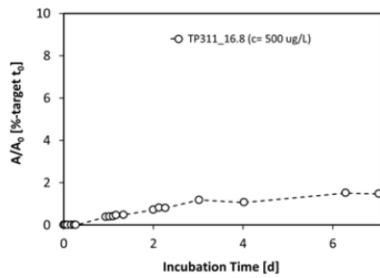| Name: TP386_21.3	Formula: C24H34O4	Precursor: [M+H]+	RT Shift: +0.3 min	Identification: Reference standard
Structure:			**Comments:** Inhibition of the Δ4-hydrogenation by C6-substituents (KUHL, 2011)	
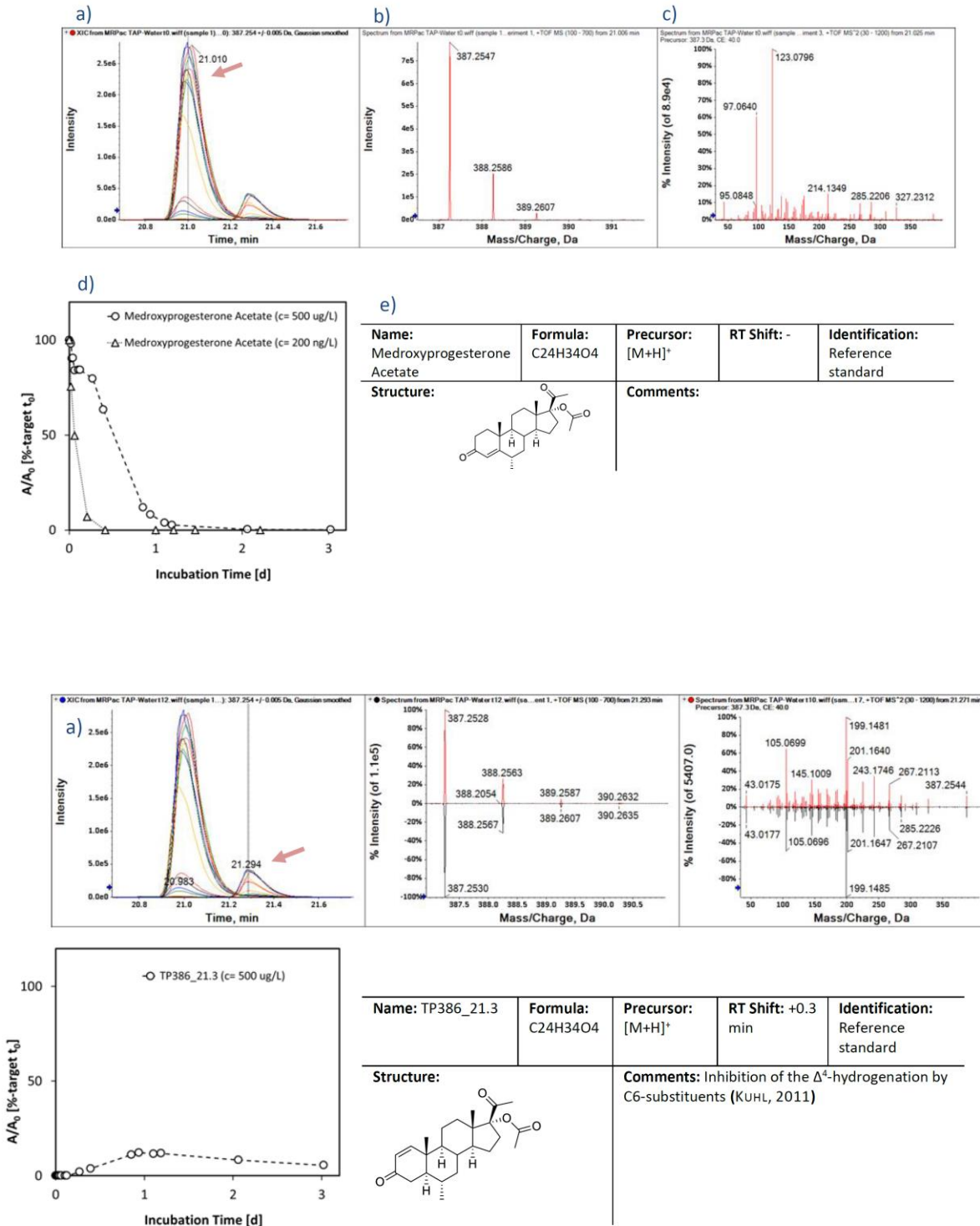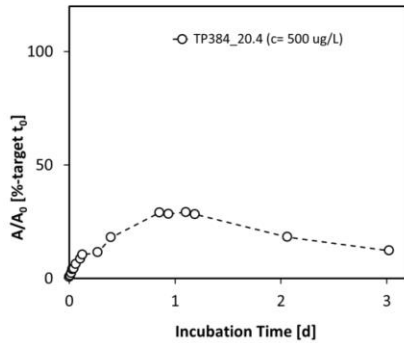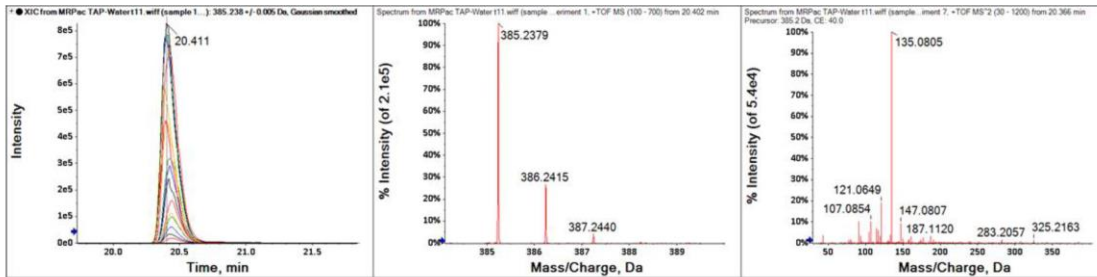
Name: TP384_20.4	Formula: C24H32O4	Precursor: [M+H]⁺	RT Shift: -0.6 min	Identification: Analogy to chlormadinone acetate
Proposed structure:			Comments: RT shift and MS fragmentation similar to delmadinone acetate. Δ¹-Dehydro TP most plausible.	
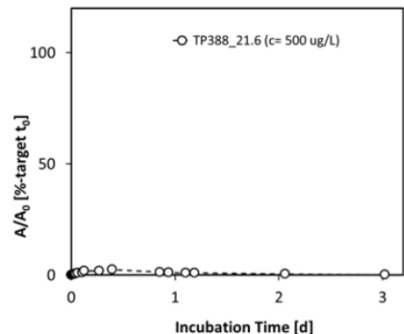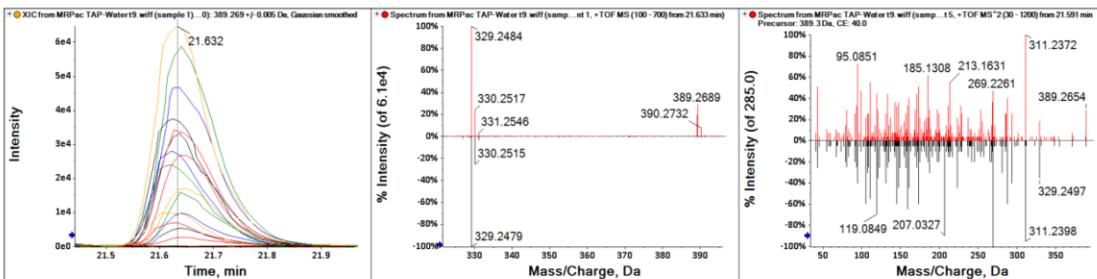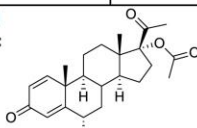
Name: TP388_21.6	Formula: C24H36O4	Precursor: [M+H]⁺	RT Shift: +0.6 min	Identification: Isotopic pattern, RT shift, MS fragmentation
Proposed structure:			Comments: Comparison with (5β)-4,5-dihydro medroxyprogesterone acetate revealed different RT. Although, the double peak and MS² indicates minor formation of the 5β-isomer. Most likely due to the C6α-methyl substituent, since only medroxyprogesterone acetate showed the double peak.	
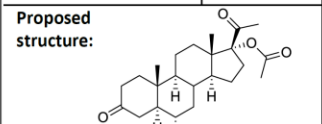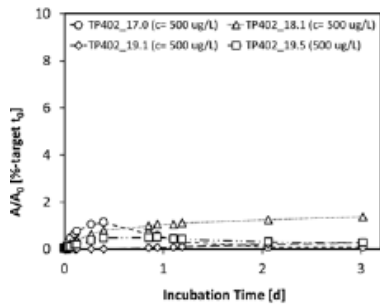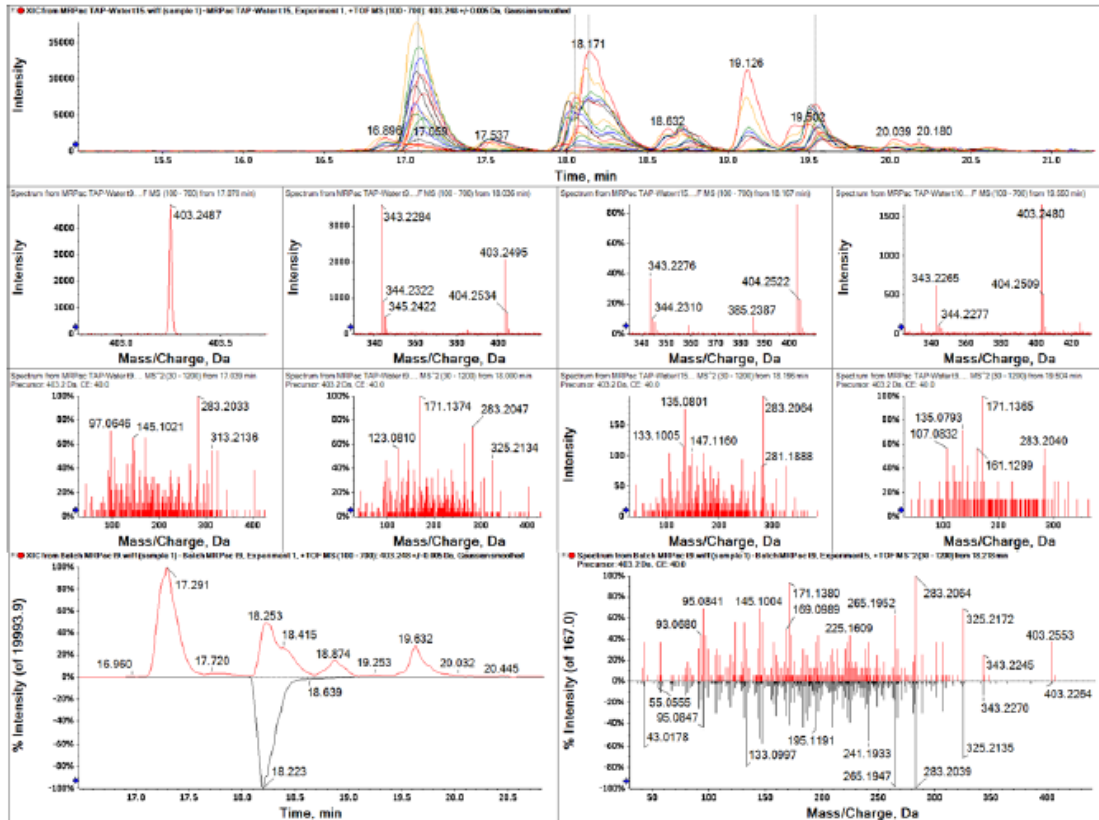
Name: TP402_17.0, (TP402_18.1), TP402_19.1, TP402_19.5	Formula: C24H34O5	Precursor: [M+H]⁺	RT Shift: -4.0 min, (-2.9 min), -1.9 min, -1.5 min	Identification: Isotopic pattern, MS fragmentation (reference standard)
(Proposed) structure: (TP402_18.1)			Comments: Formation of the hydroxylated TPs below 2%. Verification of TP402_18.1 via reference standard of 6β-hydroxy medroxyprogesterone acetate.	
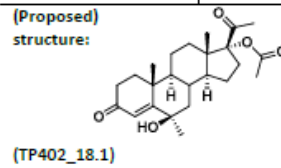
Name: TP404_19.2, TP404_20.6	Formula: C24H36O5	Precursor: [M+H]+	RT Shift: -1.8 min,- 0.4 min	Identification: Isotopic pattern, MS fragmentation (reference standard)
Structure:			Comments: Proposed structure, exact position and configuration of –OH not determined. The small RT shift of TP404_20.6 suggests an α-hydroxy ketone. Formation of the hydroxylated TPs below 3%.	
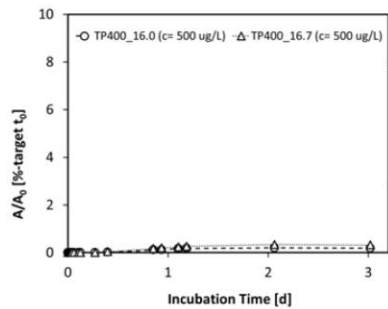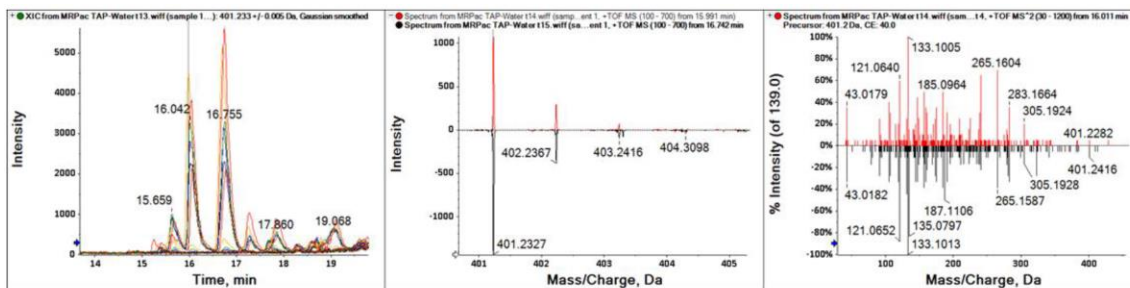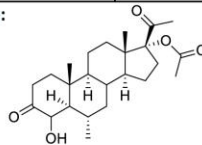
Name: TP400_16.0, TP400_16.7	Formula: C24H32O5	Precursor: [M+H]+	RT Shift: -5.0 min, -4.3 min	Identification: Isotopic pattern, MS fragmentation (reference standard)
Proposed structure:			Comments: Exact position and configuration of –OH not determined. Formation of the hydroxylated TPs below 1%.	
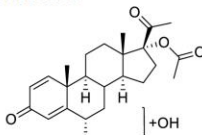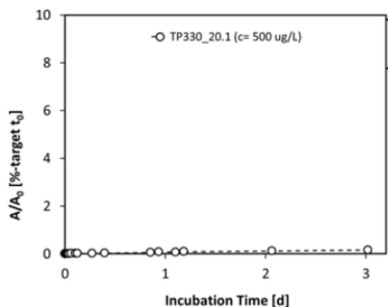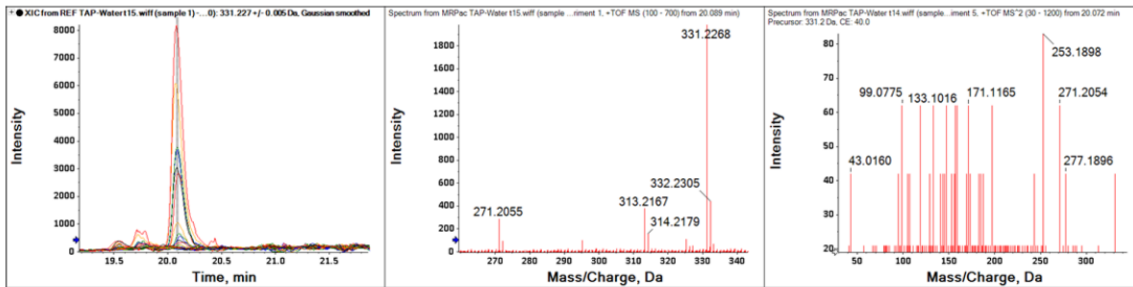
Name: TP330_20.1	**Formula:** C21H30O3	**Precursor:** [M+H]$^+$	**RT Shift:** -0.9 min	**Identification:** -
Structure: no final structure		**Comments:** Formation below 1%. TP showed cleavage of acetic acid (331<271).		
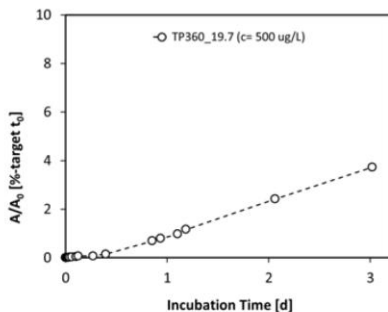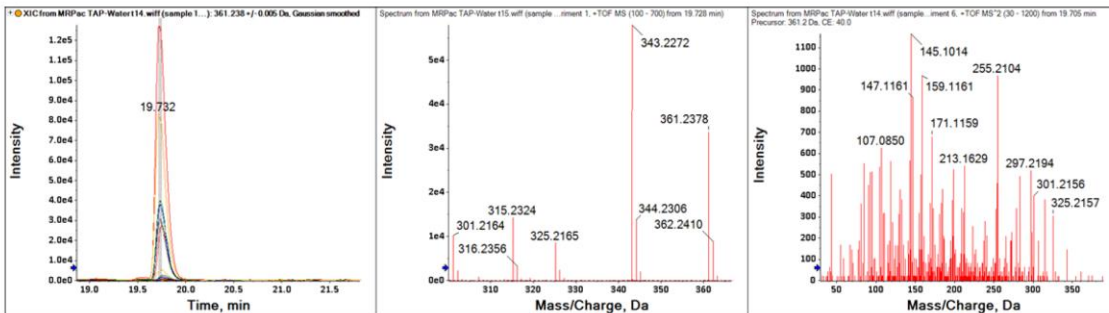
Name: TP360_19.7	**Formula:** C22H32O4	**Precursor:** [M+H]$^+$	**RT Shift:** -1.3 min	**Identification:** -
Structure: no final structure		**Comments:** Formation below 5%.		
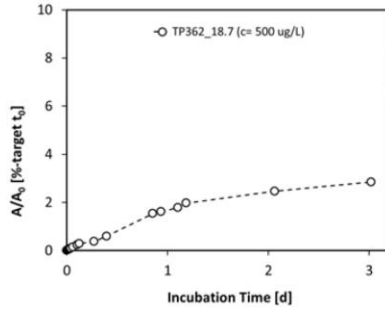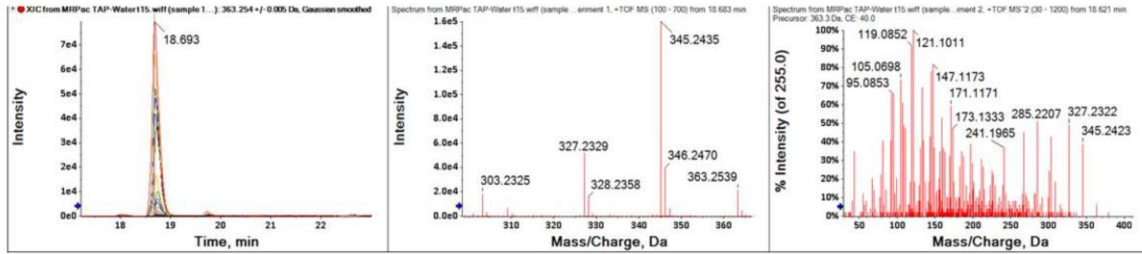
Name: TP362_18.7	Formula: C22H34O4	Precursor: [M+H]⁺	RT Shift: -2.3 min	Identification: -
Structure: no final structure		Comments: Formation below 5%.		
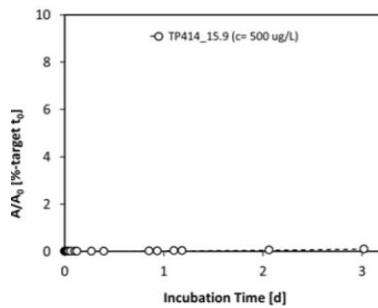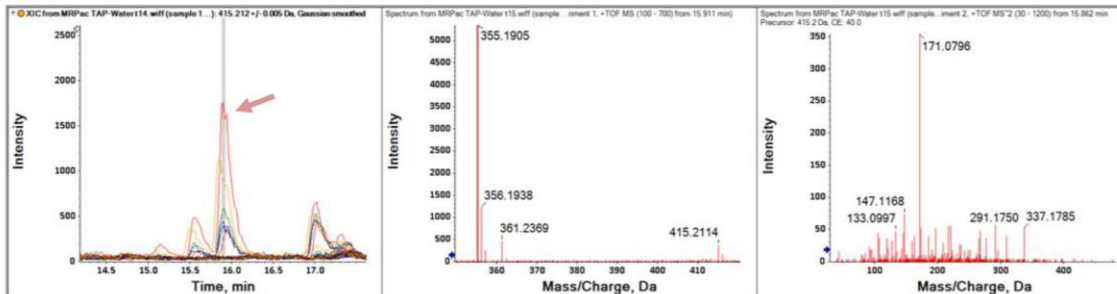
Name: TP414_15.9	Formula: C24H30O6	Precursor: [M+H]⁺	RT Shift: -5.1 min	Identification: -
Structure: no final structure		Comments: Formation below 1%. TP showed significant loss of acetic acid in ESI (415<355).		
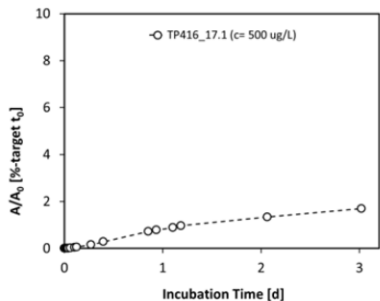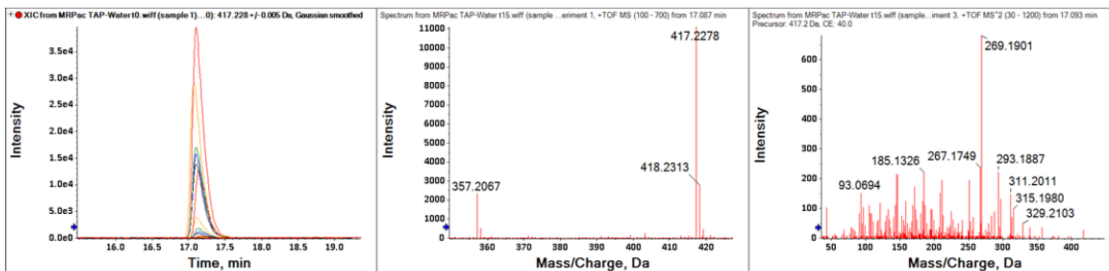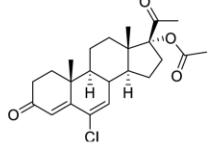
Name: TP416_17.1	Formula: C24H32O6	Precursor: [M+H]⁺	RT Shift: -3.9 min	Identification: -
Structure: no final structure		Comments: Formation below 3%. TP showed loss of acetic acid in ESI (417<357).		
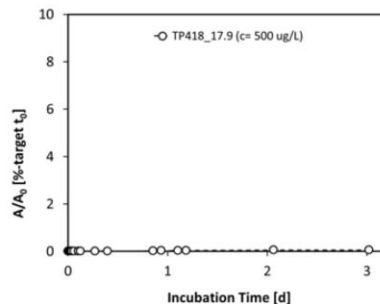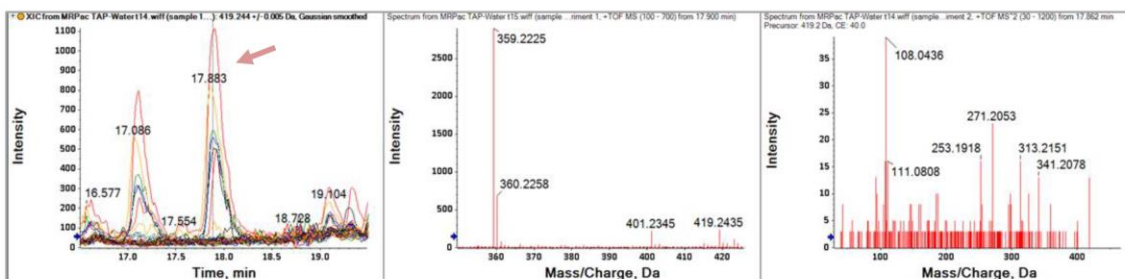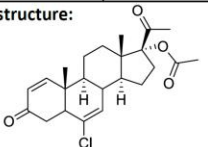
Name: TP418_17.9	Formula: C24H34O6	Precursor: [M+H]⁺	RT Shift: -3.1 min	Identification: -
Structure: no final structure		Comments: Formation below 1%. TP showed significant loss of acetic acid in ESI (419<359).		
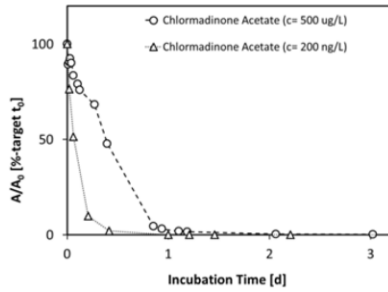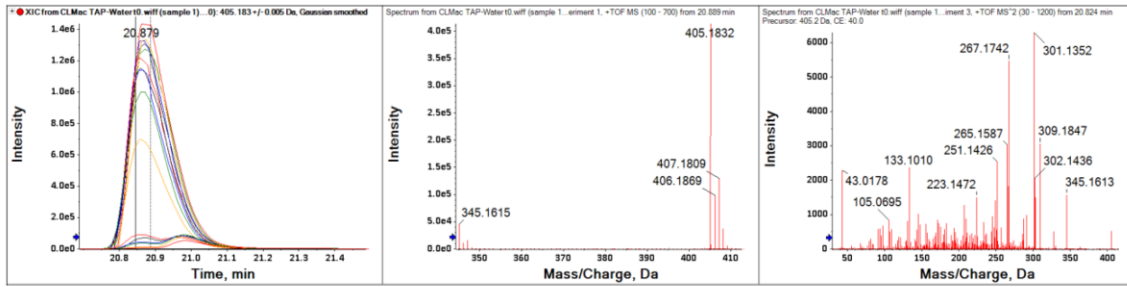
Name: Chlormadinone Acetate	Formula: C23H29ClO4	Precursor: [M+H]$^+$	RT Shift: -	Identification: Reference standard
Structure:		Comments: -		

Name: TP404_21.0	Formula: C23H29ClO4	Precursor: [M+H]$^+$	RT Shift: +0.1 min	Identification: Analogy to Medroxyprogesterone Acetate
Proposed structure:		Comments: Inhibition of the Δ^4-hydrogenation by C6-substituents (KUHL, 2011)		
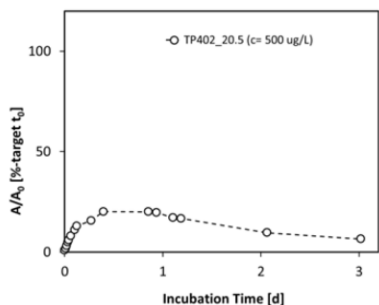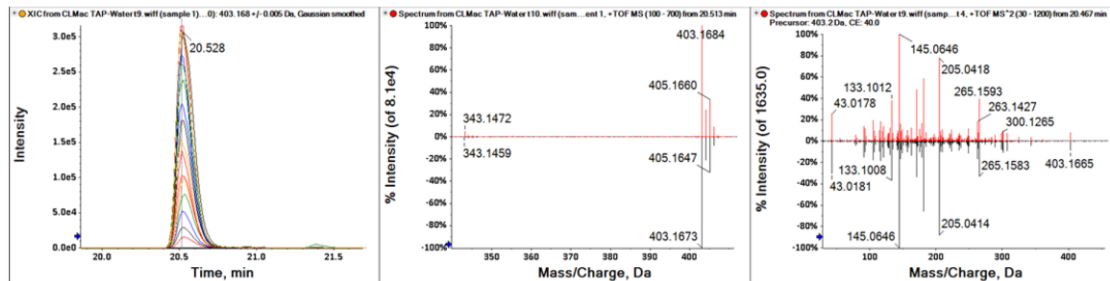
Name: TP402_20.5 (Delmadinone Acetate)	Formula: C23H27ClO4	Precursor: [M+H]⁺	RT Shift: -0.4 min	Identification: Reference standard
Structure:		Comments: Δ¹-Dehydrogenation favored initial reaction		
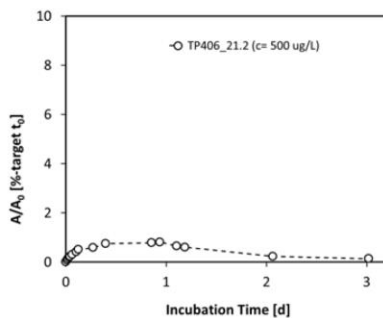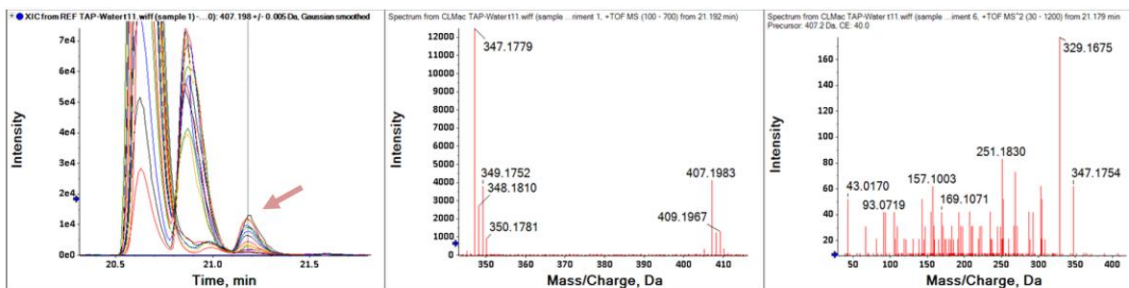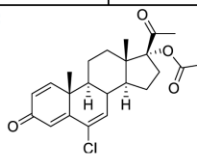
Name: TP406_21.2	Formula: C23H31ClO4	Precursor: [M+H]⁺	RT Shift: +0.3 min	Identification: Isotopic pattern, MS fragmentation, RT shift
Proposed structure:		Comments: Formation below 3%. Significant loss of acetic acid in ESI (407>347).		
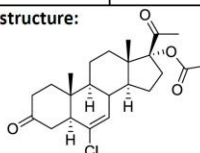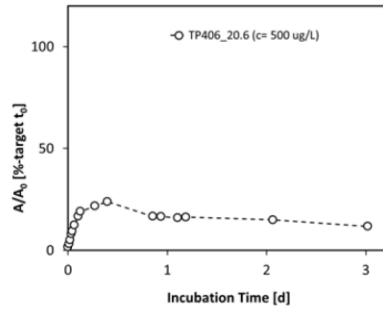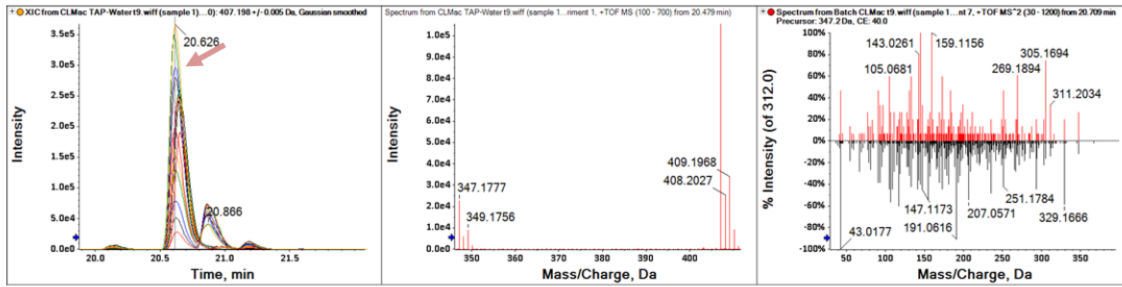
	Name: TP406_20.6	Formula: C23H31ClO4	Precursor: [M+H]⁺	RT Shift: -0.3 min	Identification: Isotopic pattern, MS fragmentation, RT shift, comparison to chlormadinol acetate reference standard
	Proposed structure:		Comments: Comparison with 3β-hydroxy-Δ⁴-derivative (chlormadinol acetate) revealed differing RT and MS². Other structures might be possible (e.g. 3α-hydroxy-Δ⁴, 3β-hydroxy-Δ¹)		
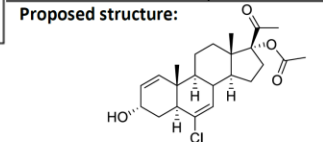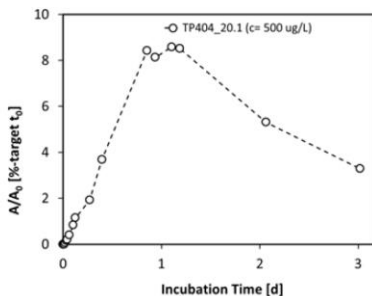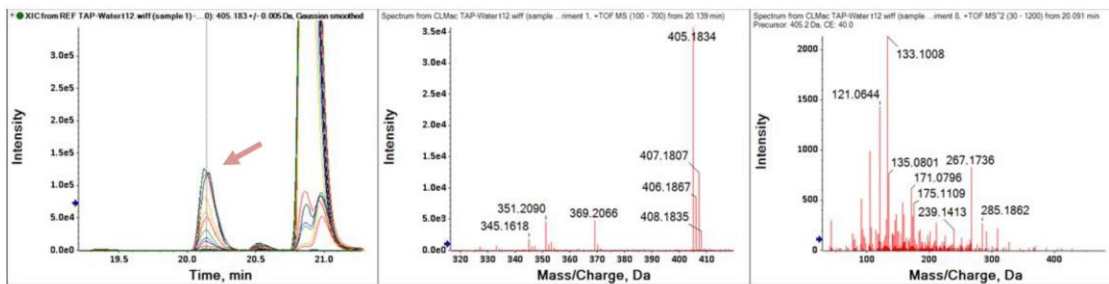
	Name: TP404_20.1	Formula: C23H29ClO4	Precursor: [M+H]⁺	RT Shift: -0.8 min	Identification: Isotopic pattern, MS fragmentation, RT shift
	Proposed structure:		Comments: Formation below 10%.		
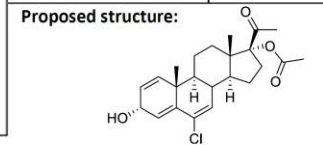
216
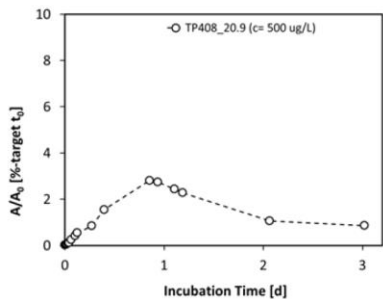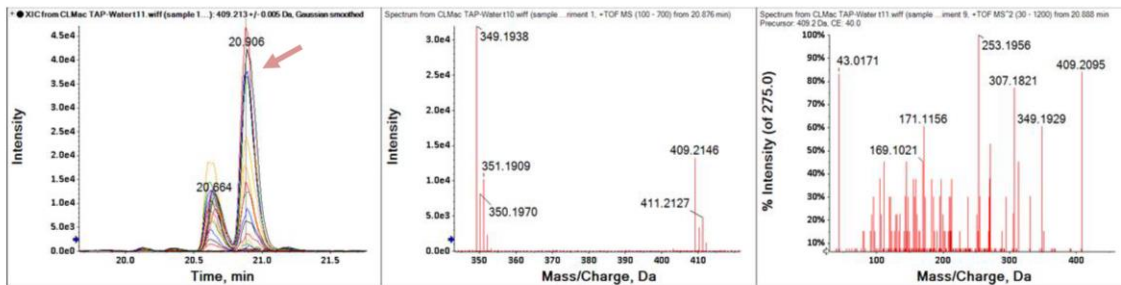
Name: TP408_20.9	Formula: C23H33ClO4	Precursor: [M+H]⁺	RT Shift: 0.0 min	Identification: Isotopic pattern, MS fragmentation, RT shift
Proposed structure:			Comments: Formation below 5%. Same RT as chlormadinone acetate.	
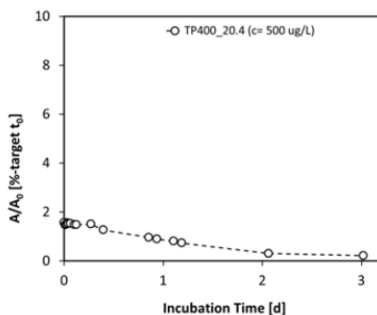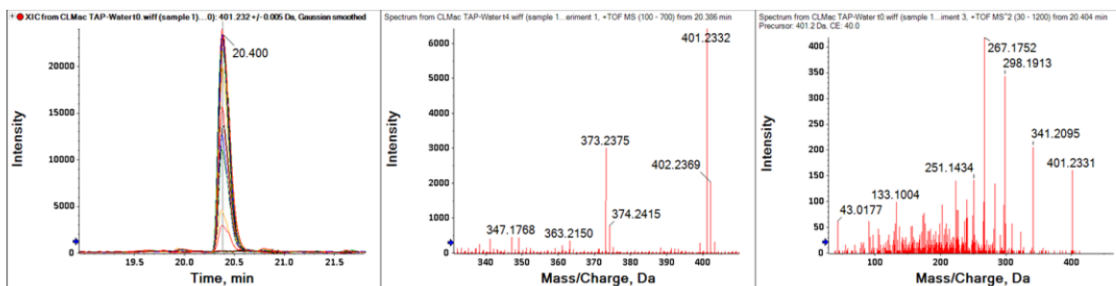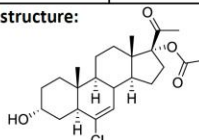
Name: TP400_20.4	Formula: C24H32O5	Precursor: [M+H]⁺	RT Shift: -0.5 min	Identification:
Structure: no final structure		Comments: Dechlorinated TP. Might be a contamination from the used reference standard, since maximum at t0. Formation below 2%.		
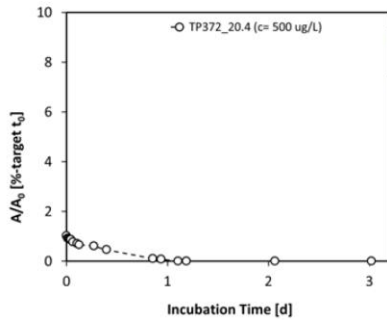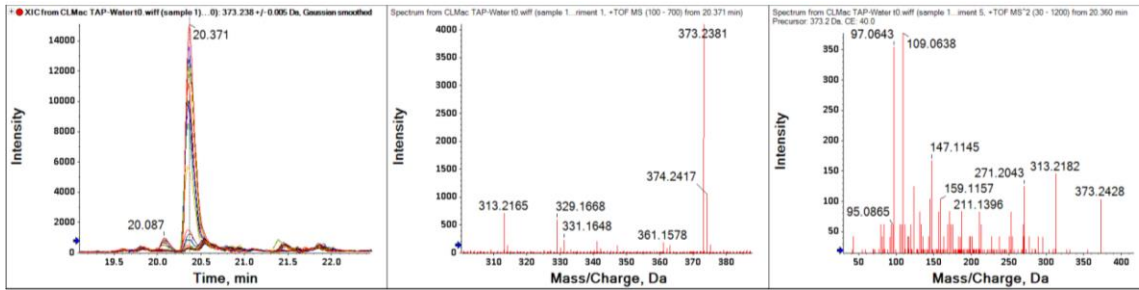
Name: TP372_20.4	**Formula:** C23H32O4	**Precursor:** [M+H]⁺	**RT Shift:** -0.5 min	**Identification:**	
Structure: no final structure		**Comments:** Dechlorinated TP. Might be a contamination from the used reference standard, since maximum at t0. Formation below 2%.			
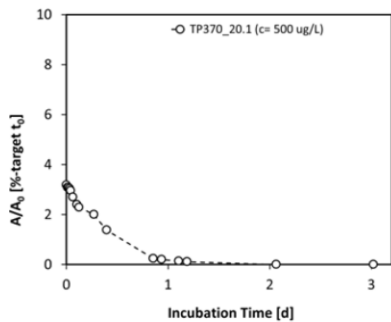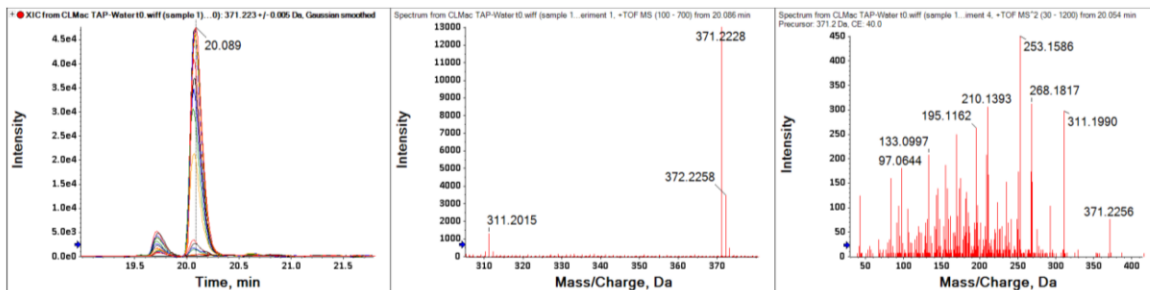
Name: TP370_20.1	**Formula:** C23H30O4	**Precursor:** [M+H]⁺	**RT Shift:** -0.8 min	**Identification:**	
Structure: no final structure		**Comments:** Dechlorinated TP. Might be a contamination from the used reference standard, since maximum at t0. Formation below 5%.			
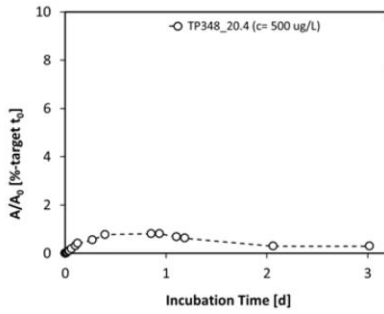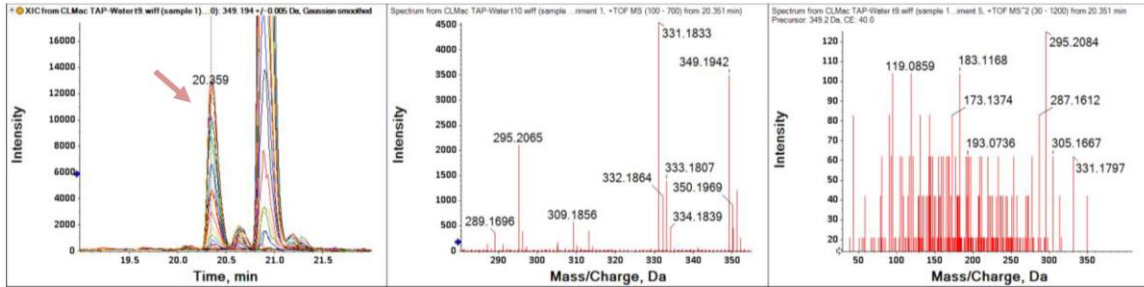
	Name: TP348_20.4	**Formula:** C21H29ClO2	**Precursor:** [M+H]+	**RT Shift:** -0.5 min	**Identification:**
	Structure: no final structure		**Comments:** Formation below 2%.		
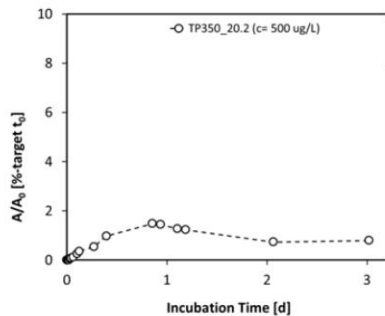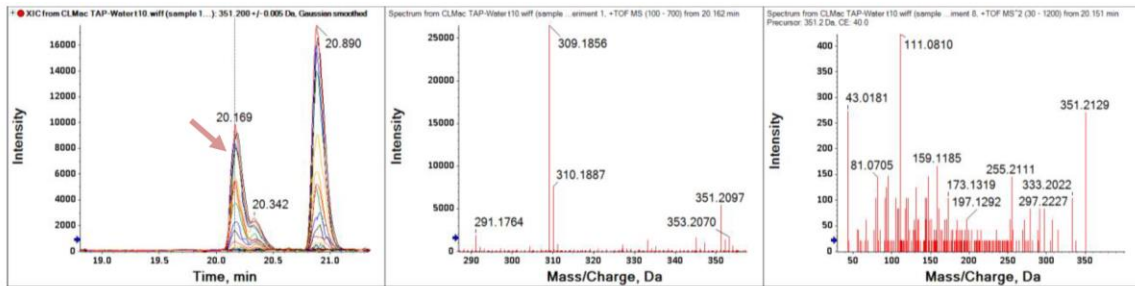
	Name: TP350_20.2	**Formula:** C21H31ClO2	**Precursor:** [M+H]+	**RT Shift:** -0.7 min	**Identification:**
	Structure: no final structure		**Comments:** Formation below 2%.		
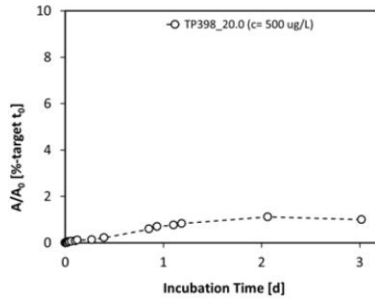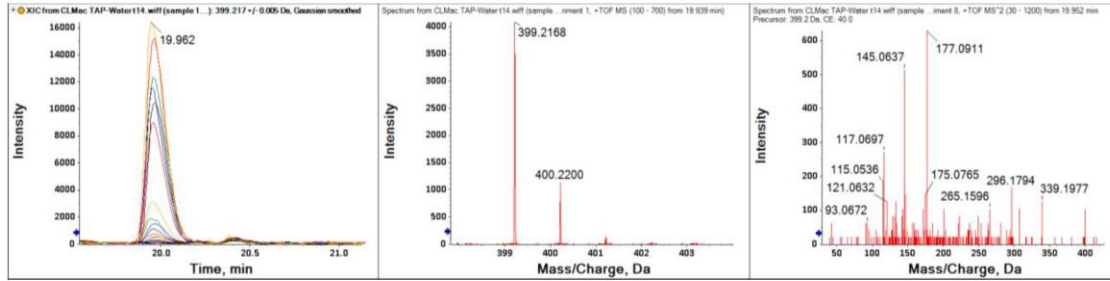
Name: TP398_20.0	**Formula:** C24H30O5	**Precursor:** [M+H]⁺	**RT Shift:** -0.9 min	**Identification:**
Structure: no final structure		**Comments:** Dechlorinated TP. Formation below 2%.		
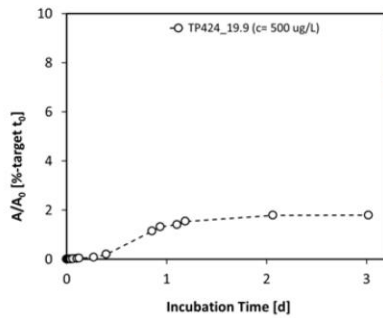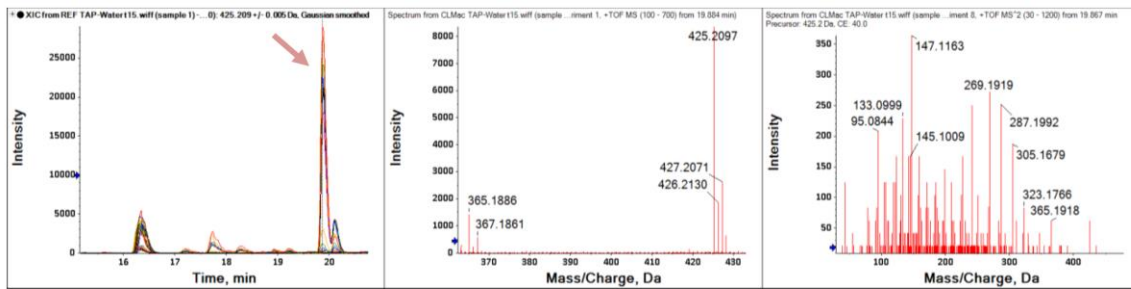
Name: TP424_19.9 & further minor TPs	**Formula:** C23H33ClO5	**Precursor:** [M+H]⁺	**RT Shift:** -1.1 min	**Identification:**
Structure: no final structure		**Comments:** Dechlorinated TP. Formation below 5%.		
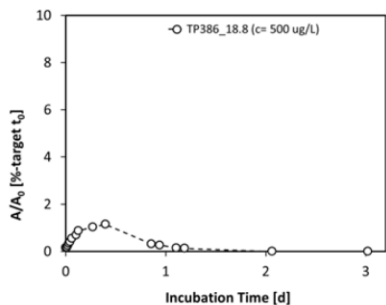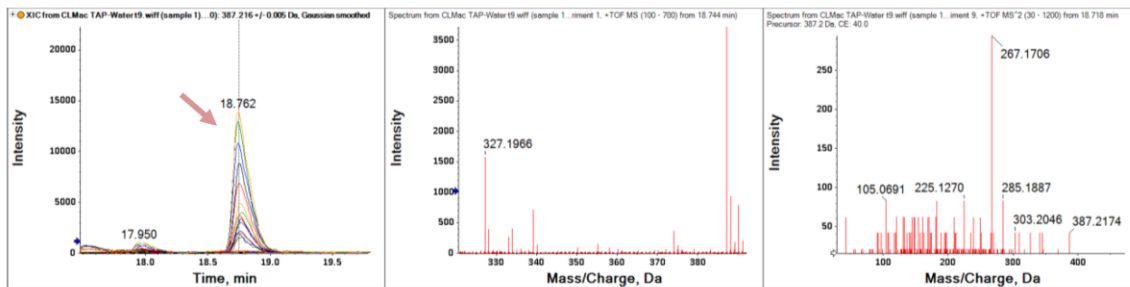
Name: TP386_18.8	Formula: C23H30O5	Precursor: [M+H]⁺	RT Shift: -2.1 min	Identification:
Structure: no final structure		Comments: Dechlorinated TP. Formation below 2%.		
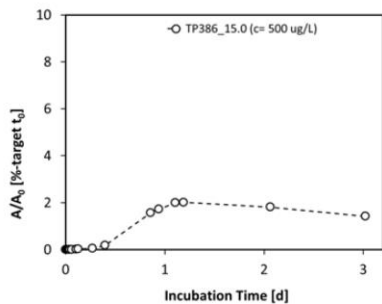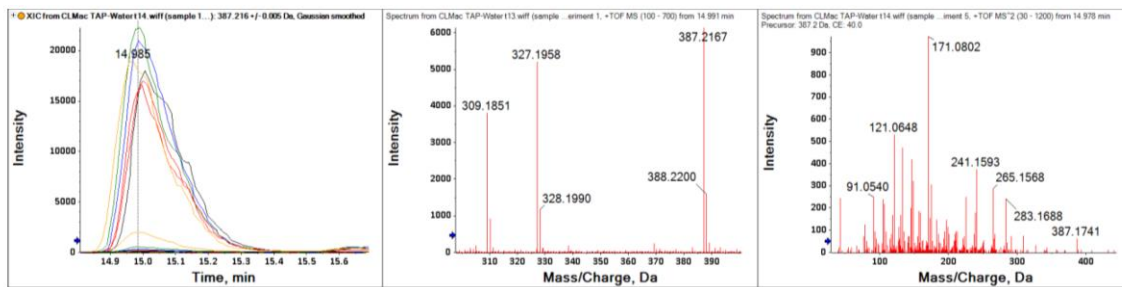
Name: TP386_15.0	Formula: C23H30O5	Precursor: [M+H]⁺	RT Shift: -2.1 min	Identification:
Structure: no final structure		Comments: Dechlorinated TP. Formation below 5%.		
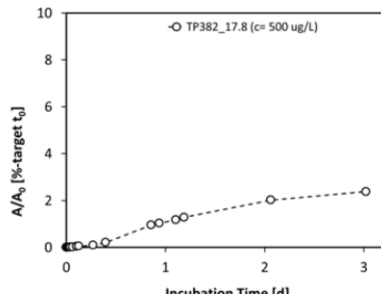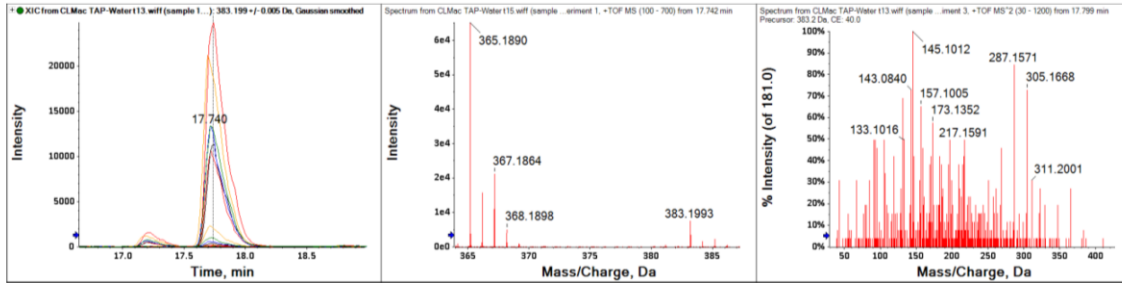
Name: TP382_17.8	Formula: C21H31ClO4	Precursor: [M+H]⁺	RT Shift: -3.1 min	Identification:
Structure: no final structure		Comments: Significant loss of H2O in ESI (383>365). Formation below 5%.		

d)

e)
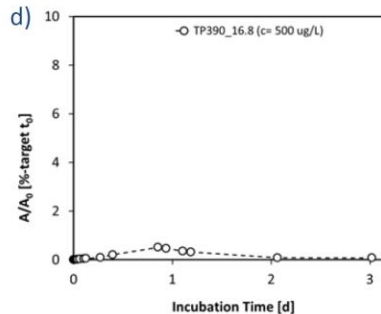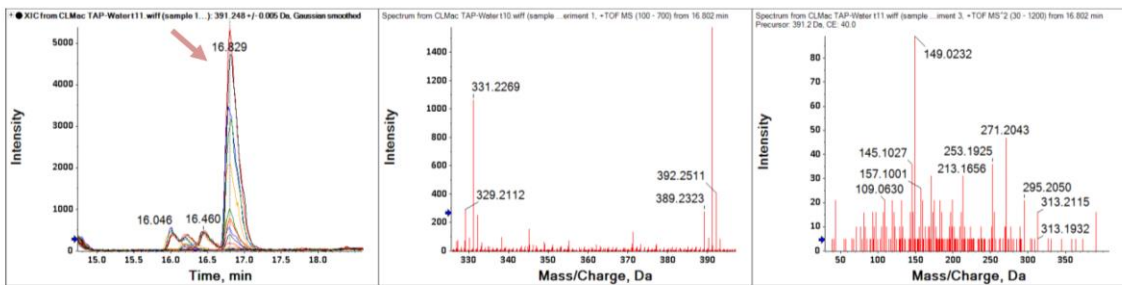
Name: TP390_16.8	Formula: C23H34O5	Precursor: [M+H]⁺	RT Shift: -4.1 min	Identification:
Structure: no final structure		Comments: Dechlorinated TP. Formation below 1%.		
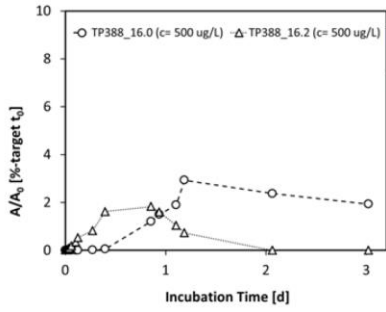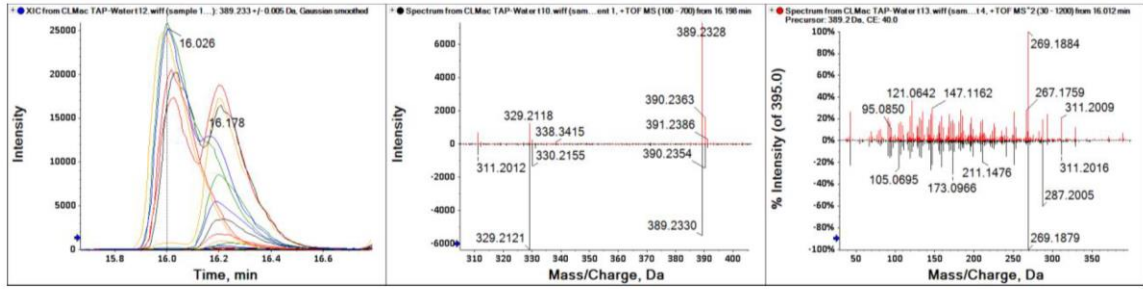
Name: TP388_16.0, TP388_16.2	Formula: C23H32O5	Precursor: [M+H]⁺	RT Shift: -2.1 min	Identification:
Structure: no final structure		Comments: Dechlorinated TPs. Formation below 5%.		
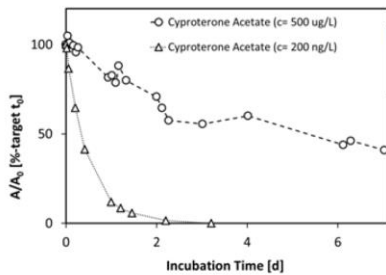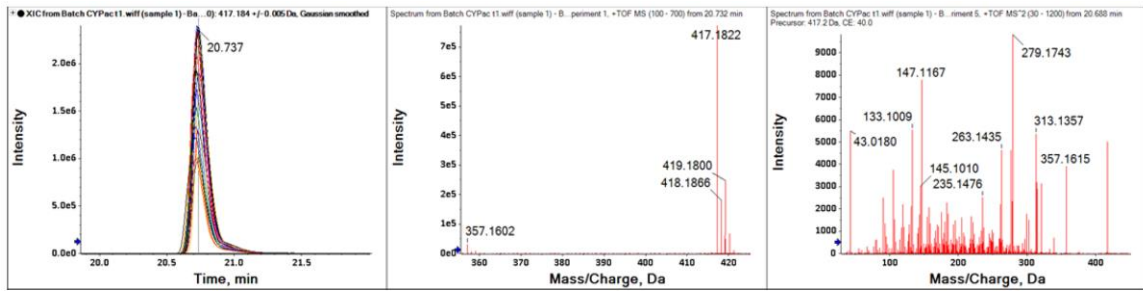
Name: Cyproterone Acetate	Formula: C24H29ClO4	Precursor: [M+H]⁺	RT Shift: -	Identification: Reference standard
Structure:		Comments: -		
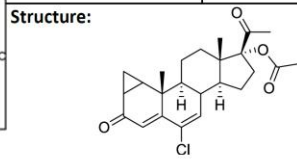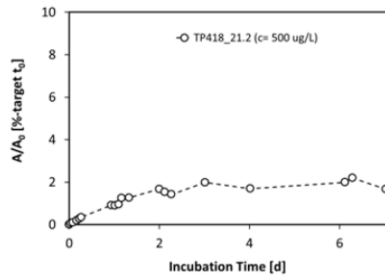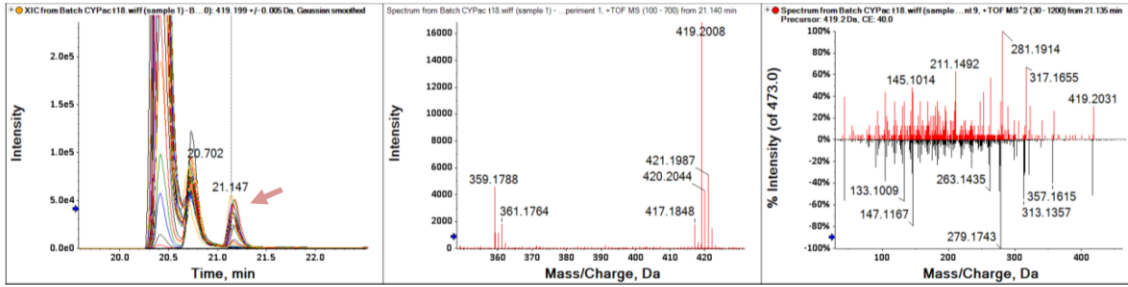
Name: TP418_21.2	Formula: C24H31ClO4	Precursor: [M+H]⁺	RT Shift: +0.5 min	Identification: Analogy to other results, isotopic pattern, MS fragmentation, RT shift
Proposed structure:			Comments: Formation below 5%.	
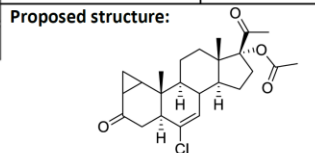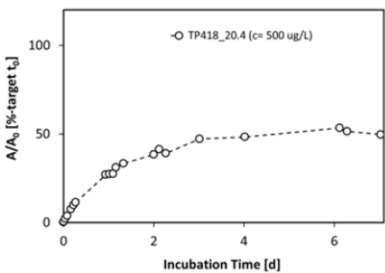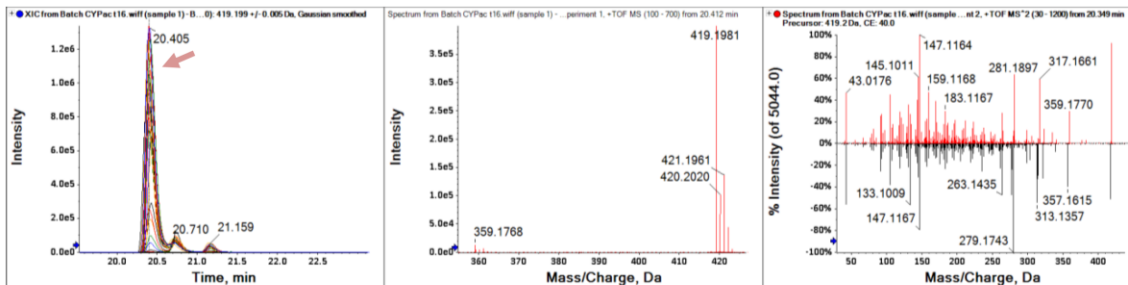
Name: TP418_20.4	Formula: C24H31ClO4	Precursor: [M+H]⁺	RT Shift: -0.3 min	Identification: Analogy to other results, isotopic pattern, MS fragmentation, RT shift
Proposed structure:			Comments: Main TP and ubiquitously detected in WWTP effluents. Reference standard is ordered and confirmation in progress.	
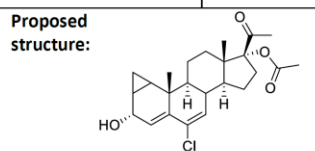
	Name: TP420_20.6	Formula: C24H33ClO4	Precursor: [M+H]⁺	RT Shift: -0.1 min	Identification: Analogy to other results, isotopic pattern, MS fragmentation, RT shift
	Proposed structure:		Comments: Formation below 3%.		

	Name: TP400_15.8, TP400_16.5, TP400_17.6	Formula: C24H32O5	Precursor: [M+H]⁺	RT Shift: -4.9 min, -4.2 min, -3.1 min	Identification: -
	Structure: no final structures		Comments: Dechlorinated TPs.		

	Name: TP384_20.1	Formula: C24H32O4	Precursor: [M+H]⁺	RT Shift: -0.6 min	Identification: -
	Structure: no final structure		Comments: Dechlorinated TP.		
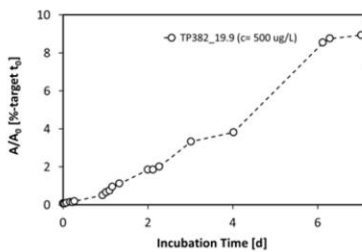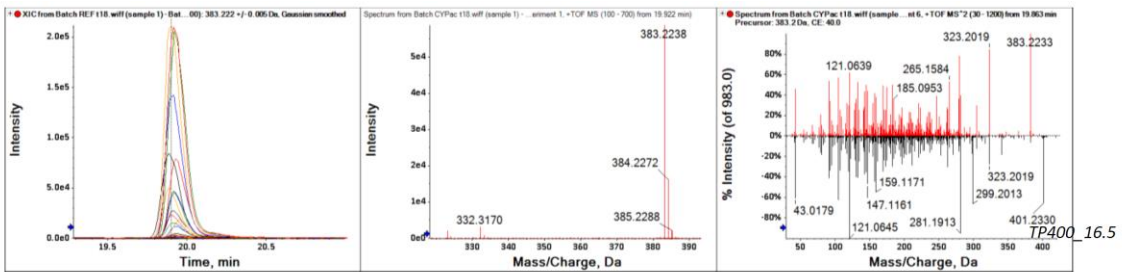
	Name: TP382_19.9	Formula: C24H30O4	Precursor: [M+H]⁺	RT Shift: -0.8 min	Identification: -
	Structure: no final structure		Comments: Dechlorinated TP.		
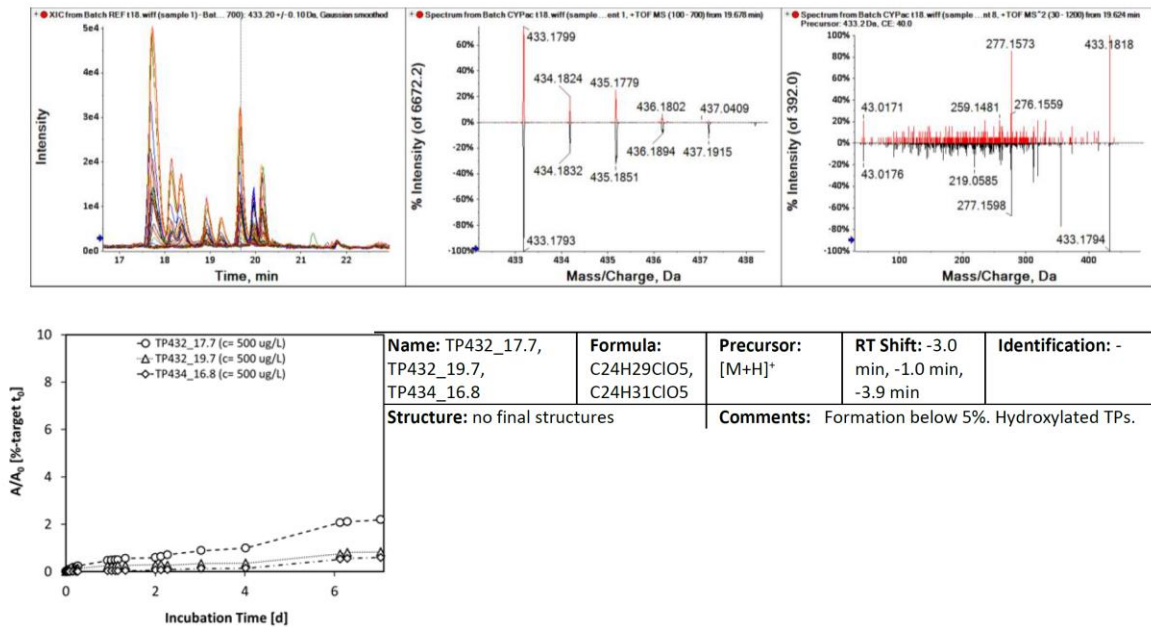
Figure C.2. Analytical results and further remarks for 19-nortestosterone derivatives and their detected TPs. a) XIC of the corresponding m/z, b) HRMS spectra, c) MS/MS spectra, d) time course of the relative intensity, e) information and comments.
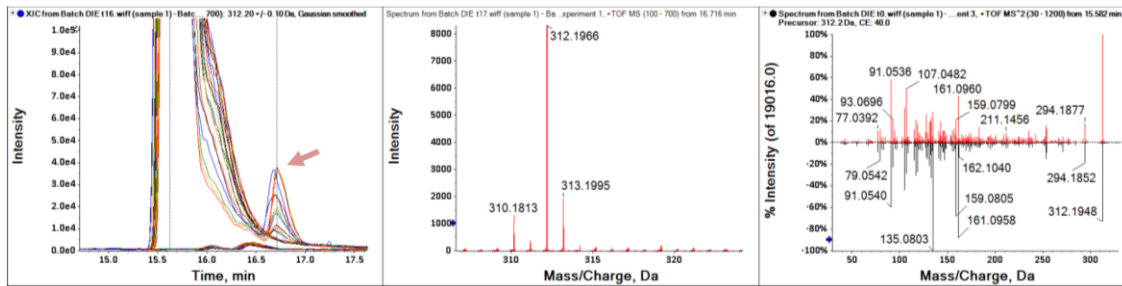
	Name: TP311_16.8	**Formula:** C20H25NO2	**Precursor:** [M+H]⁺	**RT Shift:** +1.2 min		**Identification:** Analogy to other results, isotopic pattern, MS fragmentation, RT shift, comparison with similar reference standard
	Proposed structure:			**Comments:** Comparison with reference standard of (17α)-17-Hydroxy-3-oxo-19-norpregna-5(10),9(11)-diene-21-nitrile (CAS: 106111-42-6) revealed different RT and equal MS² spectra. Formation below 3%.		
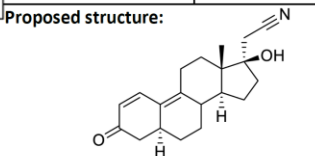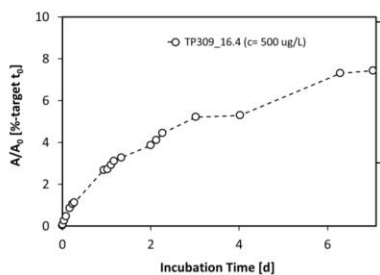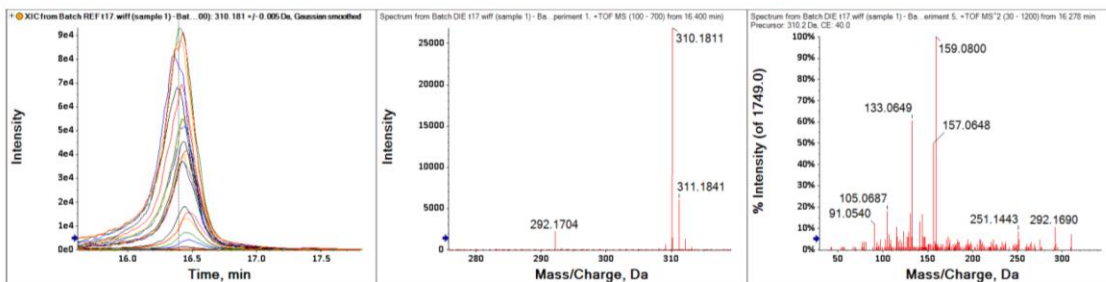
	Name: TP309_16.4	**Formula:** C20H27NO2	**Precursor:** [M+H]⁺	**RT Shift:** +0.8 min		**Identification:** Analogy to other results, isotopic pattern, MS fragmentation, RT shift
	Proposed structure:			**Comments:** Broad peak width (1.5 min) suggests isomers or equilibrium of different species. Comparison with other column/ chromatographic conditions showed similar peak symmetry and peak width.		
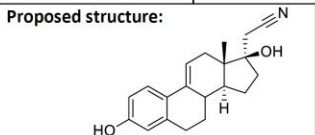
Name: TP309_11.4	Formula: C20H27NO2	Precursor: [M+H]⁺	RT Shift: -4.2 min	Identification: Isotopic pattern, MS fragmentation
Proposed structure: no final structure		**Comments:** Broad peak width (2 min) suggests isomers or equilibrium of different species. Comparison with other column/chromatographic conditions showed similar peak symmetry and peak width.		

Name: TP327	Formula: C20H29NO3	Precursor: [M+H]⁺	RT Shift: -	Identification: Reference standard, Isotopic pattern, MS fragmentation
Structure: (TP327_11.3)		**Comments:** Confirmation of TP327 with 6β-hydroxy dienogest reference standard. Several hydroxylated isomers were detected.		
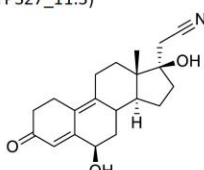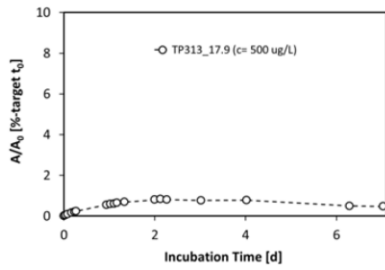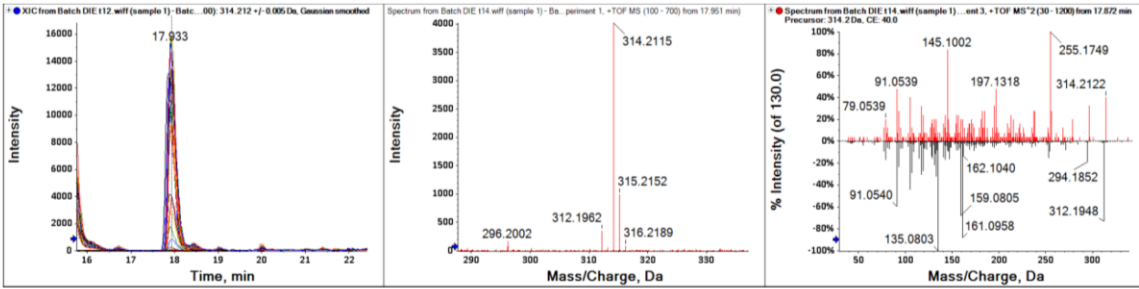
Name: TP313_17.9	Formula: C20H27NO2	Precursor: [M+H]⁺	RT Shift: +2.3 min	Identification: Isotopic pattern, MS fragmentation, analogy to other results
Proposed structure:		Comments: Formation below 5%.		
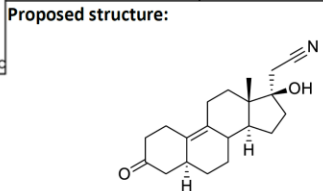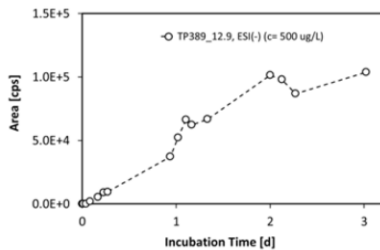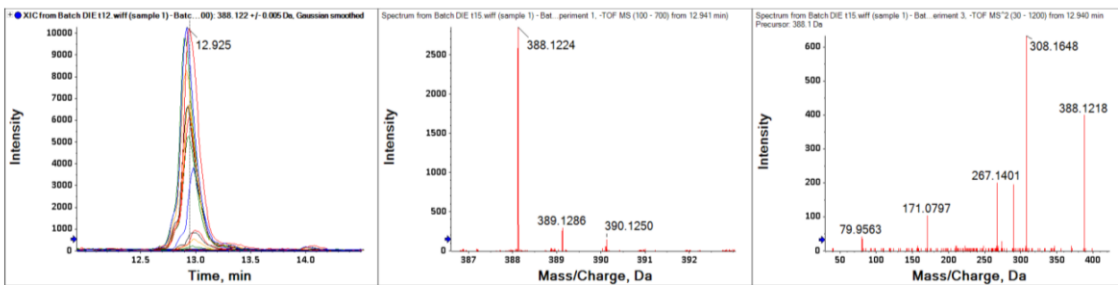
Name: TP389_12.9	Formula: C20H23NO5S	Precursor: [M-H]⁻	RT Shift: -2.7 min	Identification: Isotopic pattern, MS fragmentation, RT shift
Proposed structure:		Comments: Phenolic sulfate conjugate (388.2 > 79.9). Relative formation quantity for TPs in ESI(-) could not been estimated.		
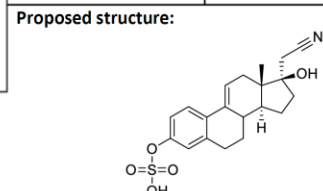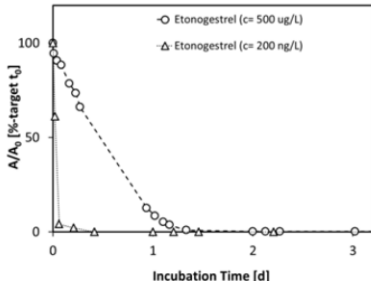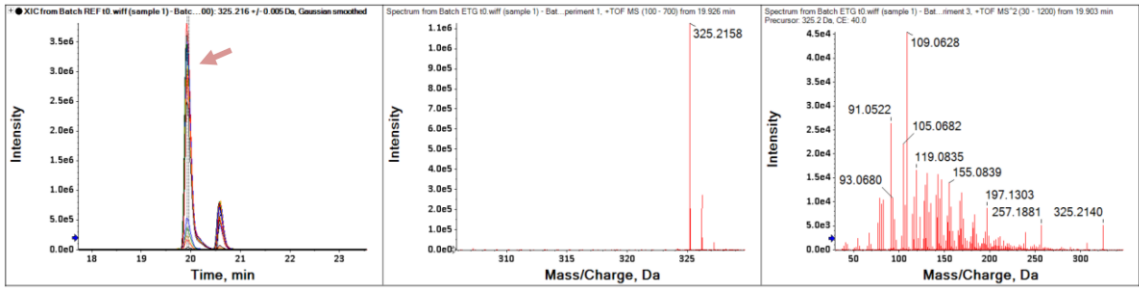
Name: Etonogestrel	**Formula:** C22H28O2	**Precursor:** [M+H]$^+$	**RT Shift:** -	**Identification:** Reference standard
Structure:		**Comments:** -		
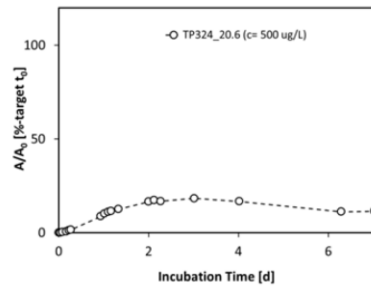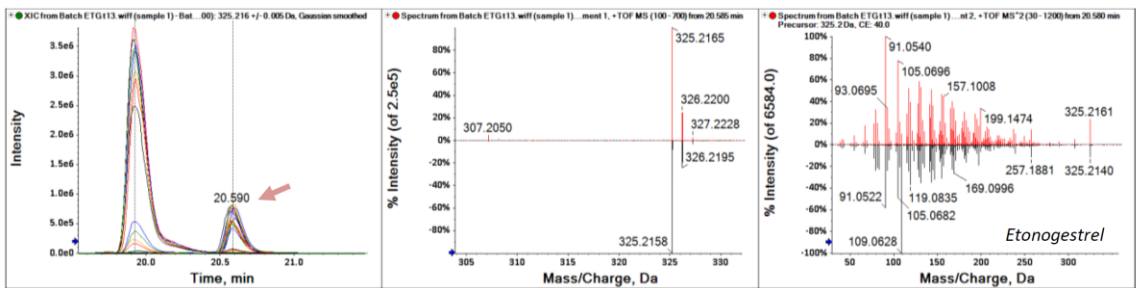
Name: TP324_20.6	**Formula:** C22H28O2	**Precursor:** [M+H]$^+$	**RT Shift:** +0.7 min	**Identification:** RT shift, isotopic pattern, MS fragmentation, analogy to other results
Proposed structure:		**Comments:**		
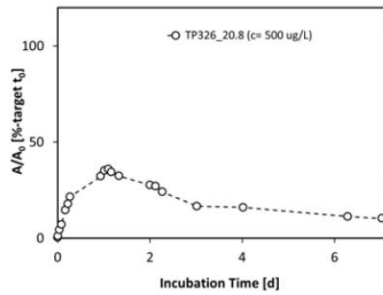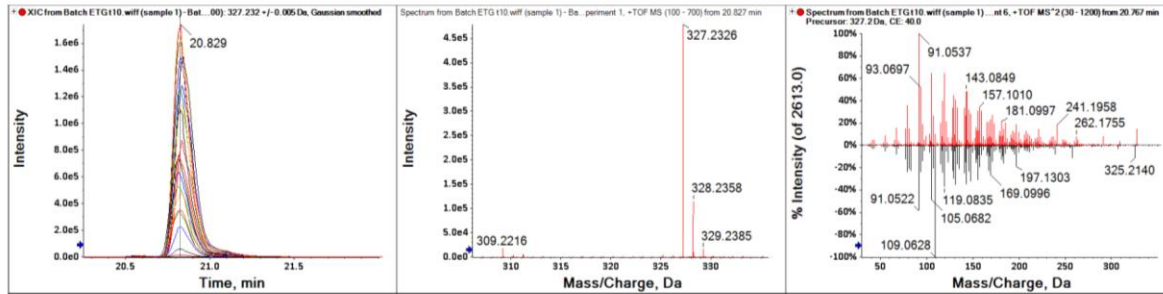
Name: TP326_20.8	Formula: C22H30O2	Precursor: [M+H]+	RT Shift: +0.9 min	Identification: RT shift, isotopic pattern, MS fragmentation, analogy to other results
Proposed structure:		Comments: Major TP.		
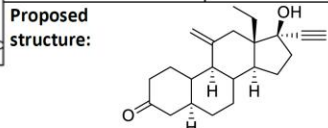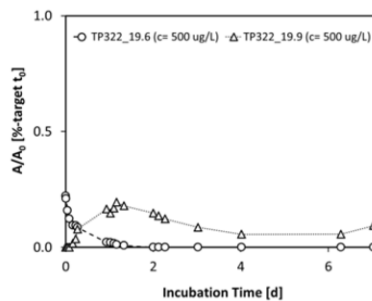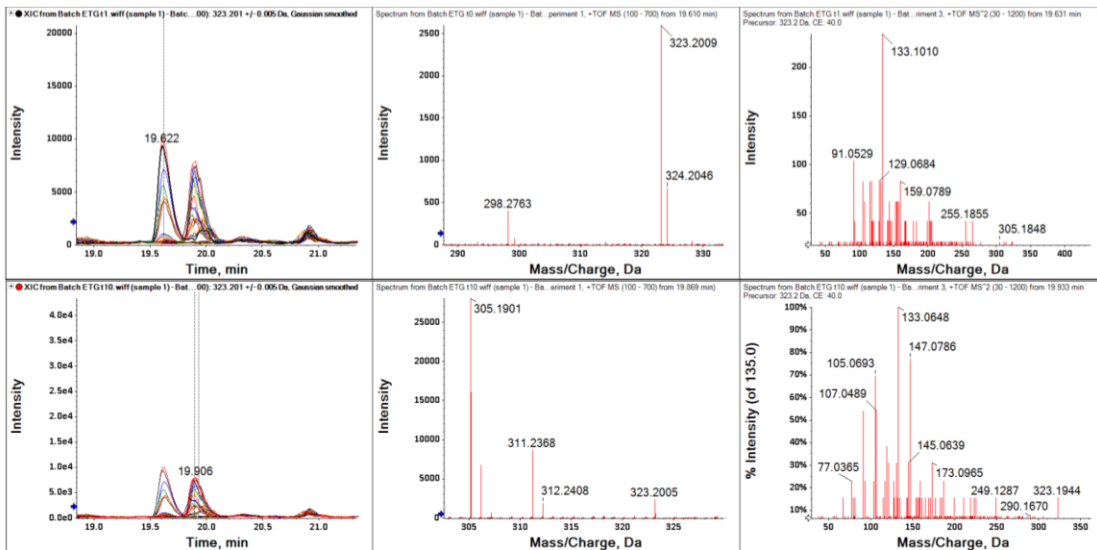
Name: TP322_19.6, TP322_19.9	Formula: C22H26O2	Precursor: [M+H]+	RT Shift: -0.3 min, -0.03 min	Identification: RT shift, isotopic pattern, MS fragmentation, analogy to other results
Proposed structure: TP322_19.6 TP322_19.9		Comments: TP322_19.9 showed typical fragmentation pattern of estrogens. MS² of the in-source fragment of TP322_19.9 matched with the MS² of the in-source fragment of EE2 (see Fig. S5)		
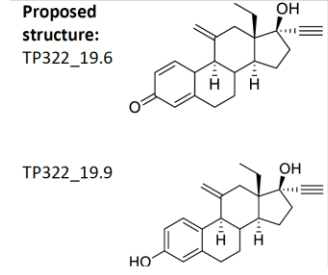
Name: TP340_14.5, TP340_14.8	Formula: C22H28O3	Precursor: [M+H]+	RT Shift: -5.4 min, -5.1 min	Identification: RT shift, isotopic pattern, MS fragmentation, analogy to other results
Proposed structure:			**Comments:** Several hydroxylated isomers were detected. Formation below 5%.	

Name: TP342_15.2, TP342_19.2, TP342_19.7	Formula: C22H30O3	Precursor: [M+H]+	RT Shift: -4.7 min, -0.7 min, -0.2 min	Identification: RT shift, isotopic pattern, MS fragmentation, analogy to other results
Proposed structure:			**Comments:** Several hydroxylated isomers were detected. Formation below 5%.	

Name: TP344_16.4	Formula: C21H28O4	Precursor: [M+H]⁺	RT Shift: -3.5 min	Identification: -
Proposed structure: no final structure		Comments: TP with incomplete steroid skeleton.		
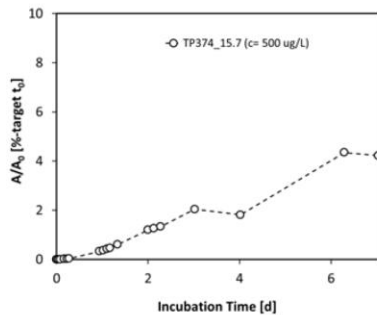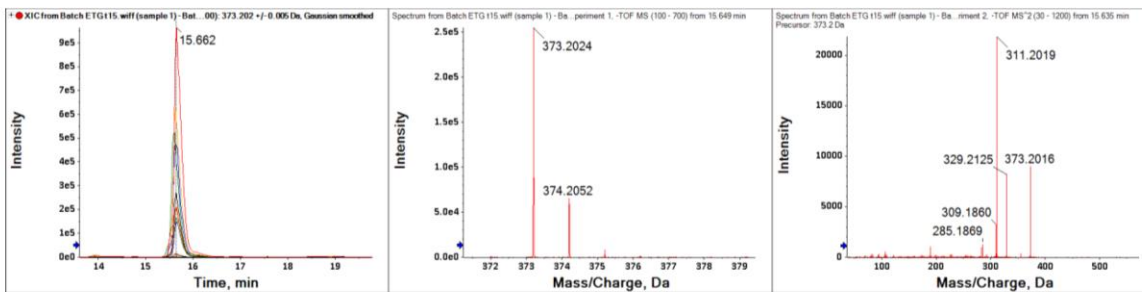
Name: TP374_15.7	Formula: C22H30O5	Precursor: [M-H]⁻	RT Shift: -4.2 min	Identification: -
Proposed structure: no final structure		Comments: MS² showed neutral loss of 44 Da, typically for carboxylic acids (373>329).		
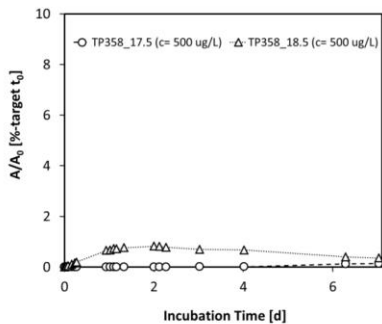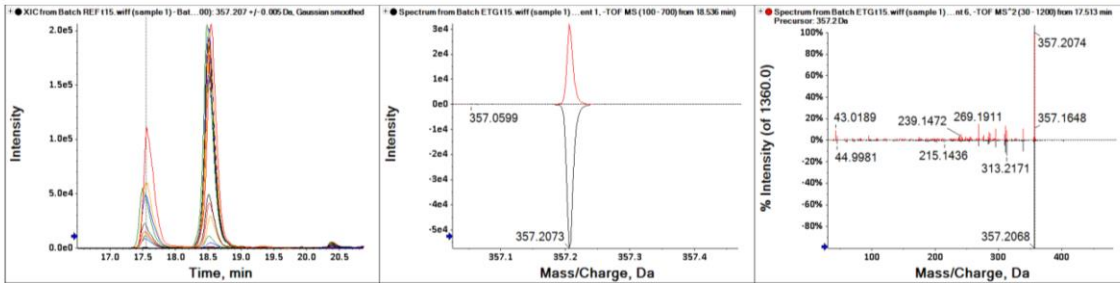
Name: TP358_17.5, TP358_18.5	Formula: C22H30O4	Precursor: [M-H]⁻	RT Shift: -2.4 min, -1.4 min	Identification: -
Proposed structure: no final structure		**Comments:** MS² showed neutral loss of 44 Da, typically for carboxylic acids (357>313).		
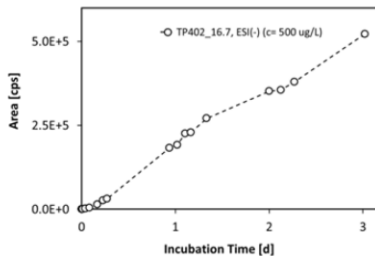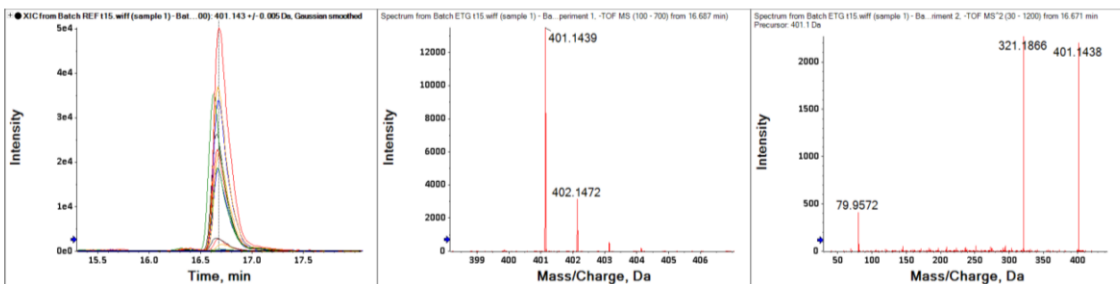
Name: TP402_16.7	Formula: C22H26O5S	Precursor: [M-H]⁻	RT Shift: -3.2 min	Identification: Isotopic pattern, MS fragmentation, RT shift, analogy to other results
Proposed structure:		**Comments:** MS² showed the fragment 79.957 Da, typically for phenolic sulfates.		
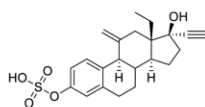
Name:	Formula:	Precursor:	RT Shift: -, -2.6	Identification:
Norethisterone acetate, Norethisterone (TP298_18.3)	C22H28O3, C20H26O2	[M+H]⁺	min	Reference standard

Structure:	Comments: Fast hydrolysis of the 17β-ester to norethisterone.

Name:	Formula:	Precursor:	RT Shift: -2.6	Identification:
Norethisterone (TP298_18.3)	C20H26O2	[M+H]⁺	min	Reference standard

| Structure:	Comments: Fast hydrolysis of the 17β-ester to norethisterone.

Name: TP298_19.2	Formula: C20H26O2	Precursor: [M+H]⁺	RT Shift: -1.7 min	Identification: Isotopic pattern, MS fragmentation, RT shift, analogy to other results
Proposed structure: 		Comments: Comparison with reference standard of 4,5(α)-dihydro norethisterone revealed strong similarities. Formation below 5%.		
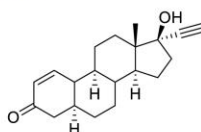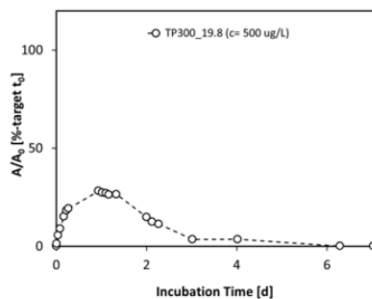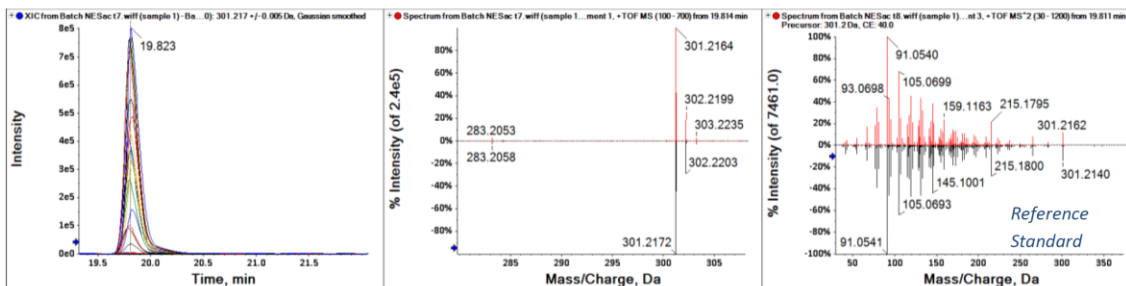
Name: TP300_19.8	Formula: C20H28O2	Precursor: [M+H]⁺	RT Shift: -1.1 min	Identification: Reference standard
Structure: 		Comments: Major TP and main human metabolite of norethisterone.		
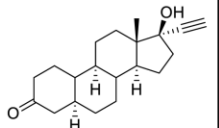
Name:	Formula:	Precursor:	RT Shift: -2.4	Identification:
TP296_18.5 (17α-ethinyl estradiol, EE2)	C20H24O2	[M+H]⁺	min	Reference standard

Structure:	Comments: Comparison of the areas in ESI(+) indicate formation below 1%, while quantitative comparison revealed the formation of 7% EE2. Ionization efficacy of estrogens in ESI(+) is significant lower than for progestogens.

Name:	Formula:	Precursor:	RT Shift: -3.4	Identification:
TP296_17.5	C20H24O2	[M+H]⁺	min	Isotopic pattern, MS fragmentation, RT shift, analogy to other results, comparison to norethisterone

Proposed structure:	Comments: Formation below 1%.

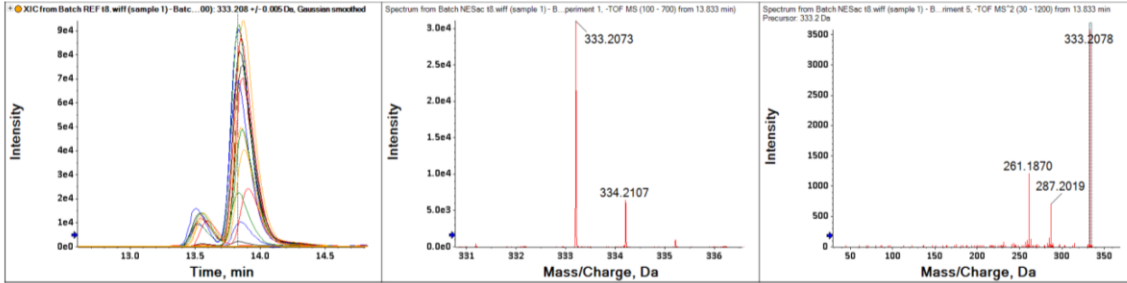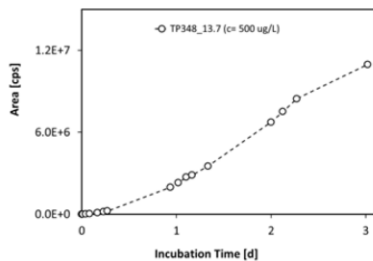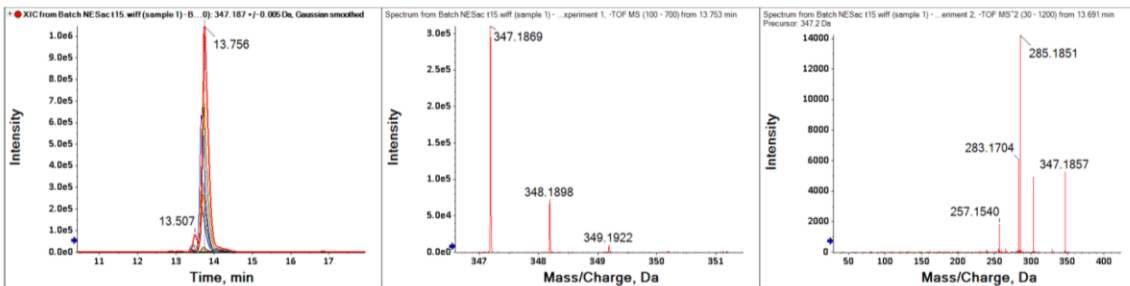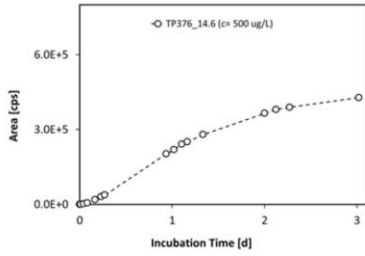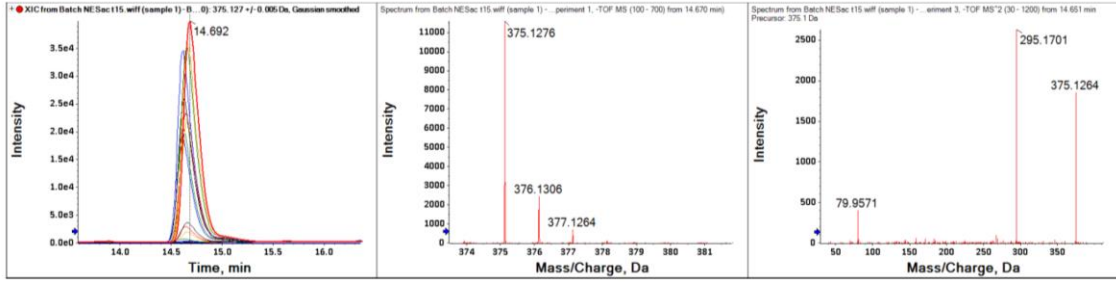| Name:	Formula:	Precursor:	RT Shift: -2.9	Identification:
TP316_18.0	C20H28O3	[M+H]⁺	min	Isotopic pattern, MS fragmentation, RT shift, analogy to other results, comparison to TP300_19.8
Proposed structure:			**Comments:** Most likely hydroxylated at C4 or C2 (α-hydroxy ketone), due to the low RT shift. MS²-fragmentation showed siniliarities to TP300_19.8. Other structure is possible. Formation below 5%.		
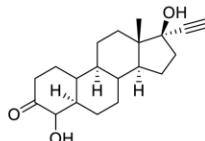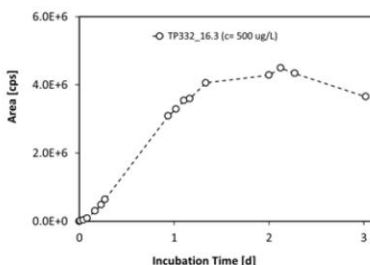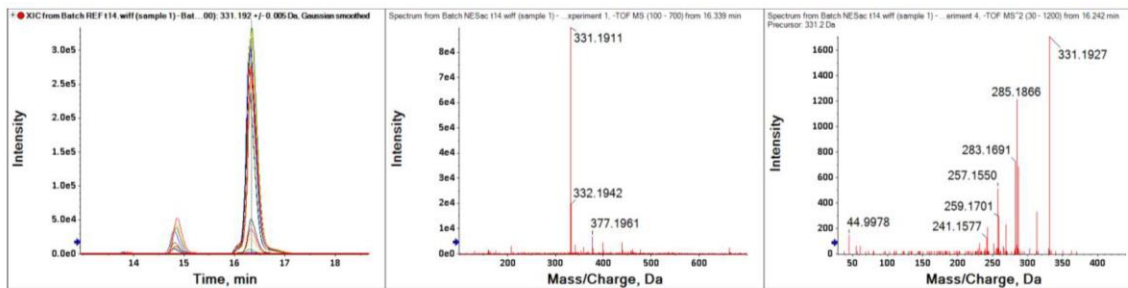
Name: TP332_16.3	Formula: C20H28O4	Precursor: [M-H]⁻	RT Shift: -4.6 min	Identification: -
Structure: no final structure		**Comments:**		
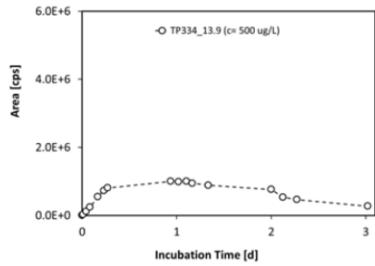
Name: TP334_13.9	**Formula:** C20H28O4	**Precursor:** [M-H]⁻	**RT Shift:** -7.0 min	**Identification:** -
Structure: no final structure		**Comments:** -		

Name: TP348_13.7	**Formula:** C20H28O5	**Precursor:** [M-H]⁻	**RT Shift:** -7.2 min	**Identification:** -
Structure: no final structure		**Comments:** MS² showed neutral loss of 44 Da, typically for carboxylic acids (347>303).		

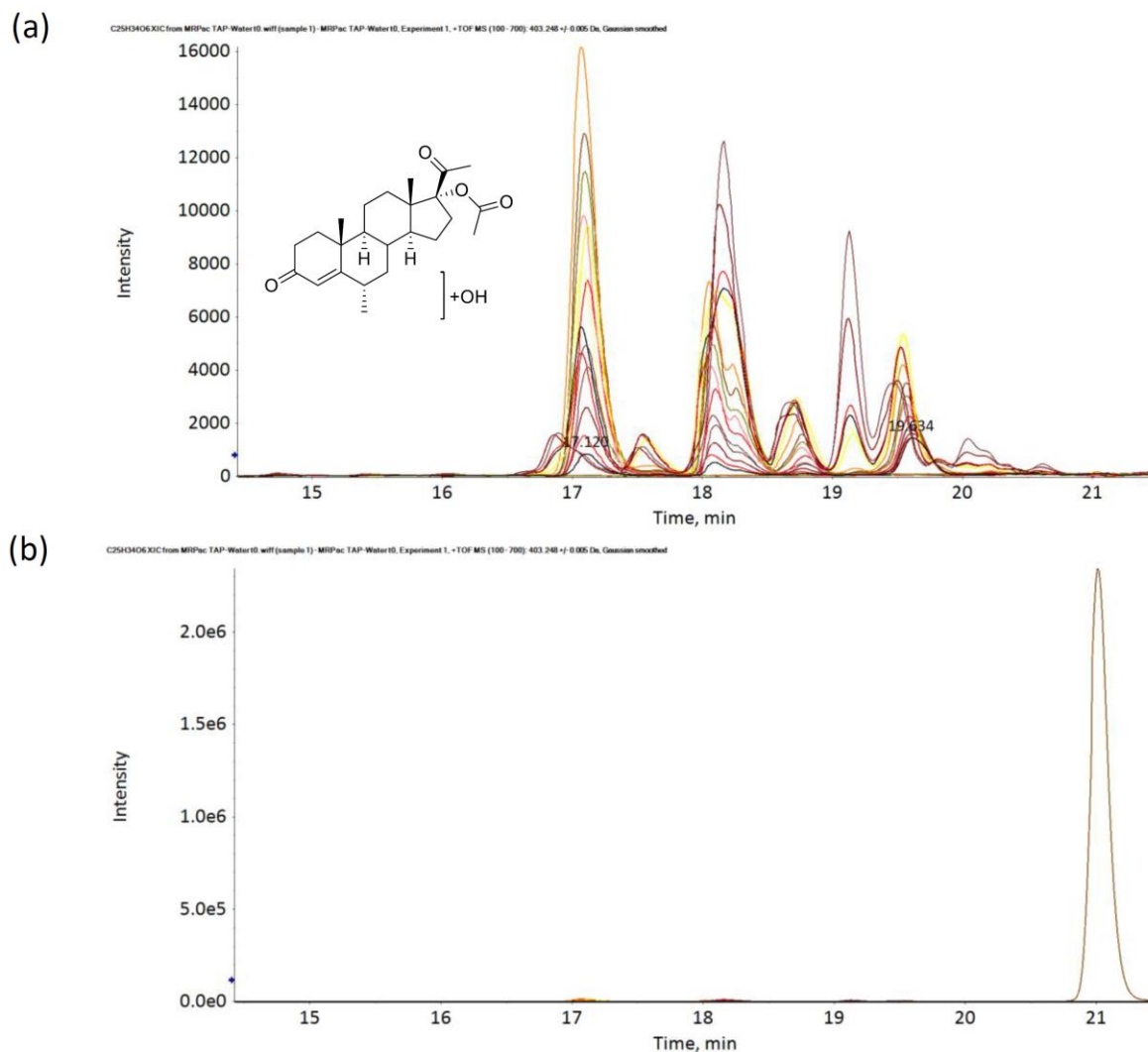| Name: TP376_14.6	Formula: C20H24O5S	Precursor: [M-H]⁻	RT Shift: -7.2 min	Identification: -
Proposed structure:		Comments: Phenolic sulfate conjugate (375.1264 > 79.9571). Relative formation quantity for TPs in ESI(-) could not been estimated.		

Additional Figures

Figure C.3. XICs of the mono hydrogenated TPs (403.248±0.005 Da) of medroxyprogesterone acetate in detail (a) and in comparison to the initial intensity of medroxyprogesterone acetate (b). Similar behavior was found for other 17α-hydroxyprogesterone derivatives.
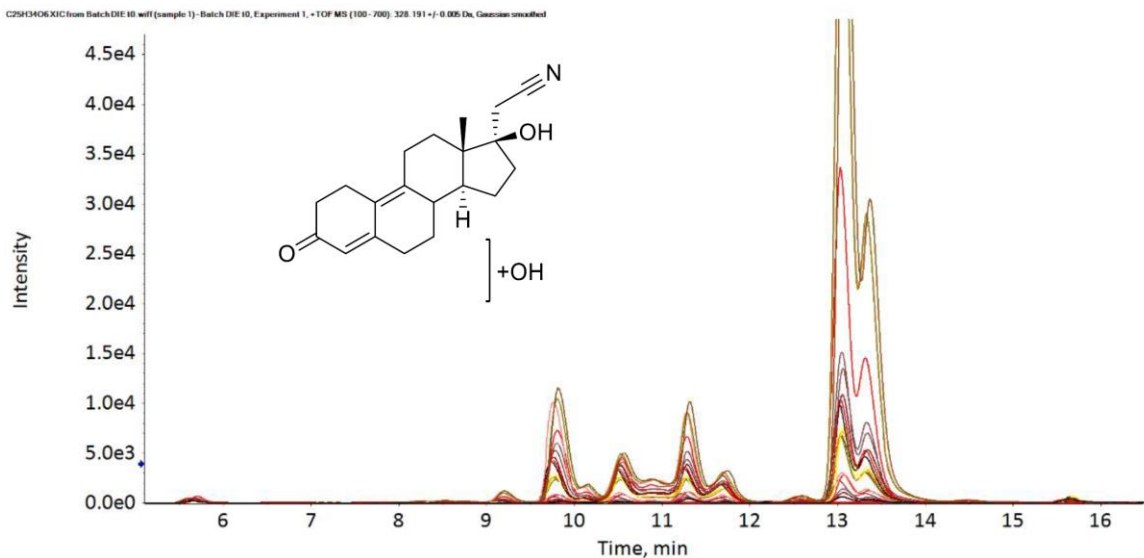
Figure C.4. XICs of the mono hydroxylated TPs (328.191 ± 0.005 Da) of dienogest. Similar behavior was found for other 19-nortestosterone derivatives.
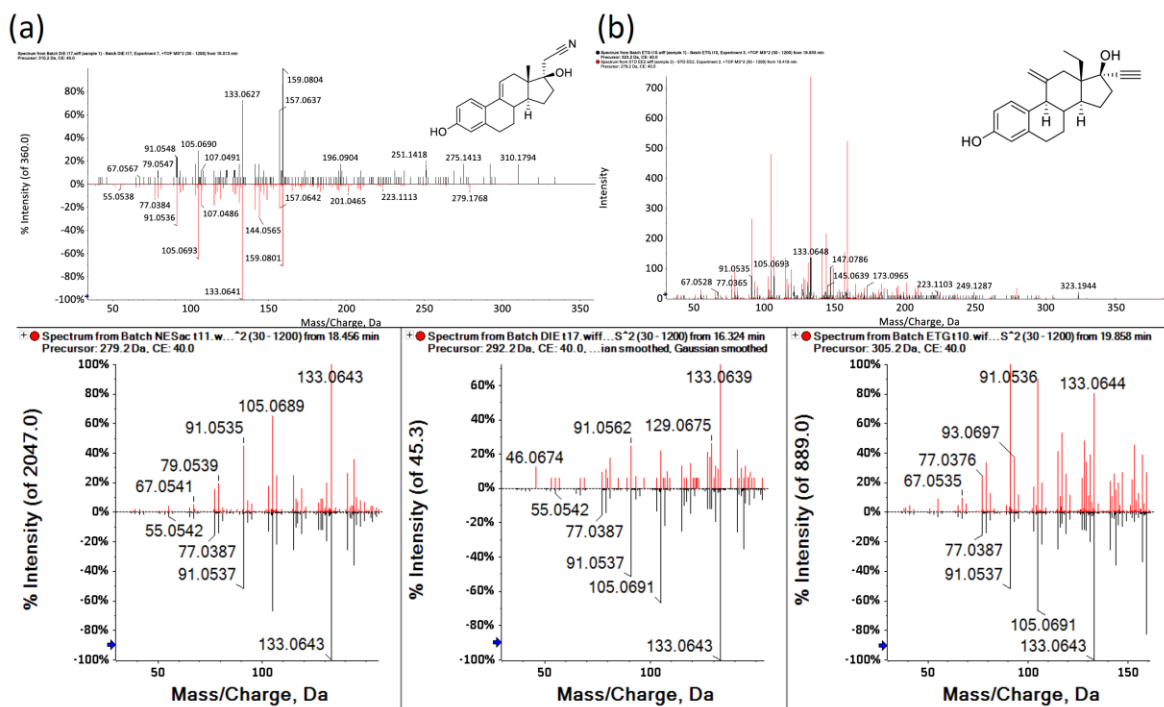

Figure C.5. Identification of the phenolic TPs of 19-nortestosterone derivatives dienogest, etonogestrel and norethisterone. (a) Comparison of MS² spectra of TP309_16.4 (dienogest TP) and 17α-ethinylestradiol. (b) Comparison of MS² spectra of TP322_19.9 (etonogestrel TP) and 17α-ethinylestradiol. (c) The characteristic in-source fragmentation and the MS² spectra of the in-source fragments [M-H2O+H]+ of the phenolic TPs and of 17α-ethinylestradiol show strong similarity.
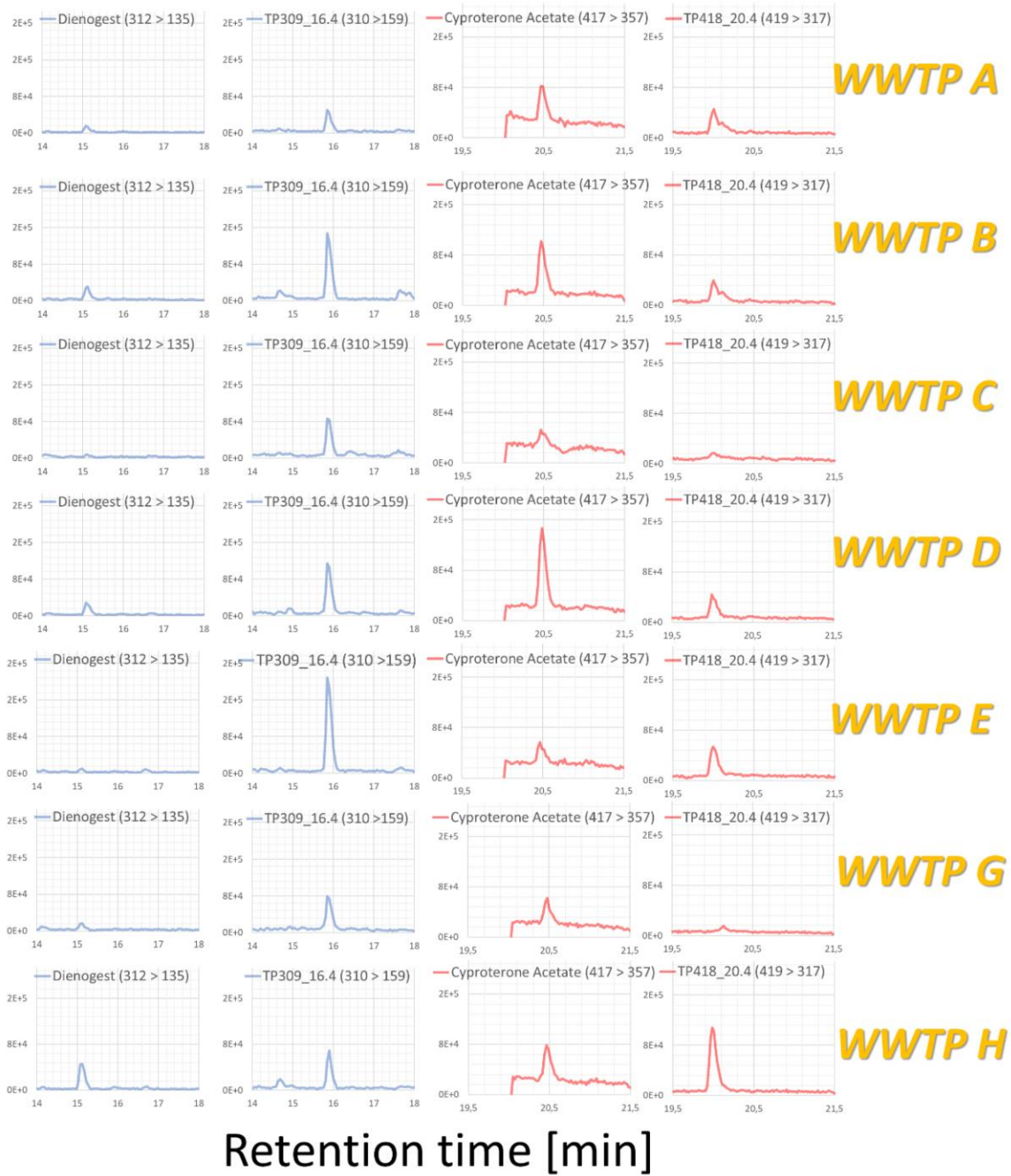
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Figure C.6. LC-MS/MS chromatograms of dienogest, TP309_16.4, cyproterone acetate and TP418_20.4 from the analyzed WWTP effluents.