Investigation of agonistic and antagonistic endocrine activity during full-scale ozonation of waste water

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Fabian Itzel

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Gutachter:	Prof. Dr. Torsten C. Schmidt
	Prof. Dr. Elke Dopp
Vorsitzender:	Prof. Dr. Bettina Siebers

Summary

The use of a wide variety of chemicals in our society, such as industrial chemicals, pharmaceuticals, personal care products, etc., leads to pollution of surface waters. Especially in densely populated urban areas such as the Ruhr catchment, sustainable water management poses a major challenge. Despite intensive use through various types of discharges (effluents of direct dischargers, municipal waste water treatment plants, industry, etc.), good water quality has always to be guaranteed in accordance to the European Water Framewor Directive. Endocrine disrupting chemicals can have an effect on aquatic organisms even at very low concentrations (pg/L range). In order to reduce the emission, ozonation was investigated as advanced waste water treatment for the elimination of organic trace compounds. An elimination performance of ≥ 80% for selected substances at specific ozone doses in the range of $z_{spec.} = 0.3 - 0.7\ mg_{O3}/mg_{DOC}$ was achieved. Since 2015, estrogens are listed on the watch-list of the European Water Framework Directive with required detection limits in the pg/L range. These detection limits cannot be achieved with current instrumental methods. Therefore, sensitive effect based methods based on modified yeast cells (*Arxula adeninivorans*), which carry the human estrogen or androgen receptor, were used as a screening tool for corresponding activities. The results showed high elimination rates of the effects already at low ozone doses ($z_{spec.} = 0.3\ mg_{O3}/mg_{DOC}$). Inhibitory effects (antagonists) were investigated in parallel. No significant reduction in antagonistic effects was observed. A comprehensive analysis method based on effect-based analysis and a non-target approach using high-resolution mass spectrometry was developed to identify unknown active compounds. Due to concerns about possible toxicity of the transformation products formed during the ozonation, biological post-treatments are recommended for further treatment. To characterize the efficiency of post-treatment, the assimilable organic carbon together with a trend analysis done by a non-target approach of unknown features formed during ozonation was established and demonstrated to be a useful tool.

Zusammenfassung

Der Einsatz verschiedenster Chemikalien in unserer Gesellschaft wie z. B. Arzneimittelwirkstoffen, Industriechemikalien, Körperpflegeprodukte, etc. führen zu einer Belastung der Oberflächengewässer. Besonders in dicht besiedelten Metropolen wie dem Ruhrgebiet stellt eine nachhaltige Bewirtschaftung der Gewässer eine große Herausforderung dar. Denn hierbei muss trotz intensiver Nutzung durch Einleitungen verschiedenster Art (Direkteinleiter, kommunale Kläranlagen, Industrie, etc.) eine stetig gute Wasserqualität nach der EU Wasserrahmenrichtline gewährleistet werden. Bei endokrin wirkenden Chemikalien wird bei bereits sehr niedrigen Konzentrationen (pg/L-Bereich) ein Effekt auf aquatische Organismen beobachtet. Um die Einträge zu verringern, wurde im Rahmen dieser Arbeit die Ozonung als erweiterte Abwasserbehandlung zur Elimination organischer Spurenstoffe untersucht. Es konnte eine Eliminationsleistung von ≥ 80% für ausgewählte Substanzen bei spezifischen Ozondosen im Bereich von $z_{spec.}$ = 0,3 - 0,7 mg_{O3}/mg_{DOC} erreicht werden. Seit 2015 sind Östrogene auf der Beobachtungsliste der EU Wasserrahmenrichtlinie mit erforderlichen Nachweisgrenzen im pg/L-Bereich aufgeführt. Diese Nachweisgrenzen sind mit derzeitigen instrumentellen Methoden nicht erreichbar. Daher wurden sensitive effektbasierte Methoden auf Basis modifizierter Hefezelle (*Arxula adeninivorans*), welche den humanen Östrogen- bzw. Androgenrezeptor enthalten, als Screening-Tool für die Messung entsprechender Aktivitäten eingesetzt. Die Ergebnisse zeigten bereits bei niedrigen Ozondosen ($z_{spec.}$ = 0,3 mg_{O3}/mg_{DOC}) hohe Eliminationsraten der hormonellen Aktivitäten. Parallel wurden inhibierende Effekte (Antagonisten) untersucht. Es konnte keine signifikante Reduktion antagonistischer Effekte beobachtet werden. Um entsprechend unbekannte Wirkstoffe zu identifizieren, wurde eine umfassende Analysemethode entwickelt, die auf der effektbasierten Analytik sowie einem non-target Ansatz mittels hochauflösender Massenspektrometrie basiert. Aufgrund von Bedenken zur möglichen Toxizität der bei der Ozonung gebildeten Transformationsprodukte, werden biologische Nachbehandlungen zur weiteren Behandlung empfohlen. Zur Charakterisierung der Effizienz einer Nachbehandlung konnte der assimilierbare organische Kohlenstoff zusammen mit einer Trendanalyse der gebildeten Features während der Ozonung als nützliches Tool etabliert werden.

Table of Content

List of abbreviations

4OH-TAM	4-Hydroxy-Tamoxifene
AA-EQS	Annual average environmental quality standard
AEF	Androgen equivalent factor
anti-A-YAS	Antagonistic *Arxula Adeninivorans* yeast androgen screen
anti-A-YES	Antagonistic *Arxula Adeninivorans* yeast estrogen screen
anti-YAS	Antagonistic yeast androgen screen
anti-YES	Antagonistic yeast estrogen screen
AOC	Assimilable organic carbon
AOPs	advanced oxidation processes
APCI	atmospheric-pressure chemical ionization
AQS	Analytical quality assurance
AR-CALUX	Androgen receptor - Chemical Activated LUciferase gene eXpression assay
Arg	Arginine
A-YAS	*Arxula Adeninivorans* yeast androgen screen
A-YES	*Arxula Adeninivorans* yeast estrogen screen
BAC	Biological activated carbon
BEQ	Biological equivalent concentration
CBMN	Cytokinesis-block micronucleus assay
CEC	chemicals of emerging concern
CHO-9	Chinese Hamster Ovary cells
DAPI	4′,6-diamidine-2′-phenylindole dihydrochloride
DBD	DNA-binding domain
DBP	Desinfection byproduct
DDT	Dichlorodiphenyltrichloroethane
DES	Diethylstilbestrol
DHT	Dihydrotestosterone
DHTEQ	Dihydrotestosterone equivalent quotient
DIN	German Institute for standardization
DMSO	Dimethylsulfoxide
DOC	Dissoved organic carbon
DOM	Dissolved organic matter
E1	Estrone
E2	17β-Estradiol
E3	Estriole
EBM	Effekt based methods
EBT	Effect based trigger value
EC_{50}	Effect concentration at 50% of the maximum effect
EDA	Effect directed analysis
EDC	Endocrine disrupting chemical
EE2	17α-ethinylestradiol
EEQ	17β-estradiol equivalent quotient
EF	Enrichment factor
EN	European Standard
EPA	Environmental Protection Aagency

EQS	environmental quality standards
ER-CALUX	Estrogen receptor - Chemical Activated LUciferase gene eXpression assay
ERE	estrogen response element
ESI	Electrospray ionisation
EU	European Union
FA	Formic acid
FCS	Fetal calf serum
FLD	fluorescence detector
FMPTS	2-fluoro-1-methyl-pyridinium p-toluenesulfonate
FOEN	Swiss Federal Office for the Environment
FPSE	fabric phase sorptive extraction
FWHM	Full width at halt maximum
GAC	Granualr activated carbon
GC	gas chromatography
GFP	Green fluorescent protein
Glu	Glutamine
GschV	Water Protection Ordinance
hAR	Human androgen receptor
HCl	Hydrochloric acid
Hep-G2	Hepatocellular carcinoma cells
hER	Human estrogen receptor
HLB	Hydrophilic/lipophilic balanced
HPLC	High performace liquid chromatography
HRMS	High-resolution mass spectrometry
HSP90	heat-shock protein 90 complex
IC_{20}	Inhibtion concentration at 20% signal reduction
IC_{50}	Inhibtion concentration at 50% signal reduction
ISO	International Organization of Standardization
JRC	Joint Research Council
LBD	ligand-binding domain
LC	liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
m/z	Mass to charge ratio
MBR	Membrane bioreactor
Min	Minutes
mJ	Millijoule
MN	Micronucleus
MS	Mass spectroscopy
MTBE	Methyl-*tert*-butylether
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOH	Sodium hydroxide
NOEL	No observed effect concentration
NOM	Natural organic matter
NRW	North Rhine Westphalia
NTD	N-terminal domain

O_2	Oxygen
O_3	Ozone
OTM	Olive Tail Moments
PAC	Powdered activated carbon
PBS	Phosphate-buffered saline
PE	Population equivalents
PFBBr	Pentafluorobenzyl bromide
PNEC	Predicted no effect concentration
PPCP	Pharmaceuticals and personal care products
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
REF	Resulting enrichment factor
RT	Retention time
SEC	Size exclusion chromatography
SEM	Standard error of mean
SERM	Selective estrogen receptor modulator
SI	Supplementary information
SIMONI	Smart Integrated Monitoring
SPE	Solid phase extraction
TAM	Tamoxifene
TCEP	tris(2-chloroethyl)phosphate
TIC	Total ion chromatogram
TOC	Total organic carbon
ToF	Time of flight
TP	Transformation product
USA	United States of America
UV	Ultraviolet light
UVEK	Swiss legal requirement for evaluation
VSA	Association of Swiss sewage and water protection specialists
EU WFD	European Water Framework Directive
WHO	World health organization
WWTP	Waste water treatment plant
YAS	Yeast androgen screen
YES	Yeast estrogen screen
YMM	Yeast minimal maltose medium
$z_{spec.}$	Quotient of mg Ozone / mg DOC

List of units and symbols

α	Alpha
β	Beta
©	Copyright
Da	Dalton
°C	Degree Celsius
∆	Delta
eV	Electronvolt
g	Gram
h	Hour
kg	Kilogram
L	Liter
log	Logrithm
µL	Microliter
mL	Milliliter
mg	Milligram
mJ	Millijoule
min	Minutes
nm	Nanometer
ng	Nanogram
ppm	Parts per million
pg	Picogram
rpm	Rounds per minute
™	Trademark
V	Volt

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1. Introduction

1.1 Water quality and waste water management

In 2017 the World Health Organization (WHO) estimated that more than 800.000 people died by diarrhoea and about 240 million suffered from schistosomiasis due to contaminated drinking water worldwide showing the need of sustainable water management. To realise the water needs in every country the UNESCO highly recommends to change the awareness of waste water and therewith the ability to re-use waste water as drinking water in regions in the world with water shortage (WWAP 2017). Due to that reasons it becomes obvious, that a sustainable use of fresh water is mandatory not only for regions with extreme water scarcity but also for water rich countries.

Also, in water rich industrialized western countries, the sustainable use of water is important to prevent local as well as global contaminations leading to ecological damages. Regions with high population densities like the “Ruhr area” in Germany, located in the federal state of North Rhine Westphalia (NRW) have an intense use of surface waters as drinking water resource. About 60% of the drinking water in NRW is produced from surface waters and at the same time 627 municipal waste water treatment plants are discharging their effluent into the same water bodies leading to a high use and stress of the water bodies (Figure 1-1).

Figure 1-1: Distribution of municipal waste water treatment plants in NRW. Green dots = District Council; White dots = District Cities. Source: ELWAS-WEB NVN, © Geobasis NRW 2013.

A further stress factor is the climate change which will affect the water quality and therewith the water management especially in highly populated regions. One example are longer periods of droughts and extreme heat events, which will lead to low tide in rivers and higher water temperatures (Gierk et al. 2010). In such a case the O_2-concentration in the river would

drop due to higher dwell time of biological degradable compounds and even further by the discharge of treated waste water. This example clearly demonstrates the need of a sustainable water management not only in regions in world with water scarcity but also for highly populated and industrialized water rich regions.

1.2 Micropollutants and effects

Due to today's social lifestyle and the associated increasing use of chemical products in western countries, chemicals for a wide range of applications like pharmaceuticals, industrial chemicals and personal care products, also called chemicals of emerging concern (CEC), are consequently reaching the environment.

Erickson (2017) published a study in which the steadily increasing amount of chemicals is illustrated, showing that even the Environmental Protection Agency (EPA) in the United States of America (USA) does not have an exact number of how many compounds are in use today. In the European Uunion the European chemicals agency (ECHA) was established to register chemicals within a new EU-wide act (EG 1907/2006) for Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH). Within REACH about 17400 compounds are listed until now (ECHA 2018). As already stated, all these known but also unknown compounds are potentially released into the environment and thereby could also reaching the surface waters to a certain amount (Salimi et al. 2017).

In terms of pharmaceuticals, CECs and other chemicals, it is known that municipal WWTPs are not specifically designed for a reduction of those compounds, leading to discharges in low (µg/L-ng/L range) concentrations (Anumol et al. 2016; Salimi et al. 2017; Gabet-Giraud et al. 2010; Morasch et al. 2010; Rechenberg 2015; Richardson and Kimura 2016; Valitalo et al. 2016). Due to the low concentration these chemical compounds are called micropollutants.

One group of micropollutants not sufficiently removed by WWTPs are endocrine disrupting compounds (EDC). Especially EDCs can lead to several effects in aquatic organisms already at very low (ng/L range) concentrations (Plahuta et al. 2017; NealeAltenburger et al. 2017; Aris et al. 2014; Caldwell et al. 2012; Kidd et al. 2007). Several studies in the last decade showed effects caused by EDCs, like the feminization of male fish and the shift in population size towards female organisms (Kidd et al. 2007; Peschke et al. 2014).

EDCs can either be of natural origin like steroid hormones reaching the WWTP by human excretion such as 17β-estradiol (E2) and testosterone as typical estrogene and androgene active compounds or they can be of synthetic origin like the estrogene active contraceptive 17α-ethinylestradiol (EE2).

Besides target synthetic EDCs designed for a desired effect, there are many other non-targeted chemicals which are also able to interact with the endocrine system.

One of the first reported endocrine effects to humans was caused by Dichlorodiphenyltrichloroethane (DDT). In the 1940's DDT was used as pesticide in agriculture and farmers had a significant loss in sperm counts as well as acute health problems due its endocrine disrupting effect (Singer 1949). Some years later, a study by Guzelian (1982) reported similar health problems of employees working in a company prodcucding the insecticide "Kepone". In 1991 the additive in plastics and antioxidant nonylphenol was found by chance during laboratory tests, to have estrogenic effects (Soto et al. 1991). A further study by Krishnan et al. (1993) showed bisphenol A, a commonly used additive in plastics as well, to be estrogenic active. First environmental investigations later showed signs of feminization of male fish close to WWTP effluents in rivers in UK which was not able to ascribe by a single substance. Thereby chemical analysis of fish showed the presence of several compounds (Colborn et al. 1993).

Today, many different classes of compounds are known to trigger an endocrine activity. One recent compound is the non-steroidal analgetic agent diclofenac which is used in high amounts worldwide and showed estrogenic effects in experiments with frogs of the species Xenopus laevis (Sedlak and Pinkston 2011; Efosa et al. 2017).

The risk for human health for example by eating contaminated fish or getting in contact by different ways with micropollutants is not known so far. In Germany the guiding idea in the environmental sector is the so called "precautionary principle" to prevent a discharged in general if possible and especially if the risk is not known.

Therefore, the EU implemented the water framework directive (WFD) to achieve and keep a good chemical and ecological status of surface waters until 2027 (EU 2000/60; EU 2008/105). To measure the goals and be able to classify water bodies with regard to their chemical status, a list of priority substances (ANNEX X) with environmental quality standards was developed (EU COM(2011)876). In 2013 a further list was added, to monitor compounds which are not listed but maybe added to the priority list in annex X of the WFD (EU 2013/39). In this monitoring list (also known as "watch-list") endocrine active compounds are listed the first time showing the raising awareness of endocrine activity in surface waters. Such compounds are the natural estrogen 17ß-estradiol as well as the synthetic analoga 17a-ethinylestradiol and the derivate estrone which was added to the watch-list in 2015 (EU 2015/495).

1.3 Advanced waste water treatment

Major point sources are municipal waste water treatment plants (WWTPs). Today's classical (WWTPs) consist of a mechanical treatment to filter raw material in a first step. As a second step a biological treatment to eliminate organic carbon and nitrogen is installed. In a last step inorganic salts like phosphorous can be removed by precipitation. Residual solid matter is settled and thereby separated from the water phase by the final clarifier and afterwards the effluent is discharged into surface waters. All these steps have the aim to remove organics as well as micro and macro nutrients to prevent the receiving water body from a eutrophication.

To reach achieve the goals of the water framework directive WFD and to ensure a sustainable water management in future especially for highly populated areas and areas in which the fraction of the waste water into the surface water is larger than the natural water, new advanced treatment technologies should to be implemented to be able to reduce the discharge of such micropollutants and potential ecological effects.

Potential new technologies to further remove micropollutants are for example reverse osmosis or the use of activated carbon which is also applied in drinking water production. Beside filtration and sorption processes, oxidative processes were further investigated in the last decades for waste water treatment. Such oxidative processes are for example UV-irradiation which can either react by direct photolysis or selective bond cleavage or the addition of ozone which reacts selective with electron rich moieties or nonspecific by forming radicals in presencet of organic matter (Gligorovski et al. 2015; Lee and von Gunten 2010). In some pilot scale studies, the combination of different reagents to enhance radical formation was investigated like UV/H_2O_2, H_2O_2/O_3 or even UV/H_2O_2/O_3 resulting in so called advanced oxidation processes (AOPs) (De la Cruz et al. 2013; Salimi et al. 2017). As such combinations are difficult to upscale especially for UV due to high particle load and therewith inefficient reaction rate with the target micropollutant as well as cost aspects, the application of ozone or activated carbon were are the technologies tested in most of the full-scale investigations today (Audenaert et al. 2014; Rechenberg 2015; Sperlich and Gnirß 2016; Mahamuni and Adewuyi 2010; Margot et al. 2013). The decision to implement ozone or activated carbon is often due to technical reasons by limitation of space to build additional facilities.

In oxidative processes such as ozone, however, there is no complete mineralization of micropollutants. Instead micropollutants get transformed into new structures which might even enhance their toxic effects (Schlüter-Vorberg et al. 2015; Knopp et al. 2016). However, there are still different studies showing also no increase in toxicity or endocrine activity by formed transformation products (Richard et al. 2014).

Due to uncertainties of toxicity by formed transformation products (TPs) formed during ozonatione, a further biological treatment or sorption by activated carbon is recommended to subsequently remove TPs (Hollender et al. 2009; DWA 2014).

1.4 Analytical tools for evaluation of treatment efficiancy

1.4.1 Chemical analysis

To investigate new advanced treatment technologies and to evaluate their removal efficiency and potential effects to organisms, highly sensitive analytical methods are needed to identify and quantify micropollutants. Chemical target analysis by liquid chromatography or gas chromatography coupled with mass spectrometry is a widely used and common tool to detect target compounds. As the detection of all produced and known compounds, which are potentially reaching the sewage system and finally WWTPs would be enormously time consuming and consequently produce high analytical costs, indicator parameters are selected like the listed compounds of the water framework directive. Typical measured indicator compounds are 1*H*-benzotriazole, carbamazepine, clarithromycin, diclofenac, sulfamethoxazole and metoprolol which are detected in the µg/L range and removed by ozone to more than 80% which is also the requirement in Switzerland for micropollutant reduction (Itzel et al. 2017; EU 2013/39; EU 2015/495; Margot et al. 2013; GschV 814.201 2016; VSA 2016; Jekel et al. 2015). Beside such widely detected and analysed compounds endocrine active compounds especially the most potent E1, E2 and EE2 which should be monitored also by the watch-list are challenging todays analytical methods to quantify or even to detected in the required low ng/L range. To reach the required limits of quantification (LOQ) of 0.035 ng/L/l for EE2 using modern chemical analysis tools is not possible till now. In a study of the joint research center (JRC) of the European commission to review current analytical methods it was stated that “to reach LOQs in the low pg/L concentration range is extremely difficult, if not impossible with current analytical methods.” (Loos 2015).

1.4.2 Effect based methods

For this reason, new methods are needed to be able to evaluate the endocrine activity during an advanced treatment process and to get a status information of the receiving water bodies. One option to overcome those limitations are the more sensitive biological effect based analysis tools. Such test systems are highly sensitive in the required range of low ng/L concentrations and are available for several endpoints like cytotoxicity, genotoxicity as well as endocrine activity (NealeMunz et al. 2017; Gehrmann et al. 2018). All these biological effect based methods have in common to express the detected effect in relation to a certain

standard compound resulting in biological equivalent concentration (BEQ). In case of endocrine effects the human estrogen- or androgen receptor (hER/hAR) is clonated into the test organism and highly potent compounds like E2 or dihydrotestosterone are used for calibration respectively. Using effect based methods for estrogenic or androgenic effects will thereby result in either ng E2 equivalent concentrations per liter (ng EEQ/L) or in ng dihydrotestosterone equivalent concentrations per liter (ng DHTEQ/L). Such generated sum parameters expressed in equivalent concentration are able to give information about the whole sample the resulting effect to aquatic organisms. Compared to classical chemical target analysis the effect based approach does not give information about the single substance relevant for the detected activity but can bridge the gap for effects of non-target and unknown compounds. However, to identify compounds acting as endocrine disruptors chemical analysis is still essential for example to identify persistent and active compounds during a treatment step or to localise a certain source to directly prevent a discharge into the environment. As effect based methods are more sensitive than current chemical methods, approaches to combine both for a prior screening and a chemical analysis for the identification in a second step are discussed at the moment (Locatelli et al. 2016; Altenburger et al. 2015; Brack et al. 2016; Brack et al. 2017; Burgess et al. 2013; Neale et al. 2015).

To correlate detected estrogenic or androgenic compounds with measured effects it is of high importance to take inhibitory antagonistic effects into account as well. As such inhibitory antagonistic effects are always in mixture, when analysising environmental samples, the detected overall effect is always the sum of agonistic and antagonisitc acting compounds. In figure 1-2 the interrelationship of agonists with respective antagonisitst is shown. In terms of the estrogen or androgen receptor, anti-estrogens or anti-androgens are able to interfere and consequently block the receptor for agonisitc estrogens resulting in an inhibition of agonists which leads to a lower equivalent concentration. Beside the signal reduction by antagonisits there are similarities in the resulting biological effects. In organisms it is known, that estrogens and anti-androgen lead to a reduced estrogen level as well as androgens and anti-estrogens lead to a lower androgen level.

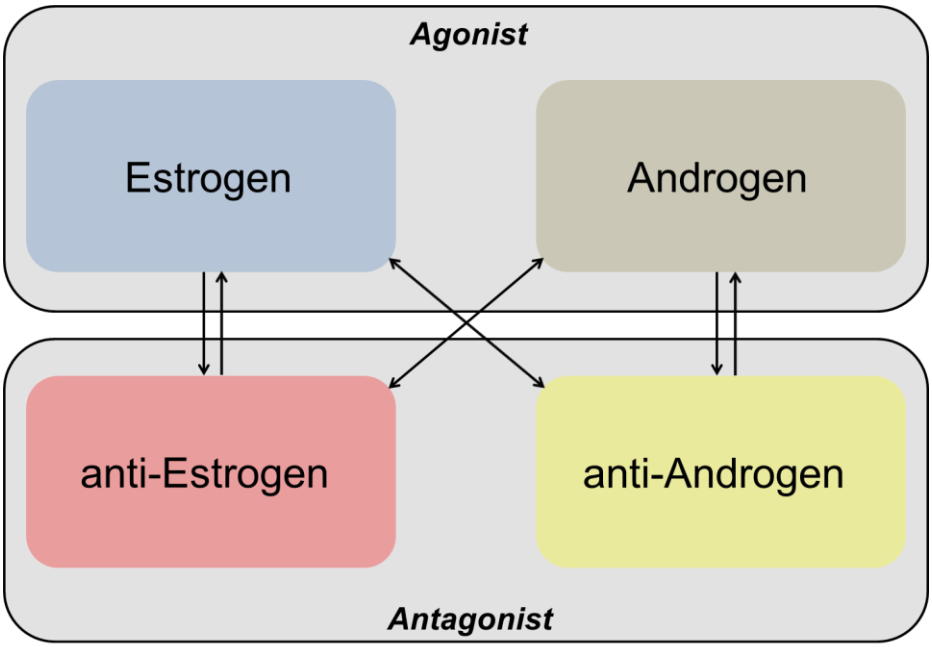

Figure 1-2: Interrelationship of agonistic and antagonistic effects.

As single substanzes have to be analysed as they are regulated a correlation to the overall measured effect is often needed. In a study by Ihara et al. (2014) it could be shown that predictions of the effect by chemical analysis was always lower than the detected effect due to antagonistic compounds. Antagonistic acting compounds can be drugs developed to block the receptor like cancer therapy drugs tamoxifen or flutamide which are acting anti-estrogenic or anti-androgenic respectively (Kiang and Kennedy 1977; Simard et al. 1986). However, there are also compounds acting antagonistic which are not specifically designed for that purpose like flame retardants (Zhang et al. 2014), pesticides (Orton et al. 2012) as well as anti-inflammatory drugs like ibuprofen or diclofenac (Ezechiáš et al. 2016). Beside the reduction of the measured effect, antagonistic acting compounds have an environmental relevance as well. In a A study by Jobling et al. (2009) aquatic organisms showed symptoms such as feminization in presence of anti-androgenic besides already known estrogenic compounds. In the last years even more studies were able to demonstrate the direct effect of antagonistic compounds to aquatic organisms in various ways (Watermann et al. 2016; Rao et al. 2014). To identify compounds relevant for the less studied antagonistic effects new approaches need to be developed. The effect directed analysis (EDA) approach which is a combination of chemical target and non-target analysis with effect based methods is one option to rapidly find relevant compounds. Thereby effect based methods should be used for pre-screening of samples and enable a reduction of relevant features during non-target analysis (Di Paolo et al. 2016; Brack 2003; Brack et al. 2016; Burgess et al. 2013).

1.5 Objectives and scope of the thesis

In this thesis ozonation as advanced waste water treatment technology was investigated with focus on endocrine effects. Therefore, effect based methods for the analysis of estrogenic, androgenic as well as anti-estrogenic and anti-androgenic were applied and combined with chemical target as well as non-target analysis. Therefore, chapter two summarizes analytical possibilities and limitations in terms of endocrine activity using chemical and effect based analysis with focus on antagonistic effects and responsible compounds (Figure 1-3).

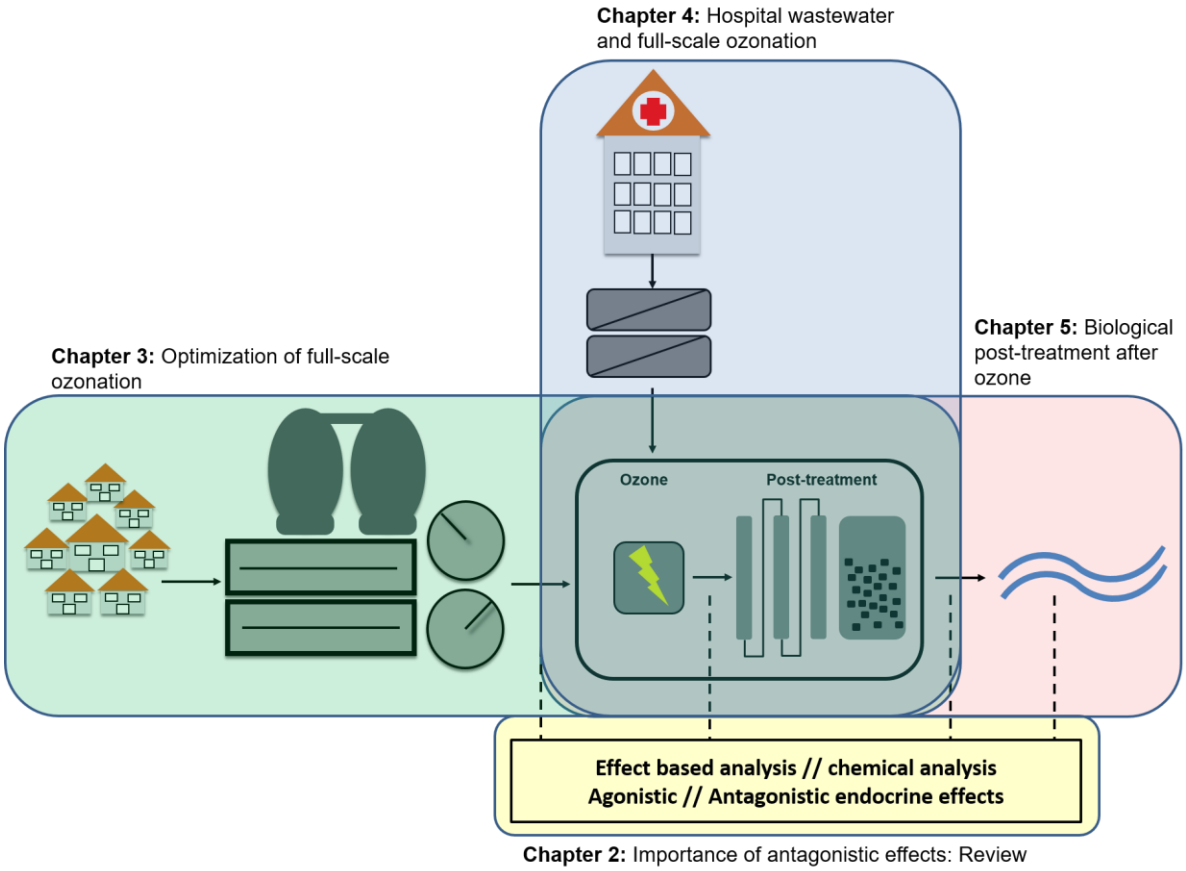

Figure 1-3: Interrelationship of individual chapters.

As ozone is one of the most applied technologies in several pilot plants the thrid chapter investigates ozone as advanced treatment technology during waste water treatment for the reduction of micropollutants with focus on estrogenic and androgenic effects. To further analyse the behaviour of antagonistic effects during advanced waste water treatment one hospital waste water treatment plant with full-scale ozonation and one with ozonation and granular activated carbon were investigated. Therefore, a comprehensive analysis using effect based and chemical non-target analysis combined with a fractionation approach was

applied to identify relevant antagonistic acting compounds in chapter four. In the last chapter five, the biological post-treatment by a moving bed reactor was further investigated with prior applied methods and tried to characterise using the assimilable organic carbon (AOC) as new parameter.

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2. Recent developments and concepts of effect based methods for the detection of endocrine activity and the importance of antagonistic effects

Submitted to: Trends in Analytical Chemistry. Fabian Itzel, Linda Gehrmann, Thorsten Teutenberg, Torsten C. Schmidt, Jochen Tuerk.

2.1 Abstract

The use of many different substances has led to an increased detection of endocrine activity in aquatic systems. Regarding endocrine disrupting chemicals, an impact on aquatic organisms at already very low concentrations (pg/L-ng/L range) is observed. Consequently, there is a need for sensitive analytical methods. Currently, no instrumental analysis methods exist that fulfil this requirement. To overcome those limitations, effect based methods (EBM) are applied for screening and quantification. Determination of agonistic effects caused by estrogen and androgen active compounds are focus of actual research activities due to environmental impacts and regulatory measures. However, also relevant antagonistic effects have to be considered. This review highlights the perspectives and limitations of EBMs as well as the relevance of antagonistic acting compounds. To apply EBMs under the EU water framework directive and therewith to overcome limitations by instrumental analysis, the usage and acceptance of recently introduced effect based trigger values is needed.

2.2 Keywords

Antagonist, Agonistic effects, anti-estrogen, anti-androgen, effect based methods (EBM), instrumental analysis, effect based trigger values (EBT), effect directed analysis (EDA)

2.3 Introduction

Thousands of compounds are used by our society for a variety of applications and are consequently discharged into the aquatic environment. Such compounds, although often present only in trace concentrations, can lead to adverse biological effects in organisms. These effects comprise genotoxicity, cytotoxicity or endocrine disruption (Kidd et al. 2007, Liu et al. 2017, Masteling et al. 2016, Neale, Ait-Aissa, et al. 2015, König et al. 2017, Zhu et al. 2018). Especially estrogenic activity was frequently observed. Indications of feminization

of male fish exposed to effluents from nearby waste water treatment plants (WWTP) were reported (Kidd et al. 2007, Gross-Sorokin et al. 2006). Subsequently, many compounds were found to interact with the estrogen receptor. These include industrial chemicals such as nonylphenol or bisphenol A, pesticides such as DDT, azo dyes used by the textile industry or analgetic agents like diclofenac (Krishnan et al. 1993, Soto et al. 1991, Singer 1949, Ezechiáš et al. 2016, Bazin et al. 2012). Recent studies have shown that only a small proportion of endocrine effects can be attributed to known substances. This fact makes an identification of effect-causing substances, if at all, only possible by very complex non-target analyses (Altenburger et al. 2015, Tousova et al. 2017). In contrast to instrumental analysis with focus on detection of specific substances, effect based methods (EBMs) could be used for integrative effect screening of the whole sample. In case of endocrine activity, different receptors are applied for different classes of hormonal activity. Typical endocrine receptors are glucocorticoid, thyroid, progesterone as well as the human estrogen or androgen receptor (Escher et al. 2014). Detecting endocrine effects using receptor based bioassays, it is important to keep in mind that a competitive binding of compounds to a certain receptor occurs. In contrast to single substance testing, environmental samples always contain a mixtures of compounds. Therefore, endocrine effects can be classified into two types. There are agonistic effects, which are promoting a certain cell function (e.g. the production of an enzyme), and there are antagonistic effects, which are inhibitory effects that block a certain receptor and prevent a certain effect in an organism (Kabir et al. 2015). To quantify these different effects, reference standards are used to derive equivalent concentrations (shown in Figure 2-1). An overview of estrogen and androgen effects as agonists as well as corresponding antagonistic effects is shown in figure 2-1. The resulting effect in an organism exposed to compounds responsible for the effect is illustrated by the different arrows. It is shown that estrogens as well as anti-androgens are leading to a similar effect in organisms. The same applies to androgen and anti-estrogen effects.

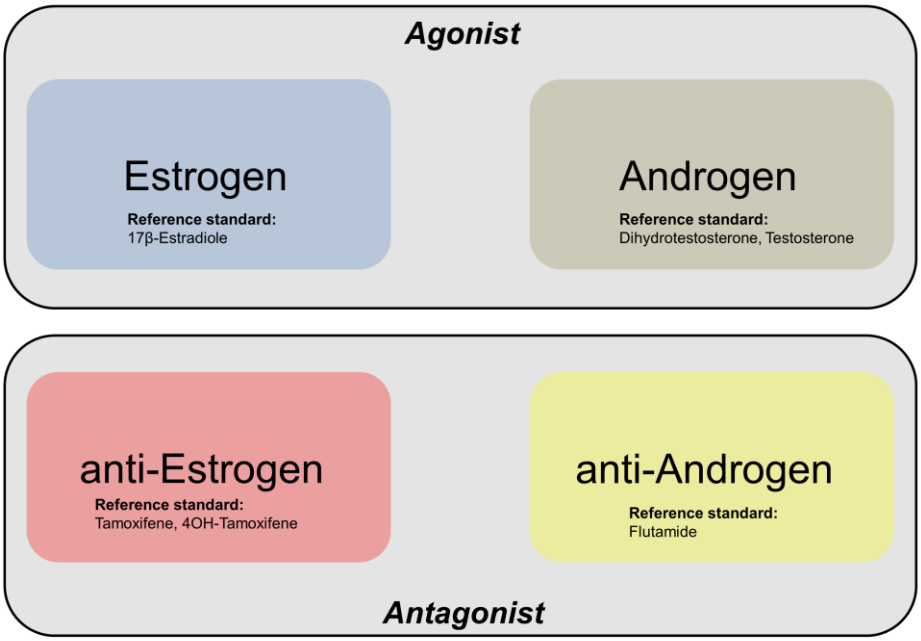

Figure 2-1: Schematic representation of two effect classes measured by in vitro bioassay using the human estrogen/androgen receptor. Commonly used standards for derivation of equivalent concentrations are listed as well.

To detect the endocrine effects, the human estrogen receptor is commonly applied in bioassays. The human estrogen receptor alpha (hERα) consists of a ligand-binding domain (LBD), the N-terminal domain (NTD), the heat-shock protein 90 complex (HSP90) and the DNA-binding domain (DBD) for transcription of the target enzyme similar to the human androgen receptor (Lewis and Jordan 2005). The principle of the receptor is explained on the example of the hERα. First, estrogen active compounds have to bind to the LBD region. In case of the natural estrogen 17β-estradiol (E2), this is done by forming hydrogen bonds to the amino acids glutamine (Glu) 353 and arginine (Arg) 394 (Figure 2). The LBD consists of 12 α-Helix structures which form a hydrophobic pocket sealing the ligand. The hydrogen bonding inside this complex to Glu353 and Arg394 is formed by the phenolic hydroxyl group of E2 leading to the conformational change of the 12 α-Helix “sealing” the whole E2-receptor complex (Brzozowski et al. 1997). Consequently, the HSP90 complex detaches the receptor complex leading to a dimerization of two bound receptors by the so called “hinge” region (Lewis and Jordan 2005). This region (D region) connects the LBD and the DBD in a flexible way to enable the dimerization of two receptor complexes (Schwabe et al. 1993). At the same time a transcription complex is formed together with different cofactors. This enables the “docking” to the estrogen response element (ERE) encoding the target enzyme. In the last step, the target enzyme is measured giving information about the concentration of

compounds bound to the receptor. The result is reported as equivalent concentration to a corresponding standard.

Similar to agonistic estrogen active compounds, antagonistically active compounds bind to the LBD region via Glu353 and Arg394. However, instead of a structural change of the 12α-Helix leading to a sealing of the receptor, antagonistically active compounds lead to a steric hindrance by the longer side chains, subsequently preventing a sealing (Brzozowski et al. 1997). Figure 2 shows the binding to the two amino acids Glu353 and Arg394 via hydrogen bonds by estradiol as well as the antagonist raloxifene. The structural differences of the LBD due to the side chain of raloxifene and the bonding to the amino acid Asp351 by the amino group leads to the blocking of the receptor (Figure 2-2, B). The mechanism of this blocking can be explained for specifically synthesised antagonists applied in medicine like the breast cancer drugs tamoxifene or raloxifene. For non-specifically manufactured substances able to act as antagonists which are to a large extent still unknown, more research is needed to identify relevant compounds and to explain their specific mode of action.

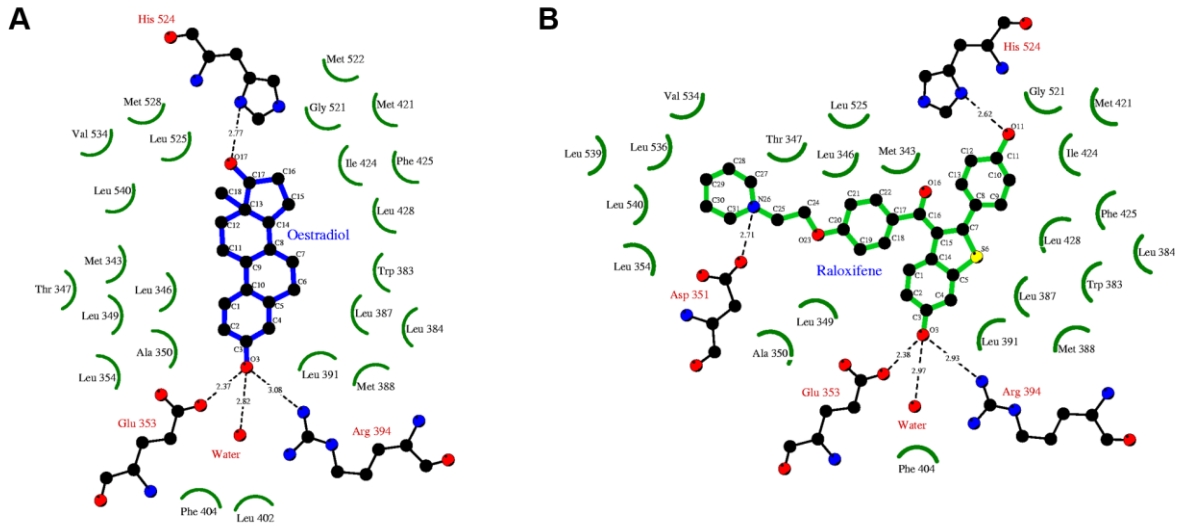

Figure 2-2: Schematic representation of the interactions by E2 (A) and raloxifene (B) within the binding cavity of the human estrogen receptor alpha. Hydrogen bonds between the amino acids of the receptor complex and the respective molecule are illustrated by dashed lines. Adapted by permission from Springer Nature: Nature, 389(6652): 753-758. Molecular basis of agonism and antagonism in the oestrogen receptor. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engström O, Ohman L, Greene GL, Gustafsson JA, Carlquist M, COPYRIGHT 1997 (Brzozowski et al. 1997).

A screening of literature databases by the keywords “anti-estrogen” or “anti-androgen” and “water” or “estrogen / androgen and water”, reveals that the investigation of antagonistic effects as well as androgenic effects is not in the focus of current research activities. In water-related research, less than 15 papers dealing with antagonists were published within

one year on average, while more than 300 publications dealt with estrogens (Figure 2-3). The focus on estrogenic effects can be explained by several cases of endocrine disruption in humans in the last decades and consequently the high public interest. One of the first cases was caused by the pesticide dichlorodiphenyltrichloroethane (DDT) which caused a reduction of sperm counts in farmers (Singer 1949). Later, estrogenic effects were detected caused by compounds such as nonylphenols or bisphenol A used as additives in plastics applying EBM (Soto et al. 1991, Krishnan et al. 1993). One reason that scientific research in the last years is more focused on estrogen active compounds and less on antagonists are studies demonstrating the environmental effect of estrogens to organisms as well as the addition of estrogen active compounds to the watch-list of the WFD (EU 2015/495, Kidd et al. 2007, Plahuta et al. 2017, Osman et al. 2015, EU 2018/840). Estrogen active compounds such as estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) are listed for an EU wide monitoring since the establishment of the watch-list in 2015 and are still listed in the updated watch-list from 2018 (EU 2015/495, EU 2018/840).

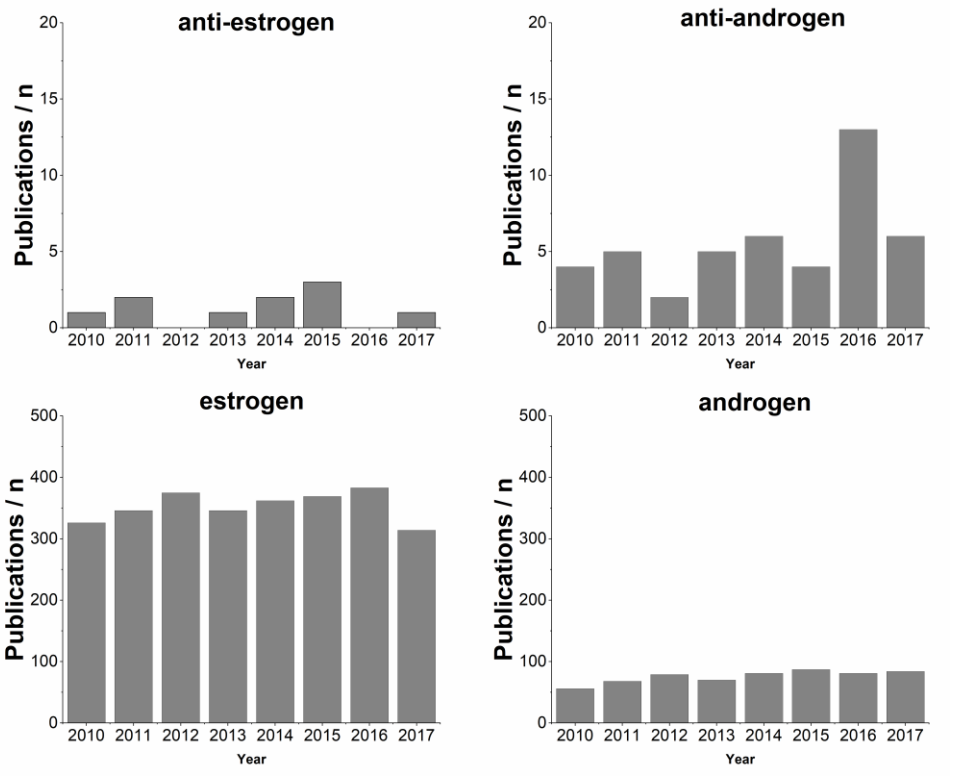

Figure 2-3: Number of publications since 2010 using the Web of Science Database (www.webofknowledge.com). Keywords used in the category “Topic” were “anti-estrogen or anti-androgen” & “water” as well as “estrogen or androgen” & “water”.

A further limitation of the search algorithm by the combination "anti-androgen” and “water” within the manuscript title, results in only one single hit (Liscio et al. 2014a). This study

investigates anti-androgen mixtures in river water in combination with chemical and EBM. The authors of that study conclude that further comprehensive investigations are needed to identify relevant compounds and corresponding effects. Because agonists and antagonists might both be present in a sample they might both contribute to the overall effect. This fact therefore needs to be taken into account when applying effect based analysis and especially when correlations to single compounds are done. As estrogenic effects are occurring even at extremely low concentrations, required LODs are 0.4 ng/L for E1 and E2, and 0.035 ng/L for EE2. Currently, no instrumental analysis methods exist based on either liquid chromatography or gas chromatography coupled to mass spectrometry that fulfil this requirement for EE2. (Loos 2015). In contrast, when applying EBMs, concentrations as low as pg/L can be detected. However, it is difficult to correlate the effects to single substances, because EBMs always determine the sum of compounds leading to the effect. This effect is expressed as equivalent concentration, for example as 17β-estradiol equivalent quotient (EEQ). When analysing environmental samples, antagonistic effects, which reduce the estrogenic activity, make it even more complicated to derive single compound concentrations of EDC. These facts illustrate that there is a need for research focusing on antagonistic effects in mixtures. Moreover, relevant compounds that contribute to an overall effect need to be identified in order to correlate the observed effects with the corresponding compounds. This review summarizes the state of the art of modern chemical detection methods and highlights the potential and limitations of EBM with special focus on antagonistic effects. As there is less data on anti-estrogenic and anti-androgenic effects, this review summarizes the information about compounds already known to act as antagonists. Furthermore, the current status and especially problems associated with correlation analysis when using instrumental methods are briefly discussed.

2.4 Instrumental analysis of endocrine active compounds

Determination of the concentration of single steroidal endocrine active compounds is usually done by gas (GC) or liquid chromatography (LC) (Locatelli et al. 2016, Grover et al. 2009). In the past, chemical methods for the detection of estrogens have mostly been based on GC coupled with mass spectrometry (MS). Detection limits (LOD) between 1 and 5 ng/L were usually achieved in waste water and surface water (Lee and Peart 1998, Cathum and Sabik 2001, Trenholm et al. 2006, Huang and Sedlak 2001). Due to further technical improvements, sensitivity and selectivity could be increased by GC-MS/MS. In a study by Ternes et al. (1999) LODs of 0.5 ng/L for 17β-estradiol (E2) and estrone (E1) were achieved by solid phase extraction with an enrichment factor of 1000 comparable toother studies with LODs of 0.3 - 0.6 ng/L using GC-MS/MS (Wozniak et al. 2014, Kuch and Ballschmiter 2001).

Also androgens like testosterone and its metabolite dihydrotestosterone could be detected with LODs in the range of 1 - 5 ng/L using GC-MS/MS (Trang et al. 2011). When using GC, a solvent exchange as well as a derivatisation step is mandatory for the analysis of hormones, which is more time consuming compared to LC methods (Grover et al. 2009, Locatelli et al. 2016). The WFD therefore recommends the use of LC-MS/MS for the detection of estrogens.

In an updated report of the first year monitoring compounds from the watch-list, the Joint Research Centre (JRC) published a report, showing that the LODs for EE2 and E2 that were obtained by applying instrumental analyses methods of institutions from different member states were still not sufficient. There are two methods able to reach the low LOD of 0.035 ng/L for EE2. One method is based on liquid/liquid extraction (LLE) and analysis with GC-MS/MS, the other method is based on a solid phase enrichment (SPE) and analysis with LC-MS/MS. In summary, laboratories from 4 countries were able to reach an LOD of 0.03 ng/L (172 samples), laboratories from 4 other countries were able to reach an LOD equal to the environmental quality standard (EQS) of 0.035 ng/L (57 samples). However, laboratories from 12 countries reported that LODs were insufficient (247 samples in total), highlighting the difficulty for an EU wide screening under the WFD requirements (Loos et al. 2018).

Differences in achievable LODs, which are in the range of 0.1 - 20 ng/L for E1, E2 and EE2 depending on the matrix load when using LC-MS/MS, are shown by many other publications (Havens et al. 2010, Valdes et al. 2015, Chang et al. 2011, Ronan and McHugh 2013). The necessity to reduce matrix interferences was already discussed in a study by Huang and Sedlak (2001) performing HPLC fractionation by repeated injections of 200 µL aliquots. The authors showed that the reduction of matrix components enhances the sensitivity reaching an LOD of 0.1 ng/L for EE2 (Huang and Sedlak 2001).

In a study by the JRC, literature was screened to find a suitable method reaching the required LODs, and it was concluded that an enrichment step by SPE is necessary (Loos 2015). The lowest reported LOD for EE2 were 0.03 ng/L by the JRC Report and 0.05 ng/L by Williams et al. (Williams et al. 2012, Loos et al. 2018). They used SPE LC-MS/MS in combination with gel permeation chromatography, again indicating the need of a clean-up to remove matrix components.

An earlier study by Schlusener and Bester (Schlusener and Bester 2005) showed LODs about 1 ng/L for the different estrogens using size exclusion chromatography (SEC) prior to LC-MS/MS analysis. Instead of the most commonly applied electrospray ionization (ESI) source, atmospheric-pressure chemical ionisation (APCI) was used. This led to less matrix interferences compared to ESI.

Within standardization efforts in Germany different methods are currently under development (Lebertz 2018). These comprise liquid/liquid extraction (LLE) as well as online and offline

SPE methods. The current LODs for LLE using standards without matrix components are in the range of 2.5 - 25 pg/L. The advantage of LLE compared to SPE is that even samples with high particle load can be enriched without blocking the SPE cartridge. A major disadvantage is of course the high use of environmentally relevant solvent. Our current in-house method consists of offline SPE (using C-18 Speediscs) prior to an online SPE (using CN material) in combination with LC-MS/MS. The LODs for surface water were 20 pg/L for E1, 50 pg/L for E2 and 30 pg/L for EE2 (Lebertz 2018). It is obvious that only with high efforts on sample enrichment and clean-up the required limits of detection can be obtained in surface waters.

Chen et al. (2016) reported limits of detection of 0.02 - 0.06 ng/L for six different estrogens by using polymeric ionic liquid-based stir cake sorptive extraction followed by high performance liquid chromatography. Although the LOD of EE2 as required by the EU WFD could not be reached, the sensitivity can be increased if the matrix is removed. In a study by Kumar et al. (Kumar et al. 2014) a novel developed enrichment technique which is referred to as selective fabric phase sorptive extraction (FPSE) was used in combination with HPLC coupled with a fluorescence detector (FLD) for the detection of E2 and EE2. LODs of 0.02 ng/L and 0.036 ng/L were obtained using 1 L of sample. Major advantages of FPSE if compared to other extraction methods are a higher sample capacity, selectivity and shorter sample preparation time, which is an advantage especially for biologically active environmental samples. However, in this study the detection limits were calculated from standards and not from spiked matrix-loaded samples. As already mentioned, matrix separation represents the greatest challenge to obtain low LODs. Therefore, it should be mandatory to report LODs from spiked matrix containing samples. Unfortunately, many studies do not precisely describe if LODs are obtained from standards in pure water or by spiked matrix loaded samples. In addition, the method to determine LODs varies in literature and in some cases is not even indicated. In the latter cases, the reported numbers are not useful since reported LODs can strongly depend on the chosen approach for their determination. A true comparison of the results of different studies is therefore difficult if not impossible at the moment.

Furthermore, in most of the publications a filtration step was included prior to sample enrichment. This is not in accordance to the EU WFD that requires investigation of the whole water sample including particles (EU 2000/60). Since suspended matter from environmental samples can lead to clogging of cartridges in standard format, special enrichment materials with a larger cross-section in a disc format are available. Solid phase enrichment is generally very time-consuming, therefore automated enrichment systems are increasingly used in many laboratories (Naldi et al. 2016, Goh et al. 2016, Viglino et al. 2008, Locatelli et al. 2016). However, automatic systems always have a higher risc of contamination if the cleaning is not done properly. Although manual SPE is more time consumimng, all plugs,

tubes and other parts in contact with the sample can be changed after each sample and the risk of cross-contamination is lower as each sample is treated independently from the other. Especially in the low pg/L concentration range this can become very important.

To increase the ionization efficiency and therefore sensitivity when using LC-MS/MS, a derivatisation can also be done for steroid hormones. Common derivatisation agents for estrogens in blood serum as well as water are dansyl chloride, 2-fluoro-1-methyl-pyridinium p-toluenesulfonate (FMPTS) or pentafluorobenzyl bromide (PFBBr) (Lin et al. 2007, Faqehi et al. 2016). However, derivatisation efficiency depends strongly on the matrix. For example, Lin et al. (Lin et al. 2007) used dansyl chloride which exhibited good signal intensity enhancement in less matrix loaded samples like drinking water, while PFBBr derivatisation was more suitable for complex matrices like surface or sewage effluent water samples. Faqehi et al. (Faqehi et al. 2016) used FMPTS as derivatisation reagent to detect estrogens in blood samples. FMPTS has the advantage to create more specific product ions compared to other derivatisation agents, leading to higher signal intensity and selectivity. LODs for E1 and E2 were 0.2 pg/sample on-column using positive electrospray ionisation (Faqehi et al. 2016). However, this was only tested in blood samples. In general, the sample pre-treatment is the most important step when aiming at very low LODs. Unfortunately, derivatisation is a time-consuming step and the efficiency is strongly depending on the sample matrix.

Compared to estrogenic substances androgens are not yet regulated and there are no precautionary limits. However, androgen active compounds are also suspected to interfere with the sexual development and endocrine system in aquatic organisms (Kolodziej et al. 2003). In some of the presented analytical methods for estrogens, androgen active compounds are also included. LODs of 0.06 - 0.03 ng/L for testosterone are reported (Vulliet et al. 2008, Chang et al. 2011). The concentration of natural androgens is usually higher compared to estrogens due to higher excretion rates by humans and animals (Shore and Shemesh 2003). However, there is a lack of data to derive trigger values, showing the need to collect more information for these compounds (Escher, Aït-Aïssa, et al. 2018, Könemann et al. 2018).

2.5 Effect based analysis

2.5.1 Agonistic effects

2.5.1.1 Relevance of agonistic effects

EBM such as in vitro or in vivo bioassays are suitable screening tools due to higher sensitivity compared to instrumental analysis (Giebner et al. 2016, Ihara et al. 2015, Leusch et al. 2018, Leusch et al. 2014, Galluba and Oehlmann 2012, Swart et al. 2011, Kunz et al.

2015, Escher, Aït-Aïssa, et al. 2018). During the last years, effect based analysis has therefore gained an increasing interest especially for estrogen effects, which is also seen by the number of articles published (Figure 2-3).

Besides estrogen effects, the environmental effects of androgenic agonistic effects are not much studied, probably due to low potencies of known androgen active chemicals leading to less detected environmental effects (Escher, Aït-Aïssa, et al. 2018). Therefore, this review focus more on estrogen effects.

Since EBM determine a sum parameter, a correlation to individual compounds is not possible. However, they are discussed as a supplement to instrumental analysis for an initial screening (Kunz et al. 2015, Brack et al. 2017, Kase et al. 2018, Wernersson et al. 2015, Escher, Aït-Aïssa, et al. 2018, Könemann et al. 2018).

Typical in vivo bioassays applied for detecting endocrine disruption are the green fluorescent protein (GFP) induction in zebrafish (Danio rerio) embryos or the expression of vitellogenin in male medeka (Oryzias latipes) (Brion et al. 2012, Reinen et al. 2010, Ihara et al. 2015). Brion et al. (Brion et al. 2012) demonstrated that the estrogenic activity is not only caused by compounds known to act estrogenic. Compared to in vivo tests using higher organisms like fish, in vitro bioassays have the advantage of simpler handling. Moreover, no ethical issues have to be considered because only cell systems are used. Furthermore, higher reproducibility is achieved due to the use of standardized cell lines and validated assays (Hettwer et al. 2018). Additionally, the result gained by typical bioassays is available in a few days compared to in vivo tests, which can take weeks. Another advantage of in vitro bioassays is the relatively small sample volume required for analysis (µL range). Especially in view of the necessity to achieve high enrichment factors, small sample volumes are of decisive advantage (Wernersson et al. 2015).

As bioassays are not detecting specific compounds, a general enrichment of known as well as unknown compounds has to be performed. Therefore, less selective hydrophilic/lipophilic balanced (HLB) material as sorbent is used most commonly (Locatelli et al. 2016). Because cartridges in a disc format are often not available for a special sorbent material, a clogging can occur for standard cartridges. In order to overcome that problem, Leusch et al. (Leusch et al. 2014) used super clean coconut charcoal cartridges that were stacked on top of an HLB column.

A further important point is the time between sampling and enrichment in the laboratory due to biological activity of environmental samples (Hillebrand et al. 2013). In some cases, samples are shipped to a laboratory which can take several days. The stability during that period is often unclear as bioassays are not analysing specific compounds. To that end, a method was recently introduced by Schulze et al. (Schulze et al. 2017) in which up to 1000 L can be enriched on-site. However, a pre-filtration to prevent a clogging by particles was

performed which is not in accordance with the guidelines of the WFD. Due to the sensitivity of commonly applied bioassays, the sample volume can also be reduced to prevent a clogging of cartridges.

There are numerous in vitro biotests available for water quality monitoring (Brack et al. 2016, Leusch et al. 2017, Gehrmann et al. 2018, Reungoat et al. 2010, Kunz et al. 2017, Neale et al. 2017, Chamas et al. 2017, Neale, Ait-Aissa, et al. 2015, Escher et al. 2014, Escher, Aït-Aïssa, et al. 2018, Könemann et al. 2018). Some typically applied tests for the investigation of estrogenic or androgenic effects in surface and waste water samples are the human cell line based ER-/AR-CALUX assay and the yeast cell based assays YES/YAS or A-YES/A-YAS (Gerlach et al. 2014, Gehrmann et al. 2018, Itzel et al. 2017, Hettwer et al. 2018). In a direct benchmark test of the ER-/AR-CALUX, YES/YAS, A-YES and A-YAS by Gehrmann et al. (Gehrmann et al. 2018) the different bioassays showed specific sensitivities towards waste water samples. In general, the human cell line based CALUX assay showed the highest sensitivity yielding LODs of 0.2 ng EEQ/L for estrogenic effects and 1.6 ng dihydrotestosterone equivalents (DHTEQ)/L for androgenic effects. However, waste water samples often led to cell death due to toxic matrix components (Gehrmann et al. 2018). As waste water contains a high variety of matrix compounds potentially cytotoxic to the applied bioassays, viability controls have to be performed in parallel to each series. Yeast based assays did not show any inhibition due to toxicity of the waste water, highlighting the robustness which is important when analysing diverse environmental samples.

The A-YES assay is based on the yeast strain Arxula adeninivorans. Compared with the YES assay it showed good limits of detection (0.003 ng EEQ/L, nominal enrichment factor 1000) for the analysis of waste water samples and a high robustness (Gehrmann et al. 2018). This assay is therefore ideally suited to analyse highly matrix loaded samples (Hettwer et al. 2018, Itzel et al. 2017, Gehrmann et al. 2018, Escher, Aït-Aïssa, et al. 2018, Kunz et al. 2017).

2.5.1.2 Effect based trigger values

In theory, sensitive bioassays could be used to determine whether the EEQ is below the required LOD for EE2 of 0.035 ng/L. Based on this, it could be assumed that EE2 is probably below the LOD as well as EE2 is the most potent estrogen towards the applied human estrogen receptor. However, mixture effects like inhibitory antagonistic effects can influence the result. Therefore, matrix effects must always be monitored in parallel by e.g. matrix calibrations to get further information about the measured equivalent concentration. If the effect is not influenced by the matrix, a statement can be made about compliance with the proposed EQS for EE2. In most cases, detected effects caused by unknown compounds

cannot be adequately explained with the help of instrumental analysis (Könemann et al. 2018). Tang et al. (Tang et al. 2013) applied an in vivo test using the organism vibrio fisheri in combination with instrumental analysis. In this study they could explain less than 1% of the detected effect by measured target compounds. In other studies, using different endpoints like oxidative stress by human breast cancer lines AREc32, less than 0.1% of the effect could be explained (Escher et al. 2013). Mehinto et al. (Mehinto et al. 2015) were able to explain just about 5% of the overall effect using the GeneBLAzer assay.

The implementation of effect based guideline values would make it possible to monitor water bodies by sensitive bioassays without the need of instrumental analysis. As EBM are based on an integrative approach and thus already include potencies of all active compounds, including unknown compounds and mixtures of compounds such as agonistic and antagonistic acting ones, a threshold value of the overall effect would be potentially more useful for an evaluation and reduce the effort to develop sensitive instrumental analysis methods. To implement effect based threshold values according to the WFD, a special taskforce was established to identify potential effect based tools. In this taskforce, in vitro bioassays were shown to be more suitable compared to in vivo tests due to their cost effectiveness and relatively fast results and are therewith seen as potential screening tool prior to instrumental analysis (Wernersson et al. 2015). However, a method would be needed to derive statistically verified and comparable trigger values, which are applicable to respective bioassays and also to different end-points like estrogen or androgen effects. Such a method was developed by Escher et al. (Escher et al. 2015) including data of many different compounds with a high response to the bioassay to derive effect based trigger (EBT) values (Escher et al. 2015). In a follow-up study the algorithm was updated based on data of 48 in vitro bioassays to derive EBT values from already existing EQS of single compounds listed in the WFD (Escher, Aït-Aïssa, et al. 2018). There it was shown that for androgen, anti-androgen and anti-estrogen effects it is not possible to derive EBT values due to a lack of effect data of compounds for specific bioassays. For this reason, data of antagonistic active compounds need to be collected in future work. A summary of proposed EBTs for estrogenic activity from different publications is shown in table 2-1.

Table 2-1: EBTs for estrogenic activity.

EBT value	Method / Assay	Information	Reference
3.8 ng EEQ/L	ER-CALUX	For human exposure*	(Brand et al. 2013)
			Human
0.1 - 0.4 ng EEQ/L	YES, ER-CALUX, MELN, T47D-KBluc, E-screen, MVLN	Derived by long-term exposure of different assays	(Jarosova et al. 2014)
0.5 - 2 ng EEQ/L	YES, ER-CALUX, MELN, T47D-KBluc, E-screen, MVLN	Derived by short-term exposure different assays	
0.4 ng/L	Direct application of EQS	EQS of E2	(Kienle et al. 2015)
0.1 - 2.15 ng EEQ/L	32 different bioassays	High variation due to results of different bioassays	(Escher, Selim Aït-Aïssae, et al. 2018)
0.5 ng EEQ/L	ER-CALUX	Derived from LOEC 3.3 ng/L	(van der Oost et al. 2017)
			Biota

*value for humans in terms of drinking water

There are mainly two approaches used in the different studies. One suggestion is the direct application of the annual average EQS (AA-EQS) for E2 of 0.4 ng/L as EBT (Kienle et al. 2015). Another suggestion is a generic method based on the AA-EQS and additional empirical data collected from many different compound studies. On that basis, estrogen effect EBTs were published as preliminary values and showed a variation of 0.1 - 2.15 ng EEQ/L using the ER-CALUX and Cyp19a1b-GFP (EASZY) assay, respectively. This variation illustrates the need of a specific EBT for a certain bioassay. However, mixture effects are not considered in previously described approaches. This could lead to false negative results in case of high concentrations of antagonists.

In a study by Van der Oost et al. (van der Oost et al. 2017) an overall strategy was published called Smart Integrated Monitoring (SIMONI) to address this problem. In a first step they recommend that contaminations of water bodies should be localised as “hot spots” by non-specific and specific toxicity end-points. For non-specific end-points organisms of different trophic levels such as zooplankton, phytoplankton and bacteria were selected. As specific end-points several toxicity tests such as cytotoxicity, genotoxicity as well as estrogen and anti-androgen activity were selected. For screening and evaluation of contaminations in addition to single substance EQS values, existing EBTs were used to indicate a contamination of the sampling sites. In the second step a risk assessment was done using EDA and advanced instrumental analysis. Therefore, besides a no-effect level of active compounds based on no observed effect concentrations (NOEC) and predicted no effect concentrations (PNEC), values were established named safe biological equivalent concentration (BEQ). The next higher value named “Background BEQ” was derived from field data in which no effects have been observed and the ecological status of the water bodies were classified as “good” with regard to the WFD. However, when classifying a water

body as “stable” system, false negative values could be derived due to single contaminations during data collection for the calculation of the background BEQ.
Due to the dependency of EBTs on the applied bioassay, standardised bioassays like the A-YES, YES and ER-CALUX (Hettwer et al. 2018) should be included in addition to corresponding EBT values as defined by the WFD as an official supplement to instrumental analysis for screening of surface waters.
In a recent study by Kase et al. (Kase et al. 2018), the use of EBM for assessing water conditions was investigated for the first time. The aim of the study was to overcome the limits of instrumental analysis using sensitive *in vitro* assays for screening. Various EBTs were tested for evaluation and finally the proposed AA-EQS of E2 (400 pg EEQ/L) was recommended.

2.5.2 Antagonistic effects

2.5.2.1 Relevance of antagonistic active compounds

The environmental relevance of antagonistic acting endocrine compounds and their contribution to disrupt the endocrine system was investigated in some projects dealing with waste water treatment plant effluents and surface waters (Jobling et al. 2009, Rao et al. 2014, Matthiessen and Weltje 2015).
In addition, current research projects have investigated the behaviour of endocrine effects during advanced waste water treatment by ozone or adsorptive processes. (Gehrmann et al. 2018, Itzel et al. 2018, Hill et al. 2010), wherein contrast to agonistic effects a significantly different trend was observed for antagonistic effects. No or only very low elimination of antagonistic effects could be achieved during advanced treatment by ozone leading to discharges into surface waters. Knoop et al. (2018) investigated the activity of formed transformation products (TPs) during ozonation of the known anti-estrogen tamoxifen. It was shown that some formed TPs still exhibited a significant anti-estrogen effect, exemplarily explaining the constant anti-estrogen activity during advanced waste water treatment. These studies showed the potential environmental importance of antagonistic effects and the need of further research.
In general, there are two important types of antagonistic effects. One is the pure antagonistic effect by blocking a receptor. For example, the medical drug fulvestrant is acting as a pure anti-estrogen at the human estrogen receptor alpha (hERα). A further effect can be observed by selective estrogen receptor modulators (SERMs) which are used as pharmaceuticals. Such compounds can act as agonist or as antagonist depending on the tissue in the human body. One example is raloxifene, which can act as agonist in bone and as antagonist in the uterus tissue (Huang and Aslanian 2012). The latter makes it difficult to predict the

environmental behaviour and especially the response in a certain bioassay. As mixtures of agonists and antagonists are always present in environmental samples, a correlation to single substances can become very difficult due to synergistic effects of agonists or as already mentioned antagonistic acting compounds leading to a signal reduction. These two cases are illustrated by figure 2-4, showing that a prediction of an effect by a single compound can lead to under- as well as overestimation of the exerted effect in a real sample.

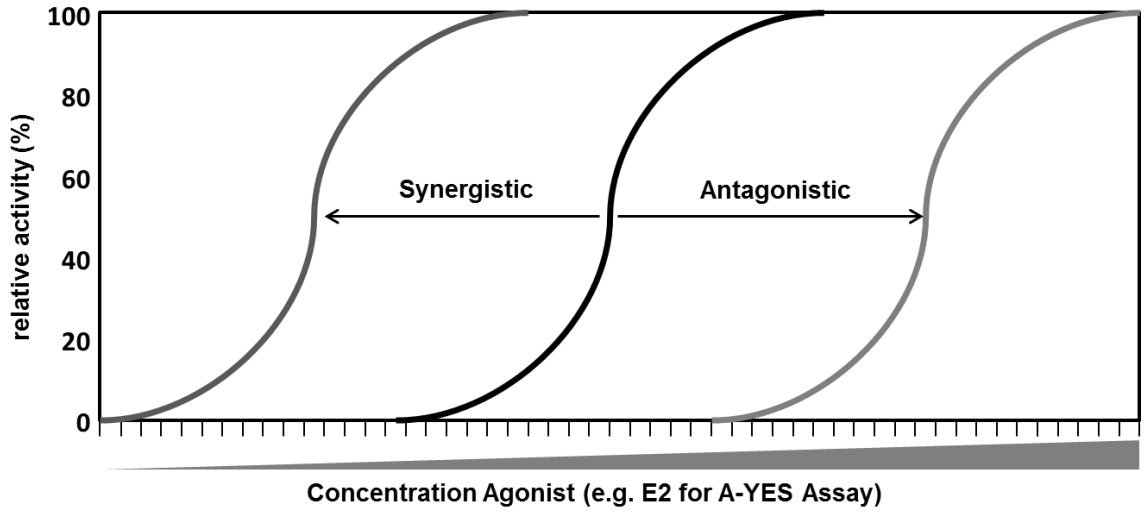

Figure 2-4: Illustration of mixture effects of agonist and antagonist active compounds. X-axis represening the concentration of a known standard increasing from left to right. Reprinted from Science of the Total Environment, 624: 1443-1454, Itzel F, Jewell KS, Leonhardt J, Gehrmann L, Nielsen U, Ternes TA, Copyright (2018) with permission from Elsevier (Itzel et al. 2018).

Beside correlation analysis, antagonistic effects can become important during evaluation of the fate of agonistic effects during waste water treatment processes, as it is done in many studies. Thereby an increase of the measured agonistic effect could be possible due to a change of the water composition (e.g. higher removal of antagonists leading to a relatively higher agonistic activity). In such a case, the effect-causing substance would be of high interest. To identify such a compound, new approaches combining biological information and chemical data are useful (Brack et al. 2017). Such an approach is called an effect directed identification approach (EDA), which was successfully used in several studies (Tousova et al. 2017, Brack et al. 2016, Comtois-Marotte et al. 2017, Kassotis et al. 2015, Chou et al. 2015, Ma et al. 2017, Leusch et al. 2014).

2.5.2.2 Anti-estrogenic effects

In terms of combining effect based and instrumental analysis as proposed by the WFD, anti-estrogenic effects and relevant compounds are important to explain the detected effect in a

certain sample. Some research on anti-estrogenic active substances has been done and showed that not only compounds produced for desired effects are responsible for the inhibition (table 2-2). Endocrine pharmaceuticals such as cancer therapy drugs are developed for the inhibitory effect. One example is tamoxifen, which is acting as estrogen receptor antagonist (Kiang and Kennedy 1977). Also substances designed for a completely different application like flame retardants (Zhang et al. 2014a), some pesticides (Orton et al. 2012), fungicides (Seeger et al. 2016) and even non-steroidal anti-inflammatory drugs such as diclofenac or ibuprofen are able to interfere and act competitively with the estrogen receptor (Ezechiáš et al. 2016). Ezechias et al. (Ezechias et al. 2016) investigated the potential of anti-inflammatory drugs, in which the highest anti-estrogenic potency was observed for diclofenac showing an inhibition concentration at 50% signal reduction (IC_{50}) of 9.45 µM compared to clofibrate with an IC_{50} of 170.84 µM using the YES-assay.

Table 2-2: Overview of compounds acting anti-estrogenic in different bioassays. Inhibitory concentration (IC) are shown in which the effect is lowered by 50% (IC50) or 20% (IC20).

Substance	Test system / Application	Effect concentration	Potencies[1]	Reference
Tamoxifene	Medicine	n.d.	n.d.	(Kiang and Kennedy 1977)
Tamoxifene citrate	Medicine MCF-7 cells	IC_{50}=0.796 µM	n.d.	(Torikai et al. 2017)
4-Hydroxy-Tamoxifene	Main human metabolite	n.d.	n.d.	(Lim et al. 2005, Lee et al. 2003)
Toremifene **Fulvestrant** **Clomifene** **Raloxifene** **Exemestame** **Endoxifene** **Fulvestrant**	Medicine	n.d.	n.d.	(Shelly et al. 2008)
Endoxifene	Medicine	n.d.	n.d.	(Lim et al. 2005)
Bezopurpurine 4B **Yellow 4G** **Direct blue 71** **Red alpacide 3BL** **Everzol navy blue** **Blue HFRL** **Direct red 89 BNL** **Blue DERF** **Everzol blue ED** **Eversol navy ED** **Everzol red**	Yeast estrogen screen BY4741	IC_{50}= 0.17 g/L IC_{50} > 1.0 g/L IC_{50} > 1.0 g/L IC_{50} > 1.0 g/L IC_{50}=0.065 g/L IC_{50}=0.017 g/L IC_{50}=0.029 g/L IC_{50} > 1.0 g/L IC_{50} > 1.0 g/L IC_{50} > 1.0 g/L IC_{50} > 1.0 g/L	1.0 0.13 0.22 0.44 0.94 1.0 1.0 0.1 0.35 0.32 0.30	(Bazin et al. 2012)
Ibuprofen **Diclofenac** **Ketoprofen** **Naproxen** **Clofibrate**	Yeast estrogen screen BMAEREluc/Erα	IC_{50}=22.2 µM IC_{50}=9.45 µM IC_{50}=100 µM IC_{50}=80.02 µM IC_{50}=170.84 µM	n.d.	(Ezechiáš et al. 2016)

Substance	Test system / Application	Effect concentration	Potencies[1]	Reference
Tonalide **Galaxolide**	Zebra fish Assay (hER)	n.d. n.d.	< 0.01 < 0.01	(Schreurs et al. 2004)
Benzophenone-3 **4-Methylbenzylidene camphor** **Octylene**	YES-Assay	n.d. n.d. n.d.	0.02 0.005 0.005	(Kunz and Fent 2006)
TCEP **TEHP**	Luciferase reporter gene assay	$IC_{20} < 10^{-6}$ M $IC_{20} < 10^{-6}$ M	n.d.	(Zhang et al. 2014b)
Dibenzonaphthyridine **BenzoA)acridine**	MCF-7 cells	0.951 µM 0.796 µM	n.d.	(Torikai et al. 2017)

n.d. = no data available
[1] Potencies relative to Tamoxifene

Table 2-2 illustrates the high variability of compounds able to interfere by inhibition of the estrogen receptor in competition with estrogenic compounds already showing the need of a more general approach such as the use of bioassays. At the same time, it shows the large variability of published information about detected effects, as different IC values are given like the IC_{50} or IC_{20}. This means that a meaningful comparison of different bioassays is rather difficult. To derive the potency of a substance based on only that information, the IC values of the reference compounds are always required. If these data are not provided, they cannot be used for other laboratories. Only in some studies relative potencies are given which helps to classify a certain compound regarding its relevance in a specific bioassay. In addition to experiments with single compounds, mixture effects should be studied using, e.g., waste water as has been done by Wu et al. (Wu et al. 2009). They were able to show by a simple fractionation approach that a chlorination of waste water significantly increased the anti-estrogenic effect. The specific reason is still unclear but it can be assumed that the chlorination prevents a hydrogen bonding to the amino acids Glu353 and Arg394 preventing a conformational change of the 12α-Helix (shown in figure 2-2). The most active fraction found in that study was the polar fraction, where aromatic amino acids (histidin, phenylalanin, prolin, tryptophan, tyrosin) were identified as the main precursors of the chlorination products. In a different study general structure activity correlations were investigated showing that aromatic ring systems with polar functional groups like hydroxyl groups are most potent anti-estrogens, especially benzo(A)acridine with an OH-group at C9 and C3 (Torikai et al. 2017).

This criterion of aromatic ring systems fits to a large number of chemical substances (e.g. several drugs like diclofenac, ibuprofen), which illustrates the need for research to further limit relevant compounds and compile a list of most potent compounds.

2.5.2.3 Anti-androgenic effects

In contrast to androgens, anti-androgenic effects are gaining more interest as they will result in a similar ecological effect on aquatic organisms compared to estrogens (Jobling et al. 2009). Watermann et al. (Watermann et al. 2016) revealed an effect by anti-androgens on reproductive organs of the copepod *Acartia tonsa*. Therewith, effects on aquatic organisms can be attributed to agonistic as well as to antagonistic acting compounds (Jobling et al. 2009, Milnes et al. 2006, Lor et al. 2015). A prominent example is the feminization of fish populations due to high discharge of estrogens by WWTPs. In addition to cancer drugs such as flutamide, which act as an androgen receptor antagonist, there are many other chemical substances that may act in the same way (Simard et al. 1986). Potential sources of anti-androgen acting compounds were identified to be effluents from livestock feedlot, paper mills, leather industry and WWTPs (Soto et al. 2004, Jenkins et al. 2004, Ellis et al. 2003, Kumar et al. 2008, Runnalls et al. 2010).

However, anti-androgenic compounds have been rarely investigated and, like anti-estrogenic agents, only a few substances are fully described in terms of their endocrine activity. Compounds reported as anti-androgenic in different bioassays are summarized in table 2-3. Here it is shown which compound classes are effective in a respective bioassay. In addition to already known active substances such as pharmaceuticals, chemicals which are not produced for a targeted anti-androgenic effect are also included. In medicine known compounds as e.g. flutamide, cyproterone, megestrol and bicalutamide are used specifically as chemotherapeutic agents for cancer treatment.

Table 2-3: **Overview of compounds acting anti-androgenic in different bioassays.**

Substance	Test system / Application	Effect concentration	Potencies[1]	Reference	EQS (µg/L)
Flutamide	Used as standard / applied in medicine	n.d.	1.00	(Kolle et al. 2011, Takashi and Hiroshi 2014)	n.d.
Cyproterone acetate	applied in medicine	n.d.	n.d.	(Green et al. 2015, Kiparissis et al. 2003, Takashi and Hiroshi 2014)	n.d.
megestrol acetate	applied in medicine	n.d.	n.d.	(Takashi and Hiroshi 2014)	n.d.
Chlormadinone acetate	Zebra Fish	n.d.	n.d.	(Siegenthaler et al. 2017)	n.d.
Spironolactone	applied in medicine	n.d.	n.d.	(Young et al. 1987)	n.d.
Oxendolone	applied in medicine	n.d.	n.d.	(Okada et al. 1988)	n.d.
Bicalutamide	applied in medicine	n.d.	n.d.	(Green et al. 2015, Takashi and Hiroshi 2014)	n.d.
Nilutamide	applied in medicine	n.d.	n.d.	(Takashi and Hiroshi 2014)	n.d.
Medrogestone	applied in medicine	n.d.	n.d.	(Schneider 2003)	n.d.
Vinclozolin	mature goldfish (Carassius auratus) MDK-kb2 Assay anti-YAS Japanese medaka (Oryzias latipes)	800 µg/L IC20 = 0.16µM	n.d.	(Hatef et al. 2012) (Kiparissis et al. 2003) (Kolle et al. 2011) (Orton et al. 2011)	n.d.
2-naphthol **2,2dihydroxybiphenyl** **Chloroxylenol** **Dichlorophene** **1-hydroxypyrene** **Chlorophene** **Oxybenzone** **di(chloromethyl)anthracene** **Triclosan** **4-nonylphenol** **Abietic acid** **Pimaric acid** **Isopimaric acid**	anti-YAS	n.d.	0.32 0.35 0.16 4.70 9.90 13.0 0.34 2.50 4.80 0.30 4.00 2.73 5.00	(Rostkowski et al. 2011)	n.d. 0.02 0.3
Bisphenol A	Anti-YAS / MDA-kb2 Assay		0.60 / n.d.	(Rostkowski et al. 2011, Kortenkamp et al. 2014)	0.24
Fludioxonil **Fenhexamid** **Ortho-phenylphenol** **Imazalil** **Dimethomorph** **Methiocarb** **Pirimiphos-methyl**	MDA-kb2 Assay MDA-kb2 Assay / anti-YAS	 IC20 = 2.02µM IC20 = 5.43µM IC20 = 3.23µM IC20 = 0.26 / 38.5µM IC20 = 6.82µM n.d.	n.d.	(Orton et al. 2012, 2011)	n.d.
Linuron **p.p`-DDE** **Bromopropylate** **Diclofol**	MDA-kb2 Assay	IC20 = 1.74µM IC20 = 0.098µM IC20 = 0.54µM IC20 = 1.43µM	n.d.	(Orton et al. 2011)	0.26 0.0013

Endosulfan **Ethoxyquin** **Tebuconazole** **Prochloraz** **Procymidone**	MDA-kb2 Assay / anti-YAS MDK-kb2 Assay / anti-YAS	n.d. IC20 = 10.7 / 11.2µM IC20 = 2.89µM IC20 = 2.39µM IC20 = 0.163 / 0.956µM			0.005 0.24
PCBs (19-209) **PCB 138** **PCB 118** **PCB 180** **PCB 153**	AR-CALUX Assay / MDA-kb2 Assay MDA-kb2 Assay	n.d.	n.d.	(Kortenkamp et al. 2014, Li et al. 2013)	n.d.
Cypermethrin	Mammalian two-hybrid Assay hAR CV-1 Assay	IC50 = 6.8×10^{-5}M	n.d.	(Pan et al. 2013) (Xu et al. 2008)	0.00008
Perfluorooctansulfonic acid (PFOS) **Benzophenone 2** **Benzophenone 3** **Enzacamene (4 MBC)** **Tonalide** **Galaxolide** **Methylparaben** **Ethylparaben** **Butylparaben** **Propylparaben** **Butylated hydroxyanisol** **3-Benzylindencampher** **Benzo[a]pyrene** **Butylhydroxytoluol (BHT)** **Pentabromdiphenylether**	MDA-kb2 Assay	n.d.	n.d.	(Kortenkamp et al. 2014)	0.00065 0.00017
Lamotrigine **Trimethoprim** **Sulisobenzone** **Clopidogrel** **Clothioapine** **Clozapine** **Venlafaxine** **Bepridil** **Chloroxyenol** **Terbutryn** **Dilfiazem** **Carbamazepine** **Citalopram** **Irbesartan** **Terbinafine** **Diethyltolunamide** **Crotamiton** **Diclofenac** **Tris-(2-chlorisopropyl)-phosphate (TCPP)** **Diazinone** **Diclofenac amid** **N-Desethylamiodarone** **Dehydrofelodipine** **Amiodarone** **Thiaminphyrophosphat (TPP)** **Tributyl phosphate (TBP)** **Triclosan** **Piperonyl butoxide**	anti-YAS Assay	n.d.	0.01 0.02 0.05 0.97 0.62 0.13 0.01 0.27 0.16 0.06 0.01 0.01 0.01 0.01 0.05 0.05 0.01 0.02 0.02 0.17 1.05 1.68 0.13 0.05 0.25 0.02 4.80 0.02	(Liscio et al. 2014b)	120 0.065 2 704 88 0.1 0.012 3.7 0.02
Parathion **Permethrin**	hAR CV-1 Assay	IC50 = 2×10^{-7}M IC50 = 5.68×10^{-5}M	n.d.	(Xu et al. 2008)	n.d.
4-Methyl-7-diethylaminocoumarin	Anti-AR-CALUX	n.d.	0.52	n.d.	n.d.

n.d. = no data available,
[1] Potencies relative to Flutamide

This summary clearly displays the high diversity of anti-androgen active compounds similar to compounds shown to be anti-estrogen active. Substances not specifically designed for an anti-androgenic effect in an organism include synthetic drugs, softeners, personal care products, industrial chemicals, phthalates and more. This high number of potentially active compounds and the even higher number of compounds that have not yet been investigated makes targeted substance analysis pointless so far, but here the effect based analysis can provide a meaningful parameter due to its integrative approach. However, in order to find the cause of an effect, it is necessary to identify relevant individual substances. Therefore, the EDA approach can be used to systematically investigate substances relevant for an effect.

Besides the already discussed effect of single compounds and the structure relationship to aromatic ring systems and polar functional groups able to interfere with the human estrogen or androgen receptor, humic substances in general could be relevant as well. Neale et al. (Neale, Escher, et al. 2015) showed a general antagonistic effect in correlation to the dissolved organic matter (DOM) content. Especially during analysis of samples with a higher DOM content such as environmental or waste water samples, the influence of the matrix should be considered (Neale, Escher, et al. 2015).

The complexity of the antagonistic effects becomes even more apparent by the comparison of substances from table 2-2 and 2-3, which can be both anti-estrogen and anti-androgen like anti-inflammatory drugs such as diclofenac or phosphorous flame retardants such as TCEP. Since *in vitro* EBM often use target enzymes to measure the effect signal, other points of interaction are in general possible. An example could be the inhibition during the enzyme translation or with the translated enzyme itself, rather than interaction with the receptor. This effect should be considered by experiments with the isolated target enzyme as well as the isolated receptor when investigating potentially relevant compounds.

Furthermore, there are substances such as DDE or 4-nonylphenol which can act as an estrogen receptor agonist and androgen receptor antagonist. This means that some substances do not necessarily trigger only one effect (Ezechias et al. 2016, Amaro et al. 2014, Gould et al. 1998).

To derive EBT values also for anti-androgenic assays, relative potencies compared to a reference standard like flutamide and the EQS values are needed of known compounds (Escher, Aït-Aïssa, et al. 2018).

The effects of some compounds are already investigated and respective potencies as well as EQS are summarized in table 2-3. However, there are many substances that have not been tested, or if they have been tested, often only in a single bioassay. A first attempt to derive EBTs for anti-androgenic assays like the AR-GeneBLAzer, MDA-kb2, AR-CALUX and AR RADAR resulted in EBTs ranging from 3.3 to 14.4 µg flutamide EQ/L (Escher, Aït-Aïssa, et al. 2018). In a different study using a less specific approach for anti-androgens an EBT of

25 µg flutamide EQ/L was derived (van der Oost et al. 2017). This variation of suggested EBTs clearly shows the need of further research on antagonistic effects and their receptor binding mechanism to specify EBT values. In order to derive robust EBTs for antagonistic activity, active compounds have to be identified as well as their occurrence in the environment. This step will be the most important and time consuming work of the future as it is not yet possible to restrict compounds to a certain compound class. One option to filter relevant compounds and collecting information would be the application of an EDA approach. To collect antagonistic compound information in future and to realise a comparability of different applied bioassays, basis data like potency and EC_{50} values have to be measured and published. After this work the most relevant compounds responsible for the detected antagonisitc activity should be considered for EBT derivation.

2.6 Conclusions

- Chemical analysis of estrogens is still limited for evaluation of proposed environmental quality standards. Therefore, the combination of chemical and biological methods for screening and quantification are necessary.
- Interpretation of measured effects and correlations with chemical analysis is often not possible due to inhibitory antagonistic effects.
- Identification of antagonistic acting compounds by an effect directed analysis approach is a promising tool.
- Effect based trigger values enable a screening with regard to the EU Water Framework Directive reducing a time-consuming chemical identification.
- Compounds acting as anti-androgens are highly diverse. As they are leading to an effect in organisms similar to estrogens, there is a particular need for research.

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3. Investigation of a full-scale ozonation at a municipal waste water treatment plant by a toxicity-based evaluation concept

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3.1 Abstract

Effluents from municipal waste water treatment plants (WWTP) are known to be point sources of micropollutants for surface waters. The aim of this study was to examine a reconstructed full-scale ozonation equipped with a pump-injector system for ozone (O_3) dosage and a fluidized moving bed reactor as biological post-treatment at a municipal WWTP utilizing an effect directed approach. This approach consists of chemical analysis in combination with toxicological tests for the evaluation of the treatment efficiency of the plant. Chemical analysis showed elimination rates > 80% for pharmaceuticals and industrial chemicals. Analysis of endocrine disruptors was limited due to substance concentrations below the limit of detection (LOD). Estrogenic activity was detected by the Arxula Adeninivorans yeast estrogen screen (A-YES) at very low concentrations (pg to ng EEQ/l range). Estrogenic activity was reduced by more than 90% after ozonation. In contrast, androgenic activity (measured in the Adeninivorans yeast androgen screen, A-YAS) was still found after O_3 treatment and after biological post-treatment which is consistent with data obtained by chemical analysis. Further, no significant genotoxic or cytotoxic effect was observed after ozonation using the alkaline comet and MTT assays, respectively. This suggests that the applied specific O_3 dose of 0.4 mg_{O3}/mg_{DOC} is a safe operation setup in terms of toxicologically relevant transformation products. In addition, no adverse effects on primary producers as evidenced by algae growth inhibition tests were detected. The monitored biofilm growth in the biological post-treatment showed a steady state after one month. On the basis of computational fluid dynamics (CFD) simulations and biomass we can conclude that O_3 did not enter the biological post-treatment in high extend and that hydraulic retention time in the O_3 reactor was sufficient. Our data demonstrate the success of a full-scale O_3 treatment in combination with a fluidized moving bed reactor as biological post-treatment for reduction of a majority of micropollutants without releasing relevant toxic transformation products as assessed by a chemical and toxicity-based approach.

3.2 Keywords

Municipal waste water treatment, advanced oxidation processes, ozonation, estrogenicity, androgenicity, micropollutant removal, (eco)toxicity

3.3 Introduction

Due to the extensive use of a variety of chemicals such as pharmaceuticals, personal care products (PPCP), biocides, pesticides and industrial chemicals, these substances are discharged continuously into the aquatic environment in low concentrations (Bergmann et al. 2011). Point sources for the entrance of these substances (micropollutants) into surface waters are waste water treatment plants (WWTP), which are not able to ensure a complete removal of these compounds during conventional treatment process (Gabet-Giraud et al. 2010; Morasch et al. 2010; Bueno et al. 2012; Michael et al. 2013; Jarosova et al. 2014b; Rechenberg 2015; Richardson & Kimura, 2016; Valitalo et al. 2016). In the last decades, fish and other aquatic organisms exhibited symptoms such as feminization of males and a decrease in population sizes (e.g. Kidd et al. 2007; Henneberg et al. 2014; Peschke et al. 2014). These effects might be traced back to endocrine disrupting compounds (EDC) such as estrogenic substances which are detected in the effluent of WWTP. These compounds are able to induce biological effects even at very low concentrations: A predicted no effect concentration (PNEC) for the synthetic estrogen EE2 of 0.1 ng/l was reported from fish reproduction studies (Bolong et al. 2009; Pal et al. 2010; Caldwell et al. 2012; Aris et al. 2014). In frame of the water framework directive (EU 2000/60; EU 2008/105; EU 2013/39) which was established by the European Union (EU) with the aim to achieve a “good chemical and ecological status” for all rivers until 2027, a “watch-list” of priority substances was established in 2013 to investigate new priority compounds and their potential risk (EU 2013/39). In addition to other chemicals three EDCs including estrone (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) were included for the first time (EU 2011/876, EU 2015/495). Androgenic active compounds are not listed. However, a comparative study by Chang et al. (2011) demonstrated that compared to estrogenic compounds, androgen active compounds constitute a significant proportion of the total EDC concentration in WWTP influents and even in the effluent and should be investigated for that reason.

These facts raised the question of the necessity of advanced treatment methods for WWTP to remove micropollutants and to prevent discharge to surface waters. For that reason, several studies including “strategy Micropoll” in Switzerland investigated strategies and treatment processes, like ozonation, for a further removal of micropollutants (Abegglen et al. 2009; Abegglen and Siegrist 2012). Ozonation as well as the application of activated carbon

turned out to be reliable technologies that may easily be integrated into existing WWTP and promising results were attained in removal efficiency and maintenance costs (Andreozzi et al. 1999; Tuerk et al. 2010; Rechenberg 2015; Sperlich and Gnirß 2016). A good overview of already installed and operating O_3 treatment units at WWTP was provided by Audenaert et al (2014). In addition, online information is available from the association of Swiss sewage and water protection specialists (VSA 2017) and from homepage of the Competence Centre Micropollutants of North Rhine Westphalia in Germany (Competence Centre Micropollutants - NRW 2016). Further studies worldwide examined the formation of transformation products from ozonation and their potential toxicity after micropollutant treatment by ozone (O_3) which have to be kept in mind when using oxidative processes for further removal (Knopp et al. 2016; Lee and von Gunten 2016; Li et al. 2016).

To evaluate an advanced treatment technology in terms of toxicity and removal efficiency more than classical analytical methods are needed. The conventional chemical analysis by GC- or LC-MS/MS is mostly focused only on target substances which are known to be relevant in the specific catchment. However, adverse effects, due to a mixture of various compounds, cannot be predicted (Brack et al. 2016). Furthermore, the number of known substances and even more of unknown compounds in complex water samples exceeds the measurable quantity. In addition, for some of these compounds it is still challenging to reach sufficiently low LOD with chemical analysis to be able to detect them at ecotoxicologically relevant concentrations (Valitalo et al. 2016).

Bioassays, which are capable of measuring the activity of the whole mixture of compounds with a certain mode of action (e.g. estrogenic or androgenic activity) are considered to be suitable to bridge this gap.

Typical bioassays for detection of EDC currently available are, amongst others, the chemical activated luciferase gene expression (CALUX) assays using human cell lines and yeast-based estrogen and androgen screens (YES/YAS) assays, which are able to detect an endocrine activity in the desired concentration ranges of pg/l to ng/l (Maletz et al. 2013; Gehrmann et al. 2016). Thus, changes in activities before and after an advanced treatment can be detected and new advanced treatment technologies evaluated in terms of endocrine activity (Gehrmann et al. 2016; Di Paolo et al. 2016). Different to chemical analytical methods, the bioassays generate a sum parameter without information on the individual substances. For these reasons, effect-based biotests are a useful supplement to chemical analysis in order to ascertain the elimination effectivity in terms of residual genotoxicity, cytotoxicity, and estrogenic and/or androgenic activity. The use of interdisciplinary approaches, combining chemical analysis with biological effect based test systems, is thus increasing (Creusot et al. 2014; Escher et al. 2014; Brack et al. 2016). Regarding the EU

water framework directive in which a “good chemical and ecological status of surface water bodies” is aimed for, an interdisciplinary investigative approach for point sources like WWTP is necessary to assess treatment technologies with respect to the receiving surface water.

The aim of this study was to evaluate a reconstructed two-line full-scale ozonation plant with a biological post-treatment to ensure a safe and efficient operation. This evaluation was done by a toxicity based approach with focus on removal of micropollutants and consequent determination of cytotoxicity and genotoxicity. In addition, endocrine activity was analyzed using the highly sensitive Arxula adeninivorans yeast estrogen and androgen screens, the first one being able to detect estrogenic activity at concentrations near the chronic environmental quality standard for EE2 (0.035 ng/l) listed in the EU water framework directive (EU 2009/90; EU 2015/495). The investigated WWTP in this study was reconstructed due to a high variable waste water inflow. Changes were made in the O_3 entry system which was altered from diffuser to a pump-injector system to reduce oxygen demand and running costs (Maus et al. 2014). Furthermore, an additional weir was tested the first time to prevent an O_3 entrance into the biological post-treatment and was evaluated by biofilm growth in the post-treatment compared to flow simulations made before.

3.4 Materials and Methods

3.4.1 Chemicals and Standards

Methanol (LC-MS grade), Methyl-tert-butylether (MTBE) and pure water (LC-MS grade) were purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). Hydrochloric acid (98% purity) and formic acid were purchased from Sigma Aldrich GmbH (Steinheim, Germany). Estrogenic, androgenic, pharmaceutical as well as industrial compounds used are listed in table 3-1 and were purchased from Sigma Aldrich GmbH (Steinheim, Germany).

Table 3-1: Standards and respective CAS numbers, limits of detection (LOD) and quantification (LOQ) for LC-MS/MS.

Substance	CAS	Internal Standard	LOD (ng/L)	LOQ (ng/L)
Estrone (E1)	53-16-7	E1_d4	0.20	0.70
17β-Estradiol (E2)	50-28-2	E2_d3	0.02	0.07
17α-Ethinylestradiol (EE2)	57-63-6	EE2_d4	0.05	0.20
Estriol (E3)	50-27-1	E1_d4	2.00	7.00
Diethylstilbestrol	56-53-1	E1-d4	0.90	3.00
Dihydrotestosteron	521-18-6	-*	0.02	0.07
Dehydroepiandrosteron	53-43-0	-*	0.02	0.07
Testosterone	58-22-0	-*	0.02	0.07
Trenbolon	10161-33-8	-*	0.02	0.07
Etiocholanon	53-42-9	-*	0.02	0.07
Boldion	897-06-3	-*	0.02	0.07
Androsteron	53-41-8	-*	0.02	0.07
1H-Benzotriazol	59-05-2	Carbamazepine_d10	0.02	0.07
5-Methyl Benzotriazol	50-18-0	Carbamazepine_d10	0.04	0.10
Carbamazepine	298-46-4	Carbamazepine_d10	0.10	0.30
Clarithromycin	81103-11-9	Carbamazepine_d10	0.10	0.30
Diclofenac	15307-86-5	Diclofenac_d4	0.06	0.20
Metoprolol	37350-58-6	Metoprolol_d7	0.04	0.10
Sulfamethoxazole	723-46-6	Sulfamethoxazole_d4	0.06	0.20
Carbamazepine_d10	132183-78-9		-	-
Diclofenac_d4	153466-65-0		-	-
Metoprolol_d7	1292906-91-2		-	-
Sulfamethoxazole_d4	1020719-86-1		-	-
E1_d4	53866-34-5		-	-
E2_d3	79037-37-9		-	-
EE2_d4	350820-06-3		-	-

*Quantified without internal standard.

3.4.2 Waste water treatment plant description

The mechanical-biological WWTP Duisburg-Vierlinden (Wirtschaftsbetriebe Duisburg AöR, Germany), designed for 30,000 population equivalents (PE), is equipped with two full-scale ozonation units (line 1 and line 2) after the final clarification. O_3 is generated on-site by pure oxygen, provided by a pure oxygen tank with an in-line evaporator and injected by a pump-injector system. The resulting ratio of dissolved organic carbon (DOC) and the ozone concentration is expressed as specific value z (zspec.). Main operation characteristics are presented in Table 3-2.

Table 3-2: Operating parameter of the ozonation plant.

Parameter	Value
Flow velocity (maximum)	200 m³/hr (each line)
Flow velocity (average, dry weather)	120 m³/hr (each line)
Hydraulic retention time (minimum)	0.5 h
pH (Influent ozone treatment)	7.4 (in average)
Ozone dosage	3 mg/L (flow proportional)
Dissolved organic carbon (DOC)	8 ± 2 mg/L
$z_{spec.}$	0.36 ± 0.15 mg_{O3}/mg_{DOC}

As biological post-treatment, a fluidized bed reactor for each line is installed. The formation of a biofilm for the biological degradation is achieved by using plastic growth bodies (2H-BCP 750, GEA 2H Water Technologies GmbH, Germany). Each reactor consists of about 1.5 million growth bodies with a specific surface area of approximately 4410 m². To prevent an aggregation and to ensure a continuous mixing of the growth bodies, compressed air was used for ventilation. Residual oxygen from ozonation in combination with the aeration should result in a nearly saturated solution with oxygen. A scheme of the ozonation unit including the reaction basin, the fluidized bed reactor and the location of the sampling points (SP) is illustrated in Figure SI 7.1-1.

A CFD simulation using ANSYS-Fluent (ANSYS Inc., Canonsburg, USA) was performed by Aixprocess (Aachen, Germany) to simulate the flow conditions inside the O_3 reactors and biological post treatment. The setup for the simulation, assuming an oxygen/ozone gas mixture, was as follows: O_2/O_3 gas flow = 6.8 Nm³/hr, O_2/O_3 bubble diameter = 0.5 mm and a total flow of 200 m³/hr.

3.4.3 Sampling and sample preparation

Samples were taken as flow proportional 24 hr composite samples at the sampling points (Influent ozone (SP1); Effluent ozone line 1 (SP2); Effluent ozone line 2 (SP4); Final effluent line 1 (SP3); Final effluent line 2 (SP5)) indicated in Figure SI 7.1-1 and stored at 4 °C for at maximum 48 hr until analysis or enrichment by solid phase extraction (SPE). Samples were collected weekly from January to mid of February 2015. An overview of the sampling dates and parameters analyzed is given in Table 3-3.

Table 3-3: Sampling and test overview of biological and chemical analysis performed.

Biological analysis	**14.01.**	**22.01.**	**28.01.**	**04.02.**	**11.02.**	**18.02.**	**21.04.**
Biofilm growth	x	x	x	x	x	x	x
Algal growth inhibition test				x	x		
A-YES	x	x	x	x	x		x
A-YAS	x	x	x	x	x		x
MTT assay				x	x		
Alkaline comet assay				x	x		
Micronucleus test				x	x		
Chemical analysis (LC-MS/MS)	**14.01.**	**22.01.**	**28.01.**	**04.02.**	**11.02.**	**18.02.**	**21.04.**
Pharmaceuticals	x	x	x	x	x	x	x
Endocrine disruptors		x	x		x		x

Sample preparation performed for the chemical and effect-based analysis of EDC, and for genotoxic and cytotoxic tests included a solid phase extraction (SPE) step, which was carried out within 48 hr after sampling. At first, the cartridge (200 mg, 6 ml Strata-XL, Phenomenex, Germany, Aschaffenburg) was conditioned (2 x 5 ml methanol) and equilibrated (2 x 5 ml water, LC-MS grade). Subsequently, for each analysis a separate enrichment was performed in order to get enough sample amounts for each analysis. Therefore, separate cartridges were loaded with 1000 ml sample for the LC-MS/MS analysis of endocrine active substances (addition of internal standards), the Arxula adeninivorans yeast estrogen/androgen screen (A-YES/A-YAS) as well as genotoxic and cytotoxic measurements, respectively. After drying the cartridges under vacuum, the loaded cartridges were stored at -18 °C until further analysis. The elution was performed with 5 x 5 ml MTBE. The solvent was evaporated (Evaporator Vapotherm Basis mobil I, Barkley, Germany) at 50 °C under a nitrogen gas stream and re-dissolved in 1 ml (LC-MS/MS) and 4 ml (A-YES/A-YAS) water (LC-MS grade), respectively. The nominal enrichment factor for LC-MS/MS analysis as well as for genotoxicity and cytotoxicity assays was 1000, respectively and 250 for the bioassays to assess estrogenicity and androgenicity (A-YES/A-YAS).

For determination of pharmaceutical and industrial compounds the samples were adjusted to pH 3 with hydrochloric acid followed by an automated solid phase extraction (GX-821, Gilson Inc., Middleton, USA). The method was set up to start with 5 ml methanol and 5 ml water for conditioning and equilibration of cartridges (150 mg, 6 ml, Oasis HLB, Waters, Germany), respectively, followed by the sample loading with a volume of 1000 ml (adding 20 ng/l isotope labelled internal standards see Table 3-1). Then a wash step was run with 5 ml water (pH 3) and 5% methanol, and cartridges dried by an air stream. For each run the flow rate was set to 20 ml/min. The extracts were than evaporated (Evaporator Vapotherm Basis mobil I, Barkley, Germany) at 50 °C under a nitrogen gas stream and re-dissolved in 1 ml pure water for LC-MS/MS analysis. In addition, 60 µl of each sample were analyzed directly, without any extraction, as described above.

3.4.4 Chemical analysis by LC-MS/MS

The analysis of pharmaceuticals, industrial compounds and EDCs was conducted using an LC-MS/MS with an Agilent 1100 LC system (Agilent Technologies, Waldbronn, Germany) and a QTRAP® 6500 (AB Sciex Deutschland GmbH, Darmstadt, Germany) for detection. A Chromolith Fast Gradient RP-18e column (Merck KGaA, Darmstadt, Germany) was employed. For measurement of pharmaceuticals and industrial compounds, a gradient of methanol and water (both with 0.1% formic acid) was applied over 15 min, starting from 1% methanol to 99% within 12 min and two min equilibration time with 1% methanol. The injection volume was set to 50 µl and column oven temperature at 40 °C. Samples analyzed were SPE extracts (enrichment 1000x) and aqueous samples by direct injection. For the analysis of EDC, a gradient of methanol and water (both with 0.1% formic acid) was applied over 14 min, starting from 30% methanol to 70% within 10 min followed by 2 min with 99% methanol and 2 min with 30% methanol for equilibration. The injection volume was set to 30 µl and column oven temperature at 40 °C. The analysis was performed only with aqueous SPE extracts (enrichment 1000x). Each sample was measured once. To ensure data quality we used a fresh calibration, quality control and blank samples with each measurement series and spiked internal standards for estrogenic active compounds (1 ng/l) and for the PPCPs (20 ng/l) see Table 3-1. For androgenic active compounds no isotope labelled internal standards were available. Therefore, these compounds were evaluated externally as mentioned in Table 3-1. To proof the quality of each analysis recovery rates were calculated by the quality control samples and need to be within the acceptable range of 80 - 120% recovery. The measured substances as well as internal standards, respective multiple reaction monitoring (MRM) transitions, collision energies and retention times are provided in the supporting information (Table SI 7.1-1, 7.1.1-2 and 7.1-3). The limit of detection (LOD)

and limit of quantification (LOQ) was calculated from a standard by a signal to noise ratio of 3:1 and 10:1 respectively.

3.4.5 Biological analysis

3.4.5.1 Biofilm detection

To monitor biofilm formation in the fluidized moving bed reactor, samples of growth bodies were taken and analyzed for the biofilm mass. Four samples (40 g each) of the exposed growth bodies were placed in a beaker and stored at 50 °C overnight. Two samples were then dried at 105 °C for 24 hr. The two other samples were washed with 50 ml hydrochloric acid (37%), followed by ultra-pure water in order to remove the biofilm from the growth bodies and then also dried at 105 °C for 24 hr. The difference in weight between both sample sets, treated with and without acid, was used to calculate biofilm mass grown on growth bodies.

3.4.5.2 Algal growth inhibition test

To investigate adverse effects on primary producers an algal growth inhibition test was performed according to DIN EN ISO 8692 (2004) with 24 hr composite samples (Table 7.1-3). Briefly, WWTP samples were homogenized by shaking and, after sedimentation for about 30 min, the supernatant was analyzed in the biotest. The influence of the samples on algal growth was investigated at four different dilutions (corresponding to a waste water proportion of 12.5, 25, 50 and 80%) for each sample in triplicate. The control samples were tested in 6 replicates. The dilutions were prepared with specific concentrated test medium, waste water and water ratios. The test using green algae Desmodesmus subspicatus was performed in microplates with a modified medium with less growth promoting effects compared to the DIN EN ISO 8692 medium. In the used test medium according to Altenburger et al. (2010) the nitrogen and micronutrient contents remained unchanged, only the phosphate buffer was markedly increased (KH2PO4 from 1.6 mg/l in the ISO medium to 181 mg/l in the modified medium; K2HPO4 from 0 mg/l to 117 mg/l). The assessed endpoint is the growth rate over an incubation period of 72 hr measured as fluorescence in 24-well microplates using the multimode microplate reader TECAN Infinite® M200 (TECAN, Männedorf, Switzerland) (excitation wavelength 440 nm, emission wavelength 685 nm). The growth rate was tested for significance as described in the statistics part.

3.4.6 Analysis of estrogenic and androgenic activity

The enriched and aqueous samples (samples without enrichment) were analysed as described in the committee draft of ISO/CD 19040-2 (2016) using the validated Arxula adeninivorans yeast estrogen and androgen screens (A YES / A-YAS, new_diagnostics, Freising, Germany) in duplicate with a variability of residuals of 5.1% (A-YES) and 4.2% (A-YAS) indicating a high robustness of the assay. Four hundred µl of sample as well as blanks, quality control and standards were pipetted into 96-deep-well plates (2.2 ml, Ratiolab, Germany) and 100 µl of supplied yeast cells, suspended in yeast minimal maltose medium (YMM) were added. The 96-deep-well plates were incubated for 20 hr at 30 °C on an orbital shaker at 750 rpm (Virbramax 100, Heidolph Instruments, Schwabach, Germany). After incubation, plates were centrifuged (900xg, GS-15R, Beckman Coulter, Krefeld, Germany) for 20 min to separate the cells from the supernatant. Fifty µl of supernatant were transferred into a 96-well microtiter plate, 50 µl 3.5 mM p-nitrophenyl phosphate in 0.1 M sodium citrate buffer (pH 3.9) were added and plates incubated for 60 min at 37 °C. Subsequently, 100 µl 3 M sodium hydroxide were added to stop the reaction and optical density measured immediately at 405 nm (TECAN Infinite® M200, Tecan Group Ltd., Maennedorf, Switzerland). Cell growth was measured by re-suspending the centrifuged cells and diluting 30 µl of cell suspension with 270 µl water. The optical density was measured at 620 nm. The results for the enriched samples were extrapolated by the respective enrichment factor of 250. Further, for blank measurements of the aqueous samples only YMM without yeast cells was simultaneously determined. This was undertaken to check for possible growth of bacteria, which might influence optical measurements. The optical absorbance was measured with a multiwell plate reader (TECAN Infinite® M200, Tecan Group Ltd., Maennedorf, Switzerland). Data evaluation was conducted using the software BioVal® (QuoData GmbH, Dresden, Germany). A calibration was performed in each test to calculate respective equivalent concentrations with 17β-estradiol and dihydrotestosterone as reference compounds for the A-YES and A-YAS respectively. Calibration levels were prepared from 1.5 to 150 ng/l E2 for the A-YES and from 10 to 350 ng/l DHT for the A-YAS. From each calibration level 400 µl together with 100 µl yeast cell suspension, as mentioned before, were pipetted in duplicate into the 96-well-plate to obtain a sigmoidal dose response curve (A-YES / A-YAS, New Diagnostics, Freisingen, Germany). Additionally, blank and quality control samples were analysed in parallel to the real water samples for the whole analytical procedure including the sample pre-treatment steps. Quality control samples (1 l LC-MS water) were spiked with 17β-estradiol (15 ng/l) or DHT (60 ng/l) for the A-YES or A-YAS assay, respectively.

3.4.7 Determination of cytotoxicity and genotoxicity

3.4.7.1 Cytotoxicity assessment in the MTT assay

The enriched samples were diluted to a concentration factor of 25 and 2.5, respectively, and examined for cytotoxic effects utilizing liver cancer cells (Hep-G2)) and Chinese hamster ovary cells (CHO-9) (both provided by ECACC, UK) in the MTT assay according to Mosmann (1983). Both cell lines were cultured at 37 ± 1.5 °C, 5 ± 0.5% CO2 and 95% humidity. CHO-9 cells were cultured and treated afterwards in HAM's F12 medium supplemented with 10% fetal calf serum (FCS), 0.5% gentamycin, and 0.5% L-glutamine. Whereas Hep-G2 cells were cultured and treated in Eagle's minimum essential medium supplemented with 10% FCS, 0.5% gentamycin, 1% L-glutamine, and 1% non-essential amino acids. 10.000 cells in 200 µl medium were seeded in well-plates and after 24 ± 1 hr exposed to enriched samples or control solutions in a 1:10 dilution (negative control: culture medium; positive control: 25% DMSO; water control: 10% cell culture water) in triplicate (CHO cells) or duplicate (HEP-G2 cells). Additional to control the SPE step a solvent blank of the SPE were performed in parallel to a solvent blank without SPE. After 24 hr exposure, cells were stained with MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and subsequently lysed for photometrical determination. The optical absorbance was measured with a Tecan Spark plate reader at 595 nm (Tecan Group Ltd., Maennendorf, Switzerland). The viability of the cells was determined in relation to the negative control and classified based upon a cytotoxicity scale (DIN EN ISO 10993-5: 2009).

3.4.7.2 Genotoxicity assessment in the alkaline comet assay

For detection of DNA damage in single CHO-9 and Hep-G2 cells, induced by enriched samples, the alkaline comet assay was applied as described by Bielak et al. (2015) with slight modifications. Therefor, 100,000 cells were seeded in 2 ml of the above described modified HAM's F12 medium. After 24 ± 1 hr of cell growth and another 24 hr exposure to 25x (Hep-G2) or 2.5x (CHO-9) enriched samples in a 1:10 dilution, cells were re-suspended, mixed with low melting agarose and transferred to agarose mini gels. After incubation of the gels in lysis solution at 4 °C overnight, and subsequent incubation in cooled alkaline electrophoresis solution, electrophoresis was performed (20 min, 300 mA, cooled) followed by neutralization and fixation of gels. The dried gels were stained (SYBR® Safe DNA Gel Stain, Thermo Fisher Scientific, USA), fixed on microscopic slides and manually evaluated under a fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). For each sample 50 data points were measured per sample in one replicate. Olive Tail Moments (OTM) with standard error of mean (SEM) were determined using the Comet Assay 4 software (Perceptive Instruments Ltd., Bury St Edmunds, UK).

3.4.7.3 Genotoxicity assessment in the micronucleus (MN) test

For the detection of DNA damage (DNA breakage and aneuploidy) in dividing CHO-9 and Hep-G2 cells, induced by enriched samples, the cytokinesis-block micronucleus assay (CBMN) was applied according to the OECD-guideline 487 (OECD, 2010) with some modifications. Cells were seeded at a density of 150,000 per 5 ml medium on microscope slides in duplicate and incubated overnight. Thereafter, the medium was replaced by exposure medium consisting of medium, Cytochalasin B, and 25x (Hep-G2) or 2.5x (CHO-9) enriched sample or 10% water as negative control and 1 µg/ml Mitomycin C as positive control. After 24 hr the exposure medium was replaced by fresh medium containing Cytochalasin B and cells were incubated for another 20 hr.

Subsequently, the slides were treated with a KCl solution and transferred first to a phosphate-buffered saline (PBS)/acetic acid/methanol solution, and then to acetic acid/methanol to fix the cells. After fixation, slides were stained with 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), washed with water and air dried. For analysis of induced MN a fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany) was employed. With CHO cells two independent tests were run (= 2 biological replicates), with Hep-G2 cells one test was performed. The results were recorded as number of MN per 1000 binuclear cells.

3.4.8 Statistics

The viability of the cells using the MTT assay was determined in relation to the negative control and classified based upon a cytotoxicity scale (DIN EN ISO 10993-5 2009). The significance of DNA damage by the alkaline comet assay as well as the number of micronuclei in the micronucleus test was calculated using a non-parametric analysis of variances (ANOVA) with a Kruskal-Wallis-test as post hoc test (Graph Pad Prism 6, GraphPad Software, Inc., La Jolla, CA, USA). Statistical data evaluation for the A-YES and A-YAS for growth correction, outliers and sigmoidal curve fitting based on a four-parameter logistic function were performed using the software BioVal® (QuoData GmbH, Dresden, Germany). The growth rate for algal inhibition was tested for significant differences between controls and treatments using a Student t-test ($p=0.05$) if normal distribution and variance homogeneity was fulfilled, otherwise the non-parametric Mann-Whitney test was used.

3.5 Results and Discussion

3.5.1 Investigation of the biological post-treatment

To evaluate the influence of an additional weir in the O_3 reaction basin, which was installed to reduce the risk of O_3 entrance into the fluidized bed reactor, simulations were conducted prior to installation. The results of the simulation showed a higher minimal retention time compared to the setup with a single weir and two mixing areas and therewith a minimal risk of O_3 entrance into the biological post-treatment (Figure 3-1).

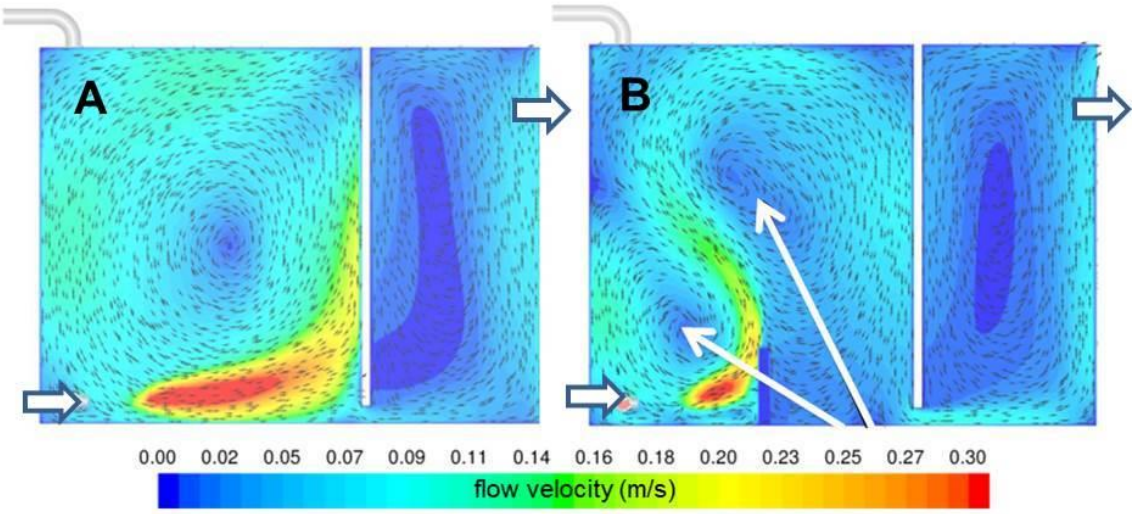

Figure 3-1: Simulated hydraulic conditions without (A) and with (B) an additional weir. The two arrows indicate the two enhanced mixing zones by the weir. The flow velocity [m/s] is indicated in colour from blue (low velocity) to red (high velocity). The flow direction is from left to right as indicated by the arrows.

To verify the simulation results in the operating process, the biofilm mass in both lines was measured. A significant biomass development of 8-10 mgbiomass/ggrowthbody in both lines was detected (Figure SI 7.1-2). In prior investigations without a weir it was assumed that lower biofilm formation would occur due to O_3 entrance into the biological post-treatment (Gruenebaum et al. 2014). Together with the predictions from the simulations it may be concluded that O_3 is not likely to enter the biological post-treatment after reconstruction, which would result in an inhibition of biofilm growth.

3.5.2 Algal growth inhibition test

Both analyzed 24 hr composite sample series showed a significantly increased algal growth of 10 - 25% at lower dilutions of 12.5 and 25% WWTP samples compared to negative control (Figure 3-2).

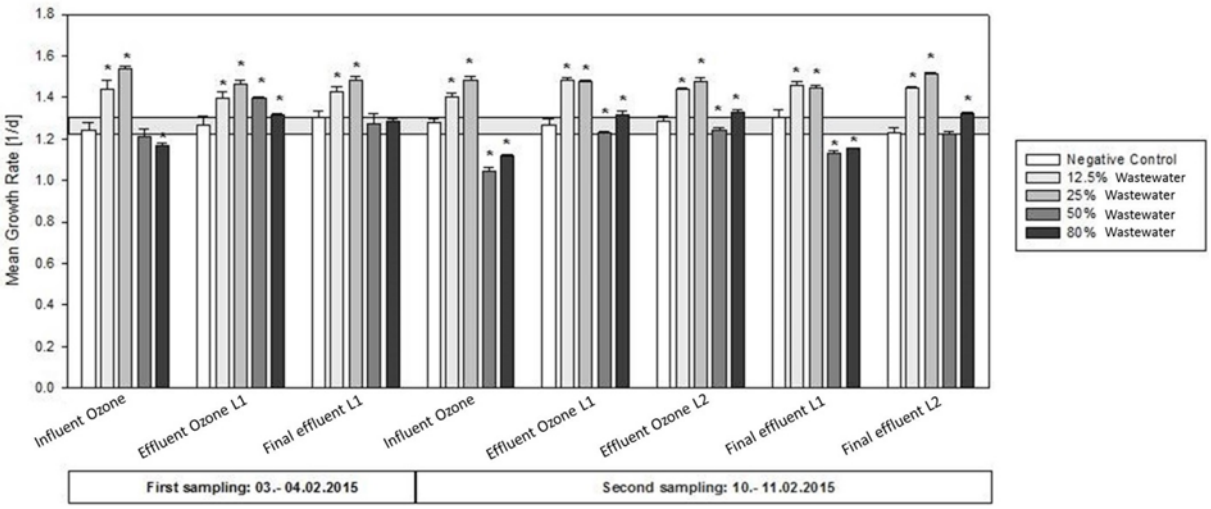

Figure 3-2: Algal growth (mean growth rate, 1/d) of the green algae Desmodesmus subspicatus in two 24hr composite sample series tested in different waste water dilutions. The stars indicate a significant difference compared to the negative control ($p < 0.05$). Columns indicate means; error bars = standard deviation; n = 3 (samples), n = 6 (controls). L1 and L2 indicate two different lines in the ozone treatment.

Evidence indicates that potential adverse effects of pollutants might be overcompensated by the presence of nutrients or other stimulating compounds in the sample, which enhance algal growth (Walsh and Bahner 1980, Abdel-Hamid and Shaaban-Dessouki 1993, Altenburger et al. 2010, OECD 2011). With higher waste water ratios up to 80% a rise of algae growth was not observed, supporting the hypothesis of interferences between nutrients and toxic compounds in the sample. In the first sample series only the less diluted samples of 80% O_3 influent waste water showed a significant inhibition in algal growth of 6 % compared to negative control. In the second sample series both, the O_3 influent and final effluent samples of line one, exhibited a significant growth reduction of 11 - 18% in the two least diluted samples (50 and 80% WWTP fractions). The effluent samples of O_3 line one and two displayed a significant inhibition of approximately 3% by 50% WWTP fractions and a numerically increased growth in the 80% WWTP fractions. In contrast, the final effluent of line two showed no marked inhibition in any dilution. Overall, this test demonstrated low algae growth inhibition in the O_3 influent, and a further reduction of the inhibitory effect after O_3 treatment. In other research studies of Thalmann et al. (2015) and Schlüter-Vorberg et al. (2015) an increased toxicity to green algae after ozonation of Ag2S-spiked effluents or ozonation of acyclovir was found. Prior investigations on the same plant as in our study by Schmidt et al. (2014) were also reporting algal growth inhibition after ozonation. Other studies demonstrated that ozonation treatment can reduce waste water toxicity to green algae (Margot et al. 2013, Escher et al. 2009). Results of the present study, indicate as well that a formation of more toxic compounds in terms of algae growth after O_3 treatment may be

excluded. However, this needs to be monitored in further sampling campaigns to attain more statistically consolidated data.

3.5.3 Pharmaceuticals and industrial chemicals

Seven selected compounds (listed in table 3-1), which were assessed by one LC-MS/MS method, were used as model compounds for efficiency analysis of the ozonation unit. This strategy was also suggested by the Swiss Federal Office for the Environment (FOEN) based upon data from previous screening analyses by Götz et al. (2010; 2011). The detected substances are typically found in high concentrations in municipal waste water and range from substances being less reactive with O_3, such as 1H-benzotriazole, which is used as anti-corrosion agent in dishwashers (Giger et al. 2006), to fast reacting compounds such as diclofenac which is widley used as analgesic agent (Boelsterli 2003). The results of the investigated pharmaceutical compounds showed high elimination rates up to 97% after ozonation and no significant further elimination after biological post-treatment (Figure 3-3).

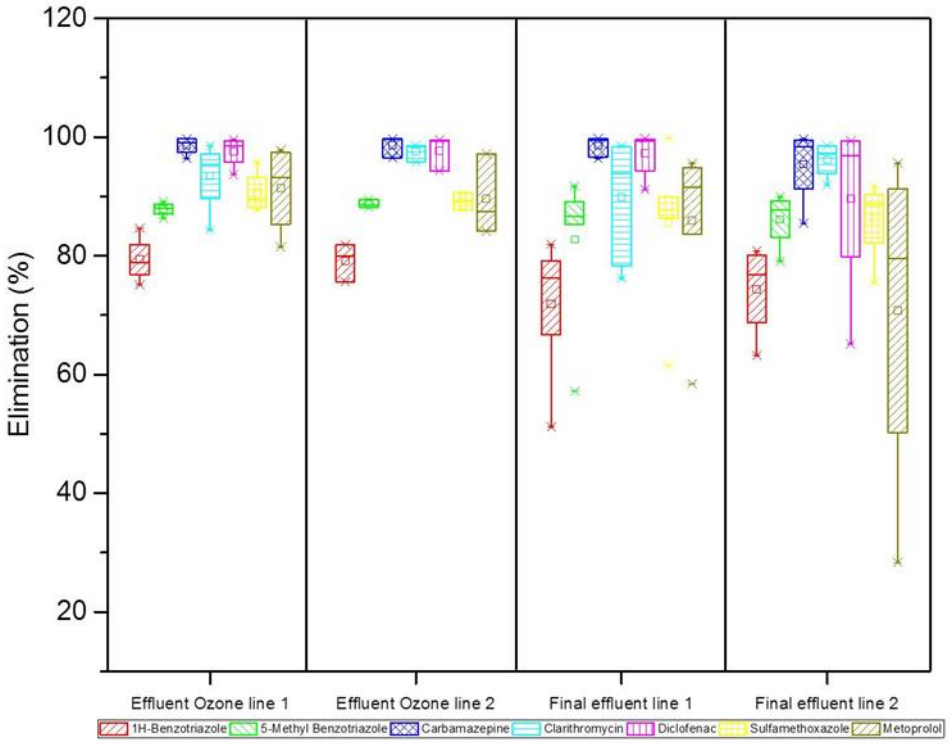

Figure 3-3: Elimination rates compared to the ozone influent (%) of 7 micropollutants after ozonation and the whole treatment including the biological post treatment measured during the whole project, respectively (n = 7). The median is symbolised by the square and the mean by the horizontal line inside the boxes. The upper and lower limit of the boxes are the 25 and 75 percentile. The whisker indicate the interquartile range (IQR) by a coefficient of 1.5 of the box.

The highest elimination was observed for carbamazepine, clarithromycin and diclofenac. Sulfamethoxazole, metoprolol, 5-methylbenzotriazole, and 1H-benzotriazole exhibited numerically lower elimination rates around 80%. The average elimination rate for substances noted was 87 ± 2% with zspec. = 0.38 mg_{O3}/mg_{DOC} which is in agreement with Hollender et al (2009) where similar specific O_3 doses (zspec. = 0.4 mgO3/mgDOC) were applied. In a prior research project on the same plant before the reconstruction, a much higher zspec. of 0.6 - 0.8 mg_{O3}/mg_{DOC} was needed to achieve comparable elimination rates of the measured substances (Gruenebaum et al. 2014). The better efficiency in the current study may be attributed to the additional weir and more efficient mixing and thereby an improved contact of O_3 with compounds in the waste water. With a mean micropollutant elimination of 80% this plant is within the elimination rates required in Switzerland (GschV 814.201: 2016), where for the most part the same substances with good and moderate elimination rates were selected (Abegglen and Siegrist 2012). Significant differences of elimination rates in line one and two were not detected. However, high variability in the elimination rate measurements was observed which is due to daily variations of the waste water influent during the 24 hr composite sampling and the sampling period which spanned two months.

3.5.4 Endocrine disrupting compounds (EDC)

The natural estrogenic hormone E2 and its active metabolite E1 were not detected in LC-MS/MS analysis above their respective LOD (Table SI 7.1-4). E3, EE2 and DES were the only estrogenically active compounds measured in some of the samples. As the method for the determination of estrogenic and androgenic active compounds was not optimised for waste water samples and especially the reduction of matrix interferences, an interpretation of the results has to be done with caution as well as a correlation to the bioassay results is difficult.

In general, E3 concentrations were found to be significantly higher compared to those of E1 and E2 in raw waste water, with ratios of E3 from 0.2 to 8.14 compared to the sum of E1 and E2 (Liu et al. 2015). One reason for higher E3 levels are the higher excretion rates by human faeces compared to E1 and E2 (Kostich et al. 2013). WWTP effluent concentrations of E3 of some research studies are found to be lower compared to E1 (Valitalo et al. 2016; Margot et al. 2013). In contrast in our study E3 was detected in only 2 sample sets with concentrations of 9.4 and 13.3 ng/l and E1 as well as E2 were below the LOD of 0.2 and 0.02 ng/l respectively. E3 was assumed to be formed by aerobic and anaerobic biological transformation processes via E1 during the conventional treatment processes which might also explains the higher levels of E3 detected in our study (Czajka & Londry 2006). However, more measurements as well as an optimized chemical analysis method regarding reduction

of matrix interferences and more sensitivity are necessary for detailed investigations of estrogens. The estrogenic activity measured with the A-YES was eliminated by up to 92% after total advanced treatment by O_3 and biological post-treatment (Figure 3-4).

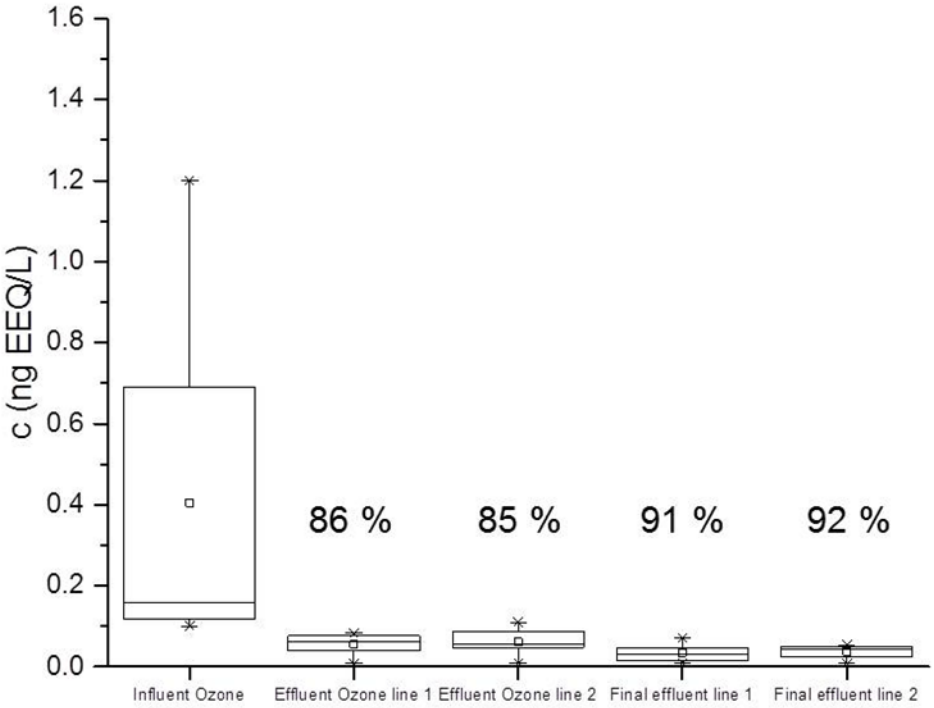

Figure 3-4: Estrogenic activity (ng DHTEQ/l) measured in six 24 hr composite samples in the A-YES. The values in percent indicate the reduction of the effect (elimination) of the respective treatment step compared to the ozone influent. The median is symbolised by the square and the mean by the horizontal line inside the boxes. The upper and lower limit of the boxes are the 25 and 75 percentile. The whisker indicates the interquartile range (IQR) by a coefficient of 1.5 of the box.

The A-YES showed comparable and reliable results for each sampling, except for samples from the influent to the ozonation plant due to daily variations of waste water properties and especially the amount of waste water treated during the 24 hr sampling. Comparing measured EEQ of prior investigations, results of the O_3 influent as well as of the O_3 effluent are in a similar range of 0.27 to < 0.04 ng EEQ/l, respectively, demonstrating a stable elimination process (Schmidt et al. 2014). Also the elimination with a reduction in estrogenic activity of 86 – 92% is comparable to another study in Switzerland in which the reduction was 89 ± 4% by ozone (Margot et al. 2013). In addition, the EEQ after ozone treatment was below the trigger value (or safe concentration level of estrogenic equivalents) of 0.4 ng EEQ/l representing a concentration that should not be exceeded in order to protect the aquatic environment (Jarosova et al. 2014a, Kienle et al. 2015, Kunz et al. 2015). Comparing the WWTP effluent activities a study by Jarosova et al. (2014b) analyzed 75 WWTP effluents with EEQ levels between 0.53 – 17.9 ng EEQ/l, and a more recent study by Välitalo et al.

(2016) presented data using the ERα-CALUX of 1 ng EEQ/l in most of the investigated municipal WWTP effluents which is comparable to the activity of 0.06 to 1.6 ng EEQ/l detected in the WWTP effluent of our study.

Comparing the detection limits of the bioassay with chemical analysis by LC-MS/MS in terms of the three estrogenic compounds listed on the watch-list of the EU WFD, our LC-MS/MS method with limits of quantification between 1 and 10 ng/l (Table 3-1) depending upon the respective substances is in a comparable range with other publications (Tavazzi et al. 2016; Vymazal et al. 2015; Sapozhnikova et al. 2011). However, due to matrix interferences we observed a drop of the LOQ by approximately 5-10 compared to a measured standard. Therewith chemical analysis was not sensitive enough to be able to determine estrogenic compounds in the range of their respective EQS (e.g. 0.035 ng/l for EE2, EU 2015/495) in environmental water samples. These matrix problems are also known from another study by Valitalo et al. (2016) and should be focused in future research. In contrast, the A-YES turned out to be more sensitive without matrix interferences with a LOD of 0.032 ng EEQ/l, reaching the concentration range of the EU WFD EQS of EE2. Beside the Arxula adeninivorans yeast screens used in this study, also other available bioassays like the YES with Saccharomyces cerevisiae (Routledge & Sumpter, 1996) or ERα-CALUX with a human cell line assay were compared in a prior study by Gehrmann et al. (2016). These assays are similar in sensitivity but differ in their robustness to the waste water samples as for example waste water was toxic to the ERα-CALUX assay in some cases (Gehrmann et al. 2016; Kunz et al. 2017). Thus, effect based methods are suitable tools to screen for estrogenic activity in WWTP samples in the range of safe concentrations of estrogenic equivalents of 0.1 - 0.4 ng EEQ/l (Wu et al. 2014; Jarosova et al. 2014a; Kienle et al. 2015). In addition, due to low LOD (0.032 ng EEQ/l) of the A-YES it can be assumed that substances like EE2 would be within the proposed limits of the EU-Water Framework Directive of 0.035 ng/l if the A-YES signal is below the LOD. An approach combining chemical and effect based analysis was shown by Buchinger et al. (2013) in which thin layer chromatography was used to isolate estrogenic active compounds from real samples. Thereby in theory EE2 could be isolated and quantified by the A-YES applied in our study below the limit of 0.035 ng EE2/l. The low detection limits compared to chemical analysis makes biotests like the A-YES a useful tool to monitor WWTP effluent as well as surface water under the EU WFD with regard to EDCs even without knowing target substance concentrations; this aspect is also discussed in other studies (e.g. Kunz et al. 2015). However, the relative potencies (e.g. EE2 = 0.94, E1 = 0.06) of single substances in the bioassay compared to the reference compound need to be considered (Kaiser et al. 2010). Additionally, antagonistic activity needs to be taken into account by analyzing WWTP samples. They play an important role as measured values in the A-YES

are a sum parameter and reflects agonistic and antagonistic activities (Gehrmann et al. 2016). The measured androgenic activity is shown in Figure 3-5.

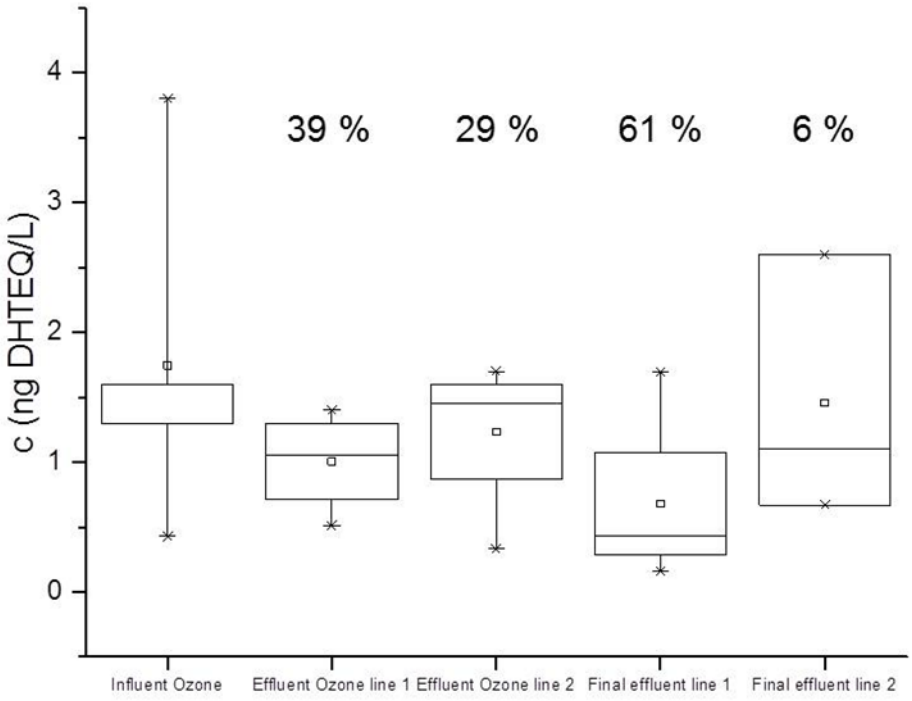

Figure 3-5: Androgenic activity (ng DHTEQ/l) measured in six 24 hr composite samples in the A-YAS, the values in percent indicate the reduction of the effect (elimination) of the respective treatment step compared to the ozone influent. The median is symbolised by the square and the mean by the horizontal line inside the boxes. The upper and lower limit of the boxes are the 25 and 75 percentile. The whisker indicates the interquartile range (IQR) by a coefficient of 1.5 of the box.

Comparing the elimination of estrogenic and androgenic activity, a lower elimination rate of androgenic activity was noted which might be due to a slower reaction of androgen active compounds with O_3. However, this needs to be investigated in more detail in further studies. Prior investigations demonstrated that O_3 depletion occurs relatively fast within less than 10 min, so this might be a possible explanation for a lower elimination (Schmidt et al. 2014). Another reason might be the higher androgen concentration in the influent, generated by 10 to 100-fold higher excretion levels compared to estrogens in human urine, leading to greater loads in waste water which was also reported by Shore and Shemesh (2003) and Chang et al. (2011). The results of the LC-MS/MS measurements (Table SI 7.1-5) displayed a higher concentration of androgens in the WWTP influent and effluent compared to estrogens especially for the most potent compounds testosterone and dihydrotestosterone. To further reduce the androgenic activity higher O_3 doses might be necessary. In this context, the formation of oxidation by-products such as bromate and N-nitrosodimethylamine (NDMA) needs to be carefully observed when applying higher O_3 doses (von Gunten and Hoigne 1994; Schindler Wildhaber et al. 2015). Oxidation by-products as well as transformation

products, which are likely formed at higher O_3 doses, were examined in prior research projects but not particularly at this plant (Schmidt et al. 2014; Lee and von Gunten 2016). Referring to a study of Brand et al. (2013), where a trigger value of 11 ng DHTEQ/l is proposed, the observations of DHT equivalents in the present study might be viewed as safe for human health. However, environmental risks that are important in case these compounds are released into surface waters via WWTP effluents are not known so far (Brand et al. 2013). Some studies investigating androgenic activities showed contradictory results to our investigation, as they did not detect androgenic activities or only low activities in waste water effluents (Neale et al. 2017; Roberts et al. 2015; Kirk et al. 2002). Kirk et al. (2002) detected androgenic activity in one out of five investigated WWTP effluents. It could be concluded that the different activities were related to the type and setup of the respective treatment plant. Compared to the A-YES, the A-YAS showed higher detection limits of 0.12 to 0.9 ng DHTEQ/l (enrichment factor of 250). As yet, no apparent trigger values or suggested threshold values are known for waste water, and thus an evaluation of potential environmental risks of measured A-YAS activity is not possible. This needs to be investigated if possible in combination with chemical analysis of androgenic compounds in future projects.

3.5.5 Cytotoxicity and genotoxicity

Cytotoxicity and genotoxicity were examined with two different cell lines in the same tests (Figure 3-6). Regarding cytotoxicity measured in the MTT assay, cell viability of the Hep-G2 cells after exposure to the 2.5 fold enriched samples was above 80% for all samples in both sampling campaigns. With regard to CHO-9 cells, the 2.5 fold enriched samples showed cytotoxic effects in the second sampling campaign (11.02.) leading to cell viability < 80%. Therefore, samples from the second sampling campaign were further diluted (0.25 fold enriched samples) to rule out cytotoxic effects as these can mask genotoxic effects (see Figure SI 7.1-4). Consequently, the resulting enrichment factors of samples for genotoxicity testing were 2.5 for Hep-G2 and 0.25 for CHO-9 cells.

In Hep-G2 cells no significant genotoxic response was induced by the enriched samples after all three treatment steps compared to the negative control after 24 hr exposure, neither in the alkaline comet assay, nor in the MN assay (see Figure SI 7.1-5 and 7.1-6). The response of CHO-9 cells was similar to that of Hep-G2 cells (Figure SI 7.1-6), except the final effluent of line two in one sampling campaign (11.02.), which demonstrated significant genotoxicity in the MN assay with CHO-9 cells compared to the negative control. Because this effect was only detected in one sample and only in effluent of one line, the test needs to be repeated with more samples to exclude a random error and to verify the results.

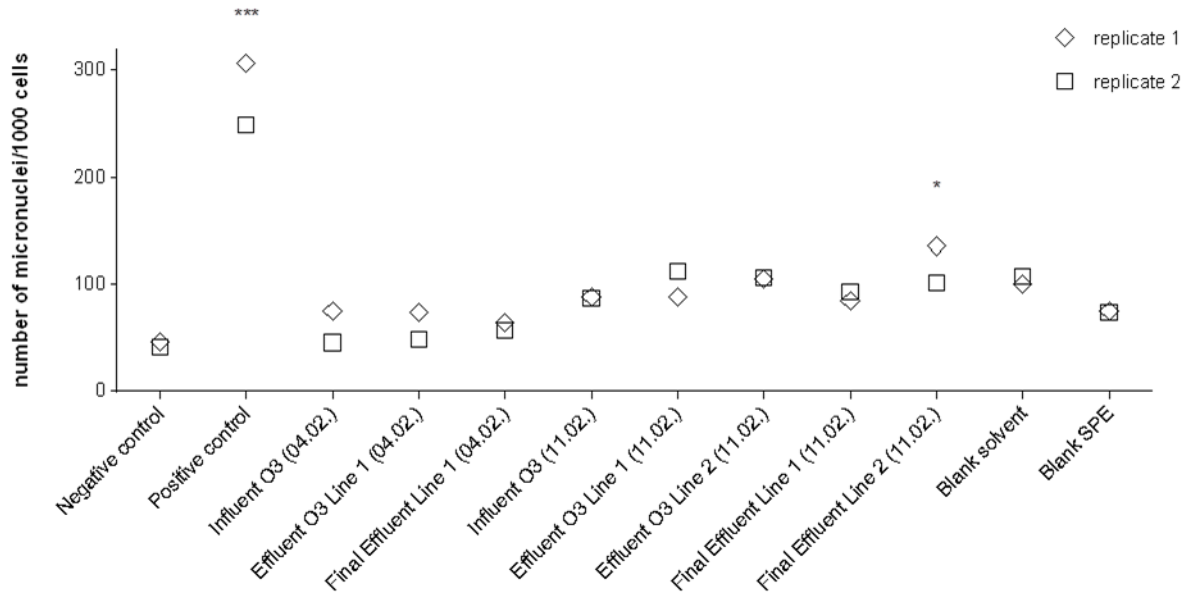

Figure 3-6: Results of the micronucleus assay with Chinese hamster ovary (CHO) cells test 0.25 fold enriched samples for the samples from the 04.02.2015 and 11.02.2015. Significances levels indicated by stars compared to negative control (*** p = 0.009, * p = 0.026). Columns indicate means; error bars = standard deviation; n = 2 replicates from independent tests (each tested in duplicate).

The findings were similar in both assays and waste water in our study may be considered as non-genotoxic before and after O3 treatment of both lines. Similar results were found in a study by Mišík et al. (2011) in which genotoxic effects were not observed after O3 treatment. The detected genotoxic potential after final treatment in the present study still has to be verified by additional investigations. Comparing the results with other studies, Maier et al. (2015) showed contrary results as a genotoxic effects in 5 of 7 WWTP effluent samples were observed. In addition another study by Magdeburg et al. (2014) showed, when applying O3 as advanced treatment genotoxic effects were increased depending on the applied O3 dosage but further reduced after a sand filtration. Prior investigations on the WWTP Duisburg-Vierlinden did not show relevant genotoxic effects in the O3 effluent suggesting that no significant genotoxic transformation products from ozonation were formed using this ozonation setup (Schmidt et al. 2014). Thus, the formation of more toxic transformation products by O3 is not expected at this plant, but considering the variations between both sampling campaigns, cell lines and different results in other publications, further monitoring seems reasonable.

3.6 Conclusions

In this study a toxicity-based approach combining chemical and (eco)toxicological analysis was applied to evaluate a full-scale O3 unit. The results showed no marked cytotoxic effects by ozonation and simultaneously high elimination rates of pharmaceuticals, industrial compounds and estrogenic activity, suggesting a successful setup of the ozonation. Currently, chemical analysis of estrogenic substances is often not sensitive enough to detect EDC in the required range, bioassays such as the A-YES represent one possibility to overcome this limitation. Reaching a LOD of 0.032 ng EEQ/l by using the A-YES, effect-based test systems are one option to monitor estrogenic activity even within the proposed environmental quality standard of 0.035 ng/l for EE2 in WWTP effluents. However, antagonistic effects need to be taken into account and more investigations regarding antagonistic and mixture effects are necessary in future studies. Androgenic activity, on the other hand, was not significantly reduced and needs to be investigated in more detail in the future. Overall, this study successfully demonstrated a possible approach combining effect based methods as well as chemical analysis to evaluate advanced waste water treatment for micropollutant elimination with regard to sustainable water protection.

3.7 Acknowledgments

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4. Comprehensive analysis of antagonistic endocrine activity during ozone treatment of hospital waste water

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4.1 Abstract

To reduce the discharge of micropollutants, advanced waste water treatment methods were investigated in the last years. Estrogenic effects were found to be reduced by ozonation. These activities are usually measured using genetically modified cell-based tests. As these bioassays are representing a sum parameter, also inhibitory effects such as antagonistic effects need to be further investigated as they are potentially reducing the detected activities. Therefore, a direct comparison of chemical target analysis and biological equivalent concentrations measured by bioassays is often difficult. To investigate the fate of antagonistic activities and their role in mixtures with agonistic activities, two hospital waste water treatment plants were studied after different treatment steps. Thereby highly enriched samples were analyzed by a combination of bioassays with chemical target and non-target analyses. In order to achieve an in-depth characterization of the antagonistic activities a fractionation of the enriched samples was performed. To identify relevant compounds an effect directed identification approach was used by combining high-resolution mass spectrometry and bioassays. The results showed a high reduction for estrogene and androgene activities. However, a constant antagonistic activity after membrane bioreactor and ozone treatment was observed. A reduction of the antagonistic activity was observed after passing an activated carbon filter. The fractionation approach showed a specific finger-print of each sample of the different treatment steps. Hereby we could show that the composition of agonistic and antagonistic active compounds is changing after each treatment step while the overall measured activity stays the same. Using fractionation and the combination of bioassays the number of relevant features detected by chemical non-target screening could be reduced by more than 85%. As a result, the phosphorous flame retardant TCEP could be identified as anti-estrogene active. Future research should be done to identify more antagonistic active compounds and potentially active transformation products after ozone treatment.

4.2 Keywords

Hospital waste water, ozone, anti-estrogenic, anti-androgenic, effect directed analysis, non-target screening

4.3 Introduction

The increased use of chemicals which include industrial chemicals, pharmaceuticals & personal care products (PPCP), biocides, X-ray contrast media and pesticides leads to a relevant discharge of those trace substances (micropollutants) into surface waters. Besides municipal waste water, one point source is hospital waste water, which is released into the sewage system and treated in municipal waste water treatment plants (WWTPs) as well. Since conventional WWTPs are not designed for a targeted removal of micropollutants, they get discharged into receiving surface water in ng/L to µg/L range (Bergmann et al., 2011; Hillenbrand et al., 2016; Gabet-Giraud et al., 2014). Micropollutants could have several cytotoxic, genotoxic, immunotoxic as well as endocrine disrupting effects on aquatic organisms, which were shown in several studies on surface water influenced by WWTP effluents (Kidd et al., 2007; König et al., 2017; Liu et al., 2017; Masteling et al., 2016; Neale et al., 2015). One important group of micropollutants are endocrine disrupting chemicals, which are naturally occurring, such as estrogens and androgens from human excretions, or of anthropogenic origin such as the contraceptive agent 17α-ethinylestradiol. Furthermore, a variety of compounds not specifically designed to act as an endocrine disruptor like phthalates, phosphorous flame retardants, biocides, industrial contaminants, anti-inflammatory as well as non-steroidal anti-inflammatory drugs and antiarrhythmic agents were found to be able to interfere with the human estrogen or androgen receptor (Ezechias et al., 2016; Ma et al., 2017; Shi et al., 2016; Yang et al., 2016; Zhang et al., 2014). Also besides endocrine effects, many different compounds are biological active in different ways and are even not typically quantified by chemical analysis which makes a bioanalytical test battery an important supplement (Neale et al., 2017; Tousova et al., 2017, Wernersson et al., 2015). Especially endocrine disrupting effects on organisms, like the feminization of male fish due to higher vitellogenin production, were detected at WWTP effluents as well as in surface waters in several studies in the last decades (Chen and Chou, 2016; Kidd et al., 2007; Peschke et al., 2014; Valitalo et al., 2016).

To minimize the discharge of micropollutants new advanced treatment technologies are needed. Therefore, first pilot and full-scale applications are tested and implemented in several research projects of municipal as well as hospital waste water treatment plants (Antoniou et al., 2013; Audenaert et al., 2014; Knopp et al., 2016; Miralles-Cuevas et al.,

2017; Nielsen et al., 2013; Verlicchi et al., 2015). The “PILLS” project showed the effectivity of advanced treatment by ozonation of hospital waste water as a point source (Boehling et al., 2012; Pills Project, 2012). Ozonation as well as the use of activated carbon are the most discussed and tested techniques (Prasse et al., 2015). Furthermore, there is a need to reduce micropollutant loads to achieve a good chemical status by 2027 as regulated by the European Water Framework Directive (EU 2000/60; EU 2009/90; EU 2013/39). Additionally, three estrogenic active compounds are listed for the first time in a “watch-list” and should be monitored to possibly be added to the list of priority substances of the European water framework directive (EU WFD). These compounds are the naturally occurring substances estrone (E1) and 17ß-estradiol (E2), as well as the synthetic contraceptive agent 17α-ethinylestradiol (EE2) with required limits of detection of 0.4 ng/L (E1, E2) and 0.035 ng/L (EE2) by the use of liquid chromatography coupled to mass spectrometry (EU 2015/495). The demanded limits of detection (LOD) are very challenging and methods need to be developed to overcome matrix effects to achieve the required LODs in real water samples (Hettwer et al., 2018; Itzel et al., 2017; Välitalo et al., 2016). An alternative could be the use of effect based methods such as the yeast cell based estrogen screening assays or the chemical activated luciferase gene expression assay (CALUX). Compared to chemical analysis, effect based bioassays have lower limits of detection and are within the required range of the EU WFD (Gehrmann et al., 2016). However, effect based methods represent a sum parameter characterizing a water sample with regard to its estrogenic or androgenic activity by an equivalent concentration and it is not possible to get the concentration of the respective single compound. Furthermore, it is of high importance to know that the applied bioassays are genetically modified cells with a human estrogen or androgen receptor respectively. Estrogen or androgen active compounds are able to bind to these receptors and result in a formation of a target enzyme, which can be photometrically detected by an enzyme-substrate reaction. Calibration of the bioassay is done by using a standard (e.g. E2 for estrogen activity) for calculation of a biological equivalent concentration (e.g. EEQ for 17ß-estradiol equivalent concentration). This mode of action is called agonistic activity as this is promoting the formation of a target enzyme. Additional to the agonistic activity, inhibitory effects, also called antagonistic activity, can occur by compounds which bind to the same receptor but instead of promoting an enzyme expression, they block the receptor which results in an inhibition of the enzyme transcription and consequently lower the detected signal (Shi et al., 2016).

To correlate measured activity with detected known endocrine disruptors and to understand the fate of endocrine effects during waste water treatment, it is important to investigate inhibitory antagonistic effects as well, as they are reducing the overall measured activity of a sample. A study by Ihara et al. (2014) showed the importance of antagonistic effects if

predictions by chemical analysis are made. In their study, they found always lower activities using a reporter gene assay (HEK 293 cells with luciferase gene as reporter vector) compared to the predicted activity calculated by known estrogens which were measured by chemical analysis. This could be partly explained by compounds acting antagonistic and are reducing the overall endocrine activity. Compounds responsible for these antagonistic effects can be target compounds developed for this effect. One example is the breast cancer therapy drug tamoxifen, which is acting as an estrogen receptor antagonist (Kiang and Kennedy, 1977) or flutamide acting as an androgen receptor antagonist (Simard et al., 1986). Other substances designed for a completely different application like flame retardants (Zhang et al., 2014), some pesticides (Orton et al., 2012) and even non-steroidal anti-inflammatory drugs such as diclofenac or ibuprofen (Ezechias et al., 2016) are able to interfere and act competitively with the estrogen or androgen receptor. This makes it even more complex to correlate effects with specific compounds in complex matrices like waste water. The environmental relevance of antagonistic-acting endocrine compounds has been shown in a few studies of WWTP effluents and surface waters in the last years in which aquatic organisms showed a disruption of their endocrine system (Jobling et al., 2009; Matthiessen and Weltje, 2015; Rao et al., 2014). A recent study by Watermann et al. (2016) revealed an effect by anti-androgens on reproductive organs of the copepod Acartia tonsa. The high diversity of substances acting antagonistically in addition to the more studied agonistic acting compounds, which are summarized by Kiyama and Wada-Kiyama (2015), makes it even more complex to identify relevant compounds by chemical target analysis. Therefore, it is of increasing importance to use effect-based analysis in combination with the classical approach by chemical analysis to find and identify antagonistic effects and relevant substances (Kunz et al., 2015, Neale et al., 2017). For this reason, new methods need to be applied with a combination of target and non-target chemical analysis together with effect-directed analysis (Di Paolo et al., 2016). Different approaches of comprehensive analysis for agonistic effects are studied in the last years and the use of bioanalytical tools for a screening is highly recommended (Brack et al., 2016; Chen et al., 2016; Di Paolo et al., 2016; König et al., 2017; Neale et al., 2015; Tousova et al., 2017, Wernersson et al., 2015).

The aim of this study was to investigate antagonistic effects in comparison to agonistic effects during advanced waste water treatment of hospital waste water using effect-based and chemical analysis. Based on prior research by Gehrmann et al. (2016) in which anti-estrogenic as well as anti-androgenic effects showed no significant reduction during different treatment stages, samples were taken from the same German hospital WWTP as well as from a Danish hospital WWTP to compare activities and different treatment procedures. The two WWTPs make it possible to study the effect after each treatment step due to the sequence setup (MBR, ozone, GAC/UV). In this study, antagonistic effects detected by

bioassays with an estrogen-/androgen-receptor were additionally analyzed using a fractionation approach by liquid chromatography. Therefore, highly enriched samples were analyzed before and after a fractionation to get a more detailed view of the antagonistic effect. Additionally, chemical target analysis, suspect screening and non-target screening were done regarding activity of fractions to reduce the amount of relevant features from high-resolution mass spectrometry (HRMS) measurements and to identify potentially responsible compounds for observed antagonistic effects.

4.4 Materials and methods

4.4.1 Chemicals and standards

The standards estrone (E1), 17β-estradiol (E2), 5α-dihydrotestosterone (DHT), etiocholanone, trenbolone, nandrolone, tamoxifen, 4-hydroxy-tamoxifene E/Z (4-OH-TAM), cyproteron acetate, flutamide, tris(2-chloroethyl)phosphate (TCEP), amidotrizoic acid, bisoprolol, ciprofloxazin, ibuprofen, gabapentin, metronidazole, phenanzone, 5-Methyl-benzotrialzole, carbamazepine, clarithromycine, diclofenac, sulfamethoxazole, hydrochloric acid (HCl), sodium hydroxide (NaOH) and formic acid (FA) were purchased from Sigma Aldrich Chemie GmbH (Taufkirchen, Germany). Testosterone, boldenone and ammonium fluoride were obtained from Fluka™ Honeywell (Germany). 17α-Ethinylestradiol (EE2) and boldione were from Riedel-de Häen™ Honeywell (Schwerte, Germany), water and methanol (LC-MS grade) from Th. Geyer GmbH & Co. KG (Renningen, Germany), methyl-tert.-butylether (MTBE) from LGC Promochem GmbH (Wesel, Germany). The substrate p-nitrophenyl phosphate from AppliChem GmbH (Darmstadt, Germany) and tri-sodium citrate dehydrate as well as citric acid monohydrate were purchased from Carl Roth GmbH & Co. KG (Karlsruhe, Germany). CAS number, limit of detection as well as limit of quantification of target substances are listed in Table SI 7.2-1.

4.4.2 Waste water treatment plant description

The two investigated hospital waste water treatment plants are the Marien-Hospital in Gelsenkirchen (Germany) and the Herlev-Hospital in Herlev (Denmark). Both plants are treating exclusively hospital waste water by membrane bioreactors (MBR) followed by a polishing with ozone and a post-treatment. The Marien-Hospital has approximately 580 beds and 75,000 patients per year. The maximum inflow is 25 m^3/hr with an average inflow of 200 m^3/day. As the ozonation was performed in bench-scale ozone concentrations are not mentioned here. The Herlev-Hospital consists of approximately 700 beds and an average inflow of 410 m^3/day. The ozone dosage at the Herlev-Hospital was set to 21 mg/L by a

dissolved organic carbon (DOC) concentration of 12 mg/L resulting in a zspec of 1.75 mg_{O3}/mg_{DOC}. Each of the three installed granular activated carbon (GAC) filters has a bed volume of 3.1 m^3 and as the density of GAC is 470 kg/m^3, the resulting amount of GAC per filter is 1,460 kg. At a last treatment step for biological disinfection UV is applied at the Herlev-Hospital WWTP. The UV reactor has a volume of 20 L and a flow rate of 10-15 m^3/h at 220 W and 5-10 mJ/cm^2.

4.4.3 Ozonation in bench-scale

MBR samples (5 L) of the Marien-Hospital were treated with ozone in bench-scale experiments to allow for replicates and to exclude variances according to changes of the waste water matrix. Ozone was produced using pure oxygen and an ozone generator (COM-AD-01, Anseros Klaus Nonnenmacher GmbH, Tübingen, Germany) which was set to a flow rate of 25 L/h and 4.4 g/Nm^3. The ozone gas was passed through a 5 L glass bottle by a frit to ensure a good distribution of ozone within the sample until the final concentration of 5 mg/L ozone was reached. The concentration in the gas-stream was measured optically using an ozone-in-off-gas analyzer (Off-gas System BMT 964 OG, BMT Messtechnik GmbH, Stahnsdorf, Germany). The DOC concentration of the MBR effluent was 9 mg/L, resulting in a zspec of 0.56 mgO3/mgDOC.

4.4.4 Sampling and sample preparation

Waste water samples were taken after different treatment steps of the two investigated hospital waste water treatment plants. The ozonation of samples from Marien-Hospital was done with MBR effluent samples in the laboratory within 12 h after sampling (see 4.4.2). The influent, MBR effluent and final effluent samples of the treatment plant at Herlev Hospital were collected as 24-h composite samples. The samples in between like after ozonation and after GAC were taken as random samples during the 24 h sampling. The sample for the GAC could only be withdrawn after UV treatment. In the following it is named only GAC as the applied UV-C should rather have an effect on microbiological disinfection than on micropollutant removal as it was not installed nor optimized for this purpose. Additionally, it is known from other studies that the applied UV dosage (low mJ/cm^2 range) is not efficient for elimination of micropollutants (Adams et al., 2002). All samples were stored during transportation and in the laboratory at 4 °C until further treatment.

A solid phase extraction (SPE) of the samples was done within 48 h after the sampling using hydrophilic / lipophilic balanced (HLB) cartridge (6 cc, 150 mg, Oasis SPE, Waters GmbH, Frechen, Germany). At first, the cartridges were conditioned (2 x 5 mL methanol) and

equilibrated (2 x 5 ml water). Subsequently 1 L of each sample was loaded for instrumental and effect based analysis. To prevent a clogging of the cartridge by the influent samples, a pre-filtration by a glass fiber filter (PALL Corporation, New York, USA) with a pore size of 1 µm was done. After drying the cartridges under vacuum, they were stored at -18 °C until further analyses. The elution of the SPE cartridge was done with 5 x 5 ml MTBE. The MTBE was evaporated (Evaporator Vapotherm Basis mobil I, Barkley GmbH & Co. KG, Leopoldshoehe, Germany) at 50 °C under a gentle nitrogen gas stream until complete dryness. Each sample was enriched 4 times on 4 separate cartridges to ensure enough volume and high enrichment factors for chemical and biological measurements (the exact enrichment factors are given at the specific analytical methods).

4.4.5 Detection of endocrine effects using Arxula adeninivorans yeast cell assays

The endocrine effects were investigated for aqueous and enriched samples as well as in different fractions after separation by liquid chromatography (section 2.6). The dried extracts (see section 2.4) were re-dissolved in 4 mL water leading to a nominal enrichment factor (EF) of 250. A dilution of the extracts was done in a first experiment to select the optimal resulting enrichment factor (REF). Thereby, a REF of 250 showed the best results and was used in this study. The effect based analysis was done using the validated Arxula adeninivorans yeast estrogen screen (A-YES) which was shown to be a robust bioassay and applicable for waste water samples (Hettwer et al., 2018). Both assays, the Arxula adeninivorans estrogen and androgen screen were purchased as ready to use kits (A-YES, A-YAS, new_diagnistics GmbH, Freising, Germany). The tests for agonistic effects were performed as previously described (Itzel et al., 2017). All tests were performed in triplicate. Limits of detection were 10% relative activity and 0.007 ng 17β-estradiol equivalents per liter (EEQ/L) for the A-YES and 0.2 ng dihydrotestosterone equivalents per liter (DHTEQ/L) for the A-YAS. Investigating antagonistic effects, the same test procedure was done with a different sample pre-treatment. Therefore, samples were spiked with a known amount of a standard solution. In case of antagonistic estrogenic effects (anti-A-YES), 20 µL 17β-estradiol solution (1000 ng/L) were added to samples (380 µL) inside the 96-well plate resulting in a 50 ng/L E2 concentration. To investigate antagonistic androgenic effects (anti-A-YAS) 100 µL DHT solution (800 ng/L) were added to the samples (300 µL) inside the 96-well plate resulting in 200 ng/L DHT concentration. As a reference, deionized water (LC-MS grade) was treated the same way for the anti-A-YES and the anti-A-YAS. To compare the effect of androgenic and estrogenic as well as the respective antagonistic effects the result is expressed as relative activity compared to the standard which is set as the maximum (Figure 4-1).

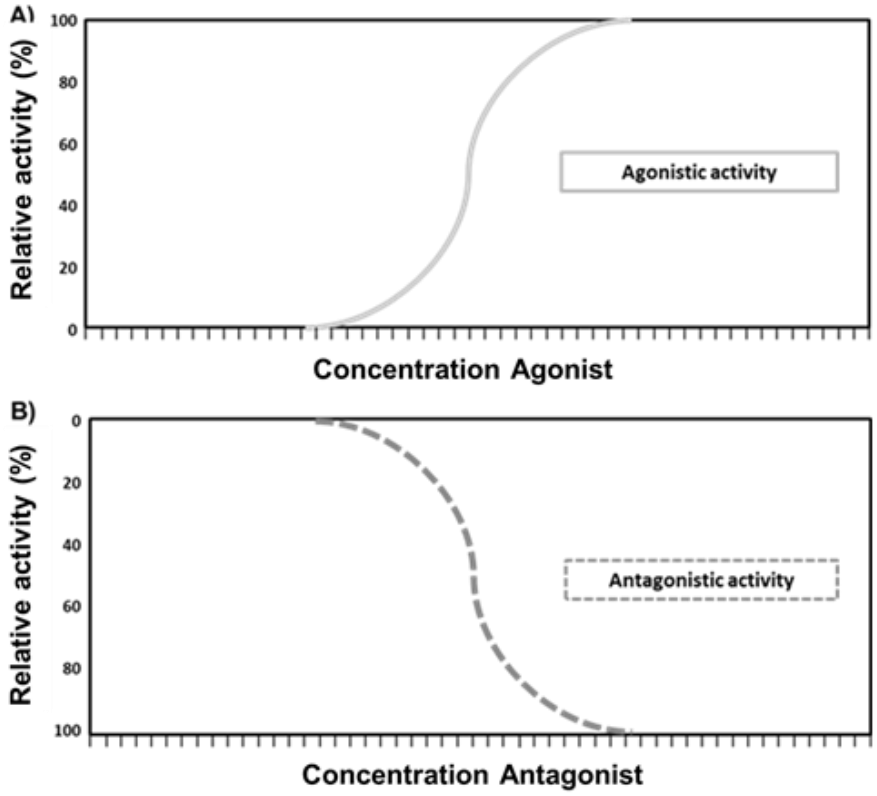

Figure 4-1: Schematic illustration of agonistic (A) and antagonistic (B) activity expressed as relative activity. Increasing agonist/ antagonist concentration from left to right.

4.4.6 Fractionation using liquid chromatography

The separation and fractionation of each sample was performed on a liquid chromatography system (Shimadzu LC-10, Deutschland GmbH, Duisburg, Germany). This instrument was equipped with two liquid chromatography pumps (Model LC-10ADvp), a degasser (Model DGU-14A), an autosampler (Model SIL-10ADvp), a column oven (Model CTO-10ASvp), diode array detector (Model SPC-M10Avp), fraction collector (Model FRC-10A) and a system controller (Model SCL-10Asp). The dried extracts (see section 2.4) obtained from each sampling point were re-dissolved in 100 µL water, leading to a resulting enrichment factor of 10,000. 40 µL of each sample were injected. For chromatography, a reversed phase C-18 column (2.5 µm 2.1 x 75 mm, xBridge™, Waters GmbH, Frechen, Germany) was applied and the solvents for the mobile phase were acetonitrile and water, each with 0.1 % formic acid. A linear gradient was set, from 20% to 99% acetonitrile over 25 min and the flow rate was set to 0.3 mL/min. The fraction collector was started with a delay of 2.5 minutes and took one fraction per minute, resulting in 0.3 mL sample for each fraction each run. To achieve a high concentration in each fraction (F) the whole procedure was repeated 4 times using 4 separate extracts of each sampling position. To estimate the retention of known substances in the method, a standard mix of different endocrine active substances (listed in 4.4.1) was injected and measured using a diode array detector at wavelength of 200-800 nm (Figure 4-

2). The fraction collector started after 2.5 minutes and the fractionation time window was set to one minute each.

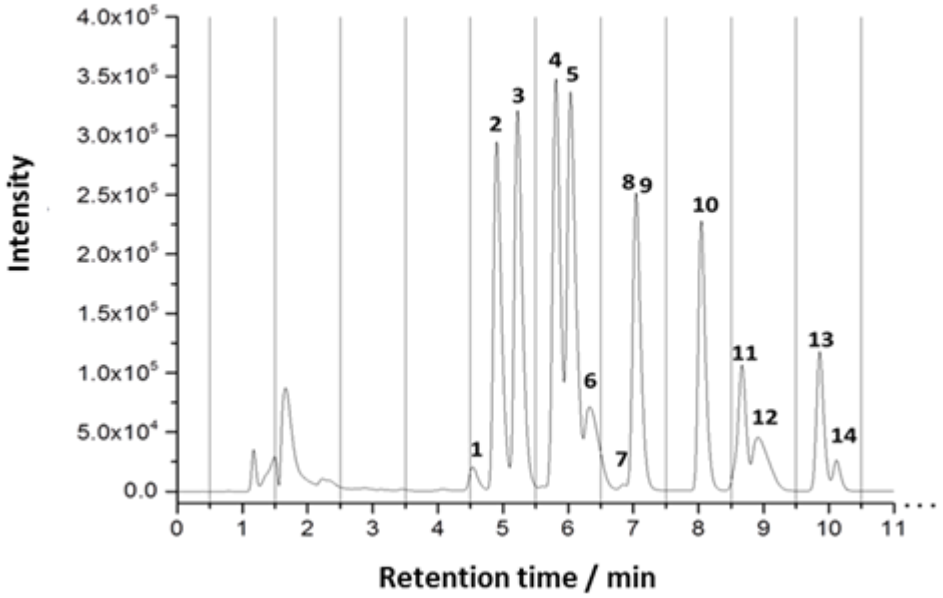

Figure 4-2: Fractionation experiment with a standard mix (14 compounds) to estimate elution of known substances. The active compounds are numbered from left to right with boldenon (1), nandrolon (2), E2 (3), boldione (4), BPA (5), testosterone (6), 4-OH-TAM (7), EE2 (8), androstendione (9), DES (10), trenbolon (11), flutamid (12), TAM (13), cyproteron acetat (14).

Single fractions (0-25) were evaporated at 60 °C to complete dryness and re-dissolved in 5 mL water (resulting enrichment factor 800). Fractions were tested again in Arxula Adeninivorans yeast cell assays (see chapter 2.5). Active fractions in the bioassay were further investigated by HRMS using target and non-target approaches.

4.4.7 Chemical analysis

4.4.7.1 Detection of endocrine substances by LC-MS/MS

The dried SPE extracts were re-dissolved in 1 mL water leading to a nominal enrichment factor of 1000. The extracts and corresponding fractions which showed a biological activity in the bioassay were further investigated using LC-MS/MS with electrospray ionization in positive and negative mode. Two different instruments and methods were used for the analysis.

The three estrogens listed in the watch-list of the WFD estron (E1), 17β-estradiol (E2) and 17α-ethinylestradiol (EE2) were measured using a LC-MS/MS system with an Agilent 1100 LC pump (Agilent Technologies Deutschland GmbH, Ratingen, Germany) and the mass

spectrometer QTRAP® 6500+ (AB Sciex Germany GmbH, Darmstadt, Germany) with electron spray ionization (ESI) in negative mode. The measured substances, respective multiple reaction monitoring (MRM) transitions, collision energies and retention times are provided in the supporting information (Table SI 7.2-2 and 7.2-3). Chromatography was done using a reversed phase C-18 column (Chromolith® FastGradient, RP-18e, 50 x 2 mm, Merck KgaA, Darmstadt, Germany). For measurement, a gradient of methanol and water (both with 0.2 mmol/L NH4F after Hindle (2013) and Baker et al., 2014) was applied over 11 min, from 20% methanol to 60% methanol within four minutes, holding 99% methanol for four minutes and 3 minutes equilibration time with 20% methanol in the end of the run. The flow rate was set to 500 µL/min and the injection volume to 30 µL. Additionally, the integrated Valco valve of the MS was set to “column outflow to waste” for the first 3 minutes and switched to “MS inlet” from 3 to 10 minutes to reduce matrix entering the MS system. To prevent the ESI needle from dryness during the first 3 minutes of the method, a continuous flow of 200 µL/min methanol / water (50/50, v/v) was set with a second LC pump (Agilent 1100 LC, Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

The measurement of the androgenic and antagonistic substances was done using a LC-MS/MS system with an Agilent 1100 G1312A pump (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) and a QTRAP® 6500 mass spectrometer (AB Sciex Germany GmbH, Darmstadt, Germany) operating in positive ionization mode. The measured substances, respective multiple reaction monitoring (MRM) transitions, collision energies and retention times are provided in the supporting information (Table SI 7.2-1 and 7.2-2). Chromatography was done using a C-18 reversed phase (Raptor ARC-18, 50 x 2.1 mm, Restek GmbH, Bad Homburg v. d. Höhe, Germany) as stationary phase and as mobile phases acetonitrile and water with 0.1% formic acid, respectively. For measurement, a gradient of acetonitrile and water (both with 0.1% formic acid) was applied over 14 min, from 30% acetonitrile to 70% acetonitrile within 10 minutes followed by a hold time at 99% acetonitrile for 2 minutes and a equilibration time at 30% acetonitrile for 2 minutes. The flow rate was set to 500 µL/min and the injection volume to 30 µL. The evaluation and quantitation for both methods was done by linear regression with weighting (1/x) using the software MulitQuant™ (Version 3.0.1, AB Sciex Germany GmbH, Darmstadt, Germany). The limit of detection (LOD) and limit of quantification (LOQ) were calculated from a standard by a signal to noise ratio of 3:1 and 10:1 respectively.

4.4.7.2 Detection of PPCPs and industrial chemicals by LC-MS/MS

The dried extracts were re-dissolved in 1 mL water leading to a nominal enrichment factor of 1000. The extracts and corresponding fractions (section 2.6) which showed activity in the

bioassays were further investigated for selected pharmaceuticals and industrial chemicals using LC-MS/MS with an Agilent 1100 LC system and a QTRAP® 6500 for detection (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). As stationary phase a reversed phase (Chromolith® FastGradient, RP-18e, 50 x 2 mm, Merck KgaA, Darmstadt, Germany) was applied. For measurement, a gradient of methanol and water (both with 0.1% formic acid) was applied over 15 min, from 1% methanol to 99% within 12 min and two min equilibration time with 1% methanol. The injection volume was set to 50 µL and column oven temperature at 40 °C.

To ensure data quality we used a fresh calibration, quality control and blank samples with each measurement and spiked internal standards for estrogenic and androgenic active compounds (1 ng/L) as well as for pharmaceutical compounds (20 ng/L). The measured substances, respective multiple reaction monitoring (MRM) transitions, collision energies and retention times are provided in the supporting information (Table SI 7.2-1 and 7.2-7). The LOD and LOQ were calculated from a standard by a signal to noise ratio of 3:1 and 10:1 respectively.

4.4.7.3 Suspect and non-target screening

Non-target analysis of individual fractions was performed using high-resolution LC ESI-QToF-MS (Sciex 5600 TripleTOF, Sciex Germany GmbH, Darmstadt, Germany). The chromatography, analysis and data evaluation methodology has been described previously (Nürenberg et al., 2015). Briefly, the method uses reversed-phase chromatography (C-18 stationary phase) with a standard water-acetonitrile gradient (both phases with 0.1% formic acid). For analysis a full scan (MS1) followed by data-dependent MS2 scans of the 8 most intense signals were performed. Non-target feature detection was performed on all samples. The peak detection and building of the peak inventory list was performed with R (R Core Team, 2015) using the package XCMS (XCMS™, The Scripps Research Institute, California, USA) using the matched-filter method with bin-size = 0.1 Da and FWHM = 6 s. Features were prioritized by only considering those found in active fractions. These were further reduced by excluding features found in both active and non-active fractions and features found in blank measurements. The filtering for relevant features by comparing different active and non-active fractions was done using R. Relevant peaks were further processed by the database searches including STOFF-IDENT, mzCloud and MassBank. Thereby the m/z-ratios, as well as respective MS2-spectra (if available), were compared with database entries with a mass tolerance of 5 ppm.

4.5 Results and Discussion

4.5.1 Endocrine effects

Endocrine activity of enriched samples, measured by the Arxula Adeninivorans yeast estrogen and androgen screen, were compared for samples of the Marien-Hospital and Herlev-Hospital WWTP. The activity after each treatment step of the two different WWTPs is shown in figure 4-3. Estrogenic activity (A-YES) showed a further reduction after O3 at both plants of >90% which is even below the LOQ (10%; 0.02 ng EEQ/L). However, comparing the Herlev-Hospital with the Marien-Hospital WWTP the estrogenic activity is slightly more reduced (> 95%), which can be explained by the significantly higher specific ozone dose of 1.7 mg_{O3}/mg_{DOC}. The specific ozone dose of the Marien-Hospital WWTP (0.56 mg_{O3}/mg_{DOC}) leading to an elimination of > 90% is comparable to other studies in which a reduction in estrogenic activity of 90% by corresponding specific O_3 concentration in the range of 0.4 and 0.80 mg_{O3}/mg_{DOC} was found (Abegglen et al., 2009; Itzel et al., 2017; Margot et al., 2013). Comparing the reduction in the plants in our study with other plants from literature a reduction of 90% seems possible even at lower specific ozone doses (e.g. 0.4 mg_{O3}/mg_{DOC}). Meaning, in terms of estrogenic activity, the specific ozone dose does not have to be as high as it is at the Herlev-Hospital WWTP.

The highest androgenic activity (A-YAS) was detected in the influent and was reduced down to 10% and 20% already after MBR treatment for Marien- and Herlev-Hospital, respectively. A further reduction of the androgenic activity by O_3 was not observed at either of the hospital WWTPs. Similar results were observed in prior investigations during full-scale ozonation at a municipal WWTP, where a strong reduction of estrogenic effects but substantially lower reduction of androgenic effects were observed (Itzel et al., 2017).

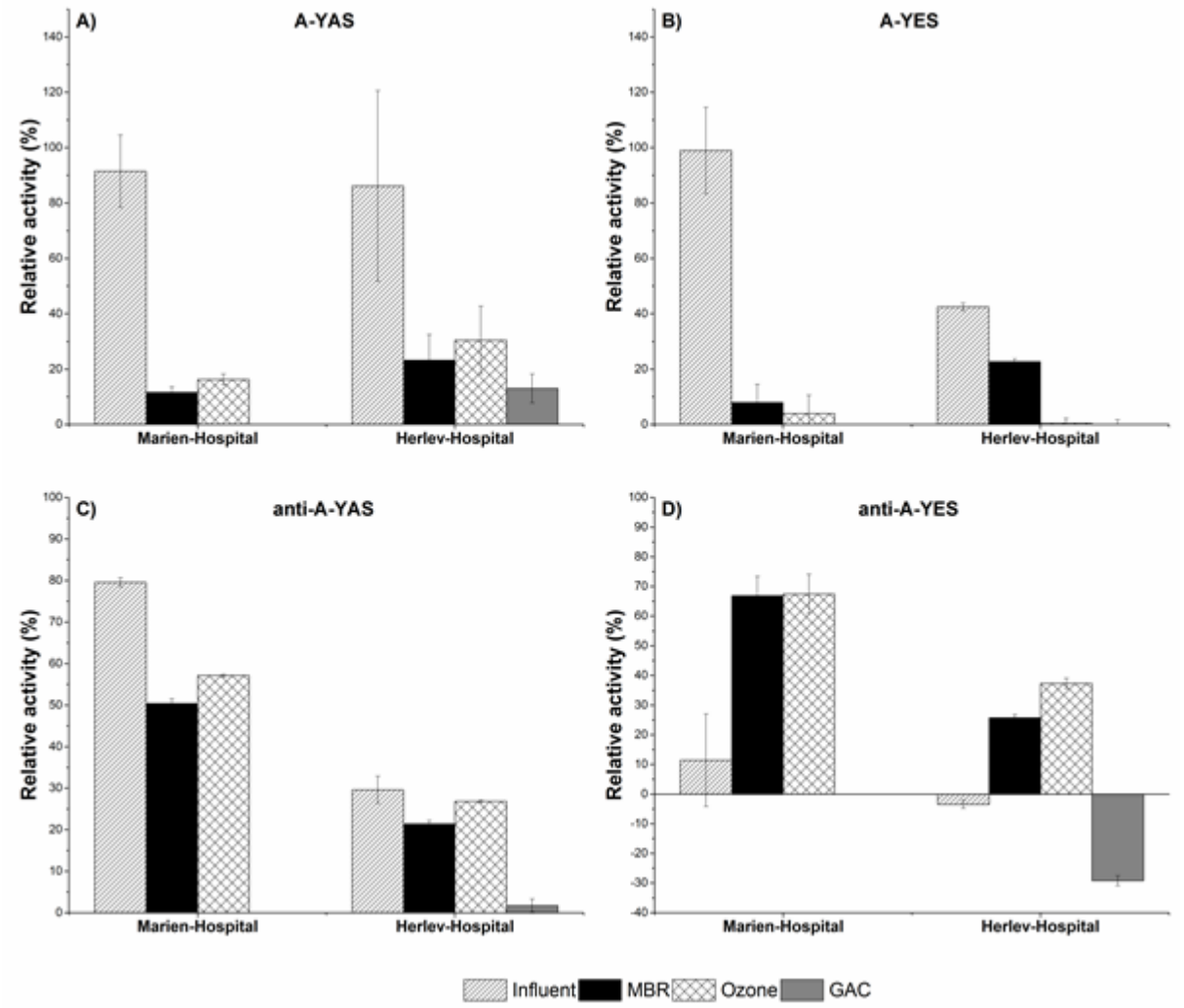

Figure 4-3: Results of estrogenic, androgenic and respective antagonistic effects of both hospital treatment plants after the different treatment steps. A) androgenic activities, B) estrogenic activities, C) anti-androgenic activities, D) anti-estrogenic activities.

In contrast to the reduced agonistic androgenic and estrogenic activity (Figure 4-3, A, B), a reduction of the respective antagonistic activity (Figure 4-3, C, D) was not observed during MBR and O_3 treatment. Comparing the influent of both plants, the anti-estrogenic activity is increasing while the anti-androgenic activity showed a reduction. Samples after additional adsorption on GAC showed a reduction of the antagonistic activity at the WWTP from Herlev-Hospital (Figure 4-3, C, D). For anti-estrogenic effects even a negative activity was detected, which means that the estrogenic activity of the sample is dominating and antagonistic active compounds are negligible in comparison to agonistic active compounds. This agonistic activity after GAC could not be confirmed by the estrogenic activity test (Figure 4-3, B) in which a positive activity should have been detected in the same range of about 30%. This effect should be further investigated. However, these results could be an indication that a combination of treatment processes is mandatory to fully reduce antagonistic effects. In case of O_3 as an advanced treatment technology, a post-treatment is already under discussion

and recommended to reduce biodegradable transformation products and potential toxic effects (Abegglen and Sigrist, 2012; Eggen et al., 2014; Knopp et al., 2016). A constant antagonistic effect during different treatment steps could also be monitored using the same biotests in prior investigations by Gehrmann et al. (2016). In our study the samples after MBR treatment from Marien-Hospital were treated with ozone in bench-scale experiments to exclude potential influences of the plant itself. However, the same trends of antagonistic activity were detected in ozone treated samples from the Marien-Hospital compared to Gehrmann et al. (2016). Furthermore, it could be shown that the increasing antagonistic activity after MBR and ozone treatment is not a specific effect of the Marien-Hospital and was also observed in the hospital WWTP in Denmark. A possible explanation for these phenomena could be the availability of both agonistic and antagonistic active compounds in the mixture. As a consequence, a competition of agonistic and antagonistic acting compounds towards the respective receptor is occurring. Thereby a masking of antagonistic effects by competing agonistic compounds or vice-versa is likely. This effect is used during cancer treatment for example by the use of 4-hydroxytamoxifen, which is binding to the estrogen receptor in competition to 17β-estradiol (Fabian et al., 1981). When agonistic compounds are removed more efficiently, as shown in our results, the antagonistic activity could increase (a generic illustration is also shown in Figure SI 7.2-1). This means that using effect based methods antagonists have to be taken into account more intensively.

4.5.2 Endocrine effects after fractionation

4.5.2.1 Androgenic and anti-androgenic effects

To further investigate the phenomena of antagonistic activity during the different treatment steps, a fractionation of samples after the different treatment steps was performed to get a more detailed view and to identify responsible substances relevant for the constant antagonistic activities using the combination of effect based methods and HRMS during treatment.

The results for the androgenic activity at Herlev-Hospital after MBR showed a high activity at F2 and an increasing activity from 80-90% in F4-9 (Figure 4-4, A). The same was observed for F7-9 after GAC treatment. Comparing the activity in the non-fractionated samples (Figure 4-3, A) the GAC showed an activity of about 15% which is substantially lower compared to the active fractions in figure 4-4. An androgenic activity was not observed after MBR or ozone treatment, which is different to the non fractionated samples in figure 4-3. During the last treatment step (GAC) an androgenic activity was observed again which could be due to the fractionation and the reduction of masking effects. In case of masking antagonistic activities, a positive relative activity should be detected in the anti-A-YAS which was not the

case. Androgenic activities are also reflected by a negative activity in the anti-A-YAS assay (Figure 4-4, B). Anti-androgenic effects were not observed in any fraction which is contrary to the results shown in figure 4-3. One reason for the contrary results can be the described mixtures effects or a potential loss of activity by unknown compounds due to the sample preparation by HPLC fractionation.

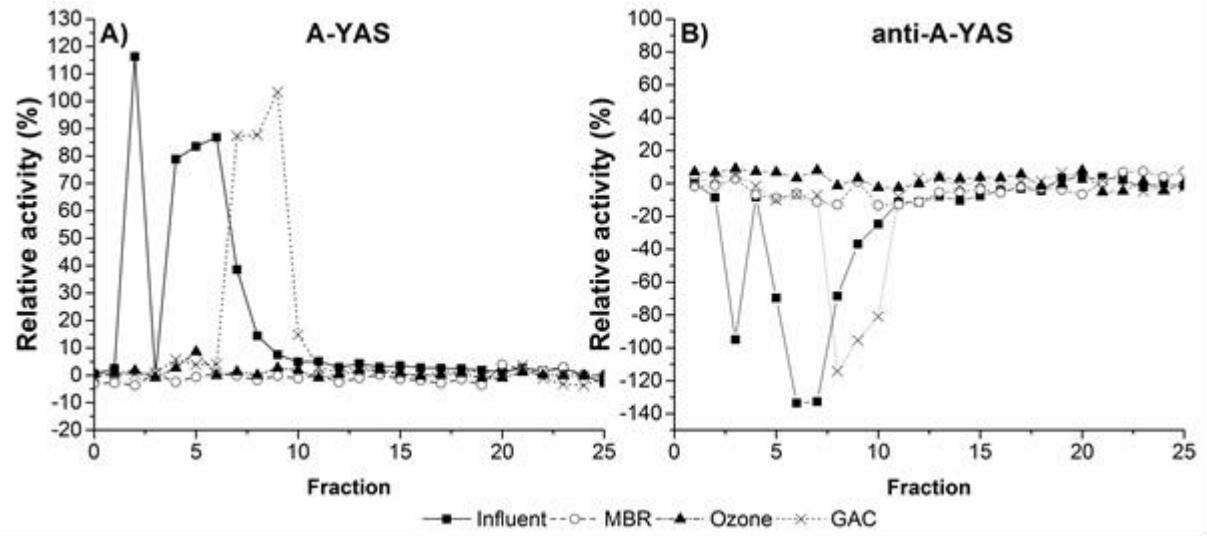

Figure 4-4: Results of the fractionation measured in the A-YAS (A) and anti-A-YAS (B) assays of the Herlev-Hospital WWTP.

Fractions of the Marien-Hospital WWTP showed a completely different pattern in active fractions for both the androgenic and anti-androgenic activities. The fractions 5-12 of the influent show an androgenic activity with a maximum in F7 of about 90% (Figure 4-5, A). However, no activity was observed in the fractions after MBR and O_3 treatment. When compared to the non-fractionated results in figure 4-3, with an activity of about 20%, an androgenic activity was expected. A possible reason could be that active substances were separated during chromatography leading to a lower total activity as it is described by Kjaerstad et al. (2010). Although fractions had a resulting enrichment factor of 800, even higher enrichment factors should be tested. Therefore, higher sampling volumes would be necessary or tools like an on-site enrichment, which was shown in a recent study by Schulze et al. (2017), could be a potential solution. However, the idea of the research was neither to quantify unknown compounds nor to estimate a mass balance, which would not be possible without reference standards of the unknown compounds. Instead this fractionation approach using effect directed methods was tested as a potential filter to reduce features during the subsequent chemical non-target screening to be able to find relevant antagonistic compounds qualitatively. Results of the anti-androgenic effects after fractionation are shown in figure 4-5, B.

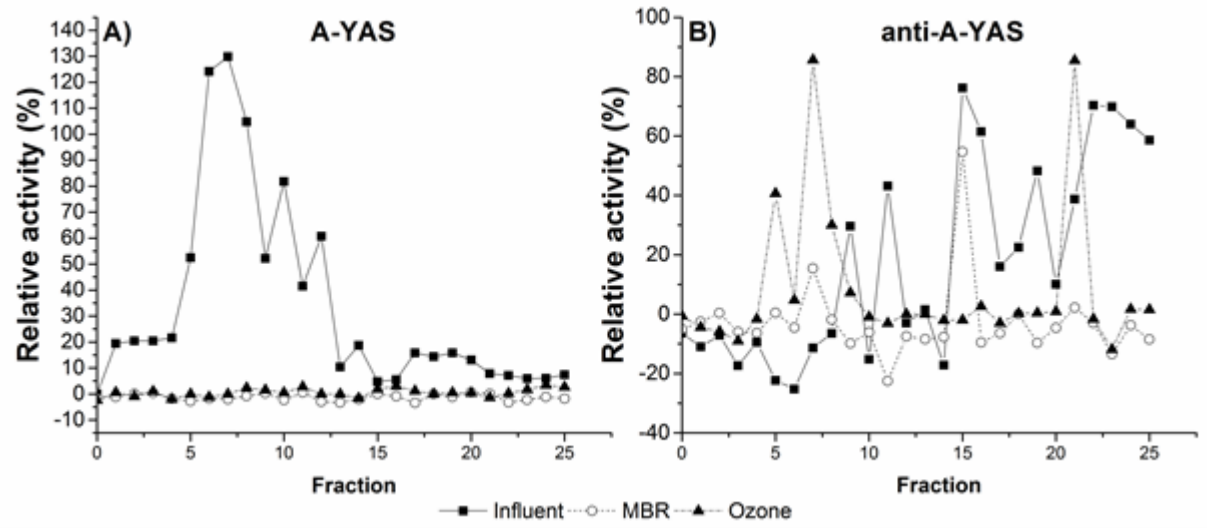

Figure 4-5: Results of the fractionation measured in the A-YAS (A) and Anti-A-YAS (B) assays of the Marien-Hospital WWTP.

Anti-androgenic activity was detected after all treatment steps. From this pattern of active and non-active fractions it becomes obvious that some active compounds seem to pass the MBR as is indicated by fraction 15. However, in this fraction the activity was reduced during ozonation below the LOQ. A reduction of the total effect would be expected in the non-fractionated ozone samples. Due to the mixture effect which was mentioned before, the increase of anti-androgenic activity could be explained by a reduction of the agonistic activity.

After O_3 treatment, fraction 5, 7 and 21, which were non-active before, showed anti-androgenic activities. This might also be explained by the formation of active transformation products (Magdeburg et al., 2014). Especially after ozone treatment more polar compounds are expected which would results in an elution by the first fraction, as a reversed phase column was used. However, even in fraction 21 a high activity is detected, which is an indication that transformation products could be highly diverse in their polarities.

4.5.3 Estrogenic and anti-estrogenic effects

The analysis of fractions from both WWTPs for estrogenic and anti-estrogenic effects using the A-YES and anti-A-YES assay also showed a successful separation of active fractions from non-active fractions.

Samples from Herlev-Hospital WWTP showed an estrogenic activity in the influent at F2 and F4 of 40% and 100%, respectively (Figure 4-6, A). After MBR treatment at Herlev-Hospital WWTP, F9 became active while F7 became active after GAC treatment. This could also be explained by masking effects during ozonation as explained in the previous section.

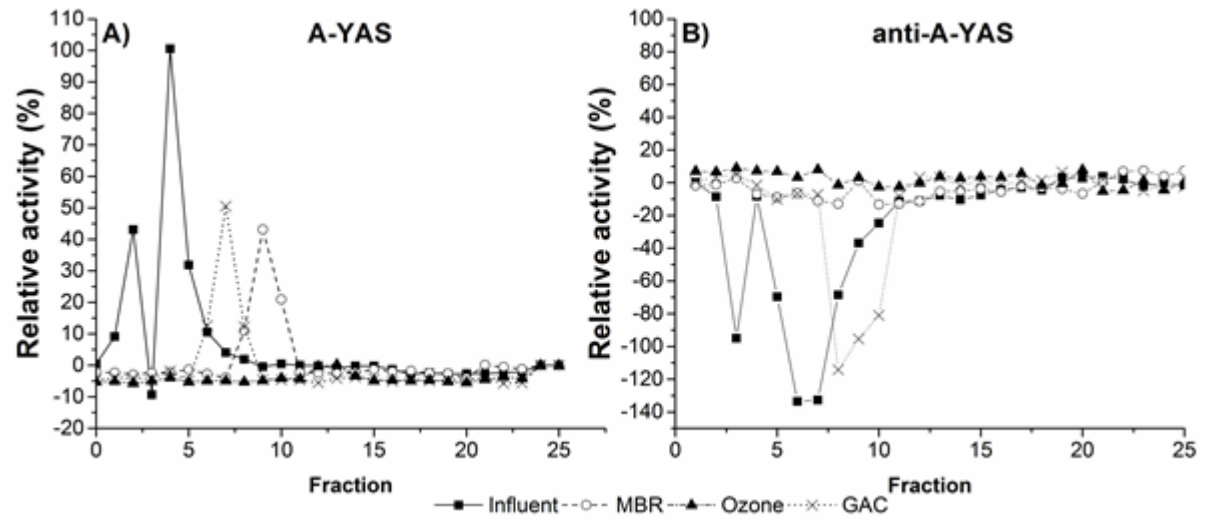

Figure 4-6: Results of the fractionation measured in the A-YES (A) and Anti-A-YES (B) assays of the Herlev-Hospital WWTP.

The negative activity in influent of F2 by the anti-A-YES confirms the agonistic activity shown by the A-YES assay in influent F2 (Figure 4-6, B). However, the absolute activity is lower which can be due to synergistic agonistic effects when spiked with E2 for the anti-A-YES. An anti-estrogenic activity could be observed in F13 (60%) and F22 (35%) after ozone treatment. However, an anti-estrogenic activity which was shown in the non-fractionated samples in section 3.1 could not be detected in fractions of the influent, MBR and ozone. This could also be due to mixtures being responsible for the effect.

The same can be observed in fractions from the Marien-Hospital analyzed by the anti-A-YES assay (Figure 4-7, B).

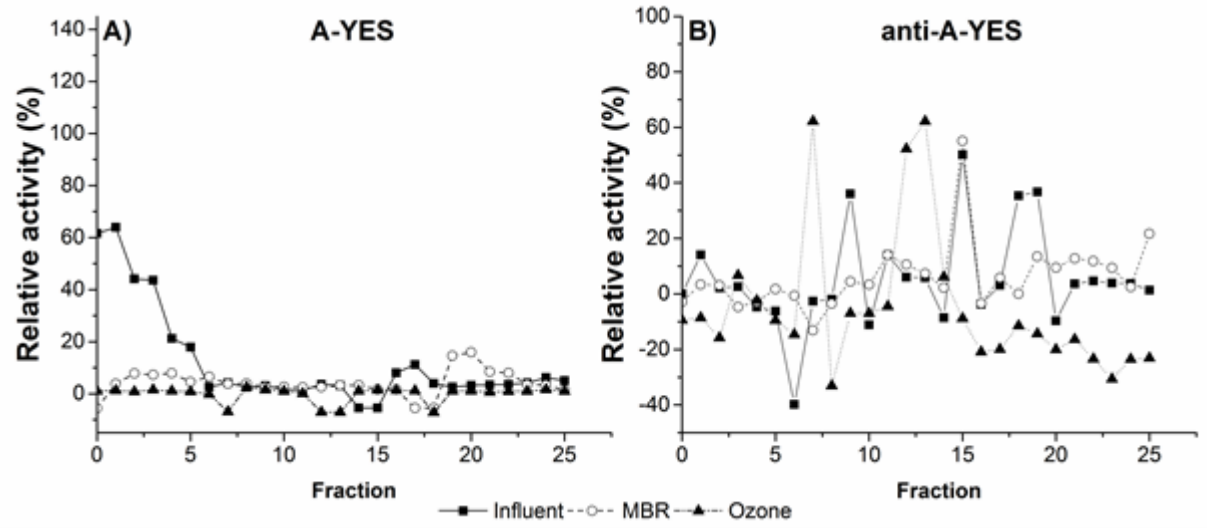

Figure 4-7: Results of the fractionation measured in the A-YES (A) and Anti-A-YES (B) assays of the Marien-Hospital WWTP.

Estrogenic effects were detected only in influent fractions with an activity of 65% in F2 (Figure 4-7, A). Anti-estrogenic activity (Figure 4-7, B) was detected in F15 of the influent sample and did not change after MBR, indicating that the anti-estrogenic activity was not eliminated by the MBR. This could indicate that compounds in fraction 15 are able to pass the MBR filter. In contrast, other active substances for example in fraction 9 are potentially removed by the MBR. After ozone treatment fractions 7, 12 and 13 showed an activity in the anti-A-YES although there was no activity in the previous treatment step. This might be explained by the formation of active transformation products as well, that masking effects by agonistic compounds are lowering the antagonistic activity. Active transformation products could also be relevant and were observed in a different study for the anti-estrogen tamoxifen metabolite 4-hydroxy-tamoxifene which showed antagonistic effects even after ozonation depending on the pH and the corresponding species of tamoxifen (Zheng et al., 2007). In general, the fractionation by HPLC combined with the bioassays showed a good separation of the active compounds and a more detailed view of a single sample from which first explanations and assumptions of effect behavior during different treatment steps can be made. Especially after ozone, transformation products or the unmasking of compounds must be responsible for the activity in fractions which were not active before treatment. Such information can be used as a filter to reduce the number of features during a subsequent chemical non-target screening. For example, if the same fraction is compared after different treatment steps and only features which are detected after ozone should be transformation products as a consequence. Compounds which are detected before and after ozone could be an indication of an unmasking due to the elimination of other compounds which are in competition in terms of binding to the receptor.

4.5.4 Chemical analysis

4.5.4.1 Target analysis

The measurements of some known estrogenic compounds showed E1 and E2 in the non-fractionated influent samples of both WWTPs and also in the fractions 2 and 3 of the Herlev-Hospital WWTP (the data for all fractionated and non-fractionated samples and detected concentrations are shown in the supplementary information, section 1.2.1, Table SI 3-4 and Table SI 7-9). Comparing the detected compounds with the standard mixture (Figure 4-2) the compound E2 should be detected in fraction 5. The shift to fraction 2-3 can be caused be the high matrix of the influent samples. Because of the decreasing activity from fraction 2 to 3 also a decreased concentration of these compounds would be expected, but in both fractions the concentration was 13 and 16 ng/L E1 as well as 3.7 - 4.4 ng/L E2, respectively. The anti-estrogenic compound tamoxifen and its metabolite 4-hydroxytamoxifen were not detected in

any sample (LOD = 1.0 ng/L). From tests with standards (Figure 4-2) tamoxifen would be expected to be in fraction 7, therewith other compounds must be responsible for the detected effect.

Androgenic compounds were found in the influent of both WWTPs (Table SI 3-5). In fraction 2 of the Herlev-Hospital WWTP DHT and etiocholanone were found in concentrations of 54 ng/L and 32 ng/L, respectively. Compared to the androgenic activity in fraction 2 of around 90 - 100% (equals 250 ng DHTEQ/L) the detected concentrations could not explain the measured activity entirely.

Androgen active compounds were not detected in fractions of the Marien-Hospital WWTP, except for testosterone in fraction 9 (influent) with a concentration of 25 ng/L. As testosterone has an androgenic equivalent factor (AEF) of 0.98 shown by Gerlach et al. (2014) a comparable potency in the A-YAS assay as for DHT is expected. For that reason, the detected concentration of 25 ng/L is in the range of the LOQ (10%, 25 ng DHTEQ/L) of the A-YAS and does not explain the activity in fraction 9 of about 50% (see figure 5).

Target analysis did not show any of the measured antagonistic substances which could have explained the detected effect in the active fraction by the bioassays. In general, known endocrine disruptors were detected in the non-fractionated influent samples and in fraction 9 of the influent of both hospitals (results are shown in table SI 7.2-1), but do not seem to be the responsible compounds for the detected effects. As known from prior studies, compounds showing a similar steroid structure are in general able to interfere with the receptor used in the bioassay. For example, pharmaceuticals like the non-steroidal anti-inflammatory drugs diclofenac and ibuprofen showed an anti-estrogenic effect (Ezechias et al., 2016). Compounds detected in highest concentrations were clarithromycin in fraction 6 (influent) of both plants and ibuprofen in fraction 2 and 3 (influent) only detected at the Herlev-Hospital WWTP (see Table SI 7.2-9 and 7.2-10). These detected compounds which are potentially relevant for the activity were tested in the bioassay but did not show any activity in the measured range (µg/L - pg/L). Diclofenac and Ibuprofen showed an anti-estrogenic activity at higher concentrations with an EC50 of 490 µg/L and 1100 µg/L respectively (Figure SI 7.2-2). The calculated AEFs in comparison to the standard 4-OH-TAM are 0.40 and 0.80 respectively. Clarithromycin did not show an effect even at higher concentrations. In a different study by Ma et al. (2017) compounds were identified using the same approach of effect directed analysis combined with fractionation and chemical analysis for WWTP effluents without ozonation and they identified pharmaceuticals, flame retardants, food additives, plasticizers and industrial chemicals to be potentially active antagonists.

Due to the wide range of compounds and the gap between the found concentrations and their contribution to the effect, unknown compounds needed to be identified by further suspected and non-target screening.

4.5.4.2 Non-target screening

A non-target screening was done for active fractions of the Marien-Hospital and the Herlev-Hospital WWTP to identify unknown compounds which might be responsible for the detected activities. The focus was on the antagonistic activity after ozone treatment as this was not removed, in contrast to agonistic activities which are known to be eliminated well (Gehrmann et al., 2016; Margot et al., 2013). As a screening for relevant masses is very time consuming and a difficult task, a method to filter for relevant features by combining chromatography with fractionation and the detection of activity by the use of bioassays was tested. Thereby active and non-active fractions were compared and relevant features could be extracted. Using this new approach, the number of relevant features could be reduced by 85 - 99% depending on the fraction (e.g. from 8000 to 32 after ozonation at Marien-Hospital WWTP, see Table SI 7.2-11).

After filtering for relevant masses, the most intense features with monitored corresponding MS^2 spectra were compared by a manual database search using mzCloud, MassBank and STOFF-IDENT.

Using STOFF-IDENT several hits were shown for these active fractions (Table SI 7.2-11). One hit was found in fraction 7 after ozone treatment (Marien-Hospital WWTP) for m/z 284.9605, which could be subsequently identified (Figure 4-8). Comparing the exact mass with an accuracy of 5 ppm and the MS^2-spectra (see Figure SI 7.2-3) with the database mzCloud, *m/z* 284.961 was identified as tris(2-chloroethyl)phosphate (TCEP) with a probability factor of 82% and later verified using a certified reference standard (see method parameters in Table SI 7.2-1 and 7.2-7).

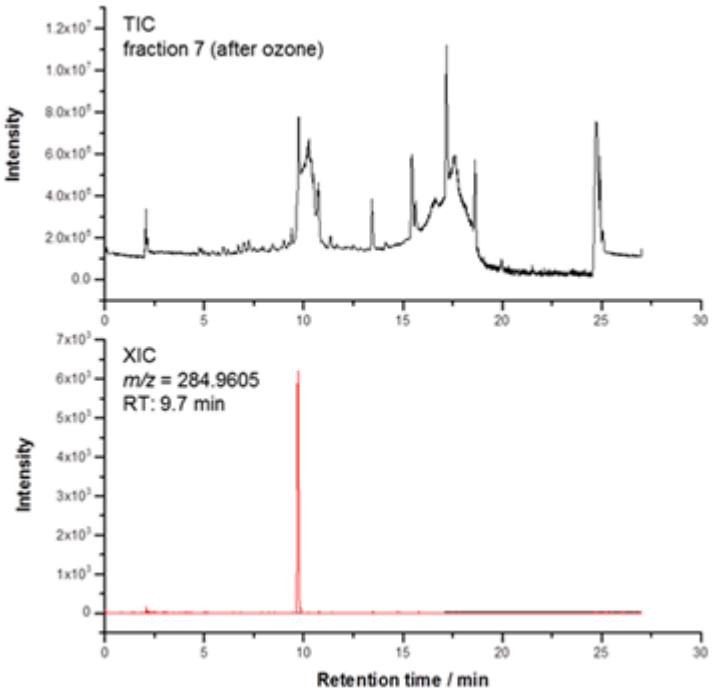

Figure 4-8: Total ion chromatogram (TIC) and extracted ion chromatogram (XIC) of m/z = 284.9605 (TCEP) of fraction 7 after ozone treatment of the Marien-Hospital WWTP.

Interestingly, TCEP was found in fraction 7 after MBR and after ozone treatment, meaning that TCEP is resistant during ozonation and due to the reduction of other masking substances, TCEP might become active after ozonation. This substance is a flame retardant and plasticizer in several products and known to be endocrine active (Zhang et al., 2014). To verify that TCEP has an effect in the used assay, TCEP was tested in different concentrations in four-fold measurements. The results showed an agonistic effect (negative activity) at lower concentrations and an antagonistic effect (positive activity) at higher concentrations (Figure 4-9). This hybrid effect behavior is comparable to that of tamoxifen, which is a known pharmaceutical in breast cancer treatment. Tamoxifen is a selective estrogen receptor modulator (SERM), which means that a molecule can act agonistically and antagonistically depending on its concentration.

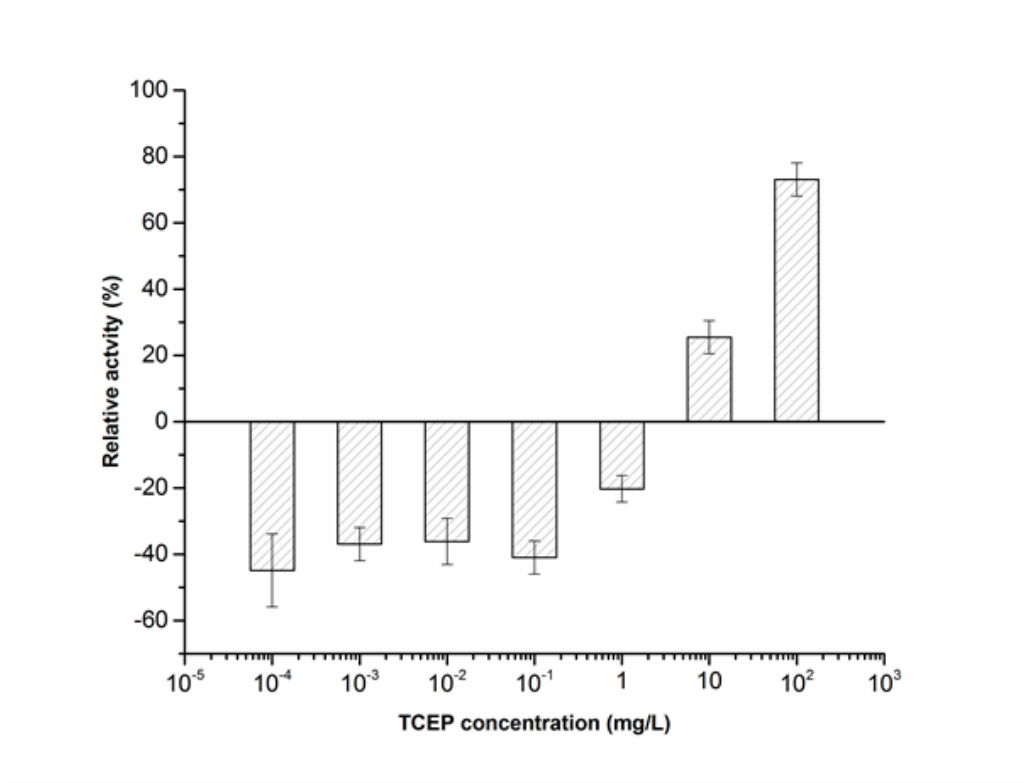

Figure 4-9: Estrogenic activity of different TCEP concentration in the A-YES assay (n = 4). Negative activity represents an agonistic activity and positive activity an antagonistic activity.

These findings are similar to those published by Zhang et al. (2014), who found strong antagonistic effects (20% inhibition) for TCEP in concentrations of 0.29 mg/L. The detected antagonistic activity in our study of ~20% at 10 mg/L (AEF = 40) compared to the study of Zhang et al. (2014) could be explained by the use of a different bioassay (luciferase reporter gene assay by CHO-K1 cells) which might have a different sensitivity towards TCEP compared to the anti-A-YES assay used in our study. The different sensitivities of bioassays are known and were compared by samples of the Marien-Hospital in a prior study, but trends are always the same (Gehrmann et al., 2016). Other compounds found by database search were pharmaceuticals like clarithromycin, which was also detected by our target analysis method but showed no effect in the bioassays. Further unknown compounds could also be relevant for the detected activity and need to be identified by future non-target analyses of different waste waters using this developed approach including effect-directed methods. Additional, databases which are needed to identify unknown compounds need to get more data of compounds in waste water matrix to include matrix influences of the respective mass spectra.

As already mentioned, transformation products might play an important role in activity and may have a more toxic potential to aquatic species compared to the respective parent compound (Schluter-Vorberg et al., 2015). However, TP's are difficult to identify in real samples with unknown parent compounds and thus ozonation as an advanced waste water

treatment step should be investigated in more detail regarding the antagonistic endocrine activities and potential active compounds which are not eliminated or which are formed during treatment. Also the addition of a sorption post-treatment step like the application of GAC filters was shown to be effective in the reduction of transformation products by Knopp et al. (2016) and should be further investigated as this study showed also a reduction of the antagonistic activity after GAC at the Herlev-Hospital WWTP.

4.6 Conclusion

Antagonistic activities and relevant compounds are of interest as they are not reduced significantly compared to respective agonists during MBR and ozone treatment and are lowering the detected agonistic activity. Therefore, antagonists should be investigated always in parallel to agonistic activities if chemical mass balances are made. The application of a GAC filter after ozonation at the Herlev-Hospital WWTP showed a significant reduction of antagonistic activity and should be further investigated as potential post-treatment. This study demonstrated the use of fractionation in combination with effect-directed and chemical target and non-target analysis for an in-depth view of antagonistic activities during different treatment steps. Next to estrogenic and androgenic effects, antagonistic effects are of high importance. Using bioassays inhibitory effects, which leads to a reduction of the total measured activity, need to be kept in mind as the result reflects a sum parameter. Additionally, the explanation of activities by chemical target analysis is not possible without considering agonistic and antagonistic activities. Application of fractionation in combination with effect based analysis showed good results in reduction of relevant features and the potential identification of unknown substances as a wide range of compounds are able to interfere with estrogen and androgen receptor. The activity profile by using bioassays showed, that transformation products after ozone treatment potentially seem to be relevant for antagonistic activities and should be investigated in future research. The transformation of micropollutants during ozonation might lead to a more favorable structure which is able to bind more efficient to the receptor and should therefore be further investigated in future work.

4.7 Achknowledgement

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5. Evaluation of a biological post-treatment after full-scale ozonation at a municipal waste water treatment plant

5.1 Abstract

To reduce the discharge of trace organic compounds into water bodies associated with potential toxic effects such as endocrine disruption, new advanced treatment methods are under investigation at several waste water treatment plants (WWTPs). One of the most studied and already implemented technology is ozonation. When ozonation is applied, however, parent compounds are oxidized and transformation products with unknown properties will be formed. In order to minimise the risk of releasing unknown, potentially toxic transformation products (TPs) into surface water, a biological post-treatment is recommended after ozonation. The aim of this study was to evaluate the efficiency of a moving bed reactor following ozonation in a full-scale plant. Therefore, different ozone dosages ($z_{spec.}$ = 0.3, 0.5, 0.7 mg_{DOC}/mg_{O3}) were investigated. To assess the biological activity of the post-treatment, the assimilable organic carbon (AOC) was determined in addition to the formed biomass. Furthermore, selected pharmaceuticals and pesticides were analysed in parallel to monitor the ozonation efficiency at the different ozone doses. Furthermore, estrogenic, androgenic as well as corresponding antagonistic effects were investigated after each treatment step using the A-YES and A-YAS assay. A non-target screening was performed to establish a trend analysis of formed TPs as well as their removal by the post-treatment. The results showed a successful design of the biological post-treatment reactor by a constant biofilm development and reduction of the AOC at high ozone dosages. Endocrine effects were removed below the LOD already after ozonation for all applied ozone doses. Antagonistic effects were not significantly reduced during ozonation and subsequent biological post-treatment. For this reason, further research is needed to evaluate different post-treatment technologies. The trend analysis from non-target screening data showed a reduction of about 95% of formed TPs by the biological post-treatment. Consequently, an assessment of the biological activity and elimination capacity of a certain biological post-treatment is thus possible by applying the AOC in combination with a non-target screening.

5.2 Introduction

As a result of today's standard of living and the associated use of chemicals (more than 100,000 of the most relevant compounds are registered in the EU under REACH), these are permanently released into the environment. A high proportion of those chemicals are compounds such as pharmaceuticals and personal care products (PPCP), biocides, pesticides and industrial chemicals, which can be detected in low concentrations in waters (Bergmann et al. 2011, European Chemical Agency 2018, Petrie et al. 2015). One major source of such trace organic compounds in surface waters are waste water treatment plants (WWTPs) (Ternes and Joss 2006). Conventional WWTPs are not designed to remove trace organic compounds during the treatment process, but to reduce inorganic nitrogen and phosphorus as well as the biological and chemical oxygen demand (Gabet-Giraud et al. 2014, Jarosova, Ersekova, et al. 2014, Gabet-Giraud et al. 2010, Morasch et al. 2010, Bueno et al. 2012, Michael et al. 2013, Jarosova, Blaha, et al. 2014, Rechenberg 2015, Richardson and Kimura 2016, Valitalo et al. 2016). As a result of the introduction of these substances into surface waters, aquatic organisms may be affected, for example by a disruption of the endocrine system. Endocrine effects can be triggered by naturally excreted hormones as well as by chemicals capable of acting endocrine. (Kidd et al. 2007, Peschke et al. 2014, Henneberg et al. 2014). Especially for non-natural endocrine disrupting compounds (EDCs) biological effects could already be observed in very low concentrations (ng/L - pg/L range) (Aris et al. 2014, Kidd et al. 2007). Since 2013 within the EU-Water Framework Directive, a watchlist was established including the estrogene active compounds 17β-estradiol (E2), estron (E1) and the synthetic analogon 17α-ethinylestradiol (EU 2013/39 , EU 2015/495 , EU 2018/840). Besides estrogene active compounds, androgene effects are relevant as well. A study by Chang et al. (2011) revealed that androgen active compounds account for a significant proportion (60%) of the total hormone load in WWTP influents as well as effluents. In contrast, estrogens account for only about 16% of the total load, which increases the potential relevance of androgen active compounds. Furthermore, besides estrogenic or androgenic effects, which are leading to an increased hormonal effect level (agonistic activity) in organisms, there are existing inhibitory effects lowering the overall effect level caused by agonists (antagonistic activity). Such an antagonistic activity was observed during treatment of hospital waste water in prior studies. However, in these studies it was not possible to reduce antagonistic activity by ozonation (Itzel et al. 2018, Gehrmann et al. 2018). Antagonistic effects, especially anti-androgen effects, can lead to similar effects in organisms compared to estrogens due to the reduction of the androgen level (Jobling et al. 2009). For this reason, the potential discharge of antagonistic endocrine active compounds could become important in terms of ecological status of water bodies.

To reduce the discharge of trace organic compounds and endocrine effects into water bodies, new advanced treatment methods are needed for WWTPs. Ozonation as well as the application of activated carbon are the technologies most studied and already implemented (Andreozzi et al. 1999, Audenaert et al. 2014, Knopp et al. 2016, Verlicchi et al. 2015, Tuerk et al. 2010, Sperlich and Gnirß 2016, Hollender et al. 2009, Margot et al. 2013). In case of ozonation, a full mineralization during waste water treatment is not achieved due to high organic matter leading to fast ozone depletion. For this reason, a transformation of parent compounds is more likely than a mineralization (Lee and von Gunten 2010). However, the toxicological characteristics of transformation products are difficult to predict and thus lead to increased uncertainty compared to the parent compound. There are various studies which show contradictory results regarding toxic effects by transformation products (Knopp et al. 2016, Lee and von Gunten 2016, Li et al. 2016, Itzel et al. 2017, Schmidt et al. 2014).

In order to minimise the risk of releasing unknown potentially toxic transformation products into surface water, a further filter is recommended after ozonation in Switzerland (GSchG 814.20 , GschV 814.201). Besides legal requirements applying to the Swiss, there are numerous studies recommending the implementation of additional post-treatments to reduce the potential risk of formed TPs during oxidative processes (Hollender et al. 2009, Prasse et al. 2015, Zimmermann et al. 2011).

Due to the fact that oxidized molecules (transformation products) are more polar, they exhibit a higher bioavailability (Hübner et al. 2014). For this reason, biological filters are one option to reduce transformation products and were already tested in the Swiss project “Re-treat” (Böhler et al. 2017). In this study, for N-nitrosodimethylamine (NDMA), a known toxic transformation product, it was shown that a biological sand filter after a prior ozone treatment at 0.5 g_{O3}/g_{DOC} could reduce the concentration by 66%, which illustrates the elimination potential of biological post-treatment.

Also other studies investigating biological post-treatment showed a removal of oxidative TPs formed during drinking water production, indicating the efficiency as well (Richardson et al. 1999, Hammes et al. 2006). Applied biological post-treatments are for example sand filters, moving bed filters and fixed bed reactors. Additionally, granular activated carbon (GAC) filters are often investigated as sorption technologies in parallel to their biological activity due to the formation of biofilm on the surface (Bourgin et al. 2018, Knopp et al. 2016, Böhler et al. 2017). The use of GAK filters as biological post-treatment by targeted cultivation of biofilm on its surface is also referred to as BAC filter (Kalkan et al. 2011).

To evaluate different technologies and especially the efficiency of a certain biological post-treatment, parameters need to be selected, which are able to give information about removal, toxicity and the bioavailability of the compounds. A common evaluation criterion is the removal efficiency of certain indicator parameters such as compounds listed within the

European Water Framework Directive (EU WFD) (2000/60/EC) or selected compounds within the Swiss legal requirement for evaluation (UVEK 2016). However, after ozonation an integrative approach would be useful, due to unknown transformation products and corresponding ecotoxicological effects.

Therefore, effect based methods are a useful tool in order to ascertain the elimination effectivity in terms of genotoxicity, cytotoxicity, and estrogenic and/or androgenic activity (Itzel et al. 2017, Valitalo et al. 2017). However, due to regulatory measures, it may sometimes be necessary to identify relevant compounds and identify certain emission sources. Thus, effect-based analysis would be used as a sensitive screening tool in the first step and subsequently more extensive instrumental analysis. This approach is already being implemented in some studies. In particular, the combination of both analysis techniques enables faster and more precise identification through decisive meta information (Weiss et al. 2011, Brack et al. 2016, Tousova et al. 2017, Comtois-Marotte et al. 2017, Neale et al. 2017, Itzel et al. 2018, Altenburger et al. 2015). Especially in terms of transformation products such an effect-based identification approach could help to identify most potent and relevant transformations products and thus their fate during treatment.

As transformation products potentially exhibit a higher bioavailability, the assimilable organic carbon (AOC) fraction of the dissolved organic carbon (DOC) could be a further important indicator parameter to assess the removal effectivity of a certain post-treatment (van der Kooij 1992). The AOC analysis method was originally developed to determine the biological regrowth potential of freshwater distribution networks (van der Kooij 1992, van der Kooij et al. 1989). The correlation of AOC and ozone dose was already observed during ozonation of drinking water, indicating the correlation to formed TPs (van der Kooij et al. 1989, Escobar and Randall 2001). As the AOC analysis method is based on isolated drinking water specific organisms (*Pseudomonas fluorescens* P17 and *Spirillum species* NOX) the influence of high DOC containing waste water samples remains a challenge. However, in a study by Bourgin et al. (2018) three different biological waste water post-treatment systems were successfully investigated using the AOC method by van der Kooij. They demonstrated the usability of the AOC as parameter for the characterisation of a post-treatment in terms of a transformation product elimination.

The aim of this study was to evaluate a full-scale ozonation plant with a biological post-treatment in terms of endocrine activities and potential transformation products. Therefore, effect based methods using *Arxula Adeninivorans* yeast cell assays were applied to determine estrogen, androgen as well as the less investigated anti-estrogen and anti-androgen effects. Parallel to effect based methods, EU WFD relevant indicator parameters were measured to characterise the full treatment process. Due to influent from a sugar beet processing factory, pesticides were investigated in parallel as some are known to potentially

be endocrine active. To determine the elimination rate of formed transformation products during a biological post-treatment, the AOC was applied. To further evaluate the elimination efficiency of transformation products by the biological post-treatment a trend analysis was done using a non-target approach based on high-resolution mass spectrometry (HRMS) measurements.

5.3 Material and Methods

5.3.1 Chemicals and Standards

Acetonitrile (LC-MS grade), methanol (LC-MS grade), methyl-tert-butylether (MTBE, for residue analysis) and pure water (LC-MS grade) were purchased from Th. Geyer GmbH & Co. KG (Renningen, Germany). Hydrochloric acid (98% purity) was purchased from Sigma Aldrich GmbH (Steinheim, Germany) and formic acid (99% purity) was purchased from VWR Chemicals (Darmstadt, Germany). 17β-estradiol, dihydrotestpsterone, 1H-benzotriazole, carbamazepine, clarithromycin, desphenyl-chloridazone, diclofenac, diflufenican, disulfoton, isoproturon, metazachlorsulfonicacid, metoprolol, N4-acetyl-sulfamethoxazole, quinmerac, sulfamethoxazole and terbutryn were purchased from Sigma Aldrich GmbH (Steinheim, Germany).

5.3.2 Waste water treatment

The mechanical-biological municipal WWTP Warburg (Stadtwerke Warburg GmbH, Warburg, Germany) is designed for 70,000 population equivalents (PE) and receives municipal as well as industrial waste water. Since November 2016, an ozonation unit is in operation. The advanced treatment unit is designed as a two-line full-scale ozonation with a moving bed reactor as biological post-treatment. A detailed scheme is shown in the supplementary information (figure SI 7.3-1). The specific operation parameters of the plant are listed in table 5-1.

Table 5-1: Operating parameters of the WWTP Warburg.

Parameter	Value
Conventional treatment	
Average Inflow raw water	19,200 m³/day
Max. Inflow raw water	1,530 m²/h
BSB_5-fraction	4,200 kg/BSB_5/day
Advanced treatment	
Flow velocity (maximum)	200 m³/h (each line)
Flow velocity (average, dry weather)	120 m³/h (each line)
Hydraulic retention time (minimum)	~ 20 min
pH (Influent ozone treatment)	7.1 (in average)
Ozone dosage	DOC dependent
Dissolved organic carbon (DOC)	6 ± 2 mg/L
Investigated ozone dose ($z_{spec.}$)	0.3, 0.5, 0.7 mg_{O3}/mg_{DOC}

5.3.3 Sampling

The samples were taken at specific ozone dosages of 0.3, 0.5 and 0.7 mg_{O3}/ mg_{DOC} over a period of three weeks. In addition to qualified grab samples, 24 h composite samples were taken to assess the general elimination efficiency of the ozonation for selected indicator parameters. Grab samples were always analysed for all parameters. A detailed sampling plan is shown in the table SI 7.3-1.

5.3.4 Sample preparation

5.3.4.1 Effect based methods

Sample preparation performed for effect-based analysis of EDC, included a solid phase extraction (SPE) step, which was carried out within 48 h after sampling. At first, the cartridge (150 mg, 6 mL, Oasis HLB, Waters, Germany) was conditioned (2 x 5 mL methanol) and equilibrated (2 x 5 mL water, LC-MS grade).

Therefore, separate cartridges were loaded with 1000 mL sample. After drying the cartridges under vacuum, the loaded cartridges were stored at -18 °C until further analysis. The elution was performed with 5 x 5 mL MTBE. The solvent was evaporated (Evaporator Vapotherm Basis mobil I, Barkley, Germany) at 50 °C under a nitrogen gas stream and re-dissolved in 2 mL (A-YES/A-YAS) and 4 mL (anti-YES/anti-YAS) water (LC-MS grade), resulting in an enrichment factor of 500 and 250 respectively.

5.3.4.2 PPCPs and Pesticides

For determination of trace organic compounds, the samples were adjusted to pH 3 with hydrochloric acid and 10 ng isotope labelled internal standards (see table SI 7.3-2) were added, followed by an automated SPE (GX-821, Gilson Inc., Middleton, USA). The method was set up to start with 5 mL methanol and 5 mL water (pH 3) for conditioning and equilibration of cartridges (150 mg, 6 mL, Oasis HLB, Waters, Germany), respectively, followed by the sample loading with a volume of 200-1000 mL (for an enrichment factor from 200 to 1000). Then a wash step was run with 5 mL water (pH 3) and 5% methanol, and cartridges dried by a nitrogen gas stream. For each run the flow rate was set to 20 mL/min. After elution with 3 x 3 mL methanol the extracts were evaporated (Evaporator Vapotherm Basis mobil I, Barkley, Germany) at 50 °C under a nitrogen gas stream and re-dissolved in 1 mL water with 1% acetonitrile and 0.1% formic acid for LC-MS/MS analysis. In addition to the SPE extracts, 50 µL of each sample were analysed directly. Those directly analysed samples were filtered with syringe filters (CHROMAFIL® RC-45/25, Macherey-Nagel, Germany) and 10 ng/mL internal standards were added before LC-MS/MS analysis.

5.3.4.3 Non-target analysis

The sample preparation for HRMS included an enrichment step using SPE cartridges (150 mg, 6 mL, Oasis HLB, Waters, Germany) which were performed as described in 2.4.1. Prior to the enrichment 10 ng of an internal standard mix (shown in table SI 7.3-2) were added for a retention time correction later on. The dried cartridges were stored at -18 °C until further sample preparation. The elution was done using 5 x 5 mL methanol (LC-MS grade) and the solvent was evaporated to complete dryness at 50 °C under a gentle nitrogen stream. Before analysis, the samples were re-dissolved in water (LC-MS grade).

5.3.5 Biological activity

5.3.5.1 Biofilm growth

To monitor biofilm formation in the fluidized moving bed reactor, samples of growth bodies (Anox™ K5, AnoxKaldnes AB, Sweden) were taken as grab samples and analysed for their biofilm mass. As the plant was already running for about 24 months, a constant biofilm mass

was expected. The protected surface area for biofilm growth is 800 m^2/m^3. Four samples (40 g each) of the exposed growth bodies were placed in a beaker and stored at 50 °C overnight. Two samples were then dried at 105 °C for 24 h. The two other samples were washed with 50 mL hydrochloric acid (37%), followed by ultra-pure water in order to remove the biofilm from the growth bodies and then also dried at 105 °C for 24 h. The calculation of the biofilm mass was based on the difference between the two sets of samples, which were treated with and without hydrochloric acid.

5.3.6 Assimilable organic carbon

The measurement of the assimilable organic carbon (AOC) was performed after van der Kooij et al. (1982) with the following modifications.

The AOC was performed with grab samples, as 24 h samples would not be representative due to microbial degradation. To prevent a biodegradation until measurement, the samples were directly filled in sterile 30-mL glass vials and pasteurised at 150 °C for 30 min in an oven within 4 h after sampling. After pasteurisation the samples were stored at -20 °C until further treatment. Prior to the sample incubation with target organisms, the cell cultures had to be prepared. Therefore, both freeze dried strains, the *Spirillum* strain NOX (ATCC, No. 49643, USA, Manassas) and the *Pseudomonas fluorescens* strain P-17 (ATCC, No. 49642, USA, Manassas) were re-suspended in 2 mL sterile drinking water (autoclaved and filtered < 0.2 µm) separately. From these suspensions 100 µL were transferred into separate 250-mL sterile Erlenmeyer flask containing 125 mL sterile drinking water. The cell cultures were incubated at room temperature (15-20 °C) until the stationary phase was reached. Therefore 100 µL of the cell cultures were spread onto R2A-Agar plates for each day over a period of one week and incubated for 3-5 days at 25°C using an incubator. Colony counting was done manually. After reaching the stationary phase, both organisms were spiked to the samples at a concentration of 500 CFU/mL and incubated for 7-10 days at room temperature (15-20 °C). To count the formed colonies, 100 µL of the samples were spread onto R2A-Agar in triplicate and incubated for 5 days. The NOX were identified as small (1-2 mm) white colonies and the P-17 as larger (3-4 mm) yellow colonies.

5.3.7 Estrogen and androgen effects

5.3.7.1 Agonistic effects

The enriched and aqueous samples (samples without enrichment) were analysed using the Arxula adeninivorans yeast estrogen and androgen screen assays (A YES, A-YAS, New_diagnostics, Freising, Germany). The measurements of estrogenic effects were done

according to the ISO 19040-2:2018 and for androgenic effects similar to our prior study (Itzel et al. 2017). The achieved LODs were 0.02 ng EEQ/L for the A-YES and 0.06 ng DHTEQ/L for the A-YAS (enrichment factor of 500 included).

5.3.7.2 Antagonistic effects

The measurement of antagonistic effects was done similar to the agonistic effects as described in 2.6.1 using the yeast *Arxula Adeninivorans*. The analyses were done after Itzel et al. (2018) by spiking a known amount of agonist to the samples. This was done for anti-estrogenic effects by spiking 50 ng of E2 and for anti-androgen effects 200 ng of DHT respectively. After performing the test, the detected concentration was set relative to the spiked amount leading to a percentage inhibition. In case of less antagonistic effects, the spiked agonist can lead to higher levels than the spiked concentration. Due to calculation relative to the spiked concentration (set as maximum) a negative result can be obtained.

5.3.8 Instrumental analysis

5.3.8.1 Dissolved organic carbon

DOC was determined with reference to DIN EN 1484:1997-08 (1997). The quality assurance is based on the factsheet no. 28 "analytical quality assurance (AQS) for water analysis in North Rhine-Westphalia (NRW)" of the Environmental Agency (LUA NRW 2001). For the measurement of DOC, 20 mL of the sample to be measured were filtered with a syringe filter (CHROMAFIL® RC-45/25, Macherey-Nagel, Germany) and acidified with hydrochloric acid c = 2 mol/L (Roth ROTIPURAN® Ultra 34 %) to a pH value < 2. Acidification was done within 24 hours after sampling. Samples were sealed and stored in the refrigerator (≤ 8°C) unitl analysis. The sample was measured within one week using a TOC-VcpN analyzer (Shimadzu Deutschland GmbH, Duisburg, Germany) by catalytic oxidation at 680 °C followed by NDIR detection. The DOC mass concentration was determined directly.

5.3.8.2 Tandem mass spectrometry

The samples for determination of PPCPs and pesticides were analysed by LC-MS/MS using a 1260 Infinity LC pump (Agilent Technologies GmbH, Waldbronn, Germany) with a HTS PLA autosampler (CTC Analytics AG, Zwingen, Switzerland) and a 6500 QTrap (Sciex GmbH, Darmstadt, Germany) tandem mass spectrometer. 50 µL of the samples were injected onto a Raptor ARC-18 column (50 x 2.1 mm, 2.7 µm) with a Raptor ARC-18 guard column (5 x 2.1 mm, 2.7 µm) purchased from Restek GmbH (Bad Homburg, Germany). A generic gradient of water and acetonitrile (both with 0.1% formic acid) at a flow rate of

500 µL/min was used for separation. The gradient starts from 1% acetonitrile to 67% within 8 min followed by 2 min with 99% acetonitrile and 3 min with 1% acetonitrile for equilibration. The column temperature was set to 40 °C. Detection via mass spectrometer (MS) was realised with the scheduled multiple reaction monitoring (sMRM) mode. The respective mass transfers, ion source setup for measured analytes as well as internal standards used for quantification are listed in table SI 7.3-2. Corresponding limits of detection (LOD) and limits of quantification (LOQ) are shown in table SI 7.3-3.

5.3.8.3 High-resolution mass spectrometry

For HRMS measurements, only samples treated with the highest ozone dose are further processed and analysed as these samples should be influenced the most by ozone transformation products and therewith the effect of the biological post-treatment should be best visible and at the same time demonstrates a worst-case scenario. After eluting the cartridges (see 5.3.4.3), the measurement was done within 48 h. Chromatographic separation was performed using a Dionex UltiMate 3000 HPLC system (Thermo Scientific, Bremen, Germany). A gradient method was carried out on a XSelect HSS T3 (2.1 mm x 75 mm, 3.5 µm particle size) column from Waters (Waters, Milford, MA, USA). The mobile phase consisted of eluent A: ultrapure water + 0.1% formic acid, and eluent B: methanol + 0.1% formic acid. After an isocratic step with 5% B for 5 min, the concentration of eluent B was raised to 95% within 10 min and kept constant for 10 min. Following, the initial conditions were reached again within 0.1 min and were kept for 5 min constant to re-equilibrate the column. The injection volume was 20 µL and the flow rate 0.35 mL/min. All samples were measured in triplicates.

Mass spectrometric detection was performed on an Orbitrap mass spectrometer (QExactive Thermo Scientific, Bremen, Germany) using positive and negative ion mode electrospray ionization in separate runs. The parameters of the ion source are summarized in table SI 7.3-4. The full scan HRMS spectra (*m/z* 100-1000) with a resolution of 70,000 was followed by data dependent MS^2 scans of the five most intense ions with a resolution of 17,500. The settings of the full scan/ddMS^2 (Top 5) measurement are shown in table SI 7:3-5. Mass calibration was performed with the calibration solution (Pierce LTQ Velos Positive/Negative Ions Calibration Solution, Thermo Scientific, Bremen, Germany) before a new measurement series was started. In addition, a lock mass (391.2843 for positive mode and 265.1476 for negative mode) was used. Further, an “exclusion list” was used to avoid fragmentation scans of continuous background signals (see table SI 7.3-6).

5.3.8.4 Non-target data evaluation

The peak processing and peak picking as well as final trend analysis were done by the R (R Core Team 2018) based software tool MZmine 2 (Pluskal et al. 2018). Therefore, the raw data from LC-HRMS analysis was loaded as mzXML files into MZmine 2 in a centroided mode. The first step was the peak detection out of the triplicates measured, therefore the noise level was set to 1.0 x 10^4 by manual view into the total ion chromatogram (TIC) of the samples to reduce the number of features from the noise. The peak detection in MZMine 2.0 is based on the R-Script XCMS with a min. time span of similar features assigned to one peak of 0.02 min and a *m/z* tolerance of 0.001. To pick relevant peaks and reduce artefacts, a chromatogram deconvolution was done by setting the max. peak duration to 0.8 min prior to the function “chromatogram builder”. The chromatogram builder generates a list for each detected peak with an *m/z* tolerance of 5 ppm and minimum signal intensity above the set noise level. The peak alignment was done to be able to track features during the treatment steps. Therefore, the retention time tolerance was set to 5%. Additional an isotope pattern comparison was done to align the right peaks. The minimum score of the isotope ratios was set to 85%. As a last step the formula prediction was done using a *m/z* tolerance of 5 ppm, the ionization type $[M+H]^+$, isotope pattern as well as MS/MS information (score threshold of 80%).

5.4 Results and Discussion

5.4.1 Biological investigations

5.4.1.1 Biofilm growth

The biofilm mass of 30 ± 2 mg per growth body was constant during the sampling campaign in both lines (data not shown). These results indicate a constant biofilm growth in the lag phase of a typical biofilm growth curve. Therefore, a potential ozone entrance from the reactor could be excluded. In a prior study, ozone withdrawal was observed by a decreasing biofilm mass (Gruenebaum et al. 2014), which supports the successful design of the treatment unit as ozone is not influencing the biological post-treatment. Comparing the biofilm growth bodies by their developed biofilm mass with prior investigations, the now used growth bodies showed a 3 times higher biofilm mass per growth body (Itzel et al. 2017). As the design of the growth bodies is different compared to Itzel et al. (2017), a normalization to the protected surface area was done for both types and resulted in 12.4 x 10-3 mg/m² (Based on manufacturers data of 800 m²/m³, 331000 pieces/m³, Type: AnoxTM K5, used in this study) and 3.2 x 10-3 mg/m² (Based on manufacturers data of 635 m²/m³, 255000 pieces/m³, Type: 2H-BCP 750, Itzel et al. 2017). This comparison demonstrates that

the biofilm growth bodies used in this study can achieve up to 4 times higher biofilm masses and are potentially more suitable for an efficient post-treatment.

5.4.1.2 Biological activity

The results of the DOC measurements showed a significant decrease to a minimum of 6 mg/L after conventional waste water treatment due to the biological assimilation. A further reduction by ozonation and subsequent moving-bed reactor was not observed. Since ozonation does not completely mineralize the organic content, a conversion of compounds to TPs is more likely, which is reflected in a constant DOC through the oxidative treatment. As smaller polar molecules are more easily accessible to microorganisms, the formation of corresponding TPs should lead to higher biological activity after ozonation (von Gunten 2003). This is shown by an increasing AOC concentration (figure 5-1) parallel to an increasing ozone dose comparable to van der Kooij et al. (1989) and Escobar and Randall (2001). The significant drop of the AOC to 100 ± 25 µg carbon/L for all ozone doses clearly demonstrates the biological activity of the post-treatment by a high assimilation of organic compounds. Especially for the highest ozone dose of $z_{spec.} = 0.7\ mg_{O3}/mg_{DOC,}$ a significant increase in activity (~ factor 2) as well as a decrease after biological post-treatment was observed. These results show that this specific biological post-treatment setup has the potential to remove the bioavailable organic carbon fraction, which originates from TPs during ozonation (Hammes et al. 2006).

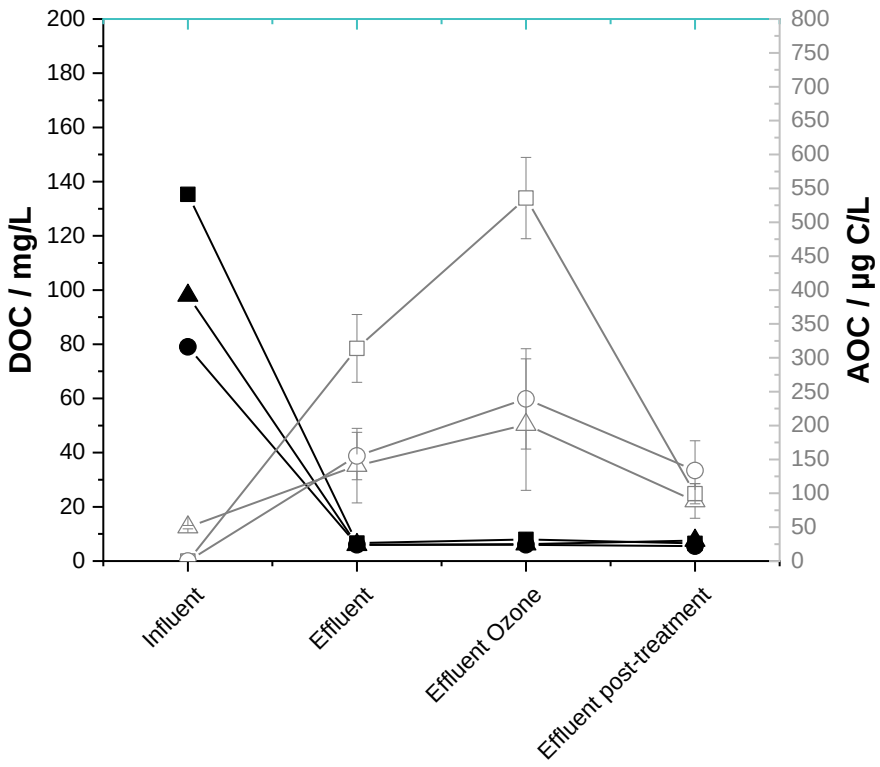

Figure 5-1: DOC (filled symbols) and AOC (open symbols) concentrations at different specific ozone doses (0.7, 0.5, 0.3 mg_{O3}/mg_{DOC}). Rectangle □ = 0.7, circle O = 0.5, triangle Δ = 0.3.

The investigation of the biological activity demonstrates that the AOC could be a powerful parameter to describe the effectivity of a certain biological post-treatment in terms of the reduction of formed TPs during ozonation. Bourgin et al. (2018) compared different biological post-treatments after ozonation at the WWTP Neugut (Dübendorf, Switzerland). A threefold increase of the AOC was observed at a specific ozone dose of 0.55 $mg_{O3}/mg_{DOC,}$ which is higher compared to the here observed increase shown in figure 5-1. This difference could be explained due to the different waste water composition at different WWTPs. The results of Bourgin et al. (2018) showed the highest AOC removal of 50-70% for GAC filters due to the additional sorption. The sand filter showed still a high abatement of 40%. The moving bed reactor had the lowest abatement of 13-17%, which is in contradiction to the results obtained in our study. However, the AOC method applied in this study after van der Kooij et al. (1982) is optimised for drinking water samples with low levels of bacteria, nutrients and DOC and not for waste water. During the experiments in the laboratory, some WWTP influent samples could not be evaluated due to too high bacterial background leading to false values. Therefore, this method needs to be adapted and optimised for an accurate and robust measurement in waste water matrix. As the applied method of van der Kooij uses organisms isolated from drinking water, a more natural inoculum isolated from waste water could help to overcome matrix problems. This approach was already done by Hammes and Egli (2005).

However, using an undefined bacterial consortium a comparability and standardization could be complicated.

5.4.2 Endocrine effects and compounds

5.4.2.1 Estrogen activity

The results of the estrogen effects (figure 5-2) measured with the A-YES assay showed the highest activity of 0.15 ng EEQ/L in week 2. This value is already below the proposed threshold value of 0.4 ng EEQ/L (Jarosova, Blaha, et al. 2014). In week 1, no estrogen activity was detected in the inlet of the treatment plant, possibly due to the type of sample (grab sample). Thus, no data on elimination by conventional treatment can be provided. The activity in week 3 was about 0.09 ng EEQ/L in the waste water treatment plant influent. The variation of the estrogenic activity between the three inlet samples is due to the fact that the experiments were carried out on different days within a period of 3 weeks (composition of the waste water has thus changed). Comparing the estrogenic activity with a prior study on a comparable municipal WWTP (0.4 ng EEQ/L on average), the influent concentration in the actual study is about 4 times lower, which may be due to a different waste water constitution and potentially higher antagonistic activities that had not been studied previously (Itzel et al. 2017).

In the effluent of the conventional treatment an effect similar to the influent was detected in week 3, showing no significant reduction except for week 2 with an elimination of about 53%. As the samples were taken as random samples within 5 h, this increase could be explained by the hydraulic retention time of the conventional treatment, which is already about 24 h. For this reason, it was not possible to take a corresponding grab sample.

The results for the ozonation showed a significant decrease, below the LOD of 0.02 ng EEQ/L in week 3 at zspec. = 0.7 and 0.5 mg_{O3}/mg_{DOC}. Such an elimination rate is comparable to prior studies using ozone in full-scale, in which a reduction below the LOD was observed as well (Itzel et al. 2017). The achieved elimination rates in relation to the effluent of the waste water treatment plant were 55%, 80% and 90% for the ozone doses of zspec. 0.3, 0.5, and 0.7 mg_{O3}/mg_{DOC}. The moving bed reactor further removed the estrogenic effect by 15% at zspec. 0.3 mg_{O3}/mg_{DOC} respectively, which was below the LOD of 0.02 ng EEQ/L. A further removal at the highest ozone dose was not observed.

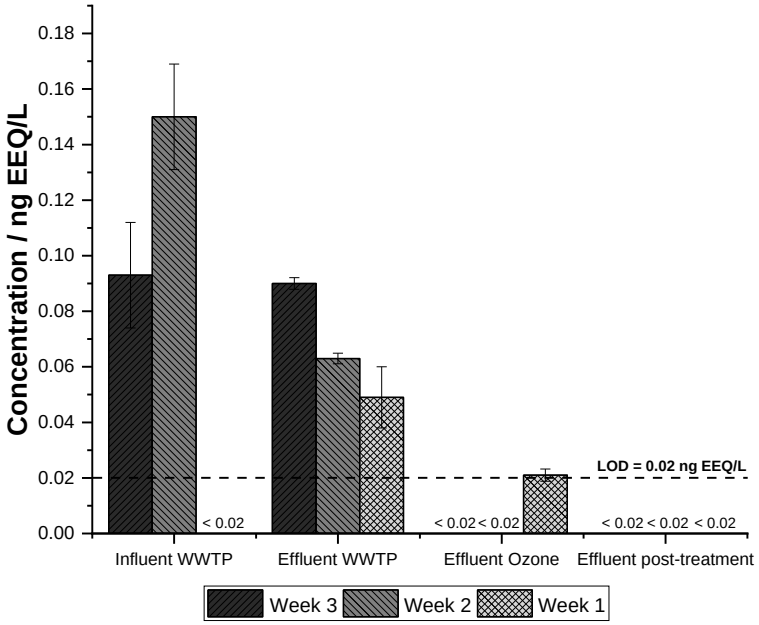

Figure 5-2: Estrogenic activity of random samples after respective treatment steps. Samples were taken for each ozone dose.

The results for anti-estrogenic effects showed the highest inhibition in the inflow of the waste water treatment plant (~90% inhibition). An anti-estrogenic effect was observed in week 1 ($z_{spec.}$ = 0.7 mg_{O3}/mg_{DOC}) after conventional treatment and ozonation, indicating a lower reduction due to ozonation compared to agonistic estrogen effects. This could be an indication that either compounds acting as anti-estrogens are not reduced by ozonation or formed TPs during ozonation could cause an anti-estrogenic effect. In a recent study by Knoop et al. (2018) in which anti-estrogen effects of TPs from the anti-estrogen pharmaceutical tamoxifene were investigated during ozonation, it could be shown that the total effect was not reduced. This is an indication that TPs contribute to the overall effect and thus compensate for the reduction of tamoxifen. Regarding the biological post-treatment, a further reduction (below 10%) could be observed for the sample in week 3 ($z_{spec.}$ = 0.7 mg_{O3}/mg_{DOC}). This is comparable to a prior study by Itzel et al. (2018), at a hospital WWTP using granular activated carbon (GAC) as post-treatment, indicating that antagonistic active compounds are rather biological available and adsorbing to surfaces which is also shown by elimination rates of already 60-70% during conventional treatment (figure 5-3).

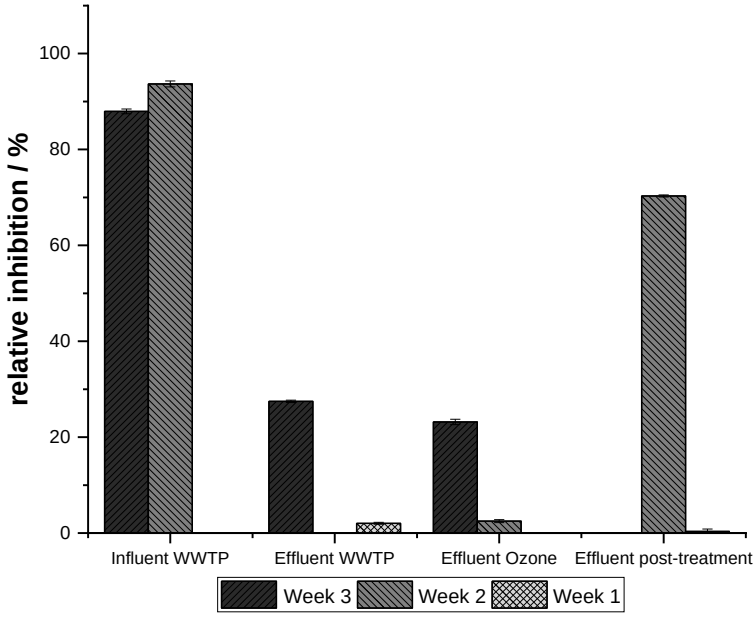

Figure 5-3: Anti-estrogene activites of random samples after respective treatment step. Samples were taken for each ozone dose.

The relatively high anti-estrogen activity, occurring after biological post-treatment, during week 2 (zspec. = 0.5 mg_{O3}/mg_{DOC}), could be due to grab sampling. As the cell growth of the bioassay was always checked, a false positive result from the assay can be excluded. Comparing the anti-estrogen activity, our results are different from prior studies investigating full-scale ozonation at hospital waste water treatment plants (Gehrmann et al. 2018, Itzel et al. 2018). In those studies, a constant anti-estrogenic effect was observed during the conventional and ozone treatment (Gehrmann et al. 2018). Furthermore, even an increasing trend during conventional treatment was observed (Itzel et al. 2018). Since the composition of hospital waste water differs from that of municipal waste water, anti-estrogenic effects could be attributed to different compounds which are characteristic for hospital waste water and municipal waste water. This fact should be investigated in more detail to identify relevant anti-estrogen active compounds. In a study by Buckley (2010) a relative inhibition of estrogenic activity by the WWTP effluent of about 50% was observed. Therewith, a further treatment combination seems to be necessary to improve the reduction of anti-estrogen effects as well. However, as the effluent activity seems to be highly variable, more samples should be investigated to make a representative statement about the inhibition by anti-estrogenic compounds.

5.4.2.2 Androgen activity

The results of androgen activity measured by the A-YAS are shown in figure 5-4. In general, the equivalent concentrations expressed as DHTEQ/L are about 10 times higher compared to estrogen activity. These higher concentrations are probably due to higher excretion rates in humans. It is known that in human urine levels of androgens are about 10-100 times higher compared to estrogens and are found in waste water in similar ratios (Shore and Shemesh 2003, Chang et al. 2011). Due to spot sampling at different weeks, the influent concentrations vary between LOD and 0.75 ng DHTEQ/L. The elimination rates for low (week1, $z_{spec.} = 0.3$), medium (week 2, $z_{spec.} = 0.5$) to high (week 3, $z_{spec.} = 0.7$) ozone dose were between 50-70% compared to the effluent of the WWTP. At high ozone concentrations the highest elimination would be expected, however the ratio seems to remain constant, which is probably due to variation prior to ozonation. These results are in accordance to those found in prior investigations with elimination rates of about 30-60% (Itzel et al. 2017).

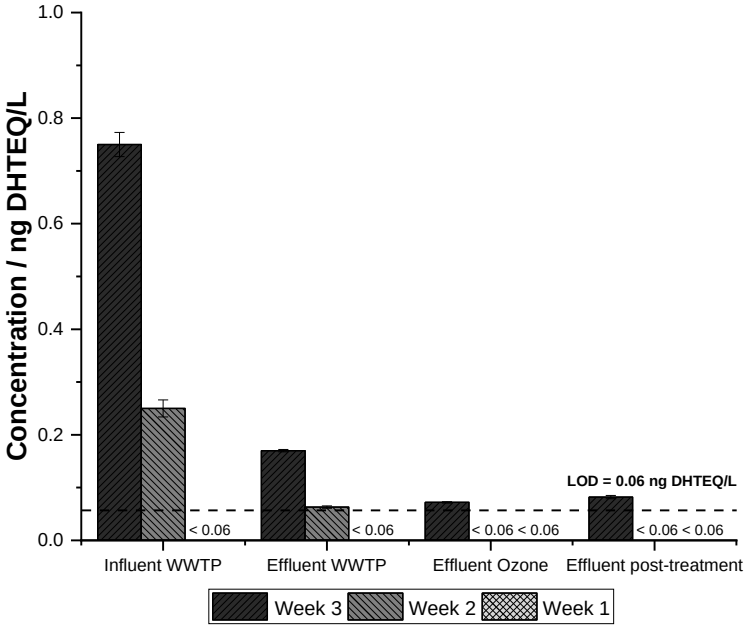

Figure 5-4: Anti-androgen activites of random samples after respective treatment step. Samples were taken for each ozone dose.

A similar trend of increased activity after ozonation was already shown in a different study investigating hospital waste water after full-scale ozonation (Gehrmann et al. 2018, Itzel et al. 2018). The release of anti-androgen active compounds to the receiving surface water can lead to ecotoxicological issues like those by estrogens. In a study by Jobling et al. (2009) it was demonstrated that anti-androgens are responsible for sexual disruption in fish of rivers in the UK. One option to further reduce anti-androgen activity after ozone treatment could be

the application of granular activated carbon (GAC) (Itzel et al. 2018). Due to the ecological relevance of anti-androgen active compounds, several studies started to identify potential candidates of known compounds. Compounds being under suspicion are highly diverse including PCBs, PAHs, flame retardants, pesticides as well as pharmaceuticals (Liscio et al. 2014, Li et al. 2013, Kortenkamp et al. 2014, Rostkowski et al. 2011).

Recent studies are working on effect-based trigger values to be able to use results of bioassays for evaluation without identification of single compounds (van der Oost et al. 2017, Escher et al. 2018, Wernersson et al. 2015). This point is discussed in more detail in chapter 2 of this thesis.

5.4.3 Chemical target analysis

5.4.3.1 PPCPs and Pesticides

In figure 5-5 the elimination results of all analysed 24 h composite samples are shown. Due to ozonation system failure at the highest ozone dose of zspec. = 0.7 mgO3/mgDOC, only random samples were taken for this setting. Elimination of 24 h composite samples would be expected to be ≥ 80% for zspec. = 0.7, as prior investigations showed elimination of 80% at a specific ozone dosage of zspec. = 0.3 - 0.4 mg_{O3}/mg_{DOC} (Götz et al. 2011, Abegglen et al. 2009, Hollender et al. 2009). For the highest ozone dose of zspec. = 0.7 mg_{O3}/mg_{DOC} the elimination for all PPCPs was around 86% and thus within the Swiss regulation of 80% annual average elimination (GschV 814.201). However, the results presented are only based on grab samples and longer periods should be monitored to provide an annual average elimination. The lower ozone dose of zspec = 0.5 mg_{O3}/mg_{DOC} showed slightly lower elimination rates of around 66% and for zspec. = 0.3 mg_{O3}/mg_{DOC} the average elimination rates were around 42%. Elimination rates of some specific compounds such as diclofenac, carbamazepin and clarithromycin are within the expected range of elimination (80%) compared to literature in which 80% elimination are realised at zspec. of 0.3 - 0.4 mg_{O3}/mg_{DOC} (Itzel et al. 2017, Hollender et al. 2009).

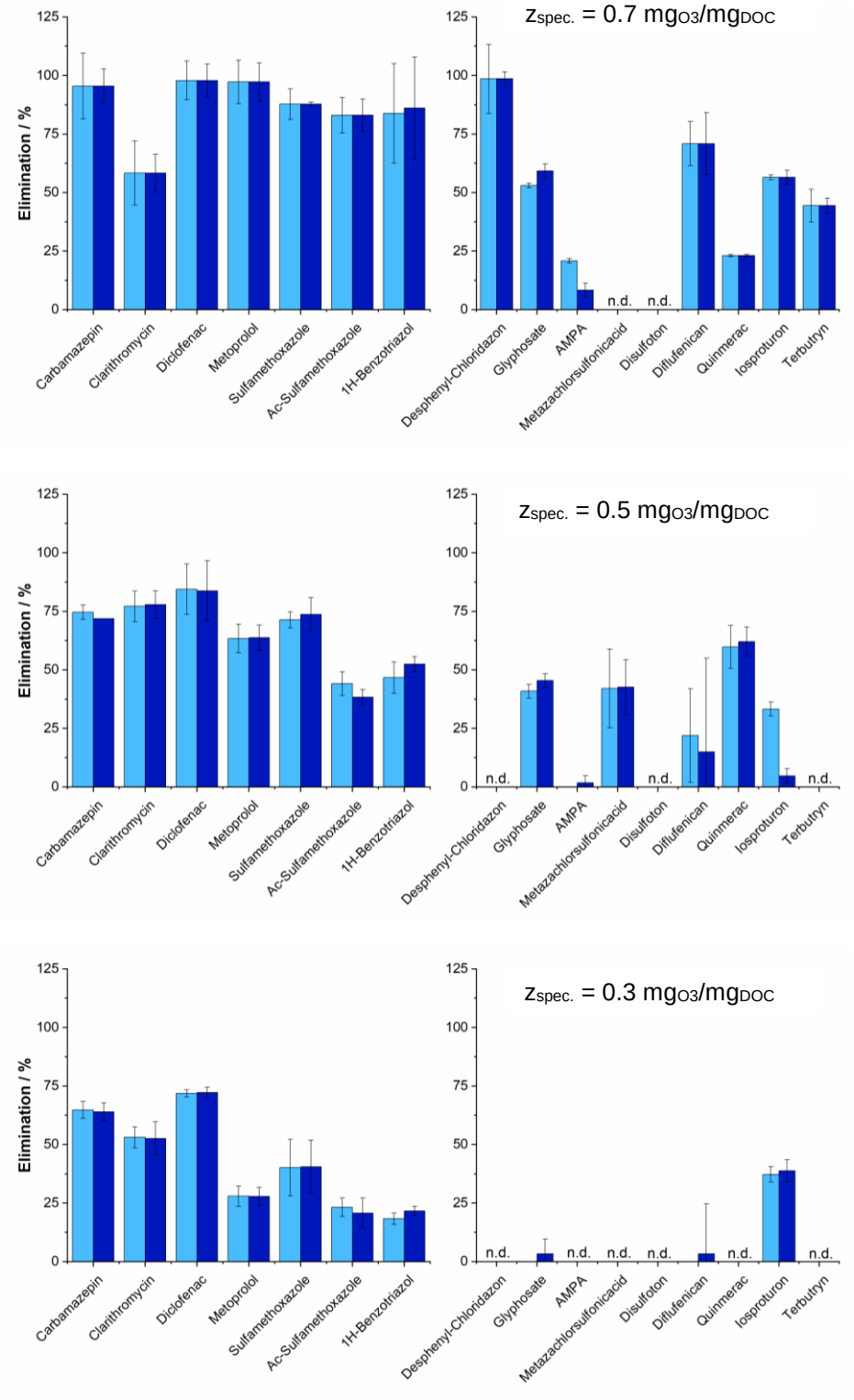

Figure 5-5: Elimination of PPCPs (left - all samples including 24 h composite samples, n = 5) and pesticides (right - random samples, n = 3) at different specific ozone concentrations (0.5, 0.3mg_{O3}/mg_{DOC}) except for 0.7 mg_{O3}/mg_{DOC}, there are random samples shown due to technical errors. Light blue bars represent the elimination after ozone. Dark blue bars represent the overall elimination including ozone and biological post-treatment. Substances for which elimination could not be calculated have the abbreviation n.d. (no data).

Since the pesticides were always collected as random samples, the results were not influenced by the failure of ozonation during the 24h sampling. As expected, the highest elimination rate is at the highest ozone dose ($z_{spec.}$ = 0.7 mg_{O3}/mg_{DOC}). The lower the ozone dose the lower are the elimination rates, which is as expected. However, the compound isoproturon has still an elimination rate of 40% at the lowest ozone dose of $z_{spec.}$ = 0.3 mg_{O3}/mg_{DOC}. Studies on the elimination of pesticides from waste water by ozone and biological post-treatment are hardly available, as these substances are more likely to be used in agriculture and investigated in surface runoff waters. However, in a study of selected pesticide fate during conventional WWTPs no elimination was found (Kock-Schulmeyer et al. 2013). The reported concentration in the inlet was in the range of 1 µg/L for most substances, which is comparable to our investigations. In a recent study by Westlund et al. (2018), pesticides were investigated during ozonation of waste water. It was found that despite the higher ozone dose applied of $z_{spec.}$ = 2 mg_{O3}/mg_{DOC} (DOC assumed to be 7 mg/L, based on empirical data from other studies), most of the investigated substances could be reduced by less than 50%. Compared with our results, pesticides seem to have a more recalcitrant nature during ozonation and the applied post-treatment. In general, a significant influence of the biological post-treatment regarding the analysed compounds was not observed. In addition to the elimination performance of selected compounds, the final concentrations must comply with respective EQS. Within this study, none of the EU WFD regulated compounds were found to exceed the EQS at any ozone dose, showing a successful ozonation in terms of the discharge of regulated compounds.

5.4.4 HRMS analysis

5.4.4.1 Non-target investigations

The non-target analysis using the software MZmine 2 resulted in 2211 features which yielded 822 detected peaks in the inflow of ozonation at the highest ozone dose of $z_{spec.}$ = 0.7. The peak alignment and following trend analysis showed a reduction of relevant compounds by 60%, resulting in 336 residual compounds. Simultaneously, a formation of 241 new compounds by ozonation was observed. As the SPE as well as the chromatographic method during the LC run was done by a balanced phase (polar/non-polar), also transformation products should be able to detect to some extent. However, this procedure also represents a certain selectivity, which limits the analytical window. Nevertheless, the 241 peaks only detected after ozonation are highly probable to be TPs. The predicted sum formulae support the assumption, by a high number of oxygen atoms compared to other peaks (Table SI 7.3-7). A further data filtration was done to evaluate the elimination efficiency of the biological post-treatment. The result showed the elimination of 230 TPs (11 residual peaks) during

ozonation, leading to a TP elimination rate of 95% by the biological post-treatment. This tracking of formed and eliminated TPs shows the efficiency of the post-treatment after ozone (figure 5-6).

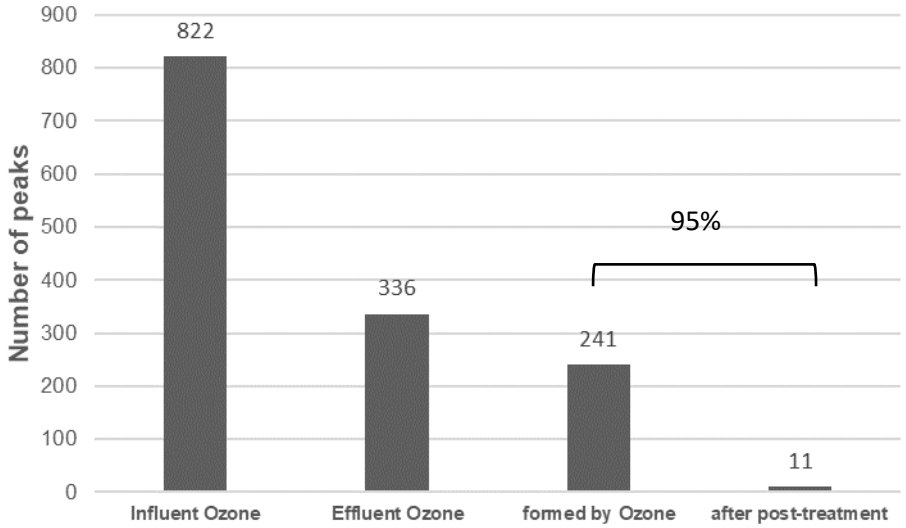

Figure 5-6: Detected peaks and potential TP elimination by biological post-treatment. Residual 336 features were detected after ozonation. 241 features were only detected after ozonation and are potential TPs.

As the 11 formed compounds are released into the receiving water, they are of major interest due to lack of information about their toxicity and structure. The corresponding MS^2 spectra, retention times as well as predicted sum formulae are shown in table SI 7.3-7. As these results are only based on a single random sample, further sampling should be done to assess the relevance of these 11 compounds. The trends of individual peaks which were detected through the whole treatment are shown in figure 8. In total, profiles could be created from influent ozone to the effluent post-treatment of all 822 peaks detected in the influent ozone. The results could be clustered into three major groups: increasing after biological post-treatment, constant during biological post-treatment and elimination during biological post-treatment (figure 5-7).

The first group comprising increasing signal area of detected features could be due to matrix interferences as the increase shown on the left of figure 5-7 is not significant when compared to the influent. The second group of constant signal area of detected features are compounds not influenced by ozonation and biological post-treatment or formed compounds not further reduced by the post-treatment, as already shown in table SI 7.3-7 by 11 residual TPs. The last group of decreasing signal area could be attributed to compounds which are biologically degradable and getting reduced by the biological post-treatment. In this case formed TPs are shown which are completely removed as well as compounds which are

rather constant during ozonation but getting reduced in the biological post-treatment. Similar results were shown by Deeb et al. (2017), in which a significant removal of formed TPs was achieved by a biological post-treatment (fluidized moving bed-reactor). These results indicate that the non-target approach in combination with meta information such as the AOC is a useful tool to evaluate the efficiency of treatment steps in case of unknown compounds.

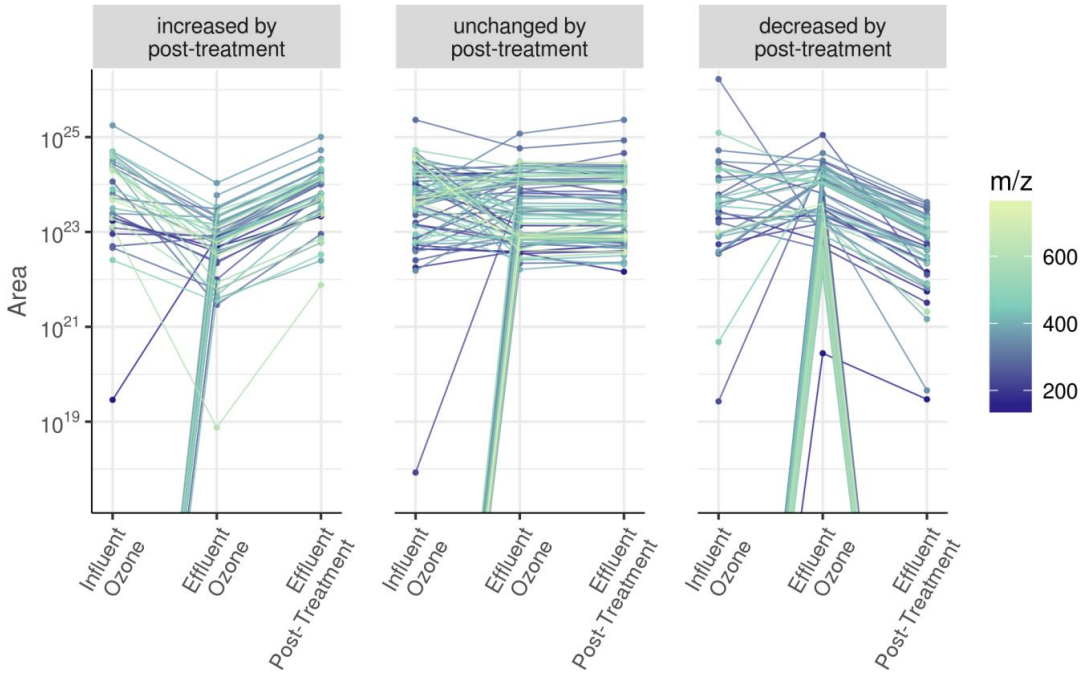

Figure 5-7: Results of the trend analysis (n = 822). Results were clustered into 3 major groups with regard to the biological post-treatment.

Comparing the formation and elimination in figure 5-6 and 5-7 with the trend of the AOC and the anti-androgen activity analysis for the ozone setup of $z_{spec.}$ = 0.7, a correlation can be observed. As the TPs are smaller, potentially more polar compounds due to oxidation, the biological assimilation is promoted so that the increase in AOC should correlate with the number of formed TPs. This correlation supports the hypothesis that TPs are relevant for the AOC result. However, correlations are based on only one sampling and should be further investigated. As peak picking and the identification of features is still difficult especially for TPs, meta information are very important to reduce the number of relevant features (Brack 2003, Itzel et al. 2018, Weiss et al. 2011).

5.5 Conclusions

This study on the efficiency and evaluation of biological post-treatment after ozonation showed, that concentrations of known target parameters such as pharmaceuticals and pesticides cannot be further reduced. However, endocrine effects could be further reduced in some samples. Especially anti-androgenic effects showed a correlation to the applied ozone dose and could be reduced by the biological post-treatment. Here, further investigations must be carried out to check a possible correlation of anti-androgenic effects to formed TPs. Antagonistic activity in general showed no significant reduction during ozonation and should be further monitored as such inhibitory antagonistic effects are also suspected to interfere with the endocrine system of aquatic organisms. The high diversity of compounds potentially able to lead to an anti-androgen effect, illustrates the need of further investigations and set up of regulations and limit values to evaluate results of anti-androgen as well as anti-estrogen activity. Since biological post-treatment steps are installed for the removal of unknown TPs formed in ozonation, the use of non-target screening can be considered very useful for evaluation. To evaluate a certain biological post-treatment in terms of its biological activity, the AOC seems to be a powerful parameter. In combination with non-target analysis, substances which are not retained and thus are potentially relevant for the receiving water body could thereby be identified in future research.

5.6 References

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6. General Conclusion and Outlook

6.1 General conclusion

The investigations of this PhD thesis demonstrate that advanced waste water treatment for micropollutant removal using ozone successfully reduces indicator parameters ≥ 80% at ozone doses in the range of $z_{spec.}$ = 0.3 - 0.7 mg_{O3}/mg_{DOC}. Therewith, the applied procedures are within the Swiss requirements of an annual average elimination rate of 80% (GschV 814.201 2016). Regarding the EU WFD, already at low ozone doses the concentrations of selected compounds were below the environmental quality standards of surface waters in the effluent of the treatment plants. These results are comparable to other studies investigating the implementation of ozone as advanced waste water treatment (Hollender et al. 2009, Götz et al. 2010, Gruenebaum et al. 2014, Knopp et al. 2016, Böhler et al. 2017). Regarding the elimination and fate of estrogen active compounds (E1, E2, EE2) listed on the watch-list of the EU WFD, evaluation is still challenging with current instrumental methods due to required limits of quantification in the pg/L range (Locatelli et al. 2016, Valitalo et al. 2016, Loos et al. 2018). Main limitations were shown to be due to matrix interferences, consequently requiring high effort during sample enrichment and clean-up. Therefore, this thesis presents an effect-based approach by a comprehensive implementation of sensitive bioassays and instrumental analysis. To evaluate an endocrine effect, the analysis of single compounds such as the most potent compounds E1, E2 and EE2 is insufficient due to instrumental detection limits and neglecting of the whole sample effect such as inhibitory effects.

Furthermore, synergistic as well as antagonistic effects are traced back to a wide range of compounds, which make an integrative approach necessary. High elimination rates for estrogenic as well as androgenic effects known from other studies could be confirmed in this thesis at low ozone doses ($z_{spec.}$ = 0.4 ± 0.1 mg_{O3}/mg_{DOC}) (Huber et al. 2004, Abegglen et al. 2009, Margot et al. 2013). However, in some samples, androgenic effects also showed significant lower elimination rates (<90%) compared to estrogenic effects and should thus be considered more carefully in future studies.

Furthermore, due to low LODs, the applied A-YES assay could be used as a screening tool for monitoring of estrogens under the EU WFD. However, it could also be shown that antagonistic effects reduce the detected agonistic effects and it is therefore important to measure them in parallel. Especially if correlations to single compounds are desired, combined effects are very important to consider due to potentially false negative or false positive results (Orton et al. 2012, Ihara et al. 2014). As antagonistic effects such as anti-estrogen and anti-androgen effects are not much studied, relevant compounds are still unknown in most cases (Tousova et al. 2017). This thesis presented also a comprehensive

approach using effect based methods in combination with non-target analysis for an identification of unknown active compounds. Typical non-target analysis leads to a high number of signals (features) from which it is hard to identify relevant compounds. Therefore, a prioritization of relevant compounds can help to filter relevant signals (Brack et al. 2016). The presented method based on a developed algorithm to exclude non relevant signals was able to reduce the number of features by about 2 orders of magnitude, which makes a manual database matching much more efficient and faster. The identified flame retardant TCEP showed together with compounds from other studies that antagonistic active compounds are highly diverse. In addition to correlation analysis, antagonistic effects are potentially relevant for the aquatic environment as they are not significantly reduced in ozonation and may lead to toxic effects in organisms (Jobling et al. 2009, Gehrmann et al. 2018). Knoop et al. (2017) investigated the formation of antagonistic active transformation products (TPs) during ozonation and could show that TPs of the antagonistic active pharmaceutical Tamoxifene still pose a significant effect. Due to the toxic uncertainty of formed TPs in general, biological post-treatments are already recommended in some studies as well as in Switzerland by legal requirements, to further eliminate those (GschV 814.201 2016, Hollender et al. 2009, Prasse et al. 2015). To be able to evaluate the treatment efficiency of biological post-treatments the assimilable organic carbon (AOC) was demonstrated to be a useful parameter to characterize the biological activity. To investigate the removal efficiency of formed TPs during the biological post-treatment, an algorithm based on non-target data was successfully developed to enable a trend analysis. Therewith it is possible to track formed features, which are potentially generated TPs, through the different treatment processes. It was shown that about 95% of the TPs were successfully removed by a moving bed biofilm reactor. Whenever applying non-target approaches, it is highly important to keep in mind that the single steps during treatment as well as the measurement are affecting the final results. Important steps are for example sample handling, enrichment and clean-up, chromatographic conditions, setup of the mass spectrometric detection and used software for data evaluation which will affect the number and type of detectable features. Therefore, results can only be interpreted relative to a certain method.

6.2 Outlook

Future studies should focus on agonistic as well as antagonistic activities during waste water treatment processes and investigate different biological post-treatments potentially able to reduce formed TPs and persistent antagonistic effects. For example, granulated activated carbon (GAC) was already shown to reduce anti-androgen activity and should be systematically investigated as one further treatment option (Ma et al. 2017).

To evaluate the biological activity of a certain biological post-treatment, the AOC was shown to be a suitable parameter. Comparable results were shown by a study of Bourgin et al. (2018). However, the method used in this thesis was originally developed for the analysis of low nutrient drinking water (van der Kooij et al. 1982, van der Kooij et al. 1989) and thus should be optimized and validated for waste water effluent matrix as well. Therefore, other organisms should be tested, which are probably more robust to waste water matrix. Hammes et al. (2008) developed an AOC protocol using flow-cytometry instead of cultivation based heterotrophic plate counts. The flow cytometry allows the analysis of considerably more samples in a shorter time and thus a higher validity of the results.

Future work should therefore be done to combine robust organisms and flow-cytometry to allow a fast evaluation of biological post-treatments.

As only limited information on detected antagonistic effects is known, an identification of the most relevant compounds should be in the focus of future investigations to be able to explain the origin of such compounds and develop potential measures to minimize the discharge. One option discussed in the scientific community is the effect directed analysis (EDA) approach by the combination of effect-based and instrumental methods (Brack et al. 2016, Di Paolo et al. 2016, Tousova et al. 2017). The developed procedure in this thesis is based on fractionation using HPLC and subsequent effect-based and HRMS analysis. Fractions were filled in separate vials and further analyzed manually. To save time and allow higher sample throughput, an automatization of all system components should be aimed at. One option could be the use of thin layer chromatography together with effect based methods utilizing different endpoints and subsequent HRMS analysis of selected bands (Morlock and Schwack 2010, Buchinger et al. 2013, Chamas et al. 2017, Stutz et al. 2017). Using more than only one endpoint will further narrow down the most relevant compounds. In addition, intelligent algorithms should be developed to add meta information and get connected to online databases. Therewith, databases, which still lack environmental data, could be easily enlarged and increase the chance of identification. In terms of non-target analysis, ion mobility spectrometry could be added as further characterization option prior to HRMS analysis potentially leading to a higher probability to be able to identify unknown compounds (Hinnenkamp et al. 2018, Lu et al. 2018).

To directly apply effect based methods within the EU WFD and therewith to overcome limitations by instrumental analysis, effect-based trigger values (EBT) need to be derived. First approaches are already done in different studies but still lack data for antagonistic activity of relevant compounds (van der Oost et al. 2017, Escher et al. 2018). However, EBTs would make it possible to screen and evaluate surface waters independent of single compounds.

6.3 References

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7. Appendix

7.1 Chapter 3 – supplementary material

7.1.1 Plant describtion

A more detailed overview and the respective locations of the sampling points as well as a schematic reactor design are shown in figure SI 7.1-1.

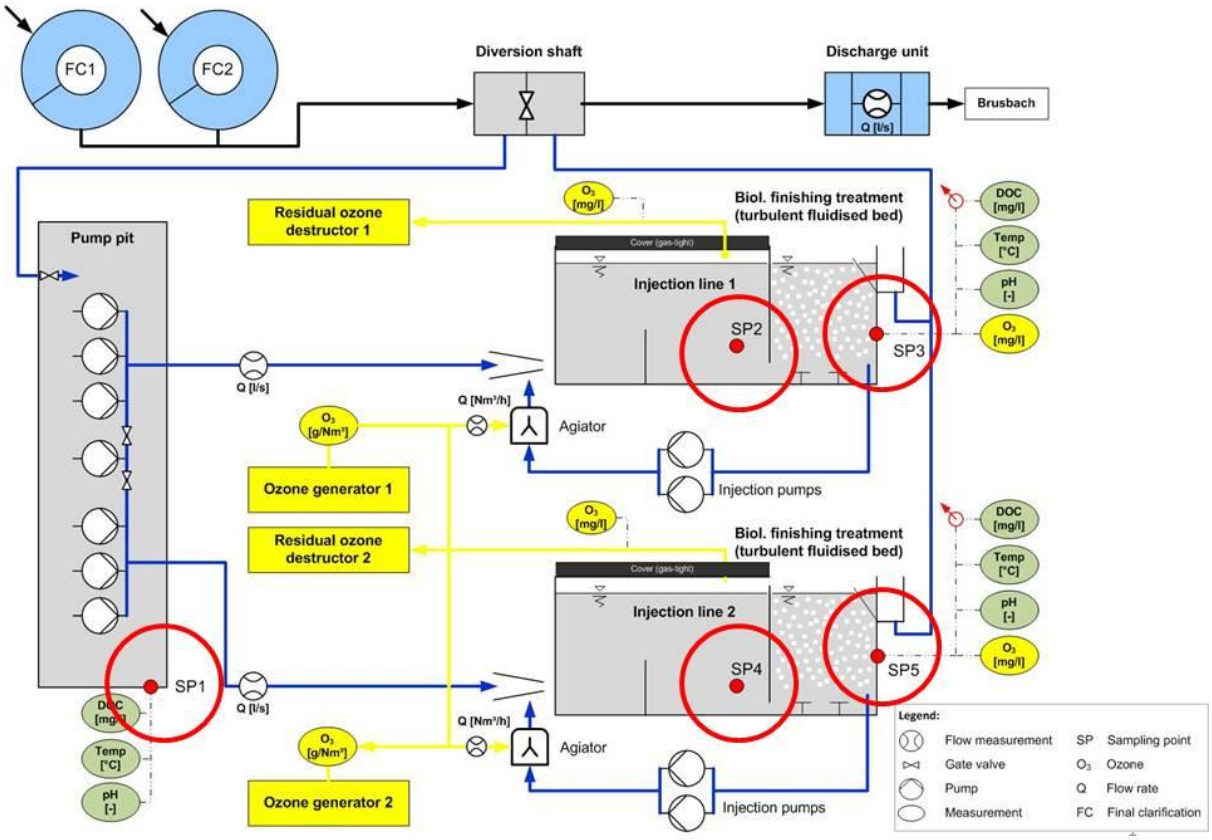


Figure SI 7.1-1: Flow-chart of the ozonation unit with two treatment lines in parallel. Sampling points (SP) are shown in circles. SP1 = influent ozonation, SP2 = effluent ozone line 1, SP3 = final effluent after biological post-treatment of line 1, SP4 = effluent ozone line 2, SP5 = final effluent after biological post-treatment of line 2.

7.1.2 Chemical analysis

Table SI 7.1-1 till 7.1-3 show the multiple reaction monitoring (MRM) transitions of the investigated substances and internal standards together with the retention times, DP, CE and CXP energies. The mass transfer for quantitation is indicated by (Q) and the mass transfer for the validation is indicated by (V) in the Tables.

Table SI 7.1-1: Retention times and MS/MS-parameters for detection of micropollutants after positive electrospray ionisation (ESI+). (Q) Quantitation-MRM, (V) Validation-MRM.

MRM m/z	Time (min)	Substance	DP (Volts)	EP (Volts)	CE (eV)	CXP (Volts)
120.1 → 65.2	2.10	1H-Benzotriazol (Q)	51	10	31	4
120.1 → 92.1	2.10	1H-Benzotriazol (V)	51	10	23	4
134.0 → 76.8	3.50	5-Methyl Benzotriazol (Q)	51	10	10	4
134.0 → 79.1	3.50	5-Methyl Benzotriazol (V)	51	10	10	4
237.1 → 194.2	5.70	Carbamazepine (Q)	126	10	28	4
237.1 → 193.1	5.70	Carbamazepine (V)	126	10	43	4
748.5 → 158.1	6.60	Clarithromycin (Q)	80	10	35	4
748.5 → 83.0	6.60	Clarithromycin (V)	80	10	50	4
296.0 → 214.1	8.20	Diclofenac (Q)	80	10	50	4
296.0 → 215.1	8.20	Diclofenac (V)	80	10	20	4
268.2 → 133.1	3.20	Metoprolol (Q)	102	10	35	4
268.2 → 103.1	3.20	Metoprolol (V)	102	10	50	4
254.1 → 156.1	2.80	Sulfamethoxazole (Q)	85	10	23	4
254.1 → 92.1	2.80	Sulfamethoxazole (V)	85	10	40	4
247.2 → 204.2	5.50	Carbamazepine_d10	51	10	29	4
300.0 → 218.0	8.20	Diclofenac_d4	26	10	41	4
275.0 → 123.1	3.10	Metoprolol_d7	41	10	27	8
258.1 → 96.1	2.70	Sulfamethoxazole_d4	170	10	46	10

For endocrine active compounds the ionisation were done in positive and negative ionisation mode (Table SI 7.1-2).

Table SI 7.1-2: Parameter setup for the MS-Detection of endocrine active compounds, (Q) Quantitation, (V) Validation, positive ionisation mode (ESI+).

MRM m/z			Time (min)	Substance	DP (Volts)	EP (Volts)	CE (eV)	CXP (Volts)
270.9	→	115.2	4.57	Trenbolon (Q)	60	11	97	4
270.9	→	165.0	4.57	Trenbolon (V)	100	11	79	4
285.1	→	121.1	4.13	Boldion (Q)	100	5.5	31	4
285.1	→	77.0	4.13	Boldion (V)	100	5.5	71	4
289.0	→	271.3	6.25	Dehydroepiandrosteron (Q)	60	7.5	13	4
289.0	→	253.4	6.25	Dehydroepiandrosteron (V)	60	7.5	17	6
291.0	→	255.3	7.05	Dihydrotestosteron (Q)	60	10.5	23	4
291.0	→	91.1	7.05	Dihydrotestosteron (V)	60	10.5	79	4
291.1	→	255.3	8.10	Etiocholanon (Q)	60	7.5	19	4
291.1	→	215.3	8.10	Etiocholanon (V)	60	7.5	23	4
291.2	→	273.5	8.09	Androsteron (Q)	60	9	15	4
291.2	→	77.0	8.09	Androsteron (V)	60	9	19	4
287.2	→	121.1	4.91	Boldenon (Q)	100	4	35	4
287.2	→	135.3	4.91	Boldenon (V)	100	4	19	4
299.4	→	109.2	5.40	19-Norethindron (Q)	100	4.5	35	4
299.4	→	91.2	5.40	19-Norethondron (V)	100	4.5	59	4
273.1	→	107.2	5.44	E2 (Q)	60	6	37	4
273.1	→	135.3	5.44	E2 (V)	60	6	23	4
297.2	→	107.0	5.36	EE2 (Q)	60	6.5	37	4
297.2	→	77.2	5.36	EE2 (V)	140	6.5	75	2
289.2	→	97.1	5.34	Testosterone (Q)	100	10	35	4
289.2	→	109.1	5.34	Testosterone (V)	100	10	35	4

Endocrine active compounds and internal standards which are measured by ESI negative mode are listed in Table SI 7.1-3.

Table SI 7.1-3: Parameter setup for the MS-Detection of endocrine active compounds, (Q) Quantitation, (V) Validation, negative ionisation mode (ESI-).

MRM m/z			Time (min)	Substance	DP (Volts)	EP (Volts)	CE (eV)	CXP (Volts)
269.4	→	144.8	5.04	E1 (Q)	-140	-2	-50	-2
269.4	→	143	5.04	E1 (V)	-140	-2	-50	-2
271.1	→	183.1	5.44	E2 (Q)	-200	-10	-54	-9
271.1	→	144.9	5.44	E2 (V)	-200	-10	-52	-15
295.1	→	145.0	5.36	EE2 (Q)	-155	-10	-48	-13
295.1	→	159.0	5.36	EE2 (V)	-155	-10	-48	-17
287.2	→	145	0.72	E3 (Q)	-140	-5.5	-56	-2
287.2	→	170.8	0.72	E3 (V)	-140	-5.5	-44	-2
267.1	→	236.9	6.23	Diethylstilbestrol (Q)	-80	-2	-36	-2
267.1	→	222.2	6.23	Diethylstilbestrol (V)	-80	-2	-42	-2
273.1	→	147.1	5.00	E1d4_1	-130	-10	-50	-17
273.1	→	145.1	5.00	E1d4_2	-130	-10	-72	-19
274.1	→	185.0	5.40	E2d3_1	-150	-10	-54	-23
274.1	→	144.9	5.40	E2d3_2	-150	-10	-52	-15
299.1	→	147.0	5.30	EE2d4_1	-135	-10	-50	-15
299.1	→	161.0	5.30	EE2d4_2	-135	-10	-46	-17

7.1.3 Detected endocrine substances

The results of the detected endocrine active compounds of all samplings are shown in Table SI 7.1-5. Compounds which were not found in any sample are not shown. In case of detected compounds below the limit of detection (LOD), the LOD is written (see Table SI 7.1-4).

Table SI 7.1-4: Limits of quantification (signal to noise ratio: 10:1) and limit of detection (signal to noise ratio: 3:1) with regard to enrichment factor of 1000. TRE=Trenbolone, BOL=Boldione, DHEA=Dihydroepiandrosterone, DHT=Dihydrotestosterone, ETIO=Etiocholanone.

	TRE	BOL	DHEA	DHT	ANDRO	TE	E2	E1	EE2	E3	DES
LOD (ng/l)	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.2	0.05	2	0.9
LOQ (ng/l)	0.07	0.07	0.07	0.07	0.07	0.07	0.07	0.7	0.20	7.00	3.00

Table SI 7.1-5: Detected Concentrations of the LC-MS/MS measurements (n= 4). TRE=Trenbolone, BOL=Boldione, DHEA=Dihydroepiandrosterone, DHT=Dihydrotestosterone, ETIO=Etiocholanone, ANDRO=Androsterone, E2=17ß-estradiol, EE2=17α-ethinylestradiol, TE=Testosterone, E1=Estrone, E3=Estriol, DES=Diethylstilbestrol. Limit of detection (LOD) = signal to noise ratio of 3:1, limit of quantification (LOQ) = signal to noise ratio of 10:1.

		TRE	BOL	DHEA	DHT	ANDRO	TE	E2	E1	EE2	E3	DES
Date	**Sampling Point**					**c (ng/l)**						
22.01.2015	**Influent Ozone-System**	42.9	3.7	65.3	3.2	<0.02	1.2	<0.02	<0.2	<0.05	13.3	20.9
	Effluent Ozone Line 1	32.9	2.2	96.9	0.5	<0.02	0.8	<0.02	<0.2	<0.05	21.7	11.6
	Effluent Ozone Line 2	21.5	2.7	66.1	2.9	<0.02	0.8	<0.02	<0.2	<0.05	<2	15.2
	Final Effluent Line 1	12.4	1.4	48.8	<0.02	<0.02	<0.02	<0.02	<0.2	<0.05	<2	5.2
	Final Effluent Line 2	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.2	<0.05	<2	<0.9
28.01.2015	**Influent Ozone-System**	<0.02	1.4	<0.02	<0.02	8.8	<0.02	<0.02	<0.2	4.4	<2	21.0
	Effluent Ozone Line 1	<0.02	1.8	<0.02	<0.02	1.8	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Effluent Ozone Line 2	<0.02	1.4	<0.02	<0.02	4.1	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Final Effluent Line 1	<0.02	1.8	<0.02	<0.02	1.5	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Final Effluent Line 2	<0.02	2.6	<0.02	<0.02	4.6	<0.02	<0.02	<0.2	<0.05	<2	<0.9
11.02.2015	**Influent Ozone-System**	0.9	1.6	<0.02	<0.02	7.5	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Effluent Ozone Line 1	<0.02	1.5	<0.02	<0.02	7.7	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Effluent Ozone-Line 2	<0.02	1.2	<0.02	<0.02	4.0	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Final Effluent Line 1	<0.02	1.5	<0.02	<0.02	5.5	<0.02	<0.02	<0.2	<0.05	<2	<0.9
	Final Effluent Line 2	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.02	<0.2	<0.05	<2	<0.9
21.04.2015	**Influent Ozone-System**	31.4	2.2	82.7	1.2	<0.02	0.9	<0.02	<0.2	<0.05	9.4	34.8
	Effluent Ozone Line 1	17.1	2.0	23.6	2.0	<0.02	0.4	<0.02	<0.2	<0.05	<2	11.3
	Effluent Ozone Line 2	23.4	2.0	62.7	<0.02	<0.02	0.7	<0.02	<0.2	<0.05	<2	8.3
	Final Effluent Line 1					*no samples*						
	Final Effluent Line 2					*no samples*						

7.1.4 Biological analysis

7.1.4.1 Biofilm growth

The results for line 1 and 2 are shown in figure SI 7.1-2. Data before reconstruction is not available for line 2 because this line was not equipped with a biological post treatment before.

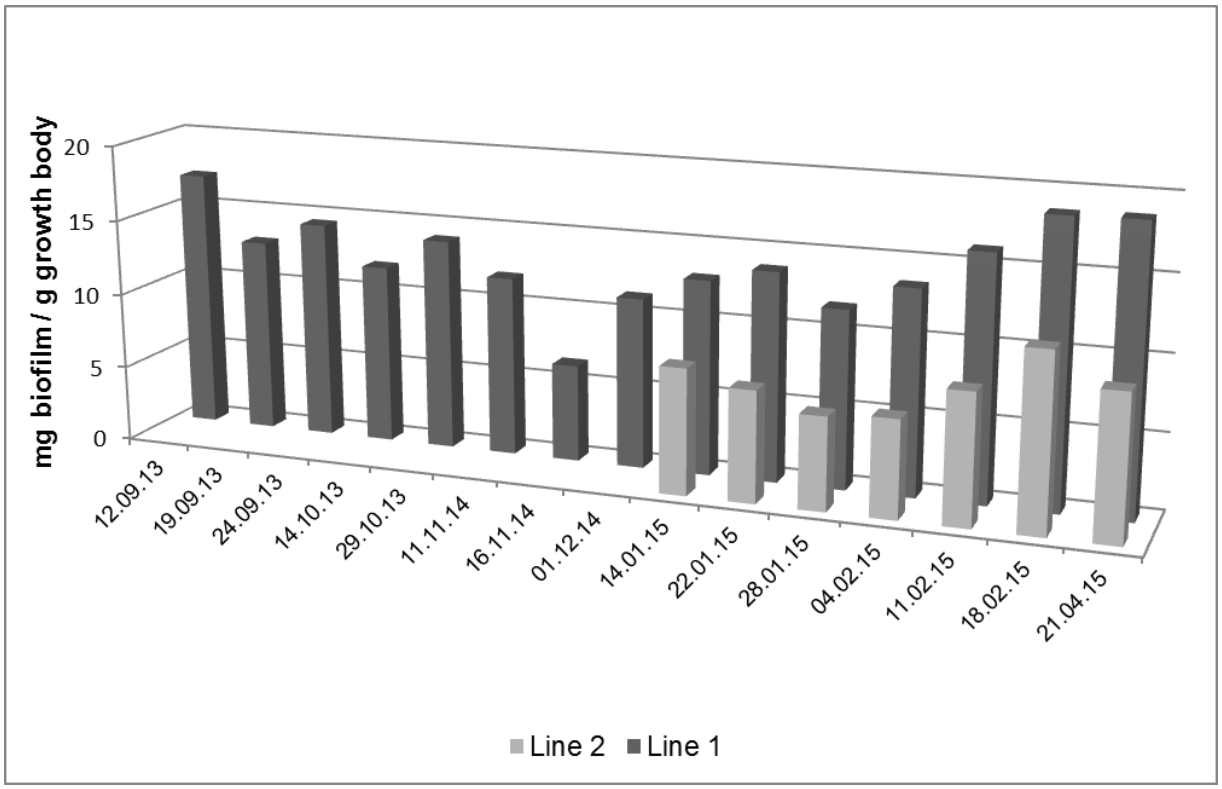


Figure SI 7.1-2: Biofilm formation on the different sampling days shown for the time before (dark grey?) and after reconstruction (light grey?). The bars represent the average biofilm mass per growth body in mg (n = 3).

7.1.4.2 A-YES test

The 17β-estradiol equivalent (EEQ) concentrations of the respective enriched samples are listed in table SI 7.1-6.

Table SI 7.1-6: Estrogenic activity measured inthe A-YES. N.a.=not available due to sampling problems. Concentrations shown as ng 17β-Estradiol equivalents (EEQ)/l.

Sampling Date	14.01.	22.01.	28.01.	04.02.	11.02.	21.04.	Mean	SD
				c (ng EEQ/l)				
Influent Ozone	0.14	1.60	1.20	0.10	0.18	0.06	0.55	0.68
Effluent Ozone Line 1	0.08	0.12	0.08	0.04	0.05	<0.03	0.06	0.04
Final Effluent Line 1	0.07	0.08	<0.10	0.04	0.05	<0.03	0.05	0.04
Effluent Line 2	n.a.	0.06	<0.10	0.10	n.a.	<0.03	0.06	0.04
Final Effluent Line 2	n.a.	n.a.	0.06	n.a.	0.05	0.03	0.04	0.01

LOD = 0.032 - 0.10 ng EEQ/L (depending on daily performance). Negative samples were considered for mean value calculation by the half of the daily LOD.

7.1.4.3 A-YAS test

Androgenic activity measured in the A-YAS assay is shown in table SI 7.1-7 as Dihydrotestosterone equivalent (DHTEQ) concentrations.

Table SI 7.1-7: Androgenic activity measured in the A-YAS . N.a. = not available due to sampling problems. Concentrations shown as ng Dihydrotestosterone equivalents (DHTEQ)/L.

Sampling Date	14.01.	22.01.	28.01.	04.02.	11.02.	21.04.	Mean	SD
				c (ng DHTEQ/l)				
Influent Ozone	1.30	1.60	3.80	1.60	0.43	1.75	1.24	0.68
Effluent Ozone Line 1	1.20	0.96	0.91	1.40	0.51	1.00	0.34	0.04
Final Effluent Line 1	0.16	0.42	0.45	1.70	0.41	0.63	0.61	0.04
Effluent Line 2	n.a.	1.40	1.70	1.50	n.a.	1.53	0.15	0.04
Final Effluent Line 2	n.a.	n.a.	1.10	n.a.	0.67	0.89	0.30	0.01

7.1.4.4 Cytotoxicity by MTT assay

The results of the MTT assay expressed as viability are shown in figure SI 7.1-3 and SI 7.1-4 using HepG2 and CHO cells respectively. Additionally, 2.5 fold enriched samples were analysed by CHO cells (test specific dilution was 1:10).

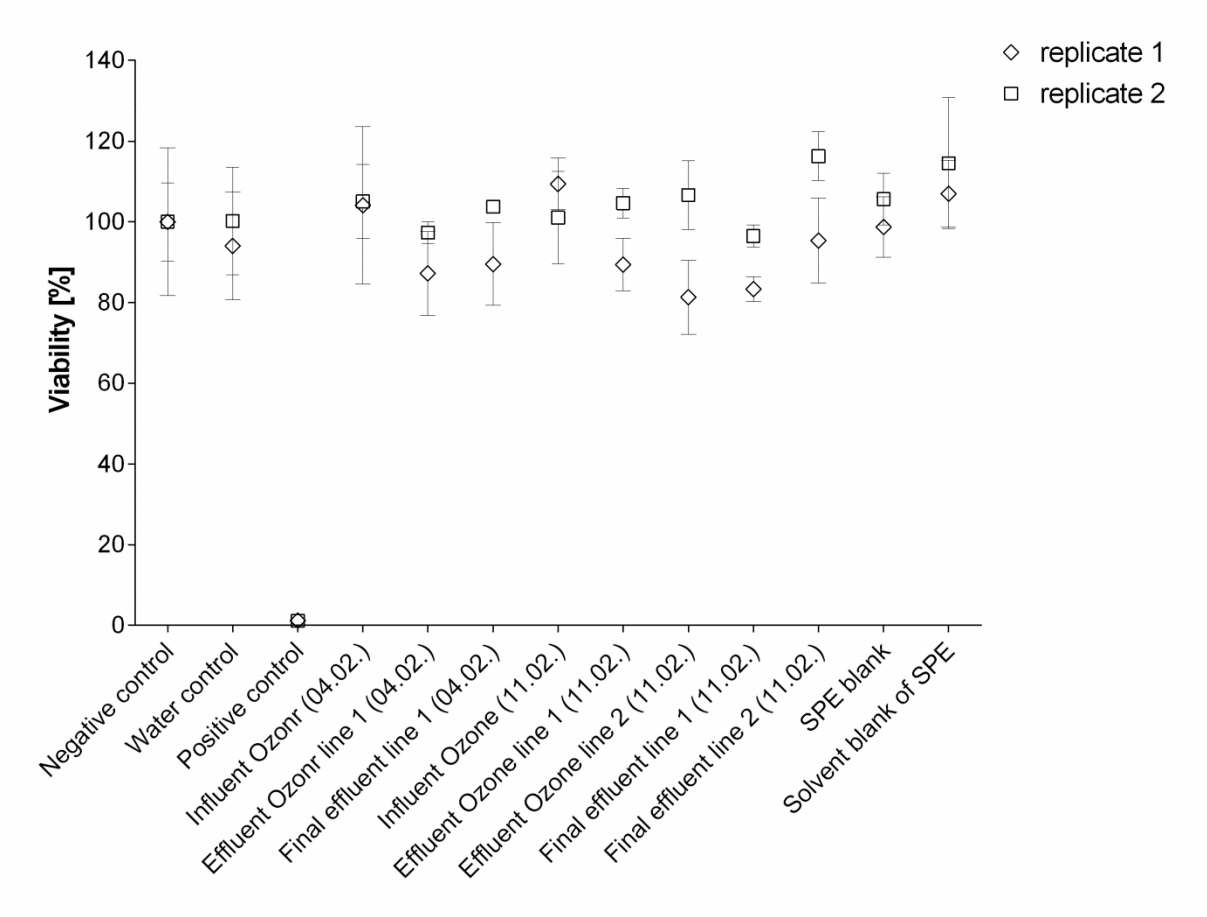


Figure SI 7.1-3: Cell viability (%) of HepG2 cells in the MTT assay for 2.5 fold enriched samples. Dots and diamonds show test results of two replicates (each in triplicate) Error bars indicating deviation of the triplicates of each test.

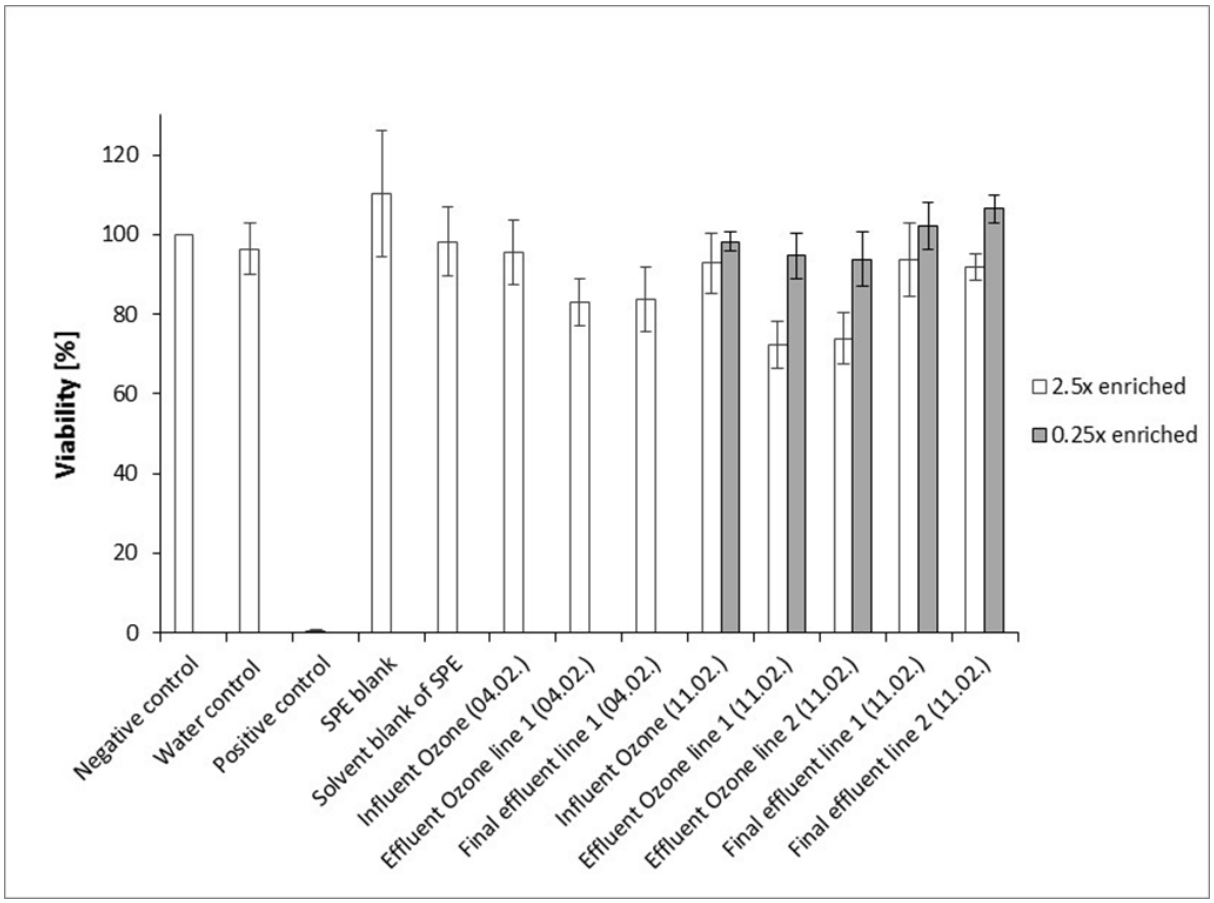


Figure SI 7.1-4: Results of CHO cells using MTT, White or grey columns indicate weighted means of 2.5 or 0.25 fold enriched samples; error bars = internal (negative control) or external (samples) consistency; n = 3 (each in triplicate).

7.1.4.5 Genotoxicity measured in the alkaline comet assay

The results of the alkaline comet assay using HepG2 and CHO cells are shown in figure SI 7.1-5.

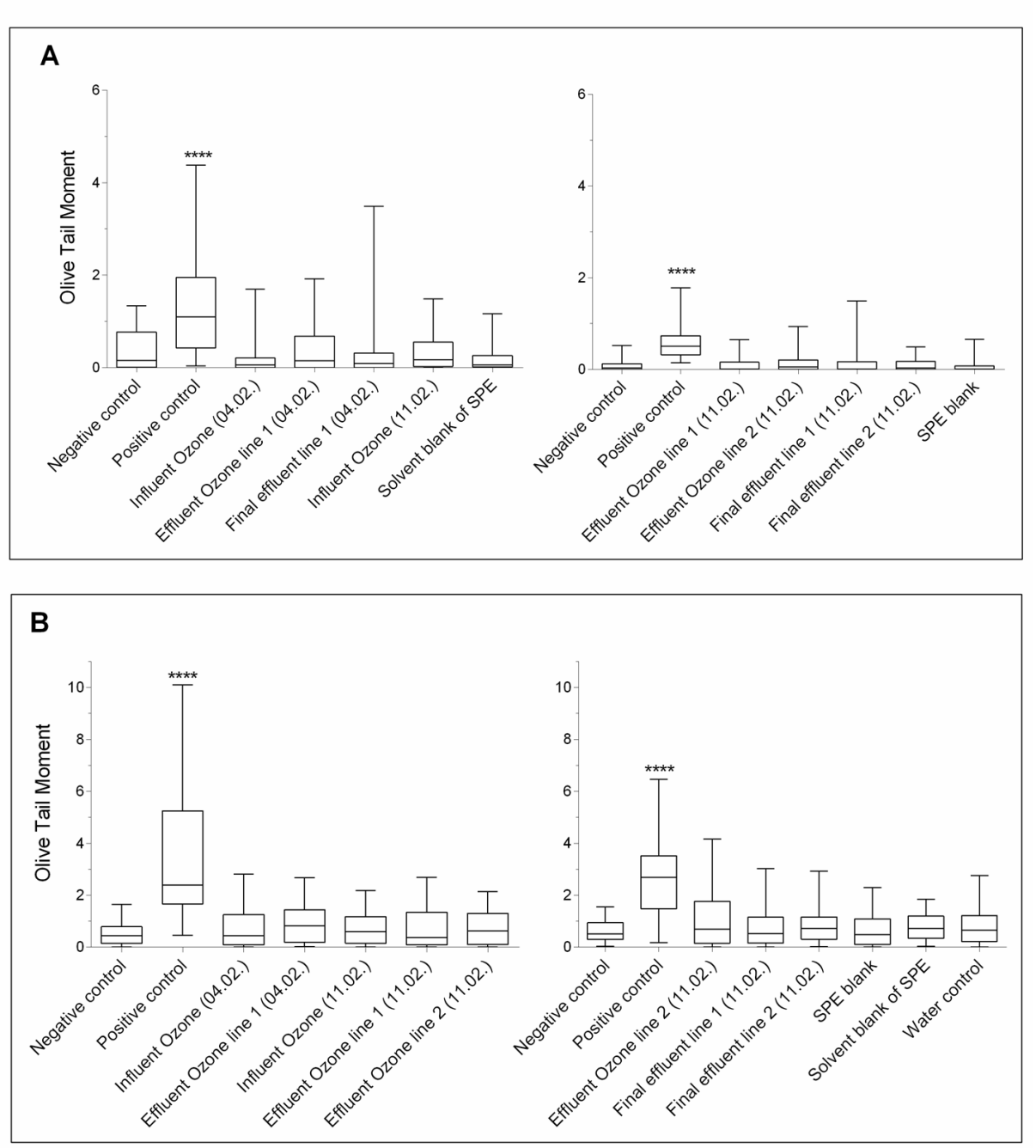


Figure SI 7.1-5: Results of the alkaline comet assay using Hep-G2 cells, 2.5 fold enriched samples (A) and CHO cells, 0.25 fold enriched samples (B). The horizontal line of boxplots represents the median of 50 data points per sample in one replicate. Whiskers show the 5-95 percentile, significance is indicated by asterisks related to negative control (**** $p < 0.0001$).

7.1.4.6 Genotoxicity measured in the micronucleus assay

Results of the micronucleus test using HepG2 cells are shown in the following figure SI 7.1-6.

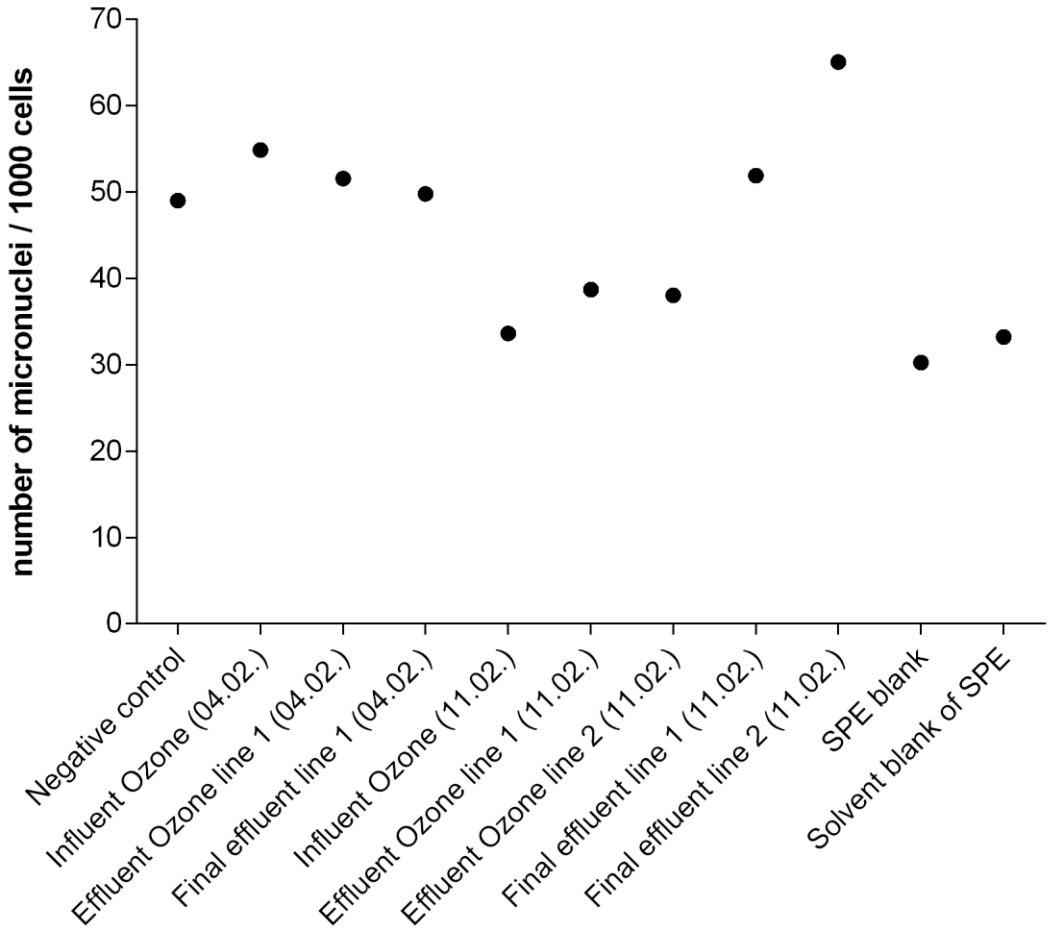


Figure SI 7.1-6: Results of the micronucleus assay using HepG2 cells, 2.5 fold enriched samples. Dots show test results of one replicate.

7.2 Chapter 4 – supplementary information

7.2.1 Chemical analysis

7.2.1.1 List of used analytical standards

Table SI 7.2-1: Standards and respective CAS numbers, limits of detection (LOD, S/N = 3:1) and quantification (LOQ, S/N = 10:1) for LC-MS/MS.

Substance	CAS	LOD (ng/l)	LOQ (ng/l)
Estrone (E1)	53-16-7	0.25	0.80
17β-Estradiol (E2)	50-28-2	0.05	0.20
17α-Ethinylestradiol (EE2)	57-63-6	0.15	0.50
Tamoxifen	10540-29-1	1.70	0.50
Hydroxytamoxifen	68392-35-8	1.00	3.30
Dihydrotestosteron	521-18-6	0.50	1.50
Testosterone	58-22-0	0.50	1.70
Trenbolon	10161-33-8	1.65	5.50
Etiocholanon	53-42-9	2.10	5.00
Boldion	897-06-3	0.90	2.80
Nandrolon	434-22-0	0.50	1.70
Boldenone	846-48-0	0.50	0.50
Flutamide	13311-84-7	0.60	2.00
Cyproterone acetate	427-51-0	0.30	1.00
5-Methyl Benzotriazol	50-18-0	1.30	4.50
Carbamazepine	298-46-4	0.30	0.90
Clarithromycin	81103-11-9	0.30	0.90
Diclofenac	15307-86-5	0.30	0.90
Ibuprofen	15687-27-1	0.70	2.25
Sulfamethoxazole	723-46-6	0.05	0.20
Gabapentin	60142-96-3	0.09	0.30
Metronidazole	443-48-1	0.23	0.80
Phenanzone	60-80-0	0.08	0.30
TCEP	115-96-8	0.50	1.60
Ciprofloxacin	85721-33-1	0.15	0.50
Bisoprolol	66722-44-9	0.01	0.03
Amidotrizoic acid	117-96-4	0.05	0.20

7.2.1.2 Endocrine substances

The results of the detected endocrine active compounds of all sampling experiments are shown in Table SI 7.2-4. Compounds which were not found in any sample are not shown. In the case of detected substances below the limit of quantification (LOQ), n.d. (not detected) is written (see Table SI 7.2-4).

Table SI 7.2-2: Parameter setup for the MS-detection of endocrine active compounds, (Q) Quantitation, (V) Validation, positive ionisation mode (ESI^+).

MRM *m/z*	Time (min)	Substance	DP (Volts)	EP (Volts)	CE (eV)	CXP (Volts)
270.9 → 115.2	4.57	Trenbolon (Q)	60	11	97	4
270.9 → 165.0	4.57	Trenbolon (V)	100	11	79	4
285.1 → 121.1	4.13	Boldion (Q)	100	5.5	31	4
285.1 → 77.0	4.13	Boldion (V)	100	5.5	71	4
291.0 → 255.3	7.05	Dihydrotestosteron (Q)	60	10.5	23	4
291.0 → 91.1	7.05	Dihydrotestosteron (V)	60	10.5	79	4
291.1 → 255.3	8.10	Etiocholanon (Q)	60	7.5	19	4
291.1 → 215.3	8.10	Etiocholanon (V)	60	7.5	23	4
417.1 → 357.3	5.24	Cyproteron acetat (Q)	60	5.5	21	6
417.1 → 279.3	5.24	Cyproteron acetat (V)	60	5.5	31	4
287.2 → 121.1	4.91	Boldenon (Q)	100	4	35	4
287.2 → 135.3	4.91	Boldenon (V)	100	4	19	4
275.2 → 109.1	2.46	Nandrolon (Q)	100	10	35	4
275.2 → 257.3	2.46	Nandrolon (V)	100	10	20	4
289.2 → 97.1	5.34	Testosterone (Q)	100	10	35	4
289.2 → 109.1	5.34	Testosterone (V)	100	10	35	4
372.2 → 72.1	4.10	Tamoxifene (Q)	120	10	40	4
372.2 → 129.1	4.10	Tamoxifene (V)	120	10	40	4
388.2 → 72.0	2.17	Hydroxytamoxifene (Q)	176	10	57	12
388.2 → 44.0	2.17	Hydroxytamoxifene (V)	176	10	95	12

Table SI 7.2-3: Parameter setup for the MS-Detection of endocrine active compounds, (Q) Quantitation, (V) Validation, negative ionisation mode (ESI-).

MRM *m/z*	Time (min)	Substance	DP (Volts)	EP (Volts)	CE (eV)	CXP (Volts)
269.4 → 144.8	5.04	E1 (Q)	-140	-2	-50	-2
269.4 → 143	5.04	E1 (V)	-140	-2	-50	-2
271.1 → 183.1	5.44	E2 (Q)	-200	-10	-54	-9
271.1 → 144.9	5.44	E2 (V)	-200	-10	-52	-15
295.1 → 145.0	5.36	EE2 (Q)	-155	-10	-48	-13
295.1 → 159.0	5.36	EE2 (V)	-155	-10	-48	-17
274.9 → 201.7	3.51	Flutamid (Q)	-100	-11	-32	-6
274.9 → 185.7	3.51	Flutamid (V)	-100	-11	-42	-2

Measured concentration of original, non-fractionated samples as well as fractions are listed in Table SI 4-6. Limits of quantification were calculated by a signal to noise (S/N) ratio of 10:1and limits of detection by a S/N of 3:1, with regard to the enrichment factor of 1000.

Table SI 7.2-4: Results of the original samples prior to fractionation.

ng/mL	Estron (E1)	17β-estradiol (E2)	17α-ethinylestradiol (EE2)	Trenbolon	Nandrolon	Boldion	Boldenon	Testosterone	Dihydrotestosteron	Etiocholanon	Tamoxifen	Hydroxytamoxifen	Cyproteron acetate	Flutamid
Marien-Hospital														
Influent original	4.30	2.50	n.d.	0.74	n.d.	130.00	7.60	22.00	79.00	140.00	n.d.	n.d.	n.d.	n.d.
Ozone original	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.06	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR original	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Herlev-Hospital														
Influent original	2.20	1.30	n.d.	3.00	n.d.	11.00	3.20	2.60	34.00	53.00	n.d.	n.d.	n.d.	n.d.
MBR original	1.30	0.47	n.d.	2.30	n.d.	0.63	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ozone original	n.d.	n.d.	n.d.	n.d.	n.d.	0.32	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC original	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected

Table SI 7.2-5: Results of the active fractions from the Marien-Hospital.

ng/L	Estron (E1)	17β-estradiol (E2)	17α-ethinylestradiol (EE2)	Trenbolon	Nandrolon	Boldion	Boldenon	Testosterone	Dihydrotestosteron	Etiocholanon	Tamoxifen	Hydroxytamoxifen	Cyproteron acetate	Flutamid
Marien-Hospital														
Influent F1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F3	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F4	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F5	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F6	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F9	n.d.	10.00	n.d.	20.00	0.50	52.00	n.d.	23.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F19	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ozone F7	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected

Table SI 7.2-6: Results of the active fractions from the Herlev-Hospital.

ng/L	Estron (E1)	17β-estradiol (E2)	17α-ethinylestradiol (EE2)	Trenbolon	Nandrolon	Boldion	Boldenon	Testosterone	Dihydrotestosteron	Etiocholanon	Tamoxifen	Hydroxytamoxifen	Cyproteron acetate	Flutamid
Herlev-Hospital														
Influent F2	16.00	4.40	n.d.	n.d.	n.d.	13.00	2.50	2.40	54.00	32.00	n.d.	n.d.	n.d.	n.d.
Influent F3	13.00	3.70	n.d.	2.80	n.d.	12.00	4.20	3.80	220.00	240.00	n.d.	n.d.	n.d.	n.d.
Influent F4	0.70	<0.05	n.d.	0.72	n.d.	1.30	0.08	0.17	69.00	105.00	n.d.	n.d.	n.d.	n.d.
Influent F5	n.d.	n.d.	n.d.	<0.03	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F6	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F7	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F8	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F9	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ozone F13	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ozone F23	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC F7	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC F8	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC F9	n.d.	n.d.	n.d.	n.d.	n.d.	<0.10	<0.25	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected

7.2.2 Pharmaceuticals and industrial compounds

Table SI 7.2-7 shows the multiple reaction monitoring (MRM) transitions of the investigated substances together with the retention times, DP, CE and CXP energies. The mass transfer for quantitation is indicated by (Q) and the mass transfer for the validation is indicated by (V) in the tables.

Table SI 7.2-7: Retention times and MS/MS-parameters for detection of micropollutants with positive electrospray ionisation (ESI^+). (Q) Quantitation-MRM, (V) Validation-MRM.

MRM *m/z*	Time (min)	Substance	DP (Volts)	EP (Volts)	CE (eV)	CXP (Volts)
134.0 → 76.8	3.50	5-Methyl Benzotriazol (Q)	51	10	10	4
134.0 → 79.1	3.50	5-Methyl Benzotriazol (V)	51	10	10	4
237.1 → 194.2	5.70	Carbamazepine (Q)	126	10	28	4
237.1 → 193.1	5.70	Carbamazepine (V)	126	10	43	4
748.5 → 158.1	6.60	Clarithromycin (Q)	80	10	35	4
748.5 → 83.0	6.60	Clarithromycin (V)	80	10	50	4
296.0 → 214.1	8.20	Diclofenac (Q)	80	10	50	4
296.0 → 215.1	8.20	Diclofenac (V)	80	10	20	4
207.1 → 161.2	6.30	Ibuprofen (Q)	65	10	17	4
207.1 → 119.1	6.30	Ibuprofen (V)	65	10	33	4
254.1 → 156.1	2.80	Sulfamethoxazole (Q)	85	10	23	4
254.1 → 92.1	2.80	Sulfamethoxazole (V)	85	10	40	4
332.1 → 231.1	2.90	Ciprofloxacin (Q)	94	10	54	4
332.1 → 288.2	2.90	Ciprofloxacin (V)	94	10	26	4
326.2 → 116.1	3.60	Bisprolol (Q)	80	10	20	4
326.2 → 74.1	3.60	Bisprolol (V)	80	10	35	4
614.8 → 360.9	2.10	Amidotrizoic acid (Q)	80	10	20	4
614.8 → 233.0	2.10	Amidotrizoic acid (V)	80	10	50	4
172.1 → 137.0	2.40	Gabapentin (Q)	90	10	20	4
172.1 → 154.2	2.40	Gabapentin (V)	90	10	19	4
172.1 → 128.1	3.10	Metronidazole (Q)	68	10	21	4
172.1 → 82.1	3.10	Metronidazole (V)	68	10	37	4
189.1 → 77.1	3.20	Phenanzone (Q)	70	10	50	4
189.1 → 56.0	3.20	Phenanzone (V)	70	10	20	4
284.9 → 222.9	1.15	TCEP (Q)	161	10	33	4
284.9 → 161.0	1.15	TCEP (V)	50	10	17	24

The results of the original samples prior to fractionation as well as active fractions are shown in the following tables 7.2-8 and 7.2-9. Limits of quantification were calculated by a signal to noise (S/N) ratio of 10:1 and limits of detection by a S/N of 3:1, with regard to the enrichment factor of 1000.

Table SI 7.2-8: Results of the original samples prior to fractionation.

ng/L	5-Methyl Benzotriazol	Amidotrizoic Acid	Bisoprolol	Carbamazepine	Ciprofloxacin	Clarithromycin	Diclofenac	Ibuprofen	Sulfamethoxazol	Gabapentin	Metronidazole	Phenazone
Marien-Hospital												
Influent original	110.00	0.09	160.00	56.00	8.20	7300.00	200.00	990.00	n.d.	0.97	120.00	13.00
Ozone original	35.00	n.d.	5.00	11.00	n.d.	250.00	1.60	n.d.	n.d.	3.40	52.00	2.60
MBR original	46.00	n.d.	4.80	120.00	n.d.	470.00	11.00	n.d.	n.d.	n.d.	50.00	n.d.
Herlev-Hospital												
Influent original	28.00	28.00	2.60	16.00	9.60	410.00	42.00	750.00	18.00	71.00	210.00	n.d.
MBR original	58.00	n.d.	6.20	72.00	n.d.	280.00	7.00	n.d.	n.d.	n.d.	48.00	2.70
Ozone original	78.00	30.00	2.40	4.70	5.80	43.00	0.37	n.d.	1.40	9.00	62.00	n.d.
GAC original	n.d.	3.80	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected

Table SI 7.2-9: Results of the active fractions from the Marien-Hospital.

ng/L	5-Methyl Benzotriazol	Amidotrizoic Acid	Bisoprolol	Carbamazepine	Ciprofloxacin	Clarithromycin	Diclofenac	Ibuprofen	Sulfamethoxazol	Gabapentin	Metronidazole	Phenazone
Influent F1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.10	100.00	17.00
Influent F2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	20.00
Influent F3	62.00	n.d.	47.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	3.20
Influent F4	230.00	n.d.	368.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.70
Influent F5	n.d.	n.d.	3.20	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.00
Influent F6	n.d.	n.d.	0.19	38.00	n.d.	2500.00	n.d.	n.d.	n.d.	n.d.	n.d.	21.00
Influent F9	n.d.	n.d.	n.d.	n.d.	n.d.	50.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F15	n.d.	n.d.	n.d.	n.d.	n.d.	3.30	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F18	n.d.	n.d.	n.d.	n.d.	n.d.	4.50	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F19	n.d.	n.d.	n.d.	n.d.	n.d.	6.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ozon F7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected,

Table SI 7.2-10: Results of the active fractions from the Herlev-Hospital.

ng/L	5-Methyl Benzotriazol	Amidotrizoic Acid	Bisoprolol	Carbamazepine	Ciprofloxacin	Clarithromycin	Diclofenac	Ibuprofen	Sulfamethoxazol	Gabapentin	Metronidazole	Phenazone
Influent F2	n.d.	13.00	n.d.	25.00	16.00	n.d.	21.00	880.00	45.00	12.00	280.00	n.d.
Influent F3	n.d.	8.90	6.90	19.00	n.d.	n.d.	29.00	870.00	43.00	8.90	170.00	n.d.
Influent F4	46.00	n.d.	1.40	n.d.	n.d.	n.d.	4.00	44.00	6.60	0.27	18.00	n.d.
Influent F5	34.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	40.00	0.53	n.d.	3.30	n.d.
Influent F6	n.d.	n.d.	n.d.	n.d.	n.d.	1000.00	n.d.	n.d.	0.08	n.d.	1.60	n.d.
Influent F7	n.d.	n.d.	n.d.	n.d.	n.d.	990.00	n.d.	n.d.	n.d.	n.d.	0.98	n.d.
Influent F8	n.d.	n.d.	n.d.	n.d.	n.d.	200.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Influent F9	n.d.	n.d.	n.d.	n.d.	n.d.	35.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F10	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F11	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
MBR F12	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	24.00	n.d.	n.d.	n.d.	n.d.	n.d.
Ozone F13	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Ozone F23	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC F7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC F8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
GAC F9	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. = not detected,

7.2.3 R script for peak selection

To filter for relevant features and to plot most intense features detected as peaks, a routine was used developed by the BfG (Bundesanstalt für Gewässerkunde in Koblenz).

The script can be provided on request.

7.2.4 Mixtures effects in bioassays

Schematic illustration of synergistic and antagonistic effects using effect based analysis is shown in Figure SI 7.2-1. Different sigmoidal curves in classical effect based analysis are shown. In the case of mixtures, compounds acting antagonistically can be present and cause a reduction in the measured activity at the same spiked agonist concentration (curve is shifted to the right). Therewith higher agonist concentrations are necessary to get the same signal compared to without antagonistic compounds. The other effects are synergistic effects in which compounds amplify the measured signal (curve is shifted to the left). In this case lower spiked agonist concentrations result in higher activity.

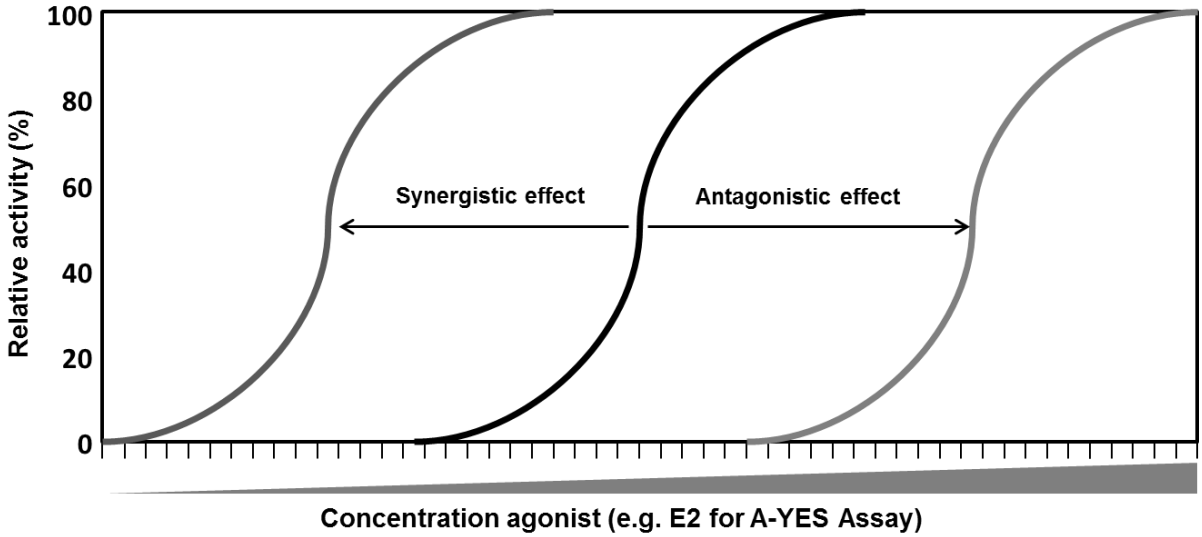


Figure SI 7.2-1: Generic illustration of sigmoidal dose response curves using effect based methods. Shown are resulting synergistic and antagonistic effects in possible mixtures, starting from a non-influenced standard curve in the middle. Synergistic effects lead to an amplification of the signal and antagonistic effect to an inhibition and reduction of the overall signal.

7.2.5 Antagonistic effect of diclofenac and ibuprofen

The results of diclofenac and ibuprofen showed significant anti-estrogenic activities at concentrations of > 100µg/L.

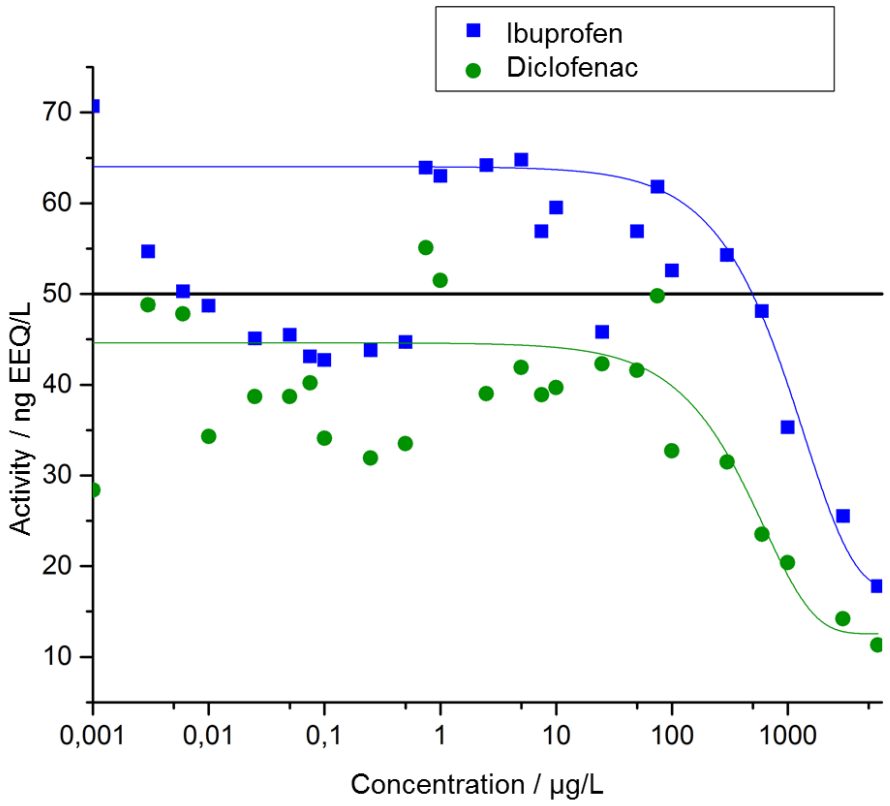


Figure SI 7.2-2: Antagonistic activities of Diclofenac and Ibuprofen in the A-YES assay. Tests were done with spiking 50 ng/L E2. EC_{50} of Diclofenac 490.0 µg/L; EC_{50} of Ibuprofen 1100.0 µg/L.

7.2.6 Non-target and suspect screening

In table SI 7.2-11 the advantage of the combination of bioassay and non-target analysis is shown by the reduction of features due to comparing fractions to exclude non-relevant features. Suspect target screening based on non-target features was performed using the database Stoff-ident by using the software PeakView (Version 2.2, Sciex, Germany) to view spectra. The relevant hits are listed in table SI 7.2-11.

Table SI 7.2-11: Reduction of relevant features

Anti-/Estrogenic effects

Marien-Hospital

Influent non-fractionated	**42103**				
Active fractions	F6	F9	F15	F18	F19
after fractionation	9711	5032	3401	3164	2334
Isotope reduction	6855	4496	3054	2744	1963
Adduct reduction	6176	3838	2421	2297	1606
Blank reduction	5811	3302	1907	1756	1088
Relevant for activity	**5496**	**2839**	**1730**	**1305**	**701**
Hits (Stoff-ident)	**2**	**6**	**2**	**4**	**4**

MBR non-fractionated	**11796**
active fractions	F15
after fractionation	7328
Isotope reduction	6399
Adduct reduction	5624
Blank reduction	4849
Relevant for activity	**95**
Hits (Stoff-ident)	0

Ozone non-fractionated	**8431**		
active fractions	F7	F12	F13
after fractionation	2132	1534	1540
Isotope reduction	1690	1232	1253
Adduct reduction	1345	944	964
Blank reduction	846	426	446
Relevant for activity	**624**	**32**	**91**
Hits (Stoff-ident)	33	0	0

Herlev-Hospital

Ozone non-fractionated	**9526**
active fractions	F13
after fractionation	7340
Isotope reduction	6028
Adduct reduction	5551
Blank reduction	303
Relevant for activity	**64**
Hits (Stoff-ident)	**0**

Anti-/Androgenic effects

Influent non-fractionated	**42103**			
Active fractions	F9	F11	F15	F19
after fractionation	5032	3839	3401	3164
Isotope reduction	4496	3453	3054	2744
Adduct reduction	3838	2885	2421	2297
Blank reduction	3302	2366	1907	1756
Relevant for activity	**2850**	**2042**	**1730**	**1305**
Hits (Stoff-ident)	**6**	**2**	**2**	**4**

MBR non-fractionated	**11796**
active fractions	F15
after fractionation	7328
Isotope reduction	6399
Adduct reduction	5624
Blank reduction	4849
Relevant for activity	**93**
Hits (Stoff-ident)	0

Ozone non-fractionated	**8431**	
active fractions	F5	F7
after fractionation	1520	2306
Isotope reduction	1225	1891
Adduct reduction	944	1496
Blank reduction	423	966
Relevant for activity	**36**	**629**
Hits (Stoff-ident)	0	33

7.2.7 Identified features with mzCloud

The MS^2 spectra of TCEP (*m/z* = 284.961) was compared with library data of mzCloud and identified with a propability factor of 82%. The figure SI 7.2-3 shows the comparison of measured and library data.

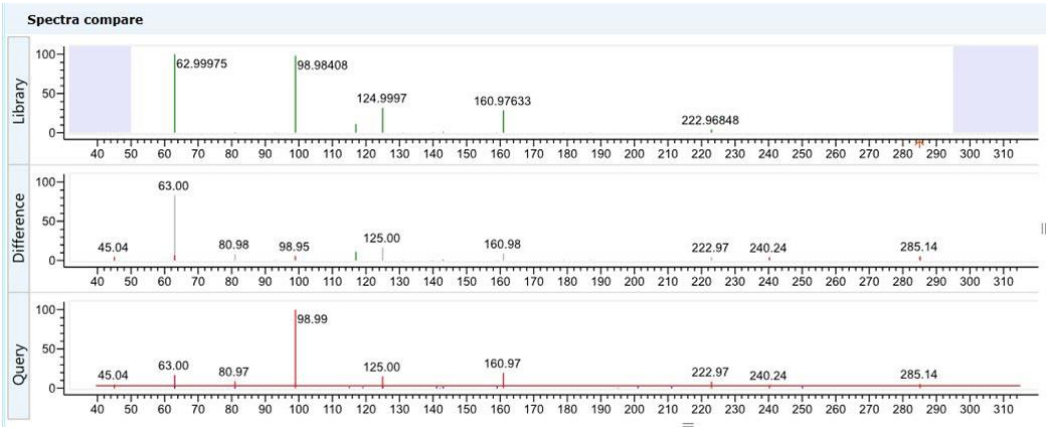


Figure SI 7.2-3: TCEP identified by MS^2 spectra and compared with libary data of mzCloud.

7.2.8 Lists of relevant features

The lists of all features detected can be provided on request.

7.3 Chapter 5 – supplementary information

7.3.1 Sampling

The following table illustrates the sampling campaign during the 3-week program. All parameter are listed and marked if analysed for a certain sample.

Table SI 7.3-1: Sample overview of biological and chemical parameters.

	Week 1; $z_{spec.}$ = 0.7 mg O3/mg DOC				Week 2; $z_{spec.}$ = 0.5 mg O3/mg DOC			Week 3; $z_{spec.}$ = 0.3 mg O3/mg DOC			
	24 h composite sample			Random sample	24 h composite sample		Random sample	24 h composite sample			Random sample
Parameter	20.-21.11.2017	21.-22.11.2017	22.-23.11.2017	22.11.2017	27.-28.11.2017	28.-29.11.2017	29.11.2017	4.-5.12.2017	5.-6.12.2017	6.-7.12.2017	6.12.2017
Chemical analysis											
PPCPs	•	•	•	•	•	•	•	•	•	•	•
Pesticides				•			•				•
Non-target-screening				•							
Sum-parameter											
DOC				•			•				•
AOC				•			•				•
Biological analysis											
A-YES				•			•				•
anti-A-YES				•			•				•
A-YAS				•			•				•
anti-A-YAS				•			•				•
AOC				•			•				•

7.3.2 Treatment plant design

The design and setup of the two-line ozone treatment unit with follow up biological post-treatment is illustrated in figure SI 7.3-1.

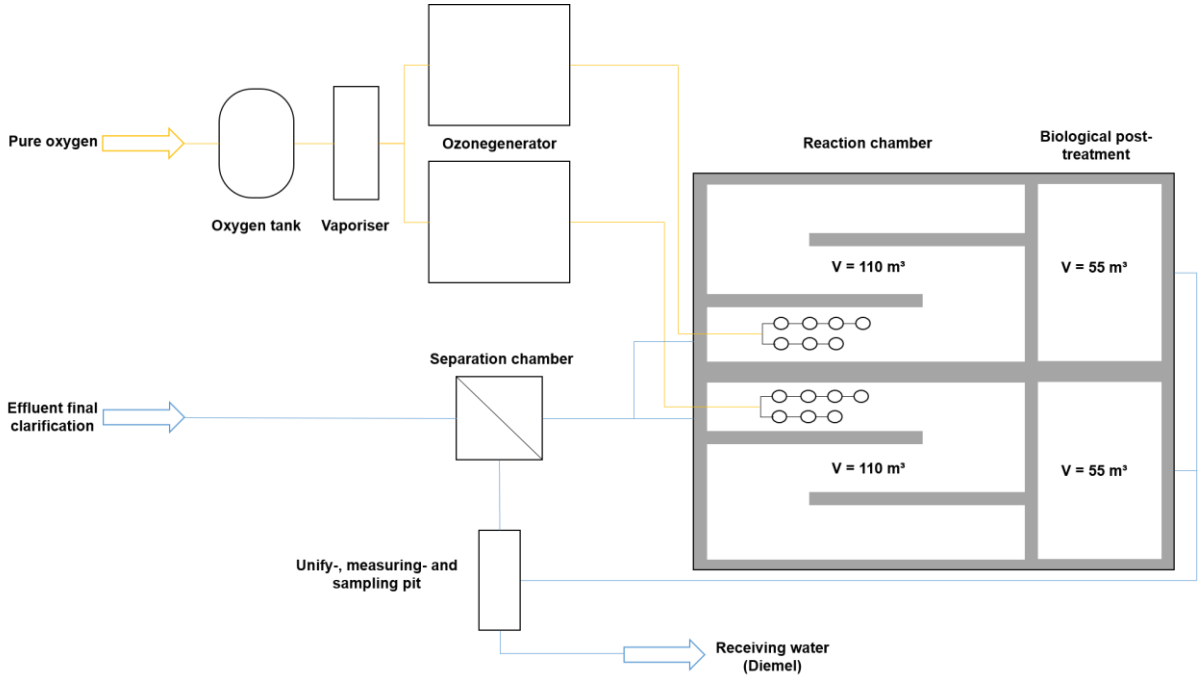


Figure SI 7.3-1: Scheme of the two-line ozone unit and biological post-treatment.

7.3.3 Chemical analysis

The MS parameter for each measured compound including respective internal standards are listed in table SI 7.3-2. The mass transfer marked with "1" was used for quantification and "2" as qualifier. The sMRM detection window was set to 60ms for all transitions. The electron potential (EP) was set constant to 10 volts.

Table SI 7.3-2: MS parameter for measured pharmaceuticals, pesticides and PPCPs**.**

Compound name and ID	Internal Standard (used for quantification)	sMRM	RT (min)	DP (V)	CE (eV)	CXP (V)
1H-Benzotriazol_1	IS_Cyclophosphamide_d_6	120.1→65.2	2.1	51	31	4
1H-Benzotriazol_2	IS_Cyclophosphamide_d_6	120.1→92.1	2.1	51	23	4
Carbamazepine_1	IS_Carbamazepine_d_{10}	237.1→194.2	4.4	126	28	4
Carbamazepine_2	IS_Carbamazepine_d_{10}	237.1→193.1	4.4	126	43	4
Clarithromycin_1	IS_Clarithromycin_ d_3	748.5→158.1	4.8	80	35	4
Clarithromycin_2	IS_Clarithromycin_ d_3	748.5→83.0	4.8	80	50	4
Diclofenac_1	IS_Diclofenac_d_4	296.0→214.1	6.2	80	50	4
Diclofenac_2	IS_Diclofenac_d_4	296.0→215.1	6.2	80	20	4
Metoprolol_1	IS_Metoprolol_d_7	268.2→133.1	2.6	102	35	4
Metoprolol_2	IS_Metoprolol_d_7	268.2→103.1	2.6	102	50	4
Sulfamethoxazole_1	IS_Sulfamethoxazole_d_4	254.1→156.1	3.0	85	23	4
Sulfamethoxazole_2	IS_Sulfamethoxazole_d_4	254.1→92.1	3.0	85	40	4
Terbutryn_1	IS_Terbutryn_d_5	242.1→186.1	4.5	64	25	4
Terbutryn_2	IS_Terbutryn_d_5	242.2→68.1	4.5	64	57	4
N4-Acetyl-sulfamethoxazol_1	IS_Sulfamethoxazole_d_4	295.9→134.1	3.3	100	31	4
N4-Acetyl-sulfamethoxazol_2	IS_Sulfamethoxazole_d_4	295.9→65.0	3.3	100	63	4

Diflufenican_1	IS_Cyclophosphamide_d_6	395.1→266.1	7.9	201	37	14
Diflufenican_2	IS_Cyclophosphamide_d_6	395.1→246.1	7.9	201	51	16
Disulfoton_1	IS_Cyclophosphamide_d_6	275.0→88.7	7.7	76	11	4
Disulfoton_2	IS_Cyclophosphamide_d_6	275.0→60.7	7.7	76	45	12
Quinmerac_1	IS_Cyclophosphamide_d_6	222.0→204.1	2.7	66	29	0
Quinmerac_2	IS_Cyclophosphamide_d_6	222.0→140.9	2.7	66	47	24
Desphenylchloridazon_1	IS_Cyclophosphamide_d_6	146.1→66.0	0.7	126	49	10
Desphenylchloridazon_2	IS_Cyclophosphamide_d_6	146.1→117.0	0.7	126	21	30
Isoproturone_1	IS_Cyclophosphamide_d_6	207.2→72.1	4.9	86	29	4
Isoproturone_2	IS_Cyclophosphamide_d_6	207.1→165.2	4.9	86	19	4
Metazachlorethane sulfonic acid_1	IS_Cyclophosphamide_d_6	324.0→134.1	3.0	146	35	12
Metazachlorethane sulfonic acid_2	IS_Cyclophosphamide_d_6	324.0→69.1	3.0	146	13	16
IS_Carbamazepine_d_{10}	-	247.2→204.2	4.4	51	29	4
IS_Clarithromycin_ d_3	-	751.5→593.3	4.8	151	29	10
IS_Cyclophosphamide_d_6	-	267.2→140.1	3.3	80	31	2
IS_Diclofenac_d_4	-	300.0→218.0	6.2	26	41	4
IS_Metoprolol_d_7	-	275.0→123.1	2.6	41	27	8
IS_Sulfamethoxazole_d_4	-	258.1→96.1	3.0	170	46	10
IS_Terbutryn_d_5	-	247.1→191.1	4.5	170	46	10

The analytical method information regarding LOD and LOQ using SPE with an enrichment factor of 1000 are listed in table SI 7.3-3.

Table SI 7.3-3: Method information regarding LOD and LOQ using SPE

Compound	LOD (ng/L)	LOQ (ng/L)
1H-Benzotriazole	0.005	0.02
Carbamazepine	0.002	0.005
Clarithromycin	0.003	0.01
Desphenyl-Chloridazone	0.07	0.2
Diclofenac	0.005	0.02
Diflufenican	0.004	0.01
Disulfoton	1	3
Isoproturon	0.01	0.04
Metazachlorsulfonicacid	0.02	0.07
Metoprolol	0.005	0.02
N4-Acetyl-sulfamethoxazole	0.005	0.02
Quinmerac	0.008	0.03
Sulfamethoxazole	0.008	0.03
Terbutryn	0.001	0.002

LOD/LOQ include the SPE enrichmentfactor (1000x).

7.3.4 HRMS analysis

7.3.4.1 Method parameters

The parameters of the ion source are listed in table SI 7.3-4.

Table SI 7.3-4: HESI Source Parameter

Sheath gas flow rate	37
Aux gas flow rate	15
Sweep gas flow rate	1
Spray Voltage	3.5 kV
Capillary Temp.	320 °C
S-Lens RF level	60
Aux gas heater Temp.	150 °C

The settings of the full scan/ddMS^2 (Top 5) measurement are shown in table SI 7.3-5.

Table SI 7.3-5: Parameter of the full scan/ddMS^2 (Top 5) method.

General	
Polarity	Positive/Negative
Runtime	28 min
Chromatographic peak width	10 s
Full MS	
Resolution	70,000
AGC Target	1E+06
Maximum injection time	100 ms
Scan Range	*m/z* 100 – 1000
dd-MS^2	
Resolution	17,500
AGC Target	5E+04
Maximum IT	50 ms
Loop Count	5*
Isolation window	2 Da
NCE	30
Intensity Threshold	2E+03

* Loop Count specifies the number N of fragmentation scans

To avoid fragmentation analysis of background signal known, an exclusion list was used shown in table SI 7.3-6.

Table SI 7.3-6: Exclusion list

Positive mode m/z	Negative mode m/z
127.01552	124.99931
141.03090	110.97410
113.00005	197.8067
301.1406	160.8410
413.2657	311.1689
803.5420	160.8410
533.4536	
663.4527	
173.0286	

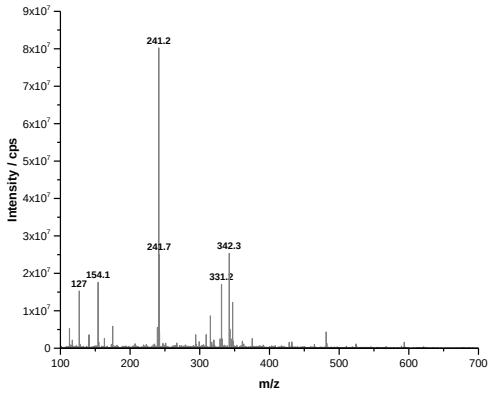
7.3.5 Non-target screening results

Due to the high amount of data, the detail feature list with proposed sum-formulas can be provided on request.

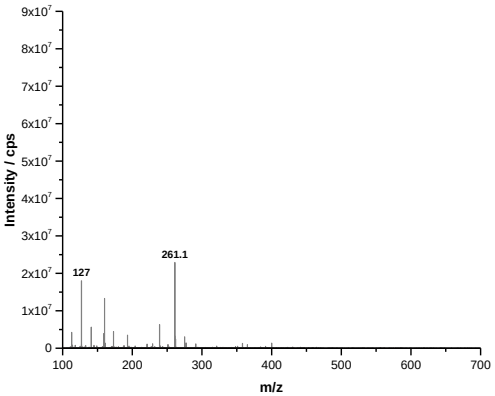
The 11 most interesting peaks filtered by the Software MZMine 2.0 after biological post-treatment are shown in more detail by their respective MS^2 spectra (Table SI 7.3-7). These peaks are potential TP's formed after ozonation and not removed during the final biological post-treatment.

Table SI 7.3-7: MS^2-speactra of 11 detected peaks after biological post-treatment. Retention times (RT), parent ion (*m/z*) as well as predicted sum formula (based on chemspider data; ppm deviation of the exact mass) are shown.

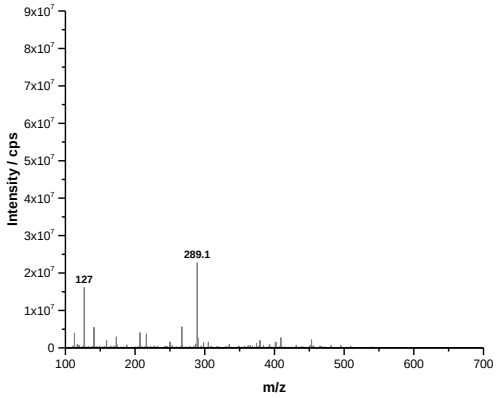
ID 42 (*m/z* = 241.7050)
RT: 12.60 min
Formula: $C_3H_{88}N_6O_2$ (0.6ppm)



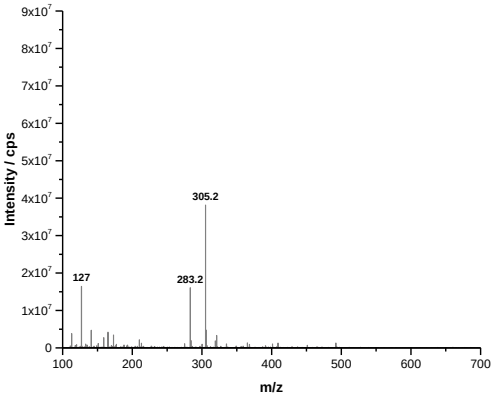
ID 61 (*m/z* = 261.1309)
RT: 9.81 min
Formula: $C_9H_{12}N_{10}$ (-1.2ppm)



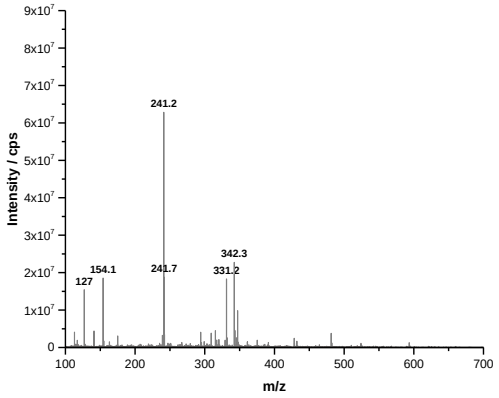
ID 79 (*m/z* = 289.1256)
RT: 10.11 min
Formula: $C_8H_{10}N_{13}$ (0.3ppm)



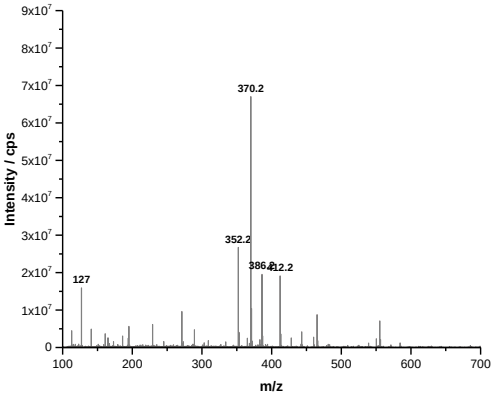
ID 90 (*m/z* = 305.1569)
RT: 9.77 min
Formula: $C_9H_{14}N_{13}$ (-0.5ppm)



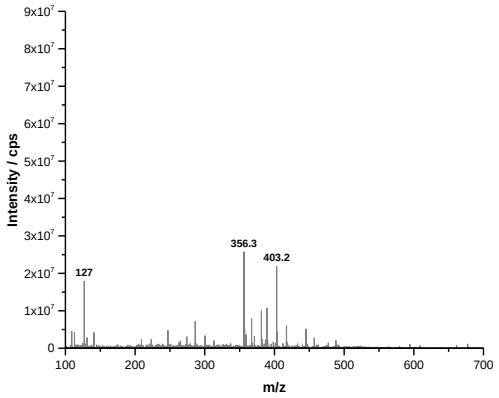
ID 113 (*m/z* = 342.2636)
RT: 12.59 min
Formula: $C_{20}H_{31}N_5$ (1.6ppm)



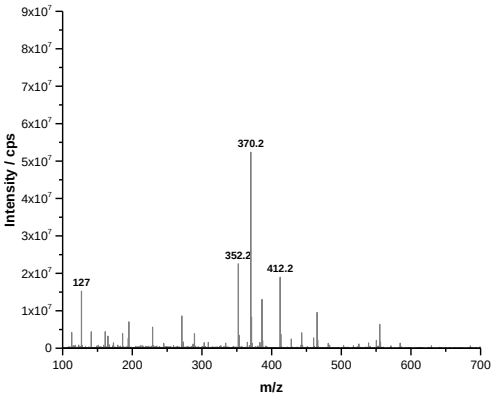
ID 124 (*m/z* = 352.1517)
RT: 11.48 min
Formula: $C_{15}H_{15}N_{10}O$ (-2.2ppm)



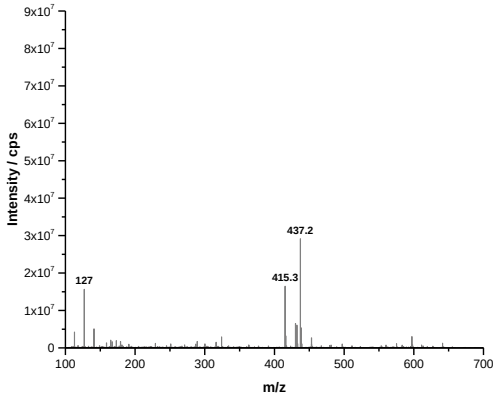
ID 127 (*m/z* = 356.2792)
RT: 13.80 min
Formula: $C_{21}H_{33}N_5$ (-0.3ppm)



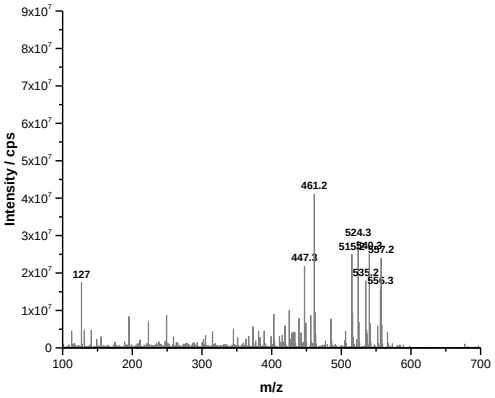
ID 157 (*m/z* = 412.2093)
RT: 11.47 min
Formula: $C_6H_{17}N_{23}$ (0.4ppm)



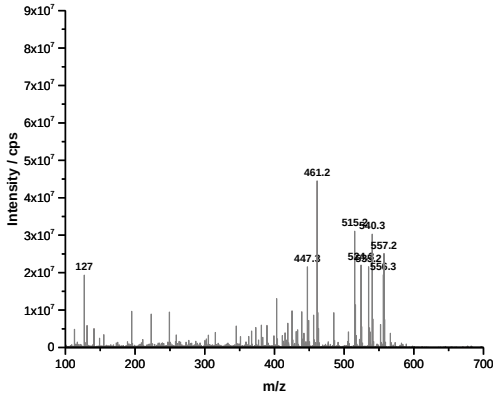
ID 172 (*m/z* = 437.2357)
RT: 10.86 min
Formula: $C_{28}H_{28}N_4O_1$ (3ppm)



ID 207 (*m/z* = 524.2616)
RT: 14.03 min
Formula: $C_{25}H_{27}N_{14}$ (0.7ppm)



ID 213 (*m/z* = 540.2564)
RT: 14.02 min
Formula: $C_{40}H_{31}N_2$ (-0.8ppm)



7.4 List of publications

7.4.1 Publications in peer-reviewed journals

Itzel, F., Gehrmann, L., Bielak, H., Ebersbach, P., Boergers, A., Herbst, H., Maus, C. Simon, A., Dopp, E., Hammers-Wirtz, M., Schmidt, T.C., Tuerk, J. (2017): Investigation of full-scale ozonation at a municipal waste water treatment plant using a toxicity-based evaluation concept, Journal of Toxicology and Environmental Health Part A, 80, 1242-1258.

Itzel, F., Jewell, K.S., Leonhardt, J., Gehrmann, L., Nielsen, U., Ternes, T.A., Schmidt, T.C., Tuerk, J. (2017): Comprehensive analysis of antagonistic endocrine activity during ozone treatment of hospital waste water. Science of the total Environment, 624, 1443-1454.

Gehrmann, L. & Bielak, H., Behr, M., Itzel, F., Lyko, S., Wagner, M., Simon, A., Dopp, E., Kunze, G., Tuerk, J. (2018): (Anti-)estrogenic and (anti-)androgenic effects in waste water during advanced treatment: comparison of three in vitro bioassays, Environmental Science and Pollution Research, 25, 4094-4104.

Knoop, O., Itzel, F., Tuerk, J., Lutze, H.V., Schmidt, T.C. (2018): Endocrine effects after ozonation of tamoxifen. Science of the Total Environment, 622-623, 71-78.

Itzel, F., Gehrman, L., Teutenberg, T., Schmidt, T.C., Tuerk, J. (2018): Recent developments and concepts of effect based methods for the detection of endocrine activity and the importance of antagonistic effects. Submitted to: Trends in Analytical Chemistry.

7.4.2 Oral presentations

Itzel, F., Gehrmann, L., Bielak, H., Behr, M., Lyko, S., Wagner, M., Simon, A., Dopp, E., Schmidt, T.C., Tuerk, J. (2015): Estrogenic and androgenic activity during full-scale treatment of hospital waste water, Micropoll & Ecohazard Conference 2015, Singapore.

Itzel, F., Gehrmann, L., Bielak, H., Behr, M., Lyko, S., Wagner, M., Simon, A., Dopp, E., Schmidt, T.C., Türk, J. (2016): Untersuchung von Krankenhausabwasser auf östrogene und androgene Effekte und deren Elimination mittels Ozon, Vortrag auf der GdCh Tagung „Wasser“, Bamberg.

Itzel, F., Gehrmann, L., Bielak, H., Behr, M., Lyko, S., Wagner, M., Simon, A., Dopp, E., Schmidt, T.C., Türk, J. (2016): Untersuchung von Krankenhausabwasser auf östrogene und androgene Effekte und weitergehende Untersuchungen zu antagonistischen Effekten, IFAT, München.

Itzel, F., Gehrmann, L., Bielak, H., Jewell, K., Behr, M., Lyko, S., Wagner, M., Simon, A., Dopp, E., Terners, T., Schmidt, T.C. Tuerk, J. (2017): Investigation of hospital waste water – focus on antagonistic endocrine effects before and after ozone treatment, ASLO 2017, Honolulu, Hawai.

Itzel, F., Jewell, K.S., Leonhardt, J., Gehrmann, L., Nielsen, U., Ternes, T.A., Schmidt, T.C., Tuerk, J. (2017): Comprehensive analysis of antagonistic endocrine activity during ozone treatment of hospital waste water. Micropoll & Ecohazard Conference 2017, Vienna

Itzel, F., Kerstein, J., Posch, T., Türk, J. (2017): Moderne Wasser-, Abwasser- und Sedimentanalytik mittels GC-MS/MS, 12. Langenauer Wasserforum, Langenau.

Itzel, F., Kerstein, J., Türk, J. (2018): Bestimmung von östrogen aktiven Stoffen aus Oberflächen- und Abwasserproben mittels GC-MS/MS. Umwelt Seminar Shimadzu. Düsseldorf.

7.4.3 Poster presentations

Itzel, F., Gehrmann, L., Portner, C., Herbst, H., Kunze, G., Schmidt, T.C., Tuerk, J. (2015): Determination of estrogenic activity at a waste water treatment plant with full scale ozonation using Arxula adeninivorans yeast estrogen screen assay, Posterbeitrag auf der International Ozone Association World Conference 2015, Barcelona

Itzel, F., Schmidt, T.C., Türk, J. (2016): Beurteilung einer Ozonanlage einer Kommunalen Kläranlage in Bezug auf hormonelle Aktivität, Posterbeitrag und Stand bei Tag der offenen Tür, Kläranlage Dülmen

Itzel, F., Schmidt, T. C., Türk, J. (2016): Beurteilung einer Ozonanlage einer Kommunalen Kläranlage in Bezug auf hormonelle Aktivität, Posterbeitrag inkl. Messestand bei der 1. Future Water Fortschrittswerkstatt Wasser, Essen

Itzel, F., Gehrmann, L., Schmidt, T. C., Türk, J. (2016): Beurteilung einer Ozonanlage einer Kommunalen Kläranlage in Bezug auf hormonelle Aktivität, Posterbeitrag inkl. Messestand bei der IFAT, München

Itzel, F., Gehrmann, L., Herbst, H., Schmidt, T. C., Türk, J. (2017): Großtechnische Ozonung als vierte Reinigungsstufe auf einer Kläranlage, Wasser International – Ozon Smposium der IOA, Berlin

Itzel, F., Jewell, K.S., Gehrmann, L., Leonhardt, J., Nielsen, U., Ternes, T.A., Schmidt, T.C., Türk, J. (2017): Bestimmung von antagonistischen hormonellen Effekten in

Krankenhausabwasser mittels *Arxula Adeninivorans* Hefezellenassays und HRMS, Langenauer Wasserforum, Langenau

Itzel, F., Bätz, N., Hohrenk, L., Gehrmann, L., Schmidt, T.C., Türk, J. (2018): Bewertung einer biologischen Stufe im Anschluss an die Ozonung zur erweiterten Abwassserbehandlung, Umwelt 2018, Münster

7.5 Curriculum vitae

Nicht in digitaler Form enthalten!

7.6 Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit mit dem Titel

„Investigation of agonistic and antagonistic endocrine activity during full-scale ozonation of waste water"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe, und dass die Arbeit in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht wurde.

Essen, im September 2018

Fabian Itzel

7.7 Danksagung

In erster Linie gilt mein Dank Dr. Jochen Türk, welcher mir die Möglichkeit gab meine Promotion am IUTA durchzuführen und mir während dieser Zeit fachlich und persönlich zur Seite stand.

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