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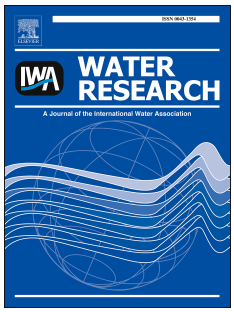

Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies

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Spoilt for choice: A critical review on the chemical and biological assessment of current wastewater treatment technologies

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Abstract

The knowledge we have gained in recent years on the presence and effects of compounds discharged by wastewater treatment plants (WWTPs) brings us to a point where we must question the appropriateness of current water quality evaluation methodologies. An increasing number of anthropogenic chemicals is detected in treated wastewater and there is increasing evidence of adverse environmental effects related to WWTP discharges. It has thus become clear that new strategies are needed to assess overall quality of conventional and advanced treated wastewaters. There is an urgent need for multidisciplinary approaches combining expertise from engineering, analytical and environmental chemistry, (eco)toxicology, and microbiology. This review summarizes the current approaches used to assess treated wastewater quality from the chemical and ecotoxicological perspective. Discussed chemical approaches include target, non-target and suspect analysis, sum parameters, identification and monitoring of transformation products, computational modeling as well as effect directed analysis and toxicity identification evaluation. The discussed ecotoxicological methodologies encompass *in vitro* testing (cytotoxicity, genotoxicity, mutagenicity, endocrine disruption, adaptive stress response activation, toxicogenomics) and *in vivo* tests (single and multi species, biomonitoring). We critically discuss the benefits and limitations of the different methodologies reviewed. Additionally, we provide an overview of the current state of research regarding the chemical and ecotoxicological evaluation of conventional as well as the most widely used advanced wastewater treatment technologies, *i.e.*, ozonation, advanced oxidation processes, chlorination, activated carbon, and membrane filtration. In particular, possible directions for future research activities in this area are provided.

Keywords:

wastewater quality assessment; conventional and advanced treatment; sewage; environmental chemistry; ecotoxicology; toxicity

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1. Introduction

The access to clean and safe water has become one of the major challenges of our modern society, due to the growing imbalance between freshwater availability and consumption (Jackson et al., 2001). Water scarcity often results from the increasing use for agricultural irrigation, industry, and domestic purposes (Jackson et al., 2001). Additionally, the quality of fresh water is threatened by a large number of pathogens (Rizzo et al., 2013) as well as anthropogenic chemicals entering the urban and rural water cycle (Schwarzenbach et al., 2006). Discharges from municipal and industrial wastewater treatment plants (WWTPs) have been identified as one of the major sources of aquatic pollution in industrialized countries (Reemtsma et al., 2006). Considering the predicted growth rate of the global population and constantly increasing number of people that are connected to WWTPs, the amount of treated wastewater (WW) is likely to increase in the future. Water shortages currently necessitate indirect non-potable and even potable reuse of treated WW. Advances in WWTP technologies are crucial to limit the burden of WW-originated contaminants, due to the importance of WWTPs as point sources for microbial and chemical contaminants entering surface waters. To date, one of the main challenges is to appropriately evaluate the different treatment technologies regarding their potential to minimize the toxicological risks for both, biota and human health.

In the past, advances in WW treatment in high-income countries have strongly improved the quality of wastewater discharged into the aquatic environment as well as minimized wastewater related human health risks. More than 100 years ago the establishment of the first WWTPs was driven by the outbreaks of waterborne diseases such as cholera and typhoid, which were caused by the contamination of drinking water resources with pathogens originating from wastewater. Similarly, nutrient removal stages were installed in the 1960s and 70s after recognizing WW as major cause for the eutrophication of surface waters due the emission of nutrients such as nitrogen and phosphorous. Until the beginning of the 1990' the scientific community focused on persistent organic pollutants (POP) such as PCBs, PAHs and heavy metals to evaluate the quality of WW and sewage sludge as well as the receiving waters. Today's WWTPs, however, are generally able to substantially reduce the emission of these contaminants. In combination with source control measures in most cases these "older" contaminants are thus less relevant today (*e.g.*, Teijon et al., 2010). In recent years, the occurrence and severe effects such as feminization in fish of the so-called contaminants of emerging concern (CECs) in WW as well as in rivers and streams downstream of WWTP discharges has led to an ongoing debate about the necessity for upgrading WWTPs with advanced treatment steps (Sumpter, 2005; Jobling et al., 1998). CECs are recently

identifized hazardous or potentially hazardous compounds. These compounds are mainly synthetic but also naturally-occurring, which are not covered by routine monitoring and regulatory programs. CECs are thus potential candidates for future regulation. This includes also their transformation products (TPs) formed in different stages of the urban and rural water cycle such as WW treatment (Escher and Fenner, 2011).

An assessment of the actual risks induced by WW discharge to surface water is challenging and often hampered by relatively low concentrations of pollutants, difficulties in identifying relevant toxicity endpoints, and the multiplicity of environmental parameters influencing ecotoxicological effects (Joss et al., 2008; Stalter et al., 2013). In a recent study, Malaj et al. (2014) pointed out that organic chemicals were likely to exert adverse effects on sensitive aquatic species at up to 43% out of 4,000 European freshwater monitoring sites. The increasing knowledge about environmental and human health effects caused by CEC's has already launched a profound discussion about the upgrade of municipal WWTPs to improve CEC removal by additional treatment steps.

This review provides an overview of the various chemical and ecotoxicological methodologies that are most commonly used for the quality assessment of WW using conventional and advanced treatment technologies. Special emphasis thereby is placed on answering the following questions:

- Which chemical and ecotoxicological tools are available to assess the quality of treated WW?
- Are current approaches sufficient to appropriately assess the quality of treated WW?
- Which chemical and ecotoxicological parameters are crucial to determine the overall WW quality?

Due to the vastness of the topic of WW and WW treatment we do not aim at completeness, but to discuss the most important aspects. Some issues however, such as antibiotic resistance, are crucial and closely linked to WW quality but will not be addressed in detail because they are beyond the scope of this review. We rather refer to other reviews in this field (e.g., Rizzo et al., 2013).

2. Chemical and ecotoxicological methods for water quality assessment

Progress in analytical chemistry has led to the development of technologies that enable detection of CECs down to the low ng/L- or even pg/L-range. Similarly, a variety of ecotoxicological tools, in particular *in vitro* and *in*

vivo assays, have been developed to detect (eco)toxicological effects on a variety of endpoints and trophic levels. In this chapter, the most commonly applied chemical and ecotoxicological methods used for the assessment of WW quality are introduced, their specific benefits and limitations are discussed, and main future research needs are highlighted.

2.1. Sampling and sample preparation

A basic requirement for the successful assessment of WW quality are appropriate sampling strategies as well as proper sample handling, as both can result in erroneous data and misleading interpretations. In addition, sample preparation is crucial to increase sensitivities and remove interfering compounds.

2.1.1. Sampling strategies and sample handling

Tailored sampling strategies according to the underlying research question(s), in particular sampling mode and frequency, are crucial to draw meaningful conclusions from obtained results. Grab sampling of raw and treated WW is sufficient whenever the mere presence of CECs or the applicability of a new analytical method is the objective of a study. However, this sampling strategy is inappropriate to determine elimination efficiencies of WWTPs, as CEC concentrations might vary significantly over time. As an example, concentrations of X-ray contrast media show a specific weekly concentration pattern, which reflects the common practice of performing X-ray examinations between Monday and Friday (Oleksy-Frenzel et al., 2000). Also meteorological conditions during and before sampling can significantly alter the results as, *e.g.*, heavy rain events may lead to a significant dilution of raw WW, a decrease of removal efficiencies, and a discharge of biomass from activated sludge tanks (Rouleau et al., 1997). Consequently, flow proportional composite samples are essential when i) treatment efficiencies of WW treatment technologies are evaluated, ii) the data is used as input parameters in modeling approaches or iii) CEC loads are calculated to estimate usage or consumption quantities of CECs (Wick et al., 2009; van Nuijs et al., 2011a). However, a recent review by Ort and co-authors (2010) evaluating WWTP sampling practices applied in 87 peer-reviewed publications, revealed that less than 5% of the reviewed studies explicitly follow internationally acknowledged guidelines or methods for the experimental design of monitoring campaigns.

A second important aspect is sample handling because inappropriate storage can lead to a degradation of CECs (Baker and Kasprzyk-Hordern, 2011; Hillebrand et al., 2013). Storage of samples over days or even weeks is often inevitable, since limited laboratory capacities prevent an immediate sample analysis. Inhibition and

187 reduction of microbial activity can be achieved by filtration (< 0.2 µm), freezing, acidification, and/or by the
188 addition of preservatives such as NaN_3 or copper sulfate. However, hydrophobic compounds can sorb to the
189 membranes (Ng and Cao, 2015), and freezing and acidification might lead to chemical degradation of specific
190 compounds, *e.g.*, via hydrolysis (Stangroom et al., 2000; Jewell et al., 2014). Furthermore, acidification and the
191 addition of preservatives cannot be used if samples are directly used (i.e. without further pretreatment) for
192 ecotoxicological analysis.
193

194 ### *2.1.2. Sample enrichment*

195 Sample extraction and enrichment is often necessary to achieve sufficient sensitivities for both chemical and
196 bioassay analysis to determine the removal of CECs and their effects during WW treatment. Additionally,
197 sample pre-treatment substantially reduces matrix effects caused by interfering constituents such as natural
198 organic matter (NOM). This is particularly important when LC-MS is applied for the detection of CECs, as
199 matrix effects, caused by co-eluting compounds, strongly alter the ionization efficiencies of target compounds.
200 Solid phase extraction (SPE) has been most widely used and a large spectrum of sorbents is available today,
201 enabling the selective enrichment of neutral, anionic, or cationic compounds. Depending on the sample matrix,
202 volumes are usually enriched ranging from several milliliters to several liters. In recent years, online-SPE
203 methodologies have been developed, which allow for the direct analysis of untreated samples after online sample
204 cleanup and/or analyte enrichment (*e.g.*, Viglino et al., 2008; Huntscha et al., 2012). Furthermore, the elevated
205 sensitivities of recent LC/MS/MS instruments even allow for a direct injection of water without any sample
206 enrichment (Backe and Field, 2012). Other methods used for the extraction of CECs from aqueous matrices are
207 liquid-liquid extraction (LLE) and solid-phase microextraction (SPME), which are predominantly used for
208 hydrophobic compounds and/or volatiles/semi-volatiles (*e.g.*, Pena-Pereira et al., 2012; Pawliszyn and Pedersen-
209 Bjergaard, 2006). As conventional LLE requires large amounts of organic solvents, liquid-phase microextraction
210 (LPME) is increasingly used and has been applied for the analysis of pesticides, pharmaceuticals, and UV filter
211 substances in raw and treated WW (Wen et al., 2004; Rodil et al., 2009; Lambropoulou and Albanis, 2007). For
212 SPME, a polymer-coated fused silica fiber is either directly immersed in a sample solution for extraction of
213 volatile and non-volatile analytes or to the headspace above the sample for the extraction of volatiles. SPME has
214 been used for the extraction of estrogenic compounds, pesticides, musk fragrances, siloxanes, bisphenol A, and
215 chlorophenols (Penalver et al., 2002; Kim et al., 2013; Vallecillos et al., 2013; Xu et al., 2013).

For bioanalytical *in vitro* studies, enrichment of water samples is often required to exceed the limit of detection as well as to provide optimum assay medium conditions and to avoid contaminating bioassays with pathogens. Effect concentrations of enriched sample extracts can be translated into equivalent concentrations of a reference compound (*e.g.*, estrogen equivalents), which can then be extrapolated to the original sample (Wagner et al., 2013b). Bioassays ideally target all contaminants present in a sample. However, the enrichment of water samples generally entails the loss of a significant fraction of the total contaminants (*e.g.*, Daughton, 2003). Enrichment methods used for bioanalytical tests systems include freeze-drying, reverse-osmosis concentration (Speth et al., 2008), liquid-liquid extraction (Pan et al., 2014), passive sampling (Jin et al., 2013), SPE, or purge and trap methods (Stalter et al., 2015b). Among these extraction techniques, SPE is most widely used with polystyrene/divinyl benzene polymers being the most frequently used type of sorbent (in particular the hydrophilic-lipophilic-balanced reversed-phase sorbent HLB). As opposed to freeze drying and reverse-osmosis, SPE allows for a good recovery of organic contaminants while removing matrix components to a wide extent. However, very polar or volatile compounds are lost during extraction. Dosing and exposure in bioassays also usually lead to the loss of volatile compounds. Accordingly, when volatile contaminants are expected to be present in a sample, extraction and bioassay methods need to be adapted to avoid underestimating the sample toxicity (Stalter et al., 2013, 2015b).

Benefits and limitations & future research needs

Further efforts should focus on the development of standardized sampling and sample handling strategies. This would significantly enhance the accuracy of analytical data which is important in terms of comparability between different studies and the modeling of the fate of CECs in WWTPs. The substantially increased sensitivities of modern LC/MS/MS instruments allow for detection and quantification of CECs without prior sample enrichment (direct-injection LC/MS/MS). For bioanalytical assessments the development of new sample enrichment methods is desirable to minimize the loss of volatile and hydrophilic contaminants.

2.2. Overview of chemical analytical methods for water quality assessment

A number of different chemical methodologies have been developed to assess WW quality including 1) the analysis of known compounds (target analysis), 2) screening for so far unknown compounds (non-target and suspect analysis), 3) investigating the fate of compounds during WW treatment (formation of transformation

products (TPs)), 4) computational modeling, and 5) the identification of toxicants (effect-directed analysis (EDA) and toxicity identfication evaluation (TIE); Fig. 1). All these approaches have specific strengths and weaknesses, which will be further discussed. However, this review does not aim to provide an exhaustive compilation of all available techniques. Rather, it focusses on most commonly used techniques among researchers. Similarly, in the chapter on target analysis we focus on emerging organic contaminants as these have recently been shown to be an important class of anthropogenic compounds detected in the effluents of WWTPs. For a detailed overview of other compounds such as priority pollutants, we refer to a number of reviews on these topics (*e.g.*, Luo et al., 2014; Verlicchi et al., 2012).

2.2.1. Target analysis

The analysis of CECs in WW dates back more than 50 years (Hignite and Azarnoff, 1977). Due to the complexity of the sample matrix and the large variety of CECs, numerous methods have been developed with gas chromatography (GC) and liquid chromatography (LC) being most widely used. The development of powerful separation technologies has been accompanied by advances in detection methods, in particular tandem mass spectrometry (MS/MS), which are specific and sensitive enough to detect CECs at concentrations typically observed in WW down to the lower ng/L range.

LC/tandem MS

Due to the high polarity of most compounds emitted from WWTPs, most advances in this field have been based on LC methodologies (Alder et al., 2006). The application of LC-MS increased exponentially in recent years and a large number of LC-MS methods have been developed to detect and quantify a huge variety of organic CECs (*e.g.*, Petrovic et al., 2003, Fatta-Kassinos et al., 2011). For chromatographic separation most frequently reversed-phase (RP) columns are used, which allow for the retention of a wide spectrum of compounds with different physico-chemical properties. However, RP columns provide only poor retention of very hydrophilic compounds. The analysis of highly polar compounds is of major importance for the assessment of the WW quality as these compounds might be formed in considerable quantities in different oxidative WW treatment processes. Examples include the formation of low molecular weight aldehydes, carboxylic acids and amines during ozonation (*e.g.*, Alvares et al., 2001; Wert et al., 2007). To tackle this problem, alternative stationary phases, in particular ion exchange chromatography (IC), hydrophilic interaction liquid chromatography (HILIC), and porous graphitic carbon chromatography have been developed. IC coupled to MS enables the separation of

charged molecules such as carboxylic acids on anion exchange columns (Bauer et al., 1999). HILIC employs traditional stationary phases known from normal phase (NP) chromatography, but uses similar mobile phases as RP-LC (Buszewski and Noga, 2012). Consequently, it allows for an improved retention of highly polar compounds compared to RP-LC, whereas hydrophobic compounds elute close to the void volume. Another alternative are porous graphitic columns (Tornkvist et al., 2003), which also provide exceptionally high sorption capacities for highly polar compounds. However, the analysis of hydrophobic compounds might be limited due to irreversible sorption to the stationary phase.

GC/MS

GC/MC is frequently used for the analysis of non-charged compounds as well as (semi)volatiles, with and without derivatization (Fatta-Kassinos et al., 2011). These include endocrine disrupting compound (EDCs), phenolic compounds, perfluorinated compounds, surfactants, musk fragrances, and siloxanes (*e.g.*, Trinh et al., 2011; Field et al., 1994; Bester, 2009). For EDCs such as hormones, phenols, and phthalates the reliable detection and quantification at very low concentrations is crucial as these compounds have been shown to cause adverse effects already at ng/L to pg/L levels (Kidd et al., 2007; Sumpter, 2005). Furthermore, two dimensional gas chromatography (GCxGC) methodologies have been developed to allow for detailed fingerprinting of WW samples (Gomez et al., 2011). However, the analysis of polar analytes is only possible after appropriate derivatization procedures. To overcome this problem, recent approaches used ionic liquids as GC stationary phases, as these allow for the analysis of polar compounds such as nitrosamines and caffeine metabolites (Reyes-Contreras et al., 2012).

Enantioselective and compound specific isotope analysis

Recently the enantioselective analysis of chiral emerging contaminants has substantially increased, using GC, LC and capillary electrophoresis (Wong, 2006). Due to the enantioselectivity of enzymes and biochemical receptors, the separation of chiral compounds is crucial in terms of biodegradation and ecotoxicity. The enantiomeric ratio can also be used to assess raw WW discharges into surface waters (*e.g.*, Buser et al., 1999; Fono and Sedlak, 2005). Furthermore, enantiomer specific analysis allows for the differentiation between biotic and abiotic degradation processes in contrast to abiotic processes, biotic processes often discriminate specific enantiomers (Kasprzyk-Hordern, 2010). Another approach is the application of compound specific isotope analysis (CSIA) which allows for the differentiation between i) abiotic and biotic processes, attributable to the

process dependent discrimination of light and heavy isotopes (Elsner et al., 2012), as well as ii) various sources of CECs such as different production facilities (Spahr et al., 2013).

Benefits, limitations & future research needs

Target analysis has become one of the major tools for the chemical assessment of WW quality. Though developments in recent years have substantially improved the capabilities of analytical instruments to detect and quantify CECs at concentrations typically observed in raw and treated WW, analytical chemists are still facing a number of challenges. These include the i) development of highly sensitive analytical methods for the detection of a specific compound or compound class known to already show adverse environmental effects at very low concentrations (e.g. 17α-ethinylestradiol), ii) development of multi-methods which allow for the simultaneous quantification of hundreds of CECs as well as their TPs, iii) development of standardized protocols for the analysis of CECs, to improve the comparability of analytical results obtained from different laboratories, iv) development of strategies for the semi-quantification of compounds for which no reference standards are available (e.g., TPs), and v) targeted approaches for the identification of new CECs based on production volume data, reported toxicities, high stability (e.g., non-biodegradable)/likelihood to be transformed into toxic TPs (e.g., based on modeling; structural alerts).

2.2.2. Non-target and suspect screening

The development and the application of so called "non-target" approaches are growing in response to the large compounds detected in environmental waters. However, as very few studies on their applicability for the evaluation of wastewater quality exist so far, only a brief introduction is provided here.

In non-target screening no *"a priori"* information about the presence of individual compounds is available (Krauss et al., 2010b). In contrast to non-target analysis, suspect screening approaches analyze the high-resolution MS data by searching for compounds suspected to be present in the samples but without a reference standard at hand (Little et al., 2012). Advances are closely linked to improvements in the accuracy of modern mass spectrometry instruments, as the determination of exact masses, and thus the assignment of chemical structures, is a major prerequisite for the identification of unknown compounds. The large amount of data generated makes it necessary to use computational software tools for further data processing such as automated identification of peaks via comparison with online databases, isotope pattern recognition, automatic recalibration, and processing of mass spectra as well as automated MS and MS/MS data interpretation

(Katajamaa and Oresic, 2007). However, the application of non-target screening to WW samples is hampered by factors such as matrix effects which complicate the comparison between different samples such as raw and treated WW. Alternative approaches may include the application of analytical techniques, which are less influenced by the sample matrix, such as high field asymmetric waveform ion mobility spectrometry (FAIMS) (Sultan and Gabryelski, 2006).

Benefits, limitations & future research needs

Non-target and suspect analysis can be very valuable in the search for unknown CECs, including TPs. Modern instruments allow for the simultaneous detection of thousands of peaks within a single run by simultaneously providing MS spectral information of the most abundant masses (data dependent acquisition). To focus only on the most abundant peaks might, however, be misleading as the ionization efficiencies strongly depend on the chemical structure of the compounds as well as the sample matrix. The latter is particularly important when the comparison of raw and treated wastewater is used for the identification of compounds which are eliminated, recalcitrant, or newly formed. As the chemical structures of compounds are unknown, new methods have to be developed allowing for their (semi)quantification.

2.2.3. Sum parameters

General wastewater characteristics

Sum parameters such as total nitrogen (N_{tot}), total phosphorous (P_{tot}), chemical and biological oxygen demand (COD, BOD), total organic carbon (TOC), dissolved organic carbon (DOC), and total suspended solids (TSS) were the first indicators used to determine the quality of treated WW. These offer the great advantage that they are i) easy to measure with standardized methods and ii) affordable with no sophisticated instrumentation needed. Consequently, they belong to the most frequently measured parameters. However, while they allow for the determination of nutrient and organic emissions from WWTPs, they do not provide any detailed information on the presence of toxic CECs.

CEC specific sum parameters

The specific UV absorbance at 254 nm ($SUVA_{254}$) has proven a useful parameter for the control of ozonation processes and the assessment of the oxidation efficiency of aromatic compounds (Weishaar et al., 2003). $SUVA_{254}$ or the fluorescence volume in excitation emission matrix fluorescence spectra might be good indicators

for potential ecotoxicological effects as several aromatic compounds, in particular those containing phenolic moieties, are known to frequently exhibit endocrine disrupting effects, (Tang et al., 2014b). To further aid the search for compounds containing heteroatoms (*e.g.*, halogens or metals), GC- or LC-MS analysis should be supplemented by complimentary techniques such as adsorbable organic halogen (AOX) analysis and/or other element specific analytical approaches (e.g. LC-ICP-MS). AOX is appropriate to cover highly persistent compounds of considerable health concern (Jekel and Roberts, 1980). Adsorbable organic fluoride (AOF) measurements in surface waters recently indicated that in surface water samples only <5% of total AOF could be attributed to PFCs. This highlights the need to identify unknown CECs bearing fluorine atoms (Wagner et al., 2013a). In order to assess the formation potential of toxicological relevant N-nitrosamines formed during oxidative (waste)water treatment via chlorination or ozonation, a total nitrosamine assay (TONO assay) has been developed to identify N-nitrosamine precursors such as natural and anthropogenic WW constituents (Mitch and Sedlak, 2004).

Benefits, limitations & future research needs

Sum parameters are very helpful to determine overall wastewater characteristics such as nutrient and organic loads. However, they do not provide any information on the presence of CECs. To take up this challenge, new sum parameters should be developed for toxicological relevant compounds such as phenols, aldehydes, or nitrosamines (so called toxicophore assays). Those methods should be easy to apply and standardized facilitate the routine application for WWTPs.

2.2.4. Identification and monitoring of transformation products

The analysis of CECs in raw and treated WW, using target, suspect, and non-target screening does not typically provide any information on the actual fate of CECs. Observed losses when comparing influent and effluent concentrations of WWTP can be caused by sorption, mineralization, volatilization as well as transformation to stable TPs. The latter is not a “real” removal, since the toxicological potential of the formed TPs can be significant. TP formation has to be considered, or the mass balances might not close, and the toxicity reduction will likely be over-estimated. TPs can be formed by a number of different processes such as biodegradation (catalyzed by enzymes) or chemical oxidation (*e.g.*, during ozonation or chlorination). A number of different methodologies exist to isolate and identify TPs formed in laboratory experiments (exposure-driven) or to monitor toxicity of the parent compound during transformation (effect-driven) (Escher and Fenner, 2011).

Laboratory transformation experiments

Transformation experiments are often carried out in batch systems, whose controlled laboratory conditions allow the investigation of factors influencing wastewater treatment, such as pH and redox potential. Elevated concentrations of CECs are frequently used to i) identify TPs using a variety of methods such as high-resolution MS and NMR, ii) quantify TPs in samples taken from WWTPs and receiving waters, and iii) investigate the fate of TPs in subsequent WW treatment steps. Experiments at environmentally relevant concentrations should always be conducted in parallel to better represent likely outcomes in the field. Furthermore, control experiments, *i.e.*, in the absence of the target compounds, are used to determine if TPs formed are originated from degradation of substances already present in the sample (e.g. NOM). Sterilized control experiments are essential for biodegradation studies to differentiate between biotic and abiotic degradation processes. This can be achieved via irradiation with gamma rays, autoclaving and/or antimicrobial additives (NaN_3, antibiotics). Autoclaving should be repeated several times to also ensure the inactivation of spores. The addition of a chemical additive (antimicrobial) should be considered as least favorable option as, *e.g.*, NaN_3 can react as a strong nucleophile with the target compounds.

Analytical tools to identify transformation products

The monitoring of the dissipation of CECs and the determination of (bio)degradation kinetics is performed by target analysis, whereas chemical structures of unknown TPs are typically identified by LC/MS/MS (with and without ion trap), LC-HRMS, ICP-MS, and NMR. Among these, HRMS is most widely used as it allows the fast scanning of samples over a wide range of m/z values by simultaneously providing information on fragmentation of formed TPs (MS^n experiments). ICP-MS has been applied for the identification of TPs formed from the artificial sweeteners cyclamate and acesulfame as well as the X-ray contrast medium diatrizoate (Scheurer et al., 2012; Zwiener et al., 2009; Redecker et al., 2014). However, the results obtained from HRMS or ICP-MS often do not allow for an unambiguous identification of the chemical structures (Creek et al., 2014; Schymanski et al., 2014a). Thus, comparison with a reference standard or NMR analysis is needed to elucidate the chemical structure of TPs formed.

In cases where an unambiguous identification of TPs is difficult, indirect approaches are used by obtaining additional information on the presence of specific functional moieties. For instance, derivatization offers the

possibility to identify the presence of specific functional moieties. Trimethylsilane (TMS) and subsequent GC/MS analysis of derivatives can be used to determine the number of acidic hydrogens from acidic, alcoholic, thiol, amine, and amide moieties. Furthermore, derivatization is applied to detect the formation of moieties which might be of toxicological relevance (toxophores) such as aldehydes, amines and N-nitrosamines (*e.g.*, Kataoka, 1996). The investigation of structural analogues provides additional information on transformation mechanisms (*e.g.*, Wick et al., 2011).

Calculation of mass balances

The calculation of mass balances based on quantification of both parent compounds and TPs in both laboratory experiments and real treatment systems is crucial to assess whether the dominant TPs have been considered. Incomplete mass balances indicate that i) the used detection methods were unsuitable to identify major TPs, ii) a complete mineralization and/or microbial uptake occurred, and/or iii) sorption of TPs took place. Calculation of mass balances based on MS peak areas is generally not suitable, as the ionization efficiencies can differ substantially even for compounds with very similar chemical structures. HPLC-UV is suitable in those cases where the main chromophore(s) remain unchanged, *e.g.*, transformation only takes place at the side chain attached to an aromatic ring system. In case of major transformations of parent compounds reference standards are thus needed which often have to be synthesized in the laboratory due to the lack of commercially available reference standards.

In cases when the mass balance is incomplete, radioactive (*e.g.*, ^{3}H, ^{14}C) or stable isotope labeled compounds (*e.g.*, ^{13}C, ^{15}N) can be used to obtain further insights. The application of radioactive labeled compounds offers the advantage that also volatile compounds such as $^{3}H_2O$ or $^{14}CO_2$ can be quantified (*e.g.*, by trapping volatiles). Furthermore, the aqueous phase can be analyzed directly using liquid scintillation counting (LSC) to determine the total amount of radioactivity present (Roslev et al., 2007). For solids, samples can be combusted, and the released $^{14}CO_2$ or $^{3}H_2O$ can be trapped and also analyzed via LSC. However, the labeling position is crucial in terms of the interpretation of the experimental results with both radioactive and stable isotope labeled compounds as the release of $^{3}H_2O$ and $^{14}CO_2$ from partially labeled compounds only indicates a mineralization of the labeled moiety but not the whole molecule. Stable isotope labeled compounds can significantly facilitate the identification of TPs in laboratory experiments when they are added together with their non-labeled analogues due to the distinct isotope patterns observed in MS detection (Badia-Fabregat et al., 2014). In addition, isotope

fractionation visible in the relative abundance of, *e.g.*, carbon and nitrogen atoms present in CECs allows for distinguishing between different degradation processes (Elsner et al., 2012) and identification of CEC degrading bacteria (Uhlik et al., 2013).

Benefits, limitations & future research needs

We are only at the very beginning in our understanding of the transformation of CECs in WW treatment. Most studies conducted so far clearly revealed that the elimination of CECs in most cases is leading to the formation of TPs. Future research should emphasize the development of methodologies targeting a more comprehensive investigation of transformation mechanisms. Furthermore, high-throughput methodologies combining different analytical techniques such as HRMS and NMR are needed to allow for the unambiguous identification of formed TPs. Instead of focusing on TPs formed from individual CECs, further emphasis should be placed on methods enabling a simultaneous assessment of potential adverse environmental effects (e.g., by combination with bioassays). Furthermore, approaches are needed to semi-quantify TP concentrations, which is crucial to calculate mass balances and determine their environmental relevance.

2.2.5. Computational modeling

Computational tools for the assessment of WW quality are likely to be of increasing importance in the future, as these can be used for the prediction of i) (bio)degradation kinetics and elimination efficiencies of CECs, ii) TP formation, and iii) toxicities of CECs and their TPs. Several structure-based biodegradation estimation methods have been developed to predict of biodegradability of organic compounds (Raymond et al., 2001; Jaworska et al., 2003). Quantitative structure biodegradability relationships (QSBRs) allow classification of chemicals according to relative biodegradability and prediction of biodegradability for newly identified CECs. These models are using molecular descriptors or specific moieties, so called biophores, to predict biodegradability (Mansouri et al., 2013). Similar approaches have also been used to assess removal efficiencies of organic compounds during oxidative treatment such as ozonation or chlorination (Deborde and von Gunten, 2008; Lee et al., 2013). Associated uncertainties of the models are still relatively high, however, and the applicability is often limited to structurally similar compounds.

A second application field of computational modeling approaches is the prediction of TPs being formed during WW treatment. Prediction tools such as UM-PPS, KEGG, Molgen, and PathPred have been successfully used to predict the formation of TPs from known compounds (see Ruecker et al., 2012 and references therein). These

predictions are, in general, based on transformation rules derived from known biochemical reactions acting on specific chemical functional groups. This information can then be used in combination with HRMS to screen for biotransformation TPs (Helbling et al., 2010). One major current drawback of these predictions is that they often lack of specificity and thus either result in a large over-prediction of formed TPs or that TPs are not predicted at all (Prasse et al., 2011). However, the increasing knowledge about transformation pathways of organic CECs will most likely lead to further refinements of relevant environmental transformation reactions and thus a higher accuracy of TP predictions.

Finally, computational tools such as quantitative structure activity relationships (QSAR) and 3D toxophore mapping also have been utilized for the prediction of the toxicological potential of CECs and their TPs. These include the prediction of binding of CECs to the estrogen receptor (Liu et al., 2006) as well as the identification of toxicophores in pesticides as well as their TPs (Sinclair and Boxall, 2003). QSAR models have also been used to assess mixture toxicity (Xu et al., 1998). These methods allow for the high-throughput screening of a large number of compounds and can thus be used as a first indication of potential toxicological effects. However, QSAR models require that each chemical must be unambiguously assigned to a specific mechanism of action, because only chemicals with the same mechanism of action share a common QSAR equation (Schwoebel et al. 2011). Structural alerts, which are frequently used in the assessment of the formation of toxic metabolites in toxicology, can also provide additional information on potential toxicity mechanisms of TPs and can be used to identify structural elements in CECs, which might lead to the formation of toxic TPs such as N,N-dimethylamine moieties, which are potential NMDA precursors (Krauss et al., 2010a).

Benefits, limitations & future research needs

Computational approaches have been shown to be capable to predict the elimination of CECs during conventional and advanced WW treatment. For the (environmental) risk assessment of chemicals, computational modeling already today plays an important role and its application is likely to increase in the future. Computational toxicity prediction may help to substantially reduce the number of animal tests and is thus also advantageous from an ethical point of view. The main future challenge is related to its applicability in terms of both behavior (i.e., transformation) and effects of CECs. Computational methods may support the identification of relevant CECs as they allow for the screening of large numbers of chemical substances for specific structural alerts with known (eco)toxicological relevance. This is also true for the prediction of compounds leading to the formation of toxic TPs.

2.2.6. Effect-directed analysis and toxicity identification evaluation

A general restriction of the methods focussing on the identification of unknown compounds is that they do not provide any information on related (eco)toxicological effects. Approaches to identify compounds which show (eco)toxicological effects are effect directed analysis (EDA) and toxicity identification evaluation (TIE) (Burgess et al., 2013; Hewitt and Marvin, 2005). These have the advantage that the large number of compounds present in environmental samples is reduced to those which specifically interact with biological systems (Brack, 2003). The main difference between both approaches is that TIE focuses on toxicological endpoints by using whole organism toxicity tests, whereas EDA utilizes *in vitro* assays to determine mode of actions such as mutagenicity or genotoxicity (Burgess et al., 2013). Detailed information on the (eco)toxicological methods are provided in the next chapter (chapter 2.3). In contrast to TIE, sample extraction and enrichment are usually performed for EDA to attain a sufficient sensitivity of applied bioassays. For EDA and TIE further analysis is only conducted in case of an observed (adverse) effect in order to identify responsible compound(s). To this end, sample fractionation or selective sample extraction is applied to reduce sample complexity and remove non-toxic compounds (Hewitt and Marvin, 2005; Brack, 2003). Both EDA and TIE have been successfully applied for the identification of toxic CECs in highly contaminated matrices such as industrial WW (de Melo et al., 2013). For municipal WW, however, so far only a limited number of studies exist showing a successful application (*e.g.*, Grung et al., 2007; Smital et al., 2011). This can be attributed primarily to much lower concentrations of toxic CECs, which often go undetected by bioassays due to insufficient sensitivities. Even though sample enrichment via SPE is frequently used, this only allows for the enrichment of compounds that can sorb appreciably on SPE materials. The use of different SPE materials (*e.g.*, RP-C18, HLB polymers, activated carbon) might allow for the enrichment of a broad spectrum of CECs (*e.g.*, neutrals, anionic and cationic CECs). Elevated concentrations of co-extracted matrix components such as NOM, however, can lead to erroneous results.

Application of EDA/TIE in laboratory experiments

In addition to laboratory degradation experiments used to elucidate the formation of TPs, the application of EDA can provide valuable information (Fenner and Escher, 2011). In cases where the toxic effect(s) of a parent compound is known, EDA can be used to follow (de)toxification during degradation (Dodd et al., 2009; Mestankova et al., 2012). When the overall toxicity increases, it is indicated that toxic TPs are formed which might trigger further studies to identity of the responsible compounds. It is important to note that this only

provides information on the specific mode of action (MoA) covered by the used bioassay. If TP(s) are formed with different MoAs than the parent compound, they may be missed.

Application of innovative bioanalytical tools for EDA

To couple analytical and ecotoxicological tools and to increase high-throughput capabilities, several advanced methods have been developed in the last years. As an example, high performance thin-layer chromatography (HPTLC) with bioactivity screening and subsequent MS analysis is increasingly used to identify toxic compounds in complex mixtures and to elucidate their chemical structures (Eberz et al., 1996; Morlock and Schwack, 2010). This approach has recently been extended to the analysis of EDCs as well as antibacterial agents in environmental samples (Spira et al., 2013; Lewis et al., 2012). Hyphenation with mass spectrometry thereby allows for the identification of those CECs that are responsible for observed effects (Morlock and Schwack, 2010). Another methodology for the elucidation of EDCs is the application of receptor affinity chromatography (Shang et al., 2014), with separation of estrogenic compounds being achieved by estrogen receptor ligand binding domain (LBD) immobilized affinity Ni–NTA columns (Jondeau-Cabaton et al., 2013).

Benefits, limitations & future research needs

Thus far for EDA and TIE, very few examples exist in the literature that successfully identified the CEC(s) causing the observed toxic effects. On the one hand, there is a need for high-throughput procedures as most of the methods currently used are labor intensive and time consuming. On the other hand, bioassays are often not sensitive enough to detect effects in the different fractions. Therefore, high sample enrichment factors (up to >5 orders of magnitude) are required for HPLC fractionation and subsequent bioanalytical assessment. However, co-enriched matrix constituents can interfere with both, the sample separation as well as the bioassay results. Thus, highly specific and effective sample enrichment methods as well as more sensitive bioassays are required to improve the success for the identification of currently unknown toxic CECs.

2.3. Overview of ecotoxicological methods for water quality assessment

The vast number and toxicological relevance of unknown contaminants in water samples (Stadler et al., 2012; Tang et al., 2013) emphasizes the need for bioanalytical water quality assessment to complement chemical analysis. Depending on the objectives, studies and experiments are designed to assess single substance effects or complex mixture interactions with different complexity levels of biological organization (*i.e.*, molecular,

cellular, organ, single species, population, community, ecosystem). The toxicological impact of substances is mainly dependent on concentration, bioavailability, duration of exposure, critical windows of exposure, and species-specific sensitivity. Regarding the latter, different test designs, biological targets, and test species might be selected to identify deficiencies in water quality.

In this chapter, we focus on water quality assessment methods, which are used to toxicologically or ecologically characterize water samples. The most commonly used approaches are categorized into four groups: i) *in vitro* bioassays, ii) *in vivo* toxicity tests, iii) *in situ* exposure or active and passive bio-monitoring, and iv) effect assessment on community level of aquatic organisms (bacteria, plants, invertebrates, fish). Each group encompasses a multitude of different test methods and analysis strategies. Here we provide an overview of the most common approaches, discuss advantages and drawbacks, as well as general challenges, without providing an exhaustive list of all methods available.

2.3.1. In vitro bioassays

We refer to *in vitro* bioassays as cell or bacteria-based assays, which can be conducted in wells of microplates aiming to assess toxicity and toxicity pathways. *In vitro* bioassays encompass simple cytotoxicity tests (ISO, 1998; Riss et al. 2011), sophisticated engineered reporter gene assays to detect adaptive stress response pathways (*e.g.*, Escher et al., 2012) or receptor interactions, as well as biomarker studies (*e.g.*, Suares Rocha et al., 2010; Gagne et al., 2013) and toxicogenomic or metabolomic methods (Van Aggelen et al., 2010). *In vitro* screening methods are increasingly preferred compared to chronic *in vivo* approaches due to logistical, cost, and time constraints as well as ethical considerations. Additionally, the level of simplification of *in vitro* test systems facilitates mechanistic studies to explore toxicity pathways (Toxcast21, Martin et al., 2010) as the multitude of existing bioassays covers numerous different MoAs from non-specific baseline toxicity to receptor-ligand interactions. However, most challenging remains to extrapolate from *in vitro* test results to the relevance to organisms and ecosystems. *In vitro* effect concentrations are often correlated to adverse *in vivo* effects (*e.g.*, Schipper et al., 2009; Stalter et al., 2015a) and provide a reliable indicator for the presence of toxicologically relevant contaminants like EDCs (Schipper et al., 2009; Escher et al., 2014) or photosynthesis inhibiting herbicides (Escher et al., 2011), while only a few of those compounds can explain more than 90% of the observed effect (Escher et al., 2011). Furthermore, it is comparably simple to translate such very specific *in vitro* endpoints to their environmental relevance, which can then be easily confirmed with field studies (intersex, phytotoxicity, biomarker). However, less specific toxicity endpoints (*e.g.*, baseline toxicity or reactive toxicity

endpoints such as genotoxicity or oxidative stress) are usually more challenging to tackle because many more substances are able to trigger respective effects (Escher et al., 2013, Tang et al., 2013).

Benefits, limitations & future research needs

The chances to detect in short order unknown properties of chemicals with the help of in vitro *assays are excellent. Generally,* in vitro *tests represent screening tools appropriate for high sample throughput. The possibilities to identify toxic CECs with* in vitro *test systems in complex mixtures are higher compared to* in vivo *assays because of the former's fast screening and high throughput capabilities. Additionally,* in vitro *tests can scan environmental samples for very specific MoAs, which can indicate the presence of specifically acting CECs in a mixture. However, the extrapolation from* in vitro *test results to toxicological relevance in organisms and ecosystems is fraught with many uncertainties, because* in vitro *assays are neither designed to model nor to assess systemic effects of substances. They are useful to determine biological activity, impact potentials, and MoAs but do not consider counter-regulatory processes within whole organisms. Furthermore,* in vitro *assays do not necessarily detect receptor-, cell-, tissue-, or organ-specific effects; metabolic mechanisms targeting biological activation; or detoxification.*

2.3.1.1. Cytotoxicity

Cytotoxicity assays measure whether a compound or sample is toxic to cells—usually by determining cell viability, cell number, or cell proliferation after a defined exposure period—and are used as predictor of potential toxicity *in vivo* (Riss et al., 2011). Cell viability can be measured by assessing the membrane integrity (*e.g.*, lactate dehydrogenase and neutral red assay), the optical density as an indicator for cell density, the caspase-3/7 activity as a marker for apoptosis, several markers for disturbances of metabolism and energy production (*e.g.*, tetrazolium reduction using MTS, resazurin reduction, ATP level, photosystem II inhibition in algae cells; Escher and Leusch 2011), and luminescence of luminescent bacteria. Cytotoxicity is most commonly referred to as non-specific toxicity. However, only the measured endpoint is unspecific (viability or proliferation), whereas the underlying MoA can be non-specific (*e.g.*, apolar narcosis or oxidative damage) as well as highly specific (*e.g.*, specific inhibition of photosystem II or ATP synthase; Escher and Leusch, 2011).

A variety of different cell lines are used to assess effects on a cellular level, *e.g.*, mammalian or fish cell lines, bacteria, or algae. One of the most commonly used and most simple approaches to assess cytotoxicity is the

633 measurement of bioluminescence inhibition of naturally bioluminescent bacteria (*e.g.*, *Aliivibrio fischeri*; ISO,
634 1998).
635
636 ***Benefits, limitations & future research needs***
637 *Cytotoxicity tests provide a quick and easy procedure to check vitality parameters of cells which are usually*
638 *affected by a broad range of substances and hence are important initial screening tools for water quality*
639 *assessment. Additionally, cytotoxicity is important to assess in combination with more specific assays (e.g.,*
640 *mutagenicity or genotoxicity assays) to check for false-negative results due to cytotoxic effects.*
641

642 ### *2.3.1.2. Genotoxicity/mutagenicity*

643 Genotoxicity can be defined as the property of chemical or physical stressors to cause DNA damage or to induce
644 an adaptive stress response preventing DNA damage repair. Direct DNA damage encompasses mutations (*i.e.*,
645 change in the DNA base-pair sequence; *e.g.*, base pair substitutions or frame shifts), structural and numerical
646 chromosomal aberrations, DNA alkylation, oxidative damage, de-purination, formation of DNA adducts, and
647 various other mechanisms (Lindahl, 1993). In organisms, DNA damage may be repaired or leads to apoptosis. If
648 left uncorrected, DNA damage can lead to uncontrolled cell proliferation and cancer. Thus, genotoxicity
649 assessment is of critical importance for public and environmental health, and genotoxicity assays are among the
650 most widely used *in vitro* bioassays in (eco-)toxicology.
651 The Ames test (Ames et al., 1975) was one of the earliest *in vitro* bioassays used for water quality assessment
652 and still plays a dominant role in genotoxicity testing (Claxton et al., 2010). The Ames test uses various bacterial
653 test strains to detect mutagenicity; some of them detect specific types of mutations (*e.g.*, TA98 for frame shift
654 mutations; TA100 for base-pair substitutions; TA102 for oxidizing mutagens; Reifferscheid et al., 2012; OECD
655 Guideline); others detect alkylation (Yamada et al. 1997) or glutathione-conjugation mediated mutagens (Thier
656 et al., 1993). To avoid false negative results, it is recommended to use at least five different strains of bacteria
657 with and without exogenous metabolic activation system such as S9 (OECD, 1997).
658 The comet assay (Singh et al., 1988) and the micronucleus assays (Countryman and Heddle, 1976) are frequently
659 applied to detect chromosomal aberrations in mammalian and other cell-lines and are important tools for water
660 quality assessment.
661 The most commonly applied assay to detect adaptive response to DNA damage is the bacterial umuC assay (Oda
662 et al., 1985). For this assay a *Salmonella* strain was modified by fusing the reporter gene lacZ to the umuC

operon, which is part of the SOS pathway cellular response to DNA damage that controls DNA repair mechanisms. Additionally, the tumor suppressor protein p53 is used to detect adaptive responses to mammalian DNA damage (Yeh et al., 2014; Stalter et al., 2015a). In any case, a set of different genotoxicity assays should be chosen for water quality assessment as single assays are prone to false negative results (Magdeburg et al., 2014).

Benefits, limitations & future research needs:

Positive genotoxicity data entail an inherent risk for carcinogenesis wherefore genotoxicity assessment is of high importance for human and environmental health. However, non-carcinogens can also induce positive results in genotoxicity assays and hence the in vivo *relevance of positive* in vitro *results needs to be evaluated for a comprehensive assessment. Additionally, also non-genotoxic mechanisms can play a role in carcinogenesis which is not detected with most* in vitro *genotoxicity tests. Cell lines applied usually derive from malignant tissue, e.g., deficient in p53 function or DNA repair. The latter suggests differences in vulnerability towards genetic disorders between, e.g., tumor and healthy cells, what makes the extrapolation of data between the two difficult. Beyond that it is an ongoing debate whether prokaryote test findings can be transferred to eukaryotic cells. To avoid false negative results a set of different genotoxicity assays and different strains of bacteria should be applied.*

2.3.1.3. Endocrine disruption

Since the discovery of hormone-like activity of many environmental contaminants and their implications for human and wildlife health (*e.g.*, Sonnenschein and Soto, 1998; Tyler et al., 1998) many bioanalytical test systems have been developed as an alternative to animal studies to assess the endocrine disrupting potency of chemicals and environmental samples. EDCs can interfere with the endocrine system via direct receptor binding mimicking an endogenous hormone (agonism), by blocking the receptor causing an antagonistic effect, or by indirect mechanisms such as interferences with the hormone biosynthesis (*e.g.*, Escher and Leusch 2011). In addition to genotoxicity and cytotoxicity assays, bioassays detecting endocrine activity are probably the most frequently applied *in vitro* assays in environmental toxicology, with the major focus on aryl-hydrocarbon receptor (AhR) agonists (*e.g.*, TCDD, PCBs, PAHs) and steroid receptor agonists and antagonists. Among the steroid hormone receptors, the estrogen (ER) and androgen receptor (AR) are most commonly studied, while effects on the progesterone, glucocorticoid, and mineralocorticoid receptors gain increasing attention. Effects on the thyroid and retinoic acid receptor are also becoming more and more relevant for water quality assessment.

The *in vitro* evaluation of environmental samples on endocrine disrupting activity became popular with the *in vitro* screening methods developed in the 1990s including the E-screen assay on estrogenicity using the human MCF7 cell line (Soto et al., 1992), the yeast estrogen and androgen screen (YES, YAS; Routledge and Sumpter, 1996; Sohoni and Sumpter, 1998) as well as a yeast screen assay on AhR activity (Miller, 1997). Additionally, a growing number of mammalian cell-based test systems have been developed (*e.g.*, ER-calux (Legler et al., 1999), AR-calux, AhR calux (Murk et al., 1996), T-screen). Yeast assays are robust, simple, and cost-efficient tools, but they are also less sensitive compared to mammalian cell based assays (Leusch et al., 2010). *In vitro* tests on endocrine activity have been shown to be good predictors for *in vivo* endocrine disruption (Sonneveld et al., 2006; Jobling et al., 2009). In recent years, bioanalytical studies investigating environmental samples encompass more and more endocrine endpoints covering antagonistic activities and a growing number of receptors (*e.g.*, Martin et al., 2010; Escher et al., 2014).

Benefits, limitations & future research needs

Endocrine disrupters interfere with endocrine systems by a wide variety of direct and indirect MoAs. The majority of in vitro *tests applied for endocrine disrupter detection are based on either hormone directed transcription of reporter genes, proliferation in hormone-responsive mammalian cell lines, or subcellular receptor ligand binding. For these mechanisms* in vitro *tests can be very promising predictive tools. However, the identification of indirect effects is not covered by most of the established* in vitro *assays, with the H295R steroidogenesis assay as one of the rare exceptions (OECD guideline 456). Furthermore, transactivation assays are often composed of artificially-engineered (yeast-) cells, many of which are provided with mammalian hormone receptors in cellular environments foreign to the species. This may affect the predictive power when assay data are generated in the context of environmental research as endocrine systems, and receptors of non-mammalian species could vary in structure and function.*

2.3.1.4. Adaptive stress response induction

In recent years, a high number of new reporter gene assays have been developed to measure the activation/inactivation of cellular stress response pathways as reviewed by Simmons et al. (2009). Adaptive stress response pathways are cellular processes which aim to minimize and repair damages to cellular infrastructure (*e.g.*, nucleic acids, lipids, proteins, DNA, membranes, organelles) with the final goal to restore homeostasis (Simmons et al., 2009). The activation of such signal transduction pathways via environmental

stressors (*e.g.*, chemical toxicity, heat stress, radiation, osmotic stress) causes the activation of cyto-protective genes and in the production of cyto-protective proteins. Accordingly, their activation occurs at lower doses or exposure times than those required to cause apical toxic effects (*e.g.*, apoptosis; Simmons et al., 2009). Adaptive stress response induction is therefore regarded as an early warning signal of exposure to toxicants and respective assays are usually more sensitive than those measuring apical endpoints such as cytotoxicity (Escher et al., 2012, JEM). Commonly, adaptive stress response assays are reporter gene assays where the reporter gene (*e.g.*, for luciferase) is activated along with the target gene encoding for the stress response machinery. Currently, the Nrf2-mediated oxidative stress response pathway is the most frequently used stress response pathway in bioassays for water quality assessment and is usually the most responsive assay whenever a battery of bioassays is applied (*e.g.*, Farré et al. 2013; Stalter et al., 2013; 2015a; Escher et al. 2014).

Benefits, limitations & future research needs

Generally the biological function of stress response is to protect the organism from harm to enhance the chances of survival. Chronic exposure to stressors is known to result in decrease of other energy-intensive functions of the body such as immune defense, reproduction, cellular repair mechanism, etc., probably ending up in cancer or infecundity. Consequently, the measurement of adaptive stress response markers provides early warning signals as their production occurs prior to impacts on apical endpoints. They are thus an important and sensitive screening tool in environmental science.

2.3.1.5. Toxicogenomics

Advances in molecular biology within the last decades have dramatically increased the knowledge about gene structure and function which provides the basis of an increasing database of genetic sequence information (Aardema and MacGregor 2002) and allows investigating responses of the gene transcript or metabolome on environmental stress. This will support the understanding of mechanisms of chemical toxicity and can be used to monitor and characterize the effect of pollutants (Van Aggelen et al., 2010). Hence, toxicogenomic methods are increasingly applied in environmental risk assessment using microorganisms, cell-lines, or animals (Van Aggelen et al., 2010). One of the key challenges, however, is how to relate genome and metabolome data to toxicity pathways and ecological outcomes.

Benefits, limitations & future research needs

Toxicogenomics offer a unique chance to explore common features and differences between species. The task would be to find out where species share similar biochemical reaction chains and where it is possible to extrapolate (eco)toxicological results across species to reduce the number of toxicity tests. Toxicogenomics may provide support for the identification of unexpected mechanisms of action for toxicologically non-characterized substances by delivering their genomic effect profiles. However, before doing so the broad variety of criteria for data interpretation and techniques applied suggest an urgent need for standardization. A major challenge will be to link genetic data and endpoints with adverse effects in test species and to establish cause-effect relationships. Comprehensive genomic information is available for some selected species only and far apart from providing the relevant data for model or ecologically relevant organisms.

2.3.2. In vivo tests

In vivo bioassays aim to assess severity, time and dose dependency of toxic effects in multiple standard and non-standard whole organisms and communities. Studies encompass dosing regimens from acute (exposure time ≤ 96 h) to chronic (exposure time > 96 h) through to life-cycle experiments and several routes of exposure (in the aquatic environment usually via food or percutaneous). Testing of single species or biocoenoses is carried out either under laboratory or field conditions (*in situ*). Mesocosm studies represent something in between and focus on exposures of artificial/wild species communities under semi-field conditions. Field monitoring studies can be divided into passive and active. Whereas passive monitoring is focused on naturally occurring organisms in the test area, active studies insert organisms under controlled conditions into monitoring sites.

Benefits, limitations & future research needs

Whole organismic tests aim for the assessment of "integrative" or "apical" effects on, e.g., mortality, development, growth, reproduction, or behavior. However, they do not necessarily provide insights into the underlying molecular and biochemical reactions nor the targets responsible for toxicant action.

A different approach to categorize biological effects of environmental pollution was proposed by Segner et al. (2014). The authors launched a discussion towards a change of paradigm in ecotoxicological research appreciating cumulative impacts of multiple stressors on a huge amount of biological targets at various biological organizational levels. Biological receptors vary in sensitivity, vulnerability, response dynamics, and function as part of interacting physiological networks. Therefore, Segner et al. (2014) encourage a focus on properties of biological receptors rather than on properties of stressors. Segner et al. (2014) proposed to start a

tiered approach with an inventory of stressors followed by an inventory of affected biological receptors. "Multistressor response profiles" of receptors and network interactions should be assessed to integrate data in a yet to be developed framework for data structuring and organizing in compliance with the "Adverse Outcome Pathway" concept of Ankley et al. (2010). Actually the concept requires considerable research and development work but represents a refreshingly different way of thinking, probably shaping future assessment activities.

2.3.2.1. Single species tests

Results of tests with single species from different trophic levels are used for risk assessment of substances, *e.g.*, according to the European Commission's technical guidance document (European Commission, 2003) or in the course of authorization procedures for chemicals. Beyond regulatory actions, single species tests form the basis for general water quality assessment purposes. Studies allow for the detection of measurable adverse effects of biological parameters in target organisms including counter-regulatory actions. Ideally, species selected for laboratory or on-site tests (*e.g.*, WWTPs) represent an ecologically relevant choice of organisms inhabiting both the matrix (water, sediment, suspended solids), as well as the water body section of interest (resident species).

In environmental research, the detection of effects caused by hazardous substance often originated from field observations that were later verified in single species tests.

A high degree of popularity achieved wildlife studies at Lake Apopka (Florida), which was heavily contaminated with a large variety of chlorinated hydrocarbon insecticides. Guillette et al. (1994) observed malformed male sexual organs in *Alligator missippiensis* accompanied by lower plasma testosterone levels. Laboratory studies with the red-eared slider turtle (*Trachemys scripta elegans*) eggs exposed to the pesticides toxaphene, dieldrin, p,p'-DDD, cis-nonachlor, trans-nonachlor, p,p'-DDE, and chlordane in a concentration range detected in alligator eggs from Lake Apopka demonstrated that these chemicals are able to override a male-producing incubation temperature in reptiles (Willingham and Crews, 1998). As a result, temperature-dependent sex determination was undermined and resulted in enhanced female hatching. At about the same time, the detection of estrogenic effects under, *e.g.*, ethinylestradiol (EE2) and nonylphenol exposure in teleost species (Christiansen et al. 1998), attracted the attention of the scientific community. First Jobling and Sumpter (1993) observed that WWTP effluents contain chemicals that induce vitellogenin synthesis in male fish. Shortly thereafter, an increased prevalence of hermaphroditism in roach (*Rutilus rutilus*) colonizing near sewage treatment discharges was detected (Sumpter and Jobling, 1995). However, the responsible estrogenic compounds in the WWTP effluents inducing these effects were largely unknown. Subsequent single species tests checked a broad range of

chemicals for cause-effect relationships. In particular, alkylphenoles, pesticides, paints, and other formulations came into focus. The alkylphenols formed in municipal WWTPs sorb to activated sludge particles, suspended matter, and accumulate in aquatic organisms (Ekelund et al. 1990). *In vivo* studies revealed that nonylphenol exposure induces elevated plasma vitellogenin levels in fish (*e.g.*, Jobling et al. 1996). Altered vitellogenin plasma levels in fish have proven to be linked to severe disorders of spermatogenesis/oogenesis and impairment of fertility. Meanwhile vitellogenin induction in fish is a well-established biomarker of exposure to estrogenic substances (*e.g.*, Cheek et al., 2001; Christiansen et al., 1998; Sumpter and Jobling, 1995).

Tests prioritizing the functioning of ecosystems may consider endpoints such as primary production, food conversion rates, and impacts on behavior or intra-/interspecific competition. Adverse effects on these endpoints allow one to draw at least initial conclusions on potential impacts on ecological cycles (food-, energy-, oxygen-, nitrogen cycles). Little et al. (1990) demonstrated that sub-lethal concentrations of pesticides alter the spontaneous swimming activity, feeding behavior, and vulnerability to predation in rainbow trout *Oncorhynchus mykiss* already after 96-h exposures. Data suggested that similar effects will also appear in natural populations inhabiting contaminated environments. Consequently, exposure-related modifications in behavior may also lead to effects on the community level.

Benefits, limitations & future research needs

Single species tests enable the detection of adverse effects on biological parameters in target organisms including counter-regulatory actions. They primarily perform their role in the context of regulatory actions and mono-substance testing. In case they are applied for the assessment of complex matrices (i.e., whole effluent testing) the choice of test species has to be done with caution and respect to species' ecology. Especially the application of laboratory animals which are sensitive to nitrogen, salinity, or suspended organic carbon can easily turn into a problem where the task is to monitor formation and removal of toxic substances. In most of these cases a differentiation between carbon/nitrogen effects and technological impacts on biological parameters (e.g., biomass, growth, reproduction) is impossible.

It is difficult to extrapolate from mono-species laboratory experiments to field conditions. The susceptibility of species to toxic impacts of chemicals greatly varies. Unfortunately, the number of adverse effects considerably exceeds the number of standardized test organisms able to model this broad range of toxicological endpoints.

2.3.2.2. Micro- and mesocosm multi species tests

Micro-/ Mesocosm studies try to bridge the gap between field and single or multi species laboratory experiments (Crossland and La Point 1992). The advantage is that species communities can be maintained under close to natural conditions retaining the advantage of control groups and replications. The obtained data integrate over multiple direct and indirect effects and provide the basis for feeding predictive ecosystem models. Nevertheless micro-/mesocosm studies are not comparable to field studies as exposure effects may also be linked to site-specific ecosystem characteristics that are outside the scope of these investigations. Microcosm studies usually represent small-scale indoor studies, whereas mesocosm studies are carried out as larger outdoor tests. Microcosm/mesocosm tests are part of higher tier risk assessment procedures (comp. EC 2003). Depending on the test design, aquatic mesocosms are composed of (several) water enclosures equipped with natural/artificial water, sediment, and biocoenoses.

Benefits, limitations & future research needs

Cost intensity of these tests normally limits the application to higher tier risk assessment procedures. Difficult recovery of species and natural fluctuations make the system prone to malfunction. Large predators (e.g., fish, some insect larvae) must be limited if not excluded at all to prevent for collapse of the testing units. Habitat sampling without disturbing populations is a challenging exercise. Micro-/mesocosm studies integrate over multiple direct and indirect effects and thereby facilitate greater understanding of toxicological effects on ecological processes.

2.3.2.3. Passive and active biomonitoring

Biological monitoring means at most a multiple and systematic investigation of environmental parameters, ecological processes, and biodiversity following a defined sampling protocol at natural sampling sites. Common to all monitoring studies is that organisms or communities respond to environmental stressors by changing somatic functions, population dynamics, and composition or intra- and interspecific interactions. Biological monitoring seeks to describe environmental state, identify pressures and impacts, quality surveillance, and early warning for pollution accidents. The diversity of guidelines is merely due to the large variety of protected goods, sampling matrices, chemical/biological methodology, and parameters (for overview compare JAMP monitoring webpage of OSPAR; OECD, 2012).

According to van Gestel and van Brummelen (1996), environmental monitoring might be carried out at four different levels of organization: the sub-organismic (determined by biomarkers), the organismic (determined by *in vivo* bioassays), the population (determined by structure and abundance analyses), and community (determined by changes in species composition, abundance, and diversity). Community relevant studies usually require broadly based data mining and evaluation. Most of the data is taken from passive monitoring studies that, *e.g.*, aim at species richness inventory using differently focused community indices like SPEAR (Species at Risk), Shannon Weaver (Biodiversity Index), etc. (for overview comp. Magurran, 2004). Community composition and diversity at sampling sites are determined by multifold variables (*e.g.*, habitat quality, substance concentrations, temperature, oxygen and organic carbon content). Therefore causalities are mostly traced to multivariate statistics and principle component analyses that may provide indications for key factors impairing biocoenoses.

Benefits, limitations & future research needs

Biological monitoring studies are of high ecological relevance but are time-consuming and require multiple and systematic investigations of environmental parameters and processes to offer more than a simple snapshot of environmental settings. Design, performance, and evaluation of these studies are often subject of methodical shortcomings and over-interpretation of data as community composition and diversity at sampling sites are determined by multifold variables. As a consequence, the derivation of clear cause-effect relationships is the exception rather than the rule.

3. Water quality assessment of individual treatment technologies

This chapter focuses on the evaluation of different water treatment technologies based on the chemical and (eco)toxicological methodologies discussed in the previous chapters. Particular focus is placed on the applicability of these methods for the assessment of treated WW quality from the individual treatment steps as this is a major prerequisite for a valid comparison between conventional and new technologies currently discussed and/or already implemented in WWTPs. This review does not provide a general overview of eliminiation efficiencies of CECs in WWTPs. For this, we refer to a number of available reviews on this topic (*e.g.*, Verlicchi et al., 2012, Luo et al., 2014).

3.1. Conventional wastewater treatment

We use the term conventional WW treatment for physical, chemical, and biological processes that remove solids, pathogens, organic matter, and nutrients. Relevant processes for the removal of CECs present in WW primarily include biodegradation, sorption to excess sludge, and volatilization. Biodegradability, via both metabolic and co-metabolic processes, strongly depends on the chemical structure of the molecules, their physico-chemical properties, and the capability of microorganisms to degrade them, *i.e.*, the expression of relevant enzymes. Sorption is the primary removal mechanism for more hydrophobic compounds, which tend to partition onto primary and secondary sludge. Ion exchange, complex formation with metal ions, and polar hydrophilic interactions can also lead to CEC binding to solids and thus a removal from the liquid phase (Rogers, 1996). Sorption is a reversible process, thus decreasing concentrations in the water phase can result in re-partitioning of compounds from the solids back into the liquid phase. Volatilization is only of minor importance for most emerging contaminants. However, for volatile compounds such as musk fragrances, stripping by aeration in aerobic sludge tanks can contribute significantly to the overall elimination from WW (Simonich et al., 2002).

Insufficient removal of most CECs in conventional treatment

The analysis of WWTP effluents using target analysis has clearly shown that conventional WW treatment is not capable to sufficiently remove CECs from treated waters. In the last two decades a large number of studies have investigated the elimination of hundreds of anthropogenic compounds, including pharmaceuticals and personal care products (PPCPs), as well as industrial and household chemicals in conventional WW treatment (*e.g.*, Snyder et al., 2003; Verlicchi et al., 2012; Luo et al., 2014). In raw WW, CECs are typically present at concentrations in the ng/L to µg/L range, but vary greatly depending on the origin of the WW such as municipal and industrial sources. Observed elimination efficiencies of CECs during conventional treatment vary considerably with compounds such as caffeine, pharmaceuticals such as ibuprofen and acetaminophen being removed to a large extent (> 90%; Luo et al., 2014) whereas others, such as the pharmaceuticals carbamazepine and diclofenac, the artificial sweeteners acesulfame and sucralose, X-ray contrast media as well as perfluorinated chemicals are only eliminated to a small proportion (< 25%) (Stasinakis et al., 2013; Scheurer et al., 2009; Kormos et al., 2011).

Concentrations of EDCs such as estrogens, steroid hormones and phthalates are reduced substantially during conventional treatment via both biodegradation and/or sorption (Schlüsener and Bester, 2008; Deblonde et al., 2011). For other EDCs, such as surfactants removal efficiencies vary widely as linear alkylbenzene sulfonates

928 are reported to be efficiently removed (>96%), while mean removal rates of nonylphenol ethoxylates were
929 significantly lower (<20%) (Camacho-Munoz et al., 2014). Due to their low effect levels, the analysis of EDCs
930 in treated WW constitutes a particularly challenging task, as the methods have to be sensitive enough to detect
931 and quantify them at low ng/L or even pg/L levels. This is especially true for EDCs, which are regulated by
932 national or international law. For example, the European Commission added the hormones 17α-ethinylestradiol
933 (EE2) and 17β-estradiol (E2) to a new 'watch list' of emerging aquatic pollutants, which will amend the revised
934 priority list of the Water Framework Directive that currently regulates 45 known pollutants. Although
935 environmental quality standard (EQS) concentrations for substances added to the priority list will not be set
936 before 2018, those discussed prior to the revision for EE2 (0.035 ng L^{-1} annual average threshold concentration)
937 and E2 (0.4 ng L^{-1} annual average threshold concentration) are extraordinary low (European Commission, 2012),
938 reflecting their high biological activity (*e.g.*, Kidd et al., 2007). Even though some of the major estrogens in raw
939 WW are removed by conventional WWTPs to a high extent already, the low EQS values would require to
940 quantify them at concentrations which only very few analytical methods can reach currently.
941 Antibiotics and antivirals are of considerable concern due to the potential development or proliferation of
942 resistant strains of bacteria and viruses (Singer et al. 2007, Hirsch et al., 1999). Conventional treatment processes
943 significantly reduce the loads of several antibiotics, but many have been reported to occur at concentrations
944 ranging from 10 to 1000 ng L^{-1} in treated effluents, including β-lactams, sulfonamides, trimethoprim, macrolides,
945 fluoroquinolones, and tetracyclines (Le-Minh et al., 2010). The same is true for antiviral drugs such as
946 oseltamivir, zidovudine, nevirapine, and acyclovir (Prasse et al., 2010). Cytostatic drugs have only very recently
947 been investigated in greater detail. They are highly toxic and have been shown to be cytotoxic, genotoxic,
948 mutagenic, carcinogenic, and teratogenic (Zounkova et al., 2007). As cytostatic drugs are usually administered in
949 very small amounts and thus are typically present at very low concentrations in raw WW, their analysis is highly
950 challenging (Kosjek and Heath, 2011). Even though very few studies exist so far, it is indicated that cytostatic
951 drugs are not significantly eliminated in conventional WW treatment plants (Buerge et al., 2006; Besse et al.,
952 2012).
953
954 *WWTP x ≠ WWTP y*
955 Most studies so far have investigated the fate of CECs in individual or in a small number of WWTPs. However,
956 the overall load and composition of CECs entering WWTPs is likely to vary considerably and depends strongly
957 on a number of factors such as: i) the proportion of municipal and industrial WW, ii) types of industries emitting

WW, iii) demographics, and iv) the number of facilities with an extended use of specific CECs (*e.g.*, for pharmaceuticals: hospitals, elderly housings). In addition, strong seasonal variations are expected (Yu et al., 2013). As applied treatment processes in WWTPs can also differ (e.g., depending on the treatment steps used as well as on sludge age) it is so far widely unclear to what extent the removal efficiencies of individual CECs vary. There is thus an urgent need for comprehensive national and international monitoring programs (Hope et al., 2012; Glassmeyer et al., 2005; Ruel et al., 2012). In an EU-wide study, Loos et al. (2013) investigated the presence of 156 organic contaminants in effluents of 90 WWTPs. Most of the compounds (80%) could be detected in the effluents with concentrations ranging from ng/L to µg/L with highest median concentration levels for the artificial sweeteners acesulfame and sucralose, benzotriazoles, several organophosphate ester flame retardants, and plasticizers, pharmaceuticals such as carbamazepine, tramadol, and diclofenac, pesticides, as well as perfluoroalkyl substances. Similar results were observed in a state-wide survey of effluents from the 52 largest municipal WWTPs and water pollution control facilities in Oregon (USA) (Hope et al., 2012). In addition to the lack of sufficient monitoring data, the insufficient standardization of analytical methods hampers the generalization of results. Yet, the large number of CECs with a vast range of physico-chemical properties makes it impossible to analyze all compounds simultaneously. As result, a great variety of methods have been developed for their analysis. In a recent inter-laboratory comparison study including 25 laboratories, 52 methods were used to determine method accuracy and comparability for 22 target compounds in surface water and drinking water (Vanderford et al., 2014). The results revealed a high degree of variability in particular for those compounds for which several analytical methods were used for quantification. Raw and treated WW represent even more challenging matrices due to high concentrations of matrix constituents and biases are likely to be even higher. Thus, standardized methods are needed to improve data quality, increase comparability between studies, and help reduce false positive and false negative rates.

Searching for unknowns

In the EU, more than 100,000 chemicals are currently on the market with 4,000 new compounds being added every year (European Chemicals Agency, 2015). Similar numbers have been reported for the US with more than 84,000 industrial chemicals, 9,000 food additives, 3,000 cosmetics ingredients, 1,000 pesticide active ingredients, and 3,000 pharmaceutical drugs being used (Benotti et al., 2009; Muir and Howard, 2006). Based on these numbers, it becomes clear that the CECs, which have been analyzed so far, most likely represent only a small fraction of the total number of anthropogenic compounds entering WWTPs. As a consequence, reliable

methods capable of detecting and identifying a large number of potentially hazardous compounds are needed. To tackle this challenge, the application of non-target and suspect-screening methods have been shown to provide valuable insights into the overall elimination of organic compounds present in raw WW (van Stee et al., 1999; Gonsior et al., 2011; Gomez et al., 2010). By specifically searching for compounds containing specific elements such as sulfur, it could be shown that a great variety of sulfur-containing compounds such as linear alkyl benzene sulfonates, their co-products as well as their biodegraded metabolites are still present in the effluents indicating their insufficient removal during treatment (Gonsior et al., 2011). A recent study conducted in Switzerland, in which the influents and effluents of ten WWTPs were analyzed using both target and non-target LC-Orbitrap MS, revealed that among the 30 most intensive peaks detected in negative ion mode only 4 target analytes were present (Schymanski et al., 2014b). This clearly confirms that a much larger number of anthropogenic compounds is present in conventionally treated WW than so far known.

Elimination ≠ Mineralization

One crucial question is the actual fate of CECs in conventional WW treatment. In terms of the quality of treated WW it is of particular importance whether a given CEC is completely mineralized or only transformed. This question was addressed in a number of studies, mainly by using laboratory batch experiments with sewage sludge (see *e.g.*, references in Evgenidou et al., 2015). As it was shown, degradation of CECs often leads to the formation of a large variety of TPs, thus resulting in an increased number of CECs present in WWTP effluents. In general, biotransformation reactions comprise simple biochemical reactions such as oxidation of alcohols and aldehydes to the respective carboxylic acids, N-dealkylation, ester cleavage, and hydroxylation. As a result, only slight modifications of the parent compounds are typically observed. For bioactive compounds, such as pharmaceuticals or biocides, this might imply that the bioactivity is being conserved (*e.g.*, Boxall et al., 2004). In addition, formed TPs often exhibit a higher polarity and an increased stability compared to parent compounds, which raises concerns in terms of their elevated mobility in the urban water cycle. The relevance of an ecotoxicological assessment of TPs can certainly be inferred from, *e.g.*, the detection of innumerable biotically/photochemically formed degradation products of pesticides detected in ground and surface waters (for overview see Schulte-Oehlmann et al. 2011). The correlation of acute toxic TPs and parent compound concentrations of selected pesticides and model organisms (algae, daphnia, fish) demonstrated that for 70% of the substances a detoxification can be assumed, whereas 30% of the generic compounds are converted to TPs that are comparable or even more toxic (Boxall et al. 2004).

The identification and ecotoxicological assessment of TPs is a highly challenging task. Elevated concentrations of target compounds are generally used to facilitate the search for TPs in batch experiments. Due to the lack of analytical standards of TPs it remains unclear in most cases how much TPs actually contribute to the overall load of chemicals emitted by WWTP effluents. Very few studies have isolated TPs in sufficient purity and quantity to enable their quantification in WWTP effluents and surface waters and to assess their potential adverse effects in ecotoxicological monosubstance testing (see Evgenidou et al., 2015 and references therein). Typically elevated concentrations (in the mg/L range) are used and formed TPs are isolated using semi-preparative HPLC which is coupled to a fraction collector.

Future efforts should thus focus on the development of alternative strategies for the generation of TP standards, in particular by using systems mimicking microbial degradation. One promising approach thereby could be the application of electrochemical systems, which are increasingly applied in pharmaceutical research for the generation of human metabolites (*e.g.*, Baumann et al., 2009). Similarly, specific fractions of mammal liver cell homogenates (S9 fraction) containing major enzymes of phase I & II metabolism as well as filamentous fungi are used to mimic drug metabolism in mammals and to produce sufficient amounts of metabolites and TPs for structural confirmation (Aberg et al., 2009; Ruan et al., 2008). These approaches offer the advantage of a high degree of standardization and thus a higher reproducibility, which ultimately enhances comparability of obtained results. However, as the enzymatic inventory of microorganisms inhabiting treatment plants and the aquatic environment may differ from mammal hepatocytes and fungi, the applicability of these *in vitro* systems to mimic environmental degradation processes still needs to be proven.

Modeling the fate of CECs in conventional treatment

Models developed to predict the fate of CECs in conventional WW treatment focus on i) degradation kinetics and elimination efficiencies and ii) TP formation. A large number of studies have been published in recent years that employed various models to predict the degradation kinetics and elimination efficiencies of CECs in biological WW treatment (Pomies et al., 2013; Plosz et al., 2013). In contrast to modeling the removal of macropollutants (*e.g.*, nutrients), the prediction of CEC elimination is complicated by the fact that co-metabolic degradation processes have to be considered due to their low concentrations (Fischer and Majewski, 2014). In addition, removal efficiencies of CECs can vary widely between different WWTPs (Helbling et al., 2012). Thus, models have to be as simple as possible, using a limited number of easily measured parameters, but also complex enough to allow for the appropriate prediction of variations due to different process conditions. In terms of WW

regulation and process control, the application of models is crucial, because the large number of CECs makes it impossible to investigate the elimination of every single compound. In addition, models can help identify compounds which conventional treatment insufficiently eliminates, thus indicating potential threats for the aquatic environment. This prioritization can then be used to decide which CECs should be implemented into monitoring programs. Most recent modelling approaches consider the formation of bio-TPs, as these have been shown to be crucial for the assessment of the environmental persistence of CECs (Ng et al., 2011). In addition, these candidate TPs can also be used in suspect screening methods and substantially facilitate the search for TPs formed in both laboratory experiments and WWTPs (Helbling et al., 2010; Kern et al., 2009). One crucial aspect, which is not appropriately considered so far, is the prediction of (eco)toxicological effects. This is essential, however, to appropriately assess the potential effects of CECs and their TPs in the environment and to select compounds included in monitoring programs.

Ecotoxicological benefits and concerns

The presence of CECs and their TPs at very low concentrations in conventionally treated WW raises questions regarding their environmental relevance. An EDA study by Smital et al. (2011) has shown that conventional activated sludge processes reduced the initial toxicity of raw WW to various extents, ranging from 28% for algal toxicity to 73% for estrogenic activity. In a survey study of 39 WWTP effluents in Australia it was shown that 75% of samples elicited a genotoxic response (Allison et al., 2012). Even though a large number of compounds were identified in the effluents, none could be unambiguously tied to the observed toxic effects. Recent studies demonstrated that 299 organic compounds analyzed in WW explained less than 3% of the observed cytotoxicity and 1% of oxidative stress responses (Tang et al. 2013; Escher et al. 2013). Toxicogenomic studies found a range of biological pathways impacted through effluent exposure (Martinovic-Weigelt et al., 2014; Berninger et al., 2014).

This demonstrates the significance of identifying toxicologically relevant mixture activities of treated WW discharges. Considering the goal of any WW purification to be protection of human and ecological health, the assessment of biologically active contaminants based on whole-effluent testing with organismic test systems has clear advantages. This is all the more relevant given that conventionally treated WW has proven to be responsible for many adverse effects observed in invertebrates, fish, amphibians, birds, and mammals. Exposure resulted in, *e.g.*, immunosuppression, reproductive disorders, endocrine disruption, behavioral changes, and population decline (comp. Liney et al. 2006; Vajda et al. 2011; Stalter et al. 2013). Amphipods exposed to WW

significantly reduced their feeding rate and showed impaired vitality parameters (Bundschuh et al. 2011b). As leaf litter breakdown performed by these crustaceans is an important ecosystem function and factor for energy supply in aquatic food webs, effects on decomposer communities endanger the conditions for long-term sustainability of the environment. The sensitivity of wild fish against sewage effluents has been described worldwide for a number of species (*e.g.*, Nichols et al. 1998; Folmar et al. 2001). Specifically, estrogenic compounds have been identified to induce intersexuality in wild roach (*Rutilus rutilus*) populations. Rodgers-Gray (2001) exposed juvenile roach for 150 days to graded concentrations (0%, 12.5%, 25%, 50%, and 100%) of treated WW effluents resulting in dose-dependent vitellogenin (VTG) induction and feminization of male gonoducts. Transplantation of primarily effluent exposed fish in clear water was able to reduce the plasma VTG titers but not to restore alterations of feminized genital systems. Habitat loss and environmental pollution have been identified as major factors threatening bat species in Europe (Temple & Terry 2007). Several authors observed an impact of WW effluents on bat populations searching for fodder along rivers (*e.g.*, Abbott et al. 2009; Kalcounis-Rueppell et al. 2007). Bat activity and prey captures of *Pipistrellus pipistrellus* and *Myotis daubentonii* were recorded upstream and downstream from 19 sewage discharges by Vaughan et al. (1996). Overall and foraging activity was reduced below treatment plant effluents by 11% (total reduction in passes) and 28% (total reduction in buzzes). Whereas *P. pipistrellus* types were less active downstream compared to upstream sites (total reduction in activity was >50%). *Myotis* spp. foraged more often at the downstream than upstream site (increase in foraging rate 112%). Up- and downstream activities of bat species near sewage outfalls may correlate well with preferences for insect prey found more abundantly at these sites. Sewage effluents have been shown to change macroinvertebrate species composition (Stalter et al. 2013), thus it is most likely that prey reduction or extinction will indirectly affect bat populations. Interestingly, Markmann et al. (2008) described one exceptional case where detrimental health effects in European starlings (*Sturnus vulgaris*), feeding on earthworms inhibiting sewage percolating filter beds of treatment plants, seemed to be compensated by other population relevant advantages. Male birds exposed to the hormone mimicking compounds identified in the worms sang longer, more often, and more complex compared to controls. Although these behavioral changes promise male starlings to become more attractive to females, their immune functions were reduced, which on the other hand could adversely affect offspring quality and their survival.

All together, ecotoxicological effect studies indicate that industrial and municipal WWTP effluents should be assessed for their overall biological water quality, including occurrence and probability of adverse effects on aquatic organisms and their expected extent of damage. *In vitro* and *in vivo* bioassays (comp. chapters 2.3.1 and

2.3.2) could provide helpful tools to check WW quality on-site before discharging into receiving waters. Active and passive biomonitoring approaches (comp. chapter 2.2.2.3) provide appropriate sum measures for all biologically active compounds including TPs. Field studies downstream of WWTP discharges are ideally suited for these approaches to provide information on human impacts that are relevant at the population level.

What's next? – Challenges for analytical chemists and ecotoxicologists

The chemical perspective:

Our knowledge about the occurrence of CECs in raw and conventionally treated WW has increased substantially in the last two decades. Thousands of anthropogenic compounds are entering WWTPs. The presence of most of these compounds in WWTP discharges indicates their insufficient removal during conventional WW treatment. One of the main challenges for the future will be the prioritization of compounds which are monitored in WWTPs. These should include compounds which are likely to i) enter WWTPs in high concentrations, ii) show a low biodegradability, and/or iii) exhibit adverse environmental and human health effects. For this, modeling approaches for the prediction of the fate and effects of CECs will most likely play a key role as these allow for the screening of a large number of compounds. For the prioritization of industrial chemicals there is an urgent need to increase the public accessibility to data on production volumes as well as information on the types of chemicals used in the various commercial products. Even though registration documents for chemicals (e.g., in REACH dossiers in the EU) contain most of this information, they are generally not publically available.

The application of selected CECs as WW indicators is an important means to determine the influence of WWTP discharges on receiving waters (Dickenson et al., 2011, Scheurer et al., 2011, Funke et al. 2015) and drinking water resources (Gasser et al., 2011). Easily biodegradable compounds such as caffeine can be used as indicators for the emission of raw WW, e.g., via sewer overflows (Buerge et al., 2003). To confirm the general applicability of these markers, however, detailed monitoring studies are necessary, which assess the presence and removal of proposed indicators on a broad scale.

Finally, we need more detailed insight into degradation and transformation processes taking place in conventional WW treatment to better understand variability in the removal of CECs between different WWTPs. For this, detailed studies with a broad range of compounds are necessary (Gulde et al., 2014). In order to better understand underlying (enzymatic) processes additional efforts in biochemical research such as 'omics'-technologies could help maximize biodegradation of CECs in WWTPs. This needs to be complemented by the

development of new and improvement of existing (eco)toxicological screening methods to address potential adverse effects of formed TPs.

The ecotoxicological perspective:

There is sufficient evidence that discharge of conventionally treated WW still leads to serious environmental problems and impacts on aquatic life in receiving waters. While it is not realistic to expect that any treatment technology is able to provide a zero discharge of pollutants, a more sophisticated adaptation of operating parameters in conventional activated sludge systems (e.g., activated sludge age, temperature, biomass activity, and process type) could help to enhance (waste)water quality without great technical efforts and monetary expenses. A study by Koh et al. (2009), for example, suggests that there is potential for enhancing the removal of EDCs by up to 7-times in conventional WWTPs.

A further alternative could be to defragment WW disposal companies. In some cases the grouping of smaller purification plants by piping of WW in large-scale WWTPs already equipped with advanced treatment technology could result in economic advantages for society and ecological benefits for the aquatic environment.

The implementation of advanced treatment processes would mean a far-sighted landmark decision already addressing global change scenarios based on changing demography, climate, and land use. It is realistic to assume that plant upgrades will prioritize large-scale WWTPs on large watercourses. Nevertheless, especially small and medium size streams and headwaters of larger streams serve as important sources of aquatic biodiversity contributing to the whatershed as a whole; thus these locations should not be neglected in the upgrade process. If important hatcheries and breeding grounds are excluded from either improved technical processing, sewage discharge reduction by WW piping, or adaptation of operating parameters, it is highly questionable whether remarkable improvements in ecological water quality can be realized.

3.2. Advanced treatment

End-of-pipe technologies - as final WW polishing steps prior to discharge into the environment - could play an important role for contamination reduction of highly polluted surface waters in the short term (Eggen et al., 2014; Malaj et al., 2014). The term “advanced treatment” is used in the following for all processes added to conventional treatment which specifically focus on the removal of CECs and associated ecotoxicological effects. This includes the application of ozone or other advanced oxidation processes (AOPs), activated carbon filtration, and dense membranes. Chlorination in WW treatment is a disinfection process that is not designed for the

removal of CECs, but the formation of toxic by-product as well as related adverse environmental effects are relevant to this review. We focus on advanced treatment technologies, which already have been or have the potential to be implemented in WWTPs worldwide. Though a number of other promising treatment technologies exist, such as wetlands or the irrigation of treated WW on agricultural fields, the applications often depend strongly on other aspects such as geographical factors and are thus not discussed in this review. However, the discussed chemical and ecotoxicological methodologies to assess quality of treated waters are also applicable to these and other emerging treatment technologies.

Advanced treatment technologies are the focus of several national and international research projects and numerous WWTPs have been upgraded with advanced treatment steps. A growing public interest in reducing pollution of surface waters and increasingly strict legislation in places like Switzerland, indicate that increasing numbers of WWTPs will be upgraded by further polishing steps such as ozonation or activated carbon treatment. This trend is most notable in Japan, where by 2004 more than 60 WWTPs had already applied ozonation to polish WWTP effluents starting in 1988 with the first ozonation plant at WWTP Oita (Takahara et al., 2006). In earlier years, the primary purpose of ozonation was decolorization, removal of odour, and disinfection, whereas the decomposition of CECs is a more recent topic. Nowadays, the reduction of trace organic CECs is the driver for the recently launched upgrade of up to 100 WWTPs in Switzerland with the goal to treat approximately 50% of the total Swiss WW load (Eggen et al., 2014).

The precautionary principle may give rise to more stringent demands on WW treatment in the future, providing incentive for a widespread upgrade of WWTPs with advanced technologies. A thorough risk-benefit analysis is critical to justify additional investments in a way that adequately articulates environmental impacts caused by an increase in energy use or infrastructure development attended by the implementation of advanced end-of-pipe technologies. For example, a study by Papa et al. (2013) demonstrated that the reduction of water pollution by ozonation is beneficial for human health to an extent on the same order of magnitude of damage caused by air pollution, casting the benefit of advanced WW treatment technologies into doubt.

3.2.1. Ozonation

Ozonation is efficient in oxidizing CECs containing electron-rich moieties

Ozonation is one of the most effective advanced WW treatment technologies as it is able to oxidize a large spectrum of CECs and dissolved organic matter while also providing disinfection properties. Ozone is a selective oxidizing agent, which readily reacts with electron rich moieties such as double bonds and deprotonated amines

(von Gunten, 2003). Besides the chemical properties of the CECs, the efficiency of ozone oxidation strongly depends on water characteristics, such as pH, type and amount of organic matter, and nitrite (*e.g.*, Wert et al., 2009). The pH is of particular importance due to the decomposition of ozone, which is accelerated under alkaline conditions, leading to the formation of OH-radicals. As a consequence, reactions with both ozone and OH-radicals have to be considered (Elovitz and von Gunten, 1999). In contrast to ozone, OH-radicals react unspecifically with CECs and reaction rates are often diffusion controlled (von Gunten and von Sonntag, 2012). However, their high reactivity leads to substantial scavenging of OH-radicals by WW organic matter. Thus, the reaction with ozone is often more relevant for the removal of CECs in WW (von Gunten and von Sonntag, 2012).

Many pollutants that are marginally affected during conventional WW treatment, are oxidized with ozonation by > 90% with ozone doses between 0.8 and 1.5 mg O_3/mg DOC (*e.g.*, diclofenac, carbamazepine, metoprolol; Hollender et al., 2009; Huber et al., 2005a; Ternes et al., 2003). These also include CECs that are of particular health concern such as EDCs and antimicrobials (Dodd et al., 2009, Mestankova et al., 2012). As the endocrine disrupting potential and antimicrobial potential of most of these CECs can be allocated to the phenolic moiety (Kuch and Ballschmiter, 2001), the efficient oxidation of the latter, *e.g.*, via hydroxylation, causes the loss of bioactivity (Hansen et al., 2010). However, a detailed investigation of the correlation between the presence of phenolic moities and overall endocrine disruption is so far missing. The general affinity of ozone to electron-rich moieties, in particular aromatic compounds, has also been utilized to monitor treatment efficiencies via monitoring of the specific UV absorbance at 254 nm ($SUVA_{254}$), a wavelength at which most organic aromatic compounds absorb light (Weishaar et al., 2003; Tang et al., 2014b). In contrast to this, CECs lacking electron-rich moieties such as X-ray contrast media, acidic pharmaceuticals, mecoprop, atrazine, and the artificial sweetener sucralose are only partially removed during ozonation (Huber et al., 2005a).

Elimination ≠ Mineralization

Even though the application of ozonation can significantly reduce CEC concentrations in treated waters, chemicals are normally not completely mineralized, but transformed to countless intermediates, which are rarely identified (Klavarioti et al., 2009). This becomes obvious from changes in overall WW characteristics, in particular $SUVA_{254}$ and DOC. Even though a substantial reduction of $SUVA_{254}$ is typically observed after the ozonation step, decrease of DOC is usually much lower (Wang et al., 2008; Reungoat et al., 2010). This can be attributed to partial oxidation of both CECs and matrix components via ozone or OH-radicals, thus leading to the

formation of reactive oxidation products (OPs) including aldehydes, ketones, keto aldehydes, carboxylic acids, keto acids, hydroxy acids, epoxides, peroxides, quinine phenols, brominated organics, alcohols, and esters all of which can be of toxicological relevance (see von Gunten and von Sonntag (2012) and references therein). As an example, concentrations of aldehydes such as formaldehyde, acetaldehyde, and glyoxal as well as carboxylic acids in ozonated WW are typically in the low to medium µg/L range (Wert et al., 2007), but can reach mg/L concentrations in high organic load WWs (Mezzanotte et al., 2013). Thus, not only complex organic compounds might be causative agents for increased toxicity as numerous reactive substances of low molecular weight form during the ozonation process. Even though several of the formed compounds are likely to be readily degradable in a subsequent biological step (*e.g.*, sand filtration), it has been indicated that a large fraction of formed OPs only shows a low biodegradability as BDOC is typically increasing only slightly, resulting in a DOC removal of <25% (Wang et al., 2008). It was also shown that BAC treatment prior and post ozonation did not result in increased DOC removal (Reungoat et al., 2011). This can most likely be attributed to the low molecular weight and high polarity of many OPs, which are formed as ozonation significantly shifts the molecular size distribution to smaller sizes (Wang et al., 2008). This also has far reaching implications regarding the used chemical and ecotoxicological assessment methodologies. In particular, SPE is frequently used as sample pretreatment to increase sensitivities. However, the increased polarities of most OPs result in substantially lower retardation on SPE sorbents. This becomes obvious by comparing DOC fractions retained on the most commonly applied SPE materials with significantly lower DOC fractions absorbed in ozone treated wastewater samples even if activated carbon is used as sorbent (Fig. 1).

<< Figure 1 >>

Among the toxic OPs known to form during ozonation, the formation of bromate from bromine containing waters is of particular concern as it has been classified as a potential carcinogen (Heeb et al., 2014). The formation of bromate takes place via a complicated multistep reaction and involves both the reaction with O_3 and OH-radicals (von Gunten and von Sonntag, 2012). Concentrations in the low µg/L-range are usually observed in low bromine WWs (Zimmermann et al., 2011; Wert et al., 2007). The reaction of intermediates formed during bromate formation such as HOBr can lead to the formation of bromo-organic by-products (von Gunten, 2003; Heeb et al., 2014). Another group of OPs of toxicological relevance formed during ozonation are nitrosamines, in particular N-nitrosodimethylamine (NDMA), a strong carcinogen. Precursors shown to yield NDMA during

ozonation include pesticides, pharmaceuticals, amine-based water treatment polymers, industrial chemicals, and NOM (*e.g.*, Mitch et al., 2003; Shah et al., 2012a; Schmidt and Brauch, 2008).

Transformation products of individual CECs

The elucidation of the fate of individual CECs during ozonation in laboratory studies has revealed the formation of a variety of ozonation OPs (*e.g.*, McDowell et al., 2005; Prasse et al., 2012). Due to the affinity of O_3 to electrophilic moieties, reactions take place primarily at double bonds, amines leading, amongst others, to the formation of aldehyde, carboxylic acid, or N-oxide functional moieties. The frequent presence of reactive functional groups such as aldehyde moieties give rise to a potentially elevated toxicity compared to the parent compound (Benner and Ternes, 2009a, b). McDowell et al. (2005) demonstrated that about 80% of carbamazepine is transformed to three new OPs during ozonation with unknown toxicity. For the estrogens estrone, 17β-estradiol, and 17α-ethinylestradiol, numerous OPs could be identified (Huber et al., 2004), and for the beta-blockers metoprolol and propranolol Benner and Ternes (2009a, b) reported several by-products occurring after the ozonation process. For propranolol, five different OPs, including aldehydes, were identified, whereas at least eight others and their isomers remained unidentified (Benner and Ternes, 2009b). Radjenović et al. (2009) detected nine OPs after ozonation of the antibiotics roxithromycin and trimethoprim. As these examples suggest, it can be assumed that ozonation of WW will multiply the number of contaminants present in effluents, and contaminant "elimination" should rather be regarded as a replacement of known compounds by unknown intermediates. It is however important to point out that the formation of intermediates represents the usual way of pollutant decomposition and ozonation most likely accelerates this process.

Existing models allow for the estimation of removal efficiencies but not for the formation of OPs

Due to the strong correlation of CEC physico-chemical properties with ozone reaction rates, several quantitative structure activity relationships (QSARs) have been developed to predict the degradation rate constants of electron-rich moieties such as phenols, anilines, and amines (Lee and von Gunten, 2012; Gerrity et al., 2012). Good correlations between predicted and observed rate constants have been observed, allowing for the prediction of elimination efficiencies of specific CECs during ozonation. Even though uncertainties were low for compounds undergoing substantial or marginal elimination, uncertainties were significantly higher for compounds with intermittent eliminations during ozonation (Lee and von Gunten, 2012). To estimate the contribution of OH-radicals, the group contribution method (GCM) can simulate the reaction rates with CECs

(Minakata et al., 2009). However, due to the aforementioned dependence of the O_3 and OH-radical exposure on the water composition, the estimation of the combined effects of both reactive species in complex WW matrices remains challenging. By determining both the ozone and the OH-radical exposure, *e.g.*, using indigo and para-chlorobenzoic acid as in situ probes, predictions can be made about the degradation of a CECs via O_3 and OH-radical reaction pathways (Lee et al., 2014). However, no model for the prediction of OPs formed during ozonation is available as of yet.

Ecotoxicological benefits and concerns

Although ozonation only partly oxidizes chemical compounds, the diminishment of biological activity of toxicants is well documented. A >90% removal of estrogenic activity in WW after ozonation is reported in several studies (*e.g.*, Escher et al. 2009, Reungoat et al. 2010; Stalter et al. 2010b, 2011). Also anti-bacterial compounds (*e.g.*, triclosan, tetracycline, sulfamethoxazole, penicillin) are sufficiently structurally modified to eliminate their anti-bacterial activities (Dodd et al., 2009). Photosystem II inhibiting herbicides lose their activity by 80–90%, and acetylcholinesterase inhibiting activity (*e.g.*, due to organophosphates or carbamates) is diminished by up to 80% (Escher et al., 2009). A genotoxicity removal of 80–98% was shown by Reungoat et al. (2010) and Magdeburg et al. (2014). Retinoic acid receptor-α (RARα) agonistic activity was nearly completely removed (Cao et al., 2009). Furthermore, anti-androgenicity and aryl-hydrocarbon receptor (AhR) agonistic activity is reduced by 78–96% as reported by Stalter et al. (2011). The mentioned toxicity endpoints are presumably of high environmental relevance, as, for example, the feminization of effluent exposed wild fish populations can lead to a reduced fertility (Jobling et al. 2002a, b). Consequently, technologies that effectively reduce endocrine activity may be greatly beneficial for aquatic wildlife.

EDC formation during ozonation is unlikely a result of the effective attack of functional groups, which are important for ligand binding activity (such as phenols with a hydrophobic moiety in the case of estrogens; Nishihara et al., 2000). Only a few studies emphasize the generation of steroid-like EDCs during WW ozonation (*e.g.*, Schrank et al., 2009). However, an activity increase most likely occurs when antagonists are more effectively oxidized than corresponding agonists or *vice versa* (Stalter et al., 2011).

Main concerns related to WW ozonation revolve around the potential formation of reactive OPs. OPs of clofibric acid and mono-chlorophenols revealed increased toxicities in bioassays with *Vibrio fischeri* and *Daphnia magna* (Rosal et al., 2009; Shang et al., 2006). Other studies found that OPs of clofibric acid, propranolol, acyclovir, and metoprolol were more toxic than the parent compound (Rosal et al., 2009; Dantas et al., 2007; Prasse et al.,

2012; Sojic et al., 2012). Due to the reactive nature of many OPs, it can be assumed that the toxicity increase is mainly a result of a non-specific reactive MoA. For that reason, the implementation of toxicity assays covering reactive toxicity endpoints is essential for the assessment of ozonated WW. Petala et al. (2006) and Rosal et al. (2009) demonstrated a toxicity increase with the bioluminescence inhibition assay using the marine bacteria *V. fischeri* and with the *Daphnia magna* acute toxicity assay. In Stalter et al. (2010b) and Magdeburg et al. (2012), the fish early life stage test (FELST) using rainbow trout in a flow-through system resulted in a significant developmental delay or increased mortality after WW ozonation. Likewise, WW ozonation caused a decreased reproduction and biomass in the *Lumbriculus* toxicity test (Magdeburg et al. 2012; Stalter et al. 2010a). A significantly increased genotoxicity was detected with the comet assay using haemocytes of the zebra mussel (Stalter et al., 2010a) or rainbow trout (Magdeburg et al., 2014). These examples emphasize the potential of ozonation to elevate the non-specific reactive toxicity of WW due to the formation of reactive oxidation by-products.

Reactive OPs after ozonation can also increase the mutagenic potency of WW. Monarca et al. (2000) and Petala et al. (2008) observed elevated mutagenic effects in solid phase extracted WW samples after ozonation in lab-scale experiments using the TA98 and TA100 *Salmonella* strains. In a study by Magdeburg et al. (2014) an ozone-dose dependent increase of mutagenicity was detected with the Ames fluctuation assay using the YG7108 strain in four different treatment plants. Sand filtration following ozonation reduced the effects only partly, which matched the effect pattern of the FELST employed in parallel. The genotoxicity decrease measured with the umuC assay in the same study might reveal an inconsistency because Reifferscheid and Heil (1996) demonstrated that chemicals, which induce the *umu* operon, can be regarded as Ames mutagens with a high degree of certainty (86%) and *vice versa*. The umuC assay detects the activation of DNA repair mechanisms by induction of the *umuC* operon. Thus, this test system reacts rather unspecifically on genotoxicants, whereas the Ames test detects very specific acting mutagens depending on the applied tester strain (*e.g.*, sensitive for base pair substitutions, frame shifts, or alkylating agents). Consequently, the genotoxicity decrease measured with the umu-test presumably masks the appearance of specific acting mutagens during ozonation. Therefore, the Ames test is required to complement the genotoxicity analysis and to detect a potential mutagenicity increase due to OP formation (Magdeburg et al., 2014).

Other studies found a removal of non-specific toxicity through WW ozonation. In a study by Margot et al. (2013) the authors found a clear reduction of toxicity after ozonation using a combined algae assay and the FELST with rainbow trout in a flow-through system. The authors attributed discrepancies with previous studies

(Stalter et al., 2010b; Magdeburg et al., 2014) to the longer ozone reaction time promoting the degradation of labile intermediate products. No adverse effects after ozonation could be observed by Altmann et al. (2012) using a 21-day fish screening assay with Japanese medaka. Studies by Bundschuh et al. (2011a, c, d) found increased feeding rates and population sizes of *Gammarus fossarum* indicating a reduced toxicity of WW through ozonation. Studies by Escher et al. (2009) and Reungoat et al. (2010) reported a removal of non-specific toxicity by WW ozonation measured with the bioluminescence inhibition assay. Both studies used solid phase extracted WW samples.

Additionally, OPs formed during ozonation are supposed to be readily degradable. Petala et al. (2006) observed a complete toxicity removal of ozonated WW after 48 h storage time when applying the *Vibrio fischeri* bio-luminescence assay. In Magdeburg et al. (2014) the ozone induced mutagenicity decreased over time with a calculated half-life of mutagenic OPs of approximately 5 days. Consequently, storage and transportation time will lead to a significant loss of toxic OPs, and thus, toxicity assays might deliver false negative results.

Post treatment of ozonation

In order to limit the emission of toxic and reactive by-products into receiving waters, a post-treatment step such as sand filtration or activated carbon treatment is usually implemented after ozonation (Stalter et al. 2010a,b). Sand filtration has been shown to only insufficiently remove ozone resistant CECs and bio-TPs from ozone treated effluents (Hollender et al., 2009; Nakada et al., 2007). This is not surprising as these compounds were not or only incompletely removed in prior activated sludge treatment. However, as both DOC and BDOC prior and after sand filtration remain fairly constant, the extensive formation of non-biodegradable TPs is indicated (Wang et al., 2008). Even though an efficient removal can be expected for products from cleavage of olefin groups and aromatic rings, hydroxylamines and N-oxides TPs, which are formed during ozonation of amines, are likely to be not or only incompletely removed during biological post treatment (Hübner et al., 2015). *In vivo* studies demonstrated that adverse effects of ozonation on rainbow trout were mitigated by downstream sand filtration (Magdeburg et al., 2012, 2014; Stalter et al., 2010a, b), indicating that sand filtration can be an effective barrier to toxic oxidation by-products. Wang & Summers (1996) were able to demonstrate that sand filtration reduces aldehyde concentrations affiliated with ozone application. An effective NDMA removal with sand filtration was observed by Schmidt and Brauch (2008) and the level of AOC is highly reduced. Hacker et al. (1994) were able to demonstrate that this is mainly an effect of biological degradation. Biologically active activated carbon filters (compare chapter 3.2.3) or membrane bioreactors (comp. chapter 3.2.4) can also act as efficient barriers for

oxidation by-product removal (Mascolo et al., 2010; Reungoat et al., 2012). To reduce the discharge of biologically active oxidation by-products ozone application should only be established in combination with a bioactive post treatment such as sand filtration.

Advanced oxidation processes (AOPs) might be of increasing importance in the future

In addition to ozonation, also advanced oxidation processes (AOPs) are objects of research for WW treatment purposes (Klavarioti et al., 2009; Yang et al., 2014). So far, AOPs have mostly been investigated on laboratory- and pilot-scale. As such, it is difficult to predict if and to which extent AOPs will be utilized in WWTPs in the future. In general, AOPs are aqueous phase oxidation methods based on the pollutant degradation by highly reactive oxygen species (ROS), in particular hydroxyl radicals. Most prominent AOPs in WW treatment research currently are photolysis via UV irradiation in combination with ozone (UV/O_3), hydrogen peroxide addition (UV/H_2O_2), photo-catalysts such as TiO_2 (UV/photocatalyst), photo-Fenton oxidation and electrochemical AOPs. Due to the unspecific high reactivity of OH-radicals, AOPs exhibit effective removal capacities of CECs (Rosenfeldt and Linden, 2004; Rosario-Ortiz et al., 2010). A number of different mechanisms are responsible for the often diffusion controlled reactivity of OH-radicals, including H-abstraction and hydroxylation reactions. Thus, compounds which are recalcitrant to oxidative attack via ozone such as X-ray contrast media and atrazine, can be degraded (Katsoyiannis et al., 2011; de la Cruz et al., 2012; Prieto-Rodriguez et al., 2013 Kim et al., 2009). Furthermore, AOPs have also been used for the removal of NMDA (*e.g.,* Landsman et al., 2007). However, depending on the type of AOPs, they can also contribute to the formation of NDMA (*e.g.,* Zhao et al., 2008). As bromate is an O_3 specific by-product, its formation can be prevented by the use of non-ozone based AOPs such as UV/H_2O_2 (von Gunten, 2003).

The unspecific reactivity of OH-radicals also accounts for substantial scavenging via reaction with natural water constituents, in particular NOM (Keen et al., 2014). As a consequence, elevated amounts of OH-radicals are needed to ensure the sufficient oxidation of CECs, which is linked to elevated energy consumption and thus costs (Rosenfeldt et al., 2006). Furtermore, this also makes a complete mineralization of CECs, which has been observed in laboratory experiments with ultrapure water (*e.g.,* Yang et al., 2008; Perez-Estrada et al., 2005), rather unlikely. Generally, the same methods as described for the assessment of ozone treated waters are applicable. However, due to the unspecific reactivity of OH-radicals generally a greater variety of OPs, in particular highly polar low molecular weight compounds, are likely to be formed.

What's next? – Challenges for analytical chemists and ecotoxicologists

The chemical perspective:

Ozonation is one of the most promising advanced treatment technologies being discussed for application in WWTPs as it allows for the removal of a large spectrum of CECs. The same is true for AOPs even though they so far have mainly been investigated on laboratory and pilot scale. The increased application of ozonation has not, however, been accompanied by significant advances in chemical methodologies to assess the quality of treated waters. This is particularly true for the analysis of formed OPs. Due to their high polarity they are often not sufficiently sorbed by typical SPE materials and show no retardation on conventional RP columns used for chromatographic separation. This has far-reaching implications regarding the applicability of EDA/ TIE approaches as well as the toxicity evaluation of treated waters as SPE is frequently applied for sample enrichment. Consequently, there is an urgent need for the development of appropriate extraction procedures. The use of alternative chromatographic separation methods such as HILIC and IC is crucial for the analysis and detection of formed OPs via target and non-target analytical approaches.

Even though NOM is present in much higher concentrations than CECs, the knowledge about its relevance for the formation of (toxic) OPs is still scarce. Modeling approaches need to be extended from the prediction of elimination efficiencies to the prediction of OP formation. In combination with toxicity and biodegradation/sorption evaluations tools this will further allow for the assessment of the potential environmental effects and CEC removal efficiencies in subsequent treatment steps. To validate these models, comprehensive studies on the fate and effects of CECs and their OPs are required, with a special emphasis on the formation of toxicologically relevant OPs such as aldehydes and hydroxylamines.

The ecotoxicological perspective:

In vitro *assays are a cost-effective way to assess the formation of toxic OPs formed during ozonation. Sample enrichment methods and bioassays should be carefully selected to avoid false-negative results. Reactive toxicity assays should be used because in most of the studies reporting a toxicity increase during ozonation, test systems that cover non-specific reactive toxicity endpoints were applied. Further research should focus on the removal capacity of filter systems to reduce the risk of toxic by-products entering the aquatic environment. Also, identification of the causative origin of an increased toxicity following WW ozonation might be indispensable for a qualitative appraisal of advanced oxidation processes and the respective post treatments. In particular the Ames assay with the tester strain YG7108 might be a promising tool for an effect-directed identification of*

mutagenic ozonation by-products and could serve as a low-cost but efficient tool to easily evaluate the efficiency of post-treatment technologies for oxidation by-product removal (Magdeburg et al., 2014).

Finally, at this stage, a fair balance of pros and cons of WW ozonation requires long-term on-site observations at WW receiving streams with a high WW load before and after establishing advanced treatment steps. For a conclusive evaluation of the risks and benefits of ozonation, plant, macroinvertebrate, fish, and microorganism community analyses as well as biomarker responses and histo-pathological endpoints in model organisms are suitable tools to draw environmentally relevant conclusions. In particular, field monitoring studies are essential as they respresent "real world" scenarios in contrast to lab studies and they comprise multiple influencing variables (contaminant mixtures as well as biotic and abiotic factors).

3.2.2.Chlorination

Disinfection of wastewater

Chlorination is an oxidative treatment technology frequently applied in WWTPs and includes the addition of Cl_2 or $Ca(OCl)_2$/NaOCl to (conventionally treated) WW. In contrast to ozonation, chlorination is primarily used for disinfection purposes and not for the oxidation of CECs. In general, chlorination offers the advantage that the reactive chlorine species (*i.e.*, free chlorine) react significantly slower with organic compounds and do not undergo self-decay, thus having a high stability. Sulfur dioxide or sodium thiosulfate are frequently used to scavenge free chlorine before discharge of treated WW into the environment. WW disinfection is regarded as critical for effluents affecting recreational waters, irrigation waters, shellfish-growing areas, and municipal water supplies to prevent waterborne diseases (CAEPA, 1993; Jacangelo and Trussell, 2002). In densely populated areas and in many high-income contries, WW disinfection is common practice to inactivate bacteria, viruses, and protozoa. In North-America the most widely used method is chlorine disinfection due to its low costs as well as disinfection efficiency while in Europe chlorine alternatives such as UV and ozone are increasingly applied (Jacangelo and Trussell, 2002).

CEC elimination as a beneficial side effect

The speciation of chlorine such as HOCl, ClO^-, and Cl_2 is strongly pH-dependent, and large differences in their reactivity with organic compounds have been observed. Reactions are restricted to specific moieties present in CECs such as reducing nucleophilic and unsaturated sites as hypochlorous acid primarily reacts via oxidation reactions, addition reactions to unsaturated bonds, and electrophilic substitution reactions at nucleophilic sites

(Deborde and von Gunten, 2008). Chlorine reactivity usually decreases in the order: reduced sulfur moieties > primary and secondary amines > phenols, tertiary amines > double bonds, other aromatics, carbonyls, amides (Deborde and von Gunten, 2008; Sharma, 2008). For pharmaceuticals containing aromatic ether functional groups such as the β-blockers atenolol and metoprolol, the rate of transformation is strongly affected by the other substituents on the ring (Pinkston and Sedlak, 2004). Similar to this, higher reaction rates with HOCl have been observed for the phenolate ion compared to the protonated species due to the increased electron density of the ionic form (Pinkston and Sedlak, 2004). For sulfonamide, tetracycline, and macrolide antibiotics reaction with ClO_2 is likely to result in a substantial elimination (Huber et al., 2005b; Le-Minh et al., 2010; Wang et al., 2011). The same is true for estrogens and other EDCs such as triclosan, bisphenol-A, and nonylphenol (Huber et al., 2005b; Noutsopoulus et al., 2013).

Disinfection by-product (DBP) formation

The reaction of chlorine with natural dissolved organic matter (DOM) has been shown to lead to the formation of a variety of undesired disinfection by-products (DBPs), with some of them being of considerable concern due to their carcinogenicity, cytotoxicity, and genotoxicity (Richardson et al., 2007; Krasner et al., 2009; Shah and Mitch, 2012b). The discharge of chlorinated WW has adverse effects on the community structure of benthic invertebrates as well as fish up to 500 m downstream of the WW discharge when chlorine residuals exceed 0.02 mg/L (CAEPA, 1993). Post treatment with dechlorination agents such as sulphur dioxide, sodium metabisulphite, sodium bisulphite, sodium sulphite, sodium thiosulphate, and hydrogen peroxide considerably reduced adverse effects (CAEPA, 1993), and hence dechlorination should be applied after chlorine disinfection.

Our knowledge about the formation of DBPs in drinking water is much more detailed compared to WW, most likely due to the potentially direct negative impact on humans. However, it has been shown that the same DBPs typically found in chlorine treated drinking water can also be formed in WW (Huang et al., 2012; Tang et al., 2012). The elevated DOM content compared to drinking water can result in much higher concentrations of DBPs in chlorine treated WW (Rebhun et al., 1997). Furthermore, emissions of DBP precursors by WWTPs might lead to DBP formation if these compounds enter drinking water treatment facilities utilizing chlorination.

To limit the formation of specific DBPs such as trihalomethanes and haloacetic acids, chloramination or chlorine dioxide are often used as alternative disinfectants (Shah and Mitch, 2012b; Le Roux et al., 2011). However, chloramination has been shown to lead to formation of toxic nitrosamines such as NDMA (Najm and Trussell, 2001; Mitch and Sedlak, 2002) with precursors including dimethylamine, NOM, as well as pharmaceuticals and

pesticides containing dimethylamine moieties (*e.g.*, Mitch and Sedlak, 2004; Shen and Andrews, 2011; Le Roux et al., 2011). NDMA formation potentials up to 6300 ng L^{-1} have been determined in secondary effluents (Mitch and Sedlak, 2004). During biological WW treatment a substantial decrease of NDMA precursors and dimethylamine (DMA) was observed (0–75%). NDMA formation cannot be explained by the presence of DMA alone, indicating the contribution of other, so far unknown, NDMA precursors (Mitch et al., 2003).

Formation of halogenated TPs from reaction with CECs

In addition to the formation of DBPs resulting from reactions with NOM, TP formation from reactions between reactive chlorine species and individual CECs has been studied. Reaction of chlorine with benzophenone-4 is leading, amongst others, to the formation of mono-, di-, and tri-chlorinated BP-4 analogues due to chlorine substitution of BP-4 (Xiao et al., 2013). Similarly, chlorgemfibrozil has been identified as the main TP during reaction of free chlorine with gemfibrozil (Bulloch et al., 2012). In terms of the toxicity, chlorination has lead to an increased toxicity of chlorinated analogues. For example, the reaction of triclosan with chlorine results in the formation of 2,4-dichlorphenol and 2,4,6-trichlorphenol, both known to be toxic and exhibiting high endocrine disruptor-activity, as well as trihalomethanes (THMs) (Fiss et al., 2007). In the reaction of acetaminophen with hypochlorite, the formation of the toxic TPs 1,4-benzoquinone and N-acetyl-p-benzoquinone imine were reported (Bedner and MacCrehan, 2006). In contrast to this, reaction of EE2 with both chlorine as well as chlorine dioxide has lead to several TPs, such as mono- and dichlorinated EE2 which exhibit a lower endocrine activity than the parent compound (Lee et al., 2008). Similar to EDCs, reaction with antibiotics such as trimethoprim exhibited a reduction of the toxic activity (Dodd and Huang, 2007). The formation of chlorinated TPs during chlorination of β-lactam (Navalon et al., 2008) and fluoroquinolone antibiotics (Wang et al., 2010), however, indicate that antibacterial activity might be conserved in some cases. Because of the often increased toxicity of chlorinated compounds, other toxic MoAs such as genotoxicity and mutagenicity have to be considered. Furthermore, the formation of anti-estrogenic TPs during chlorination of phenylalanine highlights the necessity to also take other toxicological endpoints into account, because chlorinated compounds might exhibit a different toxic mode of action than the parent compounds (Wu et al., 2010).

The presence of iodine and bromine in chlorinated WW can lead to the formation of iodinated and brominated DBPs (Sharma et al., 2014; Duirk et al., 2011). The formation of I-DBPs and Br-DBPs is of considerable health concern, as they typically exhibit a highly enhanced mammalian cell cytotoxicity and genotoxicity as compared

to their chlorinated analogues (Richardson et al., 2008). Furthermore, the presence of iodine and bromine can influence the degradation kinetics of CECs (Vikesland et al., 2013; Heeb et al., 2014).

Unscrambling the pool of halogenated compounds

Even though hundreds of DBPs have been identified, these most likely account for only a small fraction of the total organic halogens (TOX) present in chlorinated waters (Richardson et al., 2007). The analysis of DBPs is challenging due to their complex chemistry and the strong dependence of their formation on the water composition. For volatile low molecular weight compounds GC-based techniques such as GC-ECD and GC-MS have been used most frequently (Weinberg, 1999). Other compounds such as haloacetic acids or aldehydes are only amendable to GC after derivatization. Thus, LC-based methods, in particular LC-MS are increasingly applied (Zwiener and Richardson, 2005). Additionally, the application of three-dimensional excitation and emission matrix fluorescence spectroscopy has proven useful for the prediction of DBP formation (Hao et al., 2012). In general, the analysis of samples with several complementary techniques is recommended in order to account for the larger spectrum of compounds likely to be present in chlorinated waters.

To identify halogenated compounds, specific isotope patterns can be used in non-target analytical approaches, and substantially aid identification (Schymanski et al., 2014b; Martinez-Bueno et al., 2012). Cleavage of iodine (m/z 127) has been useful for the identification of iodinated compounds such as X-ray contrast media and their degradation products (Putschew and Jekel, 2003). AOX analysis can be used to determine whether all relevant compounds have been considered. Even though only few environmental studies exist so far, the application of inductive coupled plasma-MS (ICP-MS) has successfully used for investigating the fate of X-ray contrast media, iodophenols and gadolinium chelates (Profrock and Prange, 2012; Künnemeyer et al., 2009; Redeker et al., 2014) as well as for assessing DBP formation (Shi and Adams, 2009). Current limitations are mainly related to sensitivity issues, in particular for chlorine, which makes it difficult to detect chlorinated compounds at environmental concentrations. However, the application of these methods in single substance degradation laboratory studies (at elevated concentrations) could help to identify the chlorinated by-products being formed. This would also help the development of specific and highly-sensitive analytical methods (*e.g.*, using GC- or LC-MS techniques) to determine the formation of these compounds at environmental concentrations and in real systems. Even though the molecular information is lost in ICP-MS analysis, it can be used for the quantification of unknown compounds if no reference standard is available, as the response of the detector is independent of the chemical structure (Axelsson et al., 2001). Thus, ICP-MS analysis is useful for calculating mass balances of

halogen containing compounds even without an available reference standard (Profrock and Prange, 2012, Redeker et al., 2014). Together with structural information obtained from other analytical techniques such as ESI-MS, ICP-MS can be a powerful tool to identify TPs (Meermann and Sperling, 2012). Consequently, latest developments have combined chromatographic separation (*e.g.*, CE, GC or LC) with both ESI-MS and ICP-MS (Wind and Lehmann, 2004; Buchberger et al., 2003).

Modeling has been proven useful in assessing DBP formation

In order to predict the reaction of specific CECs with reactive chlorine, several QSARs have been developed and good correlations between predicted and experimentally derived second order rate constants for the reaction of CECs with ClO_2 and HOCl were obtained (Lee and von Gunten, 2012). For phenols could be shown that second-order rate constants for oxidation of the undissociated forms of substituted phenols are about six orders of magnitude smaller than the corresponding values for phenoxide anions. This indicates that only the reaction of phenoxide anions will be significant under the conditions of water treatment with chlorine dioxide (Tratnyek and Hoigne, 1994). For the prediction of DBP formation such as THMs during WW treatment, different models have been developed (see Chowdhury et al., 2009 for references). Chen and Westerhoff (2010) developed different DBP formation potential models to predict the formation of carbonaceous and nitrogenous DBPs using DOC, UVA_{254}, and bromide. DOC was used as a proxy representing the relative amount of precursor material, UVA_{254} to assess the precursors' relative reactivity toward chlorine-based disinfectants, and bromide was used as a control for the distribution among chlorinated and brominated species. Similarly, Sohn et al. (2004) observed a good correlation between THM and HAA formation in raw WWs using an empirical power function model. No models for the prediction of toxic TPs from individual CECs exist thus far.

Ecotoxicological benefits and concerns

Due to the rather transient effect of free chlorine and simple Cl_2 mitigation strategies, the main concern revolving around chlorine application for water disinfection is the formation of toxic DBPs. DBPs in WW can increase toxic effects in *in vitro* and *in vivo* bioassays. Blatchley et al. (1997) found in most cases that toxicity increased with the water flea *Ceriodaphnia dubia* after WW disinfection according to the following rank order of decreasing toxicity: chlorination/dechlorination > ozonation > UV irradiation. Monarca et al. (2000) studied the effect of chlorine, chlorine dioxide, ozone, peracetic acid, and UV radiation and found increased bacterial mutagenicity for all disinfectants. Many more studies found an effect increase after chlorine treatment using a

variety of test systems (*e.g.*, Chen et al., 2001; Fukushima et al. 2014; Schiliro et al. 2009; Pignata et al., 2012; Wang et al., 2005; Wang et al., 2007; Wei et al., 2006). While some authors observed an effective degradation of endocrine active chemicals (Noutsopoulos et al., 2013), others found a consistent increase of anti-estrogenic brominated DBPs (Tang et al., 2014a; Wu et al., 2014). Watson et al. (2012) concluded that DBPs formed in chlorinated WWs can be toxic and may have a deleterious impact on aquatic organisms, and therefore, chlorination or chlorination/dechlorination may not be adequate treatment strategies for the protection of receiving waters. Therefore, the application of less harmful alternatives is desirable. UV disinfection might be most suitable (Acher et al., 1997) because the formation of halogenated DBPs can be excluded. Additionally, two important protozoan intestine pathogens, *Giardia lamblia* (elicitor of lambliasis) and *Cryptosporidium parvum* (elicitor of cryptosporidiosis), were found to be resistant to traditional chemical disinfectants such as chlorine, while UV irradiation and membrane filtration are much more effective for their inactivation/removal (Jacangelo and Trussell, 2002).

What's next? Challenges for analytical chemists and ecotoxicologists

The chemical perspective:

Similar to DBP research in drinking water, most DBPs present in chlorinated wastewater are still unknown. Due to the toxicological relevance of many halogenated compounds, further improvements are needed for the specific analysis of halogenated compounds, e.g., by coupling IC and HRMS. Comparison with results from AOX should be used to assess to which proportion of present halogenated organic compounds have been detected. This should be extended further by the application of alternative approaches such as ICP-MS as these can provide details of the total sum of halogenated compounds. The discrepancy between total content of halogenated compounds determined by ICP-MS and AOX is most likely attributable to highly polar compounds which are not or insufficiently adsorbed by AOX sorbents. This also highlights the necessity to develop new analytical methods, similar to ozonation, which are capable to detect and quantify highly polar TPs. The identification of DBP precursors is another challenging field requiring investigation. While modeling approaches can facilitate the identification of potential precursors and the formation of TPs, further research is needed to proof their applicability in the field.

The ecotoxicological perspective:

One of the major challenges is the assessment of the unknown fraction of DBPs, since the known DBPs insufficiently explain toxic effects observed in drinking water and WW. Furthermore, volatile and very polar DBPs are usually not considered in toxicological analyses as those are lost during sample enrichment. With respect to the well-known effect increase after disinfection with chlorine, further research should focus on the assessment of alternative WW disinfection strategies like UV treatment.

3.2.3. Activated carbon (GAC, PAC) and biological activated carbon (BAC) filtration

Effective removal of non-polar and medium polar CECs

The application of activated carbon (AC) in WW treatment takes advantage of its high sorption capacities for a great variety of pollutants due to the surface area of up to 2,000 $m^2 g^{-1}$ (Boehler et al., 2012). Relevant sorption mechanisms include π- π-electron interactions (aromatic ring and graphene sheets), formation of donor-acceptor complexes, as well as electrostatic interactions and hydrogen bonds (Rivera-Utrilla et al., 2013). Consequently, sorption efficiency is affected by the properties of both the adsorbate (K_{OW}, pKa, molecular size, aromaticity versus aliphaticity, and presence of specific functional groups) and adsorbent (surface area, pore size and texture, surface chemistry, and mineral matter content) (Dabrowsky et al., 2005; Kovalova et al., 2013a). In general, AC can be used in WW treatment in two different ways: either as a packed bed filter in granular form (GAC) or as powder (PAC) which is directly added into the WW and removed in a subsequent filtration step (Snyder et al., 2007; Boehler et al., 2012). GAC is regarded as a more economic and sustainable alternative to PAC because the required amount of activated carbon in fixed bed systems is reduced, which results in lower energy requirements and operational costs (Joss et al., 2008; Walker and Weatherley, 1997). However, as AC adsorption is a slow process, equilibrium concentrations are often only attained after several hours. The application of PAC offers the additional advantage that the carbon can be circulated, similar to activated sludge, and thus remains longer in the system than the water. WW treatment with powdered activated carbon (PAC) following a conventional activated sludge system has similar efficiencies to ozonation for pollutant and effect diminishment. PAC doses of 10–20 mg/L are regarded as economically feasible (Joss et al., 2008) and sufficient for removal of many WW contaminants (Mailler et al., 2015). However, one drawback is that contaminated PAC contains high pollutant concentrations and thus has to be disposed as waste and/or incinerated or extensively recycled after usage. Both the generation and recycling of PAC is energy-intensive and the broad-scale application in WWTPs would

require large amounts of PAC (*i.e.*, 1–2 t/d for a WWTP with a flow rate of 100,000 m^3/d). Moreover, it cannot be excluded that contaminated PAC will enter the environment as subsequent sand filtration is not sufficient to retain powdered carbon particles completely which could affect the habitat of benthic organisms. Subsequent membrane filtration for PAC removal is unlikely feasible due to higher requirements of energy and technical equipment (Joss et al., 2008; Margot et al., 2013).

A large variety of compounds can be removed efficiently by AC adsorption, including non-polar and medium-polar pharmaceuticals and personal care products (Ternes et al., 2002) as well as industrial chemicals such as flame-retardants, acid dyes, and benzotriazoles (Zhang & Zhou, 2005; Nowotny et al., 2007; Walker and Weatherley, 1997; Ho et al., 2011; Grover et al., 2011; Ek et al., 2014). In contrast to this, highly polar CECs such as X-ray contrast media, sulfamethoxazole, gabapentin, irgarol, mecoprop, oxybenzone, and cytostatic drugs are only partially removed (Margot et al., 2013; Kovalova et al., 2013b; Snyder et al., 2007). Antibiotics in general show good removal efficiencies, which correlate well with K_{ow} values (*e.g.*, Rivera-Utrilla et al., 2009). The same is true for EDCs (Snyder et al., 2007). However, as shown for steroid estrogens, removal efficiencies are strongly affected by both the type of activated carbon and the composition of the water matrix (Rowsell et al., 2009). Besides energy- and cost-intensive carbonaceous adsorbent materials also low-cost adsorbent alternatives have demonstrated good removal rates for organic contaminants such as pesticides (Crini, 2006). Due to the higher surface area, activated carbon is supposed to be more effective.

Biological activated carbon (BAC) filtration combines biodegradation and sorption for the removal of CECs (Reungoat et al., 2011; Gerrity et al., 2011). In a BAC filter, a fixed bed of granular activated carbon (GAC) is used to support the growth of bacteria on its surface. BAC has been shown to substantially reduce DOC and nitrogen load of secondary treated WW with sorption to AC being dominant in the beginning and increasing importance of biodegradation over time (Reungoat et al., 2011). Within the Neptune project, a fixed bed system with biologically activated coke as sorbent material was evaluated subsequent to conventional biological activated sludge treatment. The chemical analysis revealed removal rates between 70–90% for many pharmaceuticals including the hardly degradable compounds diclofenac and carbamazepine (unpublished data, http://www.aqua-biocarbon.de/aktuelles.html). BAC has also been shown to be a cost effective alternative to UV treatment for the removal of NDMA after ozonation (Gerrity et al., 2014). The analysis of endocrine activity revealed similar results. Anti-androgenicity and aryl-hydrocarbon agonistic activity were eliminated by > 90% via BAC treatment (Fig. 2). Estrogenicity is removed by a lower rate of approximately 50% which could be a

result of the already very low estrogenicity level of ca. 0.3 ng/L E-EQ after conventional treatment. These results are based on only two samples from each treatment step and can thus not be regarded as representative.

<< Figure 2 >>

No formation of TPs

One great advantage of AC compared to the treatment technologies described so far is that removal is solely based on sorption with no TPs being formed. However, as sorption capacity of AC is limited, it has to be exchanged and/or regenerated in regular intervals. In addition, due to the above mentioned relevance of different sorption mechanisms and removal efficiencies can vary significantly. As electrostatic interactions are strongly pH dependent, removal of compounds primarily affected by this mechanism such as acetaminophen, sulfamethazine, and sulfamethoxoazole can vary significantly (Nam et al., 2014). Nguyen et al. (2013) observed a breakthrough of negatively charged compounds such as ketoprofen, naproxen, and diclofenac over time whereas neutral compounds such as carbamazepine showed a constant high removal. Furthermore, the DOC concentration in treated WW is critical due to the competition for sorption sites on the AC (Nowotny et al., 2007; Zietzschmann et al., 2014a). Even though DOC concentrations in GAC filter effluents can also be used as a surrogate (Xing et al., 2008), the low concentrations of many CECs might not lead to a significant increase in DOC when breakthrough occurs. Thus, the monitoring of highly polar compounds such as X-ray contrast media and anionic organic compounds should be used as indicators to determine the loading of AC, as these typically show the lowest removal efficiencies. If AC treatment is used as post-treatment of an oxidation step such as ozonation, TPs should be monitored as these nevertheless may pass the AC filter as well (Prasse et al., 2012). Due to the limited retention of anionic compounds, CECs containing carboxylic acid moieties are of particular relevance. In addition, carboxylic acids are frequently formed during ozonation (von Sonntag and von Gunten, 2012). However, the high polarity of compounds which can be expected to be insufficiently removed during AC treatment also makes their analysis highly challenging. In comparison to LC analysis, IC-techniques offer superior separation capacities for anionic and cationic compounds (Mascolo et al., 2005; Scheurer et al., 2012). This is especially true for compounds carrying several carboxylic acid moieties (Meyer et al., 2007). Hydrophilic interaction chromatography (HILIC) has been applied for the environmental analysis of highly polar CECs such as pharmaceuticals, pesticides, and illicit drugs (van Nuijs et al., 2011b). In addition, the hyphenation of HILIC

with RP chromatography allows for the comprehensive and simultaneous analysis of compounds spanning a wide range of polarities (Greco and Letzel, 2013).

Due to the formation of potentially toxic compounds during oxidative treatment, *e.g.*, via ozone or chlorine, AC treatment can also be applied prior to the oxidation step for the removal of precursors (Hanigan et al., 2012). However, AC has also been shown to contribute to the formation of N-nitrosamines from secondary amines (Padhye et al., 2010). In addition, hydrophilic NOM fractions, which might pass AC filtration, have been shown to exhibit higher DBP formation potentials relative to the hydrophilic fractions (Kwon et al., 2005).

Modelling of AC performance

Due to the various mechanisms relevant for removal via sorption, computational modelling of removal efficiencies is complex. Using classical sorption isotherm models, it has been shown that sorption of hydrophilic compounds to AC better fit to linear isotherms, whereas hydrophobics fit better to Freundlich isotherms (Nam et al., 2014). Furthermore, also Langmuir isotherms have been frequently used to describe the sorption of CECs on AC (Tahar et al., 2013). The application of QSARs determines parameters relevant for sorptive removal of CECs on AC (Dickenson and Drewes, 2010). As an example, Redding et al. (2009) estimated the breakthrough bed volumes of 29 EDCs and PPCPs using QSARs with good correlations for compound's 8th-order simple Chi index ($8\chi p$), and the compound's hydrophobic surface area. However, GAC adsorption is strongly dependent on the sample matrix and has been shown to depend on GAC particle size if NOM is present (Corwin and Summers, 2010). As a result, Nguyen (2013) recently observed that single-solute isotherm parameters did not demonstrate any discernible correlation individually with any of the parameters that may govern adsorption onto GAC, such as log D, number of hydrogen-bond donor/acceptor groups, dipole moment, or aromaticity ratio of the compounds. In addition, extrapolations of laboratory results are hampered by the fact that sorption has been shown to be concentration dependent, which might lead to a substantial overestimation of removal efficiencies in cases where only high concentrations are used in laboratory experiments (Yu et al., 2008). Thus, available models only allow for a first estimate of AC performance, but further improvements are necessary to also account for temporal changes of AC sorption capacities.

Ecotoxicological benefits and concerns

The toxicity of WW is effectively reduced with activated carbon filtration. Escher et al. (2009) detected a non-specific toxicity removal of 57–83% after PAC treatment (15 mg/L) compared to conventional treatment.

Photosystem II inhibitors were eliminated by more than 80% while acetylcholinesterase inhibiting activity diminished by more than 70% (Escher et al., 2009). In Stalter et al. (2011) estrogenicity was removed by 77% (20 mg/L PAC) compared to conventional treatment, whereas ozone was slightly more effective with 88% removal. Anti-androgenicity was reduced by an average of 63%, AhR agonistic activity by 82%, and cytotoxicity by 61% compared to conventional treatment (Stalter et al., 2011). The more effective non-specific toxicity reduction *in vitro* via PAC treatment was confirmed with the FELST *in vivo*, where trout mortality was significantly reduced compared to conventional treatment (Magdeburg et al., 2012). In addition, the comet assay revealed an enhanced genotoxicity removal, whereas after ozonation toxicity increased compared to conventional treatment (Stalter et al., 2010a). PAC also effectively reduced toxicity in a *Gammarus fossarum* feeding assay (Bundschuh et al., 2011d). GAC mitigated toxic effects of grey-water on aquatic invertebrate organisms (Leal et al., 2012). Extensive ecotoxicity analyses are lacking so far but are desirable, because GAC is a promising pollutant removal technology and regarded as a more sustainable approach than PAC. Based solely on an ecotoxicological perspective, activated carbon treatment might be preferable compared to ozone due to the benefit of contaminant elimination without OP formation, and hence the risk of toxicity increase can be excluded.

Activated carbon addition to the activated sludge process is also under discussion. In this case, positive effects on the cleaning capacity of WW are not only related to adsorption but also to enhanced biochemical degradation processes (Winkler et al., 1987). However, the input of activated carbon to activated sludge might make the usage of sewage sludge as fertilizer impossible and requires energy intensive sludge incineration (Püttmann et al., 2008).

What's next? – Challenges for analytical chemists and ecotoxicologists

The chemical perspective:

Activated carbon treatment offers the great advantage that no TPs are formed. However, polar compounds are often not sufficiently retained. This is of particular relevance when AC is used as post-treatment step after oxidative treatment such as ozonation. Appropriate indicators need to be developed which allow for the evaluation of a potential breakthrough of compounds and the performance of AC over time. These include the analysis of polar CECs and TPs as well as surrogate parameters such as fluorescence or UV absorbance (Anumol et al., 2015; Zietzschmann et al., 2014b). In order to accurately predict the sorption of CECs to both

GAC and PAC, further improvements of models are necessary to consider molecular interactions between CECs and the sorbent surface as well as aging of AC over time.

The ecotoxicological perspective:

From an ecotoxicological point of view, activated carbon treatment is preferable compared to AOPs due to the benefit of contaminant elimination without reactive OP formation, and hence the risk of toxicity increase is minimal. Theoretically, TPs could be formed through microbiological transformation processes on the activated carbon but no studies are known which demonstrate a toxicity increase. Potential risks from the leakage of CEC loaded PAC particles into surface waters should be considered in future research. In terms of energy and resource requirements, biological activated carbon treatment is preferable to activated carbon filtration, but further research regarding pollutant and toxicity removal is desirable.

3.2.4. Pressure-driven membrane treatment technologies

CEC removal strongly depends on physico-chemical properties and membrane characteristics

Pressure-driven membrane processes include microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), forward osmosis (FO), and reverse osmosis (RO). However, for the removal of CECs, NF, FO, and RO are most important. The rejection thereby is primarily influenced by both the physico-chemical properties of CECs (molecular weight (MW), molecular size, acid disassociation constant (pKa), hydrophobicity/hydrophilicity (log K_{ow})), and diffusion coefficient (D_p), as well as membrane characteristics (molecular weight cut-off (MWCO), pore size, surface charge, hydrophobicity/hydrophilicity (measured as contact angle), and surface morphology (Bellona et al., 2004). Additionally, feeding water composition, such as pH, ionic strength, hardness, and the presence of organic matter, influences solute rejection.

In general, only compounds with a molecular size below the MWCO (molecular weight at which 80% of the substances are prevented from membrane diffusion) are able to pass through the pores of specified membranes and thus can be retrieved in the permeate. Good retention (> 80%) of charged compounds such as sulfamethoxazole (positively charged at ambient pH), diclofenac, and bezafibrate (both negatively charged at ambient pH) are typically achieved, attributable to electrostatic repulsion as well as steric hindrance (Xie et al., 2014; Coday et al., 2014; Kimura et al., 2003). In contrast to this, retention of neutral compounds such as pentachlorophenol, caffeine, and atrazine vary significantly and depends strongly on the used membranes (Yoon et al., 2006; Xie et al., 2014). For FO it has been shown that with the exception of hydrophilic neutral

compounds, the rejection of CECs is increased by the presence of a fouling layer (Linares et al., 2011). This can be attributed to several factors such as the higher hydrophilicity of the fouled membrane and thus an increased adsorption capacity of hydrophilic compounds and reduced mass transport capacity, membrane swelling, and the higher negative charge of the membrane surface. An improved removal of negatively charged compounds is thus typically observed. Though hydrophobic nonionic compounds, such as chloroform, bromoform, and hormones, might initially be highly rejected by RO and NF due to their sorption to the membrane, partitioning of solutes through the membranes can result in decreasing removal efficiencies over time (Ng and Elimelech, 2004). Similarly, formation of a colloidal cake layer on the membrane surface can restrict back diffusion of low molecular weight organic compounds, resulting in significant decline in their rejection (Ng and Elimelech, 2004). Antibiotics generally show high removal efficiencies in NF and RO (Le-Minh et al., 2010), whereas rejection of hormones such as estrone, estradiol, and testosterone was lower (between 60 – 80 %) in distilled water but increased significantly (>90 %) in the presence of humic substances (Kojuncu et al., 2008). This can be attributed to the binding of hormones to NOM and the formation of macromolecular complexes. Thus, removal efficiencies of >90 % are usually observed in WW (Snyder et al., 2007; Homem and Santos, 2011). Other EDCs such as nonylphenol also show a good removal whereas bisphenol A is only insufficiently removed during NF (Yangali-Quintanilla et al., 2009). The same is true for the cytostatic drugs cytarabine and 5-fluorouracil. For RO treatment, however, good removal of cyclophosphamide (>90 %) has been reported (Wang et al., 2009).

Insufficient removal of small, uncharged molecules

For the removal of DBPs, RO has been shown to only insufficiently remove NDMA (maximum 49 %; Fujioka et al., 2012), whereas haloacetic acids typically show high elimination efficiencies (Linge et al., 2013; Kimura et al., 2003). The primary explanation for this is that haloacetic acids are charged at ambient pH and thus are rejected via electrostatic repulsion. For other DBPs such as haloketones and halomethane retardation has been more variable and is typically lower than for haloacetic acids (Linge et al., 2013; Agus and Sedlak, 2010). In addition to the removal of DBPs themselves, research has focused on the removal of DBP precursors by membrane filtration. Small trihalomethane precursors such as resorcinol, phloroglucinol, and 3-hydroxybenzoic acid were removed by approximately 80 % using RO (Lin et al., 2007). Similar results were obtained by Mitch and Sedlak (2004), who showed that NDMA precursors are efficiently eliminated by RO from WWTP effluents. Lin et al. (1999) observed that although the UF system is able to remove a significant portion of THMFP (trihalomethanes formation potential) in larger AMW fractions, the permeate THM in terms of mg THMs/mg

carbon is still high. Cleaning of membranes, *e.g.*, via chlorination or chloramination used to remove membrane fouling (see *e.g.*, Linge et al., 2013; Li and Elimelech, 2004) can, however, substantially contribute to the formation of DBPs.

Modelling of CEC rejection by membranes is challenging

Due to the variety of parameters influencing the rejection of CECs by membranes, modelling is highly challenging. CECs with molecular weights larger than the MWCO have been detected in the permeate (Bellona et al., 2004). Thus, molecular weights in general cannot be used as sole criterion to exclude the presence in membrane treated waters. To accommodate this challenge, molecular width and length should be used as input parameters rather than molecular weight. Using a solute transport model, Kim et al. (2007) were able to show that the transport of most investigated DBPs and pharmaceuticals through RO and NF membranes is dominated by convection, whereas diffusion is important for more hydrophobic non-polar compounds. QSARs analyses were able to demonstrate that several variables such as hydrophobicity, salt rejection, surface charge, polarity, size, and operating conditions can be used to predict CEC rejection in NF and RO (Yangali-Quintanilla et al., 2010; Libotean et al., 2008).

Ecotoxicological benefits and concerns

Cao et al. (2009) found little effect of UF on genotoxicity, RARα activity, and acute invertebrate toxicity, while RO was the most effective technology removing biological effects compared to ozonation and UF. RO is very effective in reducing toxicity often to blank level like oxidative stress, genotoxicity, endocrine effects, photosynthesis inhibition, and cytotoxicity (Escher et al., 2011; Escher et al., 2014). Libralato et al. (2010) observed enhanced toxicity removal with UF assessed with *Vibrio fischeri* and *Crassostrea gigas*. In a study by Alzahrani et al. (2013), RO was considerably more effective in removing toxic effects (*Vibrio fischeri*) compared to NF, while the latter still removed 48% of the total organic carbon.

Other approaches combine membrane filtration systems with GAC filtration. The post treatment with biological membrane assisted carbon filtration (BIOMAC) subsequent to conventional treatment revealed promising pollutant removal rates (Weemaes et al., 2010). Estrogenicity and anti-androgenicity were effectively reduced by an average of 70% and 63% while GAC was essential for the removal of endocrine activity (Weemas et al., 2010).

Treatment of brines

An important aspect to consider with pressure-driven membrane treatment is the potential environmental implication of the waste stream. Membrane treatment results in the generation of retentates (brines) which are highly enriched in CECs, salts, and NOM. Brines are often discharged into water bodies without additional treatment which is a non-sustainable practice for obvious reasons. Furthermore, oxidative cleaning (chlorination) of membranes to oppose membrane fouling may well result in the formation of toxic DBPs (compare chapter 3.2.2) originating from the reaction of reactive chlorine species with biofilm-coated compounds. Due to high CEC concentrations, the safe discharge of brine would require a post treatment for pollutant removal. The high salt content makes biodegradation difficult but electrochemical oxidation is a promising option due to the high electrical conductivity. However, the high salt concentrations might also result in an increased formation of toxic by-products, in particular chlorinated and brominated compounds (Radjenovic et al., 2011). In addition, the requirement of an additional treatment step after the already energy intensive membrane filtration makes a sustainable and affordable broad-scale application unrealistic (van der Bruggen et al., 2003; Perez-Gonzalez et al., 2012).

What's next? – Challenges for analytical chemists and ecotoxicologists

The chemical perspective:

While the assessment of CEC rejection by pressure-driven membrane technologies seems relatively easy and straight-forward, an accurate prediction is hampered by the great variability of the composition of treated wastewaters. Thus, there is a need for comprehensive studies investigating the influence of the wastewater matrix. In particular, masking or complexation might substantially lower the rejection of charged molecules, for which usually good removal is observed. The same is true for membrane fouling as this substantially influences the performance of membranes, leading to higher or lower removal efficiencies. A detailed understanding of all these factors is a major prerequisite to accurately predict the fate of CECs during membrane treatment.

The analysis of brines constitutes a major challenge due to the complexity and high concentrations of matrix components. To this end, an adequate sample pretreatment is crucial. For the evaluation of brine treatment technologies, particular focus should be placed on the potential formation of toxic TPs such as halogenated compounds, in particular if electrochemical treatment is applied. The potential use of sum parameters such as AOX to assess the extent of TP formation needs to be further investigated.

The ecotoxicological perspective:

Reverse osmosis is one of the most effective WW treatment technologies for toxicity removal. Although, membrane technologies provide a direct pollutant removal without transformation processes, toxic DBPs can be formed during the required membrane disinfection. Furthermore, the retentates require an additional treatment to avoid any ecotoxicological risk through the disposal of highly toxic brines. This has to be taken into account, together with energy, resource, and maintenance requirements for a comprehensive evaluation of membrane technologies.

The ecotoxicological assessment of reverse osmosis water and retentates as native water samples without enrichment, as usually applied for in vivo *assays, can be challenging due to too low or too high matrix concentrations. Therefore, the analysis of extracted samples is the simplest approach to avoid matrix effects. To also include compounds that are too polar for sample enrichment, native RO water could be reconstituted with a salt mix to avoid artefacts through low salinity. For a toxicological assessment of native retentate samples, dilution with ultrapure water or the use of test organisms resistant to high salinity could be feasible.*

4. Conclusions

From the critical evaluation of current chemical and ecotoxicological methodologies used for the assessment of treated wastewater quality the following conclusions can be drawn:

- Elimination of a large variety of CECs is currently used as the main basis for the evaluation of advanced wastewater treatment technologies.
- Information on the formation and toxicological relevance of transformation products, which are formed in both biological and oxidative wastewater treatment steps, is insufficient and it is widely unclear to which extent transformation products contribute to overall toxicities of treated waters.
- Capabilities of analytical methods need to be extended to highly polar compounds as these are likely to be i) formed in oxidative treatment steps and ii) insufficiently removed by activated carbon filtration. The same is true for uncharged low molecular weight compounds which are likely to be only insufficiently rejected by dense membranes.
- Sample enrichment steps for bioanalytical assessment need to be extended to highly polar and volatile compounds, which are commonly lost during conventional extraction procedures.

- There is a need for the development of new and improvement of existing methods that allow for a more specific assessment and continuous onsite monitoring of waters treated by advanced treatment technologies. This includes sum parameters for specific potentially toxic moieties such as aldehydes and nitrosamines as well as bioanalytical methods.
- On site monitoring of the aquatic community up- and downstream of a discharger and comparison to a reference site is highly desirable.
- Sensitivities and specificities of bioanalytical tools need to be further improved to allow for the allocation of ecotoxicological effects to the presence of specific CEC(s) in treated waters, when combined with chemical analysis.
- Systematic studies are needed to improve the accuracy of predictions for both transformation kinetics and formation of transformation products.
- The development of an interdisciplinary concept for handling of realistic target values and well-defined quality criteria could help to support the implementation of measures by practitioners and guarantee that ecologically relevant CECs, their TPs, as well as ecotoxicological and microbiological endpoints are taken into account appropriately.

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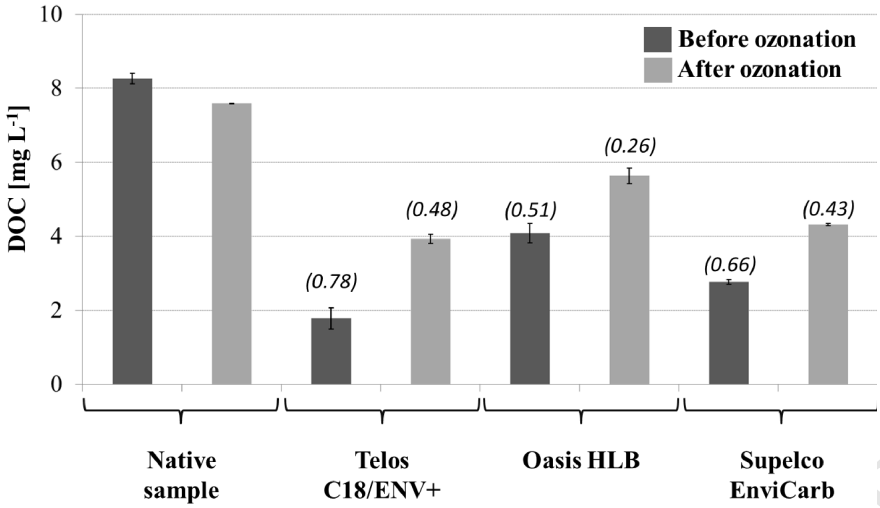

Fig.1. DOC concentrations in native influent and effluent samples of an ozonation pilot plant receiving conventional treated municipal wastewater compared to DOC remaining in the water phase after solid-phase extraction using three different sorbent materials (enriched water volume: 200 mL; sorbent amounts: Telos C18/ENV+: 200/500 mg; Oasis HLB: 200 mg; Supelco EnviCarb: 200 mg). The DOC fraction retained on the cartridges in comparison to native samples is given in parenthesis. Standard deviations (n=3) are given as error bars (own, unpublished data).

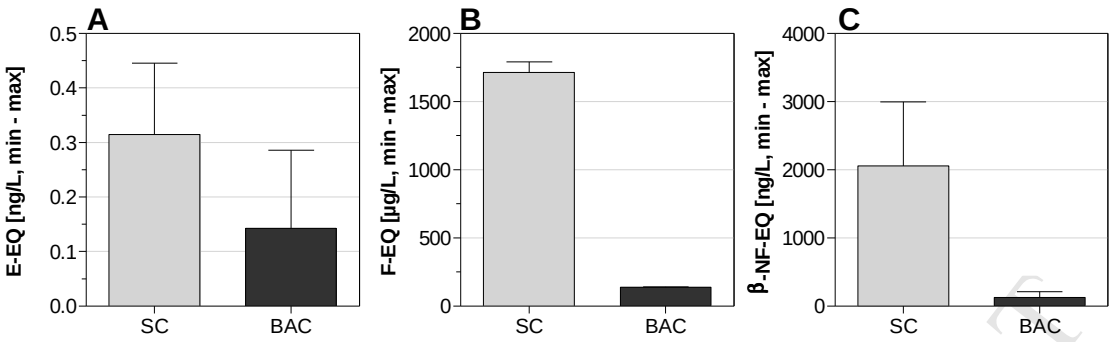

Fig. 2. Estrogenic (A, estradiol equivalents), anti-androgenic (B, flutamide equivalents) and aryl-hydrocarbon agonistic activity (C, β-naphthoflavone equivalents) before and after treatment with biologically activated carbon (BAC) using coke as carbonaceous material. Displayed are the mean values of two sampling campaigns (own, unpublished data). SC, after secondary clarifier subsequent to conventional treatment.

Research Highlights:

- Review of chemical and ecotoxicological methods to assess wastewater quality
- Critical assessment of methods including benefits and limitations
- Critical evaluation of conventional and advanced treatment technologies
- Demand for multidisciplinary assessment approaches and future research identified