Oxidative water treatment: mechanistic aspects and matrix effects

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Summary

The main emission sources of micropollutants (MPs) found in the aquatic environment are wastewater treatment plant (WWTP) effluents but also diffuse sources such as agricultural run-offs. An opportunity to reduce the load of MPs in WWTP effluents is the application of ozonation in order to degrade undesirable substances. Especially nitrogen containing (*N*-containing) substances, including many pharmaceuticals and pesticides, react fast with ozone. However, the degradation of these substances via ozone does not lead to mineralization but rather to a formation of transformation products (TPs) that can have a higher toxicity than the parent substance. Until now, reaction pathways of *N*-containing substances with ozone are still not completely understood, because they are depending on various factors such as the matrix composition. Therefore, the formation of TPs and their ecotoxicological potential is hardly predictable. Most studies investigating the degradation of MPs with ozone and the formation of TPs are performed in ultrapure water excluding the influence of matrix components.

Hence, this study wants to overcome the gap between mechanistic studies and the investigation of matrix influence. This was performed via the determination of TP formation during ozonation of three environmentally relevant substances (diclofenac (DCF), metoprolol (METO) and isoproturon (ISO)). The formed TPs were determined via target analysis, suspect and non-target screening and the toxicological potential towards aquatic organisms was evaluated. Various matrix compositions were investigated in terms of TP formation during the ozonation of DCF, METO and ISO. These included ultrapure, drinking and surface water, two different concentrations of non-purgeable organic carbon (NPOC: 2.35 mg/L and NPOC: 0.63 mg/L), two different scavengers (dimethyl sulfoxide (DMSO) and tertiary butanol (*tert*-BuOH)) and wastewater.

Within all these matrices the degradation of the three parent substances and detected stoichiometries were similar. However, even if the formation profiles of the TPs were similar (increasing formation until a certain ozone dosage and decreasing with higher ozone dosages) the yields differed among the water matrices. High differences in the TP formation were detected in the presence of scavengers. For two TPs a continuous formation without any degradation was determined either for DMSO or *tert*-BuOH but not for the other scavenger. This was striking as both scavengers are frequently applied in laboratory setups, assuming that both are leading to the same results. As this study was the first to compare the two scavengers

in terms of TP formation and revealed that the chosen scavenger can highly influence this formation further research is highly recommended.

Additionally, the influence of hypobromous acid (HOBr) formed in the reaction of bromide with ozone and the direct influence of bromide on the formation of TPs were investigated, as bromide is omnipresent in wastewater. It could be shown that HOBr and also low concentrations of bromide can influence the formation of TPs and that similar TPs could be detected in the direct reaction with HOBr compared with the samples containing bromide. This was striking as the reaction of ozone with the substances should be favored over the reaction of ozone with bromide. However, even low concentrations of bromide influenced the formation of TPs, leading to the detection of TPs not reported before.

All examined water matrices (except NPOC, drinking and surface water) in terms of TP formation were also investigated regarding the toxicity towards *D. magna*, showing that matrix composition can influence the toxic potential. After ozonation of the parent substances in ultrapure water and for all parent substances and target TPs no effect was detected. Only for the diclofenac TP 2,6-dichloroaniline an effect concentration leading to 50 % immobility of 1.02 mg/L after 48 h was observed and ozonation of DCF in the presence of DMSO led to an immobility of 95 % (48 h) of the daphnids. The reaction of HOBr with the parent substances did not reveal an effect for ISO but for metoprolol (100 % immobilization (48 h)) and diclofenac (95 % immobilization (48 h)).

Preliminary investigations performed in this study showed that simply structured *N*-containing substances, 2,2,6,6-tetramethylpiperidine and *cis*-2,6-dimethylpiperidine, do not react with ozone at pH 2 but pH 7. These two substances were chosen as they were expected to form aminyl radicals. However, stoichiometries above one have been determined for both substances at pH 7 and pH 11 which supports a postulated ozone consuming chain reaction, leading to a high ozone consumption with only low substance degradation.

The results of this study showed that even if the reaction mechanisms of *N*-containing substances during ozonation seem to be understood quite well, matrix components can highly influence these mechanisms and also the formation of TPs. Even for very well investigated substances the use of different scavengers revealed new observations within this study, underlining that further detailed research is still needed to achieve a better understanding of the influence matrix components can have on the formation of TPs and also on the toxicological potential towards aquatic organisms.

Zusammenfassung

Die Haupteintragsquelle von Mikroschadstoffen in die aquatische Umwelt sind vor allem Kläranlagen (KA), aber auch diffuse Stoffeinträge aus der Landwirtschaft können Eintragswege darstellen. Um die Schadstofflast in KA-Abläufen zu verringern und Mikroschadstoffe abzubauen kommt häufig die Ozonung als zusätzliche Reinigungsstufe zum Einsatz. Insbesondere stickstoffhaltige Substanzen (*N*-Substanzen), zu welchen auch viele Arzneimittel und Pestizide zählen, reagieren schnell mit Ozon. Allerdings können diese Reaktionen zur Bildung von Transformationsprodukten (TPs) führen, welche wiederum ein höheres toxikologisches Potential für die aquatische Umwelt haben können, als die Ausgangsubstanzen. Die TP-Bildung ist von vielen Faktoren, wie z.B. der Matrixzusammensetzung, abhängig und daher schwer abschätzbar. Vorhersagen zur Bildung von TPs und eine Einschätzung des ökotoxikologischen Potentials sind deswegen schwierig. Außerdem sind bisher die Reaktionswege von *N*-Substanzen mit Ozon nicht komplett verstanden. Des Weiteren wurde der Einfluss von Matrixbestandteilen häufig in Laborstudien nicht berücksichtigt, da diese zumeist bisher nur in Reinstwasser durchgeführt wurden.

Diese Studie soll die Lücke zwischen mechanistischen Studien und dem Einfluss der Matrix schließen. Hierzu wurde der Einfluss von Matrixbestandteilen auf die Bildung von TPs anhand von drei ökologisch relevanten Substanzen (Diclofenac (DCF), Metoprolol (METO) und Isoproturon (ISO)) mittels Target-Analytik sowie Suspect und Non-Target Screening untersucht. Außerdem wurde das akute toxikologische Potential gegenüber Daphnien bestimmt. Die Ozonung von DCF, METO und ISO erfolgte in folgenden Matrices: Reinst-, Trink- und Oberflächenwasser, zwei Konzentrationen von nicht ausblasbarem organischen Kohlenstoff (NPOC: 2.35 mg/L and NPOC: 0.63 mg/L), zwei Hydroxylradikalfängern (Dimethylsulfoxid (DMSO) und tertiäres Butanol (*tert*-BuOH)) sowie Abwasser.

Insgesamt waren der Abbau sowie die bestimmten Stöchiometrien der drei Substanzen in allen Matrices ähnlich. Bei der TP Bildung konnten zwar für fast alle TPs ähnliche Verläufe (Bildung mit ansteigender Ozondosierung und anschließender Abbau bei weiterer Erhöhung der Ozondosierungen) beobachtet werden allerdings variierten die Ausbeuten der TPs in den verschiedenen Matrices. Abhängig vom eingesetzten Radikalfänger wurden allerdings Unterschiede in der Entstehung von TPs beobachtet. Für zwei TPs wurde eine kontinuierliche Bildung ohne Abbau für entweder DMSO oder *tert*-BuOH, aber nicht für den jeweils anderen Radikalfänger beobachtet. Dies war besonders auffällig, da beide Radikalfänger häufig in Laborstudien eingesetzt werden und bisher angenommen wurde, dass beide zu vergleichbaren

Ergebnissen führen. Da diese Studie die Erste ist, welche die beiden Radikalfänger bezüglich ihres Einflusses auf die Bildung von TPs vergleicht und hierbei gezeigt wurde, dass es zu Unterschieden kommen kann, sind noch weitere Untersuchungen nötig.

Außerdem wurde auch der Einfluss von Bromid sowie hypobromischer Säure (HOBr), welche in der Reaktion von Ozon mit Bromid entsteht, auf die Bildung von TPs untersucht, da Bromid ubiquitär in Abwasser vorkommt. Hierbei konnte gezeigt werden, dass bei der Ozonung der drei Substanzen in bromidhaltigen Proben vergleichbare TPs identifiziert wurden, wie nach der direkten Zugabe von HOBr mit anschließender Ozonung. Dies verdeutlicht, dass, obwohl die Reaktion von Ozon mit den Substanzen bevorzugt stattfinden sollte, auch eine geringe Bromidkonzentration einen starken Einfluss auf die TP Bildung haben kann.

Alle untersuchten Wassermatrices (außer NPOC, Trink- und Oberflächenwasser) wurden außerdem in Bezug auf das akute toxikologische Potential gegenüber *Daphnia magna* untersucht. Dies zeigte, dass die Matrix auch hier einen Einfluss haben kann. Nach der Ozonung in Reinstwasser sowie für alle Target-TPs konnten keine Effekte nachgewiesen werden. Allerdings wurde für das DCF TP 2,6-Dichloroanilin eine Effektkonzentration von 1.02 mg/L nach 48 h bestimmt. In Gegenwart von DMSO bei der Ozonung von DCF konnte eine Immobilität von 95 % (48 h) beobachtet werden. Des Weiteren zeigte die Reaktion von HOBr mit den Ausgangssubstanzen mit anschließender Ozonung keinen Effekt für ISO aber für METO (100 % Immobilität (48 h)) und Diclofenac (95 % Immobilität (48 h)).

Vorausgehenden Experimente zur Reaktion von einfach strukturierten *N*-Substanzen, 2,2,6,6-tetramethylpiperidin und *cis*-2,6-dimethylpiperidin, mit Ozon zeigten, dass beide Substanzen bei pH 7 mit Ozon reagieren, aber nicht bei pH 2. Diese Substanzen wurden ausgewählt, da vermutet wird, dass sich bei der Reaktion mit Ozon Aminylradikale bilden. Sowohl bei pH 7 als auch pH 11 wurde eine Stöchiometrie über eins für beide Substanzen festgestellt. Diese Beobachtungen weisen darauf hin, dass bei pH 7 und pH 11 eine ozonzehrende Kettenreaktion stattfindet, welche bereits für andere *N*-Substanzen postulierte wurde und zu einem hohen Ozonverbrauch mit gleichzeitig geringem Schadstoffabbau führt.

Die Ergebnisse dieser Studie zeigen, dass auch wenn der Reaktionsmechanismus von *N*-haltigen Verbindungen scheinbar gut verstanden ist, Matrixbestandteile diesen insbesondere in Bezug auf die Bildung von TPs stark beeinflussen können. Selbst für bereits gut untersuchte Substanzen konnte gezeigt werden, dass der Einsatz von verschiedenen Radikalfängern zu neuen Ergebnissen führt. Dies zeigt, dass weitere Untersuchungen nötig sind, um ein besseres Verständnis zu entwickeln, wie Matrixbestanteile die Bildung von TPs beeinflussen und welche Auswirkungen dies auf das toxikologische Potential gegenüber Wasserorganismen haben kann.

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Chapter 1

General Introduction

1.1 Micropollutants in aqueous systems

Micropollutants (MPs) present in the aquatic environment include anthropogenic substances from various categories such as pharmaceuticals, pesticides, flame retardants, industrial chemicals and personal care products [1-7] and are frequently occurring in low concentrations of ng / L to µg / L [6-8]. They have been detected in wastewater, surface water, groundwater and even in drinking water [3, 4, 6, 9, 10]. Major sources of MPs are hospital effluents, domestic or industrial wastewater but also agricultural run-off (Figure 1-1) [3, 4, 11].

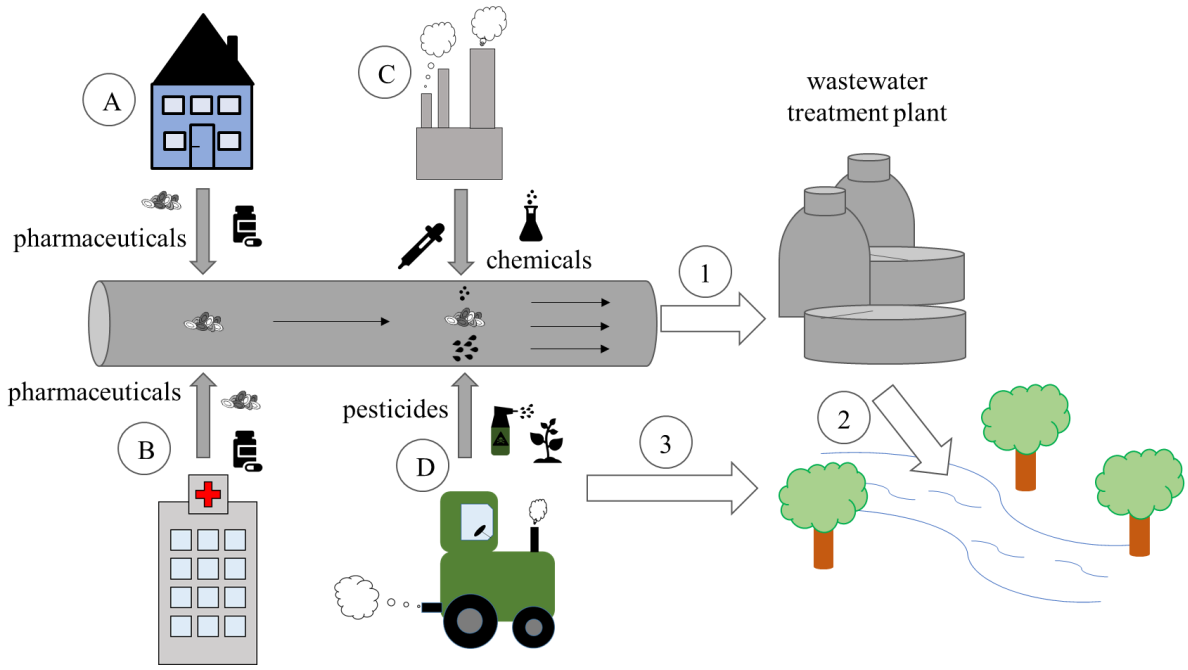

Figure 1-1 Major sources of micropollutants in aqueous systems

Domestic effluents (A), hospital effluents (B), industrial effluents (C) and emissions from agriculture (D) (can) reach the waste water system and wastewater treatment plant (WWTP) (1). From there micropollutants can also enter the environment (2) due to incomplete degradation in the WWTP. Agricultural emissions (D) can also directly reach the environment due to agricultural run-off (3).

If only taking into account human medical products used in Germany, around 1,200 substances found in the aquatic environment are considered as environmentally relevant [12]. Acute (lethal) or chronic (e.g., genotoxic) effects on the organisms can be induced by the possible uptake of substances present in the aquatic environment [13]. Even if the concentrations of substances, such as estrogens or pharmaceuticals, are already low in the wastewater treatment plant (WWTPs) effluents studies reported that also such low concentrations found in the environment can have substantial adverse effects on aquatic organisms [14, 15] and that therefore the further concentration reduction of such chemicals in

the aquatic environment is of substantial concern. Yet, even if the applications of MPs might vary, there are common structural features often present such as amine groups and aromatic ring systems [16]. A screening of the River Rhine actually outlined that around 90 % of the detected substances contained nitrogen (*N*) [17] and therefore, especially *N*-containing MPs are of high interest in water treatment and investigations of ecotoxicological potentials.

1.2 (Advanced) wastewater treatment

Removal rates of MPs in conventional wastewater treatment highly differ depending on the chemical structures of the substances and range between 12 and 100 % [3]. Due to the often incomplete elimination, WWTP effluents are the main source of MPs in the aquatic environment [3, 9].

After the wastewater from households, industry or hospitals entered the sewage system (A) it reaches the WWTP (B) (Figure 1-2). In the primary treatment (C) particles heavier than water are removed through sedimentation. This is followed by the activated sludge treatment (D) in which a degradation of the organic material via microorganisms takes place, afterwards the effluent passes through a final clarification step (G) before it reaches the environment. In a conventional WWTP the activated sludge treatment can also be followed by a chemical treatment step to remove phosphor (not shown). If a fourth treatment step is implemented the activated sludge treatment (D) can be followed by e.g., ozonation (E), the addition of activated carbon (powdered activated carbon (PAC) or a granulated activated carbon filter, both not shown) or membrane filtration (not shown) [3, 16] to increase the removal efficiency of MPs in WWTPs. After ozonation either a recirculation or a (sand) filtration (F) is normally implemented which is followed by the final clarification step (G) [16, 18].

Considering the implementation of a fourth treatment step includes the evaluation of advantages and disadvantages of the technical options. As mostly either the implementation of PAC (e.g., in Dülmen, North Rhine-Westphalia, Germany [19]) and ozonation (e.g., in Aachen-Soers, North Rhine-Westphalia, Germany [20]) are performed [21] these two will be described in detail. One possibility of PAC addition is to dose it directly to the effluent after the biological treatment followed by a contact reactor and filtration [22]. MPs adsorb onto the PAC and are therefore removed from the wastewater (> 80 % elimination efficiency [23]) and disposed with the sludge [22, 24]. However, the adsorption potential of PAC is limited and depends, e.g., on the hydrophobicity of the present substances and dissolved organic carbon

(DOC) present in the water [23, 24]. Further, the release of PAC loaded with MPs into the environment needs to be assumed as it cannot be always excluded [22]. Ozonation is degrading large fractions of MP within the wastewater treatment, leading to high removal efficiency rate (> 90 %) [24].Yet, even if ozonation is decreasing the overall amount of MPs in the wastewater, disadvantages in ozonation are the fast depletion of ozone (few minutes to an hour) and the formation of undesired oxidation by-products [25]. Indeed, ozonation is not leading to a mineralization but rather to the formation of transformation products (TPs) within the water system [16]. The reactions taking place during ozonation are not completely understood and therefore, TPs are hardly predictable.

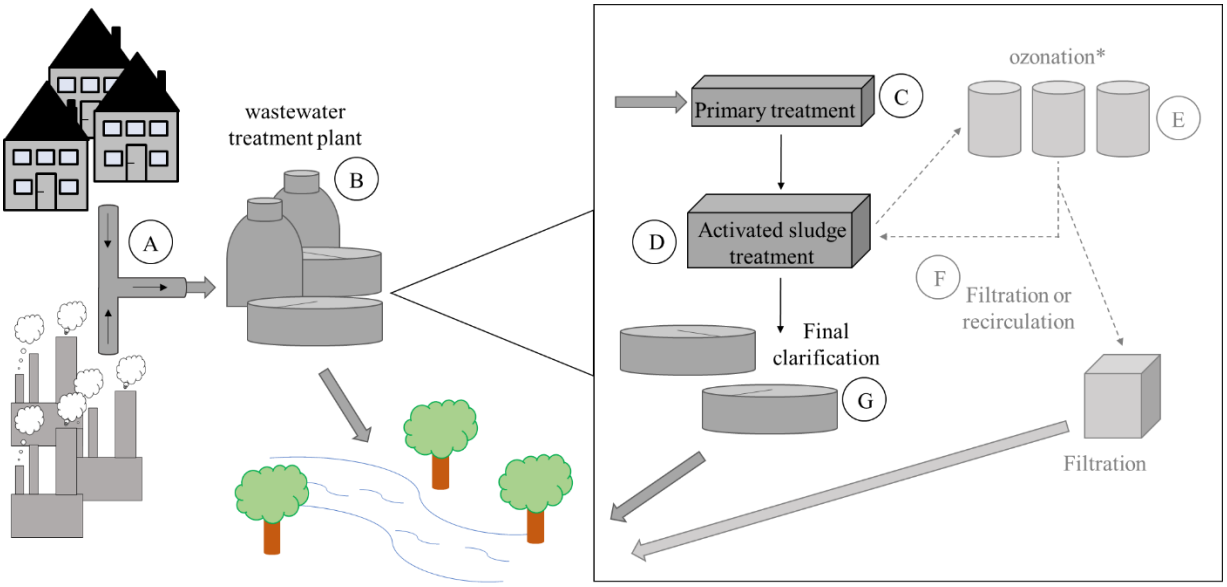

Figure 1-2 **Schematic presentation of a wastewater treatment plant (WWTP)**
Including the entrance of the wastewater into the sewerage system (A), reaching the WWTP (B), the primary treatment step (C), the activated sludge treatment (D), additional ozonation (E), possible filtration or recirculation to the activated sludge treatment (F) or a final clarification step (G) before the effluent is released into the environment. *instead of ozonation is also the addition of activated carbon or membrane filtration possible

As ozone is consumed fast by the wastewater matrix, yielding high ratios of hydroxyl radicals ($^{\bullet}OH$), it can be considered as advanced oxidation process (AOP). AOPs are always leading to high yields of $^{\bullet}OH$ which react non-selectively with a large number of organic MPs. The underlying reactions include, among others, (i) the photolysis of hydrogen peroxide (H_2O_2), (ii) the Fenton reaction [26], (iii) the peroxone process [27], (iv) the carboxone / carbozone process [16, 28] and (v) the ultra violet (UV)-light induced photolysis

of ozone (O_3) [29]. While the processes (i) and (ii) are ozone independent, processes (iii) to (v) include a reaction of ozone and are so called ozone-based AOPs [16]. Generally, ozonation and other oxidative processes have been applied in (waste)water treatment since decades, mostly for disinfection [16, 25, 30], since ozone can induce lysis of bacteria cells [31] and also leads to damages in deoxyribonucleic acid (DNA) [32]. As the wastewater effluent might be released into surface waters which are used for swimming or the reclaimed water might be reused disinfection is still of high interest. However, the field of applications, especially of ozonation, changed over the years and nowadays also includes the removal of MPs [16] and is progressively increasing implemented in wastewater treatment [33-35].

1.3 Reactions of ozone

Ozone is a selective electrophile reacting with electron-rich structures, which are often present in MPs [16, 36, 37]. These include amines, aromatic rings and double bonds, which react mostly fast with ozone [16, 37]. Nevertheless, the reactions kinetics of ozone with MPs in wastewater and the depletion of ozone over time are pH dependent [38]. Further, ozone can be consumed by the present water matrix in wastewater treatment [39], leading to a loss in degradation efficiency towards MPs (Figure 1-5.D and Figure 1-5.E). The reaction of ozone with organic compounds (especially with natural organic matter (NOM)) (Figure 1-5.D and Figure 1-5.G) and also the degradation due to an alkaline pH can yield $^{\bullet}OH$ (Figure 1-5.F and Figure 1-5.G) [38-40]. Therefore, $^{\bullet}OH$ play an important role during ozonation and need to be considered in this process as these also eliminate MPs [16]. The formed $^{\bullet}OH$ react quite fast ($k = 10^8 – 10^9\ M^{-1}s^{-1}$) and non-selectively with MPs and therefore can also degrade substances which are not likely to be degraded by ozone itself [16, 39].

Due to the structural diversity of MPs the reaction with ozone and formed $^{\bullet}OH$ is essential in the degradation of MPs. While atrazine is mostly degraded via $^{\bullet}OH$ (reported rate constants: $k_{OH} = 3 \times 10^9\ M^{-1}s^{-1}$ [41] and $k_{OH} = 1.8 \times 10^{10}\ M^{-1}s^{-1}$ [42]) rather than ozone (reported rate constants: $k_{O3} = 6\ M^{-1}s^{-1}$ [41] and $k_{O3} = 6.3\ M^{-1}s^{-1}$ [42]), 17α-ethinylestradiol is degraded in the same range by both oxidants ($k_{O3} = 7 \times 10^9\ M^{-1}s^{-1}$, $k_{OH} = 9.8 \times 10^9\ M^{-1}s^{-1}$) [34]. In order to distinguish between direct ozonation and $^{\bullet}OH$ based reactions $^{\bullet}OH$ scavengers such as dimethyl sulfoxide (DMSO), tertiary butanol (*tert*-BuOH), acetate, H_2O_2 and 2-propanol can be added [16]. Mostly applied in experimental setups considering the mechanistic investigation of reaction pathways and / or the formation of possible TPs are DMSO and *tert*-BuOH [16].

These scavengers react quickly with •OH ($k_{\bullet OH+DMSO}$ = 7 x 10^9 $M^{-1}s^{-1}$ [43] and $k_{\bullet OH+tert\text{-}BuOH}$ = 6 x 10^8 $M^{-1}s^{-1}$ [43]) and only slowly with ozone ($k_{O3+DMSO}$ = 8.2 $M^{-1}s^{-1}$ [44] $k_{O3+tert\text{-}BuOH}$ = 3 x 10^{-3} $M^{-1}s^{-1}$ [45]. Thus, scavengers are mostly dosed in concentrations scavenging 95 % of •OH but only 5 % of ozone to exclude the reactions of •OH with the substance of interest. As the main approach of both scavengers is the same (to scavenge •OH) all published studies only used one of the two (among others [36, 46-49]) and so far, no studies compared the influence of different radical scavengers in terms of TP formation.

Overall fundamental knowledge, particularly in terms of TP formation, is still lacking. This is especially the case for *N*-containing substances which can lead to very complex reaction pathways with ozone. The two reaction sites for ozone in *N*-containing structures are the aromatic ring systems and / or the lone electron pair at the nitrogen [16, 36]. Therefore, several reactions can take place and hence lead to the formation of reactive intermediates (Figure 1-3). [16, 37] in three major reaction pathways: (i) electron transfer, (ii) oxygen atom transfer and (iii) ozone addition [25]. Reported reaction rate constants of aromatic amines are k = 1.2 x 10^5 – 2.4 x 10^6 $M^{-1}s^{-1}$ [36] and of secondary heterocyclic amines k = 1.9 x 10^4 – 2.4 x 10^5 $M^{-1}s^{-1}$ [50].

Ozonation of primary and secondary amines is presumably leading to the formation of hydroxyl amines (Reaction 1, Figure 1-3) or nitrogen centered (aminyl) radicals (•NR) (via electron transfer, Reaction 2, Figure 1-3), while *N*-oxides (via oxygen atom transfer, Reactions 3 – 5, Figure 1-3) are more likely to be formed in the ozonation of tertiary amines. During ozonation of anilines a reaction at the aromatic ring is possible. Due to the high reaction rate constants (k_{O3} = 1.2 x 10^5 – 2.4 x 10^6 $M^{-1}s^{-1}$) of anilines and a derived stoichiometry of 4.5 mole ozone required for degradation of 1 mole aniline [36] a reaction of ozone with the nitrogen is also presumable (via ozone addition, Reaction 6 and 7, Figure 1-3) [16]. This reaction can lead to the formation of nitroxide radicals (•ONR_2) (Reaction 8, Figure 1-3). As the reaction of •ONR_2 with ozone can lead to the formation of •NR (Reaction 9, Figure 1-4) and vice versa an ozone consuming chain reaction has been postulated (Figure 1-4) [36, 46]. However, the formation of •ONR_2 and •NR as well as an ozone consuming chain reaction has also been postulated for the reaction of piperazine and piperidine with ozone [50].

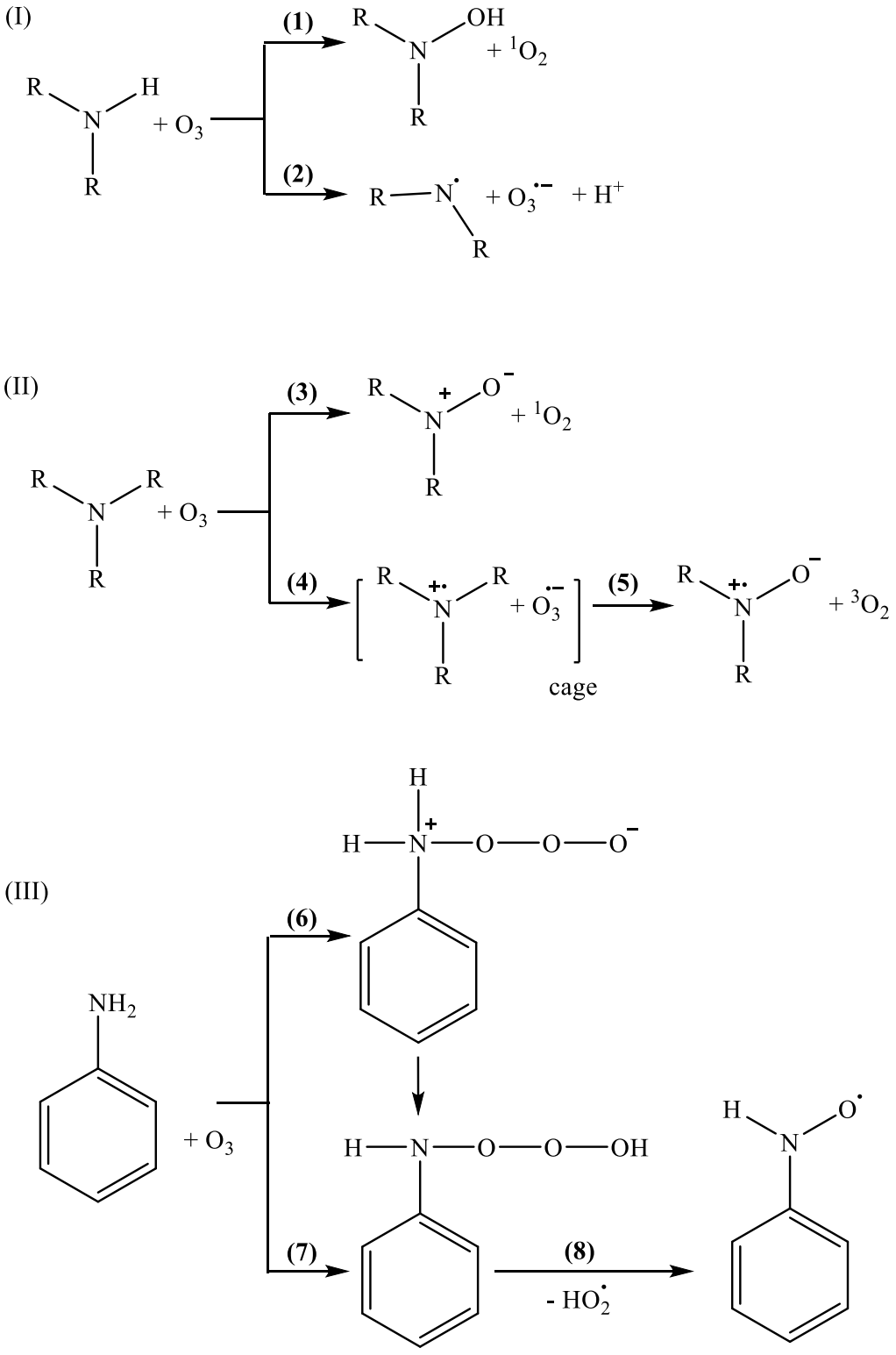

Figure 1-3 **Possible reactions during ozonation of primary and secondary amines (I), tertiary amines (II) and aromatic amines (III)**

(modified after von Sonntag and von Gunten [16] and Tekle-Röttering et al. [36])

Sein et al. [46] firstly postulated an ozone consuming chain reaction for diclofenac because it was observed that several mole ozone were needed to degrade one mole diclofenac.

In upcoming studies this has also been observed in the degradation of aniline and piperazine leading also to the postulate of an ozone consuming chain reaction [36, 50].

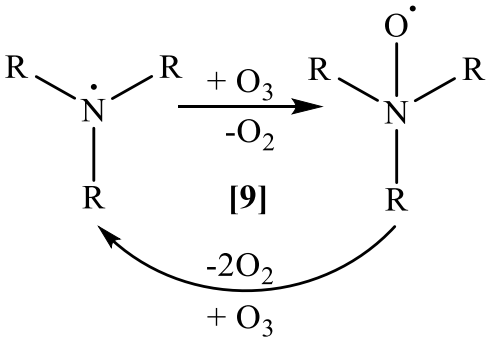

Figure 1-4 **Ozone consuming chain reaction** (modified after Tekle-Röttering et al. [36] and Sein et al. [46])

Formed intermediates during ozonation of *N*-containing substances might be involved in many side reactions, as these are postulated to be highly reactive even if reaction rate constants are still unknown [16]. Further, also the formation of unpredictable TPs through intermolecular reactions or the reactions of intermediates with matrix components are possible and not yet investigated in detail. Indeed, even if information about the reaction of $^{\bullet}$NR are still lacking it has been postulated that $^{\bullet}$NR can also react to carbon centered radicals ($^{\bullet}$CR) [36, 50, 51], which is comparable to the reaction of oxyl radicals [52]. However, the reaction to $^{\bullet}$CR can consequently also yield unwanted carbonyls [51]. Additionally, also reactive oxygen species such as H_2O_2 or singlet oxygen ($^1O_2$) can be formed and which can react with formed intermediates or the present MPs, leading to unknown TPs [16].

Generally, knowledge on reactions of *N*-containing substances during ozonation is still lacking in detail and therefore, further investigations of the reaction mechanisms of $^{\bullet}$NR and the possibly formed by-products and TPs are needed.

1.4 Influence of matrix components and the formation of transformation products during ozonation

Even though not only parameters such as temperature and pH [53], but also the composition of the water matrix can have a remarkable impact on the formation of by-products and undesired TPs [37], it is hardly addressed in literature. In fact, until now only few studies investigated the influence of matrix components on the degradation of MPs or formation of TPs [51, 54-56] and information about the influence of scavengers is also lacking.

Many matrix parameters can influence the ozonation of MPs within a WWTP [38, 39] (Figure 1-5 A-C). Three factors that mainly depend on the matrix composition are the lifetime of ozone itself, the possible reaction mechanisms and kinetics of those reactions (Figure 1-5 C). Especially possible reaction mechanism influenced by the surrounding matrix are yet not well investigated.

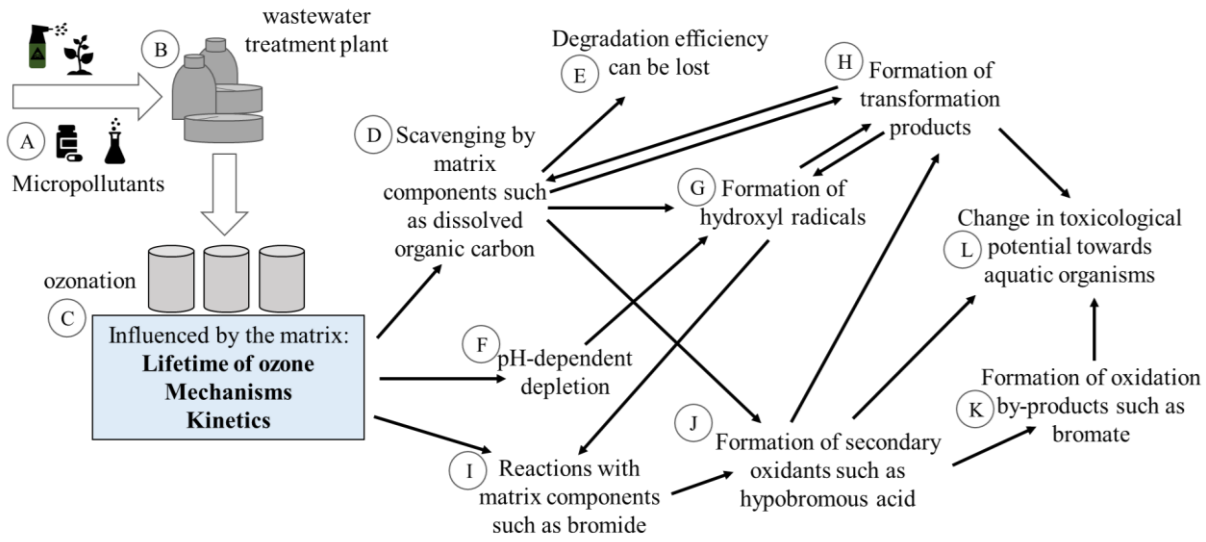

Figure 1-5 Possible influence of the water matrix on the degradation of micropollutants and the formation of transformation products during ozonation

Organic compounds or (bi)carbonate can quench $^{\bullet}$OH, while DOC yields $^{\bullet}$OH and simultaneously consumes ozone [57] (Figure 1-5 D-G). This can lead to a loss in the degradation efficiency of ozonation and also to the formation of oxidation by-products and/or secondary oxidants (Figure 1-5 E-J). The presence of e.g., bromide (Br^-) can lead to the formation of the secondary oxidant hypobromous acid (HOBr, Figure 1-5 J) which can react with organic compounds and intermediates and also influence the formation of TPs leading to an increased toxicity (Figure 1-5 L) [58, 59]. Further, present matrix components but also the presence of secondary oxidants can lead to the formation of unwanted by-products, e.g., bromate (BrO_3^-, Figure 1-5 K), which is cancerogenic and a by-product of HOBr [16, 53, 60]. In the reaction with secondary oxidants or other matrix components also reactive intermediates can be formed (Figure 1-5 H-J), leading to a change in the reactivity of certain substances. Further, DOC present in the water matrix can also quench reactive intermediates leading to the formation of different TPs which might have an increased or decreased aquatic toxicological potential (Figure 1-5 L) [61].

Next to matrix components present in real water matrices also the scavengers DMSO and *tert*-BuOH which are frequently used to scavenge $^{\bullet}$OH in laboratory studies (among others

[16, 36, 48, 50, 62-65]) need to be considered in terms of matrix effects. However, no literature is available comparing both scavengers in the same study in terms of TP formation.

The reaction of HOBr with primary and secondary amines has been reported to be faster ($k = 10^5 – 10^6$ $M^{-1}s^{-1}$) [66-68] than reactions of hypochlorous acid (HOCl) with amines ($k_{app} = 10^3 – 10^4$ $M^{-1}s^{-1}$ [69, 70]. Reactive intermediates formed during the reaction of amines with HOBr can react either with matrix components or again with residual ozone or $^{\bullet}OH$. A case in point, is the ozonation of *N*,*N*-dimethylsulfamide (DMS, Reaction 10, Figure 1-6), which is formed in the microbial degradation of the fungicide tolylfluanide, and further reacts with ozone to *N*-nitrosodimethylamine (NDMA, carcinogenic) [16, 58, 59, 69, 71, 72].

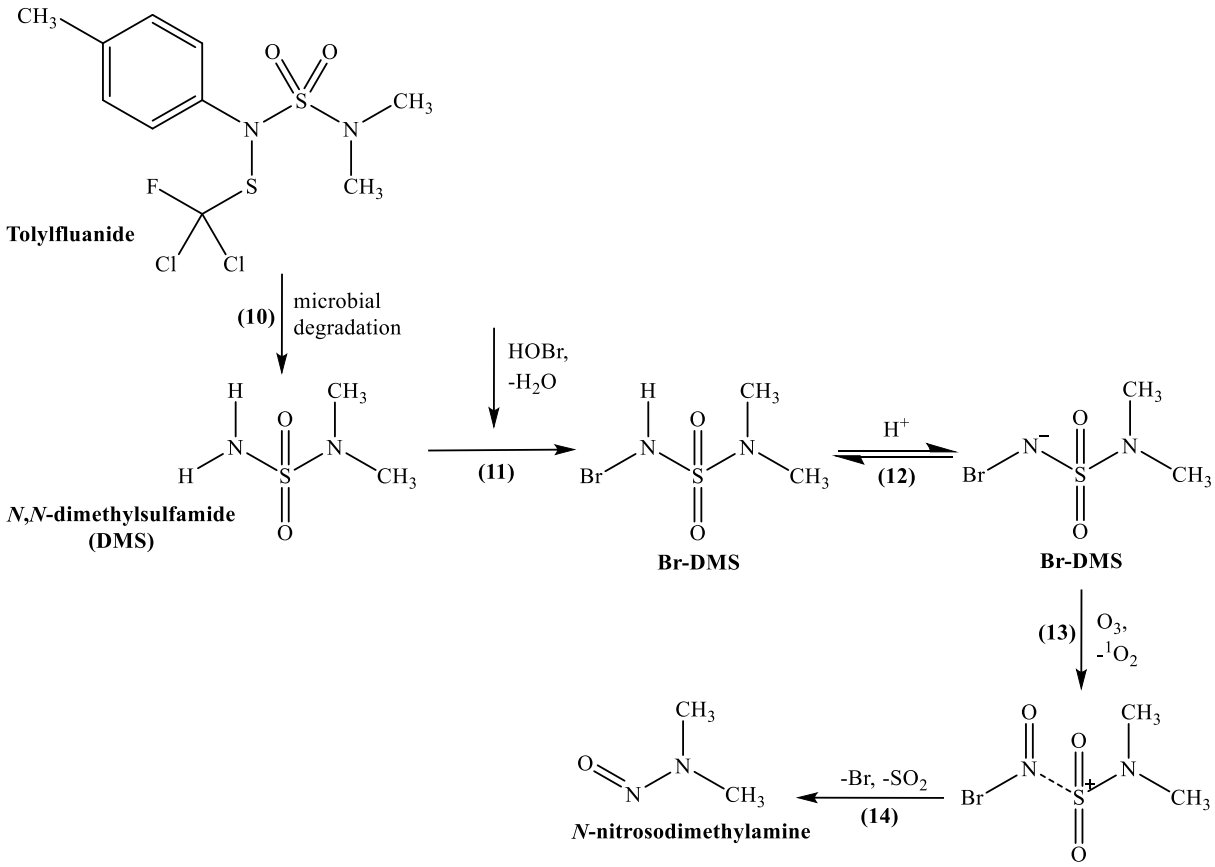

Figure 1-6 **Reaction of *N*,*N*-dimethylsulfamide to *N*-nitrosodimethylamine in the presence of bromide and ozone** (modified after Trogolo et al. [59], Schmidt and Brauch [58] and von Gunten et al. [72])

However, this reaction only takes place if Br^- is present in the water matrix, so that HOBr is formed, which further reacts with DMS to brominated DMS (BrDMS) (Reaction 11, Figure 1-6). During ozonation BrDMS is further transformed yielding NDMA (Reaction 12-14, Figure 1-6) [16, 58, 59, 72]. Even if it has been reported that NDMA can be removed via biological sand filtration [58, 73] it needs to be taken into account that matrix components can

have an influence on the formation of TPs and need to be considered more in terms of MP ozonation [37]. Indeed, some studies already showed that ozonated wastewater can have higher (eco)toxicological relevance than conventionally treated wastewater [74, 75]. However, it was not investigated which TPs were present in the analyzed wastewaters and if those were responsible for the increased toxicity. Therefore, mechanistic studies and detailed analysis of the formed TPs and possible correlations of (eco)toxicological effects are still needed.

1.5 Analysis of micropollutants and transformation products in water matrices

To identify substances present in water samples mainly three approaches are followed; target analysis, suspect and non-target screening [76, 77]. These are commonly performed with gas or liquid chromatography (LC) coupled with UV-detection, mass spectrometry (MS) or high-resolution mass spectrometry (HRMS, such as Orbitrap) [8, 78]. Especially, the use of LC-HRMS enables the fast detection of many substances with small mass deviation and is therefore frequently used in non-target screening (NTS) [78]. Most studies considering the formation of TPs during ozonation of MPs focused on the qualitative identification of TPs but revealed gaps in terms of quantitative analysis as this is very challenging in NTS [79].

Target analysis always includes the comparison of the substance of interest with the measurements of known reference standards. In suspect screening compounds which are assumed to be present in a sample are analyzed. Here mostly chemical information (e.g., exact mass or fragmentation patterns) are available and the substances might be confirmed by a reference standard [77, 78, 80]. In NTS, no a priori information about the substances within the sample is available and therefore the evaluation of the measurements includes many peaks at all *m/z* and retention times [77, 78, 80]. In Figure 1-7 the workflow of target, suspect and non-target screening is schematically outlined [76, 77]. If substances detected in suspect screening or NTS can be identified and confirmed with reference standards, these can also be considered as targets in continuing analysis [76].

As the data obtained via suspect screening and NTS with LC-HRMS is very complex (for wastewater between 10,000 and 20,000 features [81]) signal reduction involving blank subtraction, annotation or prioritization [78, 80, 81] is needed to combine and reduce the amount of data to a reasonable extent. Different software packages are available to evaluate suspect screening or NTS data such as enviMass, MZmine2 and Compound Discoverer, but

also XCMS or other R packages can be used [81, 82]. Yet, in a study comparing the results of different software packages by using the same raw data, low accordance between the optimized workflows was detected indicating how complex the data evaluation of NTS is [82].

However, to identify a substance with a high confidence still the comparison with a reference standard is needed and due to the elaborate data evaluation, it is therefore not possible to identify all substances in a sample within NTS [81]. One recently presented approach to prioritize substances obtained within a NTS, is an offline two-dimensional LC which includes an UV detection (first dimension) as well as a HRMS detection (second dimension) and was described as being successful for the prioritization of non-target substances in wastewater [83].

Still, one of the most relevant challenges within the analysis is the influence of matrix effects on the peak intensity, particularly in complex water matrices such as wastewater [84]. These can influence the signal intensity of certain substances due to ion enhancement or suppression and thus can lead to challenges in the identification of substances [78, 84, 85]. Ion suppression / enhancement does not only depend on the matrix composition but also on the structure of the substances and can be influenced by many factors such as the matrix itself, other substances within a sample, present reagents in the mobile phase or materials released from the column or extraction techniques [8, 84]. The phenomena of ion suppression / enhancement can be counteracted by the change of the ion source as electrospray ionization seems to be more affected by ion suppression / enhancement than atmospheric pressure chemical ionization. Other countermeasures are the extraction or purification of the sample, or the addition of certain concentrations of isotopic labeled (internal) standards to the samples [78, 84]. As these isotopic labeled (internal) standards have known retention times, which are similar to substances of interest, and known concentrations they are often used for corrections of the signal intensity influenced by the present matrix [78].

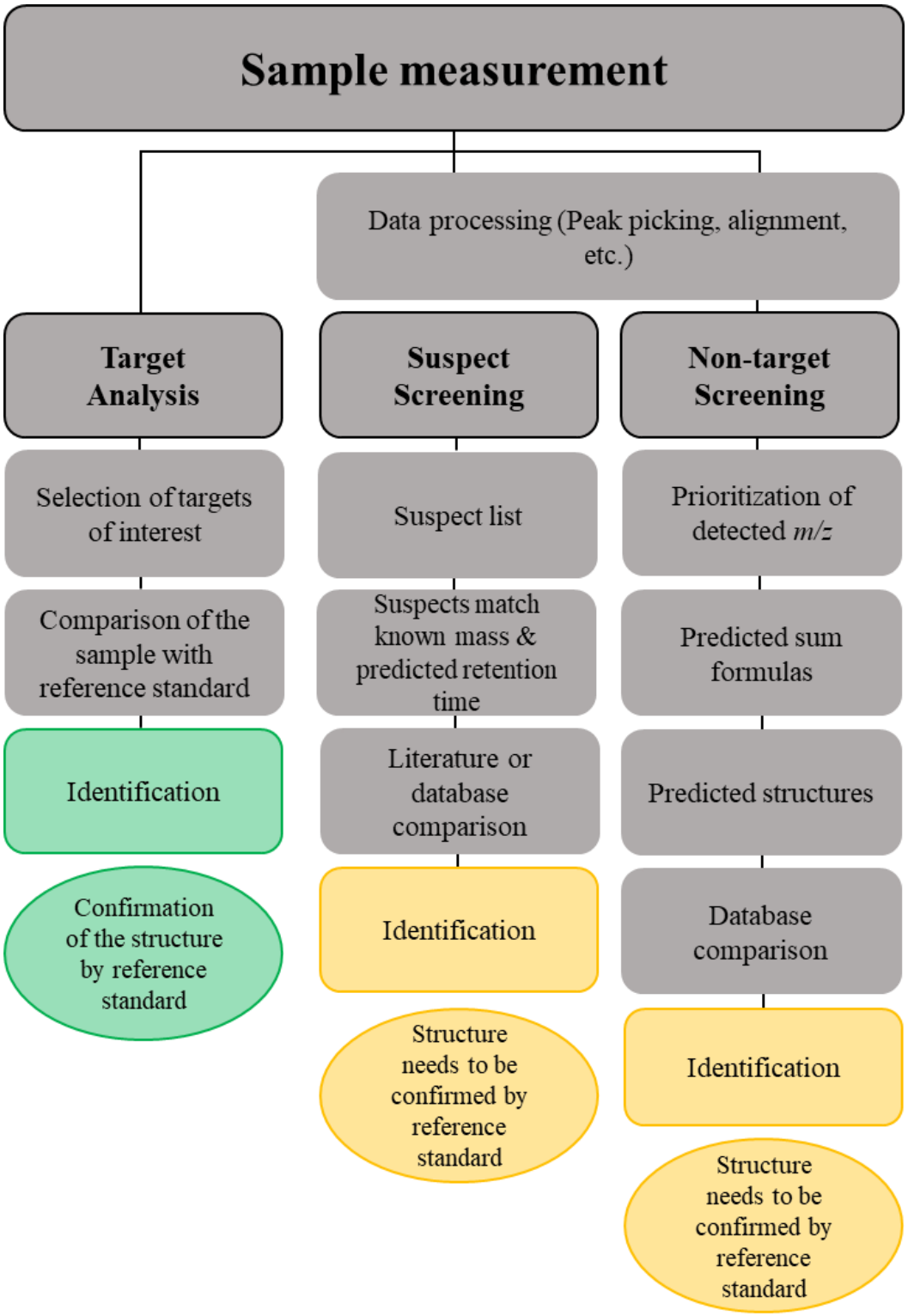

Figure 1-7 **Graphical scheme of target analysis (leading to an identification of the substance of interest, green), suspect and non-target screening (in both approaches the substance of interest needs to be confirmed, yellow) including all steps needed for identification** (based on Schymanski et al. [76] and Krauss et al. [77])

1.6 Ecotoxicological effects of micropollutants and transformation products

Many factors can influence the ecotoxicological effects of MPs and TPs formed during wastewater treatment. This can be the pH of the considered water but also different chemical structures present in the various number of MPs which are directly influencing the bioavailability and thus also the possible uptake in organisms [18]. Thus, MPs and TPs can induce a high variance of ecotoxicological effects. As a matter of fact, several studies already reported acute toxicities of MPs or TPs towards aquatic organisms [63, 86-89]. Nevertheless, as concentrations present in the aquatic environment may be too low to induce direct acute toxicity [90] it is more likely that mixtures of MPs and/or TPs lead to toxic effects [18, 90, 91] but these are mostly not considered. Further, possible chronic toxicity of certain MPs are mostly not taken into account [10]. One example considering the chronic effects is a study reporting high influence on the sexuality of fish downstream the discharge of WWTP effluents into rivers, presumably due to the presence of estrogenic substances in the effluents [92]. This assumption was further supported by another study also showing high effects of 17α-ethinylestradiol (used in birth-control pills) on the population of fathead minnow in a lake [14]. However, not only in the presence of estrogens but also pharmaceuticals chronic effects on aquatic organisms have been detected for concentrations found in the environment [15].

The evaluation of the toxicological potential of MPs and TPs towards organisms includes *in vivo* and *in vitro* assays. While *in vitro* assays include molecular and cell-based tests, *in vivo* assays determine endpoints such as immobility or mortality of aquatic organisms. *In vitro* assays are mostly faster and less expensive than *in vivo* assays. *In vivo* tests can give complimentary information on the influence of a chemical (or mixtures of chemicals) [90] such as the reproduction of a species, moving behavior or biomarkers like molecular responses and is not always leading to the death of a species [18, 90, 93]. Various aquatic organisms can be used for the toxicity assessment such as crustacea (e.g., water fleas such as *Daphnia* sp.) or fish (e.g., zebrafish) but also algae [18, 90]. Most tests to determine the aquatic toxicology of certain substances follow the guidelines provided either by the Organization for Economic Co-operation and Development (OECD) or International Organization for Standardization [18, 90]. In order to record and evaluate acute effects the effect concentration (EC) is a commonly used value. It states the concentration at which the tested individuals are expected to exhibit a certain effect. Mostly, an EC_{50} is given for the substance of interest, indicating that 50 % of the tested species showed an effect at this concentration. Ideally, the EC_{50} is calculated

from a concentration-response curve (Figure 1-8) which is obtained from a test setting including multiple concentrations in which the lowest tested concentration showed no effect while the highest concentration showed an effect of 100 %. In chronic tests concentrations inducing an effect are stated as the lowest observed effect concentration (LOEC) and highest no observed effect concentration (NOEC) [94, 95].

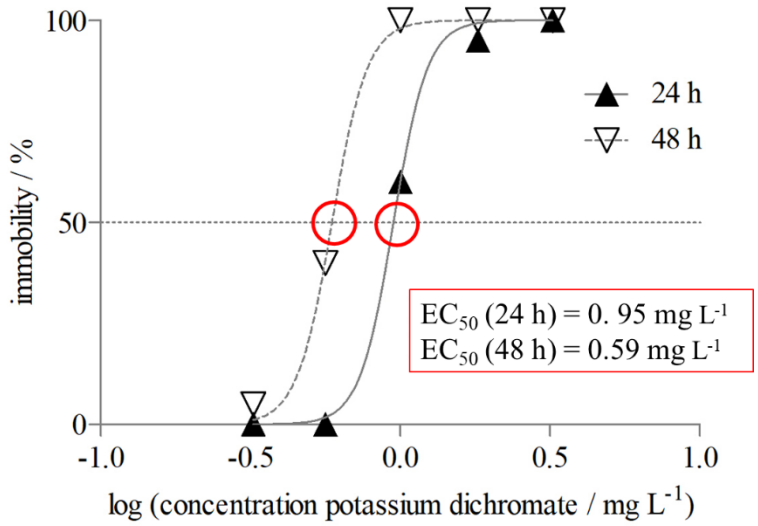

Figure 1-8 **Concentration-response curve of *Daphnia magna* towards potassium dichromate**
The effect concentration leading to 50 % death of *D. magna* (EC_{50}) is marked with red circles (after 24 h and 48 h) leading to EC_{50} (24 h) = 0.95 mg/L and EC_{50} (24 h) = 0.59 mg/L

Within possible ecotoxicity tests different endpoints are considered depending on the used organism, i.e., algae, fish or water flea (Table 1-1).

One of the most frequently applied acute or chronic toxicity tests in terms of aquatic ecotoxicology is determining the immobilization or changes in the reproduction of the water flea *Daphnia magna* (*D. magna)* [96]. The test conditions are established in the OECD Guideline 202 for the *Daphnia* sp. Acute Immobilization Test and in OECD Guideline 211 for the *Daphnia magna* Reproduction Test, respectively (Table 1-1) [94, 95].

Under ideal (laboratory) conditions female adult daphnids (approximately between three and twelve weeks old) reproduce in a parthenogenetic life cycle, leading to only female offspring with low genetic variations (Figure 1-9). The development of male daphnids is very rare under optimal laboratory conditions [97]. Male offspring only occur under stressful conditions (temperature change, food limitation etc.), leading to a sexual life cycle (not shown in Figure 1-9) [97]. Female daphnids are kept up to 12 weeks within the cycle to guarantee a steady culture. For ecotoxicological tests only daphnids younger than 24 h are used.

Table 1-1 Endpoints and test duration of different ecotoxicity tests (small overview)

Organism	Endpoint	Test duration	Guideline	Reference
Algae	Growth inhibition and biomass reduction	72 h	OECD 201	[90, 98]
Fish	Death	96 h (lethal effect concentration determined, considering the lethal concentration at which 50 % of the fish died)	OECD 203	[90, 99]
Water flea	Immobilization Reproduction	48 h 21 d	OECD 202 OECD 211	[94] [95]

Many studies already showed toxic effects on *D. magna* induced by chemicals, MPs or TPs present in the aquatic environment [19, 100-106]. Some studies reported TPs which were less biological active than the parent substances [71, 107, 108]. However, also TPs with similar or even higher biological activity or toxicity have been reported [71, 86, 88, 109, 110]. Further, even if a single substance is not of ecotoxicological interest, mixtures can have an unpredictable and increased toxicity [18, 91, 103, 111-113].

A MP very well investigated in terms of its toxicological potential is tamoxifen (TAM), a nonsteroidal selective estrogen receptor modulator, which has been detected in wastewaters [114] as well as surface waters [115]. Reported EC_{50} for TAM determined in acute tests with *D. magna* are 1530 µg / L after 24 h [116] and 210 µg / L after 48 h [117]. In addition to the acute toxicity the chronic toxicity (population growth inhibition) of TAM was investigated with *Ceriodaphnia dubia* leading to an EC_{50} of 0.81 µg / L (after 7 d) [116]. These values demonstrate that even if the acute toxicity tests show EC_{50} values far above concentrations normally found in the environment, the substances can still be of toxicological concern for aquatic organisms if long time exposures are considered. However, not only TAM but also formed TPs during ozonation were investigated in terms of their toxicological potential towards aquatic organisms. While for *D. magna* no toxicological potential was detected, in green algae tests a growth inhibition was detected for TPs of TAM formed during ozonation [63]. This indicates that even if one aquatic species is not affected by the substances, it still can have a toxicological potential for other organisms.

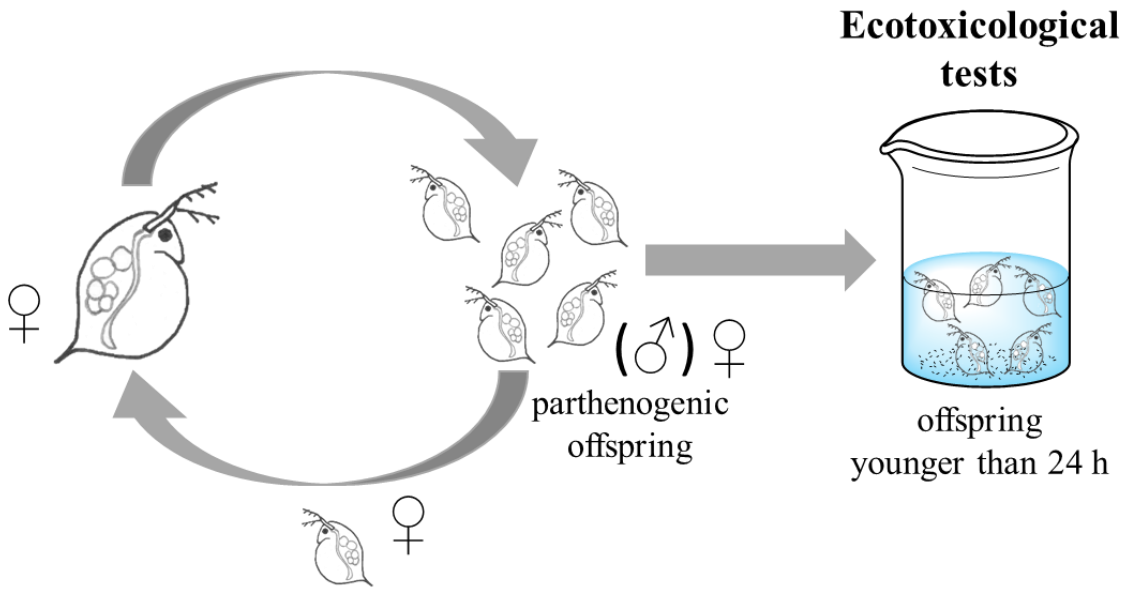

Figure 1-9 **Parthenogenetic life cycle of *Daphnia magna* under laboratory conditions** (*Daphnia magna* Figure: ©Louisa E. Rothe)

However, only few reports are available in literature considering the influence of MPs (or TPs formed during degradation) on various aquatic species within one study [56, 118]. Due to the high number of MPs present in the aquatic systems and the limitation in measurement (e.g., unknown substances or concentrations below the detection limit) full monitoring of all single MPs and their TPs is not feasible [119]. Nevertheless, one study already started investigations of the long-term influence of ozonated WWTPs on macrozoobenthos [120]. However, there is still a big gap between the mechanistic investigation of TP formation during ozonation and the corresponding estimation of the ecotoxicological potential of those TPs. Indeed, the influence of matrix effects during the implementation of ozonation have not been thoroughly studied yet and the combination of these studies with the determination of ecotoxicological relevance is to the author's best knowledge yet not addressed in literature. However, the knowledge about reactions taking place during ozonation and the possible interactions of reactive intermediates with matrix components is of high interest to minimize the formation of unwanted and possibly toxic TPs. Therefore, studies on the influence of matrix components during ozonation are highly needed to estimate possible changes within reaction pathways and for a better understanding on the formation of TPs.

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Chapter 2

Aims & Scope

The overall scope of the thesis was to investigate the influence of matrix components on the reaction of nitrogen-containing substances during ozonation and to evaluate the ecotoxicological relevance of formed transformation products (TPs). This thesis focused on (i) the reaction of simply structured nitrogen-containing substances with ozone, (ii) the influence of matrix components on the reaction and formation of TPs during ozonation of already well investigated substances (diclofenac, metoprolol and isoproturon) and (iii) the ecotoxicological relevance of these TPs. The work was divided into four chapters to achieve these aims as stated below and shown in Figure 2-1.

First, it was essential to investigate the reactions of nitrogen-containing substances in detail. Therefore, **Chapter 3** focuses on the degradation and kinetic constants of simply structured substances (2,2,6,6-tetramethylpiperidine and *cis*-2,6-dimethylpiperidine) during ozonation as a function of the pH. This chapter also aims on the investigation of a postulated ozone consuming chain reaction. Indeed, also the analysis of the reactions and formed TPs during ozonation of nitrogen-containing substances can be challenging and therefore, challenges and observations made during the analysis of 2,2,6,6-tetramethylpiperidineoxyl and 2,2,6,6-tetramethylpiperidine-1-ol will also be discussed within this chapter.

As the reaction of nitrogen-containing substances have been reported to be very complex and hardly predictable, especially in the presence of matrix components, **Chapter 4** aims to identify and quantify TPs formed during ozonation of diclofenac, metoprolol and isoproturon in the presence of matrix components, scavengers and real water matrices. This chapter also focuses on the identification of TPs formed in a wastewater treatment plant applying ozonation.

Additionally, in **Chapter 5** the focus will be on the influence of bromide, which is frequently found in water matrices, during the ozonation. As the presence of bromide can lead to the secondary oxidant hypobromous acid (HOBr) also the formation of unknown TPs directly induced by bromide or via the formed HOBr is possible and needs to be investigated.

Since TPs formed during ozonation can potentially adversely affect the aquatic environment, **Chapter 6** focuses on the acute toxicity towards the model aquatic organism *Daphnia magna* after ozonation of the three parent substances in presence of matrix components, scavengers and real water matrices. This chapter also aims to identify the acute toxicity of wastewater effluent including an ozonation step.

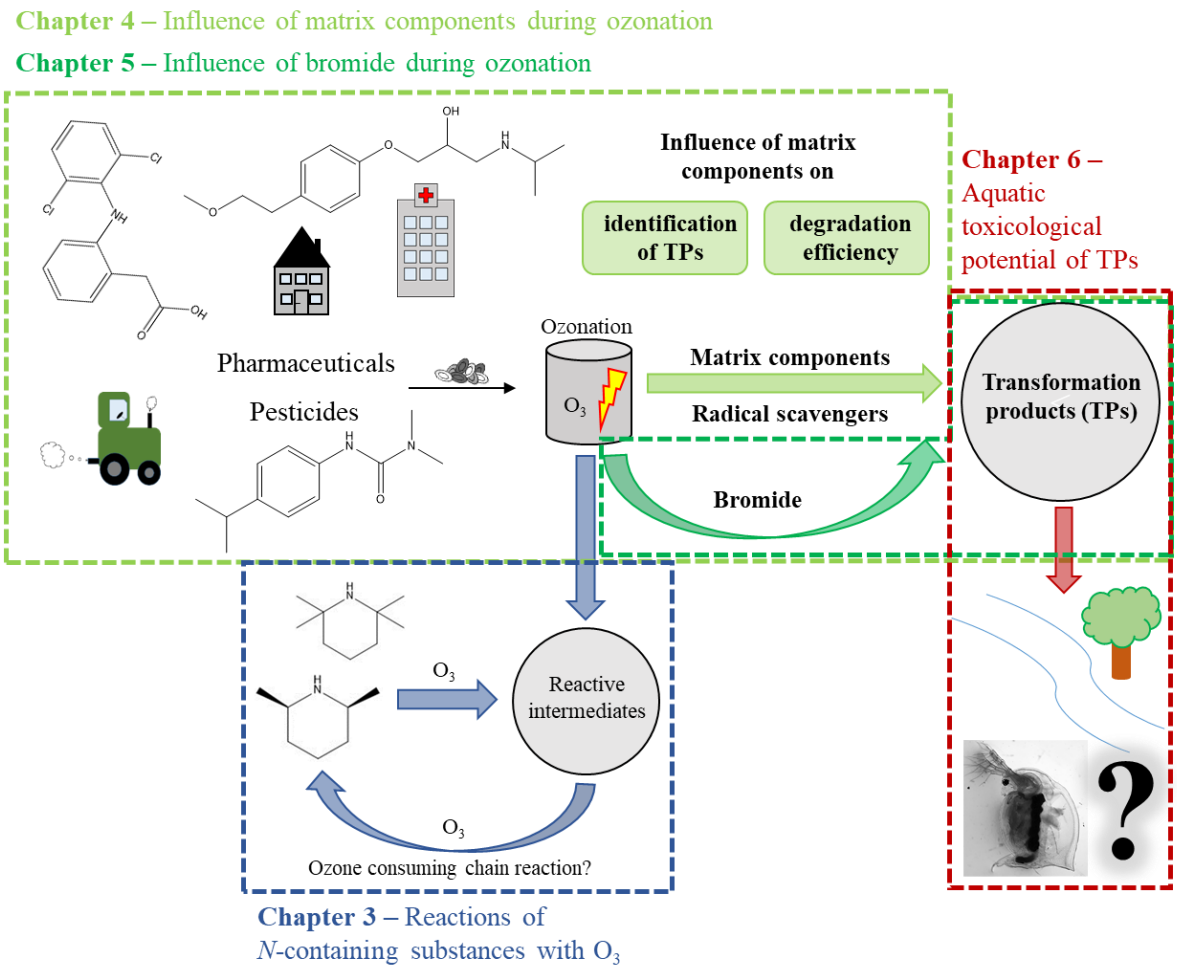

Figure 2-1 **Schematic presentation of the aims of this work and the relation of the individual chapters**

Chapter 3

Ozonation of *N*-containing substances: kinetics, stoichiometry and challenges within the analysis

3.1 Abstract

Nitrogen is found in various micropollutants present in the aquatic environment such as pharmaceuticals. Therefore, substances including nitrogen are of high interested in terms of water treatment. Most of these substances can be degraded by ozone such as the pharmaceuticals diclofenac and tamoxifen. However, the reactions of these substances are mostly still unknown as nitrogen centered radicals (aminyl radicals) might be formed which can highly influence the reaction with ozone.

To investigate the reaction pathways of nitrogen containing substances with ozone rather simple model substances (2,2,6,6-tetramethylpiperidine (TMP) and *cis*-2,6-dimethylpiperidine (DMP)), which were expected to form aminyl radicals, were used and analyzed in terms of degradation and kinetic constants as a function of the pH. Additionally, the reaction of the nitroxide radical 2,2,6,6-tetramethylpiperidineoxyl (TEMPO) was investigated in terms of the possible direct reaction to an aminyl radical.

During the analysis of TEMPO with LC-MS unaccountable observations were made as an enrichment of the substance was detected. These were increasing over time leading to the fact that the degradation and reaction of TEMPO with ozone could not be investigated further as the repeatability of the experiments could not be ensured.

The results obtained in terms of degradation of TMP and DMP with ozone as a function of the pH showed, that at pH 2 almost no degradation could be observed for both substances as no reaction with ozone is taking place at this pH value. At pH 7 and pH 11 almost complete degradation was observed. The reaction rate constants of TMP have been determined to be $k = 32.6\ M^{-1}s^{-1}$ at pH 7 and $k = 1.1 \times 10^5\ M^{-1}s^{-1}$ at pH 11. However, high stoichiometries (9 : 1 for TMP at both pH values; for DMP 5 : 1 at pH 11 and 9 : 1 at pH 7) could be determined for both substances and both pH values. As it could be shown that a reaction with ozone is taking place this could indicate that the postulated ozone consuming chain reaction is also taking place in the reaction of DMP and TMP. This reaction is assumed to lead to the formation of aminyl radicals that in a chain reaction can yield back the parent compounds while consuming ozone leading to a high ozone consumption with simultaneously low substance degradation.

3.2 Introduction

Micropollutants (MPs), mostly nitrogen containing (*N*-containing) substances, containing aromatic rings or amino groups, include various categories such as pharmaceuticals, pesticides and many others [1]. These substances are frequently detected in wastewater and, as some are not fully biodegradable, also in surface water [2-4]. Additionally, pesticides can also reach surface waters due to heavy rainfalls [5]. To remove MPs from water systems ozonation is frequently implemented in drinking water purification but also in wastewater treatment. Additional aims of ozonation are to disinfect and remove color, taste and odor from the source water [1]. In detail, the reactions occurring during ozonation of *N*-containing substances are very complex and hardly predictable, as amines can react with ozone to oxygen centered (nitroxide) radicals ($^{\bullet}ONR_2$, Reaction 1-3, Figure 3-1) and nitrogen centered (aminyl) radicals ($^{\bullet}NR$, Reaction 2-5, Figure 3-1). However, it has been postulated that $^{\bullet}ONR_2$ can also react with ozone further to $^{\bullet}NR$ in an ozone consuming chain reaction (Reaction 4, Figure 3-1) [6-8]. This reaction was suggested because a high consumption of ozone in contrast to a low substance turnover was observed for diclofenac [7], piperidine [6] and aniline [8]. The mechanism of the postulated chain reaction has not yet been proven and an experimental evidence for the formation of $^{\bullet}NR$ is still lacking.

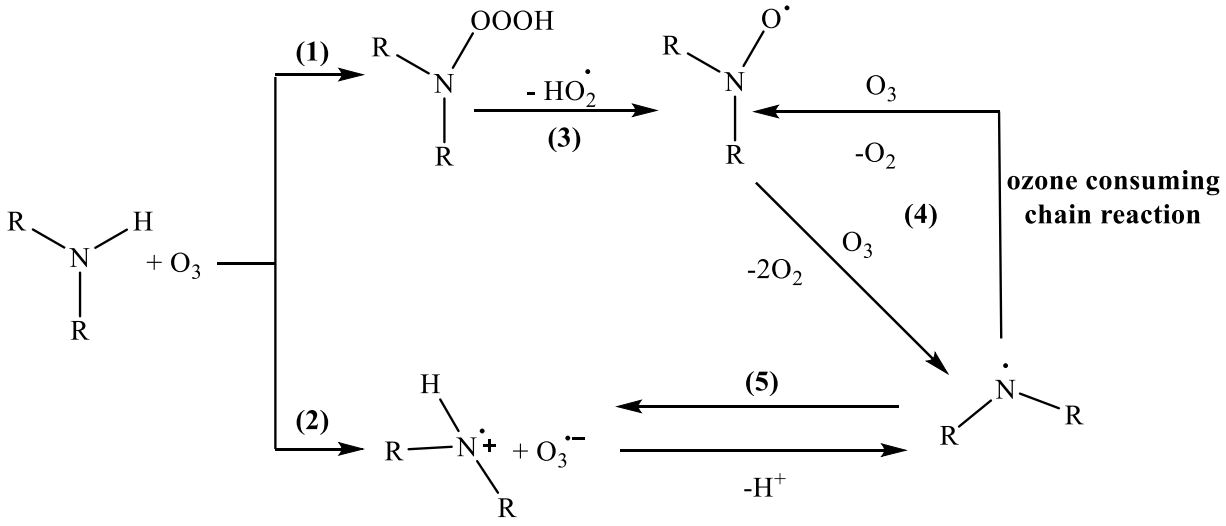

Figure 3-1 Possible reactions during ozonation of primary, secondary (I), and tertiary amines (II) including a postulated ozone consuming chain reaction for primary and secondary amines

(modified after von Sonntag and von Gunten [1] and Tekle-Röttering et al. [8])

Piperidine and piperidine similar structures, such as 2,2,6,6-tetramethylpiperidine (TMP), *cis*-2,6-dimethylpiperidine (DMP), 2,2,6,6-tetramethylpiperidineoxyl (TEMPO) and 2,2,6,6-tetramethylpiperidine-1-ol (TEMPOL), are archetypes for MPs because these structures are for example found in pharmaceuticals (e. g. ritalin, ciprofloxacin) [6]. Further, these substances are expected to form aminyl radicals and similar structures have already been investigated very well. Reactions of piperidine with ozone have been studied in detail by Tekle-Röttering et al. [6] leading to a reaction rate constant of $k = 2.4\ (\pm 0.95) \times 10^5\ M^{-1}s^{-1}$ in non-scavenged and $k = 1.4\ (\pm 0.55) \times 10^5\ M^{-1}s^{-1}$ in scavenged systems using tertiary butanol (*tert*-BuOH) or dimethyl sulfoxide (DMSO) as $^{\bullet}OH$ scavenger (both systems without pH adjustment). Additionally, a high stoichiometry for the reaction with ozone in non-scavenged (7 : 1) and scavenged (5 : 1) systems was reported, leading to the postulate of an ozone consuming chain reaction. However, the studies by Tekle-Röttering et al. [6, 8] underlined that the reactions of ozone with *N*-containing substances are hardly predictable. Thus, more research is needed to investigate possible reaction pathways which might be transferable to MPs relevant in aquatic systems.

As radicals seem to play an important role in the reaction of *N*-containing substances with ozone, in this study also TEMPO was investigated which is a commercially available nitroxide radical having a similar structure as piperidine and a modelled kinetic rate constant of $k = 10^7\ M^{-1}s^{-1}$ [9]. Being a $^{\bullet}ONR_2$, the reaction of TEMPO with ozone should directly lead to the postulated $^{\bullet}NR$ [1].

In this study four model substances (TMP, DMP, TEMPO and TEMPOL) were chosen to investigate and compare the reaction of *N*-containing substances with ozone as a function of the pH.

3.3 Materials & Methods

3.3.1 Chemicals & Equipment

All chemicals and solvents used are listed in Table 3-1. These were used as received from the according supplier. In Table 3-2 the used equipment is summarized.

Table 3-1 List of chemicals and solvents used

Chemical / Solvent	Manufacturer
2,2,6,6-tetramethyl-1-piperidineyloxyl 98 % (TEMPO)	Alfa Aesar GmbH & Co KG
2,2,6,6-tetramethylpiperidine-1-ol 95 % (TEMPOL)	Combi-Blocks
2,2,6,6-tetramethylpiperidine (TMP) ≥99 %	Sigma-Aldrich
cis-2,6-dimethylpiperidine (DMP) 98 %	Sigma-Aldrich
Dipotassium hydrogen phosphate 99 %	AppliChem GmbH
Formic acid 98 – 100 %	Merck
Methanol 100 %	VWR Chemicals
Phosphoric acid 85 %	AppliChem GmbH
Potassiumindigotrisulfonate	Sigma-Aldrich
Sodium dihydrogen phosphate > 99 %	AppliChem
Sulfuric acid >95 %	Fisher Chemical
Ultrapure water Purelab Ultra	ELGA (Celle, Deutschland)

Table 3-2 List of equipment used

Technical device	Manufacturer
HPLC-MS	
HPLC 1100 Series - G1312A Bin Pump - G1313A ALS Autosampler - G1316A Colcom Degasser 1260 Infinity	Agilent / Hewlett Packard
Mass spectrometer 6120 Quadrupole	Agilent Technologies
HPLC-column	Phenomenex
Core shell LC column Kinetex 5 µm EVO C18 100Å	
Ozone generator COM-AD-01	Anseros
Ozone generator Philaqua 802x	BMT Messtechnik
pH meter 827 pH Lab	Metrohm
UV-Vis Spectrometer UV-1650PC or UV-1800	Shimadzu

3.3.2 Generation of stock solutions

Stock solutions of the model substances (0.5 mM) were prepared in ultrapure water at three different pH values (pH 2, pH 7 and pH 11) which were adjusted with phosphate buffer (10 mM) and phosphoric acid if needed. For each experiment, an individual calibration was prepared ranging from 0.005 – 0.05 or 0.06 mM.

Ozone stock solution was prepared by enriching oxygen with an ozone generator (COM-AD-01, Anseros or Philaqua 802x, BMT Messtechnik) and bubbling into ice-cooled ultrapure water for at least 60 minutes. The UV absorption of the diluted ozone stock solution (0.5 mL ozone stock solution and 2.5 mL ultrapure water or 1 mL ozone stock solution and

2 mL ultrapure water) was measured at 258 nm, $\varepsilon_{O3} = 2950\ M^{-1}cm^{-1}$ [10] with an UV-spectrometer (UV-1650PC or UV-1800, Shimadzu) to determine the ozone concentration.

3.3.3 Reaction mechanisms of *N*-containing substances with ozone: degradation

Sample preparation

To determine the degradation of TEMPO, TMP, DMP and TEMPOL as a function of pH ozonation was performed in ultrapure water (pH 2, pH 7 and pH 11). The concentration of the reacting substance was 0.05 mM and the added ozone concentration ranged between 0 – 0.5 mM.

All experiments were performed in separate runs and samples measured via liquid chromatography mass spectrometry (LC-MS) to determine the degradation of the substances.

Sample measurement

All samples were measured by LC-MS (Agilent, HPLC 1100 Series and 6120 Quadrupole) and chromatographic separation was performed using a Kinetex C18 EVO, 5 µm, 100 x 3 mm column (Phenomenex). The column oven was set to 30 °C and as eluents water + 0.1 % formic acid and methanol (MeOH) + 0.1 % formic acid were used. Table 3-3 outlines the eluent composition, the flow rate and the measurement time for all four substances.

Table 3-3 Setting for the measurement of the four substances with HPLC-MS

Substance	Eluent composition (H_2O/MeOH)	Flow rate [mL/min]	Measurement time [min]
TMP	95/5	0.4	10
DMP	95/5	0.2	10
TEMPO	85/15	0.4	10
TEMPOL	95/5	0.4	10

Measurements were performed in positive ionization mode using scan and single-ion mode (SIM) detecting the corresponding *m/z* values of the substances (Table 3-4).

Table 3-4 Considered *m/z* values in the single-ion mode measurements with LC-MS for the measured substances

Substance	Measured *m/z*
TMP	139, 141, 142, 143
DMP	112, 113, 114, 115
TEMPO	156, 157, 158, 159, 160
TEMPOL	157, 158, 159
Piperidine	84, 85, 86, 87, 88

3.3.4 Reaction mechanisms of *N*-containing substances with ozone: kinetics

Sample preparation

To determine the reaction rate constant of TMP with ozone competition kinetics were applied with piperidine as competitor. TMP and piperidine were provided (0.05 mM each), aliquoted to a number of vials and eleven different ozone dosages (0 mM – 0.5 mM) were added. The experiments were performed at pH 2, pH 7 and pH 11 (10 mM phosphate buffer each) in the absence of a hydroxyl radical ($^{\bullet}$OH) scavenger. A calibration of both substances was performed and all measurements were done with LC-MS in duplicates or triplicates.

Sample measurement

All samples were acidified with 1 % formic acid prior to the measurements performed with a LC-MS (Agilent). A Kinetex C18 EVO, 5 µm, 100 x 3 mm column (Phenomenex) was used for chromatographic separation (column temperature 30 °C). An isocratic flow (0.4 mL / min) was used with 95 % water (acidified with 0.1 % formic acid) and 5 % methanol (MeOH, acidified with 0.1 % formic acid). The injection volume was 10 µL.

Positive ionization mode was used for all measurements which were performed in scan (70 – 170 *m/z*) and SIM detecting the corresponding *m/z* values of the substances (Table 3-4).

3.3.5 Ozone consumption over time in the absence and presence of *N*-containing substances

Sample preparation

Ozone consumption int the presence and absence of TMP and DMP was determined at pH 2 and pH 7. Therefore, indigotrisulfonate solution (10 mM) was prepared and provided in test tubes. Phosphoric acid (160 mM) was added to keep the pH stable below 4 during the reaction with ozone. Simultaneously, a reaction solution containing 0.05 mM of the substance of interest was prepared and the pH was adjusted with phosphate buffer (10 mM). The reaction solution was then placed onto a stirring plate and 0.5 mM ozone was added. 10 seconds after the ozone addition the stirring plate was stopped. Starting with 20 to 90 seconds after ozone dosage, 10 mL of the reaction solution were given to the test tubes with indigotrisulfonate solution in elongated time frames (starting with 30 seconds time frames (up to 150 seconds) and ending with an one-hour time frame). The solution was mixed once before measurement with an UV-spectrometer. As refence two samples of the solution (ultrapure water or ultrapure water + one of the two substances) were added to the indigo solution prior to the ozone addition. Additionally, also reference experiments without the addition of a model substance were performed to determine the ozone consumption in ultrapure water.

Sample measurement

All samples were measured with an UV-spectrometer (UV-1650PC or UV-1800, Shimadzu) at 600 nm and the remaining ozone concentration was determined. As indigotrisulfonate and ozone react in a stoichiometry of 1:1, the remaining ozone concentration within the samples can be calculated by the difference of the indigotrisulfonate concentration in the references and the samples (ε = 20000 $M^{-1}cm^{-1}$). The reaction product of indigotrisulfonate and ozone is isatin sulfnic acid which does not absorb at 600 nm (ε = 0 $M^{-1}cm^{-1}$, Figure 3-2) [11].

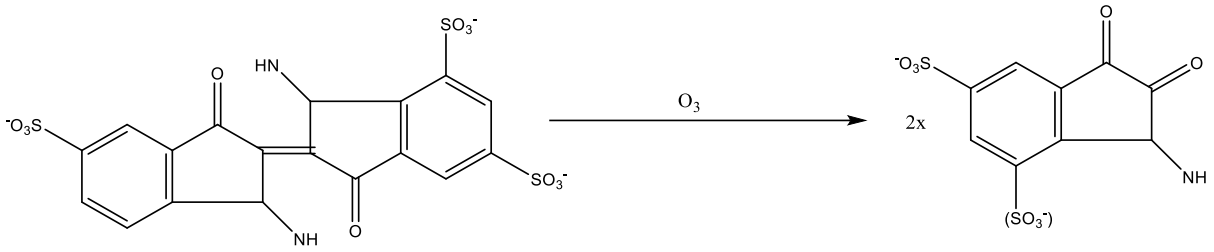

Figure 3-2 Reaction of indigotrisulfonate with ozone to isatin sufionic acid

(adapted and modified after Bader and Hoigné [11])

3.3.6 Analysis of the nitroxide radical 2,2,6,6-tetramehtylpiperidine-1-oxyl

Sample preparation

During the degradation measurement of TEMPO using the method listed in Table 3-5, some unexpected observations were made. Therefore, a troubleshooting using several measurement approaches was performed to investigate the reasons leading to these observations.

For the troubleshooting a calibration ranging from 0.005 – 0.05 mM TEMPO was prepared in ultrapure water and measured with LC-MS.

Sample measurement

All samples were measured with LC-MS (Agilent 1100 series) using a Kinetex C18 column (C18 EVO, 5 µm, 100 x 3 mm, 100 Å, Phenomenex) with a column temperature of 30 °C. TEMPO was measured with several methods shown in Table 3-5. The flow rate was always 0.4 mL / min but the gradient composition was changed to achieve better separation and peak shape. The eluents and starting parameters were chosen based on Pennington et al. [12]. All eluents contained 0.1 % of formic acid and the measurements were performed in positive mode.

These measurements were first performed at pH 2, pH 7 and pH 11. However, for all pH values increasing peak areas were detected within the measurements (data not shown). Therefore, a troubleshooting was performed using "Method 3" (Table 3-5), as the best resolution was achieved. In the troubleshooting several measurements (in total six) at pH 2 of the same samples were performed. To reduce the possibility of carry over, a washing step was

performed overnight containing 80 % methanol. Further, between the individual measurements of the calibration a washing method with 35 % methanol was added.

Table 3-5 Methods used in the troubleshooting to analyze 2,2,6,6-tetramehtylpiperidine-1-oxyl via LC-MS

	Substance	**Injection-volume[µL]**	**Column-temperature [°C]**	**Gradient-composition [H_2O %/MeOH %]**	**Total measurement time [min]**
Method 1	TEMPO	10	25	95/5	20
Method 2	TEMPO	10	25	0-4min: 95/5 4-12min: 80/20 12-20min: 95/5	20
Method 3	TEMPO	10	25	0-4min: 85/15 4-12min: 65/35 12-20min: 85/25	20
Method 4	TEMPO	10	30	85/15	10

3.4 Results & Discussion

3.4.1 Difficulties in the analysis of 2,2,6,6-tetramethyl-1-piperidineyloxyl

TEMPO has been reported to be a stable $^{\bullet}ONR_2$, which can be measured with LC-MS [12, 13] and is often used as electron spin resonance standard [1]. Further, TEMPO has been reported to react fast with ozone leading to the formation of $^{\bullet}NR$ (Figure 3-3) [1, 13] and has been assumed to be a good example for the closer investigation of the postulated ozone consuming chain reaction. However, in this study several unexpected observations were made within the measurements of TEMPO with LC-MS.

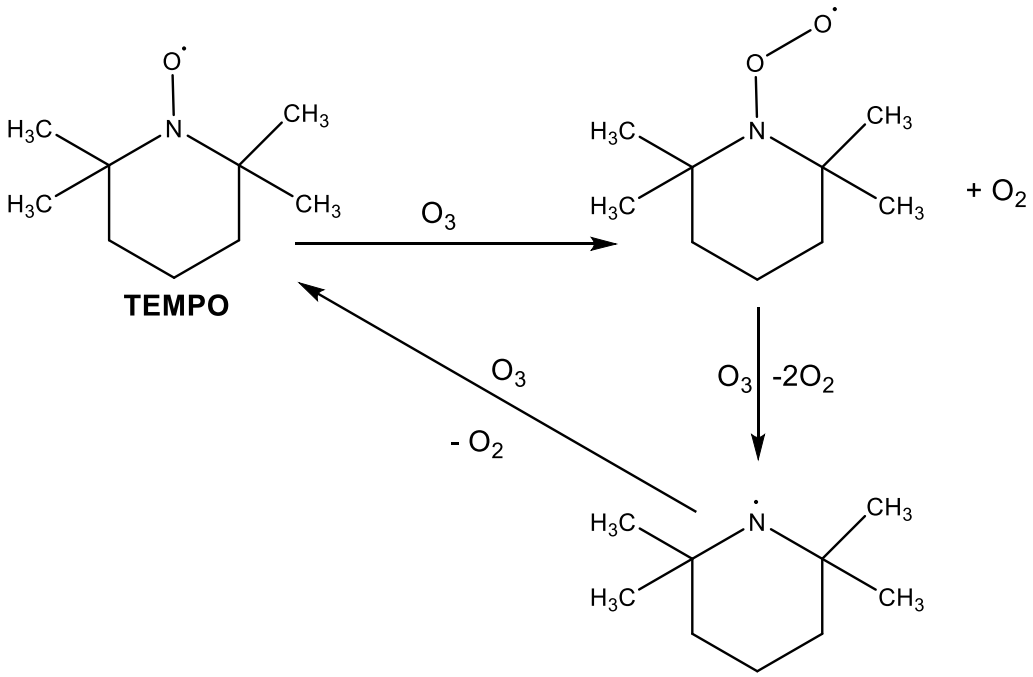

Figure 3-3 Reaction of the stable nitroxide radical 2,2,6,6-tetramethylpiperidine-1-oxyl to an aminyl radical in the reaction with ozone
(modified after von Sonntag and von Gunten [1] and Naumov and von Sonntag [13])

Increasing peak areas were detected after repeating measurements for each calibration (pH 2, pH 7 and pH 11), raising with each measurement (data not shown). Therefore, an extended washing step containing 80 % methanol was run overnight before starting the troubleshooting. However, also the six-time measurements (pH 2) including a rinsing step with 35 % methanol between the individual measurements showed continuous increasing peak areas with each measurement always taken from the same vials (Figure 3-4). First, it was assumed that this is due to a carryover of TEMPO between the measurements, but even if in the chromatograms of the washing step small TEMPO peaks were detected (data not shown), no

changes within the retention time (Figure 3-5) were determined. Therefore, a carryover can be excluded to be the reason for the increasing peak areas. Another possible explanation is that over time TEMPO is reacting to TEMPOL within the sample, leading to increasing peak areas. Further, another study considering different trouble-shooting approaches in the bioanalysis of plasma samples with LC-MS/MS also reported gradually increasing peak areas [14]. They suggested that this problem might be solved via mixing of the samples right before the measurement [14]. As all samples in this study were mixed before being placed into the autosampler and therefore had similar resting times it is not clear if the additional mixing of the samples prior to measurement would lead to comparable peak areas in the measurement of TEMPO.

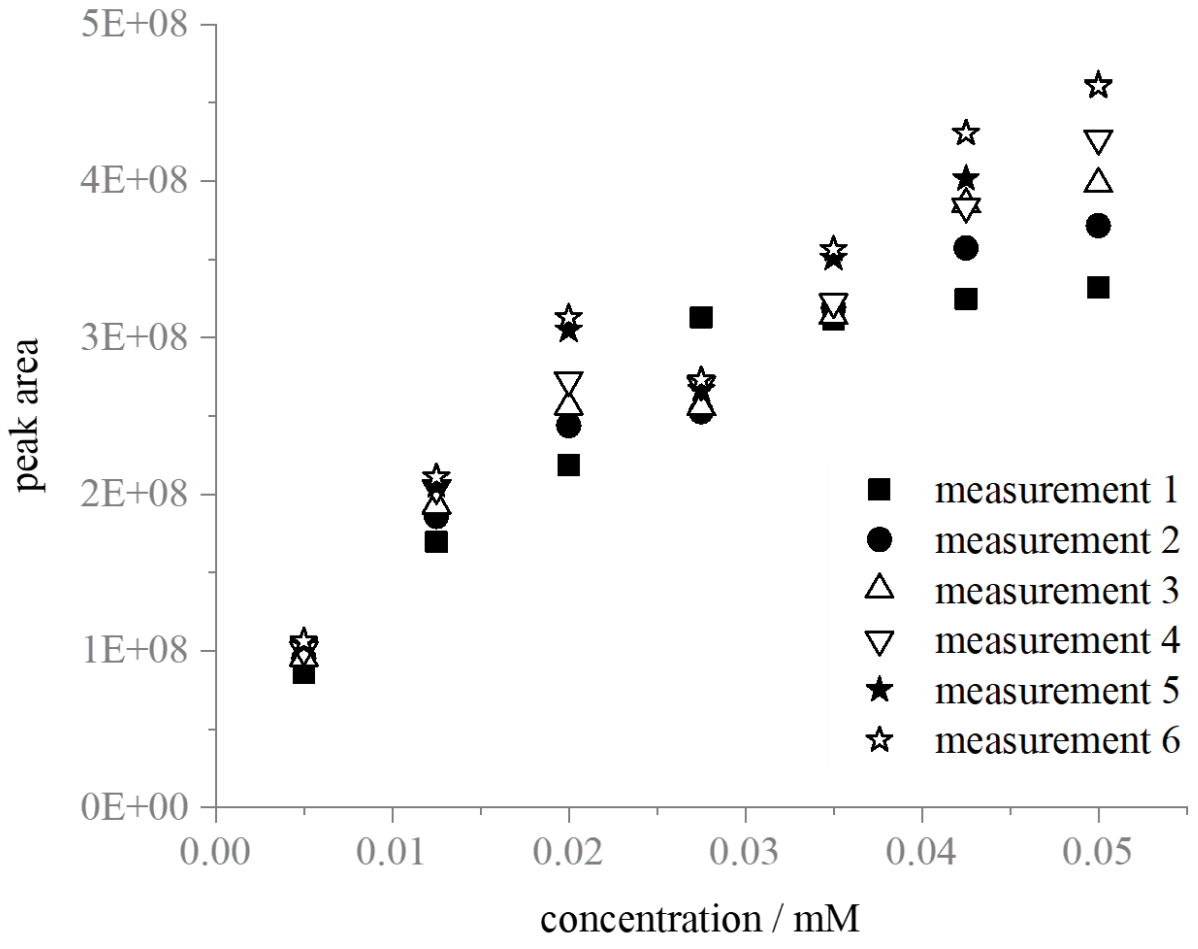

Figure 3-4 **Six-time measurement of 2,2,6,6-tetramethylpiperidine-1-oxyl calibration at pH 2**
The calibration was measured six-time measurements from the same vials. Between the individual measurements a rinsing step with 35 % methanol was included. The injection volume was set to 10 µL and the column temperature to 25 °C. Total measurement time was 20 minutes with a gradient as follows; 0-4min: 85/15 H_2O %/MeOH %, 4-12min: 65/35 H_2O %/MeOH %, 12-20min: 85/25 H_2O %/MeOH %.

However, there are several possibilities which can lead to the continuous increasing peak areas and the fact that the measurements of TEMPO were not reproducible. To overcome this problem and to explain these unexpected observations the addition of an internal standard should be considered when investigating the measurement of TEMPO with LC-MS further. An internal standard, which can be measured with LC-MS and for which a retention time is known, would point out if the observations made for TEMPO are substance or instrument related. If it is proven that the increasing peak areas are only detected in measurements of TEMPO further investigations are needed to overcome this problem.

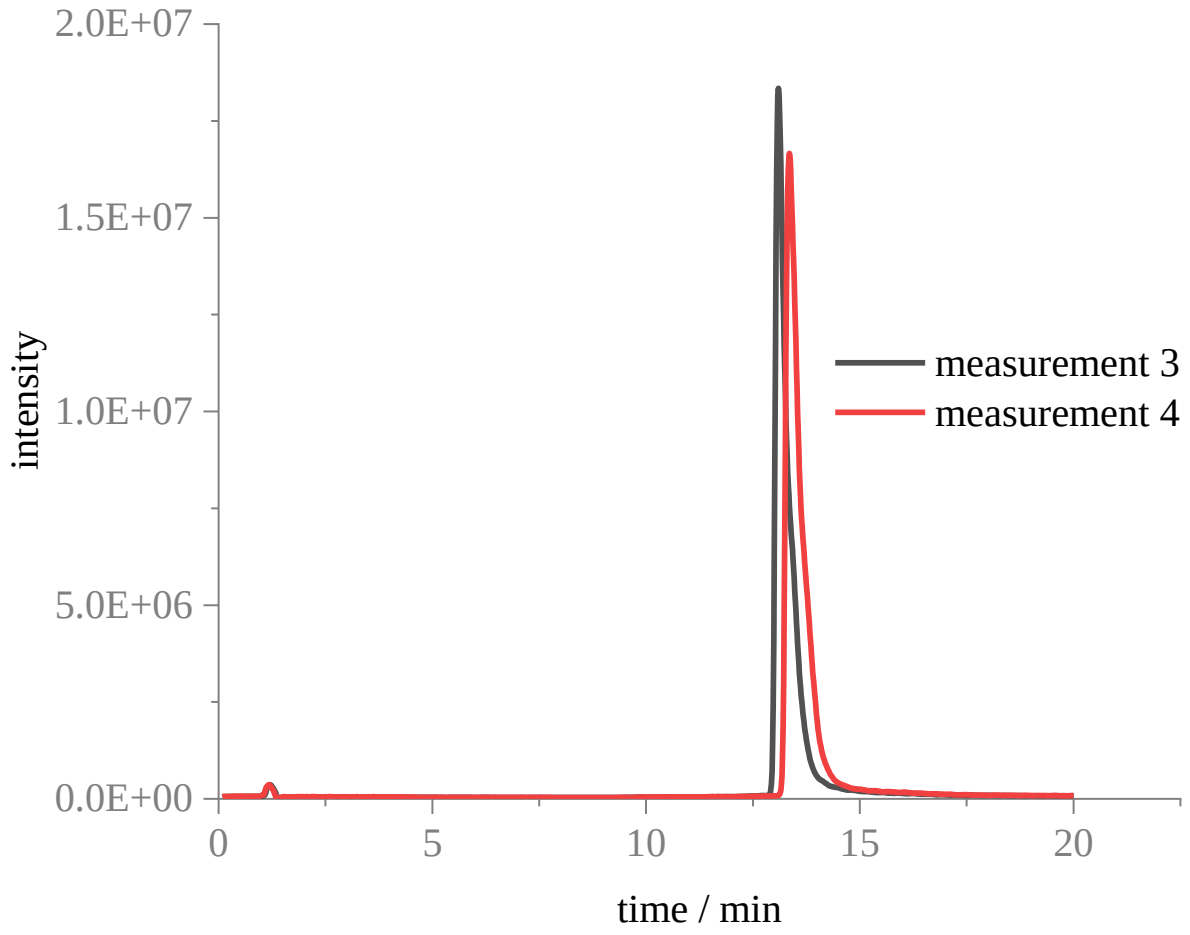

Figure 3-5 **Chromatogram of 0.05 mM 2,2,6,6-tetramethylpiperidine-1-oxyl calibration measurements three and four at pH 2**

The calibration was measured six-time measurements from the same vials. Between the individual measurements a rinsing step with 35 % methanol was included. Here the third and fourth measurement of the highest concentration (0.05 mM) are shown. The used gradient was; 0-4min: 85/15 H_2O %/MeOH %, 4-12min: 65/35 H_2O %/MeOH %, 12-20min: 85/25 H_2O %/MeOH %. Total measurement time was set to 20 minutes with an injection volume of 10 µL and a column temperature of 25 °C.

In addition to increasing peak areas within the measurements it was also observed that it is more likely that not TEMPO but rather TEMPOL was detected in the measurements with LC-MS. Pennington et al. [12] suggested that a disproportionation of TEMPO within the solution is leading to the formation of an oxidized and a reduced form during electrospray ionization (Figure 3-6).

The suggested reduced form of TEMPO shows the same structure as TEMPOL and thus also the same *m/z* (Figure 3-6). This leads to the assumption that not TEMPO was measured with LC-MS but rather TEMPOL (or the reduced form of TEMPO) as the detected peak showed a mass of *m/z* = 158. This was also reported in a previous study [12] which also detected the reduced form of TEMPO (or TEMPOL) at *m/z* = 158 in positive ionization mode. Due to these difficulties the ozone consuming chain reaction was not investigated further considering TEMPO or TEMPOL.

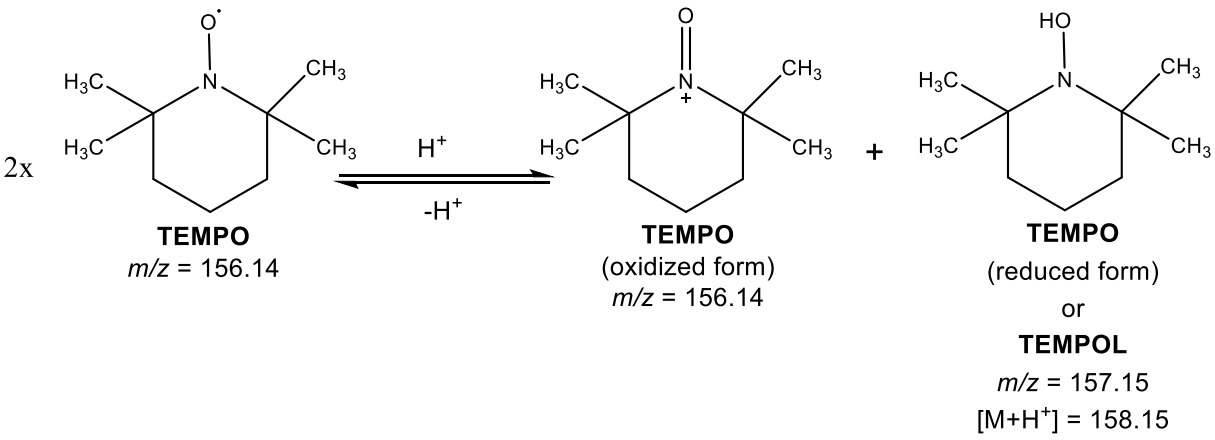

Figure 3-6 **Disproportionation of 2,2,6,6-tetramethylpiperidine-1-oxyl in solution and formation of an oxidized and a reduced form during electrospray ionization** (adapted and modified after Pennington et al. [12])

3.4.2 Ozonation of 2,2,6,6-tetramethylpiperidine and *cis*-2,6-dimethylpiperidine

Degradation of 2,2,6,6-tetramethylpiperidine and *cis*-2,6-dimethylpiperidine with ozone at different pH values

The degradation of TMP and DMP (structures shown in Figure 3-7) was strongly pH dependent.

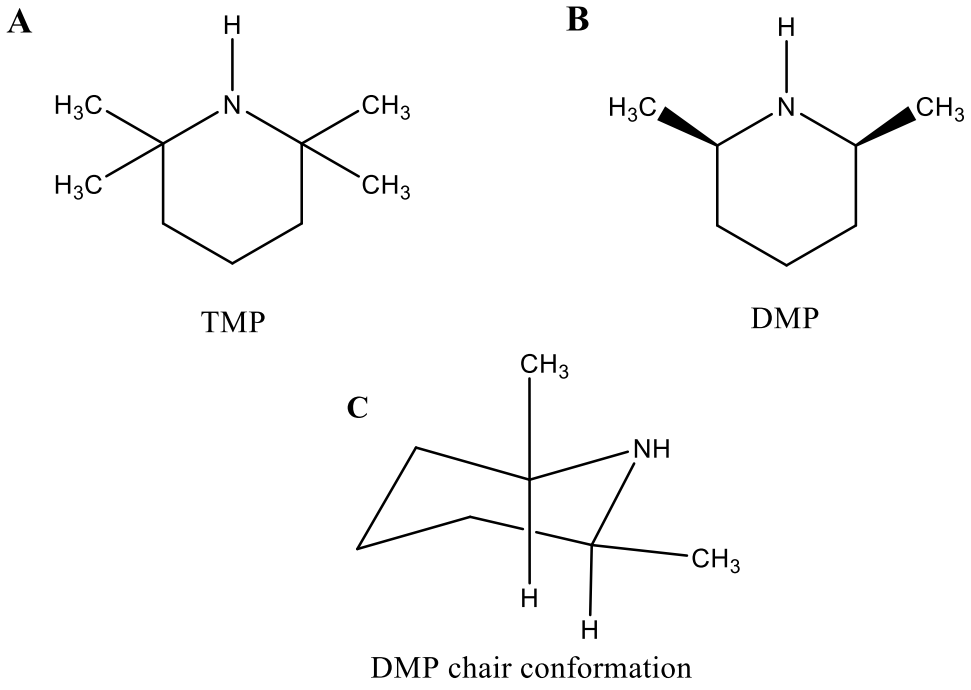

Figure 3-7 Structures of 2,2,6,6-tetramethylpiperidine (TMP, A), *cis*-2,6-dimethylpiperidine (DMP, B) and chair conformation of *cis*-2,6-dimethylpiperidine (DMP) including equatorial methyl groups (C) (chair conformation of DMP adapted and modified after Booth et al. [15])

While both substances were completely degraded after an ozone addition of 0.25 - 0.3 mM at pH 7 and pH 11 (starting substance concentration 0.05 mM), at pH 2 almost no degradation was observed (Figure 3-8). The measured stoichiometries were 9 : 1 (pH 7 and pH 11) mole ozone per degraded mole TMP, and 5 :1 (pH 11) and 9 : 1 (pH 7) mole ozone per degraded mole DMP.

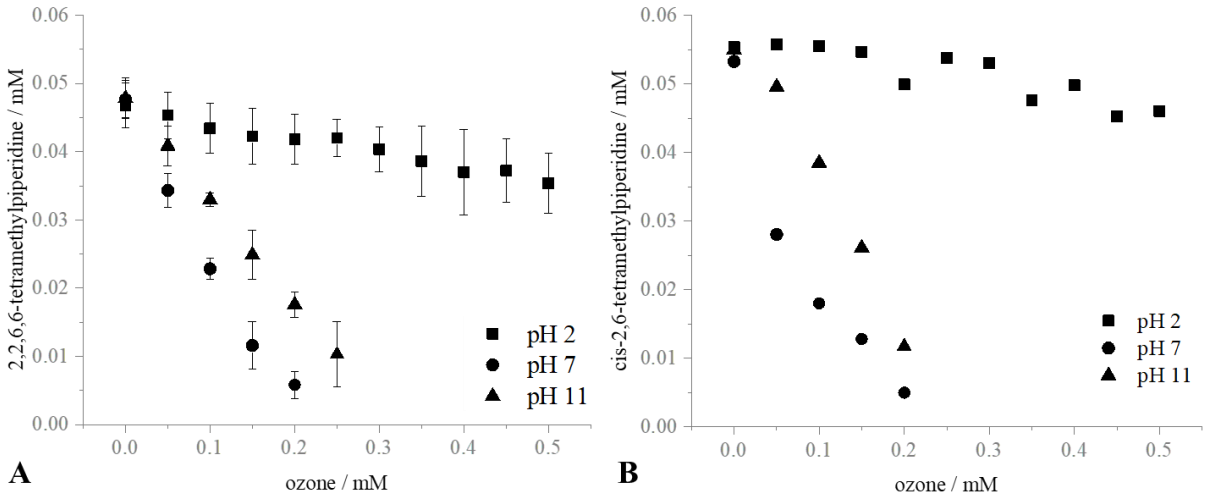

Figure 3-8 Degradation of 2,2,6,6-tetramethylpiperidine (A) and *cis*-2,6-dimethylpiperidine (B) with ozone at different pH values

The degradation of 2,2,6,6-tetramethylpiperidine and *cis*-2,6-dimethylpiperidine (0.05 mM each), respectively, with ozone (0 – 0.5 mM) was determined at different pH values (pH 2, pH 7 and pH 11) in the absence of a radical scavenger. Triplicate measurements were performed for 2,2,6,6-tetramethylpiperidine and single measurements were performed for *cis*-2,6-dimethylpiperidine.

As reported for piperidine (7 to 8 mole ozone to degrade 1 mole piperidine) [6], stoichiometries above one could indicate that an ozone consuming chain reaction is taking place in the reaction of TMP and DMP with ozone. This would explain the high ozone consumption with simultaneously low substance degradation due to the formation of $^{\bullet}$NR. However, the reactivity of *N*-containing substances with ozone can also be highly influenced by the present pH. As the nitrogen is protonated at low pH values leading to a lower reactivity towards the electrophilic ozone [1], the high stoichiometry at pH 2 can also be explained due to the low reactivity of TMP and DMP with ozone at this pH.

To verify if the high stoichiometries have been detected due to an ozone consuming chain reaction or due to the low reactivity of TMP and DMP with ozone, the ozone consumption over time as a function of the pH in the presence and absence of the substances was determined.

Ozone consumption at different pH values in the presence of 2,2,6,6-tetramethylpiperidine and *cis*-2,6-dimethylpiperidine

If TMP and DMP, respectively, are reacting with ozone, the determined ozone consumption over time in the presence of these two substances must be higher than the ozone consumption over time determined in the absence of the two substances. A similar ozone consumption in the two setups would lead to the assumption that ozone is not reacting with TMP and DMP, respectively. The ozone consumption over time was determined via the indigotrisulfonate method at pH 2 and pH 7 without the addition of a substance and in the presence of TMP and DMP, respectively (Figure 3-9).

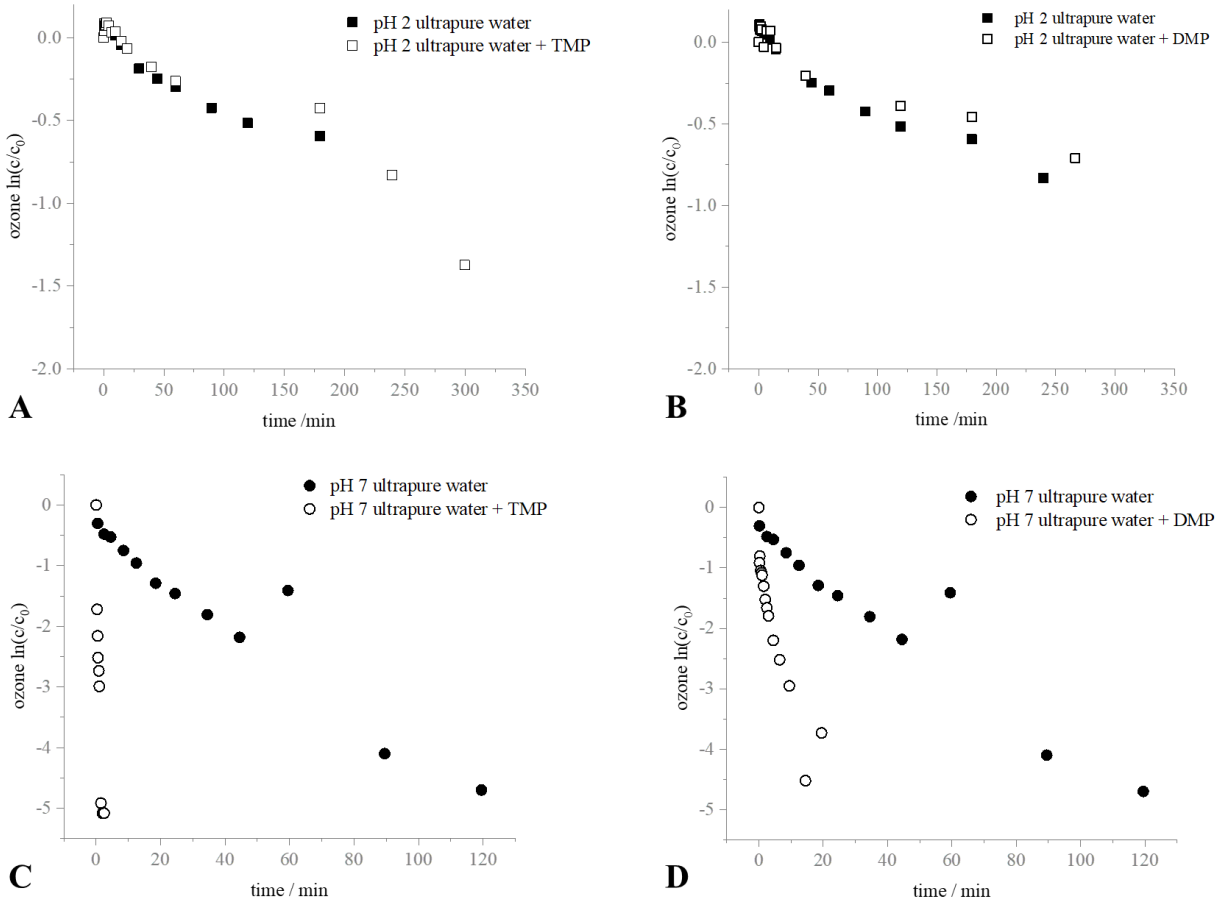

Figure 3-9 **Ozone consumption over time in the absence of a substance and in the presence of the two model substances 2,2,6,6-tetramethylpiperidine (TMP) and *cis*-2,6-dimethylpiperidine (DMP), respectively, at pH 2 (TMP A and DMP B) or pH 7 (TMP C and DMP D)**

The measured concentration is plotted as natural logarithm of the ratio of the ozone concentration at a specific time c to the initial ozone concentration c_0. All measurements have been performed as single measurements.

Unsurprisingly, ozone consumption at pH 7 was faster than at pH 2 (Figure 3-9). Only small differences could be detected between the ozone consumption in the absence or presence of the two model substances at pH 2 (Figure 3-9.A and Figure 3-9.B). Therefore, it can be concluded that TMP and DMP are not reacting with ozone at pH 2 and thus, it is also unlikely that an ozone consuming chain reaction is taking place at pH 2. However, at pH 7 different results were gained for both model substances (Figure 3-9.C and Figure 3-9.D). Differences within the ozone consumption at pH 7 could be observed after the addition of TMP and DMP, respectively, leading to a much faster ozone consumption than in ultrapure water without any substance. For TMP samples the consumption of ozone was even faster than in samples containing DMP.

These results underline the suggestion made before, that at pH 2 no reaction with the substances is taking place but at pH 7. This also supports the postulate of an ozone consuming chain reaction, as the two substances are obviously reacting with ozone at pH 7 but still high stoichiometries of 9 : 1 (mole ozone to degrade one mole substance) were detected.

3.4.3 Ozonation of 2,2,6,6-tetramethylpiperidine: kinetics

As the expected reaction rate constant of TMP was $k \geq 10^5\ M^{-1}s^{-1}$ due to reported rate constants for piperidine ($k = 2.4 \times 10^5\ M^{-1}s^{-1}$ [6] at pH 11) competition kinetics were performed. The reaction rate constant was determined using piperidine as competitor at pH 7 and pH 11 (initial concentration 0.05 mM (each) and added ozone concentrations 0.005 mM – 0.5 mM).

Known reaction rate constants for piperidine are $k = 36.2\ M^{-1}s^{-1}$at pH 7 [16] and $k = 2.4 \times 10^5\ M^{-1}s^{-1}$ at pH 11 [6]. Based on these values the reaction rate constants of TMP have been calculated leading to $k = 32.6\ M^{-1}s^{-1}$ at pH 7 (Figure 3-10.A) and $k = 1.1 \times 10^5\ M^{-1}s^{-1}$ at pH 11 (Figure 3-10.B). TMP is therefore reacting with almost the same kinetics as piperidine which was an expected result due to the similar structure and therefore similar reaction mechanism. The fast reaction at pH 11 of TMP (pKa = 11.07 [17]) was also expected due to the deprotonated species of the amines at this pH (pKa of piperidine = 11.1) [6]. As almost no data is available for TMP comparison of the results was hardly feasible. However, the obtained reaction rate constants were slightly lower as expected. The electron density of the nitrogen in TMP is higher than in piperidine, therefore also the reaction rate constant should be higher. Anyhow, this was not the case in this study, therefore it would be interesting to determine how the reaction rate constants of DMP with ozone differ from those detected for TMP and

piperidine, to investigate in more detail how the reaction rate constants are influenced. Nevertheless, the reaction rate constant observed for TMP is still higher than for aromatic *N*-containing substances which is in accordance with results reported for piperidine due to the fact that the nitrogen is less reactive in aromatic systems [6].

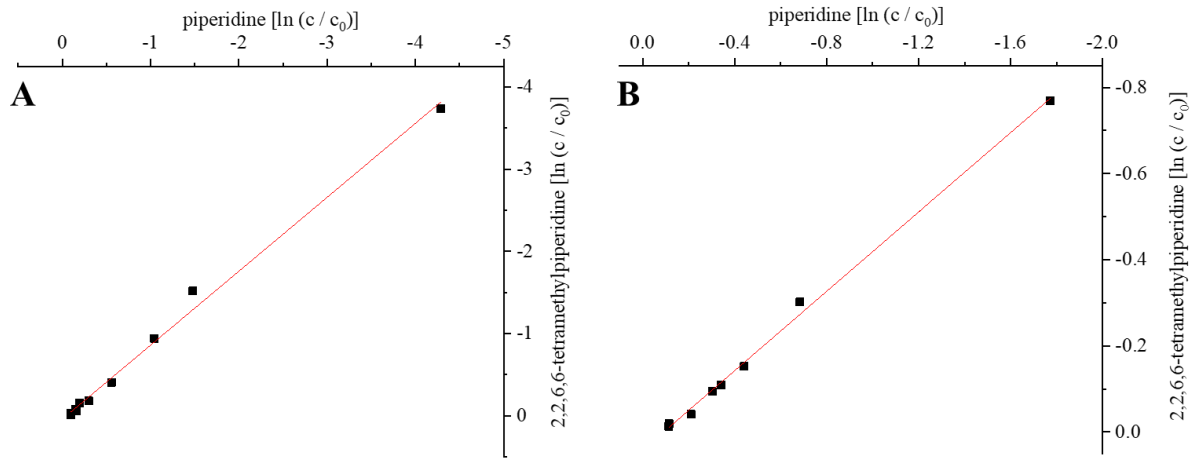

Figure 3-10 **Logarithmic scaling of the results determined in the competition kinetics experiments with piperidine and 2,2,6,6-tetramethylpiperidine at pH 7 (A) and pH 11 (B)**

Mean values of triplicate measurements are shown.

In this study it was shown that even the reaction of simple structured *N*-containing substances is very complex and highly pH dependent as it could be detected that TMP and DMP are not reacting with ozone at pH 2 but pH 7. Further, stoichiometries above one have been determined for pH 7 and pH 11. As both substances are reacting with ozone at pH 7 (which can also be assumed for pH 11) an ozone consuming chain reaction is very likely to take place explaining the high stoichiometries. However, even if it was not possible in this study to confirm the postulated ozone consuming chain reaction it is strongly supported by the observed results. The determination of the stable $^{\bullet}ONR_2$ TEMPO was not possible in this study due to difficulties within the measurements. Therefore, investigations of the formation of $^{\bullet}NR$ in the reaction of $^{\bullet}ONR_2$ with ozone still needs to be investigated. These investigations are highly recommended and needed to either confirm or deny the postulated ozone consuming chain reaction. However, the results observed in this study support the postulate of aminyl radical formation and the simultaneously occurring ozone consuming chain reaction for *N*-containing substances.

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Chapter 4

Influence of matrix composition on the formation of reaction products during ozonation of *N*-containing substances

Adapted from: Wirzberger, V., V.I. Merkus, M. Klein, L.L. Hohrenk, L.E. Rothe, N. Baetz, J. Tuerk, B. Sures, H.V. Lutze, and T.C. Schmidt, *Influence of matrix composition on the formation of reaction products during ozonation of N-containing substances*. Submitted to Environmental Science & Technology, 2020.

4.1 Abstract

The load of micropollutants, frequently detected in wastewater, can be reduced by ozonation. However, this can lead to the formation of transformation products (TPs) which can be highly dependent on the matrix composition. Yet, most studies dealing with TP formation during ozonation do not specifically address matrix influence. To overcome this gap, we investigated the effects of several matrix components, hydroxyl radical scavengers and real water matrices on the TP formation during ozonation of diclofenac, isoproturon and metoprolol.

While the degradation of the three parent substances was similar in all tested water matrices, leading to almost the same stoichiometries, it could be shown that the TP formation varies depending on the water matrix. It has been revealed that even if the formation profile of a certain TP is similar in different matrices the yields can highly variate. Unexpectedly, the use of different scavengers can strongly influence TP formation and their further degradation. For one TP of diclofenac and one of metoprolol the formation of TPs strongly depended on the used scavenger and led to a continuous formation of TPs without any degradation. As a conclusion it became clear that even in the already well-known ozonation of nitrogen-containing compounds the matrix composition can have a strong influence on TP formation.

4.2 Introduction

Micropollutants (MPs) are present in the aquatic environment in concentrations of µg/L to ng/L [1-3]. Many of these substances contain nitrogen (*N*-containing) and are used, e.g., as pharmaceuticals and pesticides. Thus, they might also have a high biological activity [3, 4]. Sources for these compounds, such as diclofenac (DCF) and metoprolol (METO) (pharmaceuticals) as well as isoproturon (ISO, pesticide), can be industrial and municipal wastewaters from wastewater treatment plants (WWTPs) as well as agricultural run-off (e.g., for ISO) [5].

In 2017, DCF and METO belonged to the Top 15 prescribed pharmaceuticals in Germany [6] and are frequently found in wastewater effluents and surface waters [1, 7, 8]. ISO was one of the most used herbicides in agriculture for the treatment of cereals [9, 10] until it was banned in the European Union in September 2016 [11]. However, it is still detected in secondary wastewater effluents [12] as well as in surface waters in Europe [13].

Depending on the chemical structure, conventional wastewater treatment removes between 12.5 and 100 % of certain MPs before the effluent is released into the aquatic environment [5]. To increase this degradation efficiency, advanced wastewater treatment by, activated carbon and ozone (O_3) are increasingly implemented in various WWTPs [5]. Ozonation is widely used for the removal of odor, taste and coloring as well as MPs and pathogens [4]. However, during ozonation MPs are not mineralized but chemically transformed which can lead to undesired transformation products (TPs). Furthermore, reactions of ozone with matrix components can form also undesired ozonation by-products.

Overall, the reactions of *N*-containing substances with ozone can be highly variable because various reactive intermediates, such as aminyl (*N*-centered radicals, $^{\bullet}NR_2$) and nitroxide radicals (O-centered radicals, $^{\bullet}ONR_2$) are formed to a yet unpredictable extent. Indeed, these intermediates might lead to a postulated ozone consuming chain reaction, resulting in high consumption of ozone albeit low substance degradation [14, 15]. This (postulated) reaction as well as possible intramolecular rearrangement (such as 1,2-H-shift) can lead to many unknown TPs. Additionally, formed intermediates such as $^{\bullet}NR_2$ and $^{\bullet}ONR_2$ can react with matrix components, such as bromide or natural organic matter (NOM), leading to the formation of TPs, which are even harder to predict. Further, the water matrix, especially wastewater, can have an influence on the formation of TPs and the corresponding yields because the matrix can lead to quenching of reactive intermediates [16]. Organic matter in the water can also consume ozone and yield $^{\bullet}OH$ [4, 17] and thus influence the formation of TPs

in a different reaction pathway. In contrast, wastewater can also act as a scavenger by quenching •OH by (bi)carbonate or organic matter [4, 18].

Despite the potential relevance of matrix components, most TP formation studies so far have been carried out in ultrapure water (at the most including •OH scavengers) and have ignored the possible influence of matrix components on TP formation in real water systems [14, 15, 19-21].

In many cases, TPs have lower biological activities than the parent compounds [22-24]. However, it was also reported that TPs can be formed with the same or even higher ecotoxicological relevance than the corresponding parent substances, such as 2,6-dichloraniline (DCA), a TP formed in the ozonation of diclofenac [15, 25, 26]. The influence of matrix components on the formation of such ecotoxicologically relevant substances has, however, not been addressed yet, as the experiments were only performed in ultrapure water [15, 26, 27].

Generally, the reactions of frequently found *N*-containing MPs during ozonation in the presence of matrix components are still not well investigated. Therefore, the aim of this study was to foster our understanding of reactions taking place during the ozonation of the relevant MPs DCF, METO and ISO in the presence of artificial matrix components (different concentrations of NOM and different •OH scavengers) and in real water matrices. These investigations are of high interest as the different matrix compositions can affect the formation of TPs which might also be relevant during ozonation in WWTPs. To that end, a twofold approach was used including target (comparison with commercially available standards) and suspect (comparison with *m/z* and structures reported before in literature) screening to detect formed TPs.

4.3 Materials & Methods

4.3.1 Chemicals

All solvents and chemicals used are listed in the Supporting Information (SI, Table 4-2) and were used as received from the according supplier. Structures of parent compounds and TPs as well as the corresponding abbreviations are outlined in Figure 4-7 (SI).

4.3.2 Equipment & Software

The used equipment and software are summarized in Table 4-3 and Table 4-4 (SI).

4.3.3 Generation of stock solution

For all tested substances as well as for available standards of TPs stock solutions were prepared in ultrapure water. For DCA and 5-hydroxyldiclofenac (5-OH DCF) max. 5 % acetonitrile were added. Stock solutions of METO (0.5 mM), DCF (0.5 mM) and ISO (0.058 mM, shaken for several days at room temperature in the dark) for ozonation experiments were prepared and stored at 4 °C. Calibrations were prepared in all matrices used and included two orders of magnitudes (0.0005 – 0.05 mM).

Ozone stock solution was prepared by bubbling ozone produced from oxygen by an ozone generator (COM (Anseros) or Philaqua 802x (BMT Mestechnik)) into ice cooled ultrapure water (≥ 60 minutes) The ozone concentration was determined by measuring the absorption of the ozone stock solution using a UV-spectrometer (UV-1650PC or UV-1800, Shimadzu, 258 nm, $\varepsilon_{O3} = 2950\ M^{-1}cm^{-1}$ [28]). The concentration of the stock solution was between 1.1 and 1.6 mM for all experiments.

Natural organic matter (NOM, 0.05 g) from the Suwannee River (2R101N, International Humic Substance Society) was dissolved in 100 mL (10 mL of NaOH (1 M) and 90 mL ultrapure water) and shaken for 24 h before filtering through 0.45 µM cellulose ester filters (Whatman®). The syringe as well as the filter paper had to be washed once with ultrapure water and 3 mL NOM solution. After filtration the non-purgeable organic carbon (NPOC) content was determined using the TOC Analyzer TOC-L (Shimadzu).

Tert-butanol (*tert*-BuOH) and dimethyl sulfoxide (DMSO) concentrations required to scavenge 90 % (ISO and METO) and 95 % (DCF) of •OH, respectively, were calculated (Equation 4-1 and Equation 4-2, SI) [29] and using the reaction rate constants listed in Table 4-5 (SI)). To minimize the reaction with ozone (≤ 5 % of ozone consumption by the scavengers) no higher concentrations could be applied.

4.3.4 Reaction of *N*-containing substances with ozone in ultrapure water, in the presence of scavengers, matrix components and real water matrices

Ozonation of DCF, METO und ISO was performed in ultrapure water and in the presence of different radical scavengers (*tert*-BuOH and DMSO). Furthermore, the three substances were spiked and ozonated in filtered WWTP effluent samples. DCF was also spiked and treated with ozone in the presence of two different NOM concentrations, in drinking water and surface water.

Sample preparation

In all experiments 0.05 mM DCF and METO, and 0.025 mM ISO were used. The addition of ozone was performed by mixing samples with different volumes of ozone stock solution to reach eleven ozone doses between 0 – 0.5 mM (DCF and METO) and 0 – 0.25 mM (ISO), respectively. Added ozone doses for DCF and METO were: 0 mM, 0.05mM, 0.1 mM, 0.15 mM, 0.2 mM, 0.25 mM, 0.3 mM, 0.35 mM, 0.4 mM, 0.45 mM and 0.5 mM and for ISO: 0 mM, 0.025 mM, 0.05 mM, 0.075 mM, 0.1 mM, 0.125 mM, 0.15 mM, 0.175 mM, 0.2 mM, 0.225 mM, 0.25 mM.

The pH for METO and ISO was adjusted to 7 by adding 10 mM phosphate buffer. For DCF and real water samples no pH adjustment was performed, to achieve comparable results to previous studies [15, 26].

The two scavengers were added separately in different runs. Applied concentrations to achieve scavenging of 90 % (ISO and METO) and 95 % (DCF) of $^{\bullet}OH$ were 3.375 mM (ISO), 6.3 mM (METO) and 20 mM (DCF) for *tert*-BuOH and 0.54 mM (METO), 0.29 mM (ISO) and 20 mM (DCF) for DMSO.

Furthermore, the influence of different NOM concentrations during the reaction of DCF with ozone was tested. The simulated surface water contained 2.35 mg/L of non-purgeable organic carbon and the NPOC in simulated drinking water was 0.63 mg/L.

Real water samples were filtered (cellulose ester filter 0.45 µm) to remove particulate matter before spiking DCF (drinking, surface and wastewater), METO (wastewater) or ISO (wastewater), respectively.

All concentrations used were higher than concentrations of the substances typically found in the aquatic environment to ensure higher yields of TPs and easier identification of those.

Sample measurement

The degradation of the parent compounds as well as formation of known TPs were measured via HPLC-DAD (LC-10 Shimadzu) and TPs without reference standard were identified by HPLC-HRMS (Dionex Ultimate 3000 UHPLC$^+$ and Orbitrap Q Exactive, Thermo Scientific). In both cases an EVO C18 column (Kinetex 5 µm EVO C18 100 Å 100 × 3.0 mm, Phenomenex) was used and formic acid (0.1 – 0.2 %) was added to DCF and METO samples before measurement. For all samples ultrapure water + 0.1 % formic acid and methanol + 0.1 % formic acid were used as eluents.

While HPLC-DAD measurements of DCF (including two target TPs) were performed with isocratic elution (Table 4-6, SI), for METO (including five target TPs) and ISO measurements a gradient elution was used (Table 4-6, SI). Wavelengths used to determine the parent substances and TPs are summarized in Table 4-7 (SI). The gradient used for ISO and METO in HPLC-HRMS measurements is outlined in Table 4-8 (SI) and for DCF in Table 4-9 (SI). All samples were measured in positive mode, as suspects considered in this study (Table 4-10, SI) were measured with positive ionization mode in previous studies [20, 21, 26, 30, 31].

Determination of hydroxyl radical yield

The yield of $^{\bullet}$OH was determined via the formation of methanesulfonic acid (MSOS) and methanesulfinic acid (MSIS) using ion-exchange chromatography. These products are formed with 92 % yield in the reaction of $^{\bullet}$OH with DMSO [32-34]. Measurements were performed using an eluent of 0.36 mM sodium carbonate and 0.34 mM sodium bicarbonate. The suppressor solution contained 0.05 mM sulfuric acid and the total measurement time was 15 minutes including a 1-minute initiation time with a flow rate of 1 mL/min. For all measurements a Metrosep A Supp 4 – 250 / 4.0 column (Metrohm) was used.

4.3.5 Detection of TPs in real water samples

Three real WWTP effluent samples (without ozonation, directly after ozonation, a mixture of ozonated and non-ozonated wastewater) were taken at two different time points. A scheme of the WWTP including the sampling sites can be found in the SI (Figure 4-8). The samples were analyzed with regard to present transformation products reported in literature

(suspect screening, compare Table 4-10, SI). The temperature, pH values and conductivity were determined for all samples on site (Table 4-11, SI).

Sample preparation

All samples were taken in glass bottles with glass stoppers and if needed the samples were stored overnight at 4 °C. A single solid phase extraction (SPE) as well as a tandem anion and cation exchange SPE were performed [35].

Single SPE was conducted using Oasis HLB cartridges (150 mg, Oasis HLB 6 cc, Waters GmbH, Eschborn, Germany) as well as a combination of Oasis MAX (150 mg, Oasis MAX 6 cc, Waters GmbH, Eschborn, Germany) and Oasis MCX (150 mg, Oasis MCX 6 cc, Waters GmbH, Eschborn, Germany) cartridges, respectively. For preconditioning 2 x 3 mL methanol and for equilibration 2 x 3 mL water were used.

Oasis MAX and Oasis MCX cartridges were connected in tandem mode following the protocol by Deeb and Schmidt [35] before one liter of each sampling station and a blank were filtered through all cartridges using vacuum suction. All samples were dried under vacuum for at least 30 min after SPE before wrapped in aluminum foil and stored at -20 °C until elution. To elute Oasis HLB cartridges 2 x 5 mL methanol and ethyl acetate, respectively, were used. Washing, elution and mixing for Oasis MAX and Oasis MCX cartridges was done following the protocol presented by Deeb and Schmidt [35].

The volume of all eluents was reduced to approximately 1 mL under a nitrogen gas stream at 60 °C. After reduction of the volume 2 x 10 mL methanol were added and dried completely before resolving in 1 mL methanol and filled into HPLC-vials.

Sample measurement

All samples were measured by HPLC-HRMS (Dionex Ultimate 3000 UHPLC$^+$ and Orbitrap Q Exactive, Thermo Scientific). For chromatographic separation an XSelect HSS T3 column (Waters XSelect HSS T3 2.5 µm 2.1 × 100 mm Column XP with a pre-column XSelect HSS T3 XP VanGuard Cartridge 2.5 µm 2.1x5 mm) was used.

For separation a gradient (Table 4-12, SI) was used including two eluents (ultrapure water + 0.1 % formic acid and methanol + 0.1 % formic acid). As described in literature reported suspects considered in this study (Table 4-10, SI) were measured with positive ionization mode [20, 21, 26, 30, 31]. Therefore, all measurements were done with positive

electrospray ionization mode. More information about settings and parameters are given in Table 4-12, SI.

4.3.6 Data evaluation

Target analysis

For quantification all parent compounds and known TPs with commercially available reference substance were analyzed with HPLC-DAD. The data generated were manually evaluated at the specific absorption maximum of the substances (Table 4-7, SI). For the known TP 3-(isopropylamino)propane-1,2-diol (3-METO) quantitative data were generated with HPLC-HRMS and processed with XCalibur 4.0.

Suspect screening

To determine the influence of matrix effects on the formation of known TPs without available standard a suspect screening was carried out. The data gathered by measurement of ozonated samples with HPLC-HRMS were processed with XCalibur 4.0. Using the tuning software of the HRMS system the monoisotopic masses of the known TPs were calculated and Qual Browser was used to determine the corresponding peaks within the samples. A processing method was written based on the *m/z* values of detected peaks and the corresponding retention times determined in Qual Browser. For all samples after ozonation and all extracted wastewater samples (prior and after ozonation) the respective processing method was used and the results were exported by Quan Browser.

4.3.7 Determination of the reaction rate constants of 2,6-dichloroaniline and des(isopropoxyethyl)bisoprolol

Reaction rate constants of ozone with the TPs 2,6-dichloroaniline (DCA) and des(isopropoxyethyl)bisprolol (DPB) in the presence and absence of scavengers were determined using competition kinetics. While aniline was chosen as competitor for 2,6-dichloroaniline, METO was used as a competitor for des(isopropoxyethyl)bisprolol. To determine the reaction rate constants both substances and the competitors were provided

(0.05 mM, each) and aliquoted to a number of vials. Different ozone doses (0.05 mM – 0.5 mM) were added to these vials. In separate runs DMSO (20 mM) for DCA and 6.3 mM *tert*-BuOH for DPB were added. The pH in the DPB experiments was adjusted to pH 7 using phosphate buffer (10 mM). For DCA no pH adjustment was done as all experiments regarding DCF were done without pH adjustment to generate comparable results to previous studies which did not perform any pH adjustment during the ozonation of DCF [15, 26]. Calibrations were performed in ultrapure water.

After a reaction time of approximately 24 h, the degradation of the substances was measured with HPLC-DAD (Shimadzu). For all measurements an EVO C18 column (Kinetex 5 µm EVO C18 100 Å 100 × 3.0 mm, Phenomenex) was used. Degradation of aniline and DCA was measured using the eluents water + 10 mM ammonium acetate (NH_4COOH) and methanol. The gradient is described in Table 4-13 (SI). The degradation of METO and DPB was also measured using a gradient (Table 4-6, SI) with ultrapure water + 0.1 % formic acid and methanol + 0.1 % formic acid as eluents.

4.4 Results & Discussion

4.4.1 Degradation of parent compounds in different water matrices

The three tested substances were degraded in all tested water matrices showing similar degradation profiles (Figure 4-9). The highest ozone dose (0.5 mM, 10 : 1 O_3 to substance ratio) was needed to reach a nearly complete degradation for DCF in the presence of NOM due to the ozone consumption by the organic water matrix. As the results for DCF did not reveal a high influence of NOM it was not tested for ISO or METO. METO and DCF were completely degraded by slightly lower ozone doses in non-scavenged systems than in scavenged systems, underlining that both substances are degraded by ozone as well as $^{\bullet}OH$. As there was a similar degradation of ISO determined during ozonation in scavenged and non-scavenged systems, it can be concluded that the main degradation is driven by ozone. Mascolo et al. [20] also proposed that $^{\bullet}OH$ play a minor role in the degradation of ISO during ozonation as only small amounts will be formed. However, in the ozonation of the three substances similar yields of $^{\bullet}OH$ were detected (25 – 35 %, Table 4-1) in the measurement with DMSO.

For all three substances stoichiometries above one were determined for a complete degradation with ozone in all water matrices (Table 4-1). Sein et al. [15] reported ozone / DCF

ratios of 5 – 8 : 1 to reach complete degradation which is similar to the values determined in this study. For METO and ISO no previous data are available for comparison. The reaction of ozone with the amine group of *N*-containing substances can lead to the formation of aminyl and nitroxide radicals [4]. It has been postulated that an ozone consuming chain reaction is leading to stoichiometries above one in the depletion of *N*-containing substances [14, 15]. Therefore, it was postulated that ozone might also react with the formed intermediates (such as aminyl radicals) leading to a higher ozone consumption with a simultaneously low substance degradation. This could indeed explain the high stoichiometries for DCF and METO in this study. For ISO this explanation cannot be applied, because the amine group is not attacked by ozone, since it is deactivated by a carbonyl (amide) [20]. However, high stoichiometries were detected independently of the water matrix indicating that not the present matrix but rather formed intermediates or highly reactive products such as aminyl and nitroxide radicals (in case of DCF and METO) or other species such as superoxide (all substances), are likely to consume ozone.

Table 4-1 Compilation of second order rate constants for the reaction with ozone, stoichiometries, hydroxyl radical yields, pKa and reactivity pKa values for isoproturon, metoprolol and diclofenac. All values are presented for ultrapure water. [a]given ranges were determined in all considered water matrices

Compound	isoproturon	metoprolol	diclofenac
k / $M^{-1}s^{-1}$	2.2 x 10^3 (pH 2) [36]	2 x 10^3 (pH 7) [30] 8.6 x 10^5 (deprotonated) [30]	6.8 x 10^5 (without pH adjustment) [15] 1.19 x 10^6 (pH 7) [26]
Stoichiometry [a] (ozone : substance)	2 – 3 : 1	4 – 5: 1	6 – 7 : 1
•OH yield (per consumed ozone)	30 %	35 %	25 %
pKa	13.79 [calculated with MarvinSketch]	9.7 [30]	< 3 (amino group) [15, 37] 4.2 (carboxyl moiety) [15, 26, 37]
Reactivity pKa	Not available	6.3 [4]	Not available

4.4.2 Identification and quantification of TPs formed in different water matrices

Target analysis

Seven standards were considered for the target analysis of TPs formed during ozonation of DCF (5-hydroxy diclofenac (5-OH DCF) and DCA) and METO (DPB; desisopropylmetoprolol; *o*-demethylmetoprolol; α-hydroxymetoprolol and 3-METO). As no standards were available for ISO TPs no quantification could be performed for this parent compound.

All targets were detected in every examined water matrix. Five out of the seven target TPs (except DCA and DPB) showed similar formation profiles in all water matrices as reported in literature [15, 30] (Figure 4-10 shows the formation of all targets). In detail, even if different concentrations of the targets were detected within the various water matrices, all targets were formed with increasing ozone doses but degraded at even higher ozone dosages. This indicates that all tested target TPs are primary TPs formed in the direct reaction of the parent substances with either ozone or •OH.

However, the formation of DCA and DPB showed striking differences in the presence of DMSO and *tert*-BuOH, respectively. Both scavengers are widely used in studies regarding the ozonation of compounds to scavenge •OH formed during ozonation [4]. Nevertheless, to the authors' best knowledge studies performed until now only used one of the two scavengers (among others [14, 15, 21, 30, 38]). In this study the influence of the two scavengers on the formation of TPs was investigated showing that the degradation of all three parent substances was similar in scavenged and non-scavenged systems (Figure 4-9), but that the formation of the TPs DCA and DPB, respectively, strongly depended on the used scavenger (Figure 4-1).

The concentration of DCA increased in ultrapure water and in the presence of *tert*-BuOH (max. yield 15 µM) until an added ozone dose of 0.25 mM (O_3 to substance ratio =5 : 1) and decreased with higher ozone doses at which DCF was completely degraded. This underlines that DCA is a primary TP, formed by a hydroxylation of the non-chlorinated ring leading to a cleavage of the two rings [15]. Yet, in the presence of DMSO the DCA concentration (yield of 12 µM at an added ozone dose of 0.25 mM, O_3 to substance ratio = 5 : 1) continued raising at ozone doses above 0.25 mM (O_3 to substance ratio = 5 : 1). Similar observations regarding the different influence of scavengers on the TP formation were also made for the formation of DPB. It was formed until an ozone dose of 0.1 mM (ultrapure water, O_3 to substance ratio = 2 : 1) and 0.2 mM (wastewater, O_3 to substance ratio = 4 : 1), respectively, and degraded at higher ozone doses. DPB was not formed in the presence of the

radical scavenger DMSO, but in the presence of *tert*-BuOH it was formed continuously with increasing ozone doses and was not degraded as in other water matrices.

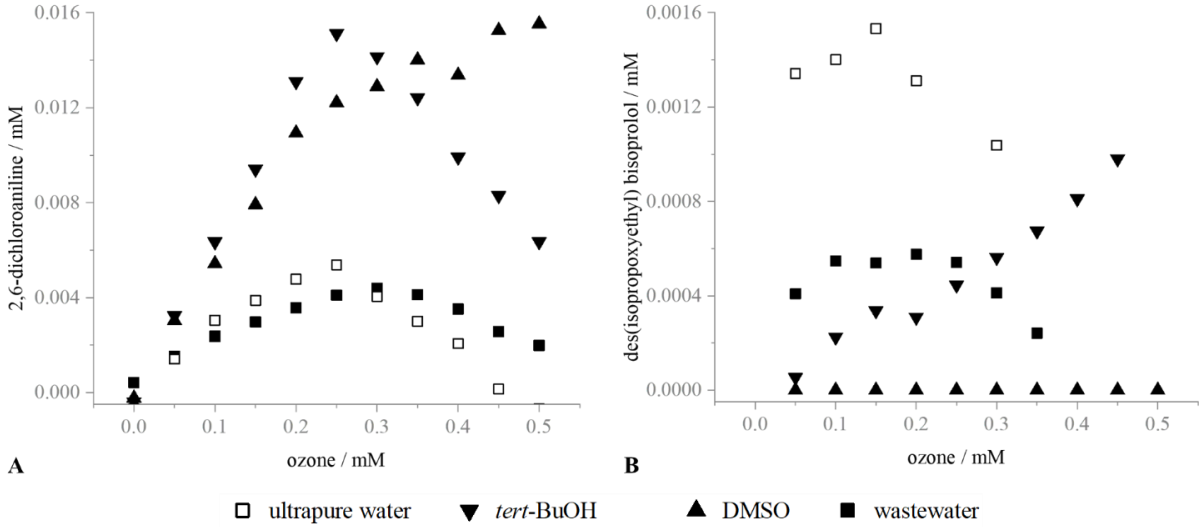


Figure 4-1 **Formation of the diclofenac transformation product (A) 2,6-dichloroaniline and the metoprolol transformation product (B) des(isopropoxyethyl) bisoprolol in ultrapure water, wastewater and in the presence of different radical scavengers (DMSO and *tert*-BuOH)**

Metoprolol and diclofenac (c_0 = 0.05 mM for both) were treated with different ozone dosages (c = 0.05 – 0.5 mM) in different water matrices. The pH was not adjusted in the experiments of diclofenac (before ozone addition: pH 7 – 8 in all samples; after ozone dosage of 0.5 mM: pH 6 – 7; in samples containing DMSO pH 4) and for metoprolol the pH was adjusted to 7 (phosphate buffer). In detail, ultrapure water □, *tert*-BuOH ▼, DMSO ▲ and wastewater ■ were tested. Mean values of two to three single experiments are shown, exception des(isopropoxyethyl) bisoprolol in wastewater n = 1. Individual uncertainties are not shown as it would lead to great complexity.

As a consequence of the different results in the presence of the two scavengers, the influence of reaction kinetics needs to be considered. One explanation for the continuing formation of DCA could be the higher reaction kinetics with ozone of DMSO ($k = 8.1\ M^{-1}s^{-1}$ at pH 7) [39] compared with *tert*-BuOH ($k = \sim 0.003\ M^{-1}s^{-1}$ at pH 6 [40]) , which may result in ozone scavenging competing for the reaction of DCA. Considering a DMSO dose of 20 mM the reaction rate of DCA has to be in the range of $< 5\ M^{-1}s^{-1}$ as the reaction of DMSO with ozone needs to be favored over the reaction of DCA to explain the results. This could not be confirmed in this study as DCA was determined to react quite fast with ozone in scavenged

($k = 1.04 \times 10^7\ M^{-1}s^{-1}$) as well as non-scavenged systems ($k = 1.06 \times 10^6\ M^{-1}s^{-1}$). This is also in accordance to the previously reported rate constant for *p*-chloroaniline ($k = 1.4 \times 10^6\ M^{-1}s^{-1}$, no pH adjustment) [14].

Although the reactions of DPB with ozone are only moderately fast ($k = 1.5 \times 10^3\ M^{-1}s^{-1}$ in scavenged and $k = 2.1 \times 10^3\ M^{-1}s^{-1}$ in non-scavenged systems), these will still not be outcompeted by the reaction of ozone with *tert*-BuOH. Therefore, the reaction of DPB with ozone should still be favored over the reaction of *tert*-BuOH with ozone and DPB should also be degraded in systems including *tert*-BuOH. However, this was not found in this study.

Sein et al. [15] also showed that scavenging of •OH radicals increases the yield of DCA (only in the presence of *tert*-BuOH), but that it is degraded at higher ozone doses. This could be also confirmed in this study for *tert*-BuOH but not for DMSO. Benner and Ternes [30] also reported similar observations for the formation of DPB (increasing DPB concentration with increasing ozone dosages and (almost) no degradation) in systems including *tert*-BuOH as presented in this study. In ultrapure water the yield of DCA is smaller than in the presence of scavengers, however, DPB shows the highest yields in ultrapure water. These observations indicate that the main formation pathway of DCA is driven by ozone while the formation of DPB includes •OH and ozone. However, DPB is not formed in the presence of DMSO but in the presence of *tert*-BuOH. These observations underline that even for very well investigated substances the TP formation can differ in presence of different scavengers independently of the reaction rate constant with ozone. Accordingly, further research is needed to investigate the influence of different •OH scavengers on TP formation.

The concentration of TPs of METO observed in in the different water matrices were compared, as here the most targets were available. It was observed, that even if the formation profiles of the TPs and the degradation of METO itself were very similar (Figure 4-9 and Figure 4-10, SI), the composition and concentrations of METO TPs differed in the examined water matrices (Figure 4-2). Primary TPs of METO formed in ultrapure water and wastewater, which were covered in the target screening, add up to around 60 % of the mass balance in both systems. Substantially smaller overall yields of TPs have been detected in the presence of radical scavengers (Figure 4-2). In samples containing radical scavengers only 10 % of the initial concentration were found as target TPs. Other TPs which have been reported before, such as hydroxylamines [4, 30], may complete the mass balance. However, as no standards were available these were not considered within the target analysis.

While *o*-demethylmetoprolol (~24 %) and 3-(METO (~24 %) are formed with the highest yields in ultrapure water at pH 7, 3-METO (~32 %) and α-hydroxymetoprolol (~28 %)

are the major reaction products after ozonation of METO in WWTP effluent. In scavenged systems all five TPs were only formed at very low concentrations (approx. 2 – 4 %).

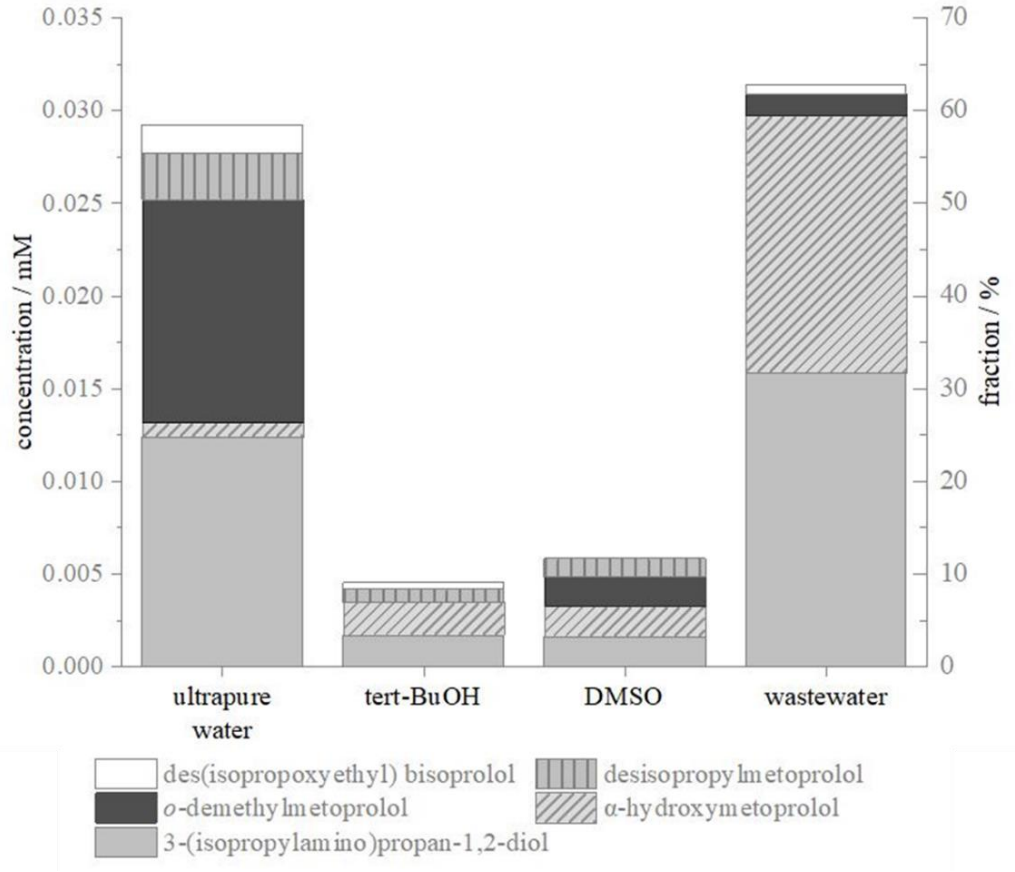


Figure 4-2 Formation of the five target transformation products of metoprolol (c_0 = 0.05 mM) in different water matrices

All experiments were performed at pH 7 and DMSO and *tert*-BuOH concentrations were 0.54 mM and 6.3 mM, respectively. The five TPs (des(isopropoxyethyl) bisoprolol, 3-(isopropylamino)propane-1,2-diol), desisopropylmetoprolol, *o*-demethylmetoprolol, and α-hydroxymetoprolol are shown for an ozone dose of 0.15 mM (only ozone dose at which α-hydroxymetoprolol was detected) and measured by HPLC-DAD. Starting concentration of metoprolol was 0.05 mM and therefore 0.05 mM was considered as 100 %. Mean values of experiments performed in duplicate or triplicate are shown.

The fact that the TP yields detected in scavenged systems are lower than in non-scavenged systems indicates that most target TPs of METO are formed in the reaction of •OH rather than molecular ozone. Benner and Ternes [30] reported similar observations for 3-METO, α-hydroxymetoprolol and *o*-demethylmetoprolol. However, the results presented in

this study lead to the assumption that desisopropylmetoprolol is also formed by the reaction of •OH. This was also proposed by Tay et al. [21] who also detected desisopropylmetoprolol only under non-scavenged conditions. However, other studies proposed an attack of molecular ozone at the amine [30, 31] because larger yields of desisopropylmetoprolol have been detected in the scavenged system compared to the ozonation in ultrapure water. Hence, the pH value seems to highly influence the reaction. Benner and Ternes [30] and Faber et al. [31] performed the experiments at pH 8, Tay et al. [21] did not use any buffer and reported a decreasing pH from 8 to 4 during the experiment. In this study, the pH was buffered at 7. With a pKa of 9.7, METO is partly deprotonated at pH 7 and 8 and the amine is mainly attacked by ozone [4]. However, also the reaction of •OH with METO may lead to the formation of desisopropylmetoprolol, which is proposed in Figure 4-3.B.

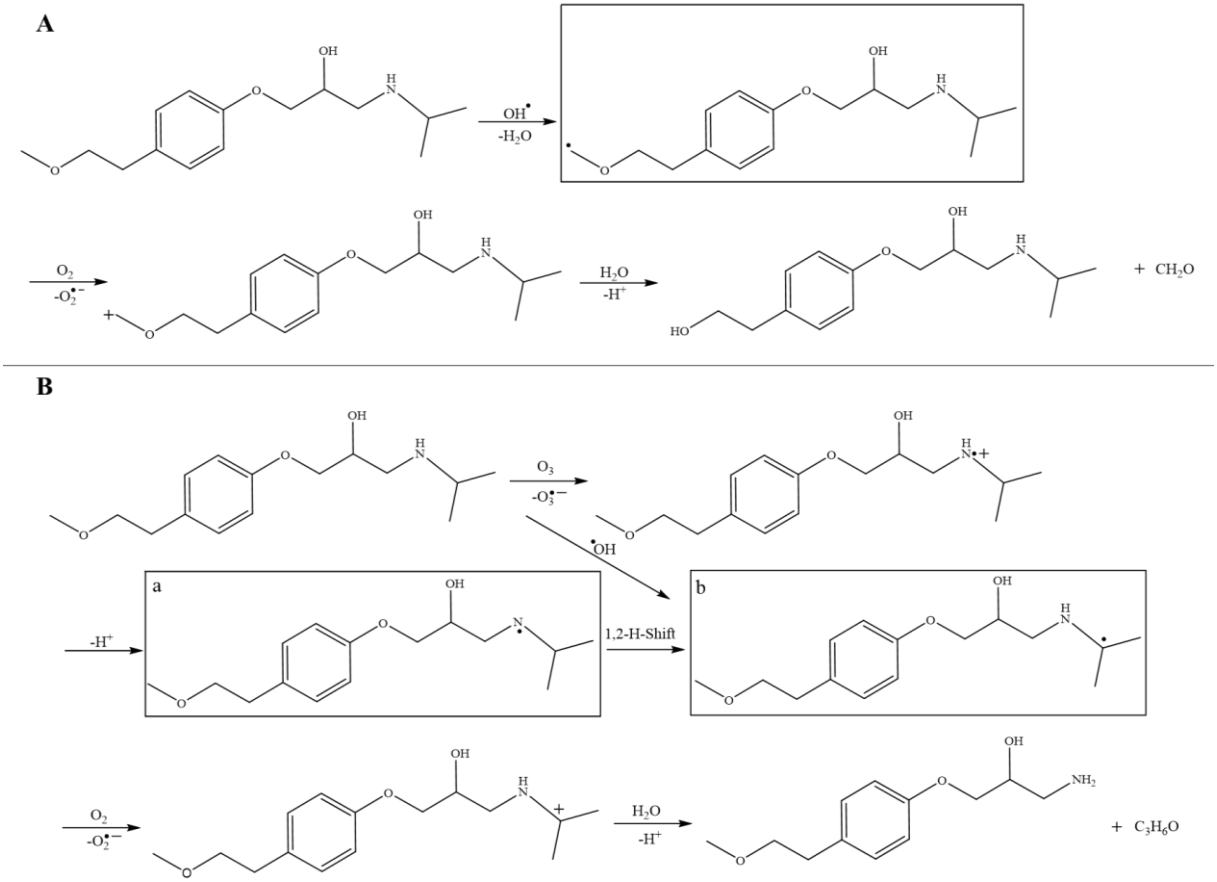


Figure 4-3 Proposed formation pathways of (A) *o*-demethylmetoprolol and (B) desisopropylmetoprolol

C-centered radical (4-3.A and 4-3.B.b) and aminyl radicals (4-3.B.a) which can be quenched by the wastewater matrix are marked with boxes.

Large differences within TP yields were not only detected in terms of used scavengers but also in wastewater compared to ultrapure water. Wastewater can have two different influences on the formation of TPs. The present organic matter can react with ozone, yielding e.g., ozonide radical anions in the following leading to •OH formation [4, 17]. In contrast, the wastewater matrix can also quench formed intermediates [16, 41], such as carbon centered radicals (Figure 4-3, example for the reaction of (A) *o*-demethylmetoprolol and (B.b) desisopropylmetoprolol) or aminyl radicals (Figure 4-3.B.a), leading to lower yields of some TPs than in ultrapure water. It should be noted that that the reaction of •OH at the aromatic ring is also important and indeed hydroxylation products were also observed by Benner and Ternes [30] and in this study. However, since •OH react with both benzenes and ethers at similar second order rate constants (within one order of magnitude [42]) the reaction with the alkyl groups of the ether may also be conceivable. As a result of the complexity of reactions taking place in water matrices, such as wastewater, a quantitative evaluation of •OH based reactions is hardly feasible to date.

Suspect screening

Besides the target analysis, the formation of all previously reported TPs of ISO, METO and DCF (Table 4-10, SI) without available standard was investigated by a suspect screening measured with HPLC-HRMS. The evaluation of the peak areas was done following the procedure described by Bader et al. [43]. In detail, the fold change (f_c = peak area$_{\text{water matrix}}$ / peak area$_{\text{ultrapure water}}$) was calculated and the results were classified in five categories: not formed ($f_c \leq 0.20$), decreased ($f_c \leq 0.50$), constant ($f_c \leq 2.00$), increased ($f_c \leq 5.00$) or formed ($f_c \geq 5.00$) (Figure 4-4, adapted and modified after [43]). Yet, all findings comparing peak areas within the suspect screening need to take into account the influence of the different matrices which might lead to ion suppression or enhancement and therefore, result in larger or smaller peak areas [44].

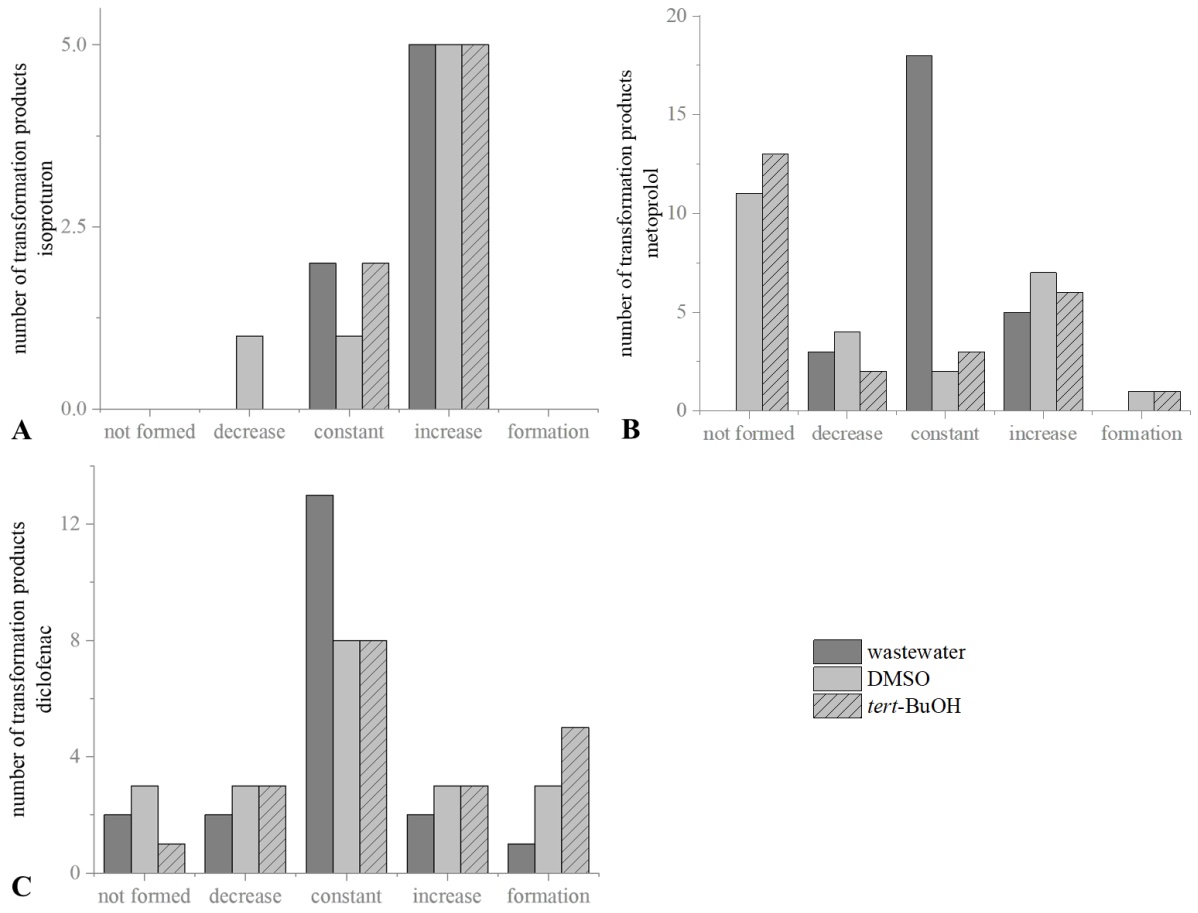


Figure 4-4 **Fold change classification of detected suspects of (A) isoproturon (c_0 = 0.025 mM), (B) metoprolol (c_0 = 0.05 mM) and (C) diclofenac (c_0 = 0.05 mM) after ozonation (c = 0.025 – 0.25 mM (isoproturon) and c=0.05 – 0.5 mM) in different water matrices compared with ultrapure water**

Measurements were performed with HPLC-HRMS and the pH was set to 7 for metoprolol and isoproturon (except in wastewater). In diclofenac samples no pH adjustment was performed. Scavenger concentrations were DMSO c = 0.29 mM and *tert*-BuOH c = 3.38 mM for isoproturon, DMSO c = 0.54 mM and *tert*-BuOH c = 6.3 mM for metoprolol and DMSO c = 20 mM and *tert*-BuOH c = 20 mM for diclofenac. Results are based on the calculated fold change (fc = peak area$_{\text{water matrix}}$ / peak area$_{\text{ultrapure water}}$). Categories are: not formed ($f_c \leq 0.20$), decreased ($f_c \leq 0.50$), constant ($f_c \leq 2.00$), increased ($f_c \leq 5.00$) or formed ($f_c \geq 5.00$) (adapted and modified after [43]). Mean fold changes of one to three single experiments were considered. Total number of considered transformation products: ISO = 10, METO = 34, DCF = 20.

Isoproturon

Mascolo et al. [20] reported for the reaction of ISO with ozone in total ten TPs, which are mainly formed by the direct ozone reaction [20]. Three of these TPs (TP 198, TP 212, and TP 228) were not detected in this study. As the nitrogen of the urea group is not reactive the direct reaction of ozone with ISO is likely to take place at the aromatic ring [4]. Yet, even if it could be confirmed, that the main reaction of ISO during ozonation is taking place with molecular ozone, the comparison of TP formation in scavenged and non-scavenged systems also suggests that several TPs are formed in the reaction of •OH with ISO. Figure 4-4.A underlines that most detected TPs showed increasing peak areas in the presence of scavengers and in WWTP effluent.

In detail, the formation of TP 222 was considered, for which three structures were postulated by Mascolo et al. [20]. Two of them contain a hydroxyl group at the aromatic suggesting a hydroxylation reaction at the isopropyl group. TP 222_1 was formed with slightly smaller peak areas in the scavenged systems (after the addition of *tert*-BuOH or DMSO) and much larger peak areas in wastewater compared to the experiment in ultrapure water. Even if ISO is reacting very fast with ozone, this result indicates that TP 222_1 is not only formed by ozone but might also be formed by •OH. A possible reaction pathway for the formation of TP 222 not only including the reaction with ozone but also considering •OH was suggested (Figure 4-5). Furthermore, TP 222_1 was formed with increasing ozone doses (until 0.075 mM, 3 : 1 O_3 to substance ratio) and degraded at even higher ozone doses (> 0.1 mM, 4 : 1 O_3 to substance ratio, Figure 4-11, SI). The largest peaks of this TP were found after ozonation in WWTP effluent. The reaction of organic compounds present in WWTP effluent (NPOC ≈ 6.7 mg/L) can react with formed intermediates and possibly giving rise to TP 222_1.

TP 180, the direct precursor of TP 196, was suggested to be a TP directly formed from ISO but also from TP 222_1 [20] (Figure 4-5, modified after Mascolo et al. [20]). This is also suggested in this study as TP 180 also showed smaller peak areas in ultrapure water than in scavenged systems while the formation profile was vice versa for TP 222_1. Yet, also larger peak areas for TP 180 were detected in WWTP effluent compared with the scavenged systems (Figure 4-11, SI). However, only low amounts of TP 196 were formed at low ozone dosages and still showed increasing peak areas at the highest ozone dosages in scavenged systems and wastewater (Figure 4-11, SI). This indicates, that TP 196 is a secondary TP of ISO, formed either from TP 180, TP 222_1 or TP 222_2/222_3 (Figure 4-5, modified after Mascolo et al. [20] and Figure 4-11, SI).

All other TPs from the suspect list were detected with larger peak areas in scavenged systems than in non-scavenged, indicating that these have been formed in the direct reaction of ozone with ISO as suggested by Mascolo et al. [20].

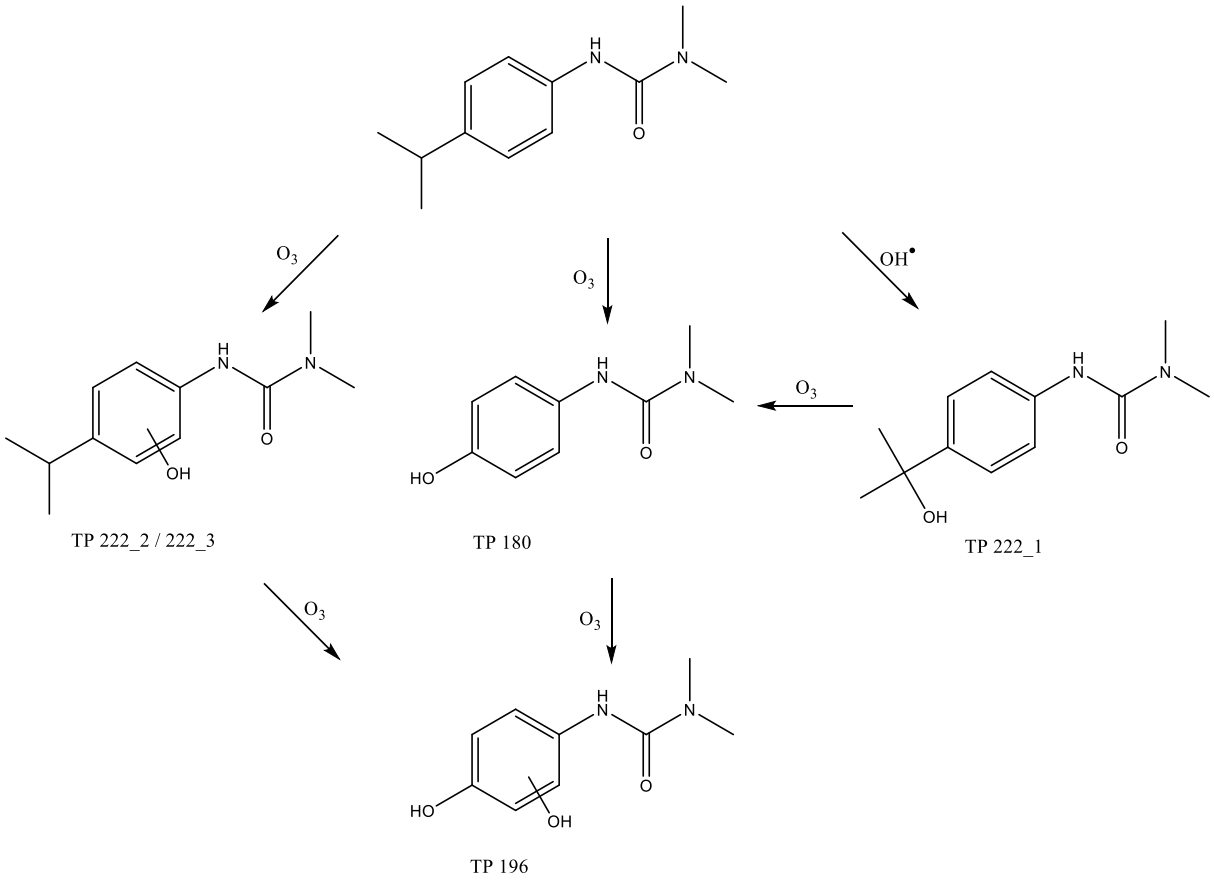


Figure 4-5 **Proposed formation pathways of the isoproturon transformation products 222_1, 222_2 / 222_3 and 180 leading as intermediates to the formation of transformation product 196** (modified after Mascolo et al. [20])

Metoprolol

In total 18 TPs have been reported for the reaction of METO with ozone [21, 30, 31]. However, as for some *m/z* more than one structure was proposed if peaks at different retention times were found, all of them were considered, leading to 34 suspects. Figure 4-4.B shows that the comparison of TPs formed in ultrapure water and wastewater led to many TPs with similar peak areas. Comparable numbers of TPs were not formed for both scavengers. However, the presence of scavengers also led to the formation of TPs, while some TPs also increased in all three matrices. It was recognized that in wastewater the peak area of fewer TPs increased than in the presence of scavengers.

Benner and Ternes [30] detected TP 273_2 and TP 299 at pH 3, however, these were also reported in a study without pH adjustment [21] and also detected in the performed experiments in this study at pH 7. At pH 3, the secondary amine is protonated so that it cannot be attacked by ozone and the ozone attack occurs at the aromatic ring. The reaction of ozone can only occur at the deprotonated amine ($pK_{a(METO)}$ = 9.7 [30]) but even if during the experiments (pH 7) the amine is partly protonated the reaction of ozone is fast enough to react with the small amount of deprotonated amine (reactivity $pK_{a(METO)}$ = 6.3 [4]). Therefore, ozone could attack at both sites but is very likely to attack at the amino group at pH 7. This pH dependent reaction and also TP formation has already been reported for tamoxifen [27].

TP 273_2 was postulated to degrade further to TP 205 (reaction meachanism shown by Tay et al. [21]) which was also confirmed in this study. Peak areas of TP 273_2 increased up to an ozone dose of 0.15 mM (3 : 1 O_3 to substance ratio, ultrapure water and *tert*-BuOH) and 0.2 mM (4 : 1 O_3 to substance ratio, DMSO and WWTP effluent), respectively, and decreased at higher ozone doses where peak areas of TP 205 started increasing simultaneously in ultrapure water and WWTP effluent. Indeed, TP 273_2 is only formed by the direct reaction of METO with ozone, while TP 205 is not formed under scavenged conditions (Figure 4-12, SI). This indicates that the reaction of $^{\bullet}$OH with TP 273_2 is cleaving a C-C bond leading to TP 205.

The main reaction product of the ozonation of METO at pH 7 is supposed to be TP 283 [4], which was also detected in this study with increasing peak areas at increasing ozone doses and completely degraded at higher ozone doses in ultrapure water and WWTP effluent but not in scavenged system. These observations underline the proposed reaction of METO and ozone to this *N*-oxide.

Small peak areas were detected under scavenged conditions for e.g., TP 133, TP 239, TP 253 and TP 283 indicating that these are mainly formed in the reaction of $^{\bullet}$OH with METO (Figure 4-12, SI). Indeed, these TPs are formed by bond cleavages taking place not at the aromatic ring (TP 133, TP 239, TP 253) or hydroxylation at the aromatic ring or carbonyl groups (TP 283). While TP 253 showed the highest peak areas in ultrapure water and WWTP effluent, the highest peak areas of TP 239 were detected in ultrapure water. TP 133 and TP 283 also showed the highest peak area in WWTP effluent (Figure 4-12, SI), which underlines the postulated reaction mechanism with $^{\bullet}$OH rather than molecular ozone. In fact, some TPs of METO are mainly formed by the direct reaction with ozone and it could be assumed that these peak areas decrease in WWTP effluent. Yet, this was only the case for TP 283. For all other TPs larger peak areas were detected, highlighting the fact that wastewater matrix can highly influence the reactions taking place during ozonation.

Diclofenac

Coelho et al. [26] reported 20 TPs for the ozonation of DCF in ultrapure water and in the presence of *tert*-BuOH. In this study, all of those suspects were detected in the different water matrices. Most TPs showed similar peak areas in wastewater but in the presence of the used scavengers the results highly differed. As it was the case for DCA some TPs showed increasing peak areas, in the presence of DMSO or *tert*-BuOH compared to peak areas in ultrapure water (Figure 4-4.C), which was not the case in wastewater. In all three water matrices the formation of some TPs was detected but some TPs were also "not formed" (Figure 4-4.C).

Detected TPs included TP 176, TP 281, TP 297 and TP 309. It has been reported before that the main degradation pathways of DCF during ozonation are hydroxylation (TP 281 and TP 309) and the cleavage of the carbon nitrogen bond (TP 177) [15, 26] (reaction mechanism has been proposed by Coelho et al. [26]). Indeed, TP 281 and TP 309 showed (slightly) larger peak areas in wastewater compared to ultrapure water but while TP 281 showed decreasing peak areas in the presence of scavengers, peak areas of TP 309 were increasing in these water matrices (Figure 4-4:C). In total the formation profiles of TP 281 and TP 309 differ within the matrices. TP 281 showed highest yields in wastewater and was almost not formed in the other three water matrices, while TP 309 showed similar formation profiles in all water matrices with the highest formation in the presence of *tert*-BuOH (Figure 4-13, SI). This indicates that TP 281 is mostly formed in the reaction of $^{\bullet}$OH rather than molecular ozone and for TP 309 the reaction pathway seems to be vice versa.

This is also correlative with the formation profile of TP 176 which showed highest peak areas in ultrapure water and in the presence of *tert*-BuOH but only small peak areas in wastewater and in the presence of DMSO. This is similar to the observations made for DCA underlining again that scavengers seem to have different influence on the TP formation. Interestingly, also similar observations for TP 297 and the reported precursor (TP 327) (reaction mechanism proposed by Coelho et al. [26]) were made, showing different ratios of detected peak areas in the presence of scavengers compared to ultrapure water (Figure 4-4.C and formation profiles in Figure 4-13, SI).

4.4.3 Determination of TPs in wastewater samples

Two wastewater effluents from a WWTP using one conventional treatment stream and one conventional treatment stream partly ozonated and recirculated were analyzed in terms of suspect transformation products. Additionally, two samples were taken directly after the ozonation and also investigated. In detail 20 TPs for DCF, 24 for METO and ten for ISO (including TPs same *m/z* but different retentions times, as TPs were detected for METO, the total number of considered TPs was smaller as in the water matrices ozonated in the experimental setup, compare Table 4-10, SI) were considered within the different sample types. As described for the suspect screening the evaluation of real water samples was also performed as reported by Bader et al. [43] (f_c = peak $area_{\text{wastewater after ozonation with or without mixing}}$ / peak $area_{\text{conventional treated wastewater}}$) (Figure 4-6) [43].

Comparison of the samples after extraction with either HLB or MCX / MAX cartridges revealed similar results. However, even if the comparison of the ozonated stream before and after recirculation were similar, also some differences could be detected (Figure 4-6).

In general, most TPs of ISO and METO showed constant peak areas in both the ozonated water with and without mixing with the conventionally treated wastewater. Most TPs considered in DCF samples showed decreasing peak areas in both wastewater types (with and without recirculation). The recirculation of the effluent and therefore additional biological treatment can lead to a degradation of TPs [4] and also to a reduction of the overall number of features detected in wastewater via post-treatment [45]. However, this was not observed in this study. Even if for many of considered TPs peak areas in the “decreased” or even “not formed” were detected, this number was very similar in ozonated wastewater with or without recirculation. Yet, peak areas in the categories “increased” or “formation” were mostly detected in ozonated wastewater without mixing. This underlines, that the biological treatment can decrease the amount of the formed TPs. However, for the suspects of METO also increasing peak areas were detected for some TPs after the biological treatment, indicating that those TPs are also formed via biological degradation. This assumption is also supported by the observed number of suspects with “constant” peak areas after mixing the ozonated wastewater with the conventional treated wastewater. This was observed for many suspects of METO but also for some suspects of ISO and for a smaller number of suspects, compared to the other two substances, also for DCF. The results are indicating that some TPs detected during the ozonation of ISO, METO or DCF are not only formed in the reaction with ozone but can also be formed via biological degradation. Indeed, as for all three substances high degradation rates in

conventional WWTP have been reported (ISO up to 63 % [46], METO up to 56 % [5] and DCF up to 80 % [5]) it is not surprising that TPs have been detected in both wastewater types. It has been reported before, that during conventional wastewater treatment also TPs are formed [47] but the possible overlap of TPs formed in conventional and ozonated wastewater has yet not been investigated in detail. Therefore, the comparison of TPs formed during biological degradation and ozonation needs to be addressed more in further studies.

TP formation in different matrix compositions was hardly addressed in literature before and this study demonstrates that even for well-known substances the ozonation in different water matrices can occur via so far unknown reaction pathways. In particular, the influence of different scavengers on the TP formation should be considered in future studies. Further, in upcoming studies also the overlap of TPs formed after ozonation compared to biological treatment of wastewater should be of high interest and investigated in more detail. This should also be investigated for other MPs as TP formation might also take place in the biological treatment leading to the same TPs reported for ozonation. In addition to this study, it is reported elsewhere whether matrix compositions can also influence toxicity to aquatic organisms, as this was not the subject of this study. Thus, not only the evaluation of formed TPs but also the ecotoxicological relevance needs to be investigated in future studies.

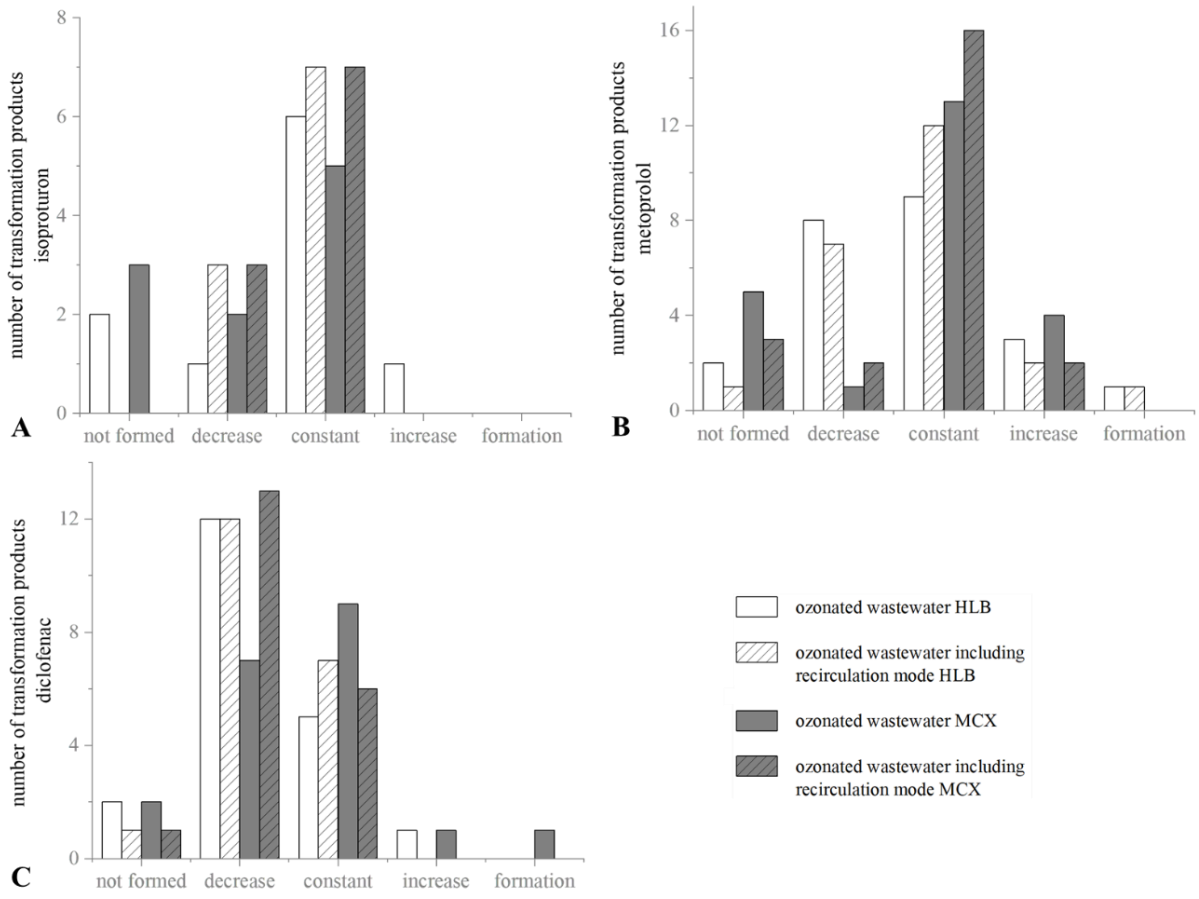


Figure 4-6 **Peak areas of detected products as suspects of (A) isoproturon, (B) metoprolol and (C) diclofenac in ozonated wastewater and ozonated wastewater after mixing with the conventional treated wastewater (recirculation mode) after extraction with HLB or MCX / MAX, respectively, in relation to peak areas detected in wastewater after conventional treatment**

Measurements were performed with HPLC-HRMS and samples were taken at two different time points. All values were blank corrected and the mean value of the two measurements is shown. Results are based on the calculated fold change (fc = peak area$_{\text{wastewater after ozonation with or without mixing}}$ / peak area$_{\text{conventional treated wastewater}}$). Categories are: not formed ($f_c \leq 0.20$), decreased ($f_c \leq 0.50$), constant ($f_c \leq 2.00$), increased ($f_c \leq 5.00$) or formed ($f_c \geq 5.00$). Total number of considered transformation products: ISO = 10, METO = 24, DCF = 20.

4.5 Supporting Information – Chapter 4

4.5.1 Materials & Methods

Table 4-2 List of chemicals and solvents used

Chemical / Solvent	Manufacturer
α-hydroxymetoprolol ≥ 98 %	Sigma Aldrich
2,6-dichloroaniline	Merck
3-(isopropylamino)propane-1,2-diol 95 %	Enamine
5-hydroxy diclofenac analytical Standard	Sigma Aldrich
Acetonitrile > 99 %	Merck / VWR Chemicals
Calibration standard TOC 1000 mg/L ± 10 mg/L	Sigma Aldrich
Cellulose membrane filter 0.45 µm	Whatman
Des(isopropoxyethyl) bisoprolol 98 %	LGC Standards GmbH
Desisopropylmetoprolol 95 %	Enamine
Diclofenac sodium salt	Sigma Aldrich
Dimethyl sulfoxide 99 %	Merck
Dipotassium hydrogenphosphate	AppliChem
Formic acid 98 – 100 %	Merck
Formic acid 99 %	VWR Chemicals
Hydrochloric acid 35 %	Bernd Kraft
Isoproturon ≥ 98 %	Sigma Aldrich
Methanesulfonic acid ≥ 99 %	Merck
Methanesulfinic acid sodium salt 95 %	Alfa Aesar
Methanol 100 %	VWR Chemicals
Methanol LC-MS grade 99.99 %	Fischer Chemicals
Metoprolol tartrate salt > 99 %	Sigma Aldrich
Natural organic matter – 2R101N (200 g)	International Humic Substance Society
o-demethylmetoprolol ≥ 97 %	Sigma Aldrich
Oxygen 99.99 % (200 bar)	Alphagaz

Continued on next page

Chemical / Solvent	**Manufacturer**
Oxygen 99.99 % (200 bar)	Alphagaz
Pierce LTQ velos ESI positive ion calibration solution	Thermo Scientific
Pierce ESI negative ion calibration solution	Thermo Scientific
Sodium dihydrogen phosphate > 99 %	AppliChem
Sodium hydrogen carbonate	Roth
Sodium hydroxide pellets	Bernd Kraft
Sulfuric acid >95 %	Fisher Chemical
tert-butanol	Merck
Ultra-purified water	-
Water LC-MS grade 99.99 %	Fischer Chemicals

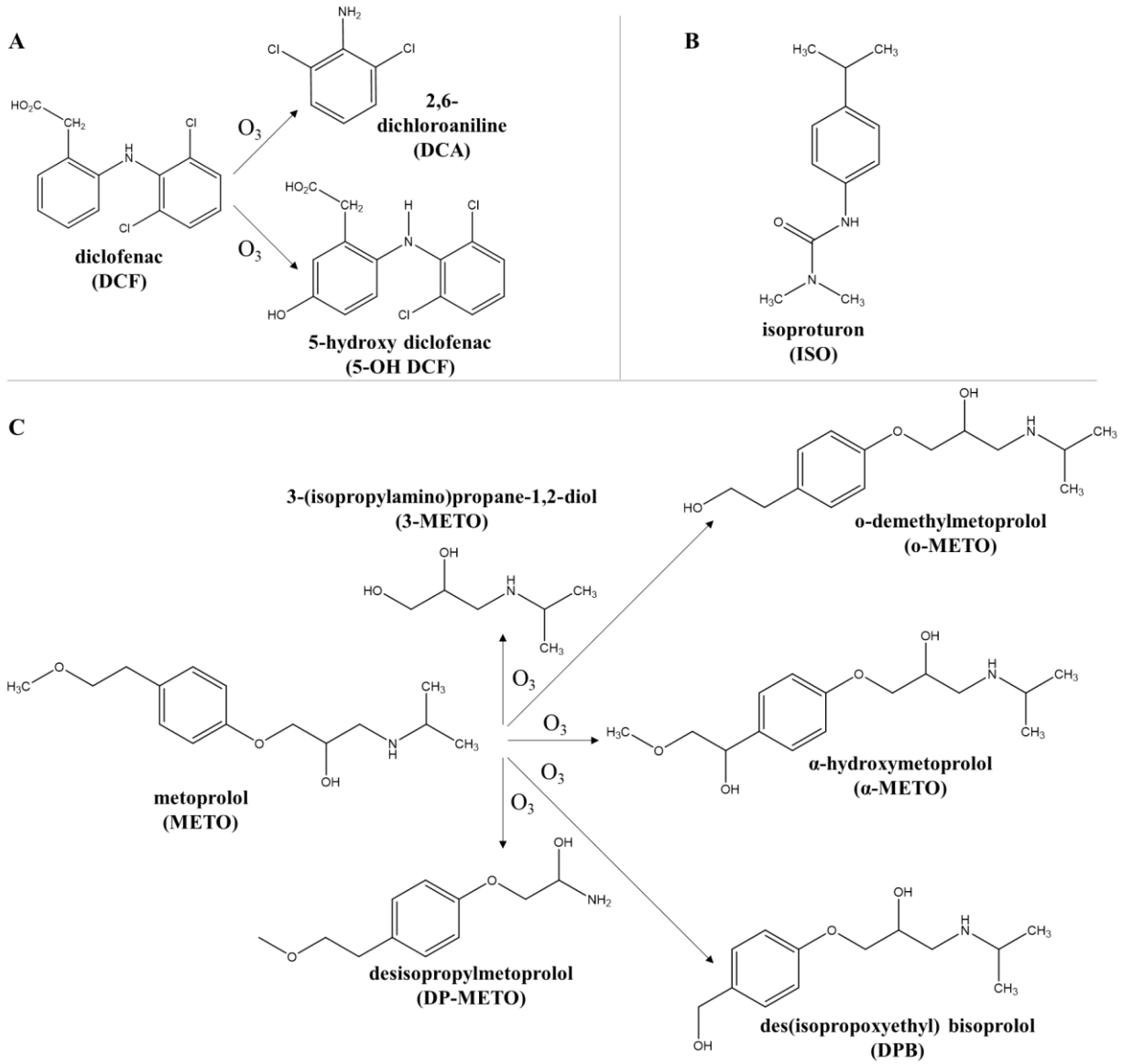


Figure 4-7 Used parent compounds and target transformation products ((A) diclofenac, (B) isoproturon, (C) metoprolol) including structures and abbreviations

Table 4-3 List of equipment used

Technical device	Model	Manufacturer
Ozone generator	COM-AD-01	Anseros
Ozone generator	Philaqua 802x	BMT Messtechnik
pH meter	827 pH Lab	Metrohm

Continued on next page

Technical device	Model	Manufacturer
Shaker	KS 260C	IKA®
Total organic carbon analyzer	TOC-L	Shimadzu
UV-Vis Spectrometer	UV-1800	Shimadzu
UV-Vis Spectrometer	UV-1650PC	Shimadzu

HPLC – DAD

Auto injector	SIL-10ADVP	Shimadzu
Column oven	CTO-10ASVP	Shimadzu
Core shell LC column	Kinetex 5 µm EVO C18 100Å	Phenomenex
Degassing unit	DGU-20A5R	Shimadzu
Diode array detector	SPD-M10AVP	Shimadzu
Fluorescence detector	RF-10AXL	Shimadzu
Liquid chromatograph	LC-10ATVP	Shimadzu
Reservoir tray	-	Shimadzu
System controller	SCL-10AVP	Shimadzu
Valve assembly	FCV-10ALVP	Shimadzu

HPLC – HRMS

Core shell LC column used for analysis of ozonated samples	Kinetex 5 µm EVO C18 100Å	Phenomenex
LC column used for wastewater samples	XSelect HSS T3 2.5 µm 2.1x100 mm	Waters
Mass Spectrometer Orbitrap	Q Exactive	Thermo Scientific
UHPLC (Pump, Autosampler)	Dionex Ultimate 3000 UHPLC^{+}	Thermo Scientific

Continued on next page

Technical device	Model	Manufacturer
IC		
IC Unit	883 Basic IC plus	Metrohm
Autosampler	863 Compact Autosampler	Metrohm
Column	Metrosep A Supp 4 – 250/4.0	Metrohm

Table 4-4 List of software used

Software	Version	Manufacturer
Chromeleon	7.2 SR5	Thermo Scientific
Compound Discoverer	3.0 and 3.1	Thermo Scientific
LC Solution	-	Shimadzu
MagIC	Net 3.2	Metrohm
Thermo XCalibur	4.0.27.10	Thermo Scientific

Equation 4-1 Calculation of minimal scavenger concentration [2]

$$c_{scavenger,min} = \frac{f_{\bullet OH+scavenger} * c_{substance} * k_{\bullet OH+substance} + f_{\bullet OH+scavenger} * c_{O_3} * k_{\bullet OH+O_3}}{k_{\bullet OH+scavenger} * (1 - f_{\bullet OH+scavenger})}$$

Equation 4-2 Calculation of maximal scavenger concentration [2]

$$c_{scavenger,max} = \frac{f_{O_3+scavenger} * c_{substance} * k_{O_3+substance}}{k_{O_3+scavenger} * (1 - f_{O_3+scavenger})}$$

Table 4-5 Reaction rate constants of relevant reactions at 20 °C

Reaction	k_2 ($M^{-1}s^{-1}$)	Reference
$^{\bullet}OH$ + Aniline	1.4×10^{10}	Buxton et al. [48]
$^{\bullet}OH$ + DCF	9.3×10^{9}	Yu et al. [49]
$^{\bullet}OH$ + DMSO	7×10^{9}	Buxton et al. [48]
$^{\bullet}OH$ + ISO	7.9×10^{9}	Benitez et al. [36]
$^{\bullet}OH$ + METO	7.3×10^{9}	Benner et al. [50]
$^{\bullet}OH$ + *tert*-BuOH	6×10^{8}	Buxton et al. [48]
O_3 + Aniline	1.4×10^{10}	Tekle-Röttering et al. [14]
O_3 + $^{\bullet}OH$	1.1×10^{8}	Sehested et al. [51]
O_3 + DCF	6.8×10^{5}	Sein et al. [15]
O_3 + ISO	2.2×10^{3}	Benitez et al. [36]
O_3 + METO	2×10^{3}	Benner et al. [50]
O_3 + DMSO	8.2	Pryor et al. [39]
O_3 + *tert*-BuOH	3×10^{-3}	Hoigné and Bader [40]

Table 4-6 Instrument settings for target analysis with HPLC-DAD

Unit	Parameter	Value
HPLC	Injection volume	50 µL
	Flow rate	0.3 mL/min (metoprolol and isoproturon) 0.5 ml/min (diclofenac)
	Eluents	A: water + 0.1 % formic acid B: methanol + 0.1 % formic acid
	Gradient	Isocratic for diclofenac 0-15 min: 40 % A, 60 % B Gradient for metoprolol and isoproturon: 0–7 min: 90 % A, 10 % B 10–13 min: 10 % A, 90 % B 13.1–30 min: 90 % A, 10 % B

Table 4-7 Wavelengths used for target analysis with HPLC-DAD

Compound	**Wavelength / nm**
isoproturon	239
metoprolol	273
α-hydroxymetoprolol	273
o-demethylmetoprolol	273
desisopropylmetoprolol	273
des(isopropoxyethyl) bisoprolol	273
3-(isopropylamino)propane-1,2-diol	not detectable via HPLC-DAD
diclofenac	280
2,6-dichloroaniline	220
5-hydroxy diclofenac	260

Table 4-8 Instrument settings LC-HRMS for measurement of metoprolol and isoproturon

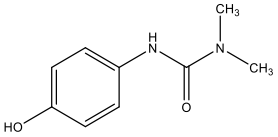
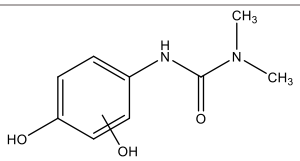
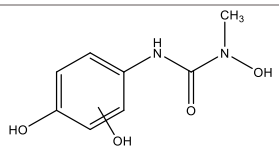
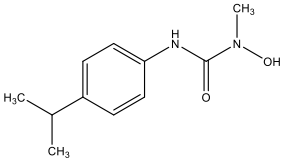
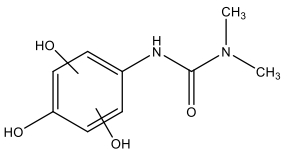
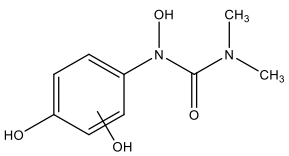
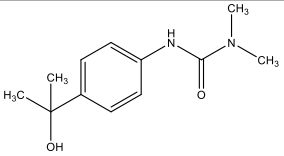
Unit	Parameter	Value
HPLC		
	Injection volume	10 µL
	Flow rate	0.3 mL/min
	Eluents	A: water + 0.1 % formic acid B: methanol + 0.1 % formic acid
	Gradient	0-4 min: 90 % A, 10 % B 7-10 min: 10 % A, 90 % B 10.1-20 min: 90 % A, 10 % B
HRMS	Scan type	Full MS
	Scan range	100.0 to 1000.0 m/z
	Fragmentation	None
	Resolution	70000
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	10 s
	AGC target	1e6
	Maximum inject time	100
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	320 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C

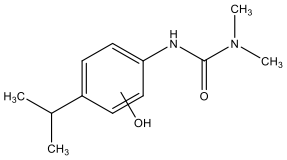
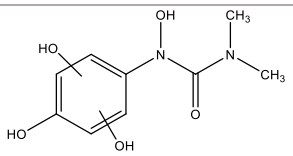
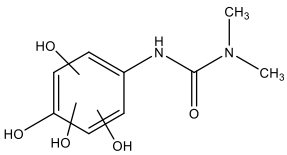
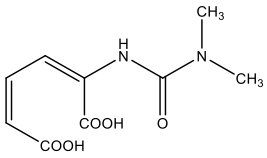
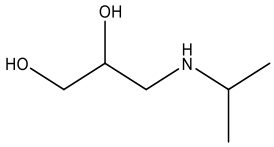
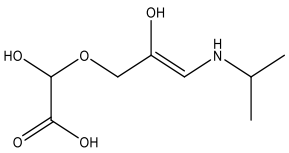
Table 4-9 Instrument settings LC-HRMS for measurement of diclofenac

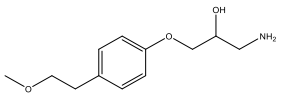
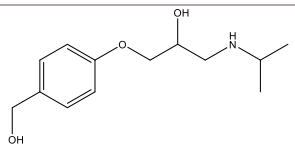
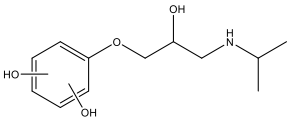
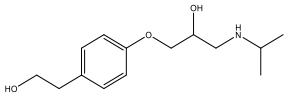
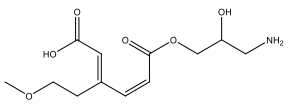
Unit	Parameter	Value
HPLC	Injection volume	100 µL
	Flow rate	0.3 mL / min
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	0-4 min: 80 % A, 20 % B
		4-10 min: 20 % A, 80 % B
		10-15 min: 80 % A, 20 % B
HRMS	Scan type	Full MS
	Scan range	100.0 to 1000.0 m/z
	Fragmentation	None
	Resolution	70000
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	10 s
	AGC target	1e6
	Maximum inject time	100
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	320 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C

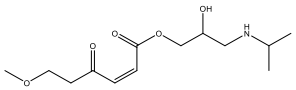
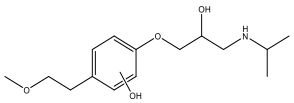
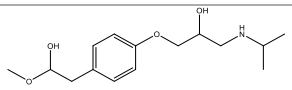
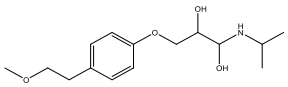
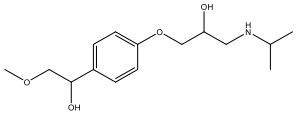
Table 4-10 **Previously reported transformation products (TPs) of isoproturon, metroprolol and diclofenac after ozonation.**

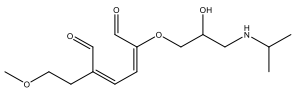
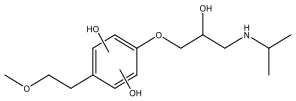
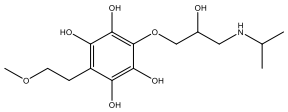
Experimental setups included different pH adjustments, addition of scavenger (w sc.) or no addition (w/o sc.). Confirmation with (A) Comparison of main fragments, (B) MS^2 spectra, (C) Comparison of fragments pattern, (D) Comparison of fragments pattern and reported before, (E) Confirmed with a reference standard in this study (red)

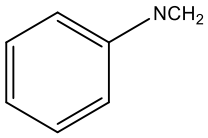
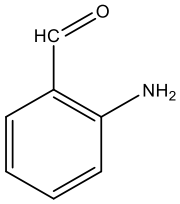
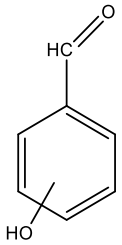
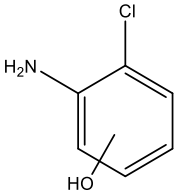
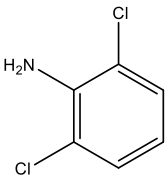
TP	[M + H$^+$]	Sum formula	Structure	Set up	Confir-mation	Reference
ISO						
TP 180	181.09715	$C_9H_{12}N_2O_2$		pH 7, w/o sc.	A	[20]
TP 196	197.09207	$C_9H_{12}N_2O_3$		pH 7, w/o sc.	A	[20]
TP 198	199.07133	$C_8H_{10}N_2O_4$		pH 7, w/o sc.	A	[20]
TP 208	209.12845	$C_{11}H_{16}N_2O_2$		pH 7, w/o sc.	A	[20]
TP 212	213.08698	$C_9H_{12}N_2O_4$	 or 	pH 7, w/o sc.	A	[20]
TP 222_1	223.14410	$C_{12}H_{18}N_2O_2$		pH 7, w/o sc.	A	[20]

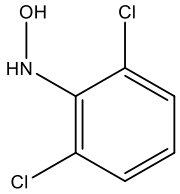
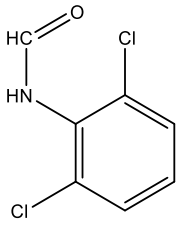
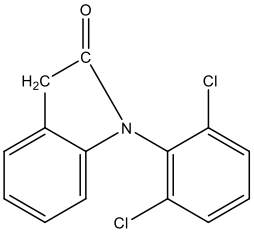
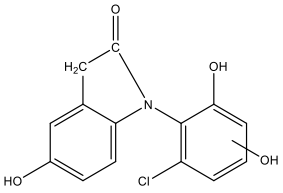
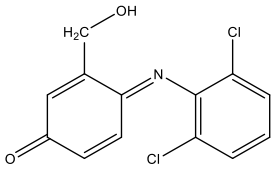
TP	[M + H⁺]	Sum formula	Structure	Set up	Confir-mation	Reference
TP 222_2 or TP 222_3	223.14410	$C_{12}H_{18}N_2O_2$		pH 7, w/o sc.	A	[20]
TP 228_1 or TP 228_2	229.08190	$C_9H_{12}N_2O_5$	 or  or 	pH 7, w/o sc.	A	[20]
METO						
TP 133	134.11756	$C_6H_{15}NO_2$		no pH, w sc. and w/o sc.	B E	[21]
TP 205	206.10230	$C_8H_{15}NO_5$		no pH, w sc. and w/o sc.	B	[21]

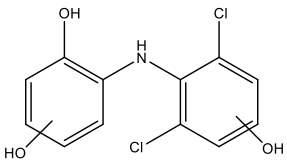
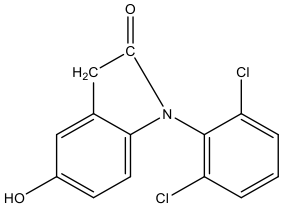
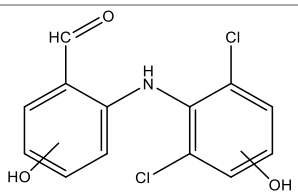
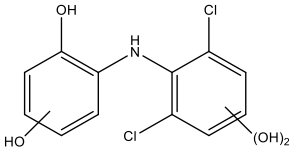
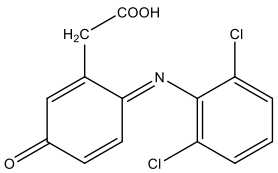
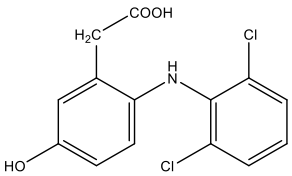
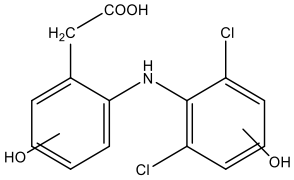
TP	[M + H$^+$]	Sum formula	Structure	Set up	Confir-mation	Reference
TP 225	226.14377	$C_{12}H_{19}NO_3$		pH 8, w sc. and w/o sc.; no pH, w/o sc.	B E	[21, 30, 31]
TP239	240.15942	$C_{13}H_{21}NO_3$		pH 8, w/o sc.	B E	[30, 31]
TP 241	242.13868	$C_{12}H_{19}NO_4$		pH 3, w sc. and w/o sc.; pH 8, w sc.	MS^2 spectra	[30, 31]
TP 253_1	254.17507	$C_{14}H_{23}NO_3$		pH 8, w/o sc.	B E	[30, 31]
TP 253_2	254.17507 or 254.13868	$C_{14}H_{23}NO_3$ or $C_{13}H_{19}NO_4$	No structure	pH 8, w sc. and w/o sc.	B	[30, 31]
TP 273_1	274.12851	$C_{12}H_{19}NO_6$		pH 3, w sc. and w/o sc.	B	[30]

TP	[M + H⁺]	Sum formula	Structure	Set up	Confir-mation	Reference
TP 273_2	274.16490	$C_{13}H_{23}NO_5$		pH 3, w sc. and w/o sc.; no pH, w sc. and w/o sc.	B	[21, 30]
TP 283_1	284.18563	$C_{15}H_{25}NO_4$		pH 8, w sc. and w/o sc.; no pH, w/o sc.	B	[21, 30, 31]
TP283_2	284.14925	$C_{14}H_{21}NO_5$	No structure	pH 8, w sc. and w/o sc.	B	[30]
TP 283_3	284.18563	$C_{15}H_{25}NO_4$	 or  or 	pH 8, w sc. and w/o sc.	B E (3rd struc-ture)	[30, 31]

TP	[M + H$^+$]	Sum formula	Structure	Set up	Confir-mation	Reference
TP 299_1	300.1855	$C_{15}H_{25}NO_5$		pH 3, w sc. and w/o sc.	B	[30]
TP 299_2	300.1855$^+$	$C_{15}H_{25}NO_5$	No structure	pH 3, w sc. and w/o sc.	B	[30]
TP 299_3	300.1855	$C_{15}H_{25}NO_5$	No structure	pH 3, w sc. and w/o sc.	B	[30]
TP 299_4	300.1855	$C_{15}H_{25}NO_5$	No structure	pH 3, w sc. and w/o sc.	B	[30]
TP 299_5	300.1806	$C_{15}H_{25}NO_5$		no pH, w sc. and w/o sc.	B	[21]
TP 331	332.17038	$C_{15}H_{25}NO_7$		no pH, w/o sc.	B	[21]

TP	[M + H⁺]	Sum formula	Structure	Set up	Confir-mation	Reference
DCF						
TP 105	106.06513	C_7H_7N		no pH, w/o sc.	C	[26]
TP 121	122.06004	C_7H_7NO		no pH, w/o sc.	C	[26]
TP 122	123.04406	$C_7H_6O_2$		no pH, w/o sc.	C	[26]
TP 133	134.06004	C_8H_7NO	No structure	no pH, w/o sc.	C	[26]
TP 143	144.02107	C_6H_6NOCl		no pH, w/o sc.	C	[26]
TP 160	161.98718	$C_6H_5NCl_2$		no pH, w sc. and w/o sc.	E	[15, 26]

TP	[M + H⁺]	Sum formula	Structure	Set up	Confir-mation	Reference
TP 176	177.98210	$C_6H_5NOCl_2$		no pH, w/o sc.	D	[26]
TP 188	189.98210	$C_7H_5NOCl_2$		no pH, w/o sc.	C	[26]
TP 240	241.97701	$C_{10}H_5NO_2Cl_2$	No structure	no pH, w/o sc.	C	[26]
TP 258	259.98758	$C_{10}H_7NO_3Cl_2$	No structure	no pH, w/o sc.	C	[26]
TP 277	278.01340	$C_{14}H_9NOCl_2$		no pH, w/o sc.	C	[26]
TP 279	280.03711	$C_{13}H_{10}NO_4Cl$		no pH, w/o sc.	C	[26]
TP 281	282.00831	$C_{13}H_9NO_2Cl_2$		no pH, w/o sc.	D	[26]

TP	[M + H$^+$]	Sum formula	Structure	Set up	Confir-mation	Reference
TP 285	286.00323	$C_{12}H_9NO_3Cl_2$		no pH, w/o sc.	D	[26]
TP 293	294.00831	$C_{14}H_9NO_2Cl_2$		no pH, w/o sc.	D	[26]
TP 297	298.00323	$C_{13}H_9NO_3Cl_2$		no pH, w/o sc.	C	[26]
TP 300	301.99814	$C_{12}H_9NO_4Cl_2$		no pH, w/o sc.	C	[26]
TP 309	310.00323	$C_{14}H_9NO_3Cl_2$		no pH, w sc. and w/o sc.	D	[15, 26]
TP 311	312.01888	$C_{14}H_{11}NO_3Cl_2$		no pH, w sc. and w/o sc.	E	[15, 26]
TP 327	328.01379	$C_{14}H_{11}NO_4Cl_2$		no pH, w/o sc.	D	[26]

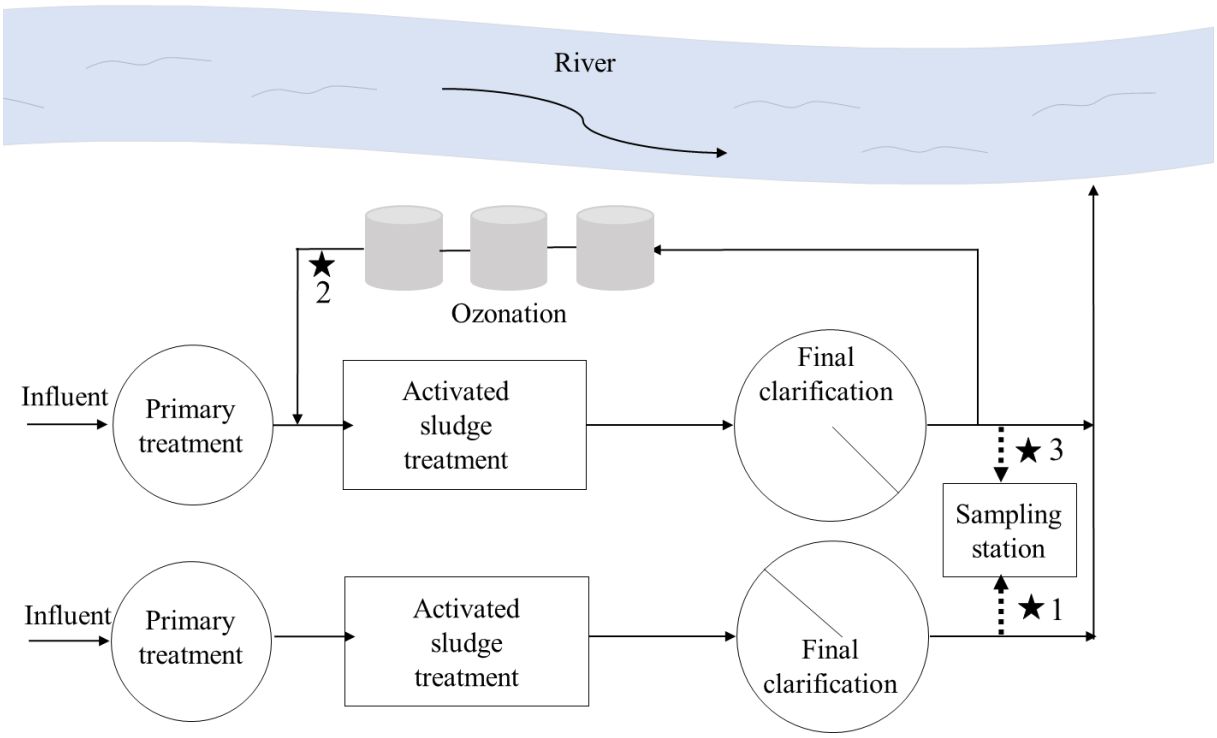


Figure 4-8 Scheme of the wastewater treatment plant including sampling sites

Table 4-11 On-site parameters of the wastewater treatment plant effluent sampling

	pH		**Conductivity [µS/cm]**		**Temperature [°C]**	
Day of measurement	1	2	1	2	1	2
Effluent conventional treatment	6.4	7.1	852	926	17.5	21.2
Effluent with oxidation step	6.6	7.1	858	911	18.1	21.7

Table 4-12 Instrument settings LC-HRMS for measurement of wastewater treatment plant effluents

Unit	Parameter	Value
HPLC	Injection volume	50 µL
	Flow rate	0.3 mL/min
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	0-2 min: 100 % A, 0 % B
		4 min: 50 % A, 50 % B
		17-22 min: 2 % A, 98 % B
		22.1-30 min: 100 % A, 0 % B
HRMS General	Scan type	Full MS / dd-MS^2
	Scan range	80.0 to 1000.0 m/z
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	6 s
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	300 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C
Full MS	Resolution	70000
	AGC target	1e6
	Maximum inject time	100 ms

Continued on next page

Unit	Parameter	Value
dd-MS² / dd-SIM	Resolution	17500
	AGC target	5e4
	Maximum inject time	50 ms
	Loop count	5
	Top N	5
	Isolation window	1.4 m/z
	NCE stepped	30,60, 80
dd settings	Minimum AGC target	8e2
	Intensity threshold	1.6e4
	Dynamic exclusion	3.0 s

Table 4-13 Instrument settings for aniline and 2,6-dichloroaniline measurements with HPLC-DAD

Unit	Parameter	Value
HPLC	Injection volume	50 µL
	Flow rate	0.5 mL/min
	Eluents	A: water + 10 mM NH_4COOH
		B: methanol
	Gradient	0–5 min: 95 % A, 5 % B
		5–8 min: 20 % A, 80 % B
		15–25 min: 95 % A, 5 % B

4.5.2 Results & Discussion

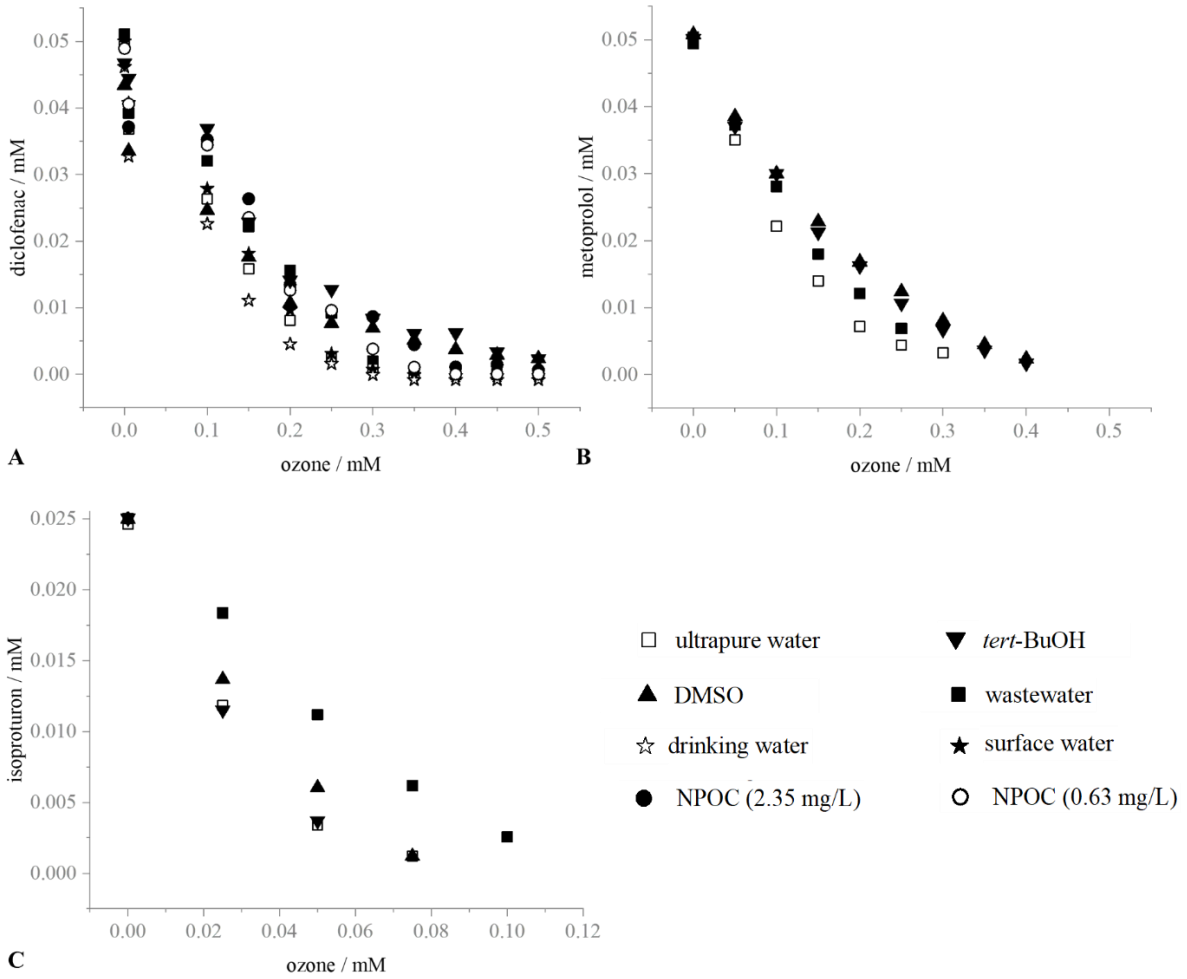
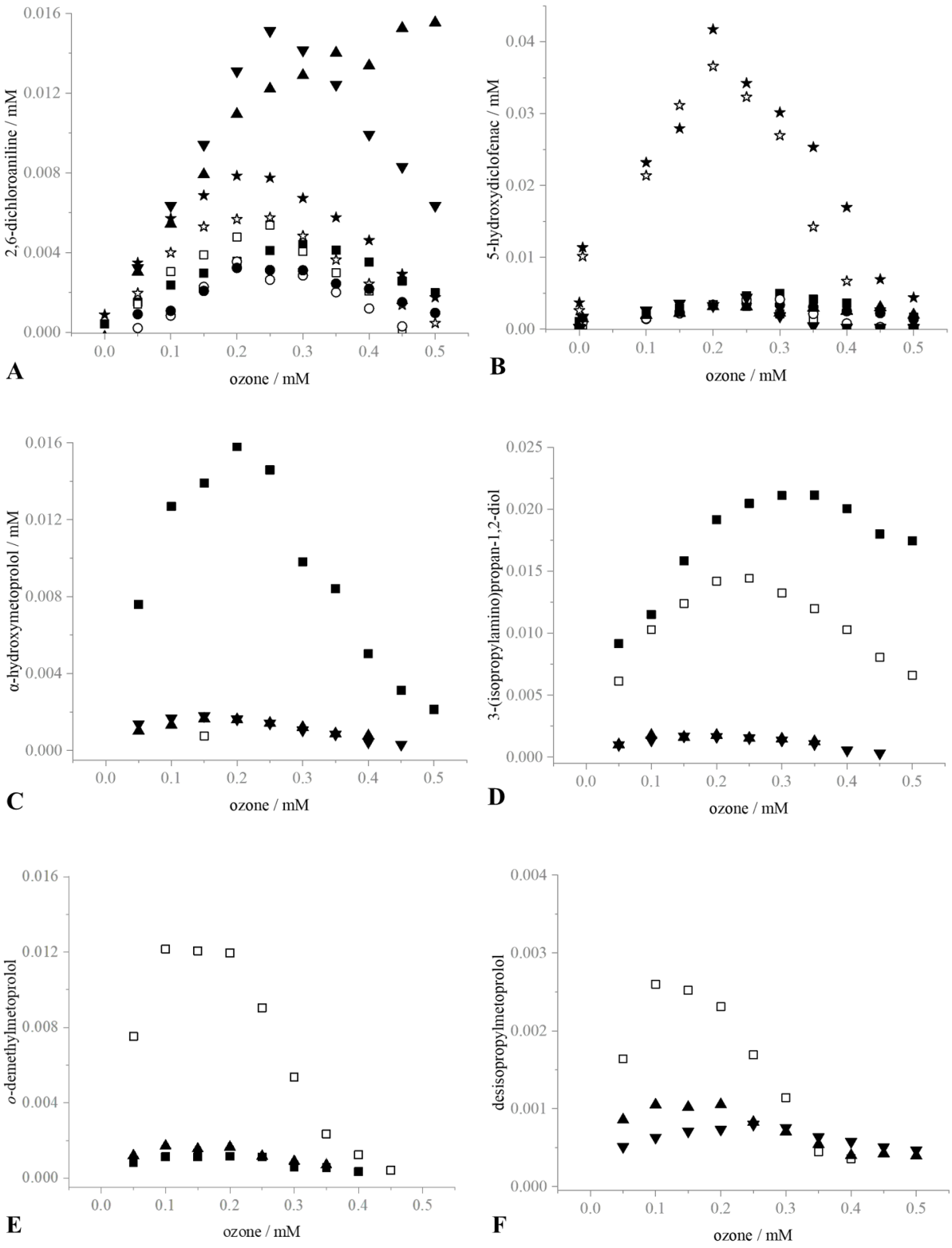


Figure 4-9 Degradation of (A) diclofenac, (B) metoprolol and (C) isoproturon in different water matrices

Starting concentration of the three parent compounds were 0.05 mM (diclofenac and metoprolol) and 0.025 mM (isoproturon), respectively. All substances were treated with different ozone concentrations between 0.05 – 0.5 mM (metoprolol and diclofenac) and 0.025 – 0.25 mM (isoproturon), respectively. The added scavenger concentrations were for DMSO 20mM in diclofenac samples and 0.54 mM in metoprolol samples. For *tert*-BuOH 20 mM in diclofenac samples and 6.3 mM in metoprolol samples were added. In ISO samples 3.375 mM *tert*-BuOH and 0.29 mM DMSO were added. In detail, ultrapure water □, *tert*-BuOH ▼, DMSO ▲ and wastewater ■ were tested. For DCF additionally drinking water ☆, surface water ★ and two NOM concentrations (NPOC: 2.35 mg/L ● and NPOC: 0.63 mg/L ○) were analyzed. Mean values of one to three single experiments are shown. Individual uncertainties are not shown as it would lead to great complexity.


2,6-dichloroaniline / mM
ozone / mM
A
5-hydroxydiclofenac / mM
ozone / mM
B
α-hydroxymetoprolol / mM
ozone / mM
C
3-(isopropylamino)propan-1,2-diol
ozone / mM
D
o-demethylmetoprolol
ozone / mM
E
desisopropylmetoprolol
ozone / mM
F

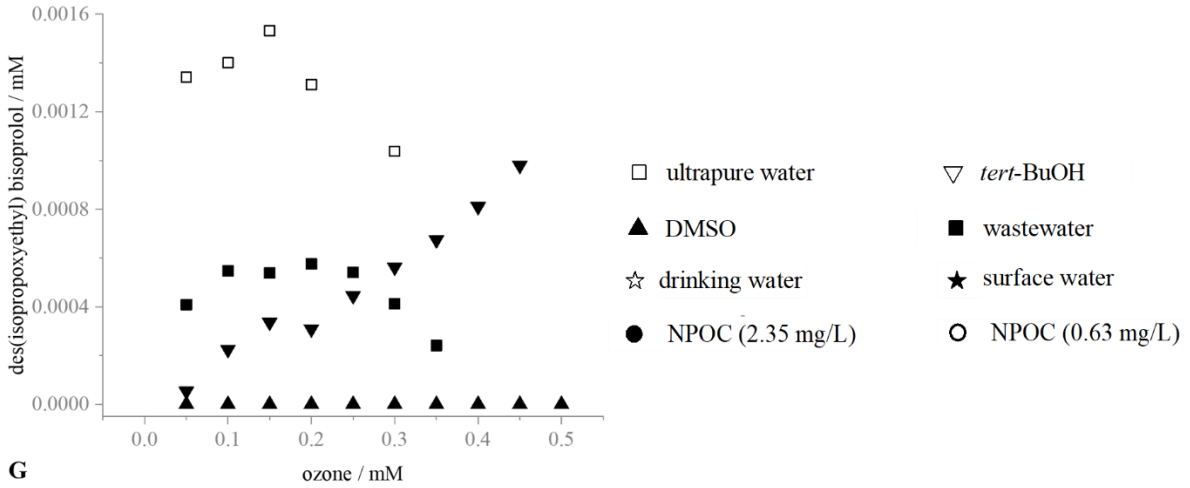


Figure 4-10 Formation of (A) 2,6-dichloroaniline, (B) 5-hydroxydiclofeanc, (C) 3-(isopropylamino)propane-1,2-diol, (D) α-hydroxymetoprolol, (E) *o*-demethylmetoprolol, (F) desisopropylmetoprolol and (G) des(isopropoxyethyl) bisoprolol in different water matrices

Starting concentration of the diclofenac and metoprolol were 0.05 mM and ozone concentrations ranged from 0.05 – 0.5 mM. While in diclofenac samples no pH adjustment was performed, the pH in metoprolol samples was set to 7. The added scavenger concentrations were for DMSO 20mM in diclofenac samples and 0.54 mM in metoprolol samples. For *tert*-BuOH 20 mM in diclofenac samples and 6.3 mM in metoprolol samples were added. Measurements were performed via HPLC-DAD and in duplicate or triplicate. In detail, ultrapure water ⊔, *tert*-BuOH ▼, DMSO ▲ and wastewater ■ were tested. For DCF additionally drinking water ☆, surface water ★ and two NOM concentrations (NPOC: 2.35 mg/L ● and NPOC: 0.63 mg/L ○) were analyzed. Mean values of one to three single experiments are shown. Individual uncertainties are not shown as it would lead to great complexity.

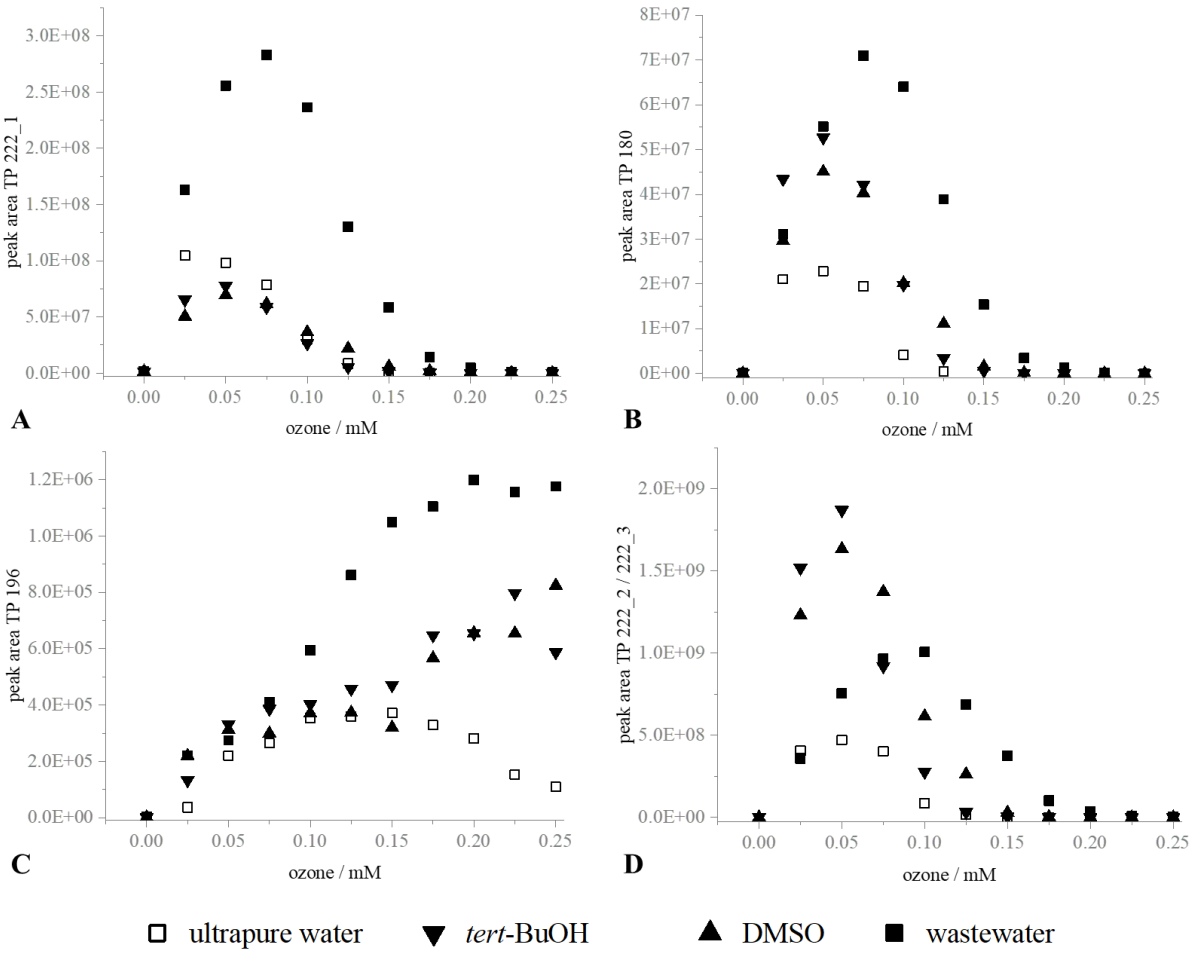


Figure 4-11 Formation of transformation products ((A) TP 222_1, (B) TP 180, (C) TP 196 and (D) TP 222_2/222_3) considered in the suspect screening of isoproturon after the ozonation in different water matrices

Starting concentration of isoproturon was 0.025 mM and ozone concentrations ranged from 0.025 – 0.25 mM. The pH was set to 7 and added scavenger concentrations were 3.375 mM *tert*-BuOH and 0.29 mM DMSO, respectively.

In detail, ultrapure water □, *tert*-BuOH ▼, DMSO ▲ and wastewater ■ were tested. Mean values of one to three single experiments are shown. Individual uncertainties are not shown as it would lead to great complexity.

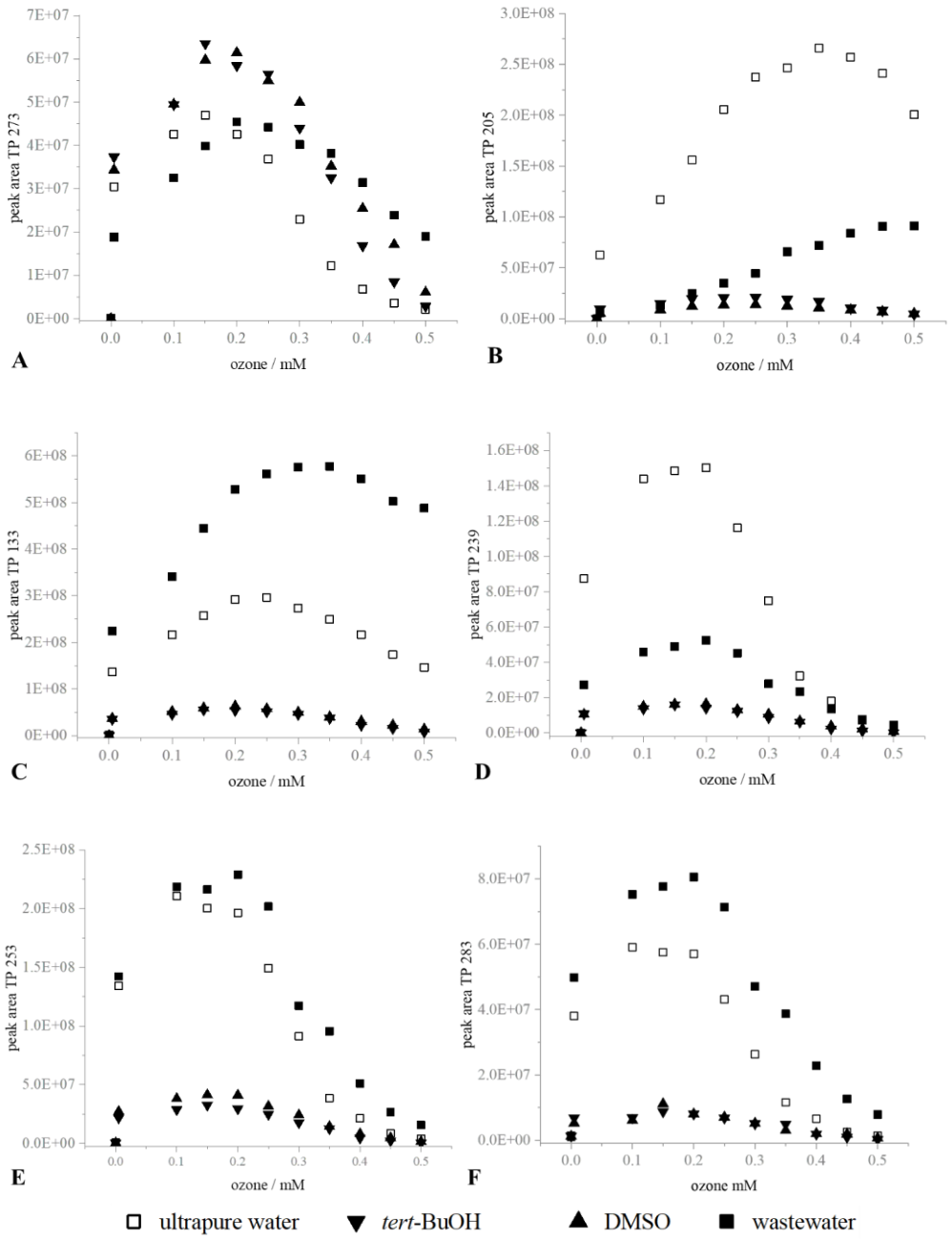


Figure 4-12 Formation of transformation products ((A) TP 273_2, (B) TP 205, (C) TP 133, (D) TP 239, (E) TP 253 and (F) TP 283) considered in the suspect screening of metoprolol after the ozonation in different water matrices

Starting concentration of metoprolol was 0.05 mM and ozone concentrations ranged from 0.05 – 0.5 mM. The pH was set to 7 and the added scavenger concentrations were 0.54 mM for DMSO and 6.3 mM for *tert*-BuOH. In detail, ultrapure water □, *tert*-BuOH ▼, DMSO ▲ and wastewater ■ were tested. Mean values of one to three single experiments are shown. Individual uncertainties are not shown as it would lead to great complexity.

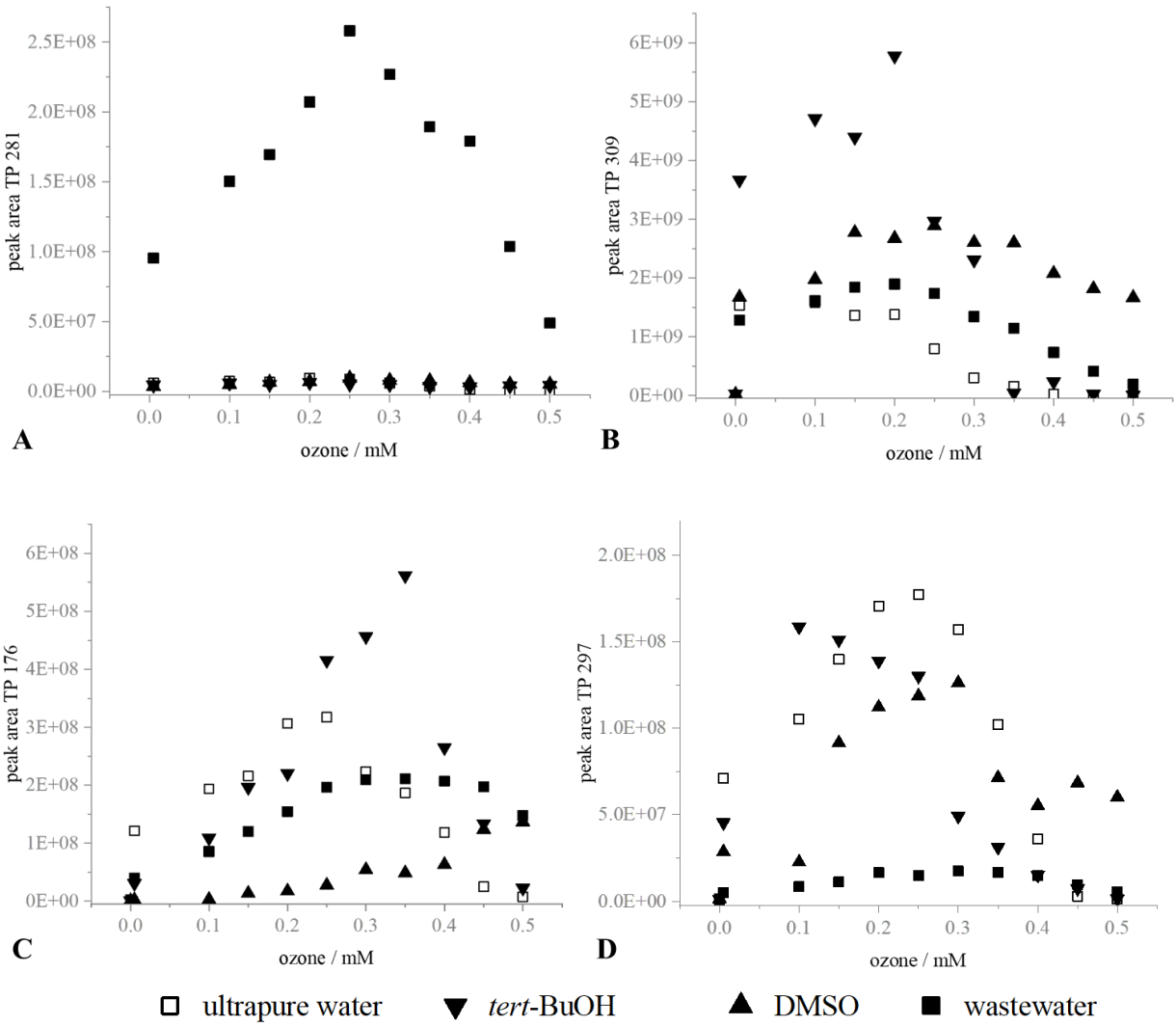


Figure 4-13 Formation of transformation products ((A) TP 281, (B) TP 309, (C) TP 176 and (D) TP 297) considered in the suspect screening of diclofenac after the ozonation in different water matrices

Starting concentration of diclofenac was 0.05 mM and ozone concentrations ranged from 0.05 – 0.5 mM. The pH was not adjusted and 20 mM were added for DMSO and *tert*-BuOH, respectively. In detail, ultrapure water □, *tert*-BuOH ▼, DMSO ▲ and wastewater ■ were tested. Mean values of one or two single experiments are shown. Individual uncertainties are not shown as it would lead to great complexity.

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Chapter 5

Influence of bromide in the water matrix on the formation of reaction products during ozonation of *N*-containing substances

5.1 Abstract

Bromide as an omnipresent matrix component in wastewater can react with ozone to form hypobromous acid (HOBr). This secondary oxidant can subsequently react with micropollutants but also with formed intermediates. Therefore, bromide and especially HOBr can highly influence the formation of transformation products (TPs). This has already been reported for the ozonation of *N*,*N*-dimethylsulfamide leading to the formation of the cancerogenic of *N*-nitrosodimethylamine only in bromide containing waters.

In this study, the influence of different bromide and ozone concentrations on the formation of TPs during the ozonation of isoproturon (ISO), metoprolol (METO) and diclofenac (DCF) were investigated. Additionally, TPs were identified, which are formed in the direct reaction of the micropollutants with HOBr with and without subsequent ozonation.

The results showed that even if the reactions of ozone with the substances should be favored bromide can highly influence the formation of TPs already at low concentrations. In summary, new TPs after the reaction with HOBr (and subsequent ozonation) could be postulated for ISO, METO and DCF. This underlines that the present water matrix can have a high influence on the formation of TPs and that these mechanisms need to be investigated further.

5.2 Introduction

Nitrogen-containing substances include various pharmaceuticals and/or pesticides such as isoproturon (ISO), metoprolol (METO) and diclofenac (DCF) and are therefore of high interest. Recently it was reported that around 90% of substances detected in a screening of the River Rhine, contained nitrogen [1]. Sources for these micropollutants (MPs) in the aquatic environment can be wastewater treatment plants (WWTP), industrial wastewaters or run-off from agriculture [2]. Especially pharmaceuticals, such as DCF and METO, are widely detected in WWTP effluents and surface waters [3-5], but also the pesticide ISO has been determined in WWTP effluents [6] and surface waters [7] despite its ban in 2016 in the European Union [8].

To remove the load of MPs next to conventional wastewater treatment an additional treatment step is increasingly implemented. This can include activated carbon, membrane filtration or advanced oxidation processes such as ozonation [2]. As ozonation can reduce the amount of MPs and is also a disinfectant, it is widely applied [9]. During ozonation MPs are chemically transformed rather than mineralized, which can lead to the formation of unwanted transformation products (TPs).

In the reaction of ozone (O_3) with nitrogen containing substances many intermediates can be formed. These are mostly highly reactive and include aminyl ($^{\bullet}NR_2$) and nitroxide radicals ($^{\bullet}ONR_2$) but also hydroxyl radicals ($^{\bullet}OH$) which can react with different components present in the water matrix. The influence of matrix components, such as natural organic matter (NOM) and bromide (Br^-), has hardly been investigated in detail yet, as most studies focus on the reaction of substances in ultrapure water, wastewater or the influence of scavengers [10-16]. However, the interaction of matrix components with MPs leads to the formation of hardly predictable and possibly also unwanted TPs. Especially, the influence of Br^- on the formation of TPs should be considered as it is omnipresent in waters at concentrations typically ranging from 0.01 to 1 mg/L [17]. The reaction of Br^- with O_3 can lead to the formation of bromate which has been reported to be possibly carcinogenic [18, 19]. Further, the essential role of bromide as matrix component has been demonstrated in a landmark study on the formation of *N*-nitrosodimethylamine (NDMA) from the ozonation of *N*,*N*-dimethylsulfamide (DMS) as the presence of Br^- was essential for NDMA formation. In detail, the reaction of Br^- with ozone leads to the formation of the secondary oxidant hypobromous acid (HOBr) which then can react with the nitrogen of DMS to form Br-DMS that reacts with O_3 in a subsequent step to the carcinogenic TP NDMA [9, 20-22]. Therefore, in the absence of bromide, NDMA formation does not occur. However, bromide is not only omnipresent but actually also acts as

catalyst within this reaction mechanisms as it is also released in the reaction of Br-DMS with ozone [22]. Therefore, the influence of bromide needs to be considered in the water treatment. Especially, as NDMA has also been detected in final drinking waters after purification [20] and thus, other unwanted TPs might also reach drinking water.

However, although this observation underlines that the formation of relevant TPs during ozonation can be highly influenced by bromide, it is seldomly addressed in literature beyond formation of bromate as oxidation by-product. To fill this gap, the influence of Br^- and in-situ formed HOBr on the TP formation during ozonation has been investigated in detail in the present study for the three common MPs ISO, METO and DCF. TPs were identified by high resolution mass spectrometry (HRMS) and possible structures tentatively suggested.

5.3 Materials & Methods

5.3.1 Chemicals

Chemicals and solvents were used as received from the according supplier and are listed in Table 5-1 (Supporting Information (SI)).

5.3.2 Equipment & Software

The used equipment and software are listed in Table 5-2 and Table 5-3 (SI).

5.3.3 Generation of stock solution

All stock solutions were prepared in ultrapure water if not stated otherwise (0.5 mM METO and DCF, 0.058 mM ISO (shaken for several days at room temperature in the dark to ensure complete dissolution)). Solutions were stored at 4 °C and calibrations included two orders of magnitudes (0.0005 – 0.05 mM, prepared in corresponding water matrix).

Oxygen was enriched by an ozone generator (COM (Anseros) or Philaqua 802x (BMT Mestechnik)) and the ozone was then bubbled into ice cooled ultrapure water (≥ 60 minutes) to prepare the ozone stock solution. In all experiments the concentration of the ozone stock

solution ranged between 1.1 and 1.6 mM and was determined by measuring the absorption with a UV-spectrometer (UV-1650PC or UV-1800, Shimadzu, 258 nm, ε_{O3} = 2950 $M^{-1}cm^{-1}$ [23]).

For the preparation of HOBr 0.8 mM sodium bromide solution were ozonated with 1 mM ozone at pH 4 (phosphate buffer) [24]. Storage and further treatment were done as described by Fischbacher et al. [25]. The spectrometric measurement was performed as described by Troy and Margerum [26].

5.3.4 Reaction of the parent compounds with hypobromous acid and in the presence of different bromide concentrations

To determine the influence of bromide (Br^-) in the water matrix two approaches were applied within this study. In the reaction of ozone with Br^- the secondary oxidant hypobromous acid (HOBr) is formed, which can react with present substances. Therefore, as an extreme scenario the reaction of HOBr with the model compounds with and without subsequent ozonation was investigated and compared with the in-situ HOBr formation in the presence of different bromide and ozone ratios.

Reaction stoichiometry of parent compounds with hypobromous acid

Prior to the experiments considering ozonation of the three model compounds after their reaction with HOBr the stoichiometry of the parent substances with HOBr was determined. To that end, DCF (0.05 mM), (METO, 0.05 mM) and isoproturon (ISO, 0.025 mM) were treated with three different molar ratios of HOBr (1 : 1, 2 : 1 and 5 : 1) and the residual concentration of the substances was measured via HPLC-DAD on the next day (data not shown). The reaction stoichiometry was calculated using the reciprocal value of the slopes from the degradation curves leading to a stoichiometry of HOBr with the substances of 2.5 : 1 (METO), 1 : 1 (ISO) and 2 : 1 (DCF), respectively. These concentration ratios were used in the following experiments considering the influence of HOBr.

Sample preparation

Solutions of DCF (0.05 mM), METO (0.05 mM) and ISO (0.025 mM) were prepared and HOBr (0.125 mM for METO, 0.025 mM for ISO and 0.1 mM for DCF) was added followed

by storage overnight at 4 °C. Afterwards, the pH was adjusted to 7 for all substances and different ozone concentrations between 0 – 0.5 mM (DCF and METO) and 0 – 0.25 mM (ISO), respectively, were added.

Furthermore, different concentrations of Br^- were added as a competitor for ozone to a solution containing single substances and treated with different ozone concentrations, leading to in-situ formation of HOBr. The experiments were performed in ultrapure water and the concentrations of the substances were kept constant (0.05 mM DCF and METO; 0.025 mM ISO) while the ozone or Br^- concentrations varied, respectively. For detailed information about the concentrations see Table 5-4 (SI).

It should be noted that concentrations present in the aquatic environment are smaller than the concentrations applied in these experiments. However, as usual in such mechanistic investigations this approach was chosen to ensure higher concentrations of TPs and therefore to simplify their detection [18].

Sample measurement

The degradation of the parent compounds was measured via HPLC-DAD (Shimadzu) at the following wavelengths: ISO 239 nm, METO 273 nm and DCF 280 nm. An isocratic eluent (Table 5-5, SI) was used for DCF measurements and a gradient was applied for METO and ISO measurements (Table 5-5, SI).

To measure the formation of TPs a HPLC-HRMS (Dionex Ultimate 3000 $UHPLC^+$ and Orbitrap Q Exactive, Thermo Scientific) was used. In this approach a gradient was used for all measurements (Table 5-6, SI, for ISO / METO and Table 5-7, SI, for DCF) and positive mode ionization was used to measure all samples.

For HPLC-DAD as well as HPLC-HRMS measurements an EVO C18 column (Kinetex 5 µm EVO C18 100 Å 100 × 3.0 mm, Phenomenex) was used. Eluents were ultrapure water + 0.1% formic acid and methanol + 0.1% formic acid. Additionally, formic acid (0.1 – 0.2%) was added to DCF and METO samples before measurement to achieve better peak shapes. This was not needed for ISO samples.

5.3.5 Data evaluation

Target analysis / Suspect screening

The degradation of the parent compounds measured with HPLC-DAD was evaluated by peak areas at the specific absorption maximum of the substances (reference standards available). All samples were also evaluated in terms of previously reported TPs formed during ozonation of ISO, METO and DCF [27]. A suspect screening was carried out as no standards were available for these TPs and evaluated with XCalibur 4.0. The *m/z* of the suspects were calculated using the tuning software of the Orbitrap system and a processing method was written based on the *m/z* values of detected peaks and the corresponding retention time including also TPs detected in the non-target screening after the reaction with HOBr. The results were exported to Quan Browser.

Non-Target data evaluation with Compound Discoverer

This study was the first to analyze TPs formed during the reaction of DCF, METO and ISO with HOBr and additional ozonation or at different O_3/Br^- ratios. Therefore, the data gained via HPLC-HRMS measurements of these experiments were analyzed with a non-target screening method. Peak processing of the non-target data was performed using "Compound Discoverer 3.0" and "Compound Discoverer 3.1" (Thermo Scientific). To process the data the workflow template "Environmental w Stats Unknown ID w Online and Local Database Searches" was used (without steps for analysis of MS^2 spectra) and the procedure presented by Hohrenk et al. [28] was followed. Fragmentation patterns (including isotope patterns) were also considered and suggested sum formulas were manually checked for suitability (containing only C, H, O, N and Br). A blank subtraction was automatically processed.

Furthermore, to reduce the number of possible features, the results were checked for peak shape and the mass spectra of features with similar retention times were compared to eliminate false positives by fragments formed in the ESI source. Proposed structures were generated based on possible reaction pathways.

5.4 Results & Discussion

5.4.1 Influences of hypobromous acid on the formation of TPs during ozonation

The reaction of ozone with bromide yields the secondary oxidant HOBr which can further lead to the formation of unknown TPs [19, 29]. As no studies were published so far considering the influence of HOBr on the formation of TPs during the ozonation of DCF, METO and ISO in this study the extreme scenario of the direct reaction with HOBr prior to ozonation was chosen and corresponding TPs were postulated. In the following, OzTPs will be referred to products which have been detected in previous studies after the reaction of ozone with ISO, METO or DCF, respectively, and are also detected after the reaction of HOBr. Products which were not reported before but formed in the reaction of HOBr with the corresponding parent substance will be referred to as hypobromous acid transformation products (HOBrTPs). As the reaction with HOBr does not always lead to a bromination but can also lead to a hydroxylation, these products will also be counted as HOBrTPs even if they do not contain bromine. Reaction products detected after subsequent ozonation of the HOBrTPs are in the following called ozonated hypobromous acid transformation products (OzHOBrTPs), even if they do not contain bromine. The results are presented in Figure 5-1 and detailed information can be found in Table 5-8 (SI).

Main reactions leading to the formation of HOBrTPs as well as OzHOBrTPs can be hydroxylation or the bromination of the aromatic ring.

As the carbonyl group is deactivating the nitrogen atoms of ISO [9, 19], the reaction of HOBr with ISO takes mainly place at the aromatic ring [19]. Indeed, ozonation without bromination is leading to a substitution of the isopropyl group at the aromatic ring in secondary TPs but with bromination hydrogen is preferably substituted by $^{\bullet}$OH. However, for some of the postulated OzHOBrTP of ISO twofold demethylation at the tertiary amine is also suggested. As it can be concluded that bromine decreases the electron density in the aromatic ring, due to its negative inductive effect and therefore, the reaction of ozone at the aromatic ring is less favorable. Consequently, this leads to an attack at the tertiary amine, which is non-reactive without the bromination at the ring system.

However, the main reactions of HOBr with METO are occurring either at the aromatic ring or at the amine group leading to an aromatic ring with a bromine substituent or bromamines, respectively [30, 31]. Nevertheless, hydroxylation of METO, which also occurs during ozonation, also needs to be considered during the reaction with HOBr [19]. Further, the

detection of some HOBrTPs implies that a bond cleavage may be also induced in the reaction of HOBr with METO.

As for METO the reaction of HOBr with DCF is likely to take place at the aromatic ring and therefore leads to a bromine substituent at the aromatic ring. However, also a hydroxylation of diclofenac can take place in the reaction with HOBr.

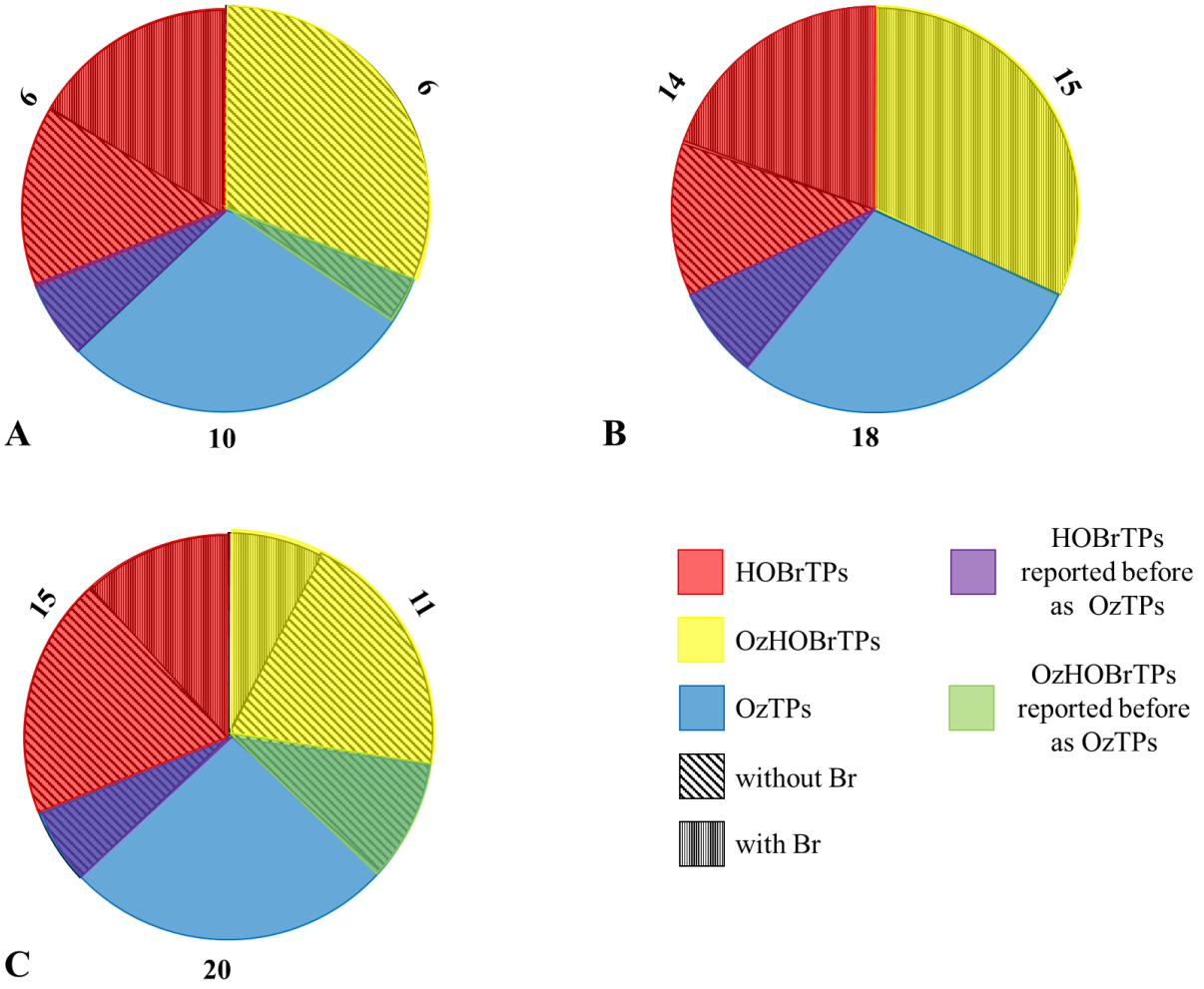


Figure 5-1 **Transformation products formed in the reaction with ozone (OzTPs), transformation products formed in the reaction with hypobromous acid (HOBrTPs) and transformation products formed in the reaction with hypobromous acid and subsequent ozonation (OzHOBrTPs) detected in the reaction of (A) isoproturon, (B) metoprolol and (C) diclofenac**

Bold numbers outside the diagrams indicate the total number of HOBrTPs, OzHOBrTPs and OzTPs, respectively, detected and considered within the diagram. Reaction products considered as reported before have been listed in [27]. Further information can be found in Table 5-8 (SI).

Isoproturon

As shown in Figure 5-1.A and Table 5-8 (SI) six HOBrTPs could be tentatively identified for ISO in the direct reaction with HOBr and four of them have not been reported before. Postulated structures of HOBrTPs are shown in Table 5-9, SI. HOBrTP 284, HOBrTP 300 and HOBrTP 361 (Figure 5-2) included a bromine substituent at the aromatic ring. However, HOBrTP 180 and HOBrTP 222 (Figure 5-2) also have been detected after ozonation of ISO in ultrapure water [14, 27] but considered as HOBrTPs in this study rather than OzTPs as they were formed in the reaction of HOBr with ISO. Indeed, HOBrTP 222 also showed the highest intensity followed by HOBrTP 220 and HOBrTP 284 (Figure 5-6, SI). Therefore, postulated formation pathways for HOBrTP 222 and HOBrTP 284 are presented in Figure 5-2. For HOBrTP 220 only a sum formula ($C_{12}H_{16}N_2O_2$) could be postulated.

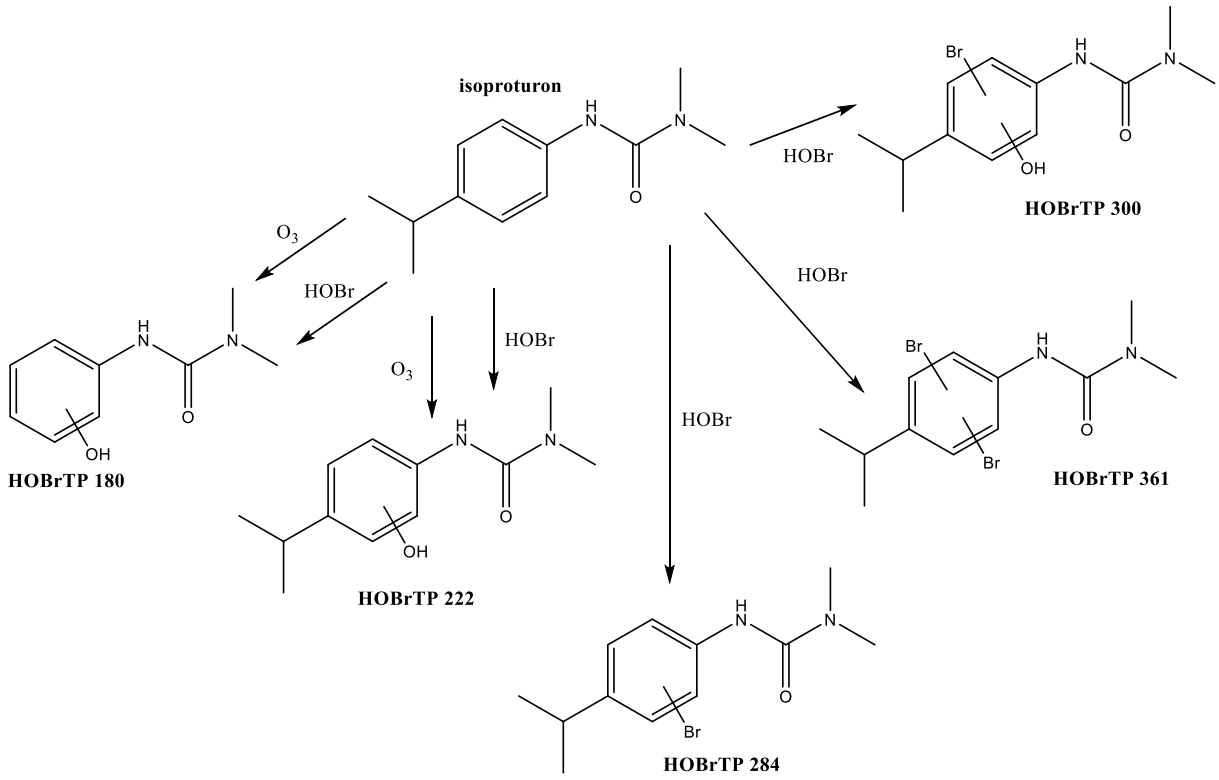


Figure 5-2 Proposed formation pathways for transformation products which can be formed via the reaction of isoproturon with ozone or hypobromous acid

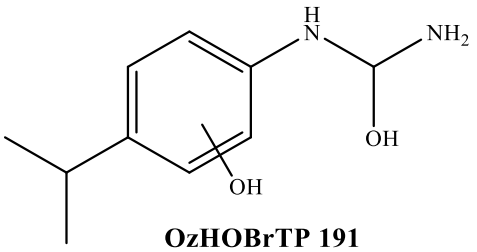
HOBrTP180 and HOBrTP 222 are reaction products which can be formed with ozone or hypobromous acid. Reaction products which are only formed in the reaction of isoproturon with hypobromous acid are HOBrTP 180, HOBrTP 222, HOBrTP 284, HOBrTP 300 and HOBrTP 361

Since HOBrTP 222 and HOBrTP 180 were also detected after ozonation of ISO in ultrapure water it can be concluded that the hydroxylation is happening due to an electron

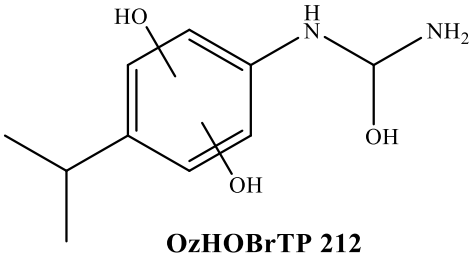
transfer during the treatment with HOBr [19]. This mechanism also applies to the formation of HOBrTP 300.

However, as HOBrTP 220, HOBrTP 222 and HOBrTP 284 were detected with the highest areas after the reaction of ISO with HOBr (compare Figure 5-6, SI) these were considered for the proposed structures of OzHOBrTPs shown in Table 5-10 (SI). In total six OzHOBrTPs were tentatively identified (Figure 5-1.A).

It was considered for the proposed structures of the HOBrTPs in Figure 5-2 and Table 5-9 (SI) that the main reaction of HOBr with ISO takes place at the aromatic ring. However, for the postulated OzHOBrTP 196 and OzHOBrTP 212 twofold demethylation at the tertiary amine is suggested, which was not detected without bromination (postulated structures in Figure 5-3, detailed information in Table 5-10, SI). As the electron density in the aromatic ring is decreased (as for HOBrTP 284, HOBrTP 300 and HOBrTP 361), the attack of ozone at the aromatic ring is less favored0 which leads to an attack at the tertiary amine. However, after the subsequent ozonation none of the detected OzHOBrTPs contained bromine. Additionally, OzHOBrTP 208 was also detected after ozonation of ISO [27] but also in experiments including the reaction of HOBr with subsequent ozonation, underlining that hydroxylation at the aromatic ring or at an alkyl chain is the main reaction pathway for the identified HOBrTPs and OzHOBrTPs.



OzHOBrTP 191



OzHOBrTP 212

Figure 5-3 Postulated ozonated bromination products (OzHOBrTPs) for the reaction of isoproturon with hypobromous acid and subsequent ozonation in ultrapure water buffered at pH 7

Metoprolol

For METO 14 HOBrTPs could be tentatively identified (Figure 5-1.B and Table 5-8, SI). These included *m/z* between 133 and 441, exact masses and proposed formulas are shown in Table 5-11 (SI).

In total, eight of the postulated HOBrTPs included a bromine substituent (Figure 5-1.B) HOBrTPs with the largest peak areas were HOBrTP 303 and HOBrTP 345 (Figure 5-7, SI) which were formed by the attack of HOBr at the amine group (HOBrTP 303) and bromination of the aromatic ring (HOBrTP 345) (Figure 5-4). However, other HOBrTPs such as HOBrTP 299 (Figure 5-4), which have previously been reported for the reaction of METO with ozone in ultrapure water [13, 27], were also detected emphasizing the possible hydroxylation of METO. HOBrTP 133, HOBrTP 136 and HOBrTP 197 are likely to be formed by bond cleavage.

Overall, various HOBrTPs have been detected which have not been reported before (Figure 5-1.B), underlining the complex reaction pathways that might occur during the reaction of METO with HOBr.

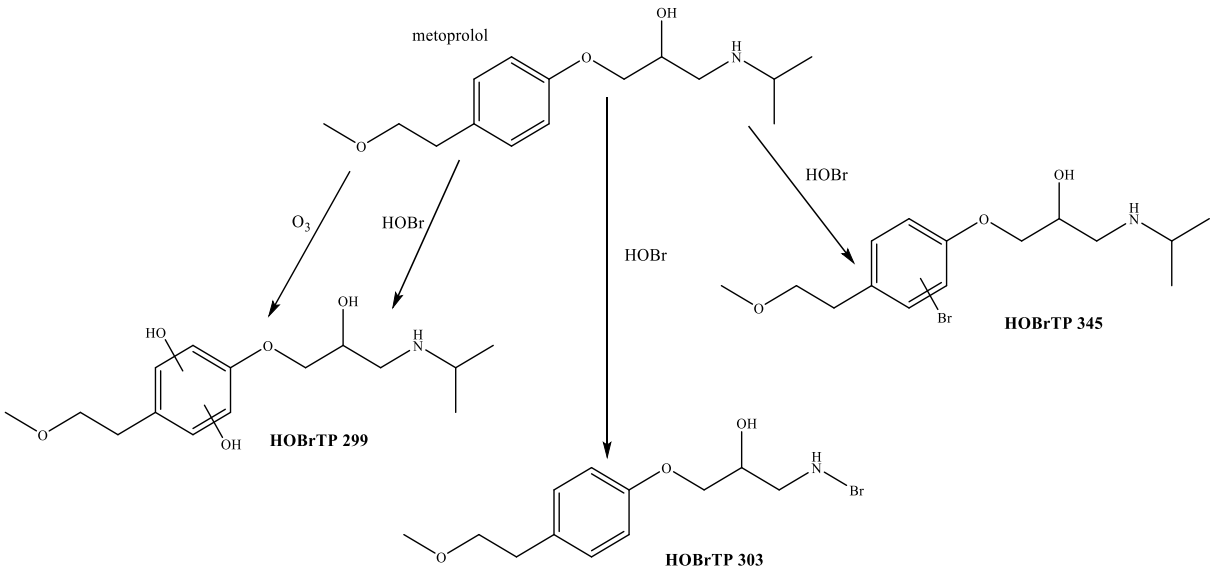


Figure 5-4 Proposed structures of the bromination products HOBrTP 299, HOBrTP 303 and HOBrTP 345 formed after the reaction of metoprolol with ozone or hypobromous acid

Since HOBrTP 303 and HOBrTP 345 were the HOBrTPs with the highest peak areas after the reaction of HOBr with METO these were considered for the proposed structures of OzHOBrTPs formed after subsequent ozonation. The data were evaluated revealing 15 OzHOBrTPs (Table 5-12, SI). However, even if all of those OzHOBrTPs were suggested to contain bromine no structures were postulated, as the sum formulas did not match theoretically excepted OzHOBrTPs based on possible reaction pathways.

Diclofenac

The influence of bromide in terms of TP formation has already been reported during chlorination of DCF [32]. However, no studies are available for its influence during ozonation. In total, 15 HOBrTPs have been tentatively identified after the reaction of HOBr with DCF (Figure 5-1.C and Table 5-8, SI). Exact masses and proposed sum formulas are shown in Table 5-13, SI.

The reaction of HOBr with DCF mainly takes place at the aromatic ring (HOBrTP 372) [32]. In addition, several HOBrTPs were found that do not include bromine but have not been reported before in the reaction of DCF with ozone, therefore, sum formulas for the detected HOBrTPs were postulated (Table 5-13, SI). After subsequent ozonation further eleven OzHOBrTPs were detected (Table 5-14, SI), which indicates that the formed HOBrTPs can react further with ozone and lead to even harder predictable TPs. However, some of these OzHOBrTPs were also found after direct DCF ozonation such as 2,6-dichloroaniline (OzTP 161), some others were already reported by another study investigating the influence of bromide during chlorination of DCF [32].

Influence of different bromide and ozone concentrations on the formation of reaction products in ultrapure water

The reaction rate constants of METO, ISO and DCF with ozone are higher ($k = 2 \times 10^3$ $M^{-1}s^{-1}$ [13], $k = 2.2 \times 10^3$ $M^{-1}s^{-1}$ [33] and $k = 6.8 \times 10^5$ $M^{-1}s^{-1}$ [10], respectively) than the reaction rate of bromide with ozone ($k = 1.6 \times 10^2$ $M^{-1}s^{-1}$) [34]. However, the concentrations of MPs in wastewater is mostly much lower (<1 µg/L) [3] than the concentration of bromide which is commonly detected in WWTPs in concentrations ranging from 0.01 to 1 mg/L but can reach concentrations up to 40 mg/L [17, 35]. Therefore, even if the reaction of ozone with MPs is kinetically favored, the reaction with bromide can still be relevant. Additionally, also the reaction of formed $^{\bullet}OH$ with bromide needs to be considered as it is very fast ($k = 1.1 \times 10^{10}$ $M^{-1}s^{-1}$ [36]) and can also lead to the formation of HOBr and thus influence the formation of TPs. To identify the reaction dynamics of ozone, bromide and the MPs, their transformation was investigated with stable concentrations of the parent substances but varying ratios of ozone and bromide. TPs formed in the direct reaction of the parent substance with ozone (suspect screening) [10, 13, 15, 16, 27, 37], products identified after the addition of HOBr (HOBrTPs) and products identified after bromination plus subsequent ozonation (OzHOBrTPs) were assessed. As hydroxylation of the parent substances may occur both in the treatment with

HOBr or ozone, reaction products which have been reported before in the ozonation of ISO, METO or DCF were considered as OzTPs. Products with the same *m/z* but different retention times were counted separately. Overall, only TPs previously identified in this study (in the reaction of HOBr and the three substances with and without subsequent ozonation) were considered (for ISO: 10 OzTPs, 5 HOBrTPs and 15 OzHOBrTPs; for METO: 24 OzTPs, 38 HOBrTPs and 32 OzHOBrTPs; for DCF: 20 OzTPs, 13 HOBrTPs and 22 OzHOBrTPs).

In all ISO samples similar numbers of OzTPs, HOBrTPs and OzHOBrTPs, respectively, have been detected independent of O_3/Br^- ratios (Figure 5-5.A). However, in almost all samples higher numbers of OzHOBrTPs were detected than OzTPs or HOBrTPs. The formation of HOBrTPs (reaction at the aromatic ring) also gives rise to OzHOBrTPs which are degraded at the highest ozone concentration. While the number of OzTPs was similar to other ratios, the highest number of HOBrTPs and the lowest number of OzHOBrTPs was detected at a 10-fold excess of ozone and Br^- compared to the concentration of ISO.

The reactions of METO with different O_3/Br^- ratios showed, that the numbers of OzTPs and HOBrTPs decrease with increasing ozone concentrations and that most OzHOBrTPs are formed at the highest ozone/Br^- concentration (Figure 5-5.B). This indicates that OzTPs and HOBrTPs are transformed with ozone to OzHOBrTPs mostly independently of the O_3/Br^- ratio. All target OzTPs were found for all ozone and Br^- ratios, except after the addition of the highest ozone and Br^- concentration. In this sample only 3-(isopropylamino)propan-1,2-diol (3-METO) was present among other identified products which is resulting from aliphatic chain degradation [15]. However, 3-METO does not contain an aromatic ring and might therefore be less reactive than other OzTPs. Interestingly, with increasing bromide concentrations and constant ozone concentration more OzTPs are formed than HOBrTPs or OzHOBrTPs, which might indicate that the reaction of METO with ozone is still favored over a reaction with Br^-. However, it cannot be excluded that some of these OzTPs are also formed in the reaction of HOBr with METO.

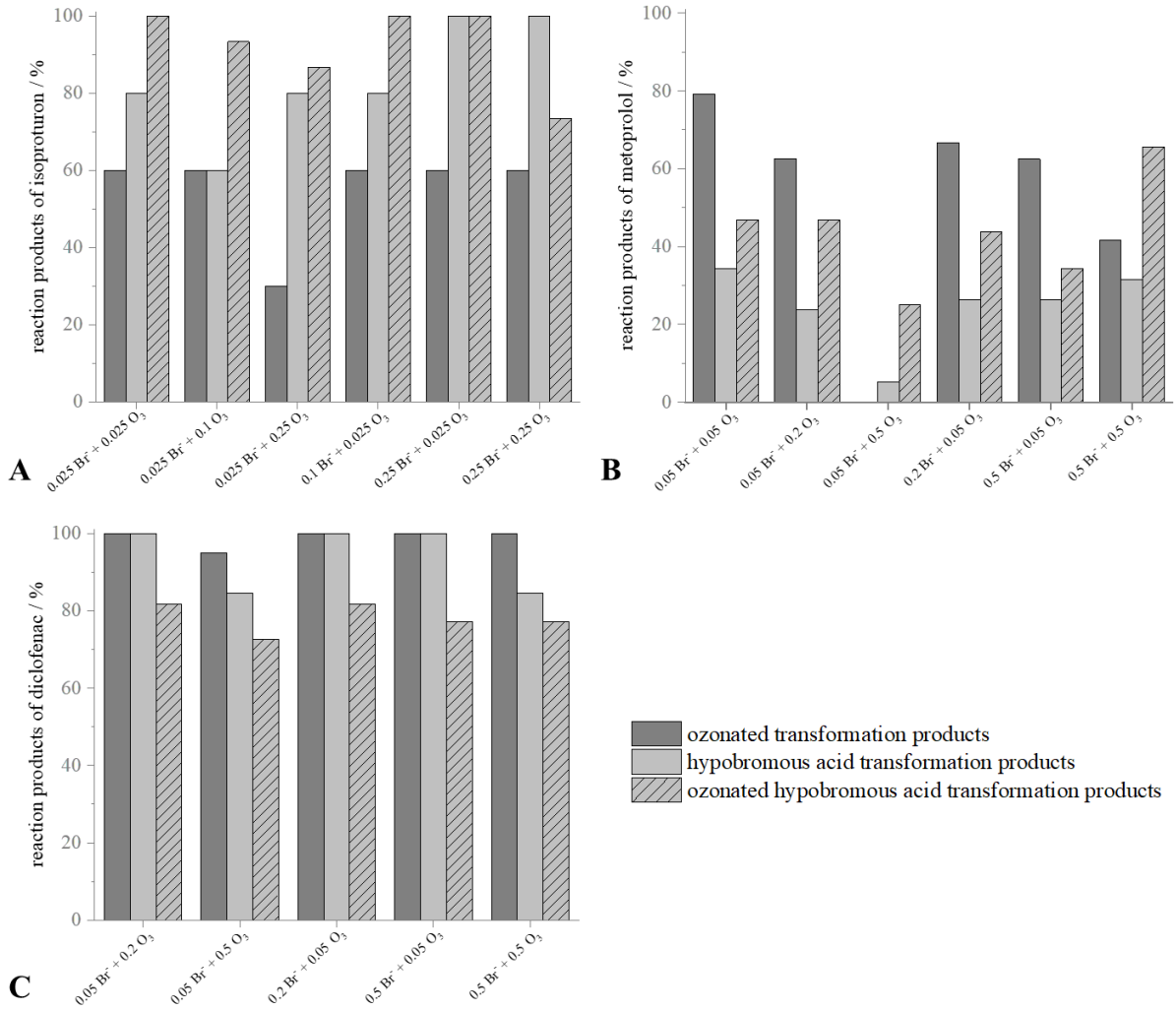


Figure 5-5 **Percentage of detected products in the reaction of (A) isoproturon (c_0 = 0.025 mM), (B) metoprolol (c_0 = 0.05 mM) and (C) diclofenac (c_0 = 0.05 mM) oxidized with different ozone (c = 0.05 – 0.5 mM for diclofenac and metoprolol, 0.025 – 0.25 mM for isoproturon) and bromide (c = 0.05 – 0.5 mM for diclofenac and metoprolol, c = 0.025 – 0.25 mM for isoproturon) concentrations in relation to the total number of transformation products (TPs), hypobromous acid transformation products (HOBrTPs) and ozonated hypobromous acid transformation products (OzHOBrTPs) identified in the reaction with HOBr and subsequent ozonation**

Measurements have been performed via HPLC-HRMS. Products of the reaction with ozone (TPs), the reaction of the parent substances with hypobromous acid (HOBrTPs) and the products of the reaction with hypobromous acid and subsequently ozonation (OzHOBrTPs) were identified. Percentages of the detected TPs, HOBrTPs and OzHOBrTPs are given in relation to the total number of considered reaction products (for ISO: 10 OzTPs, 5 HOBrTPs and 15 OzHOBrTPs; for METO: 24 OzTPs, 38 HOBrTPs and 32 OzHOBrTPs; for DCF: 20 OzTPs, 13 HOBrTPs and 22 OzHOBrTPs).

Only slight differences could be observed in the formation of OzTPs, HOBrTPs and OzHOBrTPs, respectively, at different ratios in the DCF samples (Figure 5-5.C). Even in samples with the same substance and Br^- concentrations OzHOBrTPs were formed in the reaction with ozone. Therefore, even if the reaction kinetics imply that the reactions of ozone with the substances are favored also HOBrTPs and OzHOBrTPs can be formed, which was detected for all three substances. This was also the case at ozone and substance concentration ten times lower than the Br^- concentration, emphasizing that this formation is also possible in real water systems with normally lower concentrations of MPs than concentrations of Br^-.

In fact, the bromine substituent of the formed bromamines and brominated ring systems has a negative inductive effect which reduces the electron density at the affected structure. Thus, ozone attacks structures with higher electron densities within the molecule leading to higher ratios of ozone reactions at the amine group if brominated ring systems are present. Then again, the hydroxylation increases the density due to the positive mesomeric effect of the hydroxyl group leading to an attack of ozone at the same structure. Both reaction pathways can lead to the formation of hitherto unknown TPs as it has been shown for all three parent substances. However, to understand the exact pathways leading to the formation of HOBrTPs and OzHOBrTPs, their structures need to be verified in further experiments. This is also important for the evaluation of possible adverse effects of TPs. Indeed, this was done by a simultaneously conducted study which considered the influences of matrix components during ozonation in terms of aquatic toxicological potential. This study showed that the reaction of HOBr with and without subsequent ozonation can increase the aquatic toxicological potential towards *Daphnia magna* [38].

This study was to the authors' best knowledge the first one to address the influence of bromide on the formation of TPs in terms of ISO, METO and DCF. It could be shown that not only bromide itself but also the direct addition of the secondary oxidant HOBr can influence the formation of TPs. Further, also TPs not reported prior to this study have been detected. Yet, it was not always possible to elucidate exact steps leading to the formed reaction products as not only primary products but also secondary reaction products were measured so that this needs to be investigated in future studies in detail. However, it was shown that even for very well investigated substances the presence of bromide or HOBr can lead to the formation of unknown and hardly predictable TPs. This underlines the need of further investigations in terms of matrix components and their possible influences during the reaction of MPs with ozone.

5.5 Supporting Information – Chapter 5

5.5.1 Materials & Methods

Table 5-1 List of chemicals and solvents used

Chemical / Solvent	Manufacturer
Diclofenac sodium salt	Sigma Aldrich
Dipotassium hydrogenphosphate	AppliChem
Formic acid 98 – 100 %	Merck
Formic acid 99 %	VWR Chemicals
Hydrochloric acid 35 %	Bernd Kraft
Isoproturon ≥ 98 %	Sigma Aldrich
Methanol 100 %	VWR Chemicals
Methanol LC-MS grade 99.99 %	Fischer Chemicals
Metoprolol tartrate salt > 99 %	Sigma Aldrich
Oxygen 99.99 % (200 bar)	Alphagaz
Pierce LTQ ESI positive / negative ion calibration solution	Thermo Scientific
Sodium bromide > 99 %	Fluka
Sodium dihydrogen phosphate > 99 %	AppliChem
Sodium hydroxide pellets	Bernd Kraft
Ultra-purified water	-
Water LC-MS grade 99.99 %	Fischer Chemicals

Table 5-2 List of equipment used

Technical device	Model	Manufacturer
Ozone generator	COM-AD-01	Anseros
Ozone generator	Philaqua 802x	BMT Messtechnik
pH meter	827 pH Lab	Metrohm
Shaker	KS 260C	IKA®
UV-Vis Spectrometer	UV-1800	Shimadzu
UV-Vis Spectrometer	UV-1650PC	Shimadzu
HPLC – DAD		
Auto injector	SIL-10ADVP	Shimadzu
Column oven	CTO-10ASVP	Shimadzu
Core shell LC column	Kinetex 5 µm EVO C18 100Å	Phenomenex
Degassing unit	DGU-20A5R	Shimadzu
Diode array detector	SPD-M10AVP	Shimadzu
Fluorescence detector	RF-10AXL	Shimadzu
Liquid chromatograph	LC-10ATVP	Shimadzu
Reservoir tray	-	Shimadzu
System controller	SCL-10AVP	Shimadzu
Valve assembly	FCV-10ALVP	Shimadzu
HPLC – HRMS		
Core shell LC column	Kinetex 5 µm EVO C18 100Å	Phenomenex
Mass Spectrometer Orbitrap	Q Exactive	Thermo Scientific
UHPLC (Pump, Autosampler)	Dionex Ultimate 3000 UHPLC$^+$	Thermo Scientific

Table 5-3 List of software used

Software	Version	Manufacturer
Chromeleon	7.2 SR5	Thermo Scientific
Compound Discoverer	3.0 and 3.1	Thermo Scientific
LC Solution	-	Shimadzu
Thermo XCalibur	4.0.27.10	Thermo Scientific

Table 5-4 Concentrations during the reactions of bromide with ozone and diclofenac (c_0 = 0.05 mM), metoprolol (c_0 = 0.05 mM) and isoproturon (c_0 = 0.025 mM), respectively

Parent substance [mM]	Bromide [mM]	Ozone [mM]
diclofenac and metoprolol		
0.05	0.05	0.05
0.05	0.05	0.2
0.05	0.05	0.5
0.05	0.2	0.05
0.05	0.5	0.05
0.05	0.5	0.5
isoproturon		
0.025	0.025	0.025
0.025	0.025	0.1
0.025	0.025	0.25
0.025	0.1	0.025
0.025	0.25	0.025
0.025	0.25	0.25

Table 5-5 Instrument settings for analysis of the parent compounds with HPLC DAD

Unit	Parameter	Value
HPLC	Injection volume	50 µL
	Flow rate	0.3 mL/min (metoprolol and isoproturon) 0.5 ml/min (diclofenac)
	Eluents	A: water + 0.1 % formic acid B: methanol + 0.1 % formic acid
	Gradient	Isocratic for diclofenac 0-15 min: 40 % A, 60 % B Gradient for metoprolol and isoproturon: 0–7 min: 90 % A, 10 % B 10–13 min: 10 % A, 90 % B 13.1–30 min: 90 % A, 10 % B

Table 5-6 Instrument settings LC-HRMS for measurement of metoprolol and isoproturon

Unit	Parameter	Value
HPLC	Injection volume	10 µL
	Flow rate	0.3 mL/min
	Eluents	A: water + 0.1 % formic acid B: methanol + 0.1 % formic acid
	Gradient	0-4 min: 90 % A, 10 % B 7-10 min: 10 % A, 90 % B 10.1-20 min: 90 % A, 10 % B

Continued on next page

Unit	Parameter	Value
HRMS	Scan type	Full MS
	Scan range	100.0 to 1000.0 m/z
	Fragmentation	None
	Resolution	70000
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	10 s
	AGC target	1e6
	Maximum inject time	100
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	320 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C

Table 5-7 Instrument settings LC-HRMS for measurement of diclofenac

Unit	Parameter	Value
HPLC	Injection volume	100 µL
	Flow rate	0.3 mL / min
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	0-4 min: 80 % A, 20 % B
		4-10 min: 20 % A, 80 % B
		10-15 min: 80 % A, 20 % B
HRMS	Scan type	Full MS
	Scan range	100.0 to 1000.0 m/z
	Fragmentation	None
	Resolution	70000
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	10 s
	AGC target	1e6
	Maximum inject time	100
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	320 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C

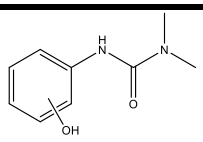
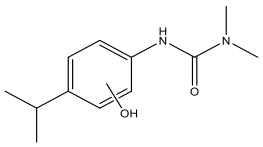
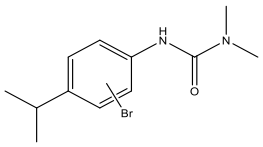
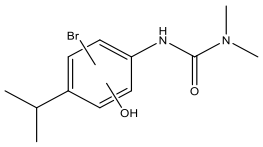
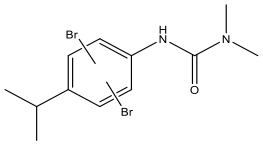
5.5.2 Results & Discussion

Table 5-8 **List of transformation products formed in the reaction with ozone (OzTPs), transformation products formed in the reaction with hypobromous acid (HOBrTPs) and transformation products formed in the reaction with hypobromous acid and subsequent ozonation (OzHOBrTPs) detected in the reaction of isoproturon, metoprolol and diclofenac**

For the in-situ experiments all OzTPs, HOBrTPs and OzHOBrTPs are listed if they have been detected after the addition of the lowest O_3 / Br^- ratio (0.05 mM O_3 / 0.05 mM Br^- for 0.05 mM DCF and METO, respectively and 0.025 mM O_3 / 0.025 mM Br^- for 0.025 mM ISO). In the in-situ experiments the total number is also stated as reaction products with same *m/z* but with different retention times were also considered. *number of considered features within the suspect screening of the in-situ samples. ** reported in [27].

	Isoproturon	**Metoprolol**	**Diclofenac**
OzTPs reported before	10 [14]	18 [13, 15, 16]	20 [10, 37]
HOBrTPs total	6	14	15
HOBrTPs reported before**	2	2	2
HOBrTPs with Br	3	8	5
HOBrTPs without Br	3	6	10
OzHOBrTPs total	6	15	11
OzHOBrTPs reported before**	1	0	4
OzHOBrTPs with Br	0	15	3
OzHOBrTPs without Br	6	0	8
In-situ OzTPs	6 (10*)	19 (24*)	20 (20*)
In-situ HOBrTPs	4 (5*)	13 (38*)	13 (13*)
In-situ OzHOBrTPs total	15 (15*)	15 (32*)	18 (22*)

Table 5-9 Features detected via Compound Discoverer 3.0 after the reaction of isoproturon (c = 0.025 mM) with hypobromous acid (c=0.025 mM) in ultrapure water buffered at pH 7 (phosphate buffer), measured with HPLC-HRMS (positive mode)

Reaction product with HOBr	Calculated m/z	Retention time [min]	Proposed sum formula by Compound Discoverer 3.0	Proposed structure	Δm [ppm]
HOBrTP 180	180.08980	7.61, 9.56, 9.70	$C_9H_{12}N_2O_2$		-0.43
HOBrTP 220	220.12108	9.97	$C_{12}H_{16}N_2O_2$	No proposed structure	-0.44
HOBrTP 222	222.13660	9.36, 9.57, 9.70, 10.16	$C_{12}H_{18}N_2O_2$		-1.02
HOBrTP 284	284.05226	10.68	$C_{12}H_{17}BrN_2O$		-0.57
HOBrTP 300	300.04717	9.81, 9.98	$C_{12}H_{17}BrN_2O_2$		-0.58
HOBrTP 361	361.96291	10.41, 11.28	$C_{12}H_{16}Br_2N_2O$		-0.07

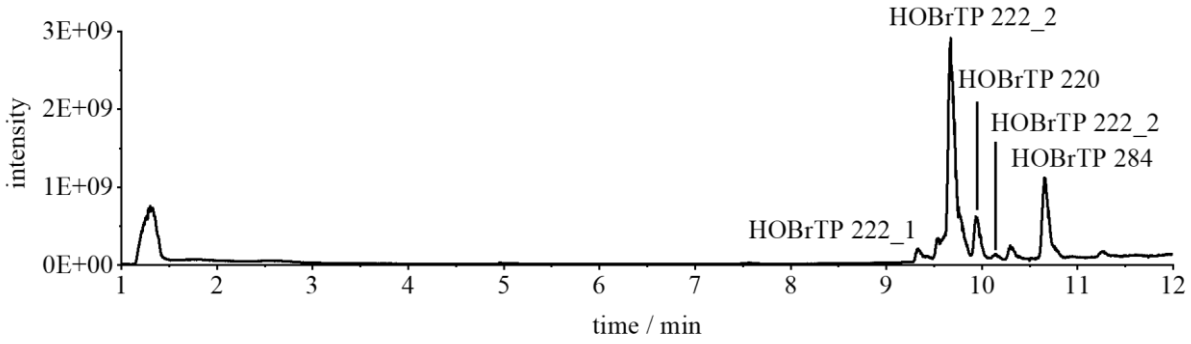


Figure 5-6 **Total ion chromatogram of the reaction products of isoproturon (c=0.025 mM) with hypobromous acid (c=0.025 mM) in ultrapure water buffered at pH 7, measured by HPLC-HRMS.**

Table 5-10 Features detected via Compound Discoverer 3.0 after the reaction of isoproturon (c = 0.025 mM) with hypobromous acid (c = 0.025 mM) and subsequent ozonation (c=0.025 - 0.25 mM) in ultrapure water buffered at pH 7 (phosphate buffer) , measured by HPLC-HRMS (positive mode)

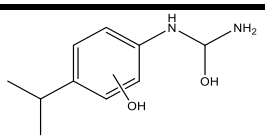
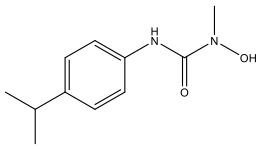
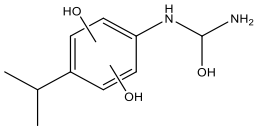
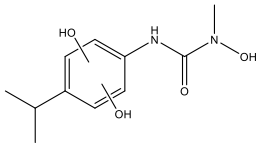
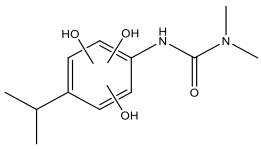
Reaction product with HOBr and subsequent ozonation	Calculated m/z	Retention time [min]	Proposed sum formula by Compound Discoverer 3.0	Proposed structure	Δm [ppm]
OzHOBrTP 196	196.12063	9.92	$C_{10}H_{16}N_2O_2$		-0.78
OzHOBrTP 208	208.12102	8.98, 9.52	$C_{11}H_{16}N_2O_2$		-0.76
OzHOBrTP 212	212.11583	8.83, 8.93, 9.02, 9.20	$C_{10}H_{16}N_2O_3$		-1.22
OzHOBrTP 230	230.12636	8.84, 9.02, 9.20	$C_{10}H_{18}N_2O_4$	No proposed structure	-1.30
OzHOBrTP 240	240.11068	8.30, 9.08	$C_{11}H_{16}N_2O_4$		-1.38
OzHOBrTP 254	254.12631	8.85, 9.09, 9.45	$C_{12}H_{18}N_2O_4$		-1.35

Table 5-11 Features detected via Compound Discoverer 3.0 after the reaction of metoprolol (c = 0.05 mM) with hypobromous acid (c = 0.125 mM) in ultrapure water buffered at pH 7 (phosphate buffer), measured with HPLC-HRMS (positive mode)

Reaction product with HOBr	Calculated m/z	Retention time [min]	Proposed sum formula by Compound Discoverer 3.0	Δm [ppm]
HOBrTP 133	133.11012	1.43	$C_6H_{15}NO_2$	-1.17
HOBrTP 136	136.05237	7.49	$C_8H_8O_2$	-0.44
HOBrTP 197	197.96791	9.90	C_8H_7BrO	-0.58
HOBrTP 225	225.09978	1.84, 2.03, 2.18, 2.70	$C_{11}H_{15}NO_4$	-1.44
HOBrTP 267	267.14656	2.51, 3.34, 3.92, 5.10	$C_{14}H_{21}NO_4$	-1.85
HOBrTP 291	291.87312	9.83	$C_8H_6Br_2O_2$	-1.13
HOBrTP 299	299.17278	2.51, 3.34, 3.92, 5.10	$C_{15}H_{25}NO_5$	-1.66
HOBrTP 303	303.04670	8.60	$C_{12}H_{18}BrNO_3$	-1.00
HOBrTP 317	317.18343	1.78, 2.51, 3.34, 3.92, 5.13	$C_{15}H_{27}NO_6$	-1.29
HOBrTP 345	345.09357	9.01	$C_{15}H_{24}BrNO_3$	-1.11
HOBrTP 347	347.07289	1.93, 2.09, 2.26, 2.70, 2.95, 3.49	$C_{14}H_{22}BrNO_4$	-0.94
HOBrTP 361	361.08849	2.25, 4.09, 5.61, 7.85, 8.18, 8.40, 8.66	$C_{15}H_{24}BrNO_4$	-1.06
HOBrTP 379	379.09886	1.87, 2.09, 2.25, 2.95, 3.50	$C_{15}H_{26}BrNO_5$	-1.51
HOBrTP 441	441.01457	8.14, 8.65, 8.77, 8.93	$C_{15}H_{25}Br_2NO_4$	-1.04

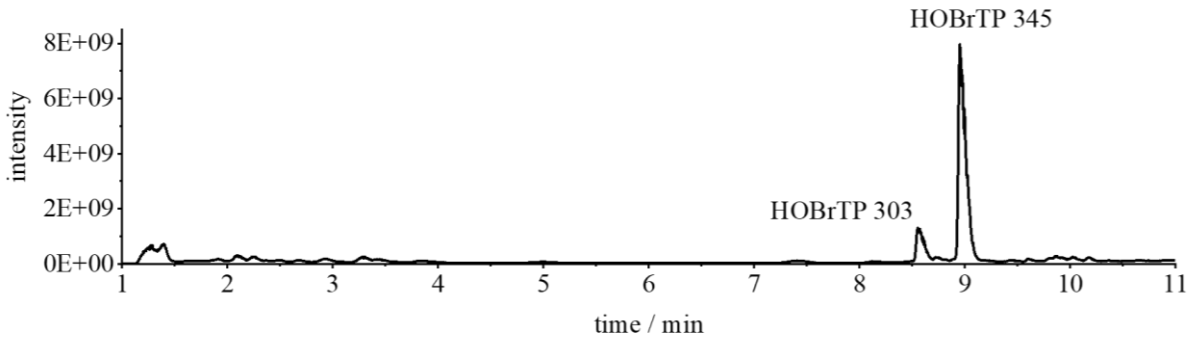


Figure 5-7 **Total ion chromatogram of the reaction products of metoprolol (c=0.05 mM) with hypobromous acid (c=0.125 mM) in ultrapure water buffered at pH 7, measured by HPLC-HRMS**

Table 5-12 Features detected via Compound Discoverer 3.0 after the reaction of metoprolol (c = 0.05 mM) with hypobromous acid (c = 0.125 mM) and subsequent ozonation (c=0.05 - 0.5 mM) in ultrapure water buffered at pH 7 (phosphate buffer), measured with HPLC-HRMS (positive mode)

Reaction product with HOBr and subsequent ozonation	Calculated m/z	Retention time [min]	Proposed sum formula by Compound Discoverer 3.0	Δm [ppm]
OzHOBrTP 221	221.96775	10.19	$C_{10}H_7BrO$	-1.23
OzHOBrTP 239	239.97831	9.86, 10.18	$C_{10}H_9BrO_2$	-1.18
OzHOBrTP 251	251.97832	10.02	$C_{11}H_9BrO_2$	-1.10
OzHOBrTP 271	271.02050	8.57, 10.19	$C_{11}H_{14}BrNO_2$	-1.08
OzHOBrTP 284	284.00445	9.95, 10.03	$C_{12}H_{13}BrO_3$	-1.25
OzHOBrTP 289	289.03098	10.17	$C_{11}H_{16}BrNO_3$	-1.32
OzHOBrTP 301	301.03094	9.94, 10.02	$C_{12}H_{16}BrNO_3$	-1.39
OzHOBrTP 315	315.04680	1.91, 2.09, 2.67, 3.93, 8.47, 8.86	$C_{13}H_{18}BrNO_3$	-0.65
OzHOBrTP 319	319.04152	9.72, 9.95, 10.02	$C_{12}H_{18}BrNO_4$	-1.25
OzHOBrTP 321	321.05748	10.18	$C_{12}H_{20}BrNO_4$	-0.28
OzHOBrTP 327	327.04656	8.88, 10.25	$C_{14}H_{18}BrNO_3$	-1.38
OzHOBrTP 333	333.05703	9.95, 10.03	$C_{13}H_{20}BrNO_4$	-1.61
OzHOBrTP 351	351.06781	9.95, 10.03	$C_{13}H_{22}BrNO_5$	-0.94
OzHOBrTP 357	357.05724	10.29	$C_{15}H_{20}BrNO_4$	-0.93
OzHOBrTP 359	359.07265	8.88, 10.25	$C_{15}H_{22}BrNO_4$	-1.59

Table 5-13 Features detected via Compound Discoverer 3.0 after the reaction of diclofenac (c = 0.05 mM) with hypobromous acid (c=0.1 mM) in ultrapure water buffered at pH 7 (phosphate buffer), measured with HPLC-HRMS (positive mode)

Reaction product with HOBr	Calculated m/z	Retention time [min]	Proposed sum formula
HOBrTP 213	213.03413	7.44, 7.68	$C_{13}H_8ClN$
HOBrTP 229	229.02914	6.81, 8.81	$C_{13}H_8ClNO$
HOBrTP 242	242.98504	6.03	$C_{10}H_7Cl_2NO_2$
HOBrTP 249	249.0107	7.45, 7.68	$C_{13}H_9Cl_2N$
HOBrTP 262	262.98996	7.20, 9. 57, 9.66	$C_{13}H_7Cl_2NO$
HOBrTP 265	265.00593	6.82	$C_{13}H_9Cl_2NO$
HOBrTP 278	278.98497	7.07	$C_{13}H_7Cl_2NO_2$
HOBrTP 281	281.00049	6.96	$C_{13}H_9Cl_2NO_2$
HOBrTP 290	290.98465	6.84	$C_{14}H_7Cl_2NO_2$
HOBrTP 293	293.00054	6.31	$C_{14}H_9Cl_2NO_2$
HOBrTP 328	328.90041	7.41	$C_{12}H_6BrCl_2NO$
HOBrTP 342	342.91621	9.28, 6.89	$C_{13}H_8BrCl_2NO$
HOBrTP 344	344.89549	7.92	$C_{12}H_6BrCl_2NO_2$
HOBrTP 358	358.91074	7.33	$C_{13}H_8BrCl_2NO_2$
HOBrTP 372	372.92647	8.59	$C_{14}H_{10}BrCl_2NO_2$

Table 5-14 **Features detected via Compound Discoverer 3.0 after the reaction of diclofenac (c = 0.05 mM) with hypobromous acid (c=0.1 mM) and subsequent ozonation (c=0.05 - 0.5 mM) in ultrapure water buffered at pH 7 (phosphate buffer), measured with HPLC-HRMS**

Reaction product with HOBr and subsequent ozonation	Calculated m/z	Retention time [min]	Proposed sum formula
OzHOBrTP 126	126.01084	5.76, 6.16	C_9H_2O
OzHOBrTP 160	160.97968	6.16	$C_6H_5Cl_2N$
OzHOBrTP 176	176.97458	6.05	$C_6H_5Cl_2NO$
OzHOBrTP 182	182.96818	5.15	C_7H_6BrN
OzHOBrTP 188	188.97456	5.76	$C_7H_5Cl_2NO$
OzHOBrTP 199	199.9469	2.19, 1.71	$C_7H_5BrO_2$
OzHOBrTP 218	218.98504	4.86	$C_8H_7Cl_2NO_2$
OzHOBrTP 240	240.96929	6.16	$C_{10}H_5Cl_2NO_2$
OzHOBrTP 241	242.985	5.69	$C_{10}H_7Cl_2NO_2$
OzHOBrTP 254	254.98505	6.39	$C_{11}H_7Cl_2NO_2$
OzHOBrTP 358	358.81095	6.56	$C_8H_5Br_2Cl_2NO$

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Chapter 6

Matrix composition during ozonation of *N*-containing substances may influence the acute toxicity towards *Daphnia magna*

Adapted from: Wirzberger, V., M. Klein, M. Woermann, B. Sures, H.V. Lutze, and T.C. Schmidt, *Matrix composition during ozonation of N-containing substances may influence the acute toxicity towards Daphnia magna*. Submitted to Science of The Total Environment, 2020.

6.1 Abstract

Micropollutants reach the aquatic environment through wastewater treatment plant effluents. Ozonation, applied in wastewater treatment for micropollutants abatement, can yield transformation products (TP), which might be of ecotoxicological concern. Previous studies on TP formation were mostly performed in ultrapure water. However, the water matrix can have a substantial influence and lead to unpredictable yields of TPs with toxicological potential.

In this study the acute toxicity (immobilization) of the parent substances (isoproturon and metoprolol) and also of available TPs of isoproturon, metoprolol and diclofenac towards *Daphnia magna* (*D. magna*) were investigated. Further, the acute toxicity of TP mixtures, formed during ozonation of isoproturon, metoprolol and diclofenac was evaluated in the following systems: in the presence of radical scavengers (*tert*-butanol and dimethyl sulfoxide) and in the presence of hypobromous acid (HOBr), a secondary oxidant in ozonation.

For all tested substances and TP standards, except 2,6-dichloroaniline (EC_{50} 1.02 mg/L (48 h)), no immobilization of *D. magna* was detected. Ozonated pure water and wastewater did not show an immobilization effect either. After ozonation of diclofenac in the presence of dimethyl sulfoxide 95 % (48 h) of the daphnids were immobile. Ozonation of parent substances, after the reaction with HOBr, showed no effect for isoproturon but a high effect on *D. magna* for diclofenac (95 % immobilization (48 h)) and an even higher effect for metoprolol (100 % immobilization (48 h)). These results emphasize that complex water matrices can influence the toxicity of TPs as shown in this study for *D. magna*.

6.2 Introduction

About 90 % of micropollutants (MPs) found in a target-screening of the River Rhine contained nitrogen, including various pharmaceuticals and pesticides [1]. Many of these MPs, especially pharmaceuticals, reach surface water via wastewater effluents [2]. Two pharmaceuticals frequently found in wastewater and surface water are metoprolol (METO) and diclofenac (DCF) [3-9]. Isoproturon (ISO) is an example of an herbicide still found in surface water [10] and secondary wastewater effluents [3], despite the fact that is was banned in the European Union in 2016 [11].

To reduce MP loads in the aquatic environment, ozonation can be used in wastewater treatment. However, MPs are not mineralized during ozonation but converted to transformation products (TPs), which might be of toxicological concern [12]. During the ozonation of *N*-containing substances reactive intermediates can be formed, which may react with the matrix and form different products compared to the ones detected in pure water studies. However, hardly any information on these reactions is available as most studies on ozonation of MPs and formation of TPs do not take matrix effects into account. The TPs formed in different matrices may also differ in their adverse impacts on the environment or even humans. One prominent example of matrix components leading to a more toxicologically relevant TP than the parent compound is the ozonation of *N*,*N*-dimethylsulfamide (DMS) leading to the carcinogenic *N*-nitrosodimethylamine (NDMA) [12-16]. This reaction only takes place in the presence of bromide, which reacts with ozone to hypobromous acid (HOBr) [16].

Aquatic organisms may take up toxicologically relevant substances present in the aquatic environment with acute (lethal) or chronic (sublethal, e.g., genotoxic) effects [17]. The chemical properties of a substance as well as the uptake routes highly influence its biological availability and therefore also subsequent adverse effects [18]. Thus, it is possible that TPs formed during ozonation have a higher aquatic toxicological potential than the parent compounds. Furthermore, since neither single substances nor TPs but mixtures are present in the aquatic environment, this may increase severity of the ecotoxicological effects. Godoy et al. [19] recently showed that a mixture of pharmaceuticals can lead to higher effects on *Daphnia similis* than individual substances, which would not be excepted by adding up the effects of the single substances.

Therefore, the present study focuses on (I) the acute toxicity of single TPs (commercially available) formed during the ozonation of METO, DCF and ISO on *D. magna* and (II) the acute toxicity of TP mixtures formed in ozonated water matrices containing

different matrix components (ultrapure water, •OH scavengers, HOBr, wastewater). In order to estimate the toxicological potential of these substances and mixtures, *Daphnia magna* tests for acute toxicity were applied as described in the OECD guideline 202 [20]. This test was chosen due to the fact that it is very well established and one of the most frequently applied tests in ecotoxicology [21]. Further, ISO, METO and DCF were chosen as toxicity data for these parent substances is already available [22-24] but knowledge about the influence of matrix components on their toxic potential is still lacking. The main aim of this study was to investigate how various matrices may influence the toxicity of TPs formed during ozonation. Thus, substances were chosen for which knowledge about the reaction with ozone and the reaction in well-established toxicity tests without the consideration of matrix effects was already present.

6.3 Materials & Methods

6.3.1 Chemicals

All chemicals and solvents used are listed in the Supporting Information (SI, Table 6-1). These were used as received from the listed suppliers. Structures of tested substances and commercially available TPs are shown in Figure 6-1.

6.3.2 Equipment

In Table 6-2 (SI) the equipment used is summarized.

6.3.3 Generation of stock solutions and media

All stock solutions of parent compounds (ISO: 0.06 mM, METO: 0.5 mM) and available TPs (METO TPs 0.5 - 0.6 mM, 2,6-dichloroaniline (DCA) 3.35 mM, 5-hydroxydiclofenac (5-OH DCF): 0.47 mM) were prepared in ultrapure water except for DCA and 5-OH DCF for which an addition of max. 1950 mg/L acetonitrile was necessary. The stock solutions were stored at 4 °C. ISO stock solution was shaken several days at room temperature in the dark until it was completely solved. Calibrations for stability measurements of the substances were performed according to DIN 38401-51.

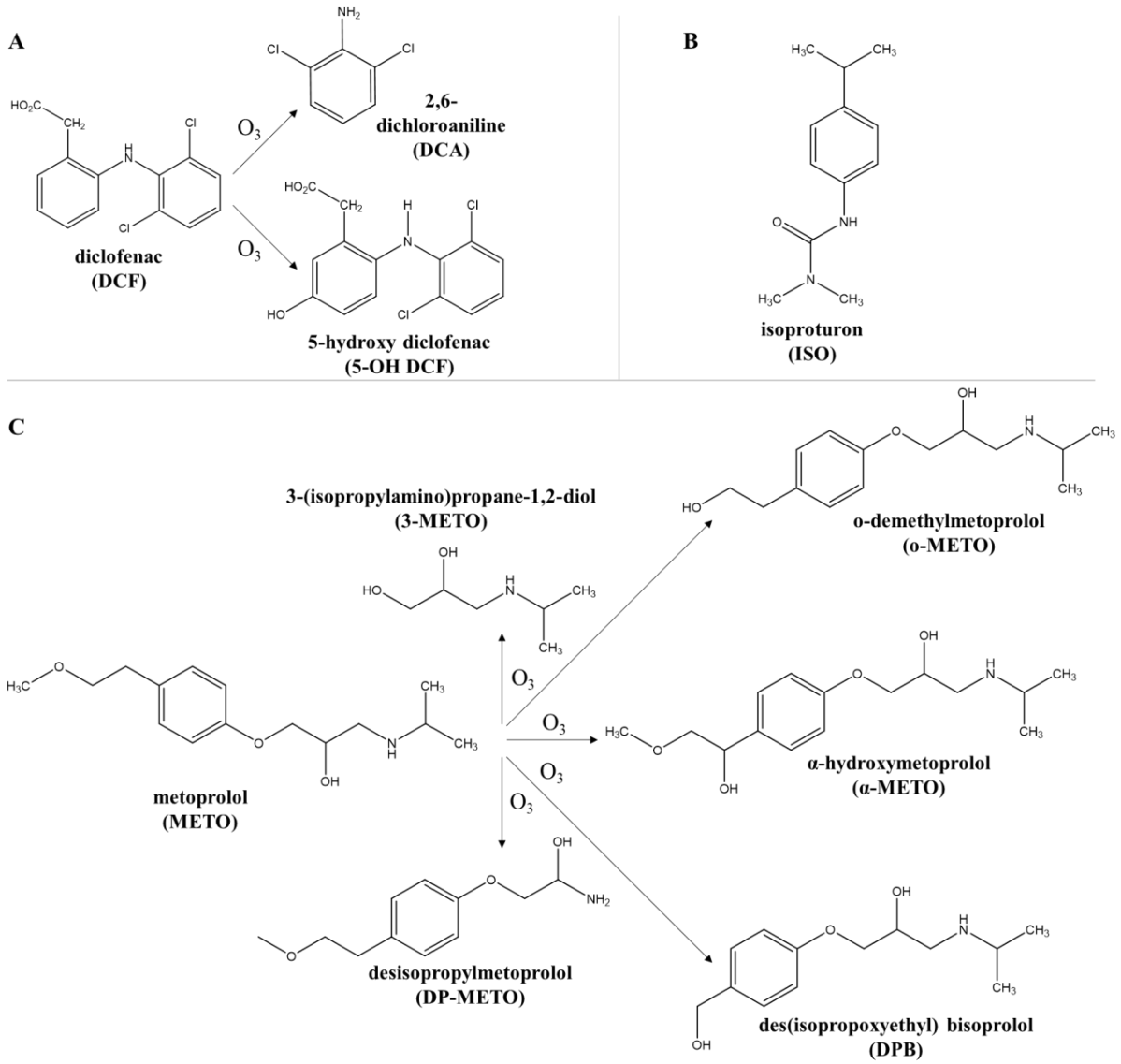

Figure 6-1 Structures of parent compounds and known transformation products

Parent compounds and transformation products (TPs) known to be formed in the ozonation of metoprolol (C) at pH 7 and diclofenac (A) without pH adjustment in ultrapure water. No standards were available for TPs formed in the ozonation of isoproturon (B).

An ozone generator (COM (Anseros) or Philaqua 802x (BMT Messtechnik)) was used to prepare ozone stock solutions. Oxygen (ALPHAGAZ™) was enriched by the generator and bubbled into ice cooled ultrapure water until stationary conditions were achieved (at least 60 minutes). The ozone concentration was determined by measuring the absorption of the ozone stock solution (0.5 mL ozone stock solution and 2.5 mL ultrapure water) using a UV-spectrometer (UV-1650PC or UV-1800, Shimadzu, 258 nm, ε_{O3} = 2950 $M^{-1}cm^{-1}$ [25]). The stock solution had for all experiments a concentration between 1.2 and 1.6 mM.

HOBr was prepared by ozonation of a 0.8 mM sodium bromide solution with 1 mM ozone at pH 4 [26]. Further preparation was performed following the protocol of Fischbacher et al. [27] and HOBr concentration in the stock solution was determined by UV-absorption according to Troy and Margerum [28].

Required concentrations of *tert*-butanol (*tert*-BuOH) and dimethyl sulfoxide (DMSO) were calculated (Equation 6-1 and Equation 6-2, reaction rate constants are listed in Table 6-3, SI) to scavenge 90 % (ISO and METO) and 95 % (DCF) of hydroxyl radicals, respectively [29]. Higher doses of scavengers were not used to minimize their reaction with ozone (≤ 5 % of ozone was consumed by the scavengers).

Klüttgen et al. [30] presented the original composition of the "Aachener Daphnia Medium" (ADaM) which was also used in this study with lower selenium dioxide concentration (Table 6-1, SI). The medium was kept in a non-transparent plastic tank, stored in the dark at 8 °C and constantly aerated with air through a 0.2 µm pore filter. Prior to use freshly prepared medium had to be aerated with oxygen for at least 30 minutes.

D. magna were cultured in 1 L glass beakers containing ~25 animals at a day/night cycle of 16/8 h at (20 ± 1 °C). Three times a week the medium was changed, the daphnids were fed with a concentrated algae solution of *Desmodesmus subspicatus.*

6.3.4 Sample preparation

Ozonation of *N*-containing substances

The three substances (DCF, METO and ISO) were ozonated in ultrapure water, in the presence of different radical scavengers (*tert*-BuOH and DMSO) and after bromination with HOBr. Two different •OH scavengers were used because Wirzberger et al. [31] showed, that these can highly influence the yields of TPs and in turn possibly also the aquatic toxicological potential. In the presence of DMSO during ozonation of DCF a continuous formation of DCA was detected even at the highest added ozone dosages. The same observation was made for the formation of des(isopropoxyethyl) bisoprolol (DPB), a TP of METO, in the presence of *tert*-BuOH. In all other tested water matrices both TPs were degraded at the same ozone dosages [31].

Starting concentrations of DCF and METO were 0.05 mM and 0.025 mM for ISO. Ozone doses were 0.25 mM and 0.5 mM (DCF and METO) as well as 0.125 mM and 0.25 mM (ISO).

In different runs, the influence of *tert*-BuOH and DMSO on the formation of TPs was determined, to simulate the quenching of •OH by organic compounds in real water matrices [12]. 6.3 mM and 3.375 mM of *tert*-BuOH were added in METO and ISO samples, respectively. The concentrations of DMSO were 0.54 mM and 0.289 mM for METO and ISO, respectively. In the ozonation of DCF 20 mM of both scavengers were added. These concentrations were determined to scavenge 90 – 95 % of •OH but only 5 - 10 % of ozone as described before by Willach et al. [29].

For METO and ISO the pH was set to 7 by adding 10 mM of phosphate buffer. Ozonation experiments of DCF were done without pH adjustment to generate comparable results to previous studies [32, 33]. However, Wirzberger et al. [31] already reported that the starting pH prior to ozonation ranged between 7 – 8 in all samples and dropped to pH 6 – 7 after ozone dosage of 0.5 mM. Only in samples containing DMSO the pH dropped to 4 but was adjusted before adding the daphnids to exclude influences of the pH towards the mobility of *D. magna*. All samples had a reaction time of at least 24 h before ecotoxicological tests were performed.

As frequently performed in other studies, investigating the influence of relevant factors during wastewater treatment, the used concentrations were higher than normally found in the environment to ensure higher yields of TPs and facilitate detection of possible influences on the mobility of *D. magna* [34].

Bromination of *N*-containing substances

During ozonation in water containing bromide, HOBr can be formed, which can then lead to the formation of brominated transformation products. To evaluate the aquatic toxicological potential of TPs arising from the reactions with HOBr all three substances were brominated directly with HOBr. To that end, DCF (0.05 mM), METO (0.05 mM) and ISO (0.025 mM) were treated with HOBr molar ratios of 2 : 1 (HOBr : DCF), 2.5 : 1 (HOBr : METO) and 1 : 1 (HOBr : ISO). These concentrations were proven to yield a complete bromination of the parent compounds, as no parent substances were detected anymore (data not shown). After a reaction time of 24 h at 4 °C 0.25 mM and 0.5 mM of ozone were added to DCF and METO samples. To ISO samples 0.125 mM and 0.25 mM of ozone were added. In these experiments the pH was adjusted to 7 in all samples prior to ozonation. In total three samples were tested for the aquatic toxicological potential towards *D. magna* (brominated

substances without further treatment and after treatment with two different ozone concentrations) 24 h after ozonation.

To determine the HOBr concentration left within the samples after a 24 h reaction time prior to ozonation the *N*,*N*-diethyl-*p*-phenylenediamine (DPD) method described in DIN EN ISO 7393-2 [35] was used. The preparation and measurements were done following the protocol by Abdighahroudi et al. [36].

Wastewater samples

To investigate if the observed results from ozonation experiments performed under laboratory conditions can also be transferred to real water samples, two wastewater samples from a wastewater treatment plant (WWTP) (conventionally treated wastewater and a recirculated mixture of conventionally treated and ozonated wastewater) were tested for aquatic toxicological potential using *D. magna* (scheme of the WWTP can be found in Figure 6-6, SI). Prior to the test, the samples were aerated with oxygen for 30 min to ensure dissolved oxygen saturation and the immobility tests were performed with the pure effluents as well as three different dilutions with ADaM medium (1:1, 1:2, 1:3).

6.3.5 Ecotoxicological test on *Daphnia magna*

Effect concentrations leading to 50 % immobility of *D. magna* (EC_{50}) were determined for the tested substances, known transformation products (in total two TPs were commercially available for DCF and five for METO) and TPs formed during ozonation of the substances in different water matrices by the acute immobilization test following OECD guideline 202 [20]. The population of the test organism, *D. magna*, had its origin in a pond at Mount Moses (Egypt). It was provided by the department of Animal Ecology, Evolution and Biodiversity at the Ruhr University Bochum. The daphnids were further cultured in the department of Aquatic Ecology at the University of Duisburg-Essen and applied frequently in previous studies [37-39]. The concentrations tested were 0.1 - 10 mg/L for ISO and 0.01 – 116.97 mg/L for METO by diluting the stock solution in ADaM. Prior to this study, Woermann and Sures [23] already tested DCF with the same culture of daphnids. For the tested TPs the concentrations ranged between 0.002– 130.03 mg/L (detailed information can be found in SI, Table 6-4). For DCA, an additional definitive test with lower concentrations (1.63 – 3.26 mg/L, SI Table 6-5) was

performed. In addition to the known TPs also samples containing either METO, ISO or DCF, were ozonated and tested for possible effects 24 h after ozonation. Additionally, one conventionally treated and one ozonated wastewater sample were tested.

Ecotoxicity tests were performed using daphnids younger than 24 h taken from cultures containing daphnids aged 3 – 12 weeks. The offspring was sorted using different mesh sized sieves. Parent substances and known TPs were tested by diluting stock solutions in at least 50 mL ADaM in a 100-mL volumetric flask. For toxicity testing of TPs formed during ozonation, 15 mL or 20 mL of ozonated samples were diluted in 65 mL or 60 mL ADaM, respectively. Due to the fact, that the final solution had to contain at least 50 % ADaM medium a fourfold concentrated stock solution of ADaM was prepared to minimize the dilution factor during the test with ozonated samples.

Immobilization tests were performed by preparing four aliquots of every sample containing 20 mL test solution each. Five daphnids (younger than 24 h) were placed into each of the aliquots by placing them on a mesh and transferring them into the aliquot to avoid dilution, resulting in 20 daphnids per tested sample. Additionally, for every test four aliquots containing the blank (ADaM only) were prepared as a negative control. The test vessels were stored in the dark at constant temperature (21 ± 1 °C) and the mobility was checked after 24 h and 48 h by visual inspection. Daphnids were considered as immobile if they did not move within 15 seconds after gentle agitation as defined by OECD guideline 202 [20]. Only tests with less than 10 % immobile daphnids in the negative control were considered as valid.

All solvents and possibly formed by-products were checked for their aquatic toxicological potential in separate tests. Additionally, a positive control using potassium dichromate ($K_2Cr_2O_7$) was prepared in concentrations ranging from 0.32 – 3.2 mg/L (detailed information in Table 6-6). All samples were prepared in ultrapure water and diluted with ADaM to reach the needed concentrations.

Acetonitrile and phosphate buffer were used for dissolving and pH adjustment, respectively. The highest used concentration of acetonitrile was 47.5 mM and for phosphate buffer 1.875 mM and therefore tested on *D. magna*. A previous study with the same *D. magna* culture also showed that a concentration of 5 mM is not harmful to *D. magna* [39]. In this study, 4 mM of DMSO were used and also tested. For *tert*-BuOH the highest used concentration and tested concentration was 3.75 mM. In the reaction of DMSO with $^{\bullet}OH$ methanesulfinic acid (MSIS) is formed, which is further oxidized to methanesulfonic acid (MSOS). Therefore, both reaction products were prepared separately and tested in the theoretically highest formed concentrations (20 mM formed, 4 mM tested in *D. magna* tests).

The theoretically highest concentration of HOBr in the test was 0.0234 mM. Thus, a test solution containing only HOBr was prepared and tested with *D. magna* in terms of immobilization. In further reaction HOBr can yield bromate (BrO_3) which was also tested in the highest possible concentration of 0.125 mM and two additionally concentrations (0.0625 mM and 0.25 mM).

Prior and after each experiment, the physicochemical properties of the samples containing daphnids were determined. For oxygen saturation and temperature, a FiveGo F4 IP67 dissolved oxygen sensor (Mettler-Toledo, Greifensee, Switzerland) was used. The pH was measured using test strips (DOSATEST® pH 6.0–10.0, VWR). In all experiments the temperature (18 – 22 °C), dissolved oxygen (> 3 mg/L) and pH (6 – 9, adjusted with HCl or NaOH) were within the ranges defined by OECD guideline 202 [20].

The EC_{50} values were calculated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com). A nonlinear regression was performed with a two-parameter logistic model. The response values were plotted against the log-transformed exposure concentrations.

6.3.6 Sample measurement

For each test performed regarding the toxicity of single substances the highest and lowest concentrations were measured after 24 h and 48 h incubation time either with HPLC-DAD (SPD-M10A VP, Shimadzu) or HPLC-HRMS (Dionex Ultimate 3000 UHPLC$^+$ focused coupled with an Orbitrap (Q Exactive Thermo Scientific)). All measurement methods were performed using an EVO C18 column (Kinetex 5 µm EVO C18 100 Å 100 × 3.0 mm). Detailed information about the measurement methods can be found for DCF in Table 6-7 (SI) and for METO and ISO in Table 6-8 (SI). METO and DCF samples were acidified with formic acid (0.2 %) prior to measurement. HPLC-DAD data was evaluated with LC Solution and for evaluation of HPLC-HRMS measurements XCalibur 4.0 software was used.

6.4 Results & Discussion

6.4.1 Toxicity of single substances

All parent compounds (except DCF) and TPs, for which reference standards were available, were tested regarding their toxicological potential towards *D. magna* (Figure 6-2 and Figure 6-4).

The derived EC_{50} values were compared with previous studies reporting EC_{50} values. For all calculations and Figures the nominal concentrations of the tests were used, except for DCA, for which the measured concentrations after 24 h and 48 h, respectively, were used. This was done, as the analytical measurements only revealed a considerable change within the concentration of DCA, the concentrations of all other TPs and parent substances stayed mostly stable over the whole duration time of the test.

Tests regarding the toxicological potential of METO towards *D. magna* did not show any effect in this study (Figure 6-2). This is in accordance with the majority of reported values in literature where also EC_{50} higher than 100 mg/L were reported (200 mg/L (48 h) [40] or even 438 mg/L [41]). However, Villegas-Navarro et al. [22] described an EC_{50} of 76.21 mg/L (48 h) [22]. This variation of EC_{50} values might be due to different origins of the daphnids, which therefore might have an influence on the test results [42]. Nevertheless, all EC_{50} values described in literature are higher than the maximum mean concentration of 1.59 µg/L in surface waters measured between 2009 and 2011 [43]. Therefore, METO can be considered as not having an effect on the immobilization of *D. magna* which suggests a low toxicological potential of this substance at least for daphnids.

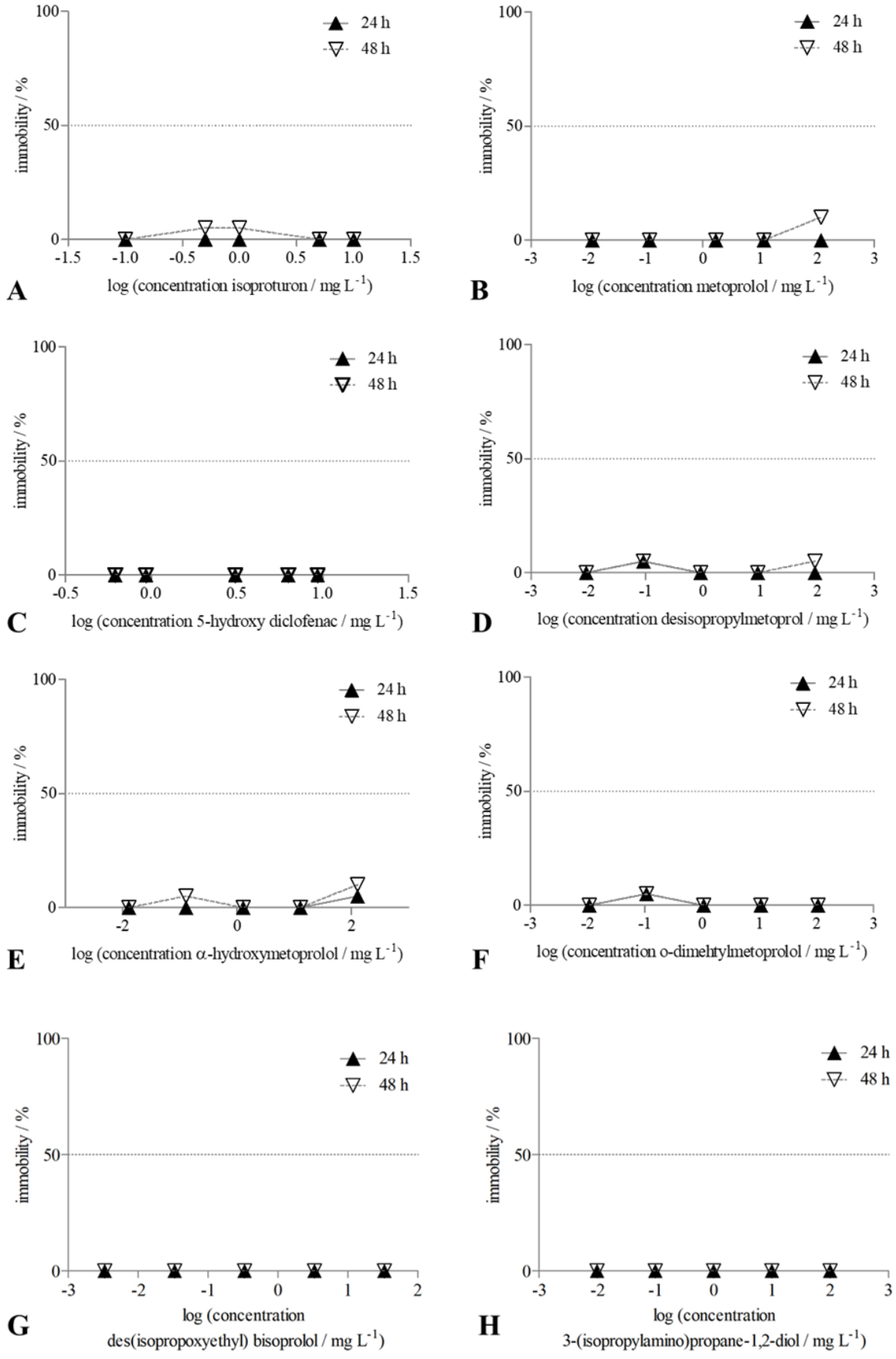
24 h
48 h
immobility / %
100
50
0
A
log (concentration isoproturon / mg L-1)
B
log (concentration metoprolol / mg L-1)
C
log (concentration 5-hydroxy diclofenac / mg L-1)
D
log (concentration desisopropylmetoprol / mg L-1)
E
log (concentration α-hydroxymetoprolol / mg L-1)
F
log (concentration o-dimehtylmetoprolol / mg L-1)
G
log (concentration
des(isopropoxyethyl) bisoprolol / mg L-1)
H
log (concentration
3-(isopropylamino)propane-1,2-diol / mg L-1)

Figure 6-2 Single toxicity results using *D. magna* acute immobilization tests of parent substances and transformation product standards

Results of the *D. magna* (n = 20) acute immobilization test two standards (isoproturon (A) and metoprolol (B)), and six transformation products (5-hydroxy diclofenac (5-OH DCF, C), desisopropylmetoprol (DP-METO, D), α-hydroxymetoprolol (α-METO, E), o-dimethylmetoprolol (o-METO, F), des(isopropoxyethyl) bisoprolol (DPB, G) and 3-(isopropylamino)propane-1,2-diol (3-METO, H). All tested concentrations ranged from 0.001 – 1000 mg/L except for isoproturon (0.01 – 100 mg/L) and 5-OH DCF (0.1 – 100 mg/L). For none of the tested concentration an effect on *D. magna* immobilization was detected. The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

In terms of ISO, the tests performed in this study did not show an aquatic toxicological potential of the substance towards daphnids (Figure 6-2). These observations confirm the results of other studies which presented no immobilization of *D. magna* up to concentrations of >1 mg/L (48 h) [24]

A recent study considering ozonation of DCF reported that the embryotoxicity towards zebrafish is decreasing after ozonation [44]. However, DCF was tested with the same culture of *D. magna* leading to an EC_{50} of 78.9 mg/L (24 h) and 52.8 mg/L (48 h), respectively [23]. The results for DCA are shown in Figure 6-4.A as it was the only substance leading to an immobility of *D. magna*. The EC_{50} values for DCA were determined as 1.84 mg/L (24 h) and 1.02 mg/L (48 h), respectively. These values are in the same order of magnitude as previously published data (EC_{50}-values of 7.1 mg/L (24 h) and 1.4 mg/L (48 h) [45]), which is why DCA is already listed as acutely and chronically toxic to aquatic organisms (category 1) [46]. Some variance in the results of *D. magna* tests always need to be considered as it is already known that aberrations can occur in these tests [42]. For short-term aquatic hazards only one category is assigned by the Classification, Labelling and Packaging (CLP) Regulation. The EC_{50} value after 48 h for crustaceans has to be ≤ 1 mg/L to belong to this category [47]. In this study the EC_{50} of DCA is very close to the threshold value and, therefore, can be considered as ecotoxicologically relevant in terms of category 1.

None of the other tested TPs did show an effect in the tested concentrations (Figure 6-2), which were already higher than levels detected in the environment.

Some of the TPs, especially DCA, showed an instability over the test duration of 48 h in ADaM medium containing daphnids. Thus, the initial concentrations were probably not available for *D. magna* throughout the whole test. However, higher initial concentrations than those found in the environment were applied (1.6 – 130 mg/L). Nevertheless, samples in every concentration of the DCA test, including one beaker without daphnids, were taken after 24 h and 48 h. The concentration of DCA was reduced by around 45 % within 48 h in samples with and without daphnids. Yet, stability tests performed in ultrapure water and ADaM medium in HPLC-vials also showed a change in the concentration. Therefore, it can be concluded that the concentration of DCA is decreases over time due to instability of the TP. However, the EC_{50} for DCA was calculated based on the measured concentrations and thus considers the instability.

However, as only an acute immobilization test was performed different results might have emerged for *D. magna* during chronic toxicity tests with the substances used in this study.

Additionally, the positive control (SI Figure 6-7) as well as the negative controls (maximum of 10 % immobility in any tests) showed satisfactory results for all tests, rendering all tests valid.

6.4.2 Toxicity of TPs formed during ozonation

All parent compounds were separately ozonated in ultrapure water to estimate the toxicological potential of TP mixtures formed during ozonation, further referred to as mixed toxicity. This was done, as it has been previously reported that mixed toxicity is hardly predictable and more complex than the toxicity of single substances [48, 49].

In the present study, no immobilization of *D. magna* was detected for any of the tested substances after ozonation in ultrapure water after 24 h (Figure 6-3). It has recently been shown that DCF is completely degraded in ultrapure water after an added ratio of ozone to parent substance of 5 : 1 while the concentrations of DCA and 5-OH DCF are increasing [31, 32]. Both TPs have the highest yield at a ratio of 5 : 1 (ozone to parent substance) ozone concentration and are degraded almost completely at a 10 : 1 ratio (ozone to parent substance) [31, 32]. However, DCA and 5-OH DCF are not the only TPs formed during ozonation of DCF, therefore, the observed immobilization of *D. magna* after 48 h at an ozone to parent substance ratio of 10 : 1 can either be due to an increasing concentration of a single toxic TP or due to mixture toxicity of TPs not degraded by higher ozone concentrations. Nevertheless, an immobilization of 10 % is too low to conclude any toxicological potential towards daphnids.

The degradation of METO und ISO follows a similar profile as DCF in ultrapure water. Most investigated TPs first show increasing yields and are degraded at higher ozone excess [31, 50]. The effect of 5 % immobilization in ISO samples at the highest ozone concentration after 48 h could also be due to a naturally occurring death rate rather than being related to TPs. The results of the acute immobilization test show that none of the TPs is leading to immobilization of *D. magna* so that these are either not toxic or not formed in concentrations high enough to have effects. Due to the fact that all concentrations used were higher than normally found in the environment it can be concluded that none of the TPs formed during the ozonation of DCF, METO and ISO in ultrapure water have any toxicological potential with respect to *D. magna* (Figure 6-3).

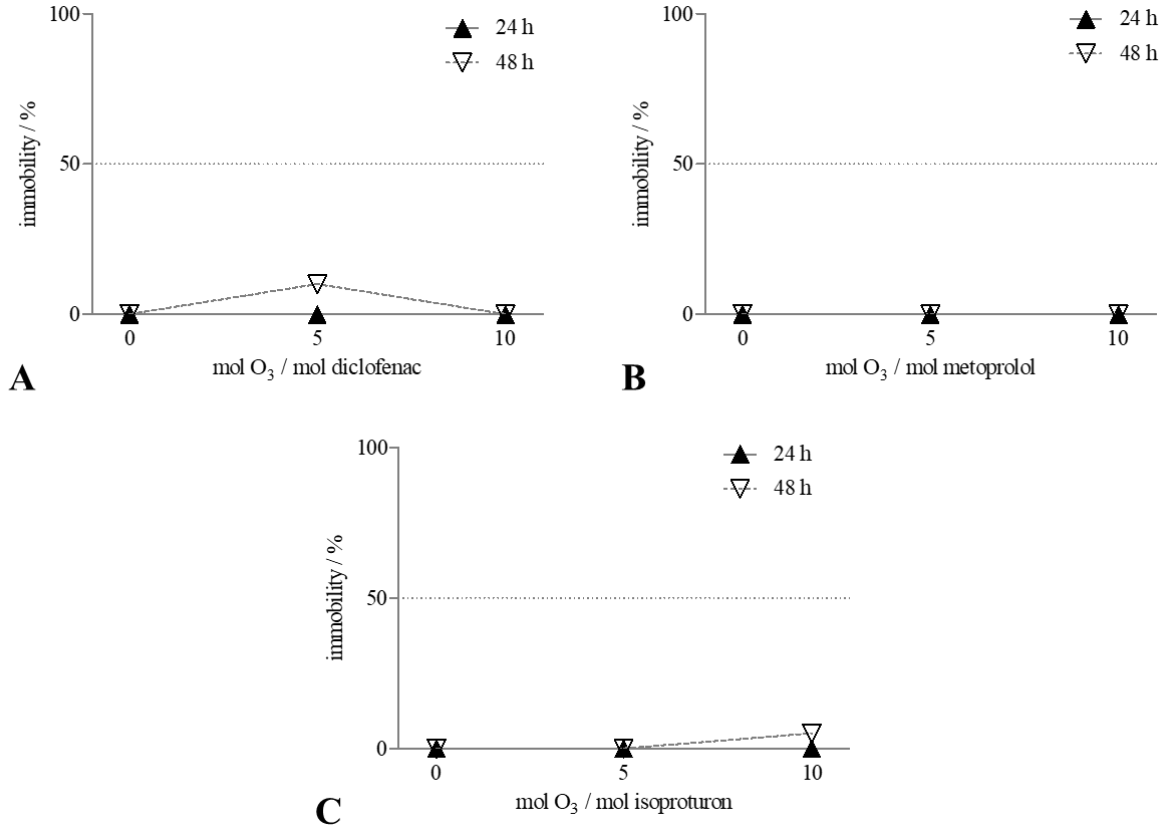

Figure 6-3 Toxicity assessment of ozonated parent substances in ultrapure water

Diclofenac (DCF, 12.5 µM, without pH adjustment, A), metoprolol (METO, 12.5 µM, pH 7, B) and isoproturon (ISO, 6.25 µM, pH 7, C) were ozonated in ultrapure water with three ozone to substance ratios (0 : 1, 5 : 1, 10 : 1) leading to different yields of transformation products which have been tested with *D. magna* (n = 20) in terms of the acute ecotoxicity. Highest immobilization detected in this test was 10 % for diclofenac with an ozone to DCF concentration ratio of 5 : 1. The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

6.4.3 Toxicity of TPs formed during ozonation in the presence of scavengers

The use of scavengers during ozonation can influence the TP formation depending on whether the main reaction pathway leading to a certain TP is an attack of molecular ozone or hydroxyl radicals [32, 39]. Quantification of •OH yields formed during ozonation is mostly performed via two assays, using either *tert*-BuOH or DMSO as scavenger [12]. However, to the authors' best knowledge, no available study focuses on the influence that the implementation of different scavengers can have on the yield of TPs. Studies available on the investigation of TP formation during ozonation only use one scavenger [32, 33, 50-56]. However, Wirzberger et al. [31] has recently shown that different scavengers can lead to differences in the yields of TP formation. Therefore, ozonation of the three parent compounds was also performed in the presence of two scavengers (DMSO and *tert*-BuOH) before testing the effects of the resulting TPs with *D. magna*.

Indeed, no immobilization of D. magna was observed after ozonation of the parent compounds in the presence of *tert*-BuOH (SI Figure 6-8). But, as presented in Figure 6-4.B, the use of DMSO as a scavenger does have an influence on the immobilization of *D. magna* in terms of ozonation of DCF. Interestingly, the concentration of DCA, which has been shown before to have an effect on *D. magna* mobility, at a ratio of 5 : 1 (ozone to parent substance) is very similar in the presence of *tert*-BuOH (15 µM DCA) and DMSO (12 µM DCA) [31], but is not degraded at higher ozone concentrations in systems containing DMSO. However, already without the addition of ozone 25 % of *D. magna* were immobile in the presence of DMSO (24 h and 48 h) and the immobility raised up to 95 % (24 h) and 100 % (48 h) after the addition of 0.5 mM ozone (Figure 6-4.B). In a previous study with the same daphnid culture it was shown, that 5 mM of DMSO is harmless towards *D. magna* [39], therefore, the concentration of 4 mM DMSO in this study cannot be the reason for the immobility of *D. magna* which was confirmed by our results. However, it has also been reported that DMSO can lead to a higher permeability of the membrane due to membrane thinning [57] which can result in a higher toxicity of formed TPs and also lead to a higher toxicity of DCF before ozonation. Therefore, the toxic effects after and even before ozonation can probably be attributed to the presence of DMSO. However, this underlines that ozonation can have an influence on the aquatic toxicological potential of substances and increase this potential.

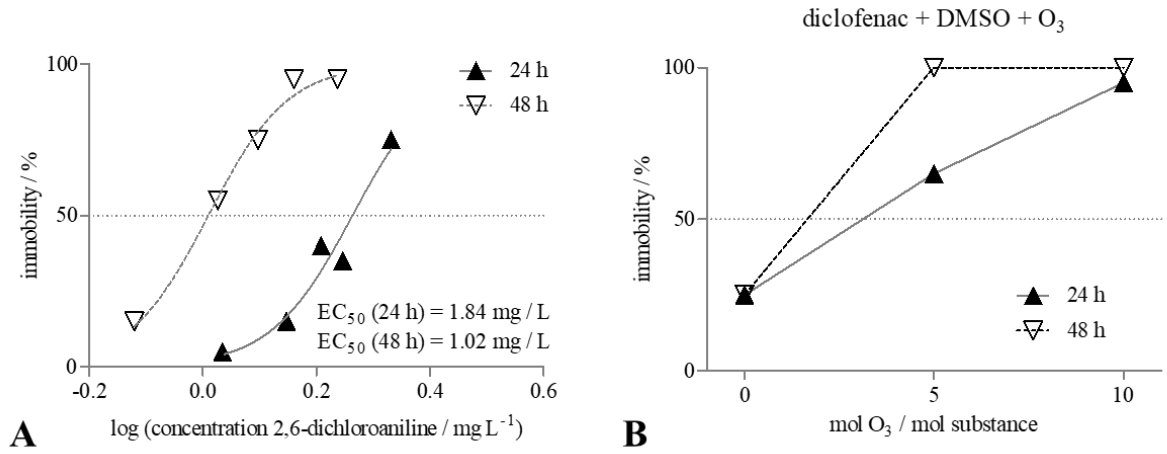

Figure 6-4 Effects of pure 2,6-dichloroaniline (DCA, A) and mixed TPs formed in the ozonation of diclofenac (DCF, 12.5 µM) in the presence of dimethyl sulfoxide (DMSO, 4 mM, B)

Acute immobilization tests were performed with *D. magna* (n = 20). Filled triangles present the results after a test duration of 24 h and open triangles after 48 h. Ozonation experiments were performed in ultrapure water without pH adjustment, a DMSO concentration to scavenge 95 % of •OH and three different ozone : substance ratios (0 : 1, 5 : 1 and 10 : 1). Tests of DCA standard lead to an EC_{50} of 1.84 mg/L (24 h) and 1.02 mg/L (48 h). In samples containing DCF and DMSO prior to ozonation an immobilization of 25 % of *D. magna* (24 h and 48 h) could be detected which raised up to an immobilization of 95 % (24 h) and 100 % (48 h) at a 10 : 1 ozone to substance concentration. The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

It has been shown that the yield of TPs formed in ozonation of METO are mainly depending on the reaction with •OH [50] and the results in this study in terms of *D. magna* correlate with these findings. The formation of TPs in the ozonation of ISO has been reported to be mainly driven by the reaction of molecular ozone [58]. However, for both substances no effect on *D. magna* could be observed in scavenged systems neither with tert-BuOH nor DMSO (SI Figure 6-8 and Figure 6-9, respectively).

Main oxidation products formed in the reaction of •OH with *tert*-BuOH or DMSO are formaldehyde and MSIS, which is further oxidized to MSOS, respectively. Indeed, an immobilization effect on *D. magna* of 20 % (24 h) and 30 % (48 h) for 4 mM MSOS as well as 35 % (24 h) and 75 % (48 h), respectively, for 4 mM MSIS (SI Figure 6-10) were detected. For all three MPs the same ozone concentration ratios have been applied and the DMSO concentration was determined to scavenge 90 – 95 % of •OH. Reported yields of •OH for all

three substances ranged from 30 – 35 % (data not shown) [31, 32] and therefore also the yields of MSIS and MSOS are expected to be similar in all samples. Note, that the reaction of DMSO with •OH also yields methyl radicals upon β-fragmentation after •OH addition. The methyl radical in turn may react in the presence of oxygen to formaldehyde and methanol (Bennett / Russel mechanism) [12, 59]. Nevertheless, for *D. magna* only a toxic effect after the ozonation of DCF in the presence of DMSO was detected, which contradicts the toxic influence of MSOS and MSIS. Therefore, it is more likely that this might be due to TPs which are formed in higher yields in the presence of DMSO – as it is the case for DCA – leading to a stronger effect on *D. magna*.

Another oxidation by-product formed in the presence of *tert*-BuOH is formaldehyde. Indeed, an EC_{50} (48 h) value of formaldehyde for *D. pulex* of 5.8 mg/L has been reported [60]. However, as no effects were detected in samples containing *tert*-BuOH it can be concluded that in this study formaldehyde is only present in small amounts not having an influence on the mobility of *D. magna*. A control of *tert*-BuOH (3.75 mM) was also performed not leading to an immobility of *D. magna*.

The influence of different scavengers on the formation of TPs is only poorly understood. This study additionally showed that also the toxicological potential can be influenced by the use of scavengers pointing out that there is still a knowledge gap in the understanding of the influence of •OH during the ozonation of MPs and even for already well investigated substances. However, it could be shown that ozonation in the presence of scavengers can increase the toxicological potential and therefore, should be also considered ion further studies.

6.4.4 Toxicity of TPs formed during bromination prior and after ozonation

In waters containing Br^- during ozonation the secondary oxidant HOBr can be formed. To investigate the influence of HOBr on the formation of TPs all parent compounds were treated with HOBr and subsequently ozonated with two different ozone concentrations (Figure 6-5). This direct oxidation by HOBr was chosen as a preliminary performed study revealed that the direct reaction of HOBr with the substances leads to the formation of similar TPs as in the reaction of the substances with different ozone and Br- concentrations. This will be studied in more detail in the future. To exclude effects of the bromination by-products such as bromate or residual HOBr these were tested separately regarding their effects using daphnids. For bromate no definitive concentration test was performed due to a too low number of *D. magna* available. However, bromate, which can be formed during ozonation of HOBr, was

tested in the highest theoretically formed concentration as well as one higher (0.25 mM) and one lower (0.0625 mM) concentration leading to an immobilization of *D. magna* in all three concentrations (24 h and 48 h) (SI Figure 6-12). The EC_{50} concentration for HOBr was determined to be 0.306 mg/L (24 h and 48 h, SI Figure 6-11).

The strongest effect on *D. magna* was determined in samples containing DCF and METO. METO samples also contained the highest concentration of residual HOBr (2.65 mg/L) while the DCF samples (0.35 mg/L) contained a similar concentration of residual HOBr as the ISO samples (0.31 mg/L). In ISO samples no effect was detected. In all tested samples the measured concentrations of residual HOBr was similar or higher than the EC_{50} but did not lead to an immobilization of *D. magna* in DCF (24 h) and ISO (24 h and 48 h) samples. However, in DCF samples an immobilization of 53 % (48 h) was detected for DCF after bromination, but decreased to 0 % at an ozone to parent substance ratio of 5 : 1 (Figure 6-5). The strongest effect for samples containing DCF and HOBr was detected after an addition of a 10 : 1 ozone to parent substance ratio with 25 % (24 h) and 95 % (48 h) immobility of *D. magna*, respectively. One possibility could be bromate formed in the reaction of HOBr with O_3 [12, 61]. However, bromate did not have such a high influence on the mobility of *D. magna* (SI Figure 6-12). Therefore, it can be assumed that the reaction of HOBr leads to reaction products which have a higher effect on the immobilization of *D. magna* that the resulting products formed after a subsequent ozonation of the samples. Continuing, at ozone to substance ratios of 10 : 1 TPs are formed which have a high effect on the mobility of daphnids.

Indeed, no effects could be detected for ISO samples including HOBr and formed ozonation products. This is contradictory to the results of DCF, in which a high effect of *D. magna* could be detected, although both samples contained similar concentrations of residual HOBr and should therefore yield similar concentrations of bromate. However, this was not the case and underlines that the toxic effect detected in DCF samples might be assigned to the overall transformation products formed at high ozone concentrations during ozonation of brominated DCF but not during ozonation of ISO.

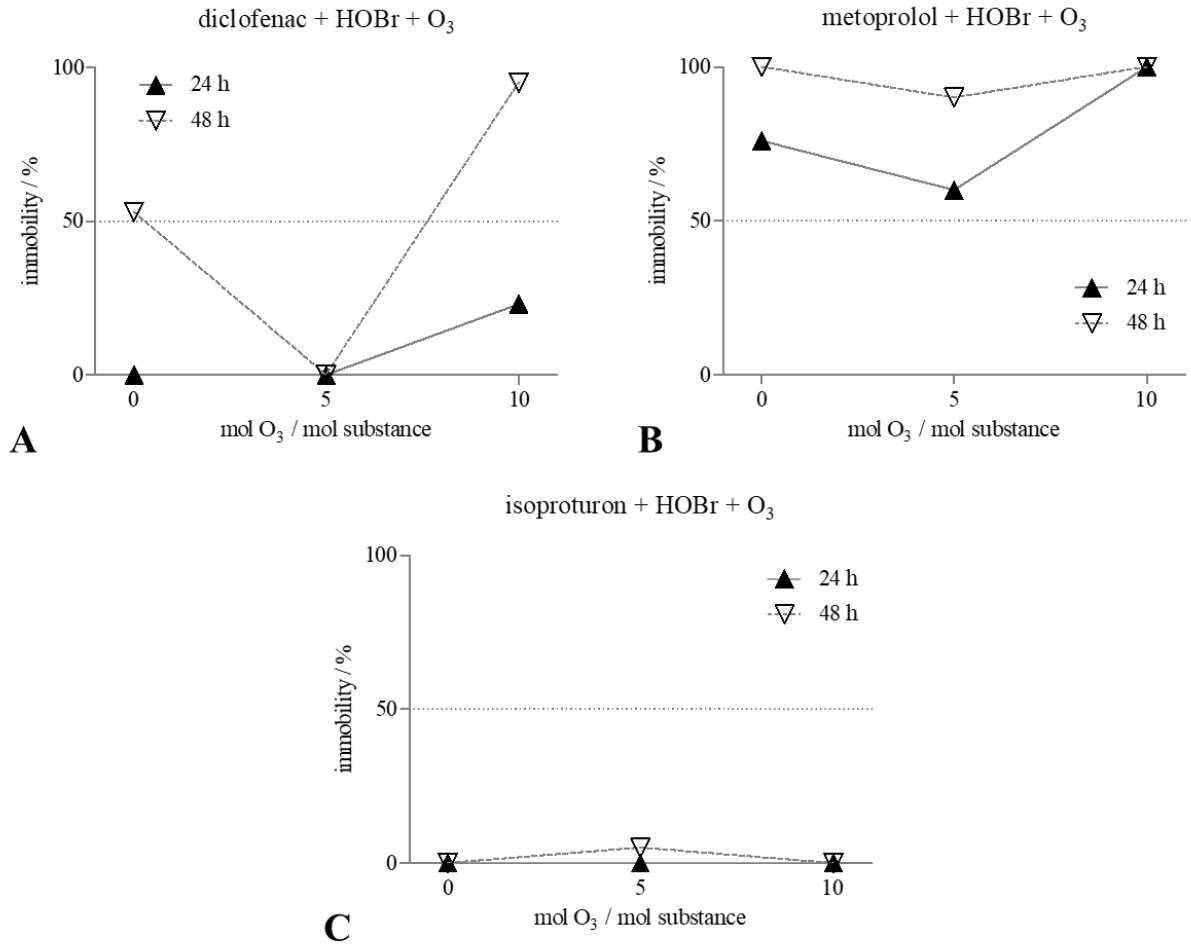

Figure 6-5 Effect of brominated and subsequently ozonated substances on the mobility of *D. magna*

Diclofenac (0.05 mM, pH 7), metoprolol (0.05 mM, pH 7) and isoproturon (0.025 mM, pH 7) were treated with different hypobromous acid to substance ratios 2 : 1 (diclofenac), 2.5 : 1 (metoprolol) and 1 : 1 (isoproturon). After a reaction time of 24 h ozone was added in ratios of 5 : 1 and 10 : 1 for all substances. Acute immobilization tests were performed with *D. magna* (n = 20) with final test concentrations of 9.4 µM (diclofenac and metoprolol) and 4.7 µM (isoproturon). After 48 h of test duration different results were detected for the three brominated parent substances in samples containing an ozone to substance ratio of 10 : 1. While for isoproturon samples no immobilization was detected, the immobilization in metoprolol samples was 100 % (48 h). For diclofenac samples 95 % (48 h) of *D. manga* were immobile. The threshold for 50 % immobilization is indicated by a dashed line in each graph.

In the case of METO brominated with HOBr the effect on *D. magna* is more likely to be due to toxic by-product formation, such as bromate or residual HOBr, rather than brominated METO and its TPs, because a higher concentration of residual HOBr was detected than in DCF and ISO samples. This was leading to an immobilization of 80 % (24 h) and 100 % (48 h), respectively (Figure 6-5). However, also TPs with a high toxicity to daphnids can be formed during ozonation of brominated METO. The immobilization of the daphnids decreased after the addition of an ozone to parent substance ratio of 5 : 1 (60 %, 24 h and 90 %, 48 h) but increased at an ozone addition of 10 : 1 to 100 % (24 h and 48 h) (Figure 6-5). Nevertheless, it needs to be kept in mind that also in ozonated samples the toxicity can be influenced by residual HOBr or formed bromate.

These results underline, that the matrix composition during ozonation can increase but also decrease the aquatic toxicological potential of MPs. This should be investigated in more detail in upcoming studies.

6.4.5 Toxicity of wastewater effluent

Since the formation of TPs can be influenced by the wastewater matrix, this study estimated the toxicological potential of the TP mixture formed in biological wastewater treatment and ozonated wastewater for aquatic organisms. Both tested wastewater types did not show any effect on the mobility of *D. magna*. Hence, it can be concluded that the concentrations of the TPs was either too low or that present TPs do not have an effect of acute toxicity towards *D. magna*. Nevertheless, only the acute test was performed and it is not known if the wastewater or TPs analyzed in this study have a chronic effect on *D. magna*. Therefore, the toxicological potential of wastewater for aquatic organisms cannot be excluded only by the acute immobilization test of *D. magna*. However, it was shown in this study that matrix components can increase or decrease the toxicological potential of *N*-containing substances towards *D. magna*.

6.5 Conclusion

- Even if in this study not a high aquatic toxicological potential of the formed TPs during the ozonation of ISO, METO and DCF in the presence of different matrix components was detected, the investigation of matrix effects, formation of TPs and the toxicological potential should be of high concern. It needs to be considered, that the variance of MPs and matrix composition can highly differ in every WWTP and therefore lead to highly variable and hardly predictable TPs.
- However, it could be shown in this study that TPs, such as DCA, have an effect on the immobilization of *D. magna* and increasing effects can be detected in different water matrices. Additionally, this study underlined that matrix components can also have an influence on the formation of possibly toxic TPs.
- Indeed, only the acute immobilization test of *D. magna* was applied not taking into account chronic or mutagenic effects, which can be induced by the formed TPs. The chronic effect of wastewater from the same WWTP after ozonation on *D. magna* and macrozoobenthos will be addressed in future work.

6.6 Supporting Information – Chapter 6

6.6.1 Materials

Table 6-1 List of chemicals and solvents used

Chemical / Solvent	Manufacturer
α-hydroxymetoprolol ≥ 98 %	Sigma Aldrich
2,6-dichlororaniline 98 %	Merck
3-(isopropylamino)propane-1,2-diol 95 %	Enamine
5-hydroxy diclofenac analytical standard	Sigma Aldrich
Acetonitrile > 99 %	Merck
Cellulose membrane filter 0.45 µm	Whatman
Des(isopropoxyethyl) bisoprolol 98 %	LGC Standards GmbH
Desisopropylmetoprolol 95 %	Enamine
Diclofenac sodium salt > 98.5 %	Sigma Aldrich
Dimethyl sulfoxide 99 %	Merck
Dipotassium hydrogenphosphate	AppliChem
EDTA disodium salt 2-hydrate	AppliChem
Formic acid 98 – 100 %	Merck
Hydrochloric acid 35 %	Bernd Kraft
Isoproturon ≥ 98 %	Sigma Aldrich
Methanesulfonic acid ≥ 99 %	Merck
Methanoesulfinic acid sodium salt 95 %	Alfa Aesar
Methanol 100 %	VWR Chemicals
Methanol LC-MS grade 99.99 %	Fischer Chemiclas

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Chemical / Solvent	**Manufacturer**
Metoprolol tartrate salt > 99 %	Sigma Aldrich
o-demethylmetoprolol ≥ 97 %	Sigma Aldrich
Oxygen 99.99 % (200 bar)	Alphagaz
Pierce LTQ ESI positive ion calibration solution	Thermo Scientific
Selenium dioxide	Sigma Aldrich
Sodium bromide > 99 %	Fluka
Sodium bromide	Roth
Sodium dihydrogen phosphate > 99 %	AppliChem
Sodium hydrogen carbonate	Roth
Sodium hydroxide pellets	Bernd Kraft
Sulfuric acid 95 %	Fischer Chemical
tert-butanol	Merck
Ultra-purified water	-
Water LC-MS grade 99.99 %	Fischer Chemicals

Daphnia media	0.333 g/L HW-Marinemix®professional,
Aachener Daphnia medium 10 L (adapted from Klüttgen et al. [30] (1994))	23 mL solution A
	22 mL solution B
	1 mL solution C
Solution A	117.6 g/L $CaCl_2$ x 2 H_2O
Solution B	25.2 g/L $NaHCO_3$
Solution C	0.07 g/L SeO_2

Table 6-2 List of used equipment

Technical device	Model	Manufacturer
Aerator Tetratec® APS 400	-	Tetra GmbH
Dissolved oxygen sensor	FiveGo F4 IP67	Mettler Toledo
HW-Marinemix® professional (sea salt)	-	Wiegand GmbH
Luminous bench Prolite 5000	-	Kaiser Fototechnik
Microscopic ocular	WHSZ 10x-H/22	Olympus
Ozone generator	COM-AD-01	Anseros
Ozone generator	Philaqua 802x	BMT Messtechnik
pH meter	827 pH Lab	Metrohm
pH test stripes 6 – 10	-	VWR
Scale	R169P	Sartonus Research
Shaker	KS 260C	IKA®
Sorting sieve 900 µm	-	Nuova
Sorting sieve 120 µm	-	Unizoo
UV-Vis Spectrometer	UV-1800	Shimadzu
UV-Vis Spectrometer	UV-1650PC	Shimadzu
Zoom Stereo Microscope	SZ51	Olympus
HPLC – DAD		
Auto injector	SIL-10ADVP	Shimadzu
Column oven	CTO-10ASVP	Shimadzu

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Technical device	Model	Manufacturer
Core shell LC column	Kinetex 5 µm EVO C18 100Å	Phenomenex
Degassing unit	DGU-20A5R	Shimadzu
Diode array detector	SPD-M10AVP	Shimadzu
Fluorescence detector	RF-10AXL	Shimadzu
Liquid chromatograph	LC-10ATVP	Shimadzu
Reservoir tray	-	Shimadzu
System controller	SCL-10AVP	Shimadzu
Valve assembly	FCV-10ALVP	Shimadzu
HPLC – HRMS		
Core shell LC column	Kinetex 5 µm EVO C18 100Å	Phenomenex
Mass Spectrometer Orbitrap	Q Exactive	Thermo Scientific
UHPLC (Pump, Autosampler)	Dionex Ultimate 3000 UHPLC$^+$	Thermo Scientific

6.6.2 Methods

Equation 6-1 Calculation of minimal scavenger concentration [29]

$$c_{scavenger,min} = \frac{f_{\bullet OH+scavenger} * c_{substance} * k_{\bullet OH+substance} + f_{\bullet OH+scavenger} * c_{O_3} * k_{\bullet OH+O_3}}{k_{\bullet OH+scavenger} * (1 - f_{\bullet OH+scavenger})}$$

Equation 6-2 Calculation of maximal scavenger concentration [29]

$$c_{scavenger,max} = \frac{f_{O_3+scavenger} * c_{substance} * k_{O_3+substance}}{k_{O_3+scavenger} * (1 - f_{O_3+scavenger})}$$

Table 6-3 Reaction rate constants of relevant reactions at 20 °C

Reaction	k_2 ($M^{-1}s^{-1}$)	Reference
$^{\bullet}OH$ + DCF	9.3×10^9	Yu et al. [62]
$^{\bullet}OH$ + DMSO	7×10^9	Buxton et al. [63]
$^{\bullet}OH$ + ISO	7.9×10^9	Benitez et al. [64]
$^{\bullet}OH$ + METO	7.3×10^9	Benner et al. [51]
$^{\bullet}OH$ + *tert*-BuOH	6×10^8	Buxton et al. [63]
O_3 + $^{\bullet}OH$	1.1×10^8	Sehested et al. [65]
O_3 + DCF	6.8×10^5	Sein et al. [32]
O_3 + ISO	$2.2 \cdot 10^3$	Benitez et al. [64]
O_3 + METO	2×10^3	Benner et al. [51]
O_3 + DMSO	8.2	Pryor et al. [66]
O_3 + *tert*-BuOH	3×10^{-3}	Hoigné and Bader [67]

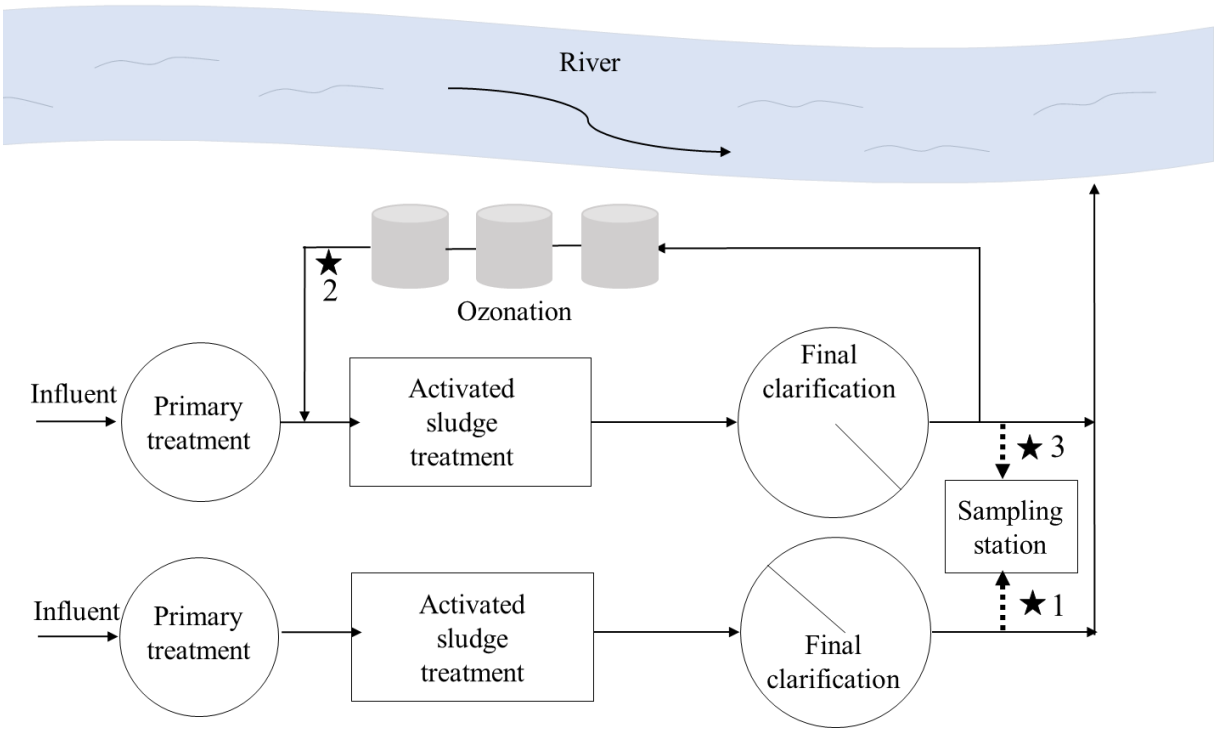

Figure 6-6 Sampling sites within the wastewater treatment plant

In June and July 2019 three samples were taken at a wastewater treatment plant. In total three samples were taken. One after the conventional wastewater treatment (1), one after ozonation of the already conventionally treated wastewater (2) and the one after mixing the ozonated with the conventionally treated wastewater (3). The three sampling sites are marked with ★.

Table 6-4 Concentrations of single parent substances and transformation products tested with *D. manga* in mg/L

All tests were performed in ADaM medium and included five different concentrations (C_1 – C_5).

[mg/L]	ISO	METO	DCA	5-OH DCF	DP-METO
C_1	10.01	116.97	16.36	9.23	90.67
C_2	5.00	11.69	1.63	6.23	9.07
C_3	1.00	1.17	0.16	3.11	0.90
C_4	0.50	0.12	0.02	0.92	0.09
C_5	0.10	0.01	0.002	0.62	0.01
[mg/L]	**O-METO**	**DPB**	**3-METO**	**α-METO**	**HOBr**
C_1	107.3	33.08	99.06	130.03	12.11
C_2	10.73	3.31	9.91	13.00	6.59
C_3	1.07	0.33	0.99	1.30	1.21
C_4	0.11	0.03	0.10	0.13	0.66
C_5	0.01	0.003	0.01	0.01	0.12

Table 6-5 Concentration of 2,6-dichloroaniline tested with *D. magna* in mg/L

The definitive test of 2,6-dichloroaniline (DCA) was performed in ADaM medium and with five different concentrations (C_1 – C_5).

	DCA [mg/L]
C_1	3.26
C_2	2.85
C_3	2.45
C_4	2.04
C5	1.63

Table 6-6 Concentrations of potassium dichromate tested on *D. magna* in mg/L

The test of potassium dichromate ($K_2Cr_2O_7$) was performed in ADaM medium and with five different concentrations (C1 – C5).

	$K_2Cr_2O_7$ [mg/L]
C_1	16.63
C_2	13.04
C_3	6.52
C_4	3.26
C5	1.63

Table 6-7 Instrument settings for analysis of diclofenac and corresponding transformation products with HPLC-DAD and HPLC-HRMS

Unit	Parameter	Value
HPLC-DAD	Injection volume	50 µL
	Flow rate	0.5 ml/min (diclofenac)
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	
		0-15 min: 40 % A, 60 % B
HPLC-HRMS		
	Injection volume	100 µL
	Flow rate	0.3 mL / min
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	0-4 min: 80 % A, 20 % B
		4-10 min: 20 % A, 80 % B
		10-15 min: 80 % A, 20 % B
	Scan type	Full MS
	Scan range	100.0 to 1000.0 m/z
	Fragmentation	None
	Resolution	70000

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Unit	Parameter	Value
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	10 s
	AGC target	1e6
	Maximum inject time	100
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	320 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C

Table 6-8 Instrument settings for analysis of metoprolol, isoproturon and corresponding transformation products with HPLC-DAD and HPLC-HRMS

Unit	Parameter	Value
HPLC-DAD		
	Injection volume	50 µL
	Flow rate	0.3 mL/min
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	0–7 min: 90 % A, 10 % B
		10–13 min: 10 % A, 90 % B
		13.1–30 min: 90 % A, 10 % B
HPLC-HRMS		
	Injection volume	10 µL
	Flow rate	0.3 mL/min
	Eluents	A: water + 0.1 % formic acid
		B: methanol + 0.1 % formic acid
	Gradient	0-4 min: 90 % A, 10 % B
		7-10 min: 10 % A, 90 % B
		10.1-20 min: 90 % A, 10 % B
	Scan type	Full MS
	Scan range	100.0 to 1000.0 m/z
	Fragmentation	None

Continued on next page

Unit	Parameter	Value
	Resolution	70000
	Polarity	Positive
	Microscans	1
	Lock masses	Off
	Chrom. peak width	10 s
	AGC target	1e6
	Maximum inject time	100
	Sheath gas flow rate	37
	Aux gas flow rate	15
	Sweep gas flow rate	1
	Spray voltage	3.5 kV
	Capillary temperature	320 °C
	S-lens RF level	50.0
	Aux gas heater temperature	50 °C

6.6.3 Results & Discussion

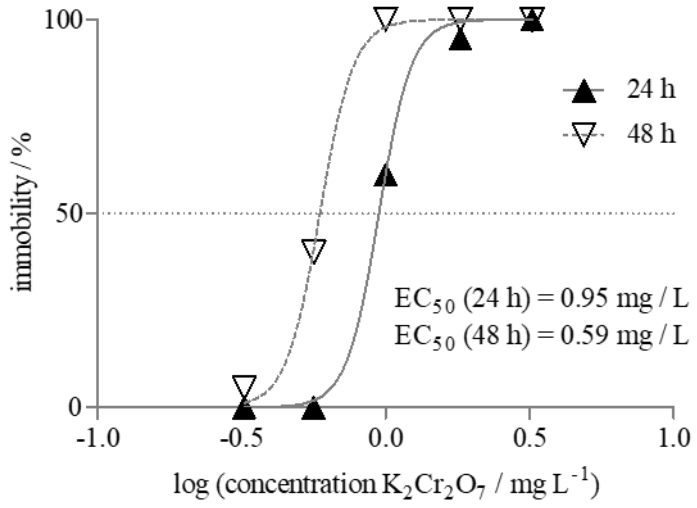

Figure 6-7 Positive control of the *D. magna* test

A positive control was performed once for all tests with 0.32 – 3.2 mg/L of potassium dichromate, leading to 50 % immobility of *D. magna* (n = 20) at a concentration of 0.95 mg/L after 24 h and 0.59 mg/L after 48 h. Additionally, a negative control was performed for every test separately containing only ADaM medium, leading for all tests to a maximum immobility of 10 %. The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

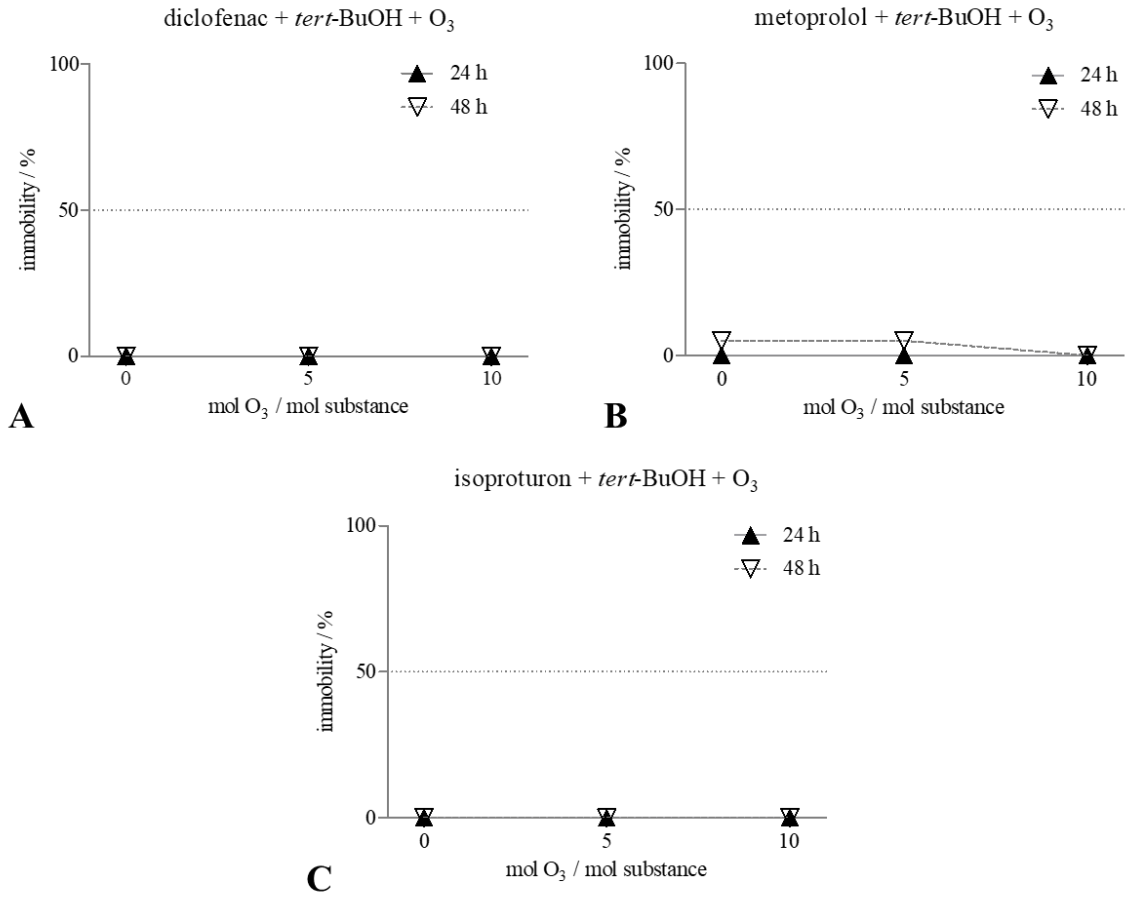

Figure 6-8 Toxicity assessment of ozonated parent substances in the presence of *tert*-BuOH

Results of diclofenac (DCF, 9.4 µM, A), metoprolol (METO, 9.4 µM, B) and isoproturon (ISO, 4.7 µM, C) and their formed transformation products at different ozone to substances ratios (0 : 1, 5 : 1, 10 : 1) in the presence of *tert*-butanol (DCF: 4 mM, METO: 1.26 mM, ISO: 0.675 mM). None of the experiments leads to immobilization of *D. magna* (n = 20). The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

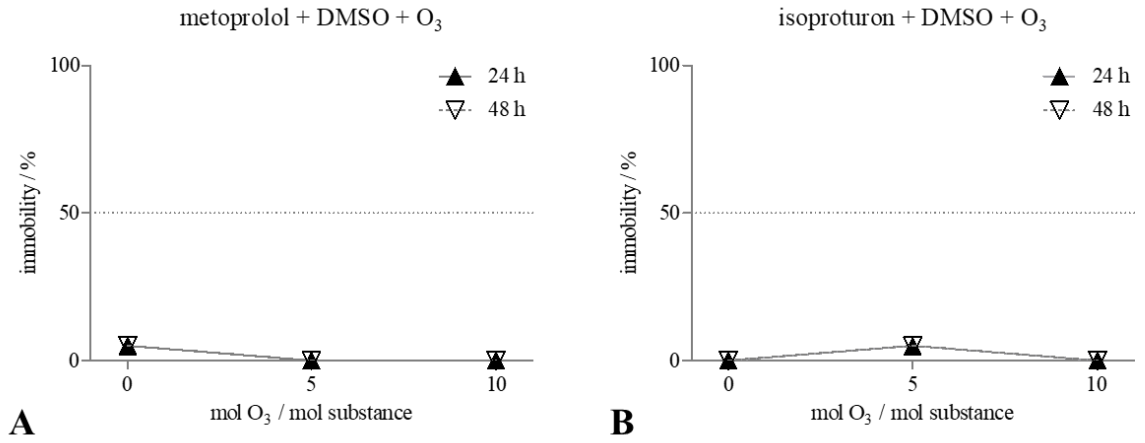

Figure 6-9 **Toxicity assessment of ozonated parent substances (metoprolol and isoproturon) in the presence of dimethyl sulfoxide (DMSO)**

Results of metoprolol (METO, 12.5 µM, A) and isoproturon (ISO, 6.3 µM, B) and their formed transformation products at different ozone to substances ratios (0 : 1, 5 : 1, 10 : 1) in presence of dimethyl sulfoxide (METO: 0.108 mM, ISO: 0.058 mM). No immobilization of *D. magna* (n = 20) was detected. The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

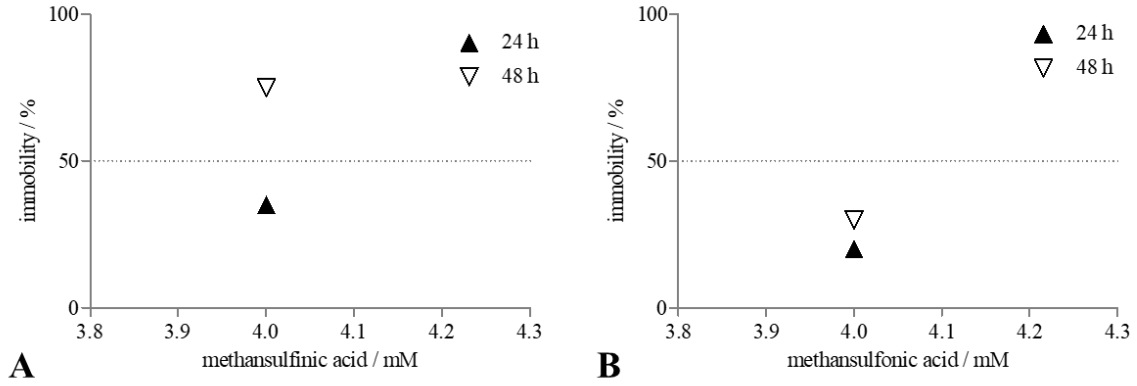

Figure 6-10 Results of the control of methanesulfinic acid (MSIS, A) and methanesulfonic acid (MSOS, B) on the mobility of *D. magna*

Effects of the by-products methanesulfinic acid (MSIS, 4 mM, A) and methanesulfonic acid (MSOS, 4 mM, B) formed during oxidation of dimethyl sulfoxide lead to an effect of 20 % (24 h) and 30 % (48 h) for MSOS and 35 % (24 h) and 75 % (48 h), respectively, for MSIS in the toxicity with *D. magna* (n = 20). The threshold for 50 % immobilization is indicated by a dashed line in each graph. Concentrations are given as used in the *D. magna* test, including dilution.

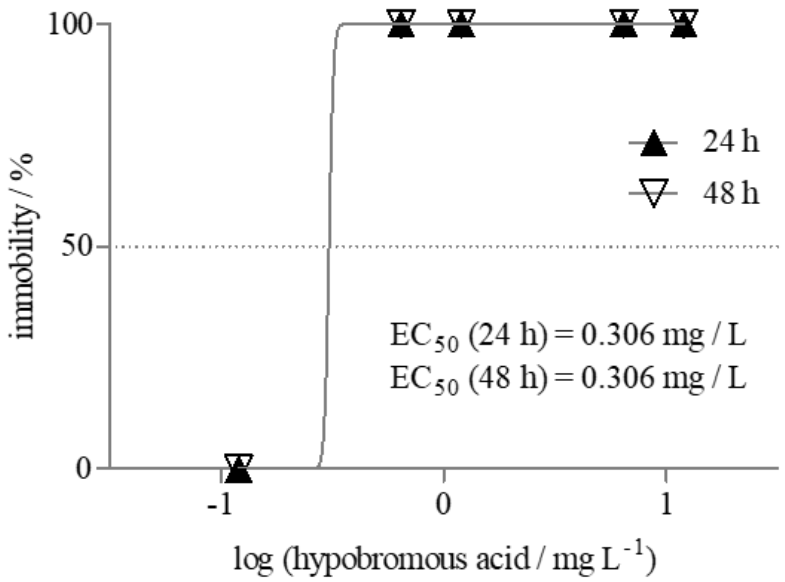

Figure 6-11 Results of the control of hypobromous acid on the mobility of *D. magna*

Five concentration of hypobromous acid (HOBr) were tested in a *D. magna* (n = 20) acute immobilization test leading to a 50 % immobilization (EC50) at 0.306 mg/L after 24 h and 48 h incubation. The threshold for 50 % immobilization is indicated by a dashed line. Concentrations are given as used in the *D. magna* test, including dilution.

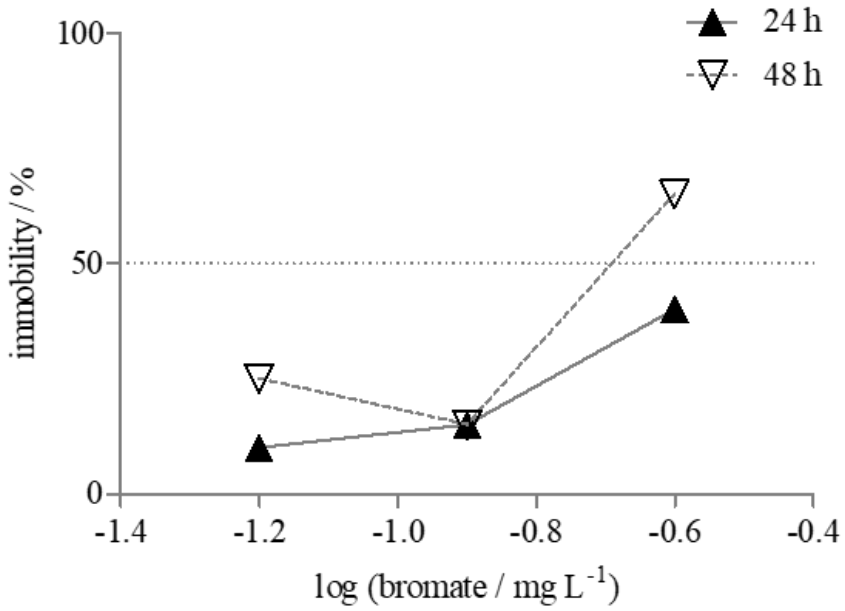

Figure 6-12 Results of the bromate control on the mobility of *D. magna*

Three different concentrations of bromate (BrO_3^-) were tested in a *D. magna* (n = 20) acute immobilization test. 0.125 mM is the highest possible formed concentration of this by-product and one higher (0.25 mM) as well as one lower concentration (0.0625 mM) were tested. All concentrations showed an effect on *D. magna* after 24 h and 48 h incubation. The threshold for 50 % immobilization is indicated by a dashed line. Concentrations are given as used in the *D. magna* test, including dilution.

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Chapter 7
Conclusion & Outlook

7.1 General Conclusion & Outlook

Ozonation of nitrogen containing (*N*-containing) substances has been of high concern since years as it is often applied in wastewater treatment to remove micropollutants (MPs) which mostly contain nitrogen [1-3]. However, the gap between mechanistic studies under laboratory conditions and the influence of matrix composition has hardly been addressed in literature until now. Within this study it was detected that the surrounding matrix is highly influencing the formation of transformation products (TPs) and also the toxicological potential of these substances towards aquatic organisms.

It could be shown that the degradation of simply structured *N*-containing substances (such as 2,2,6,6-tetramethylpiperidine (TMP) and *cis*-2,6-dimethylpiperidine (DMP)), which are very likely to form reactive intermediates (aminyl radicals), is highly pH dependent. In detail, it was observed, that these substances do not react with ozone at pH 2. Further, stoichiometries above one were detected in experiments performed at pH 7 or pH 11. These results underline the postulate of an ozone consuming chain reaction which was also supported by observations made later in this study during the degradation of more complex *N*-containing substances (isoproturon (ISO), metoprolol (METO) and diclofenac (DCF)) with ozone. This ozone consuming chain reaction was already suggested prior to the present study, to explain high ozone consumption with simultaneously low substance degradation [4-6] which was also observed in this study for the five (model) substances. Further, high stoichiometries were so far reported for ultrapure water [4-6], but could be observed in this study independently of the surrounding matrix. However, even if the obtained results support the suggested chain reaction, which includes the formation of aminyl and nitroxide radicals, experimental evidence for the formation is still lacking. As already numerous previously performed studies also suggested the formation of aminyl and / or nitroxide radicals during ozonation to explain the formation of TPs [1, 4-8] an experimental evidence of these intermediates is of high relevance to confirm the postulated reaction pathways. To verify the formation of aminyl (and / or nitroxide) radicals the direct generation of those and their reaction potential during ozonation should be investigated in detail. Generation of these radicals might be achieved through photolysis of chloramines [9, 10] or reaction of nitrogen containing substances with sulfate radicals [11] and should be of high interest in upcoming studies.

However, as it was shown within this study the measurements of substances, which might react to aminyl or nitroxide radicals, can lead to challenges (such as increasing peak areas) within the analysis. Even if it had been reported before that the stable nitroxide radical

2,2,6,6-tetramethylpiperidineoxyl can be measured with ESI-MS [12, 13], this could not be confirmed in this study. In contrast, observations made rather imply that reactive intermediates such as nitroxide radicals are not easily detectable with LC-MS. However, as those intermediates (in detail aminyl and nitroxide radicals) play an important role in the postulation of reaction pathways during ozonation [1, 4-8], it is not only of high interest to confirm their formation but also to develop a robust analytical method for their measurement. One method to determine the presence of radicals could be the combination of a Shamrock spectrometer with an ICCD camera to observe a time resolved spectrum specific for radicals [14]. It can be assumed that the detection of radicals formed during ozonation is also possible via ICCD camera.

After the formation of these intermediates has been confirmed also their reaction with matrix components should be analyzed, as in the present study these reactions have been suggested to play an important role in the formation of TPs. Hence, a confirmation of the ozonation consuming chain reaction or formed intermediates can lead to some challenges within treatment steps which need to be addressed. This can be, e.g., the adjustment of ozone dosages, as higher ozone concentrations than expected until now might be needed to degrade MPs in wastewater as a result of the ozone consumption by formed intermediates. Further, the formation of unwanted TPs due to the influence of matrix components has to be considered, as this has been shown in this study exemplary for compounds quenching $^{\bullet}$OH.

Most studies hitherto focused either on the mechanistic aspect or, if considering the influence of matrix components, on the degradation of substances during ozonation. This study combined the mechanistic studies with the investigation of TP formation based on the surrounding matrix. These investigations revealed that even if the degradation of *N*-containing substances (ISO, METO and DCF as model MPs) is independent of the surrounding matrix, the formation of TPs can highly differ. Especially, the comparison of different, frequently used $^{\bullet}$OH scavengers has yet not been addressed in literature but revealed striking results in terms of TP formation in this study. Different effects of the two used scavengers (dimethyl sulfoxide (DMSO) and *tert*-butanol (*tert*-BuOH)) could be observed, for one TP of DCF and METO, respectively. In both cases for one of the scavengers a continuous formation without subsequent degradation of one TP was observed. As the reaction rates of the two TPs with ozone were high enough to exclude the reaction of ozone with the added scavenger these observations remain still unclear. However, as this has never been investigated before, it is still unclear if this phenomenon does only occur for the chosen substances or if it generally needs to be considered for the ozonation of *N*-containing substances in the presence of scavengers. The TP of DCF,

which showed a continuous formation in the presence of DMSO, can also be considered as ecotoxicologically relevant (effect concentration leading to 50 % immobilization (EC_{50}) of *Daphnia magna* (*D. magna*) was around 1 mg/L after 48 h in the acute immobilization test) because substances for which an EC_{50} of crustaceans ≤ 1 mg/L (after 48 h) is determined are considered as ecotoxicologically relevant in terms of category 1 [15]. Therefore, the continuous formation of TPs in the presence of different radical scavengers should also be investigated in detail for other substances relevant in the aquatic environment.

Also, in the presence of bromide strong effects on the formation of TPs could be detected. As it was reported earlier, bromide and especially the formed hypobromous acid (HOBr), as a secondary oxidant, can lead to undesired TPs which can be of high ecotoxicological concern and, as it was the case for *N*-nitrosodimethylamine (NDMA), might also be detected after drinking water purification [16-18]. The influence of bromide and HOBr on the formation of TPs could be supported within the present study as new TPs were reported for the reaction of ISO, METO and DCF with ozone at different bromide concentrations. Further, for the direct reaction of ISO, METO and DCF with HOBr followed by ozonation also many TPs not previously reported could be detected. For these TPs the ecotoxicity tests performed with *D. magna* also revealed a toxicological potential towards aquatic organisms. This is similar to the results reported for *N*,*N*-dimethylsulfamide and NDMA showing that bromide, which is omnipresent in waters and ranges in concentration normally from 0.01 to 1 mg/L [19], can lead to undesired and yet mostly unknown effects on the formation of TPs.

To give an overall impression on the influence of matrix components during ozonation of *N*-containing substances also the toxic potential towards aquatic organisms was investigated within this study. As mentioned the toxicological potential of formed TPs changed in the presence of DMSO and bromide, leading to higher effects on *D. magna* than tested parent compounds or ozonated samples without matrix components. These results revealed that not only the TP formation is affected by the matrix present during ozonation but that also the toxicological evaluation can be highly influenced. So far, no other studies have been reported, combining the ozonation of ISO, METO and DCF in various water matrices with the evaluation of the toxicological evaluation towards aquatic organisms. It has been reported that the pH can influence the TP formation during ozonation and thus also the toxicological potential (algae growth inhibition) of those [7]. However, more comprehensive than this the phenomenon of matrix influences has not been investigated. As in the present study only acute toxicity tests were performed, upcoming studies should also focus on the chronic effects towards aquatic organisms as concentrations of MPs and especially TPs are normally very low in the aquatic

environment and do mostly not induce the direct death of a species [20]. Indeed, a complete risk assessment for MPs and TPs needs to consider acute and chronic effects and additionally more than one species of various trophic levels in the aquatic ecosystem. Therefore, in upcoming studies considering the influence of matrix components during ozonation on the formation of potentially toxic TPs a full screening would be recommended. This should include acute and chronic toxicity tests of single MPs, TPs and mixtures formed during ozonation performed with e.g., *D. magna*, as this test is well established and leading to fast results. Growth inhibition tests performed with algae and acute toxicity tests considering the death of fish could also be performed, to address various trophic levels in the aquatic ecosystem. These variations of tests would allow a better evaluation of the toxicological relevance of formed TPs. Thus, two approaches (an effect-driven and exposure-driven approach) should be considered in upcoming studies to investigate the influence of single TPs but also mixtures on aquatic organisms [21]. However, it needs to be kept in mind that most results of toxicity tests have been obtained in experimental setups which do not take into account environmental stressors such as changes in the availability of food, the presence of predators or the interactions between species which can highly influence the response to toxicants [22, 23]. These limits could be overcome by either adding more variables to the experimental setup, such as changes in the food availability, or by performing additional tests in field studies at, e.g., WWTPs [24] or lakes [25].

In this study a first impression of the influence of matrix composition during the formation of TPs could be given. However, as only some MPs and matrix compositions could be investigated further research is still needed. Therefore, it would be of high interest to investigate other MPs such as tamoxifen (contains nitrogen) or ibuprofen (as a relevant MP not containing nitrogen) which are frequently found in the environment and known to be degraded via ozone [7, 26-29]. Further, it is known for both substances that TPs formed in ultrapure water are of ecotoxicological concern [7, 27]. Therefore, detailed investigations are needed, considering different matrix components separately (as done in this study) but also combinations of those such as bicarbonate, bromide but also different concentrations of dissolved organic carbon. This is of high interest, because even if it has been shown that single matrix components can have a strong influence on the TP formation, effects of mixtures remain still unclear. Further, upcoming studies in terms of TP formation should also focus on the effects of different $^{\bullet}OH$ scavengers and critically question if results presented in other studies only considering one scavenger are reliable and comparable. As ozonation is frequently applied in wastewater treatment or drinking water purification [30] it should be of high interest for water suppliers to better understand the mechanistic aspects taking place during ozonation and the

influence matrix components can have. Especially, bromide should rise in interest as it has been shown that it can lead to many unknown and unwanted TPs. Therefore, it should be tested if formed TPs can also be detected in drinking water samples, as it was the case for NDMA [17], and if these are of toxicological interest.

After showing successfully within this study that the reaction of *N*-containing substances with ozone is highly depending on the surrounding matrix, the gained knowledge can immediately be used for further research on MPs found in the aquatic environment. Since ozonation is widely applied and the matrix composition differs among WWTPs, for an overall perspective on the influence of matrix components in terms of TP formation detailed research is needed. However, to be able to give a comprehensive evaluation of effects occurring during ozonation more realistic scenarios need to be investigated. This should involve the combination of mechanistic and (eco)toxicological studies to give a complete overview on the effects which the surrounding matrix can have in terms of reaction pathways and TP formation during ozonation. As most studies so far have only been performed in ultrapure water, sometimes taking into account the influence of pH but seldomly considering matrix effects, these studies are limited in terms of transferability to real scenarios. This study started filling the gap between degradation processes and TP formation observed in studies performed on laboratory scale and those which are taking place in real water systems.

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Chapter 8

Appendix

8.1 List of abbreviations

$^{\bullet}$CR	Carbon centered radicals
$^{\bullet}$NR	Aminyl radicals
$^{\bullet}$OH	Hydroxyl radicals
$^{\bullet}ONR_2$	Nitroxide radicals
α-METO	α-hydroxymetoprolol
$^1O_2$	Singlet oxygen
3-METO	3-(isopropylamino)propane-1,2-diol
5-OH DCF	5-hydroxydiclofenac
ADaM	Aachener Daphnia medium
AOP	Advanced oxidation processes
Br^-	Bromide
BrDMS	Brominated *N*,*N*-dimethylsulfamide
BrO_3^-	Bromate
CLP	Classification, Labelling and Packaging
DAD	Diode-array-detector
DCA	2,6-dichloroaniline
DCF	Diclofenac
D. magna	*Daphnia magna*
DMP	*Cis*-2,6-dimethylpiperidine
DMS	*N*,*N*-dimethylsulfamide
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Dissolved organic matter
DPB	Des(isopropoxyethyl) bisoprolol
DPD	*N*,*N*-diethyl-*p*-phenylenediamine
DP-METO	Desisopropylmetoprol
EC	Effect concentration
ESI-MS	Electrospray ionization mass spectrometry
F_c	Fold change
H_2O_2	Hydrogen peroxide
HCl	Hypochloric acid
HOBr	Hypobromous acid

HOBrTPs	Hypobromous acid transformation products
HOCl	Hypochlorous acid
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
IC	Ion chromatography
ISO	Isoproturon
k_1	(pseudo) first reaction rate constant
$K_2Cr_2O_7$	Potassium dichromate
KA	Kläranlage
k_{obs}	Reaction rate constant observed
LC	Liquid chromatography
LOEC	Lowest observed effect concentration
MeOH	Methanol
METO	Metoprolol
MPs	Micropollutants
MS	Mass spectrometry
MSIS	Methanesulfinic acid
MSOS	Methanesulfonic acid
NaOH	Sodium hydroxide
N-containing	Nitrogen containing
NDMA	*N*-nitrosodimethylamine
NH_4COOH	Ammonium acetate
NOEC	No observed effect concentration
NOM	Natural organic matter
NPOC	Non-purgeable organic carbon
N-Substanzen	stickstoffhaltige Substanzen
NTS	Non-target screening
O_3	Ozone
OECD	Organization for Economic Cooperation and Development
O-METO	o-dimethylmetoprolol
OzHOBrTPs	Ozonated hypobromous acid transformation products
OzTPs	Ozonated transformation products
PAC	Powdered activated carbon
SI	Supporting information

SIM	Single-ion mode
SPE	Solid-phase extraction
TAM	Tamoxifen
TEMPO	2,2,6,6-tetramethylpiperidineoxyl
TEMPOL	2,2,6,6-tetramethylpiperidine-1-ol
tert-BuOH	Tertiary butanol
TMP	2,2,6,6-tetramethylpiperidine
TOC	Total organic carbon
TP	Transformation product / Transformationsprodukte
UV	Ultraviolet
WWTP	Wastewater treatment plant

8.2 List of Figures

8.3 List of Tables

8.4 List of publications

8.4.1 Publications in peer-reviewed journals

Published:

Bergmann, M., V. Wirzberger, T. Krumpen, C. Lorenz, S. Primpke, M.B Tekman, and G. Gerdts, *High Quantities of Microplastic in Arctic Deep-Sea Sediments from the HAUSGARTEN Observatory*. Environmental Science & Technology, 2017. **51**(19), 11000-11010.

Submitted:

Baetz, N., L. Rothe, V. Wirzberger, B. Sures, T.C. Schmidt, J. Tuerk, *High-Performance Thin-Layer Chromatography in Combination with a Yeast-Based Multi-Effect Bioassay to Determine Endocrine Effects in Environmental Samples*. Submitted to Analytical and Bioanalytical Chemistry, 2020.

Wirzberger, V., V.I. Merkus, M. Klein, L.L. Hohrenk, L.E. Rothe, N. Baetz, J. Tuerk, B. Sures, H.V. Lutze, and T.C. Schmidt, *Influence of matrix composition on the formation of reaction products during ozonation of N-containing substances*. Submitted to Environmental Science & Technology, 2020.

Wirzberger, V., M. Klein, M. Woermann, B. Sures, H.V. Lutze, and T.C. Schmidt, *Matrix composition during ozonation of N-containing substances may influence the acute toxicity towards Daphnia magna*. Submitted to Science of the Total Environment, 2020.

8.4.2 Oral presentations

None

8.4.3 Poster presentations

Wirzberger, V., M. Klein, V.I. Merkus, L.E. Rothe, M. Woermann, N. Bätz, L.L. Hohrenk, H.V. Lutze and T.C. Schmidt, *Welche Transformationspodukte können nach der Ozonung in einer Kläranlage nachgewiesen werden und welche ökotoxikologische Relevanz haben sie?*. 3. Fortschrittswerkstatt Wasser. Heiß.Nass.Trocken. - Stürmische Zeiten für die Wasserwirtschaft?, 2020, Essen.

Wirzberger, V., S. Thebingbuß, S. Eßer, K. Kerpen, H.V. Lutze and T.C. Schmidt, *Reactions of nitrogen containing compounds with ozone.* International Young Water Professional Conference, 2019, Toronto.

Wirzberger, V., M. Klein, V.I. Merkus, K. Kerpen, L.L. Hohrenk, H.V. Lutze and T.C. Schmidt, *Ozonation of diclofenac in various water matrices.* Wasser 2019 – Jahrestagung der Wasserchemischen Gesellschaft, 2019, Erfurt.

Wirzberger, V., S. Thebingbuß, H.V. Lutze, K. Kerpen and T.C. Schmidt, *Decomposition of nitrogen containing compounds with ozone – stoichiometry and kinetics*. MWAS 2018 - 3. Wasseranalytisches Seminar, 2018, Mülheim an der Ruhr.

Wirzberger, V., M. Bergmann, C. Lorenz, S. Primpke and G. Gerdts, *Erfassung von Mikroplastik in arktischen Tiefseesedimenten mittels µFT-IR-Spektroskopie*. Wasser 2017 – Jahrestagung der Wasserchemischen Gesellschaft, 2017 Donaueschingen.

Wirzberger, V., I. Hübner, C.K. Schmidt, D. Bockmühl and G. Haase, *Eignung von MALDI-TOF MS Fingerprinting für die Identifizierung von Legionella spp. kultiviert aus Trinkwasser- und Rückkühlwässer-Proben*. Wasser 2015 – Jahrestagung der Wasserchemischen Gesellschaft, 2015 Schwerin.

8.5 Declaration of scientific contributions

Work present in this thesis has been published in cooperation with co-authors and my own contribution as declared in the following:

Chapter 4:

Wirzberger, V., V.I. Merkus, M. Klein, L.L. Hohrenk, L.E. Rothe, N. Baetz, J. Tuerk, B. Sures, H.V. Lutze, and T.C. Schmidt, *Influence of matrix composition on the formation of reaction products during ozonation of N-containing substances*. Submitted to Environmental Science & Technology, 2020.

Declaration to my contribution within this publication:

All experiments were planned and designed by Vanessa Wirzberger. Real water samples were taken and prepared for further experiments by Vanessa Wirzberger, Louisa E. Rothe, Nicolai Bätz and Valentina I. Merkus. Ozonation experiments as well as identification and quantification were experimental contributed by Valentina I. Merkus and Michelle Klein and directly supervised by Vanessa Wirzberger. Reaction rate constants of the transformation products were determined by Vanessa Wirzberger who was experimental assisted by Hieu Trung Than and Kittitouch Tavichaiyuth. Due to the interdisciplinary usage of the samples a constant exchange and discussion of the results was needed including Louisa E. Rothe, Nicolai Bätz, Lotta L. Hohrenk and Vanessa Wirzberger. Input to the discussion of the results were also contributed by Valentina I. Merkus, Michelle Klein, Dr. Jochen Türk, Prof. Bernd Sures, Prof. Holger V. Lutze and Prof. Torsten C. Schmidt. Vanessa Wirzberger wrote the manuscript and also all corrections. The whole study was supervised by Prof. Holger V. Lutze and Prof. Torsten C. Schmidt who also revised the manuscript.

Chapter 6:

Wirzberger, V., M. Klein, M. Woermann, B. Sures, H.V. Lutze, and T.C. Schmidt, *Matrix composition during ozonation of N-containing substances may influence the acute toxicity towards Daphnia magna*. Submitted to Science of The Total Environment, 2020.

Declaration to my contribution within this publication:

The experiments were designed, planned and prepared by Vanessa Wirzberger. Ozonation and ecotoxicological experiments including *Daphina magna* were performed by Michelle Klein and directly supervised by Vanessa Wirzberger and Marion Woermann. Due to the interdisciplinary work a constant exchange and discussion of the results was needed including Vanessa Wirzberger, Michelle Klein and Marion Woermann. Prof. Holger V. Lutze, Prof. Torsten C. Schmidt and Prof. Bernd Sures also contributed input to the discussion of the results. The mansuscript was written and all corrections were contributed by Vanessa Wirzberger. Prof. Holger V. Lutze and Prof. Torsten C. Schmidt supervised the whole study. The manuscript was revised by Prof. Holger V. Lutze, Prof. Torsten C. Schmidt and Prof. Bernd Sures.

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8.7 Curriculum Vitae

Aus datenschutzrechtlichen Gründen ist der Lebenslauf in der Onlineversion nicht enthalten

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8.8 Erklärung

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

„Oxidative water treatment: mechanistic aspects and matrix effects"

selbst verfasst und keine außer den angegebenen Hilfsmitteln und Quellen benutzt habe. Alle wörtlich oder inhaltlich übernommenen Stellen sind als solche gekennzeichnet und die Arbeit wurde in dieser oder ähnlicher Form noch bei keiner anderen Universität eingereicht.

Essen, 04. September 2020

_____

Vanessa Wirzberger