Biological degradation of ^{14}C-labeled micropollutants and their transformation products in ozonated wastewater

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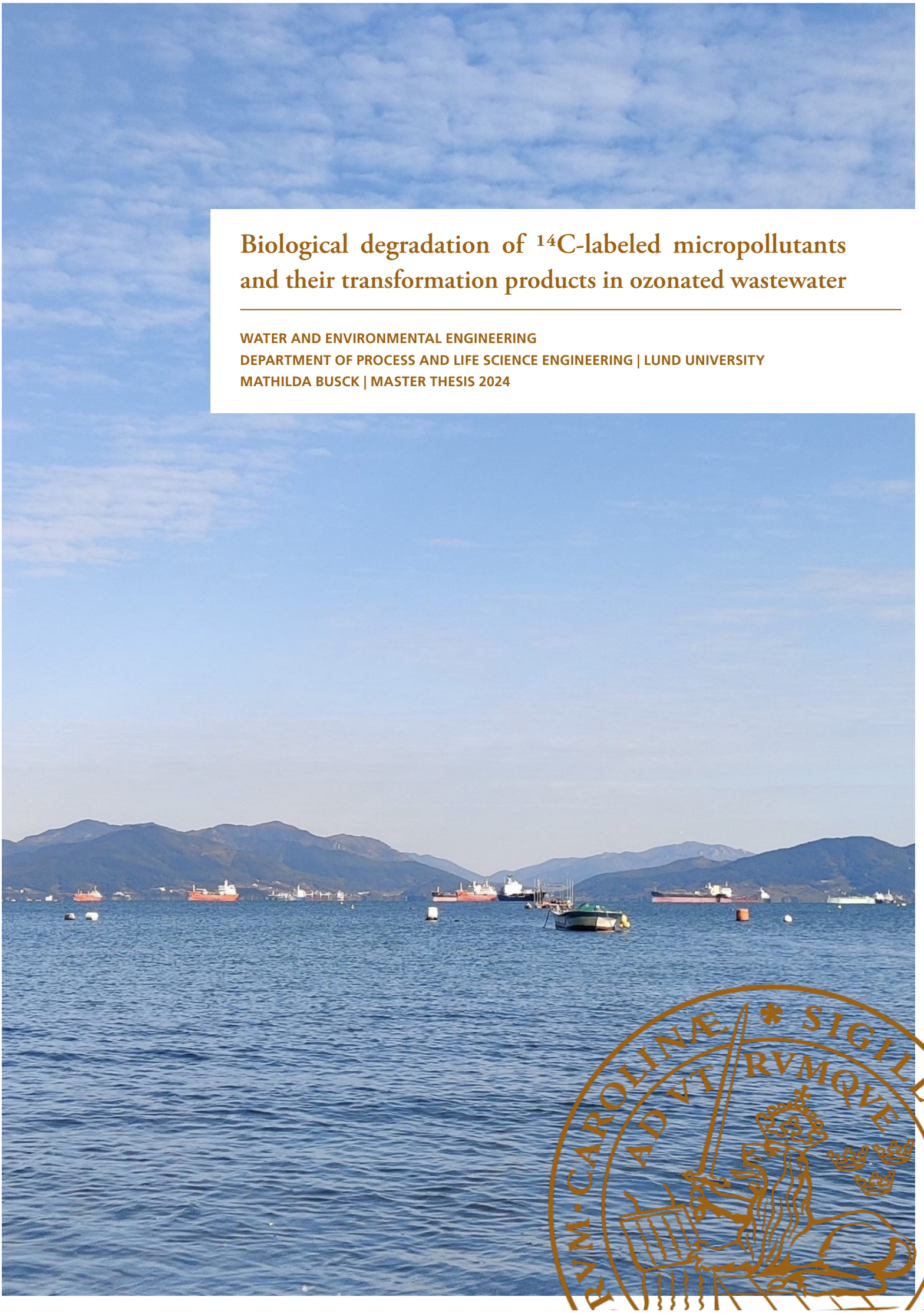

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by

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Preface

This master thesis was conducted at the Department of Process and Life Science Engineering, Lund University, as a part of two research projects: NoNo, funded by FORMAS, and XXX, funded by Vetenskapsrådet.

I would like to thank everyone who has supported me throughout this project. Firstly, to my supervisor Per Falås, for always making time for me, helping me with the experiments and showing great enthusiasm for the results. To my examiner Micheael Cimbritz, for checking in on me throughout the semester and providing your encouragement. To Ola Svanh, for helping with the micropollutant analysis and showing interest in the project. To Aina McEvoy, for your assistance in the lab, and particularly, for helping me save the last experiment. To Alexander Betsholtz, for showing me how to operate the scintillation counter and explaining quench curves. Lastly, to my friend Maria Hallström, who kindly agreed to check my writing and provided many helpful comments.

Populärvetenskaplig sammanfattning

Ozon och bakterier: en superduo för miljön?

Genom att rena avloppsvatten med en kombination av ozon och bakterier kan vi skydda miljön i våra vatten och förhindra utsläpp av läkemedelsrester och andra förorenande ämnen.

I takt med samhällets utveckling har en uppsjö av olika läkemedel, pesticider, hudvårdsprodukter och andra kemikalier kommit ut på marknaden. Du kommer säkert i kontakt med dessa produkter dagligen men har du någonsin funderat på vad som händer med dem efter användning? För de flesta upphör alla tankar på produkten så snart den uppfyllt sin funktion, men detta betyder inte att de aktiva ämnena magiskt försvinner. För att ha effekt är de i regel designade för att vara svårnedbrytbara och kommer till slut att hamna i naturen. Ett brett spektrum av läkemedelsrester och andra problematiska ämnen har kunnat påvisas i hav, sjöar och vattendrag. Där får de samlingsnamnet organiska mikroföroreningar då de ofta återfinns i låga koncentrationer men trots det kan ha en rad negativa effekter; så som att påverka vattenlevande organismer, störa akvatiska ekosystem samt riskera att förorena våra dricksvattenkällor.

I många fall anses dessa ämnen ha en sådan viktig funktion att det inte vore möjligt att förbjuda användningen av dem. För att förhindra att de gör skada ligger tyngdpunkten istället på att rena bort dem innan de släpps ut i naturen, och då avloppsreningsverk utgör viktiga utsläppsvägar för organiska mikroföroreningar är dessa i fokus. I Schweiz är det sedan 2016 redan krav på att stora reningsverk ska rena avloppsvattnet från organiska mikroföroreningar, och i EU väntas liknande regler komma på plats inom de närmsta åren.

Eftersom konventionella reningsverk inte är designade för den typen av rening krävs det nya tekniker och här lyfts ozonering fram som ett starkt alternativ. Vid ozonering tillsätts ozon till avloppsvattnet, där det reagerar med de organiska mikroföroreningarna på ett sådant sätt att deras molekylära struktur förstörs. Detta leder dock inte till att ämnet försvinner helt, utan snarare till att det omvandlas och bildar nya ämnen, så kallade transformationsprodukter. Generellt har man sett att dessa transformationsprodukter är mindre problematiska än det ursprungliga ämnet, men oklarheter kring hur giftiga de faktiskt är kvarstår. För att ta hand om transformationsprodukter och andra skadliga ämnen som kan bildas vid ozonering är det vanligt att ha ett biologiskt reningssteg där dessa kan brytas ned av bakterier och andra mikroorganismer. Det är dock svårt att fastställa hur effektiv reningen av transformationsprodukter är. En närmast oändlig uppsättning transformationsprodukter kan bildas vid ozonering, vilket gör det både komplicerat och tidsödande att identifiera dessa och sedan hålla koll på dem under den biologiska reningen.

I det här arbetet har vi undersökt vad som händer med olika transformationsprodukter vid biologisk rening genom att använda oss av organiska mikroföroreningar märkta med det radioaktiva ämnet kol-14. När de radioaktiva mikroföroreningarna ozoneras bildar de radioaktiva transformationsprodukter som vi, utan att behöva identifiera dem, enkelt kan följa genom att mäta radioaktiviteten. Dessa experiment har visat att ozonering i många fall transformerar organiska mikroföroreningar på ett sådant sätt att dessa blir lättare för bakterier att bryta ned. Vidare såg vi att denna effekt kunde uppnås även vid lägre ozondoser vilket är positivt då kostnaden ökar vid ökande doser. Biologisk rening verkar alltså som en effektiv metod för att ta hand om de transformationsprodukter som bildas vid ozonering och se till att vi inte skapar nya problem i jakten på att rena bort organiska mikroföroreningar.

Summary

Ozonation can be applied to reduce the concentration of organic micropollutants in municipal wastewater, and thereby mitigate any adverse effects these compounds may have in natural waters. As ozone is applied to wastewater, organic micropollutants, as well as other compounds present in the water matrix, will be oxidized, resulting in the formation of transformation- and byproducts. Some of these oxidation products have been proven to be toxic and biological post-treatment is recommended to reduce the toxicity of ozonated water. However, while some studies on the biodegradability of ozonation transformation products have been performed, the fate of transformation products in biological posttreatment remains largely unknown.

Through the use of ^{14}C-labeled micropollutants, biological degradation of transformation products in ozonated wastewater was studied. In a first step, selected ^{14}C-labeled micropollutants (sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac, ibuprofen, bisphenol A and trimethoprim) were ozonated to yield ^{14}C-labeled transformation products. By measuring the $^{14}CO_2$ formation, a partial mineralization of the labeled position was confirmed for all compounds, except for propyl-labeled bisphenol A. Concentration measurements of the parent compound further revealed a complete transformation of all micropollutants at the lowest ozone dose (0.3 mg O_3/mg DOC), except for ibuprofen for which the transformation was complete only at the highest dose (1 mg O_3/mg DOC). In a second step, biofilm carriers were added to the bottles holding the ozonated water and these were incubated for 72 hours. By continuous monitoring of the $^{14}CO_2$ formation, an increased mineralization of the labeled position, as compared to the parent compound, could be confirmed for the transformation products of sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac and trimethoprim. The mineralization generally increased with increasing ozone doses but was significant even at lower doses. For ibuprofen and bisphenol A, the $^{14}CO_2$ formation conversely revealed a decreased mineralization, as compared to the parent compound. The decrease was minor for ibuprofen but significant for bisphenol A, especially at lower ozone doses. The results indicate that an increased biodegradability can be expected for many transformation products, suggesting that biological posttreatment is a suitable method to remove the transformation products that are formed during ozonation.

Sammanfattning

Ozon kan användas för att reducera koncentrationen av organiska mikroföroreningar i avloppsvatten och därmed förhindra att dessa sprids i hav, sjöar och vattendrag, där deras närvaro kan medföra en rad negativa konsekvenser. Vid tillsats av ozon till avloppsvatten oxideras de organiska mikroföroreningarna, tillsammans med andra ämnen i vattenmatrisen, och bildar olika transformations- och biprodukter. Vissa av dessa oxidationsprodukter har visat sig vara giftiga och det är därför rekommenderat att efterfölja ozoneringen med ett biologiskt reningssteg. Ett fåtal studier har genomförts där nedbrytbarheten hos transformationsprodukter undersöks, men till vilken grad olika transformationsprodukter kan brytas ned i en biologisk efterbehandling är fortfarande till stor del oklart.

I det här arbetet studerades biologisk nedbrytning av transformationsprodukter från ozonering med hjälp av ^{14}C-märkta mikroföroreningar. Avloppsvatten spikat med ^{14}C-märkta mikroföroreningar (sulfametoxazol, sulfadiazin, karbamazepin, diclofenak, ibuprofen, bisfenol A och trimetoprim) ozonerades för att generera ^{14}C-märkta transformationsprodukter. Genom att mäta bildandet av $^{14}CO_2$, kunde en partiell mineralisering av den märkta positionen påvisas för alla ämnen, förutom för bisfenol A med märkning i propyl-gruppen. Mätningar av koncentrationen kvarvarande modersubstans visade vidare att en fullständig transformation uppnåddes för alla organiska mikroföroreningar vid den lägsta dosen (0,3 mg O_3/mg DOC), undantaget ibuprofen vars transformation endast var fullständig vid den högsta dosen (1 mg O_3/mg DOC). Efter ozonering tillsattes biobärare till det ozonerade vattnet och den biologiska nedbrytningen studerades över ett spann av 72 timmar. Genom kontinuerlig mätning av $^{14}CO_2$ kunde en ökad mineralisering, jämfört med modersubstansen, påvisas för transformationsprodukterna av sulfametoxazol, sulfadiazin, karbamazepin, diklofenak och trimetoprim. Mineraliseringen ökade vid ökande ozondoser men var signifikant även vid lägre doser. För ibuprofen och bisfenol A, visade bildandet av $^{14}CO_2$ däremot en minskad mineralisering hos transformationsprodukterna. Minskningen var marginell för ibuprofen men signifikant för bisfenol A, speciellt vid låga ozondoser. Det konstaterades därmed att en ökad nedbrytbarhet kan förväntas för många transformationsprodukter, vilket indikerar att biologisk efterbehandling i huvudsak är en en effektiv metod för att eliminera de transformationsprodukter som bildas vid ozonering.

List of abbreviations

WWTP	Wastewater treatment plant
p.e.	Person equivalent
DOM	Dissolved organic matter
DOC	Dissolved organic carbon
NDMA	N-nitrosodimethylamine
MBBR	Moving bed biofilm reactor
LC/MS	Liquid chromatography/mass spectroscopy
CPM	Counts per minute
TS	Total solids
COD	Chemical oxygen demand
GAC	Granular activated carbon
PAC	Powdered activated acrbon

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1 Introduction

The presence of organic micropollutants in natural waters is a growing issue and concerns have been raised about the ecological impact as well as the effect on drinking water resources. Organic micropollutants include a wide variety of substances such as pharmaceuticals, industrial chemicals, pesticides and personal care products and although their concentration in natural waters is low, typically in the range of ng-µg/L (Lopez *et al*., 2015; Petrie *et al*., 2015), their presence may have a variety of adverse effects on aquatic species (Farré *et al*., 2008). Some compounds have for instance been shown to disrupt the sexual differentiation of fish as well as to reduce their reproductive success (Parrott and Blunt, 2005; Thornton *et al*., 2016). Additionally, as organic micropollutants are present in the environment as a complex mixture, rather than as individual compounds, the risk of unfavorable synergistic effects is introduced (Petrie *et al*., 2015). Other concerns include the possible spread of antibiotic resistance due to discharge of antibiotic residues (Marti *et al*., 2014) as well as contamination of drinking water resources (Lopez *et al*., 2015).

To reduce the levels of organic micropollutants present in natural waters, a combination of different methods has been proposed, including different source control measures such as restricting the use of harmful chemicals, ecolabeling of products to inspire voluntary action and treatment prior to discharge at hospitals, industries etc. (Joss *et al*., 2008). As wastewater treatment plants (WWTPs) represent important discharge routes for organic micropollutants, implementing advanced treatment to reduce the effluent concentration is another important strategy capable of achieving a significant load reduction (Eggen *et al*., 2014). The need to upgrade WWTPs and implement measures for the abatement of organic micropollutants has been recognized in Europe, where, in a proposed update to the Urban Wastewater Treatment Directive (2022/0354/COD) the EU has introduced the obligation to remove a wide range of organic micropollutants.

Some organic micropollutants are already removed to varying degrees during conventional wastewater treatment through different pathways (Falås *et al*., 2016). However, to ensure a sufficient removal of a broad range of organic micropollutants an additional treatment step is needed. Adsorption to activated carbon and ozonation are the main methods being considered as they have the potential to achieve a significant reduction in the organic micropollutant load, while still being feasible in terms of cost and energy requirements (Eggen *et al*., 2014; Hollender *et al*., 2009).

During ozonation, organic micropollutants are oxidized resulting in the formation of transformation products. This is generally associated with a loss of the biological activity but concerns remain about the toxicity of these compounds and, in particular, the toxicity of ozonation byproducts, formed from the oxidation of organic and inorganic matter present in the water matrix (Lee and von Gunten, 2016). To mitigate any adverse effects relating to the formation of these transformation- and byproducts, ozonation is commonly followed by a biological posttreatment step such as sand filtration or different biofilm processes (Bourgin *et al*., 2018; Edefell *et al*., 2021; Hollender *et al*., 2009). The biological degradability of transformation products is, however, partially unknown and many transformation products remain undetected (Lee and von Gunten, 2016).

By labeling organic micropollutants with the radioactive isotope ^{14}C, these compounds, as well as their transformation products, can be tracked during ozonation and subsequent biological

treatment simply by measuring the radioactivity. The transformation of organic micropollutants is associated with a loss of the original structure, the radioactivity, however, will be retained so long as the ^{14}C isotope remains in the molecule. By combining measurements of the concentration of the parent micropollutant with measurements of the radioactivity remaining in the liquid phase, the transformation of organic micropollutants during ozonation can be confirmed. The detection of $^{14}CO_2$ can further be used to separate transformation, and other removal mechanisms, from complete degradation and to study the mineralization of organic micropollutants and their transformation products during ozonation and biological posttreatment. It should be noted that the results obtained using this method only pertains to the labeled position; any changes to other parts of the structure will go undetected. It is however a simple method, requiring no knowledge about the structure of the transformation products, thus providing a convenient way to gain insight into the fate of organic micropollutants and their transformation products during advanced treatment.

1.1 Aim

The overall aim of this thesis is to study the transformation and mineralization of ^{14}C-labeled micropollutants during ozonation, as well as the mineralization of the parent compound and their transformation products during biological treatment. Throughout the project, the following questions will be answered:

- To what degree are the organic micropollutants sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac, ibuprofen, bisphenol A and trimethoprim transformed during ozonation and how is the transformation affected by the ozone dose?
- How does transformation of the studied organic micropollutants affect the biodegradability?
- Does the ozone dose influence the biodegradability of the transformation products?

2 Background

In the following sections an introduction to the legislation concerning organic micropollutants in the EU will be provided, along with background on the removal of organic micropollutants during conventional wastewater treatment as well as during ozonation. The substances analyzed within the scope of this project and the methods used will also be introduced.

2.1 European legislation

2.1.1 Priority substances and watch lists

As a part of the Water Framework Directive (2000/60/EC) the EU has compiled a list of priority substances and their environmental quality standards. Member states are required to monitor the presence of these substances in surface waters and ensure that the quality standards are met. The list is to be reviewed and, if necessary, revised every 6 years. As of 2022, there is a proposal to add 25 new compounds to the list (2022/0344/COD), including PFAS, pesticides and several pharmaceuticals. In 2015, the EU also established a surface water watchlist (2015/495/EU) presenting substances that are suspected to be harmful to or via aquatic environments and that the member states are required to monitor. The watchlist has been updated several times, most recently in 2022 (2022/1307/EU), and now contains substances such as pharmaceuticals and sunscreen agents (European Commission).

2.1.2 The urban wastewater treatment directive

The EU's Urban Wastewater Treatment Directive was first adopted in 1991 (91/271/EEC) with the aim to "protect the environment from adverse effects of wastewater discharges from urban sources and specific industries". Implementation of the directive has led to significant improvements in the water quality of European rivers, lakes and seas. Still, concerns over the water quality remains and more encompassing regulations on pollution are needed. To address this, the European Commission proposed an update to the current directive in 2022 (2022/0345/COD) (European Commission).

The proposal introduces the obligation to install additional treatment processes to eliminate a broad spectrum of organic micropollutants. The additional treatment is to be applied to all urban WWTPs treating a load equal to, or greater than, 150 000 population equivalents (p.e.) by 2035. Additionally, by 2040 all facilities treating a load equivalent to 10 000 p.e., and above, are also required to apply the additional treatment if these are located in areas where the presence of organic micropollutants pose a risk to human health or the environment. This includes areas where the treated wastewater is discharged to water bodies where the dilution ratio is low or where the water body is used as bathing waters or to produce drinking water. In Annex 1 to the proposal, the requirements for advanced treatment are further specified. A set of 12 indicator substances, listed in Table 2.1, are to be removed by at least 80%. The percentage of removal must be calculated for at least six of these substances, and the average of the removal percentages should then be used to assess whether the requirement has been met.

Table 2.1. The indicator substances as specified by Annex 1 to the proposed update to the Urban Wastewater Treatment Directive (2022/0345/COD).

Indicator substance	Application
Amisulprid	Antipsychotic drug
Carbamazepine	Antiepileptic drug
Citolopram	Antidepressant
Clarithromycin	Antibiotic
Diclofenac	Anti-inflammatory drug
Hydrochlorothiazide	Diuretic drug
Metoprolol	Beta blocker
Venlafaxine	Antidepressant
Benzotriazole	Corrosion inhibitor
Candesartan	Antihypertensive drug
Irbesartan	Antihypertensive drug
4-methylbenzotriazole and 6-methylbenzotriazole	Corrosion inhibitor

2.2 Removal of organic micropollutants in conventional wastewater treatment

Organic micropollutants are already removed from wastewater to varying degrees during conventional wastewater treatment. The removal occurs through either transformation, sorption to sludge or volatilization (Falås *et al*., 2016), where the two former mechanisms are dominant (Ternes *et al*., 2004). Volatilization is typically insignificant for pharmaceuticals (Nguyen *et al*., 2021).

2.2.1 Biodegradation

Microbial degradation of organic micropollutants may result in mineralization, although transformation into varying transformation products is more common (Nguyen *et al*., 2021). The removal (either through mineralization or transformation) varies significantly between different organic micropollutants, with some compounds such as ibuprofen showing a high removal efficiency, while other compounds such as carbamazepine have proven to be persistent (Tadkaew *et al*., 2011). Some studies have found the removal efficiency to correlate with the chemical structure of the compounds and the presence of certain functional groups (Bertelkamp *et al*., 2016; Tadkaew *et al*., 2011). The presence of electron withdrawing groups (e.g. amide groups and halogens) was shown to result in poor removal, while the presence of electron donating groups (e.g. hydroxyl and amine groups) resulted in significantly higher removal efficiencies (Tadkaew *et al*., 2011). Ring structures were further found to decrease the biodegradation rate (Bertelkamp *et al*., 2016).

In addition to the chemical structure and its effect on the biodegradation rate, the removal during biological treatment has also been found to be dependent on the reactor configuration and the operational conditions with several parameters such as hydraulic retention time, solids retention time, redox conditions, pH and temperature being highlighted (Nguyen *et al*., 2021). While the biological degradation of some compounds can be enhanced by adjusting the conditions or the setup of the biological treatment, e.g. by combining different aerobic and anaerobic treatment conditions (Falås *et al*., 2016) or opting for a biofilm process rather than activated sludge (Falås *et al*., 2012), such an approach has clear limitations. Many organic micropollutants are stable in biological processes and even though the removal rate of a limited number of micropollutants

can be increased, this does not guarantee that the toxicity of the treated wastewater has been reduced. In addition, upgrading the biological treatment would likely increase the footprint of the WWTP and require a large initial investment. An upgrade of the biological treatment for enhanced removal of organic micropollutants is consequently not considered to be a viable option (Falås *et al.*, 2016).

2.2.2 Sorption

Organic micropollutants can sorb to primary as well as secondary sludge. Sorption to sludge can be divided into two main mechanisms: hydrophobic interactions and electrostatic interactions. Hydrophobic interactions occur between aliphatic and aromatic groups present on the micropollutants and the lipophilic cell membrane of microorganisms and any other lipid fractions of the sludge. Electrostatic interactions result from the attraction between positively charged groups present on the micropollutants and the negatively charged surface of the microorganisms in the sludge (Margot *et al*., 2015; Ternes *et al*., 2004). Sorption to sludge can thus be significant for micropollutants with hydrophobic or positively charged groups, particularly if the compound exhibits a low biodegradability (Margot *et al*., 2015).

Sorption to sludge can be predicted fairly well using solid-water distribution coefficients (K_d). The K_d-values vary depending on the properties of the compound, as discussed above, as well as the pH and composition of the sludge. Many pharmaceuticals have been reported to have low K_d-values, indicating that they do not sorb well to sludge (Ternes *et al*., 2004). It is worth noting that although the sorbed micropollutants will be removed from the wastewater with the sludge, the fate of the sorbed micropollutants will depend on the fate of the sludge (Margot *et al.*, 2015) and they may still end up in the environment if the sludge is used as a fertilizer in agriculture (Ternes *et al*., 2004).

2.3 Ozonation

Ozonation is widely used in drinking water treatment for disinfection, decoloration as well as to eliminate taste and odor (von Gunten, 2003). Ozone also has applications is wastewater treatment. As previously mentioned, it is, along with adsorption to activated carbon, considered to be the most viable technique for the removal of organic micropollutants (Hollender *et al*., 2009; Margot *et al*., 2015). Several full scale ozonation units for micropollutant removal are already in place, most notably in Switzerland where the legislation, as of 2016, demands that certain WWTPs employ advanced treatment for the abatement of organic micropollutants (Bourgin *et al*., 2018).

2.3.1 Reactions and mechanisms

Ozonation is a process during which organic and inorganic compounds are oxidized by reactions with ozone (O_3) or hydroxyl radicals (•OH). Ozone is an electrophilic and selective oxidant which mainly reacts with electron rich moieties such as double bonds, activated aromatic systems, amines and sulfidic groups. The reactivity varies depending on the substituents, with electron withdrawing groups such as -Cl and $-NO_2$ decreasing the reaction rate and electron donating groups such as $-CH_3$, O^- and $-OCH_3$ increasing the reaction rate. Ozone is unstable and decomposes in water due to reactions with the water matrix, forming hydroxyl radicals which, unlike ozone, are unselective and react fast with most dissolved compounds. The extent of which a compound reacts with ozone and hydroxyl radicals respectively depend on the ozone reactivity. For compounds that exhibit a high reactivity towards ozone, oxidation occurs mainly by reactions with ozone, while the oxidation of compounds exhibiting a low ozone reactivity is governed by reactions with hydroxyl radicals. For compounds that show intermediate reactivity

towards ozone, oxidation occurs through reactions with both ozone and hydroxyl radicals (von Gunten, 2003).

The effectiveness of the elimination of organic micropollutants using ozone depends on their reactivity with ozone as well as the rate of ozone consumption by the water matrix (Nöthe *et al.*, 2009). As discussed previously, ozone reactivity is governed by the chemical structure of the compound and the presence of certain reactive moieties. For compounds that can be protonated, the pH is another important variable. Protonation has been found to decrease the oxidation rate significantly due to a decrease in nucleophilicity. The protonation of amino groups for instance renders them almost nonreactive with ozone. The pH also affects the stability of ozone; as hydroxide ions initiate ozone decomposition the lifetime of ozone is reduced at elevated pHs. Alkalinity is another factor influencing the stability of ozone. Carbonate and bicarbonate act as inhibitors of ozone decomposition by interrupting radical chain reactions that would otherwise consume ozone (von Gunten, 2003).

Apart from pH and alkalinity, the major factor determining the lifetime of ozone is the presence of dissolved organic matter (DOM) (von Sonntag and von Gunten, 2012). Organic micropollutants are technically included in DOM but their contribution can be neglected. Ozone consumption, and subsequently ozone lifetime, depends entirely on reactions between the water matrix and ozone (Nöthe *et al*., 2009). DOM can consume ozone either directly, by reactions with ozone, or indirectly, by reactions with hydroxyl radicals initiating chain reactions consuming ozone. A fraction of the DOM can also, as in the case with carbonate and bicarbonate, act as inhibitors and by reacting with hydroxyl radicals terminate chain reactions that would otherwise accelerate ozone decay. It is difficult to assess the ozone stability in natural waters and impossible to estimate the fractions of DOM that inhibit or promote ozone decay (von Gunten, 2003). It has however been shown that the scavenging effect of DOM can be accounted for by normalizing the ozone dose (g O_3/L) with the concentration of dissolved organic carbon (DOC) (g O_3/g DOC). This allows for an accurate prediction of the ozone dose required to achieve a significant reduction of organic micropollutants in wastewater of varying qualities (Lee *et al*., 2013). Nitrite (NO_2^-) is another ozone scavenger which consumes ozone quickly at a 1:1 stoichiometry. If the nitrite concentration is significant the ozone dose can be corrected with respect to nitrite, as well as DOC, to ensure that there is enough residual ozone for the oxidation of the organic micropollutants (Lee *et al*., 2013).

For the compounds that exhibit a low ozone reactivity, oxidation can instead, as previously mentioned, occur by reactions with hydroxyl radicals. This pathway is, however, associated with a significant loss of oxidation capacity. As a result of the highly reactive and unselective nature of hydroxyl radicals, a large fraction of the formed radicals will rapidly be scavenged by the water matrix and thus be unavailable for the oxidation of organic micropollutants (von Gunten, 2003).

2.3.2 Ozonation by- and transformation products

Ozonation is commonly referred to as a method to remove organic micropollutants. However, it does not necessarily result in the complete mineralization of these substances, and thus their removal from the water phase, but rather the formation of transformation products which may be more or less structurally similar to the parent compound (von Sonntag and von Gunten, 2012). As previously mentioned, ozone reacts primarily with moieties such as double bonds, activated aromatic systems and amines and these groups will consequently be the main groups that are attacked and transformed by ozone, while the hydroxyl radicals will attack non-selectively (Lee and von Gunten, 2016). The formation of transformation products gives rise to the

question whether modification of the parent compound has led to the elimination of the original biological properties, and whether the transformation may have introduced new harmful functions (von Sonntag and von Gunten, 2012), especially as studies indicate that some ozonation transformation products cannot be efficiently abated in biological posttreatments (Bourgin *et al*., 2018; Edefell *et al*., 2021; Gulde *et al*., 2021a).

Another concern is the formation of byproducts resulting from oxidation of components in the water matrix. Due to the large number of dissolved compounds in the water matrix there is a wide array of possible oxidation byproducts, but two notable examples include bromate (BrO_3^-) and N-nitrosodimethylamine (NDMA), which are both human carcinogens. Bromate is formed from the oxidation of bromide and it has been shown that the yield of bromate is dependent on the ozone dose, with increasing ozone doses resulting in higher bromate yields. Bromate is not removed during conventional biological posttreatment (Bourgin *et al*., 2018). It has, however, been shown that a reduction of bromate can be achieved during posttreatment using denitrifying biofilms, although the practical applicability may be limited by the elevated oxygen concentration in ozonated water (Falås *et al*., 2022). NDMA is formed from the oxidation of precursors such as hydrazines and sulfamides. No correlation between the extent of NDMA formation and the ozone dose has been found and it has further been shown that NDMA can be abated in biological posttreatments (Bourgin *et al*., 2018). Byproducts are also formed from the oxidation of DOM, resulting in the formation of compounds such as carboxylic acids, aldehydes and ketones which are generally more biodegradable than their parent compounds (von Sonntag and von Gunten, 2012).

2.3.3 Considerations for the practical application of ozonation

The removal of organic micropollutants during ozonation (i.e. the reduction of the parent compound concentration) has been examined in many studies (e.g. Bourgin *et al*., 2018; Hollender *et al*., 2009; Margot *et al*., 2013). The ozone doses applied have typically been in the range of 0.3-1 g O_3/g DOC (Bourgin *et al*., 2018; Hollender *et al*., 2009), although higher doses have also been applied to study the oxidation of certain recalcitrant compounds (Margot *et al*., 2013). Higher doses are, however, associated with higher costs and an increased risk of bromate formation (Margot *et al*., 2013). It has been shown that organic micropollutants exhibiting a high ozone reactivity (e.g. diclofenac and sulfamethoxazole) are well abated even at lower specific ozone doses, while compounds with lower reactivities (e.g. mecoprop and benzontriazole) require higher doses. The ozone consumption by the water matrix, and subsequently the removal efficiency of organic micropollutants, is also affected by the concentration of DOC (and nitrite if present), with higher concentrations decreasing the removal rate of organic micropollutants. To ensure that the concentration of DOC is low and scavenging by DOM is minimized, ozonation is typically applied after the biological treatment and the secondary clarifier (Bourgin *et al*., 2018; Hollender *et al*., 2009; Lee *et al*., 2013; Margot *et al*., 2013).

The formation of ozonation by- and transformation products is another factor that needs to be considered when applying ozonation. In many cases it seems that ozonation can remove the biological function of the parent compound (Lee and von Gunten, 2016); a significant reduction in estrogenic activity after ozonation has for instance been reported (Margot *et al*., 2013; Stalter *et al*., 2010). Still, in some cases, toxicity has been found to increase after ozonation. It is expected that this increase in toxicity mainly stems from the formation of ozonation byproducts such as aldehydes, ketones and carboxylic acids, and it has been shown that the toxicity decreases again after subsequent biological treatment. It is consequently recommended to follow ozonation with a biological posttreatment step (Stalter *et al*., 2010). Different types of

posttreatments, such as moving bed biofilm reactors (MBBRs), sand filtration as well as solutions based on activated carbon have been suggested and tested (Betsholtz *et al.*, 2022; Edefell *et al*., 2021; Hollender *et al*., 2009).

2.4 Selected micropollutants

The organic micropollutants selected for the experiments were sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac, ibuprofen, bisphenol A and trimethoprim. The rate constants for the reactions of the selected micropollutants with ozone and hydroxyl radicals are presented in Table 2.2. No rate constants for sulfadiazine could be found, but sulfonamides are generally expected to exhibit a high ozone reactivity, with an estimated ozone rate constant of $>10^5$ M^{-1} s^{-1} (Huber *et al*., 2003).

Table 2.2. Rate constants for the reactions of the selected micropollutants with ozone (k_{O3}) and hydroxyl radicals ($k_{\bullet OH}$). References: [a](Huber et al., 2003),[b](Dodd et al., 2006), [c]Deborde et al., 2005), [d](Rosenfeldt and Linden, 2004).

Substance	k_{O3} (M^{-1} s^{-1})	$k_{\bullet OH}$ (10^9 M^{-1} s^{-1})
Sulfamethoxazole	$2.5 \cdot 10^6$ (a)	5.5 (a)
Sulfadiazine	-	-
Carbamazepine	$3 \cdot 10^5$ (a)	8.8 (a)
Diclofenac	$1 \cdot 10^6$ (a)	7.5 (a)
Ibuprofen	9.6 (a)	7.4 (a)
Bisphenol A	$1.7 \cdot 10^4$ (c)	10 (d)
Trimethoprim	$2.7 \cdot 10^5$ (b)	6.9 (b)

2.4.1 Sulfamethoxazole

Sulfamethoxazole is a bacteriostatic antibiotic that is commonly formulated with trimethoprim and used to treat a variety of bacterial infections including urinary tract infections (National Library of Medicine, n.d.). Together with sulfadiazine, sulfamethoxazole is part of a larger group of medicines known as sulfonamides. The development of the sulfonamide antibiotics began with the discovery of the antibacterial properties of *p*-aminobenzenesulfonamide in the 1930s, and since then derivatization has resulted in the synthesis of over 5000 different sulfonamide antibiotics, of which about 100 compounds have been manufactured commercially. The use of sulfonamide antibiotics have declined in recent years due to antibiotic resistance, the discovery of side effects and the availability of other antibiotics (Eckel *et al*., 1995), but the sulfonamides are still continuously used in human and veterinary medicine (Baran *et al*., 2011). According to a report compiled by the European Medicines Agency (EMA/24309/2020) in which the sale of veterinary antimicrobials in 31 European countries was examined, the sulfonamides accounted for the third most sold antibiotic in 2018.

Sulfonamides are excreted from the body via urine, approximately 10% as the parent compound and the rest in the form of metabolites. The main metabolite of sulfamethoxazole is N^4-acetylsulfamethoxazole and it is suspected that this metabolite is retransformed into the active parent compound during wastewater treatment (Göbel *et al*., 2005). Sulfamethoxazole is frequently detected in influents (Göbel *et al*., 2007; Lindberg *et al*., 2005; Margot *et al*., 2013) as well as effluents to WWTPs (Loos et al., 2013) and is generally considered to be toxic to aquatic organisms (Petrie *et al*., 2015). Sorption to sludge has been reported to be low (Göbel *et al*., 2005; Suarez *et al*., 2010) and the removal in conventional treatment varies significantly between studies with removal rates between 22 and 80 % being reported (Göbel *et al*., 2007; Lindberg *et al*., 2005; Margot *et al*., 2013; Suarez *et al*., 2010).

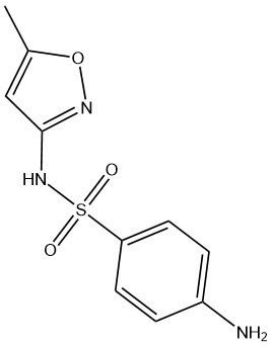


Figure 2.1. Chemical structure of sulfamethoxazole. Figure made using Chemdraw.

2.4.2 Sulfadiazine

Sulfadiazine is another sulfonamide antibiotic used for the prevention and treatment of various bacterial infections including urinary tract infections. Like sulfamethoxazole, sulfadiazine is metabolized by acetylation and known transformation products include N^4-acetylsulfadiazine (National Library of Medicine, n.d.). About 25% is excreted from the body unchanged, the rest in the form of metabolites (Göbel *et al.*, 2005). Less information about the occurrence of sulfadiazine in raw and treated wastewater is available, perhaps due to the fact that it is rarely detected in the influent to WWTPs (Göbel *et al.*, 2007). The removal during biological treatment has however been reported to reach 64% while sorption to sludge was shown to be low (Achermann *et al*., 2018).

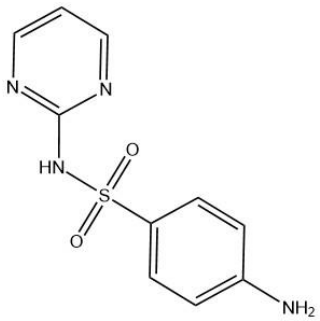


Figure 2.2. Chemical structure of sulfadiazine. Figure made using Chemdraw.

2.4.3 Carbamazepine

Carbamazepine is an antiepileptic drug which can be used to treat seizures as well as facial pain and alcohol withdrawal (FASS, 2023). The compound is commonly detected at the influent of WWTPs (Hollender *et al*., 2009; Margot *et al*., 2013) and has been reported to exhibit a low removal rate during conventional wastewater treatment, less than 10% is typically removed (Bourgin *et al*., 2018; Joss *et al*., 2005; Margot *et al*., 2013). The effluent concentrations will thus remain high, and it has been shown that carbamazepine is one of the most frequently detected pharmaceuticals in WWTP effluents (Kostich *et al.*, 2014; Loos *et al*., 2013). Carbamazepine has been reported to have a low acute toxicity towards aquatic organisms, but is suspected to have endocrine disrupting properties (Oetken *et al*., 2005).

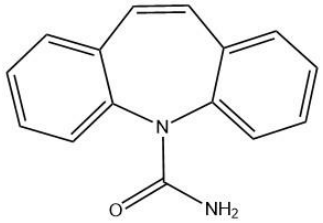


Figure 2.3. Chemical structure of carbamazepine. Figure made using Chemdraw.

2.4.4 Diclofenac

Diclofenac is an anti-inflammatory and analgesic drug which can be used for temporary treatment of rheumatic diseases, menstrual cramps and migraines (FASS, 2023). Diclofenac has been found in relatively high concentrations in the influent to WWTPs and generally exhibit a poor removal during conventional wastewater treatment (Hollender *et al*., 2009), with reported removal rates between 9-40% (Bourgin *et al*., 2018; Joss *et al*., 2005; Margot *et al*., 2013). The main removal mechanism has been reported to be biological transformation, while sorption to sludge is low (Joss *et al*., 2005). The compound is frequently detected in effluents to WWTPs (Loos *et al.*, 2013) and has been shown to be toxic to fish (Schwaiger *et al*., 2004), as well as birds (Oaks *et al.*, 2004). Diclofenac residues have been linked to a notable population decline of vultures in Pakistan (Oaks *et al.*, 2004).

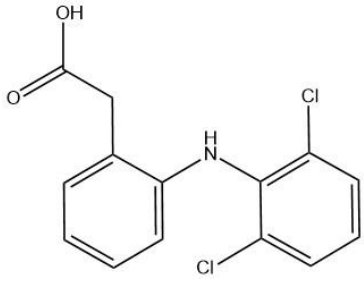


Figure 2.4. Chemical structure of diclofenac. Figure made using Chemdraw.

2.4.5 Ibuprofen

Ibuprofen is an anti-inflammatory, analgesic and fever reducing drug which can be used to treat temporary pain conditions such as migraines, menstrual cramps and fever (FASS, 2024). The drug has been found in high concentrations in raw wastewater (Margot *et al*., 2013) but is generally removed to >90% in conventional wastewater treatment (Clara *et al*., 2005; Joss *et al.*, 2005), although lower removal rates (57%) have also been reported (Margot *et al.*, 2013). Biological transformation has been shown to be the main removal mechanism with sorption to sludge being negligible (Joss *et al*., 2005; Ternes *et al*., 2004). Effluent concentrations are generally low and often fall below the limit of detection (Loos *et al*., 2013).

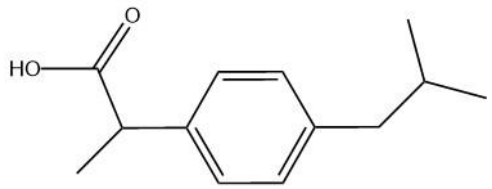


Figure 2.5. Chemical structure of ibuprofen. Figure made using Chemdraw.

2.4.6 Bisphenol A

Bisphenol A is one of the most widely produced chemicals worldwide (Vandenberg *et al*., 2009) and can be found in a variety of plastic products such as food containers, bottles and toys (Livsmedelsverket, 2024). Bisphenol A is a proven endocrine disruptor and exposure to it has been linked to a number of adverse health effects in both animals and humans (Vandenberg *et al.*, 2009). It has further been shown that bisphenol A can affect fish at concentrations as low as 1 µg/L (de Kermoysan *et al*., 2013). The compound has been found in high concentrations in raw wastewater (Clara *et al.*, 2005; Gardner *et al*., 2013; Margot *et al*., 2013), but is generally well removed in conventional wastewater treatment, with reported removal rates from 50 to over 80% (Gardner *et al.*, 2013; Margot *et al*., 2013). The removal is expected to mainly stem from biodegradation (Margot *et al.*, 2015) although a lower fraction has been found to sorb to sludge (Stasinakis *et al.*, 2008).

Figure 2.6. Chemical structure of Bisphenol A. Figure made using Chemdraw.

2.4.7 Trimethoprim

Trimethoprim is a bacteriostatic effective against common pathogens in the urinary tract (FASS, 2023) and is, as previously mentioned, commonly formulated with sulfamethoxazole (National Library of Medicine, n.d.). Approximately 50% is excreted in its original form after ingestion (FASS, 2023). Trimethoprim has been detected in raw wastewater and low removal rates, up to 20% during conventional treatment, have been reported (Göbel *et al*., 2007; Göbel *et al*., 2005; Lindberg *et al.*, 2005). The compound does not sorb well to sludge (Göbel *et al*., 2007; Göbel *et al.*, 2005) and is frequently detected in WWTP effluents (Loos *et al*., 2013).

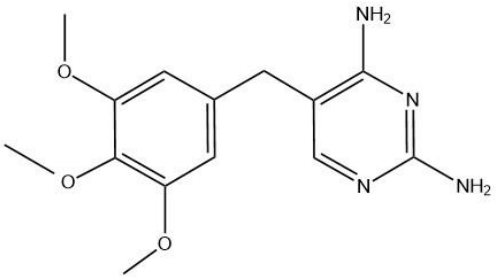


Figure 2.7. Chemical structure of Trimethoprim. Figure made using Chemdraw.

2.5 ^{14}C-labeling

^{14}C is a radioactive isotope of carbon, the decay of which is most notably used to date organic material. In recent years it has also been used to study the fate of organic micropollutants in different wastewater treatment processes (e.g. Betsholtz *et al.*, 2021; Falås *et al*., 2018; Popple *et al.*, 2016).

2.5.1 The use of ^{14}C-labeled organic micropollutants

The removal of organic micropollutants during ozonation and biological treatment is traditionally measured as the concentration difference before and after treatment (Bourgin *et al*., 2018; Hollender *et al*., 2009; Margot *et al.*, 2013). Such measurements do not allow for the biodegradability of the transformation products to be studied and any distinction between transformation and mineralization is lost. In an attempt to fill in these gaps, the concentration measurements can be complemented by screening for transformation products (Bourgin *et al*., 2018; Gulde *et al.*, 2021a; Gulde e*t al*., 2021b). The elucidation of transformation products is however complicated and time consuming, typically involving solid phase extraction followed by liquid chromatography coupled with high-resolution mass spectroscopy (LC/MS) (Gulde *et al.*, 2021b). The high number and large variety of possible transformation products further complicate the process, and detection of certain transformation products, e.g. those stemming from activated aromatic compounds and olefinic compounds, has proven to be limited (Gulde *et al.*, 2021a).

The use of ^{14}C-labeled micropollutants, i.e. organic micropollutants where at least one carbon consists of the radioactive isotope, allows for tracking of organic micropollutants and their transformation products, without any knowledge about the molecular structure of the transformation products, simply by measuring the radioactivity. Assuming that other removal mechanisms, e.g. volatilization, are negligible there are three main pathways for organic micropollutants during ozonation: they can remain unchanged in the liquid phase, they can be transformed and remain in the liquid phase as transformation products or they can be mineralized and enter the gas phase as CO_2. The remaining fraction of the parent compound as well as the transformation products retaining the ^{14}C-labeled position will account for the radioactivity in the liquid phase. To distinguish these two pathways, and determine the degree of transformation, the concentration of the parent compound can be measured. To further estimate the fraction that is mineralized during ozonation, the radioactivity in the gas phase can be determined.

The three pathways listed above are also the main pathways during biological treatment, assuming that removal mechanisms such as volatilization, sorption to biomass or incorporation into biomass are negligible. The transformation products and any remaining fraction of the parent compound can either remain unchanged in the liquid phase, be further transformed or be mineralized. It is not possible to study biotransformation using this method, however, through the partitioning of radioactivity between the liquid and gas phase the degree of mineralization can be determined. The biodegradation of ozonation transformation products can thus be studied, provided that they retain the labeled position.

2.5.2 The formation and transfer of $^{14}CO_2$

In this project measurements of the radioactivity in the gas phase were performed using a CO_2-trap. Upon mineralization, CO_2 will dissolve in the liquid as either H_2CO_3, HCO_3^- or CO_3^{2-}, the distribution of which is dependent on the pH. Release of CO_2 from the liquid phase can then occur from H_2CO_3, with a rate that is dependent on the concentration of H_2CO_3 and the partial pressure of CO_2, as described by Henry's law. The distribution of carbonate species is shifted

towards H_2CO_3 at lower pH-values. Release of CO_2 from the liquid phase is thus favored in acidic environments, while dissolution of CO_2 is favored in basic environments. By employing a CO_2-trap, in practice a glass tube filled with NaOH, the CO_2 released from the liquid phase will preferentially redissolve in the CO_2-trap. Due to the capture of CO_2 by the trap, the partial pressure is kept low, and release of CO_2 from the liquid phase can occur even at elevated pHs. It is worth noting that although the CO_2-trap will capture all CO_2 that is released, it is only the CO_2 resulting from mineralization of a labeled moiety, that is, $^{14}CO_2$, that will be measured and as such mineralization of any other moiety will not be visible using this method.

2.6 Liquid scintillation counting

Liquid scintillation counting is a method to detect and quantify radioactivity based on the ability of aromatic compounds to absorb energy from nuclear radiation and then convert it into photons. The method involves placing the radioactive sample into a scintillation vial and then adding a scintillation cocktail consisting of an organic scintillator dissolved in an organic solvent. In case the radioactive sample is dissolved in aqueous media, the scintillation cocktail also contains a surfactant to ensure that the mixture forms a homogenous solution. Because the solvent is present in greater concentration than the scintillator it will primarily be the solvent molecules that absorb the energy released upon decay of the radioactive sample. In the case of ^{14}C this energy takes the form of a beta particle. The energy is then transferred from the activated solvent molecules to the scintillator molecules whereupon the scintillator molecules quickly return to their original state by releasing the transferred energy as photons. The photon emission intensity depends on the type of nuclear decay as well as the original energy released upon decay of the radioactive sample, while the number of photon emissions per unit time is proportional to the radioactivity of the sample (L'Annunziata *et al*., 2020).

Upon emission of the photons, these are converted into electrons by photomultiplier tubes and then amplified, allowing the original photons to be detected as an electronic signal and a spectrum of the count rate (photon emissions unit time^{-1}) versus the pulse height (keV) to be obtained. Radioactivity is quantified as disintegrations per minute and to get a measure of the radioactivity the count rate must thus be converted. The count rate is however not only dependent on the radioactivity but also on the efficiency by which energy from the nuclear decay is transferred and ultimately converted into photons, and interference with these processes can result in a reduction of the count rate. This phenomenon is called quenching and can be accounted for when converting from count rate to disintegrations per minute (L'Annunziata *et al.*, 2020).

There are different types of quenching, the most common of which is chemical quenching. Chemical quenching results from the presence of chemical compounds in the sample that can absorb energy from the nuclear decay and thus interfere with the transfer of energy to the scintillation solvent. To account for the effect of quenching the counting efficiency can be determined and used to calculate the true number of disintegrations per minute and thereby accurately determine the radioactivity of the sample (L'Annunziata *et al*., 2020). In this project, the counting efficiency was high, and more importantly, uniform. As obtaining an absolute value of the radioactivity was not of interest, there was consequently no need to convert from count rate to disintegrations per minute. Instead, the count rate could be used directly to calculate the relative distribution of radioactivity in the different phases.

2.7 Determination of the ozone concentration

The concentration of ozone in water can be measured both directly and indirectly using UV spectrophotometry. For the direct method the absorbance is measured at a wavelength of 260 nm (von Sonntag and von Gunten, 2012) after which the concentration can be calculated using the Beer-Lambert law, see equation 1:

$$A = \varepsilon \cdot C_{O_3} \cdot l \quad (1)$$

where A is the absorbance, Ɛ is the molar absorption coefficient (M^{-1} cm^{-1}), C_{O3} is the ozone concentration (M) and l is the path length of the cuvette (cm). Different values of the molar absorption coefficient have been reported, ranging from 2000-3600 M^{-1} cm^{-1}. The direct method is well suited to determine the concentration of ozone in stock solutions but requires that there is no other material present in the solution (e.g. DOM) that absorbs at the same wavelength. Under such conditions, the absorbance can instead be measured indirectly using assays. One commonly used indirect method is the indigo method which relies on the rapid reaction between ozone and indigotrisulfonate, resulting in bleaching of the latter compound (von Sonntag and von Gunten, 2012). By measuring the absorption at 600 nm before and after addition of ozone, the ozone concentration $C_{O3\ stock}$ (mg/L) can be calculated according to equation 2:

$$C_{O_{3\ stock}} = \frac{\Delta A \cdot V_T}{f \cdot b \cdot V_{stock}} \quad (2)$$

where ΔA is the difference in absorbance, V_T is the total volume (ml), f is a factor with the constant value 0.42, b is the pathlength of the cuvette (cm) and V_{stock} is the volume of the ozone stock solution (ml) (Bader, 1982). The indigo method has been shown to give reliable results in the concentration range of 0.005-30 mg/L (Bader and Hoigné, 1981). Once the concentration of the stock solution has been calculated, using either equation 1 or 2, the volume of ozone stock solution to be added to a water sample to achieve the desired specific ozone concentration can be calculated according to equation 3:

$$V_{stock} = \frac{C_{O_{3\ spec}} \cdot C_{DOC} \cdot V_{sample}}{C_{O_{3\ stock}}} \quad (3)$$

where V_{stock} is the volume of the ozone stock solution (ml), $C_{O3\ spec}$ is the specific ozone concentration (mg O_3/mg DOC), C_{DOC} is the concentration of DOC in the water (mg/L), V_{sample} is the sample volume of the water (ml) and $C_{O3\ stock}$ is the concentration of the ozone stock solution (mg/L).

3 Materials and methods

Three sets of experiments were performed within the scope of this project. The experiments aimed at studying ozonation and biological degradation of organic micropollutants and their transformation products, with each experiment having the same procedure but examining a different set of compounds. The following section outlines the setup and procedure of these experiments, along with the materials used and the methods for sample preparation and analysis.

3.1 Water and carriers

Water and biofilm carriers were collected from the WWTP Nykvarnsverket in Linköping at two separate occasions, 2024-02-15 for use during the first experiment (2024-02-19) and 2024-03-22 for use during the second (2024-04-01) and third experiment (2024-04-22). The treatment at Nykvarnsverket consists of mechanical treatment to separate larger objects and particles, chemical treatment to remove phosphorus and biological treatment to remove organic material and nitrogen. The biological treatment is divided into two separate units, the first unit consisting of an activated sludge process where nitrification and reduction of organic material takes place and the second unit consisting of an MBBR where both nitrification and denitrification takes place. As of 2017, the treatment also includes an ozonation unit for the removal of organic micropollutants (Tekniska verken, n.d.). The water was collected before the ozonation unit, while the carriers (HX1LI2KLL, Christian Stöhr GmbH & Co.) were collected from the MBBR. The water and the carriers were placed in a refrigerator until use.

3.2 Organic micropollutants

Seven different ^{14}C-labeled organic micropollutants were selected for the experiments based on commercial availability: sulfamethoxazole and sulfadiazine (Izotop, Hungary), carbamazepine, diclofenac, bisphenol A, trimethoprim and ibuprofen (Hartmann Analytics, Germany). The selected micropollutants, along with the position of the ^{14}C-label, are presented in Figure 3.1. All compounds were labeled in one position, except for bisphenol A which was labeled in two different positions: the aromatic rings and the second carbon in the central propyl group. The labeling of the aromatic rings, in bisphenol A as well as in sulfamethoxazole and sulfadiazine, is uniform, meaning that each carbon atom in the ring structure has been labeled, on average, to the same extent. Sulfamethoxazole and sulfadiazine were studied in the first experiment, carbamazepine, diclofenac and ibuprofen in the second experiment and bisphenol A and trimethoprim in the third experiment.

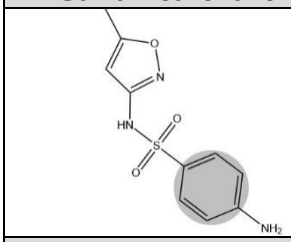
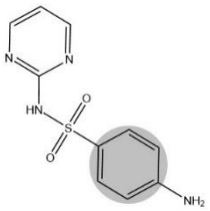
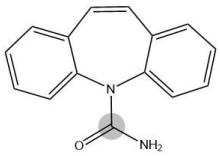
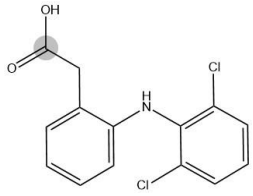
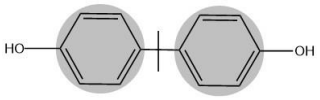
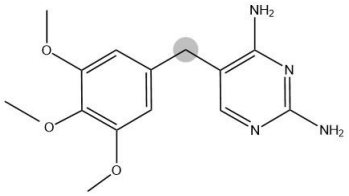
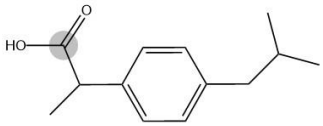
Sulfamethoxazole	Sulfadiazine	Carbamazepine
		
Diclofenac	**Bisphenol A (propyl)**	**Bisphenol A (ring)**
		
Trimethoprim	**Ibuprofen**	
		

Figure 3.1. The ^{14}C-labeled micropollutants used in the experiments with the grey area representing the position of the radiolabel. The figures were made using Chemdraw.

3.3 Ozonation and biodegradation experiments

The general setup of the ozonation and biodegradation experiments is explained in section 3.3.1, while details on the experiments are provided in section 3.3.2 and 3.3.3.

3.3.1 Experimental setup

The ozonation and biodegradation experiments were performed using glass bottles filled with wastewater and spiked with a stock solution of a ^{14}C-labeled micropollutant. The bottles were equipped with a CO_2-trap, a glass tube filled with NaOH, to dissolve the formed $^{14}CO_2$ and allow the radioactivity of the gas phase to be measured indirectly. Hereafter, samples taken from the CO_2-trap will thus be referred to as stemming from the gas phase. To seal the bottles an airtight septum was used, and after ozonation and subsequent addition of carriers, two needles were inserted through it to allow continuous sampling of the liquid and gas phase respectively. The setup can be seen in Figure 3.2.

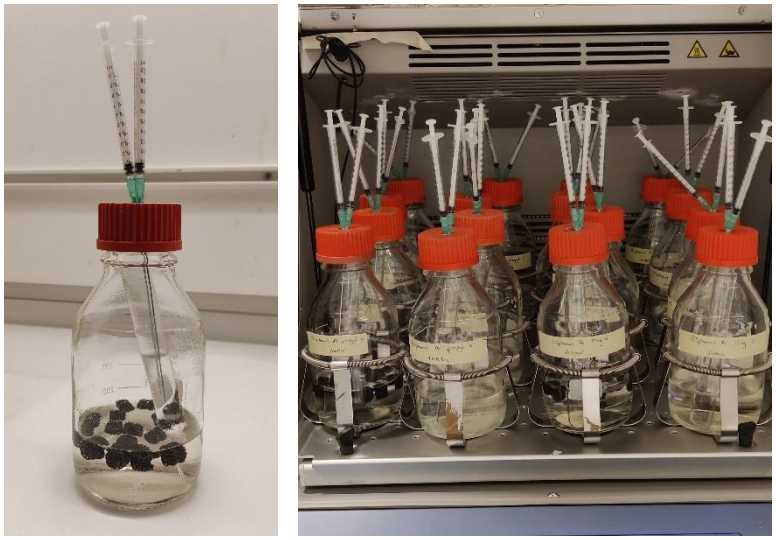


Figure 3.2. The setup used during the experiments.

The ^{14}C-labeled micropollutants were studied separately, using four biologically active bottles that had each been subjected to a different ozone dose (0, 0.3, 0.6 and 1 mg O_3/mg DOC respectively). Corresponding bottles were also prepared for background control, to which there was no addition of carriers, and for analysis of the water quality, to which there was no spiking of organic micropollutants. For each experiment, four additional beakers were also prepared, each corresponding to a different ozone dose and spiked with a cocktail of non-radiolabeled micropollutants. These bottles were sent for analysis of the parent compound and regulations regarding the handling of radioactive material necessitated the use of non-radiolabeled micropollutants.

3.3.2 Ozonation

Glass bottles of 500 ml were prepared and labeled according to the system presented in 3.3.1. The collected wastewater was buffered by addition of KH_2PO_4 to a concentration of 10 mM and adjusted to a pH of 7 by addition of NaOH/HCl. The water was then filtered using a 0.45 µm cellulose nitrate filter (Whatman). CO_2 traps were prepared by filling glass tubes with 20 ml NaOH (3 M). Stock solutions of ^{14}C-labeled micropollutants were prepared by evaporating the organic solvent of the primary stock solution and redissolving in methanol to an activity of 0.1 µCi/ml. The stock solutions were then added to the buffered and filtered wastewater to an activity of 1µCi/L and equivalent concentrations of 12.6 µg/L for sulfamethoxazole, 11.0 µg/L for sulfadiazine, 10.6 µg/L for carbamazepine, 5.3 µg/L for diclofenac, 3.8 µg/L for ibuprofen, 4.1 µg/L for the propyl-labeled bisphenol A, 1.5 µg/L for the ring-labeled bisphenol A and 5.2 µg/L for trimethoprim. To the bottles aimed for biological treatment and background control, 150 ml of wastewater spiked with radiolabeled micropollutants was added. To the bottles aimed for analysis of the water quality 150 ml of buffered and filtered wastewater was added. To the bottles aimed for analysis of the parent compound 150 ml wastewater spiked with a cocktail of non-radiolabeled micropollutants was added. A list of the non-radiolabeled micropollutants and their initial concentrations is presented in Table A.1.

An ozone stock solution was prepared by generating ozone from oxygen using an ozone generator (GM1, Primozone, Sweden), and sparging the ozone gas into a cooled contact vessel containing Milli-Q water. The concentration of the stock solution was determined by measuring the absorbance in triplicates using both the direct method and the indigo method, for details

refer to section 3.4. The mean absorbance was calculated and equation 1 and 2 respectively were used to determine the ozone concentration, after which equation 3 was used to determine the volume of stock solution to be added to each bottle to reach the desired dose (Table 3.3). For the direct method, the molar extinction coefficient in equation 1 was set to 3200 M^{-1} cm^{-1}. The additions of stock solution to the bottles were conducted under constant stirring and as the ozone concentration tends to fluctuate with subtractions from the stock solution, the concentration measurements were repeated after each dosing. The mean of the ozone concentration before and after dosing was calculated and inserted into equation 3 to determine the actual ozone dose (Table 3.3).

Table 3.3. The volume of ozone stock solution added to the bottles during each experiment and the resulting mean concentrations, calculated from the results obtained using the direct method.

Intended ozone dose (mg O_3/mg DOC)	**Added volume (ml)**	**Final ozone dose (mg O_3/mg DOC)**
Experiment 1		
0.3	10	0.33
0.6	15	0.54
1	25	0.90
Experiment 2		
0.3	10	0.26
0.6	20	0.58
1	28	0.95
Experiment 3		
0.3	7	0.31
0.6	12	0.62
1	17	0.93

Following the addition of ozone stock solution, the CO_2-traps were inserted, and the bottles were immediately sealed. To allow for transport of the formed $^{14}CO_2$ from the liquid phase to the CO_2-traps, the bottles were placed in an incubator shaker (20℃, 125 rpm) for approximately 38 h. Samples were then collected from the liquid and gas phases of the biologically active bottles and the background controls (time = 0h). The volume of each liquid sample corresponded to the volume of added ozone stock solution to ensure that all bottles contained the same volume (150 ml) at the start of the biodegradation experiments. For the bottles to which no ozone stock solution had been added, the original volume of water had been set to 155 ml, and subsequently, 5 ml was subtracted at this stage. Liquid samples were also collected from the bottles containing non-radiolabeled micropollutants and were frozen until analysis. The remaining liquid from the experiments with the non-radiolabeled micropollutants was used for analysis of the water quality after ozonation.

3.3.3 Biological treatment

After the first sampling, the CO_2-traps were removed and replaced with equivalent traps. Fifteen carriers were added to each of the biologically active bottles, after which the bottles were sealed, and two needles were inserted through the rubber septum. The bottles were returned to the incubator shaker and samples were taken from the liquid phase (1 ml) and the gas phase (0.5 ml) and transferred to Eppendorf tubes at regular intervals (time = 1h, 2h, 4h, 8h, 12h, 24h, 30h, 48h and 72h). The samples retrieved from the liquid phase were centrifuged at 13 500 rpm for

5 minutes after which the supernatant (0.8 ml) was transferred to a new Eppendorf tube while the bottom phase was discarded. This was done immediately after sampling to remove any biomass from the sample and halt the degradation. After the final sampling, the water from the control bottles was filtered and refrigerated until analysis of the water quality.

The total solids (TS) concentration for the carriers was determined by measuring the weight of five dried carriers, before and after careful removal of the biomass, and reported as the mean of triplicate measurements. The filling ratio was determined by measuring the volume occupied by the carriers per liter of liquid.

3.4 Analysis and sample preparation

The concentration of the ozone stock solution was determined spectrophotometrically (UV-VIS DR6000, Hach Lange) using both the direct method and the indigo method. For the direct method, 1 ml of ozone stock solution was mixed with 1 ml of Milli-Q water and the absorbance was measured at a wavelength of 260 nm using a cuvette with a path length of 1 cm. For the indigo method, 0.2 ml of ozone stock solution was mixed with 4.8 ml potassium indigotrisulfonate ($1.5 \cdot 10^{-4}$ M) and the absorbance was measured at a wavelength of 600 nm using a cuvette with a path length of 2 cm.

The radioactivity of the liquid and gas samples from the ozonation and biodegradation experiments was measured using liquid scintillation counting (Tri-Carb 4910 TR, PerkinElmer). The liquid samples were prepared by mixing 400 µl sample with 3.6 ml scintillation liquid (Ultima gold, PerkinElmer) while the gas samples were prepared by mixing 200 µl sample with 3.8 ml scintillation liquid. The number of counts per minute (CPM) was then measured over a time span of 5 minutes for each sample. At time = 0 h the measurements were performed in triplicates, for the other timestamps only a single measurement was made. To ensure that the values obtained from the liquid and gas samples were comparable, the background radiation was determined by recording the CPM values of five samples each of filtered wastewater and NaOH and then subtracting the mean from the liquid and gas sample values respectively. To further ensure that samples obtained at different timestamps were comparable, losses in radioactivity due to sampling were accounted for. Additionally, the effect of dilution due to addition of differing volumes of ozone stock solution was also accounted for. Analysis of the non-radiolabeled micropollutants was conducted by preparing the samples using solid-phase extraction, followed by ultra performance liquid chromatography coupled to tandem mass spectroscopy, as per the method described by Svahn and Björklund (2016; 2019).

Water quality parameters were determined prior to treatment as well as after ozonation and biological treatment. DOC and chemical oxygen demand (COD) were determined spectrophotometrically (DR2800, Hach Lange) using Hach-Lange cuvettes (LCK385 and LCK 314). UVA_{254} was measured using a spectrophotometer (UV-VIS DR6000, Hach Lange), at a wavelength of 254 nm and with a 5 cm cuvette. Ion concentrations (NO_2^--N, NO_3^--N, NH_4^+-N, Br^- and BrO_3^-) were measured in triplicates using an ion chromatograph (ECO IC, Metrohm, Switzerland). The oxygen concentration was measured using an oxygen meter (HQ40D).

4 Results and discussion

In the following sections, the results from the ozonation and biodegradation experiments will be presented and discussed. It should be noted that both radiolabeled and non-radiolabeled micropollutants were used during the experiments and that these were studied using different methods, and thus gave different types of results. For the radiolabeled micropollutants the results pertain to the labeled position, while, for the non-radiolabeled micropollutants, the results pertain to the whole compound. As such a careful distinction between the two methods is necessary, and the results should be seen as being complementary, not comparable.

4.1 Ozone concentration measurements

The results of the ozone concentration measurements are presented in Table A.2. The concentration changed between each dosing, as previously discussed, but was in the range of 60-100 mg/L, well above the suggested range of 0.005-30 mg/L for the indigo method (Bader and Hoigné, 1981). With a few exceptions the two methods gave comparable results, the percentual difference was generally below 10%. It should be noted, however, that the molar extinction coefficient used to calculate the ozone concentration for the direct method (equation 1) is not uniformly determined and values ranging between 2000 and 3600 M^{-1} cm^{-1} have been reported (von Sonntag and von Gunten, 2012). For this project, a value of 3200 M^{-1} cm^{-1} was chosen, as suggested by von Sonntag and von Gunten (2012), but should another value be used instead the calculated concentration will change. As such the error of using the indigo method outside of the suggested range is likely offset by the error introduced when choosing a molar extinction coefficient and both methods can probably be used.

4.2 Ozonation experiments

Selected water quality parameters for the filtered and buffered wastewater are presented in Table 4.1. Additional water quality parameters, before and after ozonation, can be found in Table A.4 and A.5.

Table 4.1. Selected water quality parameters determined for the filtered and buffered wastewater.

Experiment	DOC (mg/L)	NO_2^--N (mg N/L)	pH
1	14.6	0.16	7.0
2	14.6	0.41	7.0
3	11.6	0.08	7.0

The water quality parameters differed slightly between experiments, as did the applied dose, but similar trends could be observed for all of the experiments. Ozonation resulted in a slight decrease in DOC, although some inconsistencies could be observed. For COD the declining trend was more distinct, indicating a limited mineralization of DOM. UVA_{254} decreased with increasing ozone doses, consistent with the loss of double bonds and aromatic rings due to reactions with ozone. The concentration of nitrite reached concentrations below the detection limit at all ozone doses, as expected from the high reactivity between ozone and nitrite (von Sonntag and von Gunten, 2012). In experiments 1 and 2, where the original nitrite concentration was higher, this was accompanied by an increase of the nitrate concentration. Bromide could

not be detected in the raw water during any of the experiments and as such no further measurements were made of either bromide or bromate.

The results of the ozonation experiments using non-radiolabeled micropollutants are presented in Figure 4.1. As expected from the high ozone reactivity of sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac, bisphenol A and trimethoprim (Table 2.2) and as observed in a previous study (Betsholtz et al., 2022), the concentration of the parent compound approaches zero even at the lowest ozone dose, indicating a complete transformation. For ibuprofen, however, the transformation is incomplete except at the highest ozone dose, in accordance with the low ozone reactivity of ibuprofen (Table 2.2). At 0.3 mg O_3/mg DOC the transformation ranges between 62-81%, and at 0.6 mg O_3/mg DOC between 88-96%, consistent with previous results (Betsholtz et al., 2022). From Figure 4.1 it can further be concluded that a majority of the examined micropollutants (56%) are completely transformed even at the lowest ozone dose. In addition, 68% reaches a transformation rate of at least 80% at 0.3 mg O_3/mg DOC, 91% at 0.6 mg O_3/mg DOC and 94% at 1 mg O_3/mg DOC.

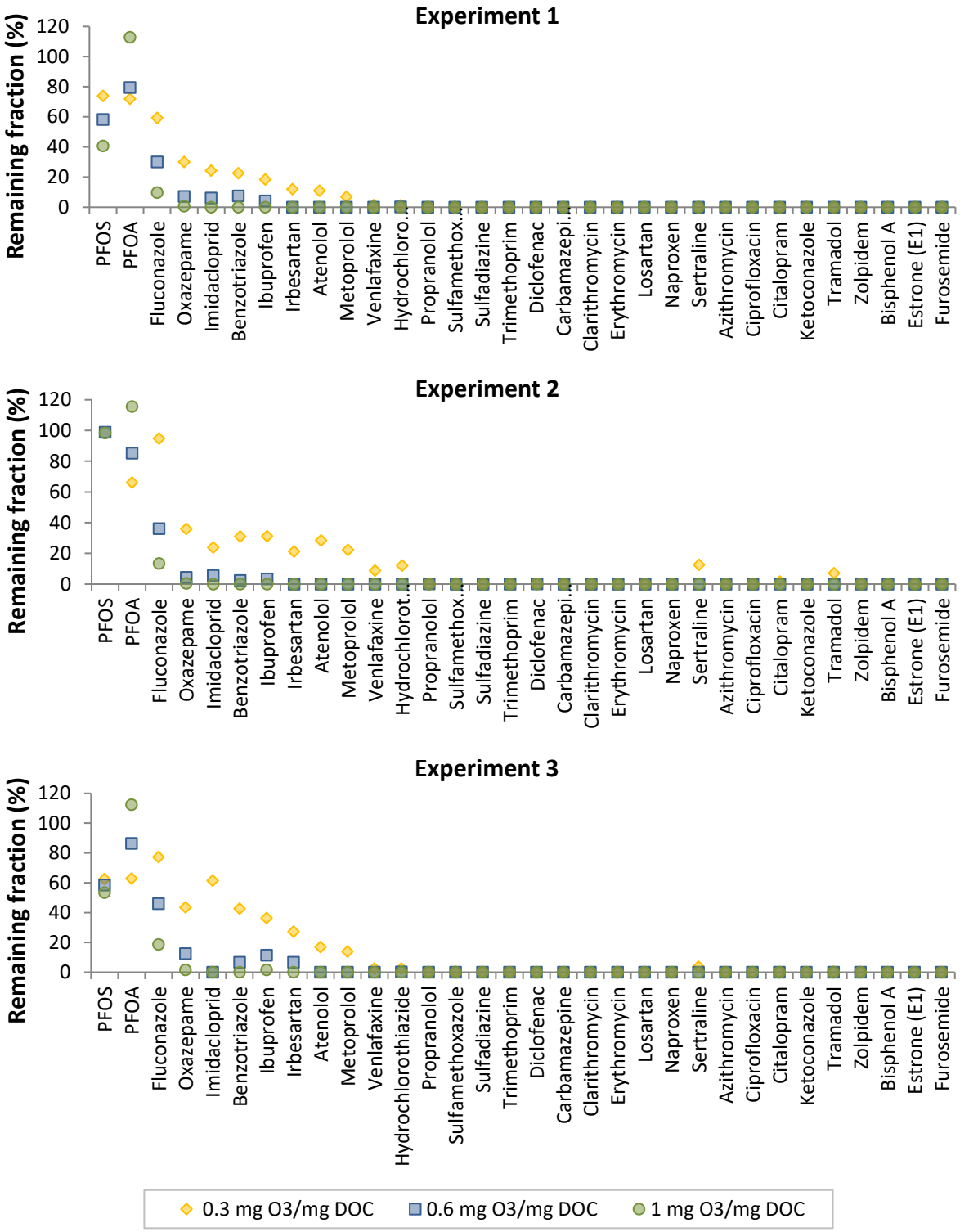


Figure 4.1. The fraction of the parent compound remaining after ozonation.

The results of the ozonation experiments using ^{14}C-labeled micropollutants are presented in Figure 4.2. For all compounds, except for propyl-labeled bisphenol A, a partial mineralization could be observed. As the ozone dose was increased the radioactivity in the liquid phase decreased while the radioactivity of the gas phase increased, corresponding to mineralization of the labeled position. As will be further discussed in section 4.4, the mass balance closed to a satisfactory degree. Of the initial activity >95% could generally be recovered in the gas and liquid phase after ozonation.

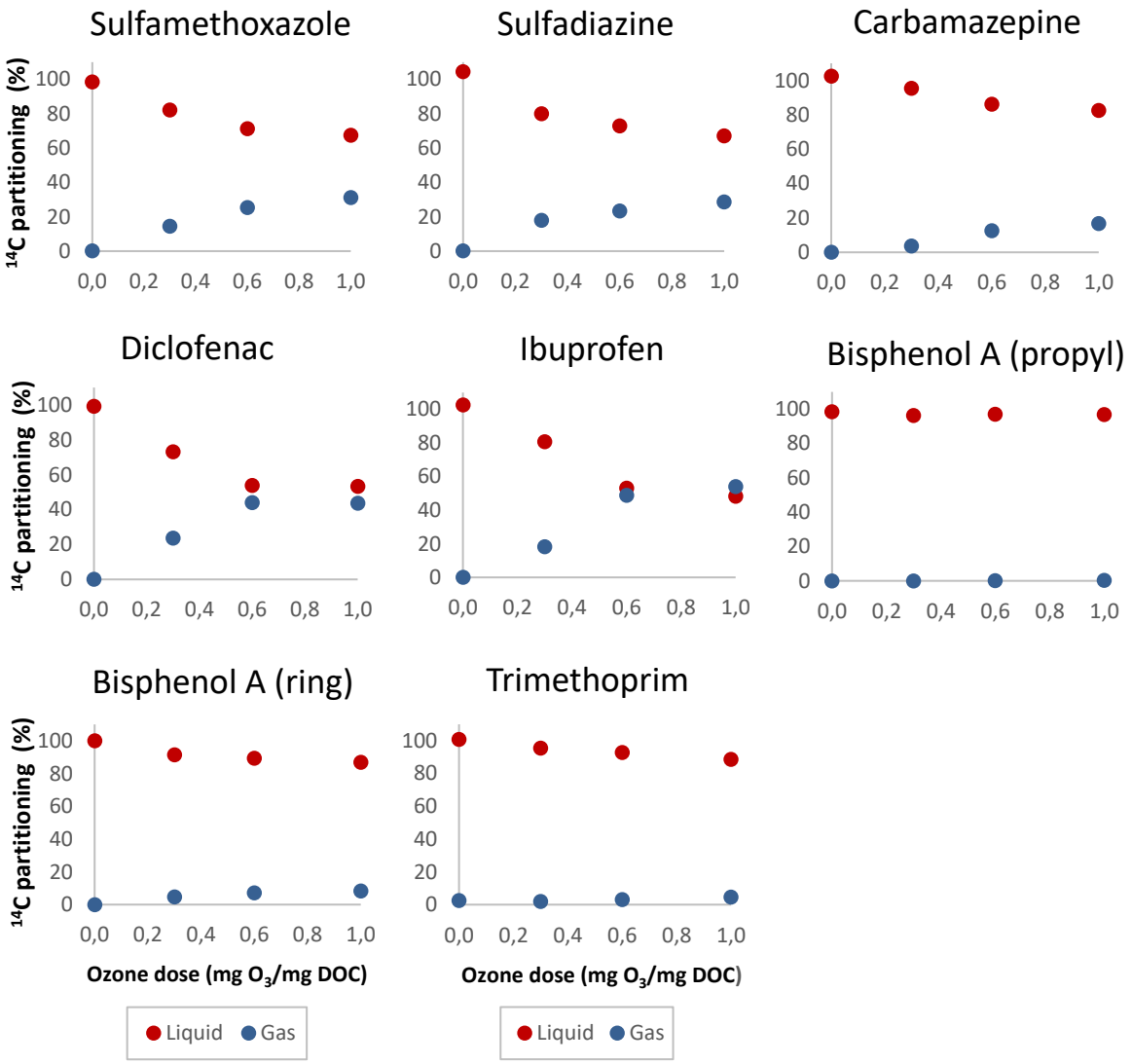


Figure 4.2. ^{14}C partitioning between the liquid and the gas phase as a function of the ozone dose.

4.2.1 Sulfamethoxazole and sulfadiazine

Sulfamethoxazole and sulfadiazine are both labeled in the aniline ring and as indicated by the $^{14}CO_2$ formation, the two compounds show nearly identical degrees of mineralization. At 0.3 mg O_3/mg DOC mineralization of the labeled aniline moiety reaches an approximate value of 15%, rising to about 30% at 1 mg O_3/mg DOC, consistent with the results obtained in another study (Betsholtz *et al.*, 2022). The complete transformation observed at all ozone doses further indicate that the fraction of ^{14}C remaining in the liquid phase after ozonation is part of different transformation products.

The aniline moiety has been identified as the preferred site of an ozone attack (Dodd *et al*., 2006) and the partial mineralization suggests that ring opening takes place as a result of reactions between the aniline moiety and ozone. Despite this, there are, to the knowledge of the writer, no known transformation products of sulfamethoxazole or sulfadiazine resulting from cleavage of the aniline ring. Several other transformation pathways have however been proposed for sulfamethoxazole, including oxidation of the amine group at the aniline ring, hydroxylation of the aniline ring, oxidation of the methyl group and the C=C bond in the isoxazole ring as well as cleavage of the C-S bond and the S-N bond (Abellán *et al*., 2008; Gómez-Ramos *et al*., 2011). The lack of detected ring opening products may be explained by the method used in these studies (and shortly explained in section 2.5.1). Transformation products resulting from ring opening are likely very polar, thus escaping enrichment during solid phase extraction and making it difficult to detect these products in the subsequent LC/MS analysis (Gulde *et al*., 2021a).

4.2.2 Carbamazapine

A mineralization of 4% could be observed at the lowest ozone dose, increasing to 17% at the highest dose, consistent with previous results (Betsholtz *et al*., 2022). A large fraction of the labeled position will thus remain after ozonation and based on the complete transformation of the parent compound, this fraction will be part of various transformation products. The radiolabel is positioned in the amide group which is not considered to be a point of attack for ozone. The high ozone reactivity of carbamazepine (Table 2.2) is instead stemming from the olefinic C-C bond in the central ring (von Sonntag and von Gunten, 2012). It has been proposed that reactions between ozone and the olefin are dominant in the beginning, resulting in ring opening followed by the formation of a secondary pyrimidine ring structure in which the labeled position is retained. Further transformation by ozone is slow but may instead take place through reactions with hydroxyl radicals (Gulde *et al*., 2021a; McDowell *et al*., 2005). From these transformation pathways a number of different transformation products have been suggested, although a majority leave the labeled position intact (Azaïs *et al*., 2017; Gulde *et al*., 2021a; Kråkström *et al*., 2020; McDowell *et al*., 2005), explaining the low mineralization.

4.2.3 Diclofenac and ibuprofen

Diclofenac and ibuprofen are both labeled in the carboxylic acid of their respective structures and display similar degrees of mineralization. At 0.3 mg O_3/mg DOC a mineralization of around 20% can be observed, increasing to 45 and 55% at 1 mg O_3/mg DOC for diclofenac and ibuprofen respectively. A similar trend was observed previously, although the reported mineralization was slightly lower (Betsholtz *et al*., 2022). The high mineralization of ibuprofen is not reflected by the compound's ozone reactivity; the ozone rate constant of diclofenac is roughly 10^6 times larger than the rate constant of ibuprofen (Table 2.2). The transformation rate of the parent compound does, however, reflect the differences in ozone reactivity. While the transformation of diclofenac is complete at all ozone doses, the transformation of ibuprofen is incomplete except at the highest ozone dose. As the transformation of ibuprofen is incomplete at 0.3 and 0.6 mg O_3/mg DOC, both the parent compound and the transformation products will contribute to the ^{14}C-activity remaining in the liquid phase after ozonation.

The labeled carboxylic acid is not associated with a high ozone reactivity. For diclofenac the main reactive sites are the nitrogen and the aromatic ring adjacent to the carboxylic acid (von Sonntag and von Gunten, 2012), while ibuprofen lacks reactive groups (Huber *et al*., 2003). As such reactions with ozone play a minor role in the oxidation of ibuprofen, with the main oxidative power coming from hydroxyl radicals (Huber *et al*., 2003). Based on the high

mineralization observed for the carboxylic acid of both compounds, it seems reasonable to assume that this is mainly stemming from reactions with hydroxyl radicals, in the case of ibuprofen as well as diclofenac.

For ibuprofen, two transformation products have been identified, corresponding to the addition of a carbonyl group and hydroxyl group respectively, and leaving the labeled position intact (Gulde *et al*., 2021a). For diclofenac, a number of transformation products have been proposed, mainly corresponding to hydroxylation of the aromatic rings. Loss of the labeled carboxylic acid has also been suggested (Alharbi *et al*., 2022; Coelho *et al*., 2009). The high mineralization observed for the labeled position would suggest that the transformation products in which the original carboxylic acid is missing are in abundance.

4.2.4 Bisphenol A

For the ozonation of bisphenol A, a slight mineralization of the aromatic rings could be observed (5 and 8% at 0.3 and 1 mg O_3/mg DOC respectively), while no $^{14}CO_2$-formation could be seen for the propyl group (<1% at all ozone doses). For the propyl group, the results are in accordance with those obtained previously, while the mineralization of the aromatic rings is considerably lower than previously reported (Betsholtz *et al*., 2022). This could perhaps be explained by differences in the water quality affecting the availability of ozone and hydroxyl radicals. Based on the low mineralization of both positions as well as the complete transformation observed for the parent compound, the ^{14}C-activity remaining in the liquid after ozonation stems from transformation products retaining much of the original structure.

The high ozone reactivity of bisphenol A (Table 2.2) is due to the equilibrium between the phenolic functions and phenolate. The phenol anion reacts rapidly with ozone, exhibiting a reaction rate that is nearly diffusion controlled (von Sonntag and von Gunten, 2012). Several transformation pathways have been proposed for the oxidation of bisphenol A, including carbonylation, hydroxylation, ring opening and cleavage of the propyl bridge. From the two former pathways, transformation products largely retaining the original structure, and thus the labeled positions, are formed. From the ring opening reaction, two carboxylic acid functions are introduced, but no loss of the carbons in the aromatic ring occurs. Cleavage of the propyl bridge results in two fragments, the first of which retains one of the aromatic rings as well as the propyl group and the second of which retains the other aromatic ring (Deborde *et al*., 2008). As such all proposed transformation products retain the labeled positions. These transformation products were, however, shown to have a low stability and are likely further transformed into smaller and more polar compounds such as acids and aldehydes (Deborde *et al*., 2008). Such reactions may be responsible for the minor mineralization of the aromatic rings, while the lack of ^{14}C-formation from the propyl group indicates that this position remains intact even if further transformation occurs.

4.2.5 Trimethoprim

Minor mineralization of the labeled position could be observed for trimethoprim, 2 and 5% at the highest and lowest ozone dose respectively, which is slightly lower than previously reported values (Betsholtz *et al*., 2022). The labeled position is thus mostly intact after ozonation, and as a complete transformation of the parent compound could be observed, it must be incorporated into transformation products. The diaminopyrimidine moiety and the trimethoxytolyl moiety have been identified as being the main ozone reactive sites (Dodd *et al*., 2006), with the preferred reaction pathways of hydroxylation, carbonylation, demethylation and methylene group cleavage (Kuang *et al*., 2013). All proposed transformation products retain the labeled position (Kuang *et al*., 2013; Radjenović *et al*., 2009) and can thusly not explain the minor

mineralization that was observed. Based on the suggestion that the identified transformation products can be further transformed (Kuang *et al.*, 2013) it is, however, possible that secondary reactions are responsible for the minor $^{14}CO_2$ formation that could be observed from the labeled position.

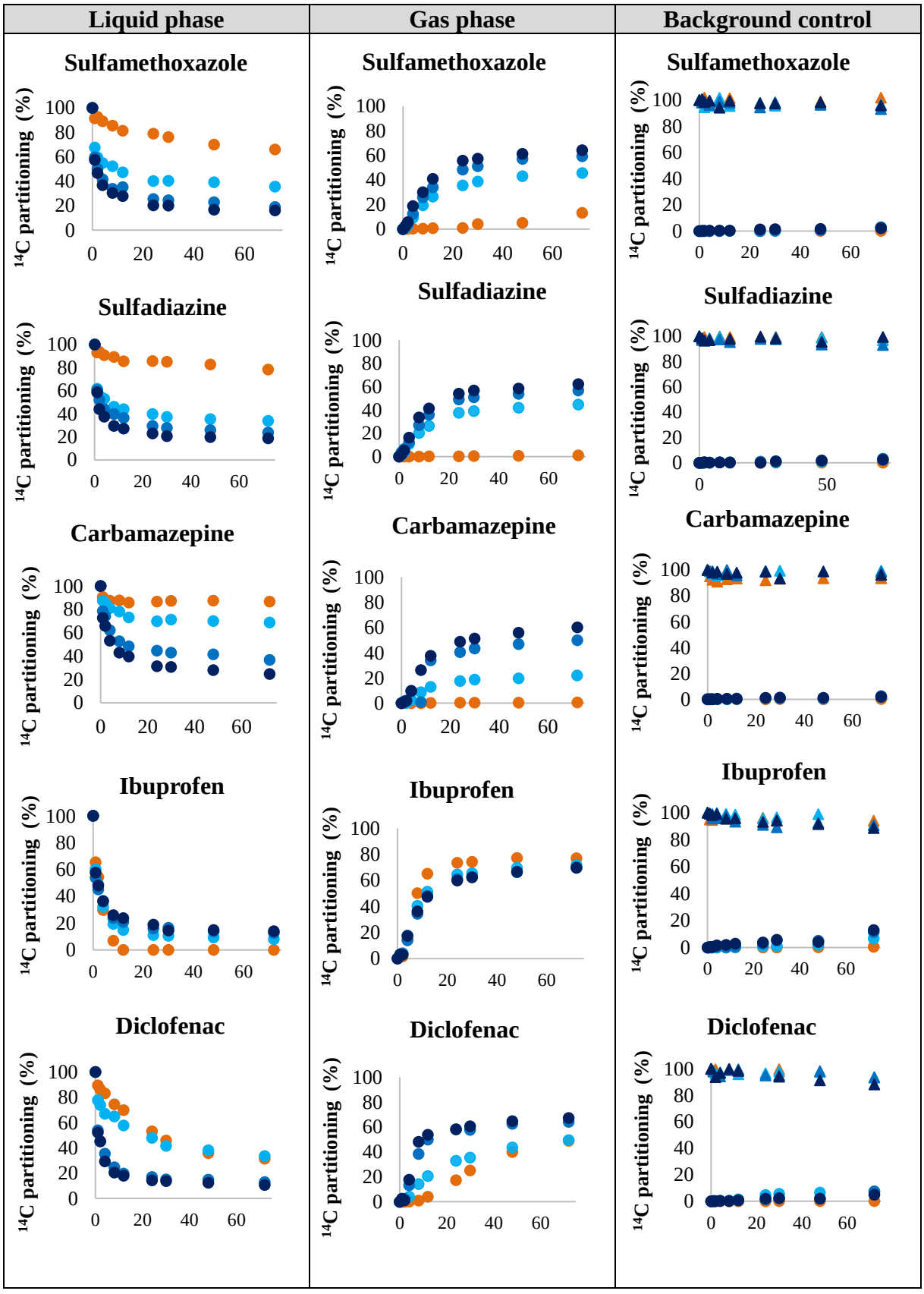
4.3 Biodegradation experiments

The biodegradation experiments were performed under aerobic conditions (Table A.4), with a TS concentration of 1.5-1.6 g/L and a filling ratio of around 23% (Table A.3). DOC and COD continued to decrease during the biological treatment (Table A.4), indicating mineralization of DOM due to biological activity. Measurements of the ion concentrations (Table A.5) further showed a decrease in the ammonium concentration coupled to an increase in the nitrate concentration, indicating nitrification.

The results of the biodegradation experiments are presented in Figure 4.3. As the experiment progressed, a decrease in the radioactivity of the liquid phase and an increase in the radioactivity of the gas phase could be observed, indicating mineralization of the labeled position. For the bottles that were not ozonated (0 mg O_3/mg DOC), the mineralization corresponds to the parent compound, while for the ozonated bottles (0.3, 0.6 and 1 mg O_3/mg DOC) the mineralization corresponds to the transformation products, except in the case of ibuprofen where transformation during ozonation was incomplete. The background controls further showed a limited mineralization, indicating negligible degradation by microorganisms not removed by the initial filtration. The mineralization increased slightly towards the end of the experiment which may correspond to regrowth.

An increased mineralization could be observed for the transformation products of sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac and trimethoprim, indicating an improved biodegradability as compared to the parent compound. For ibuprofen and bisphenol A, the opposite trend could be observed; ozonation resulted in a decreased mineralization, indicating that the transformation products are less biodegradable than the parent compounds. The results further showed a rapid decrease in the radioactivity of the liquid phase at the beginning of the experiment followed by a plateau in the liquid and gas phase at the end of the experiment. This is likely corresponding to the presence of different transformation products, some of which are easily degradable, and some of which are persistent.

In contrast to the ozonation experiments where equilibrium was allowed to be established before sampling, samples were taken continuously throughout the biodegradation experiment and as such a delay in the transfer of $^{14}CO_2$ between the liquid and the gas phase could be observed. The delay is most significant at the beginning of the experiment, as will be discussed in section 4.4. However, even at the later timestamps the decrease in the radioactivity of the liquid phase is not entirely matched by an increase in the gas phase, indicating a loss of radioactivity due to additional removal mechanisms, such as sorption or incorporation into biomass.


Liquid phase
Gas phase
Background control
Sulfamethoxazole
Sulfadiazine
Carbamazepine
Ibuprofen
Diclofenac
^{14}C partitioning (%)
0
20
40
60
80
100
50

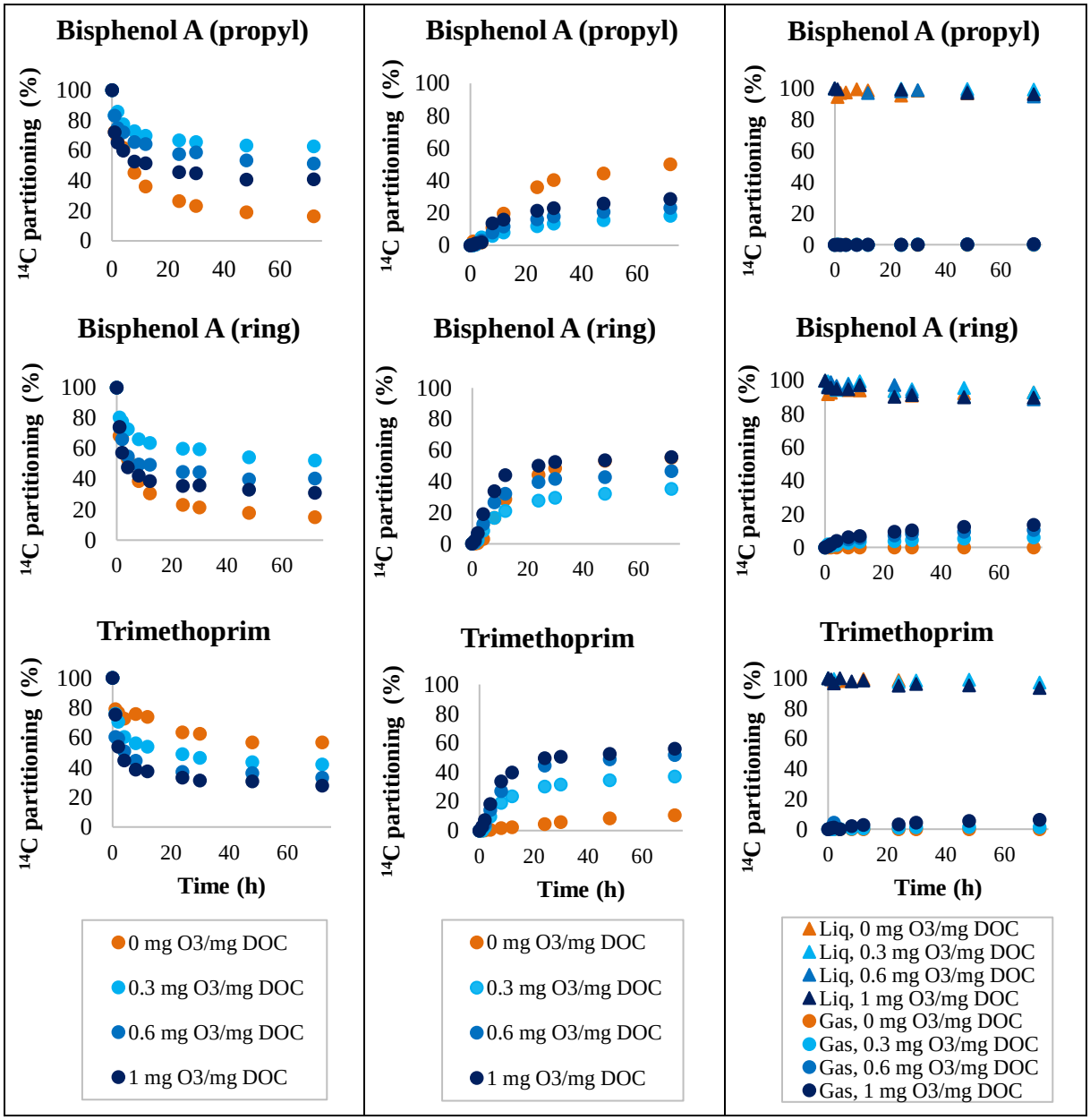


Figure 4.3. ^{14}C partitioning in the liquid and gas phase during the biodegradation experiments. For ibuprofen (0 mg O_3/mg DOC, ≥12h) the measured values in the liquid phase were not considered significant (less than three times the background) and the partitioning was set to zero.

4.3.1 Sulfamethoxazole and sulfadiazine

As indicated by the $^{14}CO_2$ formation at 0 mg O_3/mg DOC, a partial mineralization of the aniline ring in sulfamethoxazole could be observed (13%), while no mineralization of the corresponding position in sulfadiazine could be seen (<1.5%). These results can be compared with a previous study in which the biotransformation of five different sulfonamides was examined. Several transformation products were identified for both sulfamethoxazole and sulfadiazine, of which all retained the aniline moiety, except for one, stemming from sulfamethoxazole and only retaining the isoxazole ring (Achermann *et al.*, 2018).

At an ozone dose of 0.3 mg O_3/mg DOC there was a large increase in the $^{14}CO_2$ formation for both sulfonamides, resulting in a mineralization degree of around 45%. Mineralization of the labeled position continued to increase at higher ozone doses, reaching a maximal value of around 63% at 1 mg O_3/mg DOC. It should, however, be noted that the rate of improvement decreases as the ozone dose is increased and that the difference in mineralization at 0.6 and 1 mg O_3/mg DOC is marginal. It can thus be concluded that ozonation increases the bioavailability of the original aniline moiety in both sulfamethoxazole and sulfadiazine, and that this effect is significant even at lower ozone doses.

As the fraction of ^{14}C activity that remains in the liquid phase after ozonation will be part of different transformation products, this shows a general trend where the transformation products retaining the labeled position exhibit an increased biodegradability, as compared to the parent compound. It does not, however, mean that all transformation products will be more biodegradable, it has for instance been shown that the transformation product nitro-sulfamethoxazole is stable during biological treatment (Gulde *et al*., 2021a). It should also be noted that transformation due to biological activity may occur, resulting in the formation of additional transformation products that can either be further degraded or prove to be persistent.

Similarly to the trend observed during ozonation, sulfamethoxazole and sulfadiazine exhibited nearly identical behaviors during biological treatment. This can be explained by the fact that both compounds share similar structures and are labeled in the same position. Considering that the labeled aniline moiety is common to all sulfonamide antibiotics (Walsh, 2003), this may also indicate a more general trend and may further suggest elimination of the biological function. All sulfonamide antibiotics are derivatives of *p*-aminobenzenesulfonamide (Figure 4.4), with the antibacterial properties stemming from antagonistic competition with *p*-aminobenzoic acid (Figure 4.4), a structural analog of *p*-aminobenzenesulfonamide, for an enzyme involved in the bacterial synthesis of a folic acid precursor (Walsh, 2003). As such, even if the parts of sulfamethoxazole and sulfadiazine that were not labeled were to remain unchanged throughout ozonation and biological treatment, the high mineralization observed for the aniline moiety would suggest that the antibacterial properties have been largely lost.

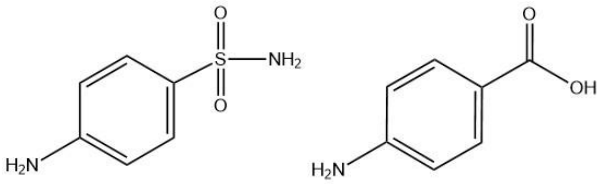


Figure 4.4. The chemical structure of p-aminobenzenesulfonamide (left) and p-aminobenzoic acid (right). Figure made using Chemdraw.

4.3.2 Carbamazepine

No $^{14}CO_2$ formation could be observed for carbamazepine at 0 mg O_3/mg DOC, consistent with previous studies identifying the compound as persistent during biological treatment (Bourgin *et al.*, 2018; Joss *et al*., 2005; Margot *et al*., 2013). Mineralization could however be observed for the ozonated bottles, indicating an increased bioavailability of the labeled position upon ozonation. The mineralization was relatively low at 0.3 mg O_3/mg DOC (22%), but increased with increasing doses (50 and 60% at 0.6 and 1 mg O_3/mg DOC respectively). As could be seen for sulfamethoxazole and sulfadiazine, the increase in mineralization did not exhibit a linear behavior. Increasing the dose from 0.3 to 0.6 mg O_3/mg DOC resulted in a significantly higher $^{14}CO_2$ formation but increasing the dose to 1 mg O_3/mg DOC only had a limited effect.

The biodegradability of the transformation products of carbamazepine have been examined in previous studies (Gulde *et al*., 2021a; Hübner *et al*., 2014). Of the four major transformation products identified by Hübner *et al*. (2014), all retaining the labeled position, three were shown to be efficiently biotransformed while the fourth proved persistent. These results were partially corroborated by Gulde *et al*. (2021a), who also identified an additional transformation product, retaining the labeled position and exhibiting an enhanced biodegradability. In addition to the biotransformation observed in these studies it can thus be concluded that a significant mineralization of the original carbonyl carbon also takes place when biological treatment is applied after ozonation.

4.3.3 Diclofenac and ibuprofen

Diclofenac and ibuprofen are labeled in the same position but unlike sulfamethoxazole and sulfadiazine, the two compounds exhibit differing behaviors during the biological treatment. A substantial mineralization of the labeled position could be observed for ibuprofen at 0 mg O_3/mg DOC (77%), indicating that the compound is readily degraded during biological treatment, in accordance with previous studies (Clara *et al.*, 2005; Joss *et al*., 2005). Applying ozonation before the biological treatment slightly decreased the degree of mineralization, with no great distinction observed for the different ozone doses. It should be noted that transformation of the parent compound was incomplete during ozonation at 0.3 and 0.6 mg O_3/mg DOC, and that the mineralization observed during the biological treatment will thus stem from the parent compound as well as the transformation products.

For diclofenac, mineralization of the labeled position at 0 mg O_3/mg DOC was lower than for ibuprofen, but still significant at 49%. Applying an ozone dose of 0.3 mg O_3/mg DOC prior to biological treatment did not increase the final degree of mineralization. It did, however, increase the mineralization rate, as evidenced by the higher $^{14}CO_2$ formation at the beginning of the experiment. At 0.6 mg O_3/mg DOC the mineralization rate as well as the final degree of mineralization (64%) increased. Further increasing the dose from 0.6 to 1 mg O_3/mg DOC resulted in no significant improvements. Considering that the residence time of a biological posttreatment step would be significantly lower than 72 hours in a practical application, the increased mineralization rate at 0.3 mg O_3/mg DOC could prove to have an effect. However, to achieve a significant improvement it is likely that a dose of 0.6 mg O_3/mg DOC is required. In all, it can be concluded that the transformation of ibuprofen results in a slightly decreased bioavailability of the labeled position and that the decrease is largely independent of the ozone dose, while the transformation of diclofenac increases the bioavailability and that this effect is most significant at 0.6 mg O_3/mg DOC.

4.3.4 Bisphenol A

A substantial $^{14}CO_2$ formation could be seen for both labeled positions of bisphenol A at 0 mg O_3/mg DOC (50 and 55% for the propyl- and the ring-label respectively). As the two labels cover most of the molecule this indicates a significant mineralization, not only of the labeled positions, but of the molecule as a whole. The high biodegradability of the parent compound is consistent with previous studies where bisphenol A has been found to be well removed during biological treatment (Gardner *et al*., 2013; Margot *et al*., 2013). The $^{14}CO_2$ formation showed a significant decrease as ozonation was applied, dropping to 18 and 35% for the propyl- and the ring-label respectively at 0.3 mg O_3/ mg DOC. However, as the ozone dose was increased the $^{14}CO_2$ formation increased as well. At 1 mg O_3/ mg DOC, mineralization of the propyl-labeled position reached a final value of 29%, while mineralization of the ring-labeled position returned to the same level as for the parent compound. As such it can be concluded that bisphenol A is

more biodegradable than its transformation products and further that the primary transformation products exhibit the lowest biodegradability, while the degradability is increased for later generation transformation products.

4.3.5 Trimethoprim

A slight $^{14}CO_2$ formation (~10%) could be observed for the labeled position at 0 mg O_3/mg DOC, indicating a limited biodegradability of the parent compound. This is consistent with previous studies that have shown that trimethoprim largely remains after biological treatment (Göbel *et al.*, 2007; Göbel *et al.*, 2005; Lindberg *et al.*, 2005). Mineralization of the labeled position was increased significantly at an ozone dose of 0.3 mg O_3/mg DOC, reaching a final value of 37%. Applying higher doses resulted in a larger $^{14}CO_2$ formation, the mineralization reached 52 and 56% at 0.6 and 1 mg O_3/mg DOC respectively. Similar to the case of sulfamethoxazole and sulfadiazine, it can thus be observed that the greatest increase in mineralization occured at the lowest ozone dose, with the difference in mineralization decreasing at increasing doses. As such it can be concluded that transformation of trimethoprim increases the bioavailability of the labeled position, and that this effect can be obtained even at low ozone doses.

4.4 Mass balance

The mass balance was calculated for both the ozonation and the biodegradation experiments, the results of which are presented in Figure 4.5 and 4.6. For the ozonation experiments, the sum of the radioactivity in the liquid and gas phase after ozonation was found to closely approach the initial activity. In general, >95% of the initial activity could be recovered. The high recovery indicates that the $^{14}CO_2$ that was formed during the experiments was efficiently transferred from the liquid phase to the CO_2-trap and consequently that the setup was successful. It should be noted however, that sampling was performed after about 38 hours, allowing sufficient time for the transfer of $^{14}CO_2$.

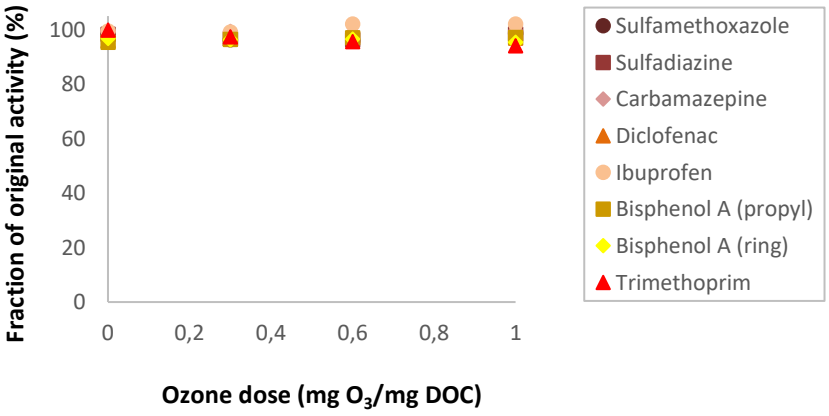


Figure 4.5. The fraction of the initial radioactivity that could be recovered in the liquid and gas phase after ozonation.

For the biodegradation experiments a significant loss of radioactivity could be observed for all ozone doses and all examined micropollutants (Figure 4.6). This trend has been observed in a previous study in which the biodegradability of ^{14}C-labeled micropollutants was examined using a similar setup (Betsholtz *et al.*, 2021). In another study, also using a similar setup, control experiments further showed that there is a delay in the capture of $^{14}CO_2$ by the CO_2-trap and that approximately 24 hours is needed to achieve a complete transfer (>95%) (Betsholtz *et al.*,

2022). This can likely explain the large discrepancy in ^{14}C-activity measured for the liquid and gas phase at the beginning of the experiment. As evidenced by the ^{14}C-partitioning in the liquid phase during biological treatment (Figure 4.3), most of the mineralization occurs within the first few hours. This is also the timeframe during which the sampling is most frequent and as such, the delayed transfer will be most visible. The large initial spike in missing radioactivity could not be observed for the organic micropollutants where no or limited mineralization of the parent compound occurred during biological treatment (sulfamethoxazole, sulfadiazine, carbamazepine and trimethoprim), further supporting the theory that delayed transfer of $^{14}CO_2$ is responsible.

However, even after the initial spike, a significant fraction of the radioactivity remains unaccounted for. As the mineralization is slow (figure 4.3) and the time between sampling is longer at this stage, the loss cannot be explained by a delayed transfer of $^{14}CO_2$. It is instead likely that other removal mechanisms such as volatilization, sorption or incorporation into the biomass are responsible. As previously discussed, removal of organic micropollutants can occur through volatilization and sorption to sludge (Falås *et al.*, 2016), and it would be reasonable to assume that the same applies to their transformation products. Although ^{14}C is present in quantities far below the non-radioactive isotope, it would further be possible that a small fraction of ^{14}C is taken up by the biomass and used as a carbon source. As the biomass is never tested, any fraction that is sorbed or incorporated would go undetected. Volatilization would also go undetected as the gas phase is only tested for $^{14}CO_2$. ^{14}C incorporated into other structures would not dissolve in the CO_2-trap and consequently not be visible. Considering that the mass balance is nearly complete for the ozonation experiments, volatilization of the parent compound and the ozonation transformation products should be negligible. It is, however, possible that volatile transformation products form during the biological treatment.

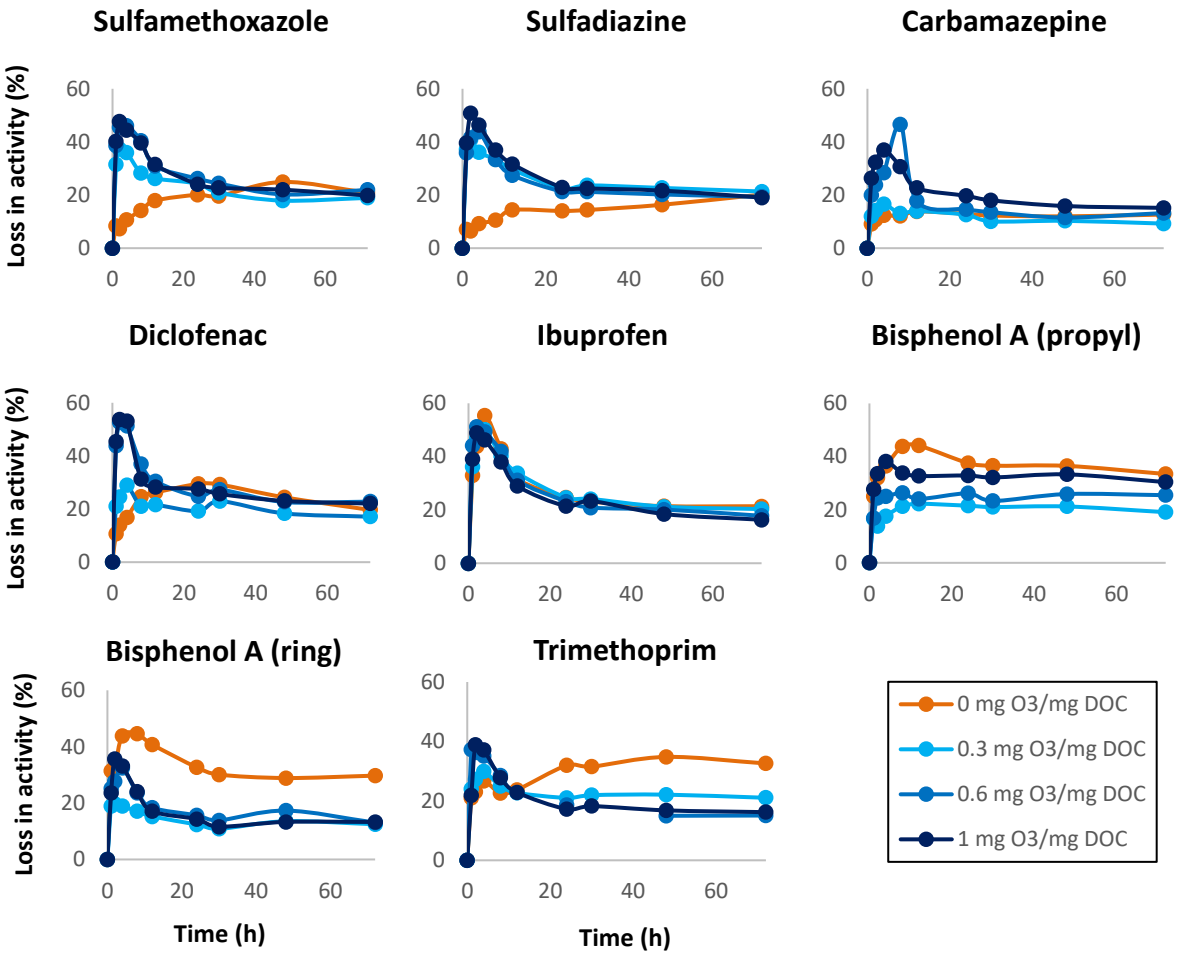


Figure 4.6. The fraction of initial activity that could not be recovered in either the liquid or the gas phase during the biodegradation experiments.

4.5 Activated carbon as a posttreatment to ozonation

As previously discussed, a biological step is not the only option as a posttreatment to ozonation. Solutions based on activated carbon, either in the form of granular activated carbon (GAC) (Edefell *et al*., 2021; Hollender *et al*., 2009) or powdered activated carbon (PAC) (Gulde *et al*., 2021b), are also being explored. Several advantages of such an approach have been suggested, where an increased removal of a larger variety of organic micropollutants is presented as a major benefit. As ozonation and adsorption to activated carbon work by different mechanisms, they are also effective against different types of organic micropollutants (Margot *et al*., 2013) and combining these two methods may thus be expected to result in a greater overall removal. The potential for removing ozonation transformation products in such a setup is, however, largely unknown.

In a previous study, in which a largely overlapping set of ^{14}C-labeled micropollutants were used to study the adsorption of organic micropollutants and their ozonation transformation products to PAC, a substantial decline in the adsorbability of the transformation products, as compared to the parent compound, was observed. It was thus concluded that adsorption to PAC may not be a viable method to achieve a wide removal of ozonation transformation products (Betsholtz *et al*., 2022). The results obtained in this study conversely show a general increase in the

biodegradability of the transformation products, as compared to the parent compound. A decreased adsorbability, but an increased biodegradability, may thus be expected for many organic micropollutants upon ozonation. This would implicate that biological treatments such as MBBRs or sand filters are suitable to employ after ozonation but would also imply that GAC may be a suitable posttreatment. The surface of GAC particles will inevitably become colonized by bacteria and other microorganisms over time (Weber *et al.*, 1978), and as shown by experiments using organic micropollutants, removal can thus occur through two mechanisms: adsorption as well as biodegradation (Betsholtz *et al.*, 2021). The results obtained in this study would thus serve to highlight the biological function of a mature GAC-filter and further studies on the removal of ozonation transformation products using GAC would be interesting.

5 Conclusion

Through the use of ^{14}C-labeling, the transformation and mineralization of seven organic micropollutants and their transformation products could be studied during ozonation and biological treatment. The main conclusions that could be drawn from the results are listed below.

- A complete transformation at all ozone doses (0.3, 0.6 and 1 mg O_3/mg DOC) was achieved for all examined micropollutants, except for ibuprofen, for which a complete transformation was only achieved at the highest ozone dose. A partial mineralization of the labeled position, up to 55% at 1 mg O_3/mg DOC, could further be observed for all examined micropollutants, except for propyl-labeled bisphenol A. The mineralization was found to increase with increasing ozone doses and could be observed for micropollutants where the labeled position is the main reactive site, such as sulfamethoxazole, as well as for those where the labeled position is not associated with a high ozone reactivity, such as diclofenac and ibuprofen.
- The transformation products of sulfamethoxazole, sulfadiazine, carbamazepine, diclofenac, and trimethoprim showed an increased mineralization of the labeled position during biological treatment, as compared to the parent compound. The mineralization increased with increasing ozone doses, the most significant effect could however be observed at 0.3-0.6 mg O_3/mg DOC, indicating that lower ozone doses are sufficient to increase the degradation rate during biological treatment.
- The transformation products of bisphenol A and ibuprofen showed a decreased mineralization of the labeled position as compared to the parent compound. For ibuprofen the decrease was marginal and showed no strong correlation to the ozone dose. For bisphenol A the decrease was more significant, especially for the propyl-label, and was found to be greatest at lower ozone doses. However, in the case of both bisphenol A and ibuprofen, the parent compound has been documented to exhibit a high biodegradability and a substantial mineralization of the labeled positions could be observed during biological treatment. As ozonation is applied after the biological treatment in practical applications, the concentration of these compounds will consequently be greatly reduced as it reaches the ozonation unit and the decreased biodegradability observed for the transformation products should thus be of minor importance.

A general trend could thus be established where most of the transformation products showed an increased biodegradability, as compared to the parent compound, indicating that biological posttreatment is largely effective in removing the transformation products that are formed upon ozonation of organic micropollutants.

6 Future studies

Three areas where further studies would be interesting have been identified and are presented below.

- With the exception of bisphenol A, the organic micropollutants studied within the scope of this project were labeled in one position. By labeling in several positions it would be possible to get a wider understanding of the extent to which different groups are mineralized during ozonation and subsequent biological treatment, and to aid in the elucidation of transformation pathways. To further differentiate by reaction pathways initiated by ozone and hydroxyl radicals respectively, ozonation experiments could be performed in the presence of a hydroxyl radical scavenger, such as t-BuOH.
- In this work biological degradation was studied in a rather wide timeframe. However, in practical applications such extended residence times would not be feasible. To put the results obtained in this study in a more realistic perspective, further studies could be performed where residence times more consistent with real applications are employed, and where the sampling interval is in the range of minutes rather than hours. As evidenced by the large deviations in the mass balancing at the beginning of the biodegradation experiments, the current setup would not prove useful in such studies. Instead, a decoupling of the formation and transfer of $^{14}CO_2$ between phases is needed in order to allow the appropriate time required for the respective process. This could be done by performing the biodegradation experiments in a continuous mode, collecting the effluent at appropriate intervals and then transferring it to bottles equipped with a CO_2-trap.
- As discussed in section 4.5, many transformation products may be expected to exhibit a decreased adsorbability but an increased biodegradability. As shown by Betsholtz *et al.* (2021), it is possible to use ^{14}C-labeled micropollutants to differentiate between adsorption and biological degradation in a GAC-filter. As that study focused on organic micropollutants, not their transformation products, it would be interesting to perform an additional study where ozonation is applied prior to the GAC-filter. This would allow for the removal of ozonation transformation products, through a combination of biodegradation and adsorption, to be studied.

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Appendix

Table A.1. The initial concentrations of the non-radiolabeled micropollutants in experiment 1 (E1), experiment 2 (E2) and experiment 3 (E3).

Compound	**Concentration E1 (ng/L)**	**Concentration E2 (ng/L)**	**Concentration E3 (ng/L)**
Atenolol	503	699	667
Azithromycin	40	63	85
Benzotriazole	415	1197	1228
Bisphenol A	10201	8830	9935
Carbamazepine	8777	9908	9659
Ciprofloxacin	52	36	54
Citalopram	18	40	41
Clarithromycin	59	33	39
Diclofenac	8632	9908	9658
Erythromycin	101	41	46
Estrone (E1)	6	15	1
Fluconazole	56	82	78
Furosemide	331	577	734
Hydrochlorothiazide	353	563	543
Ibuprofen	13729	11752	11882
Imidacloprid	3	4	3
Irbesartan	43	53	59
Ketoconazole	2829	2921	5866
Losartan	2111	2658	2845
Metoprolol	998	1330	1299
Naproxen	8213	10175	8887
Oxazepame	155	196	196
PFOA	4	6	4
PFOS	8	8	9
Propranolol	5337	6280	7118
Sertraline	14	24	41
Sulfadiazine	760	1230	4326
Sulfamethoxazole	1621	2597	6859
Tramadol	526	625	622
Trimethoprim	6405	7891	7748
Venlafaxine	263	370	371
Zolpidem	1	1	2

*Table A.2. The results of the ozone concentration measurements using both the direct method (DM) and the indigo method (IM). All concentrations were measured in triplicates and are presented here as the mean value, except for the one marked with *, for which only two measurements were made due to an error.*

Measurement	**$C_{O3\ stock}$ DM (mg/L)**	**$C_{O3\ stock}$ IM (mg/L)**	**Percentual difference**
Experiment 1			
1	68.8* (SD = 1.3)	70.9 (SD = 2.2)	3.0
2	77.3 (SD = 2.4)	85.6 (SD = 2.3)	10.2
3	80.6 (SD = 2.7)	87.9 (SD = 0.3)	8.7
4	76.3 (SD = 1.5)	83.3 (SD = 1.1)	8.8
Experiment 2			
1	58.3 (SD = 4.5)	65.6 (SD = 2.1)	11.8
2	54.5 (SD = 0.8)	70.1 (SD = 1.1)	25.0
3	72.1 (SD = 3.9)	75.2 (SD = 1.7)	4.2
4	76.0 (SD = 0.5)	78.6 (SD = 2.2)	3.4
Experiment 3			
1	70.4 (SD = 0.3)	70.6 (SD = 1.2)	0.3
2	85.6 (SD = 0.4)	81.5 (SD = 2.6)	4.9
3	93.2 (SD = 1.7)	96.1 (SD = 1.7)	3.1
4	96.3 (SD = 1.5)	93.9 (SD = 1.2)	2.5

Table A.3. The TS concentration and filling ratio used during the biodegradation experiments.

Experiment	**TS (g/L)**	**Filling ratio (%)**
1	1.6 (SD = 0.04)	~23
2	1.5 (SD = 0.03)	~23
3	1.5 (SD = 0.1)	~23

*Table A.4. Water quality parameters measured for the raw water (RW), after ozonation (AO) and after biological treatment (AB). The number denotes the ozone dose (in mg O_3/mg DOC). For the values marked with * triplicate measurements were made and the presented value is the mean.*

Sample	**DOC (mg /L)**	**COD (mg/L)**	**UVA_{254} (m^{-1})**	**pH**	**O_2 (mg/L)**
Experiment 1					
RW	14.6*	33.6	19.9*	7.0	-
AO 0	14.2	35.2	21.2	7.3	>8.0
AO 0.3	14.3	35.6	12.2	7.3	>8.0
AO 0.6	12.9	29.9	8.9	7.3	>8.0
AO 1	14.4	32.6	8.2	7.3	>8.0
AB 0	14.9	32.7	-	7.0	5.8
AB 0.3	12.7	30.5	-	7.0	5.6
AB 0.6	12.5	26.5	-	7.0	6.0
AB 1	13.4	29.6	-	7.0	6.5
Experiment 2					
RW	14.6*	33.6	20.7*	7.0	-
AO 0	13.7	31.5	22.4	7.4	>8.0
AO 0.3	14.6	30.6	16.3	7.4	>8.0
AO 0.6	13.5	27.2	13.1	7.4	>8.0
AO 1	13.5	24.6	9.1	7.4	>8.0
AB 0	13.3	30	-	6.8	5.9
AB 0.3	13.8	27.3	-	6.8	5.8
AB 0.6	12.4	23.1	-	7.0	5.4
AB 1	11.2	20.1	-	6.8	5.4
Experiment 3					
RW	11.6*	30.6*	20.6	7.0	-
AO 0	11.8	32.5	19.2	8.3	>8.0
AO 0.3	10.3	27.3	11.6	8.5	>8.0
AO 0.6	9.9	28.1	9.1	8.5	>8.0
AO 1	10.6	25.7	7.6	8.5	>8.0
AB 0	10.3	28.2	-	6.1	6.2
AB 0.3	8.6	24.1	-	6.0	6.6
AB 0.6	8.6	24.2	-	6.0	6.7
AB 1	7.5	20.9	-	6.4	6.8

Table A.5. Water quality parameters measured for the raw water (RW), after ozonation (AO) and after biological treatment (AB). The number denotes the ozone dose (in mg O_3/mg DOC). Triplicate measurements were made in all cases and the values presented are the mean values. For the values marked with < the concentration was below the limit of detection.

Sample	**NO_2^--N (mg N/L)**	**NO_3^--N (mg N/L)**	**NH_4^+-N (mg N/L)**	**Br^- (mg/L)**
Experiment 1				
RW	0.16	6.70	6.35	<0.1
AO 0	0.17	6.72	6.36	-
AO 0.3	<0.05	6.97	6.38	-
AO 0.6	<0.05	7.05	6.47	-
AO 1	<0.05	7.08	6.42	-
AB 0	<0.05	23.67	<0.1	-
AB 0.3	<0.05	25.10	<0.1	-
AB 0.6	<0.05	25.68	<0.1	-
AB 1	<0.05	26.37	<0.1	-
Experiment 2				
RW	0.41	9.60	14.67	<0.1
AO 0	0.41	9.75	14.68	-
AO 0.3	<0.05	10.23	14.74	-
AO 0.6	<0.05	10.28	14.77	-
AO 1	<0.05	10.36	14.81	-
AB 0	<0.05	33.27	<0.1	-
AB 0.3	<0.05	34.25	<0.1	-
AB 0.6	<0.05	35.34	<0.1	-
AB 1	<0.05	36.14	<0.1	-
Experiment 3				
RW	0.08	13.60	11.24	<0.1
AO 0	0.08	13.40	10.49	-
AO 0.3	<0.05	13.57	10.66	-
AO 0.6	<0.05	13.54	10.69	-
AO 1	<0.05	13.66	10.84	-
AB 0	<0.05	29.18	2.43	-
AB 0.3	<0.05	31.11	1.65	-
AB 0.6	<0.05	30.67	2.19	-
AB 1	<0.05	34.37	0.12	-