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Monitoring transformation product formation in the drinking water treatments rapid sand filtration and ozonation

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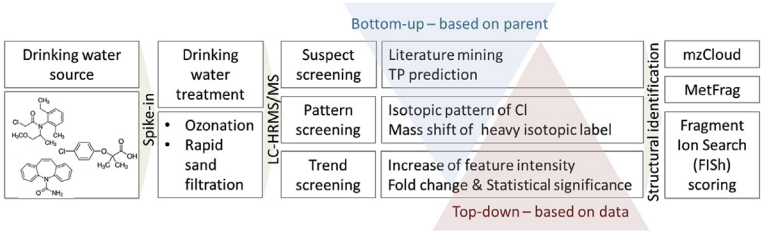
HIGHLIGHTS

- Developed workflow to monitor formation of drinking water treatment specific TPs.
- Compound transformation during ozonation and rapid sand filtration in drinking water.
- TP tracing using isotopic patterns of halogenated and isotopically labeled parents.
- Semi-automatic TP detection based on statistical testing and trend filters.
- Identification with suspect lists of *in silico* predicted and literature mined TPs.

GRAPHICAL ABSTRACT



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ABSTRACT

Transformation products (TPs) can be formed from organic micropollutants in the water cycle through both biological and technological processes. Despite the TPs' potentially altered toxicity compared to their parent compounds, transformation processes are not routinely monitored, and in particular those induced by drinking water treatment remain elusive. This lack of information is mainly due to the technical challenges in analyzing TPs, which are often unknown compounds occurring in low concentrations. Their analysis requires sophisticated analytical techniques such as non-target screening (NTS) based on high-resolution tandem mass spectrometry (HRMS/MS) methods combined with novel data analysis approaches. Here, we addressed the challenges of TP analysis and the scarcity of TP research concerning studies in drinking water. We performed lab-scale experiments to monitor TP formation of three organic micropollutants prevalent in drinking water sources, i.e. carbamazepine, clofibric acid and metolachlor, during rapid sand filtration and ozonation, two readily applied biotic and abiotic drinking water treatments, respectively. To facilitate TP identification in the NTS data, halogenated and/or isotopically labeled parent compounds were used, revealing potential TPs through their isotopic patterns. The experimental results showed that degradation of the parent compounds and TP formation were treatment and compound specific. *In silico* TP prediction and literature mining enabled suspect screening of the non-target data and thereby significantly enhanced TP identification. Overall, the developed workflow enables an efficient and more comprehensive assessment of drinking water quality changes during water treatment.

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Abbreviations

HRMS/MS	high-resolution tandem mass spectrometry
log2FC	log2 fold change
NTS	non-target screening
RSF	rapid sand filtration
RT	retention time
TP	transformation product

1. Introduction

1.1. Drinking water treatment induced transformation of organic micropollutants

Organic micropollutants can enter the aquatic environment manifold, through routes such as atmospheric deposition, waste water effluent, spills, and leakage, and consequently contaminate drinking water sources (Kolpin et al., 2002; Oenema and Pietrzak, 2002; Stackelberg et al., 2004). Drinking water treatment aims at removing these chemicals. Therefore, treatment processes are optimized and validated using compound removal rates (Guzzella et al., 2002; Tang et al., 2012; Wols et al., 2013). However, instead of complete mineralization, transformation of parent compounds can occur during treatment by (a combination of) biotic and abiotic processes such as hydrolysis, photolysis, oxidation, reductive transformation, elimination and substitution (Atkinson et al., 1999; Bletsou et al., 2015). The resulting transformation products can differ significantly in functionality and polarity from their parent compounds, and thereby removal efficiencies during drinking water treatment can be affected (Fenner et al., 2013). Moreover, TPs can potentially exhibit increased toxicity compared to their parent compounds (Escher and Fenner, 2011). Despite this having been described over thirty years ago in waste water treatment (Giger et al., 1984), current drinking water monitoring, toxicity testing, and risk assessment still mainly focus on parent compounds, while TPs are rarely assessed. However, from a health risk perspective, not the removal of a compound but the reduction of adverse health effects is the relevant drinking water parameter. It is therefore crucial to monitor the transformation processes during drinking water treatment and the resulting TPs.

1.2. Strategies for TP identification

TP monitoring remains challenging as most TPs are so-called “unknown unknowns” that is compounds of which the structure is unknown and which are not present in chemical databases. Identifying these compounds requires non-target screening (NTS) high-resolution tandem mass spectrometry (HR MS/MS) methods combined with novel data analysis approaches (Schymanski et al., 2015). Greatly simplified, there are two complementary strategies to monitor TP formation and identify TPs from NTS data. They both distinguish themselves from more general NTS strategies by taking advantage of the relationships between a parent compound and its TPs. In the case of a known parent compound, in a so-called bottom-up approach, expected TPs of a given parent compound are screened for via suspect screening. The TP suspect lists used can be compiled of previously detected TPs found through literature and data base mining, and TPs that are *in silico* predicted based on metabolic logic or transformation rules (Schollee et al., 2015; Wicker et al., 2016; Lee et al., 2017). Alternatively, in a so-called top-down approach, transformation products are identified through the statistical analysis of data patterns, such as changes of peak intensities between samples and mass shifts indicative of transformation processes (Li et al., 2017; Schollee et al., 2017) and patterns reflecting structural −fragmentation relationships (Schollee et al., 2017). In addition, the use of stable isotope labeled parent compounds spiked to experimental systems can facilitate transformation product identification (Hegeman et al., 2007; Giavalisco et al., 2009; Kolkman et al., 2015). A labeled parent compound will be transformed into a labeled TP, given that the labeled residue is still present in the TP. As both labeled and unlabeled compounds exhibit the same physico-chemical properties, they are not separated by chromatography, but can be distinguished by their mass difference when mixed in one sample or analyzed sequentially. While the use of isotopic labelling is limited to experimental systems, isotopic pattern filters can be used to identify TPs based on the distinct isotopic patterns of their parent compounds for chlorine and bromine containing compounds also in *in situ* (Nagao et al., 2014).

1.3. Applied workflow for TP monitoring in drinking water treatment

Here, we combined these strategies to monitor transformation processes in drinking water treatment. The TPs of the organic micropollutants metolachlor, clofibric acid and carbamazepine were identified in a lab-scale experiment under well-defined conditions during the biotic and abiotic drinking water treatments rapid sand filtration and ozonation, respectively. By providing 1) before and after treatment samples, data analysis could focus on the differences between the two using trend and statistical analyses, and disregard all information that was the same in both, 2) known parent compounds, potential TPs could be predicted based on literature and models, and consequently a suspect screening of the data against a list of predicted TPs could be performed, 3) the possibility to use high concentrations of spiked-in parent compounds, also TPs formed at low rates could be detected, 4) the presence of the halogen chlorine, the distinct isotopic pattern of the halogenated parent compound and TPs supported screening approaches unless dehalogenation occurred, and 5) the inclusion of isotopically labeled parent compounds, labeled TPs could readily be detected based on the mass shift of the label. To our knowledge, this is the first time that these methods have been applied together to identify TPs formed in drinking water treatment.

2. Materials and methods

2.1. Chemicals

All solvents used were of analytical grade quality. Acetonitrile (ultra-gradient HPLC grade) was purchased from Avantor Performance Materials B.V. (Deventer, the Netherlands). Formic acid, FA (50% in water) was obtained from Fluka Analytical (Sigma-Aldrich, Steinheim, Germany). The ultrapure water was obtained by purifying demineralized water in an Elga Purelab Chorus ultrapure water system (High Wycombe, UK). The internal standards atrazine-d5 and bentazon-d6 were purchased from CDN isotopes (Pointe-Claire, Canada) and LGC Standards (Wesen, Germany), respectively.

2.2. Parent compounds: incorporating halogens and labels

Parent compounds were selected based on their occurrence in drinking water sources, and the availability of an isotopically labeled parent or the presence of a halogen. The anti-epileptic and neuropathic pain medication carbamazepine (Supplementary Table 1a) is one of the pharmaceuticals most frequently detected

in the aqueous environment (Ternes, 1998). It is persistent in sand filtration (Ternes et al., 2002), but readily reacts with ozone and ozone TPs are well studied (McDowell et al., 2005). In addition, an isotopically labeled standard carbamazepine-(carboxamide-13C, 15N) (Table 1, 1b), which has a 13C and a 15N incorporated at atoms that remain in the known ozone TPs of carbamazepine, is available (McDowell et al., 2005). Clofibric acid (Tables 1 and 2) and metolachlor (Tables 1 and 3) are both herbicides that have been detected in groundwater and surface waters (Chesters et al., 1989; Masse et al., 1994; Ritter et al., 1994; Scheytt et al., 2001; Tixier et al., 2003). Clofibric acid is also a human metabolite of the cholesterol-lowering pharmaceutical clofibrate. It is a medium biodegradable pollutant (Zearley and Summers, 2012), with a number of known biotic (Kosjek et al., 2009b; Salgado et al., 2012; Brox et al., 2016) and abiotic (Doll and Frimmel, 2004) TPs. Metolachlor is susceptible to both biotic and abiotic degradation (Liu et al., 1991; Stamper and Tuovinen, 1998; Steen et al., 2000; Mersie et al., 2004; Sakkas et al., 2004; Warner and Morrow, 2007; Orge et al., 2017), and its TPs have been shown to be more toxic than the parent compound (Osano et al., 2002; Huntscha et al., 2008). Both compounds exhibit a distinct isotopic pattern due to the presence of chlorine atoms. Parent compounds were purchased from Sigma-Aldrich (Steinheim, Germany).

2.3. Drinking water treatment

Emphasis was placed on the development of a generic approach to monitor TPs that could be implemented across other drinking water treatments. As environmental TPs can be formed by either abiotic or biotic processes, a treatment representing each process was applied. The selected treatment can then serve as a model for other treatments with a similar transformation process.

2.3.1. Rapid sand filtration (RSF) as a model for biotransformation

RSF is implemented in almost every drinking water treatment plant in the world. Its main purpose is to remove particles, iron and manganese, ammonia, and part of the organic matter (Craft and Eichholz, 1970), but it also facilitates the biological degradation of a number of organic micropollutants (Zearley and Summers, 2012; Hijnen et al., 2016; Bertelkamp et al., 2017), and can lead to microbial biotransformation (Brezina et al., 2015).

RSF was simulated in laboratory-scale columns with sand obtained from a used RSF filter at the pre-treatment plant of Waternet (WRK, Nieuwegein, The Netherlands). In brief, the sand was collected, mixed and transported in a closed PE bucket at 4 °C. The sand was flushed with drinking water (KWR, Nieuwegein) prior to transfer of the sand slurry to two glass columns (di = 3,5 cm, height = 100 cm) to a final bed height of 80 cm, i.e. 770 mL. Columns were backwashed for adequate packing and air removal. Each column was fed from a 550L stainless steel tank filled with influent water from the RSF filters (WRK, Nieuwegein, The Netherlands), pre-filtered through 10 μm cartridge filters. Parent compounds were added to a final spike-in concentration of 10 μg/L, which is roughly one to two orders of magnitude higher than environmental concentrations often found in surface waters (Loos et al., 2009; Monteiro and Boxall, 2010; Schreiner et al., 2016). The water was stirred mechanically for 1 h. A flow of 4.8 L/h (velocity of 5.0 m/h) was set for both columns. Influent and effluent samples were taken at after 8 h and 96 h (4 days) of experiments, which corresponds to 437 and 5239 treated bed volumes, respectively. Transformation experiments were run with a single parent compound at a time. In the two column set-up, metolachlor and clofibric acid experiments were performed in parallel for five days, followed by seven days of column flushing with WRK water prior to labeled and unlabeled carbamazepine experiments, again performed in parallel for five days.

Table 1
Change of parent compound abundance during drinking water treatment, expressed in log2FC units between after/before treatment. Grey: not significant, Yellow: significant difference, change below cut-off (−0.25 < log2FC < 0.25. p < 0.05), red: significant increase (log2FC > 0.25. p < 0.05), Green: significant decrease (log2FC < -0.25 p < 0.05).

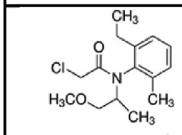
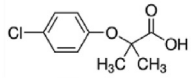
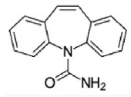
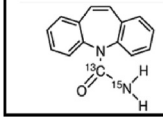
Parent compound structure	Parent compound name (ionization mode)	Feature sand filtration	Feature ozonation	Sand filtration (log2FC Effluent / influent)		Ozonation (log2FC O_3 / no O_3)			
						10 μg/L spike-in		100 μg/L spike-in	
		(M [Da] / RT [min])	(M [Da] / RT [min])	8h	4d	low O_3	high O_3	low O_3	high O_3
	Metolachlor (+)	283.1334 / 18.91	283.1335 / 18.90	0.25	-0.35	-5.08	-5.69	-11.33	-12.30
	Clofibric acid (-)	214.0394 / 15.86	214.0396 / 15.87	-0.24	-0.20	-6.56	-12.59	-5.06	-11.54
	Carbamazepine (+)	236.0949 / 13.26	236.0947 / 13.26	0.37	-0.02	-14.03	-14.01	-10.37	-15.68
	Carbamazepine-(carboxamide-^{13}C,^{15}N) (+)	238.0953 / 13.26	238.0951 / 13.26	0.05	-0.17	-13.84	-13.87	-14.58	-15.06

Table 2
Counts of potential metolachlor (top) and clofibric acid (Bottom) Transformation products.Filtering parameters were log2FC before/after treatment >1, log2FC spike-in/no spike >2, and $p < 0.05$.

Metolachlor			potential TP pos ionization mode	potential TP neg ionization mode	with Cl pos ionization mode	with Cl neg ionization mode		match with suspect list (M [Da] / RT [min])	tentative ID
sand filtration	10 µg/L spike-in	8h	1	0	0	0	2	249.17233 / 17.148	Deschlormetolachlor (CAS 126605-22-9; Norman SusDat, StoffIDENT, EnviPATH)
		4d	1	0	0	0			
ozonation	10 µg/L spike-in	low O3	57	5	38	1	212	269.11772 / 15.421	CCC1=C(C(=CC=C1)C)N(C(C)CO)C(=O)CCl (EnviPATH)
		high O3	28	3	15	0			
	100 µg/L spike-in	low O3	62	5	38	1			
		high O3	47	5	30	1			
			196	**18**	**121**	**3**			
total number			**214**		**124**				
unique features			**69**		**40**				

Clofibric acid			potential TP pos ionization mode	potential TP neg ionization mode	with Cl pos ionization mode	with Cl neg ionization mode		match with suspect list (M [Da] / RT [min])	tentative ID
sand filtration	10 µg/L spike-in	day 0	8	7	0	0	173		
		day 4	107	51	0	5		136.08849 / 9.425	CC(C)OC1=CC=CC=C1 (EnviPATH)
ozonation	10 µg/L spike-in	low O3	5	2	1	0	21		
		high O3	3	1	1	0			
	100 µg/L spike in	low O3	1	3	0	0		104.04685 / 2.299; 104.04685 / 2.456	Alpha-Hydroxyisobutyric (Salgadoac et al., EnviPATH)
		high O3	0	6	0	0			
			124	**70**	**2**	**5**			
total number			**194**		**7**				
unique features			**161**		**6**				

2.3.2. Ozonation as an example for abiotic transformation processes

Ozonation is an (advanced) oxidation process broadly applied worldwide both in drinking water and in wastewater treatment (Lawrence and Cappelli, 1977; Collivignarelli and Sorlini, 2004). The technology is known to lead mainly to transformation of compounds rather than their mineralization, and the biological effects of formed TPs have been of concern (Genena et al., 2011; Muller et al., 2012; Von Sonntag and Von Gunten, 2012; Segura et al., 2013; Tay et al., 2013). There are two distinct reactions occurring during ozonation: the direct reaction of the ozone molecule with a target compound, and the decomposition of ozone in aqueous medium, producing hydroxyl radicals which can in turn react with

Table 3
Overlap of transformation products of carbamazepine without (blue) and with label (Red). Features with 2 Da mass shift are listed in purple, features with same mass in blue.

		Feature (Molecular weight / Retention time)	total number detected	Positive ionization				Negative ionization				Suspect list match	Structural identification based on MS2 match
				10 µg/L spike- in		100 µg/L spike- in		10 µg/L spike- in		100 µg/L spike- in			
				Low O_3	High O_3	Low O_3	High O_3	Low O_3	High O_3	Low O_3	High O_3		
TP smaller than parent compound		119.037 / 8.676	4	1	1	1	1	0	0	0	0	no match	
		119.03698 / 8.674	4	1	1	1	1	0	0	0	0		
		119.07336 / 2.899	2	0	0	1	1	0	0	0	0	no match	
		119.0734 / 2.894	1	0	1	0	0	0	0	0	0		
		130.06241 / 4.747 // 130.06242 / 4.624 // 130.06243 / 4.531	4	0	0	0	0	1	1	1	1	no match	
		130.06245 / 4.499	4	0	0	0	0	1	1	1	1		
		137.04709 / 8.682 // 137.04751 / 8.676	7	1	1	1	1	1	0	1	1	no match	
		137.0471 / 8.658 // 137.04753 / 8.673	7	1	1	1	1	1	0	1	1		
		146.04782 / 5.965	2	1	0	1	0	0	0	0	0	no match	
		148.04822 / 5.967	2	1	0	1	0	0	0	0	0		
		146.0574 / 6.219	2	0	0	0	0	0	0	1	1	no match	
		146.05737 / 6.232	3	0	0	0	0	1	1	0	1		
		162.04267 / 7.132	3	1	0	1	1	0	0	0	0	no match	
		164.04276 / 7.14 // 164.04309 / 7.13	6	1	0	1	1	1	0	1	1		
		162.11565 / 2.283	3	1	1	1	0	0	0	0	0	no match	
		162.11558 / 2.287	1	1	0	0	0	0	0	0	0		
		165.07852 / 2.83 // 165.07877 / 2.895	3	0	0	1	1	0	0	1	0	no match	
		165.07878 / 2.895	1	0	1	0	0	0	0	0	0		
		180.053 / 8.685	4	0	0	0	0	1	1	1	1	no match	
		182.05339 / 8.655	4	0	0	0	0	1	1	1	1		
		195.068 / 11.198	3	0	0	0	0	1	0	1	1	acridone, 9-hydroxy-acridine	FISh scoring - no match. MetFrag 2.0 search against acridone 1/4 fragments match.
		195.06801 / 11.192	2	0	0	0	0	1	0	1	0		
		200.05025 / 10.172	3	0	0	0	0	1	1	1	0	no match	
		200.05022 / 10.184	2	0	0	0	0	0	1	0	1		
		208.01903 / 9.23	1	0	0	0	0	0	0	1	0	no match	
		208.01901 / 9.234	1	0	0	0	0	1	0	0	0		
TP bigger than parent compound		238.07397 / 11.2	3	0	0	0	0	1	0	1	1	no match	
		240.0744 / 11.192	3	0	0	0	0	1	0	1	1		
		264.05294 / 11.193	3	1	0	1	1	0	0	0	0	no match	
		266.05342 / 11.19	3	1	0	1	1	0	0	0	0		
		266.06868 / 10.307	2	1	0	1	0	0	0	0	0	1-(2-Benzaldehyde)-(1H,3H)-quinazoline-2,4-dione (BQD); BaQM	**BaQM (Azais et al.)**
		268.06918 / 10.303	2	1	0	1	0	0	0	0	0		
		266.06871 / 11.78	2	1	0	1	0	0	0	0	0	1-(2-Benzaldehyde)-(1H,3H)-quinazoline-2,4-dione (BQD); BaQM	**BQD (McDowell et al., Azais et al.)**
		268.06914 / 11.775	2	1	0	1	0	0	0	0	0		
		282.06345 / 11.194	4	1	1	1	1	0	0	0	0	1-(2-benzoic acid)-(1H,3H)-quinazoline-2,4-dione (BaQD)	**BaQD (McDowell et al., Azais et al.)**
		284.06392 / 11.19 // 284.06432 / 11.193	7	1	0	1	1	1	1	1	1		
		337.86582 / 23.606	1	0	0	0	0	0	1	0	0	no match	
		337.86583 / 23.489	1	0	0	0	0	0	1	0	0		

the target compound. In practice, both direct and indirect reactions take place simultaneously. It should be noted that the published prediction software for ozonation TPs by Lee et al. which derived 340 individual reaction rules from literature data mining to predict the TPs of micropollutants, does not predict hydroxyl radical-induced transformation products and could thus not be used for the prediction of metolachlor and clofibric acid TPs (Lee et al., 2017).

Ozonation experiments were performed using a BMT-laboratory setup consisting of the BMT 803 BT ozone generator and two BMT 964 ozone analyzers (BMT MESSTECHNIK GMBH, Stahnsdorf, Germany), and surface water from the Waternet Leiduin plant (Leiduin, The Netherlands) spiked with no, 10 µg/L and 100 µg/L final concentration of each parent compound separately. For each experiment, the ozone reactor was filled with 1L water sample. The ozone generator was started with a continuous flow of oxygen at 1 N-L/

min, and the water sample continuously recirculated in the opposite direction of the gas flow. A tap was mounted to enable sampling from the recycling stream. The low and high ozone concentration samples, 60 mL each, were taken after 1 and 6 min, respectively. Subsequently, the reactor was flushed and filled with the next water sample. Ozone concentrations and gas flow were monitored every minute to determine the ozone consumption, which amounted to an average of 5.60 ± 0.32 mg/L for low ozone and 11.78 ± 0.65 mg/L for high ozone concentrations. In blank experiments, water samples were exposed to oxygen from the oxygen concentrator without starting the ozone generator.

2.4. LC-HRMS based non-target screening

A Tribrid Orbitrap Fusion mass spectrometer (ThermoFisher Scientific, Bremen, Germany) provided with an electrospray ionization source was interfaced to a Vanquish HPLC system (ThermoFisher Scientific). For the chromatographic separation an XBridge BEH C18 XP column (150 mm × 2.1 mm I.D., particle size 2.5 μm) (Waters, Etten-Leur, The Netherlands) preceded by a 2.0 mm × 2.1 mm I.D. Phenomenex SecurityGuard Ultra column (Phenomenex, Torrance, USA) maintained at a temperature of 25 °C was used. The gradient started with 5% acetonitrile, 95% water and 0.05% formic acid (v/v/v), increased to 100% acetonitrile with 0.05% formic acid in 25 min, and was held constant for 4 min at a flow rate of 0.25 mL/min. Prior to LC-HRMS analysis, bentazon-d6, atrazine-d5 and benzotriazole-d4 were added to the water samples as internal standards with a final concentration of 1 μg/L; this allowed LC-HRMS performance evaluation and quality control based on their signal intensities, peak shapes, exact mass and retention times. Subsequently, samples were filtered using Phenex™-RC 15 mm Syringe Filters 0.2u (Phenomenex, Torrance, USA). 100 μL of filtered sample was used for injection, and samples were measured in triplicate. Blank samples of internal standards spiked into ultrapure water were run every 5–10 samples to check for carry-over and contamination. With every batch run mass calibration was performed using Pierce ESI positive and negative ion calibration solution to ensure a mass error smaller than 2 ppm. The vaporizer and capillary temperature were maintained both at 300 °C. Sheath, auxiliary and sweep gas was set to arbitrary units of 40, 10 and 5, respectively. The source voltage was set to 3.0 kV in the positive mode, and −2.5 kV the negative mode respectively. The RF lens was set to 50%. Full scan high accuracy mass spectra was acquired in the range of 50–1000 m/z with the resolution set at 120,000 FWHM and quadruple isolation were used for acquisition. Data dependent MS/MS acquisition was performed for the eight most intense ions detected in the full scan, using a High Collision Dissociation (HCD) energy at 35% and an FT resolution of 15,000 FWHM.

2.5. Data processing and analysis

The acquired data was processed using Compound Discoverer 2.1 (Thermo Scientific, San Jose, USA) for peak picking, componentization, chlorine pattern scoring, suspect screening and automatic MS2 fragment searches via mzCloud (HighChem LLC, Slovakia). Searches were performed with 5 ppm mass tolerance. Parameter settings are listed in SI 1.1. For each parent compound, suspect screening was performed against an in-house curated TP suspect list specific for the selected parent compound via the mass list node. The in-house suspect lists were generated through literature mining for known environmental TPs and metabolites (Liu et al., 1991; Stamper and Tuovinen, 1998; Steen et al., 2000; Osano et al., 2002; Ternes et al., 2002; Doll and Frimmel, 2004; Mersie et al., 2004; Sakkas et al., 2004; McDowell et al., 2005; Warner and Morrow, 2007; Huntscha et al., 2008; Kosjek et al., 2009b; Salgado et al., 2012; Brox et al., 2016; Orge et al., 2017), entries in the NORMAN SusDat (http://www.norman-network.com/?q=node/236) and the STOFF-IDENT (https://www.lfu.bayern.de/stoffident/) databases, and *in silico* prediction using EnviPATH (Wicker et al., 2016). The suspect lists for potential TPs of metolachlor, clofibric acid and carbamazepine are provided in SI 1.2.1, 1.2.2 and 1.2.3, respectively.

After processing, significance testing and fold change filtering was applied to identify potential TPs. Features were categorized as TP when their intensity increased, i.e. log2FC between after and before treatment samples was greater than 1, indicating that the feature was formed during water treatment, and when they showed significantly higher intensities in the spike-in compared to no-spike samples, i.e. the log2FC between spike-in and no spike samples was greater than 2, indicating that the feature originated from the parent compound. As the selected parent compounds could be present in the source water, but at concentrations 100x lower than the lowest spike-in concentration, this filtering step did not compromise TP identification, and greatly reduced data complexity. Significance testing and fold change filtering results were illustrated using Volcano plots displaying log2FC and the negative log 10-transformed p-values of features (Cui and Churchill, 2003) in R (R Core Team, 2017).

The TPs thereby identified were further inspected using MS1 full scan data in regard to suspect screening matches based on accurate mass, and if applicable presence of a halogen or label based on isotopic patterns or mass shift, respectively. For features matching a suspect list entry, identification was attempted using MS2 fragmentation data for spectral library searches against mzCloud (HighChem LLC, Slovakia), including spectral tree searches for fragmentation similarities, Fragment Ion search (FiSH) scoring in Compound Discoverer 2.1 (Thermo Fisher Scientific) exploiting the structural relationship of a parent compound and its transformation products, and MetFrag queries (Ruttkies et al., 2016), including MassBank of North America fragmentation similarity searches.

3. Results

3.1. Parent compound degradation

Parent compound degradation in the different drinking water treatments was assessed through comparison of peak intensities of the parent compounds between samples and calculation of log2FC values between parent compound peak areas in the before and after treatment samples. We defined a significant increase as a $\log2FC > 0.25$, a significant decrease as a $\log2FC < -0.25$, both with $p < 0.05$ with Benjamini-Hochberg correction. These cut-offs reflect a roughly 20% change between samples. Parent compounds with corresponding ionization modes, molecular weight, RT and log2FC across the different experimental conditions are summarized in Table 1.

No significant decrease of parent compounds was observed in sand filtration experiments. This was expected for carbamazepine, which initially showed a slight signal increase (~30%). However, after four days there was no longer any difference in carbamazepine signal between influent and effluent samples. This might be due to sorption or/and charging. As carbamazepine is a neutral and slightly hydrophobic compound at pH7 with a $logK_{OW}$ of 2.43 (US EPA, 2012), the column might need to first stabilize. Metolachlor and clofibric acid are known to be degraded in soil, with a DT50 of months, and weeks to month, respectively (US EPA, 2012). A significant decrease of these parent compounds was thus expected, but not observed. This could result from the continuous flow of spiked-in parent compound which might mask the decrease in

concentration. As biodegradation can depend on the bacterial population present, it could be that no degradation was observed due to the lack of appropriate microorganisms in the RSF sand (Rauch-Williams et al., 2010; Li et al., 2012, 2013, 2016; Alidina et al., 2014a, 2014b; Regnery et al., 2016). In the case of clofibric acid, a significant change between effluent and influent was observed, but did not exceed the arbitrarily defined log2FC cut-off of −0.25. This could indicate low rates of degradation and thus potential TP formation. A data-driven or inert tracer based cut-off could alleviate this issue in future experiments.

In contrast, all parent compounds showed a significant decrease in ozonation experiments, with an overall stronger decrease in parent compound signal at higher ozone concentrations. Interestingly, in the case of metolachlor ozone degradation seemed to be dependent on the spike-in concentration, with the lower spike-in concentrations showing less decrease in peak intensities. In contrast, the extent of clofibric acid degradation by ozonation seemed to be dependent on the ozone concentration, with higher ozone concentrations leading to a stronger decrease. The decrease of both labeled and not labeled carbamazepine was similar under all experimental conditions, and more pronounced than that of metolachlor and clofibric acid. The two distinct reactions occurring during ozonation might be responsible for these results, i.e. direct and indirect reaction of ozone with the target molecule. Carbamazepine is known to react directly with ozone, however, metolachlor and clofibric acid react indirectly through the hydroxyl radicals that are generated by decomposition of ozone in aqueous medium (Lee et al., 2017).

3.2. Formation of TPs

Next, it was determined whether the degradation of parent compounds resulted in mineralization or TP formation. Moreover, identification of TPs, potentially specific for the different treatment conditions was attempted. Therefore, peak areas of all features were compared between the before and after treatment groups. Subsequently, filtering steps were applied to reduce the peak number to those peaks that are potential TPs. As TPs are formed during treatment, only peaks that showed a log2FC > 1 ($p < 0.05$) between treatments were kept. Additionally, peaks with a log2FC < 2 between spike-in and no spike samples were discarded, as these peaks could potentially be TPs that are formed from other micropollutants and/or dissolved organic matter present in the source water, and thus not derived from the spiked-in parent compounds. The benefits of these filtering steps are illustrated in the volcano plots in Fig. 1. These plots show the features detected in metolachlor sand filtration and ozonation experiments, respectively, with the log2FC plotted on the x-axis against the −log 10 of the p-value on the y-axis. All features left of the y-axis decrease through treatment, the features right of it increase. Features that significantly increase with a log2FC > 1 are potential TPs. In addition, features that have a log2FC > 2 ($p < 0.05$) between spike-in and no spike samples are potential TPs of the spiked-in parent compound, i.e. here metolachlor TPs. Interestingly, although no significant degradation of metolachlor had been observed in sand filtration experiments (Table 1), a TP is observed.

3.2.1. Metolachlor: 1 biotic RSF TP versus 68 abiotic ozonation TPs

The filtering steps resulted in the identification of a total of 214 metolachlor TPs across all experiments (>90% in positive ionization mode), 124 of which exhibited an isotopic pattern suggesting the presence of a chlorine atom (Table 2, top panel). 69 of these features were unique, and 40 were dechlorinated. Sand filtration led to the formation of a single, dechlorinated TP already after 8 h, which persisted through day 4. The sand filtration TP matched with the

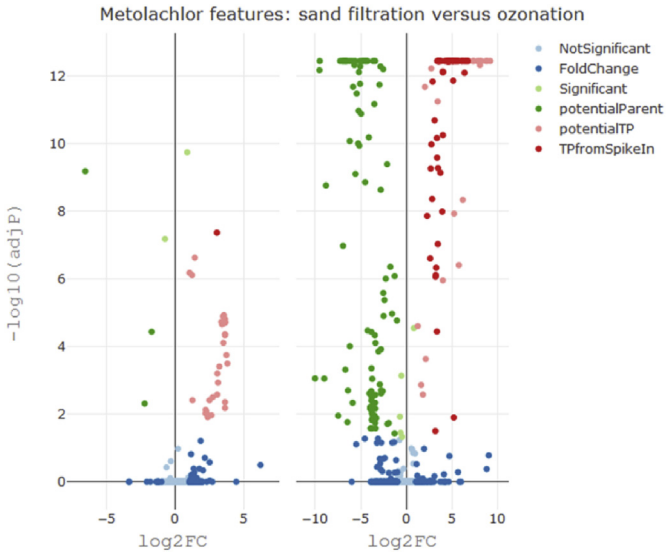


Fig. 1. Volcano plot of Metolachlor features (10 µg/L spike-in) detected in Sand filtration experiments after 8 h (left) and in ozonation experiments with low ozone concentrations (right), in positive ionization mode. Features are plotted as dots according to fold change between effluent and influent samples (x-axis) and significance value (y-axis).

suspect screening candidate deschlormetolachlor, which is listed in SusDat and STOFF-IDENT, and was predicted by EnviPATH. MS2 fragmentation data shown in Fig. 2 middle panel, allowed confirmation of the structural identification through FiSH scoring (Supplementary information 2.1.1, confidence level 2 according to Schymanski (Schymanski et al., 2014)). All other TPs were formed through ozonation, with more than half comprising the chlorine atom. There was substantial overlap in TP formation with the different spike-in and ozone concentrations (Supplementary Table 2). On average, a TP was detected in three different experimental conditions. Merely a single abiotic TP could be matched to a suspect list entry, namely an EnviPATH predicted compound with the SMILES CCC1=C(C(=CC=C1)C)N(C(C)CO)C(=O)CCl and corresponding chemical formula C14H20Cl1N1O2. As EnviPATH is a prediction tool for biodegradation reactions, an overlap between ozonation TPs and predicted biodegradation TPs was not per se expected. However, MS2 based FiSH scoring (MS2 spectrum shown in Fig. 2, lower panel, Supplementary Information 2.1.2) and MetFrag fragmentation (data not shown) confirmed the EnviPATH predicted compound (confidence level 2), while it rejected the two known compounds alachlor and acetochlor which have the same accurate mass, but no fragmentation peak at *m/z* 176.14305.

3.2.2. Clofibric acid: abundance of biotic TPs & increase of biodegradation over time

An overview of clofibric acid TP formation is shown in Table 2, bottom panel, and a detailed list of identified clofibric acid TPs can be found in Supplementary Table 3. 194 TPs were formed in all experiments together, roughly one third of the TPs was detected in negative ionization mode, the mode of ionization of the parent compound. There was minor overlap between experimental conditions, 161 of the 194 TPs detected across all conditions were unique. This is due to the fact that about 80% of all TPs were detected in sand filtration experiments, and 3 out of 4 on day 4 of the biodegradation time course. Only 5 of the 173 biotic TPs were chlorinated, indicating that dechlorination was one of the main biodegradation pathways occurring. Ozonation resulted in the formation of 13 unique TPs, only one of which was chlorinated.

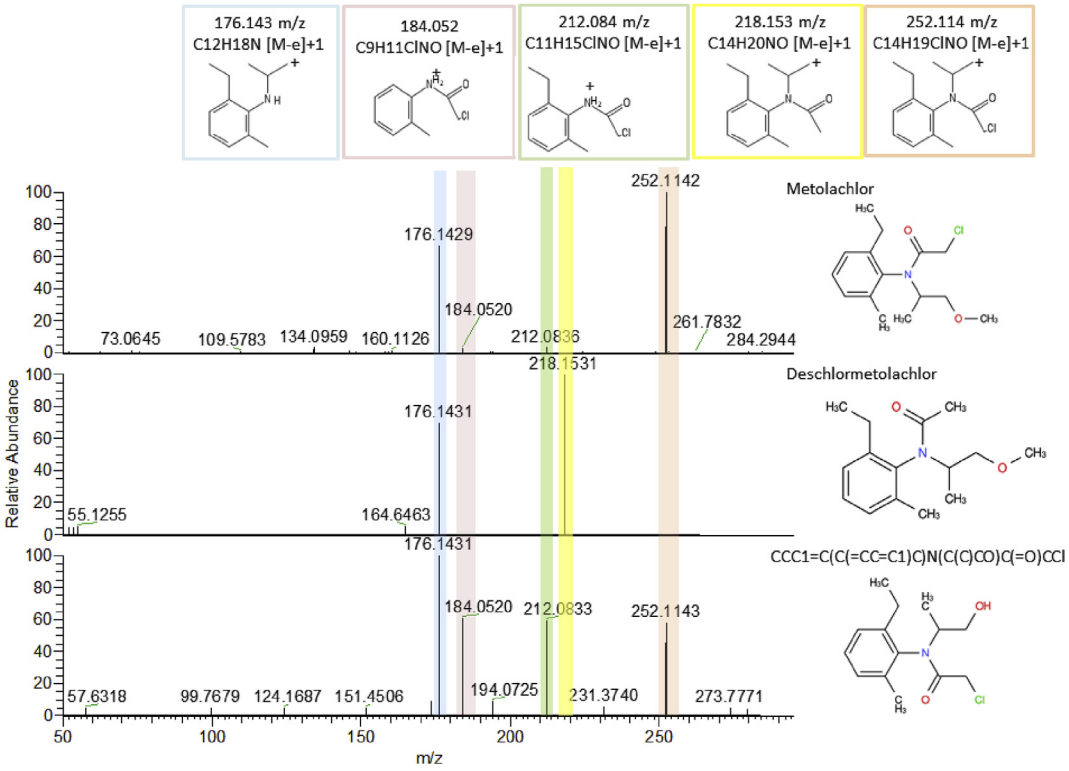


Fig. 2. MS2 Fragmentation Spectra and fragment structures of the parent compound metolachlor (top), of the biotic transformation product deschlormetolachlor (middle) and the abiotic transformation product CCC1=C(C(=CC=C1)C)N(C(C)CO)C(=O)CCl.

As was the case for metolachlor TPs, suspect list hits for clofibric acid TPs were limited. Again one single biotic and one single abiotic TP could be matched, specifically the EnviPATH predicted structure CC(C)OC1=CC=CC=C1 in sand filtration, and alpha-hydroxyisobutyric acid in ozonation. Alpha-hydroxyisobutyric acid was matched twice to two isobaric features with RTs differing by less than 0.2 min. Interestingly the abiotic TP had been described in biodegradation experiments previously (Salgado et al., 2012), and was predicted by EnviPATH. MS2 based FiSH scoring (Supplementary Information 2.2.1) structurally confirmed alpha-hydroxyisobutyric acid based on a single fragment ion(confidence level 2). However, CC(C)OC1=CC=CC=C1 could not be confirmed based on MS2 data.

3.2.3. *Carbamazepine: labeling facilitates efficient TP detection*

Finally, carbamazepine experiments were performed with carbamazepine and labeled carbamazepine, the results of which are summarized in Supplementary Tables 4 to 7. Experiments without label resulted in a total of 135 potential TPs and 78 unique features, experiments with label in 81, of which 29 unique, respectively. TPs were detected to similar extents using positive and negative ionization mode. The discrepancy in TP abundance between the non-labeled and labeled compound, which in theory should form the exact same TPs without and with the label, may be related to slightly different experimental conditions across experiments, such as actual ozone concentrations, and bacterial populations in the RSF. In addition, difficulties in data processing due to the shift in isotopic distribution for the labeled TPs might be responsible for the different results and consequently limited overlap in carbamazepine TPs. The latter could potentially be resolved in future experiments by manually adding the isotopic pattern of the label to the pattern recognition node in Compound Discoverer 2.1. The former could be addressed by including treatment replicates in addition to technical replicates in future studies.

In addition to the statistical testing and fold change filtering, the labeling strategy allowed to use an additional, more stringent criterion, i.e. the overlap between the two experimental groups, to filter for features representing TPs. An added benefit here that could also be achieved without labeling, is that the experiment is performed in duplicate which by itself will lead to more stringent results, and more confident TP identification. In the carbamazepine experiments, 19 TPs overlapped as shown in Table 3. Of these, 8 TPs showed the characteristic 2 Da mass shift, indicating that the labeled residues were still present in the compound. The eight TPs exhibiting the 2 Da mass shift were manually inspected for peak duplets in the raw data of the mixed label experiment. None of the biotic TPs showed overlap between groups, in line with what was observed for metolachlor and clofibric acid, indicating that sand filtration is more susceptible to slight changes in experimental conditions, and thus less reproducible. In case of the abiotic TPs of carbamazepine, 19 of the 24 labeled TPs from ozonation overlapped, representing 83% overlap.

For four of the twenty overlapping TPs, suspect list matches were found, for two of them multiple suspects were possible based on the accurate mass alone. MS2 based FiSH scoring (Supplementary Information 2.3) and MetFrag queries (data not shown) enabled unambiguous identification of the three TPs 1-(2-benzoic acid)-(1H, 3H)-quinazoline-2,4-dione (BaQD) (McDowell et al., 2005; Azaïs et al., 2017), 1-(2-benzaldehyde)-(1H, 3H)-quinazoline-2,4-dione (BQD) and 1-(2-benzoic acid)-4-hydro(1H, 3H)-quinazoline-2-one (BaQM, Azaïs et al., 2017) with confidence level 2. The fragmentation spectra of the fourth TP with a suspect list match could be equally well explained by *in silico* fragmentation spectra of acridone and 9-hydroxy-acridine, which are tautomers and have both been previously reported TPs of carbamazepine (Kosjek et al., 2009a) (Supplementary Information 2.3).

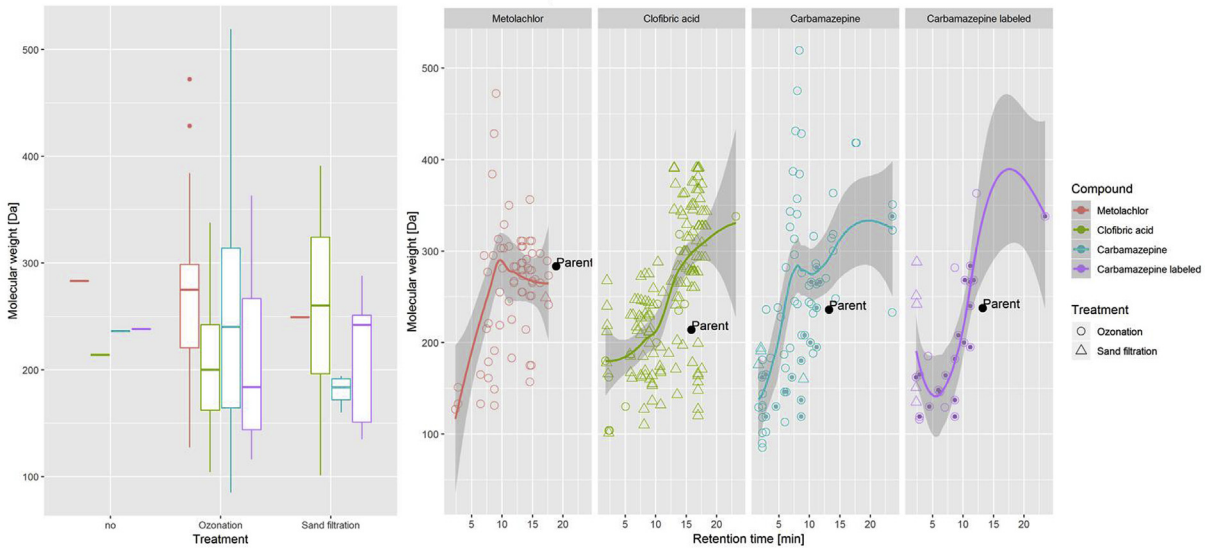


Fig. 3. Left: molecular weight distribution of parent compounds (no treatment group) and TPs from ozonation and sand filtration. Right: Comparison molecular weight and RT of parent compounds and their TPs. TPs detected in both carbamazepine labeled and unlabeled experiments are represented with filled dots.

4. Discussion: suitability and performance of the developed workflow

The developed workflow allowed monitoring of TP formation in a lab-scale experiment. TP identification based on log2FC filters and statistical significance between before and after treatment, and spike-in and no spike samples was efficient in revealing an abundance of drinking water treatment-specific TPs. Interestingly, an absence of significant parent compound degradation could still be accompanied by TP formation. The distinct isotopic pattern of chlorine in metolachlor and clofibric acid samples, as well as the 2 Da mass shift of the label in carbamazepine experiments further facilitated TP identification. Monitoring three different parent compounds and two drinking water treatments in parallel allowed to assess similarities and differences between their biotic and abiotic TPs. Prior to the start of the lab-scale experiment, it was known that carbamazepine readily reacted with ozone and was not susceptible to biodegradation. Correspondingly, the majority of carbamazepine TPs was formed during ozonation. However, sand filtration did result in the formation of a small number of TPs, which was surprising, but in line with previous research reporting minor degradation of carbamazepine in laboratory scale experiments (Duran-Alvarez et al., 2015; Dalahmeh et al., 2018), and the isolation of bacterial strains able to biodegrade the pharmaceutical (Bessa et al., 2017). Metolachlor formed one single biotic TP, which was dehalogenated. Clofibric acid TPs, in contrast, were mainly formed during sand filtration, and less than 5% contained a chlorine atom. Dehalogenation thus seemed an ubiquitous process in the biodegradation experiments performed, which is in line with dehalogenation being a thermodynamically favorable reaction (Parsons et al., 2008). In particular TPs formed by biodegradation varied between experiments, performing experiments in duplicates or triplicates would be more meaningful, but was logistically not possible for the sand filtration set-up. Another significant difference between the two halogenated parent compounds metolachlor and clofibric acid was that 2/3 of the metolachlor ozonation TPs still contained the chlorine atom, while only a single clofibric acid TP did. The structural positioning of the chlorine atom strongly influences its breakdown during ozonation experiments and thus TP formation.

TP studies often include logical filters concerning molecular weight and RT to identify TPs (Helbling et al., 2010; Escher and Fenner, 2011); the hypothesis being that TPs are more polar than their parent compound, and therefore elute earlier in RP-LC runs, and that they are smaller than their parent compounds. However, when the sand filtration and ozonation data sets were examined in regards to molecular weight and RT distribution as illustrated in Fig. 3, metolachlor was the only parent compound of which all TPs had shorter RT, and molecular weight distribution of TPs spanned from roughly 1/3 to 2x that of the parent compounds. Visual examination showed that the metolachlor sand filtration TP was smaller than the mean ozonation TP. Accordingly statistical testing showed that carbamazepine sand filtration TPs were significantly smaller than ozonation TPs. However, the small number of sand filtration TPs renders generalization difficult. Contrarily, for clofibric acid an abundance of sand filtration TPs were identified, and there was no significant difference between treatment groups. These results emphasize that filters have to be carefully selected when designing the data processing workflow. Application of a logical filter for decreased molecular weight and shorter RT here would have led to a substantial loss of identified TPs.

5. Conclusion: challenges of transformation product research and outlook

Substantial advances in TP identification have occurred through application of analytical methods combining so called “bottom-up” and “top-down” approaches, i.e. the prediction of transformation processes to create suspect lists with TPs of known parent compounds that can be searched in non-target LC-HRMS data, and statistical methods to identify patterns and similarities between unknown parent compounds and their TPs. Here, the methods developed and applied to a lab-scale experiment representing relevant drinking water treatment technologies and parent compounds allowed the detection of a multitude of TPs. However, despite current advancements in the NTS based identification of unknown compounds, the number of TP features remaining unidentified exceeds the number of annotated features by far. This is in particular due to the fact that TPs are often lacking from suspect lists and spectral databases and thus represent so called “unknown

unknowns", as well as that reference standards for their confirmation are missing. The results of the lab-scale experiments emphasize this issue, with only a minority of TPs matched to a suspect, despite the effort spent on manually creating appropriate suspect lists and the selection of 3 separately tested chemicals. More comprehensive databases will likely alleviate this issue in the future. In particular, STOFF-IDENT has increasingly been adding TPs to its database. In addition, Schollee et al. suggested that based on the relationship between structural and fragmentation similarity, spectral similarity could be used to screen for organic micropollutants and their TPs (Schollee et al., 2017). This approach could thus allow identification of TPs that are unknown unknowns based on MS2 fragmentation data. With these developments in mind, we envision that the monitoring of TPs in drinking water could become a routine task in water analyses leading to a more comprehensive assessment of drinking water quality on the long run. Moreover, by identifying TPs and connecting them to their parent compounds, sources of contaminants and treatment processes could be determined and potentially regulated.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2018.09.140.

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