

Characterization of advanced wastewater treatment with ozone and activated carbon using LC-HRMS based non-target screening with automated trend assignment

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ABSTRACT

Advanced treatment is increasingly being applied to improve abatement of micropollutants in wastewater effluent and reduce their load to surface waters. In this study, non-target screening of high-resolution mass spectrometry (HRMS) data, collected at three Swiss wastewater treatment plants (WWTPs), was used to evaluate different advanced wastewater treatment setups, including (1) granular activated carbon (GAC) filtration alone, (2) pre-ozonation followed by GAC filtration, and (3) pre-ozonation followed by powdered activated carbon (PAC) dosed onto a sand filter. Samples were collected at each treatment step of the WWTP and analyzed with reverse-phase liquid chromatography coupled to HRMS. Each WWTP received a portion of industrial wastewater and a prioritization method was applied to select non-target features potentially resulting from industrial activities. Approximately 37,000 non-target features were found in the influents of the WWTPs. A number of non-target features (1207) were prioritized as likely of industrial origin and 54 were identified through database spectral matching. The fates of all detected non-target features were assessed through a novel automated trend assignment method. A trend was assigned to each non-target feature based on the normalized intensity profile for each sampling date. Results showed that $73 \pm 4\%$ of influent non-target features and the majority of industrial features (89%) were well-removed (*i.e.*, $>80\%$ intensity reduction) during biological treatment in all three WWTPs. Advanced treatment removed, on average, an additional 11% of influent non-target features, with no significant differences observed among the different advanced treatment settings. In contrast, when considering a subset of 66 known micropollutants, advanced treatment was necessary to adequately abate these compounds and higher abatement was observed in fresh GAC (7,000–8,000 bed volumes (BVs)) compared to older GAC (18,000–48,000 BVs) (80% vs 56% of micropollutants were well-removed, respectively). Approximately half of the features detected in the WWTP effluents were features newly formed during the various treatment steps. In ozonation, between 1108–3579 features were classified as potential non-target ozonation transformation products (OTPs). No difference could be observed for their removal in GAC filters at the BVs investigated (70% of OTPs were well-removed on average). Similar amounts (67%) was observed with PAC (7.7–13.6 mg/L) dosed onto a sand filter, demonstrating that a post-treatment with activated carbon is efficient for the removal of OTPs.

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1. Introduction

Trace organic compounds, or micropollutants, originating from human activities, including pharmaceuticals, pesticides, food additives, personal care products, and industrial chemicals, have been extensively documented to be present in surface waters

around the globe (Kolpin et al., 2002; Ternes 2001; Schmidt 2018). Their input pathway into surface waters is often through treated (or sometimes untreated) wastewater (Loos et al., 2013; Schwarzenbach et al., 2006; Ternes 1998). Due to the closed water cycle, surface waters are also a source of drinking water for many communities. Therefore, it is of great interest to reduce the load of micropollutants discharging from wastewater treatment plants (WWTPs), to subsequently protect aquatic communities and drinking water resources (Joss et al. 2008; Eggen et al. 2014).

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Because primary and secondary wastewater treatment are mostly ineffective at removing polar micropollutants (Joss et al., 2005; Luo et al., 2014), advanced treatment needs to be applied for micropollutant abatement. In Switzerland, a new Water Protection Act mandates that approximately 130 of 700 WWTPs in the country be upgraded with, e.g., either ozonation or activated carbon treatment (GschV 814.201). In ozonation, compounds react with either ozone or hydroxyl radicals, leading to the degradation of parent micropollutants (Huber et al., 2003; von Sonntag and von Gunten 2012) and the formation of ozonation transformation products (OTPs) (Lee and von Gunten 2010). Additionally, the water matrix (i.e., dissolved organic matter (DOM)) is also degraded in ozonation and can lead to the formation of disinfection by-products (DBPs) (Richardson et al. 2007; von Gunten 2003). Therefore, a biologically-active post-treatment should be applied after ozonation (Hollender et al., 2009; Reungoat et al., 2012). Powdered activated carbon (PAC) treatment is an alternative method for advanced wastewater treatment (Boehler et al., 2012; Kovalova et al., 2013; Margot et al., 2013; Snyder et al. 2007) because compounds adsorb to the activated carbon without the formation of TP or DBPs. PAC treatment should also be followed by a polishing step such as sand filtration to remove particles. A third option for advanced wastewater treatment is the use of granular activated carbon (GAC) filtration, which is considered more sustainable because GAC can be reactivated (Siegrist et al., 2019). GAC filtration can remove compounds both through sorption and through the promotion of biologically active filters (also referred to as biological activated carbon (BAC)) (Altmann et al., 2016; Choi et al., 2008; Hernández-Leal et al., 2011). Efficiency of removal in GAC, however, can be expected to decrease over time, as adsorption sites become limiting (Benstoem et al., 2017), and is strongly affected by contact time (Sari et al., 2020). Due to the demonstrated advantages of both ozonation and activated carbon for the removal of micropollutants, research into coupling these technologies has been pursued and has shown promising results (Knopp et al. 2016; Lee et al., 2012; Reungoat et al., 2010; Sánchez-Polo et al., 2006). The coupling of low-dose ozonation (i.e., pre-ozonation) with GAC filtration is expected to be cost-efficient while also increasing overall removal due to the synergism of the two technologies. In the first step, pre-ozonation leads to partial abatement of micropollutants and the formation of more readily biodegradable compounds at lower energy consumption (and therefore costs) compared to ozone treatment at higher doses. In the second step, adsorption to activated carbon functions as a removal mechanism for micropollutants, while the presence of increased assimilable organic carbon (AOC) through pre-ozonation can promote biofilm formation; together these are expected to result in better removal of recalcitrant micropollutants, OTPs, and DBPs (Bourgin et al., 2018; Knopp et al., 2016). The promotion of biofilm formation and the transition to a BAC treatment (and the combination with ozonation) increases the lifetime of these treatments (Altmann et al., 2016; Piai et al., 2020). Although a high number of studies have evaluated the coupling of these technologies through target screening of known micropollutants (e.g., Bourgin et al., 2018; Knopp et al., 2016; Reungoat et al., 2012; Reungoat et al., 2010), a more comprehensive assessment of unknown compounds is lacking.

A wide variety of analytical methods have been applied to assess the occurrence and fate of micropollutants in WWTPs and in the environment (Fatta et al., 2007; Richardson and Kimura 2016). However, screening methods that focus on a limited number of target compounds provide an incomplete picture. Non-target screening (NTS) with high-resolution mass spectrometry (HRMS) can be applied to provide a more comprehensive assessment of the compounds present (Hollender et al., 2017; Ibáñez et al., 2008; Krauss et al., 2010; Petrie et al., 2015). NTS is generally used in one of the following two ways. The first, and more common method,

is to identify unknown substances by applying appropriately selected differential data analyses to prioritize a small number of unknown non-target features (Brunner et al., 2019; Hollender et al., 2017; Schymanski et al., 2014b). This analysis method is relevant especially when looking for unknown sources of toxicity, for example, or trying to narrow the knowledge gap about potential new emerging chemicals of concern. However, by choosing to focus on a limited number of prioritized non-target features, ultimately some knowledge is lost about the overall chemical burden of the samples. The second NTS strategy is to apply data mining to comprehensively characterize all detected non-target features and identify possible patterns resulting from a treatment or different environmental conditions, without the goal of compound identification. This type of non-target screening has only been applied sparingly to date (Brunner et al., 2020; Nürenberg et al., 2019; Schollée et al., 2018). Specifically, information about the formation of unknown OTPs and their fate in post-treatment is sparse (Deeb et al., 2017; Itzel et al., 2020; Schollée et al., 2018).

In this study, the two complementary NTS approaches were applied to study advanced wastewater treatment at three WWTPs across Switzerland. First, for a complete NTS characterization, trends of non-target features, which were automatically assigned with a newly developed method, were compared among different advanced wastewater treatments (i.e., ozone and/or activated carbon treatment). Second, since each WWTP received a portion of industrial wastewater, a prioritization approach modified from Anliker et al. (2020) was applied to select and identify non-target features possibly of industrial origin (i.e., NTS identification). Goals of the study were (1) to assess the fate of non-target features, including possible industrial compounds; (2) to compare the impact of advanced treatment with low-dose ozone and/or activated carbon on the formation and removal of non-target features, including evaluating the formation of TP/DBPs and (3) comparing the effectiveness of different (biological) activated carbon treatment (i.e., GAC or PAC dosed onto a sand filter) for the removal of potential OTPs.

2. Methods

2.1. Sampling locations and procedure

Samples were collected at three WWTPs in Switzerland during predominately dry weather conditions, operating with biological treatment mostly in full-scale and advanced treatment in pilot scale. On each sampling date, samples were collected at the influent of the WWTP (*INF*), after the biological treatment (*BIO*), after ozonation (*OZO*) and after post-treatment with activated carbon, at the effluent (*EFF*). The sampling locations are summarized below, the different sample settings are shown in Table 1, and details on the WWTPs can be found in the SI, Section S1.

WWTP Glarnerland (*GL*) treats the wastewater of 70,000 population equivalents (PEs), with an industrial contribution of approximately 40%. Biological treatment consists of the S::Select® technology with hydrocyclones to separate the excess sludge. A portion of this wastewater was directed to the pilot-scale plant and further treated with either GAC filtration or with a pre-ozonation, followed by GAC filtration, using a GAC empty bed contact time (EBCT) of 24 minutes during dry weather. More details on the plant can be found elsewhere (McArdell et al., 2020; Ultramare et al., in prep). Three sampling events were conducted over the course of approximately 15 months, with increasing GAC bed volumes (BVs) over the sampling period (6,720–32,853 BV; Table 1). 24-h composites were collected at the influent (*INF*), after biological treatment at the influent of the pilot plant (*BIO*), after only GAC filtration (*EFF*) and after ozonation (*OZO*; 0.18–0.22 gO₃/gDOC) followed by GAC filtration (*EFF*) (SI, Table S1 and Fig. S1).

Table 1

Summary of sampling locations and settings, including sampling dates, sampling campaign, flow at the WWTP influent, dissolved organic carbon (DOC) after biological treatment, ozone dose applied, and settings of the respective post-treatment, either granular activated carbon (GAC) filter or powdered activated carbon (PAC) dosed onto a sand filter.

	Sampling Dates	Sampling Campaign	Influent flow (m ³ /day)	DOC after BIO ² (mg/L)	Ozone dose (gO ₃ /gDOC)	GAC BV ³	
WWTP Glarnerland¹ (GAC over time – with and without pre-ozonation)	08.06.2017	GL _{no03_GAC7000}	16371	5.7	0	6720	
	16.01.2018	GL _{no03_GAC17000}	31904	8.4	0	17051	
	05.09.2018	GL _{no03_GAC31000}	16267	7.3	0	30535	
	08.06.2017	GL _{0,203_GAC8000}	16371	5.7	0.22	8415	
	16.01.2018	GL _{0,203_GAC19000}	31904	8.4	0.2	19121	
	05.09.2018	GL _{0,203_GAC33000}	16267	7.3	0.18	32853	
WWTP Altenrhein (loaded GAC with pre-ozonation)	16.07.2018	AR _{0,203_GAC44000}	14096	8.6	0.13	44483	
	17.07.2018	AR _{0,203_GAC44000}	13837	8.7	0.13	44555	
	18.07.2018	AR _{0,203_GAC44000}	13690	9.5	0.18	44627	
	03.09.2018	AR _{0,303_GAC48000} ⁴	18467	6.1	0.36	48010	
	04.09.2018	AR _{0,303_GAC48000}	16138	6.4	0.35	48082	
	05.09.2018	AR _{0,303_GAC48000}	15751	7.6	0.29	48154	
	Sampling Dates	Sampling Campaign	Pilot inflow (L/h)	DOC after BIO ² (mg/L)	Ozone dose (gO ₃ /gDOC)	PAC ⁵ (mg/L)	PAC ⁵ (g/gDOC)
WWTP ProRhen^{6,7} (PAC+sand filter with pre-ozonation)	21.02.2017	PR _{0,203_PAC7.7}	20.6	8	0.19	5.8	0.73
	02.03.2017	PR _{0,203_PAC7.7}		NA	0.27	8.8	1.1
	09.03.2017	PR _{0,203_PAC7.7}		8	0.24	8.6	1.1
	06.04.2017	PR _{0,203_PAC12.1}	21.3	8	0.2	13.6	1.7
	12.04.2017	PR _{0,203_PAC12.1}		6	0.2	9.9	1.7
	20.04.2017	PR _{0,203_PAC12.1}		7	0.21	12.9	1.8
	27.04.2017	PR _{0,103_PAC12.5}	20.4	7	0.09	9.3	1.3
	04.05.2017	PR _{0,103_PAC12.5}		7	0.09	11.8	1.7
	11.05.2017	PR _{0,103_PAC12.5}		8	0.08	16.3	2.0
	06.06.2017	PR _{no03_PAC13.6}	22.1	7	0	15.8	2.3
	15.06.2017	PR _{no03_PAC13.6}		9	0	13.4	1.5
	22.06.2017	PR _{no03_PAC13.6}		8	0	11.8	1.5
	20.07.2017	PR _{0,303_noPAC}	21.4	8	0.29	0	0
	27.07.2017	PR _{0,303_noPAC}		9	0.27	0	0
	10.08.2017	PR _{0,303_noPAC}		10	0.21	0	0

¹ McArdell et al. 2020

² dissolved organic carbon after biological treatment.

³ amount of bed volumes run in the granular activated carbon filter.

⁴ after sampling and measurement, it was determined that this sample was influenced by wet weather and it was removed from the dataset.

⁵ powdered activated carbon dose.

⁶ influent samples collected from two streams, PR-Comm and PR-Chem and mixed 90:10, respectively, prior to treatment.

⁷ obtained from Krahnstöver et al. 2018.

WWTP Altenrhein (AR) treats the wastewater of 82,000 PEs and has an industrial contribution of approximately 26%. Biological treatment consists of activated sludge and fixed bed processes operating in parallel, followed by a sand filter (SI, Fig. S2). A portion of the treated wastewater was redirected to the pilot plant, which was equipped with pre-ozonation followed by GAC filtration with an EBCT of 20 minutes. Two sampling campaigns were conducted with two different ozone doses (0.15 ± 0.03 gO₃/gDOC and 0.33 ± 0.04 gO₃/gDOC), each with 24-h composite samples collected over three consecutive days. GAC BVs during the sampling campaigns were around 44,000 and 48,000, respectively. Prior to the second sampling event at AR, 62.9 mm rainfall had been recorded in the area in the 3 days prior (Argometeo, station Thal). The first sample of this measurement campaign also had slightly higher flow (Table 1) and was thus removed from the dataset.

WWTP ProRhen (PR) treats wastewater of 470,000 PEs. A pilot plant received two influent wastewater streams. One (PR-Comm) being mostly municipal wastewater (approximately 26% industrial contributions), while a second (PR-Chem) was from nearby (mainly) pharmaceutical industries (100% industrial wastewater). The two streams were mixed 90:10 PR-Comm:PR-Chem (v:v) and treated in a sequencing batch reactor (SBR), followed by ozonation and then PAC / iron (III) chloride dosed onto a two-layer sand filter (PAC+SF; SI, Fig. S3). The sludge water containing PAC was recircu-

lated to the SBR. Details on the pilot plant can be found elsewhere (Krahnstöver et al. 2018). Five sampling campaigns were carried out, covering a range of ozone doses (0–0.27 gO₃/gDOC) and PAC doses (0–16.3 mg/L) (Table 1). For each campaign, 48-h composite samples were collected three times over the course of 2–3 weeks.

In total, 24 sampling campaigns were conducted across the three WWTPs and 140 samples were collected (for a complete list, see SI, Table S1). Samples were collected in borosilicate glass bottles, frozen and kept at -20°C until analysis. Influent samples were collected one day prior at GL and AR to account for hydraulic retention time in the WWTPs.

2.2. Chemical analysis

Samples were measured as described in Bourgin et al. (2018); further details on the analytical procedure are also available in the SI, Section S2. Briefly, samples were first thawed overnight and filtered. All samples were diluted 1:1 (w:w) with nanopure water, except influent samples, which were diluted 1:3 (w:w) to account for possible higher matrix effects. Each sample was spiked with a mixture of 147 isotopically labeled internal standards (ISs; SI, Table S2), which covered a broad mass and retention time range and were used as quality controls at multiple points in the data evaluation. After filtration and spiking, samples were stored for a maximum of 8 days at 4°C prior to measurement.

In addition to individual samples, pooled samples were produced for each matrix at each WWTP. For example, equal aliquots of all BIO samples from WWTP Altenrhein were mixed to generate an *AR-BIO-pool* (SI, Table S1). These pooled samples were also split into two sets. One set was only spiked with ISs and one set that was spiked with ISs and a mixture of reference micropollutants (*i.e.*, target compounds). For the non-target screening, the pooled samples were injected in triplicate and were used for data cleaning, as advocated for in Bader et al. (2016), through the implementation of a replicate filter (discussed in Section 2.3.1 and in SI, Section S3).

Samples were enriched with a multi-layer online solid-phase extraction (SPE) and then measured in reverse-phase liquid chromatography, followed by positive electrospray ionization and high-resolution mass spectrometry (LC-ESI-HRMS). The mass range was 100–1000 *m/z*, mass error <5 ppm, and the resolution was 140,000 for MS1 spectra and 17,500 for MS/MS spectra. Further details on the analytical method can be found in the SI, Section S2.

2.3. Data analysis

In the following, ‘non-target feature’ is used when referring to a unique mass to charge (*m/z*) / retention time (RT) combination that was detected. The term ‘profile’ is used when referring to multiple detections of the same non-target feature across samples and the corresponding detected intensities of this feature.

Analysis of LC-HRMS data entailed the following steps. Detailed information on each step is provided in the sections below. First, pre-processing of the LC-HRMS data was done to detect unique exact mass / retention time pairs (*i.e.*, a ‘non-target feature’) in each sample. Second, to analyze the trends of non-target features in the different wastewater treatment settings, an automated trend assignment method was developed and applied. Third, two lists were used for feature annotation: (1) a list of known organic micropollutants and (2) a list of known ozonation transformation products (OTPs). Fourth, a classification method was applied similar to Anliker et al. (2020) to prioritize and identify possible industrial inputs in the two influent streams in ProReno. Finally, a linkage analysis was used to screen for possible unknown OTPs and compare their removal in different post-treatment settings.

2.3.1. Pre-processing

Acquired RAW files were converted to centroid data and .mzXML files with MSConvert (v. 3.0.11781) from ProteoWizard (Chambers et al., 2012). Unless otherwise noted, data analysis was performed in R (v.3.5.0/v.3.5.3, R Development Core Team (2017)) and RStudio (v.1.1.453/v.1.2.1335). The list of R packages used is provided in the SI, Table S3 and additional functions are available at www.github.com/dutchjes. The non-target screening workflow (SI, Fig. S4) is summarized below; more detailed information and evaluation of different processing steps is available in the SI, Section S3, Schollée et al. (2015), and Schollée et al. (2018). The evaluation of the pre-processing was done with a set of 147 isotopically labeled internal standards, for the quantification of false positives and false negatives.

New pre-processing steps include the following: a ‘profile grouping’ step; a replicate filter based on the pooled samples; a profile quality control filter; annotation and removal of homologues; and an algorithm for missing value imputation. The implementation of these additional steps are discussed below; their evaluation is discussed in the Section 3.1. Further information is provided in the SI, Section S3.

Pre-processing of HRMS data was performed as follows. (1) Peak picking and (2) profiling were done with functions from *enViPick* (v.2.4, Loos (2016)), settings in SI, Section S3) and executed with *RMassScreening* (Stravs 2020). Next, a (3) profile grouping step

was implemented, to detect cases where features belonging to the same chromatographic peak were split across multiple profiles (additional information in SI, Section S3; SI, Fig. S5). Thereafter, a (4) replicate filter was applied, using the triplicate injections of the pooled samples. Non-target features were only retained if present in all 3 injections of at least one pooled sample type (Bader et al., 2016; Schulze et al., 2020). Following the replicate filter, (5) intensity normalization was performed as in Albergamo et al. (2019) (SI, Fig. S6). A (6) profile quality control filter was developed to address cases where different chromatographic peaks were incorrectly assigned to the same profile. These cases were detected based on the boxplot rule to detect outliers (Tukey 1977; further information in SI, Section S3). Next, (7) isotopes, adducts, and homologues were annotated with the *non-target* package (v.1.9, Loos (2015), <https://github.com/blosloos/nontarget>; settings in SI, Section S3; SI, Figs. S7, S8, and S9) and removed, with the aim to (a) eliminate multiple profiles associated with one compound (isotopes and adducts) and (b) exclude profiles possibly associated with the wastewater matrix (homologues). Previous studies have shown that many of the homologues detected in wastewater are related to either surfactants (García et al., 2019; Mairinger et al., 2021), to matrix components such as proteins (Mairinger 2019), or to DOM (Verkh et al., 2018), all of which were not the focus of this characterization. In (8), intensities were adjusted based on the dilution factors of the different wastewater matrices. Finally, in (9), missing value imputation was done by randomly drawing values from below the estimated global instrument LOQ based on the feature intensity distribution (SI, Fig. S10, more information in SI, Section S3). This algorithm accounts for missing data resulting from non-target features being below the LOD. It does not address missing at random cases (*e.g.*, instances where the peak picking missed a high intensity peak); however, in our experience these occurrences are rare.

A final data matrix was obtained with a row for each non-target feature (defined by the mean *m/z* and RT of a profile), a column for each sample, and populated with the respective intensities. The pre-processing was optimized with 147 ISs and with the final workflow, 100% (147 ISs, corresponding to 156 profiles) were detected (SI, Table S4).

2.3.2. Automated trend assignment

To analyze trends of non-target features across a WWTP, a method was developed to automatically assign trends to each non-target feature. For each sampling date, each profile was normalized to the maximum intensity detected in the four sampling locations (*i.e.*, INF, BIO, OZO, EFF). Domains of high (100–60%), medium (60–20%), and low intensity (<20%) were defined (SI, Fig. S11) and the intensity at each treatment step was converted to 1, 0, -1, respectively, to generate an intensity profile ‘barcode’, which could then be interpreted as profile trends. Based on four treatment steps, three intensity domains, and the normalization method, 65 unique barcodes were generated (SI, Fig. S12). These were then assigned to one of 12 ‘major’ trends (SI, Fig. S13 and SI, Table S5), 11 of which correspond to expected patterns in the WWTP (*e.g.*, “removed in biological treatment”, “removed in ozonation”, “persistent”) and a twelfth for trends that didn’t fit to expected patterns in wastewater treatment (referred to generally as ‘other’). Overall, the numbers of features classified as ‘other’ were extremely low ($1.8 \pm 0.7\%$ of the total number of features; $n_{\text{samples}}=27$) and were therefore not further considered. Trend assignment was done separately for each sampling date. It was observed that a number of potential OTPs appeared to be formed on the sampling dates in PR where no ozone had been dosed. These features were assumed to result from either analytical or experimental artifacts caused by a biofilm in the ozonation column, and were removed from the entire dataset. Overall, this method represents a significant improvement over the

trend assignment that was previously done with hierarchical cluster analysis (Chiaia-Hernández et al., 2017; Schollée et al., 2018) because (1) trends are assigned individually for each profile, rather than for an entire cluster and because (2) trend assignment is done automatically, increasing reproducibility and throughput.

2.3.3. Qualitative target and suspect screening

A qualitative target screening was done with a list of 427 organic micropollutants. Targets were confirmed through m/z and RT matches to reference compounds, using 2.5 ppm and ± 30 seconds as tolerances for a positive match. From these, a list of 66 wastewater-relevant micropollutants (MP66; SI, Table S7), which have been previously reported in Swiss wastewater (Bourgin et al., 2018) and were detected on all sampling dates, was compiled. Furthermore, to evaluate different post-treatments, a suspect screening was also conducted for 999 known OTPs obtained from the literature (SI, Table S8). These OTPs were either previously found to be formed during wastewater treatment or in laboratory experiments. The list included OTPs from 84 parent micropollutants and spanned wide m/z and RT ranges (m/z : 86.0598–764.4791; RT: 5.1–24.7 minutes). Suspect hits were tentatively identified with exact mass only (5 ppm tolerance; confidence level 3, Schymanski et al., 2014a). Screening was done with functions available in *RMassScreening* (Stravs 2020) and at www.github.com/dutchjes/.

2.3.4. Characterization of WWTP influents and possible industrial contribution

Classification of non-target features possibly of industrial origin was done in the two influent streams from ProReno using intensity spread (calculated as the ratio of the 95th / 5th profile intensity percentiles) according to a method by Anliker et al. (2020), with the following modifications. First, classification was done with non-target features detected in influent samples, rather than with effluent samples. Classifying influent features was considered an advantage for this study because parent compounds are directly measured, prior to treatment and potential transformation, and because the removal over the whole treatment train could be evaluated. Furthermore, in PR, separate influent streams were collected from industrial and municipal discharges and different advanced treatment settings were sampled, so changes in intensity in the effluents could be a result of higher/lower removal efficiency and not necessarily related to industrial inputs. However, classification of influent features also presented a challenge because the expected intensity spread is higher, since detection of industrial peaks would be restricted to the day of emission and not spread out and diluted over multiple sampling days on account of retention in the WWTP. Therefore, the cutoff criteria for classification as an industrial input was increased to an intensity spread $>1E4$ and no minimum number of consecutive daily detections was included. Exceptionally large intensity spreads were generated in cases where the 5th percentile was actually a non-detect. Therefore, a low intensity baseline was simulated for the calculation of intensity spread, where intensities of previously imputed data points (i.e., non-detects) were increased by $1E5$. This modification allowed for the detection of one-time industrial inputs, without generation of high spread values due to the lack of continuous time series data.

After classification of possible industrial non-target features, structure elucidation was done. MS2 spectra were extracted with *RMassBank* (Stravs et al., 2013) and converted to .msp files. MS2 were compared to library spectra with NIST MS Search (v.2.3; Stein and Wallace 2017); spectral libraries included MassbankEU (Schulze et al., 2012; retrieved December 2019), MoNA (MassBank of North America; <http://mona.fiehnlab.ucdavis.edu>, retrieved December 2019), CSUPMF library (Broeckling et al., 2016), and NIST HRMS 2017. Matches were visually inspected and finally a best match was selected (confidence level 2b; Schymanski et al. 2014a).

2.3.5. Linkage analysis

Linkage analysis, where parents and possible TP's are linked together, was performed as described in Schollée et al. (2015) and Schollée et al. (2018) to detect possible OTPs. The table of reactions that can be expected based on known ozonation mechanisms (e.g., addition of oxygen atoms, demethylation, SI, Table S9) was edited to include additional known transformations. Expected ozonation transformations were derived from OTPs reported in literature and from predicted OTPs (O3-PPD; Lee et al., 2017; Schollée et al., in prep). In total, 45 possible reactions were included. Possible parent compounds were any feature with a trend that decreased during ozonation, while possible OTPs were any feature assigned a trend that increased during ozonation. An m/z window of 5 ppm was used to link possible pairs.

3. Results and discussion

3.1. Evaluation of pre-processing of optimized non-target screening workflow

In accordance with discussions in the non-target screening community about data quality (Schulze et al., 2020), a number of new pre-processing steps were implemented into previously developed non-target screening workflows (Schollée et al., 2018; Schollée et al., 2015) to reduce noise and eliminate artifacts or redundant non-target features. These additional steps were evaluated with a set of 147 internal standards (ISs). The motivation and impact of these additional steps is discussed briefly below; detailed information can be found in the SI, Section S3.

Initially, after peak picking and profiling, a total of 459,867 non-target features were detected (Fig. 1). To evaluate the efficiency of the pre-processing, non-target features of the internal standards were used. Based on exact mass and RT, 1136 of non-target features matched to the expected m/z and RTs of the 147 ISs. For the purposes of the evaluation of the pre-processing, duplicate hits of the ISs were labeled as false positives (at least 989), since multiple features were detected for each IS. No false negatives were found, since at least 1 non-target feature was detected for each IS. Additional workflow steps were therefore incorporated to reduce the number of IS false positives and the overall number of non-target features detected. The first additional step was a 'profile grouping' algorithm. Using very narrow m/z and RT windows (1 ppm and 20 seconds, respectively), the grouping algorithm combined possible 'split peaks' into one profile. This step resulted in a 14% reduction in the total number of features (Fig. 1) and reduced the number of detected IS features from 1136 to 929, thereby lowering the number of false positive IS hits by 21% (from 989 to 782; SI, Table S4). The second additional step included was a replicate filter based on the pooled samples, adopted from standard methods in metabolomics (Broadhurst et al., 2018). Overall, higher numbers of features were detected in the pooled samples compared to the individual samples (SI, Fig. S15), suggesting that very few compounds fell below the LOD due to dilution in the pooled samples. Through implementation of this quality control step, the number of detected IS features decreased from 929 to 161, thereby decreasing the false positive IS features to 14 (98%). Overall, the number of non-target features was reduced by 83%. Next, intensity normalization was incorporated to account for matrix effects from the different wastewater matrices, as explained in Alberghini et al. (2019) and resulted in a similar intensity distribution across all samples (SI, Fig. S6). Subsequently, a profile quality control criterion considered the intra-profile variability of m/z and RT. The number of detected IS features decreased to 158, with 11 false positives, while the percent of detected non-target features was further decreased by 10%.

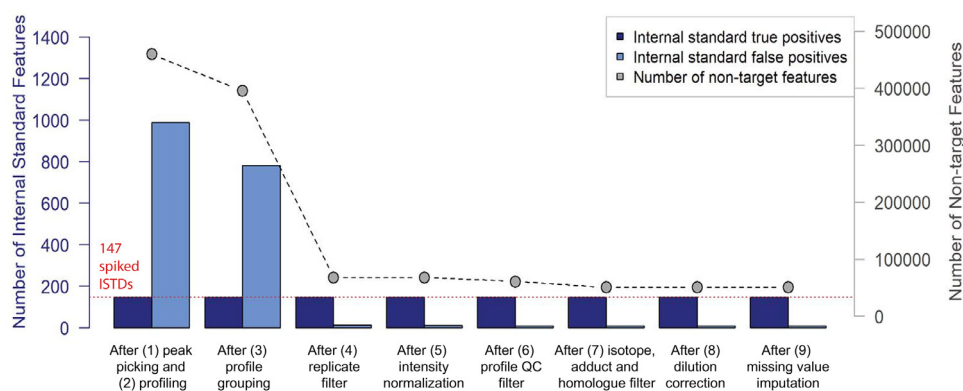


Fig. 1. Summary of the total number of non-target features and the number of internal standard features detected after each step of the non-target screening workflow. The number of true positive and false positive non-target features corresponding to internal standards are plotted in dark and light blue bars, respectively, on the primary (left) y-axis. The total number of spiked internal standards was 147 and is indicated by a horizontal dotted red line. Total number of non-target features is plotted by a black, dashed line on the secondary (right) y-axis (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

Potential interfering or redundant features, isotopes, adducts, and homologues were annotated ('non-target' package; v1.9, Loos 2015) and were removed to reduce redundancy in the data set. The number of detected ISs decreased to 156 (false positives reduced by 2); overall, the number of non-target features decreased by 17%, from 60,953 to 50,722 through implementation of this criteria. Thereafter, feature intensities were adjusted for dilution (1:3 for INF samples, 1:1 for all other samples) and missing value imputation was applied, which estimated the intensity LOD per profile and randomly sampled from below this threshold to fill non-detects. Through this optimized method, a highly curated data set was produced, with 0% false negative IS hits, 100% true positive IS hits, and 50,722 detected non-target features.

3.2. Evaluation of automated trend assignment across WWTPs

Trends were assigned to non-target features on each sampling date based on the normalized intensity profile, to indicate if the feature was removed, persistent, or formed, as described in Section 2.3.2 and in the SI, Section S3. The assignment of compounds into either one or another trend is governed by the definition of the cutoffs between the three normalized intensity domains. Therefore, a sensitivity analysis was conducted with different domain cutoffs (SI, Table S12) to determine the robustness of the trend assignment. Domain cutoffs considered were 20/60%, 30/70%, 20/80%, and 10/90%, where the first number is the cutoff between the low and middle domains and the second number is the cutoff between the middle and high domains. Differences among the percent of features assigned to a particular trend ranged only from -1.6 to 1.7% (SI, Figs. S19 and S20), establishing the method to be robust against the selection of cutoffs for the high, middle, and low domains (further details in the SI, Section S5). In the end, 20% was selected as the lower cutoff, representing a minimum intensity elimination of 80%, because it corresponds to the overall micropollutant abatement target set by the new Swiss regulation. For the upper cutoff, 60% was chosen because it equally divided the remaining portion.

This automated trend assignment was validated by comparing trends assigned to the non-target HRMS data with trends assigned to eight target compounds quantified in the same samples. Additionally, trends assigned to the internal standards were evaluated. Quantified target screening results of the so-called "Swiss indicator substances" (Götz et al., 2015) were reported elsewhere (GL: McArdell et al., 2020; Oltramare et al., in prep; AR: Bogler 2019; PR: Krahnstöver et al., 2018) and trends were assigned through the same method as for the non-target HRMS data, except with quan-

tified concentrations rather than non-target feature intensities. For each sampling day, concentrations were normalized to the maximum concentration, normalized concentrations were binned into high, middle, and low domains and a trend was assigned. In total, quantified data was available for 8-9 target compounds on 25 sampling days, resulting in 206 trend assignments for comparison. Of these, 172 trends were the same between the quantified target data and the non-target profile intensity data, with an average of $84 \pm 17\%$ consistency per sampling date (SI, Table S6), demonstrating that overall, the trends assigned to the non-target features was representative of the results obtained with quantified targets. Finally, the trend analysis was also evaluated based on 147 detected ISs, corresponding to 156 profiles. Of these, $90 \pm 3\%$ were assigned the correct trend (i.e., stable intensity across sampling points (Trend 10)); this was increased to $95 \pm 3\%$ when the 8 profiles resulting from split profiles were manually removed.

3.3. Characterization of influents and potential industrial contribution

A general characterization of the four influent streams (i.e., GL, AR, PR-Comm, and PR-Chem) was performed (further details in SI, Section S4; principal component analysis (PCA) in SI, Figs. S16 and S17). Subsequently, in PR, a classification method was applied to find influent features potentially of industrial origin. Finally, structure elucidation was performed on the selected potential industrial features.

A total of 37,427 non-target features were detected in the four influent streams ($n_{GL}=3$; $n_{AR}=5$; $n_{PR-Comm}=15$, $n_{PR-Chem}=14$), with between 17,514 (GL) and 22,862 (PR-Chem) non-target features detected in one influent stream. Almost a quarter (23%) of all influent features (11,482 features, SI, Fig. S17) were found in all four influents, indicating wastewater commonality despite different industrial inputs. In contrast, between 2–8% features were unique to one influent stream. In the PCA (SI, Fig. S17), some differences are observed among the influents. For example, samples from the first sampling campaign in AR appeared to be substantially different (SI, Fig. S17); however, it is not clear what the cause of this difference is, perhaps a change in industrial input in the catchment (e.g., due to batch production).

Classification of potential industrial inputs with the intensity spread method was carried out only on the two PR influent streams, since calculating the intensity spread was not possible in AR and GL on account of the low number of influent samples (further details in Section 2.3.4). This classification resulted in a total of 1207 non-target influent features as potential industrial features (SI, Table S10). In PR-Comm, 274 (1.3% of detected features) and in

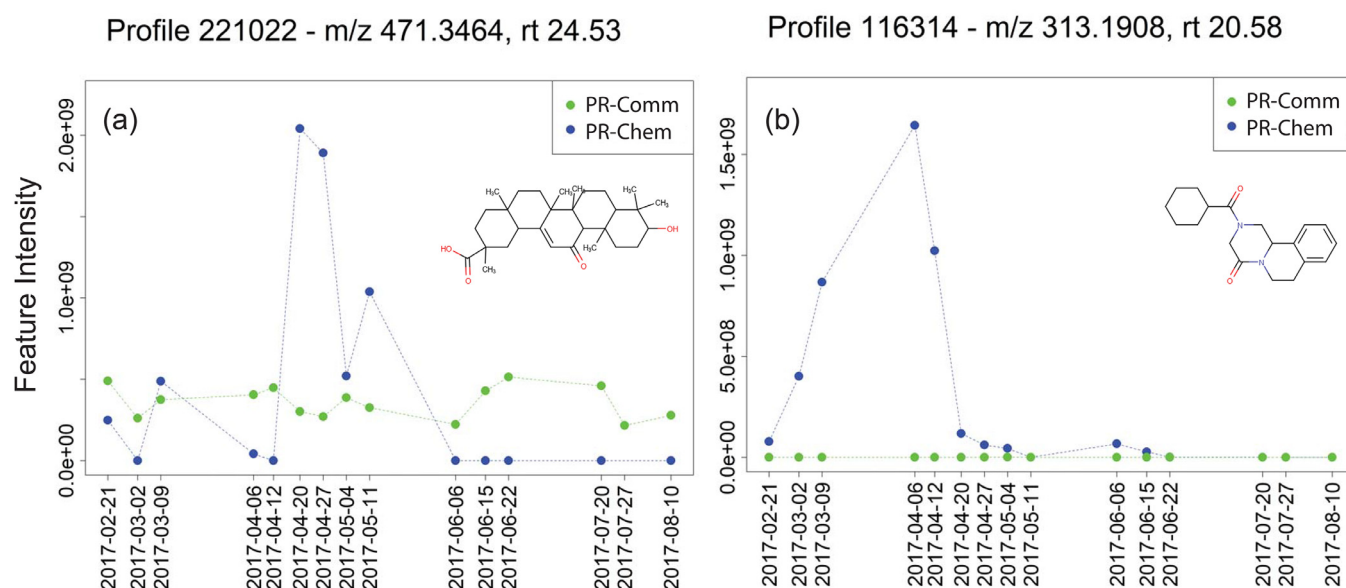


Fig. 2. Intensity profiles in the influent of two potential industrial features (based on intensity spread), identified (confidence level 2b) as (a) 18 β -glycyrrhetic acid and (b) praziquantel. Along the x-axis is sampling date (YYYY-MM-DD), while on the y-axis is the absolute intensity of the feature in the WWTP influent stream (PR-Chem is shown in blue and PR-Comm in green). In the upper right is the molecular structure of the proposed chemical. Both compounds were observed to be completely removed during subsequent wastewater treatment (18 β -glycyrrhetic acid completely in biological treatment; praziquantel partially in biological treatment (77% intensity reduction) and completely in advanced treatment with ozonation followed by PAC+SF).

PR-Chem 977 (4.3%) potential industrial features were detected (SI, Table S10 and Fig. S18; note that some features were classified as industrial in both influents).

To identify the potential industrial compounds, MS2 spectra were extracted and compared to the NIST spectral library. Of the 1207 possible industrial features, MS2 spectra was available for 754 features; this list was further reduced to features with 'clean' MS2 spectra (defined as msPurity score >40%; Lawson et al. 2017), resulting in 556 MS2 spectra that were evaluated. Matches to library spectra were found for 54 compounds (i.e., confidence level 2b; Schymanski et al., 2014a). Overall, 37 of the 54 identified compounds were present in the NORMAN Substance Database (NORMAN SusDat; Aalizadeh et al., 2020), including 21 that were on the STOFF-IDENT (Letzel et al., 2017) list of environmentally relevant compounds and included REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals; EC No 1907/2006) chemicals, pharmaceuticals, and transformation products (SI, Table S10).

Identified compounds included some that could be confirmed from the qualitative target screening, e.g., 18 β -glycyrrhetic acid, 1H-benzotriazole, atenolol, carbamazepine, crotamiton, mycophenolic acid, naproxen, N-desmethyltramadol, oseltamivir, praziquantel, and propamocarb. Intensity profiles of 18 β -glycyrrhetic acid (pharmaceutical used in dermatology and cosmetology applications) and praziquantel (a pharmaceutical only licensed for veterinary use in Switzerland) are shown in Fig. 2. In both examples, high peaks detected over 1-2 months was observed in PR-Chem samples. In the PR-Comm samples, no detections of praziquantel were observed, while for 18 β -glycyrrhetic acid, a relatively constant baseline was observed, as could be expected from domestic use.

3.4. Summary of non-target feature trends

Using the optimized non-target screening workflow and automated trend assignment, the fate of all non-target features, including potential industrial inputs and known target compounds (MP66 list), could be traced across the three WWTPs. The trends (detected through the automated trend assignment) of all non-target features

were aggregated in the different treatment steps and are shown in Fig. 3. At each sampling location, features still detected after the respective treatment step were annotated as >80% removed and <80% removed (details in the SI, Section S6). The cumulative intensities of features for each sample setting and at each sampling point are shown in SI, Fig. S22.

Overall, as is expected, the WWTPs provide large reductions in both the number and the intensity of detected non-target features, many of which are likely to be matrix (i.e., DOM). The number of features is reduced by 6-63% (median 36%) from the influent to the effluent (Fig. 3 and SI, Table S14) and the cumulative feature intensity is on average 8.5x less in the effluent than in the influent (SI, Table S14 and SI, Fig. S22). Following along each treatment step, the formation of new features is observed, as well as the elimination of non-target features. For example, after biological treatment, across all WWTPs between 36 and 52% of the detected features are newly formed (median 46%), while the other portion are influent features that have not been (completely) eliminated. These values are in agreement with those recently reported by Nürenberg et al. (2019), who found that after biological treatment, the percent of features originating from the influent was between 33-45%. Formation of features during ozonation and post-treatment with activated carbon was also observed, resulting in the effluents in a complex mixture of influent features, biological TPs, OTPs, and TPs formed during activated carbon treatment, as discussed in detail below. On average across all WWTPs, the effluent was composed of approximately 45% features that originated from the influent (including features removed by >80% and features removed <80%), between 10-20% were features formed during biological treatment, and around 10% each were features formed during either ozonation or post-treatment with GAC or PAC+SF (Fig. 3).

3.5. Fate of influent features

A comparison was made between the removal of all influent non-target features and the removal of a selected set of 66 wastewater-relevant micropollutants (i.e., the MP66 list). The

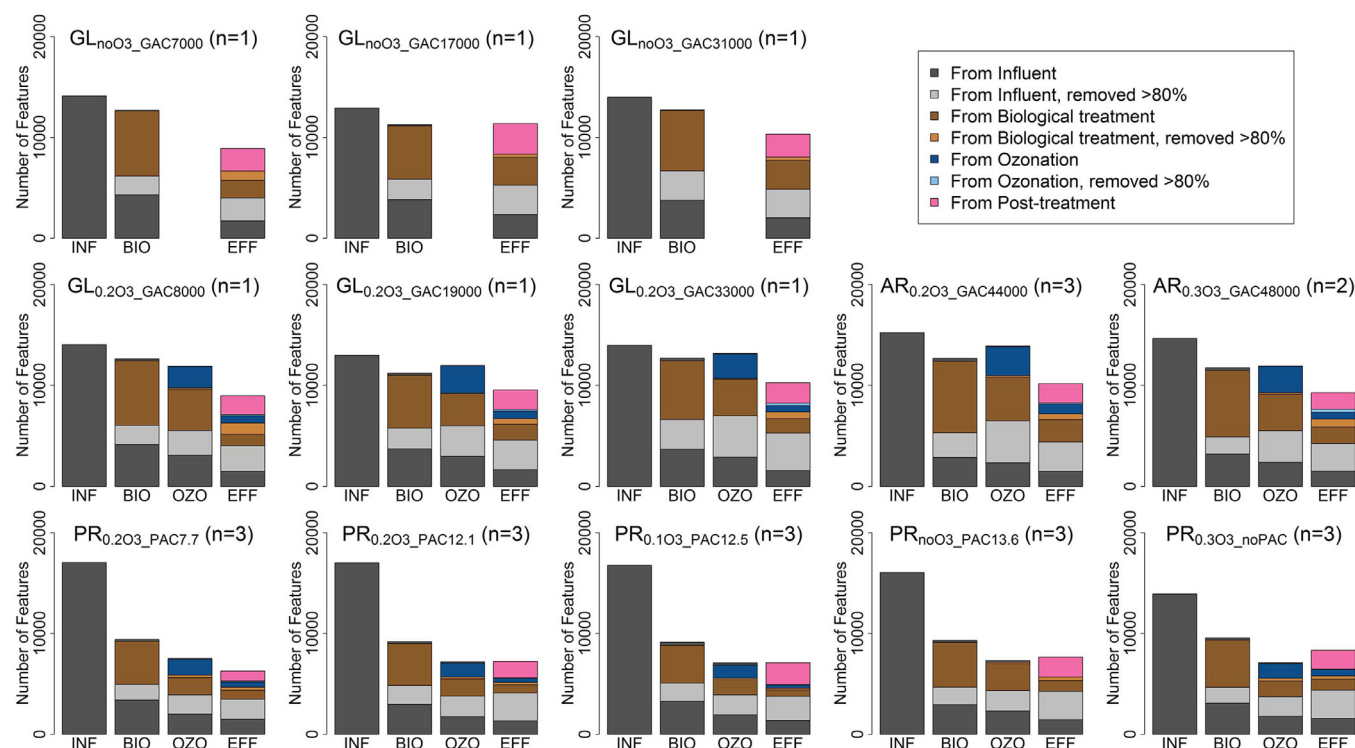


Fig. 3. Number of non-target features at each step of the WWTP (INF: influent; BIO: after biological treatment; OZO: after ozonation; EFF: effluent after post-treatment), with the average for each sample setting shown. Features present but removed by more than 80% (e.g., from influent, removed >80%) are differentiated from the features removed by less than 80% (e.g., from influent); features 100% removed (i.e., non-detect in the respective treatment step) are not shown. The WWTP and the applied advanced treatment settings are shown in the title of each graph (as in Table 1). GL: WWTP Glarnerland; AR: WWTP Altenrhein; PR: WWTP ProRheno; O₃: ozone dose in gO₃/gDOC; GAC: granular activated carbon filtration with bed volumes; PAC: powdered activated carbon in mg/L dosed onto sand filter. Colors indicate the fate of features in each treatment step, as indicated in the legend.

largest contributor to the removal of influent non-target features in all three WWTPs was biological treatment, with 66–81% removed by more than 80% (Fig. 4a). In contrast, only 6–42% of compounds from the MP66 list were removed in BIO by >80% (Fig. 4b). This difference suggests that many of the non-target features removed during BIO are matrix-related compounds and/or that the MP66 list is biased toward compounds that are persistent during biological treatment.

Nevertheless, differences in elimination were seen among the WWTPs. For example, the highest numbers of influent non-target features were well-removed during biological treatment at PR (Figs. 3, 4, SI, Tables S13 and S14), which can be expected due to recirculation of loaded PAC from the post-treatment into the SBR, providing additional sorption sites. Higher removal in biological treatment in PR was even more apparent when considering the compounds in the MP66 list (Fig. 4b), with PR removing 2–5x more target compounds than the other WWTPs.

Some misclassification of features can occur with the automated trend assignment. For example, eight target compounds were assigned the trend 'removed in ozonation' in the setting in PR where no ozone was dosed (Fig. 4b). In reality, these features were partially removed during biological treatment (approximately 50% removal), followed by a further (smaller) intensity decrease (approximately 35%) after ozonation, possibly due to matrix effects. Due to the selected cutoffs and reduction of 63 minor trends into only 12 major trends, this intensity pattern was assigned as 'removed in ozonation'. However, the original minor trend can easily be used to identify such instances and generally misclassification with this method is expected to be less than with other classification method (e.g., hierarchical cluster analysis).

During ozonation, 7–18% of influent non-target features detected after biological treatment were removed >80% in GL and

AR at a specific ozone dose of 0.21 ± 0.08 gO₃/gDOC, while 3–28% of the compounds in the MP66 list were removed. For both the non-target features and the MP66 compounds, a difference was seen between the two ozone doses in AR. For the non-target features detected after biological treatment, $6.4 \pm 2.7\%$ (n=3) were well-removed (>80%) at AR_{0.2O3_GAC44000}, while $24.8 \pm 1.4\%$ (n=2) were well-removed at AR_{0.3O3_GAC48000}. Similarly, a difference was seen for the MP66 compounds (Fig. 4b). The number of target compounds removed >80% during ozonation was 4 at the lower specific ozone dose (0.15 ± 0.03 gO₃/gDOC) and increased to 16 at the higher specific ozone dose (0.32 ± 0.04 gO₃/gDOC). In GL, removal of the MP66 compounds differed, even though similar ozone doses were applied. The second sampling date was possibly influenced by rain or snow melt, but still showed a high organic matter content (Table 1), pointing to a different matrix composition that may have influenced the removal of compounds. In PR, the samples reported to be dosed at 0.1–0.2 gO₃/gDOC did not show a clear trend for higher removal at higher ozone dose. Moreover, higher removal of influent non-target features, as well as MP66 compounds, was observed compared to AR and GL at similar specific ozone doses (between 21 and 27% of influent non-target features and between 45 and 66% of MP66 compounds were removed >80%) (Fig. 4a and b). Based on these values and on the observed abatement of quantified target compounds (Krahnstöver et al., 2018), we assume that ozone doses applied at this location were higher than reported and showed a high uncertainty.

The higher ozone dose applied at PR complicates the evaluation of the post-treatments PAC+SF. Due to increased elimination in ozonation, compounds are already close to the LOD prior to post-treatment. Any additional elimination achieved in post-treatment cannot be fully quantified and therefore, the elimination in PAC+SF is likely underestimated. At average PAC doses of 7.7 and 13.6 mg/L



Fig. 4. Non-target features in the WWTP influents and their fate in subsequent treatment steps (a) for all non-target features and (b) for a selected set of 66 micropollutants. Stacked bars are shown for each sampling date. The bar colors indicate the fate of the influent feature, i.e., if it was removed >80% in biological treatment, removed >80% during ozonation (possibly partial removal in biological treatment), removed >80% during activated carbon post-treatment (with possibly partial removal in biological treatment and/or ozonation), or if it was persistent across all treatment steps. Bars are normalized to the total number of features on the respective sampling date. The absolute number of features in each category is given in the bars. "Other" refers to influent features that did not belong to one of the indicated categories (for example, when the feature intensity increased in one of the treatment steps; see Section 2.3.2). Below each bar are the corresponding specific ozone doses (gO_3/gDOC) and granular activated carbon bed volumes (GAC BV) or powdered activated carbon dose (mg/L PAC).

(corresponding to the two sampling campaigns with reportedly similar specific ozone doses of $0.2 \text{ gO}_3/\text{gDOC}$), 8–21% of remaining non-target features are removed >80%, while 13–59% of MP66 compounds are removed, without clear differences among the different PAC doses.

A number of interesting observations were found with regard to the effect of GAC BVs and the coupling of O_3 +GAC. In GL, more of the remaining non-target features were well-removed in the freshest GAC (33%; 7,000–8,000 BV), with a relatively small decrease in removal with increasing BVs (18–25%; 17,000–33,000 BV). This pattern was observed for both all non-target features and the MP66 list and also with and without pre-ozonation, though the decrease was more pronounced in the case of GAC without pre-ozonation (removal decreased by approximately 15% without pre-ozonation, compared to approximately 5% with pre-ozonation). This difference may be due to higher biological activity on the GAC after pre-ozonation, though more research is needed on this point. The percent of well-removed features was smaller during the second sampling (GAC BVs 17,000–19,000) compared to the third sampling (GAC BVs 31,000–33,000), which was not expected. This difference may be explained by a higher influent flow (resulting in lower inflow concentrations) on the second sampling day, which

was found to decrease the sorption efficiency in GAC columns (Oltamare et al., in prep, McArdell et al., 2020). These results show that GAC alone removes quite a high number of remaining features, but a pre-ozonation can increase the overall removal under conditions where the GAC filtration may not perform optimally. The influence of the ozone dose can be seen in AR, especially when considering the MP66 list. The increase of the specific ozone dose from $0.15 \text{ gO}_3/\text{gDOC}$ to $0.32 \text{ gO}_3/\text{gDOC}$ led to a slight increase in the number of target compounds removed in the advanced treatment (i.e., O_3 +GAC) from 36 to 43 (44,000 and 48,000 BV, respectively). Previous results (Boehler et al., 2020; McArdell et al., 2020) have shown that for target compounds, pre-ozonation prior to GAC filtration can be an effective method to extend the lifetime of the GAC; these examples show that this may also be true for non-target compounds, though further research will be necessary. However, a combined treatment of course requires additional investment costs.

Finally, the fates of the 1207 potential industrial compounds prioritized in Section 3.3 were also investigated. Because the industrial inputs were sporadic and generally short-lived, the removal was evaluated only in the sample with the highest intensity. This meant that, depending on when the discharge was observed,

different advanced treatment settings may have been applied for the removal of different features. The majority of the potential industrial compounds were well-removed (*i.e.*, >80% removal) during biological treatment (SI, Table S11), indicating that traditional wastewater treatment is in many cases sufficient to remove these compounds. Ozonation was also an important removal mechanism and was responsible for elimination an additional 9% of the detected industrial features; 11% were well-removed with ozonation combined with post-treatment. Less than 3% of potential industrial features were found to be persistent. These results demonstrate that although emissions of high intensity can occur, in the overwhelming number of cases these emissions are substantially reduced through the wastewater treatment technologies applied.

3.6. Formation and fate of transformation products

During wastewater treatment, non-target features are removed, but new features (*i.e.*, TPs) are also formed, as seen in Fig. 3 and discussed above. The assessment of the formation and subsequent removal of these TPs is important for the overall evaluation of different wastewater treatment steps.

Similar to the compounds originating from the influent, TPs formed from biodegradation (*i.e.*, bio-TPs) were removed in ozonation better in PR compared to GL and AR. This is most probably due to ozone doses at PR that was higher than reported, although other factors may also be responsible (*e.g.*, different wastewater matrices). Slightly higher ozone doses in AR had also a minor effect with regards to the removal of bio-TPs (66% removal in AR_{0.103_GAC44000} vs. 74% removal in AR_{0.303_GAC48000}; $p < 0.01$, *t*-test). With regard to GAC, the highest percentage of bio-TPs were well-removed in the freshest GAC (73% in GL_{no03_GAC7000}; 48% in GL_{no03_GAC17000}; 52% in GL_{no03_GAC31000}), as was expected, and on average more bio-TPs were removed when pre-ozonation was applied ($74 \pm 7\%$ removal >80% with pre-ozonation vs. $57 \pm 13\%$ removal without pre-ozonation).

The formation of TPs in both PAC+SF and GAC post-treatment was also observed. The formation of TPs during GAC and sand filtration has also been reported in Nürenberg et al. (2019) and is not surprising, since both the GAC filter and the sand filter (used for PAC removal) can be expected to be biologically active (Reungoat et al., 2012). Between 11–40% of non-target features detected in the effluent were formed during post-treatment with AC; however, no significant differences were observed among the various post-treatments.

Of particular interest was the formation of OTPs and their fate in activated carbon post-treatment due to the novelty of coupling these technologies for wastewater treatment. It is clear that not all micropollutants or OTPs are captured through reverse-phase LC; however, this method is standard practice for the measurement of polar, organic compounds in environmental matrices and is expected to be appropriate to measure a wide variety of micropollutants and associated TPs. Through the trend assignment method, 5–13% non-target features (1911 features on average) with increasing intensities after ozonation were classified as 'non-target OTPs' (Fig. 3). A subset were suspect OTPs, based on an exact mass suspect screening with a list of 999 known OTPs (SI, Table S8; details in Section 2.3.3). Per sampling date, the average number of suspect hits with an OTP trend was 53 (min 43, max 65, $n_{\text{samples}}=20$) (SI, Table S16). The suspect OTPs were previously found to be formed from 84 relevant micropollutants in laboratory experiments or wastewater treatment, while many of the non-target OTPs are likely formed from reactions with the wastewater matrix, as well as OTPs from other micropollutants. The amount of both non-target and suspect OTPs formed during ozonation did not correlate with ozone dose (SI, Table S16). This was previously reported for non-target OTPs with a broader range of ozone doses

(Schollée et al., 2018) and is thought to result from the differing wastewater matrices, as well as different OTPs (*i.e.*, higher generation OTPs) being formed at higher ozone doses.

Overall $68 \pm 9\%$ of non-target OTPs were well-removed (*i.e.*, eliminated >80%) during post-treatment. This value was significantly lower for the suspect OTPs (Student's *t*-test, $p < 0.01$), of which $59 \pm 15\%$ were well-removed, indicating that known OTPs originating from micropollutants may be more stable during post-treatment (Fig. 5 and SI, Table S16). In both cases, the lowest removal was observed in PR with no PAC addition onto the sand filter PR_{0.303_noPAC} (Tukey's HSD, $p > 0.01$; 54% non-target OTPs and 43% suspect OTPs were well-removed).

Notable differences in OTP removal among the post-treatments could be observed (though not statistically significant; $p > 0.05$, *t*-test). In PR, a higher removal of suspect OTPs (and to a smaller extent non-target OTPs) was observed at 12–13 mg/L PAC compared to 8 mg/L PAC (Fig. 5a). Nevertheless, a high removal was also observed when no PAC was added onto the sand filter, indicating that a large part of removal is due to biological transformation. Others found that OTPs are only moderately well-removed during sand filtration and/or biofiltration without additional sorptive removal by AC (Gulde et al., 2021; Knopp et al., 2016; Schollée et al., 2018). We can, however, not exclude that removal with PAC is underestimated, as discussed in Section 3.5. Looking at GAC filtration across both GL and AR, removal of suspect OTPs was highest for the freshest GAC, as expected (65%, 47%, 59%, and 51% OTP removal at 8,000, 19,000, 33,000, and 44,000 BVs, respectively; Fig. 5b). Lower removal at 19,000 BV is likely related to higher flow on this sampling day, as discussed in Section 3.5. Contrarily, removal for non-target OTPs was not significantly different among the GAC sampled at different BVs (66% in GL_{0.203_GAC7000}; 72% in GL_{0.203_GAC17000}; 74% in GL_{0.203_GAC31000}; 67% in AR_{0.203_GAC44000}; 73% in AR_{0.203_GAC48000}). Lower sorption with higher BV may be compensated by higher biotransformation, which could also be the case for the suspect OTPs, but to a lesser extent.

A linkage analysis (Schollée et al., 2015) was performed, using 45 expected ozonation transformation reactions. The possible parent compounds were non-target features with decreasing trends during ozonation, while possible OTPs were non-target features with increasing trends during ozonation. The number of possible parent compounds, which could be linked to OTPs through the expected transformations, per sampling date ranged from 2576 to 4341, while the number of possible OTPs ranged from 1108 to 3579. It should be noted that in this analysis, parents can be matched to many OTPs and vice versa and false links may arise (Schollée et al., 2018). The reaction types detected most were +2H, -2H, +H₂O, +O, and -CH₃ (SI, Fig. S25), which have also been reported in previous linkage analyses during ozonation of non-targets (Schollée et al., 2018) or of DOM (Remucal et al., 2020). Hierarchical cluster analysis revealed one cluster where the OTPs appeared to be better removed at higher GAC BVs (SI, Fig. S26). This cluster included the transformation reactions +3O-H, +4O-H, and +5O-H and suggests that for these highly oxygenated OTPs, biological degradation is the primary removal mechanism (further details in SI, Section S7).

Finally, the characteristics of well-removed and stable OTPs were compared. On all sampling dates, the median *m/z* of stable OTPs was lower than for well-removed OTPs (SI, Fig. S23). In the GAC samples, the median RT of stable OTPs was also lower than for well-removed OTPs (SI, Fig. S24). These results imply that OTPs not well-removed in GAC filters were generally smaller and more polar, *i.e.*, less prone to sorption, as has been reported previously (Schollée et al., 2018). This RT change was not observed in most of the samples treated with PAC, possibly suggesting different removal mechanisms between the two post-treatment types. Overall, these results demonstrate that, based on the non-targets detected

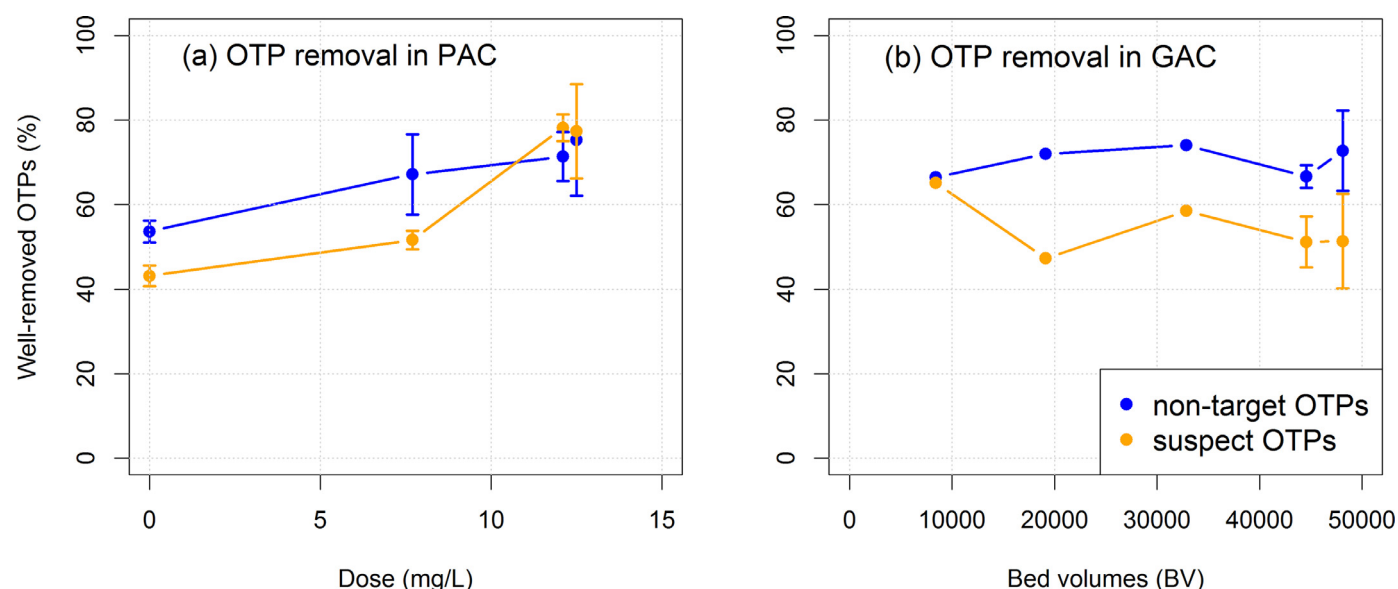


Fig. 5. OTP removal in post-treatments during (a) powdered activated carbon (PAC) + sand filter treatment in PR at different PAC doses and (b) granular activated carbon (GAC) filtration in GL (7,000–33,000 BV) and AR (44,000–48,000 BV). On the y-axis is the percent of OTPs well-removed (*i.e.*, >80% elimination) in the post-treatment. Shown are the data for both the suspect OTPs (43–65 features, in orange) and for the non-target OTPs (1108–3579 features, in blue) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

with the applied LC-HRMS method, both GAC and PAC+SF appear to be suitable for the removal of OTPs formed during ozonation.

4. Conclusions

Improvements and novel additions to previous non-target screening workflows resulted in a fully validated method for the characterization and identification of detected LC-HRMS non-target features. With this workflow, trends were automatically assigned to each feature detected in the WWTP. Subsequently, formation and elimination of features in different advanced wastewater treatments could be evaluated, both for all detected non-target features and for a subset of 66 wastewater-relevant micropollutants. Moreover, a prioritization method was applied to identify non-target features potentially of industrial origin and assess their fate during wastewater treatment. Finally, the applicability of GAC and PAC+SF as post-treatment for the removal of OTPs was evaluated.

The application of this workflow showed that:

1. The majority of non-target features and features intensities, including those potentially originating from industrial inputs, were removed during biological treatment at all three WWTPs. Approximately three-quarters of all features were well-removed (*i.e.*, >80% intensity reduction), demonstrating that this treatment step is effective for the abatement of a broad spectrum of compounds that are detected with RPLC-HRMS, including both matrix component such as DOM and polar organic micropollutants.
2. Advanced treatment provided an additional barrier and contributed to the removal of features recalcitrant in biological treatment. This step is especially important for the removal of micropollutants (measured here with a set of 66 wastewater-relevant compounds), of which 38–80% were removed by >80% during advanced treatment. During biological treatment, only 6–40% of these compounds were well-removed.
3. Between one and five percent of all non-target features detected in the influent of one WWTP were classified as potentially originating from industrial inputs; 54 compounds could be tentatively identified through spectral library database matching. More than 83% of the assigned industrial non-targets

were well-removed during biological treatment, while an additional 11% of features well-removed during advanced treatment. These results demonstrate that in the majority of cases, the applied wastewater treatment lead to a substantial reduction in potential emissions.

4. Approximately half of the features detected in the effluent originated from the influent, while the other half were transformation products formed during the various treatment steps. This result emphasizes the importance of understanding the formation and toxicity of transformation products, as well as high-intensity influent features that are not completely abated during treatment.
5. More than half of the non-target features formed during ozonation (*i.e.*, non-target OTPs) were well-removed during activated carbon post-treatment, regardless of GAC BVs or PAC dose. OTPs expected to be highly oxygenated were better removed at higher GAC BVs. In contrast, for suspect OTPs expected to be formed from micropollutants, higher removal was observed at higher PAC doses and in fresher GAC. Different reaction mechanisms, *i.e.*, sorption vs. biodegradation, influence OTP removal in GAC filters, emphasizing the need for more research on the performance of biological activated carbon filters.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2021.117209](https://doi.org/10.1016/j.watres.2021.117209).

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