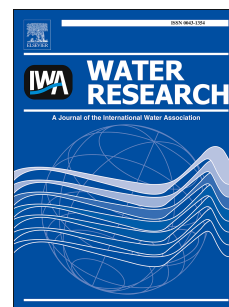# Accepted Manuscript

Non-target screening to trace ozonation transformation products in a wastewater treatment train including different post-treatments

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Feature Intensity

High

Low

Before CAS

After CAS

After ozonation

After post-treatment

Persistent in activated sludge
Removed in ozonation

Formed in ozonation
Removed in post-treatment

Formed in ozonation
Persistent in post-treatment

Non-target Screening to Trace Ozonation Transformation Products in a Wastewater Treatment Train including different Post-treatments

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1 Abstract

2 Ozonation and subsequent post-treatments are increasingly implemented in wastewater treatment

3 plants (WWTPs) for enhanced micropollutant abatement. While this technology is effective,

4 micropollutant oxidation leads to the formation of ozonation transformation products (OTPs). Target

5 and suspect screening provide information about known parent compounds and known OTPs, but for

6 a more comprehensive picture, non-target screening is needed. Here, sampling was conducted at a

7 full-scale WWTP to investigate OTP formation at four ozone doses (2, 3, 4, and 5 mg/L, ranging from

8 0.3-1.0 gO_3/gDOC) and subsequent changes during five post-treatment steps (*i.e.*, sand filter, fixed

9 bed bioreactor, moving bed bioreactor, and two granular activated carbon (GAC) filters, relatively fresh

10 and pre-loaded). Samples were measured with online solid-phase extraction coupled to liquid

11 chromatography high-resolution tandem mass spectrometry (LC-HRMS/MS) using electrospray

12 ionization (ESI) in positive and negative mode. Existing non-target screening workflows were adapted

13 to (1) examine the formation of potential OTPs at four ozone doses and (2) compare the removal of

14 OTPs among five post-treatments. In (1), data processing included principal component analysis

15 (PCA) and chemical knowledge on 31 possible oxidation reactions to prioritize non-target features

16 likely to be OTPs. Between 394 and 1328 unique potential OTPs were detected in positive ESI for the

17 four ozone doses tested; between 12 and 324 unique potential OTPs were detected in negative ESI.

18 At a specific ozone dose of 0.5 gO_3/gDOC, 27 parent compounds were identified and were related to

19 69 non-target features selected as potential OTPs. Two OTPs were confirmed with reference

20 standards (venlafaxine *N*-oxide and chlorothiazide); 34 other potential OTPs were in agreement with

21 literature data and/or reaction mechanisms. In (2), hierarchical cluster analysis (HCA) was applied on

22 profiles detected in positive ESI mode across the WWTP and revealed 11 relevant trends. OTP

23 removal was compared among the five post-treatments and 54-83% of the non-target features that

24 appeared after ozonation were removed, with the two GAC filters performing the best. Overall, these

25 data analysis strategies for non-target screening provide a useful tool to understand the behavior of

26 unknown features during ozonation and post-treatment and to prioritize certain non-targets for further

27 identification.

28 **Keywords**

29 Advanced wastewater treatment

30 Ozonation

31 Non-target screening

32 Transformation products

33 Micropollutants

34

35

36 1.1 Introduction

37 Thousands of organic compounds originating from domestic and industrial sources are discharged to

38 municipal wastewater treatment plants (WWTPs). During conventional treatment in WWTPs, these

39 compounds can be removed through sorption or degradation, or pass through treatment unchanged

40 (Loos et al. 2013, Luo et al. 2014). The subsequent discharge of treated wastewater introduces

41 compounds that are not removed during treatment into receiving waters, where they have the potential

42 to affect downstream aquatic communities or contaminate drinking water resources (Kolpin et al. 2002,

43 Reungoat et al. 2010). To enhance the abatement of micropollutants in wastewater treatment,

44 advanced treatment, with either ozonation or activated carbon-processes, such as granular activated

45 carbon (GAC) filtration or powdered activated carbon (PAC) treatment, is needed after conventional

46 activated sludge (CAS) treatment. Both technologies have been shown to successfully remove a large

47 number of compounds, including micropollutants (Boehler et al. 2012, Hernández-Leal et al. 2011,

48 Hollender et al. 2009, Snyder et al. 2006). Ozonation in combination with biological post-treatment has

49 been demonstrated to reduce the ecotoxicity of the effluent water (Escher et al. 2009, Margot et al.

50 2013, Prasse et al. 2015, Stalter et al. 2011).

51

52 During ozonation, organic compounds either react directly with ozone or the secondary oxidant

53 hydroxyl radical (Staehelin and Hoigne 1985, von Gunten 2003a). These reactions can lead to the

54 formation of ozonation transformation products (OTPs). Reactions with ozone involve electron-rich

55 moieties (*e.g.*, olefins, activated aromatic rings, neutral amines and thioethers), while reactions with

56 hydroxyl radicals are much less specific (von Sonntag and von Gunten 2012). Many types of

57 transformation reactions can occur and a variety of OTPs can be formed, *e.g.*, *N*-oxides from tertiary

58 amines, hydroxylated compounds from aromatic moieties, or aldehydes from olefins. Information on

59 mechanisms has been used to predict possible OTPs from known parent compounds (Lee et al.

60 2017). In addition to OTP formation, oxidation by-products such as *N*-nitrosodimethylamine (NDMA) or

61 bromate are (potentially) formed during ozonation (von Gunten 2003b, Wert et al. 2009, Zimmermann

62 et al. 2011). It is assumed, and for some compounds has been confirmed, that the formation of

63 oxygen-rich compounds such as aldehydes and carboxylic acids potentially leads to increased

64 biodegradability (Hübner et al. 2015, Zimmermann et al. 2011). It is therefore recommended to apply a

65   biological post-treatment step after ozonation to remove biodegradable oxidation by-products formed

66   from the organic matrix or OTPs (Hollender et al. 2009, Stalter et al. 2011, von Gunten 2003a).

67

68   Sand filtration has been used after ozonation as a biological post-treatment, where it has been shown

69   to be effective for removal of NDMA and reduction of potential ozonation-induced toxicity in full-scale

70   wastewater treatment (Hollender et al. 2009, Prasse et al. 2015). Bioreactors such as moving bed or

71   fixed bed reactors have been proposed as alternatives for biological post-treatment of wastewater

72   (Accinelli et al. 2012, Falås et al. 2012, Luo et al. 2014). Treatment with GAC can remove

73   micropollutants or OTPs through sorption, depending on the compound properties or, after a period of

74   adaptation through biological degradation (Grover et al. 2011, Simpson 2008). However, as previously

75   reported, biological post-treatments are often only moderately effective at eliminating known OTPs

76   (Margot et al. 2013) and ozonation may not always lead to an expected increase of OTPs

77   biodegradability compared to the parent compounds (Hübner et al. 2015). In the case of known OTPs,

78   it is possible to monitor their presence and removal in full-scale WWTPs (Borowska et al. 2016, Deeb

79   et al. 2017, Zimmermann et al. 2011). However, the elucidation of OTPs of single substances is very

80   labor-intensive and therefore data remain scarce. Consequently, many OTPs remain unknown,

81   making evaluation of different treatment types difficult and biased toward known compounds.

82   Therefore, application of non-target screening is promising to characterize unknown peaks in WWTPs

83   and to determine if unknown, non-target compounds are either abated or formed during wastewater

84   treatment.

85

86   Liquid chromatography coupled to high-resolution tandem mass spectrometry (LC-HRMS/MS) is

87   increasingly applied for the analysis of known and unknown emerging contaminants in water

88   (Hernández et al. 2014, Richardson and Kimura 2016). Numerous studies have shown that reverse-

89   phase LC-HRMS/MS can be used for the simultaneous analysis of hundreds of known compounds

90   including TPs (Bletsou et al. 2015, Cotton et al. 2016, Ferrer and Thurman 2012). Additionally,

91   information from HRMS/MS measurement (such as exact mass and isotope information) can be

92   acquired for suspect or non-target compounds and can then be used for structure elucidation of parent

93   compounds or TPs without reference standards (Krauss et al. 2010, Müller et al. 2012, Singh et al.

94   2015). However, the breadth of information produced from LC-HRMS/MS can no longer be analyzed

95   manually and requires the addition of data analysis strategies to prioritize unknown peaks of interest.

96 Recently, differential/statistical analysis has been used to compare removal of non-target compounds

97 in advanced oxidation processes (Müller et al. 2012, Parry and Young 2016) and to understand the

98 fate of unknown compounds in a conventional WWTP by prioritizing features for identification based

99 on intensity profiles (Getzinger 2016).

100

101 To study the impact of ozonation and five post-treatments on the fate of micropollutants and OTPs, a

102 large sampling campaign was conducted in a full-scale WWTP in 2014-2015. The WWTP is the first in

103 Switzerland upgraded with ozonation to comply with the new Swiss water protection act that aims to

104 reduce the load of micropollutants to receiving waters (Eggen et al. 2014). The aims of this study were

105 to expand on the target and suspect screening approaches used in Bourgin et al. (2018) through the

106 application and further development of existing non-target screening workflows (Chiaia-Hernández et

107 al. 2017, Schollée et al. 2015) to the expansive data set, considering four different ozone doses and

108 five post-treatments. First, non-target peaks detected either before or after ozonation were

109 characterized with principal component analysis (PCA) and potential parent compounds and potential

110 OTPs were linked together using expected ozone and/or OH radical reaction mechanisms. Second,

111 hierarchical cluster analysis (HCA) was applied to compare the efficiencies of five post-treatment

112 technologies in removing non-target features, focusing on OTPs that were detected.

113

2.1 Methods

115   *2.1 Sampling*

116 Samples were collected at the full-scale WWTP Neugut (Dübendorf, Switzerland). Wastewater

117 includes mostly residential sources as well as a number of food- and fragrance-related industries. The

118 original treatment consists of primary treatment and CAS (both aerobic and anaerobic) treatment,

119 including biological phosphorus removal. Effluent from the CAS treatment step is passed to an

120 ozonation reactor that has been in full-scale operation since March 2014, followed by treatment with a

121 sand filter. The ozonation system includes a multi-chamber reactor with a total volume of 530 m^3 and

122 an average retention time of 43 minutes during dry weather. Ozone was injected in the first reaction

123 chamber and was completely depleted at the effluent. After ozonation, a variety of post-treatment

124 technologies were applied in pilot-scale and sampled for target and non-target analyses (Bourgin et al.

125 2018).

126

127   For the investigation of the four ozone doses, sampling was carried out in September/October 2014

128   (Supporting Information (SI), Table S1) during dry weather conditions. For each ozone dose, three

129   24-h flow-proportional composite samples were collected on consecutive days at the influent and

130   effluent of the ozone reactor, for a total of 24 samples. Low variability was observed for target

131   compounds and DOC among the three sampling days, and they were therefore considered as

132   replicates for the PCA. Dissolved organic carbon (DOC) content ranged from 3.5 to 6 mg/L after CAS

133   treatment. The following four ozone doses were tested: 2, 3, 4, and 5 mg/L ozone, corresponding to

134   specific ozone doses of 0.35±0.02, 0.54±0.05, 0.67±0.03, and 0.97±0.07 $gO_3/gDOC$, respectively, for

135   the three replicates. Exact values for each sampling date are provided in Table S1.

136

137   For sampling of the complete treatment train (including post-treatment systems), samples were

138   collected as 24-h or 48-h composites at the following four locations: (1) before CAS treatment; (2) after

139   CAS treatment, at the influent of the ozone reactor; (3) after the ozone reactor, before post-treatment;

140   and (4) after one of the five post-treatments at the effluent of the treatment plant (SI, Figure S1 and

141   Table S2). Four samples were collected for each location (total 32 samples) from February to May

142   2015. Ozone dosing was set around 2.7 mg/L ozone (ranging between 2.13 and 2.89 mg/L ozone or

143   0.45-0.57 $gO_3/gDOC$). Five post-treatment setups were operated in parallel. Two GAC filters were

144   used, one relatively fresh (GAC-A – sampled at 14,000–24,000 bed volumes (BV)) and one already

145   pre-loaded prior to implementation with reduced sorption capacity but potentially stimulated biological

146   activity (GAC-B – sampled at 36,000–46,000 BV). Additionally, three biologically active systems were

147   tested – a moving bed bioreactor (MB), a fixed bed bioreactor (FB), and a full-scale sand filter (SF).

148   The post-treatment reactors were fed with the effluent of the ozonation reactor to condition them and

149   ensure the formation of a biofilm, with the exception of GAC-B which was conditioned up to 16,000 BV

150   with the effluent of a secondary treatment. Details on each of the post-treatments is available in the SI.

151   Samples were collected in 1L glass bottles and stored at 4°C until analysis. In the case of storage >7

152   days, samples were frozen at -20°C until analysis.

153

154   *2.2 Measurement by online SPE-LC-HRMS/MS*

155   Measurement details are provided in the SI, Section S2; quality control and target screening results

156   are reported in Bourgin et al. (2018). Briefly, samples were filtered, diluted with nanopure water

157 (Barnstead Nanopure, Thermo Scientific), and then spiked with 51 isotopically-labeled standards. A 20

158 mL sample was injected for measurement with online solid-phase extraction (SPE), using a multi-layer

159 cartridge (Oases HLB (Waters), Strata X-AW and Strata X-CW (Phenomenex), Env+ (Biotage),

160 followed by LC-HRMS/MS. Reverse-phase LC separation was done with an Atlantis T3 column

161 (Waters). The enrichment and measurement methods were shown to be effective for a wide range of

162 polar and medium polar organic micropollutants (Huntscha et al. 2012); however, highly polar OTPs

163 that can be formed during ozonation may not be detected with this method.

164

165 Samples were measured on an Orbitrap mass spectrometer (Q Exactive Plus, Thermo Scientific;

166 resolution 70,000 at 200 m/z) with electrospray ionization (ESI) in positive and negative modes

167 separately. Settings are listed in SI, Table S4. An external mass calibration was performed and, using

168 the spiked internal standards, the difference between measured accurate mass and expected exact

169 mass was calculated to estimate mass accuracy of the measurements. For the majority of compounds

170 and samples, mass accuracy was greater than 5 ppm, but in rare cases, deviations of up to 15 ppm

171 were recorded. Consequently, a mass recalibration was performed (see below). A Top-5 data

172 dependent acquisition method was used to trigger MS/MS measurements, using an inclusion list of 67

173 target compounds, a dynamic exclusion window of 3.0 seconds, and the "pick others" feature enabled.

174 In this way, MS/MS measurements were triggered first on masses listed on the inclusion list, and

175 second on the top 5 most abundant ions in the corresponding full scan. With the dynamic exclusion

176 window, ions that were fragmented in one scan cycle were not fragmented again for 3.0 seconds,

177 ensuring the greatest possible coverage of MS/MS fragmentation. A list of inclusion masses can be

178 found in the SI, Table S5. Matrix effects of 43 target micropollutants were evaluated and intensities

179 were found to vary by less than 10% between the different sample matrices for the individual

180 substances (Bourgin et al. 2018).

181

182

183   *2.3 Data Analysis*

184 A flowchart of the general workflow is shown in Figure 1. Samples from ozonation and post-treatment

185 analyses were processed separately because of the different sampling periods and study objectives.

186 For evaluation of the four ozone doses, principal component analysis (PCA) and so-called linkage

187 analysis were combined to investigate the possible OTPs resulting from the treatment. This workflow

188 has been demonstrated to be effective for prioritizing potential biological TPs resulting from CAS

189 treatment (Schollée et al. 2015) and was adapted here for known oxidation reactions. Potential OTPs

190 were prioritized with this workflow and their behavior in post-treatment samples was investigated. To

191 probe possible trends of non-target features, especially the formation and elimination of OTPs across

192 the entire wastewater treatment train, HCA was applied to samples from influent through CAS

193 treatment and ozonation to the effluent of the five post-treatment setups. All data analysis was

194 performed with the R software (R Development Core Team 2017). Specific information about each of

195 the elements of the workflow is outlined below.
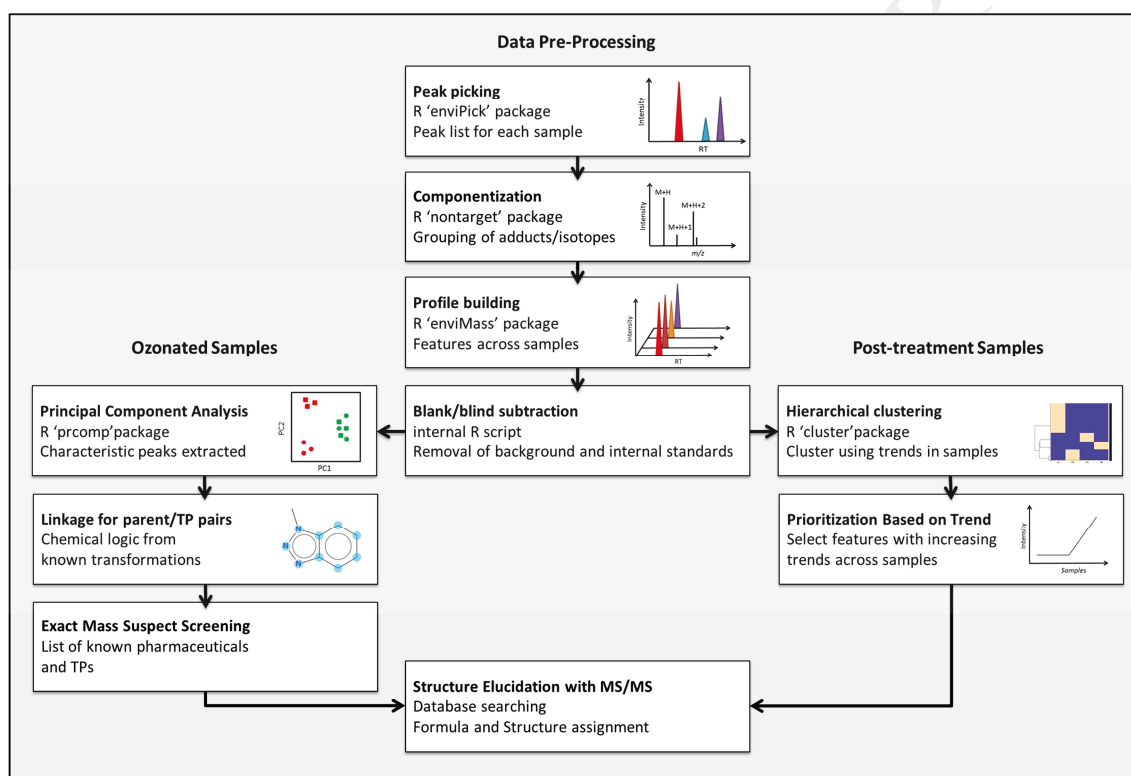
197 **Figure 1. Flowchart of data analysis with an illustration of the workflow. Pre-processing steps**

198 **were similar for ozonated and post-treatment samples, apart from different replicate filters and**

199 **a retention time alignment and mass-to-charge recalibration steps applied to the post-**

200 **treatment samples to correct for multiple measurement runs. Subsequent multivariate analyses**

201 **were different for the two sampling campaigns (ozonation (left), post-treatment (right)).**

202

203 ### *2.3.1 Pre-Processing*

204 All data files were converted and peaks centroided with the software ProteoWizard MSConvert

205 (v.3.0.8725) (Chambers et al. 2012) to ".mzXML" files. Peak picking of each file was carried out with

206 the R package 'enviPick' (Loos 2014b) (settings in SI, Table S6), whereby a feature (peak) list was

207 generated for each sample file, where each feature represents a unique *m/z* and retention time (RT)

208 pair. Measurements for the post-treatment analysis were spread out over the course of the sampling

209 campaign; therefore, *m/z* recalibration and RT alignment was performed using internal standards with

210 the R package 'enviMass' (Loos 2014a), to account for batch deviations. Results of the *m/z*

211 recalibration indicated that in one file and then in a negligible number of features were mass shifts of

212 up to 15 ppm observed. These feature lists were then analyzed with the R 'nontarget' package (Loos

213 2015), where features belonging to the same compound, such as those related to isotopes, were

214 grouped together. Thereafter, features were selected that were either classified as the monoisotopic

215 peak of a compound or not related to other features (*i.e.*, unpaired), with the goal to reduce the

216 amount of data through the removal of isotopes.

217

218 The reduced feature lists from all samples were then merged with the R package 'enviMass' (Loos

219 2014a) using the settings in SI, Table S7. The resulting data sets generated from the enviMass

220 package included a row for each feature, a column for each sample, and was populated with the

221 feature intensities. Then a blank (*i.e.*, nanopure water) and blind (*i.e.*, nanopure water spiked with

222 internal standards) subtraction was performed, where any feature detected in a blank/blind sample

223 was removed from the feature list. A blind subtraction was included to remove any potential noise or

224 contamination resulting from the measurement, as well as the spiked internal standards. Finally, a

225 restriction was applied such that a feature had to be detected in at least three samples. For the

226 ozonation analysis, the feature had to be detected in any three samples; for the post-treatment

227 analysis the restriction was applied on each sample type separately. Applying this restriction allowed

228 the removal of noise peaks from the peak list, and ensured that non-target features were relevant for

229 further investigation due to their continued presence in the WWTP.

230

231 ### *2.3.2 Principal Component Analysis*

232 Each feature was standardized (mean zero, unit variance) and PCA was performed with the R function

233 'prcomp'. The PCA results showed which samples grouped together and in which sample type

234 features were most often detected, allowing for feature characterization. Using expected trends (*i.e.*,

235 decreasing intensities for parent compounds or increasing intensities for OTPs), features could be

236 tentatively classified as potential parent compounds or potential OTPs (or neither).

237

238   *2.3.3*   **Linkage Analysis**

239 After classification with PCA, a so-called linkage analysis was done. To find possible OTPs, the

240 masses of likely OTPs were calculated using the exact mass of the potential parent compounds

241 (obtained with the LC-HRMS/MS and classified with PCA) and the mass difference resulting from a list

242 of proposed transformation reactions (see below). Proposed transformations stem from either

243 reactions of the parent compound with ozone or hydroxyl radicals (or both) since both possibilities

244 need to be considered during ozonation of real wastewaters. A summary of the reactions that were

245 considered, with example reactions, is provided in the SI, Table S8 and the necessary R functions are

246 available at https://github.com/dutchjes/TPPrioritizR. The resulting list of calculated OTP masses was

247 then screened against the masses of potential OTPs classified by the PCA, using a 15 ppm mass

248 tolerance, the maximum mass error observed in the samples. In the case of a match, a "link" was

249 established between the potential parent compound and a potential OTP. The linkage analysis was

250 carried out separately for each ozone dose so that the numbers and types of transformation reactions

251 resulting from the four ozone doses could be compared.

252

253 Thirty-one transformation reactions (SI, Table S8) were considered and included additions of 1-3

254 atoms of oxygen, losses of various alkyl groups, and dehydrogenation. Six (de)conjugation reactions,

255 as well as a number of reductive reactions, that were not expected during ozonation, were also

256 included to provide an assessment of the potential number of false positive links. The linkage analysis

257 was run through one cycle only; therefore, second- or higher-generation OTPs would not be detected,

258 although in reality many of the transformations included are likely the result of multiple reaction steps

259 (*i.e.*, addition of multiple oxygen atoms). Not all potential OTPs detected are expected to arise from

260 reactions with micropollutants, some might result as by-products from the organic matrix.

261

262   *2.3.4*   **Suspect Screening and Structure Elucidation**

263 Potential links from a specific ozone dose of 0.54 ± 0.05 gO$_3$/gDOC (2.7 mg/L ozone) were further

264 prioritized for structure elucidation. This dose was selected for the following two reasons: (1) the

265 highest number of links was detected at this dose and (2) it is the recommended specific ozone dose

266 for the required abatement of micropollutants at the WWTP and was therefore used in the post-

267 treatment analysis. Suspect screening was carried out on the parent masses present in the linkage

268 results. The suspect list that was used included 195 target micropollutants (including OTPs) monitored

269 at this site (Bourgin et al. 2018) and an additional 1022 suspected pharmaceuticals (Singer et al.

270 2016). HRMS/MS spectra from any potential parent compound that matched to a suspect compound

271 was then compared to reference standard spectra measured in-house (available at MassBank (Horai

272 et al. 2010)). In the case of a positive identification of a suspect compound, structures were proposed

273 for the associated potential OTP(s) based on the parent structure, expected transformation reaction as

274 indicated by the linkage analysis, and knowledge on ozone reactivity (von Sonntag and von Gunten

275 2012). Confidence in structure assignment is reported using the levels proposed in Schymanski et al.

276 (2014). Plausibility of OTP formation was further confirmed through prediction of OTPs with an ozone

277 pathway prediction system (O_3-PPS) (Lee et al. 2017), as well as literature searches.

278

2.3.5 *Hierarchical Cluster Analysis (HCA)*

280 For analysis of the post-treatment systems, the trends of features across the WWTP were

281 investigated, and similar feature profiles were clustered together with HCA. The majority of the links

282 and potential OTPs were found in the positive ESI mode in the PCA and linkage analysis; therefore,

283 we focused on this data for the HCA. To observe the behavior of features across the WWTP, first a

284 feature list was generated for each of the four sample types (SI, Figure S1), where feature intensity

285 corresponded to the maximum intensity detected in that sample type, multiplied by the corresponding

286 dilution factor. The feature lists were then profiled together with the R package 'enviMass' (Loos

287 2014a) and a dissimilarity matrix was calculated using intensities normalized to the maximum intensity

288 with the R package 'cluster' (Maechler et al. 2016). Finally a HCA was performed with the profiles

289 using the Euclidian distance (which is the length of the line connecting them) and Ward method

290 (designed to minimize intra-cluster variance, thereby producing compact clusters). Considering that

291 the intensities of a peak could either increase, decrease, or remain the same from one sampling point

292 to the next, there are $3^{(n-1)}$ possible trends, corresponding to 27 possible trends in this case. Therefore,

293 the resulting dendogram was cut such that 30 clusters were identified and the profiles in each of these

294 clusters visualized. Eleven trends were defined, corresponding to expected trends in the WWTP

295 (Figure 2) and each of the 30 clusters was visually assigned to a trend that best described the

296 behavior of the profiles in that cluster. Characteristics of features assigned to different trends were

297 then compared. The R-script used for cluster analysis and characterization is available at

298 https://github.com/dutchjes/TPPrioritizR. Validation of the HCA was performed with a set of eight

299   parent compounds (amisulpride, carbamazepine, cetirizine, clarithromycin, lamotrigine, ranitidine,

300   tramadol, and venlafaxine) and their respective OTPs, known to be formed during ozonation.

301

13

302
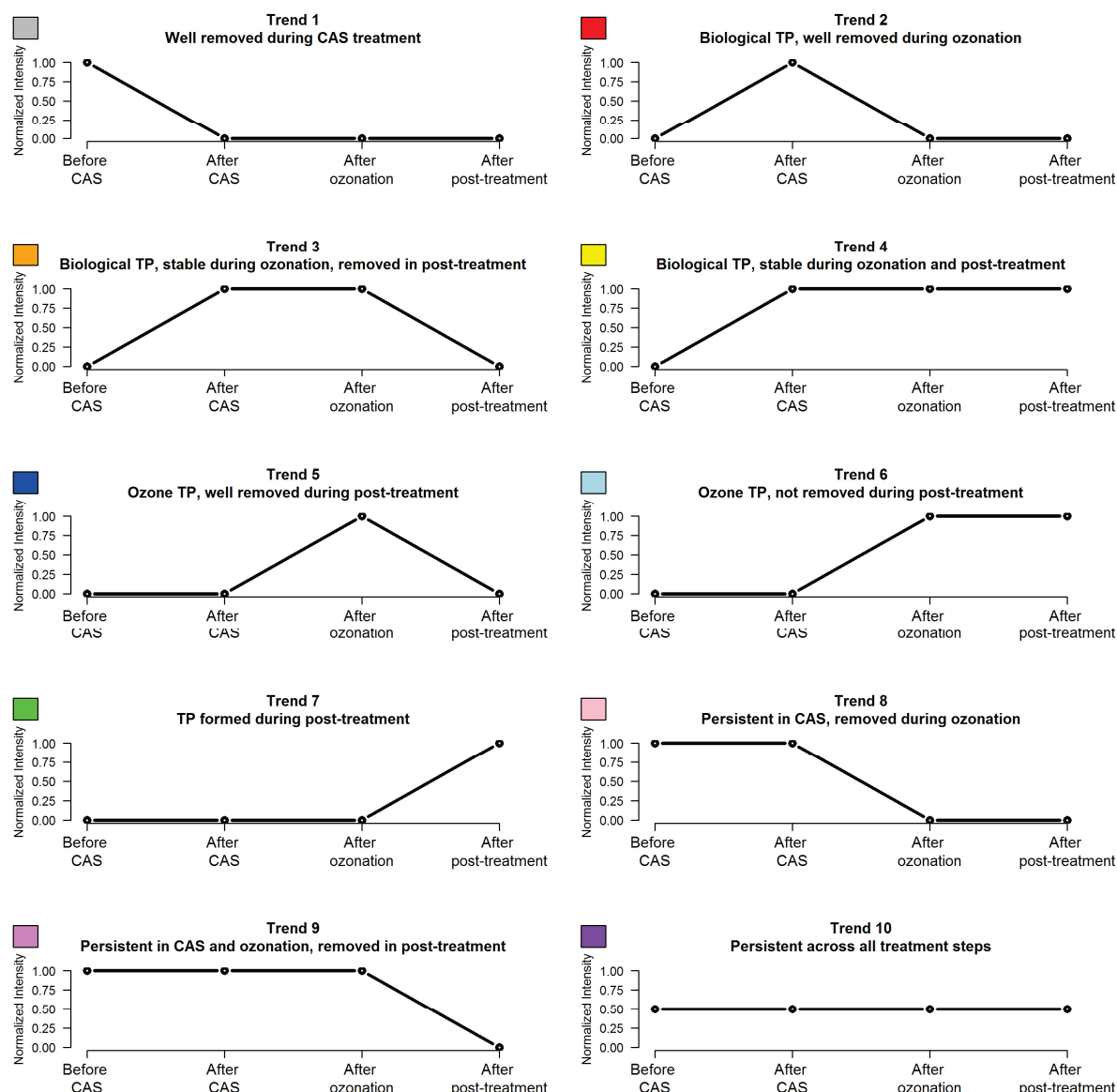
Figure 2. Examples of the ten trends that were defined to describe the intensity profiles in a cluster. Each trend can be associated with the specific behavior of a compound in the wastewater treatment plant, and is described in the title of each trend. A Trend 11 was also defined but is not shown here, including any cluster that did not fit into the other categories. Intensity profiles for each post-treatment were clustered separately and are therefore generally labeled as 'After post-treatment'. CAS refers to conventional activated sludge treatment.

309

3.1 Results and Discussion

3.1   Analysis of Ozonated Samples

3.1.1   Principal Component Analysis and Linkage Analysis

14

313   Classification of non-target features detected with LC-HRMS/MS was performed by interpretation of

314   the PCA scores and loading plots on samples collected from the four specific ozone doses (0.4, 0.5,

315   0.7, and 1.0 $gO_3/gDOC$). The total number of non-target features detected and included in the PCA for

316   the specific ozone doses 0.4, 0.5, 0.7, and 1.0 $gO_3/gDOC$ were 4260, 6207, 3798, and 2279,

317   respectively (Table 1). An example scores plot is shown for 0.5 $gO_3/gDOC$ in Figure 3a; scores plots

318   from the other ozone doses for both ionization modes are shown in SI, Figures S2 and S3. In each

319   case, the first principal component (PC1) could explain approximately 50% of the variance in the data

320   sets (SI, Tables S9 and S10), which can be attributed to the difference in sample types (*i.e.*, before vs.

321   after ozonation). PC2 (plotted along the y-axis) generally explained between 15 and 20% of the

322   variance among the samples and can be attributed to daily changes in the wastewater composition,

323   while additional PCs were related to differences in individual samples (SI, Figure S4).

324
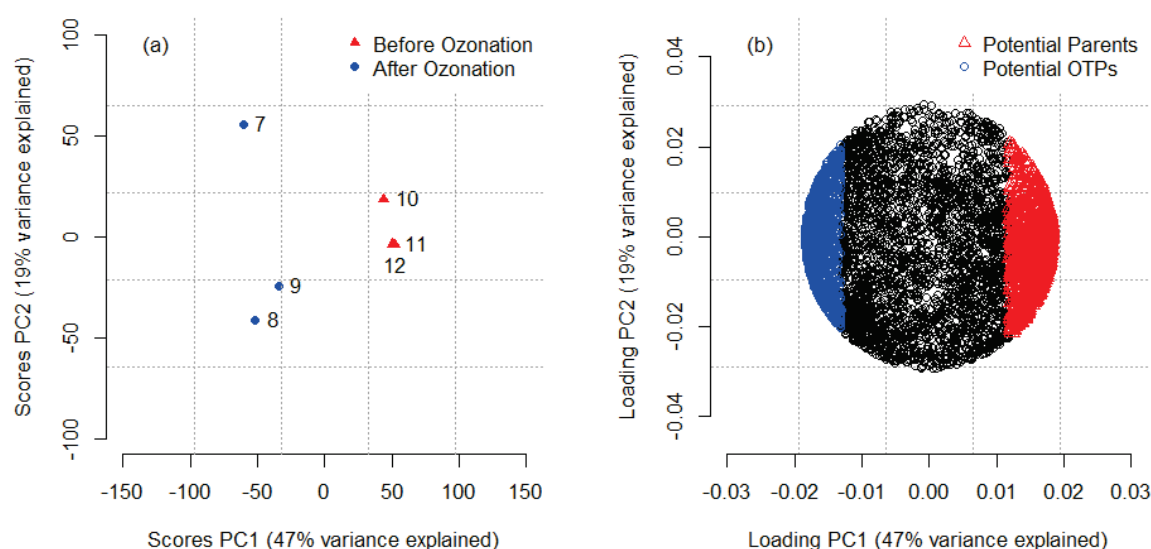
Figure 3. Principal component analysis (a) scores plot and (b) loading plot of WWTP effluent

before and after ozonation for a specific ozone dose of 0.5 gO3/gDOC. The first and second

principal components are shown, on the x- and y-axes, respectively. A good separation

between the samples before ozonation (red triangles) and after ozonation (blue circles) was

obtained along the x-axis and could explain 47% of the variance, allowing for classification of

non-target features as potential parent compounds or potential OTPs (or neither, black circles).

Similar results were obtained for the other ozone doses (SI, Figure S2). Numbers refer to the

sample IDs (Table S1).

333

334   Features were classified based on their loading in PC1 (Figure 3b; SI, Figures S6 and S7). The top

335   25% of features (*i.e.*, features with the highest PC1 loadings, and therefore characteristic of the

336   samples before ozonation) were classified as potential parent compounds. Similarly, the bottom 25%

337   of features (*i.e.*, features with the lowest PC1 loadings and therefore characteristic of the samples after

338   ozonation) were classified as potential OTPs. This procedure resulted in equal numbers of features as

339   potential parent compounds and potential OTPs. In the positive mode between 570 (1.0 gO_3/gDOC)

340   and 1552 (0.5 gO_3/gDOC) features were selected as potential parents/OTPs (Table 1). In negative

341   mode, between 68 (1.0 gO_3/gDOC) and 430 (0.5 gO_3/gDOC) features were classified as potential

342   parents/OTPs (Table 1). Wastewater treated with 0.5 gO_3/gDOC had the highest number of features,

343   indicating that with this dose possibly the highest number of OTPs was detected (SI, Tables S11 and

344   S12). This result is in agreement with previous work from Bourgin et al. (2018), which found that at this

345   dose the highest number of suspected OTPs was detected. DOC removal was observed to be

346   approximately 10%, independent of the specific ozone dose. The decrease in detected features at

347   higher specific ozone doses is likely due to the fact that these higher generation OTPs were too polar

348   to be detected with online-SPE LC-HRMS/MS. This could be caused by either poor enrichment in the

349   SPE cartridge or low retention on the reverse-phase LC column, and an alternative analytical method

350   (*e.g.*, hydrophilic interaction liquid chromatography (HILIC)) would be required to detect them.

351   Characteristics of features (*e.g.*, mass to charge ratios (*m/z*), retention times, intensities, or mass

352   defects (*i.e.*, the difference between the nominal mass and the exact mass)) detected after ozonation

353   from the four ozone doses were compared and effluent features from the 0.5 gO_3/gDOC were in some

354   cases significantly different (SI, Figures S7 and S8).

355

356   From the linkage analysis with 31 transformation reactions, the number of unique features with links to

357   potential OTPs ranged from 17—1416, depending on ionization mode and specific ozone dose, and

358   represented between 4 to 21% of all detected features (Table 1). In positive ESI mode, the highest

359   number of links were detected in samples with a specific ozone dose of 0.5 gO_3/gDOC (Figure 4a).

360   Consequently, the highest total number of links for both ESI modes (9636) was found in samples from

361   0.5 gO_3/gDOC, followed by samples from 0.4 gO_3/gDOC (5070) (SI, Table S13). Samples from

362   specific ozone doses of 0.7 or 1.0 gO_3/gDOC had the lowest number of links, namely 3983 or 1295,

363   respectively.

364 **Table 1. Summary of non-target features and links detected for the four specific ozone doses**

365 **and two electrospray ionization (ESI) modes. Number of influent and effluent peaks were**

366 **determined based on the characterization with PCA. The number of unique potential parent or**

367 **OTP features refers to unique *m/z* and retention time features for which a link was detected**

368 **through the linkage analysis.**

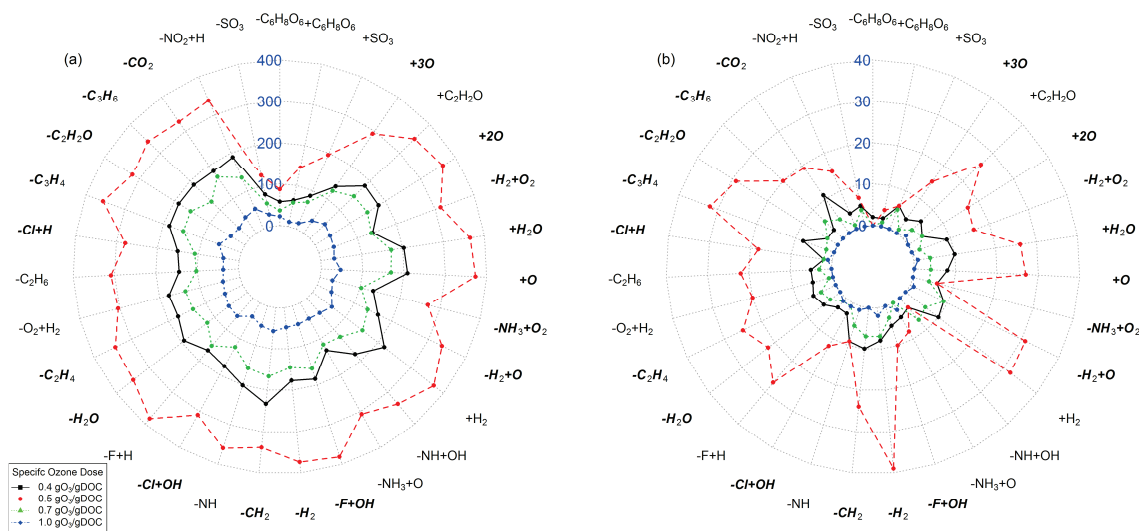|	*Positive ESI Mode*				*Negative ESI Mode*			
	0.4 gO₃/gDOC	*0.5 gO₃/gDOC*	*0.7 gO₃/gDOC*	*1.0 gO₃/gDOC*	*0.4 gO₃/gDOC*	*0.5 gO₃/gDOC*	*0.7 gO₃/gDOC*	*1.0 gO₃/gDOC*
Total number of features	4260	6207	3798	2279	1048	1719	787	270
Number of influent peaks	1065	1552	950	570	262	430	197	68
Number of effluent peaks	1065	1552	950	570	262	430	197	68
Number of unique potential parent features	979	1416	874	482	184	343	100	17
Number of unique potential OTP features	869	1328	724	394	153	324	85	12
% features as potential parents with links	23	23	23	21	18	20	13	6
% features as potential OTPs with links	20	21	19	17	15	19	11	4
Average number of links for each parent	6	7	5	3	2	4	2	2
Average number of links for each OTP	2	8	6	4	2	3	2	1

369

370 **Figure 4. Radar plot of the different linkage types detected for each ozone dose in (a) positive**

371 **ESI mode and (b) negative ESI mode. The scale, showing the number of times each**

372 **transformation type was detected, is indicated on the grey concentric circles. The number of**

373 **detections for each ozone dose is shown with the different colors and line styles. Mass**

374 **differences are ordered starting at the top from the largest addition and continuing clockwise**

17

375 **to the largest loss. Formula differences in bold and italic correspond to transformation types**

376 **that are expected to occur either through reactions with ozone or hydroxyl radicals (further**

377 **information in SI, Table S8).**

378

379 It is reasonable that the samples with the highest number of features (0.5 gO_3/gDOC) also had the

380 highest number of links, because more links are possible when more features are detected (Table 1).

381 Furthermore, each parent compound can be linked to multiple OTPs and each OTP can be linked to

382 multiple parents. On average, more OTPs per parent compound were found in the linkage analysis of

383 the 0.5 gO_3/gDOC samples (7 OTPs per parent compound) compared to the linkage analysis of the

384 1.0 gO_3/gDOC samples (3 OTPs per parent compound), perhaps also because fewer potential OTPs

385 were detected at the higher doses. Additionally, the average number of links to parent compounds for

386 each OTP, as well as the percentage of OTPs with links, was highest at intermediate specific ozone

387 doses (8 links/OTPs or 21% at 0.5 gO_3/gDOC versus 4 links/OTP or 17% at 1.0 gO_3/gDOC). Overall,

388 in the negative ESI mode, fewer links were detected, probably because fewer features were detected.

389 However, also in the negative ESI mode, the average number of links, the average number of

390 OTPs/parent compound, and the percent of OTPs with links were highest for the intermediate specific

391 ozone dose (3.3 OTPs per parent compound at 0.5 gO_3/gDOC vs. 1.2 OTPs per parent compound at

392 1.0 gO_3/gDOC).

393

394 The most frequently detected transformation types were hydrogenation (885 links), addition of a single

395 oxygen (864 links), and demethylation (861 links). Addition of a single oxygen can be related to

396 multiple possible reactions with either ozone or OH radicals, including hydroxylation, formation of *N-* or

397 *S-*oxides, or formation of an aldehyde from an olefin (Merel et al. 2017, von Sonntag and von Gunten

398 2012). Demethylation during ozonation, particularly at heteroatoms, has also been described for

399 different micropollutants (Benner and Ternes 2009, Kuang et al. 2013). The prevalence of

400 hydrogenation can currently not be explained and needs further investigation. Substitution reactions

401 involving halogens are not favorable during ozonation, and were also rarely detected in the linkage

402 analysis. The (de)conjugation transformations (*i.e.*, (de)acetylation, (de)sulfation, and

403 (de)glucuronidation), that were included to provide an estimate of false positive links, were in general

404 detected the least. Between 200-400 (de)conjugation links were detected in positive ESI mode across

405 all ozone doses, compared to 500-900 for the other linkages, demonstrating the potential for mass-

406 related peaks to be erroneously linked together as parents and OTPs. Second- or third-generation

407 OTPs incorporating multiple oxygen atoms were found less than expected, probably because they are

408 not formed under the applied ozone doses or too polar to be detected with reverse-phase LC-

409 HRMS/MS.

410

411   *3.1.2   Characterization of OTPs resulting from Suspect Screening*

412 The suspect screening was performed on the potential parent compounds detected at 0.5 gO$_3$/gDOC

413 (see above). From 159 suspect hits, 27 compounds were confirmed with HRMS/MS spectra match to

414 a reference standard measured in-house (confidence level 1, SI, Table S14). Almost all 27 compounds

415 have been described as persistent in CAS treatment, thereby making their presence in the ozone

416 reactor and consequently the possibility of OTP formation plausible. In the linkage analysis, 182

417 potential OTPs were detected for these 27 parent compounds, an average of 6 potential OTPs per

418 parent compound (SI, Table S15). Of the 182 potential OTPs, 69 were determined to be structurally

419 possible by comparing the parent structure and proposed transformation, *e.g.*, deethylation only

420 possible for parent compounds with an ethyl group. Of these 69 structurally possible OTPs, 30 were

421 predicted as possible ozonation products by a recently developed ozone pathway prediction system

422 (O$_3$-PPS) (SI, Table S15).

423

424 HRMS/MS spectra were triggered in data-dependent mode for 22 of the 69 structurally possible OTPs,

425 and the structures of three OTPs were confirmed with reference standard matches (level 1).

426 Hydrochlorothiazide was linked to chlorothiazide through a dehydrogenation (Table S16, TP structure

427 7), 2-aminobenzothiazole was linked to benzothiazole through a deamination reaction (Table S16, TP

428 structure 1), and venlafaxine was linked to venlafaxine *N*-oxide (Table S16, TP structure 22).

429 Chlorothiazide was recently found as the main OTP from hydrochlorothiazide oxidation (Borowska et

430 al. 2016). Similarly, the formation of venlafaxine *N*-oxide during ozonation has been documented

431 (Bourgin et al. 2018, Lajeunesse et al. 2013, Merel et al. 2017). The link between 2-

432 aminobenzothiazole and benzothiazole would need to be confirmed through batch experiments.

433 However, deamination on the heteroaromatic ring is unlikely during ozonation. For the other 19

434 potential OTPs with HRMS/MS spectra, probable structures (*i.e.*, level 3) were suggested for 13 of

435 them, based on the structure of the parent compound and proposed transformation (SI, Table S16).

436 Five of the suggested OTPs were also predicted by the software O$_3$-PPS, and one OTP (oxidative

19

437   replacement of fluorine in citalopram; OTP structure 3) has been described previously in literature

438   (Hörsing et al. 2012). HRMS/MS spectra were not informative enough for structure confirmation of the

439   proposed OTPs, but in most pairs, two or more fragments were shared between parent compound and

440   proposed OTP, possibly indicating related structures (Schollée et al. 2017).

441

442   For the 47 proposed OTPs where no HRMS/MS spectra were collected, 23 were also predicted by the

443   software O_3-PPS. Additionally, information about degradation during ozonation could be found in the

444   literature for many of the parent micropollutants and circumstantial evidence was found for several of

445   the proposed OTPs, as indicated in SI, Table S15. For instance, for 5-methyl-benzotriazole, an

446   addition of two oxygen atoms was found to be in agreement with Müller *et al.* (2012). The structurally

447   possible OTP suggested for DEET (*i.e.*, a deethylation) has also been reported (Tay *et al.* 2009). Two

448   proposed OTPs (both oxygen additions) for phenazone were detected by Miao et al. (2015), two OTPs

449   for metoprolol (*i.e.*, oxygen addition and dealkylation) were previously reported (Tay *et al.* 2012), and

450   one OTP for trimethoprim was previously detected by Kuang *et al.* (2013). An interesting example

451   presented itself in the case of atenolol. Molecular formulas of three structurally likely OTPs for atenolol

452   corresponded to 7 different structures which were reported by Tay *et al.* (2011) since one formula was

453   related to multiple OTPs (*e.g.*, oxygen addition at multiple locations on the molecule). Concerning the

454   OTPs reported in the literature that were not detected here, the majority of these were resulting from

455   transformations not predicted by the linkage analysis and can be incorporated in the future. For the

456   OTPs**,** which were detected here but not reported in literature, most studies on micropollutant

457   abatement during ozonation do not include identification of OTPs or only identify OTPs resulting from

458   reaction with ozone but not with OH radicals, therefore, not all OTPs which might be formed during

459   ozonation at a full-scale WWTP are known. Specifically, very little information is available on the fate

460   of biological TPs from CAS treatment in ozonation, although a number of them (*i.e.*, desethyl-atrazine

461   and *N*-desmethylvenlafaxine) were found to be possible parent compounds in the ozone analysis,

462   highlighting the need to study their behavior and potential for OTP formation. Nevertheless, the

463   numerous successful examples demonstrate that the combined PCA and linkage analysis approach to

464   analyze full-scale WWTPs lead to the detection of OTPs of known micropollutants that had previously

465   only been described in batch experiments.

466

467   For four of the 27 identified parent compounds (desethyl-atrazine, 4-acetamidoantipyrin (4-AAA),

468   carbendazim, and irbesartan), no structurally possible OTPs were suggested. Low reactivity with

469   ozone has been reported for two of these, desethyl-atrazine (k_{O3} 0.19 M^{-1} s^{-1} at pH 7 (Beltrán et al.

470   1998)), and irbesartan (k_{O3} 24 M^{-1} s^{-1} (Bourgin et al. 2018)). No second order rate constants for the

471   reaction of ozone with 4-AAA or carbendazim are available in the literature. For compounds with low

472   reactivity with ozone, the reaction with hydroxyl radicals dominates during ozonation of WWTP effluent

473   water. Since reactions with hydroxyl radicals are less specific than those with ozone, the resulting

474   transformations may not have been included in the linkage analysis and the resulting OTPs therefore

475   not been found. Additionally, the following reasons can be suggested for why OTPs were not detected

476   for these parent compounds: (i) it may be the case that first generation OTPs react further and the

477   resulting second generation products were not predicted with the linkage analysis; (ii) it is possible that

478   yields of OTPs were too low and therefore below the detection limit of the instrument; or (iii) that the

479   OTPs were not detected with online-SPE LC-HRMS/MS (*e.g.*, too polar to be retained in the SPE

480   cartridge or on the reverse-phase LC column, or not ionized in the MS). In the future, incorporation of

481   an additional LC method such as HILIC to detect highly polar OTPs would be advantageous.

482

483   *3.2*   *Analysis of Post-Treatment Sampling*

484   ***3.2.1***   ***Non-target Peak Characterization including OTPs***

485 Non-target screening was used to compare five post-treatment technologies (Figure 1). No large

486 differences were observed among the number of features detected in the effluents of the five post-

487 treatment step-ups (SI, Table S17). Approximately 3000-4000 features were detected in the effluent of

488 each post-treatment, which is a slight reduction from the number of features in the effluent of the

489 ozone reactor (4340) and in the effluent of the CAS treatment (5016). The characteristics of non-target

490 features (*i.e.*, *m/z* and RT) were significantly lower after the CAS treatment (Tukey's HSD, p<0.01, SI,

491 Figure S9). The *m/z* values of non-target features in the GAC filters were significantly lower than those

492 detected in the other steps of the WWTP (Tukey's HSD, p<0.01), likely because larger non-polar

493 compounds are expected to be removed by sorption in the GAC filters.

494

495   Of the 69 structurally possible OTPs detected from the analysis of the specific ozone dose of 0.5

496 gO_3/gDOC (section *3.1.2*), 67 were also detected in the second set of samples analyzing the different

497 post-treatments. No substantial differences between the intensity detected after ozonation and

21

498 intensity after post-treatment were observed, suggesting little to minor removal of these potential

499 OTPs; however, reference standards would be needed for proper quantification.

500

501 ### 3.2.2   *Hierarchical Clustering across the WWTP*

502 To further investigate the behavior of non-target features during wastewater treatment, hierarchical

503 clustering was applied to group features including OTPs into clusters based on intensity profiles

504 across the WWTP. For each feature, intensity profiles were constructed from the maximum intensities

505 detected in the four sample types, *i.e.*, before CAS treatment, after CAS treatment, after ozonation,

506 and after post-treatment.

507

508 The HCA was validated with a suspect screening of eight pharmaceuticals (amisulpride,

509 carbamazepine, cetirizine, clarithromycin, lamotrigine, ranitidine, tramadol, and venlafaxine) and 12

510 corresponding OTPs in the WWTP with sand filtration as post-treatment. The correct trend was

511 assigned to five of eight parent compounds and nine of twelve OTPs (SI, Table S18). ~~For~~ Of the

512 falsely assigned parent compounds, two (tramadol and ranitidine) were incorrectly grouped during pre-

513 processing and one (clarithromycin) was detected in the blind and therefore removed during pre-

514 processing. For the three falsely assigned OTPs, two were not assigned to the expected trend

515 because of their low intensities (<2E6 for amisulpride *N*-oxide and cetrizine *N*-oxide) and one

516 (clarithromycin *N*-oxide) because of large retention time shifts over the samples which were not

517 completely corrected for with the RT alignment. Four OTPs were not detected in either the target or

518 non-target screening, and were considered as true negatives (and as correctly classified) in the

519 validation.

520

521 Clustering and trend assignment was done separately for each of the five post-treatment set-ups. The

522 normalized clusters resulting from the HCA of each post-treatment are shown in SI, Figure S12. It is

523 expected that the number of features with Trend 1 or Trend 2 would not change among the five set-

524 ups, because the CAS treatment and ozonation were always the same although slight variations in the

525 total number of features may occur as a result of the data pre-processing or the HCA. Table 2

526 illustrates that the number of features assigned to Trend 1 or Trend 2 indeed did not vary substantially

527 (RSD 1.4% for Trend 1 and 5.0% for Trend 2). In all cases the largest number of features (59-61%)

528 were removed during the CAS treatment (*i.e.*, Trend 1).

529

530

Table 2. Summary of the number / percentage of non-target features removed or formed in each of the described trends in the five post-treatment set-ups. CAS treatment refers to conventional activated sludge treatment. GAC-A was relatively fresh (14,000–24,000 BV) and GAC-B pre-loaded (36,000–46,000 BV).

| Trend	Intensity Profile Behavior	GAC-A	GAC-B	Moving bed	Fixed bed	Sand filter	RSD
1	Well removed during CAS treatment	4890 / 61%	4758 / 59%	4866 / 61%	4804 / 60%	4903 / 61%	1.4%
2	Biological TP, well removed during ozonation	1442 / 18%	1304 / 16%	1395 / 17%	1302 / 16%	1419 / 18%	5.0%
3	Biological TP, stable during ozonation, removed in post-treatment	155 / 2%	125 / 2%	22 / 0.3%	26 / 0.3%	15 / 0.2%	97%
4	Biological TP, stable during ozonation and post-treatment	35 / 0.4%	200 / 3%	230 / 3%	304 / 4%	236 / 3%	50%
5	Ozone TP, well removed during post-treatment	597 / 8%	452 / 6%	385 / 5%	393 / 5%	374 / 5%	21%
6	Ozone TP, not removed during post-treatment	122 / 2%	231 / 3%	327 / 4%	305 / 4%	312 / 4%	33%
7	TP formed during post-treatment	269 / 3%	276 / 3%	303 / 4%	298 / 4%	266 / 3%	5.7%
8	Persistent in CAS, removed during ozonation	184 / 2%	123 / 2%	105 / 1%	181 / 2%	152 / 2%	23%
9	Persistent in CAS and ozonation, removed in post-treatment	0 / 0%	0 / 0%	0 / 0%	0 / 0%	0 / 0%	0.0%
10	Persistent across all treatment steps	13 / 0.2%	11 / 0.1%	45 / 0.6%	30 / 0.4%	39 / 0.5%	55%
11	Other	283 / 4%	524 / 7%	361 / 5%	394 / 5%	268 / 3%	28%
	Total number of features detected	7990	8004	8039	8037	7984	0.3%

535

The number of features in the Trends 3 to 11 show differences in the post-treatments. Features with Trend 5 represent compounds that are likely formed during ozonation, and are subsequently removed in the post-treatment. The sand filter, moving bed, and fixed bed post-treatments each had relatively low percentages of features with this trend (5%), indicating possibly low removal of OTPs. The two GAC filters had slightly more features with this trend (8% in GAC-A and 6% in GAC-B). In addition to biological degradation, sorption can also occur on the GAC filters, possibly accounting for the increased removal potential in the GAC filters. The better performance of GAC-A compared to GAC-B may be explained by the fact that GAC-A was only run to approximately half of the BVs and therefore exhibits a higher sorption capacity, which was also shown in target analysis (Bourgin et al. 2018). DOC abatement was also higher in the two GAC filters (15-20%) compared to the fixed bed and moving bed reactors (5 and 6%, respectively). Conversely, the number of features assigned to Trend 6 (likely OTPs not removed in post-treatment) was highest in the sand filter and two bioreactors (4%), and lowest in the two GAC filters (2-3%). Only a minor portion of features detected appeared to be

24

549   formed during ozonation (Trend 5+6;–9-10%), and from these, 54% to 83% were removed in post-

550   treatment, with highest removal efficiencies being observed in the two GAC filters. Other research

551   based on target micropollutants has also shown that GAC may be more efficient at removing OTPs

552   compared to solely biological filters (Knopp et al. 2016) but further systematic investigations would be

553   needed to assess the fate of OTPs in GAC filters, *e.g.*, over a longer run time. It has to be noted that

554   many of the clustered observed peaks formed during ozonation arise from the reaction of ozone with

555   matrix components and not with micropollutants and need to be further evaluated. Therefore, further

556   evaluation is necessary to determine the efficiency of post-treatments on OTP removal.

557

558   The characteristics of non-target features in each trend type were compared for each post-treatment

559   analysis (shown for the sand filter in Figure 5 and SI, Figure S13, and the other post-treatments in the

560   SI, Figures S14-S17) to determine if specific feature characteristics were indicative of, *e.g.*, better

561   biodegradability. In almost all cases there was no significant difference (Tukey's HSD, p<0.05) in mass

562   defect of non-target features in each of the 11 trends (SI, Figures S18-S22), indicating that, *e.g.*,

563   chlorinated compounds with negative mass defect were not less prone to transformation. In all five

564   post-treatments, OTPs that were removed had significantly higher RTs (p<0.05, Mann Whitney U test)

565   than OTPs which were not well removed, indicating possible recalcitrance of some polar OTPs (Figure

566   6).

567
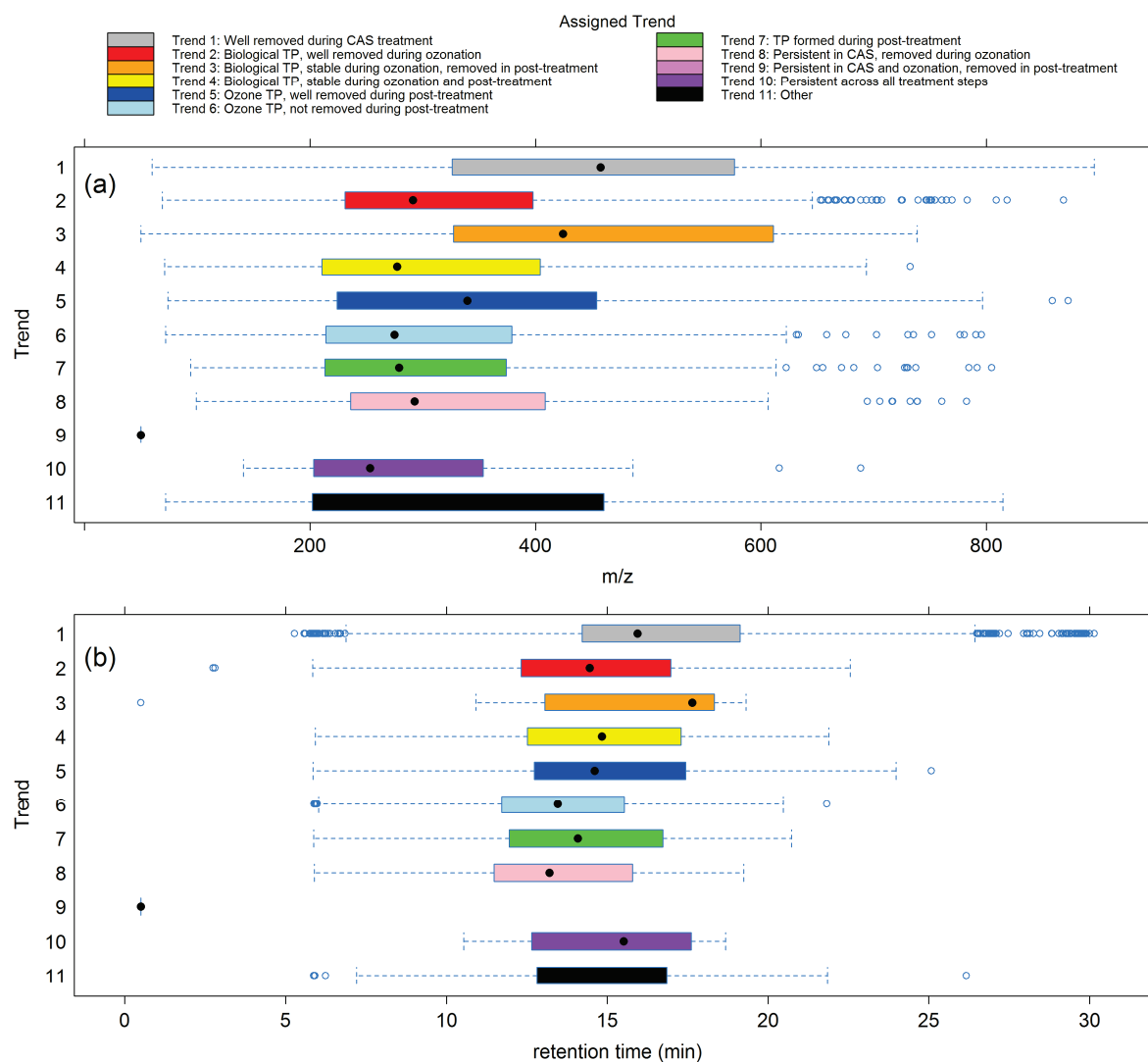
Figure 5. Box-whisker plots of the (a) *m/z* and (b) retention time in each of the 11 defined trends with sand filtration as post-treatment step for positive ESI. Colors represent the trends, also indicated on the y-axis.

571

26
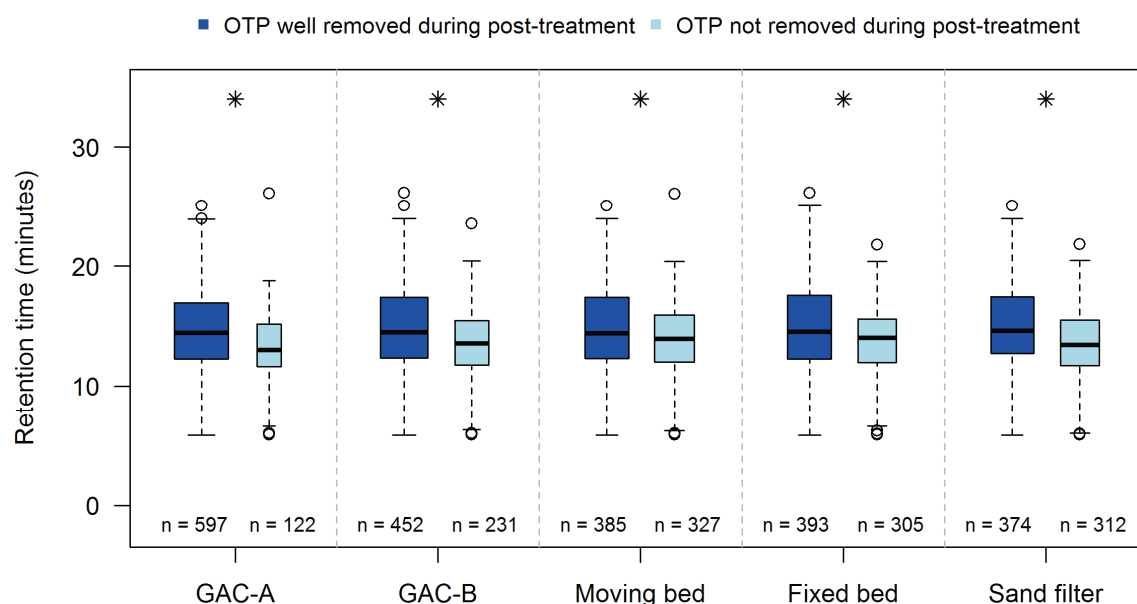
Figure 6. Box-whisker plots of the retention times of OTPs removed or not removed during five post-treatment steps, measured in positive ESI. OTPs removed were features classified with Trend 5 and OTPs not removed were features classified with Trend 6 in the cluster analysis. The number of features (n) in each box is also represented by the width of the box (*i.e.*, large box indicates more features). Within one post-treatment sample set-up, the retention times of OTPs in Trend 5 and Trend 6 were in all cases significant (Mann Whitney U test, p<0.05), as indicated by a star.

The clusters with trends relevant to OTP formation can be compared to the potential OTPs from the linkage analysis to prioritize possible OTP peaks for future structure elucidation. From the linkage analysis with an specific ozone dose of 0.5 gO_3/gDOC, 1328 unique non-target features were suggested as potential OTPs (SI, Table S12). With the HCA, 686 non-target features were assigned either to Trend 5 or Trend 6 using the sand filter post-treatment, indicating possible formation during ozonation. With these two prioritization methods, 131 non-target features overlapped using a 15 ppm *m/z* tolerance and 1 minute RT window. While this overlap may appear to be quite small, wastewater inputs vary considerably, as can treatment efficiency. Taking into account that the samples were collected in some cases more than six months apart, these features may represent relevant OTPs that warrant further attention due to their frequent detection.

4.1 Conclusions

592

- 593 The combination of PCA and chemical logic (*i.e.*, linkage analysis) made it possible to gain an
- 594 overview of the types of non-target peaks present in wastewater effluent treated with different
- 595 ozone doses. Results suggested that treatment with a specific ozone dose of 0.5 gO_3/gDOC
- 596 led to the formation of the highest number of observable OTPs (1552), supporting previous
- 597 findings, although other enrichment or chromatography methods may be necessary to detect
- 598 highly polar OTPs.

- 599 Chemical logic was able to suggest 69 structurally plausible OTPs originating from 27 parent
- 600 compounds, and three OTPs could be confirmed by reference standards. A number of the
- 601 suggested OTPs had previously been detected in ozonation batch experiments and some
- 602 were also predicted to be likely by mechanistic information using an ozone pathway prediction
- 603 system (O_3-PPS), providing further evidence of their presence in the full-scale WWTPs.

- 604 The presented workflow, combining HRMS data with statistical methods such as PCA,
- 605 chemical logic and mechanistic information, provides an efficient approach to select and
- 606 prioritize potential parent compounds for further studies. The ability to monitor OTPs in a full-
- 607 scale WWTP is critical, because it is not possible to conduct batch experiments on all
- 608 chemicals. Intensities of the potential OTPs in the effluent of five post-treatment technologies
- 609 suggest incomplete removal, although further studies are needed for quantification.

- 610 From hierarchical cluster analysis across all treatment steps, a small portion of the non-target
- 611 features (9%) was found to be formed during ozonation and 54% to 83% of these features
- 612 were removed during post-treatment. Compared to the other post-treatment options, the two
- 613 GAC filters evaluated appeared to perform slightly better in removing non-target features
- 614 formed during ozonation.

5.1 Acknowledgements

Appendix A – Supplementary data

Supplementary data is available as indicated in the text.

29

6.1 References

628

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1 **Highlights**

2 • Non-target screening with HRMS was applied to a wastewater treatment train

3 • Statistics were used to prioritize potential ozonation transformation products OTPs

4 • 69 plausible OTPs were found; 34 agreed with literature data or reaction mechanisms

5 • 54 to 83% of potential OTPs were removed in the five post-treatments

6