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Removal of pharmaceuticals, toxicity and natural fluorescence through the ozonation of biologically-treated hospital wastewater, with further polishing via a suspended biofilm

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Abstract:

In the present study, a pilot-scale ozonation system was introduced as post treatment to reduce the pharmaceuticals and toxicity in the effluent of a pilot-scale Moving Bed Biofilm Reactor (MBBR) treating hospital wastewater. The ozonated effluent was polished further by suspended biofilm carriers to remove biodegradable organic matter and toxicity generated from ozonation by-products. A laboratory ozonation system was used to mimic the experiments, which were carried out at the pilot plant so that the removal of pharmaceuticals in the pilot and laboratory experiments could be compared. Delivered ozone dose achieved 90% removal of pharmaceutical was obtained and it was normalised to dissolved organic carbon (DOC). These normalised results show that trimethoprim was eliminated by ozone easier than other pharmaceuticals.

Fluorescence was found to be highly correlated to the removal of pharmaceuticals, and fluorescence with a wavelength of 275 nm of excitation and 310 nm of emission had the closest correlation. After polishing MBBR was introduced into the ozonated wastewater, half of its protein-like fluorophore was removed. The toxicity of the hospital wastewater during MBBR treatment was measured by *Vibrio fischeri*, the inhibition of which decreased from 80% to 50%. By applying ozonation, this inhibition reduced to 20%.

30 **Keywords:** Hospital wastewater; Ozonation; Toxicity; Fluorescence; Pharmaceuticals.

31 1. Introduction

32 Hospital wastewater containing various pharmaceuticals mostly discharge into the municipal sewage system
33 and is successively treated by municipal wastewater treatment plants (WWTPs). However, conventional
34 WWTPs are not designed to eliminate all pharmaceuticals, and thus non-biodegradable compounds can be
35 released into the aquatic environment [1–3]. As one of the biological treatment processes which are
36 potentially alternative to conventional activated sludge (CAS), a moving bed biofilm reactor (MBBR)
37 operates with biomass grown on suspended plastic carriers [4]. Previous studies have demonstrated that
38 MBBR treatment achieves similar or better biodegradation efficiencies for pharmaceuticals than CAS [5,6].

39 Although higher pharmaceutical removal has been found in MBBRs than CAS, they can be compound-
40 specific, so some investigated pharmaceuticals in hospital wastewater are not completely degraded by
41 MBBR (e.g. iohexol, metoprolol and carbamazepine) [6]. To enhance residual pharmaceutical reduction
42 from MBBR effluent, polishing methods, such as ozonation, are currently being studied. Ozone is a strong
43 oxidant that decomposes in water into hydroxyl radicals, which act as a stronger oxidising agent than ozone
44 itself. The oxidation of pharmaceuticals may occur either as direct ozonation targeting of certain functional
45 groups of organic molecules, or as indirect ozonation, via the less selective hydroxyl radicals [7].

46 Ozonation as a post-treatment process, in conjunction with biological treatment, has been reported to remove
47 pharmaceuticals in wastewater, thus indicating that it is a promising technology for efficiently oxidizing
48 these compounds [8–12]. Moreover, ozone is more cost-efficient compared to UV or UV/H₂O₂ and powdered
49 activated carbon in terms of oestrogen and pharmaceutical removal from wastewater effluent [13–15].

50 Although ozone can remove pharmaceuticals efficiently, ozonated wastewater still retains some degree of
51 toxicity [16]. Furthermore, a potential disadvantage of ozone is the formation of unknown reactive by-
52 products, resulting in possibly even higher toxicity compared with the original compounds, as well as

53 making hardly degradable organic matter more biodegradable, which increases the biological oxygen
54 demand of the effluent [17–19].

55 To address the issue of frequent micropollutant (i.e. pharmaceutical) analysis, which is both expensive and
56 time-consuming, fluorescence monitoring has been suggested as an alternative approach, since it is a rapid
57 and reagentless technique requiring no sample preparation prior to analysis. The fluorescence excitation
58 emission matrices comprise fluorescence signals at several thousand pairs of excitation/emission
59 wavelengths (Ex/Em) for each sample, thus providing in depth information on the samples. Thus, this
60 technique can provide a tool to source fractions of DOC and examine its transformation during ozonation
61 [20]. Furthermore, a few studies have used UV_{254} and fluorescence with the excitation wavelength at 254 nm
62 as surrogate parameters, finding that reductions in UV_{254} and fluorescence intensity are correlated with
63 decreased concentrations of endocrine-disrupting chemicals, pharmaceuticals and personal care products
64 during ozonation [21,22]. Thus, fluorescence could be considered a tool for the indirect determination of the
65 degree of micropollutant reduction.

66 The aims of this work were as follows. First, it sought to investigate the effect of ozone dosage on
67 pharmaceutical removal in the effluent of a pilot MBBR. In order to verify ozone performance, the same
68 MBBR effluent was treated with a laboratory-scale ozone equipment by applying the same dosages as those
69 applied in the pilot experiments. The ozone demand for eliminating specific amounts of pharmaceuticals was
70 based on the pilot and verified laboratory experiments. Second, in parallel with the previous aim, the
71 fluorescence intensity of ozonated effluent was measured and the correlation between fluorescence intensity
72 and pharmaceutical concentration were investigated. Third, the toxicity of the wastewater in the MBBR and
73 ozonation was explored. The influence of ozone dosage on toxicity in MBBR effluent was also studied.
74 Finally, in order to address residual toxicity in ozonated effluent, a laboratory-scale MBBR was used to
75 polish ozonated wastewater. The effect of time development on toxicity and fluorescence intensity in
76 ozonated effluent, with or without a polishing MBBR, was also investigated.

2. Materials and Methods

2.1 Chemicals

Pharmaceuticals were obtained from different suppliers, and these are presented in the Supplementary Information (Table S1). The compounds analysed in this study were a) antibiotics (ciprofloxacin, sulfadiazine, sulfamethizole, sulfamethoxazole, trimethoprim, azithromycin and the sulfadiazine metabolite acetyl-sulfadiazine); b) blood pressure regulators (atenolol, metoprolol, propranolol and sotalol); c) analgesics (diclofenac, ibuprofen, phenazone and tramadol); d) antidepressants (venlafaxine and carbamazepine) and e) X-ray contrast media (iopromide, iohexol and iopamidol). Formic acid and HPLC-gradient grade methanol were purchased from Merck (Darmstadt, Germany). Potassium indigotrisulfonate was purchased from Sigma-Aldrich (Brøndbyvester, Denmark).

2.2 MBBR effluent

Wastewater from the new Aarhus University Hospital at Skejby, Aarhus, was treated with a five-stage MBBR demonstration plant, which had a denitrification tank of 900 L (M1), a nitrification tank of 900 L (M2), a nitrification tank of 900 L (M3A and M3B), a denitrification tank of 500 L (M4) and a nitrification tank of 500 L (M5) (Fig. 1, left). AnoxKaldnes™ K5 carriers (AnoxKaldnes, Lund, Sweden) were used in each tank with a filling ratio of 50%. The inlet flow rate was 300 L/h and the return flow from the M3 effluent was 500 L/h. More detailed descriptions with regard to the staged MBBR pilot can be found in G. Ooi et al [23]. The average values of dissolved oxygen (DO), pH, total organic carbon (TOC), NH₄-N and NO₃-N during the whole operation period are presented in Table S2.

2.3 Ozone equipment in the pilot and at the laboratory

The ozone equipment (Fig.1, left) was supplied by Air Liquide (Krefeld, Germany) and consisted of an effluent pump, an ozone generator (max. 10 g/h, Degremont Technologies, France), a reaction column, an ozone analyser (BMT, Germany) and an ozone destructor. MBBR effluent was pumped from top to bottom through the reaction column, and ozone was produced from an ozone generator which was supplied by compressed oxygen; the delivered dosage was controlled through a gas-flow regulator. Thus, the oxygen-

102 ozone mixture was injected into the bottom of the reaction column. The hydraulic retention time (HRT) of
 103 the reactor was 13.1 min, based on a 1 L/min MBBR effluent flow rate and the volume of the reaction
 104 column of 13.1 L. The residual mixture gas was transferred to the ozone destructor, where the remaining
 105 ozone was depleted. However, in the pilot, the off-gas ozone concentration was constantly equal to zero
 106 while performing the experiments.

107 The laboratory ozone set-up was based on a 20 g/h ozone generator from O₃-Technology AB (Vellinge,
 108 Sweden). Generated ozone from the ozone generator was dissolved in Milli-Q water (Purelab flex 2, Elga-
 109 Veolia, High Wycombe, UK), which was stored in a collection bottle, and then immersed in an ice bath to
 110 enhance ozone solubility, thus producing an ozone stock solution. To increase ozone solubility further, a
 111 manometer and a needle valve were placed on the outlet of the collection bottle, thereby maintaining higher
 112 internal pressure (around 1.4 barG), which made it possible to create an ozone stock solution with a
 113 concentration between 100 and 120 mg O₃/L.

115 Fig. 1. Left (pilot): A schematic diagram of a five-stage pilot-scale MBBR demonstration plant in conjunction with a pilot-scale
 116 ozonation system. M0 stands for the influent from hospital wastewater. Right (laboratory): MBBR effluent was treated by a
 117 laboratory-scale ozonation, and ozonated effluent was polished further by a laboratory-scale MBBR in the laboratory.

118 2.4 Fluorescence and MicroTox[®]

119 2.4.1 Fluorescence

120 Fluorescence intensity identifies the presence and the amount of specific molecules in a medium, and it was
 121 determined by a fluorometer (Cary Eclipse, Varian, Santa Clara, US) and expressed in arbitrary units (Au).

122 Six transition pairs were used, ranging from 213 nm to 335 nm and from 310 nm to 450 nm, respectively

123 (Hudson et al. [20]) (Table 1). Basically, five fundamental fluorescence peaks from the peak-picking method

124 have been observed in freshwater aquatic samples, and these have been classified according to Coble [24]
 125 and are presented in Table 1, below: Peak A ($\lambda_{\text{excitation}}$, emission: $\lambda_{249,450}$), Peak B: ($\lambda_{231,315}$, $\lambda_{275,310}$), Peak C:
 126 ($\lambda_{335,450}$), Peak T₁: ($\lambda_{275,340}$) and Peak T₂: ($\lambda_{231,360}$). Peaks A and C stand for the humic-like fluorophore, while
 127 peaks B, T₁ and T₂ stand for the protein-like fluorophore.

128 Table 1: Excitation, emission wavelength pairs for fluorophores based on Hudson et al. [20].

Fluorophore type	Fluorophore name [24]	Excitation, Emission wavelength (nm)
Protein-like (Tyrosine)	B	231,315
Protein-like (Tryptophan)	T ₂	231,360
Humic-like	A	249,450
Protein-like (Tyrosine)	B	275,310
Protein-like (Tryptophan)	T ₁	275,340
Humic-like	C	335,450

129 Samples were transferred in a quartz cuvette and subjected to further analysis, and there was no need for
 130 dilution. In case of turbidity, samples were left to settle prior to fluorescence scanning. The dilution of
 131 fluorescence intensity in samples ozonated in the laboratory by adding ozone stock solution was corrected by
 132 adjusting for the calculated extra volume from the ozone stock solution.

133 2.4.2 MicroTox[®] test

134 The MicroTox[®] test is based on light emissions (luminescence) from the bacterial marine organism *Vibrio*
 135 *fischeri*, which is a widely used test that is internationally recognised and standardised through ISO (2007)
 136 [25]. Dried bacteria for MicroTox[®] test were obtained from ABOATOX, Finland. Toxicity tests with
 137 wastewaters were performed in duplicate.

138 2.5 Quantification

139 2.5.1 Determination of ozone dosage

140 Delivered ozone dosage was quantified by using the indigo method [26]. Details regarding the modified
 141 indigo method conducted for this study can be found in Hansen et al.. (2016).

142 2.5.2 Pharmaceutical analysis (HPLC-MS/MS)

143 In both experiments 1 and 2, a 10 mL sample was transferred into a glass vial containing 3 mL methanol and
 144 then stored in a fridge at 4°C. After completing all of the experiments, the samples were transported to the
 145 laboratory in cool conditions (ice boxes). Once in the laboratory, the samples were stored at -20°C. HPLC-
 146 MS/MS was used for analysing concentrations of pharmaceuticals in the wastewater. The HPLC was
 147 equipped with dual low-pressure mixing ternary gradient system Ultimate 3000 from Dionex, consisting of a
 148 pump, a column oven and a degasser, all of which were 3000 series, and a 3000 TSL auto-sampler (WPS
 149 3000 TSL). The mass spectrometer was an API 4000 (ABSciex, Framingham, MA, USA). The samples were
 150 quantified using multi reaction monitoring (MRM). From the spiking experiments, the spiked concentrations
 151 (around 10 µg/L) and the determined concentrations were compared. Generally speaking 98% of the
 152 theoretical concentration was recovered. Detailed procedures regarding sample preparation and analysed by
 153 HPLC-MS/MS, and conditions of HPLC-MS/MS instrument were based on Escolà Casas et al. (2015), while
 154 the limits of quantifications (LOQs) are shown in Table S3. The term applied in this article related to
 155 pharmaceutical levels above the LOQ was defined based on the following principles. If there are two points
 156 of normalised concentrations above the LOQ or more than two points, this pharmaceutical is regarded as
 157 belonging to the group above the LOQ (e.g. clarithromycin in the laboratory experiments, Fig. 2). However,
 158 if there are fewer than two points of normalised concentrations above the LOQ, this pharmaceutical is
 159 regarded as belonging to the group below the LOQ (e.g. clarithromycin in the pilot experiments, Fig.2).

160 2.6 Experiments performed

161 In this study, MBBR effluent was treated with various ozone dosages in both pilot-scale (in the field) and
 162 lab-scale (laboratory) conditions. To investigate the effects of ozone dosages on the removal of
 163 pharmaceuticals, the intensity of fluorescence and microtoxicity, five experiments were conducted under
 164 different initial conditions (Table 2).

165 Table 2. Outline of experiments in the pilot and laboratory set-ups.

	Type	Pharmaceuticals		Fluorescence		Toxicity	
		Pilot	Laboratory	Pilot	Laboratory	Pilot	Laboratory
Exp 1	MBBR effluent was treated with pilot-scale ozonation through six dosages of ozone	+		+		+	

Exp 2	MBBR effluent was treated with laboratory-scale ozonation through nine dosages of ozone	+	+	
Exp 3	Flow through of the pilot-scale MBBR treatment train			+
Exp 4	Laboratory-scale ozonation with three dosages of ozone		+	+
Exp 5	Laboratory-scale ozonation with one dosage of ozone, followed with or without a laboratory-scale polishing MBBR		+	+

166

167 Exp 1: MBBR effluent was treated with six ozone dosages in the ozonation pilot (2.4, 10, 18, 34, 42 and 54
168 mg O₃/L). For each dosage, samples for pharmaceutical analysis were collected and preserved until analysis,
169 as described in Section 2.5.2, and samples for fluorescence measurements were stored at -18°C. These
170 samples were then brought up to room temperature overnight, before measuring fluorescence (Section 2.4.1)
171 and bacterial toxicity (Section 2.4.2). The average DOC of MBBR effluent during campaigns was 40 mg/L
172 and its standard deviation (±) was 5.7%.

173 Exp 2: MBBR effluent was treated with nine ozone dosages in the laboratory-scale ozonation (2, 4, 6, 10, 12,
174 15, 20, 30 and 40 mg O₃/L). Despite the ozone being completely consumed after 1-2 mins, due to high DOC
175 (40 mg/L), the ozonated samples always stood for 30 min prior to taking subsamples for pharmaceutical
176 measurement according to the method mentioned in Section 2.5.2. Furthermore, the fluorescence intensity of
177 the ozonated samples was measured over time, after the ozone was applied.

178 Exp 3: Conducted to investigate the effect of configuring the wastewater treatment process on toxicity,
179 toxicity measurements using MicroTox[®] were conducted for samples taken from each tank in the pilot-scale
180 MBBR treatment train.

181 Exp 4: MBBR effluent was treated with three ozone dosages (2, 4 and 6 mg O₃/L) in the laboratory. To
182 investigate the effect of time development after ozonation on the toxicity of ozone by-products, the
183 MicroTox[®] test was performed for the three ozonated samples over time. In parallel, fluorescence intensity
184 for these samples was measured.

185 Exp 5: To study whether MBBR carriers have the capacity to reduce the sample toxicity generated from
186 ozonation by-products. MBBR effluent was treated with 4 mg O₃/L in the laboratory. The ozonated samples

stood for 20 min before transferring each 40 mL sample to two 50 mL blue cap glass bottles (with and without carriers). A filling ratio of 50% was used in the bottle with carriers (Fig.1, right), and these carriers, with 3.3 g/L of biomass, were the same as those in the last nitrification reactor of the pilot MBBR, which acted as a polishing step. Then, samples under these two conditions, together with a 40 mL sample of MBBR effluent as a control in the third bottle (without ozonation and carriers), were tested using MicroTox[®] and fluorescence over the same time intervals.

2.7 Data treatment

To identify the delivered ozone dosage that achieved 90% removal of each pharmaceutical in the effluent, the normalised concentrations of each pharmaceutical and delivered ozone dosage were plotted by Eq. 1.

$$\frac{C}{C_0} = 10^{-\left(\frac{DO_3}{DDO_3}\right)} \quad (1)$$

where the remaining concentration of pharmaceuticals (C) is related to the initial concentration (C₀). After the relevant reaction with a specific delivered ozone dosage (DO₃, mg O₃/L), a decadic dosage of ozone (DDO₃, mg O₃/L), which is a compound-associated constant explaining the required ozone dosage needed to remove 90% of the respective pharmaceutical[9,15,27].

3. Results and discussion

3.1 Removal of pharmaceuticals with the delivered ozone dosage

In this study, we detected 22 pharmaceuticals present in the actual effluent of a pilot MBBR treating hospital wastewater, without spiking. According the defined term for LOQ in section 2.5.2, 15 pharmaceuticals were above the limit of quantification, while a further seven were only qualitatively detected but could not be quantified (Fig. 2, see all compounds in Fig. S1). Despite some points coming in below the LOQ in the defined pharmaceutical that was above the LOQ, it did not significantly affect accuracy when generating a fitting curve to affirm conversely specific ozone doses to eliminate 90% of the pharmaceuticals.

209 Concentrations of the investigated compounds decreased in line with increasing ozone dosages, except for
 210 iopromide, which was not removed, even at the highest ozone dosage in either the pilot or the laboratory
 211 experiment. The poorer fit for carbamazepine, clarithromycin, sulfamethizole, propranolol and trimethoprim,
 212 as well as the steep decrease in line with lower ozone doses, is explained by the two mechanisms for ozone
 213 reaction with chemicals. The reactions of compounds to ozonation involve non-direction reaction (i.e. $\cdot\text{OH}$)
 214 and direct reaction (i.e. ozone molecule). The first reaction happens instantly, as $\cdot\text{OH}$ has higher reaction rate
 215 constant with compounds compared to the rate constant of ozone molecule to compounds. Hence, in Fig.2,
 216 there was a steep decrease at the beginning, when low dose of ozone was delivered, thus indicating that $\cdot\text{OH}$
 217 contributed more to removing pharmaceuticals than the ozone molecule. After increasing the ozone dose,
 218 more ozone molecules became involved in the removal of pharmaceuticals, leading to a flat fitting curve.

219
 220 Fig. 2. Comparison of pharmaceutical removal by ozone in the effluent of a staged MBBR demonstration plant at Skejby Hospital,
 221 using the onsite continuous pilot ozonation system and batch treatment in a laboratory. The dashed lines in the figure stand for the
 222 limit of quantification (LOQ) of pharmaceuticals by HPLC-MS/MS. Error bars represent the standard deviations.

223 To evaluate removal efficiencies for individual pharmaceuticals, DDO_3 are derived by curve fitting as shown
 224 in Fig. 2 and the results for which are shown in Table 3. DOC is a water quality parameter which

225 significantly affects the performance of the ozone process [28]. Hence, DDO_3 was normalised to the effluent
226 DOC ($Z_{90}=DDO_3/DOC$) which creates a site unspecific parameter for the sensitivity of each pharmaceutical
227 to ozonation, (Table 3) as previously described by Hansen et al., 2016 [9]. Of the investigated
228 pharmaceuticals, propranolol, tramadol, trimethoprim and venlafaxine had the lowest Z_{90} , which means that
229 they can be considered easier to degrade by ozone than the other substances. Trimethoprim and iopromide
230 had the lowest and highest Z_{90} , respectively, indicating that they reacted the slowest and the fastest with
231 ozone, respectively. Nevertheless, the lowest and highest Z_{90} obtained in the laboratory experiment were
232 carbamazepine and iopamidol, respectively. Although factors about ozone set-up and temperature can be
233 explained for the discrepancies identified when eliminating pharmaceuticals in the pilot and laboratory
234 experiments, the effects caused by thawing and unthawing the MBBR effluents, before carrying out the
235 laboratory experiments, also cannot be neglected. Furthermore, the deviations between these experiments
236 were attributed to the dosage method. For the laboratory method, ozone was added at once resulting in a
237 peak with a higher concentration than resulting from a dosage in the pilot column, where ozone gas and
238 wastewater were added in the countercurrent flow, resulting in a smaller peak concentration, as ozone was
239 transferred and simultaneously consumed over the height of the column. The main purpose of including both
240 pilot and laboratory experiments was to investigate whether the predication of ozone dosage from the
241 laboratory fitted with the pilot experiments, namely the flow-through experiment, or not. Additionally, by
242 including the laboratory experiments, we aimed to verify the field measurement system of the ozone dosage,
243 which we were not able to calibrate on site. While, the good overall agreement with dosages indicated the
244 measurement system was reasonably correct.

245 In previous field studies, pilot- or full-scale ozonation has also been applied to eliminate pharmaceuticals in
246 either biologically-treated hospital wastewater or WWTP effluent [29,30]. Discrepancies regarding the
247 results of normalised ozone dosages with DOC (Z_{90}) between this pilot experiment and previous field studies
248 can be attributed to several factors: i) wastewater matrices, including hospital wastewater treated with a
249 membrane bioreactor or an MBBR and municipal wastewater effluent, can affect pharmaceutical removal
250 efficiency, which in turn can lead to different levels of Z_{90} . ii), the DOC in the literature [29,30] was below

10 mg/L, while DOC was 40 mg/L in this study. Theoretically, a long HRT ensures a sufficient reaction time between ozone and the pharmaceutical. However, ozone transfer efficiency in the literature was 66-78%, while in this pilot study it was nearly 100%, as no ozone was detected in the off-gas.

In previous laboratory studies, Hansen et al. [9] studied the effect of the DOC on the removing spiked pharmaceuticals in effluent from a staged MBBR with laboratory ozone equipment, finding that the Z_{90} values from effluent with different DOCs were close to the Z_{90} that was obtained in this laboratory study for most of pharmaceuticals, except for carbamazepine, diatrizoic acid, iomeprol, iopromidol and sulfadiazine. Furthermore, Antoniou et al. [31] investigated removal differences in effluent from six WWTPs on the removal of spiked pharmaceuticals in laboratory ozonation. When comparing Z_{90} with the present study, it was found that pharmaceuticals like metoprolol, sulfamethoxazole and trimethoprim in our study have similar or even higher removal.

Thus, Z_{90} can be considered an important index on which to base ozone dosage for eliminating pharmaceuticals and evaluating their removal efficiency via ozonation.

Table 3. Ozone dosage for 90% removal of pharmaceuticals (DDO_3 , mg O_3 /L) in a pilot study and in laboratory conditions, and the normalisation of ozone dosage to the relevant DOC (mg/L) condition ($Z_{90}=DDO_3/DOC$, mg O_3 / mg DOC). DOC in this study is 40 mg/L. Error bars represent the standard deviation.

	Pilot		Laboratory		Z_{90}						
	DDO_3	R^2	DDO_3	R^2	Pilot	Laboratory	A	B	C	D	E
Ac-sulfadiazine	<LOQ ^a	<LOQ									
Atenolol	103±19	0.89	46±7	0.91	2.6±0.5	1.1±0.2		1.1		0.6	0.5
Azithromycin	<LOQ	<LOQ	<LOQ	<LOQ							
Carbamazepine	147±53 ^c	0.63	7.4±1.1	0.96	3.7±1.3	0.18±0.03	0.61	0.58	0.6	0.6	0.25
Ciprofloxacin	<LOQ	<LOQ	<LOQ	<LOQ							
Clarithromycin	<LOQ	<LOQ	39±6	0.92		0.98±0.14		0.75	0.6	0.6	
Diatrizoic acid	<LOQ	<LOQ	<LOQ	<LOQ				4.7			
Ibuprofen	<LOQ	<LOQ	46±8	0.78		1.1±0.2	1.61	1.3			1.0
Iohexol	151±42	0.77	110±30	0.69	3.8±1.0	2.7±0.7		1.8		1.8	
Iomeprol	No fit ^b	0.41	No fit	0.44				1.9	1.9		
Iopamidol	No fit	0.01	No fit	0.34				2.6	1.8		
Iopromide	<LOQ	<LOQ									
Metoprolol	52±6	0.94	54±10	0.75	1.3±0.2	1.3±0.2	1	0.89	0.6	0.6	
Phenazone	<LOQ	<LOQ	<LOQ	<LOQ				0.77			
Propranolol	42±9	0.79	35±2	0.96	1.0±0.2	0.88±0.06		0.6	1.1	0.6	

Sotalol	<LOQ	<LOQ	<LOQ	<LOQ						
Sulfadiazine	<LOQ	<LOQ					0.5			
Sulfamethizole	50±12	0.862	<LOQ	<LOQ	1.2±0.03		0.77	0.52		
Sulfamethoxazole	56±9	0.74	68±14	0.66	1.4±0.2	1.7±0.3		0.52	0.6	0.6
Tramadol	33±19	0.64	31±9	0.77	0.81±0.47	0.8±0.2		0.97	0.6	
Trimethoprim	51±12	0.61	29±2	0.96	1.3±0.3	0.73±0.06	0.55	0.55		0.6
Venlafaxine	44±7	0.90	50±9	0.77	1.1±0.2	1.2±0.2	0.91	1.4	0.6	0.6

^a: If concentration was below the limit of quantification (LOQ), indicated as <LOQ.

^b: If $R^2 < 0.5$, it indicates 'no fit'.

^c: If $0.5 < R^2 < 0.7$, it is considered a poor fit and is indicated in italic.

A indicates the reference [31].

B indicates the reference [9].

C indicates the reference [30].

D indicates the reference [29].

E indicates the reference [32].

3.2 Use of natural fluorescence to estimate ozone dosage

3.2.1 Comparison of fluorescence results in pilot and laboratory studies

Fig. 3. (a)-(d): Comparison of fluorescence in ozonated M5 effluents between the pilot and laboratory experiments. (e): the correlation of two fluorescence signals at peak T₁ (λ_{275,340}) and peak C (λ_{335,450}) in ozone-treated effluent between pilot and laboratory studies, fitted by a straight line.

The decrease in fluorescence intensity was continuous with increase of ozone dosages in both pilot and laboratory experiments (Fig. 3a-d). This decreased fluorescence intensity was attributed to the depletion of aromatic structures and the increase in electron withdrawing groups such as -COOH in aromatic compounds [33,34]. For the pilot study, the intensity of all protein-like fluorescent peaks (Peak B, T₁ and T₂) decreased significantly at ozone dosages around 2-10 mg O₃/L, whereas, for the laboratory experiments, the intensity of

287 all protein-like fluorescence peaks decreased gradually by up to 40 mg O₃/L. However, the intensity of
 288 humic-like fluorescence could not be removed in all instances, even at high ozone concentrations (40 or 50
 289 mg O₃/L), because it represents (besides others) humic substances in wastewater which have complex
 290 chemical properties aligned with high stability [35]. Spiliotopoulou et al. [36] proved that fluorescence can
 291 be used as a monitoring tool to determine indirectly ozone in recirculating aquaculture system water.
 292 Therefore, in order to predict and control water quality in online systems, less ozone is required when aiming
 293 at removing protein-like fluorescence, while high levels of ozone are needed when looking to remove humic-
 294 like fluorescence.

295 In terms of the investigated fluorescence wavelengths, peak T₁ and peak C illustrated the highest intensity
 296 (Fig.3. a-d). In previous studies [37,38], peak T₁, was considered a tracer for dissolved organic matter, yet
 297 Riopel et al. [38] demonstrated that peak C, with a higher intensity compared to peaks T₁, T₂ and B, can be
 298 used when wastewater is characterised by fluorescence spectra. Comparing either peak T₁ or peak C is more
 299 pertinent in terms of expressing fluorescence spectra characteristics in wastewater, and so the correlation was
 300 made between the intensity of peak T₁ and the intensity of peak C under relevant ozone dosage in pilot and
 301 laboratory experiments (Fig. 3e). Results from the fitting curve in the pilot experiments, with delivered
 302 ozone levels ranging from 2 to 34 mg O₃/L, show that intensity at peak T₁ was much sensitive than at peak C,
 303 while intensity results were opposite when the delivered ozone dosage ranged from 42 to 54 mg O₃/L. For
 304 results from the fitting curve in the laboratory experiments, intensity at peak T₁ and C was very similar, even
 305 with different ozone dosages ranging from 2 to 40 mg O₃/L. The fitting lines in the pilot and the laboratory
 306 had good linear ability with R² of 0.72 and 0.97, respectively, thereby indicating that either peak T₁ or peak C
 307 in well-controlled laboratory conditions could be used as a surrogate for water clarity (i.e. DOM).

3.2.2 Correlation of fluorescence intensity and the removal of pharmaceuticals

Fig. 4. Linear correlation between normalised concentrations of selected pharmaceuticals ($\Delta C/C_0$) and normalised intensities of six fluorescence ($\Delta A/A_0$) signals.

The correlation between the reduction of fluorescence intensity at six wavelengths and the normalised concentration of selected pharmaceuticals was fitted by straight lines. The fitting results are presented in Fig. 4 (for all pharmaceuticals, see Fig. S2). For the selected pharmaceuticals, fluorescence was linearly correlated to their removal, and the fitting line was linear, i.e. compound-specific. Based on the fitting results illustrated in Table S4, among all of investigated peaks, peak T_1 had had the closest correlation for ten out of the 19 pharmaceuticals, if counting the numbers for the highest R^2 that it achieved. This was followed by peak T_2 , where seven out of the 19 investigated pharmaceuticals correlated closest. However, if the numbers for the second highest R^2 were also counted, peak B had ten out of the 19 investigated pharmaceuticals. Additionally, if counting the numbers of the biggest slope from the fitting results, peak A had 18 out of the 19 investigated pharmaceuticals with the biggest slope, although peak A had only two out of the 19 investigated pharmaceuticals with the highest R^2 . The biggest slope demonstrated that it was more sensitive to reflecting pharmaceutical removal instantly compared to other peaks, which indicated that with peak A more pharmaceutical removal can be detected alongside less reduction in fluorescence intensity. Hence, peak

325 T_1 can be used as a surrogate for the accurate expression of the removal of pharmaceuticals in wastewater,
 326 since it had closest correlation with pharmaceutical concentration.

327 Previous studies have found that there is good correlation between fluorescence and common parameters (e.g.
 328 biochemical oxygen demand (BOD) and TOC) for treated or untreated sewage, and thus fluorescence can be
 329 considered an indicator in this regard [40]. It has been also found that fluorescence with the excitation
 330 wavelength at 254 nm correlates with trace organic contaminant oxidation ($R^2=0.50-0.83$) and can be used as
 331 an indicator for target compound elimination [21]. Hence, alongside the similar findings in this study, we can
 332 assume that fluorescence intensity as a surrogate has the potential ability to trace pharmaceutical oxidation.

333 3.3 Effects observed with Microtox[®] during treatment

334 3.3.1 Effects observed with Microtox[®] during the staged biological treatment 335

336 Fig. 5. Toxicity (MicroTox[®]) at three assay times (5, 15 and 30 min) in the pilot-scale MBBR treatment train (a) and pilot ozonation
 337 (b). M0: hospital wastewater fed into the pilot-scale MBBR treatment train. RF: return flow from M3B marked with an open symbol.
 338 M5: effluent from the MBBR treatment train, which was also the initial ozonation inlet (O₃ =0 mg/L).
 339

340 A comparison of the inhibition of *Vibrio fischeri* in wastewater, taken from different pilot-scale MBBR
 341 treatment processes, followed by ozonation, is presented in Fig. 5. The inhibition percentage of *Vibrio*
 342 *fischeri* generally increased in line with increasing assay time, from 5 min to 30 min. In Fig. 5a, the
 343 inhibition percentage decreased tank by tank, except for M4, thereby indicating that toxicants were removed
 344 during the MBBR treatment train. Reduced toxicity was attributed to the removal of biodegradable fractions
 345 of organic content in the wastewater. The higher toxicity in M1 than in M0 could possibly indicate that

346 substances with higher toxicities were produced in the M1 denitrification tank (anaerobic status), either by
347 hydrolysis of conjugated substances or via reductive biotransformation. Moreover, the inhibition percentage
348 of *Vibrio fischeri* increased from M3B to M4, indicating an overdosing of ethanol used as a supplementary
349 carbon source for denitrification in M4. Farre et al. [41] established that the inhibition percentage of *Vibrio*
350 *fischeri* increased between raw influent and primary sedimentation effluent, due to chemicals added in the
351 primary sedimentation step. It was found that the inhibition percentage of *Vibrio fischeri* in MBBR effluent
352 (M5) was around 60% for an assay of 30 min, but the water was still toxic, thus indicating that influent
353 pollutants were not biodegradable and were difficult to remove completely with the MBBR system.
354 Moreover, these pollutants can inhibit the metabolic activity of microbes [42].

355 The MBBR had the capacity to reduce effluent toxicity down to around 50%, corresponding to the toxicity
356 noted in the effluent (M5, Fig.5b). However, the toxicity of the ozonated effluent treated by 2.4 to 18 mg
357 O₃/L first increased and then decreased (Fig. 5b). In this case, we might assume that an ozonated by-product
358 with the highest concentration formed at 10 mg O₃/L, when organic matter in the wastewater interacted with
359 ozone.

360 3.3.2 Microtox[®] developed in ozonated MBBR effluent, following wastewater treated by a suspended
 361 biofilm.

362 Fig. 6. Comparison of Microtox[®] with three assay times (5, 15 and 30 min) under different concentrations of ozone from the pilot,
 363 laboratory and polishing processes (MBBR) over time. (a) Wastewater effluent treated by various dosages of ozone in the pilot, (b)
 364 wastewater effluent treated by three dosages of ozone in the laboratory, (c) wastewater effluent treated by one dosage of ozone in the
 365 laboratory, followed with or without a polishing MBBR.
 366

367 The effect of developed time in ozone reaction (0.3 2, 6 and 24 h) on the inhibition of *Vibrio fischeri* was
 368 investigated, and the results are presented in Fig. 6. The inhibition percentage of *Vibrio fischeri* in Fig. 6a-c
 369 increased in line with an increase in the assay time of ozonated effluent to *Vibrio fischeri*, from 5 min to 30
 370 min. In Fig. 6a, the inhibition of *Vibrio fischeri* in effluent gradually increased from 20% for an assay time of
 371 5 min to 70% for an assay time of 30 min, while ozone at various dosages was able to reduce toxicity
 372 existing in effluent. At an assay time of 30 min, the inhibition percentage of *Vibrio fischeri* in the ozonated
 373 effluent, with dosages ranging from 2.4 mg O₃/L to 54 mg O₃/L, was similar, thereby indicating no

374 significant effect of ozone dosage on the inhibition of *Vibrio fischeri*. Therefore, low ozone dosages were
375 spiked in the present laboratory study (2, 4 and 6 mg O₃/L).

376 In Fig. 6b, at an assay time of 15 min, the inhibition percentage of *Vibrio fischeri* in each sample increased
377 along with an increase in ozone reaction time; however, for 30 min, the inhibition percentage in each sample
378 initially increased until 6 hours had passed and then decreased to a lower level at 24 h. In addition, we also
379 observed that the inhibition in ozonated wastewater with a different assay time was not reduced in line with
380 an increasing ozone dose, which was converse to what we observed in the pilot study (Fig. 6a). This could be
381 assumed that the effluent property had been changed during the freezing and thawing process, before
382 conducting the ozone experiments in the laboratory. Due to the residual toxicity in the ozonated effluent, a
383 laboratory-scale MBBR was introduced as a polishing method after ozonation.

384 The effects on toxicity of adding MBBR carriers in ozone-treated effluent (4 mg O₃/L) were examined (Fig.
385 6c). At an assay time of 5 min, the inhibition percentage of *Vibrio fischeri* in each sample increased over
386 time, while at an assay time of 15 min, it was found that ozonated effluent treated with a polishing MBBR
387 was able to reduce more toxicity than effluent treated only with ozone, when the developed time was at
388 either 2 or 6 h. At an assay time of 30 min, the inhibition percentage of *Vibrio fischeri* in ozonated effluent
389 treated with the polishing MBBR was around 20% less than effluent treated only with ozone, when the
390 developed time was at 6 h. The influence of assay time on the trend of toxicity in samples over time could be
391 attributed to the fact that *Vibrio fischeri* hadn't completely adapted to the tested wastewater in such a short
392 assay time (e.g. 5 mins). When the assay time increased, *Vibrio fischeri* gradually started to adapt to the
393 tested wastewater and expressed more illuminance, giving an accurate assessment of wastewater toxicity.
394 The toxicity increasing after 2 h occur in all three water samples. It appears to be a variation in the bioassay,
395 though we have no foundation on which to base any explanation as to why it happened. However, the key
396 point of the results was that after 6 h, the polishing MBBR was capable of reducing toxicity compared to
397 untreated effluent or only ozonated effluent.

Hence, although ozone can reduce to some extent the toxicity of MBBR effluent, a subsequent polishing
 MBBR after ozonation can further reduce any residual toxicity in ozonated effluent.

3.4 Fluorescence intensity in the MBBR polishing of ozonated effluent

Fig. 7. Development of fluorescence of MBBR effluent treated by ozone with and without suspended biofilm treatment (MBBR). 0 mg O₃/L stands for MBBR effluent; 0 mg O₃/L (MBBR) stands for MBBR effluent treated with only a polishing MBBR; 2 mg O₃/L stands for MBBR effluent treated with 2 mg O₃/L; 2 mg O₃/L (MBBR) stands for MBBR effluent treated with 2 mg O₃/L followed with a polishing MBBR.

In Fig. 7, protein-like fluorescence intensities of ozonated effluent treated by the polishing MBBR decreased significantly over time, especially after 6 h, while the fluorescence intensities of effluent and the ozonated effluent did not change to a great extent. The humic-like fluorescence intensities in ozonated effluent and ozonated effluent polished with MBBR first decreased, but ultimately they increased in ozonated effluent polished with MBBR. The decreased fluorescence could be due to ozone having oxidized hardly degradable organic carbon wastewater into some biodegradable organic carbon, thereby causing BOD to increase in the wastewater. The highest BOD was found in the ozonated wastewater, followed by ozonated wastewater polishing by MBBR and MBBR effluent. Its BODs were 52.5, 44.0 and 18.4 mg/L, respectively. In contrast, the higher the BOD, the lower humic-like fluorescence. Increased fluorescence could be attributed to the fraction of leaching from the attached biofilm in carriers that might be humic-like after 24 hours. Hence, Fig.

416 7 demonstrates further that ozone followed with a polishing MBBR can reduce both organic matter and
417 toxicants in the initial effluent and by-products from the ozonation process.

418 4. Conclusion

419 Pharmaceutical concentrations decreased in line with an increase in ozone dosage, and Z_{90} , based on a ratio
420 of a delivered ozone dosage achieving 90% removal of pharmaceuticals and DOC in wastewater, was
421 considered to be an indicator of the removal efficiency of pharmaceuticals in wastewater by ozonation.
422 Fluorescence intensity was related positively to pharmaceuticals in wastewater, and less ozone was needed
423 when aiming to reduce the fluorescence intensity of protein-like fluorophores, while high amounts of ozone
424 were required when reducing the fluorescence intensity of humic-like fluorophores. Pilot-scale staged
425 MBBRs were capable of reducing toxicity inhibitions in wastewater, reactor by reactor, and subsequent
426 ozone further reduced by half any toxicity remaining in the MBBR effluent. A laboratory-scale polishing
427 MBBR could reduce further the toxicity and fluorescence intensity of protein-like fluorophores from
428 ozonated wastewater.

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