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1 Ecotoxicological risk assessment of micropollutants from treated urban wastewater effluents for  
2 watercourses at a territorial scale: application and comparison of two approaches.

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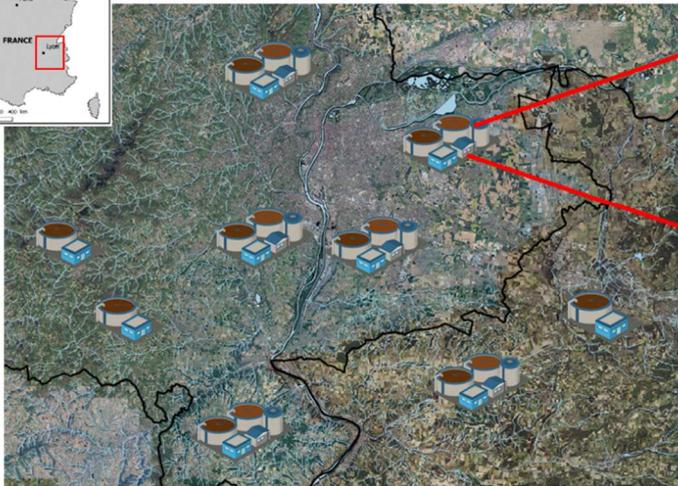
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## 10 Abstract

11 In most cases, urban Wastewater Treatment Plants (WWTP) only partially abate **pollutants occurring**  
12 **in the influent**. Treated effluents can thus contain a complex mixture of ecotoxic pollutants, such as  
13 heavy metals, detergents, disinfectants, plasticizers, pharmaceuticals residues or pesticides. In this  
14 context, Ecotoxicological Risk Assessment (ERA) provide essential decision-making tools to public  
15 authorities for establishing environmental policies and conducting territorial planning. The present  
16 work aims to develop a territorial-scale ERA methodology using two complementary approaches  
17 based on a Risk Quotient (RQ) calculation: (1) the first, based on the risk linked to each individual  
18 pollutant (single substances ERA); (2) the second, considering all pollutants present, and the “cocktail  
19 effect” (mixture ERA). This research was performed at 33 urban WWTPs of in a highly urbanized part  
20 of France (Lyon area). Initial minimum, median and maximum pollutant concentrations in treated  
21 effluents were obtained from a literature review of physico-chemical analysis studies, to reconstitute  
22 “typical” effluents. The classical approach (single substances ERA) identified the riskiest substances  
23 (e.g. endocrine disruptors, as the Estrone with RQ up to 593.75), and showed the risks for each  
24 WWTP. The mixture ERA approach revealed new risks, which were not highlighted in the classical  
25 ERA approach, thus increasing the number of WWTPs identified as at risk. This study shows the  
26 importance of accounting for the cocktail effect, which is not considered in current regulatory  
27 decisions. Finally, this methodology allowed us to identify the riskiest situations (often medium sized  
28 WWTPs, releasing into small streams), that could worsen in the context of climate change.



## Territory-scale approach



## Ecotoxicological risk assessment

Substance approach: risks

$$RQ_x = \frac{PEC_x}{PNEC_x} \quad RQ_y = \frac{PEC_y}{PNEC_y} \quad RQ_z = \frac{PEC_z}{PNEC_z}$$

Mixture approach: cumulative risks

$$RQ_{\text{Mix}} = \sum_{x=1}^n \frac{PEC_x}{PNEC_x}$$

29 Graphical abstract

30

31 Keywords

32 Urban Wastewater

33 Micropollutants

34 Pharmaceuticals

35 Ecotoxicological Risk Assessment

36 Mixture Risk

37

38 Highlights

- 39 • Micropollutants are often **not effectively removed and so** released by the urban WWTPs.
- 40 • A territorial approach is essential to evaluate the risks linked to WWTP effluents.
- 41 • Ecotoxicological Risk Assessment (ERA) for the WWTPs of the Grand Lyon territory was
- 42 carried out.
- 43 • Two different ERA approaches were used for WWTP risk assessment.
- 44 • Mixture ERA revealed hidden risks compared to the classic single substance approach.

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## 1. Introduction

55 In recent decades, many studies have detected and reviewed the presence of (in-)organic  
56 (micro-)pollutants in surface water (Luo et al., 2014; Pal et al., 2010). This pollution stems from  
57 intense urban and industrial activities leading to the release of liquid effluents. Among the various  
58 sources of contamination, the discharge of treated urban wastewater appears to be a major source of  
59 potentially ecotoxic pollutants including pharmaceuticals, personal care products, surfactants,  
60 plasticizers, heavy metals and pesticides (Deblonde et al., 2011; Loos et al., 2013; Luo et al., 2014; Pal  
61 et al., 2010; Teijon et al., 2010). These results can be explained by the frequent low efficiency of urban  
62 wastewater treatment plants (WWTP) in micropollutant abatement (Falas et al., 2016; Grand-Clément  
63 et al., 2017) and occasionally, the creation of (more) ecotoxic by-products (Bertanza et al., 2013;  
64 Rosal et al., 2009; Stalter et al., 2010). The significant toxic potential linked to each of these pollutants  
65 has already been evaluated (Orias and Perrodin, 2014; Gosset et al., 2017).

66 However, the chemical characterization of treated WWTP effluents is not currently sufficient,  
67 as these data do not account for the exposure of aquatic organisms. To understand the impact on these  
68 organisms, performing Ecotoxicological Risk Assessment (ERA) on WWTP effluents is crucial. These  
69 traditionally compare the measured presence of each pollutant within the receiving ecosystems  
70 through Predicted Environmental Concentration (PEC) or Measured Environmental Concentration  
71 (MEC) values, and their potential effects on the aquatic ecosystems through Predicted No Effect  
72 Concentration (PNEC) values (Perrodin et al., 2011). To this end, many investigations have been  
73 performed on urban WWTP effluents and receiving watercourses (Kosma et al., 2014; Yan et al.,  
74 2014; Perrodin et al., 2013; Pereira et al., 2015; Singh et al., 2014).

75 But, in many cases, ERA are performed on isolated WWTPs, and ecotoxicological risks at the  
76 territorial scale are not taken into account. Loiseau et al. (2012) indicated that finding methods for  
77 environmental assessment of a territory are essential, because they provide useful decision support  
78 tools and information to help public authorities in designing policies for territorial planning. This is

79 why spatialized environmental assessment methods have been developed for some years (Carafa et al.,  
80 2011; Loiseau et al., 2012; [Pereira et al., 2015](#); Stelzenmüller et al., 2015; Vulliet and Cren-Olivé,  
81 2011; Yang et al., 2010). One critical development in territorial ERA has been identified: assessing the  
82 risk linked to multiple WWTPs for the receiving aquatic watercourses in a territory. An initial study  
83 was carried out by Brus and Perrodin (2017). They developed an ERA methodology to evaluate the  
84 ecotoxicological risks of 18 WWTPs, scattered over a territory, on receiving watercourses for  
85 micropollutants (pharmaceuticals, plasticizers, pesticides, ...) not often examined, at realistic  
86 concentrations reviewed from scientific literature. They uncovered a high risk for many of them.

87 One other limitation was highlighted from their methodology: the non-consideration of  
88 “cocktail effect” in the regulatory-based risk assessment procedure based on European Technical  
89 Guidance Document (TGD, 2003) that they employed. [It has been now widely demonstrated that](#)  
90 [aquatic organisms exposed to a mixture of pollutants at low doses and individually not supposed to](#)  
91 [induce negative impacts \(concentration under the No Observed Effect Concentration \(NOEC\)\), can](#)  
92 [lead significantly intoxicated \(Beyer et al., 2014; Kortenkamp, 2007\). This joint toxic effects have](#)  
93 [been for example observed with organism’s exposure to endocrine disruptors, pharmaceuticals,](#)  
94 [pesticides, or surfactants \(Beyer et al., 2014; Kortenkamp, 2007; Panouillères et al., 2007; Vasquez et](#)  
95 [al., 2014\). Thus,](#) this problematic has emerged for now several years in various worldwide ERA  
96 studies (Brus and Perrodin, 2017; Gros et al., 2010; Jean et al., 2012; Kosma et al., 2014; Orias and  
97 Perrodin, 2014; Perrodin et al., 2011). Research is currently moving toward the development of new  
98 methods to evaluate mixture toxicity (Riva et al., 2019) and risk. Several studies [have recently applied](#)  
99 mixture ERA, most of the time through an additive model for risk quotient (RQ) calculation, on  
100 different families of wastewater pollutants, and demonstrated its interest (Backhaus and Faust, 2012;  
101 Backhaus and Karlsson, 2014; Escher et al., 2011; Kase et al., 2018; Riva et al., 2019; Thomaidi et al.,  
102 2015).

103 In summary, the aim of the present study was to evaluate the influence of the “cocktail effect”  
104 consideration for the ecotoxicological risk assessment of urban wastewaters for receiving watercourses  
105 at a territorial scale. For that, a methodology was developed and applied, employing two compared

106 approaches based on Risk Quotient (RQ) calculation: (1) the first, studying the risk linked to each  
107 individual pollutant (single substance ERA) (2) the second, considering all pollutants present (the  
108 “cocktail effect”), into the receiving watercourses (mixture additive ERA). Initial minimum, median  
109 and maximum pollutant concentrations in treated effluents used for this study were obtained from a  
110 literature review of physico-chemical analyses studies to reconstitute “typical” effluents. This work  
111 was then applied to 33 urban WWTPs of the highly urbanized Lyon region in France. Finally, the  
112 interest of this territory-scale methodology based on single and mixture ERA was discussed in a  
113 context of climatic change that could worsen the identified risk situations.

## 114 2. Materials and methods

### 115 2.1. Presentation of the territory and WWTPs

116 This work studies the highly urbanized territory of Grand Lyon (France) and the surrounding  
117 suburban municipalities. This area was selected for several reasons: the presence of available data for  
118 WWTP and the watercourse flow rates, the diversity (WWTP/watercourse flow rate ratios) of each  
119 WWTP and the highly anthropized situation (and so, a priori, high risk).

120 A total of 85 WWTPs were identified in the territory. Sufficient information (WWTP and  
121 watercourse flow rates) to perform an ERA were available for 41 WWTPs. These WWTPs are  
122 presented in Table 1. Among them, 32 correspond to WWTPs releasing treated effluents into  
123 watercourses, and 9 in the soil. These 32 WWTPs were selected to evaluate the ecotoxicological risk  
124 of wastewater discharges for aquatic ecosystems in the Lyon area. The WWTP locations, flow rate and  
125 receiving watercourses are presented in Table 1 and Figure 1. These WWTP flows present a large  
126 diversity in the territory, from 4 to 215092 m<sup>3</sup>/day.

### 127 2.2 Implementation of the methodologies for ERA evaluation on the selected territory

#### 128 2.2.1 Pollutant ecotoxicity, and concentration in WWTP effluents and watercourses

129 Two approaches exist when conducting ERA. The first, theoretical, is based on the compilation  
130 of data obtained from peer-reviewed literature, and the calculation of a maximum, median and/or  
131 minimal “typical” concentration observed for each researched pollutant in the various studies. The

132 second, more realistic, is based on the measurement of selected compounds in collected WWTP  
133 effluent samples. However, the latter approach is very expensive (Orias and Perrodin, 2014), and  
134 particularly when territorial ERA are performed. This is why the first approach was employed to  
135 perform the ERA evaluations in the selected territory. It assumes that the pollutant concentration  
136 ranges detected in different countries are similar (Thomaidi et al., 2015). Thus, 55 pollutants were  
137 examined in the study: 7 analgesic and anti-inflammatory pharmaceuticals (Diclofenac, Ibuprofen,  
138 Indomethacine, Ketoprofen, Naproxen, Paracetamol (Acetaminophen), Salicylic acid), 1 antiepileptic  
139 (Carbamazepine), 4 lipid regulators (Fenofibrate, Bezafibrate, Gemfibrozil, Clofibrate), 9 antibiotics  
140 (Amoxicillin, Ciprofloxacin, Doxycyclin, Erythromycin, Norfloxacin, Ofloxacin, Roxithromycin,  
141 Sulfamethoxazole, Trimethoprim), 3  $\beta$ -blockers (Atenolol, Metoprolol, Propanolol), 4 steroid  
142 hormones (Estrone, Estradiol (17 $\beta$ -Estradiol), 17 $\alpha$ -Ethinylestradiol, Estriol), 4 antineoplastics and  
143 immunomodulant agents (tamoxifène, 5-fluorouracil, Cyclophosphamide, Ifosfamide), 1 disinfectant  
144 (Triclosan), 2 detergents (Nonylphenol, Octylphenol), 4 plasticizers (Bisphenol A, DBP (DiButyl  
145 Phthalate), DEHP (Di(2-ethylhexyl) phthalate), DMP (Dimethyl phthalate)), 2 fire retardants (TCEP  
146 (Tris (2-chlorethyl) phosphate), TCPP (TRIS(2-Chloro-1-methylethyl) Phosphate)), 7 metallic trace  
147 elements (Cadmium, Chromium, Copper, Nickel, Mercury, Lead, Zinc), 6 pesticides (Atrazine,  
148 Diuron, Diazinon, Cypermethrin, Clotrimazole, Tebuconazole), and **Ammonia**. Their ecotoxicity  
149 (through Predicted No Effect Concentration (PNEC) values) and typical concentrations (minimum,  
150 median and maximum) in WWTP treated effluents have already been calculated in Brus and Perrodin  
151 (2017) and were used in this study to realize the 2 ERA methodologies in the present territory. Briefly,  
152 PNEC values were obtained from international database (e.g. INERIS, ECHA or OSPAR  
153 Commission), or from scientific literature on pollutant concentrations. These data are supplied in  
154 supplementary data (Table S1).

155 The Predicted Environmental Concentration (PEC) of the selected pollutants in the watercourses  
156 of our territory, **which are presented in Figure 2**, were based on WWTP discharge rates, their typical  
157 concentration in effluents and watercourse flows during the low-water period (to apply the

158 precautionary principle in the most critical period of the year) and calculated using the following  
159 equation (1):

$$160 \quad PEC_{x,y} = \frac{WWTP_x \text{ flow rate}}{Watercourse_x \text{ flow rate}} Conc_y \quad (1)$$

161 With  $PEC_{x,y}$ : Predicted Environmental Concentration the pollutant y for the WWTPx in  $\mu\text{g/L}$ ;  
162 WWTP<sub>x</sub> flow rate: the flow rate of the WWTPx in  $\text{m}^3/\text{s}$ ; Watercourse<sub>x</sub> flow rate: the flow rate of the  
163 WWTPx's receiving watercourse in  $\text{m}^3/\text{s}$  and  $Conc_y$  : the (minimum, median or maximum)  
164 concentration of the pollutant y in the urban WWTP effluent.

### 165 2.2.2 Evaluation of the ecotoxicological risk of single micropollutants 166 (classic approach)

167 The involvement of each pollutant in the ecotoxicological risk of each WWTP discharge was  
168 calculated using its ecotoxicity data (PNEC) and its final concentration in the receiving watercourse  
169 (PEC), in accordance with the guidelines of the Technical Guidance Document (TGD) of the European  
170 Chemical Bureau (ECB, 2003), following the equation (2) below:

$$171 \quad RQ_{x,y} = \frac{PEC_{x,y}}{PNEC_y} \quad (2)$$

172 With  $RQ_{x,y}$ : Risk Quotient of the pollutant y for the WWTP<sub>x</sub>;  $PEC_{x,y}$ : Predicted Environmental  
173 Concentration of the pollutant y for the WWTP<sub>x</sub> in  $\mu\text{g/L}$  and  $PNEC_y$ : Predicted No Effect  
174 Concentration of the pollutant y in  $\mu\text{g/L}$ . An  $RQ_{x,y}$  value below 1 was associated with a nonexistent  
175 ecotoxicological risk, and a  $RQ_{x,y}$  value above 1 to a potential ecotoxicological risk for watercourses  
176 (ECB, 2003 ; Perrodin et al., 2011) following the ranking categories described in Table 2.

### 177 2.2.3 Evaluation of the cumulative ecotoxicological risk of the 178 micropollutant mixtures

179 Several methods have been developed to assess the cumulative ecotoxicological risk of a  
180 mixture of micropollutants for watercourses (Backhaus and Faust, 2012; Backhaus and Karlsson,  
181 2014). These have been applied in various water contamination contexts related, for instance, to

182 pesticide or pharmaceutical **residues** (Palma et al., 2014; Riva et al., 2019). Two concepts of mixture  
183 risk assessment have been designed based on the interaction of chemicals in the water samples: The  
184 Concentration Addition (CA) and the Independent Action (IA) (Junghans et al., 2006; Backhaus and  
185 Faust, 2012). The former (CA) assumes a similar action and an identical site of action for all the  
186 chemicals on aquatic organisms. The **latter** (IA) assumes a dissimilarity in the mechanisms of the  
187 chemicals' action in the mixture (Junghans et al., 2006; Riva et al., 2019). Consequently, the CA  
188 concept can lead to an overestimation of the mixture's risk while the IA concept can lead to an  
189 underestimation. Even if the CA concept is considered by some authors to be a conservative method  
190 (worst-case scenario), it remains relevant for preliminary mixture risk assessment (Palma et al., 2014)  
191 and is usually employed. The CA concept-based risk assessment is based on the summing of all the  
192 risk quotients of each pollutant (Riva et al., 2019) detected in a water sample. Finally, mixture risk  
193 quotients were calculated in this study following eq. (3):

$$194 \quad RQ_{\text{Mix},x} = \sum_{y=1}^n \frac{PEC_{x,y}}{PNEC_y} = \sum_{y=1}^n RQ_{x,y} \quad (3)$$

195 With  $RQ_{\text{Mix},x}$  : Risk Quotient for the cumulative risk of the pollutant mixture for WWTPx ;  
196  $RQ_{x,y}$ : Risk Quotient of the pollutant y for the WWTPx;  $PEC_{x,y}$ : Predicted Environmental  
197 Concentration of the pollutant y for the WWTPx in  $\mu\text{g/L}$  and  $PNEC_y$ : Predicted No Effect  
198 Concentration of the pollutant y in  $\mu\text{g/L}$ . Similarly, to the  $RQ_{x,y}$  calculated for each pollutant alone, a  
199  $RQ_{\text{Mix}}$  value below 1 was associated with a nonexistent ecotoxicological risk, and a  $RQ_{\text{Mix}}$  value above  
200 1 with a potential ecotoxicological risk for watercourses following the ranking categories described in  
201 Table 2.

### 202 2.3 Data representation and WWTP risk ranking and comparison of the methodologies

203 For each ERA methodology and each WWTP, Risk quotients (RQ) were determined using  
204 minimum, median and maximum concentrations in order to calculate better, median and worst case  
205 ecotoxicological risks for receiving watercourses (Palma et al. 2014). For the individual pollutant risk  
206 assessment, WWTPs were ranked from the least risky WWTP to riskiest, in order to identify the most  
207 significant risk situation in the territory. Cumulative risks were presented cartographically, using a

208 Geographical Information System (GIS), with the dedicated QGIS free and open-source desktop  
209 software (<http://www.qgis.org>). A comparison between the two different approaches was performed,  
210 evaluating the number of WWTPs identified as risky for their respective receiving watercourses, and  
211 the RQ obtained in each case.

212

213

### 214 3. Results and discussion

#### 215 3.1 Pollutant ecotoxicity, and concentration in WWTP effluents and watercourses

##### 216 3.1.1 Predicted No Effect Concentration (PNEC) values

217 The ecotoxicity of each of the studied pollutants (PNEC) employed in the present paper is  
218 presented in supplementary data Table S1. A wide variability of PNEC values can be noted. The three  
219 most toxic compounds recorded were the steroid hormones (Estrone, 17 $\beta$ -Estradiol and 17 $\alpha$ -  
220 Ethynylestradiol), with calculated PNEC values between 0.008 and 0.16 ng/L. Their very high toxicity  
221 is directly linked to the significant endocrine disruption properties of these molecules for aquatic  
222 organisms, such as fish, with very high reproduction and embryonic development interference at very  
223 low concentrations (Lang et al., 2001; Santos et al., 2014). Other very ecotoxic micropollutants  
224 families were the pyrethroid pesticides (e.g. Cypermethrin, PNEC = 3 ng/L), the antibiotics (e.g.  
225 Amoxicillin, PNEC = 0.78 ng/L), and anticancer drugs (e.g. fluorouracil, PNEC = 1 ng/L). The least  
226 ecotoxic pollutants detected were Tris(2-Chloro-1-methylethyl) Phosphate, a fire retardant, and  
227 Indomethacine, an analgesic with PNEC values of 260 and 16.14  $\mu$ g/L, respectively.

228 The authors would like to draw attention to the fact that the PNEC (7.4  $\mu$ g/L), and RQ values  
229 for ammonia nitrogen must be treated with caution in the present work. The toxicity of ammonia is  
230 highly dependent on pH and water hardness (Emmanuel et al., 2005; Perrodin et al., 2013), which can  
231 influence the equilibrium between ammonia (NH<sub>3</sub>, the most toxic form) and ammonium ions (NH<sub>4</sub><sup>+</sup>,  
232 the least toxic form) in aqueous samples. In the case in urban effluents, slightly alkaline pH (most of

233 the time between 7.5 and 8.5 (Teijon et al., 2010; Stalter et al., 2010)) allows the nitrogen to be present  
234 in both ammonium and ammonia forms (Clément and Merlin, 1995), and thus to contribute  
235 significantly to the ecotoxicological risk linked to WWTP effluents. Following the equation from  
236 Clément and Merlin (1995), a 10%/90%  $\text{NH}_3/\text{NH}_4^+$  ratio was so considered to calculate the  $\text{NH}_3$  PEC  
237 into the watercourses and so the RQ values by comparison with its ecotoxicological threshold (PNEC).

238

239

### 240 3.1.2 Pollutant concentrations in effluents and in watercourses (PEC)

241 Table S1 presents the minimal, median and maximal pollutant concentrations in the effluents.  
242 Ten of the 55 surveyed pollutants were not detected in the best case, with a maximum concentration of  
243 100 ng/L for organic contaminants (Tris(2-Chloro-1-methylethyl) Phosphate). The majority were  
244 detected at a concentration below 50 ng/L (e.g. Triclosan, 13 ng/L). A large range has been reviewed  
245 for median and maximal concentrations of pollutants. Dimethyl phthalate was detected with the lowest  
246 concentrations (resp. 0.19 and 115 ng/L), and conversely, Ammonia was observed with the highest  
247 concentrations (resp. 104.44 and 600  $\mu\text{g/L}$ ). Some heavy metals, such as Nickel and Zinc occurred at  
248 high concentrations in treated urban effluents (median concentration of 21 and 105  $\mu\text{g/L}$  respectively).  
249 This presence can be explained either by a low removal efficiency in treatment plants, as noticed by  
250 scientific community (da Silva Oliveira et al., 2007; Shafer et al., 1998), or by a very important initial  
251 concentration into the influent, depending of the WWTP. Pharmaceutical drugs represent the main  
252 family of organic micropollutants in the reviewed literature in treated effluents. Incoming raw  
253 wastewater concentrations due to their specific consumption by patients and fluctuation of removal  
254 efficiency rates lead to a significant variability in the concentration of each of these substances (e.g.  
255 Clotrimazole and Salicylic acid have median concentration of 4 ng/L and 1.1  $\mu\text{g/L}$  respectively)  
256 (Verlicchi et al., 2014; Yang et al., 2017).

257 As detailed in a previous section (2.2.1), PEC values were calculated for the lowest flow  
258 period of the year. On that basis, dilution factors were calculated comparing WWTP and watercourse

259 flow rates. The calculation led to a wide range of effluent dilutions (between 0,0042 % of effluent  
260 discharged into the freshwater for WWTP N° 30 and 100 % for N°1) depending on the plant. Thus, a  
261 wide range of risk situations is to be expected. All the detailed data are presented in Table S2.

262 Finally, every predicted concentration in each watercourse is provided in Table S2. The  
263 comparison between pollutant concentrations remains identical to the one for effluents, as in this  
264 study, the “typical” effluent was applied to all WWTPs in the territory. It is worth noting that the final  
265 pollutant PEC range calculated for most of the WWTPs is in accordance with the concentrations  
266 detected in French and worldwide streams (most of the time between 0 and 500 ng/L depending on the  
267 substance and the stream) (Chiffre et al., 2016; Patrolecco et al., 2015; Petrie et al., 2015; Vulliet et al.,  
268 2011), showing that the present methodology is environmentally realistic.

### 269 3.2 Single substance approach

270 The ecotoxicological risk quotients for each substance and WWTP are provided in the  
271 supplementary data Table S2. The Figures 3a, b and c represent the risk quotients for the 11 main  
272 pollutants for the best, median and worst cases (Ammonia, Amoxicillin, Copper, Diazinon Diclofenac,  
273 Ethynylestradiol, Estradiol, Estrone, Nickel, Norfloxacin, Roxithromycin, Tamoxifene, Trimethoprim  
274 and Zinc) involved in the 15 riskiest WWTPs identified in the Lyon territory for receiving  
275 watercourses.

276 In short, in the most favorable case, 7 of the 33 WWTPs constituted a risk for receiving  
277 freshwater, with higher quotients for 17 $\beta$ -Estradiol (up to an RQ value equal to 37.5 for the riskiest  
278 WWTP), 17 $\alpha$ -Ethinylestradiol, Copper, Nickel, Trimethoprim and Zinc. For median concentrations,  
279 16 of the 33 represented a risk with higher values for 17 $\beta$ -Estradiol (up to an RQ value equal to 187  
280 for the riskiest WWTP), 17 $\alpha$ -Ethinylestradiol, Amoxicillin, Estrone, Nickel, Roxithromycin and  
281 Trimethoprim. Finally, in the worst-case scenario, 28 of the 33 WWTPs led to a risk (RQ above 1),  
282 with the highest values for 17 $\beta$ -Estradiol (up to an RQ value equal to 3750 for the riskiest WWTP),  
283 17 $\alpha$ -Ethinylestradiol, Dianizon, Estrone, Nonylphenol and Trimethoprim. On the contrary, the lowest

284 RQ<sub>med</sub> and RQ<sub>max</sub> were computed for Dimethyl phthalate, Clotrimazole, Cyclophosphamide and  
285 Ifosfamide.

286 The present results are in accordance with previous studies. For example, Riva et al. (2019)  
287 conducted research on the risks linked to 47 emergent pollutants in 3 rivers in a highly urbanized zone,  
288 contaminated by WWTP effluents, runoffs, etc. Among the riskiest pollutants, Amoxicillin, 17 $\beta$ -  
289 Estradiol and Estrone were identified respectively with RQ values up to 11.33, 158 and 903.6  
290 depending of the sampling site. In their case, as in the present study, even if the predicted/measured  
291 concentrations of estrogens in the receiving streams were very low, the very high toxicity of these  
292 compounds (biocides and endocrine disruptors) resulting in very low PNEC values, led to high RQ  
293 values. Our results are also corroborated by several studies that conducted ERA for urban WWTPs,  
294 and contaminated rivers by emerging micropollutants (pharmaceuticals, plasticizers, pesticides, ...) in  
295 various regions of the world such as Greece, Portugal, Spain and the USA. The authors demonstrated  
296 significant risks (RQ above 1) for Diclofenac (Kosma et al., 2014; Pereira et al., 2015), Trimethoprim  
297 (Kosma et al., 2014; Valcarcel et al., 2011), 17 $\alpha$ -Ethinylestradiol (Barber et al., 2013) and  
298 Amoxicillin (Thomaidi et al. 2015). Zinc, Copper and Nickel risks are linked to their high  
299 concentrations in effluents, which can be attributed to the low efficiency of WWTP in removing them.  
300 Finally, all of these results show that the current theoretically proposed methodology is in accordance  
301 with all the recent research and could be used as an a priori risk tool for WWTP management in a  
302 territory.

### 303 3.3 Mixture approach

304 To obtain a more representative view of the risks linked to the pollutants that do not have an  
305 independent toxic action, but rather in interaction with one other, a mixture risk assessment was  
306 performed. To our knowledge, the present paper is the first to investigate the cumulative risk of a  
307 “representative” mixture of micropollutants from urban wastewater treatment plant effluents, based on  
308 literature review, for aquatic ecosystems. Results are presented in Figure 4. The smallest circles  
309 correspond to the cumulative risk for the minimal concentrations, medium circles for median  
310 concentrations and the biggest circles for maximal reviewed pollutant concentrations in effluents.

311 To identify the number of WWTPs presenting a significant risk (above 1), the supportive  
312 situation led to identify 8 WWTPs as compared to 7 for the single substance approach; 19 against 16  
313 for median situation and 32 against 28 for worst-case situation. As expected, in all cases, the number  
314 of WWTPs with a significant mixture risk is much greater than for the single substance approach, as  
315 well as the RQ values (up to 9270.55 for WWTP N°1 and maximum concentrations). Hence, these  
316 results support the hypothesis that classical single substance ERA analyses can mitigate and  
317 overshadow some of the risks and thus demonstrate the importance of performing mixture ERA. This  
318 observation has already been attested by Riva et al. (2019). A recent study reported the mixture risk of  
319 emerging organic micropollutants from several WWTP effluents, for Greek rivers (Thomaidi et al.,  
320 2015). Authors evaluated the mixture RQ linked to the maximum concentrations of each pollutant  
321 detected (worst-case scenario), and calculated RQ values were up to 1000 for most of the rivers  
322 (16/25). These results are precisely in agreement with the present work.

323 Nevertheless, if the mixture approach has the advantage of taking into account the cumulative  
324 risk of many substances, it remains an additive approach that does not consider the synergic and  
325 antagonistic ecotoxic effects of pollutants, which have been demonstrated to occur in many surveys,  
326 for example, the mixture of antibacterial compounds on the microalgae *P. subcapitata* (Yang et al.,  
327 2008), or veterinary intestinal pest control drugs on *D. magna* (Puckowski et al., 2017), and must be  
328 refined.

#### 329 3.4 ERA evaluation at highly urbanized territory scale and further climate change influences

330 In assessing the territorial risks of WWTP effluents, results highlighted very diverse risks  
331 depending on exposure factors. The mixture approach has pointed out that eight (N° 1, 2, 4, 9, 10, 12,  
332 13 and 18) of the thirty-three WWTPs led to a significant risk regardless of the pollutant concentration  
333 considered (minimal, median or maximal). Figure 5 represents the RQ<sub>mix</sub> for median pollutant  
334 concentrations in relation to each WWTP and watercourse flow rates. It demonstrates that most of the  
335 time, the RQ<sub>mix</sub> is higher for small and median WWTPs rejecting effluents into small/very small  
336 watercourses (e.g. N° 1, 2, 4, 9, 10, 12, 13 and 18), and at the opposite end, WWTP dumping into the  
337 high flow rates lead to limited/very weak risks (e.g. N° 23, 24 and 30). It is important to note that the

338 riskiest situation corresponds to a WWTP (N°1) discharging into a small dried-up (intermittent)  
339 watercourse during the driest period of the year, leading to a flow composed of 100% wastewater, as  
340 pointed out in a previous study (Brus and Perrodin, 2017).

341         The present methodology may be useful for further WWTP placement in a selected territory,  
342 and to identify the already installed plants that need to be adjusted, for example strengthening the  
343 treatments or reducing the inbound/outbound loads. Nevertheless, one must be aware that the current  
344 exposure situations will evolve in a context of climate change, which must be taken into account. In  
345 many cases, implemented WWTPs have been designed in a hydrological context, and without taking  
346 into account possible evolution of watercourse flow rates due to climate change (Zouloubis and  
347 Tolkou, 2015). Several studies have focused on the evolution of river flow regimes in various world  
348 regions (e.g. Europe, Africa), and all have illustrated a long-term flow rate decrease, especially during  
349 the summer (Bodian et al., 2018; Schneider et al., 2013). This could lead to an increase in  
350 micropollutant (e.g. pharmaceutical) concentrations in receiving watercourses (Gooré Bi et al., 2015;  
351 Schlüsener et al., 2015; Schneider et al., 2013; Zouloubis and Tolkou, 2015), due to lower dilution.  
352 Moreover, receiving water may be subjected to an increase in temperature and pH due to the decrease  
353 of their flow rate. This could enhance the toxicity of some pollutants, their biodegradation and by-  
354 product creation (Schlüsener et al., 2015), and thus ultimately influence the ecotoxicological risks.

355         Finally, in the present case, if one situation has been described as critical in our study  
356 compared to others (dried-up watercourse), a similar description would fit several other watercourses,  
357 which currently present a very low flow rate (e.g. N°2, 4).

### 358 Conclusion and perspectives

359 In the present work, an ecotoxicological risk assessment evaluation was performed for 33 urban  
360 wastewater treatment plants located on a highly urbanized territory (the 2nd most in France). Our  
361 research increased the knowledge and the methods of WWTP effluent ecotoxicological risk  
362 assessment, at a territorial scale, combining two approaches to assess the individual (classic ERA) and  
363 “cocktail” effects of pollutants (mixture ERA), better evaluating the real risk for receiving

364 watercourses. The classical single substance approach highlighted a significant risk for many of the  
365 WWTPs. However, the cumulative risk approach showed its usefulness in evaluating the mixture of  
366 micropollutants in treated effluents, indicating a higher risk for some receiving watercourses. A map  
367 view of the theoretical risk of multiple WWTP effluents was produced, ranking the WWTPs' risks, on  
368 the various watercourses of an urbanized area for better territorial management.

369 Nevertheless, from this work and previous studies (Brus and Perrodin, 2017), four limits have been  
370 identified and should be overcome in further research to achieve a completely realistic ERA of the  
371 different types of urban liquid effluents on receiving aquatic ecosystems. Thus, the following trends  
372 should be adopted:

373 (1) The use of **experimental physico-chemical data** at the various WWTPs of the urbanized territory  
374 during different seasons (climatic influence).

375 (2) The consideration of organism exposure parameters (e.g. half-life time and bioaccumulation  
376 factors) for a more realistic risk assessment.

377 (3) The consideration of other types of potentially impacted ecosystems, such as lakes or groundwater  
378 through soil and sedimentation contamination and transfer.

379 (4) The inclusion of other types of urban effluents (stormwater and combined sewer overflow) that  
380 may be a significant hazard for aquatic ecosystems, with a significant, highly variable, and partially  
381 similar pollution (Gosset et al., 2017).

382 (5) The use of new analytical chemistry methods, such as “non-target screening” (Blum et al., 2017) or  
383 “suspect-screening” (Pinasseau et al., 2019) to overcome the fact that we find only what we are  
384 looking for.

385 In conclusion, the present research opens up perspectives in terms of territorial management  
386 for public and private partners. Applying the proposed methodology to real effluents would allow the  
387 ranking of WWTPs in a territory in terms of ecotoxicological risk and link ecotoxic pollution to socio-  
388 economic characteristics of each WWTP territory, with the goal of reducing emissions at the source.

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Figure 1: Mapping representation of the WWTP's location and discharge flow rates.

Figure 2: Mapping representation of the WWTP's receiving watercourse flow rates.

Figure 3: Ecotoxicological risk quotients (RQ) calculated for 11 main pollutants involved in the 15 riskiest WWTPs identified of the Lyon territory : (A) considering minimal pollutant concentration ; (B) median pollutant concentrations and (C) maximum pollutant concentrations. Red lines represent the significant risk schershold (RQ = 1).

Figure 4: Ecotoxicological Risk Assessment (ERA) of WWTP effluent pollutant mixture for receiving watercourses. Little circles correspond to the mixture risk quotient (RQ<sub>mix</sub>) for the minimal concentrations, medium circles for median concentrations and biggest circles for maximal pollutant concentrations.

Figure 5: RQ<sub>mix</sub> for median pollutant concentrations in relation to each corresponding WWTP and watercourse flow rate.

N°	Coordinates of the WWTP (Latitude / Longitude)		Number of drained municipalities	WWTP population equivalent (PE)	Design flow rate of the WWTP (m <sup>3</sup> /day)	Main treatment process	Name of the receiving watercourse
1	45° 30' 4.59"	4° 45' 5.859"	1	113	52	Bacterial bed*	Ruisseau de bassemont
2	45° 36' 9.743"	4° 47' 0,69"	1	3724	1430	Activated sludges	Ruisseau du Bullion
3	45° 41' 11.55"	4° 36' 55.026"	1	13	6	Sand filters	L'Artilla
4	45° 41' 52.728"	4° 41' 50.612"	3	8599	6812	Activated sludges	Le Garon
5	45° 43' 33.943"	4° 44' 58.303"	1	30	12	Vertical flow constructed wetland	Yzeron
6	45° 36' 9.743"	4° 31' 42.058"	1	157	68	Vertical flow constructed wetland	La Coise
7	45° 36' 21.428"	4° 30' 43.53"	1	137	102	Natural lagoon	La Coise
8	45° 36' 44.548"	4° 33' 8.579"	1	27	11	Vertical flow constructed wetland	La Coise
9	45° 49' 4.08"	4° 36' 24.753"	2	5192	1736	Activated sludges	La Brévenne
10	45° 49' 10.9"	4° 59' 39,062"	3	36200	2160	Activated sludges	Sereine
11	45° 53' 27.563"	4° 43' 48.45"	1	1120	785	Activated sludges	l'Azergues
12	45° 54' 1.949"	4° 43' 32.624"	1	1789	1566	Activated sludges	l'Azergues
13	45° 52' 19.34"	4° 38' 57.819"	8	6824	4000	Activated sludges	L'Azergues
14	45° 33' 15.365"	4° 57' 42.224"	2	3735	195	Bacterial bed	La Véga
15	45° 39' 30.904"	5° 9' 24.302"	4	5101	960	Activated sludges	Le Catelan
16	45° 52' 30.546"	4° 43' 21.733"	2	1419	791	Activated sludges	L'Azergues
17	45° 52' 18.044"	4° 42' 55.162"	2	2059	1573	Activated sludges	L'Azergues
18	45° 39' 31.97"	5° 8' 14.835"	9	109292	29540	Activated sludges	La Bourbre
19	45° 42' 21.266"	5° 11' 23.189"	1	676	90	Natural lagoon	La Bourbre
20	45° 48' 57.842"	5° 4' 12.349"	6	17477	9020	Activated sludges	Canal de Miribel
21	45° 49' 53.882"	4° 51' 9.84"	12	47250	9670	Biofilter	La Saône
22	45° 52' 2.215"	4° 50' 18.915"	4	30892	18000	Activated sludges	La Saône
23	45° 55' 2.726"	4° 46' 25.863"	1	2262	1000	Activated sludges	La Saône
24	45° 53' 22.628"	4° 48' 18.451"	2	3665	900	Activated sludges	La Saône
25	45° 54' 34.818"	4° 48' 46.271"	4	13624	5904	Activated sludges	La Saône
26	45° 29' 21.474"	4° 49' 29.85"	22	77834	18000	Activated sludges	Rhône
27	45° 41' 33.04"	4° 50' 5.364"	34	524325	300000	Activated sludges	Rhône
28	45° 41' 28.235"	4° 50' 55.267"	20	622800	554000	Activated sludges	Rhône
29	45° 34' 22.504"	4° 47' 59.939"	18	80232	17845	Biofilter	Rhône
30	45° 34' 8.344"	4° 48' 59.773"	3	27216	3000	Activated sludges	Rhône
31	45° 47' 43.464"	5° 2' 12.133"	7	21800	9900	Activated sludges	Rhône - Canal de Jonage
32	45° 47' 28.366"	4° 59' 44.352"	2	25732	8730	Biofilter	Rhône - Canal de Jonage
33	45° 47' 15.042"	4° 53' 21.753"	10	179772	91000	Activated sludges	Rhône
34	45° 33' 56.953"	4° 44' 52.98"	1	72	18	Sand filters	Soil
35	45° 36' 19.286"	5° 6' 29.502"	1	280	60	Vertical flow constructed wetland	Soil
36	45° 36' 8.389"	5° 7' 2.35"	1	638	120	Vertical flow constructed wetland	Soil
37	45° 40' 15.965"	5° 5' 10.447"	1	808	255	Natural lagoon	Soil
38	45° 44' 35.439"	5° 6' 14.384"	1	54	15	Natural lagoon	Soil
39	45° 34' 27.378"	4° 38' 13.881"	1	33	15	Vertical flow constructed wetland	Soil
40	45° 35' 8.114"	4° 34' 21.425"	1	3	15	Vertical flow constructed wetland	Soil
41	45° 32' 9.405"	4° 41' 9.883"	1	5	2	Sand filters	Soil

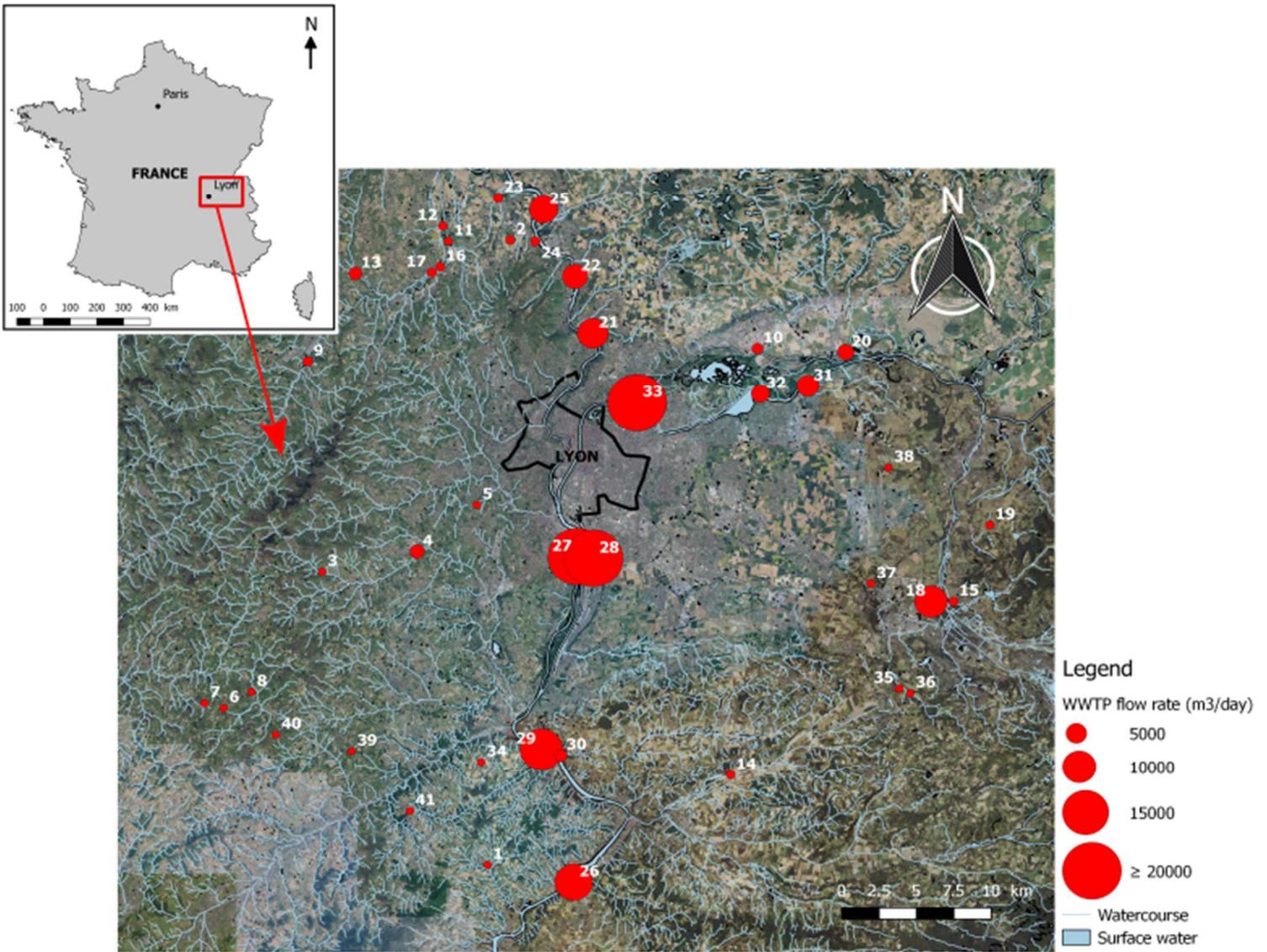
633 Table 1: Characteristics of the WWTPs considered on the territory.

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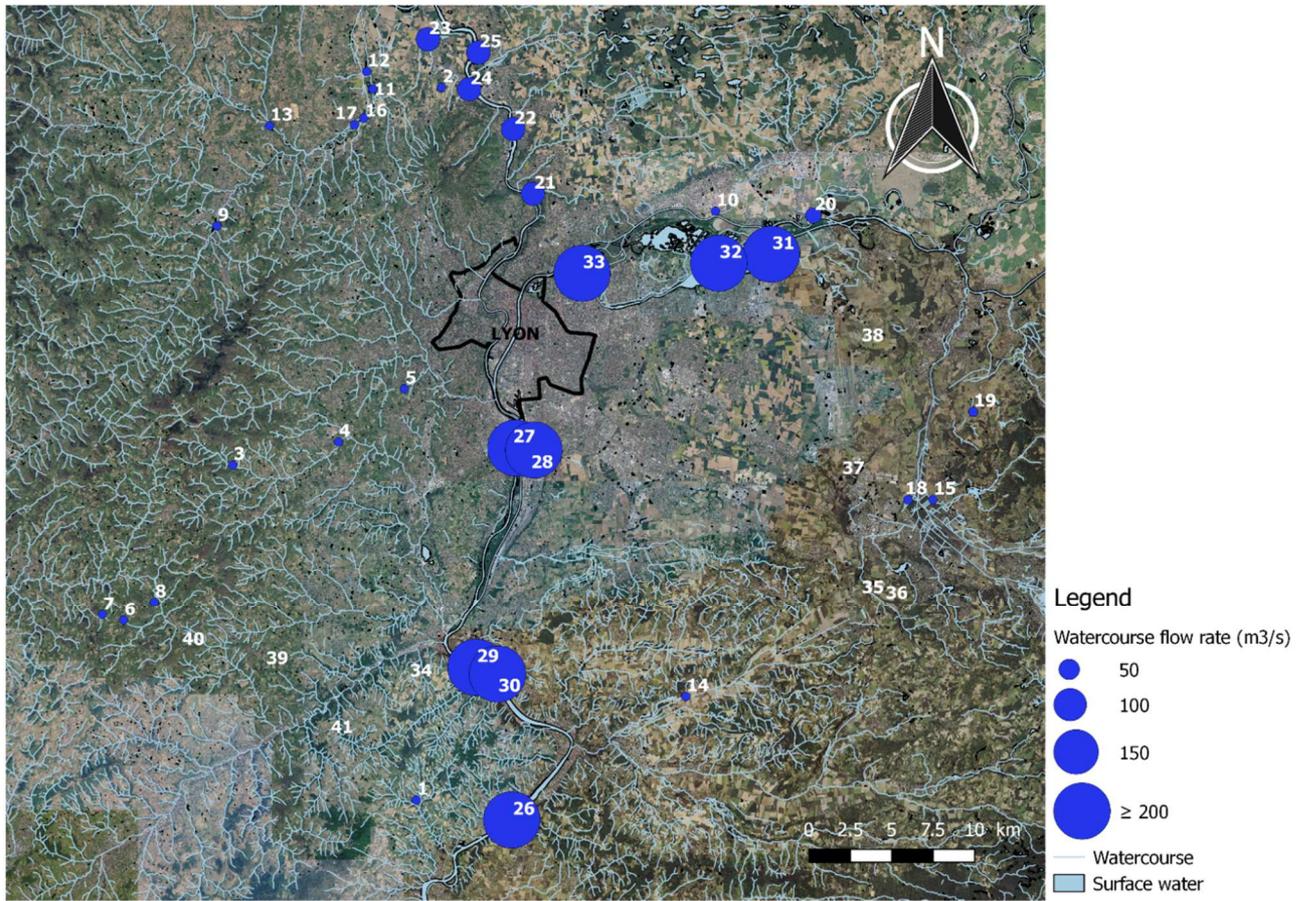
Table 2: Ecotoxicological risk ranking based on the risk quotient (RQ) values.

Risk Quotient (RQ) value	Ranking categories
$RQ < 1$	Negligible risk
$1 < RQ < 10$	Low risk
$10 < RQ < 100$	Medium risk
$100 < RQ < 1000$	High risk
$RQ > 1000$	Very high risk



660 Figure 1: Mapping representation of the WWTP's location and discharge flow rates.

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672 Figure 2: Mapping representation of the WWTP's receiving watercourse flow rates.

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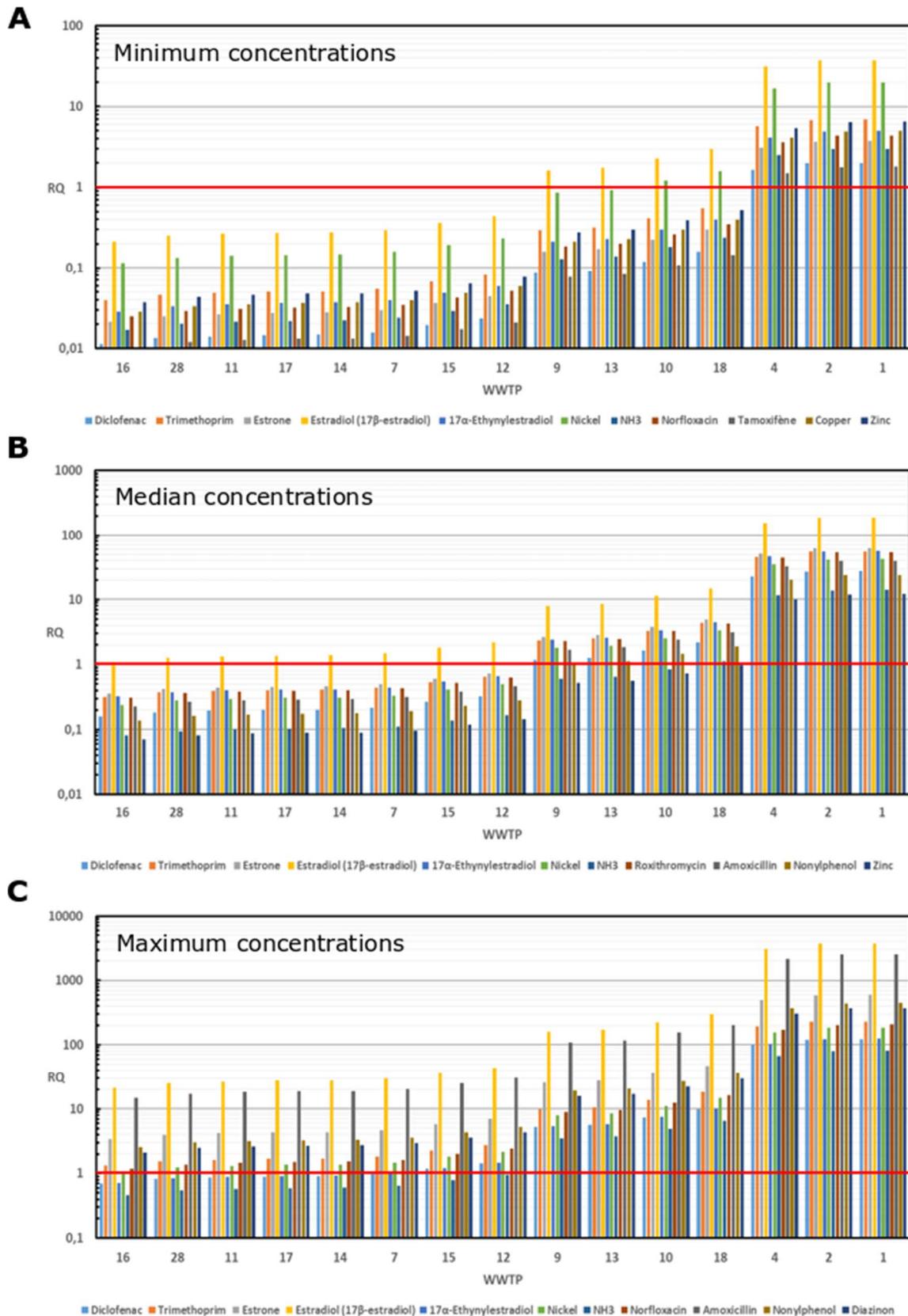
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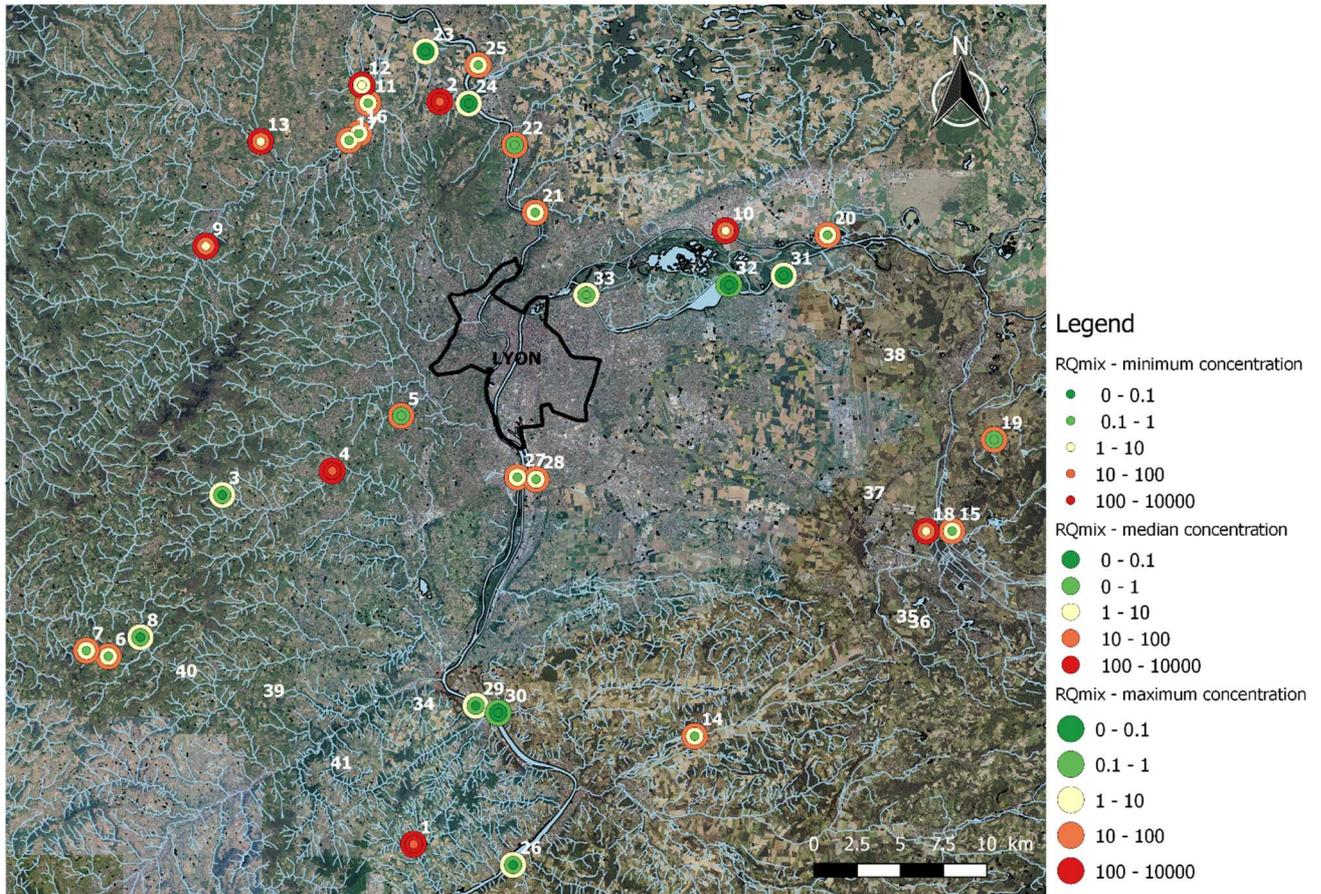
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693 Figure 4: Ecotoxicological Risk Assessment (ERA) of WWTP effluent pollutant mixture for receiving  
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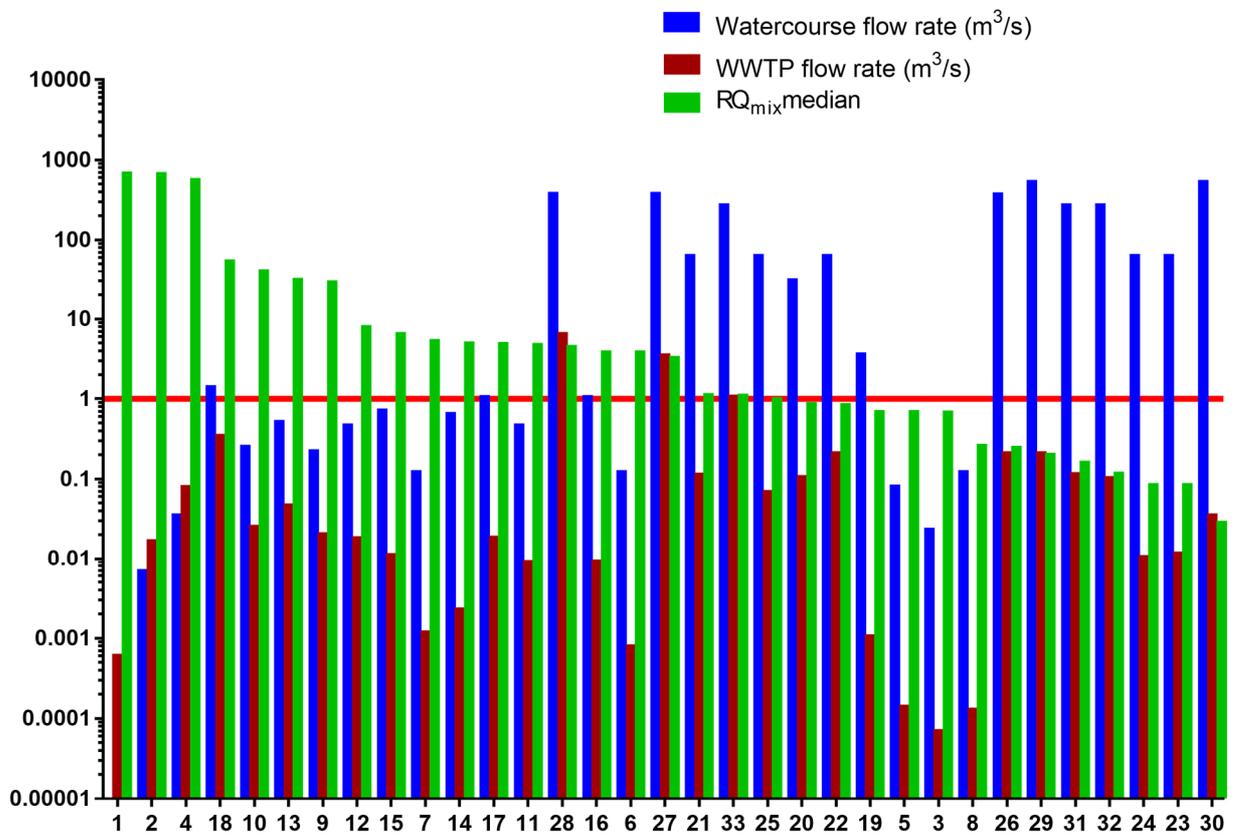


Figure 5: RQ<sub>mix</sub> for median pollutant concentrations in relation to each corresponding WWTP and watercourse flow rate.

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