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**Chlorination in a wastewater treatment plant: acute toxicity effects of the effluent and of the recipient water body**

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# UNIVERSITÀ DEGLI STUDI DI TORINO

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1 **CHLORINATION IN A WASTEWATER TREATMENT PLANT: ACUTE TOXICITY**  
2 **EFFECTS OF THE EFFLUENT AND OF THE RECIPIENT WATER BODY.**

3

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28

29 **ABSTRACT**

30 This study investigates the impact of wastewater treatment plant (WWTP) effluent on  
31 the toxicity of the recipient water body and the effectiveness of the disinfection  
32 treatment applied (sodium hypochloride) to assure the compliance of both  
33 microbiological and toxicological emission limits. No toxicity was found in the majority  
34 of samples collected from the recipient river, upstream and downstream of the WWTP,  
35 using three different toxicity tests (*Vibrio fischeri*, *Daphnia magna*, *Pseudokirchneriella*  
36 *subcapitata*). Only three samples presented Toxic Unit (TU) values with *V. fischeri*, and  
37 one presented TU with *S. capricornutum*. The influent toxicity ranged from slightly toxic  
38 to toxic (TU = 0.68 - 4.47) with *Vibrio fischeri*, while only three samples presented TU  
39 values with the other tests. No toxicity was found in the absence of chlorination, while  
40 the mean toxicity was  $3.42 \pm 4.12$  TU with chlorination in the effluent. Although no  
41 toxicity or very slight toxicity was found in the receiving water, its residual toxicity was  
42 higher than the U.S. EPA Quality Standard in two samples. *E. coli* concentration had a  
43 lower mean value in the chlorinated effluent:  $13,993 \pm 12,037$  CFU/100 mL vs.  $62,857$   
44  $\pm 80,526$  CFU/100 mL for the non-chlorinated effluent. This difference was shown to be  
45 significant ( $p < 0.05$ ). *E. coli* in ten chlorinated samples was higher than the limit  
46 established by European and Italian Legislation. The mean highest Trihalomethanes  
47 (THMs) value was found in the influent samples ( $2.79 \pm 1.40$  µg/L), while the mean  
48 highest disinfection by products (DBPs) was found in the effluent samples ( $1.85 \pm 2.25$   
49 µg/L). Significant correlations were found between toxicity, sodium hypochlorite, THMs,  
50 DBPs, *E. coli* and residual chlorine.

51 In conclusion, this study highlighted that the disinfection of wastewater effluents with  
52 sodium hypochlorite determines the increase of the toxicity, and sometimes is not  
53 enough to control the *E. coli* contamination.

54

55 Keywords: Wastewater; Chlorination; Toxicity; Trihalomethanes; *Escherichia coli*.

56

57 **1. Introduction**

58 Industrial wastewater, effluent of sewage treatment plants and run-off from agriculture  
59 are major sources of surface water pollution. Wastewater is a complex mixture of  
60 various organic and inorganic compounds; in addition to the unknown products  
61 discharged into the wastewater treatment plants, other substances are formed during  
62 the treatment processes (Farrè et al. 2001; Ricco et al. 2004). Moreover, in recent  
63 years, the incidence of human-use compounds, such as pharmaceuticals and drugs, in  
64 aquatic environments has been recognized as an important issue in environmental  
65 chemistry. Some of these compounds enter the aquatic environment, mostly via the  
66 effluents of municipal sewage treatment plants, unaltered or as slightly transformed  
67 metabolites (Huerta-Fontela et al. 2008; Watkinson et al. 2009). Due to the presence of  
68 several chemical pollutants, no useful monitoring or screening of surface water can be  
69 based only on chemical analysis of a limited number of toxic compounds. Therefore,  
70 biological tests prove to be indispensable for the assessment of cytotoxic and  
71 genotoxic potential in surface water. Because of the variety of aquatic organisms and  
72 the heterogeneous condition in aquatic environments, there is no single biotest for  
73 detecting toxic and genotoxic effects. Only a set of bioassays with prokaryotic and  
74 eukaryotic organisms can be applied to estimate accurately the effects of toxicants in  
75 surface waters (Dizer et al. 2002; **Persoone et al. 2003**).

76 One of the objectives of the European Community's (EC) environmental regulations is  
77 to reduce the pollution of surface water caused by municipal waste (see the Council  
78 Directive 91/271/EEC as amended by the Commission Directive 98/15/EEC of 27  
79 February, 1998). This requires the European Union (EU) member states to ensure that  
80 discharge of urban wastewater and its effects are monitored (Farrè et al. 2001; Mantis  
81 et al. 2005, see also Council Directive 2000/60/EC).

82 In order to prevent sanitary hazards related to the uses of recipient water bodies, the  
83 current Italian regulations prescribe WWTP effluent emission limits for a wide range of  
84 chemical compounds, toxicity and bacterial discharge (i.e., *Escherichia coli*). In order to

85 meet the bacterial discharge limit, WWTPs can introduce a wastewater disinfection  
86 step; however, disinfectants may induce chemical reactions, leading to the production  
87 of disinfection by-products (Decree Italian Law 152/2006).

88 The microbiological emission limit of *E. coli* is not stated by the national regulation at a  
89 general level, but it should be established by local authorities in each specific discharge  
90 licence with respect to the public health situation, and to the foreseen uses of the  
91 recipient water body. In the case of the WWTP investigated, the local authority  
92 (Piedmont Region) has evaluated the introduction of a concentration limit for *E. coli* of  
93 20,000 CFU/100ml, while the Council Directive 2000/60/EC has introduced a  
94 concentration limit for *E. coli* of 5,000 CFU/100ml. In order to respect this limit, many  
95 WWTPs apply a wastewater disinfection process, because sometimes the *E. coli*  
96 concentration at the end of the purification process is higher than the limit established  
97 by the local authorities and by European legislation. However, disinfectants can induce  
98 chemical changes in these systems, thus resulting in changes that will not be restricted  
99 to the microbial population. One possible outcome of these chemical changes is a  
100 change of the effluent toxicity, as demonstrated by Blatchley et al. (1997), Monarca et  
101 al. (2000), Wang et al. (2007) and Wu et al. (2010). Chemical disinfectants are effective  
102 for killing harmful microorganisms in water, but they are also powerful oxidants,  
103 oxidizing the organic matter, anthropogenic contaminants, and bromide/iodide naturally  
104 present in most source waters (rivers, lakes, and groundwater). Chlorine, ozone,  
105 chlorine dioxide, and chloramines are the most common disinfectants in use today:  
106 each produces its own suite of DBPs in water, with overlapping constituents. In the 30  
107 years since the THMs were identified as DBPs in drinking water, significant research  
108 efforts have been directed toward increasing the understanding of DBP formation,  
109 occurrence, and health effects. Although more than 600 DBPs have been reported in  
110 the literature, only a small number has been assessed either in quantitative occurrence  
111 or health-effects studies (Richardson et al. 2007). Toxicity of water disinfection and  
112 DBPs was studied intensively. Many chlorinated by-products showed dose. response

113 relationships with DNA and chromosome damage, cytotoxicity and apoptosis in vivo  
114 (Lu et al. 2002; Richardson et al. 2007; Yuan et al. 2006) or in vitro (Boorman et al.  
115 1999; Lu et al. 2004; Yuan et al. 2005; Shi et al. 2009).

116 The aim of the present work was to investigate the impact of a WWTP effluent on the  
117 recipient water body, with particular respect to its toxicity, and to verify the  
118 effectiveness of disinfection treatment with sodium hypochlorite (NaOCl). This was to  
119 ensure the compliance with both microbiological and toxicological emission limits, using  
120 three different toxicity tests (*Vibrio fischeri*, *Daphnia magna*, *Pseudokirchneriella*  
121 *subcapitata*). Finally, the presence of disinfection by-products (trihalomethanes) and *E.*  
122 *coli* was measured to evaluate the correlation with acute toxicity and the efficiency of  
123 the chlorination process.

124

## 125 **2. Material and methods**

126

### 127 *2.1. Features of the sewage treatment plant*

128 The considered WWTP is a consortium plant that treats civil and industrial discharges  
129 from the municipal districts of Collegno, Grugliasco, Rivoli and Villarbasse (Torino,  
130 Piedmont Region, Italy), a metropolitan area in Northern Italy, with a total population  
131 equivalent of about 400,000. The mean treated flow is around 42,000 m<sup>3</sup>/day. The  
132 plant comprises a water and sludge treatment system. The former includes primary  
133 sedimentation, active sludge oxidation with nitrification/denitrification processes, and a  
134 section for the recovery and reutilization of treated water. The mean COD of the  
135 influent and of the effluent is 844.16 mg/L and 40.5 mg/L. In order to limit and to  
136 evaluate microbiological emissions, 15 of the 22 effluent samples were chlorinated with  
137 sodium hypochlorite (3 mg/L) at a mean dosage of 34 L/h. The final effluent was then  
138 discharged into the Dora Riparia River (one of the tributaries of the Po River, the  
139 largest Italian river) which has a **mean** flow rate of 26 m<sup>3</sup>/s.

140

141 *2.2. Sampling of sewage and water*

142 Twenty-four hour composite samples of the influent (IN) and final WWTP effluent  
143 (OUT) were taken during ten different sampling events from February 2005 to  
144 November 2005 (first sampling period) and twelve different sampling events from  
145 September 2006 to May 2007 (second sampling period). On the same dates, grab  
146 samples of water were collected from the recipient river, 2 km upstream (US) and 2 km  
147 downstream (DS) of the WWTP. The samples (4 . 14 L) were divided into four aliquots  
148 and stored in brown glass flasks at 4°C. In each sample, an aliquot of 1 L was used for  
149 the toxicological analysis, and another 200 mL aliquot was utilised for the  
150 microbiological analysis. Another 1 L aliquot was used for trihalomethanes (THMs)  
151 analysis (only during the second sampling period), and the remainder was stored at  
152 4°C until the end of the analyses. All the analysis were performed within 24 hours from  
153 the sampling. Also grab disinfected effluent samples (100 ml) were collected for  
154 immediate analysis of the residual chlorine.

155

156 *2.3. Microbiological analysis*

157 Determination of *E. coli* was performed using the membrane filter technique (AWWA  
158 1998), which is highly reproducible, can be used to test relatively large sample  
159 volumes, and yields numerical results more rapidly than the multiple-tube procedure.  
160 The results are expressed in Colony Forming Unit (CFU)/100 mL.

161

162 *2.4. Biological assays*

163 *Microtox™ test*

164 After the screening test, the BASIC test (90%) was applied following the procedure  
165 described in the Microtox™ manual (Azur Environmental 1995). The principle of this  
166 system is based on the evaluation of the luminous energy naturally emitted by *V.*  
167 *fischeri* bacteria (Azur Environmental, Carlsbad, CA, USA). Luminescence was  
168 measured at time zero and after 5, 15 and 30 minutes, and compared to the control.



169 The final expression of the toxic potentials of samples is the Effective Concentration at  
170 30 minutes, EC50, showing the sample concentration factor which caused a 50%  
171 brightness decrease of the bacteria population. Each test was analysed using a  
172 Microtox<sup>®</sup> reference toxicant (phenol) as quality control.

173

#### 174 *Daphnia magna* test

175 This test is based on the evaluation of the immobilization of 10 organisms in the  
176 presence of stress sources against a control. The dormant eggs of the crustacean and  
177 stock solution for preparation of the standard freshwater (International Organization for  
178 Standardization) medium were taken from the commercial test system, DaphToxkit F<sup>™</sup>  
179 *magna* (MicroBioTests, Nazareth, Belgium). The hatching of ephippia and the  
180 preparation of standard freshwater were performed according to the manufacturer's  
181 instructions. The ephippia were transferred to hatching petri dishes with 50 mL pre-  
182 aerated standard freshwater, thereafter covered and incubated for 72 hours, at 20.  
183 22°C under continuous illumination of 6000 lux. A dilution series of treated and  
184 untreated water samples was prepared by serial 1:1 dilution with standard freshwater.  
185 Assays were carried out in 24-well plates. Five neonates were transferred into each  
186 well, which each contained a 10ml water sample. Freshwater controls were included in  
187 every test. Tests were performed in quadruplicate. The plates were covered and  
188 incubated at 20°C in the dark. After 24 hours and 48 hours of incubation, the number of  
189 dead and immobilized neonates was recorded, and the percent mortality was  
190 calculated (Cao et al. 2009). The toxic potential of the sample is expressed with EC50,  
191 showing the concentration of the sample which causes the immobilization of the 50% of  
192 the organisms against the control (OECD 1984a).

193

#### 194 *Pseudokirchneriella subcapitata* test

195 The algal culture and stock solution for the preparation of growth media were taken  
196 from the commercial test system AlgalToxkit F<sup>™</sup> (MicroBioTests, Nazareth, Belgium).

197 Water and wastewater samples were supplemented with mineral nutrients, and  
198 incubated with *P. subcapitata* at 23°C ± 2°C under constant uniform illumination (8000  
199 lux) for 72 hours in disposable long cells in polystyrene (volume 25 mL). The test was  
200 run in triplicate for both samples and controls. Algal growth was followed by optical  
201 density (OD) at 670 nm after 24, 48 and 72 hour exposure to the samples. The algal  
202 growth inhibition was calculated from these data by integrating the mean values, from  
203 time zero to time 72 hours, for each concentration tested, including control. **The toxic**  
204 **potential** of the samples is expressed **with** EC50 (OECD 1984b). The toxicity test is  
205 considered acceptable when the number of algae in the control test vials increases at  
206 least by a factor of 16 during the 72 hour test period and the pH does not change by  
207 more than one unit.

208

#### 209 *Final expression of the toxicity results*

210 The EC50 values of the three tests were subsequently converted in toxic units (TU)  
211 that are proportional to toxicity:

$$212 \text{ TU} = (1/\text{EC50}) \times 100$$

213 **Considering the hazard classification system for wastes discharge into aquatic**  
214 **environment described by Persoone et al. (2003)** the judgment of toxicity depends on  
215 the values shown in Table 1.

216 Without specific information concerning the persistence of toxicity, it is recommended  
217 that effluent toxicity is limited to dilution estimates and that toxicity is assumed to be  
218 additive and conservative. For rivers, the following dilution equation should be used,  
219 assuming completely mixed conditions:

$$220 C = (C_s Q_s + C_e Q_e) / (Q_e + Q_s)$$

221 C = downstream toxicity concentration (TU)

222 C<sub>s</sub> = upstream toxicity concentration (TU)

223 Q<sub>s</sub> = upstream mean flow

224 C<sub>e</sub> = effluent toxicity concentration (TU)

225  $Q_e$  =effluent mean flow (U.S. EPA, 1991).

226 The downstream toxicity concentration (C) was calculated considering the highest TU  
227 value of the three tests applied.

228

### 229 2.5. Trihalomethanes analysis

230 Trihalomethanes (THMs), composed of disinfection by-products (DBPs) chloroform,  
231 bromoform, chlorodibromomethane, bromodichloromethane and other THMs, 1,1,1-  
232 trichloroethane, trichloroethylene, carbon tetrachloride, 1,2-dichloroethane,  
233 trichloroethylene, and tetrachloroethene, were analysed by headspace combined with  
234 gas chromatography coupled to an electron capture detector (GC-ECD) (Ottavini and  
235 Bonadonna 2000), with a detection limit of 0.1 µg/L in the samples collected during the  
236 second sampling period (Sep 2006 . May 2007).

237

### 238 2.6. Residual chlorine analysis

239 The residual chlorine concentrations of the effluent samples were analysed as reported  
240 in the Standard Methods for the Examination of Water and Wastewater (AWWA 1998).

241

### 242 2.7. Statistical analysis

243 The statistical analyses were performed with the statistical package SPSS 17.0 (SPSS  
244 for Windows, Chicago, IL, USA) using Spearman's test, ANOVA, Probit regression  
245 analysis and T-test.

246

## 247 3. Results

248

### 249 3.1. Toxicity

250 Tables 2 - 5 report the toxicity of the 22 different water samples in the four sampling  
251 sites. The samples collected from the Dora Riparia River, upstream (table 2) and  
252 downstream (table 5) of the WWTP, were not toxic with the three toxicity tests adopted

253 (Microtox™, *Daphnia magna*, *Pseudokirchneriella subcapitata*), not even when the  
254 chlorination process of the final effluent was started (June 2005) during the first  
255 sampling period. But, during the second sampling period, we found acute toxicity in  
256 some samples. The sixteenth sample US, and the sixteenth and eighteenth samples  
257 DS exhibited slight acute toxicity with Microtox™. Moreover, the fifteenth sample DS  
258 exhibited acute toxicity (TU = 1.55) with *P. subcapitata*. As reported in table 3, all the  
259 influent samples exhibited TUs and the toxicity ranged from slight acute toxicity to  
260 acute toxicity (TU = 0.68 - 4.47) with *V. fischeri*, while only the eleventh and the  
261 twentieth samples presented TUs values with *D. magna* (TU sample 11 = 1.09, TU  
262 sample 20 = 2.05), while the twenty-second sample presented TU = 1.18 with *P.*  
263 *subcapitata*. So *V. fischeri* was confirmed to have a different sensitivity in the toxicity  
264 evaluation of wastewater (Tizler and Zagorc-Kon an 1999; Ricco et al. 2004). As  
265 reported in table 4, TUs (*V. fischeri*) were often detected in the WWTP effluent samples  
266 ranging from 0.40 to 13.83. Using the hazard classification system reported by  
267 Persoone et al. (2003), the OUT site was classified from not toxic to highly toxic.  
268 Moreover, four effluent samples presented TUs with *D. magna* ranging from 1.68 to  
269 8.30, and five samples presented TUs with *P. subcapitata* ranging from 1.75 to 4.19. In  
270 two cases (the eighth and the tenth samples), the sample concentration and the  
271 inhibition of algal growth were inversely proportional. The presence of a high  
272 concentration of nutrients for algae in wastewater could have been one of the possible  
273 reasons for that. Throughout the 72 hour exposure time, the adverse effects of  
274 toxicants could have been masked by the ameliorating effects of the nutrient  
275 compounds that stimulate algae growth (Manusad0ianas et al. 2003). The mean  
276 highest TUs value (*V. fischeri*) was found in the effluent samples ( $2.27 \pm 3.65$ ), and the  
277 results of the linear regression analysis (ANOVA) suggested that there were significant  
278 differences in the TUs between sites ( $F = 7.84$  and  $p < 0.001$ ). The post-hoc Tukey test  
279 of the ANOVA results indicated that the difference between effluent and both US and  
280 DS TU values was significant, while there was no statistical difference between the US

281 and DS TU values. As shown in Fig. 1, the TUs mean values were higher in the effluent  
282 samples then in the influent for the three toxicity tests applied, and this means that the  
283 toxicity generally increased in the effluent.

284 The evaluation of the overall toxic concentration following the ecotoxicological  
285 approach is shown in Fig. 2 (U.S. EPA 1991). Eight DS samples, taken during the  
286 disinfection period, exhibited an appreciable toxicity (C), although only the ninth and  
287 the sixteenth samples exceeded the U.S. EPA acceptance limit for acute toxicity (TU =  
288 0.3).

289 In relation to the effect of the chlorination process on the toxicity of the effluent, no  
290 toxicity was found in the absence of chlorination, while the mean toxicity was  $3.42 \pm$   
291  $4.12$  TU with chlorination, considering the highest TU values of the three tests applied.

292

### 293 3.2. Microbiological analyses

294 Microbiological analyses (Tables 2 - 5) highlighted that there was generally a difference  
295 between the four sampling sites (IN:  $7,622,700 \pm 6,227,340$  CFU/100 mL; US:  $42,700 \pm$   
296  $23,400$  CFU/100 mL; OUT:  $34,700 \pm 67,000$  CFU/100 mL; DS:  $39,000 \pm 29,200$   
297 CFU/100 mL). The results of the linear regression analysis (ANOVA) suggested that  
298 these differences in *E. coli* concentration between sites were significant ( $F = 31.629$   
299 and  $p < 0.0001$ ). The post-hoc Tukey test of the ANOVA results indicated that the  
300 difference between influent and both US and DS samples values was significant, while  
301 there was no statistical difference between the *E. coli* concentrations of the other three  
302 sites (OUT, US and DS). Microbiological analyses have highlighted the efficiency of  
303 the WWTP in the removal of *E. coli* from the influent. The mean removal was  $97.83\% \pm$   
304  $7.03\%$  at the end of the process; however, sometimes this was not sufficient to reduce  
305 the *E. coli* concentration below 20,000 CFU/100 mL, which is the concentration limit  
306 established by the local authorities, or below 5,000 CFU/100 mL, which is the  
307 concentration limit established by the Decree Italian Law 152/2006. In relation to the  
308 effect of the chlorination process on the *E. coli* concentration of the WWTP effluent, we

309 found a lower mean value for the chlorinated effluent:  $13,993 \pm 12,037$  CFU/100 mL vs.  
310  $62,857 \pm 80,526$  CFU/100 mL for the non-chlorinated effluent (Figure 3). This  
311 difference was shown to be significant with the *T*-test ( $p < 0.05$ ). However, *E. coli* in ten  
312 chlorinated samples was higher than 5,000 CFU/100 mL (Decree Italian Law  
313 152/2006).

314

### 315 *3.3. Trihalomethanes concentration*

316 THMs expressed as the sum of disinfection by-products (DBPs) chloroform,  
317 bromoform, chlorodibromomethane, bromodichloromethane and other THMs, 1,1,1-  
318 trichloroethane, trichloroethylene, carbon tetrachloride, 1,2-dichloroethane,  
319 trichloroethylene, and tetrachloroethene (Tables 2 - 5) were detected at all of the  
320 sampling sites at concentrations ranging from  $<0.10$  to  $7.72$   $\mu\text{g/L}$ . The highest mean  
321 THMs value was found in the influent samples ( $2.79 \pm 1.40$   $\mu\text{g/L}$ ), while the mean  
322 highest DBPs value was found in the effluent samples ( $1.85 \pm 2.25$   $\mu\text{g/L}$ ), and the  
323 results of the linear regression analysis (ANOVA) suggest that there were significant  
324 differences in DBPs mean concentrations between sites ( $F = 5.44$  and  $p < 0.01$ ). The  
325 post-hoc Tukey test of the ANOVA results determined that the mean DBP  
326 concentration of the WWTP effluent differs significantly from the mean DBP  
327 concentrations of the US and DS samples; however, the mean DBP concentrations of  
328 the US and DS samples are not significantly different from one another. The DBP  
329 values of the effluent exhibited a higher mean value ( $2.52 \pm 2.52$   $\mu\text{g/L}$ ) in the presence  
330 of chlorination, as shown in figure 4. Despite this, the t-test performed between the  
331 DBP values with and without chlorination showed that this difference was not  
332 significant (t-test,  $p > 0.05$ ), which could be a result of the small sample size.

333

### 334 *3.4. Residual chlorine concentration*

335 The residual chlorine concentrations of the effluent samples (Table 4) ranged from  
336 <0.05 mg/L to 1.01 mg/L. In six samples it exceeded the limit ( $\leq 0.2$  mg/L) established by  
337 the Decree Italian Law 152/2006 for the effluent discharged into surface waters.

338

339 **3.5. Comparison of toxicity, *E. coli*, NaOCl, residual chlorine and DBPs**

340 Spearman correlations were calculated between toxicity and the other parameters  
341 considered in this study. Significant correlations were found for TU vs. DBPs ( $r = 0.632$ ,  
342  $p < 0.01$ ), TU vs. *E. coli* ( $r = 0.254$ ,  $p < 0.05$ ), and DBPs vs. *E. coli* ( $r = 0.570$ ,  $p < 0.01$ ).  
343 These relationships become closer if one only considered the effluent site. All the data  
344 are reported in table 6.

345

#### 346 **4. Discussion**

347 In the absence of effluent chlorination, the WWTP investigated in this study has a good  
348 efficiency in removing the influent toxicity. This evidence is confirmed by the absence  
349 of toxicity with all the tests utilized in the recipient water body both downstream and  
350 upstream of the plant discharge, except for the fifteenth sample from DS site that  
351 presented a TU value with *P. subcapitata*. Whereas, in the second sampling period  
352 (2006 - 2007), we found a low toxicity in one US sample and in two DS samples **after**  
353 **the effluent treatment with NaOCl**; the disinfection of these samples might have used  
354 the highest concentrations of sodium hypochlorite (4.58 and 5.00 mg/L). Furthermore,  
355 during the first sampling period (2005), the effluent toxicity did not change in summer,  
356 even when the disinfection had been applied. However, with the lowering of effluent  
357 temperature in October, toxicity increased significantly, showing the maximum value in  
358 the eighth sample (October 2005). This was probably due to the high temperatures  
359 observed that summer in Northern Italy. This phenomenon probably caused a high  
360 evaporation rate of oxidising volatile compounds, and minimised the formation and  
361 residence time in the water phase of disinfection by-products, as reported in the study  
362 of Matamoros et al. (2007), where it was observed that the THMs production

363 decreased with higher temperatures, and that this decrease could be attributed to the  
364 increase of ammonia nitrogen concentration observed during summer. Moreover, the  
365 increase in the toxicity value from summer to autumn could also depend on the change  
366 of quality of wastewater entering the plant. Ra et al. (2007) reported a seasonal  
367 variation in the toxicity which was lower in summer compared to winter, but it was due  
368 to the rainfall. The calculated toxicity (C) of the Dora Riparia was obtained by taking  
369 into account the toxicities and flow rates of both WWTP discharge and its recipient  
370 water body, and it resulted in being above the water quality standard established by  
371 U.S. EPA (1991) for acute toxicity in two samples. This result was not in accordance  
372 with the measured toxicity in the river downstream of the WWTP outlet, but it has to be  
373 considered that this was based on grab sampling, so the results are not completely  
374 representative. Moreover, the toxicity with *V. fischeri* presented a significant correlation  
375 with the NaOCl concentration, the THMs and the DBPs concentration as reported in  
376 other studies (Petala et al. 2008; Zouboulis et al. 2007; Monarca et al. 2000), but we  
377 did not find correlation with the effects on *D. magna* and *P. subcapitata*. Cao et al.  
378 (2009) found an increased mortality of neonates (*D. magna*) after chlorination, but the  
379 disinfectant dosages used were higher than 5 mg/L.

380 The WWTP reached a good percentage removal of the bacterial concentration, but the  
381 disinfection process applied can be considered less effective: in eight effluent samples  
382 (four in absence of chlorination and four in presence of chlorination), *E. coli* exceeded  
383 the concentration of 20,000 CFU/100 mL (the limit established by the local authority)  
384 and exceeded the concentration of 5,000 CFU/100 mL (Decree Italian Law 152/2006)  
385 in all the effluent samples not disinfected and in eleven disinfected samples, even if the  
386 *E. coli* concentration in the effluent presented a significant correlation with the  
387 disinfectant dosage and with the residual chlorine. It is interesting to highlight that, even  
388 if the *E. coli* effluent concentrations were higher than the established limits, we  
389 observed no impact on the recipient river because the mean *E. coli* concentration  
390 upstream was  $42,667 \pm 23,422$  CFU/100 mL. Also, the study of Gaki et al. (2007)



391 reported that the chlorination applied was unable to produce the required effluent  
392 standard.

393 THMs and DBPs in both the chlorinated and the non-chlorinated samples were  
394 acceptable under Italian legislation (Decree Italian Law 152/2006), which restricts  
395 chlorinated solvents of WWTP effluents to 1 mg/L. THMs and DBPs presented a  
396 significant correlation with the disinfectant dosage, residual chlorine and toxicity, as  
397 reported in the study by Matamoros et al. (2007), where the concentrations of THMs  
398 found were comparable with the ones reported in this study.

399 Regarding the hygienic. sanitary evaluation of the impact of the disinfection practice on  
400 the recipient water body, we observed that the chlorination with sodium hypochlorite  
401 seems inadequate to comply with the foreseen microbiological emission limit;  
402 moreover, it produces an increase in the toxicity of the effluent and the overcoming of  
403 the limit established by Italian Law for the residual chlorine concentration. Thompson  
404 and Blatchley (1999) studied the toxicity response of wastewater effluent samples  
405 exposed to  $\gamma$ -radiation compared with chlorinated and municipal wastewater effluent  
406 samples not disinfected. The chlorinated effluent samples often showed a statistically  
407 significant increase in toxicity as compared to those not disinfected and to the  $\gamma$ -  
408 irradiated samples. This type of disinfection system is more expensive than  
409 chlorination, so it is not as widespread. In another study, Emmanuel et al. (2004)  
410 showed that the addition of NaOCl to wastewater can reduce bacterial pollution, but  
411 highlighted considerable acute toxicity with *D. magna* (TU = 9.8 . 116.8) and *V.*  
412 *fischeri* (TU = 2.47 . 4.15). Petala et al. (2008) evaluated different ozone treatments  
413 applied to secondary effluents by combination of bioassays (*V. fischeri*) using different  
414 end-points and physicochemical parameters. The study of toxicity of pre-concentrated  
415 samples showed that ozonation may either increase or decrease the toxic potential of  
416 secondary effluents. The application of low ozone doses induced a decrease of toxicity,  
417 whereas ozone doses higher than 5.0 mg O<sub>3</sub>/L resulted in an increase of toxicity of  
418 treated wastewater, and this was due to the formation of ozonation by-products.

419 Moreover Gagnè et al., (2008) evaluated the immunotoxic potential of a primary treated  
420 municipal effluent following enhanced disinfection by ozonation on freshwater mussels.  
421 They found that this disinfection process successfully reduced microbial loading, but  
422 increased the inflammatory properties of the effluent.

423 The studies on wastewater effluents indicated that all toxicity tests have a variable role  
424 to play in monitoring and control of water quality, and demonstrated that there is no  
425 single method that can constitute a comprehensive approach to aquatic life protection.  
426 For this reason, toxicity tests containing sensitive microorganisms should be applied in  
427 battery form, so the tests can complement each other, in addition to complementing the  
428 chemical analysis (Hemming et al. 2002; Sponza 2003).

429 In conclusion, this study highlighted that the disinfection of wastewater effluents with  
430 sodium hypochlorite determines the increase of the toxicity, and sometimes is not  
431 enough to control the *E. coli* contamination; the effluent toxicity after the chlorination  
432 process seems to be due to the concentration of the DBPs. The toxicity assessment of  
433 the wastewater (influent and effluent) and of the surface water provides a real  
434 approach to assess the effluent risk, and enables confirmation of the efficiency of the  
435 WWTP to remove toxic compounds. The toxicity tests can be considered as useful  
436 analytical tools for the screening of chemical analysis, and as an early warning system  
437 to monitor the WWTPs (Hernando et al. 2005). The identification of different  
438 disinfectants, such as peracetic acid, ozone or UV, and the study of the ideal  
439 concentration for reaching the toxicological and the microbiological standard for WWTP  
440 effluent seems to be a research issue that could facilitate the management of the  
441 surface water bodies.

442

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445

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614 **FIGURE CAPTIONS**

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616 **Fig. 1** TU mean values of the influent (IN) and effluent (OUT) samples.

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618 **Fig. 2** Calculated Toxicity (C) of the recipient water body (Dora Riparia River,  
619 Collegno, Torino, Italy) expressed in Toxic Unit (TU) and U.S. EPA acceptance limit.

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621 **Fig. 3** *E. coli* concentration in effluent samples chlorinated and not-chlorinated.

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623 **Fig. 4** Total THMs, DBPs, and industrial THMs in effluent samples chlorinated and not-  
624 chlorinated.

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Table 1 Hazard classification system for wastes discharged into the aquatic environment proposed by Persoone et al. (2003).

| TU              | Class     | Toxicity                 |
|-----------------|-----------|--------------------------|
| $< 0.4$         | Class I   | No acute toxicity        |
| $0.4 < TU < 1$  | Class II  | Slight acute toxicity    |
| $1 < TU < 10$   | Class III | Acute toxicity           |
| $10 < TU < 100$ | Class IV  | High acute toxicity      |
| $TU > 100$      | Class V   | Very high acute toxicity |

Table 2 Toxicity with Microtox™, *D. magna*, *P. subcapitata*, *E. coli*, THMs concentration in the Upstream WWTP (US) sampling point.

| Site and<br>sampling | <i>V. fischeri</i><br>(TU) | <i>D. magna</i><br>(TU) | <i>P. subcapitata</i><br>(TU) | <i>E. coli</i><br>(CFU/100 ml) | THMs<br>(µg/L) |
|----------------------|----------------------------|-------------------------|-------------------------------|--------------------------------|----------------|
| Upstream             |                            |                         |                               |                                |                |
| 1 (2005)             | N.T.                       | N.T.                    | N.T.                          | 30,000                         | N.D.           |
| 2 (2005)             | N.T.                       | N.T.                    | N.T.                          | 15,000                         | N.D.           |
| 3 (2005)             | N.T.                       | N.T.                    | N.T.                          | 33,000                         | N.D.           |
| 4 (2005)             | N.T.                       | N.T.                    | N.T.                          | 10,000                         | N.D.           |
| 5 (2005)             | N.T.                       | N.T.                    | N.T.                          | 48,000                         | N.D.           |
| 6 (2005)             | N.T.                       | N.T.                    | N.T.                          | 10,000                         | N.D.           |
| 7 (2005)             | N.T.                       | N.T.                    | N.T.                          | 150,000                        | N.D.           |
| 8 (2005)             | N.T.                       | N.T.                    | N.T.                          | 33,000                         | N.D.           |
| 9 (2005)             | N.T.                       | N.T.                    | N.T.                          | N.D.                           | N.D.           |
| 10 (2005)            | N.T.                       | N.T.                    | N.T.                          | 30,000                         | N.D.           |
| 11 (2006)            | N.T.                       | N.T.                    | N.T.                          | 69,000                         | 0.11           |
| 12 (2006)            | N.T.                       | N.T.                    | N.T.                          | 87,000                         | <0.10          |
| 13 (2006)            | N.T.                       | N.T.                    | N.T.                          | 61,000                         | 0.24           |
| 14 (2006)            | N.T.                       | N.T.                    | N.T.                          | 37,000                         | 0.53           |
| 15 (2006)            | N.T.                       | N.T.                    | N.T.                          | 34,000                         | 0.56           |
| 16 (2007)            | 0.69                       | N.T.                    | N.T.                          | 31,000                         | 0.47           |
| 17 (2007)            | N.T.                       | N.T.                    | N.T.                          | 30,000                         | 0.68           |
| 18 (2007)            | N.T.                       | N.T.                    | N.T.                          | 25,000                         | 0.64           |
| 19 (2007)            | N.T.                       | N.T.                    | N.T.                          | 18,000                         | 0.27           |
| 20 (2007)            | N.T.                       | N.T.                    | N.T.                          | 29,000                         | 0.37           |
| 21 (2007)            | N.T.                       | N.T.                    | N.T.                          | 18,000                         | 0.45           |
| 22 (2007)            | N.T.                       | N.T.                    | N.T.                          | 73,000                         | 0.65           |

N.T. = not toxic; N.D. = not determined

Table 3 Toxicity with Microtox™, *D. magna*, *P. subcapitata*, *E. coli*, THMs concentration in the WWTP Influent (IN) sampling point.

| Site and<br>sampling | <i>V. fischeri</i><br>(TU) | <i>D. magna</i><br>(TU) | <i>P. subcapitata</i><br>(TU) | <i>E. coli</i><br>(CFU/100 ml) | THMs<br>(µg/L) |
|----------------------|----------------------------|-------------------------|-------------------------------|--------------------------------|----------------|
| Influent             |                            |                         |                               |                                |                |
| 1 (2005)             | 1.08                       | N.T.                    | N.T.                          | 270,000                        | N.D.           |
| 2 (2005)             | 1.18                       | N.T.                    | N.T.                          | 1,600,000                      | N.D.           |
| 3 (2005)             | 0.76                       | N.T.                    | N.T.                          | 1,900,000                      | N.D.           |
| 4 (2005)             | 1.46                       | N.T.                    | N.T.                          | 12,000,000                     | N.D.           |
| 5 (2005)             | 1.70                       | N.T.                    | N.T.                          | 9,200,000                      | N.D.           |
| 6 (2005)             | 1.08                       | N.T.                    | N.T.                          | 13,000,000                     | N.D.           |
| 7 (2005)             | 3.86                       | N.T.                    | N.T.                          | 4,500,000                      | N.D.           |
| 8 (2005)             | 2.63                       | N.T.                    | N.T.                          | 12,000,000                     | N.D.           |
| 9 (2005)             | 2.44                       | N.T.                    | N.T.                          | 11,000,000                     | N.D.           |
| 10 (2005)            | 4.47                       | N.T.                    | N.T.                          | 8,400,000                      | N.D.           |
| 11 (2006)            | 0.88                       | 1.09                    | N.T.                          | 770,000                        | 1.27           |
| 12 (2006)            | 1.05                       | N.T.                    | N.T.                          | 980,000                        | 3.15           |
| 13 (2006)            | 1.01                       | N.T.                    | N.T.                          | 11,000,000                     | 4.77           |
| 14 (2006)            | 1.14                       | N.T.                    | N.T.                          | 12,000,000                     | 4.60           |
| 15 (2006)            | 0.68                       | N.T.                    | N.T.                          | 14,000,000                     | 4.98           |
| 16 (2007)            | 1.02                       | N.T.                    | N.T.                          | 9,800,000                      | 1.39           |
| 17 (2007)            | 1.11                       | N.T.                    | N.T.                          | 24,000,000                     | 3.64           |
| 18 (2007)            | 1.15                       | N.T.                    | N.T.                          | 11,000,000                     | 1.86           |
| 19 (2007)            | 1.91                       | N.T.                    | N.T.                          | 1,300,000                      | 2.03           |
| 20 (2007)            | 3.72                       | 2.05                    | N.T.                          | 110,000                        | 2.52           |
| 21 (2007)            | 1.92                       | N.T.                    | N.T.                          | 8,700,000                      | 1.96           |
| 22 (2007)            | 0.93                       | N.T.                    | 1.18                          | 170,000                        | 1.28           |

N.T. = not toxic; N.D. = not determined

Table 4 Toxicity with Microtox™, *D. magna*, *P. subcapitata*, *E. coli*, NaOCl, residual

Chlorine (RCHL) and THMs concentration in the WWTP effluent (OUT) sampling point.

| Site and<br>sampling | <i>V. fischeri</i><br>(TU) | <i>D. magna</i><br>(TU) | <i>P. subcapitata</i><br>(TU) | <i>E. coli</i><br>(CFU/100 ml) | NaOCl<br>(mg/L) | RCHL<br>(mg/L) | THMs<br>(µg/L) |
|----------------------|----------------------------|-------------------------|-------------------------------|--------------------------------|-----------------|----------------|----------------|
| Effluent             |                            |                         |                               |                                |                 |                |                |
| 1 (2005)             | N.T.                       | N.T.                    | N.T.                          | 50,000                         | 0.00            | N.D.           | N.D.           |
| 2 (2005)             | N.T.                       | N.T.                    | N.T.                          | 27,000                         | 0.00            | N.D.           | N.D.           |
| 3 (2005)             | N.T.                       | N.T.                    | N.T.                          | 19,000                         | 0.00            | N.D.           | N.D.           |
| 4 (2005)             | N.T.                       | N.T.                    | N.T.                          | 37,000                         | 3.32            | 0.30           | N.D.           |
| 5 (2005)             | N.T.                       | N.T.                    | 2.24                          | 12,000                         | 3.69            | 0.17           | N.D.           |
| 6 (2005)             | N.T.                       | N.T.                    | N.T.                          | 41,000                         | 3.88            | 0.17           | N.D.           |
| 7 (2005)             | N.T.                       | N.T.                    | N.T.                          | 3,500                          | 3.66            | N.D.           | N.D.           |
| 8 (2005)             | 13.83                      | 3.53                    | 4.17                          | 18,000                         | 3.52            | 0.62           | N.D.           |
| 9 (2005)             | 3.17                       | N.T.                    | N.T.                          | 18,000                         | 1.80            | 0.22           | N.D.           |
| 10 (2005)            | 5.20                       | 1.68                    | 3.15                          | 8,500                          | 2.00            | N.D.           | N.D.           |
| 11 (2006)            | N.T.                       | N.T.                    | N.T.                          | 2,400,000                      | 0.00            | N.D.           | 0.44           |
| 12 (2006)            | N.T.                       | N.T.                    | N.T.                          | 16,000                         | 2.44            | <0.05          | 1.51           |
| 13 (2006)            | N.T.                       | N.T.                    | N.T.                          | 17,000                         | 0.00            | N.D.           | 0.63           |
| 14 (2006)            | N.T.                       | N.T.                    | N.T.                          | 21,000                         | 2.89            | <0.05          | 0.90           |
| 15 (2006)            | N.T.                       | N.T.                    | N.T.                          | 18,000                         | 0.00            | N.D.           | 0.99           |
| 16 (2007)            | 3.31                       | N.T.                    | 1.75                          | 1,800                          | 4.58            | 0.63           | 7.72           |
| 17 (2007)            | N.T.                       | N.T.                    | N.T.                          | 69,000                         | 0.00            | N.D.           | 1.05           |
| 18 (2007)            | 7.01                       | 8.30                    | N.T.                          | 1,700                          | 5.00            | 1.01           | 2.85           |
| 19 (2007)            | 0.40                       | N.T.                    | N.T.                          | 9,900                          | 3.61            | N.D.           | 0.85           |
| 20 (2007)            | 3.78                       | N.T.                    | N.T.                          | 8,300                          | 4.07            | 0.59           | 1.54           |
| 21 (2007)            | 5.68                       | N.T.                    | N.T.                          | 200                            | 3.38            | 0.51           | 5.14           |
| 22 (2007)            | 7.59                       | 7.28                    | 4.19                          | 13,000                         | 2.82            | 0.08           | 1.40           |

Table 5 Toxicity with Microtox™, *D. magna*, *P. subcapitata*, *E. coli*, THM concentration in the Downstream WWTP (DS) sampling point.

| Site and<br>sampling | <i>V. fischeri</i><br>(TU) | <i>D. magna</i><br>(TU) | <i>P. subcapitata</i><br>(TU) | <i>E. coli</i><br>(CFU/100 ml) | THMs<br>(µg/L) |
|----------------------|----------------------------|-------------------------|-------------------------------|--------------------------------|----------------|
| Downstream           |                            |                         |                               |                                |                |
| 1 (2005)             | N.T.                       | N.T.                    | N.T.                          | 20,000                         | N.D.           |
| 2 (2005)             | N.T.                       | N.T.                    | N.T.                          | 16,000                         | N.D.           |
| 3 (2005)             | N.T.                       | N.T.                    | N.T.                          | 16,000                         | N.D.           |
| 4 (2005)             | N.T.                       | N.T.                    | N.T.                          | 29,000                         | N.D.           |
| 5 (2005)             | N.T.                       | N.T.                    | N.T.                          | 39,000                         | N.D.           |
| 6 (2005)             | N.T.                       | N.T.                    | N.T.                          | 10,000                         | N.D.           |
| 7 (2005)             | N.T.                       | N.T.                    | N.T.                          | 100,000                        | N.D.           |
| 8 (2005)             | N.T.                       | N.T.                    | N.T.                          | 29,000                         | N.D.           |
| 9 (2005)             | N.T.                       | N.T.                    | N.T.                          | N.D.                           | N.D.           |
| 10 (2005)            | N.T.                       | N.T.                    | N.T.                          | 26,000                         | N.D.           |
| 11 (2006)            | N.T.                       | N.T.                    | N.T.                          | 120,000                        | 0.15           |
| 12 (2006)            | N.T.                       | N.T.                    | N.T.                          | 44,000                         | 0.15           |
| 13 (2006)            | N.T.                       | N.T.                    | N.T.                          | 46,000                         | 0.40           |
| 14 (2006)            | N.T.                       | N.T.                    | N.T.                          | 34,000                         | 0.51           |
| 15 (2006)            | N.T.                       | N.T.                    | 1.55                          | 39,000                         | 0.65           |
| 16 (2007)            | 0.94                       | N.T.                    | N.T.                          | 24,000                         | 0.53           |
| 17 (2007)            | N.T.                       | N.T.                    | N.T.                          | 31,000                         | 0.68           |
| 18 (2007)            | 0.68                       | N.T.                    | N.T.                          | 26,000                         | 1.03           |
| 19 (2007)            | N.T.                       | N.T.                    | N.T.                          | 12,000                         | 0.25           |
| 20 (2007)            | N.T.                       | N.T.                    | N.T.                          | 13,000                         | 0.38           |
| 21 (2007)            | N.T.                       | N.T.                    | N.T.                          | 19,000                         | 0.74           |
| 22 (2007)            | N.T.                       | N.T.                    | N.T.                          | 61,000                         | 0.44           |

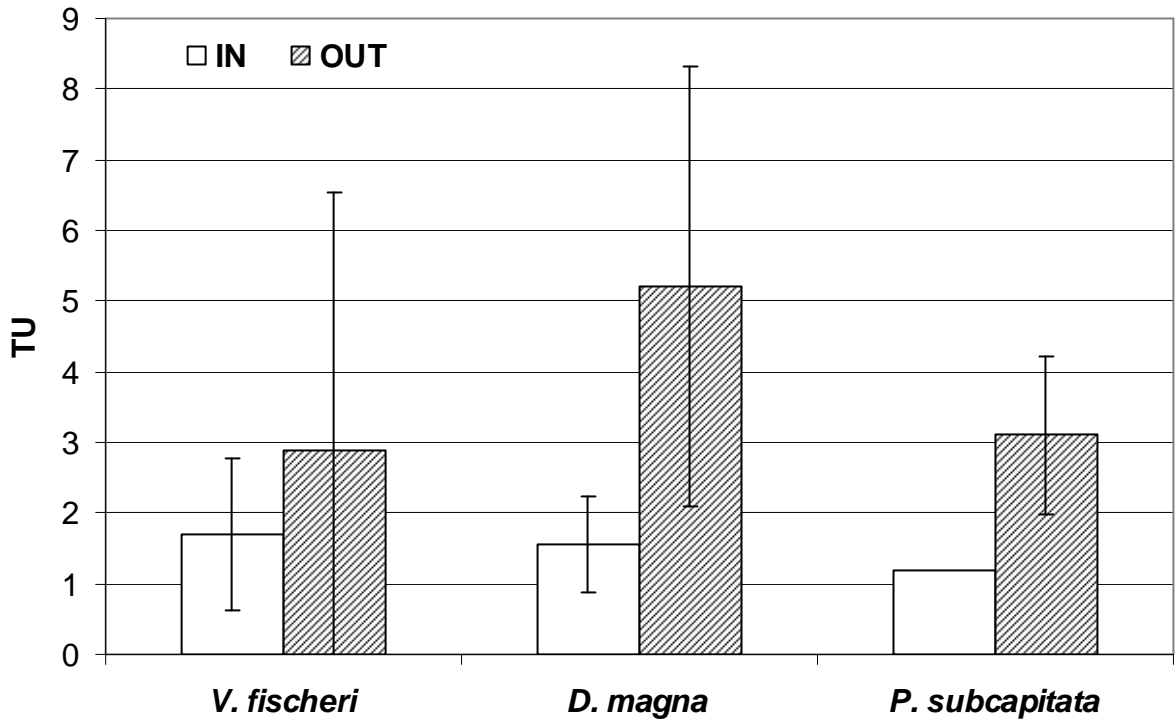
N.T. = not toxic; N.D. = not determined

642 Table 6 Comparison of toxicity (TU), *E. coli*, NaOCl, THMs, DBPs and residual chlorine  
 643 (RCHL) in the effluent site (OUT).

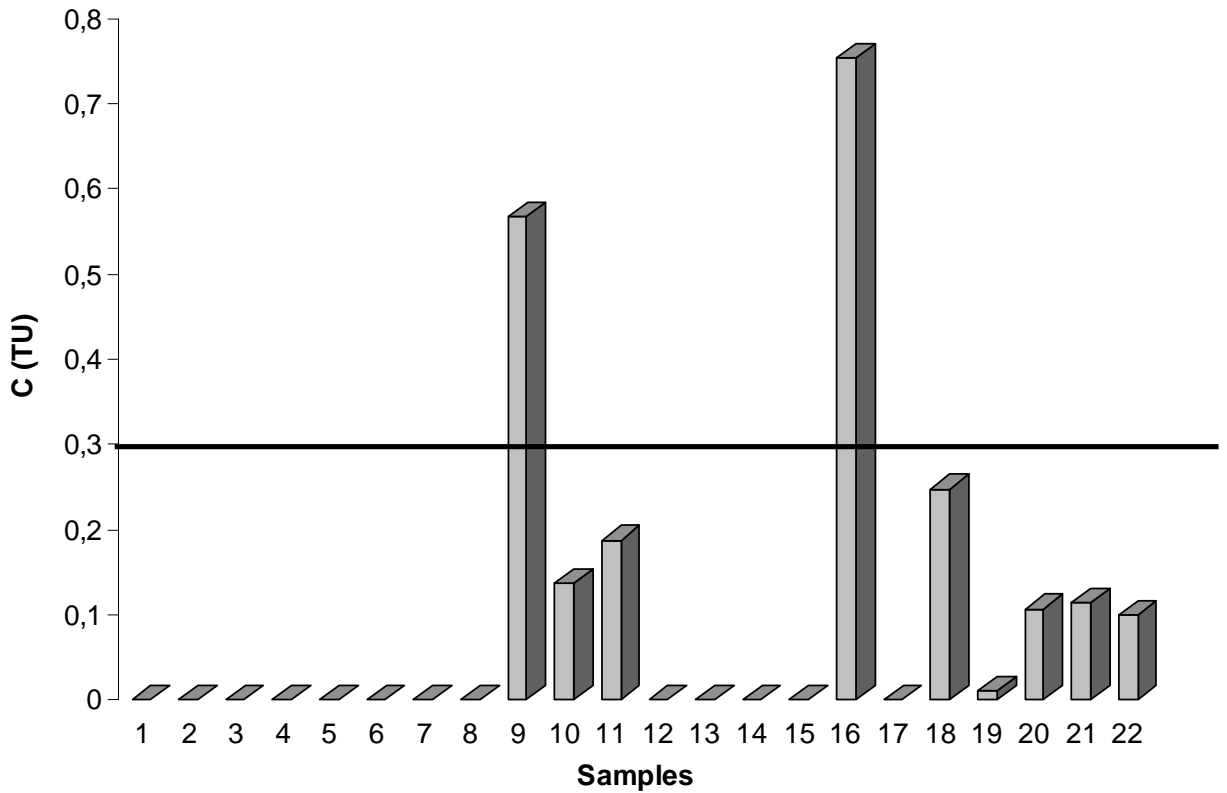
| Spearman correlations   | r     | p      |
|-------------------------|-------|--------|
| TU vs NaOCl             | 0.539 | < 0.01 |
| TU vs THMs              | 0.664 | < 0.05 |
| TU vs DBPs              | 0.788 | < 0.01 |
| TU vs <i>E. coli</i>    | 0.660 | < 0.01 |
| TU vs RCHL              | 0.657 | < 0.01 |
| THMs vs <i>E. coli</i>  | 0.850 | < 0.01 |
| THMs vs RCHL            | 0.865 | < 0.01 |
| NaOCl vs <i>E. coli</i> | 0.631 | < 0.01 |
| NaOCl vs THMs           | 0.676 | < 0.05 |
| NaOCl vs DBPs           | 0.715 | < 0.01 |
| NaOCl vs RCHL           | 0.740 | < 0.01 |
| RCHL vs <i>E. coli</i>  | 0.428 | < 0.05 |
| RCHL vs DBPs            | 0.835 | < 0.01 |

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654 **Figure 1**  
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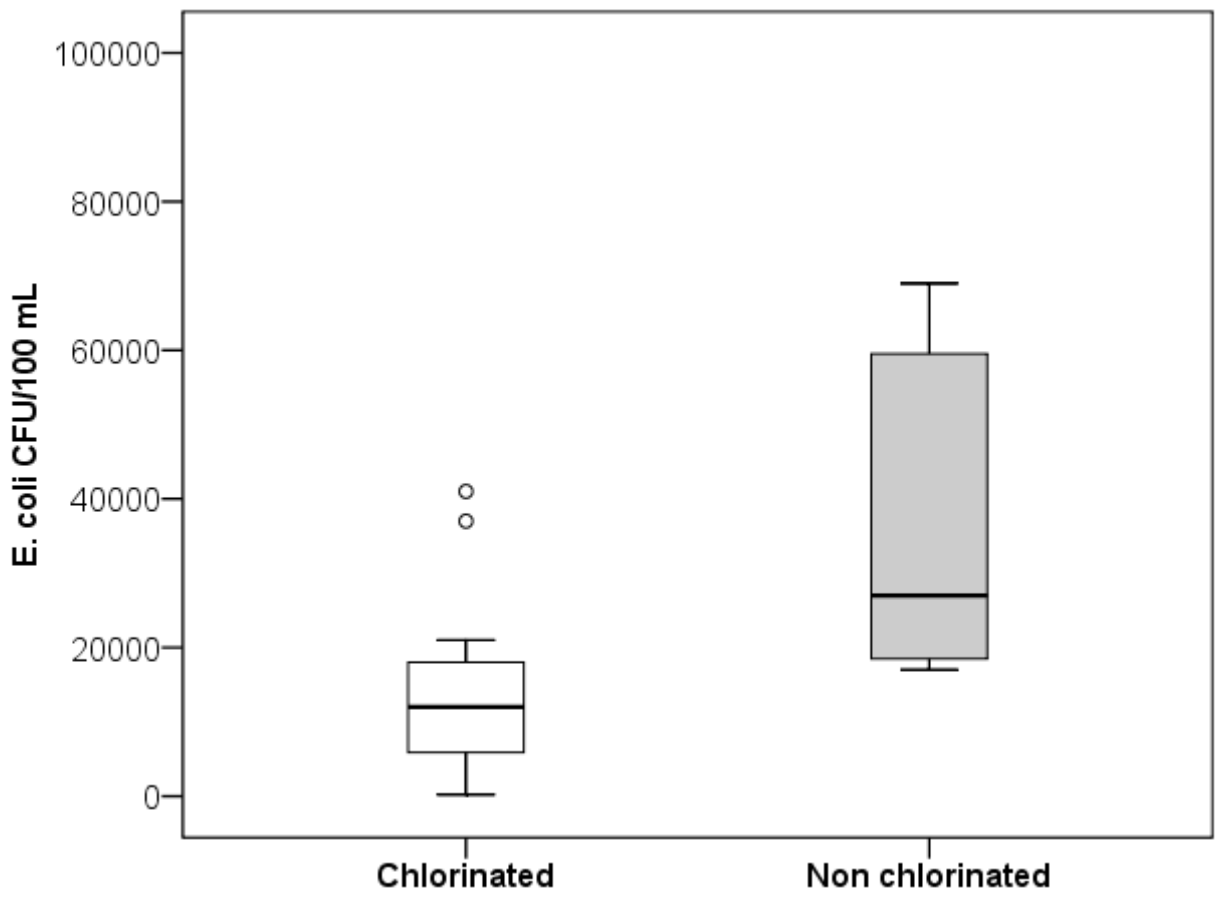
656 **Figure 2**  
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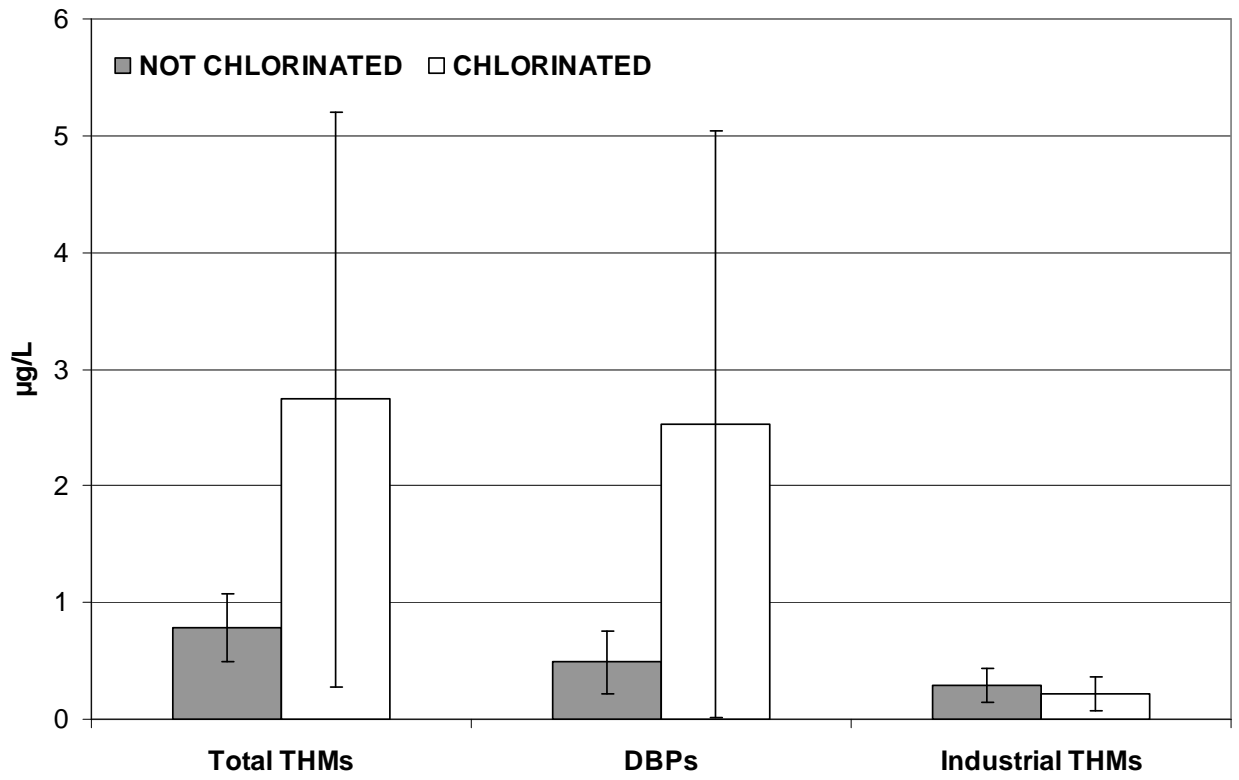


661 Figure 3



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687 Figure 4  
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