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1 **Assessment of long term ecotoxicity of urban stormwaters using a multigenerational**
2 **bioassay on *Ceriodaphnia dubia*: A preliminary study**

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4 Antoine Gosset^{1,3*}, Adriana Wigh¹, Sylvie Bony^{1,2}, Alain Devaux^{1,2}, Rémy Bayard³, Claude
5 Durrieu¹, Melissa Brocart¹, Marine Applagnat¹, and Christine Bazin⁴

6
7 ¹Université de Lyon, ENTPE, CNRS, UMR 5023 LEHNA, 2 Rue Maurice Audin,
8 69518 Vaulx-en-Velin, France

9
10 ²INRA, USC LEHNA 1369, ENTPE, F-69518 Vaulx-en-Velin, France

11
12 ³Université de Lyon, INSA Lyon, DEEP Laboratory, EA7429, F-69621 Villeurbanne cedex,
13 France

14 ⁴PROVADEMSE, Boulevard Niels Bohr, CS 52132, 69603 Villeurbanne Cedex, France

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17 _____
18 **corresponding author*. Antoine Gosset, Université de Lyon, ENTPE, CNRS, UMR 5023
19 LEHNA, 3 Rue Maurice Audin, 69518 Vaulx-en-Velin, France.

20 Tel.: +33 4 72 04 72 90; Fax: +33 4 72 04 77 43.

21 E-mail address: antoine.gosset@entpe.fr

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ABSTRACT

Standardized ecotoxicity bioassays show some limits to assess properly long-term residual toxicity of complex mixture of pollutants often present at low concentration, such as stormwaters. Among invertebrate organisms used for ecotoxicity testing, the microcrustacean *Ceriodaphnia dubia* (*C. dubia*) is considered as one of the most sensitive, especially regarding reproduction impairment as a toxicity endpoint. Consequently, the present work explores the interest to perform a multi-generational assay based on the study of the reproduction of *C. dubia* to assess long-term ecotoxicity of complex mixture, using stormwater samples. With this in mind, a battery of standardized bioassays (*Daphnia magna* mobility, *Pseudokirchneriella subcapitata* population growth, *Heterocypris incongruens* growth and one generation *C. dubia* reproduction inhibition assays) was performed in parallel to a three generation *C. dubia* reproduction inhibition assay on 2 stormwater samples. Results highlighted that while all standardized bioassays failed to reveal residual toxicity in the stormwater samples, the *C. dubia* multi-generational assay exhibited an higher sensitivity than the previous ones. No adverse effect was observed for the first exposed generation, but an increase in mortality and a reproduction disturbance was obtained in the second and third exposed generation depending of the sample. Further experiments are now needed to optimize the exposure protocol of this multi-generational assay.

KEYWORDS: Stormwater; Bioassay battery; Daphnid reproduction; life-history trait; Multigenerational; Micropollutant

55 INTRODUCTION

56

57 In Europe, the Urban Wastewater Directive (91/271/EEC) requires the management of all
58 urban wastewaters *i.e.* effluents and stormwaters, even though available treatments (mainly retention
59 and/or infiltration basins) are relatively limited in efficiency notably due to the large volumes to be
60 treated. Furthermore, European Water Framework Directive (EWFD, 2000/60/EC) requires to
61 maintain the good ecological status of water bodies receiving such effluents. To achieve this goal, it
62 remains of value to implement effective monitoring methods such as bioassays likely to reveal the
63 residual ecotoxicity of chemically complex mixtures of contaminants at low concentrations such as
64 stormwater.

65 Many standardized bioassays are currently used to assess the ecotoxicity of urban
66 stormwaters. 24-h *Daphnia magna* (*D. magna*) immobilization, 72-h *Pseudokirchneriella subcapitata*
67 (*P. subcapitata*) growth, 6-d *Heterocypris incongruens* (*H. incongruens*) and 7-d *Lemna minor* (*L.*
68 *minor*) growth assays are the most common ones. However, it has been shown that such standardized
69 bioassays often fail to properly assess stormwater toxicity, mainly due to their low contamination
70 level, and/or to the presence of nutrients interfering with the measured endpoint, in particular
71 growth.^[1,2] Angerville et al.^[1] showed that *C. dubia* reproduction bioassay was the most sensitive to
72 assess the toxicity of this kind of matrixes. Moreover, previous studies using cladocerans (*D. magna*)
73 highlighted that many environmental pollutants (pesticides, heavy metals, pharmaceuticals, phthalates,
74 ...) led to reproduction impairment with different modes of toxic action: estrogenic and anti-
75 estrogenic, androgenic and anti-androgenic activities through activation/blocking of specific receptors,
76 modulation of steroid metabolism (others than estrogens and androgens), disturbance of ecdysis or
77 juvenile hormone mimicry.^[3,4,5] Endocrine disrupting activities can lead to adverse effects on exposed
78 organisms, but also on their progeny, creating adverse effects on subsequent generations.^[6,7]
79 Nowadays, there is undoubtedly an important concern about the long term ecotoxicity assessment of
80 single chemicals and of multi-contaminated matrixes and the scientific community is actively working

81 to create harmonization (OECD guidelines) of multigenerational bioassays on cladocerans (*D. magna*)
82 to detect long-term impairments, as done recently by Barata et al.^[6]

83 Multigenerational (3 to 5 generations) reproduction inhibition tests have already been
84 developed on *C. dubia*. Rose et al.^[8,9] used such an approach for testing the long-term reproduction
85 toxicity of low concentration of 3,4-dichloroaniline (3,4 DCA), a by-product of the herbicide diuron.
86 These authors observed that 3,4-DCA at environmentally realistic concentrations led to a stimulation
87 of the reproduction (overproduction of neonates) starting at the second generation exposed (total of 4
88 generations studied), resulting in tolerance (physiological acclimation or genetically-based resistance)
89 to 3,4 DCA at concentration up to 2.5 µg/L. On the contrary, Lamichhane et al.^[10,11] observed that two
90 pharmaceuticals (carbamazepine and sertraline) led to a decrease in offspring production in three
91 successive *C. dubia* generations exposed respectively to 196.7 and 4.8 µg/L.

92 The main objective of this study was to implement an ecotoxicological battery of bioassays,
93 comprising *D. magna* acute bioassay and *P. subcapitata*, *H. incongruens* and *C. dubia* chronic
94 bioassays, as well as a multigenerational bioassay carried out on *C. dubia*, to assess the toxicity of two
95 stormwater effluents. For the later, *C. dubia* reproduction assay was derivated from the standardized
96 bioassay (NF ISO 20665^[12]) and applied in three successive generations in order to assess with a good
97 reliability a possible long-term toxicity of urban stormwater samples collected.

98

99 **MATERIALS AND METHODS**

100

101 **Sampling Sites**

102 Two samples of stormwaters were collected. The first one (CHA) stemming from a sub-urban
103 zone of Lyon City (France), corresponded to the outfall of a retention–infiltration basin receiving
104 stormwater from a highly urbanized industrial area of 185 ha, with an imperviousness rate of 75%.^[13]
105 The second one (MIR) was collected in the overflow of three interconnected retention ponds, in the

106 North-East of Lyon City corresponding to a small watershed of 11 ha draining a sub-urban area under
107 high agricultural pressure. The two samples were collected between March and April 2015 and the
108 main characteristics of the corresponding storm events are summarized in Table 1.

109 Grab stormwater samples were collected manually during the storm events, using 2L high-
110 density polyethylene flasks previously washed three times with the discharge. Samples were
111 transported immediately to the laboratory and was filtered (0.45 μm pore size) or not, depending on
112 the assay. Then, 120 mL aliquots (filtered and non-filtered) were stored at -20°C until testing.

113

114

Tab 1

115 **Physico-Chemical Analyses of Samples**

116 Several physico-chemical parameters (pH, Conductivity, Dissolved Organic Carbon (DOC), Total
117 suspended Solids (TSS), etc.) were measured according to European standards as described by
118 Angerville et al.^[1] Using 0.45 μm filtered samples, a total of 102 organic compounds were analyzed by
119 an accredited laboratory (CARSO laboratory in Venissieux) according to European standards (34
120 pesticides, 15 Polycyclic Aromatic Hydrocarbons (PAHs), 22 Volatile Organic Compounds (VOCs),
121 16 Benzene derivatives, 12 Phenol derivatives and 3 others). A list of all organic compounds and
122 analytical methods employed are summarized in Supplementary Table 1. Additionally, eight major
123 metals (Pb, Cr, Ni, Cd, Cu, Fe, Ca and Zn) were analyzed by ICP-MS (Hitachi spectrophotometer).

124

125 **Algal (*Pseudokirchneriella subcapitata*) Culture and Growth Inhibition Test**

126 The algal strain was purchased from the French National Museum of Natural History (Paris, France).
127 *P. subcapitata* were cultured in Lefebvre-Czarda medium.^[14] Algae cultures were transplanted weekly
128 under sterile condition, using sterilized glassware and medium (autoclaved during 20 min, at 130°C ,
129 under 1.3 bar), with the aim of using fresh algae in their growth phase. Algae were maintained at 21°C
130 in a culture room under a 16h/8h light/dark cycle (5000 lux illumination). Growth inhibition assays

131 were realized following the OECD guidelines,^[15] and EC₂₀ 72h values were calculated using the Hill
132 probit model (Regtox®, E. Vindimian, <http://normalesup.org/~vindimian/>).

133

134 **Ostracode (*Heterocypris Incongruens*) Lethality And Growth Inhibition Test**

135 A 6-day subchronic toxicity test was conducted following the NF EN ISO 14371 standard.^[16]
136 Organisms were exposed to 50% (standard value) or to 95% v/v of stormwater with minor
137 modifications. Briefly, test was carried out using the OSTRACODTOXKIT F®. Cysts were kept for
138 48 h in standard freshwater at 25°C and under a 3000-4000 lux illumination to hatch. Ten 4h-post
139 hatching neonates (6 replicates of 10 organisms) were sampled and their survival and body length
140 were measured after 6-day exposure using the image analysis Motic® software.

141

142 **Cladoceran Stock Culture**

143 Water flea *D. magna* and *C. dubia* (IRSTEA, France) were bred (weekly transplantation, continuous
144 parthenogenetic reproduction) in two liter glass jars, under 500±200 lux illumination and at a
145 temperature of 25±2°C for *C. dubia* and at 20±2 °C for *D. magna*, under a 16h light/8h dark
146 photoperiod. *D. magna* was cultured in Volvic® water. *C. dubia* was cultured in Evian® water
147 supplemented by 75 µg/L thiamine chlorhydrate, 1 µg/L cyanocobalamine and 0.75 µg/L biotine.
148 According to the NF EN ISO 20665 standard,^[12] organisms were daily fed on *P. subcapitata* algae
149 culture (40.10⁶ cells/mL) and on fish food (2.5g/L JBL NovoRed® solution).

150

151 ***Daphnia Magna* Mobility Inhibition Test**

152 The mobility inhibition bioassay (24 and 48h acute toxicity test) was conducted following the NF EN
153 ISO 6341 standard.^[17] EC₂₀ values were calculated using the Hill probit model (Regtox®).

154

155 ***Ceriodaphnia Dubia* Multigenerational Reproduction Inhibition Test**

156 *C. dubia* reproduction test was performed on three successive generations following a modified
157 protocol derived from the standardized one generation-reproduction bioassay NF ISO 20665.^[12] Due
158 to the rather small volume of stormwater samples available, bioassay was developed and conducted in
159 6-well plates. In each well, one organism was exposed during 10 days in 10 mL (instead of using 50
160 mL in the ISO standard) of either 0.45 µm filtered stormwater sample at a concentration of 90% (v/v)
161 or to the control medium (see 2.5). Main physico-chemical characteristics (pH and conductivity) of
162 control medium and of tested stormwaters were in the same range what enabled to expose organisms
163 up to 90% of stormwater. Exposure medium consisted of 9 mL of sample (stormwater or control
164 water) mixed with 1 mL of *C. dubia* food suspension. Exposure medium was renewed every two days
165 with thawed stormwater in order to insure constant exposure conditions. Twelve replicate organisms
166 were used per tested condition, each replicate consisting of one neonate (age < 24h) obtained from the
167 stock culture of *C. dubia* and considered as the first generation (F0). Every two days when exposure
168 medium was renewed, neonates were counted and removed. Organisms were then fed and parent
169 mortality measured. Neonates from the second brood (of the twelve replicates) were mixed and
170 randomly used to produce the next generation (F1) exposed again to the wastewater samples or to the
171 control medium. Experiment was repeated for three successive generations (Figure 1). Two parameters
172 were followed at each generation (*i.e.* after 10 days of exposure): first, the population growth
173 expressed as the total number of neonates per mother (sum of neonates produced along the successive
174 broods), and second, the reproduction success expressed as the number of neonates per alive mother at
175 each generation, enabling to take into account a possible effect of mother mortality on reproduction
176 success. Regarding the NF ISO 20665 standard, a reproduction inhibition and a mortality lower than
177 30 and 20% respectively, were considered as non-significant.

178

Fig 1

179

180 **Statistical Analysis**

181 Data from *H. incongruens* and *C. dubia* assays were analyzed using GraphPad Prism version 7 for
182 Windows (GraphPad software, San Diego, CA, USA). Distribution normality and homoscedasticity
183 were tested for each parameter using Shapiro-Wilk and Brown-Forsythe tests. When these conditions
184 were respected, comparisons between groups were carried out using the ANOVA test followed by
185 post-hoc tests, and if not, by using the non-parametric Kruskal-Wallis test followed by post-hoc tests.

186

187 **RESULTS**

188

189 **Chemical Analyses**

190 Table 2 illustrates physico-chemical analyses of the stormwaters. Metal concentrations were lower
191 than the Predicted No Effect Concentration (PNEC) values established by INERIS (National Institute
192 for the Industrial Environment and Risks, France), except for copper and zinc. Regarding organic
193 micropollutants, some pesticides were detected in the µg/L range: for example the fungicide fosetyl-
194 aluminium in CHA sample (2.6 µg/L), the herbicide glyphosate (69.25 µg/L, higher than the PNEC
195 value) and one of its degradation by-product AMPA (acide 2-amino-3-(5-méthyl-3-hydroxy-1,2-
196 oxazol-4-yl)propanoïc) in MIR sample (20.15 µg/L). In CHA sample, two other organic pollutants,
197 benzyl chloride and tributyl phosphate, were detected but concentration remained below their PNEC
198 values. Other compounds, such as the PAH fluorene, were found in both samples. All other
199 compounds among the 105 analyzed were under their limit of quantification.

200

Tab 2

201 ***D. magna*, *P. subcapitata* And *H. incongruens* Bioassays**

202 As illustrated in Table 3, EC₂₀ value was above 90 % whatever the stormwater sample, suggesting a
203 lack of acute toxicity towards *D. magna* (24 and 48 hours of exposure), as well as towards *P.*
204 *subcapitata* (72h exposure).

205

Tab 3

206 Results of the ostracod growth assay are presented in Figure 2. After exposure to a 50 % stormwater
207 concentration, ostracod growth was not significantly affected neither by CHA nor by MIR sample.
208 With 95 % of sample in the exposure medium, both samples led to a slight growth stimulation ($p <$
209 0.05). No mortality was observed at the end of the assay.

210

Fig 2

211 *C. dubia* Multigenerational Bioassay

212

213 *Validation of the method*

214 The NF ISO 20665 standard requires some validation criteria for the reproduction *C. dubia*
215 bioassay in control exposure conditions: less than 20 % mortality of parents, at least three broods at
216 the end of exposure for 60 % of the parents, less than 20 % of males in parent generation and at least
217 15 neonates produced per parent. As presented in Table 4, mortality of parents at each generation
218 remained null in the control. Furthermore, no male was detected whatever the generation. It must be
219 stressed that probably due to the reduction of the exposure volume (10 mL vs 50 mL), the number of
220 broods and neonates produced per mother did not respect the standard. However, values remained
221 closed to the requested criteria (2.33; 3.33 and 3 broods per mother; 11.1; 10.7 and 12.5 neonates per
222 mother for F1, F2 and F3 generations respectively).

223

Tab 4

224 *First generation effect on Ceriodaphnia dubia*

225 Studying the first generation of *C. dubia* corresponds to the standard chronic reproduction bioassay,
226 despite minor modifications applied to the protocol as stated before. In control and in exposed
227 organisms, mortality of the parents was under or equal to 8.3%, and consequently was considered as
228 not significant. Responses of *C. dubia* to the different stormwater samples are given in Figure 3 and

229 Table 4. No chronic toxicity (population growth and reproduction) was observed at the first exposed
230 generation (F1).

231

232 *Second and third generations*

233 CHA stormwater sample led to a strong inhibition of *C. dubia* population growth (60.81% of control)
234 and of reproduction (53.14% of control) at the second generation F2 (p-value=0.0151 and p-
235 value=0.0188, respectively), and to a high parent mortality rate (54.54% increase compared to
236 control). On the contrary, a significant stimulation of both growth and reproduction was obtained at
237 the third generation (F3) of daphnids exposed to CHA stormwater (Figure 3).

238 An opposite trend was observed after exposure to MIR stormwater sample, *i.e.* a significant
239 stimulation of reproduction (38.71%) at generation F2 (p-value=0.0347) and a high and significant
240 inhibition at the third generation (F3) regarding both population growth (64.67% p-value<0.0001) and
241 reproduction (52%; no statistics possible because of the high parent mortality rate (80%) resulting in
242 only 2 available replicates).

243

Fig 3

244 DISCUSSION

245

246 Chemical analyses (realized once and considered representative of the 10 day-exposure since
247 medium was renewed every other day) show that for the two sampled stormwaters, several pollutants
248 were detected, the chemical pattern depending on the characteristics of the corresponding watershed.
249 Concerning organic micropollutants, their presence can be easily explained by the different human
250 activities along the watersheds studied. Here, high concentration of glyphosate, regarding its PNEC
251 value, and of AMPA (respectively 69.25 and 20.15 µg/L) found in MIR sample may be related to a
252 high agricultural pressure in the corresponding watershed. For the CHA sample, the presence of
253 tributyl phosphate and of benzyle chloride could arise from plastics and wood industry settled in the
254 Chassieu industrial area. Moreover, heavy metals (here Cu, Cd, Cr, Ni or Zn), and PAHs (here

255 fluorene) are commonly found in urban stormwater discharges,^[18,19] due to road traffic, vehicle brake
256 emissions and atmospheric depositions.

257 The absence of acute toxicity in *D. magna*. (mobility) observed in both samples tested is in
258 accordance with previous results of Waara et al.^[20], Angerville et al.^[11], or Bi et al.^[21], obtained after
259 exposure to urban wet weather discharges, confirming the tendency that acute toxicity bioassays are
260 not enough sensitive for stormwater toxicity evaluation.

261 When conducting standard chronic assays with algae and ostracods, no toxicity was observed
262 either. A slight growth stimulation of *H. uncongruens* was even recorded when organisms were
263 exposed to 95% of MIR or CHA sample. Although not commonly described in the case of
264 stormwaters, such an effect was observed by Wigh et al.^[22] with wastewater effluents stemming from a
265 treatment plant. Several authors as Sterner^[23], Elser et al.^[24] or Civitello et al.^[25] have suggested that
266 enrichment due to nutrients such as phosphorus and nitrogen can stimulate the growth of producers
267 and consumers and their reproduction. Here, nutrients could have contributed to stimulate the micro-
268 algal growth and consequently, to increase the amount of available food for primary consumers (*e.g.*
269 7.0 mg.L⁻¹ NO₃⁻ in MIR sample dissolved phase). .

270 The main objective of the present work was to overcome the issues encountered in the
271 ecotoxicity assessment of complex matrixes such as stormwater with standardized methods. Therefore,
272 a *C. dubia* multigenerational assay was performed. The choice of this organism and of reproduction as
273 an end point was driven by previous results obtained in our laboratory by Angerville et al.^[11] They
274 observed that *C. dubia* reproduction bioassay was the most sensitive one among a rather broad
275 ecotoxicity test battery applied to urban combined sewer overflows (acute bioassays on : *Vibrio*
276 *Fischeri*, *D. magna*, *Danio rerio* ; chronic bioassays on : *P. subcapitata*, *D. magna*, *Brachionus*
277 *calyciflorus*, *C. dubia*).

278 The novelty of the assay performed here is the threefold repetition (*i.e.* on 3 successive generations) of
279 the one generation NF ISO 20665 assay in a miniaturized version (10 vs 50 mL exposure volume).
280 This was done in an attempt to evaluate long term toxic events. Sufficient fulfilment of most of the

281 validity criteria supported by the standardized method was achieved excepted for the number of brood
282 that was slightly lower than three in average. In 2003, Pandard.^[26] attempted to perform the *C. dubia*
283 reproduction assay in 25 mL instead of 50 mL and described the same trend. This point certainly
284 deserves further improvements or a reevaluation of a minimum valid brood number in the perspective
285 of a refined assay.

286 As performed here, one can first stress that the *C. dubia* reproduction bioassay carried out with
287 stormwater samples did not highlight any significant toxic effect (mortality or reproduction
288 impairment) at the first generation. This result is in accordance with the studies of Kayhanian et al.^[27],
289 McQueen et al.^[28] and Angerville et al.^[1], showing with the standardized assay that for a majority of
290 urban runoffs samples, no effect was detected after one single *C. dubia* generation exposure. As
291 hypothesized, when second and third generations were exposed, acute and chronic toxicity appeared
292 after both MIR and CHA sample exposure. Two kinetics of toxicity response of *C. dubia* were
293 obtained: the one observed after exposure to MIR sample, is a stimulation of population reproduction
294 at the second generation followed by an acute toxicity and reproduction impairment at the third
295 generation. The second one observed after exposure to CHA sample, is a possible
296 resistance/acclimation of organisms at the third generation, expressed as a stimulation of reproduction
297 in F2 mothers, after an increase in mortality and reproduction inhibition measured at the second
298 generation. Caution must be taken when considering the physico-chemistry of the samples to explain
299 such results because of the possible interaction between chemicals and the fact that chemical analysis
300 is limited to some compounds. Nevertheless, regarding MIR sample we can hypothesize that the
301 results could be partially related to the presence of glyphosate and its degradation product AMPA,
302 glyphosate concentration being much higher than its PNEC value. This herbicide disturbs aromatase
303 activity involved in the synthesis of estrogens and thus has been identified as an endocrine
304 disruptor.^[29] Cuhra et al.^[30] observed a reduction of the fecundity and an increased abortion rate
305 (determined counting the number of aborted eggs along the time) after exposure to 1.35 mg/L of
306 glyphosate and a reduction of offspring length at lower concentrations (50 µg/L) in *D. magna*.
307 Trends observed after CHA sample exposure are more complex to interpret, but heavy metals could be
308 responsible for the observed effects. Chemical analyses (Table 2) showed rather high concentrations of

309 heavy metals regarding their PNEC values (in particular Cu and Zn, at respectively 12.48 and 80
310 $\mu\text{g/L}$). Cooper et al.^[31] observed that a mixture of the three metals Cu, Pb, and Zn could lead to a
311 significant reproduction impairment on 7-days exposed *C. dubia* at concentrations of 1.3 (Cu), 1.1
312 (Pb) and 13.0 (Zn) $\mu\text{g/L}$, and to a high mortality at 10.6 (Cu), 9.0 (Pb) and 101.1 (Zn) $\mu\text{g/L}$,
313 respectively. In CHA sample, Cu, Pb and Zn concentrations although being slightly lower than in
314 Cooper et al.^[31] could have led to the delayed (F2) reproductive defect showed in *C. dubia*. On the
315 contrary, some studies observed a tolerance/acclimation of cladocerans to copper (1-35 $\mu\text{g/L}$) and zinc
316 (50 $\mu\text{g/L}$) at environmental realistic concentration as found in CHA samples.^[32,33] Considering the
317 high mortality rate observed in F1 parents (56%), it could be possible that a resistance/acclimation of
318 the neonates borne during the stormwater exposure appeared and led to a higher production of
319 neonates at the last (F3) generation.

320 We can also note that a significant concentration (considering its PNEC value) of fluorene was
321 measured in the two samples (respectively 3.45 $\mu\text{g/L}$ and 5.95 $\mu\text{g/L}$ in CHA and MIR samples). If the
322 acute and chronic (*e.g.* reproduction) ecotoxicity of fluorene is known (*e.g.* *C. dubia* reproduction
323 bioassay CE_{10} at 25 $\mu\text{g/L}$)^[36], its mechanism of action remains unclear and its toxicity is difficult to
324 predict because depending for example on light exposure (possible UV photo-activation of PAHs.^[37]
325 The role of epigenetic events to explain obtained results cannot be excluded since it was demonstrated
326 by Vandegheuchte et al.^[38,39] in another cladoceran organism (*D. magna*) that sub-lethal concentrations
327 of some heavy metals (such as zinc) or organic compounds (such as the 5-azacytidine drug) could
328 modify the methylation pattern and subsequent transcription of several genes without stability among
329 several generations.

330

331 **CONCLUSION**

332

333 A battery of acute and chronic bioassays was implemented for the ecotoxicity assessment of
334 stormwaters. No toxic effect was observed neither with any of the acute and chronic standardized
335 bioassays applied (*D. magna* mobility, *P. subcapitata* and *H. incongruens* growth), nor with the *C.*

336 *dubia* reproduction bioassay performed on one generation (NF ISO 20665 slightly amended). We
337 modified the *C. dubia* reproduction bioassay to perform the test over three successive generations in
338 order to evaluate if this could improve the battery sensitivity. This study is the first attempt to apply a
339 multigenerational reproduction bioassay on *C. dubia*, for the toxicity detection of complex urban
340 matrixes such as urban stormwaters. This work contributes to an issue of growing interest: the
341 pollutant long-term effect assessment through a multi-generational approach, as recently proposed as
342 part of a new OECD guideline targeting *D. magna* reproduction.^[6] Present results show that the *C.*
343 *dubia* three generation reproduction bioassay appears to be a relevant approach, since exposure of the
344 second and third generations enables to reveal a disturbance of some fitness parameters (reproduction
345 and mortality). In order to confirm the interest and thus the added value of such a multigenerational
346 reproduction assay it is now necessary to complete the development of the assay (*e.g.* by studying
347 changes in the size, weight, and/ or other physiological defects of *C. dubia* parents and neonates).

348

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350

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354

355 **CONFLICT OF INTEREST**

356 The authors declare that they have no competing interests.

357

358

359

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FIGURES and TABLES CAPTION LIST

488 **Table 1:** Characteristics of the rain events corresponding to stormwater samples.

489 **Table 2:** Chemical analyzes of urban stormwater samples.

490 **Table 3:** *Daphnia magna* immobilization and *P. subcapitata* growth inhibition tests: EC₂₀ values after
491 24 and 48 h exposure of *D. magna* (n=4) and 72 h exposure of *P. subcapitata* (n=3).

492 **Table 4:** Percentage of mortality in *Ceriodaphnia dubia* parents (F0, F1 and F2) along the exposure to
493 wastewater samples and to control medium. Limit of significance is 20 % (from AFNOR standard,
494 2009).

495 **Figure 1:** Exposure protocol of three successive generations in *Ceriodaphnia dubia*.

496 **Figure 2:** *Heterocypris incongruens* growth inhibition assay. Six replicates of 10 organisms were
497 performed per assay. Histograms represent the mean growth of control neonates or neonates exposed
498 to stormwater. Bars show standard errors (n = 6). Asterisks indicate a significant difference from the
499 control (* p < 0.05, ** p < 0.01, *** p < 0.001).

500 **Figure 3:** Multigenerational *Ceriodaphnia dubia* bioassay. The stormwater concentration tested is
501 90%. Effect on population growth is expressed as the mean of variation percentage compared to
502 control of 12 replicates ± standard deviation. Effect on population reproduction is expressed as the
503 mean of variation percentage compared to the control for the n surviving mothers ± standard deviation.
504 Dotted lines represent the significance limit of 30% established in NF ISO 20665 standard. Asterisks
505 indicate a statistical significant difference from the control (* p<0.05, ** p<0.01, *** p<0.001).

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510 **Table 1:** Characteristics of the rain events corresponding to stormwater samples.

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Sample name	Rain event duration (hh:min)	Rain height (mm)	Rain intensity (mm.h ⁻¹)	Dry period before rain event (dd:hh:min)
CHA	20:06	17.2	0.86	03:03:48
MIR	04:30	5.4	1.20	01:22:30

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530 **Table 2:** Chemical analyzes of urban stormwater samples.

Parameter	Unit	CHA sample	MIR sample	Freshwater Aquatic PNEC ^[36] (From INERIS)
pH	-	8.0	7.2	/
Conductivity	μS/cm	103.6	105.1	/
TSS	mg/L	82	56.26	/
DOC	mg/L	7.1	6.53	/
NO ₃ ⁻	mg/L	1.6	7.0	/
NH ₄ ⁺	mg/L	1.1	0.3	/
SO ₄ ²⁻	mg/L	0.3	3.4	/
Cl ⁻	mg/L	0.7	1.6	/
PO ₄ ³⁻	mg/L	0.01	0.3	/
K ⁺	mg/L	1.27	3.3	/
<u>Metals</u>				
Pb	μg/L	0.10	<0.05*	5
Cr	μg/L	0.63	0.31	4.7
Ni	μg/L	1.23	0.59	1.7
Cd	μg/L	0.09	<0.002	0.19
Cu	μg/L	12.48	4.38	1.6
Fe	mg/L	<0.005	<0.006	/
Ca	mg/L	20,42	25,51	/
Zn	mg/L	0.08	0.02	0.0078
<u>Organic compounds#</u>				
Benzyle chloride	μg/L	< 0.1	0.5	1
Fluorene	μg/L	3.45	5.93	0.25
AMPA	μg/L	0.98	20.15	80
Glyphosate	μg/L	< 0.5	69.25	28
Fosetyl-aluminium	μg/L	2.6	< 0.5	590
Tributyl phosphate	μg/L	0.12	< 0.05	82

531 PNEC (Predicted No Effect Concentration), TSS (Total Suspended Solids), DOC (Dissolved Organic
532 Carbon), AMPA (2-Amino-3-(3-hydroxy-5-Methyl-isoxazol-4-yl)Propanoic Acid), / : no data available.
533 * : < limit of quantification. # : organic chemicals listed here are those detected at a level ≥ limit of
534 quantification among 105 compounds analyzed.

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536 **Table 3:** *D. magna* immobilization and *P. subcapitata* growth inhibition tests: EC₂₀ values after 24
 537 and 48 h exposure of *D. magna* (n=4) and 72 h exposure of *P. subcapitata* (n=3).

	CHA sample	MIR sample
<u><i>Daphnia magna</i> bioassay</u>		
EC _{20-24h}	> 90 %	> 90 %
EC _{20-48h}	> 90 %	> 90 %
<u><i>Pseudokirchneriella subcapitata</i> bioassay</u>		
EC _{20-72h}	> 90 %	> 90 %

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541 **Table 4:** Percentage of mortality in *Ceriodaphnia dubia* parents (F0, F1 and F2) along the exposure to
 542 wastewater samples and to control medium. Limit of significance is 20 % (from AFNOR standard,
 543 2009).

	Control	CHA	MIR 544
F0	0	8.33	8.33
F1	0	54.54	8.33 ⁵⁴⁵
F2	0	0	80 ⁵⁴⁶

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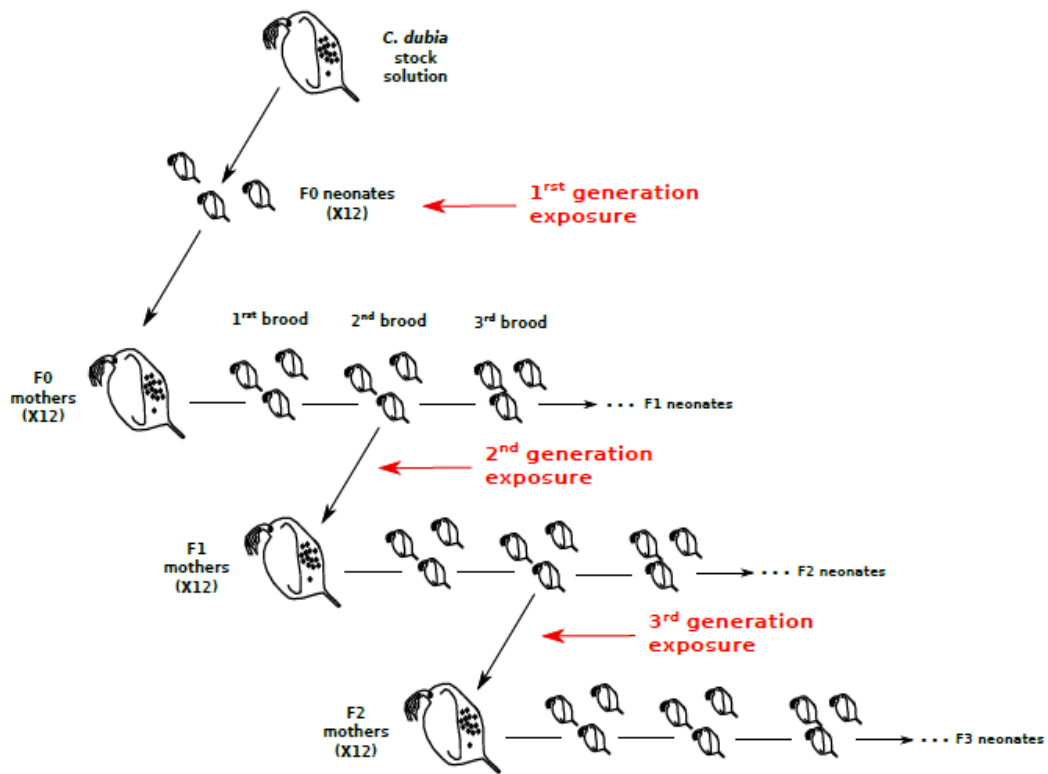
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566 **Figure 1:** Exposure protocol of three successive generations in *Ceriodaphnia dubia*.

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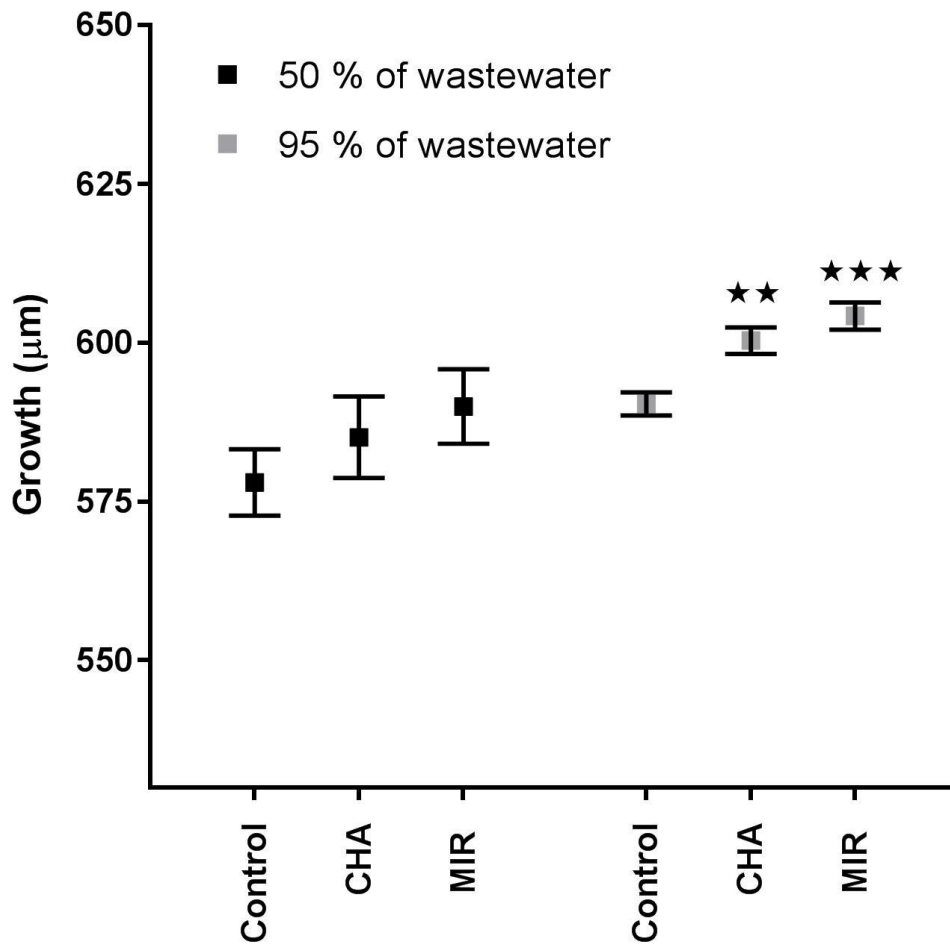
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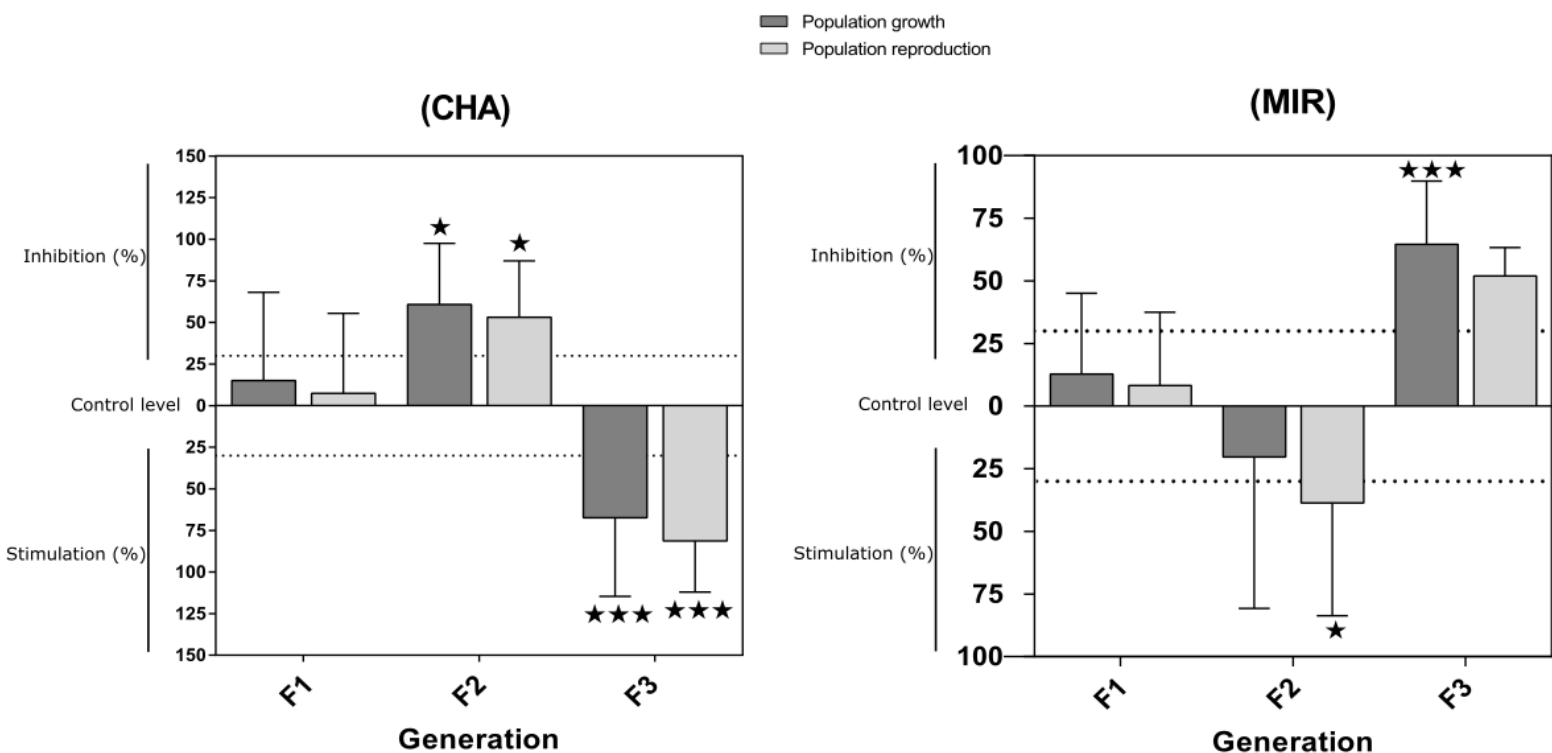
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579 to stormwater. Bars show standard errors (n = 6). Asterisks indicate a significant difference from the
580 control (* p < 0.05, ** p < 0.01, *** p < 0.001).

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586 **Figure 3:** Multigenerational *Ceriodaphnia dubia* bioassay. The stormwater concentration tested is
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 588 control of 12 replicates \pm standard deviation. Effect on population reproduction is expressed as the
 589 mean of variation percentage compared to the control for the n surviving mothers \pm standard deviation.
 590 Dotted lines represent the significance limit of 30% established in NF ISO 20665 standard. Asterisks
 591 indicate a statistical significant difference from the control (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

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600 **Supplementary table 1:** List of the organic compounds, analytical methods and standards used for
 601 stormwater physico-chemical analyses in this study.

Compound	Analytical method used	Standard
VOC : Volatil Organic Compounds		
<u>Organohalogenated solvents</u>		
1,1,2,2-tetrachloroethane	HS/GC/MS	NF EN ISO 10301
1,1,1-trichloroethane	HS/GC/MS	NF EN ISO 10301
1,1,2-trichloroethane	HS/GC/MS	NF EN ISO 10301
1,1-dichloroethane	HS/GC/MS	NF EN ISO 10301
1,1-dichloroethylene	HS/GC/MS	NF EN ISO 10301
1,2-dichloroethane	HS/GC/MS	NF EN ISO 10301
Cis 1,2-dichloroethylene	HS/GC/MS	NF EN ISO 10301
Trans 1,2-dichloroethylene	HS/GC/MS	NF EN ISO 10301
1-chloronaphtalene	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
2-chloronaphtalene	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Vinyl chlorid	HS/GC/MS	NF EN ISO 10301
Dichloromethane	HS/GC/MS	NF EN ISO 10301
Hexachlorobutadiene	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Pentachloroethane	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Tétrachloroethylene	HS/GC/MS	NF EN ISO 10301
Carbon tetrachloride	HS/GC/MS	NF EN ISO 10301
Trichloroethylene	HS/GC/MS	NF EN ISO 10301
Sum of tri and tetrachlorethylene	HS/GC/MS	NF EN ISO 10301
Sum of 1,2-dichloroethylene	HS/GC/MS	NF EN ISO 10301
<u>Autres</u>		
Dichlorobenzidine	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Benzidine	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
Benzyl chlorid	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
benzylidene chlorid	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
4-chloroacetanilide	GC/MS after CH ₂ Cl ₂ liquid-liquid extraction	Internal method
PAH : Polycyclic Aromatic		
Hydrocarbons		
Fluoranthene	HPLC/FLUO	NFT90-115
Benzo (b) fluoranthene	HPLC/FLUO	NFT90-115
Benzo (k) fluoranthene	HPLC/FLUO	NFT90-115
Benzo (a) pyrene	HPLC/FLUO	NFT90-115
Benzo (ghi) perylene	HPLC/FLUO	NFT90-115
Indeno (1,2,3 cd) pyrene	HPLC/FLUO	NFT90-115
Anthracene	HPLC/FLUO	NFT90-115
Acénaphtene	HPLC/FLUO	NFT90-115
Chrysene	HPLC/FLUO	NFT90-115
Dibenzo (a,h) anthracene	HPLC/FLUO	NFT90-115
Fluorene	HPLC/FLUO	NFT90-115
Naphtalene	HPLC/FLUO	NFT90-115
Pyrene	HPLC/FLUO	NFT90-115
Phenanthrene	HPLC/FLUO	NFT90-115
Benzo (a) anthracene	HPLC/FLUO	NFT90-115
Pesticides		
<u>Nitrogen Pesticides</u>		
Atrazine	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Simazine	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
<u>Organochlorine Pesticides</u>		
Lindane (γ -HCH)	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
α -HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Aldrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Diieldrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Endrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
2,4'-DDT	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
4,4'-DDT	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695

Isodrin	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
β-HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
δ-HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Endosulfan	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Heptachlor	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
2,4'-DDD	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
4,4'-DDD	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
2,4'-DDE	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
4,4'-DDE	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
(cis+trans) Chlordane	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Sum of HCH isomers (except ε-HCH)	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
ε-HCH	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Chlordecone	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
<u>Organophosphorus pesticides</u>		
Chlorfenvinfos	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
Ethyl-Chlorpyrifos	GC/MS/MS after liquid-liquid extraction	ISO 6468, NF EN 12918, ISO 10695
<u>Pesticides (others)</u>		
AMPA	HPLC/MS/MS after direct injection	Internal method
Glyphosate	HPLC/MS/MS after direct injection	Internal method
Fosetyl-aluminium	HPLC/MS/MS after direct injection	Internal method
Alachlor	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Trifluralin	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Oxadiazon	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Biphenyl	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
<u>Substituted urea</u>		
Diuron	HPLC/DAD after liquid-liquid extraction	Internal method
Isoproturon	HPLC/DAD after liquid-liquid extraction	Internal method
Chlorotoluron	HPLC/DAD after liquid-liquid extraction	Internal method
Linuron	HPLC/DAD after liquid-liquid extraction	Internal method
<u>Benzene derivatives</u>		
<u>Chlorobenzenes</u>		
1,2,4,5-tetrachlorobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Hexachlorobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Pentachlorobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Monochlorobenzene	HS/GC/MS	NF EN ISO 11423-1
Bromobenzene	HS/GC/MS	NF EN ISO 11423-1
2-chlorotoluene	HS/GC/MS	NF EN ISO 11423-1
3-chlorotoluene	HS/GC/MS	NF EN ISO 11423-1
4-chlorotoluene	HS/GC/MS	NF EN ISO 11423-1
1,2-dichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,3-dichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,4-dichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,2,4-trichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
1,3,5-trichlorobenzene	HS/GC/MS	NF EN ISO 11423-1
Sum of trichlorobenzenes	HS/GC/MS	NF EN ISO 11423-1
Sum of dichlorobenzenes	HS/GC/MS	NF EN ISO 11423-1
Sum of monochlorotoluenes	HS/GC/MS	NF EN ISO 11423-1
<u>Chloronitrobenzenes</u>		
1-chloro, 4-nitrobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
1-chloro, 2-nitrobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
1-chloro, 3-nitrobenzene	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
<u>Phenol derivatives</u>		
<u>Chlorophenols</u>		
2-chlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
3-chlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
4-chlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
2,4-dichlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
2,4,5-trichlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
2,4,6-trichlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
Pentachlorophenol	GC/MS/MS after acetylation and liquid-liquid extraction	Internal method following NF EN 12673
<u>Alkylphenols</u>		
4-n nonylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
4-n octylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
4-tert octylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Sum of octylphenols	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Nonylphenol (CAS 25154-52-3)	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1

Sum of nonylphenols 4-tert butylphenol	GC/MS/MS after liquid-liquid extraction GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1 Internal method following NF EN ISO 18857-1
<u>Other compounds</u> 4,4'-biphenol Tributyl phosphate Hexachlorocyclopentadiene	GC/MS/MS after CH ₂ Cl ₂ liquid-liquid extraction GC/MS/MS after liquid-liquid extraction GC/MS/MS after liquid-liquid extraction	Internal method Internal method following NF EN 6468 Internal method following NF EN 6468

DAD (Diode-Array Detection), FLUO (Fluorescence), GC (Gas Chromatography), HPLC (High Performance Liquid Chromatography), HS (HeadSpace), MS (Mass Spectrometry).

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