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

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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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Sampling Sites

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

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

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

Tab 1

Physico-Chemical Analyses of Samples

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

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

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

were realized following the OECD guidelines,[15] and EC_{20} 72h values were calculated using the Hill probit model (Regtox®, E. Vindimian, http://normalesup.org/~vindimian/).

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

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

Cladoceran Stock Culture

Water flea *D. magna* and *C. dubia* (IRSTEA, France) were bred (weekly transplantation, continuous parthenogenetic reproduction) in two liter glass jars, under 500±200 lux illumination and at a temperature of 25±2°C for *C. dubia* and at 20±2 °C for *D. magna*, under a 16h light/8h dark photoperiod. *D. magna* was cultured in Volvic® water. *C. dubia* was cultured in Evian® water supplemented by 75 µg/L thiamine chlorhydrate, 1 µg/L cyanocobalamine and 0.75 µg/L biotine. According to the NF EN ISO 20665 standard,[12] organisms were daily fed on *P. subcapitata* algae culture (40.10^6 cells/mL) and on fish food (2.5g/L JBL NovoRed® solution).

***Daphnia Magna* Mobility Inhibition Test**

The mobility inhibition bioassay (24 and 48h acute toxicity test) was conducted following the NF EN ISO 6341 standard.[17] EC_{20} values were calculated using the Hill probit model (Regtox®).

Ceriodaphnia Dubia Multigenerational Reproduction Inhibition Test

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

Fig 1

Statistical Analysis

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

RESULTS

Chemical Analyses

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

Tab 2

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

As illustrated in Table 3, EC_{20} value was above 90 % whatever the stormwater sample, suggesting a lack of acute toxicity towards *D. magna* (24 and 48 hours of exposure), as well as towards *P. subcapitata* (72h exposure).

Tab 3

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

Fig 2

***C. dubia* Multigenerational Bioassay**

Validation of the method

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

Tab 4

First generation effect on Ceriodaphnia dubia

Studying the first generation of *C. dubia* corresponds to the standard chronic reproduction bioassay, despite minor modifications applied to the protocol as stated before. In control and in exposed organisms, mortality of the parents was under or equal to 8.3%, and consequently was considered as 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=0.0.0188, 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

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

The absence of acute toxicity in *D. magna*. (mobility) observed in both samples tested is in accordance with previous results of Waara et al.[20], Angerville et al.[1], or Bi et al.[21], obtained after exposure to urban wet weather discharges, confirming the tendency that acute toxicity bioassays are not enough sensitive for stormwater toxicity evaluation.

When conducting standard chronic assays with algae and ostracods, no toxicity was observed either. A slight growth stimulation of *H. uncongruens* was even recorded when organisms were exposed to 95% of MIR or CHA sample. Although not commonly described in the case of stormwaters, such an effect was observed by Wigh et al.[22] with wastewater effluents stemming from a treatment plant. Several authors as Sterner[23], Elser et al.[24] or Civitello et al.[25] have suggested that enrichment due to nutrients such as phosphorus and nitrogen can stimulate the growth of producers and consumers and their reproduction. Here, nutrients could have contributed to stimulate the micro-algal growth and consequently, to increase the amount of available food for primary consumers (*e.g.* 7.0 $mg.L^{-1}$ NO_3^- in MIR sample dissolved phase). .

The main objective of the present work was to overcome the issues encountered in the ecotoxicity assessment of complex matrixes such as stormwater with standardized methods. Therefore, a *C. dubia* multigenerational assay was performed. The choice of this organism and of reproduction as an end point was driven by previous results obtained in our laboratory by Angerville et al.[1] They observed that *C. dubia* reproduction bioassay was the most sensitive one among a rather broad ecotoxicity test battery applied to urban combined sewer overflows (acute bioassays on : *Vibrio Fischeri, D. magna, Danio rerio* ; chronic bioassays on : *P. subcapitata, D. magna, Brachionus calyciflorus, C. dubia*).

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

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

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

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

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

CONCLUSION

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

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

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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FIGURES and TABLES CAPTION LIST

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

Table 2: Chemical analyzes of urban stormwater samples.

Table 3: *Daphnia magna* immobilization and *P. subcapitata* growth inhibition tests: EC_{20} values after 24 and 48 h exposure of *D. magna* (n=4) and 72 h exposure of *P. subcapitata* (n=3).

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

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

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

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

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

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

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	/
Metals				
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
Organic compounds#				
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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Table 3: *D. magna* immobilization and *P. subcapitata* growth inhibition tests: EC_{20} values after 24 and 48 h exposure of *D. magna* (n=4) and 72 h exposure of *P. subcapitata* (n=3).

	CHA sample	MIR sample
Daphnia magna bioassay		
$EC_{20\text{-}24h}$	> 90 %	> 90 %
$EC_{20\text{-}48h}$	> 90 %	> 90 %
Pseudokirchneriella subcapitata bioassay		
$EC_{20\text{-}72h}$	> 90 %	> 90 %

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

	Control	CHA	MIR
F0	0	8.33	8.33
F1	0	**54.54**	8.33
F2	0	0	**80**

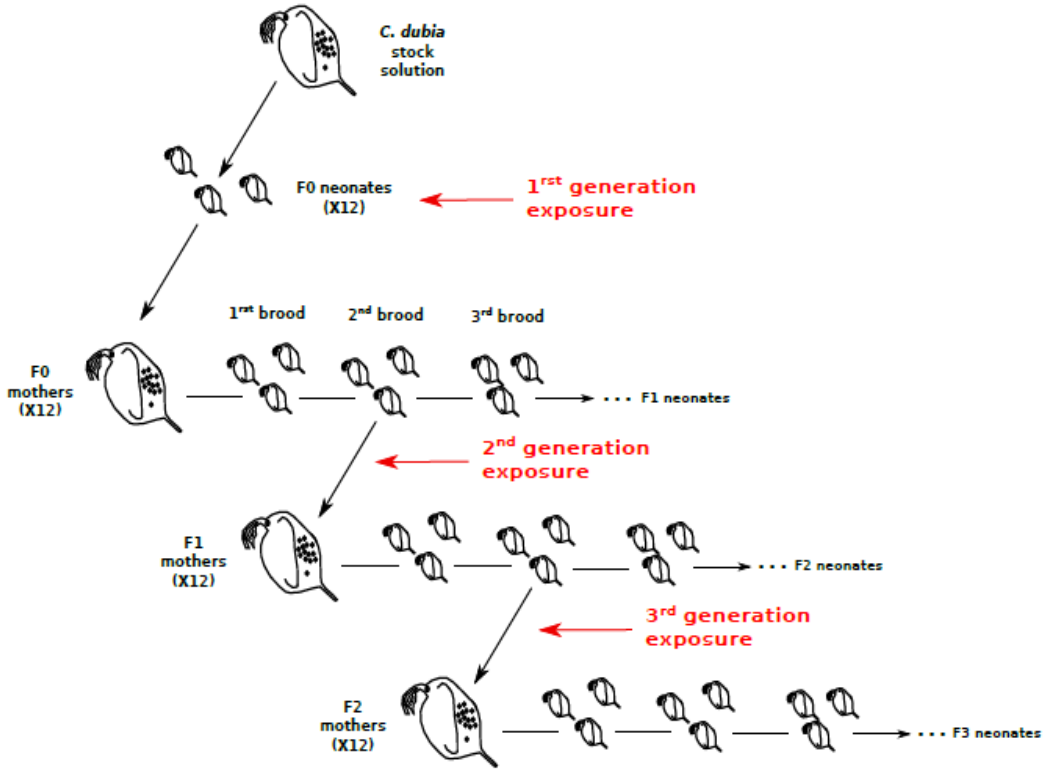


Figure 1: Exposure protocol of three successive generations in *Ceriodaphnia dubia*.

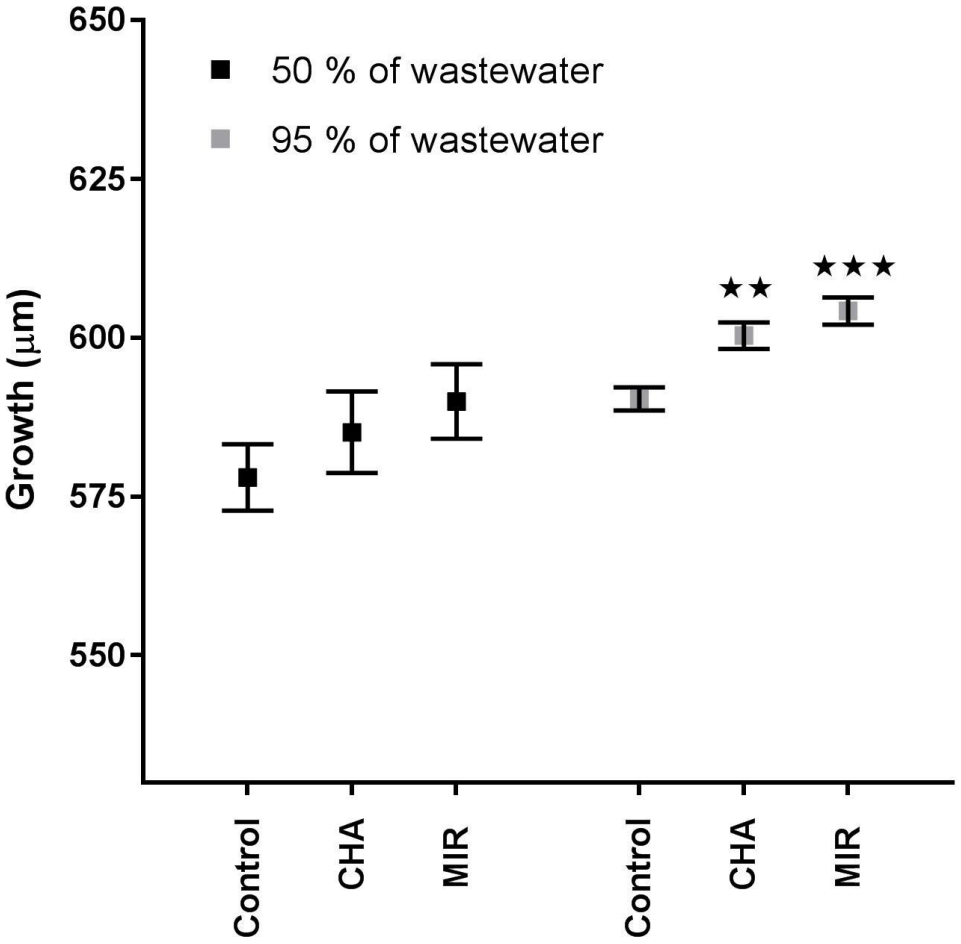
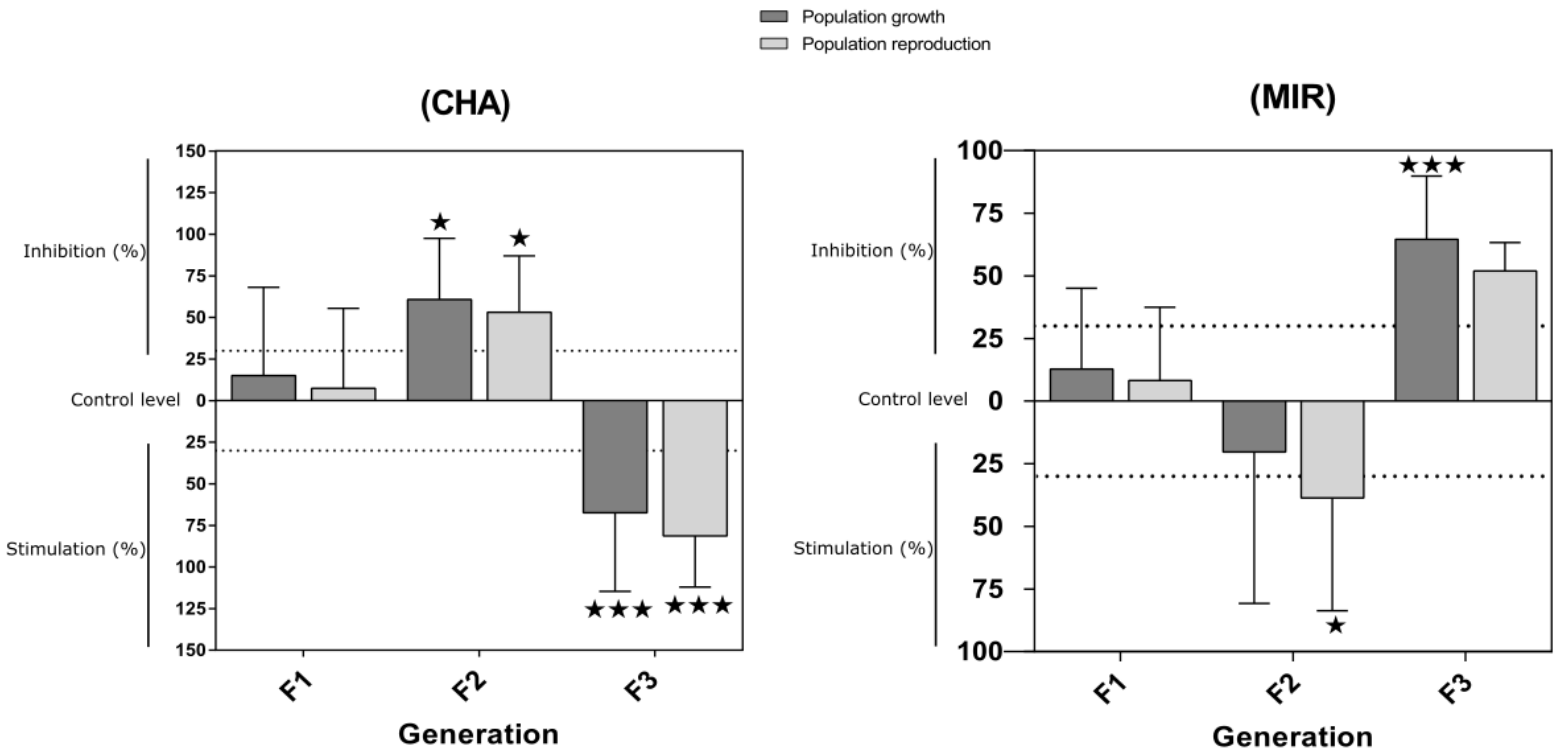


Figure 2: *Heterocypris incongruens* growth inhibition assay. Six replicates of 10 organisms were performed per assay. Histograms represent the mean growth of control neonates or neonates exposed to stormwater. Bars show standard errors (n = 6). Asterisks indicate a significant difference from the control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



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586 **Figure 3**: Multigenerational *Ceriodaphnia dubia* bioassay. The stormwater concentration tested is
587 90%. Effect on population growth is expressed as the mean of variation percentage compared to
588 control of 12 replicates ± standard deviation. Effect on population reproduction is expressed as the
589 mean of variation percentage compared to the control for the n surviving mothers ± 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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599 **Supplementary material**

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		
Organohalogenated solvents		
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-dichloroéthylene	HS/GC/MS	NF EN ISO 10301
Trans 1,2-dichloroethylene	HS/GC/MS	NF EN ISO 10301
1-chloronaphtalene	GC/MS after CH2Cl2 liquid-liquid extraction	Internal method
2-chloronaphtalene	GC/MS after CH2Cl2 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 CH2Cl2 liquid-liquid extraction	Internal method
Pentachloroethane	GC/MS after CH2Cl2 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
Autres		
Dichlorobenzidine	GC/MS after CH2Cl2 liquid-liquid extraction	Internal method
Benzidine	GC/MS after CH2Cl2 liquid-liquid extraction	Internal method
Benzyl chlorid	GC/MS after CH2Cl2 liquid-liquid extraction	Internal method
benzylidene chlorid	GC/MS after CH2Cl2 liquid-liquid extraction	Internal method
4-chloroacetanilide	GC/MS after CH2Cl2 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		
Nitrogen Pesticides		
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
Organochlorine Pesticides		
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
Dieldrin	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
Organophosphorus pesticides		
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
Pesticides (others)		
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
Substituted urea		
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
Benzene derivatives		
Chlorobenzenes		
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
Chloronitrobenzenes		
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
Phenol derivatives		
Chlorophenols		
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
Alkylphenols		
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	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
4-tert butylphenol	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN ISO 18857-1
Other compounds		
4,4'-biphenol	GC/MS/MS after CH2Cl2 liquid-liquid extraction	Internal method
Tributyl phosphate	GC/MS/MS after liquid-liquid extraction	Internal method following NF EN 6468
Hexachlorocyclopentadiene	GC/MS/MS after liquid-liquid extraction	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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