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# Miniaturization of an extraction protocol for the monitoring of pesticides and polar transformation products in biotic matrices

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## 1 1. Introduction

2 Pesticides, or plant protection products (PPPs), are widely used across the world to increase  
3 agricultural yields. As only some of the applied dose reaches target organisms, a significant amount  
4 of active substances ends up in the environment, depending on the PPPs, environmental conditions,  
5 and agricultural practices (Gaillard et al. 2016b). Aquatic ecosystems play the role of final  
6 receptacles for organic micropollutants, with concentration levels ranging from ultra-traces to  $\mu\text{g.L}^{-1}$   
7 (Gaillard et al. 2016a; Nowell et al. 2018). Gaillard et al. (2016a) estimated PPP concentrations of up  
8 to  $20 \mu\text{g.L}^{-1}$  in streams located in headwater agricultural catchments. Moreover, PPPs can affect the  
9 health of wild organisms such as macroinvertebrates (MIs) (Macchi et al. 2018), amphibians (Slaby  
10 et al. 2019), fish (Clasen et al. 2018), and birds (Stanton et al. 2018; Brain and Anderson 2019), and  
11 biodiversity in general (McLaughlin and Mineau 1995; Geiger et al. 2010). Bioaccumulation and  
12 bioamplification along the trophic chain are of particular concern as they may result in increased  
13 contamination levels in top predators (Konwick et al. 2006; Rostron 2010). To overcome these  
14 phenomena, more polar PPPs have been synthesized. However, they can reach aquatic ecosystems  
15 and contaminate wild organisms even more easily because of their intrinsic mobility. In addition,

16 PPPs can undergo biotic and abiotic processes in the environment that lead to the generation of  
17 transformation products (TPs) being usually more stable, polar, and mobile (Reemtsma et al. 2016),  
18 and sometimes having more effects on organisms (Sinclair and Boxall 2003; Tousova et al. 2017)  
19 than their parent compounds. There is a current gap in knowledge on these polar mobile organic  
20 chemicals (PMOCs) since, compared to historic PPPs, their nature and dynamics in the environment  
21 are poorly known (Reemtsma et al. 2016) despite their potential toxicity at low concentrations.  
22 Filling this gap would require the use of specific analytical tools and method such as ion  
23 chromatography, that are usually not compatible with multi-residues analysis of medium to non-  
24 polar compounds.

25 The QuEChERS (quick, easy, cheap, effective, rugged, and safe) method (Anastassiades et al. 2003)  
26 is one of the most universal extraction approach for multi-residue analysis of pesticides, including  
27 polar pesticides. It usually ensures good extraction rates (ERs) for a large number of substances  
28 (Knoll et al. 2020). This method was successfully applied to solid matrices with different levels of  
29 complexity such as fruit and vegetables (Lehotay et al. 2010; Koesukwiwat et al. 2010), meat and  
30 fish (Lazartigues et al. 2011; Lichtmannegger et al. 2015; Oliveira et al. 2018), or food from animal  
31 origin (Giroud et al. 2013; Golge et al. 2018; Song et al. 2019). However, owing to their nature, more  
32 polar substances such as TPs present other analytical challenges. Indeed, they are more soluble in the  
33 aqueous phase than in acetonitrile, even if the latter is saturated with buffer, resulting in low or no  
34 recoveries. (Dufour et al. 2020).

35 Another disadvantage of most current extraction methods is the use of high sample masses, usually  
36 greater than 1 g, that can be very difficult to obtain for many environmental matrices. This hinders  
37 the study of PPP transfers in food webs for two reasons: (1) it may not be possible to collect enough  
38 biomass to analyze pesticides in smaller organisms such as insects or small fish (Knoll et al. 2020);  
39 or (2) individuals must be pooled, which may lead to less accurate results (Roche et al. 2009). This is  
40 typically the case for MIs (e.g., insect or crustacean larvae), which have small individual masses but

41 play a key role in trophic chains by ingesting plant matter and then being consumed by predators  
42 (Four et al. 2019). Developing analytical methods to quantify the contamination of this trophic level  
43 is a major challenge for modeling PPP transfers in ecosystems. Moreover, it may also represent an  
44 interesting tool for the monitoring of marine mammals, reptiles and apex predators from a biopsy,  
45 avoiding euthanasia or invasive surgery to obtain sufficient material for analysis, which is an actual  
46 need concerning the assessment of micropollutants in marine wildlife (Sanganyado et al., 2020).  
47 Providing a robust, reliable, and sensitive analytical method based on low matrix masses could  
48 increase the number of studies in that research field and benefit the understanding of the global  
49 dynamics of organic micropollutants in the environment. In this context, the aim of this work was to  
50 study the effect of the miniaturization of a previously developed extraction protocol (Dufour et al.,  
51 2020) on analytical performance and to test its applicability on two matrices: fish and MIs. As  
52 masses of MI samples are very low, the effect of miniaturization was first investigated on fish, and  
53 then applied and validated on MIs. Special attention was paid to the validation phase in order to  
54 guarantee the reliability and accuracy of the method as it is well known that decreasing the reduction  
55 of the sample mass increases the variability of the measurement (Han et al., 2018; Lehotay et al.,  
56 2018).

57

## 58 **2. Materials and methods**

### 59 *2.1. Standards and reagents*

60 Ultrapure water (Fisher Chemical, Geel, BE), acetonitrile (ACN) (Honeywell, Seelze, DE), and  
61 heptane (Merck, Darmstadt, DE) were of LC-MS quality. Magnesium sulfate ( $MgSO_4$ ) was  
62 purchased from Sigma-Aldrich (Saint Louis, USA). Formic acid (FA) and ammonium sulfate were  
63 purchased from Biosolve-chemicals (Dieuze, FR). Fifty-milliliter polypropylene metal-free  
64 centrifuge tubes were purchased from VWR (Fontenay-sous-Bois, FR) and 2 mL polypropylene  
65 safe-lock centrifuge tubes were obtained from Eppendorf (Montesson, FR). Forty-one analytical

66 standards and 15 isotopically labeled standards (Table S1) were purchased from A2S (Martignas-sur-  
67 Jalle, FR), Honeywell (Seelze, DE), HPC standards (Cunnersdorf, DE), LGC group (Teddington,  
68 UK), Sigma-Aldrich (Saint-Louis, USA), and TCI Europe (Anvers, BE). All were of high purity  
69 (> 96%) with most > 99%. Individual solutions made in acetonitrile (100 mg.L<sup>-1</sup>) were stored at -  
70 18°C. They were mixed to obtain a 5 µg.L<sup>-1</sup> solution used to develop and validate the extraction  
71 protocol. Mix solutions were regularly characterized to check their stability over time and data were  
72 compiled in a control chart. Stored at -18°C, they were stable for at least 6 months.

73

## 74 2.2. Sampling strategy and pre-treatment

75 Young tench (*Tinca tinca*) (n = 13) were provided by the experimental platform of aquaculture  
76 (URAFPA, University of Lorraine) and were used as a blank reference matrix for fish. The MI  
77 reference matrix consisted in chironomid larvae reared under laboratory conditions, kindly provided  
78 by INRAE (ECOTOX team, Centre Lyon-Villeurbanne, FR).

79 For this purpose, fish and MIs were both sampled in November 2018 in two fishponds of the  
80 Dombes region (FR), over 2 distinct days. Those fishponds have watershed strongly influenced by  
81 conventional farming with the presence of either grassland or maize, wheat or rape crops. Fish and  
82 MI are expected to be exposed to diverse pesticides at important levels, and more especially  
83 herbicides like metazachlor and metolachlor, which are often applied to this type of crop, as well as  
84 to their TPs. Three fish species were selected for their representativeness of the local production:  
85 carp (*Cyprinus carpio*, n = 6), roach (*Rutilus rutilus*, n = 6), and rudd (*Scardinius erythrophthalmus*,  
86 n = 5). On average ( $\pm$  SEM), they weighed  $247.5 \pm 11.2$ ,  $58.3 \pm 3.5$ , and  $37.6 \pm 12.2$  g wet weight  
87 (ww), respectively. Concerning MIs, organisms were pooled to constitute 19 samples according to  
88 taxonomic classification. They were sorted into Anisoptera (n = 2), Chironomidae (n = 5), Corixidae  
89 (n = 5), Daphniidae (n = 2), Dystiscidae (n = 1), and Physidae (n = 4) groups. All blank reference  
90 matrices and samples (whole organisms) were frozen at -20°C in aluminum tray. Then, they were

91 freeze-dried (Reacti-Vap, PIERCE, Bellafonte, USA), ground, and stored at room temperature until  
92 extraction.

93

### 94 *2.3. Initial protocol*

95 The initial protocol (IP), based on a previous work by Dufour et al. (2020) and designed for the  
96 analysis of pesticides and their degradation products in high-mass environmental samples (e.g.,  
97 eggs), is illustrated in Fig. 1. Briefly, 500 mg of sample was placed in a 50 mL centrifuge tube and  
98 spiked with 50  $\mu\text{L}$  of a 500  $\mu\text{g}\cdot\text{L}^{-1}$  mix solution containing all standards to constitute artificial  
99 samples. The solvent of the solution was evaporated under gentle nitrogen flux for 10 min and the  
100 spiked matrix was mixed with 5 mL of LC-MS water at 16 Hz for 10 min (SamplePrep 2010  
101 Geno/Grinder from SPEX, Costa Mesa, USA). Then, 5 mL of heptane and 10 mL of ACN (+  
102 0.2% FA) were added and the whole sample was mixed again at 16 Hz for 10 min before  
103 centrifugation at 9,500 RCF (Relative Centrifugal Force) at 20°C for 5 min. Six milliliters of the  
104 lower layer (ACN/water mix) was dehydrated over 2.5 g of  $\text{MgSO}_4$  in a 50 mL centrifuge tube and  
105 manually mixed before being centrifuged again for 2 min (20°C, 9,500 RCF). The final extract was  
106 diluted 10 times in ultrapure water + 0.1% FA before injection in LC-MS/MS.

107

### 108 *2.4. Miniaturized protocol*

109 In order to make the analysis of small organisms (e.g., insect larvae, small fish) possible, a  
110 miniaturized protocol (MP) was developed (Fig. 1). For this MP, a 2 mL polypropylene centrifuge  
111 tube was filled with 30 mg of matrix spiked with 50  $\mu\text{L}$  of a mix solution containing all analytes  
112 (30  $\mu\text{g}\cdot\text{L}^{-1}$ ) to constitute artificial samples (concentrations and volumes used specifically for  
113 validation are given in section 2.6). Before extraction, the added solvent was evaporated under gentle  
114 nitrogen flux for 10 min. Next, 0.5 mL of LC-MS water was added, and the whole sample was  
115 shaken for 10 min at 16 Hz using Geno/Grinder. The tube was then completed with 1 mL of ACN +

116 0.2% FA and 0.5 mL of heptane before being shaken again for 10 min at 16 Hz. It was centrifuged  
117 for 5 min at 20°C and at 9,500 RCF. Then, 0.6 mL of the lower layer (ACN/water mix) was sampled  
118 and dehydrated in a new centrifuge tube containing 0.3 g of MgSO<sub>4</sub> and 3 zirconium oxide 3 mm  
119 grinding balls (Fisher Scientific, Illkirch, FR). Grinding balls were used to break up crystals formed  
120 in these small tubes, which slow down the dehydration process and lead to poor recoveries for the  
121 most polar analytes (e.g. -ESA or -OXA TPs). The tube was shaken for 1 min at 16 Hz to favor the  
122 reaction and was centrifuged again at 9,500 RCF and 20°C for 2 min. Finally, the extract was diluted  
123 10 times in ultrapure water + 0.1% FA before injection in LC-MS/MS.

124

## 125 *2.5. LC-MS/MS*

126 The development of the LC-MS/MS method was described in Dufour et al. (2020). Analysis of the  
127 41 PPP residues was performed using a 1290 Infinity UPLC system (Agilent Technologies, Massy,  
128 FR) coupled with a 5500 QTRAP mass spectrometer (Sciex, Villebon-sur-Yvette, FR) in both  
129 positive and negative modes using electrospray ionization. Forty microliters of sample was injected,  
130 and separation was performed at 0.4 mL.min<sup>-1</sup> and at 50°C on an XSelect® HSS T3 column (2.1 ×  
131 150 mm, 2.5 µm, Waters, Saint Quentin-en-Yvelines, FR). The column was protected with a  
132 KrudKatcher Ultra filter (Phenomenex, Le Pecq, FR) and an XSelect® HSS T3 precolumn (2.1 × 5  
133 mm, 2.5 µm, Waters, Saint Quentin-en-Yvelines, FR). Ultrapure water + 0.1% FA (A) and ACN +  
134 0.1% FA (B) were used as mobile phases in positive mode, while 1 mM ammonium acetate in  
135 ultrapure water (A) and ACN (B) were used in negative mode. The gradient was the same for both  
136 methods: it started with 10% B for 1 min, increased to 100% B over 7 min and held for 6 min, then  
137 decreased back to 10% B over 1 min and held for 4 min. The temperatures of the mass spectrometer  
138 source, the nebulizer gas pressure, and heating gas pressure were set at 550°C, 40 psi, and 50 psi,  
139 respectively. Ion spray voltages were 5500 V for positive ionization and -4500 V for negative  
140 ionization. Analytes and analytical parameters are available in Table S1.

141

## 142 *2.6. Method validation*

143 The MP was validated for the 41 molecules (21 PPPs and 20 TPs) for the fish matrix, using matrix-  
144 matched calibration and following European guidance recommendations (European Commission  
145 DG-SANTE 2019). The reference matrix spiked at 6 different concentration levels, namely 1 (C1), 2  
146 (C2), 5 (C3), 10 (C4), 15 (C5), and 20 (C6) times the initial concentration (C1) (available in Table  
147 1), and a protocol blank (C0), made up with reference matrix only, were extracted 5 times following  
148 the whole MP to validate response linearity. The matrix was spiked at C2, C4, and C6 before  
149 extraction and the corresponding areas were compared to the matrix spiked after extraction to  
150 calculate ERs. The areas in the matrix spiked after extraction were also compared to those of  
151 standard solutions of equivalent concentrations to calculate matrix effects (MEs). ER and ME  
152 calculations are available in Supplementary information (SI). Inter-day precision was determined  
153 over 5 days with the daily extraction in triplicate of one of the following concentration levels: C2 (n  
154 = 3), C4 (n = 2), and C6 (n = 3).

155 Because only low masses of the reference MI matrix were available, the validation of the method for  
156 MIs could not follow European guidelines (European Commission DG-SANTE 2019). Two  
157 triplicates of matrix spiked at C4 and 2 protocol blanks were extracted on 2 different days to assess  
158 protocol performance on this specific matrix. The limits of quantification (LOQs) were estimated to  
159 be 10 times the signal-to-noise ratio on C4 samples. No ER estimation could be performed but  
160 recoveries (RECs, Eq. 3 in SI), which reflect the trueness of the method, were calculated instead for  
161 each molecule on the C4 samples (n = 6), using matrix-matched calibration (C1 to C6).  
162 Chromatograms of both reference matrices spiked with monitored molecules at concentration C6 are  
163 available in SI (Fig. S1).

164

## 165 *2.7. Method application*



166 The developed miniaturized method was applied to 17 fish from 3 different species and 19 MI  
167 samples from 6 different taxa (see Section 2.2 for details) to characterize contamination in field  
168 samples. Following European guidelines (European Commission DG-SANTE 2019), quantification  
169 was performed with a 6-point matrix-matched calibration curve (described in Section 2.6). A  
170 protocol blank and a reference matrix spiked at C2 for fish or C4 for MIs were extracted with each  
171 sample series to avoid false positives and control RECs of each extraction series. When protocol  
172 blanks showed contamination, the LOQs were increased to 3 times their quantified concentrations to  
173 avoid false positives.

174

### 175 *2.8. Statistical analysis*

176 Isotope-labeled standards (D11-acetochlor, D5-acetochlor-ESA, D5-atrazine, D5-atrazine-2-  
177 hydroxy, D6-bentazone, D4-carbendazim, <sup>13</sup>C-fipronil, D4-imidacloprid, D3-isoproturon, D5-  
178 metazachlor, D6-metazachlor-ESA, D10-simazine, D9-tebuconazole, D5-terbutryn) were introduced  
179 into the MP in parallel with the analytes in the artificial samples, or instead of them for the analysis  
180 of the field samples (50 µL of a 30 µg.L<sup>-1</sup> mix solution). They are too few and do not cover all the  
181 chemical families researched to be used as internal standards, or they would probably have led to a  
182 decrease of the accuracy and an increase of uncertainties (Han et al., 2018; Lehotay et al., 2018).  
183 However, they can be used for evaluating the complexity of the matrices analyzed and tracing matrix  
184 effects.

185 The area ratio between the reference matrix and the field samples was calculated for each  
186 isotopically labeled standard. This parameter allowed to evaluate matrix complexity because  
187 quantities introduced in all samples were the same. Principal component analyses (PCAs) were  
188 performed on this ratio, for both fish and MIs, to evaluate if clusters of matrix complexity could be  
189 determined based on species (fish) and taxonomic groups (MIs). PCAs were based on Pearson  
190 regression ( $\alpha = 0.05$ ). All statistical analyses and graphical PCA illustrations were performed using

191 the software R (v. 4.0.0, © The R Foundation, 2020) with the following packages: “ggplot2”  
192 (Wickham 2016), “factoextra” (Kassambara and Mundt 2020), “FactoMineR” (Lê et al. 2008), and  
193 “RColorBrewer” (Neuwirth 2014).

194

### 195 **3. Results and discussion**

#### 196 *3.1. Performance comparison between the initial protocol and the miniaturized protocol*

197 The IP and the MP were applied to a fish matrix (n = 5) with a C4 concentration level to assess  
198 protocol performance (ERs and LOQs). The results are shown in Fig. 2. ERs ranged from 53%  
199 (terbutryn) to 103% (benzamide) with the IP and from 50% (pro sulfocarb) to 120% (methsulfuron-  
200 methyl) with the MP. According to European guidance recommendations (European Commission  
201 DG-SANTE 2019), the ERs were considered good (70% < ERs < 120%) for 39 and 38 molecules,  
202 respectively. They were similar for both protocols, with a slight advantage for the MP. The most  
203 noticeable increases in ER were observed for desethylterbuthylazine-hydroxy (61% to 97% for IP  
204 and MP, respectively), terbuthylazine (73% to 87%), and terbutryn (53% to 69%). The only outlier  
205 that did not benefit from the change in protocol was pro sulfocarb with respective ERs of 54% and  
206 50%. LOQs ranged from 0.1 (fipronil, fipronil sulfone, metolachlor) to 221.8 ng.g<sup>-1</sup> dw  
207 (metazachlor-OXA) with the IP and from 0.1 (metolachlor) to 221.2 ng.g<sup>-1</sup> dw (metazachlor-OXA)  
208 for the MP. Fourteen molecules showed LOQs of up to 10 ng.g<sup>-1</sup> dw for the IP, as against 18  
209 molecules for the MP, with 4 and 5 molecules having LOQs of up to 50 ng.g<sup>-1</sup> dw, respectively. The  
210 LOQs were very similar for both protocols, following a near-perfect linear regression (R<sup>2</sup> = 0.958).  
211 However, they were on average 1.4 times higher with the MP than with the IP. As proportions of  
212 reactants were conserved between the two protocols, obtaining similar performance results in terms  
213 of LOQ and ER did not come as a surprise. Miniaturizing the IP yielded equivalent ERs and LOQs,  
214 and allowed trace-level quantification of pesticide residues using 30 mg instead of 500 mg of matrix.

215

216           3.2. *Performance of the miniaturized protocol in fish*

217 The performance of the MP was fully evaluated for fish, as described in Section 2.6. The results are  
218 shown in Table 1. Linear regression was determined for each molecule and R<sup>2</sup> values were always  
219 greater than 0.99. Only 6 chemicals out of the 41 tested had R<sup>2</sup> values below 0.99: acetochlor-OXA  
220 (0.987), chlorotoluron (0.988), fenthion (0.950), flufenacet-ESA (0.987), metolachlor-ESA (0.989),  
221 and terbutryn (0.989). However, second-order polynomial regressions were more adapted for those  
222 molecules and their R<sup>2</sup> values were found to be 0.994, 0.996, 0.991, 0.992, 0.999, 0.993, and 0.996,  
223 respectively.

224 Among the 41 targeted substances, 38 had LOQs lower than or equal to 60 ng.g<sup>-1</sup> dw, including 19  
225 below 10 ng.g<sup>-1</sup> dw. Taking into account the dehydration of the matrix (82% ± 11%), these limits  
226 corresponded to about 2 and 10 ng.g<sup>-1</sup> ww, respectively. The overall performance was consistent  
227 with that reported in the literature (Lazartigues et al. 2011; Kaczyński et al. 2017; Inostroza et al.  
228 2017; Barbieri et al. 2019) although some LOQs were higher probably because of the low matrix  
229 masses used to perform the extraction (0.9 to 10 g in the literature, as against 0.03 g in the present  
230 study).

231 MEs ranged from -77% (fenthion) to 131% (imidacloprid-desnitro). Signal suppression was observed  
232 for 11 molecules and signal enhancement concerned 8 substances (out of a -20/+20 range). This was  
233 consistent with other studies reporting that signal suppression is the most commonly observed ME in  
234 complex matrices (Lazartigues et al. 2011; Barbieri et al. 2019). The monitoring of this parameter  
235 was important for validating the method even though matrix-matched calibration is supposed to  
236 compensate for those effects.

237 ERs at C2 ranged from 57% (pro sulfocarb) to 108% (TCP). According to the European guidelines  
238 (European Commission DG-SANTE 2019) 35 of the 42 molecules presented good ERs (between  
239 70% and 120%), including 12 of up to 80%. At C4, acceptable ERs for fish and MI matrices were  
240 obtained for 31 and 25 molecules, respectively. The inter-day precision was considered good for 32

241 chemicals at C2 according to the European guidelines previously mentioned (< 20%). This  
242 variability tended to decrease for higher concentration levels as the inter-day precision was lower  
243 than or equal to 20% for 31 molecules at C4, which is in agreement with European guidelines  
244 (European Commission DG-SANTE 2019). Moreover, if we leave aside the nature of the tested  
245 matrix, the inter-day precision is better with IP (Dufour et al., 2020) than with MP. This was most  
246 probably due to the decrease of the sample mass which led to an increase of the measurement  
247 uncertainties, as shown by Han et al. (2018) and Lehotay et al. (2018) on food samples towards  
248 pesticide contaminations. The developed analytical method was suitable for accurate quantification,  
249 except for fenthion for which inter-day precision was of up to 50% for the 3 tested concentration  
250 levels.

251 A signal-to-noise ratio of more than 3 was observed in protocol blanks for acetochlor-alachlor-ESA,  
252 bentazon, benzamide, boscalid, CGA 50267, fipronil, fipronil sulfone, isoproturon, metolachlor,  
253 prosulfocarb, and terbutryn. Contamination ranged from 0.1 to 8.8 ng depending on the molecule  
254 considered. The quantification frequency in blanks was usually below 50%, but reached 60% for  
255 fipronil and prosulfocarb, while metolachlor and terbutryn were systematically quantified. The LOQs  
256 were increased to 3 times the contamination levels for these molecules to ensure the reliability of the  
257 method.

258

### 259 *3.3. Performance of the miniaturized protocol in macroinvertebrates*

260 Performance of the MP was good for fish and compatible with trace analysis, so the protocol was  
261 tested on MI samples. As very low mass was available for the reference matrix (chironomid larvae),  
262 only linearity, RECs, and LOQs were assessed (Fig. 3). The tested concentration domains, ERs,  
263 LOQs, and blank contamination of each molecule are shown in Table S2. The quantification of most  
264 molecules followed a linear regression with  $R^2$  values greater than 0.99; only fenthion (0.986),  
265 flufenacet (0.943), and TCP (0.961) were slightly below, reaching 0.994, 0.961, and 0.992,

266 respectively, when a second-order polynomial regression was applied. Protocol blanks were clean (n  
267 = 4) but traces ( $\leq 0.8$  ng) of dimethenamid-ESA, MCPA, and terbutryn were quantified  
268 (quantification frequency below 0.5). LOQs ranged from 0.1 (fipronil, fipronil sulfone, metolachlor)  
269 to 356 ng.g<sup>-1</sup> dw (metazachlor-OXA); taking into account the dehydration (85% on average for this  
270 matrix), the LOQs ranged from 0.015 to 53 ng.g<sup>-1</sup> ww. Among the 41 tested molecules, 29 had LOQs  
271 below 10 ng.g<sup>-1</sup> dw, 14 of which being below 1 ng.g<sup>-1</sup> dw. These values were of the same order of  
272 magnitude as those reported in the literature (Haroune et al. 2015; Althakafy et al. 2018; Miller et al.  
273 2019). Most tested molecules had good RECs (80%-120%) and values above 120% were obtained  
274 with four of them (i.e., bentazone 122%, boscalid 122%, desethylterbutylazine-hydroxy 121%, and  
275 metazachlor-OXA 131%). The latter values were close to the acceptable limits, except for  
276 metazachlor-OXA for which a correction was necessary for the quantification of MI field samples. It  
277 confirmed that most pesticide residues can be monitored in complex biotic matrices with low matrix  
278 masses using a single method that provides equivalent levels of performance.

279

### 280 *3.4. Field samples*

281 In order to validate the performance of the method, 17 fish (3 species) and 19 MI (6 orders) samples  
282 were collected and analyzed with the MP. All samples were spiked before extraction with 14  
283 isotopically labeled standards (see section 2.8). The areas of these molecules were compared  
284 between field samples and reference matrices. The average ratio ranged from 0.9 (D5-acetochlor-  
285 ESA, D6-bentazone, D6-metazachlor-ESA, D5-atrazine-2-hydroxy, D9-tebuconazole) to 1.4 (D5-  
286 terbutryn) for fish and from 0.8 (D5-acetochlor-ESA, D6-metazachlor-ESA) to 1.5 (D11-acetochlor)  
287 for MIs. The relative standard deviation (%RSD) was lower than 30% for all molecules, except for  
288 D5-atrazine (34%) and 13C-fipronil (32%) in fish, and D5-acetochlor-ESA (50%) in MIs. Taking  
289 into account that a single matrix was used to quantify 3 different fish species, and another single  
290 matrix for 6 different MI groups, these ratios close to 1 confirmed the potential for the protocol to be

291 transposed to other biotic matrices with similar relative levels of fat and protein. PCAs performed on  
292 area ratios illustrated 77.6% and 66.5% of total variability for fish and MI matrices, respectively  
293 (Fig. 4). Clusters could be identified for fish, showing that differences in matrix complexity exist  
294 between the 3 species, probably based on their physiological constitution. However, there was  
295 considerable overlap between the clusters, indicating relatively similar responses for internal  
296 standards. The same overlap was observed for MIs, confirming that the chosen reference matrix is  
297 suitable for matrix-matched calibration. However, hindsight is relatively poor for Anisoptera,  
298 Daphniidae, and Dytiscidae as their number of samples is  $\leq 2$ .

299 Concentrations monitored in field samples are shown in Table 2. Among the 40 targeted molecules  
300 (fenthion being excluded because of its unreliability, see Section 3.3), 7 were detected: benzamide,  
301 imidacloprid-desnitro, fipronil, imidacloprid, prosulfocarb, tebuconazole, and terbutryn. Benzamide,  
302 imidacloprid-desnitro, and prosulfocarb were almost systematically quantified in fish (from 42 to  
303 237  $\text{ng.g}^{-1}$  dw, 3  $\text{ng.g}^{-1}$  dw, and from 30 to 165  $\text{ng.g}^{-1}$  dw, respectively) and in MIs (from 62 to 438  
304  $\text{ng.g}^{-1}$  dw, from 2 to 6  $\text{ng.g}^{-1}$  dw, and from 15 to 29  $\text{ng.g}^{-1}$  dw, respectively). Considering the  
305 dehydration ratio, the average quantified concentrations of these three molecules were of 21, <LOQ,  
306 and 12  $\text{ng.g}^{-1}$  ww in fish, respectively, and of 26, 0.4, and 3  $\text{ng.g}^{-1}$  ww in MIs, respectively.

307 Prosulfocarb is a herbicide known to drift away, leading to the contamination of non-target crops and  
308 waterbodies (Devault et al. 2019). As it possesses a log P of 4.5, it is likely to accumulate in  
309 organisms, which seemed to be the case in this study for MIs and fish. It has already been reported in  
310 ponds at concentrations of up to 0.5  $\mu\text{g.L}^{-1}$  (Gaillard et al. 2016a). Benzamide is a TP of folpet that  
311 accumulates in both fish and MIs. Despite having a lower log P value than prosulfocarb (log P =  
312 0.6), it was found in higher concentrations. Imidacloprid-desnitro, a TP of imidacloprid, was  
313 quantified in 5% of the fish samples and in 42% of the MI samples, while the quantification  
314 frequency of the active substance was 0% and 16%, respectively. There is a lack of data in the  
315 literature investigating the toxicity of imidacloprid-desnitro in aquatic organisms. Nevertheless,

316 research revealed that it has lower toxicity than imidacloprid in bees (Suchail et al. 2001). It should  
317 be noted that, in the present study, field sampling was carried out in May 2018. A few months later,  
318 in September 2018, the outdoor use of the herbicide was banned by the European Commission, so  
319 biotic concentrations are expected to decrease over time. However, the monitoring of TPs remain  
320 relevant as they sometimes have higher environmental concentrations than their parent molecules,  
321 and there is little evidence for their safety (Kiefer et al., 2019).

322

#### 323 **4. Conclusion**

324 A miniaturized extraction protocol was successfully developed and applied to two complex matrices:  
325 fish and MIs. It allowed a sensitive and consistent analysis of PPPs even when only low sample mass  
326 was available. A decrease in the required mass of matrix slightly increased the LOQs of the MP in  
327 fish compared to the IP, but final LOQs were still suitable for trace-level analysis with most below  
328 10 ng.g<sup>-1</sup> dw. Moreover, ERs were in the range 70%-120% for 35 of the 41 molecules. The  
329 transposition of IP to MP did not altered its performances. The application of this protocol in the  
330 field has revealed low contamination of aquatic organisms, but it has also shown that they are  
331 contaminated by a cocktail of molecules and TPs. The harmlessness of such a combination of  
332 contaminants is not guaranteed, and it is important to take it into consideration in order to ensure the  
333 good status of aquatic ecosystems. The herbicide prosulfocarb and the transformation product  
334 benzamide were almost systematically quantified in both matrices. The MP, developed in this study,  
335 represents a potential tool for the characterization of polar pesticides in small complex biotic  
336 samples.

337

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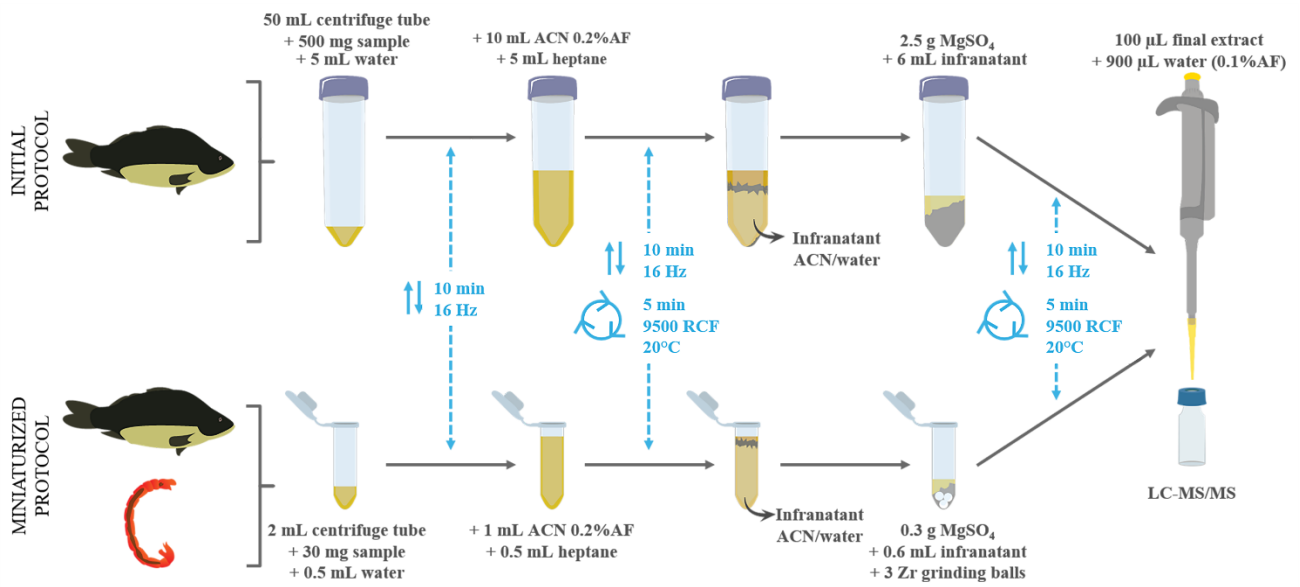
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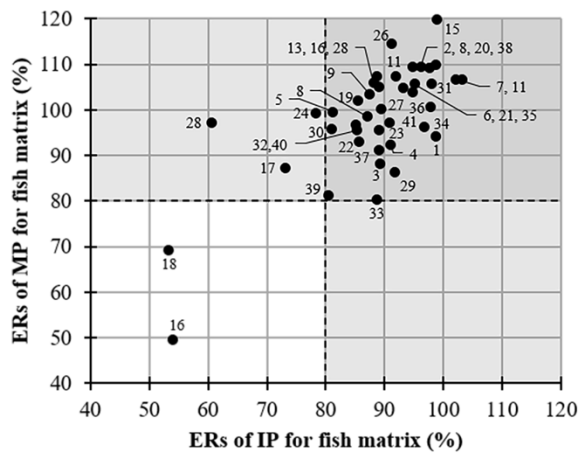


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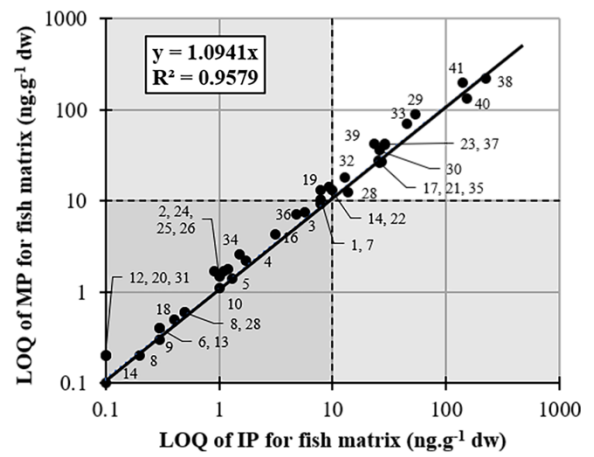
492 Fig. 1. Step comparison between the initial and miniaturized protocol (ACN: acetonitrile, FA: Formic acid)

493

494



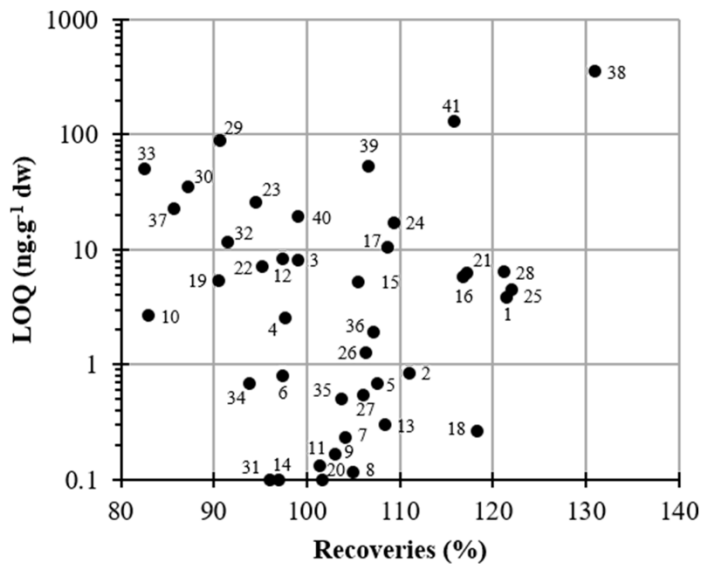
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496 Fig. 2. Comparison between the initial (IP) and miniaturized protocol (MP) in terms of extraction rates (ERs)  
497 and limits of quantification (LOQs) obtained for fish blank matrix spiked at C4 (n=5); see Table 1 for the  
498 molecules associated with the numbers

499

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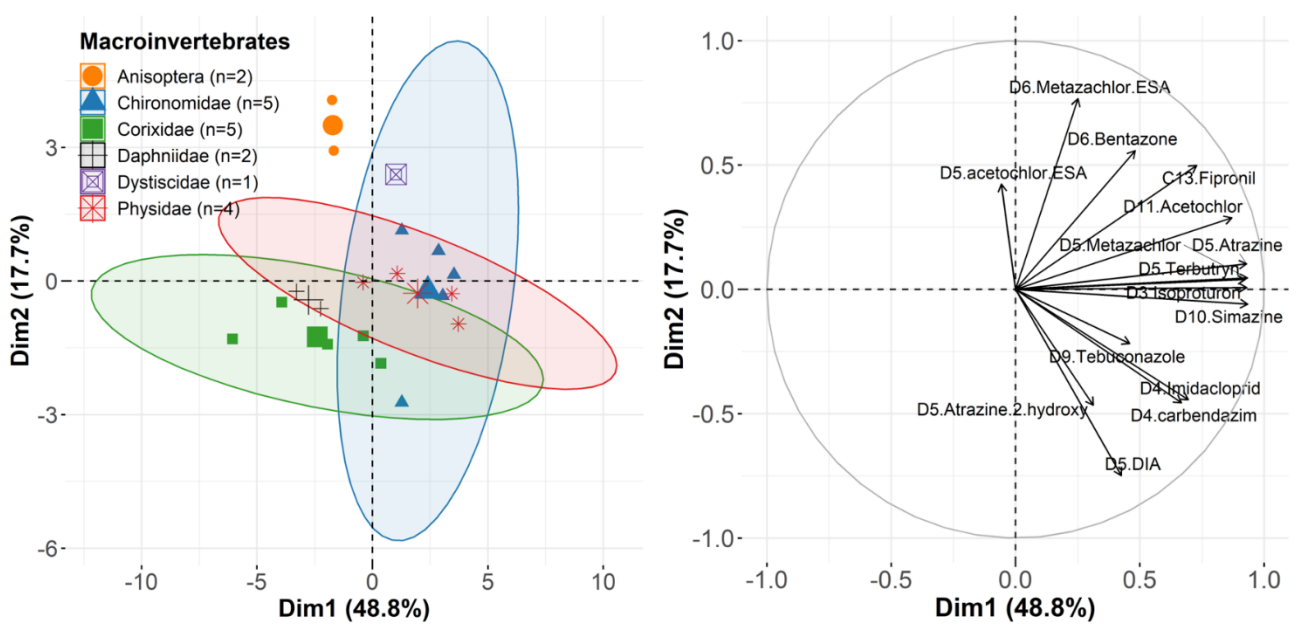
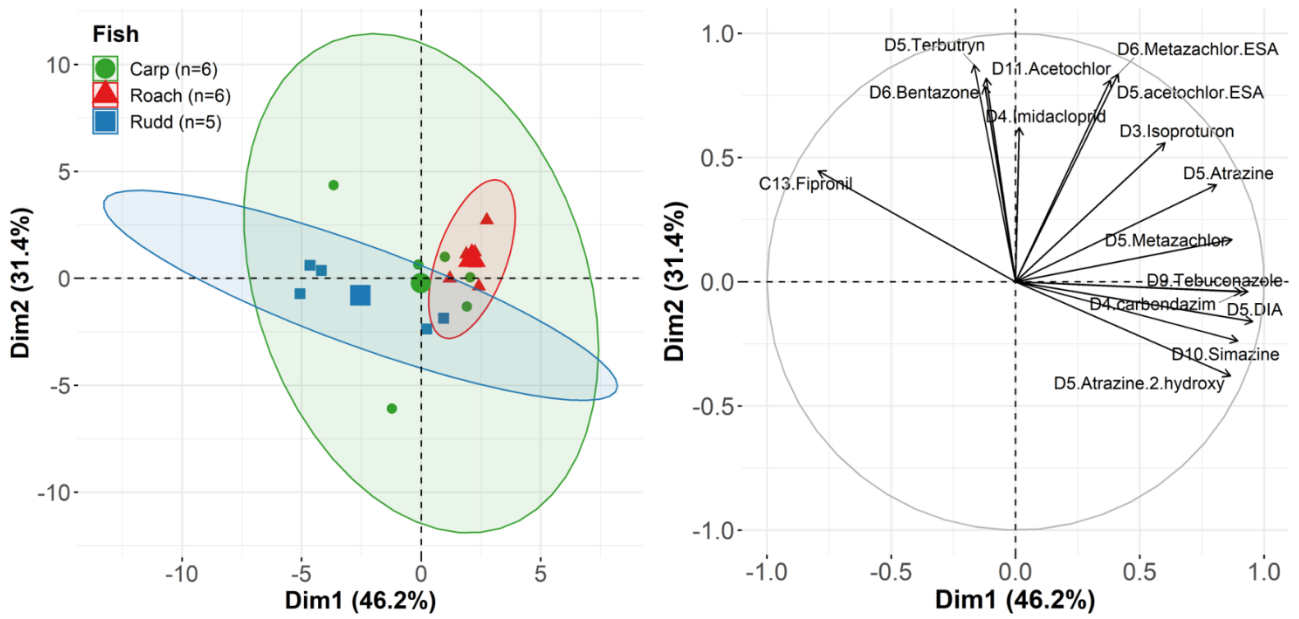


501

502 Fig. 3. Quantification recoveries (%) and limits of quantification (LOQs) ( $\text{ng}\cdot\text{g}^{-1}\text{ dw}$ ) obtained for  
503 macroinvertebrates spiked at C4 (n=6); see Table 1 for the molecules associated with the numbers

504





506

507 Fig. 4. Representation of isotope-labeled standard area ratios between reference and field samples for fish and  
 508 macroinvertebrates; PCA based on Pearson regression ( $\alpha = 0.05$ )

509 (USE COLORS FOR THIS FIGURE)

510

511 Table 1. Analytical performance of the validated method in fish

n°	Chemicals	Domain (ng.g <sup>-1</sup> dw)	r <sup>2</sup> ± %RSD (n = 5)	LOQ (ng.g <sup>-1</sup> dw)	Matrix effect ± SD (%)	Recovery ± %RSD (%)			Inter-day precision (%RSD)			Blanks (n = 5)	
						C2 (n = 6)	C4 (n = 3)	C6 (n = 6)	C2 (n = 5)	C4 (n = 5)	C6 (n = 5)	Min - Max (ng)	QF
<b>Fungicides</b>													
1	Boscalid	60 - 1200	0.996 (4)	60	-6 ± 12	80 ± 15	87 ± 4	93 ± 8	27	18	23	5.1 - 5.1	0.2
2	Tebuconazole	3 - 60	0.993 (2)	3	-18 ± 15	69 ± 20	98 ± 17	89 ± 8	26	15	24	-	-
<b>Herbicides</b>													
3	Acetochlore	60 - 1200	0.995 (4)	60	-2 ± 14	76 ± 12	77 ± 2	80 ± 4	27	20	22	-	-
4	Alachlor	30 - 600	0.995 (3)	30	-10 ± 12	77 ± 9	79 ± 6	79 ± 7	24	19	22	-	-
5	Atrazine	3 - 60	0.996 (2)	3	-31 ± 12	74 ± 13	82 ± 5	86 ± 7	22	18	20	-	-
6	Bentazon	3 - 60	0.992 (8)	3	-8 ± 16	69 ± 33	88 ± 6	88 ± 3	22	17	18	0.1 - 0.1	0.2
7	Chlorotoluron	6 - 120	0.988 (14)	6	-20 ± 16	83 ± 19	86 ± 10	92 ± 7	20	21	62	-	-
8	Dimethachlor	6 - 120	0.996 (2)	6	-14 ± 17	78 ± 22	89 ± 5	89 ± 3	27	21	21	-	-
9	Dimethenamide	6 - 120	0.995 (1)	6	-20 ± 14	76 ± 25	86 ± 5	88 ± 10	22	20	22	-	-
10	Flufenacet	6 - 120	0.996 (4)	6	-6 ± 17	74 ± 18	85 ± 6	91 ± 5	27	18	21	-	-
11	Isoproturon	3 - 60	0.993 (4)	3	-23 ± 15	77 ± 18	92 ± 9	93 ± 7	22	18	19	0.1	0.2
12	MCPA	30 - 600	0.993 (4)	30	16 ± 9	72 ± 29	89 ± 1	86 ± 7	26	21	21	-	-
13	Metazachlor	3 - 60	0.992 (5)	3	-10 ± 16	83 ± 19	90 ± 10	92 ± 9	25	18	20	-	-
14	Metolachlor	3 - 60	0.997 (3)	3	-6 ± 12	88 ± 8	82 ± 12	83 ± 4	26	18	19	0.1 - 0.2	1.0
15	Metsulfuron-methyl	30 - 600	0.994 (5)	30	48 ± 29	71 ± 31	101 ± 7	106 ± 7	27	21	21	-	-
16	Prosulfocarb	30 - 600	0.99 (6)	30	-57 ± 16	102 ± 63	36 ± 11	45 ± 13	23	24	23	0 - 8.8	0.6
17	Terbuthylazine	3 - 60	0.994 (4)	3	-41 ± 23	84 ± 24	67 ± 16	74 ± 7	19	17	21	-	-
18	Terbutryn	3 - 60	0.989 (7)	3	-43 ± 7	86 ± 26	63 ± 10	66 ± 6	26	16	20	0.1 - 0.2	1.0
<b>Insecticides</b>													
19	Fenthion	20 - 400	0.95 (76)	80	-77 ± 21	87 ± 33	82 ± 8	121 ± 88	62	59	60	-	-
20	Fipronil	3 - 60	0.997 (2)	3	-13 ± 8	74 ± 22	85 ± 5	90 ± 6	23	16	19	0.1	0.6
21	Imidacloprid	30 - 600	0.993 (8)	30	19 ± 11	101 ± 70	94 ± 6	94 ± 4	27	19	23	-	-

Transformation products													
22	Acetochlor OXA	30 - 600	0.987 (10)	30	27 ± 12	70 ± 24	76 ± 10	78 ± 12	27	19	24	-	-
23	Alachlor-Acetochlor ESA	30 - 600	0.993 (5)	30	33 ± 11	77 ± 17	69 ± 5	76 ± 7	34	18	22	0.5 - 0.5	0.2
24	Atrazine-2-hydroxy	3 - 60	0.996 (2)	3	-3 ± 16	68 ± 17	83 ± 8	82 ± 6	26	18	19	-	-
25	Benzamide	30 - 600	0.991 (5)	30	61 ± 93	96 ± 16	105 ± 10	98 ± 10	16	18	22	0.2 - 0.3	0.4
26	CGA 50267	3 - 60	0.995 (4)	3	-10 ± 13	72 ± 25	92 ± 3	91 ± 10	25	19	19	0.2 - 0.2	0.2
27	Desethylterbutylazine	3 - 60	0.995 (3)	3	-18 ± 14	75 ± 18	86 ± 6	89 ± 5	23	14	20	-	-
28	Desethylterbutylazine -OH	50 - 1000	0.994 (4)	50	-4 ± 8	71 ± 20	86 ± 10	77 ± 10	24	23	62	-	-
29	Dimethachlor ESA	30 - 600	0.994 (3)	60	10 ± 12	79 ± 7	67 ± 14	70 ± 15	29	18	22	-	-
30	Dimethenamide ESA	30 - 600	0.99 (9)	30	15 ± 12	87 ± 25	60 ± 8	69 ± 11	33	17	22	-	-
31	Fipronil sulfone	3 - 60	0.99 (10)	3	-43 ± 21	78 ± 14	82 ± 7	87 ± 11	27	15	22	0.1	0.6
32	Flufenacet ESA	6 - 120	0.987 (10)	12	11 ± 15	66 ± 34	64 ± 7	71 ± 12	42	13	24	-	-
33	Flufenacet OXA	30 - 600	0.991 (8)	30	20 ± 16	74 ± 28	76 ± 10	73 ± 8	35	18	23	-	-
34	Imidacloprid-desnitro	3 - 60	0.994 (3)	3	131 ± 108	71 ± 28	89 ± 12	90 ± 6	20	14	20	-	-
35	Isoproturon-desmethyl	6 - 120	0.992 (4)	6	-22 ± 14	77 ± 27	87 ± 9	91 ± 8	31	20	23	-	-
36	Me-Desphenyl-Chloridazon	30 - 600	0.995 (3)	30	-20 ± 12	77 ± 21	83 ± 6	91 ± 5	18	19	21	-	-
37	Metazachlor ESA	30 - 600	0.99 (7)	30	11 ± 10	73 ± 23	68 ± 8	71 ± 15	62	21	22	-	-
38	Metazachlor OXA	30 - 600	0.993 (2)	120	35 ± 35	77 ± 35	62 ± 12	77 ± 10	33	22	28	-	-
39	Metolachlor ESA	30 - 600	0.989 (8)	30	14 ± 9	85 ± 36	88 ± 17	70 ± 12	20	13	19	-	-
40	Metolachlor OXA	30 - 600	0.993 (5)	60	30 ± 15	59 ± 15	97 ± 11	76 ± 7	29	13	20	-	-
41	Trichloropyridinol	60 - 1200	0.991 (4)	480	-7 ± 10	108 ± 30	80 ± 11	82 ± 12	31	22	21	-	-

n°: identification of molecules in Fig. 2 and Fig. 3, **Domain**: range of tested concentrations (ng.g<sup>-1</sup> dw), **dw**: dry weight, **LOQ**: estimated limit of quantification (ng.g<sup>-1</sup> dw), **ME**: matrix effect (%), **ER**: extraction rate (%), **QF**: quantification frequency, **C2 to C6**: tested concentration levels.

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514 Table 2. Pesticide residues quantified in fish and macroinvertebrates in ng.g<sup>-1</sup> dw

Molecule	Fish (n = 17)			Macroinvertebrates (n = 19)		
	QF (%)	Min - Max	Average ± SEM	QF (%)	Min - Max	Average ± SEM
Benzamide	100	42 - 237	116 ± 12	100	62 - 438	174 ± 23
Imidacloprid-desnitro	5	3	-	42	2 - 6	3 ± 1
Fipronil	0	-	-	5	4	-
Imidacloprid	0	-	-	16	12 - 37	21 ± 8
Prosulfocarb	86	30 - 165	67 ± 8	100	15 - 29	21 ± 1
Tebuconazole	0	-	-	11	3 - 8	-
Terbutryn	5	61	-	0	-	-

QF: quantification frequency, **dw**: dry weight, **Min - Max**: minimal and maximal values quantified, **SEM**: standard error of the mean.

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