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Thierno Diallo, Julia Leleu, Julien Parinet, Thierry Guérin, Hélène Thomas,  
Adélaïde Lerebours

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1 Approaches to determine pesticides in marine bivalves

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3 Thierno Diallo<sup>1,2§</sup>, Julia Leleu<sup>1§</sup>, Julien Parinet<sup>1</sup>, Thierry Guérin<sup>3</sup>, Hélène Thomas<sup>2</sup>, Adélaïde  
4 Lerebours<sup>2\*</sup>

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6

7 <sup>1</sup> ANSES, Laboratory for Food Safety, F-94701 Maisons-Alfort, France

8 <sup>2</sup> Littoral Environnement et Sociétés (LIENSs), UMR 7266, CNRS-Université de La Rochelle,  
9 2 rue Olympe de Gouges, F-17042 La Rochelle Cedex 01, France.

10 <sup>3</sup> ANSES, Strategy and Programmes Department, F-94701 Maisons-Alfort, France

11

12

13 §: co-authors

14 \*: **Corresponding author:**

15 Adélaïde Lerebours, phone number: +33 (0)5 46 50 67 83, email address:  
16 adelaiderebours@univ-lr.fr

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24 methods and techniques

25 **Abstract**

26 Due to agricultural runoff, pesticides end up in aquatic ecosystems and some accumulate in  
27 marine bivalves. As filter feeders, bivalves can accumulate high concentration of chemicals in  
28 their tissue representing a potential risk for the health of human and aquatic ecosystems. So  
29 far, most of the studies dealing with pesticides contamination in marine bivalves, for example,  
30 in the French Atlantic and English Channel coasts, have focused on old generation of  
31 pesticides. Only a few investigated the new emerging pesticides partly due to methodological  
32 challenges. A better understanding of the most sensitive and reliable methods is thus essential  
33 for accurately determining a wide variety of environmentally relevant pesticides in marine  
34 bivalves. The review highlighted the use of more environmentally friendly and efficient  
35 materials such as sorbents and the “Quick Easy Cheap Effective Rugged Safe” extraction  
36 procedure to extract pesticides from bivalve matrices, as they appeared to be the most  
37 efficient while being the safest. Moreover, this method combined with high-resolution mass  
38 spectrometry (MS) technique offers promising perspectives by highlighting a wide range of  
39 pesticides including those that are not usually sought. Finally, recent developments in the field  
40 of ultrahigh-performance liquid chromatography coupled to MS, such as two-dimensional  
41 chromatography and ion mobility spectrometry will improve the analysis of pesticides in  
42 complex matrices.

## 43           **Introduction**

44   The contamination of the aquatic environment by pesticides may have deleterious effects on  
45   species of ecological and economic importance such as mussels and oysters [1–4]. Those  
46   organisms filter large volumes of water (30 to 40 ml/min) [5] and can thus efficiently  
47   bioaccumulate various chemicals including pesticides released by agricultural runoff in their  
48   digestive gland [6–9].

49           The agricultural production makes intensive use of pesticides which comprises a wide  
50   variety of molecules to protect crops against weeds (herbicides), fungi (fungicides), or insects  
51   (insecticides) [10]. Those pesticides are divided into classes based on their chemical  
52   properties: organochlorines (OCs), organophosphates (OPs), carbamates (Carb), pyrethroids  
53   (Pyr) and neonicotinoids (Neo) [11]. Organochlorines, organophosphates and carbamates are  
54   of the older generations, and pyrethroids and neonicotinoids of the newer [11–13]. Most of  
55   the studies led in marine bivalves from coastal areas of France (Table S1, Text S1) and other  
56   countries [14,15] mainly focused on old generation of pesticides (OCs, OPs and Carb), and  
57   thus, missed the newer generation of pesticides.

58           OCs are the most frequent pesticides detected in bivalves from the French Atlantic  
59   coast (Table 2), although most of them have been restricted or banned [16]. Indeed, OCs were  
60   detected in bivalves from all sampling sites, highlighting a persistent contamination along the  
61   French Atlantic coast (Figure 1, Table 1) [1,8,17]. However, some of them were detected at  
62   very low concentration and tend to decline over the years. For instance, aldrin concentrations  
63   decreased from 2.71 ng/g dry weight (dw) in the scallops (*Mimachlamys varia*) in March  
64   2014 [1] to values < LOQ of 1.67 ng/g in oysters (*C. gigas*) or < LOD of 0.5 ng/g in mussels  
65   (*M. edulis*) from the Pertuis Charentais littoral area, in February 2019 [18] (Table 2). This  
66   decrease over the years was expected as after being widely used in the 50s–60s, this  
67   insecticide was banned in France, in 1998.

68           The highest level of OC pesticides found in bivalves from the French Atlantic coast  
69 was measured for metolachlor in mussels with concentrations that ranged from 349 to 398  
70 ng/g dw [17]. Those concentrations seemed particularly high since this herbicide of low half-  
71 life was prohibited in France in 2003. However, after its prohibition, metolachlor was  
72 replaced by the active enantiomer S-metolachlor, which is actually the molecule measured by  
73 classical analytical approaches (LC or GCMS). More recently, a study found lower  
74 metolachlor concentrations in oysters and mussels from the Pertuis Charentais. They were  
75 below 0.33 (LOQ) or up to 0.38 ng/g wet weight (ww) in Pacific oysters, and varied between  
76 0.5 (LOD) and 1.67 ng/g ww (LOQ) in blue mussels [18] (Table 2). It would have been  
77 interesting to know the concentration levels of metolachlor metabolites: ESA and OXA  
78 metolachlor, to allow a better understanding of the fate of this pesticide in marine biota.  
79 However, studies on the contamination of the marine environment by pesticide metabolites  
80 are rare. Contamination of bivalves by the OC pesticide acetochlor, came second after  
81 metolachlor with higher concentrations found in mussels from Chausey Island coast (125 ng/g  
82 dw) than in mussels from the Seine estuary (53 ng/g dw) [17] or from the Pertuis seas (< LOD  
83 of 0.5 ng/g) [18]. The concentrations of endosulfan beta and metazachlor in mussels from the  
84 Seine estuary were lower and reached 15.2 and 24 ng/g dw respectively [17]. A few years  
85 later, the endosulfan beta and metazachlor concentrations in mussels from the Pertuis seas,  
86 were even lower, < LOD of 1 and 0.5 ng/g ww, respectively [18] (Table 2). Therefore,  
87 bivalves from the Chausey archipelago were more contaminated with OCs pesticides than  
88 those from the Seine estuary and the Pertuis seas. Other OCs, such as lindane (banned in  
89 1998) and DDT (banned in 2010), which are highly persistent pollutants in the environment,  
90 were monitored in previous studies. Lindane concentrations found in mussels and oysters  
91 ranged from 0.2 to 2.2 ng/g dw in the Pertuis seas region [8,18] (Table 2). The concentrations  
92 of 2,4- and 4,4-DDT are now very low, in the range of 0.4 - 0.9 ng/g dw [8,18] in bivalves

93 from the Gironde estuary and the Pertuis seas. However, significant accumulation of its  
94 metabolites were found in oysters collected in 2008 in the same region, with concentrations  
95 reaching 3.7 and 5.5 ng/g dw of 4,4'- and 2,4'- DDE, respectively [8].

96 Another class of widely studied pesticides are the OPs such as ethoprophos (31 ng/g)  
97 and tributyl phosphate (14 ng/g) which were quantified in bivalves from the Vendée region  
98 [19] (Table 2). Despite their significant accumulation in marine bivalves and their authorized  
99 status, there is only one recent study available in the literature on those two molecules  
100 regarding the French Atlantic coast. A recent study reported much lower concentrations of  
101 parathion (between 0.56 and 0.69 ng/g) banned in 2001, chlorpyrifos (between 0.79 and 0.93  
102 ng/g) banned in 2020, and chlorfenvinphos (between 1.65 and 2.12 ng/g) banned in 2007, in  
103 the Pacific oysters sampled in the Pertuis Charentais littoral area [18] (Table 2). As banned  
104 molecules found today at very low concentrations, those pesticides represent a minor concern  
105 for marine bivalves and human health.

106 Interestingly, more recent pesticides such as bifenthrin (48 ng/g), cypermethrin (10.9  
107 ng/g) and deltamethrin (11.2 ng/g) were quantified for the first time in bivalves from the  
108 French Atlantic coast in 2015 by Menet-Nedelec et al. (Table 2) [17]. Other emerging  
109 pesticides such as neonicotinoids could not be identified due to their low concentrations in  
110 marine bivalves from the French Atlantic and English Channel coasts [17]. However, their  
111 toxicity has been evidenced in bivalves exposed under laboratory conditions [20]. Altogether,  
112 those results highlighted the presence of old generation of pesticides in marine biota and  
113 significant concentrations of their metabolites, but data on the new generation of pesticides  
114 such as pyrethroids and neonicotinoids are lacking. Those new molecules deserve to be  
115 monitored further. Indeed, contrary to the old generation of pesticides, most of the new  
116 generation of pesticides are currently authorized. Secondly, by considering only old  
117 pesticides, studies underestimate the contamination by the environmentally realistic “cocktail”

118 of pesticides that includes the new generation of pesticides. Actually, quantifying those  
119 molecules at environmental concentrations remains a methodological and technical challenge.  
120 Therefore, this underlines the need to apply the most efficient procedures to accurately  
121 determine the wide variety of environmentally relevant pesticides that accumulate in marine  
122 bivalves.

123 The present review firstly compares the latest methods used for tissue preparation and  
124 extraction, and to clean-up samples. Advices to overcome the matrix effect specific to bivalve  
125 tissues are also provided. Finally, the review discusses the most relevant and promising  
126 analytical techniques to use and highlights the good validation procedures to apply.

## 127 **Methodologies**

### 128 *Sample pretreatment*

129 In most studies, samples were homogenized, freeze-dried or lyophilized and stored at -20°C  
130 before proceeding to the next steps (Table 1). Research focused on whole individuals or  
131 specific tissues such as the digestive gland, a key tissue to assess chemical contaminant in  
132 bivalves [7]. Then, due to low concentrations in bivalves, effective extraction procedures are  
133 used to isolate and concentrate pesticides (Table 1).

### 134 *Extraction of pesticides from bivalve samples*

135 The Matrix Solid-Phase Dispersion (MSPD) refers to one of the older extraction procedures  
136 that is less used nowadays, with 9.1% of the studies employing it since 2015 (Table 1),  
137 although it is a suitable sample preparation technique for solid and semi-solid biological  
138 samples. Carro *et al.*, (2005) was evaluated MSPD procedure using an asymmetrical  
139 experimental design [21]. The authors tested and validated the optimal condition using  
140 multispecies biological reference material from the International Atomic Energy Agency  
141 (IAEA-406), with adequate precision and accuracy. The validated procedure was applied to  
142 the analysis of several samples of aquaculture feed and marine species. MSPD consists in

143 mixing a sample with a solid support (usually a silica-based material), to obtain an  
144 homogeneous mixture [22]. The resulting mixture is placed in a solid-phase extraction (SPE)  
145 column or cartridge, followed by elution with suitable organic solvents (with different elution  
146 powers and polarities) and collection of the analytes by fractionation. The advantages of this  
147 method include the consumption of small amounts of sample and solvents, simple application,  
148 low cost and simultaneous extraction and clean-up in one step [21,22]. However, the MSPD is  
149 fairly labor intensive, requiring the sample to be ground up with the solid matrix and packed  
150 into a column for extraction since automation cannot be done [22]. The Pressurized Liquid  
151 Extraction (PLE), also known as the Accelerated Solvent Extraction (ASE), and the Selective  
152 Pressurized Liquid Extraction (SPLE) were used to extract pesticides in 27.3% of the studies  
153 (Tables 1 and 2) [8,23]. PLE is an instrument-based extraction procedure that extracts  
154 analytes (including POPs, pesticides or pharmaceuticals) from solid and semi-solid  
155 matrices at high temperature (40–200°C) and pressure (500–3000 psi) [24–26]. High  
156 temperatures enhance the solubility of non-thermolabile analytes [27]. The advantages of PLE  
157 are: less solvent used, time per sample reduced and processes fully automated. However, the  
158 disadvantage is the presence of coextracted non-targeted interfering compounds - such as  
159 proteins or lipids and the degradation of thermolabile analytes. Thus, the PLE  
160 technique requires a clean-up before quantification [28]. Selective PLE (SPLE) (or enhanced  
161 PLE, in-cell clean-up, in-situ clean-up) combines the extraction and clean-up  
162 processes [27]. Solid-phase adsorbents can be layered in the PLE cell to avoid interference.  
163 They produce clean extracts which, in most cases, are directly injected in the analytical  
164 system. Thus, no post-extraction clean-up is required. Adsorbents - for in-cell clean-up PLE -  
165 are commonly used to extract non-polar analytes, such as OCs in animal tissues. In this  
166 regard, PLE utilises a layer of fat-removal adsorbents such as Florisil, alumina (basic, acidic  
167 or neutral), silica gel and sulphuric acid-silica gel [29]. Another extraction procedure that has



168 gained considerable importance in the recent years because of its "greener" (it uses less  
169 solvent) and more efficient extraction capacity is microwave-assisted extraction (MAE)  
170 [30,31]. This approach simply involves placing the sample with the solvent in specialized  
171 vessels and heating the solvent using microwave energy. MAE has many advantages, such as  
172 reduced extraction time and solvent consumption, as well as the ability to analyse multiple  
173 samples [32]. However, the need for special equipment, the low selectivity and the  
174 unavoidable high temperature reaction are considered as disadvantages of MAE [30]. Since  
175 2003, an alternative extraction procedure, Quick, Easy, Cheap, Effective, Rugged and Safe  
176 (QuEChERS) was introduced by Anastassiades *et al.*, (2003) [33] but the first publication on  
177 the extraction of pesticides from bivalves using the QuEChERS procedure appeared in 2016  
178 [34]. In the QuEChERS procedure, the most commonly used solvent is acetonitrile. The use  
179 of this solvent allows the extraction of a wide polarity (non-polar and medium polar) of  
180 pesticides [24]. Indeed, acetonitrile leads to much less interferences from compounds that can  
181 be co-extracted such as lipophilic compounds, lipids and pigments than other extraction  
182 solvents such as acetone and ethyl acetate [33]. The solid sample is usually ground and  
183 homogenized using an Ultra-Turrax homogenizer [35]. The  $MgSO_4$  and  $NaCl$  are the original  
184 salts used for liquid-liquid phase separation by binding to water [33].  $MgSO_4$ , as well as  
185  $Na_2SO_4$ , has a good water absorption and allows the release of acetonitrile (the most  
186 commonly used extraction solvent), while  $NaCl$  affects the ionic force of the extraction  
187 solvents and, therefore, increases the selectivity of the extraction [24]. To avoid ionization or  
188 degradation of pesticides due to pH changes during extraction, two modifications of the  
189 original QuEChERS procedure were developed: (1) AOAC Method (acetate buffer) [36] and  
190 (2) EN Method 15662 (citrate buffer) [37]. Both methods stabilize the pH at 5–5.5, which  
191 facilitates the extraction of pesticides sensitive to low pH or those with stability problems.  
192 This procedure is currently replacing those traditional time-consuming extractions, requiring

193 larger amount of toxic reagents, organic solvents and samples. Because the QuEChERS  
194 method allows a fast, reliable extraction and uses less toxic solvents, it is a better method than  
195 MAE, PLE or MSPD. However, the number of studies using the QuEChERS procedure in  
196 bivalves is still low: 18 against 353 studies investigating other matrices in 2020. In the  
197 principles of green analytical chemistry, other extraction techniques such as magnetic solid  
198 phase extraction (MSPE) and stir bar sorptive extraction (SBSE) should be considered. MSPE  
199 is based on the addition of magnetic nanoparticles such as C18 functionalized to the sample  
200 solution for preconcentration of moderate and non-polar pesticides [38]. In addition to being  
201 environmentally friendly, MSPE is a technique that has the advantage of using a limited  
202 number of steps (limiting cross-contaminations), a small amount of sorbent, as well as the  
203 reuse of sorbent [39]. However, MSPE procedure requires vortex, shaker, magnetic stirrer and  
204 selection of suitable sorbents [39]. In SBSE, a stir bar coated with polydimethylsiloxane  
205 (PDMS) phase is added to a vial containing the sample. SBSE consists of two steps:  
206 extraction and desorption. An aqueous sample is extracted by stirring for a predetermined  
207 time with a PDMS-coated stir-bar. The stir-bar is subsequently removed from the aqueous  
208 sample and the absorbed compounds are then thermally desorbed and analysed by GCMS  
209 [40]. The advantage of the SBSE is its simplicity, robustness, cost effectiveness and  
210 environmental friendliness [39]. However, its important limitation is only commercial  
211 availability of PDMS coating material exists at present. Besides, due to the non-polar  
212 character of PDMS, SBSE technique is mainly applied to extract non-polar and weakly polar  
213 compounds (logarithm of octanol-water partitioning coefficient lower than 3) but failed in the  
214 extraction of strongly polar compounds [41]. In addition, SBSE procedure can only be applied  
215 to medium-high volatility and medium-high thermo-stability analytes if a thermal desorption  
216 has to be employed [42].

218 Bivalves contain lipids (between 2 and 10%) and proteins (between 10 and 18%) that can  
219 affect the analysis of target compounds, especially at low concentrations [35]. Matrix effect is  
220 caused by interactions (via van der Waals, dipolar-dipolar or electrostatic forces) between  
221 pesticides and co-extractives that could suppress or enhance the ionisation of the analytes  
222 [43]. This may result in a higher or lower signal, which affects the accuracy of the  
223 quantitative results. Thus, this matrix effect needs to be reduced to avoid bias in the overall  
224 analysis. To overcome this challenge, several interesting approaches have been used and  
225 deserved attention to minimise the matrix components such as using internal standards,  
226 improving the chromatography separation (reducing coelution with matrix components),  
227 making serial dilution, or performing extensive sample clean-up [44,45], as follows. For  
228 clean-up of sample extracts, the SPE method, previously mentioned for extracting pesticides,  
229 can be used [46]. In SPE method, the sorbents are chosen according to their properties  
230 (affinity, capacity and selectivity) towards the analytes of interest and matrix interferences [46].  
231 The most popular clean-up strategy to avoid interference involves the SPE with a reverse-  
232 phase octadecylsilane (C18) for isolation and preconcentration of nonpolar compounds [47]  
233 whereas silica-SPE retains polar compounds in the sample [8,48,49]. The primary secondary  
234 amine (PSA) is used to remove interferences such as fatty acids, organic acids, pigments,  
235 sugars and metal ions [34,50]. This SPE method offers the best compromise between time,  
236 difficulty and performances. Other clean-up procedures are less frequently used and are more  
237 complicated to set up, such as those using adsorption columns [8,51] and gel permeation  
238 chromatography [48]. The dispersive SPE (dSPE) and cartridge SPE (cSPE) methods can also  
239 be used for the clean-up. They are much more rapid, easy to set up and efficient. dSPE is  
240 based on the dispersion of a sorbent in the liquid samples [44,52]. After centrifugation, the  
241 sorbent separates the analytes from the matrix, eliminating interfering compounds and

242 reducing wastewater [53,54]. dSPE provides faster, simpler and more adaptable clean-up than  
243 traditional techniques and has been widely applied [45,47,50,53,55], however the  
244 centrifugation step may lengthen the procedure. Moreover, during sorbent adsorption or  
245 concentration, pesticides might be lost. In the cSPE procedure, the analytes are eluted from  
246 the cartridge and interfering compounds are eluted before the analytes or remained on the  
247 cartridge [54]. Compared to dSPE, the cSPE is time-consuming, adding experimental steps  
248 such as cartridge conditioning, sample loading, interference elution and analyte elution [54].  
249 Therefore, the dSPE associated to the QuEChERS method appeared to be the most reliable  
250 and efficient, although cSPE is automatable and remains the most used procedure in the  
251 routine laboratory. Indeed, this combination presents the best compromise between cost, time,  
252 and easiness to use, especially if looking after a large set of pesticides potentially present in  
253 marine bivalves. As with the SPE method, the most commonly used dSPE sorbents for extract  
254 clean-up in bivalves are C18 (especially for fat removal) and PSA, often in the presence of  
255  $MgSO_4$ . The latter is commonly used as a drying salt for acetonitrile extraction as it  
256 effectively removes residual water before injection into chromatographic systems. The  
257 zirconia based sorbent, Z-Sep is based on zirconium oxide and Z-Sep/C18 is composed of  
258 zirconium oxide and C18. This mixture reduces fat and pigment levels in matrices and it is  
259 recommended for matrices containing more than 18% of lipid [56]. In 2015, Agilent  
260 Technology introduced for the first time Enhanced Matrix Removal of lipids (EMR-lipid).  
261 Unlike other d-SPE sorbents, EMR-lipid is a novel sorbent designed specifically for lipid  
262 removal. The composition of EMR-Lipid is a proprietary secret, and it does not function as a  
263 conventional sorbent, but it dissolves to saturation the sample extract solution, and it selects  
264 compounds according to their size and hydrophobic properties [57]. More recently, novel  
265 materials based on the principle of green chemistry are being used as sorbents. Despite their  
266 effectiveness, these sorbents are not always commercially available, so they have to be

267 synthesized in the laboratory. These include molecularly imprinted polymers (MIPs), metal-  
268 organic frameworks (MOFs) and carbon-based sorbents. The principle of MIPs is based on  
269 the affinity of a polymer with specific cavities for chosen template molecules. Therefore,  
270 MIPs are highly selective for those target molecules or structural analogs. The target  
271 molecules are recognized via hydrophobic interactions, hydrogen bonding, van der Waals  
272 forces, or electrostatic interactions [58]. The MIPs sorbent was also used for pigment removal  
273 [59]. This sorbent, in combination with PSA, demonstrated their effectiveness in clean-up  
274 bivalve extracts, with matrix effects within the range of  $\pm 20\%$  for 100% of the herbicides  
275 analysed [59]. The MOFs as sorbents are based on three-dimensional structures of  
276 coordination networks consisting of a metal ion bound to organic ligands [60]. The main  
277 advantage of MOFs is their large specific surface area, enormous porosity and the possibility  
278 to easily adjust their pore size and shape from microporous to mesoporous scale, which can  
279 lead to high extraction efficiency [61]. In addition, the high capacity to functionalize the pores  
280 with functional groups such as  $-\text{NH}_2$ ,  $-\text{CO}$ ,  $-\text{CHO}$ ,  $-\text{COOH}$  and  $-\text{OH}$ , make them good  
281 candidates for adsorption of interferents through hydrophilic or hydrophobic interactions [61].  
282 However, the main limitations of MOFs is their low selectivity and stability in water [62].  
283 Moreover, the limited availability of MOF-based adsorbents prevents the widespread  
284 application of MOFs in the analysis and preconcentration of pesticides. The main reason is  
285 that only a few chemical companies (e.g. the German chemical company BASF) have started  
286 to produce MOFs on a commercial scale [63]. Furthermore, not all research laboratories have  
287 the capacity to produce MOFs. Finally, carbon-based materials (e.g., activated carbon, carbon  
288 nanotube and graphene) are used as sorbents due to their large surface area, high porosity and  
289 surface reactivity, and they can be easily functionalized [64,65].

## 290 **Matrix effects and challenge to overcome them**

291 Although matrix effects are not always considered among the performance parameters for  
292 method validation, they must be considered as they can influence the experimental results.  
293 Despite the clean-up of extracts and LC or GC separation step, it is impossible to eliminate all  
294 matrix effects. Matrix effects generally lead to signal suppression in LC-MS/MS applications.  
295 This is the case in the study by Álvarez-Muñoz *et al.*, (2019) where the authors noted ion  
296 suppression after extraction, purification and UHPLC-HRMS analysis of 16, 42 and 51% for  
297 mussels, oysters and cockles, respectively [66]. In this work, matrix matched calibration  
298 together with isotopically labelled internal standards were performed to minimise interference  
299 and to avoid underestimation during quantification of targeted compounds. In contrast to LC-  
300 MS/MS, matrix effects in GC-MS/MS mainly result in signal enhancement. Using GC-  
301 MS/MS, Yu *et al.* achieved less than 20% of matrix effects for the 14 herbicides quantified in  
302 mussels, scallops, and cockles after purification of extracts using PSA and MIPs as dSPE  
303 sorbents [59]. In the study performed by Cruzeiro *et al.*, (2016), an average signal suppression  
304 of 89% was obtained for 80% of the studied pesticides [34]. To overcome matrix effects, the  
305 authors evaluated the addition of two analyte protectors such as 3-ethoxy-1,2-propanediol and  
306 D-sorbitol before injection. Both proved to enhance the signal by more than 50% for all the  
307 selected compounds.

## 308 **Detection and identification techniques**

309 Several analytical techniques enable the determination of contaminants in seafood samples  
310 (Table 1). Liquid (LC) or gas chromatography (GC) separation method with tandem mass  
311 spectrometry (MS/MS) detection are suitable confirmatory techniques for organic residues  
312 and contaminants in foodstuffs [67]. The majority of the studies used GC or LC, coupled with  
313 MS/MS, to determine organic compounds in bivalves (Table 2). GC-ECD (electron capture

314 detection) comes second (Table 2) [51,68]. Unlike MS, which is a universal detector, ECD is  
315 a specific detector for halogenated organic molecules such as organochlorine (OC) pesticides  
316 [8,51]. Thus, GC-ECD can only detect halogenated pesticides, whereas MS can detect all  
317 types of pesticides provided they are ionised. In addition, GC allows a greater selectivity and  
318 resolution than LC in determining contaminants in complex matrices, such as farmed fish and  
319 shellfish [46]. Ziarrusta *et al.*, (2015) implemented GC, coupled to triple quadrupole MS  
320 (GC–MS/MS), to determine pesticides in farmed mollusk bivalves representing complex  
321 matrices such as blue mussels, mangrove oysters, radial purse oysters or ark clams [49]. The  
322 study reported lower limits of detection (LODs) and quantification (LOQs) using GC–MS/MS  
323 than GC-MS (49). Other analytical configurations include ultra-high-performance LC  
324 (UHPLC) (Table 1), which uses particles of smaller diameter (around 1.7  $\mu\text{m}$ ) in the  
325 stationary phase and short columns [59]. High pressures induce narrow LC peaks (5–10 s)  
326 with shorter analysis [23,50,69]. Compared to the HPLC-based methods, UHPLC uses less  
327 solvent in general. Furthermore, coupled with MS, UHPLC triggers greater throughput and  
328 improves resolution [46]. The UHPLC method separation as well as the previously described  
329 HPLC and GC coupled to triple quadrupole MS operating in multiple reaction monitoring  
330 (MRM) mode have two major advantages: selectivity and sensitivity. However, the use of  
331 triple quadrupole instruments has proven to have some limitations, such as a limited number  
332 of compounds targeted during the analysis [70,71]. For large screening purposes, an  
333 alternative to MS/MS is the application of full scan techniques based on high-resolution mass  
334 spectrometry (HRMS), using Orbitrap MS-based instruments and time-of-flight (ToF) MS  
335 systems [72]. This analytical technique can perform simultaneous target, suspect, and non-  
336 target downstream data analyses to increase the number of analytes that can be included in  
337 multi-residue methods. Moreover, it allows retrospective analysis by reprocessing acquired  
338 data (as new knowledge is gained) and the ability to use samples in multiple studies without

339 having to reinject them [73]. Despite the advantages described, only two studies have used  
340 HRMS for the determination of pesticides in bivalves. One performed target analysis of 13  
341 pesticides in mussels, oysters, cockles and razor shell [66]. More recently, Diallo *et al.*,  
342 (2022) carried out a simultaneous targeted analysis of 204 pesticides in seven different  
343 commercial bivalve species and a suspect screening analysis on real samples of oysters and  
344 mussels collected in south-west France [35]. However, studies using HRMS for “non-target”  
345 pesticide analysis in bivalve tissues are still scarce despite offering promising perspectives for  
346 detecting a wide range of pesticides in marine bivalves. This is especially relevant to  
347 characterize an environmentally realistic exposure to a cocktail of contaminants. Furthermore,  
348 the performance of HRMS systems, including automatic data processing, has improved  
349 sufficiently to make their implementation in routine analysis a realistic option, particularly  
350 when used in conjunction with conventional GC and LC-MS/MS methods. Moreover, the  
351 sensitivity of recent HRMS systems has now increased. It should be noted that the objective  
352 of HRMS is not to replace conventional methods but to perform a diagnosis for the presence  
353 of unexpected contaminants in the sample, which can then be confirmed by LC-MS/MS. This  
354 could have prevented recent food safety crises such as the presence of fipronil (which was not  
355 expected) in European eggs in summer 2017 [74].

356 Along with the advent of HRMS, there have been several developments in the field of  
357 UHPLC-MS in recent years. The most important of these are two-dimensional  
358 chromatography (2D-GC or 2D-LC) and ion mobility spectrometry (IMS). The 2D  
359 chromatography use both the comprehensive (GC) and heart cutting (LC) approaches [75,76].  
360 In the comprehensive approach, the entire effluent from the first column is transferred to the  
361 second one. The heart cutting technique involves selecting a fraction of the co-eluting zone  
362 from the first column in a sample and re-inject it to the second column for separation and  
363 detection in the MS instrument. Compared to 2D-LC, 2D-GC is more frequently used for the



364 determination of a wide mixture of pesticides [77–79]. The advantages over conventional  
365 chromatography are increased peak capacity, generation of unique structured chromatograms,  
366 better resolution of thousands of peaks in a chromatographic run and the possibility to reduce  
367 matrix interference [79]. The HRMS technique also offers the possibility of performing non-  
368 targeted analyses of various compounds in complex matrices [79]. As an alternative to 2D  
369 chromatography, IMS can be considered for the analysis of very complex mixtures. In IMS,  
370 ions are transported and separated according to their collisions with a neutral drift gas, such as  
371 helium or nitrogen, allowing the separation of ions according to their three-dimensional  
372 structure [80]. By coupling chromatographic separations, ion mobility separations (IMS) and  
373 HRMS, the overall peak capacity of the system is increased and allows for the separation of  
374 compounds (isomeric and isobaric) that would otherwise be detected as a single component in  
375 a system without IMS [81]. One difference between IMS and 2D chromatography is that  
376 when the latter is used, the user can optimize the peak capacity of the method by selecting the  
377 appropriate separation selectivity of the two separation dimensions involved. When IMS is  
378 used, the user loses this flexibility as drift times or derived collision cross section (CCS) are  
379 compound dependent and relative drift times can only be altered to a certain degree by  
380 modifying the drift gas composition. UHPLC coupled with travelling-wave (UHPLC-  
381 TWIMS-QTOF-MS) was successfully used by Regueiro *et al.*, (2017), for qualitative  
382 screening of 156 pesticides in salmon feed [82]. The authors found that the use of the CCS as  
383 an additional identification point greatly improved the method selectivity while decreasing the  
384 number of false positive detections (from 42 to only 1).

### 385 **Method validation**

386 To ensure the reliability of analytical information, a validation protocol must be  
387 followed in every study. This protocol evaluates precision, trueness, specificity, recovery,  
388 LOD and LOQ and sensitivity as established by the European Commission in 2021

389 (SANTE/11312/2021) [83]. The precision is actually evaluated through the repeatability and  
390 reproducibility of the analytical response by estimation of the relative standard deviation  
391 ( $RSD \leq 20\%$ ) for each spike level tested [83]. The trueness is expressed in terms of bias,  
392 which is the average recovery (70-120%) for each spike level tested. The recovery is  
393 expressed in percentage and represents the proportion of analyte remaining at the point of the  
394 final determination. The specificity is the ability of the detector to provide signals that  
395 effectively identify the analyte [83].

396 A satisfactory validation protocol in marine bivalves was applied by Ziarrusta *et al.*,  
397 (2015) using the SANCO/10684/2009 guideline (one of the former SANTE/11312/2021  
398 guideline) [49]. The authors assessed the repeatability by preparing four replicates of mussels  
399 at 50 ng/g and analysing them in the same day. Then, for reproducibility, eight replicates were  
400 analysed during three different days. The RSD values varied from 1% to 17% for  
401 repeatability, and from 4% to 33% for reproducibility. The LOD and LOQ values of several  
402 organophosphorus and organochlorine pesticides ranged from 0.34 to 13 ng/g and 0.83 to 29  
403 ng/g respectively [49]. These values were acceptable and comparable to the values found in  
404 other studies that focused on the MSPD extraction of trace organic pollutants [4,84–86]. To  
405 evaluate the accuracy and precision, Rodrigues *et al.* used two different concentrations,  $3 \times$   
406 MQL (method quantification limit) and  $10 \times$  MQL (European Commission 2002/657/CE)  
407 [23]. Another reliable method to evaluate accuracy and precision was used by Cruzeiro *et al.*,  
408 (2016) who made three different pools of bivalve homogenate and injected them at three  
409 concentrations: LOQ,  $2 \times$  LOQ, and  $10 \times$  LOQ (SANCO/825/00 rev 8.1) [34].

#### 410 **Recommendations and future perspectives**

411 A large variety of pesticides can bioaccumulate in bivalves potentially affecting their survival  
412 and human health. It is thus essential to refine the methods and techniques used to accurately

413 characterize the contamination of bivalves by organic chemicals to sustain a good ecological  
414 status.

415 Recommendations for sample preparation are the use of QuEChERS using MgSO<sub>4</sub> or  
416 Na<sub>2</sub>SO<sub>4</sub> and enhanced by AOAC or EN method, innovative techniques respecting the  
417 principles of green analytical chemistry, such as SBSE and MSPE and the use of new  
418 materials as sorbents. Finally, we recommend dSPE and analytical protectors to reduce matrix  
419 effect.

420 Recommendations for detecting of a wide range of pesticides and their residues in  
421 bivalves are the use of LC/GC-HRMS and UHPLC-MS followed by 2D-GC, 2D-LC or IMS.  
422 Specifically, HRMS can perform a diagnosis for the presence of unexpected contaminants in  
423 the sample, which can then be confirmed by GC or LC-MS/MS.

424 Those approaches will allow a more rigorous characterisation of the real cocktail of  
425 contaminants existing in the coastal environment.

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#### 433 **Compliance with ethical standards**

434 The author declares that there is no conflict of interest.

#### 435 436 **Table and Figure Legend.**

437 **Table 1.** Analytical methods used to evaluate pesticide concentrations in bivalve species.

438 **Table 2.** Minimum (min), mean, maximum (max), and pesticide concentrations in ng/g dw  
439 (dry weight), quantified in bivalves (collected at least at one location) collected along the  
440 French Atlantic coast. The class of pesticides, the year of sampling and the number of samples  
441 are presented. \*ww (wet weight)

442 **Figure 1.** Map indicating the location of the sampling sites along the French Atlantic and  
443 English Channel coasts (source: CAPENA).

444  
445

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801 Table 1.

Species	Sample	Pretreatment	Sample mass (g,ww)	Extraction method	Clean-up	Detection	LODs (ng/g)	LOQs (ng/g)	Recovery (%)	Reference
Peppery furrow shell ( <i>Scrobicularia plana</i> )	-	Homogenized	5	QuEChERS	PSA dSPE	GC-MS/MS (IT) in SIM	0.22-3.4	0.33-10.3	78-119	[34]
Blue mussel ( <i>Mytilus edulis</i> ), Oysters ( <i>Crassostrea rhizophorae</i> and <i>Isognomon radiatus</i> ), Clams ( <i>Polymesoda solida</i> , <i>Anadara tuberculosa</i> and <i>Anadara grandis</i> )	Pool	Homogenized and freeze-dried	0.5	MSPD	Silica SPE	GC(EI)-MS(QqQ) in SRM and SIM	0.34-13	0.83-29	55-117	[49]
						GC(EI)-MS/MS(QqQ) in SRM and SIM	0.010-2.7	0.017-3.9	64-122	
Pacific oyster ( <i>Crassostrea gigas</i> )	Pool	Homogenized and freeze-dried	0.5	ASE, PLE	Acidic silica gel colum	Quantified: GC-ECD Confirmed: GC-MS/MS	0.1-0.2	0.3-0.6	72-116	[8,51]
Mediterranean Mussel ( <i>Mytilus galloprovincialis</i> ), oyster ( <i>Crassostrea gigas</i> ), cockle ( <i>Cerastoderma edule</i> )	Pool	Homogenized and freeze-dried	1.0	QuEChERS	C18 dSPE	UHPLC-QTOF MS/MS	0.01-0.5	0.02-1.00	96-107	[66]

Brown mussel ( <i>Perna perna</i> ), Pacific oyster ( <i>C. gigas</i> )	-	-	-	-		LC-MS/MS	0.01-0.7	-	-	[68]
Mussels ( <i>M. galloprovincialis</i> , <i>M. edulis</i> , <i>Mytilus chilensis</i> and <i>Perna canaliculus</i> ), Clams ( <i>Venerupis philippinarum</i> , <i>Tapes semidecussatus</i> , <i>Meretrix meretrix</i> and <i>Meretrix lyrata</i> )	Pool	Homogenized and stored at -20°C	5	QuEChERS EN method	500mg Z-Sep	GC-MS/MS	-	5	70-120	[47]
Blue mussel ( <i>M. edulis</i> ), Salmon ( <i>Salmo salar</i> )	-	-	-	QuEChERS	GPC followed by silica SPE	GC-HRMS	11-680	-	90-110	[48]
Blue mussel ( <i>M. edulis</i> )		Lyophilised	0.5 - 1	1) SPE acetonitril	Florisil	GC-MS/MS	-	10	-	[19]
	-	-	-	2) SPE dichloromethane/ethyl acetate	cSPE oasis HLB	LC-MS/MS	-	10	-	
	-	-	-	3) SPE dichloromethane/ethyl acetate in acid environment		-	-	-	-	
Blue mussel ( <i>M. edulis</i> ), Pacific oyster ( <i>C. gigas</i> )	Individual	Homogenized	1 - 3	QuEChERS		GC-MS/MS		0.1-0.5	0.33-1.67	[18]

Peppery furrow shell ( <i>S. plana</i> )	Pool	Homogenized and stored at - 20°C	0.5	SPLE	The Oasis <sup>®</sup> on-line columns	UPLC-ESI- MS/MS	3.7-97	11-290	68-125	[23]
Clams ( <i>T. decussatus</i> )	Pool	Homogenized and freeze- dried	3 - 5	MAE	Florisil	GC	-	-	63-98	[31]

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803 Table 2.

Pesticide	Class	CAS Number	Species	Concentration (ng/g, dw)			Sites	Reference	Year	Sample number
				Min	Mean	Max				
Acetochlor	OC	34256-82-1	Mediterranean mussel ( <i>M. galloprovincialis</i> )		125		Chausey archipelago	[17]	2015	1
					67.4		Bay of Veys			1
				29.7		53.0	Seine estuary			1
Aldrin	OC	309-00-2	Variegated scallops ( <i>Mimachlamys varia</i> )		2.24		Loix en Ré	[1]	2014	1
					2.71		Les Minimes			1
				<LOD		<LOQ	Les Palles			1
			Mediterranean mussel ( <i>M. galloprovincialis</i> )		1.8		Chausey archipelago	[17]	2015	1
					3.3		Bay of Veys			1
				1.2		4.7	Seine estuary			1
4,4'-DDD	OC	2-54-8	Mediterranean mussel ( <i>M. galloprovincialis</i> )		<0.1		Chausey archipelago	[17]	2015	1
					<0.1		Bay of Veys			1
				0.2		0.3	Seine estuary			1
				1.5	2.3	Bouin	[8]	2008	2	
				1.9	2.8	Boyard			2	
	2.0	3.7	Les Palles	2						
		1.0	1.9	Mus du loup			2			
2,4'-DDE	OC	3424-82-6	Mediterranean mussel ( <i>M. galloprovincialis</i> )		<1		Chausey archipelago	[17]	2015	1
					<1		Bay of Veys			1
				<1		2.4	Seine estuary			1
			Pacific oyster ( <i>C.</i> )	2.2	2.5		Bouin	[8]	2008	2

			<i>gigas</i>	2.4	5.5	Boyard			2
				1.5	2.4	Les Palles			2
				nd	0.3	Cordouan			2
4,4'DDE + Dieldrin	-	-	Pacific oyster ( <i>C. gigas</i> )	1.2	1.4	Bouin			2
				3.6	3.8	Boyard			2
				2.4	3.7	Les Palles	[8]	2008	2
				0.8	0.9	Mus du loup			2
				1.9	3.0	Cordouan			2
4,4'-DDT	50-29-3		Mediterranean mussel ( <i>M. galloprovincialis</i> )		0.2	Chausey archipelago			1
				<0.1		Bay of Veys	[17]	2015	1
				2.5	2.9	Seine estuary			1
Dieldrin	OC	60-57-1	Mediterranean mussel ( <i>M. galloprovincialis</i> )		6.4	Loix en Ré			1
					12.4	Les Minimes	[1]	2014	1
					nd	Port-neuf			1
					4.2	Les Palles			1
					<1	Chausey archipelago			
					2.7	Bay of Veys	[17]	2015	1
	2.1	2.3	Seine estuary			1			
alpha-Endosulfan	OC	33213-65-9	Mediterranean mussel ( <i>M. galloprovincialis</i> )	<0.2*	0.27*	Le Martray			11
				<0.2*		Charente estuary	[18]	2019	5
				<0.2*		Seudre estuary			5
beta-Endosulfan	OC	959-98-8	Mediterranean mussel ( <i>M. galloprovincialis</i> )	1.5		Chausey archipelago			1
				1.7		Bay of Veys	[17]	2015	1
				1.1	1.4	Seine estuary			1
beta-Endosulfan	OC	959-98-8	Mediterranean mussel ( <i>M. galloprovincialis</i> )	<1		Chausey archipelago			1
				14		Bay of Veys	[17]	2015	1
				15.2		Seine estuary			1

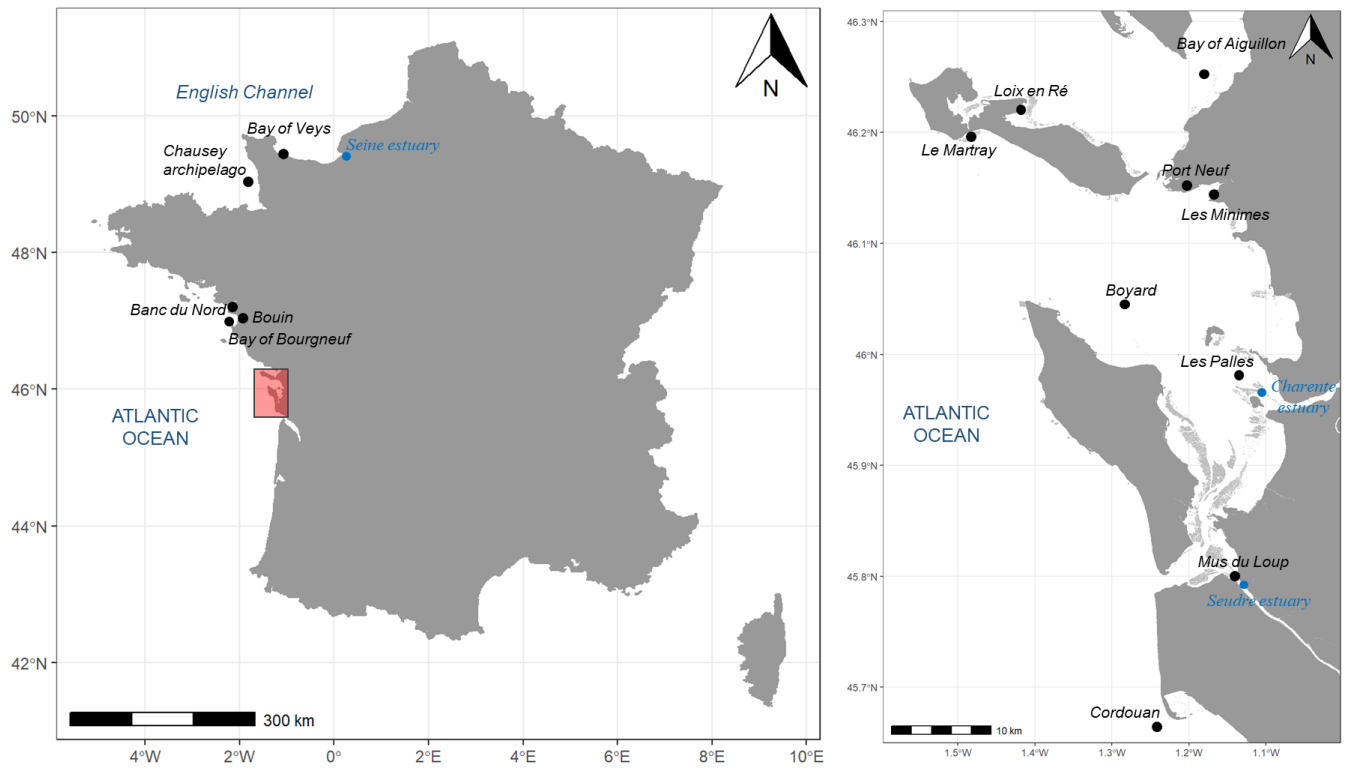
Heptachlor	OC	76-44-8	Pacific oyster ( <i>C. gigas</i> )	nd	0.3	Bouin	[8]	2008	2
				0.8	1.6	Boyard			2
				0.6	1.9	Les Palles			2
				nd	0.5	Mus du loup			2
				nd	0.7	Cordouan			2
Heptachlor endo-epoxide	OC	1024-57-3	Mediterranean mussel ( <i>M. galloprovincialis</i> )	4.1		Chausey archipelago	[17]	2015	1
				1.1		Bay of Veys			1
				1.4	2.3	Seine estuary			1
Heptachlor exo-epoxide	OC	1024-57-3	Mediterranean mussel ( <i>M. galloprovincialis</i> )	1.0		Chausey archipelago	[17]	2015	1
				2.3		Bay of Veys			1
				1.1	1.2	Seine estuary			1
Lindane	OC	58-89-9	Pacific oyster ( <i>C. gigas</i> )	2.1	2.2	Bouin	[8]	2008	2
				0.5	0.6	Boyard			2
				0.4	1.3	Les Palles			2
				nd	0.6	Mus du loup			2
				nd	0.7	Cordouan			2
alpha-BHC	OC	319-84-6	Pacific oyster ( <i>C. gigas</i> )	0.6*	0.62*	Le Martray	[18]	2019	11
				0.62*		Charente estuary			5
				0.72*		Seudre estuary			5
				0.26*		Le Martray			5
				0.31*		Charente estuary			4
Metazachlor	OC	67129-08-2	Mediterranean mussel ( <i>M. galloprovincialis</i> )	12.3		Chausey archipelago	[17]	2015	1
				10.8		Bay of Veys			1
				11.8	24.0	Seine estuary			1
Metolachlor	OC	51218-45-2	Mediterranean mussel ( <i>M. galloprovincialis</i> )	398		Chausey archipelago	[17]	2015	1
				151		Bay of Veys			1

				284	349	Seine estuary			1
			Pacific oyster ( <i>C. gigas</i> )	<0.33*	0.38*	Le Martray			11
				<0.33*		Charente estuary	[18]	2019	5
				<0.33*		Seudre estuary			5
Phtalimide	OC	-	Blue mussel ( <i>M. edulis</i> )		30	Banc du Nord			8
						Bay of Bourgneuf	[19]	2015-2017	6
						Bay of Aiguillon			12
trans-Nonachlor	OC	5103-73-1	Pacific oyster ( <i>C. gigas</i> )	nd	0.6	Bouin			2
				0.4	0.7	Cordouan	[8]	2008	2
Ethoprophos	OP	13194-48-4	Blue mussel ( <i>M. edulis</i> )		31	Banc du Nord			8
						Bay of Aiguillon	[19]	2015-2017	12
Chlorfenvinphos	OP	470-90-6	Pacific oyster ( <i>C. gigas</i> )	1.65*	1.89*	Le Martray			11
						Charente estuary	[18]	2019	5
						Seudre estuary			5
Chlorpyrifos	OP	2921-88-2	Pacific oyster ( <i>C. gigas</i> )	0.79*	0.80*	Le Martray			11
						Charente estuary	[18]	2019	5
						Seudre estuary			5
Parathion	OP	56-38-2	Pacific oyster ( <i>C. gigas</i> )	0.56*	0.69*	Le Martray			11
						Charente estuary	[18]	2019	5
						Seudre estuary			5
Tributyl phosphate	OP	126-73-8	Blue mussel ( <i>M. edulis</i> )		14	Bay of Bourgneuf			6
						Bay of Aiguillon	[19]	2015-2017	12
Bifenthrin	Pyr	82657-04-3	Blue mussel ( <i>M. edulis</i> )		48	Bay of Bourgneuf			6
						Bay of Aiguillon	[19]	2015-2017	12
Cypermethrin	Pyr	52315-07-8	Mediterranean mussel ( <i>M. galloprovincialis</i> )		10.9	Chausey archipelago			1
				<10		Bay of Veys	[17]	2015	1
				<10		Seine estuary			1

Deltamethrin	Pyr	52918-63-5	Mediterranean mussel ( <i>M. galloprovincialis</i> )	<10	11.2	13.0	Chausey archipelago Bay of Veys Seine estuary	[17]	2015	1 1 1
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805 Figure 1.



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808 **Supplementary material.**

<b>Pesticide</b>	<b>Class</b>	<b>Statute in Europe</b>	<b>Log Kow</b>	<b>K<sub>oc</sub> (L/kg)</b>	<b>Water solubility (mg/L)</b>	<b>Vapor pressure at 25°C (mPa)</b>	<b>DT<sub>50</sub> soil (days)</b>	<b>PNEC (mg/L)</b>
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810 Table S1. Physicochemical properties of pesticides found in bivalves from the French Atlantic  
811 and English Channel coasts.

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Acetochlor	OC	Banned	4.14	208.93	233	0.22 (20 °C)	20– 100	2.6.10 <sup>-6</sup>
Aldrine	OC	Banned	6.50	49,000	0.027	0.12	9490	-
DDT	OC	Banned	6.91	204,000	0.0055	2.1.10 <sup>-11</sup>	4–30	-
Dieldrin	OC	Banned	5.40	12,000	0.195	0.78 .10 <sup>-3</sup>	>7	-
Endosulfan	OC	Banned	3.83 (alpha); 3.62 (beta)	11,500 (1.06)	0.32	0.83	50	5.10 <sup>-7</sup>
Heptachlor	OC	Banned	6.10	24,000	0.18	4.0.10 <sup>-4</sup>	250	3.10 <sup>-8</sup>
Lindane	OC	Banned	3.72	1,480	7.3	4.5.10 <sup>-5</sup>	15	2.10 <sup>-6</sup>
Metazachlor	OC	Banned	2.49	-	450	9.10 <sup>-7</sup> (20 °C)	0.1– 25.3	2.10 <sup>-6</sup>
Metolachlor	OC	Banned	3.13	288.40	488	4.18.10 <sup>-4</sup>	11-31	-
Phtalimide	OC		1.15	-	360	1.33.10 <sup>-4</sup>		-
trans- Nonachlor	OC	Banned	6.20	56,000	8.2e-3	1.26.10 <sup>-10</sup>	-	-
Ethoprophos	OP	Banned	3.59	70.79	750	0.05	3-56	-
Tributyl phosphate	OP	-	4.0	-	280	1.5.10 <sup>-7</sup>	-	0.0082
Bifenthrin	Pyr	Banned	6.00	236,610	0.001	0.0178	26	1.9.10 <sup>-9</sup>
Cypermethrin	Pyr	Approved	6.60	156,250 (2.19)	0.009	0.00023	60	-
Deltamethrin	Pyr	Approved	6.20	10,240,000	0.0002	1.24.10 <sup>-5</sup>	13	1.10 <sup>-7</sup>
Naphtalene	PAH	Banned	3.30	1.1.10 <sup>-5</sup>	31	1.1.10 <sup>-5</sup>	-	0.000325

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818 Text S1. OCs are synthetic organic compounds containing at least one chlorine atom [87].  
819 Due to their hydrophobic nature, OCs are persistent, i.g., dichlorodiphenyltrichloroethane  
820 (DDT) with a Log Kow of 6.91 [88] . They tend to accumulate in sediments, bioaccumulate in  
821 human and animal tissue, and biomagnify in food chains. OCs are considered as persistent  
822 organic pollutants (POPs) (DT 50 up to 9490 days) [6,51,89,90]. From 1940 to the 1970s,  
823 they were widely used, before being formally banned [91].

824 OPs and Carb – second generation insecticides – act similarly. They inhibit the  
825 acetylcholinesterase (AChE) by releasing esters. AChE is an enzyme that catalyzes the  
826 hydrolysis of the neurotransmitter acetylcholine (ACh) [87,92]. Therefore, OPs and Carb are  
827 highly toxic to insects and mammals [87,92] and are as persistent in the environment as OCs  
828 [87]. For instance, ethoprophos has a 81-day half-life of open seawater [93]. OPs and Carb,  
829 including diazinon, parathion-methyl and carbofuran are water-soluble [13].

830 Pyr and Neo – third-generation of pesticides – were respectively produced in the 1970/80s  
831 and 1990s [11,13,94]. They have a lower bioaccumulation capacity than OCs [95] . However,  
832 these pesticides are extremely toxic to non-target aquatic invertebrates and fish species [96] .  
833 Pyr are characterized by their low solubility in water and their high organic carbon-water  
834 partition coefficients ( $K_{OC}$ ) (see Log Kow values and Koc in Table S1) [13]. Finally, Neo are  
835 water-soluble [13].