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Approaches to determine pesticides in marine bivalves

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Abstract

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

Introduction

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

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

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

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

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

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

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

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

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

Methodologies

Sample pretreatment

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

Extraction of pesticides from bivalve samples

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

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

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

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

Matrix effects and challenge to overcome them

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

Detection and identification techniques

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

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

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

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

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

Method validation

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

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

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

Recommendations and future perspectives

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

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

Recommendations for sample preparation are the use of QuEChERS using MgSO_4 or Na_2SO_4 and enhanced by AOAC or EN method, innovative techniques respecting the principles of green analytical chemistry, such as SBSE and MSPE and the use of new materials as sorbents. Finally, we recommend dSPE and analytical protectors to reduce matrix effect.

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

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

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Compliance with ethical standards

The author declares that there is no conflict of interest.

Table and Figure Legend.

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).

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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 [®] 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]

802

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
			Pacific oyster (<i>C. gigas</i>)	1.5		2.3	Bouin	[8]	2008	2
				1.9		2.8	Boyard			2
				2.0		3.7	Les Palles			2
2,4'-DDE	OC	3424-82-6	Mediterranean mussel (<i>M. galloprovincialis</i>)				Mus du loup	[8]	2008	2
					<1		Chausey archipelago			1
					<1		Bay of Veys			1
				<1		2.4	Seine estuary			1
			Pacific oyster (<i>C.</i>	2.2		2.5	Bouin	[8]		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	[8]	2008	2
				3.6	3.8	Boyard			2
				2.4	3.7	Les Palles			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	[17]	2015	1	
			<0.1		Bay of Veys			1	
			2.5	2.9	Seine estuary			1	
Dieldrin	OC	60-57-1	Variegated scallops (<i>M. varia</i>)	6.4		Loix en Ré	[1]	2014	1
				12.4		Les Minimes			1
				nd		Port-neuf			1
				4.2		Les Palles			1
			Mediterranean mussel (<i>M. galloprovincialis</i>)	<1		Chausey archipelago	[17]	2015	1
				2.7		Bay of Veys			1
				2.1	2.3	Seine estuary			1
			Pacific oyster (<i>C. gigas</i>)	<0.2*	0.27*	Le Martray	[18]	2019	11
				<0.2*		Charente estuary			5
				<0.2*		Seudre estuary			5
alpha-Endosulfan	OC	33213-65-9	Mediterranean mussel (<i>M. galloprovincialis</i>)	1.5		Chausey archipelago	[17]	2015	1
				1.7		Bay of Veys			1
				1.1	1.4	Seine estuary			1
beta-Endosulfan	OC	959-98-8	Mediterranean mussel (<i>M. galloprovincialis</i>)	<1		Chausey archipelago	[17]	2015	1
				14		Bay of Veys			1
				15.2		Seine estuary			1

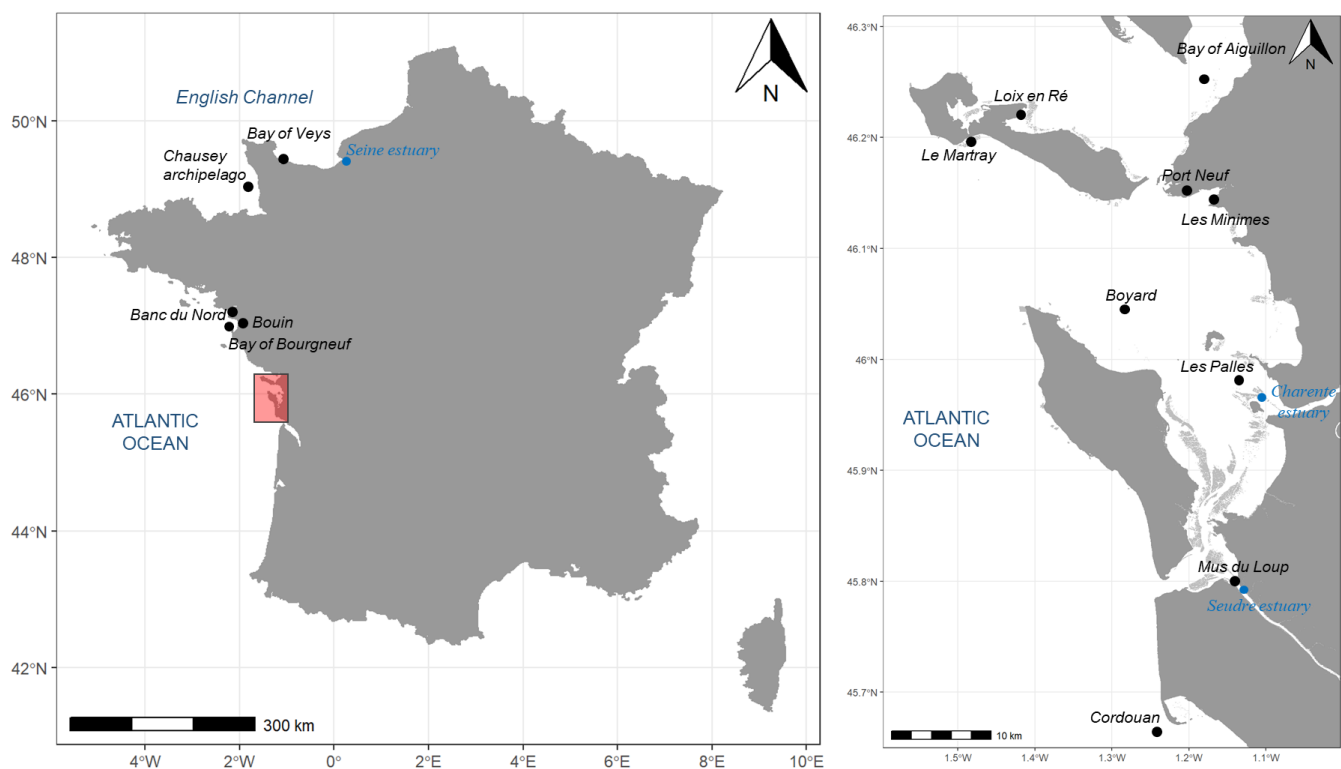
Heptachlor	OC	76-44-8	Pacific oyster (<i>C. gigas</i>)	nd	0.3	Bouin	[8]	2008	2
				0.8	1.6	Boyard			2
				0.6	1.9	Les Palles			2
				nd	0.5	Mus du loup			2
				nd	0.7	Cordouan			2
Heptachlor endo-epoxide	OC	1024-57-3	Mediterranean mussel (<i>M. galloprovincialis</i>)		4.1	Chausey archipelago	[17]	2015	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
			Blue mussel (<i>M. edulis</i>)		0.26*	Le Martray	[18]	2019	5
					0.31*	Charente estuary			4
					0.24*	Sèvre estuary			6
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
				<0.33*	0.38*	Le Martray				11
Pacific oyster (<i>C. gigas</i>)				<0.33*		Charente estuary	[18]	2019	5	
				<0.33*		Seudre estuary				5
Phtalimide	OC	-	Blue mussel (<i>M. edulis</i>)				Banc du Nord	[19]	2015-2017	8
							Bay of Bourgneuf			6
							Bay of Aiguillon			12
trans-Nonachlor	OC	5103-73-1	Pacific oyster (<i>C. gigas</i>)	nd	0.6	Bouin	[8]	2008	2	
				0.4	0.7	Cordouan			2	
Ethoprophos	OP	13194-48-4	Blue mussel (<i>M. edulis</i>)				Banc du Nord	[19]	2015-2017	8
							Bay of Aiguillon			12
Chlorfenvinphos	OP	470-90-6	Pacific oyster (<i>C. gigas</i>)	1.65*	1.89*	Le Martray	[18]	2019	11	
					1.72*	Charente estuary			5	
					2.12*	Seudre estuary			5	
Chlorpyrifos	OP	2921-88-2	Pacific oyster (<i>C. gigas</i>)	0.79*	0.80*	Le Martray	[18]	2019	11	
					0.81*	Charente estuary			5	
					0.93*	Seudre estuary			5	
Parathion	OP	56-38-2	Pacific oyster (<i>C. gigas</i>)	0.56*	0.69*	Le Martray	[18]	2019	11	
					0.63*	Charente estuary			5	
					0.61*	Seudre estuary			5	
Tributyl phosphate	OP	126-73-8	Blue mussel (<i>M. edulis</i>)				Bay of Bourgneuf	[19]	2015-2017	6
							Bay of Aiguillon			12
Bifenthrin	Pyr	82657-04-3	Blue mussel (<i>M. edulis</i>)				Bay of Bourgneuf	[19]	2015-2017	6
							Bay of Aiguillon			12
Cypermethrin	Pyr	52315-07-8	Mediterranean mussel (<i>M. galloprovincialis</i>)				Chausey archipelago	[17]	2015	1
							Bay of Veys			1
							Seine estuary			1

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

804

805 Figure 1.



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Pesticide	Class	Statute in Europe	Log Kow	K_{OC} (L/kg)	Water solubility (mg/L)	Vapor pressure at 25°C (mPa)	DT₅₀ soil (days)	PNEC (mg/L)
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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 ⁻⁶
Aldrine	OC	Banned	6.50	49,000	0.027	0.12	9490	-
DDT	OC	Banned	6.91	204,000	0.0055	2.1.10 ⁻¹¹	4–30	-
Dieldrin	OC	Banned	5.40	12,000	0.195	0.78 .10 ⁻³	>7	-
Endosulfan	OC	Banned	3.83 (alpha); 3.62 (beta)	11,500 (1.06)	0.32	0.83	50	5.10 ⁻⁷
Heptachlor	OC	Banned	6.10	24,000	0.18	4.0.10 ⁻⁴	250	3.10 ⁻⁸
Lindane	OC	Banned	3.72	1,480	7.3	4.5.10 ⁻⁵	15	2.10 ⁻⁶
Metazachlor	OC	Banned	2.49	-	450	9.10 ⁻⁷ (20 °C)	0.1–25.3	2.10 ⁻⁶
Metolachlor	OC	Banned	3.13	288.40	488	4.18.10 ⁻⁴	11-31	-
Phtalimide	OC		1.15	-	360	1.33.10 ⁻⁴		-
trans-Nonachlor	OC	Banned	6.20	56,000	8.2e-3	1.26.10 ⁻¹⁰	-	-
Ethoprophos	OP	Banned	3.59	70.79	750	0.05	3-56	-
Tributyl phosphate	OP	-	4.0	-	280	1.5.10 ⁻⁷	-	0.0082
Bifenthrin	Pyr	Banned	6.00	236,610	0.001	0.0178	26	1.9.10 ⁻⁹
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 ⁻⁵	13	1.10 ⁻⁷
Naphtalene	PAH	Banned	3.30	1.1.10 ⁻⁵	31	1.1.10 ⁻⁵	-	0.000325

815

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817

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 K_{OC} in Table S1) [13]. Finally, Neo are
835 water-soluble [13].