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## Evaluating pesticide mixture risks in French Mediterranean coastal lagoons waters

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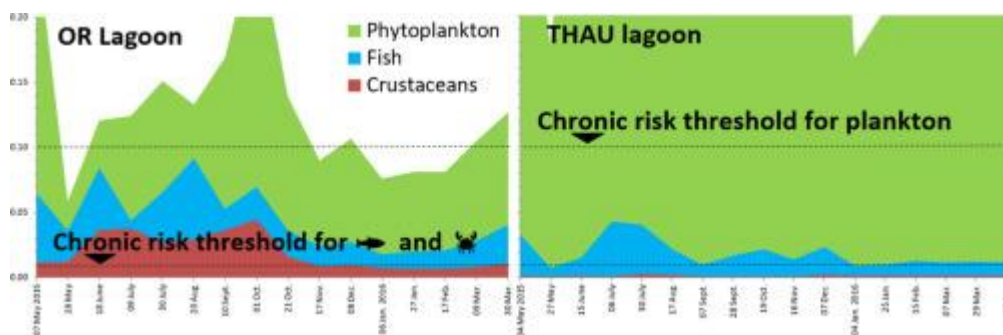
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### Abstract :

To assess the risk of pesticide mixtures in lagoon waters, this study adopted a multi-step approach using integrative passive samplers (POCIS) and concentration addition (CA) toxicological models. Two French Mediterranean lagoons (Thau and Or) were monitored for a range of 68 pesticides continuously over a period of a year (2015–16). The findings revealed mixtures of dissolved pesticides with varying composition and levels over the year. The Or site contained more pesticides than Thau site (37 vs 28 different substances), at higher concentrations (0.1–58.6 ng.L<sup>-1</sup> at Or vs <0.1–9.9 at Thau) and with overall higher detection frequencies. All samples showed a potential chronic toxicity risk, depending on the composition and concentrations of co-occurring pesticides. In 74 % of the samples, this pesticide risk was driven by a few single substances (ametryn, atrazine, azoxystrobin, carbendazim, chlorotoluron, irgarol, diuron and metolachlor) and certain transformation products (e.g. DPMU and metolachlor OA/ESA). Individually, these were a threat for the three taxa studied (phytoplankton, crustaceans and fish). Yet even a drastic reduction of these drivers alone (up to 5 % of their current concentration) would not eliminate the toxicity risks in 56 % of the Or Lagoon samples, due to pesticide mixtures. The two CA-based approaches used to assess the combined effect of these mixtures, determined chronic potential negative impacts for both lagoons, while no acute risk was highlighted. This risk was seasonal, indicating the importance of monitoring in key periods (summer, winter and spring) to get a more realistic picture of the pesticide threat in lagoon waters. These findings suggest that it is crucial to review the current EU Water Framework Directive's risk-assessment method, as it may incorrectly determine pesticide risk in lagoons.

## Graphical abstract



## Highlights

► Up to 37 pesticides were quantified in French Mediterranean coastal lagoons waters. ► Temporal and between-site differences were found in pesticides mixtures and concentrations. ► Both mixtures and single pesticides posed a chronic threat to aquatic lagoon life. ► Non-priority substances as metolachlor and its TPs were also risk drivers. ► Proposals to improve the WFD regulatory monitoring of lagoons were made.

**Keywords :** POCIS, Thau Lagoon, Or Lagoon, Water Framework Directive, environmental risk assessment, phytopharmaceuticals

# 1. Introduction

Coastal lagoons are transitional ecosystems exposed to a wide range of anthropogenic pressures that can degrade water quality, disrupt ecological restoration, and endanger the sustainability of socio-economic activities (Newton et al., 2018; Yamamuro, 2012). Of these pressures, chemical contamination is a particular concern, especially from dissolved organic pesticides, widely used in nearby watersheds and reported various adverse effects on marine species (Behrens et al., 2016; de Caralt et al., 2020; Hannachi et al., 2022).

In France, more than 30 lagoons extend along the Mediterranean coastline. The monitoring of pesticides in these lagoons began in 2012, with the implementation of the European Water Framework Directive (WFD) (European Commission, 2000). The WFD requires each member state to monitor the chemical contamination of its water bodies. Each state is free to use its own monitoring methodology as long as it provides relevant information on the 41 regulated priority chemicals that are used to define the chemical status of water bodies (European Commission, 2000). The current sampling strategy of the WFD concerning dissolved contaminants in French Mediterranean coastal lagoons involves integrative passive sampling for three weeks in the spring every three years (French Ministry of an Ecological Transition, 2018). The devices employed— polar organic chemical integrative samplers (POCIS) – have proven effective. Compared to spot sampling, POCIS enhance the capability of monitoring dissolved compounds in waters (Poulier et al., 2014), especially in highly diluted coastal

ecosystems (Munaron et al., 2012). During the three-week in-situ exposure, a POCIS device integrates both pollution events and base-level concentrations, increasing the detection of a broad range of dissolved chemicals – especially pesticides – even at very low concentrations (Tapie et al., 2011). While the WFD represents a step forward in assessing the risks of dissolved organic pesticides in French Mediterranean coastal lagoons, its current monitoring presents three major limitations.

The first is sampling frequency: no study has shown that the three-week spring window is representative of pesticide risk in French lagoons. Indeed, studies of several other lagoons around the world have shown that pesticide contamination is sometimes higher in other seasons (Cruzeiro et al., 2015; Griffero et al., 2019; Mhadhbi et al., 2019). A second limitation is the narrow list of organic pesticides monitored. Currently, the directive defines only 22 pesticides or groups of pesticides as priority pollutants to be monitored, most of which are prohibited for 20-50 years. Of these, only 3 compounds are authorized for plant protection and 4 for biocidal uses. Yet, 294 active substances are currently used for phyto-pharmaceutical purposes in France (ANSES, 2020a) and dozens for biocidal purposes (ANSES, 2020b). The third limitation of current WFD monitoring is that while the WFD assesses individually the effect of priority substances, by comparing the level of each priority substance to its regulatory benchmark – the environmental quality standard (EQS) – (European Union, 2013), the combined effects of substances are not considered. While the impact of pesticide cocktails is not well known, recent studies indicate that the combined effects of pesticides worsen the impact on aquatic ecosystems (Covert et al., 2020; Dupraz et al., 2019; Schmidt et al., 2022). Even at individual concentrations below their EQS, it has been shown under laboratory conditions that priority chemicals contribute to higher joint toxicity in mixtures (Carvalho et al., 2014). These three limitations of current WFD monitoring in French Mediterranean coastal lagoons could lead to an erroneous environmental risk assessment (ERA) and highlight the need for more representative monitoring strategy and risk assessment concerning dissolved organic pesticides in lagoons (Kortenkamp et al., 2019).

Considering the large number of substances co-occurring in waters at trace levels and the range of possible effects on diverse aquatic organisms with widely differing sensitivities, predictive ERA methods are required to describe ecotoxicological effects complexity. Among the existing methods, the concentration addition (CA) theoretical toxicological model (Loewe and Muischnek, 1926) has been found to fit well to describe the overall direct toxicity of a mixture, and is considered a suitable first-tier conservative approach (as a worst-case scenario) to deal with mixtures of chemicals (Backhaus and Faust, 2012; Belden et al., 2007; Kortenkamp et al., 2009). It is based on the toxicity of each individual compound, assuming there is no interaction between them, with respect to the same toxic endpoint on the same species, considering all constituents of a mixture as dilutions of each other. This model cannot predict the precise biological toxicity of a chemical mixture, but it gives a reliable picture of its possible direct additive consequences on tested organisms (or those with similar sensitivity within the same trophic level). So, it is considered to correctly describe the effect of

mixtures of compounds with the same mode of action (MOA). Backhaus et al. (2003) found that the more contaminants in a mixture, the more reliable the CA model was, even if they did not all have the same MOA. Several methods derived from the CA model (Chèvre et al., 2006; Nowell et al., 2018, 2014) have subsequently assessed the effect of pesticide mixtures in streams. Another method combined CA and the response addition model (Bliss, 1939) in a more complex two-stage model (De Zwart and Posthuma, 2005) which was used to predict the effect of pesticide mixtures on macro-invertebrates (Schäfer et al., 2013). Backhaus and Faust (2012) proposed a ‘funnel’ approach, starting with an extensive application of the CA model to obtain an initial pragmatic prediction of risk (using ecotoxicological data that may come from different trophic levels), to roughly rank the relative risk between sample. Then in a biologically more relevant second step, they refined the risk assessment by applying the strict CA model (specifically on a single trophic level or species). To date, this range of proposed approaches to assess the toxicity of pesticide mixtures have not been used in lagoon ecosystems.

According to the latest WFD assessment, the 22 French Mediterranean lagoons investigated were considered to have a good chemical status (Bouchoucha et al., 2019). Yet, due to the limitations of the current monitoring strategy, we hypothesize that this status is based on partial and possibly erroneous information regarding dissolved organic pesticides. This study sought to investigate this, employing continuous integrative passive sampling over one year in two Mediterranean lagoons and then analysing the risks of individual and mixed pesticides by both extensive and strict CA models. The aims were to: (i) monitor a range of dissolved pesticides, not restricted to WFD priority substances, in order to better define the exposure of Mediterranean coastal lagoons to pesticides, and their seasonal variability, (ii) identify the periods of highest pesticide risk in waters, from individual substances and from mixtures, which substances pose the highest risk, and which taxonomic groups are potentially the most impacted, and (iii) compare these results with the WFD regulatory chemical status assessment, and propose a complementary monitoring strategy for Mediterranean lagoons that more widely considers the risks of pesticides in waters, including those of pesticides cocktails.

## **2. Materials and methods**

### **2.1 Study area**

The euhaline Thau Lagoon and the mesohaline Or Lagoon are nano-tidal, semi-enclosed hydro-systems located on the Gulf of Lion on the French Mediterranean coast (Fig. 1). These lagoons are subject to high annual variations in temperature and salinity typical of the Mediterranean climate. They were selected for their contrasting characteristics in terms of morphology, hydrodynamics, ecological status and watershed uses (see Supplementary Information, S11). One site per lagoon was monitored: TH site in the Thau Lagoon and OR site in the Or Lagoon (Fig. 1). These sites were chosen based on current WFD sampling sites in the lagoons.

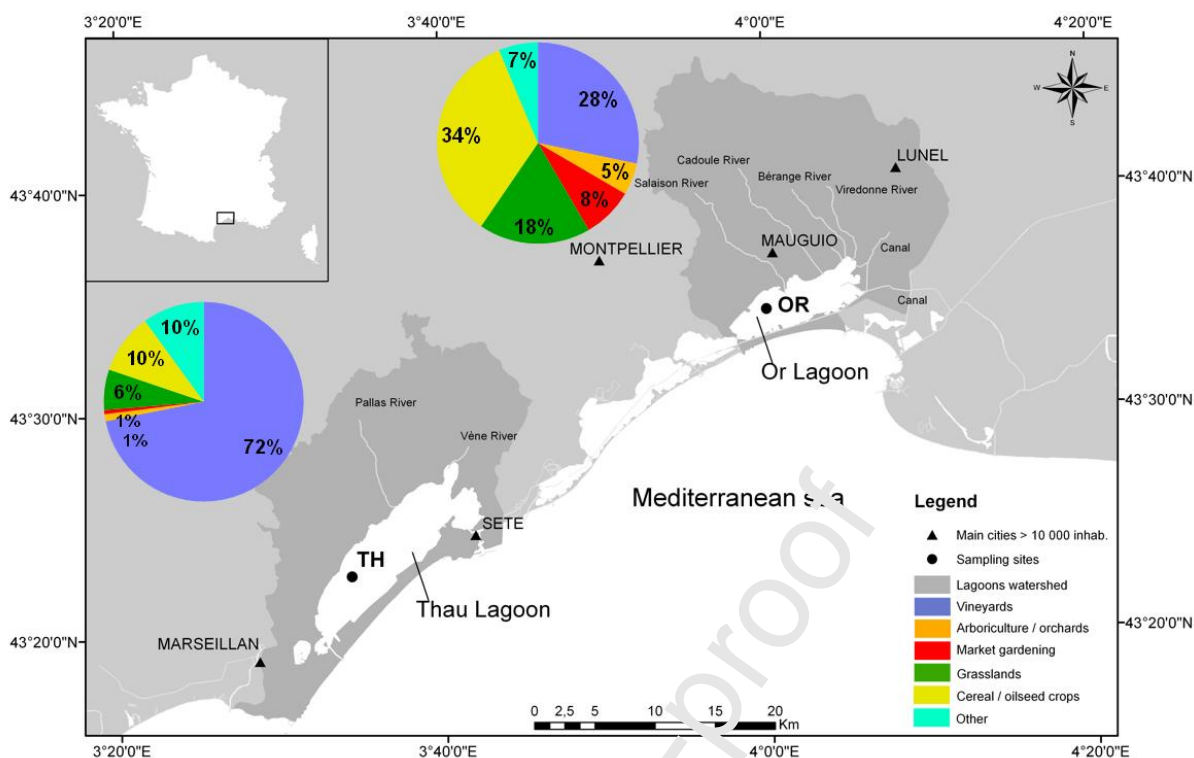


Fig. 1. The sampling sites – Thau Lagoon (TH) and Or Lagoon (OR) – on the Mediterranean coast of France (Occitanie region) and the agricultural land use in their respective watersheds shown in pie charts (sources: General Agricultural Census 2010, map: IGN 2007). See SI1 for geographical coordinates of sampling sites.

## 2.2 Monitoring strategy

### 2.2.1. Temporal monitoring strategy & in-situ measurements

Each site was continuously sampled with passive integrative samplers (POCIS) over a period of one year, from May 2015 to May 2016 for the TH site, and from May 2015 to April 2016 for the OR site. Three POCIS were immersed at each site for a period of about three weeks at mid-depth in the water column (samplers' depth at TH: 2.5 m; at OR: 0.8 m). At the end of each exposure period, these were replaced by three new devices that were exposed for about three weeks, and so on until the end of the study. This continuous monitoring with POCIS allowed to integrate each contamination event, providing an overview of the lagoons pesticide exposure throughout the year and its seasonal variability.

Spot temperature and salinity measurements were carried out at both sites during each installation and removal of a POCIS with WTW® probes immersed at the same depth (Table SI2).

### 2.2.2. Substance selection

The active substances investigated were chosen based on: (i) their occurrence in previous regional monitoring surveys (2010–14) of approximately 460 pesticides carried out in selected rivers in these watersheds across the year (French Water Agency, unpublished), (ii) previous spring studies on local

coastal water contamination (Munaron et al., 2013, 2012), and (iii) sampling and analytical criteria, mainly the adsorption capacity of the sorbent used in the POCIS devices. We selected 68 organic pesticides to monitor (35 herbicides, 7 fungicides, 3 biocides, 9 insecticides and 14 transformation products –TP–) (Table SI3), which have all been detected in previously studied rivers or lagoons with an average detection frequency > 1%. These included 7 hydrophilic substances deemed priority substances by the WFD: alachlor, atrazine, cybutryne (irgarol), diuron, isoproturon, simazine and terbutryn. Note that when the herbicide metolachlor is mentioned hereafter, it concerns the sum (r+s) of the two enantiomers of this compound. The certified reference materials and analytical standards were obtained from Cluzeau Info Labo (Ste-Foy-la-Grande, France; purity > 96%). The other hydrophobic priority pesticides were not considered in this study because they were not detected in mussels in the last WFD monitoring in French Mediterranean lagoons (Rouchouca et al., 2019). The only priority substances the last survey found in mussels at the TH site were DDE and DDT (at trace levels far below environmental and health quality standards). However this strongly hydrophobic organochlorine insecticide and its TP do not adsorb to POCIS so could not be sampled in this water monitoring.

### **2.2.3. POCIS preparation, transport and analysis**

#### **2.2.3.1. POCIS preparation and transport**

The 99 POCIS devices used in this study (IR: 51; OR: 48) were homemade samplers based on the standard ‘pharmaceutical’ configuration of commercial samplers. Two micro-porous polyether sulfone (PES) membranes (0.1 µm pore size, 90 mm ID) from VWR (Fontenay-sous-Bois, France) containing a monophasic sorbent were held by two stainless steel rings (54 mm ID). Each POCIS contained 200 mg of Oasis®HLB sorbent (60 µm particle size) from Waters (Guyancourt, France). Previous to use, the sorbent was spiked with deuterated compound (DIA-d5) as a performance reference compound (PRC) to control in-situ conditions the POCIS were exposed to, as described in Belles et al. (2014). The POCIS were stored at -25°C, then transported at +4°C for deployment in the field. After exposure, each POCIS was rinsed with ultrapure water, carefully wiped and frozen at -25°C before laboratory analysis.

#### **2.2.3.2. POCIS extraction and analysis**

Methanol (MeOH) and dichloromethane (DCM) (HPLC reagent grade) were purchased from ICS (Belin Beliet, France). The POCIS extraction at laboratory was performed as described in Tapie et al. (2011). The POCIS sorbent was transferred into an empty glass SPE tube with polyethylene frits and dried using the Visprep SPE vacuum manifold (Supelco) for 30 min. The pesticides were eluted successively with 10 mL MeOH, 10 mL MeOH/DCM (50/50; v/v) mixture, and 10 mL DCM in a receiving vial containing internal standards (Table SI3). The extract was concentrated to 200 µL of MeOH under nitrogen flow for analysis. The recovery was optimized with 200 mg of the sorbent

spiked with pesticide standards laid on the top of the polyethylene frit (10 ng of each). A blank procedure was performed to assess possible contamination, which was never detected.

The POCIS extracts were analysed by liquid chromatography and tandem mass spectrometry detection (LC-MS/MS) following Belles et al. (2014). The analyses were carried out in selective multiple reaction monitoring mode in accordance with the European requirement 2002/657/CE for analytical procedures (European Commission, 2002). Two transitions were analysed per compound – a quantification and a confirmation – to ensure compound identification and quality assurance. Quality controls and procedural blanks were performed during each step of the preparation–extraction–analysis process. Likewise, the performance of the analytical method was verified by the extraction of a spiked sample in each series of analyses. Analysed compounds and the PRC were quantified using internal standard calibration (Tapie et al., 2011). The analytical conditions used, the limits of quantification (LOQ, between 0.03 and 42.3 ng.g<sup>-1</sup>) and the extraction recoveries (between 71% and 123%) obtained are reported in Table SI3. Detection limits of the analytical method were defined as LOQ/2.

#### 2.2.3.3. POCIS theory and TWAC calculation

The time-weighted average concentration (TWAC) of pesticides in water (defined hereafter as  $C_w$ , ng.L<sup>-1</sup>) was calculated based on the POCIS sampling rates for each analyte previously determined in standard laboratory conditions ( $RS_{standard}$ , L.day<sup>-1</sup>) from Belles (2012), according to the following equation (1):

$$C_w = C_{Pocis} \times M_{sorberent} \times RS_{in-situ}^{-1} \times t^{-1} \quad (1)$$

where  $C_{pocis}$  represents the concentration of each analyte in the POCIS (ng.g<sup>-1</sup>),  $M_{sorberent}$  the mass of the receiving phase (g),  $t$  the deployment period (days), and  $RS_{in-situ}$  the in-situ sampling rate (L.day<sup>-1</sup>) corrected by the in-situ elimination rate constant ( $k_{e\ in-situ}$ , per days) of the DIA-d5 spiked in the sorbent as PRC, as follows (2):

$$RS_{in-situ} = RS_{standard} \times k_{e\ in-situ} \times k_{e\ standard}^{-1} \quad (2)$$

where  $k_{e\ standard}$  represents the elimination rate constant of DIA-d5 (per days) obtained under standard laboratory conditions. The PRC correction is assumed to correct differences in pesticide sampling rates between field exposure and laboratory calibrations of POCIS regarding environmental conditions (hydrodynamics, temperature, etc.), adjusting the  $RS_{standard}$  in inverse proportion to PRC elimination (Belles et al., 2014). The  $RS_{standard}$ , and  $k_{e\ standard}$  values used were determined by Belles (2012) under laboratory conditions and are reported in Tables SI2 and SI3.

### 2.3 Environmental risk assessment



To assess pesticide monitoring strategy effectiveness in considering the risks of dissolved pesticides in Mediterranean lagoon waters, several ERA methods were compared. All exposure values below detection limits were considered as zero values during ERAs.

### **2.3.1. Current WFD procedure**

Following current WFD methodology, an initial ERA was carried out for individual substances, comparing the concentration of each priority substance detected in the water to its annual average environmental quality standard limit (AA-EQS) (European Union, 2013). Since integrative samplers were used to estimate the TWAC in water, we assumed that chronic AA-EQS were more relevant than acute maximum annual concentration EQS (MAC-EQS) for the purposes of comparison. If a single priority substance exceeds its threshold value, the water body is considered to have bad chemical status. In this risk assessment, non-priority substances were not considered.

### **2.3.2. Alternative methods**

To go beyond what the WFD proposes to determine environmental risk, the effects of pesticides were investigated using different methods, each attempting to better represent environmental complexity. The first assessed a larger number of individual substances, and the two others studied the effect of pesticide mixtures: one with an extensive CA approach and one with a strict CA approach. Before applying these last methods, the relevance of applying a mixture model to a dataset was assessed.

#### **2.3.2.1. Environmental risk assessment of individual substances**

This assessment refined the WFD regulatory method by considering more individual priority and non-priority substances using the toxic units (TUs) concept. In this approach, individual effects are defined as toxic units, each of which is the ratio of the measured or predicted environmental concentration (PEC) of each contaminant to its water quality criteria (WQC): a threshold value usually derived from standard ecotoxicological endpoints as e.g.  $ECx_i$  (representing the concentration of component  $i$  that would cause  $x\%$  effect on a targeted species or taxonomic group). For priority substances, the WQCs used were the regulatory AA-EQS. For non-priority substances, WQCs were based on predicted no-effect concentrations (PNECs). The PNECs were derived from the deterministic approach using the assessment factor (AF) applied to a critical ecotoxicity value as proposed by the European Technical Guidance Document for deriving AA-EQS for the saltwater community (European Commission, 2018). Each PNEC was calculated by dividing the lowest available chronic or acute aquatic toxicity data by the AF depending on the size of the available aquatic toxicity dataset. Assuming that the ecotoxicity base set (i.e. 3 short-term and 3 long-term results from freshwater or saltwater each representatives of the 3 taxonomic groups: algae, crustaceans and fish) gives an AF value of 100, the greater the amount of long-term marine toxicity data from additional trophic levels (echinoderms, molluscs, etc.), the smaller the AF, with a minimum value of 10 (European Commission, 2018). Conversely, if the ecotoxicity base set is not complete, the AF will be greater than 100, up to  $10^4$ . The

aquatic toxicity data we used came from ecotoxicological databases reviewed in January 2021 (INERIS in France: <https://substances.ineris.fr/fr/>, and the EPA in the United States: <https://cfpub.epa.gov/ecotox/search.cfm>). Only validated data from ecotoxicological studies reporting multiple and satisfactory controls of experimental conditions were retained for the analysis. When validated data was missing, open-source literature was also used. Data was lacking for several TPs or metabolites, so in this case the WQC of the parent active substance was used as the PNEC if applicable. Details of the selected chronic (NOEC) and acute (EC50) aquatic toxicity values and WQCs are reported in Table SI4. A TU > 1 indicates a potential chronic risk for the considered substance, according to current ecotoxicological knowledge.

### 2.3.2.2. Suitability of using a mixture-effect model

If the risk in the mixture is primarily from a single compound, assessing the effect of this compound might be sufficient for an effective assessment of the overall effect. Price and Han (2011) recommend determining the maximum cumulative ratio (MCR), defining it as follows (3):

$$MCR = RQ \times TU_{max}^{-1} \quad (3)$$

where RQ is the so-called risk quotient defined as the sum of TUs, and  $TU_{max}$  is the highest toxic unit among all compounds in the mixture. When all compounds contribute equally to the RQ, the MCR reaches its theoretical maximum, which equals the number of compounds in the mixture. In this case, the use of a mixture-effect model to predict the toxicity of the sample is relevant. Conversely, if the risk is dominated by a single substance, the MCR tends towards 1, with an MCR of 2 meaning that a single substance accounts for half the sample toxicity. Samples with an MCR between 1 and 2 are thus considered to have one pesticide primarily contributing to overall sample toxicity, so a mixture-effect model is less suitable as a first approximation, i.e. without considering possible synergistic effects. Before applying the extended and strict CA approaches, the MCR was calculated to determine the suitability of using a mixture-effect model.

### 2.3.2.3. Environmental risk assessment of pesticide mixtures

On samples for which it was deemed suitable, the effects of pesticide mixtures were investigated using the CA model. This assumes that the overall effect (RQ) is the sum of the individual effects of each compound in the mixture, defined by their toxic units (TUs). This relatively simple method to assess the environmental risk of mixtures has been widely applied (Covert et al., 2020; Nowell et al., 2014; Sandstrom et al., 2022).

This ERA was carried out in two steps as recommended by Backhaus and Faust (2012). First, the CA concept was applied in an extensive way, as a first-tier estimation of the overall toxicity of the pesticide mixture on the lagoon ecosystem. Then in a second step, the ERA was refined through the strict application of the CA concept, making it possible to determine: (i) which taxonomic group

(phytoplankton, crustaceans or fish) was potentially most affected by a pesticide mixture, (ii) which pesticides in the mixture were risk drivers, i.e. contributed most to overall risk and thus require mitigation measures in priority, and (iii) whether or not more advanced risk assessment of mixture effects needed to be carried out.

In the first-tier risk assessment, we applied the method recommended by Backhaus and Faust (2012). This is based on an extensive CA approach, using the risk quotient  $RQ_{TWAC/WQC}$  as follows (4):

$$RQ_{TWAC/WQC} = \sum_{i=1}^n (TU_i) = \sum_{i=1}^n (TWAC_i / WQC_i) \quad (4)$$

where  $TU_i$  is the toxic unit of each pesticide  $i$  of a mixture of  $n$  compounds. The  $TWAC_i$  obtained from in-situ POCIS measurements (mean concentration of triplicates,  $ng.L^{-1}$ ) and  $WQC_i$  ( $ng.L^{-1}$ ) is the WQC of each compound  $i$ . Thus, the ERA of pesticide mixtures is based on a binary critical threshold in which  $RQ_{TWAC/WQC} < 1$  indicates ‘low or no risk’, while  $RQ_{TWAC/WQC} \geq 1$  indicates a potential risk warranting further investigation (in our case, triggering the second step of analysis). While this extensive approach has been recommended as a pragmatic and justifiable approximation of CA (Altenburger et al., 2013; Backhaus and Faust, 2012), it is conceptually different from the original CA concept, as the WQCs may be based on different toxicological endpoints and/or species from different trophic levels, meaning that the  $RQ_{TWAC/WQC}$  has no relevant biological significance. While it can rank the relative risk between samples, it does not indicate that toxicity will necessarily occur. This is why we used it as a first-tier prioritization system to compare samples, periods and lagoon sites according to the potential risk of pesticide mixtures, and then applied a strict CA model to improve the relevance of the risk assessment.

In this second step, the acute and chronic  $RQ_{STU}$  were defined as the sum of the TUs (acute and chronic) of each pesticide detected, focusing on the three standard taxonomic groups ( $RQ_{STU\text{Group}} = \sum TU_{\text{Group}}$ ), where group is used for phytoplankton, crustaceans and fish, which are representative of three trophic levels. For each trophic level, the acute (respectively chronic) TUs were first calculated according to equation (4) for each substance in the mixture using the lowest available EC50 values (respectively NOEC and/or EC10 values for chronic) of the considered trophic level instead of the WQCs. These acute (respectively chronic) TUs were then summed to obtain the  $RQ_{STU\text{ acute}}$  for the given trophic level (respectively  $RQ_{STU\text{ chronic}}$ ). Due to lack of data for each trophic level, freshwater and seawater taxa were mixed. In all, 10 taxa were used for phytoplankton, 6 taxa for crustaceans and 5 taxa for fish. As exposure was determined by integrative samplers, acute toxicity endpoints may not always be relevant for use with TWACs, as reported by the European Food Safety Agency (EFSA, 2013). So, of the 6  $RQ_{STU}$  values calculated (3  $RQ_{STU\text{ acute}}$  and 3  $RQ_{STU\text{ chronic}}$ ), we considered that  $RQ_{STU\text{ chronic}}$  values were likely to be the most relevant. Critical thresholds were then defined as 0.1 for phytoplankton and 0.01 for crustaceans and fish, as recommended by EFSA guidelines (2013) for extrapolating ecotoxicological data from laboratory experiments to the field. Above these thresholds, a

potential toxic effect for the trophic level cannot be ruled out. This toxic effect can be due to one or more substances exceeding its ecotoxicity effect threshold or to the cumulative effect of the mixture if no substance exceeds its ecotoxicity effect threshold.

## 2.4 Statistical analysis

### 2.4.1. *Seasonality and site differences in overall contamination footprint*

To determine potential differences in the contamination footprint and address our first objective, two-way ANOVAs were conducted. The contamination footprint was defined as the sum of pesticide concentrations grouped by the usual families (e.g.  $\Sigma$ herbicides,  $\Sigma$ fungicides, etc. categorized by the main use of each substance, see Table SI3), by sampling site (OR, TH) and by sampling season (spring, summer, autumn or winter). Each sampling period was assigned to the relevant calendar season as described in Table SI2. If a sampling period straddled two seasons, it was assigned to the one with which it shared more time. When differences were detected, a Fisher's protected LSD multiple comparison test was used to determine which means were significantly different pairwise. This procedure verifies the familywise error rate as this test is only applied following an ANOVA for which a null hypothesis is rejected. Further data analyses were conducted in the same way to determine potential differences in mixture risk level ( $RC_{TWAC/WQC}$ ) according to the sampling site and sampling season.

### 2.4.2. *Toxic unit composition and level*

Subsequent data processing was carried out to assess differences in the time period pertaining to risk, focusing on individual pesticide toxic units ( $TU_{TWAC/WQC}$ ). To analyse differences in the risk of pesticide mixtures and the individual level of risk between samples over time, between the two lagoons and within each lagoon, three complementary multivariate analyses were carried out on the dataset, crossing samples (rows) and pesticide toxic units (columns). First, to investigate the difference in TU composition and, to a lesser extent, TU level, between the two lagoons, a between-group correspondence analysis was performed (Legendre and Legendre, 1998). In this analysis, a group is a categorical factor (here, the two sampled lagoons were modalities) used as an instrumental variable (here, to test inter-lagoon variability in TU composition). In the first stage, the between-group analysis sought axes that best differentiated the centres of gravity of each lagoon. The second stage involved the projection of the different samples (as supplementary rows), highlighting the variability of each sample around the centre of gravity of its corresponding lagoon. In addition, a Monte-Carlo permutation test with 999 permutations was carried out to test the null hypothesis of no difference in the TU composition profile between the two lagoons. In a second stage, to specifically assess temporal variability in TU composition between samples within each lagoon, a correspondence analysis (CorA) was computed separately for both sites. A trajectory plot, graphically linking samples in the order of sampling dates, was computed. Both between-group CorA and CorA were based on the chi-square

distance, resulting in a typology based on the similarity of pesticide composition between samples, weighted by the level of TUs. Finally, to investigate differences (i) within each lagoon and (ii) between samples from the different lagoons according to their level of individual TUs, a principal component analysis (PCA) for each lagoon, and a between-group PCA among lagoons were performed, respectively. As a PCA is based on Euclidean distance, this allowed an assessment of the difference between samples according to the extent/level of individual pesticide TUs, and is thus complementary to a CorA based on the chi-square distance (Legendre and Legendre, 1998).

For all statistical tests, null hypothesis  $H_0$  was rejected when  $p < 0.05$ . All statistical analyses were performed using R software (R Development Core Team, 2020).

### 3. Results

#### 3.1 Pesticide mixtures in lagoons

##### 3.1.1. *A wide range of pesticides detected beyond priority substances*

Of the 68 substances investigated, 37 were detected in the lagoon waters (only detected substances are presented in Table 1; for the complete dataset see Table S1). All 37 substances were detected in the OR site, and 28 in the TH site (Table 1). The compounds detected in both sites with 100% detection frequency were two fungicides (carbendazim and metalaxyl-M), five herbicides (metolachlor, chlorotoluron, diuron, terbuthylazine and prometryn) and some degradation products (simazine-2-hydroxy and DET), and the two biocides irgarol and DMST (Table 1). Some compounds (mainly herbicides) had 100% detection frequency only at the OR site: metolachlor OA, terbutryn, prometryn and simazine and its TP (DIA). In contrast, the herbicides atrazine and isoproturon, and the biocide DMSA, were systematically detected at the TH site, while were less frequently detected at the OR site. Of the pesticides not detected in 100% of the samples, indicating high seasonality in waters, bentazone and metolachlor ESA were those with the highest concentrations. The herbicide bentazone was specific to the OR site. Of the seven WFD priority substances investigated in this study, only the herbicide alachlor was never detected. There was a 100% detection frequency of the other six priority compounds in the two sites, apart from simazine (76%) and terbutryn (71%) at the TH site, and atrazine and isoproturon (both 94%) at the OR site (Table 1).

Carbendazim, metolachlor ESA, simazine-2-hydroxy, bentazone, metolachlor OA, metolachlor and chlorotoluron were the pesticides found in the highest concentrations at the OR site (higher than 13.2 ng/L; the highest concentration was found for carbendazim at 58.6 ng/L). The priority pesticides detected at this site were all found with maximum concentrations lower than 4.1 ng/L. At the TH site, metalaxyl-M, DMSA and metolachlor ESA were the pesticides with the highest concentrations (higher than 4.0 ng/L; the highest concentration was found for metalaxyl-M at 9.9 ng/L). The priority pesticides detected at this site were also found in lower concentrations (maximum lower than 1.9 ng/L: Table 1).

Table 1. Detection frequencies (%) and mean, minimum and maximum TWACs (ng.L<sup>-1</sup>) of the studied pesticides measured in the Or and Thau lagoons (n = number of sampled periods), and AA-EQS (ng.L<sup>-1</sup>) of priority substances detected. Substances under the analytical limit of detection are shown as <LD. H: herbicide; F: fungicide; B: biocidal; I: insecticide; TP: transformation product.

Active substance	Code	Use	Or Lagoon (n=16)				Thau Lagoon (n=17)				AA-EQS
			Freq (%)	Mean	Min	Max	Freq (%)	Mean	Min	Max	
Carbendazim	CAR	F	100	23.6	6.9	58.6	100	1.9	0.9	3.4	
Metolachlor OA	MTOoa	TP (H)	100	15.2	8	24.6	65	1.3	<LD	2.3	
Simazine-2-hydroxy	HSMZ	TP (H)	100	9.3	2.7	30.6	100	1.3	0.4	2.7	
Metolachlor	MTO	H	100	4.8	0.6	16.7	100	0.6	0.1	0.8	
Chlorotoluron	CHL	H	100	4.4	0.2	13.2	100	0.4	0.1	0.9	
Metalaxyl-M	MTX	F	100	2.6	0.7	5.8	100	2.7	0.6	9.9	
Simazine	SMZ	H	100	2.5	1.8	4.1	76	0.2	<LD	0.4	1000
N,N-Dimethyl-N'-p-tolylsulphamide	DMST	TP (B)	100	1.5	0.3	3.8	100	1	0.4	3	
Terbuthylazine-desethyl	DET	TP (H)	100	1.3	0.8	2	100	0.3	0.2	0.8	
Diuron	DIU	H	100	1.3	0.5	2.5	100	0.7	0.3	1.9	200
Terbutryn	TBY	H	100	1	0.4	1.8	71	0.1	<LD	0.3	6.5
Deisopropylatrazine	DIA	TP (H)	100	0.8	0.1	1.7	18	0.3	<LD	0.4	
Terbuthylazine	TBZ	H	100	0.7	0.2	1.9	100	0.3	0.1	0.8	
Ametryn	AME	H	100	0.4	0.2	1	100	0.1	0.03	0.8	
Prometryn	PRY	H	100	0.1	0.1	0.2	41	0.0	<LD	0.1	
Irgarol / Cybutryn	IRG	B	100	0.1	0.1	0.4	100	0.6	0.3	1.6	2.5
Atrazine	ATZ	H	94	1	<LD	3.2	100	0.8	0.3	1.3	600
Isoproturon	ISO	H	94	0.1	<LD	0.2	100	0.1	0.01	0.2	300
Azoxystrobin	AZO	F	81	1.9	<LD	7	0	-	<LD	-	
N,N-dimethyl-N'-phenylsulphamide	DMSA	TP (B)	31	1.4	<LD	3.1	100	1.7	0.3	5.3	
Metolachlor ESA	MTOea	TP (H)	75	21.4	<LD	41.8	41	2.5	<LD	4	
Atrazine-2-hydroxy	HATZ	TP (H)	75	4.7	<LD	9.7	94	1.5	<LD	2.2	
Propachlor	PRO	H	75	0.1	<LD	0.1	94	0.1	<LD	0.2	
Propiconazole	PZL	F	50	1	<LD	1.2	0	-	<LD	-	
Desethylatrazine	DEA	TP (H)	44	6.5	<LD	7.6	0	-	<LD	-	
Hexazinone	HEX	H	44	0.1	<LD	0.2	35	0.1	<LD	0.1	
Norflurazon	NOR	H	44	0.01	<LD	0.01	0	-	<LD	-	
Fluzilazole	FLU	F	38	0.1	<LD	0.1	0	-	<LD	-	
Bentazone	BEN	H	31	20	<LD	27.6	0	-	<LD	-	
1-(3,4-Dichlorophenyl)-3-methylurea	1,3-DMU	TP (H)	31	0.4	<LD	0.7	6	0.4	<LD	0.4	
Fosthiazate	FOS	I	31	0.03	<LD	0.1	0	-	<LD	-	
Flazasulfuron	FJA	H	25	3.6	<LD	6.6	0	-	<LD	-	
Propazine	PRZ	H	19	0.1	<LD	0.1	6	0.02	<LD	0.02	
Acetochlor ESA	ACEea	TP (H)	13	2	<LD	2.8	29	1.4	<LD	3.5	
Thiamethoxam	THX	I	13	0.3	<LD	0.4	12	0.1	<LD	0.2	
Metazachlor	MTA	H	13	0.04	<LD	0.1	41	0.02	<LD	0.05	
Dimethachlor	DIM	H	6	0.1	<LD	0.1	0	-	<LD	-	

### 3.1.2. Pesticide exposure between sites and seasons

Over the year-long monitoring period, the OR site had a greater occurrence of hydrophilic pesticides than the TH site, in terms of the number of chemicals detected, detection frequency and concentrations in the water. A total of 23 compounds at the OR site had a detection frequency higher than 75% (17 at the TH site), 16 of which had a detection frequency of 100% (14 at the TH site). The median concentration of detected pesticides was 2.7 times higher at the OR site, and the average concentration 4.5 times higher than the TH site. The TWACs were in the range of 0.1–58.6 ng.L<sup>-1</sup> at the OR site and

<0.1–9.9 at the TH site. The maximum differences between the two sites were found for the herbicide simazine, the fungicide carbendazim, and the degradation product metolachlor OA, with average concentrations that were respectively 12.5, 12.4 and 11.7 times higher at the OR site than the TH site. Only the fungicide metalaxyl-M and the two biocides irgarol and DMSA showed higher levels in TH waters (respectively 1.1, 6.0 and 1.2 times higher than in the OR site). With the exception of irgarol, the other priority pesticides detected had higher or equal concentrations at the OR site.

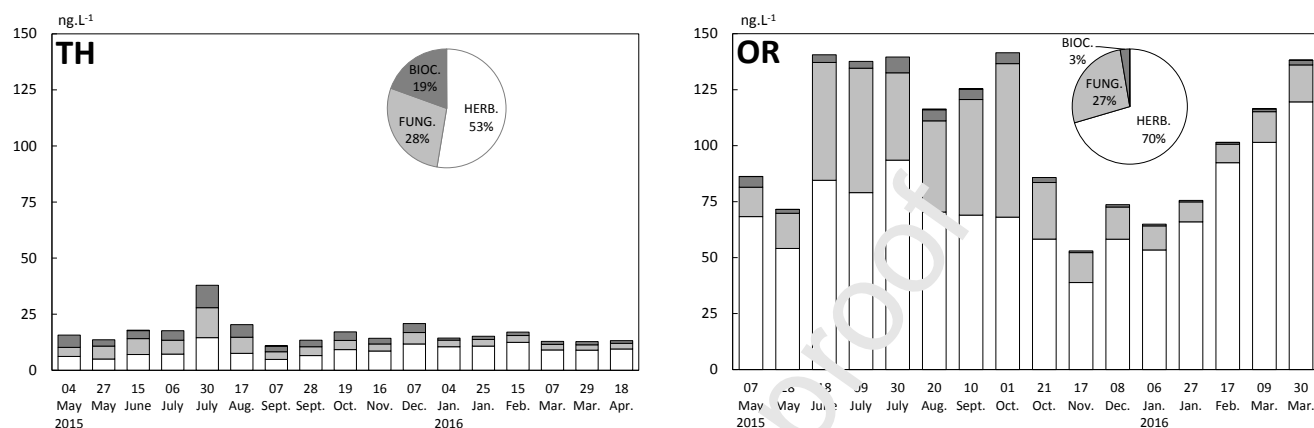


Fig. 2. Cumulative time-weighted average concentration of pesticides in waters (ng.L<sup>-1</sup>), grouped by type of pesticide in TH (left) and OR (right) sites from May 2015 to April-May 2016. Each date is the starting date of a POCIS deployment period (about three weeks), thus the subsequent date marks the end of the previous deployment period. The last deployment finished on 2 May 2016 at the TH site and 20 April 2016 at the OR site. The pie charts represent the proportion of each type of pesticide or its respective contribution to the total sum of concentration (Herbicides; Fungicides; Biocides). Insecticides represented < 0.1% of total concentration in each lagoon).

In both lagoons, herbicides were the main pesticide family detected (70% of the total sum of concentrations at OR site vs 53% at TH site) (Fig. 2). On average over all POCIS exposure periods, levels of  $\Sigma$ herbicides were significantly higher at the OR site than the TH site (73.5 vs 8.8 ng.L<sup>-1</sup>,  $p < 0.001$ ). No relevant seasonality of  $\Sigma$ herbicides was highlighted in either site ( $p = 0.26$ ). The lagoon waters were found to be contaminated with herbicides throughout the year. Fungicides were also markedly present in the waters of both lagoons (27% at OR site vs 28% at TH site). Average levels of  $\Sigma$ fungicides by period were significantly higher at the OR site than the TH site (26.0 vs 4.5 ng.L<sup>-1</sup>,  $p < 0.001$ ). A significant seasonality of  $\Sigma$ fungicides was revealed, with higher mean concentrations reported in summer in the two lagoons (27.7 ng.L<sup>-1</sup>,  $p < 0.001$ ) (Fig. 2). Biocides were little detected in OR waters (<3%) in contrast to TH waters (19%). There was a lower mean concentration of  $\Sigma$ biocides (2.6 and 3.1 ng.L<sup>-1</sup> for OR and TH sites, respectively), and no significant difference was noted between sampling sites ( $p = 0.41$ ). As with fungicides, significant seasonality in  $\Sigma$ biocides was revealed with higher average concentrations reported in summer (4.9 ng.L<sup>-1</sup>,  $p = 0.001$ ) (Fig. 2). Lastly,  $\Sigma$ insecticides represented < 0.1% of the total concentration in the two sites, with no significant difference between lagoons ( $p = 0.32$ ) or seasons ( $p = 0.11$ ).

## 3.2 Assessing environmental risk

### 3.2.1. Focusing on single priority pesticides

An initial risk assessment was carried out following WFD guidelines by comparing the exposure data (TWACs) for each of the 6 hydrophilic priority substances detected with their average annual environmental quality standard (AA-EQS) (Table 1). None exceeded its AA-EQS during the monitoring period. Thus, the WFD would classify these lagoons as good chemical status regarding these pesticides. However, we found between 16 and 24 additional co-occurring substances at the OR site, and between 11 and 18 at the TH site, not considered in this regulatory risk assessment.

### 3.2.2. Other potential risk drivers

Beyond the priority WFD substances, other individual pesticides detected in the lagoons may pose a chronic risk for these ecosystems. To determine this, we calculated the toxic unit (TU) of each substance detected at each sampling date and each lagoon. The figure 3 shows TUs summed to obtain the risk quotient  $RQ_{TWAC/WQC}$  (the latter will be described here after, in the next section).

At the OR site, with the exception of the 17 November sampling period (in which all individual  $TU < 1$ ), in each period between one and five pesticides were found to exceed their WQCs (i.e. individual  $TU > 1$ ), representing a total of eight substances (3 herbicides: metolachlor, chlorotoluron and ametryn; 2 fungicides: carbendazim and azoxystrobin; and 3 transformation products: acetochlor ESA, metolachlor OA and metolachlor ESA). Based on their WQCs, ametryn and acetochlor ESA were a potential threat to aquatic life at this site in early October and July, respectively. The two fungicides were found to be a potential chronic threat to aquatic life between June and late October, exceeding their WQC seven and four times, respectively. Chlorotoluron exceeded its WQC only in winter (three times between December and March), and metolachlor twice between March and April. The two metolachlor TPs were found to be a potential chronic threat to aquatic life throughout the monitoring period, exceeding their WQC eight and nine times respectively. The temporal distribution of these individual pesticide and their levels show that they were a risk to aquatic life at the OR site in all monitoring periods (except one). These eight non-priority substances contributed to 85.5% of the overall risk (the sum of all TUs across all sampling periods) at this site (Fig. 3).

At the TH site, throughout the monitoring period, acetochlor ESA (the TP of the herbicide acetochlor) was the only pesticide that exceeded its WQC. It exceeded it only once, in the 30 July sampling period. This single compound contributed to 16% of the overall risk at this site. The risk found in other periods (when  $RQ_{TWAC/WQC} > 1$ ) (Fig. 3), were caused by the effect of mixtures of pesticides.

While it is not possible to act directly on the occurrence of TPs in waters, the following five parent pesticides (the herbicides metolachlor, chlorotoluron and ametryn and the fungicides carbendazim and azoxystrobin) can be considered risk drivers for these two lagoons (Faust et al., 2019). Currently, with the exception of ametryn, all have at least one approved use (plant protection and/or biocidal use) (Table SI3). These five parent pesticides explained 53% of the overall risk for the Or Lagoon and 38%



for the Thau Lagoon. Although they did not exceed their WQC in the latter during monitoring, they still contributed significantly to its overall risk. In contrast to the WFD assessment, the ERA considering all co-occurring substances (individually) in lagoon waters indicated a potential threat to aquatic life caused by non-priority pesticides, changing qualitatively over time and by lagoon.

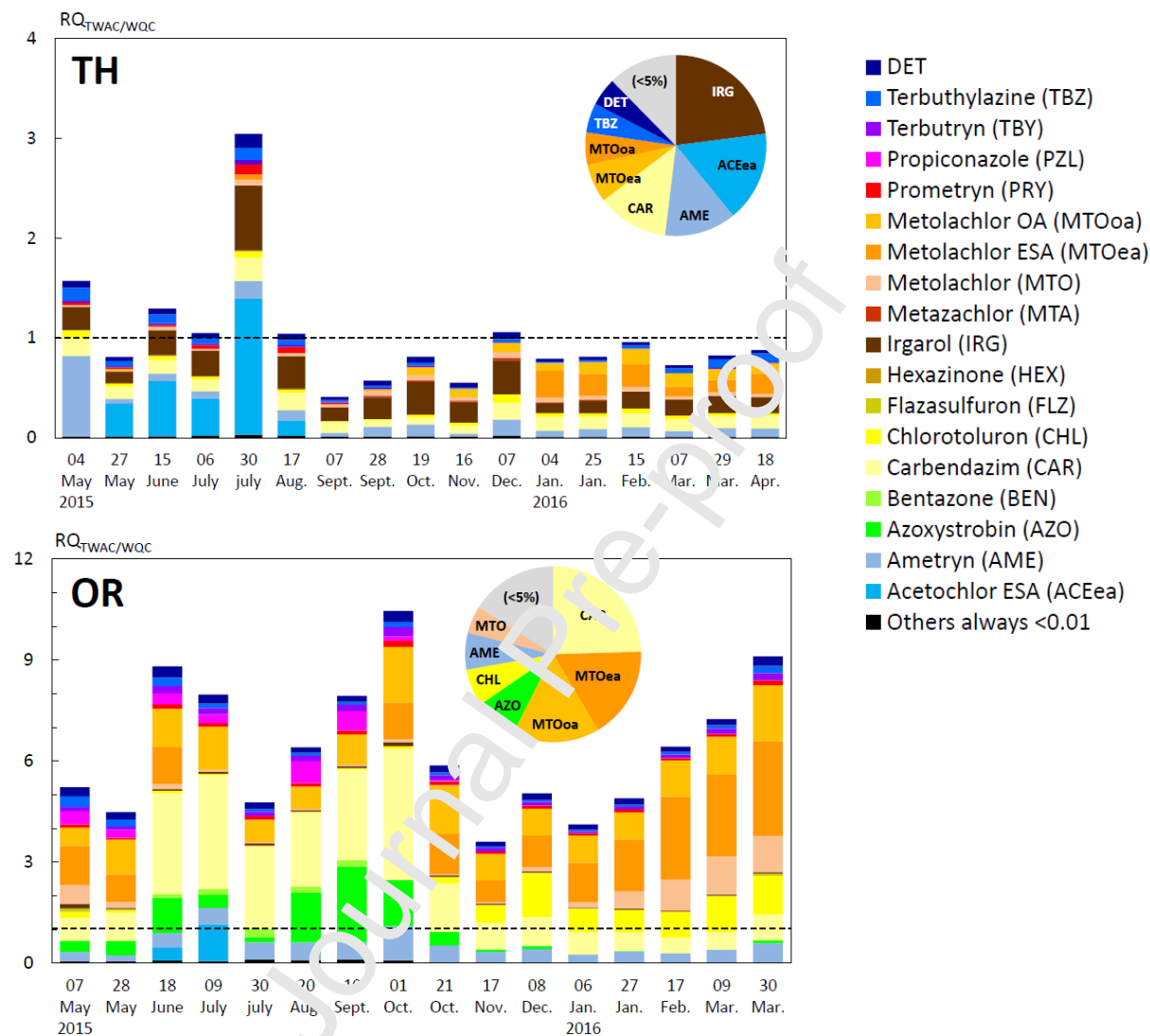


Fig. 3. Risk quotients ( $RQ_{TWAC/WQC}$ ) derived from pesticide concentrations (TWACs) measured with POCIS devices at TH site (Thau Lagoon) and OR site (Or Lagoon) during 2015–16 and from water quality criteria (WQC) reported in Table SI4. Each colour represents the toxic unit (TU) of each detected pesticide. The black dotted line represents the limit beyond which potential damage to ecosystems cannot be ruled out (risk > 1) due to pesticide mixtures in waters. The pie charts represent the relative contribution of each pesticide to the total sum of  $RQ_{TWAC/WQC}$  during the entire monitoring period. The relative contributions of compounds < 5% were summed.

### 3.2.3. Assessing the risk of pesticide mixtures

We also investigated the effect of pesticide mixtures using two variations of the CA model. First, the extensive CA approach from risk quotients  $RQ_{TWAC/WQC}$  (Fig. 3), then the strict application of the  $RQ_{STU}$ -based CA model by taxonomic group (Fig. 4).

### *Extensive CA approach*

The MCR was in the range of 1.9–4.8, indicating that in 97% of the samples (32 out of 33), no pesticide alone contributed more than 50% to the overall estimated toxicity, confirming the relevance of this mixture-effect model to assess the toxicity of these cocktails. Only one sample had a MCR < 2 (1.9 at TH site, 4 May 2015), with the herbicide ametryn contributing over 50% of its overall toxicity.

The extensive CA approach showed that risk from the mixture of pesticides was present throughout the year in the OR site ( $RQ_{TWAC/WQC} > 1$  in all samples), and between May and August in the TH site (when 6 of 17 samples had  $RQ_{TWAC/WQC} > 1$ ). At the OR site,  $RQ_{TWAC/WQC}$  was in the range of 3.6–10.5 with a maximum in early October 2015. At the TH site, it was in the range of 0.4–3.0, with a maximum in early August 2015. No significant seasonal effect of the  $RQ_{TWAC/WQC}$  was identified ( $p = 0.54$ ).

The pesticides contributing to the risk were significantly different between the two sites in composition as well as in level of individual risk (TU) (Monte-Carlo test,  $p < 0.001$ ) (see also the between-lagoon PCA in Fig. SI6). As the extensive CA approach was based on the sum of individual TUs, the previously highlighted risk drivers contributed to the mixture risk in lagoons. Even if the effect of these drivers was completely ruled out (i.e. in their TUs = 0), the effect of the pesticide mixture would still concern 37.5% of the samples at the OR site and 18% at the TH site. Thus, other pesticides were also identified for their significant contribution to overall risk, even if they did not exceed their WQC. For example, irgarol was among the highest contributors to overall risk at the TH site (23%), though it never exceeded its WQC. It was the only priority substance in that case (its TUs were always lower than 1, with a maximum of 0.65 in July 2015). In Thau Lagoon, the eight most contributing compounds (individual contribution > 5%) explained 88% of the overall risk (Fig. 3), while priority compounds explained 26.4% (irgarol was in both lists). In Or Lagoon, the seven most contributing pesticides (individual contribution > 5%) explained 84% of the overall risk, while priority compounds explained less than 4%. Based on this approach, pesticide mixtures therefore appear to pose a threat to aquatic life in Or Lagoon throughout the year, and in Thau Lagoon mainly during spring and summer.

### *Strict CA approach*

As a biologically more relevant method, a strict CA model was then applied to identify potentially threatened biological targets based on three taxonomic groups. This found no acute risk for either lagoon or any taxon, so here we discuss only the chronic risk quotients ( $RQ_{stu\ chronic}$ ) (Fig. 4). The findings showed a chronic risk for at least one taxon in all samples: phytoplankton was at risk in 75% of the samples, crustaceans at risk in 30%, and fish in 90%. Two out of three taxa were at risk in both sites over the study period (there was no risk for crustaceans at the Thau Lagoon site). In contrast to the extensive CA approach, MCRs recalculated with separate data for the three taxa were in the range of 1.04–2.11, representing 5 of 33 samples for which a mixture-effect model was suitable for

describing toxicity ( $MCR > 2$ ). The strict CA model showed that in 84% of samples (all taxa combined), toxicity could be explained by the effect of a single compound.

Concerning phytoplankton, we found a potential chronic risk for this taxon ( $RQ_{stu\ phytoplankton} > 0.1$ ) all year at the TH site, and frequently at the OR site (for 11 of 16 samples). This risk was mainly related to the priority substance irgarol, which is the most toxic compound in the mixture for phytoplankton (with the lowest  $NOEC = 0.0017\ \mu\text{g}\cdot\text{L}^{-1}$ ) from currently available data reported in the literature (Dupraz et al., 2018). Irgarol contributed between 88% and 96% to the overall phytoplankton risk at the TH site (median: 93.3%) and between 48% and 93% at the OR site (median: 54.5%). As this substance contributed overwhelmingly to the pesticide mixture risk at the TH site, a mixture effect on phytoplankton was only detected in three samples, all from the OR site ( $MCR > 2$ ). Irgarol was between 1.6 and 9.9 times more concentrated at the TH site (on average 6 times) (Table 1), so the average chronic  $RQ_{stu\ phytoplankton}$  was significantly higher at the TH site than the OR site ( $p < 0.001$ ). In contrast,  $RQ_{stu\ phytoplankton}$  showed no significant seasonal effect ( $p = 0.41$  for OR and 0.12 for TH).

For crustaceans, the potential risk was predicted only at the OR site (10 of 16 samples for which  $RQ_{stu\ crustaceans} > 0.01$ ). This risk was highly seasonal, occurring mostly in summer ( $p < 0.005$ ), and no mixture effect was found ( $MCR < 2$ ). The only  $RQ_{stu\ crustaceans}$  driver highlighted by this model was the fungicide carbendazim, which explained both the seasonal and inter-lagoon differences. The concentrations of carbendazim at the OR site were between 3.9 and 64 times higher than at the TH site (on average 12 times) (Table 1), and were higher in summer than in other seasons ( $p < 0.001$ ), as found for  $\sum\text{Fungicides}$ . Carbendazim contributed between 58% and 91% to the overall crustacean risk at the OR site and between 60% and 85% at the TH site (both medians: 79%), although it did not have the lowest  $NOEC$  value for crustaceans. This was reported for the fungicide azoxystrobin ( $NOEC = 0.98\ \mu\text{g}\cdot\text{L}^{-1}$ ), the second main contributor to chronic  $RQ_{stu\ crustaceans}$  at the OR site (this fungicide was not found in Thau Lagoon; its relative median contribution at the OR site was 3%).

For fish, chronic  $RQ_{stu\ fish}$  showed levels exceeding 0.01 at both OR and TH sites, all year round and especially in summer ( $p = 0.001$ ), potentially resulting in sub-lethal effects in lagoon ecosystems. Only two samples showed a pesticide mixture risk for fish, both at the OR site ( $MCR > 2$ ). Risks to fish were mainly explained by single compounds. Only three samples from the TH site showed no risks to fish (either from the mixture or from individual substances). The average chronic  $RQ_{stu\ fish}$  was significantly higher at the OR site than the TH site ( $p < 0.001$ ). Four compounds primarily contributed to this chronic risk for fish. Of these, the priority herbicide diuron and its TP (DPMU) together contributed to between 53% and 85% of the relative chronic  $RQ_{stu\ fish}$  at the OR site (median: 62.8%) and between 57% and 94% at the TH site (median: 71.7%). These two herbicides contributed to a chronic risk to fish all year round at the OR site, and between May and December at the TH site. They were the most toxic compounds in the mixture for fish, considering the available ecotoxicological data. The lowest  $NOEC$  of the fish dataset was for DPMU ( $0.016\ \mu\text{g}\cdot\text{L}^{-1}$ ), and the second lowest for

diuron ( $0.057 \mu\text{g}\cdot\text{L}^{-1}$ ). Two other herbicides also contributed to the chronic risk to fish at the OR site: the priority herbicides atrazine (relative median contribution to the chronic risk to fish = 16.8%) and ametryn (13.0%).

These results showed that for the same overall risk level, different taxa may be impacted due to the pesticide mixtures composition. Six pesticides were identified as the main contributors to chronic risk for the three taxa studied: irgarol (for phytoplankton), carbendazim (for crustaceans), and DPMU, diuron, atrazine and ametryn (for fish). These were considered the chronic risk drivers.

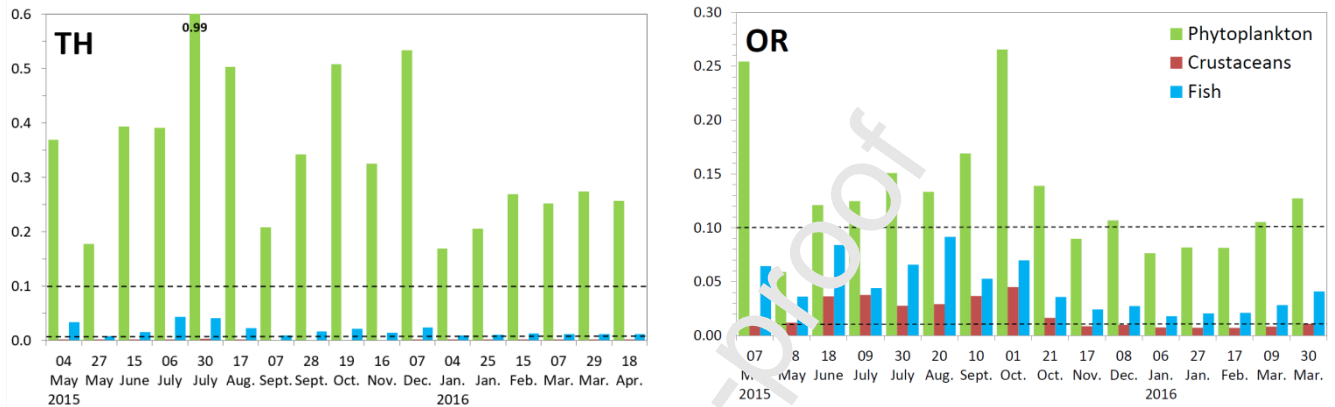


Fig. 4. Chronic risk quotients ( $RQ_{STU}$ ) for phytoplankton, crustaceans and fish at Thau Lagoon (TH) (left) and Or Lagoon (OR) (right) during 2015–16 monitoring. Black dotted lines represent the limit beyond which potential chronic damage to phytoplankton (top line:  $RQ_{STU} > 0.1$ ) or to crustaceans and fish (bottom line:  $RQ_{STU} > 0.01$ ) cannot be ruled out, due to the toxicity of pesticide mixtures in waters.

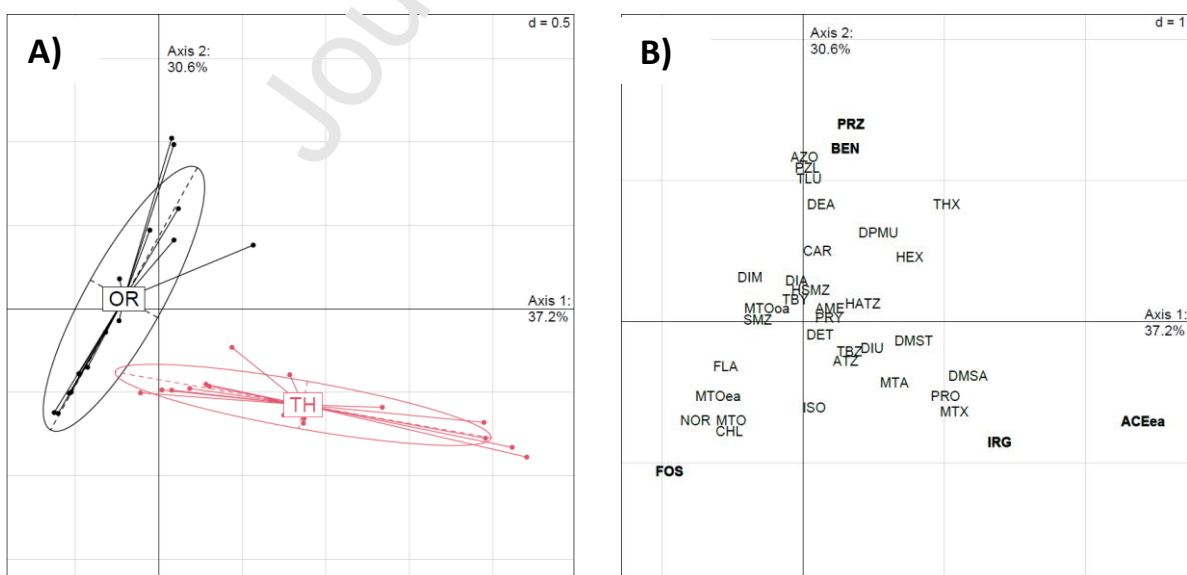
### 3.2.4. Pesticide composition and seasonality of the risk

These methods allowed us to identify the risk drivers and the periods when the level of mixture risk ( $RQ_{twac/wqc}$ ,  $RQ_{stu\ chronic}$ ) was highest, but not to define the composition of co-occurring pesticides of concern and the temporal evolution of this composition in the lagoons. To obtain this information, a correspondence analysis (CorA) was carried out. Figure 5A shows the barycenter of all the samples sorted by lagoon, confirming the results of the Monte-Carlo test: the two sites were different in both pesticide composition and levels of TUs, as the overlap between the two shapes is very low.

The first axis of the between-group CorA was highly correlated with acetochlor ESA and to a lesser extent with irgarol, DMSA and metalaxyl-M (Fig. 5B). This axis ordines TUs along a clear seasonal risk gradient (Fig. 5C & D) related to particular uses: on the right are summer risks (irgarol [IRG] and DMSA from antifouling biocides, metalaxyl-M [MTX] from fungicide treatments of vineyards, and acetochlor ESA [ACEea] from herbicide treatments of cereal crops) and on the left are two winter risks (chlorotoluron [CHL] and metolachlor [MTO] from herbicide treatments of cereal crops, and fosthiazate [FOS] from insecticide treatments of potatoes) (ANSES, 2020a, 2020b). While fosthiazate has a low contribution to the overall risk (it is not a risk driver and its  $TU_{max}$  was very low:  $1.15 \times 10^{-5}$ ), it changes seasonally over the year. This singular behaviour was highlighted as a marker of a specific

use (insecticide treatment of potatoes) by the correspondence analysis, but not as a risk marker. The Thau Lagoon samples are distributed horizontally along this first axis, mostly on the right side of the correspondence plot (Fig. 5A). This shows that the pesticide composition risk changed seasonally in this lagoon, from antifouling biocide uses, fungicide treatments of vineyards and herbicide treatments of cereals in the summer to herbicide treatments of wheat in the winter (Fig. 5C & D).

The second axis of the between-group CorA was highly correlated with the herbicides bentazone and propazine and with the fungicides azoxystrobin, propiconazole and fluzilazole (Fig. 5B). This axis ordinated TUs according to another seasonal risk gradient (Fig. 5C & D) related to plant protection uses: at the top are summer risks (bentazone [BEN] and propazine [PRZ] from herbicides used on cereal crops and market gardens, and azoxystrobin [AZO], propiconazole [PZL], flusilazole [FLU] and carbendazim [CAR] from fungicide treatments of cereal crops, market gardens, flowers and orchards) (ANSES, 2020a), and at the bottom are winter risks. Overall, winter risk composition is the same for the two lagoons, while the two shapes converge towards the left bottom side of the plot. The Or Lagoon samples were distributed relatively vertically along this second axis (Fig. 5A), showing highly seasonal pesticide risk composition in this lagoon, between summer at the top and winter at the bottom (Fig. 5C & D). This is consistent with the higher proportion of cereal and oilseed crops in the Or Lagoon's watershed (Fig. 1). Between these two extremes, the spring and autumn risks are clustered in the centre, as they result in a quite similar risk composition. This cluster mainly includes herbicide TPs (DIA, DEA, HSMZ, HATZ, DET, MTOoa) and banned priority herbicides (SMZ, TBY, DIU, ATZ) whose current water concentrations are not related to recent uses. Their location in the centre of the correspondence plot thus reveals neither lagoon nor season-specific pesticide composition, but rather a background pesticide composition that is relatively homogeneous in time and space.



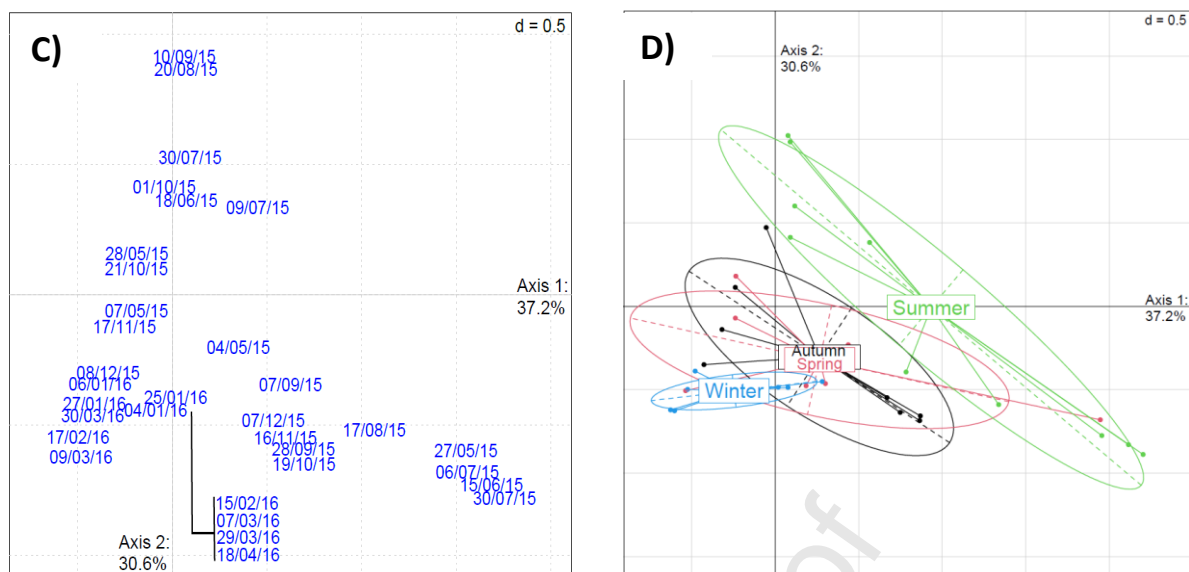


Fig.5. Between-group correspondence analysis (CorA) of the 37 toxic unit ( $TU_{TWAC/WQC}$ ) of detected pesticides from the two sites (TH and OR) in the 17 (TH) and 16 (OR) sampling periods. The same CorA plot is used to show projections of the centres of gravity of: (A) all the samples from each site, (B) the  $TU_{TWAC/WQC}$  values for the 37 pesticides (see Table 1 for code meanings), (C) the sampling dates (start of the passive sampling exposure period), and (D) the seasons. The d-values give the scale (i.e. the size of the grid in grey).

## 4. Discussion

### 4.1 Improving assessment of lagoon exposure to pesticides cocktails

In comparison with WFD regulatory method, our monitoring approach allowed a more detailed qualitative, quantitative and temporal characterization of the dissolved pesticides present in the sampled lagoon waters.

#### *From a qualitative concern*

Between 22 and 30 substances were detected simultaneously at the OR site (37 different substances throughout the monitoring), and 17 to 24 at the TH site (28 different substances) (Table 1). The WFD focuses on 22 priority pesticides or pesticides groups, including 10 hydrophilic pesticides. Of these, only six priority pesticides were detected in this work (atrazine, simazine, terbutryn, irgarol, diuron and isoproturon). Dozens more co-occurring non-priority pesticides were also found in the two sampling sites. These are not in the current WFD guidelines, but they could pose a potential threat to biodiversity and the ecological functioning of ecosystems. We argue that they should be considered in future ERAs. The list of monitored substances was suited to coastal lagoons, although this could be improved, as the greater the number of substances investigated, the more robust the risk assessment. Backhaus et al. (2003) showed that as the number of assessed compounds increases, the uncertainty of CA-based risk predictions decreases. A holistic assessment of exposure is essential for a realistic picture of risk. While our study investigated 68 substances, many pesticides currently used in

watersheds in France are not usually monitored in lagoons (e.g. glyphosate, 2,4-D, MCPA, etc.). Currently, their physicochemical properties (very strong polarity or acidity) make them undetectable with standard integrative samplers (such as the OASIS HLB POCIS we used), but alternative sorbents should soon allow them to be sampled even at low concentrations in transitional waters (Berho et al., 2017; Fauvelle et al., 2012). Moreover, integrative samplers have recently been combined with new high-resolution mass spectrometry detection techniques to screen for contaminants with no prior knowledge of their presence in waters (Guibal et al., 2015). This new coupled method should help future assessments reveal the true exposure of ecosystems to contaminants (Fauvelle et al., 2018).

#### *From a quantitative concern*

Substances below defined quantification limits add significant uncertainty in an ERA (especially if they are recorded as null because of high quantification limits). This may markedly underestimate risk, as has been shown in a study on streams in Sweden (Gustavsson et al., 2017). This problem can be avoided with integrative sampling, which captures trace levels of co-occurring pesticides in waters. In our study, the POCIS samplers used were able to quantify all compounds detected, even at levels below  $\text{ng.L}^{-1}$  (Table 1). These devices are very useful in marine contexts and transitional waters such as coastal lagoons (Mhadhbi et al., 2019), where high dilution can limit the relevance of grab sampling (Perez-Ruzafa et al., 2000). The time-weighted average concentrations they provide smooth potential concentration peaks, so the integrative data are likely to be more environmentally relevant for chronic risks than acute risks. POCIS integrative samplers (which are also used for WFD monitoring of French coastal Mediterranean lagoons) are clearly effective to sample a wide range of pesticides at low levels and their use could be extended to monitor other lagoons around the world (Mhadhbi et al., 2019).

#### *Towards a common approach to characterize pesticide exposure in lagoons*

The literature on pesticide water contamination of coastal lagoons is scarce (Table SI7). Hydrophilic priority substances are the most frequently searched (atrazine being the only one investigated in 100% of the studies). They were quantified in other Mediterranean lagoons in the same concentration range as in the Thau and Or lagoons (from a few  $\text{ng.L}^{-1}$  up to several tens of  $\text{ng.L}^{-1}$ ). However, many differences in the lists of substances searched for and sampling methodologies prevent a comparative ERA between the lagoons as it was done with Thau and Or. For example, some fungicides not investigated in our study (epoxiconazole and tebuconazole) were frequently detected in Bizerte, Ria Formosa and Vaccarès waters (Cruzeiro et al., 2015; Espel et al., 2019; Mhadhbi et al., 2019). The herbicide glyphosate, frequently detected in Vaccarès lagoon (which watershed is dominated by rice crops), and pyrethroid insecticides, detected in Ria Formosa lagoon (high proportion of citrus crops), have not yet been investigated in the other lagoons. Conversely, carbendazim and metalaxyl-M, the fungicides most frequently found in our study, were also detected in Bizerte waters, but not investigated elsewhere. In the future, harmonizing the lists of monitored substances and sampling

methodologies (with a preference for integrative sampling for the reasons mentioned above) would make inter-lagoon comparisons far easier, and would likely allow a better investigation of geographic differences in risk, use, regulation or transfer of pesticides to lagoons.

#### 4.2 Relevance and complementary of risk assessment methods

While the WFD regulatory approach concluded no risk from the priority pesticides in Thau and Or lagoons, alternative ERA methodologies revealed risk in all samples depending on the composition of co-occurring pesticides. The results of the ERA methods investigated are summarized in Table 2. This potential chronic toxicity was often driven by a few single substances either currently used or prohibited. These risk drivers resulted from a range of uses – biocides (irgarol), herbicides (ametryn, metolachlor, diuron, atrazine, chlorotoluron) and fungicides (carbendazim, azoxystrobin) – and should all be priority substances candidates if they are not considered as such already. They individually pose a threat to lagoon ecosystems. Several examples of this have been reported: during chronic exposure at environmental concentrations, irgarol can modify the structure of microalgal communities in lagoons, as has been shown in experimental conditions (Devila et al., 2005; Magnusson et al., 2012). Carbendazim and azoxystrobin have been found to have direct and indirect effects on crustaceans; they are able to alter the structure of freshwater communities (Gustafsson et al., 2010) and have endocrine-disrupting effects (Miracle et al., 2011). Metolachlor has been found to induce larval abnormalities and DNA damage in a population of exposed Pacific oysters at environmentally relevant concentrations in Arcachon Bay (France) (Mai et al., 2012). Diuron has been widely studied for these same effects on shellfish (Akcha et al., 2012; Behrens et al., 2016), which may even be transmitted from generation to generation (Barranger et al., 2015, 2014). Drastically restricting the use of these eight main risk drivers would be an important first step, but may not be sufficient to rule out risk, especially in the Or Lagoon, due to the effect of the overall mixture. Even drastic risk mitigation measures that limited each individual risk to 5% of the current risk for these drivers and their TPs would only reduce the median  $RQ_{TWAC/WQC}$  from 6.1 to 1.1 at the OR site and from 0.8 to 0.2 at TH site. The pesticide mixture risk threshold would still be exceeded (i.e.  $RQ_{TWAC/WQC} > 1$ ) in more than 56% of the samples of the OR site.

The two CA-based risk assessment approaches highlighted that pesticide cocktails also put lagoon ecosystems at risk, regardless of individual substance risk. However, some differences were noted between the two methods. The extensive CA method predicted a mixture effect in both lagoons, while the strict CA method predicted this only in the Or Lagoon for phytoplankton and fish. These differences originate from the theory behind these two methods. The extensive CA model is useful as a first step to provide a pragmatic risk assessment of mixture effects (Backhaus and Faust, 2012). However, it entails using a shortcut that distorts the very theory of the CA model, which does not allow biological interpretation of its results. Nonetheless, it provides an overview of the potential pesticide risk, highlighting chronic risk drivers in the mixture without excluding any a priori, using



100% of the ecotoxicological information currently available. It is best used as an indicator of relative risk, making it possible to prioritize risks between lagoons and the risk drivers within lagoons, allowing scientists to focus on the most relevant pesticides for their future ecotoxicological studies and stakeholders to support their environmental management actions. The strict CA model is based on a biologically more reliable approach, considering separately the different taxa. Of the 28 pesticides detected at the TH site, only between 11 and 16 (depending on the taxon considered) had a base ecotoxicological dataset available for deriving  $RQ_{STU}$ ; the same was true for only between 17 and 22 substances of the 37 detected at the OR site (Table 2). Phytoplankton was the taxon with the most ecotoxicological data, and crustaceans the taxon with the least (Table 2). So, while this method theoretically represents a real advance compared to the extensive CA model and the current WFD risk assessment, the lack of ecotoxicological data meant that 43–60% of compounds detected at the TH site and 41–54% at the OR site could not be considered – that is, the toxicity of about half of the dissolved pesticides detected was not assessed. This likely explains why the MCR was systematically lower than 2 with this model, and why the mixture effect, calculated with only half of the substances present, was not demonstrated for any of the three taxa. Dataset availability was the main factor of uncertainty in our risk assessment. A more extensive ecotoxicological dataset would improve the value of a strict CA model regarding marine and lagoon species and also decrease the assessment factors, thus reducing overall uncertainty (Faust et al., 2019; Kortenkamp et al., 2019). In a study looking at several ways of deriving EQS, Merrington and Van Sprang (2014) concluded that seven chronic values in three trophic levels provided the best relationship between strong protection of the whole ecosystem and low uncertainty in ERAs. Unfortunately, this amount of ecotoxicological information is rarely available apart for certain priority substances. Even their recommendation that the base dataset should include at least three acute values from three trophic levels by default to reasonably reduce uncertainty in deriving EQS is challenging, as to date, this ‘optimal’ dataset is not complete for all pesticides. In our study, the strict CA model did not determine the herbicides metolachlor and chlorotoluron as single risk drivers, while the extensive CA did. This difference was not due to a poor ecotoxicological dataset, as both substances had a base dataset, but to the calculation methods for deriving TU values and risk limits that differ between the two methods. For example, the maximum TWAC quantified for chlorotoluron was 13.2 ng/L (OR site, 08 December 2015). With the extensive CA method, its WQC was 10 ng/L. Thus, its TU was higher than 1 (1.32), indicating a chronic risk. With the strict CA method, its lower NOEC was reported for a seawater phytoplankton species (~1000 ng/L). So its TU was lower than 0.1 (0.0132), indicating no chronic risk for phytoplankton. These examples suggest an area of uncertainty in these methods near their respective risk limits. The ‘safest’ option would be to consider these two pesticides as potential risk drivers until the ecotoxicological datasets are enriched, reducing the uncertainty. Despite the limitations mentioned above, in a review of experiments measuring pesticide mixture toxicity, Belden et al. (2007) reported that the strict CA model was broadly applicable. These authors found that the difference between the toxicity predicted by this

model and observed toxicity was in the range of 0.5–2.0 in 88% of experiments. Given this acceptable margin of error, this indicates that this type of ERA can provide useful complementary information to the current WFD method to better assess the effect of mixtures.

Our results show that the two CA methods are complementary. The extensive CA method allows a first broad screening of the risk using all available ecotoxicological data, so that sites and substances can be prioritized for further investigation. The strict CA method provides more biologically detailed information, but relies only on half the available exposure data, due to the lack of specific ecotoxicological data for some taxa. In our case, it was carried out only on three taxa and thus cannot be considered representative of the whole ecosystem. Therefore, we recommend using them together to improve the risk assessment. As both methods indicated a theoretical risk related to pesticide mixtures in lagoon waters, subsequent risk assessment steps (response addition model, modelling) could be applied in the future (Backhaus and Faust, 2012; Faust et al., 2019).

The two CA methods used to assess mixture toxicity in this study assumed the absence of interaction between pesticides. Several recent studies have reported synergistic effects between pesticides (mainly in binary mixtures) and particularly between biocides (Dupraz et al., 2019, 2018; Koutsaftis and Aoyama, 2006), although Cedergreen (2014) concluded that true synergistic interactions between chemicals seem to be rare and occur mainly at high concentrations. An earlier study by Belden et al. (2007) determined that the CA model has a relatively small likelihood of underestimating effects due to interactions. Yet a recent study reported that the presence of adjuvants tends to markedly increase the toxicity of active substances contained in the market formulation compared to active pesticide substances alone (Nagy et al., 2020). These adjuvants often act as synergizers; however, they are not currently considered in ERAs. Further investigations are needed to assess if these synergistic interactions act in a similar way to interactions between pesticides.

Another potential risk we did not assess is two historical priority insecticides, DDT and its metabolite DDE, which were detected in the flesh of mussels during the 2018 WFD campaign in the Thau Lagoon (Bouchoucha et al., 2019). The quantification in water of these extremely lipophilic insecticides is complex. Other types of integrative passive samplers (Ellis et al., 2008) or models based on bioconcentration factors (Wang et al., 2022) would be needed to extrapolate their concentration in lagoon waters. Although they were found to be present as traces (maximum value of the sum of the two compounds:  $3.34 \mu\text{g}\cdot\text{kg}^{-1} \text{ ww}$ ), these substances may constitute an additional risk for the Thau Lagoon organisms that merits further study.

While the CA models are considered as a valid initial approximation of a theoretical worst case of toxicity, in reality they may still not to be a worst case. As outlined above, the limitations include: (i) the number of substances monitored, which is still far from the real number of substances present in waters, (ii) the absence of assessment of very lipophilic substances such as DDT, (iii) the potential

synergistic effect between active substances and between these and their adjuvants, which is not considered, and (iv) the need for more – and more environmentally relevant – ecotoxicological data regarding lagoon ecosystem species. The CA methods used provided much useful information both on new risk drivers (not just priority substances) that may have an individual effect on lagoon ecosystems and on pesticide mixture effects. They are therefore totally complementary to the WFD regulatory risk assessment.

Table 2. A comparison of the results of the ERA methods applied to assess risk in the Thau and Or lagoons: (1) WFD for individual priority pesticides, (2) application of TUs of all single pesticides, (3) extensive CA model for pesticide mixtures, and (4) strict CA model for pesticide mixtures. \*: Number of single pesticides exceeding their EQS or WQC / Number of pesticides used for assessment; \*\*: Number of pesticides for which the TU exceeded 0.1 (for phytoplankton) or 0.01 (for crustaceans and fish) / Number of pesticides used for assessment. Background colours indicate no risk (green) or chronic risk from individual pesticides or mixtures (red).

Lagoon	Pesticide exposure		Environmental risk assessment methods						
	Number of pesticides	Source of the risk	WFD	TUs	Extensive CA model RQ <sub>TWACW</sub>	Strict CA model RQ <sub>STU</sub>	for phytoplankton?	crustaceans?	fish?
Thau	68	28	Single pesticides	No (0/10)*	Yes (1/8)*, in summer	Yes (1/28)*, in summer	Yes (1/16)**, all year round	No (0/11)**	Yes (2/12)**, between spring and autumn
			Mixtures of pesticides	Not assessed	Not assessed	Yes, in spring and summer	No mixture effect	No mixture effect	No mixture effect
Or	68	37	Single pesticides	No (0/10)*	Yes (8/37)* all year round	Yes (8/37)*, all year round	Yes (1/22)**, occasionally	Yes (1/17)**, in summer and autumn	Yes (4/18)**, all year round
			Mixtures of pesticides	Not assessed	Not assessed	Yes, all year round	Yes, in winter	No mixture effect	Yes, occasionally in summer

### 4.3 Increasing pesticide monitoring frequency to consider risk seasonality and risk levels in lagoons

The results of our risk assessments clearly showed that the cumulative and/or single effects of pesticides may lead to chronic toxicity for different organisms in lagoon ecosystems. This directly depends on the composition of co-occurring pesticides, and their concentration in the mixture, which change over time, seasonally (Fig. 5), and according to the lagoons, depending on use in watersheds and on weather. Risks related to single pesticides exist throughout the year in both lagoon ecosystems we investigated, while mixture risks are a threat throughout the year in Or Lagoon and between May and August (in spring and summer) in Thau Lagoon (Fig. 4, Table 2).

As WFD monitoring of the chemical status of transitional waters is currently carried out once every three years and only in spring (French Ministry of an Ecological Transition, 2018), this is clearly insufficient to determine the most realistic annual risk, when the real risk cannot be determined. We found that the risk in spring is representative only of recurrent substance risks throughout the year (which can be significant), but is not indicative of specific summer or winter risks (Fig. 5, Table 2). So, monitoring solely during spring leaves information gaps regarding both mixture composition and pesticide risk drivers, which could result in erroneous assessment of chemical status and underestimating the risk. This is the case for crustaceans in the Or Lagoon, for example, for which we found the risk to be highly season-dependent (Fig. 4, Table 2), the summer being the main season at risk for this taxon. A too limited monitoring frequency could thus lead to an incomplete or biased picture of the pesticide mixture risk. To avoid this, we recommend three important sampling periods (Fig. 5): (i) summer to measure specific biocidal and phytosanitary pesticides, (ii) winter to measure specific winter phytosanitary treatments, and (iii) spring for an overview of all chemicals in waters (banned or not) at a period when many treatments from various uses (urban as well as agricultural) are combined and to get continuity with previous monitoring. Monitoring with integrative samplers during these three periods could enhance knowledge of the dissolved pesticide mixture in lagoon waters and inform subsequent risk assessments. This seasonal time-sampling strategy would greatly improve qualitatively, quantitatively and over time the annual representativeness of the WFD risk assessment.

## 5. Conclusion

Our findings indicate that the current WFD chemical risk assessment in French Mediterranean lagoons does not provide a realistic picture of potential pesticide risk in waters. Pesticide cocktails, mainly composed of non-priority substances, were found in lagoons. In both lagoons, the composition and levels of pesticides changed over time, but all year round revealed potential chronic toxicity for lagoon organisms (phytoplankton, crustaceans and fish), often driven by a few single active substances or transformation products. The risk drivers were a range of current or banned biocides (irgarol), herbicides (ametryn, atrazine, chlorotoluron, diuron, metolachlor) and fungicides (carbendazim, azoxystrobin), which should be serious candidates for priority substances if they are not already. While drastically restricting the use of these substances is an important first step in reducing the overall risk, this may not be sufficient, especially in Or Lagoon, due to mixture effects, which are not considered by the WFD.

To improve the environmental relevance of the current regulatory chemical risk assessment in lagoons, we recommend: (i) investigating more pesticides, including the risk drivers highlighted in this study as well as widely used substances such as glyphosate, (ii) increasing sampling frequency in key seasons, (iii) using integrative samplers to measure pesticide mixtures at trace levels, (iv) analysing their combined effects with both extensive and strict CA models, and (v) increasing pesticide

ecotoxicological data, both quantitatively and qualitatively, i.e. using specific coastal or lagoon species that currently do not have standardized tests. These steps would allow a more realistic assessment of pesticide risk in transitional waters. In a context of global biodiversity loss, with environmental pollution reported one of the major causes, it is urgent to improve risk assessment to better protect lagoon ecosystems. This monitoring methodology could be usefully extrapolated for risk assessment in other lagoon ecosystems.

### **Competing financial interests**

The authors declare that they have no conflicts of interest concerning this work.

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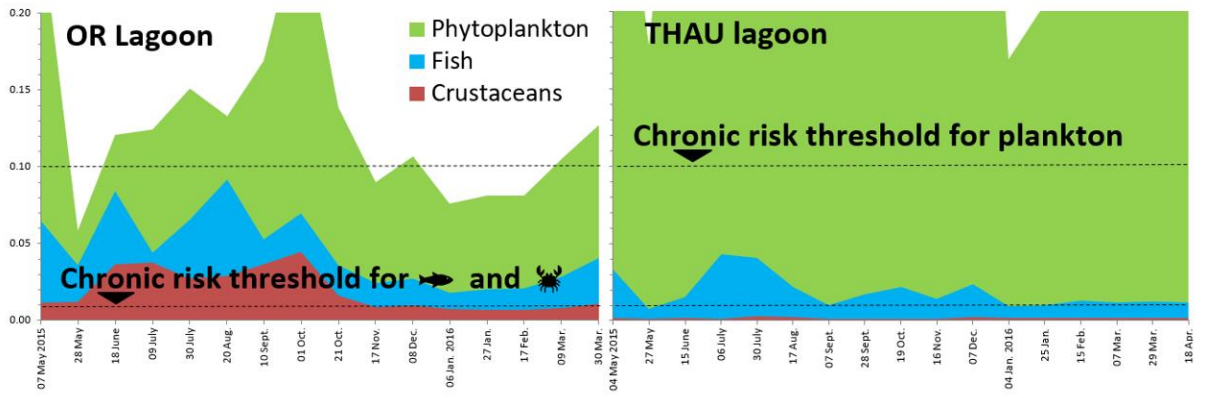
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## Highlights

- Up to 37 pesticides were quantified in French Mediterranean coastal lagoons waters
- Temporal and between-site differences were found in pesticides mixtures and concentrations
- Both mixtures and single pesticides posed a chronic threat to aquatic lagoon life
- Non-priority substances as metolachlor and its TPs were also risk drivers
- Proposals to improve the WFD regulatory monitoring of lagoons were made

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