EVALUATION OF THE OCCURRENCE AND FATE OF PESTICIDES IN A TYPICAL MEDITERRANEAN DELTA ECOSYSTEM (EBRO RIVER DELTA) AND RISK ASSESSMENT FOR AQUATIC ORGANISMS

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ABSTRACT

Delta ecosystems are areas of high ecologic and economic values, where wildlife commonly shares the territory with intensive agricultural activities, particularly, rice cultivation and seafood production. This work aimed at evaluating the occurrence of a wide spectrum of pesticides and transformation products in the water of irrigation and drainage channels of the Ebro River Delta (NE Spain) during the main rice-growing season, when pesticide application is at its peak. Furthermore, the impact that these contaminants may have on local ecosystems and seafood production activities was assessed. A total of 35 pesticides, mainly associated with rice cultivation, out of the 66 analyzed were detected. Bentazone, propanil, MCPA, acetamiprid, and triallate were found at the µg/L level. Cybutryne was measured for the first time in the area and at concentrations above its environmental quality standard (11-49 ng/L), despite being banned in the European Union. Sixteen additional banned pesticides were also detected at trace levels, likely due to their desorption from soil and sediment particles. Bentazone, cybutryne, dicofol, imidacloprid, MCPA, and propanil posed a moderate to high risk for aquatic organisms at the normal concentration levels measured during the rice-growing season. The co-occurrence of pesticides may pose a high risk for aquatic organisms in all sampling locations. Despite its dilution when discharged into the bay, this study demonstrates that the agricultural use of pesticides may have important effects on water quality and may pose a serious hazard for aquatic non-target organisms, despite other factors such as temperature and salinity may play also a relevant role in pesticide toxicity. The finding of imidacloprid and acetamiprid, including in the Watch List, at concentrations above their maximum acceptable method detection limit, calls for control of their use and revision of their legal status.

Keywords: plant protection products, water analysis, agriculture impact, pollutant mixtures, bentazone, propanil, wetlands

Capsule: Agricultural activities in delta ecosystems may pose a risk to non-target aquatic organisms. Banned pesticides may still be of relevance due to sediment desorption.

1. Introduction

Pesticides have been widely used since the mid-twentieth century to control pests and improve agricultural production. However, the global use of pesticides has resulted in their widespread presence in the environment (Fenner et al., 2013). In regions with intensive agricultural activities, thousands of pesticide residues are continuously released in the aquatic environment. Pesticide pollution of water is ruled by different mechanisms, viz., physical-chemical and biological degradation, sorption-desorption into solid particles, surface run-off, soil leaching, plant uptake, volatilization and atmospheric deposition. These mechanisms are, at the same time, linked to the pesticide properties (*e.g.,* solubility, hydrophobicity), environmental factors (*e.g.,* salinity, temperature, precipitation events), type of soil/sediment (*e.g.,* organic carbon content, microbial activity), and agricultural management practices (*e.g.,* type of crops, pesticide application rate) (de Souza et al., 2020; Geissen et al., 2015; Hintze et al., 2020).

Wetlands, which are often scenario of intensive agriculture, play also an important role in removing excess pesticides (Lizotte et al., 2012), although with some limitations, because the fate and effects of pesticides in these ecosystems are largely unpredictable and far from being fully known (Rooney et al., 2020). Therefore, the monitoring of these contaminants in areas with intense agriculture, wetlands and adjacent bays remain a subject of scientific interest, provided that pesticides can persist in water, accumulate in sediments and biota, and hence, affect non-target organisms (Bustos et al., 2019; de Souza et al., 2020; Iturburu et al., 2019).

The Ebro River Delta (Catalonia, NE Spain) is one of the largest wetlands in the western Mediterranean (320 Km^2). Similarly to other Mediterranean areas, this delta has been used for agricultural purposes.

Most of the pesticides used in this area are commonly employed also at European level for the control of pests in rice crops, e.g., the herbicides bentazone, molinate, and propanil, mixtures of MCPA with propanil or bentazone (EC, 2003), or the pyrethroids insecticides cypermethrin and deltamethrin (Feo et al., 2010), and occasionally fenitrothion and malathion (Kuster et al., 2008). These pesticides are less persistent and bioaccumulative than the classical organochlorine pesticides, whose use has been banned for years in many countries. However, because they are produced and applied in high quantities, they still represent a potential threat to the aquatic ecosystems (Aguilar et al., 2017; Montiel-León et al., 2019; Parsons et al., 2010). Their behaviour in the environment is strongly linked to their physical-chemical properties. As a general rule, polar compounds are likely to remain in the aqueous phase and potentially leach into aquifers, while less polar compounds are persistent and tend to be sorbed into sediments and bioaccumulate in living organisms. Moreover, once in the environment, all pesticides can degrade through chemical, physical or biological processes and transform into other compounds (Fenner et al., 2013; Ji et al., 2020), which are usually more polar, and hence more mobile. In some cases, these transformation products (TPs) become more persistent and even more toxic for the aquatic environment than their corresponding parent compound (Bustos et al., 2019; Buttiglieri et al., 2009; Gutowski et al., 2015; Hensen et al., 2020; Sinclair and Boxall, 2009).

In the context of the Water Framework Directive (Directive 2000/60/EC) for the protection of freshwater resources (EC, 2000), 24 pesticides or biocides have been identified as hazardous substances for the environment and are considered as priority substances (EC, 2013). This means that their concentrations in inland surface waters and biota should be below environmental quality standards (EQS). Furthermore, six additional pesticides are included in the European Watch List of substances for Union-wide monitoring (EC, 2018), to gather information to decide on their consideration as priority substances. All the existing regulations issued to protect water quality are focused on the presence of single compounds and do not consider the co-occurrence of multiple contaminants. Indeed, most of the studies to assess pesticide toxicity are conducted with individual pesticides, and possible cumulative or synergic exposure effects caused by compounds of different nature are neglected (Cedergreen, 2014; Verro et al., 2009). In this regard, some studies have already demonstrated that the toxicity effects caused by the co-exposure to a pesticide mixture can be much higher than those derived from the corresponding additive exposure to the single compounds (Backhaus et al., 2004; Gatidou et al., 2015; Junghans et al., 2006). Thus, the real ecotoxicological impact of pesticide mixtures is still largely unknown to date (Kuzmanović et al., 2016).

Within this context, the objectives of this study were to (i) investigate the simultaneous presence of 66 pesticides in the Ebro River Delta freshwaters, in terms of their spatial distribution and fate in the study area, and (ii) assess the potential ecotoxicological risk of individual pesticides and the pesticide mixtures. All results obtained were finally integrated with the ultimate goal of identifying the main hazards for aquatic organisms and for the seafood production that takes place in the Ebro River Delta during the rice-growing season.

2. Materials and methods

2.1 Chemicals and standards

High purity (96-99.9%) standards of the 66 selected pesticides and 48 isotopically labeled compounds used as internal standards (IS) were purchased from Fluka (Honeywell Specialty Chemicals Seelze GmbH, Germany), Sigma Aldrich (Merck KGaA, Darmstadt, Germany), Toronto Research Chemicals (North York, ON, Canada), Cambridge Isotope Laboratories (Tewksbury, MA, USA), or Dr. Ehrenstorfer (LGC Standards, Teddington, UK). The target compounds are listed in Table S1 in Supplementary Material (SM). The list includes: five acidic pesticides (2,4-D, bentazone, fluroxypyr, MCPA, mecoprop), two anilides (diflufenican, propanil), two azoles (cyproconazole, triadimefon), three carbamates (methiocarb, molinate, triallate), two chloroacetanilides (alachlor, metolachlor), two dinitroanilines (pendimethalin, trifluralin), five neonicotinoids (acetamiprid, clothianidin, imidacloprid, thiacloprid, thiamethoxam), nine organochlorides (2,4’-DDD, 4,4’-DDD, 2,4-DDE, 4,4’-DDE, 2,4’-DDT, 4,4’-DDT, dicofol, heptachlor epoxide, oxadiazon), thirteen organophosphates (azinphos ethyl, azinphos-methyl, azinphos-methyl oxon, chlorfenvinphos, chlorpyrifos, diazinon, dichlorvos, dimethoate, fenitrothion, fenitrothion oxon, fenthion, malaoxon, malathion), five organothiophosphates (fenthion oxon, fenthion oxon sulfone, fenthion oxon sulfoxide, fenthion sulfone, fenthion sulfoxide), four phenylureas (chlortoluron, diuron, linuron, isoproturon), two pyrethroids (cyhalotrhin, cypermethrin), eight triazines (atrazine, cyanazine, cybutryne, deisopropilatrazine, desethylatrazine, simazine, terbuthylazine, terbutryn), and four pesticides of other chemical classes (bromoxynil, oxyfluorfen, quinoxyfen, thifensulfuron methyl). The 66 target pesticides were selected considering their current legislation and use in Europe, Spain, Catalonia, and rice crops, according to previous studies and information from local authorities.

Individual stock solutions were prepared at a concentration of 1000 µg/mL in methanol (MeOH) in the case of polar compounds or 100 µg/mL in ethyl acetate (EtAc) in the case of nonpolar compounds. As an exception, simazine and its IS analog were prepared in dimethyl sulfoxide to overcome solubility problems. Intermediate working mixture solutions containing all the standards and/or the IS were prepared by appropriate dilution of the individual stock solutions in MeOH or EtAc. These mixtures were used for the preparation of the calibration solutions and in the validation studies. All these solutions were stored in amber glass bottles at -20 °C protected from light. Pesticide-grade solvents used, i.e., MeOH, EtAc, acetonitrile (ACN), dichloromethane (DCM), hexane, and LC-grade water were supplied by Merck (Darmstadt, Germany).

2.2 Extraction procedures and instrumental analysis

Two different analytical approaches had to be employed to cover all analytes. Medium to highly polar compounds were analyzed according to Barbieri et al. (2020), using a method based on on-line solid-phase extraction-liquid chromatography-tandem mass spectrometry (SPE-LC-MS/MS). Briefly, water samples (5 mL) were preconcentrated onto previously conditioned CHROspe cartridges (divinylbenzene polymer, 10 mm x 2 mm i.d., 25-35 µm particle size) (Axel Semrau GmbH & Co. KG, Srockhövel, Germany) using an automated on-line SPE sample processor Prospekt-2 (Spark Holland, Emmen, The Netherlands) at a flow rate of 1 mL/min. After sample loading, the cartridges were washed with 1 mL of LC-grade water and the analytes were eluted with the LC mobile phase onto the chromatographic column. LC-MS/MS analysis was performed using a reversed-phase Purospher® STAR RP-18 end-capped column (100 mm x 2 mm i.d., 5 µm particle size) from Merck, a 1525 binary HPLC pump (Waters, Milford, MA, USA), and a TQD triple-quadrupole mass spectrometer (Waters) equipped with an electrospray ionization (ESI) source operated in both positive and negative modes. Mass acquisition was done in the selected reaction monitoring (SRM) mode.

Nonpolar pesticides were analyzed following a previously optimized method based on liquid-liquid extraction (LLE) and gas chromatography-tandem mass spectrometry (GC-MS/MS) detection (Peris and Eljarrat, 2020). Water samples (50 mL) were manually extracted twice with 25 mL of EtAc/chloroform (1:1) mixture by classical LLE in a 100 mL separatory funnel. The extract obtained was evaporated under a gentle stream of nitrogen: firstly, to an approximate volume of 1 mL using a Turbovap (Biotage, Sweden), and then, to dryness using a needle evaporator (Reacti-Vap III, Pierce, USA). The dried extract was reconstituted in 50 µL of EtAc (1000x concentration factor). GC-MS/MS determination was performed using a 7890B GC system coupled to a 7000C triple quadrupole (Agilent Technologies, Santa Clara, CA, USA) detector. For chromatographic separation, a DB-5MS column (30 m x 250 µm x 0.25 µm) was used.

Table S2 in SM summarizes the analytical limits of detection (LODs) and quantification (LOQs) achieved for the target pesticides with the methodologies employed. Table S3 in SM reports the main LC-MS/MS and GC-MS/MS acquisition parameters.

2.3 Study area

The Ebro River Delta is an area of high ecological and agricultural value. This delta is characterized by two lateral spit bars NW and SW of the river mouth that form two bays, namely Fangar (NW) and Alfacs (SW). Although fishing and aquaculture are economic activities of relevance, agriculture is the main occupation, with 80% of the land devoted to rice cultivation (Köck et al., 2010). Rice cultivation in the Ebro River Delta extends for 22,000 ha and as for this, an extensive network of irrigation and drainage channels has been constructed. Two main channels, located on each side of the river, move water into the fields for irrigation, and also collect the water excess (Ochoa et al., 2012). Thus, this channel network plays an important role in the transport of pesticides from rice crops to the bays, where aquaculture is also an important activity, especially for the cultivation of oysters and mussels. Moreover, there are several chemical industries in the area that may also affect the quality of the water in the Delta (Gusmaroli et al., 2019). In addition to the impact that these activities may have in the Delta ecosystem, hydrological changes derived from climate change have also an effect on the chemical status of its water (Batalla et al., 2004; Ccanccapa et al., 2016). In this regard, the Ebro Delta, due to its Mediterranean character, is a very vulnerable area that has faced major changes since the last century as a consequence of global warming (Taller d'Enginyeria Ambiental SL, 2008; Somoza and Rodríguez-Santalla, 2014).

2.4 Sampling

Sampling was conducted in June 2017, during the main rice-growing season, and hence the highest use of pesticides in the area, reflecting the worst-case scenario. The Ebro Delta area and the sampling sites are shown in Figure 1 (coordinates of sampling locations are provided in Table S4 as SM). The freshwater sites sampled included nine locations in the northern part (Fangar bay) and nine in the southern part (Alfacs bay). Most of these samples were collected from drainage channels (fourteen samples), whereas five of them were taken from irrigation channels. The irrigation channels were sampled at different sites with an increasing proportion of recovered water from the fields (Terrado et al., 2007).

Samples were collected in amber polyethylene terephthalate (PET) bottles, transported in refrigerated containers to the laboratories, and frozen at -20 °C until their analysis. Before sample extraction, the samples were spiked with the mixture of IS, and, for the analysis of medium to highly polar compounds, which were only measured in the aqueous phase, they were also centrifuged at 3500 rpm at room temperature for 10 minutes to remove suspended particles (centrifuge 5810 R, Eppendorf Ibérica, Spain).

2.5 Statistical analysis

For statistical purposes, the concentration of non-detected values was set to half the LOD, while the non-quantifiable values (<LOQ) were assigned a concentration of LOQ/2. Furthermore, those pesticides with low detection frequencies (<30%) were excluded. A detailed analysis of the investigated variables and the statistical analyses performed is provided as supporting information (Text S1 and Table S5).

The relationships among pesticides occurrence in the study area was investigated through pairwise correlations using the Spearman's rank test. A significance level of 0.05 was established. Principal-component analysis (PCA) was used to extract useful information from the data, e.g., to investigate multivariate correlations between the concentrations of the different pesticides, and their geographical distribution.

All statistical analyses were performed using the R statistical software interface R-Studio (R version 3.6.3). The R code used is stored in the GitHub repository https://github.com/albamgarces/PCA_EbrePesticides_2020.

2.6 Risk assessment

The environmental risk that the presence of pesticides in the freshwater samples may pose to aquatic organisms was assessed using the hazard quotient (HQ) approach. The risk quotient of a single pesticide (HQi) was calculated using the equation $\mathrm{HQi} = \frac{\mathrm{MEC}}{\mathrm{PNEC}}$, where MEC is the measured environmental concentration and PNEC is the lowest concentration at which toxic effects are not expected (predicted no-effect concentration). In this study, the average concentration measured for each pesticide was used as MEC to assess the general risk and the maximum concentration was used to evaluate the worst-case scenario. PNEC values were obtained from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/) (Dulio and Von der Ohe, 2013), and corresponded to the lowest value predicted by QSAR models or obtained experimentally. Moreover, to have a complete view of water pollution by pesticides in the area and the associated ecotoxicological risk, an additive model was applied. For this, the HQ of each sampling site (HQs) was calculated as the sum of the HQ of the various pesticides present in the corresponding sample (HQi), following the equation: $\mathrm{HQs} = \sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{HQ}_i$. Such an additive model allows to evaluate the potential ecotoxicological risk derived from the co-occurrence of various pesticides in a specific location, although with some limitations, as it does not consider unpredictable synergism or antagonism effects. HQ values below 1 indicate zero or low risk, while HQ values between 1 and 10 anticipate moderate risk, and HQ values above 10 suggest high environmental risk.

3. Results and discussion

3.1 Occurrence of individual pesticides

The occurrence of the investigated pesticides in surface waters of the Ebro River Delta is summarized in Table 1 and detailed in Table S8 in the SM. Moreover, the pairwise correlations among pesticide concentrations in the investigated samples (Figure 2; Figures S1 and S2 Table S6), have been also evaluated to further explain the results observed.

More than half of the target pesticides (35 out of 66) were detected in the Ebro River Delta surface waters. It is important to highlight that in the case of medium and highly polar pesticides, their occurrence was investigated only in the aqueous phase, as suspended particles were removed before sample extraction. However, in the case of apolar pesticides, amounts sorbed onto suspended particles may have eventually been recovered during the sample extraction process, although the determination in the solid phase of freshwater was not the objective of the study.

The compound that was found at the highest concentration was the herbicide bentazone, found in all investigated samples at the maximum concentration of 18×10^4 ng/L. Individual concentrations of propanil, MCPA, acetamiprid, and triallate also reached the µg/L level, with maximum values of $61\text{x}10^3$, 8200, 4000, and 1000 ng/L, respectively. Imidacloprid and 2,4-D could be also highlighted among the most abundant pesticides in the investigated area, with concentrations above 100 ng/L, and occurrence peaks of 700 and 440 ng/L, respectively.

Among the most abundant pesticides aforementioned, bentazone and triallate were the two most widely distributed herbicides, found in all sampling sites. The remaining most abundant compounds were detected in more than half of the samples (61-83 %), except 2,4-D that was present in 50 % of the samples (Table 1). The presence of these pesticides is related to their agricultural use in rice crops. Bentazone, propanil, and MCPA are indeed among the herbicides mostly applied at the European level for the control of pests in rice crops and, consequently, their presence in the Ebro Delta surface waters has been previously documented. Similar bentazone concentrations (up to $13\text{x}10^4$ ng/L) were reported in a study conducted in this area during the rice-growing season ten years ago (Kuster et al., 2008). This finding suggests a repeated pattern of high bentazone levels in the Ebro Delta during the same period throughout the years. Its physical-chemical properties (Table S1 in SM) - high solubility (7112 mg/L) and polarity (log K_{ow} -0.46), relatively high half-life time in water (DT_{50}) (80 days), and rather low organic carbon-water partition coefficient (K_{oc} 55) - denote its preference to remain in the water, and thus, justify the high concentrations found after its application. Similar conclusions could be drawn for MCPA and propanil, pesticides continuously found in the Ebro Delta surface waters (Köck et al., 2010; Kuster et al., 2008). Despite propanil was withdrawn from the EU market in 2008 (EC, 2008), its detection is still possible because the Spanish Government annually issues an exceptional authorization for the use of this active substance in rice crops during the growing season (May to July) (Spanish Ministry of Agriculture, 2017).

Although bentazone concentrations were not strongly correlated with those found for MCPA or propanil, these two showed a significant and strong positive correlation (Figure 2 and Figures S1 and S2). This could suggest a similar application pattern of MCPA and propanil in this area, which differs from that of bentazone. Note that contrary to MCPA (auxin synthesis inhibitor), bentazone and propanil share a common mode of action (photosynthesis inhibition). MCPA and propanil were negatively correlated with triazine herbicides like terbuthylazine and cybutryne, and the carbamate herbicide triallate. Terbuthylazine and triallate may be more commonly applied in crops other than rice in the area, while cybutryne is no longer approved for use and residual concentrations may be released from the sediments/soils from past use as an antifouling paints for ships and boats. Triallate, scarcely investigated in the Ebro Delta freshwaters in previous studies, was recently reported to occur in this area at very low concentrations (<2 ng/L, in 24% of the analyzed samples) (Gusmaroli et al., 2019). The herbicide triallate showed also a high and significant correlation with cybutryne and chlorpyrifos (Table S6, Figure 2 and Figures S1 and S2). While the correlation with cybutryne is difficult to explain, since its use is banned, that with chlorpyrifos, an organophosphate insecticide used to control foliage and soil-borne insect pests in a variety of crops, especially in corn and other cereal fields, could be explained by the simultaneous application of these two pesticides in the study area.

The herbicide 2,4-D, largely used in cereal crops, presented an average concentration of 41 ng/L in this study. This herbicide was also found at similar concentrations in previous studies conducted in this area by Terrado et al. (2007) (mean of 22 ng/L) and Barata et al. (2007) (mean of 24 ng/L).

In addition to bentazone and triallate, other pesticides like chlorpyrifos, cybutryne, and metolachlor can be classified among the most ubiquitous in the area of study (detection frequency of 100%), but they presented much lower concentrations (up to 27, 49 and 73 ng/L, respectively) than the aforementioned pesticides bentazone and triallate. Chlorpyrifos has already been reported as one of the most commonly detected pesticides in the Ebro River in several studies (Claver et al., 2006; Navarro et al., 2010), where it has been found at concentrations higher than those measured in the present work, in spite that it presents a low solubility in water (1.05 mg/L) and is considered not persistent in the water phase (DT50 of 5 days). The concentrations found in this study were always below the EQS of 100 ng/L set for this pesticide in surface water (EC, 2013), in contrast to the concentrations up to 312 ng/L reported in the previously cited study of Claver et al. (2006). On the contrary, the herbicidal biocide cybutryne, from the triazines group, exceeded its EQS of 16 ng/L in 72% of the sampled locations. As previously mentioned, the presence of this compound is not linked to agriculture practices, but to its application as an antifouling agent in paints for boats and other water vessels to control slimes, molds, mosses, and algae (Lewis et al., 2006). Since 2016 its use in antifouling products is no longer allowed in the European Union (EC, 2016) and, therefore, its detection could be attributed to illegal use of cybutryne stocks or, what it is more likely, to resuspension from the Delta sediments. According to its properties (low water solubility 7 mg/L, high log K_{ow} 3.95, and high K_{oc} 1569) this compound is likely to sorb onto particles, which is supported by the report of its presence in freshwater sediments of Mediterranean areas (Barbieri et al., 2019). To the best of our knowledge, cybutryne has never been investigated before in the Ebro River Delta and hence the results obtained cannot be compared with historical data. Metolachlor, an herbicide usually found in the Ebro Delta freshwaters, was also detected in this study, at concentrations similar to those reported in previous works (Claver et al., 2006; Köck et al., 2010), even though it is no longer commercially available in the EU market (EC, 2002).

A large proportion of the pesticides identified in the study area are currently banned for use (17 out of 35 detected pesticides). Strong positive correlations were also found between banned pesticides (chlorfenvinphos + diazinon, chlorfenvinphos + terbutryn, or diazinon + terbutryn) (Table S6, Figure S3, and Figures S1 and S2). Like cybutryne, these positively correlated pesticides present a Log K_{ow} above 3.6, and thus, a high tendency to sorb onto soil and sediment particles, and a GUS index above 1.5 (Table S1). Thus, their presence in the area could also result from desorption of solid particles where they may be accumulated.

As for the neonicotinoid insecticides detected at the highest concentrations in this study, *viz*., imidacloprid (up to 700 ng/L) and acetamiprid (up to 4×10^3 ng/L), the former was also found in previous studies conducted in the Ebro Delta, at maximum concentrations of 182 ng/L (Gusmaroli et al., 2019), 16 ng/L (Borrull et al., 2019) and 15 ng/L (Ccanccapa et al., 2016), while the latter was measured for the first time at maximum concentrations of 58 ng/L in a few samples in the recent study conducted by Gusmaroli et al. (2019). Since acetamiprid is the neonicotinoid with the shortest half-life DT50 (4.7 days) and imidacloprid is the neonicotinoid with the highest K_{oc} (6719) and moderate water solubility (610 mg/L), the high concentrations detected indicate that these two insecticides are extensively applied in the Ebro Delta to control sucking insects on crops like rice, cereals, potatoes, and sugar beet.

3.2 Spatial distribution of pesticide contamination patterns in the Ebro River Delta

The contribution of each pesticide class to the total pesticide levels in the investigated samples is illustrated in Figure 3. As can be seen, the profile of pesticide contamination in the Ebro Delta is overall characterized by the dominant presence of acidic pesticides (85 % contribution to total pesticide levels on average, with 82 % corresponding to bentazone) and anilides (14 %). The contribution of all the remaining pesticide classes was lower than 2 % in each case. Triazines and organophosphate pesticides, although at lower levels than acidic pesticides and anilides, were also among the most detected and ubiquitous pesticide groups in the investigated waters, with 2 to 5 different compounds present in each sample. PCA was used to statistically investigate pesticide contamination patterns (Figure 4) and the geographical distribution of the identified contamination patterns in the Ebro Delta during the main rice-growing season (Figure 5) (further details in SM, Figures S3 and S4). Up to 65 % of the data variance could be explained with four principal components (PCs) (Table S7). Overall, diffuse contamination patterns were identified by each PC, with several different pesticides (including banned pesticides) contributing in each case. However, all four PCs describe a contamination pattern mainly coming from rice-growing fields (due to the presence of MCPA, bentazone and/or propanil) that is inversely correlated to pesticides coming from other sources (different agricultural activities in the area or main river transport from upstream activities) (Figure 4). In the case of PC4, which explains only 9% of the total variance, pesticides used for rice cultivation were inversely correlated, and thus, this PC may describe a contamination pattern generated by small local changes in the use of pesticides (Figure 4).

Although the spatial distribution of pesticide pollution was variable (Figure 2), overall waters in the Alfacs bay (south of the Delta) were more contaminated than in the Fangars bay (north), in terms of co-occurrence of pesticides and total pesticides loads. PCA scores plots also indicate indeed a different pesticide pattern in Alfacs bay compared to Fangars bay (Figure 5). Overall, all PCs point a contamination pattern coming from rice-growing fields in most sampling locations of the Alfacs bay, although some locations of the Fangars bay were also exceptionally included in each case. This type of contamination was found in both drainage and irrigation channels. PC4 indicated that MCPA and propanil use was relevant in ACD1, ACD6 and ACE2 locations of the Alfacs bay, while bentazone application was predominant in other locations of this bay (ACD2, ACD3, and ACD5) and in FCD3 of the Fangars bay.

The most contaminated sites of the Alfacs bay were ACD1 and ACD6, which correspond with drainage channels, located close to the main course of the river and south of Deltebre town, that collect water from the fields located nearby. Both sites were highly contaminated with bentazone, MCPA, and propanil, due to the use of these pesticides for rice and cereal cultivation in the surrounded areas. s. The pesticide contamination pattern described by PC3 and PC4 was similar in these two locations, but those described in PC1 and PC2 were exclusive for ACD1 and ACD6, respectively (Figure 5). This could be explained because there is less rice cultivated area upstream (ACD1) than downstream (ACD6). The farmland upstream is also devoted to the cultivation of fruit trees, especially citrus fruits such as orange and tangerine, which would also explain the presence of pesticides that are not commonly applied in rice field. This hypothesis could be confirmed by the increasing bentazone and MCPA concentrations in the downstream direction.

In the irrigation and drainage system network further south, water pollution by pesticides increased in the direction to the bay (from ACD2 to ACD5 sampling sites). Each water sample collected in the Alfacs drainage channels presented a total co-occurrence of more than 20 pesticides, which reflects the high use of pesticides in the area during the sampling period. While ACD4 receives water from ACD2, which may explain the increased levels found in ACD4 (both showing positive scores in PC1). ACD3 and ACD5 are independent channels that do not receive water from the aforementioned channels, and thus, pesticide pollution found in their waters has its origin on the drained crop fields (ACD3 has a similar contamination pattern though than ACD2 and ACD4 according to PC1, and ACD5 has a similar contamination pattern than ACD4 according to PC2, though) (Figure 5).

The least contaminated site on the SW side of the delta was the irrigation channel ACE1. In this site, the water is not affected by pesticide application because it comes directly from the right-hand channel of the Ebro River without receiving any input (Terrado et al., 2007) from drainage channels. On the other hand, and contrary to expected, the irrigation channel ACE2, coming directly from Sant Carles de la Rapita town, presented considerable contamination, which may result from runoff events from nearby agricultural fields.

Overall, pesticide contamination patterns in the Fangar bay (NW) was driven by herbicides other than bentazone, MCPA and propanil (e.g., triallate in PC1 and PC3, terbuthylazine in PC2) and a variety of other pesticides. Overall, higher pesticide loads were present in the drainage channels, particularly, in those located nearby the bay, than in the irrigation ditches. The water from FCD5 and to a smaller extent the water from FCD6 feed a green filter designed to improve the quality of the water drained from the rice fields into the bay. The effluent of the filter is discharged into FCD4; however, the sampling location was located before the discharge point, and therefore the results observed cannot be used to evaluate the performance of the filter in terms of pesticide removal.

3.3 Pesticides in the Ebro Delta under the current legal framework

Nowadays, 17 of the 35 detected compounds (see Table 1 and S1 in SM) are currently banned by the European Commission for their use. Of these, propanil is the only one whose exceptional use in rice crops is annually authorized in Spain during the growing season. Thus, the presence of the remaining banned pesticides in the Ebro Delta waters is unexpected and could be attributed to illegal use of existing stock solutions or what is more likely, their release from soils/sediments. Looking at the physical-chemical properties of these compounds (Table S1) (i.e, low water solubility, high octanol-water partition coefficient (log K_{ow}), high organic carbon-water partition coefficient (K_{oc}) and a long half-life time (DT50)), they are not expected to be found in the aqueous phase and they are likely to sorb onto suspended particles and accumulate into soil and sediments. This is particularly true, in the case of the banned pesticides with high log K_{ow} values (>3), i.e, 4,4-DDD, alachlor, azinphos ethyl, chlorfenvinphos, diazinon, dicofol, linuron, oxadiazon, terbutryn, and triadimefon (Table 1). Due to the apolar character of these compounds, they are also likely to bioaccumulate in aquatic organisms, which points out the importance of assessing the environmental risk associated with their occurrence in water but also their occurrence in aquatic organisms, paying special attention to those intended for human consumption.

The only priority pesticide found to occur at concentrations above the established EQS in surface water was the herbicide cybutryne (EQS of 16 ng/L) (100 % of detection frequency and concentration > 16 ng/L in 72 % of the samples) (Table 1). Cybutryne contributed moderately to the pesticide pollution pattern described by PC1 and PC3, observed mainly in most of the Fangar bay samples (Figures 4 and 5).The remaining priority pesticides targeted were either not detected or measured at a concentration below their corresponding EQS (EC, 2013).

As for the pesticides included in the Watch List, the neonicotinoids imidacloprid and acetamiprid, with maximum measured concentrations of 700 ng/L and 4×10^3 ng/L, respectively, largely exceeded the maximum acceptable LOD of 8.3 ng/L set for them in the regulation (EC, 2018). Also, methiocarb was found to exceed its LOD (2 ng/L) in two sampling locations (up to 3.3 ng/L, frequency of detection of 50 %). LOD values set in the legislation for the detection of these substances match their PNECs in water, and therefore, undesired effects on aquatic organisms at the measured concentrations could be expected.

3.4 Environmental risk assessment

To evaluate the impact of the pesticides in the Ebro River Delta ecosystem, the hazard quotient approach was employed, by comparing the maximum and mean measured concentrations of each pesticide with its corresponding lowest PNEC (extracted from the NORMAN ecotoxicology database) (Dulio and Von der Ohe, 2013). The results obtained have been summarized in Table 2. Only 10 out of the 35 pesticides detected in the Ebro Delta presented a certain risk in both investigated contamination scenarios. Bentazone, dicofol, imidacloprid, and propanil exhibited the highest HQ values (HQ > 10) under normal (average) and worst-case contamination scenarios, whereas MCPA and cybutryne only may pose a high risk under the worst contamination scenario. The potential high risk obtained for these pesticides is mostly attributed to the high concentrations measured in the samples, except for dicofol and cybutryne, detected at relatively low concentrations (< 7.3 ng/L in the case of dicofol and 11-49 ng/L in the case of cybutryne). Thus, the high-risk values obtained for these pesticides are mainly driven by their very low PNEC values (0.032 ng/L for dicofol and 3.5 ng/L for cybutryne).

A moderate risk (HQ>1) was obtained for MCPA and cybutryne under a normal (average) contamination scenario and 4,4'-DDD, acetamiprid, azynphos ethyl and diflufenican under the worst-case contamination scenario. Except for acetamiprid, which was detected at high concentrations in the investigated area, the risk associated with 4,4’DDD, azynphos ethyl, and diflufenican can be attributed to their low PNEC values (< 10 ng/L).

Further risk assessment analyses were conducted in this study to evaluate the environmental risk associated with the pesticide mixtures present in each sampling site. As shown in Figure 6 (detailed in Table S9 in the SM), the HQ values obtained suggest a high risk in all sampling locations (HQ>10), even in those with the lowest pesticide loads. The approach used is a simple additive model, and consequently, it may underestimate the real risk because synergistic effects that may occur among co-occurring contaminants are not considered. Despite this, the findings obtained highlight the need for conducting risk assessment studies with pollutant mixtures. For mixtures of substances that do not share a common mechanism of action, effects on joint toxicity can be expected. This hypothesis has been already confirmed in several studies that investigated the co-exposure to pesticide mixtures and their synergistic effects on non-target organisms. For instance, the exposure of carps to organophosphates and carbamate pesticides produced neurotoxicity (Wang et al., 2015), by inhibiting the activity of acetylcholinesterase (AChE) and interfering with the normal behavior of this species. The combined presence of organochlorine and organophosphate pesticides resulted in a synergistic effect that decreased the immune response capacity of white shrimps (Abad-Rosales et al., 2019; Bautista-Covarrubias et al., 2020). A recent study has also demonstrated that mixtures of pyrethroids and neonicotinoids exhibit a synergistic effect in the enzyme activity and gene expression of embryonic zebrafish (Wang et al., 2020). Additional evidence of additive toxicity has been observed after exposure of duckweeds to phenylureas and algal plants to triazines, which resulted in the blockage of the transport of photosynthetic electrons at the level of the photosystem II (Faust et al., 2001; Gatidou et al., 2015).

In the Ebro River Delta, two studies have investigated the potential toxic effects of some pesticides on non-targeted organisms (Álvarez-Muñoz et al., 2019; Ochoa et al., 2012). In 2017, analysis of shellfish specimens dead in the course of mortality episodes that took place in the Ebro

Delta between April and November showed the presence of small concentrations of metolachlor, atrazine, bentazone, and acetamiprid. In this study, mortality events were not associated with any particular chemical present in the water but with other causes such as the presence of potential pathogens (Álvarez-Muñoz et al., 2019). In a previous study, a similar approach conducted in this case to find out the possibles causes of mortality events affecting oysters cultivated in the Ebro Delta bays showed the presence of bentazone and propanil in the dead organisms and its relation with markers of tissue damage during DNA strand breakage (Ochoa et al., 2012). However, many other factors need to be considered when assessing possible causes that lead to organism mortality, since aspects such as temperature, salinity, runoff, as well as temporal trends of exposure may influence the synergistic effects of pesticides and therefore, their risk to aquatic organisms.

4. Conclusions

The monitoring of 66 pesticides in the Ebro River Delta during the rice-growing season in 2017 revealed the presence of 35 compounds in the Ebro Delta surface waters. Bentazone was found to be the herbicide with the highest concentrations (up to $18x10^4$ ng/L) in all the samples analyzed, followed by propanil, MCPA, acetamiprid, triallate, imidacloprid, and 2,4-D. The occurrence of all these pesticides in the waters is related to their agricultural use and has continuously been documented in this area. Different diffuse pesticide contamination patterns were identified using PCA. All PCs obtained describe a contamination pattern mainly coming from rice-growing fields (due to the presence of MCPA, bentazone and/or propanil) that was inversely correlated to pesticides coming from other sources (different agricultural activities in the area or main river transport from upstream activities). According to the last decade data, total loads of pesticides show an increasing trend, particularly associated with the presence of acidic pesticides and anilides. The neonicotinoids acetamiprid and imidacloprid were measured at concentrations that largely exceeded the LOD established for the analysis of Watch List substances in water. Thus, this study provides relevant information for the revision of the Commission Implementing Decision (EU) 2018/840 (EC, 2018), and based on the results obtained, reduced and controlled use of the neonicotinoids imidacloprid and acetamiprid is recommended. Cybutryne was the only pesticide found above its EQS in surface water, despite being banned. A total of 17 banned pesticides (including propanil, whose use is exceptionally authorized in Spain) were found in Ebro Delta waters, at trace concentrations. Their presence is explained by desorption from soil and sediment particles, where they are likely accumulated due to their physical-chemical characteristics.

The environmental risk assessment carried out indicates that bentazone, propanil, MCPA, imidacloprid, dicofol, and cybutryne pose a moderate to high risk for aquatic organisms at the average contamination levels found. The co-occurrence of different pesticides results in a high potential risk (HQ>10) for organisms in all investigated sites, even in those with the lowest pesticide loads. Although the approach applied to investigate mixture toxicity is a simple additive model and does not consider synergistic effects, it highlights the need of evaluating the effect of all contaminants present in a sample. This work, to the best of our knowledge, is the most complete assessment of pesticide contamination in waters from a delta ecosystem because it assesses the co-occurrence of low to highly polar pesticides and some of their TPs. The results of this study demonstrate that the agricultural use of pesticides has important effects on water quality and may pose a serious hazard for aquatic non-target organisms. However, many other factors need to be considered to link pesticide occurrence with mortality episodes of aquatic organisms in the area, because aspects like temperature and salinity may be also relevant, and even affect the toxic effects of pesticides. Long-term toxicological studies are required to assess the real risk of the pesticide mixtures for the health of wetlands ecosystems.

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Figure 1. Map of the Ebro River Delta, with a detail of the sampling locations. F: Fangar zone; A: Alfacs zone; CD: drainage channel (red spots); CE: irrigation channel (blue spots).

Figure 2. Pairwise correlations between pesticides concentrations found in the study area (after Spearman's Rank test, α= 0.05)).

Figure 3. a) Total concentration of pesticides in the analyzed water samples of the Ebro River Delta; b) contribution of each class to the total pesticide levels. Pesticides included under "Others": azoles, chloroacetanilides, organochlorines, organophosphates, organothiophosphates, pendimethalin, phenylureas. Bentazone is shown outside the class of acids to show its amount separately. (Sample codes: F: Fangar zone, A: Alfacs zone, CD: drainage channel, CE: irrigation channel).

Figure 4. Amount of variance explained by each PC and loading plots showing the main pesticide contamination patterns identified by PCA in the Ebro Delta.

Figure 5. Scores plot of the four contamination patterns identified by PCA showing the spatial distribution of pesticide pollution.

Figure 6. Cumulative hazard quotients (HQs) calculated for the various investigated sampling locations as the sum of the HQ of each pesticide found in the sample, following the equation: $\mathrm{HQs} = \sum_{\mathrm{i}=1}^{\mathrm{n}} \mathrm{HQ}_{i}$.

Table 1. Minimum, maximum, and mean concentration in ng/L of the detected individual pesticides and frequency of detection in the investigated water samples.

Class	***Name***	***Concentration (ng/L)***			
		Min	**Max**	**Mean**[α]	**Frequency**[β] **(%)**
Acidics	2,4-D	10	440	41	50
	Bentazone	150	180×10^3	53×10^3	100
	MCPA	130	8210	1700	61
Anilides	Diflufenican	2.0	19	4.2	50
	Propanil*	21	61×10^3	9000	83
Carbamates	Methiocarb	0.74	3.3	1.0	56
	Molinate*	5.7	48	16	61
	Triallate	41	1000	310	100
Chloroacetanilides	Alachlor*	1.4	1.6	0.17	11
	Metolachlor*	10	73	38	100
Dinitroaniline	Pendimethalin	1.0	1.0	0.61	61
Neonicotinoids	Acetamiprid	0.25	4000	420	67
	Imidacloprid	23	700	130	61
	Thiacloprid	0.11	2.7	0.43	44
Organochlorines	4,4’-DDD*	1.2	1.2	0.07	6
	Dicofol*	3.7	3.7	1.8	50
	Oxadiazon*	0.35	47	18	89
	Triadimefon*	2.0	4.9	1.2	50
Organophosphates	Azinphos ethyl*	0.70	5.6	0.94	33
	Chlorfenvinphos*	0.40	6.3	1.9	72
	Chlorpyrifos	0.75	27	15	100
	Diazinon*	1.0	4.8	2.1	89
	Malaoxon	0.25	0.57	0.060	17
Organothiophosphates	Fenthion oxon	0.81	2.5	0.41	28
	Fenthion oxon sulfoxide	0.22	3.2	0.11	28
	Fenthion sulfoxide	0.70	4.5	0.43	22
Phenylureas	Chlortoluron	7.5	14	1.2	11
	Diuron	5.2	12	5.7	67
	Isoproturon*	13	13	0.70	6
	Linuron*	1.0	13	1.1	22
Triazines	Atrazine*	0.45	2.5	0.19	17
	Cybutrine*	11	49	29	100
	Simazine*	0.55	6.7	1.5	44
	Terbuthylazine	11	41	21	72
	Terbutryn*	1.7	6.6	2.7	89

[α] Mean calculated considering values <LOQ as LOQ/2 and values <LOD as zero.

[β] % of positive samples, including compounds with values <LOQ.

*Compounds currently prohibited for their use in Europe. The exceptional use of propanil is annually allowed in Spain.

Table 2. Hazard Quotient (HQ) for the worst-case (HQ-Max) and the normal (HQ-mean) contamination scenarios.

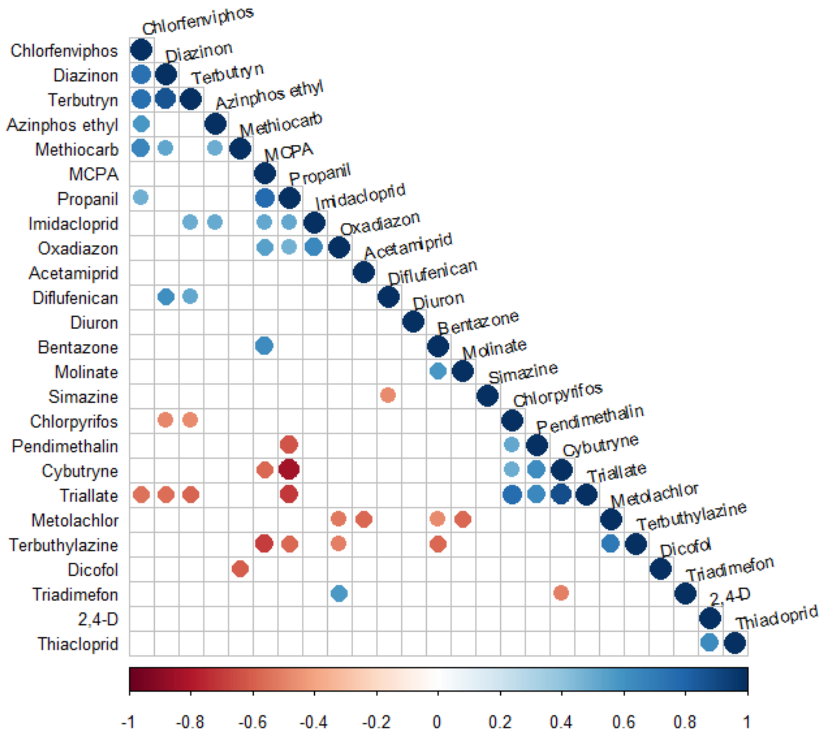
Class	***Name***	**PNEC* (µg/L)**	**MEC-Max (µg/L)**	**MEC-Mean (µg/L)**	**HQ-Max**	**HQ-Mean**
Acidics	2,4-D	12.4	0.441	0.041	0.036	0.003
	Bentazone	0.1	177.4	53.04	**1774**	**530**
	MCPA	0.5	8.212	1.704	**16.4**	**3.41**
Anilides	Diflufenican	0.009	0.019	0.004	**2.07**	0.463
	Propanil*	0.2	61.21	8.968	**306**	**44.8**
Carbamates	Methiocarb	0.01	0.003	0.001	0.332	0.097
	Molinate*	3.8	0.048	0.016	0.013	0.004
	Triallate	10	1.011	0.306	0.101	0.031
Chloroacetanilides	Alachlor*	0.3	0.002	0.0002	0.005	0.001
	Metolachlor*	0.2	0.073	0.038	0.364	0.189
Dinitroaniline	Pendimethalin	0.018	0.001	0.001	0.056	0.034
Neonicotinoids	Acetamiprid	3.74	3.993	0.421	**1.07**	0.112
	Imidacloprid	0.0083	0.703	0.127	**84.7**	**15.3**
	Thiacloprid	0.01	0.003	0.0004	0.266	0.043
Organochlorines	4,4’-DDD*	0.0005	0.001	0.0001	**2.4**	0.135
	Dicofol*	0.000032	0.004	0.002	**114.1**	**57.03**
	Oxadiazon*	0.088	0.047	0.018	0.532	0.207
	Triadimefon*	1.86	0.005	0.001	0.003	0.001
Organophosphates	Azinphos ethyl*	0.0011	0.006	0.001	**5.13**	0.851
	Chlorfenvinphos*	0.1	0.006	0.002	0.063	0.019
	Chlorpyrifos	0.03	0.027	0.015	0.900	0.513
	Diazinon*	0.01	0.005	0.002	0.481	0.211
	Malaoxon	0.31	0.001	0.0001	0.002	0.0002
Organothiophosphates	Fenthion oxon	0.2	0.003	0.0004	0.013	0.002
	Fenthion oxon sulfoxide	n/a	0.001	0.00011	-	-
	Fenthion sulfoxide	10	0.004	0.0004	0.0004	0.00004
Phenylureas	Chlortoluron	7.25	0.014	0.001	0.002	0.0002
	Diuron	0.2	0.012	0.006	0.059	0.029
	Isoproturon*	0.3	0.013	0.001	0.042	0.002
	Linuron*	0.1	0.013	0.001	0.131	0.011
Triazines	Atrazine*	0.6	0.002	0.0002	0.004	0.0003
	Cybutrine*	0.0035	0.049	0.029	**14.03**	**8.294**
	Simazine*	1	0.007	0.001	0.007	0.001
	Terbuthylazine	0.06	0.041	0.021	0.686	0.352
	Terbutryn*	0.065	0.007	0.003	0.101	0.042

*Lowest PNEC extracted from the NORMAN Ecotoxicology Database (https://www.norman-network.com/nds/ecotox/)

*MEC
n/a: not available


41
L'Ampolla
El Perelló
L'Ampolla
Golf de Sant Jordi
EL BAIX EBRE
41
Tortosa
L'Aldea
41
Amposta
St. Carles
de la Ràpita
Camarles
L'Aldea
FCD4
FCD5
FCD6
FCD7
FCE1
FCE2
FCD3
FCD2
FCD1
ACD1
Deltebre
Urbanització Riumar
Balada
l'Ebre
Amposta
TV-3405
ACD6
Sant Jaume d'Enveja
EL MONTSIÀ
TV-3405
Els Muntells
ACD2
ACE1
ACD7
Urbanització l'Eucaliptus
ACD4
ACD5
Poble del Delta
ACE2
ACD3
Sant Carles de la Ràpita
mar Mediterrània
© FRANK I&D, S.L. 977 46 95 60 E-Mail: frank@dsi.es - 2003
0
1
5
10
15 km

Figure 1


Chlorfenviphos
Diazinon
Terbutryn
Azinphos ethyl
Methiocarb
MCPA
Propanil
Imidacloprid
Oxadiazon
Acetamiprid
Diflufenican
Diuron
Bentazone
Molinate
Simazine
Chlorpyrifos
Pendimethalin
Cybutryne
Triallate
Metolachlor
Terbuthylazine
Dicofol
Triadimefon
2,4-D
Thiacloprid
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1

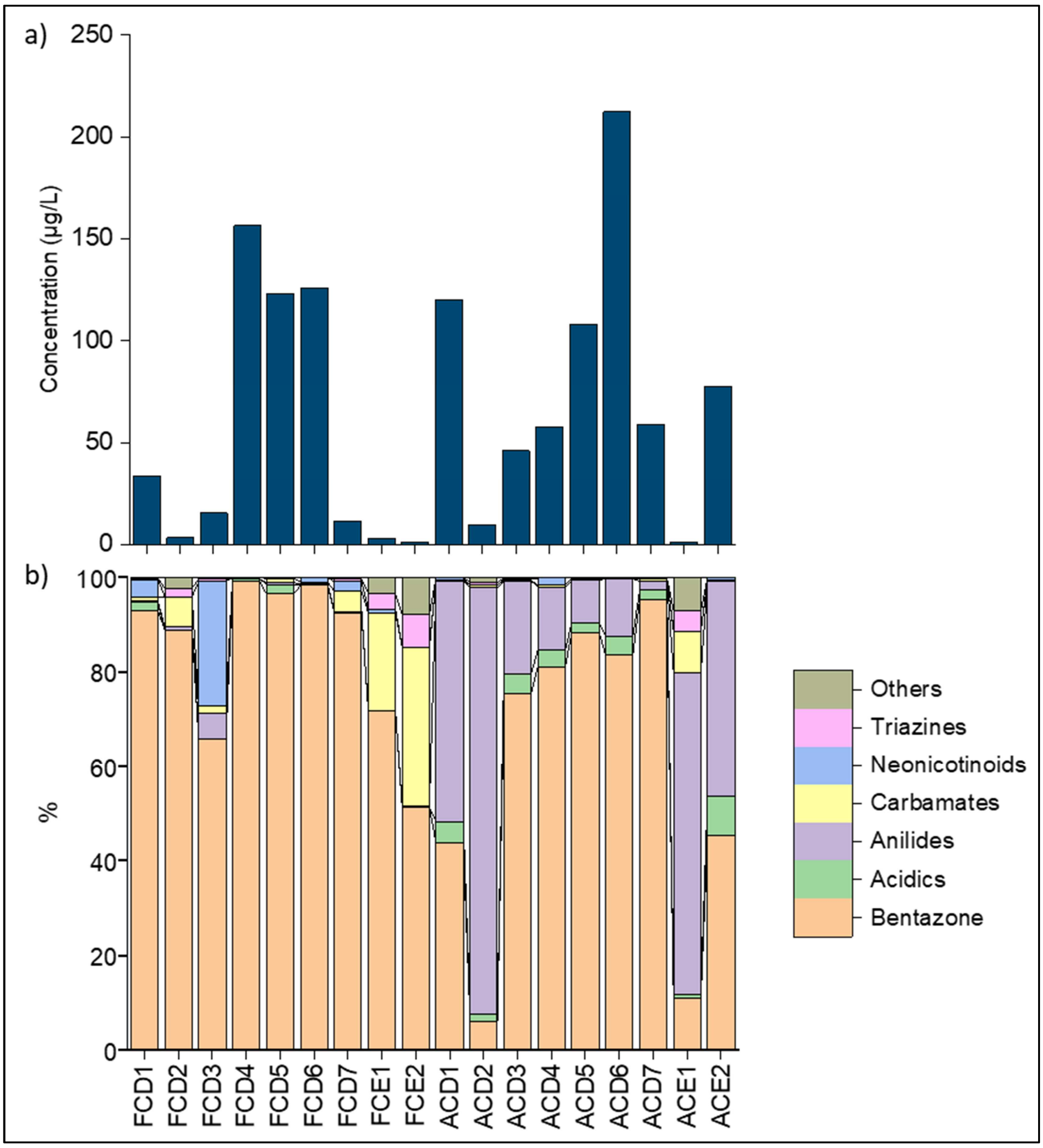
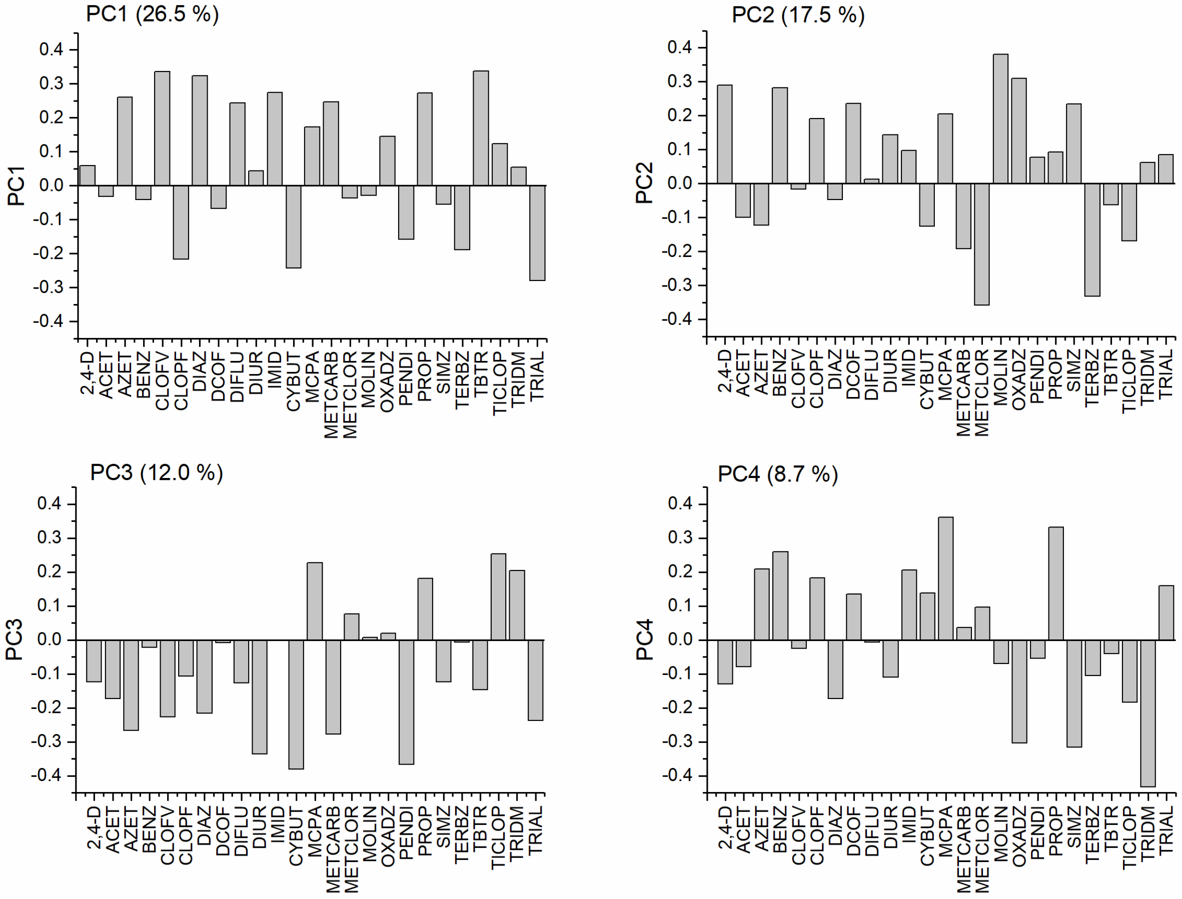
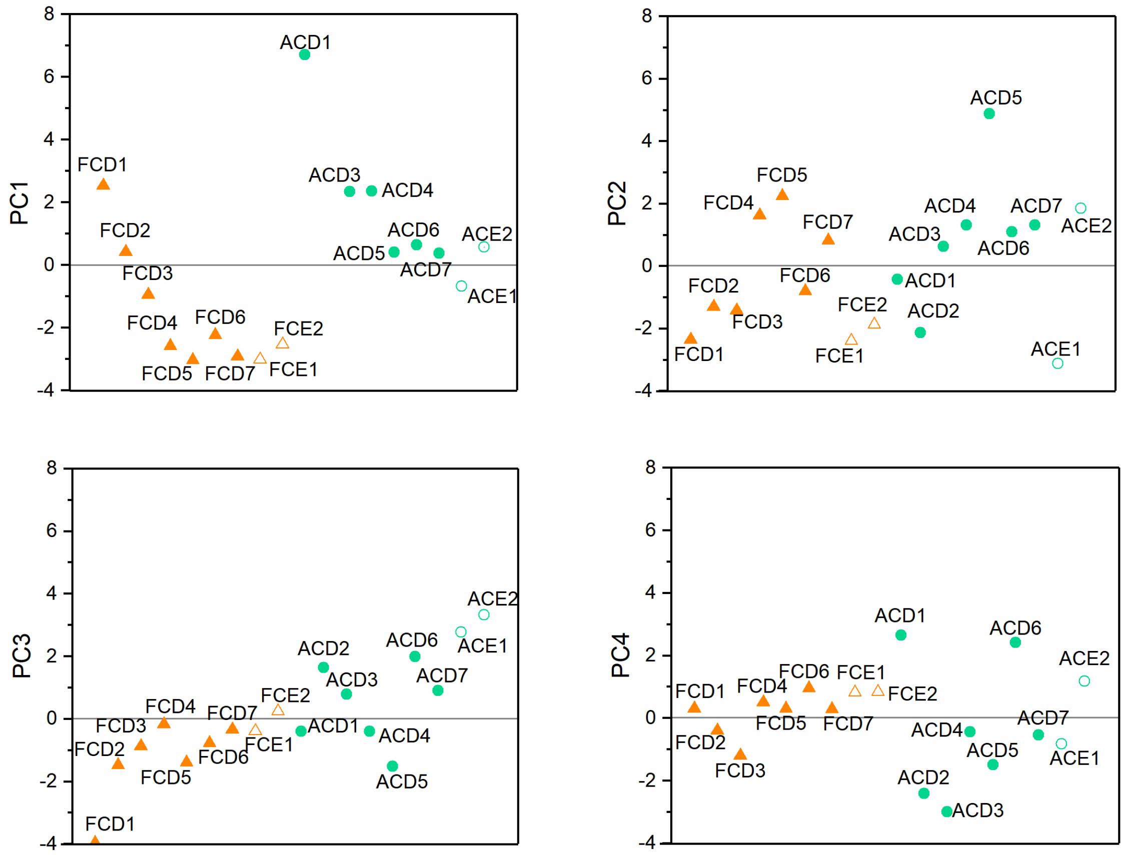
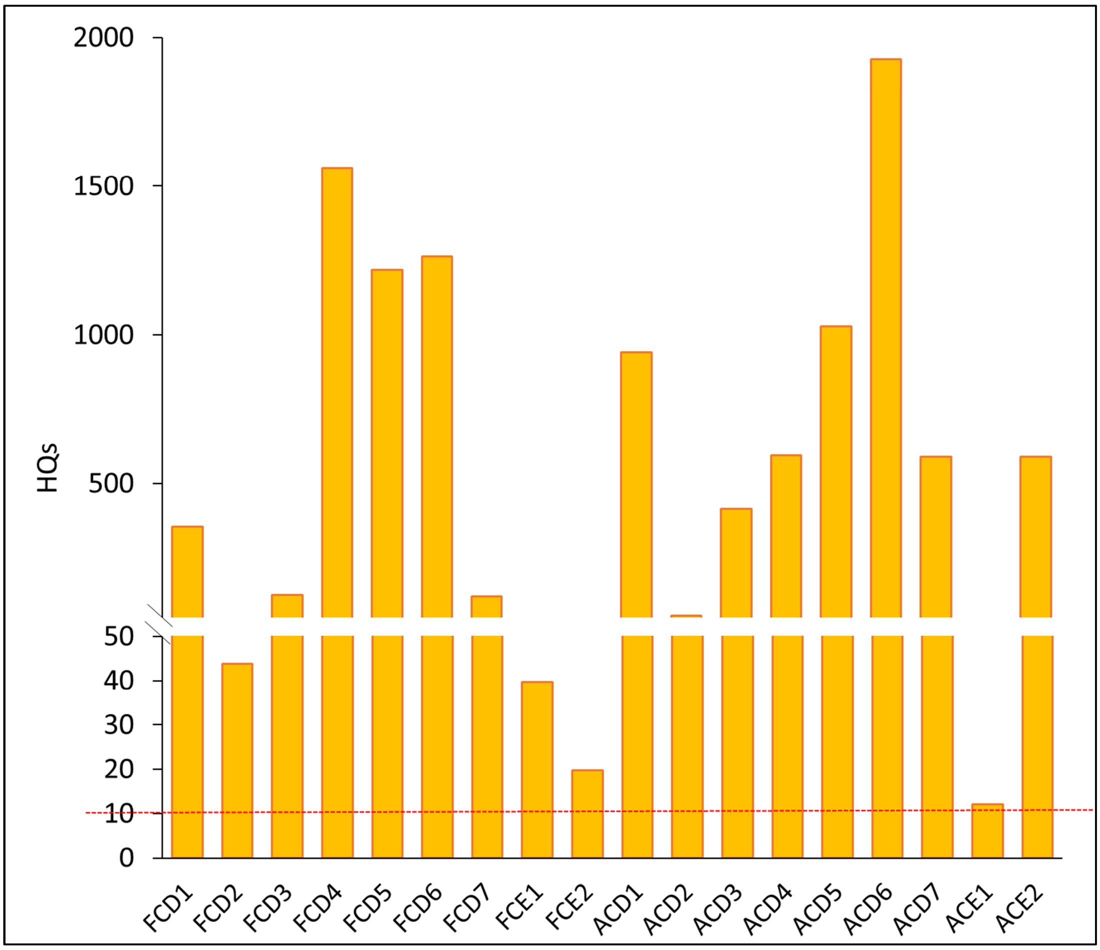

a)
b)
Concentration (µg/L)
%
0
50
100
150
200
250
0
20
40
60
80
100
FCD1
FCD2
FCD3
FCD4
FCD5
FCD6
FCD7
FCE1
FCE2
ACD1
ACD2
ACD3
ACD4
ACD5
ACD6
ACD7
ACE1
ACE2
Others
Triazines
Neonicotinoids
Carbamates
Anilides
Acidics
Bentazone

Figure 2.


PC1 (26.5 %)
PC2 (17.5 %)
PC3 (12.0 %)
PC4 (8.7 %)
PC1
PC2
PC3
PC4
0.4
0.3
0.2
0.1
0.0
-0.1
-0.2
-0.3
-0.4
2,4-D
ACET
AZET
BENZ
CLOFV
CLOPF
DIAZ
DCOF
DIFLU
DIUR
IMID
CYBUT
MCPA
METCARB
METCLOR
MOLIN
OXADZ
PENDI
PROP
SIMZ
TERBZ
TBTR
TICLOP
TRIDM
TRIAL


PC1
8
6
4
2
0
-2
-4
ACD1
FCD1
ACD3
ACD4
FCD2
ACD6
ACE2
ACD5
ACD7
FCD3
ACE1
FCD4
FCD6
FCE2
FCD5
FCD7
FCE1
PC2
ACD5
FCD5
FCD4
ACD4
ACD7
ACE2
FCD7
ACD3
ACD6
FCD6
ACD1
FCD2
FCE2
FCD3
ACD2
FCD1
FCE1
ACE1
PC3
ACE2
ACD6
ACE1
ACD2
ACD3
ACD7
FCE2
FCD4
FCD7
FCD3
ACD1
ACD4
FCD2
FCE1
FCD6
FCD5
ACD5
FCD1
PC4
ACD1
ACD6
ACE2
FCD6
FCE1
FCD1
FCD4
FCE2
FCD5
FCD7
ACD7
FCD2
ACD4
ACD5
ACE1
FCD3
ACD2
ACD3



HQs
2000
1500
1000
500
50
40
30
20
10
0
FCD1
FCD2
FCD3
FCD4
FCD5
FCD6
FCD7
FCE1
FCE2
ACD1
ACD2
ACD3
ACD4
ACD5
ACD6
ACD7
ACE1
ACE2

Figure 6.

Highlights:

- Thirty-five out of 66 pesticides were detected, 17 of them currently banned for use.
- Bentazone was the most ubiquitous and abundant pesticide (up to 180 µg/L).
- Acidic pesticides and anilides (used to grow rice) were the most abundant pesticides.
- The presence of cybutryne, imidacloprid, and acetamiprid is of concern.
- The co-occurrence of pesticides may pose a high risk for non-target organisms.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: