

Ecological risk assessment of pesticides in the Mijares River (eastern Spain) impacted by citrus production using wide-scope screening and target quantitative analysis

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Abstract

The widespread use of pesticides, especially in agricultural areas, makes necessary to control their presence in surrounding surface waters. The current study was designed to investigate the occurrence and ecological risks of pesticides and their transformation products in a Mediterranean river basin impacted by citrus agricultural production. Nineteen sites were monitored in three campaigns distributed over three different seasons. After a qualitative screening, 24 compounds were selected for subsequent quantitative analysis. As expected, the lower section of the river was most contaminated, with total concentration $> 5 \mu\text{g/L}$ in two sites near to the discharge area of wastewater treatment plants. The highest concentrations were found in September, after agricultural applications and when the river flow is reduced. Ecological risks were calculated using two mixture toxicity approaches (Toxic Unit and multi-substance Potentially Affected Fraction), which revealed high acute and chronic risks of imidacloprid to invertebrates, moderate-to-high risks of diuron, simazine and 2,4-D for primary producers, and moderate-to-high risks of thiabendazole for invertebrates and fish. This study shows that intensive agricultural production and the discharge of wastewater effluents containing pesticide residues from post-harvest citrus processing plants are threatening freshwater biodiversity. Further actions are recommended to control pesticide use and to reduce emissions.

Keywords

Pesticides; Surface water; Chromatography coupled to mass spectrometry; Ecological risk assessment; Mixture toxicity

Highlights

- Pesticides were monitored in the Mijares River (Spain) in different seasons
- A screening method was applied to select compounds for quantitative analysis
- Quantitative analyses detected high concentrations of thiabendazole and imazalil
- Wastewater effluents contributed to increase pesticide concentrations in the river
- High risks were calculated for imidacloprid, diuron and thiabendazole

1. INTRODUCTION

Pesticides are considered as a key tool to increase agricultural crop yields while reducing man power (Cooper & Dobson, 2007). Agricultural pesticides are prone to enter surface water ecosystems, and have been considered as one of the main causes of the freshwater biodiversity decline (Beketov et al., 2013). Spain is the second largest consumer of pesticides within the European Union (Eurostat, 2019). In 2018, the total amount of active substances commercialized was 73,286 Ton, which represents an increase of almost 2% as compared to the previous year (Ministerio de Agricultura Pesca y Alimentación, 2017). At present, more than 2,000 compounds are authorized, containing several hundreds of active ingredients. The largest group of active substances commercialized for agricultural use corresponds to fungicides and bactericides (52%), followed by herbicides (23%), molluscicides and other plant protection products (16%), and insecticides (9%).

Surface water runoff, spray-drift and leaching are considered as the main entry routes for pesticides into surface water ecosystems. An alternative, less-researched pathway are wastewater treatment plants (WWTPs), which can discharge pesticide mixtures coming from washing equipment used in agriculture, fruit and vegetable post-harvest processing, and/or urban and domestic applications (Aguirre-Martínez and Martín-Díaz, 2020). In general, pesticides are poorly eliminated by conventional wastewater treatments (Gago-Ferrero et al., 2020). Possible explanations for the poor or even negative removal rates are deconjugation of metabolites and/or transformation products (TPs) of the pesticides, and desorption from particulate matter during wastewater treatment (Köck-Schulmeyer et al., 2013).

The European Union established a list of 45 priority substances that have been identified amongst those that pose a significant risk to the aquatic environment (Directive 2013/39/EU). The fact that about 50% of these substances are pesticides indicates concerns about their potential negative side effects in freshwater ecosystems. However, many of these pesticides are obsolete compounds or have been recently banned. In 2018, the European Commission updated the Watch List of the Water Framework Directive (European Commission 2018/840, 2018), including seven pesticides, five of them neonicotinoids. In the time of writing this paper, the Watch List was

updated again, removing all the previous pesticides except metaflumizone, and adding 10 azole compounds, famoxadone and dymoxystrobin (European Commission 2020/1161, 2020). In Spain and other southern European countries, the efforts to monitor the exposure to and risks of pesticides other than those included as part of the Water Framework Directive (WFD) are commonly insufficient. As discussed by several authors, post-registration monitoring is needed to assess the occurrence of pesticide mixtures and their retrospective risks for freshwater biodiversity, as well as to identify potential misuse practices and flaws of the prospective risk assessment framework (Rico et al., 2021; Vijver et al., 2017).

Comprehensive data on pesticide occurrence in water are crucial for a good characterization of the water quality. However, the large number of compounds that could be potentially present in water makes this task challenging. Most studies on pesticide risk assessment are based on concentration data obtained by target quantitative methods applied for a limited list of compounds. In the last years, gas chromatography-mass spectrometry (GC-MS) methods have moved to methods based on liquid chromatography-mass spectrometry (LC-MS), due to the change in the use of pesticides, from non-polar and volatile compounds to more polar and less volatile compounds, which are commonly less toxic and persistent in the environment. Nowadays, analytical techniques based LC coupled to tandem mass spectrometry (MS/MS) are widely applied for the determination of target pesticides and their TPs in water, at very low concentration levels (ng/L) (Marín et al., 2009; Sancho et al., 2004; Wille et al., 2012). As an integral complement to target MS/MS analysis, more limited in the scope, screening methods based on high resolution mass spectrometry (HRMS) allow the detection of a wide list of compounds and help to prioritize those that are more frequently found in environmental samples (Della-Flora et al., 2019; Hernández et al., 2015; Masiá et al., 2013; Pitarch et al., 2016). The application of HRMS-based screening methods is of great help to focus the subsequent quantitative analysis to those compounds that have been previously identified in water by wide-scope screening.

To date, only few studies have investigated the potential toxicological effects of pesticide mixtures at river catchment scale (Arenas-Sánchez et al., 2019; Le et al., 2017).

One of the most commonly used mixture toxicity methods is the Toxic Unit (TU) approach, which relies on the summation of the individual toxic pressures exerted by each compound to a given standard test species assuming concentration addition (Liess and Ohe, 2005; Sprague, 1971). The major benefit of this approach is that it requires a limited number of toxicity data for each compound. On the other hand, several studies demonstrate that the standard test species used for the calculation of TUs are not necessarily within the most sensitive taxa for some pesticide classes, potentially rendering the method estimations under protective for some species (Brock et al., 2016; Rico and Van den Brink, 2015). Another mixture toxicity method that is increasingly used is the multi-substance Potentially Affected Fraction (ms-PAF) approach (de Zwart and Posthuma, 2005; Posthuma et al., 2019) calculated on the basis of Species Sensitivity Distributions (SSDs) (Posthuma et al., 2002). One of its major strengths is that it uses as many toxicity data as possible to represent the sensitivity range of species assemblages in freshwater ecosystems, and provides probability estimates as regards to the number of species that will be affected given one or several pesticide concentrations. Its major limitation is that it requires a larger number of toxicity values to represent the sensitivity of different species of the ecosystem and to achieve robust risk estimates (Maltby et al., 2005; Wheeler et al., 2002). For some pesticides, the number of toxicity data available is often too limited, which forces researchers to exclude some compounds from the ms-PAF analysis (e.g. Rämö et al., 2018), thus hampering the risk characterization for all compounds contained in the pesticide mixture.

The objectives of this study were to investigate the occurrence of pesticides and their TPs into a Mediterranean river (Mijares River) by applying complementary analytical techniques and to assess their ecological risks. The Mijares River is directly impacted by WWTPs and, in some areas, it is expected to receive high loads of pesticides used in citrus production and post-harvest processing. A total of 57 surface water samples were collected in three different sampling campaigns over one year. After a preliminary screening to identify the most relevant pesticides, the samples were quantitatively analyzed by LC-MS/MS for the determination of 24 selected analytes. The results of the quantitative analysis were used to assess the risks of each pesticide individually and in

mixtures for aquatic ecosystems making use of the TU and the ms-PAF approaches. This study highlights single compounds and pesticide mixtures that are posing an ecotoxicological risk to Mediterranean freshwater ecosystems in areas with predominant citrus production, and discusses the pros and cons of the implementation of each mixture toxicity techniques.

2. EXPERIMENTAL

2.1 Pesticide standards and reagents

A total of 19 pesticides and 5 TPs were selected for the quantitative analysis. More details regarding the standards and reagents used for their chemical analyses can be found in the Supplementary Material (SM).

2.2 Study area

The study was carried out along the Mijares River, which is situated in the East coast of Spain (see **Figure 1**). It is a relative small river (156 km) that originates at a height of 1,600 m in the Sierra de Gúdar, in the municipality of El Castellar, Teruel province (TE), and discharges into the Mediterranean Sea between Almassora and Borriana, Castellón province (CS). Its basin covers 5,466 km², which represents 13% of the total demarcation of the Júcar Water Basin Authority. The area is composed of featured reservoirs such as the one in Toranes (TE) and two others in Arenós and Sitjar (CS). The river regime is characterized by a period of moderate-to-high flow in February and June in the low course of the river, which is surpassed in October (the season with the highest water flow), and noticeable descents in January and especially in August.

The use of the river water for irrigation is highly important in this area. A total of 43,530 ha benefit from its water (94% corresponds to CS and the remaining 6% to TE). Most of the irrigated area (77%) is located in the lower section of the river (Plana Baixa region), where citrus fruits are the predominant crop with a percentage close to 87% of the irrigated area. In the rest of the sections of the catchment, especially the upper one, more than 80% of land cover is occupied by forested areas and a large part of the existing cultivation areas are abandoned (González, 2017).

Currently, in the upper part, a fertilizer factory and a fish farm are located in the neighbouring municipality of Sarrión (TE). Moreover, there are four WWTPs which discharge their treated effluents to the river. They are located in four municipalities, two small ones, Montanejos and Toga, and two of bigger size, Vila-real and Almassora, which are considered as important sources of contamination in the surrounding environment (Fonseca et al., 2020). In addition, in the middle section of the river, there is a Solid Waste Treatment Plant (SWTP) in Onda (CS).

2.3 Sample collection

The Mijares River was sampled in 19 different sites (see **Figure 1**), selected according to their characteristics and/or accessibility (see **Table S1** in SM). Sites 1-6 were selected in the upper section, among which stand out site 2 (next to the fertilizer factory), sites 3 and 4 (close to the fish farm) and site 5 (Toranes reservoir). In the middle part, eight locations were studied corresponding to Arenós reservoir (sites 7-8), Montanejos WWTP (sites 9-10), Toga WWTP (site 11), Sitjar reservoir (site 12) and two sampling sites (13-14) located downstream of the SWTP. In the lower section, four sampling locations (sites 15-18) were selected. Sites 17 and 18 are close to the discharge point of the WWTPs of Vila-real and Almassora, respectively. The last sampling site 19 is located in the estuary of the river.

In order to evaluate potential seasonal variation in the Mijares River, three sampling campaigns were conducted along different seasons, summer (June 2018), autumn (September 2018) and winter (February 2019). In every campaign, 19 surface water samples were collected, one from each sampling site, in polyethylene bottles, transported in refrigerated isothermal containers and stored in the dark at -20 °C until their analysis. A total of 57 river water samples were collected.

2.4 Instrumentation

2.4.1 QTOF MS

A hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (Xevo G2 QTOF) was interfaced to a Waters Acquity ultra-high performance liquid chromatography (UHPLC) system using an orthogonal Z-spray electrospray ionization (ESI) interface or to an Agilent 7890A GC system using an atmospheric pressure chemical ionization source (APCI), all within a single instrument. For further details see the SM.

2.4.2. LC-MS/MS (QqQ)

Waters Acquity UPLC™, equipped with a quaternary pump system, interfaced to a Xevo TQ-S™ triple quadrupole (QqQ) mass spectrometer was used. See the SM for further details.

2.5 Sample treatment in screening analysis

Sample extraction and pre-concentration was made by solid-phase extraction (SPE). The procedure applied was based on the method previously developed by our research group (Pitarch et al., 2016). Briefly, a volume of 100 mL of centrifuged water sample was passed through an Oasis HLB cartridge (150 mg, Waters) and then, the analytes were eluted with 5 mL of methanol. The extract was divided into 2 aliquots (2.5 mL each), which were evaporated to dryness and reconstituted with 50 μ L of hexane (for GC analysis) and 100 μ L of methanol:water (10:90, v/v) (for LC analysis). Finally, 1 μ L of the hexane extract and 20 μ L of the methanol-water extract were injected into GC-QTOF MS and LC-QTOF MS, respectively.

2.6 Screening by QTOF MS

The presence of pesticides in water samples was firstly investigated by complementary wide-scope screening using both GC- and LC-QTOF MS (**Table 1**). The purpose was to identify the main pesticides and TPs present in surface water from the river in order to design the subsequent quantitative analysis and to apply it to those compounds that are actually present in water.

Pesticides and TPs investigated were included in a customized home-made database (around 550 compounds in LC and 425 in GC) (Fonseca et al., 2019; Pitarch et al., 2016). Detection and identification was based on mass accuracy, isotopic pattern (typically due to the presence of bromine or chlorine atoms), retention time (Rt) deviation and presence of fragment ions. For more details about the strategy employed, see (Hernández et al., 2014; Ibáñez et al., 2017) and the SM.

2.7 Sample treatment in quantitative analysis

Quantitative determination of pesticides was performed without any pre-treatment of the sample, except centrifugation, i.e. using direct injection of the water sample (Boix et al., 2015; Botero-Coy et al., 2018; Fonseca et al., 2020). Briefly, 2 mL of water was centrifuged at 12,000 rpm for 10 min. Subsequently, in an injection vial, 50 μ L of an isotopic labelled internal standard (ILIS) solution of 1 ng/mL was added to 950 μ L of the centrifuged water sample. Finally, 100 μ L were injected into the LC-MS/MS system.

2.8 Quantitative analysis by LC-MS/MS

For the quantitative analysis of samples by LC-MS/MS, 24 target pesticides and TPs from different physicochemical families were selected. The experimental conditions are shown in **Table S2**.

Between samples, quality control (QC) were included. The QCs consisted on 3 surface water samples, selected among those analysed in this study, each one fortified at three concentration levels: 0.01, 0.1 and 1 µg/L. QCs recoveries were accepted between 60 and 140% (SANTE, 2019), giving in this way validity to the results obtained.

As samples were analysed by direct injection without any pre-concentration step, the lowest calibration level (LCL) was considered as the limit of quantification in water analysis (1 ng/L for all compounds). A compound was considered as “detected” when its concentration was below 1 ng/L and at least one q/Q ratio was accomplished. For the constructions of graphs, risk assessment evaluation, and for discussion of results obtained, the cut-off value used for detected positives was half the LCL (0.5 ng/L for all compounds).

2.9 Ecological risk assessment

Aquatic toxicity data for the pesticides that were detected in this study were retrieved from the US EPA ECOTOX database (ECOTOX, 2020) and the Pesticide Properties Database (PPDB, 2020). The toxicity data selection criteria (i.e., selected endpoints, exposure duration) for primary producers, invertebrates and vertebrates were based on those described by Rico et al. (Rico et al., 2019b), and are described in the SM.

Risks for freshwater ecosystems were calculated following the TU approach and the msPAF approach for primary producers, invertebrates (acute and chronic) and vertebrates (acute and chronic), separately. TUs were calculated by dividing the measured concentration in each sampling site by the toxicity value of standard test species representing the different taxonomic groups and exposure durations. TUs for primary producers were calculated with the EC50-72/96h for the green algae *Raphidocelis subcapitata*. TUs for acute and chronic exposure to aquatic invertebrates were calculated with EC50-48h and NOEC-21d for the microcrustacean *Daphnia magna*, and TUs for acute and chronic exposure to vertebrates were calculated with LC50-96h and NOEC-28d values for the fish *Oncorhynchus mykiss*, respectively (**Table 2**). When

chronic toxicity data for *D. magna* or *O. mykiss* were not available, it was estimated by dividing the acute toxicity value by an extrapolation factor of 10. The TUs for the evaluated pesticide TPs were calculated based on the available toxicity data for the parent compounds. Finally, the mixture toxicity assessment following the TU approach was performed by summing the individual TUs for each compound measured in the sample assuming additivity of the measured substances. Aquatic risks were classified as low or insignificant when the sum of TUs was lower than 0.1, moderate when the sum of TUs was between 0.1 and 1, and high when the sum of TUs was above 1.

Aquatic risks following the msPAF approach were also calculated separately for each taxonomic group and exposure duration. First, an SSD was calculated for each pesticide that was detected at least once in the samples. The SSD parameters μ (median of log-transformed available toxicity values) and σ (standard deviation of log-transformed toxicity values or slope) were calculated assuming a log-normal distribution on the basis of the available toxicity data when there were at least three available data points. In such way, SSDs parameters could be calculated for 42 pesticide-taxonomic group and exposure duration combinations, out of the 75 needed (**Table 3**). Some extrapolation rules were applied to increase the number of compounds that could be evaluated following this approach. For example, acute μ were calculated based on the chronic ones (when these were available) by summing 1 to the chronic μ value and maintaining the same σ , while chronic μ were extrapolated by subtracting 1 to the acute μ value and maintaining the same σ . The summation or subtraction of 1 to the chronic and acute μ correspond to an extrapolation factor of 10 between acute and chronic toxicity data, and allowed the evaluation of 9 additional cases (i.e., pesticide-taxonomic group and exposure duration combinations; **Table 3**). As for TPs, the same SSD parameters as for the parent compounds were used due to the lack of toxicity data to construct TP-specific SSDs.

The monitored pesticides were classified into seven Toxicological Modes of Action (TMOAs) following the classifications described by the Insecticide (IRAC, 2020), Fungicide (FRAC, 2020) and Herbicide (HRAC, 2020) Resistance Action Committees (**Table 3**). After this, the σ of the SSDs of the pesticides belonging to the same TMOA were compared, and when differences were larger than 10% they were assigned to a

different TMoA. The toxic pressure of the compounds within each TMoA and their mixtures was calculated for each sample following the methods described by (de Zwart and Posthuma, 2005; Fonseca et al., 2020). First, the Hazard Unit (HU) was calculated for each compound in each sampling site by dividing the logarithm of the measured concentration by the μ of the corresponding SSD. Then, the msPAF corresponding to each TMoA ($msPAF_{TMoA}$) in each sample was calculated assuming concentration addition and using the Microsoft Excel© function (Eq. 1).

$$msPAF_{TMoA} = NORM.DIST (HU_{TMoA}, 0, \sigma_{TMoA,1}) \quad \text{Eq. 1}$$

Where HU_{TMoA} is the sum of the HUs within each TMoA, and σ_{TMoA} is the average σ for all compounds within the same TMoA. Subsequently, the total toxicity of the sample ($msPAF_{Total}$) was calculated using the response addition model (Eq. 2).

$$msPAF_{Total} = \prod_{i=1}^n (1 - msPAF_{TMoA,i}) \quad \text{Eq. 2}$$

The $msPAF_{Total}$ for each sample was represented together with the relative contribution of each pesticide to the total toxic pressure. The PAF and the $msPAF_{Total}$ represent the fraction of aquatic species within each taxonomic group that will be affected by the exposure to an individual compound or the pesticide mixture, respectively. Usually, the Hazardous Concentration for 5% of species (HC5), which is expected to protect the 95% of species, is taken as concentration threshold for ecological risk assessment (Posthuma et al., 2002). However, other authors have used the HC1 or the lower confidence limit of the HC5 as suitable threshold value (Maltby et al., 2009). In our study, ecological risks resulting from PAFs or msPAFs above 5% were classified as high, while values between 1% and 5% were classified as moderate, and values below 1% were classified as low or insignificant.

3. RESULTS AND DISCUSSION

The present study was designed to collect only water samples, focusing the analyses in the pesticides dissolved in water. This means that no sediment and suspended particulate matter (SPM) samples were collected. Although sediment and SPM analysis have been reported to be essential to better understand the type of pollution encountered (contemporary or historical pesticides input) (Barbieri et al., 2020; Zhang et al., 2020), the fact that most pesticides applied at present in the studied area are medium-high polar (i.e. better fitting with LC-MS analysis) implies that sorption on solid samples should be less important with respect to classical/old pesticides, which were mostly of low polarity (i.e. better fitting with GC-MS analysis). This may be certainly seen as a study limitation. However, data presented in this work on pesticides dissolved in the aqueous phase allow to have a good picture on the current situation of the study area. The strategy applied in this work can be considered as an starting point for future monitoring programmes focused on those pesticides actually used and posing the largest ecological risks.

3.1 Preliminary QTOF screening

After a general SPE pre-concentration step, the samples were screened for a list of more than 800 compounds (550 compounds in LC and 425 in GC), using the combined approach described in section 2.6. The list contained a total of 337 compounds for which the reference standards were available. For the remaining compounds, only the exact mass and fragments reported in the literature were available. **Table 1** shows the pesticides and TPs identified in the screening. Most of compounds were identified/confirmed by agreement of R_t and experimental fragments with their reference standards. Only two fungicides, fluapyram and pyrimethanil, were tentatively identified (their reference standards were not available at our laboratory) based on the information obtained by QTOF MS (i.e. accurate mass of the protonated molecule and of fragment ions), which was compared with online database, such as MassBank or MetLin, and/or with reported bibliography (Valera-Tarifa et al., 2020).

A total of 32 compounds (28 pesticides and 4 TPs) were identified using both QTOF MS methodologies (**Table 1**). As an example, **Figures S1** and **S2** illustrate the identification

of thiabendazole (by LC-QTOF MS) and terbutryn (by GC-QTOF MS). Among parent compounds, there were 9 insecticides, 9 fungicides and 10 herbicides, the three groups of compounds commonly present in surface water (de Souza et al., 2020). Most pesticides found in the Mijares River have been reported in different studies of Mediterranean river catchments in Spain (Ccanccapa et al., 2016; Fonseca et al., 2019; Hernández et al., 2008; Marín et al., 2006; Masiá et al., 2013; Moreno-González et al., 2013; Rico et al., 2019a; Rousis et al., 2017). As regards to TPs, four compounds were herbicide triazines' TPs: atrazine desethyl (DEA), terbuthylazine 2-OH and –desethyl, and terbumeton desethyl, which are frequently detected in surface water and even groundwater (Ccanccapa et al., 2016; Marín et al., 2006; Masiá et al., 2013). From the results obtained after the wide-scope screening methodology, a list of pesticides was elaborated to carry out the next stage of target quantitative analysis. A total of 24 compounds (pesticides and TPs) were selected for the monitoring in the three subsequent campaigns. We prioritized the application of a LC-MS/MS method against a GC-MS/MS method, as most of the compounds found in the samples were LC-MS amenable. A few more compounds were added to the list of target analytes (atrazine-desisopropil, 2,4-D, linuron, prometryn, propamocarb, pyridaphention), based on our previous experience on surface water analysis, and on availability of reference standards in our laboratory. Several of the compounds selected are widely used in citrus production and processing (e.g. imazalil, metalaxyl, tebuconazole, thiabendazole).

3.2 Target analysis by LC-MS/MS (QqQ)

A total of 57 surface water samples were analysed by LC-MS/MS (QqQ) for the quantification of 24 target pesticides and TPs. QCs were analysed together (see **Table S3**). The results of the analysed samples are shown in **Tables S4, S5** and **S6**, which correspond to the 1st (June 2018, summer), 2nd (September 2018, autumn) and 3rd (February 2019, winter) campaigns, respectively.

Table 4 shows the frequency of detection (% positive samples) for the compounds under study. The majority of compounds selected (19 out of 24) were found at least once in the analysed samples. The fungicide thiabendazole (26%) and the insecticide imidacloprid (23%) were the most commonly detected, with concentrations above the cut-off value of 0.5 ng/L. Four out of five pesticides (diuron, atrazine, terbutryn and

simazine) included in the list of priority substances in the field of water policy were detected, but in all cases at concentrations levels below the maximum allowable concentration for surface waters (Directive 2013/39/EU). Four out of the five investigated TPs were found, being the TPs of terbumeton (-desethyl) and terbuthylazine (-desethyl and -OH) those with the highest detection frequencies (9-10%). The maximum concentration level for TPs corresponded to terbuthylazine-OH (0.066 µg/L) in the mouth of the river (sample 19) during the first campaign (June 2018). The use of three triazine herbicides detected (atrazine, terbutryn and terbumeton) is prohibited in the countries of the European Union, so finding residues of these compounds or their TPs confirms their persistence in the environment, and hence the need to phase them out of the markets.

Only four pesticides exceeded in some occasions the value of 0.1 µg/L, namely 2,4-D, imidacloprid, thiabendazole and imazalil. The latter fungicides were detected at the highest concentrations, being thiabendazole the one that reached the highest concentration reported in this study (34.5 µg/L, sampling site 18, 2nd campaign). Both, thiabendazole and imazalil, are widely used in the post-harvest processing of citrus fruits in the Mediterranean area (Sanitat Vegetal. Generalitat Valenciana., 2020). Discharges of effluents from WWTPs that treat wastewater from agro-food industries can be an important route of water contamination in the investigated zone. In fact, previous studies have shown the presence of thiabendazole in effluents from these industries (Sánchez Pérez et al., 2014). These fungicides have been frequently detected in surface water samples collected in this area (Ccanccapa et al., 2016; Fonseca et al., 2019; Hernández et al., 2015; Rousis et al., 2017), in untreated wastewaters (Marín et al., 2009) and occasionally in ground water (Hernández et al., 2008). Furthermore, the neonicotinoid insecticide imidacloprid has been recently prohibited for outdoor agricultural applications in the European Union (European Commission 2018/783, 2018) and is currently included in the Watch List of European Commission (European Commission 2018/840, 2018). The presence of imidacloprid in the surface water under study could be explained by its high persistence in sediments and soils (Bonmatin et al., 2015), and because its application as pest control and consequent environmental discharge by WTP effluents (Sadaria et al., 2017).

The total accumulated concentrations of all pesticides in each sampling site are shown in **Figure 2**. As expected, the upper section of the river was the less contaminated one and none of the sampling sites showed pesticide residue concentrations above the limit of quantification. The incidence of agriculture on water pollution in this area is presumably low, as more than 80% of land cover is occupied by forested areas and large part of the existing cultivation areas are abandoned (González, 2017). As expected, the fertilizer factory near to site 2 did not have any pesticide contribution to the river. The nearest sampling sites to the fish farm (sites 3 and 4) did not appear to influence the river pesticide exposure either.

With regards to the middle section of Mijares River, only two sites produced positive detections above the limit of quantification (sites 8 and 10) and the rest of sampling sites, did not show any remarkable positives. The concentration sum of pesticides downstream the Arenós reservoir (site 8) was above 600 ng/L, mainly due to the contribution of the phenoxyacetic herbicide 2,4-D, found in the second campaign at a relatively high concentration (611 ng/L) probably due to spray drift during its application in the late summer season. This herbicide is normally used to control dicotyledons, especially in wheat and barley crops. In the locations sampled downstream of WWTPs, Montanejos (site 10) showed some contamination by pesticides (total concentration 42 and 32 ng/L, in the first and the second campaign respectively). Although surface runoff from agricultural areas is expected to be one of the main sources of pesticide pollution together with spray drift, the contribution of WWTPs cannot be neglected (Fairbairn et al., 2016; Gago-Ferrero et al., 2020; Köck-Schulmeyer et al., 2013; López-Pacheco et al., 2019). However, the sampling site 11 (downstream WWTP Toga) did not appear to be contaminated, probably due to the small size of this village (only 100 inhabitants) and the limited agricultural production. In addition, the water samples collected downstream of the treatment plant of solid waste in Onda (sites 13 and 14) did not present any positive results, indicating that this treatment plant cannot be considered a pollution source for pesticides. This is in agreement with our previous study (Pitarch et al., 2016), which shows that the major contamination source for groundwater in the surroundings of that SWTP corresponded to pesticides used in intensive citrus agriculture.

The lower section of the Mijares River was the most contaminated one, especially near to the river mouth. The sites with the highest pollution ($>5,000$ ng/L) were located downstream of the WWTPs of Vila-real (site 17) and Almassora (site 18). Besides, these two sampling sites were among those with the highest number of positives (up to 10 pesticides were identified at site 18 in the 1st and 2nd campaigns), the next sampling site (19, Gola Almassora), the mouth of Mijares River, still presented an important pollution level. In this site, however, the total sum of concentrations was lower than 500 ng/L, indicating some natural pesticide attenuation.

Finally, it can be observed from **Figure 2**, that the highest total concentration of pesticides in all the sampling sites was found in the second campaign (September 2018). This is in line with the pesticide application schemes, as many pesticides are reported to be applied until the end of summer (Planas et al., 2006), but may also be explained by a lower flow and dilution capacity of the river during that period. In relation to pesticide groups, it is worth noting the high concentration of fungicides with respect to that of herbicides and insecticides (**Figure 3**). Total fungicide concentrations as high as 59 $\mu\text{g/L}$ were found in September. From the remaining groups, herbicides showed the second largest concentrations, and the only insecticide found did not exceed 0.5 $\mu\text{g/L}$.

An important finding of the present study was the high pesticide levels found in the surface water downstream of WWTP, which could be associated to the discharges of effluents from agro-food industries. Our results demonstrate that WWTP effluents can be considered important sources of pesticides into the aquatic environment, which is in agreement with previous findings (Gago-Ferrero et al., 2017; Golovko et al., 2021). Therefore, advanced technologies for removal of contaminants from wastewater are necessary to protect freshwater ecosystems (Prada-Vásquez et al., 2020).

3.3 Aquatic risk assessment

The results of the ecological risk assessment following the TU approach show that mixture toxicity risks for primary producers were low or insignificant, with the sum of TUs being below 0.1 in all cases (**Figure 4, Table S7**). However, following the msPAF approach, moderate-to-high risks were calculated for sampling sites 8 (September), 10 (September), 17 (September and February) and 18 (in all sampling seasons). Toxicity was dominated by the herbicide diuron in the majority of samples, except for sampling

site 18 in June, which was dominated by a combination of diuron and simazine, and sampling site 8 in September, which was dominated by 2,4-D (**Figure 5, Table S8**). Diuron has been identified as one of the main pesticides causing risks for primary producers in Mediterranean watersheds (Arenas-Sánchez et al., 2019; Kuzmanović et al., 2015). It is particularly toxic to macrophytes such as *Myriophyllum spicatum* and *Apium nodiflorum* (Lambert et al., 2006), which explains the differences encountered between the results of the TU evaluation (based on the surrogate algae species *R. subcapitata*) and the ms-PAF evaluation (which also includes macrophytes). Similarly, the toxicity values for *Myriophyllum aquaticum* NOEC-7d (length) and *Myriophyllum sibiricum* NOEC-14d (root growth) for 2,4-D have been found to be about 4-5 orders of magnitude lower than the calculated NOEC-96h (growth rate) for *R. subcapitata* (**Table 2**) (Ebke et al., 2013; Roshon, 1997). The results of the acute risk assessment for aquatic invertebrates based on the TU approach showed low or insignificant risks, while moderate risks were calculated for chronic exposure in sampling sites 17 (in September) and 18 (in June and September), with a maximum sum of TUs of 0.83. In all cases, the toxic pressure was dominated by the fungicide thiabendazole (**Figure 4, Table S9**). On the other hand, the results of the ms-PAF approach for acute exposure showed moderate risks for aquatic invertebrates in sites 17 (in September) and 18 (June and February), and high risks in site 18 in September. Chronic risks calculated with the ms-PAF approach were found to be moderate in sampling site 10 (in June and September) and high in sampling sites 17 (in September and February), 18 (all sampling seasons) and 19 (in September). The maximum calculated msPAF_{Total} was 22% in sampling site 18 in September. According to the ms-PAF approach, toxicity was dominated by the insecticide imidacloprid in all cases (**Figure 5, Table S10**), which was not identified by the TU analysis. Imidacloprid, as well as other neonicotinoid insecticides, has been found to be orders of magnitude more toxic to aquatic insects as compared to *D. magna* (Morrissey et al., 2015; Roessink et al., 2013; Van den Brink et al., 2016), which explains the different results offered by the two approaches for this substance; and usually show larger than usual differences between acute and chronic toxicity values due to their particular toxic mode of action (Sánchez-Bayo and Tennekes, 2020). In a mesocosm study performed under Mediterranean conditions, Rico et al. (Rico et al., 2018) found a significant decline of the aquatic insects *Cloeon dipterum* and chironomids, and the

zooplankton Cyclopoida, after the application of one single dose of 0.20 µg/L of imidacloprid, which is very close to the highest concentration measured in our study (0.27 µg/L, **Table 4**), indicating a very likely abundance reduction of these taxonomic groups. It should be noted that risks of thiabendazole to invertebrates could not be evaluated following the ms-PAF approach due to lack of sufficient toxicity data (**Table 3**). However, previous studies have pointed towards a high toxicity of benzimidazole fungicides to aquatic invertebrates (Zubrod et al., 2019), and studies performed with another benzimidazole compound (carbendazim) highlighted flatworms, oligochaetes, amphipods and cladocerans as particularly sensitive to prolonged exposure regimes (van Wijngaarden et al., 1998) such as the one found in our study.

Regarding the TU approach, acute toxicity to vertebrates was found to be insignificant. However, chronic mixture toxicity was found to be moderate in three samples (17 in February, and 18 in June and February), and high in sampling sites 17 and 18 during September, with a maximum sum of TUs of 1.7 (sampling site 18 in September). In these cases, toxicity was clearly dominated by thiabendazole, although the fungicide imazalil also had a small contribution (**Figure 4, Table S11**). The analysis performed with the ms-PAF approach showed similar results, with thiabendazole triggering moderate-to-high risks, with a maximum msPAF_{Total} of 9% in sampling site 18 during the sampling performed in September (**Figure 5, Table S12**). It should be taken into account, however, that the chronic SSD for thiabendazole was extrapolated from the acute one (**Table 3**), which makes the ms-PAF assessment less accurate. However, the thiabendazole's NOEC-21d for *O. mykiss* (21 µg/L, **Table 2**) was well below the concentrations measured in this study (34 µg/L, **Table 4**), suggesting that thiabendazole may be posing a potential risk for fish populations. Thus, further experiments should be dedicated to assess the long-term effects of this fungicide to invertebrate and fish populations in the Mijares River.

Our study shows that, in the majority of cases, moderate-to-high risks for the different taxonomic groups were triggered by only one compound or two. This is in line with other mixture toxicity assessments performed in surface water ecosystems, which indicate that only a very limited number of compounds are responsible for biodiversity impacts in small-to-medium sized water basins (Arenas-Sánchez et al., 2019; Gustavsson

et al., 2017; Munz et al., 2017; Verro et al., 2009). Our study also demonstrates that given the current data availability, risk characterization studies performed using solely the TU approach may disregard substances for which the standard test species is not sufficiently sensitive and/or for which there is wide variation in sensitivity among the species of the same taxonomic group (Morrissey et al., 2015). On the other hand, the implementation of the ms-PAF approach is limited by the number of toxicity data available, particularly for chronic risk assessments, and cannot be applied for all substances. Therefore, the combination of both methods seems a reasonable choice to identify compounds and mixtures that are posing an unacceptable risk, at least until further experimental (or extrapolated) toxicity data become available to expand the application of the ms-PAF approach and its statistical power.

4. CONCLUSIONS

A comprehensive investigation was performed on the occurrence and risks of pesticides along a Mediterranean river impacted by citrus production and WWTPs in Spain. The upper section of the river presented low contamination, explained by the little agricultural activity, while the lower section, where citrus fruits are predominantly cultivated, was the most contaminated one, especially near the river mouth. The impact of wastewater effluents was evidenced by a notable increase of pesticide concentrations and by the higher number of compounds detected in the water samples collected downstream of WWTP discharges, especially in the lower section of the river. The highest total concentration of pesticides corresponded to the second campaign performed in September 2018, as most pesticide applications are made until late summer and dilution rates are lower during that period. The ecological risk assessment performed showed that imidacloprid poses high acute and chronic risks to aquatic invertebrates. Furthermore, moderate-to-high risks were calculated for primary producers due to diuron, and mixtures containing diuron, simazine and 2,4-D; and moderate-to-high risks were calculated for invertebrate and vertebrate species due to chronic exposure to the fungicide thiabendazole. This study demonstrates that, while intensive agricultural production is the main source of pesticide contamination in the Mijares River, freshwater biodiversity is primarily threatened in areas near to WWTPs and downstream of post-harvest citrus processing plants. Further actions are needed to control pesticide use and environmental emissions in agricultural areas of eastern Spain dominated by citrus production.

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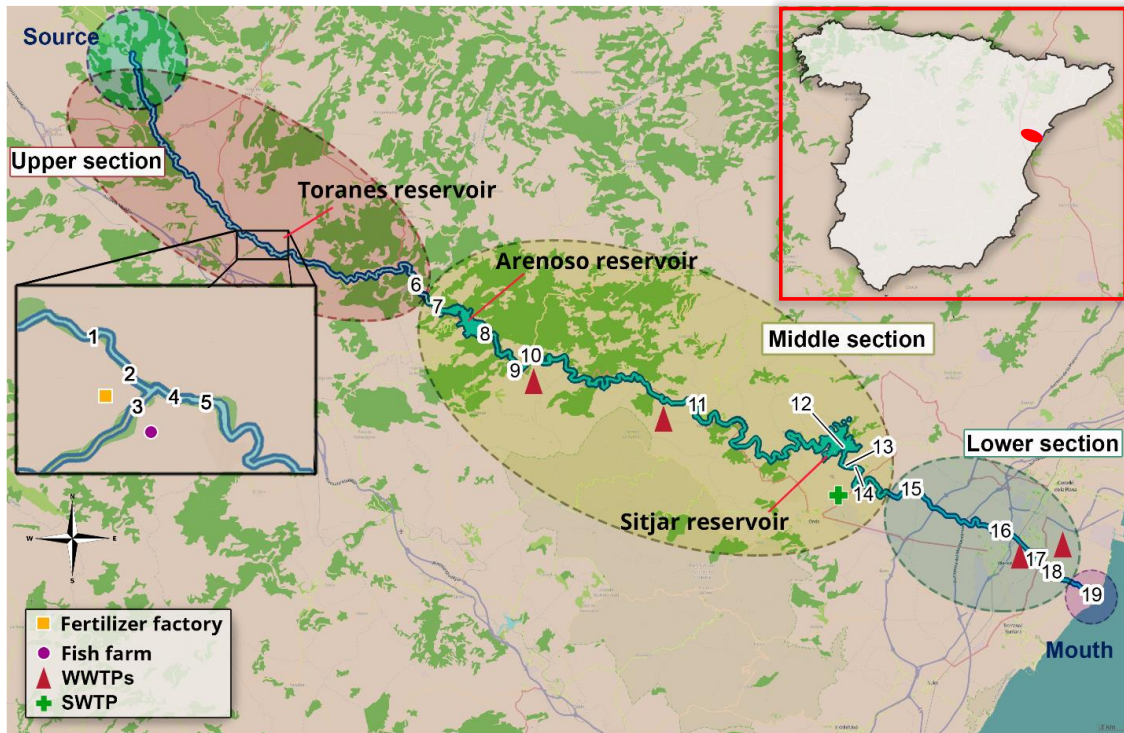


Figure 1. Sampling sites (1-19) in the Mijares River. The bottom left inset shows the location of the Mijares River basin within Spain. The location of the fertilizer factory, fish farm, WWTPs and SWTP in the surrounding of the river are also displayed.

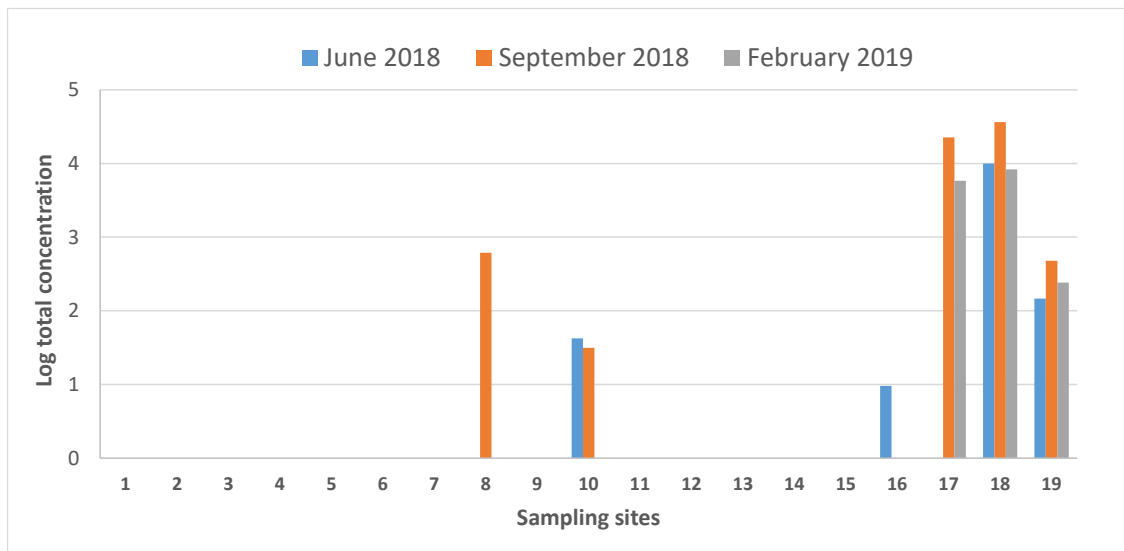


Figure 2. Log total pesticide concentrations in the Mijares River in every sampling campaign (1st: June 2018; 2nd: September 2018, 3rd: February 2019).

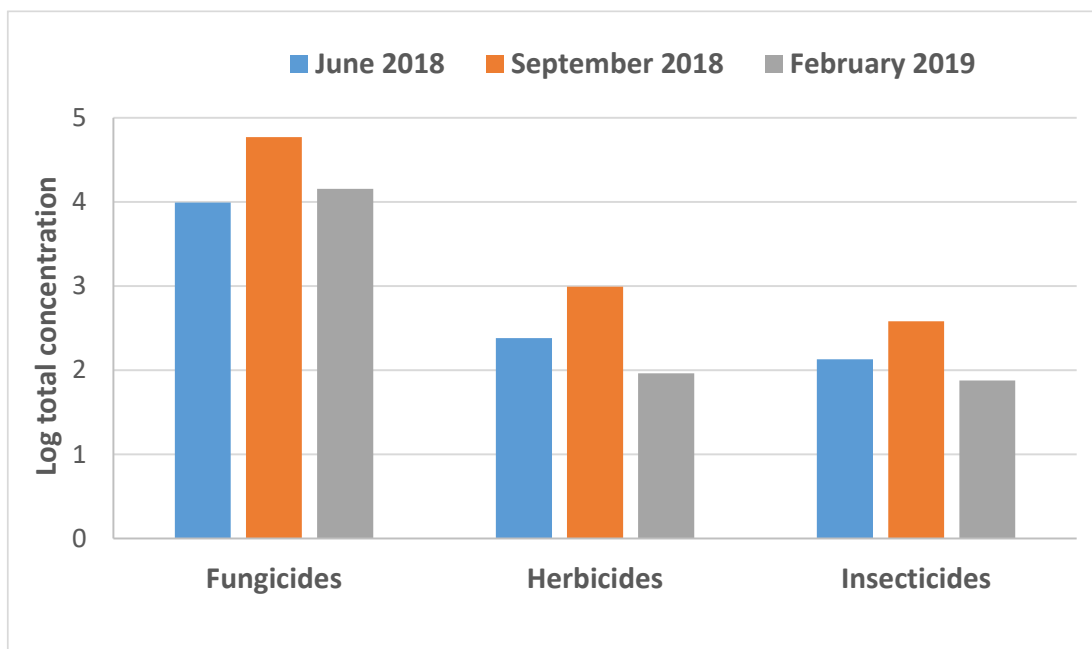


Figure 3. Log total concentration of pesticide groups in the Mijares River in every sampling campaign (1st: June 2018; 2nd: September 2018, 3rd: February 2019).

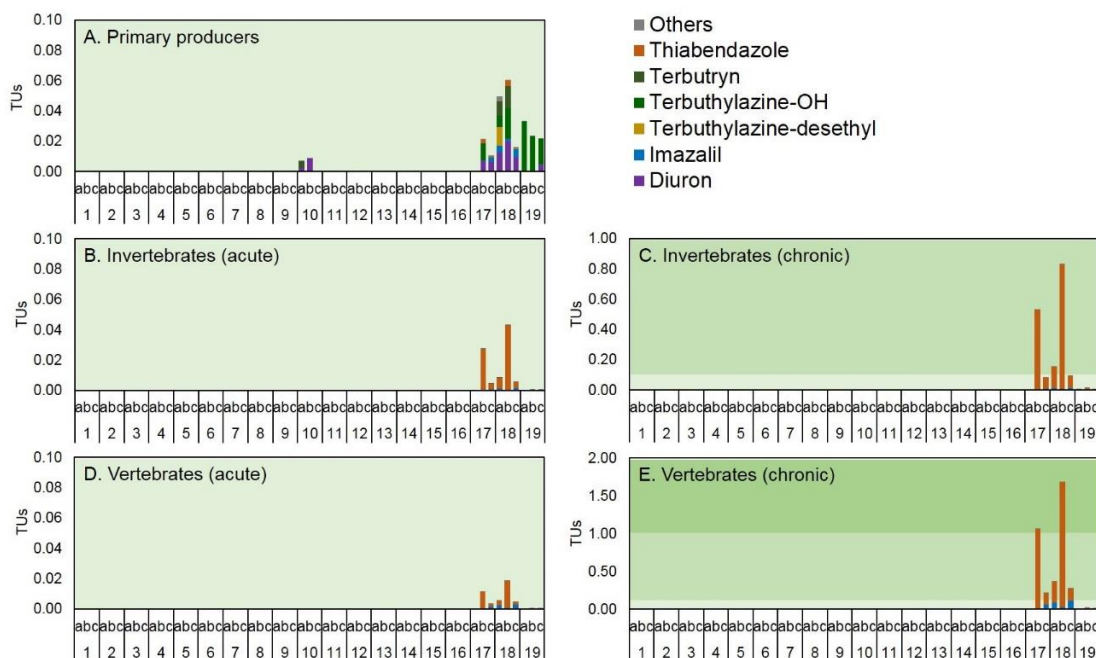


Figure 4. Calculated sum of TUs and relative contribution of each pesticide to the total mixture toxicity. Only pesticides with TUs above 0.01 in at least one sampling site are shown. The shaded area in light, medium and intense green indicate low (sum of TUs < 0.1), moderate (0.1 ≤ sum of TUs ≤ 1), and high (sum of TUs > 1) ecological risks, respectively. a, b, c refer to the samples taken in the first, second and third sampling campaigns (1st: June 2018; 2nd: September 2018; 3rd: February 2019).

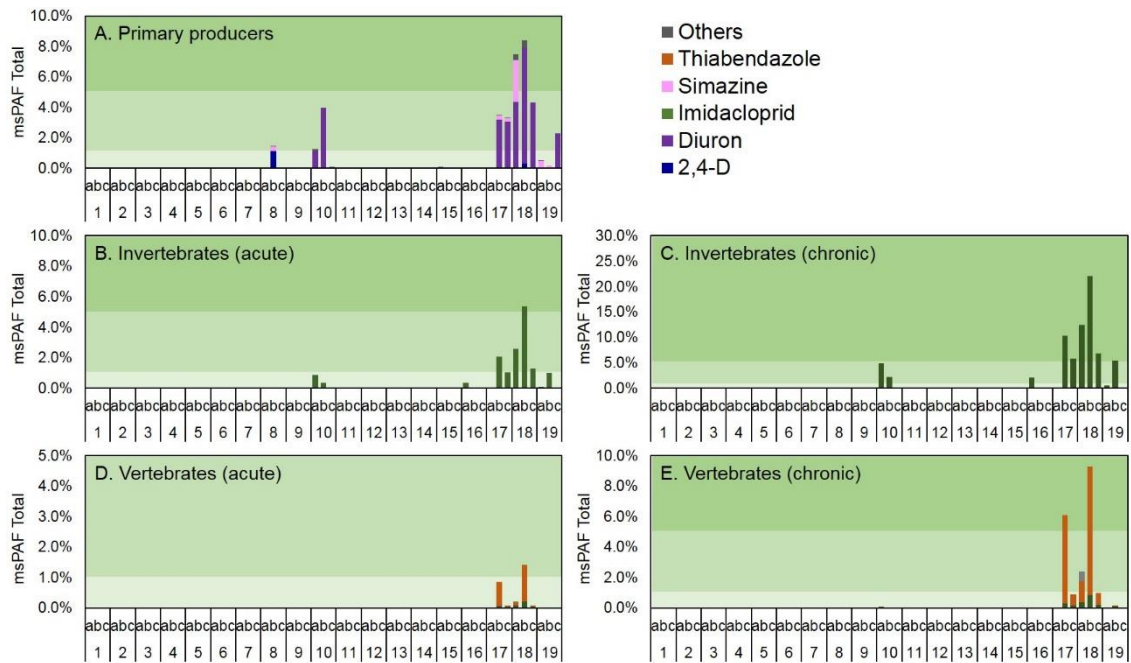


Figure 5. Calculated msPAF_{Total} for each sample and relative contribution of each pesticide to the mixture toxicity. Only pesticides with a maximum calculated PAF of 1% in at least one sampling site are included. The shaded area in light, medium and intense green indicate low (msPAF_{Total} < 1%), moderate (1% ≤ msPAF_{Total} ≤ 5%), and high (msPAF_{Total} > 5%) ecological risks, respectively. a, b, c refer to the samples taken in the first, second and third sampling campaigns (1st: June 2018; 2nd: September 2018; 3rd: February 2019).

Supplementary Material

Ecological risk assessment of pesticides in the Mijares river (eastern Spain) impacted by citrus production using wide-scope screening and target quantitative analysis

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2. EXPERIMENTAL

2.1 Pesticide standards and reagents

Reference standards of pesticides, transformation products and isotopically labelled reference standards were purchased from Dr. Ehrenstorfer (Augsburg, Germany), Fluka (Buchs, Switzerland), or Sigma-Aldrich (Madrid, Spain). Stock standard solutions (around 500 mg/L) were prepared in acetone and were stored at -20°C. Twenty-two mixtures of pesticide standards (individual concentration of each pesticide around 50 mg/L) were prepared by dilution of stock individual solutions in acetone. Working standard solutions containing all pesticides were prepared by dilution of mixtures with acetone (for sample fortification in GC), hexane (GC injection), methanol (for sample fortification in LC) and water (instrument injection in LC).

Acetonitrile and methanol (HPLC grade) were purchased from Scharlab (Barcelona, Spain). HPLC-grade water (resistivity of 18 MΩ cm) was obtained by purifying demineralised water (Millipore Ltd., Bedford, MA, USA). Formic acid (HCOOH, content > 98%) and ammonium acetate (NH₄Ac, reagent grade) were supplied by Scharlab.

Cartridges used for solid phase extraction were 150 mg Oasis HLB (Waters, Milford, MA, USA)

2.3 Sample collection

Table S1. Description and location of the sampling sites along the Mijares River

Sampling site	Source	Location	Province	Observations	UTM X	UTM Y	UTM Zone
1	Upper	Azud de Babor, Mora de Rubielos	Teruel	-	689781	4448005	30T
2	Upper	La Escaleruela, Sarrión	Teruel	Fertilizer factory	691742	4446731	30T
3	Upper	La Escaleruela, Sarrión	Teruel	Fish farm	691816	4446682	30T
4	Upper	La Escaleruela, Sarrión	Teruel	Fish farm (downstream)	691833	4446727	30T
5	Upper	Toranes, Albentosa	Teruel	Reservoir	692172	4446656	30T
6	Upper	Pozo de las Palomas (Los Cantos) Puebla de Arenoso	Castellón	-	703509	4443872	30T
7	Middle	Arenós, Puebla de Arenoso	Castellón	Upstream Reservoir	705419	4442293	30T
8	Middle	Arenós, Puebla de Arenoso	Castellón	Downstream Reservoir	708738	4440125	30T
9	Middle	Montanejos	Castellón	Upstream WWTP	712148	4438332	30T
10	Middle	Montanejos	Castellón	Effluent WWTP	712150	4438356	30T
11	Middle	Toga	Castellón	Effluent WWTP	725126	4435377	30T
12	Middle	Sitjar, Onda	Castellón	Reservoir	736137	4432693	30S
13	Middle	Onda	Castellón	Downstream SWTP, Power-plant	736525	4431287	30S
14	Middle	Onda	Castellón	Downstream SWTP, Gauging station	736713	4431282	30S
15	Lower	Onda	Castellón	-	740931	4429492	30S
16	Lower	Vila-real	Castellón	Santa Quiteria	748201	4426699	30S
17	Lower	Vila-real	Castellón	Effluent WWTP	751127	4424425	30S
18	Lower	Almassora	Castellón	Effluent WWTP	751477	4424111	30S
19	Mouth	Almassora	Castellón	Gola	755367	4421890	30S

UTM: Universal Transverse Mercator (hemisphere Northern)

2.4. Instrumentation

2.4.1 QTOF MS

LC-QTOF MS

A Waters Acquity UPLC system (Waters, Milford, MA, USA) was interfaced to a hybrid quadrupole-orthogonal acceleration-TOF mass spectrometer (XEVO G2 QTOF, Waters Micromass, Manchester, UK), using an orthogonal Z-spray-ESI interface, operating in both positive and negative ionisation modes. The chromatographic separation was performed using a Cortecs C₁₈ analytical column (2.1 i.d. × 100 mm length, 2.7 μm particle size) from Waters, at a flow rate of 300 μL/min. The mobile phases used were (A) H₂O with 0.01% HCOOH and (B) MeOH with 0.01% HCOOH. The initial percentage of B was 10%, which was linearly increased to 90% in 14 min, followed by a 2 min isocratic period and, then, returned to initial conditions during 2 min. Nitrogen was used as drying and nebulizing gas. The gas flow was set at 1000 L/h. TOF-MS resolution was approximately 20,000 at full width half maximum (FWHM) at *m/z* 556. MS data were acquired over an *m/z* range of 50–1000. Capillary voltages of 0.7 and 3.0 kV were used in positive and negative ionisation modes, respectively. A cone voltage of 20 V was selected for both ionisation modes. Collision gas was argon 99.995% (Praxair, Valencia, Spain). The desolvation temperature was set to 600 °C and the source temperature to 130 °C. The column temperature was set to 40 °C.

For MS^E experiments, two acquisition functions with different collision energies were created. The low energy function (LE), selecting a collision energy of 4 eV, and the high energy (HE) function, with a collision energy ramp ranging from 15 to 40 eV, in order to obtain a greater range of fragment ions. The LE and HE functions settings were for both a scan time of 0.4 s.

Calibrations were automatically conducted from *m/z* 50 to 1000 with a 1:1 mixture of 0.05M NaOH:5% HCOOH, twenty five-fold diluted with ACN:H₂O (80:20). For automated accurate mass measurement, a solution of leucine enkephalin (10 μg/mL) in ACN:H₂O (50:50) with 0.1% HCOOH was used as lock mass and pumped at a flow rate of 20 μL/min. The (de)protonated molecule of leucine enkephalin (*m/z* 556.2771 in positive

mode, m/z 554.2615 in negative mode) was used for recalibrating the mass axis and ensuring a robust accurate mass measurement at any time. Mass data were acquired with MassLynx v 4.1 (Waters) and processed by ChromaLynx application manager (within MassLynx v 4.1).

GC-QTOF MS

An Agilent 7890A GC system (Palo Alto, CA, USA) equipped with an Agilent 7683 autosampler was coupled to the Xevo G2 QTOF, operating in APCI mode. The GC separation was performed using a fused silica DB-5MS capillary column with a length of 30 m x 0.25 mm i.d. and a film thickness of 0.25 μm (J&W Scientific, Folsom, CA, USA). The oven temperature was programmed as follows: 90 °C (1 min); 5 °C/min to 300 °C (2 min). Pulsed splitless (50 psi) injections of 1 μL of sample extracts were carried out with an injector temperature of 280 °C and with a splitless time of 1 min. Helium 99.999 % (Praxair, Valencia, Spain) was used as carrier gas at a constant flow of 2 mL/min. The interface and source temperatures were set to 310 °C and 150 °C, respectively. The desolvation gas (N_2) was set at 300 L/h flow and the cone gas at 16 L/h. The voltage of the sampling cone was set at 20 V, the voltage of the extraction cone was 4 V, and the APCI corona pin was fixed at a current 1.7 μA . The ionization process occurred within an enclosed ion volume, which enabled control over the protonation/charge transfer processes. TOF MS resolution was approximately 20,000 (FWHM) at m/z 614. A scan time of 0.4 s was selected. MS data were acquired over an m/z range of 50-650. Heptacose was used for the daily mass calibration. Continuous internal calibration was performed using a background ion coming from the GC-column bleed as lock mass ($[\text{M}-\text{H}]^+$ of octamethylcyclotetrasiloxane, m/z 297.0830). Two injections were performed for sample: the first one promoting the formation of the molecular ion, and the second one, promoting the formation of the protonated molecule

2.4.2. LC-MS/MS (QqQ)

A triple quadrupole mass spectrometer was interfaced to a Waters ACQUITY ultra performance liquid chromatography (UPLC™) system (Waters Corp., Milford, MA, USA), equipped with a quaternary pump system. Chromatographic separation was carried out

using an ACQUITY UPLC BEH C₁₈ column (100 x 2.1 mm i.d., particle size 1.7 μm) (Waters). An optimized gradient was applied at a constant flow rate of 0.4 mL/min using methanol LC-MS (solvent A) and water LC-MS (solvent B), both 0.01% HCOOH and 1 mM ammonium acetate. The gradient elution was: 0 min, 5% A; 0-7 min linear from 5 to 90% A; 7-8 min, 90% A; 8-8.1 min linear from 90 to 5 % A, return to initial conditions; 8.1–10 min 5% A, equilibration of the column. The injection volume was 100 μL.

A Xevo TQ-S™ triple quadrupole mass spectrometer (Waters Micromass, Manchester, UK), equipped with ESI source was used. Determination of analytes was performed using ESI source in both positive and negative ion modes. Drying gas as well as nebulising gas was nitrogen (Praxair, Valencia, Spain). The cone gas flow rate was optimized at 250 L/h and the desolvation gas flow was set to 1200 L/h. The desolvation temperature was 650 °C. For operation in MS/MS mode, collision gas was Argon 99.995% (Praxair, Valencia, Spain) with a flow of 0.15 mL/min in the collision cell. Electrospray needle capillary voltage was fixed at 3.5 kV and 2 kV in positive and negative ionisation modes, respectively. The source temperature was set to 150 °C. The column temperature was maintained at 40 °C.

MassLynx software v 4.1 (Waters Corporation) was used to acquire data. TargetLynx application manager was used to quantify the concentration levels of the target analytes.

2.6 Screening by QTOF MS

When the reference standard was available, a compound was classified as “detected” when the molecular ion (i.e. in GC analysis), the (de)protonated molecule (in both GC and LC analysis) or a relevant adduct was observed (together with its characteristic isotopic pattern, if exists) at the expected Rt (±0.1 min deviation respect to a reference standard) with accurate mass (mass error ≤ 5 ppm). Then, the detected compound was considered as “identified/confirmed” when the molecular ion or (de)protonated molecule and at least one fragment ion were observed, both with mass errors below 5 ppm at the appropriate Rt. On the other hand, a compound (for which reference standard was not available at our laboratory) was considered as “tentatively identified”

when one or more fragment ions were compatible with the chemical structure of the candidate or in agreement with previously reported data in the literature.

2.8 Quantitative analysis by LC-MS/MS

In order to allow the simultaneous quantification and reliable identification of the positive findings, two selected reaction monitoring (SRM) transitions (Q for quantification and q for confirmation) were acquired for every compound (see **Table S2**). In addition, 8 isotopically labelled internal standard (ILIS) were used for matrix effects correction.

Quantification of pesticides was made using the Q transition and external calibration with standards in solvent, using relative areas with ILIS for several compounds. At least seven-points calibration curves (between 0.001-10 µg/L) were injected at the beginning and the end of each sequence. Linearity was assumed when regression coefficient was >0.99 with residuals lower than 30%. As samples were analysed by direct injection without any pre-concentration step, the lowest calibration level (LCL) was considered as the limit of quantification in water analysis (1 ng/L for all compounds).

The reliable identification of pesticides found in the samples was based on the ion ratio (peak area) calculation between both transitions (q/Q). The finding was considered as positive when the ion-ratio and the Rt of the compound in sample were within the tolerance ranges ($\pm 30\%$ for ion ratio, ± 0.1 min for Rt) in comparison with the reference standards injected in the calibration.

Table S2. LC-MS/MS conditions for pesticide quantification. Quantification (Q) and confirmation (q) transitions. Collision energy (CE).

Compounds	ESI	Cone (V)	Transition (Q)	CE (eV)	Transition (q)	CE (eV)
Atrazine	+	50	216.1 > 174.1	20	216.1 > 96.2	25
Atrazine-desethyl (DEA)	+	10	188.3 > 146.3	15	188.3 > 104.2	25
Atrazine-desisopropyl (DIA)	+	10	174.3 > 132.2	15	174.3 > 96.3	15
Carbaryl	+	30	202.0 > 145.1	20	202.0 > 127.2	30
Chlorpyrifos	+	10	350.0 > 97.0	30	350.0 > 198.0	20
2,4-D	-	10	219.0 > 125.0	25	219.0 > 161.0	15
Diuron	+	30	233.1 > 72.2	20	233.1 > 160.1	20
Imazalil	+	30	298.1 > 159.0	20	298.1 > 256.0	20
Imidacloprid	+	20	256.1 > 209.2	10	256.1 > 175.1	10
Linuron	+	10	249.0 > 160.0	15	249.0 > 182.0	15
Metalaxyl	+	35	280.1 > 220.1	15	280.1 > 160.1	25
Metolachlor	+	50	284.2 > 252.0	15	284.2 > 176.1	25
Prometryn	+	10	242.0 > 158.0	20	242.0 > 200.0	15
Propamocarb	+	10	189.0 > 102.0	15	189.0 > 144.0	10
Pyridaphention	+	20	340.9 > 189.2	20	340.9 > 205.2	20
Simazine	+	10	202.3 > 132.1	15	202.3 > 124.2	15
Tebuconazole	+	10	308.0 > 70.0	15	308.0 > 125.0	30
Terbumeton	+	50	226.0 > 170.2	20	226.0 > 113.9	15
Terbumeton-desethyl	+	50	198.3 > 142.2	15	198.3 > 86.1	20
Terbutylazine	+	10	230.3 > 174.2	15	230.3 > 104.2	25
Terbutylazine-desethyl	+	20	202.2 > 145.9	25	202.2 > 79.0	25
Terbutylazine-OH	+	30	212.2 > 156.1	15	212.2 > 86.1	20
Terbutryn	+	30	242.1 > 91.2	30	242.1 > 186.2	30
Thiabendazole	+	20	202.1 > 131.2	40	202.1 > 175.1	40
ILIS						
Atrazine-d ₅	+	40	221.1 > 179.2	20		
Chlorpyrifos methyl-d ₆	+	10	328.0 > 130.5	20		
Diuron-d ₆	+	30	239.1 > 78.2	20		
Imazalil-d ₅	+	30	302.0 > 159.0	20		
Metolachlor-d ₆	+	25	290.2 > 258.2	15		
Simazine-d ₅	+	30	207.2 > 137.0	20		
Terbutylazine-d ₅	+	10	235.2 > 179.2	15		
Thiabendazole-d ₆	+	20	208.1 > 137.2	40		

2.9 Ecological risk assessment

The toxicity data selection criteria for primary producers, invertebrates and vertebrates were:

For primary producers, NOEC (No Observed Effect Concentration) values based on growth rate or yield obtained after an exposure period of 3-5 days and 7-28 days, for algae and macrophytes, respectively, were selected.

Acute toxicity data for invertebrates consisted of EC50s (mortality or immobilization) calculated after an exposure period of 2-4 days, while chronic toxicity data for invertebrates consisted of NOECs (growth rate, feeding inhibition, reproduction, mortality) calculated after an exposure period of 21 days.

Acute toxicity data for aquatic vertebrates (i.e., fish and amphibians) consisted of LC50s calculated after an exposure period of 2-4 days. Chronic toxicity data for vertebrates consisted of NOEC values (growth rate, development, behaviour, mortality) calculated after an exposure period of 21 days.

In the case that more than one toxicity value was available for a given taxon and pesticide combination, the geometric mean was calculated for the same endpoint and exposure duration, and the lowest value was conservatively selected.

3. RESULTS AND DISCUSSION

Table S3. Mean recoveries and RSD (in brackets) obtained for QCs after application of the LC-MS/MS procedure, corresponding to the three sampling campaigns. A total of 9 replicates (n=3 at each concentration level) were analyzed. The ILIS used for each compound is also shown.

Compounds	ILIS	QC		
		0.01 µg/L	0.1 µg/L	1 µg/L
Atrazine	Atrazine-d ₅	101 (4)	99 (3)	101 (3)
Atrazine-desethyl (DEA)	Atrazine-d ₅	107 (13)	96 (16)	96 (14)
Atrazine-desisopropyl (DIA)	Atrazine-d ₅	-	115 (18)	111 (20)
Carbaryl	-	82 (23)	67 (18)	64 (14)
Chlorpyrifos	-	158 (38)	80 (23)	71 (21)
2,4-D	-	-	96 (20)	94 (18)
Diuron	Diuron-d ₆	116 (17)	102 (3)	106 (7)
Imazalil	Atrazine-d ₅	148 (24)	105 (4)	107 (4)
Imidacloprid	Thiabendazole-d ₆	116 (30)	113 (28)	111 (29)
Linuron	Diuron-d ₆	104 (13)	109 (7)	120 (9)
Metalaxyl	-	81 (9)	80 (9)	84 (17)
Metolachlor	Metolachlor-d ₆	84 (6)	86 (8)	92 (10)
Prometryn	Terbuthylazine-d ₅	102 (8)	99 (9)	94 (5)
Propamocarb	-	111 (21)	97 (7)	100 (23)
Pyridaphention	-	85 (30)	83 (22)	81 (21)
Simazine	Simazine-d ₅	120 (20)	109 (4)	110 (5)
Tebuconazole	Metolachlor-d ₆	127 (19)	120 (14)	119 (12)
Terbumeton	Terbuthylazine-d ₅	112 (9)	98 (13)	92 (6)
Terbumeton-desethyl	Simazine-d ₅	104 (6)	97 (9)	98 (10)
Terbuthylazine	Terbuthylazine-d ₅	109 (13)	103 (9)	105 (11)
Terbuthylazine-desethyl	Atrazine-d ₅	97 (12)	103 (12)	107 (9)
Terbuthylazine-OH	Terbuthylazine-d ₅	123 (21)	109 (12)	99 (5)
Terbutryn	Terbuthylazine-d ₅	83 (14)	88 (10)	89 (14)
Thiabendazole	Thiabendazole-d ₆	121 (22)	106 (7)	103 (6)

- Not available due to the lack of sensitivity

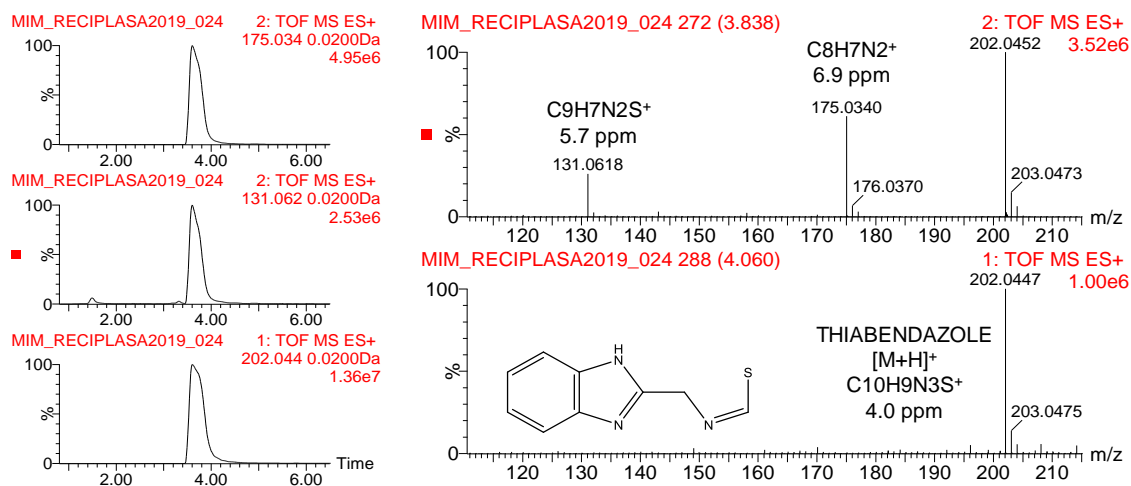


Figure S1. Detection and identification of fungicide thiabendazole in a surface water sample by LC-QTOF MS. Left: nw-XICs at 0.02 Da mass window for [M+H]⁺ in LE function and main fragments in HE function. Right: LE (bottom) and HE (top) TOF mass spectra for the chromatographic peak at 3.9 min

The LE spectrum of the chromatographic peak at 3.9 min showed an abundant signal at m/z 202.0447 (**Figure S1**) at the expected retention time of thiabendazole (C₁₀H₉N₃S⁺, 4 ppm mass error). The HE spectrum also showed its characteristic main fragment ions at m/z 175.0340 (C₈H₇N₂⁺, 6.9 ppm) and 131.0618 (C₉H₇N₂S⁺, 5.7 ppm), confirming the identity of the pesticide.

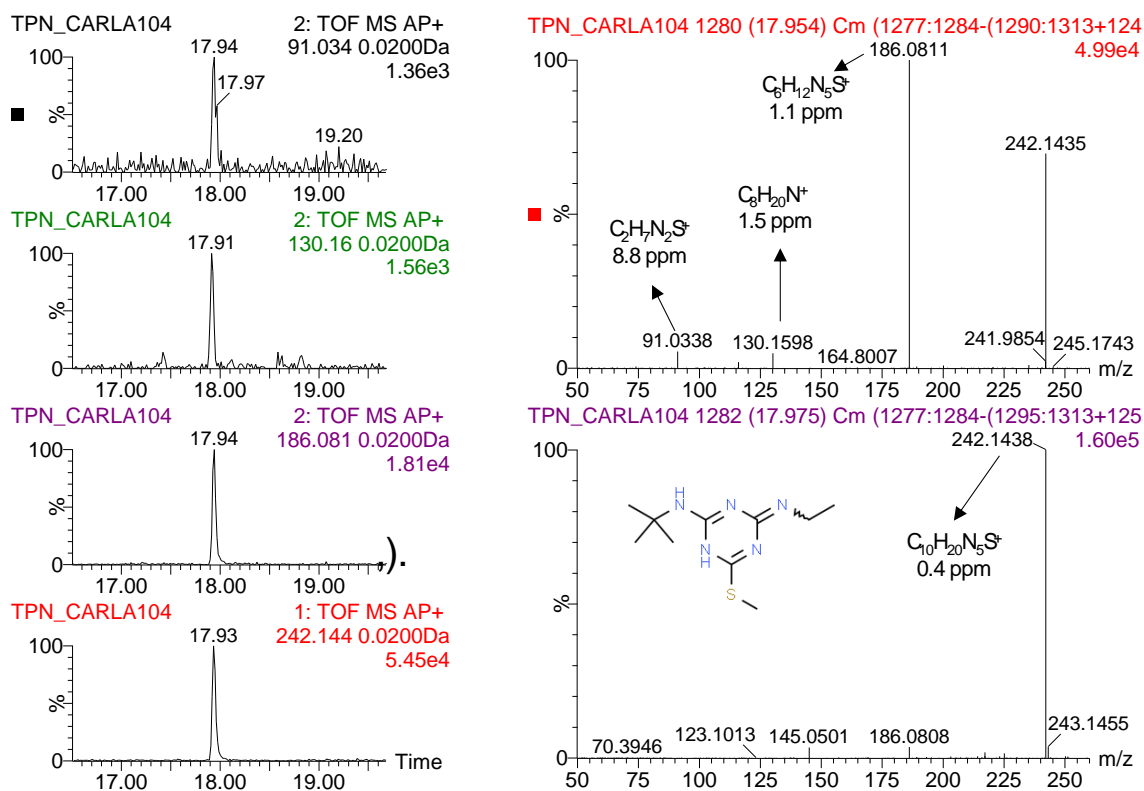


Figure S2. Detection and identification of terbutryn in a surface water sample by GC-QTOF MS. Left: nw-XICs at 0.02 Da mass window for $[M+H]^+$ in LE function and main fragment in HE function. Right: LE (bottom) and HE (top) TOF mass spectra for the chromatographic peak at 17.93 min

The LE spectrum of the chromatographic peak at 17.93 min showed an abundant signal at m/z 242.1439 (**Figure S2**) which could fit with the herbicide terbutryn ($C_{10}H_{20}N_5S^+$, 0.4 ppm mass error). The HE spectrum showed the characteristic main fragment ions of this compound at m/z values 186.0811 ($C_6H_{12}N_5S^+$, 1.1 ppm), 130.1598 ($C_8H_{20}N^+$, 1.5 ppm) and 91.0338 ($C_2H_7N_2S^+$, 8.8 ppm). Moreover, the retention time deviation with respect to the reference standard was lower than 0.1 min.

Table S4. Concentration levels (ng/L) of pesticides in samples from the first campaign after applying LC-MS/MS (QqQ)

Compounds	Samples																		
	1a	2a	3a	4a	5a	6a	7a	8a	9a	10a	11a	12a	13a	14a	15a	16a	17a	18a	19a
Atrazine	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Atrazine-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	47	-
Atrazine-desisopropil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbaryl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,4-D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Diuron	-	-	-	-	-	-	-	-	-	5.3	-	-	-	-	d	-	-	31	-
Imazalil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3921	28
Imidacloprid	-	-	-	-	-	-	-	-	-	26	-	-	-	-	-	9.6	-	97	2.2
Linuron	-	-	-	d	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metalaxyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Metolachlor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Prometryn	-	-	-	-	-	d	-	-	-	-	-	-	-	-	-	-	d	-	-
Propamocarb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyridaphention	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	11	2.3
Tebuconazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.2	-
Terbumeton	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terbumeton-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1.3
Terbuthylazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terbuthylazine-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	25	-
Terbuthylazine-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	15	66
Terbutryn	-	-	-	-	-	-	-	-	-	11	-	-	-	-	-	-	-	22	-
Thiabendazole	-	-	-	-	-	-	-	-	d	-	-	-	-	-	-	-	-	5823	47

d. detected, concentration below to the limit value used for the quantification (LCL) and at least one q/Q ratio accomplished

Table S5. Concentration levels (ng/L) of pesticides in samples from the **second campaign** after applying LC-MS/MS (QqQ)

Compounds	Samples																		
	1b	2b	3b	4b	5b	6b	7b	8b	9b	10b	11b	12b	13b	14b	15b	16b	17b	18b	19b
Atrazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Atrazine-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Atrazine-desisopropil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbaryl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,4-D	-	-	-	-	-	-	-	611	-	-	-	-	-	-	-	-	-	136	-
Diuron	-	-	-	-	-	-	-	-	-	21	-	-	-	-	-	-	17	50	d
Imazalil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	395	1353	22
Imidacloprid	-	-	-	-	-	-	-	-	-	10	-	-	-	-	-	-	74	268	30
Linuron	-	-	-	-	-	-	-	d	-	-	-	-	-	-	-	-	d	-	-
Metalaxyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	d
Metolachlor	-	-	-	-	-	-	-	d	-	-	-	-	-	-	-	-	-	-	-
Prometryn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propamocarb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyridaphention	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simazine	-	-	-	-	-	-	-	d	-	-	-	-	-	-	-	-	d	-	d
Tebuconazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terbumeton	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terbumeton-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	d
Terbuthylazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Terbuthylazine-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	-
Terbuthylazine-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	23	40	46
Terbutryn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	35	-
Thiabendazole	-	-	-	-	-	-	-	-	-	d	-	-	-	-	-	-	22065	34547	377

d. detected, concentration below to the limit value used for the quantification (LCL) and at least one q/Q ratio accomplished

Table S6. Concentration levels (ng/L) of pesticides in samples from the **third campaign** after applying LC-MS/MS (QqQ)

Compounds	Samples																		
	1c	2c	3c	4c	5c	6c	7c	8c	9c	10c	11c	12c	13c	14c	15c	16c	17c	18c	19c
Atrazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Atrazine-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Atrazine-desisopropil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Carbaryl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Chlorpyrifos	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2,4-D	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Diuron	-	-	-	-	-	-	-	-	-	d	-	-	-	-	-	-	16	23	11
Imazalil	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2647	4940	67
Imidacloprid	-	-	-	-	-	-	-	-	-	d	-	-	-	-	d	-	33	41	d
Linuron	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Metalaxyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d
Metolachlor	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Prometryn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Propamocarb	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pyridaphention	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Simazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	-	-
Tebuconazole	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Terbumeton	-	-	-	-	d	d	-	d	d	-	-	-	-	-	-	-	-	-	-
Terbumeton-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	d	d
Terbuthylazine	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d
Terbuthylazine-desethyl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	d	d	d
Terbuthylazine-OH	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	34
Terbutryn	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Thiabendazole	d	d	d	-	-	-	-	d	d	-	-	-	-	-	-	-	3123	3307	127

d. detected, concentration below to the limit value used for the quantification (LCL) and at least one q/Q ratio accomplished